CHEMICAL DISRUPTION OF WNT SIGNALING AND TELOMERE LENGTH MAINTENANCE

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DEDICATION

For my beloved spouse Kerem Kulak

CHEMICAL DISRUPTION OF WNT SIGNALING AND TELOMERE LENGTH MAINTENANCE

by

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A nearly universal feature of colorectal cancer (CRC) incidents is the presence of genetic alterations that promote deviant activation of the TCF/LEF transcriptional regulators. The TCF/LEF proteins control the transcriptional output of intercellular signaling mediated by the Wnt family of secreted ligands. Several chemical screening efforts devoted to disrupting deviant TCF/LEF activity have converged on two vulnerabilities in the Wnt pathway – the poly-ADP-ribose polymerases, Tankyrase 1 and 2 (Tnks1 and 2) that control the threshold response levels to Wnt ligands, and the Wnt acyltransferase Porcupine that provides an essential fatty acyl adduct to all nineteen Wnt ligands. My thesis focuses on the chemical biology of one of these strategies – the Tnks enzymes - with the goal of understanding the

strengths and limitations of drugging the Tnks proteins for achieving therapeutic goals in regenerative medicine and cancer. Given the previously assigned role of Tnks enzymes in telomere maintenance, I have also devoted considerable effort to understanding the cell biological effects of disrupting Tnks activity on telomere integrity. Finally, I mined a high-confidence collection of Wnt pathway inhibitors with previously unidentified mechanisms of action to identify novel small molecules that directly target the TCF/LEF transcriptional apparatus. This effort netted a chemical approach for disabling deviant transcriptional activity in CRC that is distinct from the one afforded by Tnks and Porcn inhibitors. Taken together, my thesis establishes a chemical toolkit for interrogating the inner workings of Wnt-mediated signaling and also reveals new avenues for disabling deviant Wnt responses in cancer and normal Wnt responses in tissue engineering.

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CRC-Colorectal Cancer

FAP-Familial adenomatous polyposis

APC-Adenomatous Polyposis Coli

ACF-Aberrant crypt focus

Wg-Wingless

GSK3beta-Glycogen synthase kinase 3b

Dvl-Disheveled

LEF-Lymphocyte enhancement factor

TCF-T cell factor

MBOAT-Membrane-bound O-acyl-transferase

Porcn-Porcupine

Wls-Wntless

Fzd-Frizzled

CK1-casein kinase 1

LRP-low-density lipoprotein related protein

TA cells-transit-amplifying cells

PP2A-Protein phosphatase 2A

KAP3-kinesin superfamily-associated protein 3

ASEF-APC-stimulated guanine nucleotide exchange factor

CTNNB1- Beta-catenin

EB1-End-binding protein 1

DLG-Drosophila disc large tumor suppressor gene

PTP-BL-protein tyrosine phosphatase

MCR-Mutation cluster region

Tnks-Tankyrase

Parp-Poly-ADP-ribose polymerase

IWR-Inhibitor of Wnt response

IWP-Inhibitor of Wnt production

PAR-Poly-ADP-ribose

Parg-Poly-ADP-ribose glycohydrolase

NAD-Nicotinamide adenine dinucleotide

TA cells- Transit-amplifying cells

Bmi1- B cell-specific Moloney murine leukemia virus integration site 1

Lgr5- Leucine-rich repeat-containing G-protein coupled receptor 5

NSAID-Non-steroidal anti-inflammatory drug

COX2-Cyclooxygenase 2

Rnf146-Ring finger protein 146

Terf1-Telomeric repeat binding factor-1

NuMa-Nuclear mitotic apparatus protein

IRAP- Insulin-responsive aminopeptidase (IRAP), myeloid cell leukemia 1 (Mcl1)

CHAPTER ONE
Introduction and Literature Review

APC – The Most frequently Mutated Gene In CRC

In 1950, researchers Eldon Gardner and Fayette Stephens exploited the meticulous genealogical records of relatively isolated communities found in Utah to identify a family with an extremely high incidence (66%) of colorectal cancer (CRC) (1). Follow up medical examinations of the kindred established dominant inheritance of multiple intestinal polyps as well as carcinoma disposition (2). This syndrome was dubbed familial adenomatous polyposis or FAP.

In order to identify the gene responsible for FAP, Herrera (3) and Solomon (4) utilized chromosomal spreading and restriction fragment length polymorphism analysis and assigned the locus for the FAP to chromosomal region 5q21. Subsequent studies showed that alleles from the same region are often lost in sporadic tumors (3,4). Thus, familial and sporadic CRC likely take a shared genetic path for disease emergence.

Since sporadic CRC is the third leading cause of cancer deaths in the United States (5), the possibility of a common genetic path for sporadic and inherited forms of CRC galvanized a search for the genetic basis of FAP. Cosegregation of mutant APC alleles in affected kindreds indicated that the *Adenomatous Polyposis Coli (APC)* gene was responsible for FAP (6,7). Subsequent studies revealed that somatic mutations in *APC* occur in 50-80% of the

sporadic colorectal and that these mutations typically result in production of truncated proteins (8,9).

A genetic confirmation of APC's role as a tumor suppressor came from a forward genetic screen in mice that netted a strain with an autosomal dominant predisposition to multiple intestinal tumors (Min mice) (10). A truncating mutation in the murine homolog of the *Apc* gene similar to that found in many FAP patients was identified as the cause of cancer susceptibility by linkage analysis and chromosomal walking experiments (11). Min mice not only inherited the mutation in a dominant fashion as observed in FAP patients but also exhibited similar histopathological changes in the gut such as the formation of multiple benign adenomas in the intestines. This convergence in the inheritance pattern and symptoms in humans and mice established a causal relationship between *APC* mutations and colorectal cancer.

APC as a suppressor of a cancer-promoting genetic program

From studying FAP patients, we understood that benign tumors of the intestines (also known as benign polyps) with time could become invasive cell masses (12,13). The ability to track this transformation process in the intestinal epithelium has made CRC a powerful model system for understanding the sequence of genetic changes that promote cancer biogenesis (14). Histological and molecular analysis combined with clinical data support a multi-hit genetic model of sporadic CRC biogenesis thus revealing potential strategies for thwarting tumorigenesis premised about countering one or more of these genetic changes (14,15).

CRC can arise as a consequence of at least four sequential genetic changes: activating mutations in the *KRAS* oncogene and loss of function mutations in three tumor suppressor genes (*APC*, *SMAD4* and *TP53*) (Figure 1.1). *APC* mutations are found with the same frequency in the early neoplastic lesions known as aberrant crypt focus (ACF) as the late stage CRC suggesting that *APC* mutations occur early during the intestinal transformation process (16). Kras is a small GTPase that is mutated in 7% of all cancers. Activating mutations of Kras is found in ~50% of intestinal adenomas and carcinomas thus revealing that deviant activity of Kras contributes to cancers of the gut (17,18). Acquisition of inactivating mutations in components of the growth promoting TGF-beta pathway such as SMAD4 was found in intermediate to late stage adenomas. Loss of function mutations in the tumor suppressor p53 is observed in carcinomas rather than benign lesions. *TP53* mutations promote genetic instability due to cell cycle checkpoint loss and subsequent induction of aneuploidy and chromosomal aberrations (15). Thus, loss of p53 function is probably essential for progression rather than initiation of tumorigenesis.

The temporal order of the genetic alterations involving these key tumor suppressor genes and oncogenes appears to be critical for CRC. For example, patients with germline p53 mutations do not develop multiple polyps or CRC (19). In addition, gut epithelial cells harboring only Kras mutations exhibit increased proliferation, but not dysplastic changes that are observed in *APC* mutant cells (20). These observations suggest that *APC* mutations initiate a sequence of genetic events that result in carcinoma in the intestinal epithelium (15). Thus targeting cellular processes that are engaged as a consequence of compromised APC may be beneficial for all CRC cases.

Despite the emergence of a molecular framework for understanding CRC biogenesis from genetic studies, a more complete inventory of genetic changes associated with CRC is now being assembled with the advent of deep sequencing technology. So far, this sequencing effort has resulted in the identification of a handful of highly mutated genes (thus confirming previous hypotheses) and a relatively large number of less frequently mutated genes. As expected the most frequently mutated genes were the "usual suspects": APC, p53 and Kras with mutation frequencies of 90%, 50% and 40% respectively (21-23). At the same time, the identification of less frequent mutations in other genes that may collaborate with the "usual suspects" reveals whole cellular processes that could be exploited to identify vulnerabilities in cancerous cells.

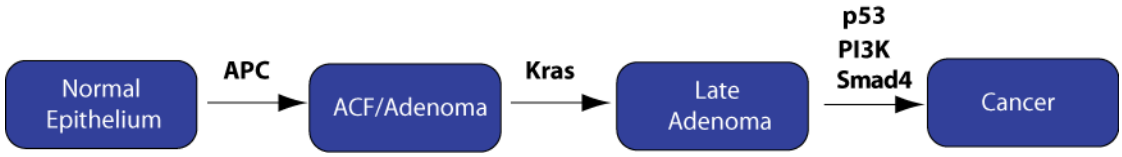


Figure 1.1 A genetically based model of colorectal cancer progression. Mutations in APC are required for tumor initiation. Accumulation of genetic alterations in Kras, Smad4, and p53 genes is associated with tumor progression (Adapted from Fearon and Vogelstein)

The identification of APC as a Wnt Signaling component

The findings from two studies constitute a turn around point with respect to our understanding of how APC suppresses cell growth. The first was the identification of a binding partner for APC known as beta-catenin using biochemical approaches (24,25). The second was the discovery that the *Drosophila* homologue of beta-catenin, Armadillo (Arm), is essential to embryonic patterning (26-29).

The beta-catenin association links APC to two apparently unrelated cellular processes. The beta-catenin protein was initially identified as a cytoplasmic partner of the

transmembrane protein E-cadherin that mediates adhesion between cells (30-32). Thus, APC might also function in cellular adhesion (33). This hypothesis was supported by evidence that loss of cadherin-mediated adhesion can contribute to metastasis, but it was less consistent with evidence that suggested that loss of APC contributed to the early stages of intestinal tumorigenesis (15). There were additional observations that were not consistent with an adhesion-based model of APC function. For example, beta-catenin was not only present at the cell membrane; it was also present in the nucleus indicating a possible role in transcription (34). At the same time, injection of antibodies raised against beta-catenin induced dorsal axis duplication in *Xenopus* (35). These observations suggested that the beta-catenin must have functions other than those supporting cell-to-cell adhesion.

The binding of beta-catenin to APC also revealed a potential link between APC and the Wnt signal transduction pathway. In the late 1980s the *Drosophila* homolog of beta-catenin, Armadillo was identified as a part of a signaling cascade that conveyed cell-to-cell information by the Wingless (Wg) secreted protein. Wg was initially identified as a segment polarity gene from general screens executed by Nusslein-Volhard and Wieschaus in *Drosophila* with the goal of identifying genes essential for embryonic patterning (27). The *Drosophila* embryonic body plan consists of a series of homologous segments. Every segment is separated into anterior and posterior compartments by boundaries. The anterior segment is marked by a band of tooth-like projections called denticles while the posterior portion is naked (36). Wg mutations result in loss of naked cuticles and segmental boundaries. The mammalian homologue of this segment polarity gene, Integration 1 (Int1)

was previously defined as a proto-oncogene in mouse mammary tissue (37). The name Wnt is in fact derived from the fusion of the "Int" and "wg" gene names (38).

Subsequent studies in *Drosophila* mapped *Armadillo* downstream of Wnt gene as the primary effector of this pathway. Further genetic mapping studies placed *arm* downstream of scaffolding protein Dvl, and Zeste-White 3 (zw3), *Drosophila* homolog of glycogen synthase kinase 3-beta (GSK3-beta). APC was found in a complex with beta-catenin together with GSK3-beta (39). Subsequent biochemical studies showed that APC regulates beta-catenin levels through GSK3beta mediated beta-catenin phosphorylation (40,41). Transcriptional mediators of beta-catenin dependent signaling – the LEF/TCF transcription factors - emerged from yeast-two-hybrid screens and subsequent biochemical studies (42-44). Since nuclear levels of beta-catenin are regulated by APC, a possible link between APC and Wnt signaling was proposed. Subsequent studies confirmed that beta-catenin and TCF/LEF mediated transcription is indeed regulated by APC levels (45).

The role of APC in Wnt signal transduction

Many vertebrate genomes harbor 19 Wnt genes with each Wnt gene contributing some non-redundant role in development (46). The biosynthesis of all 19 Wnt proteins is dependent upon their fatty acylation, a biochemical event essential to their exiting the secretory pathway (Figure 1.2 a). Porcupine (Porcn), a member of the membrane-bound O-acyl-transferase (MBOAT) enzyme family, adds a palmitoleic acid to the N-terminus of Wnt precursor molecules (47,48). Only palmitoleoylated Wnts are transported to the cell surface with the help of a G protein–coupled receptor like protein known as, Wntless (Wls) (49). Thus, the lipidation of Wnt proteins represents a secretory pathway checkpoint that ensures

the release of duly processed Wnt molecules. At the same time, thee palmitoleic acid adduct found on Wnt molecules is also essential for engaging a groove found in the Frizzled (Fzd) family of Wnt receptors to secure ligand-receptor interactions (50). Thus, lipidation of Wnt proteins signifies a point of attack to achieve Wnt pathway inhibition.

In the absence of Wnt ligands, a large cytoplasmic complex comprised of the cytoplasmic proteins Axin and APC scaffolds a destruction complex that promotes the destruction of beta-catenin (Figure 1.2 b). Axin and APC recruit two kinases, casein kinase 1 (CK1) and GSK3-beta, that mark beta-catenin in a sequential fashion for destruction by the ubiquitin/proteasome protein destruction system. (51-54). In the absence of beta-catenin available for binding to TCF/LEF DNA binding proteins, the transcriptional activities of its binding partners are thus silenced. (55-58). Somatic mutations of the APC gene are found in more than 90% of CRC cases. Truncation of APC leads to the inappropriate stabilization and nuclear translocation beta–catenin by mechanism described above and drives Wnt target gene transcription.

Although a superficial interpretation of the genetic observations would assign to APC a role as a suppressor of the Wnt pathway, more in-depth probing of APC cellular activities using genetic and biochemical approaches reveal that APC also acts as a positive player likely by the promoting the degradation of Axin (59,60) APC-mediated Axin degradation depends on the N-terminal part of APC that is not involved in beta-catenin degradation. Since CRC cells are rarely null for APC but rather retain the amino-terminal half of APC, the truncated forms of APC have been assigned pro-tumorigenic functions including potentially

enhancing the transcriptional activities already heightened by the accumulation of beta-catenin protein (61).

In the presence of Wnt ligands, Wnt proteins bind to a receptor complex consisting of a member of the seven-transmembrane GPCR-like Frizzled family (62-65) and a member of the low-density lipoprotein related protein (LRP) family (66-68). This ligand-receptor complex recruits the Dishevelled (Dvl) signaling molecules, Axin and other members of the beta-catenin destruction complex to the cell surface (69,70). Inhibition of beta-catenin degradation results in the accumulation and nuclear translocation of beta-catenin (71,72). TCF/LEF proteins have a DNA binding domain but lack a transactivation domain. Beta-catenin, which lacks a DNA-binding domain but contains an activation domain serves as a co-activator of TCF/LEF proteins and activates target gene transcription (42,43,73-75). Forms of Wnt signaling that do not utilize beta-catenin, non-canonical Wnt responses, additionally occur and include Wnt induced signal transduction through intracellular calcium, JNK, and RhoA and Rac1 that constitute components of the Wnt/PCP pathway (76,77).

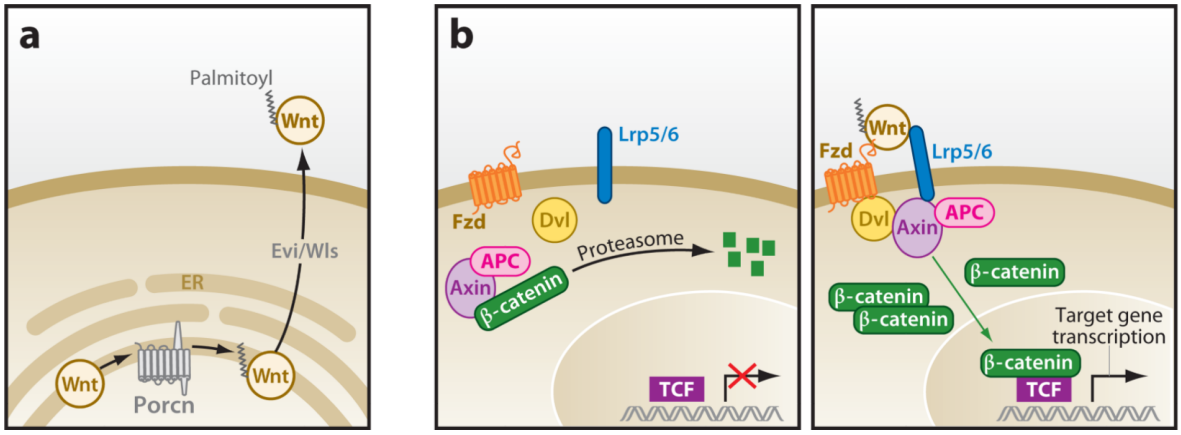


Figure 1.2 Wnt/beta-catenin mediated signal transduction in mammals (a) Wnt ligand production. Production of Wnt proteins depends on palmitoleoylation by Porcupine (Porcn) in the ER. Palmitoleoylated Wnt is then chaperoned by Wntless to the extracellular space. (b) Activation of the Wnt/beta-catenin pathway. In the absence of Wnt secreted protein a cytoplasmic complex composed of Axin, APC, CKI (not shown) and GSK3-beta (not shown) binds to and phosphorylates the transcriptional co-activator beta-catenin, thus marking it for proteasome-mediated degradation (right). In the absence of beta-catenin, members of the TCF/LEF family of transcriptional regulators mainly function as transcriptional repressors. In the presence of Wnt ligand, Wnt, Lrp5/6, and Fzd receptor complex recruit the Axin/APC and Dvl scaffolding protein to the membrane, thus abolishing destruction of beta-catenin (left). Accumulated beta-catenin activates members of the TCF/LEF transcription factor family. Modified from Dodge, 2011(78).

Functions of Wnt signaling in the intestinal tissue homeostasis and cancer

The intestinal epithelium is the fastest proliferating tissue in adults with a self-renewal cycle of 4-5 days (79). The intestinal surface consists of projections that protrude from the epithelial lining known as villus and invaginations between the villi termed crypts. The colonic epithelium has crypts similar to intestine but instead of villi, it forms a flat surface. Stem cells reside at the bottom of the crypts and produce progenitor transit-amplifying (TA) cells. Dividing TA cells migrate upwards onto the villi where they differentiate to terminal epithelial cells (80). This physical organization allows proliferating stem cells to push their progeny up towards villi. Differentiated cells migrate all the way through the villus tip where they undergo apoptosis and then shed into the intestinal lumen (80). The integrity of intestinal tissue architecture depends on the coordination of proliferation and differentiation throughout this intestinal self-renewal process.

The activity of the Wnt pathway is indispensable for the coordination of proliferation and differentiation processes in the gut. Disruption of Wnt pathway response at the level of ligand/receptor interaction or transcription inhibits the formation of downstream epithelial lineages resulting in a loss of the epithelial lining and altered tissue structure (81-83). Thus, Wnt signaling maintains crypt stem cell/progenitor compartments. Approximately 98% of all colorectal cancer incidents in humans harbor mutations that cause aberrant Wnt pathway activation, predominantly due to mutations causing truncated APC (23). Consistently, mouse models generated either by loss of function mutations in APC or gain of function mutations in beta-catenin develop numerous intestinal polyps indicating that APC acts through beta-

catenin in tumorigenesis (24,25,45,84). Loss of function mutations in APC stimulates aberrant induction of beta-catenin mediated transcription of the growth-promoting genes Myc and Cyclin D1(85-87). Conditional deletion of Myc rescues increased proliferation and crypt size and loss of differentiation in APC-mutant intestines suggesting that effects of APC mutation is mediated by beta-catenin dependent transcriptional activation (85) . Several other components of the Wnt pathway are mutated in CRC including Axin, Tcf4, and beta-catenin (22,23,88,89). Perturbations of the Wnt/beta-catenin pathway converge on similar phenotypic changes supporting the idea that Wnt pathway is critical for intestinal tissue homeostasis.

Cell replacement of the intestinal epithelium is sustained by stem cells located just above the crypt base in the small intestine and at the crypt base in the colon (90). The identity and the marker of the intestinal stem cells is a matter of debate. Two different molecular markers, the Polycomb group protein Bmi1 and the G protein-coupled receptor Lgr5 represent two different populations of intestinal stem cells residing in the crypts (91-93). Both of these cell populations have the ability to functionally repopulate the intestinal epithelium as demonstrated by *in vivo* lineage tracing experiments in mice. Furthermore lineage-tracing experiments suggested that Bmi1 marks quiescent, injury-inducible stem cells while Lgr5 marks stem cells that are important for homeostatic regeneration (93). Inhibition of Wnt signaling at the ligand/receptor level resulted in complete loss of Lgr5 positive cells although Bmi1 positive cells persist in the crypt suggesting that Wnt signaling is essential for the maintenance of Lgr5 positive stem cells and intestinal homeostasis.

Isolated single Lgr5 stem cells have the capacity to form mini-organs (so called organoids) ex vivo. These cultured cells multiply indefinitely and form spheres with a central

lumen and protruding buds (94). These buds contain all differentiated epithelial cell types and display a crypt like morphology. The integrity of the stem cells in this ex vivo culturing system is dependent upon Wnt ligands as the addition of a Porcn inhibitor abolishes the renewal of Lgr5+ cells (95). In this regard, these cultured organoids are faithful models of gut regeneration in vivo.

To investigate the cellular origin of intestinal cancer Barker et al deleted APC in Lgr5 positive stem cells, which resulted in rapid transformation of the gut epithelium (96). The transformed cells remained at the bottom of the crypts and gave rise to microadenoma with high levels of nuclear beta-catenin. Subsequently, these microadenoma developed into macroscopic adenomas throughout the small intestine and colon. However deletion of APC in transit-amplifying cells did not result in transformation suggesting that Lgr5 positive stem cells are likely a cell of origin in intestinal cancer (96). The shared role of Wnt pathway responses in tissue homeostasis and cancer indicating that Wnt pathways has overlapping roles in the maintenance of stem cell populations in both normal and pathological settings.

Domains, binding partners and other cellular functions of APC

The functional role of APC in the Wnt signaling pathway is well known, and it is widely accepted that inappropriate activation of Wnt pathway through loss of APC function contributes to the progression of colon cancers (14,97,98). However, APC participates in several cellular processes including cell migration, cell adhesion, spindle assembly, cell cycle control and chromosome segregation. Thus, other functions of APC may have a role in CRC pathogenesis. I will briefly discuss protein domains found in APC, the binding partners of APC and cellular processes associated with these interactions (Figure 1.3).

Human APC encodes a 312-kilodalton protein consisting of 2843 amino acids. The sequence of APC proteins is highly conserved in *Drosophila*, C. elegans, *Xenopus*, zebrafish and mammals. *Drosophila*, mouse, and humans also harbor a second APC gene that is predicted to encode a shorter protein (99). These two paralogs differ in their expression patterns: while APC is ubiquitously expressed, APC2 is primarily expressed in the nervous system. Whereas the role of APC2 protein has been studied in *Drosophila*, its role in cancer is unclear.

APC is a multi-domain protein with the ability to interact with a large number of proteins. APC has an oligomerization domain that mediates homo or hetero-dimerization. In CRC, truncated forms of APC retain the ability to dimerize with wild type APC. Therefore, it has been proposed that truncated APC has a dominant-negative function that supports tumor development. (100). This hypothesis was tested in mouse by overexpressing N-terminal region of APC in intestinal epithelium. This intervention did not lead to polyposis suggesting that the hypothesis does not likely explain the prevalence of these truncating mutations in intestinal tumorigenesis (101).

The armadillo repeat region of APC interacts with components of the Wnt signal transduction pathway (PP2A), the microtubule cytoskeleton (KAP3) and F-actin cytoskeleton (ASEF). APC protects beta-catenin from dephosphorylation by protein phosphatase 2A (PP2A), thereby enhancing beta-catenin phosphorylation and degradation (102). Complete loss of APC may lead to a decrease in beta-catenin phosphorylation and degradation by this mechanism. APC localizes in puncta at the plus end tips of microtubules (103,104). Kinesin superfamily-associated protein 3 (KAP3), a linker protein for motor kinesins, has a role in

transportation of APC to the microtubule ends (105). APC-stimulated guanine nucleotide exchange factor (ASEF) is a Rac-specific guanine nucleotide exchange factor, and its overexpression with APC promotes cell migration-related phenotypes like cell flattening, membrane ruffling and lamellipodia formation (106). Expression of truncated APC in cell culture activates ASEF while full length APC expression does not show this activity suggesting that truncated APC stimulate inappropriate cell migration (107).

APC contains three 15 amino acid repeats (15R) and seven 20 amino acid repeats (20R). These repeats bind to beta-catenin and mediate inhibitory activity of APC in Wnt signaling. Binding of cytoplasmic beta-catenin to the 20R is modulated by phosphorylation via GSK3-beta. 20R and 15R domains are essential for binding and degradation of beta-catenin. There are three SAMP repeats within the 20R domain of APC. SAMP repeats bind to destruction complex scaffold Axin and mediates beta-catenin Axin binding. A majority of germline and somatic mutations in APC lie in the first half of the coding region within the mutational cluster region (MCR). These mutations result in the expression of a truncated protein lacking most of the 20R beta-catenin binding domains and Axin binding domains (15). Thus, mutations in APC leads to inappropriate beta-catenin stabilization and Wnt target gene transcription.

APC has two nuclear localization signals (NLS), and several nuclear export signals (NES) at the N-terminus within the 20-amino-acid repeats of APC. Both types of signals are well conserved in metazoa. While NLS is essential for importing cytoplasmic beta-catenin to nucleus, NES sequences are critical for exporting nuclear beta-catenin into the cytoplasm (108). The absence of these NES sequences leads to nuclear beta-catenin accumulation and

subsequent activation of Wnt target genes (86). Loss of the NES may contribute to APC-associated tumorigenesis in CRC.

The basic domain consists of arginine, lysine, and proline residues. The basic domain of APC is essential for direct interaction with microtubules in epithelial cells and mediates tubulin polymerization by binding to unassembled tubulin and promoting the assembly of branched microtubule arrays (109,110). In cell culture, APC depletion leads to a decrease in overall microtubule stability and loss of polarization of acetylated microtubules towards the leading edge suggesting that cells can not migrate in a guided fashion (111). This observation is supported by *in vivo* data; differentiating epithelial cells in mouse intestine migrate upwards from the crypts and truncation of APC leads to a decrease in upward movement (86). This migration defect may be acquired due to loss of APC basic domain or loss of association with binding partners that mediate microtubule attachment. The cytoskeletal interaction of APC can also contribute to the function of the microtubule-based mitotic spindle. This hypothesis is supported by localization of APC to kinetochores and centrosomes (112). Loss of APC leads to defects in spindle alignment, chromosome segregation defects and aneuploidy (113). Hence, APC loss cause changes in cytoskeletal regulation that affects microtubules and cytoskeleton thus leading defects in directed cell migration and polarity of the cell (111,113,114).

The C-terminal region of APC may play a role in cell cycle progression or growth control through binding to at three different proteins; end-binding protein 1 (EB1), Drosophila disc large tumor suppressor gene (DLG), and Protein tyrosine phosphatase (PTP-BL) (115). EB1 is a tubulin binding protein, and it is also associated with the microtubule cytoskeleton of the

mitotic spindle where it is important for spindle assembly throughout the cell cycle. DLG is involved in the maintenance of cell polarity, migration and mitotic spindle orientation in epithelial cells (116). PTP-BL and APC interaction may indirectly modulate the steady-state levels of tyrosine phosphorylation of APC-associated protein like beta-catenin. In CRC, mutated APC proteins lack C-terminal tail as well as SAMP motifs and most of 20R. In order to investigate contribution of C-terminal region in intestinal tumorigenesis, researchers knocked-out just the C-terminal region of APC protein but keep the 20R and SAMP domain intact. Truncation of the C-terminal part of APC did not result in a polyposis phenotype suggesting that APC's association with DLG, EB1, and PTP-BL is not essential for intestinal tumorigenesis (117).

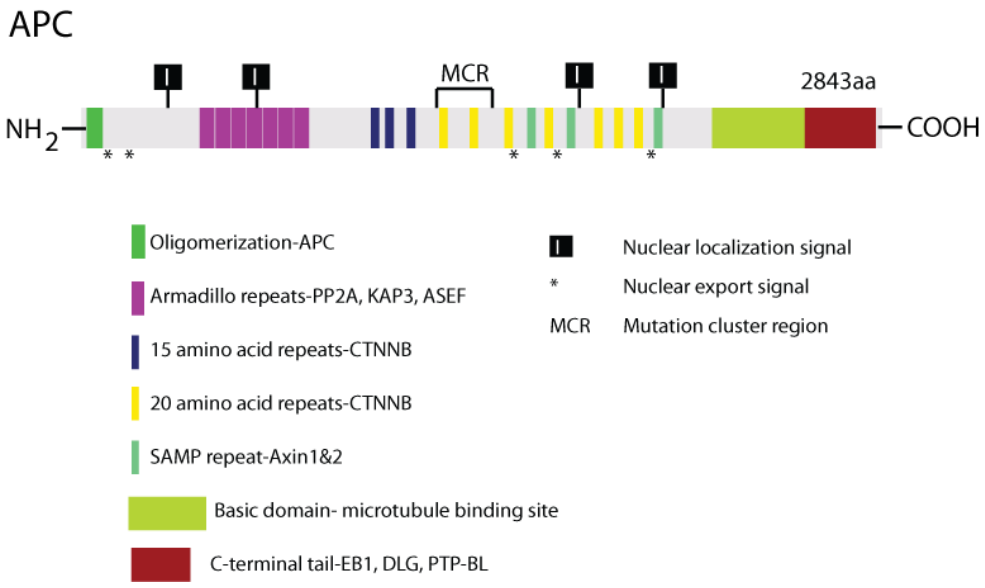


Figure 1.3 APC protein domains and binding partners Domains of APC and binding partners are shown. Binding partners are protein phosphatase 2A (PP2A), kinesin superfamily-associated protein 3 (KAP3), APC-stimulated guanine nucleotide exchange factor (ASEF), beta-catenin (CTNNB1), end-binding protein 1 (EB1), *Drosophila* disc large tumor suppressor gene (DLG), and protein tyrosine phosphatase (PTP-BL). Most of the APC mutations lie in mutation cluster region (adapted from McCartney and Nathke).

Drugging the Wnt pathway

Wnt pathway is crucial to many aspects of embryonic development and homeostasis in almost every adult tissue. During early vertebrate embryonic development Wnt signaling guides gastrulation, establishment of the anterior-posterior axis, and convergent extension movements (118-120). Wnt signaling is also essential for proper formation of several organ systems including the gut, skin, heart, mammary gland, brain, eye, bone, cartilage, spinal cord, lung, teeth, liver, kidney, pancreas and the hematopoietic system (97,121,122). Furthermore, Wnt signaling governs cell proliferation and differentiation in the adults especially in self-renewing tissues such as hair, skin, intestinal homeostasis and hematopoietic system (123-125). It is also involved in pulmonary and hepatic repair after injury (126-128). Given the significant role of Wnt signaling in the processes mentioned, chemical tools that enable temporal control of Wnt signaling could be useful for basic studies focused on its role in many aspects of physiology and for tissue engineering agendas.

In tissues where Wnt signaling balances the fine equilibrium between proliferation and differentiation, exploitation of these signaling pathways may lead to a variety of pathologies including carcinogenesis. Aberrant Wnt signaling has been associated with fibrosis, metabolic disease and neurodegenerative disorders (129-131). Furthermore, Wnt pathway activation is associated with a multitude of tumors including CRC. Despite a general convergence of oncogenic processes upon Wnt pathway activation, the prevalence of genetic mutations that give rise to changes to pathway misactivation differs from one tissue to another. For example, oncogenic beta-catenin mutations are observed with a frequency of 31% in endometrial cancers and 5% in medulloblastoma, 8 % in melanoma, 5% in CRC and

3% of lung adenocarcinomas (17,23,132-135). Loss of activity mutations in the Wnt inhibitor Axin1 is detected in 38% of biliary tract tumors and 11% of hepatic cancers but is not frequent in other cancers (136). Thus, inhibitors that directly target the beta-catenin/TCF transcriptional complex would presumably benefit a significant number of cancer patients.

Given the nearly universal participation of Wnt signaling in tissue homeostasis and the diseases described above, tremendous effort have been invested in gaining control of this pathway. Several screens by many independent groups have yielded a portfolio of agents that target different underlying mechanisms of signal transduction in the Wnt pathway (137-140). A small number of druggable Wnt pathway components have been identified so far. At the same time, the mechanism of action for many chemicals with on-target Wnt pathway inhibitory activity remains unknown.

In 2009, Chen et al identified two classes of compounds that target distinct Wnt-pathway components (138). The first class inhibits the membrane-bound O-acyl-transferase Porcn enzyme (termed IWP compounds), thus disrupting fatty acylation and activity of Wnt proteins. The second class inhibits Wnt/beta-catenin pathway response (named IWR compounds) by stabilizing Axin proteins, thereby blocking accumulation of beta-catenin protein that results from either ligand-dependent pathway activation, or loss of the APC tumor suppressor function. Specifically, the IWR compounds as well as another Wnt pathway inhibitor described six months after the IWR compounds, XAV939 attack the Tankyrase (Tnks) enzymes, two members of the poly-ADP-ribose polymerase (PARP) gene family that regulate Axin protein stability (139). In the following five years several different groups developed chemically diverse Porcn and Tnks inhibitors suggesting that identification

of a pathway specific druggable note was the rate limiting process for drug discovery efforts (141-145).

Mutations in different genes can cause aberrant Wnt pathway activation in different cancerous contexts. For example deviant Wnt pathway activation in CRC is mostly caused by APC mutations while in endometrial cancer this activation is observed due to activating beta-catenin mutations (17,135). Therefore, inhibiting the pathway activity at the transcription level by blocking beta-catenin/TCF-LEF1 or other transcriptional co-activators represents a point of attack, which cannot by bypassed by cellular compensatory mechanisms. Several different groups conducted *in vitro, in vivo* and *in silico* screens to identify molecules that can disrupt interaction between beta-catenin/TCF-LEF1 transcription factors (137,146-149). The molecular targets of these chemicals have not been identified (137,146-149). Small molecules that prevent interaction of beta-catenin with transcriptional co-activator proteins like B-cell lymphoma 9 (BCL9) and CREB binding protein (CBP) have also been identified. The use of small-molecule inhibitors that target a co-activator protein with multiple partners such as BCL9 and CBP can raise concerns about specificity of this approach. Despite these concerns, a small molecule (PRI-724) that prevent interaction of beta-catenin with transcriptional co-activator protein CREB binding protein (CBP) recently entered clinical testing (140). Results of this clinical testing can be used to infer the utility of targeting transcriptional co-activators in other disease scenarios.

Targeting the interaction between the Fzd receptors and their cytoplasmic signaling molecules, the Dvl proteins, has also been attempted as a means for disrupting Wnt-dependent signaling. Here, screens monitoring direct protein–protein interactions and in

silico methodology led to the identification of several compounds with high half maximal inhibitory concentrations (150-152). Given the challenges associated with achieving greater potency and specificity of the lead compounds netted from these efforts, the overall strategy has not yielded clinical candidates thus far.

A number of small molecules developed for the management of non-Wnt associated diseases have also been found to exhibit activity within the Wnt pathway. For example, lithium chloride, a drug to treat bipolar disorders for decades, inhibits the constitutively active kinase GSK-3beta, thereby stabilizing beta-catenin and activating WNT signaling (153). Non-steroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase 2 (COX2) inhibitors can inhibit beta-catenin dependent transcription in colorectal cancer and reduce adenoma formation in patients with FAP (154-159). The clinical data gained via using non-specific Wnt pathway inhibitory agents can provide insights for utilizing specific Wnt pathway inhibitors.

The Role of Tnks in the Wnt Pathway

Molecular insight into the functional role of Tnks in Wnt pathway emerged from two high throughput screens intended to discover chemicals that interfered with Wnt-stimulated transcription (138,139). The hits from these two independent screens, IWRs and XAV939 blocked Wnt-stimulated accumulation of beta-catenin by increasing the levels of the Axin1 and Axin2 proteins. Subsequent work established that these Wnt pathway inhibitors regulate Axin levels via inhibition of Tnks 1&2, both members of the poly-ADP-ribose polymerase (PARP) protein family. Tnks proteins bind directly to Axin proteins and regulate Axin levels via poly-ADP-ribosylation (PARsylation) and ubiquitination.

In the absence of Wnt protein, a cytoplasmic complex composed of Axin, APC, CKI and GSK3-beta binds to and phosphorylates the transcriptional co-activator beta-catenin, thus marking it for proteasome-mediated degradation. Axin proteins are thought to be rate limiting for destruction complex activity, as their cellular levels are low compared to other complex components (60,61). Tnks proteins PARsylate Axin proteins that are subsequently recognized by an E3 ubiquitin ligase called ring finger protein 146 (RNF146). RNF146 ubiquitylates PARSylated Axin and targets Axin for destruction by the proteasome (160). Inhibition of Tnks abrogates Wnt pathway activity by stabilizing Axin proteins, thereby blocking accumulation of beta-catenin protein that results from either ligand-dependent pathway activation, or loss of the APC tumor suppressor function.

Other Cellular Functions of Tnks

Tankyrase 1 and 2 are homologous multi-domain proteins that contain 20 ankyrin repeats arranged in 5 ankyrin repeat clusters (ARC), a sterile alpha motif (SAM) domain, and a PARP domain (161). PARP domain utilizes NAD (+) to catalyze the addition of poly-ADP-ribose chains onto acceptor proteins. This modification, PARsylation, confers a large negative charge to the modified protein and consequently alters protein function and interactions (162). The ankyrin repeats and SAM domains both mediate protein-protein interactions. The SAM domain enables Tnks proteins to form polymers. The polymerization is disrupted by auto-PARsylation, suggesting that Tnks can act as a scaffolding molecule according to its PARsylation status (163). As Tnks recognize a degenerate hexapeptide motif through their ankyrin repeats clusters, and they interact with a variety of regulatory proteins and substrates like Axin, telomeric repeat binding factor-1 (Trf1), nuclear mitotic apparatus

protein NuMA, insulin-responsive aminopeptidase (IRAP), myeloid cell leukemia 1 (Mcl1) and 3BP2 (139,163-166).

Binding partners of Tnks determine the major roles and cellular localization of these enzymes. Some of the cellular processes linked to Tnks function include Wnt signaling, telomere length maintenance, mitosis and vesicle transport associated with glucose metabolism. Tankyrase-mediated PARsylation of telomere protecting protein complex component TRF1 targets it for degradation, and thus leads to telomere elongation by enabling telomerase to access the telomere ends (167). During mitosis Tnks localizes to mitotic spindle poles, where PARsylation of microtubule organizing protein NUMA and other mitotic proteins is required for normal spindle formation and sister chromatid segregation (168). Tnks regulates insulin mediated glucose transporter 4 (GLUT4) vesicle localization to cell membrane probably through PARsylation of another vesicle resident protein IRAP (169).

Clinical testing of LGK-974, an agent targeting the Wnt acyl-transferase Porcupine

Achieving chemical control of Wnt pathway responses is a shared goal of both regenerative medicine and anti-cancer efforts. In 2012 one of the first small molecules targeting Wnt pathway called LGK-974 entered a phase I clinical trials with the ultimate goal of ameliorating the ill effects stemming from a number of Wnt-associated maladies. LGK-974 targets the membrane bound O-acyl-transferase, Porcn thus inhibits of Wnt palmitoleoylation, secretion and activity (145). Porcn was identified as the most druggable protein for Wnt pathway from a chemical screen using cultured cells that report cell autonomous Wnt signaling just three years earlier before the clinical trials (138).

Identification of this Porcn as a molecular target is success for governmental drug identification efforts that elevated by subsequent industrial investment.

Although Porcn is a family member of several related MBOAT enzymes, functional studies revealed no substrate overlap between Porcn and other MBOATs suggesting that Porcn acts as a Wnt pathway dedicated acyl-transferase (170,171). In humans, loss of function mutations in Porcn is associated with a X-linked dominant disorder called focal dermal hypoplasia (172). Similar to observations in humans, loss of Porcn in mice leads to cell-autonomous defect in Wnt ligand secretion and mesoderm generation failure, a phenotype associated with loss of Wnt signaling (173,174). These observations confirm a conserved role of Porcn in Wnt signaling across metazoa.

Therapeutic agents that target crucial developmental signaling pathways like Wnt, Hedgehog or Notch may have devastating effects on embryonic patterning. For example, the naturally occurring Hedgehog inhibitor cyclopamine was discovered because of its cyclopia inducing effect in lambs (175). However, these developmental signaling pathways are also essential in the regulation of stem cell and progenitor cell proliferation and differentiation in adult tissues (176). Given the role of Wnt pathway in maintenance of fast renewing tissues like intestine, potential adverse effects in the gut was expected (140,177). Nevertheless, Porcn inhibitors dosed in mice were well tolerated at concentrations sufficient for inducing anti-cancer activity. In particular, the absence of gut toxicity suggested such compounds could be used in humans for anti-cancer agendas (145).

Aberrant Wnt pathway activity in cancer most frequently arises from mutational defects in cytoplasmic regulators of Wnt signaling such as APC and beta-catenin (17). Despite

intense research, targeting Wnt signaling in cancers harboring downstream pathway mutations remains challenging because of the lack of tractable targets. Nevertheless a small group of cancers may be sensitive to inhibitors of Wnt ligand production. Indeed, Porcn inhibitors are now being tested in phase one trials in patients with these tumors. One example of such a tumor type is cystic pancreatic cancer, which is associated with mutations in RNF43, a transmembrane ubiquitin ligase that promotes the turnover of Fzd receptors (145,178,179).

Biologics vs. small molecules: comparison of Wnt pathway inhibitors

Small molecules represent over 90% of the drugs on the market today. Small molecules have several advantages over biologics: they can be synthesized chemically in large quantities, often have little batch-to-batch variation, can be cheaply produced and oftentimes can be orally delivered. By contrast, biologics are therapeutic proteins; they are produced in biotechnological processes via genetically modified cells of microorganisms such as bacteria, yeasts or in mammalian cell lines. Biologics may have limited tissue penetration and longer half-lives compared to small molecules. Furthermore biologics typically must be administered by injection or infusion. The additional costs associated with their production and the challenges associated with patient self-administration result in increased costs and reduced compliance for the patient.

Three Wnt pathway inhibitory molecules that inhibits Wnt signaling pathway at the receptor/ligand level are in clinical trials. These molecules are a Porcn inhibitor called LGK-974 and two biologics called OMP-18R5 and OMP-54F28. OMP-18R5 is a humanized monoclonal antibody that binds to extracellular domain of 5 Fzd family members. The

second agent, OMP-54F28 is a fusion protein comprised of the cysteine-rich domain of frizzled family receptor 8 (Fzd8) fused to the human immunoglobulin Fc domain. OMP-18R5 and OMP-54F28 target a subgroup of Fzd receptors while LGK-974 inhibits all Wnt ligand dependent activities. The near simultaneous initiation of clinical testing of these agents with similar modes of action (targeting ligand-dependent Wnt signaling) affords a unique opportunity to compare first hand the strengths and weaknesses of small molecule and biologics as anti-cancer agents.

CHAPTER TWO
Chemically targeting TNKS

Introduction

Despite the nearly universal acceptance that loss of the adenomatous polyposis coli (APC) tumor suppressor is the most common initiating event in colorectal cancer, no clinical candidates have emerged for countering the deleterious effects that stem from this phenomenon. A mechanistic model of disease premised upon compromised transcriptional regulation as a consequence of *APC* loss forms the basis of a major drug discovery front (176). APC scaffolds a destruction complex that promotes turnover of the transcriptional co-activator beta-catenin. Beta-catenin-induced activation of TCF/LEF DNA binding proteins as a consequence of compromised APC activity results in deviant transcription of growth-promoting genes such as *Myc*.

From several independent chemical-screening efforts with the shared intent to counteract compromised *APC* activity, the Tnks enzymes (Tnks1 and 2) have emerged as highly druggable regulators of beta-catenin abundance (138,139,180). Tnks inhibitors induce stabilization Axin proteins, thereby blocking accumulation of beta-catenin protein that results from either ligand-dependent pathway activation, or loss of the APC tumor suppressor function. Here, I have identified at least three distinct groups of Tnks inhibitors based on their mode of target engagement from a portfolio of Wnt/beta-catenin pathway inhibitors with ill defined mechanisms-of-action. These three groups of inhibitors target Tnks *in vitro* and in cultured cells that exhibit deviant Wnt signaling due to compromised APC activity.

Using a panel of recombinant PARP family members, two of these compounds emerged as being highly selective for Tnks enzymes (IWR-1 and IWR-8). Moreover, one of these molecules (IWR-8) represents a chemotype that is amenable to a variety of chemical modifications thus making it a viable starting point for drug development purposes.

Diverse pharmacophores supporting Tnks inhibition identified from a portfolio of Wnt pathway inhibitors

From screening a 200K small molecule library in cells with autonomous Wnt signaling, we previously identified ~60 compounds that disrupted cellular response to Wnt ligands (termed Inhibitors of Wnt Response or IWR compounds) (138). The identification of the Tnks enzymes as the target of two IWR compounds (IWR-1 and -2) suggested that other IWR compounds also disable Wnt signaling by the same mechanism (78,181). Inhibition of Tnks-mediated auto-PARsylation, a biochemical trigger for its proteasome-mediated destruction, results in accumulation of Tnks in cultured cells thus affording a straightforward screen for potentially novel Tnks inhibitors (Figure 2.1) (139). The loss of beta-catenin and accumulation of Axin1&2 proteins additionally serves as a useful marker of chemically induced Tnks inhibition. After subjecting all of the IWR compounds to these biochemical tests for Tnks inhibitory activity in cultured cells, we uncovered six additional Tnks antagonists (IWR-3 to -8; Figure 2.2). Thus, a total of four chemotypes supporting Tnks inhibition emerged from our original Wnt pathway antagonist screen (Figure 2.2). We note that IWR-6 and -7 resemble the previously identified Tnks inhibitor XAV-939 whereas IWR-8 represents a novel chemotype with such activity.

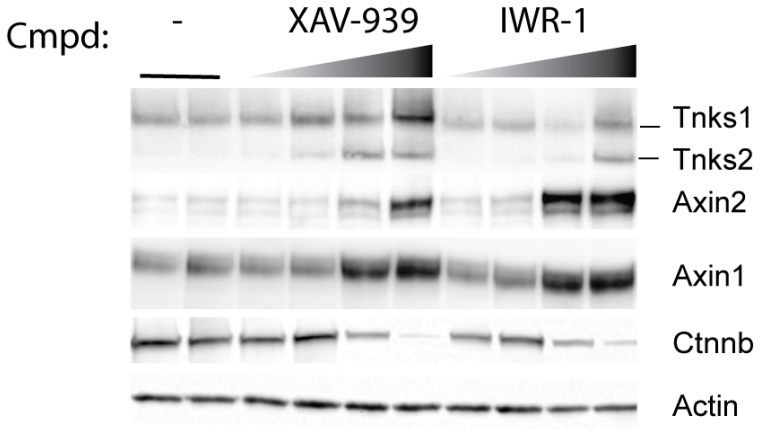


Figure 2.1 Biochemical changes with Tnks inhibition reveals a screening method for Tnks inhibitors. Biochemical markers of Tnks inhibition associated with the Wnt/beta-catenin pathway. The Tnks inhibitors XAV-939 and IWR-1 induce stabilization of both Tnks enzymes and Axin proteins but promote destruction of beta-catenin (Ctnnb1).

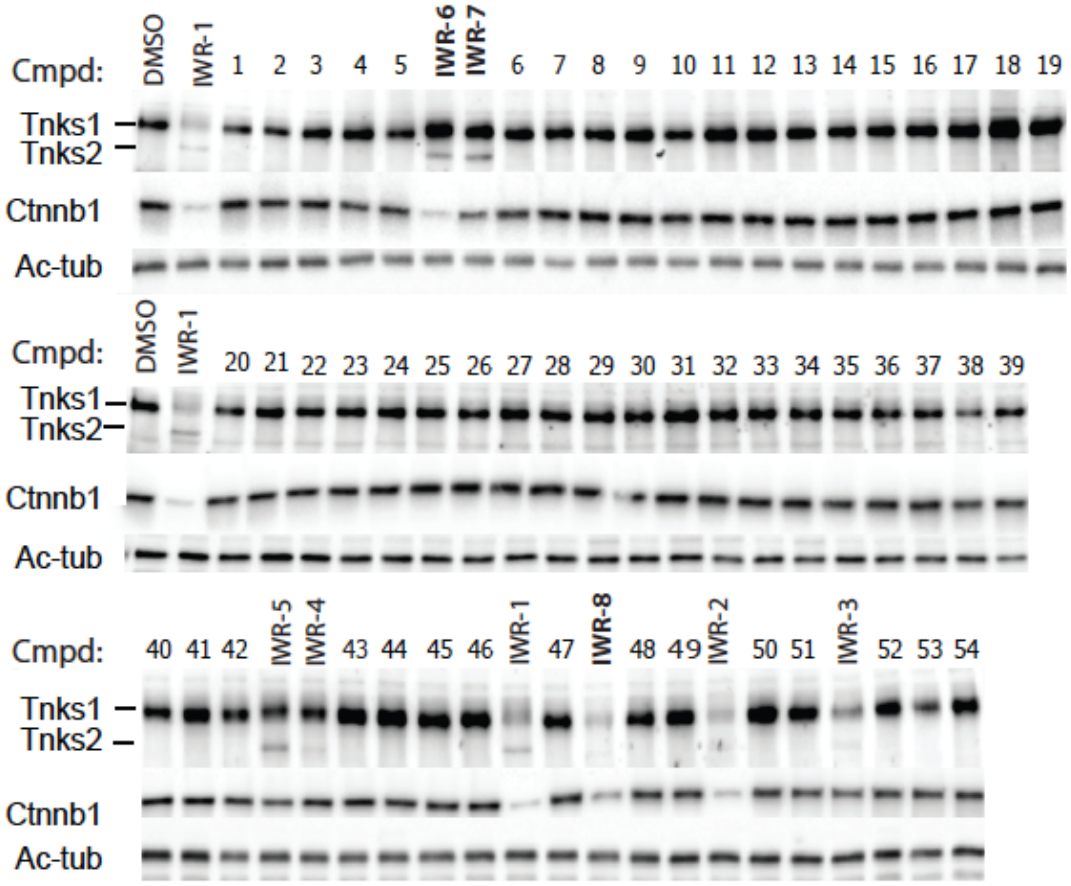


Figure 2.2 Identification of additional Tnks inhibitors from the IWR collection of Wnt pathway inhibitors. A biochemical screen to identify additional Tnks inhibitors from the IWR collection of Wnt pathway inhibitors. The effects of the majority of IWR compounds on Tnks 1 and 2, beta-catenin, and acetylated tubulin (Ac-Tub) expression levels were determined in SW-480 cells by Western blot analysis. Tnks inhibitors prevent destruction of Tnks enzymes while promoting beta-catenin turnover. IWR-1 to -2 are known Tnks inhibitors whereas IWR-3 to -5 were previously shown to induce beta-catenin destruction. Novel Tnks inhibitors identified by this screen were re-named IWR-6 to -8 (in bold).

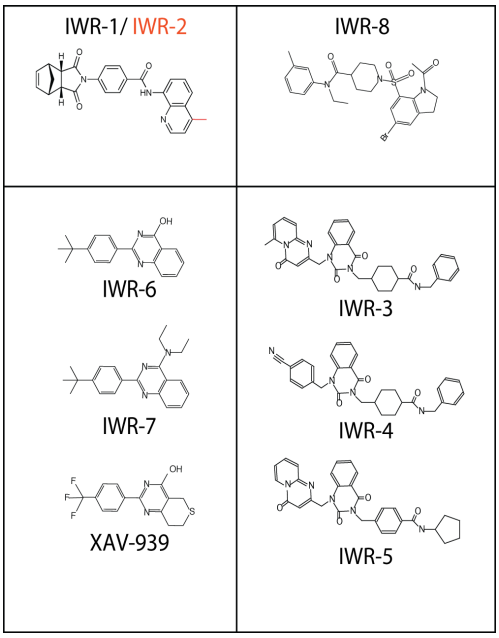


Figure 2.3 Chemotype-based grouping of potential Tnks inhibitors identified from the IWR collection. IWR-6 and -7 are similar to XAV-939. IWR-8 is a novel chemical scaffold. Thus, despite the absence of resemblance to other previously described Tnks inhibitors.

We confirmed that molecules representative of each chemotype found in this chemical set were able to directly target Tnks using a recombinant Tnks protein assay that utilizes histone PARsylation as a read out (Figure 2.4a). Given the intense interest in developing Tnks inhibitors as anti-cancer drugs, we reasoned that the novel chemical scaffold supporting Tnks inhibition revealed by IWR-8 might provide new opportunities for achieving a drug-like molecule. Indeed, similar to other established Tnks inhibitors IWR-8 disrupted deviant Wnt signaling in cells lacking normal APC activity in a dose-dependent fashion (Figure 2.4b). IWR-8 exhibits both in vitro and in vivo activities associated with a bona fide Tnks inhibitor.

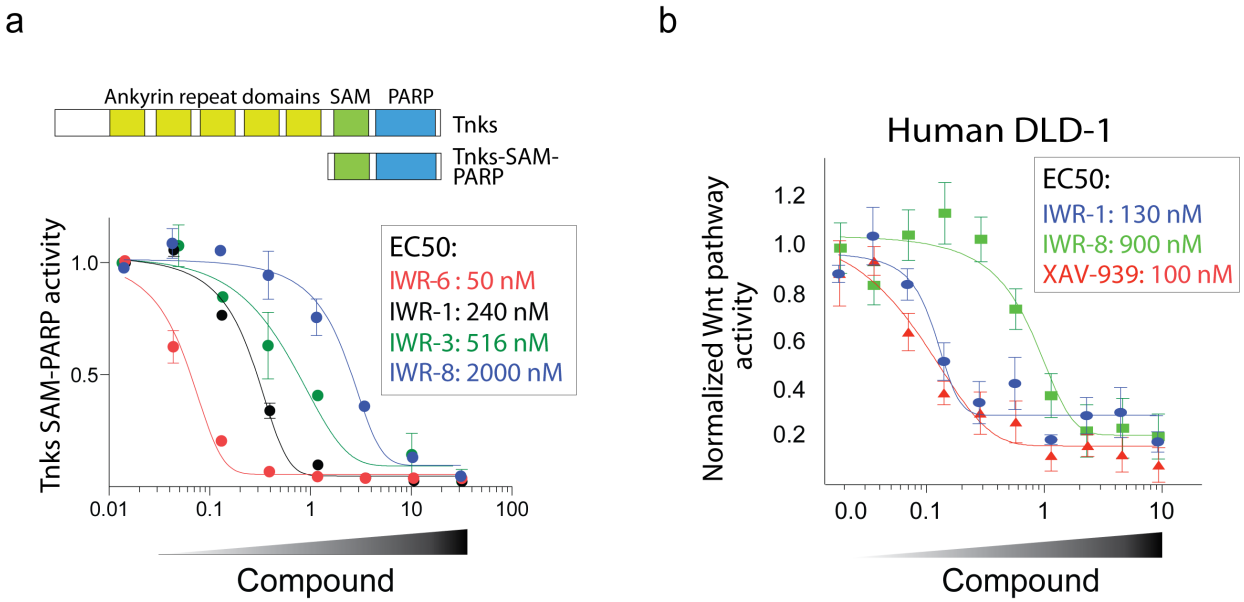


Figure 2.4 The novel Tnks inhibitor chemotype IWR-8 directly inhibits Tnks enzymes and disrupts b-catenin-dependent transcriptional activity induced by loss of APC function. (a) IWR-3, -6 and -8 directly target Tnks. Representative IWRs from each chemotype were evaluated for their ability to inhibit purified recombinant Tnks1 protein (Tnks1 SAM-PARP protein). Parsylation of immobilized histone protein was determined by colorimetric detection of incorporated biotinylated NAD+ in 96 well format. (b) IWR-8 inhibits b-catenin activity induced by loss of APC function. EC50 of IWR-8 as well as IWR-1 and XAV-939 were measured in DLD-1 cells using a Wnt/b-catenin specific luciferase reporter.

Crystal Structures Of Structurally Diverse Tnks Inhibitors

Tnks enzymes accommodate their ADP-ribose donor substrate, nicotinamide adenine dinucleotide (NAD+), in two sub-pockets – one binds the nicotinamide (NI) and another the adenosine (AD) moiety. IWR-1 was previously shown to be a first in class inhibitor that targets the AD-binding pocket rather than the NI binding pocket (182). We selected representative compounds from each group and determined their mode of attack using structural analyses. First, we confirmed observations that IWR-1 engages the AD-binding pocket unlike prototypical PARP inhibitors represented here by the NI-binding pocket inhibitor XAV-939 (Figure 2.5a, Table 1). IWR-2, a derivative of IWR-1, also binds Tnks1 in this mode (183). Despite their chemical dissimilarity, IWR-8 like IWR-1 exploits a histidine uniquely found in the D-loop of Tnks enzymes (His1201 in Tnks1; His1048 in Tnks2) to engage the AD pocket while concomitantly inducing a change in the D-loop conformation (Figure 2.5b). Instead of a quinolone group found in IWR-1 that mediates aromatic stacking with His1201, IWR-8 achieves a similar chemical protein interaction using an indoline group. We noted from the crystallographic and NMR-based evidence that the structure of IWR-8 is actually a regioisomer of the structure that was described in the first report of its synthesis (See Chapter 5 Figure 5.1). Perhaps not surprising given its size, IWR-3 engages both NI- and AD-binding pockets (Figure 2.5c). In the end, these Tnks antagonists can be organized based on their pocket binding preference (Figure 2.5d).

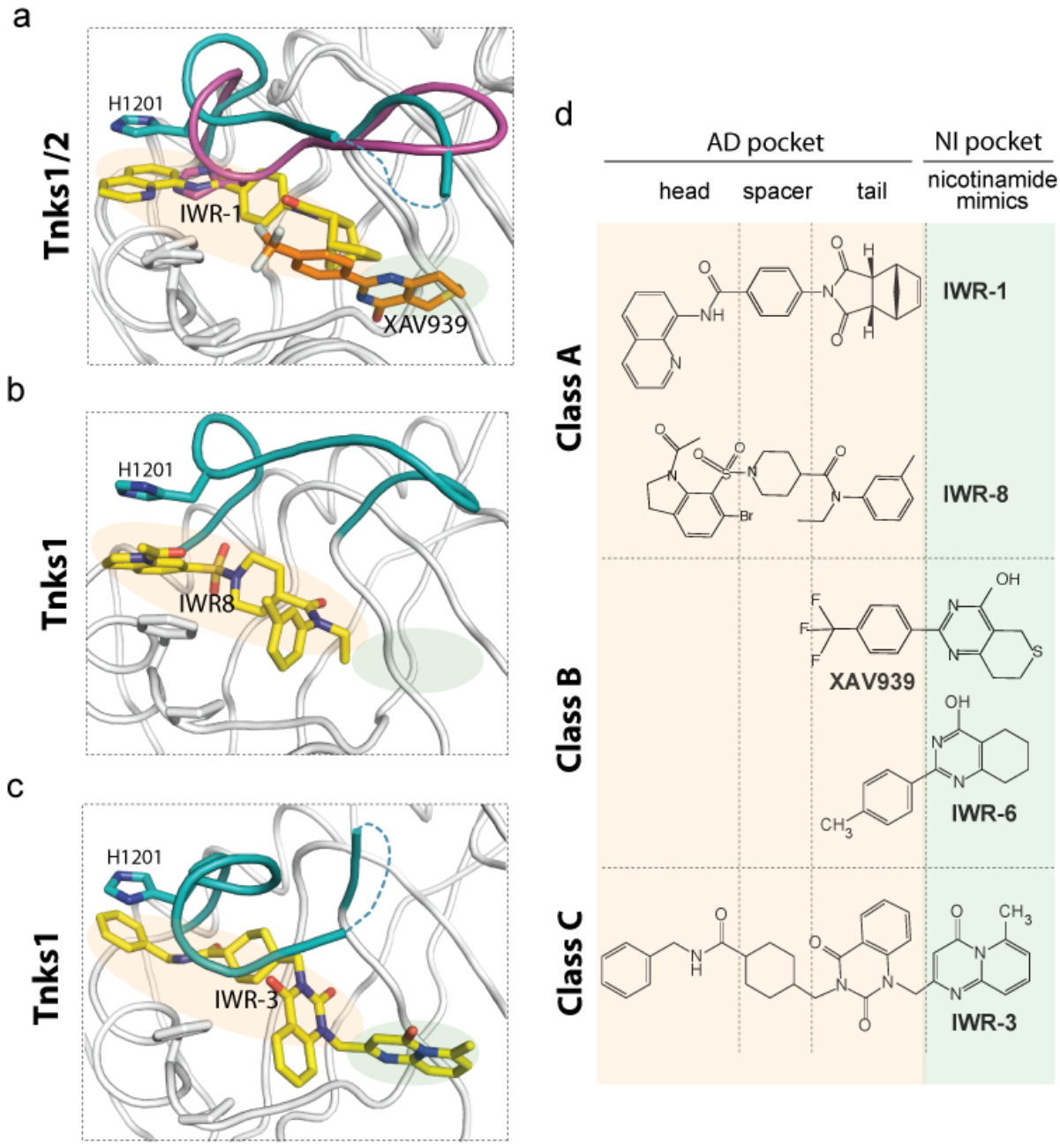

a
Tnks1/2
H1201
IWR-1
XAV939
b
Tnks1
H1201
IWR8
c
Tnks1
H1201
IWR-3
d
AD pocket
NI pocket
head
spacer
tail
nicotinamide mimics
Class A
IWR-1
IWR-8
Class B
XAV939
IWR-6
Class C
IWR-3

Figure 2.5 Crystallographic studies reveal distinct modes of chemical attack by members of the IWR collection of Tnks inhibitors. (a) Crystal structure of IWR-1/Tnks1. The structure of XAV-939, a prototypical PARP inhibitor, bound to Tnks2 (PDB ID: 3KR8) is superimposed for comparison with respect to chemical binding pocket preference and ability to induce conformational change in the D-loop. (b) Crystal structure of IWR-8/Tnks1. (c) Crystal structure of IWR-3/Tnks1. The D-loop of Tnks1 (1196-1211) or Tnks2 (1043-1058) is shown in turquoise and cyan, respectively. His1201 that interacts with all three IWR compounds and facilitates chemical-specific D-loop conformational changes is highlighted. Adenosine (AD) and nicotinamide (NI) binding sites are shown in pink and green, respectively. (d) Summary of binding pocket preference of IWR compounds that target Tnks enzymes. Given the structural similarity of IWR-6 and -7 to XAV-939, these novel molecules likely engage the NI pocket of Tnks. (Panel by H. Chen& H. He& D. Borek& Z.Otwinowski& X. Zhang)

Data collection	TNKS1/IWR-1	TNKS1/IWR-3	TNKS1/IWR-8
Space group	$P6_2$	$P\ 2_12_12_1$	$P\ 6_2$
Cell dimensions			
a, *b*, *c* (Å)	107.94, 107.94, 121.92	48.20, 81.17, 114.16	108.38, 108.38, 122.22
α, β, γ (°)	90, 90, 120	90, 90, 90	90, 90, 120
Resolution (Å)	50.0-2.3(2.34-2.30)*	31.0-1.8(1.87-1.80)	37.4-1.5(1.55-1.50)
R_{sym}	11.2(51.3)	7.3(>100)	8.3(95.1)
$I/\sigma I$	15.7(2.4)	38.8(1.5)	26.3(1.3)
Completeness (%)	99.7 (94.7)	99.3 (94.5)	99.1 (92.0)
Redundancy	7.3 (5.2)	7.0 (6.2)	6.2(3.0)
$CC_{1/2}$ at the highest resolution shell	0.86	0.84	0.60
Refinement			
Resolution (Å)	2.30	1.80	1.50
No. reflections	34662	41860	128406
Completeness (%)	97	99	99
R_{work}/ R_{free} (%)	22.6/28.3	18.1/21.1	17.7/20.7
No. atoms	7048	3522	7804
Protein	6568	3262	6820
Ligand/ion	128	44	163
Water	352	216	821
B-factors			
Protein	43.7	45.7	22.0
Ligand/ion	40.6	37.3	26.2
Water	39.5	49.9	33.7
R.m.s deviations			
Bond lengths (Å)	0.004	0.007	0.007
Bond angles (°)	0.77	1.01	1.08
Ramanchandran plot			
Favored (%)	98.2	99.0	99.0
Allowed (%)	1.8	1.0	1.0
Disallowed (%)	0	0	0

*Highest resolution shell is shown in parenthesis.

Table 1. Data collection and refinement statistics for IWR compound/Tnks1 crystal structure. (Panel by H. Chen& H. He& D. Borek& Z.Otwinowski& X. Zhang)

AD-binding pocket targeted compounds exhibit high specificity for Tnks1&2

The higher level of conservation between the NI-binding pocket in PARPs as compared to the AD-binding pocket likely imposes greater challenges to achieving specificity with NI-binding pocket inhibitors (184). Indeed the ~100-fold greater specificity of IWR-1 compared to XAV-939 for Tnks enzymes over several other PARPs has been attributed to their difference in their mode of attack (182,183). At the same time, the discovery of structurally distinct chemicals both capable of binding the AD-binding pocket in a similar fashion (IWR-1 and IWR-8) afforded an opportunity to evaluate the strength of this hypothesis. We profiled two AD-binding pocket compounds (IWR-1 and IWR-8), one NI-binding pocket compound (IWR-6), and one dual pocket inhibitor (IWR-3) against a panel of recombinant PARP proteins that covers 70% of the protein family (Table 2). In support of the hypothesis, the AD-binding chemicals exhibited greater specificity than the NI-binding chemical. Surprisingly, despite having to engage both pockets, IWR-3 exhibited poor selectivity. In the end, our overall effort to glean novel Tnks antagonists from our IWR chemical collection identified two highly specific Tnks inhibitors – IWR-1 and IWR-8 – with on-target activities confirmed using biochemical, structural, and cell biological analyses described in chapter three.

	% inhibition				
	AD pocket inhibitor		NI pocket inhibitor	Dual pocket inhibitor	Control
	IWR-1	IWR-8	IWR-6	IWR-3	Olaparib
PARP1	0	0	44	0	100
PARP2	5	3	71	27	100
PARP3	6	11	60	11	100
TNKS1	100	99	100	98	N/A
TNKS2	100	99	100	100	N/A
PARP6	0	0	0	6	100
PARP7	7	8	9	20	87
PARP8	1	0	0	21	96
PARP10	18	9	8	49	99
PARP11	2	12	32	17	96
PARP12	11	14	36	31	84
PARP15	0	0	0	28	82

Table 2. Specificity of novel IWR Tnks inhibitors. Indicated IWR compounds (10mM) and the control pan-PARP inhibitor Olaparib (20nM) were incubated with indicated recombinant proteins and parsylation of immobilized histone measured.

A potential role for an inducible adenosine-binding pockets in the regulation of Tnks activity

The Tnks enzymes regulate their own stability by auto-PARsylation. Biochemical evaluation of Tnks levels in cells treated with Tnks inhibitors revealed two outcomes that segregated based on their subpocket binding preference (Figure 2.1). AD-binding compounds such as IWR-1 and IWR-8 resulted in loss of Tnks protein while NI-binding compounds represented by IWR-3 and IWR-6 induced Tnks accumulation (Figure 2.2).

As discussed above, AD- but not NI-binders induce a conformational change in the Tnks D-loop that forms the "roof" of the adenosine-binding pocket. Could the flexibility observed in the D-loop in chemical studies support a physiologically relevant conformational change? Notably, a post-translation modification (phosphorylation of Tyr1203) maps to the D-loop sequence (185) (Figure 2.6). One possibility then is that phosphorylation of Tyr1203 locks

the D-loop into a conformation that makes the active site more accessible to substrate and NAD (+). At the same time, the proximity of the catalytic domains to the D-loop sequences revealed in a crystal structure that captured dimerized Tnks molecules could support a dimerization-dependent mechanism for controlling Tnks activity. Further biochemical studies focused on identifying the basis for AD-binding inhibitor-induced Tnks destruction should improve our ability to interrogate the potential functional relevance of the D-loop flexibility in Tnks molecules.

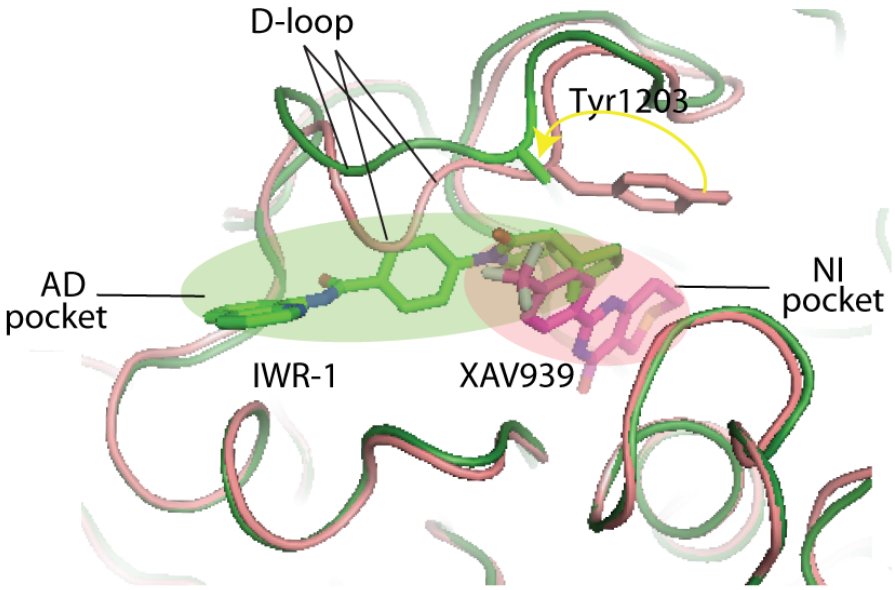


Figure 2.6 IWR-1 induced conformational changes generate variation in the protein surface exposure of the potential key Tyr residue. IWR-1 engages adenosine (AD)-binding pocket (green) and induces a conformational change in the D-loop. On the other hand, XAV-939 occupies the nicotinamide (NI)-binding pocket and does not induce structural changes in the target (pink). Yellow arrow indicates movement of Tyr1203 due to the IWR-1 induced conformational change.

Leveraging our understanding of Tnks inhibitors for targeting other PARPs

According to crystallographic evidence, His1201 in the Tnks D-loop is stacked with the quinolone and indoline group of IWR-1 and IWR-8, respectively (Figure 2.5). Sequence alignment of PARP D-loops indicated that a tyrosine residue in the D-loop of Parp9 and Parp14 aligns with the histidine used for IWR-1/IWR-8 stacking (Figure 2.7). Taken into consideration that Tyr and His could presumably a similar π− π stacking interaction with the aromatic rings of IWR-1and IWR-8, it is conceivable that IWR-1 and IWR-8 could also inhibit Parp9 and Parp14 activities. As a first step to evaluate this hypothesis, first we assessed the impact of IWR-1 on the total cellular levels of Parp9 and Parp14 with the logic that IWR-1 may directly alter their stability. Indeed, treatment of cells with IWR-1 resulted in the loss of Parp9 and Parp14 proteins suggesting that this compounds may be IWR-1 targets (Figure 2.8). Further studies are required for testing the direct effects of AD-binding Tnks inhibitors on Parp9 and Parp14 activity.

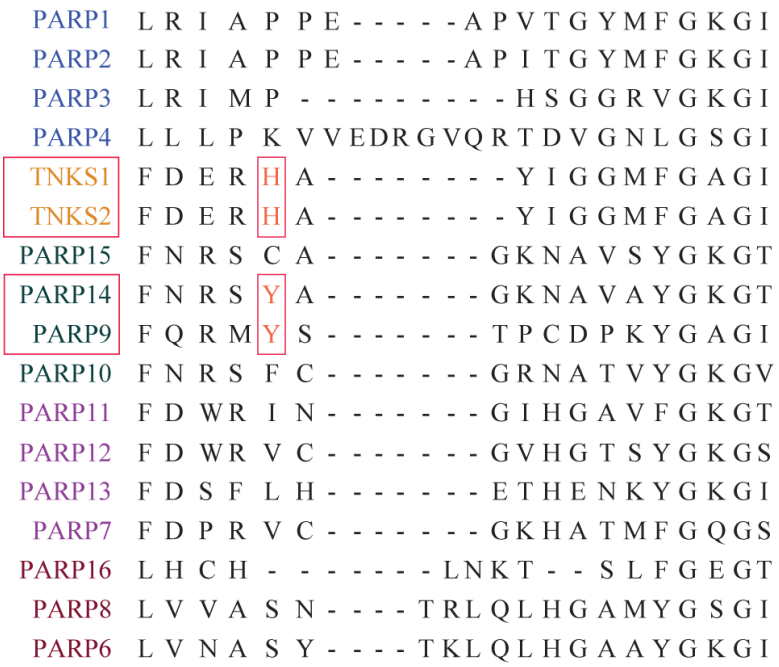


Figure 2.7 Alignment of D-loops of PARP family members indicated that AD-binding pocket Tnks inhibitors could inhibit Parp14. Color-coding indicates subgrouping according to catalytic domain sequence. Alignment data is modified from Otto, 2005 (186)

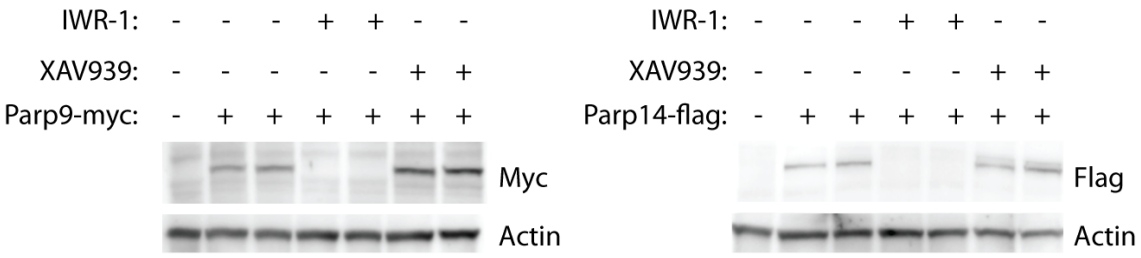


Figure 2.8 IWR-1 treatment induces loss of Parp14 and Parp9 protein suggesting that AD-binding pocket Tnks inhibitors may inhibit these enzymes. Cells overexpressing Parp9 and Parp14 were treated with either IWR-1 or XAV-939, and cellular protein levels evaluated by Western blot analysis. Whereas XAV-939 either increased or induced a slower-migrating form of Parp9 and Parp14 respectively, IWR-1 induced protein loss in both cases. Given the difference in action of these two classes of Tnks inhibitors, we assume that these observations are not a consequence of Tnks inhibition but rather direct protein-chemical interactions.

Conclusions & Future Directions

The IWP compounds that collectively disable Wnt protein production were netted from the same screen that identified the IWR compounds (138). Despite the structural diversity of the chemicals that constitute the IWP chemicals, these compounds nevertheless appear to converge on a single target - Porcn (187). Taken into consideration the absence of a universal target for the IWR compounds revealed here, our overall chemical effort to define Wnt pathway inhibitors suggests that Porcn is the most druggable entry point within this signaling system. At the same time, our findings suggest that mechanisms for disabling beta-catenin activity in addition to Tnks inhibition may be revealed by exploration of other IWR compounds. These additional compounds could form the basis for more universally specific probes of Wnt/beta-catenin pathway activity or for bypassing any potential detrimental effects associated with Tnks inhibition.

From mining a high confidence collection of small molecules that disrupt Wnt-mediated responses, we have identified additional Tnks inhibitors that together with previously discovered molecules delineate three strategies for disabling PARPs – targeting the ADP ribose- and nicotinamide-binding pockets alone, or simultaneously both pockets. The structures of the Tnks/IWR-1 and Tnks/IWR-8 complexes reveal a selective approach for inhibiting Tnks and other PARP family members through targeting the D-loop, which is diverse in sequence among the PARP family members.

CHAPTER THREE
Interrogation of Tnks activity in other cancer relevant pathways

Introduction

The discovery of Tnks antagonists emerged from efforts to counter the deleterious effects of compromised APC activity in CRC (188). In the absence of Tnks activity, a beta-catenin destruction complex scaffolded by the Axin proteins accumulates thus restoring the homeostatic rate of beta-catenin destruction in cells with altered Apc activity. Distinct from their utility in probing pathological signaling events in CRC, Tnks inhibitors have also benefited areas of research focused on Wnt signaling in embryonic development, tissue regeneration, and tumorigenesis at other organs (138,189-194).

Despite the abundance of evidence that disabling Tnks activity can achieve specific anti-Wnt/beta-catenin signaling effects (78,139,191,195), several other therapeutically relevant Tnks substrates have been identified suggesting that Tnks inhibitors may be useful in non-Wnt associated diseases (167,196-199). The telomeric repeat binding factor (Terf1, also known as Trf1) is essential to telomere integrity and is a well-established target of Tnks enzymatic activity (167). Conditional loss of TERF1 results in cellular senescence and telomeric fragility (200,201). The effects of chemically based inhibition of Tnks on telomere biology are not well understood and could reduce confidence in Tnks inhibitors as probes for Wnt/beta-catenin signaling. At the same time, such molecules may disrupt the immortalizing effects of deviant telomere-length maintenance in cancerous cells. The potential existence of reciprocally re-enforcing Wnt-telomere signals further suggests that Tnks inhibitors could

support a single agent strategy for disabling cancerous cells with stem-like characteristics (202).

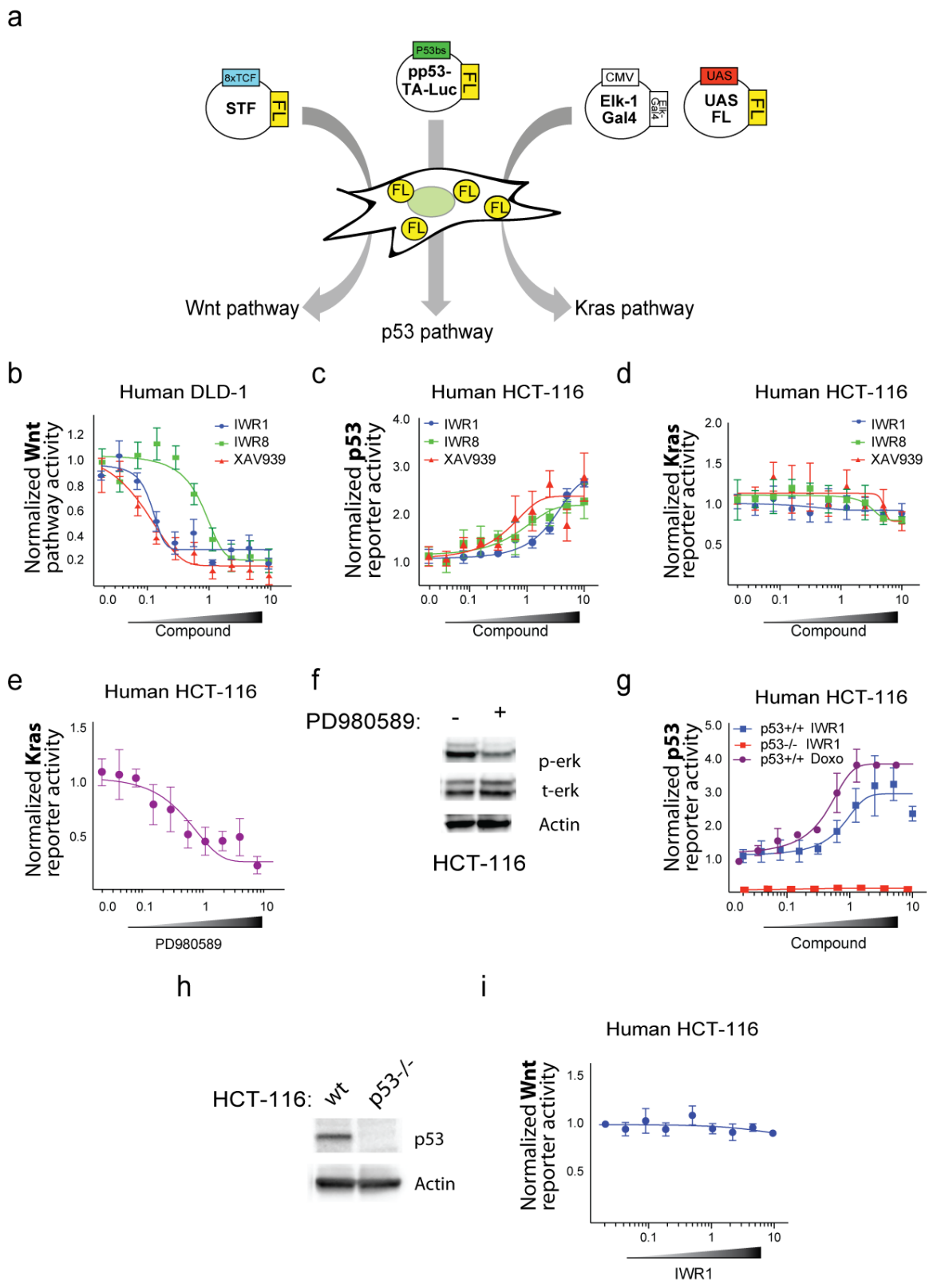
I utilized three distinct groups of Tnks inhibitors based on their mode of target engagement to define consensus effects of Tnks inhibition on three pathways frequently exploited in colorectal cancer – the Wnt, p53, and Kras pathways. In addition to inhibiting beta-catenin-associated transcriptional responses, all of these chemicals promoted p53 activity likely by perturbing Terf1 function at telomeres. I further show that this chemically induced telomeric stress is accompanied by progressive telomere shortening. I discuss the implications of coupled Wnt/beta-catenin pathway- and telomere-antagonism inherent to Tnks inhibitors for their clinical development and use as research probes.

Induction of p53 activity by Tnks inhibition revealed by transcriptional reporter profiling

In order to understand the consequences of Tnks inhibition on CRC signaling, we monitored the activities of IWR-1, IWR-8, and XAV-939 (representing three different chemotypes) in three pathways that frequently collaborate in CRC using highly validated transcriptional reporter systems (the Wnt, p53, and Kras pathways) (Figure 3.1a) (203). As anticipated, all Tnks inhibitors evaluated disrupted beta-catenin activity in the CRC-derived DLD-1 cell line that harbors a truncated form of APC (Figure 3.1b). Since DLD-1 cells do not have functional p53 activity, we evaluated the effects of the Tnks inhibitors on p53 signaling in HCT-116 cells that express elevated levels of beta-catenin due to a mutation in its glycogen synthase kinase beta (GSK3b) phosphorylation site, as well as wild-type p53 protein (45,204). Surprisingly, all three Tnks inhibitors also induced p53 reporter activity but

left unperturbed KRAS signaling (Figure 3.1c). The ability of a MEK inhibitor (PD980589) to reduce KRAS reporter activity confirms previous accounts of reporter fidelity and activated KRAS signaling in HCT-116 cells (203) (Figure 3.1d,e,f). We also demonstrated the specificity of the p53 reporter using the DNA-damaging agent doxorubicin and HCT-116 cells engineered for p53 deletion (Figure 3.g, h). Thus, subjecting our Tnks inhibitor portfolio to a reporter-based transcriptional profiling strategy revealed an unexpected influence of Tnks inhibition on p53 activity.

Since HCT-116 cells express a destruction-resistant form of beta-catenin, Tnks inhibitors do not promote beta-catenin turnover as a consequence of Axin stabilization in these cells (205). Indeed, we observed little change in the Wnt reporter in cells treated with IWR-1 (Figure 3.1i). Thus, the effects of Tnks inhibition on p53 are independent of those stemming from Wnt/beta-catenin pathway inhibition. Given the well-established role for the Tnks substrate Terf1 in suppressing telomere-initiated DNA damage responses (200,201), we reasoned IWR-1 associated p53 activation could be an indirect consequence of induced telomeric stress. Since Tnks inhibitors have little or no effect on the growth of HCT-116 cells (205), their induction of p53 activity is also not likely attributable to mitotic defects previously associated with loss of Tnks activity (196,199,206).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b
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Normalized Wnt
pathway activity
IWR1
IWR8
XAV939
Compound
c
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Figure 3.1 Induction of p53 activity by Tnks inhibition revealed by transcriptional reporter profiling. *(a)* A transcriptional reporter profiling strategy to elucidate chemical-cellular interactions. The status of three cellular processes frequently altered in colorectal cancer (CRC) - Wnt/beta-catenin, p53, and Kras signaling – is monitored with luciferase-coupled reporters of pathway-dependent transcriptional activity. *(b,c,d)* Tnks inhibitors induce p53 activity. Representatives from three different chemotypes were tested for their activity in the Wnt/beta-catenin, p53, and Kras pathways in the given CRC cell lines using transiently transfected pathway-specific reporters (see "a") and a control reporter (SV40-Ren luc). *(e)* The MEK inhibitor PD980589 disrupts luciferase reported Kras signaling in a dose-dependent manner. HCT-116 cells transiently transfected with the Ras reporter and SV40-Ren luc DNAs were treated with increasing amounts of PD980589. *(f)* PD980589 inhibits ERK phosphorylation. *(g)* Specificity controls for the p53 reporter system. HCT-116 cells devoid of p53 expression exhibit little p53 reporter activity in the absence or presence of IWR-1. On the other hand, p53 reporter activity in HCT-116 cells can be induced by the DNA-damaging agent doxorubicin (Dox). *(h)* Western blot confirmation of p53 loss in HCT-116 (TP53-/-) cells. *(i)* IWR-1 does not inhibit Wnt/beta-catenin signaling in HCT-116 cells, which express a destruction-resistant form of beta-catenin.

Tnks inhibitors induce telomeric stress and shortening in human cells independently of Wnt/beta-catenin pathway inhibition

Terf1 (also known as Trf1) is essential to telomere integrity by binding double stranded TTAGGG telomere repeats and is a well-established target of Tnks enzymatic activity (167). Each round of cell doubling engenders loss of some telomeric sequence due to the inability of the DNA replication machinery to achieve complete chromosomal end duplication (the "end replication" problem) (207). When telomeric sequences become sufficiently shortened, chromosomal ends become "uncapped" and are unable to properly engage the shelterin protective components such as Terf1 thereby resulting in a DNA damage response and cellular senescence or apoptosis. Cancerous cells overcome this cell growth braking mechanism largely by invoking telomerase expression (208). We exploited the diversity of pharmacophores and their different modes of supporting target engagement embodied by the IWR chemical collection to define consensus effects of Tnks inhibition on telomere end protection. As controls, we employed chemicals targeting Porcn, an acyltransferase essential for Wnt-instructed beta-catenin activation, as a means of influencing beta-catenin without directly disabling Tnks (Figure 3.2a). Telomere Induced Foci (TIF) are markers of telomeric damage scored by the appearance of co-localizing shelterin component Terf2/Trf2 and phosphorylated H2A.X (gamma H2A.X), a biochemical marker of damaged DNA (209). Whereas the Tnks inhibitors markedly induced TIF formation in a cervical carcinoma cell line (HeLa cells), the Porcn inhibitors failed to do the same (Figure 3.2b,c). Yet, true to their previously assigned activities, both type of chemicals disrupted the expression of *Axin2*, a well-validated target gene of the Wnt/beta-catenin pathway (Figure 3.2d). Thus, the chemical

induction of telomeric damage is independent of the effects stemming from the dampening of Wnt/beta-catenin pathway activity. We further confirmed specific activities of Tnks inhibitors by measuring the ability of these chemicals to promote Tnks and Axin protein stabilization, a consequence of direct chemical attack on Tnks enzymes (Figure 3.2e). We also confirmed the activity of Porcn inhibitors by measuring the phosphorylation status of Dvl2, a signaling molecule that is phosphorylated upon engagement of Frizzled receptors by lipidated Wnt molecules (210,211) (Figure 3.2e). Thus, the chemical induction of telomeric damage is independent of the effects stemming from the dampening of Wnt/beta-catenin pathway activity.

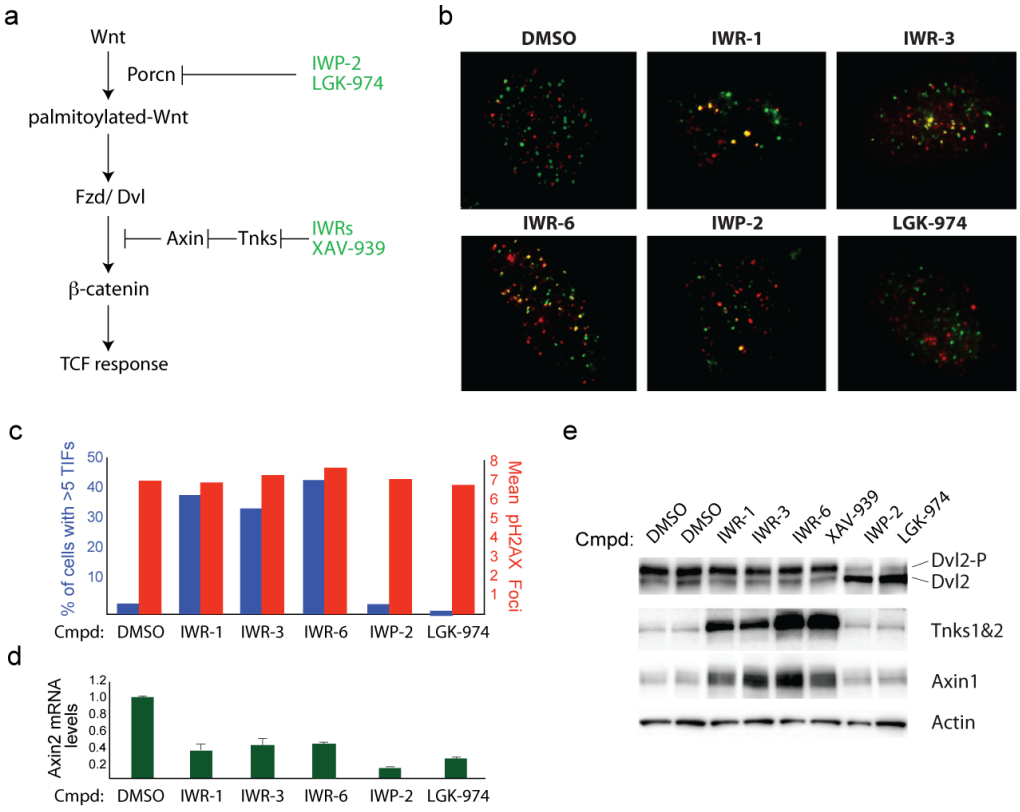


Figure 3.2 Tnks inhibitors induce telomeric stress in human cells independently of Wnt/beta-catenin pathway inhibition. (a) Porcn inhibitors afford a Tnks-independent approach for disrupting Wnt/beta-catenin signaling. Chemicals targeting the Wnt acyltransferase Porcn abrogate Wnt palmiteoylation which in turn results in loss of activity of the Frizzled (Fzd) family of Wnt receptors. Fzd receptors directly induce activation of the cytoplasmic signaling molecule Dishevelled (Dvl) in a ligand-dependent manner, which in turn results in activation of b-catenin and transcription mediated by the TCF family of DNA binding proteins. IWP-2 and the clinical candidate LGK-974 represent two structurally distinct Porcn inhibitors. (b) Tnks but not Porcn inhibitors induce DNA damage at telomeres. The appearance of co-localized Terf2 and pH2A.X (an indicator of DNA damage at the telomere ends; Telomere Induced Foci or TIFs) was scored using indirect immunofluorescence. Green: Terf2, Red: pH2A.X, Yellow:TIFs (c) Quantification of results represented in “b”. Cells with >5 TIFs were scored as positive for telomeric damage. Mean total phosphorylated H2A.X (pH2A.X) was used to measure general DNA damage responses. (d) Tnks and Porcn inhibitors disrupt beta-catenin-dependent transcription in HeLa cells. qPCR analysis of Axin2, a target gene of Wnt/beta-catenin signaling, reveals both classes exert influence on beta-catenin activity. (e) Biochemical evidence for on-target activities of Tnks and Porcn inhibitors in HeLa cells. IWP-2 but not IWR-1 inhibits phosphorylation of Dishevelled 2 (Dvl-2), a biochemical marker of Wnt-dependent activation of the Frizzled receptors. On the other hand, IWR-1 but not IWP-2 induces Tnks and Axin1 stabilization.

The removal of Terf1 by Tnks-mediated PARsylation is a prerequisite for the accession of telomeric DNA by the telomere-extending ribonucleoprotein enzyme complex (telomerase) and thus essential to normal telomere length maintenance in cancerous cells (167,212). Using an in vitro reconstitution assay, we demonstrated IWR-1, IWR-6 and IWR-3, representing AD-binding, NI-binding and dual pocket binding inhibitors, prevents Tnks-mediated PARsylation of Terf1 (Figure 3.3a). Taken together with the substantial biochemical and genetic evidence that Tnks-Terf1 interaction is essential to telomere regulation, our observations demonstrate that IWR-dependent induction of TIFs is a consequence of impaired Tnks regulation of Terf1.

We next evaluated the effects of Tnks inhibition on telomere length maintenance in human HeLa cells treated for an extended period of time with IWR-1 (up to 40 population doublings). IWR-1 shortened telomere length in a dose-dependent manner, whereas the Porcn inhibitor IWP-2 did not (Figure 3.3b). Our data is consistent with Tnks inhibitor-induced telomeric shortening independent of changes to Wnt signaling status in this cell line. We also ruled out inadvertent direct inactivation of telomerase by IWR-1 as the cause of telomeric shortening using an in vitro assay for telomerase activity (Figure 3.4). Taken together, our findings reveal a single agent strategy for inducing telomerase shortening that is distinct from current efforts devoted to directly targeting telomerase. At the same time, we uncovered an inseparable biological impact on Wnt/beta-catenin signaling and telomere length maintenance stemming from the chemical attack of Tnks enzymes.

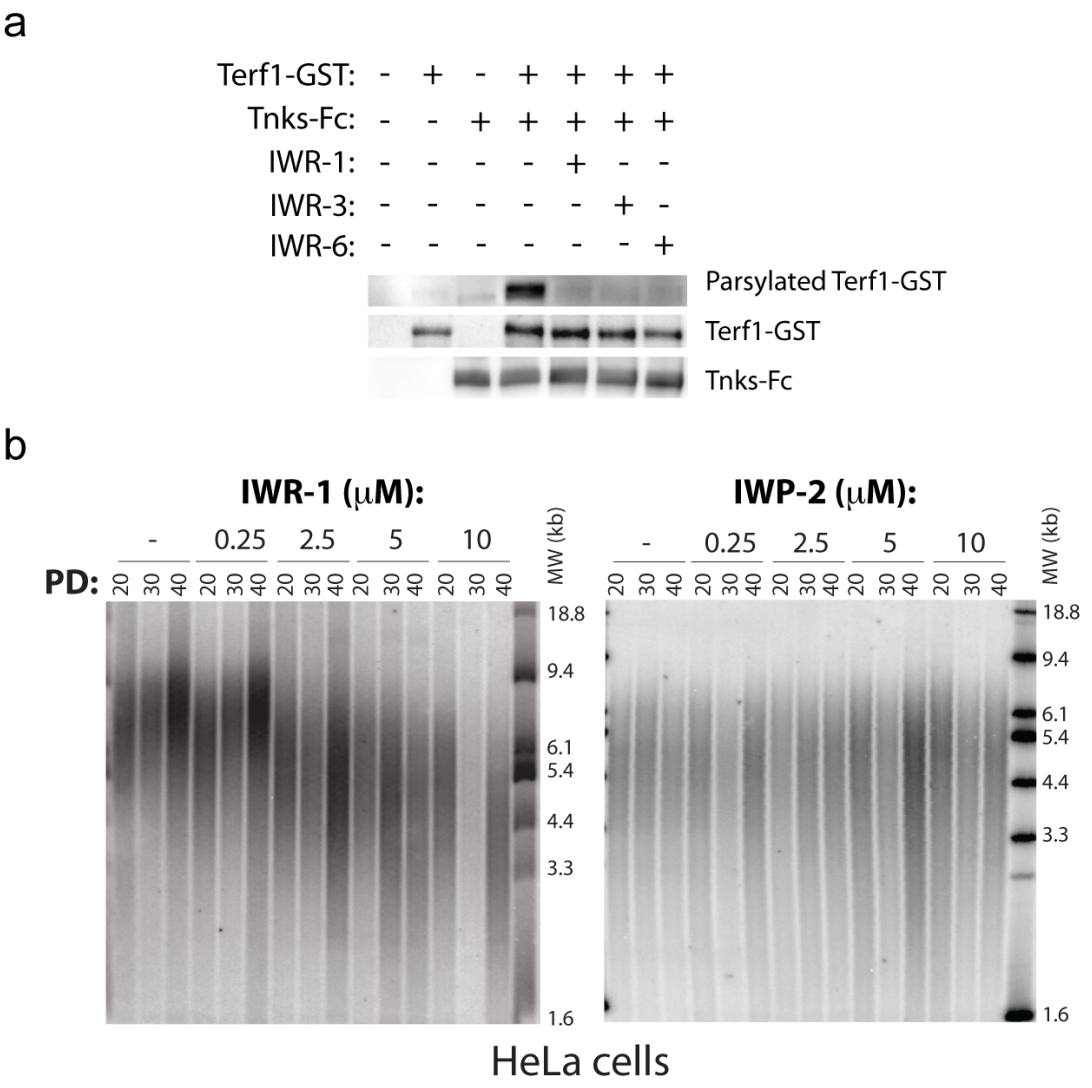


Figure 3.3 Tnks inhibitors induce telomeric shortening. (a) IWR compounds inhibit Tnks-mediated parsylation of Terf1 *in vitro*. Tnks1-Fc fusion protein purified using protein A sepharose from HEK293 cells transiently transfected with Tnks1-Fc DNA was incubated with recombinant Terf1-GST protein, NAD-Biotin (2.5mM), and indicated compounds (5 mM). (b) IWR-1 but not IWP-2 induces telomere shortening. HeLa cells were incubated with either IWR-1 or IWP-2 for the indicated number of population doublings (PD) and telomere length measured by terminal restriction fragment (TRF) analysis. Panel b by Brody Holohan & Ozlem Kulak.

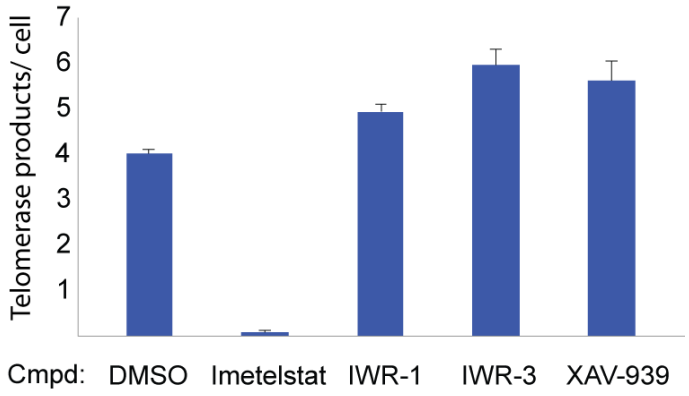


Figure 3.4 Tnks inhibitors do not inhibit telomerase activity. Digital droplet-based telomerase extension analysis of genomic DNA isolated from HeLa cells treated for 24 hrs with indicated compounds. Study by Brody Holohan & Ozlem Kulak.

A Tnks binding motif in Terf1 dictates species-dependent p53 induction by Tnks inhibitors

Dependency of telomere integrity on Tnks activity is dictated by the presence of a Tnks binding sequence in Terf1 (164,213). We aligned Terf1 sequence from model organisms routinely used for research and drug development in order to identify animals potentially susceptible to telomeric damage upon exposure to Tnks inhibitors (Figure 3.5a). Using induction of p53 reporter response as a surrogate assay for Tnks inhibitor-induced telomeric stress, we evaluated the strength of our predictions using a mouse-derived cell line (NIH-3T3 cells; Figure 3.5b,c). Doxorubicin but not IWR-1 was able to induce p53 reporter activity as predicted based on the absence of a Tnks binding motif in mouse Terf1 protein. Taken together, our observations reveal that disruption of telomere integrity with Tnks inhibitors is superimposed upon Wnt/beta-catenin pathway inhibition in cells that harbor a Tnks-binding motif such as those derived from humans (Figure 3.6).

Differences in the response to genetic and chemical perturbations between rodents and humans have long challenged the tenet that such animals should be used as front line informants of drug potential (214-216). Given the highly conserved mechanisms that support Wnt/beta-catenin signaling across metazoan animals, the inability of Tnks inhibitors to elicit loss of Wnt/beta-catenin signaling in model organisms was not anticipated to be a limitation to a clinical development path. Rather, our transcriptional reporter profiling of Tnks inhibitors coupled with a prior understanding of Tnks cellular functions revealed a species-specific telomeric response superimposed on the effects of Wnt/beta-catenin pathway depression in chemically treated cells.

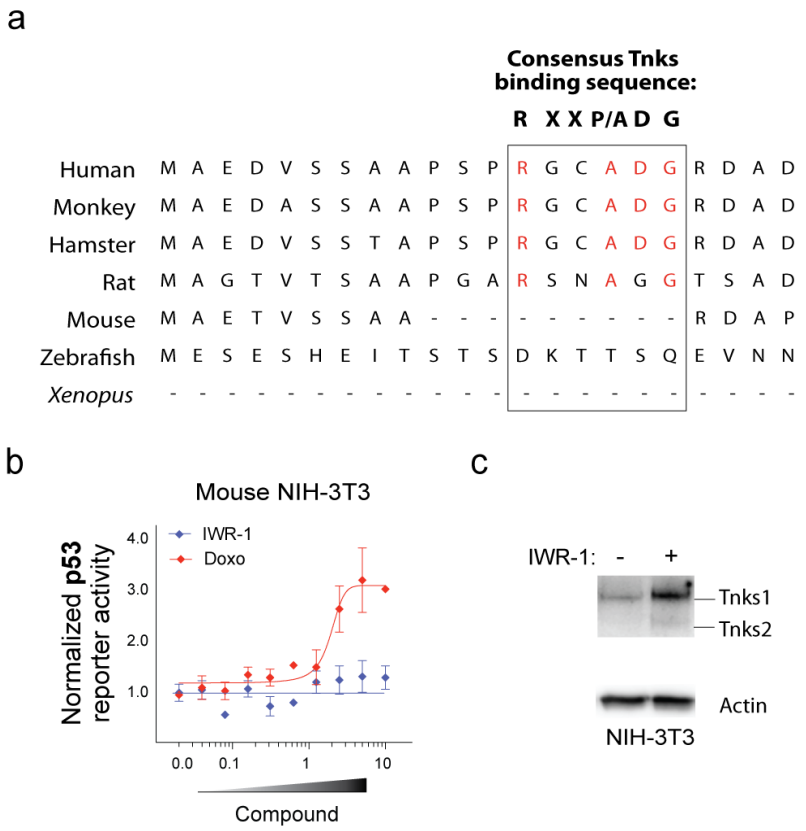


Figure 3.5 A Tnks binding motif in Terf1 dictates species-dependent p53 induction by Tnks inhibitors. (a) Alignment of Terf1 protein sequence from human and model organisms reveals potentially useful animals for studying Tnks associated telomeric activity. (b) IWR-1 does not induce p53 activity in mouse NIH-3T3 cells. NIH-3T3 cells transiently transfected with the pp53-6x-TA-Luc reporter were treated with either IWR-1 or doxorubicin (positive control). (c) IWR-1 inhibits auto-destruction of Tnks enzymes NIH-3T3 cells.

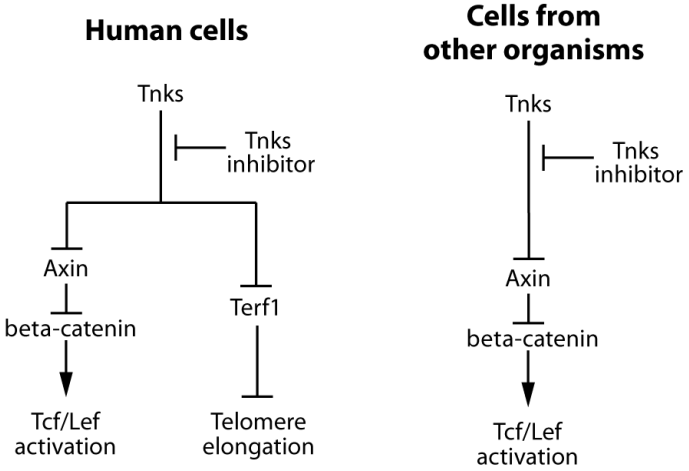


Figure 3.6 Species-dependent responses to Tnks inhibition. Cellular response to Tnks inhibitors in cells with different dependencies on Tnks-regulated telomere stability.

Conclusions And Future Directions

Chemically based efforts to disable deviant Wnt/beta-catenin signaling in cancer have converged on two major strategies that target either the Tnks or Porcn enzymes (217). Our findings reveal that suppression of telomere lengthening and Wnt/beta-catenin signaling are coincident in cells treated with highly selective Tnks inhibitors (Figure 3.6). In the context of cancer, Tnks inhibitors may afford a single agent synthetic lethal strategy for targeting cancer initiating cells that may rely on both cell stemness associated processes. At the same time, loss of Wnt/beta-catenin signaling invoked by short or prolonged chemical attack of Porcn spares telomeres suggests that previous observations linking Wnt/beta-catenin mediated transcription to telomere regulation may not be universal (218-220) (Figure 3.6).

Tnks and Porcn inhibitors including IWR-1 and IWP-2 are now widely used for the *in vitro* engineering of various tissues including cardiomyocytes, retinal pigmented epithelial cells, pneumocytes, and dopaminergic neurons (190,193,195,221-225). Given the transient nature of the chemical exposure in these protocols (typically 2-3 days) and similar efficacy of both Tnks and Porcn inhibitors for directing cell fate outcome in a direct comparison (193), we assume the chemical induction of these cells types are due to suppression of Wnt/b-catenin transcriptional responses and not telomeric stress or shortening. Further studies will be required to address whether or not concomitant induction of telomeric stress associated with the Tnks inhibition has any adverse affects on tissue engineering agendas.

The recent identification of specific Tnks inhibitors is timely given the development of Imetelstat, an oligonucleotide-based inhibitor representing the only small molecule targeting telomerase to advance in clinical testing, has stalled due to hematological and hepatoxic dose

limiting side effects (226,227). Whereas PARP inhibitors have been posed to be useful for sensitizing telomeres for chemical attack using other agents (228,229), our study using selective Tnks inhibitors suggest a single agent strategy can achieve the same endpoint. Moreover, the AD-binding pocket of Tnks can accommodate diverse pharmacophores as demonstrated here and from other efforts thus providing a more versatile starting point by comparison to Imetelstat with respect to medicinal chemistry goals (230). Recent advances in genetic testing have also uncovered new patient cohorts associated with long telomeric length that may benefit from a Tnks inhibitor including those with mutations in the shelterin component POT1 in familial melanoma and chronic lymphocytic leukemia (231-233), the catalytic subunit of telomerase Tert in familial and sporadic melanoma (234), and single nucleotide polymorphisms near the promoters for Tert and the RNA component of telomerase Terc in glioma (235). Thus, the clinical development path for Tnks inhibitors as anti-cancer agents should not only include consideration of the status of Wnt/beta-catenin signaling in various diseases but also the potential contribution of telomere-associated genetic alterations to drug sensitivity.

CHAPTER FOUR
Disabling the Wnt transcriptional apparatus by direct chemical attack

Introduction

Despite the success in the identification of underlying genetic alterations associated with cancer, current therapeutic approaches to treat this disease complex rely on agents that target not the molecular basis of the disease, but rather mechanisms that generally support both normal and cancerous cell growth. The recent success of Vismodegib, a molecule that inhibits hedgehog signaling in basal cell carcinoma, has galvanized efforts to produce similarly targeted therapeutic agents for all cancer types. A broad range of cancerous cell types frequently exhibit growth-dependency on cellular programs controlled by the Wnt family of secreted signaling molecules (135). Colorectal cancer represents the most intensely studied association between Wnt signaling and cancer.

In order to elucidate the mechanisms that underpin Wnt-dependent cellular responses supporting CRC cell growth, we employed concerted genetic and chemical screens in cultured cells. We mined an early draft of the colorectal cancer genome to uncover previously unrecognized proteins that are functionally linked to Wnt/beta-catenin pathway. In parallel we utilized a high-confidence collection of Wnt pathway inhibitors with previously unidentified mechanisms of action to reveal novel small molecules that directly target the TCF/LEF transcriptional apparatus. Collectively, these efforts netted a novel chemical approach for disabling deviant transcriptional activity in CRC.

Functional annotation of candidate (CAN) genes reveals Tcf4 to be a tumor suppressor in CRC

A major impasse for the effective use of cancer genome sequencing data for therapeutic anti-cancer intervention is the delineation of common denominator cellular processes that support cancer development and that could be chemically exploited. Efforts focused on the colorectal cancer (CRC) genome have revealed that Wnt/beta-catenin pathway is altered in >98% of cancer incidents suggesting Wnt/beta catenin pathway is the key driver of cellular transformation in the gut (236).

Using this understanding as a framework for assigning function to CAN genes identified from CRC cancer genome sequencing efforts (22), we measured the impact of RNAi reagents targeting CAN genes on the Wnt/beta-catenin activity using a cultured cell-based reporter system. We utilized three different cell lines with different Wnt pathway activities; RKO cells had little or no aberrant Wnt/beta-catenin pathway activity while HCT116 and DLD-1 cells both exhibited aberrant pathway activities due to an activating mutation in beta-catenin and an inactivating mutation in APC, respectively. Thus, these cell lines allowed us to interrogate gene function with respect to the Wnt/beta-catenin pathway activity with two of these cells recapitulating cancerous states associated with aberrant pathway responses.

Not surprisingly, APC functions as a suppressor of Wnt signaling as revealed from our RNAi screen in HCT-116 and RKO cell lines (Figure 4.1). Surprisingly, we also identified APC as a pathway suppressor from DLD-1 cells, which have been shown to express a truncated form of APC, suggesting that the APC protein retains some pathway suppressor function in these cells. Indeed, complete loss of APC results in greater tumorigenesis in mice

(237). Also, consistent with the lack of aberrant Wnt/beta-catenin pathway activity in RKO cells, we failed to isolate beta-catenin using this technique as an outlier in our screen in contrast to the case in DLD-1 and HCT-116 cells. We also identified two known Wnt pathway regulators TIAM-1 and Bcl9 in DLD-1 cells. Our ability to predictably identify these established components of aberrant Wnt/beta-catenin pathway response suggests our approach is robust and can be used to provide mechanistic insight into the cancer-associated roles of CAN genes. Finally, Tcf7l2 (formerly Tcf4) emerged as a major suppressor of Wnt/beta−catenin pathway activity thus corroborating previous studies that suggest it functions as a tumor suppressor (238). At the same time, the finding re-enforces previous calls for small molecules that directly target TCF/LEF family members that likely engage pro-growth genes de-repressed in the absence of Tcf7l2.

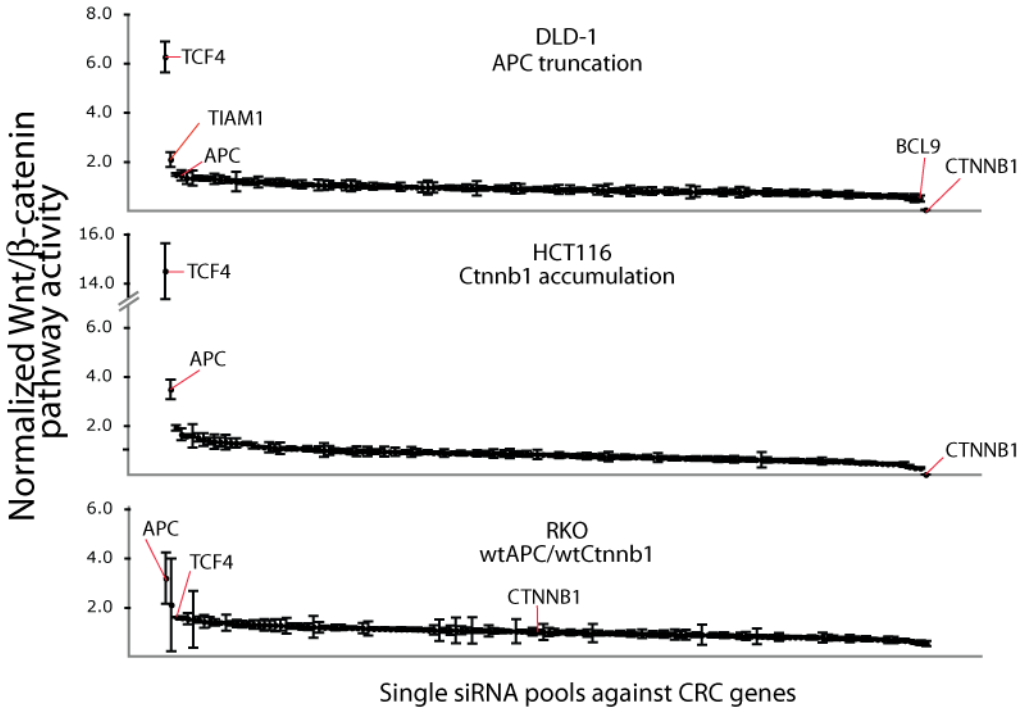


Figure 4.1 Identification of genetic alterations in colorectal cancer that may be countered with IWR compounds. Tcf7l2/Tcf4 is a potent suppressor of Wnt/beta-catenin signaling in colorectal cancer cells. To mechanistically assign functional roles to genes identified from a draft CRC genome, we examined the effects of decreasing cancer gene function by RNAi in three different CRC cell lines harboring the Wnt-responsive STF reporter. The status of APC and/or beta-catenin in each cell line is indicated. Loss of Tcf4 resulted in the most potent de-repression of pathway activity in DLD-1 and HCT116 cells. Study by Wei Tang& Ozlem Kulak.

Targeting nuclear effectors in the Wnt pathway

Identification of IWR-16, a chemical that disrupts TCF/LEF binding to DNA

Even though the loss of the APC tumor suppressor is the predominant Wnt pathway-activating event in colorectal cancer (CRC), mutations in other downstream pathway components such as Axin and beta-catenin are also common (21-23). In other cancer types, these non-APC associated mutations are more predominant over those found in APC. For example mutations in beta-catenin, Axin1 and APC are observed in 31%, 15% and 4% of hepatocellular carcinomas, respectively (239). Similarly, beta-catenin mutations have been linked to up to 50% of endometrial cancer (134,240). Therefore, agents directly targeting the transcriptional apparatus would have broad implications for Wnt pathway-dependent cancers.

Previously Chen et al. identified two classes of synthetic molecules that inhibit Wnt-dependent cellular responses from screening a diverse chemical library using cultured cells (138) (Figure 4.2a). The first class inhibits the acyltransferase Porcn (termed IWP compounds), thus disrupting fatty acylation and activity of most if not all Wnt proteins. The second class inhibits Wnt/beta-catenin pathway response (named IWR compounds), and five members of this class inhibit poly-ADP ribosylase enzymes Tnks1&2 (Figure 4.2b). Chemical antagonism of Tnks proteins induces the destruction of the transcriptional co-activator beta-catenin by promoting the stability of an Axin-scaffolded beta-catenin destruction complex. Although the targets of the most IWP compounds were identified as Porcn (187), the mechanism of action for the majority of so-called IWR compounds remain elusive.

To identify IWRs that may more directly target nuclear components of Wnt signaling, we examined the effects of a subgroup of IWRs representing all the different chemical structures in a DNA-TCF/LEF binding assay (Figure 4.3a). From this effort IWR-16 emerged as an inhibitor of DNA-TCF4 binding (Figure 4.3b). IWR-16 also inhibited DNA-LEF1 binding in a dose-dependent manner (Figure 4.4a). This result suggests that IWR-16 acts as a DNA-TCF/LEF binding inhibitor by either inhibiting direct binding or modifying the affinity of TCF/LEF1 to the DNA. Since molecular size of IWR-16 is smaller than known protein-protein interaction inhibitors, we hypothesize that IWR-16 affects transcription through altering post-translational modification (PTM) status of TCF/LEF proteins (Figure 4.5). Sequence alignment analysis of TCF/LEF transcription factors indicated presence of two shared PARsylation sites and a small ubiquitin-like modifiers (SUMO) modification site (Figure 4.4b). Mutational analysis of the two PARsylation and one SUMOylation residue suggested that these residues are essential for DNA-TCF/LEF binding (Figure 4.4c). Of note one of the PARsylated residue (the amino acids corresponding to E322 of Tcf4) lies inside the SUMOylation motif. Considering the close interplay between PARsylation and SUMOylation modifications (241) PARsylation status of TCF/LEF proteins may regulate SUMOylation status of the protein or vice versa. Taken together, we hypothesize that IWR-16 can affect PARsylation/ SUMOylation status of TCF/LEF transcription factors (Figure 4.5).

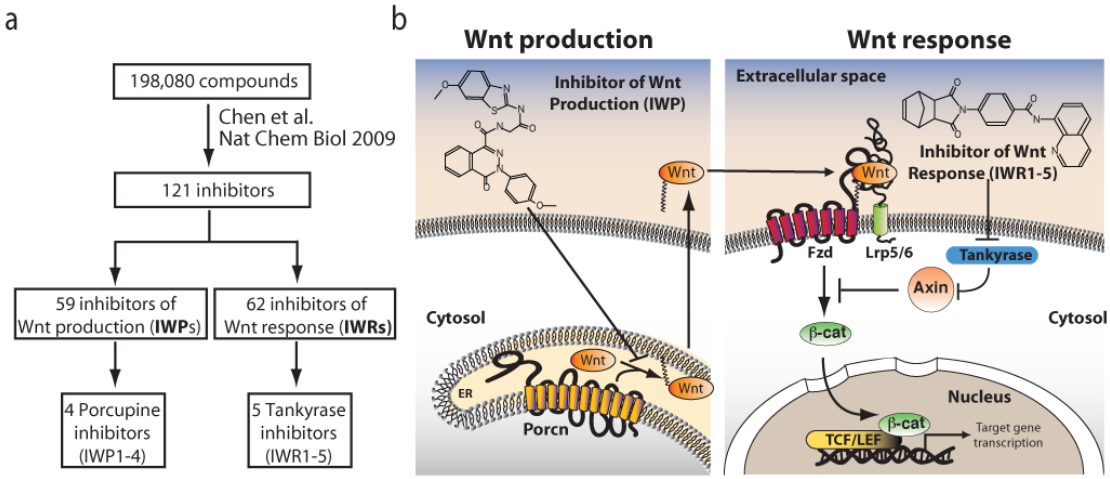


Figure 4.2 A high confidence collection of small molecules targeting Wnt/beta-catenin signaling. (a) A small molecule library screen identifies a small collection of highly specific Wnt pathway inhibitors. The majority of compounds that disrupt ligand production (IWP compounds) target the Wnt acyltransferase Porcupine (Porcn) whereas a handful of molecules that target Wnt response (IWR compounds) disable the poly-ADP ribose polymerase Tankyrase (Tnks). (b) Mechanistic models of IWP and IWR action. IWP1-4 inhibit Porcupine (Porcn), a membrane-bound acyltransferase that is important for Wnt protein production. IWR1-5 inhibit Tankyrase activity causing stabilization of Axin, a scaffolding protein that functions in the destruction complex for beta-catenin. Although most of the IWPs found to be Porcn inhibitors the mechanism of action for the rest of the IWRs are not identified yet.

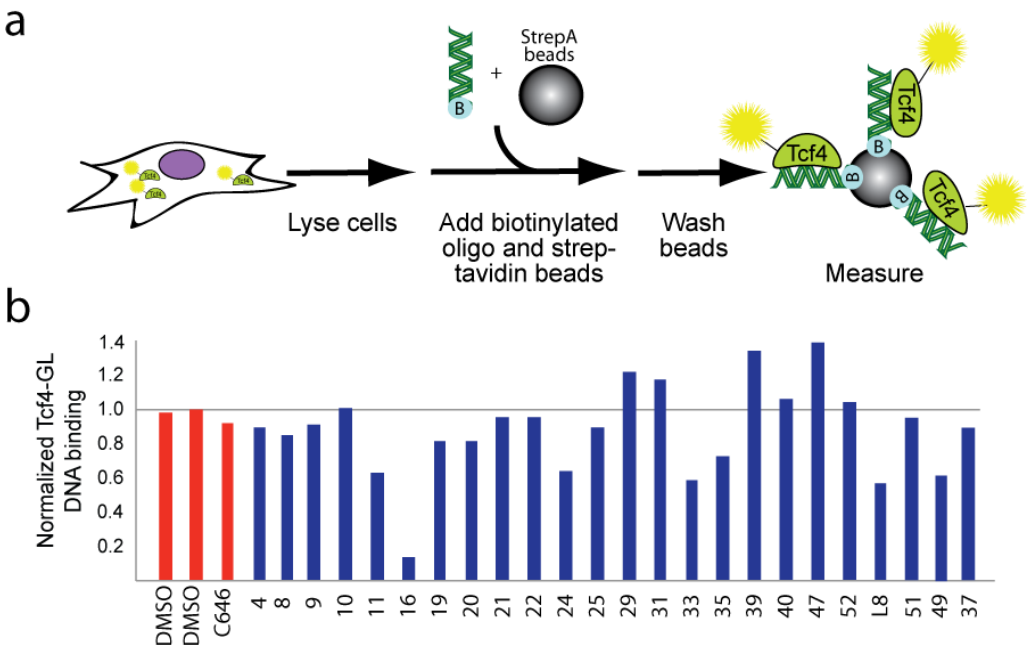


Figure 4.3 Identification of a small molecule that disrupts TCF/DNA binding (a) Schematic representation of a high-throughput platform for quantifying interactions between TCF/LEF and DNA. Oligonucleotides with TCF binding elements are used for pulling down luciferase-tagged Tcf4. (b) In order to identify inhibitors that work at the transcriptional level, we re-evaluated representatives of structurally different the IWR groups in the assay "a". IWR-16 exhibited the most potent inhibitory activity.

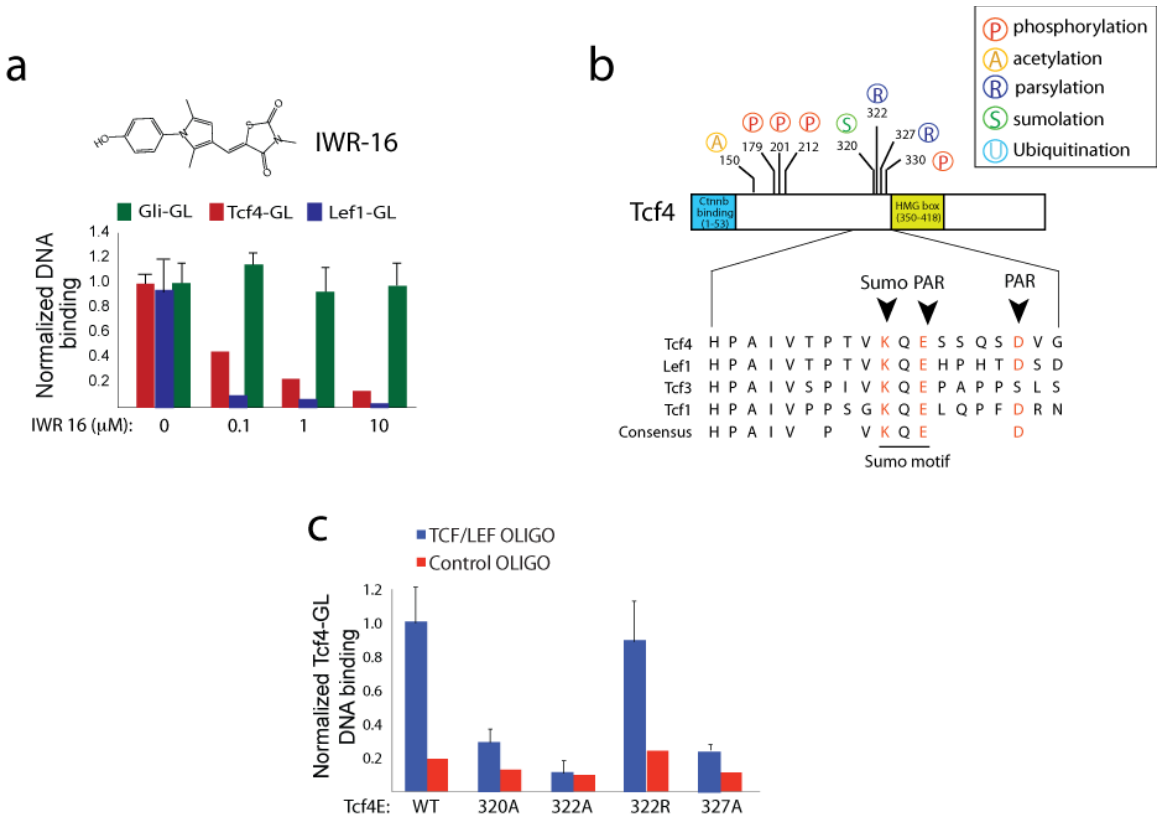


Figure 4.4 Disruption of a putative TCF/LEF PARsylation site eliminates DNA binding. (a) IWR-16 inhibits binding of DNA to different TCF/LEF transcription factors. On the other hand, hedgehog transcription mediator GLI binding to DNA is not effected. Study by Xiaofeng Wu & Ozlem Kulak (b) Conserved PARsylation sites for TCF/LEF transcription factors implies a potential point of attack for all family members. Tcf4 PARsylation at E322 is detected in a proteomics screen (242). A putative PARsylation site is embedded within a SUMOylation motif (243) suggesting that SUMOylation status can be affected by PARsylation of the protein or vice versa. (c) Genetic ablation of PARsylation or SUMOylation sites inhibits DNA binding activity of Tcf4. An E322R mutant retains PARsylation capacity suggesting that protein binding is influenced PARsylation.

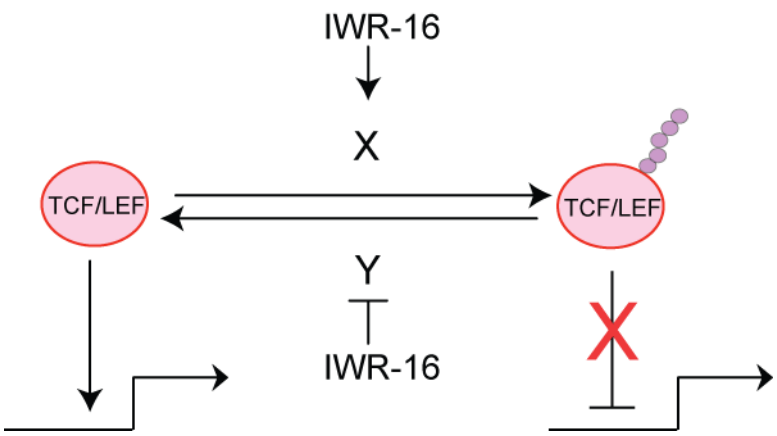


Figure 4.5 SUMOylation and PARsylation of TCF/LEF transcription factors may alter their DNA binding affinity. X represents an enzyme that either SUMOylates or PARsylates TCF/LEF molecules whereas Y represents an enzyme that either deSUMOylates or dePARsylates TCF/LEF molecules. Thus, IWR-16 may affect the DNA binding activity of TCF/LEF proteins either by activating X or inhibiting Y.

IWR-16 induces cellular PARsylation and SUMOylation

Given the influence of PARsylation or SUMOylation on TCF/LEF binding to DNA, we conjectured that IWR-16 might affect either of these cellular processes. Surprisingly, cellular levels of PARsylated and SUMOylated proteins increased upon IWR-16 treatment while levels of ubiquitinated protein did not change (Figure 4.6) suggesting that IWR-16 may either inhibit enzymes that remove PARsylation or Sumoylation, or activate enzymes that mediate these biochemical changes. Given these biochemical changes are often subject to mechanisms that support their co-regulation, conceivably IWR-16 targets an enzyme that participates in both cellular processes.

IWR-16 was identified as a Wnt/beta-catenin pathway inhibitor from a cell based screen using a pathway-specific transcriptional reporter. Gene expression analysis of IWR-16 treated cells demonstrated that transcriptional levels of known Wnt targets genes such as

Axin2 and Myc decreased upon IWR-16 treatment thus confirming the initial discovery observations (Figure 4.7a). As a first step towards generating a form of IWR-16 suitable for in vivo studies, we leveraged the Axin2 qPCR assay to establish structure-activity relationships (SAR) to define chemical features of IWR-16 that can be modified without compromising its activity (Figure 4.8). From these studies, the phenol group of IWR-16 emerged as critical for supporting Wnt/beta-catenin pathway inhibitory activity. The phenol group is expected to be readily biodegraded in vivo (personal communication with Chuo Chen) thus substitution of the hydroxyl group with an inert chemical adduct will likely be required.

We also evaluated the on-target activities of IWR-16 by evaluating its ability to induce cardiomyocyte (CM) differentiation from embryonic stem (ES) cells (193,244,245). Consistent with the on-target activities assigned to IWR-16 and the CM promoting activity associated with Tnks and Porcn inhibitors, IWR-16 but not a Hedgehog pathway inhibitor promoted the induction of CMs following chemical treatment (Figure 4.7b). Thus, IWR-16 appears to be a fairly specific Wnt/beta-catenin pathway inhibitor based on its behavior in several highly validated reporters of Wnt/beta-catenin signaling.

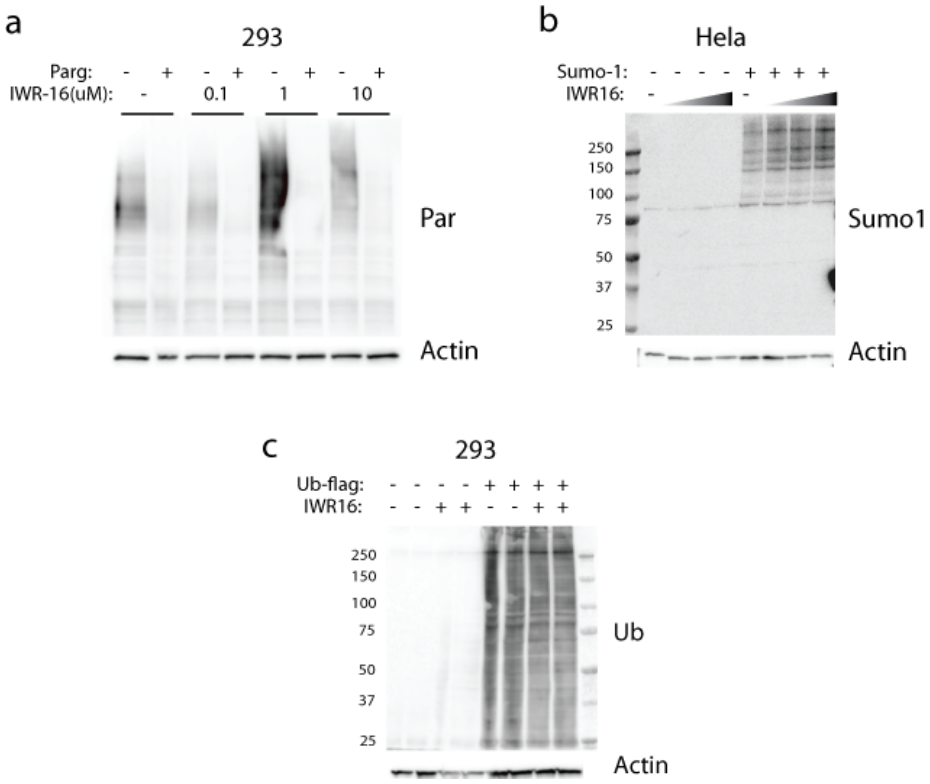


Figure 4.6 IWR-16 treatment increases total cellular levels of PARsylated and SUMOylated proteins. IWR-16 induces PARsylated and SUMOylated protein levels in a dose dependent manner (a, b). Overexpression of PARG, the major enzyme responsible for the catabolism of poly (ADP-ribose) in cells, abolished the high molecular weight bands induced by IWR-16 treatment. On the other hand, ubiquitination did not change with IWR-16 treatment (c).

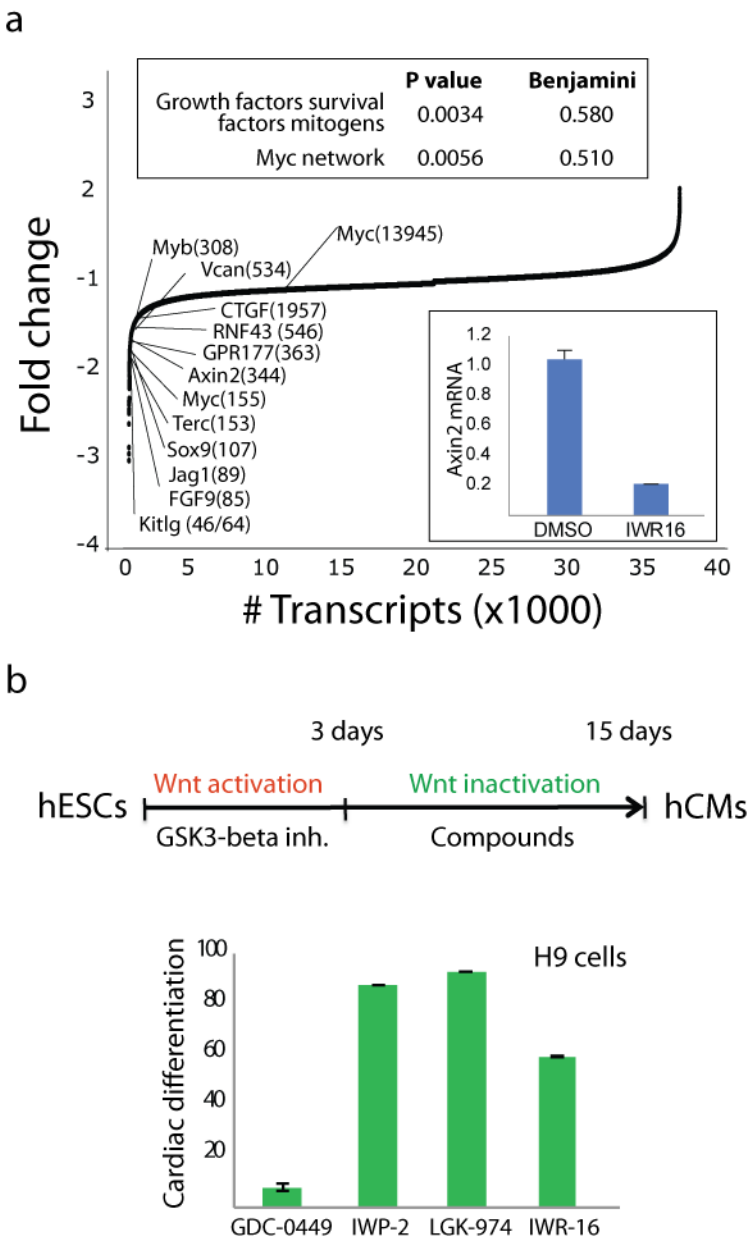


Figure 4.7 IWR-16 is a Wnt pathway inhibitor. a) Microarray analysis of IWR-16 activity in HCT116 cells confirms its anti-beta-catenin/TCF activity. The KEGG pathway was used to analyze microarray expression data. Microarray results were validated by QPCR (box). (b) Specificity of IWR-16 is assessed in a Wnt pathway dependent cardiomyocyte differentiation assay using the human embryonic stem cell (ESC) line H9. Human ESCs treated with a GSK-beta inhibitor for 3 days followed by IWR-16 for 12 days give rise to cardiomyocytes. The number of contracting embryoid bodies was visually assessed using a microscope with a 37°C-heated stage. Xiaoping Bao & Sean Palecek provided data in panel b.

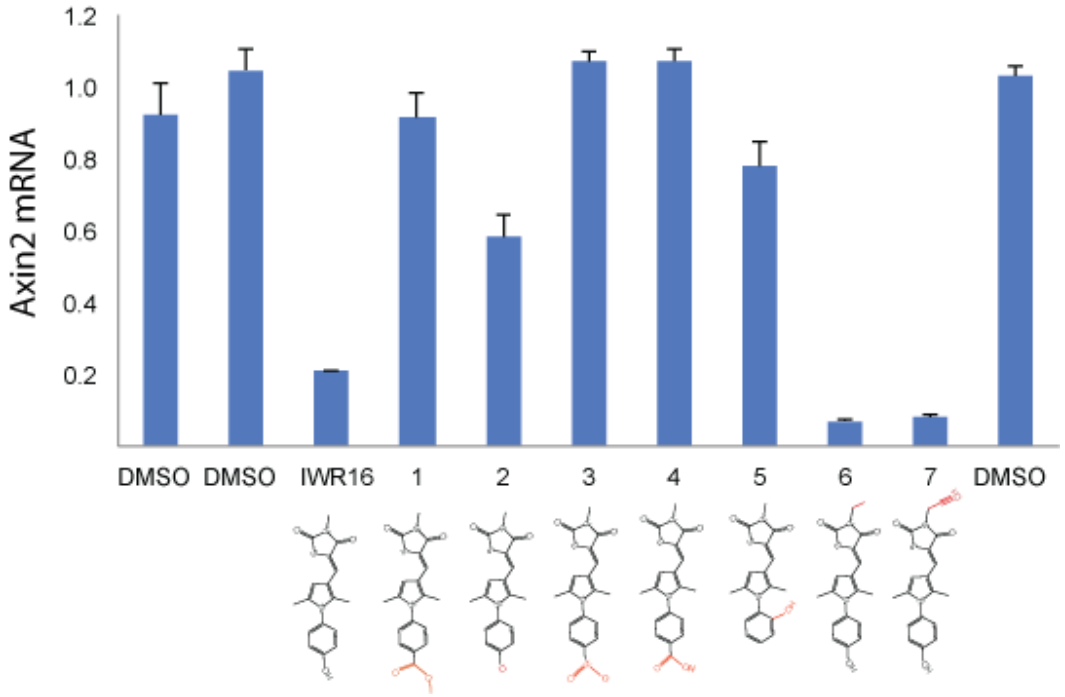


Figure 4.8 An SAR study of IWR-16 reveals a critical role for the phenol in supporting Wnt/beta-catenin pathway inhibitory activity.

Conclusions and future directions

The identification of Tnks as a chemical vulnerability in Wnt signaling arguably represents the most promising advance in efforts to drug APC-induced transcriptional responses in CRC from several decades of research. However, Tnks inhibitors have not moved forward in clinical development as anti-CRC agents likely due their inconsistent action against APC loss. This failing may relate to their indirect mechanism of targeting TCF/LEF activity that leaves open a window for cellular compensatory mechanisms. Whereas Porcn inhibitors and anti-Fzd antibodies that have advanced in clinical testing may be useful for other Wnt-related cancers, they are not likely to be useful for countering the effects of mutations in APC given their inability to restore homeostatic beta-catenin/TCF

activity induced by loss of APC function. In addition to CRC, several other cancers are associated with similar management challenges including liver and endometrial cancers, which frequently harbor mutations in beta-catenin that result in abrogation of its turnover. Thus, it remains imperative that we uncover a new strategy for directly targeting the transcriptional effectors in the Wnt pathway. In this regard, IWR-16 may succeed where Tnks and Porcn inhibitors have failed. To realize the full potential of IWR-16 as an anti-cancer agent, we must first identify the target of IWR-16 and develop a variant with drug-like properties. Studies are underway to accomplish these goals.

CHAPTER FIVE
Materials & Methods

Reagents

Cell lines

Human colorectal cancer cell lines DLD-1, RKO, HCT116 (TP53+/+), HCT116 (TP53-/- and SW-480 cells were acquired from ATCC. Human cervical cell line, Hela cells were provided by Jerry Shay.

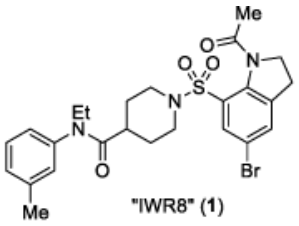
Antibodies

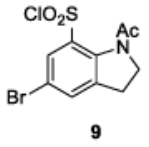
Antibodies were purchased from the following sources: BD Biosciences (Ctnnb1), Sigma (beta-Actin and Acetylated tubulin), Santa Cruz Biotechnology (Tnks, GST, p53), Cell Signaling Technology (Axin1, Axin2, and Dvl2), Millipore [phospho-Histone H2A.X (Ser139); gamma H2A.X], and Epitomics (Terf2).

Chemicals

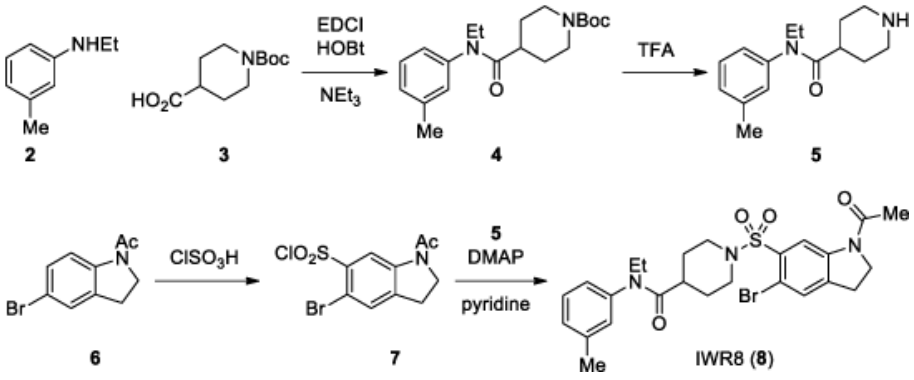
IWR-1 and IWP-2 were synthesized as previously described (138). Sources of chemicals: IWR-3 to -8 (ChemDiv or ChemBridge), XAV-939 (Maybridge) and LGK-974 (ActiveBiochem).

IWR-8 synthesis





Synthesis scheme:



Compound **3**. To a 25 mL vial charged with **1** (287 µL) and DCM (5 mL) was added **2** (229 mg), NEt_3 (697 µL), HOBt (270 mg) and EDCI (383 mg). Upon completion, the reaction was diluted with DCM, the organic layer was washed with 1N HCl, water and dried over Na_2SO_4, filtered, concentrated and purified by HPLC to afford **3** (24 mg).

Compound **4**. To a 4 mL vial charged with **3** (12 mg) was added 20% TFA in DCM (0.4 mL). Upon completion, the solvent was removed, the residue was dissolved in EA, the organic layer was washed with sat. $NaHCO_3$, dried over Na_2SO_4, filtered and concentrated to afford **4** (6.2 mg).

Compound **6**. To a 4 mL vial charged with **5** (200 mg) was added $ClSO_3H$ (200 µL). The reaction was stirred at 70 °C for 2 days, it was quenched by adding into ice-cold water slowly. The solid was washed with H_2O, filtered and dried under vacuum to afford a mixture

of **5** and **6** (170 mg) (**5**/**6** = 1/2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.99 (s, 1H), 7.60 (s, 1H), 4.17 (t, *J* = 8.6 Hz, 2H), 3.31 (t, *J* = 8.6 Hz, 2H), 2.25 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.2, 143.0, 142.2, 140.1, 132.0, 118.3, 114.2, 49.0, 27.9, 24.2.

Compound **IWR8**. To a 4 mL vial charged with **4** (6.2 mg) obtained above and DCM (0.5 mL) was added Py (12.1μL), mixture of **5** and **6** (25.4 mg) and DMAP (3.0 mg). Upon completion, the reaction was diluted with EA, the organic layer was washed with 0.5N HCl, water and dried over Na_2SO_4, filtered and concentrated, the residue was purified by HPLC to afford IWR8 (4.1 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 (s, 1H), 7.45 (s, 1H), 7.27 (t, *J* = 7.6 Hz, 1H), 7.15 (d, *J* = 7.5 Hz, 1H), 6.93 – 6.84 (m, 2H), 4.09 (t, *J* = 8.4 Hz, 2H), 3.75 (d, *J* = 11.9 Hz, 2H), 3.66 (q, *J* = 7.1 Hz, 2H), 3.21 (t, *J* = 8.4 Hz, 2H), 2.54 (t, *J* = 12.3 Hz, 2H), 2.36 (s, 3H), 2.25 – 2.15 (m, 1H), 2.20 (s, 3H), 1.89 – 1.73 (m, 2H), 1.65 – 1.55 (m, 2H), 1.05 (t, *J* = 7.1 Hz, 3H).

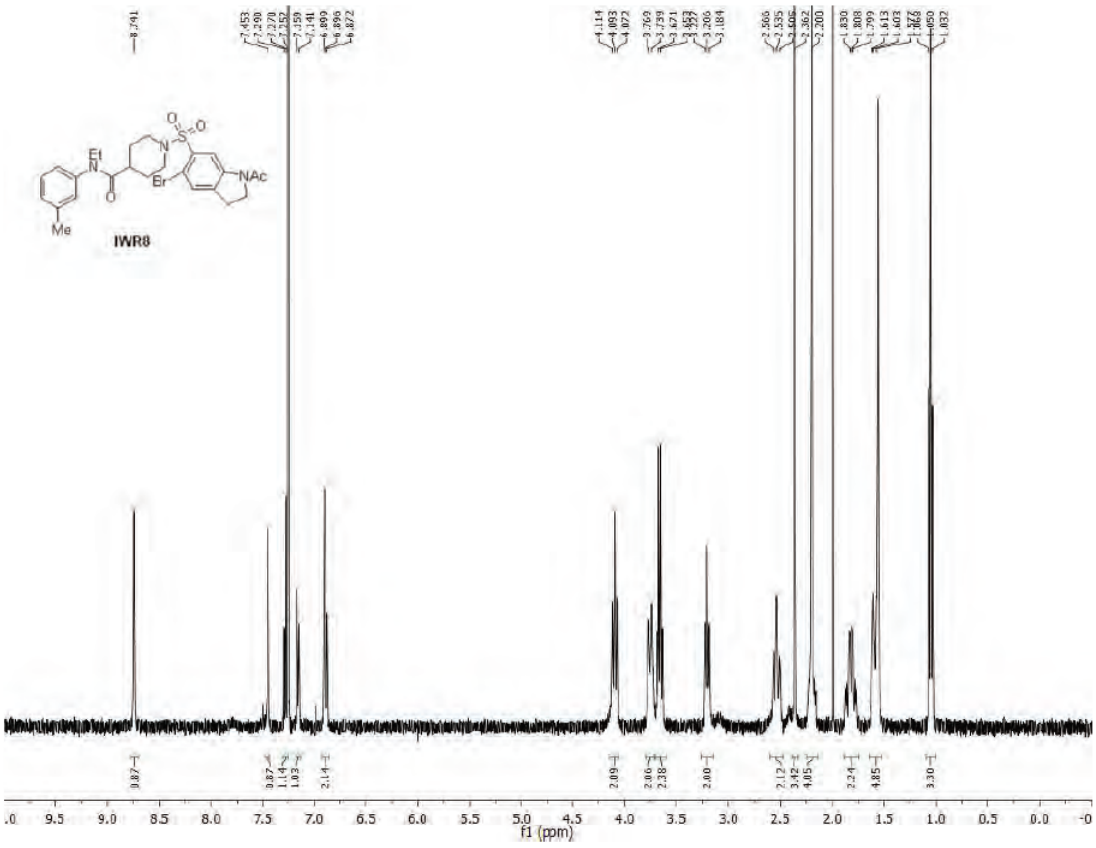


Figure 5.1 Synthesis of IWR-8. Scheme for the synthesis of IWR-8 and NMR data for IWR-8. We followed the procedure reported by ChemDiv (246) to synthesize the presumed structure of IWR8 (**1**) that was originally purchased from ChemDiv (scheme). While ^{1}H NMR spectrum of the final product is the same as that of the library compound, crystallographic studies indicate that the correct structure of IWR8 is **8** instead of **1**. The ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC studies also support that sulfonylation of **6** gave **7** but not **9**, consistent with the findings reported by Herchen, Petersen, and co-workers (247).

Western blot analysis

Cell lysates were generated with either PBS-NP40 buffer [phosphate-buffered saline (PBS)/1% NP40], or Tris-Triton X-100 buffer [50mM Tris (pH 8), 200mM NaCl, 1mM EDTA, 1% Triton X-100, 10% glycerol, 1mM DTT, 0.5mM deoxycholate]. Both buffers were supplemented with protease inhibitor cocktail (Sigma).

Luciferase reporter assay

Assays were executed as described using a Dual Luciferase kit (Promega), and SuperTopFlash and control SV40-driven Renilla luciferase reporters (SV40-Ren luc) (138,203).

Telomere dysfunction induced foci (TIF) assay

. Cells were treated with IWR-1/IWP-2 for 24 hrs before fixation [2% formaldehyde and permeabilized in 0.5% (v/v) of NP-40], and then incubated with gamma H2A.X and Terf2 antibodies and secondary antibodies (mouse fluorescein isothiocyanate–conjugated or Alexa Fluor 488–conjugated antibodies). Primary and secondary antibodies were diluted in PBS, 0.2% fish gelatin, and 0.5% BSA. Cells were imaged using a Zeiss LSM 780 confocal/multiphoton microscope and 3D co-localization assessed using Bitplane Imaris software. The TIF index was determined by assessment of co-localization of pH2A.X and Terf2 for 90-100 cells in 10 random areas within each slide.

Terminal restriction fragment (TRF) telomere length assay

$1x10^6$ cells were collected and washed with PBS. DNA was isolated using the manufacturer's instructions (Qiagen). 2.5 μg DNA was digested with six different restriction

enzymes (HhaI, HinfI, MspI, HaeIII, RsaI, AluI) (New England Biolabs) and incubated at 37°C overnight. Digested DNA was separated on a 0.7% agarose gel overnight at 70V. The terminal restriction fragment (TRF) gel was denatured for 20 mins in denaturation solution (0.5 M NaOH, 1.5 M NaCl, pH 13.2) and dried on Whatman 3MM paper under vacuum for 3 hrs at 56°C. The gel was neutralized for 15 mins in neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) and then probed with a radiolabeled telomeric probe (C-rich) for 16 hrs at 42°C in 5x SSC buffer, 5xDenhardt's solution, 10 mmol/L Na_2HPO_4, and 1 mmol/L $Na_2H_2P_20_7$. The gel was washed once with 2x SSC, 0.1% SDS, twice with 0.5x SSC, 0.1% SDS and then twice with 0.5x SSC, 1% SDS at room temperature for 15 mins. Gels were exposed to a PhosphorImager screen overnight and analyzed using a Typhoon PhosphorImager scanner system (Molecular Dynamics).

Protein expression and purification

The catalytic domain of human Tnks1 (residues 1105-1313) was expressed by using a modified pET28 vector, which encodes an N-terminal His6-tag and a cleavage site for the Tobacco Etch Virus (TEV) protease. Protein expression was induced by 0.1 mM IPTG at 15°C when the culture of the bacteria strain BL21 (DE3) transformed with the plasmid reached O.D. of 1.5. Cells were harvested 12 hrs after induction and lysed by Frenchpressing. The lysate was cleared by centrifugation at 15000 rpm for 1 hr. The protein was purified by Ni-NTA-based affinity chromatography and ion exchange chromatography. The N-terminal His6-tag was removed by overnight TEV protease treatment at 4°C. Purified protein was concentrated and stored in -80°C.

Crystallization, data collection and structure determination

The purified Tnks1 catalytic domain at 5 mg/ml (0.22 mM) mixed with IWR-1 (the exo-form), IWR-3 and IWR-8 at 0.25 mM were subjected to crystallization trials by using sitting-drop 96-well plates. Following initial hits of crystallization, large crystals were obtained by hanging- drop vapor diffusion in conditions optimized based on the initial conditions. Crystals of the Tnks1/IWR-1 complex were grown at 20°C in 0.1 M Bis-Tris (pH5.3), 0.15 M $MgCl_2$ and 20-23% PEG3350. Crystals of the Tnks1/IWR-3 complex were grown at 20°C in 0.1 M Tris (pH 7.5), 0.2 M sodium acetate and 30% PEG4000. Crystals of the Tnks1/IWR-8 complex were grown at 20°C in 0.1 M Bis-Tris propane (pH8.0), 0.2 M sodium bromide and 25% PEG3350. PDB IDs for structures are as follows: Tnks1/IWR-1 (4OA7), Tnks1/IWR-3 (4TOS), and Tnks1/IWR-8 (4TOR).

Crystals were cryo-protected in the crystallization buffer supplemented with 25% glycerol and flash-cooled in liquid nitrogen. Diffraction data were collected at 100K on beamline 19ID at the Advanced Photon Source (Argonne National Laboratory). Data were indexed, integrated and scaled by using HKL2000. The structure of the Tnks1 catalytic domain in the apo state (PDB ID: 2RF5)(xxx) was used as the search model for molecular replacement by using the Phaser module in the Phenix package (xxx). Iterative model building and refinement were performed by using the Phenix and Coot programs, respectively (xxx). The IWR compounds were placed when the Rfree was below 32% and the position of the compound was well defined by the electron density map. Comprehensive model validation was performed by using MolProbity (xxx). Detailed statistics of data

collection and refinement are listed in Supplemental Table 1. Structure figures were rendered in PyMOL (the PyMOL Molecular Graphics System, Schrodinger).

Quantitative PCR

Total RNA was purified using RNeasy Plus Mini Kit (Qiagen), and reverse transcribed by ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs) according to the manufacturer's protocol. qPCR was performed using FastStart SYBR Green Master (Roche) on a Light Cycler 480 System. Primers used for RT-PCR are as follows:

hAXIN2 – F ccacacccttctccaatcc

hAXIN2 – R tgccagtttctttggctctt

Actin-b – F ggatgcagaaggagatcactg

Actin-b – R cgatccacacggagtacttg

In vitro Tnks activity assays

The HT Universal Color PARP Assay Kit w/ Histone Coated Strip Wells (Trevigen) was used to monitor the activity of purified Tnks1 SAM-PARP protein (1 mg/96 well reaction; provided by Herwig Schüler) according to the manufacturers' protocol. Tnks-Fc construct was generated by PCR-based cloning of human Tnks1 sequence in frame into pcDNA3-hIgG-Fc vector using *BglII* restriction sites (248). Overexpressed Tnks-Fc protein immobilized on Protein A sepharose was incubated with human Terf1-GST protein (Abnova) and NAD biotin (2.5 mM; Trevigen) for 30 min. at RT prior to Western blot analysis. Parsylated Terf1 protein was detected using streptavidin-HRP. Terf1-GST or Tnks1-Fc proteins were detected by Western blot analysis with anti-GST or anti-Fc antibodies, respectively.

Digital droplet PCR

. The ddTRAP assay was performed as previously described (249). Briefly, pellets of 100,000 fresh sample cells were lysed on ice for 30 mins in NP-40 lysis buffer (10mM Tris-HCl, at pH 8.0, 1mM EDTA, 1mM MgCl2, 1% (vol/vol) NP-40, 0.25 mM sodium deoxycholate, 150mM NaCl, 10% (vol/vol) glycerol, 5mM β-mercaptoethanol, 0.1mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride)]. 1250 cell equivalents of lysate were added to the telomerase extension reaction in TRAP buffer (20mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$) with 0.4 mg/ml BSA, the telomerase extension substrate (TS, 200nM HPLC purified 5'-AATCCGTCGAGCAGAGTT), and 2.5mM dNTPs. The extension was incubated at 25°C for 40 mins followed by heat killing at 95°C for 5 mins. The PCR reaction was performed in 1x EvaGreen ddPCR Supermix v2.0 (Bio-Rad) with 50 nM TS primer and 50nM ACX reverse primer and 50 cell-equivalents of the extension reaction as input. Droplets were produced following the manufacturer's instructions in the droplet generator (Bio-Rad), and the emulsions were transferred to a 96-well plate for amplification (95°C for 5 min, 40 cycles, 95°C for 30 s, 54°C for 30 s, 72°C for 30 s, hold at 12°C). Fluorescence was quantitated using a QX200 droplet reader (Bio-Rad) in the 6-Fam channel. Thresholds for quantitation were determined by comparison to a telomerase negative fibroblast cell line and no cell input negative control samples and untreated Hela cell positive control samples.

PARP profiling. Performed by BPS Bioscience Inc., San Diego by following the BPS PARP or TNKS assay kit protocols. The enzymatic reactions were conducted in duplicate at room temperature for 1 hr in a 96 well plate coated with histone substrate. 50ml of reaction

buffer (Tris·HCl, pH 8.0) contains NAD^+, biotinylated NAD^+, activated DNA, recombinant PARP enzyme and 10mM of the test compound. Olaparib used at 20nM concentration. After enzymatic reactions, 50µl of Streptavidin-horseradish peroxidase was added to each well and the plate was incubated at room temperature for an additional 30 min. 100µl of developer reagents were added to wells and luminescence was measured using a BioTek Synergy 2 microplate reader.

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APPENDIX A

Multiplexed Luciferase Reporter Assay Systems

US008569002B2

(12) **United States Patent**
Lum et al.

(10) **Patent No.: US 8,569,002 B2**
(45) **Date of Patent: Oct. 29, 2013**

(54) **MULTIPLEXED LUCIFERASE REPORTER ASSAY SYSTEMS**

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(73) Assignee: **The Board of Regents of the University of Texas System**, Austin, TX (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 320 days.

(21) Appl. No.: **12/908,754**

(22) Filed: **Oct. 20, 2010**

(65) **Prior Publication Data**

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(51) **Int. Cl.**
C12Q 1/66 (2006.01)
C12Q 1/68 (2006.01)
G01N 33/567 (2006.01)

(52) **U.S. Cl.**
USPC **435/8**; 435/6.1; 435/7.21

(58) **Field of Classification Search**
USPC 435/8
See application file for complete search history.

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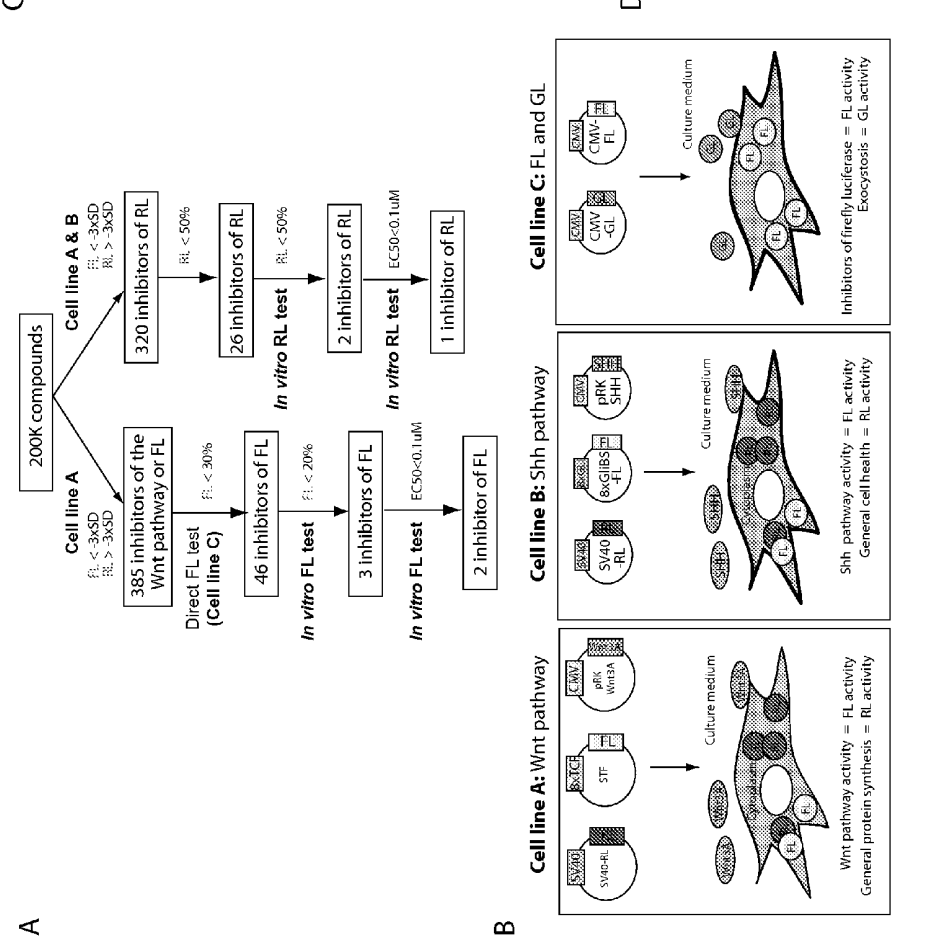
(57) **ABSTRACT**

Inhibitors of luciferase enzymes are disclosed and find use in multiplexed assays using multiple luciferases and multiple inhibitors, in both in vitro and in vivo embodiments.

18 Claims, 6 Drawing Sheets

Figure 1


A
200K compounds
Cell line A
FL: < -3xSD
RL: > -3xSD
Cell line A & B
FL: < -3xSD
RL: > -3xSD
385 inhibitors of the Wnt pathway or FL
320 inhibitors of RL
Direct FL test (Cell line C)
FL: < 30%
RL < 50%
46 inhibitors of FL
26 inhibitors of RL
In vitro FL test
FL: < 20%
In vitro RL test
RL < 50%
3 inhibitors of FL
2 inhibitors of RL
In vitro FL test
EC50<0.1uM
In vitro RL test
EC50<0.1uM
2 inhibitor of FL
1 inhibitor of RL
B
Cell line A: Wnt pathway
SV40-RL
8xTCF
STF
FL
CMV
pRK Wnt3A
Culture medium
Wnt pathway activity = FL activity
General protein synthesis = RL activity
Cell line B: Shh pathway
SV40 RL
8xGliBS -FL
CMV
pRK SHH
Culture medium
SHH
Shh pathway activity = FL activity
General cell health = RL activity
Cell line C: FL and GL
CMV -GL
CMV-FL
Culture medium
Inhibitors of firefly luciferase = FL activity
Exocystosis = GL activity
C
FL Inhibitor #1
IC50: 0.01 µM
Normalized FL activity
compound [µM]
FL Inhibitor #2
IC50: 0.1 uM
Normalized FL activity
compound [µM]
D
RL inhibitor #1
IC50:0.02 µM
Normalized RL activity
compound [µM]

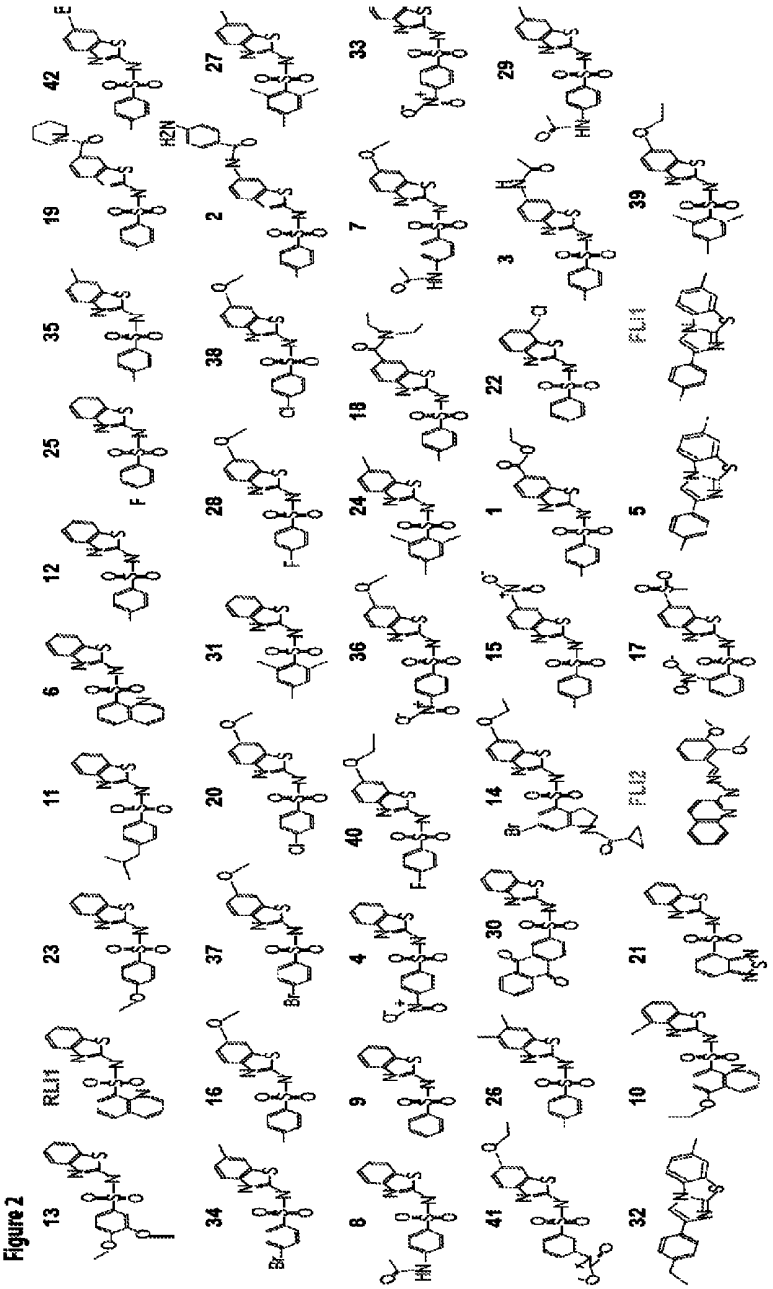


Figure 2
RLI1
RLI2
RLI1

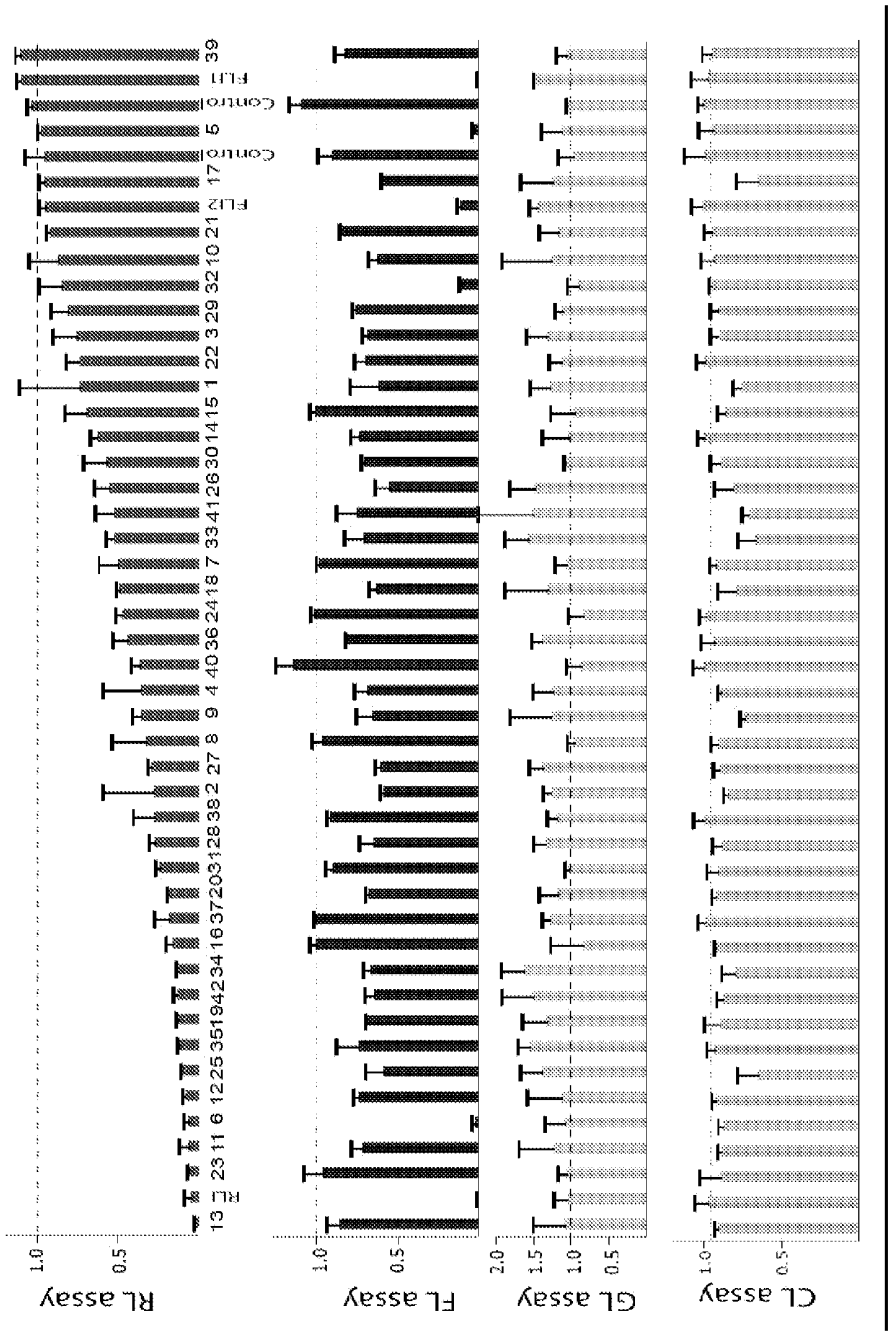
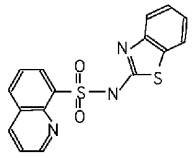


Figure 2 (cont.)

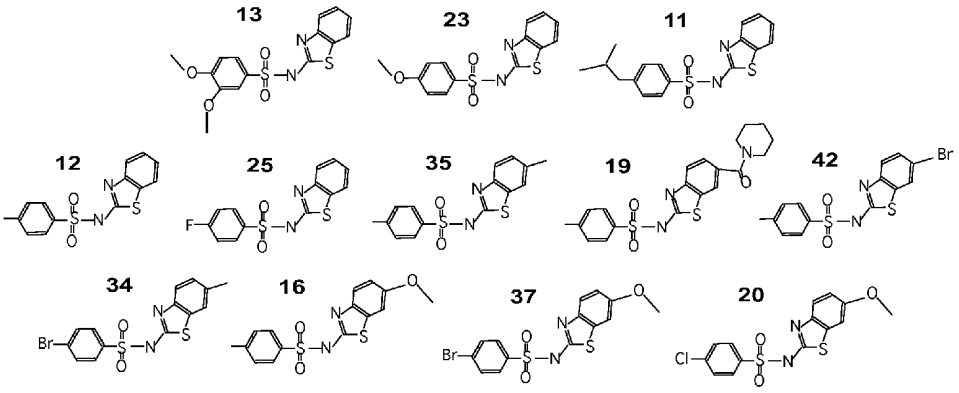
Figure 3

A

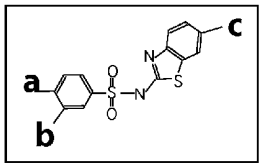
RLI (inhibits both RL and FL; henceforth called RLFLI)



RL inhibitors (RLIs)



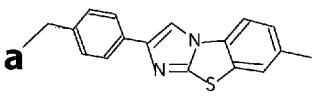
Scaffold for potent RLIs



a=alkyl, alkoxy, amide, halide
b=nothing, alky, alkoxy, amide, halide
c=nothing, alkyl, alkoxy, amide, halide

B

Scaffold for FLI (class I)



a=nothing, alkyl, alkoxy, amide, halide

FLI2

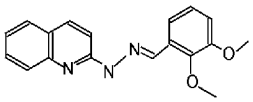
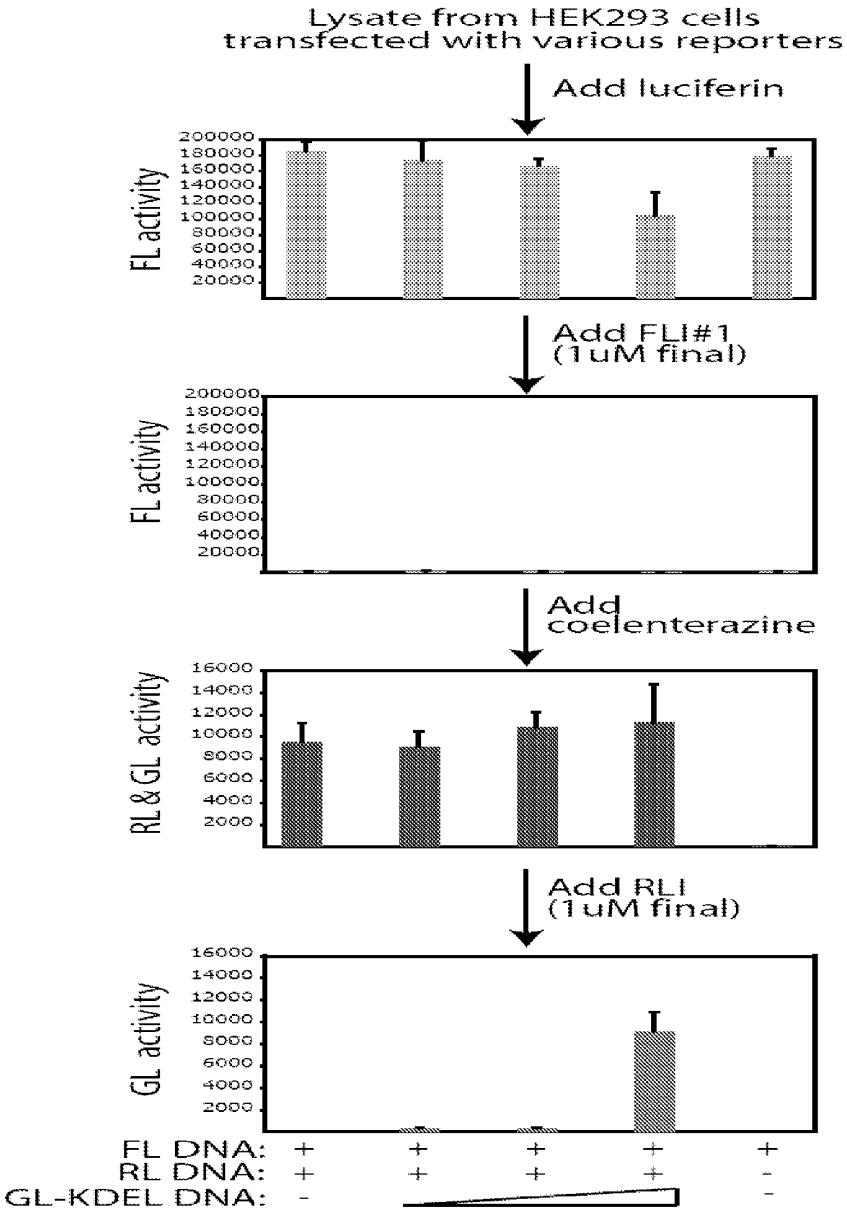
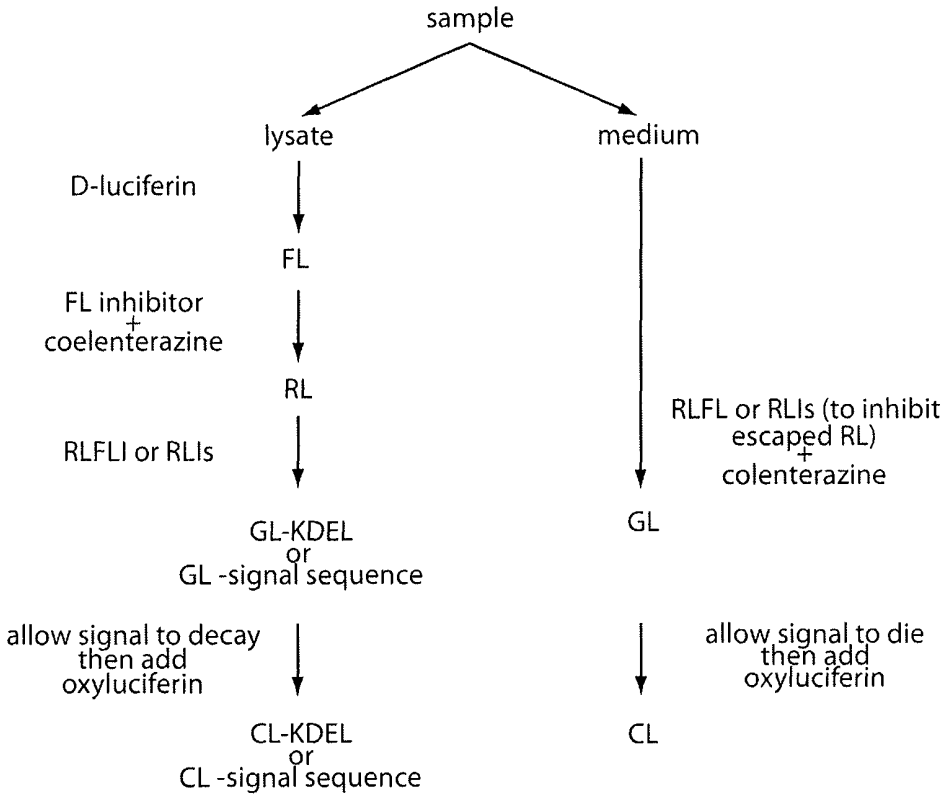


Figure 4


Lysate from HEK293 cells
transfected with various reporters
Add luciferin
FL activity
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
Add FLI#1
(1uM final)
FL activity
Add
coelenterazine
RL & GL activity
16000
14000
12000
10000
8000
6000
4000
2000
Add RLI
(1uM final)
GL activity
FL DNA: + + + + +
RL DNA: + + + + -
GL-KDEL DNA: - -



FL: firefly luciferase (cytoplasmic)
RL: Renilla luciferase (cytoplasmic
GL: Gaussia luciferase (secreted)
GL-KDEL: Gaussia luciferase with ER retention signal
GL--signal sequence: Gaussia luciferase expressed in cytoplasm
CL: Cypridina luciferase (secreted)
CL-KDEL: Cypridina luciferase with ER retention signal
CL--signal sequence: Cypridina luciferase expressed in cytoplasm
coelenterazine: substrate for RL and GL
luciferin: substrate for FL
oxyluciferin: substrate for CL

FIG. 5

MULTIPLEXED LUCIFERASE REPORTER ASSAY SYSTEMS

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made in part with government support under Grant No. 1R01GM076398 from the National Institutes of Health. The United States Government has certain rights in the invention.

TECHNICAL FIELD

The present invention relates to the fields of molecular biology, biochemistry, and drug screening. More particularly, the present invention relates to the use of multiple luciferases and multiple inhibitors thereof to create a multiplexed system permitting analysis of multiple targets at the same time and in sequence.

BACKGROUND OF THE INVENTION

Reporter genes have become an invaluable tool in studies of gene expression. They are widely used in biomedical and pharmaceutical research and also in molecular biology and biochemistry. A key feature in reporter assays is an expression cassette comprising a coding region for marker of interest and a transcriptional regulatory region, i.e., a promoter regulatory region, that drives expression of the reporter. Any agent can then be tested for direct or indirect effects on the promoter, thereby identifying agents with potential value in directing control of products naturally driven by the promoter regulatory region. Common reporters are β-galactosidase, β-glucuronidase and luciferase, which rely upon various readouts including luminescence, absorbance and fluorescence.

Luciferase is a generic term for the class of oxidative enzymes used in bioluminescence and is distinct from a photoprotein. The name is derived from Lucifer, the root of which means "light-bearer." The advantages of a luciferase assay are the high sensitivity, the absence of luciferase activity inside most of the cell types, the wide dynamic range, rapidity and low costs. One example is the firefly luciferase from the firefly *Photinus pyralis*, and this protein requires no post-translational modification for enzyme activity; it is not even toxic in high concentration (in vivo) and can be used in pro- and eukaryotic cells. Firefly luciferase catalyzes the bioluminescent oxidation of the luciferin in the presence of ATP, magnesium and oxygen.

In many assay formats, it is useful to examine multiple effects using distinct reporter cassettes. This is termed "multiplexing," i.e., using multiple readouts to simultaneous assess various effects. By using multiplexed assays, one can acquire information much more quickly. While there exist different luciferase enzymes, current assays only permit looking at two different reporters within the confines of a single assay. Thus, there remains a need to develop reagents that permit higher levels of multiplex luciferase assays.

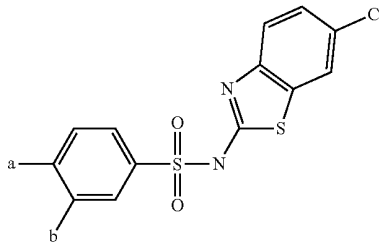
SUMMARY OF THE INVENTION

Thus, in accordance with the present invention, there is provided a method of detecting a plurality of distinct luciferase molecules comprising (a) providing a plurality of distinct luciferase molecules and substrates therefor; (b) providing a plurality of distinct luciferase inhibitors, wherein the plurality of inhibitors inhibit at least two of the plurality of distinct luciferase molecules; (c) sequentially measuring

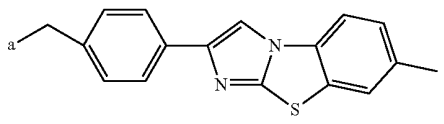
chemiluminescence from each of the plurality of distinct luciferase molecules, wherein other of the plurality of the distinct luciferase molecules are inhibited or lack substrate. The plurality of distinct luciferase molecules may be provided sequentially or simultaneously. The plurality of distinct luciferase molecules may comprise 2, 3, 4, 5 or 6 distinct luciferase molecules. The plurality of distinct luciferase inhibitors may comprise 2, 3, or 4 distinct luciferase inhibitors.

The plurality of distinct luciferase molecules may comprise two or more or each of firefly luciferase, *renilla* luciferase, gaussia luciferase, *cypridina* luciferase, gaussia luciferase lacking a secretion signal sequence or having an endoplasmic retention sequence, or *cypridina* luciferase lacking a secretion signal sequence or having an endoplasmic retention sequence. The plurality of luciferases may be expressed from cells in culture. The method may further comprise separating the cells from cell medium, such as by disrupting the cells to form a cell lysate.

The substrates may comprise two or all three of D-luciferin, oxyluciferin, and coelenterazine. The inhibitor may be selective or specific for *renilla* luciferase, such as:



wherein a is substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide, b is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide, and c is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide. Alternatively, the inhibitor may be selective or specific for firefly luciferase, such as:

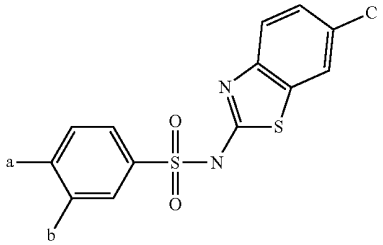


wherein a is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide. The inhibitor may be cross-reactive with *renilla* luciferase and firefly luciferase.

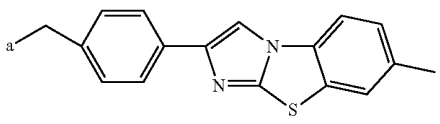
In another embodiment, there is provided a biological system comprising (a) a plurality of distinct luciferase molecules and substrates therefor; and (b) a plurality of distinct luciferase inhibitors, wherein the plurality of inhibitors inhibit at least two of the plurality of distinct luciferase molecules. The plurality of distinct luciferase molecules may comprise 2, 3, 4, 5 or 6 distinct luciferase molecules. The

plurality of distinct luciferase inhibitors may comprise 2, 3, or 4 distinct luciferase inhibitors. The plurality of distinct luciferase molecules comprises two or more or each of firefly luciferase, *renilla* luciferase, gaussia luciferase, *cypridina* luciferase, gaussia luciferase lacking a secretion signal sequence or having an endoplasmic retention sequence, or *cypridina* luciferase lacking a secretion signal sequence or having an endoplasmic retention sequence.

The system may further comprise cells that express the plurality of luciferase molecules. The substrates may comprise two or all three of D-luciferin, oxyluciferin, and coelenterazine. The inhibitor may be selective or specific for *renilla* luciferase, such as:



wherein a is substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide, b is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide, and c is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide. Alternatively, the inhibitor may be selective or specific for firefly luciferase, such as:

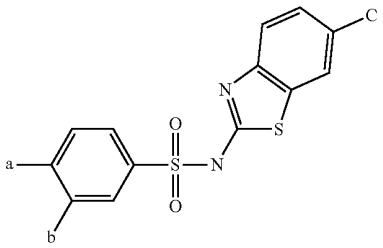


wherein a is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide. The inhibitor may be cross-reactive with *renilla* luciferase and firefly luciferase.

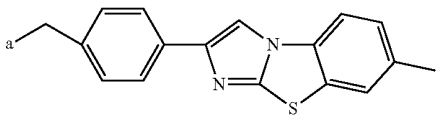
In yet another embodiment, there is provided a kit comprising (a) a plurality of distinct luciferase substrates; and (b) a plurality of distinct luciferase inhibitors. The plurality of distinct luciferase substrates may comprise D-luciferin, oxyluciferin, and coelenterazine. The plurality of distinct luciferase inhibitors may comprise inhibitors for firefly luciferase and *renilla* luciferase. The kit may further comprise cells for expression of luciferase molecules. The kit may further comprise a plurality of expression constructs comprising sequences for expressing a plurality of luciferase molecules.

In a further embodiment, there is provided a method of imaging a cell in vivo comprising (a) providing a non-human animal comprising a cell expressing a plurality of distinct luciferase molecules; (b) providing to the animal (i) luciferase substrates; and (ii) a plurality of distinct luciferase inhibitors, wherein the plurality of inhibitors inhibit at least two of the plurality of distinct luciferase molecules; (c) sequentially measuring chemiluminescence from each of the plurality of distinct luciferase molecules, wherein other of the plurality of the distinct luciferase molecules are inhibited or lack substrate. The cell may be a cancer cell. The plurality of distinct luciferase molecules may be provided sequentially or simultaneously. The plurality of distinct luciferase molecules may comprise 2, 3, 4, 5 or 6 distinct luciferase molecules. The plurality of distinct luciferase inhibitors may comprise 2, 3, or 4 distinct luciferase inhibitors.

The plurality of distinct luciferase molecules may comprise two or more or each of firefly luciferase, *renilla* luciferase, gaussia luciferase, *cypridina* luciferase, gaussia luciferase lacking a secretion signal sequence or having an endoplasmic retention sequence, or *cypridina* luciferase lacking a secretion signal sequence or having an endoplasmic retention sequence. The substrates may comprise two or all three of D-luciferin, oxyluciferin, and coelenterazine. The inhibitor may be selective or specific for *renilla* luciferase, such as:



wherein a is substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide, b is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide, and c is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide. Alternatively, the inhibitor may be selective or specific for firefly luciferase, such as:



wherein a is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide. The inhibitor may be cross-reactive with *renilla* luciferase and firefly luciferase.

Providing may comprise introducing into the non-human animal a cell previously transfected or transformed with one or more expression cassettes comprising one or more promoters driving the plurality of luciferase molecules, such as into a cancer cell. Providing may also comprise use of a transgenic non-human animal generated from stem cells transfected or transformed with one or more expression cassettes comprising one or more promoters driving the plurality of luciferase

molecules. Providing may alternatively comprise transfecting or transforming a cell of the non-human animal in vivo with one or more expression cassettes comprising one or more promoters driving the plurality of luciferase molecules, such as by transfecting or transforming with viral infection of the non-human animal.

The method may further comprise providing to the non-human animal a candidate substance, for example, wherein step (c) is performed after provision of the candidate substance, or wherein step (c) is performed before provision of the candidate substance, and further comprising, after provision of the candidate substance, sequentially measuring chemiluminescence from each of the plurality of distinct luciferase molecules, wherein other of the plurality of the distinct luciferase molecules are inhibited or lack substrate. The non-human animal may be a mouse.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

These, and other, embodiments of the invention will be better appreciated and understood when considered in conjunction with the following description and the accompanying drawings. It should be understood, however, that the following description, while indicating various embodiments of the invention and numerous specific details thereof, is given by way of illustration and not of limitation. Many substitutions, modifications, additions and/or rearrangements may be made within the scope of the invention without departing from the spirit thereof, and the invention includes all such substitutions, modifications, additions and/or rearrangements.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIGS. **1**A-D. Identification of FL and RL inhibitors from high-throughput chemical library screens. (FIG. **1**A) Schematic of two screens using the UTSW chemical library to identify firefly luciferase (FLuc) and *Renilla* luciferase (RLuc) inhibitors. (FIG. **1**B) Cell lines used in various screening steps of the screens. (FIG. **1**C) Structure and in vitro IC50 of firefly luciferase inhibitors 1 and 2 (FLI1 and FLI2). (FIG. **1**D) Structure and in vitro IC50 of *Renilla* luciferase inhibitor 1 (RLI1).

FIG. **2**. Analysis of FLIs and RLI specificity and Identification of second generation RL inhibitors with specificity for RL activity. Chemical structures of FLI- and RLI-related compounds tested in vitro for *Renilla*, firefly, Guassia, and *Cypridina* luciferase activity (RLuc, FLuc, GLuc, and CLuc activity, respectively). Note that RLI has both RLuc and FLuc inhibitory activity (henceforth called RLFLI) whereas other compounds tested have either FLuc or RLuc inhibitory activity alone. No inhibitors of GLuc or CLuc were identified from this approach.

FIG. **3**A-B. Assembling a chemical scaffold that supports specific RL and FL inhibitory activity. Compounds related to RLFLI that inhibited RL (FIG. **3**A) activity greater than 20% of control and with no activity against FLuc, GLuc, or CLuc, are shown. A general scaffold that supports specific RLuc inhibitory activity emerges. (FIG. **3**B) A general scaffold that supports specific FLuc inhibitory activity is shown.

FIG. **4**. A multiplexed screening platform premised upon sequential analysis of multiple luciferase-based read-outs. HEK293 cells transfected with indicated FLuc, RLuc or GLuc-KDEL reporters driven by a CMV promoter were lysed and luciferase activities analyzed with the following sequence of addition for substrate and inhibitors: 1) luciferin, 2) FLI1, 3) coelenterazine (reveals both RLuc and GLuc activities), and 4) an RLI (to reveal GLuc signal). GL-KDEL protein is a secreted protein but harbors an endoplasmic reticulum retention signal and is therefore found in the cell lysate as opposed to the culture medium.

FIG. **5**. A general schematic for conducting multi-luciferase assays using FLIs, RLFL, and RLIs.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. For purposes of the present invention, the following terms are defined below.

An inhibitor is defined as a molecule that is able to reduce the activity of a luciferase molecule to no more than about 20% of the normal activity level, as measured in a similar assay without the inhibitor.

A selective inhibitor is one that inhibits a particular luciferase by at least X-fold over any other luciferase, or one that has less than Y % inhibition of any other luciferase.

A specific inhibitor is one that inhibits a particular luciferase by at least Z-fold over any other luciferase, or one that has less than Y % inhibition of any other luciferase.

When used in the context of a chemical group, "hydrogen" means —H; "hydroxy" means —OH; "oxo" means =O; "halo" means independently —F, —Cl, —Br or —I; "amino" means —NH_2 (see below for definitions of groups containing the term amino, e.g., alkylamino); "hydroxyamino" means —NHOH; "nitro" means —NO_2; imino means =NH (see below for definitions of groups containing the term imino, e.g., alkylimino); "cyano" means —CN; "azido" means —N_3; in a monovalent context "phosphate" means —OP(O)$(OH)_2$ or a deprotonated form thereof; in a divalent context "phosphate" means —OP(O)(OH)O— or a deprotonated form thereof; "mercapto" means —SH; "thio" means =S; "thioether" means —S—; "sulfonamido" means —NHS$(O)_2$— (see below for definitions of groups containing the term sulfonamido, e.g., alkylsulfonamido); "sulfonyl" means —$S(O)_2$— (see below for definitions of groups containing the term sulfonyl, e.g., alkylsulfonyl); "sulfinyl" means —S(O)— (see below for definitions of groups containing the term sulfinyl, e.g., alkylsulfinyl); and "silyl" means —SiH_3 (see below for definitions of group(s) containing the term silyl, e.g., alkylsilyl).

The term "amido" (acylamino), when used without the "substituted" modifier, refers to the group —NHR, in which R is acyl, as that term is defined above. A non-limiting example of an acylamino group is —$NHC(O)CH_3$. When the term amido is used with the "substituted" modifier, it refers to groups, defined as —NHR, in which R is substituted acyl, as that term is defined above. The groups —$NHC(O)OCH_3$ and —$NHC(O)NHCH_3$ are non-limiting examples of substituted amido groups.

The term "alkyl" when used without the "substituted" modifier refers to a non-aromatic monovalent group with a saturated carbon atom as the point of attachment, a linear or branched, cyclo, cyclic or acyclic structure, no carbon-carbon double or triple bonds, and no atoms other than carbon and hydrogen. The groups, —CH_3 (Me), —CH_2CH_3 (Et), —$CH_2CH_2CH_3$ (n-Pr), —$CH(CH_3)_2$ (iso-Pr), —$CH(CH_2)_2$ (cyclopropyl), —$CH_2CH_2CH_2CH_3$ (n-Bu), —$CH(CH_3)CH_2CH_3$ (sec-butyl), —$CH_2CH(CH_3)_2$ (iso-butyl), —$C(CH_3)_3$ (tert-butyl), —$CH_2C(CH_3)_3$ (neo-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, and cyclohexylmethyl are non-limiting examples of alkyl groups. The term "substituted alkyl" refers to a non-aromatic monovalent group with a saturated carbon atom as the point of attachment, a linear or branched, cyclo, cyclic or acyclic structure, no carbon-carbon double or triple bonds, and at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S. The following groups are non-limiting examples of substituted alkyl groups: —CH_2OH, —CH_2Cl, —CH_2Br, —CH_2SH, —CF_3, —CH_2CN, —$CH_2C(O)H$, —$CH_2C(O)OH$, —$CH_2C(O)OCH_3$, —$CH_2C(O)NH_2$, —$CH_2C(O)NHCH_3$, —$CH_2C(O)CH_3$, —CH_2OCH_3, —$CH_2OCH_2CF_3$, —$CH_2OC(O)CH_3$, —CH_2NH_2, —CH_2NHCH_3, —$CH_2N(CH_3)_2$, —CH_2CH_2Cl, —CH_2CH_2OH, —CH_2CF_3, —$CH_2CH_2OC(O)CH_3$, —$CH_2CH_2NHCO_2C(CH_3)_3$, and —$CH_2Si(CH_3)_3$.

The term "alkenyl" when used without the "substituted" modifier refers to a monovalent group with a nonaromatic carbon atom as the point of attachment, a linear or branched, cyclo, cyclic or acyclic structure, at least one nonaromatic carbon-carbon double bond, no carbon-carbon triple bonds, and no atoms other than carbon and hydrogen. Non-limiting examples of alkenyl groups include: —$CH{=}CH_2$ (vinyl), —$CH{=}CHCH_3$, —$CH{=}CHCH_2CH_3$, —$CH_2CH{=}CH_2$ (allyl), —$CH_2CH{=}CHCH_3$, and —$CH{=}CH{-}C_6H_5$. The term "substituted alkenyl" refers to a monovalent group with a nonaromatic carbon atom as the point of attachment, at least one nonaromatic carbon-carbon double bond, no carbon-carbon triple bonds, a linear or branched, cyclo, cyclic or acyclic structure, and at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S. The groups, —$CH{=}CHF$, —$CH{=}CHCl$ and —$CH{=}CHBr$, are non-limiting examples of substituted alkenyl groups.

The term "alkynyl" when used without the "substituted" modifier refers to a monovalent group with a nonaromatic carbon atom as the point of attachment, a linear or branched, cyclo, cyclic or acyclic structure, at least one carbon-carbon triple bond, and no atoms other than carbon and hydrogen. The groups, —$C{\equiv}CH$, —$C{\equiv}CCH_3$, —$C{\equiv}CC_6H_5$ and —$CH_2C{\equiv}CCH_3$, are non-limiting examples of alkynyl groups. The term "substituted alkynyl" refers to a monovalent group with a nonaromatic carbon atom as the point of attachment and at least one carbon-carbon triple bond, a linear or branched, cyclo, cyclic or acyclic structure, and at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S. The group, —$C{\equiv}CSi(CH_3)_3$, is a non-limiting example of a substituted alkynyl group.

The term "alkoxy" when used without the "substituted" modifier refers to the group —OR, in which R is an alkyl, as that term is defined above. Non-limiting examples of alkoxy groups include: —OCH_3, —OCH_2CH_3, —$OCH_2CH_2CH_3$, —$OCH(CH_3)_2$, —$OCH(CH_2)_2$, —O— cyclopentyl, and —O— cyclohexyl. The term "substituted alkoxy" refers to the group —OR, in which R is a substituted alkyl, as that term is defined above. For example, —OCH_2CF_3 is a substituted alkoxy group.

II. LUCIFERASES

Luciferase is a generic term for the class of oxidative enzymes used in bioluminescence and is distinct from a photoprotein. One famous example is the firefly luciferase from *Photinus pyralis*. "Firefly luciferase" as a laboratory reagent usually refers to *P. pyralis* luciferase, although recombinant luciferases from several other species of fireflies are also commercially available.

In luminescent reactions, light is produced by the oxidation of a luciferin (a pigment):

$$\text{luciferin}+O_2 \rightarrow \text{oxyluciferin}+\text{light}$$

The most common luminescent reactions release CO_2 as a product. The rates of this reaction between luciferin and oxygen are extremely slow until they are catalyzed by luciferase, sometimes mediated by the presence of cofactors such as calcium ions or ATP. The reaction catalyzed by firefly luciferase takes place in two steps:

$$\text{luciferin}+\text{ATP} \rightarrow \text{luciferyl adenylate}+PP_i$$

$$\text{luciferyl adenylate}+O_2 \rightarrow \text{oxyluciferin}+\text{AMP}+\text{light}$$

The reaction is very energetically efficient: nearly all of the energy input into the reaction is transformed into light. As a comparison, the incandescent light bulb loses about 90% of its energy to heat. Photon emission can be detected by light sensitive apparatus such as a luminometer or modified optical microscopes. This allows observation of biological processes.

A subtle structural difference in luciferase has been discovered to be the cause of the change in bioluminescence emission color from a yellow-green to red. The structure of wild-type luciferase and red mutant (S286N) luciferase from the Japanese Genji-Botaru (*Luciola cruciata*) in complex with an intermediate analogue 5'-O—[N-(dehydroluciferyl)-sulfamoyl]adenosine (DLSA) was examined and studies showed that the wild-type luciferase complexed with DLSA exhibited a 'closed form' of the active site, where the side chain of amino acid isoleucine 288 moved towards the benzothiazole ring of DLSA, creating a rigid hydrophobic pocket. The 'closed form' wild-type luciferase bound the excited state of oxyluciferin in a highly rigid and nonpolar microenvironment, minimizing energy loss before emitting yellow-green light. The S286N luciferase complexed with DLSA exhibited an 'open form' of the active site, where the amino acid side chain of isoleucine 288 did not move towards the benzothiazole ring of DLSA, creating a less rigid and less hydrophobic microenvironment. The 'open form' S286N luciferase had a less rigid microenvironment allowing some energy loss from the excited state of oxyluciferin, which resulted in the emission of low-energy red light.

A variety of organisms regulate their light production using different luciferases in a variety of light-emitting reactions. The most famous are the fireflies, although the enzyme exists in organisms as different as the Jack-O-Lantern mushroom (*Omphalotus olearius*) and many marine creatures. In fireflies, the oxygen required is supplied through a tube in the abdomen called the abdominal trachea. The luciferases of fireflies, of which there are over 2000 species, and of the Elateroidea (fireflies, click beetles and relatives) in general, are diverse enough to be useful in molecular phylogeny. The most thoroughly studied luciferase is that of the Photinini firefly *Photinus pyralis*, which has an optimum pH of 7.8.

Also well studied is the luciferase from *Renilla reniformis*. In this organism, the luciferase is closely associated with a luciferin-binding protein as well as a green fluorescent pro-

tein (GFP). Calcium triggers release of the luciferin (coelenterazine) from the luciferin binding protein. The substrate is then available for oxidation by the luciferase, where it is degraded to coelenteramine with a resultant release of energy. In the absence of GFP, this energy would be released as a photon of blue light (peak emission wavelength 482 nm). However, due to the closely associated GFP, the energy released by the luciferase is instead coupled through resonance energy transfer to the fluorophore of the GFP, and is subsequently released as a photon of green light (peak emission wavelength 510 nm). The catalyzed reaction is:

$$\text{coelenterazine}+O_2 \rightarrow \text{coelenteramine}+CO_2+\text{photon of light}$$

Newer luciferases have recently been identified that, unlike *Renilla* or Firefly luciferase, are naturally secreted molecules. One such example is the *Metridia* luciferase (MetLuc) that is derived from the marine copepod *Metridia longa*. The *M. longa* secreted luciferase gene encodes a 24 kDa protein containing an N-terminal secretory signal peptide of 17 amino acid residues. The sensitivity and high signal intensity of this luciferase molecule proves advantageous in many reporter studies. Some of the benefits of using a secreted reporter molecule like MetLuc is its no-lysis protocol that allows one to be able to conduct live cell assays and multiple assays on the same cell.

Gaussia luciferase (GLuc) is a 20 kDa luciferase from the marine cocepod *Gaussia princeps*. This luciferase, which does not require ATP, catalyzes the oxidation of the substrate coelenterazine in a reaction that produces light (480 nm), and has considerable advantages over other luminescent reporter genes. It is normally secreted from the cells and its secretion signal also functions very efficiently in mammalian cells. GLuc offers the advantage of a greatly increased bioluminescent signal relative to the commonly used firefly (Fluc) and *Renilla* luciferases (RLuc). GLuc was determined to emit light with a specific activity of 4.2×10^{24} photons/s/mol, the highest reported activity for any characterized luciferase.

Cypridina luciferase (CLuc) is isolated from the marine ostracod *Cypridina noctiluca* and is efficiently secreted from mammalian cells. CLuc differs from Fluc in the form of luciferin it uses as a substrate (*Cypridina* luciferin), it its independence from ATP to achieve light emission. CLuc does not react with coelenterazine, a common substrate of marine luciferases. This allows the simultaneous detection of both of FLuc, Rluc, or GLuc with CLuc expressed from the same cells provided the cell-derived samples can be divided and independently analyzed for each enzymatic activity, or if a method for sequentially measuring individual enzymatic activities from the same cell-derived sample is in place.

Luciferase can be produced in the lab through genetic engineering for a number of purposes. Luciferase genes can be synthesized and inserted into organisms or transfected into cells. Mice, silkworms, and potatoes are just a few organisms that have already been engineered to produce the protein. In biological research, luciferase commonly is used as a reporter to assess the transcriptional activity in cells that are transfected with a genetic construct containing the luciferase gene under the control of a promoter of interest. Luciferase can also be used to detect the level of cellular ATP in cell viability assays or for kinase activity assays. Additionally, pro-luminescent molecules that are converted to luciferin upon activity of a particular enzyme can be used to detect enzyme activity in coupled or two-step luciferase assays. Such substrates have been used to detect caspase activity and cytochrome P450 activity, among others.

Whole animal imaging (referred to as in vivo or, occasionally, ex vivo imaging) is a powerful technique for studying cell populations in live animals, such as mice. Different types of cells (e.g., bone marrow stem cells, T-cells) can be engineered to express a luciferase allowing their non-invasive visualization inside a live animal using a sensitive charge-couple device camera (CCD camera). This technique has been used to follow tumorigenesis and response of tumors to treatment in animal models. However, environmental factors and therapeutic interferences may cause some discrepancies between tumor burden and bioluminescence intensity in relation to changes in proliferative activity. The intensity of the signal measured by in vivo imaging may depend on various factors, such as D-luciferin absorption through the peritoneum, blood flow, cell membrane permeability, availability of co-factors, intracellular pH and transparency of overlying tissue, in addition to the amount of luciferase.

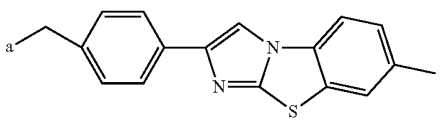
Luciferase can be used in blood banks to determine if red blood cells are starting to break down. Forensic investigators can use a dilute solution containing the enzyme to uncover traces of blood remaining on surfaces at a crime scene. Luciferase is a heat sensitive protein that is used in studies on protein denaturation, testing the protective capacities of heat shock proteins. The opportunities for using luciferase continue to expand.

III. LUCIFERASE INHIBITORS

In accordance with the present invention, a variety of luciferase inhibitors are now provided. As shown in FIG. **2**, a number of inhibitors have been tested and can be used in accordance with the present invention to inhibit firefly luciferase, *renilla* luciferase or both. FIG. **3** shows one of the inhibitors from FIG. **2**, which is a cross-inhibitor, and a number of additional inhibitors that are specific or selective for *renilla* luciferase, as well as a generic structure indicating the core for such inhibitors.

A. Firefly Luciferase

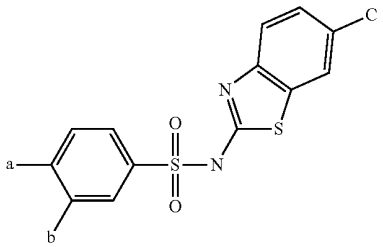
Firefly luciferase selective inhibitors are identified as compounds 32, FLI1 (also named compound 5), and FLI2. A scaffold for such compounds is shown below and in FIG. **3**:



wherein a is H, alkyl substituted or unsubstituted (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide.

B. *Renilla* Luciferase

Renilla luciferase selective inhibitors are identified as compounds 13, 23, 11, 12, 25, 35, 19, 42, 34, 16, 37 and 20. A scaffold for such compounds is shown below and in FIG. **3**:



wherein a is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide, wherein b is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide, and c is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide.

C. Cross-Inhibitors

An inhibitor effective on both *renilla* and firefly luciferase are identified as compound RFLI/6.

IV. ASSAY COMPONENTS

A. Expression Constructs

The present invention may involve using expression constructs to luciferase molecules in the context of screening assays. In certain embodiments, it is contemplated that the expression construct comprises nucleic acid sequences encoding luciferase polypeptides, as discussed above. Generally, such methods involve the generation of expression constructs containing, for example, a heterologous nucleic acid sequence encoding the luciferase and a means for its expression, replicating the vector in an appropriate cell.

Luciferase enzymes may be fused to proteins of interest in order to interrogate changes in the proteins' half-lives in response to various cellular perturbations including addition of signaling proteins (such as Wnt, Hh, FGF proteins), chemicals, or siRNAs. Luciferase-fusion proteins co-expressed in cells by introduction of appropriate expression constructs could also be used to examine protein-protein interactions, when a specific protein of interest is immunoprecipitated or otherwise isolated from cells expressing multiple luciferase-fusion proteins and co-purified luciferase activities measured as a determinant of interaction between luciferase-fusion proteins and the target protein.

Yet further, in certain embodiments, it is contemplated that nucleic acid or proteinaceous sequences may be co-expressed with other selected nucleic acid or proteinaceous sequences in the same host cell. Co-expression may be achieved by co-transfecting the host cell with two or more distinct recombinant vectors. Alternatively, a single recombinant vector may be constructed to include fused coding regions for luciferase and other products.

1. Regulatory Sequences

As used in the present invention, the term "expression vector" refers to any type of genetic construct comprising a nucleic acid sequence coding for luciferase polypeptides. In some cases, DNA molecules are then translated into a protein, polypeptide, or peptide. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host cell.

In certain embodiments, the nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units, or from the cytomegalovirus (CMV). These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins. Other promoters that can be used, include IPTG-inducible promoter.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box.

In the bacterial genome, there are several conserved features in a bacterial promoter: the start site or point, the 10-35 bp sequence upstream of the start site, and the distance between the 10-35 bp sequences upstream of the start site. The start point is usually (90% of the time) a purine. Upstream of the start site is a 6 bp region that is recognizable in most promoters. The distance varies from 9-18 bp upstream of the start site, however, the consensus sequence is TATAAT. Another conserved hexamer is centered at 35 bp upstream of the start site. This consensus sequence is TTGACA. Additional promoter elements regulate the frequency of transcriptional initiation. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another.

By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized or assessed. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the gene product. In certain embodiments, the promoter will be selected based on an interest in the regulation of a coding sequence naturally associated with the promoter, and the ability of candidate substance to alter the activity of that promoter, once linked to a luciferase coding region, will be assessed.

In certain embodiments of the invention, the cells contain expression cassettes of the present invention, a cell may be selected using a selectable marker in the expression construct. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (tk) or chloramphenicol acetyltransferase (CAT) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed

simultaneously with the nucleic acid encoding a gene product. Further examples of selectable markers are well known to one of skill in the art.

In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions such as replications of origin, transcription termination signals, multipurpose cloning sites, internal ribosome entry site, etc. It is contemplated in the present invention, that virtually any type of vector may be employed in any known or later discovered method to deliver nucleic acids encoding a luciferase polypeptide. Where incorporation into an expression vector is desired, the nucleic acid luciferase polypeptides may also comprise a natural intron or an intron derived from another gene. Such vectors may be viral or non-viral vectors as described herein, and as known to those skilled in the art.

2. Vectors

In particular embodiments of the invention, a plasmid vector is contemplated for use to transform a host cell. In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences that are capable of providing phenotypic selection in transformed cells. Plasmid vectors are well known and are commercially available. Such vectors include, but are not limited to, pWL-NEO, pSV2CAT, pOG44, PXT1, pSG (Stratagene) pSVK3, pBSK, pBR322, pcDNA3 and pUC vectors.

Yet further, prokaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available. One skilled in the art is aware of the various prokaryote-based expression systems. Exemplary systems from PROMEGA include, but are not limited to, pGEMEX®-1 vector, pGEMX®-2 Vector, and Pinpoint control Vectors. Examples from STRATAGENE® include, but are not limited to, pBK Phagemid Vector, which is inducible by IPTG, pSPUTK In vitro Translation Vector, pET Expression systems, Epicurian Coli® BL21 Competent Cells and pDual™ Expression System.

3. Transfection/Transformation

In order to effect expression of constructs, the expression construct must be delivered into a cell. This delivery may be accomplished in vitro, as in laboratory procedures for transforming cells lines using well developed procedures. Transformation of cell lines can be achieved using a variety of techniques, although the techniques generally fall into either viral or non-viral methods. These techniques and modifications are well known in the art. Thus, it is well within the scope of the present invention that a cell line may be transformed by any available transformation procedure or modification thereof.

Once the cell is transformed with the vector, the cells are cultured such that the cells multiply resulting in production of the desired protein. In certain embodiments, the cells that are transformed can be bacterial cells. Thus, a skilled artisan is cognizant that the development of microorganisms in culture media is dependent upon a number of very important factors, e.g., the proper nutrients must be available; oxygen or other gases must be available as required; a certain degree of moisture is necessary; the media must be of the proper reaction; proper temperature relations must prevail; the media must be sterile; and contamination must be prevented.

A satisfactory microbiological culture contains available sources of hydrogen donors and acceptors, carbon, nitrogen, sulfur, phosphorus, inorganic salts, and, in certain cases, vitamins or other growth promoting substances. The addition of peptone provides a readily available source of nitrogen and carbon. Furthermore, different media results in different growth rates and different stationary phase densities. A rich media results in a short doubling time and higher cell density at a stationary phase. Minimal media results in slow growth and low final cell densities. Efficient agitation and aeration increases final cell densities. A skilled artisan will be able to determine which type of media is best suited to culture a specific type of microorganism. For example, since 1927, the DIFCO manual has been used in the art as a guide for culture media and nutritive agents for microbiology.

Non-viral Methods. Suitable methods for nucleic acid delivery for transformation of an organelle, a cell, a tissue or an organism for use with the current invention are believed to include virtually any method by which a nucleic acid (e.g., DNA) can be introduced into an organelle, a cell, a tissue or an organism, as described herein or as would be known to one of ordinary skill in the art. Such methods include, but are not limited to, direct delivery of DNA such as by injection (U.S. Pat. Nos. 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859, each incorporated herein by reference), including microinjection (Harland and Weintraub, 1985; U.S. Pat. No. 5,789,215, incorporated herein by reference); by electroporation (U.S. Pat. No. 5,384,253, incorporated herein by reference); by calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe et al., 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer et al., 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley et al., 1979; Nicolau et al., 1987; Wong et al., 1980; Kaneda et al., 1989; Kato et al., 1991); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Pat. Nos. 5,610,042; 5,322,783, 5,563,055, 5,550,318, 5,538,877 and 5,538,880, and each incorporated herein by reference); by agitation with silicon carbide fibers (Kaeppler et al., 1990; U.S. Pat. Nos. 5,302,523 and 5,464,765, each incorporated herein by reference); or by PEG-mediated transformation of protoplasts (Omirulleh et al., 1993; U.S. Pat. Nos. 4,684,611 and 4,952,500, each incorporated herein by reference); by desiccation/inhibition-mediated DNA uptake (Potrykus et al., 1985). Through the application of techniques such as these, organelle(s), cell(s), tissue(s) or organism(s) may be stably or transiently transformed.

In certain embodiments, a nucleic acid may be delivered to an organelle, a cell, a tissue or an organism via one or more injections (i.e., a needle injection), such as, for example, either subcutaneously, intradermally, intramuscularly, intervenously or intraperitoneally. Methods of injection of vaccines are well known to those of ordinary skill in the art (e.g., injection of a composition comprising a saline solution). Further embodiments of the present invention include the introduction of a nucleic acid by direct microinjection. Direct microinjection has been used to introduce nucleic acid constructs into *Xenopus* oocytes (Harland and Weintraub, 1985).

In certain embodiments of the present invention, a nucleic acid is introduced into an organelle, a cell, a tissue or an organism via electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge. In some variants of this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells (U.S. Pat. No. 5,384,253, incorporated herein

by reference). Alternatively, recipient cells can be made more susceptible to transformation by mechanical wounding.

Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter et al., 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa et al., 1986) in this manner.

To effect transformation by electroporation in cells such as, for example, plant cells, one may employ either friable tissues, such as a suspension culture of cells or embryogenic callus or alternatively one may transform immature embryos or other organized tissue directly. In this technique, one would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Examples of some species which have been transformed by electroporation of intact cells include maize (U.S. Pat. No. 5,384,253; Rhodes et al., 1995; D'Halluin et al., 1992), wheat (Zhou et al., 1993), tomato (Hou and Lin, 1996), soybean (Christou et al., 1987) and tobacco (Lee et al., 1989).

One also may employ protoplasts for electroporation transformation of plant cells (Bates, 1994; Lazzeri, 1995). For example, the generation of transgenic soybean plants by electroporation of cotyledon-derived protoplasts is described by Dhir and Widholm in International Patent Application No. WO 9217598, incorporated herein by reference. Other examples of species for which protoplast transformation has been described include barley (Lazerri, 1995), sorghum (Battraw and Hall, 1991), maize (Bhattacharjee et al., 1997), wheat (He et al., 1994) and tomato (Tsukada, 1989).

In other embodiments of the present invention, a nucleic acid is introduced to the cells using calcium phosphate precipitation. Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe et al., 1990).

In another embodiment, a nucleic acid is delivered into a cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal, 1985).

Additional embodiments of the present invention include the introduction of a nucleic acid by direct sonic loading. LTK$^-$ fibroblasts have been transfected with the thymidine kinase gene by sonication loading (Fechheimer et al., 1987).

In a further embodiment of the invention, a nucleic acid may be entrapped in a lipid complex such as, for example, a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated is an nucleic acid complexed with Lipofectamine (Gibco BRL) or Superfect (Qiagen).

Liposome-mediated nucleic acid delivery and expression of foreign DNA in vitro has been very successful (Nicolau and Sene, 1982; Fraley et al., 1979; Nicolau et al., 1987). The feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells has also been demonstrated (Wong et al., 1980).

In certain embodiments of the invention, a liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda et al., 1989). In other embodiments, a liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato et al., 1991). In yet further embodiments, a liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In other embodiments, a delivery vehicle may comprise a ligand and a liposome.

Still further, a nucleic acid may be delivered to a target cell via receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis that will be occurring in a target cell. In view of the cell type-specific distribution of various receptors, this delivery method adds another degree of specificity to the present invention.

Certain receptor-mediated gene targeting vehicles comprise a cell receptor-specific ligand and a nucleic acid-binding agent. Others comprise a cell receptor-specific ligand to which the nucleic acid to be delivered has been operatively attached. Several ligands have been used for receptor-mediated gene transfer (Wu and Wu, 1987; Wagner et al., 1990; Perales et al., 1994; Myers, EPO 0273085), which establishes the operability of the technique. Specific delivery in the context of another mammalian cell type has been described (Wu and Wu, 1993; incorporated herein by reference). In certain aspects of the present invention, a ligand will be chosen to correspond to a receptor specifically expressed on the target cell population.

In other embodiments, a nucleic acid delivery vehicle component of a cell-specific nucleic acid targeting vehicle may comprise a specific binding ligand in combination with a liposome. The nucleic acid(s) to be delivered are housed within the liposome and the specific binding ligand is functionally incorporated into the liposome membrane. The liposome will thus specifically bind to the receptor(s) of a target cell and deliver the contents to a cell. Such systems have been shown to be functional using systems in which, for example, epidermal growth factor (EGF) is used in the receptor-mediated delivery of a nucleic acid to cells that exhibit upregulation of the EGF receptor.

In still further embodiments, the nucleic acid delivery vehicle component of a targeted delivery vehicle may be a liposome itself, which will preferably comprise one or more lipids or glycoproteins that direct cell-specific binding. For example, lactosyl-ceramido, a galactose-terminal asialganglioside, have been incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes (Nicolau et al., 1987). It is contemplated that the tissue-specific transforming constructs of the present invention can be specifically delivered into a target cell in a similar manner.

Viral delivery. The capacity of certain viral vectors to efficiently infect or enter cells, to integrate into a host cell genome and stably express viral genes, have led to the development and application of a number of different viral vector systems (Robbins et al., 1998). Viral systems are currently being developed for use as vectors for ex vivo and in vivo gene transfer. For example, adenovirus, herpes-simplex virus, retrovirus and adeno-associated virus vectors are being evaluated currently for treatment of diseases such as cancer, cystic fibrosis, Gaucher disease, renal disease and arthritis (Robbins and Ghivizzani, 1998; Imai et al., 1998; U.S. Pat. No. 5,670,488). The various viral vectors described below, present specific advantages and disadvantages, depending on the particular gene-therapeutic application.

In particular embodiments, an adenoviral expression vector is contemplated for the delivery of expression constructs. Adenoviruses comprise linear, double-stranded DNA, with a genome ranging from 30 to 35 kb in size (Reddy et al., 1998; Morrison et al., 1997; Chillon et al., 1999). An adenovirus expression vector according to the present invention comprises a genetically engineered form of the adenovirus. Advantages of adenoviral gene transfer include the ability to infect a wide variety of cell types, including non-dividing cells, a mid-sized genome, ease of manipulation, high infectivity and the ability to be grown to high titers (Wilson, 1996). Further, adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner, without potential genotoxicity associated with other viral vectors. Adenoviruses also are structurally stable (Marienfeld et al., 1999) and no genome rearrangement has been detected after extensive amplification (Parks et al., 1997; Bett et al., 1993).

A common approach for generating an adenoviruses for use as a gene transfer vector is the deletion of the E1 gene (Er), which is involved in the induction of the E2, E3 and E4 promoters (Graham and Prevec, 1995). Subsequently, a therapeutic gene or genes can be inserted recombinantly in place of the E1 gene, wherein expression of the therapeutic gene(s) is driven by the E1 promoter or a heterologous promoter. The $E1^-$, replication-deficient virus is then proliferated in a "helper" cell line that provides the E1 polypeptides in trans (e.g., the human embryonic kidney cell line 293). Thus, in the present invention it may be convenient to introduce the transforming construct at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. Alternatively, the E3 region, portions of the E4 region or both may be deleted, wherein a heterologous nucleic acid sequence under the control of a promoter operable in eukaryotic cells is inserted into the adenovirus genome for use in gene transfer (U.S. Pat. Nos. 5,670,488; 5,932,210, each specifically incorporated herein by reference).

In certain embodiments of the invention, the use of retroviruses for gene delivery are contemplated. Retroviruses are RNA viruses comprising an RNA genome. When a host cell is infected by a retrovirus, the genomic RNA is reverse transcribed into a DNA intermediate which is integrated into the chromosomal DNA of infected cells. This integrated DNA intermediate is referred to as a provirus. A particular advantage of retroviruses is that they can stably infect dividing cells with a gene of interest (e.g., a therapeutic gene) by integrating into the host DNA, without expressing immunogenic viral proteins. Theoretically, the integrated retroviral vector will be maintained for the life of the infected host cell, expressing the gene of interest.

A recombinant retrovirus of the present invention may be genetically modified in such a way that some of the structural, infectious genes of the native virus have been removed and replaced instead with a nucleic acid sequence to be delivered to a target cell (U.S. Pat. Nos. 5,858,744; 5,739,018, each incorporated herein by reference). After infection of a cell by the virus, the virus injects its nucleic acid into the cell and the retrovirus genetic material can integrate into the host cell genome. The transferred retrovirus genetic material is then transcribed and translated into proteins within the host cell. As with other viral vector systems, the generation of a replication-competent retrovirus during vector production or during therapy is a major concern. Retroviral vectors suitable for use in the present invention are generally defective retroviral vectors that are capable of infecting the target cell, reverse transcribing their RNA genomes, and integrating the reverse transcribed DNA into the target cell genome, but are incapable of replicating within the target cell to produce infectious retroviral particles (e.g., the retroviral genome transferred into the target cell is defective in gag, the gene encoding virion structural proteins, and/or in pol, the gene encoding reverse transcriptase). Thus, transcription of the provirus and assembly into infectious virus occurs in the presence of an appropriate helper virus or in a cell line containing appropriate sequences enabling encapsidation without coincident production of a contaminating helper virus.

Herpes simplex virus (HSV) type I and type II contain a double-stranded, linear DNA genome of approximately 150 kb, encoding 70-80 genes. Wild type HSV are able to infect cells lytically and to establish latency in certain cell types (e.g., neurons). Similar to adenovirus, HSV also can infect a variety of cell types including muscle (Yeung et al., 1999), ear (Derby et al., 1999), eye (Kaufman et al., 1999), tumors (Yoon et al., 1999; Howard et al., 1999), lung (Kohut et al., 1998), neuronal (Gamido et al., 1999; Lachmann and Efstathiou, 1999), liver (Miyatake et al., 1999; Kooby et al., 1999) and pancreatic islets (Rabinovitch et al., 1999).

For use in therapeutic gene delivery, HSV must be rendered replication-defective. Protocols for generating replication-defective HSV helper virus-free cell lines have been described (U.S. Pat. Nos. 5,879,934; 5,851,826, each specifically incorporated herein by reference in its entirety). One IE protein, Infected Cell Polypeptide 4 (ICP4), also known as alpha 4 or Vmw175, is absolutely required for both virus infectivity and the transition from IE to later transcription. Thus, due to its complex, multifunctional nature and central role in the regulation of HSV gene expression, ICP4 has typically been the target of HSV genetic studies.

Phenotypic studies of HSV viruses deleted of ICP4 indicate that such viruses will be potentially useful for gene transfer purposes (Krisky et al., 1998a). One property of viruses deleted for ICP4 that makes them desirable for gene transfer is that they only express the five other IE genes: ICP0, ICP6, ICP27, ICP22 and ICP47 (DeLuca et al., 1985), without the expression of viral genes encoding proteins that direct viral DNA synthesis, as well as the structural proteins of the virus. This property is desirable for minimizing possible deleterious effects on host cell metabolism or an immune response following gene transfer. Further deletion of IE genes ICP22 and ICP27, in addition to ICP4, substantially improve reduction of HSV cytotoxicity and prevented early and late viral gene expression (Krisky et al., 1998b).

Adeno-associated virus (AAV), a member of the parvovirus family, is a human virus that is increasingly being used for gene delivery therapeutics. AAV has several advantageous features not found in other viral systems. First, AAV can infect a wide range of host cells, including non-dividing cells. Second, AAV can infect cells from different species. Third, AAV has not been associated with any human or animal disease and does not appear to alter the biological properties of the host cell upon integration. For example, it is estimated that 80-85% of the human population has been exposed to AAV. Finally, AAV is stable at a wide range of physical and chemical conditions which lends itself to production, storage and transportation requirements.

AAV has been engineered to deliver genes of interest by deleting the internal non-repeating portion of the AAV genome and inserting a heterologous gene between the ITRs. The heterologous gene may be functionally linked to a heterologous promoter (constitutive, cell-specific, or inducible) capable of driving gene expression in target cells. To produce infectious recombinant AAV (rAAV) containing a heterolo-

gous gene, a suitable producer cell line is transfected with a rAAV vector containing a heterologous gene. The producer cell is concurrently transfected with a second plasmid harboring the AAV rep and cap genes under the control of their respective endogenous promoters or heterologous promoters. Finally, the producer cell is infected with a helper virus.

Once these factors come together, the heterologous gene is replicated and packaged as though it were a wild-type AAV genome. When target cells are infected with the resulting rAAV virions, the heterologous gene enters and is expressed in the target cells. Because the target cells lack the rep and cap genes and the adenovirus helper genes, the rAAV cannot further replicate, package or form wild-type AAV.

Lentiviruses are complex retroviruses, which, in addition to the common retroviral genes gag, pol, and env, contain other genes with regulatory or structural function. The higher complexity enables the virus to modulate its life cycle, as in the course of latent infection. Some examples of lentivirus include the Human Immunodeficiency Viruses: HIV-1, HIV-2 and the Simian Immunodeficiency Virus: SIV. Lentiviral vectors have been generated by multiply attenuating the HIV virulence genes, for example, the genes env, vif, vpr, vpu and nef are deleted making the vector biologically safe.

Other viral vectors such as poxvirus; e.g., vaccinia virus (Gnant et al., 1999; Gnant et al., 1999), alpha virus; e.g., sindbis virus, Semliki forest virus (Lundstrom, 1999), reovirus (Coffey et al., 1998) and influenza A virus (Neumann et al., 1999) are contemplated for use in the present invention and may be selected according to the requisite properties of the target system.

In certain embodiments, vaccinia viral vectors are contemplated for use in the present invention. Vaccinia virus is a particularly useful eukaryotic viral vector system for expressing heterologous genes. For example, when recombinant vaccinia virus is properly engineered, the proteins are synthesized, processed and transported to the plasma membrane. Vaccinia viruses as gene delivery vectors have recently been demonstrated to transfer genes to human tumor cells, e.g., EMAP-II (Gnant et al., 1999), inner ear (Derby et al., 1999), glioma cells, e.g., p53 (Timiryasova et al., 1999) and various mammalian cells, e.g., P-450 (U.S. Pat. No. 5,506,138). The preparation, growth and manipulation of vaccinia viruses are described in U.S. Pat. Nos. 5,849,304 and 5,506,138 (each specifically incorporated herein by reference).

Chimeric or hybrid viral vectors are being developed for use in therapeutic gene delivery and are contemplated for use in the present invention. Chimeric poxyiral/retroviral vectors (Holzer et al., 1999), adenoviral/retroviral vectors (Feng et al., 1997; Bilbao et al., 1997; Caplen et al., 1999) and adenoviral/adeno-associated viral vectors (Fisher et al., 1996; U.S. Pat. No. 5,871,982) have been described.

These "chimeric" viral gene transfer systems can exploit the favorable features of two or more parent viral species. For example, Wilson et al., provide a chimeric vector construct which comprises a portion of an adenovirus, AAV 5' and 3' ITR sequences and a selected transgene, described below (U.S. Pat. No. 5,871,983, specifically incorporate herein by reference).

B. Host Cells

As used herein, the terms "cell," "cell line," and "cell culture" may be used interchangeably. All of these terms also include their progeny, which is any and all subsequent generations formed by cell division. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. A host cell may be "transfected" or "transformed," which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny. As used herein, the terms "engineered" and "recombinant" cells or host cells are intended to refer to a cell into which an exogenous nucleic acid sequence, such as, for example, luciferase molecules or a construct thereof. Therefore, recombinant cells are distinguishable from naturally occurring cells that do not contain a recombinantly introduced nucleic acid. In particular embodiments of the present invention, the host cell is a eukaryotic cell that has been transformed with one or more luciferase encoding constructs.

C. Purification of Proteins

In certain embodiments, luciferase proteins may be are expressed using expression systems and may further be purified using standard techniques. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamido gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting

the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi et al., 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, etc. There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength).

A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Conconavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography.

V. MULTIPLEXED ASSAY FORMATS

A. Transcriptional Reporter Assays

In a first embodiment, the present invention is drawn to transcriptional assays where the luciferase gene is linked to a promoter of interest. When active, the promoter drives transcription of the luciferase mRNA, which is subsequently translated into an active enzyme and activity can be measured. The number of different formats which utilize this basic format are myriad.

One version involves the use of candidates that merely assess their ability to activate pathways upstream of the promoter, such as by increasing or decreasing the amount of transcription factors. Alternatively, the transcriptional readout may be artificial in the sense that the promoter is chosen merely for convenience, and the assay measures another biological event that results in subsequent activation of the promoter. This would include various forms of the "two-hybrid" transcription assay, where the interaction of two molecules is measured by joining each to transcriptional regulatory factors that must act in tandem to promote transcription—in the absence of an interaction, the promoter remains inactive. Many other embodiments exist.

B. Protein-Protein Interaction Assays

Interaction of luciferase fusion proteins can be measured from immunoprecipitation of a target protein from cell lysates expressing additionally expressing interacting proteins fused to various luciferase enzymes.

C. Protein Stability Assays

The levels of several proteins of interest can be monitored by fusing said proteins with luciferase enzymes and monitoring luciferase activities in cells treated with various perturbagens including signaling molecules, small molecules, or siRNAs. Protein stability assays can be also combined with transcription-based reporters using a multiplexed luciferase system.

D. Target Cleavage Assays

The present invention also contemplates assays where the luciferase signal is dependent upon the cleavage of a target molecule by one or more enzymatic agents, such as a protease. In one such format, the luciferase is rendered non-functional by inclusion of a protease site that, when cleaved, results in restoration of functionality. One version of this kind of assay involves use of a peptide to circularize the luciferase. Contact with an appropriate protease will result in the linearization of the enzyme, and its subsequent activation.

E. In Vivo Methods

In a further embodiment, the assays of the present invention may be employed in vivo in animals who have cells expressing luciferase molecules. The assays effectively operate as those discussed above, except that the cells must be transformed in vivo or be transgenic (i.e., the luciferase must be integrated into a cell that populates the animal). Such assays find particular use in determining the ability of agents reach a target tissue and to enter target cells, such as cancer cells engineered to express a plurality of luciferases.

VI. KITS

In still further embodiments, the present invention concerns kits for use with the luciferase inhibitors described above. In addition to the inhibitiors, one may also include expression constructs encoding various luciferase molecules, as well as cells for expressing the luciferases, either transformed or untransformed with expression constructs. Another component that may be included in the kits is a luciferase substrate. Further suitable reagents for use in the present kits include buffers, diluents, and containers for growing cells, such as flasks or multi-well plates.

The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the reagent may be placed, or preferably, suitably aliquoted. The kits of the present invention will also typically include a means for containing the reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

VII. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

Identification of RLFLI, FLI, RLI inhibitors. The UTSW 200K chemical library was screened in either L-Wnt-STF cells (Cell line A, Chen et al., 2009) or NIH3T3 cells stably harboring a Hh responsive FLuc reporter and a control RLuc reporter (Cell line B) (FIGS. **1**A-B). Compounds that inhibited FLuc activity in L-WNT-STF cells were further tested in cells that express a constitutive FLuc reporter (Cell line C) in order to distinguish between compounds with activity against the Wnt/β-catenin pathway and potential FLuc inhibitors. Two candidate FLuc inhibitors (FLI 1 and 2) were identified (FIG. **1**C). Candidate RLuc inhibitors was identified by considering the results from screening both cell lines A and B. Candidate RLuc inhibitors were further tested using lysate from cells expressing RLuc.

Example 2

Candidate FL and RL inhibitors exhibit potent activity in vitro. The two FLIs and one RLI are able to inhibit FLuc and RLuc activity, respectively, in the low μM range (FIG. **1**C).

Example 3

Identification of Rluc inhibitors with no activity against FLuc, RLuc, or GLuc. Compounds structurally similar to RLI1 or FLI1 were identified from the UTSW chemical library and tested for inhibitor activity against RLuc, FLuc, GLuc, and CLuc in lysate derived from cells transfected with the corresponding DNA reporter construct (FIG. **2**). RLI1 exhibited activity against FLuc (henceforth RLFLI) but many other variants exhibited activity only against RLuc activity and not FLuc. None of the new RLIs exhibited activity against GLuc or CLuc. An additional FLI with similarity to FLI1 was also identified from this exercise (compound 32).

Example 4

A chemical scaffold for inhibitors of RLuc with little activity against other luciferase enzymes identified from compounds with similarity to RLFLI (FIG. **3**).

Example 5

FLI and RLI can be used to facilitate multiplexing of luciferase-based assays (FIGS. **4** and **5**).

Example 6

Prophetic

Methods. Human cervical carcinoma cells (HeLa cells) are transiently transfected with a Wnt/β-catenin pathway responsive Fluc reporter, a p53 responsive RLuc reporter, a K-ras pathway responsive GLuc-KDEL reporter, and a constitutively expressed secreted Cluc reporter as a control where the ratio of RLuc to GLuc DNA transfected is 5:1. One day post-transfection, transfected cells are split into 96 well culture plates and 94 individual chemicals from a diverse synthetical chemial library added per well. Two wells are reserved for addition of chemical carrier (in this case DMSO) alone. Two days post-addition of chemicals, culture medium is replica-plated in a white-opaque 96 well plate. Remaining cells are washed and lysed in passive lysis buffer. Luciferin is added and FLuc activity (reflecting Wnt/β-catenin pathway activity) measured using a standard luminometer with plate-reading capacity. FL1 is added to quench the Fluc activity prior to addition of coelenterazine to measure both RLuc and GLuc activities (reflecting p53 and Kras pathway activity respectively). Given that the ratio of Rluc to Gluc based reporter is 5:1, luciferase signal detected using coelenterazine predominantly measures Rluc activity. RL1 is added to lysate to quench RLuc activity and to evaluate Gluc activity alone. Cluc activity in the culture medium is analyzed with the addition of *Cypridina* luciferin.

Results. The effects of any invidual compound on Wnt/β-catenin, p53, or K-ras pathway activities will be determined as follows. Datasets for a particular luciferase activity are normalized to controls (lysate from cells treated only DMSO). Chemicals that either inhibit protein secretion or induce cellular toxicity based on loss of CLuc activity in culture medium are eliminated from further consideration. Chemicals that inhibit or augment activity of any particular pathway can be determined by considering the effects of that compound on each luciferase activity. Alternatively, compounds with multiple activities against these pathways can be identified with the same type of analysis.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue

experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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U.S. Pat. No. 5,464,765
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What is claimed is:

1. A method of detecting a plurality of distinct luciferase molecules comprising:

(a) providing a plurality of distinct luciferase molecules in combination with substrates therefor, wherein said plurality of distinct luciferase molecules includes at least *renilla* luciferase and firefly luciferase;

(b) providing a plurality of distinct luciferase inhibitors, at least one of which selectively inhibits *renilla* luciferase and one of which selectively inhibits firefly luciferase molecules;

(c) sequentially measuring chemiluminescence from said plurality of distinct luciferase molecules prior to and after combination of said plurality of distinct luciferase inhibitors with said plurality of distinct luciferase molecules,

whereby the addition of said *renilla* luciferase inhibitor permits the detection of chemiluminescence from non-*renilla* luciferase molecules, and the addition of said firefly luciferase inhibitor permits the detection of chemiluminescence from non-firefly luciferase molecules.

2. The method of claim **1**, wherein said plurality of distinct luciferase inhibitors are provided sequentially.

3. The method of claim **1**, wherein said plurality of distinct luciferase inhibitors are provided simultaneously.

4. The method of claim **1**, wherein said plurality of distinct luciferase molecules comprises 2, 3, 4, 5 or 6 distinct luciferase molecules,

5. The method of claim **1**, wherein said plurality of distinct luciferase inhibitors comprises 2, 3, or 4 distinct luciferase inhibitors.

6. The method of claim **1**, wherein said plurality of distinct luciferase molecules further comprises one or more of gaussia luciferase, *cypridina* luciferase, gaussia luciferase lacking a secretion signal sequence or having an endoplasmic retention sequence, or *cypridina* luciferase lacking a secretion signal sequence or having an endoplasmic retention sequence.

7. The method of claim **1**, wherein said plurality of luciferases are expressed from cells in culture.

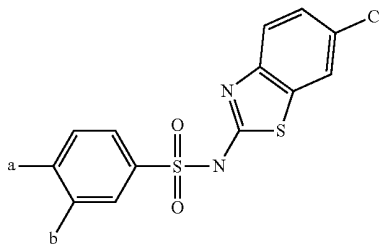
8. The method of claim **7**, further comprising separating said cells from cell medium.

9. The method of claim **8**, further comprising disrupting said cells to form a cell lysate.

10. The method of claim **1**, wherein said substrates comprise two or all three of D-luciferin, oxyluciferin, and coelenterazine.

11. The method of claim **1**. wherein an inhibitor is selective or specific for *renilla* luciferase.

12. The method of claim **11**, wherein said inhibitor selective or specific for *renilla* luciferase has the formula:

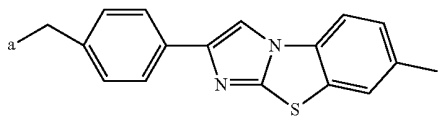


wherein a is substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), amido or halide, b is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), amido or halide, and c is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), amido or halide.

13. The method of claim **1**, wherein an inhibitor is cross-reactive with *renilla* luciferase and firefly luciferase.

14. The method of claim **1**, wherein an inhibitor is selective or specific for firefly luciferase.

15. The method of claim **14**, wherein said inhibitor selective or specific for firefly luciferase has the formula:



wherein a is H, substituted or unsubstituted alkyl (C_1-C_{10}), substituted or unsubstituted alkyoxy (C_1-C_{10}), substituted or unsubstituted alkenyl (C_1-C_{10}), substituted or unsubstituted alkynyl (C_1-C_{10}), amido or halide.

16. The method of claim **1**, wherein said method comprises measuring firefly lueiferase, *renilla* luciferase, and one or more of gaussia luciferase, *cypridina* luciferase, gaussia luciferase lacking a secretion signal sequence or having an endoplasmic retention sequence, or *cypridina* luciferase lacking a secretion signal sequence or having an endoplasmic retention sequence.

17. The method of claim **1**, wherein said method is peformed in an intact cell.

18. The method of claim **1**, wherein said method is peformed in a cell-free system.

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