IDENTIFICATION OF SMALL MOLECULE INHIBITORS TARGETING TRUNCATED ADENOMATOUS POLYPOSIS COLI (APC) AS A NOVEL THERAPEUTIC STRATEGY IN COLORECTAL CANCER

APPROVED BY SUPERVISORY COMMITTEE

_____
Jerry W. Shay, Ph.D., Mentor

_____
Woodring E. Wright, M.D., Ph.D., Mentor

_____
Ralph Deberardinis, M.D., Ph.D., Chair

_____
Michael Roth, Ph.D.

_____
Michael White, Ph.D.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my mentors Dr. Jerry Shay and Dr. Woodring Wright, for their support, encouragement, valuable guidance and instrumental criticism throughout my graduate study. Thank you for sharing your knowledge with me and providing me a strong foundation for my future career as a research scientist.

I would like to thank my thesis committee members, Dr. Ralph Deberardinis, Dr. Michael Roth and Dr. Michael White for their invaluable suggestions and constructive feedbacks.

I would also like to thank all the current and former lab members of the Shay/Wright lab. In particular, I would like to thank my colleagues Dr. Sang Bum Kim, Dr. Ugur Eskiocak and Dr. Guido Stadler for their experimental guidance and assistance. Likewise, thanks to Sze Wong, Ilgen Mender, Ejun Huang and Gaoxiang Jia for their kind friendship and encouragement. Special thanks to Kevin Kennon for his administrative support.

Finally, I would like to thank my parents, Jie Wang and Bin Zhang, and my husband Wenhao Chen for their unconditional support, encouragement and everlasting love. Thank you for being with me every step throughout my life.

IDENTIFICATION OF SMALL MOLECULE INHIBITORS TARGETING TRUNCATED ADENOMATOUS POLYPOSIS COLI (APC) AS ANOVEL THERAPEUTIC STRATEGY IN COLORECTAL CANCER

By

Lu Zhang

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of Doctor Of

DOCTOR OF PHILOSOPHY

University Of Texas Southwestern Medical Center

Dallas, Texas

August, 2014

IDENTIFICATION OF SMALL MOLECULE INHIBITORS TARGETING TRUNCATED ADENOMATOUS POLYPOSIS COLI (APC) AS A NOVEL THERAPEUTIC STRATEGY IN COLORECTAL CANCER

Lu Zhang, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2014

Jerry W.Shay, Ph.D., Woodring E. Wright, M.D./Ph.D.

Abstract

Adenomatous polyposis coli (*APC*) is a tumor suppressor gene that is mutated in the vast majority of colorectal tumors. Inactivation of this gene is a key and early event in colorectal tumorigenesis. APC primarily acts as a negative regulator of Wnt pathway by aiding in degradation of β-catenin. Further studies have suggested that APC plays important roles in several other cellular processes, including cell adhesion and migration, organization of actin and microtubule networks, spindle formation and chromosome segregation. Mutations in *APC* gene generate truncated gene products, leading to deregulation of these processes. Accumulating evidence suggest that these C terminally truncated APC may have gain of function properties beyond their loss of tumor suppressive function. Both the gain and

loss of function of APC truncations contribute to CRC initiation and progression. Utilizing a series of isogenic immortalized human colonic epithelial cells (HCECs), we have screened a 200K compound library for small molecules that exhibited selective cytotoxicity towards HCECs expressing truncated APC. We identified a compound, TASIN-1(Truncated APC Selective Inhibitor-1), which showed selective cytotoxicity towards HCECs and colorectal cancer (CRC) cells with APC truncations. TASIN-1 induces apoptotic cell death in APC truncated cells and its effect is independent of canonical Wnt pathway activity. Short hairpin RNA (shRNA) mediated knockdown of truncated APC confers resistance to TASIN-1. Additionally, TASIN-1 can interact with *in vitro* translated APC fragments, implicating truncated APC in the mechanism of action of TASIN-1. TASIN-1 inhibits tumor growth in both xenograft and genetic CRC mouse models. TASIN-1 may represent a novel therapeutic strategy for colon cancer prevention and intervention.

Table of Contents **Page**

Prior Publications

Zhang L, Komurov K, Wright WE, Shay JW. Identification of Novel Driver Tumor Suppressors Through Functional Interrogation of Putative Passenger Mutations in Colorectal Cancer. *International Journal of Cancer*. 132: 732-737. 2013.

Kim SB, Ly P, Kaisani A, **Zhang L**, Wright WE, Shay JW. Mitigation of Radiation-Induced Damage by Targeting EGFR in Noncancerous Human Epithelial Cells. *Radiation Research*. 180: 259–267. 2013.

Kim SB, **Zhang L**, Barron S, and Shay JW. Inhibition of microRNA-31-5p protects human colonic epithelial cells against ionizing irradiation. *Life Sciences in Space Research*. 1:67-73. 2014.

List of Figures **Page**

List of Tables **Page**

List of Abbreviations

ABPP	activity-based protein profiling
ACF	aberrant crypt foci
APC	adenomatous polyposis coli
BER	base excision repair
CIMP	CpG island methylator phenotype
CIN	chromosome instability
CK2	casein kinase 2
CPC	Chromosomal passenger complex
CRC	colorectal cancer
CSMD1	CUB and shushi multiple domain 1
CtBP	C terminal binding protein
DART	Drug affinity responsive target stability
DNMT1	DNA methyl transferase 1
DRI	DNA repair inhibitory
DNAPK	DNA-dependent protein kinase
DSB	double stranded break
ENU	ethylnitrosourea
FAP	familial adenomatous polyposis
GEF	guanine exchange factor
HBEC	human bronchial epithelial cell
HCEC	human colonic epithelial cell
HIP	haploinsufficiency profiling
HNPCC	hereditary non-polyposis colorectal cancer
HOP	Homozygous profiling
HTS	high-throughput screening

HURP	hepatoma upregulated protein
IQGAP1	IQ-motif-containing GTPase activation protein 1
ITC	isothermal titration calorimetry
iTRAQ	isobaric tags for relative and absolute quantitation
JNK	c-Jun N-terminal protein kinase
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LMF	ligation-mediated amplification
LOH	loss of heterozygous
LP-BER	long patch BER
MCR	mutation cluster region
Min	multiple intestinal neoplasia
MMR	mismatch repair
MS	mass spectrometry
MSI	miscrosatellite instability
MSP	multicopy suppression profiling
Nampt	nicotinamide phosphoribosyltransferase
NGS	Next generation sequencing
EGFR	epidermal growth factor receptor
PHB1	protein prohibitin 1
PIP_3	phosphatidylinositol-3,4,5-triphosphate
Pol-β	polymerase β
PPIN	Protein-protein interaction
PTPRT	Protein tyrosine phosphatase receptor type T
RNAi	RNA interference
SAR	structure-activity relationship
SCF	SKP1-CUL1-F-box protein

SILAC	stable isotope labeling by amino acids in cell culture
siRNA	small interfering RNA
shRNA	short hairpin RNA
SNPs	Single nucleotide polymorphisms
TASIN-1	truncated APC selective inhibitor-1
TGF-β	transforming growth factor β
TGFβ IIR	TGF-β type II receptor
TNKS1	tankyrase 1
TNKS2	tankyrase 2
TSG	tumor suppressor genes
VDAC	voltage-dependent anion channel
VEGF	vascular endothelial growth factor

Chapter 1 .Introduction

Colorectal cancer (CRC)

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and third leading cause of cancer related mortality in the United States. It is estimated that 136,830 new cases of colon and rectal cancer will occur in 2014. Of these, 50,310 will result in death (Society, 2014 American Cancer Society). Thus, 1 in 19 Americans will be diagnosed with CRC in their lifetime for an overall risk of 5.4%. The etiological factors underlying CRC development appear to be complex and heterogeneous. Contributory mechanisms include dietary and lifestyle factors and genetic mutations (Fearon, 2011). Although it remains to be precisely determined the specific lifestyle and environment factors that contribute to CRC, there has been significant progress in identifying the gene defects underlying hereditary as well as sporadic CRC cases (Fearon, 2011).

CRC is categorized into two forms: familial or hereditary cases and sporadic cases. The two most common hereditary types are hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP). HNPCC (also known as Lynch syndrome) accounts for about 3% to 5% of all colorectal cancers. It is caused by mutations in genes encoding DNA mismatch repair. FAP accounts for about 1% of all CRC cases. It arises almost exclusively from germline mutations in the adenomatous polyposis coli (APC) gene. Virtually all FAP patients will develop CRC without intervention (Fearon, 2011).

The adenoma-carcinoma sequence

It is widely accepted that sporadic CRC occur through acquisition of genetic alterations in a specific oncogene or tumor suppressor, the so-called “adenoma-carcinoma” sequence (Kinzler and Vogelstein, 1996). Both mutational activation of oncogenes together with inactivation of tumor suppressor genes (TSG) contribute to colorectal tumor formation. It has been proposed that at least four sequential genetic alterations are required for colorectal cancer evolution, including one oncogene (*KRAS*) and three TSGs

(*APC, SMAD4, TP53* as the main targets (Fodde et al., 2001b) (Figure 1-1). The dominant or recessive nature of these genes predict that at least seven mutations or alterations (*KRAS* and six additional ones) are required for complete inactivation of the aforementioned tumor suppressor genes' function (Fodde et al., 2001b). The TSG mutations occur in most tumors, whereas *KRAS* mutations are found in approximately 50% of sporadic adenomas and carcinomas (Bos et al., 1987; Forrester et al., 1987). It is believed that the temporal order of the mutational events is of great importance for determining tumor morphology and the potential of tumor progression (Fodde et al., 2001b) .

Major somatic oncogene and TSG mutations in CRC

APC mutations are found in the earliest lesions and its mutation frequency remains constant as tumors progress from very small adenomas to advanced adenomas or carcinomas (Powell et al., 1992). Therefore, it is believed that mutation in *APC* gene is one of the earliest events that are required to initiate clonal evolution. APC mutations are closely associated with the degree of dysplasia of the small lesions (Jen et al., 1994; Smith et al., 1994). Both allelic loss and point mutations have been identified in chromosome 5q21 where APC is localized (Okamoto et al., 1988; Powell et al., 1992; Solomon et al., 1987). Given the frequency of APC mutation in CRC and the implication that APC inactivation may play a central role in initiating adenoma-carcinoma pathway, it has been argued (Kinzler and Vogelstein, 1996) that APC acts as a gatekeeper in normal colorectal epithelial cells, the inactivation of which provide cells with selective growth advantage and genetic instability to allow for tumorigenesis.

The Ras family of small G-proteins are involved in transmitting signals downstream of growth factor receptors (Malumbres and Barbacid, 2003). Its three family members-KRAS, HRAS, and NRAS-are common targets for mutation in various types of cancers (Malumbres and Barbacid, 2003). Activating mutation in *KRAS* are found largely in nondysplastic lesions that are less likely to progress to malignant tumors, such as nondysplastic aberrant crypt foci (ACF) and hyperplastic polyps (Jen et al., 1994; Pretlow et al., 1993). The vast majority of *KRAS* mutations occur in codon 12, a subset affect codon 13, and rare mutations affect colon 61 (Fearon and Vogelstein, 1990; Wood et al., 2007). It is possible that the synergistic effects of mutations in *APC* and *KRAS* genes underscore the initial clonal evolution in the

early colorectal tumors. Since only 50% of the tumors harbor *KRAS* mutations, mutations in other oncogenes would be predicted to play a role in the rest of the cases. Alternatively, additional epigenetic and/or mutation mechanism might contribute to CRC tumorigenesis in co-operation with *APC* mutations.

Loss of chromosome 17p, primarily centered around tumor suppressor gene *TP53*, is found in approximately 75% of colorectal carcinomas, but infrequently in benign lesions, indicating that mutations in *TP53* play important roles in later stage of CRC tumor progression instead of initiation (Rodrigues et al., 1990; Vogelstein et al., 1988). Importantly, loss of heterozygosity (LOH) of chromosome 17p is almost always associated with the missense mutation in the remaining TP53 allele, consistent with the KNUDSON'S TWO-HIT MODEL for tumor suppressor genes (Baker et al., 1989). The p53 protein functions as a critical transcriptional regulator of genes involved in cell-cycle checkpoint control, apoptosis, and angiogenesis (Vousden and Prives, 2009). As such, selection for p53 defects may represent a counter stress response of tumor cells in the face of cellular stresses such as double stranded breaks (DSBs), hypoxia, lack of nutrition, and other factors (Fearon, 2011). Mutations in *TP53* may facilitate continued tumor growth and acquisition of invasive properties under various kinds of stress that may otherwise affect tumor cell survival at the adenoma-carcinoma transition.

Similar LOH frequency (~70%) has been found in chromosome 18q. The *SMAD2* and *SMAD4* genes are now regarded as the most likely candidate tumor suppressor genes localized at this region. *SMAD4* encodes a key signaling molecule in the growth-suppressing transforming growth factor β (TGF-β) pathway (Blobe et al., 2000). The function of TGF-β pathway response is highly context-dependent (Massague, 2000). In the colorectum, TGF-β and related cytokines exhibit potent inhibitory effects on cell growth (Blobe et al., 2000). Loss of function, by LOH, has been found in pancreatic and colorectal carcinoma (Hahn et al., 1996; Thiagalingam et al., 1996). *SMAD2*, encodes another member of TGF-β pathway, is specifically mutated in a subset of colorectal carcinoma (Eppert et al., 1996). Inactivating mutations in other TGF-β pathway components such as *SMAD3* and TGF-β type II receptor (*TGFβIIR)* are found in approximately 5% and 25% of CRC, respectively. Mutations in members of this pathway have been regarded as a rate limiting event in CRC (Blobe et al., 2000).

Somatic mutations in *PIK3CA, CMYC* and *FBXW7*

PIK3CA encodes the catalytic subunit of one of the four class I PI3Ks that catalyze formation of phosphatidylinositol-3,4,5-triphosphate (PIP_3), a key second messenger that affect cell proliferation, survival, and other cellular processes (Zhao and Vogt, 2008). Activating mutations in *PIK3CA* occur in approximately 15-25% of CRC cases that increases the production of PIP_3 in affected cells (Carson et al., 2008; Samuels et al., 2004; Wood et al., 2007). Even though PIK3CA functions downstream of K-Ras protein, there appears to be a tendency for *KRAS* mutations to co-segregate with mutations in *PIK3CA* to some extent (Baldus et al., 2010; Wood et al., 2007). One possible explanation for this observation is that mutant K-Ras protein is not very efficient in activating PI3K signaling (Li et al., 2004).

The c-Myc protein is a transcriptional factor that regulates genes involved in cell cycle progression, cell survival and other aspects of cellular metabolism in both normal and neoplastic cells (Dang et al., 2009; Eilers and Eisenman, 2008; Ruggero, 2009). High copy amplification of the *CMYC* gene is found in approximately 5-10% of CRCs (Leary et al., 2008). A moderate increase in c-*Myc* gene copy number and c-Myc protein expression is observed in more than 30% of CRC cases (Camps et al., 2009). Since c-*Myc* is one of the major β-catenin/TCF regulated downstream genes, the up-regulation of c-*Myc* expression may be partially due to activation of Wnt signaling pathway induced by perturbation of *APC* inactivation (He et al., 1998; van de Wetering et al., 2002).

FBXW7 encodes an F-box protein that serves as the substrate recognition component of a SCF (SKP1-CUL1-F-box protein) E3 ubiquitin-protein ligase complex which mediates the ubiquitination and subsequent proteasomal degradation of target proteins (Tan et al., 2008). Mutations in this gene are found in approximately 20% of CRCs (Akhoondi et al., 2007; Wood et al., 2007). Loss of FBXW7 function may be selected for due to its regulation of oncogenic factors, such as c-Myc, c-Jun, Notch protein, cyclin E with resultant dysregulation of multiple oncogenic pathways (Tan et al., 2008).

Mismatch repair (MMR) gene defects in CRC

Microsatellite instability (MSI) is a hypermutable phenotype caused by defects in the MMR system (Boland and Goel, 2010). At least six different proteins are involved in the MMR system. These proteins include hMSH2, hMLH1, hPMS1, hPMS2, hMSH3 and hMSH6 (Narayan and Roy, 2003). MSI is detected in about 15% of all colorectal cancers. Approximately 12% of the cases are caused by acquired hypermethylation of the MLH1 gene promoter, which occurs in tumors with the CpG island methylator phenotype (Boland and Goel, 2010). *TGFβIIR, hMSH3, hMSH6,* are examples of the few well-defined gene targets of MMR that may play a role in CRC tumorigenesis (Narayan and Roy, 2003).

Based on the "adenoma-carcinoma" sequence model for CRC, inactivation of both alleles of *APC* triggers the tumorigenesis process and provides the nascent tumor initial selective growth advantage to allow for clonal expansion. Subsequently, an oncogene mutation, mostly in *KRAS*, is required for adenoma growth and progression. Continued tumor growth and ensuing malignant transformation is driven and maintained by mutations in additional tumor suppressor genes, namely *TP53, SMAD4*, etc. It is also possible that mutations in *PIK3CA, CMYC, FBXW7* and other unidentified genes are also required for CRC progression and maintenance (Fodde et al., 2001b).

Therapeutic intervention in CRC

The main treatments for CRC are surgery, radiofrequency ablation, cryosurgery, radiation therapy, chemotherapy and targeted therapy. Depending on the stages of CRC, two or more of these types of treatment may be combined or used one after another. For late stage colorectal cancer, chemotherapy and/or targeted therapy has been used as neoadjuvant, adjuvant or palliative therapy, among which 5 Fluorouracil (5-FU) based chemotherapy regimen has been used as a first-line adjuvant treatment. Other chemotherapeutic drugs include leucovorin, oxaliplatin, capecitabine, and irinotecan (Society, 2014 American Cancer Society). As with other chemo drugs that act with minimal specificity for the underlying genetic basis of disease, these reagents exert side effects such as hair loss, mouth sores, loss of appetite, nausea/vomiting and low blood counts.

Most chemotherapeutic agents are limited in their therapeutic index due to their adverse effects on normal cells. Over the last decade, a better understanding of the underlying genetic basis of

tumorigenesis has led to the development of a new class of drugs called targeted therapy. To date, three targeted agents have been approved by the U.S. Food and Drug Administration for clinical use in advanced CRC: bevacizumab, an antibody against the vascular endothelial growth factor A (VEGF-A) ligand, and cetuximab and panitumumab, antibodies against the epidermal growth factor receptor (EGFR). Studies of these drugs used as either single agent or in combinations have produced controversial results and dose limiting increased toxicities have been reported with these agents (Knijn et al., 2010; Ortega et al., 2010). Therefore, development of novel targeted therapeutic agents for CRC intervention is critically needed.

Adenomatous polyposis coli (*APC*)

The *APC* gene

The *APC* gene is located on chromosome 5q21-q22, consists of 8535 nucleotides and spans 21 exons. The APC gene encodes a 310 kDa protein which is composed of 2843 amino acids. Approximately 75 percent of the coding sequence is located on exon 15, which appears to be the most common region for both germline and somatic mutations of APC (Beroud and Soussi, 1996). Several alternative splicing variants exist, including the non-coding exons 0.1, 0.2, 03 and BS and coding exons 1,3-4, 7, 9, 10A and 14 which lead to the generation of multiple protein variants with molecular weight ranging from 90 to 300 kDa. However, it remains unclear whether these splicing variants exert any biological functions (De Rosa et al., 2007).

Hypermethylation of the APC gene promoter has been observed in colorectal, melanoma, lung, oesophogeal, gastric, pancreatic, and hepatic cancers (Clement et al., 2004; Esteller et al., 2000; Grote et al., 2004; Tsuchiya et al., 2000). It is reported that DNA methyl transferase 1 (DNMT1) induces changes in APC by methylating the CpG region around the CCAAT-box in the promoter region of *APC* gene. Methylated chromatin undergoes conformational changes which block the binding of the transcription factor CBF to the CCAAT box, silencing *APC* gene expression (Deng et al., 2004). Interestingly,

DNMT1 gene expression has been found to be regulated by APC mutations in an indirect manner (Campbell and Szyf, 2003). These findings indicate that hypermethylation of the APC promoter may provide an alternative mechanism of *APC* gene inactivation during early stages of CRC tumorigenesis.

APC protein and its many functions

APC is a multi-domain protein and is present in both the cytoplasm and nucleus. It serves multiple functions through different binding partners. From the N terminus to C terminus, there is an oligomerization domain, an armadillo repeat-domain, a15- or 20-residue repeat domain important for binding to β-catenin, SAMP repeats for axin binding, a basic domain for microtubule binding and C-terminal domains that bind to EB1 and DLG proteins (Aoki and Taketo, 2007). The oligomerization domain has been shown to be a binding site for APC mutants. It has been reported that APC mutant proteins that retain at least the first 171 amino acids are able to bind to this region and produce a dominant negative effect (Dihlmann et al., 1999; Su et al., 1993a). However, a study showing that the overexpression of a truncated APC protein in a mouse model did not lead to tumor formation seemed to contradict these findings (Fodde et al., 1999). The armadillo repeat domain is the most conserved domain and has been shown to bind to IQ-motif-containing GTPase activation protein 1 (IQGAP1), PP2A, Asef, and KAP 3 (Jimbo et al., 2002; Kawasaki et al., 2000; Seeling et al., 1999; Watanabe et al., 2004). These interactions contribute to stimulation of cell migration and cell adhesion. The following 15-, 20-residue repeat domain and SAMP repeats play central roles in negative regulation of canonical Wnt signaling pathway by aiding in proteasomal degradation of β-catenin. The basic and C-terminal domains that can bind to microtubule directly or indirectly through interaction with EB1 are important for microtubule stabilization, kinetochore functions and chromosomal segregation. Due to its numerous interactions with a variety of proteins, APC protein is involved in cellular processes related to cell cycle control, migration, differentiation, and apoptosis (Baeg et al., 1995; Browne et al., 1998; Chen et al., 2004; Chen et al., 2003; Kaplan et al., 2001; Kroboth et al., 2007; Nathke et al., 1996; Sansom et al., 2004; van de Wetering et al., 2002) (Figure 1-2).

Role of APC in regulating the actin network

APC interacts with IQGAP1 (Watanabe et al., 2004), an effector of Rac1 and Cdc42 (Briggs and Sacks, 2003). IQGAP1 is a scaffold protein that modulates actin filaments and microtubules. IQGAP1 and APC localize interdependently at the leading edge of migrating Vero cells, where they form a complex with CLIP-170, the microtubule stabilizing protein, and with activated Rac1/Cdc42 (Watanabe et al., 2004). Depletion of either IQGAP1 or APC inhibits actin meshwork formation and polarized migration, and also results in mislocalization of CLIP-170 (Watanabe et al., 2004). Since these analyses have been performed in Vero cells which were isolated from monkey kidney epithelial cells, further investigations are warranted using cells derived from different tissues and/or species.

More recent studies show that APC might also directly bind to individual actin filaments as well as bundles of actin filaments through its C-terminal domain, which also mediates interaction with microtubules through EB1 (Moseley et al., 2007; Polakis, 1997). Interaction of APC and EB1 can directly inhibit APC bundling of actin *in vitro* and its association with the actin cytoskeleton in cells (Moseley et al., 2007). Therefore, EB1might control the APC mediated regulation of actin and microtubules. Additional studies, such as loss of function experiments, are needed to determine whether there is a direct link between APC and its regulation of actin.

Role of APC in regulating microtubules

APC is found at the ends of microtubules (Nathke et al., 1996) and can bind to and stabilize them (Munemitsu et al., 1994). Depletion of APC using Small interfering RNA (siRNA) has been shown to inhibit cell migration and protrusion formation. Loss of APC results in a decrease in overall microtubule stability and also post-translationally modified microtubules at the migrating edge of the cell (Kroboth et al., 2007). Similarly, APC is also reported to form complex with kinetochore proteins and regulate local microtubule dynamics at kinetochore with the evidence that the distances between kinetochore sister chromatids are decreased in cells depleted of APC (Draviam et al., 2006; Fodde et al., 2001a). Additionally, the role of APC in regulating microtubule dynamics in cytokinesis is supported by the

observation that cells expressing APC(1-1450) have a significant number of cells with no cytokinetic furrows. Depletion of APC leads to formation of tetra- and polyploidy cells (Dikovskaya et al., 2007)

In addition to direct interaction, APC has also been shown to mediate stabilization and polymerization of microtubule by interaction with EB1 (Morrison et al., 1998; Nakamura et al., 2001; Su et al., 1995). It has been shown that APC and EB1bind to the formin family protein, mDia, downstream of Rho in microtubule stabilization (Wen et al., 2004). Since EB1, APC and mDia form a complex at stable microtubule ends, mDia may function as a scaffold protein for EB1 and APC to stabilize microtubule and promote cell migration (Wen et al., 2004).

A potential role of APC in orienting the spindle poles is supported by studies in several organisms. In *Drosophila*, depletion of *dAPC2* in the embryonic epidermis leads to asymmetric division in those cells (Lu et al., 2001). In vertebrate, RNAi of APC in mitotic cells results in loss of astral microtubules and mis-positioning of spindles (Green et al., 2005). However, the role of APC in spindle positioning in mammalian cells remains unclear.

APC is also found to localize to centrosome and this localization is conserved in several organisms (Louie et al., 2004; Miller and Rose, 1998; Yamashita et al., 2003). Mouse embryonic stem cells with mutant APC have multipolar spindle and other centrosome abnormalities in mitotic cells (Fodde et al., 2001a). In addition to APC, several other regulatory components of Wnt signaling pathway also localize to centrosomes, including β-catenin and GSK3β as well as components of the SCF ubiquitin ligase complex(Freed et al., 1999; Huang et al., 2007; Kaplan et al., 2004; Wakefield et al., 2003). Little is known about the exact functions of APC at the centrosome, these observations, however, suggest that Wnt signals may coordinate with the cytoskeleton to exert specific functions.

Role of APC in the cell cycle

Similar to other tumor suppressors, such as pRB or TP53, APC plays a role in controlling cell cycle progression. Overexpression of *APC* in NIH3T3 fibroblasts and CRC cell lines inhibits progression of the cells from G_0/G_1 to S phase of the cell cycle in response to serum stimulation (Baeg et al., 1995;

Heinen et al., 2002) . It has been reported that transient overexpression of a GFP-APC fusion protein induces G1/S arrest in the APC-deficient colon cancer cell line SW480 (Heinen et al., 2002). This arrest can be partially alleviated by overexpression of constitutively active β-catenin or components of the Rb pathway (Heinen et al., 2002). These experiments indicate that maintenance of the G_1-S checkpoint by APC is mediated through its effect on components of the Rb pathway and is attributable, at least in part, to regulation of β-catenin/TCF–mediated transcription of S-phase regulators such as *cyclin D1* and c-*Myc* (Heinen et al., 2002).

A direct role for APC in mitosis is also likely given the observations that full length, but not truncated APC is hyperphosphorylated during M-phase (Bhattacharjee et al., 1996), accumulates at the microtubule organizing center (Olmeda et al., 2003), associates with kinetochore in dividing cells (Fodde et al., 2001a; Kaplan et al., 2001) and is a target of the M-phase kinase $p34^{cdc2}$ (Trzepacz et al., 1997). APC has also been found to interact with topoisomerase II α, a critical regulator of G2/M transition. Overexpression of the15-amino acid repeat region of APC that is required for this interaction induces G2/M arrest in HCT116 cells, suggesting a possible role of APC in G2/M transition (Wang et al., 2008). APC is found to associate with both casein kinase 2 (CK2) α- and β- subunits in immunoprecipitates as well as in pull-down assays, preferentially with tetrameric CK2 holoenzyme (Homma et al., 2002), an abundant protein kinase implicated in cell proliferation and cell survival (Homma and Homma, 2005). This association is cell cycle dependent, which peaked at G2/M phase. Immunoprecipitates containing full length APC inhibited CK2 kinase activity *in vitro*, whereas immunoprecipitates of truncated APC from SW480 cells did not affect CK2 activity (Homma et al., 2002). The inhibitory region was further mapped to the C terminus of APC. These results suggest that full length APC might exert growth inhibitory effects through regulation of CK2 activity (Homma et al., 2002) and that APC C-terminus truncations may lose this resulting in increased proliferation.

Role of APC in DNA replication and repair

APC is primarily located in cytoplasm, yet it has also been found to shuttle into the nucleus (Henderson, 2000; Neufeld et al., 2000) to regulate nuclear functions, including DNA replication and DNA repair. Transient knockdown of both full length and truncated APC impairs cell proliferation and DNA replication in 293 and SW480 colon cancer cell line through down-regulating polymerase β and other cell cycle components (Schneikert and Behrens, 2006). Full length APC can directly bind to polymerase β (Pol-β), FEN1 endonuclease and APE1 endonuclease, inhibiting the assembly of base excision repair (BER) proteins on damaged DNA and blocking long patch BER (LP-BER) (Jaiswal et al., 2006; Jaiswal and Narayan, 2011; Narayan et al., 2005). The DNA repair inhibitory (DRI) domain of APC, which is the binding site for Pol-β and FEN1, is located in the N terminal region and is retained in APC mutant. Thus most truncated APC proteins are still capable of modulating BER , possibly to different extent though (Jaiswal and Narayan, 2008). It has been reported that LoVo, colon cancer cells expressing truncated APC protein, shows an accelerated assembly of BER proteins, as well as higher activity of APE1, FEN1 and Pol β (Jaiswal and Narayan, 2011). Increased APE1 activity results in unbalancing of the BER pathway and may favor CIN and cancer progression (Tudek and Speina, 2012). APC has also been shown to interact with replication protein A 32 (RPA32) to modulate the replication stress response (Brocardo et al., 2011) and has been reported to be involved in DSB repair as a part of nuclear complex containing DNA-dependent protein kinase (DNAPK) (Kouzmenko et al., 2008).

APC mutation in sporadic CRC

APC is mutated in more than 80% of colon tumors and less frequently in other cancer types, such as hepatoblastoma (Fodde et al., 2001b; Goss and Groden, 2000; Laurent-Puig and Zucman-Rossi, 2006). Mutation of the APC gene is an early, if not an initiating event for sporadic colorectal tumorigenesis (Powell et al., 1992). Although both alleles are mutated in colorectal cancer cells, these mutations do not cause complete loss of APC protein. Instead, over 90% of APC mutations generate premature stop codons, resulting in truncated gene products. Most (~60%) of the chain terminating mutations occur in a region

referred to as mutation cluster region (MCR) that is located between codons 1280-1513(Miyoshi et al., 1992; Polakis, 1997). The C-terminally truncated proteins present in CRC lack the domains that are required for binding to microtubules, EB1 and β-catenin, thus leading to the induction of chromosomal instability, activation of proliferation, and inhibition of differentiation (Aoki and Taketo, 2007) (Figure 1-3). Genetic studies using a wide variety of Apc mutant mouse models demonstrated that mutations in *Apc* gene are responsible for intestinal tumorigenesis (Zeineldin and Neufeld, 2013b).

Comprehensive screening of the entire coding region of APC in 41 colorectal cancer cell lines reveals that there appears to be interdependency between the "two hits" at APC in both sporadic colorectal tumors as well as FAP. Specifically, tumors with *APC* mutation within the "mutation cluster region," especially those close to codon 1,300, are coupled with allelic loss, whereas tumors with mutations outside this region tend to harbor truncating mutations (Rowan et al., 2000).

While loss of APC tumor suppressing functions due to the mutational loss of APC C-terminal sequence has been regarded as a critical event in the initiation of colon cancer, there is also evidence suggesting that APC truncations exert dominant functions that might contribute to colon tumorigenesis.

Loss of tumor suppressive function of APC in CRC

Colorectal carcinomas arise through a series of histological changes as a result of the acquisition of genetic alterations in a specific oncogene or tumor suppressor (Kinzler and Vogelstein, 1996). Mutational loss of APC C-terminal sequence is regarded as essential to initiation of colon cancer through loss of APC tumor suppressive functions. The following sections will review the well-established tumor suppressive role of APC in CRC.

APC mutant and Wnt pathway

APC is a key negative regulator of canonical Wnt signaling pathway that controls the coordinated cell proliferation and differentiation in the intestine (van de Wetering et al., 2002). Loss of *APC1* and *APC2* genes in *Drosophila* results in increased Wnt/ β-catenin activity (Ahmed et al., 2002). Additionally,

mutations of *APC* in Zebrafish and mice also lead to elevated incidence of colorectal cancer with increased Wnt activity in the tumors (Haramis et al., 2006; Taketo, 2006). Aberration in Wnt signaling pathway contributes to a variety of cancer types, in particular CRC via a number of different genetic alterations (Polakis, 2012). APC is a critical component of the β-catenin "destruction complex", which consists of APC, Axin2, GSK3, CK1 and PP2A(Kimelman and Xu, 2006). The primary mechanism through which APC inhibits β-catenin/TCF-dependent transcription is by providing a scaffold for the destruction complex that stimulates phosphorylation and subsequent ubiquitin-dependent degradation of β-catenin (Rubinfeld et al., 1996). Other regulatory mechanisms include reducing nuclear β-catenin levels by promoting export of β-catenin (Henderson and Fagotto, 2002; Neufeld et al., 2000), blocking interaction with TCF by directly binding to β-catenin (Neufeld et al., 2000; Rosin-Arbesfeld et al., 2003), facilitating C terminal binding protein (CtBP)-mediated repression of Wnt-target genes through direct interaction with a repression complex (Hamada and Bienz, 2004; Sierra et al., 2006). Mutations in APC lead to activation of β-catenin/TCF transcriptional activity by both increasing nuclear β-catenin level and attenuating CtBP-mediated inhibition of the repression complex. This activation leads to up-regulation of downstream targets, such as cyclin D1 and c-Myc, which are known to be involved in tumor formation due to their roles in cell proliferation, apoptosis and cell-cycle progression (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999). Interestingly, there is a strong selection pressure favoring the retention of the first 20 amino acid repeat (Fodde et al., 2001b) that can bind to β-catenin and regulate its transcriptional activity (Schneikert et al., 2007). One explanation of this phenotype is the "just-right" signaling model which proposed that selection for APC genotypes is aimed at a specific degree of β-catenin that is optimal for tumor formation (Albuquerque et al., 2002). According to this model, deletion of all of the β-catenin binding sites are less favorable because constitutive activation of β-catenin pathway leads to extensive changes in gene regulation that results in increased risk of genomic instability and cell death. In contrast, retaining some of the β-catenin binding sites allows for its partial down-regulation thus providing sufficient proliferation advantage without risking cell death. There is evidence showing that overexpression of β-catenin induces apoptosis independent of its transactivation function

with LEF-1 or the downstream major G1 cell cycle regulators (Kim et al., 2000), lending support to this model. In addition, analysis of APC genotypes in sporadic CRC cases demonstrate "fine-tuned" interdependency of hits by types and location to achieve the "optimal" β-catenin thresholds for proximal and distal CRCs (Christie et al., 2013). Besides, circumstantial evidence comes from a study where it has been shown that knockdown of APC protein using truncated APC specific shRNAs results in up-regulation of the transcriptional activity of β-catenin in 5 out of 6 CRC cell lines, suggesting that truncated APC can still modulate wnt signaling activity (Chandra et al., 2012).

APC mutant and its role in cell adhesion

Inactivation of APC has also been proposed to promote tumorigenesis through loss of adhesion (Bienz and Hamada, 2004). APC interacts with β-catenin which links E-cadherin to α-catenin and the actin cytoskeleton (Kemler, 1993; Rubinfeld et al., 1993; Su et al., 1993b). Enterocytes and tumor cells from $Apc^{min/+}$ mice which has one copy of mutant *Apc* showed decreased level of E-cadherin at the cell membrane as well as decreased association between β-catenin and E-cadherin (Carothers et al., 2001). Expression of full length APC results in increased levels of E-cadherin at the cell membrane and causes translocation of β-catenin from nucleus and cytoplasm to the cell periphery, which enhances cell adhesion in CRC cell harboring truncated APC (Faux et al., 2004). Therefore, APC regulates cell adhesion by controlling the distribution of β-catenin and E-cadherin between the cytoplasm and the cell membrane. APC mutants lack the binding domains for β-catenin, resulting in weakened cell adhesion.

APC mutant and its role in chromosome instability (CIN)

APC can bind to and stabilize microtubules directly or through its association with EB1, a microtubule binding protein that is enriched at microtubule plus end (Deka et al., 1998; Juwana et al., 1999; Nathke et al., 1996; Su et al., 1995; Zumbrunn et al., 2001). Both APC and EB1 have been implicated in maintaining proper spindle positioning in the developing nervous system of *Drosophila* (Lu et al., 2001; McCartney et al., 2001; Rogers et al., 2002). APC is found to localize to kinetochores in

mitotic cells and form a complex with checkpoint proteins Bub1 and Bub3 (Kaplan et al., 2001). Removal of APC in U2OS and HCT116 cells decreased association of checkpoint proteins Bub1 and BubR1with kinetochores, altered mitotic progression and increased mitotic slippage (Dikovskaya et al., 2007). Cells carrying a truncated APC gene (Min) are defective in chromosome segregation (Kaplan et al., 2001). It has also been shown that depletion of APC in mitotic *Xenopus* egg extracts results in weaker spindles (Dikovskaya et al., 2004). siRNA-mediated knockdown of APC and/or EB1 causes defects in mitotic spindle and chromosome alignment (Green et al., 2005). Aberrant spindle structures and weakened kinetochore-microtubule attachments have been observed in CRC cells harboring APC truncations (Fodde et al., 2001a; Green and Kaplan, 2003). Taken together, the C terminal truncated APC that lack the binding domains for microtubules and EB1 can lead to spindle dysfunction, contributing to CIN in CRC cells.

Dominant-negative effects of APC

On the basis of the correlation of FAP severity with specific truncating *APC* mutations, and the association of mutant APC proteins with WT APC proteins *in vitro* as well as *in vivo*, it has been proposed that APC truncations may inactivate WT APC in a dominant negative manner (Su et al., 1993a). Exogenous expression of a mutant *APC* that is truncated at codon 1309 antagonized WT APC induced reduction in TCF-mediated transcriptional activity (Dihlmann et al., 1999). Increased β-catenin levels, a decrease of the proliferation rate and decreased cell migration have been reported in histologically normal Min/+ mucosa compared with that of +/+ mice (Mahmoud et al., 1997). Evidence against the dominant negative effects comes from a study in mice carrying a transgene encoding APC amino acid 1-716 ($Apc^{\Delta 716/+}$). Since no difference was seen in terms of tumor number, distribution or morphology in $Apc^{\Delta 716/+}$ mice with or without an extra truncated Apc transgene, it was concluded from this study that Apc truncation does not act in a dominant negative manner (Oshima et al., 1995). These studies provide controversial evidence as to the dominant-negative effects of APC truncations. This conundrum has not yet been completely resolved.

APC truncation: Switch from tumor suppressor to oncogene?

Although both alleles are mutated in APC-defective colorectal tumors, homozygous deletions of *APC* seem to be very rare or absent. Analysis of tumors from FAP patients and colorectal tumors revealed that there appears to be an interdependence of the "two hits" at *APC.* Specifically, germline mutations within the MCR are associated with allelic loss of the remaining wild-type allele whereas mutations occurring outside of this region are associated with truncating mutations (Lamlum et al., 1999; Rowan et al., 2000). A correlation between the presence of a germline mutation in the MCR and the severity of polyposis has also been noted (Ficari et al., 2000; Nagase et al., 1992; Wu et al., 1998). Particularly, complete deletion of *APC* has been reported only rarely and is not associated with the most severe phenotype (Herrera et al., 1986; Sieber et al., 2002). The strong selection for these truncated APC proteins might be explained by the requirement for the optimal level of β-catenin activity as discussed earlier, APC protein stability or retained N terminal functions (Albuquerque et al., 2002; Rowan et al., 2000) . There is emerging evidence supporting that APC mutants have gain of function properties that promotes tumor progression beyond simple loss of function. The following parts will discuss the reported dominant functions of APC mutants in terms of cell survival, cell migration, and chromosomal stability.

Role of APC truncation in cell survival

It has been shown that overexpresstion of full length APC promote apoptosis in colon cancer cells (Morin et al., 1996). More recent studies show that APC plays a role in apoptosis through both transcription-dependent and transcription–independent mechanisms via caspase cleavage of APC (Qian et al., 2007). The caspase-cleaved APC fragment directly associates with the mitochondrial protein hTID-1 isoforms to promote cell sensitivity to apoptosis (Qian et al., 2010). In contrast, APC mutants have been reported to harbor anti-apoptotic function in colon cancer cell lines through a transcription-independent mechanism. It has been shown that the addition of APC truncation 1309 into cell-free *Xenopus* egg extract decelerates apoptosis-associated caspase cleavage through a mechanism that is independent of β-catenin-mediated effects on transcription (Steigerwald et al., 2005). In addition, truncated APC proteins,

unlike full length APC, are resistant to cleavage by group II caspase and are unable to accelerate apoptosis *in vitro* or *in vivo* (Qian et al., 2007). In addition to aiding in evading apoptosis, APC truncations promote cell survival through other mechanisms. Full length APC is primarily located in cytoplasm, yet it has also been found to shuttle into the nucleus (Henderson, 2000; Neufeld et al., 2000) to regulate nuclear functions. Truncated APC mutants have been shown to be preferentially targeted to mitochondria by Western Blot and immunocytochemistry and promote cell survival through regulation of Bcl-2 at mitochondria (Brocardo et al., 2008). Exogenous expression of APC truncation but not full-length APC protect SW480 cells against sulindac-induced apoptosis whereas transient knockdown of APC truncation in SW480 cells induced about A two-fold increase of apoptotic cell death with concurrent down-regulation of Bcl-2 at mitochondria. Other studies showed that transient knockdown of APC in SW480 compromises DNA replication and cell proliferation through down-regulation of cell cycle components (Schneikert and Behrens, 2006). Altogether, these data can be interpreted to suggest a pro-survival function of truncated APC that is essential for tumor cell survival.

Role of APC truncation in cell migration

APC regulates cell migration through different mechanisms, including control of the actin cytoskeleton (Akiyama and Kawasaki, 2006), regulation of the microtubule network (Nathke, 2006; Nathke, 2004), and interactions with Asef, a Rac-specific guanine exchange factor (GEF) (Kawasaki et al., 2003; Kawasaki et al., 2000). Overexpression of APC induces disordered migration of epithelial cells in the small intestine (Wong et al., 1996). In contrast, inactivation of APC impairs cell migration (Andreu et al., 2005; Sansom et al., 2004) and leads to formation of a benign polyp consisting of a single layer of adenoma cells (Oshima et al., 1997).

APC was initially identified as a binding partner with Asef1 through its armadillo repeat domain. This interaction enhances the GEF activity of Asef1 and stimulates Asef-mediated cell flattening, membrane ruffling and lamellipodia formation in MDCK cells (Kawasaki et al., 2000). Also, APC interacts with a close homolog of Asef, termed Asef2 that exhibits significant structural and functional

similarity to Asef (Kawasaki et al., 2007). Since the armadillo repeat domain is N terminal to the MCR region of APC, the C terminally truncated APC found in most CRC can still bind and activate Asef. Interestingly, exogenous expression of APC truncated proteins, but not full length APC, stimulate Asef-mediated cell migration in MDCK cells. Additionally, short hairpin RNA (shRNA)-mediated knockdown of truncated APC proteins inhibited cell migration in SW480 and WiDr cells whereas expression of shRNA-APC in HCT116 and LS180 cells with WT APC did not affect cell migration. These results can be interpreted to suggest that truncated APC, but not full length APC, is an effective activator of Asef. Therefore, expression of truncated APC could lead to aberrant cell migration in cancer cells (Kawasaki et al., 2007; Kawasaki et al., 2003).

Role of APC truncation in chromosome instability (CIN)

Certain forms of APC truncations have been reported to have dominant effects on proliferation, spindle checkpoint control, survival and chromosome stability (Green and Kaplan, 2003; Tighe et al., 2004) . Expression of mutant APC (1-1450) dominantly disrupts spindle microtubules, interferes with microtubule plus-end attachments and induces abnormal chromosome segregation in 293 cells (Green and Kaplan, 2003).Cells expressing N-terminal 750 amino acids of APC show weakened kinetochore-microtubule interactions and exhibit a phenotype more typical of CIN lines. The expression of this APC fragment reduces accumulation of mitotic cells and accelerates mitotic exit following spindle damage, suggesting a dominant effects on spindle checkpoint control. Additionally, the expression of N750 enhances survival following prolonged mitotic arrest and the survivors contain chromosome aberrations and are highly aneuploid (Tighe et al., 2004). Unlike the N750 mutants, expression of N1309 did not affect checkpoint function or accelerate mitotic progression. In another study, APC mutants dominantly inhibit cytokinesis by blocking initiation of the cytokinetic furrow (Caldwell et al., 2007) . Similar mitotic defects are observed in dividing crypt cells in the small intestines of $Apc^{Min/+}$ mice. Therefore, these results provide direct evidence that the dominant functions of APC mutants induce aneuploidy *in vivo* (Caldwell et al., 2007). Although it remains to be answered whether CIN is a participant or side

effects of oncogenic processes, there is increasing evidence suggesting that CIN may play a role in tumor formation (Dai et al., 2004; Fujiwara et al., 2005). Therefore, the dominant effects on chromosome stability of APC truncations may facilitate colorectal tumorigenesis.

Additional support for a dominant function of APC truncations

Until recently there has been limited, if any, cell-based studies on truncated APC's oncogenic properties mostly due to a lack of optimum cellular reagents. The majority of previous studies used cultured cell models of CRC of malignant origin containing multiple cytogenetic changes which imposes difficulties in assigning linear genetic relationships from gene function studies. As part of our efforts to elucidate the molecular underpinnings of colon cancer tumorigenesis, our lab has developed a series of human colonic epithelial cell (HCEC) lines immortalized with telomerase and CDK4 (Roig et al.,2010). We showed that these cells are non-transformed, karyotypically diploid and have multipotent differentiation characteristics. When placed in Matrigel® in the absence of a mesenchymal feeder layer, individual cells divide and form self-organizing, crypt-like structures with a subset of cells exhibiting markers associated with mature epithelium (Roig et al., 2010). Utilizing these immortalized HCECs, our group showed that truncated APC proteins are able to confer HCECs tumorigenic properties on normal (manuscript in preparation). The two isogenic progressed HCEC cell lines harbor *TP53* and *APC* knockdowns, as well as ectopic expression of oncogenic $KRAS^{V12}$ with or without the ectopic expression of truncated APC1309. Ectopic expression of APC1309 confers a moderate proliferative advantage and enhancement of soft agar growth as well as invasion through Matrigel® compared to its matched parental cell lines (Figure 1-4). However, stable knockdown of >90% WT APC does not have any of these effects, demonstrating that loss of APC function by itself does not drive colon cancer progression. Additionally, transient knockdown of truncated APC proteins in DLD1 CRC cells which express a truncated APC significantly slowed cell proliferation, induced G2/M phase arrest and affected S phase progression. These observations lent support to the notion that APC truncations can promote tumorigenic properties in advanced lesions with additional genetic alterations. CRC cells harboring truncated APC may gradually

become “addicted” to this truncated protein for cell survival and maintaining the tumorigenic phenotype (Figure 1-5). It is entirely possible that APC truncations interact with novel protein partners due to different conformational changes from the conformation of full length APC. It would be interesting to investigate the downstream effectors/pathways that contribute to the progression of normal HCECs to tumor-cell-like HCECs.

Lessons from genetic mouse models

For the past two decades, extensive efforts have been made in generating and characterizing Apc mouse and rat models to investigate APC functions in intestinal homeostasis and tumor suppression (Kwong and Dove, 2009; McCart et al., 2008; Zeineldin and Neufeld, 2013a). The first hereditary mouse model of colon cancer, designated as multiple intestinal neoplasia (*Min*), was identified in an ethylnitrosourea (ENU) mutagenesis screen. The $Apc^{Min/+}$ mice harbor a nonsense mutation resulting in APC truncation at codon 851and have been used extensively to study APC tumor suppressive functions. These mice develop polyps and benign adenomas with most of them present in the small intestine (Moser et al., 1990; Su et al., 1992). After the description of the $Apc^{Min/+}$ mouse model, a wide variety of additional mouse models expressing truncated Apc protein longer or shorter than Apc^{min} have been generated. Most, if not all, of these models develop adenomas (polyps) with varying numbers and varying intestinal distributions. However, there is no distinct correlation between the mutation types and intestinal phenotypes (Zeineldin and Neufeld, 2013a; Zeineldin and Neufeld, 2013b). Of note, *CPC;Apc* mice is one of the few Apc mouse models that mainly develops colorectal tumors. These mice carry a *CDX2P-NLS* Cre recombinase transgene and a *lox*P-targeted *Apc* allele that deletes exon 14, leading to a frame shift at codon 580 and a truncated APC protein. The tumors developed in these mice showed biallelic *Apc* inactivation, Wnt pathway dysregulation, global DNA hypomethylation and aneuploidy (Hinoi et al., 2007). Studies of these mouse models of intestinal tumors have aided in discovery of important pathways involved in colon tumorigenesis but have also raised several unanswered questions. In particular, significant phenotypic variation has been observed among the different models and even in

the same model analyzed by different laboratories, which has been recently reviewed (Zeineldin and Neufeld, 2013b). Some possible explanations for these observed variations include different rates and mechanisms of WT Apc loss, differences in Wnt signaling, varying contributions of genetic modifiers or environmental factors, and differences in technologies used (Zeineldin and Neufeld, 2013a; Zeineldin and Neufeld, 2013b). Recently, a mouse model with a complete deletion of all 15 *Apc* exons ($Apc^{\Delta e1\text{-}15}$) was generated to test the requirement of truncated APC for tumor formation. $Apc^{\Delta e1\text{-}15}$ mice develop intestinal polyps of the same distribution and histology as with $Apc^{Min/+}$ mice but at higher frequency in female mice. Colon tumors from these mice seem to have lower levels of Wnt pathway activation compared with $Apc^{Min/+}$ mice (Cheung et al., 2010). This observation is consistent with the "just right" hypothesis that submaximal level of β catenin level is advantageous for tumor formation, yet the underlying mechanism remains unknown. This study, at least superficially, argues against the pro-tumorigenic functions of truncated APC, however, there are several caveats to this interpretation. First, the more severe polyposis phenotype is only observed in female $Apc^{\Delta e1\text{-}15}$ mice on a C57BL/6 background but not in male $Apc^{\Delta e1\text{-}15}$ mice. In addition, this has not been tested in mice on different backgrounds suggesting gender and strain modifiers may be important. Thus, it would be premature to draw any conclusion as to the tumorigenic functions of truncated Apc based on this mouse model. Since the phenotypes of CRC mouse models are influenced by many factors, the use of standardized conditions to compare phenotype of different Apc mutant mouse models would be more appropriate for reliable genotype-phenotype analysis (Zeineldin and Neufeld, 2013b). Additionally, since these germline Apc mutation mice develop polyps mainly in the small intestine, observations from these studies do not warrant definitive conclusions regarding CRC tumorigenesis in humans, especially since human almost never present with small intestinal tumors. It would be worthwhile to investigate the effect of complete loss of APC on tumor formation using conditional mouse models developing colorectal tumors, which may be more reflective of CRC in human patients. Moreover, these mouse models harbor Apc germline mutations whereas CRC patients harbor somatic APC mutations which are stochastic. As with most mouse models, these Apc mice are more representative of human cancer–predisposition syndromes (such as FAP) rather than sporadic cancers

(Cheon and Orsulic, 2011). In addition, it is important to remember that the mouse is different from humans in many aspects, such as size, life span, organ morphology and physiology. In particular, some tissues in the mouse have active telomerase which prevents the modeling of genomic instability in mice unless one uses a telomerase knockout mouse model. Besides, the overall mechanism predisposing to tumor formation may be different in mice and humans. Therefore, cautions should be taken when interpreting observations from mouse studies.

Target-based versus chemical genetics drug discovery approaches

Traditional target-based drug discovery approaches rely on a predefined protein target. High-throughput screening (HTS) can be performed to search for modulators of a given protein once the correlation and/or causal relationship between the protein and a specific disease setting is established. The identification of novel and validated targets remains a top priority in current target-based drug discovery fields. Both forward and reverse genetics have been utilized to reveal functions of undefined genes. Recent advances in RNA interference (RNAi) technology has allowed for the loss-of-function screen in a genome-wide scale. However, determination of feasible drug targets based solely on depletion of a certain protein is an oversimplified strategy, considering the diverse regulatory mechanisms of a protein's function, such as phosphorylation and dephosphorylation. Additionally, the regulation of the activity of a certain protein, by the assembly of a protein complex or by epigenetics mechanisms, can be both spatial and temporal and thus cannot be recapitulated with deletion (Cong et al., 2012). Other confounding factors include compensatory pathways such as protein redundancy.

Chemical genetic drug discovery approaches generally employ a cell-based phenotypic assay to identify bioactive small molecule by HTS as a first step. The implementation of a counter screen and secondary screens are essential to filter nonspecific hits in order to narrow the candidate hits list. Structure-activity relationships (SAR) analysis using in silico methods, together with profiling and data mining assist in hit selection/prioritization. Then medicinal chemistry efforts are initiated to optimize the potency and physical properties of the lead compound based on the cellular assay readout without the

prior knowledge of the exact target. Meanwhile, chemical probes with a linker are synthesized for the subsequent affinity purification based target identification, followed by validation studies using genetic manipulations. This unbiased approach allows for the discovery of novel proteins/mechanisms that are otherwise elusive. This approach improves efficacy of the drug discovery process since the potency and off target effects of the small molecule is assessed in the early stages. Once the target is validated, the newly identified target and its small molecule inhibitor are ready to be used in the subsequent drug discovery process. Additionally, the newly identified biological probe can be employed as a valuable tool to study the function of its protein target and to provide insight into the signaling network of the specific disease state. This tool has its unique advantages compare to genetic screen strategy. Firstly, since perturbation of a protein's function via a small molecule is conditional and tunable, this method enables temporal study of a biological process and minimizes the compensatory mechanisms that often complicate the interpretation of experiment results. Moreover, a small molecule typically can recognize the binding sites of homologs and thus can overcome gene redundancy problem, which is inherent in genetic screen (Cong et al., 2012).

Chemical proteomics for protein target identification in chemical genetics drug discovery

The identification of the targets of the small molecule has generally been the "bottleneck" for chemical genetics drug screen approach. Current strategies for target identification can be roughly grouped into three categories: affinity-based (such as the chemical proteomics approach), transcriptional profiling-based, and genetics-based. At present, the chemical proteomic approach has been one of the most versatile methods to directly and comprehensively identify the cellular protein target of a chemical compound (Katayama and Oda, 2007). This approach is an unbiased and large-scale method in that it probes the entire proteome instead of relying on a predefined set of recombinant protein, and it can be utilized in any cell or tissue of interest. Also, it does not rely on generation of a target hypothesis since it identifies the direct binding proteins of the small molecule. Chemical proteomics approach consists of the classical drug-affinity chromatography and modern mass spectrometry (MS) analysis for protein

identification. The drug-affinity chromatography starts with the preparation of a linker derivative of the small molecule that retains biological activity as well as an inactive derivative as a negative control. The procedure includes immobilization of linker analog onto a solid-phase resin through a spacer arm, and incubation of the compound-immobilized resin with cell lysates from cells or tissues or subcellular fractions, followed by separation and purification of the interacting protein target. The enriched specific proteins are then proteolytically digested and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) for protein identification (Figure 1-6). Recent developed quantitative MS methods, such as stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002) and isobaric tags for relative and absolute quantitation (iTRAQ) (Ross et al., 2004) are critical for distinguishing specific and nonspecific binding partners, which has been the most significant challenge for the chemical proteomics approach.

One commonly used approach to discriminate specific and nonspecific binding partners is the implementation of parallel pull-down using inactive analog that lack cellular activity. Proteins pulled down by active molecule but not by inactive molecule are regarded as candidate direct binding targets. Utilization of this strategy has led to the identification of mitochondria protein prohibitin 1 (PHB1) as the target of aurilide, a potent cytotoxic marine natural product that induces apoptosis in cultured cells (Sato et al., 2011). Additional successful examples include, but not limited to, the identification of multifunctional protein 2 as the target of chromeceptin (Choi et al., 2003), cytosolic malate dehydrogenase as the target of E7070, an anticancer drug under clinical evaluation (Oda et al., 2003).

In addition to utilizing an inactive linker derivative to rule out nonspecific binding proteins, an in-solution competition approach represents another strategy to distinguish specific and nonspecific binding partners. In this approach, cell lysates are pretreated with excess amount of active free molecules before incubation with the affinity matrix. The active molecules in solution can efficiently block capture of specific protein targets by the affinity matrix, so comparative analysis of parallel samples should uncover specific binders. Using this approach, Huang et al. has identified tankyrase 1 (TNKS1) and tankyrase 2 (TNKS2) as the direct target of XAV939, a potent Wnt pathway inhibitor (Huang et al., 2009). Another

successful example of using this competition approach to rule out nonspecific binders is the identification of nicotinamide phosphoribosyltransferase (Nampt) as the cellular target of CB30865, a potent and selective cytotoxic compound with unknown mechanism of action (Fleischer et al., 2010). In addition, this competitive approach was used to identify Class Ia PI3K subunits p85a and p85b as the cellular target of quinostatin (Yang et al., 2007) and KEAP-1 as the cellular target of antioxidant response element activator AI-1(Hur et al., 2010).

The synthesis of linker derivative bioactive small molecules for immobilization has been the critical and also the rate limiting step for protein identification in earlier stages of chemical proteomics. The high molecular weight of the reporter group (fluorophore, biotin, or both), may limit the cellular uptake and distribution in *vivo* (Speers and Cravatt, 2004). The low affinity between the small molecule and its target also poses an obstacle for protein target identification (Speers and Cravatt, 2004). To circumvent these problems, an activity-based protein profiling (ABPP) strategy has been developed in which the "bait" is linked to a reaction group which can be crosslinked to a protein target using a photoaffinity reaction (Speers and Cravatt, 2004). Then one or more reporter groups can be added for detection and isolation of probe-labeled proteins using "click chemistry" which employs efficient, selective and versatile chemical reaction (Lutz and Zarafshani, 2008). This approach has been used to successfully detect cancer linked galectin-3 (Ballell et al., 2006). It remains to be determined the applicability of this strategy in protein target identification.

As mentioned above, the synthesis of the active linker derivatives require extensive chemical efforts and is very time consuming. Based on photoaffinity reactions, a nonselecive method has been developed to attach small molecules to a solid surface for target enrichment while sparing the need for compound modification (Kanoh et al., 2005). Drug affinity responsive target stability (DART) is a recently developed drug target identification method based on the observation that binding to a drug often stabilizes the target protein, thereby reducing its protease sensitivity. This method was validated by identification of eIF4A as the target of resveratrol (Lomenick et al., 2009).

Another challenge for the chemical proteomics approach is the identification of low abundance protein targets. Several strategies have been developed to overcome this problem by artificially increasing the concentration of target proteins (Terstappen et al., 2007), including three-hybrid systems (Becker et al., 2004; Caligiuri et al., 2006), mRNA display (McPherson et al., 2002), phage display (Boehmerle et al., 2006; Kim et al., 2007; Shim et al., 2004) and protein microarray (Huang et al., 2004). Despite their unique advantages, it remains to be seen whether these methods can be put into wide application.

Additional approaches for target identification-transcriptional profiling

Transcriptional profiling has been demonstrated as a useful method for target identification (Butcher and Schreiber, 2005; Gunther et al., 2005; Stoughton and Friend, 2005). This method requires the generation of database of transcriptional profiles for various compounds and genetic alterations. Perturbations targeting the same protein or pathway are likely to generate similar transcriptional profiles; therefore, their gene expression profile would cluster in a similar manner. Comparison of the profiles of novel compounds with profiles of a well-characterized compound or a specific gene perturbation can reveal important insight into the mechanism of action of these compounds (Cong et al., 2012). On the basis of the assumption that drug treatment and deletion of the drug target should have similar transcriptional profile, Hughes et al. (Hughes et al., 2000) identified ERG2 as the target of the anesthetic dyclonine by comparing a collection of expression profiles of 300 yeast deletion strains and cells treated with various kinds of drugs. Based on the same concept, Lamb et al. (Lamb et al., 2006) created a compendium of gene expression profiles of several human cancer cell lines treated with bioactive small molecules and generated a connectivity map using pattern matching software. Using this method, they identified the targets of compounds of unknown mechanism of action. Although encouraging, the high cost of genome-wide transcriptional profiling and complex bioinformatics analyses has limited its application. cDNA microarray analysis shows that a subset of gene signature profiles can be used to categorize compounds with different mechanisms of action (Gunther et al., 2003; Gunther et al., 2005). Recently developed gene signature assays such as bead-based LMF (ligation-mediated amplification)

technology are more suitable for large scale compound profiling due to its lower cost compared to whole genome-wide transcriptional profiling (Peck et al., 2006; Stegmaier et al., 2007).

Additional approaches for target identification-genetics screening

In addition to transcriptional profiling-based approach, genetics screening has also proven to be a valuable tool for target deconvolution. The genetic tractability of the budding yeast *Saccharomyces cerevisiae* has allowed for the development of a comprehensive yeast deletion collection consisting of deletion strains of all 6,000 *S. cerevisiae* genes (Giaever et al., 2002; Smith et al., 2010; Winzeler et al., 1999). Each deletion strain is bar-coded which enables examination of competitive growth of the entire collection in a single culture. Drug-induced haploinsufficiency profiling (HIP) is an assay based on the observation that decreased dosage of a drug target gene can increase drug sensitivity (Giaever et al., 1999).This method is unbiased and does not require the knowledge of the mechanism of action of a compound, but it requires the compound to have growth inhibitory activity. The HIP assay uncovers both the direct target and other relevant targets in the same pathway and has been shown to be a robust assay through identification of the targets of well-studied compounds as well as unknown compounds (Giaever et al., 2004; Hillenmeyer et al., 2008; Lum et al., 2004; St Onge et al., 2007). Homozygous profiling (HOP) is similar to HIP, except that the strains have complete deletion for nonessential genes. In most cases, the HOP assay reveals genes that are involved in the drug target pathway instead of the direct target since it is creating a synthetic lethality between a deleted gene and a drug (Cong et al., 2012). Multicopy suppression profiling (MSP) is basically the reverse of HIP and HOP. It is based on the idea that increasing the dosage of a drug target can confer resistance to drug's effects. HIP, HOP and MSP are complementary assays and integration of the results from each of these assays provides a more broad and comprehensive understanding of the mechanism of drug action in cells. Successful examples include the identification of guanine monophosphate synthase, poly (A) polymerase and FKBP12 as targets of several compounds (Heitman et al., 1991; Jiang et al., 2008; Rodriguez-Suarez et al., 2007). Advancement in generating systematic collections of human cDNA clones and genome-wide RNAi studies can potentially

be used in the same way as these assay to identify drug targets in mammalian cells (Baross et al., 2004; Fire et al., 1998; Gerhard et al., 2004; Sachse and Echeverri, 2004).

Purpose and significance

Until recently there has been limited, if any, cell-based studies on truncated APC's oncogenic properties mostly due to a lack of optimum cellular reagents. The majority of previous studies used cultured cell models of CRC of malignant origin containing multiple cytogenetic changes which imposes difficulties in assigning linear genetic relationships from gene function studies. Although defects in APC occur in a high fraction of colon cancer cases, there has been few reported therapeutics targeting vulnerabilities resulting from these defects. As part of our efforts to elucidate the molecular underpinnings of colon cancer tumorigenesis, our lab has developed a series of human colonic epithelial cell (HCEC) lines immortalized with telomerase and CDK4 (Roig et al.,2010). We showed that these cells are non-transformed, karyotypically diploid and have multipotent differentiation characteristics. When placed in Matrigel® in the absence of a mesenchymal feeder layer, individual cells divide and form self-organizing, crypt-like structures with a subset of cells exhibiting markers associated with mature epithelium (Roig et al., 2010). We also developed progressed APC truncated HCECs which harbor *TP53* and *APC* knockdowns, as well as ectopic expression of oncogenic $KRAS^{V12}$ with or without truncated APC1309. The primary purpose of my studies in this dissertation research was to identify small molecule that are selectively toxic to cells harboring truncated APC. By performing a 200K small molecule high throughput screening, I identified a genotype-selective compound, TASIN-1(Truncated APC selective inhibitor-1) that can induce apoptotic cell death in authentic CRC cells and HCECs harboring APC truncations while sparing cells with wild type APC. TASIN-1 can inhibit tumor growth *in vivo* in human xenograft experiments and in transgenic mouse models of CRC. Additional SAR study has led to the identification of more potent and selective analogs. These analogs will serve as a platform for further translational development as putative drugs for clinical testing and represent a paradigm shift for targeted

therapy in CRC. Additionally, lead compounds can serve as biological probes to gain important insights into the potential dominant functions of APC truncations in colon cancer initiation and/or progression.

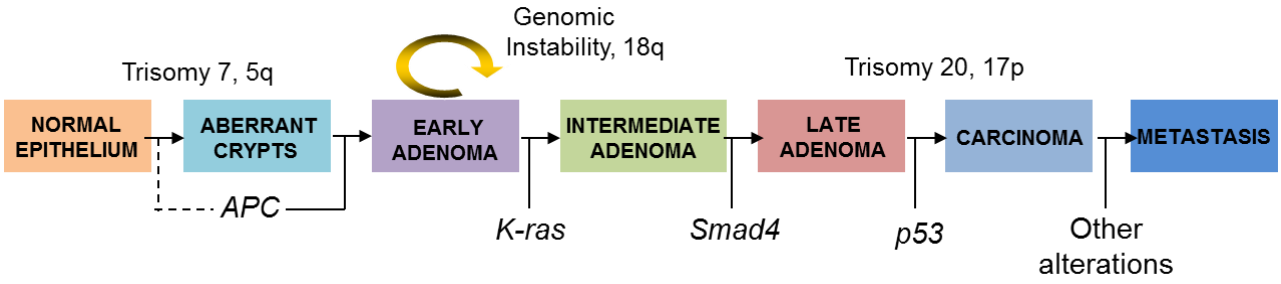


Figure 1-1.The adenoma-carcinoma sequence of colorectal cancer (CRC).-adapted from Fearon and Vogelstein. APC mutation is an early event in CRC tumorigenesis. At least four sequential genetic alterations are required for colorectal cancer evolution, including one oncogene (*KRAS*) and three tumor suppressor genes (*APC*, *SMAD4*, *TP53*). The tumor suppressor gene mutations occur in most tumors, whereas *KRAS* mutations are found in approximately 50% of cases.

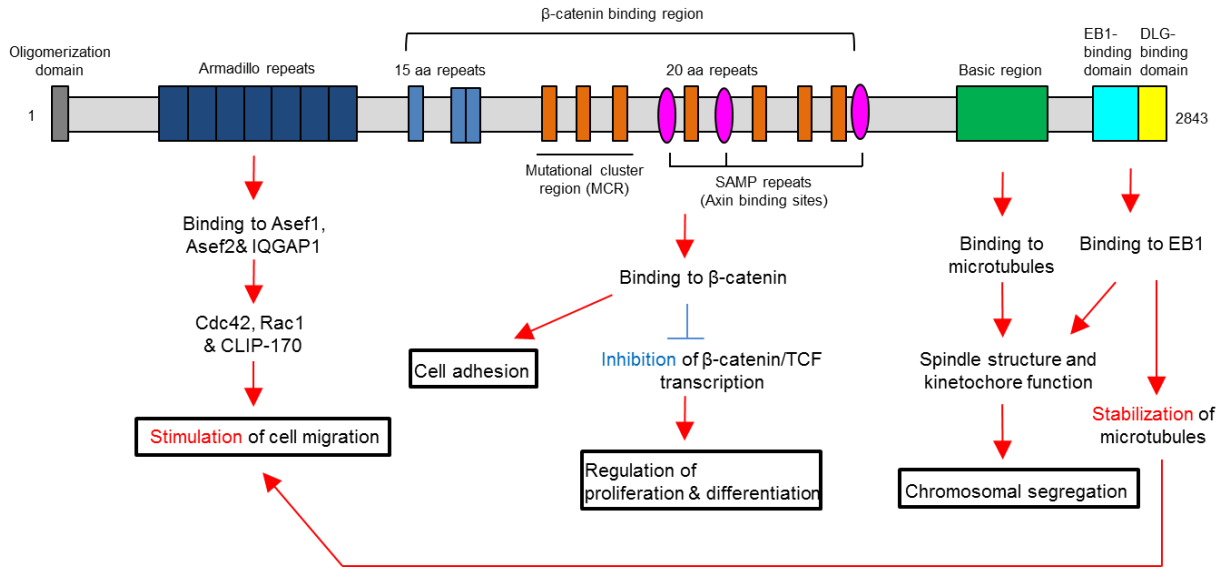


Figure 1-2.Structure and functions of full length APC. Full length APC proteins contain multiple domains including oligomerization domain, an armadillo repeat-domain, a15- or 20-residue repeat domain important for binding to β-catenin, SAMP repeats for axin binding, a basic domain for microtubule binding and C-terminal domains that bind to EB1 and DLG proteins. Due to its numerous interactions with a variety of proteins, APC are involved in cellular processes related to cell migration, adhesion, proliferation, differentiation, and chromosomal segregation.

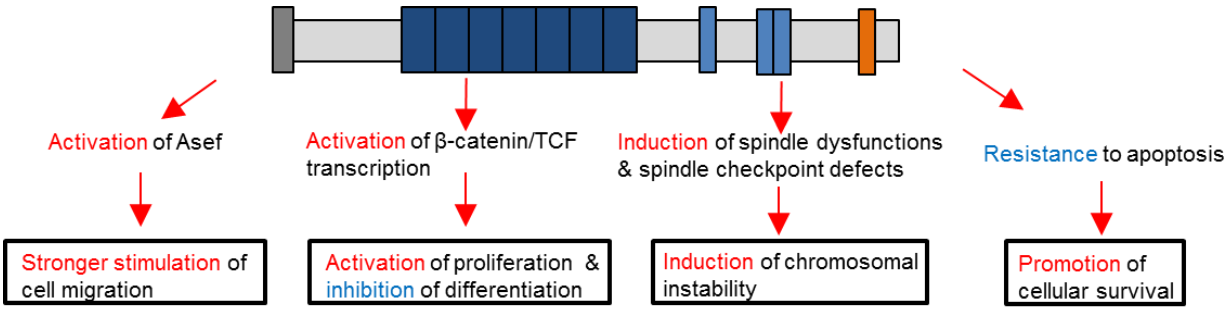


Figure 1-3. Structure and main functions of C-termianlly truncated APC. The C-terminally truncated proteins present in CRC lack the domains that are required for binding to microtubule, EB1 and β-catenin, thus leading to the induction of chromosomal instability, activation of proliferation, and inhibition of differentiation. There is also evidence suggesting that truncated APC may have dominant properties that lead to stronger stimulation of cell migration and promotion of cellular survival.

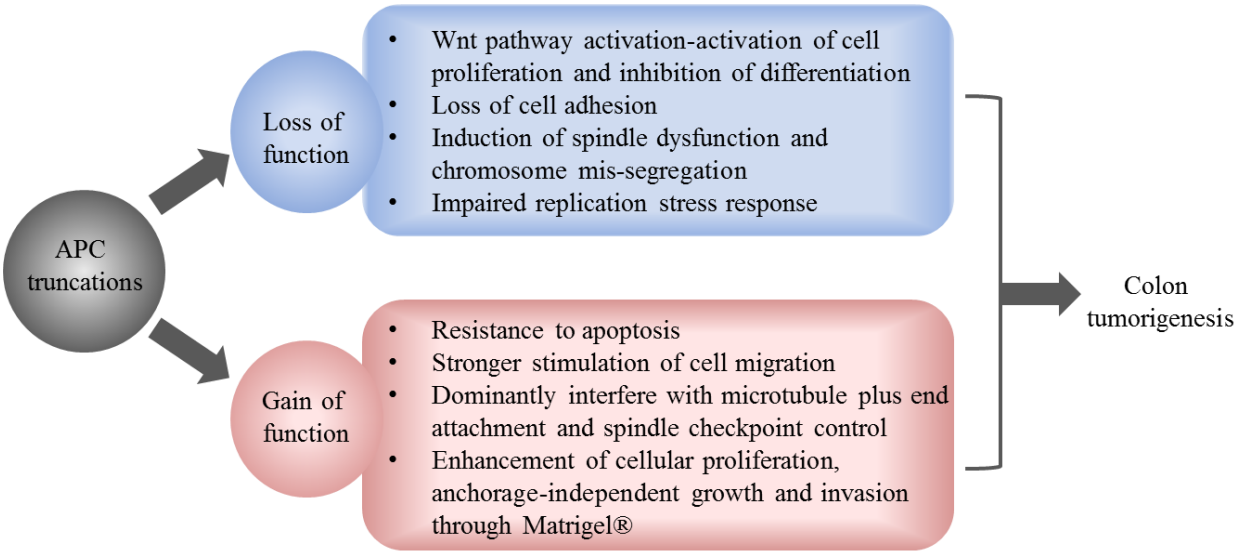


Figure 1-4. Dual roles of APC truncations in colorectal tumorigenesis. Both the loss of tumor suppressive functions and gain of functions of truncated APC proteins contribute to the initiation, progression and maintenance of colorectal cancer.

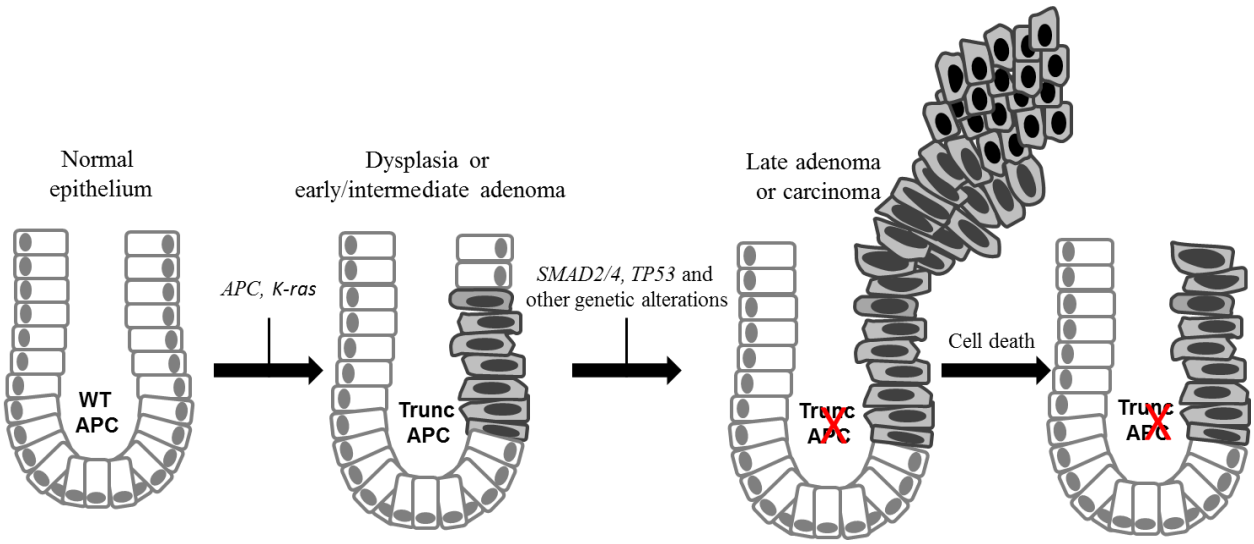


Figure 1-5. Dependency of CRC cells on truncated APC for cell growth and survival. In early stage of CRC tumorigenesis, APC truncations can promote tumorigenic properties in the presence of other genetic alterations. Gradually, the late stage CRC cells with altered signaling network become "addicted" to truncated APC for cell survival and maintaining tumorigenic properties. Interfering with expression of truncated APC can affect cellular proliferation and tumor cell survival. Trunc: truncated.

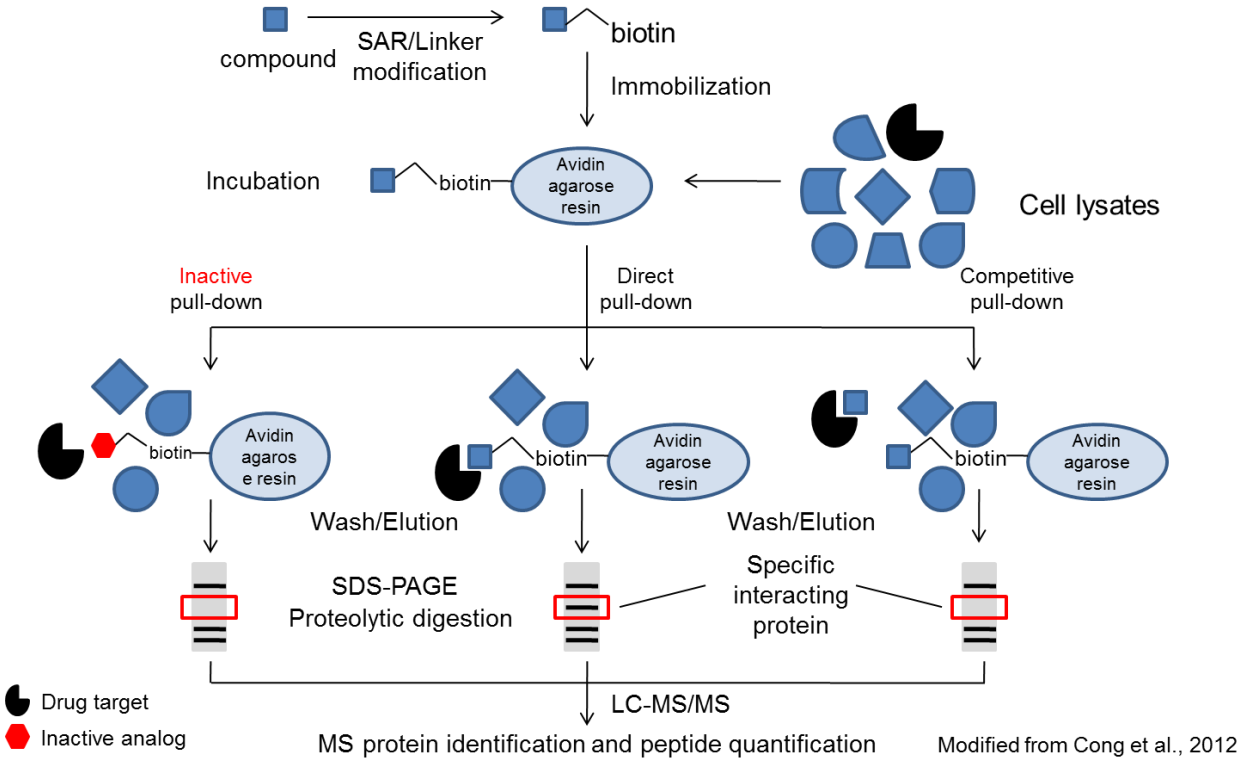


Figure 1-6. Flowchart of chemical proteomics for target identification. A linker derivative that retains biological activity is immobilized onto a solid-phase resin through a spacer arm. IN direct pull-down mode (middle), the compound-immobilized resin is incubated with cell lysates from cells or tissues or subcellular fraction, followed by separation and purification of interacting protein target. The enriched specific bands are then proteolytically digested and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) for protein identification. To distinguish specific binding proteins from nonspecific binders, incubation with inactive molecule (left) or free parental compound (right) were performed in parallel. In both of these methods, only nonspecific binders will apprear on the gel. Subsequent quantitative MS methods such as SILAC or iTRAQ can be used for comparative analysis of these experiments.

Chapter 2 . Cell Survival Pathways in Colorectal Cancer (CRC) Cells: Targeting Mutant Adenomatous Polyposis Coli (APC)

The work presented in this chapter was performed by Lu Zhang unless otherwise noted in the text and/or figure legends.

Introduction

Adenomatous polyposis coli (APC) is a multifunctional tumor suppressor gene that is mutated in more than 80% of colon tumors (Goss and Groden, 2000). APC mutation is believed to be one of the earliest events that contribute to colon cancer initiation (Powell et al., 1992). The primary function of APC has been attributed to the negative regulation of canonical Wnt signaling pathway through proteasomal degradation of β-catenin (Roberts et al., 2011). Additionally, it has been reported that APC functions beyond WNT pathway regulation, such as cellular processes related to cell cycle control, migration, differentiation, and apoptosis, all of which might contribute to colon cancer (Baeg et al., 1995; Browne et al., 1998; Chen et al., 2004; Chen et al., 2003; Kaplan et al., 2001; Kroboth et al., 2007; Nathke et al., 1996; Sansom et al., 2004; van de Wetering et al., 2002). Although both alleles are altered in APC-defective colorectal tumors, homozygous deletions of *APC* seem to be very rare. Instead, over 90% of APC mutations generate premature stop codons, resulting in truncated gene products, among which mutations at codons 1309 and 1450 are the most highly represented (Miyoshi et al., 1992; Polakis, 1997). While loss of APC tumor suppressing functions due to the mutational loss of the APC C-terminal sequence has been regarded as a critical event in the initiation of colon cancer, there is increasing evidence suggesting that APC truncations exert dominant functions that might contribute to colon tumorigenesis. These include enhancement of cell migration, interference with spindle formation, and induction of chromosome instability (Green and Kaplan, 2003; Kawasaki et al., 2003; Kawasaki et al., 2000; Tighe et al., 2004). Despite being such a highly frequent mutational event in CRC, there is no APC truncations targeted therapeutics for treating CRC patients. Here, we report the identification of a genotype-selective compound, TASIN-1(Truncated APC selective inhibitor-1) that can induce apoptotic

cell death in authentic CRC cells harboring APC truncations and also inhibit tumor growth in human xenografts and genetically engineered mouse models of CRC. This compound and its analogs serve as a platform for further translational development as putative drugs for clinical testing and may represent a novel therapeutic approach for prevention and intervention of the vast majority of human sporadic colon cancers.

Materials and Methods

Compound screen

The UT Southwestern chemical screening library (~230,000 small molecules) is composed of compounds purchased from ChemBridge Corporation (75,000), Chemical Diversity Labs (100,000), ComGenex (22,000), TimTek (1200), Prestwick (1100), and NIH (clinical collection, 450) as well as those submitted for screening by UTSW chemistry labs (2,500 synthetic compounds and ~30,000 natural product fractions from marine bacteria). The library is arrayed in DMSO in 384-well plates at a concentration of 0.5mM and stored at -20^{o}C. For each HTS experiment, cells were seeded at a density of 400 cells/well in 384-well plates. After 24 hours, compounds were added to a final concentration of 2.5 µM in 0.5% (v/v) DMSO in a one-compound-one well format (single replicate). Experimental samples (compounds) were limited to columns 3 to 22, with vehicle controls (0.5% DMSO only) in second and last two columns and a positive control consisting of 1µM of bardoxolone methyl (in 0.5% DMSO) in the first column. Plates were incubated for 96 hours at physiologic oxygen conditions (5% O_2) after which the Cell Titer-Glo® assay was performed to measure ATP levels as a surrogate for cell viability. Primary HTS, confirmation, and dose response data was analyzed using GeneData's Screener^{TM} software suite (GeneData, Inc.) as described previously(Kim et al., 2013). Compounds with Z score lower than -3 were identified as primary hits which were cherry picked and confirmed (3 replicates per compound). For high-throughput dose response curve analyses, cells were seeded in 384-well plate and treated with TASIN-1 at 12-point half log dilution series for 72 hours. Cell viability was determined using CTG assay.

Each value was normalized to cells treated with DMSO. Cheminformatics analysis was performed using the chemistry collection of Pipeline Pilot™ (Accelrys, Inc) and Instant J Chem™ (ChemAxon, Inc.).

Cell culture, cell synchronization and drug treatment

The culture conditions of HCECs and their isogenic series have been reported elsewhere (Roig et al., 2010). Briefly, HCECs were maintained under 2% oxygen and 5% carbon dioxide on Primaria (BD Biosciences) plates in 4:1 high-glucose Dulbecco modified Eagle medium/medium 199 with 2% cosmic calf serum (Hyclone) plus growth supplements: epidermal growth factor (EGF; 20 ng/ml; Peprotech), hydrocortisone (1 mg/ml), insulin (10 mg/ml), transferrin (2 mg/ml), and sodium selenite (5 nM) (all Sigma). All cancer cell lines and virus-producing cell lines (293FT, Phoenix A) were acquired from American Type Culture Collection (ATCC; Manassas, VA) and were cultured in basal medium supplemented with 10% serum and grown at 37˚C in a humidified 5% CO_2 atmosphere. All the cell lines are tested as mycoplasma free. 1,4'-Bipiperidine, 1'-[(4-methoxyphenyl) sulfonyl]-4-methyl-, ethanedioate (TASIN-1) is purchased from Chembridge and also synthesized in house and validated for its cytotoxicity. For dose response curve analyses, cells were seeded in 96-well plate in triplicate and treated with TASIN-1 at 10-point 3-fold dilution series for 72 hours. Cell viability was determined using CTG assay. Each value was normalized to cells treated with DMSO and the IC_{50} values are calculated using Graphpad Prism software. For cleaved PARP1 detection and caspase 3 activity assay, cells were treated with 2.5 μM of TASIN-1 for 72 h or 5 μM of doxorubicin (Sigma) alone for 6 h as positive controls. For mitotic synchronization, cells were treated with 100ng/mL of nocodazole in the presence or absence of 2.5 μM of TASIN-1 for 16 h. Cells were then treated with 2.5μM of TASIN-1 or 0.1% DMSO vehicle for additional 1h immediately following nocodazole washout.

Synthesis of TASIN-1 and analog 210

For the synthesis of TASIN-1, a mixture of 4-methyl-1,4'-bipiperidine (0.3664 g, 2.01 mmol), 4-methoxybenzenesulfonyl chloride (0.4787 g, 2.32 mmol), *N*, *N*-diisopropyl ethylamine (1.80 ml, 10.35 mmol), and CH_2Cl_2 (6 ml) was stirred at room temperature overnight. The reaction solution was then poured into saturated $NaHCO_3$ (40 ml) and extracted by CH_2Cl_2 (3 x 40 ml).The combined organic layers

were dried by Na_2SO_4, filtered and concentrated under reduced pressure. Flash chromatography (0.5:9.5 MeOH/CH_2Cl_2) provided the desired product as yellow solid (0.6674 g, 94%). A scheme of the synthetic route for TASIN-1 and analog 210 and characterization of these compounds are shown in Figure 2-15 and 2-16, respectively.

Plasmids

CDK4, hTERT, pSRZ-shTP53 and pBABE-hyg-*KRAS*^{V12} were described previously (Eskiocak et al., 2011). shRNAs targeting APC were in pLKO.1 vector. The lentiviral APC constructs were cut from pcDNA5 plasmids and cloned into pLenti6 vector.

Knockdown using shRNA

HCEC cells and DLD1 cells were infected with lentiviruses expressing shRNAs targeting APC(Table 2-2). HCECs were infected with retroviral vectors expressing shRNA against p53 (Sato et al., 2006).

Viral transductions

For retrovirus production, 2 mg of the appropriate vector was transfected into Phoenix A cells in 6-cm dishes with Effectene reagent (Qiagen) according to manufacturer's instructions. For lentivirus production, 1 mg of the appropriate vector together with 1mg of helper plasmids (0.4 mg pMD2G and 0.6 mg psPAX2) were transfected into 293FT cells with Effectene reagent (Qiagen). Viral supernatants were collected 48 hours after transfections and cleared through 0.45-mm filter. Cells were infected with viral supernatants containing 4 mg/mL polybrene (Sigma) and selected with appropriate antibiotics.

Growth rate assay

At 3 day intervals, HCEC cells were seeded at 10^5 cells/well in a 6-well plate in triplicate for a period of 27 days. Population doubling was calculated based on the cell counts of each cell suspension.

Cytochrome *c* release

DLD1 cells were seeded at a density of $5X10^5$ in 10cm dish and treated with DMSO or 2.5 μM TASIN-1 for 72 hours. At the time of harvesting, cells were trypsinized and collected in combination with

culturing media. After washing, cells were separated into mitochondria and cytoplasmic fractions using Q-proteome Mitochondria Isolation Kit (Qiagen). Cytoplasmic supernatant and mitochondrial pellets were solubilized in S.D.S–polyacrylamide gel electrophoresis loading buffer and analyzed by western blotting.

Soft agar assay

Cells were suspended in 0.375% Noble agar (Difco) in supplemented basal medium at a density of 250 cells containing DMSO or 2.5 μM of TASIN-1 and overlaid on 0.75% Noble agar in 24-well plates. Each sample was seeded in triplicate and each assay was performed at least twice using cells from different cell suspensions at different times. Colony formation efficiency was calculated by average number of colonies counted per well divided by number of seeded cells. Colonies larger than 0.1 mm were measured and counted after 7 days' growth and the average of those counts was used. Data was plotted as fold change compared with DMSO treated cells.

Invasion assays

Briefly, 10^5 cells were serum-starved overnight, suspended in basal medium and plated onto 8.0-μm pore Matrigel® transwell (BD Biosciences). Five hundred microliters of medium containing 2% serum and growth supplements was added to the bottom well. Non-migratory cells were scraped off 24 hours later, and migratory cells were stained with 4',6-diamidino-2-phenylindole (DAPI). Experiments were performed in triplicate transwells for biological triplicates and quantified by averaging the number of stained cells per 4x field of view counting five fields per chamber.

Western blot

Total cell lysates were prepared by harvesting cells in Laemmli S.D.S reducing buffer (50 mM Tris-HCl (pH 6.8), 2% S.D.S, and 10% glycerol), boiled and resolved on an 8% to 10% polyacrylamide gel, and transferred to polyvinylidinefluoride. The following antibodies were used: anti-APC (1:100, EMD Biosciences, OP44), anti-p53 (1:1000, Santa Cruz, sc-263), anti-cleaved PARP1 (1:1000, Cell Signaling, 9541S), anti-cytochrome c (1:1000, Cell Signaling, Beverly, MA), anti-β-actin (1:20,000, Sigma, A1978), HRP-conjugated goat anti-mouse or anti-rabbit (Jackson ImmunoResearch) were used as

secondary antibodies at 1:5000 and detected with SuperSignalWest Pico or Femto Chemiluminescent Substrate Kit (Thermo Scientific).

Caspase 3/7 activity

Cells were treated with vehicle control or 2.5 μM of TASIN-1 for 72 hours and subjected to Caspase-Glo 3/7 assay according to the manufacturer's instructions in 96-well plates (Promega).

Immunocytochemistry

Cells were fixed using 3.7% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min and incubated with blocking solution (10% goat serum and 3% BSA in PBS containing 0.1% Triton X-100) for 60 min. Cells were then incubated with primary antibodies diluted in blocking solution for an hour. The following antibodies are used: anti-HURP (1:50, Santa Cruz Biotechnology, sc-377004), anti-α-tubulin (1:100, Cell Signaling, 2125S). After incubation using secondary antibodies labeled with Alexa-568 or Alexa-488 (Invitrogen), slides were mounted with Mowiol 4-88 (Calbiochem) solution (Longin et al., 1993). Cells were observed under a fluorescence microscope, Axiovert 200M (Carl Zeiss). Nuclei were counterstained with DAPI (Vectashield, Vector Laboratories). Metaphase plate width, spindle width and cell width was determined using the line measurement tool in ImageJ software.

SuperTopFlash assay

SuperTopflash reporter constructs were kindly provided by Dr. Lawrence Lum from UT Southwestern and the assay was performed as described previously with minor modifications(Ren et al., 2011). Briefly, DLD1 cells were transiently transfected with DNA constructs encoding firefly luciferase (FL) and Gaussia luciferase (GL) proteins driven by a CMV promoter with Effectene reagent (Qiagen) according to manufacturer’s instructions and analyzed for GL and FL activities, respectively 48 hours’ later. DMSO, TASIN-1 or IWR1 were added 24 hours before assaying.

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated from mouse tissue using RNeasy Plus Universal Mini kit (Qiagen) according to the manufacturer's protocol. Then 1 μg RNA was converted to cDNA using a First Strand

cDNA Synthesis Kit (Roche). Real-time quantitative PCR reactions were set up in triplicate with Ssofast Master Mix (Biorad) and run on a LightCycler® 480 (Roche). Restriction fragment length polymorphism (RFLP) analysis for detection of mutant Kras was performed as described previously (Sato et al., 2006).All the primer sequences are listed in Table 2-3.

***In vivo* pharmacokinetic analysis**

CPC;Apc mice with mixed gender were injected i.p. with 20 mg/kg TASIN-1formulated as 5% DMSO, 5% cremophor EL, and 90% D_5W (5% dextrose in water) pH 7.4. Whole blood was harvested using acidified citrate dextrose (ACD) as the anticoagulant. Plasma was processed from whole blood by centrifugation of the ACD treated blood. Large intestines with contents were harvested and large intestine contents (LIC) were removed for further analysis. All harvested tissues were weighed and snap frozen in liquid nitrogen. PBS was used to homogenize large intestinal tissue and LIC and the resulting homogenate or plasma was subject to a simple organic extraction to precipitate protein and release total TASIN-1 compound. The supernatant was then analyzed by LC-MS/MS using a Sciex (Framingham, MA) 3200 Qtrap mass spectrometer coupled to a Shimadzu (Columbia, MD) Prominence LC with the mass spectrometer in MRM (multiple reaction monitoring) mode by following the precursor to fragment ion transition 354.2 → 171.0 (pos. mode; $M+H^+$). Non treated mice were used to collect plasma and tissue to prepare blank homogenates for standards and quality control samples (QCs).

Animal experiments

Subcutaneous (s.c.) xenografts were established in 5- to 6-week-old female athymic nude mice (NCI) by inoculation of $2X10^6$ CRC cells into both dorsal flanks of each mouse. When the tumors grew to 2 to 3 mm in diameter, the mice were injected i.p. with TASIN-1 at a dose of 40 mg/kg (dissolved in 0.2 mL solvent containing 10% DMSO, 10% cremophor) or solvent alone twice daily until the tumors grew to about 15 mm in diameter in the control group. Tumor volumes were measured using calipers and calculated using formula $l \times w^2 \times 0.5$, where l and w represented the length and width of the tumor, respectively. The colorectal transgenic cancer mouse model, *CDX2P-NLS Cre;Apc*$^{+/loxP}$ (*CPC;Apc*) mouse, was provided from Dr. Eric Fearon (Hinoi et al., 2007) and bred and housed in our facilities. Male

CPC;Apc mice ~110 days old were injected i.p. with either solvent or 20 mg/kg/injection of TASIN-1 twice a week for 90 days. Weights were measured every 15 days over the treatment period. These studies were performed according to the guidelines of the UT Southwestern Institutional Animal Care and Use Committee.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Scholl et al., 2007) with the following antibody: anti-cleaved caspase-3 (1:400, Cell Signaling, 9664P), anti-Ki-67 (1:1000, Cell Signaling, 12202S), anti-cyclin D1 (1:100, Cell Signaling, 2978S). Negative control slides were processed in the absence of the primary antibody.

Statistical analysis

Results are described as mean±s.e.m. or mean±s.d. Comparisons of different groups for statistical significance were analyzed by two-tailed, unpaired Student's *t*-test or multiple t-test using Graphpad Prism software. A *p*-value of 0.05 or less was considered significant.

Results

Generation and characterization of isogenic HCEC cell lines

To investigate the functions of truncated APC protein in CRC tumorigenesis, we developed a unique series of isogenic immortalized human colonic epithelial cell (HCEC) lines (Figure 2-1(A)). 1CT is the normal HCECs immortalized with telomerase and cyclin-dependent kinase 4 (CDK4), and we previously showed that these cells are non-transformed, karyotypically diploid, have multipotent stem-like characteristics, and can differentiate in 3D culture conditions(Roig et al., 2010). 1CTRPA A1309 harbors *TP53* and *APC* knockdowns (>90%), as well as ectopic expression of oncogenic $KRAS^{V12}$ and truncated APC1309, whereas 1CTRPA has the same genetic alterations except lacking the ectopic expression of truncated APC (Figure 2-1(B)). Mutations in genes *TP53, Kras* and *APC* are key molecular events that contribute to the initiation and progression of the vast majority of CRC (Fearon and Vogelstein, 1990). Specifically, this APC truncation (A1309) is strongly selected for in colon

cancers(Miyoshi et al., 1992). We found that ectopic expression of APC truncation promotes a moderate increase in proliferation, enhances soft agar growth, and increases migration/invasion through Matrigel® (Figure 2-3). Importantly, ~90% stable knockdown of WT APC does not have any of these effects, demonstrating that loss of APC function by itself does not drive colon cancer progression in this experimental cell culture model. These observations support the notion that APC truncations can promote tumorigenic properties in the presence of other genetic alterations.

Identification of genotype selective compounds targeting truncated APC

Utilizing this series of isogenic HCEC lines we carried out a toxicity screen to identify small molecules that can selectively inhibit cell growth of APC-truncated HCECs (1CTRPA A1309) within a 200,000 compound library. The primary positive hits were counter screened against 1CTRPA A1309, its isogenic precursor 1CTRPA and normal HCECs (1CT), which yielded 14 candidate compounds that selectively killed 1CTRPA A1309 cells. Dose response analysis in two authentic CRC cell lines: HCT116 (WT APC) and DLD1 (truncated APC), led to identification of the lead compound TASIN-1 (truncated APC selective inhibitor) (Figure 2-1(D)). The overall screening strategy is shown in Figure 1C. This compound exhibited potent and selective toxicity towards DLD1 cells (IC_{50} = 83 nM) but not towards HCT116 cells (IC_{50}>50 μM) (Figure 2-1(E)). Sustained treatment of TASIN-1 inhibited soft agar growth in DLD1 but not in HCT116 cells (Figure 2-1(F)).

Truncated APC is required for TASIN-1's cytotoxic effect

To validate APC truncation dependency, we generated two independent stable knockdown DLD1 cell lines expressing shRNAs against truncated APC. Knockdown of truncated APC expression desensitized DLD1 cells to TASIN-1 with a protein reduction of >90% (Figure 2-2(A)), supporting that APC or a protein(s) in a relevant APC-dependent pathway is the target of TASIN-1. Similar effects were observed in HT29 cells depleted of truncated APC protein (Figure 2-4(A)). In addition, ectopic expression of truncated APC partially sensitized HCT116 and HCT116 p53 null cells to TASIN-1 (Figure 2-2(B)). We next tested TASIN-1 in a panel of CRC cell lines with varied APC status. The expression of truncated APC proteins in the CRC cell lines harboring APC mutation were confirmed by Western Blot (Figure

S2B). Despite the highly heterogeneous genetic backgrounds, the results showed a consistent correlation between TASIN-1 sensitivity and truncated APC status (Figure 2-2(C)). TASIN-1 did not affect the viability of HCECs, human bronchial epithelial cells (HBEC) and BJ fibroblast cells which are derived from normal tissues as well as other cancer cell types with WT APC except for a breast cancer cell line, DU4475, which expresses truncated APC (Schlosshauer et al., 2000), again supporting APC truncation dependency (Figure 2-4(C) and 2-2(D), Table S2-1). These results are consistent with the view that APC truncation itself is directly implicated in the mechanism of action of TASIN-1. We discovered that TASIN-1 slightly inhibits cell growth of DLD1 cells but has little effect on the growth rate of HCT116 cells (Figure 2-5(A&B)). Moreover, TASIN-1 caused poly (ADP ribose) polymerase 1 (PARP1) cleavage, cytochrome c release from mitochondria, and induced caspase 3/7 activity in DLD1 cells but not in HCT116 cells (Figure 2-5(C-E)), indicative of induction of apoptosis.

TASIN-1 induces JNK-dependent apoptotic cell death

The mitogen-activated protein kinases (MAPKs) are the family of kinases that relay extracellular signals to the nucleus in response to a wide range of stimuli. Thus, the MAPK signaling pathways modulate diverse cellular programs including mitosis, proliferation, metabolism, apoptosis, etc (Wada and Penninger, 2004). To explore the potential role of MAPKs in TASIN-1's cytotoxicity, we treated cells with TASIN-1 and collect cell lysates at different time points for Western blot. TASIN-1 treatment led to activation of JNK after 48 hours in DLD1 cells but not in HCT116 cells and this activation persists until 72 hours (Figure 2-6 (A)). To determine if activation of JNK is the cause of TASIN-1 induced cell death, we treated DLD1 cells with either TASIN-1 alone or together with JNK inhibitor SP600125. Cotreatment with SP600125 attenuated TASIN-1's effects (Figure 2-6(B)). As shown in Figure 2-6(C&D), JNK inhibitor efficiently inhibited JNK activation and abolished cleavage of PARP as well as caspase3/7 activation. Collectively, these data demonstrate that TASIN-1 induced JNK-dependent apoptotic cell death in DLD1 cells.

TASIN-1 does not affect canonical Wnt pathway activity

Currently, intense efforts has been focused on targeting the transcriptional regulatory role of APC in β-catenin /Tcf activity in CRC (Goss and Groden, 2000; Huang et al., 2009; Lau et al., 2013; Waaler et al., 2012). We found that TASIN-1 does not affect Tcf/Lef transcriptional activity using the Topflash assay (Figure 2-7) as does IWR1, which is an inhibitor of Wnt response via tankyrase interaction and identified from a compound screen using the same chemical library (Chen et al., 2009a). None of our top 14 candidate hits overlap with the collection of high confidence Wnt pathway inhibitors from this previous screen (Chen et al., 2009a). Microarray analysis of TASIN-1 and IWR1 treated DLD1 cells showed that among a subset of critical Wnt genes down-regulated by IWR1, most are not affected by TASIN-1(data not shown). These results demonstrate that the effects of TASIN-1 are independent of canonical WNT signaling.

TASIN-1 interferes with mitotic spindle formation in DLD1 cells

APC truncation has been reported to exert a dominant function on microtubule kinetochore attachment (Green and Kaplan, 2003). We found that in metaphase synchronized DLD1cells (APC mutant) treated withTASIN-1, there is a broader metaphase plate and a reduced ratio of the spindle width:total cell width at the cell equator (Figure 2-8(A&B)), indicative of TASIN-1's effects on chromosome congression and mitotic spindle formation. Additionally, TASIN-1 treated DLD1 cells have abnormal staining of the K-fibre marker, HURP. TASIN-1 disrupted HURP staining in 62.6% ±14% of metaphase cells (Figure 2-8(C)). Also, TASIN-1 treatment compromised chromosome segregation as indicated by the appearance of lagging chromosomes and anaphase bridges in cells containing APC truncations (Figure 2-8(D)). TASIN-1 also affects HURP localization in HT29 cells (Figure 2-9(A)). Therefore, TASIN-1 disrupts mitotic spindle organization by disrupting K-fibres, leading to chromosome alignment defects. However, similar effects were not observed in HCT116 cells with WT APC (Figure 2-9(B)), suggesting that TASIN-1 is causing synthetic lethality in APC truncated cells partially through interference with microtubule function.

TASIN-1 inhibits tumor growth in xenograft mouse model

We next examined the *in vivo* antitumor activity of TASIN-1 in a xenograft mouse model. Nude mice with established DLD1 tumors were injected intraperitoneally with either solvent (control) or 40 mg/kg of TASIN-1 twice daily for 18 days. TASIN-1 treatment reduced the size of tumor xenografts (Figure 2-10(A)) and reduced tumor growth rates (Figure 2-10(B)) compared with control mice. No overt toxicity and no statistically significant differences were observed in the body weight of mice between the control and TASIN-1 treated groups (Figure 2-11(A)). Similar antitumor activity was observed in HT29 xenografts, which also harbors truncated APC and demonstrated a similar sensitivity as DLD1 *in vitro* (Figure 2-10(C)). However, TASIN-1 did not inhibit tumor growth in HCT116 (WT APC) xenografts (Figure 2-10(D)), demonstrating that TASIN-1 maintains selectivity *in vivo*. H&E staining and immunohistochemical analyses of excised tumors showed that TASIN-1 treatment led to appearance of apoptotic cells with fragmented nuclei and induction of the apoptotic marker, cleaved caspase 3 (Figure 2-11(B)). TASIN-1 treated tumor lysates from DLD1 xenografts also showed less truncated APC protein expression (Figure 2-11(C)), suggesting that the surviving DLD1 cells with less APC protein may be less sensitive to TASIN-1, thus not killed by TASIN-1.

TASIN-1 inhibits tumor growth in a genetic CRC mouse model

Considering that TASIN-1 is metabolically stable and has a long retention time in the mouse large intestinal tissue (Figure 2-13(A)), we further tested its antitumor effects in *CPC;Apc* mice, a genetically engineered mouse model that mainly develops colorectal tumors. These mice carry a *CDX2P-NLS* Cre recombinase transgene and a *lox*P-targeted *Apc* allele that deletes exon 14, leading to a frame shift at codon 580 and a truncated APC protein (Hinoi et al., 2007). Mice ~110 days old were injected intraperitoneally with solvent or 20 mg/kg TASIN-1 twice a week for 90 days. TASIN-1 treatment resulted in significant reduction in tumor formation in the colon of *CPC;Apc* mice (Figure 2-12(A&B)). Benign tumors (polyps) that developed in TASIN-1 treated *CPC;Apc* mice were much smaller compared to the control group (Figure 2-12(C)). Unlike control mice that lack weight gain over the 90 days' treatment, TASIN-1 treated mice gained weight probably due to reduced tumor burden (Figure 2-12(D)). In addition, tumors isolated from TASIN-1 treated mice showed suppressed expression of a panel of

inflammatory response genes (Figure 2-13(B)). Following toxicity study showed that TASIN-1 did not induce obvious histological changes on livers, kidneys or spleens (Figure 2-13(C)). Nor did it have any effects on cell blood counts or serum alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatine and NaCl levels (data not shown). Taken together, these *in vivo* experiments show that TASIN-1 efficiently attenuates tumorigenesis in both human xenografts and genetically engineered CRC mouse models without noticeable toxicity.

***In vitro* and *in vivo* evaluation of potent analogs of TASIN-1**

To develop more potent analogs with better drug-like properties, other compounds were designed and synthesized to evaluate their ability to induce selective cell death in APC truncated cells. We prepared a set of 53 analogs of TASIN-1to probe structure-activity relationship (SAR). The dose response curve studies showed that 23 out of the 53 analogs have lower IC_{50} than TASIN-1 (Table 2-4). The four most potent analogs showed selective toxicity towards A1309 and DLD1 cells (Figure 2-14(A)). As observed with TASIN-1, these analogs induced cleavage of PARP as well as activation of caspase 3/7 activity (Figures 2-14 (B&C)), demonstrating the association of the cytotoxicity of these potent analogs with apoptosis. Since analog 014 showed much greater efficacy than TASIN-1 *in vitro*, we next investigate its antitumor effects in xenograft mouse model. As shown in Figure 2-14 (D), 014 significantly inhibits tumor growth in DLD1 xenografts at lower dose than TASIN-1, but no growth inhibitory effects on HCT116 xenografts (Figure 2-14 (E)).

Discussion

There have been increasing reports suggesting that APC truncations may exert dominant functions that contribute to colon cancer tumorigenesis (Green and Kaplan, 2003; Kawasaki et al., 2003; Schneikert and Behrens, 2006; Tighe et al., 2004). We provide evidence showing that ectopic expression of APC truncations confer tumorigenic properties compared to its isogenic precursors (Figure 2-3). We also observed that transient knockdown of truncated APC in DLD1 CRC cells slowed down proliferation rates and induced apoptosis (data not shown), suggesting that expression of truncated APC protein can

lead to cell survival pathways in CRC cells which they gradually become "addicted" to for cell survival. However, there is no therapeutics directly targeting truncated APC proteins currently available. In our study, we have identified a small molecule, TASIN-1 that specifically kills HCECs and CRC cells with truncated APC while sparing cells expressing WT APC. TASIN-1 affects CRC cells through induction of apoptotic cell death both *in vitro* and *in vivo* in a Wnt pathway-independent manner. The strong selectivity for truncated APC harboring cells (Figure 2-2(C) and Figure 2-4(D)) and the observation that stable knockdown of truncated APC confer resistance to TASIN-1 in surviving cells (Figure 2-2(A) and Figure 2-4(A)) support that APC itself or a protein(s) in a relevant APC-dependent pathway is the direct target of TASIN-1. Our preliminary attempts to identify the protein target of TASIN-1 using chemical proteomics show that active analog of TASIN-1 can interact weakly with endogenous truncated APC protein in DLD1 cells and *in vitro* translated truncated APC protein (data not shown). Whether APC is the direct target of TASIN-1 or it is in a complex that TASIN-1 recognizes remains to be determined. Biophysics methods, such as isothermal titration calorimetry (ITC), can be applied to assess direct binding using recombinant protein. The future optimization of the chemical proteomics approach using more potent analog as well as immunoprecitation of HA-tagged truncated APC will reveal insights into the direct target of TASIN-1. Considering the high prevalence of APC mutations in patients with CRC, targeting truncated APC represent an ideal therapeutic strategy for prevention and intervention of CRC as well as a potential enrollment enrichment biomarker for future personalized medicine clinical trials. Therefore, TASIN-1 represents a paradigm shift for targeted therapy in CRC.

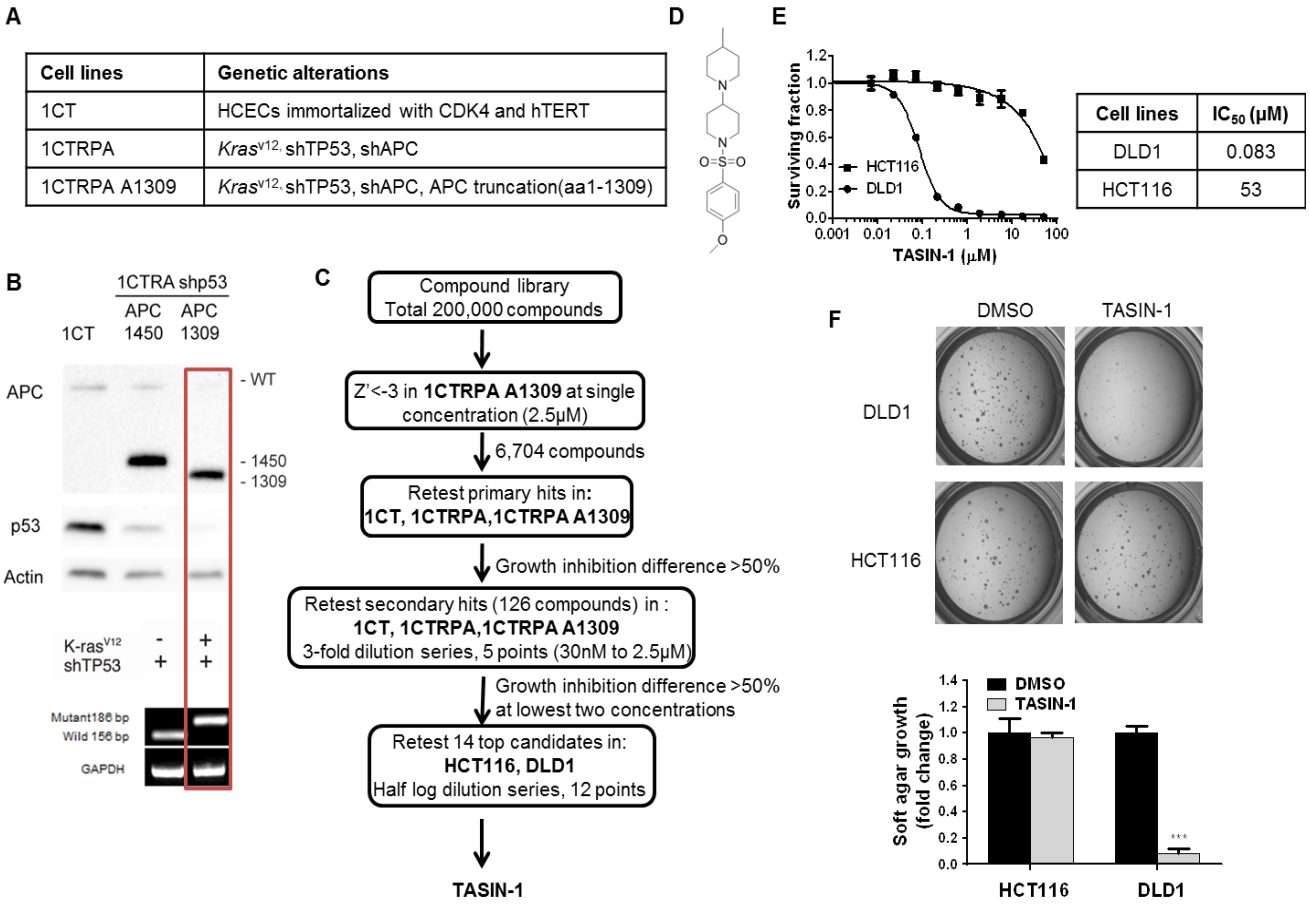


Figure 2-1. Identification of TASIN-1 through a 200,000 small molecule high-throughput screen. (**A**) List of isogenic HCECs used in the high-throughput screen. (**B**) Validation of ectopic expression of APC truncation, knockdown of WT APC, and p53 and expression of oncogenic Kras^{v12} by Western Blot or a restriction digestion assay. The cell line used in our primary screen is highlighted in the red box. (**C**) Flowchart of overall screening strategy. (**D**) Chemical structure of TASIN-1. (**E**) Dose response curve of TASIN-1 in HCT116 and DLD1 cells. The table (**E**) lists the IC_{50} value for each cell line. (**F**) Representative photographs and quantification of HCT116 and DLD1 cells grown in soft agar in the presence or absence of 2.5 μM of TASIN-1 for 7 days. Data represent mean±s.d., n=2. Student's *t*-test, ***P<0.001.

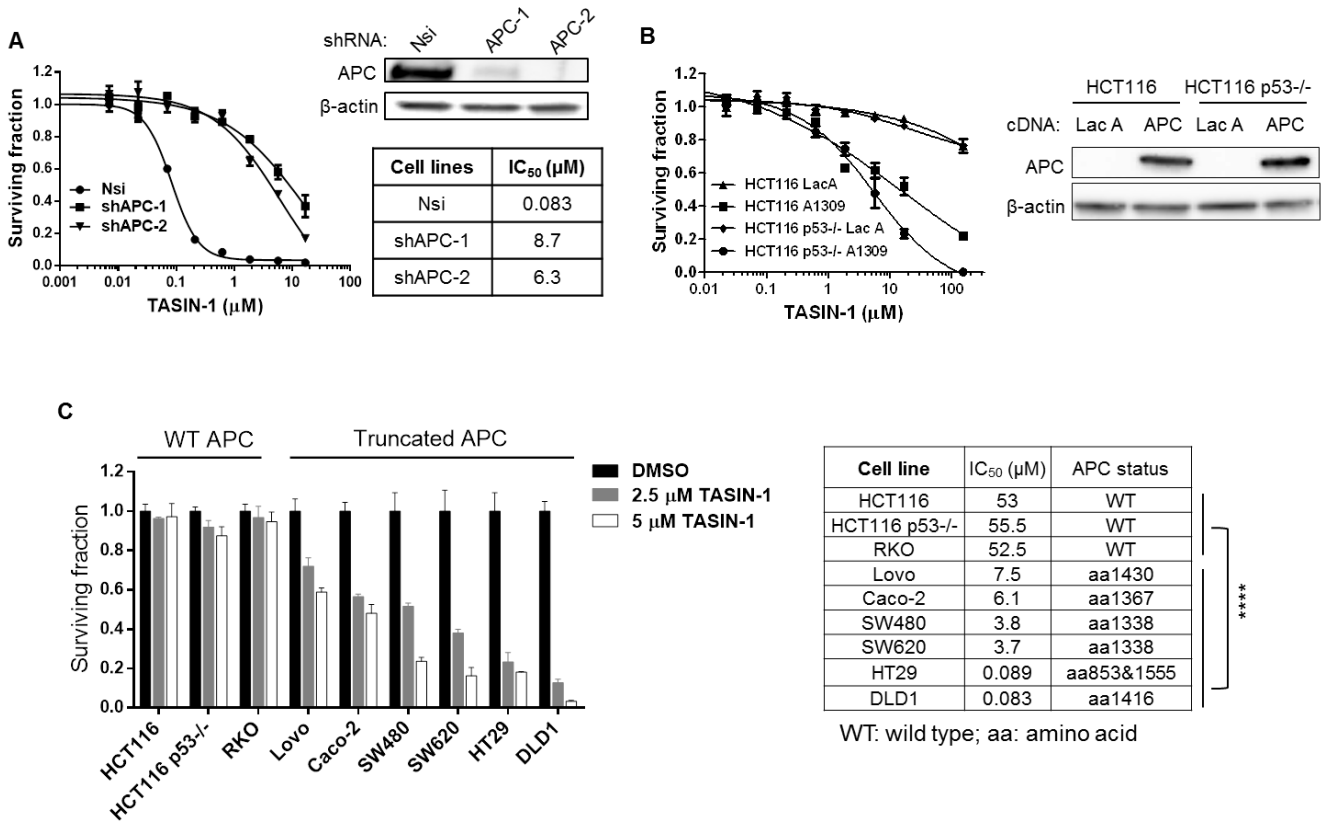


Figure 2-2. APC truncation is required for TASIN-1's cytotoxic effects. (**A**) Dose response curve of DLD1 cells expressing non-silencing (Nsi) shRNA or shRNAs against APC. The table (**A**) lists the IC_{50} value for each cell line. (**B**) Dose response curve of HCT116 and HCT116 p53-/- cells infected with a lentiviral vector expressing truncated APC A1309. Data represent mean±s.d., n=2. Knockdown and ectopic expression were demonstrated by Western blot. β-actin was used as the loading control. (**C**) Left bar graph shows the surviving fraction for each cell line treated with DMSO, 2.5μM or 5μM of TASIN-1 for 72h. Right table (**C**) lists the average IC_{50} values for each cell line from two biological replicates. Statistical significance was determined between average of IC_{50} values of the cell lines with WT APC and cell lines with truncated APC. Student's *t*-test, ****P<0.0001. The status of APC in each cell line is based on published data(Chandra et al., 2012) and the Cancer Genome Project Database (www.sanger.ac.uk/genetics/CGP).

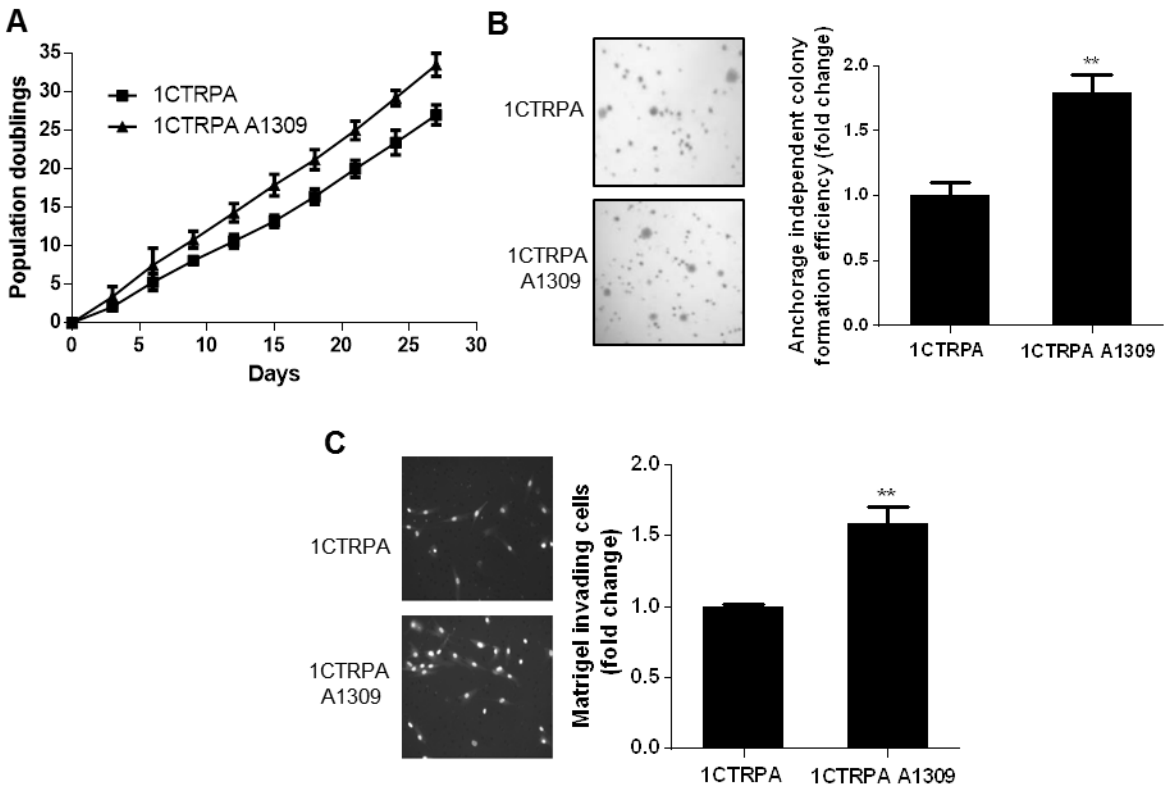


Figure 2-3. Ectopic expression of APC truncation confers tumorigenic properties. (**A**) Ectopic expression of APC truncation increased growth rate in 1CTRPA A1309 cells compared to 1CTRPA. Data represent mean±s.d. (**B**) Fold change in soft agar colony formation efficiency in 1CTRPA A1309 cells compared to 1CTRPA. Data represent mean±s.d., n=2. Student's *t*-test. ***P*<0.01. (**C**) Fold change in invasion through Matrigel® in 1CTRPA A1309 cells compared to 1CTRPA. Data represent mean±s.d., n=2. Student's *t*-test, ***P*<0.01.

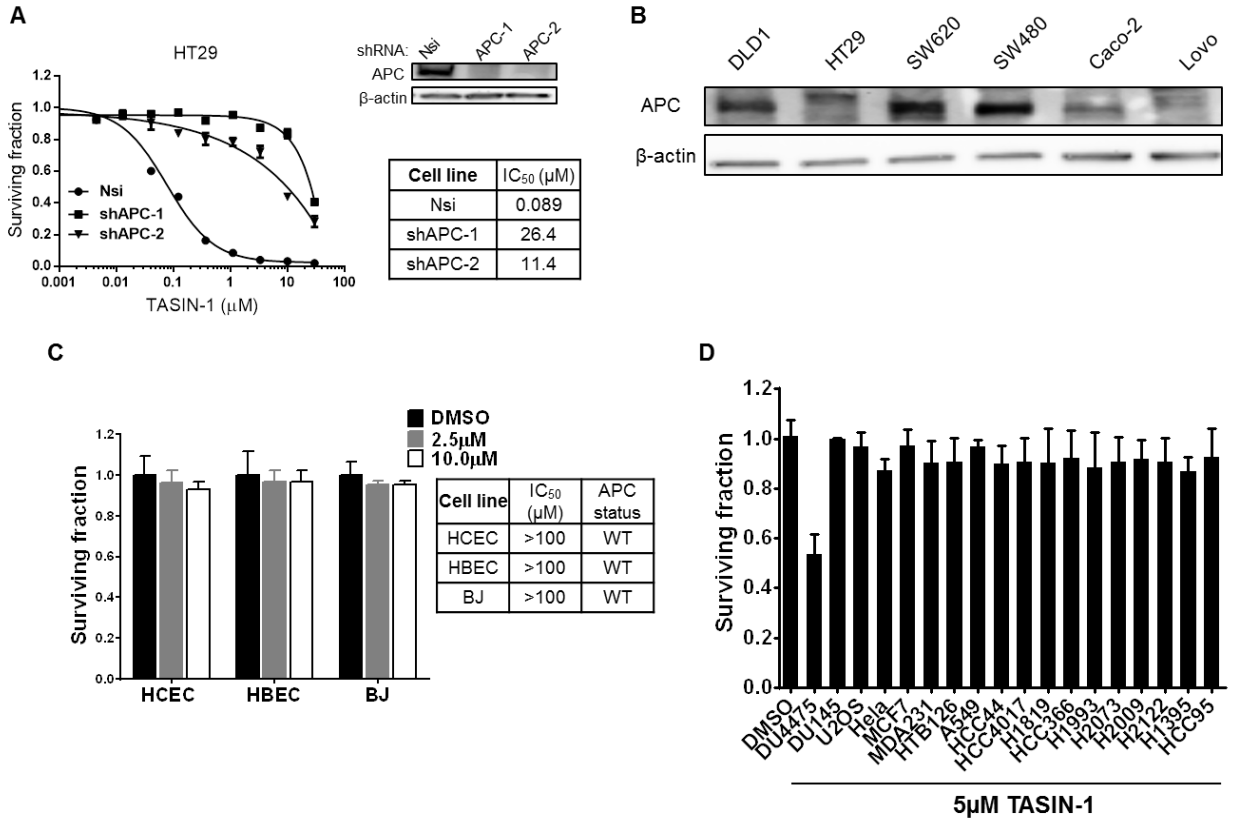


Figure 2-4. TASIN-1 is selectively toxic towards cells with truncated APC. (**A**) Depletion of truncated APC protein desensitizes HT29 cells to TASIN-1. The table (**A**) lists the IC_{50} value for each cell line. Knockdown efficiency was demonstrated by Western blot. β-actin was used as the loading control. (**B**) Western blot for truncated APC protein in CRC cell lines harboring APC mutation. β-actin was used as the loading control. (**C**) TASIN-1 does not affect viability of normal cell lines. Data represent mean±s.d., n=2. (**D**) TASIN-1 does not affect viability of other cancer type cells with WT APC but kills DU4457 cells with APC truncation mutation at a concentration of 5 μM. Data represent mean±s.d., n=2.

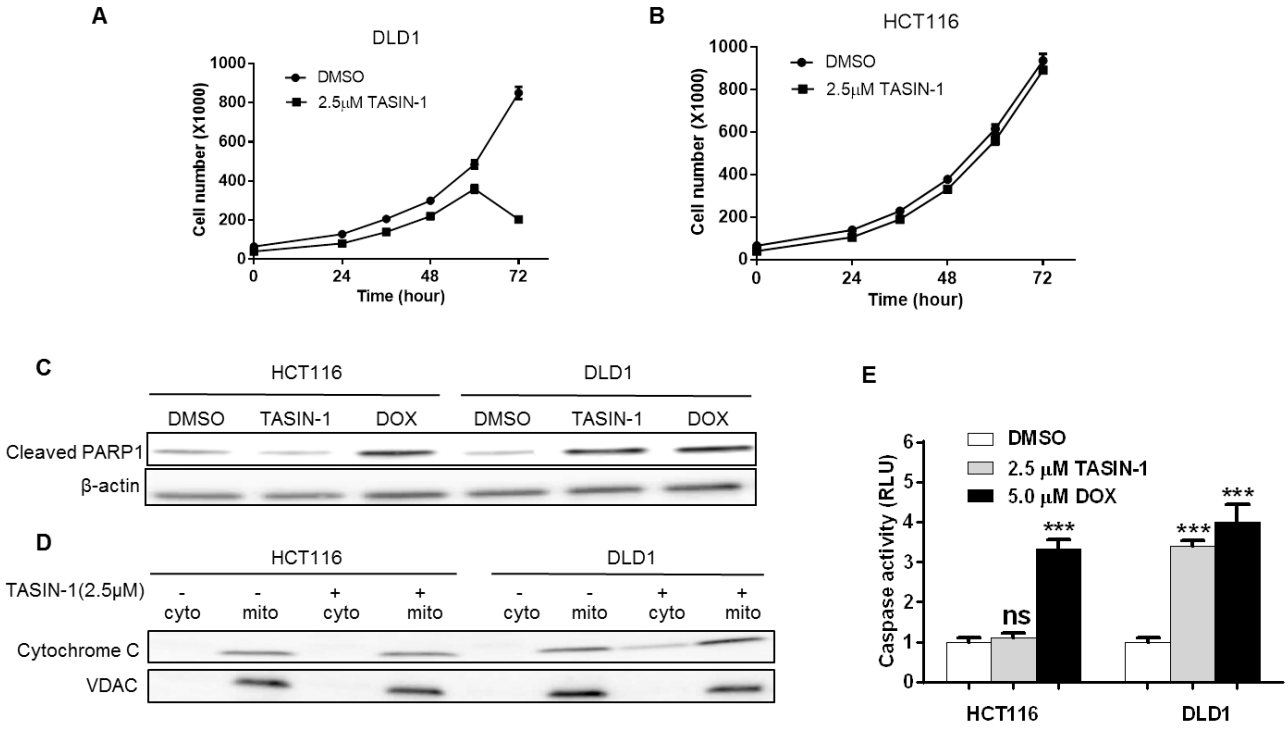


Figure 2-5. TASIN-1 suppresses cell proliferation and induces apoptosis in DLD1 but not in HCT116 cells. (**A**-**B**) HCT116 and DLD1 cells were seeded at 40,000 cells/well in 12-well plate at time 0 and treated with DMSO or 2.5 μM of TASIN-1. Cell counts were measured at indicated interval. (**C**) Incubation with TASIN-1for 72 hours induces cleavage of PARP1. β-actin was used as the loading control. (**D**) Incubation with TASIN-1for 72 hours induces release of cytochrome c from mitochondria into cytoplasm. VDAC was used as a marker for mitochondria. (**E**) TASIN-1 induces caspase 3 activation in DLD1 cells after 72 hours' treatment. Doxorubicin (DOX) was used as positive control for apoptosis. The values represent fold induction of caspase3/7 activity normalized to DMSO treated cells. RLU: relative luciferase unit. Student's *t*-test, ***P<0.001.

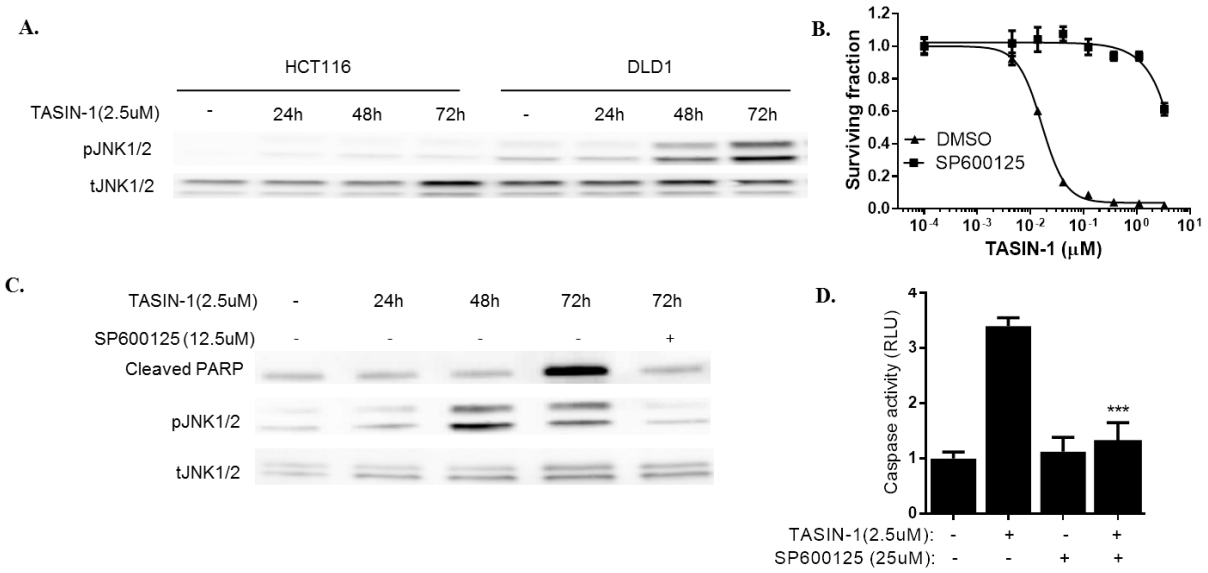


Figure 2-6. TASIN-1induces JNK-dependent apoptosis.(**A**) TASIN-1 treatment induced phosphorylation of JNK after 48h in DLD1 cells and this induction persisted until 72h. (**B**). Cotreatment of TASIN-1 with SP600125 reversed TASIN-1's killing effects in DLD1 cells. DLD1 cells were treated in the presence or absence of 12.5uM of SP600125 for 72 hours. Data represents mean±SD of triplicate samples. (**C**-**D**). DLD1 cells were treated with 2.5uM of TASIN-1 in the presence or absence of 12.5uM of SP600125. Cotreatment of TASIN-1 with SP600125 efficiently inhibited phosphorylation of JNK, abolished cleavage of PARP induced by TASIN-1 treatment (**C**) and reduced caspase 3/7 activity (**D**). For **D**, Column, mean of three experiments. Bars, S.E.M. Student's *t*-test, ***P*<0.01, ****P*<0.001. Abbreviation: DOX: doxorubicin.

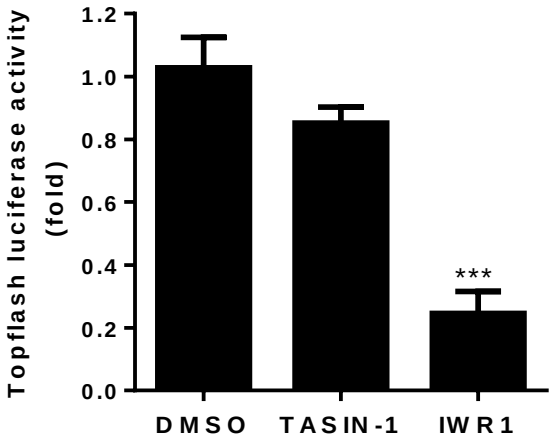


Figure 2-7.TASIN-1 does not affect Wnt pathway activity in DLD1 cells. TASIN-1 did not affect Tcf/Lef transcriptional activity using the SuperTopFlash reporter. IWR (Chen et al., 2009a) was used as a positive control. Data represent mean±s.d., n=2. Student's *t*-test, ***P<0.001.

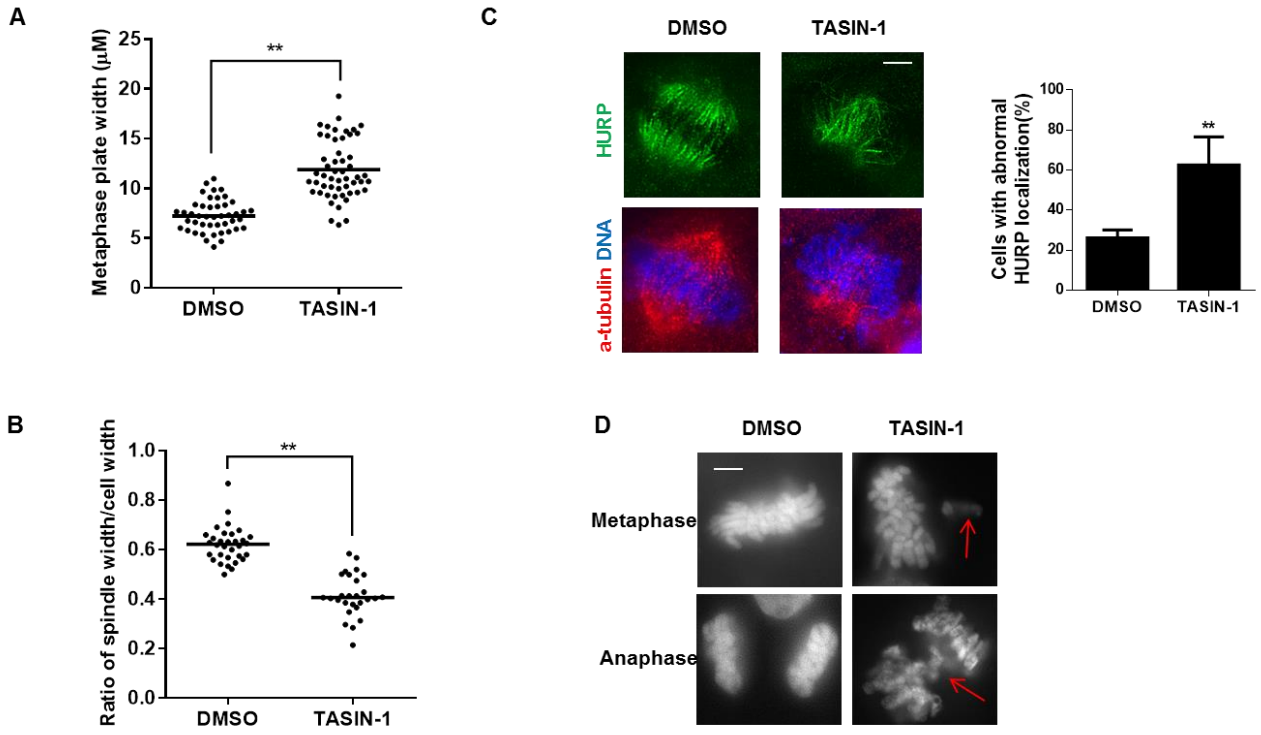


Figure 2-8. TASIN-1 disrupts mitotic spindles, chromosome alignment and K-fiber organization during mitosis in metaphase synchronized DLD1 cells. (**A**) The width of the metaphase plate. Student's *t*-test, ***P*<0.01. (**B**) The ratio of spindle width/total cell width of metaphase cells. Each dot represents a cell. Mean is indicated by the solid black line. Student's *t*-test, ***P*<0.01. (**C**) Representative images for metaphase synchronized cells stained for α-tubulin (magenta), HURP (green), and DNA (DAPI, blue). The graph shows the percentage of TASIN-1 treated cells with abnormal HURP localization. Data represent mean±s.e.m. of 50 cells scored from 3 biological triplicates. Student's *t*-test, ***P*< 0.01. (**D**) TASIN-1 treatment induces lagging chromosome and anaphase bridges as indicated by the red arrow. All scale bars: 10μM.

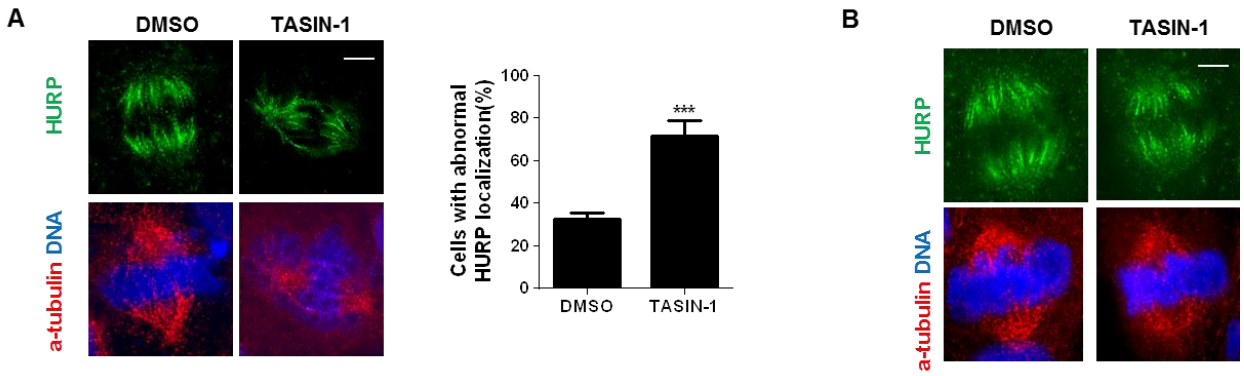


Figure 2-9.TASIN-1 affects HURP localization in HT29 cells but not in HCT116. HT29 and HCT116 cells were treated as in Figure 2-8. (**A**) Representative images for metaphase synchronized HT29 cells stained for α-tubulin (magenta), HURP (green), and DNA (DAPI, blue). The graph shows the percentage of TASIN-1 treated cells with abnormal HURP localization. Data represent mean±s.e.m. of 50 cells scored from 3 biological triplicates. Student's *t*-test, ***P*< 0.01. (**B**) Representative images for metaphase synchronized HCT116 cells stained for α-tubulin (magenta), HURP (green), and DNA (DAPI, blue). All scale bars: 10μM.

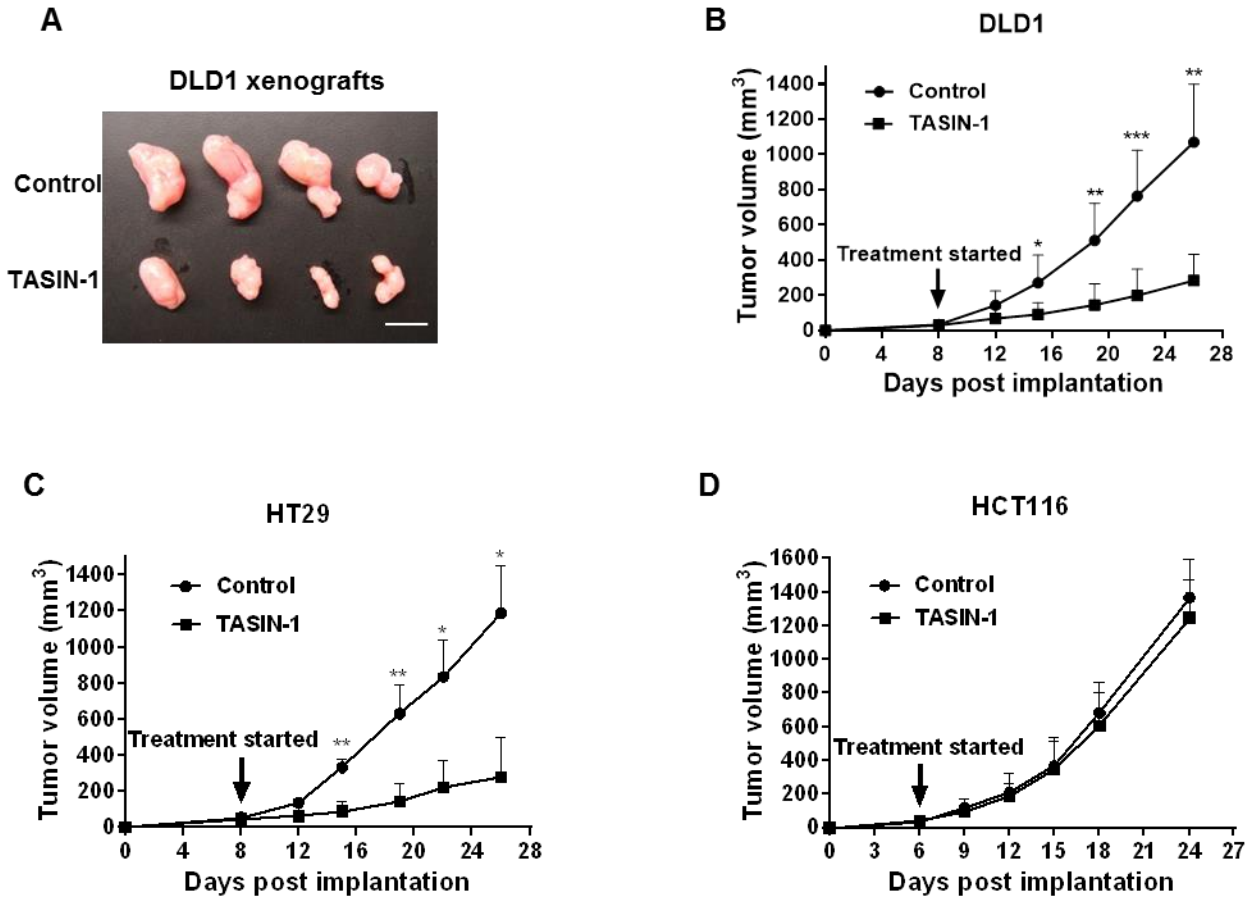


Figure 2-10. TASIN-1 selectively inhibits tumor growth in xenografts with APC truncation. (**A**) Tumor sizes of TASIN-1 treated DLD1 xenografts (below) are smaller than those of control mice (above). Scale bar, 10mm. (**B**) TASIN-1 significantly reduces tumor growth rate of DLD1 xenografts. Data represent mean±s.d. of 8 tumors. Student's *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001. (**C**) TASIN-1 significantly reduces tumor growth rate of HT29 xenografts. Data represent mean±s.d. of 8 tumors. Student's *t*-test, **P*<0.05, ***P*<0.01. (**D**) TASIN-1 does not reduce tumor growth rate of HCT116 xenografts.

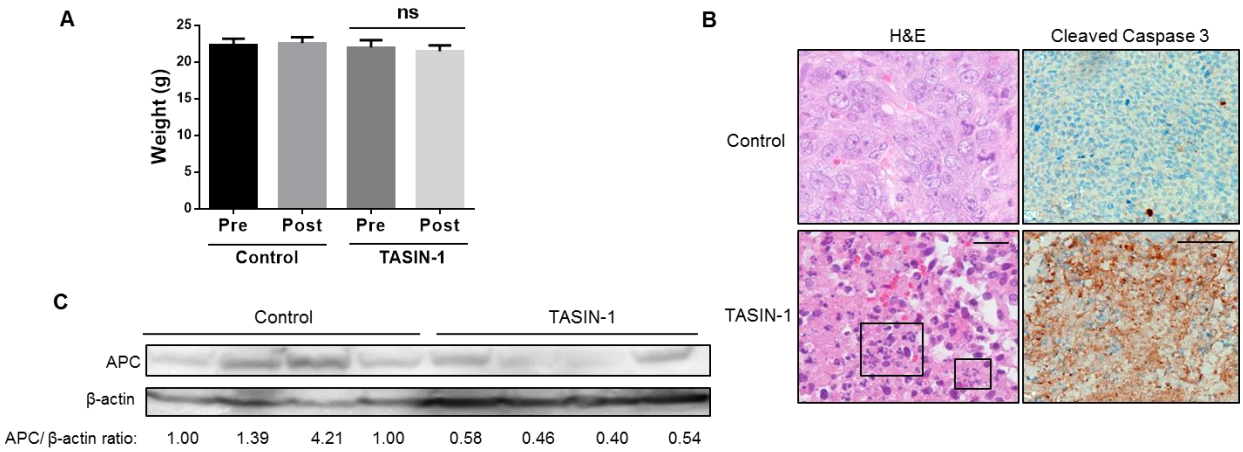


Figure 2-11.TASIN-1 inhibits tumor in xenograft mouse model through induction of apoptosis without noticeable toxicity. (**A**) Body weights before and after treatment in control or TASIN-1- treated nude mice. (**B**) Representative H&E staining images and immunohistochemistry (IHC) for cleaved caspase 3 in TASIN-1 or solvent treated DLD1 xenografts. Scale bar (left), 50μM; (right), 200μM. TASIN-1 treated tumors showed areas of apoptotic tumor cells (marked by box, lower left) and were positive staining for cleaved caspase 3 (lower right). (**C**) Western Blot was performed on tumor lysates from control and TASIN-1 treated DLD1 xenografts.

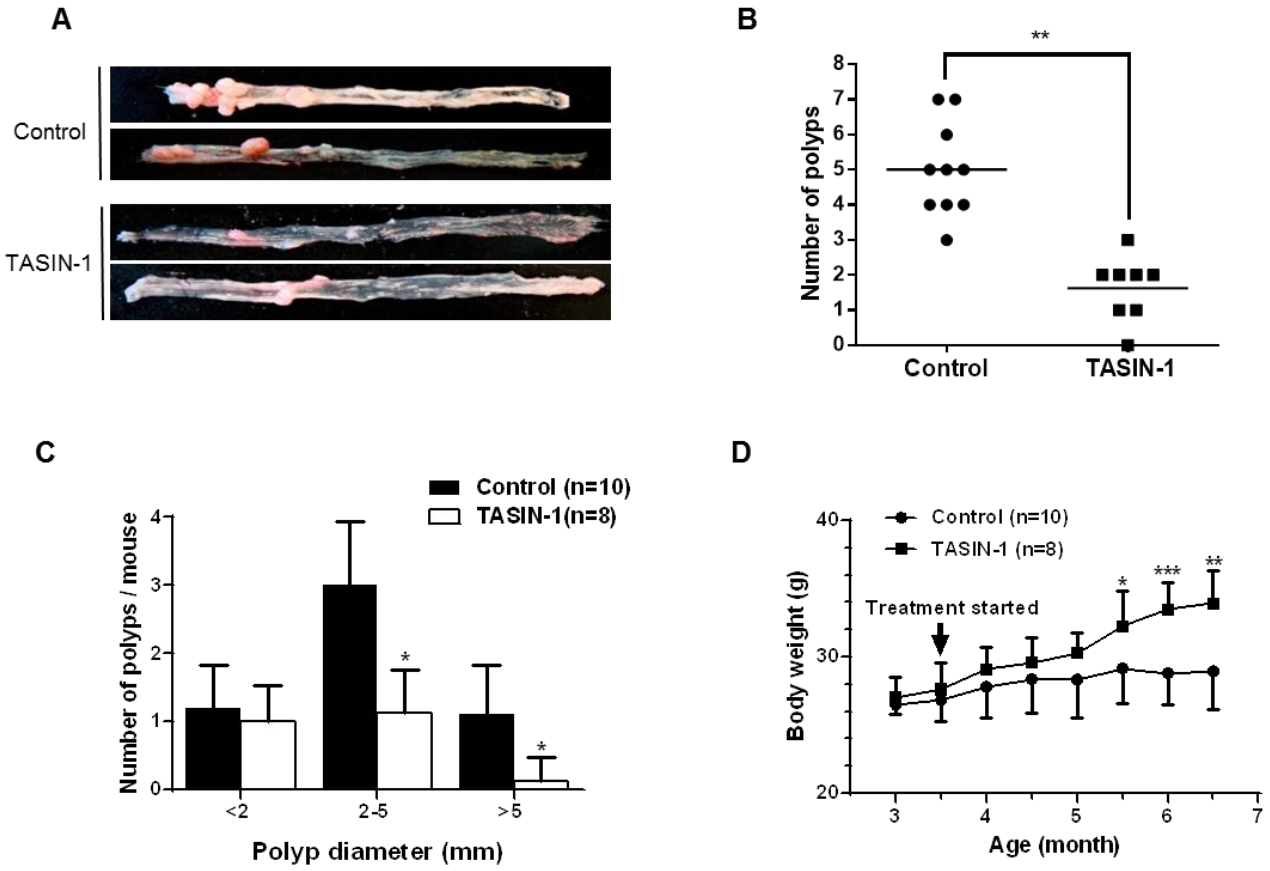


Figure 2-12. TASIN-1 reduces tumorigenicity in a genetic CRC mouse model. (**A**) Representative pictures of colons isolated from control or TASIN-1 treated *CPC; Apc* mice. (**B**)TASIN-1 treatment significantly reduces the number of benign (polyps) tumors. Each dot represents one mouse. Mean is indicated by the solid black line. Student's *t*-test, ***P*<0.01. (**C**) TASIN-1 significantly decreases polyp size in *CPC;Apc* mice. Data represent mean±s.d. of 8-10 mice. Multiple t test (FDR=1%), **P*<0.05. (**D**) TASIN-1 did not inhibit the growth of mice. Data represent mean±s.d. of 8-10 mice. Student's *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001.

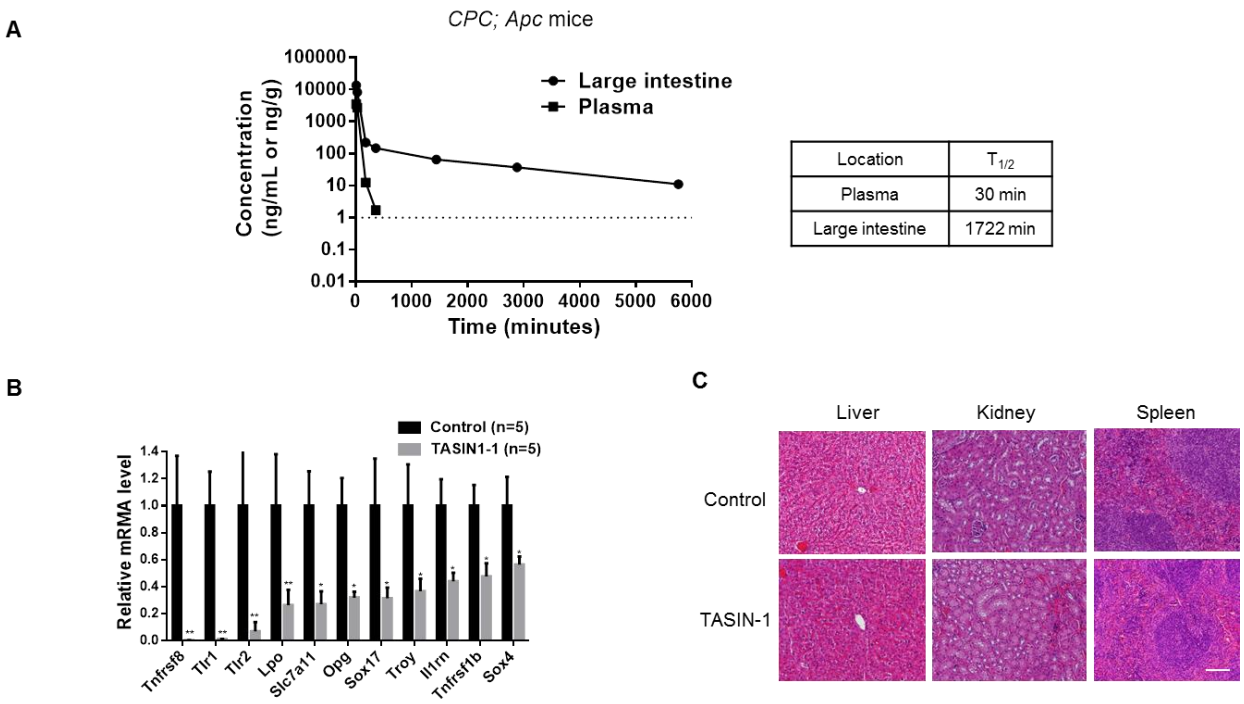


Figure 2-13.TASIN-1 reduces tumorigenicity in a genetically engineered CRC mouse model with little effects on liver, kidney and spleen. (**A**) TASIN-1 is mainly retained in large intestine tissue after i.p. injection. The half-life values of the compound in plasma and large intestine are listed in the table. This work is performed by Dr. Noelle Williams and Lorraine Morlock. (**B**) TASIN-1 suppresses expression of inflammatory gene set *in vivo*. RNA was extracted from tumor lysates of control and TASIN-1 treated group, cDNA synthesized and subjected to qPCR analysis. Data represent mean±s.d. of 5 mice. Student's *t*-test, **P*<0.05, ***P*<0.01. (**C**) H&E staining was performed on livers, kidneys and spleens collected at the end of study from both control and TASIN-1 treated groups. Scale bar, 50μM.

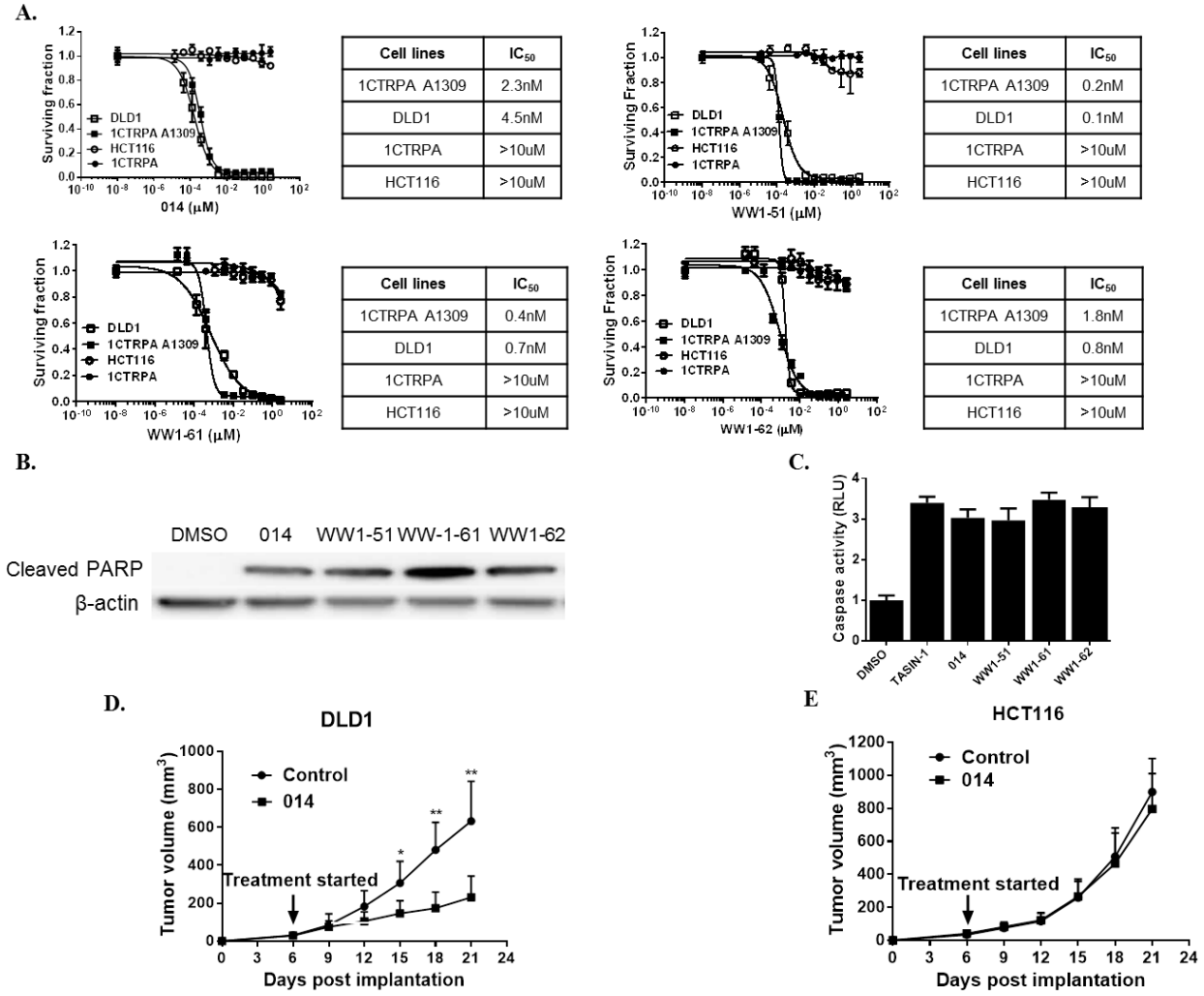
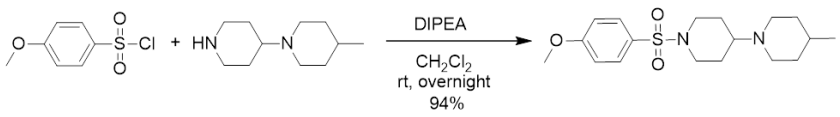
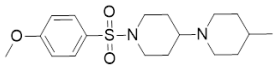


Figure 2-14.Evaluation of potency, selectivity, apoptosis-inducing effects and in vivo antitumor activity of the most potent analogs. (**A**). The four most potent analogs of TASIN-1 can potently and selectively kill DLD1 and 1CTRPA A1309 cells. The tables list the IC_{50} value of each cell line for each compound. Data are represented as mean mean±SD. These analogs were synthesized by Dr. Jef De Brabander, Wentian Wang and Priyabrata Das. (**B-C**). Evaluation of apoptotic cell death inducted by these analogs. As with TASIN-1, these four analogs induced cleavage of PARP (**B**) and activation of caspase 3 activity (**C**). Data are represented as mean mean±SD. (**D**). 014 significantly reduces tumor growth rate of DLD1 xenografts. Data are represented as mean mean±SD. Student's *t*-test, **P*<0.05, ***P*<0.01. (**E**) 014 does not reduce tumor growth rate of HCT116 xenografts. Data are represented as mean mean±SD.

A



B



^{1}H NMR (400 MHz,$CDCl_3$) δ 7.67 (d, *J* = 8.8 Hz, 2H), 6.97 (d, *J* = 8.8 Hz, 2H), 3.85 (s, 3H), 3.83 – 3.74 (d, *J* = 12.0 Hz, 2H), 2.76 (d, *J* = 11.7 Hz, 2H), 2.28 – 2.04 (m, 5H), 1.88 – 1.75 (d, *J* = 12.9 Hz, 2H), 1.72 – 1.54 (m, 4H), 1.28 (m, 1H), 1.23 – 1.08 (m, 2H), 0.87 (d, *J* = 6.3 Hz, 3H).; **^{13}C NMR** (100MHz, $CDCl_3$) δ 162.9, 129.8, 127.6, 114.1, 61.5, 55.6, 49.5, 46.2, 34.6, 31.0, 27.3, 21.9. **MS** (ES^+) calcd for $C_{18}H_{29}N_2O_3S$ $(M+H)^+$ 353.2, found.

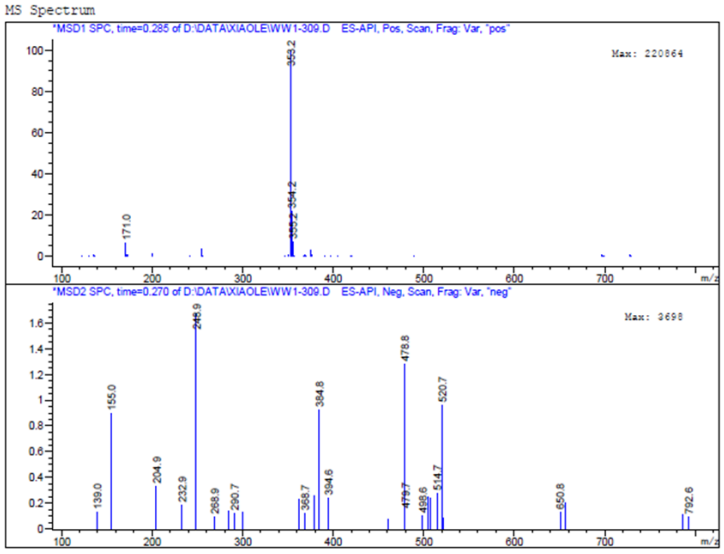


Figure 2-15. Synthesis and chemical characterization of TASIN-1. (**A**) Synthetic scheme for TASIN-1. (**B**) Chemical characterization of TASIN-1.

Table 2-1.APC status and origin of other cancer types.

Cell line	APC status	Tumor type
DU4475	aa1577	Breast carcinoma
MCF7	WT	Breast carcinoma
MDA231	WT	Breast carcinoma
HTB126	WT	Breast carcinoma
U2OS	WT	Osteosarcoma
DU145	WT	Prostate carcinoma
Hela	WT	Cervical carcinoma
A549	WT	Lung carcinoma
HCC366	WT	Lung carcinoma
HCC44	WT	Lung carcinoma
HCC4017	WT	Lung carcinoma
HCC95	WT	Lung carcinoma
H1819	WT	Lung carcinoma
H1993	WT	Lung carcinoma
H2073	WT	Lung carcinoma
H2009	WT	Lung carcinoma
H2122	WT	Lung carcinoma
H1395	WT	Lung carcinoma

Table 2-2. Sequence of shRNAs against APC.

shRNA	Mature sense sequence	Mature antisense sequence
APC-1	TAATGAACACTACAGATAGAA	TTCTATCTGTAGTGTTCATTA
APC-2	CCCAGTTTGTTTCTCAAGAAA	TTTCTTGAGAAACAAACTGGG

Table 2-3. qPCR primer sets for inflammatory genes.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
Il1rn	TTGTGCCAAGTCTGGAGATG	TTCTCAGAGCGGATGAAGGT
Lpo	TGACCTTGCTCCAGACTGC	TTGACCCAGACCTTGACCTC
Opg	ATGAACAAGTGGCTGTGCTG	TCACACAGGAGCTGATGACC
Slc7a11	TCTGGTCTGCCTGTGGAGTA	CAAAGGACCAAAGACCTCCA
Sox4	AATTGCACCAACTCCTCAGC	TCGATTGCAGTTCACGAGAG
Sox17	TGAAATATGGCCCACTCACA	CTGTCTTCCCTGTCTTGGTTG
Tlr1	GGACCTACCCTTGCAAACAA	TATCAGGACCCTCAGCTTGG
Tlr2	GAGCATCCGAATTGCATCA	ACAGCGTTTGCTGAAGAGGA
Tnfrsf1b	GTCTTCGAACTGCAGCTGTG	TACCCAGGTTCCGGTTTGTA
Tnfrsf8	GAGACTCGGGAAGCCAAGAT	GGTGGTCTTGAGTGGTCGAT
Troy	CGCTGCCATTCTCTTCCTAC	TCGATCCTTGAATTCCTGCT

Table 2-4. IC_{50} values for analogs of TASIN-1.

Analog	001	002*	005	006	007*	008	010*	011*
IC_{50} (nM)	**243**	**30.8**	**366**	**254**	**29**	**105**	**4.5**	**0.7**
Analog	012*	013*	014*	015	017*	018*	019*	020*
IC_{50} (nM)	**40.9**	**0.65**	**0.03**	**253**	**23**	**4.8**	**10**	**29**
Analog	021	022*	023*	024	025	026	027	029
IC_{50} (nM)	**426**	**56**	**19**	**421**	**147**	**501**	**205.4**	**338**
Analog	030	037*	038	042*	043*	044*	045	ww1-18
IC_{50} (nM)	**2940**	**4**	**206.9**	**8.2**	**39.5**	**26.8**	**97.5**	**293**
Analog	ww1-31*	ww1-35	ww1-36	ww1-37	ww1-38	ww1-41	ww1-42	ww1-44
IC_{50} (nM)	**1.2**	**7963**	**ND**	**3203**	**ND**	**172**	**374**	**3896**
Analog	ww1-46	ww1-48	ww1-50	ww1-51*	ww1-52	ww1-53*	ww1-54*	ww1-56
IC_{50} (nM)	**463**	**84**	**69**	**0.1**	**2300**	**2**	**0.96**	**1100**
Analog	ww1-58	ww1-59	ww1-61*	ww1-62*	ww1-63			
IC_{50} (nM)	**ND**	**225**	**0.6**	**0.6**	**96**			

Analogs highlighted with * has lower IC_{50} value than TASIN-1.

Chapter 3 . Identification of Novel Driver Tumor Suppressors through Functional Interrogation of Putative Passenger Mutations in Colorectal Cancer

The work presented in this chapter has been published in *International Journal of Cancer*. 132: 732-737. Experiments were performed by Lu Zhang unless otherwise noted in the text and/or figure legends.

Introduction

Cancer genome sequencing efforts are leading to the identification of an abundance of mutated genes in many cancer types (Stratton, 2011). Although these efforts provide a list and frequency of mutant genes, their functional relevance remains elusive (Pleasance et al., 2010). Genes frequently mutated across tumor samples are often considered to be driver mutations whereas those detected at a lower frequency are treated as passenger (or incidental) mutations. Driver mutations are defined as being causally involved in the neoplastic process and are positively selected for during tumorigenesis whereas passenger mutations are thought to provide no selective advantage but are retained by chance during clonal expansion (Greenman et al., 2007). Discriminating between driver and passenger mutations is critical for understanding the signaling pathways and molecular mechanisms underlying cancer initiation, progression and maintenance. Thus far, the efforts to subdivide driver from passenger mutations have largely, if not entirely, relied upon bioinformatic tools, and have not been subjected to rigorous experimental testing. These frequency-based approaches have raised concerns and debates as to the classification of driver and passenger mutations due to the lack of any experimental evidence (Parmigiani et al., 2009). Therefore, complementary functional studies are regarded as being critical for validation of driver mutations identified from large-scale genomic screens (Chin and Gray, 2008; Frohling et al., 2007).

The extreme diversity of mutations present in cancer genomes has contributed to the concept that pathways rather than individual genes govern tumorigenesis. Transcriptomic and proteomic analyses suggest a convergence onto a finite number of pathways that are involved in those mutational and

epigenetic events (Liu, 2008). Interactome network analysis has been shown to be a powerful tool in predicting protein functions, delineating pathways involved in human disease and identifying novel therapeutic targets for drug development (Chautard et al., 2009; Tewari et al., 2004). This approach thereby represents an important step towards a systematic and comprehensive understanding of biological networks.

Initial efforts to sequence the colorectal cancer (CRC) genome detected 151 highly mutated candidate genes (*CAN*-genes) and approximately 700 mutations occurring at a lower frequency (passenger mutations)(Wood et al., 2007). *CAN*-genes were determined primarily based on the total number of mutations per nucleotide sequenced (Wood et al., 2007). With few exceptions, almost all of these mutations occur at very low frequencies. Anchorage-independent growth is considered one of the hallmarks of cancer progression and one of the most reliable markers for *in vitro* transformation as well as *in vivo* human tumorigenic and metastasis potential (Mori et al., 2009). We previously functionally interrogated CRC mutations by conducting a soft agar-based short hairpin RNA (shRNA) screen within the cohort of *CAN*-genes. Knockdown of 65 of the 151 *CAN*-genes enhanced anchorage-independent growth in hTERT and CDK4 immortalized karyotypically diploid human colonic epithelial cells (HCECs) either expressing a shRNA targeting *TP53* and/or oncogenic *K-Ras*^{V12} (Eskiocak et al., 2011). We have now probed the extent of proclaimed passenger mutations and delineated the important pathways involved by constructing an interaction map of all 65 confirmed driver mutations with the additional less frequently mutated genes (Figure 3-1). We then screened for novel tumor suppressors of anchorage-independent growth within the cohort of passenger mutations. We found that knockdown of those passenger mutated genes that interacted with the confirmed *CAN*-genes enhanced anchorage-independent growth at a significantly higher frequency compared with those that did not interact. Additionally, there were more tumor suppressors than predicted by current statistical models even among the non-interacting low frequency mutated genes compared with random genes not involved in CRC. This study has identified a pool of novel driver mutations causally involved in anchorage-independent growth among the putative passenger mutations, demonstrating the need for biological functional assays to distinguish driver

from passenger mutations. These studies may lead to better predictive models and identification of novel therapeutic targets.

Materials and Methods

Cells

HCEC growth media and tissue culture conditions are described elsewhere (Roig et al.). HCECs isolated from normal colonic biopsies were immortalized by successive infections of CDK4 and hTERT followed by selection with respective antibiotics—G418 (250 μg/mL) and blastocidin (2.5 μg/mL). shRNAs against p53 were introduced with retroviruses and p53 knockdown efficiency was verified as described elsewhere (Eskiocak et al., 2011). Human colon cancer cell lines (HCT116, DLD-1, RKO) and virus-producing cell lines (293FT, Phoenix A) were cultured in basal medium supplemented with 10% serum. The identity of all cell lines was verified by DNA fingerprinting.

Viral transduction

1 μg of shRNA together with 1 μg of helper plasmids (0.4 μg pMD2G and 0.6 μg psPAX2) were transfected into 293FT cells with Polyjet reagent (SignaGen). Viral supernatants were collected 48 hours after transfection and cleared through 0.45-μm filter. Cells were infected with viral supernatants containing 4 μg/mL polybrene (Sigma) at multiplicity of infection (MOI) of approximately 1. Successfully infected cells were selected with 1ug/mL puromycin for 3 days. Clone IDs for each shRNA used in this study are listed in Table 1-8.

qRT-PCR

Total RNA was isolated from cells using RNeasyMinikit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Then 1 μg RNA was converted to cDNA using a First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN). Real-time quantitative PCR reactions were set up in triplicate with Ssofast Master Mix (Biorad) and run on a LightCycler® 480 (Roche). All the primers (Sigma) used in this study are listed in Table1-9.

Soft agar assay

Stably knockdown cells were suspended in 0.375% Noble agar (Difco, Detroit, MI) in supplemented basal medium at two densities (one thousand and two thousand) and overlaid on 0.75% Noble agar in 24-well plates. Each density was seeded in triplicate and each assay was performed at least twice using cells from different cell suspensions at different times. Colony formation efficiency was calculated by average number of colonies counted per well divided by number of seeded cells. Non-silencing shRNA expressing cells were seeded with each assay at the same density to be used as normalization control to correct for plate-to-plate variations. Colonies larger than 0.1 mm were measured and counted after 3 weeks growth and the average of those counts was used. Data was plotted as fold change compared to non-silencing shRNA expressing cells. GraphPad Prism 5 (GraphPad Software, Inc.) was used to plot data and perform two tailed Student's t-tests.

Network construction

Network construction was done in the NetWalker suite (Komurov et al, submitted) (http://netwalkersuite.org), using the comprehensive network of biomolecular relationships of protein-protein, gene regulation, neighboring and metabolic reactions described earlier (Komurov, 2012).

Results

A large number of putative passenger mutated genes enhanced anchorage-independent growth upon depletion

To discover novel tumor suppressors within the putative passenger genes, we first mapped the confirmed *CAN*-genes (from our previous screen that enhanced anchorage-independent growth upon depletion) onto the human protein interaction network. We then identified which of approximately 700 passenger CRC mutations had direct interactions with the confirmed *CAN*-genes (Figure 3-1). The soft agar-based shRNA screen was performed in a one-shRNA-one-well format using hTERT and CDK4 immortalized diploid HCECs with *TP53* knockdown. *TP53* is one of the most frequent mutations found in CRC. This premalignant HCEC derivative was not tumorigenic (Eskiocak et al., 2011), thus providing an ideal sensitized background that might allow for discovery of novel tumor suppressors. In the present

studies, we tested 25 less frequently mutated genes that interacted with at least one confirmed *CAN*-gene within the interaction map and 55 putative passenger genes that did not. Knockdown efficiency for all the tested genes was confirmed using quantitative real-time PCR except for those with non-detectable mRNA levels. Genes with at least two shRNAs that reduced mRNA levels were subjected to the soft agar assay and colony formation efficiency. Results were quantified, after normalizing to non-silencing shRNA controls (Figure 3-2(A)). We discovered that knockdown of 15 out of 25 less frequently mutated genes that interacted with confirmed *CAN*-genes (60%) promoted soft agar growth (Figure 3-2(B)) whereas knockdown of 7 out of 55 less frequently mutated genes that did not interact with any of the confirmed *CAN*-genes enhanced soft agar growth (12.7%) (Figure 3-2(C)). In our previous screen, we tested 362 random shRNAs and only 5 of them enhanced soft agar growth, thus giving a non-specific background rate of 1.4% (Eskiocak et al., 2011). Statistical analyses using Fisher's exact test revealed that the percentage of shRNAs scoring positive within either interacting (56.7%) or non-interacting (11.5%) category was higher than that of the random shRNAs, and a higher fraction of the interacting passenger mutated genes were causally involved in anchorage-independent growth (Table 3-1). Almost all of the scored hits exhibited a good correlation between knockdown levels and soft-agar growth enhancement, suggesting a dosage effect of these tumor suppressors. This supports the recently proposed continuum model of tumor suppression (Berger et al., 2011). In addition, the stable knockdown HCECs expressing shRNAs against the most potent hits also exhibited enhanced invasion through Matrigel® (Figure 3-3(A)). We examined the relevance of these observations to authentic cancer cell lines by overexpressing the gene (*ITPR1*) whose knockdown had produced the greatest effect on invasion through extracellular matrices in our test cell lines. Ectopically expressed wild type *ITPR1* cDNA reduced invasion efficiency in all three colon cancer cell lines tested (Figure 3-3(B)). Sequencing analysis of these three cancer cell lines revealed that there are no nonsynonymous mutations within the coding sequences where the mutations in those CRC patients have been identified. One possibility is that other related protein components within this pathway are mutated, rendering these cell lines sensitive to the restoration of

ITPR1 function. Future experiments will further investigate the tumor-suppressive function of this gene in CRC.

Newly identified driver mutations are highly represented across CRC tumor samples

A closer analysis of the genomic-wide sequenced CRC tumor samples from Wood *et al* (Wood et al., 2007) revealed that on average, at least 9.4% of all mutated genes in any individual colon cancer tumor are involved in anchorage independent growth (Table 3-2) and this percentage could be underestimated considering that our approach is not at all exhaustive. Furthermore, analysis of the most recently available Cancer Genome Atlas (TCGA) CRC dataset (http://www.cbioportal.org/public-portal/) showed that the 22 newly identified suppressor genes are altered in 29% of all 193 cases (Table 3-3). Of note, ITPR1, one of the most potent hits scored in our screen, is altered in 5.7% of all CRC cases, higher than almost all the other cancer types, supporting its important role in CRC tumorigenesis (Table 3-4). Most of the mutations identified in CRC patients occur within the six functional domains in the coding sequence and are predicted to have medium or high functional impact (http://www.cbioportal.org/public-portal/).These analyses suggest that a large fraction of mutations present within a tumor are causally involved in tumorigenesis rather than incidental events and each of these mutations might provide an additive fitness for pre-cancerous cells to progress into a more malignant state.

Sub-networks involving DNA repair pathways and cyclic nucleotide metabolic process are enriched within the network map

Considering that *PTEN, TP53* and *FBXW7* are among the most frequent mutations in colon cancer, we focused on the interacting passenger mutations that link these genes. Surprisingly, we found that all the interacting less frequently mutated genes for which adequate shRNAs were available within this small sub-network scored positive in our screen (Figure 3-4). Four of them (*RPA3, RFC, LIG1* and *HUWE1),* together with *TP53* and *ERCC6* have established roles in DNA repair. We next placed all the positive hits from both of our screens as the central nodes and explored additional interactors among those less frequently mutated CRC genes by extending the interaction map using the same network analysis (Figure 3-5). Enrichment analysis for all the genes within this extended map using Molecular Signature

Database showed that 7 out of all the enriched pathways are related to this process, such as double-strand break repair, homologous recombination repair, and nucleotide excision repair (Table 3-5). Although it's well established that deficiency in these pathways contribute to tumorigenesis (Hung et al., 2005; Jiricny and Marra, 2003; Khanna and Jackson, 2001), it remains largely controversial whether these proteins function as tumor suppressors (Chen et al., 2006; Givalos et al., 2007; Ljuslinder et al., 2007). Thus, our study provides the first direct evidence for the suppressive role of these proteins in tumorigenesis.

Functional enrichment analysis for all the genes within this extended interaction map revealed the enhancement for various aspects of biological processes among which cyclic nucleotide metabolic process is the most highly enriched (Table 3-6). All the tested genes involved in this process (*GNAS, ADCY9, ADORA1, GUCY1A2*) scored positive in our screen, supporting the important role of cyclic nucleotide processes in colon tumor development. These two sub-networks (TP53 and cyclic nucleotides) are representative of the central components of the regulatory network underlying CRC tumorigenesis (Figure 3-6). We then performed mutual exclusivity/co-occurrence analysis based on TCGA CRC dataset using odds ratio and Fisher's exact tests. We found that there was a strong tendency towards co-occurrence for most pairs of the genes present in DNA repair sub-network ($p<0.05$), suggesting that these genes harbor additive effects in tumor development and disruption of various aspects of DNA repair processes might contribute to tumorigenesis, or they may play a role in other pathways beyond DNA repair. In contrast, no such strong tendency was observed among the genes implicated in cyclic nucleotide metabolic process. Notably, *GNAS* and *GHRHR* tended to be mutually exclusive with the other 5 genes within this sub-network (Table 3-7(A&B)). Similar analysis performed for another small transcriptional control central hub consisting of *TP53, TGFBR2, SMAD2*, which were among the most frequent mutations present in CRC and *HUWE1*, a newly identified driver mutation from our screen showed that *TGFBR2* and *TP53* tended to be mutually exclusive whereas *TGFBR2* and *HUWE1* tended to co-occur in CRC tumor samples (Table 3-7(C)). Taken together, these observations indicate the important roles of DNA repair pathways, cyclic nucleotide metabolism and many other biological processes in the regulation of colon tumor development and revealed molecular relationships among the protein

components within the representative central sub-networks. These results provide a platform for future system-level analyses as well as detailed mechanistic investigations.

Discussion

This study functionally interrogated the putative passenger mutated genes in the CRC genome by using a relevant biological transformation assay in combination with network analysis. We identified a number of novel driver tumor suppressors among the genes that are otherwise considered passengers. We discovered that driver mutations are enriched in the putative passenger mutations interacting with confirmed *CAN*-genes, and there are far more "drivers" even among the non-interacting "passengers". Additionally, we demonstrated that a subset of newly identified candidate tumor suppressors are also involved in invasion through the basement membrane and thus may play important roles in multiple stages of CRC tumorigenesis. These multifunctional low frequency mutated genes may represent a pool of novel potential drug targets that were previously unknown or misclassified as passenger mutations. For example, *ADORA1*, a newly identified tumor suppressor from the present study, has recently been shown to suppress CW2 human colonic cancer growth by inducing apoptosis (Saito et al., 2010). Thus *ADORA1*might be a novel drug target considering the availability of highly selective and potent agonists against this adenosine receptor (Lohse et al., 1988). In conclusion, the observation that a large fraction of the low frequently mutated genes have been misclassified as passengers reveals that frequency-based biostatistic models performed poorly for parsing driver mutations and demonstrate the need for implementing biologically relevant functional filters to distinguish between driver and passenger mutations instead of solely depending on bioinformatics.

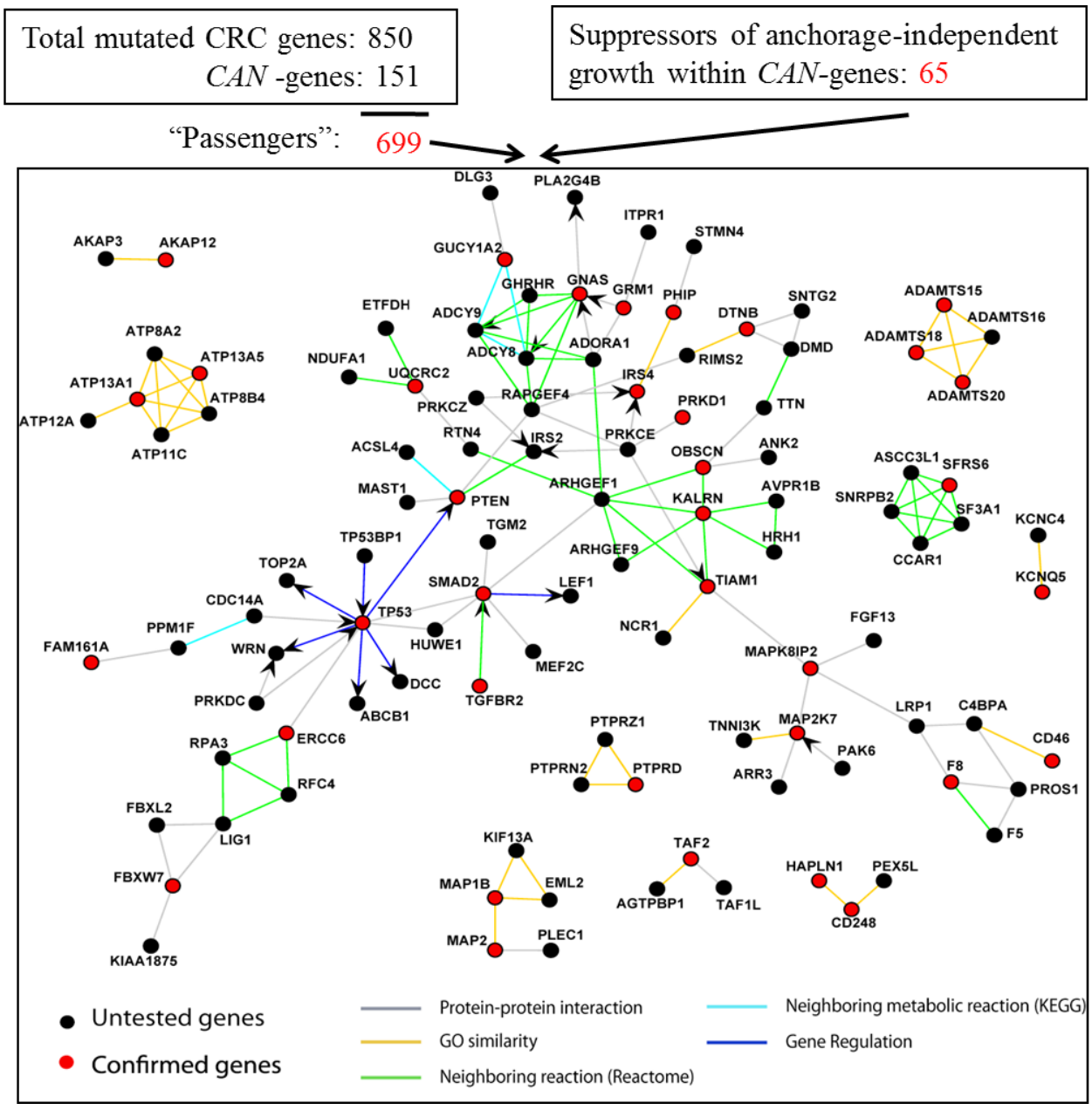


Figure 3-1. Discovering novel tumor suppressors from passenger mutations by interaction mapping. Data on the interaction of confirmed *CAN*-genes (red nodes) and less frequently mutated genes (black nodes) are from Wood *et al*. Interactions are colored according to type of interactions shown in the color key. (Adapted from Fig.4 in (Eskiocak et al., 2011))

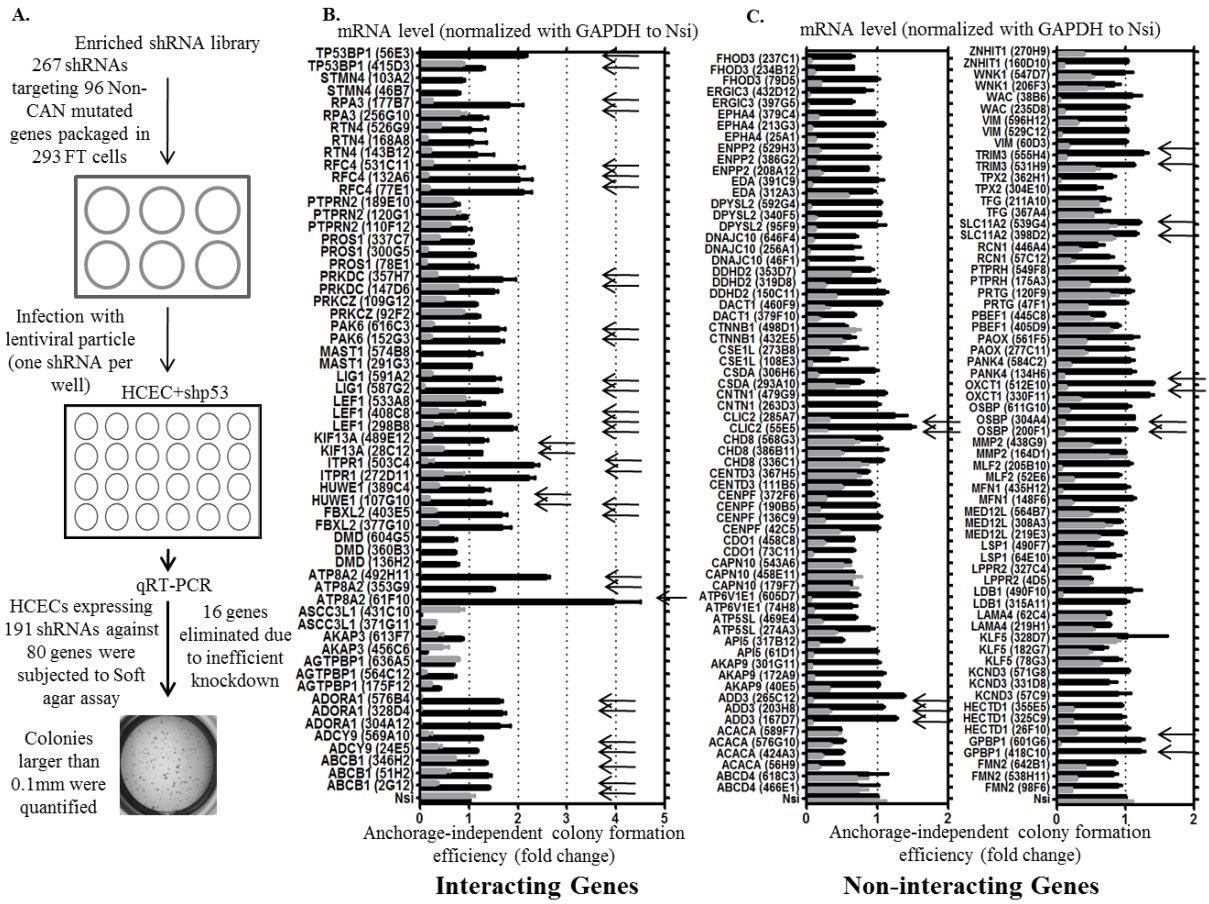


Figure 3-2. Identification of novel driver mutations among putative passenger mutations. (A) Schematic representation of the overall screening strategy. HCECs expressing shRNA against p53 were infected with lentiviral shRNA constructs in a "one-shRNA-one-well" format, knockdown efficiency measured by quantitative RT-PCR and colonies were quantified after 3 weeks. (B) Quantitative validation of shRNA knockdown efficiency (grey bars) and soft agar growth enhancement (black bars) for passenger mutated genes that interact with confirmed *CAN*-genes in shTP53 expressing HCECs. (C) Same analysis as in Figure 2B for passenger mutated genes that did not interact with confirmed *CAN*-genes. "1-fold" represents no change compared to non-silencing shRNAs for both mRNA levels and anchorage independent growth. Each black bar represents 9 data points (triplicates from three separate experiments). Arrows denote shRNAs that enhance anchorage-independent growth in a statistically significant manner (two tailed Student's T-test, compared to non-silencing, mean ± s.e.m, $P<0.05$). Nsi, non-silencing.

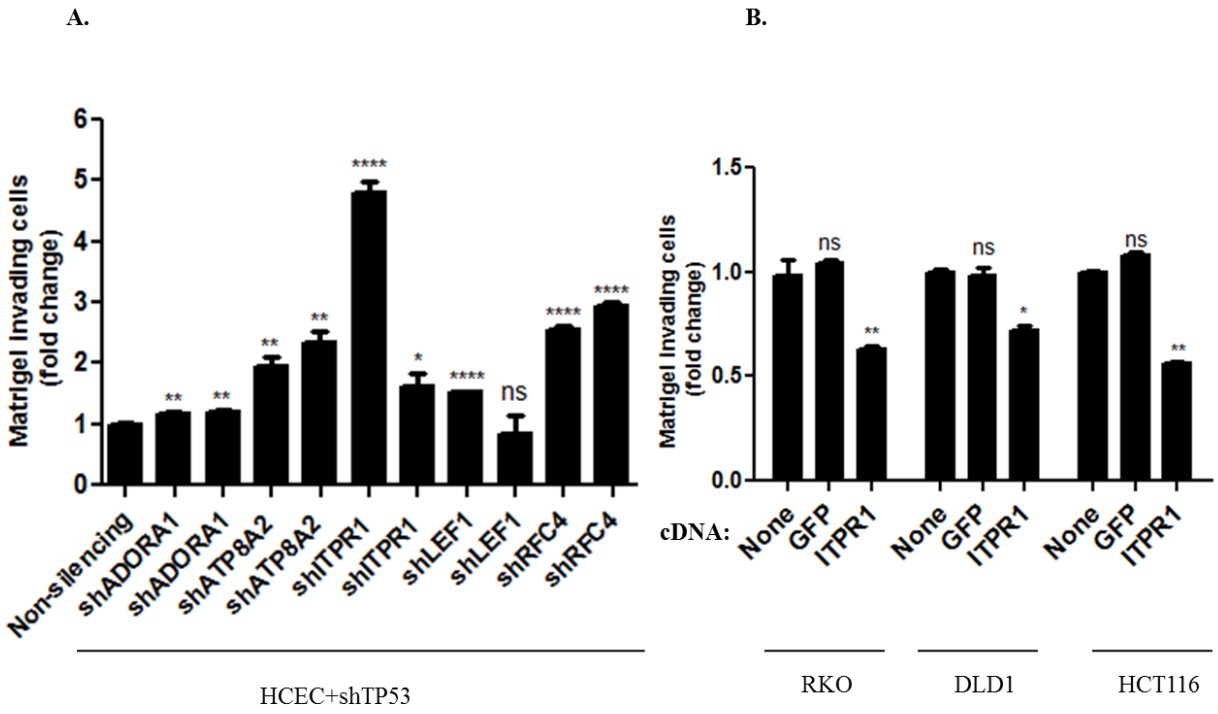


Figure 3-3. A subset of the positive hits are also causally involved in invasion through Matrigel®. (A) Stably knockdown cell lines expressing shRNAs against the most potent hits scored in soft agar screenshowed enhanced invasion through Matrigel®. (B) Transient expression of wild type cDNA of ITPR1 reduced invasion in colon cancer cell lines. Triplicates from 2 separate experiments, 2-tailed Student *t* test, Compared with non-silencing or none, mean ± s.e.m. *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$; ns, nonsignificant.

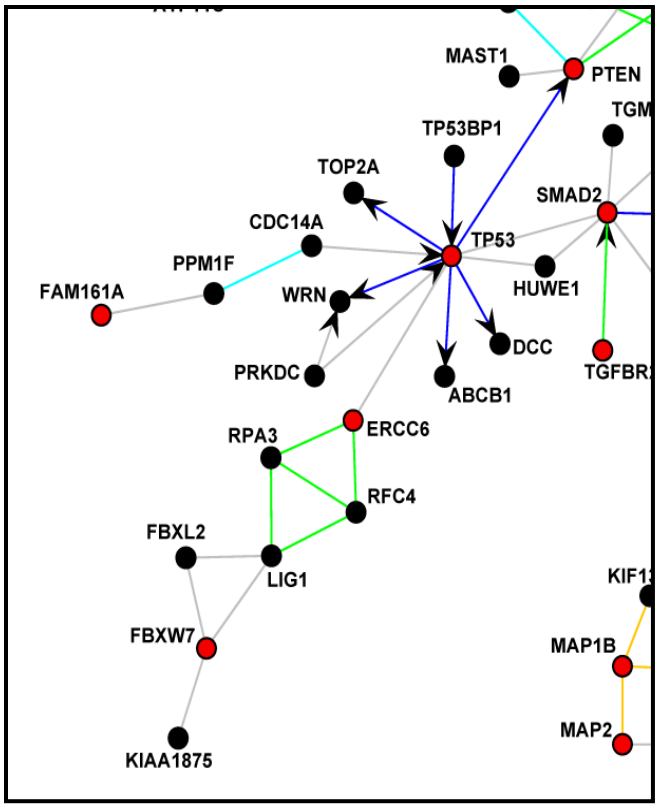


Figure 3-4. Knockdown of eight passenger genes that interact with either TP53, ERCC6 or FBXL7 enhanced anchorage-independent growth. The genes scored positive were marked with red circle.

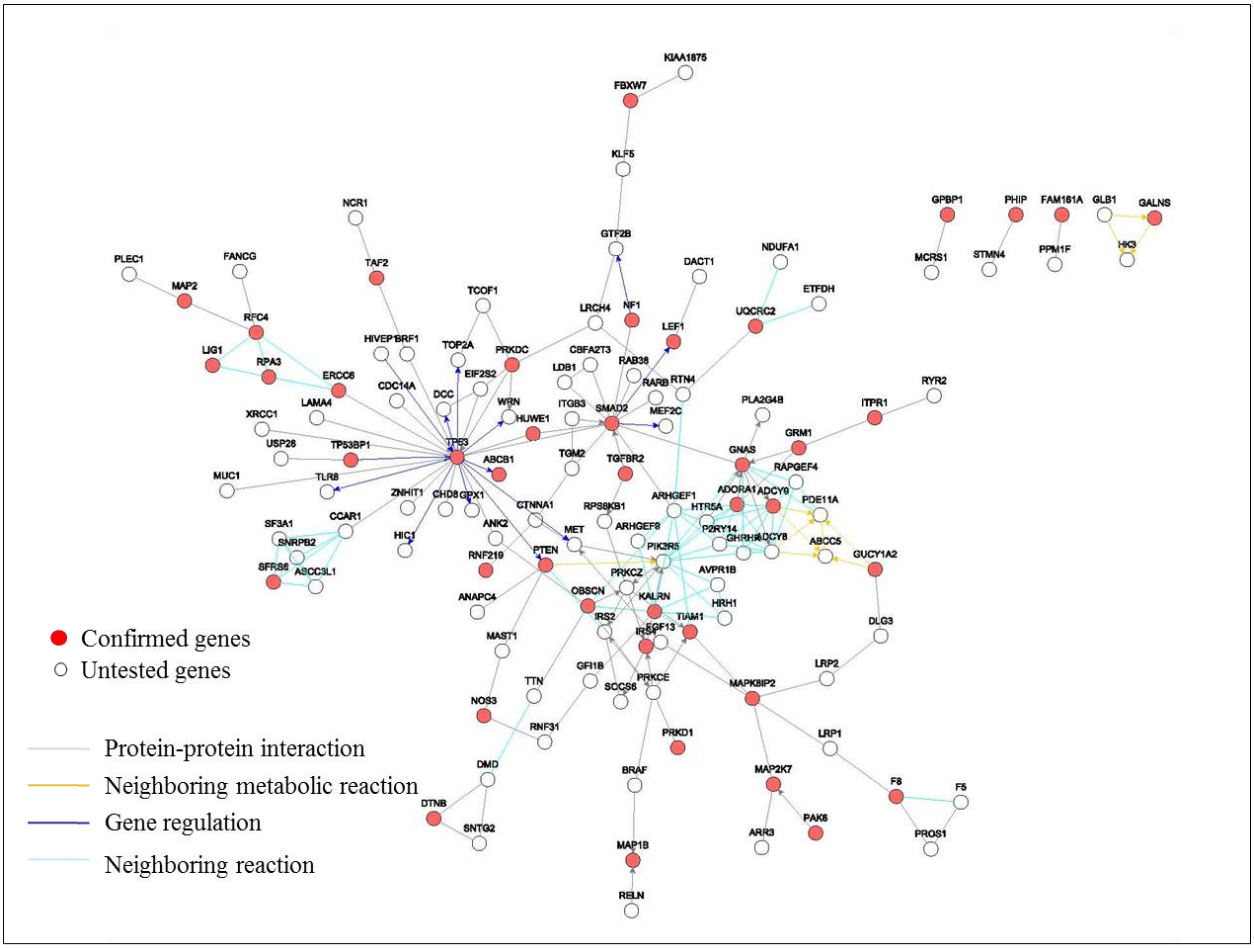


Figure 3-5. Extended interaction map of the confirmed genes from both our previous screen and this screen (red nodes) and the rest of untested CRC less frequently mutated genes (white nodes). Interactions are (colored according to type of interactions as shown in the color key.

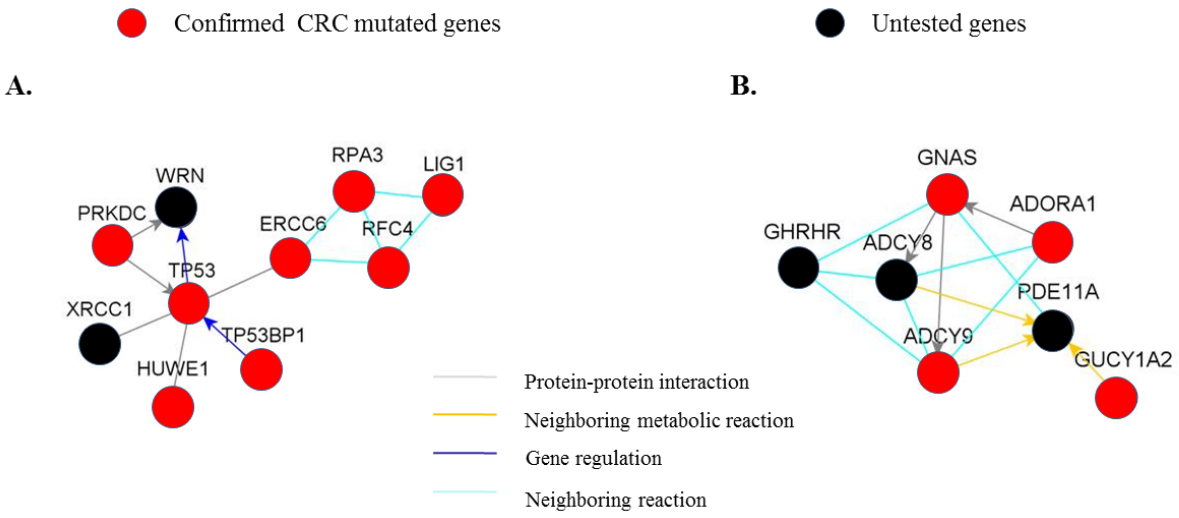


Figure 3-6. Representative interactions of the enriched sub-networks within full extended network map. (A) Sub-network showing the enriched DNA repair pathways. (B) Sub-network showing enriched cyclic nucleotide metabolic process. Red nodes denote the positive hits scored from our screens and black nodes denote the untested CRC less frequently mutated genes. All the genes tested within these two sub-networks scored positive in our screens.

Table 3-1. Summary of low frequency mutated genes scored positive in the screen.

shRNA set	Number of shRNAs screened	# AI enhancing	% positive
Interacting	60	34	56.7
Non-interacting	131	15	11.5
Random	362	5	1.4

Gene set	Number of genes screened	# AI enhancing	% positive
Interacting	25	15	60
Non-interacting	55	7	12.7

Expression of shRNAs against "passenger" mutated genes that interact with confirmed CAN-genes enhanced anchorage-independent growth at higher fraction than those that do not interact in HCECs with TP53 knockdown. Genes scored with at least two different shRNAs are considered as positive hits. Data for random shRNAs were from our previous screen (Eskiocak et al 2011). Higher fraction of interacting passenger mutated genes scored positive than non-interacting ones. The percentage of positive hits in both interacting and non-interacting categories is higher than that of the background rate. Fisher's exact test, ****, $p<0.0001$.

Table 3-2. Distribution of anchorage-independent genes for individual tumor sample.

Tumor *	All mutated genes*	AI genes~	AI gene symbol**	AI gene symbol***
co108	82	6	OBSCN,NTNG1,NOS3,IRS4	ADCY9,ADORA1
co74	123	10	TP53,TGFBR2,SYNE1,SFRS6,PRKD1,MLL3,GANLS,FBXW7,CNTN4	OSBP
co92	90	6	TP53,MAP2,KRT73,HAPLN1,AKAP12	ADD3
mx22	69	6	ZMYM4,TCERG1L,MAP1B,KIAA0182,GNAS	RFC4
mx27	86	7	TIAM1,MYO19,KALRN,IGSF22,F8,DTNB	N/A
mx30	57	3	SMAD2,PRDM9,MAP2K7	N/A
mx32	76	7	UHRF2,TP53,SYNE1,NAV3,KCNQ5,FAM161A,ATP13A5,ADAMTS18	SLC11A2
mx38	77	10	TP53,SH3TC1,RNF219,NF1,NAV3,KIAA2022,ITGAE,ERCC6	RPA3,OXCT1
mx41	99	19	UQCRC2,TCF7L2,TAF2,SYNE1,PHIP,OBSCN,LMO7,GUCY1A2,GRM1,COL3A1,CD248,ATP13A1,ADAMTS20,ADMATS15	CLIC2,ABCB1,LEF1,ATP8A2,TP53BP1
mx42	81	9	TP53,PTPRD,MAPK8IP2,KIAA0556,DSCAML1,C15orf2	GPBP1,PAK6,FBXL2
mx43	102	7	SYNE1,SLC29A1,PTEN,HISTH1B,CHL1	ITPR1,TRIM3

*Data from Wood et.al. **Data from Eskiocak et al. ***Data from this study
~ Data from Eskiocak et al and this study AI:Anchorage-independent growth
This table lists 11 completely sequenced colon cancer samples with the number of all mutated genes (column2) and genes scored positive in anchorage-independent growth from our previous screen (Eskiocak et. Al.,2011) and this screen are listed in column 3. On average at least 9.4% of all mutated genes are involved in anchorage-independent growth.

Table 3-3. Occurrence of newly identified driver mutations in colon tumor samples

Gene symbol	# of cases altered	percent of cases altered	Gene symbol	# of cases altered	percent of cases altered
HUWE1	14	7.30%	ADD3	4	2.10%
ATP8A2	13	6.70%	FBXL2	4	2.10%
PRKDC	13	6.70%	SLC11A2	4	2.10%
ITPR1	11	5.70%	TRIM3	4	2.10%
ABCB1	9	4.70%	LEF1	3	1.60%
TP53BP1	9	4.70%	GPBP1	2	1%
ADORA1	8	4.10%	OSBP	2	1%
KIF13A	7	3.60%	RFC4	2	1%
LIG1	6	3.10%	PAK6	1	0.50%
OXCT1	6	3.10%	CLIC2	0	0%
ADCY9	5	2.60%	RPA3	0	0%

Data from http://www.cbioportal.org/public-portal/
This table lists the incidence and percentage of the 22 newly identified driver mutations among the 193 colon tumor samples from TCGA database. Totally, these genes are altered in 29% of all cases. The absence of occurrence of CLIC2 and RPA3 in CRC cases is due to the fact that the sequencing information of the 11 tumors from Wood et al. is not included in TCGA CRC dataset.

Table 3-4. Occurrence of ITPR1 in 22 cancer types

Cancer type	percent of cases altered	Cancer type	percent of cases altered
Colorectal Carcinoma	5.70%	Thyroid Carcinoma	0%
Glioblastoma	0%	Kidney Renal Papillary Cell Carcinoma	0%
Prostate Cancer	0%	Liver Hepatocellular Carcinoma	0%
Sarcoma	2%	Lung Adenocarcinoma	0%
Serous Ovarian Cancer	4.10%	Lung Squamous Cell Carcinoma	0%
Bladder Urothelial Carcinoma	2.90%	Pancreatic Adenocarcinoma	0%
Brain Lower Grade Glioma	0%	Prostate Adenocarcinoma	1.20%
Breast Invasive Carcinoma	2.50%	Stomach Adenocarcinoma	3.40%
Cervical Squamous Cell Carcinoma	0%	Kidney Renal Clear Cell Carcinoma	5.90%
Head and Neck Squamous Cell Carcinoma	0%	Uterine Corpus Endometrioid Carcinoma	0.50%

Data from http://www.cbioportal.org/public-portal/
This table lists the percentage of ITPR1 alterations in 22 cancer types. The percentage is higher in CRC than in all other cancer types except kidney renal clear cell carcinoma.

Table 3-5. Enriched pathways of genes within interaction map.

Gene Set Name	# Genes in Gene Set (K)	Description	# Genes in Overlap (k)	k/K	p value
REACTOME_SIGNALLING_BY_NGF	215	Genes involved in Signalling by NGF	14	0.0651	2.70E-05
KEGG_VASCULAR_SMOOTH_MUSCLE_CONTRACTION	115	Vascular smooth muscle contraction	10	0.087	3.62E-05
KEGG_COLORECTAL_CANCER	62	Colorectal cancer	7	0.1129	1.09E-04
KEGG_TYPE_II_DIABETES_MELLITUS	47	Type II diabetes mellitus	6	0.1277	1.73E-04
KEGG_ENDOMETRIAL_CANCER	52	Endometrial cancer	6	0.1154	3.05E-04
REACTOME_DOUBLE_STRAND_BREAK_REPAIR	21	Genes involved in Double-Strand Break Repair	4	0.1905	4.65E-04
REACTOME_DNA_REPAIR	104	Genes involved in DNA Repair	8	0.0769	5.20E-04
BIOCARTA_CTCF_PATHWAY	23	CTCF: First Multivalent Nuclear Factor	4	0.1739	6.69E-04
SIG_PIP3_SIGNALING_IN_CARDIAC_MYOCTES	63	Genes related to PIP3 signaling in cardiac myocytes	6	0.0952	8.67E-04
KEGG_DILATED_CARDIOMYOPATHY	92	Dilated cardiomyopathy	7	0.0761	1.25E-03
KEGG_CALCIUM_SIGNALING_PATHWAY	178	Calcium signaling pathway	10	0.0562	1.28E-03
KEGG_LONG_TERM_DEPRESSION	70	Long-term depression	6	0.0857	1.51E-03
KEGG_MELANOMA	71	Melanoma	6	0.0845	1.63E-03
REACTOME_HOMOLOGOUS_RECOMBINATION_REPAIR	15	Genes involved in Homologous Recombination Repair	3	0.2	2.19E-03
REACTOME_TRKA_SIGNALLING_FROM_THE_PLASMA_MEMBRANE	103	Genes involved in TRKA signalling from the plasma membrane	7	0.068	2.40E-03
REACTOME_DOWNSTREAM_EVENTS_IN_GPCR_SIGNALING	448	Genes involved in Downstream events in GPCR signaling	17	0.0379	2.49E-03
REACTOME_G_ALPHA_12_13_SIGNALLING_EVENTS	54	Genes involved in G alpha (12/13) signalling events	5	0.0926	2.68E-03
REACTOME_PLC_GAMMA1_SIGNALLING	35	Genes involved in PLC-gamma1 signalling	4	0.1143	3.35E-03
REACTOME_P75_NTR_RECEPTOR_MEDIATED_SIGNALLING	82	Genes involved in p75 NTR receptor-mediated signalling	6	0.0732	3.39E-03
BIOCARTA_IGF1MTOR_PATHWAY	20	Skeletal muscle hypertrophy is regulated via AKT/mTOR pathway	3	0.15	5.13E-03
REACTOME_LAGGING_STRAND_SYNTHESIS	20	Genes involved in Lagging Strand Synthesis	3	0.15	5.13E-03
KEGG_GAP_JUNCTION	90	Gap junction	6	0.0667	5.37E-03
KEGG_PATHWAYS_IN_CANCER	328	Pathways in cancer	13	0.0396	5.69E-03
REACTOME_FORMATION_OF_PLATELET_PLUG	186	Genes involved in Formation of Platelet plug	9	0.0484	6.02E-03
ST_DIFFERENTIATION_PATHWAY_IN_PC12_CELLS	42	Differentiation Pathway in PC12 Cells; this is a specific case of PAC1	4	0.0952	6.51E-03
BIOCARTA_INTRINSIC_PATHWAY	23	Intrinsic Prothrombin Activation Pathway	3	0.1304	7.67E-03
BIOCARTA_P53HYPOXIA_PATHWAY	23	Hypoxia and p53 in the Cardiovascular system	3	0.1304	7.67E-03
BIOCARTA_PTDINS_PATHWAY	23	Phosphoinositides and their downstream targets.	3	0.1304	7.67E-03
KEGG_MISMATCH_REPAIR	23	Mismatch repair	3	0.1304	7.67E-03
KEGG_NUCLEOTIDE_EXCISION_REPAIR	44	Nucleotide excision repair	4	0.0909	7.68E-03
REACTOME_TRANSCRIPTION_COUPLED_NER	44	Genes involved in Transcription-coupled NER (TC-NER)	4	0.0909	7.68E-03
KEGG_PANCREATIC_CANCER	70	Pancreatic cancer	5	0.0714	8.16E-03
KEGG_GNRH_SIGNALING_PATHWAY	101	GnRH signaling pathway	6	0.0594	9.32E-03

REACTOME_NRAGE_SIGNALS_DEATH_THROUGH_JNK	47	Genes involved in NRAGE signals death through JNK	4	0.0851	9.69E-03
REACTOME_PLATELET_ACTIVATION	167	Genes involved in Platelet Activation	8	0.0479	1.00E-02
KEGG_ADHERENS_JUNCTION	75	Adherens junction	5	0.0667	1.09E-02
REACTOME_NUCLEOTIDE_EXCISION_REPAIR	49	Genes involved in Nucleotide Excision Repair	4	0.0816	1.12E-02
SIG_INSULIN_RECEPTOR_PATHWAY_IN_CARDIAC_MYOCYTES	49	Genes related to the insulin receptor pathway	4	0.0816	1.12E-02
KEGG_INSULIN_SIGNALING_PATHWAY	137	Insulin signaling pathway	7	0.0511	1.13E-02
KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC	76	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	5	0.0658	1.15E-02
REACTOME_GS_ALPHA_MEDIATED_EVENTS_IN_GLUCAGON_SIGNALLING	27	Genes involved in G(s)-alpha mediated events in glucagon signalling	3	0.1111	1.20E-02
ST_INTEGRIN_SIGNALING_PATHWAY	78	Integrin Signaling Pathway	5	0.0641	1.27E-02
BIOCARTA_RNA_PATHWAY	10	Double Stranded RNA Induced Gene Expression	2	0.2	1.31E-02
REACTOME_EXTENSION_OF_TELOMERES	28	Genes involved in Extension of Telomeres	3	0.1071	1.33E-02
KEGG_THYROID_CANCER	29	Thyroid cancer	3	0.1034	1.46E-02
KEGG_NON_SMALL_CELL_LUNG_CANCER	54	Non-small cell lung cancer	4	0.0741	1.56E-02
REACTOME_ADENYLATE_CYCLASE_ACTIVATING_PATHWAY	11	Genes involved in Adenylate cyclase activating pathway	2	0.1818	1.58E-02
KEGG_SMALL_CELL_LUNG_CANCER	84	Small cell lung cancer	5	0.0595	1.71E-02
REACTOME_DNA_STRAND_ELONGATION	31	Genes involved in DNA strand elongation	3	0.0968	1.75E-02
KEGG_PROGESTERONE_MEDIATED_OOCYTE_MATURATION	86	Progesterone-mediated oocyte maturation	5	0.0581	1.88E-02

This table lists all the enriched pathways based on Molecular Signature Database. P value is calculated using hypergeometric test.

Table 3-6. Enriched molecular functions of genes within interaction map.

GO Term	# Genes in Gene Set (K)	**GO Ontology**	# Genes in Overlap (k)	k/K	p value
cyclic nucleotide metabolic process	151	BP	9	0.0596026	1.03E-06
positive regulation of protein kinase activity	283	BP	12	0.0424028	1.56E-06
cyclic nucleotide biosynthetic process	133	BP	8	0.0601504	3.07E-06
activation of protein kinase activity	139	BP	8	0.057554	4.42E-06
positive regulation of phospholipase activity	105	BP	7	0.0666667	4.60E-06
regulation of lymphocyte apoptosis	17	BP	3	0.1764706	1.36E-05
induction of apoptosis by intracellular signals	41	BP	4	0.097561	3.15E-05
positive regulation of phospholipase C activity	100	BP	6	0.06	3.22E-05
activation of adenylate cyclase activity	42	BP	4	0.0952381	3.55E-05
positive regulation of adenylate cyclase activity by G-protein signaling pathway	42	BP	4	0.0952381	3.55E-05
cAMP biosynthetic process	109	BP	6	0.0550459	5.61E-05
regulation of cAMP metabolic process	109	BP	6	0.0550459	5.61E-05
regulation of cAMP biosynthetic process	109	BP	6	0.0550459	5.61E-05
glucose import	32	BP	3	0.09375	1.85E-04
ATPase activity, coupled	225	MF	7	0.0311111	9.68E-04
ATPase activity	235	MF	7	0.0297872	0.00127948
glucose transport	71	BP	3	0.0422535	0.003816711
regulation of peptidyl-tyrosine phosphorylation	88	BP	3	0.0340909	0.008151978
helicase activity	96	MF	3	0.03125	0.010995278
protein ubiquitination	260	BP	5	0.0192308	0.030102176
chromatin modification	263	BP	5	0.0190114	0.031586307
calcium ion transport	139	BP	3	0.0215827	0.036820768
covalent chromatin modification	209	BP	4	0.0191388	0.040532341
regulation of protein serine/threonine kinase activity	283	BP	5	0.0176678	0.042706554
proteasomal ubiquitin-dependent protein catabolic process	151	BP	3	0.0198675	0.047468896

This table lists all the enriched functional groups based on GO annotations. P value is calculated using hypergeometric test.

Table 3-7. Mutual exclusivity/co-occurrence analysis of genes within representative central subnetworks.

A. Genes within DNA repair subnetwork

Gene	TP53	HUWE1	PRKDC	WRN	ERCC6	TP53BP1	LIG1	XRCC1	RFC4	RPA3
TP53	---	0.057471	0.429709	0.019931	0.566493	0.064336	0.613561	0.214749	0.72744	1
HUWE1		---	0.008621	0.004345	0.157307	0.131268	0.062111	0.683423	0.140166	1
PRKDC			---	0.003201	0.021921	0.001323	0.004296	0.70295	0.00421	1
WRN				---	0.01339	0.000025	0.039192	0.743341	0.002968	1
ERCC6					---	0.000014	0.000053	0.022309	0.002429	1
TP53BP1						---	0.001325	0.214431	0.001943	1
LIG1							---	0.000166	0.00081	1
XRCC1								---	0.948726	1
RFC4									---	1
RPA3										---

B. Genes within cyclic nucleotide metabolic process subnetwork

Gene	GNAS	ADCY8	GUCY1A2	ADORA1	PDE11A	ADCY9	GHRHR
GNAS	---	0.600541	0.325656	0.409657	0.459013	0.575174	0.803001
ADCY8		---	0.138708	0.011095	0.072725	0.29705	0.130505
GUCY1A2			---	0.057372	0.044267	0.022309	0.898802
ADORA1				---	0.028542	0.19261	0.91861
PDE11A					---	0.170307	0.928595
ADCY9						---	0.948726
GHRHR							---

C. Genes within transcriptional control subnetwork

Gene	TP53	TGFBR2	SMAD2	HUWE1
TP53	---	0.002038	0.238832	0.057471
TGFBR2		---	0.548042	0.000096
SMAD2			---	0.355045
HUWE1				---

Legend

tendency toward mutual exclusivity

no association

tendency toward co-occurrence

zero events recorded for one or both genes

This analysis was performed based on the Cancer Genome Atlas CRC dataset from http://www.cbioportal.org/public-portal/. Tendency toward mutual exclusivity or co-occurrence was colored as described in legend based on odds ratio test. p-values <0.05, as derived via Fisher's Exact test are outlined in red. p-values are not adjusted for FDR.

Table 3-8. List of all screened shRNAs targeting CRC-mutated genes.

Gene symbol	Clone ID	shRNA scored positive	Gene symbol	Clone ID	shRNA scored positive
ABCB1	V2LHS_131346	+	CENPF	V2LHS_115504	
ABCB1	V2LHS_131345	+	CENPF	V2LHS_115505	
ABCB1	V2LHS_131347	+	CENPF	V2LHS_115500	
ABCD4	V2LHS_19118		CENPF	V2LHS_115502	
ABCD4	V2LHS_19117		CENTD3	V2LHS_80761	
ACACA	V2LHS_111896		CENTD3	V2LHS_80758	
ACACA	V2LHS_111901		CHD8	V2LHS_201382	
ACACA	V2LHS_266202		CHD8	V2LHS_103252	
ACACA	V2LHS_111898		CHD8	V2LHS_201084	
ADCY9	V2LHS_6513	+	CLIC2	V2LHS_113081	+
ADCY9	V2LHS_231143	+	CLIC2	V2LHS_113079	+
ADD3	V2LHS_154117	+	CNTN1	V2LHS_150704	
ADD3	V2LHS_154114	+	CNTN1	V2LHS_150708	
ADD3	V2LHS_154118	+	CSDA	V2LHS_27462	
ADORA1	V2LHS_111949		CSDA	V2LHS_27392	
ADORA1	V2LHS_111947	+	CSE1L	V2LHS_113224	
ADORA1	V2LHS_111948	+	CSE1L	V2LHS_225798	
AGTPBP1	V2LHS_81419		CTNNB1	V2LHS_151023	
AGTPBP1	V2LHS_260469		CTNNB1	V2LHS_151022	
AGTPBP1	V2LHS_81422		DACT1	V2LHS_135444	
AKAP3	V2LHS_214488		DACT1	V2LHS_238757	
AKAP3	V2LHS_216862		DDHD2	V2LHS_91092	
AKAP9	V2LHS_5828		DDHD2	V2LHS_91091	
AKAP9	V2LHS_198616		DDHD2	V2LHS_91088	
AKAP9	V2LHS_199608		DMD	V2LHS_84118	
API5	V2LHS_197671		DMD	V2LHS_84116	
API5	V2LHS_199238		DMD	V2LHS_84115	
ASCC3L1	V2LHS_60297		DNAJC10	V2LHS_51235	
ASCC3L1	V2LHS_60298		DNAJC10	V2LHS_51233	
ATP5SL	V2LHS_155343		DNAJC10	V2LHS_51234	
ATP5SL	V2LHS_155345		DPYSL2	V2LHS_113549	
ATP6V1E1	V2LHS_132873		DPYSL2	V2LHS_113545	
ATP6V1E1	V2LHS_132872		DPYSL2	V2LHS_228290	
ATP8A2	V2LHS_134858		EDA	V2LHS_113604	
ATP8A2	V2LHS_134855	+	EDA	V2LHS_113602	
ATP8A2	V2LHS_134859	+	ENPP2	V2LHS_58937	
CAPN10	V2LHS_190404		ENPP2	V2LHS_58932	
CAPN10	V2LHS_194527		ENPP2	V2LHS_58935	
CAPN10	V2LHS_192459		EPHA4	V2LHS_101745	
CDO1	V2LHS_150496		EPHA4	V2LHS_101750	
CDO1	V2LHS_150495		EPHA4	V2LHS_17861	
FBXL2	V2LHS_202584	+	MAST1	V2LHS_202375	
FBXL2	V2LHS_195222	+	MED12L	V2LHS_260590	
ERGIC3	V2LHS_262029		MED12L	V2LHS_89441	
ERGIC3	V2LHS_134570		MED12L	V2LHS_89443	

FHOD3	V2LHS_137119		PANK4	V2LHS_156350	
FHOD3	V2LHS_234601		PANK4	V2LHS_156349	
FHOD3	V2LHS_137120		PAOX	V2LHS_65001	
FMN2	V2LHS_111174		PAOX	V2LHS_65003	
FMN2	V2LHS_111173		PRKCZ	V2LHS_263092	
FMN2	V2LHS_111176		PRKCZ	V2LHS_170475	
GPBP1	V2LHS_116350	+	PRKDC	V2LHS_2435	+
GPBP1	V2LHS_116349	+	PRKDC	V2LHS_231208	+
HUWE1	V2LHS_262858	+	PBEF1	V2LHS_5824	
HUWE1	V2LHS_154890	+	PBEF1	V2LHS_209138	
HECTD1	V2LHS_120182		PROS1	V2LHS_229104	
HECTD1	V2LHS_149726		PROS1	V2LHS_92312	
HECTD1	V2LHS_149725		PROS1	V2LHS_92309	
ITPR1	V2LHS_235012	+	PRTG	V2LHS_121241	
ITPR1	V2LHS_133540	+	PRTG	V2LHS_179418	
KCND3	V2LHS_239045		PTPRH	V2LHS_170993	
KCND3	V2LHS_61437		PTPRH	V2LHS_170992	
KCND3	V2LHS_61438		PTPRN2	V2LHS_222995	
KIF13A	V2LHS_202861	+	PTPRN2	V2LHS_171023	
KIF13A	V2LHS_262720	+	PTPRN2	V2LHS_171025	
KLF5	V2LHS_150118		RCN1	V2LHS_33166	
KLF5	V2LHS_150120		RCN1	V2LHS_240735	
KLF5	V2LHS_150116		RFC4	V2LHS_32862	+
LAMA4	V2LHS_133879		RFC4	V2LHS_32863	+
LAMA4	V2LHS_133876		RFC4	V2LHS_32866	+
LDB1	V2LHS_52998		RTN4	V2LHS_211277	
LDB1	V2LHS_52997		RTN4	V2LHS_204901	
LEF1	V2LHS_224400	+	RTN4	V2LHS_214535	
LEF1	V2LHS_115188	+	RPA3	V2LHS_32105	+
LEF1	V2LHS_115192	+	RPA3	V2LHS_65113	+
LIG1	V2LHS_76784	+	SLC11A2	V2LHS_111723	+
LIG1	V2LHS_76783	+	SLC11A2	V2LHS_111725	+
LPPR2	V2LHS_98261		STMN4	V2LHS_116885	
LPPR2	V2LHS_98262		STMN4	V2LHS_116881	
LSP1	V2LHS_134079		TFG	V2LHS_15557	
LSP1	V2LHS_134075		TFG	V2LHS_70308	
MAST1	V2LHS_62038		TP53BP1	V2LHS_56193	+
MFN1	V2LHS_277148		TP53BP1	V2LHS_56192	+
MFN1	V2LHS_174555		TPX2	V2LHS_24989	
MLF2	V2LHS_69468		TPX2	V2LHS_24986	
MLF2	V2LHS_69465		TRIM3	V2LHS_251148	+
MMP2	V2LHS_48434		TRIM3	V2LHS_250531	+
MMP2	V2LHS_48430		VIM	V2LHS_171948	
OSBP	V2LHS_152505	+	VIM	V2LHS_171949	
OSBP	V2LHS_152508	+	VIM	V2LHS_171946	
OSBP	V2LHS_152509		WAC	V2LHS_135342	
OXCT1	V2LHS_93027	+	WAC	V2LHS_135340	
OXCT1	V2LHS_93026	+	WNK1	V2LHS_51379	
PAK6	V2LHS_194065	+	WNK1	V2LHS_51380	
PAK6	V2LHS_192793	+	ZNHIT1	V2LHS_198940	
			ZNHIT1	V2LHS_198872	

+ denotes enhanced growth in soft agar (in at least 6 replicates)
Clone ID could be used to retrieve shRNA sequences from Open Biosystem's website

Table 3-9. List of qRT-PCR primers for all screened genes.

Gene symbol	Forward primers	Reverse primers
ABCB1	aaggcatttacttcaaacttgtca	tggattcatcagctgcatttt
ABCD4	ctggctgtcatgctcattgt	aggtccttcctccagctcac
ACACA	gtctttgccaactggagagg	caaacttcagcacttggtcgt
ADCY9	cgagcataaaaacagcacca	agcccgttctgagttttgg
ADD3	ggacaatcgaacgtaaacaaca	tgaatttgtgaaacagatgaagc
ADORA1	gtcaagatccctctccggta	tcccaccacgaaggagag
AGTPBP1	cagatggtgtcatcaatggaa	gactttgccactgcctattca
AKAP3	tttgctggtgatgatgaagg	cacactgcatcctttgtcca
AKAP9	catggaggacgaggagagac	ctttgtcgaaactgggcaag
API5	aagctcaaagatttcaaaatcagg	ttttaaggcctcacccgttt
ASCC3L1	gctccttggtgcaggagat	cagcaacagtgatgccataag
ATP5SL	tccaaagggagatgtacaagg	ccgtatggaccatgttgct
ATP6V1E1	aggagtcctacctgcctgaag	ggaaaccttattttacgatctcca
ATP8A2	gaaggaggactctttggatgc	gcaaattcccaaggtcagtg
CAPN10	atcgcaaccaggattgaca	actgccatgacggagacct
CDO1	gatgattctctgttggggtga	agcatcttcagaaagcagtgg
CENPF	gagtcctccaaaccaacagc	tccgctgagcaactttgac
CENTD3	accacctggaaggacgtg	gggagctgctgctctatataaact
CHD8	tgaagacgtagccatcttgc	ccttggggacctccagac
CLIC2	aacagacttcattaaaattgaggagtt	tgggactcaggtgagggta
CNTN1	gatgactggaaagatgcaaaga	catgggattaagtccactgct
CSDA	cttcacaagatggcaaagagg	gagcctggtgttactcagca
CSE1L	tgtaaacctaactgagttctttgtgaa	ccgtcagctttaaggacagg
CTNNB1	gctttcagttgagctgacca	caagtccaagatcagcagtctc
DACT1	gtgacctgagactggatgtagaaa	aagccccatcactcagctc
DDHD2	gccatgcagtgtcctcct	gcactgatgacatttcgctct
DMD	cctccactcgtacccacact	tcccagcaagttgtttgagtc
DNAJC10	ggtgtggaccttgccaga	tccagctttcacttttcctttaat
DPYSL2	ctgagccgacctgaggag	ggcagttggtctggttgg
EDA	ccaagggtcagcaattcaag	tgatgcgagaccagtcattg
ENPP2	aagaggctggacctcctgat	caaagtcatggcagcaactg

Gene symbol	Forward primers	Reverse primers
EPHA4	catgtcccgagtgcttgag	cagtccaccggataggaatc
FBXL2	tcctgataaccgacagcaca	cacagtgggacaggctcag
ERGIC3	tcgctgtgagagctgctatg	cgcacatcttcacaggtgtt
FHOD3	aggccaggttggaaaggt	tctgctgccagtgactcttg
FMN2	ttcaagccaaaattgaccaag	ccgtggtctccaaaaagc
GPBP1	aaaatctcccaagctcctctct	tgggaatccagatagctgtaagt
HUWE1	agagagcggctgacagagg	cactaacccactcaggtcagg
HECTD1	agcagggagccgacctat	cgtcctcctttagtttcgtactct
ITPR1	cctcaatgatatcaatcctttgg	caacttcgaggcattgttctt
KCND3	cagccctgatatgtcagcaa	cagcttggagttagttcagcaa
KIF13A	ggcaagactgtctccgatg	aagctgtggcctgaggag
KLF5	ggctttactcaagcagatctcatc	cccttacccatgttgagacg
LAMA4	tgcacaggaccttcaacaag	tgtaccagcccgttcatatct
LDB1	gaagaagagcccagctagcac	cccaccaccatcacatcag
LEF1	tgcatcaggtacaggtccaa	cgttgggaatgagcttcg
LIG1	ggcatgatcctgaagcagac	agcagcacggggataattc
LPPR2	catcatcccctgctttgtct	ggtaagcaagcaggatcacaa
LSP1	tccctaggcgtcccatct	ggcaacaggaagcaacttct
MAST1	caaggagggccaggagag	tcgactgcttcgtgatcttc
MED12L	cagccgagtggctatgttc	tctgctgtagagcctgatgg
MFN1	gggtgctcctaggattatcaga	tatctggcgttgctggagt
MLF2	ggcaggactatatcaacctgga	actcaagccgccgaaact
MMP2	ccacgtgacaagcccatggggcccc	gcagcctagccagtcggatttgatg
OSBP	cgggctcctgagctactaca	aggagtcctccacggtgat
OXCT1	ctggggcaacctggtaca	aaaacttggtatggcgatgag
PAK6	caagctgaaaaactctcacaagg	ctgggctgtggctctctct
PANK4	ggggaatgtcttcgactgg	ccaaagtaggggtcggattc
PAOX	atcgtcaccgtgcccttа	tcccaaagcctatcttcctg
PRKCZ	ccttcctggtcggattacac	cgttgacgtactcaatgacca
PRKDC	acacgttcattggcgagag	tgaagactgggcttcagtacc
PBEF1	gccagcagggaattttgtta	gccattcttgaagacagtatgga

Gene symbol	Forward primers	Reverse primers
PROS1	acatacctgggtggccttc	tccagatccaactgtacaccat
PRTG	cactccaggatgcgtgtct	catagagatggtggggtggt
PTPRH	gacggagtaaatagctctgtgga	agcctccactgtcaggtttc
PTPRN2	gcatggtccagacgaagg	ccttgaggatggcgttca
RCN1	tatgaaggaaattgtggttttgg	aacccatccccgttcttg
RFC4	tacagacattgccggggta	ccacagcttctagtttgtcaaaag
RTN4	gagcctgtgatacgctcctc	ttgaccagccgaaatagtgtt
RPA3	ggaaaatgaggctgaaatgg	ggtgctctgctgcttggt
SLC11A2	caccgtcagtatcccaaggt	ccgatgatagccaactccac
STMN4	gccgatcccctgaataagtc	tctttccgctggctttca
TFG	ccattcagcggagacctg	ggtggactccaggatgttcta
TP53BP1	tcaggacagtctttccacgaa	cgataaaaggagtagatcggaaag
TPX2	acatctgaactacgaaagcatcc	ggcttaacaatggtacatccctta
TRIM3	gaattcaccaatttacaaggtgtg	acttgaactggccctcattg
VIM	acaccctgcaatctttcagaca	gattccactttgcgttcaaggt
WAC	cctgtttcatcacagccaaa	ggttcatgaccttgctgctt
WNK1	acaagctgctgaggttcca	gtactgtggtggcagtcgag
ZNHIT1	gcctcagtttgatgacgatg	tgatcacctcgggttttctt
All of these primers were designed based on Universal Probe Library design cencer.		

Chapter 4 . Exome Sequencing of Normal and Isogenic Transformed Human Colonic Epithelial Cells (HCECs) Reveals Novel Genes Potentially Involved in the Early Stages of Colorectal Tumorigenesis

The work presented in this chapter was performed by Lu Zhang unless otherwise noted in the text and/or figure legends.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and third leading cause of cancer related mortality in the United States. It is well established that sporadic colorectal cancer (CRCs) arises through the acquisition of a series of sequential genetic mutations in both tumor suppressor genes and oncogenes (Kinzler and Vogelstein, 1996). Mutational activation of oncogenes together with inactivation of tumor suppressor genes (TSG) contributes to colorectal tumor formation. It has been proposed that a minimum of four sequential genetic alterations are required for colorectal cancer evolution, including one oncogene (*KRAS*) and three TSGs (*APC, SMAD4, TP53)* as the main targets (Fodde et al., 2001b). The dominant or recessive nature of these genes predict that at least seven mutations (*KRAS* and six additional ones) are required for complete inactivation of important TSG function (Fodde et al., 2001b). The TSG mutations occur in most tumors, whereas *KRAS* mutations are found in approximately 50% of sporadic adenomas and carcinomas (Bos et al., 1987; Forrester et al., 1987). However, additional changes are required to convert a normal colonic epithelial cell into a malignant carcinoma. While most CRCs have ~100 or more genomic changes, many of these are believed to be incidental or "passenger" alterations, and it is estimated that up to 15 "driver" oncogenic changes are required for transforming into full malignancy (Wood et al., 2007). Many of these changes are not frequently observed in CRC and thus it remains to be determined which less frequently mutated genes are involved in CRC initiation and development.

Recent advances in next generation sequencing (NGS) technology have allowed for rapid and efficient analysis of causative mutations in rare Mendelian disorders (Ng et al., 2010). Several studies

have demonstrated the utility of exome sequencing in identifying novel driver mutations in various cancer types (Varela et al., 2011; Wang et al., 2011; Wei et al., 2011; Yan et al., 2011). In particular, the whole

exome and even the whole genome sequencing of colorectal tumors have delineated a comprehensive mutational landscape of genetic alterations in CRC (Bass et al., 2011; Sjoblom et al., 2006; Wood et al., 2007). However, the mutational events that contribute to CRC initiation are less well-studied, partly due to the lack of appropriate cellular reagents for validating important changes. We reasoned that examination of the landscape of genomic changes as early events in CRC initiation could be determined by introduction of specific alterations in the background of normal diploid HCECs. In the present study, we applied exome sequencing on a series of isogenically-derived immortalized human colonic epithelial cell (HCEC) lines generated from the same individual with defined genetic manipulations. Analysis of the mutation spectrum of these cell lines reveal expected changes and a list of novel candidate genes that may be involved in early stage of CRC tumorigenesis.

Materials and Methods

Cell culture

The culture conditions of HCECs and their isogenic series have been reported elsewhere (Roig et al., 2010). Briefly, HCECs were maintained under 2% oxygen and 5% carbon dioxide on Primaria® (BD Biosciences, San Jose, CA) plates in 4:1 high-glucose Dulbecco modified Eagle medium/medium 199 with 2% cosmic calf serum (Hyclone, Logan, UT) plus growth supplements: epidermal growth factor (EGF; 20 ng/ml; Peprotech, Rocky Hill, NJ), hydrocortisone (1 mg/ml), insulin (10 mg/ml), transferrin (2 mg/ml), and sodium selenite (5 nM) (all Sigma, St Louis, MO).

DNA and RNA Extraction

DNA and RNA were extracted from cell lysates using a DNeasy Blood & Tissue Kit or RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Genomic DNA was used for exome capture.

qRT-PCR

Total RNA was isolated from cells using RNeasyMinikit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Then 1 μg RNA was converted to cDNA using a First Strand cDNA

Synthesis Kit (Roche, Indianapolis, IN). Real-time quantitative PCR reactions were set up in triplicate with Ssofast Master Mix (Biorad, Hercules, CA) and run on a LightCycler® 480 (Roche, Indianapolis, Indiana).

Sanger sequencing

PCR was performed on cDNA from each cell line and purified PCR products were directly sequenced. Each read was aligned with reference sequence at Nucleotide BLAST website (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The forward and reverse primers for INCENP are: 5'-tctgcagggcagcaagag-3' and 5'-tcctccttcatctgctccac-3'.

Whole-exome sequencing

Exome capture using 3 µg of genomic DNA from each cell line was performed using the TargetSeq (TM) Exome Enrichment system (A14061) from Life Technologies according to the manufacturer's protocol. Sequencing was performed on the SOLiD(TM) 5500XL platform. Mapping to the hg19 version of the human genome and single nucleotide variations as well as small indels identification was performed using default settings of the LifeScope software (Life Technologies, Carlsbad, CA). High quality variants (with coverage >=10x and MQV>=20) were annotated and filtered using the SNP and Variation Suite (SVS) version 7 from Golden Helix. Novel and rare variants (with MAF <1%) were filtered against the NHLBI exome project database. SNVs were predicted damaging using the SIFT, Poly-Phen or the Mutation Taster software within the SVS7 pipeline.

Results

Characteristics of the sequenced HCEC lines

The HCEC 1CT line used in these experiments was derived from non-malignant colonic tissue from a patient with a previous history of CRC who was undergoing routine colonoscopy screening. The cells derived from explants were immortalized with ectopic expression of CDK4 and hTERT as previously described (Roig et al., 2010). This cell line maintains a stable normal karyotype (46, XY)

when continuously propagated in 2% oxygen and medium containing 2% serum (Roig et al., 2010). 1CT7 cells were spontaneously generated from 1CT cells after prolonged passage under serum-free condition (Ly et al., 2011). Trisomy in chromosome 7 is one of the earliest events occurring in up to ~ 40% of colonic benign adenomas (Bomme et al., 2001; Habermann et al., 2007; Ried et al., 1996). 1CT7 cells have enhanced cell migration (in a scratch-wound assay) compared to 1CT cells when cultured under normal oxygen conditions (data not shown). Additionally, 1CT7 cells have significant up-regulation of EGFR and c-Met, which are two chromosome 7-located receptor tyrosine kinase compared to 1CT cells (Ly et al., 2013). 1CTRPA A1309 (abbreviated as A1309) is a partially transformed cell line harboring *TP53* and *APC* knockdowns (>90%), as well as ectopic expression of oncogenic $KRAS^{V12}$ and truncated APC1309, all of which are common mutations detected in CRC tumors (Fodde et al., 2001b; Kinzler and Vogelstein, 1996). This cell line exhibits enhancement in cellular proliferation, anchorage independent growth as well as invasion through Matrigel® compared with the 1CT line which does not have any detectable tumorigenic characteristics. Both 1CT7 and A1309 cell line are not fully transformed because they lack the ability to form tumors in immunocompromised mice (data not shown).

Exome capture and sequencing results

Exome capture was performed on the three isogenic 1CT cell lines using SOLiD(TM) 5500XL platform. A summary of the sequencing result is provided in Table 4-1. On average, 57.6 % of the bases were covered to 10X within the targeted bases. After mapping to the hg19 version of human genome (http://genome.ucsc.edu), we obtained the average depth of each read in the target region as ~19X, 21X, 11X for each sample. The average number of observed variants for three samples is 11582. To filter out neutral variants, SIFT and Poly-Phen or the Mutation Taster analysis were performed to predict the functional consequences of all the mutations. We focused our analysis on the 240 and 280 genes with a minimum of three "deleterious" variant reads that are specifically mutated in 1CT7 and A1309 cells, respectively (Table 4-2). There are 32 genes altered found in common in both cell types.

Mutation spectrum of the isogenic 1CT cell lines

To examine if the mutations identified in 1CT7 and A1309 cells are relevant in CRC initiation or progression, we compared the high confidence mutations specifically present within 1CT7 or A1309 cells as listed in Table 4-2 (at least six "deleterious" reads) with the TCGA CRC tumor dataset for 212 cases (http://www.cbioportal.org/public-portal/). This analysis shows that the 1CT7 specific mutated genes are altered in 30.4% of all CRC cases whereas A1309 specific mutated genes are altered in 73.6% of all CRC cases, among which five genes are known cancer genes, i.e. *PBRM1, MYB, PRDM16, BCR* and *NUP214* (Figure 4-1). In particular, *PTPRT* (protein tyrosine phosphatase receptor type T), one of the 1CT7 specific mutated gene that may be involved in cell adhesion is altered in 16.7% of the CRC cases (Wang et al., 2004). Another example is the A1309 specific mutated gene *CSMD1* (CUB and shushi multiple domain 1), that is a TSG altered in 15.6% of the CRC cases (Farrell et al., 2008). The other frequently mutated genes, such as *SYNE1, MUC16*, etc. have been found to be mutated in other cancer types and may be novel candidate driver mutations in early stage of CRC tumorigenesis (Lee et al., 2010; Masica and Karchin, 2011).

Previously, exome sequencing of 24 randomly selected colorectal adenomas revealed mutations involved in multiple known CRC related pathways, such as Wnt signaling, cadherin signaling, integrin signaling, inflammation, and angiogenesis (Nikolaev et al., 2012). Comparison of cell autonomous specific mutations found in the present study shows the overlap of a subset of the genes implicated in these pathways (Table 4-3). Additionally, the distribution of all the high confidence hits in the present study exhibited similar pattern of biological processes to that of mutations detected in those adenomas (Figure 4-2). Among these activities, metabolic, cell communication and transport are the most highly represented processes. Interestingly, a subset of mutations detected in 1CT7 or A1309 cells clustered on chromosome 11p15 (Table 4-4), consistent with a previous report of numerous aberrations detected on chromosome 11 in colorectal adenocarcinoma (Tagawa et al., 1996). Taken together, these results can be interpreted to suggest that existence of trisomy 7 and the other introduced genetic alterations lead to the acquisition of additional mutations that may drive CRC initiation and progression.1CT7 and partially transformed A1309 cells may harbor the genetic background mimicking early stages CRC. In addition,

since many of these mutations are detected sequentially in an experimental *in vitro* manipulated setting, it suggests these mutations occur in a cell autonomous manner and are not dependent on the extracellular microenvironment that occurs *in vivo*.

Identification of novel candidate genes involved in CRC tumorigenesis

To identify the genetic alterations that may be most relevant in CRC tumorigenesis, we prioritized our candidate genes using the ToppGene suite. This web-based tool has been shown to be a useful portal in identifying novel disease candidate genes (Chen et al., 2009b; Chen et al., 2007). We built the training gene set using the 24 colorectal adenoma sequencing data (Nikolaev et al., 2012) and the test set using the high confidence 1CT7 or A1309 specific mutations. Protein-protein interaction (PPIN)-based methods, including K-Step Markov, Hits with Priors, and PageRank with Priors, as well as functional annotation-based prioritization were used for the analyses (Table 4-5). The intersection of the top 20 genes using each method for 1CT7 or A1309 is represented as a Venn diagram in Figure 4-3. This analysis reveals 13 genes in 1CT7 cells and 14 genes in A1309 cells that can be designated as hits using more than three methods. To investigate whether this collection of top ranked novel candidate genes are potentially important in CRC biology, we compare these 27 genes with the TCGA dataset. We found that the 27 genes are altered in 35% of all the CRC cases and the cases with these alterations show poorer overall survival in Kaplan-Meier Plot analyses (Figure 4-4) compared to the cases without these alterations. We then placed the 27 genes as central nodes and overlaid them with the TCGA dataset. This leads to the generation of an interaction network as shown in Figure 4-5. Within this network 15 out of 27 genes interact either directly or indirectly and most, if not all of the interactors are altered in CRC tumors. A subset of these interacting genes are known to be involved in CRC tumorigenesis, such as *AXIN2, FBXW7*and *PIK3CA* whereas the rest of the interacting genes do not have established roles in CRC. Further Gene Set Enrichment Analysis (http://www.broadinstitute.org/gsea/msigdb/annotate.jsp) of these 27 genes using C2 (except chemical and genetic perturbation category) curated gene sets reveals the enrichment in multiple pathways, such as EGFR, endocytosis, FGFR, splicesome and apoptosis (Table 4-6). Taken together, these results could be interpreted to suggest that these 27 genes and their interactors

may be novel candidate genes that are involved in CRC tumorigenesis. Further mechanistic investigations of these genes and the pathways they are implicated in may give insights into their role in CRC initiation and progression and perhaps the identification of novel therapeutic targets.

Identification of INCENP polymorphism in 1CT isogenic series

The exome capture identified 3 single nucleotide polymorphisms (SNPs) in the *INCENP* gene in 1CT7 cells and one of the variants, p.M506T is predicted to be deleterious using SIFT analysis (Table 4-7). INCENP is a member of chromosomal passenger complex (CPC) which also consists of Aurora B, Survivin and Borealin (Carmena et al., 2012). Overexpression of INCENP is observed in several colorectal cancer cell lines (Adams et al., 2001). Validation by Sanger sequencing confirmed that variant p.M506T is present in all 1CT series, i.e. 1CT, 1CT7 and A1309 as well as its pre-immortalized HCEC1 cells and this variant occurs at a highly conservative position (Figure 4-6). Interestingly, this INCENP variant does not occur in 2CT cell line which is an independent CDK4 and hTERT immortalized colonic epithelial cell line derived from a patient with no CRC history. This cell line did not acquire trisomy 7 as does 1CT when cultured under the same serum deprived culture conditions. Since INCENP plays important roles in mitosis (Wheatley et al., 2001), it is possible that mutations in this gene may be one of the contributing factors that lead to aneuploidy and the occurrence of trisomy 7 cells in 1CT cell population. Further functional investigation is warranted to delineate its potential role in aneuploidy and as an early event in CRC initiation.

Discussion

We performed whole exome sequencing of a series of isogenically derived human colonic epithelial cell lines (HCECs), including the non-cancerous diploid parental 1CT cells, the 1CT7 cells with spontaneously occurring trisomy 7 which is frequently observed as an early event in CRC, and partially transformed A1309 cells harboring commonly found mutations in CRC. On average, ~60 % of the bases were covered to 10X within the targeted bases with over 10,000 variants detected in these samples. The reason we chose 1CT as the control cell line but not 2CT or another known independent cell line is

because 1CT, 1CT7 and A1309 are isogenically derived from the original pre-immortalized patient cells. Thus, the genes specifically mutated in 1CT7 and A1309 cells are more likely to be candidate "driver" genes instead of "passengers" involved in CRC tumorigenesis.

Based on the TCGA datasets examined, the mutations unique to 1CT7 occurs in 30.4% of all CRC cases whereas A1309 specific mutated genes are altered in 73.6% of all CRC cases. 1CT7 is a premalignant cell line containing only one early molecular change that occurs in about 40% of CRC cases. Additionally, many of the mutations found in this cell line are likely to be incidental events. Therefore, it is very likely amplification of chromosome 7 is an important early event in a reasonable fraction of sporadic CRC. The top ranked genes, *PTPRT* and *CSMD1*, which are unique to 1CT7 and A1309 cells, respectively, have previously been reported to be mutated in colorectal tumors (Farrell et al., 2008; Wang et al., 2011). Comparison of our sequencing data with a previous exome sequencing study for 24 colorectal adenomas reveals the overlap of a subset of genetic mutations involved in CRC related pathways. These results suggest that the existence of trisomy 7 and the introduction of other genetic manipulation can lead to acquisition of additional genetic mutations that may contribute to CRC progression. Interestingly, knockdown of TP53 and expression of K-Ras^{V12} in 1CT7 cells results in the emergence of trisomy 20, another nonrandom aneuploidy observed in ~85% of CRC (Ly et al., 2011). Therefore, 1CT7 and partially transformed A1309 cells may harbor the genetic background mimicking susceptibility to early stage colon cancer initiation and progression. These cell lines represent an ideal cell autonomous model to delineate the molecular events that contribute to CRC tumorigenesis.

Utilizing the ToppGene portal, we prioritized the candidate gene list based on protein-protein interactions and other functional annotations. A total of 27 genes are putative CRC hits using more than three methods. Many of these genes are frequently mutated in CRC tumors and patients with alteration in these genes exhibit overall poorer survival. Network analysis of these genes reveals additional and perhaps novel interactors that are also altered in CRC tumors. Therefore, this set of genes and their interacting partners may play important role in CRC tumorigenesis.

Epigenetic regulation of gene silencing is another pathway by which tumor suppressor genes are inactivated (Lao and Grady, 2011). Aberrant DNA methylation has been reported to contribute to colon cancer progression through CpG Island Methylator Phenotype (CIMP) (Goel and Boland, 2012; Lao and Grady, 2011). We can speculate that 1CT7 and A1309 cell lines may harbor a higher presence of aberrantly methylated genes compared to their normal isogenic counterpart, 1CT cells. Future investigation of the epigenetic signatures of these cell lines compared to preimmortalized normal epithelial cells as well as the authentic CRC samples are warranted.

In conclusion, the present study revealed the comprehensive mutation spectrums of a series of isogenically-derived HCEC lines. This has led to the identification of known CRC genes as well as a collection of novel candidate CRC genes, demonstrating the potential of utilizing these isogenic HCEC lines to unravel the early cell autonomous events that contribute to CRC initiation and progression. These newly identified important CRC "driver" genes can be potentially utilized as biomarkers for the diagnostic and prognostic applications. A collection of these candidate genes may be further pursued as novel therapeutic targets for CRC prevention and intervention.

Availability of supporting data:

Raw sequencing data can be retrieved from DOI**:** 10.6070/H44M92HV.

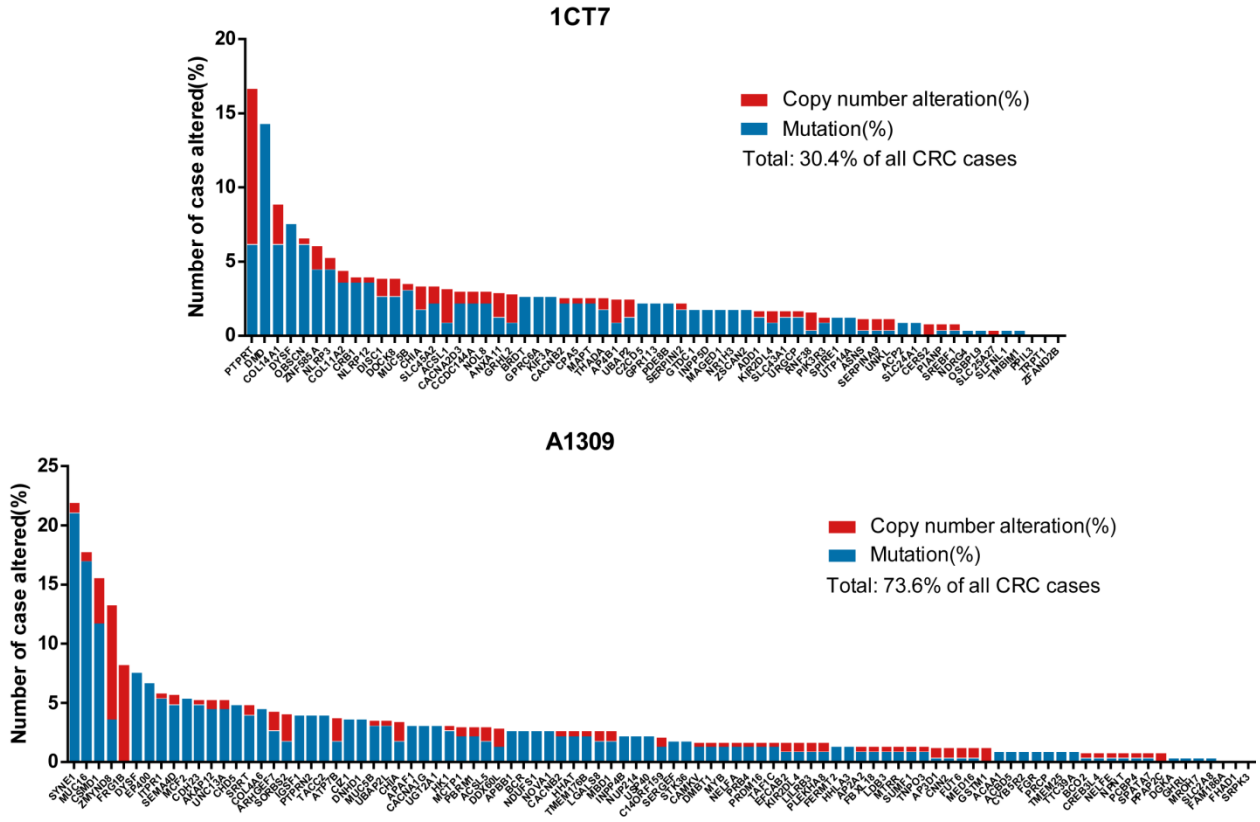


Figure 4-1. Mutations specifically occurred in 1CT7 or A1309 cells are highly present in colorectal tumor samples. These two bar graphs display the frequency of mutation or copy number alteration for each gene that have over six "deleterious" reads and are uniquely detected in 1CT7 or A1309 cells. Data was retrieved from the TCGA database. (http://www.cbioportal.org/public-portal/)

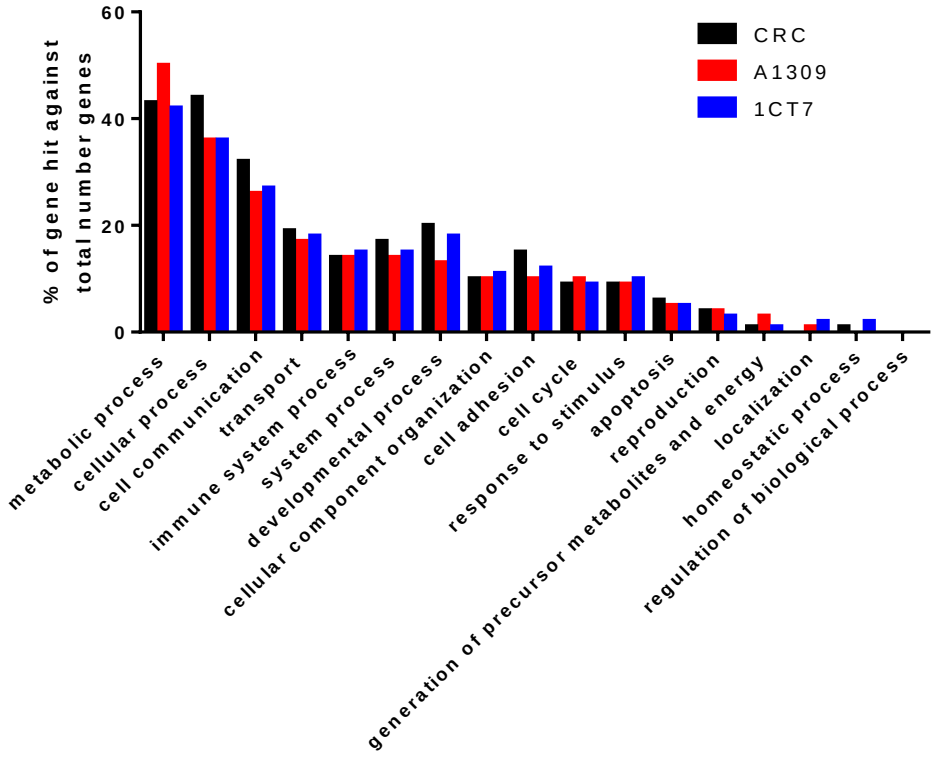


Figure 4-2. Distribution of mutations specifically occurred in 1CT7 or 1CTRPA A1309 cells as well as colorectal adenomas in different biological processes. Red and blue bars display the frequency of the A1309 or 1CT7 specific mutations that have over three “deleterious” reads in each biological process category. Black bar displays the mutations detected in colorectal adenomas from a previous study (Nikolaev et al., 2012).

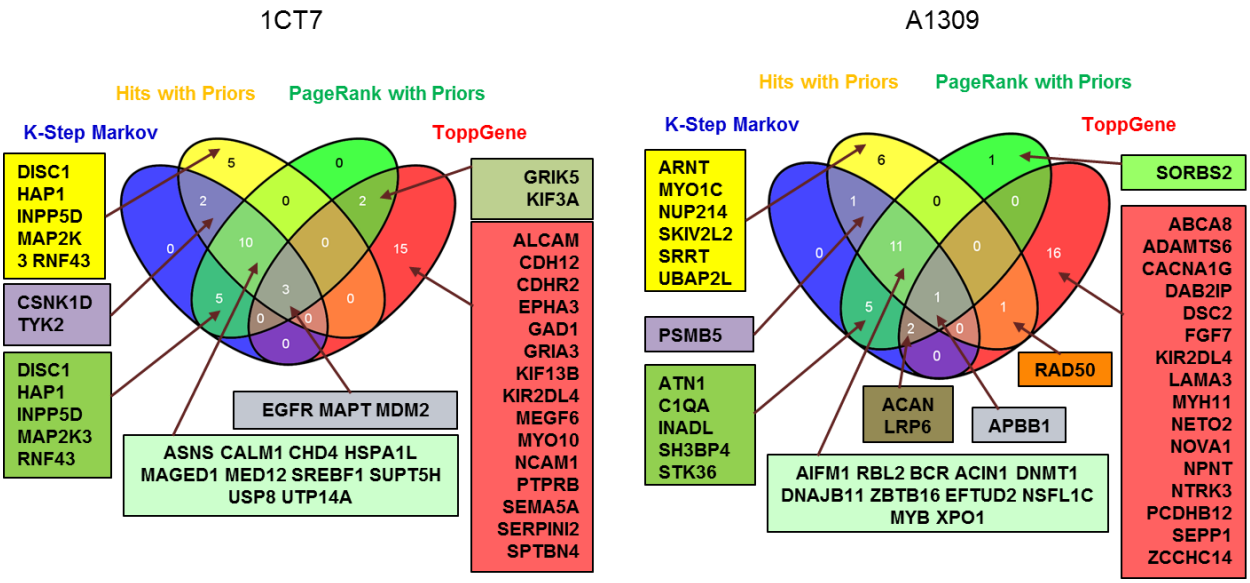


Figure 4-3. Venn Diagram comparing the top 20 ranked candidate genes for CRC tumorigenesis derived from 1CT7 and A1309 sequencing data using functional annotation- and protein-protein interaction (PPIN)- based methods. Functional annotation-based prioritization was done using ToppGene server. For PPIN-based methods, K-Step Markov, Hits with Priors, and PageRank with Priors were used.

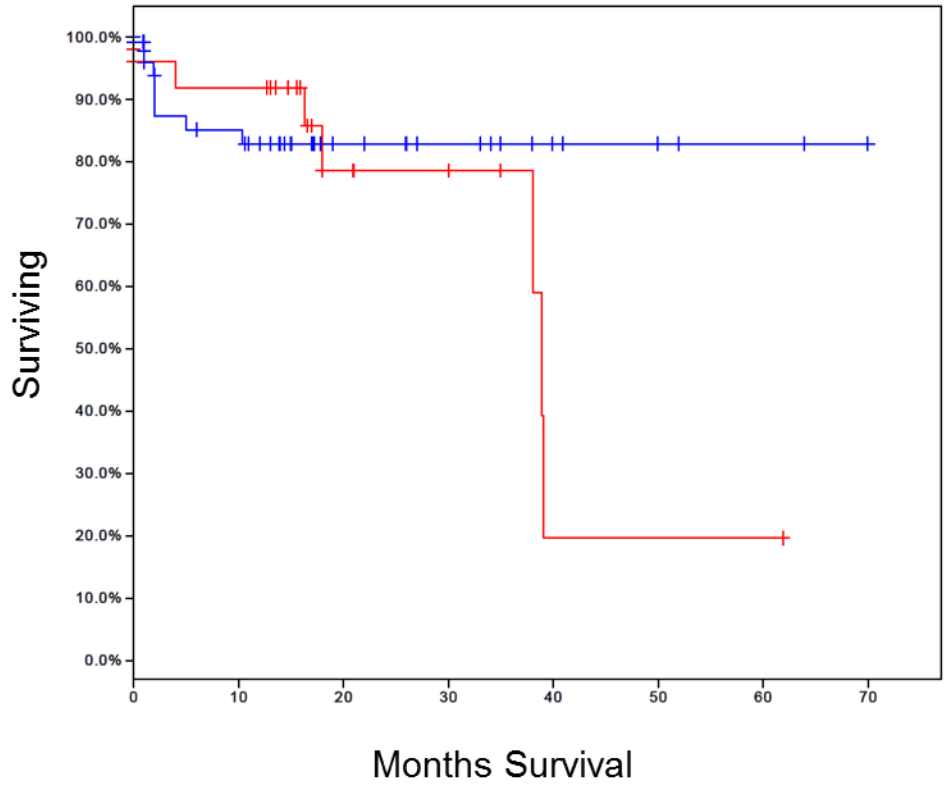


Figure 4-4. Kaplan-Meier plot of cases with or without the 27 genetic alterations. Cases with these alterations show poorer overall survival. Data was retrieved from the TCGA database. (http://www.cbioportal.org/public-portal/)

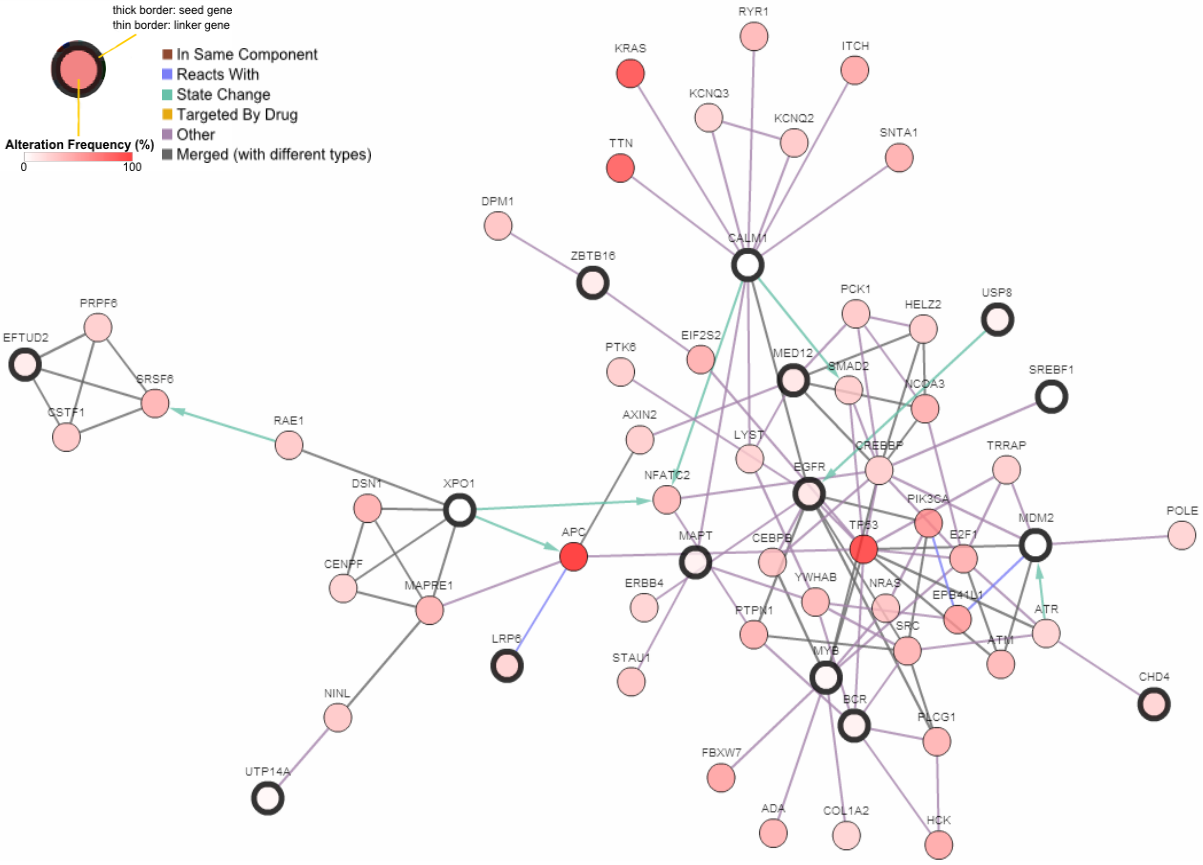


Figure 4-5. Interaction map of prioritized genes identified from our cells (thick border) and the genes found to be mutated in CRC tumor samples (thin border). Interactions were colored according to the type of interactions as shown in the color key. This map is constructed from TCGA portal. (http://www.cbioportal.org/public-portal/)

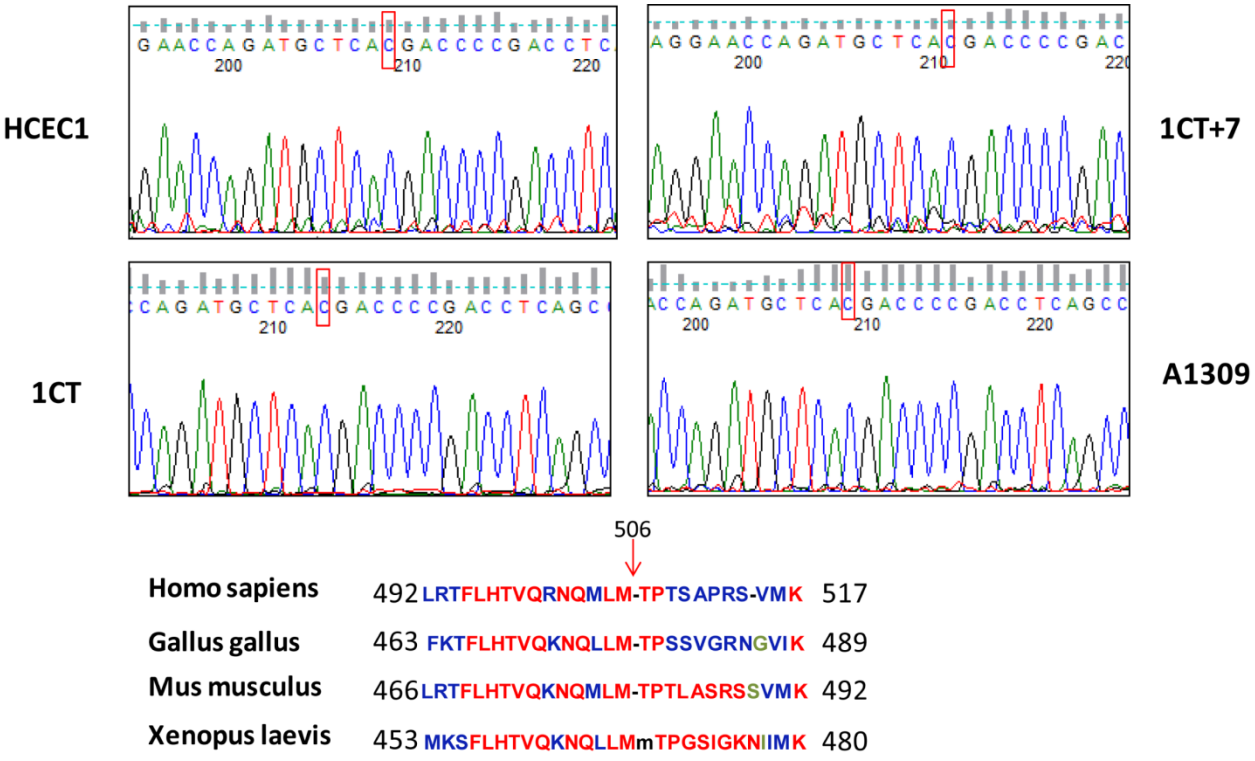


Figure 4-6. Validation of "deleterious" INCENP variants in ICT series. Predicted "deleterious" mutation p.M506T was confirmed by Sanger sequencing of PCR products from 1CT series. c. T1517>C mutation (highlighted in red box) was detected in all 1CT series as well as the pre-immortalized HCEC1 cells and it occurs at a highly conserved position.

Table 4-1. Summary of exome sequencing results of three isogenic HCEC cell lines.

Parameter	**1CT**	**1CT7**	**A1309**
Total reads	40157067	40491020	33924594
Total yield (bp)	2066960672	2078084457	1412998222
Mappable reads	33533312	33767043	25863504
Mappable yield (bp)	2514998400	2532528225	1939762800
On-target reads	14514230	13119148	8353753
On-target yield (bp)	37262779	37262779	37262779
Coverage of target region (more than 10x)	66.18%	65.34%	42.24%
Mean read depth of targeted region	20.99	19.06	10.79
Mean read depth of called variants	23.4	23.1	21.7
Number of total variants	14466	13791	6491
Number of coding variants	9841	9723	4990
Number of missense, nonsense, splice, and indel variants	4910	4895	2583
After NHLBI ESP6500 Exomes filtering	4071	3990	2050

Table 4-2. List of genes specifically mutated in 1CT7 or A1309 cells with at least three or six “deleterious” variant reads.

At least 3 "deleterious" reads				At least 6 "deleterious" reads	
1CT7 specific		**A1309 specific**		**1CT7 specific**	**A1309 specific**
ACP2	LEPREL4	AASS	UVSSA	ACP2	AC016757.3.1
ACSL1	LILRA6	ABCA8	KIF21B	ACSL1	ACAA1
ADD1	LILRB2	ABCC3	KIF24	ADD1	ACBD5
AGER	LOXHD1	ACAA1	KIR2DL4	ANXA11	ACSL5
AIPL1	LRIG3	ACAN	KLK10	AP4B1	AKAP12
AKT3	LRRC31	ACBD4	KRTAP5-5	ASNS	AP2A2
ALCAM	LRRC8A	ACBD5	LAMA3	BRDT	AP3D1
ALS2	LRRN3	ACIN1	LAMC3	C12orf53	APAF1
ANO3	LTK	ACSL5	LDB3	CACNA2D3	APBB1
ANXA11	LYSMD4	ADAMTS6	LGALS8	CACNB2	ARHGEF7
AP4B1	MAGED1	ADH7	LIG1	CCDC144A	ATP7B
APC	MAP2K3	AHNAK2	LILRA6	CERS2	BCO2
APOL4	MAPT	AIFM1	LILRB3	CHIA	BCR
ARMC10	MBD6	AKAP12	LRP6	COL11A2	C14orf159
ARMC3	MDM2	AKR1C2	LRRK1	COL14A1	CACNA1G
ASNS	MECP2	AKR7A2	LTF	CPA5	CACNB2
ATHL1	MED12	ANKDD1A	LYVE1	CRB1	CAMKV
ATP8B3	MEGF6	AP2A2	MAEL	DISC1	CDH23
BAHD1	MICAL2	AP3D1	MAN2B2	DMD	CHD5
BCL9L	MTHFR	APAF1	MAPKBP1	DOCK8	CHIA
BRDT	MTUS1	APBB1	MBD1	DYSF	CIZ1
C10ORF32	MYO10	ARAP1	MCF2	GPR113	CNN2
C10ORF68	MYOM3	ARHGEF7	MCTP1	GPRC6A	COL4A6
PIANP	N4BP2	ARNT	MED16	GRHL2	CREB3L4
C14ORF105	NAA16	ARSD	MFSD4	GTDC1	CSMD1
PCNXL4	NACA	ASPM	MICAL2	INPP5D	CYB5R2
C17ORF80	NARFL	ATG2A	MTHFD2	KIAA0528	DDX60L
C1ORF170	NCAM1	ATN1	MTRF1L	KIF3A	DGKA
C3ORF18	NDRG4	ATP7B	MTRR	KIR2DL4	DMBT1
C5ORF30	NFU1	TEX38	MXD3	MAGED1	DNHD1
C6ORF165	NLRP12	B3GAT3	MYB	MAPT	DYSF
ARHGEF39	NLRP3	BBS1	MYBPC3	MUC5B	EFCAB2
TMEM245	NOC3L	BCO2	MYH11	NDRG4	EP400
CA6	NOL8	BCR	MYO1C	NLRP12	FAM186A

CACNA2D3	NOMO1	C10ORF129	MYO5B	NLRP3	FBXL18
CACNB2	NOTCH2NL	C14ORF159	NCF2	NOL8	FERMT2
CALM1	NPAS2	SYNE3	NDUFS1	NR1H3	FGR
CCDC144A	NR1H3	NUTM1	NEK10	OBSCN	FHAD1
CCDC92	NUMBL	C16ORF93	NETO2	OSBPL9	FRG1B
CCP110	OBSCN	C1ORF198	NHSL2	PDE8B	FUT6
CD22	OSBPL9	C1QA	NOS1	PIK3R3	GHRL
CDH12	PAH	C3ORF18	NOVA1	PPIL3	GSTM1
CDHR2	PCSK7	EOGT	NPNT	PTPRT	HEATR8
CDK11A	PDE8B	CAB39	NSFL1C	RNF38	HHAT
CEACAM21	PHYH	CACNA1G	NTRK3	SERPINA9	HHLA3
CECR2	PIGC	CACNB2	NUP214	SERPINI2	HK1
CERS2	PIK3CB	CAMKV	PBRM1	SLC24A1	IGSF1
CHD4	PIK3R3	CAPN2	PCBP4	SLC25A27	INPP4B
CHIA	PPIL3	CAPZA2	PCDHB12	SLC43A1	ITPR1
CLEC4A	PPP2R3A	CCDC108	PDE7A	SLC45A2	KIR2DL4
COL11A1	PRAMEF2	CCDC167	PFAS	SLFNL1	LDB3
COL11A2	PRG2	CD209	PGS1	SPIRE1	LGALS8
COL14A1	PRRT4	CDC25B	PKP2	SREBF1	LILRB3
COL6A5	PTPRA	CDH23	PLEKHA8	THADA	MBD1
CPA5	PTPRB	CECR2	PLEKHG2	TMBIM1	MCF2
CRB1	PTPRT	CEP120	PLXNB1	TRPT1	MCTP1
CRYM	QRICH2	CEP128	POLR1B	UBAP2	MED16
CSNK1D	QSER1	CHD5	PPAP2C	UNKL	MTRR
PIH1D3	RANBP3	CHIA	PRB4	URGCP	MUC16
CYP4B1	RBMS1	CHST10	PRCP	UTP14A	MUC5B
DISC1	RBMXL3	CIZ1	PRDM16	ZFAND2B	MYB
DMD	RNASEL	CLCN7	PRSS1	ZNF585A	NDUFS1
DNAAF2	RNF38	CNN2	PRUNE2	ZSCAN2	NOVA1
DNAH12	RNF43	COASY	PSMB5		NPNT
DNMT3L	ROM1	COL4A6	PTPRN2		NUP214
DOCK2	SCN3A	CREB3L4	RAB15		PBRM1
DOCK6	SEC14L4	CRIP1	RABGGTB		PCBP4
DOCK8	SEMA5A	CSMD1	RAD50		PLEKHA8
DYSF	SERPINA9	CYB5R2	RBL2		PPAP2C
EDN3	SERPINI2	CYP2R1	NELFE		PRB4
EGFR	SETX	DAAM2	RELL2		PRCP
EML3	SLC23A2	DAB2IP	RGL4		PRDM16
EPHA3	SLC24A1	DAGLB	RNF213		PTPRN2
EPN3	SLC25A27	DDX4	RYR1		RDBP
EXOG	SLC27A1	DDX42	SDSL		SEMA4D

FAM135A	SLC43A1	DGKA	SEPP1		SERGEF
FAM186A	SLC45A2	DIO2	SERGEF		SLC2A8
FAM204A	SLCO1C1	DMBT1	SERPINA3		SORBS2
FLNA	SLFNL1	DNAAF1	SH3BP4		SPATA7
FLT3	SMC6	DNAAF2	SKIV2L2		SRPK3
FMO3	SNRNP25	DNAJB11	SLC2A8		SRRT
FRG1B	SPIRE1	DNMT1	SLC43A2		STK36
FRMD7	SPTBN4	DOT1L	SLC45A1		SUMF1
GAD1	SREBF1	DSC2	SLC4A11		SYNE1
GALNT8	SRP9	DSCAML1	SLC4A4		TACC2
GBE1	SRSF4	DYSF	SORBS2		TAF1C
GBP6	SUPT5H	EFCAB2	SORBS3		TMEM176B
GCC2	TCN2	EFTUD2	SORL1		TMEM25
GCFC2	THADA	EHBP1	SPATA7		TNPO3
GFRA2	THSD7B	EIF2AK4	SRPK3		TTC39A
GK	TIGIT	EIF2B4	SRRT		UBAP2L
GOLGA4	TIMELESS	EP400	SSX5		UGT2A1
GPR116	TLR3	EPB41L5	STARD13		UNC13A
GPRC6A	TMBIM1	ESRRA	STK31		USP40
GRHL2	TMC6	EXTL2	STK36		WHSC2
GRIA3	TNRC6A	FAM132B	STOX1		ZMYND8
GRIK5	TOP3A	FAM186A	STRADA		
GTDC1	TRIM66	FBXL18	SUCLG2		
HAP1	TRPM8	FERMT2	SUMF1		
HKR1	TRPT1	FGF7	SYNE1		
HLA-DRB1	TTC24	FGR	TACC2		
HPS4	TYK2	FHAD1	TAF1C		
HSPA1L	UBAP2	FRG1	TMEM159		
IBTK	UNKL	FRG1B	TMEM176B		
IL1F10	URGCP	FRG2B	TMEM25		
INMT	USP47	FUT3	TNPO3		
INPP5D	USP8	FUT6	TP53INP2		
ITGA11	UTP14A	GAL3ST4	TPD52L3		
JMJD1C	VCAN	GALNT14	TPTE		
KHNYN	VSIG4	GFM2	TRIM38		
C2CD5	VWDE	GHRL	TRO		
KIAA1324	DAW1	GJB3	TTC24		
KIF13B	MAP3K19	GLIPR1	TTC39A		
KIF3A	ZFAND2B	GLOD4	UBAP2L		
KIR2DL4	ZNF222	GPER1	UCP2		
KRT13	ZNF28	GRAMD1C	UGT2A1		

KRT72	ZNF281	GRTP1	UNC13A		
KRTAP5-4	ZNF585A	GSTM1	UNC80		
KRTAP5-5	ZNF606	HCRTR1	UQCRC2		
	ZNF780A	HDHD1	USP37		
	ZSCAN2	HDX	USP40		
		MROH7	UTP18		
		HHAT	VPS53		
		HHLA3	WAPAL		
		HK1	WDFY4		
		HLX	NELFA		
		HMGXB4	XPO1		
		HPS4	ZBTB16		
		HPS5	ZBTB7B		
		HPSE2	ZCCHC14		
		HS1BP3	ZFP37		
		HYLS1	ZMYND8		
		IGSF1	ZNF2		
		IL1F10	ZNF335		
		INADL	ZNF418		
		INPP4B	ZNF438		
		IQCH	ZNF623		
		IVD	ZFP69B		
		AREL1	ZNF721		
			ZNF780A		
			ZNF793		

Functional consequence of the variants were predicted using SIFT, Poly-Phen or the Mutation Taster softwares. Genes with variants that have more than three or six “deleterious” or “damaging” reads in either one of these analyses but are not present in 1CT cells were selected.

Table 4-3. Comparison of exome sequencing data of 24 colorectal adenomas with our sequencing data.

24 Colorectal adenoma *						1CT7 **						A1309 **					
Angiogenesis	p53	Wnt	Integrin	Inflammation	Cadherin	Angiogenesis	p53	Wnt	Integrin	Inflammation	Cadherin	Angiogenesis	p53	Wnt	Integrin	Inflammation	Cadherin
AKT1	AKT1	APC	ACTL9	ACTL9	ACTL9	AKT3	AKT3	APC	COL11A1	AKT3	CDH12	AIFM1	APAF1	CDH23	COL4A6	MYH11	CDH23
APC	APAF1	ARID1A	ARPC5L	ADCY2	CDH10	APC	MDM2	CDH12	COL11A2	COL14A1	CHDR2	APAF1	HMGXB4	EP400	LAMA3		PCDHB12
BRAF	CDC25C	ARID1B	BRAF	ADCY8	CDH13	EPHA3	PIK3CB	CDHR2	COL11A2	COL6A5	EGFR		TPTE	LRP6	LAMC3		PKP2
CTNNB1	CDKN2A	CDH10	CAV1	AKT1	CDH23	PIK3CB	PIK3R3	CSNK1D	COL11A2	PIK3CB				PCDHB12			
HSPB2	COL6A6	CDH13	COL11A1	ARPC5L	CDH9	PIK3R3			COL14A1	TYK2							
KRAS	CREBBP	CDH23	COL27A1	BRAF	CTNNB1	PTPRB			COL6A5								
NRAS	PIK3C2A	CDH9	COL5A1	CASK	CTNND2				FLNA								
PIK3C2A	PIK3C2G	CREBBP	COL6A3	COL6A3	ERBB2				ITGA11								
PIK3C2G	PIK3R3	CTNNB1	COL6A6	COL6A6	ERBB4				MAP2K3								
PIK3R3	TP53	FAT1	COL7A1	GNB3	FAT1				PIK3CB								
SHC1		FAT3	FLNA	KRAS	FAT3				PIK3R3								
TCF7L2		FBXW11	FN1	MAP3K4	FZD10												
		FZD10	ITGA6	MYH1	PCDH10												
		GNB3	ITGAD	MYH13	PCDH15												
		MYH1	ITGAX	MYO3A	PCDH20												
		MYH13	KRAS	NFATC1	PCDH7												
		NFATC1	LAMA2	NRAS	PCDHA1												
		PCDH10	LAMA4	PIK3C2G	PCDHA11												
		PCDH15	LAMA5	PREX1	PCDHA12												
		PCDH20	LAMB1	SHC1	PCDHA13												
		PCDH7	LAMC3	VWF	PCDHA3												
		PCDHA1	MAP3K4		PCDHA4												
		PCDHA11	MAPK13		PCDHA5												
		PCDHA12	NRAS		PCDHB1												
		PCDHA13	PIK3C2A		PCDHB10												
		PCDHA3	PIK3C2G		PCDHGA2												
		PCDHA4	PIK3R3		PCDHGA7												
		PCDHA5	SHC1		PCDHGA8												
		PCDHB1	TLN1		PCDHGC4												
		PCDHB10			TCF7L2												
		PCDHGA2			WNT3A												
		PCDHGA7			YES1												
		PCDHGA8															
		PCDHGC4															
		PYGO2															
		SMARCA5															
		SMARCD3															
		TBL1Y															
		TCF7L2															
		TP53															
		TTBK1															
		WNT3A															

* Exome sequencing data of 24 colorectal adenomas were retrieved from Sergey et al. Functional classification of these genes was performed using PANTHER web-based software.

**High confidence mutations with over three "deleterious" reads were compared to 24 adenoma sequencing data.

This table lists the genes mutated in colorectal adenoma samples, 1CT7 or A1309 cells that belong to the five known CRC related pathways. Overlapped genes are highlighted in yellow or green.

Table 4-4. Summary of mutations detected in 1CT7 and A1309 cells on chromosome 11p15.

1CT7	A1309
ch11p15	**ch11p15**
TRIM66	TRIM66
OR52J3	OR52J3
OR52E6	OR52E6
MICAL2	MICAL2
ATHL1	SERGEF
SCGB1C1	LYVE1
COPB1	AP2A2
USP47	HPS5
OR51I2	OR52W1
OR52N2	CYP2R1
OR52N1	MUC5B
DCHS1	DNHD1
	KCNC1
	OR51M1
	OR56B1

Table 4-5. CRC candidate genes prioritization.

Cell line	1CT7				A1309			
Rank	PPIN-based ranking (K-Step Markov)	PPIN-based ranking (Hit with Priors)	PPIN-based ranking (PageRank with Priors)	Integrative function annotation based ranking (ToppGene)	PPIN-based ranking (K-Step Markov)	PPIN-based ranking (Hit with Priors)	PPIN-based ranking (PageRank with Priors)	Integrative function annotation based ranking (ToppGene)
1	ASNS	ASNS	ASNS	ALCAM	ACAN	ACIN1	ACAN	ABCA8
2	CALM1	CALM1	CALM1	CDH12	ACIN1	AIFM1	ACIN1	ACAN
3	CHD4	CHD4	CHD4	CDHR2	AIFM1	APBB1	AIFM1	ADAMTS6
4	CSNK1D	CSNK1D	DISC1	EGFR	APBB1	ARNT	APBB1	APBB1
5	DISC1	EGFR	EGFR	EPHA3	ATN1	BCR	ATN1	CACNA1G
6	EGFR	HSPA1L	GRIK5	GAD1	BCR	DNAJB11	BCR	DAB2IP
7	HAP1	MAGED1	HAP1	GRIA3	C1QA	DNMT1	C1QA	DSC2
8	HSPA1L	MAPT	HSPA1L	GRIK5	DNAJB11	EFTUD2	DNAJB11	FGF7
9	INPP5D	MDM2	INPP5D	KIF13B	DNMT1	MYB	DNMT1	KIR2DL4
10	MAGED1	MED12	KIF3A	KIF3A	EFTUD2	MYO1C	EFTUD2	LAMA3
11	MAP2K3	NACA	MAGED1	KIR2DL4	INADL	NSFL1C	INADL	LRP6
12	MAPT	NR1H3	MAP2K3	MAPT	LRP6	NUP214	LRP6	MYH11
13	MDM2	RANBP3	MAPT	MDM2	MYB	PSMB5	MYB	NETO2
14	MED12	SREBF1	MDM2	MEGF6	NSFL1C	RAD50	NSFL1C	NOVA1
15	RNF43	SRP9	MED12	MYO10	PSMB5	RBL2	RBL2	NPNT
16	SREBF1	SRSF4	RNF43	NCAM1	RBL2	SKIV2L2	SH3BP4	NTRK3
17	SUPT5H	SUPT5H	SREBF1	PTPRB	SH3BP4	SRRT	SORBS2	PCDHB12
18	TYK2	TYK2	SUPT5H	SEMA5A	STK36	UBAP2L	STK36	RAD50
19	USP8	USP8	USP8	SERPINI2	XPO1	XPO1	XPO1	SEPP1
20	UTP14A	UTP14A	UTP14A	SPTBN4	ZBTB16	ZBTB16	ZBTB16	ZCCHC14

This table lists the top 20 ranked genes using each prioritization method in 1CT7 or A1309 cells.

Table 4-6. The pathways that the 27 prioritized genes are enriched in.

Gene Set Name	# Genes in Gene Set (K)	Description	# Genes in Overlap (k)	k/K	p-value	FDR q-value
REACTOME_SIGNALING_BY_ERBB2	101	Genes involved in Signaling by ERBB2	4	0.0396	2.68E-07	3.54E-04
KEGG_ENDOCYTOSIS	183	Endocytosis	4	0.0219	2.88E-06	1.90E-03
KEGG_GLIOMA	65	Glioma	3	0.0462	6.07E-06	2.67E-03
BIOCARTA_EGFR_SMRTE_PATHWAY	11	Map Kinase Inactivation of SMRT Corepressor	2	0.1818	1.56E-05	4.11E-03
PID_RANBP2PATHWAY	11	Sumoylation by RanBP2 regulates transcriptional repression	2	0.1818	1.56E-05	4.11E-03
KEGG_PATHWAYS_IN_CANCER	328	Pathways in cancer	4	0.0122	2.86E-05	5.42E-03
REACTOME_SIGNALING_BY_EGFR_IN_CANCER	109	Genes involved in Signaling by EGFR in Cancer	3	0.0275	2.87E-05	5.42E-03
REACTOME_SIGNALING_BY_FGFR_IN_DISEASE	127	Genes involved in Signaling by FGFR in disease	3	0.0236	4.53E-05	6.81E-03
KEGG_SPLICEOSOME	128	Spliceosome	3	0.0234	4.64E-05	6.81E-03
REACTOME_APOPTOSIS	148	Genes involved in Apoptosis	3	0.0203	7.15E-05	9.43E-03

Gene set enrichment analysis was performed for the 27 top ranked candidate genes using C2 (except chemical and genetic perturbation category) curated gene sets.

Table 4-7. Characteristics of INCENP variants detected in this study.

Cell line	Genomic positions	Nucleotide change	Amino acid change	Type	Evolutionary conservation	SIFT analysis*
1CT7	q12.3	c. T1517>C	p. M506T	Single AA change	Yes	0.03
1CT7	q12.3	c.G1931>C	p. E644D	Single AA change	Yes	0.101
1CT7	q12.3	c. T1187>C	p.N396N	Synonymous	No	0.894

* Prediction of a change to be deleterious (<0.05) or tolerated.

Chapter 5 . Discussion and Future Perspectives

It has been extremely challenging to establish long-term culture of colonic epithelial cells from non-malignant colonic tissues. Prior to our study, most, if not all, of the colon cell lines are derived from colorectal cancer tissue and/or contain multiple cytogenetic changes and thus are not appropriate for *in vitro* study of normal colonic biology or colon cancer initiation and progression. As described in this thesis, we have developed and utilized a unique cell culture model to study colon cancer progression by step-wise introduction of commonly found mutations in colon cancer patients. We generated a series of isogenic immortalized HCEC lines with genetic alterations such as ectopic expression of oncogenic $Kras^{V12}$, shRNA mediated down regulation of p53 or APC, alone or in various combinations. These oncogenic changes confer HCECs subtle tumorigenic properties as demonstrated by transformation assays such as anchorage independent colony formation and Matrigel® invasion/migration. These cell lines with defined genetic alterations represent an ideal cell autonomous model to delineate the molecular events that contribute to CRC tumorigenesis.

Mutations in tumor suppressor gene *APC* is a highly frequent and early event in CRC tumorigenesis (Goss and Groden, 2000; Powell et al., 1992). Interestingly, shRNA mediated down regulation of WT APC alone (>90% stable knockdown) does not progress HCECs. Most APC mutations generate premature stop codons, resulting in truncated gene products. While it's not clear as to the exact functions of these truncated APC proteins, it has been reported that truncated APC is required for cell proliferation and DNA replication in APC-defective CRC cells and truncated APC proteins but not full-length APC can efficiently activate Asef, a Rac-specific guanine nucleotide exchange factor, resulting in enhanced cell migration (Kawasaki et al., 2003; Schneikert and Behrens, 2006). Additionally, truncating APC mutations have been shown to interfere with spindle functions in a dominant manner (Green and Kaplan, 2003; Tighe et al., 2004). To better mimic the clinical representation of CRC patients, we also generate HCECs stably expressing the two most frequently observed truncated forms of APC, together

with *TP53* and *APC* knockdowns (>90%), as well as ectopic expression of oncogenic $KRAS^{V12}$ (1CTRPA A1309) . Consistent with the previous reports, we provide evidence showing that ectopic expression of APC truncations confer tumorigenic properties in terms of cellular proliferation, anchorage-independent growth and enhanced invasion/migration through Matrigel® compared to its isogenic precursors. Interestingly, even the HCECs with all the alterations in CDK4, hTERT, KRAS, p53 and APC were not tumorigenic when injected into multiple different models of immune-compromised mice. These results suggest that more than 5 genetic changes are required to fully transform normal human colonic epithelial cells and it remains to be explored what and how many other genetic alterations are needed.

To determine which less frequently mutated genes are involved in CRC initiation and development, we took advantage of our immortalized diploid HCECs with *TP53* knockdown with a sensitized genetic background and functionally interrogated the CRC genome using an shRNA screen and a relevant biological assay. In combination with network analysis, we identified a number of novel driver tumor suppressors among the genes that are otherwise considered "passengers". Driver mutations are defined as being causally involved in the neoplastic process and are frequently mutated across tumor samples whereas passenger mutations are thought to provide no selective advantage but are retained by chance during clonal expansion (Greenman et al., 2007). We found that knockdown of those passenger mutated genes that interacted with the confirmed frequently mutated genes enhanced anchorage-independent growth at a significantly higher frequency compared with those that did not interact and a large fraction of the low frequently mutated genes have been misclassified as "passengers". This is due to the limitation of frequency-based biostatistic models and the difficulty of sequencing enough individuals to generate data with significant predicting power. Therefore, it is necessary to implement biologically relevant functional filters to distinguish between driver and passenger mutations instead of solely depending on bioinformatics. Our work provides a framework for combining bioinformatics and experimental testing to investigate the functions of those less frequently mutated genes.

Despite the improved understanding of the mutational landscape of CRCs, the mutational events that contribute to CRC initiation are less well-studied, partly due to the lack of appropriate cellular

reagents for validating important changes. In order to further unravel the critical players involved in CRC tumorigenesis, we applied exome sequencing on the series of isogenically-derived HCEC lines. Analysis of the mutation spectrum of these partially progressed HCEC lines reveal known CRC genes as well as a list of novel candidate genes that may be involved in early stage of CRC tumorigenesis, demonstrating that these isogenically-derived HCECs may harbor the genetic background mimicking susceptibility to early stage colon cancer initiation and progression. Therefore, they can potentially be utilized to unravel the early cell autonomous events that contribute to CRC tumorigenesis in other different experimental settings.

Taken together, these studies reveal a collection of newly identified important CRC "driver" genes that may represent new biomarkers for diagnostic and prognostic applications. A subset of these candidate genes may be further pursued as novel therapeutic targets for CRC prevention and intervention. Future efforts can be extended on the investigation of the biological functions of these "drivers" and the additional pathways involved in CRC. Small molecule high-throughput screening can also be performed to identify potential drug leads targeting these vulnerabilities.

Although APC mutations occur at such high frequency, there has been no reported therapeutics targeting the vulnerabilities resulting from these mutations. By taking a synthetic lethality screening approach utilizing the series of isogenic progressed HCECs with defined genetic alterations, we have identified a small molecule, named TASIN-1 that can selectively kill HCECs and a panel of CRC cells expressing APC truncations while sparing normal HCECs and CRC cells with WT APC. While stable knockdown of truncated APC initially results in a subset of cells dying, the surviving DLD1 cells with over 90% of truncated APC knockdown are resistant, suggesting that APC truncation is required for TASIN-1's effects. However, HCECs with knockdown of WT APC and expression of truncated APC are not sensitive to TASIN-1, suggesting that existence of truncated APC alone is not sufficient for TASIN-1's cytotoxicity (data not shown). Additionally, there are differential responses to TASIN-1 among different CRC lines. Therefore, TASIN-1's cytotoxicity is highly context-dependent. The oncogenic background targeted by TASIN-1remains to be determined and is challenging due to the intrinsic highly

heterogeneous genetic background of cancer cells. Future efforts including exome sequencing or gene expression analysis of this panel of CRC lines may reveal some insights in this aspect.

c-Jun N-terminal protein kinase (JNK) is a subfamily of the mitogen activated protein kinase (MAPK) (Hibi et al., 1993; Liu and Lin, 2005). Many pieces of evidence shows that JNK can function as a proapoptotic kinase although it remains debatable whether it is an intrinsic component of the apoptotic machinery or a modulator of apoptosis (Davis, 2000; Lin, 2003; Liu and Lin, 2005). Several DNA damaging or microtubule-interfering agents have been shown to induce JNK activation that contribute to cell death (Chen et al., 1996a; Chen et al., 1996b; Sanchez-Perez et al., 1998; Wang et al., 1999; Wang et al., 1998). Truncated APC has been shown to interfere with microtubule plus-end attachments, spindle checkpoint control and chromosome stability in a dominant manner (Green and Kaplan, 2003; Tighe et al., 2004). We provide evidence that TASIN-1 disrupts proper spindle formation and exerts its cytotoxic effects through induction of JNK-dependent apoptotic cell death. It's likely that TASIN-1is causing synthetic lethality in APC truncated cells that have defects in mitotic spindles and the mitotic abnormalities induced by TASIN-1 activates JNK pathway. However, it remains to be determined how TASIN-1 mediates these mitotic interfering effects and whether these effects are direct or indirect.

Extensive efforts to pinpoint the protein target of TASIN-1 and unravel the mechanism of action of TASIN-1 have been underway in our laboratory. Our preliminary attempts to identify the protein target of TASIN-1 using chemical proteomics show that an active analog of TASIN-1 can interact weakly with endogenous truncated APC protein in DLD1and HT29 cells and *in vitro* translated truncated APC protein (data not shown). Whether APC is the direct target of TASIN-1 or it is in a complex that TASIN-1 recognizes remains to be determined. Biophysical methods, such as isothermal titration calorimetry (ITC), can be applied to assess direct binding using recombinant protein. Future optimization of the chemical proteomics approaches using more potent analogs should reveal insights into the direct target of TASIN-1 and are the subject of our current studies. Additional biochemical investigations to unravel novel binding partners of truncated APC and whether TASIN-1 disrupts any of those interactions are warranted.

Considering the high prevalence of APC mutations in patients with CRC, targeting truncated APC represent an ideal therapeutic strategy for prevention and intervention of CRC as well as a potential enrollment enrichment biomarker for future personalized medicine clinical trials. We have shown that *in vivo* administration of TASIN-1 dramatically reduces tumor size and inhibits tumor growth in both xenograft mouse model and a genetic engineered CRC mouse model without noticeable tissue or hematological cell toxicities, demonstrating its potential use as an antitumor agent for CRC patients. Additional SAR analysis is needed to identify more potent and selective analogs of TASIN-1 with optimized drug-like properties.These analogs will serve as a platform for further translational development as putative drugs for clinical testing and represent a paradigm shift for targeted therapy in CRC.

The tumor suppressive function of APC has long been a focus of research in the field of colorectal cancer, whereas emerging evidence has called attention to its oncogenic functions. Accumulating evidence suggest that both loss of tumor suppressive function and gain of function of APC mutants play critical roles in CRC tumorigenesis. As tumorigenesis progresses, tumor cells undertake altered pattern of signaling networks when they gradually become dependent on oncogenic function of the truncated APC proteins for survival or maintenance of their malignant phenotypes. Despite the increasing knowledge of dominant functions of APC truncations, comprehensive understandings of APC mutants' oncogenic properties are still lacking and worth pursuing. We can envision that the utilization of proteomics as well as the advanced genome-wide approaches may aid in identifying the novel binding partners of truncated APC and the downstream signaling events involved. Our work aids in the development of therapeutic agents targeting CRC cells harboring truncated APC. More importantly, TASIN-1 and its analogs can be utilized as biological probes to unravel the refined diagram of signaling network in CRC cells harboring truncated APC. Further understanding the comprehensive functions of these truncated APC mutants will shed light on the molecular mechanism of CRC initiation, progression and maintenance, ultimately providing an opportunity to allow for the development of targeted therapeutics for prevention and intervention of colon cancer.

References

Adams, R. R., Eckley, D. M., Vagnarelli, P., Wheatley, S. P., Gerloff, D. L., Mackay, A. M., Svingen, P. A., Kaufmann, S. H., and Earnshaw, W. C. (2001). Human INCENP colocalizes with the Aurora-B/AIRK2 kinase on chromosomes and is overexpressed in tumour cells. Chromosoma *110*, 65-74.

Ahmed, Y., Nouri, A., and Wieschaus, E. (2002). Drosophila Apc1 and Apc2 regulate Wingless transduction throughout development. Development *129*, 1751-1762.

Akhoondi, S., Sun, D., von der Lehr, N., Apostolidou, S., Klotz, K., Maljukova, A., Cepeda, D., Fiegl, H., Dafou, D., Marth, C., *et al.* (2007). FBXW7/hCDC4 is a general tumor suppressor in human cancer. Cancer Res *67*, 9006-9012.

Akiyama, T., and Kawasaki, Y. (2006). Wnt signalling and the actin cytoskeleton. Oncogene *25*, 7538-7544.

Albuquerque, C., Breukel, C., van der Luijt, R., Fidalgo, P., Lage, P., Slors, F. J., Leitao, C. N., Fodde, R., and Smits, R. (2002). The 'just-right' signaling model: APC somatic mutations are selected based on a specific level of activation of the beta-catenin signaling cascade. Human molecular genetics *11*, 1549-1560.

Andreu, P., Colnot, S., Godard, C., Gad, S., Chafey, P., Niwa-Kawakita, M., Laurent-Puig, P., Kahn, A., Robine, S., Perret, C., and Romagnolo, B. (2005). Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. Development *132*, 1443-1451.

Aoki, K., and Taketo, M. M. (2007). Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene. Journal of Cell Science *120*, 3327-3335.

Baeg, G. H., Matsumine, A., Kuroda, T., Bhattacharjee, R. N., Miyashiro, I., Toyoshima, K., and Akiyama, T. (1995). The tumour suppressor gene product APC blocks cell cycle progression from G0/G1 to S phase. The EMBO journal *14*, 5618-5625.

Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., vanTuinen, P., Ledbetter, D. H., Barker, D. F., Nakamura, Y., *et al.* (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science *244*, 217-221.

Baldus, S. E., Schaefer, K. L., Engers, R., Hartleb, D., Stoecklein, N. H., and Gabbert, H. E. (2010). Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases. Clinical cancer research : an official journal of the American Association for Cancer Research *16*, 790-799.

Ballell, L., van Scherpenzeel, M., Buchalova, K., Liskamp, R. M., and Pieters, R. J. (2006). A new chemical probe for the detection of the cancer-linked galectin-3. Organic & biomolecular chemistry *4*, 4387-4394.

Baross, A., Butterfield, Y. S., Coughlin, S. M., Zeng, T., Griffith, M., Griffith, O. L., Petrescu, A. S., Smailus, D. E., Khattra, J., McDonald, H. L., *et al.* (2004). Systematic recovery and analysis of full-ORF human cDNA clones. Genome research *14*, 2083-2092.

Bass, A. J., Lawrence, M. S., Brace, L. E., Ramos, A. H., Drier, Y., Cibulskis, K., Sougnez, C., Voet, D., Saksena, G., Sivachenko, A., *et al.* (2011). Genomic sequencing of colorectal adenocarcinomas identifies a recurrent VTI1A-TCF7L2 fusion. Nature genetics *43*, 964-968.

Becker, F., Murthi, K., Smith, C., Come, J., Costa-Roldan, N., Kaufmann, C., Hanke, U., Degenhart, C., Baumann, S., Wallner, W., *et al.* (2004). A three-hybrid approach to scanning the proteome for targets of small molecule kinase inhibitors. Chemistry & biology *11*, 211-223.

Berger, A. H., Knudson, A. G., and Pandolfi, P. P. (2011). A continuum model for tumour suppression. Nature *476*, 163-169.

Beroud, C., and Soussi, T. (1996). APC gene: database of germline and somatic mutations in human tumors and cell lines. Nucleic acids research *24*, 121-124.

Bhattacharjee, R. N., Hamada, F., Toyoshima, K., and Akiyama, T. (1996). The tumor suppressor gene product APC is hyperphosphorylated during the M phase. Biochemical and biophysical research communications *220*, 192-195.

Bienz, M., and Hamada, F. (2004). Adenomatous polyposis coli proteins and cell adhesion. Current opinion in cell biology *16*, 528-535.

Blobe, G. C., Schiemann, W. P., and Lodish, H. F. (2000). Role of transforming growth factor beta in human disease. The New England journal of medicine *342*, 1350-1358.

Boehmerle, W., Splittgerber, U., Lazarus, M. B., McKenzie, K. M., Johnston, D. G., Austin, D. J., and Ehrlich, B. E. (2006). Paclitaxel induces calcium oscillations via an inositol 1,4,5-trisphosphate receptor and neuronal calcium sensor 1-dependent mechanism. Proceedings of the National Academy of Sciences of the United States of America *103*, 18356-18361.

Boland, C. R., and Goel, A. (2010). Microsatellite instability in colorectal cancer. Gastroenterology *138*, 2073-2087 e2073.

Bomme, L., Lothe, R. A., Bardi, G., Fenger, C., Kronborg, O., and Heim, S. (2001). Assessments of clonal composition of colorectal adenomas by FISH analysis of chromosomes 1, 7, 13 and 20. International journal of cancer Journal international du cancer *92*, 816-823.

Bos, J. L., Fearon, E. R., Hamilton, S. R., Verlaan-de Vries, M., van Boom, J. H., van der Eb, A. J., and Vogelstein, B. (1987). Prevalence of ras gene mutations in human colorectal cancers. Nature *327*, 293-297.

Briggs, M. W., and Sacks, D. B. (2003). IQGAP proteins are integral components of cytoskeletal regulation. EMBO reports *4*, 571-574.

Brocardo, M., Lei, Y., Tighe, A., Taylor, S. S., Mok, M. T., and Henderson, B. R. (2008). Mitochondrial targeting of adenomatous polyposis coli protein is stimulated by truncating cancer mutations: regulation of Bcl-2 and implications for cell survival. The Journal of biological chemistry *283*, 5950-5959.

Brocardo, M. G., Borowiec, J. A., and Henderson, B. R. (2011). Adenomatous polyposis coli protein regulates the cellular response to DNA replication stress. The international journal of biochemistry & cell biology *43*, 1354-1364.

Browne, S. J., MacFarlane, M., Cohen, G. M., and Paraskeva, C. (1998). The adenomatous polyposis coli protein and retinoblastoma protein are cleaved early in apoptosis and are potential substrates for caspases. Cell Death Differ *5*, 206-213.

Butcher, R. A., and Schreiber, S. L. (2005). Using genome-wide transcriptional profiling to elucidate small-molecule mechanism. Current opinion in chemical biology *9*, 25-30.

Caldwell, C. M., Green, R. A., and Kaplan, K. B. (2007). APC mutations lead to cytokinetic failures in vitro and tetraploid genotypes in Min mice. The Journal of cell biology *178*, 1109-1120.

Caligiuri, M., Molz, L., Liu, Q., Kaplan, F., Xu, J. P., Majeti, J. Z., Ramos-Kelsey, R., Murthi, K., Lievens, S., Tavernier, J., and Kley, N. (2006). MASPIT: three-hybrid trap for quantitative proteome fingerprinting of small molecule-protein interactions in mammalian cells. Chemistry & biology *13*, 711-722.

Campbell, P. M., and Szyf, M. (2003). Human DNA methyltransferase gene DNMT1 is regulated by the APC pathway. Carcinogenesis *24*, 17-24.

Camps, J., Nguyen, Q. T., Padilla-Nash, H. M., Knutsen, T., McNeil, N. E., Wangsa, D., Hummon, A. B., Grade, M., Ried, T., and Difilippantonio, M. J. (2009). Integrative genomics reveals mechanisms of copy number alterations responsible for transcriptional deregulation in colorectal cancer. Genes, chromosomes & cancer *48*, 1002-1017.

Carmena, M., Wheelock, M., Funabiki, H., and Earnshaw, W. C. (2012). The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. Nature reviews Molecular cell biology *13*, 789-803.

Carothers, A. M., Melstrom, K. A., Jr., Mueller, J. D., Weyant, M. J., and Bertagnolli, M. M. (2001). Progressive changes in adherens junction structure during intestinal adenoma formation in Apc mutant mice. The Journal of biological chemistry *276*, 39094-39102.

Carson, J. D., Van Aller, G., Lehr, R., Sinnamon, R. H., Kirkpatrick, R. B., Auger, K. R., Dhanak, D., Copeland, R. A., Gontarek, R. R., Tummino, P. J., and Luo, L. (2008). Effects of oncogenic p110alpha subunit mutations on the lipid kinase activity of phosphoinositide 3-kinase. The Biochemical journal *409*, 519-524.

Chandra, S. H., Wacker, I., Appelt, U. K., Behrens, J., and Schneikert, J. (2012). A common role for various human truncated adenomatous polyposis coli isoforms in the control of beta-catenin activity and cell proliferation. PloS one *7*, e34479.

Chautard, E., Thierry-Mieg, N., and Ricard-Blum, S. (2009). Interaction networks: from protein functions to drug discovery. A review. Pathol Biol (Paris) *57*, 324-333.

Chen, B., Dodge, M. E., Tang, W., Lu, J., Ma, Z., Fan, C. W., Wei, S., Hao, W., Kilgore, J., Williams, N. S., *et al.* (2009a). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nature chemical biology *5*, 100-107.

Chen, C., Seth, A. K., and Aplin, A. E. (2006). Genetic and expression aberrations of E3 ubiquitin ligases in human breast cancer. Mol Cancer Res *4*, 695-707.

Chen, J., Bardes, E. E., Aronow, B. J., and Jegga, A. G. (2009b). ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. Nucleic acids research *37*, W305-311.

Chen, J., Xu, H., Aronow, B. J., and Jegga, A. G. (2007). Improved human disease candidate gene prioritization using mouse phenotype. BMC bioinformatics *8*, 392.

Chen, T., Turner, J., McCarthy, S., Scaltriti, M., Bettuzzi, S., and Yeatman, T. J. (2004). Clusterin-mediated apoptosis is regulated by adenomatous polyposis coli and is p21 dependent but p53 independent. Cancer Res *64*, 7412-7419.

Chen, T., Yang, I., Irby, R., Shain, K. H., Wang, H. G., Quackenbush, J., Coppola, D., Cheng, J. Q., and Yeatman, T. J. (2003). Regulation of caspase expression and apoptosis by adenomatous polyposis coli. Cancer Res *63*, 4368-4374.

Chen, Y. R., Meyer, C. F., and Tan, T. H. (1996a). Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in gamma radiation-induced apoptosis. J Biol Chem *271*, 631-634.

Chen, Y. R., Wang, X., Templeton, D., Davis, R. J., and Tan, T. H. (1996b). The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. J Biol Chem *271*, 31929-31936.

Cheon, D. J., and Orsulic, S. (2011). Mouse models of cancer. Annual review of pathology *6*, 95-119.

Cheung, A. F., Carter, A. M., Kostova, K. K., Woodruff, J. F., Crowley, D., Bronson, R. T., Haigis, K. M., and Jacks, T. (2010). Complete deletion of Apc results in severe polyposis in mice. Oncogene *29*, 1857-1864.

Chin, L., and Gray, J. W. (2008). Translating insights from the cancer genome into clinical practice. Nature *452*, 553-563.

Choi, Y., Kawazoe, Y., Murakami, K., Misawa, H., and Uesugi, M. (2003). Identification of bioactive molecules by adipogenesis profiling of organic compounds. The Journal of biological chemistry *278*, 7320-7324.

Christie, M., Jorissen, R. N., Mouradov, D., Sakthianandeswaren, A., Li, S., Day, F., Tsui, C., Lipton, L., Desai, J., Jones, I. T., *et al.* (2013). Different APC genotypes in proximal and distal sporadic colorectal cancers suggest distinct WNT/beta-catenin signalling thresholds for tumourigenesis. Oncogene *32*, 4675-4682.

Clement, G., Bosman, F. T., Fontolliet, C., and Benhattar, J. (2004). Monoallelic methylation of the APC promoter is altered in normal gastric mucosa associated with neoplastic lesions. Cancer Res *64*, 6867-6873.

Cong, F., Cheung, A. K., and Huang, S. M. (2012). Chemical genetics-based target identification in drug discovery. Annual review of pharmacology and toxicology *52*, 57-78.

Dai, W., Wang, Q., Liu, T., Swamy, M., Fang, Y., Xie, S., Mahmood, R., Yang, Y. M., Xu, M., and Rao, C. V. (2004). Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency. Cancer Res *64*, 440-445.

Dang, C. V., Le, A., and Gao, P. (2009). MYC-induced cancer cell energy metabolism and therapeutic opportunities. Clinical cancer research : an official journal of the American Association for Cancer Research *15*, 6479-6483.

Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. Cell *103*, 239-252.

De Rosa, M., Morelli, G., Cesaro, E., Duraturo, F., Turano, M., Rossi, G. B., Delrio, P., and Izzo, P. (2007). Alternative splicing and nonsense-mediated mRNA decay in the regulation of a new adenomatous polyposis coli transcript. Gene *395*, 8-14.

Deka, J., Kuhlmann, J., and Muller, O. (1998). A domain within the tumor suppressor protein APC shows very similar biochemical properties as the microtubule-associated protein tau. European journal of biochemistry / FEBS *253*, 591-597.

Deng, G., Song, G. A., Pong, E., Sleisenger, M., and Kim, Y. S. (2004). Promoter methylation inhibits APC gene expression by causing changes in chromatin conformation and interfering with the binding of transcription factor CCAAT-binding factor. Cancer Res *64*, 2692-2698.

Dihlmann, S., Gebert, J., Siermann, A., Herfarth, C., and von Knebel Doeberitz, M. (1999). Dominant negative effect of the APC1309 mutation: a possible explanation for genotype-phenotype correlations in familial adenomatous polyposis. Cancer Res *59*, 1857-1860.

Dikovskaya, D., Newton, I. P., and Nathke, I. S. (2004). The adenomatous polyposis coli protein is required for the formation of robust spindles formed in CSF Xenopus extracts. Molecular biology of the cell *15*, 2978-2991.

Dikovskaya, D., Schiffmann, D., Newton, I. P., Oakley, A., Kroboth, K., Sansom, O., Jamieson, T. J., Meniel, V., Clarke, A., and Nathke, I. S. (2007). Loss of APC induces polyploidy as a result of a combination of defects in mitosis and apoptosis. The Journal of cell biology *176*, 183-195.

Draviam, V. M., Shapiro, I., Aldridge, B., and Sorger, P. K. (2006). Misorientation and reduced stretching of aligned sister kinetochores promote chromosome missegregation in EB1- or APC-depleted cells. The EMBO journal *25*, 2814-2827.

Eilers, M., and Eisenman, R. N. (2008). Myc's broad reach. Genes & development *22*, 2755-2766.

Eppert, K., Scherer, S. W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L. C., Bapat, B., Gallinger, S., Andrulis, I. L., *et al.* (1996). MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. Cell *86*, 543-552.

Eskiocak, U., Kim, S. B., Ly, P., Roig, A. I., Biglione, S., Komurov, K., Cornelius, C., Wright, W. E., White, M. A., and Shay, J. W. (2011). Functional parsing of driver mutations in the colorectal cancer genome reveals numerous suppressors of anchorage-independent growth. Cancer Res *71*, 4359-4365.

Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M. A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S. J., *et al.* (2000). Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res *60*, 4366-4371.

Farrell, C., Crimm, H., Meeh, P., Croshaw, R., Barbar, T., Vandersteenhoven, J. J., Butler, W., and Buckhaults, P. (2008). Somatic mutations to CSMD1 in colorectal adenocarcinomas. Cancer biology & therapy *7*, 609-613.

Faux, M. C., Ross, J. L., Meeker, C., Johns, T., Ji, H., Simpson, R. J., Layton, M. J., and Burgess, A. W. (2004). Restoration of full-length adenomatous polyposis coli (APC) protein in a colon cancer cell line enhances cell adhesion. J Cell Sci *117*, 427-439.

Fearon, E. R. (2011). Molecular genetics of colorectal cancer. Annual review of pathology *6*, 479-507.

Fearon, E. R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell *61*, 759-767.

Ficari, F., Cama, A., Valanzano, R., Curia, M. C., Palmirotta, R., Aceto, G., Esposito, D. L., Crognale, S., Lombardi, A., Messerini, L., *et al.* (2000). APC gene mutations and colorectal adenomatosis in familial adenomatous polyposis. British journal of cancer *82*, 348-353.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

Fleischer, T. C., Murphy, B. R., Flick, J. S., Terry-Lorenzo, R. T., Gao, Z. H., Davis, T., McKinnon, R., Ostanin, K., Willardsen, J. A., and Boniface, J. J. (2010). Chemical proteomics identifies Nampt as the target of CB30865, an orphan cytotoxic compound. Chemistry & biology *17*, 659-664.

Fodde, R., Kuipers, J., Rosenberg, C., Smits, R., Kielman, M., Gaspar, C., van Es, J. H., Breukel, C., Wiegant, J., Giles, R. H., and Clevers, H. (2001a). Mutations in the APC tumour suppressor gene cause chromosomal instability. Nature cell biology *3*, 433-438.

Fodde, R., Smits, R., and Clevers, H. (2001b). APC, signal transduction and genetic instability in colorectal cancer. Nature reviews Cancer *1*, 55-67.

Fodde, R., Smits, R., Hofland, N., Kielman, M., and Meera Khan, P. (1999). Mechanisms of APC-driven tumorigenesis: lessons from mouse models. Cytogenetics and cell genetics *86*, 105-111.

Forrester, K., Almoguera, C., Han, K., Grizzle, W. E., and Perucho, M. (1987). Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. Nature *327*, 298-303.

Freed, E., Lacey, K. R., Huie, P., Lyapina, S. A., Deshaies, R. J., Stearns, T., and Jackson, P. K. (1999). Components of an SCF ubiquitin ligase localize to the centrosome and regulate the centrosome duplication cycle. Genes & development *13*, 2242-2257.

Frohling, S., Scholl, C., Levine, R. L., Loriaux, M., Boggon, T. J., Bernard, O. A., Berger, R., Dohner, H., Dohner, K., Ebert, B. L., *et al.* (2007). Identification of driver and passenger mutations of FLT3 by high-throughput DNA sequence analysis and functional assessment of candidate alleles. Cancer Cell *12*, 501-513.

Fujiwara, T., Bandi, M., Nitta, M., Ivanova, E. V., Bronson, R. T., and Pellman, D. (2005). Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. Nature *437*, 1043-1047.

Gerhard, D. S., Wagner, L., Feingold, E. A., Shenmen, C. M., Grouse, L. H., Schuler, G., Klein, S. L., Old, S., Rasooly, R., Good, P., *et al.* (2004). The status, quality, and expansion of the NIH full-length cDNA project: the Mammalian Gene Collection (MGC). Genome research *14*, 2121-2127.

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B., *et al.* (2002). Functional profiling of the Saccharomyces cerevisiae genome. Nature *418*, 387-391.

Giaever, G., Flaherty, P., Kumm, J., Proctor, M., Nislow, C., Jaramillo, D. F., Chu, A. M., Jordan, M. I., Arkin, A. P., and Davis, R. W. (2004). Chemogenomic profiling: identifying the functional interactions of small molecules in yeast. Proceedings of the National Academy of Sciences of the United States of America *101*, 793-798.

Giaever, G., Shoemaker, D. D., Jones, T. W., Liang, H., Winzeler, E. A., Astromoff, A., and Davis, R. W. (1999). Genomic profiling of drug sensitivities via induced haploinsufficiency. Nature genetics *21*, 278-283.

Givalos, N., Gakiopoulou, H., Skliri, M., Bousboukea, K., Konstantinidou, A. E., Korkolopoulou, P., Lelouda, M., Kouraklis, G., Patsouris, E., and Karatzas, G. (2007). Replication protein A is an independent prognostic indicator with potential therapeutic implications in colon cancer. Mod Pathol *20*, 159-166.

Goel, A., and Boland, C. R. (2012). Epigenetics of colorectal cancer. Gastroenterology *143*, 1442-1460 e1441.

Goss, K. H., and Groden, J. (2000). Biology of the adenomatous polyposis coli tumor suppressor. J Clin Oncol *18*, 1967-1979.

Green, R. A., and Kaplan, K. B. (2003). Chromosome instability in colorectal tumor cells is associated with defects in microtubule plus-end attachments caused by a dominant mutation in APC. The Journal of cell biology *163*, 949-961.

Green, R. A., Wollman, R., and Kaplan, K. B. (2005). APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. Molecular biology of the cell *16*, 4609-4622.

Greenman, C., Stephens, P., Smith, R., Dalgliesh, G. L., Hunter, C., Bignell, G., Davies, H., Teague, J., Butler, A., Stevens, C., *et al.* (2007). Patterns of somatic mutation in human cancer genomes. Nature *446*, 153-158.

Grote, H. J., Schmiemann, V., Kiel, S., Bocking, A., Kappes, R., Gabbert, H. E., and Sarbia, M. (2004). Aberrant methylation of the adenomatous polyposis coli promoter 1A in bronchial aspirates from patients with suspected lung cancer. International journal of cancer Journal international du cancer *110*, 751-755.

Gunther, E. C., Stone, D. J., Gerwien, R. W., Bento, P., and Heyes, M. P. (2003). Prediction of clinical drug efficacy by classification of drug-induced genomic expression profiles in vitro. Proceedings of the National Academy of Sciences of the United States of America *100*, 9608-9613.

Gunther, E. C., Stone, D. J., Rothberg, J. M., and Gerwien, R. W. (2005). A quantitative genomic expression analysis platform for multiplexed in vitro prediction of drug action. The pharmacogenomics journal *5*, 126-134.

Habermann, J. K., Paulsen, U., Roblick, U. J., Upender, M. B., McShane, L. M., Korn, E. L., Wangsa, D., Kruger, S., Duchrow, M., Bruch, H. P., *et al.* (2007). Stage-specific alterations of the genome, transcriptome, and proteome during colorectal carcinogenesis. Genes, chromosomes & cancer *46*, 10-26.

Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science *271*, 350-353.

Hamada, F., and Bienz, M. (2004). The APC tumor suppressor binds to C-terminal binding protein to divert nuclear beta-catenin from TCF. Developmental cell *7*, 677-685.

Haramis, A. P., Hurlstone, A., van der Velden, Y., Begthel, H., van den Born, M., Offerhaus, G. J., and Clevers, H. C. (2006). Adenomatous polyposis coli-deficient zebrafish are susceptible to digestive tract neoplasia. EMBO reports *7*, 444-449.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. Science *281*, 1509-1512.

Heinen, C. D., Goss, K. H., Cornelius, J. R., Babcock, G. F., Knudsen, E. S., Kowalik, T., and Groden, J. (2002). The APC tumor suppressor controls entry into S-phase through its ability to regulate the cyclin D/RB pathway. Gastroenterology *123*, 751-763.

Heitman, J., Movva, N. R., Hiestand, P. C., and Hall, M. N. (1991). FK 506-binding protein proline rotamase is a target for the immunosuppressive agent FK 506 in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America *88*, 1948-1952.

Henderson, B. R. (2000). Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. Nature cell biology *2*, 653-660.

Henderson, B. R., and Fagotto, F. (2002). The ins and outs of APC and beta-catenin nuclear transport. EMBO reports *3*, 834-839.

Herrera, L., Kakati, S., Gibas, L., Pietrzak, E., and Sandberg, A. A. (1986). Gardner syndrome in a man with an interstitial deletion of 5q. American journal of medical genetics *25*, 473-476.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes & development *7*, 2135-2148.

Hillenmeyer, M. E., Fung, E., Wildenhain, J., Pierce, S. E., Hoon, S., Lee, W., Proctor, M., St Onge, R. P., Tyers, M., Koller, D., *et al.* (2008). The chemical genomic portrait of yeast: uncovering a phenotype for all genes. Science *320*, 362-365.

Hinoi, T., Akyol, A., Theisen, B. K., Ferguson, D. O., Greenson, J. K., Williams, B. O., Cho, K. R., and Fearon, E. R. (2007). Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. Cancer Res *67*, 9721-9730.

Homma, M. K., and Homma, Y. (2005). Regulatory role of CK2 during the progression of cell cycle. Molecular and cellular biochemistry *274*, 47-52.

Homma, M. K., Li, D., Krebs, E. G., Yuasa, Y., and Homma, Y. (2002). Association and regulation of casein kinase 2 activity by adenomatous polyposis coli protein. Proceedings of the National Academy of Sciences of the United States of America *99*, 5959-5964.

Huang, J., Zhu, H., Haggarty, S. J., Spring, D. R., Hwang, H., Jin, F., Snyder, M., and Schreiber, S. L. (2004). Finding new components of the target of rapamycin (TOR) signaling network through chemical genetics and proteome chips. Proceedings of the National Academy of Sciences of the United States of America *101*, 16594-16599.

Huang, P., Senga, T., and Hamaguchi, M. (2007). A novel role of phospho-beta-catenin in microtubule regrowth at centrosome. Oncogene *26*, 4357-4371.

Huang, S. M., Mishina, Y. M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G. A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S., *et al.* (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature *461*, 614-620.

Hughes, T. R., Marton, M. J., Jones, A. R., Roberts, C. J., Stoughton, R., Armour, C. D., Bennett, H. A., Coffey, E., Dai, H., He, Y. D., *et al.* (2000). Functional discovery via a compendium of expression profiles. Cell *102*, 109-126.

Hung, R. J., Hall, J., Brennan, P., and Boffetta, P. (2005). Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. Am J Epidemiol *162*, 925-942.

Hur, W., Sun, Z., Jiang, T., Mason, D. E., Peters, E. C., Zhang, D. D., Luesch, H., Schultz, P. G., and Gray, N. S. (2010). A small-molecule inducer of the antioxidant response element. Chemistry & biology *17*, 537-547.

Jaiswal, A. S., Balusu, R., Armas, M. L., Kundu, C. N., and Narayan, S. (2006). Mechanism of adenomatous polyposis coli (APC)-mediated blockage of long-patch base excision repair. Biochemistry *45*, 15903-15914.

Jaiswal, A. S., and Narayan, S. (2008). A novel function of adenomatous polyposis coli (APC) in regulating DNA repair. Cancer letters *271*, 272-280.

Jaiswal, A. S., and Narayan, S. (2011). Assembly of the base excision repair complex on abasic DNA and role of adenomatous polyposis coli on its functional activity. Biochemistry *50*, 1901-1909.

Jen, J., Powell, S. M., Papadopoulos, N., Smith, K. J., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. (1994). Molecular determinants of dysplasia in colorectal lesions. Cancer Res *54*, 5523-5526.

Jiang, B., Xu, D., Allocco, J., Parish, C., Davison, J., Veillette, K., Sillaots, S., Hu, W., Rodriguez-Suarez, R., Trosok, S., *et al.* (2008). PAP inhibitor with in vivo efficacy identified by Candida albicans genetic profiling of natural products. Chemistry & biology *15*, 363-374.

Jimbo, T., Kawasaki, Y., Koyama, R., Sato, R., Takada, S., Haraguchi, K., and Akiyama, T. (2002). Identification of a link between the tumour suppressor APC and the kinesin superfamily. Nature cell biology *4*, 323-327.

Jiricny, J., and Marra, G. (2003). DNA repair defects in colon cancer. Curr Opin Genet Dev *13*, 61-69.

Juwana, J. P., Henderikx, P., Mischo, A., Wadle, A., Fadle, N., Gerlach, K., Arends, J. W., Hoogenboom, H., Pfreundschuh, M., and Renner, C. (1999). EB/RP gene family encodes tubulin binding proteins. International journal of cancer Journal international du cancer *81*, 275-284.

Kanoh, N., Honda, K., Simizu, S., Muroi, M., and Osada, H. (2005). Photo-cross-linked small-molecule affinity matrix for facilitating forward and reverse chemical genetics. Angewandte Chemie *44*, 3559-3562.

Kaplan, D. D., Meigs, T. E., Kelly, P., and Casey, P. J. (2004). Identification of a role for beta-catenin in the establishment of a bipolar mitotic spindle. The Journal of biological chemistry *279*, 10829-10832.

Kaplan, K. B., Burds, A. A., Swedlow, J. R., Bekir, S. S., Sorger, P. K., and Nathke, I. S. (2001). A role for the Adenomatous Polyposis Coli protein in chromosome segregation. Nature cell biology *3*, 429-432.

Katayama, H., and Oda, Y. (2007). Chemical proteomics for drug discovery based on compound-immobilized affinity chromatography. J Chromatogr B Analyt Technol Biomed Life Sci *855*, 21-27.

Kawasaki, Y., Sagara, M., Shibata, Y., Shirouzu, M., Yokoyama, S., and Akiyama, T. (2007). Identification and characterization of Asef2, a guanine-nucleotide exchange factor specific for Rac1 and Cdc42. Oncogene *26*, 7620-7267.

Kawasaki, Y., Sato, R., and Akiyama, T. (2003). Mutated APC and Asef are involved in the migration of colorectal tumour cells. Nat Cell Biol *5*, 211-215.

Kawasaki, Y., Senda, T., Ishidate, T., Koyama, R., Morishita, T., Iwayama, Y., Higuchi, O., and Akiyama, T. (2000). Asef, a link between the tumor suppressor APC and G-protein signaling. Science *289*, 1194-1197.

Kemler, R. (1993). From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. Trends in genetics : TIG *9*, 317-321.

Khanna, K. K., and Jackson, S. P. (2001). DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet *27*, 247-254.

Kim, H., Deng, L., Xiong, X., Hunter, W. D., Long, M. C., and Pirrung, M. C. (2007). Glyceraldehyde 3-phosphate dehydrogenase is a cellular target of the insulin mimic demethylasterriquinone B1. Journal of medicinal chemistry *50*, 3423-3426.

Kim, Hyun S., Mendiratta, S., Kim, J., Pecot, Chad V., Larsen, Jill E., Zubovych, I., Seo, Bo Y., Kim, J., Eskiocak, B., Chung, H., *et al.* (2013). Systematic Identification of Molecular Subtype-Selective Vulnerabilities in Non-Small-Cell Lung Cancer. Cell *155*, 552-566.

Kim, K., Pang, K. M., Evans, M., and Hay, E. D. (2000). Overexpression of beta-catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. Molecular biology of the cell *11*, 3509-3523.

Kimelman, D., and Xu, W. (2006). beta-catenin destruction complex: insights and questions from a structural perspective. Oncogene *25*, 7482-7491.

Kinzler, K. W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. Cell *87*, 159-170.

Knijn, N., Tol, J., and Punt, C. J. (2010). Current issues in the targeted therapy of advanced colorectal cancer. Discovery medicine *9*, 328-336.

Komurov, K. (2012). Modeling community-wide molecular networks of multicellular systems. Bioinformatics *28*, 694-700.

Kouzmenko, A. P., Takeyama, K., Kawasaki, Y., Akiyama, T., and Kato, S. (2008). Truncation mutations abolish chromatin-associated activities of adenomatous polyposis coli. Oncogene *27*, 4888-4899.

Kroboth, K., Newton, I. P., Kita, K., Dikovskaya, D., Zumbrunn, J., Waterman-Storer, C. M., and Nathke, I. S. (2007). Lack of adenomatous polyposis coli protein correlates with a decrease in cell migration and overall changes in microtubule stability. Molecular biology of the cell *18*, 910-918.

Kwong, L. N., and Dove, W. F. (2009). APC and its modifiers in colon cancer. Advances in experimental medicine and biology *656*, 85-106.

Lamb, J., Crawford, E. D., Peck, D., Modell, J. W., Blat, I. C., Wrobel, M. J., Lerner, J., Brunet, J. P., Subramanian, A., Ross, K. N., *et al.* (2006). The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. Science *313*, 1929-1935.

Lamlum, H., Ilyas, M., Rowan, A., Clark, S., Johnson, V., Bell, J., Frayling, I., Efstathiou, J., Pack, K., Payne, S., *et al.* (1999). The type of somatic mutation at APC in familial adenomatous polyposis is determined by the site of the germline mutation: a new facet to Knudson's 'two-hit' hypothesis. Nature medicine *5*, 1071-1075.

Lao, V. V., and Grady, W. M. (2011). Epigenetics and colorectal cancer. Nature reviews Gastroenterology & hepatology *8*, 686-700.

Lau, T., Chan, E., Callow, M., Waaler, J., Boggs, J., Blake, R. A., Magnuson, S., Sambrone, A., Schutten, M., Firestein, R., *et al.* (2013). A novel tankyrase small-molecule inhibitor suppresses APC mutation-driven colorectal tumor growth. Cancer Res *73*, 3132-3144.

Laurent-Puig, P., and Zucman-Rossi, J. (2006). Genetics of hepatocellular tumors. Oncogene *25*, 3778-3786.

Leary, R. J., Lin, J. C., Cummins, J., Boca, S., Wood, L. D., Parsons, D. W., Jones, S., Sjoblom, T., Park, B. H., Parsons, R., *et al.* (2008). Integrated analysis of homozygous deletions, focal amplifications, and sequence alterations in breast and colorectal cancers. Proceedings of the National Academy of Sciences of the United States of America *105*, 16224-16229.

Lee, W., Jiang, Z., Liu, J., Haverty, P. M., Guan, Y., Stinson, J., Yue, P., Zhang, Y., Pant, K. P., Bhatt, D., *et al.* (2010). The mutation spectrum revealed by paired genome sequences from a lung cancer patient. Nature *465*, 473-477.

Li, W., Zhu, T., and Guan, K. L. (2004). Transformation potential of Ras isoforms correlates with activation of phosphatidylinositol 3-kinase but not ERK. The Journal of biological chemistry *279*, 37398-37406.

Lin, A. (2003). Activation of the JNK signaling pathway: breaking the brake on apoptosis. BioEssays : news and reviews in molecular, cellular and developmental biology *25*, 17-24.

Liu, E. T. (2008). Functional genomics of cancer. Curr Opin Genet Dev *18*, 251-256.

Liu, J., and Lin, A. (2005). Role of JNK activation in apoptosis: a double-edged sword. Cell research *15*, 36-42.

Ljuslinder, I., Golovleva, I., Palmqvist, R., Oberg, A., Stenling, R., Jonsson, Y., Hedman, H., Henriksson, R., and Malmer, B. (2007). LRIG1 expression in colorectal cancer. Acta Oncol *46*, 1118-1122.

Lohse, M. J., Klotz, K. N., Schwabe, U., Cristalli, G., Vittori, S., and Grifantini, M. (1988). 2-Chloro-N6-cyclopentyladenosine: a highly selective agonist at A1 adenosine receptors. Naunyn Schmiedebergs Arch Pharmacol *337*, 687-689.

Lomenick, B., Hao, R., Jonai, N., Chin, R. M., Aghajan, M., Warburton, S., Wang, J., Wu, R. P., Gomez, F., Loo, J. A., *et al.* (2009). Target identification using drug affinity responsive target stability (DARTS). Proceedings of the National Academy of Sciences of the United States of America *106*, 21984-21989.

Longin, A., Souchier, C., Ffrench, M., and Bryon, P. A. (1993). Comparison of anti-fading agents used in fluorescence microscopy: image analysis and laser confocal microscopy study. J Histochem Cytochem *41*, 1833-1840.

Louie, R. K., Bahmanyar, S., Siemers, K. A., Votin, V., Chang, P., Stearns, T., Nelson, W. J., and Barth, A. I. (2004). Adenomatous polyposis coli and EB1 localize in close proximity of the mother centriole and EB1 is a functional component of centrosomes. J Cell Sci *117*, 1117-1128.

Lu, B., Roegiers, F., Jan, L. Y., and Jan, Y. N. (2001). Adherens junctions inhibit asymmetric division in the Drosophila epithelium. Nature *409*, 522-525.

Lum, P. Y., Armour, C. D., Stepaniants, S. B., Cavet, G., Wolf, M. K., Butler, J. S., Hinshaw, J. C., Garnier, P., Prestwich, G. D., Leonardson, A., *et al.* (2004). Discovering modes of action for therapeutic compounds using a genome-wide screen of yeast heterozygotes. Cell *116*, 121-137.

Lutz, J. F., and Zarafshani, Z. (2008). Efficient construction of therapeutics, bioconjugates, biomaterials and bioactive surfaces using azide-alkyne "click" chemistry. Advanced drug delivery reviews *60*, 958-970.

Ly, P., Eskiocak, U., Kim, S. B., Roig, A. I., Hight, S. K., Lulla, D. R., Zou, Y. S., Batten, K., Wright, W. E., and Shay, J. W. (2011). Characterization of aneuploid populations with trisomy 7 and 20 derived from diploid human colonic epithelial cells. Neoplasia *13*, 348-357.

Ly, P., Kim, S. B., Kaisani, A. A., Marian, G., Wright, W. E., and Shay, J. W. (2013). Aneuploid human colonic epithelial cells are sensitive to AICAR-induced growth inhibition through EGFR degradation. Oncogene *32*, 3139-3146.

Mahmoud, N. N., Boolbol, S. K., Bilinski, R. T., Martucci, C., Chadburn, A., and Bertagnolli, M. M. (1997). Apc gene mutation is associated with a dominant-negative effect upon intestinal cell migration. Cancer Res *57*, 5045-5050.

Malumbres, M., and Barbacid, M. (2003). RAS oncogenes: the first 30 years. Nature reviews Cancer *3*, 459-465.

Masica, D. L., and Karchin, R. (2011). Correlation of somatic mutation and expression identifies genes important in human glioblastoma progression and survival. Cancer Res *71*, 4550-4561.

Massague, J. (2000). How cells read TGF-beta signals. Nature reviews Molecular cell biology *1*, 169-178.

McCart, A. E., Vickaryous, N. K., and Silver, A. (2008). Apc mice: models, modifiers and mutants. Pathology, research and practice *204*, 479-490.

McCartney, B. M., McEwen, D. G., Grevengoed, E., Maddox, P., Bejsovec, A., and Peifer, M. (2001). Drosophila APC2 and Armadillo participate in tethering mitotic spindles to cortical actin. Nature cell biology *3*, 933-938.

McPherson, M., Yang, Y., Hammond, P. W., and Kreider, B. L. (2002). Drug receptor identification from multiple tissues using cellular-derived mRNA display libraries. Chemistry & biology *9*, 691-698.

Miller, R. K., and Rose, M. D. (1998). Kar9p is a novel cortical protein required for cytoplasmic microtubule orientation in yeast. The Journal of cell biology *140*, 377-390.

Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992). Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Human molecular genetics *1*, 229-233.

Mori, S., Chang, J. T., Andrechek, E. R., Matsumura, N., Baba, T., Yao, G., Kim, J. W., Gatza, M., Murphy, S., and Nevins, J. R. (2009). Anchorage-independent cell growth signature identifies tumors with metastatic potential. Oncogene *28*, 2796-2805.

Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1996). Apoptosis and APC in colorectal tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America *93*, 7950-7954.

Morrison, E. E., Wardleworth, B. N., Askham, J. M., Markham, A. F., and Meredith, D. M. (1998). EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle. Oncogene *17*, 3471-3477.

Moseley, J. B., Bartolini, F., Okada, K., Wen, Y., Gundersen, G. G., and Goode, B. L. (2007). Regulated binding of adenomatous polyposis coli protein to actin. The Journal of biological chemistry *282*, 12661-12668.

Moser, A. R., Pitot, H. C., and Dove, W. F. (1990). A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science *247*, 322-324.

Munemitsu, S., Souza, B., Muller, O., Albert, I., Rubinfeld, B., and Polakis, P. (1994). The APC gene product associates with microtubules in vivo and promotes their assembly in vitro. Cancer Res *54*, 3676-3681.

Nagase, H., Miyoshi, Y., Horii, A., Aoki, T., Ogawa, M., Utsunomiya, J., Baba, S., Sasazuki, T., and Nakamura, Y. (1992). Correlation between the location of germ-line mutations in the APC gene and the number of colorectal polyps in familial adenomatous polyposis patients. Cancer Res *52*, 4055-4057.

Nakamura, M., Zhou, X. Z., and Lu, K. P. (2001). Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization. Current biology : CB *11*, 1062-1067.

Narayan, S., Jaiswal, A. S., and Balusu, R. (2005). Tumor suppressor APC blocks DNA polymerase beta-dependent strand displacement synthesis during long patch but not short patch base excision repair and increases sensitivity to methylmethane sulfonate. The Journal of biological chemistry *280*, 6942-6949.

Narayan, S., and Roy, D. (2003). Role of APC and DNA mismatch repair genes in the development of colorectal cancers. Molecular cancer *2*, 41.

Nathke, I. (2006). Cytoskeleton out of the cupboard: colon cancer and cytoskeletal changes induced by loss of APC. Nature reviews Cancer *6*, 967-974.

Nathke, I. S. (2004). The adenomatous polyposis coli protein: the Achilles heel of the gut epithelium. Annual review of cell and developmental biology *20*, 337-366.

Nathke, I. S., Adams, C. L., Polakis, P., Sellin, J. H., and Nelson, W. J. (1996). The adenomatous polyposis coli tumor suppressor protein localizes to plasma membrane sites involved in active cell migration. The Journal of cell biology *134*, 165-179.

Neufeld, K. L., Zhang, F., Cullen, B. R., and White, R. L. (2000). APC-mediated downregulation of beta-catenin activity involves nuclear sequestration and nuclear export. EMBO reports *1*, 519-523.

Ng, S. B., Buckingham, K. J., Lee, C., Bigham, A. W., Tabor, H. K., Dent, K. M., Huff, C. D., Shannon, P. T., Jabs, E. W., Nickerson, D. A., *et al.* (2010). Exome sequencing identifies the cause of a mendelian disorder. Nature genetics *42*, 30-35.

Nikolaev, S. I., Sotiriou, S. K., Pateras, I. S., Santoni, F., Sougioultzis, S., Edgren, H., Almusa, H., Robyr, D., Guipponi, M., Saarela, J., *et al.* (2012). A single-nucleotide substitution mutator phenotype revealed by exome sequencing of human colon adenomas. Cancer Res *72*, 6279-6289.

Oda, Y., Owa, T., Sato, T., Boucher, B., Daniels, S., Yamanaka, H., Shinohara, Y., Yokoi, A., Kuromitsu, J., and Nagasu, T. (2003). Quantitative chemical proteomics for identifying candidate drug targets. Analytical chemistry *75*, 2159-2165.

Okamoto, M., Sasaki, M., Sugio, K., Sato, C., Iwama, T., Ikeuchi, T., Tonomura, A., Sasazuki, T., and Miyaki, M. (1988). Loss of constitutional heterozygosity in colon carcinoma from patients with familial polyposis coli. Nature *331*, 273-277.

Olmeda, D., Castel, S., Vilaro, S., and Cano, A. (2003). Beta-catenin regulation during the cell cycle: implications in G2/M and apoptosis. Molecular biology of the cell *14*, 2844-2860.

Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Molecular & cellular proteomics : MCP *1*, 376-386.

Ortega, J., Vigil, C. E., and Chodkiewicz, C. (2010). Current progress in targeted therapy for colorectal cancer. Cancer control : journal of the Moffitt Cancer Center *17*, 7-15.

Oshima, H., Oshima, M., Kobayashi, M., Tsutsumi, M., and Taketo, M. M. (1997). Morphological and molecular processes of polyp formation in Apc(delta716) knockout mice. Cancer Res *57*, 1644-1649.

Oshima, M., Oshima, H., Kobayashi, M., Tsutsumi, M., and Taketo, M. M. (1995). Evidence against dominant negative mechanisms of intestinal polyp formation by Apc gene mutations. Cancer Res *55*, 2719-2722.

Parmigiani, G., Boca, S., Lin, J., Kinzler, K. W., Velculescu, V., and Vogelstein, B. (2009). Design and analysis issues in genome-wide somatic mutation studies of cancer. Genomics *93*, 17-21.

Peck, D., Crawford, E. D., Ross, K. N., Stegmaier, K., Golub, T. R., and Lamb, J. (2006). A method for high-throughput gene expression signature analysis. Genome biology *7*, R61.

Pleasance, E. D., Cheetham, R. K., Stephens, P. J., McBride, D. J., Humphray, S. J., Greenman, C. D., Varela, I., Lin, M. L., Ordonez, G. R., Bignell, G. R., *et al.* (2010). A comprehensive catalogue of somatic mutations from a human cancer genome. Nature *463*, 191-196.

Polakis, P. (1997). The adenomatous polyposis coli (APC) tumor suppressor. Biochim Biophys Acta *1332*, F127-147.

Polakis, P. (2012). Wnt signaling in cancer. Cold Spring Harbor perspectives in biology *4*.

Powell, S. M., Zilz, N., Beazer-Barclay, Y., Bryan, T. M., Hamilton, S. R., Thibodeau, S. N., Vogelstein, B., and Kinzler, K. W. (1992). APC mutations occur early during colorectal tumorigenesis. Nature *359*, 235-237.

Pretlow, T. P., Brasitus, T. A., Fulton, N. C., Cheyer, C., and Kaplan, E. L. (1993). K-ras mutations in putative preneoplastic lesions in human colon. Journal of the National Cancer Institute *85*, 2004-2007.

Qian, J., Perchiniak, E. M., Sun, K., and Groden, J. (2010). The mitochondrial protein hTID-1 partners with the caspase-cleaved adenomatous polyposis cell tumor suppressor to facilitate apoptosis. Gastroenterology *138*, 1418-1428.

Qian, J., Steigerwald, K., Combs, K. A., Barton, M. C., and Groden, J. (2007). Caspase cleavage of the APC tumor suppressor and release of an amino-terminal domain is required for the transcription-independent function of APC in apoptosis. Oncogene *26*, 4872-4876.

Ren, Y., Lee, M. Y., Schliffke, S., Paavola, J., Amos, P. J., Ge, X., Ye, M., Zhu, S., Senyei, G., Lum, L., *et al.* (2011). Small molecule Wnt inhibitors enhance the efficiency of BMP-4-directed cardiac differentiation of human pluripotent stem cells. Journal of molecular and cellular cardiology *51*, 280-287.

Ried, T., Knutzen, R., Steinbeck, R., Blegen, H., Schrock, E., Heselmeyer, K., du Manoir, S., and Auer, G. (1996). Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. Genes, chromosomes & cancer *15*, 234-245.

Roberts, D. M., Pronobis, M. I., Poulton, J. S., Waldmann, J. D., Stephenson, E. M., Hanna, S., and Peifer, M. (2011). Deconstructing the sscatenin destruction complex: mechanistic roles for the tumor suppressor APC in regulating Wnt signaling. Mol Biol Cell *22*, 1845-1863.

Rodrigues, N. R., Rowan, A., Smith, M. E., Kerr, I. B., Bodmer, W. F., Gannon, J. V., and Lane, D. P. (1990). p53 mutations in colorectal cancer. Proc Natl Acad Sci U S A *87*, 7555-7559.

Rodriguez-Suarez, R., Xu, D., Veillette, K., Davison, J., Sillaots, S., Kauffman, S., Hu, W., Bowman, J., Martel, N., Trosok, S., *et al.* (2007). Mechanism-of-action determination of GMP synthase inhibitors and target validation in Candida albicans and Aspergillus fumigatus. Chemistry & biology *14*, 1163-1175.

Rogers, S. L., Rogers, G. C., Sharp, D. J., and Vale, R. D. (2002). Drosophila EB1 is important for proper assembly, dynamics, and positioning of the mitotic spindle. The Journal of cell biology *158*, 873-884.

Roig, A. I., Eskiocak, U., Hight, S. K., Kim, S. B., Delgado, O., Souza, R. F., Spechler, S. J., Wright, W. E., and Shay, J. W. Immortalized epithelial cells derived from human colon biopsies express stem cell markers and differentiate in vitro. Gastroenterology *138*, 1012-1021 e1011-1015.

Roig, A. I., Eskiocak, U., Hight, S. K., Kim, S. B., Delgado, O., Souza, R. F., Spechler, S. J., Wright, W. E., and Shay, J. W. (2010). Immortalized epithelial cells derived from human colon biopsies express stem cell markers and differentiate in vitro. Gastroenterology *138*, 1012-1021 e1011-1015.

Rosin-Arbesfeld, R., Cliffe, A., Brabletz, T., and Bienz, M. (2003). Nuclear export of the APC tumour suppressor controls beta-catenin function in transcription. The EMBO journal *22*, 1101-1113.

Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., *et al.* (2004). Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Molecular & cellular proteomics : MCP *3*, 1154-1169.

Rowan, A. J., Lamlum, H., Ilyas, M., Wheeler, J., Straub, J., Papadopoulou, A., Bicknell, D., Bodmer, W. F., and Tomlinson, I. P. (2000). APC mutations in sporadic colorectal tumors: A mutational "hotspot" and interdependence of the "two hits". Proceedings of the National Academy of Sciences of the United States of America *97*, 3352-3357.

Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., and Polakis, P. (1996). Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. Science *272*, 1023-1026.

Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S., and Polakis, P. (1993). Association of the APC gene product with beta-catenin. Science *262*, 1731-1734.

Ruggero, D. (2009). The role of Myc-induced protein synthesis in cancer. Cancer Res *69*, 8839-8843.

Sachse, C., and Echeverri, C. J. (2004). Oncology studies using siRNA libraries: the dawn of RNAi-based genomics. Oncogene *23*, 8384-8391.

Saito, M., Yaguchi, T., Yasuda, Y., Nakano, T., and Nishizaki, T. (2010). Adenosine suppresses CW2 human colonic cancer growth by inducing apoptosis via A(1) adenosine receptors. Cancer Lett *290*, 211-215.

Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S. M., Riggins, G. J., *et al.* (2004). High frequency of mutations of the PIK3CA gene in human cancers. Science *304*, 554.

Sanchez-Perez, I., Murguia, J. R., and Perona, R. (1998). Cisplatin induces a persistent activation of JNK that is related to cell death. Oncogene *16*, 533-540.

Sansom, O. J., Reed, K. R., Hayes, A. J., Ireland, H., Brinkmann, H., Newton, I. P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I. S., *et al.* (2004). Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes & development *18*, 1385-1390.

Sato, M., Vaughan, M. B., Girard, L., Peyton, M., Lee, W., Shames, D. S., Ramirez, R. D., Sunaga, N., Gazdar, A. F., Shay, J. W., and Minna, J. D. (2006). Multiple oncogenic changes (K-RAS(V12), p53 knockdown, mutant EGFRs, p16 bypass, telomerase) are not sufficient to confer a full malignant phenotype on human bronchial epithelial cells. Cancer Res *66*, 2116-2128.

Sato, S., Murata, A., Orihara, T., Shirakawa, T., Suenaga, K., Kigoshi, H., and Uesugi, M. (2011). Marine natural product aurilide activates the OPA1-mediated apoptosis by binding to prohibitin. Chemistry & biology *18*, 131-139.

Schlosshauer, P. W., Brown, S. A., Eisinger, K., Yan, Q., Guglielminetti, E. R., Parsons, R., Ellenson, L. H., and Kitajewski, J. (2000). APC truncation and increased beta-catenin levels in a human breast cancer cell line. Carcinogenesis *21*, 1453-1456.

Schneikert, J., and Behrens, J. (2006). Truncated APC is required for cell proliferation and DNA replication. Int J Cancer *119*, 74-79.

Schneikert, J., Grohmann, A., and Behrens, J. (2007). Truncated APC regulates the transcriptional activity of beta-catenin in a cell cycle dependent manner. Human molecular genetics *16*, 199-209.

Scholl, F. A., Dumesic, P. A., Barragan, D. I., Harada, K., Bissonauth, V., Charron, J., and Khavari, P. A. (2007). Mek1/2 MAPK kinases are essential for Mammalian development, homeostasis, and Raf-induced hyperplasia. Developmental cell *12*, 615-629.

Seeling, J. M., Miller, J. R., Gil, R., Moon, R. T., White, R., and Virshup, D. M. (1999). Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. Science *283*, 2089-2091.

Shim, J. S., Lee, J., Park, H. J., Park, S. J., and Kwon, H. J. (2004). A new curcumin derivative, HBC, interferes with the cell cycle progression of colon cancer cells via antagonization of the Ca2+/calmodulin function. Chemistry & biology *11*, 1455-1463.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proceedings of the National Academy of Sciences of the United States of America *96*, 5522-5527.

Sieber, O. M., Lamlum, H., Crabtree, M. D., Rowan, A. J., Barclay, E., Lipton, L., Hodgson, S., Thomas, H. J., Neale, K., Phillips, R. K., *et al.* (2002). Whole-gene APC deletions cause classical familial adenomatous polyposis, but not attenuated polyposis or "multiple" colorectal adenomas. Proceedings of the National Academy of Sciences of the United States of America *99*, 2954-2958.

Sierra, J., Yoshida, T., Joazeiro, C. A., and Jones, K. A. (2006). The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. Genes & development *20*, 586-600.

Sjoblom, T., Jones, S., Wood, L. D., Parsons, D. W., Lin, J., Barber, T. D., Mandelker, D., Leary, R. J., Ptak, J., Silliman, N., *et al.* (2006). The consensus coding sequences of human breast and colorectal cancers. Science *314*, 268-274.

Smith, A. J., Stern, H. S., Penner, M., Hay, K., Mitri, A., Bapat, B. V., and Gallinger, S. (1994). Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons. Cancer Res *54*, 5527-5530.

Smith, A. M., Ammar, R., Nislow, C., and Giaever, G. (2010). A survey of yeast genomic assays for drug and target discovery. Pharmacology & therapeutics *127*, 156-164.

Solomon, E., Voss, R., Hall, V., Bodmer, W. F., Jass, J. R., Jeffreys, A. J., Lucibello, F. C., Patel, I., and Rider, S. H. (1987). Chromosome 5 allele loss in human colorectal carcinomas. Nature *328*, 616-619.

Speers, A. E., and Cravatt, B. F. (2004). Profiling enzyme activities in vivo using click chemistry methods. Chemistry & biology *11*, 535-546.

St Onge, R. P., Mani, R., Oh, J., Proctor, M., Fung, E., Davis, R. W., Nislow, C., Roth, F. P., and Giaever, G. (2007). Systematic pathway analysis using high-resolution fitness profiling of combinatorial gene deletions. Nature genetics *39*, 199-206.

Stegmaier, K., Wong, J. S., Ross, K. N., Chow, K. T., Peck, D., Wright, R. D., Lessnick, S. L., Kung, A. L., and Golub, T. R. (2007). Signature-based small molecule screening identifies cytosine arabinoside as an EWS/FLI modulator in Ewing sarcoma. PLoS medicine *4*, e122.

Steigerwald, K., Behbehani, G. K., Combs, K. A., Barton, M. C., and Groden, J. (2005). The APC tumor suppressor promotes transcription-independent apoptosis in vitro. Molecular cancer research : MCR *3*, 78-89.

Stoughton, R. B., and Friend, S. H. (2005). How molecular profiling could revolutionize drug discovery. Nature reviews Drug discovery *4*, 345-350.

Stratton, M. R. (2011). Exploring the genomes of cancer cells: progress and promise. Science *331*, 1553-1558.

Su, L. K., Burrell, M., Hill, D. E., Gyuris, J., Brent, R., Wiltshire, R., Trent, J., Vogelstein, B., and Kinzler, K. W. (1995). APC binds to the novel protein EB1. Cancer research *55*, 2972-2977.

Su, L. K., Johnson, K. A., Smith, K. J., Hill, D. E., Vogelstein, B., and Kinzler, K. W. (1993a). Association between wild type and mutant APC gene products. Cancer Res *53*, 2728-2731.

Su, L. K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A., and Dove, W. F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science *256*, 668-670.

Su, L. K., Vogelstein, B., and Kinzler, K. W. (1993b). Association of the APC tumor suppressor protein with catenins. Science *262*, 1734-1737.

Tagawa, Y., Sawai, T., Nakagoe, T., Morinaga, M., Yasutake, T., Ayabe, H., and Tomita, M. (1996). Numerical aberrations of chromosomes 11 and 17 in colorectal adenocarcinomas. Surgery today *26*, 869-874.

Taketo, M. M. (2006). Mouse models of gastrointestinal tumors. Cancer science *97*, 355-361.

Tan, Y., Sangfelt, O., and Spruck, C. (2008). The Fbxw7/hCdc4 tumor suppressor in human cancer. Cancer letters *271*, 1-12.

Terstappen, G. C., Schlupen, C., Raggiaschi, R., and Gaviraghi, G. (2007). Target deconvolution strategies in drug discovery. Nature reviews Drug discovery *6*, 891-903.

Tetsu, O., and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature *398*, 422-426.

Tewari, M., Hu, P. J., Ahn, J. S., Ayivi-Guedehoussou, N., Vidalain, P. O., Li, S., Milstein, S., Armstrong, C. M., Boxem, M., Butler, M. D., *et al.* (2004). Systematic interactome mapping and genetic perturbation analysis of a C. elegans TGF-beta signaling network. Mol Cell *13*, 469-482.

Thiagalingam, S., Lengauer, C., Leach, F. S., Schutte, M., Hahn, S. A., Overhauser, J., Willson, J. K., Markowitz, S., Hamilton, S. R., Kern, S. E., *et al.* (1996). Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. Nature genetics *13*, 343-346.

Tighe, A., Johnson, V. L., and Taylor, S. S. (2004). Truncating APC mutations have dominant effects on proliferation, spindle checkpoint control, survival and chromosome stability. Journal of cell science *117*, 6339-6353.

Trzepacz, C., Lowy, A. M., Kordich, J. J., and Groden, J. (1997). Phosphorylation of the tumor suppressor adenomatous polyposis coli (APC) by the cyclin-dependent kinase p34. The Journal of biological chemistry *272*, 21681-21684.

Tsuchiya, T., Tamura, G., Sato, K., Endoh, Y., Sakata, K., Jin, Z., Motoyama, T., Usuba, O., Kimura, W., Nishizuka, S., *et al.* (2000). Distinct methylation patterns of two APC gene promoters in normal and cancerous gastric epithelia. Oncogene *19*, 3642-3646.

Tudek, B., and Speina, E. (2012). Oxidatively damaged DNA and its repair in colon carcinogenesis. Mutation research *736*, 82-92.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A. P., *et al.* (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell *111*, 241-250.

Varela, I., Tarpey, P., Raine, K., Huang, D., Ong, C. K., Stephens, P., Davies, H., Jones, D., Lin, M. L., Teague, J., *et al.* (2011). Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature *469*, 539-542.

Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., and Bos, J. L. (1988). Genetic alterations during colorectal-tumor development. The New England journal of medicine *319*, 525-532.

Vousden, K. H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

Waaler, J., Machon, O., Tumova, L., Dinh, H., Korinek, V., Wilson, S. R., Paulsen, J. E., Pedersen, N. M., Eide, T. J., Machonova, O., *et al.* (2012). A novel tankyrase inhibitor decreases canonical Wnt signaling in colon carcinoma cells and reduces tumor growth in conditional APC mutant mice. Cancer Res *72*, 2822-2832.

Wada, T., and Penninger, J. M. (2004). Mitogen-activated protein kinases in apoptosis regulation. Oncogene *23*, 2838-2849.

Wakefield, J. G., Stephens, D. J., and Tavare, J. M. (2003). A role for glycogen synthase kinase-3 in mitotic spindle dynamics and chromosome alignment. J Cell Sci *116*, 637-646.

Wang, K., Kan, J., Yuen, S. T., Shi, S. T., Chu, K. M., Law, S., Chan, T. L., Kan, Z., Chan, A. S., Tsui, W. Y., *et al.* (2011). Exome sequencing identifies frequent mutation of ARID1A in molecular subtypes of gastric cancer. Nature genetics *43*, 1219-1223.

Wang, T. H., Popp, D. M., Wang, H. S., Saitoh, M., Mural, J. G., Henley, D. C., Ichijo, H., and Wimalasena, J. (1999). Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-

Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. J Biol Chem *274*, 8208-8216.

Wang, T. H., Wang, H. S., Ichijo, H., Giannakakou, P., Foster, J. S., Fojo, T., and Wimalasena, J. (1998). Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. J Biol Chem *273*, 4928-4936.

Wang, Y., Azuma, Y., Moore, D., Osheroff, N., and Neufeld, K. L. (2008). Interaction between tumor suppressor adenomatous polyposis coli and topoisomerase IIalpha: implication for the G2/M transition. Molecular biology of the cell *19*, 4076-4085.

Wang, Z., Shen, D., Parsons, D. W., Bardelli, A., Sager, J., Szabo, S., Ptak, J., Silliman, N., Peters, B. A., van der Heijden, M. S., *et al.* (2004). Mutational analysis of the tyrosine phosphatome in colorectal cancers. Science *304*, 1164-1166.

Watanabe, T., Wang, S., Noritake, J., Sato, K., Fukata, M., Takefuji, M., Nakagawa, M., Izumi, N., Akiyama, T., and Kaibuchi, K. (2004). Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration. Developmental cell *7*, 871-883.

Wei, X., Walia, V., Lin, J. C., Teer, J. K., Prickett, T. D., Gartner, J., Davis, S., Program, N. C. S., Stemke-Hale, K., Davies, M. A., *et al.* (2011). Exome sequencing identifies GRIN2A as frequently mutated in melanoma. Nature genetics *43*, 442-446.

Wen, Y., Eng, C. H., Schmoranzer, J., Cabrera-Poch, N., Morris, E. J., Chen, M., Wallar, B. J., Alberts, A. S., and Gundersen, G. G. (2004). EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. Nature cell biology *6*, 820-830.

Wheatley, S. P., Carvalho, A., Vagnarelli, P., and Earnshaw, W. C. (2001). INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis. Current biology : CB *11*, 886-890.

Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., *et al.* (1999). Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science *285*, 901-906.

Wong, M. H., Hermiston, M. L., Syder, A. J., and Gordon, J. I. (1996). Forced expression of the tumor suppressor adenomatosis polyposis coli protein induces disordered cell migration in the intestinal epithelium. Proceedings of the National Academy of Sciences of the United States of America *93*, 9588-9593.

Wood, L. D., Parsons, D. W., Jones, S., Lin, J., Sjoblom, T., Leary, R. J., Shen, D., Boca, S. M., Barber, T., Ptak, J., *et al.* (2007). The genomic landscapes of human breast and colorectal cancers. Science *318*, 1108-1113.

Wu, J. S., Paul, P., McGannon, E. A., and Church, J. M. (1998). APC genotype, polyp number, and surgical options in familial adenomatous polyposis. Annals of surgery *227*, 57-62.

Yamashita, Y. M., Jones, D. L., and Fuller, M. T. (2003). Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. Science *301*, 1547-1550.

Yan, X. J., Xu, J., Gu, Z. H., Pan, C. M., Lu, G., Shen, Y., Shi, J. Y., Zhu, Y. M., Tang, L., Zhang, X. W., *et al.* (2011). Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nature genetics *43*, 309-315.

Yang, J., Shamji, A., Matchacheep, S., and Schreiber, S. L. (2007). Identification of a small-molecule inhibitor of class Ia PI3Ks with cell-based screening. Chemistry & biology *14*, 371-377.

Zeineldin, M., and Neufeld, K. L. (2013a). More than two decades of Apc modeling in rodents. Biochim Biophys Acta *1836*, 80-89.

Zeineldin, M., and Neufeld, K. L. (2013b). Understanding phenotypic variation in rodent models with germline Apc mutations. Cancer Res *73*, 2389-2399.

Zhao, L., and Vogt, P. K. (2008). Class I PI3K in oncogenic cellular transformation. Oncogene *27*, 5486-5496.

Zumbrunn, J., Kinoshita, K., Hyman, A. A., and Nathke, I. S. (2001). Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3 beta phosphorylation. Current biology : CB *11*, 44-49.