COLON CANCER INITIATION AND PROGRESSION

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DEDICATION

Dedicated to my lovely wife Banu for her everlasting love and support.

COLON CANCER INITIATION AND PROGRESSION

by

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DISSERTATION / THESIS

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COLON CANCER INITIATION AND PROGRESSION

UGUR ESKIOCAK, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2011

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Landmark cancer genome resequencing efforts are leading to the identification of mutated genes in many types of cancer. The extreme diversity of mutations being detected presents significant challenges to subdivide causal from coincidental mutations in order to elucidate how disrupted regulatory networks drive cancer processes. Given that a common early perturbation in solid tumor initiation is bypass of matrix-dependent proliferation restraints we sought to functionally interrogate candidate colorectal cancer genes (*CAN*-genes) to identify driver tumor-suppressors. We have developed an isogenic human colonic epithelial cell (HCEC) model to identify suppressors of anchorage-independent growth by conducting a soft agar based shRNA screen within the cohort of *CAN*-genes. Remarkably, depletion of 65 of the 151 *CAN*-genes tested collaborated with ectopic expression of *K-RAS*^{V12} and/or TP53 knockdown to promote anchorage-independent proliferation of HCECs. In contrast only 5 out of 362 random shRNAs (1.4%) enhanced soft agar growth. We have identified additional members of an extensive gene network specifying matrix-dependent proliferation, by constructing an interaction map of these confirmed progression suppressors with the ~700 mutated genes

that were excluded from *CAN*-genes, and experimentally verifying soft-agar growth enhancement in response to depletion of a subset of these genes. Collectively, this study revealed a profound diversity of nodes within a fundamental tumor suppressor network that are susceptible to perturbation leading to enhanced cell-autonomous anchorage-independent proliferative fitness. Tumor suppressor network fragility as a paradigm within this and other regulatory systems perturbed in cancer, could in large part, account for the heterogeneity of somatic mutations detected in tumors.

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LIST OF ABBREVIATIONS

[^{3}H]dT – Tritiated Thymidine

3D – 3-Dimensional

ALT – Alternative Lengthening of Telomeres

APC – Adenomatous Polyposis Coli

BIO – 6-bromoindirubin-3-oxime

Bmi1 – B Lymphoma Mo-MLV Insertion Region 1 Homolog

BrdU – 5-Bromodeoxyuridine

BSA – Bovine Serum Albumin

CAN-genes – Candidate Colorectal Cancer Genes

CDK4 – Cyclin Dependent Kinase 4

cDNA – Complementary DNA

CGA – Chromogranin A

CIN – Chromosomal Instability

CRC – Colorectal Cancer

DAPI – 4',6-Diamidino-2-Phenylindole

DNA – Deoxyribonucleic Acid

DPP4 – Dipeptyl Peptidase 4

EDTA – Ethylenediaminetetraacetic Acid

EGF – Epidermal Growth Factor

FAP – Familial Adenomatous Polyposis

GPCR – G-Protein-Coupled Receptor

GSK-3β – Glycogen Synthase Kinase-3 Beta

HCEC – Human Colonic Epithelial Cell

HNPCC – Hereditary Non-Polyposis Colorectal Cancer

HPV – Human Papillomavirus

HRP – Horseradish Peroxidase

hTERT – Human Telomerase Reverse Transcriptase

HUVEC – Human Umbilical Vein Endothelial Cell

JNK – c-Jun N-Terminal Kinase

kb – Kilobase Pairs

LGR5 – Leucine-Rich-Repeat-Containing G-Protein-Coupled Receptor 5

LiCl – Lithium Chloride

M0 – Mortality Stage 0

M1 – Mortality Stage 1

M2 – Mortality Stage 2

MIN – Microsatellite Instability

mRNA – Messenger RNA

miRNA – Micro RNA

MOI – Multiplicity of Infection

MUC1 – Mucin-1

MUC2 – Mucin-2

NF1-GRD – Neurofibromin1 GTPase-Activating Protein Related Domain

NF-kB – Nuclear Factor-Kappa-Light-Chain-Enhancer of Activated B Cells

PARP – Poly ADP-Ribose Polymerase

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PD – Population Doubling

PVDF – Polyvinylidene Fluoride

PFA – Paraformaldehyde

qRTPCR – Quantitative Reverse Transcription PCR

Rb – Retinoblastoma

RNA – Ribonucleic Acid

RNAi – RNA Interference

SDS - Sodium Dodecyl Sulfate

siRNA – Short Interfering RNA

shRNA – Short Hairpin RNA

SV40 – Simian Virus 40

tGFP – Turbo Green Fluorescent Protein

TRAP – Telomeric Repeat Amplification Protocol

TRF – Telomere Restriction Fragment

CHAPTER ONE

INTRODUCTION

Organization of human colonic crypts

The primary function of the human colon is to absorb water from the remaining indigestible food matter, absorb vitamins created by the colonic bacteria and store the remaining waste material for excretion from the body. The crypt is the basic functional unit within the colon and is formed by invaginations of the single epithelial sheet layer of the colonic surface into the underlying connective tissue (Humphries and Wright 2008). Millions of crypts together make up a colon and each of these crypts is believed to be monoclonal arising from a stem cell located at the stem cell niche near the base of the crypt (Figure 1-1).

Positional identification of stem cells in the intestine relied on label retention experiments in which [^{3}H]dT (tritiated thymidine) or bromodeoxyuridine (BrdU) marked DNA is retained in the stem cells up to 10-15 rounds of division (Potten et al. 2009). Given the fast turnover of the crypt (5-7 days) this retention ability point to stem cell activity. However, this does not necessarily mean that stem cells of the intestine are quiescent. In fact, experiments in which the tritiated thymidine labeled cells is further labeled with BrdU estimated that the putative stem cell divide once a day (Potten et al. 2003). Interestingly, in these experiments labels were differentially segregated. One label disappearing from the putative stem cells to the progeny with in the first few divisions where as the other is retained through many divisions (Potten et al. 2003). These results supports the immortal strand hypothesis in which stem cells, in order to protect their

genetic material from replication induced errors, always retain the template strand of DNA.

There have been multiple markers suggested to identify the stem cells of the intestine. However only two markers, Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5) (Barker et al. 2007) and Bmi1 (B lymphoma Mo-MLV insertion region 1 homolog) (Sangiorgi and Capecchi 2008), functionally demonstrate multipotency by marking an individual clone and demonstrating that cell was able to clonally replace all the cell lineages in that crypt. As in most adult stem cell markers of various tissues, the functions of intestinal stem cell markers are not clear. Lgr5 belongs to a family of class A rhodopsin-like seven trans membrane domain, G protein-coupled receptors (GPCRs) (Hsu, Liang, and Hsueh 1998) and until recently was an orphan receptor. However, simultaneous findings from two groups de-orphanized Lgr5 and identified R-spondins as its ligands (Carmon et al. 2011; de Lau et al. 2011). These observations suggest that Lgr5 plays an important role in stem cell biology by regulating Wnt pathway in response to R-spondins (de Lau et al. 2011).

Although much of the evidence relating to the clonality of the crypts has been obtained from rodent studies there are few experiments in human tissues pointing to the same conclusions. For instance, analysis of a tissue taken from a patient with familial adenomatous polyposis (FAP) who is a mosaic for XO/XY showed that colonic crypts were exclusively either XO or XY (Novelli et al. 1996). Thus in this rare example, using a probe that hybridizes to sequences on Y chromosome, researchers have been able to show that each crypt is likely to be derived from a single stem cell that is either XO or XY.

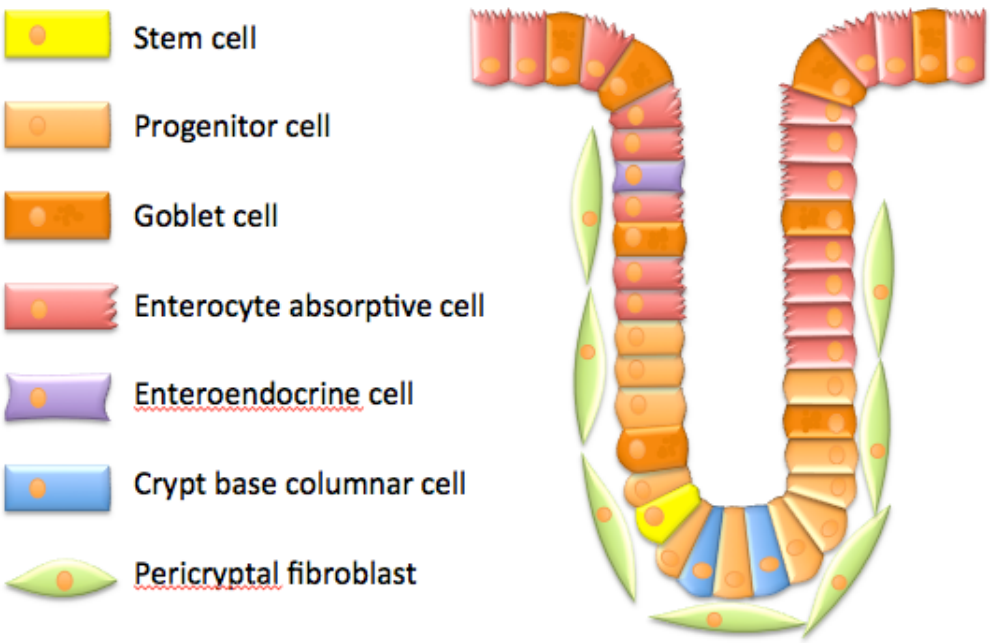

Figure 1-1. The organization of the colonic crypt. Adapted from (Medema and Vermeulen 2011)

The stem cells in the colon give rise to four different terminally differentiated cell types (Figure 1-1). These include the absorptive cells, the mucus-secreting goblet cells, the peptide hormone-secreting enteroendocrine cells and the crypt base columnar epithelial cells. The remainder of the crypt is occupied by the progenitor cells (also known as transit-amplifying cells), which proliferate in an estimated rate of two doublings a day and are crucial for rapid turnover of the colonic epithelium (Crosnier, Stamataki, and Lewis 2006). Pericryptal fibroblasts are mesenchymal cells that surround and support the colonic crypt. Although these cells are outside of the crypt they are thought to provide important signaling cues, including that of WNT, BMP and HGF

signaling, for stem cell maintenance and differentiation (Humphries and Wright 2008; Medema and Vermeulen 2011).

Cell culture models of human colonic epithelial cells

Many of the cell culture models available to study colonic biology are established from colorectal cancer tissue (Bjerknes and Cheng 2006) and thus are not appropriate to study either normal colon biology or initiation of disease states such as colorectal cancer. Given that the majority of the colonic epithelial cells in vivo are in a differentiated state and are replaced every 4-6 days, the challenge of establishing a normal human colonic epithelial cell culture system is understandable. However, although in minority, there are still a number of transit amplifying and stem cells that might be able to continue to proliferate in culture if adequate growth factors and substrates are provided. Even in this extended in vitro proliferative capacity these cells will likely to stop growing after a while as normal somatic cells do not have unlimited growth potential.

The maximum number of times that a normal mammalian somatic cell can divide is known as the Hayflick limit (Hayflick 1965) at which one or more critically shortened telomeres induce cell cycle exit, a stage referred as mortality stage 1 (M1) (Wright, Pereira-Smith, and Shay 1989). The M1 cell cycle check point can be bypassed by inactivation of critical tumor suppressor genes such as p53, p16 and RB in which case the cells continue to divide and continue to lose telomeres until mortality stage 2 (M2) (Counter et al. 1992; Shay et al. 1993) occurs. M2 is characterized by a crisis in which most of the cells die due to critically short telomeres. There are two mechanisms that can replenish the telomeric loss: 1) activation of telomerase, a reverse transcriptase that

synthesizes de novo telomeric repeats using an RNA template (Greider and Blackburn 1989); or 2) the alternative lengthening of telomeres (ALT) pathway which is caused by homologous recombination events between telomeric repeats (Bailey, Brenneman, and Goodwin 2004; Dunham et al. 2000).

This model of replicative senescence has been discovered and found to be true in fibroblasts such that reactivation of telomerase is sufficient to immortalize fibroblasts (Mathon and Lloyd 2001). However, realization that some epithelial cells undergo growth arrest earlier than it would be predicted to be caused by replicative senescence and telomerase reactivation alone is not sufficient to immortalize these cells suggests that there is yet another mechanism regulating life span of epithelial cells. This senescence is believed to result from various stress stimuli resulting in increased p16 levels and has been observed both *in vitro* and *in vivo* (Campisi and d'Adda di Fagagna 2007). It has been given multiple names including mortality stage 0 (M0), premature senescence, stress-induced senescence or oncogene-induced senescence and is believed to be a major tumor suppressive mechanism that blocks the proliferation of aberrant cells (Campisi and d'Adda di Fagagna 2007). Supporting this notion, there are *in vivo* data indicating that senescence is activated in most benign lesions (Cichowski and Hahn 2008).

Stress-induced senescence in culture results from less than optimal growth conditions and can be prevented by inactivation of the p16 response. The primary function of p16 is to bind to cyclin-dependent kinase 4 (CDK4) and inhibit formation of the active CDK4/cyclin D complex (Serrano, Hannon, and Beach 1993). There are different strategies to overcome premature-senescence however the majority of them involves viral oncoproteins such as large T antigen of simian virus 40 (SV40) and E6/E7

of high-risk strains of human papillomavirus (HPV) (Hahn et al. 1999; Shay et al. 1993). The major disadvantage for using such viral oncoproteins is their strong transforming ability and therefore losing the ability to examine normal biology. Additionally, these viral oncoproteins tend to have multiple functions some of which are yet to be identified. For these reasons our group developed an immortalization protocol that does not rely on viral oncoproteins but instead prevents stress-induced senescence by titering out p16 with ectopic expression of CDK4 (Ramirez et al. 2004).

Similarly, human colonic epithelial cells could be immortalized by ectopic expression of CDK4 to overcome stress-induced senescence and hTERT to overcome replicative senescence. This approach generates cells that more closely resemble parental cells as opposed to immortalization with viral oncoproteins (Ramirez et al. 2004).

Colorectal cancer

Colorectal cancer is the third most common and the third leading cause of cancer related deaths in United States (Siegel et al. 2011). There are ~1.2 million new cases every year (Ferlay et al. 2010). It is estimated that every other person living in the United States will develop colorectal tumors by the age of 70 and about 10% of these individuals will progress to malignancy (Kinzler and Vogelstein 1996). It is believed that environmental factors can strongly influence the incidence of colorectal cancer. Sedentary lifestyle, high-fat diet and obesity have been found to increase the risk of colorectal cancer (Lieberman et al. 2003). Supporting this hypothesis, the highest incidence of colorectal cancer in the world is seen in the United States and Europe.

In addition to environmental factors, genetic predisposition has also been linked to increased risk of colorectal cancer as demonstrated in familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome) patients. The likelihood of developing colorectal cancer is 100% for FAP and 80% for HNPCC patients. Interestingly, FAP patients have an accelerated tumor initiation (5-20 years as opposed to 30-50 years) but normal tumor progression (10-20 years) whereas HNPCC patients have normal initiation but accelerated progression (1-3 years) (Grady and Carethers 2008). These syndromes account for less than 10% of all colorectal cancer cases whereas the majority of the colon cancer cases occur sporadically (Komarova et al. 2002).

There are two general classes of colorectal cancers divided on the basis of the type of genomic instability they present: chromosomal instability (CIN) or microsatellite instability (MIN). Around 85% of colorectal cancers have the CIN phenotype whereas the remaining ~15% of the cases are characterized as MIN (Grady and Carethers 2008).

Genetic alterations

Cancer results from deregulated cell growth due to activation of oncogenes or inactivation of tumor suppressor genes. Although these genes might differ greatly in their function, these alterations allow cancer cells to acquire a similar set of capabilities commonly referred as the hallmarks of cancer. These hallmarks were first summarized a decade ago and included self-sufficiency in proliferative signaling, evading anti-growth signals, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg 2000). Research in the last

decade added two emerging hallmarks (deregulating cellular energetics and avoiding immune destruction) to the list (Hanahan and Weinberg 2011). Furthermore, two characteristics of cancers, genome instability and mutation and tumor-promoting inflammation, have been recognized as enabling characteristics for multiple of these hallmarks to emerge (Hanahan and Weinberg 2011).

Acquiring mutations is one of the most common ways for oncogenes and tumor suppressor genes to be abnormally regulated. In order to become cancer, normal cells accumulate mutations in multiple genes that will help them acquire hallmark capabilities. Colorectal cancer has been one of the better-defined cancers in terms of their genetic make-up due to availability of large numbers of tumors at various stages of cancer progression from very small adenomas to large metastatic carcinomas. For instance colonoscopy of FAP patients will reveal hundreds of small polyps, multiple adenomas and a few carcinomas all from the same patient (Kinzler and Vogelstein 1996) thereby enabling researcher to dissect all the genetic changes required to develop carcinoma. In fact, on the basis of histopathological, clinical and molecular data generated from the analysis of such samples, a model for colon cancer initiation, development and progression has been proposed over two decades ago (Fearon and Vogelstein 1990) (Figure 1-2). This is a very simple concept of an orderly progression of cancer, with each step involving a distinct gene. Although this model has been helpful to understand colorectal carcinogenesis researchers now recognize that it is an over simplification. The data I will present in this thesis will provide evidence that it isn't just a simple matter of having a single gene at each stage of cancer progression, but there are probably multiple genes that when altered enables the cancer cell to progress.

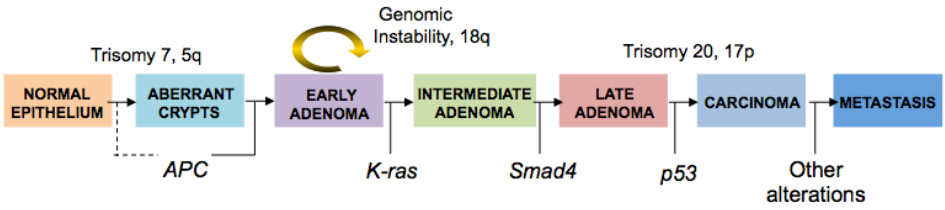

Figure 1-2. Colorectal carcinogenesis model. Adapted from (Fearon and Vogelstein 1990).

Advances in sequencing technologies enabled tremendous international efforts to sequence cancer genomes. These studies are leading to identification of thousands of mutated genes in many different cancer types. A major challenge is to identify driver mutations that are causally involved in initiation, progression and maintenance of cancers from passenger (incidental) mutations that are acquired by chance and do not have any selective advantage. Another challenge is to understand the functional contributions of each of these mutated genes to cancer initiation, progression and maintenance in order to investigate ways to leverage this information for therapeutic benefit.

Initial colorectal cancer sequencing efforts identified 850 genes that are mutated (Sjöblom et al. 2006; Wood et al. 2007). However, on the basis of frequency of mutations per unit length, only 150 of these genes were classified as drivers (Sjöblom et al. 2006; Wood et al. 2007). Interestingly, only a handful of these genes are mutated in more than 10% of the cases. These frequently mutated genes were already known to be important in carcinogenesis and include *APC* (85%), *KRAS* (45%), *TP53* (47%), *FBXW7* (13%)and *PI3KCA* (13%). The remainder of the putative driver gene list was observed to be mutated in only 5-10% of the cases as opposed to ~700 passenger mutations that are

mutated in 2-5% of the cases. The biostatistic algorithms used to come to these conclusions have been widely criticized (Getz et al. 2007; Trent and Touchman 2007) and the need for functionally test these genes' transforming ability instead of relying solely on biostatistics has emerged.

CHAPTER TWO

DEVELOPMENT AND CHARACTERIZATION OF NORMAL HUMAN COLONIC EPITHELIAL CELLS

Introduction

It has been extremely challenging to derive epithelial cell cultures that are capable of long-term propagation from non-malignant colonic tissues. The development of human colonic epithelial cell lines (HCECs) not derived from tumor specimens would provide valuable cellular models to test and validate *in vitro* paradigms of colonic diseases, such as colon cancer progression. For the past 30 years, cell biologists have attempted to grow normal HCECs in long-term culture but the results have been largely disappointing, with many of the cells losing their replicative capacity or dying shortly after placing them in the tissue culture environment (Kaeffer 2002; Whitehead, Brown, and Bhathal 1987; Whitehead et al. 1999; Aldhous et al. 2001; Buset, Winawer, and Friedman 1987). This is mainly the result of the difficulties in establishing adequate conditions to permit continuous cell proliferation unhampered by acute and chronic culture-induced cellular stress and death, overgrowth of other cell types such as fibroblasts, and bacterial contamination. To our knowledge, all currently available colon cell lines are of malignant origin and/or contain multiple cytogenetic changes, thus limiting their utility for the *in vitro* study of normal colonic epithelial cell biology and initiation and progression of colonic diseases, such as colon cancer.

Over the past 10 years, there has been substantial progress in elucidating the epithelial stem and progenitor cell localization and behavior in various organs. With

respect to the digestive tract, putative markers for stem cells in position +4 (Bmi1, Msi1, and Dcamkl1) and crypt base cells (Lgr5, Msi1, and Dcamkl1) have been identified *in vivo* in murine intestinal cells and colonic cells. These cells display self renewal and tissue maintenance capacity, hallmark features of stem cells (Barker et al. 2007; May et al. 2008; Potten et al. 2003; Sangiorgi and Capecchi 2008; Kayahara et al. 2003). The relation of these stem cell markers with the cellular self renewal and differentiation *in vitro* has been recently demonstrated. For example, Lgr5-positive cells derived from mouse intestine form crypt-like organoids in 3-dimensional (3D) Matrigel^{TM} culture and cellular progeny corresponding to the various intestinal lineages (Sato et al. 2009). In addition, Lgr5- and Bmi1-positive cells are present in mouse neonatal intestinal epithelial cells capable of sustained self-renewal and differentiation in 3D collagen culture (Ootani et al. 2009).

With respect to humans, multiple stem cell markers have been elucidated in colonic tissues and cells. LGR5, BMI1, MSI1, DCAMKL1 markers have recently been identified in the epithelial cells of normal human colons (Samuel et al. 2009; Becker, Huang, and Mashimo 2008). Perhaps more well characterized with respect to self renewal capacity and differentiation are the β1-integrin (CD29), CD44, CD133, and CD166 markers. β1-integrin is present in undifferentiated normal colonic epithelial cells that display a high self-renewal capacity in short term clonogenic assays (Fujimoto, Beauchamp, and Whitehead 2002). CD29, CD44, CD133, and CD166 are also present in the side populations of cells isolated form colorectal cancer specimens. These cells have an increased self renewal capacity, and increased ability to form tumors in mice with

histology similar to that of the cancer of origin (Dalerba et al. 2007; O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Vermeulen et al. 2008).

With this rich background of recent advances in our understanding of the cellular biology of the gastrointestinal tract, we reasoned that the long-term propagation of adult human colonic epithelial cells could be accomplished by obtaining cells enriched with most, or at least a subset, of these stem cell markers, and by growing those cells in an appropriate cell culture environment with adequate nutrients and growth factors. The stem cell properties of such cultures could be confirmed, not only by the identification of colonic epithelial cell specific proteins, but also by the demonstration that single cells have the ability to self renew and differentiate.

Thus we have been able to develop methods that successfully expand adult HCECs extracted from normal colonic biopsy specimens taken from patients undergoing screening colonoscopy. In addition, we have also demonstrated that those cells can be immortalized and continue to express a panel of putative gastrointestinal stem cell markers. Importantly, we further demonstrate that, under appropriate *in vitro* conditions, individual immortalized cells can proliferate and differentiate into crypt-like structures (similar to the adult colonic crypt) and express colonic differentiation specific markers.

Results

Primary Cultures of Human Colonic Epithelial Cells (HCECs)

The crypt extraction procedure using collagenase and dispase (Figure 2-1) provides a greater yield of crypt attachment and initial epithelial cell viability compared to previously described EDTA chelation methods (data not shown) (Whitehead, Brown,

and Bhathal 1987). With this enzymatic protocol 40%-50% of isolated crypts attach to the substrate with viable cells 24 hours after isolation. Cell culture conditions are critical for the initial and long-term proliferation of the HCEC populations and included optimizing atmospheric (Wright and Shay 2006), substrate (Ince et al. 2007), and media conditions. In the setting of low oxygen environment (2%-5% O_2), Primaria® plastic culture dishes, and half confluent human colon fibroblast-feeder layers, small subsets of cuboidal-shaped cells in nests appear by 10 days after crypt seeding (Figure 2-1, 10 day explant).

Expansion of nests does not occur in normoxic/atmospheric (21%) conditions and extended proliferation is absent in conventional plastic cell culture dishes. To deter fibroblast over-growth, the 2% serum used in the initial crypt seeding was gradually removed from the culture medium over the first 6 days. Despite the removal of serum, cell nests are still able to proliferate in basal medium supplemented with a variety of growth factors (see methods) for approximately 20 days in the presence of human fibroblast feeder layers until first passage. At that time, the cells are subcultured in the presence of 2% serum but in the absence of feeder layers. Cells from these primary cultures can continuously divide in these conditions for approximately 40 population doublings under low oxygen conditions on Primaria® plates (Figures 2-2A-B).

Immortalization of Cells

Cells obtained from the two patient specimens were transduced with both cyclin dependent kinase 4 [*Cdk4,* bypasses cell culture associated stresses that frequently lead to premature senescence (Ramirez et al. 2001; Ramirez et al. 2004)] and the catalytic

component of the human ribonucleoprotein enzyme telomerase (*hTERT;* maintains telomere length and prevents replicative senescence (Bodnar et al. 1998)). The immortalized cells were termed HCEC 1CT (patient one; "C" for cdk4 and "T" for telomerase) and HCEC 2CT (patient 2). Both lines have been subcultured for greater than 100 population doublings, an indication that they have been successfully immortalized (Figures 2-2A-B). Cessation of growth of non-immortalized cells is accompanied by a flattened and enlarged morphological phenotype typical of cells undergoing replicative senescence (Figure 2-2C, right panel). Compared to the flattened and enlarged shape of the non-immortalized controls at senescence, the compact appearance of immortalized HCEC CTs PD 90 (Figure 2-2D, right panel) is similar to the morphology of earlier passaged cells in log-phase growth (Figure 2-2D, left panel). Immortalization is only obtained after transduction of both *Cdk4* and *hTERT*. Cells from both patients still undergo senescence with only *hTERT* or had a slightly extended lifespan with only *Cdk4* (Figure 2-2A-B). The order of *hTERT* or *Cdk4* introduction into cells was not a factor for successful immortalization. Western blots showed increased Cdk4 expression in HCEC CTs for both patients compared to non-infected HCECs, indicating successful integration and expression of *Cdk4* (Figure 2-3A). Telomere restriction fragment length analysis (TRF assays) showed that the telomere length of normal HCECs progressively shorten with increased population doublings (Figure 2-3B**)** and while heterogeneous in length, the shortest telomeres ranged from 1.8 to 2.6 Kb at senescence. In contrast, the immortalized HCECs maintained telomere lengths well above 10 Kb. Telomerase activity (TRAP) assays for the immortalized HCEC lines (HCEC 1CT and HCEC 2CT) show activity levels comparable to that in a control tumor cell line (HeLa) indicating successful *hTERT*

integration and expression (Figure 2-3C**)**. In contrast, absence of the processive telomerase ladders in the TRAP assay in normal HCEC1 and 2 parental controls indicates lack or very low levels of telomerase activity that are insufficient to maintain telomere length. HCEC CTs are able to be cryopreserved at a wide range of passages, and have been reintroduced into cell cultures without complications.

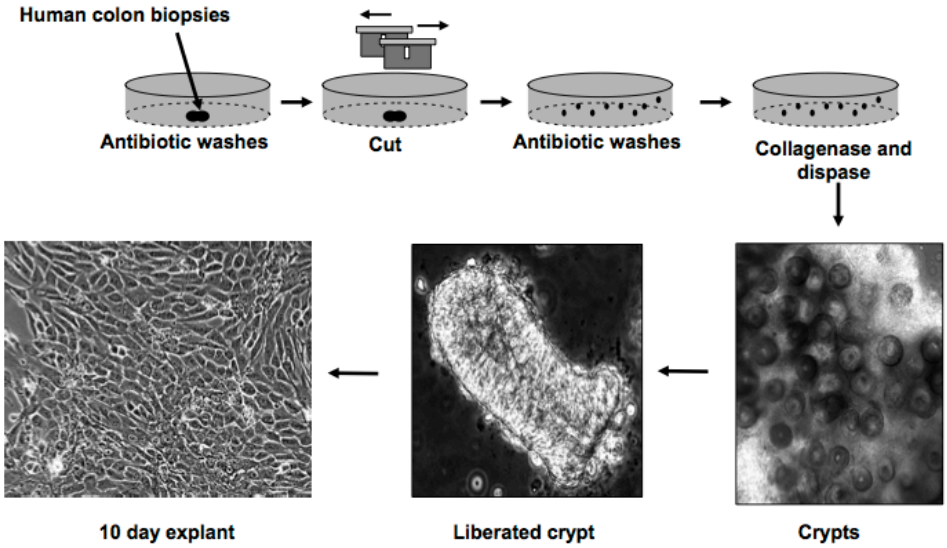

Figure 2-1. Crypt extraction sequence. Crypts are extracted from processed biopsies and seeded onto a Primaria® dish with colonic fibroblast feeder layers. The dishes are immediately placed in 3% to 5% oxygen tension. 10 days after seeding, nests of epithelial cells can be identified. Images obtained at 20x magnification.

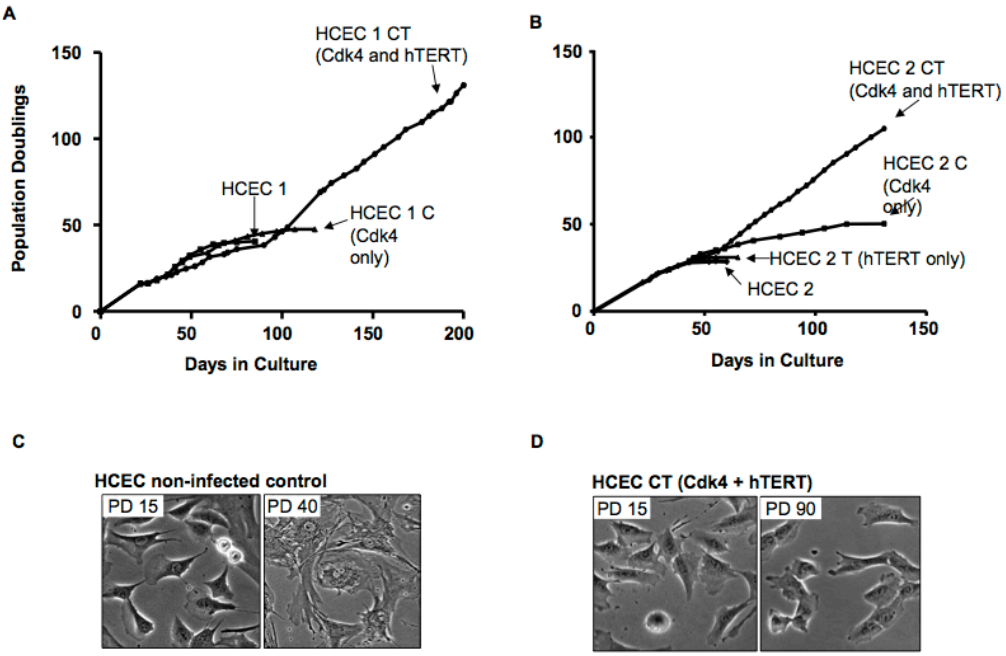

Figure 2-2. Immortalization of colonic epithelial cells. **(A, B)** Growth curves for cells derived from patient 1 and patient 2. (**C, D**) Expression of CDK4 and hTERT maintains a compact and healthy appearance of HCEC CTs compared to non-infected HCEC CTs at population doubling 40. Images 20x magnification.

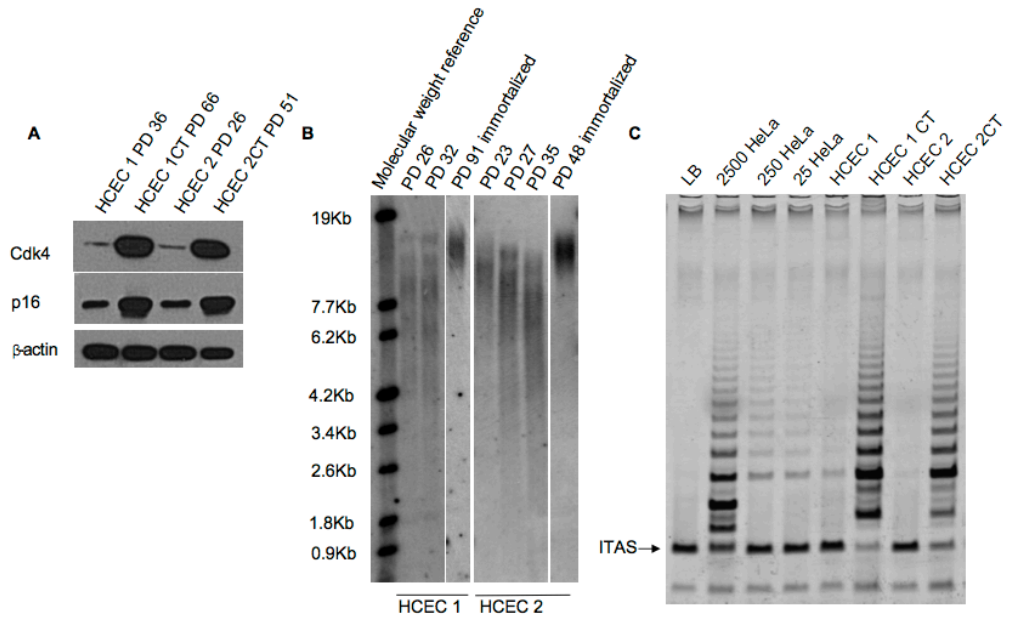

Figure 2-3. Cdk4 and telomerase expression in immortalized HCECs. (**A**) Western blots showing prominent Cdk4 and p16 bands. (**B**) TRF gel showing overall increased telomere length in immortalized HCECs. (**C**) TRAP assay showing positive telomerase activity in both HCEC 1CT and HCEC 2CT.

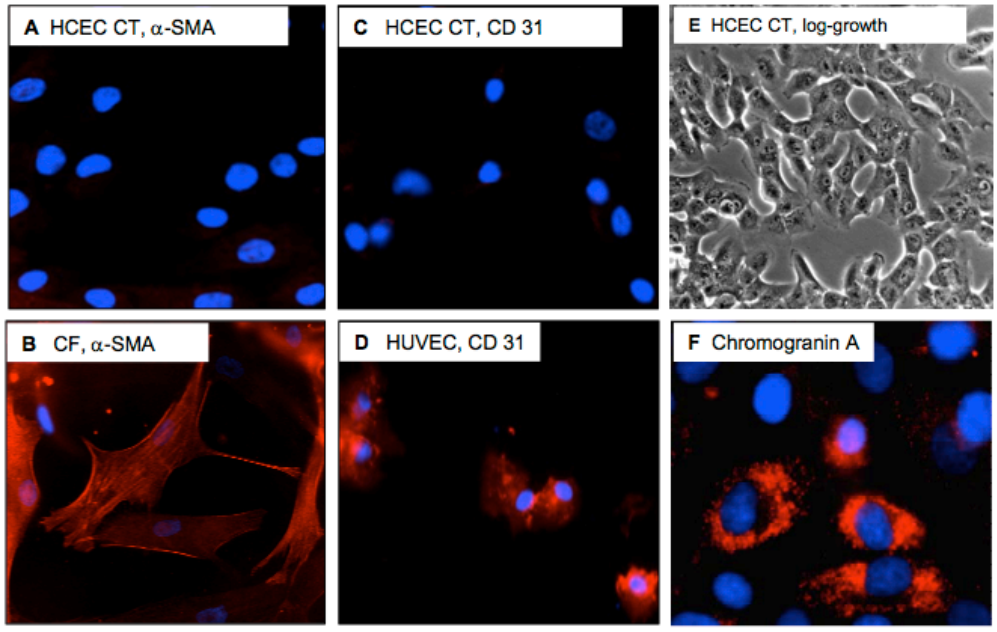

Figure 2-4. HCEC CTs are not pericryptal fibroblasts or endothelial cells. (**A, B**) HCEC CTs do not stain for α-smooth muscle actin as do the control colonic fibroblasts. (**C, D**) HCEC CTs do not stain for CD31 as do the control human umbilical vein endothelial cells (HUVECs). (**E**) Appearance of HCEC CT cells in log-phase growth. (**F**) Chromogranin A staining in log-phase growth cells. DAPI was used to stain nuclei (blue). All images obtained at 40x magnification.

Characterization of HCECs as Colonic Epithelial Cells

HCEC CTs were immunostained for markers to exclude the possibility that non-epithelial cells, such as pericryptal fibroblasts and endothelial cells may have contaminated the populations of cell cultures. Cells did not stain for α-smooth muscle actin (Figure 2-4A) or CD31 (Figure 2-4C) demonstrating that the cells are not likely to be pericryptal fibroblasts or endothelial cells, respectively, compared to their appropriate controls (Figure 2-4B, D). Log phase growing HCEC CTs in 2% serum supplemented media at about 50% confluency consist of both cuboidal and spindle-shaped cells (Figure 2-4E). Log phase growth HCEC CTs from both patients reveal approximately 5% to 8% of the population of cells stain positively for the neuroendocrine marker chromogranin A (CGA) (Modlin et al. 2006) (Figure 2-4F). 3 day exposure to reduced serum (0.1%) and EGF (0.5 ng/ml) media containing 5 μM of the glycogen synthase kinase-3 beta (GSK-3β) inhibitor 6-bromoindirubin-3-oxime (BIO) (Meijer et al. 2003) induces subconfluent HCEC CTs undergo growth arrest and display cuboidal morphology. Importantly, the immortalized colonic epithelial cells can self assemble into circular (crypt-like) structures with a central lumen (Figure 2-5A). A similar finding was also observed with lithium chloride exposure for 5 days (data not shown). In this growth arrested state approximately 30% of cells express CK18 (Figure 2-5B), and over 90% of cells express a juxtanuclear pattern for CK20 (an intermediate filament normally present in the cytoplasm of cells located in the apical portion of differentiated epithelial cells in colonic crypts (Wildi et al. 1999)) (Figure 2-5C**)**. Evidence of tight junction formation (membranous ZO-1 staining) is also seen (Figure 2-5D). β-catenin, a component of adherens junctions localizes to the cell membranes, the cytoplasm, and is absent in the nucleus (Figure 2-5E). The colon

epithelial cell specific marker A33 antigen, a transmembrane glycoprotein specific to human intestinal and colonic epithelial cells (Johnstone et al. 2000), is observed in the cytoplasm and cell membranes, a site characteristic for this antigen (Ackerman et al. 2008) (Figure 2-5F). These epithelial markers or their pattern of expression is less abundant in rapidly proliferating cells under serum and normal EGF conditions. Reduced cell proliferation is necessary to demonstrate the increased cuboidal morphology with characteristic epithelial staining. In this growth arrested state immunostaining revealed prominent perinuclear and cytoplasmic mucin-1 (MUC1, a transmembrane mucin typically expressed in to the lower third of colonic crypts corresponding to the compartment where undifferentiated colonic epithelial cells reside (Ajioka, Allison, and Jass 1996; Ajioka et al. 1997)) in over 90% and 70% of HCEC 1CTs and 2CTs, respectively. Evidence of membranous localizations for MUC1 is seen in HCEC CTs (Figure 2-5G**)** as has previously been described in other epithelial cells *in vitro* and *in vivo* (van Klinken et al. 1996; Chopra et al. 1994; Cao et al. 1997). Villin staining reveals a perinuclear and cytoplasmic distribution primarily in HCEC 1CT (Figure 2-5H). Approximately 15% of HCEC CTs in the growth arrested state show perinuclear and cytoplasmic staining of the goblet cell marker mucin-2 (MUC2) (Lugli et al. 2007) (Figure 2-5I). Approximately 20% of HCEC CTs stain for the colonocyte specific marker dipeptyl peptidase 4 (DPP4) (Whitehead, Zhang, and Hayward 1992; Young et al. 1992) (**Figure 5J**). FITC and Rhodamine controls are shown, respectively (Figure 2-5 K, L). Although immortalized HCECs from both patients displayed MUC1, A33, MUC2, DPP4, and CGA, the signal intensity for all these proteins was overall higher in HCEC 1CT than HCEC 2CT. In summary, the above data suggest that the populations of HCEC

CTs from both patients consist of epithelial cells with undifferentiated progenitor features (MUC1), with a subset of the immortalized population expressing the lineage specific markers DPP4, MUC2, and CGA.

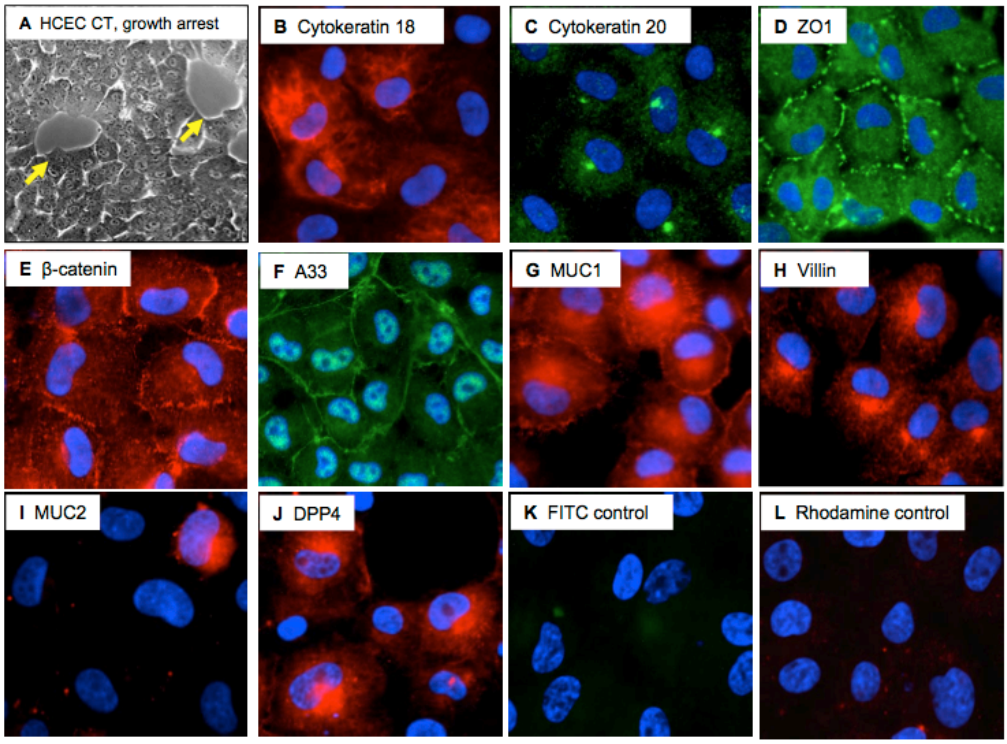

Figure 2-5. Representative images characterizing HCEC CTs in growth arresting conditions. (**A**) Appearance of HCEC CTs in growth arrested state; yellow arrows indicate circular crypt-like openings. (**B-J**) Cytokeratin 18, cytokeratin 20, ZO-1, β-catenin, A33, MUC1, villin, MUC2, and DPP4 staining. (**K, L**) FITC and Rhodamine controls, respectively. All images obtained at 40x magnification.

HCECs Express Stem Cell Markers

Given that HCEC CTs express MUC1, a marker for undifferentiated colonic epithelial cells, we then investigated if HCEC CTs express any of the recently described colonic and intestinal stem cell markers. A microarray analysis was used as an initial approach to characterize the stem cell marker expression of immortalized HCEC (HCEC 1CT and HCEC 2CT) lines. This analysis suggested that the HCEC 1CT and HCEC 2CT have relatively high mRNA expression of the stem cell markers CD29 (β1-integrin), CD44, CD166, BMI-1, and lower expression of LGR5 (data not shown). We then proceeded to stain log-phase cells with BMI-1 and LGR5 with validated antibodies (see methods). Staining with the Genetex LGR5 antibody revealed prominent expression of LGR5 in HCEC 1CTs and lower expression in HCEC 2CTs (Figure 2-6A, B**)**. Human colon (C26Ci) and skin (BJ) fibroblasts do not show LGR5 staining under equal camera exposure times (Figure 2-6C, D). Staining with the Abcam LGR5 antibody revealed similar staining (data not shown). Significant expression was also observed for both BMI1 in both HCEC CTs (Figure 2-6E, F). Expression of the stem cell markers CD29 and CD44 were validated via western blots for both HCEC 1CT and 2CT (data not shown).

HCEC CTs proliferate into self-organizing cysts in Matrigel™ and express absorptive and mucus secreting markers

Given that HCEC CTs express stem cell markers and exhibit other lineage specific markers in 2D monolayer culture, we next addressed if HCEC CTs can

differentiate into various epithelial lineages of the colon (absorptive, mucus secreting, and neuroendocrine cells) in 3D organotypic culture in Matrigel™. To assess for stem-like properties, populations of HCEC CTs from both patients were grown inside Matrigel™ in serum supplemented media in the absence of feeder layers to assess for self-renewal and multipotent capacity, as has been previously demonstrated with human mammary epithelial cells (Lee et al. 2007), and mouse progenitor intestinal epithelial cells from single LGR5 stem cells (Sato et al. 2009). HCEC CT cells were seeded at low densities inside Matrigel™ and subsets of individual cells were observed to proliferate and form spherical organoid structures (Figure 2-6G). Some of these organoids continue to grow beyond 7 days and make larger organoids with a hollow interior. Organoids were stained with a variety of colon specific differentiation markers at different time points. As visualized in a confocal microscope small organoids exhibit continuous ZO-1 surrounding the cells and in between cells (Figure 2-6H). Villin staining is also observed in a membranous polarized distribution (Figure 2-6I**)**. At later time points villin expression is observed lining the central lumen of the organoid, again suggesting a polarized distribution. MUC2 is observed in a subset of cells comprising the cysts. An isolated clone from HCEC 2CT was seeded in Matrigel™ and this also showed the ability to form spherical organoids with MUC2 and villin staining (data not shown). These results suggest that individual cells are able to self renew and give rise to the absorptive (villin expressing) and goblet cell (MUC2 expressing) lineages of the colon.

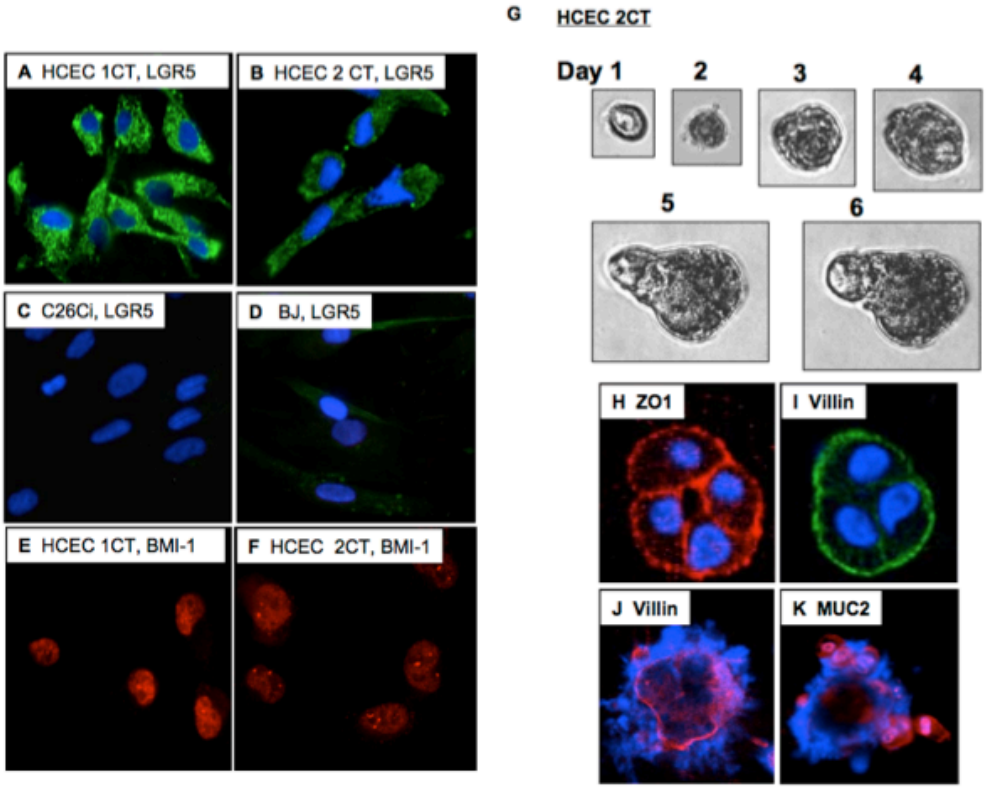

Figure 2-6. Stem cell markers, and organoid formation and differentiation in 3D Matrigel™ culture. (**A, B**) Cytoplasmic (green) LGR5 staining in HCEC 1CT and HCEC 2CT, respectively. (**C, D**) LGR5 staining in C26Ci human colon fibroblasts and BJ human skin fibroblasts, respectively. (**E, F**) Nuclear (red) BMI1 staining in HCEC 1CT and HCEC 2CT, respectively. (**G**) In Matrigel™ culture, individual HCEC CTs cells self renew and form multicellular crypt-like structures within 6 days. (**H**) Crypt -like structures show evidence of ZO1 formation . (**I**) ViIlin staining is observed in a membranous polarized distribution. (**J**) Villin expression lining the central lumen of the organoid at later time points. (**K**) MUC2 in a subset of cells comprising the cysts. All images obtained at 40x magnification, except for (G) (20x).

Karyotype of Immortalized HCEC CTs and DNA Sequencing Analysis

The karyotype of HCEC CTs from both patients is diploid (Figure 2-7A). DNA sequencing of mutational hotspots in *APC*, *KRAS*, and *TP53* (genes important for colon cancer progression) was performed for both populations of HCEC CTs and revealed no amino acid changing mutations in either cell line. HCEC 1CT and 2CT do not display tumorigenic properties, such as anchorage independent growth compared to a colon cancer cell line HCT116 (Figure 2-7B) or tumor formation in nude mice (data not shown), consistent with previous reports showing that immortalization with *CDK4* and *hTERT* does not transform cells (Morales et al. 1999; Ramirez et al. 2004).

Discussion

During the last 30 years, culture systems have been established for the long-term growth of colonic epithelial cells derived from rodents, fetal human intestine, and colon cancer. Although these cell lines have proven extremely useful for studying multiple normal and disease processes of the colon, their utility is limited because they contain key genetic aberrations, such as altered p53 and Rb. To study human diseases such as colon cancer progression, and to conduct basic research to develop new treatment modalities focused on tissue reconstitution from stem and progenitor cell pools, culture models consisting of cytogenetically normal and non-tumorigenic cell lines need to be created. To our knowledge, this is the first report to describe a system that reproducibly establishes the long-term growth in monolayer culture of adult HCECs derived from colonoscopically obtained biopsy specimens of non-cancerous tissues.

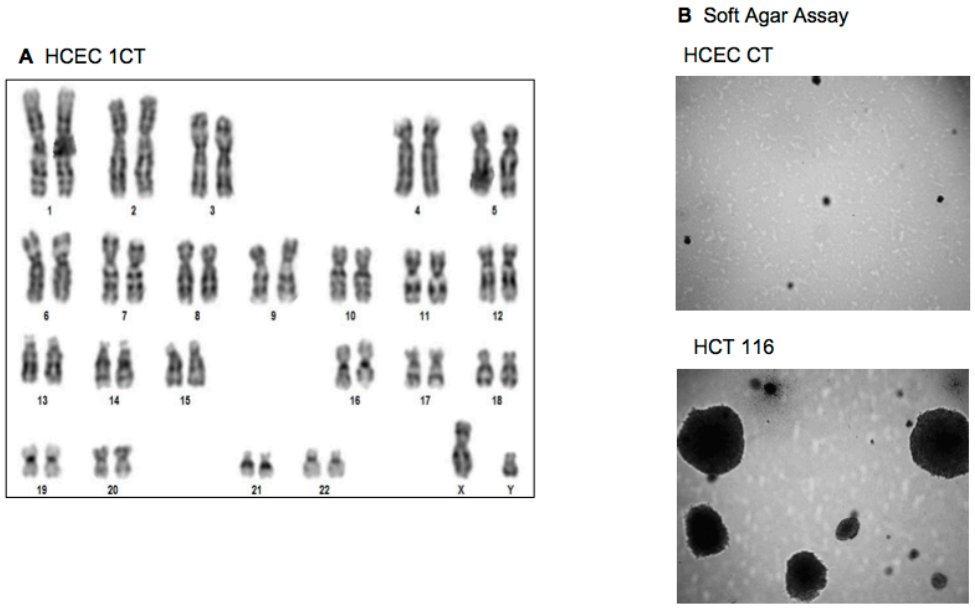

Figure 2-7. HCEC CTs are diploid and do not have tumorigenic features. (**A**) Karyotype of HCEC 1CT. (**B**) HCEC CTs do not make colonies in soft agar (top panel) as do the transformed colonic epithelial cell line HCT 116.

We attribute the initial expansion and long-term propagation of our colonic epithelial cells to the combination of a low oxygen environment and the use of Primaria® plates. It has previously been shown that a tissue culture environment kept under low oxygen (2%-5%) extends the life-time of normal fetal and adult human diploid cells (Forsyth et al. 2003; Packer and Fuehr 1977; Ramirez et al. 2004). We did not observe robust expansion of colonic epithelial nests in normoxic (21%) conditions during the first 12 days after crypt seeding. Once the cells were immortalized with *CDK4* and *hTERT* (HCEC CTs), cells continued to require a combination of low oxygen and Primaria® culture dishes for long-term growth. Therefore, we emphasize that the initial expansion

and successful long-term proliferation of human colonic epithelial cells is dependent on appropriate substrate and low oxygen conditions.

The evidence we have gathered suggests that HCEC CTs have features of the undifferentiated epithelial stem cells found in the bottom of the colonic crypt. For example HCEC CTs express CD29, CD44, LGR5, and BMI1, as do putative stem cells in the lower portions of the intact intestinal and colonic crypts. In addition, a large fraction of HCEC CTs stains for LGR5. Since LGR5 has been described in the actively replicating cells of the intestine and colon (Barker et al. 2007), it may not be surprising that the majority of undifferentiated epithelial cells with proliferative capacity in culture express this marker. Although BMI-1 has been reported to be a stem cell marker of intestinal quiescent cells of mice, we have observed BMI-1 in actively replicating undifferentiated HCECs. This may reflect a fundamental difference between human and mouse intestinal cells or, perhaps, a difference between the small intestine and the colonic stem cell compartments. A second indication that HCEC CTs are undifferentiated precursors of the colon mucosa is their expression of MUC1, as shown by both western blotting and immunostaining. MUC1 has previously been demonstrated to be expressed *in vivo* in cells of the lower third of human crypts, the site where undifferentiated epithelial cells reside (Ajioka, Allison, and Jass 1996; Ajioka et al. 1997). As colonic epithelial cells migrate upwards in the colonic crypt and differentiate into the functional cells of the colon, MUC1 expression diminishes. Thus, the presence of the stem cell markers LGR5, BMI1, CD29, and CD44, and the expression of the transmembrane MUC1 protein, all suggest an undifferentiated stem cell nature of HCEC CTs.

The 3D Matrigel™ environment promoted organotypic differentiation of HCEC CTs and further demonstrated that HCEC CTs have stem cell features. Prior to the Matrigel™ culture experiments, we isolated clones from both HCEC CTs to determine if subsets of daughter cells from one single originating cell have the ability to express multiple lineage associated markers seen in the immortalized populations (DPP4, MUC2, and CGA). With the clonal derivative we did not observe chromogranin A (CGA) in log phase growth cells, and MUC2 and DPP4 were not observed in the growth arrested clones. Instead, varying levels of MUC1 and A33 were observed in the clones (Data not shown).The replication of single HCEC CT cells in Matrigel™ culture into organoid cysts similar to that seen by Sato et al. (Sato et al. 2009) strongly supports a self renewal and self-organizing capacity for the HCECs. Moreover, the presence of apical villin and MUC2 expression in a subset of cells in the organoids indicates a multipotent ability, because villin and MUC2 are lineage markers associated with absorptive and goblet cells, respectively. Our demonstrations of the self-renewal and self-organizing capacities of single cells, as well as the ability to differentiate into various cell lineages all support the stem cell nature of HCEC CTs.

In conclusion, we have successfully immortalized non-neoplastic human colonic epithelial cells from two different patients, have maintained these cells in long-term culture for over a year, and have cryopreserved them for future experimentation. These cells express gastrointestinal stem cell proteins, and have the ability to self-renew, self-organize, and differentiate in 3D Matrigel™ culture. These cells should serve as valuable

reagents for future studies in human colonic epithelial stem cell biology, and for studies on the initiation and progression of colonic diseases, such as colon cancer.

Materials and Methods

Colonic Tissues

This study was approved by the Institutional Review Board on human studies at the Dallas VA Medical Center. Multiple random colon biopsies (20 to 30 samples, ~ 0.5 cm^3) from tissue not involved with endoscopically visible adenomas were obtained from patients undergoing routine screening colonoscopy after obtaining informed consent.

Growth Media and Tissue Culture Substrate

Cells were grown on basal media consisting of 4 parts high glucose DMEM and 1 part medium 199 (HyClone, Logan, UT; from here on referred to as "X" media) supplemented with EGF (100ng/ml) (PreproTech, Inc.; Rocky Hill, NJ), hydrocortisone (1µg/ml), insulin (10 µg/ml), transferrin (2 µg/ml), sodium selenite (5 nanomolar) (all from Sigma Chemical, St Louis, MO), and antibiotic/antimycotic solution consisting of penicillin (10,000 IU/mL), streptomycin (10,000 mg/ml), and amphotericin B (5,000 IU/mL); (Gibco, Gaithersburg, MD); and gentamicin sulfate (50 µg/ml). Cells were cultured in Primaria® tissue culture flasks (BD Biosciences, San Jose, CA). Primaria® is a specially treated plastic substrate that improves attachment via addition of a variety of nitrogen-containing functional groups into the surface in addition to the negatively charged oxygen-containing groups found on standard tissue culture surfaces. HCT 116, human umbilical vein endothelial cells (HUVEC), BJ human skin fibroblasts, and immortalized human colonic fibroblast cells (C26Ci, PD 150) (Forsyth et al. 2004) were

maintained in X media supplemented with 10% cosmic calf serum (HyClone, Logan, UT). C26Ci cells were treated with 10μg/ml mitomycin C (Sigma) for 2 hours and used as feeder layers from the point of initial crypt attachment until the first passage. All cultures were grown in atmosphere consisting of 2%-5% oxygen and 7% carbon dioxide.

Cell Isolation and Immortalization

The intestinal cell isolation techniques previously described were used with some modifications (Booth C 2002; Evans et al. 1992). Briefly, colonic biopsies were immersed in cold X medium containing antibiotic/antimycotic solution and brought to the lab within 40-60 minutes after colonoscopy. Specimens were copiously washed with phosphate buffered saline (PBS) containing antibiotic/antimycotic agents, cut with apposing blades into multiple small pieces (~1mm in size), then exposed to collagenase 150 units/ml (Worthington Biochemical, Lakewood, NJ) and dispase 40 μg/ml (Roche, Germany) for digestion at 37° C for a total of 2.5 hours. After enzymatic digestion the crypts were resuspended in basal X medium with growth supplements including 2% serum, and plated in Primaria® culture dishes seeded 48 hours previously with 50% confluent colonic fibroblast feeder layers. Primary cultures arising from attached cells were allowed to expand in the same media under low oxygen conditions. During the first ten days after attachment cells were fed every 3 days, reducing the serum by 1% each change until 0% to prevent growth of unwanted cells such as pericryptal fibroblasts and endothelial cells. Once small nests of expanding epithelial cells were easily observed, cells were transduced with a retroviral vector containing *Cdk4* [retroviral parent vector pSRαMSU ($G418^+$) expressing mouse *Cdk4* (Charles J. Sherr, St. Jude Children's

Research Hospital, Memphis, TN)] in the presence of 2 µg/mL polybrene (Sigma). This was followed 48 hours later by a retroviral infection with the catalytic component of human telomerase *(hTERT*). When numerous epithelial cell nests were observed (usually 3-4 weeks after initial crypt seeding), the cells were trypsinized and reseeded into another Primaria® culture vessel. Feeder layers were not needed or used for routine tissue culture after the first passage. After viral transduction and first passage, 2% serum was added back to the supplemented basal media, permitting more rapid growth of the cells. Cells were subcultured approximately every 7 days or when confluent using trypsin/EDTA (0.025% and 0.01% respectively; Cascade Biologics, Portland, OR).

Conditions to Induce Growth Arrest of Colonic Epithelial Cells

HCEC CTs seeded at a density of 5.0 x 10^5 on Deckglasser cover-slips (Germany) inside 24 well plastic dishes (BD Biosciences) were allowed to proliferate until sub-confluent and were then exposed to lithium chloride (LiCl 30mM; Sigma) for 8 days or 6-bromoindirubin-3-oxime days (BIO 5.0 µM; CalBiochem) for 3 days in the HCEC supplemented X media with the serum decreased to 0.1% and EGF to 5ng/ml. Depending on the primary antibody to be used for staining, cells were fixed with either a 1:1 mixture of cold methanol and acetone for 5 minutes or neutral buffered formalin for 10 minutes at 2 days after BIO exposure and stored in 4 degrees until staining.

Telomeric Repeat Amplification Protocol

After dissociation from the culture plates, 10^5 cells were aliquoted into a DNase-free, RNase-free 1.5-mL microfuge tube. This aliquot of cells was pelleted by

centrifugation using a tabletop centrifuge (5415D; Eppendorf) at 6,000 rpm for 5 minutes at room temperature. The supernatant was aspirated and discarded, and the remaining cell pellet was frozen at -80°C. The TRAP (Telomere Repeat Amplification Protocol) assay was performed as previously described (Ramirez et al. 2004).

Western Blotting

Cell pellets were washed once in PBS, lysed in an appropriate volume of RIPA or SDS buffer supplemented with protease inhibitors. For detection of CDK4, p16, and MUC1 by western blotting, ~25 μg of cell extract were separated by SDS-PAGE and transferred to PVDF membrane. The primary antibodies used were mouse antibodies against Cdk-4, p16INK4A (both from Chemicon), MUC1 (BD Biosciences), and anti β-actin. All antibodies were diluted in 5% non-fat dry milk/PBST. Primary antibodies were detected using HRP-conjugated secondary antibodies and chemiluminescent substrate (ECL Plus, GE Healthcare, UK).

Immunocytochemistry

Primary antibodies used were mouse monoclonal antibodies against cytokeratin 18 and 20, chromogranin A (Santa Cruz Biotechnology, CA), α-smooth muscle actin (Sigma), β-catenin (Abcam, Cambridge, MA), villin (BD Biosciences), BMI1(Abcam); rabbit polyclonal antibody against ZO-1 (Zymed Laboratories, South San Francisco, CA), A33 (Santa Cruz Biotechnology, CA), LGR5 (Gentex, Irvine, CA), and CD31 (DAKO, Carpinteria, CA) and rabbit monoclonal against GPR49 (LGR5) (Abcam). With the exception of MUC1 and BMI1, cells on coverslips were fixed with a 1:1 mixture of cold

acetone and methanol for 5 minutes, permealized with 0.5% Triton for 10 minutes, and blocked with 5% bovine serum albumin (BSA) (Sigma) for 30 minutes. Cells for MUC1 and BMI1 staining were fixed with neutral buffered formalin for ten minutes and processed the same as above. A drop of Vectashield with DAPI (Vector Laboratories, Burlingame, CA) was added to coverslips and these were subsequently mounted onto glass slides for visualization with an epifluorescence microscope (Zeiss Axiovert 200M).

Tumorigenic Assays

For growth in soft agar, one thousand cells per well were suspended in 0.37% Noble agar (Difco) in supplemented X media and overlaid over 0.5% Noble agar in triplicate 12-well plates. The number of macroscopically visible colonies was counted after 3-5 weeks of growth. For assessment of tumor formation in nude mice 5 x 10^6 cell were injected subcutaneously and maintained in a germ free barrier for up to 6 months or until tumor size required euthanasia.

Matrigel™ Culture

After HCEC CT cultures were trypsinized into a single cell suspension between 200 to 1000 cells were mixed with 100 µL of undiluted Matrigel™ (BD Biosciences) and seeded on top of Deckglasser coverslips. The coverslips with Matrigel™ were submerged under 0.5 ml of supplemented X media for up 15 days. Media changes were performed every 3 days. Organoid fixation was performed *in situ* with 4% paraformaldehyde (PFA). *In situ* processing for immunofluorescence staining with antibodies was performed per standard procedures. Coverslips were mounted on top of

glass slides using Mowiol mounting medium. A Zeiss Axiovert 200M epifluorescence microscope and Leica TCS SP5 confocal microscope were used to visualize organoids.

CHAPTER THREE

FUNCTIONAL PARSING OF DRIVER MUTATIONS IN THE COLORECTAL CANCER GENOME REVEALS NUMEROUS SUPPRESSORS OF ANCHORAGE-INDEPENDENT GROWTH

Introduction

Normal epithelial cells require attachment to the extracellular matrix components for differentiation, proliferation and viability. Loss of matrix anchorage results in a type of programmed cell death called anoikis that is regulated by a variety of different signaling pathways including ERK, JNK and AKT (Chiarugi and Giannoni 2008). In order to become cancer cells, normal epithelial cells need to acquire the ability to grow anchorage-independently, a hallmark feature of cancer and one of the most faithful in vitro indications of tumorigenicity. In contrast, how epithelial tumors acquire anchorage-independent abilities is poorly understood and therapeutic strategies exploiting this process are almost non-existing. Advances in RNA interference techniques have enabled loss-of-function screens to identify tumor suppressors in mammalian cells. These studies have identified genes that restrain anchorage-independent growth in SV40 T-antigen and hTERT immortalized partially transformed fibroblasts (Kolfschoten et al. 2005) and mammary epithelial cells (Westbrook et al. 2005). Lack of overlapping genes from these two screens and identification of different tumor suppressor profiles from in vivo lymphoma (Bric et al. 2009) and liver cancer (Zender et al. 2008) shRNA screens suggest that tumor suppressors are highly context dependent and emphasize the importance of loss-of-function screens using different tissue specific cell types.

Similar to other "hallmarks of cancer" the ability to grow anchorage-independently could be acquired by progressive genetic alterations and represented by some of the mutations already established to occur in tumors. Since the first published cancer genome-sequencing project (Sjöblom et al. 2006), thousands of cancer genomes have been sequenced. One ongoing point of debate concerning these efforts is the cost versus benefit imbalance (Ledford 2010). Many point mutations, duplications, deletions, or small insertions have been reported that had not previously been associated with cancer. The functional role of the vast majority of these mutated genes in cancer initiation, progression or maintenance is unknown. It is believed that many of these mutated genes may be incidental or passenger mutations and thus not driving oncogenic processes. Efforts to identify "drivers" within the cohort of mutated genes have largely been *in silico* (Carter et al. 2009; Youn and Simon 2011) and have not been subjected to rigorous experimental testing using biologically relevant functional assays. To this end, we set out to identify suppressors of anchorage-independent growth within reported colorectal cancer (CRC) mutated genes (Sjöblom et al. 2006; Wood et al. 2007) using otherwise isogenic K-*ras*^{V12} expressing or p53 knocked-down hTERT immortalized diploid human colonic epithelial cells (HCECs) (Roig et al. 2010). This approach revealed a profound enrichment for driver tumor suppressors within CRC *CAN*-genes.

Results and Discussion

Anchorage-independent growth suppressor screen

With the exception of *APC* truncation mutations, *K-RAS*^{V12} and *TP53* alterations represent two of the most frequent mutations in CRC (Wood et al. 2007; Sjöblom et al.

2006) and either alone or in combination slightly enhance colony formation efficiency of HCECs in soft-agar. However, even in HCECs with both of these oncogenic alterations, soft-agar growth is considerably less robust compared to that of an established CRC cell line, HCT116 (Figure 3-1A,B). Furthermore, none of these HCEC derivatives formed tumors when injected either subcutaneously or under the renal capsule of immunocompromised mice (data not shown). Therefore, these premalignant HCECs provide a sensitized background that might allow discovery of context-dependent (i.e. cooperation with a specific oncogenic alteration) and context-independent (i.e. cooperation with both oncogenic alterations) tumor suppressors.

A soft-agar based shRNA screen against *CAN*-genes (Sjöblom et al. 2006; Wood et al. 2007) in a "one-shRNA-one-well" format (Figure 3-1C) revealed 49 context-independent and 16 context-dependent progression suppressors (Figure 3-1D, Appendix A). Although our approach by its nature was not exhaustive, the number of identified progression suppressors was unexpectedly high. To test if the high rate of tumor suppressors discovered within *CAN*-genes was due to a more permissive cellular system, we assayed 4 plates from the whole genome shRNA library each plate containing at least one shRNA against one of the confirmed genes from primary screen. All five shRNAs that scored in the primary screen were also identified in this screen whereas only 5 out of 362 random shRNAs enhanced soft agar growth (Appendix B) – a background rate of 1.4% as opposed to 48% observed against CRC-*CAN* genes. These results provide strong evidence against off target effects or artifacts that may have resulted from global perturbation of miRNA biogenesis and suggest that the assay itself is extremely robust.

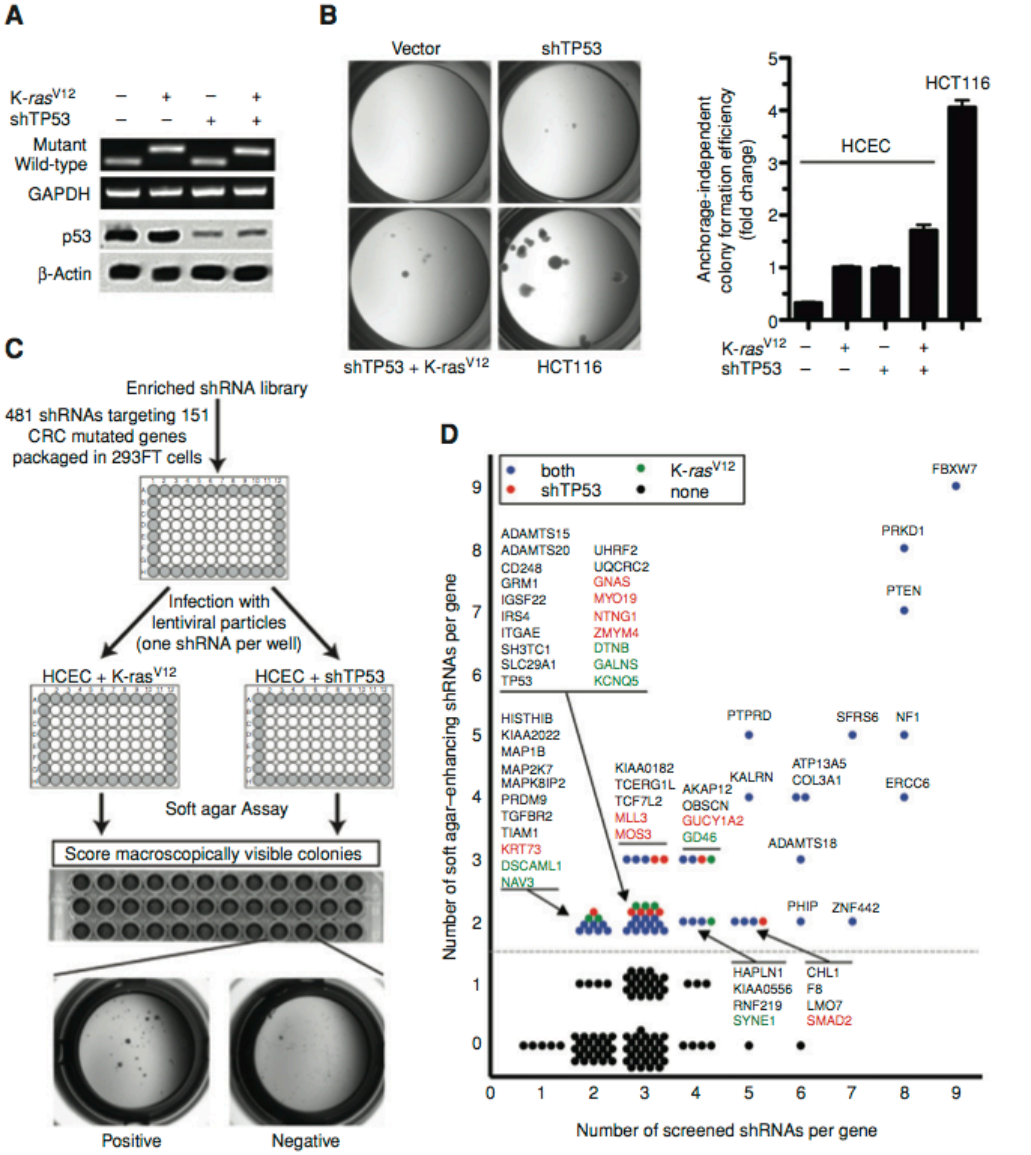

Figure 3-1. Identification of tumor suppressors within CRC *CAN*-genes with an enriched shRNA library using isogenic HCECs. A, total RNA from immortalized HCECs and their oncogenicly progressed derivatives expressing K-*ras*^{V12}, shTP53 or both were subjected to RT-PCR followed by a diagnostic restriction digestion assay to detect wild type and mutant *KRAS* alleles. Whole-cell extracts from same cells were immunoblotted for expression of p53. B, each cell line were cultured in soft-agar in 96-well plates for three weeks and photographed with a stereomicroscope. Quantification of colonies (>0.1 mm) in. Bars represent 12 data points (3 separate experiments in quadruplicates), mean ± s.e.m. C, schematic representation of the strategy. HCECs expressing K-*ras*^{V12} or shRNA against p53 were infected with lentiviral shRNA constructs in "one-shRNA-one-well" format and the ability of cells to form macroscopic soft-agar colonies were assessed after three weeks. D, scatter plot showing the overall results of the screens. Each circle represents a gene. Context-dependent anchorage-independent growth suppressors are shown in green (K-*ras*^{V12} specific hits) and red (shTP53 specific hits) where as context-independent suppressors are shown in blue. All genes, except those isolated only in K-*ras*^{V12} context, were plotted according to results obtained from shTP53 context.

The observation that oncogenic Ras expressing cells are more resistant to p53-dependent apoptosis (Ries et al. 2000) may account, in part, for the large number of overlapping hits in mutant *KRAS*^{V12} and *TP53* knockdown backgrounds. Accordingly, *KRAS*^{V12} expressing HCECs failed to activate p53 induced pro-apoptotic targets such as p21 and Bax despite having wild type levels of p53 and were able to phosphorylate p53 upon radiation exposure (Figure 3-2). These findings suggest that shRNAs identified in both *KRAS*^{V12} and *TP53* knockdown backgrounds may cooperate with deregulated p53 signaling.

Suppressors of anchorage-independent growth identified in this screen fall under a variety of different signaling pathways and biological processes (Table 3-1). Furthermore, on average 37% of all *CAN*-genes in any individual colon cancer are involved in matrix dependent proliferation (Table 3-2). Candidate tumor suppressors identified in this study were uniformly contributed from all samples rather than a few tumors with highly deregulated matrix dependent proliferation.

In order to further minimize off-target effects, we only considered genes as candidates if two or more shRNAs enhanced soft agar growth. Quantitative re-testing of soft-agar enhancing shRNAs confirmed all tested genes with at least one shRNA with comparable or greater enhancement to K-*ras*^{V12} and an attrition rate of 8% (3 out of 36 shRNAs did not confirm, arrows, Figure 3-3). Importantly, shRNAs against well-known tumor suppressors (*FBXW7*, *NF1*, *PTEN*, *TGFBR2* and *TP53*) scored positive whereas genes with established oncogenic gain of function (*KRAS*, *PIK3CA* and *RET*) did not scored in the loss of function assay as might have been expected. Analysis of the non-

scoring, well-described tumor suppressor genes such as *APC* and *SMAD4* showed that none of the screened shRNAs against these two genes were effective in decreasing

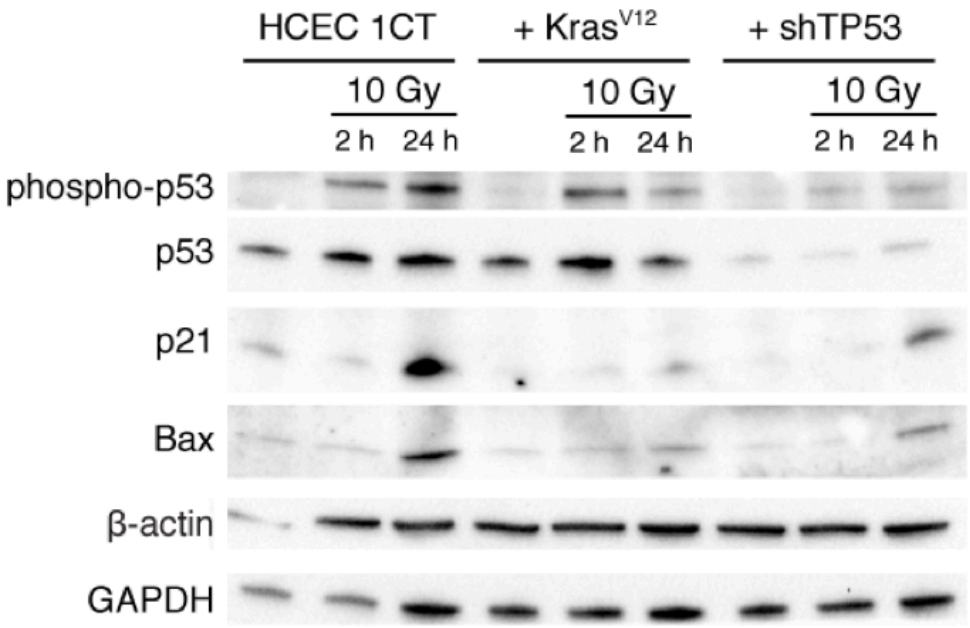

Figure 3-2. K-ras^{V12} expressing cells are resistant to p53 induced apoptosis. Whole-cell extracts from immortalized K-ras^{V12} or p53 down regulated HCECs were immunoblotted with p53 and its down-stream effectors after 10 Gy gamma-radiation.

Common function	Candidate tumour suppressor
Apoptosis	CHL1, CNTN4, DSCAML1[#], MAP2, OBSCN, PRKD1, PTEN, TP53
ATPases	ATP13A1, ATP13A5, ERCC6, MYO19*
Cell adhesion	ADAMTS15, ADAMTS18, ADAMTS 20, CD248, CHL1, CNTN4, COL3A1, DSCAML1[#], F8, HAPLN1, IGSF22, ITGAE, OBSCN, PTEN, PTPRD, TCERG1L
Chromatin remodelling & transcription	ERCC6, HIST1H1B, KRT73*, MLL3*, PRDM9, SFRS6, SMAD2*, TAF2, TCERG1L, TCF7L2, TP53, UHRF2, ZMYM4*
Cytoskeleton remodelling	AKAP12, DTNB#, LMO7, MAP1B, MAP2, MYO19*, SYNE1[#]
ECM remodelling	CD248, COL3A1, ADAMTS15, ADAMTS18, ADAMTS20, F8, HAPLN1, PTPRD
JNK signalling	MAP1B, MAP2, MAP2K7, MAPK8IP2, TP53
Nitrous oxide signalling	GUCY1A2*, NOS3*
Small GTPase regulators	IGSF22, KALRN1, NF1, MYO19*, OBSCN, TIAM1
TGF-beta signalling	SMAD2*, TGFBR2
Ubiquitin-protein ligase	FBXW7, UHRF2
Uncategorized	CD46[#], GALNS[#], GNAS*, GRM1, IRS4, KCNQ5[#], NAV3[#], NTNG1*, SLC29A1, UQCRC2
Unknown	C15orf2, FAM161A, KIAA0182, KIAA0556, KIAA2022, PHIP, RNF219, SH3TC1

Table 3-1. Categorization of candidate tumor suppressor genes. This table categorize candidate tumor suppressor genes into a common function or process based on gene ontology molecular function, biological process and/or literature curation. ECM: extracellular matrix. * enhanced soft agar growth only in p53 down-regulated cells or [#] only in K-ras^{V12} expressing cells.

Tumor*	All mutated genes*	CAN genes*	AI genes~	AI gene symbols~
co108	82	15	4	OBSCN, NTNG1, NOS3, IRS4
co74	123	27	9	TP53, TGFBR2, SYNE1, SFRS6, PRKD1, MLL3, GALNS, FBXW7, CNTN4
co92	90	15	4	TP53, MAP2, KRT73 HAPLN1, AKAP12
mx22	69	14	5	ZMYM4, TCERG1L, MAP1B, KIAA0182, GNAS
mx27	86	16	6	TIAM1, MYO19, KALRN, IGSF22, F8, DTNB
mx30	57	9	3	SMAD2, PRDM9, MAP2K7
mx32	76	19	8	UHRF2, TP53, SYNE1, NAV3, KCNQ5, FAM161A, ATP13A5, ADAMTS18
mx38	77	14	8	TP53, SH3TC1, RNF219, NF1, NAV3, KIAA2022, ITGAE, ERCC6
mx41	99	26	14	UQCRC2, TCF7L2, TAF2, SYNE1, PHIP, OBSCN, LMO7, GUCY1A2, GRM1, COL3A1, CD248, ATP13A1, ADAMTS20, ADAMTS15
mx42	81	14	6	TP53, PTPRD, MAPK8IP2, KIAA0556, DSCAML1, C15orf2
mx43	102	18	5	SYNE1, SLC29A1, PTEN, HISTH1B, CHL1

Table 3-2. Distribution of anchorage independent genes for each sequenced sample. This table lists 11 completely sequenced colon cancer samples with the number of all genes (Column 2) and *CAN*-genes (Column 3) mutated in each sample. Genes involved in anchorage independent growth that were identified through a loss of function screen in immortalized human colonic epithelial cells expressing either mutant K-*ras* or shRNA against *TP53* are listed in column 4. Approximately 37% of all *CAN*-genes in an individual tumor enhance anchorage independent growth when downregulated in these sensitized non cancer cells. * Data from Wood et. al., ~ Data from this study. AI: Anchorage independent growth.

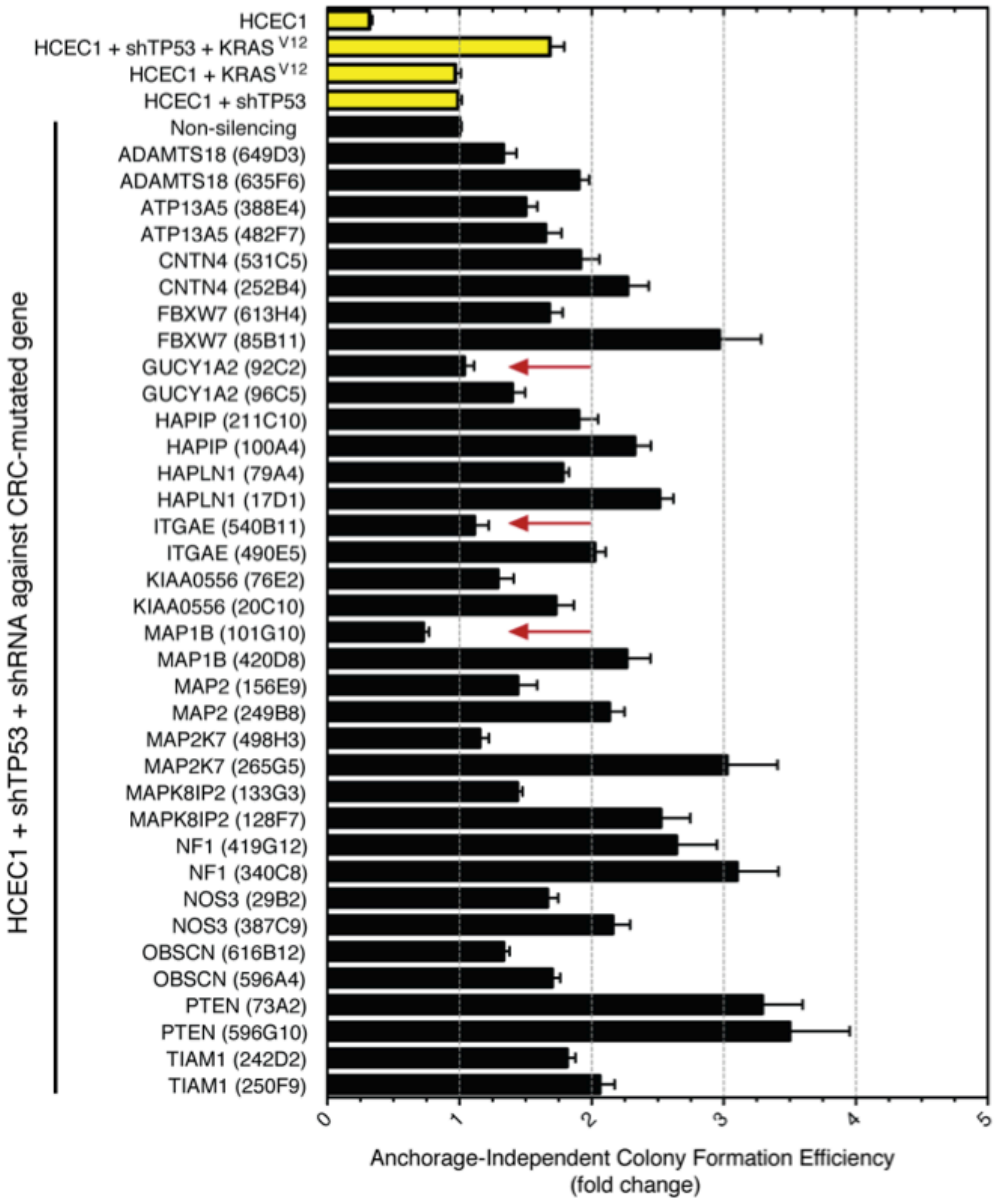

Figure 3-3. Quantitative validation of selected shRNAs for their ability to enhance soft-agar growth of immortalized shTP53 expressing HCECs. Each bar represents 8 data points (quadruplicates from two separate experiments). Arrows denote shRNAs that failed to enhance anchorage-independent growth in a statistically significant manner. Enhancement for all other shRNAs were significant (two tailed Student's t-test, compared to none, mean ± s.e.m., $P<0.05$).

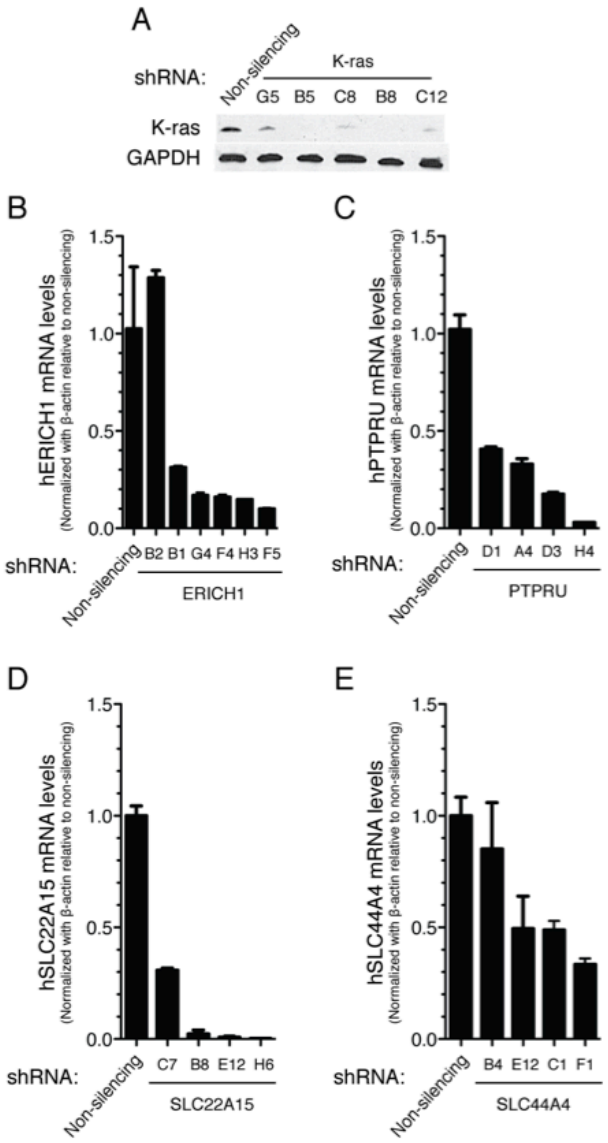

Figure 3-4. Ability of shRNAs to knockdown expression was demonstrated by A, immunoblotting for K-ras or B-E, Quantitative RT-PCR for ERICH1, PTPRU, SLC22A15 and SLC44A4 48 hours after transfection into 293FT cells. Two out of 23 tested shRNAs did not provide any knockdown.

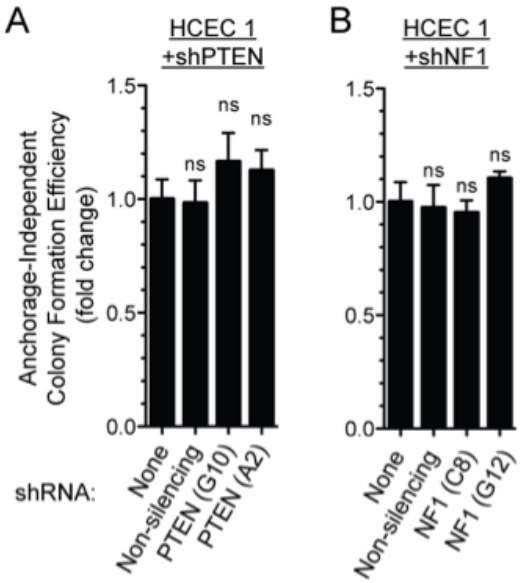

Figure 3-5. shRNAs against A, PTEN and B, NF1 do not enhance soft agar growth in HCECs without oncogenic manipulations (Student's t-test, compared to none, mean ± s.e.m., ns= non-significant).

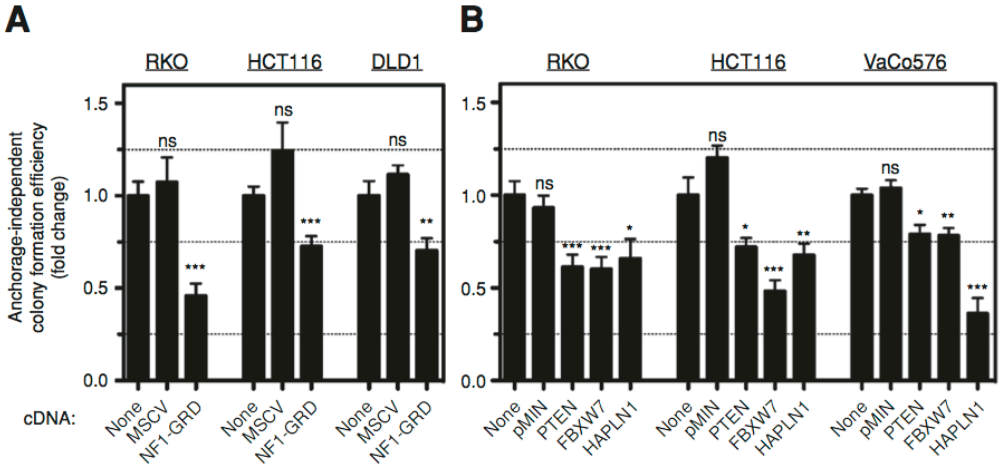

Figure 3-6. Functional validation of candidate tumor-suppressors. Ectopic expression of candidate tumor suppressors by A, retroviral or B, lentiviral vectors in colon cancer cell lines leads to decreased soft-agar growth. Triplicates from two separate experiments, two tailed Student's t-test, compared to none, mean ± s.e.m., ns= non-significant, **P*<0.05, ***P*<0.01, ****P*<0.001.

protein levels (data not shown). This is an exception within the library, as analyses of 23 non-scoring shRNAs for their ability to down regulate their target gene revealed only two non-functional shRNAs (Figure 3-4). Quantitative testing of PTEN and NF1 (Figure 3-5) or screening of approximately half of the shRNA library using immortalized HCECs without $KRAS^{V12}$ or *TP53* alterations did not reveal any gene that enhanced anchorage-independent growth when knocked-down (data not shown). This suggests that candidate tumor suppressors identified in this screen are important for progression but not initiation of CRC.

Ectopic expression of most potent candidates

We next ectopically expressed some of the most potent candidate tumor suppressors, selected by quantitative re-testing (Figure 3-3) in established colon cancer lines and measuring their ability to decrease anchorage-independent growth. Almost all tested cDNAs in all tested colon cancer lines decreased soft-agar growth with varying degrees (Figure 3-6). An interesting genotype-phenotype correlation emerged from NF1 GTPase-activating protein related domain (GRD) expression studies. NF1-GRD has been shown to be sufficient to inhibit wild type KRAS (Hiatt et al. 2001). Accordingly, we observed a 50% reduction in anchorage-independent growth in RKO cells with two wild type *KRAS* alleles whereas the reduction was only 25% in HCT116 and DLD1 which carry one mutant (G13D) and one WT allele (Figure 3-6A) without any change in their monolayer growth kinetics (data not shown). shRNAs against NF1 enhanced soft-agar growth in both p53 down-regulated and, surprisingly, in oncogenic KRAS expressing

HCECs with similar potencies that are proportional to the level of NF1 knockdown (Figure 3-7). Considering the majority of KRAS transcripts in these cells are the mutant form (Figure 3-1A) that cannot be inactivated by NF1, involvement of NF1 in an additional pathway to enhance soft-agar growth is suggested by these observations. Recently, it has been shown that another Ras GTPase activating protein, DAB2IP2, can activate both Ras and nuclear factor-kappaB (NF-kB) pathways (Min et al. 2010). However, examination of shNF1 expressing cells did not reveal increased NF-kB activation in HCECs (Figure 3-7B). The mechanism underlying increased anchorage-independent growth in response to NF1 knockdown in oncogenic KRAS expressing cells has yet to be identified. Importantly, we have also confirmed the ability of *HAPLN1*, a novel candidate tumor suppressor, to decrease anchorage-independent growth in a variety of colon cancer cell lines (Figure 3-6B). The magnitude of inhibition was greatest when wild type *HAPLN1* was introduced into cells carrying *HAPLN1* mutations (VaCo576, Figure 3-6B).

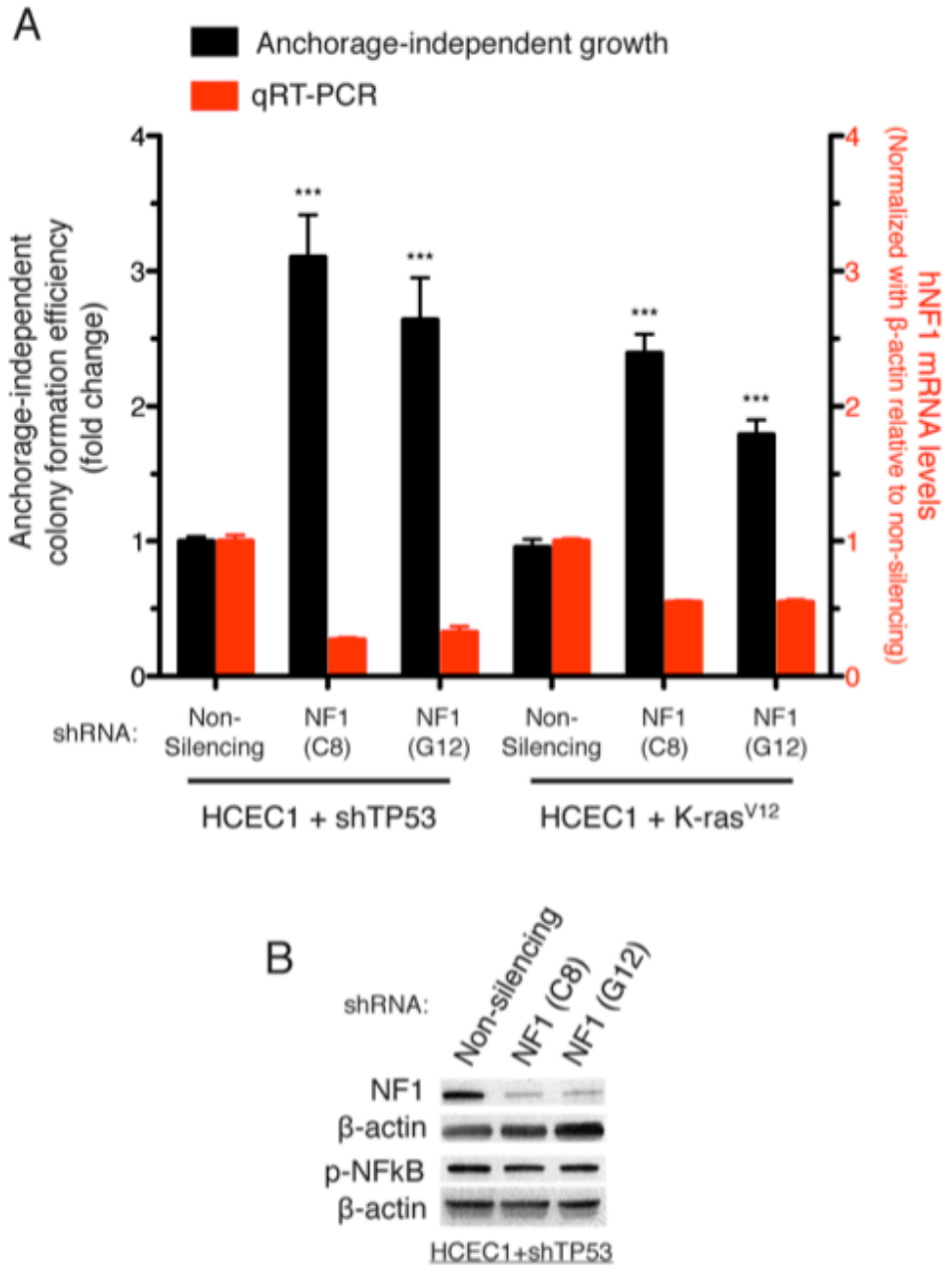

Figure 3-7. A, shRNAs against NF1 enhance soft agar growth HCECs both in shTP53 and oncogenic K-ras background (left axis, black bars, two tailed Student's t-test, compared to non-silencing, mean ± s.e.m., ***P<0.0001) and provide knock down measured by quantitative RT-PCR analysis for human NF1 mRNA (right axis, red bars). B, Whole-cell extracts from shTP53 expressing cells were immunoblotted for the indicated antibodies.

JNK signalling is a master suppressor of anchorage-independent growth

As another proof of concept, we next focused on c-JUN N-terminal kinase (JNK) signaling pathway not only because it was the most enriched pathway (Figure 3-8A) but also because *MAP2K7* and *MAPK8IP2* were the most potent hits without previously established tumor suppressive functions (Figure 3-3). The role of JNK signaling in cancer is controversial with evidence for both oncogenic and tumor suppressor activities largely due to opposing roles of JNK targets (Wagner and Nebreda 2009; Bode and Dong 2007). These opposing effects are also regulated by the duration of JNK activating signal: transient activations promote survival, while sustained JNK activity (1-6 hours) promotes apoptosis (Ventura et al. 2006). Accordingly, switching HCECs to detached culture conditions results in rapid phosphorylation of JNK, which is sustained for 24 hours (Figure 3-8B). JNK activation increases p53 levels and stabilizes MAP2, which in turn leads to apoptosis (Soltani et al. 2005) as demonstrated by increased cleaved PARP levels (Figure 3-8B). shRNAs against upstream activating kinase MAP2K7 and scaffold protein MAPK8IP2 enhances anchorage-independent growth by 3 and 2.5 fold, respectively (Figure 3-8C). Importantly, anchorage-independent growth induced JNK activation was abrogated in HCECs expressing shRNAs against *MAP2K7* or *MAPK8IP2* (Figure 3-8D). Taken together these results suggest that HCECs normally undergo JNK induced apoptosis in response to loss of attachment (anoikis) and that down-regulation of *MAP2K7* and *MAPK8IP2* enhances anchorage-independent growth by preventing activation of JNK. These results were corroborated in HCT116 cells with *MAP2K7* siRNA knockdown or over-expression (Figure 3-9). Interestingly, shRNAs against *MAP2K7*, but not *MAPK8IP2*, also enhanced invasion through Matrigel^{TM} by 3-fold in

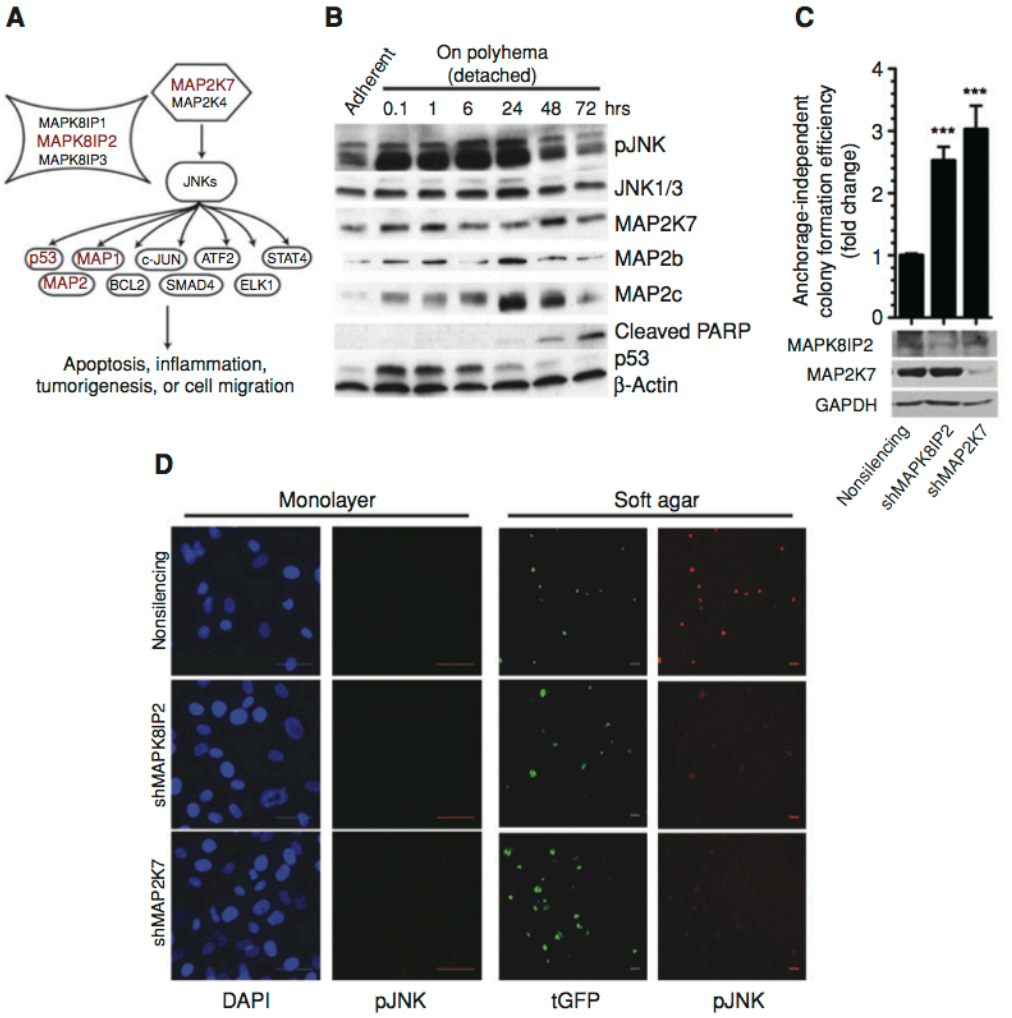

Figure 3-8. Identification of JNK pathway as suppressor of anchorage-independent growth. A, schematic representation of JNK pathway. shRNAs against five members of this pathway (red) was found to enhance soft agar growth. B, whole-cell extracts from immortalized HCECs were immunoblotted for JNK pathway members in response to loss of attachment. C, Fold change in soft agar colony formation efficiency in response to MAPK8IP2 or MAP2K7 depletion in shTP53 expressing HCECs (bars represent 16 data points from two separate experiments, two tailed Student's t-test, mean ± s.e.m., ***P<0.0001). D, Phosphorylated (active) JNK immunofluorescence staining of cells in C, either in monolayer or soft-agar culture (bars: 50 µm, tGFP is encoded from the shRNA vector, 24h after seed).

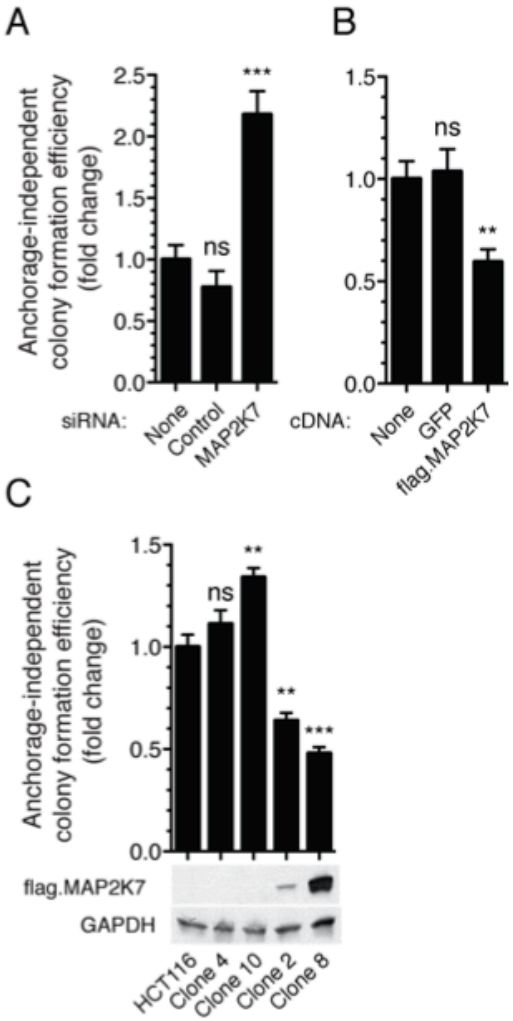

Figure 3-9. A, HCT116 cells were transfected with control or MAP2K7 siRNAs, cultured in soft agar for two weeks and colonies larger than 0.2mm were counted. Each bar represents 8 data points (quadruplicates from two separate experiments). B, HCT116 cells were transfected with control or flag tagged MAP2K7 cDNAs, cultured in soft agar for two weeks and colonies larger than 0.2mm were counted. Each bar represents 8 data points (quadruplicates from two separate experiments). C, Cells in B selected for G418 resistance that is co-expressed from flag.MAP2K7 cDNA vector. Parental cells, two clones without detectable flag expression (as a control for cloning protocol and clonal variation) and two with varying flag expression were cultured in soft agar for a week and colonies larger than 0.2mm were counted. Each bar represents 6 data points (triplicates from two separate experiments, two tailed Student's t-test, compared to none, mean ± s.e.m., ns= non-significant, ***P*<0.001, ****P*=0.0001).

HCECs (Figure 3-10) supporting the idea that a different set of genes is involved in invasion through basement membrane, a later stage in carcinogenesis.

Discovery of additional tumor suppressors using network analysis

Given the high degree of enrichment in JNK signaling pathways we next asked whether other hits could be used as stepping-stones to identify novel tumor suppressors. Initial CRC genome resequencing efforts identified ~850 mutated genes; however only ~150 of these mutated genes were included in *CAN*-genes and considered drivers based on frequency (Sjöblom et al. 2006; Wood et al. 2007). To identify relevant mutated tumor suppressors within genes that are otherwise considered "passengers", we constructed an interaction map of the candidate tumor suppressors identified in this anchorage-independent screen with ~700 additional CRC mutated genes (Figure 3-11). Importantly, down regulation of 5 of the 6 tested mutated genes (*FBXL2, HUWE1, PAK6, PRKDC1* and *TP53BP1*) that interacted with confirmed hits in the primary screen also enhanced soft agar growth (Figure 3-12). These enhancements were comparable to that of ectopic expression of oncogenic K-*ras* in p53 down-regulated HCECs (Figure 3-3). This analysis provides a rationale framework to further test additional mutated genes to potentially identify more "druggable", colon cancer candidate tumor suppressors and demonstrate the need for biological assays to distinguish between driver and passenger genes instead of relying solely on bioinformatics.

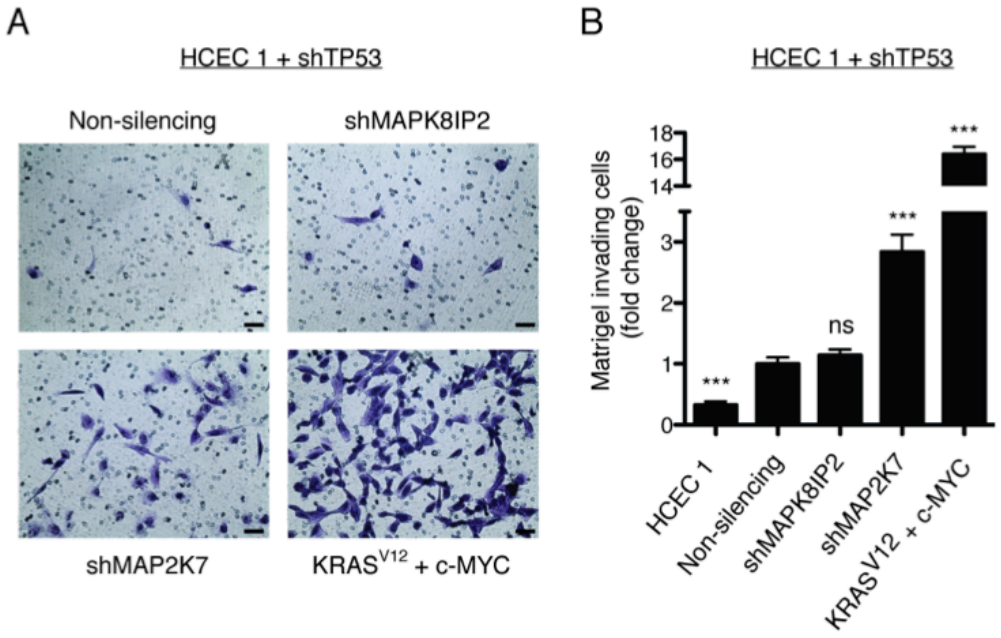

Figure 3-10. shRNAs against *MAP2K7*, but not *MAPK8IP2*, enhance matrigel invasion of HCECs**.** A, Representative images (bars: 50 μm) B, quantification of A (two tailed Student's t-test, compared to non-silencing, mean ± s.e.m., ***P<0.0001).

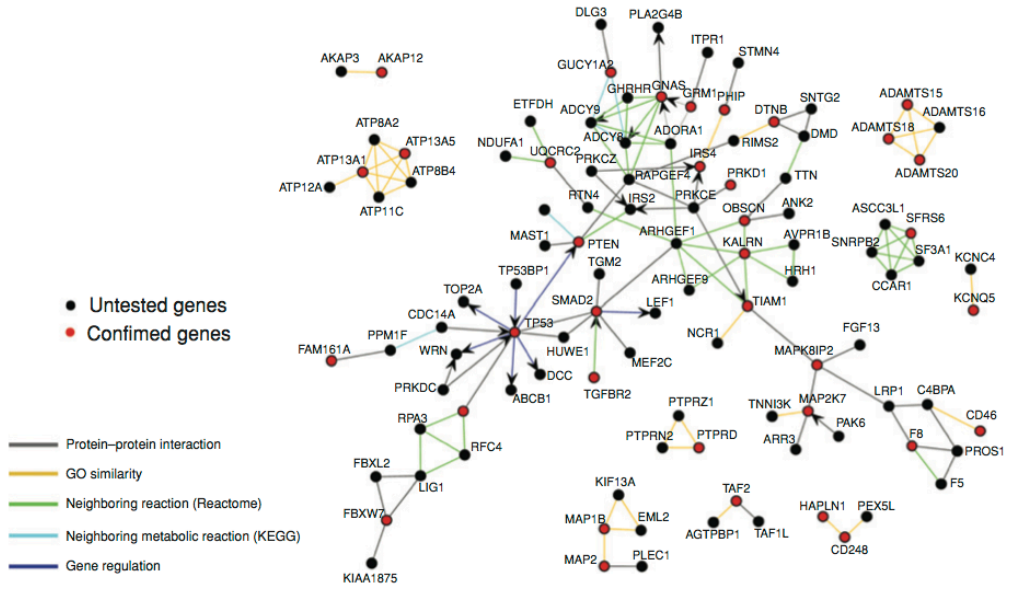

Figure 3-11. Discovering tumor suppressors from less frequently mutated genes with interaction mapping. Plot of the network of interactions between the confirmed genes (red nodes) and other mutated genes from Wood *et al*. Interactions are colored according to the source and type of interactions as shown in the color key. Untested genes were included in the network plot only if they had an interaction with at least one of the confirmed genes, and genes with no interactions (confirmed or untested) were removed for the purpose of clarity.

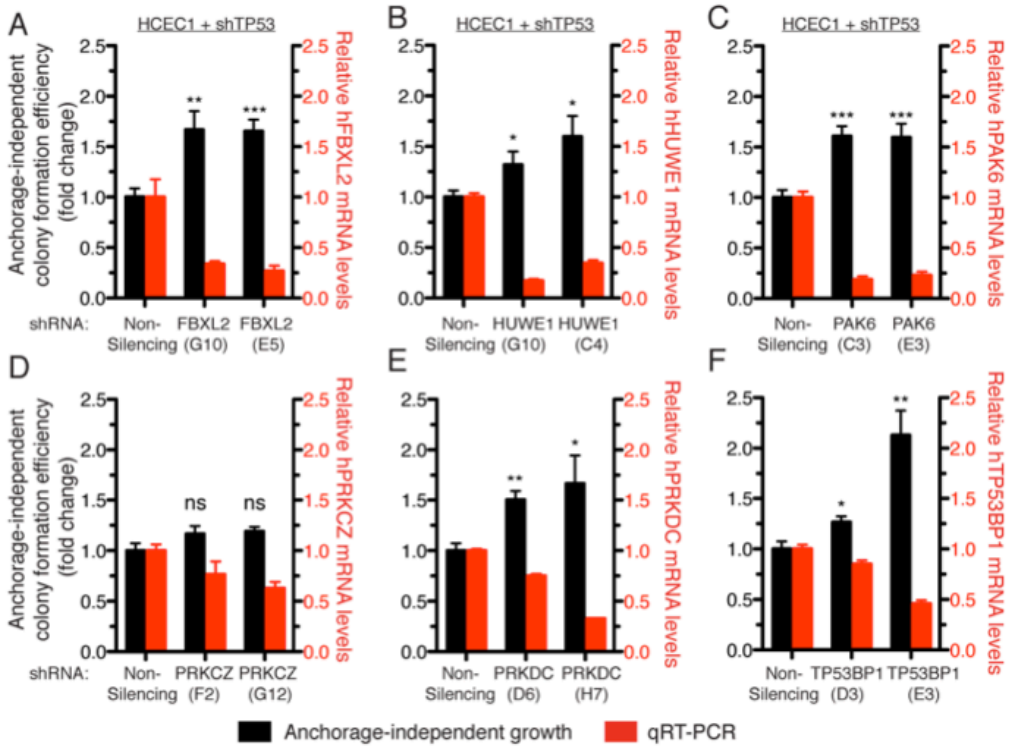

Figure 3-12. shRNAs against A, *FBXL2*; B, *HUWE1*; C, *PAK6*; D, *PRKCZ;* E, *PRKDC* and F, *TP53BP1* were tested for their ability to enhance anchorage-independent growth in p53 down-regulated cells (left axis, black bars, two tailed Student's t-test, compared to non-silencing, mean ± s.e.m., **P*<0.05, ***P*<0.005, ****P*<0.0001)) and provide knockdown measured by quantitative RT-PCR analysis for human mRNA levels (right axis, red bars).

In summary, this study functionally interrogated genes mutated in CRC and showed that cancer genome sequencing provides a valuable enrichment for cancer driver genes. More specifically our study discovered (using a relevant transformation assay) that a remarkable fraction of *CAN*-genes are tumor suppressors involved in cell autonomous anchorage-independent growth. Highly fragile tumor suppressor processes could, in part, explain the extensive heterogeneity observed in primary cancers: there are multiple independent genes abrogating various pathways that pre-cancerous cells acquire in order to progress. This approach not only permits identification of causal cancer genome mutations, but also provides a roadmap for the interrogation and identification of important tumor suppressors in the much larger list of putative cancer genes and reveals the need to implement functional significance filters when identifying driver cancer genes.

Materials and Methods

Plasmids.

CDK4 was expressed together with G418 from retroviral pSRaMSU vector. hTERT was cloned from pGRN145 to pMIN-Ub-IRES-Blast lentiviral vector co-expressing blastocidin resistance marker (both vectors were provided by Geron Corporation). pSRZ-shTP53 and pBABE-hyg-KRAS^{V12} are described elsewhere (Sato et al. 2006). Flag tagged MAP2K7 was in pcDNA3.1 neo backbone (a gift from L. Lum). MSCV-GRD-Pac retroviral vector was kindly provided by W. Clapp and used to express

NF1 GTPase-activating protein related domain. PTEN cDNA was obtained from mammalian gene collection and cloned in to pMIN based lentiviral vector. FBXW7 and HAPLN1 cDNAs were gifts from L. Lum and were cloned in to pMIN lentiviral vector. All constructs were sequence verified.

Viral transductions.

For retrovirus production 2 μg of the appropriate vector was transfected into Phoenix A cells in 6cm dishes with Effectene reagent (Qiagen). For lentivirus production 1 μg of the appropriate vector together with 1 μg of helper plasmids (0.4 μg pMD2G and 0.6 μg of psPAX2) were transfected into 293FT cells with Effectene reagent (Qiagen). Viral supernatant were collected at 48h after transfections and cleared through 0.45 μm filter. Cells were infected with viral supernatants containing 4 μg ml^{-1} poybrene (Sigma) and selected with appropriate antibiotics.

Cells.

HCEC growth media and tissue culture conditions are described elsewhere (Roig et al. 2010). 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^{-1}) and blastocidin (2.5 μg ml^{-1}). $KRAS^{V12}$ and shRNA against p53 were introduced with retroviruses and oncogenic KRAS expression was verified as described (Eskiocak et al. 2010; Sato et al. 2006). Human colon cancer cell lines (HCT116, DLD-1, RKO and LoVo) and virus producing cell lines (293FT, Phoenix A) were cultured in basal medium supplemented with 10% serum. Fully sequenced colon

cancer cell lines (VaCo) were provided by J. Wilson and cultured as described (Sjöblom et al. 2006). Identity of all cell lines were verified by DNA fingerprinting.

Screen.

481 shRNAs against CRC mutated genes were arrayed in 96-well plates and transfected into 293FT cells together with helper plasmids – pMD2G and psPAX2. Viral supernatants were collected 48 hours later and were passed through Multiscreen HTS 0.45μm filter plates (Milipore). HCECs were infected with filtered viral supernatants containing 4 μg ml^{-1} of polybrene (Sigma) at multiplicity of infection (MOI) of ~1. Successfully infected cells were selected with 1 μg ml^{-1} puromycin and seeded in 0.375% Noble agar (Difco) on top of 0.5% pre-solidified agar in 96-well plates in "one-shRNA-one well" format. After 21 days wells were stained with 0.005% crystal violet and scored for macroscopically visible colonies. For the random shRNA screen, we assayed 4 plates from the whole genome shRNA library as purchased from Open Biosystems. These 4 plates were chosen because they each contained at least one shRNA that scored in our primary screen. This setting ensured consistency with the primary screen and enabled us to screen 362 random shRNAs. Clone IDs for each shRNA used in this study is provided in Supplementary Tables S1 and S2, and could be used to retrieve target sequence as well as detailed information about the vectors from Open Biosystems.

Anchorage-independent colony formation assay.

Generated cells were seeded in soft-agar as described (Eskiocak et al. 2010) with following modifications: cells were seeded in two different densities (adjusted depending

on the cell type) in 24-well plates, each density was seeded at least in triplicates and each assay was performed at least from two 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 to be used as normalization control to correct for plate-to-plate variations. Data is plotted as fold change compared to non-silencing or vector only infected cells. GraphPad Prism 5 (GraphPad Software, Inc.) was used to plot data and perform two tailed Student's t-tests.

Transient transfections.

For cDNA expression experiments 2 million HCT116 cells were transfected with 1 µg of flag.MAP2K7 or pMAX.GFP construct using Effectene as described above. Cells were collected and seeded in soft agar 24 hours after transfection, cultivated for 10 days and analysed as described above. Remaining cells were selected with 800 µg ml^{-1} G418 to isolate clones with stable integrations. For siRNA experiments, 100nM pooled siMAP2K7 (M004016, Dharmacon) or siControl (D001206, Dharmacon) was transfected to HCT116 cells using RNAiMAX (InVitrogen) according to manufacturer's instructions. Cells were collected and seeded in soft agar 24 hours after transfection, cultivated for 10 days and analysed as described above.

Immunofluorescence.

Cells were either seeded in monolayer or in soft-agar in chamber slides. Twenty four hours after seeding, cells were fixed and stained as previously described (Eskiocak et al. 2010) with phospho-JNK antibody (Cell Signalling, 9251).

*Immunobloting***.**

Immunoblotting studies were performed as described previously (Eskiocak et al. 2010) with following antibodies: p53 (Biomeda), phospho-JNK (Cell Signalling, 9251), JNK1/3 (Santa Cruz, SC-474), MAP2K7 (Santa Cruz, SC-25288), MAP2 (BD, 5190021018), Cleaved PARP (Cell Signalling, 9541), b-actin (Sigma, A1978), MAPK8IP2 (Abcam, 65211), GAPDH (Cell Signalling, 2118), APC (Calbiochem, OP44), KRAS (Santa Cruz, SC-30), PTEN (Cell Signalling, 9552), Flag (Sigma, F3165), NF1 (Santa Cruz, SC-67), phospho-NF-kB (Cell Signalling, 3031).

qRT-PCR.

qRT-PCR assays were performed as described previously(Eskiocak et al. 2010) for the following target genes: ERICH1 (5p primer, tgagccagaaacatgctgag; 3p primer, ccgctggcagtgtagagc), PTPRU (5p primer, ggagcaagtgcgaatcca; 3p primer, gaagtgttgaccatcaagtaggag), SLC22A15 (5p primer, ttgtcttattgtaatgtttcttccaga; 3p primer, gttaaaggcagcactgatggt), SLC44A4 (5p primer, cctggtattggattcttgttgc; 3p primer, ggcgcagaagcaagataaac), NF1 (5p primer, tgtacccatctattcaagcaaaaa; 3p primer, agtacaacatcaagcagatctgtaatc).

Network analysis.

The comprehensive network of human genes was previously described (Komurov et al. 2010). Briefly, the network was constructed by compiling protein-protein interactions in HPRD (Mishra et al. 2006), Gene (Maglott et al. 2007), BIND (Bader et al. 2001) and IntAct (Kerrien et al. 2007). Signaling interactions were compiled from

Biocarta and KEGG (Kanehisa and Goto 2000) as well as through manual curation. Transcription factor-target interactions were obtained from ORegAnno (Griffith et al. 2008) and TRANSFAC (Wingender et al. 2000). Functional interactions between genes were constructed based on the significance of overlap of their Gene Ontology (Ashburner et al. 2000). These interactions were supplemented by neighbouring interactions from the Reactome (Joshi-Tope et al. 2005) and KEGG metabolic interactions. For the latter, an interaction to a pair of genes was assigned if the reactions performed by their respective enzymes shared a metabolite (e.g. Hexokinase II and glucokinase, shared metabolite: glucose phosphate).

CHAPTER FOUR

DISCUSSION AND FUTURE DIRECTIONS

Prior to our work, previous reports have demonstrated that primary human colonic epithelial cells in conventional monolayer cultures are short-lived, with a majority of the explanted epithelial cells being committed to a differentiated phenotype and dying within forty-eight hours after isolation (Kaeffer 2002). One possible explanation for the failure to sustain human colonic cells with epithelial characteristics in long-term monolayer cell culture is the inability to reproduce in the tissue culture environment the complex interplay of various signaling pathways (Hardwick et al. 2008; Katoh 2007; Reya and Clevers 2005; Mishra et al. 2005) responsible for promoting epithelial properties and cellular differentiation in the colonic crypt. It is also possible that once a colonic cell is exposed to the multiple crypt signals and committed to differentiation it would have a similarly short lifetime in cell culture as seen *in vivo,* where the usual transit time from the bottom of the crypt to apoptosis and sloughing at the top takes 5-7 days (Marshman, Booth, and Potten 2002). Therefore, successful long-term growth of human colonic epithelial cells may only be possible with stem-like progenitor cells that have not been exposed to the interplay of crypt signals and committed to differentiation.

The isolation of such cells with long-term growth capacity as well as a reliable procedure to accomplish immortalization sets the basis for our work in establishing a new human colonic epithelial cell culture model system to investigate physiological processes of the colonic epithelium and mechanisms of disease development and progression.

As described in this thesis, we have developed and utilized a unique cell culture model to investigate colorectal cancer progression by step-wise introduction of defined oncogenic changes that are frequently seen in colorectal cancer patients. These oncogenic changes, such as ectopic expression of $Kras^{V12}$, ectopic expression of cMYC, shRNA mediated down regulation of p53 or PTEN, alone or in various combinations progressed HCECs subtly in transformation assays such as anchorage independent colony formation and Matrigel™ invasion. However, even the HCECs with all these alterations in CDK4, hTERT, KRAS, cMYC, p53 and PTEN were not tumorigenic when injected into multiple different models of immunocompromised mice either sub-cutaneously or under the renal capsule. These results suggest that it takes more than 6 genetic events to fully transform normal human colonic epithelial cells and our results are consistent with findings from works of others on human bronchial epithelial cells and keratinocytes (Sato et al. 2006; Dickson et al. 2000) but contradict findings from mammary and kidney epithelial cells in which 3 such alterations was sufficient to turn them tumorigenic (Elenbaas et al. 2001; Hahn et al. 1999). The explanation of the latter finding is that one of the alterations was the simian virus 40 large-T oncoprotein which can de-regulate a multitude of different cellular processes all at once.

Interestingly shRNA mediated efficient down regulation of APC did not progress HCECs. APC is considered to be the major tumor suppressor in colorectal cancer mutated in 85% of all cases (Wood et al. 2007). It is a large (312 kDa) multifunctional protein involved in transcription, cell cycle control, migration, differentiation and apoptosis (Goss and Groden 2000). Despite being considered as the gatekeeper tumor suppressor of colorectal cancer (Gupta, Pretlow, and Schoen 2007) homozygous deletions are not

observed in APC rather loss of function occurs almost always through truncating mutations that renders the protein half of its original size (Fodde, Smits, and Clevers 2001). Therefore it is not surprising that robust APC knock down is not transforming in HCECs. In fact some groups have proposed that truncated APC proteins might have dominant effects (Dihlmann et al. 1999; Green and Kaplan 2003; Green, Wollman, and Kaplan 2005; Kouzmenko et al. 2008) or might even be essential for survival of the colon cancer cells (Schneikert and Behrens 2006).

In order to better mimic the clinic presentations of APC mutations I have generated HCECs stably expressing two of the most commonly observed truncated versions in the presence or absence of various other oncogenic changes mentioned above. Initial results confirm that truncated APC proteins have dominant functions independent of regulation of Wnt pathway (data not shown). HCECs expressing mutant APC showed enhanced invasion through Matrigel^{TM} even in the presence of WT APC and in the absence of Wnt deregulation. Systematic efforts to pinpoint contributions of these mutations to transformation of HCECs are currently underway. Of note, we have also started small molecule screens with the hopes of finding anti-proliferative compounds that are specific to HCECs expressing truncated APC but not the wild-type APC. So far I've screened ~12,000 molecules and identified one APC-truncated selective anti-proliferative compound (ATSAC). Since APC is mutated in 85% of the cases and since currently there is no therapeutic approach targeting vulnerabilities that may be arising from these mutations, discovery of potent ATSACs has the potential to have immediate translational implications for colorectal cancer therapy.

I have further utilized the HCEC progression model to functionally interrogate the colorectal cancer genome using an shRNA screen and biological transformation assays. While there has been a tremendous investment in sequencing cancer genomes, we are left with few deliverables as far as identifying novel tumor suppressors or oncogenes. What we are left with is a list of mutated genes that rarely overlap between different cancers and with the unsettling realization that there is likely to be multiple pathways to cancer development even within one tumor type such as colorectal cancer. The central question is how are we to deal with the complexities of the cancer genome knowing that each cancer cells has 80-100 mutated genes?

Work described in this thesis provides a framework for beginning to understand biological functions of these genes. Our results provide strong evidence that cancer genome sequencing provides a valuable enrichment. More specifically this study is the first to discover that a high fraction of mutated genes are tumor suppressors that are monogenicly and cell autonomously involved in anchorage independent growth. Furthermore, this enrichment was specific to the mutated genes and the background rate was very low when random shRNAs were tested. The observation that so many of the mutated genes are driver tumor suppressors that are involved in anchorage independent growth partly explains the reason why cancers are so heterogeneous: there are numerous ways a cancer can acquire a transforming ability.

Even though there was a high validation rate in the genes that are considered to be drivers and thus arguing that cancer genome sequencing is accurately identifying functionally relevant genes, they fail to identify all. This is due to biostatistical limitations and difficulty of sequencing enough individuals to generate data with

sufficient predicting power. In this thesis, I also provide a framework involving a combination of bioinformatics and experimental testing to identify relevant genes within the much larger (~700) list of mutated genes that are otherwise considered to have incidental or passenger mutations. Five out of 6 genes tested with this approach also enhanced soft-agar growth when knocked down. These results demonstrate the need for implementing functional assay to distinguish between driver versus passenger genes instead of relying solely on bioinformatics. What is the extent of drivers within genes considered to be passengers? How much of this enrichment is due to network analysis? Efforts to answer these questions are currently on going in our laboratory.

Given that most of the available shRNAs do not provide a complete knockout phenotype tumor suppressors identified in this study do not follow the Knudson's two-hit hypothesis. They are likely to be haplo-insufficient where 50% reduction in the tumor suppressor level is sufficient for induction of the phenotype. Alternatively, as in the case of PTEN, they could be quasi-sufficient where even subtle decreases in expression is enough to elicit tumor associated phenotypes (Alimonti et al. 2010). Taken together, these data suggest that colon cancer suppression follows a continuum model (Berger, Knudson, and Pandolfi 2011) rather than the classical two-hit model.

Similarly, the continuum model can be applied to the colon cancer progression. Oncogenic changes identified in this study provided incremental increases in tumor associated phenotypes rather than discrete large gains. Additionally, each of the fully sequenced tumor sample contained mutations in 3 to14 anchorage-independent genes (Table 3-2), providing further *in vivo* support that these genes alone are not sufficient to provide full anchorage-independent abilities. It is likely that initial mutations in

anchorage-independent genes will allow an abnormal cell to go through initial expansion however for continued growth additional mutations are required to accumulate in these genes to continuously enhance anchorage-independent capabilities.

A natural extension of this work will be to use different biologically relevant end points (i.e. invasion through extracellular matrices such as basement membrane or tumorigenicity in xenograft experiment) to further interrogate cancer genomes. These experiments have also been initiated in our lab. Completion of this and similar studies will generate a biological catalogue of cancer mutations, so we can better understand the pathways used by cells to gain cancer associated functions such as anchorage independent growth, invasion through extracellular matrices, tumor formation, and metastasis in xenograft models. This will ultimately help us make informed decision about therapy.

APPENDIX A
List of All Screened shRNAs Targeting CRC-*CAN* genes

Gene Symbol	Clone ID	1CTR	1CTP
ABCA1	V2LHS_64364		
ABCA1	V2LHS_64362		
ABCA1	V2LHS_64365		
ABCB11	V2LHS_23959		
ABCB11	V2LHS_23961		
ABCB11	TRCN0000059362		
ACAN	V2LHS_193758		
ACAN	TRCN0000071732		
ACAN	V2LHS_193758		
ACSL5	V2LHS_115033		
ACSL5	V2LHS_115032		
ADAM29	V2LHS_252416		
ADAM29	V3LHS_397683		
ADAM29	V3LHS_397686		
ADAMTS15	V2LHS_70921	+	+
ADAMTS15	V3LHS_364310	+	
ADAMTS15	V3LHS_364309	+	
ADAMTS18	V2LHS_123010	+	+
ADAMTS18	V2LHS_70909		
ADAMTS18	V2LHS_123009		
ADAMTS18	V2LHS_70905		+
ADAMTS18	V2LHS_70907	+	
ADAMTS18	V2LHS_70906		+
ADAMTS20	V2LHS_272489		
ADAMTS20	V2LHS_136558	+	+
ADAMTS20	V3LHS_302842	+	+
ADAMTSL3	V2LHS_54992		
ADAMTSL3	V2LHS_54991	+	
ADAMTSL3	V2LHS_54989		
ADARB2	V2LHS_26757		
ADARB2	V2LHS_26756		+
ADARB2	V2LHS_26755		
AKAP12	V2LHS_220134		
AKAP12	V2LHS_221508	+	+

Gene Symbol	Clone ID	1CTR	1CTP
AKAP12	V2LHS_50557	+	+
AKAP12	V2LHS_50556		+
AKAP6	V2LHS_27894		+
AKAP6	V2LHS_27895		
ALK	V2LHS_70308		
ALK	V2LHS_15557		
APC	V2LHS_153492		
APC	V2LHS_240271		+
APC	V2LHS_153494		
APC	V2LHS_89656		
ARHGEF10	V2LHS_80181		
ARHGEF10	V2LHS_80180		
ATP11A	V2LHS_249221	+	
ATP11A	V2LHS_61340		
ATP11A	V2LHS_61338		
ATP13A1	V2LHS_34066		
ATP13A1	TRCN0000051608	+	
ATP13A1	TRCN0000051609	+	+
ATP13A5	V2LHS_179229		
ATP13A5	V2LHS_23942	+	+
ATP13A5	V2LHS_164725		+
ATP13A5	V2LHS_179230	+	+
ATP13A5	V2LHS_164724	+	
ATP13A5	V2LHS_23938	+	+
BCL9	V2LHS_15242	+	
BCL9	V2LHS_287024		
BCL9	V2LHS_287023		
C10orf137	TRCN0000116737		
C10orf137	TRCN0000144797		
C14orf115	V2LHS_156410		
C15orf2	V2LHS_51792	+	
C15orf2	V2LHS_51795		+
C15orf2	V2LHS_245404	+	
CACNA2D3	V2LHS_175409		
CACNA2D3	V2LHS_175404		
CD109	V2LHS_99968	+	+
CD109	TRCN0000073648		

Gene Symbol	Clone ID	1CTR	1CTP
CD109	TRCN0000073649		
CD248	V2LHS_34217		+
CD248	TRCN0000053453	+	+
CD248	TRCN0000053454		
CD46	V2LHS_151639		
CD46	V2LHS_151637	+	
CD46	V2LHS_151638	+	
CD46	V2LHS_151636	+	
CD93	V2LHS_28878		
CD93	TRCN0000029084		
CD93	TRCN0000029085		
CHL1	V2LHS_80198	+	+
CHL1	V2LHS_258073	+	+
CHL1	V2LHS_260545		
CHL1	V2LHS_91574		
CHL1	V2LHS_91578	+	+
CLSTN2	V2LHS_201036		+
CLSTN2	V2LHS_200976	+	
CLSTN2	V2LHS_11827		
CNTN4	V2LHS_38255	+	+
CNTN4	V2LHS_245484		
CNTN4	V2LHS_38256	+	
COL3A1	V2LHS_88556		
COL3A1	V2LHS_259244		+
COL3A1	TRCN0000003295	+	+
COL3A1	TRCN0000003293	+	
COL3A1	TRCN0000003294	+	+
COL3A1	TRCN0000091487	+	+
CPAMD8	V2LHS_225403		
CPAMD8	V2LHS_114636		+
CSMD3	V2LHS_166348		
CSMD3	V2LHS_119271		
CSMD3	V2LHS_119274	+	
CUX1	V2LHS_151077		
CUX1	V2LHS_151079		
DPP10	V2LHS_213795		+
DPP10	TRCN0000046663		

Gene Symbol	Clone ID	1CTR	1CTP
DPP10	TRCN0000046664		
DSCAML1	V2LHS_57527	+	
DSCAML1	V2LHS_57526	+	
DTNB	V2LHS_74472	+	
DTNB	V2LHS_74470		
DTNB	V2LHS_74471	+	
EPHA3	V2LHS_43360		
EPHA3	V3LHS_639472		
EPHA3	V3LHS_639471		
EPHB6	V2LHS_17714		
EPHB6	V2LHS_17715	+	+
EPHB6	V2LHS_17717		
EPHB6	V2LHS_17717		
ERCC6	V2LHS_182155		
ERCC6	V2LHS_182151		+
ERCC6	V2LHS_182153	+	
ERCC6	V2LHS_83786		+
ERCC6	V2LHS_100066	+	
ERCC6	V2LHS_83785	+	
ERCC6	V2LHS_182154		+
ERCC6	V2LHS_83784	+	+
ERGIC3	V2LHS_262029		
ERGIC3	V2LHS_134570		
ERICH1	V2LHS_163215		
ERICH1	V2LHS_163219		
ERICH1	V2LHS_91292		
ERICH1	V2LHS_91293		
ERICH1	V2LHS_163216		
ERICH1	V2LHS_91291		
EVL	V2LHS_266512		
EVL	V2LHS_115473		
EXOC4	V2LHS_81246		
EXOC4	V2LHS_81245		
EYA4	V2LHS_172982		
EYA4	V2LHS_172983		
EYA4	V2LHS_172985		
F8	V2LHS_255926	+	

Gene Symbol	Clone ID	1CTR	1CTP
F8	V2LHS_83667		+
F8	V2LHS_67564	+	+
F8	V2LHS_259026		
F8	V2LHS_256236		
FAM161A	V2LHS_216416	+	
FAM161A	V2LHS_254445	+	+
FAM161A	V3LHS_330732		
FAM193B	V2LHS_176467		
FAM193B	V2LHS_176466		
FBN2	V2LHS_151526		
FBN2	V2LHS_151530		
FBXW7	V2LHS_14559	+	+
FBXW7	V2LHS_254965	+	+
FBXW7	V2LHS_202810		+
FBXW7	V2LHS_203045		+
FBXW7	V2LHS_89326		+
FBXW7	V2LHS_89328		+
FBXW7	V2LHS_203036	+	+
FBXW7	V2LHS_202932	+	+
FBXW7	V2LHS_254965	+	+
FLNC	V2LHS_238475		+
FLNC	V3LHS_327303		
FLNC	V3LHS_327300		
FN1	V2LHS_113935		
FN1	V2LHS_113940		
FN1	V3LMM_433067		
GALNS	V2LHS_93416	+	
GALNS	V2LHS_93414	+	
GALNS	V3LHS_358773		
GJD4	V2LHS_39029	+	+
GJD4	TRCN0000074124		
GLI3	V2LHS_82965	+	+
GLI3	V2LHS_82970		
GLI3	V3LHS_372606		
GNAS	V2LHS_93436		+
GNAS	V2LHS_268469		+
GNAS	V2LHS_202713		

Gene Symbol	Clone ID	1CTR	1CTP
GPR112	V2LHS_215633		
GPR112	V2LHS_211038		
GPR158	V2LHS_90354		
GPR158	TRCN0000062923		
GPR158	TRCN0000062924		
GRID1	V2LHS_222616		
GRID1	TRCN0000063034		
GRID1	TRCN0000063033		
GRM1	V2LHS_130872	+	+
GRM1	V2LHS_130874		
GRM1	V2LHS_130870		+
GUCY1A2	V2LHS_130956		+
GUCY1A2	V2LHS_130958		+
GUCY1A2	V2LHS_130955		+
GUCY1A2	V2LHS_237217		
HAPLN1	V2LHS_150924	+	
HAPLN1	V2LHS_150925		
HAPLN1	V2LHS_191024	+	+
HAPLN1	TRCN0000150533	+	+
HIST1H1B	V2LHS_37955	+	+
HIST1H1B	V2LHS_37954	+	+
IGFBP3	V2LHS_111629	+	
IGFBP3	V2LHS_111628		+
IGFBP3	V2LHS_225584		
IGSF22	V2LHS_178658		
IGSF22	V2LHS_178659		+
IGSF22	V2LHS_178660	+	+
IRS4	V2LHS_28086	+	+
IRS4	V2LHS_28015	+	
IRS4	V2LHS_28085		+
ITGAE	V2LHS_275031	+	
ITGAE	V2LHS_133454	+	+
ITGAE	V2LHS_133451	+	+
KALRN	V2LHS_199006		
KALRN	V2LHS_263240		+
KALRN	V2LHS_262322	+	+
KALRN	V2LHS_46992	+	+

Gene Symbol	Clone ID	1CTR	1CTP
KALRN	V2LHS_46991		+
KCNQ5	V2LHS_34566		
KCNQ5	V2LHS_34562	+	
KCNQ5	V2LHS_34563	+	
KIAA0182	V2LHS_274950	+	+
KIAA0182	V2LHS_139767		+
KIAA0182	V2LHS_139766	+	+
KIAA0556	V2LHS_15592	+	+
KIAA0556	V2LHS_15593	+	
KIAA0556	V2LHS_240500	+	+
KIAA0556	V2LHS_241707		
KIAA1409	V2LHS_161077		
KIAA1409	V2LHS_161079		
KIAA2022	V2LHS_86535		+
KIAA2022	V2LHS_86537	+	+
KRAS	V2LHS_178283		
KRAS	V2LHS_202204		
KRAS	V2LHS_169384		
KRAS	V2LHS_203252		
KRAS	V2LHS_275818		
KRT73	V2LHS_286579		+
KRT73	V2LHS_193029		+
LAMA1	V2LHS_76891		
LAMA1	V2LHS_76887		
LCN9	V2LHS_284827		
LCN9	V2LHS_284623		
LGR6	V2LHS_28493		
LMO7	V2LHS_114789		
LMO7	V2LHS_114791	+	+
LMO7	V2LHS_114786	+	
LMO7	V2LHS_114787		
LMO7	V2LHS_114790		+
LRP2	V2LHS_48526		
LRP2	V2LHS_48528	+	+
MAP1B	V2LHS_209336	+	+
MAP1B	V2LHS_197483	+	+
MAP2	V2LHS_151558	+	

Gene Symbol	Clone ID	1CTR	1CTP
MAP2	V2LHS_151557	+	+
MAP2	V2LHS_151560	+	
MAP2K7	V2LHS_19417	+	+
MAP2K7	V2LHS_262276	+	+
MAPK8IP2	V2LHS_50504		+
MAPK8IP2	V2LHS_50507	+	+
MCM3AP	V2LHS_47913		
MCM3AP	V2LHS_47912		
MKRN3	V2LHS_56062		
MKRN3	V2LHS_56060		
MKRN3	V2LHS_56057		
MLL3	V2LHS_60368		+
MLL3	V2LHS_282577		+
MLL3	V2LHS_248916		+
MMP2	V2LHS_48434		
MMP2	V2LHS_48430		
MMP2	V2LHS_48431		
MYO18B	V2LHS_159519		
MYO18B	TRCN0000117367		
MYO18B	TRCN0000117368		
MYO19	V2LHS_137025		
MYO19	V2LHS_137024		+
MYO19	V2LHS_137020		+
MYO5C	V2LHS_26316		
NAV3	V2LHS_95965	+	
NAV3	V2LHS_95967	+	
NF1	V2LHS_189526	+	
NF1	V2LHS_193129	+	
NF1	V2LHS_76028		+
NF1	V2LHS_76027	+	+
NF1	V2LHS_260806	+	+
NF1	V2LHS_76029		+
NF1	V2LHS_190255		+
NF1	V2LHS_76032	+	
NOS3	V2LHS_111653		+
NOS3	V2LHS_111652		+
NOS3	V2LHS_264475		+

Gene Symbol	Clone ID	1CTR	1CTP
NTNG1	V2LHS_96052		+
NTNG1	V2LHS_96051		+
NTNG1	V2LHS_96054		
NUP210	V2LHS_176870		
NUP210	V2LHS_219299		
NUP210	V2LHS_176868		
OBSCN	V2LHS_28312	+	
OBSCN	V2LHS_238841		+
OBSCN	V2LHS_202462		+
OBSCN	V2LHS_202481	+	+
OR51E1	V2LHS_20776		
OR51E1	V2LHS_20775		
P2RX7	V2LHS_152543	+	+
P2RX7	V3LHS_379761		
P2RX7	V3LHS_379765		
P2RY14	V2LHS_95836		
P2RY14	V2LHS_95837		
P2RY14	V2LHS_95834	+	+
PCDH11X	V2LHS_86840	+	+
PCDH11X	V2LHS_86845		
PCDH11X	V3LHS_337355		
PCDHA9	V3LHS_336241		
PCDHA9	V3LHS_306867		+
PCDHA9	V3LHS_306865		
PHIP	V2LHS_55558	+	+
PHIP	V2LHS_174594	+	
PHIP	V2LHS_174592		+
PHIP	V2LHS_174591		
PHIP	V2LHS_174593		
PHIP	V2LHS_55555		
PIK3CA	V2LHS_1227		
PIK3CA	V2LHS_58550		
PKNOX1	V2LHS_47744		
PKNOX1	V2LHS_47746		
PLB1	V2LHS_22403		
PLCG2	V2LHS_262144		
PLCG2	V2LHS_170087		

Gene Symbol	Clone ID	1CTR	1CTP
PRDM9	V2LHS_189753		+
PRDM9	V2LHS_191050	+	+
PRKD1	V2LHS_170466		+
PRKD1	V2LHS_170467	+	+
PRKD1	V2LHS_170464	+	+
PRKD1	V2LHS_170465	+	+
PRKD1	V2LHS_170466		+
PRKD1	V2LHS_170467	+	+
PRKD1	V2LHS_170464	+	+
PRKD1	V2LHS_170465	+	+
PRUNE2	V2LHS_212836		
PRUNE2	V2LHS_204508	+	
PRUNE2	V2LHS_253448		
PRUNE2	V2LHS_207055		+
PTEN	V2LHS_119551	+	+
PTEN	V2LHS_265808		+
PTEN	V2LHS_231477	+	+
PTEN	V2LHS_92317	+	+
PTEN	V2LHS_92314		+
PTEN	V2LHS_92319		
PTEN	V2LHS_228508	+	+
PTEN	V2LHS_192536		+
PTPRD	V2LHS_221647	+	+
PTPRD	V2LHS_170974	+	+
PTPRD	V2LHS_211074	+	+
PTPRD	V2LHS_170972	+	+
PTPRD	V2LHS_170976	+	+
PTPRS	V2LHS_171032		
PTPRS	V3LHS_634063		
PTPRS	V3LHS_634062		
PTPRU	V2LHS_197528		
PTPRU	V2LHS_239079	+	
PTPRU	V2LHS_197656		
PTPRU	V2LHS_196429		
RASGRF2	V2LHS_201735		
RASGRF2	V2LHS_203362		
RASGRF2	V2LHS_202571		

Gene Symbol	Clone ID	1CTR	1CTP
RET	V2LHS_203395		
RET	TRCN0000040024		
RNF219	V2LHS_136271	+	+
RNF219	V2LHS_136273		+
RNF219	V3LHS_644374		
RNF219	V3LHS_644375		
ROBO1	V2LHS_32195		
ROBO1	V2LHS_32196		
ROBO1	V2LHS_32197		
RUNX1T1	V2LHS_15002		
RUNX1T1	TRCN0000096598		
SCN3B	V2LHS_175419		
SCN3B	V2LHS_175417		
SCN3B	V3LHS_358981		
SEMA3D	V2LHS_38000		
SEMA3D	TRCN0000063214		
SEMA3D	TRCN0000063213		
SFRS6	V2LHS_203007		
SFRS6	V2LHS_263096	+	+
SFRS6	V2LHS_263152	+	+
SFRS6	V2LHS_201643	+	
SFRS6	V2LHS_20637	+	+
SFRS6	V2LHS_20638		+
SFRS6	V2LHS_263096	+	+
SH3TC1	V2LHS_51198		
SH3TC1	TRCN0000062608	+	+
SH3TC1	TRCN0000062609		+
SHANK1	V2LHS_97792	+	+
SHANK1	TRCN0000136791		
SHANK1	TRCN0000138405		
SLC22A15	V2LHS_276310		
SLC22A15	V2LHS_275692		
SLC22A15	V2LHS_175529		
SLC22A15	V2LHS_175527		
SLC29A1	V2LHS_249880	+	
SLC29A1	V2LHS_61922	+	+
SLC29A1	TRCN0000043643	+	+

Gene Symbol	Clone ID	1CTR	1CTP
SLC44A4	V2LHS_158453		
SLC44A4	V2LHS_158451		
SLC44A4	V2LHS_158455		
SLC44A4	V2LHS_158456		
SMAD2	V2LHS_213986		
SMAD2	V2LHS_197144		+
SMAD2	V2LHS_206703		
SMAD2	V2LHS_285936		
SMAD2	V2LHS_216709		+
SMAD3	V2LHS_215032	+	
SMAD4	V2LHS_37199		
SMAD4	V2LHS_37196		
SMAD4	V2LHS_37198		
SMAD4	V2LHS_37195		
SMTN	V2LHS_94901		
SMTN	TRCN0000123229		
SMTN	TRCN0000123230		
SORL1	V2LHS_153328		
SORL1	TRCN0000062948		
SORL1	TRCN0000062949		
STAB1	V3LHS_386813		
STAB1	V3LHS_386815		
SYNE1	V2LHS_79914	+	
SYNE1	V2LHS_79910		
SYNE1	V2LHS_79911	+	
SYNE1	V3LHS_334366		
SYT14L	V2LHS_165008		
SYT14L	V3LHS_353747		
SYT14L	V3LHS_353743	+	+
TAF2	V2LHS_153737	+	+
TAF2	V2LHS_153736	+	
TAF2	V2LHS_153738		
TBX22	V2LHS_154236	+	
TBX22	V2LHS_154237		
TCERG1L	V2LHS_41904		+
TCERG1L	V2LHS_41900	+	+
TCERG1L	V2LHS_244266		+

Gene Symbol	Clone ID	1CTR	1CTP
TCF7L2	V2LHS_116684		+
TCF7L2	V2LHS_116688	+	+
TCF7L2	V2LHS_116683		+
TGFBR2	V2LHS_153994	+	+
TGFBR2	V3LHS_645438	+	+
TGM3	V2LHS_154012	+	+
TGM3	V2LHS_154017		
TGM3	TRCN0000036129		
TIAM1	V2LHS_154064	+	+
TIAM1	V2LHS_154060	+	+
TLR9	V2LHS_154388		
TLR9	V3LHS_635783		
TLR9	V3LHS_636051		
TNN	V2LHS_77556		
TNN	V2LHS_77554		+
TP53	V2LHS_93615	+	+
TP53	V2LHS_93613	+	+
TP53	TRCN0000003753		
TTLL3	V2LHS_223927		
TTLL3	TRCN0000048529		
TTLL3	TRCN0000048528		
UHRF2	V2LHS_149460	+	+
UHRF2	V2LHS_149462	+	+
UHRF2	V2LHS_149463		+
UQCRC2	V2LHS_220312	+	+
UQCRC2	V2LHS_276849		+
UQCRC2	V2LHS_171876		
ZMYM4	V2LHS_17818		
ZMYM4	V2LHS_17815		+
ZMYM4	V2LHS_241770		+
ZNF442	V2LHS_117028	+	
ZNF442	V2LHS_225430		
ZNF442	V2LHS_226701		+
ZNF442	V2LHS_190261	+	
ZNF442	V2LHS_30713		+
ZNF442	V2LHS_117027		
ZNF442	V2LHS_117029	+	

Gene Symbol	Clone ID	1CTR	1CTP
ZNF521	V2LHS_233336	+	
ZNF521	V2LHS_96617	+	
ZNF521	V2LHS_96615		

+ denotes enhanced growth in soft agar (in at least 6 out of 8 replicates).
Clone ID could be used to retrive shRNA sequences from Open Biosystem's website.

APPENDIX B
List of All Screened shRNAs Targeting Random Genes

Clone ID	Gene Symbols	1CTP
V2LHS_101114	HSF5	
V2LHS_101618	-	
V2LHS_101768	-	
V2LHS_103872	SLC35E2, RP11-345P4.4	
V2LHS_104531	-	
V2LHS_105645	-	
V2LHS_108415	-	
V2LHS_109255	-	
V2LHS_110154	-	
V2LHS_110452	-	
V2LHS_110633	-	
V2LHS_110767	-	
V2LHS_111327	RP11-151A6.2	
V2LHS_111896	ACACA	
V2LHS_112050	ALDH3A1	
V2LHS_112366	CHRNB1	
V2LHS_113117	CCR1	
V2LHS_113258	CSTF2	
V2LHS_113545	DPYSL2	
V2LHS_113750	EPAS1	
V2LHS_113857	FHIT	
V2LHS_114304	GTF2E2	
V2LHS_116102	MRPL51, RAB3IP	
V2LHS_116821	GPR63	
V2LHS_118342	MAS1L	
V2LHS_119091	ELOVL7	
V2LHS_119132	MORC3	
V2LHS_120978	LOC100130673, LOC100134684	
V2LHS_123189	ZNF624	
V2LHS_123448	-	
V2LHS_123619	-	
V2LHS_124058	-	
V2LHS_124550	-	
V2LHS_125451	-	

Clone ID	Gene Symbols	1CTP
V2LHS_126622	-	
V2LHS_128925	-	
V2LHS_129834	-	
V2LHS_132025	GPR39	
V2LHS_133095	HMGB2	
V2LHS_134135	LY75	
V2LHS_134397	NOP5/NOP58	
V2LHS_135471	FOLR1	
V2LHS_135551	SOBP	
V2LHS_137268	DEPDC2	
V2LHS_137913	ARPC5L	
V2LHS_138593	C8orf53	
V2LHS_138639	CHCHD6	
V2LHS_138988	PLAT	
V2LHS_139519	EBF2	
V2LHS_140914	-	
V2LHS_141304	RSBN1L	
V2LHS_141373	LOC285501	
V2LHS_142260	-	
V2LHS_144752	RSHL3	
V2LHS_144815	-	
V2LHS_145056	-	
V2LHS_145188	LOC100131353	
V2LHS_145271	-	
V2LHS_146481	-	
V2LHS_147158	-	
V2LHS_148300	-	
V2LHS_150657	CLTCL1	
V2LHS_150823	COX7A2	
V2LHS_150860	CPM	
V2LHS_152058	MYD88	
V2LHS_152085	MYH11	
V2LHS_152804	SEPW1	
V2LHS_153498	SRP54, LOC650638	
V2LHS_15365	DNLZ	
V2LHS_153812	TCEB3	
V2LHS_154118	ADD3	

Clone ID	Gene Symbols	1CTP
V2LHS_156230	HHAT	
V2LHS_156875	ZCCHC6	
V2LHS_157244	BBS10	
V2LHS_157296	C6orf103	
V2LHS_157493	MYH14	
V2LHS_158349	C10orf81	
V2LHS_162266	KIAA2026	
V2LHS_163209	FBXO43	
V2LHS_165401	-	
V2LHS_165590	-	
V2LHS_165596	-	
V2LHS_16782	FBP2	
V2LHS_168539	-	
V2LHS_169173	-	
V2LHS_169387	-	
V2LHS_169577	-	
V2LHS_16967	GOLGB1	
V2LHS_169974	SERPINB8	
V2LHS_171239	RAP1A	
V2LHS_171350	TLR4	
V2LHS_171493	TPM4	
V2LHS_17212	-	
V2LHS_172145	ZNF264	
V2LHS_17240	TMC4	
V2LHS_172493	TAF15	
V2LHS_174041	C21orf55	
V2LHS_174072	LPCAT2	
V2LHS_174609	FLJ20712	
V2LHS_175228	RSBN1	
V2LHS_175239	CNO	
V2LHS_176261	RNF216	
V2LHS_176549	ING3	
V2LHS_176630	CRLS1	
V2LHS_177218	C19orf48	
V2LHS_179779	MOXD1	
V2LHS_18068	LOC149684	
V2LHS_180778	-	
V2LHS_181420	PGAP1	
V2LHS_181966	TMEM17	

Clone ID	Gene Symbols	1CTP
V2LHS_182233	-	
V2LHS_182438	-	
V2LHS_182486	-	
V2LHS_183592	LOC284865	
V2LHS_184128	ORAI2	
V2LHS_184307	LOC286114	
V2LHS_184442	-	
V2LHS_184523	LOC286254	
V2LHS_185846	-	
V2LHS_18592	DYNC1I1	
V2LHS_186617	-	
V2LHS_18664	TBC1D20	
V2LHS_188068	LOC643166	
V2LHS_190449	LOC344328, LOC728162	
V2LHS_19083	LOC146325	
V2LHS_191378	-	
V2LHS_192413	PRO2012	
V2LHS_193874	-	
V2LHS_19417	MAP2K7	+
V2LHS_195354	-	
V2LHS_196157	USP26	
V2LHS_196370	LHFP	
V2LHS_196442	WWP2	
V2LHS_197782	LHX6	
V2LHS_199955	HYOU1	
V2LHS_20081	ZNF597	
V2LHS_202139	STK33	
V2LHS_204157	-	
V2LHS_204426	RNF151	
V2LHS_20445	RAD9B	
V2LHS_20531	MYO1B	
V2LHS_20685	LOC441086	
V2LHS_207008	-	
V2LHS_207961	-	
V2LHS_208532	-	
V2LHS_208575	-	
V2LHS_209406	-	
V2LHS_21029	HMGB4	
V2LHS_211123	-	
V2LHS_214308	INTS1	

Clone ID	Gene Symbols	1CTP
V2LHS_214638	GPRIN1	
V2LHS_21650	-	
V2LHS_216983	LYRM7	
V2LHS_21728	-	
V2LHS_217322	CDGAP	
V2LHS_218229	PLN	
V2LHS_218270	SMPD4, FLJ41352	
V2LHS_218527	TSPAN4	
V2LHS_218834	SLC16A10	+
V2LHS_219156	OR5K4	
V2LHS_219922	LOC130355	
V2LHS_220477	-	
V2LHS_220779	ORC1L	+
V2LHS_221197	ZNF85	
V2LHS_221407	ACTBL2	
V2LHS_221662	SERPINF1	
V2LHS_221966	PINX1	
V2LHS_222117	WDR44	
V2LHS_222727	LOC284262	
V2LHS_222883	UBE2V2	
V2LHS_222945	DEPDC1	
V2LHS_223321	PLS1	
V2LHS_223430	PIGA	
V2LHS_22475	-	
V2LHS_225011	NAP1L6	
V2LHS_225587	RCBTB2	
V2LHS_227034	C6orf32	
V2LHS_227310	CXCL12	
V2LHS_228842	ADSS	
V2LHS_229142	LETMD1	
V2LHS_230644	KRT10	
V2LHS_230654	NAT5	
V2LHS_23124	-	
V2LHS_232882	PKD2L1	
V2LHS_235586	HEATR5B	
V2LHS_235702	LY75	
V2LHS_236794	LOC283435	
V2LHS_237284	-	
V2LHS_23774	SRD5A2L2	
V2LHS_238193	POLR2B	

Clone ID	Gene Symbols	1CTP
V2LHS_238546	TRIM46	
V2LHS_238690	VPS54	
V2LHS_238825	RAD54B	
V2LHS_239255	NR0B1	
V2LHS_239291	HNF4G	
V2LHS_240019	GTF3C5	
V2LHS_240036	ZFP62, LOC643836	
V2LHS_241222	-	
V2LHS_241276	GTF2A2	
V2LHS_242130	OR51E2	
V2LHS_242428	-	
V2LHS_242962	GPR39, LYPD1	
V2LHS_243155	ZFP62, LOC643836	
V2LHS_24350	C10orf6	
V2LHS_243686	-	
V2LHS_244007	RAB3GAP2	
V2LHS_245782	ZNF648	
V2LHS_246137	-	
V2LHS_246972	UBIAD1	
V2LHS_247631	-	
V2LHS_247865	-	
V2LHS_247951	EXOD1	
V2LHS_248572	-	
V2LHS_249542	UCHL3	
V2LHS_24967	LOC285401	
V2LHS_250042	-	
V2LHS_250215	PDZK1IP1	
V2LHS_251838	-	
V2LHS_25191	GLRX	+
V2LHS_251919	C3orf43	
V2LHS_252337	ZMYM6	
V2LHS_253357	LOC114227	
V2LHS_25459	-	
V2LHS_254594	USP8	
V2LHS_254879	HECTD3	
V2LHS_254965	FBXW7	+
V2LHS_255392	ALAD	
V2LHS_25671	-	
V2LHS_257412	LOC440131	
V2LHS_257476	STARD3	

Clone ID	Gene Symbols	1CTP
V2LHS_257994	SPIN1	
V2LHS_258949	-	
V2LHS_25956	-	
V2LHS_259783	NF2	
V2LHS_260651	-	
V2LHS_261232	-	
V2LHS_262372	ST5	
V2LHS_262537	VCAM1	
V2LHS_262629	ACYP2	
V2LHS_263096	SFRS6	+
V2LHS_264104	CCDC80	
V2LHS_264773	-	
V2LHS_265855	-	
V2LHS_268217	ARF4	
V2LHS_26908	-	
V2LHS_269098	-	
V2LHS_276463	MFSD5	
V2LHS_277826	POLR1B	
V2LHS_27935	LY86	
V2LHS_27966	RAE1	
V2LHS_28203	-	
V2LHS_285279	HSN2	
V2LHS_285980	C1orf49, C1orf220	
V2LHS_286436	RBM11	
V2LHS_29175	RNF183	
V2LHS_29248	C19orf40	
V2LHS_29339	RPUSD2	
V2LHS_29824	FLJ16124	
V2LHS_30865	MXD1	
V2LHS_31264	-	
V2LHS_31648	-	
V2LHS_31708	RAVER1	
V2LHS_32326	TTF2	
V2LHS_33383	SLC30A8	
V2LHS_33437	HIST1H4C	
V2LHS_33452	-	
V2LHS_35058	DEFA5	
V2LHS_3553	DUSP8	
V2LHS_36444	SLC16A7	
V2LHS_36857	SSPO	

Clone ID	Gene Symbols	1CTP
V2LHS_37991	-	
V2LHS_38134	GRB10	
V2LHS_40790	-	
V2LHS_40923	-	
V2LHS_44557	-	+
V2LHS_45778	-	
V2LHS_45803	-	
V2LHS_47453	MBD3	
V2LHS_47505	MBD4	
V2LHS_47557	-	
V2LHS_47605	-	
V2LHS_49921	NR1D2	
V2LHS_49989	CCS	
V2LHS_50715	C11orf30	
V2LHS_51042	PMEPA1	
V2LHS_51716	SMARCAD1	
V2LHS_51754	C11orf60	
V2LHS_51796	DAZAP1	
V2LHS_52116	-	
V2LHS_52452	-	
V2LHS_52876	LOC127295	
V2LHS_53720	CNTNAP2	
V2LHS_54334	-	
V2LHS_54537	-	
V2LHS_55636	-	
V2LHS_56193	TP53BP1	+
V2LHS_56749	STK3	
V2LHS_58426	-	
V2LHS_59014	PDE4D	
V2LHS_59352	C9orf32	
V2LHS_60022	METTL7A	
V2LHS_60927	-	
V2LHS_61314	-	
V2LHS_61719	IDE	
V2LHS_61795	FUS	
V2LHS_62078	DEFB4, DEFB4P	
V2LHS_65159	LOH3CR2A	
V2LHS_66511	ANGPTL7	
V2LHS_66661	MRPL23	
V2LHS_68122	PRELID2	

Clone ID	Gene Symbols	1CTP
V2LHS_68738	LOC157381	
V2LHS_69357	EOMES	
V2LHS_69401	-	
V2LHS_69715	LYG2	
V2LHS_70215	-	
V2LHS_70516	UHRF1, LOC100133565	
V2LHS_70718	-	
V2LHS_70802	CCDC104	
V2LHS_71965	B4GALNT2	
V2LHS_72116	-	
V2LHS_72154	-	
V2LHS_72199	-	
V2LHS_72249	CAMK1G	
V2LHS_72587	-	
V2LHS_74033	HDAC9	
V2LHS_74041	C2orf43	
V2LHS_74615	ASXL1	
V2LHS_75292	LOC121906	
V2LHS_75840	-	
V2LHS_76027	NF1	+
V2LHS_78198	-	
V2LHS_80182	DMN	
V2LHS_81798	-	
V2LHS_82128	-	
V2LHS_82529	HK2	
V2LHS_82896	IRX6	
V2LHS_83245	FLJ32682	
V2LHS_83376	ANKRD57	
V2LHS_83817	ERCC3	
V2LHS_83945	EPB42	
V2LHS_83962	-	
V2LHS_84218	-	
V2LHS_84707	SYPL1	
V2LHS_86605	KIAA0922	
V2LHS_86870	-	
V2LHS_88470	CP	
V2LHS_88888	-	
V2LHS_89932	PSEN1	
V2LHS_89945	-	
V2LHS_90115	-	

Clone ID	Gene Symbols	1CTP
V2LHS_90366	NMU	
V2LHS_91329	EML5	+
V2LHS_91553	-	
V2LHS_91573	-	
V2LHS_91751	KLHL18	
V2LHS_92232	PLA2G2A	
V2LHS_93320	COL17A1	
V2LHS_93663	DCX	
V2LHS_94561	APBB1	
V2LHS_95305	KIAA0141	
V2LHS_96867	LOC26010	
V2LHS_96902	PTCD1	
V2LHS_97427	HDGFRP3	
V2LHS_97516	EIF3EIP	
V2LHS_98768	NUDT9	
V2LHS_99249	SEH1L	
V2LHS_99336	TEX11	

+ denotes enhanced growth in soft agar (in at least 6 out of 8 replicates). Yellow highlight denotes positive controls that were idenitified in our original screen. Green highlight denotes soft-agar enhancing random shRNAs.
Clone ID could be used to retrive shRNA sequences from Open Biosystem's website.

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