Functional parsing of driver mutations in the colorectal cancer genome reveals numerous suppressors of anchorage-independent growth

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Edited Précis: Many mutated genes in cancer that are tumor suppressors may also be involved in anchorage-independent growth, perhaps helping explain the roots of tumor heterogeneity.

Running title: Parsing driver mutations in the colorectal cancer genome

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Abstract

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 employed 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\textsuperscript{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.

**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 (1). 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 (2) and mammary epithelial cells (3). Lack of overlapping genes from these two screens and identification of different tumor suppressor profiles from in vivo lymphoma (4) and liver cancer (5) 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 (6), thousands of cancer genomes have been sequenced. One ongoing point of debate concerning these efforts is the cost versus benefit imbalance (7). 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 (8, 9) 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 (6, 10) using otherwise isogenic K-rasV12 expressing or p53 knocked-down hTERT immortalized diploid human colonic epithelial cells (HCECs) (11). This approach revealed a profound enrichment for driver tumor suppressors within CRC CAN-genes.

Materials and Methods

Plasmids. CDK4 was expressed together with G418 from retroviral pSRaMSU vector. hTERT was cloned from pGRN145 to pMIN-Ub-IRE-Blast lentiviral vector co-expressing blastocidin resistance marker (both vectors were provided by Geron Corporation). pSRZ-shTP53 and pBABE-hyg-KRASV12 are described elsewhere (12). 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 (11). 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). KRAS³¹² and shRNA against p53 were introduced with retroviruses and oncogenic KRAS expression was verified as described (12, 13). 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 (6). 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, Transient transfections, Immunofluorescence, Immunobloting, qRT-PCR, Network analysis.**

See Supplementary Data.

**Results and Discussion**

**Anchorage-independent growth suppressor screen**

With the exception of APC truncation mutations, K-RAS⁴¹² and TP53 alterations represent two of the most frequent mutations in CRC (6, 10) 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 (Fig. 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 (6, 10) in a “one-shRNA-one-well” format (Fig. 1C) revealed 49 context-independent and 16 context-dependent progression suppressors (Fig. 1D, Supplementary Table S1). 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 (Supplementary Table S2) – 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.

The observation that oncogenic Ras expressing cells are more resistant to p53-dependent apoptosis (14) 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 (Supplementary Fig. S1). 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 (Supplementary Table S3). Furthermore, on average 37\% of all \( CAN \)-genes in any individual colon cancer are involved in matrix dependent proliferation (Supplementary Table S4). 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, Supplementary Fig. S2). Importantly, shRNAs against well-known tumor suppressors (\( FBXW7, NFI, PTEN, TGFBRII \) and \( TP53 \)) scored positive whereas genes with established oncogenic gain of function (\( KRAS, PIK3CA \) and \( RET \)) did not score 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 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 (Supplementary Fig. S3). Quantitative testing of PTEN and NF1 (Supplementary Fig. S4) or screening of approximately half of the shRNA library using immortalized HCECs without KRAS<sup>V12</sup> 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 (Supplementary Fig. S2) 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 (Fig. 2). 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 (15). 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 (Fig. 2A) 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 (Supplementary Fig. S5A). Considering the majority of KRAS transcripts in these cells are the mutant form (Fig. 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 (16). However, examination of shNF1 expressing cells did not reveal increased NF-kB activation in HCECs (Supplementary Fig. S5B). 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 (Fig. 2B). The magnitude of inhibition was greatest when wild type *HAPLN1* was introduced into cells carrying *HAPLN1* mutations (VaCo576, Fig. 2B).

**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 (Fig. 3A) but also because *MAP2K7* and *MAPK8IP2* were the most potent hits without previously established tumor suppressive functions (Supplementary Fig. S2). 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 (17, 18). 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 (19). Accordingly, switching HCECs to detached culture conditions results in rapid phosphorylation of JNK, which is sustained for 24 hours (Fig. 3B). JNK activation increases p53 levels and stabilizes MAP2, which in turn leads to apoptosis (20) as demonstrated by increased cleaved PARP levels (Fig. 3B). shRNAs against upstream activating kinase MAP2K7 and scaffold protein MAPK8IP2 enhances anchorage-
independent growth by 3 and 2.5 fold, respectively (Fig. 3C). Importantly, anchorage-independent growth induced JNK activation was abrogated in HCECs expressing shRNAs against MAP2K7 or MAPK8IP2 (Fig. 3D). 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 (Supplementary Fig. S6). Interestingly, shRNAs against MAP2K7, but not MAPK8IP2, also enhanced invasion through Matrigel™ by 3-fold in HCECs (Supplementary Fig. S7) 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 (6, 10). 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 (Fig. 4). 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 (Supplementary Fig. S8). These enhancements were comparable to that of ectopic expression of oncogenic K-
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.

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References

Figure Legends

**Figure 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 oncogenically 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) whereas 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.

**Figure 2.** 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, \(*P<0.05, **P<0.01, ***P<0.001.\)
**Figure 3.** 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 4.** 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.
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