Genome-Wide Study of Hypomethylated and Induced Genes in Patients with Liver Cancer Unravels Novel Anticancer Targets

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Abstract

Purpose: We utilized whole-genome mapping of promoters that are activated by DNA hypomethylation in hepatocellular carcinoma (HCC) clinical samples to shortlist novel targets for anticancer therapeutics. We provide a proof of principle of this approach by testing six genes short-listed in our screen for their essential role in cancer growth and invasiveness.

Experimental Design: We used siRNA- or shRNA-mediated depletion to determine whether inhibition of these genes would reduce human tumor xenograft growth in mice as well as cell viability, anchorage-independent growth, invasive capacities, and state of activity of nodal signaling pathways in liver, breast, and bladder cancer cell lines.

Results: Depletion of EXOSC4, RNMT, SENP6, WBSCR22, RASAL2, and NENF effectively and specifically inhibits cancer cell growth and cell invasive capacities in different types of cancer, but, remarkably, there is no effect on normal cell growth, suggesting a ubiquitous causal role for these genes in driving cancer growth and metastasis. Depletion of RASAL2 and NENF in vitro reduces their growth as explants in vivo in mice. RASAL2 and NENF depletion interferes with AKT, WNT, and MAPK signaling pathways as well as regulation of epigenetic proteins that were previously demonstrated to drive cancer growth and metastasis.

Conclusion: Our results prove that genes that are hypomethylated and induced in tumors are candidate targets for anticancer therapeutics in multiple cancer cell types. Because these genes are particularly activated in cancer, they constitute a group of targets for specific pharmacologic inhibitors of cancer and cancer metastasis. Clin Cancer Res; 20(12); 3118–32. ©2014 AACR.

Introduction

Cancer initiation and progression are driven by concurrent changes in expression of multiple genes via genetic and epigenetic alterations leading to activation of oncogenes and prometastatic genes, silencing of tumor suppressor genes and to genome rearrangements and instability (1–3). As extensive research revealed methylation-mediated silencing of tumor suppressor genes to be common in cancer, therapeutic strategies have been developed with the goal of decreasing DNA methylation using inhibitors of DNA methyltransferases (DNMT), enzymes catalyzing DNA methylation reaction (1, 4–7).

However, in contrast with the overall focus of the field on hypermethylation both from the therapeutic and diagnostic perspectives, genome-wide high-resolution studies have shown that DNA hypomethylation is as frequent and persistent as hypermethylation in cancer and it targets not just repetitive sequences as has been previously well documented (8) but also promoters of genes involved in cell invasion and metastasis (2, 9–12). A recent comprehensive study demonstrated that zebularine, a DNA methylation inhibitor, exhibited a dual mode of action on hepatocellular carcinoma (HCC) cells depending on the cellular context (7). In zebularine-sensitive cells, the drug caused demethylation and activation of tumor suppressor genes as well as genes involved in regulation of apoptosis and cell-cycle
Translational Relevance

Our study provides evidence that aberrant DNA promoter hypomethylation in cancer leads to activation of genes that are required for continued cancer cell proliferation and invasiveness. These sets of genes are candidate targets for anticancer therapy. We show, for the first time, that silencing of RASAL2, NENF, EXOSC4, SENP6, and WBSCR22 in human liver cancer cell lines inhibits cell growth and invasive properties. Depletion of RASAL2 and NENF reduces anchorage-independent growth in vitro and human subcutaneous tumor xenograft growth in mice. We establish the critical role of RASAL2 and NENF in multiple human cancer cell lines and provide insights into the mechanism of action. Our studies describe a general workflow for identifying novel anticancer therapeutic targets by selecting genes hypomethylated in cancer.

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...progression. This was concomitant with inhibition of proliferation and induction of apoptosis. However, the same drug led to demethylation and activation of oncogenes and oncogenic pathways in resistant cells. This differential action was independent of the fact that DNMT1 inhibition was observed in both sensitive and resistant cells (7). Genes that are activated in cancer and are required for cancer growth and cancer metastasis are likely targets for novel drug development that could also overcome resistance to existing chemotherapeutics. Moreover, genome-wide analyses might reveal genes that are commonly hypomethylated in many cancers and are thus candidates for novel broad-spectrum anticancer and antimetastatic agents. Thus, DNA hypomethylation analysis should unravel novel targets for anticancer therapeutics that were unrealized to date.

We tested this hypothesis by examining a dataset of 230 genes that were hypomethylated and induced in HCC in comparison with normal adjacent tissue (≥1.5 fold change in promoter methylation and in expression, P ≤ 10E−3) as we have recently delineated (2). Within these 230 hypomethylated genes, we focused on 111 genes with promoters of high CpG density as they showed overall the most robust induction in expression in tumors (≥2-fold, P ≤ 10E−4; ref. 2). We used GO, KEGG, and NCBI databases in order to establish biologic processes, functions, and pathways that these genes are involved in. We found a large body of literature that documented the involvement of 91 of these genes in biologic processes, functions, and/or pathways characteristic of cancer and carcinogenesis. The role of the remaining 20 genes in cancer has been unknown. Although some of the genes have unknown biologic functions, hypomethylation and induction of these genes in tumors compared with normal tissue suggest their potential role as novel anticancer targets and warranted further validation and elaboration on their effects. Pathway analysis of these 20 new candidates showed possible involvement in functional networks, which might be crucial to cancer such as cell proliferation and differentiation, cell-cycle progression, signal transduction, transcriptional regulation, DNA and RNA modifications. In this study, we selected 6 genes from this list, Ras-GTPase-activating protein (RASAL2), neuron-derived neurotrophic factor (NENF, neudesign), Williams–Beuren syndrome chromosome region 22 (WBSCR22), SUMO1/sentrin-specific peptidase 6 (SENP6), exosome complex component RRP41 (EXOSC4), RNA (guanine-7-) methyltransferase (RNMT), and provided evidence that their depletion with selective siRNA attenuates cell growth and invasiveness of HCC cancer cell line HepG2. RASAL2 is a protein containing the GAP-related domain (GRD) that is a characteristic domain of GTPase-activating proteins (GAP) that were shown to be crucial for AKT activity and bind to AKT inducing its phosphorylation via integrin-linked kinase (13). RASAL2 was recently shown to interact with ECT2, a guanine nucleotide exchange factor that is overexpressed in primary astrocytomas, activating promyelotoid RHO family members (14). Knockdown of RASAL2 decreases ECT2 activity toward RHO in astrocytoma cells and leads to mesenchymal–amoeboid transition, altering the invasive properties of the cells. NENF is a secreted protein with neurotrophic activity expressed abundantly in the developing brain and spinal cord (15). It was shown to induce phosphorylation of ERK1/2 and AKT in primary cultured mouse neurons and MCF-7 breast cancer cells (15). NENF is also highly expressed in several cancers and its overexpression in MCF-7 breast cancer cells increases their tumorigenicity and invasiveness (16). WBSCR22 that contains a methyltransferase domain and is part of the chromosomal region deleted in Williams syndrome, a multisystem developmental disorder (17), was shown to be overexpressed in invasive breast cancer. Ectopic expression of WBSCR22 in nonmetastatic cells enhances metastasis formation without affecting cell growth and motility and its knockdown in tumor cells reverses metastasis in breast cancer cells (18). SENP6 is a member of a family of sumoylation proteases (19) that were shown to be elevated in several kinds of tumors and contribute to cancer through altering the balance of sumoylation of important transcription regulators (20). For SENP6 however, a few reports show downregulation in breast cancer (20). EXOSC4, is a noncatalytic member of RNA exosome complex and is involved in RNA degradation with no documented role to our knowledge in cancer (21). RNMT is involved in methylating mRNA cap structures (22). mRNA cap methylation is rate limiting for translation of the c-MYC oncogene in epithelial cells (23).

We showed for the first time that depletion of 5 of the 6 genes (excluding RNMT) in liver cancer cell lines caused attenuation of cancer growth and invasiveness, suggesting that the genome-wide approach we have undertaken should point to potentially cancer critical genes with high efficiency. We further focused on 2 of these genes, RASAL2 and NENF, as several reports suggest their involvement in regulation of key oncogenic signal transduction pathways, MAPK and AKT (13, 15). Our findings show that depletion of RASAL2 or NENF decreases tumorigenic properties of different cancer cells, including liver, bladder, and breast
cancer, and reduces the ability of liver cancer cells to form tumor xenografts in mice. This was associated with reduced phosphorylation of key members of MAPK, AKT/mTOR, and WNT oncogenic signaling cascades and downregulation of DNMT1 and MBD2, proteins that were shown to be involved in tumorigenesis and metastasis (4, 5). Our present work provides proof of principle that hypomethylated genes in tumors are broad-spectrum candidate anticancer targets and describes a method for identifying these targets using genome-wide DNA methylation and transcriptome screens in human tumors.

Materials and Methods

Tissue samples, cell lines, and transfection with siRNA
Cancerous and normal adjacent tissue samples were obtained from 11 patients with HCC in Chinese National Human Genome Center at Shanghai, China (Dr. Z.-G. Han). All patients provided written informed consent, and the Ethics Committee from Chinese National Human Genome Center at Shanghai approved all aspects of this study. Human HCC HepG2, HCC Hep3B, adenocarcinoma SkHep1, bladder cancer T24, and breast cancer MDA-MB-231 cells were purchased from ATCC (HB8065, HB8064, HTB52, HTB4, and HTB26, respectively), whereas human untransformed hepatocytes (normal hepatocytes, NorHep) were obtained from Celprogen (3,003-02). HepG2, Hep3B, SkHep1, and MDA-MB-231 cells were maintained in MEM medium (Gibco) and T24 cells in McCoy’s 5A medium, supplemented with 2 mmol/L glutamine (Sigma-Aldrich), 10% fetal bovine serum (FBS; Gibco), 1 U/mL penicillin, and 1 mg/mL streptomycin (Gibco). NorHep cells were maintained in human hepatocyte cell culture complete medium (Celprogen). Cells were grown in a humidified atmosphere of 5% carbon dioxide at 37°C. Twenty-four hours before siRNA treatment, cells were plated at a density of 3 to 6 × 10^5 per a 10-cm tissue culture dish. The following siRNA, obtained from Dharmacon, were used in this study (see Supplementary Table S1A for the sequences): control siRNA (siCtrl), human RASAL2 siRNA (siRASAL2, M-009411-02-0020, and siRASAL2-1), human NENF siRNA (siNENF, M-016079-00-0020, siNENF-1), human EXOSC4 siRNA (siEXOSC4, M-013760-01-0020), human RNMT siRNA (siRNMT, M-019525-00-0020), human DNMT1 siRNA (siDNMT1, M-013760-01-0020), and human MBD2 siRNA (siMBD2, M-013760-01-0020).

Viability, invasion, and anchorage-independent growth assays
Cell viability was determined by the Trypan blue (Sigma-Aldrich) exclusion test. Cells were harvested after transfection with siRNA on day 3, 6, and 9. Following 3 to 5 minute incubation with Trypan blue, the viable cells were counted under the microscope.

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The ability of cells treated with siCtrl and siRASAL2 or siNENF to invade through extracellular matrix was evaluated by the Cell Invasion Assay Kit (Chemicon Int.). The kit utilizes a reconstituted basement membrane matrix of proteins derived from Engelbreth–Holm–Swarm (EHS) mouse tumor. Briefly, 50,000 cells resuspended in serum-free media were added to the inserts dipped in the lower chamber containing complete media. Following 24-hour incubation at 37°C, invasive cells were stained and counted under the microscope.

The effects of transfection with siRNA are transient. To measure long-term impact of depletion of these genes, we generated RASAL2-, or NENF-depleted stable HepG2, SkHep1, and NorHep cell lines with shRNA-expressing lentivirus (see Supplementary Materials and Methods for details on lentivirus production and cell transduction and Supplementary Table S1D for shRNA sequences). To determine anchorage-independent growth on soft agar, an in vitro measure of oncogenesis (24), 6,000 to 12,000 live cells with stable knockdown of RASAL2 or NENF were seeded into soft agar and plated in triplicate in a 6-well plate for 21 days as previously described (25). The number of colonies (>10 cells/colony) in 5 random fields (40×) per well, throughout all planes of the triplicate wells, was counted under the microscope.

RNA extraction and quantitative real-time PCR
Total RNA was isolated using Trizol (Invitrogen, Life Technologies) according to the manufacturer’s protocol. One micrograms of total RNA served as a template for cDNA synthesis using 20 U of AMV reverse transcriptase (Roche Diagnostics), as recommended by the manufacturer. The quantitative real-time PCR (QPCR) reaction was carried out in Light Cycler 480 machine (Roche) using 2 μL of cDNA, 400 nmol/L forward and reverse primers listed in Supplementary Table S1B, and 10 μL of Light Cycler 480 SybrGreen I Master (Roche) in a final volume of 20 μL. Amplification was performed using the following conditions: denaturation at 95°C for 10 minutes, amplification for 60 cycles at 95°C for 10 seconds, annealing temperature for 10 seconds, 72°C for 10 seconds, and final extension at 72°C for 10 minutes. Quantification was performed using a standard curve and analyzed by the Roche LightCycler 480 software.

Pyrosequencing
Bisulfite conversion was performed as previously described (26). Specific bisulfite converted promoter sequences were amplified with HotStar Taq DNA polymerase (Qiagen) using biotinylated primers listed in Supplementary Table S1C. The biotinylated DNA strands were pyrosequenced in the PyroMarkTMQ24 instrument.
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Western blot analyses

Total protein extract was obtained by dissolving in RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1× complete protease inhibitors; Roche Diagnostics). The total protein yield was determined using Bradford reagent (Bio-Rad). A total of 50 to 100 μg of proteins were loaded on a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were immunoblotted with anti-RASAL2 (Bethyl Laboratories Inc., A302-109A) or anti-NENF (Abcam, ab74474) antibody at 1:1,000 and 1:500 dilution, respectively, followed by a secondary anti-rabbit (Santa-Cruz Biotechnology; sc-2004) IgG antibody at 1:5,000 or 1:4,000 dilution, respectively. The membranes were blotted with an anti-β-actin antibody as a loading control (Sigma-Aldrich).

Determination of kinase phosphorylation

Relative levels of phosphorylation of a set of kinases were determined using Human Phospho-MAPK Array Kit (ARI002B, R&D Systems, Inc.). The kit utilizes control and capture antibodies carefully selected and spotted in duplicate on nitrocellulose membrane. Briefly, cell lysates were diluted, mixed with a cocktail of biotinylated detection antibodies, and incubated overnight with the Proteome Profiler Human Phospho-MAPK array membrane. The membranes were washed to remove unbound material. Streptavidin-horseradish peroxidase and chemiluminescent detection reagents were then applied and a signal was produced at each capture spot corresponding to the amount of phosphorylated protein bound. The densitometric analysis of the spot intensity on developed film was performed using ImageJ software. A signal from a clear area of the array was used as a background value. An average background signal was subtracted from each positive spot and the resulting value constituted a relative phosphorylation level.

Human tumor xenographs in NOD/SCID mice

For xenograft studies, 4 to 6 weeks old male NOD/SCID mice (The Jackson Laboratory) were divided randomly into 6 groups of 6 animals each. We used stable RASAL2 or NENF-depleted HepG2 and SkHep1 cell lines in the in vivo study to verify long-term knockdown (see Supplementary Materials and Methods for details on lentivirus production and cell transduction). Two million viable cells transduced with sh-Scr (nontargeting negative control shRNA), shRASAL2 (sequence No. 7), or shNENF (sequence No. 2) expressing lentivirus were injected subcutaneously into the mice in 50% matrigel. The experimental protocol was approved by the Institutional Animal Care Committee. We also repeated the study with transiently transfected cells to measure the long-term impact of transient depletion of these genes, which would mimic a pharmacologic treatment if these data were translated. HepG2 and SkHep1 cells were treated with 56 nmol/L siRASAL2, siNENF, or siCtrl as described above. After 48 hours, the transfection was repeated and the cells were harvested and counted 24 hours later. A small pool of 10^5 cells was saved for QPCR analysis in order to confirm knockdown of the target genes. Viable cells in amount of 2×10^6 of HepG2 and 1×10^6 of SkHep1 cells were injected subcutaneously into the mice in 50% matrigel. The next steps were identical to the protocol with stable cell lines.

Statistical analysis

Statistical analysis was performed using an unpaired t test. Each value represents the mean ± SD of 3 determinations in either 2 or 3 independent experiments. The results were considered statistically significant when P < 0.05.

Results

Biologic functions of novel liver cancer candidate genes hypomethylated and induced in HCC

We reasoned that genes that are potentially critical for cancer growth and metastasis but not for normal tissue survival and physiology would be epigenetically reprogrammed and activated in tumors in comparison with normal noncancerous tissue. Our previous study of the landscape of DNA methylation in tumors from patients with liver cancer revealed a group of 230 genes that were induced and whose promoters were hypomethylated in HCC in comparison with the matched adjacent tissue (2). We shortlisted 20 genes that were not previously shown to be anticancer targets (Table 1). The analysis of their functions based on publicly available datasets such as Gene Ontology (GO), KEGG, and NCBI revealed biologic processes and pathways that are crucial to carcinogenesis (Table 1). The Pharmacogenomics Knowledgebase (PharmGKB) and Therapeutic Target Database were used to verify whether any of these 20 genes is already a target of a known drug or acts within a known drug pathway. We did not find any information about drug development for any of the candidates listed in Table 1. To define the functional role of the identified novel putative cancer targets in cellular transformation, we used shRNA/shRNA-mediated depletion in liver cancer cell lines as a tractable cellular model for human liver tumors where these genes were found to be hypomethylated and activated. We first focused on 2 genes, RASAL2 and NENF because previous literature data suggested their involvement in MAPK and/or AKT signal transduction pathways and their likely important role in carcinogenesis and metastasis (14, 16).

RASAL2 and NENF are regulated by DNA promoter methylation in HCC tumors and liver cancer cell lines and are overexpressed in several types of human cancer

Promoter methylation microarrays and Affymetrix expression microarray data indicated that RASAL2 and NENF promoters are hypomethylated and that their expression is induced in HCC samples compared with adjacent normal tissue (Fig. 1A–C; see Supplementary Materials and Methods for details on microarray analyses). Statistically significant hypomethylation of several CG sites in the

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<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Chromosome</th>
<th>Pathway/function/biologic process</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASD1</td>
<td>CAS1 domain containing 1, O-acetyltransferase</td>
<td>7</td>
<td>Not known, integral to membrane</td>
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<tr>
<td>CCDC138</td>
<td>Coiled-coil domain containing 138</td>
<td>2</td>
<td>Not known</td>
</tr>
<tr>
<td>CSPP1</td>
<td>Centrosome and spindle pole-associated protein 1</td>
<td>8</td>
<td>Positive regulation of cell division, cell-cycle-dependent microtubule organization</td>
</tr>
<tr>
<td>EXOSC4</td>
<td>Exosome complex component RRP41</td>
<td>8</td>
<td>mRNA metabolic process, positive regulation of cell growth</td>
</tr>
<tr>
<td>FAM83D</td>
<td>Family with sequence similarity 83, member D</td>
<td>20</td>
<td>Chromosome congression and alignment during mitosis, cell division</td>
</tr>
<tr>
<td>GDPD1</td>
<td>Glycerophosphodiester phosphodiesterase domain containing 1</td>
<td>17</td>
<td>Lipid metabolic process, glycerol metabolic process</td>
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<tr>
<td>IFT81</td>
<td>Intraflagellar transport 81 homolog (Chlamydomonas), carnitine deficiency-associated gene expressed in ventricle 1</td>
<td>12</td>
<td>Development of the testis and spermatogenesis, cell differentiation, cardiac hypertrophy caused by carnitine deficiency</td>
</tr>
<tr>
<td>JPH3</td>
<td>Junctophilin 3, trinucleotide repeat-containing gene 22 protein</td>
<td>16</td>
<td>Formation of junctional membrane complexes, cross-talk between the cell surface and intracellular calcium release channels, regulation of neuronal synaptic plasticity</td>
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<td>KCTD2</td>
<td>Potassium channel tetramerization domain containing 2</td>
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<td>Protein homooligomerization, voltage-gated potassium channel activity</td>
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<tr>
<td>NEIL3</td>
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<td>DNA N-glycosylase activity, base-excision repair, damaged DNA binding</td>
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<td>NENF</td>
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<td>Activation of MAPK and AKT signaling pathways, growth factor activity</td>
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<td>PAQR4</td>
<td>Progestin and adipoQ receptor family member IV</td>
<td>16</td>
<td>Not known, integral to membrane, putative receptor activity</td>
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<td>Carbohydrate metabolic process</td>
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<tr>
<td>RASAL2</td>
<td>RAS protein activator like 2</td>
<td>1</td>
<td>Regulation of small GTPase-mediated signal transduction, GTPase activator activity, inhibitory regulator of the Ras/cyclic-AMP pathway, putative activator of the AKT pathway, somatic mutations identified in colorectal cancer mRNA-capping methyltransferase, hepatocyte growth factor receptor (HGFR), putative proto-oncogenic receptor tyrosine kinase</td>
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<td>RNMT</td>
<td>RNA (guanine-7-) methyltransferase</td>
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<td>SENP6</td>
<td>SUMO1/sentrin specific peptidase 6</td>
<td>6</td>
<td>Regulation of transcription, deconjugation of SUMO1, SUMO2 and SUMO3 from targeted proteins</td>
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<td>SMYD5</td>
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<td>SRRT</td>
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<td>Cell proliferation, regulation of transcription, RNA-mediated gene silencing by miRNAs</td>
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<td>Not known, cell redox homeostasis</td>
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<td>WBSCR22</td>
<td>Williams–Beuren syndrome chromosome region 22, Williams–Beuren candidate region putative methyltransferase</td>
<td>7</td>
<td>Methyltransferase activity putatively acting on DNA</td>
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</table>

NOTE: Functional analyses were performed using GO, KEGG, and NCBI databases.
promoters of these genes in the tumors group in comparison with the control tissue group was confirmed by pyrosequencing of DNA samples from all 11 patients as shown in Fig. 1D and E (see Supplementary Fig. S1 for extensive methylation analysis). Interestingly, \textit{RASAL2} and \textit{NENF} were also upregulated in HepG2 HCC and SkHep1 liver adenocarcinoma cell lines as compared with nontransformed normal hepatocytes (NorHep; Fig. 1F) and their promoters were hypomethylated at the same CpG sites as in patients with HCC (Fig. 1G and H; see Supplementary Fig. S1 for extensive methylation analysis). The cell lines were chosen for further experiments as a suitable model to study the functional role of these genes in cellular transformation and invasiveness using siRNA/shRNA-mediated depletion.

To determine causal relationship between promoter hypomethylation and activation of these genes in HCC, we tested whether the DNA methylation inhibitor 5-aza-2’-deoxycytidine (5-azaCdR) would induce these genes in HepG2 liver cancer cells as well as in NorHep cell culture. NorHep were included in this experiment because of high methylation state of promoters of the tested genes. Both \textit{RASAL2} and \textit{NENF} were induced upon treatment of HepG2 cells with 1.0 \textmu g/ml 5-azaCdR for 3 days, which was accompanied with strong hypomethylation of their promoters (Fig. 1I; see Supplementary Fig. S1 for extensive methylation analysis). In NorHep, hypomethylation of \textit{NENF} promoter was associated with its upregulation just after 3-day treatment, whereas hypomethylation and induction of \textit{RASAL2} were observed after 20-day exposure to 5-azaCdR (Supplementary Fig. S2; see Supplementary Fig. S1 for extensive methylation analysis, note no cytotoxic effect of the treatment in NorHep in Supplementary Fig. S2A). These results support regulation of transcription of these candidates by DNA methylation.

We reasoned that hypomethylation should target genes that are fundamental to the transformation process and that, therefore, there should be a common set of genes that is hypomethylated in several cancer types. Consistent with this hypothesis we show that overexpression of \textit{RASAL2} and \textit{NENF} is not specific only to liver cancer. Using publicly available expression microarray data (Oncomine), we demonstrate that the genes show increased expression in lymphoma, breast, colorectal, lung (\textit{NENF}), pancreatic (\textit{RASAL2}), and an independent set of HCC tumors (different from the set analyzed by our group; Supplementary Fig. S3A). The data are consistent with a general role for these proteins in many different types of cancer. This is consistent with the hypothesis that these proteins may play a fundamental role in either cancer progression or metastasis and with recent data, suggesting a role for \textit{RASAL2} and \textit{NENF} in cancer (14, 16).

\textbf{Invasiveness of liver cancer is dependent on \textit{RASAL2}, \textit{NENF}, \textit{EXOSC4}, \textit{SENP6}, or \textit{WBSCR22} expression}

To test whether \textit{RASAL2} and \textit{NENF} play a causal role in cellular transformation and invasiveness, we first used a human liver cell culture model, a cancer cell type where the epigenetic activation of these genes was first discovered (Fig. 1A and B). We depleted \textit{RASAL2} or \textit{NENF} mRNA in HepG2 HCC and SkHep1 liver adenocarcinoma cell lines, as well as in non-transformed normal hepatocytes (NorHep) to test whether the effects are cancer specific. For long-term studies such as soft-agar assay, stable HepG2, SkHep1, and NorHep cell lines with \textit{RASAL2} or \textit{NENF} depletion were established and used rather than siRNA-transfected cells (see Supplementary Materials and Methods for details on lentivirus production and cell transduction). Depletion of either \textit{RASAL2} or \textit{NENF} expression in the liver cancer cell lines (Fig. 2A and E and Supplementary Fig. S4 and S5C, S5D, S5G, and S5H) resulted in inhibition of the rate of cancer cell growth (Fig. 2B and F and Supplementary Fig. S5A and S5E), cell invasive capacities (Fig. 2C and G and Supplementary Fig. S5B and S5F) and anchorage independent growth as compared with control nontreated cells (Fig. 2D and H). Importantly, using other sets of siRNAs (si\textit{RASAL1} and si\textit{NENF-1}) or shRNAs (sh\textit{RASAL-9} and sh\textit{NENF-1}), we observed similar effects on cell growth and metastatic properties in both HepG2 and SkHep1 cells (Supplementary Figs. S3 and S5). These data demonstrate that the results we present are not an idiosyncrasy of the siRNA sequence used and provide high confidence beyond a reasonable statistical doubt that we are measuring effects of \textit{RASAL2} and \textit{NENF} depletion. We also confirmed the role of \textit{RASAL2} and \textit{NENF} in HCC by examining the consequences of knockdown of these genes in a different HCC cell line that bears an integrated hepatitis B virus (HBV) genome, Hep3B (Supplementary Fig. S6A). Depletion of \textit{RASAL2} and \textit{NENF} with siRNAs in Hep3B cells significantly inhibited cell growth and invasive properties.

The effects of siRNA/shRNA-mediated depletion of the tested candidates on cell growth, anchorage-independent growth, and invasiveness differentiate between cancer and normal cell lines. NorHep cells that were treated with siRNAs/shRNAs targeting \textit{RASAL2} or \textit{NENF} proliferated at almost the same rate as control cells with a slight decrease only after triple si\textit{NENF} treatment (9 days; Fig. 2B and F and Supplementary Fig. S5A and S5E). There was no significant effect on invasiveness or anchorage independent growth in either of the treatments (Fig. 2 and Supplementary Fig. S5). These data confirm the cancer-specific requirement for upregulation of these genes and that targeting these genes for inhibition has a profound anticancer effect demonstrating their potential as targets for anticancer and antimetastatic drugs.

We then evaluated whether 4 other genes from the list of 20 novel putative cancer candidates (Table 1) such as \textit{EXOSC4}, \textit{RNMT}, \textit{SENP6}, and \textit{WBSCR22} have a functional role in liver cancer and are potential targets of anticancer therapies. Our previous genome-wide studies revealed hypomethylation of promoters of these genes and induction of their expression in HCC tumors in comparison with adjacent normal tissue (Fig. 3A and B). The genes were depleted in HepG2 HCC cells using siRNA as described in Materials and Methods (Fig. 3C) followed by assessing the functional effects. Cell growth was impaired by 53% to 74%
on day 6 of treatment with siEXOSC4, siSENP6, and siWBSCR22 as compared with siCtrl (Fig. 3D). The number of cells invaded through extracellular matrix was strongly reduced by 80% to 90% (Fig. 3E). Interestingly, we did not observe any changes after depletion of RNMT, which was previously shown to have a putative oncogenic role (23). Taken together, downregulation of 5 of the 6 genes selected for further testing from the list of
hypomethylated and induced genes had an anticancer effect, suggesting high efficacy of this selection method of anticancer targets.

Functional consequences of RASAL2 or NENF depletion in bladder and breast cancer cells

We then showed that targeting NENF and RASAL2 has an effect beyond liver cancer confirming previous findings (14, 16). Depletion of RASAL2 or NENF in invasive T24 bladder and MDA-MB-231 breast cancer cells (Fig. 3F) exerted robust effects on cancer cell growth and invasiveness. The number of viable cells was reduced to 30% and 8% in T24 and to 20% and 40% in MDA-MB-231 cells after siNENF or siRASAL2 treatment, respectively, as compared with siCtrl after double siRNA treatment (Fig. 3G). The invasiveness of viable cells was impaired by 90% and 70% in MDA-MB-231 after either NENF or RASAL2 knockdown, respectively, and almost completely suppressed in T24 cells (Fig. 3H).

Depletion of RASAL2 or NENF causes impairment of MAPK, WNT/β-catenin, and PI3K/AKT/mTOR signaling pathways by alterations in phosphorylation of their effectors

Because RASAL2 and NENF were previously implicated in cellular signaling pathways (14–16), we measured phosphorylation levels of a set of kinases in invasive SkHep1 cells treated with siCtrl and either siRASAL2 or siNENF to determine whether depletion of these proteins affects PI3K/AKT/mTOR and MAPK pathways in cancer cells. Significant changes exceeding 10% are demonstrated in Table 2, whereas all observed alterations are listed in Supplementary Table S2. RASAL2 and NENF depletion reduced phosphorylation of several kinases downstream to PI3K; AKT 1, 2, and 3 and p70S6 kinase whereas mTOR phosphorylation was slightly affected (22% reduction) only by NENF depletion. NENF and RASAL2 depletion reduces phosphorylation of RSK1 and RSK2, which are downstream to both PI3K and MAPK and in turn regulate transcription of several genes via phosphorylation of transcription factors such as CREB, CREBP, NF-kB, c-FOS, and ERz (ref. 28; Fig. 4A). Kinases of RSK p90 subfamily were also shown to phosphorylate GSK3 and activate WNT signaling (28), a critical pathway for oncogenesis. Phosphorylation of GSK3β at serine 9 inactivates its enzymatic activity and results in induction of the WNT/β-catenin pathway. Upon treatment with siRASAL2 or siNENF, GSK3β phosphorylation is attenuated by 15% to 20% that is presumably a result of AKT and RSK1/2 decreased activities, which may lead to downregulation of WNT1 signal transduction.

The MAPK signaling pathway includes 3 main branches of kinase cascades: MEK(1,2)/ERK(1,2), MKK(3,6)/p38, and MKK(4,7)/JNK (Fig. 4A; ref. 29). Depletion of RASAL2 or NENF affects phosphorylation of JNK and MKK6 (Table 2 and Fig. 4A) without any effect on ERKs (Supplementary Table S2). The most relevant 40% to 80% reduction in phosphorylation was seen for JNK3 and MKK6 in response to either RASAL2 or NENF depletion. Depletion of NENF had a more robust action on MAPK signaling than RASAL2 depletion, resulting in 15% to 35% attenuation of p38β and MSK2 phosphorylation. MSK2 is required for MAPK-induced phosphorylation and activation of CREB and ATF transcription factors (30) and for phosphorylation of histone H3, which is important for the rapid induction of immediate-early genes (31). In summary, depletion of NENF and RASAL2 affects the nodal PI3K and MAPK pathways at different points as well as the WNT/β-catenin pathway. Because these signaling pathways converge on regulatory circuitries that control cell-cycle progression, apoptosis, and metastasis, these data provide an explanation for the anticancer effect of NENF and RASAL2 depletion in cancer cells (Fig. 4A).

Diminished expression of RASAL2 or NENF causes reduction of human liver tumor xenograft growth in NOD/SCID mice

To address the question of whether depletion in vitro of RASAL2 and NENF would block the ability of liver cancer cells to grow as explants in vivo, we established stable RASAL2- or NENF-depleted HepG2 and SkHep1 cell lines using lentiviral transduction in vitro, as described in Supplementary Materials and Methods (Supplementary Fig. S5), and then injected the cells subcutaneously into NOD/SCID mice (The Jackson Laboratory). Tumor growth was monitored and the results are presented in Fig. 4B. Depletion in vitro of RASAL2 or NENF had a significant effect on the ability of the cells to grow as explant tumors in vivo. The ability of both HepG2 and SkHep1 cells to form tumors in the mice was blocked after RASAL2 and NENF depletion with slightly greater extent of reduction after RASAL2 knockdown (Fig. 4B). Similar results were obtained when transiently depleted cells were injected into mice (Supplementary Fig. S6B), suggesting a long-term effect of transient depletion of these genes through possibly reversal of the transformed phenotype of the cells.

Discussion

The programmatic changes in gene expression that trigger cancer growth and metastasis involve both activation and suppression of gene expression. The focus in the field to date has been on targeting hypermethylated and silenced genes in cancer. It is extremely difficult to specifically activate a gene silenced in cancer. Pharmacologic agents are usually more effective in inhibiting protein function than activating proteins that are poorly expressed, particularly when gene-silencing results in almost complete deficiency of a protein. Therefore, different strategies were used to develop inhibitors that target the epigenetic mechanisms that control gene silencing such as histone methyltransferases, DNMT1s and histone deacetylases rather than targeting the particular silenced genes and their products (5). However, these methods are inherently nonspecific as each of these enzymes controls numerous gene expression programs, including potentially those that are essential for cellular differentiation and cell physiology.
Figure 2. RASAL2 or NENF depletion decreases cancer cell growth and invasive capacities in vitro with selectivity for cancer versus nontransformed cells. A and E, expression of the depleted genes, RASAL2 (A) and NENF (E), quantified by QPCR after first (I), second (II), and third (III) transfection and by Western blot analysis after second transfection of HepG2, SkHep1, and NorHep with scrambled siRNA (siCtrl) and siRNA directed to RASAL2 (siRASAL2) or NENF (siNENF). (Continued on the following page.)
Pharmacologic inhibitors could be developed to target proteins that are specifically and persistently activated in cancer. The challenge is identifying targets that are qualitatively selective for cancer cells rather than normal tissue. DNA methylation is a stable mark of epigenetic programming that participates in defining the differentiated identity of cells (32). Genes that are methylated in differentiated normal cell types encompass a repertoire that is not required for their normal physiology and are therefore stably silenced. Epigenetic reprogramming and activation of these genes by DNA hypomethylation should result in a stable repertoire of cancer-specific genes that are likely candidates to be particularly required for the cancer state and therefore serve as ideal candidates for specific inhibitors that act as anticancer agents. Therefore, we hypothesized that genes that are activated by DNA hypomethylation in cancer and silenced by methylation in normal cells should be prime targets for inhibition in cancer. We tested this hypothesis by delineating the landscape of hypomethylation in liver cancer (2). Using transcription and promoter methylation (Continued). B and F, effect on cell growth after first (day 3), second (day 6), and third (day 9) transfection with siRASAL2 (B) or siNENF (F). C and G, effect on cell invasion as measured by Boyden chamber invasion assay after second transfection with siRASAL2 (C) or siNENF (G). For SkIhep1 cells transfected with siRASAL2 or siNENF, evaluation of expression on mRNA and protein levels after third transfection was not possible because of the reduced cell number after 3 transfections. Therefore, all the comparisons and invasive assays measurements were performed after the second transfection for all 3 cell lines. D and H, effect on anchorage independent growth as measured by soft-agar assay in stable cell lines with RASAL2 (G) or NENF (H) depletion (see Supplementary Materials and Methods for details on lentivirus production and cell transduction). All results represent mean ± SD of 3 determinations in either 2 or 3 independent experiments; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 4. Impact of RASAL2 and NENF depletion on phosphorylation state of effectors of the MAPK and PI3K/AKT signaling pathways and on xenograft tumor volume in NOD/SCID mice in vivo. A, parallel determination of the relative levels of phosphorylation of MAPK and other serine/threonine kinases was performed in SkHep1 cells using Human Phospho-MAPK Array Kit (ARY002B; R&D Systems, Inc.). Red squares indicate kinases whose phosphorylation was significantly attenuated upon siRASAL2 and siNENF treatments as compared with siCtrl-treated cells, except p38, MSK2, and mTOR kinases that were affected only by siNENF treatment. B, stable RASAL2 or NENF depleted HepG2 and SkHep1 cell lines were established using lentiviral transduction (see Supplementary Materials and methods for details on lentivirus production and cell transduction), and then injected subcutaneously into the NOD/SCID mice as described in the Materials and Methods. Each group was composed of 6 male mice 4 to 6 weeks old. Differences in tumor volume were monitored starting from postinjection week 2 until the mice were sacrificed and are demonstrated in charts. Results represent the mean ± SEM for each group of animals; ***, P < 0.001; **, P < 0.01; *, P < 0.05.
genome-wide microarrays we unraveled a shortlist of genes that are induced and hypomethylated in HCC tumors with no or little data on their implications in cancer (Fig. 1A–C, Fig. 3A and B, Table 1). We determined using publicly available datasets such as GO, KEGG, and NCBI that biologic processes and pathways that play a key role in carcinogenesis are enriched within this group of genes and several of these genes were indeed just recently shown to play different roles in cancer metastasis such as NENF (16), RASAL2 (14), and WBSCR22 (ref. 18; Table 1). Because none of the genes was found among targets of known drugs to date, such genes could be considered as new targets for cancer drug development. In this study, we tested using siRNA/shRNA-mediated gene depletion whether inhibition of these targets that were identified in samples from patients with liver cancer would have anticancer and anti-invasive effects in 2 liver cancer cell lines in vitro and whether these effects would be specific to liver cancer cells or whether they would also affect nontransformed liver cells (Fig. 1B and Fig. 3B).

Our data provide supports for the approach we have taken. We show that a genome-wide analysis of hypomethylated and activated genes in cancer could yield new anticancer drug targets with high efficiency. We have tested 6 candidates, EXOSC4, RNMT, SENP6, WBSCR22, RASAL2, and NENF that are likely to be involved in signal transduction, DNA methylation, and regulation of transcription. We demonstrated for the first time that depletion of 5 of the 6 tested candidates reduced cancer cell growth and invasive capacities in liver cancer (Figs. 2 and 3 and Supplementary Fig. S5). The effects on cell invasion cannot be just explained as a consequence of cell death or inhibition of proliferation as the number of cells was corrected for the number of viable cells and it is well known that proliferation and invasiveness are controlled by different pathways (33). Thus, our approach focusing on genes activated by hypomethylation in tumors resulted in a high yield of candidate anticancer drug targets whose pharmacologic inhibition could block cellular transformation and invasiveness.

Surprisingly, we did not observe any anticancer effects by depleting RNMT that was previously shown to enhance human mammary epithelial cell transformation (23). RNMT increased the number and size of colonies only in combination with c-Myc. It is possible that RNMT may be critical for cancer only in specific cancer subsets that express particular genomic programs. We then focused on 2 of the genes RASAL2 and NENF that were shown previously to trigger oncogenic signal transduction pathways, MAPK and AKT (13, 15). Both genes were hypomethylated and induced in HCC tumors and liver cancer cell lines compared with normal tissue and normal nontransformed hepatocyte culture (NorHep), respectively, providing an in vitro model to test whether blocking these targets will have a specific anticancer effect (Fig. 1). We examined whether expression of these genes could differentiate HCC from chronic hepatitis B infection. Although the results were inconclusive because of a small number of patients, they warrant further investigation on higher number of samples in order to determine whether these genes could serve as predictive markers in chronic hepatitis B samples (Supplementary Fig. S3H). Please note that RASAL2 possesses 2 isoforms with their transcription start sites more than

**Table 2. A list of kinases whose phosphorylation was significantly attenuated in SkHep1 liver cancer cells after RASAL2 or NENF depletion**

<table>
<thead>
<tr>
<th>Name</th>
<th>Signaling pathway</th>
<th>siRASAL2 (% control)</th>
<th>siNENF (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT1</td>
<td>PI3K/AKT/mTOR, WNT</td>
<td>69.9</td>
<td>54.7</td>
</tr>
<tr>
<td>AKT2</td>
<td>PI3K/AKT/mTOR, WNT</td>
<td>58.0</td>
<td>52.4</td>
</tr>
<tr>
<td>AKT3</td>
<td>PI3K/AKT/mTOR, WNT</td>
<td>42.9</td>
<td>46.2</td>
</tr>
<tr>
<td>AKT pan</td>
<td>PI3K/AKT/mTOR, WNT</td>
<td>41.6</td>
<td>37.4</td>
</tr>
<tr>
<td>GSK3α, β</td>
<td>PI3K/AKT, WNT</td>
<td>84.9</td>
<td>80.6</td>
</tr>
<tr>
<td>GSK3β</td>
<td>PI3K/AKT, WNT</td>
<td>107.4</td>
<td>78.3</td>
</tr>
<tr>
<td>JNK1</td>
<td>MAPK</td>
<td>106.5</td>
<td>67.4</td>
</tr>
<tr>
<td>JNK3</td>
<td>MAPK</td>
<td>65.4</td>
<td>48.3</td>
</tr>
<tr>
<td>M KK3</td>
<td>MAPK</td>
<td>94.6</td>
<td>88.5</td>
</tr>
<tr>
<td>M KK6</td>
<td>MAPK</td>
<td>20.9</td>
<td>53.4</td>
</tr>
<tr>
<td>MSK2</td>
<td>MAPK</td>
<td>87.1</td>
<td>66.7</td>
</tr>
<tr>
<td>p38/β</td>
<td>MAPK</td>
<td>104.0</td>
<td>84.0</td>
</tr>
<tr>
<td>p70S6 kinase</td>
<td>PI3K/AKT/mTOR</td>
<td>56.4</td>
<td>36.2</td>
</tr>
<tr>
<td>RSK1</td>
<td>PI3K/AKT/mTOR, WNT</td>
<td>47.4</td>
<td>78.2</td>
</tr>
<tr>
<td>RSK2</td>
<td>PI3K/AKT/mTOR, WNT</td>
<td>58.6</td>
<td>66.7</td>
</tr>
<tr>
<td>mTOR</td>
<td>PI3K/AKT/mTOR</td>
<td>95.5</td>
<td>77.7</td>
</tr>
</tbody>
</table>

NOTE: The level of phosphorylation is expressed as a percentage of phosphorylation in the control—cells transfected with scrambled siRNA (siCtrl). Phosphorylation of a panel of 26 kinases was assessed using Human Phospho-MAPK Array Kit (ARY002B; R&D Systems Inc.).
100 kb apart. Methylation differences between tumors and normal liver tissue were detected by the microarray approach and validated by pyrosequencing for transcript variant 2. Thus, our data indicate that DNA methylation seems to regulate the activity of this transcript variant of RASAL2. Interestingly, expression of these 2 isoforms is upregulated upon treatment of HepG2 and NorHep cells with a demethylating agent, 5-azaCdr (Supplementary Fig. S8). It suggests that both isoforms might be regulated by demethylation. It is plausible that the fragment tested in pyrosequencing that is located in intron 1 of variant 2 downstream of a CpG island (note that no differences were detected within the CpG-rich region upstream of CpG: 1; Supplementary Fig. S1), may act as a regulatory region for both transcripts. This issue requires extensive studies and remains to be elucidated in future experiments. We demonstrated that there is causal relationship between hypomethylation of these genes and their activation. Treatment of HepG2 liver cancer cells and normal hepatocytes with a demethylating agent 5-azaCdr increased expression of the genes and reduced DNA methylation in their regulatory regions that is consistent with methylation-dependent regulation of their transcription (Fig. 11 and Supplementary Figs. S1 and S2). We then determined that both genes are overexpressed in other human cancer types as shown in Supplementary Fig. SS, which suggested that inhibitors of these proteins would have a broad spectrum of anticancer action. Our results confirmed this proposition and show that RASAL2 and NENF depletion on cancer cells (Fig. 2 and Supplementary Fig. S5). Stable inhibition of RASAL2 and NENF has an impact on tumorigenic properties of liver cancer cells as explants in NOD/SCID mice in vivo significantly reducing the rate of tumor growth in vivo (Fig. 4B). Furthermore, depletion of RASAL2 or NENF in both HepG2 and SK-Hep1 liver cancer cells led to changes in expression of several genes involved in the DNA methylation machinery and implicated in cancer and cancer metastasis. As shown in Supplementary Fig. S7, siRNA-mediated knockdown of RASAL2 or NENF diminished expression of DNMT1 and MDB2 and changed DNMT3A and DNMT3B levels.

A remarkable observation is the exquisite specificity of the effects of RASAL2 or NENF depletion on cancer cells (Fig. 2 and Supplementary Fig. S5). One possible explanation is that these genes when overexpressed affect transcription and/or activity of downstream effectors with tumorigenic properties and thereby their depletion attenuates cancer growth and invasiveness. Because RASAL2 and NENF are expressed at low levels in normal cells, their depletion would have no impact on the effectors. These data support the idea of focusing on gene targets that are epigenetically silenced in normal cells.

We demonstrate that Ras/Raf/MAPK, PI3K/akt/mTOR, and WNT/β-catenin signaling pathways are affected by depletion of RASAL2 or NENF. These pathways form a complex network that promotes cell proliferation, cell-cycle progression, survival, migration, and metastatic properties. RAS and RAF are mutated in many types of cancers, including colon and pancreatic cancer, melanoma, and HCC that result in the constitutive activation of the pathway and hyperproliferative state (34). Activation of PI3K/akt is also observed in numerous human cancers and leads to stimulation of WNT and NF-κB signals through regulating GSK3β and IκBβ (34). The WNT pathway is frequently switched on in cancers as a result of inactivation of its negative regulators such as APC and GSK3 (34). In normal cells, GSK3 in a complex with APC and axin promotes proteolytic degradation of β-catenin in cytoplasm and prevents β-catenin nuclear translocation and activation of pro-proliferative genes. Depletion of RASAL2 and NENF seems to simultaneously target key kinases of all these 3 pathways as well as downregulate proteins involved with the DNA methylation machinery such as DNMT1 and MDB2 (Supplementary Fig. S7). DNMT1 expression was found to be upregulated in cancer (35), and ectopic expression of DNMT1 resulted in cellular transformation (36). Inhibition of DNMT1 reversed the growth of cancer cells (37) and had antitumorigenic effects in animal models (4, 5, 38, 39). MDB2 was shown to be involved in demethylation and activation of metastatic genes in cancer (40, 41), such as MMP2, PLA2, S100A5, and NUPR1 (2, 5). Knockdown of MDB2 inhibits tumorigenicity (42) and M6B2 depletion inhibits colorectal neoplasia in mice (43). Knockdown of MDB2 decreased cancer cell growth and invasiveness in breast, prostate, and liver cancer cells with concomitant suppression of genes implicated in carcinogenesis (2, 40, 41). Taken together, these effects on signaling pathways and DNA methylation enzymes provide a mechanism of action for the anticancer effects of RASAL2 and NENF inhibition positioning these genes as excellent anticancer drug targets.

Our study lays down a workflow for identifying drug target candidates for anticancer therapy. This workflow involves identifying hypomethylated and activated genes in tumor samples from several types of cancer that are methylated and silenced in normal tissue and have putative important roles in cancer and/or cancer metastasis. We determined from the shortlist of these genes the functional role of RASAL2, NENF, and several other genes in cancer and provided evidence that these genes were potential targets for anticancer drug development as they are not essential for normal cell growth but are required for continued cancer cell proliferation and invasiveness. Our results established for the first time the role of RASAL2 and NENF in multiple cancers and defined the potential functional role of DNA hypomethylation in activation of these genes. The fact that these genes were found to reduce cell growth and invasiveness of cancers derived from vastly different tissues suggested that these proteins may be involved in the most fundamental and common pathways that are required for cellular transformation and cancer metastasis and are candidate targets for anticancer inhibitors.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Genome-Wide Study of Hypomethylated and Induced Genes in Patients with Liver Cancer Unravels Novel Anticancer Targets

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