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Gene signature critical to cancer phenotype as a paradigm for anti-cancer drug discovery

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Abstract

Malignant cell transformation commonly results in the deregulation of thousands of cellular genes, an observation that suggests a complex biological process and an inherently challenging scenario for the development of effective cancer interventions. To better define the genes/pathways essential to regulating the malignant phenotype, we recently described a novel strategy based on the cooperative nature of carcinogenesis that focuses on genes synergistically deregulated in response to cooperating oncogenic mutations. These so-called “cooperation response genes” (CRGs) are highly enriched for genes critical for the cancer phenotype, thereby suggesting their causal role in the malignant state. Here we show that CRGs play an essential role in drug-mediated anti-cancer activity and that anti-cancer agents can be identified through their ability to antagonize the CRG expression profile. These findings provide proof-of-concept for the use of the CRG signature as a novel means of drug discovery with relevance to underlying anti-cancer drug mechanisms.

Introduction

Differential gene expression associated with malignant cell transformation aids development of molecular diagnostic and prognostic applications for the cancer clinic. For example, genomic gene expression signatures can distinguish between tumors of different tissue of origin (1), and segregate various cancer sub-types with distinct clinical features, as for example in B-cell lymphoma (2), breast cancer (3), or lung cancer (4). Further, genomics-based tumor sub-typing, in several instances, has improved disease outcome predictions previously merely based on histological classification (5–8), and gene expression signatures

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can be used as a surrogate to identify signaling pathway activity associated with specific cancer gene mutations (9). Notably, cancer gene expression profiling can also help to match drugs with drug-sensitive cancers (6–7, 10), and mimetic drugs can be identified based on genomic comparison of gene signatures following treatment with a variety of compounds (11–18). Due to the high complexity of differential gene expression patterns associated with cancer, however, the causal relevance of available expression signatures for the cancer phenotype remains largely unknown, thus confining their application to correlative approaches. Utilization of gene expression signatures for discovery of novel intervention strategies and mechanistic analysis has thus remained limited.

Key features of the cancer cell phenotype, such as uncontrolled proliferation, cell motility, and invasiveness, only emerge as a result of the interplay between multiple cooperating oncogenic mutations (19–27). This suggested that oncogenic mutations cooperate through converging mechanisms that involve synergistic regulation of key downstream mediators. Recent work from our laboratory directly supports this idea, as we have shown that multiple oncogenic mutations cooperate in malignant cell transformation through synergistic deregulation of a relatively small group of approximately 100 downstream genes, termed cooperation response genes (CRGs). These genes were sub-selected from the list of genes differentially expressed between young adult mouse colon (YAMC) cells and their malignant derivatives transformed with a pair of cooperating oncogenic mutations, i.e. mutant p53 and activated Ras. Synergistic regulation of CRGs was detected by comparing gene expression levels in cells harboring either mutant p53 or activated Ras, or both oncogenic mutations combined. Remarkably, we found that reversing the deregulation of individual CRGs in malignant cells via genetic perturbation induced strong inhibition of tumor growth at a frequency of approximately 50%. In contrast, equivalent perturbations of differentially expressed non-CRGs yielded tumor inhibition at a frequency near 5%. CRGs thus effectively pinpoint a multitude of drivers of malignant cell transformation downstream of oncogenic mutations among the complex patterns of differential gene expression arising as a consequence of the cancer phenotype (28).

The causal role of CRGs in malignant transformation indicates that modulation of CRG expression is functionally relevant to the cancer phenotype. We thus reasoned that reversal of CRG expression in response to pharmacological agents may be a powerful indicator of anti-cancer activity. In addition, CRGs are implicated in the control of virtually all features of cancer cells, such as proliferation, survival, invasiveness and metabolism, suggesting that cooperating oncogenic mutations drive malignant cell transformation by simultaneously enabling multiple cancer cell traits through modulation of CRG expression (28). Accordingly, modulation of the CRG expression signature by anti-cancer agents should provide insight into underlying mechanisms of drug action.

Results

Compounds antagonizing the CRG signature

Reprogramming expression of individual CRGs in cancer cells to levels found in normal parental cells via genetic perturbation inhibits tumor formation with high efficacy (28). We thus hypothesized that pharmacological agents capable of antagonizing the CRG signature also may inhibit tumor growth, presumably through altering CRG expression. To identify compounds with such potential, we interrogated the Connectivity Map database (CMap) (14, 29) searching for compounds that reversed the CRG expression signature, i.e. suppressed up-regulated CRGs and activated down-regulated CRGs. Among the compounds that maximally antagonized CRG expression, we found that 9 out of the top 20 represented histone deacetylase inhibitors (HDI) (Figure 1A, Table S1), compounds with known anti-cancer effects (30). HDI thus emerged as an example to test our hypothesis that CRGs can

be used as a causally relevant multi-dimensional tool for identification of anti-cancer agents together with underlying mechanisms of action. Given the strong enrichment for HDI as antagonizing the CRG signature in multiple human cancer cell lines and the strong negative connectivity scores (see also below), we chose to focus on HDI for proof-of-principle experiments testing a causal relationship between pharmacological modulation of the CRG signature and the biological outcome of such modulation.

Notably, HDIs demonstrated an overall tendency to antagonize rather than enhance the CRG expression signature (Figure 1B). Nevertheless, given that HDIs affect the expression of numerous genes, we next sought to estimate the significance and specificity of our findings. To this end, we challenged the CMap with 1000 randomly generated signatures, each comprising 76 genes as the CRG signature, and found that none of these simulated signatures could demonstrate the overall level of antagonism as the CRG signature (Figure 1C; $p < 0.001$). Next, we determined whether CMap identification of HDI instances, negatively connected to the CRG signature, was due to HDI being general antagonists of gene expression signatures arising from perturbation of most any pathway. To explore this possibility, we used CMap2, which contains gene expression signatures arising from 5842 non-HDI chemical perturbations and hence is reflective of many pathway perturbations. The 24 most up-regulated and 54 most down-regulated genes from each CMap2 non-HDI instance were selected and used to query CMap1. The frequency of negative connectivity of the same magnitude as the CRG signature to HDIs by any of the 5842 signatures was very low ($< 0.017\%$; Figure 1D). Overall, these observations strongly suggested a very specific and non-random relationship between the CRGs and the influence of HDIs upon them.

A variety of natural and synthetic compounds function as HDI (30) and induce cell cycle arrest, differentiation, and apoptosis selectively in murine and human cancer cell lines *in vitro* and inhibit tumor formation *in vivo* (31–35). These drugs inhibit the function of histone deacetylases (HDACs), that remove acetyl groups from lysine residues on histone tails, thus inducing chromatin condensation (36), associated with heterochromatin formation and transcriptional silencing (37–38). HDI are currently under clinical evaluation as single agents (39–45) or in combination with existing chemotherapeutic agents (46–49).

Exposure of a variety of human cancer cells, such as PC3 prostate carcinoma cells, MCF-7 breast carcinoma cells, or HL-60 acute myeloid leukemia cells, to the CMap-identified HDI valproic acid (VA) (14), caused alterations in CRG expression levels, resulting in an inverted pattern as compared to the CRG expression signature (SFig 1). The cell lines indicated carry Ras and loss-of-function p53 mutations that can cooperatively control CRG expression, in the form of loss of p53 and mutation of N-Ras in HL-60 cells, or mutations in key components of the Ras and p53 pathways, such as loss of p53 and PTEN in PC3 cells, and mutation in PI3-Kinase and loss of p16/ARF in MCF-7 cells (50).

HDI reverse cooperation response gene signature in mp53/Ras cells

Because CRGs are critical for the malignant state, our finding that HDI antagonize the CRG signature in multiple human cancer cell contexts suggested that this class of compounds, at least in part, may act through resetting CRG expression. In order to test this prediction, we first examined the sensitivity to HDI of young adult mouse colon (YAMC) cells (51) transformed with activated Ras and mutant p53 (mp53/Ras cells), where CRG expression is known to be essential for tumor formation (28). The relevance of CRG modulation for the biological effects of HDI on cancer cells was tested based on the notion that HDI can inhibit tumor formation *in vivo* (34, 52–53), a context where the role of CRGs is well defined. Either of the HDI, VA and sodium butyrate (NB), a naturally occurring HDI structurally related to VA, strongly inhibited the ability of treated cells to form tumors when grafted onto immune-compromised mice (Figure 2A). In particular, HDI treatment reduced the

frequency of tumor take after implantation by ~75%, i.e. the number of tumors growing larger than the 0.1 cm³ cell transplant in four weeks (Table 1). For these experiments, cells were exposed to a single dose of either VA or NB for 72 hr in tissue culture, while further functional testing was carried out in absence of drug (53). This procedure allowed for monitoring of CRG expression following HDI pre-treatment, prior to cell transplantation into animals. Under these conditions, HDI treatment induced a cytostatic effect without apoptosis in cell culture (SFig 2, SFig 3A). HDI treatment apparently did not act by targeting the initiating oncogenes, as mp53 and Ras protein levels virtually remained unaltered (SFig 4). Similarly, the GTP-binding activity of mutant Ras also remained unaffected by either mutant p53 or VA and NB (SFig 5), consistent with the idea that modulation of CRG expression acts downstream of oncogenic mutations.

Because CRGs are critical to tumor formation capacity of transformed cells, we next examined the CRG expression response in mp53/Ras cells treated with VA or NB, as compared to untreated controls, using TaqMan Low-Density Arrays (TLDA) with probes to 76 of 95 CRGs, based on probe set availability. Notably, the expression of 39 (~50%) of the 76 CRGs tested responded to HDI exposure, affecting CRG expression in a demonstrably antagonistic manner (Figure 2B and C, SFig 6 and 7; $p < 0.05$, unadjusted t-test). The responses to both VA and NB were highly similar, with 30 out of 39 of the responding genes in common between the two drugs. Moreover, genes with increased expression in response to HDI also showed an increase in histone acetylation at their promoters, while genes whose expression was unaffected show virtually no alteration in promoter acetylation upon drug treatment (SFig 8). In contrast, HDI treatment of normal colon cells (YAMC) did not induce similar changes in CRG expression, significantly altering levels of only 15 CRGs (Figure 2D). Furthermore, only 11 genes responded in both mp53/Ras cells and YAMC cells to HDI treatment (Figure 2D and 2E), demonstrating that HDI can have specific effects on CRG expression in transformed mp53/Ras cells. CRGs are associated with a variety of biological functions in the cell, and HDI-sensitive CRGs are drawn from all of these functional classes (Figure 2F). HDI thus appear to reset the CRG expression signature in cancer cells to a pattern resembling a state in-between cancer and normal cells, in agreement with the CMap prediction.

CRG induction is essential for tumor inhibition by HDI

In order to test for a potential contribution of CRG modulation to the anti-cancer effects of HDI, we selected a subset of five CRGs that were induced following HDI exposure in a cancer cell-specific manner (SFig 9), i.e. Dapk1, Fas, Notch3, Noxa and Perp. Moreover, HDI-mediated induction of Dapk1 (54), Fas (55) and Noxa (56) has also been reported in other settings. To test whether increased expression of any of these five CRGs contributes to the biological effects of HDI treatment in transformed colon cells, we generated mp53/Ras cells in which HDI-mediated gene induction was blocked or significantly inhibited by stable expression of shRNA (SFig 10). Tumor initiation capacity of these cells with and without HDI exposure was tested following engraftment into nude mice. Because both of the HDI, VA and NB, show similar effects on CRG expression (Figure 2B), *in vivo* experiments were limited to NB treatment. Remarkably, knock down of any of the five CRGs, Dapk1, Fas, Notch3, Noxa or Perp, recovered the tumor growth potential of NB-treated mp53/Ras cells (Figure 3A, Table 1, SFig 11), thus demonstrating a causal role for each of these genes in tumor suppression by HDI. Notably, induction of Dapk1, Notch3, Noxa or Perp expression by HDI becomes evident only between 48 and 72 h following drug exposure, suggesting that relatively late alterations in gene expression mounted in response to HDI are essential to generating HDI-sensitivity of tumor formation capacity (SFig 12). CRG perturbations had no significant effect, however, on HDI-mediated inhibition of cell accumulation *in vitro*

(SFig 3B), suggesting that CRGs are integral to anti-tumor effects of HDI, but not necessarily anti-proliferative effects *in vitro*.

The roles of Dapk1, Notch3, Noxa, Perp or Fas, in HDI-mediated tumor inhibition appear distinct. Induction of Dapk1 expression by HDI appears essential and specific for establishing tumor-sensitivity towards HDI. ShRNA-mediated suppression of Dapk1 mRNA expression completely abrogated HDI-mediated tumor inhibition, i.e. generates HDI resistance, while having no effect on tumor growth in the absence of HDI. Notch3, Noxa and Perp play an intermediate role, with each of the respective knock down cell populations exhibiting reduced NB sensitivity, as compared to genetically unperturbed controls. In contrast, Fas and HDI suppress tumor formation through independent mechanisms. Both HDI-treated and untreated cells formed significantly larger tumors following knock down of Fas expression, and conversely HDI sensitivity of Fas-perturbed cells was similar to that of unperturbed cells (Figure 3A). CRG induction by HDI thus contributes to HDI anti-tumor activity through multiple mechanisms.

Gene knock down appears to be specific for the targeted CRGs and affects cell regulation downstream of oncogenic mutations. Multiple independent shRNAs for each of the genes consistently led to increased tumor growth (SFig 11). In addition, rescue of Noxa or Perp gene expression in knock down cells with shRNA-resistant cDNAs (SFig 10) restored HDI sensitivity and anti-tumor activity in these cells (Figure 3B). Moreover, interference with Elk3 or Etf1 expression did not alter tumor formation in HDI-treated mp53/Ras cells (Figure 3C), demonstrating that tumor formation is not altered by shRNA-mediated gene knock down *per se*. In addition, neither HDI treatment by itself, nor interference with CRG re-expression upon HDI treatment affected expression of the mp53 or Ras oncogenes (SFig 13), indicating that RNA interference with HDI-mediated gene induction elicits its biological effects downstream of the initiating oncogenic mutations.

CRG induction predicts and mediates HDI sensitivity of tumor growth in human cancer cells

Given the essential role for CRGs in mediating HDI anti-tumor activity in murine colon cancer cells, shown above, we wanted to test whether CRGs may play a similar role in human colon cancer cells. HDI treatment induced expression of potentially biologically relevant CRGs in SW480 cells but not in SW620 and DLD-1 cells (Figure 4A). Notably, HDI responsiveness of these CRGs correlated with HDI anti-tumor effects, as HDI pre-treatment suppressed tumor formation of SW480 cells, but not SW620 or DLD-1 cells (Figure 4B). As suggested above, CRG modulation by HDI, however, does not appear to track with HDI effects on proliferation of human cancer cells *in vitro*, as proliferation of any of the human colon cancer cell lines tested, i.e. SW480, SW620 and DLD-1, is inhibited by HDI in tissue culture (SFig. 3C). Notably, we find a similar scenario, following exposure of murine mp53/Ras cells to the second generation HDI, suberoylanilide hydroxamic acid (SAHA). As expected, SAHA inhibited the proliferation of mp53/Ras cells *in vitro*. However, neither CRG response, nor anti-tumor effects were observed following SAHA treatment of these cells (SFig 14), possibly owing to differences in spectrum of HDAC targeting or to off-target effects of VA and NB versus SAHA (57). Our observations thus suggest that antagonistic regulation of CRG expression by pharmacological agents can serve as an indicator for anti-tumor effects.

As demonstrated below, regulation of CRG expression plays a causal role in HDI anti-tumor activity in human colon cancer cells as well as in transformed murine colon cells. Overall measurement of the CRG response to HDI in SW480 cells with available probes to 64 of the 95 CRGs revealed altered expression of 60% of the CRG signature following exposure to either VA or NB (Figure 5A, Supplementary Table 2), with 21 genes responding similarly to

both VA and NB, and 16 of these genes showing differential expression at a statistically significant level (Figure 5B, $p < 0.05$, t-test). Among the nine genes responsive to NB and VA in SW480 and mp53/Ras cells were DAPK1, NOTCH3 and PERP. Because of the important role for Dapk1 in HDI effects on murine colon cells, and strong induction by both VA and NB in SW480 cells, we tested the role of DAPK1 in the anti-tumor activity of HDI on human cells. Similar to mp53/Ras cells, DAPK1 knock down (Figure 5C and D; SFig 15 – indicating two independent shRNA constructs) renders tumor formation capacity of SW480 cells virtually resistant to NB, while genetically unperturbed control cells show a significant reduction in tumor growth in response to NB (Figure 5E; SFig 15). As shown above, the modulation of the HDI response by CRG perturbation is specific to HDI anti-tumor activity, as the HDI ability to inhibit cell proliferation in tissue culture was not affected by knock down of Dapk (SFig. 3D). The effects of HDI and DAPK1 shRNA are not mediated by altered expression of the initiating oncogenic mutant proteins. Levels of oncogenic Ras proteins were virtually unaffected by either HDI treatment or DAPK1 knock-down in SW480 cells (SFig 16). Furthermore, SW480 cells are null for wild type p53 (58). HDI sensitivity at the level of tumorigenicity thus depends on DAPK1 expression in human as well as in murine cancer cells. Taken together, we conclude that modulation of CRG expression plays a causal role in the anti-tumor effects of HDI in both murine and human cancer cells.

Discussion

Here we provide proof-of-concept that a gene expression signature comprising genes essential to the cancer phenotype has utility for discovery of anti-cancer agents and their underlying mechanisms of action. Previously we have shown that drivers of the cancer phenotype downstream of oncogenic mutations can be identified based on their synergistic deregulation by cooperating oncogenic mutations. We now demonstrate that these so-called “cooperation response genes” (CRGs) play an essential role in drug-mediated anti-cancer activity and that anti-cancer agents can be identified through their ability to reverse the CRG expression profile. Notably, reversal of CRG expression by anti-cancer agents appears integral to underlying drug mechanism. Our data thus reveal the CRG expression profile as a powerful probe to identify anti-cancer agents acting downstream of multiple cancer gene mutations.

Specifically, we show that the CRG signature is antagonized by histone deacetylase inhibitors (HDI) in a cancer cell-specific manner, and that such reversal of CRG expression is essential for HDI anti-cancer activity. Treatment of mp53/Ras cells with VA or NB, two carboxylic acid HDI, partially reversed expression of 50% of the 76 CRGs tested. Among the HDI-regulated CRGs are a number of genes that are repressed in transformed cells and reactivated by HDI. These include Dapk1, Fas, Notch3, Noxa and Perp. Notably, Dapk1, Notch3, Noxa or Perp each play causal roles in the anti-tumor activity of HDI, as shRNA-mediated knock down of any of these four genes decreases cancer cell sensitivity to HDI. Conversely, Fas suppresses tumor growth independent of HDI sensitivity, a feature that previously remained undetected (35). Up-regulation of individual CRGs by HDI thus contributes to HDI anti-tumor activity involving multiple paths. We thus demonstrate that reversal of CRG expression by pharmacological agents is integral to the underlying anti-cancer drug mechanism and that the mechanism involved may be multi-dimensional in nature.

In addition to providing insight into mechanisms underlying anti-tumor activity of pharmacological agents, our findings also identify a means by which cancer cells may acquire resistance to previously effective compounds (59). Inhibition of HDI-mediated induction of the five CRGs mentioned above significantly restored tumor formation capacity

of transformed cells. This indicates that tumors can evade the effects of HDI through multiple paths, with modulation of gene regulation as a general theme. Such a mechanism of mounting drug resistance may be effective against a series of pharmacologic agents. In fact, such acquired resistance to therapeutic agents through multiple routes has also been recently observed in an *in vitro* model of non-small cell lung cancer (60). Conversely, this type of resistance may be overcome by drug combinations, composed of agents that perhaps, as discussed below, each modulate diverse sub-sets of CRGs. Alternatively, drug resistance may be circumvented by compounds that boost expression of tumor inhibitory CRGs that act independent of drug resistance, as, for example, shown here for Fas expression.

Our observation that reversion of the CRG signature is essential for the tumor inhibitory activity of anti-cancer compounds has important practical implications. First, the CRG signature provides a new means for anti-cancer drug discovery based on insights relating to drug mechanism. We thus anticipate that the CRG signature will allow functional classification of compounds with anti-cancer activity based on the distinct CRG expression patterns they elicit. In this context, it is also worth noting that the CRG signature effectively identified tumor inhibitory compounds across gene expression profiles from drug-treated breast, prostate and myeloid cancer cells. Preliminary observations in our laboratory show antagonism of the CRG signature by other known anti-cancer compounds, with the relevance of such change in CRG expression currently under investigation. CRG expression patterns thus may serve as an indicator for anti-tumor activity, as a surrogate for tumor cell behavior *in vivo*, thereby facilitating identification of potential anti-cancer compounds, perhaps with relevance to multiple types of human cancer. Second, we envisage that the responsiveness of the CRG signature to pharmacologic agents can also function as a diagnostic indicator of cancer cell sensitivity to such agents, wherever CRGs contribute to the cancer phenotype.

Cooperation response genes (CRGs) have emerged as a highly valuable resource for understanding mechanisms essential to both the cancer phenotype and anti-cancer drug activity. CRG selection specifically detects genes that are regulated by multiple oncogenic mutations. Moreover, as CRGs are essential to the cancer phenotype, they represent key elements in the molecular architecture underlying malignant transformation. CRGs thus provide fundamental insight into the mechanisms generating and maintaining the malignant state and indicate associated cancer cell vulnerabilities as points for intervention downstream of oncogenic mutations. Moreover, as shown here, reversal of the CRG expression signature by pharmacological agents pinpoints key components in underlying drug mechanism. In addition, the considerable number of CRGs offers access to developing cancer interventions in a multi-dimensional manner providing rationale for combinations of molecularly targeted drugs. These are exciting prospects for cancer intervention strategies with high cancer selectivity and with broader application.

Materials and Methods

Interrogation of the Connectivity Map Database

To facilitate cross-species queries, a local version of the CMap database was downloaded and remapped to gene symbols (14). Where multiple Affymetrix IDs mapped to a single gene symbol, the median fold change for that gene was utilized. This local gene symbol-based version of the CMap performed similarly to the Affymetrix ID-based version originally described.

The query signature consisted of 24 up-regulated CRGs and 52 down-regulated CRGs for which gene symbol annotation was present in the CMap data set. This 76-gene subset of the CRG signature was used to interrogate the Connectivity Map database according to the

Connectivity Score (CS) metric, previously described (14). The Kolmogorov-Smirnov-based gene set enrichment analysis (GSEA) algorithm (29) was used to obtain enrichment scores for both up-regulated (ES_{up}) and down-regulated (ES_{down}) CRGs for each CMap drug treatment instance. The values of ES_{up} and ES_{down} were combined to generate a CMap connectivity score as described. Drugs that mimic the CRG signature attain a positive connectivity score whereas drugs that antagonize the CRG signature attain a negative connectivity score.

To test the randomness of results, 1000 random gene 76-gene signatures consisting of the same proportion of up-regulated and down-regulated genes as the CRG signature were generated. Each random signature was used to interrogate the CMap database. The non-normalized CSs for the relevant HDI instances were summed together and multiplied by -1 to make the value positive (negative summed non-normalized CS; $-\text{sum}(nCS)$). Positively connected instances and non-connected instances were assigned a value of zero. The p-value indicates the number of random comparisons achieving as extreme a summed score or greater.

To determine specificity, we obtained fold changes for all genes resulting from 5842 non-HDI chemical perturbations in CMap2. From these data, 5842 76-gene signatures were generated according to fold change, using the 24 most up-regulated and 54 most down-regulated genes. Each 76-gene signature was used to interrogate CMap1 and the $-\text{sum}(nCS)$ was computed as above.

Cell Culture, Retroviral Infections and Tumor Formation Assays

The YAMC cell system (51) and transformation of these cells by both $p53^{175H}$ and $HRas^{V12}$ (mp53/Ras) are described elsewhere (27–28). YAMC and mp53/Ras cells were cultured for two days at 39 °C in RPMI with 10% FBS on collagen IV-coated dishes. Cells were then replated on collagen IV-coated dishes into the same medium containing either 2.5 mM NB, 2.5 mM VA, 5 or 10 μM SAHA or no drug for 72 hours at a density of 4.58×10^5 cells per 15-cm dish at 39 °C. SW480, SW620 and DLD-1 cells were grown at 37°C in DMEM with 10% FBS and antibiotics. For HDI treatment of human colon cancer cells, cells were plated into medium containing either 2.5 mM NB, 2.5 mM VA or no drug for 72 hours at a density of 1.37×10^6 cells per 15-cm dish. For all cell types, following HDI treatment, cells were harvested for RNA isolation, or used for tumor formation studies as described below.

Polyclonal cell populations stably expressing individual shRNA molecules targeting each of the tested CRGs were generated as previously described (28). shRNA target sequences are listed in Supplementary Table 3. For each up-regulated gene, we identified 2–3 independent shRNA target sequences yielding at least 50% reduction in gene expression with the goal to guard against off-target effects (SFig 10). The specificity of Noxa and Perp knock-down was independently confirmed by expression of cDNA for either of these genes, rendered shRNA-resistant by introduction of appropriate silent mutations. Gene expression was achieved using the pBabe-hygro retroviral vector to introduce either cDNA into appropriate mp53/Ras cells harboring Noxa or Perp shRNA using the methods described above.

For tumor formation studies, cells were treated with HDI, then trypsinized, counted and injected sub-cutaneously into the flanks of CD-1 nude mice at a multiplicity of 5×10^5 cells per injection for mp53/Ras cells, 1×10^6 cells per injection for SW620 and DLD-1 cells, and 5×10^6 cells per injection for SW480 cells. Mice were observed and tumors measured for up to 6 weeks post-injection by caliper.

Real-time quantitative PCR

RNA was harvested and extracted as described above for TLDA analysis and SYBR Green QPCR. TaqMan Low-Density Array (Applied Biosystems) and quantitative PCR reactions were prepared as previously described (28). All primers sets, listed in Supplementary Table 4, used an annealing temperature of 58 °C. PCR reactions were run on an iCycler (Bio-Rad).

Biological process analysis of gene sets

Gene ontology classification of CRGs and oncogenes/tumor suppressors was assigned by mapping Affymetrix probe set IDs to GO biological process categories for each gene via the Affymetrix NetAffx tool. NetAffx reported GO biological processes were compared to the NCBI Gene database to check for completeness and consistency.

Chromatin immunoprecipitation and promoter QPCR

Cells were incubated at 37 °C for 15 minutes in the presence of 1% formaldehyde. This reaction was stopped with the addition of glycine to a final concentration of 0.125 M and incubation at room temperature for 5 minutes. Cells were then washed 2x with ice-cold PBS, scraped, pelleted and stored at –80 °C until ready for lysis and sonication. An Acetyl-Histone H3 Immunoprecipitation (ChIP) Assay Kit (Millipore) was then used according to the manufacturer's protocol. SYBR Green-based quantitative PCR was run using 1x Bio-Rad iQ SYBR Green master mix, 0.2 mM forward and reverse primer mix, with gene-specific qPCR primers for each gene tested. Reactions were run on the iCycler (Bio-Rad), as follows: 5 min at 95°C, 45 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds to amplify products, followed by 40 cycles of 94°C with 1°C step-down for 30 seconds to produce melt curves.

Western blotting

mp53/Ras cells were grown at 39°C for 2 days, followed by plating into 2.5 mM VA or NB for 3 days prior to lysis for Western blots. SW480 cells were grown in standard conditions, then plated into 2.5 mM VA or NB for 3 days prior to Western analysis. Cell pellets were lysed for 20 min at 4°C with rotation in RIPA buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCL, 1% NP-40, 5 mM EDTA, 0.1% SDS, 0.5% deoxycholic acid, protease inhibitor cocktail tablet). Lysates were clarified by centrifugation at 13,000g for 10 min at 4°C and quantitated using Bradford protein assay (Bio-Rad). 25 µg of protein lysate was separated by SDS-PAGE and transferred to PVDF membrane (Millipore). Immunoblots were blocked in 5% non-fat dry milk in PBS with 0.2% Tween-20 for 1 hour at RT, probed with antibodies against p53 (FL-393, Santa Cruz) for all cell lines, H-Ras (C-20, Santa Cruz) for mp53/Ras cells, Ras (Ab-1, Calbiochem) for SW480 cells, and tubulin (H-235, Santa Cruz) for all cell lines. Bands were visualized using the ECL+ kit (Amersham).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The CRG gene expression pattern is antagonized by HDAC inhibitors

A 76-gene subset of the CRG signature was used to interrogate the CMap database, based on availability of probes to CRG homologues. (A) HDI instances among the top 20 results are shown. The Connectivity Score (CS) is indicated as well as the enrichment score for the up-regulated genes (up), down-regulated genes (down), and the specific CMap instance (Instance). (B) Bar plot indicating the distribution of HDI along the 453 CMap instances ordered according to similarity to the CRG signature. Horizontal black lines represent an instance of any of the HDI valproic acid, trichostatin A, HC toxin, or sodium phenylbutyrate. The green region indicates instances achieving positive connectivity scores (i.e. similar to CRG signature). The gray region indicates instances with no relationship to the CRG signature. The red region indicates instances of compounds with negative connectivity scores (i.e. antagonize the CRG signature). (C) Distribution of 1000 random 76-gene signatures. The vertical axis indicates frequency. The horizontal axis shows the negative sum of non-normalized CS for HDI instances in panel A. The dashed vertical line represents the negative summed non-normalized CS for these HDIs. (D) Distribution of

5842 signatures from CMap2. The vertical axis indicates frequency. The horizontal axis shows the negative sum of non-normalized CS for HDI instances in panel A. The dashed vertical line represents the negative summed non-normalized CS for the HDIs.

Figure 2. HDI antagonism of CRG expression signature correlates with inhibition of tumor formation

(A) Histogram showing mean volume of tumors $\pm$ SEM formed by untreated mp53/Ras cells ($n=6$), or by mp53/Ras cells pre-treated with either 2.5 mM VA ($n=4$) or 2.5 mM NB ($n=8$) at four weeks post-injection; *, $p<0.05$, Mann-Whitney test. **(B–D)** RNA from mp53/Ras or YAMC cells treated with 2.5 mM VA or NB for 3 days, four replicates per condition, was analyzed for changes in CRG expression via TLDA (*, unadjusted $p<0.05$, t -test).

Histograms indicate relative levels of CRG expression, represented by the t statistic, as follows: **(B)** mp53/Ras cells as compared to normal YAMC cells. **(C)** HDI-treated mp53/Ras cells as compared to untreated mp53/Ras (VA, green bars; NB, blue bars). **(D)** HDI-treated YAMC cells as compared to untreated YAMC (VA, green bars; NB, blue bars). **(E)** Venn diagram showing distinct and overlapping gene responses to HDI treatment in mp53/Ras cells and YAMC cells (NB, blue circles, VA, green circles). **(F)** Pie charts show the percentage of CRGs associated with specific biological processes for all CRGs and those associated with CRGs that are HDI-responsive, according to the Gene Ontology database.

Figure 3. Induction of CRG expression essential for HDI anti-tumor activity in mp53/Ras cells
Cells with shRNA-mediated repression of indicated CRGs were pre-treated with 2.5 mM NB and injected sub-cutaneously into nude mice. Histograms show mean tumor volume, $\pm$ SEM, for each CRG perturbation at four weeks post-injection. Injection numbers are identical for both untreated and NB-treated instances of each perturbation, indicated below histograms. *, $p < 0.05$ versus untreated empty vector cells; #, $p < 0.05$ versus NB-treated empty vector cells, Mann-Whitney test. (A) Tumor formation by mp53/Ras cells with indicated perturbations. (B) Tumor formation by mp53/Ras cells with indicated perturbations and genetic rescue. (C) Tumor formation by mp53/Ras cells with indicated perturbations.

Figure 4. CRG induction correlates with tumor formation capacity of HDI treated human colon cancer cell lines

(A) SW480, SW620 or DLD-1 cells were treated with 2.5 mM NB or VA for 3 days. RT-QPCR was performed to assess changes in Dapk1, Notch3, Noxa, and Perp expression, relative to untreated cells. Shown is the mean expression, $\pm$ SEM. *, $p < 0.05$ versus untreated cells; t-test. (B) SW480, SW620 or DLD-1 cells were pre-treated with 2.5 mM NB and injected sub-cutaneously into nude mice. Tumor formation was measured at 4 weeks post-injection, $n=6$ for each condition; *, $p < 0.05$ versus untreated cells; Mann-Whitney test.

Figure 5. Induction of the CRG DapK1 is essential for anti-tumor effects of HDI in SW480 human colon cancer cells

(A) Differential CRG expression induced by HDI in SW480 cells in comparison with CRG expression pattern in mp53/Ras cells (*, unadjusted $p < 0.05$, t-test). Histograms indicate relative levels of CRG expression, represented by the *t statistic*, in mp53/Ras cells compared to normal YAMC cells (white bars), and VA- or NB-treated SW480 cells versus untreated SW480 (VA, green bars; NB, blue bars). RNA from SW480 cells treated for 3 days with 2.5 mM NB and untreated controls, four replicates per condition, was analyzed for changes in CRG expression via TLDA. (B) Venn diagram showing distinct and overlapping gene responses to HDI treatment in mp53/Ras cells and SW480 cells (NB, blue circles, VA, green circles). (C, D) SW480 cells stably expressing shRNAs targeting DapK1 were treated with 2.5 mM NB for 3 days. RT-QPCR was performed to assess changes in DapK1 expression, relative to empty vector cells. Shown is the mean reduction in expression for two independent shRNAs, $\pm$ SEM, for untreated cells in (B) and following NB treatment in (C). (E) SW480 cells with shRNA-mediated repression of DapK1 were pre-treated with 2.5 mM NB and injected sub-cutaneously into nude mice. Tumor formation was scored at 4 weeks

post-injection. Injection numbers are indicated below the histogram. *, $p < 0.05$ versus untreated empty vector cells; #, $p < 0.05$ versus NB-treated empty vector cells; Mann-Whitney test.

Table 1

Tumor formation following implantation of HDI-treated mp53/Ras cells

Sample	Treatment?	Tumors/Injections
Vector	UT	32/32
	NB	8/32
Dapk shRNA	UT	11/14
	NB	13/14
Fas shRNA	UT	7/7
	NB	8/8
Notch3 shRNA	UT	12/12
	NB	12/12
Noxa shRNA	UT	7/8
	NB	8/8
Perp shRNA	UT	8/8
	NB	8/8
Elk3 shRNA	UT	4/4
	NB	0/4
Etv1 shRNA	UT	4/4
	NB	0/4
Noxa cDNA	UT	4/4
	NB	1/4
Noxa shRNA/Noxa cDNA	UT	4/4
	NB	0/4
Perp cDNA	UT	4/4
	NB	4/4
Perp shRNA/Perp cDNA	UT	4/4
	NB	0/4

Columns indicate targets and manner of perturbation (gene knock-down or over-expression) of indicated CRGs, and the number of tumors of size greater than 0.1 cm³ at 4 weeks post-implantation per number of implantations attempted. Each gene perturbation represents data from multiple shRNA constructs, as shown in supplementary figures.