Chemical Research in Toxicology

Chemical Profile

pubs.acs.org/crt

Transcriptomic Responses of Cancerous and Noncancerous Human Colon Cells to Sulforaphane and Selenium

Simona Constantinescu,[†,‡] Katrin Hecht,[§] Nadine Sobotzki,[∥] Melanie M. Erzinger,[§] Cédric Bovet,[§] Jerry W. Shay,[⊥] Bernd Wollscheid,[∥] Shana J. Sturla,[§] Giancarlo Marra,[#] and Niko Beerenwinkel*[,†,‡]

[†]Department of Biosystems Science and Engineering, ETH Zurich, Basel 4058, Switzerland

[‡]SIB Swiss Institute of Bioinformatics, Basel 4058, Switzerland

[§]Department of Health Science and Technology, Institute of Food, Nutrition & Health, ETH Zurich, Zurich 8092, Switzerland

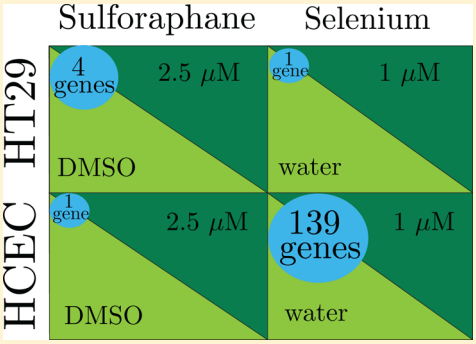
[∥]Institute of Molecular Systems Biology, ETH Zurich, Zurich 8093, Switzerland

[⊥]Department of Cell Biology, Division of Digestive and Liver Diseases, University of Texas Southwestern Medical Center, Dallas, Texas 75390, United States

[#]Institute of Molecular Cancer Research, University of Zurich, Zurich 8057, Switzerland

Supporting Information

ABSTRACT: Diets enriched with bioactive food components trigger molecular changes in cells that may contribute to either health-promoting or adverse effects. Recent technological advances in high-throughput data generation allow for observing systems-wide molecular responses to cellular perturbations with nontoxic and dietary-relevant doses while considering the intrinsic differences between cancerous and noncancerous cells. In this chemical profile, we compared molecular responses of the colon cancer cell line HT29 and a noncancerous colon epithelial cell line (HCEC) to two widely encountered food components, sulforaphane and selenium. We conducted this comparison by generating new transcriptome data by microarray gene-expression profiling, analyzing them statistically on the single gene, network, and functional pathway levels, and integrating them with protein expression data. Sulforaphane and selenium, at doses that did not inhibit the growth of the tested cells, induced or repressed the transcription of a limited number of genes in a manner distinctly dependent on the chemical and the cell type. The genes that most strongly responded in cancer cells were observed after treatment with sulforaphane and were members of the aldo-keto reductase (AKR) superfamily. These genes were in high agreement in terms of fold change with their corresponding proteins (correlation coefficient $r^2 = 0.98$, $p = 0.01$). Conversely, selenium had little influence on the cancer cells. In contrast, in noncancerous cells, selenium induced numerous genes involved in apoptotic, angiogenic, or tumor proliferation pathways, whereas the influence of sulforaphane was very limited. These findings contribute to defining the significance of cell type in interpreting human cellular transcriptome-level responses to exposures to natural components of the diet.



INTRODUCTION

Associations between diet and carcinogenesis have indicated strong negative correlations between fruit and vegetable consumption and tumor incidence,[1] most consistently for colorectal cancer and cruciferous vegetables.[2−4] Colorectal cancer is the third-most common cancer worldwide, and its global pattern of incidence (10-fold higher in developed than in developing countries) may in part be explained by diet-related factors.[5−7] Natural products obtained from cruciferous vegetables have demonstrated anticancer activity in cell cultures,[8−12] animal models,[13] and human clinical trials.[14,15] In understanding the impact of dietary agents on cancer progression, one challenge is that food contains a mixture of active compounds that may initiate a complex array of molecular responses in the various cell types of the human body. These effects can be characterized on the basis of systems-wide measurements and comparative data analysis. Here, we analyzed and compared the in vitro gene expression perturbations that two food components, sulforaphane and selenium, trigger in cancerous versus noncancerous epithelial cells of the human colon.

Selenium (sodium selenite) is widely recognized as an essential redox-active trace element for humans, and it is typically found in nuts, cereals, fish, and eggs as well as cruciferous vegetables.[16,17] Plasma levels of selenium in healthy individuals have been reported to be between 0.6 and 2 μM.[16,18] Variations in these concentrations are related to the

Special Issue: Systems Toxicology

Received: November 14, 2013
Published: January 2, 2014

amount of selenium in the diet and control the intracellular levels of selenoproteins, including thioredoxin reductase (TrxR) and glutathione peroxidase (GPX).[19] For example, increasing cellular selenium by supplementing cell culture medium with sodium selenite (Na_2SeO_3) induces the expression of TrxR in the cancer cell lines MCF-7, HepG2, and HeLa.[20−22] Moreover, when HeLa cells were cultured in media supplemented with as low as 1 μM selenium for 3 days, cellular TrxR protein levels and TrxR activity increased 4-fold, and cellular sensitivity to the bioreductive cancer drug (hydroxymethyl)acylfulvene was enhanced.[22,23]

On the basis of these data, we became interested in the therapeutic relevance of this pathway and whether the regulation of selenoenzymes might differ between target cancer cells and noncancerous cells. Because selenium may influence protein levels and activity by both transcriptional activation and as an essential elemental component of the enzymes, it is of interest to compare the differential influences of selenium with those of a nonessential food compound with a well-characterized pathway for transcriptional activation of related redox-regulating enzymes. Thus, sulforaphane is an ideal comparison because its underlying transcriptional activation mechanism is well-characterized and it is known to induce detoxifying and antioxidant enzymes such as glutathione S-transferases (GSTs) as well as selenoproteins such as thioredoxin reductase-1 (TrxR1)[21,24,25] and gastrointestinal glutathione peroxidases (GI-GPX).[26] Sulforaphane is a glucosinolate-derived isothiocyanate produced in cruciferous vegetables.[27] In humans, plasma levels of sulforaphane and its metabolites have been measured in concentrations of up to 2.2 μM after consumption of a single portion of broccoli sprouts.[28,29] The above-mentioned enzyme-induction patterns associated with sulforaphane are mediated by the nuclear factor E2-related factor-2 (NRF2)-regulated transcriptional activation of genes encoding carcinogen detoxification and antioxidant enzymes.[30−34] Mechanistically, sulforaphane acts by covalently binding to the protein Kelch-like ECH-associated protein 1 (KEAP1)[35] and disrupts its physical interaction with NRF2, thus promoting the translocation of NRF2 into the nucleus[36,37] where it induces the transcription of genes under the control of an antioxidant response element in their promoter region.[33]

The global influence of sulforaphane on transcription and translation has been evaluated in vivo and in vitro through mRNA profiling and untargeted proteomic measurements. Transcriptome analysis of human gastric mucosa after consumption of a single meal of sulforaphane-rich high-glucosinolate broccoli soup indicated that several genes were significantly, but modestly, differentially expressed (absolute log fold changes less than 1.1). Most notably, upregulation was reported in the aldo-keto reductase family 1, members C1 (*AKR1C1*) and C2 (*AKR1C2*), thioredoxin reductase (*TrxR*), carbonyl reductase 1 (*CBR1*), and glutamate-cysteine ligase, modifier subunit (*GCLM*).[14] A subset of these genes, together with *AKR1C3*, were also highly expressed in various human cell lines exposed to sulforaphane, such as colon cancer Caco-2 and LS-174 cells and human skin keratinocyte HaCaT cells (treated with 5 μM sulforaphane).[38,39] Furthermore, similar upregulation effects on the mRNA and protein levels were reported in nontumorigenic human breast epithelial MCF10A cells treated with 15 μM sulforaphane.[40]

For selenium, cellular impacts are finely controlled by dose, as demonstrated by a large number of studies. For example, at low concentrations (<0.1 μM), selenium stimulates cell proliferation, whereas at higher concentrations (1−1000 μM), it inhibits proliferation in the colon cancer cell lines Caco-2, HRT18, and HT29.[12] A different study showed that treatment of HT29 cells with medium doses (>5 μM) of selenium decreased cell growth and increased cell detachment, whereas treatment with high doses (>10 μM) increased cell differentiation and apoptosis.[41]

Gene expression analysis of a large collection of selenium-treated cancerous cell lines and tissue samples at various concentrations (≥5 μM) revealed that selenium altered several genes in an antitumorigenic manner. More specifically, genes related to phase II detoxification enzymes, selenium-binding proteins, and apoptosis were upregulated, including *TP53*, cyclin-dependent kinase inhibitor 1A (*CDKN1A*) and NAD(P)H dehydrogenase, quinone 1 (*NQO1*), whereas genes related to phase I detoxification enzymes, cell proliferation, and arrest at G1 were downregulated, including cyclin A1 (*CCNA1*), cyclin D1 (*CCND1*), and cyclin-dependent kinase 4 (*CDK4*).[11]

Despite extensive information available, our understanding of how diet-related chemicals, such as sulforaphane and selenium, mediate their potentially toxic or protective effects is limited by several practical constraints. First, in vitro experiments are commonly performed with chemical concentrations that inhibit cell growth and are much higher than may be achieved physiologically from the diet or supplements.[8,11,18,28,38,39] Second, experimental read-outs are often limited to a few specific genes or proteins and do not offer a comprehensive picture of the perturbed cellular system. Third, molecular perturbations are almost always characterized only in cancerous cells, neglecting the treatment effects on noncancerous cells, where the transcriptional machinery may be differently impacted.

In this study, we performed large-scale molecular transcriptome profiling of both a human colon cancer cell line (HT29) and a noncancerous HCEC line. HCECs are immortalized but share many characteristics with normal colon epithelial cells and were therefore used in this study as an in vitro model for noncancerous colon tissue. They were established from colon tissue remote from endoscopically visible adenomas, and they express epithelial as well as stem cell markers.[42] Their karyotype is diploid, and DNA sequencing revealed no amino acid changing hotspot mutation in genes such as *APC*, *KRAS*, or *TP53*.[42] Additionally, they were shown to not have tumorigenic properties such as tumor formation in nude mice or anchorage-independent growth.[42] Both cell types were treated with two chemicals, sulforaphane and selenium, at doses where no significant decrease in cell viability occurred.[9] The transcriptomic responses were analyzed and compared at the single-gene, network, and pathway levels, with emphasis on the specific gene expression patterns triggered by treatment with each of the two food components in both the cancerous and the noncancerous cell lines.

MATERIALS AND METHODS

Materials. HT29 cells were obtained from the Leibniz-Institut DSMZ GmbH (Braunschweig, Germany), and human colon epithelial cells (HCECs) were provided by Jerry W. Shay (University of Texas SW, Dallas, TX, USA). Cell culture media (DMEM-GlutaMax, M199 Earle), fetal bovine serum (FBS), penicillin/streptomycin, trypsin/EDTA, and 1× PBS were purchased from Invitrogen (Life Technologies Ltd., Paisley, UK). Cosmic calf serum HyClone was purchased from ThermoScientific (Waltham, MA, USA), and epidermal growth factor, hydrocortisone, insulin, transferrin, sodium selenite, and gentamicin were purchased from Sigma-Aldrich (St.

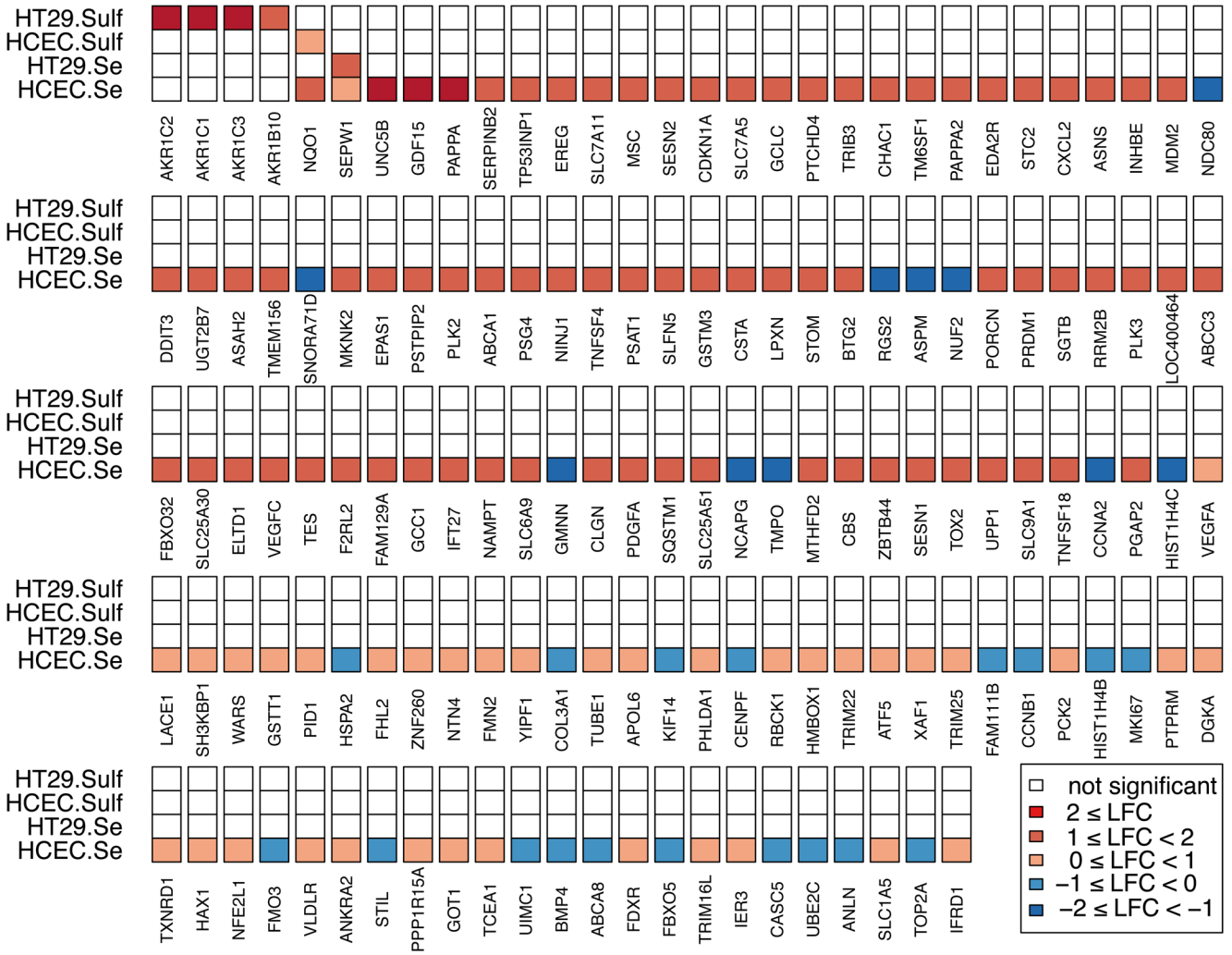


Figure 1. Full characterization of the differentially expressed genes on the basis of an absolute LFC of 0.5 and a q value of 0.01 in the four comparisons (HT29 cells and HCECs treated with either 2.5 μM sulforaphane or 1 μM selenium). The y axis depicts the four comparisons (e.g., HT29.Sulf denotes the differential expression testing between HT29 cells treated with 2.5 μM sulforaphane and the respective DMSO controls). Each large vertical rectangle is labeled according to the gene it represents, and its four subdivisions are colored according to the regulation pattern displayed by the gene in the above-described comparisons. Selenium triggers a high number of differentially expressed genes in HCECs but only a very limited response in HT29, whereas the converse is true for sulforaphane. The AKR family 1 shows a highly specific upregulation signal in HT29 cells following sulforaphane treatment.

Louis, MO, USA). Stock solutions of (*R*)-sulforaphane (LKT Laboratories, St. Paul, MN, USA) were prepared in DMSO (Sigma-Aldrich), and stock solutions of sodium selenite were prepared in water.

Cell Culture and Sample Preparation. HT29 cells were grown in DMEM-GlutaMax medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. HCECs were grown in medium as described previously in Roig et al.[42] The cells were seeded in 150 mm Nunc dishes (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 5 × 10^6 (HT29) or 2 × 10^6 (HCEC) cells per dish at 37 °C and 5% carbon dioxide. Twenty-four hours after seeding they were treated with 2.5 μM sulforaphane or 0.01% DMSO (control) or with 1 μM sodium selenite or water (control) for 48 h. Cells were trypsinized and washed with 1× PBS. The pellets were frozen and stored at −80 °C. Total RNA extraction and processing were performed as described in Cattaneo et al.[43] Human Gene 1.1 ST Array Strips (Affymetrix, Santa Clara, CA, USA) were used for hybridization in the GeneAtlas Microarray System (Affymetrix, Santa Clara, CA, USA). All experiments were conducted in triplicate.

Statistical Analysis. All statistical analyses were performed in the statistical computing environment R.[44] The transcriptome data was preprocessed using the R package oligo, v. 1.26,[45] by removing all of the features on the chip that mapped to duplicate Entrez IDs as well as the ones exhibiting less than 0.5 standard deviation. From an initial 33 297 expressed features, 10 964 remained after preprocessing. These features mapped to 10 099 unique HUGO gene identifiers. All absolute intensities were $\log_2$-transformed. For detecting differentially expressed genes, a linear model with a single effect for each comparison, implemented in the R package limma, v. 3.18,[46] was fitted to the preprocessed expression intensities. p values were adjusted for multiple testing using the Benjamini−Hochberg (BH) method.[47]

Data from a SILAC (stable isotope labeling by amino acids in cell culture)-based proteome-wide study in which HT29 cells were treated with either 2.5 μM sulforaphane or 0.01% DMSO (control) for 48 h were reanalyzed to assess the relationship between the transcriptome and the proteome.[9] Data preprocessing and statistical analysis for detecting differential expression of proteins were performed using the same filtering criteria as stated in Bovet et al.,[9] with the exception that here we required the existence of at least one unique peptide in only one of the three replicates, whereas previously this condition had to be met in all three replicates. After filtering, 3032 proteins mapping to nonunique HUGO gene identifiers remained, 3003 of which mapped uniquely. In cases of ambiguous mappings, proteins with the highest log fold change were kept. Among the 3003 proteins identified in this

dx.doi.org/10.1021/tx400427t | *Chem. Res. Toxicol.* 2014, 27, 377−386

manner, 1848 corresponded to genes also present in the preprocessed microarray data set and were used for the integration of microarray and SILAC data.

The pathway-level analysis was done with the R package piano, v. 1.2.1.[48] A separate analysis was performed on the gene-level statistics (BH[47]-adjusted *p* value and log fold change) resulting from each of the four comparisons: HT29 cells and HCECs treated with sulforaphane or selenium. The complete Gene Ontology Database (GO)[49] of biological processes (referred to as pathways here) was used as input. All of its members were tested for either up- or downregulated significant enrichment by considering the respective gene subsets separately and by scoring each subset according to the proportion of significant genes. The GO term-level statistic used was the mean of the gene-level statistics for the genes belonging to that GO term. A permutation test was used to assess statistical significance. Using the mean as the gene-level statistics has been shown to be often more powerful[50] than the modified Kolmogorov–Smirnov statistic used in the classical GSEA.[51] A GO term was considered to be differentially regulated in a certain direction if the corresponding adjusted *p* value was smaller than 0.01. The biomaRt R package, v. 2.18,[52] was used for mapping between the Affymetrix probe IDs and the GO terms.

Network Analysis. For network analysis, the interactions between the top differentially expressed genes were investigated using the GeneMania, v.3.3,[53] plugin of Cytoscape, v. 3.0.2.[54] For each comparison of cell line and treatment with food component for which more than one differentially expressed gene was detected, an input gene list was created that included only those genes with an absolute log fold change higher than 1.5 (with the exception of the *PTCHD4* gene, which was not recognized by GeneMania). These gene lists were extended by 15 functionally similar genes identified by GeneMania using transcriptomics, genomics, and proteomics databases. The similarity criteria used were coexpression (two genes are linked if they were found to be coexpressed in previous studies), shared protein domains (the two gene products have the same protein domain), and pathway (evidence of participation in the same functional pathway). The remaining GeneMania and Cytoscape options were set to their default values. In the resulting networks, the size of a node was inversely proportional to its rank in a sorted score list, assigned by a label-propagation algorithm.[53]

RESULTS AND DISCUSSION

Sulforaphane- and Selenium-Induced Transcriptome Perturbations. We quantified gene expression after treatment of HT29 cells and HCECs with sulforaphane and selenium, and performed genewise tests for detecting differential expression in each of the four comparisons. Quantitative transcriptomic changes, on the basis of an absolute log fold change (LFC) cutoff of 0.5 and an adjusted *p*-value (*q*-value) cutoff of 0.01, are represented in Figure 1 and are listed in the Supporting Information (Table S2). As expected, no differential expression between the negative control samples for sulforaphane experiments (i.e., DMSO treated) and those for selenium experiments (i.e., H_2O treated) was detected (data not shown).

Principal component analysis (PCA) revealed that the first component, explaining 46% of the variation in the data, perfectly separates the samples by their cell line of origin (Figure S1). The second component hardly explains any of the transcriptome-wide variation (2%). When comparing the transcription profiles of untreated HT29 cells and HCECs, 1088 genes were differentially expressed at an absolute LFC cutoff of 2 and a *q*-value cutoff of 0.01 (data not shown). Conversely, treatment-induced differential gene expression was not observed transcriptome-wide but rather for specific genes and gene families. This previously observed effect[8,11,38,39] was likely enhanced by the low compound doses with which the cells were treated.

In HT29 cells, four members of the AKR family 1 (*AKR1C2*, *AKR1C1*, *AKR1C3*, and *AKR1B10*) were significantly overexpressed at the transcript level after sulforaphane exposure (Figure 2A). The fold changes in expression of these genes

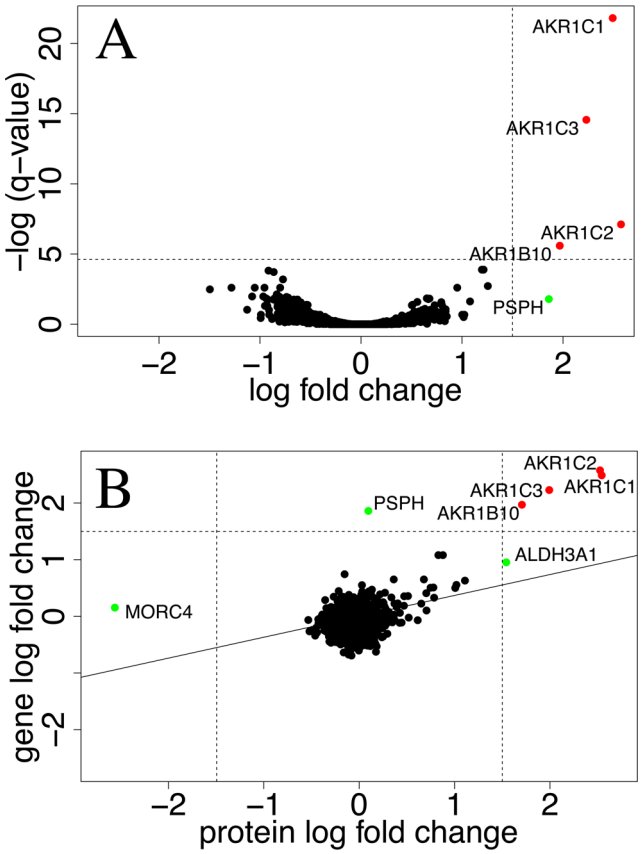


Figure 2. Gene- and protein-level changes in HT29 cells after sulforaphane treatment. (A) Volcano plot of the differentially expressed genes in HT29 cells after exposure to sulforaphane. The horizontal dotted line denotes a significance level of 0.01, and the vertical one denotes a LFC of 1.5. The four genes above these two thresholds are members of the AKR family 1, colored in red. Their specific upregulation effect is clearly visible. The LFC of the green-colored gene was higher than 1.5; however, it did not achieve statistical significance. (B) Integration of the transcriptome and proteome (SILAC) data sets. The horizontal dotted line represents a LFC cutoff of 1.5 on the gene level, and the two vertical dotted lines represent an absolute LFC cutoff of 1.5 on the protein level. Colored in red, the four members of the AKR family 1 (*AKR1C1*, *AKR1C2*, *AKR1C3*, and *AKR1B10*) are the only species with fold changes higher than the imposed thresholds at both levels. Colored in green are species with fold changes above or below only one of the thresholds. The solid black line denotes the linear fit between the two data sets ($r^2 = 0.44$, $p < 0.01$).

were similar to those previously observed in the immortal human keratinocyte cell line HaCaT following the knockdown of *KEAP1*, the negative regulator of NRF2.[39] Our results reinforced the fact that the AKR family 1 is particularly inducible by sulforaphane and that these changes are mediated by NRF2.[39] Similar stimulation of NRF2-activated genes has been reported previously for several chemicals, including *tert*-butylhydroquinone or beta-naphthoflavone in various cell lines, including HT29.[39,55,56] In the case of HT29 cells treated with sulforaphane, no additional genes were detected as either up- or downregulated at a 0.01 significance cutoff.

dx.doi.org/10.1021/tx400427t | *Chem. Res. Toxicol.* 2014, 27, 377–386

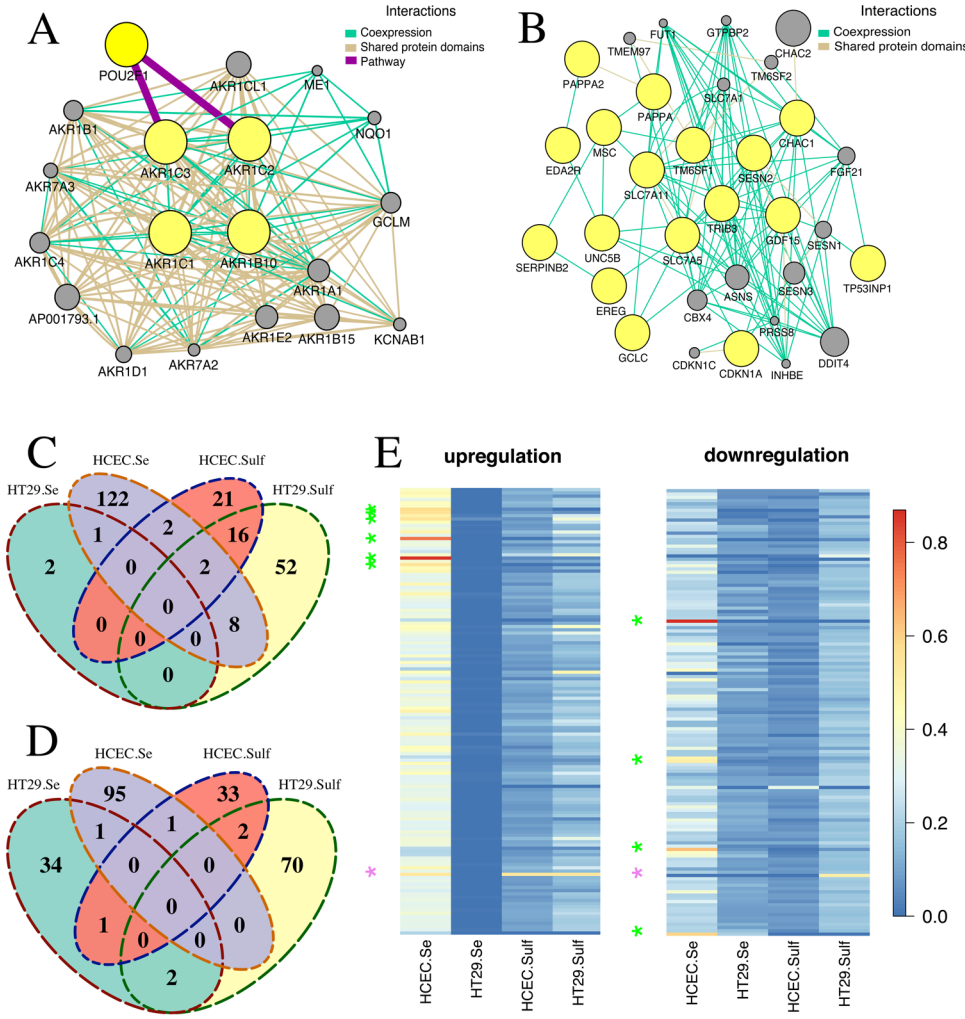


Figure 3. Network and pathway analyses of differentially expressed genes. Gene−gene interaction networks for the top differentially expressed genes with absolute LFC > 1.5 are shown for HT29 cells treated with sulforaphane (A) and HCECs treated with selenium (B). The networks were produced with the GeneMania plugin of Cytoscape. Yellow highlighted nodes correspond to the top differentially expressed input genes and gray nodes correspond to the 15 closest related genes on the basis of the similarity measures coexpression, shared protein domains, and pathway. The size of a gray node is inversely proportional to the rank of the corresponding gene in a relevance-based sorted GeneMania score list, and the thickness of the edges is proportional to the relevance of the interaction between two genes.[53] Members of the AKR superfamily, highly connected by coexpression and shared protein domains links, dominate the network after sulforaphane treatment in HT29 (A), whereas a sparser network, mostly based on coexpression, best characterizes the top differentially expressed genes in HCECs following selenium treatment (B). (C, D) Venn diagrams of the 226 upregulated (C) and 239 downregulated (D) GO terms following functional-pathway analysis on the transcriptome levels are displayed. Selenium triggered the highest pathway-level perturbations in HCECs and the lowest perturbations in HT29 cells. (E) Heatmaps of up- and downregulation scores of each of the 138 GO Slim (GOS) terms in the four comparisons of interest. The green stars correspond to highly upregulated or downregulated supergroups in HCECs after selenium treatment (upregulation, from bottom to top: RNA binding, secondary metabolic process, structural molecule activity, transferase activity, transferring glycosyl groups, translation factor activity, nucleic acid binding, and transmembrane transport; downregulation, from bottom to top: aging, cilium, histone binding, and pigmentation). The violet stars correspond to the cell−cell signaling supergroup, displaying the highest upregulation effect in both cell lines as well as the highest downregulation effect in HT29 cells.

However, although sulforaphane stimulated the upregulation of several NRF2-controlled genes in cancerous HT29 cells, the corresponding response of noncancerous HCECs (Figure 1) amounted to the differential regulation of only one gene, namely, *NQO1* (LFC 0.9). Its expression has been demonstrated previously to also be induced by sulforaphane under the control of NRF2 in HaCaT keratinocytes.[39] However, we did not find the *NQO1* gene upregulated in the colon cancer cell line exposed to sulforaphane, likely because of the nontoxic and physiologically relevant dose used here for treatment, at which the noncancerous cells were more responsive.

Selenium had a converse effect on the two cell lines: HCECs showed a rather large transcriptomic response (139 differentially expressed genes at an absolute LFC cutoff of 0.5), whereas in HT29 cells, only the expression of one gene (*SEPW1*) was perturbed at the low dose used (LFC 1.2). *SEPW1*, also upregulated in HCECs (LFC 0.7), codes for the most abundant member of the human selenoproteome, selenoprotein W, a highly conserved small thioredoxin-like protein required for cell cycle progression. *SEPW1* is expressed in all tissues, at levels proportional to the dietary selenium intake,[57] and is considered a selenium status biomarker.[58] The transcriptional activation of *SEPW1* and its role in carcinogenesis are still poorly understood.[59]

An examination of the 139 differentially expressed genes (109 upregulated and 30 downregulated) in selenium-treated

dx.doi.org/10.1021/tx400427t | *Chem. Res. Toxicol.* 2014, 27, 377−386

noncancerous HCECs supports the notion that selenium triggers apoptotis-, cell cycle-, and angiogenesis-related responses.[19,60,61] The most upregulated genes were the netrin receptor *UNC5B* (LFC 2.6) and the growth differentiating factor 15, *GDF15* (LFC 2.4). In cancer cells, these genes inhibit angiogenesis and act as tumor suppressors and apoptosis inducers,[62−64] but their role in noncancerous cells is largely unknown. Selenium treatment likely induced cell cycle arrest and apoptosis also through the overexpression of *TP53INP1* (LFC 1.8) while effecting regulation of the cell cycle progression at G1 and inhibition of proliferation through the overexpression of *CDKN1A* (LFC 1.7). These two genes are transcriptionally activated under stress conditions by p53,[65,66] a well-known tumor-suppressor protein with DNA-binding and transcriptional-activation properties that activates apoptosis in response to DNA damage. p53 also induces the upregulation of *SESN2*[67] (LFC 1.0), coding for sestrin 2, which was shown to protect against neurotoxicity.[68]

Two interesting, strongly upregulated genes not previously associated with selenium are *PAPPA* and *PAPPA2*, which code for the pregnancy-associated plasma proteins A and A2, two critical growth regulatory factors during fetal development.[69,70] Other newly identified selenium-associated upregulated genes in noncancerous cells are involved in development (*EREG*, *EDA2R*), protease inhibition (*SERPINB2*), amino acid transport (*SLC7A11*), protein kinase inhibition (*TRIB3*), and unfolded protein response (*CHAC1*).

The specific differential regulation of genes related to cell cycle modulation, apoptosis induction, tumor invasion, or angiogenesis inhibition in the HCEC line and not in the cancer cells, as previously reported,[19,60,61] provides a molecular basis for the low therapeutic selectivity of selenium. Namely, at dietary-relevant doses, the noncancerous cells experienced the upregulation of toxicity pathways, whereas the cancer cells were less responsive and more resistant to the promotion and induction of these pathways.

The top downregulated genes were predominantly related to signaling, cell division, proliferation, and cell cycle regulation. More specifically, during cell division, the proteins encoded by *CCNA2* (LFC −1.0), *NDC80* (LFC −1.4), and *NUF2* (LFC −1.1) were previously demonstrated to play important regulatory roles in chromosome segregation.[71−73] *GMNN* (LFC −1.0) codes for the critical cell cycle regulator Geminin (alias, DNA replication inhibitor), usually expressed during the S and G2 phases.[74,75] This gene has been reported to be positively correlated with cell proliferation because of its upregulation in several cancer cell lines, including colon.[76,77] None of these genes have previously been found to be responsive to selenium treatment.

Integrative Analysis of Sulforaphane-Treated HT29 Cells. A comparison of the transcriptome data described herein (Figure 2B) with a SILAC-derived proteome-wide data set for HT29 cells treated with 2.5 μM sulforaphane[9] showed very close agreement between the log fold changes of AKR family 1 genes and their respective protein products following sulforaphane treatment (linear correlation r^2 = 0.98, p = 0.01). Even though as expected, many of the quantified genes and proteins displayed negligible changes, the linear correlation between the two data sets was r^2 = 0.44 (p < 0.01).

In a previous study,[40] nontumorigenic human breast epithelial MCF10A cells were treated with 15 μM sulforaphane and gene and proteome (SILAC) expression levels were assessed. Similar to our results, *AKR1C1*, *AKR1C2*, *AKR1C3*, and *AKR1B10* were the highest upregulated on both the transcript and protein levels. However, the magnitudes of the log fold changes were much higher, very likely because of the high dose of sulforaphane employed.

Network and Pathway Analysis. In addition to the single gene- and protein-centric analyses described earlier, we also investigated the effects of cellular perturbations on the level of network interactions and functional pathways. For analyzing the connections between the top differentially expressed genes, we generated gene−gene interaction networks using the GeneMania[53] plugin of Cytoscape[54] (Figure 3A,B). In the case of HT29 cells treated with sulforaphane (Figure 3A), the input set consisted of *AKR1C1*, *AKR1C2*, *AKR1C3*, and *AKR1B10*. Nine out of the top 15 related genes were also members of the AKR superfamily (*AKR1A1*, *AKR1B1*, *AKR1B15*, *AKR1C4*, *AKR1CL1*, *AKR1D1*, *AKR1E2*, *AKR7A2*, and *AKR7A3*), highly connected by coexpression and shared protein domains. *GCLM*, *NQO1*, and *ME1* are known NRF2-regulated genes, and *GCLM* and *NQO1* have been previously shown to share similar upregulation patterns as members of the AKR superfamily.[14,39,78] A very strong pathway link was detected between *POU2F1* and *AKR1C2* or *AKR1C3*, genes jointly responsive to cigarette smoke condensate in different human oral cell phenotypes.[79,80]

In the case of HCECs treated with selenium (Figure 3B), the top differentially regulated genes belonged to a sparser interaction network, with most connections representing coexpression links and the few remaining ones representing shared protein domains. Among the input set of top 17 genes with absolute LFC > 1.5, *CHAC1*, *SESN2*, *SLC7A5*, and *SLC7A11* were the highest connected. Among the top 15 related genes, the strongest connected were *FUT1*, coding for a protein involved in the formation of blood group antigens, *GTPBP2*, coding for a GTP binding protein, *DDIT4*, a regulator of cell growth and proliferation whose upregulation was shown to induce apoptosis,[81] and *INHBE*, shown to be involved in pancreatic exocrine cell growth and proliferation.[82]

To detect differences in transcriptome responses after moderate perturbations with increased statistical power, we further employed a functional pathway-level approach, which can identify affected biological processes rather than individual genes or proteins. At a significance cutoff of 0.01, 226 upregulated and 239 downregulated GO terms were detected in at least one of the four comparisons (HT29 cells or HCECs treated with either sulforaphane or selenium), among which 24 were simultaneously perturbed in both directions (Figure 3C,D).

At the pathway level, selenium triggered the highest regulation signal in HCECs (228 differentially regulated GO terms) and the lowest regulation signal in HT29 cells (41 differentially regulated GO terms). In the case of the cancerous cells treated with selenium, downregulation (38 GO terms) was much more common than upregulation (3 GO terms), whereas in HCECs treated with selenium, the opposite was true (135 GO upregulated and 97 downregulated GO terms). Table S3 displays the complete list of significant effects on the pathway level. Of note, the process of aldo-keto reductase (NADP) activity appeared upregulated on the pathway level in both cell lines after treatment with sulforaphane, whereas at the gene level, the aldo-keto reductases themselves were significantly upregulated only in HT29 cells. This is a consequence of the pathway-level analysis involving multiple genes simultaneously. Moreover, many NADP-dependent pathways were upregulated

in HT29 cells after sulforaphane treatment, confirming the existing link between NRF2 and various NADP-dependent proteins.[33,83]

To analyze further the large number of differentially regulated GO terms, we narrowed the pathway space by mapping the 441 GO terms to more general supersets named GO Slim (GOS) terms. These supergroups contain multiple conceptually related GO terms, for example, those describing similar aspects of the same parent process (the correspondence can be retrieved via biomaRt).[52] After mapping, we found 138 differentially expressed GOS terms in at least one of the four comparisons. We assigned up- and downregulation scores to the perturbation of each GOS term in each of the four comparisons (Table S4) by normalizing the count of up- or downregulated members of a supergroup in a given comparison to the total count of supergroup members detected across all comparisons.

Consistent with the perturbation responses on the single-gene level, the highest differential regulation scores on the pathway level corresponded to the case of HCECs following selenium treatment (Figure 3E). Pathways related to RNA binding, secondary metabolic process, structural molecule activity, transferase activity, transferring glycosyl groups, translation factor activity, nucleic acid binding, and transmembrane transport were the highest upregulated supergroups, whereas aging, cilium, histone binding, and pigmentation were the highest downregulated supergroups. HT29 cells treated with selenium showed almost no upregulation and only modest downregulation at the pathway level. After sulforaphane exposure, the cell−cell signaling supergroup displayed the highest upregulation effect in both cell lines as well as the highest downregulation effect in HT29 cells.

CONCLUSIONS

In this study, we employed a data-driven approach to compare the specific perturbation responses of cancerous (HT29) and noncancerous (HCEC) human colon cells to nontoxic, dietary-relevant doses of sulforaphane and selenium, two food components with well-documented anticancer properties. With our experimental design and data-analysis strategy, we addressed often-encountered shortcomings of previous studies assessing the impact of dietary supplements on carcinogenesis, namely, too high of a dosage of the food components, lack of large-scale quantification of molecular profiles, and exclusive use of cancerous cells when assessing the impact of dietary supplements on carcinogenesis.

We quantified and investigated molecular responses in human colon cells by employing single-gene as well as network and functional pathway-level approaches. The transcriptome data revealed a highly specific gene-level effect of sulforaphane on the HT29 cancerous cell line, evidenced through significant upregulation of four of the AKR family 1 members (*AKR1C1*, *AKR1C2*, *AKR1C3*, and *AKR1B10*), whereas only small changes were observed following selenium treatment. The expression levels of these four genes were in good agreement with those obtained in a protein-level SILAC analysis conducted for HT29 cells treated with the same dose of sulforaphane.[9] Conversely, in the noncancerous epithelial cell line HCEC, selenium triggered the differential regulation of many genes, including the upregulation of apoptosis activators, cell cycle regulators, and tumor inhibitors. These findings were also reflected in the transcriptome-based pathway analysis, where the strongest perturbations were triggered by selenium treatment in HCECs and hardly any perturbations were observed after sulforaphane treatment of HT29 cells.

Thus, integrated analysis of perturbations induced by the two food components sulforaphane and selenium on the two colon cell lines HT29 and HCEC provided an in vitro characterization of molecular-profile changes related to diet and colon cancer. Despite the focused experimental design in which a single dose of each compound was used in two contrasting cell lines, our data-driven approach provided a comprehensive picture of the particularities of differential regulation in the studied setups. Further studies addressing temporal- and dose-related aspects of this work as well as extension to different cell lines will broaden our understanding on the impact of diet in colon cancer.

ASSOCIATED CONTENT

Supporting Information

PCA analysis on the transcriptome data set; list of differentially expressed genes (q value < 0.01, absolute LFC > 0.5) in the four comparisons consisting of each of the two cell lines (HT29 and HCEC) treated with each of the two food components (selenium and sulforaphane) sorted by their absolute LFC; description and differential regulation pattern (q value < 0.01) of the identified GO terms in at least one of the four comparisons; and description and differential regulation scores of the mapped GO Slim (GOS) terms in the four comparisons. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: niko.beerenwinkel@bsse.ethz.ch.

Funding

This work was financially supported by the Swiss National Science Foundation (Sinergia project 136247).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Heidi Dahlmann for helpful comments on the manuscript and Ritva Haider for technical assistance.

ABREVIATIONS

LFC, log fold change; Se, selenium; Sulf, sulforaphane

REFERENCES

(1) Steinmetz, K. A., and Potter, J. D. (1996) Vegetables, fruit, and cancer prevention. *J. Am. Diet. Assoc. 96*, 1027−1039.

(2) Trock, B., Lanza, E., and Greenwald, P. (1990) Dietary fiber, vegetables, and colon cancer: Critical review and meta-analyses of the epidemiologic evidence. *J. Natl. Cancer Inst. 82*, 650−661.

(3) Willett, W. C., Stampfer, M. J., Colditz, G. A., Rosner, B. A., and Speizer, F. E. (1990) Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. *N. Engl. J. Med. 323*, 1664−1672.

(4) Verhoeven, D. T., Goldbohm, R. A., van Poppel, G., Verhagen, H., and van den Brandt, P. A. (1996) Epidemiological studies on brassica vegetables and cancer risk. *Cancer Epidemiol., Biomarkers Prev. 5*, 733−748.

(5) *Colorectal Cancer Incidence, Mortality and Prevalence Worldwide in 2008*, Report from the International Agency for Research on Cancer, Lyon, France.

(6) Key, T. J., Schatzkin, A., Willett, W. C., Allen, N. E., Spencer, E. A., and Travis, R. C. (2004) Diet, nutrition and the prevention of cancer. *Public Health Nutr. 7*, 187−200.
(7) Martínez, M. E., and Giovannucci, E. (1997) Diet and the prevention of cancer. *Cancer Metastasis Rev. 16*, 357−376.
(8) Gamet-Payrastre, L., Li, P., Lumeau, S., Cassar, G., Dupont, M.-A., Chevolleau, S., Gasc, N., Tulliez, J., and Tercé, F. (2000) Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. *Cancer Res. 60*, 1426−1433.
(9) Bovet, C., Erzinger, M. M., Hecht, K., Winiker, P., Constantinescu, S., Sobotzki, N., Beerenwinkel, N., Wollscheid, B., Marra, G., and Sturla, S. J. (2013) Unpublished work.
(10) Andělová, H., Rudolf, E., and Červinka, M. (2007) In vitro antiproliferative effects of sulforaphane on human colon cancer cell line SW620. *Acta Med. 50*, 171−176.
(11) El-Bayoumy, K., and Sinha, R. (2005) Molecular chemoprevention by selenium: A genomic approach. *Mutat. Res., Fundam. Mol. Mech. Mutagen. 591*, 224−236.
(12) Nano, J. L., Czerucka, D., Menguy, F., and Rampal, P. (1989) Effect of selenium on the growth of three human colon cancer cell lines. *Biol. Trace Elem. Res. 20*, 31−43.
(13) Corpet, D. E., and Taché, S. (2002) Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutr. Cancer 43*, 1−21.
(14) Gasper, A. V., Traka, M., Bacon, J. R., Smith, J. A., Taylor, M. A., Hawkey, C. J., Barrett, D. A., and Mithen, R. F. (2007) Consuming broccoli does not induce genes associated with xenobiotic metabolism and cell cycle control in human gastric mucosa. *J. Nutr. 137*, 1718−1724.
(15) Clark, L. C., Combs, G. F., Turnbull, B. W., Slate, E. H., Chalker, D. K., Chow, J., Davis, L. S., Glover, R. A., Graham, G. F., and Gross, E. G. (1996) Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. *J. Am. Med. Assoc. 276*, 1957−1963.
(16) Navarro-Alarcon, M., and Cabrera-Vique, C. (2008) Selenium in food and the human body: A review. *Sci. Total Environ. 400*, 115−141.
(17) (2012) *Selenium: Its Molecular Biology and Role in Human Health* (Hatfield, D. L., Berry, M. J., and Gladyshev, V. N., Eds.) Springer, New York.
(18) (2005) *Vitamin and Mineral Requirements in Human Nutrition*, World Health Organization, Geneva, Switzerland.
(19) Zeng, H. (2009) Selenium as an essential micronutrient: Roles in cell cycle and apoptosis. *Molecules 14*, 1263−1278.
(20) Gallegos, A., Berggren, M., Gasdaska, J. R., and Powis, G. (1997) Mechanisms of the regulation of thioredoxin reductase activity in cancer cells by the chemopreventive agent selenium. *Cancer Res. 57*, 4965−4970.
(21) Zhang, J., Švehlíková, V., Bao, Y., Howie, A. F., Beckett, G. J., and Williamson, G. (2003) Synergy between sulforaphane and selenium in the induction of thioredoxin reductase 1 requires both transcriptional and translational modulation. *Carcinogenesis 24*, 497−503.
(22) Liu, X., Pietsch, K. E., and Sturla, S. J. (2011) Susceptibility of the antioxidant selenoenyzmes thioredoxin reductase and glutathione peroxidase to alkylation-mediated inhibition by anticancer acylfulvenes. *Chem. Res. Toxicol. 24*, 726−736.
(23) Tanasova, M., and Sturla, S. J. (2012) Chemistry and biology of acylfulvenes: Sesquiterpene-derived antitumor agents. *Chem. Rev. 112*, 3578−3610.
(24) Hintze, K. J., Wald, K. A., Zeng, H., Jeffery, E. H., and Finley, J. W. (2003) Thioredoxin reductase in human hepatoma cells is transcriptionally regulated by sulforaphane and other electrophiles via an antioxidant response element. *J. Nutr. 133*, 2721−2727.
(25) Wang, W., Wang, S., Howie, A. F., Beckett, G. J., Mithen, R., and Bao, Y. (2005) Sulforaphane, erucin, and iberin up-regulate thioredoxin reductase 1 expression in human MCF-7 cells. *J. Agric. Food Chem. 53*, 1417−1421.
(26) Banning, A., Deubel, S., Kluth, D., Zhou, Z., and Brigelius-Flohé, R. (2005) The GI-GPx gene is a target for Nrf2. *Mol. Cell. Biol. 25*, 4914−4923.
(27) Boddupalli, S., Mein, J. R., Lakkanna, S., and James, D. R. (2012) Induction of phase 2 antioxidant enzymes by broccoli sulforaphane: Perspectives in maintaining the antioxidant activity of vitamins A, C, and E. *Front. Genet. 3*, 7.
(28) Cramer, J. M., Teran-Garcia, M., and Jeffery, E. H. (2011) Enhancing sulforaphane absorption and excretion in healthy men through the combined consumption of fresh broccoli sprouts and a glucoraphanin-rich powder. *Br. J. Nutr. 13*, 1−6.
(29) Gasper, A. V., Al-Janobi, A., Smith, J. A., Bacon, J. R., Fortun, P., Atherton, C., Taylor, M. A., Hawkey, C. J., Barrett, D. A., and Mithen, R. F. (2005) Glutathione S-transferase M1 polymorphism and metabolism of sulforaphane from standard and high-glucosinolate broccoli. *Am. J. Clin. Nutr. 82*, 1283−1291.
(30) Hecht, S. S. (2000) Inhibition of carcinogenesis by isothiocyanates. *Drug Metab. Rev. 32*, 395−411.
(31) Brigelius-Flohé, R., and Banning, A. (2006) Part of the series: From dietary antioxidants to regulators in cellular signaling and gene regulation. Sulforaphane and selenium, partners in adaptive response and prevention of cancer. *Free Radical Res. 40*, 775−787.
(32) Juge, N., Mithen, R. F., and Traka, M. (2007) Molecular basis for chemoprevention by sulforaphane: A comprehensive review. *Cell. Mol. Life Sci. 64*, 1105−1127.
(33) Thimmulappa, R. K., Mai, K. H., Srisuma, S., Kensler, T. W., Yamamoto, M., and Biswal, S. (2002) Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res. 62*, 5196−5203.
(34) Sharma, R., Sharma, A., Chaudhary, P., Pearce, V., Vatsyayan, R., Singh, S. V., Awasthi, S., and Awasthi, Y. C. (2010) Role of lipid peroxidation in cellular responses to D, L-sulforaphane, a promising cancer chemopreventive agent. *Biochemistry 49*, 3191−3202.
(35) Hu, C., Eggler, A. L., Mesecar, A. D., and van Breemen, R. B. (2011) Modification of Keap1 cysteine residues by sulforaphane. *Chem. Res. Toxicol. 24*, 515−521.
(36) Hong, F., Freeman, M. L., and Liebler, D. C. (2005) Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. *Chem. Res. Toxicol. 18*, 1917−1926.
(37) Zhang, D. D., and Hannink, M. (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol. Cell. Biol. 23*, 8137−8151.
(38) Bonnesen, C., Eggleston, I. M., and Hayes, J. D. (2001) Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. *Cancer Res. 61*, 6120−6130.
(39) MacLeod, A. K., McMahon, M., Plummer, S. M., Higgins, L. G., Penning, T. M., Igarashi, K., and Hayes, J. D. (2009) Characterization of the cancer chemopreventive NRF2-dependent gene battery in human keratinocytes: Demonstration that the KEAP1−NRF2 pathway, and not the BACH1−NRF2 pathway, controls cytoprotection against electrophiles as well as redox-cycling compounds. *Carcinogenesis 30*, 1571−1580.
(40) Agyeman, A. S., Chaerkady, R., Shaw, P. G., Davidson, N. E., Visvanathan, K., Pandey, A., and Kensler, T. W. (2012) Transcriptomic and proteomic profiling of KEAP1 disrupted and sulforaphane-treated human breast epithelial cells reveals common expression profiles. *Breast Cancer Res. Treat. 132*, 175−187.
(41) Stewart, M. S., Davis, R. L., Walsh, L. P., and Pence, B. C. (1997) Induction of differentiation and apoptosis by sodium selenite in human colonic carcinoma cells (HT29). *Cancer Lett. 117*, 35−40.
(42) Roig, A. I., Eskiocak, U., Hight, S. K., Kim, S. B., Delgado, O., Souza, R. F., Spechler, S. J., Wright, W. E., and Shay, J. W. (2010) Immortalized epithelial cells derived from human colon biopsies express stem cell markers and differentiate in vitro. *Gastroenterology 138*, 1012−1021.

(43) Cattaneo, E., Laczko, E., Buffoli, F., Zorzi, F., Bianco, M. A., Menigatti, M., Bartosova, Z., Haider, R., Helmchen, B., Sabates-Bellver, J., Tiwar, A., Jiricny, J., and Marra, G. (2011) Preinvasive colorectal lesion transcriptomes correlate with endoscopic morphology (polypoid vs. nonpolypoid). *EMBO Mol. Med. 3*, 334−347.
(44) R Core Team (2013) *R: A Language and Environment for Statistical Computing*, R Foundation for Statistical Computing, Vienna, Austria.
(45) Carvalho, B. S., and Irizarry, R. A. (2010) A framework for oligonucleotide microarray preprocessing. *Bioinformatics 26*, 2363−2367.
(46) Smyth, G. K. (2005) Limma: Linear models for microarray data, in *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*, pp 397−420, Springer, New York.
(47) Benjamini, Y., and Hochberg, Y. (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc., Ser. B 1*, 289−300.
(48) Väremo, L., Nielsen, J., and Nookaew, I. (2013) Enriching the gene set analysis of genome-wide data by incorporating directionality of gene expression and combining statistical hypotheses and methods. *Nucleic Acids Res. 41*, 4378−4391.
(49) Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., and Eppig, J. T. (2000) Gene ontology: Tool for the unification of biology. *Nat. Genet. 25*, 25−29.
(50) Efron, B., and Tibshirani, R. (2007) On testing the significance of sets of genes. *Ann. Appl. Stat. 1*, 107−129.
(51) Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., and Lander, E. S. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U.S.A. 102*, 15545−15550.
(52) Durinck, S., Moreau, Y., Kasprzyk, A., Davis, S., De Moor, B., Brazma, A., and Huber, W. (2005) BioMart and Bioconductor: A powerful link between biological databases and microarray data analysis. *Bioinformatics 21*, 3439−3440.
(53) Warde-Farley, D., Donaldson, S. L., Comes, O., Zuberi, K., Badrawi, R., Chao, P., Franz, M., Grouios, C., Kazi, F., and Lopes, C. T. (2010) The GeneMANIA prediction server: Biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res. 38*, W214−W220.
(54) Cline, M. S., Smoot, M., Cerami, E., Kuchinsky, A., Landys, N., Workman, C., Christmas, R., Avila-Campilo, I., Creech, M., and Gross, B. (2007) Integration of biological networks and gene expression data using Cytoscape. *Nat. Protoc. 2*, 2366−2382.
(55) Li, J., Lee, J.-M., and Johnson, J. A. (2002) Microarray analysis reveals an antioxidant responsive element-driven gene set involved in conferring protection from an oxidative stress-induced apoptosis in IMR-32 cells. *J. Biol. Chem. 277*, 388−394.
(56) Devling, T. W. P., Lindsay, C. D., McLellan, L. I., McMahon, M., and Hayes, J. D. (2005) Utility of siRNA against Keap1 as a strategy to stimulate a cancer chemopreventive phenotype. *Proc. Natl. Acad. Sci. U.S.A. 102*, 7280−7285.
(57) Hawkes, W. C., and Alkan, Z. (2011) Delayed cell cycle progression from SEPW1 depletion is p53- and p21-dependent in MCF-7 breast cancer cells. *Biochem. Biophys. Res. Commun. 413*, 36−40.
(58) Kipp, A. P., Frombach, J., Deubel, S., and Brigelius-Flohe, R. (2012) Selenoprotein W as biomarker for the efficacy of selenium compounds to act as source for selenoprotein biosynthesis. *Methods Enzymol. 527*, 87−112.
(59) Reszka, E. (2012) Selenoproteins in bladder cancer. *Clin. Chim. Acta 413*, 847−854.
(60) Zeng, H. (2002) Selenite and selenomethionine promote HL-60 cell cycle progression. *J. Nutr. 132*, 674−679.
(61) Zeng, H., and Combs, G. F., Jr. (2008) Selenium as an anticancer nutrient: roles in cell proliferation and tumor cell invasion. *J. Nutr. Biochem. 19*, 1−7.
(62) Castets, M., Coissieux, M.-M., Delloye-Bourgeois, C., Bernard, L., Delcros, J.-G., Bernet, A., Laudet, V., and Mehlen, P. (2009) Inhibition of endothelial cell apoptosis by netrin-1 during angiogenesis. *Dev. Cell 16*, 614−620.
(63) He, K., Jang, S.-W., Joshi, J., Yoo, M.-H., and Ye, K. (2011) Akt-phosphorylated PIKE-A inhibits UNC5B-induced apoptosis in cancer cell lines in a p53-dependent manner. *Mol. Biol. Cell 22*, 1943−1954.
(64) Breit, S. N., Johnen, H., Cook, A. D., Tsai, V. W. W., Mohammad, M. G., Kuffner, T., Zhang, H. P., Marquis, C. P., Jiang, L., and Lockwood, G. (2011) The TGF-β superfamily cytokine, MIC-1/GDF15: A pleotrophic cytokine with roles in inflammation, cancer and metabolism. *Growth Factors 29*, 187−195.
(65) Tomasini, R., Seux, M., Nowak, J., Bontemps, C., Carrier, A., Dagorn, J.-C., Pébusque, M.-J., Iovanna, J. L., and Dusetti, N. J. (2005) TP53INP1 is a novel p73 target gene that induces cell cycle arrest and cell death by modulating p73 transcriptional activity. *Oncogene 24*, 8093−8104.
(66) Löhr, K., Möritz, C., Contente, A., and Dobbelstein, M. (2003) p21/CDKN1A mediates negative regulation of transcription by p53. *J. Biol. Chem. 278*, 32507−32516.
(67) Han, M.-K., Song, E.-K., Guo, Y., Ou, X., Mantel, C., and Broxmeyer, H. E. (2008) SIRT1 regulates apoptosis and nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization. *Cell Stem Cell 2*, 241−251.
(68) Daixing, Z., Chengye, Z., Qiang, Z., and Shusheng, L. (2013) Upregulation of sestrin-2 expression via P53 protects against 1-methyl-4-phenylpyridinium (MPP+) neurotoxicity. *J. Mol. Neurosci. 51*, 967−975.
(69) Conover, C. A., Bale, L. K., Overgaard, M. T., Johnstone, E. W., Laursen, U. H., Füchtbauer, E.-M., Oxvig, C., and van Deursen, J. (2004) Metalloproteinase pregnancy-associated plasma protein A is a critical growth regulatory factor during fetal development. *Development 131*, 1187−1194.
(70) Christians, J. K., Hoeflich, A., and Keightley, P. D. (2006) PAPPA2, an enzyme that cleaves an insulin-like growth-factor-binding protein, is a candidate gene for a quantitative trait locus affecting body size in mice. *Genetics 173*, 1547−1553.
(71) McCleland, M. L., Gardner, R. D., Kallio, M. J., Daum, J. R., Gorbsky, G. J., Burke, D. J., and Stukenberg, P. T. (2003) The highly conserved Ndc80 complex is required for kinetochore assembly, chromosome congression, and spindle checkpoint activity. *Genes Dev. 17*, 101−114.
(72) Kabeche, L., and Compton, D. A. (2013) Cyclin A regulates kinetochore microtubules to promote faithful chromosome segregation. *Nature 502*, 110−113.
(73) Nabetani, A., Koujin, T., Tsutsumi, C., Haraguchi, T., and Hiraoka, Y. (2001) A conserved protein, Nuf2, is implicated in connecting the centromere to the spindle during chromosome segregation: A link between the kinetochore function and the spindle checkpoint. *Chromosoma 110*, 322−334.
(74) McGarry, T. J., and Kirschner, M. W. (1998) Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell 93*, 1043−1053.
(75) Zhu, W., Chen, Y., and Dutta, A. (2004) Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint. *Mol. Cell. Biol. 24*, 7140−7150.
(76) Bravou, V., Nishitani, H., Song, S. Y., Taraviras, S., and Varakis, J. (2005) Expression of the licensing factors, Cdt1 and Geminin, in human colon cancer. *Int. J. Oncol. 27*, 1511.
(77) Wohlschlegel, J. A., Kutok, J. L., Weng, A. P., and Dutta, A. (2002) Expression of geminin as a marker of cell proliferation in normal tissues and malignancies. *Am. J. Pathol. 161*, 267−273.
(78) Chorley, B. N., Campbell, M. R., Wang, X., Karaca, M., Sambandan, D., Bangura, F., Xue, P., Pi, J., Kleeberger, S. R., and Bell, D. A. (2012) Identification of novel NRF2-regulated genes by ChIP-Seq: Influence on retinoid X receptor alpha. *Nucleic Acids Res. 40*, 7416−7429.

(79) Wu, G., Feng, X., and Stein, L. (2010) A human functional protein interaction network and its application to cancer data analysis. *Genome Biol. 11*, 1−23.
(80) Nagaraj, N. S., Beckers, S., Mensah, J. K., Waigel, S., Vigneswaran, N., and Zacharias, W. (2006) Cigarette smoke condensate induces cytochromes P450 and aldo-keto reductases in oral cancer cells. *Toxicol. Lett. 165*, 182−194.
(81) Knowles, L. M., Yang, C., Osterman, A., and Smith, J. W. (2008) Inhibition of fatty-acid synthase induces caspase-8-mediated tumor cell apoptosis by up-regulating DDIT4. *J. Biol. Chem. 283*, 31378−31384.
(82) Hashimoto, O., Ushiro, Y., Sekiyama, K., Yamaguchi, O., Yoshioka, K., Mutoh, K.-I., and Hasegawa, Y. (2006) Impaired growth of pancreatic exocrine cells in transgenic mice expressing human activin βE subunit. *Biochem. Biophys. Res. Commun. 341*, 416−424.
(83) Papaiahgari, S., Kleeberger, S. R., Cho, H.-Y., Kalvakolanu, D. V., and Reddy, S. P. (2004) NADPH oxidase and ERK signaling regulates hyperoxia-induced Nrf2-ARE transcriptional response in pulmonary epithelial cells. *J. Biol. Chem. 279*, 42302−42312.