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Classification of Environmental Toxicants Using HazChem Human Array V2

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Abstract

Toxicogenomics using microarray technology offers the ability to conduct large-scale detections and quantifications of mRNA transcripts, particularly those associated with alterations in mRNA stability or gene regulation. In this study, we developed the HazChem Human Array V2 using the Agilent SurePrint technology-based custom array, which is expected to facilitate the identification of environmental toxicants. The array was manufactured using 600 VOCs and PAHs-specific genes identified in previous studies. In order to evaluate the viability of the manufactured HazChem human array V2, we analyzed the gene expression profiles of 9 environmental toxicants (6 VOCs chemicals and 3 PAHs chemicals). As a result, nine toxicants were separated into two chemical types-VOCs and PAHs. After the chip validations with VOCs and PAHs, we conducted an expression profiling comparison of additional chemical groups (POPs and EDCs) using data analysis methods such as hierarchical clustering, 1-way ANOVA, SAM, and PCA. We selected 58 genes that could be classified into four chemical types via statistical methods. Additionally, we selected 63 genes that evidenced significant alterations in expression with all 13 environmental toxicants. These results suggest that the HazChem Human Array V2 will expedite the development of a screening system for environmentally hazardous materials at the level of toxicogenomics in the future.

Keywords: Toxicogenomics, PAHs, VOCs, POPs, EDCs, Microarray, HazChem

The risks of chemicals on human health and the environment can be evaluated by combining the chemical-specific hazard data (toxicity or other potential to cause harm) and the estimation of the extent to which man and other organisms are exposed to chemicals. Environmental exposure is generally expressed in terms of environmental concentration in air, water, sediment, or soil. Environmental exposures have been shown to modify the genome, resulting in birth malformations and diseases, including cancer[1]. Such adverse effects of environmental exposures are generally surmised to result from mutations in the DNA sequence that will, in turn, influence gene regulation. Many toxic chemicals can be grouped according to their characteristics.

Volatile Organic Compounds (VOCs) are important indoor air contaminants that evaporate into the atmosphere at room temperature. It appears that high concentrations of VOC principally cause toxic effects[2] and at low exposure levels, VOCs can still exert direct harmful effects on the environment or the human body, or can occasionally create secondary pollutants[3,4].

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that contain two or more fused aromatic rings. PAHs are one of the most widespread organic pollutants. In addition to their presence in fossil fuels, they are also formed via the incomplete combustion of carbon-containing fuels including wood, coal, diesel, fat, tobacco, or incense[5]. Additionally, as they have been recognized as endocrine disruptors, special care must be taken when considering environmental controls.

Persistent Organic Pollutants (POPs) are organic compounds, and are resistant to environmental degradation via chemical, biological, and photolytic processes. POPs have been noted to persist in the environment, to be capable of long-range transport, to bioac-

cumulate in human and animal tissues, to biomagnify in food chains, and to exert potentially significant impacts on both human health and the environment.

Endocrine-disrupting chemicals (EDCs) originate from a variety of man-made sources, including plastics, pharmaceuticals, ordinary household chemicals, and industrial chemicals. They adjust the hormonal functions of various species by functioning as sex hormones, which prevent normal hormonal binding and the breakdown of natural hormones.

Toxicogenomics technologies using microarray technology provide a comprehensive means for the profiling of cell-wide changes in gene expression after exposure to a toxin[6]. Changes in gene expression associated with toxicity are frequently more sensitive and characteristic of the toxic response than are currently employed endpoints of pathology[7].

The principal objective of this study was the biomarker identification of environmental toxicant groups and common expression gene discovery in environmental chemical-treated cells using a manufactured HazChem Human array V2. In this study, we assessed 6 of VOCs, 3 PAHs, 2 POPs and 2 EDCs chemicals.

HazChem Human Array V2

Previously, in the studies of Oh *et al.* (2008) and Park *et al.* (2008), the Hazchem human array V1 was developed, consisting of 300 VOCs- and PAHs-specific genes. We have also conducted further studies, selecting more than 300 genes including VOCs and PAHs commonly expressed genes as well as specific genes (gene list not shown). The HazChem human array V2 was manufactured using the Agilent custom array platform. In the HazChem human array V2, the total number of genes is 600 and the oligonucleotide probes are approximately 15,000. The number of probe per gene was 25.

Validation and Identification of HazChem Human Array V2 at VOCs and PAHs

Firstly, we assessed the whole gene expression profile, and used the data for a definitive classification of VOCs and PAHs (Figure 1). This indicates that the gene contents on HazChem human array V2 are appropriate for the classification of the two groups. In order to conduct a more accurate classification, 135 genes were selected by the statistical method, 1-way ANOVA (p-value cutoff 0.05, multiple testing correction: Benjamini and Hochberg False Discovery Rate). The expression profiles of these genes were more clearly clustered to two chemical types than the total gene expression profile. Their hierarchical clustering data (Figure 2A) showed differences in gene expression

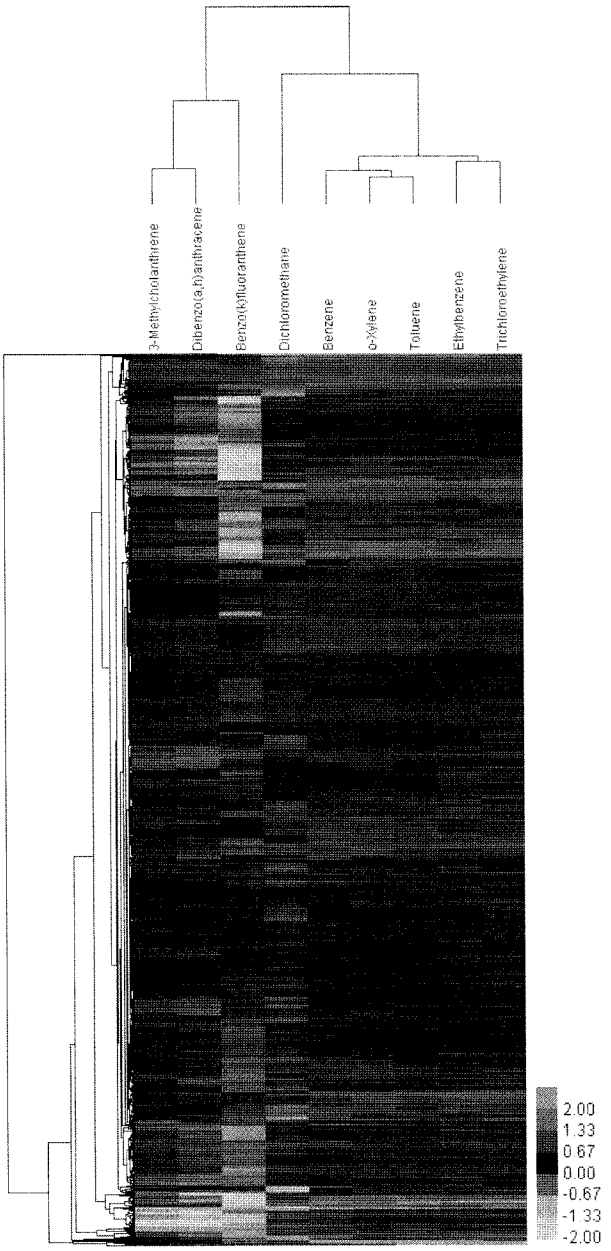
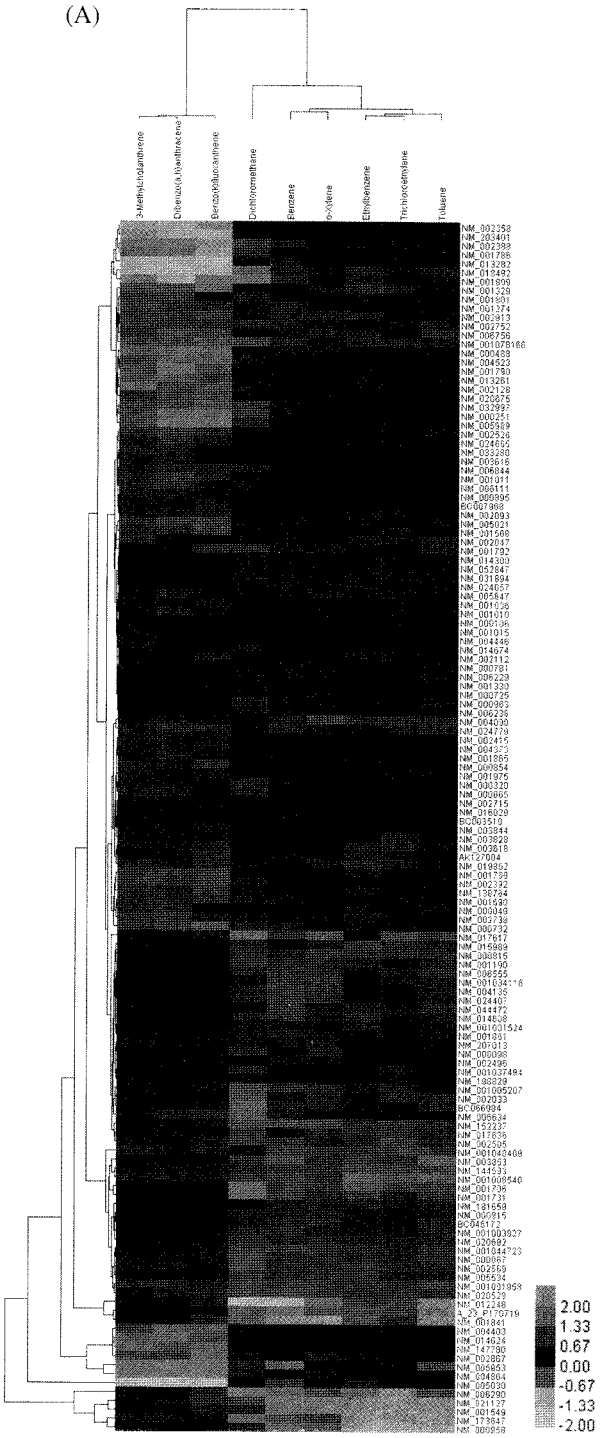


Figure 1. Heat map of total gene expression profile. A total of 600 genes with similar expression patterns are clustered. Similarity Measure is a Euclidean distance, the clustering algorithm is an average linkage. Expression profiles of 6 VOCs samples are separate with 3 PAHs samples. Red color indicates overexpression, green color indicates downregulation and a black color is normal.

patterns. Additionally, the principal components analysis (PCA) data (Figure 2B) shows the relevant classifications.

Significant Expressed Gene Analysis

Human cells were treated with VOCs, PAHs, POPs



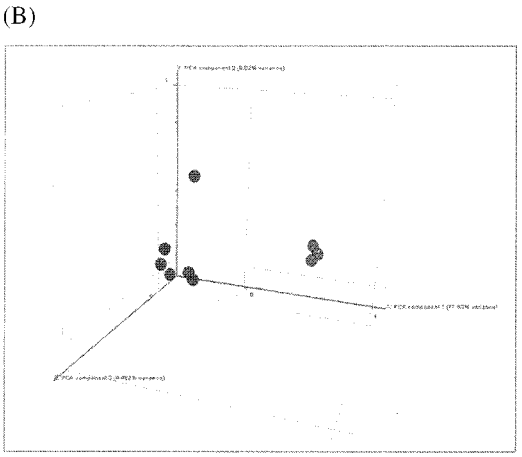


Figure 2. Classification of two chemical group using genes selected by 1-way ANOVA. 135 genes selected by statistical method, 1-way ANOVA (p-value cutoff 0.05, multiple testing correction: Benjamini and Hochberg False Discovery Rate). Expression profile of these genes more clearly clustered to two chemical types relative to the total gene expression profile. (A) Hierarchical clustering, (B) Principle Component Analysis (blue: VOCs, green: PAHs).

and EDCs, after which the RNA was subjected to human HazChem array analysis. Changes in gene expression were analyzed via comparison with the treated and control groups, via the SAM method (q-value < 0.0001). In all chemical groups, a total of 63 genes were selected (Figure 3): 36 genes were upregulated, and 27 genes were downregulated. In the gene ontological data, these genes were associated with cell cycle, response to chemical stimuli, response to stress, M phase, regulation of protein kinase activity, etc. (Table 1).

Classification of 4 Chemical Groups

We compared the expression profiles of additional chemical groups (POPs and EDCs) via data analysis. We selected 58 genes that could be classified into four chemical types using statistical methods such as 1-way ANOVA. Genes from all genes with statistically significant differences when grouped by chemical type; parametric test. Variances were calculated using on-chip replicates. P-value cutoff 0.03, multiple testing correction: Benjamini and Hochberg False Discovery Rate. The following hierarchical clustering data (Figure 4A) evidenced a similar pattern. Moreover, in our PCA analysis, 4 chemical groups were classified with the 58 selected genes (Figure 4B).

Discussion

The principal objective of this study was to validate the HazChem Human Array V2 on environmental toxicants. The HazChem Human Array V2 design was based on VOCs and PAHs groups of environmental toxicants. In previous studies, the manufactured HazChem Human Array chip was validated[8,9]. Therefore, in this study, we tested the chip using 13 differ-

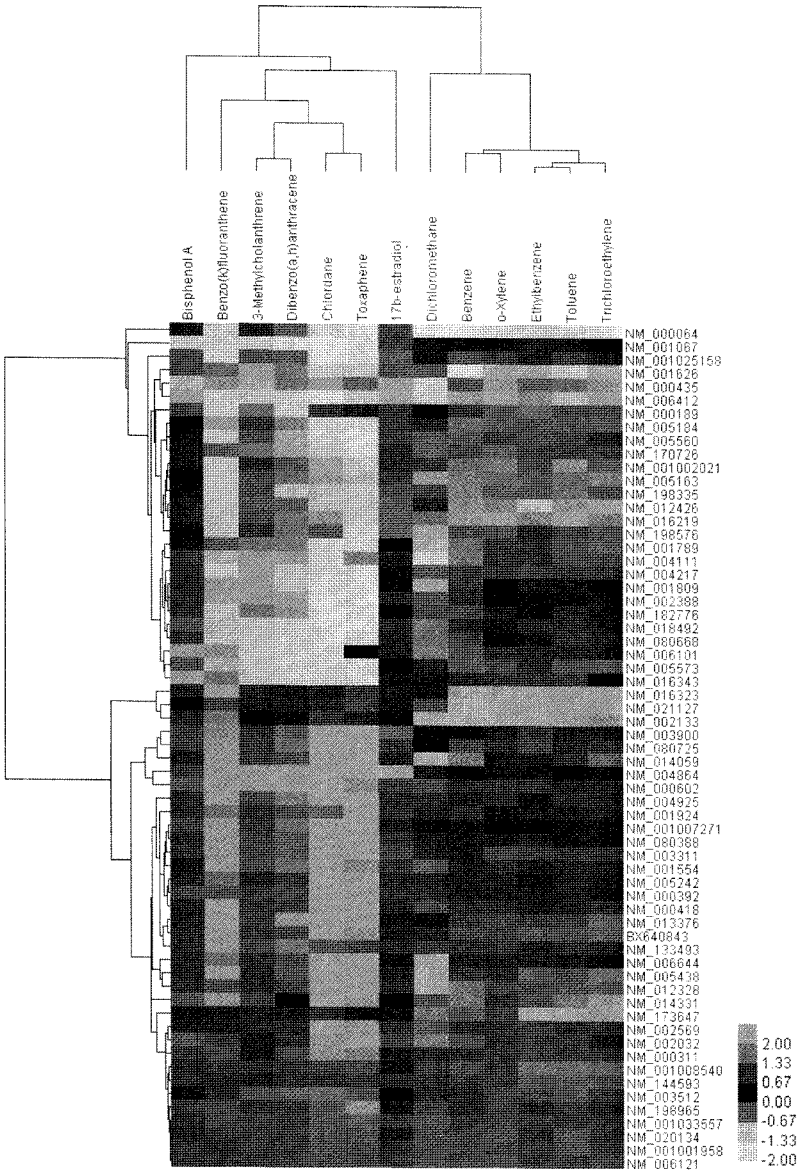


Figure 3. Heat map of significantly changed gene through total 13 chemicals. Significant changed 63 genes are selected via the statistical method, SAM (Significant Analysis of Microarray) (q-value (%) cutoff < 0.0005, multiple testing correction: Benjamini and Hochberg False Discovery Rate) in 4 chemical type, such as VOCs, PAHs, EDCs, POPs. Red color indicates overexpression, green color indicates downregulation and the black color is normal.

Table 1. GO ontology classification of significant changed 63 genes.

GO ontology	Genes in list in category	% of genes in list in category	*P*-value
GO:7049: cell cycle	15	25.42	1.62E-06
GO:42221: response to chemical stimulus	10	16.95	1.92E-05
GO:6950: response to stress	15	25.42	3.94E-05
GO:279: M phase	7	11.86	8.74E-05
GO:45859: regulation of protein kinase activity	5	8.475	0.000892
GO:1525: angiogenesis	4	6.78	0.00153
GO:8283: cell proliferation	9	15.25	0.0027
GO:6979: response to oxidative stress	3	5.085	0.00329
GO:9605: response to external stimulus	9	15.25	0.00524
GO:50801: ion homeostasis	4	6.78	0.0073
GO:7599: hemostasis	3	5.085	0.00803
GO:6915: apoptosis	8	13.56	0.00924
GO:48513: organ development	9	15.25	0.0175
GO:19725: cell homeostasis	4	6.78	0.0184
GO:6952: defense response	9	15.25	0.0198

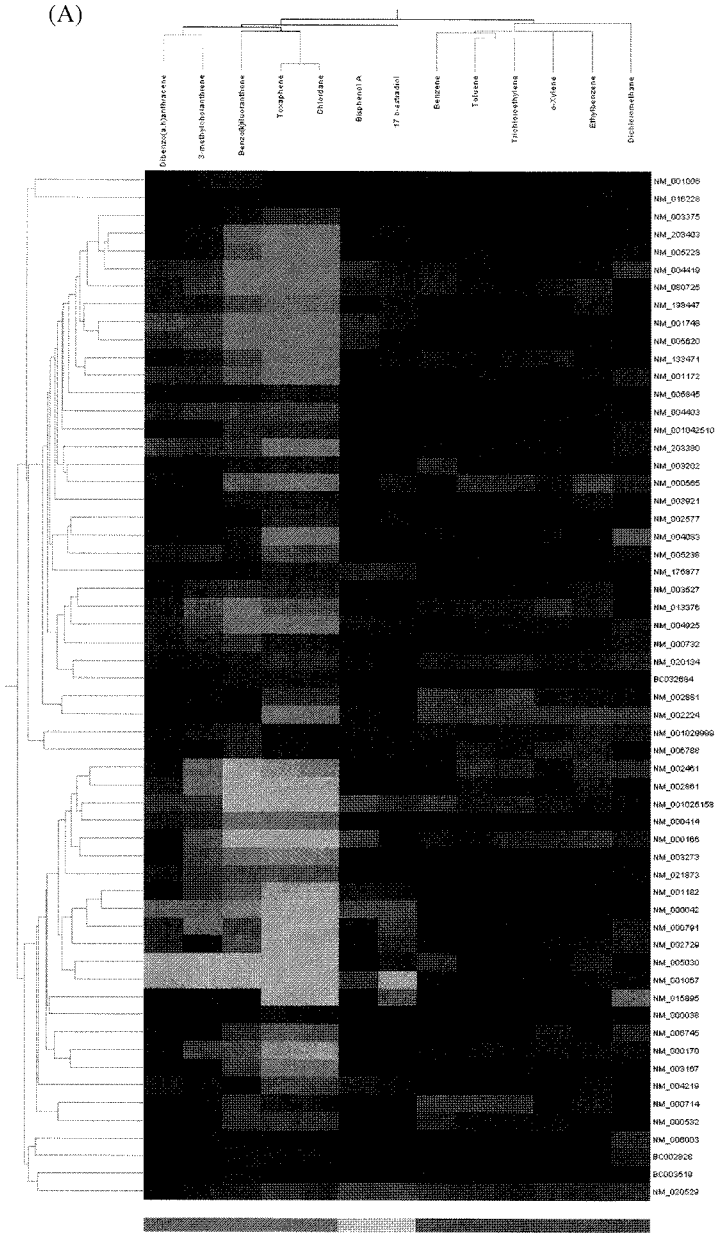


Figure 4. Classification of 4 chemical groups using gene selected by 1-way ANOVA. 58 genes selected by statistical method, 1-way ANOVA (p-value cutoff 0.05, multiple testing correction: Benjamini and Hochberg False Discovery Rate). 13 chemicals accurately classified to 4 chemical types using 58 selected genes. (A) Hierarchical clustering (blue bar: VOCs, green bar: PAHs, red bar: POPs, light blue bar: EDCs), (B) Principal Component Analysis (blue ball: VOCs, green ball: PAHs, red ball: POPs, gray ball: EDCs).

ent chemicals. In addition to the gene expression data, much of the analysis data showed that the manufactured chip could effectively sort out each of the chemical groups.

Genomic approaches have been applied extensively to elucidate the molecular mechanisms of toxicological phenomena, as well as for toxicity assessments[10-13]. Changes in gene expression associated with toxicant characteristics have been previously described via microarray analysis. This method is also useful for the rapid classification of toxicants[14-16].

The HazChem Human Array chip was designed for the purpose of assessing toxicant action in humans[7]. In this study, we used a HazChem human array to identify the significantly differentially expressed genes induced by several chemicals in human cell lines. The results of our microarray data analysis demonstrated differentially expressed gene patterns resulting from exposure to VOCs (benzene, o-xylene, toluene, ethylbenzene, dichloromethane, trichloroethylene), PAHs (dibenzo[a,h]anthracene, 3-methylchlolanthrene, benzo[k]fluoranthene), POPs (chlordane, toxaphene), and EDCs (bisphenol A, 17b-estradiol) in a human cell line. In this study, the data were compiled from authentic sources through the added or changed chemicals from the previous data. We found 63 com-

mon expressed genes associated with all types of environmental toxicants, on the basis of the gene expression profiles and genetic distances (Figure 3). Furthermore, different analysis methods such as 1-way ANOVA, SAM, and PCA were also utilized for the validation and classification of the HazChem Human Array chip.

In summary, the above data shows that the HazChem Human Array V2 may, in the future, facilitate the development of an efficient system for the screening of environmentally hazardous materials at the level of toxicogenomics.

Materials & Methods

Chemicals

Toxaphene and chlordane were purchased from TCI-EP (Japan), benzene, toluene, o-xylene, ethylbenzene, trichloroethylene, dichloromethane, dibenzo[a,h]anthracene, and 3-methylcholanthrene were purchased from the Sigma-Aldrich Chemical Company (USA).

Cell Culture and Determination of Cell Viability

The human hepatocellular carcinoma cell line (HepG2) and the human promyelocytic leukemia HL-60 cell line culture were referred to in the paper[8]. Additionally, the MTT cell viability assay experiment was conducted as described by Park *et al.*[9].

Preparation of HazChem Human Array and Hybridization

HazChem Human array V2 is manufactured using SurePrint technology with the Agilent custom array. Approximately 600 genes were selected from previous VOCs and PAHs toxicogenomic studies[17]. Genes were specifically expressed on each type of chemicals or commonly expressed on two types. Normalization was conducted with probe DNA at a density of 50 pmol, then moved into 384-well plates. The reproducibility, reliability, and accuracy of the HazChem array were evaluated using the control materials in accordance with the internal guidelines.

Each extracted total RNA sample (15 μg) was labeled with Cyanine (Cy3) or Cyanine (Cy5) conjugated dCTP (Amersharm, Piscataway, NJ) via a reverse transcription reaction using reverse transcriptase, SuperScrip II (Invitrogen, Carlsbad, California). The labeled cDNA mixture was then concentrated via ethanol precipitation. The concentrated Cy3- and Cy5-labeled cDNAs were resuspended in 10 μL of hybridization solution (GenoCheck, Korea). The two labeled cDNAs were then mixed, placed on a HazChem array Human V2 (Agilent, Korea) and covered Validation of Human HazChem Array 49 with a MAUI M4 chamber (Biomicro Systems, Inc. UT). The slides were hybridized for 12 hr at 62°C using a MAUI system (Biomicro Systems, Inc. UT). The hybridized slides were washed in 2XSSC, 0.1% SDS for 2 min, 1XSSC for 3 min, and then 0.2XSSC for 2 min at room temperature. The slides were dried via 20 seconds of centrifugation at 3,000 rpm to dry.

Microarray Data Analysis

The hybridized slides were scanned with an Axon Instruments GenePix 4000B scanner and the scanned images were analyzed using the GenePix Pro 5.1 program (Axon, CA) and GeneSpring GX 7.3.1 (Agilent Technologies, CA). Spots that were adjudged as substandard via the visual examination of each slide were flagged and excluded from further analysis. Spots harboring dust artifacts or spatial defects were manually flagged and excluded. In an effort to filter out the unreliable data, spots with signal-to-noise (signal-background-background SD) ratios below 10 were not included in the data. Data were normalized by Global, lowess, print-tip and scaled normalization for data reliability. Fold change filters included the requirement that the genes be present in at least 200% of the controls for upregulated genes and lower than 50% of controls for the downregulated genes. The data were clustered into groups of genes that behaved similarly across the drug treatment experiments using GeneSpring GX 7.3.1 software (Agilent Technologies, CA). We utilized an algorithm based on the Pearson's correlation to separate genes exhibiting similar patterns.

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