

Subchronic Exposure to TCDD, PeCDF,
PCB126, and PCB153: Effect on Hepatic
Gene Expression

Chad M Vezina, Nigel J Walker, James R Olson
doi:10.1289/txg.7253 (available at <http://dx.doi.org/>)
Online 22 September 2004



The National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Department of Health and Human Services

Subchronic Exposure to TCDD, PeCDF, PCB126, and PCB153: Effect on Hepatic Gene Expression

Chad M Vezina,¹ Nigel J Walker,² James R Olson³

¹cmvezina@pharmacy.wisc.edu, UW Madison, School of Pharmacy, Madison, WI 53735

²walker3@niehs.nih.gov National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

³jolson@buffalo.edu, University at Buffalo, Department of Pharmacology and Toxicology, Buffalo, NY 14214.

Corresponding Author: James Olson

University at Buffalo

Department of Pharmacology and Toxicology

102 Farber Hall

3435 Main Street

Buffalo, NY 14214

Phone: (716) 829-2319

Fax: (716) 829-2800

Email: jolson@buffalo.edu

Running Title

Gene Expression Analysis of Dioxin and Related Compounds.

Key Words

TCDD, Liver, microarray, PCB, *AhR*, HAH

Abbreviations

AhR: aryl hydrocarbon receptor, *ARNT*: *AhR* nuclear transporter, *CAP2*: adenylate cyclase-associated protein 2, *C-CAM4*: carcinoembryonic-cell adhesion molecule 4, *CYP*: cytochrome P450, DRE: dioxin response element, HAH: Halogenated aromatic hydrocarbon, NTP: National Toxicology Program, PCA: Principal Components Analysis, PCB: polychlorinated biphenyl, PCB126: 3,3',4,4',5-pentachlorobiphenyl, PCB153: 2,2',4,4',5,5'-hexachlorobiphenyl, PCDD: polychlorinated dibenzo-*p*-dioxin, PCDF: polychlorinated dibenzofuran, PeCDF: 2,3,4,7,8-pentachlorodibenzofuran, PTM: Pavlidis Template Matching, SD: Sprague Dawley, TCDD: 2,3,7,8 tetrachlorodibenzo-*p*-dioxin, TEF: Toxic Equivalency Factor.

Acknowledgements

Tissues for this study were provided to us by the National Toxicology Program (<http://ntp-server.niehs.nih.gov>) as part of a series of chronic 2-year rat bioassays examining the relative potencies for carcinogenicity of individual and mixtures of dioxin-like compounds. These studies were supported in part by NIESH ES09440, the University at Buffalo, and the Environment and Society Institute, University at Buffalo. The authors declare they have no competing financial interests.

Outline of Section Headers

- I. Abstract
- II. Introduction
- III. Materials and Methods
 - A. Sample procurement
 - B. RNA isolation and hybridization
 - C. Data analysis
 - D. Real-time RT-PCR validation of gene expression
- IV. Results
 - A. Dosimetry and liver pathology from the NTP cancer bioassay
 - B. Effects of HAH exposure on global hepatic gene expression
- V. Discussion
- VI. References

ABSTRACT

We have employed DNA microarray to identify unique hepatic gene expression patterns associated with subchronic exposure to TCDD and other halogenated aromatic hydrocarbons (HAHs). Female Harlan Sprague-Dawley rats were exposed for 13 weeks to toxicologically equivalent doses of four different HAHs based on the toxic equivalency factor of each chemical: 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD; 100 ng/kg/day), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF; 200 ng/kg/day), 3,3',4,4',5-pentachlorobiphenyl (PCB126; 1000 ng/kg/day), or 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153; 1000 µg/kg/day). Global gene expression profiles for each exposure, which account for 8,799 gene probe sets contained on Affymetrix RGU34A GeneChips, were compared by principal components analysis (PCA). The aryl hydrocarbon receptor (*AhR*) ligands TCDD, PeCDF, and PCB126 produced very similar global gene expression profiles that were unique from the non-*AhR* ligand, PCB153, underscoring the extensive impact of *AhR* activation and/or the resulting hepatic injury on global gene expression in female rat liver. Many genes were co-expressed during the 13 wk TCDD, PeCDF, or PCB126 exposures, including classical *AhR* regulated genes and some genes not previously characterized as being *AhR* regulated, such as carcinoembryonic-cell adhesion molecule 4 (*C-CAM 4*) and adenylate cyclase-associated protein 2 (*CAP2*). Real time RT-PCR confirmed the increased expression of these genes in TCDD, PeCDF, and PCB126 exposed rats as well as the up- or downregulation of several other novel dioxin-responsive genes. In summary, DNA microarray successfully identified dioxin responsive genes expressed following exposure to *AhR* ligands (TCDD, PeCDF, PCB126), but not following exposure to the non-*AhR* ligand, PCB153. Together, these findings may help to elucidate some of the fundamental features of dioxin toxicity and may further clarify the biological role of the *AhR* signaling pathway.

INTRODUCTION

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin, TCDD) is a persistent environmental contaminant, a human and rodent carcinogen, and the most potent ligand for the aryl hydrocarbon receptor (*AhR*) (Fingerhut et al. 1991; Gu et al. 2000; Kociba et al. 1978; McGregor et al. 1998). The *AhR* also displays affinity for structurally related xenobiotics, including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (PCBs) (Denison et al. 2002). Ligand binding and activation of the *AhR* induces nuclear localization and heterodimerization with the *AhR* nuclear transporter (*ARNT*) protein (Whitlock 1999). This activated heterodimer binds to cognate *cis*-acting sequences (dioxin response elements or DREs), located in the 5'-regulatory region of target genes.

A specific subgroup of genes are activated by an *AhR*-dependent mechanism during dioxin exposure, including (but not limited to) cytochrome P450 (*CYP*) *1A1*, *1A2*, *1B1*, aldehyde dehydrogenase, NADPH-quinone-oxidoreductase, glutathione-s-transferase (*GST*) Ya, and UDP-glucuronosyltransferase 1A1 (Manjunath and Dufresne 1988; Mimura and Fuji-Kuriyama 2003; Schrenk 1998; Sutter and Greenlee 1992). *AhR*-dependent transcription is required for dioxin toxicity (Bunger et al. 2003), but it is unclear how activation of *AhR*-dependent genes produces the multiplicity of toxic responses characteristic of dioxin exposure.

As an attempt to characterize *AhR*-dependent genes and signaling pathways responsible for subchronic dioxin toxicity, the current study evaluated differential hepatic gene expression in female Harlan Sprague Dawley (SD) rats exposed subchronically (13 wks) to toxicologically equivalent doses of the *AhR* ligands 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 100 ng/kg/day) 2,3,4,7,8-pentachlorodibenzofuran (PeCDF; 200 ng/kg/day), 3,3',4,4',5-pentachlorobiphenyl

(PCB126; 1000 ng/kg/day), or the non-*AhR* ligand 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153; 1000 µg/kg/day). This gene expression study was performed in conjunction with a cancer bioassay conducted by the National Toxicology Program (NTP), which included interim sacrifices (13, 30, and 52 wks) to investigate tissue dosimetry, histopathology, and other biochemical and molecular responses throughout the 2-year study.

Subchronic TCDD exposure is associated with numerous toxic responses and many of these responses may be *AhR*-dependent. Kociba et al. (1976) showed previously that SD rats exposed to high levels of TCDD (1 µg/kg/day) for 13 weeks were subject to mortality, chloracne, thymic atrophy, and a 'wasting syndrome' characterized by rapid weight loss and fat redistribution. Antioxidant enzyme expression was enhanced in rats exposed to lower doses of TCDD for 13 weeks (10-46 ng/kg/day; Hassoun et al. 1998) and the hepatotoxic biomarkers serum bilirubin and alkaline phosphatase were elevated in rats exposed to intermediate doses for the same exposure period (100 ng/kg/day TCDD; Kociba et al. 1976). Rats exposed to TCDD for 13 weeks (100 ng/kg/day) also developed cachexia, hepatic hypertrophy, and altered hepatic foci (Kociba et al. 1976), while chronic exposure (104 weeks) at this dose resulted in porphyria and cancer of the liver, lung, and oral mucosa (Kociba et al. 1978; National Toxicology Program 2004a). While *AhR* activation likely contributes to the many toxicological effects produced by subchronic and chronic TCDD exposures, little is known about *AhR* and non-*AhR* signaling mechanisms mediating these effects.

The TCDD, PeCDF, and PCB126 exposure doses utilized in this study were carcinogenic to female SD rats, but tumors and other hepatotoxic effects were not evident until several months after the 13 wk interim sacrifice (National Toxicology Program 2004a, 2004b, 2004c). Thus, evaluation of differential gene expression patterns following 13 wk subchronic HAH exposures

may yield important clues about the mechanisms by which these chemicals produce their chronic toxicological effects, including cancer. While there have been several attempts to evaluate TCDD-dependent gene expression *in vitro* and *in vivo* (Fisher et al. 2004; Frueh et al. 2001; Martinez et al. 2002; Puga et al. 2000), to our knowledge, this is the first of such to characterize gene expression during long-term, subchronic exposure to carcinogenic doses of TCDD and other dioxin-like chemicals.

MATERIALS AND METHODS

Sample procurement

Tissues for this study were provided by the National Toxicology Program (2004a, 2004b, 2004c) as part of a 2-year bioassay for relative carcinogenic potencies of dioxin-like chemicals. Female Harlan SD rats were exposed 5 days a week by oral gavage to toxicologically equivalent doses of TCDD (TEF = 1.0; 3, 10, 22, 46, 100 ng/kg/day), PeCDF (TEF = 0.5; 6, 20, 44, 92, 200 ng/kg/day), PCB126 (TEF = 0.1; 10, 30, 100, 175, 300, 550, 1000 ng/kg/day), PCB153 (TEF = 0, N/A; 10, 100, 300, 1000 µg/kg/day), or corn oil:acetone (99:1; vehicle control). Toxicological dose equivalence was based on the current World Health Organization TEF recommendations (Van den Berg et al. 1998). Subgroups of rats were sacrificed on study weeks 14, 31 and 53 weeks (corresponding to 13, 30, or 52 weeks duration of exposure) and target organs were removed, flash frozen in liquid nitrogen, and stored at -70°C for mechanistic studies.

RNA isolation and hybridization

The present study utilized liver from female rats exposed to vehicle control or the highest dose of each compound for 13 weeks to ensure that hepatic gene expression was evaluated in the context of carcinogenic exposure doses for TCDD, PeCDF, and PCB126. Frozen hepatic tissue was disrupted by homogenization with a rotor stator homogenizer and total RNA was isolated with Qiagen RNeasy columns. There were a total of six rats in each exposure group. Three pools of RNA were created from each exposure group ($n = 2$ rats per pool), similar to the experimental design of Yechoor et al. (2002). Pooled total RNA was further purified using the Qiagen Poly(A)RNA Isolation Kit. RNA integrity was assessed by the Agilent Bioanalyzer 2100. This study employed high quality RNA that displayed two distinct, sharp peaks and a

28S/18S ribosomal RNA ratio greater than 1. Poly(A) RNA was transformed into labeled cRNA by the Roswell Park Cancer Institute Microarray Core Facility. cRNA from each pool was fragmented and its quality evaluated with Affymetrix GeneChip Test3 arrays by comparing 3'/5' signal ratios of housekeeping genes. High quality cRNA (3'/5' signal ratio near 1) was subsequently hybridized to Affymetrix RGU34A GeneChips and chips were scanned with the Affymetrix 428 scanner.

Data analysis

Cell Intensity Files (.CEL) files were generated with Affymetrix Microarray Suite 5.0 software and probe level data were background subtracted, normalized, and gene expression was summarized using the MAS 5.0 algorithm included in the Bioconductor Affy package for R, version 1.6.1 (Ihaka and Gentleman 1996). Gene expression data from $n=3$ GeneChips in each exposure group were averaged and changes in gene expression were calculated as the average change versus gene expression for the $n=3$ GeneChips from the vehicle-treated control group. Cluster analysis was performed with TIGR Microarray Experiment Viewer (Saeed et al. 2003). The gene expression profiles associated with TCDD, PeCDF, PCB126, and PCB153 exposures were assessed by Principal Components Analysis with the covariance value distance metric (PCA; Raychaudhuri et al. 2000) to evaluate relationships between exposure groups. Genes co-expressed during various exposure conditions were identified by Pavlidis Template Matching (PTM; Pavlidis and Noble 2001). For each PTM analysis, gene expression profile templates were constructed by designating relative gene expression ratios for each exposure condition. Gene expression data was filtered for genes that matched each template based on Pearson Correlation ($R \geq 0.9$). Template matching genes were subjected to Euclidean distance

hierarchical clustering. Genes were annotated with GenBank accession numbers by Affymetrix Microarray Suite Software 5.0 and TIGR Resourcerer gene annotation tool (Tsai et al. 2001) and official gene names were provided by the Rat Genome Database. Expressed Sequence Tags without annotation were filtered from PTM outputs, thus restricting gene sets to annotated genes. Promoters of selected genes were mapped for DREs using MatInspector Professional (Quant et al. 1995). Quantitative gene expression estimates obtained by microarray analysis were validated by two-step real-time RT-PCR for selected genes.

Real-time RT-PCR validation of gene expression

Reverse transcriptase reactions (80 μ l) contained 20 μ g total RNA, 0.5 mM dNTP Mix, and 15 ng/ μ l random primers, 1X first Strand buffer, 10 mM dithiothreitol, 27 U Rnasin® RNase inhibitor (Promega, Madison WI), and 800 U superscript reverse transcriptase (Invitrogen, Carlsbad CA). A mixture containing total RNA, dNTPs, and random primers was heated to 65°C for 5 min to denature the RNA and then immediately placed on ice. The remaining components of the reaction mixture were then added to the RNA and cDNA synthesis was performed at 42°C for 60 min. Reactions were terminated by heating to 70°C for 10 min.

PCR primers were selected from GenBank database sequences with Primer3 software (Rozen and Skaletsky 2000). Primer sequences were between 20 and 22 bp, contained at least one 3'-GC clamp, displayed a maximal T_m difference of 1, a maximal Poly-X value of 3, maximal 3'-complimentarity of 2, and a T_m between 60 and 62°C. Non-specific mispriming was managed by a mispriming threshold of 10.0 in the rodent mispriming library. The primer sequences utilized in the current study are shown in Table 1.

Real-time PCR was performed with the Applied Biosystem's SYBR Green PCR kit according to the manufacture's instructions. PCR reactions (25 μ l) contained diluted cDNA, 1X SYBR Green Buffer, 3 mM MgCl₂, 0.2 mM dNTP mix, 0.2 μ M of left and right primers, 10 nM fluorescein, and 1.1 U of Amplitaq Gold polymerase. The reaction was initiated by incubation at 95°C for 10 min, and followed by 40 cycles of denaturation at 95°C for 15 sec and primer annealing/extension at 60°C for 1 min. Sample fluorescence was evaluated during the annealing/extension step. Upon completion of thermocycling, the specificity of each reaction was evaluated by melting analysis. Samples were heated to 95°C for 2 minutes and cooled to 55°C. The temperature was maintained at 55°C for 15 sec to analyze sample fluorescence, and the temperature was increased by increments of 0.5°C followed by 15 sec of fluorescence analysis for a total of 80 cycles.

The efficiency of each primer set was validated over a range of cDNA concentrations. Primer pairs that demonstrated reaction efficiencies between 85-103%, concentration vs. fluorescence slope factors between -1.3 to -1.7, and concentration vs. fluorescence correlation coefficients between 0.98-1.0 were accepted for further use. Subsequent to primer validation, PCR reactions were performed with a single cDNA concentration and the threshold cycle (C_t) was determined for each reaction. The difference (ΔC_t) between the threshold cycle for the target gene and endogenous control gene (18S RNA) was calculated for each sample, and the 18S normalized relative expression of each gene was calculated by the comparative method according to the Applied Biosystem's User Bulletin # 2 for the ABI Prism Sequence Detection System (Applied Biosystems, Foster City, CA), as described by Imasato et al. (2002).

RESULTS

Dosimetry and liver pathology from the NTP cancer bioassay.

Tissue dosimetry and liver pathology for each 13 wk HAH exposure are detailed in Table 2. The dosimetry values indicate that the *AhR* ligands (TCDD, PeCDF, PCB126) exhibit more pronounced hepatic accumulation, relative to administered dose, than the non-*AhR* ligand PCB153. It is important to note that the exposure to PCB153 is in $\mu\text{g}/\text{kg}/\text{day}$, while the level of PCB153 in the liver is in units of ng/g liver, supporting the greatly reduced relative hepatic accumulation of PCB153. Preferential hepatic accumulation of TCDD, PeCDF and PCB126 is consistent with *CYP1A2* serving as a sequestering protein for *AhR* ligands (Van den Berg, 1994). There was a statistically significant increase in the incidence of hepatic hypertrophy for all exposures compared to the respective vehicle control animals. Liver hypertrophy was mild to moderate among female rats from the TCDD exposure group, minimal to mild in the PCB126 exposure group and minimal in rats exposed to PeCDF or PCB153. A limited, but non-significant number of animals exposed to TCDD for 13 wks displayed multinucleated hepatocytes.

There was an increased incidence and severity of liver hypertrophy for the TCDD and PCB126 exposures relative to the other *AhR* ligand, PeCDF. Pavlidis Template Matching (PTM) was employed to identify genes associated with the more pronounced hepatic hypertrophy in the TCDD and PCB126 groups relative to the PeCDF group (Fig. 1). We were unable to identify genes that were selectively repressed, but we did identify a limited subset of genes selectively induced by TCDD and PCB126 compared to PeCDF. These genes were functionally classified as neurotransmitter and endocrine signaling genes.

Effects of HAH exposure on global hepatic gene expression

TCDD, PeCDF, and PCB126 activate the *AhR*, while PCB153 displays little or no binding affinity for the *AhR*. Principal components analysis (PCA) was employed to determine whether the relationship between global gene expression profiles for each chemical would be related to their *AhR* affinities (Fig. 2). The principal components for TCDD, PeCDF, and PCB126 exposures were spatially co-localized along the *x*- axis of the PCA plot (PeCDF and PCB126 were localized to the same quadrant, while TCDD was proximally located in the adjacent quadrant). The principal component for the PCB153 exposure was unique from those of *AhR* ligands.

Since the global gene expression profile associated with PCB153 exposure differed from that of TCDD, PeCDF, and PCB126, PTM was employed to identify genes that were selectively induced or repressed by PCB153 (Fig. 3). PCB153 does not bind to the *AhR* and genes activated or repressed during PCB153 exposure are likely regulated by an *AhR*-independent mechanism. Liver from PCB153 exposed rats exhibited a unique class of differentially expressed genes compared to rats exposed to *AhR* ligands, with cytochrome P450 2B1 being the most upregulated by PCB153. PCB153 exposure also produced the differential expression of proinflammatory genes interleukin 2 and interleukin 1 and myxovirus (influenza virus) resistance (*MXI*), and apoptosis related genes B-cell leukemia/lymphoma 2 (*BCL-2*) and weel1.

Global hepatic gene expression profiles of animals exposed to PeCDF more closely resembled those of PCB126 exposed animals than TCDD exposed animals, indicating PeCDF and PCB126 may co-regulate a unique group of genes that are not differentially expressed relative to TCDD exposure. This subgroup of genes may be activated by an *AhR*-independent mechanism unique to PeCDF and PCB126. PTM was utilized to identify genes that were

selectively activated by PeCDF and PCB126 compared to TCDD (Fig. 4). A total of 29 different genes were identified, all of which were mutually induced by PeCDF and PCB126. These genes included metabolic enzymes (cytochrome P450 15-beta gene, cytochrome P450 2C39, NADH dehydrogenase) and oxidative stress response genes (catalase, cytochrome c oxidase).

PTM was also employed to identify genes co-expressed during TCDD, PeCDF, and PCB126 exposures, since differential expression of these genes may be associated with *AhR*-mediated pathology (Fig. 5). Many of the TCDD-inducible genes identified by PTM represented classical dioxin-inducible genes, including *CYP1A1*, *CYP1B1*, *CYP1A2*, NAD(P)H-menadione oxidoreductase, Immunoglobulin M, and UDP glycosyltransferase 1 family, polypeptide A1. While cytochrome c oxidase has been associated with TCDD induction, *COX8H* represents a novel dioxin-inducible isoform of this gene. *C-CAM4* and *CAP2* were also induced during TCDD, PeCDF, and PCB126 exposures. Several genes were coordinately downregulated by TCDD, PeCDF, and PCB126, but not PCB153, including epidermal growth factor and mitochondrial thioesterase.

C-CAM4 and *CAP2* were highly induced by all three *AhR* ligands but have not been linked to *AhR*-mediated transcriptional regulation. The enhanced expression of *C-CAM4* and *CAP2* was verified by real-time RT-PCR (Table 3). To determine whether *C-CAM4* and *CAP2* are direct *AhR* target genes, the 5'-regulatory sequences of these genes were modeled by MatInspector Professional to identify potential *cis*-acting DRE sequences within these gene promoters. Neither gene promoter contained DRE consensus sequences.

The expression of several additional genes was also verified by real-time RT-PCR (Table 3). In general, there was relatively good association between the up- or downregulation of genes

according to Affymetrix GeneChip analysis and real time RT-PCR methods. *CYP1B1*, *C-CAM4*, and *CAP2* were consistently increased in livers of rats exposed to *AhR* ligands (TCDD, PeCDF, PCB126), while exhibiting little or no change in livers of PCB153 exposed rats. Cytochrome P450 3A9 (*CYP3A9*) and serine proteinase inhibitor, clade A (*SERPIN7A*) were down-regulated by exposure to *AhR* ligands, while somatostatin was down-regulated by exposure to *AhR* ligands and PCB153. Real time RT-PCR also suggests that carboxylesterase 3 was down-regulated by *AhR* ligands.

DISCUSSION

The TEF classification scheme has been used for many years to facilitate risk assessment for individual congeners and mixtures of dioxin-like PCDDs, PCDFs, and PCBs (Becher et al. 1998; Finley et al. 2003; Flesch-Janys et al. 1998; Van den Berg, 1998). However, there is some question about whether TEF values are predictive of long-term toxicological endpoints, including cancer (Safe 1994). The current study evaluated hepatic gene expression during a 13 wk interim sacrifice from a two year chronic toxicity and carcinogenicity study of TCDD, PeCDF, PCB126, and PCB153 in female Harlan SD rats. The TCDD, PeCDF, and PCB126 exposures from this study produced cholangiocarcinoma, hepatocellular adenoma, toxic hepatopathy, multinucleated hepatocytes, diffuse fatty change, and liver pigmentation after two years of exposure (National Toxicology Program 2004a, 2004b, 2004c). Liver tumor incidence was not equivalent among *AhR* ligands, but instead was higher in animals exposed to TCDD and PCB126 compared to animals exposed to PeCDF at toxicologically equivalent doses. Similar results were seen for the incidence and severity of hepatic hypertrophy at 13 wks where PeCDF produced less hypertrophy than TCDD and PCB126 (Table 2). The 13 wk PeCDF exposure also induced substantially less *CYP1B1* mRNA compared to TCDD and PCB126 as shown by RT-PCR in Table 3. Thus, it is possible that enhanced *AhR* activation by TCDD and PCB126 may be responsible for the increased liver pathology of TCDD and PCB126 exposed rats compared to PeCDF exposed rats. Consistent with these findings, our laboratory previously reported more CYP1-mediated ethoxyresorufin-*o*-deethylase activity in liver microsomes from TCDD and PCB126 exposed female SD rats compared to PeCDF exposed rats after 13 wks of exposure (Shubert et al. 2002). Thus, the 200 ng/kg/day PeCDF exposure is less effective towards

activating *AhR*-dependent gene expression and less toxic with regard to hepatic hypertrophy and carcinogenicity compared to 100 ng/kg/day TCDD and 1000 ng/kg/day PCB126 exposures.

An alternative possibility of the enhanced toxicity of TCDD and PCB126 exposures compared to PeCDF is that these chemicals activate a unique subgroup of genes that is not activated by PeCDF, thus enhancing toxicity. In support of this possibility, DNA microarray analysis revealed a subset of differentially expressed genes in TCDD and PCB126 exposed rat liver that were relatively unchanged during PeCDF exposure (Fig. 1). These genes were functionally related to neurotransmitter and neuroendocrine signaling. Activation of neuroendocrine signaling by TCDD has been demonstrated in other tissues, including monkey hypothalamus and rodent pituitary and adrenal (Pitt et al. 2000; Shridhar et al. 2001). Subchronic exposure to TCDD and PCB126 may therefore stimulate hepatic neuroendocrine signaling. Activation of hepatic neuroendocrine cells by these chemicals may also be a symptom or contributor to chemical-induced liver hypertrophy, which was substantially less in PeCDF exposed rats.

AhR activation by PCDDs, PCDFs, and coplanar PCBs is a hallmark of exposure to dioxin-like chemicals and is likely implicated in their toxicity. *AhR* activation has also been attributed to the direct activation of some dioxin-responsive genes (Mimura and Fuji-Kuriyama 2003; Schrenk 1998; Sutter and Greenlee 1992). However, there is little knowledge regarding how many genes are activated or repressed in an *AhR*-dependent mechanism during subchronic exposure to *AhR* ligands. The current study utilized PCA to address relationships between exposure to traditional *AhR* ligands (TCDD, PeCDF, and PCB126) and the non-*AhR* ligand PCB153. PCA has been previously employed to compare genomic profiles of toxicants (Heijne et al. 2003), identify common molecular effects among potential drug candidates (Shi et al.

2000), and predict treatment prognosis (Ringberg et al. 2001). PCA revealed a unique association among hepatic gene expression profiles produced by exposure to dioxin-like toxicants, where global gene expression profiles for rats exposed to PeCDF and PCB126 were very similar and closely related to the gene expression profile for TCDD exposure (Fig. 2). The global gene expression profile for exposure to the non-coplanar PCB153; however, was substantially different from that of the dioxin-like *AhR* ligands. Prominent differences between PCB153-mediated and *AhR*-ligand mediated gene expression suggests subchronic exposure to TCDD, PeCDF and PCB126 has an extensive impact on global hepatic gene expression that involves many genes. Furthermore, it is important to note that the expression or repression of the genes examined may result from direct regulation by the AhR, events further downstream from a direct regulation of some gene by the AhR, and/or a response to the tissue injury resulting from the genes directly or indirectly regulated by the dioxin-like chemicals via the AhR.

PCB153 is the most prevalent PCB congener in biological tissues (Kimbrough 1995; Krogaenas et al. 1998; Safe 1994). It is also extraordinarily persistent and its half-life may exceed 100 years in marine sediments (Jonsson et al. 2003). PCB153 exposure has been associated with various biological effects including developmental toxicity (Kuchenhoff et al. 1999) and induction of *CYP2b1* and other phenobarbital responsive genes (Connor et al. 1995). While PCB153 does not bind to the *AhR* and produced minimal hepatic hypertrophy after 13 wks of subchronic exposure, PCB153 did promote differential expression of several biomarker genes for liver injury (Fig. 3). Thus, gene expression profiling may be a more sensitive gauge of PCB153 toxicity than standard histology, and this hypothesis will be tested with low dose PCB153 exposures in future experiments. PCB153 activates an acquired immune response in

mice (Smialowicz et al. 1997). PCB153 exposure in this study was associated with differential gene expression of proinflammatory cytokines, including interleukin 1, interleukin 2, and the immune response gene *MX1*. PCB153 exposure decreased expression of the apoptotic genes *BCL-2* and *Wee1*. *BCL-2* and *Wee1* are responsive to p53 and are downregulated during apoptosis (Bishay et al. 2000; Leach et al. 1998). PCB153 exposure selectively enhanced expression of cAMP response element modulator (*CREM*) protein. *CREM* gene activation is a signature response to liver regeneration after hepatocyte injury. *CREM* mRNA is increased following partial hepatectomy in wild type mice and liver regeneration is inhibited in *CREM*^(-/-) mice (Servillo et al. 1998). The identification of these and other PCB153 responsive genes in the current study provides new targets for future mechanistic studies of PCB153 toxicity.

The mutual *AhR* binding affinity of TCDD, PeCDF, and PCB126 is likely responsible for strong similarity between global gene expression profiles produced by these chemicals. However, PCA also revealed that gene expression profiles associated with PCB126 and PeCDF exposures were more closely related to each other than to TCDD. Based on this finding, it is possible that PeCDF and PCB126 activate a unique group of genes not activated during TCDD exposure.

We identified a limited subset of genes activated by PeCDF and PCB126 but not TCDD (Fig. 4). Induction of catalase, cytochrome b5, and cytochrome c oxidase oxidatative stress response genes (Poulsen et al. 2000) suggests PeCDF and PCB126 exposures are capable of inducing oxidative stress. PeCDF and PCB126 also induced *Gadd45* expression, a DNA damage-inducible gene product (Sheikh et al. 2000). Induction of *Gadd45* during PeCDF and PCB126 exposures may indicate oxidative DNA damage in liver from animals exposed to these toxicants. Oxidative stress was previously reported during *AhR* activation (Dalton et al. 2002).

Interestingly, however; oxidative stress response genes were not activated in livers from animals exposed to TCDD in the current study. It is interesting that PeCDF produced less hypertrophy than TCDD, yet was more effective towards activating the expression of oxidative stress response genes. These data may indicate that PeCDF and PCB126 exposures promote oxidative stress through a unique, *AhR*-independent mechanism and /or that the responses are secondary to liver injury produced during the subchronic exposure.

AhR activation plays a critical role in many endpoints of TCDD toxicity (ATSDR 1998; Hahn 2002; Mimura and Fujii-Kuriyama 2003; VandenBerg 1998). PCA from the current study suggests that subchronic exposure to *AhR* ligands causes differential expression of numerous genes. While a few *AhR* target genes have been identified in previous studies, many members of the *AhR* gene battery remain unknown. Genomic and proteomic approaches provide valuable opportunities to elucidate additional genes involved in *AhR* signal transduction and hepatotoxic responses to dioxin-like chemicals.

PTM revealed genes specifically induced or repressed by *AhR* ligands TCDD, PeCDF, and PCB126, but not by PCB153 (Fig. 5). Many of these genes were previously classified as being dioxin responsive, which validated the efficacy of the PTM approach and further verified RNA sample integrity. PTM also revealed several genes, including *C-CAM4*, *CAP2*, *SERPIN7A*, carboxylesterase, and ESTs not yet associated with the *AhR* signaling pathway (Table 3).

C-CAM4 represents a novel dioxin-responsive gene. In the current study, *C-CAM4* was selectively induced in rats exposed to *AhR* ligands but not in rats exposed to PCB153 and was among the genes most highly upregulated by TCDD exposure (Table 3, Fig. 5). *C-CAM4* does not contain a promoter-based DRE sequence, which suggests it may not be regulated directly by the *AhR*.

C-CAM4 is a recent addition to the carcinoembryonic antigen (CEA) family of cell adhesion molecules (Earley et al. 1996). CEA immunoglobulins demonstrate important roles in growth and differentiation. While most soluble CEA molecules are unable to mediate intercellular associations, *C-CAM4* actively promotes cell adhesion (Lin et al. 1998). *C-CAM1* is a secretory paralog of *C-CAM4*, is expressed during hepatocyte differentiation (Cheung et al. 1993; Thompson et al. 1993) and is selectively down regulated in hepatocellular carcinoma (Cheung et al. 1993; Thompson et al. 1993). TCDD has been associated with hepatocellular carcinoma in female Spartan Sprague Dawley rats (Kociba et al. 1978) and with hepatocellular adenoma and cholangiocarcinoma in the recent NTP study of female Harlan Sprague Dawley rats (National Toxicology Program 2004a). The NTP study also found evidence of other hepatotoxic responses after two 2 yrs of chronic exposure to TCDD, including: hepatocyte hypertrophy, multinucleated hepatocyte, diffuse fatty change, bile duct hyperplasia, bile duct cyst, oval cell hyperplasia, necrosis, pigmentation, inflammation, nodular hyperplasia, portal fibrosis, cholangiofibrosis, and toxic hepatopathy (National Toxicology Program 2004a). No major evidence for hepatotoxicity was observed after 13 wks of exposure to TCDD, but livers from female rats exposed for this time period were hypertrophic. The increased expression of secreted *C-CAM4* in these rats may suggest that TCDD initiates a cellular transition from membrane-bound *C-CAM1* expression in normal tissue to secreted *C-CAM4* expression during hypertrophy and neoplastic transformation. While *C-CAM4* may be a potential marker for disrupted cell differentiation in livers of animals exposed to dioxin-like toxicants, it will be important in future studies to investigate protein expression in conjunction with gene expression

Like *C-CAM4*, Adenylate cyclase-associated protein 2 (*CAP2*) is a novel dioxin-responsive gene that exhibits dynamic activation in the presence of *AhR* ligands (Table 3, Fig. 5).

The *CAP2* promoter is also devoid of XRE sequences. *CAP2* mRNA is expressed at moderate to low levels in normal rat liver tissue (Swiston et al. 1995), but is markedly induced by TCDD and related compounds. It is unclear whether *CAP2* activity is implicated in the preneoplastic hepatotoxic effects of dioxin in rats, but a yeast homolog of *CAP2* associates with the actin cytoskeleton (Lila and Drubin 1997) and is responsible for the post-translational processing of Ras and serves as an effector for Ras-dependent activation of adenylyl cyclase (Shima et al. 1997). Ras expression was increased in altered hepatic foci from a diethyl nitrosamine/TCDD tumor initiation and promotion study (Sills et al. 1994). A Ras-related mechanism suppressed *CYP1A1* expression, potentially serving as a negative feedback mechanism that rectifies *CYP1A1* levels in the presence of TCDD (Reiners et al. 1997). Although the *AhR* pathway may indeed cross talk with the adenylyl cyclase pathway, the specific details of these interactions are unclear. *CAP2* may play an important role in this signaling cross talk and warrants further characterization. As with *CAM4*, future studies will investigate protein expression in conjunction with the expression of this gene.

This study represents one of the first attempts to characterize hepatic gene expression in the context of subchronic exposure to carcinogenic doses of dioxin-like chemicals. DNA microarray and/or RT-PCR successfully identified novel dioxin responsive genes that were either up- or down-regulated following exposure to *AhR* ligands (TCDD, PeCDF, PCB126), but not following exposure to the non-*AhR* ligand, PCB153. Future studies are needed to assess the species- and tissue-specific expression of these genes and their respective functional proteins to establish whether these differentially expressed genes may be a response to and/or lead to the carcinogenic and/or non-carcinogenic effects of these compounds in humans and laboratory animals. Together, these findings may help to elucidate some of the fundamental features of

dioxin toxicity and may further clarify the biological role of the enigmatic *AhR* signaling pathway.

REFERENCES

ATSDR. 1998. Toxicological Profile for Chlorinated Dibenzo-p-dioxins (Update). Agency for Toxic Substances and Disease Registry, U.S. Department of Health and Human Services.

Becher H, Steindorf K, Flesch-Janys D. 1998. Quantitative cancer risk assessment for dioxins using an occupational cohort. *Environ Health Perspect.* 106(Suppl 2): 663-670.

Bishay K, Ory K, Lebeau J, Levalois C, Olivier M-F, Chevillard S. 2000. DNA damage-related gene expression as biomarkers to assess cellular response after gamma irradiation of a human lymphoblastoid cell line. *Oncogene* 19:916-923.

Bunger MK, Moran SM, Glover E, Thomae TL, Lahvis GP, Lin BC et al. 2003. Resistance to 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity and abnormal liver development in mice carrying a mutation in the nuclear localization sequence of the aryl hydrocarbon receptor. *J Biol Chem* 278:17767-17774.

Cheung PH, Thompson NL, Earley K, Culic O, Hixon D, Lin S-H. 1993. Cell-CAM105 isoforms with different adhesion functions are coexpressed in adult rat tissues and during liver development. *J Biol Chem* 268:6139-6146.

Connor K, Safe S, Jefcoate CR, Larsen M. 1995. Structure-dependent induction of CYP2B by polychlorinated biphenyl congeners in female Sprague-Dawley rats. *Biochem Pharmacol* 50: 1913-1920.

Dalton TP, Puga A, Shertzer HG. 2002. Induction of cellular oxidative stress by aryl hydrocarbon receptor activation. *Chem-Biol Interact* 141:77-95.

Denison MS, Pandini A, Nagy SR, Baldwin EP, Bonati L. 2002. Ligand binding and activation of the Ah receptor. *Chem Biol Interact* 141:3-24.

Earley K, Weiping L, Yuhong Q, Thompson NL, Chou J, Hixson DC, et al. 1996. Identification of a new isoform of cell-cell adhesion molecule 105 (C-CAM), *C-CAM4*: a secretory protein with only one Ig domain. *Biochem J* 315:799-806.

Fingerhut MA, Halperin WE, Marlow DA, Piacitelli LA, Honchar PA, Sweeney MH, et al. 1991. Cancer mortality in workers exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *N Engl J Med* 324:212-218.

Finley BL, Connor KT, Scott PK. 2003. The use of toxic equivalency factor distributions in probabilistic risk assessments for dioxins, furans, and PCBs. *J Toxicol Environ Health* 66:533-550.

Fisher MT, Nagarkatti M, Nagarkatti PS. 2004. Combined screening of thymocytes using apoptosis-specific cDNA array and promoter analysis yields novel gene targets mediating TCDD-induced toxicity. *Toxicol Sci* 78:116-124.

- Flesch-Janys D, Steindorf K, Gurn P, Becher H. 1998. Estimation of the cumulated exposure to polychlorinated dibenzo-p-dioxins/furans and standardized mortality ratio analysis of cancer mortality by dose in an occupationally exposed cohort. *Environ Health Perspect* 106(Suppl 2):655-662.
- Frueh FW, Hayashibara KC, Brown PO, Whitlock JP Jr. 2001. Use of cDNA microarrays to analyze dioxin-induced changes in human liver gene expression. *Toxicol Lett* 122:189-203.
- Gu YZ, Hogenesch JB, Bradfield CA. 2000. The PAS superfamily: sensors of environmental and developmental signals. *Annu Rev Pharmacol Toxicol* 40: 519-561.
- Hahn ME. 2002. Aryl hydrocarbon receptors: diversity and evolution. *Chem Biol Interact* 141:131-160.
- Hassoun EA, Al-Ghafri M, Abushaban A. 2003. The role of antioxidant enzymes in TCDD-induced oxidative stress in various brain regions of rats after subchronic exposure. *Free Radic Biol Med* 35:1028-1036.
- Heijne WH, Stierum RH, Slijper M, van Bladeren, PJ, van Ommen B. 2003. Toxicogenomics of bromobenzene hepatotoxicity: a combined transcriptomics and proteomics approach. *Biochem Pharmacol* 65:857-875.
- IARC. 1997. Polychlorinated dibenzo-para-dioxins and polychlorinated dibenzofurans, Monogr Eval Carcinog Risks Hum 69:33-343.
- Ihaka R, Gentleman R. 1996. R: A Language for Data Analysis and Graphics. *J Comput Graph Stat* 5:299-314.
- Imasato AC, Han J, Kai H, Cato ACB, Akira S, Li J-D. 2002. Inhibition of p38 MAPK by Glucocorticoids via Induction of MAPK Phosphatase-1 Enhances Nontypeable Haemophilus influenzae-induced Expression of Toll-like Receptor 2. *J Biol Chem* 277:47444-47450.
- Jonsson A, Gustafsson O, Axelman J, Sundberg H. 2003. Global accounting of PCBs in the continental shelf sediments. *Environ Sci Technol* 37:245-255.
- Kimbrough R. 1995. Polychlorinated biphenyls (PCBs) and human health: an update. *Crit Rev Toxicol* 25:133-163.
- Kociba RJ, Keeler PA, Park CN, Gehring PJ. 1976. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD): Results of a 13-week Oral Toxicity Study. *Toxicol Appl Pharmacol* 35:553-574.
- Kociba RJ, Keyes DG, Beyer JE, Barreon RM, Wade CE, Dittener DA, et al. 1978. Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-Tetrachlorodibenzo-p-dioxin in rats. *Toxicol Appl Pharmacol* 46:279-303.

Krogaenas AK, Nafstad I, Skare JU, Farstad W, Hafne AL. 1998. In vitro reproductive toxicity of polychlorinated biphenyls congeners 153 and 126. *Reprod Toxicol* 12:575-580.

Kuchenhoff A, Eckard R., Buff K, Fischer B. 1999. Stage-specific effects of defined mixtures of polychlorinated biphenyls on in vitro development of rabbit preimplantation embryos. *Mol Reprod Develop* 54:126-134.

Leach SD, Scatena CD, Keefer CJ, Goodman HA, Song SY, Yang L, et al. 1998. Negative regulation of Wee1 expression and Cdc2 phosphorylation during p53-mediated growth arrest and apoptosis. *Cancer Res* 58:3231-3236.

Lila T, Drubin DG. 1997. Evidence for physical and functional interactions among two *Saccharomyces cerevisiae* SH3 domain proteins, an adenylyl cyclase-associated protein and the actin cytoskeleton. *Mol Biol Cell* 8:367-385.

Lin SH, Chen G, Earley K, Luo W, Chou J. 1998. Demonstration of adhesion activity of the soluble Ig-domain protein *C-CAM4* by attachment to the plasma membrane. *Biochem Biophys Res Comm* 245:472-477.

Manjunath GS, Dufresne MJ. 1988. Evidence that 2,3,7,8-tetrachlorodibenzo-p-dioxin induces NADPH cytochrome c (P-450) reductase in rat hepatoma cells in culture. *Cell Biol Int Rep* 12:41-51.

Martinez JM, Afshari CA, Bushel PR, Masuda A, Takahashi T, Walker NJ. 2002. Differential toxicogenomic responses to 2,3,7,8-tetrachlorodibenzo-p-dioxin in malignant and nonmalignant human airway epithelial cells. *Toxicol Sci* 69:409-423.

McGregor DB, Partensky C, Wilbourn J, Rice JM. 1998. An IARC evaluation of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans as risk factors in human carcinogenesis. *Environ Health Perspect* 106(Suppl 2):755-760.

Mimura J, Fujii-Kuriyama Y. 2003. Functional role of *AhR* in the expression of toxic effects by TCDD. *Biochimica Biophysica Acta* 1619:263-268.

National Toxicology Program. 2004a. Toxicology and Carcinogenesis Studies of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (CAS No. 1746-01-6) in Female Harlan Sprague-Dawley Rats (Gavage Studies). Technical Report 521 (Draft). Research Triangle Park, NC: National Toxicology Program.

National Toxicology Program 2004b. Toxicology and Carcinogenesis Studies of 2,3,4,7,8-Pentachlorodibenzofuran (PeCDF) (CAS No. 57117-31-4) in Female Harlan Sprague-Dawley Rats (Gavage Studies). Technical Report 525 (Draft). Research Triangle Park, NC: National Toxicology Program.

National Toxicology Program 2004c. Toxicology and Carcinogenesis Studies of 3,3',4,4',5-Pentachlorobiphenyl (PCB 126) (CAS No. 57465-28-8) in Female Harlan Sprague-Dawley Rats

(Gavage Studies). Technical Report 520 (Draft). Research Triangle Park, NC: National Toxicology Program.

Pavlidis P, Noble WS. 2001. Analysis of strain and regional variation in gene expression in mouse brain. *Genome Biol* 2:0042.1-0042.15.

Pitt JA, Buckalew AR, House DE, Abbott BD. 2000. Adrenocorticotropin (ACTH) and corticosterone secretion by perfused pituitary and adrenal glands from rodents exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Toxicology* 151:25-35.

Poulsen HE, Jensen BR, Weimann A, Jensen SA, Sorensen M, Loft S. 2000. Antioxidants, DNA damage and gene expression. *Free Radical Res* 33(Suppl):S33-39.

Puga A, Maier A, Medvedovic M. 2000. The transcriptional signature of dioxin in human hepatoma HepG2 cells. *Biochem Pharmacol* 60:1129-1142.

Quant K, Frech K, Karas H, Wingender E, Werner T. 1995. MatInd and MatInspector- new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 23:4878-4884.

Raychaudhuri S, Stuart JM, Altman RB. 2000. Principal components analysis to summarize microarray experiments: application to sporulation time series. *Pac Symp Biocomput* 455-466.

Reiners JJ, Jones CL, Hong N, Clift RE, Elferink C. 1997. Downregulation of aryl hydrocarbon receptor function and cytochrome P450 1A1 induction by expression of Ha-ras oncogenes. *Mol. Carcinogenesis* 19:91-100.

Ringberg A, Anagnostaki L, Anderson H, Idvall I, Ferno M. 2001. South Sweden Breast Cancer Group. Cell biological factors in ductal carcinoma in situ (DCIS) of the breast-relationship to ipsilateral local recurrence and histopathological characteristics. *Eur J Cancer* 37:1514-1522.

Rozen S, Skaletsky HJ. 2000. Primer3 on the WWW for general users and for biologist programmers In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. (Krawetz S, Misener S, eds). Totowa, NJ: Humana Press, 365-386.

Safe S. 1994. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses and implications for risk assessment. *Crit Rev Toxicol* 24:87-147.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N et al. 2003. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 34:374-378.

Schrenk D. 1998. Impact of dioxin-type induction of drug-metabolizing enzymes on the metabolism of endo- and xenobiotics. *Biochem Pharmacol* 55:1155-1162.

Servillo G, Della Fazia MA, Sassone-Corsi P. 1998. Transcription factor CREM coordinates the timing of hepatocyte proliferation in the regenerating liver. *Genes Dev* 12:3639-3643.

Shi LM, Fan Y, Lee JK, Waltham M, Andrews DT, Scherf U, et al. 2000. Mining and visualizing large anticancer drug discovery databases. *J Chem Info Comp Sci* 40:367-379.

Sheikh MS, Hollander MC, Fornance AJ Jr. 2000. Role of Gadd45 in apoptosis. *Biochem Pharmacol* 59:43-45.

Shima F, Yamawaki-Kataoka Y, Yanagihara C, Tamada M, Okada T, Kariya K, et al. 1997. Effect of association with adenylyl cyclase-associated protein on the interaction of yeast adenylyl cyclase with Ras protein. *Mol Cell Biol* 17:1057-1064.

Shridhar S, Farley A, Reid RL, Foster WG, Van Vugt DA. 2001. The effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on corticotrophin-releasing hormone, arginine vasopressin, and pro-opiomelanocortin mRNA levels in the hypothalamus of the cynomolgus monkey. *Toxicol Sci* 2:181-188.

Shubert DE, Chi LH, Manns DC, Olson JR. 2002. 17 β -estradiol metabolism and EROD activity in rat liver and lung as biomarkers of chronic exposure to TCDD and related compounds [Abstract]. *Toxicol Sci* 66(1-s):830.

Sills RC, Goldsworthy TL, Sleight SD. 1994. Tumor-promoting effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin and phenobarbital in initiated weanling Sprague-Dawley rats: a quantitative, phenotypic, and ras p21 protein study. *Toxicol Pathol* 22:270-281.

Smialowicz RJ, DeVito MJ, Riddle MM, Williams WC, Birnbaum LS. 1997. Opposite effects of 2,2',4,4',5,5'-hexachlorobiphenyl and 2,3,7,8-tetrachlorodibenzo-p-dioxin on the antibody response to sheep erythrocytes in mice. *Fund Appl Toxicol* 37:141-149.

Sutter TR, Greenlee WF. 1992. Classification of members of the Ah gene battery. *Chemosphere* 25:223-226.

Swiston J, Hubberstey A, Yu G, Young, D. 1995. Differential expression of CAP and *CAP2* in adult rat tissues. *Gene* 165:273-277.

Thompson NL, Panzica MA, Hull G, Lin S-H, Curran TR, Ruppuso PA, et al. 1993. Spatiotemporal expression of two cell-cell adhesion molecule 105 isoforms during liver development. *Cell Growth Differ* 4:257-268.

Tsai J, Sultana R, Lee Y, Perteau G, Karamycheva S, Antonescu V, et al. 2001. Resourcerer: a database for annotating and linking microarray resources within and across species. *Genome Biol* 2:0002.1-0002.4.

Yeboor VK, Patti M-E, Saccone R, Kahn CR. 2002. Coordinated patterns of gene expression for substrate and energy metabolism in skeletal muscle of diabetic mice. *Proc Natl Acad Sci USA* 99:10587-10592.

United States Environmental Protection Agency. 2000. Draft Exposure and Human Health Reassessment of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) and Related Compounds. Washington, DC: US Environmental Protection Agency Office of Research and Development.

Van den Berg, M., de Jongh, J., Poiger, H., and Olson, J.R. 1994. The Toxicokinetics and Metabolism of Polychlorinated Dibenzop-dioxins (PCDDs) and Polychlorinated Dibenzofurans (PCDFs) and their relevance for Toxicity. *CRC Critical Reviews in Toxicology* 24:1-74.

Van den Berg M, Birnbaum L, Bosveld ATC, Brunstrom B, Cook P, Feeley M, et al. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ Health Perspect* 106:775-791.

Whitlock JP Jr. 1999. Induction of Cytochrome P4501A1. *Annu Rev Pharmacol Toxicol* 39:103-125.

TABLES

Table 1. Primer sequences for real-time RT-PCR.

GenBank Ac#	Gene	Primer Sequences (5' to 3')
U09540	Cytochrome P450 1B1	CGTCTGATGCTTTCAGCAAAGG GCAGGCTTTCCTCAACTAAGCCAG
U23055	C-CAM4 protein	CTCGTCTCCTCAGAGGGCAGATTC ACAGCGTCTACGGTGACTTGGG
A1145367	Adenylate cyclase-associated protein 2	ATCACCGTCGATAACTGCAAG CCCATTACCTGGATCTGAATG
U46118	Cytochrome P450 3A9	CCACCAGCATGAAAGACATC GTCCTGTGGGTTGTTAAGGG
M63991	Serine proteinase inhibitor, clade A	TCTGGCTCTAGCACCCAAAC GATCAAATGCTGGAAGCCC
M25890	Somatostatin	CAGAACTGCTGTCTGAGCCC AGTCCAGCCTCATCTCGTC
X65296	Carboxylesterase 3	GGCCATTTCTGAGAGTGGTGTG GCAGGCAATGAACCATAACAGC
V01270	Ribosomal 18s RNA	GAGCGAAAGCATTTGCCAAG GGCATCGTTTATGGTCGGAA

Table 2. Summary of the 13 Week Dosimetry and Liver Pathology Data from the NTP Cancer Bioassay.

	TCDD		PeCDF		PCB126		PCB153	
	Control	100 ng/kg/day	Control	200 ng/kg/day	Control	1000 ng/kg/day	Control	1000 µg/kg/day
Liver levels (ng/g)	BLOQ	18.3±0.8	BLOQ	132.9±47.4	BLOQ	412.6±73.5	BLOQ	9250±2061
Liver-hypertrophy	1 (1.0)	10*(2.3)	0	7 *(1.0)	0	10*(1.7)	0	9* (1.2)
Multinucleated hepatocytes	0	3 (1.0)	0	0	0	0	0	0
Diffuse fatty change	0	2 (1.0)	0	2 (1.0)	0	0	0	0
Pigmentation	0	0	0	2 (1.0)	0	0	0	0

Dosimetry values are the mean ± SD of 10 rats per group. Values for each pathological endpoint represent the incidence or number of rats (out of 10) which exhibit the response. The mean severity score is given in parenthesis (1=minimal, 2=mild, 3= moderate, 4=marked).

BLOQ=Below Level of Quantification. N/A=Not Applicable.

*Denotes a statistically significant (p<0.05) increase in the incidence of a given pathological response relative to the respective control group.

Table 3. Differential gene expression in liver from rats exposed subchronically (13 weeks) to halogenated aromatic hydrocarbons. The average change in hepatic mRNA for TCDD, PeCDF, PCB126, or PCB153 exposures compared to vehicle exposure was determined by real-time RT-PCR in order to validate microarray results. Each value represents the average (\pm 1 S.D.) of three independent RNA pools (RT-PCR) or three independent GeneChips (microarray). Real-time RT-PCR values were normalized to 18s rRNA as described in Materials and Methods.

		TCDD	PeCDF	PCB126	PCB153
Cytochrome P450 1B1	RT-PCR	256 (236.3 to 277.3)	59.7 (48.3 to 73.8)	106.4 (98.2 to 115.3)	-1.2 (-1.6 to 1)
	Microarray	676.3 (470.8 to 881.8)	493.3 (310.1 to 676.5)	317.1 (41.1 to 593.1)	2.1 (1 to 3.2)
C-cam4 protein	RT-PCR	4.1 (3.8 to 4.4)	2.5 (2.3 to 2.7)	3.5 (2.6 to 4.7)	-1.1 (-1.3 to 1)
	Microarray	133.4 (112.1 to 154.7)	37.4 (25.5 to 49.3)	25.1 (4 to 46.2)	1.1 (-0.7 to 1.5)
Adenylate cyclase-associated protein 2	RT-PCR	168.9 (147.9 to 192.9)	20.2 (17.8 to 22.9)	119.4 (104 to 137.2)	-1.3 (-1.5 to -1.1)
	Microarray	53.6 (31.1 to 76.1)	10.9 (4 to 17.8)	7.6 (2 to 13.2)	1.1 (-0.8 to 1.4)
Cytochrome P450 3A9	RT-PCR	-1024 (-1201.8 to -872.5)	-4.2 (-5 to -3.5)	-28.5 (-30.2 to -26.9)	-1.8 (-2.1 to -1.5)
	Microarray	-35.4 (-53.4 to -17.4)	-2.3 (-3.3 to -1.3)	-4.5 (-5.8 to -2.2)	1.6 (0.8 to 2.4)
Serine proteinase inhibitor, clade A	RT-PCR	-26.6 (-30.2 to -23.4)	-29.9 (-32 to -27.9)	-8.4 (-12 to -5.9)	-1.2 (-1.5 to 1)
	Microarray	-11.5 (-17.2 to -5.8)	-2.7 (-3.6 to -1.8)	-4 (-4.9 to -3.1)	1.6 (1.3 to 1.9)
Somatostatin	RT-PCR	-36.8 (-46.4 to -29.1)	-1.6 (-2 to -1.2)	-1.5 (-1.6 to -1.4)	-3 (-3.5 to -2.5)
	Microarray	-8 (-10.5 to -5.5)	-8.3 (-11.1 to -5.5)	-6.4 (-9.4 to -3.4)	-9.5 (-10.1 to -8.9)
Carboxylesterase 3	RT-PCR	-4.8 (-5.2 to -4.4)	-2.8 (-2.5 to -3.1)	-1.4 (-1.5 to -1.3)	2.4 (2.1 to 2.6)
	Microarray	-8.1 (-9.9 to -6.3)	-2.8 (-3.8 to -1.8)	-4 (-4.4 to -3.6)	1.3 (1 to 1.6)

FIGURE LEGENDS

Figure 1. Hepatic genes differentially expressed during TCDD and PCB126 exposures but not during PeCDF exposure. PTM was utilized to identify genes co-expressed after exposure to TCDD and PCB126 but not PeCDF for 13 wks. Matching genes conformed to a template where the relative expression ratios for toxicant versus vehicle exposure were PeCDF = 0.2, TCDD = 1, and PCB126 = 1 ($r^2=0.9$). The PTM output was further refined to include only those genes differentially expressed ≥ 2 -fold in livers of rats exposed to TCDD and PCB126 compared to vehicle control animals. (Left) PTM diagram showing co-expressed genes. (Right) Average fold-changes for $n = 3$ Affymetrix GeneChips (each chip represents pooled RNA from two animals). The color key indicates the magnitude of change.

Figure 2. Relationship between global hepatic gene expressions resulting from TCDD, PeCDF, PCB126, or PCB153 exposures. The global hepatic gene expression profiles for rats exposed to each toxicant were analyzed by principal components analysis. The principal components for the gene expression of rats exposed to *AhR* ligands TCDD, PeCDF, and PCB126 were localized near a single PCA quadrant, while the principle component for the non-*AhR* ligand PCB153 was located in a distant quadrant.

Figure 3. Hepatic genes differentially expressed during PCB153 exposure. PTM was employed to identify differentially expressed genes in livers of rats exposed to PCB153 compared to rats exposed to *AhR* ligands. Matching genes conformed to a template where the relative expression ratios for toxicant versus vehicle exposure were TCDD = 0, PeCDF = 0, PCB126 = 0, and PCB153 = 1 ($r^2=0.9$). The PTM output was further refined to include only those genes differentially expressed ≥ 2 -fold in livers of rats exposed to PCB153 compared to vehicle control animals. (Left) PTM diagram showing genes differentially expressed during PCB153 exposure. (Right) Average fold-changes for $n = 3$ Affymetrix GeneChips (each chip represents pooled RNA from two animals). The color key indicates the magnitude of change.

Figure 4. Hepatic genes activated or repressed during PeCDF and PCB126 exposure but not during TCDD exposure. PTM was utilized to identify genes co-expressed after exposure to PeCDF and PCB126 but not TCDD for 13 wks. Matching genes conformed to a template where the relative expression ratios for toxicant versus vehicle exposure were TCDD = 0.2, PeCDF = 1, and PCB126 = 1 ($r^2=0.9$). The PTM output was further refined to include only those genes differentially expressed ≥ 2 -fold in livers of rats exposed to PeCDF and PCB126 compared to vehicle control animals. (Left) PTM diagram showing co-expressed genes. (Right) Average fold-changes for $n = 3$ Affymetrix GeneChips (each chip represents pooled RNA from two animals). The color key indicates the magnitude of change.

Figure 5. Identification of novel dioxin responsive genes. Gene expression data were clustered by Pavlidis Template Matching (PTM) to identify co-expressed genes in livers of rats exposed to *AhR*-ligands TCDD, PeCDF, and PCB126 for 13 weeks. Matching genes conformed to a template where the relative expression ratios for toxicant versus vehicle exposure were TCDD = 0.8, PeCDF = 0.8, PCB126 = 0.8, and PCB153 = 0.1 ($R^2 = 0.9$). Each row represents a separate gene, each column specifies the toxicant treatment ($n = 3$ replicate arrays per toxicant). The PTM output was further refined to include only genes differentially expressed ≥ 2 -fold in livers of

rats exposed to PeCDF and PCB126 compared to sham operated control animals. The color key indicates the magnitude of change. Italicized genes were previously shown to respond to dioxin.

FIGURES

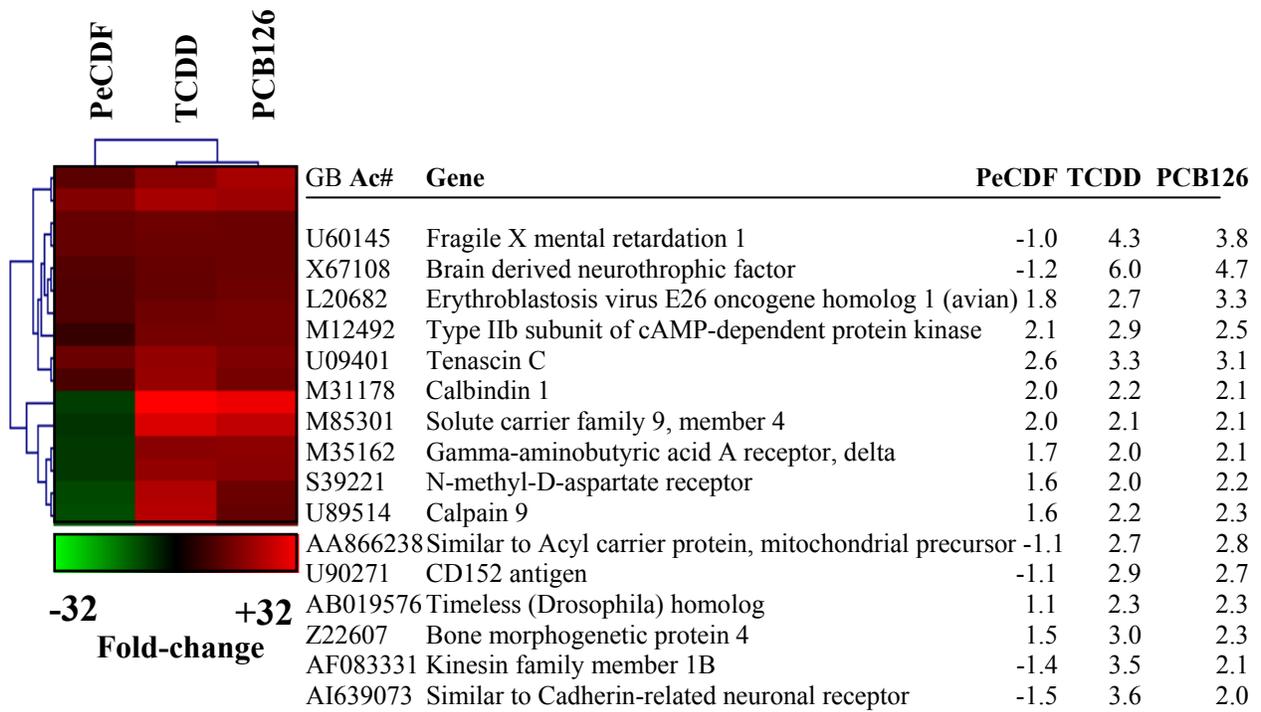


Figure 1.

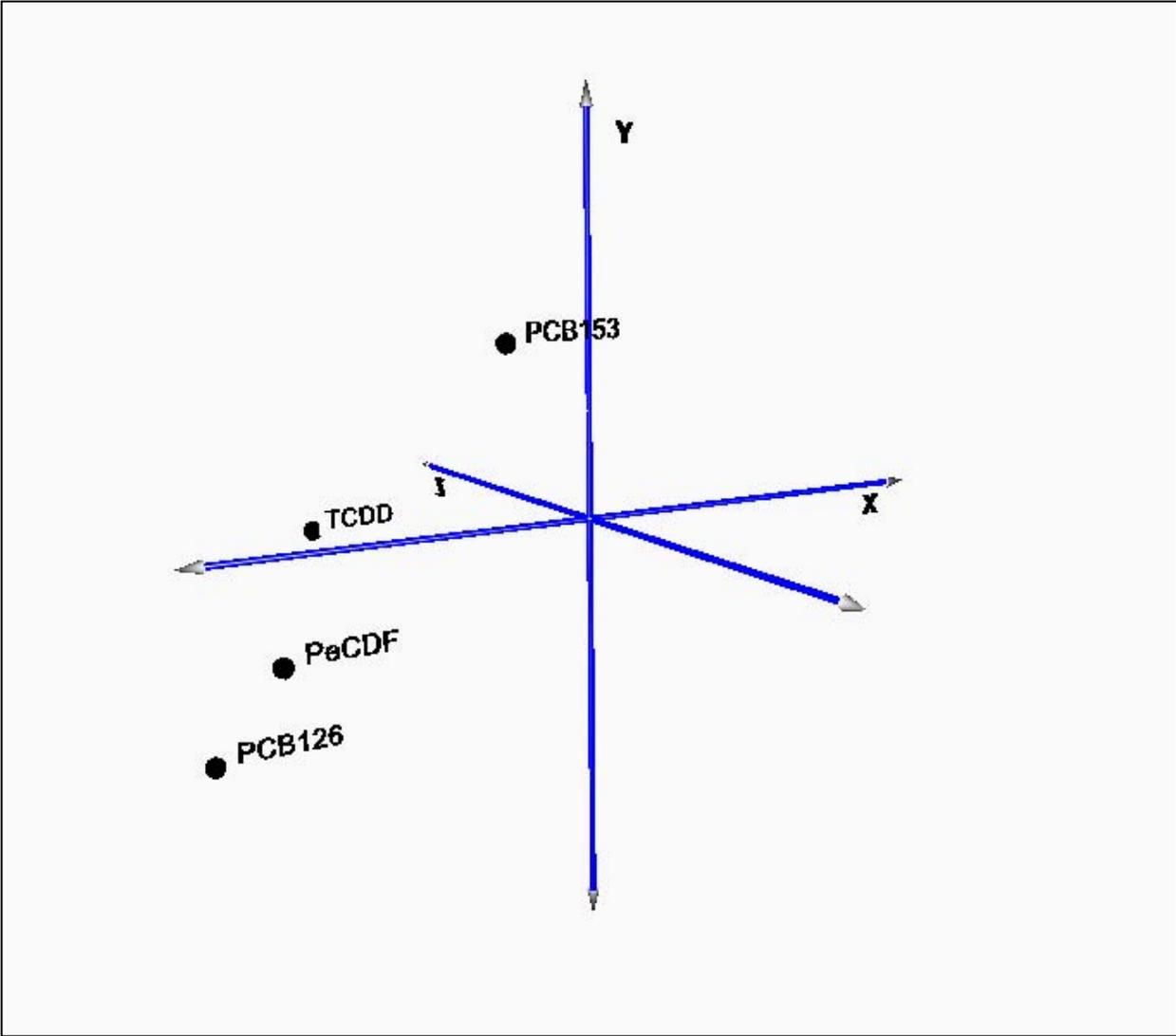


Figure 2.

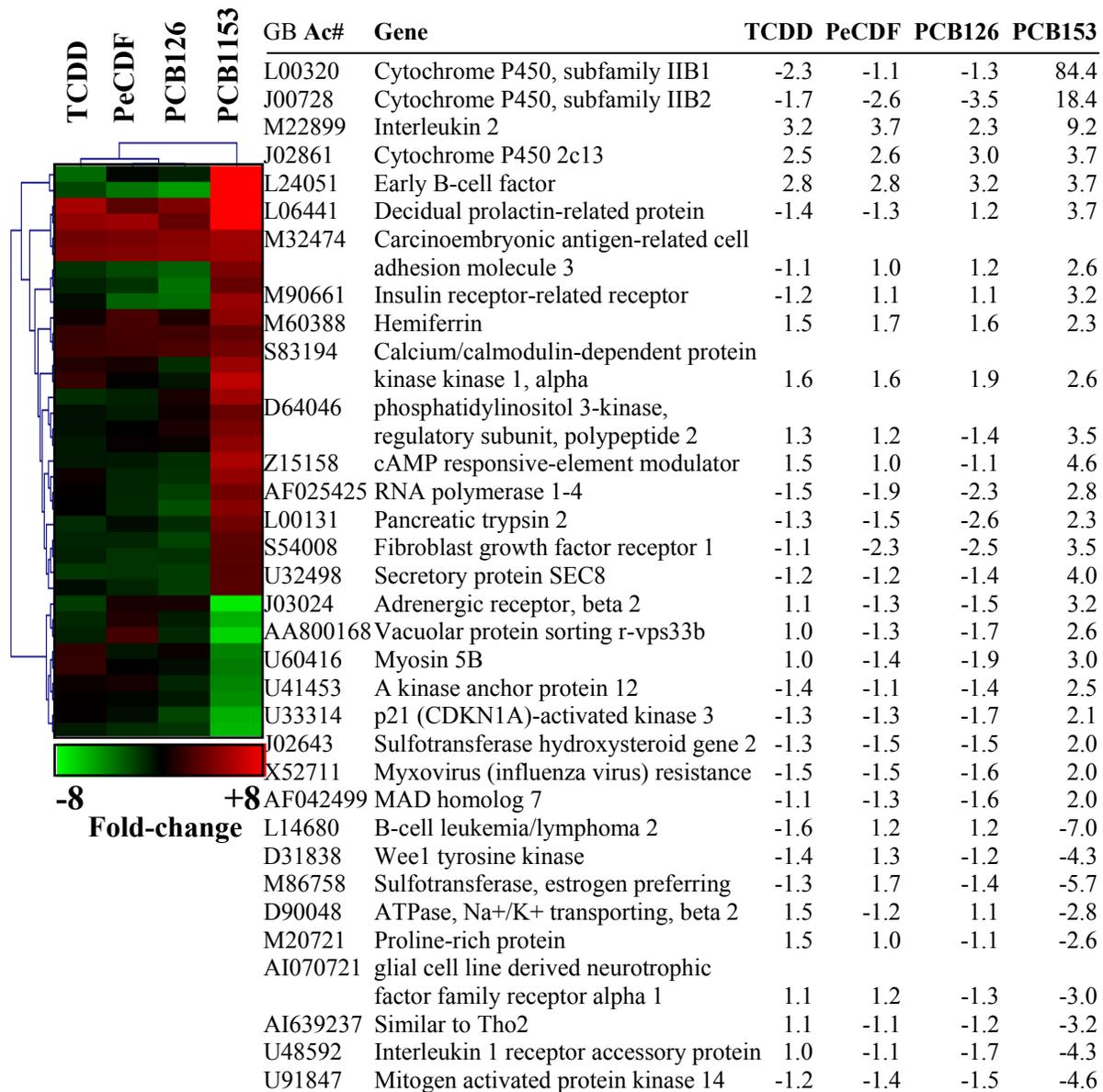


Figure 3.

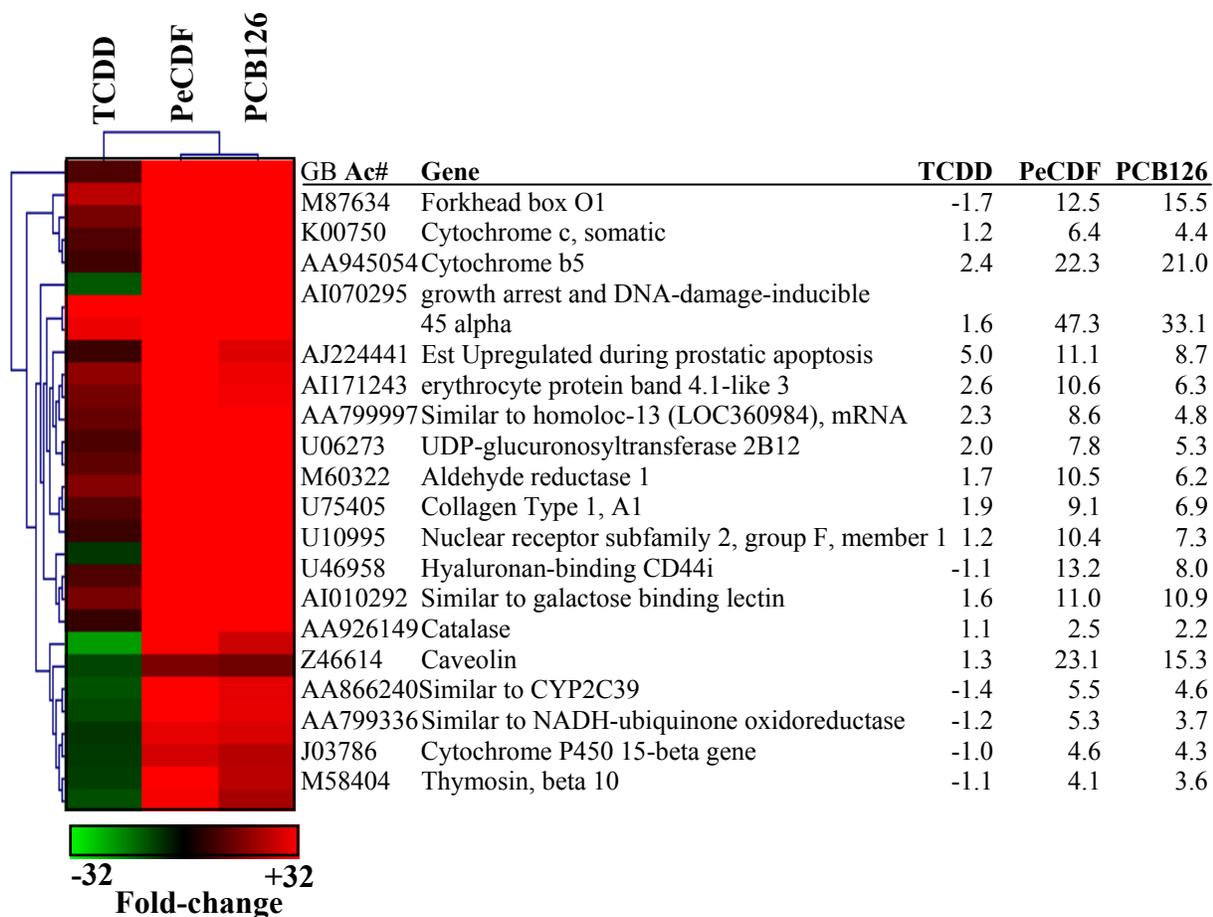


Figure 4.

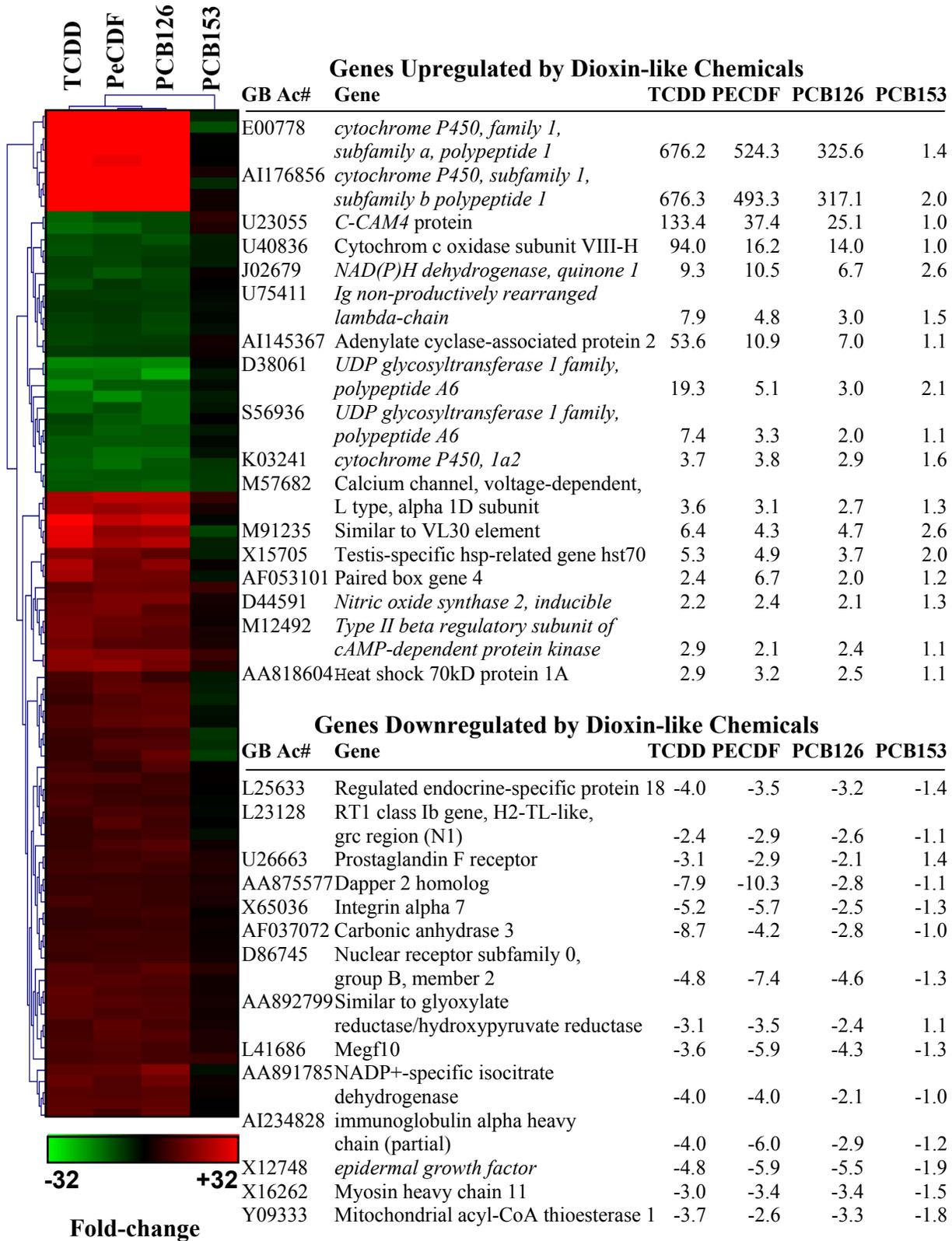


Figure 5.