Toxicology and Applied Pharmacology 260 (2012) 135–145

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Inter-strain heterogeneity in rat hepatic transcriptomic responses to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)

Cindy Q. Yao [a], Stephenie D. Prokopec [a], John D. Watson [a], Renee Pang [a], Christine P'ng [a], Lauren C. Chong [a], Nicholas J. Harding [a], Raimo Pohjanvirta [c,d], Allan B. Okey [e], Paul C. Boutros [a,b,*]

[a] *Informatics and Biocomputing Platform, Ontario Institute for Cancer Research, Toronto, Canada*
[b] *Department of Medical Biophysics, University of Toronto, Toronto, Canada*
[c] *Laboratory of Toxicology, National Institute for Health and Welfare, Kuopio, Finland*
[d] *Department of Food Hygiene and Environmental Health, University of Helsinki, Finland*
[e] *Department of Pharmacology and Toxicology, University of Toronto, Toronto, Canada*

ARTICLE INFO

Article history:
Received 8 November 2011
Revised 30 January 2012
Accepted 1 February 2012
Available online 9 February 2012

Keywords:
TCDD
Dioxin
Aryl hydrocarbon receptor
mRNA abundance
Time-course study
Microarray
Real-time RT-PCR

ABSTRACT

The biochemical and toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have been the subject of intense study for decades. It is now clear that essentially all TCDD-induced toxicities are mediated by DNA–protein interactions involving the Aryl Hydrocarbon Receptor (AHR). Nevertheless, it remains unknown which AHR target genes cause TCDD toxicities. Several groups, including our own, have developed rodent model systems to probe these questions. mRNA expression profiling of these model systems has revealed significant inter-species heterogeneity in rodent hepatic responses to TCDD. It has remained unclear if this variability also exists within a species, amongst rodent strains. To resolve this question, we profiled the hepatic transcriptomic response to TCDD of diverse rat strains (L-E, H/W, F344 and Wistar rats) and two lines derived from L-E × H/W crosses, at consistent age, sex, and dosing (100 μg/kg TCDD for 19 h). Using this uniquely consistent dataset, we show that the majority of TCDD-induced alterations in mRNA abundance are strain/line-specific: only 11 genes were affected by TCDD across all strains, including well-known dioxin-responsive genes such as *Cyp1a1* and *Nqo1*. Our analysis identified two novel universally dioxin-responsive genes as well as 4 genes induced by TCDD in dioxin-sensitive rats only. These 6 genes are strong candidates to explain TCDD-related toxicities, so we validated them using 152 animals in time-course (0 to 384 h) and dose–response (0 to 3000 μg/kg) experiments. This study reveals that different rat strains exhibit dramatic transcriptional heterogeneity in their hepatic responses to TCDD and that inter-strain comparisons can help identify candidate toxicity-related genes.

Introduction

Chlorinated dioxins are a large class of environmental contaminants produced by industrial processes ranging from incineration, recycling of electronics, pesticide manufacturing and paper bleaching (Schecter et al., 2006). Dioxins cause a wide variety of toxic effects and are the subject of intense study due to concerns around widespread human exposures, particularly through the ingestion of contaminated food (Pohjanvirta and Tuomisto, 1994). While the outcomes of exposure in humans are controversial and difficult to determine, short-term dioxin toxicities in adult laboratory animals include hepatic lesions, endocrine and immune imbalances, body wasting, augmented oxidative stress, and acute lethality (reviewed in Pohjanvirta and Tuomisto, 1994).

Most studies of dioxins have focused on the most potent and toxic congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Although the mechanisms of dioxin toxicity have not been fully elucidated, several key steps common to all members of this chemical family are known. Many studies show that the toxicity of TCDD, related halogenated aromatic hydrocarbons, and polycyclic aromatic hydrocarbons (PAHs) is mediated by a ligand-activated transcription factor — the aryl hydrocarbon receptor (AHR) (Bunger et al., 2003; Okey, 2007; Walisser et al., 2004). This mechanism is sometimes referred to as the "classic action pathway". In the absence of an appropriate ligand, the AHR sits quiescent in the cytoplasm in a complex of proteins that includes heat-shock protein 90, p23 and X-associated protein 2 (Furness et al., 2007; Harper et al., 2006; Petrulis and Perdew, 2002). Ligand-binding triggers a conformational change, leading the complex to translocate to the nucleus and dissociate (Lin et al., 2007; McMillan and Bradfield, 2007). Nuclear AHR then forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Reyes et al., 1992). The AHR:ARNT complex then recognizes and binds to DNA response element called AHRE-I and AHRE-II (Aryl

* Corresponding author at: MaRS Centre, South Tower, 101 College Street, Toronto, Ontario, Canada M5G 0A3. Fax: +1 416 673 8564.
E-mail address: Paul.Boutros@oicr.on.ca (P.C. Boutros).

0041-008X
doi:10.1016/j.taap.2012.02.001

Hydrocarbon Response Element I and II) and enhances transcription of genes such as *Cyp1a1* (Boutros et al., 2008; Lusska et al., 1993; Mimura and Fujii-Kuriyama, 2003).

Several lines of evidence prove that the aryl hydrocarbon receptor (AHR) is essential for TCDD toxicity. First, when the AHR is genetically knocked out in mice, they demonstrate exceptional resistance to TCDD-induced toxicities, including acute lethality, thymic atrophy, main features of the liver lesion, teratogenicity (cleft palate and hydronephrosis), developmental toxicity to male reproductive organs, reduced plasma thyroxin levels, disrupted vitamin A homeostasis, porphyria, and immune toxicity (humoral and cell-mediated immune reactions) (Davies et al., 2008; Fernandez-Salguero et al., 1995; Lin et al., 2002; Mimura and Fujii-Kuriyama, 2003; Nishimura et al., 2005; Schmidt et al., 1996; Vorderstrasse et al., 2001). They are also refractory to transcriptional responses (Boutros et al., 2009; Tijet et al., 2006). Second, mice with mutations in the AHR that prevent nuclear translocation (Bunger et al., 2003) or binding to AHREs (Bunger et al., 2008) were non-responsive to all impacts of TCDD examined including hepatomegaly and thymic atrophy. Finally, mice hypomorphic for ARNT exhibited attenuated thymic atrophy and hepatotoxicity but unaffected *Cyp1a1* induction (Walisser et al., 2004). Taken together, these data suggest that DNA-binding of the ligand-activated AHR:ARNT complex is essential for major toxic outcomes of TCDD.

Beyond transgenic mice, several other model systems have been used to study dioxin toxicity. Of particular importance, Long-Evans (*Turku A/B*) (L-E) and Han/Wistar (*Kuopio*) (H/W) rats have been extensively exploited in mechanistic studies because of their striking differential susceptibilities to TCDD toxicity. L-E rats are sensitive to TCDD, with an LD_{50} of 10–20 μg/kg (Pohjanvirta et al., 1993). In contrast, a large deletion in the AHR transactivation domain (Pohjanvirta et al., 1998) induces remarkable resistance to TCDD ($LD_{50} > 10{,}000$ μg/kg) in H/W rats (Unkila et al., 1994). However, in spite of this mutation, H/W rats remain responsive to TCDD treatment: for example, thymic atrophy occurs in both L-E and H/W rats after TCDD-exposure (Pohjanvirta et al., 1989; Tuomisto et al., 1999; Viluksela et al., 2000). Responses that are similar in sensitive and resistant strains are termed "Type-I" responses, while those that differ, such as acute lethality, are known as "Type-II" responses (Pohjanvirta et al., 2011; Simanainen et al., 2002, 2003). These pathologic differences are also evident at the molecular level: many AHR-regulated genes such as *Cyp1a1*, *Cyp1a2*, and *Nqo1* respond equally in sensitive and resistant rats (Boutros et al., 2011; Moffat et al., 2010).

Previously, we identified transcriptional changes that are concurrent with the onset of dioxin toxicities by contrasting mRNA abundances in mice and rats treated with TCDD (Boutros et al., 2008). We found very dramatic inter-species heterogeneity, with approximately 90% of dioxin-responsive genes being species-specific. Similarly, when we compared dioxin-sensitive L-E versus dioxin-resistant H/W rats 19, 96, and 240 h following exposure to TCDD (Boutros et al., 2011; Moffat et al., 2010), we found that the vast majority of genes exhibited altered mRNA abundances in only one rat strain (Boutros et al., 2011; Moffat et al., 2010).

Here, we extend our previous studies by considering six rat strains/lines: H/W, L-E, line-A, line-C, Fischer 344 (F344) and Wistar (Wis) rats. Lines A and C are derived from F1 crosses of H/W × L-E rats (Tuomisto et al., 1999). F344 rats are moderately resistant to TCDD but their LD_{50} values vary depending on the supplier (from 164 to 340 μg TCDD/kg body weight) (Walden and Schiller, 1985). Wis rats, on the other hand, exhibit a mixed population of AHR genotypes, consisting of either $AHR^{wt/wt}$, $AHR^{wt/hw}$, or $AHR^{hw/hw}$. Wis rats' sensitivities to TCDD vary according to the genotype that they carry (Kawakami et al., 2009). All the Wis rats employed in the present study were of the homozygous wildtype *AHR* genotype and are thus more sensitive than H/W rats (see Methods). Our goals here are two-fold. First, we survey for the first time the inter-strain heterogeneity of rat transcriptomic responses to TCDD within a single consistent experiment. Second, we exploit the genetic diversity amongst these rat strains to identify genes that show Type-I and Type-II responses to TCDD. Type-I genes might regulate common dioxin-induced toxicities in both sensitive and resistant rats; Type-II genes are candidates to explain dioxin toxicities unique to sensitive rats and not observed in resistant rats. We hypothesize that the genetic "filter" imposed by inter-strain variability will facilitate identification of candidate genes for AHR-regulated toxicities.

Methods

Animal treatment. Male rats of four strains and two lines were examined: Long-Evans (L-E), Han/Wistar (*Kuopio*) (H/W), Fischer 344 (F344), Wistar (Wis), Line-A (LnA) and Line-C (LnC). Animals were either treated with 100 μg/kg TCDD or corn-oil vehicle (4 mL/kg by gavage) at the age of 11–15 weeks. The treatment dose chosen is lethal to all animals in dioxin-sensitive strains but not to any animals in dioxin-resistant strains (Fig. 1) (Pohjanvirta and Tuomisto, 1994; Tuomisto et al., 1999; Walden and Schiller, 1985). We confirmed that all Wistar animals possessed wild-type AHR by PCR analysis of liver cDNA as previously described (Pohjanvirta, 2009). The rats were housed singly in stainless steel wire-mesh cages and given access to R36 feed (Ewos, Södertälje, Sweden) and water. Animals were fed during the early light hours daily. Artificial illumination was provided in the rooms with light and dark cycles every 12 h with lights on daily at 07:00. The room temperature was maintained at 21.5 ± 1 °C and humidity at 55 ± 10%. In total, 208 animals (56 for microarray only and the remaining 152 for PCR validation) were used. Animals in the microarray experiments were euthanized 19 h after treatment with TCDD or corn oil vehicle. Animals in the time-course experiments were given either 100 μg/kg TCDD or corn-oil vehicle and their liver excised at different time intervals (from 0 to 384 h) and animals in the dose–response experiments were treated with different doses of TCDD (from from 0 to 3000 μg/kg) or corn-oil vehicle and their livers removed at 19 h post-treatment. Samples excised from time-course and dose–response experiments were used for subsequent PCR validation. Approval from the Animal Experiment Committee of the University of Kuopio and the Provincial Government of Eastern Finland was obtained for all animal study plans.

Microarray hybridization. Following euthanasia, liver tissues were excised, sliced, and snap-frozen. The tissues were later homogenized and total RNA was extracted using Qiagen RNeasy kits according to the manufacturer's instructions (Qiagen, Mississauga, Canada) as previously described (Boutros et al., 2011). The isolated RNA was assayed on Affymetrix RAT230-2 (Wis and F344; performed with six biological replicates each) or RAE230-A (L-E, H/W, LnA, and LnC; performed with four biological replicates each) arrays at The Centre for Applied Genomics at The Hospital for Sick Children (Toronto, Canada). The two platforms RAT230-2 and RAE230-A differ by the number of probe sets contained on the array. The platform RAE230-A is a subset of RAT230-2 and hence shares many of the same genes as RAT230-2. Our statistical comparisons were performed within the same platform; thus any variability is balanced and no bias is introduced. We rigorously assessed the technical quality of each array and none were excluded from subsequent data analyses. Animal handling and reporting comply with ARRIVE guidelines (Kilkenny et al., 2010).

Microarray data pre-processing and statistical analysis. Raw quantitated array data (CEL files) were loaded into the R statistical environment (v2.12.2) using the affy package (v1.28.0) of the BioConductor library (Gentleman et al., 2004). Data were screened for spatial and distributional homogeneity and none were excluded from this study. Data were pre-processed with a sequence-specific version of RMA algorithm – GCRMA – as implemented in R (gcrma package v2.22.0). Probes were remapped to Entrez Gene IDs using rat2302rnentrezgcdf (v13.0.0)

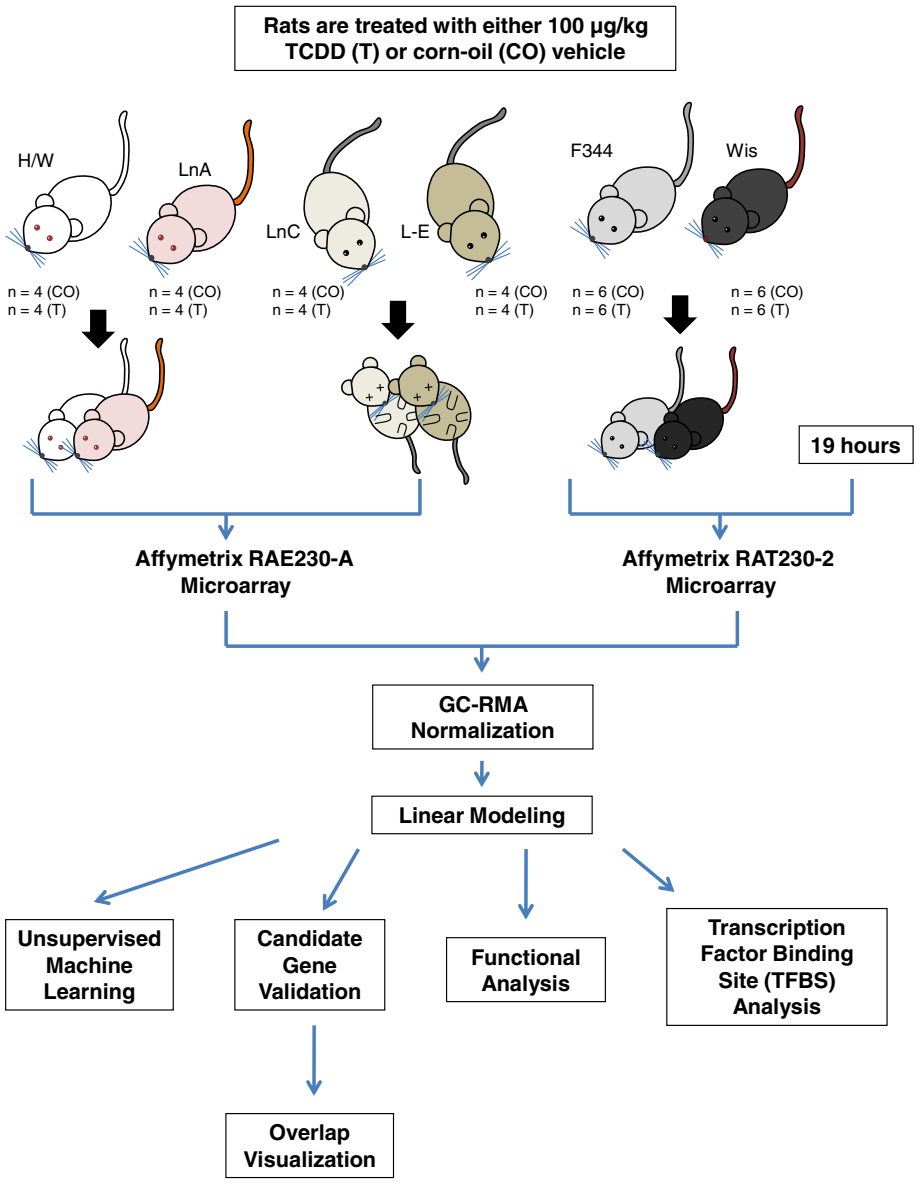

Fig. 1. Experimental design. We analyzed responses of Long-Evans (*Turku A/B*) (L-E), Han/Wistar (*Kuopio*) (H/W), Fischer344 (F344), and Wistar (Wis), Line-A (LnA), Line-C (LnC) rats. Animals were treated with either 100 µg/kg TCDD or corn-oil vehicle. After 19 h, the animals were euthanized and their livers excised and RNA extracted. Samples from F344 and Wis rats were hybridized onto Affymetrix RAT230-2 microarrays and those from L-E, H/W, LnA, and LnC rats onto Affymetrix RAE230-A microarrays. A total of 65 animals were studied. The number of animals for each strain or line is shown. The resulting data underwent a variety of statistical analyses including unsupervised machine learning, statistical modeling, functional analysis and TFBS enrichment analysis as described in Methods.

and rae230arnentrezgcdf (v13.0.0) R packages (Dai et al., 2005). Entrez Gene annotation was downloaded from NCBI on 2011-02-22. Individual strains were treated as separate cohorts and animals within a cohort were pre-processed together to avoid confounding effects from co-normalization of diverse strains. Raw and pre-processed microarray data are available in the GEO repository under accession GSE31411.

Following pre-processing, we employed general linear-modeling to identify genes affected by TCDD treatment relative to the vehicle control. The expression profiles across all animals within a cohort were determined using a per-gene linear model that assesses both basal levels and TCDD-induced effects. Coefficients were fit to terms representing each effect and the standard errors of the coefficients were adjusted using an empirical Bayes moderation of the standard error (Smyth, 2004). To test if each coefficient was statistically different from zero, we applied model-based t-tests, followed by a false-discovery rate adjustment for multiple-testing (Storey and Tibshirani, 2003). A full list of genes that are common between the two platforms and their fitted coefficients is shown in Supplementary Table 1. For most downstream analyses, genes were deemed statistically significant if the multiple-testing-adjusted probability that they were falsely-deemed altered by TCDD (i.e. α, the false-positive probability) was below that of our positive control gene, *Cyp1a1*, in any of the rat strains. Thus our effective p-value threshold was the maximum adjusted p-value observed for the well-characterized dioxin-responsive gene *Cyp1a1*. All statistical analyses were performed in the limma package (v3.6.9) for the R environment (v2.12.2).

Visualization. Unsupervised agglomerative hierarchical clustering with complete linkage was employed to visualize patterns in mRNA expression across rat strains, using Pearson's correlation as the similarity metric. The lattice and latticeExtra packages were used for visualization (v0.19–24 and v0.6–15 respectively) in the R statistical environment (v2.12.2). Venn diagrams were created using the VennDiagram R package (v1.0.0) (Chen and Boutros, 2011). We applied the hypergeometric test to assess statistical significance of gene overlaps.

Table 1
Genes for PCR validation.

A list of 6 genes was selected from this study to be carried forward into PCR validation. Here, coefficients represent the fold-change of expression in $\log_2$ scale. The genes chosen showed significant responsiveness in all four strains or only in the sensitive strains after treatment with TCDD for 19 h. Further, they all have changes in mRNA abundances that are at least 2-fold.

Gene ID	Symbol	Long-Evans rats		Han/Wistar rats		Fischer 344 rats		Wistar rats		Name
		Coefficient	p	Coefficient	p	Coefficient	p	Coefficient	p	
85332	Prkcdbp	2.05	7.36×10^{-3}	1.85	7.50×10^{-2}	1.45	9.28×10^{-4}	1.91	3.03×10^{-2}	Protein kinase C, delta binding protein
64045	Glrx1	1.71	3.43×10^{-4}	1.74	2.95×10^{-2}	1.97	1.55×10^{-4}	1.52	9.16×10^{-3}	Glutaredoxin 1
24162	Acp2	1.55	4.39×10^{-3}	1.41	6.39×10^{-2}	1.35	2.70×10^{-6}	1.06	3.85×10^{-3}	Acid phosphatase 2, lysosomal
298652	Ube4b	1.61	8.38×10^{-4}	1.13	3.32×10^{-2}	1.42	3.13×10^{-2}	1.4	8.26×10^{-3}	Ubiquitination factor E4B (UFD2 homolog, yeast)
29573	Slc37a4	−1.36	1.71×10^{-2}	−1.01	1.57×10^{-1}	−1.03	1.47×10^{-2}	−1.61	5.29×10^{-2}	Solute carrier family 37 (glucose-6-phosphate transporter), member 4
25216	Sdc1	−2.07	2.27×10^{-2}	−0.59	5.21×10^{-1}	−1.41	7.06×10^{-3}	−1.76	2.49×10^{-2}	Syndecan 1

Pathway analysis. Pathway analysis was conducted using GOMiner software (Zeeberg et al., 2003). We used build 269 of the GOMiner application, with database build 2009-09. We checked our genes of interest against a randomly drawn sample from the dataset with a false discovery rate (FDR) threshold of 0.1, 1000 randomizations, all rat databases and look-up options, all GO evidence codes and ontologies (molecular function, cellular component and biological process) and a minimum of five genes for a GO term. Separate ontological analyses were run for genes differentially expressed in each rat strain. Subsequently, RedundancyMiner (Zeeberg et al., 2011) was used to de-replicate enriched GO categories and to refine pathway analysis. A CIM file generated from GOMiner was loaded into R statistical environment (v2.13.1). Input files for RedundancyMiner were created by concatenating categories when FDR ≤ 0.20 in at least 4 strains. This relaxed p-value threshold was chosen to allow for biological variability between strains; the emphasis on at least 4 strains allowed the genetic model to form the primary filter, while allowing flexibility for biological variability and allowing for false negatives. There are two parameters used to collapse the matrix: compression and biological interpretation. Generally, more permissive p-values offer greater compression but can concatenate many of the same GO categories into different groups, thereby producing another type of redundancy. For each dataset, p-values were empirically chosen to ensure sufficient compression that GO categories with biological functions could be interpreted correctly. Based on these selection criteria, 32 GO categories were chosen. The input matrix was collapsed to obtain 20 final categories and a compression ratio of 1.60. Visualization of RedundancyMiner results was done using lattice package (0.19-31) for R (v2.13.1).

Transcription-factor binding site analysis. To determine if changes in mRNA expression levels were associated with differences in transcription factor binding sites (TFBS) (Wasserman and Sandelin, 2004), we conducted motif-recognition analysis by searching −10 kbp to +5 kbp region from our significantly altered genes against AHRE-I (core), AHRE-I (extended), AHRE-I (full), and AHRE-II, with sequences: GCGTG (Denison and Whitlock, 1995), TNGCGTG (Denison and Whitlock, 1995), [T|G]NGCGTG[A|C][G|C]A (Denison and Whitlock, 1995), and CATG{N6}C[T|A]TG (Sogawa et al., 2004), respectively. Genes were annotated with transcription start-site (TSS) using REFLINK and REFFLAT tables downloaded from the UCSC genome browser data on August 23, 2010 (Karolchik et al., 2003). Within each AHRE-motif, a PhyloHMM conservation score was calculated across different species. The scores vary from 0 to 1, with a score of 0 meaning that there is a minimal conservation and 1 meaning that there is a strong conservation. Motifs that are evolutionarily well conserved are particularly likely to be functional (Siepel and Haussler, 2004).

PCR quantitation of selected genes. Primers and probes were designed using the real-time PCR Assay Design Tool on the Integrated DNA Technologies (IDT, Coralville, IA) website (http://www.idtdna.com/Scitools/Applications/RealTimePCR). To ensure specificity, probes were compared with *Rattus norvegicus* nr/nt database using nucleotide Basic Local Alignment Search Tool (BLAST). Similarly, primer pairs were compared to the same database using Primer BLAST (http://www.ncbi.nlm.nih.gov/blast) (Altschul et al., 1990, 1997). Total RNA samples were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). In addition, for each reverse-transcribed sample, a similar preparation was made where all the reagents were included except for the reverse transcriptase. The above reactions were conducted with 1 μg of RNA as outlined in the manufacturer's instruction. PCR reactions were prepared with 5 ng of cDNA using the TaqMan Gene Expression Master Mix (Applied Biosystems) with gene-specific primers/probes (Table 1). A total of 84 biological replicates were analyzed for H/W rats and 68 replicates for L-E rats, each performed with two technical replicates in a 10 μL reaction volume. PCR reactions were run on the 7900HT Fast Real-Time PCR System (Applied Biosystems) using default settings for relative quantification calculated from comparative Ct values. qPCR results were then collected using Sequence Detection System Software v2.3 (Applied Biosystems) and the quantitated data were loaded into the R statistical environment (v2.12.2). These data were then processed and normalized as previously described (Barsyte-Lovejoy et al., 2006) using the previously validated housekeeping genes *Gapdh* and *Pgk1* (Pohjanvirta et al., 2006). Normalized data were $\log_2$-transformed and visualized across the different doses and time points tested for both L-E and H/W rats. ANOVAs were used to identify differences in expression between the strains across all time-points and doses. Student's t-tests were performed *post hoc* when $p_{ANOVA} < 0.1$.

Results

Animal models

In previous studies we examined two rat strains (L-E and H/W) and two inbred lines (LnA and LnC) derived from L-E × H/W crosses (Boutros et al., 2011; Franc et al., 2008; Moffat et al., 2010). Here we expand our search for association genes responsible for mediating dioxin-sensitivity phenotypes by including two other rat strains with wildtype AHR (and greater dioxin sensitivity than H/W rats). These strains – Fischer 344 (F344) and Wistar (Wis) – were selected because of their wide use in toxicology and pharmacology. We previously showed that mRNA abundances vary substantially between sensitive and resistant rat strains at late time-points (4 and 10 days post treatment) (Boutros et al., 2011), so we designed our current experiment to examine the effects of TCDD at a time consistent with the onset of

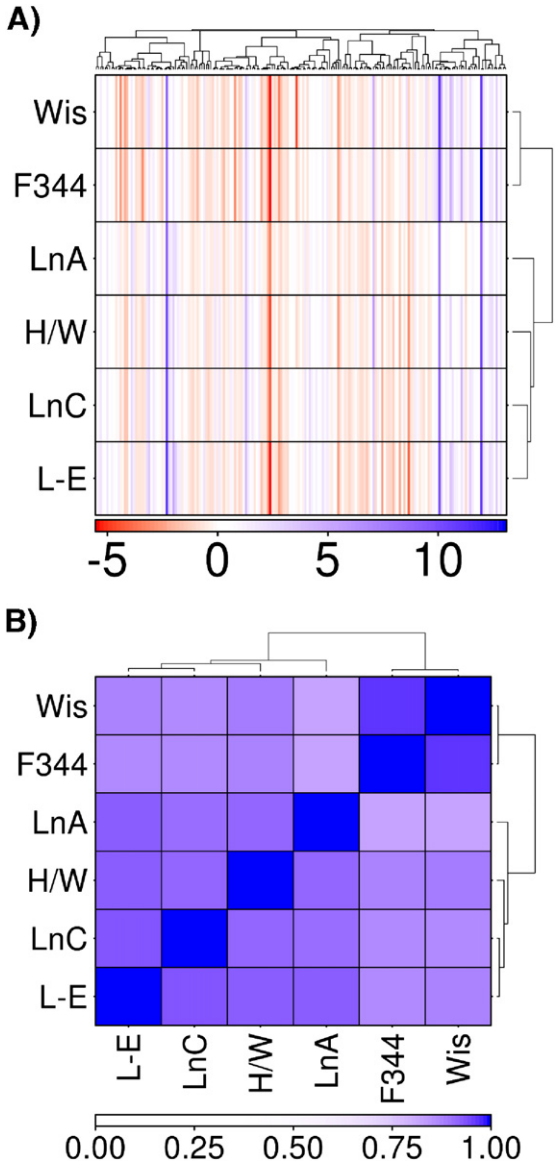

Fig. 2. Transcriptomic summary. A) We visualized the fold-changes using unsupervised machine-learning to identify innate patterns within the dataset. Each row represents a strain and each column represents a gene. The red-to-blue scale at the bottom represents the fold-changes, going from repression to induction. We applied a $p_{adjusted}$ cut-off of 0.01. Rats that possess wild-type AHR (L-E and LnC), have similar sensitivity to TCDD and cluster tightly on the heatmap while resistant rats (H/W and LnA) cluster together. B) We next looked at the inter-strain correlation of fold-changes at the same $p_{adjusted}$ threshold. Shading in the bottom panel represents the correlation (white is low correlation and blue is high). Again, we see clustering amongst rat strains that have similar sensitivities to TCDD toxicity. Further, the fold-changes between different rat strains/lines overall are highly correlated.

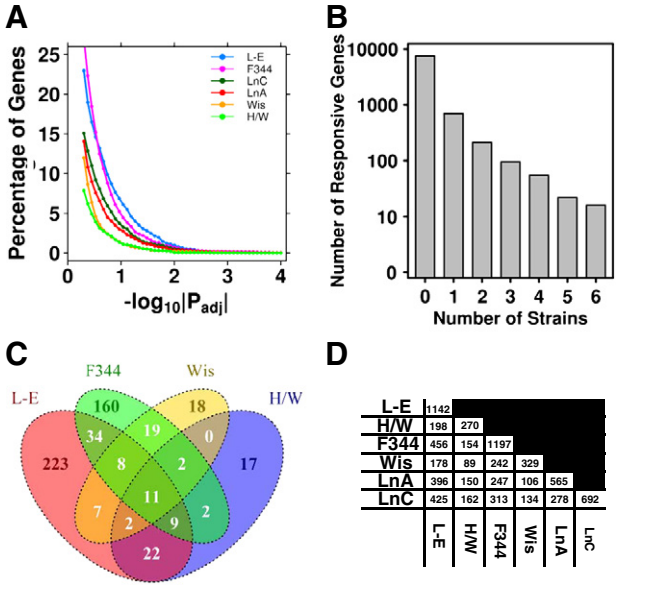

Fig. 3. Inter-strain variability. A) We first examined the percentage of genes significantly altered by TCDD in each rat strain at different p-value cut-offs. The greatest number of changes was observed in L-E rats, independent of the threshold selected. H/W rats displayed the fewest gene responses to TCDD. Other rat strains showed intermediate numbers of responses. B) We then assessed the number of responsive genes across rat strains. The number of responsive genes decreases as the number of strains increases. C) After addressing how many genes were responsive to TCDD within each strain, we determined the number of responsive genes between or across strains. We applied a $p_{adjusted}$ cut-off that equals to the maximum p-value across all strains for *Cyp1a1*, a classic TCDD-induced gene. A set of 11 genes showed significant responses across all strains. D) Outside of this core, genes vary significantly across strains in their responses to TCDD.

dioxin toxicity (19 h) and at a dose that distinguishes sensitive from resistant strains (100 μg/kg; Fig. 1).

Overall transcriptomic responses to TCDD in the strains

Following data pre-processing and linear modeling, we first evaluated our dataset using unsupervised machine-learning to identify the strongest trends within the dataset in an unbiased way (Boutros and Okey, 2005). We applied an adjusted p-value threshold of 0.01 to remove genes that showed small or no differential expression in response to TCDD. We found that rat strains clustered together according to their dioxin sensitivity (Fig. 2A). Sensitive L-E and LnC clustered tightly together on the heatmap, as do the resistant H/W and LnA resistant pair of strains and the F344 and Wis rats are of intermediate sensitivity and also cluster together. Thus, the strongest trend in gene expression changes after a single high dose of TCDD is dioxin-sensitivity rather than general inter-strain variability. We also examined the correlation of gene expression between all possible pairings of rat strains and again found that strains with similar dioxin-sensitivity shared similar patterns and clustered tightly together (Fig. 2B).

This type of co-clustering could be caused by either a small global alteration in a large number of genes or by large changes in a small number of genes. To assess which of these two possibilities was occurring, we asked what fraction of genes was significantly altered by TCDD exposure in each rat strain. To do so in a threshold-independent manner, we evaluated the number of changes at different p-value cut-offs (Fig. 3A). The highest number of genes altered was observed in L-E rats (blue curve), followed by F344 rats (purple curve). Wis and H/W rats showed the smallest number of TCDD-responsive genes (yellow and light green curves, respectively). LnC (dark green) and LnA (red) were intermediate amongst the other strains. All effects were independent of the p-value threshold, indicating that the variation in the number of responsive genes across strains is a real biological phenomenon, not an artifact of statistical methodology.

These data indicate that large magnitude changes in a small number of genes probably accounted for the co-clustering seen in Fig. 2. To determine the overlap of these TCDD-responsive genes across the different strains/lines, we analyzed the number of responsive genes across strains. We merged the data from RAT230-2 and RAE230-A microarrays by keeping genes common to both and visualized those that were TCDD-responsive (Fig. 3B). There is a log-decrease in the number of responsive genes as the number of strains

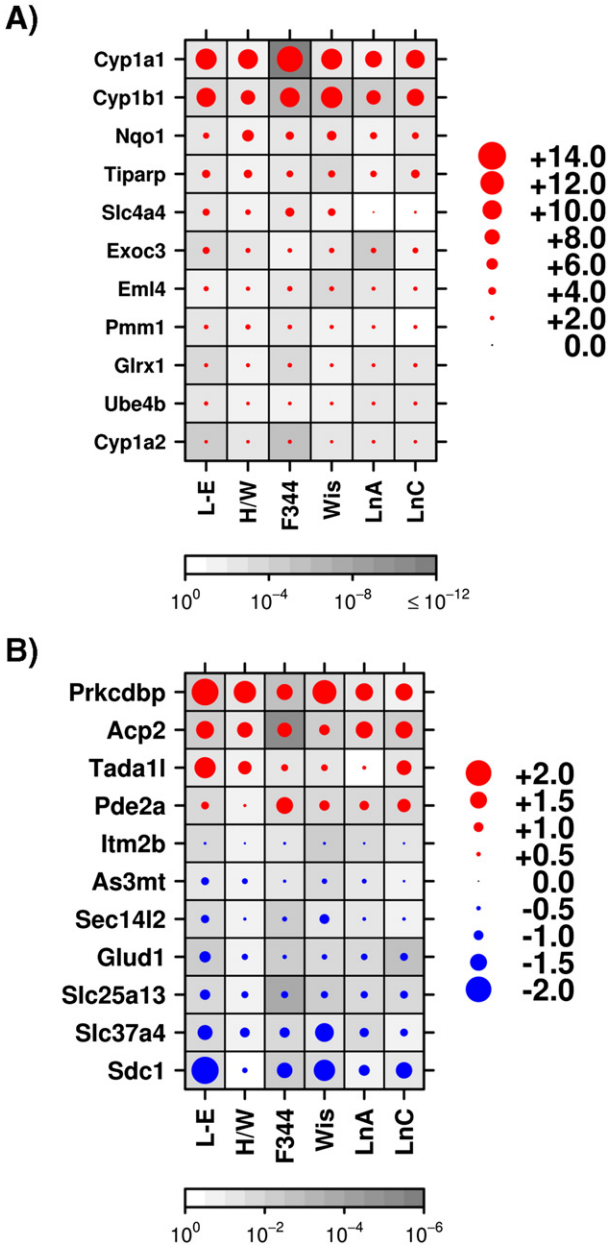

Fig. 4. Dotmap of TCDD-responsive genes. A) We focused on the 11 genes with conserved responses across all four rat strains and visualized their mRNA abundances in log_2-scale using a dotmap. These include genes that are well-known AHR-regulated genes, including cytochromes P450, ubiquitination factors, and glutaredoxin. B) Conversely, we identified a set of 11 genes that showed significant responses in L-E, F344, and Wis rats but not in the resistant H/W rats. All genes showed the same direction of change, but the magnitude of changes varied significantly amongst the different strains. The size of the circle corresponds to the magnitude of the fold-change and the colours represent up- (red) or down- (blue) regulation. Shading in the background represents adjusted p-values in -log10 scale with gray having an adjusted p-value of 10^{-6} and white as a value of 1.

increases, indicating very large inter-strain differences in the number of TCDD-responsive genes. We found a set of 11 genes that responded significantly to TCDD in all six strains/lines (Fig. 3C). Outside of this core, strains differ significantly in their responses to TCDD and there is minimal overlap between them (Fig. 3D). Interestingly, F344 rats showed greater similarity to L-E rats (25.3% overlap) than to H/W rats (9.8% overlap); Wis rats had similar numbers of gene alterations as H/W rats but greater similarity in specific genes to L-E (41.8% overlap) than to H/W rats (22.4% overlap).

Conserved transcriptomic responses to TCDD

We previously contrasted the transcriptomic responses to TCDD between L-E and H/W rats at 4 and 10 days following TCDD treatment at 100 μg/kg and found considerable overlap between the two strains at both time points (Boutros et al., 2011). Similarly, we looked for overlap between different rat strains at an early time point (19 h) to identify genes that may have critical roles in triggering TCDD toxicity. Consistent with our previous data, the 11 genes that exhibited the greatest magnitude of response and were most consistent across all 6 strains/lines at the onset of TCDD toxicity are classic AHR-regulated genes, such as *Cyp1a1*, *Cyp1b1*, *Nqo1*, and *Tiparp*. All 11 of these genes exhibited consistent directions and magnitudes of change across all six strains and lines (Fig. 4A).

Responses to TCDD that are specific to sensitive rats

We hypothesized that genes showing differential gene expression between sensitive and resistant rat strains are strong candidates to mediate susceptibility to dioxin lethality. To test this hypothesis, we focused on genes that showed divergent responses between rat strains with differing TCDD-sensitivity. We identified genes that were altered specifically in highly or moderately TCDD-sensitive L-E, F344, and Wis rats but not in resistant H/W rats (Fig. 4B). Here we see that although multiple genes showed the same directionality of change across all 6 strains, there are differences in the magnitude of response across the different strains, with some genes having a 4-fold difference in gene expression between strains.

Functional analysis of TCDD-responsive genes

To examine whether genes identified from the above analysis belong to a specific pathway, perhaps leading to conserved strain-independent TCDD toxicity, we employed functional analysis for the 100 genes that showed the smallest adjusted p-values for each strain. We examined GO terms that have FDR of less than or equal to 0.01 and visualized their overlap across different rat strains (Fig. 5A for statistical significance; Fig. 5B for enrichment). Processes that pertain to oxidation–reduction were commonly dysregulated in L-E, H/W, LnA, and LnC rats but not in F344 and Wis rats, perhaps implying different mechanisms that animals possess for handling TCDD. By contrast toxin metabolic processes were significantly enriched across all six strains, and many core TCDD-responsive genes (e.g. *Cyp1a1*) lie within this highly enriched category.

In order to gain additional insight into the functional processes of the candidate genes, we performed RedundancyMiner analysis. Redundant GO categories were eliminated and parent categories were weighted to prevent over-representation. Redundant GO terms were collapsed into groups; GO categories that were recognized as statistically significant from GOMiner analysis were also significant after application of RedundancyMiner. Oxidoreductase activity and toxin metabolic process showed significant enrichment before and after RedundancyMiner analysis (FDR<0.01), indicating the robustness of the results (Fig. 5C).

Transcription factor binding site analysis

To provide additional mechanistic insight into how this functional diversity of TCDD responses is generated, we hypothesized that a small number of transcriptional regulators were at play. We therefore analyzed the occurrence of transcription factor binding sites (TFBSs) in TCDD-responsive genes using enrichment analysis as previously described (Boutros et al., 2011). We plotted the number of occurrences and the maximal conservation scores of each motif against the number of rat strains in which the gene was affected by TCDD treatment. AHRE-I has been found to reside on common AHR-

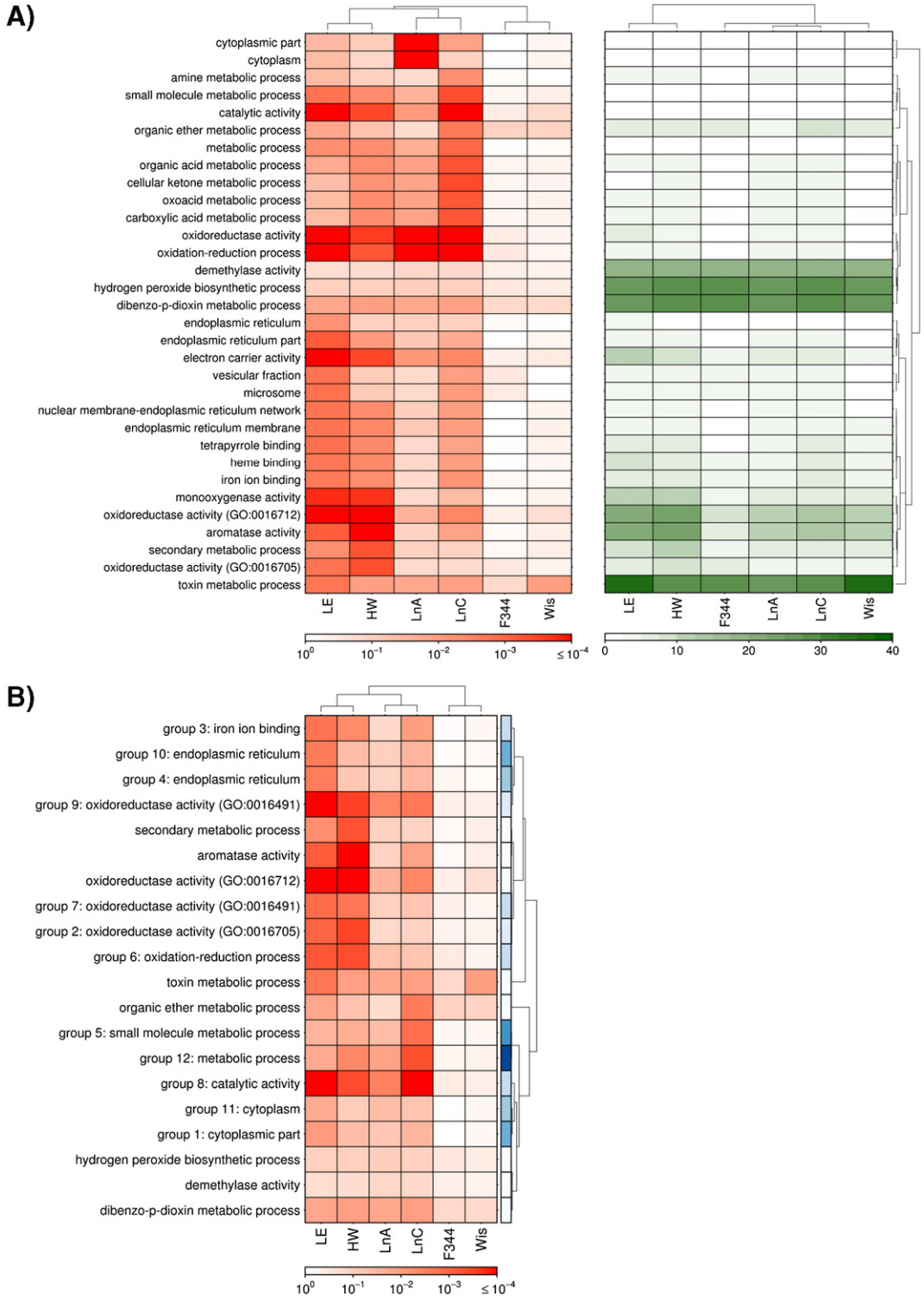

Fig. 5. Functional analysis. To assess whether genes that responded to TCDD within each rat strain might be regulated by a common pathway, we selected the top 100 genes from each rat strain that have the smallest adjusted p-values and ran them through GO Miner (see Methods section for selection parameters used). A) The majority of the GO categories were significantly enriched in L-E, H/W, LnA, and LnC but not in F344 and Wis rats (left panel). Of the significantly expressed GO categories, toxin metabolic process and dibenzo-*p*-dioxin metabolic process showed the highest enrichment (right panel; GO terms clustered according to left panel data). B) To further investigate pathways involved in dioxin toxicities, we employed RedundancyMiner, a tool that dampens the over-representation of specific GO terms. We collapsed our 32 GO categories into 20 functional groups. The row-annotation table represents the number of GO categories that were grouped into each functional group (going from white to dark blue: 1 to 8 categories). A number of processes that react to toxins or toxicants showed consistently significant enrichment before and after the application of RedundancyMiner; these include dibenzo-*p*-dioxin metabolic process, toxin metabolic process, oxidoreductase activity, and catalytic activity.

regulated genes such as *Cyp1a1* where it binds the ligand–AHR–ARNT complex and enhances transcription. More recently, several studies have revealed that the AHRE-II motif aids transcription of *Cyp1a2* and some other TCDD-responsive genes (Boutros et al., 2004; Sogawa et al., 2004). We analyzed the number and conservation of each motif across the strains (Figs. 6A–D). AHRE-I motifs were

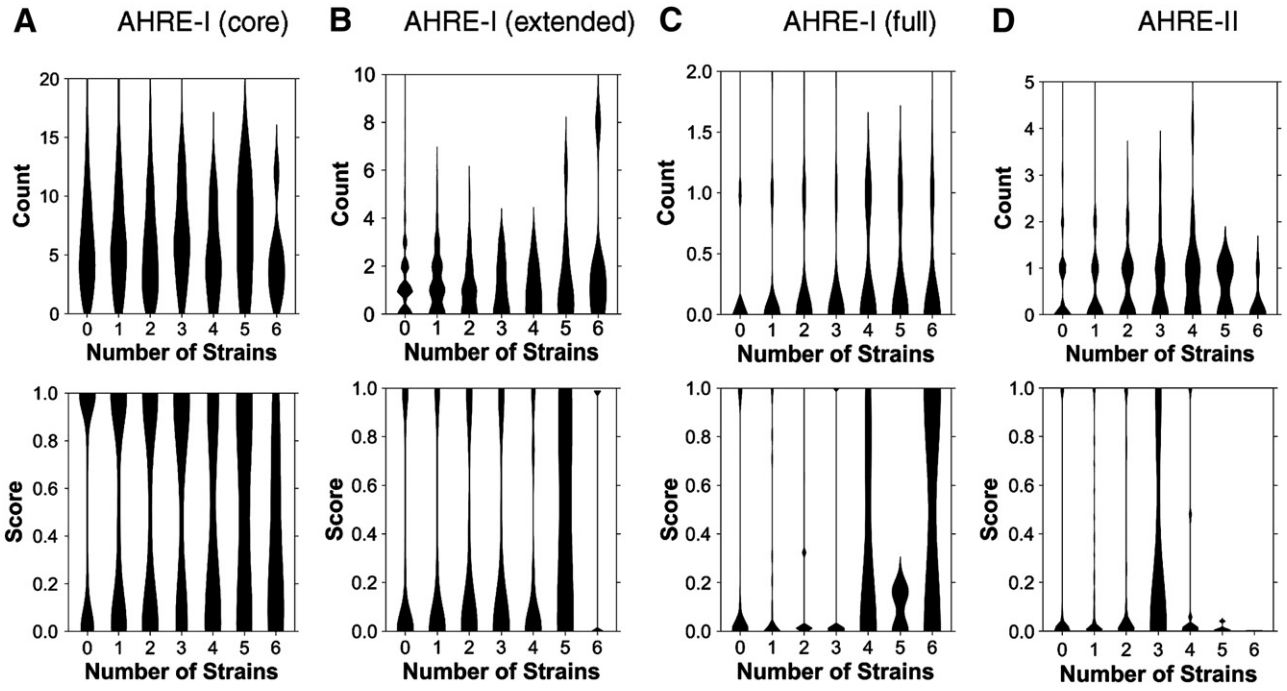

Fig. 6. TFBS analysis. We performed a TFBS enrichment analysis that assesses the match between our genes of interest and the occurrence of 4 different motifs: A) AHRE-I (core); B) AHRE-I (extended); C) AHRE-I (full); and D) AHRE-II (the sequence for each is described in Methods). The genes were selected at a P_{adj} threshold equaling to the maximum P_{adj} of *Cyp1a1* as described in Methods. We then visualized both the counts and conservation scores in all genes of interests across different numbers of rat strains; that is, 0 represents genes that are not significantly altered across any number of rat strains and 6 represents genes that are responsive to TCDD across all six rat strains. We found that AHRE-I was conserved in genes that were significantly altered by TCDD in all six rat strains whereas AHRE-II was not conserved in responsive genes across strains.

conserved within genes that were significantly altered across all six strains, whereas AHRE-II motifs were not conserved across the rat strains that we tested.

Validation of TCDD-responsive genes

Finally, to examine potential roles of the selected genes in mediating TCDD toxicity and to check whether the responsiveness of these genes is regulated in a time- or dose-dependent way, we conducted PCR analysis on six genes across 152 animals (84 H/W rats and 68 L-E rats) in both time-course (from 0 to 384 h) and dose–response experiments (from 0 to 3000 μg/kg). Experiments involving different time points were used to determine whether the genes exhibit acute or downstream effects; dose–response experiments were used to observe patterns of expression with increasing dose that might relate to doses that evoke hepatic toxicity. The genes analyzed by PCR showed significant mRNA abundance changes with a magnitude of 2-fold or larger in response to TCDD either in all rat strains or in all sensitive rat strains. Aside from *Sdc1*, all of the selected genes showed both time-dependent and dose-dependent responses to TCDD (Fig. 7). As expected, we observed fewer differences in the expression of the tested genes in the dose–response experiments than in the time-course experiments due to the short duration of exposure (19 h). Results from *Sdc1* were not interpretable due to a discrepancy between the time- and dose–response. However, of the five genes that showed time- and dose-dependent responses, *Acp2*, *Glrx1*, *Slc37a4*, and *Ube4b* showed differential responses to TCDD between L-E and H/W rats around and after the onset of TCDD toxicity (19 h post-treatment), potentially suggesting their roles in determining sensitivity or resistance to TCDD.

Discussion

We previously compared transcriptomic responses of sensitive L-E rats to those of resistant H/W rats in response to TCDD. Liver samples were collected at 19, 96 or 240 h post treatment to allow comparison of changes in mRNA abundances around or after the onset of toxicity (Boutros et al., 2011; Moffat et al., 2010). In the current study, we expanded this comparison by including additional rat strains that are moderately sensitive to TCDD, F344 and Wis. The two main goals of this study were to identify transcriptomic responses that are conserved across rat strains along with responses that differ between sensitive and resistant strains at a time near the onset of the first manifestations of TCDD toxicity. TCDD-induced toxicities include hepatic lesions, endocrine imbalances, immunosuppression, and wasting syndrome (reviewed in Pohjanvirta and Tuomisto, 1994).

Our results show that the vast majority of dioxin-induced changes in mRNA abundances are not conserved across strains, at least in liver, and at dose of 100 μg/kg and exposure time of 19 h. One mechanistic explanation for AHR activity is the “classic action pathway” wherein TCDD binds to the AHR and elicits a series of downstream effects which ultimately results in the activation of transcription of AHR-regulated genes such as *Cyp1a1*, *Cyp1a2*, etc. (Okey, 2007). Recently, some groups have proposed an alternative mechanism of the AHR's involvement in TCDD toxicity, particularly inflammatory responses, in a ligand-independent way. The ligand-independent pathway does not involve the presence of ARNT and is said to be “non-genomic” (Dong and Matsumura, 2008; Li and Matsumura, 2008; Li et al., 2010; Sciullo et al., 2008). Our data support the “classic action pathway” as the main mechanistic determinant of AHR toxicity, as those few genes consistently altered by TCDD across strains are significantly enriched for AHR DNA binding-motifs.

The set of common AHR regulated genes that showed differential expression amongst multiple rat strains and at multiple doses and time-points includes common dioxin responsive genes such as *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Tiparp*, and *Nqo1*. These genes might partially explain some of the Type-I responses to TCDD that are observed in both sensitive and in resistant animals. Amongst the other genes, Ube4b was shown to be responsive to TCDD across all four rat strains as well as the two lines, LnA and LnC. *Ube4b* encodes for an

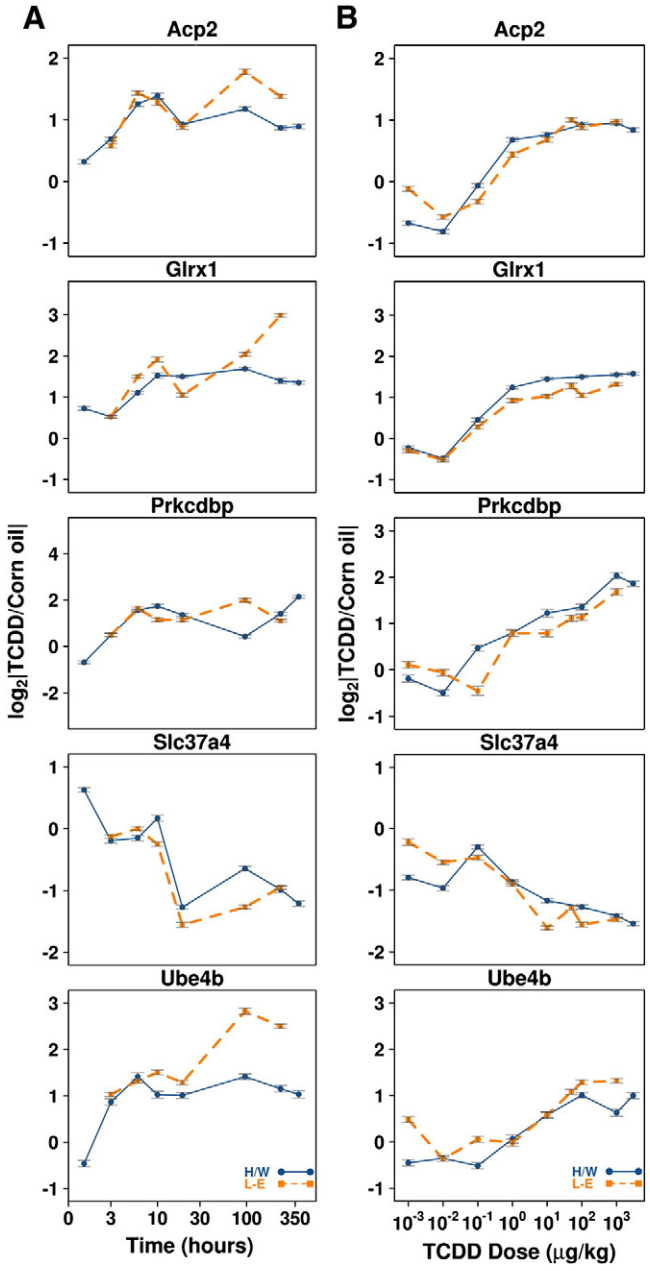

Fig. 7. PCR validation using time-course and dose–response experiments. Finally, to verify if the novel TCDD-responsive genes identified in this study (Table 1) are candidates to mediate specific TCDD toxicities, we studied their A) time-course and B) dose–response. The time of exposure ranges from 0 to 16 days (shown in $\log_{10}$ scale). L-E and H/W rats were treated with either 100 μg/kg TCDD or corn-oil vehicle for each time point in the time-course study. Five of the six genes displayed classic time-course response where the fold-change increases with prolonged time of exposure. Results from our dose–response experiments are shown in B). The doses range from 0 to 3000 μg/kg and animals were uniformly treated with TCDD or corn-oil vehicle and liver collected 19 h following the treatment. Interestingly all five genes that exhibited a time-course response also showed a dose-dependent change in mRNA levels.

ubiquitination factor E4B, which binds to the ubiquitin moieties and accelerates ubiquitin chain assembly in synchrony with factors E1, E2, and E3, which subsequently tags aberrant proteins for degradation (Koegl et al., 1999). We found that *Ube4b* is consistently dysregulated by TCDD treatment (2-fold induction). It is unclear what role it plays in dioxin toxicity but it could be a protective mechanism that is elicited in response to exposure to xenobiotics. Interestingly, the AHR was recently shown to act as a ligand-dependent ubiquitin E3 ligase targeting e.g. sex hormone receptors and β-catenin for proteasomal degradation (Ohtake and Kato, 2011).

Glrx1, another gene whose mRNA abundances were statistically different between the treated and untreated rats across all four rat strains, is a glutaredoxin that catalyzes deglutathionylation of protein-SS-glutathione mixed disulfides. *Glrx1* was induced more than 2-fold across all rat strains and lines. It is involved in protecting cells against oxidative stress (Terada et al., 2010); up-regulation of *Glrx1* may be a protective mechanism since other studies have also suggested its potential role in regulating apoptosis in cardiomyocytes (Gallogly et al., 2010) and controlling autocrine and paracrine proinflammatory responses in retinal glial cells (Shelton et al., 2009). Since L-E rats, which are much more sensitive to TCDD-induced liver tumor promotion than H/W rats (Viluksela et al., 2000), exhibited an upward trend in *Glrx1* expression at the latest time-points analyzed (Fig. 7), dysregulation of *Glrx1* might have a role in the hepatocarcinogenicity of TCDD in rats. On the other hand, the enhanced *Glrx1* expression coincides with aggravation of lipid peroxidation (an index of oxidative stress) in lethally TCDD-treated L-E rats (Pohjanvirta et al., 1990).

Another trend that was also consistent with our previous finding is that outside of the set of "classic" AHR-responsive genes, genes vary significantly in their responses to TCDD across the different rat strains. We identified a set of genes whose expression was significantly altered by TCDD in the sensitive rat strains but not the resistant H/W rats. These genes may represent predisposing genes that give rise to the observed toxicities to TCDD as mentioned above in the sensitive strains. For example, *Slc37a4* encodes a transporter protein that transports glucose-6-phosphate to the microsomal lumen where hexose-6-phosphate dehydrogenase hydrolyses it to glucose and inorganic phosphate (P_i) (Marcolongo et al., 2007). Deficiencies in the protein have been associated with disturbed glucose homeostasis and glycogen storage diseases (Chou et al., 2002; Pan et al., 2009). In our experiment, *Slc37a4* was significantly down-regulated by 2-fold in the sensitive rat strains but not in the resistant H/W rats. This is consistent with our previous finding where H/W rats showed minimal or no change in body weight following TCDD treatment whereas significant weight loss was observed for the sensitive L-E strain after both time points (Boutros et al., 2011), as is a more prominent decrease in plasma glucose upon TCDD treatment in L-E than H/W rats (Viluksela et al., 1999). Dysregulation of *Slc37a4* could be involved in the differential energy and feed metabolism between sensitive and resistant strains and the resulting wasting syndrome observed in the sensitive strains but not in resistant strains (Boutros et al., 2011; Pohjanvirta and Tuomisto, 1994).

Endocrine imbalance is another acute effect that follows TCDD treatment (Pohjanvirta and Tuomisto, 1994). Some portion of endocrine disruption may be due to altered synaptic transmission and communication from neurons to the endocrine system. *Acp2*, a gene that is consistently induced by 2-fold in the sensitive strains but not in the resistant strain, encodes a lysosomal acid phosphatase that catalyzes p-nitrophenyl phosphate hydrolysis. The abundance of the phosphatase in the nerve endings suggests its potential role in synaptic transmission (Tanino et al., 1999). In other studies, *Acp2* was found to play a role in acute pancreatitis (Lakowska et al., 2001). It is difficult to evaluate the role of *Acp2* in TCDD toxicity due to the insufficient characterization of its physiological functions, but the increase in *Acp2* expression may have a role in the imbalance in the endocrine system of rats that are exposed to TCDD (Pohjanvirta and Tuomisto, 1994).

Long-term exposure to TCDD leads to cancer formation in liver and other organs (Viluksela et al., 2000). *Prkcdbp* encodes a protein kinase-binding protein that may be involved in the control of cell growth mediated by protein kinase C (Izumi et al., 1997). *Prkcdbp* showed greater than 3-fold induction in the sensitive strains but did not reach statistical significant in the resistant H/W strain. Impaired control of cell growth could well contribute to the carcinogenic effect of TCDD in sensitive animals. On the other hand, *Sdc1*, a mouse homolog

that is found to promote cell–cell adhesion, showed significant repression by at least 3-fold in all the sensitive strains but remained unperturbed in the resistant H/W strain. *Sdc1* has been previously shown to be implicated in hepatocellular cancer (HCC). Both the gene and protein expression of *Sdc1* was significantly reduced in HCC with extra-hepatic metastasis in comparison with those without (Matsumoto et al., 1997). This suggests that *Sdc1* may play a role in determining metastatic potential.

A highly characteristic feature of the acute toxicity of TCDD is its delayed emergence. Even after supralethal doses of TCDD, the exposed animals do not die immediately but only after 2–5 weeks (Pohjanvirta and Tuomisto, 1994). Therefore, it is conceivable that the critical phase of TCDD toxicity that differs between sensitive and resistant animals is not – in contrast to the case of many other toxicants – the early primary response but rather its later repercussions. This view would be in keeping with the poor segregation of changes in individual gene expression levels with TCDD sensitivity in the present study. We recently also showed that the number of dissimilar transcriptomic responses between L-E and H/W rats increases as a function of time (Boutros et al., 2011).

TCDD exposure and subsequent toxicity are an important issue that could directly affect human health. We focused our experiments on liver tissue because there is extensive hepatotoxicity in rats following exposure to TCDD. The ultimate target organ for lethality remains unknown; however, large hepatic differences exist in toxic end-points between the sensitive L-E and the resistant H/W rats, making liver a good candidate organ for involvement in systemic TCDD toxicities.

The role of the AHR genotype in regard to liver toxicity is especially well demonstrated in a study conducted by Pohjanvirta, where transgenic C57BL/6 mice that express the rat wild-type isoform of the AHR showed significantly higher expression of AHR and CYP1A1 in comparison to non-transgenic mice, particularly in liver (Pohjanvirta, 2009). That study also demonstrated that liver is a major target for TCDD's toxic effects; hence, studying differential gene expression in liver is critical to the overall understanding of TCDD toxicity. By combining existing genetic models with microarray analysis, we have identified key novel candidate genes that are worthy of further investigation for differential expression at the protein level and ultimately in mechanistic studies to connect altered expression to subsequent overt toxicity.

Supplementary materials related to this article can be found online at doi:10.1016/j.taap.2012.02.001.

Conflict of interest statement

ABO has served as a paid consultant to the Dow Chemical Company as a member of their Dioxin Scientific Advisory Board. Other authors declare that they have no conflicts of interest.

Funding sources

This work was supported by the Canadian Institutes of Health Research (grant number MOP-57903 to ABO and PCB), the Academy of Finland (grant number 123345 to RP), the Ontario Genomics Institute (to CP) and with the support of the Ontario Institute for Cancer Research to PCB through funding provided by the Government of Ontario. The study sponsors had no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Acknowledgments

The authors thank Hanbert Chen, Alexander Wu, Ashley Smith, Janne Korkalainen, Arja Moilanen, and Virpi Tiihonen for excellent technical assistance and support. We are also obliged to Dr. Jouni T. Tuomisto for providing us LnA and LnC rats.

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