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Sex-related differences in murine hepatic transcriptional and proteomic responses to TCDD

Stephenie D. Prokopec [a], John D. Watson [a], Jamie Lee [a,b], Raimo Pohjanvirta [c,d], Paul C. Boutros [a,b,e,*]

[a] Informatics and Bio-computing Program, Ontario Institute for Cancer Research, Toronto, Canada
[b] Department of Pharmacology & Toxicology, 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, Helsinki, Finland
[e] Department of Medical Biophysics, University of Toronto, Toronto, Canada

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an environmental contaminant that produces myriad toxicities in most mammals. In rodents alone, there is a huge divergence in the toxicological response across species, as well as among different strains within a species. But there are also significant differences between males and females animals of a single strain. These differences are inconsistent across model systems: the severity of toxicity is greater in female rats than males, while male mice and guinea pigs are more sensitive than females. Because the specific events that underlie this difference remain unclear, we characterized the hepatic transcriptional response of adult male and female C57BL/6 mice to 500 μg/kg TCDD at multiple time-points.

The transcriptional profile diverged significantly between the sexes. Female mice demonstrated a large number of altered transcripts as early as 6 h following treatment, suggesting a large primary response. Conversely, male animals showed the greatest TCDD-mediated response 144 h following exposure, potentially implicating significant secondary responses. *Nr1i3* was statistically significantly induced at all time-points in the sensitive male animals. This mRNA encodes the constitutive androstane receptor (CAR), a transcription factor involved in the regulation of xenobiotic metabolism, lipid metabolism, cell cycle and apoptosis. Surprisingly though, changes at the protein level (aside from the positive control, CYP1A1) were modest, with only FMO3 showing clear induction, and no genes with sex-differences. Thus, while male and female mice show transcriptional differences in their response to TCDD, their association with TCDD-induced toxicities remains unclear.

Introduction

Chlorinated dioxins are a class of chemically-similar compounds produced as byproducts of industrial processes such as low temperature incineration and electronics recycling (Shen et al., 2009), as well as historically through the manufacture of herbicides and pesticides (Schecter et al., 2006). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent dioxin, eliciting a range of toxic and biochemical responses including chloracne, thymic atrophy, hepatotoxicity, wasting syndrome and cancer (Pohjanvirta and Tuomisto, 1994). In addition, gestational exposure results in various reproductive abnormalities in mice, such as reduced sperm count and decreased uterine weight (Theobald and Peterson, 1997), and rats, including abnormal lung morphology (Kransler et al., 2009) and an altered transcriptome of the developing sex organs (Magre et al., 2012). After a single exposure, TCDD accumulates within the liver and adipose tissues of rats and mice (Pohjanvirta et al., 1990; Diliberto et al., 1995) and causes dose-dependent toxicities, such as increased liver weight (Boverhof et al., 2005).

Essentially all of the TCDD-associated toxic effects are mediated through its interaction with the aryl hydrocarbon receptor (AHR). Activation of this receptor results in nuclear translocation and heterodimerization with the AHR nuclear translocator (ARNT). This complex then recognizes and binds to aryl hydrocarbon response elements (AHREs) and regulates transcription. Evidence that TCDD toxicities occur via the AHR come from studies examining the significantly diminished toxic outcomes observed in both AHR knockout mice (Fernandez-Salguero et al., 1996; Mimura et al., 1997) and mice with mutant AHREs (Bunger et al., 2003). In addition, studies of hepatocyte-specific ARNT-null mice observed reduced hepatotoxicity following exposure to TCDD (Nukaya et al., 2010).

There is a wide disparity in the toxic effects of TCDD among different animal models. For example acute lethality, as measured by the LD_{50}, is highly variable for different species: ~1 and 5100 μg/kg for guinea pigs

Abbreviations: AHRE, Aryl hydrocarbon response element; ARI, Adjusted Rand Index; FDR, False discovery rate; GEO, Gene Expression Omnibus; H/W, Han/Wistar rats; L-E, Long-Evans rats; TCDD, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin.

* Corresponding author at: Ontario Institute for Cancer Research, Suite 510, 661 University Avenue, Toronto, Ontario, M5G 0A3, Canada.
E-mail address: Paul.Boutros@oicr.on.ca (P.C. Boutros).

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Table 1
Candidate genes summary.
Table shows if the candidate gene was significantly altered in a given comparison ($q < 0.01$). Candidate genes were identified as those targets significantly differentially expressed between TCDD-treated and corn-oil control groups at 3 or more time-points in either male or female mice. A total of 20 candidate genes were identified.

Comparison	Male $_{(T-C)}$				Female$_{(T-C)}$				Male $_{(C)}$–Female$_{(C)}$			
Time-point	6	24	72	144	6	24	72	144	6	24	72	144
Smcp	+	+	+	−	+	−	−	−	−	−	−	−
Dclk3	+	+	+	+	+	−	−	−	−	−	−	−
Fmo3	+	+	+	+	−	−	−	+	+	+	+	+
Fmo2	+	+	+	+	+	−	−	−	−	+	−	+
Nup155	+	+	+	+	+	−	−	−	−	−	−	−
Slc18a2	+	−	+	+	+	−	−	−	−	−	−	−
Atoh8	+	−	+	+	+	−	−	−	−	−	−	−
Nr1i3	+	−	+	+	+	−	−	−	−	−	+	−
Gadd45b	−	+	+	+	−	−	−	+	−	−	−	−
Sec61a2	+	−	+	+	+	−	−	−	−	−	−	−
Serpina6	+	−	+	+	−	−	−	−	−	−	−	−
Prhoxnb	+	−	+	+	+	−	−	−	−	−	−	−
Ugt1a9	−	+	+	+	−	−	−	+	−	−	−	−
Tmem62	+	−	+	+	−	−	−	+	−	−	−	−
Ces1b	−	+	+	+	−	−	−	−	−	−	−	−
Tlr5	−	+	+	+	+	−	−	−	−	−	−	−
Aig1	−	+	+	+	+	−	−	−	−	−	−	−
Ces4a	−	+	+	+	−	−	−	−	+	−	−	−
Klhdc7a	−	+	+	+	−	−	−	+	−	−	−	−
Gpd2	−	+	+	+	−	−	−	−	−	−	−	−

and hamsters respectively (Schwetz et al., 1973; Henck et al., 1981). Similarly, toxicity varies among different strains within a species: the LD_{50} for Long-Evans (L-E) rats is ~10 μg/kg while Han/Wistar (H/W) rats can tolerate upwards of 9600 μg/kg with minimal effects (Pohjanvirta et al., 1999). In many of these cases, the difference in sensitivities is a result of variations within the *Ahr* gene. A point mutation leads to splicing-generated alteration of the transactivation domain of the H/W rat AHR rendering it refractory to TCDD toxicities while the wild-type L-E rat is one of the most sensitive responders (Pohjanvirta et al., 1998). These differing sensitivities have allowed researchers to compare and contrast the transcriptomic responses to TCDD across species (Boverhof et al., 2006; Boutros et al., 2008; Dere et al., 2011; Forgacs et al., 2013; Nault et al., 2013) and between strains/lines within a species (Franc et al., 2008; Pohjanvirta, 2009; Yao et al., 2012).

But differences in sensitivities have also been identified within a single strain, between male and female animals (Pohjanvirta et al., 1993; Enan et al., 1996; Silkworth et al., 2008). In L-E rats, female animals are more sensitive to the acute lethality of TCDD ($LD_{50} = 9.8$ μg/kg) while males are more resistant ($LD_{50} = 17.7$ μg/kg) (Pohjanvirta et al., 1993). Alternatively in guinea pigs, male animals are more susceptible than females (Enan et al., 1996). Recently, it was discovered that, unlike in rats and similar to guinea pigs, female mice are less sensitive than males and develop fewer toxicities (Pohjanvirta, 2009). The pattern in mice did not extend to all other sub-strains examined (Pohjanvirta, 2009), however was later confirmed through extensive analyses of the physiological effects of TCDD on these mice (Pohjanvirta et al., 2012). Furthermore, this differential response is caused, at least in part, by the sex hormones; ovariectomized mice proved more sensitive while castrated mice are more resistant to TCDD-induced lethality than intact mice (Pohjanvirta et al., 2012). Here we attempt to identify the specific TCDD-mediated transcriptional events in the liver responsible for the divergent sensitivity phenotypes between male and female mice.

Results

Experimental design

We have characterized the specific hepatic transcriptional modifications associated with the differential TCDD-toxicity phenotypes observed in male and female C57BL/6 mice. Adult male and female mice were treated with either TCDD in corn oil vehicle or corn oil alone, and transcriptional profiling was performed on hepatic tissue collected 6, 24, 72 or 144 h after treatment. Animals were followed along this time-course to evaluate the progressive transcriptomic changes. In this way we sought to identify both the primary and secondary TCDD:AHR-mediated responses. The experimental approach is outlined in Fig. 1 and sample information, including identification of outliers, is in Supplementary Table 1.

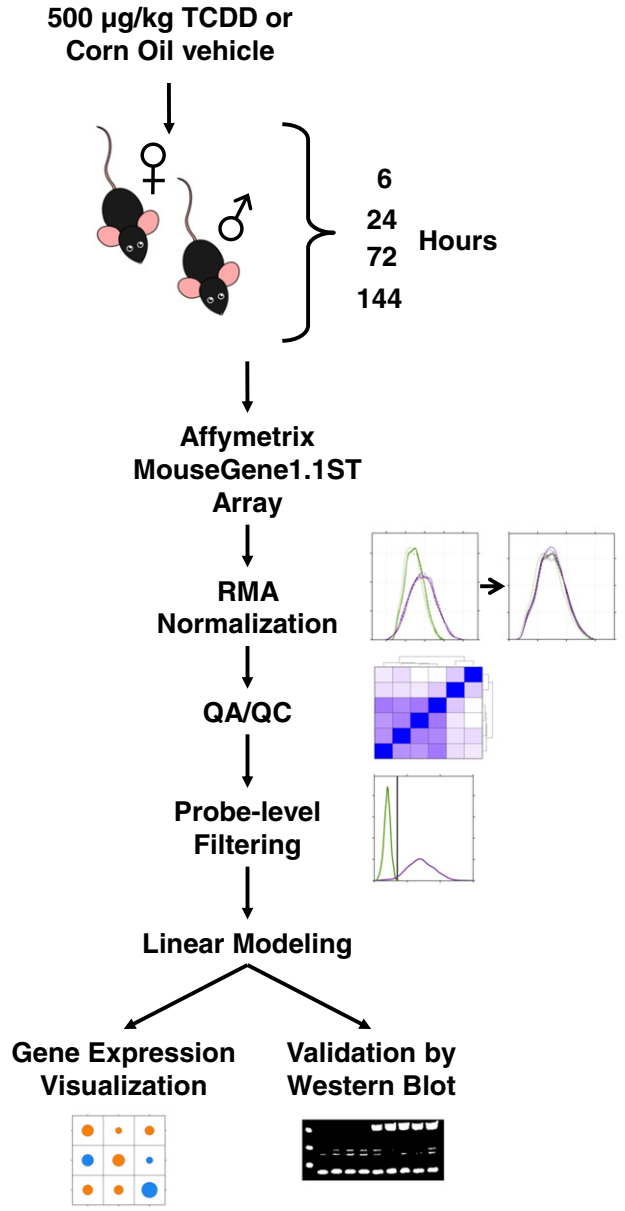

Fig. 1. Experimental design. Outline of the experimental design: 65 adult male and female C57BL/6 mice were treated with either 500 μg/kg TCDD or corn oil and euthanized 6, 24, 72 or 144 h post-treatment. Liver tissue was harvested and RNA isolated. Samples were hybridized onto Affymetrix MouseGene1.1ST arrays. Data were pre-processed using RMA normalization. Quality control identified outliers which were removed; data were then re-normalized. Probe-level filtering was performed based on intensity levels of chromosome Y probes in female animals. Data underwent statistical analyses, results were visualized and validation of potential toxicity-related targets performed by western blot.

Overview of transcriptomic responses

We first assessed the overall transcriptomic responses of the liver by examining those transcripts with highest variance across our samples

(Fig. 2A). Adjusted Rand Index (ARI) indicated essentially random partitioning of samples, based on these transcripts alone (ARI_{Sex} = 0.06; $ARI_{Treatment}$ = 0.06; ARI_{Time} = 0.01). Following linear modeling (see Methods), a p-value sensitivity analysis was conducted to determine the optimal threshold for downstream analyses (Fig. 2B, Supplementary Fig. 5). Venn diagrams were used to visualize overlap of significantly altered transcripts across the time-course. Comparison of male and female vehicle-treated animals indicated only 28 genes differentially abundant at all time-points, and 47 genes altered at two or more (Fig. 2C). An evaluation of the TCDD-dependent changes revealed 18 genes differentially abundant in male liver (Fig. 2D) and 7 genes altered in female liver (Fig. 2D) at all four time-points. These analyses also showed that, in females, the largest number of dysregulated transcripts occurred at the earliest time-point, suggesting a strong primary hepatic response, while in males, the largest number of dysregulated transcripts occurred at the latest time-point indicating considerable secondary responses within the liver. A comparison between male and female TCDD-dependent responses at each time-point revealed statistically significantly more overlap than expected by chance alone (Fig. 2F). For example, overlap between male and female mice at the 144 hour time-point (n = 150) is significantly higher than expected by chance (n = 17; $p < 0.01$).

TCDD-mediated transcriptional changes

As there are clear differences in the phenotypic sensitivity between male and female mice as early as 1 day after exposure, such as weight loss and the emergence of discrete necrotic foci on the liver in males (Pohjanvirta et al., 2012), we examined transcripts differentially-abundant at multiple time-points. Importantly, transcripts significantly altered following exposure to TCDD in both male and female mice are not of interest here. We focused on transcripts significantly altered ($p_{adj} < 0.01$) at 3+ time-points in either male or female mice. In total, 20 genes met this criterion, all of which were altered in male livers

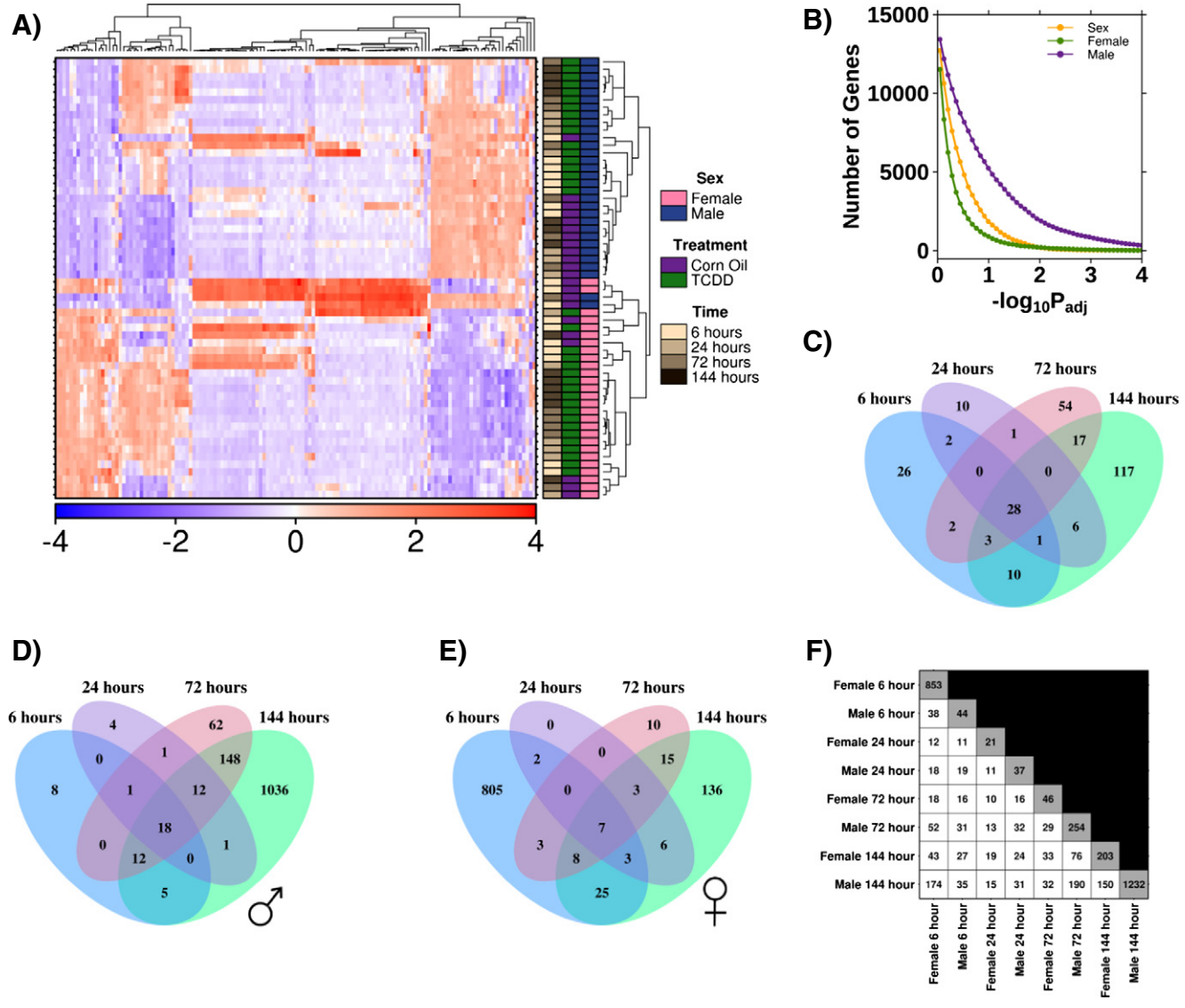

Fig. 2. Overview of transcriptional profiles. A) After normalization, samples were clustered based on transcriptomic profiles of the most variable transcripts across the study; the color key along the x-axis indicates the scaled normalized abundance values (see Methods for details). Clear patterns can be observed, differentiating between male and female mice, as well as TCDD-treated and control samples. B) P-value sensitivity analysis was performed following linear modeling; the number of genes showing differential transcription was calculated at various $p_{adjusted}$ cutoffs, regardless of direction of change. Points labeled 'Male' and 'Female' indicate the comparisons between TCDD and corn-oil treated groups, while 'Sex' indicates differences between male and female corn-oil treated groups. At the 144 hour time-point, there are significantly more altered transcripts in males, relative to females, regardless of p_{adj}-value threshold. Venn diagrams were generated across the time-course to visualize each comparison: C) sex-dependent, TCDD-independent differences, D) TCDD-dependent in male and E) in female mice. For each comparison, genes were identified as differentially expressed ($p_{adj} < 0.01$ and $\log_2|FC| > 1$). F) Overlap of transcripts altered by TCDD ($p_{adj} < 0.01$ and $\log_2|FC| > 1$) in male and female mice across the study.

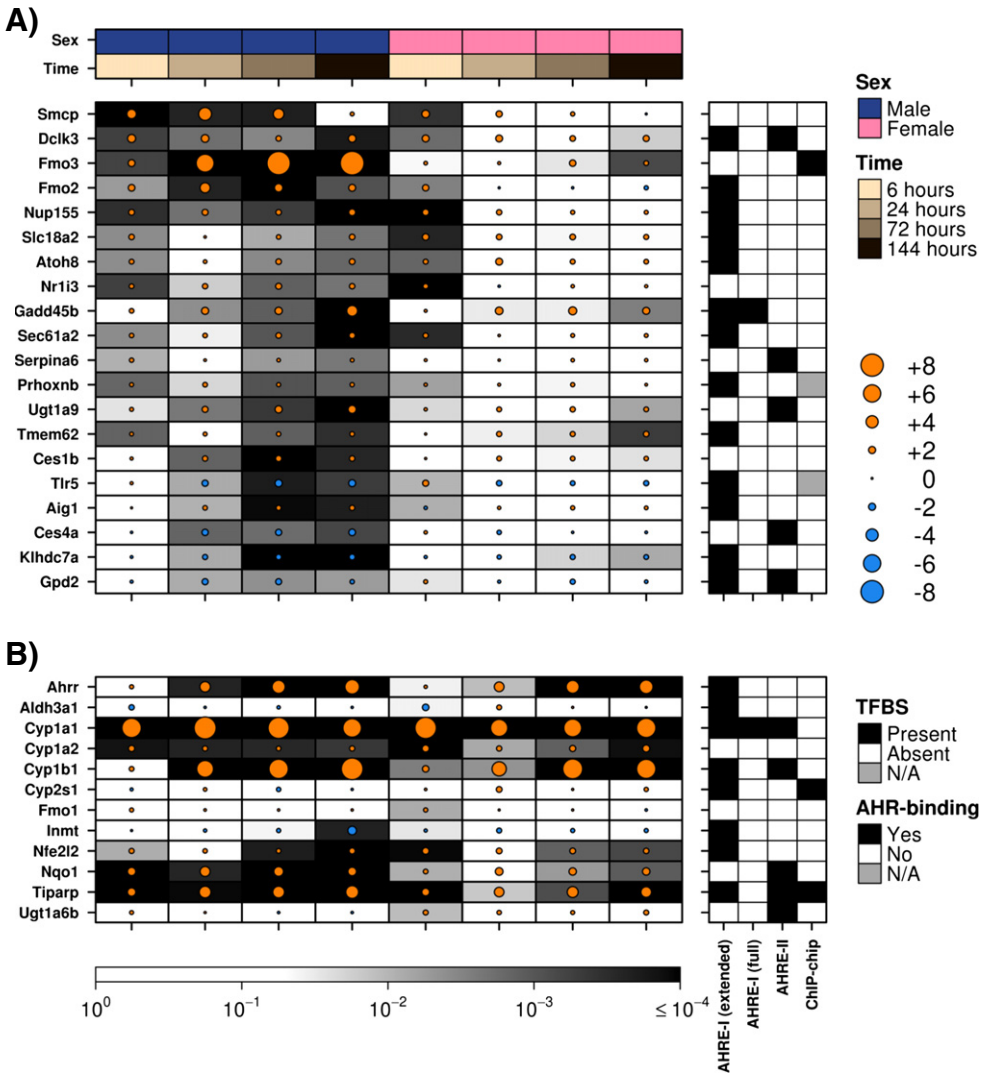

Fig. 3. Response to TCDD by candidate genes. Coefficients and significance level of A) the top 20 candidate genes, as determined by differential expression in male mice at multiple time-points with only minimal changes in female mice, and B) the AHR-core genes. Dot size represents magnitude of change (between TCDD treated and control groups) in $\log_2$-space, while background shading represents significance (FDR-adjusted p-values). Covariates indicate whether the gene contains each given transcription factor binding site (AHREI-extended, ARHEI-full or AHREII) or whether differential binding of AHR occurred between TCDD treated and control groups (as determined using ChIP-chip data Sartor et al., 2009).

only (Table 1, Fig. 3A). To determine whether these changes could be attributed to the AHR, both the occurrences of transcription factor binding sites and presence of AHR-binding for these candidates were assessed (Fig. 3A, covariate bars at the right). Despite the occurrence of local AHRE motifs for most candidates (16/20), ChIP-chip analysis indicated that only one candidate displayed direct AHR-binding. Interestingly, there was no overlap between the presence of AHRE motifs and AHR-binding, suggesting the involvement of additional transcription factors or regulatory mechanisms. Finally, to verify that the observed transcriptomic differences between sexes are in fact TCDD-dependent, we examined those candidates identified above, including *Fmo2*, *Fmo3*, *Nr1i3* and *Ces4a*, within the sex-dependent/TCDD-independent comparison (Supplementary Fig. 6A). In this comparison, coefficients represent differences between livers of male and female vehicle control mice at each time-point, with a positive value indicating increased abundance in the hepatic tissue of males relative to females. We do in fact observe statistically significant differences between sexes in certain candidate targets, specifically *Fmo2* and *Fmo3*. However, the abundance of these targets is significantly lower in males relative to females in the vehicle control group, while being higher in the male TCDD-treated mice, relative to females. This further implicates TCDD in the induction of these candidates in only males, the more TCDD-sensitive sex — a result which had been previously observed (Celius et al., 2008). *Nr1i3* shows a similar pattern; however, with a more modest, though statistically significant, induction following exposure to TCDD in males. Alternatively, *Ces4a* shows increased basal abundance in the hepatic tissue of males relative to females. Following TCDD exposure however, it is significantly repressed in male liver while remaining unchanged in females.

Response of classic 'AHR-core' genes

As the differential sensitivity of male and female C57BL/6 mice to TCDD has previously been shown (Pohjanvirta et al., 2012), we examined classic AHR-responsive genes (termed "AHR-core" genes) within our study. A set of 12 genes previously shown to respond to TCDD across multiple tissues in mice and/or rats (*Ahrr*, *Aldh3a1*, *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Cyp2s1*, *Fmo1*, *Inmt*, *Nfe2l2*, *Nqo1*, *Tiparp*, *Ugt1a6*) (Nebert et al., 1993; Korkalainen et al., 2004; Boutros et al., 2008; Yeager et al., 2009; Deb and Bandiera, 2010; Moffat et al., 2010; Watson et al., 2014) were examined (Fig. 3B). In general, these genes are similarly altered in both male and female hepatic tissue following TCDD-treatment across the experiment. There was little difference in the sex-dependent/TCDD-independent levels of these genes (Supplementary Fig. 6B).

Pathway analysis

In order to better understand the functional roles of those genes altered by TCDD exposure in the liver, identification of enriched pathways was performed using GoMiner software (Zeeberg et al., 2005). In females, one pathway, toxin metabolic process, was significantly enriched in hepatic tissue at all time-points following exposure to TCDD (Supplementary Fig. 7A). Interestingly, enrichment and significance of this process increased from 6 to 24 h before steadily decreasing towards the end of the study. In male liver, four pathways were altered at all time-points following treatment, (Supplementary Fig. 7B). Of these, enrichment of altered genes within the hydrogen peroxide biosynthetic process pathway may indicate a mechanism by which TCDD toxicity occurs. Pathways which were found to be significantly altered at 3 or more time-points in either sex are shown in Supplementary Fig. 7C.

Candidate gene validation by Western blot

To assess the functional consequences of differential transcriptomic dysregulation, we examined protein abundance of our candidates by Western blot. Of the top 20 candidates, antibodies for 12 were available. However following initial testing, only 5 were sufficiently specific, with minimal non-specific binding and/or detection of protein at the expected size (Fig. 4, Supplementary Fig. 8). Two additional targets were assessed: CYP1A1 was deemed a suitable positive control to identify treated and control samples and CES1 was chosen as numerous carboxylesterase species have been identified as being altered by TCDD, both in the current and previous studies (Matsubara et al., 2012). CYP1A1, CES1 and FMO3 all showed the similar responses at the mRNA and protein levels. ATOH8, which increased in mRNA abundance following TCDD exposure in the livers of both sexes, actually decreased in protein abundance. *Nr1i3* mRNA was significantly altered in males at all time-points; however, differences in protein abundance were not deemed statistically significant.

Discussion

As sensitivity to TCDD-induced toxicities is highly variable among animal models, characterization of transcriptomic alterations occurring in either sensitive or resistant animals would improve our understanding of how these toxicities arise. The identification of specific genes, and proteins, altered in target tissues following TCDD exposure would potentially allow for the development of treatment options for the toxic effects of TCDD and other dioxins. To this end, we have sought to identify TCDD-mediated transcriptional changes in C57BL/6 mice in which males are the more sensitive sex to TCDD-induced toxicities. Additionally, as changes in the transcriptional activity of these genes may not be themselves causative of toxicities, we have examined the protein abundance of candidate genes.

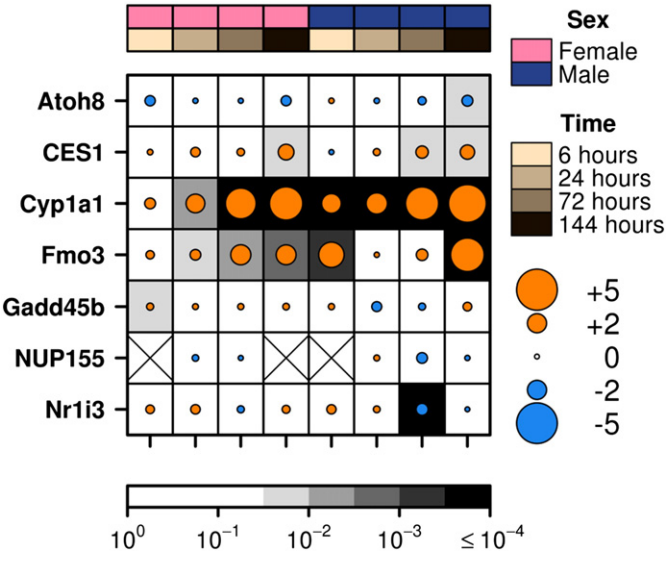

Fig. 4. Western blot validation of candidate targets. Six candidates as well as CYP1A1 and CES1 were carried forward for further evaluation of protein abundance, as measured by western blot. Dot size represents magnitude of change by TCDD treated relative to control animals while background shading refers to significance level (FDR-adjusted p-values). Fields marked with an X indicate protein abundance could not be measured.

Time-course analyses were performed to assess the transcriptional effects of TCDD in hepatic tissue of male and female C57BL/6 mice. Analysis of the expression of AHR-core genes confirmed treatment; however it also highlighted differences in *Cyp1a1* expression between the sexes occurring basally (Supplementary Fig. 6). Interestingly, we observed increased (though of modest magnitude) expression of this gene in females relative to males, at two time-points (significantly at 24 h) despite previous suggestions that *Cyp1a1* is repressed by ERα (Marques et al., 2013). The differences observed across the time-course may be explained as circadian effects as *Cyp1a1* has been shown to exhibit differences in expression that vary across a circadian cycle in both sexes (Lu et al., 2013); however, as samples for the various sexes were not time-matched (i.e., collected at different times of day), more work is required to confirm this. Additionally, a similar evaluation of the candidate genes revealed significant sex-dependent differences hepatic abundance of *Fmo3*, *Fmo2*, *Nr1i3* and *Ces4a*. Of these, differences in the flavin containing monooxygenase species have been previously identified as being unrelated to diurnal variation (Celius et al., 2008). Additionally, *Fmo3* has been shown to be negatively regulated by testosterone, with nearly absent levels in normal male liver. Upon castration, hepatic levels of *Fmo3* rise to mimic those detected in normal female liver, independent of the availability of estrogen (Falls et al., 1997). Similarly, *Nr1i3*, also known as CAR (constitutive androstane receptor) is known to be normally inhibited by some androstane derivatives (Forman et al., 1998) and has been shown to follow circadian rhythm in rats (Kanno et al., 2004). To date, *Ces4a* has been poorly studied and further studies are required to reveal the underlying cause of these sex-dependent differences.

With regard to TCDD-dependent transcriptional regulation in the liver, we identified clear differences in the transcriptomic response between the sexes, the most obvious of which is the time-point at which each sex shows the maximal number of altered genes (Figs. 2D–E). Transcript abundance was greatly affected immediately following treatment in female liver while the greatest response in male liver was observed at the latest time-point, possibly implicating a primary, defensive response in females and significant secondary responses in males that may be causative of TCDD toxicity. While the sex hormones are known to play a key role in mediating this toxicity in mice (Pohjanvirta et al., 2012), additional factors must be at play in order to explain the contrary sensitivities between sexes across species.

Numerous genes were observed to be significantly altered at multiple time-points in a TCDD-dependent manner in the liver of male mice, with only minimal changes in females. *Fmo2* and *Fmo3* have been previously shown to be significantly induced by TCDD as early as 2 h after exposure in only male C57BL/6 mice (Celius et al., 2008). Previous studies of *Ahr*-null mice have shown that this induction is a direct result of TCDD-activation of the AHR (Tijet et al., 2006). Two species of carboxylesterases, *Ces1b* and *Ces4a*, showed statistically significant variation along the time-course; however, with differing directions of change: *Ces1b* showed significantly increased hepatic expression while *Ces4a* decreased in expression in TCDD-treated males relative to control animals (Fig. 3). There has been little or no documentation to date on the contribution of either *Ces1b* or *Ces4a* on toxic outcomes. However a related species, *Ces1d* (CES3), has been previously shown to decrease in expression following treatment with TCDD. This reduction has been attributed to secondary signaling events, and was

associated with steatohepatitis (Matsubara et al., 2012). Also of particular interest is *Nr1i3*, which encodes CAR, a nuclear receptor that aids in transcriptional regulation of genes associated with xenobiotic metabolism (including *Cyp1a1* and *Cyp1a2*), cell cycle, apoptosis and lipid metabolism (Tojima et al., 2012). Activated CAR has been shown to mediate the induction of c-Myc (Blanco-Bose et al., 2008), with chronic activation leading to hepatocarcinoma and liver injury (Huang et al., 2005; Yamazaki et al., 2011). Interestingly, CAR has been also been shown to induce expression of another of our candidate genes, *Gadd45b* (Yamamoto et al., 2010; Tojima et al., 2012) and this induction has been previously implicated in liver growth (Tian et al., 2011) and hepatic tumor promotion (Yamamoto et al., 2010). Furthermore, GADD45β has been shown to be an inducible coactivator of CAR (Tian et al., 2011). Finally, CAR has also been suggested to play a role in transcriptional regulation of carboxylesterases (Staudinger et al., 2010). Complicating the role of CAR in TCDD-induced hepatotoxicity is the lack of significant induction of *Cyp2b10* as it is known to be induced following activation of CAR (Honkakoski et al., 1998). However, as CAR is also known to regulate genes involved in lipid metabolism and energy homeostasis, particularly during times of metabolic stress (Maglich et al., 2004, 2009), these results may be confounded by the use of corn oil as a vehicle control. Further studies are required to better understand this relationship.

Here we have shown a significant divergence in the hepatic transcriptional profiles of TCDD-treated male and female mice that may correspond to the phenotypic difference of TCDD-toxicity. The nuclear receptor *Nr1i3*, in connection with *Gadd45b* and carboxylesterase species, may have a role in the development of toxic effects in the more TCDD-sensitive males. Further studies in additional species, particularly in male and female rats in which the sensitivity profiles are reversed, could shed light on their complex relationships and roles in TCDD-mediated toxicity. Specifically, studies examining the transcriptional role of *Nr1i3*, through binding site analysis and/or ChIP-chip targeting of CAR, as well as CAR-knockouts could be especially beneficial.

Methods

Animal handling. Male and female wild-type C57BL/6J mice were obtained from the National Public Health Institute, Division of Environmental Health, Kuopio, Finland. The current substrain (C57BL/6Kuo) was generated through multiple years of inbreeding. While male mice reach maturity at 34–38 days, female mice do not reach maturity until 6–8 weeks of age. Therefore, to maintain similarity between groups, mice were 12–15 weeks old at the time of this experiment. Animals were housed in Macrolon cages with pelleted Altromin 1314 feed (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and tap water available *ab libitum*. The housing environment was maintained at 21 °C with 50 ± 10% relative humidity and artificial illumination on a 12/12 hour light/dark cycle. Animals were housed individually to avoid aggressive behavior. No attempt was made to verify the stage of the estrus cycle in female mice at termination. All study plans were approved by the Finnish National Animal Experiment Board (Eläinkoelautakunta, ELLA; permit code: ESLH-2008-07223/Ym-23).

Experimental design. A total of 65 animals (34 female and 31 male) were employed in this study. Animals were divided into experimental and control groups and treated by oral gavage with a single bolus of either TCDD dissolved in corn oil vehicle or corn oil alone (10 mL/kg). Mice in the experimental group received a 500 μg/kg dose of TCDD. This dose was selected as it discriminates the sexes with respect to fatality: the approximate LD_{50} for male C57BL/6 mice is 305 μg/kg while females can tolerate over 5000 μg/kg TCDD (Pohjanvirta et al., 2012). Animals were euthanized at 6, 24, 72 or 144 h post-treatment with carbon dioxide, followed immediately by cardiac exsanguination. Livers were excised and frozen in liquid nitrogen. Tissue was shipped on dry ice to the analytical laboratory and stored at −80 °C or colder. Liver was selected as the tissue of interest as it exhibits numerous TCDD-induced toxicities and is the site of xenobiotic metabolism. All animal handling and reporting comply with ARRIVE guidelines (Kilkenny et al., 2010). Each experimental group (sex, treatment, time-point) contained between 3 and 5 animals (Supplementary Table 1). The overall experimental design is outlined in Fig. 1.

Microarray hybridization. RNA was isolated as described previously (Prokopec et al., 2013). Briefly, tissue samples were ground to a fine powder in liquid nitrogen using a mortar and pestle, followed by addition of lysis buffer and rapid homogenization using a Brinkmann Polytron (Polytron PT1600E with a PT-DA 1607 generator). RNA was isolated using an RNeasy Mini Kit (Qiagen, Mississauga, Canada) following the manufacturer's instructions and quantification was performed using a NanoDrop UV spectrophotometer (Thermo Scientific, Mississauga, ON). RNA integrity was verified using RNA 6000 Nano kits on an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, Canada); all samples had an RNA integrity number above 8.5 and were used for downstream analyses. RNA was assayed on Affymetrix MouseGene1.1ST arrays by The Centre for Applied Genomics (TCAG) at The Hospital for Sick Children (Toronto, Canada).

Microarray pre-processing. Raw array data (CEL files) were loaded into the R statistical environment (v3.0.1) using the affy package (v1.38.1) of the BioConductor library (Gentleman et al., 2004). Gene annotation was performed using mogene11stmmentrezgcdf (v17.1.0), a custom EntrezGene ID map (Dai et al., 2005). Data for all samples were pre-processed and normalized together using the RMA algorithm (Irizarry et al., 2003). Data were assessed for distributional homogeneity and visualized using the lattice (v0.20-23) and latticeExtra (v0.6-26) packages (Supplementary Fig. 1); variability was observed and further analyses performed to identify outliers. Expression levels of various sex specific genes (Conforto and Waxman, 2012), as well as genes found on chromosome Y, were used to verify sex of the animals. DIANA hierarchical clustering using Pearson's correlation as a similarity metric was used to classify animal sex; seven arrays documented to be female were classified as male (Supplementary Fig. 2A). Similarly, expression of *Cyp1a1* was used to verify sample treatment; three of the above arrays (untreated) also displayed *Cyp1a1* levels equivalent to TCDD-treated animals (Supplementary Fig. 2B). To identify the point at which sample mix-ups may have occurred, qPCR was performed to compare *Cyp1a1* levels in cDNA prepared in house to that prepared and returned by TCAG for all samples. In all cases, samples prepared in house displayed the expected results, while those from TCAG did not (data not shown). In addition, gDNA was isolated from stored tissue for samples identified above, as well as a subsample of the remaining samples for comparison purposes, and *Sry* detection performed by PCR to validate the sample sex. Again, all samples as the expected sex (data not shown), indicating a sample mix-up error immediately prior to analysis. In total, seven arrays (1 treated, 6 control, all female) were excluded from the analysis. Remaining arrays were re-normalized as above and quality re-assessed; no outliers were detected (Supplementary Fig. 3). Probe-level filtering was performed to remove probes with intensity levels below background intensity, identified by evaluation of chromosome Y probes in female animals (Supplementary Fig. 4). Probes with greatest variance (variance > 2.0) were visualized using a heatmap; normalized intensity values were scaled (mean-centered with standard deviation-scaling) and both probes and samples were clustered using DIANA, as described above. Sample and array information are provided in Supplementary Table 1. Raw and pre-processed microarray data are available in the GEO repository under accession GSE61037.

Statistical analysis and visualization. Statistical analyses were performed to identify differentially expressed genes using the limma package (v3.16.7) for R (v3.0.1). Specifically, linear modeling was performed separately for each time-point using a nested interaction design [Y = Sex + Sex: TCDD] to identify genes with differential expression, such that *Sex* evaluates genes in a sex-dependent/TCDD-independent manner and *Sex:TCDD* identifies differentially expressed genes in a sex- and TCDD-dependent manner. Standard errors of the coefficients were adjusted using an empirical Bayes moderation of the standard error (Smyth, 2004) and model-based t-tests were applied to the coefficients, followed by false-discovery rate adjustment for multiple testing (Storey and Tibshirani, 2003). A full list of annotated genes with coefficients representing magnitude of differential expression and FDR-corrected p-values (p_{adj}-values) is provided in Supplementary Table 2. For downstream analyses, a threshold of $p_{adj} < 0.01$ and absolute $\log_2$-foldchange > 1 was used to define statistically significant genes. Venn diagrams were generated using the VennDiagram package (v1.6.7) to visualize overlap between groups (Chen and Boutros, 2011), and hypergeometric testing used to determine significance of overlap (data not shown). Coefficients and significance of candidate genes were visualized using lattice (v0.20-29) and latticeExtra (v0.6-26) packages for R.

Analysis of AHR-binding. In order to determine whether TCDD alters binding of the AHR to candidate genes, we performed an analysis of AHR-DNA interactions using publicly available chromatin immunoprecipitation DNA microarray (ChIP-chip) data. Raw CEL files for TCDD-treated (GSM299310, GSM299311) and control (DMSO-treated; GSM299306, GSM299307) mouse liver cells were downloaded from the GEO repository (Series GSE11850) (Sartor et al., 2009). Data were RMA normalized using the oligo package (v1.28.2) in R (v3.1.0) and probes were annotated to genomic locations using the binary probe map (NCBI build 35) provided by Affymetrix. Probes were further mapped to specific gene symbols (TSS ± 1000 bp) using cisGenome (Ji et al., 2008) and refFlat tables (mm7, downloaded on June 2, 2014) (Karolchik et al., 2003). Student's t-tests were used to identify AHR enrichment between TCDD treated and control samples. For each gene, the probe with the lowest p-value was reported (Supplementary Table 3).

Transcription factor binding site analysis. To further verify the role of the AHR in TCDD-mediated transcriptional regulation, we performed a transcription-factor binding site analysis by searching for typical AHR-binding motif sequences. Specifically, we examined 4 motifs: AHRE-I (core), AHRE-I (extended), AHRE-I (full) (Denison and Whitlock, 1995), and AHRE-II (Sogawa et al., 2004), with sequences: GCGTG, TNGCGTG, [T|G]NGCGTG[A|C][G|C]A, and CATG{N6}C[T|A]TG respectively. Transcription start sites for each gene (TSS ± 3000 bp) were identified using refLink and refFlat tables (mm9; obtained from the UCSC genome browser on July 15, 2014), and sequences were examined for the above motifs. Counts for each motif in each gene are provided in Supplementary Table 4.

Pathway analysis. Pathway analysis was performed using GoMiner software (Zeeberg et al., 2005). Specifically, the web interface tool for High-Throughput GOMiner analysis (application build 454, database build 2011-01) was used to associate those genes significantly altered by TCDD exposure (thresholds described above) in each experiment with known gene ontologies. Genes of interest were checked against a random sample of the dataset using a FDR threshold of 0.1 and 1000 randomizations. All mouse databases and look-up options were used, as were all GO evidence codes and ontologies. A minimum of five genes was required to identify enrichment. Results were visualized using the VennDiagram (v1.6.9), lattice (v0.20-29), and latticeExtra (v0.6-26) packages for R.

Protein analysis. Western blot analyses were performed as described previously (Prokopec et al., 2014). Briefly, total protein was isolated using Tissue Extraction Reagent I (Life Technologies, Burlington, ON) supplemented with cOmplete protease inhibitor cocktail (Roche, Laval, QC). Bradford reagent (Sigma-Aldrich, Oakville, Canada) was used for quantification, and protein was diluted with additional extraction reagent to a final concentration of 10 μg/μL. Protein was loaded into each well of a Novex 4–12% Bis–Tris midi-gel system (65 μg/sample) and electrophoresed for 40 min at 200 V with MES running buffer (Life Technologies). Protein was subsequently transferred to PVDF membrane using the iBlot system (program P0 for 7 minutes, Life Technologies). Blots were blocked using 5% non-fat milk or LI-COR blocking buffer (Mandel Scientific, Guelph, ON) for 60 min at room temperature. Primary antibodies were purchased from Abcam (Abcam Inc., Toronto, ON). Antibodies were diluted to the specified concentrations in 5% non-fat milk or LI-COR blocking buffer supplemented with 0.1% Tween-20 and blots were incubated overnight at 4 °C or for 2 h at room temperature. Blots were washed three times with PBS supplemented with 0.1% Tween-20 at room temperature for 5 min each, followed by incubation with secondary antibody (LI-COR IRDye-labeled secondary antibody) at a 1:10,000 dilution in the same buffer as for the primary incubation with 0.01% SDS, for 4 h at 4 °C or 1 h at room temperature. Blots were washed as above and scanned using the Odyssey quantitative western blot near-infrared system (LI-COR Biosciences, Lincoln, NE, USA) using default settings. Average band intensities were first adjusted for background levels, followed by normalization with HPRT and ACTIN (Prokopec et al., 2014). Ordering information and specific incubation conditions for each antibody (as determined through extensive optimization) are provided in Supplementary Table 5. Unlike for the transcriptomic analyses, all 65 animals were used for proteomic validation as tissue samples validated as expected.

Authors' contributions

Animal handling: RP
Sample preparation: SDP, JDW
Performed statistical and bioinformatics analyses: SDP; JL
Wrote the first draft of the manuscript: SDP
Initiated the project: RP, PCB
Supervised research: RP, PCB
Approved the manuscript: all authors

Conflict of interest statement

All authors declare that they have no conflicts of interest.

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References

Blanco-Bose, W.E., Murphy, M.J., Ehninger, A., Offner, S., Dubey, C., Huang, W., Moore, D.D., Trumpp, A., 2008. C-Myc and its target FoxM1 are critical downstream effectors of constitutive androstane receptor (CAR) mediated direct liver hyperplasia. Hepatology 48, 1302–1311.

Boutros, P.C., Yan, R., Moffat, I.D., Pohjanvirta, R., Okey, A.B., 2008. Transcriptomic responses to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in liver: comparison of rat and mouse. BMC Genomics 9, 419.

Boverhof, D.R., Burgoon, L.D., Tashiro, C., Chittim, B., Harkema, J.R., Jump, D.B., Zacharewski, T.R., 2005. Temporal and dose-dependent hepatic gene expression patterns in mice provide new insights into TCDD-Mediated hepatotoxicity. Toxicol. Sci. 85, 1048–1063.

Boverhof, D.R., Burgoon, L.D., Tashiro, C., Sharratt, B., Chittim, B., Harkema, J.R., Mendrick, D.L., Zacharewski, T.R., 2006. Comparative toxicogenomic analysis of the hepatotoxic effects of TCDD in Sprague Dawley rats and C57BL/6 mice. Toxicol. Sci. 94, 398–416.

Bunger, M.K., Moran, S.M., Glover, E., Thomae, T.L., Lahvis, G.P., Lin, B.C., Bradfield, C.A., 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.

Celius, T., Roblin, S., Harper, P.A., Matthews, J., Boutros, P.C., Pohjanvirta, R., Okey, A.B., 2008. Aryl hydrocarbon receptor-dependent induction of flavin-containing monooxygenase mRNAs in mouse liver. Drug Metab. Dispos. 36, 2499–2505.

Chen, H., Boutros, P.C., 2011. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. BMC Bioinformatics 12, 35.

Conforto, T.L., Waxman, D.J., 2012. Sex-specific mouse liver gene expression: genome-wide analysis of developmental changes from pre-pubertal period to young adulthood. Biol. Sex Differ. 3, 9.

Dai, M., Wang, P., Boyd, A.D., Kostov, G., Athey, B., Jones, E.G., Bunney, W.E., Myers, R.M., Speed, T.P., Akil, H., Watson, S.J., Meng, F., 2005. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. Nucleic Acids Res. 33, e175.

Deb, S., Bandiera, S.M., 2010. Characterization of a new cytochrome P450 enzyme, CYP2S1, in rats: its regulation by aryl hydrocarbon receptor agonists. Toxicology 267, 91–98.

Denison, M.S., Whitlock Jr., J.P., 1995. Xenobiotic-inducible transcription of cytochrome P450 genes. J. Biol. Chem. 270, 18175–18178.

Dere, E., Lee, A.W., Burgoon, L.D., Zacharewski, T.R., 2011. Differences in TCDD-elicited gene expression profiles in human HepG2, mouse Hepa1c1c7 and rat H4IIE hepatoma cells. BMC Genomics 12, 193.

Diliberto, J.J., Akubue, P.I., Luebke, R.W., Birnbaum, L.S., 1995. Dose-response relationships of tissue distribution and induction of CYP1A1 and CYP1A2 enzymatic activities following acute exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice. Toxicol. Appl. Pharmacol. 130, 197–208.

Enan, E., Overstreet, J.W., Matsumura, F., VandeVoort, C.A., Lasley, B.L., 1996. Gender differences in the mechanism of dioxin toxicity in rodents and in nonhuman primates. Reprod. Toxicol. 10, 401–411.

Falls, J.G., Ryu, D.Y., Cao, Y., Levi, P.E., Hodgson, E., 1997. Regulation of mouse liver flavin-containing monooxygenases 1 and 3 by sex steroids. Arch. Biochem. Biophys. 342, 212–223.

Fernandez-Salguero, P.M., Hilbert, D.M., Rudikoff, S., Ward, J.M., Gonzalez, F.J., 1996. Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity. Toxicol. Appl. Pharmacol. 140, 173–179.

Forgacs, A.L., Dere, E., Angrish, M.M., Zacharewski, T.R., 2013. Comparative analysis of temporal and dose-dependent TCDD-elicited gene expression in human, mouse, and rat primary hepatocytes. Toxicol. Sci. 133, 54–66.

Forman, B.M., Tzameli, I., Choi, H.S., Chen, J., Simha, D., Seol, W., Evans, R.M., Moore, D.D., 1998. Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. Nature 395, 612–615.

Franc, M.A., Moffat, I.D., Boutros, P.C., Tuomisto, J.T., Tuomisto, J., Pohjanvirta, R., Okey, A.B., 2008. Patterns of dioxin-altered mRNA expression in livers of dioxin-sensitive versus dioxin-resistant rats. Arch. Toxicol. 82, 809–830.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., Zhang, J., 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.

Henck, J.M., New, M.A., Kociba, R.J., Rao, K.S., 1981. 2,3,7,8-tetrachlorodibenzo-p-dioxin: acute oral toxicity in hamsters. Toxicol. Appl. Pharmacol. 59, 405–407.

Honkakoski, P., Zelko, I., Sueyoshi, T., Negishi, M., 1998. The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. Mol. Cell. Biol. 18, 5652–5658.

Huang, W., Zhang, J., Washington, M., Liu, J., Parant, J.M., Lozano, G., Moore, D.D., 2005. Xenobiotic stress induces hepatomegaly and liver tumors via the nuclear receptor constitutive androstane receptor. Mol. Endocrinol. 19, 1646–1653.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., Speed, T.P., 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249–264.

Ji, H., Jiang, H., Ma, W., Johnson, D.S., Myers, R.M., Wong, W.H., 2008. An integrated software system for analyzing ChIP-chip and ChIP-seq data. Nat. Biotechnol. 26, 1293–1300.

Kanno, Y., Otsuka, S., Hiromasa, T., Nakahama, T., Inouye, Y., 2004. Diurnal difference in CAR mRNA expression. Nucl. Recept. 2, 6.

Karolchik, D., Baertsch, R., Diekhans, M., Furey, T.S., Hinrichs, A., Lu, Y.T., Roskin, K.M., Schwartz, M., Sugnet, C.W., Thomas, D.J., Weber, R.J., Haussler, D., Kent, W.J., 2003. The UCSC genome browser database. Nucleic Acids Res. 31, 51–54.

Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 8, e1000412.

Korkalainen, M., Tuomisto, J., Pohjanvirta, R., 2004. Primary structure and inducibility by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) of aryl hydrocarbon receptor repressor in a TCDD-sensitive and a TCDD-resistant rat strain. Biochem. Biophys. Res. Commun. 315, 123–131.

Kransler, K.M., McGarrigle, B.P., Swartz, D.D., Olson, J.R., 2009. Lung development in the Holtzman rat is adversely affected by gestational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Sci. 107, 498–511.

Lu, Y.F., Jin, T., Xu, Y., Zhang, D., Wu, Q., Zhang, Y.K., Liu, J., 2013. Sex differences in the circadian variation of cytochrome p450 genes and corresponding nuclear receptors in mouse liver. Chronobiol. Int. 30, 1135–1143.

Maglich, J.M., Watson, J., McMillen, P.J., Goodwin, B., Willson, T.M., Moore, J.T., 2004. The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction. J. Biol. Chem. 279, 19832–19838.

Maglich, J.M., Lobe, D.C., Moore, J.T., 2009. The nuclear receptor CAR (NR1I3) regulates serum triglyceride levels under conditions of metabolic stress. J. Lipid Res. 50, 439–445.

Magre, S., Rebourcet, D., Ishaq, M., Wargnier, R., Debard, C., Meugnier, E., Vidal, H., Cohen-Tannoudji, J., Le Magueresse-Battistoni, B., 2012. Gender differences in transcriptional signature of developing rat testes and ovaries following embryonic exposure to 2,3,7,8-TCDD. PLoS ONE 7, e40306.

Marques, M., Laflamme, L., Gaudreau, L., 2013. Estrogen receptor alpha can selectively repress dioxin receptor-mediated gene expression by targeting DNA methylation. Nucleic Acids Res. 41, 8094–8106.

Matsubara, T., Tanaka, N., Krausz, K.W., Manna, S.K., Kang, D.W., Anderson, E.R., Luecke, H., Patterson, A.D., Shah, Y.M., Gonzalez, F.J., 2012. Metabolomics identifies an inflammatory cascade involved in dioxin- and diet-induced steatohepatitis. Cell Metab. 16, 634–644.

Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T.N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M., Fujii-Kuriyama, Y., 1997. Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. Genes Cells 2, 645–654.

Moffat, I.D., Boutros, P.C., Chen, H., Okey, A.B., Pohjanvirta, R., 2010. Aryl hydrocarbon receptor (AHR)-regulated transcriptomic changes in rats sensitive or resistant to major dioxin toxicities. BMC Genomics 11, 263.

Nault, R., Kim, S., Zacharewski, T.R., 2013. Comparison of TCDD-elicited genome-wide hepatic gene expression in Sprague–Dawley rats and C57BL/6 mice. Toxicol. Appl. Pharmacol. 267, 184–191.

Nebert, D.W., Puga, A., Vasiliou, V., 1993. Role of the Ah receptor and the dioxin-inducible [Ah] gene battery in toxicity, cancer, and signal transduction. Ann. N. Y. Acad. Sci. 685, 624–640.

Nukaya, M., Walisser, J.A., Moran, S.M., Kennedy, G.D., Bradfield, C.A., 2010. Aryl hydrocarbon receptor nuclear translocator in hepatocytes is required for aryl hydrocarbon receptor-mediated adaptive and toxic responses in liver. Toxicol. Sci. 118, 554–563.

Pohjanvirta, R., 2009. Transgenic mouse lines expressing rat AH receptor variants—a new animal model for research on AH receptor function and dioxin toxicity mechanisms. Toxicol. Appl. Pharmacol. 236, 166–182.

Pohjanvirta, R., Tuomisto, J., 1994. Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals: effects, mechanisms, and animal models. Pharmacol. Rev. 46, 483–549.

Pohjanvirta, R., Vartiainen, T., Uusi-Rauva, A., Monkkonen, J., Tuomisto, J., 1990. Tissue distribution, metabolism, and excretion of 14C-TCDD in a TCDD-susceptible and a TCDD-resistant rat strain. Pharmacol. Toxicol. 66, 93–100.

Pohjanvirta, R., Unkila, M., Tuomisto, J., 1993. Comparative acute lethality of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-p-dioxin and 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin in the most TCDD-susceptible and the most TCDD-resistant rat strain. Pharmacol. Toxicol. 73, 52–56.

Pohjanvirta, R., Wong, J.M., Li, W., Harper, P.A., Tuomisto, J., Okey, A.B., 1998. Point mutation in intron sequence causes altered carboxyl-terminal structure in the aryl hydrocarbon receptor of the most 2,3,7,8-tetrachlorodibenzo-p-dioxin-resistant rat strain. Mol. Pharmacol. 54, 86–93.

Pohjanvirta, R., Viluksela, M., Tuomisto, J.T., Unkila, M., Karasinska, J., Franc, M.A., Holowenko, M., Giannone, J.V., Harper, P.A., Tuomisto, J., Okey, A.B., 1999. Physicochemical differences in the AH receptors of the most TCDD-susceptible and the most TCDD-resistant rat strains. Toxicol. Appl. Pharmacol. 155, 82–95.

Pohjanvirta, R., Miettinen, H., Sankari, S., Hegde, N., Linden, J., 2012. Unexpected gender difference in sensitivity to the acute toxicity of dioxin in mice. Toxicol. Appl. Pharmacol. 262, 167–176.

Prokopec, S.D., Buchner, N.B., Fox, N.S., Chong, L.C., Mak, D.Y., Watson, J.D., Petronis, A., Pohjanvirta, R., Boutros, P.C., 2013. Validating reference genes within a mouse model system of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) toxicity. Chem. Biol. Interact. 205, 63–71.

Prokopec, S.D., Watson, J.D., Pohjanvirta, R., Boutros, P.C., 2014. Identification of reference proteins for western blot analyses in mouse model systems of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) toxicity. PLoS ONE 9 (10), e110730.

Sartor, M.A., Schnekenburger, M., Marlowe, J.L., Reichard, J.F., Wang, Y., Fan, Y., Ma, C., Karyala, S., Halbleib, D., Liu, X., Medvedovic, M., Puga, A., 2009. Genomewide analysis of aryl hydrocarbon receptor binding targets reveals an extensive array of gene clusters that control morphogenetic and developmental programs. Environ. Health Perspect. 117, 1139–1146.

Schecter, A., Birnbaum, L., Ryan, J.J., Constable, J.D., 2006. Dioxins: an overview. Environ. Res. 101, 419–428.

Schwetz, B.A., Norris, J.M., Sparschu, G.L., Rowe, U.K., Gehring, P.J., Emerson, J.L., Gerbig, C.G., 1973. Toxicology of chlorinated dibenzo-p-dioxins. Environ. Health Perspect. 5, 87–99.

Shen, C., Chen, Y., Huang, S., Wang, Z., Yu, C., Qiao, M., Xu, Y., Setty, K., Zhang, J., Zhu, Y., Lin, Q., 2009. Dioxin-like compounds in agricultural soils near e-waste recycling sites from Taizhou area, China: chemical and bioanalytical characterization. Environ. Int. 35, 50–55.

Silkworth, J.B., Carlson, E.A., McCulloch, C., Illouz, K., Goodwin, S., Sutter, T.R., 2008. Toxicogenomic analysis of gender, chemical, and dose effects in livers of TCDD- or aroclor 1254-exposed rats using a multifactor linear model. Toxicol. Sci. 102, 291–309.

Smyth, G.K., 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3 (Article3).

Sogawa, K., Numayama-Tsuruta, K., Takahashi, T., Matsushita, N., Miura, C., Nikawa, J., Gotoh, O., Kikuchi, Y., Fujii-Kuriyama, Y., 2004. A novel induction mechanism of the rat CYP1A2 gene mediated by Ah receptor-Arnt heterodimer. Biochem. Biophys. Res. Commun. 318, 746–755.

Staudinger, J.L., Xu, C., Cui, Y.J., Klaassen, C.D., 2010. Nuclear receptor-mediated regulation of carboxylesterase expression and activity. Expert Opin. Drug Metab. Toxicol. 6, 261–271.

Storey, J.D., Tibshirani, R., 2003. Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. U. S. A. 100, 9440–9445.

Theobald, H.M., Peterson, R.E., 1997. In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin: effects on development of the male and female reproductive system of the mouse. Toxicol. Appl. Pharmacol. 145, 124–135.

Tian, J., Huang, H., Hoffman, B., Liebermann, D.A., Ledda-Columbano, G.M., Columbano, A., Locker, J., 2011. Gadd45beta is an inducible coactivator of transcription that facilitates rapid liver growth in mice. J. Clin. Invest. 121, 4491–4502.

Tijet, N., Boutros, P.C., Moffat, I.D., Okey, A.B., Tuomisto, J., Pohjanvirta, R., 2006. Aryl hydrocarbon receptor regulates distinct dioxin-dependent and dioxin-independent gene batteries. Mol. Pharmacol. 69, 140–153.

Tojima, H., Kakizaki, S., Yamazaki, Y., Takizawa, D., Horiguchi, N., Sato, K., Mori, M., 2012. Ligand dependent hepatic gene expression profiles of nuclear receptors CAR and PXR. Toxicol. Lett. 212, 288–297.

Watson, J.D., Prokopec, S.D., Smith, A.B., Okey, A.B., Pohjanvirta, R., Boutros, P.C., 2014. TCDD dysregulation of 13 AHR-target genes in rat liver. Toxicol. Appl. Pharmacol. 274 (3), 445–454.

Yamamoto, Y., Moore, R., Flavell, R.A., Lu, B., Negishi, M., 2010. Nuclear receptor CAR represses TNFalpha-induced cell death by interacting with the anti-apoptotic GADD45B. PLoS ONE 5, e10121.

Yamazaki, Y., Moore, R., Negishi, M., 2011. Nuclear receptor CAR (NR1I3) is essential for DDC-induced liver injury and oval cell proliferation in mouse liver. Lab. Investig. 91, 1624–1633.

Yao, C.Q., Prokopec, S.D., Watson, J.D., Pang, R., P'ng, C., Chong, L.C., Harding, N.J., Pohjanvirta, R., Okey, A.B., Boutros, P.C., 2012. Inter-strain heterogeneity in rat hepatic transcriptomic responses to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Toxicol. Appl. Pharmacol. 260, 135–145.

Yeager, R.L., Reisman, S.A., Aleksunes, L.M., Klaassen, C.D., 2009. Introducing the "TCDD-inducible AhR-Nrf2 gene battery". Toxicol. Sci. 111, 238–246.

Zeeberg, B.R., Qin, H., Narasimhan, S., Sunshine, M., Cao, H., Kane, D.W., Reimers, M., Stephens, R.M., Bryant, D., Burt, S.K., Elnekave, E., Hari, D.M., Wynn, T.A., Cunningham-Rundles, C., Stewart, D.M., Nelson, D., Weinstein, J.N., 2005. High-Throughput GoMiner, an 'industrial-strength' integrative gene ontology tool for interpretation of multiple-microarray experiments, with application to studies of Common Variable Immune Deficiency (CVID). BMC Bioinformatics 6, 168.