

MOL PHARM # 45658

Phenotype of the *Cyp1a1/1a2/1b1*(-/-) Triple-Knockout Mouse

***Nadine Dragin, *Zhanquan Shi, Rajat Madan, Christopher L. Karp, Maureen A. Sartor,**

Chi Chen, Frank J. Gonzalez and Daniel W. Nebert**

Department of Environmental Health, and the Center for Environmental Genetics (CEG),

University Cincinnati Medical Center, Cincinnati, OH 45267-0056

(N.D., M.L.M., Z.S., M.A.S., D.W.N.)

Division of Molecular Immunology, Cincinnati Children's Hospital Research Foundation,

Cincinnati, OH 45229-0054

(R.M., C.K.)

Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute,

National Institutes of Health, Bethesda, MD 20892

(C.C., F.J.G.)

Running Title: “Phenotype of the *Cyp1* Triple-Knockout”

**Address correspondence to: Daniel W. Nebert, MD, Department of Environmental Health, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati OH 45267-0056, U.S.A. Tel. 513-558-4347; Fax 513-558-3562; email dan.nebert@uc.edu

Pages of Text = 20

Tables = 5

Figures = 6

References = 70

Words in **Abstract** = 250

Words in **Introduction** = 452

Words in **Discussion** = 1621

Abbreviations used :

Alanine-aminotransferase, ALT

Aspartate-aminotransferase, AST

Benzo[*a*]pyrene, BaP

Cytochrome oxidase, subunit VIb, polypeptide 2, COX6B2

Cytochrome P450 1A1, CYP1A1

Cytochrome P450 1A2, CYP1A2

Cytochrome P450 1B1, CYP1B1

Gene ontology, GO

NAD(P)H:quinone oxidoreductase, NQO1

Polycyclic aromatic hydrocarbons, PAHs

Principal components analysis, PCA

Real-time quantitative-polymerase chain reaction, Q-PCR

Suppressor of cytokine-signaling-2, SOCS2

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, TCDD or “dioxin”

Ultra-performance liquid chromatography quantitative time-of-flight mass spectrometry, UPLC-QTOFMS

Abstract

Crossing the *Cyp1a1/1a2*(-/-) double-knockout with the *Cyp1b1*(-/-) single-knockout, we generated the *Cyp1a1/1a2/1b1*(-/-) triple-knockout mouse. In this triple-knockout, statistically significant phenotypes (with incomplete penetrance) included slower weight gain and greater risk of: embryolethality before gestational day 11, hydrocephalus, hermaphroditism, and cystic ovaries. Oral benzo[*a*]pyrene (**BaP**) daily for 18 days in the *Cyp1a1/1a2*(-/-) produced the same degree of marked immunosuppression as seen in the *Cyp1a1*(-/-) mouse; we believe this reflects the absence of intestinal CYP1A1. Oral BaP-treated *Cyp1a1/1a2/1b1*(-/-) mice showed the same “rescued” response as that seen in the *Cyp1a1/1b1*(-/-) mouse; we believe this reflects the absence of CYP1B1 in immune tissues. Urinary metabolite profiles were dramatically different between untreated triple-knockout and wild-type; principal components analysis showed that the shifts in urinary metabolite patterns in oral BaP-treated triple-knockout and wild-type mice were also strikingly different. Liver microarray cDNA differential expression (comparing triple-knockout with wild-type) revealed at least 89 genes up- and 62 genes down-regulated (P -value ≤ 0.00086). Gene Ontology “classes of genes” most perturbed in the untreated triple-knockout (compared with wild-type) include: lipid, steroid and cholesterol biosynthesis and metabolism; nucleosome and chromatin assembly; carboxylic and organic acid metabolism; metal-ion binding; and ion homeostasis. In the triple-knockout compared with the wild-type, response to zymosan-induced peritonitis was strikingly exaggerated, which may well reflect down-regulation of *Socs2* expression. If a single common molecular pathway is responsible for all of these phenotypes, we suggest that functional effects of the loss of all three *Cyp1* genes could be explained by perturbations in CYP1-mediated eicosanoid production, catabolism and activities.

(Introduction)

Cytochrome P450 (**CYP**) proteins are heme-thiolate enzymes involved in innumerable cellular functions: eicosanoid synthesis and degradation; cholesterol, sterol, lipid, and bile acid biosynthesis; steroid synthesis and metabolism; biogenic amine synthesis and degradation; vitamin D₃ synthesis and metabolism; and hydroxylation of retinoic acid and probably other morphogens. A few CYP enzymes still have no unequivocally identified functions (Nebert and Russell, 2002; Nelson et al., 2004). The mouse and human *CYP* gene superfamilies contain 102 and 57 protein-coding genes, respectively (Nelson et al., 2004). Drugs, environmental procarcinogens and toxicants—as well as the more than 130 eicosanoids—are metabolized largely by enzymes in the CYP1, CYP2, CYP3 and CYP4 families (Nebert and Dalton, 2006).

Among the 18 mammalian CYP families, CYP1 comprises three orthologous members in human and mouse: CYP1A1, CYP1A2 and CYP1B1. The three *CYP1* genes are up-regulated via the aryl hydrocarbon receptor (**AHR**), a transcription factor that binds as a heterodimer with the AHR nuclear transporter (**ARNT**) to DNA motifs known as AHR response elements (Nebert and Russell, 2002; Nelson et al., 2004; Nebert et al., 2004). CYP1 inducers usually are ligands that activate the AHR, thereby stimulating the receptor to migrate from cytosol to the nucleus (Tukey et al., 1982); these ligands include benzo[*a*]pyrene (**BaP**) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (**TCDD**) (Nebert et al., 2004; Nebert and Dalton, 2006). Several classes of endogenous compounds that activate the AHR have been reported: [a] tryptophan metabolites and other indole-containing molecules; [b] tetrapyrroles such as bilirubin and biliverdin; [c] sterols such as 7-ketocholesterol and equilenin; [d] fatty acid metabolites, including several prostaglandins and lipoxin A₄; and [e] the ubiquitous second-messenger cAMP (McMillan and Bradfield, 2007). However, the dissociation constant of

binding (K_d) for most of these compounds is not as low as one would expect for physiologically relevant ligands of the AHR.

The *Cyp1a1*(-/-) (Dalton et al., 2000), *Cyp1a2*(-/-) (Liang et al., 1996), and *Cyp1b1*(-/-) (Buters et al., 1999) knockout mouse lines have been generated; from these, straightforward genetic crosses were performed to create the *Cyp1a1/1b1*(-/-) and *Cyp1a2/1b1*(-/-) double-knockouts (Uno et al., 2006). The *Cyp1a1* and *Cyp1a2* genes are located on mouse chromosome 9 at cM 31.0, while the mouse *Cyp1b1* gene is located on mouse chromosome 17. The *Cyp1a2* gene arose from a *Cyp1a1* duplication event ~450 million years ago. The two mouse genes are oriented head-to-head, sharing a 13,954-bp bidirectional promoter; therefore, creation of the *Cyp1a1/1a2*(-/-) double-knockout line was successful by means of an interchromosomal Cre/*loxP*-mediated excision of 26,173 bp (Dragin et al., 2007). A straightforward genetic cross between this double-knockout and the *Cyp1b1*(-/-) line has now generated the *Cyp1a1/1a2/1b1*(-/-) triple-knockout animal. Herein we describe the phenotype observed in this mouse line—in which for the first time all three *Cyp1* gene activities have been ablated.

Materials and Methods

Chemicals. BaP and zymosan A were purchased from Sigma Chemical Company (St. Louis, MO). TCDD was bought from Accustandard, Inc. (New Haven, CT). All other chemicals and reagents were obtained from either Aldrich Chemical Company (Milwaukee, WI) or Sigma as the highest available grades.

Animals. The generation of the *Cyp1a1*(-/-) (Dalton et al., 2000), *Cyp1a2*(-/-) (Liang et al., 1996) and *Cyp1b1*(-/-) (Buters et al., 1999) mouse lines and studies with the *Cyp1a1/1b1*(-/-) and *Cyp1a2/1b1*(-/-) double-knockout lines (Uno et al., 2006) have been described. The *Cyp1a1/1a2*(-/-) double-knockout was generated via Cre-mediated interchromosomal excision (Dragin et al., 2007). All these genotypes have been backcrossed into the C57BL/6J background for eight generations, ensuring that the knockout genotypes reside

in a genetic background that is >99.8% C57BL/6J (Nebert et al., 2000a). Age-matched C57BL/6J *Cyp1*(+/+) wild-type mice from The Jackson Laboratory (Bar Harbor, ME) therefore make comparable controls. Breeding of the *Cyp1a1/1a2*(-/-) with the *Cyp1b1*(-/-) mouse produced the *Cyp1a1/1a2/1b1*(-/-) triple-knockout line in the >99.8% C57BL/6J background. Except for the breeding studies, all other experiments were carried out in males and begun at 6 ± 1 weeks of age. In some instances, pretreatment with intraperitoneal TCDD (15 μ g/kg; as the prototypical *Cyp1* inducer) in corn oil was given as a single dose 48 h before sacrifice. TCDD is known to up-regulate dozens of genes that have AHR response elements in their regulatory regions. All animal experiments were approved by, and conducted in accordance with, the National Institutes of Health standards for the care and use of experimental animals and the University Cincinnati Medical Center Institutional Animal Care and Use Committee.

Breeding, In Utero Deaths, and Teratology. Various combinations of female and male genotypes were crossed, and the intrauterine contents were examined at gestational day 11 (**GD11**), GD13, GD15, GD17, GD19 and within hours of birth. GD0 was the day on which a vaginal plug was first detected. Genotyping for the ablated *Cyp1a1_1a2* locus (Dragin et al., 2007) and absence of the *Cyp1b1* gene (Buters et al., 1999) was carried out in embryos and fetuses (living or dead), resorbed fetal material, newborns, and weanlings.

Dietary BaP Experiments. BaP (125 mg/kg) was given orally (Uno et al., 2004; Uno et al., 2006; Uno et al., 2008). Lab rodent chow (Harlan Teklad; Madison, WI) was soaked in BaP-laced corn oil (10 mg/ml) for at least 24 h before presentation to mice; BaP at this concentration was calculated to be equivalent to ~125 mg/kg/day (Robinson et al., 1975). After 5 days in some mice, a 30- μ L blood sample was drawn from the saphenous vein, and total blood BaP was measured; also after 5 days of oral BaP, LC-MS studies of urinary metabolite profiles were determined. For all other studies, mice were sacrificed after 18 days of oral BaP; tissues (liver, spleen and thymus) were removed, weighed, and frozen as quickly as possible in liquid nitrogen (or prepared for pathology analysis). Peripheral blood and bone marrow smears were made for white cell differential counts. Levels of alanine-aminotransferase (**ALT**) and aspartate-aminotransferase (**AST**) activities,

in mouse plasma were also determined (Uno et al., 2004; Uno et al., 2006). Tissues were removed between 9:00 a.m. and 10:00 a.m. to exclude any circadian-rhythm effects. For each group, N = 4 to 6 mice.

Detection of BaP in Blood. BaP levels in whole blood were quantified by modification of previously described methods (Garcia-Falcon et al., 1996; Kim et al., 2000). Whole blood (30 μ L) was extracted three times with ethyl acetate/acetone mixture (2:1, v:v). The organic extracts were pooled and dried under argon, and the residue resuspended in 250 μ L of acetonitrile. An aliquot (100 μ L) was injected onto a Nova-Pak C₁₈ reverse-phase column (4- μ m, 150 x 3.9 mm i.d.; Waters Associates; Boston, MA). HPLC analysis was conducted on a WatersTM Model 600 solvent controller, equipped with a fluorescence detector (F-2000, Hitachi). Isocratic separation was performed using an acetonitrile:water (85:15, v:v) mobile phase at a flow-rate of 1 ml/min. Excitation and emission wavelengths were 294 and 404 nm, respectively. BaP concentrations in blood were calculated by comparing the peaks of samples with those of control blood that had been spiked with different known concentrations of BaP. The calibration curve for BaP showed excellent linearity (correlation coefficient $r > 0.998$); four major and several minor BaP metabolites were found to run far ahead of BaP on the column, and thus did not interfere. The detection limit (defined as 3 times the signal-to-noise ratio) was 0.05 pg/ μ L, and the limit of BaP quantification was determined to be 0.20 pg/ μ L. The intra-day and inter-day precision of repeated analyses (N=4) gave us coefficients of variation of $\leq 12\%$.

Biohazard Precaution. BaP and TCDD are highly toxic chemicals and regarded as likely human carcinogens. All personnel were instructed in safe handling procedures. Lab coats, gloves and masks were worn at all times, and contaminated materials were collected separately for disposal by the Hazardous Waste Unit or by independent contractors. BaP- and TCDD-treated mice were housed separately, and their carcasses considered as contaminated biological materials.

Urine Collection. Untreated, or oral BaP (125 mg/kg/5 days)-treated, *Cyp1*(+/+) and *Cyp1a1/1a2/1b1*(-/-) mice were placed individually in metabolic cages overnight, with food and water provided ad libitum. Urine was collected for 24 h and then frozen in liquid nitrogen. For each of the four groups, urine samples were collected from N = 6 individual mice.

LC-MS-based analysis of urinary metabolite profiles. Urine samples from untreated versus oral BaP-treated *Cyp1*(+/+) and *Cyp1a1/1a2/1b1*(-/-) mice were prepared for UPLC-QTOFMS analysis by mixing 50 μ L of urine with 200 μ L of 50% aqueous acetonitrile and centrifuging at $18,000 \times g$ for 5 min to remove protein and particulates. A 200- μ L aliquot of the supernatant fraction was transferred to an auto-sampler vial and a 5- μ L aliquot of each sample was injected into the UPLC-QTOFMS system (Waters; Milford, MA). An Acquity UPLC™ BEH C₁₈ column (Waters) was used to separate urinary metabolites at 30°C. The mobile-phase flow-rate was 0.5 mL/min, with a gradient ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid during a 10-min run. The QTOF Premier™ mass spectrometer was operated in the positive electrospray ionization mode. Capillary voltage and cone voltage were maintained at 3 kV and 20 V, respectively. Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as both the cone gas (50 L/h) and the desolvation gas (600 L/h), and argon as the collision gas. For accurate mass measurement, the QTOFMS was calibrated with sodium formate solution (m/z range 100-1000) and monitored in real time by intermittent injections of the lock mass sulfadimethoxine ($[M+H]^+ = 311.0814 m/z$).

Mass chromatograms and mass spectral data were acquired by MassLynx™ software in centroided format, and then deconvoluted by MarkerLynx™ software (Waters) to generate a multivariate-data matrix. The intensity of each ion was calculated as the percentage of total ion counts in the whole chromatogram. Further, the data matrix was exported into SIMCA-P+™ software (Umetrics; Kinnelon, NJ), and transformed by mean-centering and *Pareto* scaling, a technique that increases the importance of low abundance ions without significant amplification of noise. Principal components were generated by multivariate-data analysis to represent the major latent variables in the data matrix and were described in a scores-scatter plot.

Microarray Hybridization. Six wild-type and six triple-knockout untreated mice (6-week-old males) provided the liver RNA; RNA from two mice comprised each group, meaning there were three groups of wild-type and three of triple-knockout. The microarray experiments were carried out essentially as described elsewhere and referenced therein (Sartor et al., 2004). The mouse 70-mer MEEBO oligonucleotide library version 1.05 (25,130 unique gene symbols on the array; Invitrogen; Carlsbad, CA)

was suspended in 3X SSC at 30 μ M and printed at 22°C, with 65% relative humidity, on aminosilane-coated slides (Cel Associates, Inc.; Pearland, TX), using a high-speed robotic Omnigrid machine (GeneMachines; San Carlos, CA) with Stealth SMP3 pins (Telechem; Sunnyvale, CA). The complete gene list can be viewed at <http://www.invitrogen.com>. Spot volumes were 0.5 nl, and spot diameters 75-85 μ m. The oligonucleotides were cross-linked to the slide substrate by exposure to 600 mJ of ultraviolet light.

Fluorescence-labeled cDNAs were synthesized from total RNA, using an indirect aminoallyl labeling method via an oligo(dT)-primed reverse-transcriptase reaction. The cDNA was decorated with mono-functional reactive cyanine-3 and cyanine-5 dyes (**Cy3** and **Cy5**; Amersham; Piscataway, NJ). The details and a complete description of the slide preparation can be found at <http://microarray.uc.edu>.

Imaging and data generation were carried out using a GenePix 4000A and GenePix 4000B (Axon Instruments; Union City, CA) and associated software from Axon Instruments, Inc. (Foster City, CA). The microarray slides were scanned with dual lasers having the wavelength frequencies to excite Cy3 and Cy5 fluorescence emittance. Images were captured in *.jpg and *.tif files, and DNA spots captured by the adaptive circle segmentation method. Information extraction for a given spot is based on the median value for the signal pixels and the median value for the background pixels, to produce a gene-set data file for all the DNA spots. The Cy3 and Cy5 fluorescence signal intensities were normalized.

Microarray Data Normalization and Analysis. We sought to identify differentially-expressed genes between untreated *Cyp1a1/1a2/1b1*(-/-) and *Cyp1*(+/+) wild-type mice. Three biological-replicate arrays, with one dye-flip, were carried out. Analysis was performed using R statistical software and the *limma* Bioconductor package (Smyth, 2004). Data normalization was conducted in two steps for each microarray separately (Sartor et al., 2004). First, background-adjusted intensities were log-transformed and the differences (M) and averages (A) of log-transformed values were calculated as $M = \log_2(X1) - \log_2(X2)$ and $A = [\log_2(X1) + \log_2(X2)]/2$, where X1 and X2 denote the Cy5 and Cy3 intensities, respectively. Second, normalization was performed by fitting the array-specific local regression model of M as a function of A. Normalized log-intensities for the two channels were then calculated by adding half the normalized ratio to A

for the Cy5 channel and subtracting half the normalized ratio from A for the Cy3 channel. Statistical analysis was performed by first fitting the following analysis-of-variance model for each gene separately: $Y_{ijk} = \mu + A_i + S_j + C_k + \epsilon_{ijk}$, where Y_{ijk} corresponds to the normalized log-intensity on the i^{th} array, with the j^{th} treatment, and labeled with the k^{th} dye ($k = 1$ for Cy5, and 2 for Cy3); μ denotes the overall mean log-intensity, A_i is the effect of the i^{th} array, S_j is the effect of the j^{th} treatment, C_k is the gene-specific effect of the k^{th} dye, and ϵ_{ijk} is the error term for the i^{th} array with the j^{th} treatment, and labeled with the k^{th} dye. Estimated fold-changes were calculated from the ANOVA models, and resulting t-test statistics from each comparison were modified using an intensity-based empirical Bayesian method (IBMT) (Sartor et al., 2004). This method, an extension of (Smyth, 2004), obtains more precise estimates of variance by pooling information across genes and accounting for the dependency of variance on probe-intensity levels. Identification of significant genes was accomplished from two avenues. First, the false discovery rate (**FDR**) was calculated (Reiner et al., 2003); genes with an FDR value of ≤ 0.10 are considered as significantly differentially expressed. Next, discovery of gene categories enriched with differentially-expressed genes was performed using DAVID software (Dennis, Jr. et al., 2003) with a P -value of <0.01 significance cutoff for genes. The biological process and molecular-function branches of the Gene Ontology (**GO**) database (Harris et al., 2004) were tested for enrichment, and genes belonging to those GO terms having a calculated FDR ≤ 0.10 were considered for further analysis. The mRNA expression of 22 genes of interest was corroborated by Q-PCR studies.

Total RNA Preparation. Untreated wild-type versus triple-knockout mice, or TCDD-pretreated wild-type versus triple-knockout mice, were always compared. Total RNA from frozen liver was isolated using TRIzol (Invitrogen). The quantity of RNA was determined spectrophotometrically by the A260/A280 ratio (SmartSpec 3000, Bio-Rad; Hercules, CA). The quality of RNA was confirmed by separation on a denaturing formaldehyde/agarose/ethidium bromide gel, and then quantified using an Agilent Bioanalyzer (Quantum Analytics; Foster City, CA).

Reverse Transcription. Total RNA (2 μ g) was added to a reaction containing 3.8 μ M oligo(dT)₂₀ and 0.77 mM dNTP—to a final volume of 13 μ l. Reactions were incubated at 65°C for 5 min, then 4°C for 2 min. To the reaction mixture we added 7 μ l of solution containing 14 mM dithiothreitol, 40 units RNaseOUT Recombinant RNase inhibitorTM (Invitrogen; CA, U.S.A), and 200 units SuperScript IIITM (Invitrogen). Reactions were incubated at 50°C for 50 min, followed by 75°C for 10 min (to inactivate the reverse transcriptase). Distilled water (80 μ l) was added to the isolated cDNA; these samples were then stored at –80°C until use.

Real-Time Quantitative PCR (Q-PCR). Using the Superscript II RNase H-reverse transcriptase kit (Carlsbad, CA), hepatic total RNA was reverse-transcribed. Following this, Q-PCR was conducted using Brilliant SYBR Green Q-PCR (Stratagene; La Jolla, CA). Data were normalized to RT-PCR detection of β -actin mRNA. Primers used in RT-PCR analysis of all genes examined are available upon request.

Glucose and Lipids Assays. Animals (N = 6 mice per group) were fasted overnight, and 200 μ L of total blood removed from the saphenous vein. Blood samples were place on ice and centrifuged for 10 min at 800 rpm. Plasma glucose and lipids were determined by the National Mouse Metabolic Phenotyping Center (University Cincinnati).

Zymosan Challenge. An inflammatory response was induced with 1 mg of zymosan per mouse, as described (Kolaczowska et al., 2006). Zymosan (an insoluble carbohydrate from yeast cell wall) was freshly prepared (2 mg/ml) in sterile 0.9% NaCl, and 0.5 ml was injected intraperitoneally into each mouse; controls received vehicle only. At the appropriate time-points, each peritoneal cavity was washed with 5.0 ml of phosphate-buffered saline, and as much lavage fluid as possible was recovered. One portion (200 μ L) was used for cell counting, and another (100 μ L) taken for preparing histology slides. The amount of lavage fluid recovered per mouse was recorded so that, after centrifugation (3000 \times g for 3 min), total peritoneal cell numbers (plus neutrophils, macrophages and lymphocytes) per mouse could be determined. For each group, 4 to 8 mice were used.

Histology. From the oral BaP studies, bone marrow smears were obtained at sacrifice by dissecting the femurs free and removing muscle. Following removal of the proximal and distal epiphyses, a tiny polyethylene tube was affixed to one end of the bone shaft; the marrow was gently blown onto a glass slide, and a second slide was used to squash the droplet of marrow onto the slide. The peritoneal cells after zymosan challenge, marrow smears and peripheral blood smears were air-dried on glass slides. All slides were stained with Wright-Giemsa (University Hospital Bone Marrow Lab). Differential counts of the peripheral blood and peritoneal exudate were performed. Percent of different cell types was calculated, based on a minimum of 100 lymphocytes per sample.

Statistical analysis. Statistical significance between groups was determined by analysis-of-variance among groups, Student's *t*-test between groups, and Fisher's test between groups with very low frequencies. All assays were performed in duplicate or triplicate, and repeated at least twice. Statistical analyses were performed with the use of SAS[®] statistical software (SAS Institute Inc.; Cary, NC) and Sigma Plot (Systat Software, Inc., Point Richmond, CA).

Results

Embryolethality. One overt phenotype of the *Cyp1a1/1a2/1b1*(-/-) triple-knockout was a noticeably decreased litter size (Fig. 1A). Therefore, we carried out eight different (female × male) crosses of various combinations (Table 1). In every case, Hardy-Weinberg distribution was skewed, showing less than the expected number of triple-knockout newborns; these data indicate that no particular maternal or paternal genotype favored viability of the triple-knockout pup. Sufficient numbers for each cross were generated in order to show *P*-values of <0.05; when all breeding experiments were combined, the expected number (58.25 triple-knockout pups) was very significantly (*P* <0.001) different from the observed number (30 viable pups).

Interestingly, however, a small number of *Cyp1a1/1a2/1b1*(-/-) pups survived the neonatal period (Fig. 1B) and lived long enough to produce offspring (Table 1). Although the litter size and weight gain (in both F₁ males and females) was less in the triple-knockout than in wild-type, the triple-knockout mouse line (in a >99.8% C57BL/6J background) has now been sustained for >10 generations. We conclude that significant embryoletality, with incomplete penetrance, is a phenotype of the *Cyp1a1/1a2/1b1*(-/-) F₁ mouse.

At what gestational age does the lethality occur? We examined four litters each of GD11, GD13, GD15, GD17 and GD19 (not shown) and found no significant differences in Hardy-Weinberg distribution. We conclude that in utero deaths, when they occur, happen in the F₁ embryo—prior to GD11.

Birth defects. Among 264 littermates that were not homozygous for both the *Cyp1a1_1a2*(-) and *Cyp1b1*(-) alleles, one *Cyp1*(+/+) wild-type and one *Cyp1a1/1a2*(-/-) double-knockout exhibited hydrocephalus, and none showed hermaphroditism or cystic ovaries. In C57BL/6J mice (Kanno et al., 1987; Biddle et al., 1991), the “average” rates of occurrence for hydrocephalus or hermaphroditism is one in ~500 (~0.2%), and for cystic ovaries one in 200 (~0.5%). Among 30 triple-knockout F₁ pups, four exhibited hydrocephalus ($P < 0.001$), two hermaphroditism ($P < 0.01$), and two cystic ovaries ($P < 0.01$, all by Fisher’s test). We conclude that significant increased risks of hydrocephalus, hermaphroditism and cystic ovaries, with incomplete penetrance, are phenotypes of the *Cyp1* triple-knockout F₁ mouse.

Pathology report. Gross and microscopic evaluations of organs and tissues—including heart, lung, spleen, thymus, kidney, liver, cerebrum, cerebellum, eye, Harderian gland, testis, ovary, uterus, prostate, tongue, esophagus, pancreas, abdominal aorta, forestomach, glandular stomach, duodenum, jejunum, ileum and colon—revealed no overt abnormalities in eight “normal”-appearing, healthy 6-

week-old *Cyp1a1/1a2/1b1*(-/-) mice. Mice with overt hydrocephalus showed severe hemorrhage of the meninges and cortex with necrosis of the cortex and dilation of the ventricles and died within a few days of birth. Overt hermaphrodites showed microscopic, as well as gross, evidence of Müllerian and Wolffian duct remnants. Cystic ovaries usually occurred bilaterally and were confirmed microscopically.

Effects of dietary BaP. Previously it was shown that *Cyp1a1*(-/-) knockout mice ingesting BaP (125 mg/kg/day) die after ~28 days with severe immunosuppression, whereas *Cyp1*(+/+) wild-type mice for one year on this diet remain as healthy as untreated wild-type mice; it was concluded that BaP-induced intestinal and perhaps liver CYP1A1 are more important in detoxication than metabolic activation of oral BaP (Uno et al., 2004). On the other hand, oral BaP-treated *Cyp1a1/1b1*(-/-) mice are “rescued” and appear similar to the wild-type phenotype; this was interpreted as the CYP1B1 enzyme in immune tissues being necessary and sufficient to metabolically activate BaP and cause immunosuppression (Uno et al., 2006). *Cyp1a2*(-/-), *Cyp1b1*(-/-), and *Cyp1a2/1b1*(-/-) respond to the oral BaP regimen similarly to (untreated or oral BaP-treated) wild-type mice. After 5 days of oral BaP, the total blood BaP of the *Cyp1a1*(-/-) and *Cyp1a1/1b1*(-/-) is ~25 and ~75 times greater, respectively, than that of the wild-type mouse—demonstrating that the total body burden of an environmental toxicant can be independent of target-organ damage (Uno et al., 2006).

Fig. 2 shows that blood BaP levels of the *Cyp1* triple-knockout are ~90-fold higher than that of the wild-type. In the triple-knockout, compared to the wild-type, liver size is significantly greater and thymus weight smaller ($P < 0.01$); these parameters are prototypic signs of AHR activation and independent of CYP1 metabolism. The triple-knockout revealed elevated serum ALT and AST levels ($P < 0.05$), but no significant differences in spleen weight, or relative percent of neutrophils or lymphocytes (Fig. 2). These findings are all consistent with mild damage in the oral BaP-treated

Cyp1a1/1a2/1b1(-/-)—to about the same degree as that seen in the oral BaP-treated *Cyp1a1/1b1*(-/-) “rescued” double-knockout (Uno et al., 2006).

Histology of the bone marrow (Fig. 3) confirmed the Fig. 2 data. Whereas there was substantial bone marrow hypocellularity in oral BaP-treated *Cyp1a1*(-/-) and *Cyp1a1/1a2*(-/-) mice, the oral BaP-treated *Cyp1a1/1a2/1b1*(-/-) mouse was “rescued” and looked close to that of the BaP-treated wild-type and the corn oil-treated controls. Curiously, in the peripheral blood of the triple-knockout, there appeared to be an increased number of binucleated lymphocytes, both in untreated as well as oral BaP-treated animals.

Urinary metabolite profiles. The phenotypic differences (described above) between untreated triple-knockout and wild-type mice imply the physiological importance of CYP1 enzyme-mediated endogenous metabolism. To examine this further, we compared, via LC-MS, the urinary metabolite profiles of untreated *Cyp1a1/1a2/1b1*(-/-) and *Cyp1*(+/+) mice. PCA revealed that the metabolite profiles from untreated triple-knockout and wild-type mice were distinctively separated in a two-component model (Fig. 4A), suggesting striking endogenous metabolism differences between the two genotypes.

Oral BaP-treated versus the untreated urinary metabolite profiles (Fig. 4B) were examined by partial least squares-discriminant analysis (PLS-DA) of the LC-MS data. The distribution and clustering pattern of the four groups in this three-component model revealed that not only were there significant compositional differences among the four groups of urine samples, but also suggested that the triple-knockout and wild-type mice respond differently to BaP treatment—because the urinary metabolite profiles of *Cyp1a1/1a2/1b1*(-/-) and *Cyp1*(+/+) mice shifted in distinctly different directions, *i.e.* from the untreated profiles to the oral BaP-treated profiles (*bold arrows*, Fig. 4B).

Hepatic cDNA expression microarray analysis. Because we saw differences in endogenous metabolism between the triple-knockout and wild-type (Fig. 4A), we conducted differential liver gene expression by microarray analysis; although some of this urinary metabolite profile likely reflects extrahepatic tissues, the vast majority of metabolism is found in liver.

If we used the simple “overly-relaxed” $P < 0.05$ cut-off, there were 676 genes up-regulated and 437 genes down-regulated, comparing triple-knockout to wild-type. If we used the combination of the overly-relaxed $P < 0.05$ plus a fold-change of ≥ 1.5 as the cut-off, there were 565 genes up- and 366 genes down-regulated. At the stringent false-discovery rate (**FDR**) cut-off of ≤ 0.10 , which gave P -values of ≤ 0.00076 , at least 89 genes were up- (Table 2) and 62 genes down-regulated (Table 3); the complete lists are available in **Supplementary Data**). The genes are ranked in order of fold-increase or fold-decrease; *Cox6b2* and *Chrna4* showed the largest increases (7.09- and 5.47-fold, respectively) in the triple-knockout, whereas *Snora65* and *St3gal4* were the most decreased (7.58- and 5.32-fold, respectively). The GO categories (Table 4) for these 151 “most significantly perturbed” genes include: lipid, steroid and cholesterol biosynthesis and metabolism; nucleosome and chromatin assembly; carboxylic and organic acid metabolism; metal-ion binding; and ion homeostasis.

Because we saw increased gene expression in many lipid pathways for the untreated triple-knockout compared with the wild-type, we examined these pathways further in fasting animals (N = 6 per group). Comparing the *Cyp11a1/1a2/1b1*(-/-) with *Cyp11*(+/+), we found a trend of decreases in the triple-knockout mouse but no statistically ($P > 0.05$) significant differences in: serum cholesterol (129 ± 8.9 vs 132 ± 12 mg/dL), triglycerides (35.5 ± 10 vs 41.7 ± 2.7 mg/dL), phospholipids (152 ± 1.4 vs 159 ± 4.4 mg/dL), non-esterified fatty acids (0.79 ± 0.03 vs 0.89 ± 0.09 mEq/L), or glucose (116 ± 5.2 vs 145 ± 33 mg/dL), respectively.

Hepatic mRNA expression by Q-PCR analysis. To substantiate the microarray expression data, we performed Q-PCR analysis on 22 genes (Table 5) to see if their mRNA levels could be confirmed as up- or down-regulated—as had been determined by microarray expression. As expected, wild-type mice carried the three *Cyp1* genes, which were dioxin-inducible, whereas the triple-knockout had no detectable transcripts (*see footnote to Table 3*). *Cox6b2*, *Mid1* and *Vldlr* expression was up-regulated in the microarray (Table 2), and this was confirmed by Q-PCR (Table 5). *Nqo1*, *Ugt1a6b* and *Ugt1a7c* are dioxin-inducible genes, and thus we analyzed those genes by Q-PCR in both untreated and oral BaP-treated mice; mRNA levels of all three genes were significantly elevated in the untreated triple-knockout compared with the untreated wild-type.

Expression of the *St3gal4*, *Ccne*, *Trpm8* and *Slco1a1* genes were down-regulated in the untreated triple-knockout (Table 4); the former two were verified by Q-PCR analysis (Table 5); in the cases of *Trpm8* and *Slco1a1*, the trend was downward but would require a larger sample size to prove these genes are down-regulated, as had been found in the microarray data.

Mt1, *Mt2* and *Mt4* expression was down-regulated in the untreated triple-knockout (Table 4), and this was confirmed by Q-PCR (Table 5). Because the *Mt* genes are up-regulated under conditions of oxidative stress, we tested three additional oxidative-response genes: *Hmox1* and *Gclm* were down-regulated in untreated triple-knockout mice, but *Gclc* was not (Table 5). Curiously, TCDD treatment of *Cyp1*(+/+) mice caused significant down-regulation in the *Hmox1*, *Mt1*, *Mt2* and *Mt4* genes.

Zymosan-induced peritonitis. Members of the CYP1, CYP2, CYP3 and CYP4 families have been shown to be involved in eicosanoid biosynthesis and metabolism (Nebert and Russell, 2002). Several dozen of the 151 “most significantly perturbed genes” (**Supplementary Data**) are involved in lipid mediator and inflammation pathways. For these reasons, we therefore decided to compare the inflammatory response of *Cyp1a1/1a2/1b1*(-/-) mice with *Cyp1*(+/+) mice. No differences in

peritoneal cells (total cell numbers, or numbers of neutrophils and macrophages) between the two genotypes were found in untreated animals (Fig. 5). Following zymosan intraperitoneal injection, however, the triple-knockout displayed an exaggerated response (peaking at 6 h) compared with that in the wild-type, with significant increases in neutrophil, macrophage and total cell infiltration into the peritoneal cavity.

Discussion

In this study we have described multiple outcomes in the triple-knockout F₁ mouse—having all three *Cyp1* genes ablated—as compared with wild-type mice: embryoletality before GD11; significantly increased risk of hydrocephalus, hermaphroditism, and cystic ovaries; striking differences in urinary endogenous metabolite profiles detected by LC-MS analysis; dramatic differences in urinary metabolite profiles detected by LC-MS analysis following oral BaP treatment; at least 89 and 62 genes very significantly up- and down-regulated, respectively; gene categories most perturbed being lipid, steroid and cholesterol biosynthesis and metabolism; nucleosome and chromatin assembly; carboxylic and organic acid metabolism; metal-ion binding; and ion homeostasis; and an exaggerated response to zymosan-induced peritonitis.

CYP1-mediated eicosanoid metabolism. All of the above-described phenotypic alterations may well be the result of alterations in the production, catabolism and/or function of eicosanoids: bioactive mediators derived from arachidonic acid via ω -6 fatty acids (including prostaglandins, prostacyclins, leukotrienes, thromboxanes, hepoxilins, and lipoxins); and bioactive mediators derived from eicosapentaenoic acid and docosahexaenoic acid via ω -3 fatty acids, (including resolvins, docosatrienes, eoxins and neuroprotectins). Eicosanoids exert largely unappreciated complex control over virtually all physiological processes: inflammation (Chiang et al., 2005; Serhan, 2007; Seubert

et al., 2007; Leone et al., 2007; Mariotto et al., 2007), resolution phase of inflammation (Serhan, 2007), innate immunity (Ballinger et al., 2007), cardiopulmonary and vascular functions (Moreland et al., 2007; Seubert et al., 2007), angiogenesis (Inceoglu et al., 2007; Fleming, 2007), sensor of vascular pO₂ (Sacerdoti et al., 2003), bowel motility (Proctor et al., 1987), regulation of lipid metabolism and insulin sensitivity (Larsen et al., 2007; Spector and Norris, 2007; Nigam et al., 2007), central nervous system functions (Miyata and Roman, 2005; Jakovcevic and Harder, 2007), modulation of non-neuropathic pain (Inceoglu et al., 2007), neurohormone secretion and release (Inceoglu et al., 2007), fibrinolysis (Westlund et al., 1991; Jiang, 2007), inhibition of platelet aggregation (Westlund et al., 1991; Jiang, 2007), reproductive success (Weems et al., 2006; Cha et al., 2006), blastocyst implantation (Cha et al., 2006; Kennedy et al., 2007), early embryonic as well as fetal development (Cha et al., 2006), stimulation of tyrosine phosphorylation (Chen et al., 1998), G protein-signaling (Inceoglu et al., 2007), modulation of NFκB (Inceoglu et al., 2007), cation and anion homeostasis (Sacerdoti et al., 2003; Inceoglu et al., 2007; Spector and Norris, 2007; Nüsing et al., 2007; Plant and Strotmann, 2007; Hao and Breyer, 2007; Xiao, 2007), and cell division, proliferation and chemotaxis (Inceoglu et al., 2007; Spector and Norris, 2007; Fleming, 2007; Nieves and Moreno, 2007; Medhora et al., 2007).

Eicosanoids can be quickly released by most cell types (often stored in red blood cells) and act as autocrine or paracrine mediators, which are then rapidly inactivated. Eicosanoid biosynthesis involves metabolism by the 5-, 12- and 15-lipoxygenases and cyclooxygenases-1 and -2—as well as most, if not all, CYP1, CYP2, CYP3 and CYP4 enzymes. These same CYP enzymes also participate in the rapid inactivation/degradation of eicosanoids. We propose that the absence of all three CYP1 enzymes in the *Cyp1a1/1a2/1b1*(-/-) mouse perturbs: reproductive success; normal implantation and early embryogenesis, leading to a greater incidence of embryoletality (Table 1); proper development

of ventricle valves in the central nervous system, leading to hydrocephalus; physiological differentiation of the Müllerian and Wolffian ducts, causing hermaphroditism; normal development of the ovary during fetogenesis, leading to cystic ovaries; many of the genes in the GO categories of lipid, steroid and cholesterol biosynthesis and metabolism; nucleosome and chromatin assembly; carboxylic and organic acid metabolism; metal-ion binding; and ion homeostasis (Table 4); and the pro-inflammatory and pro-resolution processes—leading to an exaggerated response to zymosan-induced peritonitis (Fig. 5).

In fact, the *Ahr*(-/-) knockout mouse displays patent ductus venosus and other arteriovenous-shunt problems (Lahvis et al., 2005), along with immune dysregulation (Fernandez-Salguero et al., 1995), and increased susceptibility to infection (Shi et al., 2007)—probably caused also by perturbation of eicosanoid function. Indeed, six different prostaglandins (albeit at relatively high concentrations) have been shown to activate the AHR and induce the CYP1 enzymes (Seidel et al., 2001). Furthermore, a differential gene-expression microarray between *Ahr*(-/-) and wild-type mice (Yoon et al., 2006) shows perturbation of genes in all the categories listed above that reflect eicosanoid functions. Whereas the *Ahr*(-/-) mouse has no functional AHR and therefore all downstream genes regulated by the AHR would be affected, the *Cyp1a1/1a2/1b1*(-/-) mouse has a functional AHR while having just the three CYP1 enzyme functions genetically removed. Therefore, using these two mouse lines we should be able to distinguish between AHR-dependent functions and AHR-regulated CYP1-dependent functions in the intact mouse.

Microarray cDNA Expression Data. The mouse 70-mer MEEBO library version 1.05 has 25,130 genes, which is supposed to cover close to the entire genome. In several dozen instances, there are two sets of primers for the same gene, which serves as a rigorous check on the accuracy of the expression data. However, with only three replicates (**Supplementary Data**), we realize that we

could still be missing dozens of additional relevant genes having important differential expression differences between untreated *Cyp1a1/1a2/1b1*(-/-) and *Cyp1*(+/+) mice.

The *Cox6b2* gene is ~7-fold higher in triple-knockout than in wild-type mice (Tables 2 & 5). *Cox6b2* transcripts are ubiquitous (Taanman et al., 1990). O₂ consumption is known to increase in liver mitochondria prepared from *Ahr*(-/-) mice, but not from *Cyp1a1*(-/-) or *Cyp1a2*(-/-) mice (Senft et al., 2002). TCDD decreases the cytochrome oxidase rate-constant, but increases O₂ consumption and increases CoQ-cytochrome *c* reductase activity (Shertzer et al., 2006). Could it be that the mitochondrial CYP1 enzymes are part of the cytochrome-oxidase complex which divert, or supply, electrons from (or to) O₂? Hence, if all three CYP1 enzymes are absent, then the cytochrome-oxidase complex might compensate by a striking elevation of the COX6B2 subunit.

The *Stegal4* gene is ~5.3-fold lower in untreated triple-knockout than in wild-type mice (Tables 3 & 5). Sialyltransferases modulate the increased expression of surface-sialylated structures during the generation of dendritic cells derived from monocytes (Videira et al., 2008), which is likely to be associated with eicosanoid-mediated changes in various cell functions.

The *Socs2* gene, whose hepatic expression is down-regulated ~2-fold in the triple-knockout (Tables 3 & 5), is of special interest. Both endogenous and exogenous ligands have been shown to up-regulate SOCS2 expression. TCDD has been shown to drive SOCS2 expression in lymphocytes in an AHR-dependent fashion (Boverhof et al., 2004). Inhibition of dendritic cell pro-inflammatory cytokine production by lipoxins (which are pro-resolution eicosanoids) is dependent on AHR-driven up-regulation of SOCS2 expression (Machado et al., 2006). The decreased expression of SOCS2 observed in the triple-knockout suggests an obvious potential mechanism for the exaggerated inflammatory response seen in response to zymosan challenge, as well as the possibility that these CYP1 enzymes play an important role in the generation of endogenous eicosanoid ligands for the

AHR. Activation of the AHR by the lipoxins triggers expression of SOCS2, which causes the ubiquitinylation of TNF α -receptor-association factors (**TRAFs**), encoded by each of seven *Traf* genes in the mouse.

The [Ah] gene battery. The *Nqo1*, *Ugt6b* and *Ugt7c* genes were 2.20-, 2.15- and 2.07-fold increased, respectively (Tables 2 & 5). These data are particularly intriguing, because various cell culture studies (Nebert et al., 2000b) had shown that several members of the [Ah] gene battery become up-regulated when CYP1 enzyme activity is absent; moreover, addition of a mouse CYP1A1 or human CYP1A2 expression vector restores expression of these [Ah] member genes to their low basal wild-type phenotype status (RayChaudhuri et al., 1990). Long ago, these findings were interpreted as CYP1 enzymes being required to degrade a putative endogenous ligand of the AHR; when all CYP1 activity is extinguished, the AHR is highly activated (Robertson et al., 1987). This hypothesis has been supported experimentally by studies using CYP1- and AHR-deficient cells in culture (Chang and Puga, 1998) and by studies comparing the lung of *Ahr*(-/-) versus *Ahr*(+/+) mice (Chiaro et al., 2007).

Curiously, a number of xenobiotic-metabolizing enzymes (**XMEs**) are overexpressed in the triple-knockout (Table 2): all three *Gstm* genes; the sulfotransferase *Sult3a1*; *Cyp17a1* (important in steroid biosynthesis), *Cyp2b20*, and *Cyp26a1* (important in metabolism of the morphogen retinoic acid); and two *Ugt1* genes. Feedback inhibition and interactions of XMEs and their XME-related transporters (**XRTs**) during inflammation and tumorigenesis have been reviewed (Nebert and Dalton, 2006; Zhou et al., 2006). It is tempting to speculate that some (or all) of these genes might also be members of the [Ah] gene batter.

Incomplete penetrance. Lastly, we found embryolethality—as well as the risk of the hydrocephalus, hermaphroditism, and cystic ovaries—to be inherited as incomplete-penetrance traits

(Table 1, Fig. 1), despite the *Cyp1a1/1a2/1b1*(-/-) genotype having been placed directly into a >99.8% C57BL/6J genetic background. Rather than explaining incomplete penetrance due to a heterogeneous genetic background, therefore, more likely it can be explained by redundancy, *i.e.* the AHR-controlled basal and inducible CYP1 expression levels and their downstream functions must overlap with expression levels and functions of other genes and gene products.

Most interestingly, as we have continued to breed the triple-knockout F₁ homozygote survivors for more than 10 generations, the embryoletality and birth defects have disappeared. Note the trend of more pups per litter, even between the F₁ and F₂ generations (Fig. 1A). We believe this can be explained by natural selection: as the healthiest animals survive and are chosen for breeding in the next generation—genetic and epigenetic factors associated with embryoletality and birth defects give way to those associated with improved viability and high reproductive performance. Accordingly, we are maintaining in our mouse colony the double-heterozygote and single-heterozygote as mating pairs (Fig. 6); it is very clear that pups from these two breeding combinations provide animals with greatly affected phenotypes, compared with that seen in pups derived from the continued inbreeding of homozygous triple-knockout mice. To our knowledge, this effect of natural selection during subsequent brother × sister matings (when one sees F₁ embryoletality or other phenotypes having incomplete penetrance) has not been considered previously in knockout-mouse studies. We are certain, however, that this must commonly occur.

Future studies. The microarray expression data described herein represent a gold mine of opportunities to try to determine the various downstream genes and their functions—when all three *Cyp1* genes have been ablated. Future comparisons between the *Ahr*(-/-) mouse and the *Cyp1* triple-knockout mouse should uncover new exciting findings. We propose that studies such as these will provide us with a greater understanding of AHR-dependent versus AHR-regulated CYP1-dependent

eicosanoid biosynthesis and degradation, as well as their concomitant autocrine and paracrine functions. The *Cyp1a1/1a2/1b1*(-/-) mouse line is available to any investigator who might be interested.

Acknowledgments

We thank Howard Shertzer and other colleagues for valuable discussions and critical readings of the manuscript. We appreciate very much the earlier help of Shige Uno and Tim Dalton in creating the *Cyp1a1/1a2*(-/-) double-knockout line. We are also grateful to Mario Medvedovic for statistical advice and especially to Marian L. Miller for her participation in histology, microscopy, as well as all graphics. These data were presented at the 27th (March 2007) Annual Meeting of the Society of Toxicology, Charlotte, NC. These studies were supported, in part, by NIH Grants R01 ES08147 (D.W.N.), R01 ES014403 (D.W.N.), and P30 ES06096 (M.L.M., C.K. & D.W.N.).

References

- Ballinger MN, McMillan T R and Moore B B (2007) Eicosanoid Regulation of Pulmonary Innate Immunity Post-Hematopoietic Stem Cell Transplantation. *Arch Immunol Ther Exp (Warsz)* **55**:1-12.
- Biddle FG, Eales B A and Nishioka Y (1991) A DNA Polymorphism From Five Inbred Strains of the Mouse Identifies a Functional Class of Domesticus-Type Y Chromosome That Produces the Same Phenotypic Distribution of Gonadal Hermaphrodites. *Genome* **34**:96-104.
- Boverhof DR, Tam E, Harney A S, Crawford R B, Kaminski N E and Zacharewski T R (2004) 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin Induces Suppressor-of-Cytokine-Signaling-2 in Murine B Cells. *Mol Pharmacol* **66**:1662-1670.
- Buters JT, Sakai S, Richter T, Pineau T, Alexander D L, Savas U, Doehmer J, Ward J M, Jefcoate C R and Gonzalez F J (1999) Cytochrome P450 CYP1B1 Determines Susceptibility to 7,12-Dimethylbenz[*a*]Anthracene-Induced Lymphomas. *Proc Natl Acad Sci U S A* **96**:1977-1982.
- Cha YI, Solnica-Krezel L and DuBois R N (2006) Fishing for Prostanoids: Deciphering the Developmental Functions of Cyclooxygenase-Derived Prostaglandins. *Dev Biol* **289**:263-272.
- Chang CY and Puga A (1998) Constitutive Activation of the Aromatic Hydrocarbon Receptor. *Mol Cell Biol* **18**:525-535.
- Chen JK, Falck J R, Reddy K M, Capdevila J and Harris R C (1998) Epoxyeicosatrienoic Acids and Their Sulfonimide Derivatives Stimulate Tyrosine Phosphorylation and Induce Mitogenesis in Renal Epithelial Cells. *J Biol Chem* **273**:29254-29261.
- Chiang N, Arita M and Serhan C N (2005) Anti-Inflammatory Circuitry: Lipoxin, Aspirin-Triggered Lipoxins and Their Receptor ALX. *Prostaglandins Leukot Essent Fatty Acids* **73**:163-177.
- Chiaro CR, Patel R D, Marcus C B and Perdew G H (2007) Evidence for an Aryl Hydrocarbon Receptor-Mediated Cytochrome P450 Autoregulatory Pathway. *Mol Pharmacol* **72**:1369-1379.
- Dalton TP, Dieter M Z, Matlib R S, Childs N L, Shertzer H G, Genter M B and Nebert D W (2000) Targeted Knockout of *Cyp1a1* Gene Does Not Alter Hepatic Constitutive Expression of Other Genes in the Mouse [Ah] Battery. *Biochem Biophys Res Commun* **267**:184-189.
- Dennis G, Jr., Sherman B T, Hosack D A, Yang J, Gao W, Lane H C and Lempicki R A (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* **4**:3.
- Dragin N, Uno S, Wang B, Dalton T P and Nebert D W (2007) Generation of "Humanized" HCYP1A1_1A2_*Cyp1a1/1a2*(-/-) Mouse Line. *Biochem Biophys Res Commun* **359**:635-642.
- Fernandez-Salguero P, Pineau T, Hilbert D M, McPhail T, Lee S S, Kimura S, Nebert D W, Rudikoff S, Ward J M and Gonzalez F J (1995) Immune System Impairment and Hepatic Fibrosis in Mice Lacking the Dioxin-Binding Ah Receptor. *Science* **268**:722-726.
- Fleming I (2007) Epoxyeicosatrienoic Acids, Cell Signaling and Angiogenesis. *Prostaglandins Other Lipid Mediat* **82**:60-67.

- Garcia-Falcon MS, Gonzalez-Amigo S., Lage-Yusty M A, Lopez de Alda Villaizan MJ and Simal-Lozano J. (1996) Determination of Benzo[a]Pyrene in Lipid-Soluble Liquid Smoke (LSLS) by HPLC-FL. *Food Addit Contam* **13**:863-870.
- Hao CM and Breyer M D (2007) Physiologic and Pathophysiologic Roles of Lipid Mediators in the Kidney. *Kidney Int* **71**:1105-1115.
- Harris MA, Clark J, Ireland A, Lomax J, Ashburner M, Foulger R, Eilbeck K, Lewis S, Marshall B, Mungall C, Richter J, Rubin G M, Blake J A, Bult C, Dolan M, Drabkin H, Eppig J T, Hill D P, Ni L, Ringwald M, Balakrishnan R, Cherry J M, Christie K R, Costanzo M C, Dwight S S, Engel S, Fisk D G, Hirschman J E, Hong E L, Nash R S, Sethuraman A, Theesfeld C L, Botstein D, Dolinski K, Feierbach B, Berardini T, Mundodi S, Rhee S Y, Apweiler R, Barrell D, Camon E, Dimmer E, Lee V, Chisholm R, Gaudet P, Kibbe W, Kishore R, Schwarz E M, Sternberg P, Gwinn M, Hannick L, Wortman J, Berriman M, Wood V, de la C N, Tonellato P, Jaiswal P, Seigfried T and White R (2004) The Gene Ontology (GO) Database and Informatics Resource. *Nucleic Acids Res* **32**:D258-D261.
- Inceoglu B, Schmelzer K R, Morisseau C, Jinks S L and Hammock B D (2007) Soluble Epoxide Hydrolase Inhibition Reveals Novel Biological Functions of Epoxyeicosatrienoic Acids (EETs). *Prostaglandins Other Lipid Mediat* **82**:42-49.
- Jakovcevic D and Harder D R (2007) Role of Astrocytes in Matching Blood Flow to Neuronal Activity. *Curr Top Dev Biol* **79**:75-97.
- Jiang H (2007) Erythrocyte-Derived Epoxyeicosatrienoic Acids. *Prostaglandins Other Lipid Mediat* **82**:4-10.
- Kanno T, Nakamura T, Jain V K and Sugimoto T (1987) An Experimental Model of Communicating Hydrocephalus in C57 Black Mouse. *Acta Neurochir (Wien)* **86**:111-114.
- Kennedy TG, Gillio-Meina C and Phang S H (2007) Prostaglandins and the Initiation of Blastocyst Implantation and Decidualization. *Reproduction* **134**:635-643.
- Kim HS, Kwack S J and Lee B M (2000) Lipid Peroxidation, Antioxidant Enzymes, and Benzo[a]Pyrene Quinones in the Blood of Rats Treated With Benzo[a]Pyrene. *Chem Biol Interact* **127**:139-150.
- Kolaczowska E, Chadzinska M, Scislawska-Czarnecka A, Plytycz B, Opdenakker G and Arnold B (2006) Gelatinase B/Matrix Metalloproteinase-9 Contributes to Cellular Infiltration in a Murine Model of Zymosan Peritonitis. *Immunobiology* **211**:137-148.
- Lahvis GP, Pyzalski R W, Glover E, Pitot H C, McElwee M K and Bradfield C A (2005) The Aryl Hydrocarbon Receptor Is Required for Developmental Closure of the Ductus Venosus in the Neonatal Mouse. *Mol Pharmacol* **67**:714-720.
- Larsen BT, Campbell W B and Gutterman D D (2007) Beyond Vasodilatation: Non-Vasomotor Roles of Epoxyeicosatrienoic Acids in the Cardiovascular System. *Trends Pharmacol Sci* **28**:32-38.
- Leone S, Ottani A and Bertolini A (2007) Dual Acting Anti-Inflammatory Drugs. *Curr Top Med Chem* **7**:265-275.
- Liang HC, Li H, McKinnon R A, Duffy J J, Potter S S, Puga A and Nebert D W (1996) *Cyp1a2*(-/-) Null Mutant Mice Develop Normally but Show Deficient Drug Metabolism. *Proc Natl Acad Sci U S A* **93**:1671-1676.

- Machado FS, Johndrow J E, Esper L, Dias A, Bafica A, Serhan C N and Aliberti J (2006) Anti-Inflammatory Actions of Lipoxin A4 and Aspirin-Triggered Lipoxin Are SOCS-2 Dependent. *Nat Med* **12**:330-334.
- Mariotto S, Suzuki Y, Persichini T, Colasanti M, Suzuki H and Cantoni O (2007) Cross-Talk Between NO and Arachidonic Acid in Inflammation. *Curr Med Chem* **14**:1940-1944.
- McMillan BJ and Bradfield C A (2007) The Aryl Hydrocarbon Receptor *Sans* Xenobiotics: Endogenous Function in Genetic Model Systems. *Mol Pharmacol* **72**:487-498.
- Medhora M, Dhanasekaran A, Gruenloh S K, Dunn L K, Gabrilovich M, Falck J R, Harder D R, Jacobs E R and Pratt P F (2007) Emerging Mechanisms for Growth and Protection of the Vasculature by Cytochrome P450-Derived Products of Arachidonic Acid and Other Eicosanoids. *Prostaglandins Other Lipid Mediat* **82**:19-29.
- Miyata N and Roman R J (2005) Role of 20-Hydroxyeicosatetraenoic Acid (20-HETE) in Vascular System. *J Smooth Muscle Res* **41**:175-193.
- Moreland KT, Procknow J D, Sprague R S, Iverson J L, Lonigro A J and Stephenson A H (2007) Cyclooxygenase-1 and -2 Participate in 5,6-Epoxyeicosatrienoic Acid-Induced Contraction of Rabbit Intralobar Pulmonary Arteries. *J Pharmacol Exp Ther* **321**:446-454.
- Nebert DW and Dalton T P (2006) The Role of Cytochrome P450 Enzymes in Endogenous Signalling Pathways and Environmental Carcinogenesis. *Nat Rev Cancer* **6**:947-960.
- Nebert DW, Dalton T P, Okey A B and Gonzalez F J (2004) Role of Aryl Hydrocarbon Receptor-Mediated Induction of the CYP1 Enzymes in Environmental Toxicity and Cancer. *J Biol Chem* **279**:23847-23850.
- Nebert DW, Dalton T P, Stuart G W and Carvan M J, III (2000a) "Gene-Swap Knock-in" Cassette in Mice to Study Allelic Differences in Human Genes. *Ann N Y Acad Sci* **919**:148-170.
- Nebert DW, Roe A L, Dieter M Z, Solis W A, Yang Y and Dalton T P (2000b) Role of the Aromatic Hydrocarbon Receptor and [Ah] Gene Battery in the Oxidative Stress Response, Cell Cycle Control, and Apoptosis. *Biochem Pharmacol* **59**:65-85.
- Nebert DW and Russell D W (2002) Clinical Importance of the Cytochromes P450. *Lancet* **360**:1155-1162.
- Nelson DR, Zeldin D C, Hoffman S M, Maltais L J, Wain H M and Nebert D W (2004) Comparison of Cytochrome P450 (CYP) Genes From the Mouse and Human Genomes, Including Nomenclature Recommendations for Genes, Pseudogenes and Alternative-Splice Variants. *Pharmacogenetics* **14**:1-18.
- Nieves D and Moreno J J (2007) Epoxyeicosatrienoic Acids Induce Growth Inhibition and Calpain/Caspase-12 Dependent Apoptosis in PDGF-Cultured 3T6 Fibroblast. *Apoptosis* **12**:1979-1988.
- Nigam S, Zafiriou M P, Deva R, Ciccoli R and Roux-Van der M R (2007) Structure, Biochemistry and Biology of Hepoxilins: an Update. *FEBS J* **274**:3503-3512.
- Nüsing RM, Schweer H, Fleming I, Zeldin D C and Wegmann M (2007) Epoxyeicosatrienoic Acids Affect Electrolyte Transport in Renal Tubular Epithelial Cells: Dependence on Cyclooxygenase and Cell Polarity. *Am J Physiol Renal Physiol* **293**:F288-F298.
- Plant TD and Strotmann R (2007) TRPV4. *Handb Exp Pharmacol* 189-205.

- Proctor KG, Falck J R and Capdevila J (1987) Intestinal Vasodilation by Epoxyeicosatrienoic Acids: Arachidonic Acid Metabolites Produced by a Cytochrome P450 Monooxygenase. *Circ Res* **60**:50-59.
- RayChaudhuri B, Nebert D W and Puga A (1990) The Murine *Cyp1a1* Gene Negatively Regulates Its Own Transcription and That of Other Members of the Aromatic Hydrocarbon-Responsive [*Ah*] Gene Battery. *Mol Endocrinol* **4**:1773-1781.
- Reiner A, Yekutieli D and Benjamini Y (2003) Identifying Differentially Expressed Genes Using False Discovery Rate-Controlling Procedures. *Bioinformatics* **19**:368-375.
- Robertson JA, Hankinson O and Nebert D W (1987) Autoregulation Plus Positive and Negative Elements Controlling Transcription of Genes in the [*Ah*] Battery. *Chem Scripta* **27A**:83-87.
- Robinson JR, Felton J S, Levitt R C, Thorgeirsson S S and Nebert D W (1975) Relationship Between "Aromatic Hydrocarbon Responsiveness" and the Survival Times in Mice Treated With Various Drugs and Environmental Compounds. *Mol Pharmacol* **11**:850-865.
- Sacerdoti D, Gatta A and McGiff J C (2003) Role of Cytochrome P450-Dependent Arachidonic Acid Metabolites in Liver Physiology and Pathophysiology. *Prostaglandins Other Lipid Mediat* **72**:51-71.
- Sartor M, Schwanekamp J, Halbleib D, Mohamed I, Karyala S, Medvedovic M and Tomlinson C R (2004) Microarray Results Improve Significantly As Hybridization Approaches Equilibrium. *Biotechniques* **36**:790-796.
- Seidel SD, Winters G M, Rogers W J, Ziccardi M H, Li V, Keser B and Denison M S (2001) Activation of the Ah Receptor Signaling Pathway by Prostaglandins. *J Biochem Mol Toxicol* **15**:187-196.
- Senft AP, Dalton T P, Nebert D W, Genter M B, Puga A, Hutchinson R J, Kerzee J K, Uno S and Shertzer H G (2002) Mitochondrial Reactive Oxygen Production Is Dependent on the Aromatic Hydrocarbon Receptor. *Free Radic Biol Med* **33**:1268-1278.
- Serhan CN (2007) Resolution Phase of Inflammation: Novel Endogenous Anti-Inflammatory and Proresolving Lipid Mediators and Pathways. *Annu Rev Immunol* **25**:101-137.
- Seubert JM, Zeldin D C, Nithipatikom K and Gross G J (2007) Role of Epoxyeicosatrienoic Acids in Protecting the Myocardium Following Ischemia/Reperfusion Injury. *Prostaglandins Other Lipid Mediat* **82**:50-59.
- Shertzer HG, Genter M B, Shen D, Nebert D W, Chen Y and Dalton T P (2006) TCDD Decreases ATP Levels and Increases Reactive Oxygen Production Through Changes in Mitochondrial F₀F₁-ATP Synthase and Ubiquinone. *Toxicol Appl Pharmacol* **217**:363-374.
- Shi LZ, Faith N G, Nakayama Y, Suresh M, Steinberg H and Czuprynski C J (2007) The Aryl Hydrocarbon Receptor Is Required for Optimal Resistance to *Listeria Monocytogenes* Infection in Mice. *J Immunol* **179**:6952-6962.
- Smyth GK (2004) Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray Experiments. *Stat Appl Genet Mol Biol* **3**:Article 3.
- Spector AA and Norris A W (2007) Action of Epoxyeicosatrienoic Acids on Cellular Function. *Am J Physiol Cell Physiol* **292**:C996-1012.

- Taanman JW, Schrage C, Ponne N J, Das A T, Bolhuis P A, de V H and Agsteribbe E (1990) Isolation of CDNAs Encoding Subunit VIb of Cytochrome c Oxidase and Steady-State Levels of CoxVIb mRNA in Different Tissues. *Gene* **93**:285-291.
- Tukey RH, Hannah R R, Negishi M, Nebert D W and Eisen H J (1982) The *Ah* Locus: Correlation of Intracellular Appearance of Inducer-Receptor Complex With Induction of Cytochrome P₁-450 MRNA. *Cell* **31**:275-284.
- Uno S, Dalton T P, Derkenne S, Curran C P, Miller M L, Shertzer H G and Nebert D W (2004) Oral Exposure to Benzo[a]Pyrene in the Mouse: Detoxication by Inducible Cytochrome P450 Is More Important Than Metabolic Activation. *Mol Pharmacol* **65**:1225-1237.
- Uno S, Dalton T P, Dragin N, Curran C P, Derkenne S, Miller M L, Shertzer H G, Gonzalez F J and Nebert D W (2006) Oral Benzo[a]Pyrene in *Cyp1* Knockout Mouse Lines: CYP1A1 Important in Detoxication, CYP1B1 Metabolism Required for Immune Damage Independent of Total-Body Burden and Clearance Rate. *Mol Pharmacol* **69**:1103-1114.
- Uno S, Dragin N, Miller M L, Dalton T P, Gonzalez F J and Nebert D W (2008) Basal and Inducible CYP1 MRNA Quantitation and Protein Localization Throughout the Mouse Gastrointestinal Tract. *Free Radic Biol Med* **44**:570-583.
- Videira PA, Amado I F, Crespo H J, Algueró M C, Dall'olio F, Cabral M G and Trindade H. (2008) Surface α 2-3- and α 2-6-Sialylation of Human Monocytes and Derived Dendritic Cells and Its Influence on Endocytosis. *Glycoconj J* **in press**.
- Weems CW, Weems Y S and Randel R D (2006) Prostaglandins and Reproduction in Female Farm Animals. *Vet J* **171**:206-228.
- Westlund P, Palmblad J, Falck J R and Lumin S (1991) Synthesis, Structural Identification and Biological Activity of 11,12-Dihydroxyicosatetraenoic Acids Formed in Human Platelets. *Biochim Biophys Acta* **1081**:301-307.
- Xiao YF (2007) Cyclic AMP-Dependent Modulation of Cardiac L-Type Ca²⁺ and Transient Outward K⁺ Channel Activities by Epoxyeicosatrienoic Acids. *Prostaglandins Other Lipid Mediat* **82**:11-18.
- Yoon CY, Park M, Kim B H, Park J Y, Park M S, Jeong Y K, Kwon H, Jung H K, Kang H, Lee Y S and Lee B J (2006) Gene Expression Profile by 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin in the Liver of Wild-Type *Ahr*(+/+) and Aryl Hydrocarbon Receptor-Deficient *Ahr*(-/-) Mice. *J Vet Med Sci* **68**:663-668.
- Zhou C, Tabb M M, Nelson E L, Grun F, Verma S, Sadatrafiei A, Lin M, Mallick S, Forman B M, Thummel K E and Blumberg B (2006) Mutual Repression Between Steroid and Xenobiotic Receptor and NF-KB Signaling Pathways Links Xenobiotic Metabolism and Inflammation. *J Clin Invest* **116**:2280-2289.

FOOTNOTES

**Address correspondence to: Daniel W. Nebert, Department of Environmental Health, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati OH 45267-0056, U.S.A. Tele. 513-821-4664; Fax 513-558-0925; email dan.nebert@uc.edu

*These two authors contributed equally to this study.

Supported in part by NIH Grants R01 ES08147 (D.W.N.), R01 ES014403 (D.W.N.), and P30 ES06096 (M.L.M., C.K. & D.W.N.).

Mouse (and rat) genes are italicized with only the first letter capitalized (*e.g. Cyp1a1, Ahr*), whereas human and other non-rodent or generic genes are italicized with all letters capitalized (*e.g. CYP1A1, AHR*). Rodent, human and generic cDNA/mRNA/protein/enzyme activities are never italicized and all letters always capitalized (*e.g. CYP1A1, AHR*).

Figure Legends

Fig. 1. Comparison of litter size and weight gain in *Cyp1*(+/+) wild-type and *Cyp1a1/1a2/1b1*(-/-) triple-knockout mice. **A**, Number of pups per litter—viable at birth; more than half of those born alive died within the first 24 h. F1 denotes the generation when the triple-knockout genotype was initially obtained. F2 denotes litters derived from the *Cyp1a1/1a2/1b1*(-/-) × *Cyp1a1/1a2/1b1*(-/-) intercross. **B**, weight gain in F₁ males and F₁ females, aged 1 through 16 weeks. The body weights of triple-knockout (males and females) were significantly ($P < 0.05$) lower than wild-type mice at age 5, 6, 10 and 12 weeks; the body weights of triple-knockout males (but not females) were significantly ($P < 0.05$) less than wild-type mice at age 16 weeks. Values and brackets represent means \pm S.E., respectively (N = 4 to 5 litters in **A**; N = 6 mice in **B**).

Fig. 2. Comparison of wild-type and triple-knockout mice that have received oral BaP (125 mg/kg/day). Histograms of total blood BaP concentration; mg liver, spleen and thymus wet weight per g total body weight (**BW**); serum ALT and AST activities; and relative percent of neutrophils and lymphocytes in peripheral blood. Blood BaP levels were determined after 5 days of oral BaP; all other parameters were measured after 18 days of oral BaP. Values and brackets represent means \pm S.E., respectively (N = 6 mice). Differences between the two genotypes were significant ($P < 0.01$) in liver and thymus weight and ($P < 0.05$) in ALT and AST activities. The BaP-treated *Cyp1a1/1a2*(-/-) mouse was previously shown (Dragin et al., 2007) to exhibit severe immunosuppression similar to that seen in the BaP-treated *Cyp1a1*(-/-) mouse, whereas the BaP-treated *Cyp1b1*(-/-) mouse responds similarly to the BaP-treated *Cyp1*(+/+) wild-type mouse (Uno et al., 2006). All untreated genotypes show no differences (except total blood BaP) from the BaP-treated wild-type (Uno et al., 2006).

Fig. 3. Representative bone marrow histology, comparing untreated (oil) with oral BaP-treated wild-type (*top*) and triple-knockout (*bottom*) mice after 18 days of BaP. Marrows of the *Cyp1a1*(-/-) and *Cyp1a1/1a2*(-/-) mice are included as positive controls, showing oral BaP-induced massive hypocellularity—especially with loss of

lymphoid precursors. Marrows of untreated or oral BaP-treated *Cyp1a2*(-/-), *Cyp1b1*(-/-), and *Cyp1a2/1b1*(-/-) mice have previously been shown to exhibit the same normal cellularity as marrows of the *Cyp1*(+/+) wild-type, with or without oral BaP treatment (Uno et al., 2004; Uno et al., 2006). *Bar, upper left panel*, 50 microns.

Fig. 4. Multivariate data analysis of urine samples from untreated (U) and oral BaP-treated wild-type (WT) and triple-knockout (TKO) mice. **A**, Scores-scatter plot using the PCA model to compare urine samples from the two untreated genotypes (N = 6). The t[1] and t[2] values represent the scores of each of the 12 samples in principal component 1 and 2, respectively; fitness (R^2 value) of the model to the acquired dataset is 0.468, and predictive power (Q^2 value) of the model is 0.184. **B**, 3-dimensional scores-scatter plot of the partial least-squares-discriminant analysis (PLS-DA) model on the four groups of urine samples (N = 6). The t[1], t[2] and t[3] values represent the scores of each of the 24 samples in principal component 1, 2 and 3, respectively. The R^2 value of the model to the acquired dataset is 0.859, and the Q^2 value of the model is 0.743. The model was validated through the recalculation of R^2 and Q^2 values after the permutation of sample identities. *Arrows* indicate the metabolite profile directional changes, from untreated, to that induced by BaP treatment.

Fig. 5. Kinetic analysis of zymosan-induced peritonitis in wild-type and triple-knockout mice. *Top*, Total number of peritoneal cells. * P = 0.02. ** P = 0.0002. *Middle*, Total number of peritoneal neutrophils. * P = 0.02. ** P = 0.0001. *Bottom*, Total number of macrophages. * P = 0.04. ** P = 0.002. In untreated mice at time zero, *Cyp1*(+/+) and *Cyp1a1/1a2/1b1*(-/-) mice exhibited $1.14 \pm 0.12 \times 10^6$ and $1.25 \pm 0.44 \times 10^6$, respectively (P = 0.62). Values are expressed as means \pm S.E., using Student's two-tailed t-test (N = 4 to 8 mice per time-point per group).

Fig. 6. Schematic diagram to illustrate that those triple-knockout pups derived from the double-heterozygote mating (*left*) and from the single-heterozygote mating (*middle*) will show incomplete-penetrance traits that

differ dramatically from pups derived from the continual double-homozygote mating (*right*), *i.e.* maintenance of the *Cyp1a1/1a2/1b1*(-/-) triple-knockout mouse line.

TABLE 1

Chi-square analysis of in utero lethality in triple-knockout pups

Genetic cross ^a	Total number of pups	Observed number of triple k.o. pups	Expected number of triple k.o. pups	chi-square value	<i>P</i> value
<i>Aabb</i> X <i>aaBb</i>	87	11	21.75	4.35	<0.05>0.02
<i>Aabb</i> X <i>Aabb</i> <i>aaBb</i> X <i>aaBb</i>	49	6	12.25	2.63	<0.20>0.10
Totals of two above	136	17	34	6.97	<0.01>0.001
<i>Aabb</i> X <i>AaBb</i> <i>AaBb</i> X <i>aaBb</i>	106	4	13.25	5.40	<0.025>0.01
<i>aabb</i> X <i>aaBb</i> <i>aaBb</i> X <i>aabb</i>	22	9	11	0.37	<1.0>0.50
Totals of all four above	264	30	58.25	10.85	<0.001
^b <i>aabb</i> X <i>aabb</i>	11	11	11	0	1.0

^aGenetic crosses are designated as [*mother genotype* × *father genotype*]. *A* denotes *Cyp1a1/1a2*(+) wild-type allele, and *a* denotes *Cyp1a1/1a2*(-) knockout allele. *B* denotes *Cyp1b1*(+) wild-type allele, and *b* denotes *Cyp1b1*(-/-) knockout allele.

^bOf the 30 triple-knockout F₁ pups that survived, only two females and one male lived to adulthood and were able to breed successfully.

TABLE 2

Microarray analysis of liver: selection of a subset of genes most significantly **up**-regulated in the untreated *Cyp1a1/1a2/1b1*(-/-) versus untreated *Cyp1*(+/+) mouse^a

Symbol	Gene biochemical name	Fold increase	P-value	FDR ^b
^c <i>Cox6b2</i>	Cytochrome c oxidase, subunit VIb, polypeptide 2 (electron transport)	7.09	$<10^{-12}$	$<10^{-12}$
<i>Chrna4</i>	Cholinergic receptor, nicotinic, α -polypeptide-4 (extracellular ligand-gated ion channel activity, B cell activation)	5.47	$<10^{-12}$	$<10^{-12}$
<i>Pwp1</i>	PWP1 homolog [<i>S. cerevisiae</i>] (activity in nucleus?)	4.21	1×10^{-11}	2×10^{-6}
<i>Slc46a3</i>	Solute-carrier family 46, member 3 (unknown cation transporter)	3.95	4×10^{-13}	6×10^{-10}
<i>Cd3e</i>	CD3 antigen, ϵ -polypeptide (lymphocyte activation)	3.63	2×10^{-8}	2×10^{-5}
^c <i>Sult3a1</i>	Sulfotransferase, family 3A, member 1 (SO ₄ conjugation)	3.62	1×10^{-11}	2×10^{-8}
<i>Acot11</i>	Acyl-coA thioesterase-11 (fatty acid metabolism, signal transduction)	3.00	9×10^{-8}	0.00005
^c <i>Mid1</i>	Midline-1 (ligase activity; metal-ion binding)	2.93	2×10^{-8}	0.00001
<i>Gstm3</i>	Glutathione S-transferase, μ 3 (GSH conjugation)	2.71	7×10^{-7}	0.00009
<i>Cyp17a1</i>	Cytochrome P450, family 17, subfamily A, member 1 (monooxygenation)	2.70	2×10^{-7}	0.00009
^c <i>Vldlr</i>	Very low-density lipoprotein receptor (Ca ²⁺ -binding, lipid transporter, cholesterol metabolism)	2.59	1×10^{-4}	0.018
<i>Shank2</i>	SH3/ankyrin domain gene-2 (neuronal cell differentiation)	2.52	2×10^{-4}	0.018
<i>Cyp26a1</i>	Cytochrome P450, family 26, subfamily A, member 1 (monooxygenation)	2.47	3×10^{-6}	0.0011
^c <i>Nqo1</i>	NAD(P)H:quinone oxidoreductase (regulated by CYP1 activity)	2.20	0.00004	0.0097
<i>Gstm5</i>	Glutathione S-transferase, μ 5 (GSH conjugation)	2.17	$<1 \times 10^{-5}$	0.0011

^c <i>Ugt1a6b</i>	UDP glucuronosyltransferase, family 1, subfamily A, member 12 (glucuronide conjugation)	2.15	0.00002	0.0062
^c <i>Ugt1a7c</i>	UDP glucuronosyltransferase, family 1, subfamily A, member 13 (glucuronide conjugation)	2.07	0.00013	0.022
<i>Cyp2b20</i>	Cytochrome P450, family 2, subfamily B, member 20 (monooxygenation)	1.97	0.00028	0.042
<i>Gstm7</i>	Glutathione <i>S</i> -transferase, μ 7 (GSH conjugation)	1.92	0.00055	0.067
<i>Insc</i>	Inscuteable homolog [<i>Drosophila</i>] (differentiation, developmental)	1.92	0.00050	0.062
<i>Sid12</i>	SID1 transmembrane family, member 2 (early embryo and postnatal expression)	1.90	0.00073	0.079
<i>Ethel</i>	Ethylmalonic encephalopathy-1 (hydrolase activity, metal-ion binding)	1.88	0.00086	0.090

This is a partial list of 22 selected genes; the entire list of 89 up-regulated genes can be found in the **Supplementary Data**.

^aThese data exclude the *NeoR* pRev Tet-Off vector which, because it is present in the genome of the triple-knockout mouse, is 12.7-fold “up-regulated”. Mouse mammary tumor virus, complete genome, was also excluded.

^bFalse discovery rate, or adjusted *P*-value. One out of ten adjusted *P*-values ≤ 0.10 would be expected to be a false positive. In this and subsequent tables, the *P*-values are dependent on both the measurements of fold-change, as well as how consistent they are (variance).

^cConfirmed via Q-PCR to be up-regulated.

TABLE 3

Microarray analysis of liver: selection of a subset of genes most significantly **down**-regulated in the untreated *Cyp1a1/1a2/1b1*(-/-) versus untreated *Cyp1*(+/+) mouse^a

Symbol	Gene biochemical name	Fold decrease	P-value	FDR ^b
<i>Snora65</i>	Small nucleolar RNA, H/ACA box-65 (part of ribonucleoprotein complex)	7.58	$<10^{-16}$	$<10^{-12}$
^c <i>St3gal4</i>	ST3 β -galactoside α 2,3-sialyltransferase (amino-acid glycosylation)	5.32	$<10^{-16}$	$<10^{-12}$
<i>Cachd1</i>	Cache domain-containing-1 (Ca ²⁺ -ion binding)	4.75	9×10^{-16}	2×10^{-12}
<i>Rgs16</i>	Regulator of G protein signaling-16 (GTPase activator, signal transduction)	4.28	1×10^{-16}	2×10^{-11}
<i>Cbx3</i>	Chromobox homolog-3 [<i>Drosophila</i> HP1- γ] (chromatin-binding)	3.89	1×10^{-11}	5×10^{-7}
^c <i>Mt2</i>	Metallothionein-2 (metal-ion binding)	3.66	5×10^{-12}	7×10^{-9}
^c <i>Mt1</i>	Metallothionein-1 (metal-ion binding)	3.61	2×10^{-13}	3×10^{-10}
<i>Igfbp2</i>	Insulin-like growth factor-binding protein-2 (growth-factor binding)	3.33	2×10^{-10}	2×10^{-7}
<i>Rbbp4</i>	Retinoblastoma-binding protein-4 (cell cycle, chromatin modification)	2.73	2×10^{-7}	0.0001
<i>Ccnd1</i>	Cyclin D1 (cell cycle, protein kinase regulator)	2.54	4×10^{-9}	4×10^{-6}
<i>Etohd3</i>	Ethanol decreased-3 (activity in blastocyst)	2.35	0.00023	0.037
<i>Il28ra</i>	Interleukin-28 receptor- α (inflammatory signaling pathways)	2.29	0.00004	0.0084
<i>Marco</i>	Macrophage receptor with collagenous structure (PO ₄ transport)	2.20	0.00004	0.0098
<i>Snora70</i>	Small nucleolar RNA, H/ACA box-70 (part of ribonucleoprotein complex)	2.19	0.00004	0.0098
<i>Qk</i>	Quaking (axon ensheathment, nucleic-acid binding)	2.15	0.00008	0.016
<i>Wee1</i>	wee-1 homolog [<i>S. pombe</i>] (cell cycle, kinase activity)	2.14	0.00007	0.015
<i>Ifi27</i>	Interferon, α -inducible protein 27 (response to virus)	2.10	0.00007	0.014
<i>Peci</i>	Peroxisomal Δ_3, Δ_2 -enoyl-coA isomerase (peroxisome assembly, biogenesis)	2.10	0.00007	0.015

^d <i>Trpm8</i>	Transient receptor potential cation channel, subfamily M, member 8 (Ca ²⁺ channel activity)	2.08	0.00064	0.074
^d <i>Slco1a1</i>	Solute-carrier organic anion transporter family 1, member 1 (organic anion transport)	2.02	0.00010	0.018
^c <i>Socs2</i>	Suppressor of cytokine-signaling-2 (chemokine signal transduction, fat cell differentiation)	1.97	0.00035	0.048
^c <i>Mt4</i>	Metallothionein-4 (metal-ion binding)	1.91	0.00067	0.076

This is a partial list of 22 selected genes; the entire list of 62 down-regulated genes can be found in the **Supplementary Data**.

^aThese data exclude the *Cyp1a1*, *Cyp1a2* and *Cyp1b1* genes, which, because they were genetically ablated, are strikingly (>95-fold) “down-regulated”. Interestingly, one of two *Cyp1b1* primer sets that exist in the mouse 70-mer MEEBO oligonucleotide library was detectable and showed up-regulation, but this is interpreted as a genomic transcript that had not been ablated in generating the *Cyp1b1*(-/-) knockout mouse line (Buters et al., 1999). There were six murine virus genomes significantly (FDR ≤0.10) down-regulated, which were also excluded.

^bFalse discovery rate, or adjusted *P*-value. One out of ten adjusted *P*-values ≤0.10 would be expected to be a false positive.

^cConfirmed twice via Q-PCR to be down-regulated.

^dChecked twice by Q-PCR and not found to be statistically significantly down-regulated.

TABLE 4

Gene ontology (GO) classes in microarray analysis of liver: comparison of the untreated *Cyp1a1/1a2/1b1*(-/-) with the untreated *Cyp1*(+/+) with mouse line

GO classification of genes most perturbed (up- and down-regulated, combined) ^a			
Class	Gene count	P-value	False discovery rate ^b
Lipid biosynthesis	16	<10 ⁻⁵	0.0015
Steroid biosynthesis	15	0.00004	0.0067
Cellular lipid metabolism	22	0.00006	0.0083
Nucleosome	8	0.00008	0.0095
Lipid metabolism	24	0.00011	0.011
Oxidoreductase activity	29	0.00013	0.012
Steroid metabolism	11	0.00019	0.012
Carboxylic acid metabolism	21	0.00020	0.012
Organic acid metabolism	21	0.00020	0.012
Cofactor binding	9	0.00024	0.013
Lyase activity	12	0.00027	0.013
Xenobiotic metabolism	9	0.00050	0.023
Cholesterol biosynthesis	5	0.00067	0.029

Glutathione transferase activity	5	0.00080	0.030
Sterol biosynthesis	5	0.0011	0.040
Alcohol metabolism	13	0.0013	0.042
Intramolecular oxidoreductase activity	6	0.0013	0.042
Nucleosome assembly	7	0.0013	0.042
Tyrosine metabolism	7	0.0028	0.079
Chromatin	9	0.0029	0.079
Chromatin assembly or disassembly	8	0.0029	0.079
Chromatin assembly	7	0.0030	0.079
Transferase activity (alkyl or aryl)	6	0.0031	0.079
Cholesterol metabolism	6	0.0036	0.086
Tryptophan metabolism	8	0.0037	0.086
Isomerase activity	9	0.0041	0.089

^a There are 140 GO biological process categories that were tested. Those categories with an FDR <0.10 are listed.

^b Adjusted *P*-value. One out of ten adjusted *P*-values ≤ 0.10 would be expected to be a false positive.

TABLE 5

Expression of hepatic mRNA, examining genes different between untreated or TCDD-treated wild-type and triple-knockout mice

Gene name	<i>Cyp1</i> (+/+), U	<i>Cyp1</i> (+/+), TCDD	<i>Cyp1a1/1a2/1b1</i> (-/-), U	<i>Cyp1a1/1a2/1b1</i> (-/-), TCDD	TKO/WT (microarray)
<i>Cyp1a1</i>	1.0 ± 1.1	6,010 ± 51 ^a	ND	ND	NA
<i>Cyp1a2</i>	450 ± 39	2200 ± 180 ^a	ND	ND	NA
<i>Cyp1b1</i>	6.2 ± 0.59	62 ± 6.3 ^a	ND	ND	NA
<i>Cox6b2</i>	1.0 ± 0.1	0.39 ± 0.1 ^a	39 ± 0.1 ^b	13 ± 0.2 ^a	+7.09
<i>Sult3a1</i>	1.00 ± 0.2	0.42 ± 0.2 ^a	0.90 ± 0.1	0.29 ± 0.2 ^a	+3.62
<i>Mid1</i>	1.0 ± 0.6	1.1 ± 0.4	4.1 ± 0.7 ^b	0.87 ± 0.03 ^a	+2.93
<i>Vldlr</i>	1.0 ± 0.02	2.3 ± 0.1 ^a	3.8 ± 0.2 ^b	2.8 ± 0.02 ^a	+2.59
<i>Nqo1</i>	1.0 ± 0.1	4.5 ± 0.3 ^a	2.0 ± 0.2 ^b	3.7 ± 0.4 ^a	+2.20
<i>Ugt1a6b</i>	1.0 ± 0.1	1.88 ± 0.3 ^a	2.4 ± 0.4 ^b	4.6 ± 0.4 ^a	+2.15
<i>Ugt1a7c</i>	1.0 ± 0.5	4.5 ± 0.4 ^a	4.0 ± 0.2 ^b	4.6 ± 0.4	+2.07
<i>Gsta1</i>	0.83 ± 0.1	4.2 ± 0.06 ^a	0.29 ± 0.3	1.3 ± 0.1 ^a	NA
<i>Hmox1</i>	1.0 ± 0.3	0.43 ± 0.1 ^a	0.15 ± 0.1 ^b	0.17 ± 0.4	NA
<i>Gclc</i>	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.2	0.87 ± 0.3	NA
<i>Gclm</i>	1.0 ± 0.1	1.5 ± 0.1	0.55 ± 0.2	0.60 ± 0.3	NA
<i>St3gal4</i>	1.2 ± 0.03	0.48 ± 0.1 ^a	0.29 ± 0.2 ^b	0.69 ± 0.1 ^a	-5.86
<i>Mt1</i>	1.0 ± 0.3	0.35 ± 0.2 ^a	0.21 ± 0.1 ^b	0.24 ± 0.4	-3.61
<i>Mt2</i>	1.0 ± 0.2	0.09 ± 0.4 ^a	0.41 ± 0.2 ^b	0.56 ± 0.4	-3.66

<i>Mt4</i>	1.0 ± 0.01	0.71 ± 0.4 ^a	0.78 ± 0.2	0.48 ± 0.2	-1.91
<i>Trpm8</i>	1.0 ± 0.3	4.7 ± 0.4 ^a	0.94 ± 0.4	0.60 ± 0.2	-2.08
<i>Slco1a1</i>	1.0 ± 0.2	0.42 ± 0.2 ^a	0.78 ± 0.2	0.20 ± 0.3	-2.02
<i>Socs2</i>	1.00 ± 0.2	0.42 ± 0.2 ^a	0.90 ± 0.1	0.29 ± 0.2 ^a	-1.97
<i>Ccne1</i>	1.0 ± 0.03	0.05 ± 0.3 ^a	0.22 ± 0.1 ^b	0.08 ± 0.3	-1.56 ^c

TCDD is regarded as the prototypical *Cyp1* inducer and, when given intraperitoneally as a 15 µg/kg dose, induces hepatic CYP1 mRNA and protein levels to the same maximal levels as intraperitoneal BaP at a 100 mg/kg dose. Values are expressed as the means ± S.E. mRNA levels, relative to β-actin mRNA (N = 6). **ND**, not detectable by Q-PCR. **NA**, not applicable.

^aDenotes $P < 0.05$, comparing Q-PCR measurements of liver RNA from TCDD-treated with untreated (U) controls of same genotype.

^bDenotes $P < 0.05$, comparing Q-PCR in *Cyp1a1/1a2/1b1*(-/-) versus *Cyp1*(+/+) mice.

^c*Ccne1*, quite significant after two dye-flips, fell beyond the FDR ≤ 0.10 significance cutoff after three dye-flips; yet, by way of Q-PCR this mRNA was significantly decreased in untreated *Cyp1a1/1a2/1b1*(-/-) compared with untreated *Cyp1*(+/+) mice.

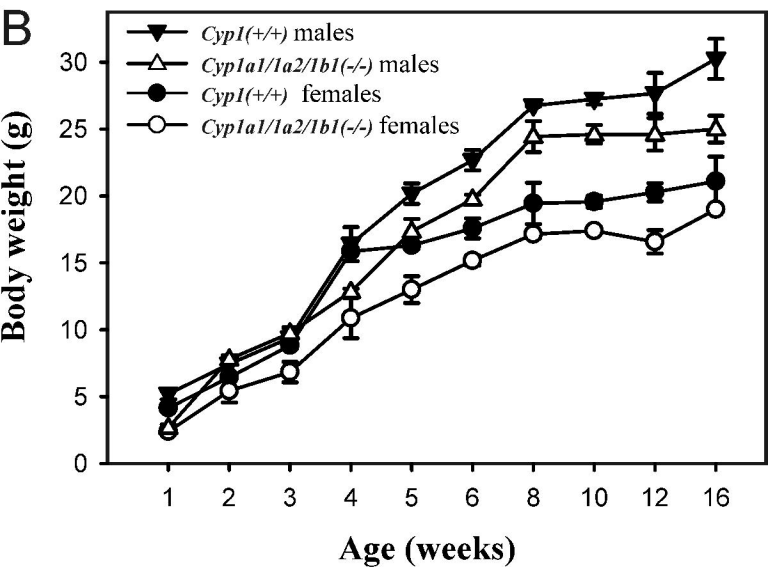
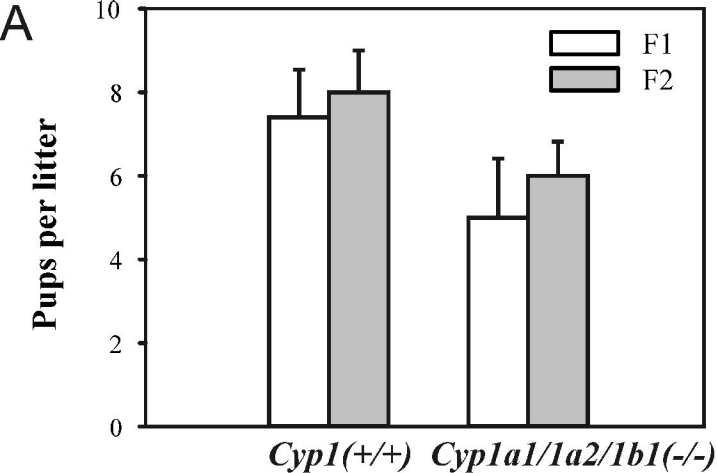


Figure 1

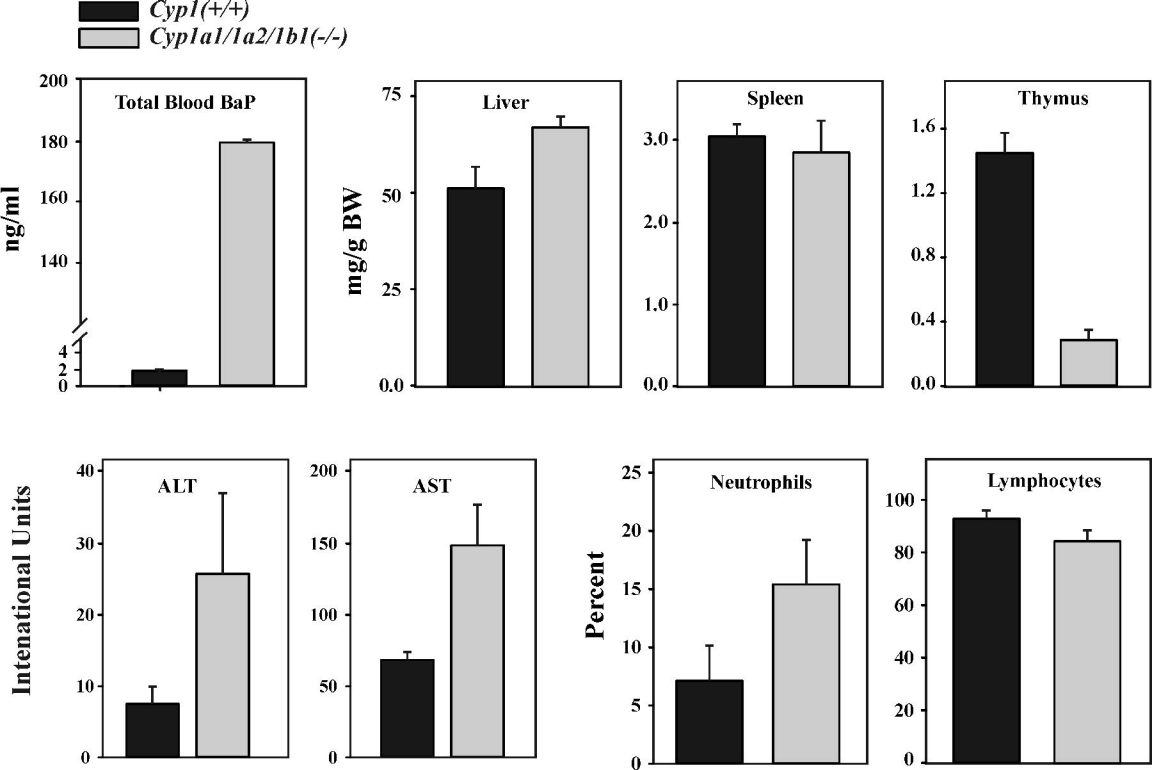
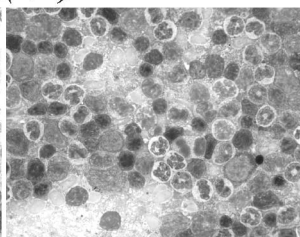
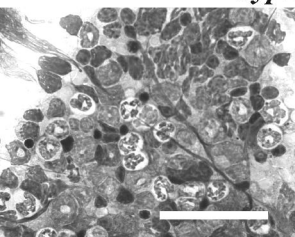


Figure 2

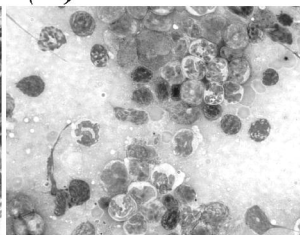
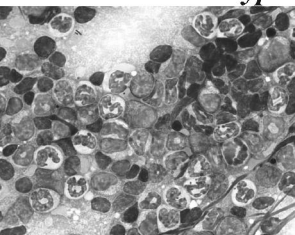
Oil

BaP

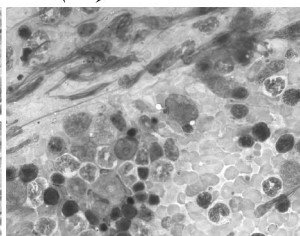
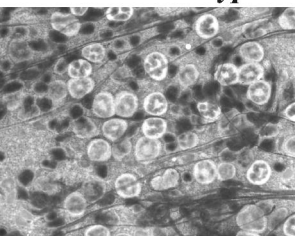
Cyp1(+/+)



Cyp1a1(-/-)



Cyp1a1/1a2(-/-)



Cyp1a1/1a2/1b1(-/-)

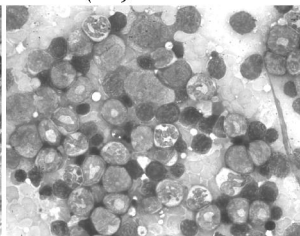
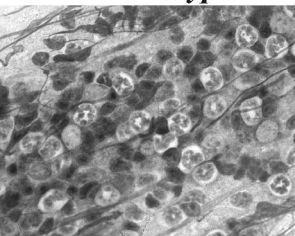


Figure 3

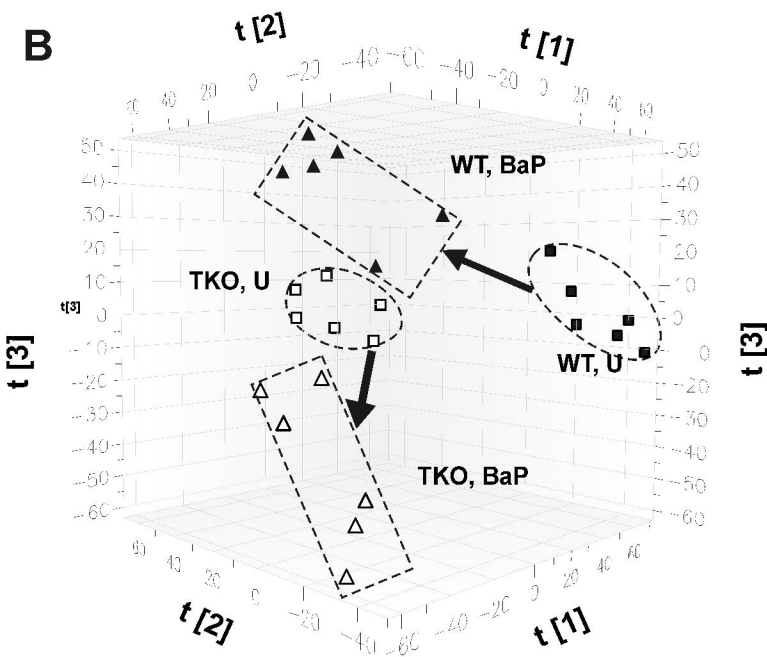
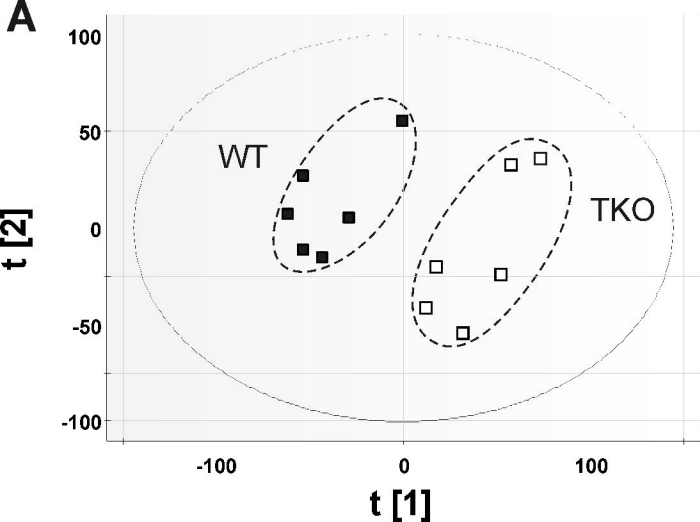


Figure 4

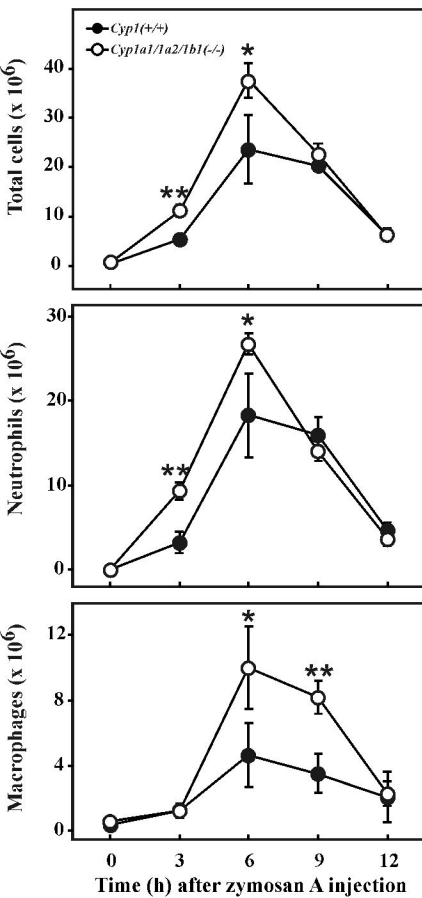


Figure 5

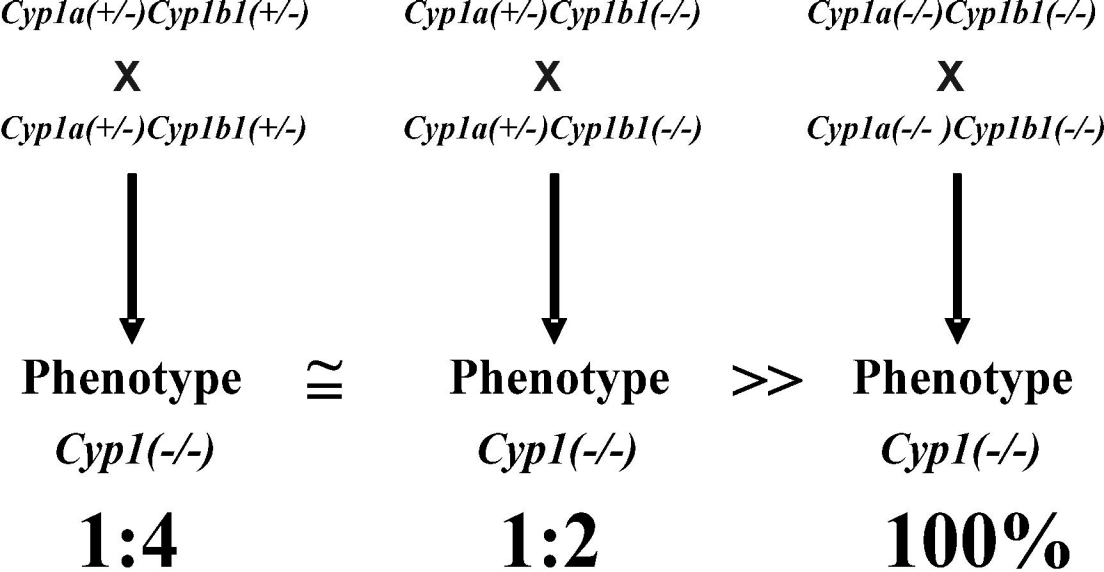


Figure 6