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

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.
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 (Kd) 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/lb1(-/-) and Cyp1a2/lb1(-/-) 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/lb1(-/-) and Cyp1a2/lb1(-/-) 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 µg/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-µL 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 μL) 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 μL of acetonitrile. An aliquot (100 μL) was injected onto a Nova-Pak C_{18} reverse-phase column (4-μm, 150 x 3.9 mm i.d.; Waters Associates; Boston, MA). HPLC analysis was conducted on a Waters™ 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/μL, and the limit of BaP quantification was determined to be 0.20 pg/μL. The intra-day and inter-day precision of repeated analyses (N=4) gave us coefficients of variation of ≤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/a2/b1(-/-) mice were prepared for UPLC-QTOFMS analysis by mixing 50 µL of urine with 200 µL of 50% aqueous acetonitrile and centrifuging at 18,000 × g for 5 min to remove protein and particulates. A 200-µL aliquot of the supernatant fraction was transferred to an auto-sampler vial and a 5-µL 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 µM 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 µm. 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 monofunctional 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 = log2(X1) – log2(X2) and A = [log2(X1) + log2(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.
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: 

\[ Y_{ijk} = \mu + A_i + S_j + C_k + \varepsilon_{ijk}, \]

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); \( \mu \) 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 \( \varepsilon_{ijk} \) 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 \( \leq 0.10 \) 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 \( \leq 0.10 \) 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 µg) was added to a reaction containing 3.8 µM oligo(dT)$_{20}$ and 0.77 mM dNTP—to a final volume of 13 µl. Reactions were incubated at 65°C for 5 min, then 4°C for 2 min. To the reaction mixture we added 7 µl of solution containing 14 mM dithiothreitol, 40 units RNaseOUT Recombinant RNase inhibitor™ (Invitrogen; CA, U.S.A), and 200 units SuperScript III™ (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 µl) 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 µL 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 (Kolaczkowska 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 µL) was used for cell counting, and another (100 µL) taken for preparing histology slides. The amount of lavage fluid recovered per mouse was recorded so that, after centrifugation (3000 × 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 embryolethality, 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 $\geq 1.5$ as the cut-off, there were 565 genes up- and 366 genes down-regulated. At the stringent false-discovery rate (FDR) cut-off of $\leq 0.10$, which gave $P$-values of $\leq 0.00076$, 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 Cyp1a1/1a2/1b1(-/-) with Cyp1(+/+), 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: embryolethality 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, hepxoilins, 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 embryolethality (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 $\text{Cyp1al1/1a2/1b1(-/-)}$ and $\text{Cyp1(+/+)}$ mice.

The $\text{Cox6b2}$ gene is ~7-fold higher in triple-knockout than in wild-type mice (Tables 2 & 5). $\text{Cox6b2}$ transcripts are ubiquitous (Taanman et al., 1990). $\text{O}_2$ consumption is known to increase in liver mitochondria prepared from $\text{Ahr(-/-)}$ mice, but not from $\text{Cyp1al1(-/-)}$ or $\text{Cyp1a2(-/-)}$ mice (Senft et al., 2002). TCDD decreases the cytochrome oxidase rate-constant, but increases $\text{O}_2$ 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) $\text{O}_2$? Hence, if all three CYP1 enzymes are absent, then the cytochrome-oxidase complex might compensate by a striking elevation of the COX6B2 subunit.

The $\text{Stegal4}$ 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 $\text{Socs2}$ 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 TNFα-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 battery.

**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 embryolethality 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 embryolethality 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 embryolethality 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


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.


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 ± 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 ± 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(-/-)* was 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. 

Marrow 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 (R² value) of the model to the acquired dataset is 0.468, and predictive power (Q² 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 R² value of the model to the acquired dataset is 0.859, and the Q² value of the model is 0.743. The model was validated through the recalculation of R² and Q² 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 ± 0.12 × 10⁶ and 1.25 ± 0.44 × 10⁶, respectively (P = 0.62). Values are expressed as means ± 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 \textit{(right)}, \textit{i.e.} maintenance of the \textit{Cyp1a1/1a2/1b1(-/-)} triple-knockout mouse line.
### TABLE 1

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

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

<sup>a</sup>Genetic 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.

<sup>b</sup>Of 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

<table>
<thead>
<tr>
<th><strong>Symbol</strong></th>
<th><strong>Gene biochemical name</strong></th>
<th><strong>Fold increase</strong></th>
<th><strong>P-value</strong></th>
<th><strong>FDR</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>c</strong>Cox6b2</td>
<td>Cytochrome c oxidase, subunit VIb, polypeptide 2 (electron transport)</td>
<td>7.09</td>
<td>&lt;10(^{-12})</td>
<td>&lt;10(^{-12})</td>
</tr>
<tr>
<td>Chrna4</td>
<td>Cholinergic receptor, nicotinic, α-polypeptide-4 (extracellular ligand-gated ion channel activity, B cell activation)</td>
<td>5.47</td>
<td>&lt;10(^{-12})</td>
<td>&lt;10(^{-12})</td>
</tr>
<tr>
<td>Pwp1</td>
<td>PWPI homolog [S. cerevisiae] (activity in nucleus?)</td>
<td>4.21</td>
<td>1 \times 10^{-11}</td>
<td>2 \times 10^{-6}</td>
</tr>
<tr>
<td>Slc46a3</td>
<td>Solute-carrier family 46, member 3 (unknown cation transporter)</td>
<td>3.95</td>
<td>4 \times 10^{-13}</td>
<td>6 \times 10^{-10}</td>
</tr>
<tr>
<td>Cd3e</td>
<td>CD3 antigen, ε-polypeptide (lymphocyte activation)</td>
<td>3.63</td>
<td>2 \times 10^{-8}</td>
<td>2 \times 10^{-5}</td>
</tr>
<tr>
<td><strong>c</strong>Sult3a1</td>
<td>Sulotransferase, family 3A, member 1 (SO(_4) conjugation)</td>
<td>3.62</td>
<td>1 \times 10^{-11}</td>
<td>2 \times 10^{-8}</td>
</tr>
<tr>
<td>Acot11</td>
<td>Acyl-coA thioesterase-11 (fatty acid metabolism, signal transduction)</td>
<td>3.00</td>
<td>9 \times 10^{-8}</td>
<td>0.00005</td>
</tr>
<tr>
<td><strong>c</strong>Mid1</td>
<td>Midline-1 (ligase activity; metal-ion binding)</td>
<td>2.93</td>
<td>2 \times 10^{-8}</td>
<td>0.00001</td>
</tr>
<tr>
<td>Gstm3</td>
<td>Glutathione S-transferase, μ3 (GSH conjugation)</td>
<td>2.71</td>
<td>7 \times 10^{-7}</td>
<td>0.00009</td>
</tr>
<tr>
<td>Cyp17a1</td>
<td>Cytochrome P450, family 17, subfamily A, member 1 (monoxygenation)</td>
<td>2.70</td>
<td>2 \times 10^{-7}</td>
<td>0.00009</td>
</tr>
<tr>
<td><strong>c</strong>Vldlr</td>
<td>Very low-density lipoprotein receptor (Ca(^{2+})-binding, lipid transporter, cholesterol metabolism)</td>
<td>2.59</td>
<td>1 \times 10^{-4}</td>
<td>0.018</td>
</tr>
<tr>
<td>Shank2</td>
<td>SH3/ankyrin domain gene-2 (neuronal cell differentiation)</td>
<td>2.52</td>
<td>2 \times 10^{-4}</td>
<td>0.018</td>
</tr>
<tr>
<td>Cyp26a1</td>
<td>Cytochrome P450, family 26, subfamily A, member 1 (monoxygenation)</td>
<td>2.47</td>
<td>3 \times 10^{-6}</td>
<td>0.0011</td>
</tr>
<tr>
<td><strong>c</strong>Nqo1</td>
<td>NAD(P)H:quinone oxidoreductase (regulated by CYPI activity)</td>
<td>2.20</td>
<td>0.00004</td>
<td>0.0097</td>
</tr>
<tr>
<td>Gstm5</td>
<td>Glutathione S-transferase, μ5 (GSH conjugation)</td>
<td>2.17</td>
<td>&lt;1 \times 10^{-5}</td>
<td>0.0011</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Fold Change</td>
<td>Adjusted P-value</td>
<td>Q-value</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------</td>
<td>------------------</td>
<td>---------</td>
</tr>
<tr>
<td>cUgt1a6b</td>
<td>UDP glucuronosyltransferase, family 1, subfamily A, member 12 (glucuronide conjugation)</td>
<td>2.15</td>
<td>0.00002</td>
<td>0.0062</td>
</tr>
<tr>
<td>cUgt1a7c</td>
<td>UDP glucuronosyltransferase, family 1, subfamily A, member 13 (glucuronide conjugation)</td>
<td>2.07</td>
<td>0.00013</td>
<td>0.022</td>
</tr>
<tr>
<td>Cyp2b20</td>
<td>Cytochrome P450, family 2, subfamily B, member 20 (monooxygenation)</td>
<td>1.97</td>
<td>0.00028</td>
<td>0.042</td>
</tr>
<tr>
<td>Gstm7</td>
<td>Glutathione S-transferase, µ7 (GSH conjugation)</td>
<td>1.92</td>
<td>0.00055</td>
<td>0.067</td>
</tr>
<tr>
<td>Insc</td>
<td>Insuteable homolog [Drosophila] (differentiation, developmental)</td>
<td>1.92</td>
<td>0.00050</td>
<td>0.062</td>
</tr>
<tr>
<td>Sidt2</td>
<td>SID1 transmembrane family, member 2 (early embryo and postnatal expression)</td>
<td>1.90</td>
<td>0.00073</td>
<td>0.079</td>
</tr>
<tr>
<td>Ethel</td>
<td>Ethylmalonic encephalopathy-1 (hydrolase activity, metal-ion binding)</td>
<td>1.88</td>
<td>0.00086</td>
<td>0.090</td>
</tr>
</tbody>
</table>

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

a These 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.

b False 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).

c Confirmed 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

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene biochemical name</th>
<th>Fold decrease</th>
<th>P-value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snora65</td>
<td>Small nucleolar RNA, H/ACA box-65 (part of ribonucleoprotein complex)</td>
<td>7.58</td>
<td>&lt;10^{-16}</td>
<td>&lt;10^{-12}</td>
</tr>
<tr>
<td><strong>St3gal4</strong></td>
<td>ST3 β-galactoside α2,3-sialyltransferase (amino-acid glycosylation)</td>
<td>5.32</td>
<td>&lt;10^{-16}</td>
<td>&lt;10^{-12}</td>
</tr>
<tr>
<td>Cachd1</td>
<td>Cache domain-containing-1 (Ca^{2+}-ion binding)</td>
<td>4.75</td>
<td>9 \times 10^{-16}</td>
<td>2 \times 10^{-12}</td>
</tr>
<tr>
<td>Rgs16</td>
<td>Regulator of G protein signaling-16 (GTPase activator, signal transduction)</td>
<td>4.28</td>
<td>1 \times 10^{-16}</td>
<td>2 \times 10^{-13}</td>
</tr>
<tr>
<td>Cbx3</td>
<td>Chromobox homolog-3 [Drosophila HP1-γ] (chromatin-binding)</td>
<td>3.89</td>
<td>1 \times 10^{-11}</td>
<td>5 \times 10^{-7}</td>
</tr>
<tr>
<td><strong>Mt2</strong></td>
<td>Metallothionein-2 (metal-ion binding)</td>
<td>3.66</td>
<td>5 \times 10^{-12}</td>
<td>7 \times 10^{-9}</td>
</tr>
<tr>
<td><strong>Mt1</strong></td>
<td>Metallothionein-1 (metal-ion binding)</td>
<td>3.61</td>
<td>2 \times 10^{-13}</td>
<td>3 \times 10^{-10}</td>
</tr>
<tr>
<td>Igfbp2</td>
<td>Insulin-like growth factor-binding protein-2 (growth-factor binding)</td>
<td>3.33</td>
<td>2 \times 10^{-10}</td>
<td>2 \times 10^{-7}</td>
</tr>
<tr>
<td>Rbbp4</td>
<td>Retinoblastoma-binding protein-4 (cell cycle, chromatin modification)</td>
<td>2.73</td>
<td>2 \times 10^{-7}</td>
<td>0.0001</td>
</tr>
<tr>
<td>Ccnd1</td>
<td>Cyclin D1 (cell cycle, protein kinase regulator)</td>
<td>2.54</td>
<td>4 \times 10^{-9}</td>
<td>4 \times 10^{-6}</td>
</tr>
<tr>
<td>Etohd3</td>
<td>Ethanol decreased-3 (activity in blastocyst)</td>
<td>2.35</td>
<td>0.00023</td>
<td>0.037</td>
</tr>
<tr>
<td>Il28ra</td>
<td>Interleukin-28 receptor-α (inflammatory signaling pathways)</td>
<td>2.29</td>
<td>0.00004</td>
<td>0.0084</td>
</tr>
<tr>
<td>Marco</td>
<td>Macrophage receptor with collagenous structure (PO₄ transport)</td>
<td>2.20</td>
<td>0.00004</td>
<td>0.0098</td>
</tr>
<tr>
<td>Snora70</td>
<td>Small nucleolar RNA, H/ACA box-70 (part of ribonucleoprotein complex)</td>
<td>2.19</td>
<td>0.00004</td>
<td>0.0098</td>
</tr>
<tr>
<td>Qk</td>
<td>Quaking (axon ensheathment, nucleic-acid binding)</td>
<td>2.15</td>
<td>0.00008</td>
<td>0.016</td>
</tr>
<tr>
<td>Wee1</td>
<td>wee-1 homolog [S. pombe] (cell cycle, kinase activity)</td>
<td>2.14</td>
<td>0.00007</td>
<td>0.015</td>
</tr>
<tr>
<td>Ifi27</td>
<td>Interferon, α-inducible protein 27 (response to virus)</td>
<td>2.10</td>
<td>0.00007</td>
<td>0.014</td>
</tr>
<tr>
<td>Peci</td>
<td>Peroxisomal Δ₁,Δ₂-enoyl-coA isomerase (peroxisome assembly, biogenesis)</td>
<td>2.10</td>
<td>0.00007</td>
<td>0.015</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Adj. P-value</td>
<td>FDR</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------------------------------------------------------------</td>
<td>--------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td><strong>d</strong></td>
<td><strong>Trpm8</strong> Transient receptor potential cation channel, subfamily M, member 8 (Ca²⁺ channel activity)</td>
<td>2.08</td>
<td>0.00064</td>
<td>0.074</td>
</tr>
<tr>
<td><strong>d</strong></td>
<td><strong>Slco1a1</strong> Solute-carrier organic anion transporter family 1, member 1 (organic anion transport)</td>
<td>2.02</td>
<td>0.00010</td>
<td>0.018</td>
</tr>
<tr>
<td><strong>c</strong></td>
<td><strong>Soxs2</strong> Suppressor of cytokine-signaling-2 (chemokine signal transduction, fat cell differentiation)</td>
<td>1.97</td>
<td>0.00035</td>
<td>0.048</td>
</tr>
<tr>
<td><strong>c</strong></td>
<td><strong>Mt4</strong> Metallothionein-4 (metal-ion binding)</td>
<td>1.91</td>
<td>0.00067</td>
<td>0.076</td>
</tr>
</tbody>
</table>

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

**a** These 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.

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

**c** Confirmed twice via Q-PCR to be down-regulated.

**d** Checked 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

<table>
<thead>
<tr>
<th>GO classification of genes most perturbed (up- and down-regulated, combined)\textsuperscript{a}</th>
<th>Gene count</th>
<th>P-value</th>
<th>False discovery rate\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid biosynthesis</td>
<td>16</td>
<td>&lt;10\textsuperscript{-4}</td>
<td>0.0015</td>
</tr>
<tr>
<td>Steroid biosynthesis</td>
<td>15</td>
<td>0.00004</td>
<td>0.0067</td>
</tr>
<tr>
<td>Cellular lipid metabolism</td>
<td>22</td>
<td>0.00006</td>
<td>0.0083</td>
</tr>
<tr>
<td>Nucleosome</td>
<td>8</td>
<td>0.00008</td>
<td>0.0095</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>24</td>
<td>0.00011</td>
<td>0.011</td>
</tr>
<tr>
<td>Oxidoreductase activity</td>
<td>29</td>
<td>0.00013</td>
<td>0.012</td>
</tr>
<tr>
<td>Steroid metabolism</td>
<td>11</td>
<td>0.00019</td>
<td>0.012</td>
</tr>
<tr>
<td>Carboxylic acid metabolism</td>
<td>21</td>
<td>0.00020</td>
<td>0.012</td>
</tr>
<tr>
<td>Organic acid metabolism</td>
<td>21</td>
<td>0.00020</td>
<td>0.012</td>
</tr>
<tr>
<td>Cofactor binding</td>
<td>9</td>
<td>0.00024</td>
<td>0.013</td>
</tr>
<tr>
<td>Lyase activity</td>
<td>12</td>
<td>0.00027</td>
<td>0.013</td>
</tr>
<tr>
<td>Xenobiotic metabolism</td>
<td>9</td>
<td>0.00050</td>
<td>0.023</td>
</tr>
<tr>
<td>Cholesterol biosynthesis</td>
<td>5</td>
<td>0.00067</td>
<td>0.029</td>
</tr>
<tr>
<td>Category</td>
<td>Count</td>
<td>Adjusted P-value</td>
<td>FDR</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-------</td>
<td>------------------</td>
<td>------</td>
</tr>
<tr>
<td>Glutathione transferase activity</td>
<td>5</td>
<td>0.00080</td>
<td>0.030</td>
</tr>
<tr>
<td>Sterol biosynthesis</td>
<td>5</td>
<td>0.0011</td>
<td>0.040</td>
</tr>
<tr>
<td>Alcohol metabolism</td>
<td>13</td>
<td>0.0013</td>
<td>0.042</td>
</tr>
<tr>
<td>Intramolecular oxidoreductase activity</td>
<td>6</td>
<td>0.0013</td>
<td>0.042</td>
</tr>
<tr>
<td>Nucleosome assembly</td>
<td>7</td>
<td>0.0013</td>
<td>0.042</td>
</tr>
<tr>
<td>Tyrosine metabolism</td>
<td>7</td>
<td>0.0028</td>
<td>0.079</td>
</tr>
<tr>
<td>Chromatin</td>
<td>9</td>
<td>0.0029</td>
<td>0.079</td>
</tr>
<tr>
<td>Chromatin assembly or disassembly</td>
<td>8</td>
<td>0.0029</td>
<td>0.079</td>
</tr>
<tr>
<td>Chromatin assembly</td>
<td>7</td>
<td>0.0030</td>
<td>0.079</td>
</tr>
<tr>
<td>Transferase activity (alkyl or aryl)</td>
<td>6</td>
<td>0.0031</td>
<td>0.079</td>
</tr>
<tr>
<td>Cholesterol metabolism</td>
<td>6</td>
<td>0.0036</td>
<td>0.086</td>
</tr>
<tr>
<td>Tryptophan metabolism</td>
<td>8</td>
<td>0.0037</td>
<td>0.086</td>
</tr>
<tr>
<td>Isomerase activity</td>
<td>9</td>
<td>0.0041</td>
<td>0.089</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Cyp1(+/+), U</th>
<th>Cyp1(+/+), TCDD</th>
<th>Cyp1a1/1a2/1b1(-/-), U</th>
<th>Cyp1a1/1a2/1b1(-/-), TCDD</th>
<th>TKO/WT (microarray)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp1a1</td>
<td>1.0 ± 1.1</td>
<td>6.010 ± 51^a</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Cyp1a2</td>
<td>450 ± 39</td>
<td>2200 ± 180^a</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Cyp1b1</td>
<td>6.2 ± 0.59</td>
<td>62 ± 6.3^a</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Cyp1a1</td>
<td>1.0 ± 0.1</td>
<td>0.39 ± 0.1^a</td>
<td>39 ± 0.1^b</td>
<td>13 ± 0.2^a</td>
<td>+7.09</td>
</tr>
<tr>
<td>Sult3a1</td>
<td>1.00 ± 0.2</td>
<td>0.42 ± 0.2^a</td>
<td>0.90 ± 0.1</td>
<td>0.29 ± 0.2^a</td>
<td>+3.62</td>
</tr>
<tr>
<td>Mid1</td>
<td>1.0 ± 0.6</td>
<td>1.1 ± 0.4</td>
<td>4.1 ± 0.7^b</td>
<td>0.87 ± 0.03^a</td>
<td>+2.93</td>
</tr>
<tr>
<td>Vdldr</td>
<td>1.0 ± 0.02</td>
<td>2.3 ± 0.1^a</td>
<td>3.8 ± 0.2^b</td>
<td>2.8 ± 0.02^a</td>
<td>+2.59</td>
</tr>
<tr>
<td>Nqo1</td>
<td>1.0 ± 0.1</td>
<td>4.5 ± 0.3^a</td>
<td>2.0 ± 0.2^b</td>
<td>3.7 ± 0.4^a</td>
<td>+2.20</td>
</tr>
<tr>
<td>Ugt1a6b</td>
<td>1.0 ± 0.1</td>
<td>1.88 ± 0.3^a</td>
<td>2.4 ± 0.4^b</td>
<td>4.6 ± 0.4^a</td>
<td>+2.15</td>
</tr>
<tr>
<td>Ugt1a7c</td>
<td>1.0 ± 0.5</td>
<td>4.5 ± 0.4^a</td>
<td>4.0 ± 0.2^b</td>
<td>4.6 ± 0.4</td>
<td>+2.07</td>
</tr>
<tr>
<td>Gsta1</td>
<td>0.83 ± 0.1</td>
<td>4.2 ± 0.06^a</td>
<td>0.29 ± 0.3</td>
<td>1.3 ± 0.1^a</td>
<td>NA</td>
</tr>
<tr>
<td>Hmox1</td>
<td>1.0 ± 0.3</td>
<td>0.43 ± 0.1^a</td>
<td>0.15 ± 0.1^b</td>
<td>0.17 ± 0.4</td>
<td>NA</td>
</tr>
<tr>
<td>Gclc</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>0.87 ± 0.3</td>
<td>NA</td>
</tr>
<tr>
<td>Gclm</td>
<td>1.0 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>0.55 ± 0.2</td>
<td>0.60 ± 0.3</td>
<td>NA</td>
</tr>
<tr>
<td>St3gal4</td>
<td>1.2 ± 0.03</td>
<td>0.48 ± 0.1^a</td>
<td>0.29 ± 0.2^b</td>
<td>0.69 ± 0.1^a</td>
<td>-5.86</td>
</tr>
<tr>
<td>Mt1</td>
<td>1.0 ± 0.3</td>
<td>0.35 ± 0.2^a</td>
<td>0.21 ± 0.1^b</td>
<td>0.24 ± 0.4</td>
<td>-3.61</td>
</tr>
<tr>
<td>Mt2</td>
<td>1.0 ± 0.2</td>
<td>0.09 ± 0.4^a</td>
<td>0.41 ± 0.2^b</td>
<td>0.56 ± 0.4</td>
<td>-3.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Mt4</td>
<td>1.0 ± 0.01</td>
<td>0.71 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.78 ± 0.2</td>
<td>0.48 ± 0.2</td>
<td>−1.91</td>
</tr>
<tr>
<td>Trpm8</td>
<td>1.0 ± 0.3</td>
<td>4.7 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94 ± 0.4</td>
<td>0.60 ± 0.2</td>
<td>−2.08</td>
</tr>
<tr>
<td>Slco1a1</td>
<td>1.0 ± 0.2</td>
<td>0.42 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.78 ± 0.2</td>
<td>0.20 ± 0.3</td>
<td>−2.02</td>
</tr>
<tr>
<td>Socs2</td>
<td>1.00 ± 0.2</td>
<td>0.42 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90 ± 0.1</td>
<td>0.29 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−1.97</td>
</tr>
<tr>
<td>Ccne1</td>
<td>1.0 ± 0.03</td>
<td>0.05 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08 ± 0.3</td>
<td>−1.56&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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.

<sup>a</sup>Denotes *P* <0.05, comparing Q-PCR measurements of liver RNA from TCDD-treated with untreated (U) controls of same genotype.

<sup>b</sup>Denotes *P* <0.05, comparing Q-PCR in *Cyp1a1/a2/b1/(-/-)* versus *Cyp1(+/+)* mice.

<sup>c</sup>*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/a2/b1(−/−)* compared with untreated *Cyp1(+/+)* mice.
Figure 1

(A) Pups per litter for different genotypes: Cyp1(+/+) and Cyp1a1/1a2/1b1(-/-). The graph shows a comparison between F1 and F2 generations.

(B) Body weight (g) over age (weeks) for different genotypes: Cyp1(+/+) males, Cyp1(+/+) females, Cyp1a1/1a2/1b1(-/-) males, and Cyp1a1/1a2/1b1(-/-) females. The data points are shown with error bars indicating the variability in the measurements.
Figure 2

The figure shows bar charts comparing the levels of Total Blood BaP and enzyme activities (ALT, AST) in different tissues and organs between two genotypes: Cyp1(+/+) and Cyp1a1/1a2/1b1(-/-). The data is presented in ng/mL, mg/g BW, and percent. The error bars indicate the standard error of the mean.
Figure 3
Figure 5

Graph showing the total cell numbers, neutrophils, and macrophages over time after zymosan A injection.
Figure 6

Cyp1a(+/-) Cyp1b1(+/-)  \[\times\]  Cyp1a(+/-) Cyp1b1(-/-)  \[\times\]  Cyp1a(-/-) Cyp1b1(-/-)

\[\Downarrow\]

Phenotype \[\cong\] Phenotype \[\gg\] Phenotype

\[Cyp1(-/-)\]  \[1:4\]  \[1:2\]  \[100\%\]