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Hepatocyte-specific deletion of TIPARP, a negative regulator of the aryl hydrocarbon receptor, is sufficient to increase sensitivity to dioxin-induced wasting syndrome

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3 **Hepatocyte-specific deletion of TIPARP, a negative regulator of the aryl**
4 **hydrocarbon receptor, is sufficient to increase sensitivity to dioxin-induced wasting**
5 **syndrome**
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23 Running Title: Hepatocyte-specific deletion of TIPARP increases dioxin toxicity
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Abstract

The aryl hydrocarbon receptor (AHR) mediates the toxic effects of dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD), which include thymic atrophy, steatohepatitis, and a lethal wasting syndrome in laboratory rodents. Although the mechanisms of dioxin toxicity remain unknown, AHR signaling in hepatocytes is necessary for dioxin-induced liver toxicity. We previously reported that loss of TCDD-inducible poly(ADP-ribose) polymerase (TIPARP/PARP7/ARTD14), an AHR target gene and mono-ADP-ribosyltransferase, increases the sensitivity of mice to dioxin-induced toxicities. To test the hypothesis that TIPARP is a negative regulator of AHR signaling in hepatocytes, we generated *Tiparp*^{fl/fl} mice in which exon 3 of *Tiparp* is flanked by loxP sites, followed by Cre-lox technology to create hepatocyte-specific (*Tiparp*^{fl/fl}*Cre*^{Alb}) and whole-body (*Tiparp*^{fl/fl}*Cre*^{CMV}; *Tiparp*^{Ex3-/-}) *Tiparp* null mice. *Tiparp*^{fl/fl}*Cre*^{Alb} and *Tiparp*^{Ex3-/-} mice given a single injection of 10 µg/kg dioxin did not survive beyond day 7 and 9, respectively, while all *Tiparp*^{+/+} mice survived the 30-day treatment. Dioxin-exposed *Tiparp*^{fl/fl}*Cre*^{Alb} and *Tiparp*^{Ex3-/-} mice had increased steatohepatitis and hepatotoxicity as indicated by greater staining of neutral lipids and serum alanine aminotransferase activity than similarly treated wild-type mice. *Tiparp*^{fl/fl}*Cre*^{Alb} and *Tiparp*^{Ex3-/-} mice exhibited augmented AHR signalling, denoted by increased dioxin-induced gene expression. Metabolomic studies revealed alterations in lipid and amino acid metabolism in liver extracts from *Tiparp*^{fl/fl}*Cre*^{Alb} mice compared with wild-type mice. Taken together, these data illustrate that TIPARP is an important negative regulator of AHR activity, and that its specific loss in hepatocytes is sufficient to increase sensitivity to dioxin-induced steatohepatitis and lethality.

Keywords: aryl hydrocarbon receptor, wasting syndrome, ADP-ribosylation, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD-inducible poly-ADP-ribose polymerase

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) is a highly toxic environmental contaminant produced during waste incineration and other high-temperature industrial processes, and it remains a global health concern. The toxic effects of dioxin are mediated through its binding to and activation of the aryl hydrocarbon receptor (AHR), which is a member of the basic helix-loop-helix (bHLH) Period-AHR nuclear translocator (ARNT)-Single-minded (PAS) family of transcription factors. In the canonical AHR signaling pathway, ligand binding to cytosolic AHR causes its translocation to the nucleus where it dimerizes with ARNT (also known as hypoxia-inducible factor 1 β ; HIF1 β). The AHR:ARNT heterodimer then binds DNA sequence elements (termed AHREs or DREs) located within the regulatory regions of its target genes, which include *cytochrome P4501A1* (*CYP1A1*), *CYP1B1* and TCDD-inducible ADP-ribose polymerase (TIPARP)(Ma *et al.*, 2001; Whitlock, 1999; Zhang *et al.*, 1998). In addition to its roles in dioxin toxicity, xenobiotic metabolism and vascular development (Stevens *et al.*, 2009), AHR has important roles in T-cell differentiation, in the defense against bacterial infections and in gut homeostasis (Moura-Alves *et al.*, 2014; Quintana *et al.*, 2008). To date, more than 400 AHR ligands have been identified, including dietary compounds (3,3'-diindolylmethane), various endogenous ligands (kynurenine, 6-formylindolo(3,2b)carbazole; FICZ), and environmental contaminants such as dioxin (Denison *et al.*, 2003).

Dioxin causes diverse toxic effects in laboratory rodents including immunosuppression, steatohepatitis, impaired reproduction and a lethal wasting syndrome (Birnbaum, 1994, 1995; Poland *et al.*, 1982). A single dose of dioxin induces a lethal starvation-like syndrome, which includes decreased gluconeogenesis, liver damage, steatohepatitis and dyslipidaemia, ultimately leading to lethality (Linden *et al.*, 2010; Seefeld *et al.*, 1984). Acute lethality varies widely both among species and between rodent strains. For example, the median lethal dose (LD₅₀) for guinea pigs is 1-2 μ g/kg dioxin, whereas the LD₅₀ for hamsters is >5000 μ g/kg (Pohjanvirta *et al.*, 1994; Poland, *et al.*, 1982). In most strains of mice, lethality occurs only 2-3 weeks after a single dose of 115-300 μ g/kg of dioxin (Pohjanvirta, *et al.*, 1994; Poland, *et al.*, 1982). There is a roughly 10-fold difference in susceptibility between the high sensitivity C57BL/6 (*Ahr*^{bl} allele) and low sensitivity DBA/2 (*Ahr*^d allele) mouse strains, which is due to polymorphic variations in their ligand-binding domains (Poland *et al.*, 1994). The molecular mechanisms of dioxin-induced wasting syndrome remain obscure, but transgenic mice overexpressing AHR show increased sensitivity to dioxin toxicities, whereas *Ahr*^{-/-} null and *Ahr*^{dbd} mice, which express a mutant AHR that does not bind to AHREs, are resistant to the effects of dioxin (Fernandez-Salguero *et al.*, 1996; Lee *et al.*, 2010; Walisser *et al.*, 2005).

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3 TIPARP (PARP7/ARTD14) is an AHR-regulated gene and a member of the poly-
4 adenosine diphosphate (ADP)-ribose) polymerase (PARP) family. PARPs are
5 nicotinamide adenine dinucleotide (NAD⁺)-dependent enzymes that use NAD⁺ as a
6 substrate to transfer one molecule of ADP-ribose, referred to as mono-ADP-ribosylation
7 (MARylation), or several ADP-ribose moieties, referred to as poly-ADP-ribosylation
8 (PARylation), to specific amino acid residues on themselves and on target proteins
9 (Hottiger *et al.*, 2010). Mono- and poly-ADP-ribosylation are reversible post-translational
10 modifications involved in several biological processes, such as immune cell function,
11 regulation of transcription, protein expression and DNA repair (Kraus *et al.*, 2013). We
12 previously reported that TIPARP is a mono-ADP-ribosyltransferase that functions as part
13 of a negative feedback loop to repress AHR activity through a mechanism that involves
14 reduced ligand-induced AHR protein levels and that requires TIPARP's catalytic activity
15 (MacPherson *et al.*, 2013). Moreover, *Tiparp*^{-/-} mice exhibit increased AHR activity but
16 also increased sensitivity to dioxin-induced toxicities, including steatohepatitis,
17 hepatotoxicity and lethal wasting syndrome (Ahmed *et al.*, 2015). Although the
18 mechanisms of dioxin-induced toxicity remain incompletely understood, AHR expression
19 in hepatocytes is needed to generate the adaptive as well as toxic response to dioxin
20 exposure (Walisser, et al., 2005).
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27 Here, we describe the generation of *Tiparp* conditional mutant mice in which
28 exon 3 of the *Tiparp* gene is flanked by loxP sites, and the subsequent creation of both
29 hepatocyte-specific (*Tiparp*^{fl/fl}*Cre*^{Alb}) and whole-body (*Tiparp*^{fl/fl}*Cre*^{CMV}; *Tiparp*^{Ex3-/-})
30 TIPARP knockout mice. These mice were used to further investigate the role of TIPARP
31 in AHR signaling and dioxin-induced toxicity.
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Materials and Methods

Generation of conditional Tiparp null mice - $Tiparp^{fl/fl}$ mice, where exon 3 of *Tiparp* was flanked by loxP sites, were generated from embryonic stem (ES) cells purchased from European Conditional Mouse Mutagenesis (EUCOMM; $Tiparp^{tm1a(EUCOMM)Wtsi}$). ES cells were expanded by the Toronto Centre for Phenogenomics (TCP). Correct targeted recombination was confirmed in 2 of 5 ES cell clones purchased. Briefly, SphI-digested genomic DNA was used in Southern blotting to produce 8.9 kb (wild-type) and 12.3 kb ($tm1a$) fragments spanning the 5' region of the targeted sequence of *Tiparp*, and in the targeted allele, including the neomycin-resistance (Neo) cassette. The Neo probe was used to reveal additional or random integrations of the targeting vector in the genomes (fragments of incorrect sizes); these ES clones were excluded. PCR primers used to generate the respective probes are provided in **Supplementary Table S1**. Based on the positive Southern blot results, ES cell clones D3 and G3 were chosen for aggregation and implantation into pseudopregnant surrogate females (performed by TCP). Chimeric $Tiparp^{+/tm1a}$ mice were bred to C57BL/6 albino females and pups were genotyped to identify those with germline transmission of the conditional mutant *Tiparp* allele ($Tiparp^{+/tm1a}$); only ES clone G3 resulted in successful germline transmission. Some $Tiparp^{+/tm1a}$ mice were bred to B6.C-Tg(CMV-cre)1Cgn/J (Jackson Labs, Bar Harbor, ME) to remove the Neo cassette and the targeted exon and leave the *lacZ* reporter gene (conversion to *tmlb* allele) and then outbred once to C57BL/6N to remove Tg-(CMV-cre). Mice heterozygous for the *tmlb* allele were then intercrossed to make homozygous *tmlb* mice ($Tiparp^{tmlb/tmlb}$, referred to as $Tiparp^{Ex3-/-}$). Other $Tiparp^{+/tm1a}$ mice were bred to B6(C3)-Tg(Pgk1-FLPo)10Sykr/J (Jackson Labs, Bar Harbor, ME) to remove the *lacZ* and *Neo* cassettes and leave the targeted exon flanked by LoxP sites (conversion to *tmlc* allele) and then outbred once to C57BL/6N to remove Tg-(Pgk1-FLPo) ($Tiparp^{+/tmlc}$). $Tiparp^{+/tmlc}$ mice were bred to B6.C-Tg(CMV-cre)1Cgn/J to remove the targeted exon (conversion to *tmld* allele) and then outbred once to C57BL/6N to remove Tg-(CMV-cre). Mice heterozygous for the *tmld* allele were then intercrossed to make homozygous *tmld* mice ($Tiparp^{tmld/tmld}$; referred to as $Tiparp^{Ex3-/-}$). $Tiparp^{+/tmlc}$ mice were also bred to B6N.Cg-Tg(Alb-cre)21Mgn/J (Jackson Labs, Bar Harbor, ME) to create hepatocyte-specific *Tiparp* knock-out mice ($Tiparp^{tmlc/tmlc}Cre^{Alb}$, referred to as $Tiparp^{fl/fl}Cre^{Alb}$). This colony was maintained such that $Tiparp^{fl/fl}$ female mice were paired with $Tiparp^{fl/fl}Cre^{Alb}$ male mice. Genotypes of all mice were determined by PCR analysis of tail biopsies using PCR primers shown in **Supplementary Table S1**. The specificity of excision events in $Tiparp^{fl/fl}Cre^{Alb}$ mice was determined by quantitative real time PCR (qPCR) using primer pairs specific for exon 3 of *Tiparp* compared with primer pairs specific for intron 1 (**Supplementary Table S1**). Genotype controls used in experiments for the $Tiparp^{fl/fl}Cre^{Alb}$ line are $Tiparp^{fl/fl}$ (observed to be phenotypically equivalent to *wild-type* (WT) mice), and for the $Tiparp^{Ex3-/-}$ are $Tiparp^{+/+}$. Both *tmlb* and *tmld* lines were used.

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5 *In vivo dioxin treatment studies* - All experiments used 8-10 week old male mice. For the
6 acute 6 hr exposure studies, *Tiparp*^{Ex3-/-} or *Tiparp*^{fl/fl}*Cre*^{Alb} mice and their respective
7 strain-specific WT control mice were treated with a single intraperitoneal (i.p.) injection
8 of 100 µg/kg dioxin, and livers were excised and flash frozen 6 hr later. For the subacute
9 dioxin toxicity studies, mice were treated with a single i.p. injection of 10 or 100 µg/kg of
10 dioxin and sacrificed on day 6. The 10 µg/kg dose of dioxin was dissolved in a mixture of
11 corn oil and dimethyl sulfoxide (CO:DMSO; 90:10, referred to as CO), while the 100
12 µg/kg dose of dioxin was dissolved in pure DMSO. For the survival studies, mice were
13 followed for up to 30 days after a single injection of 10 or 100 µg/kg of dioxin. Control
14 mice received equivalent weight-adjusted volumes of CO or DMSO. The time point for
15 euthanization was determined based on endpoint criteria for our study: a loss of 20%
16 body weight or indications of acute distress. All control mice were euthanized to match
17 the endpoints of dioxin-sensitive mice. For food intake studies, mice were housed
18 individually and provided intact pellets of food that were weighed daily; a baseline was
19 determined for each mouse by monitoring for one week prior to treatment as described
20 previously (Ahmed, et al., 2015). Whole blood was obtained from the saphenous vein for
21 serum alanine aminotransferase (ALT) analysis as described previously (Ahmed, et al.,
22 2015). Hepatic glycogen levels were determined from 10 mg of frozen liver using the
23 Glycogen Assay Kit II (Abcam). Liver, thymus, white and brown adipose tissue (WAT
24 and BAT) were dissected and weighed. Livers from *Tiparp*^{Ex3-/-} or *Tiparp*^{fl/fl}*Cre*^{Alb} mice
25 treated with vehicle, 10 or 100 µg/kg dioxin were collected either on day 6 or on the day
26 of euthanization in the survival studies. Care and treatment of animals followed the
27 guidelines set by the Canadian Council on Animal Care, and all protocols were approved
28 by the University of Toronto Animal Care Committee.

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38 *Hepatocytes* - *Tiparp*^{fl/fl}*Cre*^{Alb} or *Tiparp*^{fl/fl} male mice (8-10 weeks old) were used to
39 isolate primary hepatocytes. Mouse liver was perfused with liver perfusion medium
40 (Invitrogen) for 10 min followed by liver digestion medium for 10 min. Freshly prepared
41 hepatocytes were seeded at a final density of 0.5×10^6 cells/well onto type I collagen
42 coated six-well plates in attachment medium (William's E media, 10% dextran-coated
43 charcoal (DCC) stripped fetal bovine serum (FBS), 1× penicillin/streptomycin, and 10
44 nM insulin). The medium was changed 2 h after plating, and all experiments were
45 performed on the second day. Ligands were added to the cells in M199 media with 5%
46 DCC-FBS and cells were harvested 16 h after ligand treatment for RNA extraction.

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51 *RNA extraction and gene expression analysis* - Livers were removed, washed in ice-cold
52 PBS, weighed, and flash frozen in liquid nitrogen. Frozen livers were homogenized in
53 TRIZOL reagent (Life Technologies) and total RNA was isolated using the Aurum RNA
54 isolation kits (BioRad) and reverse transcribed as previously described (Ahmed, et al.,
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2015). Primers used to amplify target transcripts are provided in **Supplementary Table S1** or described elsewhere (Ahmed, et al., 2015). All genes were normalized to TATA binding protein (Tbp) levels and analyzed using the comparative C_T ($\Delta\Delta C_T$) method.

ChIP assays - ChIP assays were performed as previously described (Lo *et al.*, 2011). Briefly, approximately 100 mg of frozen mouse liver was homogenized in 1% formaldehyde in PBS and incubated for 10 min at room temperature. The homogenate was centrifuged at 8,000xg for 5 min at 4°C. Pellet was washed in ice-cold PBS, centrifuged, and resuspended in 900 μ L of TSEI (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate) + 1x Protease Inhibitor Cocktail (Sigma, St. Louis, MO). Samples were sonicated 10 times for 30 sec ON/30 sec OFF on the high setting using a Bioruptor (Diagenode). The supernatants were transferred to new microcentrifuge tubes and incubated with rabbit IgG (5 μ g; Sigma) and anti-AHR (5 μ g; SA-210, Enzo) overnight at 4°C under gentle agitation. ChIP samples were washed, the DNA and the ChIP-qPCR was performed as previously described (Lo *et al.*, 2011).

Histology – Hematoxylin and Eosin and Oil-Red-O/Hematoxylin staining were performed following standard methods with representative images provided. Paraformaldehyde-fixed or OCT-embedded tissues were provided to the Histology Core Facility at the Princess Margaret Cancer Centre, Toronto, Ontario, for all histology sample processing, staining and scanning of stained slides.

Western blotting - For hepatic AHR protein detection, whole cell extracts were prepared by homogenizing 100 mg of liver tissue in RIPA lysis buffer. Ten micrograms of total protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with anti-AHR antibody (SA-210) and stripped and then incubated with anti- β -actin antibody (Sigma A-2228).

Metabolomics – *Tiparp*^{fl/fl} and *Tiparp*^{fl/fl}*Cre*^{Alb} mice were treated with a single intraperitoneal injection of CO:DMSO or 10 μ g/kg dioxin. Liver tissue (100 mg) was collected on day 3 and flash frozen in liquid nitrogen. The frozen tissue was extracted and metabolomic analyses were performed by Metabolon (Durham, NC). Raw data received from Metabolon were preprocessed to input missing values and normalized using logarithmic transformation. Shapiro's test was used to test for normality and Levene's test was used to test for the homogeneity of variances. ANOVA was used to analyze the differences in group means, followed by Tukey's-HSD for post-hoc correction. The FDR method was used to adjust the *p*-values for multiple ANOVA tests (one for each metabolite). At an FDR of 10%, all the group comparisons that exhibited a statistically

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3 significant difference (post-hoc corrected p -values < 0.05) were considered significant.
4 All the analyses were performed in R version 3.4.1.
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7 *Statistical Analysis* - All data were presented as means and S.E.M. Two-way analysis of
8 variance (ANOVA) followed by Sidak's post-hoc test or one-way ANOVA followed by
9 Tukey's post-hoc test were used to assess statistical significance ($P < 0.05$) using
10 GraphPad Prism 6 Software (San Diego, CA) or R version 3.4.1.
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13 Results

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16 **Generation of Conditional *Tiparp*^{fl/fl} Mice** – Mice harboring the conditional *Tiparp*^{fl}
17 allele were generated from *Tiparp*^{tm1a(EUCOMM)Wtsi} ES cells purchased from EUCOMM. A
18 partial map of both the *Tiparp* WT and *Tiparp* tm1a alleles is shown in **Fig. 1A**. Southern
19 blotting of genomic DNA isolated from 4 different ES cell clones confirmed the correct
20 integration of the tm1a allele in the ES cell clones D3 and G3 (**Fig. 1B**). Mice generated
21 in this study were from ES cell clone G3. Mice harboring the conditional *Tiparp*^{fl} allele
22 were generated from the *Tiparp* tm1a allele by excision of the neomycin and LacZ genes
23 through crosses with B6(C3)-Tg(Pgk1-FLPo)10Sykr/J mice, leaving the targeted exon
24 flanked by LoxP sites. Mice heterozygous for the tm1c allele were then intercrossed to
25 make homozygous tm1c mice (*Tiparp*^{fl/fl}; tm1c allele).
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31 **Complete and Hepatocyte-Specific Excision of the *Tiparp*^{fl/fl} Allele** – Using genetrap
32 targeted TIPARP null mice in which a LacZ gene is inserted in front of exon 1 in the
33 *Tiparp* gene (Schmahl *et al.*, 2007), we reported that loss of TIPARP expression
34 increased the sensitivity of mice to dioxin-induced steatohepatitis and lethality (Ahmed,
35 *et al.*, 2015). These findings supported the notion that TIPARP protects against dioxin-
36 induced toxicity by negatively regulating AHR action. Because the type of gene knockout
37 targeting strategy can impact the phenotypes observed from targeting the same gene, we
38 created a complete TIPARP knockout by removal of exon 3 of *Tiparp* (*Tiparp*^{Ex3-/-}) as
39 described in Materials and Methods. To generate a complete TIPARP null mouse in
40 which exon 3 is removed (*Tiparp*^{Ex3-/-}) we used two different strategies. In the first
41 approach, mice heterozygous for the tm1c allele were bred to B6.C-Tg(CMV-cre)1Cgn/J
42 mice to remove the targeted exon (*Tiparp*^{Ex3-/-}; tm1d allele). In second approach, mice
43 carrying the tm1a allele were bred to B6.C-Tg(CMV-cre)1Cgn/J mice to remove the Neo
44 cassette and the targeted exon and leave the lacZ reporter (*Tiparp*^{Ex3-/-}; tm1b allele). A
45 map of the *Tiparp* tm1c, tm1d and tm1b alleles is shown in **Fig. 1C**. The correct genotype
46 was confirmed by PCR analysis of genomic DNA (**Fig. 1D**). *Tiparp*^{Ex3-/-} mice represent a
47 distinct TIPARP null strain compared with *Tiparp* targeted knockout using a genetrap
48 approach (Ahmed, *et al.*, 2015; Schmahl, *et al.*, 2007). To determine whether the loss of
49 TIPARP in hepatocytes is sufficient to increase sensitivity to dioxin-dependent toxicity,
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we generated mice in which *Tiparp* was deleted in hepatocytes, *Tiparp^{fl/fl}Cre^{Alb}* (**Fig. 2A**). *Tiparp^{fl/fl}Cre^{Alb}* mice were created by breeding mice homozygous for the tm1c allele (*Tiparp^{fl/fl}*) with B6N.Cg-Tg(Alb-cre)21Mgn/J mice to remove the targeted exon specifically in hepatocytes (Walisser, et al., 2005). For the *Tiparp^{fl/fl}Cre^{Alb}* line, WT mice were referred to as *Tiparp^{fl/fl}*, whereas for the *Tiparp^{Ex3-/-}* line, WT mice were referred to as *Tiparp^{+/+}* for simplicity.

To examine the specificity of excision events in *Tiparp^{fl/fl}Cre^{Alb}* mice, we analyzed various tissues by determining the relative ratios of *Tiparp* exon 3 compared with intron 1 using qPCR. *Tiparp* exon 3 was efficiently excised in liver tissue and in isolated hepatocytes (Hepa) in the presence of Cre^{Alb}, but not in the other tissues examined (**Fig. 2A**). *Tiparp* exon 3 was not detected in liver tissue isolated from *Tiparp^{Ex3-/-}* mice (**Fig. 2B**). In the absence of Cre^{Alb}, the *Tiparp^{fl/fl}* mice showed only the *Tiparp^{fl}*-unexcised allele in all tissues and isolated hepatocytes examined (**Fig. 2A**).

Cloning and DNA sequencing revealed that the conditional inactivation of the *Tiparp^{fl}* allele accomplished by Cre-mediated deletion of exon 3 (169 bp) results in splicing and fusion between exon 2 (961 bp) and exon 4 (161 bp), leading to the insertion of a premature stop codon. This truncated version of TIPARP contains 311 amino acids, of which the last 4 are the result of a frameshift (the full-length protein is 657 a.a.), and it lacks its WWE and catalytic domains (MacPherson, et al., 2013). In agreement with a previous study, cloning and transient transfection of the truncated TIPARP protein failed to inhibit AHR-dependent and dioxin-induced CYP1A1 reporter gene activity (**Supplementary Fig. S1**).

Consistent with TIPARP's role as a negative regulator of AHR activity, exposure of hepatocytes from *Tiparp^{fl/fl}Cre^{Alb}* mice for 6 hr to 10 nM dioxin increased mRNA expression levels of the AHR target genes *Cyp1a1*, *Cyp1a2* and *Cyp1b1* compared with similarly treated hepatocytes from *Tiparp^{fl/fl}* mice (**Fig. 2C**). *Tiparp* mRNA expression levels were markedly decreased in hepatocytes isolated from *Tiparp^{fl/fl}Cre^{Alb}* mice compared with *Tiparp^{fl/fl}* mice. We next examined the effect of TIPARP loss on AHR target gene expression in liver and lung tissues isolated from *Tiparp^{Ex3-/-}*, *Tiparp^{fl/fl}Cre^{Alb}* and WT mice 6 h after treatment with 100 µg/kg dioxin. Higher *Cyp1a1* and *Cyp1b1* mRNA levels were observed in both liver and lung from *Tiparp^{Ex3-/-}* mice than in *Tiparp^{Ex3+/+}* mice (**Fig. 3A and B**). *Tiparp* mRNA levels were not detected in liver or lung from *Tiparp^{Ex3-/-}* mice compared with *Tiparp^{+/+}* mice (**Fig. 3C**). Significantly increased dioxin-induced *Cyp1a1* and *Cyp1b1* mRNA levels above those observed in *Tiparp^{fl/fl}* mice were only observed in liver and lung from *Tiparp^{fl/fl}Cre^{Alb}* mice (**Fig. 3D and E**). As expected, TIPARP expression levels were reduced in liver, but not in lung in *Tiparp^{fl/fl}Cre^{Alb}* compared with *Tiparp^{fl/fl}* mice (**Fig. 3F**).

Tiparp^{Ex3-/-} Mice Exhibit Increased Sensitivity to Dioxin-Induced Toxicity and Lethality – To determine the sensitivity of *Tiparp^{Ex3-/-}* mice to dioxin-induced toxicity,

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3 these mice and their respective WT controls were injected with a single dose of 10 or 100
4 $\mu\text{g}/\text{kg}$ dioxin and monitored for up to 30 days, as previously described (Ahmed, et al.,
5 2015). All *Tiparp*^{Ex3+/+} mice were normal in physical appearance at the end of the 30-day
6 observation period (**Fig. 4A**), while no dioxin-treated *Tiparp*^{Ex3-/-} mice (tm1d or tm1b)
7 survived the 30-day experiment (**Fig. 4A**). *Tiparp*^{Ex3-/-} mice treated with 100 $\mu\text{g}/\text{kg}$ dioxin
8 became weakened and moribund and were humanely euthanized between days 2 and 3,
9 while those treated with 10 $\mu\text{g}/\text{kg}$ dioxin were euthanized at day 7. *Tiparp*^{Ex3-/-} mice
10 treated with 10 $\mu\text{g}/\text{kg}$ dioxin had lost significant body weight by 5 days after treatment
11 (**Fig. 4B**); however, no decrease in food intake was observed (**Fig. 4C**). No changes in
12 food intake or body weight were seen in dioxin-exposed *Tiparp*^{+/+} mice. Serum ALT
13 activity, a marker of hepatotoxicity, was significantly increased in dioxin-treated
14 *Tiparp*^{Ex3-/-} mice, while no increase above controls was observed in *Tiparp*^{+/+} mice (**Fig.**
15 **4D**). Increased liver weight was observed in dioxin-treated *Tiparp*^{+/+} mice but not in
16 *Tiparp*^{Ex3-/-} mice (**Fig. 4E**). Thymic involution, an endpoint associated with dioxin
17 toxicity, responded as expected in both genotypes at day 7 (**Fig. 4F**). A significant
18 decrease in epididymal white adipose tissue (WAT) weight was seen in *Tiparp*^{Ex3-/-} mice
19 but not in WT mice (**Fig. 4G**). No differences in brown adipose tissue (BAT) weight
20 were observed (data not shown). Hepatic glycogen stores were lower in dioxin-treated
21 *Tiparp*^{Ex3-/-} mice than in WT mice (**Fig. 4H**). These data support the importance of
22 TIPARP in regulating AHR action and show that loss of its expression in mice increases
23 their sensitivity to dioxin toxicity.
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Hepatocyte-Specific Loss of TIPARP Results in Increased Sensitivity to Dioxin-Induced Toxicity and Lethality

33 – Because AHR expression in hepatocytes is required for
34 dioxin-induced liver toxicity, we hypothesized that the loss of TIPARP expression in
35 hepatocytes would enhance dioxin-dependent liver toxicity. To test this hypothesis, we
36 treated *Tiparp*^{fl/fl}*Cre*^{Alb} and *Tiparp*^{fl/fl} mice with a single i.p. injection of 10 or 100 $\mu\text{g}/\text{kg}$
37 dioxin and monitored the mice for up to 30 days. As expected, all *Tiparp*^{fl/fl} mice were
38 normal in physical appearance at the end of the 30-day observation period (**Fig. 5A**). No
39 dioxin-treated *Tiparp*^{fl/fl}*Cre*^{Alb} mice survived the 30-day experiment (**Fig. 5A**).
40 *Tiparp*^{fl/fl}*Cre*^{Alb} mice treated with 100 $\mu\text{g}/\text{kg}$ dioxin appeared weakened and moribund
41 and were humanely euthanized between day 3 and 5, while those treated with 10 $\mu\text{g}/\text{kg}$
42 dioxin were humanely euthanized at day 9. Sensitivity to dioxin-induced lethality was
43 significantly different between *Tiparp*^{fl/fl}*Cre*^{Alb} and *Tiparp*^{Ex3-/-} mice at both 10 and 100
44 $\mu\text{g}/\text{kg}$ dioxin, suggesting that cells other than hepatocytes also contribute to dioxin
45 lethality in these models. *Tiparp*^{fl/fl}*Cre*^{Alb} mice treated with 10 $\mu\text{g}/\text{kg}$ dioxin had lost
46 significant body weight by 6 days after treatment (**Fig. 5B**), while no decrease in food
47 intake was observed (**Fig. 5C**). No change in food intake or body weight was seen in
48 *Tiparp*^{fl/fl} mice. Serum ALT was significantly increased in dioxin-treated *Tiparp*^{fl/fl}*Cre*^{Alb}
49 mice at days 3 and 6, but no increase was observed in *Tiparp*^{fl/fl} mice (**Fig. 5D**). Increased
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3 liver weight was observed in dioxin-treated *Tiparp^{fl/fl}* mice but not in *Tiparp^{fl/fl}Cre^{Alb}*
4 mice (**Fig. 5E**). Decreased thymus weight was seen in both genotypes at day 9 (**Fig. 5F**).
5 Consistent with dioxin-treated *Tiparp^{Ex3-/-}* mice, *Tiparp^{fl/fl}Cre^{Alb}* mice had significantly
6 decreased epididymal WAT levels compared with WT mice (**Fig. 5G**). No difference in
7 BAT weight was observed (data not shown). Hepatic glycogen stores were also decreased
8 in dioxin-treated *Tiparp^{fl/fl}Cre^{Alb}* mice compared with *Tiparp^{fl/fl}* mice (**Fig. 5H**). These
9 findings show that hepatocyte-specific deletion of TIPARP is sufficient to increase
10 dioxin-induced toxicity and lethality.
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14 As an independent measure of liver toxicity, livers were sectioned and stained with
15 hematoxylin and eosin. Vehicle-treated *Tiparp^{fl/fl}* and *Tiparp^{fl/fl}Cre^{Alb}* mice had
16 histologically normal liver architecture (**Fig. 6A**). On day 9, dioxin-treated *Tiparp^{fl/fl}*
17 livers exhibited slight hepatocyte cytoplasmic clearing within periportal regions and
18 inflammatory cell infiltration (**Fig. 6A**). In contrast, day 9 *Tiparp^{fl/fl}Cre^{Alb}* livers were
19 characterized by inflammatory infiltration and a predominant microvesicular steatosis.
20 Six days after dioxin treatment, livers from *Tiparp^{fl/fl}* mice displayed distinct
21 inflammatory cell infiltration and increased clearing of the cytoplasm with the
22 appearance of large vacuoles within hepatocytes. Similar findings were observed in livers
23 isolated from dioxin-treated *Tiparp^{+/+}* and *Tiparp^{Ex3-/-}* mice (**Supplementary Fig. 2A**).
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26 We next determined the mRNA levels of AHR-regulated cytokines and the
27 macrophage marker F4/80 (Casado *et al.*, 2011; Matsubara *et al.*, 2012). Hepatic
28 interleukin 6 (*Il6*) levels were unaffected by dioxin treatment in both genotypes (**Fig.**
29 **6B**). However, dioxin-treated *Tiparp^{fl/fl}Cre^{Alb}* mice had increased hepatic expression of
30 *Serpine 1* (also known as plasminogen activator inhibitor-1; PAI-1), chemokine (C-X-C
31 motif) ligand 2 (*Cxcl2*) and *F4/80* when compared with *Tiparp^{fl/fl}* mice (**Fig 6C-E**).
32 Similar findings were also seen in livers isolated from dioxin-treated *Tiparp^{+/+}* and
33 *Tiparp^{Ex3-/-}* mice (**Supplementary Fig. 2B-E**). The increased cytokine and F4/80 levels
34 increased hepatic inflammation in dioxin-treated *Tiparp^{fl/fl}Cre^{Alb}* compared with wild-
35 type mice.
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42 ***Hepatocyte-Specific Loss of TIPARP Increases Dioxin-Induced Steatohepatitis*** - Livers
43 from vehicle-treated *Tiparp^{fl/fl}* and *Tiparp^{fl/fl}Cre^{Alb}* mice were macroscopically normal
44 (**Fig. 7A**). Livers from *Tiparp^{fl/fl}* mice were enlarged but only slightly pale in color 9 days
45 after dioxin treatment (**Fig. 7A**). Livers from dioxin-treated *Tiparp^{fl/fl}Cre^{Alb}* mice were
46 markedly pale in color at 9 days, suggesting a high level of lipid accumulation. We tested
47 for the presence of neutral lipids by Oil-Red-O staining (**Fig. 7B**). Livers from vehicle-
48 exposed *Tiparp^{fl/fl}* and *Tiparp^{fl/fl}Cre^{Alb}* mice were negative for Oil-Red-O staining. On day
49 9 after dioxin treatment, small droplets of lipid were seen in the livers of *Tiparp^{fl/fl}* mice,
50 while those from similarly treated *Tiparp^{fl/fl}Cre^{Alb}* mice had substantial intracytoplasmic
51 lipid accumulation. Comparable findings were observed in livers isolated from dioxin-
52 treated *Tiparp^{+/+}* and *Tiparp^{Ex3-/-}* mice (**Supplementary Fig. 3A & B**).
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We then analyzed the hepatic levels of transcripts encoding genes involved in lipid uptake, lipogenesis and cholesterol/bile acid metabolism. Consistent with previous studies (Lee, et al., 2010; Lu *et al.*, 2011), the lipid uptake transporter, scavenger receptor encoded by cluster of differentiation 36 (*Cd36*), was increased 3-fold by dioxin treatment in *Tiparp^{fl/fl}* mice and to a greater extent (8-fold) in similarly treated *Tiparp^{fl/fl}Cre^{Alb}* mice (Fig. 7C). Hepatic expression of lipogenic genes including sterol regulatory element-binding transcription factor 1 (*Srebp1*), and stearoyl-CoA desaturase (*Scd1*) were significantly decreased in dioxin treated *Tiparp^{fl/fl}Cre^{Alb}* mice compared with *Tiparp^{fl/fl}* mice (Fig. 7D & E). Peroxisome proliferator activating receptor α (*Ppara*) and *Cyp7a1*, the rate limiting enzyme bile acid synthesis, were also significantly decreased in *Tiparp^{fl/fl}Cre^{Alb}* mice compared with *Tiparp^{fl/fl}* mice (Fig. 7F-G). Similar findings were seen in livers isolated from dioxin-treated *Tiparp^{+/+}* and *Tiparp^{Ex3-/-}* mice (Supplementary Fig. 3C-G). These data suggest that the increased sensitivity of *Tiparp^{fl/fl}Cre^{Alb}* and *Tiparp^{Ex3-/-}* mice to dioxin-induced steatohepatitis is due to increased lipid uptake rather than increased hepatic lipogenesis.

Increased AHR Regulated Gene Expression in *Tiparp^{fl/fl}Cre^{Alb}* Mice after Treatment with 10 μ g/kg Dioxin – To identify AHR genes and/or changes in metabolites that might provide insight into the molecular mechanisms regulating the increased dioxin sensitivity of *Tiparp^{fl/fl}Cre^{Alb}* mice, we analyzed changes in dioxin-induced hepatic mRNA and metabolite levels 3 days after dioxin treatment. Day 3 was chosen because we observed significant increases in serum ALT activity in *Tiparp^{fl/fl}Cre^{Alb}* mice, and we reasoned that this time point could be used to identify early changes in AHR target gene expression and/or metabolite levels prior to more severe toxicities that ultimately led to death. *Tiparp^{fl/fl}Cre^{Alb}* mice treated with 10 μ g/kg dioxin exhibited increased mRNA expression levels of many AHR target genes including *Cyp1a1*, *Cyp1a2*, *Ahrr*, *Nqo1*, *Nfe2l2* and *Serpine1* compared with similarly treated *Tiparp^{fl/fl}* mice (Fig. 8 A, C-H). *Cyp1a2* expression was slightly increased in *Tiparp^{fl/fl}Cre^{Alb}* compared with *Tiparp^{fl/fl}* mice, but this difference was not statistically significant (Fig. 8B). No significant increase in AHR recruitment to *Cyp1a1* was observed (Fig. 8I). Significantly higher levels of AHR were recruited to *Cyp1b1* in liver extracts from *Tiparp^{fl/fl}Cre^{Alb}* mice compared with *Tiparp^{fl/fl}* mice (Fig. 8J). Consistent with our previous study, we observed reduced dioxin-induced proteolytic degradation of total AHR protein in liver extracts from *Tiparp^{fl/fl}Cre^{Alb}* mice compared with *Tiparp^{fl/fl}* mice (Fig. 8K).

We next did comparative metabolomic analyses on liver extracts from *Tiparp^{fl/fl}Cre^{Alb}* and *Tiparp^{fl/fl}* mice. Principal component analysis (PCA) revealed significant treatment-based separations among the samples, with clear distinctions between the vehicle- and dioxin-treated animals in each genotype. Separations were also apparent between the dioxin-treated *Tiparp^{fl/fl}* and *Tiparp^{fl/fl}Cre^{Alb}* animals (Fig. 9A). Of the total of 679 named metabolites examined, 213 were significantly altered ($P < 0.05$) by

dioxin treatment of *Tiparp^{fl/fl}Cre^{Alb}* mice compared with 124 in similarly treated *Tiparp^{fl/fl}* mice (**Table 1**; **Supplementary Tables S2 & S3**). Of the 124 metabolites, 74 overlapped with those identified in dioxin-treated *Tiparp^{fl/fl}Cre^{Alb}* mice (**Fig. 9B**). Only 7 metabolites were significantly changed in vehicle-treated *Tiparp^{fl/fl}Cre^{Alb}* compared with *Tiparp^{fl/fl}* mice, showing that the loss of TIPARP had little effect on basal liver metabolism (**Supplementary Tables S4**). Consistent with previous studies, dioxin treatment resulted in significant lipidomic changes, with accumulation of several classes of free fatty acids and metabolites linked to complex lipid homeostasis (Nault *et al.*, 2016b). Increased lipids were observed in both the *Tiparp^{fl/fl}* and *Tiparp^{fl/fl}Cre^{Alb}* animals, with a few classes of lipids that were differentially expressed in the two groups (**Supplementary Table S2 and S3**). These included certain long-chain acylcarnitines, fatty acid dicarboxylates, and complex lipids such as plasmalogens and sphingolipids. However, 129 metabolites were altered in dioxin-treated *Tiparp^{fl/fl}Cre^{Alb}* compared with *Tiparp^{fl/fl}* mice (**Table 1**; **Supplementary Table S5**). Altered metabolite levels in *Tiparp^{fl/fl}Cre^{Alb}* and *Tiparp^{fl/fl}* mice were analyzed for pathway over-representation (enrichment) and connectivity within related metabolites (impact) using MetaboAnalyst (Xia *et al.*, 2016) (**Fig. 9C & D**). Only glycerophospholipid metabolism was common among the top 5 or 6 significant pathways (**Supplementary Tables S6 and S7**). Dioxin-treated *Tiparp^{fl/fl}Cre^{Alb}* mice also differed with regard to increased γ -glutamyl- ϵ -lysine levels (12.5-fold; **Fig 9E**), which may reflect increased transglutaminase activity, and polyamine metabolism (*N*-acetylputrescine; **Fig 9F**, and putrescine; **Fig. 9G**). Because dioxin toxicity has been tightly linked with NAD^+ levels, its precursors and metabolites, and because TIPARP activity is dependent on NAD^+ , we examined NAD^+ metabolism (Diani-Moore *et al.*, 2010; Diani-Moore *et al.*, 2017; He *et al.*, 2013). Dioxin-dependent increases in nicotinamide ribonucleoside (**Fig. 9H**) and nicotinamide (**Fig 9I**) were observed in *Tiparp^{fl/fl}Cre^{Alb}* and *Tiparp^{fl/fl}* mice, respectively. Significant decreases in intrahepatic NAD^+ levels were only observed in dioxin-treated *Tiparp^{fl/fl}Cre^{Alb}* mice (**Fig. 9J**).

Discussion

We previously reported that TIPARP acts as part of negative feedback loop to regulate AHR activity, and that global loss of TIPARP expression increases sensitivity to dioxin-induced toxicities such as steatohepatitis and wasting syndrome (Ahmed, et al., 2015; MacPherson, et al., 2013). Since hepatocyte-specific deletion of AHR prevents dioxin-induced hepatotoxicity, we reasoned that hepatocyte-specific deletion of TIPARP would result in increased dioxin-induced hepatotoxicity. We therefore generated a hepatocyte-specific TIPARP deletion (*Tiparp^{fl/fl}Cre^{Alb}*) mouse strain. We also generated a whole-body knockout TIPARP (*Tiparp^{Ex3-/-}*) strain in which *Tiparp* is deleted by the removal of exon 3, making it distinct from other TIPARP null lines (Ahmed, et al., 2015; Kozaki *et al.*, 2017; Schmahl, et al., 2007). Here we show that *Tiparp^{Ex3-/-}* and *Tiparp^{fl/fl}Cre^{Alb}* mice are both more sensitive than WT mice to dioxin-induced hepatotoxicity and lethality. These findings provide further support for the importance of

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3 TIPARP in AHR signaling and for its role in protecting against dioxin-induced toxicity
4 (Ahmed, et al., 2015; Matthews, 2017), as well as demonstrating that the expression of
5 TIPARP in hepatocytes plays a key role in the manifestations of this toxicity.
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7 *Tiparp*^{fl/fl}*Cre*^{Alb} mice treated with dioxin lost significant body weight without any
8 reduction in food intake. Hepatic glycogen and epididymal WAT levels were also
9 reduced, pointing to a possible deficiency in the efficiency of intestinal nutrient
10 absorption resulting in altered metabolism. Dioxin-induced hypophagia contributes to
11 body weight and adipose tissue loss in many species, but numerous studies using pair-
12 feeding or total parenteral nutrition have failed to identify a single explanation for the
13 weight loss (Linden, et al., 2010; Seefeld, et al., 1984). The severe hepatotoxicity and
14 extensive hepatosteatosis in dioxin-treated *Tiparp*^{fl/fl}*Cre*^{Alb} and *Tiparp*^{Ex3-/-} mice, would
15 cause impaired liver function that could result in reduced intestinal nutrient absorption
16 and impaired liver homeostasis (Kalaitzakis, 2014).
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18 Dioxin-treated *Tiparp*^{fl/fl}*Cre*^{Alb} and *Tiparp*^{Ex3-/-} mice exhibit many of the
19 alterations in lipid homeostasis, increased hepatic inflammation and other toxic endpoints
20 that have been reported in other studies (Boverhof *et al.*, 2006; Duval *et al.*, 2017).
21 *Tiparp*^{fl/fl}*Cre*^{Alb} and *Tiparp*^{Ex3-/-} mice exhibited increased hepatosteatosis due to increased
22 expression of genes regulating lipid uptake (*Cd36*), but decreased expression of those
23 involved in fatty acid β -oxidation and *de novo* lipogenesis (*Scd1*, *Srebp1*, *Ppara*)
24 (Ahmed, et al., 2015; Duval, et al., 2017; Lee, et al., 2010). *Scd1* expression was
25 increased in *Tiparp*^{fl/fl} mice, but decreased in *Tiparp*^{fl/fl}*Cre*^{Alb} mice. One possible
26 explanation is that the increased hepatosteatosis in *Tiparp*^{fl/fl}*Cre*^{Alb} mice results in
27 negative regulation of *Scd1* by other factors or hormones (Mauvoisin *et al.*, 2011).
28 *Srebp1* expression levels were decreased in *Tiparp*^{fl/fl}*Cre*^{Alb} and *Tiparp*^{Ex3-/-} mice,
29 supporting findings from a recent high-dose dioxin exposure study (Duval, et al., 2017).
30 *Srebp1* expression is positively regulated by the liver X receptor (LXR) and PPAR α .
31 TIPARP is an LXR coactivator, so the loss of *Tiparp* expression combined with the
32 reduced PPAR α expression levels could also contribute to reduced SREBP1 levels
33 (Bindesboll *et al.*, 2016).
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35 CYP7A1 plays a critical role in the control of bile acid and cholesterol
36 homeostasis as the rate-limiting enzyme in the classic bile acid synthesis pathway (Gupta
37 *et al.*, 2001). Overexpression of mouse CYP7A1 protects against high-fat diet induced
38 obesity, fatty liver and insulin resistance (Li *et al.*, 2010), whereas in humans, genetic
39 deficiency of CYP7A1 leads to hyperlipidemia (Pullinger *et al.*, 2002). The decrease in
40 CYP7A1 expression levels in treated *Tiparp*^{fl/fl}*Cre*^{Alb} and *Tiparp*^{Ex3-/-} mice is in agreement
41 with studies of male C57BL/6 mice treated with 0.01 to 30 μ g/kg dioxin every 4 days for
42 28 days (Fader *et al.*, 2017), and could be a contributing factor to the increased
43 hepatosteatosis and reduced WAT levels observed, through reduced lipid absorption
44 resulting from altered bile acid homeostasis (Fader, et al., 2017). In support of this, we
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3 observed increased hepatic levels of taurochenodeoxycholic acid and taurocholic acid in
4 dioxin-treated *Tiparp^{fl/fl}Cre^{Alb}* mice but not in *Tiparp^{fl/fl}* mice.

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6 Metabolomic profiling studies identified significant changes in metabolite levels
7 in dioxin-treated *Tiparp^{fl/fl}Cre^{Alb}* mice compared with *Tiparp^{fl/fl}* mice. Many of the major
8 treatment-based changes were conserved in the two cohorts (*i.e.*, accumulated fatty acids,
9 changes related to nucleotides and polyamines). However, the extent of change of the
10 affected metabolites differed between the *Tiparp^{fl/fl}* and *Tiparp^{fl/fl}Cre^{Alb}* groups. Dioxin-
11 treated *Tiparp^{fl/fl}Cre^{Alb}* mice, however, exhibited significant increases in gamma-
12 glutamyl-epsilon lysine levels compared with their untreated counterparts or dioxin-
13 treated *Tiparp^{fl/fl}* mice. This metabolite is formed by tissue transglutaminase (TG2),
14 which catalyzes crosslinks between glutamine and lysine residues of proteins (Iismaa *et*
15 *al.*, 2009). TG2 activity increases following acute and chronic liver injury, and aberrant
16 TG2 activation has been implicated in the development of fibrosis and cancer (Iismaa, *et*
17 *al.*, 2009). In contrast, retinoid-induced TG2 mRNA up-regulation is reduced by dioxin
18 treatment in a human squamous cell carcinoma cell line (Krig *et al.*, 2000). The increased
19 γ -glutamyl- ϵ -lysine observed in *Tiparp^{fl/fl}Cre^{Alb}* mice suggests that TIPARP might
20 influence TG2 activity following dioxin-induced liver damage.

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22 We observed that the expression of most of the AHR target genes examined was
23 increased in response to dioxin in *Tiparp^{fl/fl}Cre^{Alb}* and *Tiparp^{fl/fl}* compared with WT mice,
24 including *Cxcl2* (macrophage inflammation protein-2) and *Serpine1* genes (Son *et al.*,
25 2002). CXCL2 is a member of the CXC subfamily of chemokines that are crucial for
26 neutrophil recruitment to sites of inflammation following hepatic injury (Marra *et al.*,
27 2014). Levels of plasminogen activator inhibitor-1 (PAI-1), the product of *Serpine1* gene,
28 were higher in *Tiparp^{fl/fl}Cre^{Alb}* mice than in *Tiparp^{fl/fl}* mice. PAI-1 is a physiologic
29 inhibitor of plasminogen activators that regulate fibrosis via regulation of the
30 extracellular matrix. PAI-1 expression is increased in dioxin-induced fibrosis (Nault *et*
31 *al.*, 2016a).

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33 The mRNA levels of AHR repressor (AHRR), a negative regulator of AHR
34 (Mimura *et al.*, 1999), were increased in *Tiparp^{fl/fl}Cre^{Alb}* compared with *Tiparp^{fl/fl}* mice.
35 AHRR is a potent inhibitor of AHR activity *in vitro* (Karchner *et al.*, 2009) that exhibits
36 gene- and tissue-specific inhibition of AHR signaling in mice (Hosoya *et al.*, 2008).
37 Although the effect of *Ahrr* loss on dioxin-induced wasting syndrome has not been
38 reported, AHRR transgenic mice are protected from dioxin-induced lethality and
39 hepatotoxicity (Vogel *et al.*, 2016).

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41 Increased TIPARP and PARP1 activity have been proposed to be important in
42 augmenting dioxin toxicity through the depletion of NAD⁺. Indeed, NAD⁺ repletion or
43 treatment with the pan-PARP inhibitor PJ34 can prevent dioxin-induced thymic atrophy
44 and hepatosteatosis in a chicken embryo model (Diani-Moore, *et al.*, 2017). However, we
45 observed decreased hepatic NAD⁺ levels in dioxin-treated *Tiparp^{fl/fl}Cre^{Alb}* mice,
46 suggesting that PARP1 or an NAD⁺-consuming enzyme other than TIPARP is
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3 responsible for the reduced NAD⁺ levels after dioxin treatment in mice. Another possible
4 explanation is that there are species differences in the AHR-TIPARP signaling axis, such
5 that TIPARP protects against dioxin toxicity in mice but enhances it in avian species.
6 Further studies using gene targeting methods to delete TIPARP in non-murine models are
7 needed to explain these discrepancies.
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10 AHR is also a key regulator of gut homeostasis, inflammation, immunity and T
11 cell differentiation (Stockinger *et al.*, 2014). AHR is required for the maintenance of
12 intraepithelial lymphocytes (IELs), which are the first line of immune defense in the
13 intestine. Loss of AHR or reduced exposure to dietary AHR ligands compromises these
14 cells, leading to increased microbial load, immune activation and epithelial damage (Li *et*
15 *al.*, 2011). Moreover, AHR activation by dietary indoles (indole-3-carbinol) improves
16 colitis and protects against experimental autoimmune encephalomyelitis (EAE), a murine
17 model of multiple sclerosis (MS) (Lamas *et al.*, 2016; Li, et al., 2011; Monteleone *et al.*,
18 2011; Rouse *et al.*, 2013). An unanswered question is whether TIPARP also regulates
19 endogenous AHR signaling and if so, how would its loss affect the protective role of the
20 AHR signaling pathway in models of inflammatory disease? Kynurenine, an endogenous
21 AHR ligand, was reported to repress type-I-IFN responses during viral infection in an
22 AHR- and TIPARP-dependent manner, supporting the notion that TIPARP has a broad
23 role in AHR biology and regulates endogenous ligand-induced AHR activation (Yamada
24 *et al.*, 2016).
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30 In summary, we provide evidence from two additional mouse models that the loss
31 of TIPARP expression increases sensitivity to dioxin toxicity and lethality. Hepatocyte-
32 specific loss of TIPARP is sufficient to increase sensitivity to dioxin hepatotoxicity,
33 steatosis and lethality, highlighting the importance of liver damage in the dioxin-induced
34 wasting syndrome. Our results provide further support for the importance of the AHR-
35 TIPARP axis in regulating dioxin toxicity, and potentially in regulating the biological
36 actions of AHR following its activation by endogenous or dietary ligands.
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Declaration of Interest

The authors have nothing to declare.

Supplemental Data

Data available from the Dryad Digital Repository <https://doi.org/10.5061/dryad.rc5b58m>

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Figure Legends

Figure 1. Generation of the conditional *Tiparp*^{fl/fl} mice. **(A)** Schematic diagram of *Tiparp* WT allele and the allele with successful recombination of the tm1 targeting construct (*Tiparp* tm1a allele) and corresponding Southern blot data. Exon numbers reflect known coding exons; white arrowheads represent FRT sites; grey arrowheads represent LoxP sites; LacZ represents lacZ reporter gene; N represents the neomycin (Neo) resistance cassette; S, SphI site; dashed lines indicate the fragment of DNA generated by SphI digestion that was detected with the radiolabeled probes (5' probe and Neo probe); letters indicate genotyping primers. **(B)** Southern blots show *Tiparp* allele fragments detected with the radiolabeled probes: ES clones D3 and G3 show correct homologous recombination of the targeting construct (12.3 kb fragment detected with 5' probe) without additional random integrations (only the 12.3 kb band detected with Neo probe). **(C)** Schematic diagram of *Tiparp* tm1 alleles following excision of sequence by CRE or FLP recombinases. **(D)** PCR data from mouse tail biopsies. The *Tiparp* tm1a allele was converted to tm1b using CRE recombinase to remove the Neo cassette and exon 3 located between the LoxP sites. Primer pair D-E amplify a product spanning the excision sites to show that the floxed sequences were removed (420 bp). The *Tiparp* tm1a allele was converted to tm1c using FLP recombinase to remove the LacZ and Neo cassettes located between the FRT sites. Primer pair A-B amplify a product spanning the excision site to show that the sequence was flipped out (750 bp). The *Tiparp* tm1c allele was converted to tm1d using CRE recombinase to remove the floxed exon 3. Primer pair A-E amplify a product spanning the floxed region to show that the exon is removed (310 bp). Sample genotypes: + indicates the *Tiparp* WT allele; letters indicate the *Tiparp* tm1 corresponding allele (tm1a, tm1b, tm1c, tm1d).

Figure 2. Specificity of Cre^{Alb}-mediated excision of the *Tiparp*^{fl} allele. **(A)** Specificity of *Tiparp*^{fl} excision by Cre^{Alb} was determined by quantitative real-time PCR-based genotyping for excised and unexcised alleles of *Tiparp*^{fl} in genomic DNA isolated from various tissues and hepatocytes obtained from *Tiparp*^{fl/fl} and *Tiparp*^{fl/fl}Cre^{Alb} mice. * p < 0.05 compared with tissue matched *Tiparp*^{fl/fl} mice. **(B)** *Tiparp*^{fl} excision by Cre^{CMV} was determined from genomic DNA isolated from livers of *Tiparp*^{+/+} and *Tiparp*^{Ex3-/-} mice. Hep, hepatocytes; WAT, white adipose tissue. **(C)** Increased AHR regulated Cyp1a1, Cyp1a2, Cyp1b1 and *Tiparp* mRNA levels in expression in hepatocytes isolated from *Tiparp*^{fl/fl} and *Tiparp*^{fl/fl}Cre^{Alb} mice treated with 10 nM dioxin for 6 h. RNA and qPCR were performed as described in the materials and methods section. * p < 0.05 compared with genotyped match control treated, # p < 0.05 compared with dioxin-treated *Tiparp*^{fl/fl}, n = 3.

Figure 3. AHR regulated transcript levels in liver and lung tissue isolate from control and dioxin-treated *Tiparp*^{Ex3-/-}, *Tiparp*^{fl/fl}*Cre*^{Alb} and their respective WT mice. Mice were treated with a single injection of 100 µg/kg dioxin in DMSO, or DMSO vehicle alone, and euthanized 6 h later. RNA and qPCR were performed as described in the materials and methods section. * p < 0.05 compared with genotype matched control-treated; # p < 0.05 compared with dioxin-treated *Tiparp*^{+/+} (A-C) or *Tiparp*^{fl/fl} (D-F), n = 4.

Figure 4. Loss of TIPARP increases dioxin-induced hepatotoxicity and lethal wasting syndrome in male mice. (A) Kaplan-Meier survival curves for male *Tiparp*^{+/+} and *Tiparp*^{Ex3-/-} mice treated with a single 10 or 100 µg/kg i.p. injection of dioxin and monitored for 30 days. (B) Body weight, (C) food intake, (D) serum alanine aminotransferase (ALT) activity, (E) liver, (F) thymus and (G) WAT weight expressed as percentage of total body weight, and (H) hepatic glycogen levels were measured from *Tiparp*^{+/+} and *Tiparp*^{Ex3-/-} mice treated with 10 µg/kg dioxin. Data shown are the mean ± SEM. n = 4-5. For (B-G) *P < 0.05, two-way ANOVA followed by Tukey's post-hoc test compared with genotype-matched control treated mice. For (H) *P < 0.05, Student's t-test.

Figure 5. Hepatocyte-specific loss of TIPARP increases dioxin-induced hepatotoxicity and lethal wasting syndrome in male mice. (A) Kaplan-Meier survival curves for male *Tiparp*^{fl/fl} and *Tiparp*^{fl/fl}*Cre*^{Alb} mice treated with a single 10 or 100 µg/kg i.p. injection of dioxin and monitored for 30 days. (B) Body weight, (C) food intake, (D) serum alanine aminotransferase (ALT) activity, (E) liver, (F) thymus and (G) WAT weight expressed as percentage of total body weight, and (H) hepatic glycogen levels were measured from *Tiparp*^{fl/fl} and *Tiparp*^{fl/fl}*Cre*^{Alb} mice treated with 10 µg/kg dioxin. Data shown are the mean ± SEM. n = 4-5. For (B-G) *P < 0.05, two-way ANOVA followed by Tukey's post-hoc test compared with genotype-matched control treated mice. For (H) *P < 0.05, Student's t-test.

Figure 6. Increased liver inflammation and cytokine levels in dioxin-treated *Tiparp*^{fl/fl}*Cre*^{Alb} compared with *Tiparp*^{fl/fl} mice. (A) Representative H&E staining of livers from *Tiparp*^{fl/fl} and *Tiparp*^{fl/fl}*Cre*^{Alb} mice (n=4). Control animals were injected with CO and were euthanized on day 9. The asterisks (*) indicate focal inflammatory infiltration, and the arrowheads indicate microvesicular steatosis. All images are to the same scale. Hepatic (B) *Il6*, (C) *Serpine 1*, (D) *Cxcl2*, and (E) *F4/80* mRNA levels were determined as described in the methods. Data represent the mean ± SEM (n=4). *P<0.05 two-way ANOVA compared with genotyped-matched control treated mice. #P<0.05 two-way ANOVA compared with dioxin-treated *Tiparp*^{fl/fl} mice.

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5 **Figure 7.** Dioxin-induced steatosis is increased in *Tiparp*^{fl/fl}*Cre*^{Alb} mice. **(A)** Livers from
6 male *Tiparp*^{fl/fl} and *Tiparp*^{fl/fl}*Cre*^{Alb} mice given a single i.p. injection of CO or 10 µg/kg
7 dioxin and euthanized after 9 days (*n*=5). **(B)** Oil-Red-O and hematoxylin stained liver
8 sections from *Tiparp*^{fl/fl} and *Tiparp*^{fl/fl}*Cre*^{Alb} mice. All images are to the same scale.
9 Hepatic mRNA levels of *Cd36* **(C)**, *Scd1* **(D)**, *Srebp1* **(E)**, *Ppara* **(F)** and *Cyp7a1* **(G)**
10 were determined as described in the methods. Data represent the mean ± SEM (*n*=4). For
11 all data, *P* < 0.05 was determined by Two-way ANOVA followed by Tukey's post-hoc
12 test comparison. Significantly different compared with genotype-matched *DMSO- or
13 #dioxin-treated *Tiparp*^{fl/fl} mice.
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18 **Figure 8.** Hepatocyte-specific loss of TIPARP increases dioxin-dependent regulation of
19 hepatic AHR target gene expression. Hepatic mRNA levels of *Cyp1a1* **(A)**, *Cyp1a2* **(B)**,
20 *Cyp1b1* **(C)**, *Tiparp* **(D)**, *Ahrr* **(E)** *Nqo1* **(F)** *Nfe2l2* **(G)** *Serpine 1* **(H)** were determined
21 after 3 days of exposure to corn oil or 10 µg/kg dioxin as described in experimental
22 procedures (*n*=4). Recruitment of AHR to *Cyp1a1* **(I)** and *Cyp1b1* **(J)**. Data represent the
23 mean ± SEM. Representative AHR **(K)**, and β-actin protein levels were detected by
24 Western blotting after 3 days of treatment. AHR proteins levels were normalized to β-
25 actin levels, *n*=4) **(L)**. For all data, *P* < 0.05 was determined by Two-way ANOVA
26 followed by Tukey's post-hoc test comparison. Significantly different compared with
27 genotype-matched *DMSO- or #dioxin-treated *Tiparp*^{fl/fl} mice.
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33 **Figure 9.** Dioxin-induced hepatic metabolomic disruption. **(A)** Principal Component
34 Analysis (PCA) of hepatic metabolomic analysis after 3 day treatment of *Tiparp*^{fl/fl} (*fl/fl*)
35 and *Tiparp*^{fl/fl}*Cre*^{Alb} with corn oil (CO) or 10 µg/kg dioxin. **(B)** Venn diagram of the
36 overlapping metabolites that were significantly altered between dioxin treated *Tiparp*^{fl/fl}
37 and *Tiparp*^{fl/fl}*Cre*^{Alb}. Metabolic pathway enrichment analysis of altered hepatic
38 metabolites (*P* < 0.05) in dioxin-treated *Tiparp*^{fl/fl}*Cre*^{Alb} **(C)** and *Tiparp*^{fl/fl} mice **(D)**.
39 Hepatic levels of gamma-glutamyl-epsilon lysine **(E)**, N-acetylputrescine **(F)**, putrescine
40 **(G)**, nicotinamide ribonucleoside **(H)**, nicotinamide **(I)** and NAD⁺ **(J)**. Data represent the
41 mean ± SEM (*n*=6-8).
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Table 1. A summary of the numbers of biochemicals that achieved statistical significance ($p \leq 0.05$) among the different comparisons.

ANOVA Contrasts	Statistical Comparisons			
	$\frac{\text{Tiparp}^{\text{fl/fl}} \text{ TCDD}}{\text{Tiparp}^{\text{fl/fl}} \text{ CO}}$	$\frac{\text{Tiparp}^{\text{fl/fl}} \text{ Cre}^{\text{Alb}} \text{ TCDD}}{\text{Tiparp}^{\text{fl/fl}} \text{ Cre}^{\text{Alb}} \text{ CO}}$	$\frac{\text{Tiparp}^{\text{fl/fl}} \text{ Cre}^{\text{Alb}} \text{ CO}}{\text{Tiparp}^{\text{fl/fl}} \text{ CO}}$	$\frac{\text{Tiparp}^{\text{fl/fl}} \text{ Cre}^{\text{Alb}} \text{ TCDD}}{\text{Tiparp}^{\text{fl/fl}} \text{ TCDD}}$
Total Biochemicals $p < 0.05$	124	213	7	129
Biochemicals (up down)	75 49	163 50	4 3	111 18

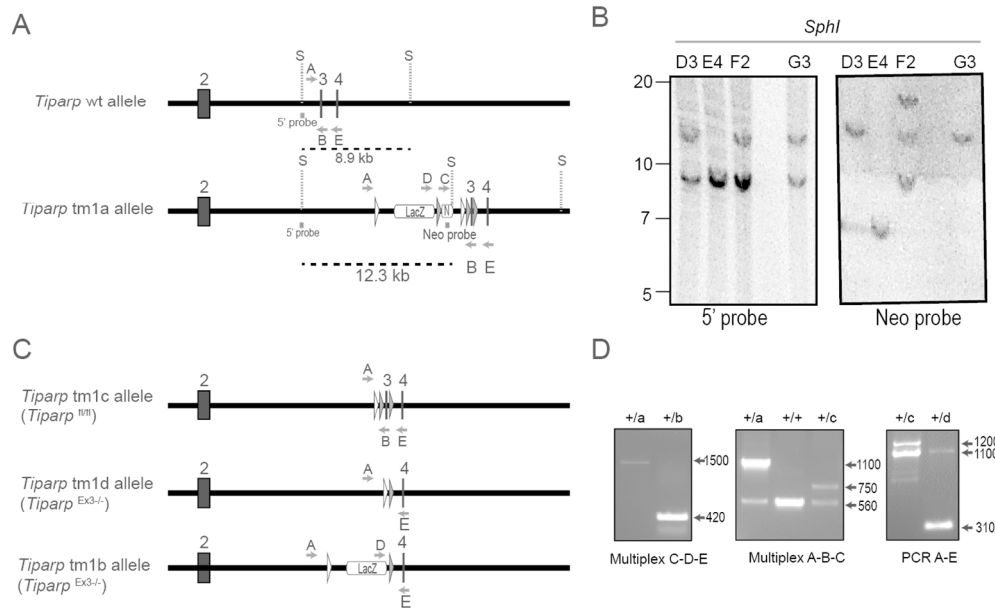


Figure 1. Generation of the conditional *Tiparp*fl/fl mice. (A) Schematic diagram of *Tiparp* WT allele and the allele with successful recombination of the tm1 targeting construct (*Tiparp* tm1a allele) and corresponding Southern blot data. Exon numbers reflect known coding exons; white arrowheads represent FRT sites; grey arrowheads represent LoxP sites; LacZ represents lacZ reporter gene; N represents the neomycin (Neo) resistance cassette; S, SphI site; dashed lines indicate the fragment of DNA generated by SphI digestion that was detected with the radiolabeled probes (5' probe and Neo probe); letters indicate genotyping primers. (B) Southern blots show *Tiparp* allele fragments detected with the radiolabeled probes: ES clones D3 and G3 show correct homologous recombination of the targeting construct (12.3 kb fragment detected with 5' probe) without additional random integrations (only the 12.3 kb band detected with Neo probe). (C) Schematic diagram of *Tiparp* tm1 alleles following excision of sequence by CRE or FLP recombinases. (D) PCR data from mouse tail biopsies. The *Tiparp* tm1a allele was converted to tm1b using CRE recombinase to remove the Neo cassette and exon 3 located between the LoxP sites. Primer pair D-E amplify a product spanning the excision sites to show that the floxed sequences were removed (420 bp). The *Tiparp* tm1a allele was converted to tm1c using FLP recombinase to remove the LacZ and Neo cassettes located between the FRT sites. Primer pair A-B amplify a product spanning the excision site to show that the sequence was flipped out (750 bp). The *Tiparp* tm1c allele was converted to tm1d using CRE recombinase to remove the floxed exon 3. Primer pair A-E amplify a product spanning the floxed region to show that the exon is removed (310 bp). Sample genotypes: + indicates the *Tiparp* WT allele; letters indicate the *Tiparp* tm1 corresponding allele (tm1a, tm1b, tm1c, tm1d).

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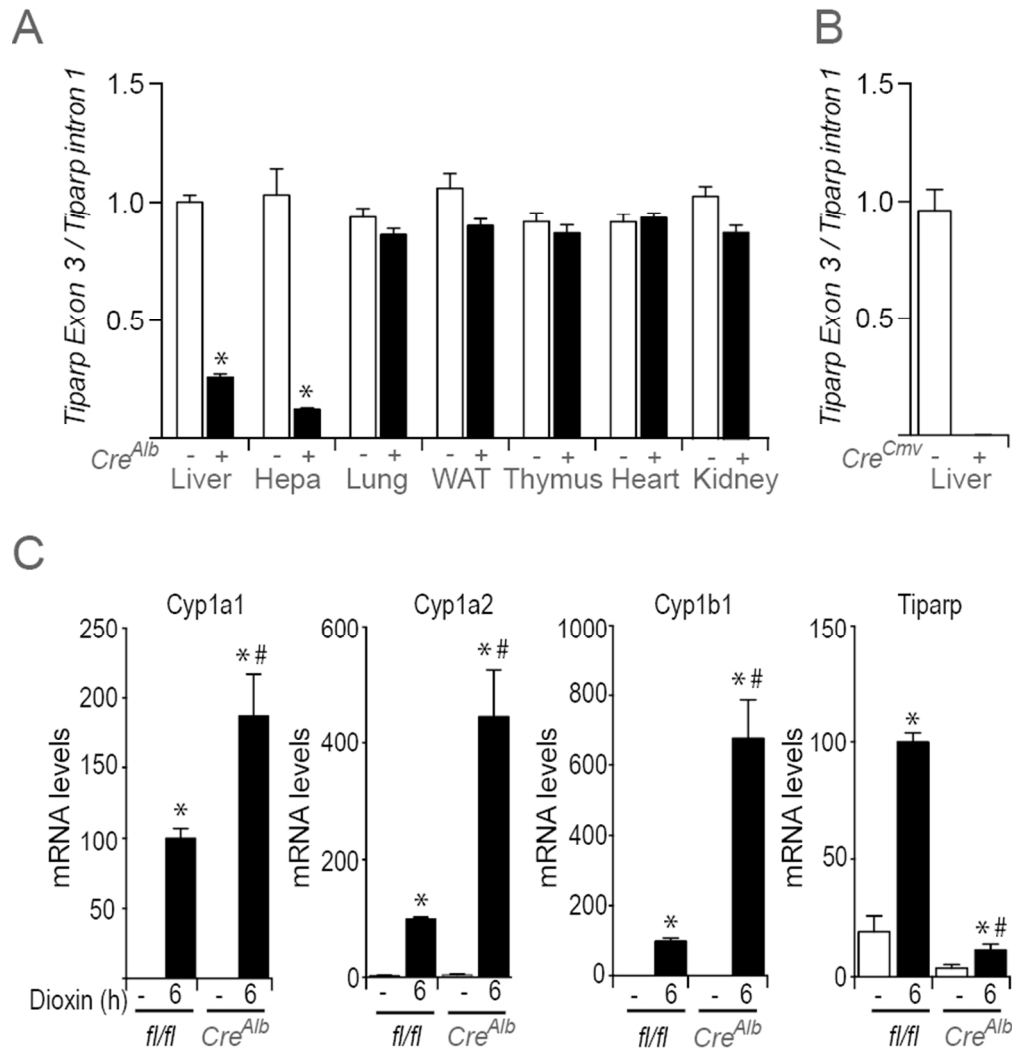


Figure 2. Specificity of CreAlb-mediated excision of the Tiparpfl allele. (A) Specificity of Tiparpfl excision by CreAlb was determined by quantitative real-time PCR-based genotyping for excised and unexcised alleles of Tiparpfl in genomic DNA isolated from various tissues and hepatocytes obtained from Tiparpfl/fl and Tiparpfl/flCreAlb mice. * $p < 0.05$ compared with tissue matched Tiparpfl/fl mice. (B) Tiparpfl excision by CreCMV was determined from genomic DNA isolated from livers of Tiparp+/+ and TiparpEx3-/- mice. Hep, hepatocytes; WAT, white adipose tissue. (C) Increased AHR regulated Cyp1a1, Cyp1a2, Cyp1b1 and Tiparp mRNA levels in expression in hepatocytes isolated from Tiparpfl/fl and Tiparpfl/flCreAlb mice treated with 10 nM dioxin for 6 h. RNA and qPCR were performed as described in the materials and methods section. * $p < 0.05$ compared with genotyped match control, # $p < 0.05$ compared with dioxin-treated Tiparpfl/fl, $n = 3$.

75x78mm (300 x 300 DPI)

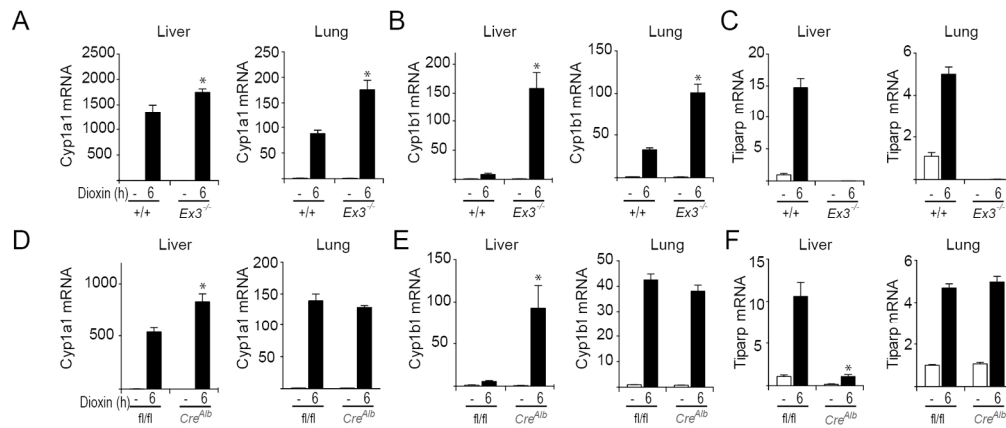


Figure 3. AHR regulated transcript levels in liver and lung tissue isolate from control and dioxin-treated *TiparpEx3^{-/-}*, *Tiparpfl/flCreAlb* and their respective WT mice. Mice were treated with a single injection of 100 $\mu\text{g}/\text{kg}$ dioxin in DMSO, or DMSO vehicle alone, and euthanized 6 h later. RNA and qPCR were performed as described in the materials and methods section. * $p < 0.05$ compared with genotype matched control-treated; # $p < 0.05$ compared with dioxin-treated *Tiparp+/+* (A-C) or *Tiparpfl/fl* (D-F), $n = 4$.

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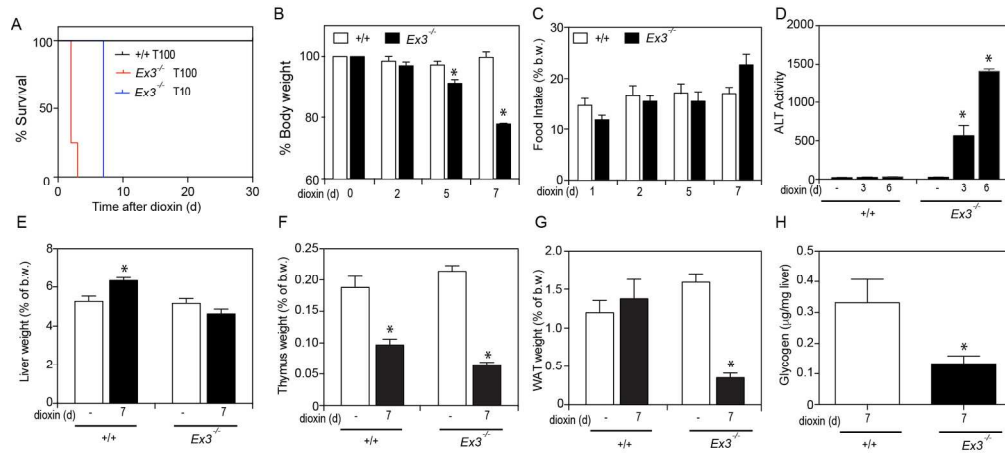


Figure 4. Loss of TIPARP increases diosin-induced hepatotoxicity and lethal wasting syndrome in male mice. (A) Kaplan-Meier survival curves for male Tiparp^{+/+} and Tiparp^{Ex3^{-/-}} mice treated with a single 10 or 100 μg/kg i.p. injection of diosin and monitored for 30 days. (B) Body weight, (C) food intake, (D) serum alanine aminotransferase (ALT) activity, (E) liver, (F) thymus and (G) WAT weight expressed as percentage of total body weight, and (H) hepatic glycogen levels were measured from Tiparp^{+/+} and Tiparp^{Ex3^{-/-}} mice treated with 10 μg/kg diosin. Data shown are the mean ± SEM. n = 4-5. For (B-G) *P < 0.05, two-way ANOVA followed by Tukey's post-hoc test compared with genotype-matched control treated mice. For (H) *P < 0.05, Student's t-test.

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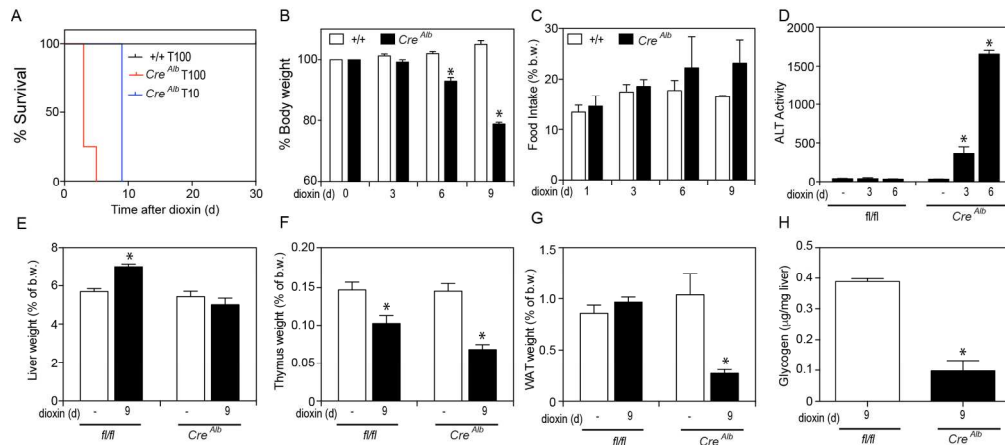


Figure 5. Hepatocyte-specific loss of TIPARP increases dioxin-induced hepatotoxicity and lethal wasting syndrome in male mice. (A) Kaplan-Meier survival curves for male *Tiparpfl/fl* and *Tiparpfl/flCreAlb* mice treated with a single 10 or 100 $\mu\text{g}/\text{kg}$ i.p. injection of dioxin and monitored for 30 days. (B) Body weight, (C) food intake, (D) serum alanine aminotransferase (ALT) activity, (E) liver, (F) thymus and (G) WAT weight expressed as percentage of total body weight, and (H) hepatic glycogen levels were measured from *Tiparpfl/fl* and *Tiparpfl/flCreAlb* mice treated with 10 $\mu\text{g}/\text{kg}$ dioxin. Data shown are the mean \pm SEM. $n = 4-5$. For (B-G) * $P < 0.05$, two-way ANOVA followed by Tukey's post-hoc test compared with genotype-matched control treated mice. For (H) * $P < 0.05$, Student's t-test.

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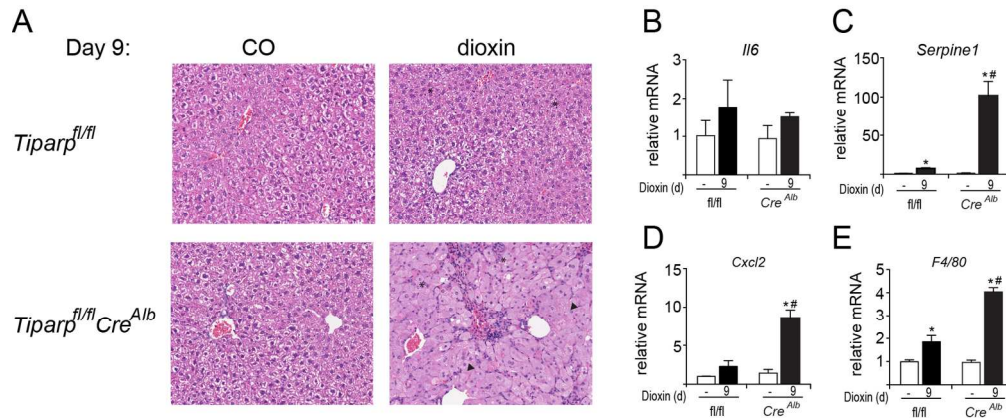


Figure 6. Increased liver inflammation and cytokine levels in dioxin-treated *Tiparpfl/flCreAlb* compared with *Tiparpfl/fl* mice. (A) Representative H&E staining of livers from *Tiparpfl/fl* and *Tiparpfl/flCreAlb* mice (n=4).

Control animals were injected with CO and were euthanized on day 9. The asterisks (*) indicate focal inflammatory infiltration, and the arrowheads indicate microvesicular steatosis. All images are to the same scale. Hepatic (B) *Il6*, (C) *Serpine 1*, (D) *Cxcl2*, and (E) *F4/80* mRNA levels were determined as described in the methods. Data represent the mean \pm SEM (n=4). * $P < 0.05$ two-way ANOVA compared with genotyped-matched control treated mice. # $P < 0.05$ two-way ANOVA compared with dioxin-treated *Tiparpfl/fl* mice.

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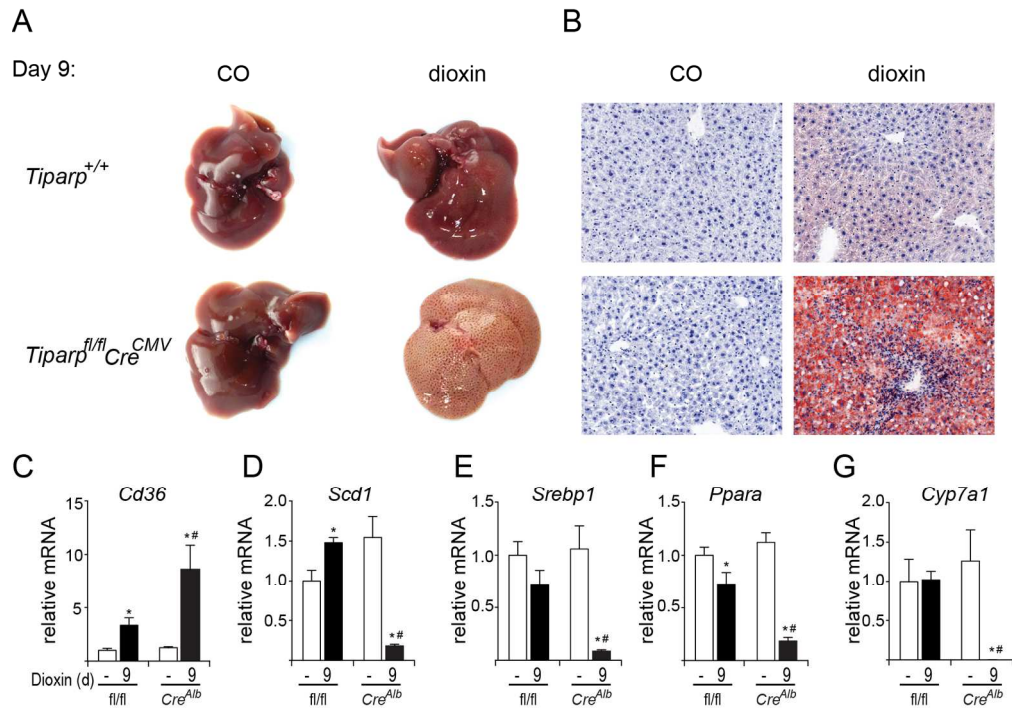


Figure 7. Dioxin-induced steatosis is increased in *Tiparpfl/fl* and *Tiparpfl/flCreAlb* mice given a single i.p. injection of CO or 10 $\mu\text{g}/\text{kg}$ dioxin and euthanized after 9 days ($n=5$). (B) Oil-Red-O and hematoxylin stained liver sections from *Tiparpfl/fl* and *Tiparpfl/flCreAlb* mice. All images are to the same scale. Hepatic mRNA levels of *Cd36* (C), *Scd1* (D), *Srebp1* (E), *Ppara* (F) and *Cyp7a1* (G) were determined as described in the methods. Data represent the mean \pm SEM ($n=4$). For all data, $P < 0.05$ was determined by Two-way ANOVA followed by Tukey's post-hoc test comparison. Significantly different compared with genotype-matched *DMSO- or #dioxin-treated *Tiparpfl/fl* mice.

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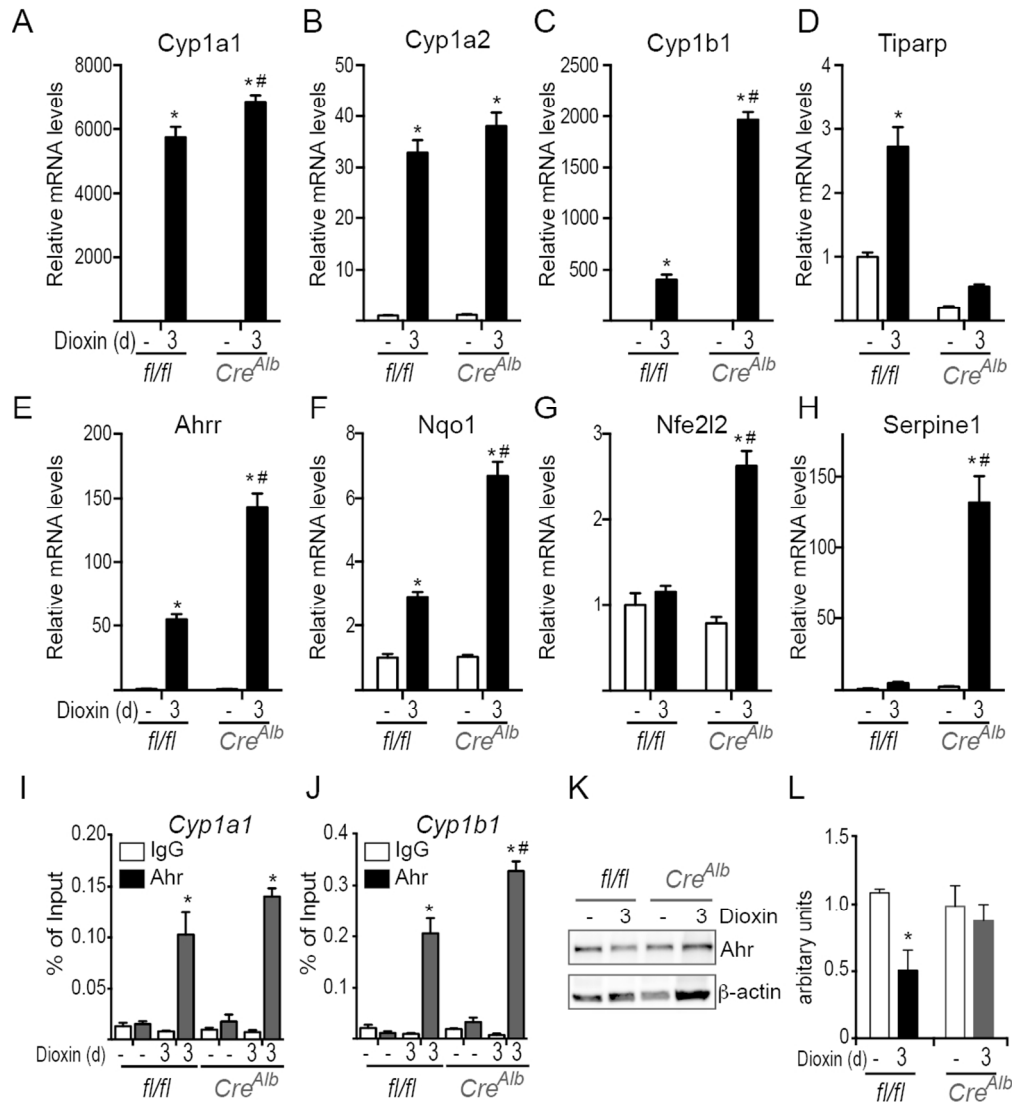


Figure 8. Hepatocyte-specific loss of TIPARP increases dioxin-dependent regulation of hepatic AHR target gene expression. Hepatic mRNA levels of Cyp1a1 (A), Cyp1a2 (B), Cyp1b1 (C), Tiparp (D), Ahrr (E) Nqo1 (F) Nfe2l2 (G) Serpine 1 (H) were determined after 3 days of exposure to corn oil or 10 μ g/kg dioxin as described in experimental procedures (n=4). Recruitment of AHR to Cyp1a1 (I) and Cyp1b1 (J). Data represent the mean \pm SEM. Representative AHR (K), and β -actin protein levels were detected by Western blotting after 3 days of treatment. AHR proteins levels were normalized to β -actin levels, n=4) (L). For all data, $P < 0.05$ was determined by Two-way ANOVA followed by Tukey's post-hoc test comparison. Significantly different compared with genotype-matched *DMSO- or #dioxin-treated Tiparpf/f/f mice.

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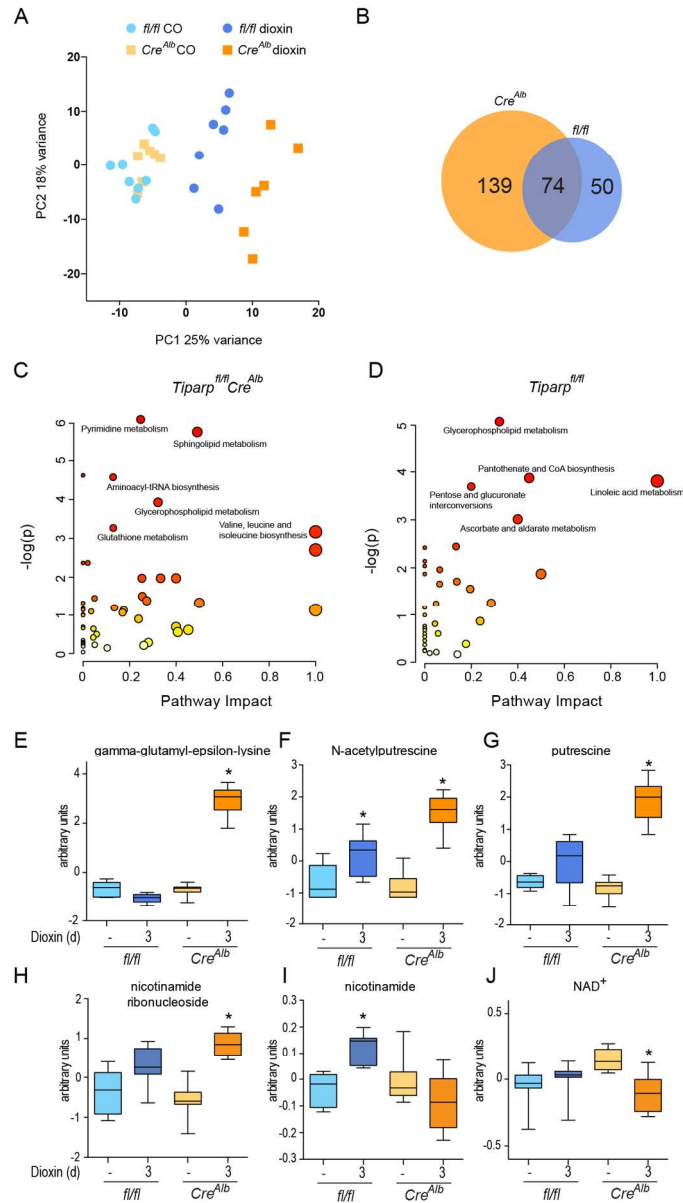


Figure 9. Dioxin-induced hepatic metabolomic disruption. (A) Principal Component Analysis (PCA) of hepatic metabolomic analysis after 3 day treatment of *Tiparpfl/fl* (*fl/fl*) and *Tiparpfl/flCreAlb* with corn oil (CO) or 10 $\mu\text{g}/\text{kg}$ dioxin. (B) Venn diagram of the overlapping metabolites that were significantly altered between dioxin treated *Tiparpfl/fl* and *Tiparpfl/flCreAlb*. Metabolic pathway enrichment analysis of altered hepatic metabolites ($P < 0.05$) in dioxin-treated *Tiparpfl/flCreAlb* (C) and *Tiparpfl/fl* mice (D). Hepatic levels of gamma-glutamyl-epsilon lysine (E), N-acetylputrescine (F), putrescine (G), nicotinamide ribonucleoside (H), nicotinamide (I) and NAD⁺ (J). Data represent the mean \pm SEM ($n=6-8$).

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