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NRF2-independent regulation of intestinal CAR by the pro-oxidant's cadmium and isothiocyanate in *hUGT1* mice

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NRF2-independent regulation of DMEs by cadmium and ITCs

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Abbreviations: CAR, constitutive androstane receptor; CCRP, cytoplasmic car retention protein; CYP, cytochrome P450; ERK1/2, extracellular signal-regulated kinases 1 and 2; HSP70/90, heat shock proteins 70 and 90; KEAP1, Kelch-like ECH associated protein 1; NRF2, nuclear factor, erythroid 2 like 2; P38, mitogen-activated protein kinase 11; TSB, total serum bilirubin

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Abstract

Environmental toxicants such as heavy metals from contaminated water or soil and isothiocyanates (ITC) from dietary sources act as pro-oxidants by directly generating reactive oxygens species (ROS) or through depleting cellular antioxidants such as glutathione. Toxicants can alter drug metabolism and it was reported that CYP2B10 and UGT1A1 are induced by phenethyl isothiocyanate (PEITC) through the constitutive androstane receptor (CAR). The possibility that NRF2, the master regulator of the antioxidant response, could co-activate CAR was investigated in neonatal *hUGT1/Nrf2*^{-/-} mice. Neonatal mice were treated with PEITC or cadmium (Cd²⁺) by oral gavage for two days. Both PEITC and Cd²⁺ induced UGT1A1 RNA and protein in intestinal tissues in both *hUGT1/Nrf2*^{+/-} and *hUGT1/Nrf2*^{-/-} neonates, indicating NRF2-independent regulation of UGT1A1. Increases in CYP2B10 RNA in intestinal tissues were observed following PEITC or Cd²⁺ exposure. Activation of intestinal CAR by Cd²⁺ exposure was directly assessed by nuclear fractionation and Western blot analyses at 0.5, 1, 2, and 4 hours after treatment in *hUGT1* neonates and after 48 hours in *hUGT1/Nrf2*^{+/-} and *hUGT1/Nrf2*^{-/-} neonates. CAR localized to the nucleus independent of NRF2 48 hours after exposure. Substantial CAR localization to the nucleus occurred at the 2- and 4-hour time points, coinciding with a decrease in cytoplasmic ERK1/2 phosphorylation and a nuclear increase in P38/p-P38 content. This suggests a novel oxidative stress-MAPK-CAR axis exists with phenotypic consequences.

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Significance Statement

Pro-oxidant toxicants can alter drug metabolism through activation of CAR, independent of the NRF2-KEAP1 signaling pathway. The changes in proteins associated with drug metabolism are likely mediated through an oxidative stress-MAPK-CAR axis that have been linked to increases in intestinal maturation.

Introduction

Exposure to certain environmental toxicants is capable of altering drug metabolism through the activation of selective transcription factors, such as xenobiotic and nuclear receptors (XRs and NRs). The activation of XRs and NRs typically occurs through direct ligand binding to the receptor or through post translation modifications. In vitro models enable medium- to high- throughput screening of compounds abilities to activate nuclear receptors while in vivo models provide more complex details such as absorption, elimination, and tissue specific metabolism.

An in vivo model that lends itself well to reverse genetic approaches is the humanized *UDP-glucuronosyltransferase 1 (hUGT1)* mouse model due to the readily quantifiable phenotype that changes following XR or NR activation (Fujiwara et al., 2010; Chen and Tukey, 2018). The phenotype is presented as neonatal hyperbilirubinemia, a condition unique to humans in which increased turnover of red blood cells causes heme degradation to bilirubin which, accumulates systemically (Pearson, 1967). The accumulation of bilirubin occurs because hepatic expression of UGT1A1, the only enzyme capable of conjugating bilirubin for excretion (Bosma et al., 1994), is developmentally delayed at the transcriptional level (Burchell et al., 1989). However, we have demonstrated that control of intestinal UGT1A1 during development plays a key role in the clearance of serum bilirubin during the neonatal period (Fujiwara et al., 2010; Liu et al., 2016; Chen et al., 2017). Induction of the *UGT1A1* gene in *hUGT1* mice can be mediated by multiple XRs and NRs, such as the pregnane X-receptor (PXR) (Chen et al., 2012), constitutive androstane receptor (CAR) (Sugatani et al., 2001), peroxisome proliferator-activated receptor α (PPAR α) (Senekeo-Effenberger et al., 2007), as well as

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environmental toxicant sensors such as the aryl hydrocarbon receptor (AhR) (Yueh et al., 2005; Bonzo et al., 2007), and the nuclear factor erythroid 2-related factor 2 (NRF2) (Yueh and Tukey, 2007). Following exposure, activation of one or more of these XRs and NRs induces *UGT1A1* gene expression resulting in a reduction of total serum bilirubin (TSB) levels in *hUGT1* mice (Yueh et al., 2017; Chen and Tukey, 2018). Known transcriptional targets can be examined to determine which XR or NR is inducing *UGT1A1*, for example CAR induces the *Cyp2b10* (Honkakoski et al., 1996) gene while activated NRF2 can induce genes such as *Gsta1/2* (McMahon et al., 2001), which are sensitive to oxidative stress. The receptor mediated induction of *UGT1A1* can be confirmed in neonatal *hUGT1* mice that express a null allele for the *XR- or NRs*, where elevated TSB levels following exposure can be directly linked to a dependency on the deleted receptor (Chen et al., 2013; Liu et al., 2016; Yoda et al., 2017).

Recently, it was reported that the dietary pro-oxidants isothiocyanates (ITCs) induce hepatic *UGT1A1* in a CAR-dependent fashion (Yoda et al., 2017). This is significant because the mechanisms of direct and indirect activation of CAR are well characterized (Yoshinari et al., 2003; Osabe and Negishi, 2011; Mutoh et al., 2013; Timsit and Negishi, 2014) but have never directly implicated oxidative stress as a CAR activator. Additionally, other environmentally relevant toxicants, such as cadmium (Cd^{2+}) and arsenic (As^{3+}) that increase reactive oxygen species (ROS) have been reported to induce intestinal *Cyp2b10* and *UGT1A1* gene expression in *hUGT1* mice (Fujiwara et al., 2010; Yoda et al., 2017). However, *UGT1A1* induction by pro-oxidants has been linked to NRF2 (Yueh and Tukey, 2007), the master regulator of the antioxidant response (Moi et al., 1994). The induction mechanism of intestinal *UGT1A1* in *hUGT1* mice by agents such as

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Cd²⁺ and ITCs are examined with respect to their link towards activation of NRF2 and CAR.

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Materials & Methods

Animal Studies.

All animals were housed at the University of California San Diego Animal Care Facility with access to food and water ad libitum. All animal use including experimental procedures, handling, and protocols were approved by the UCSD Animal Care and Use Committee (IACUC) and adhere to the National Institute of Health Guide for the Care and Use of Laboratory Animals. UCSD's Animal Care Program is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Humanized *UGT1* (*hUGT1*) mice were previously generated as described. *Nrf2*^{-/-} mice were purchased from Jackson Laboratory (Stock No: 017009), and *hUGT1/Nrf2*^{+/-} mice were generated and crossed for comparison to *hUGT1/Nrf2*^{+/+} mice, utilizing both males and females. Additional studies involved breeding *hUGT1/Nrf2*^{+/-} with *hUGT1/Nrf2*^{-/-} to generate male and female mice used for neonatal studies. Neonates were treated on day 10 and 11 with CdCl₂·2 ½ H₂O (Cd²⁺) or NaAsO₂ (As³⁺) (Sigma-Aldrich) at 10mg/kg or PEITC (Sigma Aldrich) at 200 mg/kg by oral gavage or with water or corn oil (5µL/g bw) for vehicle control. The dosages for CdCl₂ and PEITC treatments were selected from dose-response curves where both the greatest reduction in total serum bilirubin levels and no observable behavioral or gross anatomical phenotypes of toxicity were obtained. Animals were anesthetized with isoflurane and euthanized by cervical dislocation.

Real-time PCR.

Intestines were collected and briefly washed with ice cold PBS 3 times before snap freezing in liquid nitrogen. Livers were collected and washed once with ice cold PBS before snap freezing in liquid nitrogen. RNA was extracted with TRIzol™ Reagent

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(ThermoFisher Scientific) as described by the manufacturer's protocol. cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real-time PCR was performed using a CFX96™ Real-Time PCR Detection system (Bio-Rad) and SSoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) as described by the manufacturer's protocol. All values represent independent biological replicates and technical singlets as analyzed by the $\Delta\Delta CT$ method. Primers utilized for real time PCR analysis of selected genes can be found in Table 1.

Western Blot Analysis.

Whole lysate was obtained from homogenization of pulverized intestinal tissues in RIPA buffer (Millipore Sigma Cat. No. 20-188) containing protease (ThermoFisher Scientific™ Cat. No. 78429) and phosphatase (ThermoFisher Scientific™ Cat. No. 78420) inhibitors. Pulverized tissues in lysis buffer (1:4 wt/vol) were blade homogenized and incubated on ice for 15 min before centrifugation (10,000xg, 4°C, 20 min) and supernatant storage at -80°C.

Nuclear fractionation used a two-buffer extraction. Buffer A contained 10mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.05% Igepal at pH 7.9 with protease and phosphatase inhibitors. Buffer B contained 5mM HEPES, 1.5mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 26% glycerol (v/v) at pH 7.9 with protease and phosphatase inhibitors. Pulverized tissues were homogenized in buffer A (1:4 wt:vol) using a Potter-Elvehjem homogenizer for 10 passes. Homogenates were incubated on ice for 10 minutes prior to centrifugation (1500 x g, 4°C, 10 min). The supernatant containing the cytosol was stored at -80°C. The pellet containing the nuclear contents was washed with 200 uL of buffer A before resuspension in 400 uL of Buffer B. After

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resuspension, NaCl was added to a final concentration of 300mM and the solution was sonicated for 5 sec and this repeated three times. Samples were incubated on ice for 30 minutes before centrifugation (21,000xg, 4°C, 20 min). The supernatant containing the nuclear fraction was stored at -80°C. Protein concentration was determined *via* Bradford assay (Bio-Rad), and BSA (New England BioLabs) protein standards were created for simple linear regressions.

Samples were run on NuPAGE™ 4-12% Bis-Tris Protein Gels (Invitrogen) before transferring to PVDF membranes. Membranes were blocked with 5% nonfat milk or BSA for phospho-antibodies in TBST and incubated with primary antibodies overnight at 4°C and secondary antibodies for 1 hour at RT. Imaging was performed on a ChemiDoc™ Touch imaging system (Bio-Rad) using Clarity™ Western ECL substrate (Bio-Rad). All antibodies used for protein expression and localization analyses can be found in Table 2. All samples represent biological replicates and technical singlets.

Bilirubin measurement.

Approximately 50 µL of blood was obtained from the submandibular vein. Blood was centrifuged for 2 minutes at 16,000 x g and the supernatant was measured using a Unistat Bilirubinometer (Reichert Inc.) to determine total serum bilirubin (TSB) levels.

Statistical analysis

All statistical analyses were performed using GraphPad Prism's (V 6.07) two-way ANOVA with multiple comparisons. Averages +/- SEM are shown. Differences were considered statistically significant when $p < 0.05$. Statistical significance is represented by the following: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

Cd²⁺ treatment induces oxidative stress markers in an NRF2-dependent manner

Intestinal tissues were collected from *hUGT1/Nrf2^{+/+}*, *hUGT1/Nrf2^{+/-}*, and *hUGT1/Nrf2^{-/-}* neonates treated orally with 10mg/kg Cd²⁺ and gene targets known to be induced by NRF2 in response to oxidative stress examined by RT-qPCR (**Fig 1**). Statistically significant inductions were detected in Cd²⁺ exposed *hUGT1/Nrf2^{+/+}* and *hUGT1/Nrf2^{+/-}* neonates for *Nqo1* (2.7- and 1.7-fold), *Ho-1* (6.8- and 4.3-fold), *Gsta1* (37.8- and 33.0-fold), *Gsta2* (9.3- and 7.9-fold), and *Gstm3* (15.8- and 8.7-fold) genes. A gene dosage effect comparing responses between *hUGT1* and *hUGT1/Nrf2^{+/-}* neonatal mice was observed for the induction of the *Nqo1*, *Ho-1*, and *Gstm3* genes. This indicates haploinsufficiency related to NRF2's induction of antioxidant response genes. When we examined the induction of these genes in *hUGT1/Nrf2^{-/-}* mice following Cd²⁺ treatment, there was no statistically significant difference from the vehicle treatment of *hUGT1* mice, confirming that the induction of these genes in the gastrointestinal tract originates from the generation of ROS in the intestines. With oral Cd²⁺ treatment, there was no induction of these genes in hepatic tissue.

Cd²⁺ induces intestinal UGT1A1 and lowers TSB levels in an NRF2 independent fashion.

It was previously determined that oral Cd²⁺ treatment to neonatal *hUGT1* mice led to the induction of intestinal UGT1A1 (Liu et al., 2016). To determine if induction of the intestinal *UGT1A1* gene by Cd²⁺ was dependent upon the generation of ROS and activation of the NRF2-KEAP1 pathway, *hUGT1/Nrf2^{+/-}* mice were mated and 10-day old

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newborns treated with 10mg/kg Cd²⁺ or water by oral gavage once per day for two days before collecting blood and intestinal tissue. With *hUGT1*, *hUGT1/Nrf2^{+/-}*, and *hUGT1/Nrf2^{-/-}* mice, Cd²⁺ treatment eliminated circulating TSB levels within two days, indicating that a mechanism had been activated to induce intestinal UGT1A1 (**Fig. 2A**). Similar reductions were observed for PEITC and As³⁺ treated neonatal mice (**Fig. S1**). Analysis of UGT1A1 expression both at the gene and protein level firmly establish that Cd²⁺ exposure induces intestinal UGT1A1 (Fig. 2B and C). Unlike the Cd²⁺ initiated induction of the *Nqo1*, *Ho-1*, *Gsta1*, *Gsta2*, and *Gstm3* genes (**Fig. 1**), which are dependent upon NRF2, induction of the intestinal *UGT1A1* gene by Cd²⁺ is not linked to the NRF2-KEAP1 pathway. It should be noted that an antioxidant-response element (ARE) has previously been identified flanking the *UGT1A1* gene in the phenobarbital-response enhancer module region (PBREM), and has been demonstrated to bind the antioxidant *tert*-butylhydroquinone (tBHQ) inducible NRF2 in treated HepG2 cells (Yueh and Tukey, 2007). In addition, the intraperitoneal administration of tBHQ to adult *TgUGT1* mice led to induction of intestinal UGT1A1, implicating the NRF2-KEAP1 pathway as a regulator of the *UGT1A1* gene (Yueh and Tukey, 2007). However, the oral administration of Cd²⁺ and induction of intestinal UGT1A1 in *hUGT1* neonatal mice does not require NRF2-KEAP1 signaling.

Intestinal *Cyp2b10* is positively regulated by Cd²⁺ exposure and induces nuclear accumulation of CAR

The nuclear receptors that induce *UGT1A1* were examined for activation by RT-qPCR analysis of their known target genes (**Fig. S2**). Only *Cyp2b10* was substantially

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induced by Cd^{2+} in *hUGT1/Nrf2*^{+/+} (8.4-fold), *hUGT1/Nrf2*^{+/-} (13.5-fold), and *hUGT1/Nrf2*^{-/-} (9.8-fold) neonates, however only heterozygotes reached statistical significance (**Fig. 3A**). Furthermore, Western blot analysis of CYP2B10 was inconclusive with no apparent detection in either vehicle or Cd^{2+} treated mice. Given that basal expression of CYP2B10 in neonatal intestinal tissue is low, CAR activation was assayed more directly via nuclear localization.

Intestinal tissues taken from *hUGT1/Nrf2*^{+/-} and *hUGT1/Nrf2*^{-/-} neonates exposed to Cd^{2+} were fractionated and examined for nuclear CAR accumulation by Western blot analysis (**Fig. 3B**). There was a statistically significant increase in the nuclear accumulation of CAR in *hUGT1/Nrf2*^{+/-} (3.7-fold) and in *hUGT1/Nrf2*^{-/-} mice (1.8-fold) following exposure to Cd^{2+} . This localization can occur independently of NRF2 but is 2-fold less in *hUGT1/Nrf2*^{-/-} compared to *hUGT1/Nrf2*^{+/-} neonatal mice. This indicates that NRF2 is not responsible for the activation of CAR by Cd^{2+} .

In vivo Cd^{2+} exposure induces mitogen-activated protein kinase (MAPK) phosphorylation and CAR localization

The regulation of CAR localization makes use of ERK1/2 and P38 phosphorylation, which was examined by Western blot analysis. Neonatal *hUGT1* mice were treated orally with a single dose of Cd^{2+} (10mg/kg) or water and intestinal tissues were collected 30 minutes, 60 minutes, 120 minutes, and 240 minutes after exposure. Following treatment, the neonatal mice experienced dramatic changes in MAPK phosphorylation, where a large increase in cytoplasmic phosphorylated ERK1/2 occurs in intestinal tissue between 0 and 30 minutes after oral treatment before decaying (**Fig. 4B**). Additionally, there is an

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accumulation of P38 and phosphorylated-P38 in the nucleus (**Fig. 4A**). Similar phosphorylation patterns were observed in adult mice (data not shown). Consistent increases in CAR accumulation in the nucleus were observed in neonatal mice after 2 hours (**Fig. 4C**) coinciding with the dephosphorylation of ERK1/2 and the nuclear accumulation of P38. The observed changes in MAPK activation are consistent with known regulatory mechanisms for CAR by ERK (Koike et al., 2007) and P38 (Hori et al., 2016), supporting the observation that Cd²⁺ exposure activates intestinal CAR. The increased oxidative state following Cd²⁺ treatment, the activation of MAPK pathways, and the commensurate nuclear accumulation of CAR underlies the induction of intestinal UGT1A1.

PEITC induction of UGT1A1 retains CAR-dependency pattern in *hUGT1/Nrf2*^{-/-} mice.

The observation of CAR-dependent regulation of hepatic UGT1A1 by pro-oxidants was observed using neonatal *hUGT1/Car*^{-/-} mice treated with PEITC (Yoda et al., 2017). The tissue-specific induction patterns of hepatic and intestinal *UGT1A1* and *Cyp2b10* genes were observed in the *hUGT1/Nrf2*^{-/-} neonatal mice. The increased clearance of bilirubin in neonates treated with PEITC occurred concomitantly with increases in liver and intestinal expression of *UGT1A1* and *Cyp2b10* (**Fig 5**). The inducibility of intestinal *UGT1A1* by PEITC was reduced in *hUGT1/Nrf2*^{-/-} (16.2-fold) mice compared to *hUGT1/Nrf2*^{+/-} mice (30.3-fold) (**Fig 5**). No difference was observed in liver *UGT1A1* induction between *hUGT1/Nrf2*^{+/-} and *hUGT1/Nrf2*^{-/-} neonates and the induction of *Cyp2b10* was greatest in the liver (**Fig 5**). These observations are consistent with the

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previous report on CAR-dependency of hepatic *UGT1A1* induction and CAR-independent intestinal *UGT1A1* induction by PEITC (Yoda et al., 2017).

Discussion

It has been previously demonstrated that ITCs are capable of CAR-dependent induction of liver UGT1A1 (Yoda et al., 2017). It is well-established that NRF2 is the master regulator of the antioxidant response and can induce UGT1A1 (Yueh and Tukey, 2007). Nuclear receptors are susceptible to high levels of mutual regulation (Ratman et al., 2013) from shared protein interaction partners or through binding interactions at regulatory elements, therefore it was hypothesized that oxidative stress could be co-activating CAR through NRF2. The role of oxidative stress in the regulation of NRF2 and CAR target genes in *hUGT1/Nrf2*^{-/-} mice was investigated. As anticipated, NRF2-deficient mice do not generate the antioxidant response when challenged with Cd²⁺. However, the *hUGT1/Nrf2*^{+/-} mice exhibited a diminished antioxidant response in comparison to WT mice, suggesting haploinsufficiency.

By breeding *Nrf2*^{-/-} mice with *hUGT1* mice, pro-oxidants could be screened for their ability to reduce TSB levels and examine the impact of this action in an NRF2-dependent fashion. The NRF2-KEAP1 pathway is not required for Cd²⁺ cadmium and ITCs to induce intestinal UGT1A1 in *hUGT1* mice to reduce TSB levels. This supports the hypothesis that CAR can be activated by oxidative stress (Yoda et al., 2017). In analysis of NR activity, we observed suppression of target genes for PXR and PPAR α and slight induction of the AhR target gene, *Cyp1a1*, in WT mice (**Fig. S1**). The reason for the suppression is unclear, but the latter induction could have resulted from co-regulation of *Cyp1a1* by NRF2 and the AhR (Shin et al., 2007). The loss of this induction in the heterozygous mice can be linked to haploinsufficiency of NRF2, and there was little change in the *Nrf2*^{-/-} mice. From the examined NRs, there was evidence for CAR's

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activation from induction of the *Cyp2b10* gene. Attempts were made to confirm protein induction of CYP2B10, but the expression levels were too low for detection. Subsequently, CAR's activation was assayed more directly. In the absence of a readily available antibody for phosphorylated CAR, fractionated intestinal samples from neonates treated with Cd²⁺ were analyzed for CAR nuclear localization. After an acute cadmium exposure, CAR localized to the nucleus in an NRF2-independent manner, which indicates that co-activation of CAR by NRF2 is not responsible for the induction of intestinal UGT1A1 and the reduction of TSB levels.

The increase in ERK1/2 phosphorylation is a confounding factor in the mechanism of CAR's activation, given that p-ERK1/2 inhibits the activation of p-Thr38-CAR, keeping it sequestered in the cytoplasm (Osabe and Negishi, 2011). However, in cardiac ventricular myocytes, it has been reported that ERK1/2 can be dephosphorylated by PP2A activity that is dependent upon the activation of P38 (Liu and Hofmann, 2004). Furthermore, the phosphorylation status of ERK1/2 and P38 can be increased by H₂O₂ and As³⁺ respectively, both of which are linked to oxidative stress. The observation of a consistent nuclear accumulation of CAR during the decay of the p-ERK signal at the 2- and 4-hour time points suggests the reduction in ERK1/2 activity is sufficient to permit CAR activation, which occurs concurrently with the nuclear accumulation of P38/p-P38. In order for CAR to translocate to the nucleus, it must first be dephosphorylated by PP2A (Yoshinari et al., 2003) in order to interact with P38 to increase CAR's transactivation activity (Hori et al., 2016). Activated P38 phosphorylates CAR at the conserved Thr38 residue to export CAR from the nucleus. These changes in the phosphorylation status of

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P38 and ERK1/2 were also observed in whole-cell lysate from a *hUGT1* adult time course (data not shown), supporting our hypothesis of the oxidative stress-MAPK-CAR axis.

It has been reported that intestinal differentiation and crypt formation involves ROS and the activation of P38 (Houde et al., 2001; Rodriguez-Colman et al., 2017). Intestinal maturation is essential to both nutrient uptake and metabolic waste/toxicant removal. From studies in *hUGT1* mice, early intestinal expression of UGT1A1 is important to metabolize bilirubin while hepatic UGT1A1 expression is developmentally delayed (Fujiwara et al., 2010). Maturation of intestinal epithelial cells can be estimated by the decreases in genes related to cell stemness and the increase of functional genes related to the differentiated cell types. For example, the brush border glucosidase known as sucrose isomaltase, SIS, is only expressed in maturing and mature duodenum and jejunum enterocytes (Rubino et al., 1964). Alternatively, the *Lrp2* gene encodes for a protein known as megalin, which mediates nutrient uptake by endocytosis, and its expression in the jejunum and ileum is down regulated as those tissues develop (Vázquez-Carretero et al., 2014). These and other genes, like *Glb1* and *Krt20* are markers for intestinal maturation when down- and up-regulated, respectively (Chen et al., 2017). Intestinal maturation markers were examined and it was determined that low doses of Cd²⁺ may induce intestinal maturation as inferred by inhibition of the *Glb1* and *Lrp2* genes and the induction of the *Sis* and *Krt20* genes (**Fig. S3**). Recently, it was reported that the nuclear receptor corepressor 1 (NCoR1), which regulates CAR activity at the PBREM (Mäkinen et al., 2002), is also capable of regulating intestinal maturation (Chen et al., 2017). Intestinal epithelial cell specific knockout of NCoR1 leads to changes in maturation markers and induction of UGT1A1 similar to what we have observed. Thus, it may be

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possible that Cd^{2+} cadmium inhibits NCoR1, which increases maturation and de-represses CAR's transcriptional activity.

The most obvious implication of the oxidative stress-MAPK-CAR signaling axis resides in changes of xenobiotic metabolism from pro-oxidant activation of CAR. This could impact therapeutic outcomes in patients exposed to environmental pro-oxidants, such as heavy metals from smoking or contaminated drinking water and soil, or from indirect antioxidants found in certain diets and commercial supplements. It is possible that future studies making use of RNA-seq, metabolomics, and exposure data may reveal a correlation between pro-oxidant, toxicant exposure, and an individual variability in xenobiotic metabolism stemming from CAR activity.

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Acknowledgments

Author Contributions

Participated in research design: Paszek, Tukey

Conducted experiments: Paszek

Performed data analysis: Paszek

Wrote or contributed to the writing of the manuscript: Paszek, Tukey

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Footnotes

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Legends for Figures

Figure 1. NRF2-dependency of antioxidant response genes in neonatal *hUGT1* mice. Ten-day old *hUGT1/Nrf2*^{+/+}, *hUGT1/Nrf2*^{+/-}, and *hUGT1/Nrf2*^{-/-} neonatal mice were treated with vehicle (blue circles) or Cd²⁺ (10 mg/kg od. po.) (red squares) for two days before collecting intestinal tissue for RT-qPCR analysis of oxidative stress markers. The genes examined include *Nqo1*, *Ho-1*, *Gsta1*, *Gsta2*, and *Gstm3*. (Mean +/- SEM, Two-way ANOVA; * P<0.05, *** P<0.001, **** P<0.0001)

Figure 2. Cd²⁺ reduces TSB levels in *hUGT1* neonatal mice in an NRF2 independent fashion. Ten-day old *hUGT1/Nrf2*^{+/+}, *hUGT1/Nrf2*^{+/-}, and *hUGT1/Nrf2*^{-/-} neonatal mice were treated with vehicle (blue circles) or Cd²⁺ (10 mg/kg od. po.) (red squares) for two days before collecting blood and intestinal tissues. (A) Changes in TSB levels (mg/dL) from mice treated with Cd²⁺. (B) Changes in the expression of the *UGT1A1* gene. (C) Changes in the expression of the UGT1A1 protein as visualized by Western blot analysis and quantified by normalizing to GAPDH expression and set relative to vehicle treated *hUGT1/Nrf2*^{+/-} mice. (Mean +/- SEM, Two-way ANOVA; * P<0.05, ** P<0.01, **** P<0.0001)

Figure 3. NRF2-independent changes of intestinal *Cyp2b10* gene expression and CAR localization. Ten-day old *hUGT1/Nrf2*^{+/+}, *hUGT1/Nrf2*^{+/-}, and *hUGT1/Nrf2*^{-/-} neonatal mice were treated with vehicle (blue circles) or Cd²⁺ (10 mg/kg od. po.) (red squares) for two days before collecting intestinal tissues. (A) Changes in the expression of the

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Cyp2b10 gene were observed by RT-qPCR. (B) Nuclear localization of CAR was determined by Western blot analysis of fractionated intestinal protein extracts. Expression of CAR was quantified by normalizing to HDAC-1 and set relative to vehicle treated *hUGT1/Nrf2*^{+/-} mice. (Mean +/- SEM, Two-way ANOVA; * P<0.05, ** P<0.01, *** P<0.001)

Figure 4. Changes in intestinal MAPK phosphorylation and CAR localization following Cd²⁺ treatment in *hUGT1* neonates. Twelve-day old neonatal *hUGT1* mice were treated with vehicle or Cd²⁺ (10 mg/kg po.) and intestinal tissues were collected 30, 60, 120, and 240 minutes after exposure. Intestinal protein extracts were fractionated into nuclear and cytosolic components, and changes in (A) nuclear P38 content and phosphorylation status, (B) cytosolic ERK1/2 content and phosphorylation status, and (C) CAR localization were visualized by Western blot analysis with H3 and tubulin for nuclear and cytoplasmic loading controls. Quantifications and statistical analyses for A, B, and C can be found in supplementary figures 4, 5, and 6, respectively.

Figure 5. Hepatic and intestinal induction of *UGT1A1* and *Cyp2b10* in *hUGT1/Nrf2* neonatal mice. Ten-day old *hUGT1/Nrf2*^{+/-} and *hUGT1/Nrf2*^{-/-} neonates were treated with vehicle (blue circles) or PEITC (200 mg/kg od. po.) (red squares) for two days before collecting liver and small intestine. Gene expression for *UGT1A1* and *Cyp2b10* in each tissue were examined by RT-qPCR. (Mean +/- SEM, Two-way ANOVA; * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001)

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Tables

Table 1. Primers utilized for real-time PCR analysis of selected genes

Gene	Forward (5'→3')	Reverse (5'→3')
<i>hUGT1A1</i>	CCA TCA TGC CCA ATA TGG TT	CCA CAA TTC CAT GTT CTC CA
<i>Cyp1a1</i>	TGC CCT TCA TTG GTC ACA TG	CAC GTC CCC ATA CTG CTG ACT
<i>Cyp2b10</i>	AAG GAG AAG TCC AAC CAG CA	CTC TGC AAC ATG GGG GTA CT
<i>Cyp3a11</i>	ACA AAC AAG CAG GGA TGG AC	CCC ATA TCG GTA GAG GAG CA
<i>Cyp4a10</i>	CAC ACC CTG ATC ACC AAC AG	TCC TTG ATG CAC ATT GTG GT
<i>Nqo1</i>	TTT AGG GTC GTC TTG GCA AC	GTC TTC TCT GAA TGG GCC AG
<i>Ho-1</i>	ACA GGG TGA CAG AAG AGG CTA AGA C	ATT TTC CTC GGG GCG TCT CT
<i>Gsta1</i>	AGC CCG TGC TTC ACT ACT TC	TCT TCA AAC TCC ACC CCT GC
<i>Gsta2</i>	AAT CAG CAG CCT CCC CAA T	TCC ATC AAT GCA GCC ACA CT
<i>Gstm3</i>	AAA CCT GAG GGA CTT CCT GG	AAC ACA GGT CTT GGG AGG AA
<i>Il-1b</i>	GCA ACT GTT CCT GAA CTC AAC	ATC TTT TGG GGT CCG TCA ACT
<i>Il-6</i>	ACC AGA GGA AAT TTT CAA TAG GC	TGA TGC ACT TGC AGA AAA CA
<i>Tnfa</i>	GAT CGG TCC CCA AAG GGA TG	GGC TAC AGG CTT GTC ACT CG
<i>Glb1</i>	CAC TGC TGC AAC TGC TGG	ATG TAT CGG AAT GGC TGT CC
<i>Lrp2</i>	CCA GGA TTC TGG TGA TGA GG	CGG GAA CTC CAT CAC AAA CT
<i>Krt20</i>	CCT GCG AAT TGA CAA TGC TA	CCT TGG AGA TCA GCT TCC AC
<i>Sis</i>	ACC CCT AGT CCT GGA AGG TG	CAC ATT TTG CCT TTG TTG GAT GC
<i>Cph</i>	CAG ACG CCA CTG TCG CTT	TGT CTT TGG AAC TTT GTC TGC

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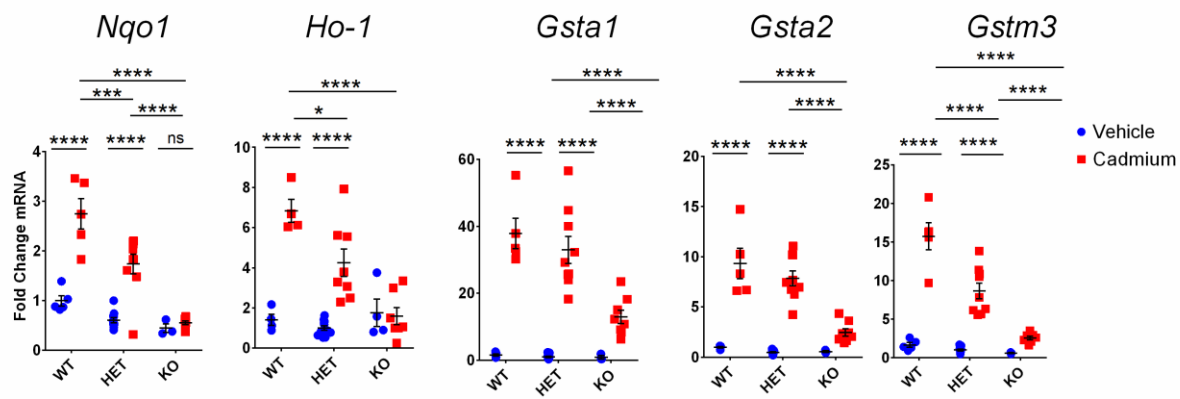
Table 2. Antibodies utilized for Western blot analysis

Antibody	Supplier	Product #
Anti-mouse IgG, HRP-linked	Cell Signaling Technology	#7076
Anti-rabbit IgG, HRP-linked	Cell Signaling Technology	#7074
CAR	ABclonal	A1970
ERK1/2	Cell Signaling Technology	#9102
Phospho-ERK1/2	Cell Signaling Technology	#9101
GAPDH	Santacruz Biotechnology	sc-47724
HDAC-1	Santacruz Biotechnology	sc-6298
Histone 3	ABclonal	A2352
P38	Cell Signaling Technology	#9212
Phospho-P38	Cell Signaling Technology	#9211
Tubulin	Sigma-Aldrich	T9026
UGT1A1	Abcam	Ab170858

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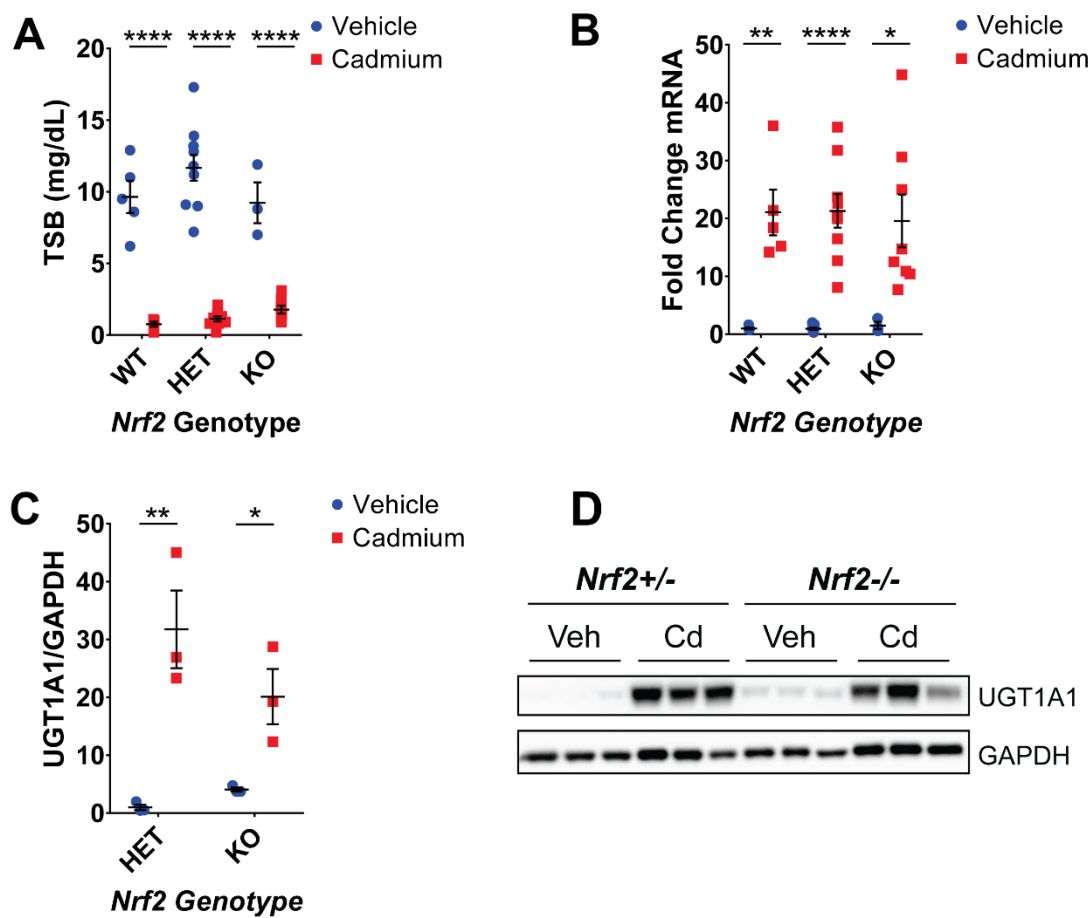
Figures

Figure 1



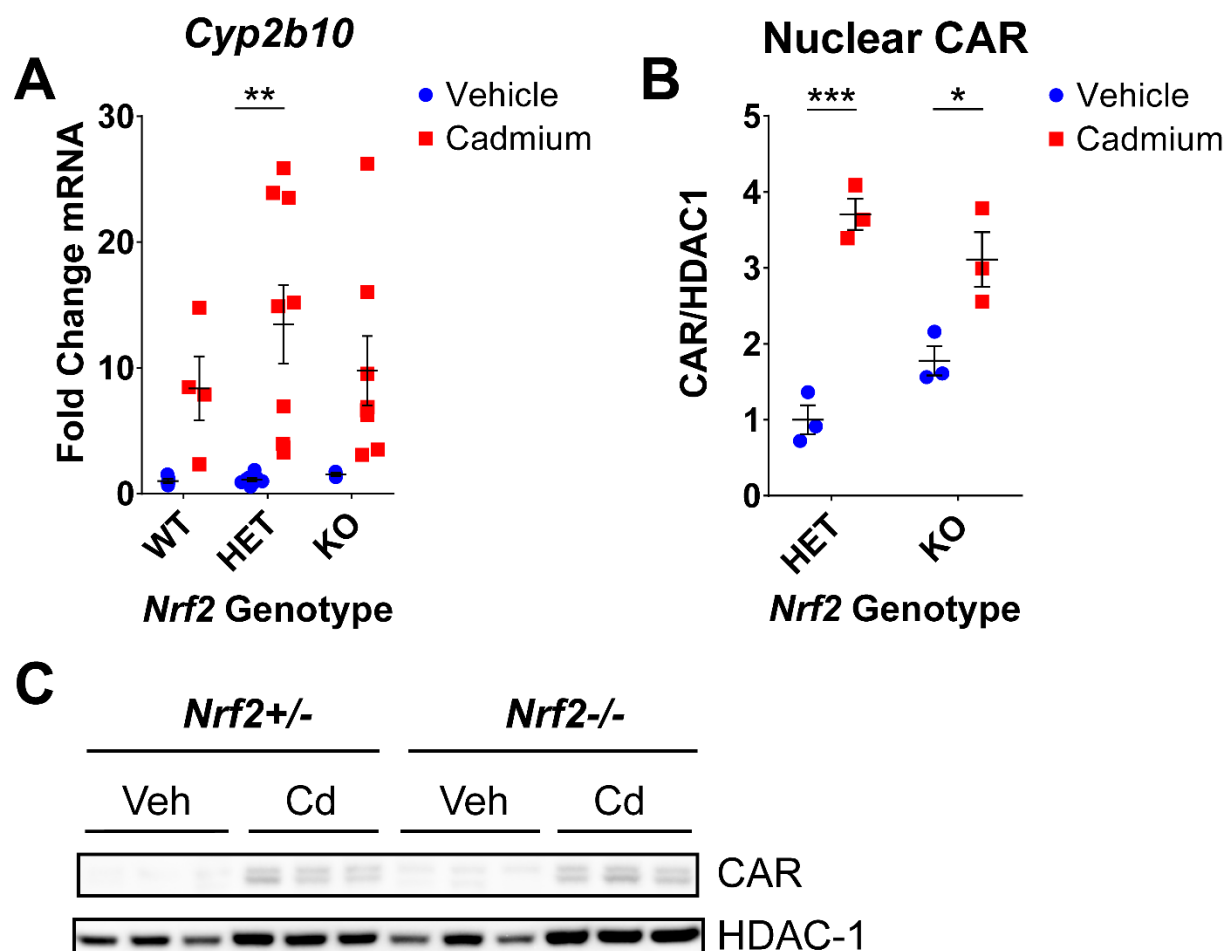
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Figure 2.



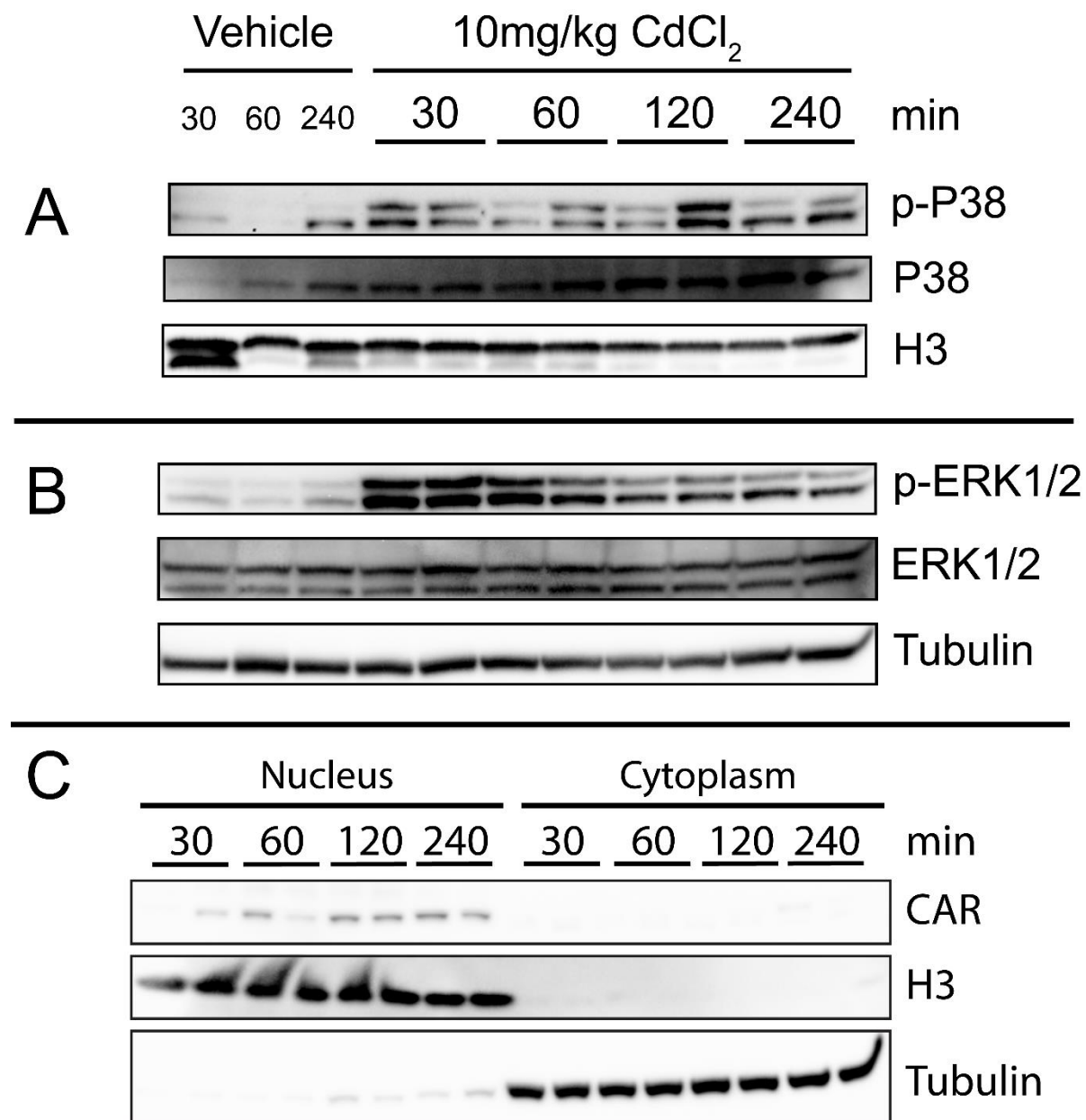
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Figure 3.



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Figure 4.



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Figure 5.

