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Research Article

A CRISPR/Cas9 Whole-Genome Screen Identifies Genes Required for Aryl Hydrocarbon Receptor-Dependent Induction of Functional CYP1A1

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ABSTRACT

Environmental pollutants including halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons, including benzo[a]pyrene, exert their deleterious effects through the activation of the aryl hydrocarbon receptor (AHR) and by the resulting transcription of genes not yet fully identified. Ligand-bound AHR translocates from cytoplasm to nucleus, where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) protein. The AHR/ARNT dimer binds to enhancer regions of responsive genes to activate transcription. AHR also mediates carcinogenesis caused by PAHs, likely via CYP1A1, CYP1A2, and CYP1B1, which are massively induced by activated AHR in many tissues and generate carcinogenic electrophilic derivatives of PAHs. In the current study, we have used the mouse GeCKOv2 genome-wide CRISPR/Cas9 library to identify novel genes in the AHR pathway by taking advantage of a B[a]P selection assay that we previously used to identify core AHR pathway genes in Hepa-1c1c7 murine hepatoma cells. Besides *Ahr*, *Arnt*, and *Cyp1a1*, we report the identification of multiple additional putative AHR pathway genes including several that we validated. These include cytochrome P450 reductase (*Por*), which mediates redox regeneration of cytochromes P450, and 5 genes of the heme biosynthesis pathway: delta-aminolevulinate synthase 1 (*Alas1*), porphobilinogen deaminase (*Hmbs*), uroporphyrinogen decarboxylase (*Urod*), coproporphyrinogen oxidase (*Cpox*), and ferrochelatase (*Fech*): heme being an essential prosthetic group of cytochrome P450 proteins. Notably, several of these genes were identified by GeCKO screening, despite not being identifiable by reverse genetics approaches. This indicates the power of high-sensitivity genome-wide genetic screening for identifying genes in the AHR pathway.

Key words: aryl hydrocarbon receptor; GeCKO CRISPR/Cas9; CYP1A1; *Por*; heme biosynthesis.

The aryl hydrocarbon receptor binds a number of important environmental carcinogens, including halogenated aromatic hydrocarbons (HAHs), such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (B[a]P) (Murray *et al.*, 2014). TCDD is a potent genotoxic carcinogen (tumor promoter). Unlike TCDD, carcinogenicity by B[a]P mainly depends on its metabolism to mutagenic derivatives (principally by CYP1A1, CYP1A2, and CYP1B1). Several endogenous ligands for AHR have also been identified, including the tryptophan catabolite kynurenine, which is overproduced in several human tumors, and enhances tumor progression (Opitz *et al.*, 2011). Several bacterial pigments can activate AHR, and thereby initiate an antibacterial response (Moura-Alves *et al.*, 2014). TCDD can induce Treg development and suppress T_H17 differentiation. Kynurenine probably plays a role in the regulation of these T cell subsets *in vivo* (Gutiérrez-Vázquez and Quintana, 2018). Certain vegetable constituents exhibit AHR agonist activities and can regulate intestinal

immunity via AHR (Lamas *et al.*, 2018; Veldhoen and Brucklacher-Waldert, 2012). The AHR also controls endotoxin tolerance via its activation by kynurenine (Bessede *et al.*, 2014). Humans and other mammals are therefore exposed to a variety of AHR ligands, many of which are deleterious, but some of which may have beneficial effects.

Unliganded AHR is located in the cytosol complexed with p23, XAP2, and 2 molecules of heat shock protein 90 (HSP90). After binding agonist, the AHR translocates to the nucleus, releases its chaperone proteins, and dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). In the nucleus, AHR/ARNT dimers bind to xenobiotic responsive elements (XREs) in the enhancer region of the *Cyp1A1* gene (and other responsive genes), and recruit a number of coactivator proteins, which remodel, relocate, or dissociate nucleosomes in chromatin at the enhancer and promoter, leading to recruitment of RNA polymerase II and resulting in transcriptional initiation (Hankinson, 2012).

The levels of AHR and the maximal level that each CYP1 family members can be induced by TCDD varies greatly between different tissues/organs (Harper *et al.*, 2006; Uno *et al.*, 2008). In addition, different CYP1 family members are inducible in different tissues. AHR is overexpressed and constitutively active in a proportion of many types of cancer, and CYP1A1 and CYP1B1 are overexpressed in many cancers, further reinforcing the notion that these proteins are intimately involved in cancer progression (Androutsopoulos *et al.*, 2009; Murray *et al.*, 2014).

This laboratory previously isolated spontaneous or mutagen-induced clones of the mouse hepatoma cell line, Hepa-1c1c7 (Hepa1), by selecting for resistance to B[a]P toxicity. These clones all exhibited loss of induction of CYP1A1 by TCDD. (In wild-type cells, B[a]P binds the AHR, thereby inducing CYP1A1, which then metabolizes B[a]P to cytotoxic derivatives. Hepa1 cells do not express CYB1A2 or CYP1B1 [Beedanagari *et al.*, 2010].) Recessive mutants from these screens were assigned to 4 complementation groups (A through D). The A and D mutants proved to be mutated in the *Cyp1A1* and *Ahr* genes, respectively (Hankinson, 1994). The C mutant provided the means for cloning *Arnt* (Hoffman *et al.*, 1991; Reyes *et al.*, 1992). The B gene has not been identified. B-mutant cells express much reduced levels of the AHR mRNA. Further studies indicated that the B clones are either mutated in a transcription factor required for expression of the endogenous *Ahr* gene or are mutated in a protein involved in the generation of an open chromatin configuration over the *Ahr* gene that is required for its expression (Zhang *et al.*, 1996).

We previously screened an inhibitory RNA (RNAi) library targeting about 25% of known protein coding genes in the mouse to identify proteins necessary for the AHR-dependent induction of CYP1A1-dependent enzymatic activity by TCDD in Hepa1 cells (Solaimani *et al.*, 2013). We thereby identified 12 novel proteins that appeared to be required for TCDD induction of CYP1A1 activity, and fully validated a role for one of these gene products, SIN3A (Solaimani *et al.*, 2014). The validation data for each of the other 11 siRNA hits were less convincing.

We describe here the use of a CRISPR/Cas9 system to identify genes required for expression or function of the *Ahr*, *Arnt*, or *Cyp1A1* genes in the Hepa1 cell line. In this system, Cas9 is programmed to induce a DNA double-stranded break at specific genomic loci dictated by the 20 nt target sequences encoded within synthetic-guide RNA (sgRNA) molecules. Subsequent repair via the nonhomologous end-joining pathway creates small deletions and insertions which, when located in coding regions of genes, can generate frameshift mutations, resulting in loss of function. We used the 2-vector mouse GeCKOv2 library system as described by Sanjana and coworkers. The 2 vectors together contain all components of the CRISPR/Cas9 system including optimized sgRNA encoding sequences (Sanjana *et al.*, 2014). The GeCKOv2 library was introduced by lentiviral transduction into Hepa1 cells, and the cells then selected in B[a]P for clones deficient in CYP1A1 activity. Importantly, the GeCKOv2 library contains sgRNAs targeting 20 611 mouse genes (with 6 targeting constructs per gene) and thus nearly all protein coding genes in the mouse. The GeCKOv2 library also targets 1178 mouse microRNAs. An important advantage of the CRISPR/Cas9 system is that it can completely inactivate targeted genes.

The Hepa1 cell line is aneuploid and possesses a modal chromosome number (58) greater than the diploid number in the mouse (40) (Hankinson, 1979). This suggests that the superdiploid chromosome number of the Hepa1 cell line may have limited the variety of recessive mutagen-induced or spontaneous B[a]P-resistant clones that we previously isolated. The efficiency with which recessive mutants can be isolated with the CRISPR/Cas9 system is minimally affected by the copy number of the corresponding gene in transduced cells, because this system can efficiently inactivate multiple copies of a gene. This suggested to us that additional types of B[a]P-resistant clones might be isolated using our CRISPR/Cas9 approach. Furthermore, as alluded to below and discussed more fully below, our CRISPR/Cas9 approach also has a number of advantages over the siRNA approach we previously used, suggesting that it may identify different/additional novel genes affecting CYP1A1 induction or activity.

MATERIALS AND METHODS

Cells. Cultures of mouse Hepa-1c1c7 hepatoma cells (Hankinson, 1979) containing fewer than 10^{-7} B[a]P-resistant cells (Hepa1 MC3 cells) were grown in Alpha MEM, 10% fetal bovine serum (FBS), 1× penicillin/streptomycin, 1× fungizone, at 37°C in a humidified incubator with 5% CO_2, unless otherwise stated.

Preparation of Cas9 lentivirus and GeCKOv2 lentivirus sgRNA libraries. Lentiviruses were made by co-transfecting HEK293T cells with pMD.G (VSV-G envelope), psPAX2 (gag/pol) and a third, virus-specific plasmid to produce infectious self-inactivating third-generation lentivirus particles. All plasmids were obtained from Addgene. The viruses of the 2-vector GeCKOv2 libraries were made using lentiCas9-Blast to produce Cas9 virus, and pooled GECKO v2.0 lentiGuide-Puro plasmid libraries to generate sgRNA viruses (Sanjana *et al.*, 2014). Single-vector CRISPR/Cas9 viruses were made using sequence verified clones of pLentiCRISPRv2 (Sanjana *et al.*, 2014) into which a gene-specific 20 nt target sequence had been cloned (Supplementary Table 1). The "scrambled" nontargeting control sgRNA sequence was obtained from Origene. Mouse GeCKOv2 CRISPR knockout pooled library and pLentiCRISPRv2 were gifts from Feng Zhang (Addgene nos. 1000000053 and 52961).

GeCKO sgRNA library lentivirus was made in HEK293T cells using Invitrogen Virapower Mix (K4975) and Lipofectamine and Plus Reagent (Cat. no. 15338-100) according to the manufacturer's protocol. For single gene target virus production, 5×10^6 HEK293T cells were plated in 100 mm plates in 10 ml standard growth media (DMEM, 10% BS—Gibco and Hyclone). After overnight incubation, each of three 100 mm dishes of nonconfluent HEK293T cells were co-transfected with 6.5 μg of psPAX2, 3.5 μg of pMD.G, and 10 μg of virus-specific plasmid by the $CaPO_4$-DNA

coprecipitation method (Chen and Okayama, 1987; Sakoda *et al.*, 1992). The next day, the medium was removed, cells were washed with 1× DPBS and media was replaced with 6 ml fresh medium containing 20 mM Hepes and 10 mM sodium butyrate. Cells were incubated for 6–8 h to obtain high-titer virus production as described previously (Sakoda *et al.*, 1999). After the incubation, cells were washed and incubated in 5 ml fresh medium without sodium butyrate. The virus containing medium was collected 18–20 h later and passed through 0.45 μm filters. For low concentration viral stocks (gene-specific CRISPR/Cas9 viruses), aliquots of the virus were stored at −80°C. High concentration viral stocks (Cas9 virus and GeCKO sgRNA library viruses) were made by ultracentrifugation of filtered medias at 24 000 RPM for 2 h at 4°C that were resuspended in standard growth media at 4°C overnight. This generated, approximately, 300× concentrated viral stocks. Viral titers for gene-specific viruses were determined by assaying viral p24 antigen concentration by ELISA (the Alliance HIV-I p24 ELISA Kit, Perkin Elmer) and expressed as μg of p24 equivalent units per milliliter. High concentration virus titers were determined by infecting Hepa1 MC3 cells, selecting transduced cells on 3 μg/ml puromycin or 7 μg/ml blasticidin, respectively, 2 days later, and determining infectious units per microliter (ifu/μl) by Poisson distribution calculation.

Transduction of Hepa1 cells for GeCKO library screening and target validation. Hepa1 MC3 cells were virally transduced by plating cells with either high or low concentration lentiviral stocks in 100 mm dishes. All transductions were performed in normal growth media (MEM Alpha, 10% FBS, 1× Pen/Strep, 1× Fungizone) with the addition of 8 μg/ml polybrene (Santa Cruz Biotech). To generate cells that expressed Cas9 (Hepa1•Cas9 cells), Hepa1 MC3 cells were transduced with virus made from lentiCas9-Blast and selected by growth on blasticidin (7 μg/ml) 2 days later. Because lentiCas9-Blast encodes Cas9 as a P2A fusion protein upstream of basticidin-S deaminase (*bsd*), cells that express *bsd* also express Cas9. To generate populations of GeCKO library-transduced cells, 3×10^7 Hepa1•Cas9 cells were transduced with 4×10^6 infectious units (*ie*, transduced at an MOI = 0.13) of high concentration GeCKO sgRNA virus. Because library transductions were performed at high cell densities, individually transduced plates were split the following day into 3 plates, and library-transduced cells were selected with puromycin (3 μg/ml) 1 day later (2 days after transduction),.

Selection in benzo[a]pyrene and harvesting of B[a]P selected cells. To select for benzo[a]pyrene-resistant cells, we plated 2×10^5 to 4×10^5 GeCKO library-transduced Hepa1•Cas9 cells (Hepa1•Cas9•GeCKO cells) in 100 mm dishes supplemented with 16 μM B[a]P. Each transduction was selected on 10–12 B[a]P-selection plates. Thus for each transduction we selected a total of 2×10^6–4.8×10^6 cells. Because cells were allowed to expand for 6 days after transduction before B[a]P selection, we had sufficient number of cells from each transduction to perform multiple selection assays on this scale. Cells were allowed to grow for 10 days under B[a]P selection in 12 ml of media per plate, by which time B[a]P-resistant clones were seen to form visible colonies. Surviving cells were harvested by washing plates with 6 ml DPBS and then trypsinizing with 0.75 ml 0.05% Trypsin-EDTA (Gibco), scraping and collecting plate contents, and then washing plates with 0.75 ml DPBS that was combined with the previously collected trypsinized material. Cells were pelleted by centrifugation in a microcentrifuge for 1 min at 11 000 × *g* at 4°C.

Preparation of DNA and next-generation sequencing. Total DNA was purified from cell pellets by spin column purification (NucleoSpin Tissue, Machery-Nagel), and purified DNAs from all 10–12 selection plates of a given transduction were combined. These pooled DNAs represented all of the cells that survived B[a]P exposure from the 2×10^6–4.8×10^6 cells of a given transduction that were exposed to B[a]P.

Next-generation sequencing (NGS) libraries were prepared by amplifying 317 bp products from the sgRNA regions of integrated GeCKO library viruses using NGS Library Primary PCR primers (Shalem *et al.*, 2014), 5 μg of pooled template DNA and PrimeSTAR Max DNA polymerase (Takara) in a 50 μl PCR reactions that underwent 18 cycles of amplification. These reaction products were column purified (QIAquick PCR—Qiagen), and barcoded NGS adaptor sequences (Shalem *et al.*, 2014) were attached by a 24-cycle second round of PCR using 5 μl of purified 1° PCR product as template and single stranded Ultramer DNAs as primers. Standard primers and Ultramer primers were purchased from IDT. NGS reactions were performed on an Illumina HiSeq 2500 platform. Data were exported in qseq format and processed on the UCLA Hoffman2 cluster.

Cas9/sgRNA ribonucleoprotein gene targeting. Targeted gene knockouts were created by transfecting Cas9/sgRNA ribonucleoprotein complexes into Hepa1 MC3 cells. sgRNAs were produced by *in vitro* transcription using the EnGen sgRNA synthesis kit (NEB). sgRNA 20 nt target sequences are listed in Supplementary Table 1. Non-GeCKO target sequences were designed using either Desktop Genetics (https://www.deskgen.com) or Sigma Genosys sgRNA design tools. In each case, purified sgRNAs (Zymo Research, RNA Clean and Concentrator) were complexed, *in vitro*, with Cas9 protein (PNA Bio) and transfected into cells using TransIT-CRISPR (Sigma-Aldrich) transfection reagent and protocol. All sgRNAs were delivered individually as well as in combination with other sgRNAs targeting the same gene.

CYP1A1 activity assay. CYP1A1 enzyme activity was analyzed using a cell-based ethoxyresorufin-O-deethylase (EROD) assay (Solaimani *et al.*, 2013, 2014). Cells were plated in a 96-well format at a density of 5000 cells per well and grown in Opti-MEM, 5% Nu-Serum IV supplemented with or without 10 nM TCDD to activate AHR. Two days later media was exchanged with EROD assay media (phenol-red-free Opti-MEM, 5% Nu-Serum IV, 10 μM ethoxyresorufin, 500 μM dicumarol) and cells were incubated for 1 h at 37°C, 5% CO_2. CYP1A1 activity was determined by measuring resorufin fluorescence (530 nm excitation/590 nm emission) on a Modulus Microplate fluorescence plate reader (Turner Biosystems). Fluorescent readings were normalized to the average reading from cell-free wells incubated with EROD assay media and then normalized to MTT readings (Cell Proliferation Kit I [MTT]—Roche) obtained from replicate plates to control for different growth rates between knockout cell lines.

Determination of heme concentrations. Total heme concentrations (free and protein bound) in cell growth medias were determined by testing FBS and Nu-Serum using an Abnova Heme Assay Kit (catalog number KA1617) according to the manufacturer's protocol. Assays were performed in optical quality 96-well plates and absorbance at 400 nm was measured on a Modulus Microplate (Turner Biosystems) plate reader.

Safe use of benzo[a]pyrene and lentivirus vectors. Preparation of stock solutions of benzo[a]pyrene was done in a fume hood.

Waste solutions containing B[a]P or TCDD were sent to the UCLA Division of Environmental of Health and Safety for disposal. Work with the lentivirus vectors was performed in Type IIA laminar flow hoods in laboratories designated BSL2+ and approved for use with the third-generation CRISPR/Cas9 lentivirus vectors. Use of the lentivirus vectors was approved by the UCLA Department of Environmental Health and Safety.

RESULTS

Generation of Cas9-Expressing Hepa1 Cells

Our approach for identifying AHR pathway genes was to screen the genome-wide mouse GeCKOv2 sgRNA library in mouse Hepa1 cells using a benzo[a]pyrene selection assay that we had previously developed to isolate mutations in genes that disrupt normal AHR pathway function (see Figure 1). The key aspect of this assay is that Hepa1 cells with a functionally intact AHR pathway are inducible for CYP1A1 and are therefore killed by cytotoxic intermediates produced from CYP1A1 metabolism of B[a]P. Hepa1 cells that harbor defects in AHR pathway genes and cannot induce functional CYP1A1 survive B[a]P treatment.

The GeCKOv2 library is a lentivirus-based system that introduces *S. pyogenes* Cas9 and gene-specific Cas9 sgRNAs into cells by viral transduction. We opted to perform these studies using the 2-component GeCKOv2 libraries in which Cas9 and the mouse-specific sgRNAs libraries are transduced on independent vectors. We started our screens with cultures of Hepa1 cells that we had previously found to contain fewer than 10^{-7} cells resistant to 16 μM benzo[a]pyrene (Hepa1 MC3 cells). We transduced these cells with a Cas9-expressing lentivirus (produced from lentiCas9-Blast) and selected cells with blasticidin to obtain a population of cells that stably expressed Cas9 (Hepa1•Cas9 cells).

Introduction of the GeCKOv2 sgRNAs

The GeCKOv2 sgRNA library is divided into 2 sublibraries, A and B, that each target the same genome-wide collection of protein coding genes, but with distinct sets of sgRNAs. Each GeCKO sgRNA virus is designed to express an sgRNA (driven by an hU6 promoter) and puromycin N-acetyltransferase (PAC—driven by an EF1α promoter) in transduced cells. Both sublibraries contain 3 sgRNAs per gene targeting 20 661 protein coding genes, with sublibrary A containing an additional 4 sgRNAs per miRNA targeting 1175 distinct miRNAs (Sanjana *et al.*, 2014). We introduced the GeCKOv2 sgRNA sublibraries individually into Hepa1•Cas9 cells to generate populations of cells (Hepa1•Cas9•GeCKO cells) that harbored an integrated Cas9 construct and one of the GeCKO sgRNA sublibraries. Overall, we performed 19 independent transductions of the GeCKO sublibraries (9 transductions of sublibrary A and 10 transductions of sublibrary B). For each transduction, we infected 3×10^7 Hepa1•Cas9 cells with 4×10^6 infectious units (ifu) of library to ensure representative transduction. These transductions were, therefore, performed at an average multiplicity of infection (MOI) of 0.13. These conditions were chosen so that most cells that were transduced (88%) were only transduced by a single virus. Because the mouse GeCKOv2 library contains 67 405 total sgRNA constructs in sublibrary A and 62 804 sgRNA constructs in sublibrary B, individual sgRNAs were transduced into approximately 63 cells per transduction. This equates to an overall depth of coverage of approximately 600-fold of the complete mouse GeCKOv2 library.

Selection of Benzo[a]Pyrene-Resistant GeCKOv2 Transductants

Two days after GeCKO library transduction, cells were subjected to puromycin selection. After an additional 4 days, we selected cells in 16 μM B[a]P. These periods of growth were to allow first, for expression of GeCKO virus encoded PAC (puromycin

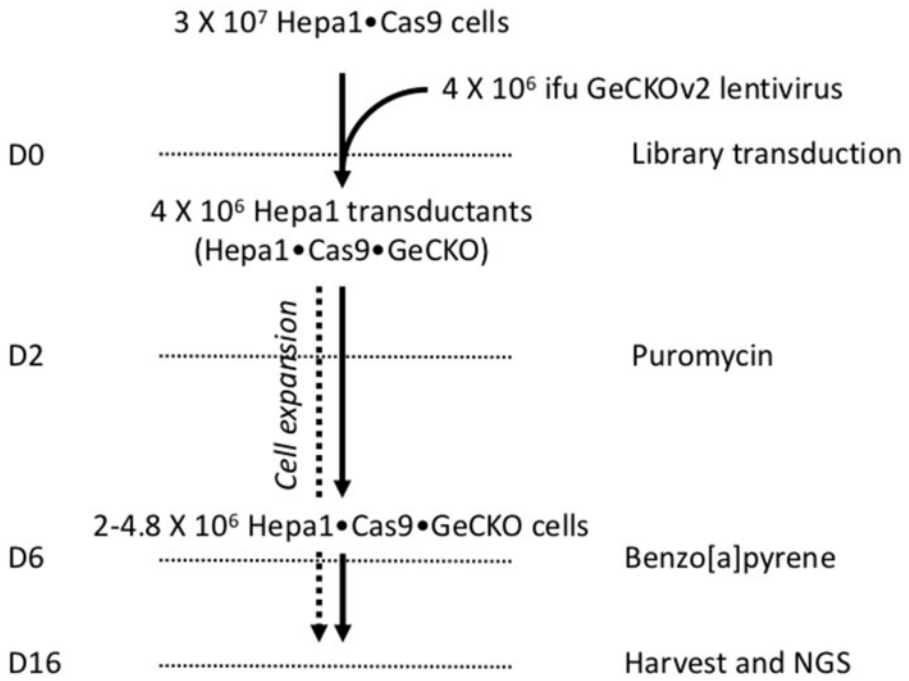

Figure 1. GeCKOv2 sgRNA library screening in Hepa1 cells. Hepa1 cells transduced with Cas9-expressing lentivirus (Hepa1•Cas9 cells) were transduced with 4×10^6 ifu of GeCKOv2 sgRNA library virus at day 0 (D0). Transductants were grown for 2 days before being selected on puromycin (D2) and then expanded for another 4 days (until D6), to allow for gene editing, before 2–4.8 $\times 10^6$ cells were plated in B[a]P-containing media to select for AHR pathway mutants. After 10 days of B[a]P selection (D16), cells were harvested and total DNAs were extracted for NGS analysis.

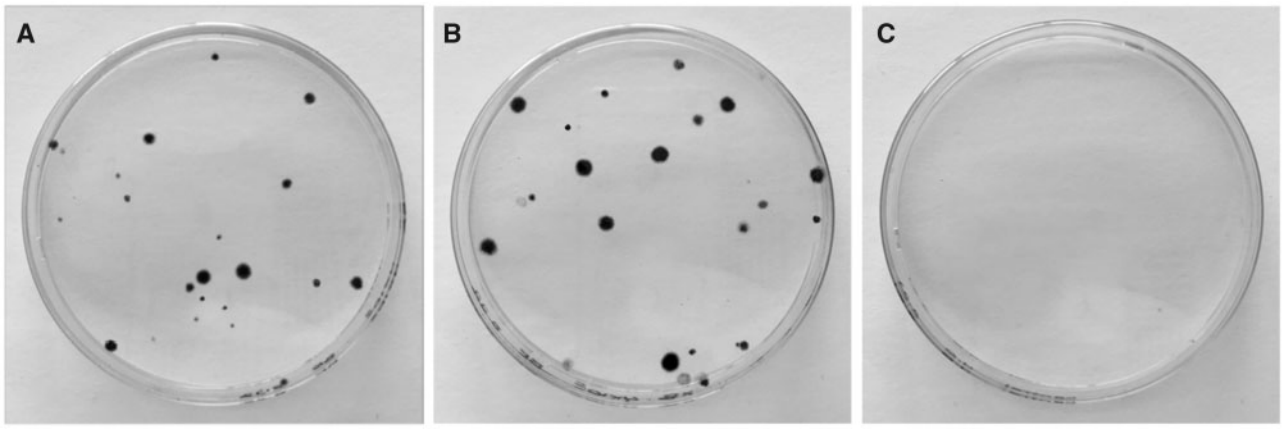

Figure 2. B[a]P-selected GeCKO sgRNA library-transduced cells. Hepa1•Cas9 cells were transduced with a GeCKO sgRNA sublibrary or mock transduced were selected on puromycin (except for mock transduced), were plated at 2×10^5 cells/plate on 100 mm dishes with 16 μM B[a]P, and were allowed to grow for 10 days before being fixed and stained with crystal violet. Panel (A) GeCKO sublibrary A transduced Hepa1•Cas9 cells. Panel (B) GeCKO sublibrary B transduced Hepa1•Cas9 cells. Panel (C) Control, mock-transduced Hepa1•Cas9 cells (not selected with puromycin).

resistance) and sgRNAs, and then, for CRISPR/Cas9 gene editing and for cellular degradation of target gene products that had been produced prior to genome editing. Each B[a]P selection assay was performed by plating 2×10^6–4.8×10^6 cells in 16 μM B[a]P across 10–12 individual 100 mm plates at densities of 2×10^5–4×10^5 cells per dish. B[a]P cell selection is concentration and density dependent, and we had previously found these conditions to be optimal for selecting B[a]P-resistant clones. We also knew from previous work that mutation of core genes in the AHR pathway, such as *Ahr*, *Arnt*, or *Cyp1A1*, gives rise to B[a]P-resistant colonies that are visible after 10 days of growth in B[a]P-containing media. We, thus, allowed GeCKO library-transduced cells to grow for 10 days under B[a]P selection before harvesting. On an average, we observed 20–40 B[a]P-resistant colonies per selection plate versus 0–4 colonies on mock-transduced control plates after the 10-day B[a]P selection period (Figure 2).

Identification of sgRNAs Contained in Benzo[a]Pyrene-Resistant Transductants

We used an NGS approach to identify integrated GeCKO viruses in cells that survived B[a]P selection. To do this we harvested cells *en masse* from B[a]P-treated plates and extracted total DNA. For each transduction, we pooled purified DNAs from each selection plate belonging to a particular transduction and used the pooled DNAs as template for a 2-step nested PCR amplification. The first PCR reaction amplified across the sgRNA regions of all integrated GeCKO viruses in B[a]P-selected cells. This was followed by a second PCR reaction that attached adaptor and bar code sequences for multiplex NGS sequencing (Sanjana *et al.*, 2014). We sorted and ranked our sequencing results based on sgRNA prevalence in each selection assay. Our criteria for identifying candidate AHR pathway genes were that they were either represented by more than 1 sgRNA or, if they were represented by a single sgRNA, that this had been selected across multiple transductions.

Most Prevalent Genes Identified by GeCKO Library Screening

In general, the number of reads that were obtained, per sgRNA, dropped off significantly after the first 15–20 sgRNAs identified from each transduction. Accordingly, we focused our analysis on sgRNAs that had a relative prevalence of ≥0.1% per transduction. From this analysis, we identified 10 targets (genes) that met our selection criteria: *Ahr*, *Cyp1A1*, *Arnt*, lipocalin 12 (*Lcn12*), serine protease 27 (*Prss27*), mitogen-activated protein kinase 15 (*Mapk15*), cytochrome P450 reductase (*Por*), and 2 genes encoding proteins involved in heme biosynthesis: Uroporphyrinogen decarboxylase (*Urod*) and porphobilinogen deaminase (*Hmbs*). *Ahr* was the top hit in each transduction, and, with 1 exception (*cf* below), either *Arnt* or *Cyp1A1* was the second most prevalent target in all of these transductions (Figure 3A). Figure 3B shows the total number of hits, per gene, from all transductions. Because we performed 19 independent transductions of the GeCKO sublibraries, there were a maximum of 57 possible hits per coding gene. We observed 46 hits for *Cyp1A1* and 23 and 26 hits for *Ahr* and *Arnt*, respectively (Figure 3B). The sequences of the sgRNAs represented in Figure 3B are listed in Supplementary Table 1. Figure 3C shows the normalized total number of NGS reads per gene (*ie*, reads of all recovered GeCKO sgRNAs targeting a specific gene) across all transductions. *Ahr* was the most frequently sequenced target. *Ahr* sgRNAs were 3 times more prevalent (number of reads) than the combined prevalence of all the recovered GeCKO sgRNAs that targeted *Cyp1A1*. Finally, Figure 3D shows how many unique sgRNAs were recovered for each of these 10 targets. Out of 6 GeCKO sgRNAs per gene, we recovered all 6 sgRNAs for *Cyp1A1*, whereas we only recovered 4 sgRNAs for *Ahr* and 4 for *Arnt*. We speculate that the missing *Ahr* and *Arnt* sgRNAs were incapable of targeting these genes in Hepa1 cells, either because of differences between the sequences used to design the GeCKOv2 sgRNAs (which were designed according to the NCBI RefSeq transcripts [Sanjana *et al.*, 2014]) and the genetic background of Hepa1 cells (which are derived from a C57L/J mouse tumor [Bernhard *et al.*, 1973; Mizejewski *et al.*, 1979]), or because of inherent differences in the efficiencies of Cas9 loaded with different guide RNAs.

Cyp1A1 or *Arnt* was normally the second most prevalent target gene in all our transductions, but in one of our transductions we recovered cells harboring a transmembrane protein 37 (*Tmem37*)-targeting virus as the second most common integrant. This observation replicated after reselecting and resequencing selected cells from this transduction. However, no other *Tmem37*-targeting sgRNAs were recovered from any of our transductions. Thus, we deemed *Tmem37* to represent a false positive. We hypothesize that this *Tmem37* virus was co-transduced into a cell that had also been transduced with a

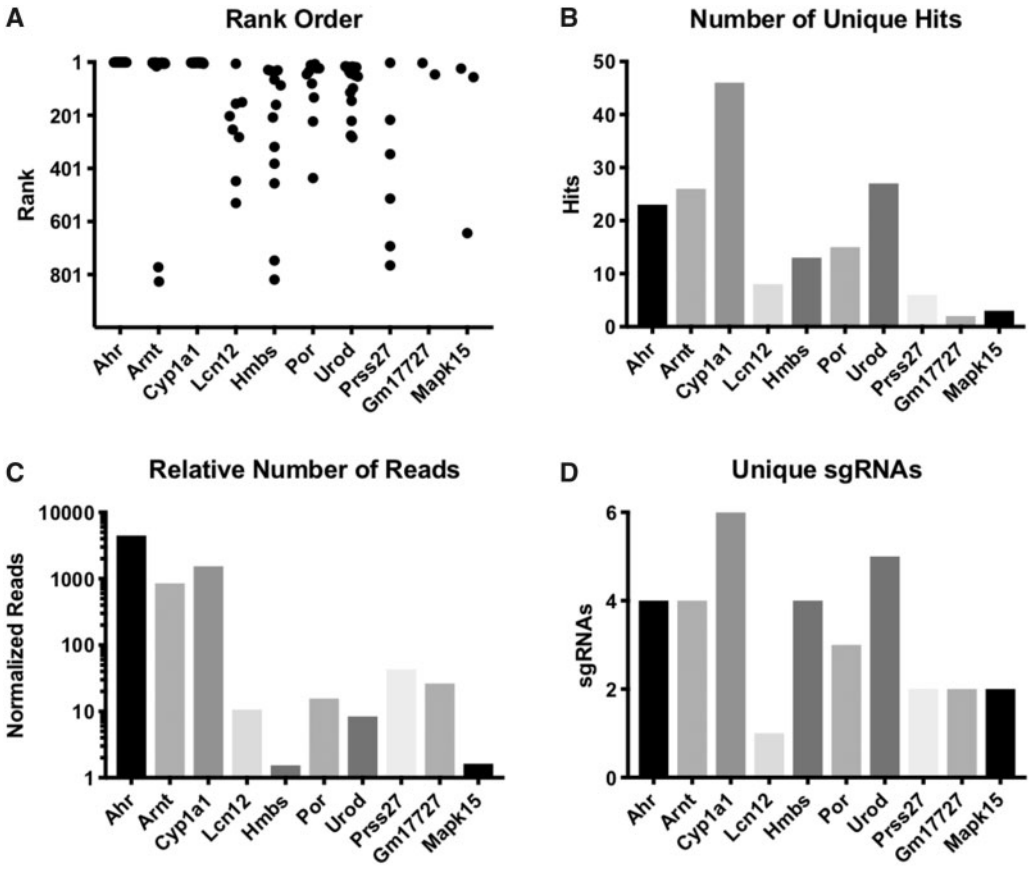

Figure 3. Gene targets identified from B[a]P screening of the GeCKOv2 library. Plotted together here are characteristics of the 10 most prevalent gene targets recovered from 19 independent transductions of Hepa1 cells transduced with 1 of the mouse GeCKOv2 sgRNA sublibraries (sublibrary A or sublibrary B). Panel (A) shows the rank order of the highest ranked sgRNA targeting the indicated gene from each of the 19 transductions. Panel (B) shows the total number of times that an sgRNA targeting the indicated gene was recovered from all 19 transductions. Panel (C) shows the relative normalized number of reads for all recovered sgRNAs targeting an indicated gene from all 19 transductions. Reads per gene target per transduction were normalized to a total of 10 000 reads per transduction and then averaged across all 19 transductions. Panel (D) shows the total number of unique sgRNA sequences targeting an indicated gene recovered across all 19 transductions.

virus targeting a *bona fide* AHR pathway gene, and that the *Tmem37* integrant was able to become prevalent by clonal expansion based on its association with an integrant that imparted B[a]P resistance. These observations highlighted the importance of assessing our findings based on the selection of sgRNAs from independent transductions rather than based on rank or prevalence alone.

Additional Candidate Hits

We noted that 3 of our novel gene targets were genes that are associated with cytochrome P450 biology. One was the gene encoding cytochrome P450 oxidoreductase (*Por*). The other 2 targets were genes that encode proteins in the heme biosynthesis pathway (*Hmbs* and *Urod*). Heme is an essential cofactor for the redox activity of cytochrome P450 proteins. We therefore searched for the presence of additional heme biosynthesis pathway genes that had been enriched in our screens that we might have missed in our initial analysis. Of the 6 other gene products involved in heme biosynthesis, delta-aminolevulinate synthase 1 and 2 (*Alas1*/*Alas2*), delta-aminolevulinic acid dehydratase (*Alad*), uroporphyrinogen III synthase (*Uros*), coproporphyrinogen oxidase (*Cpox*), protoporphyrinogen oxidase (*Ppox*), and ferrochelatase (*Fech*), we found multiple instances of sgRNAs targeting *Alas1*, *Cpox*, and *Fech*. In contrast, we only observed a single recovery of an sgRNA targeting *Alad* and recovered no sgRNAs that targeted *Uros* or *Ppox*. Figure 4 shows the rank order (panel 4A), the total number of hits per target (panel 4B), the total number of reads per target (panel 4C), and the number of sgRNAs recovered per gene (panel 4D) for each of the heme biosynthesis genes that we found. These data are shown in a similar manner to the data shown in Figure 3 and include the data previously shown in Figure 3 for *Hmbs* and *Urod*.

Validating Hits from the Screen

In order to validate the putative AHR pathway genes that our screens of the GeCKOv2 library uncovered, we performed reverse-genetics Cas9-generated knockouts of these genes and tested for the ability of these gene disruptions to elicit a B[a]P-resistant phenotype. For each gene, we synthesized *in vitro* transcribed sgRNAs and transfected these as Cas9 ribonucleoprotein (RNP) complexes into Hepa1 MC3 cells. We performed all sgRNA/Cas9 RNP transfections in parallel with transfections of *Ahr*, *Arnt*, and *Sin3A* sgRNA/Cas9 RNPs to control for efficient RNP delivery and gene editing. We also included sgRNAs consisting of the specific 20 nt sequences that had been enriched from our screens of the GeCKOv2 mouse libraries. Thus, multiple sgRNAs were made to each target gene. After transfection, we grew cells for 4 days in order to allow for CRISPR-mediated gene deletion and for degradation of already existent target gene products, before plating cells to select for B[a]P-resistance clones.

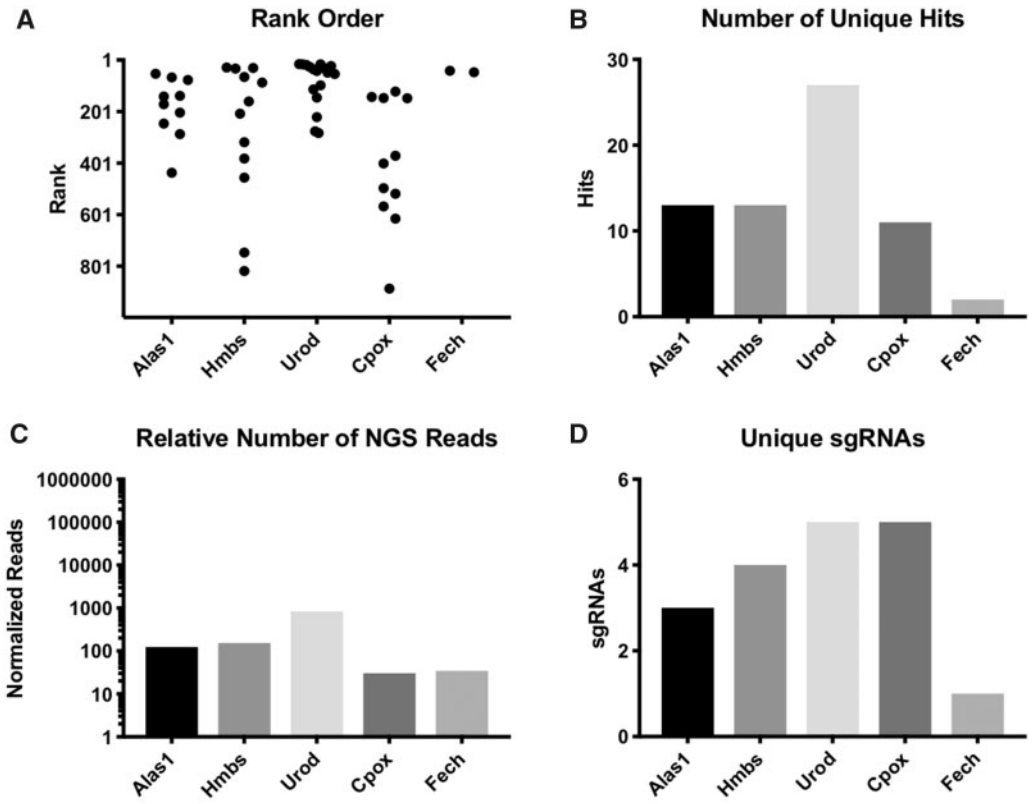

Figure 4. Heme biosynthesis genes identified from B[a]P screening of the GeCKOv2 library. Five of the 8y heme biosynthesis genes were identified from our screen of the GeCKOv2 library. The data shown for *Hmbs* and *Urod* were previously shown in Figure 2 but are included here for comparison. Panel (A) shows the rank order of the highest ranked sgRNA targeting the indicated gene from each of the 19 transductions. Panel (B) shows the total number of times that an sgRNA targeting the indicated gene was recovered from all 19 transductions. Panel (C) shows the relative normalized number of reads for all recovered sgRNAs targeting an indicated gene from all 19 transductions. Reads per gene target per transduction were normalized to a total of 1 000 000 reads per transduction and then averaged across all 19 transductions. Panel (D) shows the total number of unique sgRNA sequences targeting an indicated gene recovered across all 19 transductions.

sgRNAs targeting *Por*, *Sin3a*, *Ahr*, and *Arnt* generated B[a]P-resistant colonies, but none of the other sgRNAs that we tested by this method (*Lcn12, Prss27, Gm17727, Mapk15*, *Alas1*, *Alad*, *Hmbs*, *Uros*, *Urod, Cpox*, *Ppox*, *and Fech*) generated such colonies. *Urod*-targeted cells survived B[a]P-selection when grown under different growth conditions (*cf* "Role of heme biosynthesis in B[a]P toxicity,"). The *Por*-specific sgRNAs (both GeCKO and non-GeCKO sequences) generated robustly growing B[a]P-resistant colonies that were indistinguishable both in number and in morphology from those generated by the *Ahr* or *Arnt*-specific sgRNAs. In contrast, the *Sin3a* sgRNA generated B[a]P-resistant microcolonies. However, these colonies were present in approximately the same numbers as the colonies that arose from cells treated with the *Ahr*, *Arnt*, or *Por* sgRNAs, indicating that the respective sgRNAs functioned with similar efficiencies at directing Cas9-mediated gene editing. We included *Sin3A* as an additional positive control based on our earlier observations that targeting *Sin3A* effected a reduction of AHR-dependent induction of CYP1A1 (Solaimani *et al.*, 2014) and generated B[a]P-resistant cells. Nevertheless, we noted that cells targeted for *Sin3A* exhibited a profound growth defect regardless of the presence or absence of B[a]P. This growth defect was consistent with reports from others indicating that fibroblasts derived from *Sin3a*$^{-/-}$ mouse embryos grow poorly (Dannenberg *et al.*, 2005). In this sense, *Sin3A* served as a model of gene disruption that affects general cell growth parameters in addition to imparting B[a]P resistance.

Role of Heme Biosynthesis in B[a]P Toxicity

Directly targeting the heme biosynthesis genes that we identified from our screens using sgRNA/Cas9 RNPs failed to generate cells that exhibited B[a]P-resistant growth. However, we ascertained that our standard growth media provided a source of exogenous heme due to the 10% FBS that was added as a component of the media. To determine if reducing the quantities of heme in the growth media would allow heme biosynthesis mutants to survive B[a]P exposure, we selected cells transfected with *Alas1*, *Hmbs*, *Urod*, and *Cpox* sgRNA/Cas9 RNPs on Opti-MEM, 5% Nu-Serum IV (OptiMEM/NuSerum). The final heme concentrations in our growth media were determined to be $1.57 \pm 0.1\,\mu M$ in our standard media containing FBS versus $0.25 \pm 0.03\,\mu M$ in OptiMEM/NuSerum. Cells treated with *Alas1*, *Hmbs*, *Urod*, and *Cpox* sgRNAs all grew robustly in OptiMEM/NuSerum, but only cell that had been treated with *Urod* sgRNAs formed visible colonies after 10 days of growth when grown in OptiMEM/NuSerum plus B[a]P. The B[a]P-resistant colonies formed by *Urod* sgRNA-treated cells grown in OptiMEM/NuSerum/B[a]P consisted of poorly growing cells that were numerically and morphologically inferior to the poorly growing cells that we observed in colonies formed by *Sin3a* sgRNA/Cas9 RNP-treated cells.

We suspect that the reason that heme mutants were recovered at low frequency from our screen of the GeCKO library was 2-fold: (1) These mutants likely retained partial CYP1A1 activity due to scavenging of exogenous heme present in the growth media which, in turn, led to B[a]P toxicity and only partial B[a]P

Table 1. MTT-Normalized EROD Activity of Cells Disrupted for Heme Biosynthesis Genes

	Uninduced			Induced		
Target	Mean	SD	Two-tail *p*	Mean	SD	Two-tail *p*
Alas1	−8	10	.2560	5767	261	**.0110**
Hmbs	−15	20	.1812	3763	401	**<.0001**
Urod	−78	17	**.0003**	2588	488	**<.0001**
Cpox	−97	37	**.0022**	3101	676	**<.0001**
Ahr	−50	26	**.0112**	268	59	**<.0001**
MC3	9	21	.6474	6712	1197	.8995
Scrambled	3	13		6787	759	

Hepa1 cells transduced with CRISPR/Cas9 lentivirus targeting *Alas1*, *Hmbs*, *Urod*, or *Cpox* (or *Ahr* control) were tested for CYP1A1 activity by EROD assays in a 96-well plate format. Cells were uninduced or induced for 48 h with 10 nM TCDD. EROD readings from gene-targeted cells were normalized to readings from Hepa1 cells transduced with a CRISPR/Cas9 control virus expressing a nontargeting scrambled sgRNA, and final values were normalized to cell density per well determined by MTT assays of replica plates. Uninduced, $N = 4$. Induced, $N = 6$.

Bold indicates significance of < 0.05

resistance. (2) Because heme is a cofactor for many critical cellular functions, its exhaustion at later timepoints during the 10-day selection window may have negatively impacted the growth rates and overall survival of cells rendered deficient in heme biosynthesis.

Analysis of Inducible CYP1A1 Activity in Cells Disrupted for Heme Biosynthesis Genes

Based on our previous results, we wanted to measure inducible CYP1A1 activity in gene-targeted cells. We made lentivirus-based CRISPR/Cas9 gene targeting reagents using the lentiCRISPR v2 (Sanjana *et al.*, 2014) single virus system to target the 4 highest ranked heme biosynthesis genes (*Alas1*, *Hmbs*, *Urod*, and *Cpox*) by viral transduction. As opposed to the ribonucleoprotein approach that we used above, the use of a lentiviral approach for gene targeting provided a puromycin resistance marker that allowed us to select for cells that had been targeted. Thus, 2 days after viral transduction, we selected with puromycin and then allowed an additional 4 days of growth for gene knockout and loss of gene product. We used a cell-based, EROD assay (normalized for cell numbers between samples using MTT assay readings) to determine the CYP1A1 activity levels of mixed populations of gene-targeted cells grown in the presence and absence of TCDD, an AHR agonist. Table 1 shows the effects on CYP1A1 activity of targeting *Alas1*, *Hmbs*, *Urod*, *Cpox*, and *Ahr* by CRISPR/Cas9 lentiviral transduction. All 4 heme biosynthesis mutants exhibited reduced TCDD-inducible EROD activity when cells were grown in low heme media with the most dramatic reduction being exhibited by *Urod*. The *Ahr* positive control virtually eliminated inducible EROD activity in comparison to untransduced cells (Hepa1 MC3) and relative to cells transduced with control virus that expressed a scrambled, nontargeting, sgRNA. It should be noted that our *Ahr* sgRNA targeted the basic region of AHR which is essential for AHR function.

DISCUSSION

We identified 10 genes encoding proteins required for CYP1A1 induction or activity in hepatoma cells. Among these was *Por* which encodes cytochrome P450 reductase. Knockout of cytochrome P450 reductase in mice is lethal, whereas conditional knockout of *Por* in mouse liver has been shown to reduce activities of P450s (Gu *et al.*, 2003). Furthermore, knockout of intestinal *Por* in mice reduces metabolism of B[a]P (Fang and Zhang, 2010).

We also identified some but not all genes in the heme biosynthetic pathway. Some of the sgRNAs targeting these genes were identified at considerably lower frequencies than those for *Ahr*, *Arnt*, and *Cyp1A1*. One potential explanation of this is that cells knocked out for these genes had reduced viability even in the absence of benzo[a]pyrene. Another possibility is that heme found in the medium (provided by the fetal calf serum) partially rescued CYP1A1 activity in these cells leading to only partial B[a]P resistance. Notably, the reduced EROD activity that we observed for heme biosynthetic knockouts only reached the reported significance when cells were grown in low heme conditions.

Although we did not elucidate the mechanisms by which several of the other targets that we identified modified AHR pathway function, the means by which knockout of these genes allowed cells to survive B[a]P-selection in the GeCKO assay pose compelling questions for further study. For instance, *Lcn12* encodes a lipocalin. Lipocalin proteins are shown to bind small hydrophobic ligands (Suzuki *et al.*, 2004). *Mapk15* encodes a protein kinase that has been shown to have multiple distinct roles regulating processes that include autophagy, protein trafficking, and genomic integrity (Colecchia *et al.*, 2012; Groehler and Lannigan, 2010; Zacharogianni *et al.*, 2011).

In our previous siRNA screen, we identified 39 hits that reduced AHR-dependent CYP1A1 activity with a p value of $<.005$ and that were substantiated in a secondary screening. Several general transcription and translation factors and several transcriptional coactivators known to be required for AHR-dependent transcriptional activation of the CYP1A1 gene, were among the 39 genes so identified. Although the 9 different siRNAs we so tested knocked down expression of their mRNAs by an average of only 77% in our siRNA screening study (Solaimani *et al.*, 2013), the CRISPR-Cas9 system probably totally eliminates the functionality of target genes. This complete elimination of function probably explains why we did not identify these classes of genes in our CRISPR/Cas9 screen, because complete loss of activity of these proteins is incompatible with cell viability. Importantly, whereas our CRISPR/Cas 9 screen targeted nearly all the coding genes of the mouse, our siRNA screen only targeted about 1 quarter of these genes.

Interestingly, the genetic basis for the tolerance of certain populations of wild killifish to the toxic effects of pollutants mainly consisting of PAHs and “TCDD-like” polychlorinated compounds that bind the AHR has been investigated. Tolerance was ascribable to genetic and polymorphisms at the AHR, ARNT, CYP1A1, XAP, and HSP90 genes or combinations thereof. Thus, there was some overlap between the genes identified in the killifish study and identified in our CRISPR/Cas9 screen. Resistance in the fish study was associated in some cases with duplications of the CYP1A1 gene, rather than its inactivation (Reid *et al.*, 2016) as observed in our studies. The apparent ability of CYP1A1 to confer resistance to the pollutants in fish rather than sensitivity is probably related to the complex metabolism of PAHs in the whole organism.

Our CRISPR/Cas9 screen can potentially be modified in future experiments to identify additional genes required for AHR, ARNT, or CYP1A1 expression or function. For example, we could use a similar CRISPR/Cas9 screen to identify genes required for CYP1A1 induction in cells from other species and/or cells derived from other tissues. This may lead to the identification of a different suite of genes required for CYP1A1 induction, for

example, genes required uniquely in the human, or required in particular tissue types. To this end, we have found that the human hepatoma cell lines HepG2 and Hep3B exhibit similar sensitivity to B[a]P as Hepa-1 cells (unpublished). We have also found that HepG2 cells are very sensitive to TCDD toxicity (Yamaguchi and Hankinson, 2018), and selection with this agent after CRISPR/Cas9 library transduction could lead to the identification of genes specifically required for TCDD toxicity. Another very interesting approach would be to screen a CRISPR library of sgRNAs directed toward human long noncoding RNAs (lncRNAs), to identify any which may be involved in CYP1A1 induction. Such libraries are readily available. A potential approach to identify genes whose upregulation confers resistance to B[a]P (or TCDD) toxicity would be to screen a CRISPR Transcription Activation library, in which Cas9 is fused to a constitutive activation domain is recruited to the proximal promoters of known genes, thereby individually activating transcription of these genes. In this regard, it is interesting that the observations of Reid *et al.* suggest that upregulation of the phase II PAH-metabolizing enzyme glutalthione S-transferase theta-1 (GSTT1) as well as CYP1A1 can confer resistance to the industrial pollutants discussed above in wild populations of killifish (Reid *et al.*, 2016).

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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