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Genome-Wide RNAi High-Throughput Screen Identifies Proteins Necessary for the AHR-Dependent Induction of CYP1A1 by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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The aryl hydrocarbon receptor (AHR) has a plethora of physiological roles, and upon dysregulation, carcinogenesis can occur. One target gene of AHR encodes the xenobiotic and drug-metabolizing enzyme CYP1A1, which is inducible by the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) via the AHR. An siRNA library targeted against over 5600 gene candidates in the druggable genome was used to transfect mouse Hepa-1 cells, which were then treated with TCDD, and subsequently assayed for CYP1A1-dependent ethoxyresorufin-o-deethylase (EROD) activity. Following redundant siRNA activity (RSA) statistical analysis, we identified 93 hits that reduced EROD activity with a *p* value ≤ .005 and substantiated 39 of these as positive hits in a secondary screening using endoribonuclease-prepared siRNAs (esiRNAs). Twelve of the corresponding gene products were subsequently confirmed to be necessary for the induction of CYP1A1 messenger RNA by TCDD. None of the candidates were deficient in aryl hydrocarbon nuclear translocator expression. However 6 gene products including UBE2i, RAB40C, CRYGD, DCTN4, RBM5, and RAD50 are required for the expression of AHR as well as for induction of CYP1A1. We also found 2 gene products, ARMC8 and TCF20, to be required for the induction of CYP1A1, but our data are ambiguous as to whether they are required for the expression of AHR. In contrast, SIN3A, PDC, TMEM5, and CD9 are not required for AHR expression but are required for the induction of CYP1A1, implicating a direct role in *Cyp1a1* transcription. Our methods, although applied to *Cyp1a1*, could be modified for identifying proteins that regulate other inducible genes.

Key Words: CYP1A1; AHR; RNAi; high-throughput screening; TCDD; esiRNA.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor, which has been shown to have a plethora of physiological roles including those pertaining to development, the endocrine system, and the immune response (Fujii-Kuriyama and Kawajiri, 2010; Kawajiri and Fujii-Kuriyama, 2007). Toxicity mediated by AHR lies principally in the dysregulation of its target genes (Okey, 2007). *CYP1A1* in particular has been studied as the paradigm target gene of AHR ligand–mediated gene induction, and it is well recognized that xenobiotic metabolism by CYP1A1 can result in the conversion of procarcinogens into carcinogens and, depending upon the route of administration, can also protect certain organs against procarcinogen toxicity (Nebert *et al.*, 2000; Shi *et al.*, 2010; Uno *et al.*, 2004). Furthermore, in drug development, ligand activation of AHR has been shown to increase the expression and activity of several xenobiotic drug efflux pump target genes, including P-glycoprotein. The P-glycoprotein efflux transporter works at the blood-brain barrier and effectively reduces central nervous system (CNS) levels of therapeutic drugs (Wang *et al.*, 2011). Further characterization of the mechanism(s) of AHR-dependent induction of gene expression is an important research objective.

Although AHR is promiscuous with regard to ligand binding, some of the most prominent AHR ligands are environmental pollutants and toxicants, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the polycyclic aromatic hydrocarbon (PAH), benzo[a]pyrene (B[a]P). Humans encounter B[a]P from a wide range of sources in the environment including automobile exhaust, industrial by-products, charbroiled foods, and tobacco smoke (Ma and Lu, 2007). Upon ligand binding, AHR dissociates from its chaperone proteins and enters the nucleus where it forms a heterodimer with the aryl hydrocarbon nuclear translocator (ARNT) protein. The AHR/ARNT complex binds xenobiotic response element (XRE) sequences within genomic DNA and thereby activates expression of target genes (Hankinson, 2005; Monostory *et al.*, 2009; Nukaya and Bradfield, 2009). Adverse consequences can occur when the ensuing CYP1A1 protein acts to bioactivate PAHs to bay region epoxide metabolites that are highly reactive with DNA. Subsequent mutations can lead to carcinogenesis, making the

study of *CYP1A1* regulation a potentially important anticancer target (Androutsopoulos *et al.*, 2009). It is well accepted that additional studies are necessary for a complete understanding of the molecular events governing *CYP1A1* induction (Androutsopoulos *et al.*, 2009; Fujii-Kuriyama and Kawajiri, 2010; Hankinson, 2005).

In this study, we performed a high-throughput siRNA library screening to further characterize AHR ligand–mediated induction of *Cyp1a1* gene expression. With the use of an RNA interference (RNAi) library, we identified 93 proteins with a *p* value ≤ .005 whose expression appeared to be necessary for TCDD-mediated induction of CYP1A1-dependent ethoxyresorufin-o-deethylase (EROD) activity, and through a secondary screening using in-house produced endoribonuclease-prepared siRNAs (esiRNAs), we validated the results for 39 of these hits. Additional quantitative PCR (qPCR) analysis revealed 12 previously unidentified proteins, SIN3A, ARMC8, PDC, TCF20, TMEM5, CD9, UBE2i, RAB40C, CRYGD, DCTN4, RBM5, and RAD50, which are necessary for the induction of CYP1A1. These proteins are not required for expression of ARNT, and although UBE2i, RAB40C, CRYGD, DCTN4, RBM5, and RAD50 are required for expression of AHR (data for ARMC8 and TCF20 are ambiguous), we found that SIN3A, PDC, TMEM5, and CD9 are not required for expression or activity of AHR, implicating a likely direct role in transcription of the *Cyp1a1* gene.

MATERIALS AND METHODS

Cell culture and treatment. Hepa1c1c7 (Hepa-1) cells were grown as a monolayer in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 1% pen/strep, and 1% fungizone at 37°C in 5% CO_2. Cells were treated with TCDD dissolved in dimethyl sulfoxide (DMSO) for 24 h at a 10nM concentration, giving a final concentration of 0.01% DMSO.

Construction of esiRNAs. To generate templates for *in vitro* transcription, a 2-step PCR procedure was performed with Hepa-1 complementary DNA (cDNA) used as the primary PCR template. Primers for each esiRNA were chosen from the Web site RIDDLE (http://cluster-12.mpi-cbg.de/cgi-bin/riddle/search), and a T7 anchor tag (GGGCGGGT) was added at their 5′ ends to enable annealing of a T7 promoter primer in the second round of PCR. The 400–600 bp amplicon was then used for T7 RNA polymerase (NEB, Ipswich, Massachusetts) mediated *in vitro* transcription. PCR cycling conditions and reaction contents were performed as described (Fazzio *et al.*, 2008). Each double-stranded RNA (dsRNA) was digested with endoribonuclease RNaseIII to generate a pool of 21 bp fragments and then purified using a 2 filter purification mirVANA PARIS kit according to the manufacturer's protocols (Ambion/ABI, Foster City, California). The GST-RNaseIII cDNA was received in the pGEX-2T plasmid as a generous gift from Dun Yang at the University of California San Francisco (Yang *et al.*, 2002). Briefly, the plasmid was transformed into *Escherichia coli* strain BL21 for propagation, isolated with the PureLink Quick Plasmid Miniprep Kit (Life Technologies, Carlsbad, California) according to the manufacturer's instructions, and then digested with restriction enzymes to verify the presence of the GST-RNaseIII insert. For protein expression, the plasmid was transformed into TOP10 cells (Life Technologies) and GST-RNaseIII expression was induced by a 3-h Isopropyl β-D-1-thiogalactopyranoside (IPTG) treatment. RNaseIII was then purified using reduced glutathione beads (GE Healthcare Life Sciences).

Transfection of esiRNA or siRNA. Hepa-1 cells were reverse transfected with 25nM siRNA or 25nM esiRNA using Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's recommendations for 96-well, 6-well, and 10-cm dishes, as appropriate. In a reverse transfection, the cells are added to a dish that already contains the siRNA and Lipofectamine RNAiMax. In the 384 multiwell format, black plates with clear bottom (Greiner Bio-One, Monroe, North Carolina) were prespotted with 4 unique siRNAs for each target gene in the druggable genome, which included a kinase and a G-protein-coupled receptor (GPCR) library (Thermo Scientific Dharmacon siRNA Libraries, Lafayette, Colorado, catalog numbers GU-014600, GU-013600, and GU-013500). The druggable genome is defined by Thermo Scientific as genes that are considered potential targets for therapeutics according to recent publications and gene ontology databases. A duplicated set (in some cases, a quadruplicated set) of 4 siRNAs targeting CYP1A1 (Qiagen, Valencia, California) on each plate served as positive controls. Each well contained 2 μl of 0.5 pmol/μl (or 25nM) siRNA, and every plate was run in duplicate. RNAiMax was diluted in OptiMEM at a 1:100 dilution, and 10 μl was added to each well using a multidrop dispenser. After the diluted RNAiMax was allowed to incubate with the oligonucleotides for 10–20 min, 1500 Hepa-1 cells were seeded per well in a 20 μl volume.

EROD assay. To assay for CYP1A1 enzymatic activity, cells were treated with 10μM ethoxyresorufin and 500μM dicumarol (Sigma-Aldrich, St Louis, Missouri) in phenol red–free MEM supplemented with 10% FBS, 1% L-glutamate, and 1% pen/strep. Phenol red–free media reduces light scattering and background fluorescence, whereas dicumarol prevents further metabolic degradation of the fluorescent product, resorufin. Reactions were incubated for the optimized time of 45–60 min at 37°C, and resorufin related fluorescence (530ex/590em) was detected in a multiwell fluorescence reader (FlexStation 3, Molecular Devices).

Cell viability assays. Cells were lysed and subjected to an ATP-dependent cell viability assay for each esiRNA/siRNA examined. Cell Titer Glo reagent was used according to the manufacturers protocols (Promega, Madison, Wisconsin) in a 1:3 ratio of Cell Titer Glo to medium.

Quantitative real-time PCR. RNA was isolated using RNEasy Mini columns (Qiagen) according to the manufacturer's instructions. cDNA synthesis of 2 μg of total RNA was performed with Superscript III reverse transcriptase (Life Technologies) according to the manufacturer's protocols using random hexamer primers (Life Technologies). Synthesized cDNA was used as a template for qPCR at a 1:10 dilution and was performed using SYBR Green (Qiagen) according to standard protocols on an Applied Biosystems 7500 real-time PCR machine. Primers for qPCR were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, California) and were synthesized by Fisher Scientific, Inc.

Western blotting. Cellular lysates were resolved on a 4%–12% SDS gel in a minigel apparatus (Life Technologies) and transferred to a nitrocellulose membrane with a semidry apparatus (Thermo Scientific Pierce, Rockford, Illinois). Blots were then blocked with 3% nonfat milk and incubated with an antibody against CYP1A1 (Santa Cruz Biotechnology, Santa Cruz, California) at 1:200, AHR (Wang *et al.*, 2006) at 1:200, or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) at 1:2000. Donkey anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase were used as secondary antibodies at 1:10 000 or 1:5000, respectively. Horseradish peroxidase was then detected with a chemiluminescent kit (GE Healthcare, Piscataway, New Jersey), and densitometry calculations were performed using ImageJ software (NIH).

Transduction for EROD rescue. Retrovirus was made by cotransfection of 293T cells with the pMSCV-ires/Green Fluorescent Protein (GFP) retroviral expression vector (Wang *et al.*, 2007) (containing either no cDNA as an empty vector negative control or containing the indicated human cDNAs) and a pCL-Eco packaging plasmid, using the BioT (Bioland Scientific LLC, Paramount, California) transfection reagent. The following day, the 293T cells were treated with 10mM Na-butyrate for 9 h to enhance viral production, and after an additional 24 h, the viral suspension was harvested. Hepa-1 cells plated in 6-well culture dishes were treated with 2 ml of the viral suspension in

α-MEM supplemented with 8 µg/ml polybrene and then centrifuged at 37°C for 1.5 h at 2500 rpm. An additional 2 ml of fresh α-MEM was added, and after 48 h, the cells were trypsinized and plated in growth medium on a 10-cm plate. Once confluent, cells successfully transduced and stably expressing human cDNA were isolated by fluorescence-activated cell sorting (FACS) of GFP-expressing cells. Reverse transfections and EROD experiments were carried out as described above.
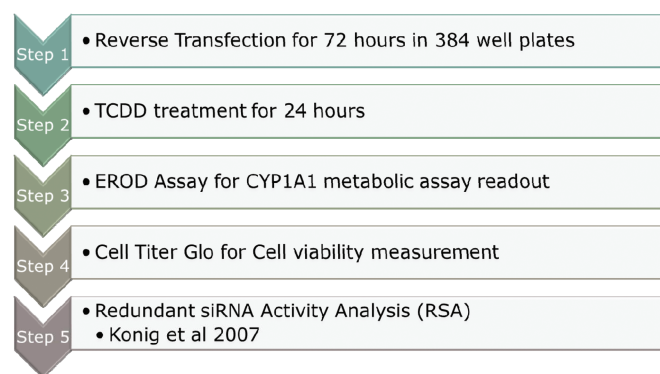
Transient transfections and reporter gene assay. Hepa-1 cells cultured in 6-well plates were cotransfected with siRNA and expression plasmids by using the Lipofectamine 2000 (Life Technologies) transfection reagent. The indicated siRNA was cotransfected with Renilla luciferase reporter (*pRL-TK*) (Promega) and firefly luciferase reporter (pGL-*CYP1A1*) (Beischlag *et al.*, 2002) constructs. The TK cDNA in *pRL-TK* is driven by the Herpes simplex virus thymidine kinase promoter, whereas the CYP1A1 cDNA in *pGL-CYP1A1* is driven by 4.2 kb of the 5′ upstream regulatory region of the rat *CYP1A1* gene, including its promoter and multiple XREs. Cells were harvested and lysed in passive lysis buffer, and luciferase activities were measured using the Dual-Luciferase system (Promega) according to the manufacturer's recommended protocols. Firefly luciferase activity was then normalized to Renilla luciferase activity.

Statistical analysis. Student's 2-tailed *t* test was performed to determine statistical significance, and data are presented as mean ± SD. Graphs and blots are representative of at least 2 separate experiments. Analysis of screening results was performed using the redundant siRNA activity (RSA) analysis algorithm, which was specifically designed for siRNA screens (Konig *et al.*, 2007). Briefly, relative fluorescence units (RFU) and relative luminescence units (RLU) from each well were normalized to their corresponding plate median to reduce variations in plate signals. The normalized RFU pertaining to CYP1A1 EROD activity was then normalized to its respective normalized RLU from Cell Titer Glo viability assays to account for differences in cell densities. A weighting factor (WK) was calculated to represent the quality of the assay on each plate using positive controls, negative controls, and average SDs. From this, a Score' was determined that utilized WK to calculate a weighted average of each siRNA from the duplicated plates. Utilizing a weighted average permitted that an siRNA would get a higher Score' when the assay on the plate was of greater quality. Lastly, Score' was scaled based on positive controls from the individual plates such that all positive control signals were 0.1 while keeping the plate median at 1.0. This further reduced plate signal variations and the resulting Score of each siRNA was used for gene ranking in the RSA algorithm with a cutoff threshold of values 3 times the average SD.

RESULTS

Development of an RNAi-Based Screen Identifies Proteins Potentially Necessary for Dioxin-Induced CYP1A1 EROD Activity

The basis of our procedure is that in 384-well plates, Hepa-1 cells are reverse transfected with siRNAs for 72 h, treated with TCDD for the last 24 h, and then assayed for CYP1A1-dependent EROD activity (Fig. 1). In the EROD assay, the chemical ethoxyresorufin is metabolized into the fluorescent product resorufin in the presence of CYP1A1, and thus the amount of resorufin can be measured as a relative indicator of CYP1A1 activity. Diminished levels of RFU indicate that the targeted gene is necessary for CYP1A1 EROD activity and thereby may reveal factors necessary for induction of *Cyp1a1* gene expression. The RNAi library we used contained 5600 gene targets of the mouse druggable genome, which included those encoding proteins for transcriptional regulation, GPCR

Step 1 • Reverse Transfection for 72 hours in 384 well plates

Step 2 • TCDD treatment for 24 hours

Step 3 • EROD Assay for CYP1A1 metabolic assay readout

Step 4 • Cell Titer Glo for Cell viability measurement

Step 5 • Redundant siRNA Activity Analysis (RSA)
• Konig et al 2007

FIG. 1. RNAi high-throughput screening methods. Methods used for the high-throughput screening are summarized in this flowchart. Abbreviations: EROD, ethoxyresorufin-o-deethylase; RNAi, RNA interference; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

signaling, cell cycle, transporters, nuclear receptors, translational regulators, enzymes, kinases, apoptosis signaling, antiapoptosis, transmembrane receptors, autophagy, and senescence. These gene candidates were chosen based on recent publications and bioinformatics that identified them as potential druggable targets (Thermo Scientific Dharmacon siRNA Libraries).

To ensure the most robust results and optimal methodology, we ran several preliminary RNAi experiments on the 384-well plates to calculate the quality of the assay (Z′) by comparing results from the 4 individual siCYP1A1 positive controls with mock negative controls (control plates). To this end, the effects of various cell densities on CYP1A1 EROD activity were determined (Supplementary Figure 1a), as well as the number of viable cells at these densities (Supplementary Figure 1b). The EROD signal and expansion in cell number increased approximately linearly up to a cell density of 1500 cells seeded per well. This number was thus determined to represent the optimal number to be used in the screening. The reproducibility of the assay between plates on the same day was established after performing technical replicates of our control plates, and the reproducibility of the assay between days was established after performing biological replicates of our control plates (Supplementary Figure 1c).

The data for the initial screening are shown in Supplementary Table 1. Mock and siCYP1A1 controls were run on each plate to determine the quality of the assay throughout the screening. The Z′ average for all the plates was 0.56. The incidence of false positives due to low cell density was diminished by normalizing EROD activity to cell viability for each well. Data were analyzed with the RSA analysis algorithm, which ranked each candidate gene based on the results from duplicated plates containing a group of 4 individual siRNAs (Konig *et al.*, 2007). The results from this screening revealed 93 hits that reduced TCDD-induced EROD activity from over 5600 candidates in the druggable genome with a *p* value ≤ .005 (logp of −2.26) exhibiting a diminished CYP1A1 EROD activity signal.

Validation of Screening Results With esiRNA Exposes Top Candidate Protein Hits Required for TCDD-Induced CYP1A1 EROD Activity and Messenger RNA Expression

To confirm our hits from the high-throughput assay, a secondary RNAi screen was performed in 96-well plates using esiRNA. esiRNAs are a pool of siRNAs targeting 1 gene transcript and are produced in-house with a 2-step PCR procedure using cDNA as a template, followed by *in vitro* transcription, and finally a digestion of the resulting dsRNA by an endoribonuclease into 21 bp fragments. During the production of the esiRNAs, agarose gels were run to determine the quality of the PCR products (Supplementary Figure 2a) and dsRNAs (Supplementary Figure 2b). RNaseIII-digested dsRNA was purified to collect the esiRNA and was run on a 6% nondenaturing polyacrylamide gel to assess their quality compared with a chemically synthesized Mitogen-activated protein Kinase (MapK) siRNA positive control (Supplementary Figure 2c). Poor quality or missing PCR/esiRNA products were omitted from the esiRNA screening, and the corresponding genes were followed up with individual siRNAs if necessary.

Once production of the esiRNAs was established, we performed validation experiments in 96-well plates (using mock negative control and esiCYP1A1 positive control treatments) to determine optimal cell density for both the maximal diminution of TCDD induction of EROD activity and for the minimal diminution in cell viability, using Z′ as a measure of the assay quality (data not shown). These experiments were necessary due to the fact that the 96-well plates contain much larger wells than the 384-well plates we used in the druggable genome screening. Optimal conditions were established at 5000 cells per well. Hepa-1 cells were reverse transfected with 25nM esiRNA for 72 h, treated with TCDD for the last 24 of these h, and assayed for CYP1A1 EROD activity. Diminished CYP1A1-dependent EROD activity was observed in esiCYP1A1 treatments at concentrations as low as 5nM, but maximum knockdown occurred at 25nM concentrations, and this concentration was therefore used in our validation experiments (data not shown).

The results of our initial assays on our 93 top hits are presented in Supplementary Table 2. Candidate genes with a single hit (1 hit of the 4 possible siRNA hits) in the original screen are at high risk of being false positives (Echeverri and Perrimon, 2006; Tiedemann *et al.*, 2010), and so most of such hits (18 of 22, see column H in Supplementary Table 2, light blue rows, tan rows, and teal rows) were excluded from the esiRNA screening and from further consideration. We were not successful in generating esiRNAs for 9 of the remaining 75 genes, and thus 66 of the 93 original hits were analyzed with esiRNAs. Hepa-1 cells treated with esiRNAs to each of these genes were analyzed for TCDD induction of EROD activity (Supplementary Table 2, column J), and 18 of these were also analyzed for TCDD induction of CYP1A1 messenger RNA (mRNA) (Supplementary Table 2, column K). Forty of the esiRNAs knocked down TCDD-induced EROD activity to a similar degree as the siRNAs knocked down EROD activity in the original screening. However, 26 of the esiRNAs were not as efficient at knocking down EROD activity as were the siRNAs in the original screening (column I in Supplementary Table 2, purple rows, red rows, and dark blue rows). These last genes were therefore excluded from further examination. In addition, 11 hits that exhibited a high cell death of >80% were removed from additional analysis (Supplementary Table 2, yellow rows and tan rows) due to the fact that cytotoxicity can cause alterations in the biochemistry of the cells and produce phenotypes different from the distinct phenotype resulting from the siRNA (Ovcharenko *et al.*, 2005). Sixteen of the genes tested with esiRNAs represented general transcription factors (eg, subunits of RNA polymerase II) or general translation factors and were excluded from further consideration. AHR, ARNT, and p300, which are known to be required for CYP1A1 induction (Hankinson, 2012), were also found among our top 93 hits and were excluded from further analysis. After completion of these various tests, 16 candidate genes remained (some genes were eliminated for more than 1 reason).

At this point, we added for further consideration an additional 4 potentially interesting gene products, CRYGD, DCTN4, RBM5, and RAD50, which had *p* values between .005 and .01 in the original screening, leading to 20 genes for follow-up. To further validate these genes, we treated Hepa-1 cells with the corresponding siRNA from the original screening that was most effective at reducing EROD activity and analyzed TCDD induction of EROD (Supplementary Table 2, column L) and CYP1A1 mRNA (Fig. 2a; Supplementary Table 2, column M). For 8 of the above 20 genes, the corresponding siRNA did not knock down EROD activity and/or CYP1A1 mRNA levels as efficiently as did the 4 siRNAs in the original screening or as did the corresponding esiRNAs. These genes were therefore excluded from further consideration. Five of the 8 gene products we excluded (CFC1, SORL1, CDC20, AGTR2, and PYGL) appeared from these last studies to be required for TCDD induction of EROD but not to be required or to be required to a lesser degree for TCDD induction of CYP1A1 mRNA and may represent genes that encode proteins required for the translation of CYP1A1 mRNA or that are required for EROD activity, such as cytochrome P450 reductase. This left the following 12 genes as likely to be involved in the induction of CYP1A1 mRNA: SIN3A, ARMC8, PDC, TCF20, TMEM5, CD9, UBE2i, RAB40C, CRYGD, DCTN4, RBM5, and RAD50 (Fig. 2a; Table 1; Supplementary Table 2, white rows). For 9 of these 12 gene products, we also examined the efficiency by which the siRNA knocked down the levels of its corresponding mRNA. Knockdown efficiencies for these 9 genes ranged between 54% and 94% and averaged 77% (Fig. 2b; Table 1; Supplementary Table 2, column R), thus confirming the efficiencies of their siRNAs.

Influence of the siRNAs on Induction of ALDH3A1 and NQO1 and on the Expression or Activity of ARNT and AHR

To shed light on mechanisms of action and determine whether our 12 candidates impact the induction of other AHR-inducible

(a)

(b)

(c)

(d)
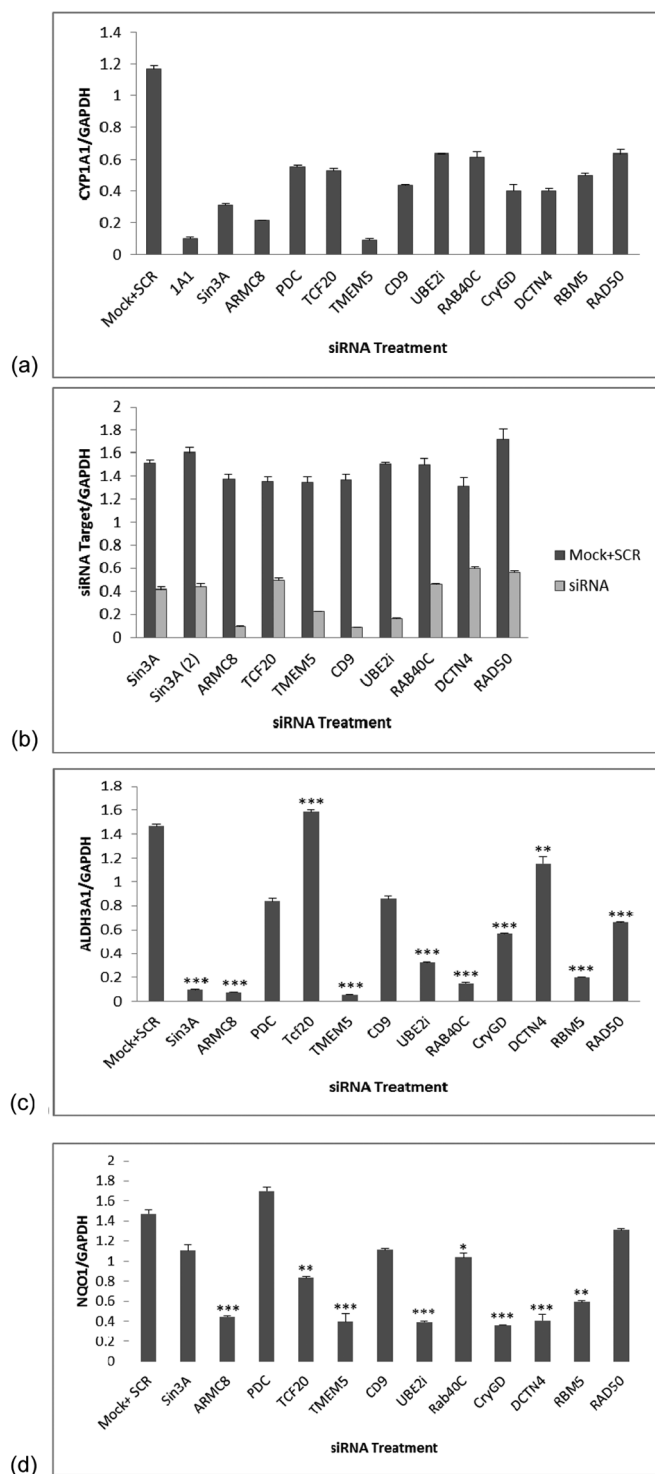
FIG. 2. Transcript levels of CYP1A1, siRNA targets, ALDH3A1, and NQO1 after siRNA-mediated knockdown and TCDD induction. The most efficient siRNA of the 4 used in the high-throughput screening was selected to use for future follow-up experiments. Effects each siRNA had on induced CYP1A1 mRNA expression (a), and efficiency of knockdown by individual siRNAs (b) was assessed by quantifying each corresponding targeted transcript. Effects each siRNA had on induced ALDH3A1 (c), and NQO1 (d) mRNA expression was also determined. Transcripts were measured using SYBR Green real-time

genes, we quantified transcript levels of ALDH3A1 (Fig. 2c; Table 1; Supplementary Table 2, column O) and NQO1 (Fig. 2d; Table 1; Supplementary Table 2, column N) after treatments with siRNA and TCDD. ALDH3A1 and NQO1 are part of the "AHR battery" of genes that are known for their inducibility by TCDD (Dewa *et al.*, 2008). The results from these studies revealed that ARMC8, TMEM5, RAB40C, UBE2i, CRYGD, and RBM5 are needed for induced expression of both ALDH3A1 and NQO1, which suggested that they may be required for the expression of AHR-inducible genes in general. The other hits did not appear to be required for the induction of either ALDH3A1 or NQO1, or both, suggesting that their deficiency may differentially affect the induction of these genes and of CYP1A1.

To investigate the possibility that the results we observed were downstream events due to effects on AHR or ARNT, we quantified AHR and ARNT mRNA levels after siRNA-mediated knockdown of our candidate genes (Table 1; Supplementary Table 2, columns P and Q). The transcript levels of ARNT were not markedly diminished by any siRNA. However, AHR mRNA was reduced to approximately the same degree as induced CYP1A1 mRNA levels when ARMC8, TCF20, UBE2i, RAB40C, CRYGD, DCTN4, RBM5, and RAD50 were knocked down (Table 1; Supplementary Table 2, column P), suggesting that these genes affect CYP1A1 induction via their requirement for AHR expression. To further test this notion, we next transduced Hepa-1 cells with a pMSCV-ires/GFP retroviral expression vector containing the human AHR cDNA. In previous studies, 89% of tested mouse genes were not targeted by the siRNAs against the corresponding human genes due to the DNA sequence divergence between mouse and human (Neumann *et al.*, 2010). In particular, the mouse-specific siRNA sequence was BLASTed against the human genome to ensure that human AHR expression would be unaffected. Isolated transduced cells were then transfected with siRNAs to certain genes of interest to see if the TCDD-induced CYP1A1-dependent EROD activity was rescued by overexpression of AHR (Fig. 3 illustrates this approach; PDC, UBE2i, and DCTN4 were not tested). Consistent with the observed decrease in AHR mRNA levels, AHR overexpression rescued TCDD-induced CYP1A1-dependent EROD activity in cells with diminished levels of ARMC8, RAB40C, CRYGD, RBM5, and RAD50 (Fig. 4; Supplementary Table 2, column U). On the contrary, EROD activity was not rescued by overexpression of AHR in the presence of siRNAs targeting SIN3A, TCF20, TMEM5, and CD9 (Fig. 4; Supplementary Table 2, column U). These results are also consistent with our observations for

PCR, and each sample was normalized to the constitutively expressed glycolytic GAPDH gene. All results shown on (a) and (b) are statistically significant with a p value ≤ .005 relative to mock and scrambled (SCR) negative controls. SCR refers to a scrambled siRNA negative control. *$p < .05$, **$p < .01$, and ***$p < .001$ compared with mock + SCR controls. Abbreviations: mRNA, messenger RNA; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

TABLE 1
Confirmed Hits Data Summary

Rank	Gene Product	Extended Gene Name	Function	EROD (%KD)	qPCR (%KD)						
					CYP1A1	siRNA Target Transcript	AHR	ARNT	NQO1	ALDH3A1
7	SIN3A	SIN3 homolog A	Transcription regulator	63	73	72/77	32	2	25	93
23	ARMC8	Armadillo repeat containing 8	Other	61	82	93	70	−109	70	95
31	PDC	Phosducin	Other	55	53		23	−2	−16	43
32	TCF20	Transcription factor 20 (AR1)	Transcription regulator	62	55	64	43	27	43	−8
54	TMEM5	Transmembrane protein 5	Other	77	93	84	49	−100	73	96
76	CD9	CD9 molecule	Other	65	63	94	−10	−13	56	90
87	UBE2i	Ubiquitin-conjugating enzyme E2I	Enzyme	70	46	89	55	−35	73	78
88	RAB40C	RAB40C, RAS oncogene family	Enzyme	58	47	70	52	−5	29	90
99	CRYGD	Crystallin, gamma D	Other	79	66		72	14	75	61
103	DCTN4	Dynactin 4 (p62)	Other	55	66	54	70	−95	72	21
113	RBM5	RNA binding motif protein 5	Other	50	58		54	−32	60	86
123	RAD50	RAD50 homolog	Enzyme	49	46		33	−7	11	55

A secondary screening confirmed the discovery of 12 novel proteins, which are necessary for TCDD-induced CYP1A1 EROD activity and mRNA expression. Additional qPCR experiments were performed to determine effects of siRNA on the expression of *Aldh3a1*, *Nqo1*, *AHR*, and *ARNT* mRNAs. Data from these experiments are expressed as percentage knockdown (%KD) compared with scrambled and mock siRNA controls.
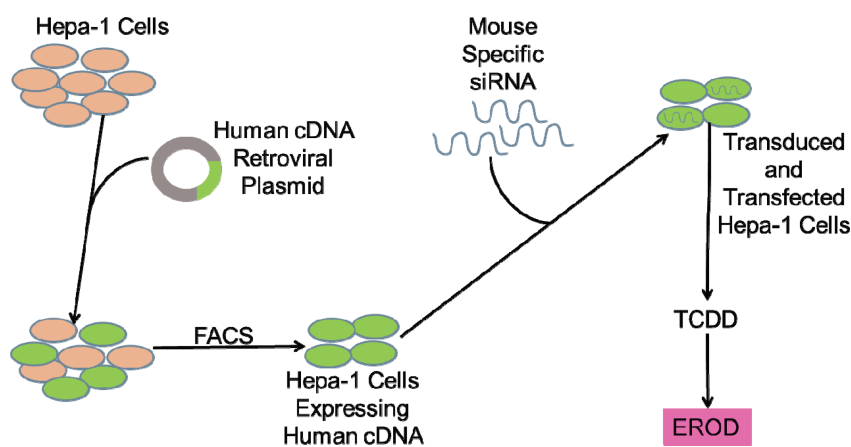

FIG. 3. EROD rescue methods. Hepa-1 cells were transduced with a retroviral pMSCV-ires/GFP vector expressing a human cDNA of interest, and GFP positive cells were isolated using fluorescent-activated cell sorting (FACS). Isolated cells were then transfected with siRNA directed at the mouse cDNA counterpart, treated with TCDD, and assayed for CYP1A1 EROD activity to see if the effects of the siRNA were rescued by overexpressing the cDNA. Abbreviations: cDNA, complementary DNA; EROD, ethoxyresorufin-o-deethylase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

SIN3A, TMEM5, and CD9 with regard to AHR mRNA levels. In the case of TCF20, we found that its expression is necessary for AHR mRNA expression, but overexpression of AHR was not sufficient to rescue EROD activity. Contradictions such as these are discussed later and also addressed in Table 3.

We performed additional studies on ARMC8, TMEM5, RAB40C, and CRYGD, consisting of densitometry analysis of Western blots to determine CYP1A1 and AHR protein levels after treatment with the corresponding siRNAs (Figs. 5 and 6; Supplementary Table 2, columns S and T). A decrease in AHR protein levels in all the TCDD-treated samples was observed, consistent with the observations of other investigators that

TCDD treatment leads to proteolysis of AHR (Davarinos and Pollenz, 1999; Giannone *et al.*, 1998; Ma *et al.*, 2000). Analysis of the CYP1A1 and AHR proteins revealed decreased expression of both proteins in the presence of siRAB40C and siC-RYGD (Figs. 6a and 6b; Supplementary Table 2, columns S and T), consistent with our observations that these siRNAs diminished the levels of both the corresponding mRNAs, and also diminished TCDD-inducible EROD activity, consistent with our observation that ectopic expression of AHR rescued TCDD-induced EROD activity in Hepa-1 cells treated with these siRNAs. Conversely, AHR protein levels were diminished less than induced CYP1A1 protein levels in samples treated
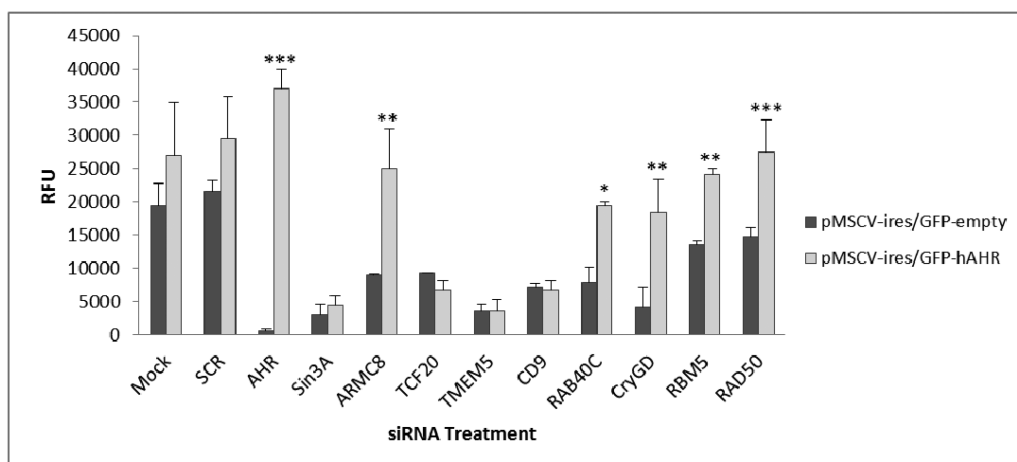
FIG. 4. Overexpresing AHR rescues EROD activity in Hepa-1 cells treated with certain siRNAs. Hepa-1 cells were transduced with a pMSCV-ires/GFP retroviral expression vector containing human cDNA to overexpress AHR, treated with siRNAs to the individual mouse genes, and subsequently treated with 10nM TCDD. CYP1A1 EROD activity was measured to determine if overexpression was enough to rescue CYP1A1 EROD activity in the presence of each siRNA (SCR refers to a scrambled siRNA negative control). $*p < .05$, $**p < .01$, and $***p < .001$ compared with empty vector control. Abbreviations: AHR, aryl hydrocarbon receptor; cDNA, complementary DNA; EROD, ethoxyresorufin-o-deethylase; RFU, relative fluorescence units; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.
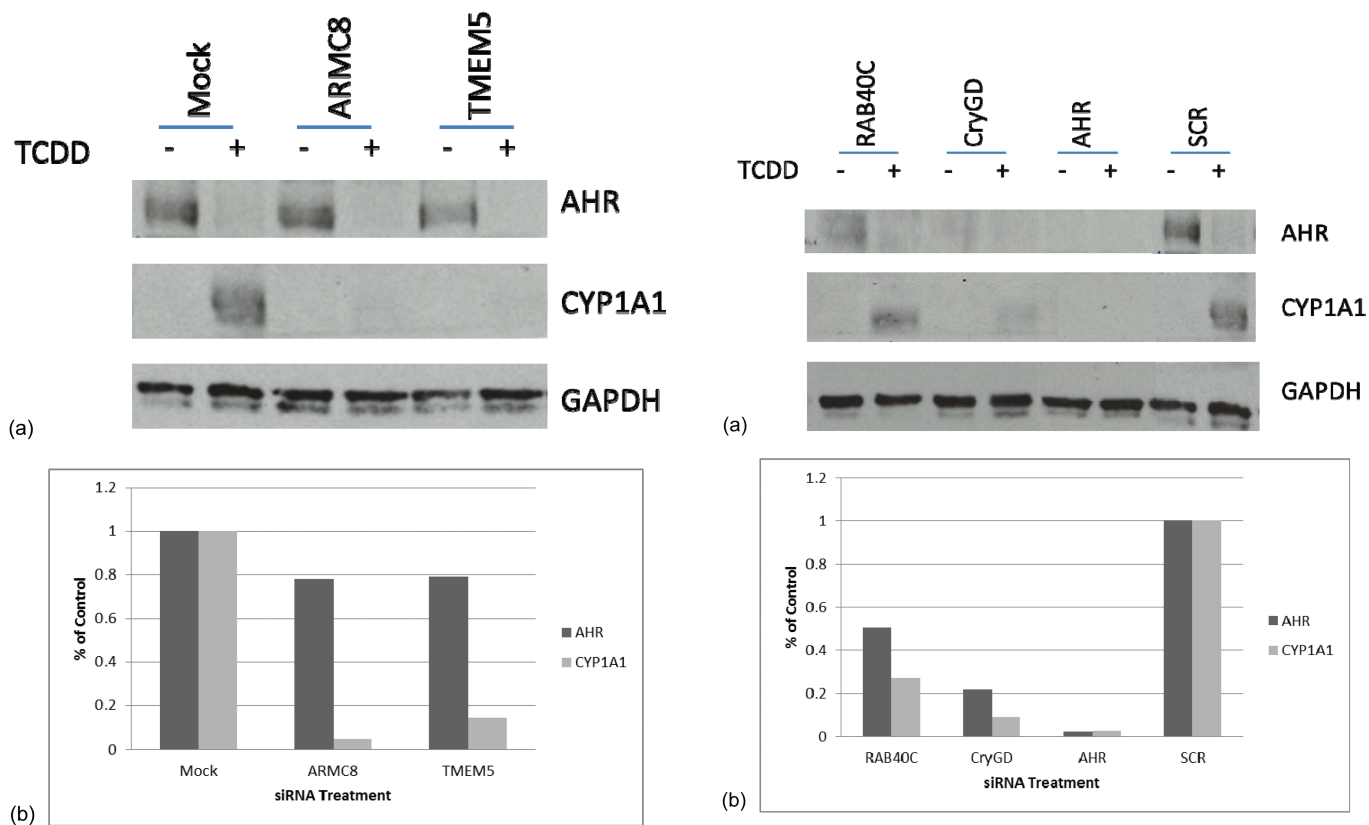
FIG. 5. ARMC8, SIN3A, and TMEM5 are necessary for TCDD-induced CYP1A1 protein expression but not for AHR protein expression. a, Western blots of CYP1A1 and AHR were performed after transfection of Hepa-1 cells with siRNAs to the indicated genes in the presence of DMSO (−) or TCDD (+) (SCR refers to a scrambled siRNA negative control). b, Densitometry quantification of Western blots (normalized to GAPDH) revealed the need for ARMC8, SIN3A, and TMEM5 in CYP1A1 but not AHR protein expression. Abbreviations: AHR, aryl hydrocarbon receptor; DMSO, dimethyl sulfoxide; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

FIG. 6. RAB40C and CRYGD are necessary for TCDD-induced CYP1A1 protein expression and AHR protein expression. a, Western blots of CYP1A1 and AHR were performed after transfection of Hepa-1 cells with siRNAs to the indicated genes in the presence of DMSO (−) or TCDD (+) (SCR refers to a scrambled siRNA negative control). b, Densitometry quantification of Western blots (normalized to GAPDH) revealed the need for RAB40C and CRYGD in both CYP1A1 and AHR protein expression. Abbreviations: AHR, aryl hydrocarbon receptor; DMSO, dimethyl sulfoxide; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

with siRNA targeting TMEM5 (Figs. 5a and 5b; Supplementary Table 2, columns S and T), consistent with our observations that this siRNA had only a modest effect on AHR mRNA levels and consistent with the observations that ectopic expression of AHR did not restore TCDD-induced EROD activity to Hepa-1 cells treated with these cDNAs. These data suggest that TMEM5 acts on the CYP1A1 gene directly and independently of any possible effect on AHR expression. The ARMC8 siRNA knocked down the TCDD-induced CYP1A1 protein levels but had a minimal effect on AHR protein levels (Supplementary Table 2, columns S and T). This suggests that ARMC8 also acts directly on the CYP1A1 gene and its effect is not mediated through AHR. However, as mentioned above, the siRNA to ARMC8 efficiently knocked down AHR mRNA levels (Supplementary Table 2, column P) and ectopic expression of AHR did rescue TCDD-inducible EROD activity in Hepa-1 cells treated with the ARMC8 siRNA (Fig. 4; Supplementary Table 2, column U), suggesting that the effect of ARMC8 on CYP1A1 induction is mediated via an effect on AHR. The data for ARMC8 are therefore not internally consistent, and the question as to whether the effect of ARMC8 on CYP1A1 induction is or is not mediated by effects on AHR remains to be determined.

Effect of Knocking Down Genes in Induction of a CYP1A1-Driven Reporter Gene

To further understand the mechanism by which our genes function in *Cyp1a1* induction and/or AHR expression, we cotransfected Hepa-1 cells with individual siRNAs to the genes in question and the pGL-*CYP1A1* firefly luciferase reporter construct. After 24 h of treatment with TCDD, we looked at luciferase expression, which was under control of the *CYP1A1* promoter, to determine if any particular gene candidate exerted its effects on the *CYP1A1* promoter and is thereby necessary for CYP1A1 expression (Fig. 7). When compared with pGL-*CYP1A1* cotransfected with either a mock negative control or siAHR and siARNT positive controls, we saw a significant influence on luciferase expression in all samples. The most remarkable decrease in luciferase activity was observed with siARMC8 and siCD9 treatments, whereas a more moderate decrease in luciferase expression was observed in samples treated with siPDC. Luciferase expression was diminished the least, or in some cases increased, in cells treated with siRNA targeting SIN3A, TMEM5, UBE2i, RAB40C, CRYGD, DCTN4, and RAD50. Because the pGL-*CYP1A1* construct is not in the native/endogenous chromatin configuration in transfected cells, an undiminished luciferase signal in the presence of an siRNA may indicate the need for the corresponding protein or its downstream effectors for chromatin remodeling, whereas a diminished luciferase signal may indicate a direct role for the protein or its downstream factors in binding 5′-flanking *CYP1A1* promoter elements. One might expect proteins that are necessary for AHR expression to display a diminished luciferase signal in these experiments, which was not always the case. However, a lack of effect of a particular siRNA could be because the induction of the luciferase reporter gene is more sensitive and therefore requires less of the corresponding protein than induction of the endogenous *Cyp1a1* gene.
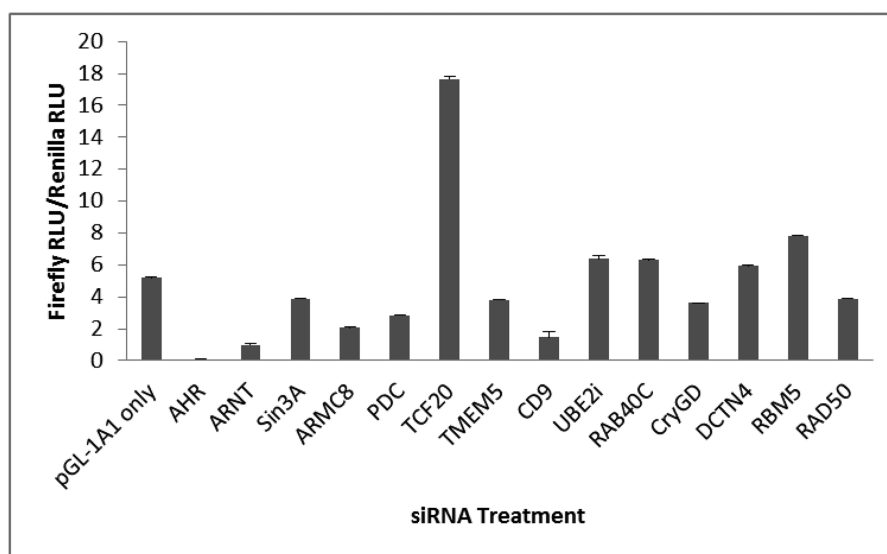
FIG. 7. Induction of luciferase activity by the pGL-CYP1A1 reporter construct in the presence of siRNAs to the individual genes of interest. Luciferase activity was measured in Hepa-1 cells that were cotransfected with pGL-*CYP1A1* and the indicated siRNAs to investigate the potential effects of the corresponding gene products of the *CYP1A1* promoter. Results revealed a significant influence of each siRNA on luciferase activity, with a most noticeable decrease for ARMC8 and CD9 compared with the untreated control. In the case of TCF20, there was a substantial increase in luciferase activity. Data are representative of 3 experiments and all results shown are statistically significant with a *p* value ≤ .001 relative to pGL-1A1 negative controls. Abbreviation: RLU, relative luminescence units.
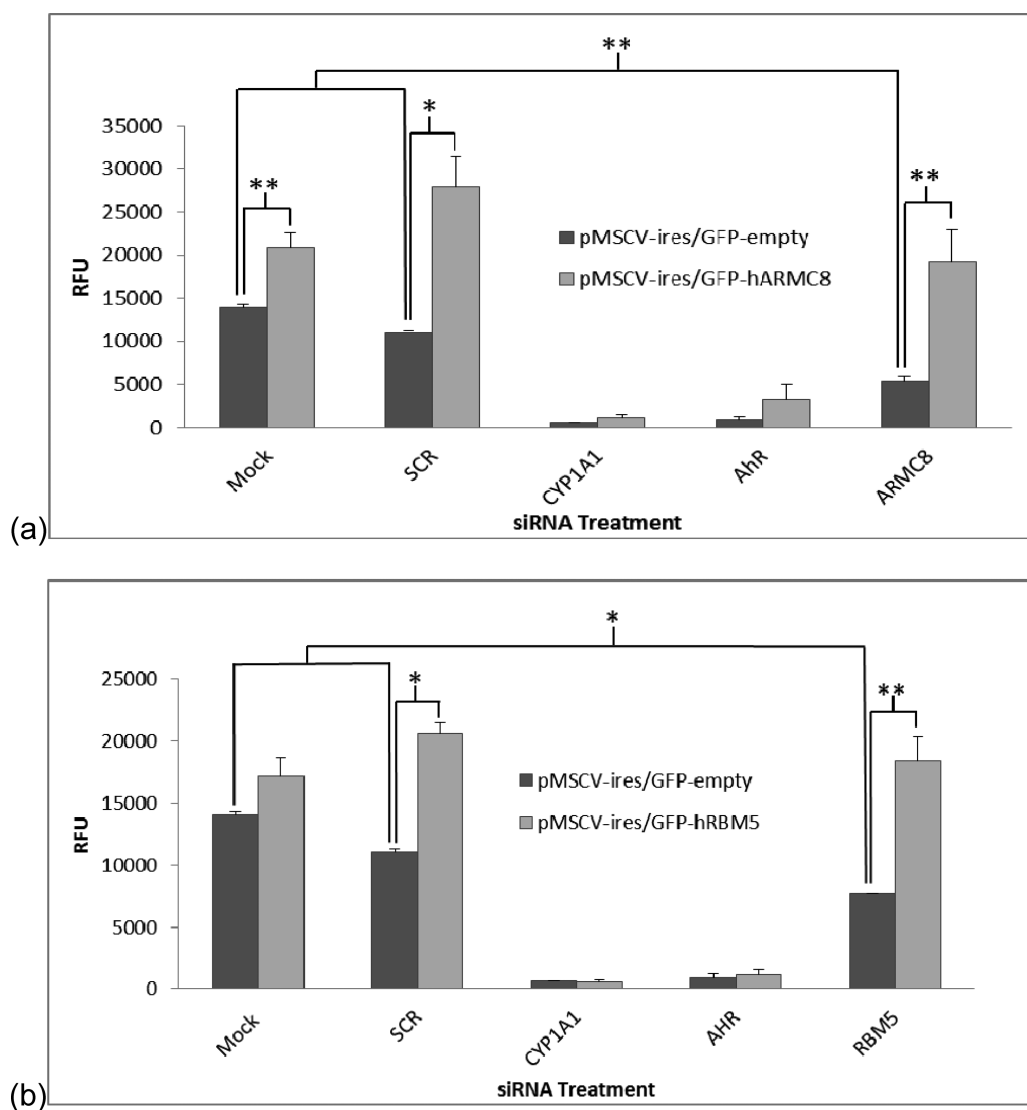
Ectopic Expression of ARMC8 and RBM5 Demonstrates That the Effects of the Corresponding siRNAs Are Not Due to Off-Target Effects and Confirms That These Gene Products Are Required for CYP1A1 Induction by TCDD

To exclude the possibility that the observed results with ARMC8 and RBM5 were due to off-target effects from the corresponding siRNAs, we transduced Hepa-1 cells with the pMSCV-ires/GFP retrovial expression vector containing human ARMC8, human RBM5 cDNA, or no insert to serve as a negative control, and isolated successfully transduced cells by FACS of GFP positive cells. These cells were then transfected with the murine siRNA counterparts. Murine siRNA sequences were BLASTed against the human genome to ensure that human ARMC8 or RBM5 expression would

be unaffected. Expression of both human ARMC8 (Fig. 8a) and human RBM5 (Fig. 8b) efficiently rescued EROD-induced levels in cells treated with the corresponding siRNAs when compared with empty vector controls. In addition, expressing ARMC8 or RBM5 in cells not transfected with the corresponding siRNAs significantly increased TCDD-induced CYP1A1 EROD activity relative to empty vector controls, further validating the involvement of these proteins in CYP1A1 expression.

DISCUSSION

In this study, we performed a series of optimization experiments to develop and implement procedures for an RNAi

FIG. 8. Ectopic expression of ARMC8 and RBM5 rescues CYP1A1 EROD activity. Hepa-1 cells were transduced with a pMSCV-ires/GFP retroviral expression vectors containing human cDNAs for ARMC8 (a) and RBM5 (b). Cells were subsequently treated with the corresponding siRNA followed by 10nM TCDD, and CYP1A1 EROD activity then was measured to determine if overexpression was enough to rescue CYP1A1 EROD activity in the presence of siRNA. The siRNAs to CYP1A1 and AHR served as positive controls. SCR refers to a scrambled siRNA negative control. $*p < .05$, $**p < .01$, and $***p < .001$ compared with empty vector control or compared with the mock and SCR controls combined. Abbreviations: AHR, aryl hydrocarbon receptor; cDNA, complementary DNA; EROD, ethoxyresorufin-o-deethylase; RFU, relative fluorescence units; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

high-throughput screening, which successfully allowed the discovery of novel gene products required for TCDD-induced CYP1A1 expression. High-throughput screenings have gained popularity, but few of these screenings have focused on transcriptional regulation. The application of our methods could be adapted to study the regulation of other inducible genes whose roles and regulations are not clearly understood.

In this study, we used the RSA algorithm that assigns a LogP value to a set of siRNAs targeting a particular gene and takes into account not only the quantity of hits but also the quality of the assay on each plate. This method of analysis for RNAi screens has proven to be more effective than standard ranking methods and has higher confirmation rates in secondary screenings (Konig *et al.*, 2007). In addition, we applied a stringent *p* value of ≤ .005 to minimize false positives to less than 1 in every 200 hits, although an additional 4 interesting candidates (*p* value ≤ .01), DCTN4, RBM5, and RAD50, which serve as nuclear proteins involved in DNA/RNA binding, and CRYGD, whose function is largely unknown, were later hand-selected for follow-up based on their potential relevance. As 1 step in validating/invalidating our screening results, we utilized esiRNAs, which offer an efficient, cost-effective, and specific alternative to siRNAs. esiRNAs have been shown to be just as effective if not more effective then synthesized siRNAs with regard to degree of silencing yet have a 12-fold reduction in off-target effects (Kittler *et al.*, 2007).

Many of the hits we identified were general transcriptional or translational factors (Supplementary Table 2), as well as known transcriptional regulators of CYP1A1 (Table 2). There were 9 known CYP1A1 regulators tested in our screen and for 7 of these, TBP (Beedanagari *et al.*, 2010), AHR, ARNT, eP300, BRG1, TRIP230, and ESR1 (Hankinson, 2012), the siRNAs in the original screening knocked down induced EROD activity with a *p* value < 0.1 (Table 2). Tan and coworkers (2004) also found that JNK was necessary for TCDD-stimulated CYP1A1 expression, which is consistent with our findings that JNK (*p* value = .02) is necessary for TCDD-induced CYP1A1 EROD activity (Supplementary Table 1). Even though general and known regulators were excluded from additional evaluation, their presence among our hits attests to the robustness of our screening procedures.

We next investigated which of our candidate hits are necessary for the induction of the CYP1A1 mRNA and in some cases, the CYP1A1 protein. From qPCR and/or Western blot densitometry analysis, we found 12 novel proteins that fit this category, namely SIN3A, ARMC8, PDC, TCF20, TMEM5, CD9, UBE2i, RAB40C, CRYGD, DCTN4, RBM5, and RAD50 (Table 1). It should be noted that all candidate genes we tested are expressed in Hepa-1 cells (except for PDC, which was not tested) as determined by microarray analysis performed by Dere and coworkers (2006, 2011). Our studies not only provided insight into the regulation of *Cyp1a1* but also the AHR-inducible genes, *Nqo1* and *Aldh3a1*, and *AhR* itself. Research

TABLE 2
RNAi High-Throughput Screening Results From Known CYP1A1 Transcriptional Regulators

	Gene Product	siRNA Hits	*p* Value	Rank
1	AHR	3	.001	29
2	eP300	4	.002	33
3	ARNT	4	.003	63
4	TBP	3	.007	107
5	BRG-1	2	.06	482
6	TRIP230	3	.09	791
7	ESR1	2	.09	805
8	BRCA1	2	.42	2618
9	MED1	1	.69	3448
10	CoCoA	Not available in library		
11	GAC63	Not available in library		
12	RIP 140	Plates contaminated		
13	NCOA1	Plates contaminated		
14	NCOA2	Plates contaminated		
15	NCO3	Plates contaminated		

All known regulators of CYP1A1 induction (Hankinson, 2012) were considered, and several were found to be a part of the druggable genome RNAi library. We therefore examined if the screening revealed their requirement in induction of CYP1A1 EROD activity. A total of 9 known regulators were screened, each of which demonstrated to have at least 1 siRNA hit out of the possible 4. The most statistically significant hits included AHR, eP300, and ARNT.

on AHR-mediated regulation has primarily focused on downstream target genes, so little information is available regarding the regulation of the *AhR* gene itself (Garrison and Denison, 2000). Although the levels of AHR mRNA are known to vary markedly in different tissues, to change during development, and to be altered in many tumors, very little is known about the mechanism(s) regulating these changes (Harper *et al.*, 2006). Our analysis indicated that UBE2i, RAB40C, CRYGD, DCTN4, RBM5, and RAD50 affect the expression of AHR, whereas SIN3A, PDC, TMEM5, and CD9 may have direct effects on the *Cyp1a1* gene. (The data for ARMC8 and TCF20 are ambiguous—see below.) Table 3 presents a summary of our data relating to the possibility the activity of each protein is mediated through its effect on AHR expression. We established that knocking down expression of TMEM5 and CD9 decreased the induction of both NQO1 and ALDH3A1 while having little or no influence on AHR expression, thus implicating a role for these proteins in the regulation of AHR-inducible genes in general. CRYGD and RBM5, which appear to be required for CYP1A1 induction via their involvement in AHR expression, were also necessary for NQO1 and ALDH3A1 induction. Although the data for each protein are generally internally consistent, this is not true in some cases where interpretation is less straightforward. For example, ARMC8 is necessary for both the expression of AHR mRNA and for TCDD induction of luciferase activity from the pGL-*CYP1A1* construct. Furthermore, in the presence of siARMC8, the human AHR cDNA rescued CYP1A1-dependent EROD activity. These results suggest that ARMC8 is necessary for AHR expression. However,

TABLE 3
AHR Data Summary

siRNA Target	Reduced Levels of AHR mRNA	Reduced Levels of AHR Protein	hAHR Expression Rescues EROD Induction
SIN3A	−		−
ARMC8	+	−	+
PDC	−		
TCF20	+/−		−
TMEM5	−/+	−	−
CD9	−		−
UBE2i	+		
RAB40C	+	+	+
CRYGD	+	+	+
DCTN4	+		
RBM5	+		+
RAD50	+/−		+

A summary table of experimental results addresses the potential that the activity of each protein is mediated through AHR. Data specify if the siRNA knockdown of each target gene affects (+) or does not affect (−) AHR mRNA expression or AHR protein expression and specify if the human AHR rescues (+) or does not rescue (−) EROD activity in the presence of the corresponding siRNA (Supplementary Table 2, columns P and M, respectively). In the first column, observations for AHR mRNA expression levels were relative to that of CYP1A1 mRNA expression levels in the presence of each siRNA and are defined as: − corresponding to the decrease in AHR mRNA levels being less than half the decrease in CYP1A1 mRNA levels; + corresponding to the decrease in AHR mRNA levels being 80% or more of the decrease in CYP1A1 mRNA levels; −/+ corresponding to AHR mRNA levels being between 50% and 65% of the decrease in CYP1A1 mRNA levels; and +/− corresponding to the decrease in AHR mRNA levels being between 65% and 80% of the decrease in CYP1A1 mRNA levels.

inconsistent with these observations is the fact that siARMC8 treatments did not lead to a reduction in AHR protein levels. Treatments with siTCF20 resulted in a diminution in expression of AHR mRNA, suggesting that TCF20 is also necessary for AHR expression. However, in the presence of siTCF20, human AHR cDNA did not rescue CYP1A1-dependent EROD activity, and we also saw a significant increase in the TCDD induction of luciferase activity from the pGL-*CYP1A1* construct suggesting, in contrast, that TCF20 is not involved in AHR expression.

To define the underlying biology behind our data and outline known pathways relevant in our screening, we analyzed our results with GeneGo, Inc. This software contains a compilation of data from published sources and therefore serves as a data mining tool for high-throughput screenings to identify the most relevant and known interactions between screening hits, as well as their resulting biological functions. This information can be used both for excluding hits and for determining the most relevant hits. As long as the p values are ≤ .05, it is recommended by the developers that approximately 300 hits be uploaded into GeneGo, Inc for analysis in order to deliver the most comprehensive networks. Supplementary Table 3 summarizes the most relevant pathway maps from our top 300 screening hits (p values ≤ .03). From these maps, we found 4 significantly relevant transcription-based pathways, the top 2 of which both contain one of our most promising hits, SIN3A. In addition

to these transcription-based pathways, our pathway analysis revealed that a calcium calmodulin–dependent kinase (Ca^{2+}/CaM/CaMKIα) pathway contained a significant number of hits, including MAPK11 (p value = .005 in the original screening) and MAP3K2 (p value = .01). Previous studies have shown that several MAP kinases are necessary for CYP1A1 induction (Tan *et al.*, 2002). However, these studies were performed using inhibitors that likely block several members of a kinase family simultaneously, whereas siRNAs only block 1 member, which means that one may not see an effect on CYP1A1 induction with an individual siRNA. The relevance of the Ca^{2+}/CaM/CaMKIα pathway as indicated from our hits is consistent with recent studies in a number of laboratories, which have identified that the upregulation of AHR target genes is dependent on the Ca^{2+}/CaM/CaMKIα pathway (Han *et al.*, 2009; Lin *et al.*, 2008; Monteiro *et al.*, 2008). Moreover, a recent kinome screening by Gilot *et al.* (2011) identified that 4 of their 22 kinase hits for proteins necessary for TCDD-induced CYP1A1-dependent EROD activity bind calmodulin. Seventeen of the 22 kinases Gilot and coworkers identified as hits were present in our siRNA library, and of the 17 kinases that we screened, 4 kinases, SPHK1, CCT2, AK3, and HIPK1, exhibited statistical significance, but because each had a .005 ≥ p value ≤ .05, we did not investigate them further. SPHK1 in particular was first isolated based on its calmodulin-binding ability (Olivera *et al.*, 1998).

Despite the overlap in the kinome portion of these studies, Gilot and coworkers screened only 712 candidate genes for TCDD-dependent EROD activity. Our screening covered nearly 5000 more targets. In addition, we performed extensive secondary screenings, which are necessary to validate potential hits irrespective of the statistical methodology that is employed (Birmingham *et al.*, 2009; Hirsch, 2010). Gilot and coworkers did not perform secondary screenings on their kinome hits nor did they analyze their most promising candidates nearly as thoroughly as we did. For example, they did not test their siRNAs on the transcript and protein levels of CYP1A1. EROD data are solely an indicator of protein activity and are not sufficient to determine the need of any particular gene for CYP1A1 expression. In addition, Gilot and coworkers did not explore the potential effects of their siRNA hits on AHR or ARNT expression. We performed such experiments and demonstrated that they are essential to discern between a direct involvement of a hit in the induction of AHR target genes or a by-product of effects on upstream events. Overall, our studies provide a more complete analysis than those of Gilot and coworkers and give valuable insight into proteins modulating the induction of CYP1A1.

Along with outlining known pathway maps, we used GeneGo, Inc software to identify networks of known interactions between our top 300 hits. This analysis revealed that PKC physically and functionally associates with one of our top proteins, CD9. Induction of PKC activity enhances TCDD-mediated CYP1A1 expression, whereas inhibition

of PKC blocks CYP1A1 induction (Carrier *et al.*, 1992; Chen and Tukey, 1996). However, the mechanism by which this occurs is still debated (Machemer and Tukey, 2005). CD9 helps in the association of PKC with specific integrins responsible for regulatory signaling, including gene induction (Akabane *et al.*, 2007; Zhang *et al.*, 2001), and this may be a mechanism by which CD9 influences CYP1A1 expression. It has also been determined that the phosphorylation and subsequent activation of our confirmed hit PDC is dependent on Ca^{2+}/CaM (Willardson *et al.*, 1996). Once active, PDC influences the activity of a known transcription factor (CRX) through direct binding and translocation of the complex into the nucleus (Zhu and Craft, 2000). Further experiments could help determine if PDC influences transcription of *Cyp1a1* in a similar manner by binding and thereby facilitating translocation of AHR. Although network analysis can only begin to help identify how each protein influences CYP1A1 expression, it does provide a valuable avenue for determining essential experiments that will define mechanisms of action.

In summary, we developed and implemented an RNAi highthroughput screening and successfully generated esiRNAs to use as a cost-effective tool for subsequent data validation. Our study has established that these procedures are effective methods for investigating the regulation of CYP1A1 induction. This is confirmed by the presence of known *CYP1A1* regulators as some of our top hits. We demonstrated that SIN3A, ARMC8, PDC, TCF20, TMEM5, CD9, UBE2i, RAB40C, CRYGD, DCTN4, RBM5, and RAD50 are cellular factors necessary for TCDD-dependent CYP1A1 mRNA expression, and upon additional examination, we found that 7 of these gene products, TCF20, UBE2i, RAB40C, CRYGD, DCTN4, RBM5, and RAD50, and also possibly ARMC8 are necessary for AHR expression. Our studies provide evidence that SIN3A, PDC, TMEM5, and CD9 are crucial for the induction of CYP1A1 independent of any effects on AHR expression or function. These proteins have not previously been implicated in CYP1A1 expression and as such this discovery provides novel insights into the AHR-mediated induction of gene expression.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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