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In Vivo Hepatic Enhancer Elements in the Human *ABCG2* Locus [S]

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ABSTRACT

ABCG2 encodes the mitoxantrone resistance protein (MXR; breast cancer resistance protein), an ATP-binding cassette (ABC) efflux membrane transporter. Computational analysis of the ~300 kb region of DNA surrounding *ABCG2* (chr4:88911376-89220011, hg19) identified 30 regions with potential cis-regulatory capabilities. These putative regulatory regions were tested for their enhancer and suppressor activity in a human liver cell line using luciferase reporter assays. The in vitro enhancer and suppressor assays identified four regions that decreased gene expression and five regions that increased expression >1.6-fold. Four of five human hepatic in vitro enhancers were confirmed as in vivo liver enhancers using the mouse hydrodynamic tail vein injection assay. Two of the in vivo liver enhancers (ABCG2RE1 and ABCG2RE9) responded to 17β-estradiol or rifampin in human cell lines, and ABCG2RE9 had ChIP-seq evidence to support the binding of several transcription factors and the transcriptional coactivator p300 in human hepatocytes. This study identified genomic regions surrounding human *ABCG2* that can function as regulatory elements, some with the capacity to alter gene expression upon environmental stimulus. The results from this research will drive future investigations of interindividual variation in ABCG2 expression and function that contribute to differences in drug response.

Introduction

The mitoxantrone resistance protein (MXR; breast cancer resistance protein/ABCG2) is an efflux membrane transporter that is expressed apically in selected tissues, including liver, kidney, breast, and intestine (Robey et al., 2009). Transport activity, tissue distribution, and cellular localization of MXR suggest that it plays a pivotal role in endogenous substrate disposition and protection from xenobiotics (Jonker et al., 2005; Noguchi et al., 2009; Robey et al., 2009). In the liver, MXR is highly expressed on the canalicular membrane, where it transports substrates and their conjugates into the bile, increasing their elimination

from the body (Maliepaard et al., 2001). Susceptibility to drug-induced side effects has been linked to hepatic MXR expression and coding and noncoding single nucleotide polymorphisms in *ABCG2*, the gene encoding MXR (Poonkuzhali et al., 2008; Mo and Zhang, 2012; Prasad et al., 2013). Therefore, understanding the mechanisms regulating hepatic expression of MXR is necessary to elucidate individual susceptibility to cancer progression and drug side effects.

ABCG2 expression varies between tissues (Maliepaard et al., 2001) and has significant tissue-specific variability, including in intestine (Zamber et al., 2003; Urquhart et al., 2008), liver (Poonkuzhali et al., 2008), and blast cells (Ross et al., 2000). The ability to alter phase I–III expression in response to xenobiotic or toxin exposure is an important mechanism for detoxification. ABCG2 expression is altered by many stimuli, including hypoxia (Cheng and To, 2012), inflammation (Pradhan et al., 2010), hormones (Zhang et al., 2006; Wang et al., 2008), and nutrients (Lemos et al., 2008, 2009). Additionally, ABCG2 expression is modulated by nuclear receptor ligands including rifampin and estrogen (Ee et al., 2004; Jigorel et al., 2006), indicating that several enhancer elements are important for altering ABCG2 expression in different contexts. Nuclear response elements (NREs) in the proximal promoter of *ABCG2* contribute minimally to ABCG2 expression since the methylation of a CpG island over the *ABCG2* promoter blocks the access of nuclear receptors to their recognition sequence (Wiench et al., 2011; Mo and Zhang, 2012). Additionally, nuclear receptors prefer binding to *cis*-elements over proximal promoters (Wiench et al., 2011), making *cis*-elements important for nuclear receptor response. With the availability of genetic and tissue expression databases, the ability to interpret and correlate expression data to genetic variation and drug

response or toxicity depends on the identification of constitutive and inducible regulatory elements (Smith et al., 2012). The studies described here test the hypothesis that there are *cis*-regulatory elements in the human *ABCG2* gene locus that are active in vivo and aim to identify and characterize those regulatory regions both in vitro and in vivo.

Liver-specific enhancers of transporter genes, such as *ABCB11* and *SLCO1A2*, have been identified by analysis of the evolutionary conservation and prediction of conserved transcription factor binding sites (TFBSs) (Pennacchio et al., 2007; Kim et al., 2011). Studies have focused on sequence conservation of noncoding regions because many developmental enhancers, tissue-specific enhancers and nuclear receptor response elements are unchanged through different species (Ahituv et al., 2004; Woolfe et al., 2005; Pennacchio et al., 2006, 2007; Loots, 2008; So et al., 2008). However, not all enhancer regions are conserved (King et al., 2007). Since *cis*-regulatory elements contain binding sites for multiple transcription factors, nonconserved regulatory elements could be identified by transcription factor clusters (So et al., 2007; Wang et al., 2007; Weltmeier and Borlak, 2011). Thus, identifying *cis*-regulatory elements, especially for tissue-specific absorption, distribution, metabolism, and excretion genes, could be achieved by combining complementary genomic data sets that include evolutionary conservation and both conserved and nonconserved TFBS clusters.

In this study, putative *cis*-regulatory elements in the *ABCG2* locus were identified by layered in silico analysis incorporating conserved sequence and TFBS predictions, combined with the clustering of nonconserved TFBSs. Regions with predictions for, or ChIP-seq evidence of, hepatic-specific transcription factor binding were prioritized. Putative regulatory regions were cloned into enhancer or suppressor luciferase vectors, which were previously validated for the identification of clinically correlated human liver enhancer variants (Kim et al., 2011; Matsson et al., 2012), and their activity was assayed in transiently transfected cell lines of liver, kidney, intestine, and breast origin. Positive in vitro enhancer elements were screened for in vivo liver-enhancer activity through hydrodynamic tail vein injection adapted for liver-enhancer screening (Liu et al., 1999; Kim et al., 2011; Kim and Ahituv, 2013). The ABCG2RE9-positive in vivo enhancer element and additional enhancers with predicted nuclear receptor binding were screened for their response to 17β-estradiol (E$_2$) or rifampin. Identified in vivo enhancers and in vitro suppressors in the *ABCG2* gene locus could be involved in tissue-specific or nuclear receptor–dependent expression of ABCG2.

Materials and Methods

Chemicals and Materials. The vectors pGL4.23 [*luc2*/minP], pGL4.74 [*hRluc*/TK], pGL4.13 [*luc2*/SV40], pGL3-promoter [*luc+*/SV40], and pGL4.11b [*luc2*P], and the Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). The human embryonic kidney (HEK293T/17), colorectal carcinoma (HCT116), hepatocellular carcinoma (HepG2), and breast adenocarcinoma (MCF-7) cell lines were purchased from the American Type Culture Collection (Manassas, VA). High-glucose Dulbecco's modified Eagle's medium, Opti-Minimal Essential Medium, and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Dimethylsulfoxide (DMSO), penicillin, and streptomycin were purchased from the University of California, San Francisco, Cell Culture Facility (San Francisco, CA). XhoI, Acc65I, NheI, and HindIII were purchased from New England BioLabs (Ipswich, MA). Rifampin, E$_2$, placental genomic DNA, and 10% charcoal-stripped fetal bovine serum (FBS) were all purchased from Sigma-Aldrich (St. Louis, MO). FBS (Axenia BioLogix, Dixon, CA), GenElute HP Endotoxin-Free Maxiprep Kits (Sigma-Aldrich), Improved Minimum Essential Medium without phenol red (Mediatech Inc, Manassas, VA), PolyJet DNA *In Vitro* Transfection Reagent (SignaGen Laboratories, Rockville, MD), TransIT EE *In Vivo* Gene Delivery System (Mirus Bio, Madison, WI), CD1 mice (Charles River Laboratories, Wilmington, MA), and PfuTurbo DNA Polymerase (Agilent Technologies, Santa Clara, CA) were purchased from the indicated manufacturers. The CYP3A4 xenobiotic responsive

enhancer module (XREM) (Goodwin et al., 1999) in the pGL4.23 [*luc2*/minP] vector and human pregnane X receptor (PXR) plasmid (hPXR)-pcDNA3.1 were a gift from Kathy Giacomini (University of California, San Francisco, San Francisco, CA).

In Silico Analysis of the ABCG2 Locus. The *ABCG2* gene locus, defined as a ~300,000-base pair (bp) region stretching one gene upstream (*PPM1K*) and downstream (*PKD2*) of *ABCG2* (chr4:88911376-89220011, hg19), was scanned for regions >100 bp and ≥70% conservation of human to mouse using the Evolutionary Conserved Region (ECR) and Vista browsers (Frazer et al., 2004; Ovcharenko et al., 2004). Conservation alignments from the ECR Browser were submitted to rVista for the identification of conserved TFBSs using all vertebrate transcription factor matrices from TRANSFAC professional (Wingender et al., 2000; Loots and Ovcharenko, 2004). Regardless of conservation, the *ABCG2* locus was examined for cluster regions of predicted TFBSs, using the default setting of the Cister program (Frith et al., 2001). Cister matrices used included preprogrammed matrices (TATA, Sp1, CRE, estrogen response element (ERE), Nf-1, E2F, Mef-2, Myf, CCAAT, AP-1, Ets, Myc, GATA, LSF, SRF, and Tef) and several additional matrices obtained from TRANSFAC for nuclear receptors, hepatocyte nuclear factors (HNFs), and HNF forkhead homologs (Supplemental Table 1) (Wingender et al., 2000). Regions consisting of repeat coding elements or gene promoters were eliminated from further analysis, and overlapping regions from the conservation and Cister plot analyses were combined into single putative regulatory regions.

The top 30 putative regulatory regions were determined based on their percentage of identity from human to mouse, the number of predicted TFBSs per length of region, and the total number of TFBSs, with extra weight given to TFBSs associated with *ABCG2* and liver or kidney gene expression (Supplemental Table 1). Extra weight was also given to regions with ChIP-seq data supporting the association with transcription factors, histone 3 lysine 27 acetylation, histone 3 lysine 4 methylation, and DNaseI sensitivity from TRANSFAC and the Encyclopedia of DNA Elements (ENCODE) databases (Wingender et al., 2000; ENCODE Project Consortium, 2011).

Cloning of Putative Regulatory Elements. Primers were designed for the top 30 putative regulatory regions with extensions added for the restriction sites Acc65I (forward primers) and XhoI (reverse primers) for all regions except ABCG2RE1, for which NheI (forward primer) and HindIII (reverse primer) were used, to ensure that the antistrand sequence of the enhancer element would be oriented in the same manner as the element is to the *ABCG2* promoter (Supplemental Table 2). The region of interest was amplified from human placenta genomic DNA using PfuTurbo DNA polymerase, gel purified, and cloned into the pGL4.23 luciferase vector. Endotoxin-free DNA for the selected enhancer plasmids, empty pGL4.23, *ApoE*-pGL4.23 (Simonet et al., 1993), pGL3-promoter with and without suppressor elements, pGL4.11b with and without the *ABCG2* promoter, pGL4.13, hPXR-pcDNA3.1, and pGL4.74 vectors (Supplemental Table 3) were isolated using the GenElute HP Endotoxin-Free Maxiprep Kit following the protocol of the manufacturer. For follow-up studies on suppressive elements, regions were reamplified from the pGL4.23 vectors and cloned, using the same restriction sites, into the pGL3-promoter vector. Plasmids were sequenced to verify their identity and orientation in the luciferase vectors.

Cell Culture and Transfections. HEK293T/17, HCT116, and HepG2 cell lines were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The MCF-7 cell line was grown in Improved Minimum Essential Medium without phenol red, supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin; for use in the nuclear receptor ligand assays, the 10% FBS was charcoal stripped. All cell lines were grown in a 5% CO$_2$ incubator at 37°C. For the in vitro luciferase assays, the HEK293T/17, HepG2, and HCT116 cells were seeded into a 96-well plate at 1.8×10^4 cells/well and transfected when they reached 80% confluency with 0.5 μL of Lipofectamine 2000 mixed with 80 ng of plasmid DNA (pGL4.23, Enhancer-pGL4.23, pGL3 promoter, Suppressor-pGL3 promoter, *ApoE*-pGL4.23, or pGL4.13) plus 20 ng of pGL4.74, following guidelines suggested in the protocol of the manufacturer. MCF-7 cells were seeded at 2.5×10^4 cells/well and transfected once they reached 95% confluency with 75 ng of *ABCG2* plasmid, 25 ng of pGL4.74, and 0.4 μL of PolyJet DNA *In Vitro* Transfection Reagent following the protocol of the manufacturer. Cells were lysed with passive lysis buffer 18–24 hours after transfection and measured for firefly and *Renilla* luciferase activity using the Dual-Luciferase Reporter Assay System in a GloMax 96 microplate Dual Injector Luminometer (Promega) following the protocol of the manufacturer. Each

experiment also included the empty pGL4.23 or pGL3-promoter vector as the negative control and the *ApoE*-pGL4.23 or pGL4.13 vector as the positive control. The activity for each plasmid was calculated as the ratio of its normalized firefly activity to that of the empty vector.

Mouse Hydrodynamic Tail Vein Enhancer Assay. Positive in vitro enhancer elements were screened for in vivo liver enhancer activity using the mouse hydrodynamic tail vein injection adapted for enhancer element screening (Liu et al., 1999; Kim et al., 2011; Kim and Ahituv, 2013). Each plasmid was injected into the tail vein of four to five mice using the TransIT EE *In Vivo* Gene Delivery System following the protocol of the manufacturer. Briefly, 10 μg of pGL4.23 vector with or without enhancer element, or the *ApoE*-positive control liver enhancer (Simonet et al., 1993), along with 2 μg of pGL4.74 were injected into the tail vein of male CD1 mice (Charles River Laboratories). After 24 hours, mice were euthanized, and their livers were harvested and homogenized in passive lysis buffer, followed by centrifugation at 4°C for 30 minutes at 21,000g. The supernatant was diluted 1:20 with lysis buffer and measured for firefly and *Renilla* luciferase activity using the dual-luciferase reporter assay system according to the protocol of the manufacturer in a Synergy 2 (BioTek Instruments, Winooski, VT) microplate reader. The enhancer firefly activity was normalized to the *Renilla* activity and expressed as fold activation relative to pGL4.23. All mouse work was performed following a protocol approved by the University of California, San Francisco, Institutional Animal Care and Use Committee.

Nuclear Receptor Ligand Treatment. The rifampin and E_2 assays were adapted from previously published protocols (Goodwin et al., 1999; Chen et al., 2004; Ee et al., 2004; Wang et al., 2006; Tan et al., 2011). A plasmid with the XREM region that induces CYP3A4 expression upon rifampin treatment and an *ABCG2* promoter construct that includes a known ERE were used as positive controls (Goodwin et al., 1999; Chen et al., 2004; Ee et al., 2004; Wang et al., 2006; Tan et al., 2011). HepG2 cells were transiently transfected as above with pGL4.23, Enhancer-pGL4.23, or XREM-pGL4.23 and cotransfected with hPXR-pcDNA3.1. Cells were treated for 24 hours with 25 μM rifampin or 0.1% DMSO before being assayed for luciferase activity. MCF-7 cells were transiently transfected as above with pGL4.23, Enhancer-pGL4.23, *ABCG2* promoter-pGL4.11b, or pGL4.11b. Cells were treated for 48 hours with 100 nM E_2 or 0.2% DMSO before being assayed for luciferase activity.

Statistical Analysis. Putative enhancer elements were considered to have statistically significant enhancer activity over the empty pGL4.23 vector activity if the analysis of variance (ANOVA) analysis, followed by a Bonferroni multiple comparison *t* test, had a *P* value <0.05 in each replicate experiment (three to six wells per plasmid). Elements with significant enhancer activity >1.6-fold above the empty vector were chosen for follow-up in vivo testing. For analysis of in vivo results, normalized enhancer activity from four to five mice per plasmid was compared with the empty pGL4.23 vector using a Student's *t* test and was considered positive in vivo at *P* < 0.05. Constructs were chosen for in vitro suppressor follow-up in the pGL3-promoter vector if they exhibited a 75% reduction in luciferase activity from empty vector in one cell line and at least a 50% reduction in an additional cell line. Results from each transfection (5–10 wells per plasmid) were analyzed with an ANOVA followed by a Bonferroni multiple comparison *t* test to compare suppressor constructs to the empty pGL3-promoter vector with significance determined if the *P* value was <0.05. All statistics were run using GraphPad Prism 5 software (GraphPad, San Diego, CA).

Results

Identification of High-Priority Putative cis-Regulatory Elements. A list of 30 high-priority elements (Table 1) was generated by the in silico analysis of the *ABCG2* gene locus. Figure 1 illustrates a snapshot of evolutionary conservation, the clustering of predicted *cis*-elements from Cister plot and ChIP-seq data available from ENCODE and TRANSFAC databases (Wingender et al., 2000; Frith et al., 2001; ENCODE Project Consortium, 2011). There were five regions that appeared in both the evolutionary conservation and Cister plot analyses, all of which were tested for enhancer activity. Also included were regions with preliminary ENCODE ChIP-seq data; for example, ABCG2RE8 had FOXA1 and GATA3 peaks in T-47D cells and p300 peaks in HeLa cells, and ABCG2RE23 had CTCF peaks in many cell

TABLE 1
High-priority putative liver enhancer regions in the *ABCG2* locus

Region	Genomic Position (hg19)	Length	Relative Luciferase Activity[1]	
			HepG2	In Vivo
ABCG2RE1	chr4:88923800-88924397	598	0.42↑	123*
			nt	nt
ABCG2RE2	chr4:88938903-88940086	1184	1.27	nt
			nt	nt
ABCG2RE3	chr4:88971418-88971681	264	0.23	nt
			1.77	nt
ABCG2RE4	chr4:88978037-88979518	1482	0.24	nt
			1.53	nt
ABCG2RE5	chr4:88988855-88989735	881	0.17	nt
			0.80	nt
ABCG2RE6	chr4:89010953-89011479	527	11.4*	2.45*
			nt	nt
ABCG2RE7	chr4:89015499-89016936	1438	0.13	nt
			0.19*	nt
ABCG2RE8	chr4:89025882-89026701	819	1.30	5.20*
			nt	nt
ABCG2RE9	chr4:89028437-89029406	969	2.41↑*	16.5*
			nt	nt
ABCG2RE10	chr4:89052340-89053333	994	0.35	nt
			nt	nt
ABCG2RE11	chr4:89056009-89057394	1386	0.17	nt
			0.67*	nt
ABCG2RE12	chr4:89062110-89062322	213	0.35	nt
			nt	nt
ABCG2RE13	chr4:89067439-89068791	1353	0.25	nt
			nt	nt
ABCG2RE14	chr4:89073171-89073422	251	1.63*	5.16*
			nt	nt
ABCG2RE15	chr4:89083363-89084199	837	1.15	nt
			nt	nt
ABCG2RE16	chr4:89088086-89089248	1163	0.17	nt
			0.84	nt
ABCG2RE17	chr4:89092791-89094046	1256	0.29	nt
			nt	nt
ABCG2RE18	chr4:89111990-89113522	1533	0.23	nt
			nt	nt
ABCG2RE19	chr4:89114835-89115893	1059	0.31	nt
			1.30	nt
ABCG2RE20	chr4:89117681-89117906	226	0.28	nt
			nt	nt
ABCG2RE21	chr4:89126078-89127298	1221	0.23	nt
			nt	nt
ABCG2RE22	chr4:89127612-89127877	266	2.37*	1.79
			nt	nt
ABCG2RE23	chr4:89132993-89133637	644	0.25	nt
			nt	nt
ABCG2RE24	chr4:89144816-89144989	174	0.37	nt
			nt	nt
ABCG2RE25	chr4:89163261-89164415	1155	0.23	0.80
			nt	nt
ABCG2RE26	chr4:89189503-89190458	956	2.98*	7.02*
			nt	nt
ABCG2RE27	chr4:89190822-89192730	1909	0.15	nt
			0.81	nt
ABCG2RE28	chr4:89197444-89198133	690	0.37	nt
			nt	nt
ABCG2RE29	chr4:89199069-89200406	1338	0.10	nt
			0.68*	nt
ABCG2RE30	chr4:89211846-89212768	923	0.29	1.2
			0.63*	nt

nt, not tested.
[1]Enhancer activity in pGL4.23 (unshaded) or suppressor activity in pGL3-promoter (shaded) expressed as the ratio of firefly to *Renilla* luciferase activity and normalized to the empty vector (pGL4.23 or pGL3-promoter). Values are the mean activities from a representative experiment with three to five wells/plasmid.
↑Activity is significantly increased after treatment with rifampin (HepG2).
*P < 0.05

lines, including HepG2 (Supplemental Fig. 1). The high-priority putative enhancer elements were located throughout the *ABCG2* gene locus and ranged from 174 to 1909 bp (Table 1). These regions were screened for in vitro enhancer activity.
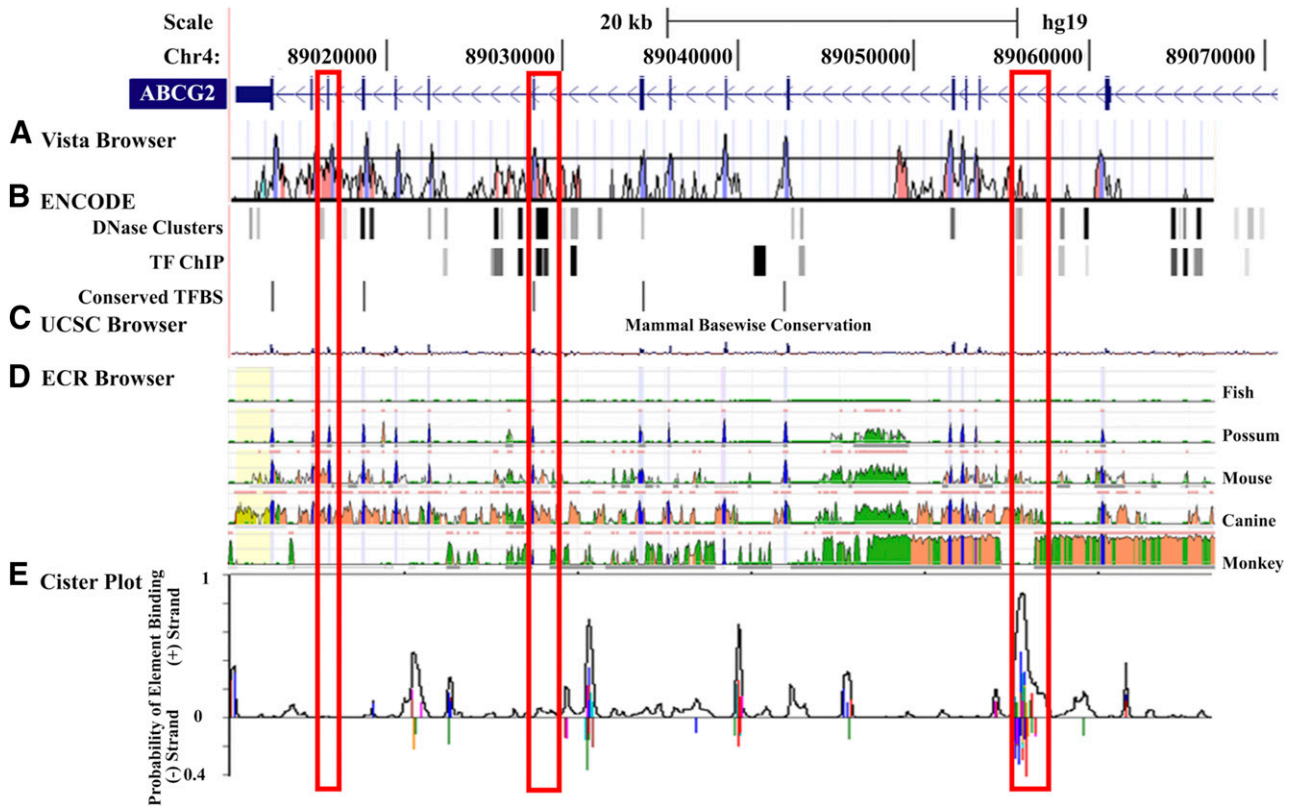
Fig. 1. Snapshot from *ABCG2* locus illustrating representative results from bioinformatic analyses. Red boxes indicate high-priority putative *cis*-regulatory regions that were chosen for further study based on conservation as determined by the Vista Browser (A); UCSC (University of California, Santa Cruz) Genome Browser mammal basewise conservation (C); or ECR browser conservation to fish, possum, mouse, canine, and monkey (D). Regions were also chosen based on clusters of transcription factor elements determined by Cister plot and overlap of these regions (E) with ENCODE DNaseI, ChIP-seq, and conserved TFBS data (B).

In Vitro Enhancer and Suppressor Identification. The 30 high-priority putative enhancer elements (Table 1) were transiently transfected into HepG2 (liver) cell lines, and their luciferase activity was quantified. Cells were cotransfected with *Renilla* as a transfection control, and in each experiment the empty vector (pGL4.23) and the pGL4.13-positive control vector were also transfected as a negative and positive control, respectively. The pGL4.13 vector had >1000-fold activity over the pGL4.23 vector (Fig. 2). Enhancers were binned according to their "strong" (≥4-fold activation), moderate (2- to 4-fold activation) and weak (1.5- to 2-fold activation) enhancer activities. Five regions had significant enhancer activity in the HepG2 cell line, with ABCG2RE6 having the strongest HepG2 enhancer activity (Fig. 2). The ABCG2RE9, ABCG2RE22, and ABCG2RE26 regions had moderate enhancer activity. The ABCG2RE14 region had weak enhancer activity. Since these elements have the potential to be enhancers in other tissues, they were also screened in HEK293T (kidney), HCT116 (intestine), and MCF-7 (breast) cell lines (Supplemental Fig. 2; Supplemental Table 2). Interestingly, the strongest enhancer in HepG2 cells (ABCG2RE6) was also the strongest enhancer in renal, intestinal, and breast cells. The ABCG2RE22 and ABCG2RE26 moderate liver enhancers also showed moderate to high enhancer activity in HEK293T cells. Based on HepG2 data, five regions with significantly increased enhancer activity >1.6-fold warranted in vivo follow-up. From the collective data across all of the cell lines, an additional four putative enhancer elements were also tested for in vivo activity.

Many of the 30 regions analyzed in the enhancer screen showed a significant decrease in luciferase activity (Fig. 2; Supplemental Fig. 2; Table 1). Nine regions exhibited a 75% decrease in luciferase activity in one cell line and at least a 50% decrease in two additional cell lines, and

these were selected for follow-up in a suppressor assay. The ABC-G2RE30 region, which was suppressive in two cell lines, was also chosen for follow-up because it exhibited enhancer activity in the renal cell line. HepG2 suppressor activity for 4 of 10 selected regions was confirmed by cloning into the pGL3-promoter vector, transfecting into
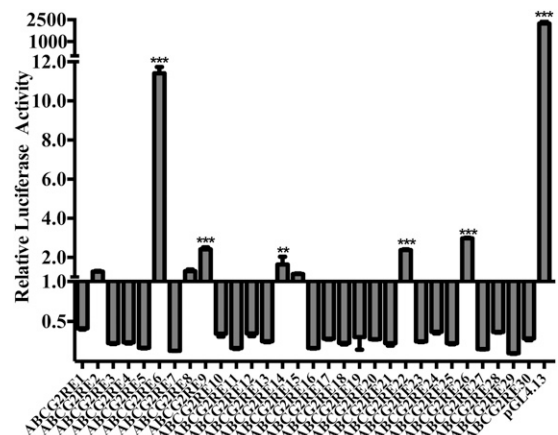

Fig. 2. Activity of putative hepatic enhancer elements in vitro. Luciferase activity was measured in the transiently transfected liver (HepG2) cell line. Enhancer activity is expressed as the ratio of firefly to *Renilla* luciferase activity normalized to the empty vector activity (pGL4.23). ECRs are displayed respective to their genomic orientation. Data are expressed as the mean ± SEM from a representative experiment (*n* = 3 wells per plasmid). Differences between putative enhancer elements and empty vector were tested by an ANOVA followed by a post hoc Bonferroni multiple comparison *t* test: ***P < 0.0001, **P < 0.001.

the HepG2 cell line, and measuring the resulting luciferase activity relative to empty vector (Fig. 3). pGL3-promoter is a firefly luciferase vector that is designed to accept a putative suppressor element upstream of the strong SV40 promoter; without a suppressor, this promoter drives strong expression of luciferase. Since regions could potentially have tissue-specific suppressor activity, they were also screened in kidney, intestinal, and breast cell lines. ABCG2RE7 was the strongest and most consistent suppressor, with a >75% decrease in luciferase activity in HepG2 and HCT116 cells. In HEK293T and MCF-7 cells, ABCG2RE7 also had a >50% decrease in luciferase activity (Supplemental Fig. 3). The ABCG2RE11, ABCG2RE29, and ABCG2RE30 also had significant suppressor activity in the HepG2 cell line. Eleven genomic regions (37%) showed enhancer activity, and 7 showed suppressor activity (23%) in at least one of the four cell lines, including ABCG2RE30, which had enhancer activity in HEK293 cells and suppressor activity in the HepG2 and HCT116 cell lines. Thus, a total of 17 regions (57%) had significant regulatory activity in vitro.

In Vivo Enhancer Activity. The five HepG2 in vitro enhancers were tested for in vivo activity in mice using the hydrodynamic tail vein injection technique (Kim and Ahituv, 2013). The *ApoE* liver enhancer (Simonet et al., 1993; Kim et al., 2011), previously shown to be a strong enhancer in vivo and an effective control for the hydrodynamic tail vein injection, had >40-fold enhancer activity (Fig. 4). Of the five HepG2 enhancer elements tested in vivo, four of them had significant enhancer activity (Fig. 4, Table 1), giving an 80% rate of positive in vivo activity when picking enhancers based on in vitro HepG2 enhancer activity. The ABCG2RE6 was the weakest enhancer in vivo, showing 2.45-fold activation. ABCG2RE14 and ABCG2RE26 had ~7-fold relative enhancer activation. Of the HepG2 putative elements, ABCG2RE9 was the strongest in vivo enhancer. Two of an additional four regions, screened in vitro based on their activity in other cell lines and predicted or ChIP-seq evidence of hepatic transcription factor binding, also had positive enhancer activity (Supplemental Fig. 4). Overall the strongest in vivo enhancer was the ABCG2RE1 region; it had consistent activation >120-fold, which is almost three times that of the positive *ApoE* control.

Nuclear Receptor Ligand Response. The ability of ABCG2RE9 activity to respond to the PXR ligand rifampin or an estrogen receptor (ER) ligand E_2 was tested in transiently transfected HepG2 and MCF-7

cells, respectively. ABCG2RE9 had a 3-fold induction of its basal enhancer activity with rifampin treatment (Fig. 5A; Table 1). The basal transcriptional activity of the ABCG2RE9 region was reduced ~50% upon treatment with E_2 (Fig. 5B; Table 1). Additionally, eight regions with both in vitro enhancer activity in at least one cell line and bioinformatic evidence that either PXR or ER were bound to it (data not shown) were tested for their response to rifampin and E_2. One additional enhancer region was responsive to rifampin, and three showed altered activity in response to E_2 (Supplemental Fig. 5).

Transcription Factor Binding via ChIP-Seq. The ABCG2RE9 enhancer has extensive transcriptional marks in ENCODE, including histone 3 lysine 4 methylation, DNaseI sensitivity clusters, and many transcription factor ChIP-seq peaks in HepG2, including HNF4α, HNF4γ, retinoid X receptor (RXR), and p300 (Fig. 6). Specifically for these studies, FOXA1, a dimer partner of ER and RXRα, binds to ABCG2RE9. A ChIP-seq experiment by our laboratories (Smith et al., 2014) showed that p300 binds ABCG2RE9 in human primary hepatocytes treated both with and without rifampin, providing strong evidence that this is a transcriptionally active region in human liver (Fig. 6). The ABCG2RE8 and ABCG2RE23 putative enhancer elements also have ENCODE ChIP-seq data supporting transcription factor binding to these regions (Supplemental Fig. 1).

Discussion

These studies provide strong evidence in support of ABCG2RE9 as a nuclear receptor responsive element and *cis*-regulatory enhancer. Aside from NREs in the *ABCG2* proximal promoter, very little is known about the transcriptional regulation of *ABCG2*. Regulatory control of MXR expression gives cells the ability to adapt to elevated or reduced levels of substrates. The identification of tissue-specific *cis*-regulatory elements of *ABCG2* could have implications for ABCG2 variation within those tissues and link clinical phenotypes with noncoding genetic variants. In the liver, MXR effluxes its substrates into the bile, where altered hepatic expression would affect drug elimination and thus pharmacokinetics. Characterizing hepatic regulation of ABCG2 is important in understanding how expression impacts both systemic and target site drug exposure.

In the present study, regulatory elements in the *ABCG2* gene locus were identified and characterized through in silico, in vitro, and in vivo
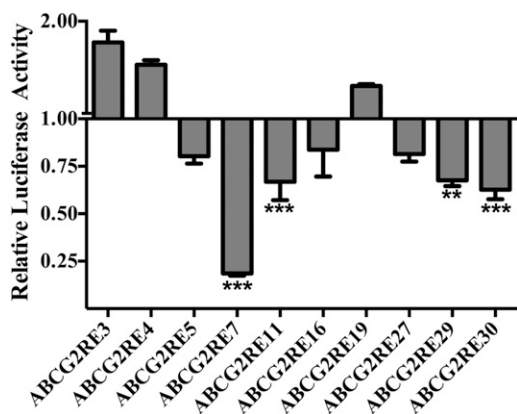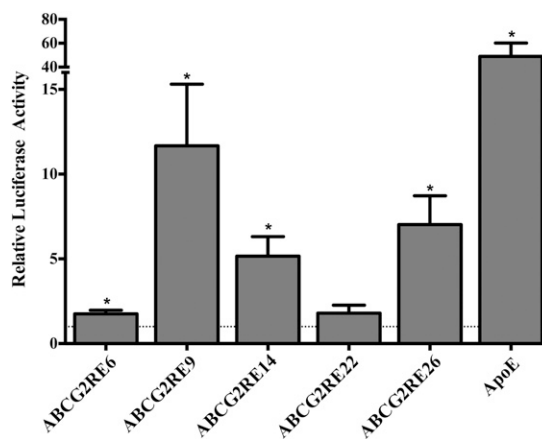
Fig. 3. Activity of putative hepatic suppressor elements in vitro. Luciferase activity of selected regions cloned into the pGL3-promoter vector and transiently transfected into the liver (HepG2) cell line. Suppressor activity is expressed as the ratio of firefly to *Renilla* luciferase activity normalized to the empty vector activity (pGL3-promoter). Data are expressed as the mean ± SEM from a representative experiment (n = 5 wells per plasmid). Differences between putative suppressor elements and empty vector were tested by an ANOVA followed by a post hoc Bonferroni multiple comparison t test: ***P < 0.0001, **P < 0.001.

Fig. 4. In vivo liver-enhancer activity in mice. Luciferase activity in liver homogenates was measured 24 hours after plasmid injection into the tail vein of mice. Enhancer activity is expressed as the ratio of the firefly to *Renilla* luciferase activity normalized to the empty vector activity (pGL4.23). Data are expressed as the mean ± SEM for four to five mice. Differences between enhancer elements and empty vector were tested by an unpaired Student's t test; *P < 0.05. The *ApoE* construct was injected as a positive control liver-specific enhancer (Kim and Ahituv, 2013).
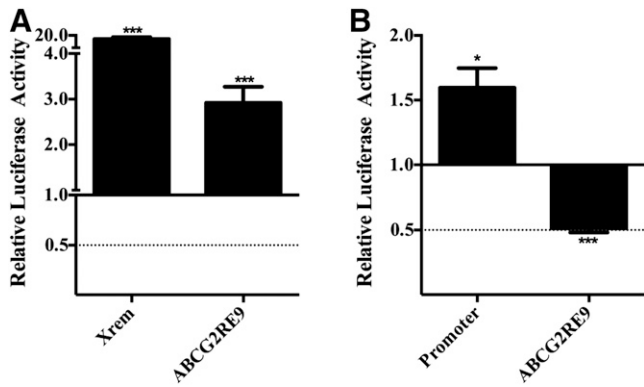
Fig. 5. Effect of rifampin and E_2 treatment on ABCG2RE9 enhancer activity. Luciferase activity of regulatory elements in transiently transfected liver (HepG2) cells 24 hours after 25 μM rifampin treatment and cotransfected with a human PXR plasmid (hPXR-pcDNA3.1) (A) and in breast (MCF-7) cells (B) 48 hours after 100 nM E_2 treatment. A known rifampin response element (XREM) and an ERE (*ABCG2* promoter) were used as positive controls (Goodwin et al., 1999; Chen et al., 2004; Ee et al., 2004; Wang et al., 2006; Tan et al., 2011). Enhancer activity is expressed as the ratio of firefly to *Renilla* activity in the presence of ligand to the same ratio after DMSO treatment. Data are expressed as the mean $\pm$ SEM from a representative experiment (n = 6–8 wells per treatment). Differences between the enhancer activity in the absence and presence of ligand were tested by an unpaired Student's t test: ***$P < 0.0001$, *$P < 0.05$.

methods. Through our in silico analysis, considering DNA and TFBS conservation between human and mice, transcription factor clustering, and ChIP-seq data, we chose 30 high-priority putative regulatory regions

to test for *cis*-regulatory activity. Starting from in silico predictions, five regions had enhancer activity and four regions had suppressor activity in the HepG2 cell line; overall, nine regions (30%) were identified with in vitro human hepatic regulatory activity. Besides the liver, MXR has variable expression in other tissues (Maliepaard et al., 2001), so it is possible that these regions have tissue-specific enhancer activity. When screened in additional cell lines, six more in vitro enhancers and three additional suppressors were identified.

The in vitro assays allowed the use of stringent criteria for selecting enhancer elements for in vivo follow-up. Based on cumulative cell line data, nine regions were screened in the in vivo hydrodynamic tail vein assay, and six regions (67%) had positive in vivo enhancer activity. Importantly, 80% (four of five regions) of the positive enhancer regions in HepG2 cells in vitro were confirmed as in vivo liver enhancers, suggesting a strong correlation between enhancer activity in human liver cell lines and mouse hepatic tissue. This high correlation may be biased by the use of DNA conservation and the presence of conserved consensus sequences from mouse to human as important selection criteria, and may miss human-specific regulatory elements. Of the 30 regions identified for characterization in vitro, 6 (20%) were positive in vivo liver enhancers. The in vivo success rate was 2-fold higher than a previous in vivo liver membrane transporter enhancer screen that used conservation and liver-specific TFBSs as screening criteria, suggesting that the addition of in vitro cell-based screening or consideration of ChIP-seq data could improve the selection process for in vivo enhancer assays (Kim et al., 2011). In general, enhancer activity in nonhepatic cells was not predictive of in vivo liver regulatory activity; one exception
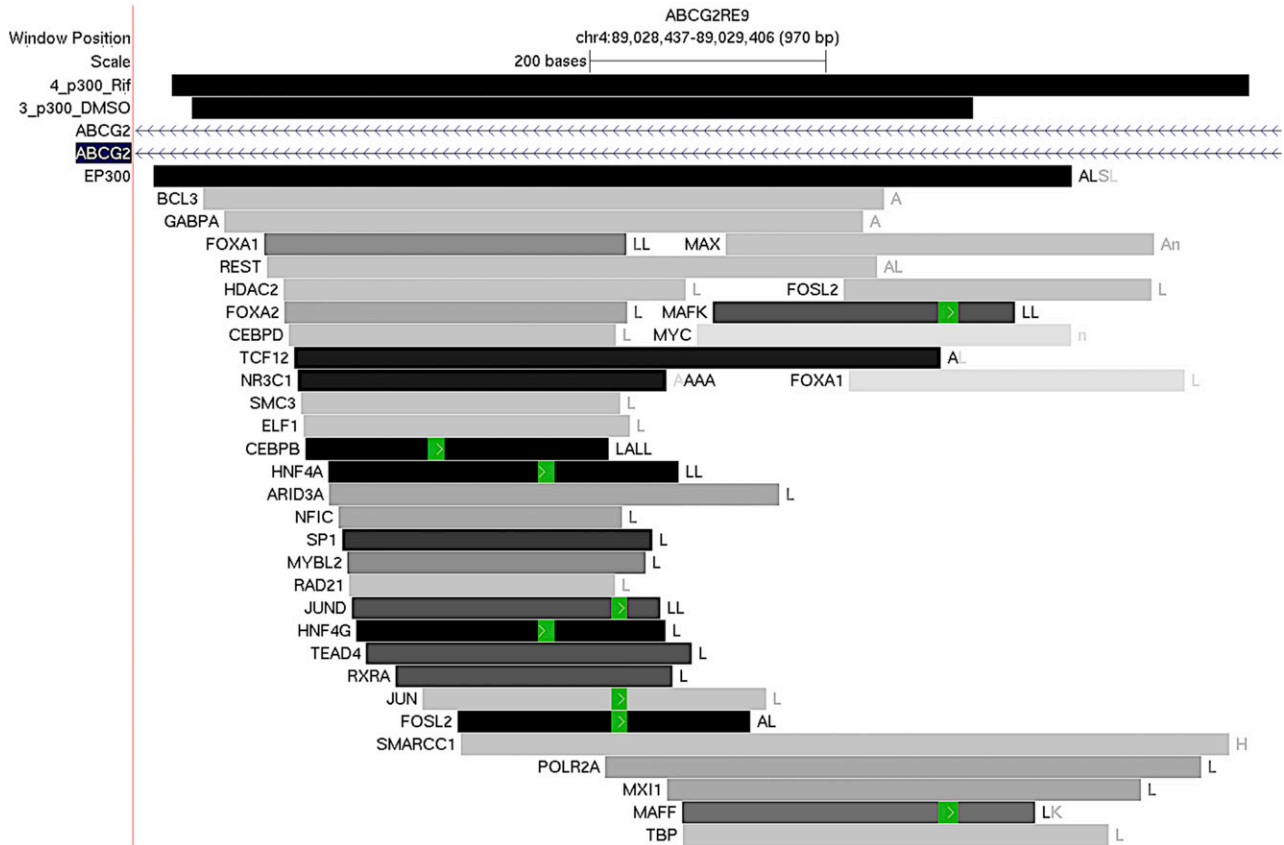

Fig. 6. ABCG2RE9 ChIP-seq peaks in human cell lines and hepatocytes. UCSC (University of California, Santa Cruz) Genome Browser snapshot of p300 ChIP-seq data from rifampin- and DMSO-treated hepatocytes (Smith et al., 2014) and ENCODE ChIP-seq data (ENCODE Project Consortium, 2011) within ABCG2RE9. The cell lines in which each of the peaks from ENCODE were found are identified as follows after each bar: A549 (A), HeLa (H), K562 (K), HepG2 (L), NB4 (n), and SK-N-HS with retinoic acid (S). Intensity of signal is indicated by the shade of gray, with black being the strongest. Green arrow boxes indicate the presence of a consensus motif for respective transcription factor. Transcription factors of note include FOXA1 (ER partner), NR3C1 (glucocorticoid receptor), HNF4α, HNF4γ, and RXRα, and p300.

was the strongest in vivo liver enhancer (ABCG2RE1), which was inactive in liver cells, but was active in kidney, intestine, and breast cell lines.

In vivo liver-enhancer activity ranged from >120-fold activation (ABCG2RE1), three times that of the positive *ApoE* control, to 2.45-fold (ABCG2RE6); the remaining enhancers showed 5- to 16.5-fold relative activation. This degree of activation is in the range of the strong enhancer elements previously discovered for membrane transporters (Kim et al., 2011). Three of the in vivo enhancers were within *ABCG2* introns [ABCG2RE8 (intron 10), ABCG2RE9 (intron 9), and ABCG2RE14 (intron 1)], which is consistent with the finding that 40% of enhancers are within intronic regions (Heintzman et al., 2009). An in vivo enhancer (ABCG2RE26) encompassing exon 4 of *PPM1K* was also identified. Since exon 4 of *PPM1K* is relatively short, ABCG2RE26 was not excluded from our analysis because it also had numerous conserved and nonconserved predicted TFBSs (data not shown). There are other examples of coding exons working as an enhancer to regulate the tissue-specific expression of a neighboring gene that were identified though ChIP-seq data sets (Birnbaum et al., 2012). However, these examples have been infrequent, since in silico pipelines to identify conserved enhancer elements often eliminate coding regions under the assumption that they are conserved to preserve protein function, not due to *cis*-regulatory activity. These data illustrate that the addition of TFBS clustering, regardless of conservation or genomic region, could improve the enhancer selection process.

To investigate the possible mechanisms driving the in vivo activity of enhancers, the ENCODE and TRANSFAC databases were mined for ChIP-seq data or predicted sites of transcription factor binding. TRANSFAC analysis of ABCG2RE1 predicted binding sites for constitutive androstane receptor, liver X receptor, PXR, vitamin D receptor, and the aryl hydrocarbon receptor (data not shown). The in vitro enhancer activity of ABCG2RE1 was increased by the PXR ligand rifampin, indicating that it has both constitutive and inducible enhancer activity. The ABCG2RE1 enhancer is ~4000 bp upstream of the *PKD2* promoter and could be regulating the expression of PKD2 or be working as a loci enhancer element to regulate the expression of PKD2, PPM1K, and/or ABGC2 in the kidney where they are all expressed.

ENCODE analysis found ChIP-seq signals for many transcription factors bound to the ABCG2RE9 region in liver cells, including RXRα and HNF4α. Both transcription factors are important in liver gene expression, and they interact with each other and with ERα (Lee et al., 1998; Schrem et al., 2002; Germain et al., 2006). Although constitutive androstane receptor and PXR cross talk with FOXA2 (another transcription factor that binds ABCG2RE9) to regulate hepatic genes (Konno et al., 2008), the presence of glucocorticoid receptor, FOXA1, RXRα, and p300 ChIP-seq signals, and the current data showing that ABCG2RE9 responds to both PXR and ER ligands, indicate a likely role of this regulatory region in hormone response and possibly the expression of ABCG2 in the intestine, liver, and placenta.

Some experiments in the ENCODE ChIP-seq database include before and after treatment with nuclear receptor ligands, providing an excellent resource for future regulatory element searches (ENCODE Project Consortium, 2011). It is worth noting that not all of the regions with ChIP-seq data were enhancers. ABCG2RE23, for example, has many ChIP-seq peaks, especially strong and reproducible signals for CTCF (data not shown), but showed neither enhancer nor suppressor activity in most of the cell lines. This could be because CTCF can act as a general transcription factor, but it is best characterized for its ability to act as an insulator and modulator of chromatin structure (Phillips and Corces, 2009). The in vivo enhancer with highest activity, ABCG2RE1, was devoid of ChIP-seq marks, but many transcription factors have either not

been characterized or do not have reliable antibodies for use in ChIP-seq experiments, thus restricting the interpretation of data in ENCODE.

Many NREs work in coordination with other transcription factors, like p300, to remodel chromatin, and the chromatin context is extremely important to transcription factor activity (Wiench et al., 2011). Without the complex chromatin context, which was not assayed in our study, and the possibility that certain transcription factors are absent in the studied cell lines, some of the putative regions could still be in vivo *cis*-regulatory elements. This is most evident from the results of the ABCG2RE1 regulatory region, which had weak activity in vitro yet the strongest activity in vivo. Similarly, ABCG2RE6 had strong in vitro activity yet weak activity detected in vivo. Other limitations of the hydrodynamic tail vein assay include discordance between mouse and human TFBSs, inability to detect nuclear factor response elements without the ligand present, and the limited ability to detect enhancer activity outside the liver. Additional induction assays would be needed to detect regulatory elements that are active only with drug treatment, and the evaluation of enhancer regions in other model systems such as zebrafish might be useful to confirm non–liver-enhancer activity. Potential tissue-specific regulatory elements are highlighted in Supplemental Table 2. Although individual steps of the screening pipeline have limitations, taken together they robustly identified several *cis*-regulatory elements with in vivo liver enhancer activity in the *ABCG2* gene locus that are strongly supported by ChIP-seq data.

Through detailed in silico, in vitro, and in vivo *cis*-regulatory assays, multiple regions in the *ABCG2* gene locus that function as enhancers or suppressors were identified. These regions have evidence for transcription factor binding that link them with tissue-specific or nuclear receptor responsive expression of ABCG2. Overall, ABCG2RE9 has the most evidence supporting its role as a nuclear receptor responsive element and *cis*-regulatory enhancer. It has constitutive hepatic activity in vitro and in vivo, nuclear receptor–inducible activity in the human HepG2 cell line, and binding of transcription factors determined by ChIP-seq in human hepatocytes. These results indicate that computational genetics coupled with in vitro and in vivo assays are capable of finding global and liver-specific enhancers. With increasing evidence for the effects of *cis*-regulatory regions on drug disposition and efficacy (Smith et al., 2012), the identification of these elements can help to elucidate how genetic variants in noncoding regions of the genome cause clinical variation in drug transporter gene expression that result in altered pharmacokinetic and pharmacodynamic properties.

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