

## Title Page

### Rare Variants in the *ABCG2* Promoter Modulate *In Vivo* Activity

Rachel J. Eclov, Mee J. Kim, Robin Smith, Nadav Ahituv and Deanna L. Kroetz

Department of Bioengineering and Therapeutic Sciences, University of California San Francisco,  
San Francisco, CA (R.J.E., M.J.K., R.P.S., X.L., N.A., D.L.K.); Institute for Human Genetics,  
University of California San Francisco, San Francisco, CA (N.A., M.J.K., R.S., D.L.K.)

## Running Title Page

### *ABCG2* Promoter Variants Modulate *In Vivo* Activity

Corresponding Author:

Deanna L. Kroetz

1550 4<sup>th</sup> Street RH584E

San Francisco, CA 94158-2911

Phone: (415) 476-1159

Fax: (415) 514-4361

deanna.kroetz@ucsf.edu

Pages of Text: 19

Number of Tables: 1

Number of Figures: 4

Number of References: 50

Number of Words in the Abstract: 187

Number of Words in the Introduction: 667

Number of Words in the Discussion: 1257

Abbreviations: ABC, ATP-binding cassette; ApoE, apolipoprotein E; BCRP, breast cancer resistance protein; ChIP-seq, chromatin immunoprecipitation coupled with sequencing; ENCODE, encyclopedia of DNA elements; ERE, estrogen response element; HFH, HNF

forkhead homologue; HNF, hepatic nuclear factor; NRE, nuclear response element; RXR, retinoid X receptor; SNP, single nucleotide polymorphism.

## Abstract

*ABCG2* encodes the breast cancer resistance protein (BCRP), an efflux membrane transporter important in detoxification of xenobiotics. In the present study, the basal activity of the *ABCG2* promoter in liver, kidney, intestine and breast cell lines was examined using luciferase reporter assays. The promoter activity of reference and variant *ABCG2* sequences were compared in HepG2, HEK293T, HCT116 and MCF-7 cell lines. The *ABCG2* promoter activity was strongest in the kidney and intestine cell lines. Four variants in the basal *ABCG2* promoter (rs76656413, rs66664036, rs139256004 and rs59370292) decreased the promoter activity by 25-50% in at least three of the four cell lines. The activity of these four variants were also examined *in vivo* using the hydrodynamic tail vein assay, and two SNPs (rs76656413 and rs59370292) significantly decreased *in vivo* liver promoter activity by 50-80%. Electrophoretic mobility shift assays confirmed a reduction in nuclear protein binding to the rs59370292 variant probe, while the rs76656413 probe had a shift in transcription factor binding specificity. While both rs59370292 and rs76656413 are rare variants in all populations, they could contribute to patient-level variation in *ABCG2* expression in the kidney, liver and intestine.

## Introduction

The breast cancer resistance protein (BCRP, ABCG2) is an efflux membrane transporter and part of the ATP-binding cassette (ABC) transporter family. It transports a variety of dietary toxins, endogenous nutrients and pharmaceutical compounds (Ni *et al.*, 2010). BCRP is expressed in the side population of hematological stem cells, endothelium of veins and capillaries (including in the brain), intestinal and colon epithelium, placental syncytiotrophoblasts, ducts and lobules of the breast, the bile canalicular membrane of hepatocytes and to a lesser extent in renal cortical tubules (Robey *et al.*, 2009). BCRP is essential for detoxification processes, transport of nutrients into milk and protection of vital organs and tissues like the brain, fetus, prostate and eye (Leslie *et al.*, 2005). Inter-individual expression of ABCG2 mRNA is highly variable, with reports of 500-fold differences among human livers without detectable copy number variation (Poonkuzhali *et al.*, 2008), 1000-fold in leukemic blast cells (Ross *et al.*, 2000) and 1.8- to 78-fold in human intestine (Zamber *et al.*, 2003; Urquhart *et al.*, 2008). Additionally, high ABCG2 expression has been linked to decreased disease-free survival in cancer (Mao and Unadkat, 2015). Understanding the mechanisms that regulate the expression of ABCG2 can help to predict cancer outcomes, drug response and toxicity. These mechanisms could become clinical targets of epigenetic inhibitors to downregulate transporter expression and enhance the efficacy of pharmacotherapy (Chen *et al.*, 2016).

BCRP is transcribed by *ABCG2*, which spans over 66 kb on the anti-strand of chromosome 4q22 (Allikmets *et al.*, 1998). The basal *ABCG2* promoter is a TATA-less promoter identified as the 312 base pairs upstream of the transcription start site (TSS) (Bailey-Dell *et al.*, 2001), while the 5' promoter regulatory region has been described as >100 kb (Poonkuzhali *et al.*, 2008). The

basal promoter includes a CCAAT box and numerous specificity protein 1 (SP1), activator protein (AP) 1 and AP2 sites (Figure 1) (Bailey-Dell *et al.*, 2001). The proximal promoter of *ABCG2* has a functional aryl hydrocarbon receptor response element (Tan *et al.*, 2010; To *et al.*, 2011) that overlaps with progesterone (H Wang *et al.*, 2008) and estrogen (Ee *et al.*, 2004) response elements (ERE, Figure 1). It also has an NF- $\kappa$ B response element which works in concert with estrogen to increase *ABCG2* expression (Pradhan *et al.*, 2010), a hypoxia inducible factor (HIF) 1 $\alpha$  response element (HRE) (Krishnamurthy, 2004) and an antioxidant response element (ARE) (Singh *et al.*, 2010) (Figure 1). A large CpG island covers most of the *ABCG2* proximal promoter (Figure 1) (Tan *et al.*, 2010). To date, there are no systematic evaluations of the effect of genetic variation on the activity of the *ABCG2* promoter.

Genetic polymorphisms in the proximal promoter of transporter genes have been linked to variation in gene expression (Ha Choi *et al.*, 2009; Hesselson *et al.*, 2009; Yee *et al.*, 2009; L Li *et al.*, 2009). Additionally, genetic variation in promoters for transporters and enzymes have been linked to adverse drug reactions (Innocenti *et al.*, 2004; D Wang *et al.*, 2008; Kenna *et al.*, 2009; McLeod *et al.*, 2010; Toffoli *et al.*, 2010). Previous studies of the effect of regulatory variants on *ABCG2* expression (Zamber *et al.*, 2003; Poonkuzhali *et al.*, 2008; Eclov, Kim, Chhibber, *et al.*, 2017) have only considered regions outside the primary promoter. In the present study, the basal activity of the major *ABCG2* promoter (-499 to +21 bp relative to the TSS) was investigated in transiently transfected kidney, liver, intestine and breast cell lines. The activity of eleven variant *ABCG2* promoters was characterized in these same cell lines to identify SNPs that alter *ABCG2* promoter activity. Rare variants were included in this study because they have recently been shown to contribute significantly to individual gene expression profiles (X Li *et al.*, 2014). Variants that caused significant *in vitro* reductions in *ABCG2* promoter activity were

also tested in the mouse hydrodynamic tail vein assay for their effect on *in vivo* promoter activity. EMSAs were performed on SNPs with significantly altered *in vivo* activity to understand how sequence affects transcription factor binding.

## Materials and Methods

**Chemicals and Materials.** The vectors pGL4.11b [*luc2P*], pGL4.74 [*hRluc*/TK], pGL4.13 [*luc2*/SV40] and the Dual-Luciferase<sup>®</sup> Reporter Assay System were purchased from Promega (Madison, WI). The human embryonic kidney (HEK293T/17), human colorectal carcinoma (HCT116), human hepatocellular carcinoma (HepG2) and human breast adenocarcinoma (MCF-7) cell lines were purchased from the American Type Culture Collection (Manassas, VA). High-glucose Dulbecco's modified Eagle's medium (DMEM), Opti-Minimal Essential Medium (Opti-MEM) and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Penicillin and streptomycin were purchased from the University of California San Francisco (UCSF) Cell Culture Facility (San Francisco, CA). Phusion High-Fidelity DNA Polymerase, *NheI*, *HindIII* and *DpnI*, were purchased from New England Biolabs (Ipswich, MA). Fetal bovine serum (FBS) (Axenia BioLogix, Dixon, CA), GenElute HP Endotoxin- Free Maxiprep Kits (Sigma Aldrich), Improved Minimum Essential Medium (IMEM) without phenol red (Mediatech Inc, Manassas, VA), TransIT EE In Vivo Gene Delivery System (Mirus Bio, Madison, WI), CD1 mice (Charles Rivers Laboratories, Wilmington, MA), PolyJet<sup>™</sup> DNA *In Vitro* Transfection Reagent (SignaGen Laboratories, Rockville, MD), Odyssey EMSA buffer kit (Lincoln, Nebraska, USA), HepG2 Nuclear Extract (Abcam, Cambridge, MA) and PfuTurbo DNA Polymerase (Agilent Technologies, Santa Clara, CA) were purchased from the indicated manufacturers.

***ABCG2* Promoter Plasmid Construction.** A 524 bp region of the *ABCG2* promoter (chr4:89079995-89080518, hg19) was PCR amplified using the forward primer 5'-TCAGGCTAGCAAGCATCCACTTTCTCAGA-3' and reverse primer



5'-TTATAAAGCTTCAGGCAGCGCTGACACGAA-3'. This region included the proximal promoter (-312 bp upstream of the TSS), adjacent transcription factor response elements and the CpG island that extends to ~500 bp upstream of the TSS (Figure 1) (Bailey-Dell *et al.*, 2001). The sequences for restriction sites *NheI* and *HindIII* were added to the forward and reverse primers, respectively (underlined in above sequences). The region was amplified from human placenta genomic DNA using PfuTurbo DNA polymerase following the manufacturer's protocol. PCR conditions were 95°C for 2 min, followed by 35 cycles of 30 sec at 95°C, 30 sec at 60°C and 1 min at 68°C, then a final extension of 10 min at 72°C. The 542 bp PCR product was gel purified, enzyme digested and ligated into the pGL4.11b vector. The reaction was purified, transformed into competent cells and colonies containing the reverse promoter (forward transcription direction) orientation were isolated. DNA for pGL4.11b promoter plasmids and empty pGL4.11b, pGL4.74 and pGL4.13 vectors were isolated using the GenElute HP Endotoxin-Free Maxiprep Kit following the manufacturer's protocol.

**Genetic Analysis of *ABCG2* Promoter Region.** SNPs in the *ABCG2* promoter region were retrieved for all available ethnic populations from publicly available databases, including 1000 Genomes (phase 1 release 02/14/2012) (1000 Genomes Project Consortium *et al.*, 2010), dbSNP build 137, and HapMap release 28 (International HapMap Consortium, 2007). These SNPs were combined with sequencing results from the *ABCG2* promoter region (-674 to +85 bp) of the SOPHIE cohort and reported in the Pharmacogenetics of Membrane Transporter Database (UCSF, San Francisco, CA) (Hesselson *et al.*, 2009; Kroetz *et al.*, 2010). Haplotypes were determined by downloading genotype and information files from the 1000 Genomes browser (phase 1 release 05/21/2011) for all available ethnic groups combined and analyzed with Haploview version 4.2 (Barrett *et al.*, 2005).

**Site-Directed Mutagenesis.** *ABCG2* promoter SNPs were introduced into the reference promoter plasmid using the Phusion High-Fidelity DNA Polymerase, following the manufacturer's protocol. Reaction conditions for all site-directed mutagenesis primers (Supplemental Table 1) except rs139256004 are as follows: an initial cycle for 30 sec at 98°C, followed by 20 cycles of 10 sec at 98°C, the primer pair melting temperature for 30 sec and 3 min at 72°C, then a final extension for 10 min at 72°C. The deletion SNP rs139256004 was introduced into the *ABCG2* promoter using a special protocol for deletion mutagenesis (Liu and Naismith, 2008). PCR reaction components were the same as above with PCR conditions as follows: an initial cycle of 5 min at 95°C, then 12 cycles of 95°C for 1 min, 45°C for 1 min and 72°C for 9 min, with a final cycle of 1 min at 36°C and 30 min at 72°C. Promoter SNP rs57327643 was also attempted via this protocol, but no colonies were isolated. The site-directed mutagenesis PCR reactions were digested with the *DpnI* enzyme, purified and transformed into competent cells. Plasmids were isolated and sequenced to confirm the presence of the SNP. All DNA used for the *in vitro* and *in vivo* luciferase assays was endotoxin-free.

**Cell Culture, Transfections and Luciferase Assays.** HEK293T/17, HCT116 and HepG2 cells were grown in high-glucose DMEM supplemented with 10% FBS, 100 U/mL of penicillin and 0.1 mg/mL of streptomycin. The MCF-7 cell line was grown in IMEM without phenol red, supplemented with 10% FBS, 100 U/mL of penicillin and 0.1 mg/mL of streptomycin. All cell lines were grown in a 5% CO<sub>2</sub> incubator at 37°C. Transient transfections were performed as previously described (Eclov, Kim, Smith, *et al.*, 2017) and the firefly and *Renilla* luciferase activity of cell lysates were measured using the Dual-Luciferase<sup>®</sup> Reporter Assay System in a GloMax 96 microplate Dual Injector Luminometer (Promega, Madison, WI) following the manufacturer's protocol. Each experiment included the empty pGL4.11b vector as

the negative control and the pGL4.13 vector as the positive control. Promoter plasmid firefly activity was normalized to *Renilla* activity and then displayed relative to the normalized activity of empty pGL4.11b.

**Hydrodynamic Tail Vein Assay.** Positive *in vitro* variant promoter plasmids were screened for their effect on *in vivo* promoter activity through the hydrodynamic tail vein injection as previously described (Eclov, Kim, Smith, *et al.*, 2017). Briefly, 10 µg of promoter plasmid or the *ApoE* (Simonet *et al.*, 1993) positive control liver enhancer, along with 2 µg of pGL4.74 was injected into the tail vein of 4-5 male CD1 mice (Charles River) weighing 21-25 g using the TransIT EE *In Vivo* Gene Delivery System following the manufacturer's protocol. After 24 hr, liver lysates harvested from the euthanized mice were measured for firefly and *Renilla* luciferase activity using the Dual-luciferase<sup>®</sup> reporter assay system following the manufacturer's protocol in a Synergy 2 (BioTek Instruments, Winooski, VT) microplate reader. Each sample's firefly activity was normalized to *Renilla* activity and expressed as fold activation relative to pGL4.11b. All mouse work was approved by the UCSF Institutional Animal Care and Use Committee.

**Electrophoretic Mobility Shift Assay.** Electrophoretic mobility shift assays (EMSA) were performed using 2.5 nM 5' IRDye 700 labeled probe (sequences available in the supplemental materials and methods), incubated with 5 µg of HepG2 nuclear extract using the Odyssey EMSA buffer kit as previously described (Eclov, Kim, Chhibber, *et al.*, 2017). Competition assays were performed by adding 40-fold molar excess of unlabeled reference oligonucleotide. DNA/protein complexes were separated from free probe by gel electrophoresis and imaged using the Licor system (Odyssey, Lincoln, NE). Transcription factors that bound over the ABCG2 promoter and its SNPs were obtained from ChIP-seq data from the Encyclopedia of DNA Elements (ENCODE) database (ENCODE Project Consortium, 2011).

**Statistical Analysis.** Normalized promoter activity is expressed relative to pGL4.11b. Basal forward and reverse promoter activity in the HEK293T and HepG2 cell lines were tested for significance ( $P < 0.05$ ) from empty pGL4.11b vector with an ANOVA analysis followed by a Bonferroni's multiple comparison *t*-test. Basal promoter activity in the HCT116 and MCF-7 cell lines was tested for significance ( $P < 0.05$ ) from the empty pGL4.11b vector with a Student's *t*-test. Variant *ABCG2* promoter sequences selected for *in vivo* testing were significantly different ( $P < 0.05$ ) from the reference promoter in three of four cell lines. Variant promoter plasmids tested *in vitro* or *in vivo* were tested for significance ( $P < 0.05$ ) from the reference *ABCG2* promoter with an ANOVA analysis followed by a Bonferroni's multiple comparison *t*-test. Reference promoter and the *ApoE* enhancer were tested for difference from the empty vector sequence *in vivo* using an unpaired Students' *t*-test. All statistics were run using the GraphPad Prism 5 program (San Diego, CA).

## Results

**Genetic Polymorphisms of the *ABCG2* Promoter.** Twelve variants (Table 1) were obtained for the *ABCG2* promoter region from publicly available databases and from sequencing of the SOPHIE cohort. Of the twelve variants, rs57327643, rs2231134, rs45604438 and rs59172759 had a minor allele frequency (MAF) above 4% in at least one ethnic population (Table 1). The *ABCG2* promoter variants included the single nucleotide insertion rs66664036 and two multiple base pair deletions, rs57327643 and rs139256004. Attempts to construct the rs57327643 variant promoter plasmid were unsuccessful and this variant was not evaluated in the functional assays. There was no notable linkage disequilibrium between variants in the *ABCG2* promoter.

**Activity of the *ABCG2* Promoter *In Vitro*.** The activity of the *ABCG2* promoter sequence (chr4:89079947-89080567, hg19), cloned into the firefly luciferase reporter vector pGL4.11b, was investigated in transiently transfected HEK293T, HepG2, HCT116 and MCF-7 cell lines (Figure 2). This region includes the basal promoter of *ABCG2* and the structural elements displayed in Figure 1. The reverse *ABCG2* promoter activity was strongest in HEK293T and HCT116 cells, with average activation of 11- and 17-fold, respectively; in the HepG2 and MCF-7 cells promoter activity was weaker, with average activation of 2.4- and 1.5-fold, respectively (Figure 2).

**Variant *ABCG2* Promoter Activity *In Vitro*.** The effect of eleven variants (Table 1) on the basal *ABCG2* promoter activity was investigated in all four cell lines. Five variants had significantly decreased activity in HEK293T cells; two of them (rs59370292 and rs76656413) had over a 50% decrease in promoter activity ( $P < 0.05$ , Figure 2A). Of the three variants with decreased activity in HepG2, only rs76656413 had over a 50% decrease ( $P < 0.05$ , Figure 2B). In

HCT116 cells, five variants had decreased activity, including a 50% decrease with rs76656413 and rs139256004 and >75% decrease with rs59370292 ( $P < 0.05$ , Figure 2C). All four variants with significant decrease in activity in MCF-7s cells, (rs76656413, rs66664036, rs139256004 and rs59370292) had at least a 50% decreased activity ( $P > 0.05$ , Figure 2D). The rs66664036 and rs139256004 variants both had 25-50% decreased activity in three of the four cell lines and the rs76656413 SNP had over a 50% decreased activity in all four of the cell lines. The rs59370292 SNP was the most detrimental variant in both HEK293T and HCT116 cell lines with an almost 75% decreased activity; it also had 25-50% decreased activity in HepG2 and MCF-7 cells. Due to their decreased promoter activity in at least three of four cell lines, rs66664036, rs139256004, rs76656413 and rs59370292 were chosen for follow up in the *in vivo* hydrodynamic tail vein injection assay.

**Variant ABCG2 Promoter Activity *In Vivo*.** Four variants (two SNPs, one single base insertion, and a four base deletion) were screened for their effect on *in vivo* ABCG2 promoter activity using the hydrodynamic tail vein injection assay. In this assay the *ApoE* liver enhancer (Simonet *et al.*, 1993) positive control had more than 200-fold activation over empty vector. The ABCG2 promoter plasmid exhibited a strong 35-fold activation over pGL4.11b. Two of the four promoter variants significantly decreased promoter activity *in vivo* ( $P < 0.05$ , Figure 3). The rs59370292 SNP decreased promoter activity over 80%, while rs76656413 resulted in a 70% decrease in promoter activity *in vivo*.

**Variant ABCG2 Promoter DNA Binding to Nuclear Protein.** The ABCG2 variants rs59370292 and rs76656413 DNA probes were tested for alteration in binding to nuclear proteins via EMSA. Reference DNA probes at these SNP locations showed strong HepG2 nuclear protein binding with specific DNA/protein bands susceptible to competition by unlabeled

oligonucleotide probes (Figure 4). The rs59370292 SNP showed reduced binding to HepG2 nuclear proteins compared to its reference DNA sequence (Figure 4A). The rs76656413 SNP lost the specific DNA/protein binding of the reference sequence, but gained a separate DNA/protein binding interaction (Figure 4B).

## Discussion

Previous research has highlighted the role of the *ABCG2* promoter in the regulation of BCRP expression (Robey *et al.*, 2009). Although the *ABCG2* proximal promoter has many transcription factor response elements, variants within the *ABCG2* promoter have not yet been associated with its mRNA levels. In this study, the activity of the *ABCG2* promoter and the effects of variants on that activity were investigated in liver, kidney, intestine and breast cell lines. Variants with a consistent *in vitro* effect on *ABCG2* promoter activity were tested for their effect in the *in vivo* mouse tail vein assay. The *ABCG2* promoter was highly active in intestinal and kidney cell lines, had medium activity in liver and low activity in breast cell lines. Despite modest *in vitro* liver activity, the *ABCG2* promoter had strong *in vivo* liver activity. These results correlate with high expression of *ABCG2* in intestine and liver and more moderate expression of *ABCG2* in kidney (Maliepaard *et al.*, 2001). In contrast, the low promoter activity in the MCF-7 cells is inconsistent with the high expression of *ABCG2* in breast tissue (Maliepaard *et al.*, 2001).

In earlier work, luciferase assays on the -628/+362 *ABCG2* promoter segment indicated suppressed activity in MCF-7 cells, whereas the -312/+362 promoter segment was highly active. It is possible that a suppressor element within the -499 to -312 segment reduces the activity of our *ABCG2* promoter construct in MCF-7 cells (Bailey-Dell *et al.*, 2001). An interferon-gamma activated sequence at -448/-422 has been shown to increase the *ABCG2* promoter activity upon stimulation of the JAK2/STAT5 pathway by prolactin (Wu *et al.*, 2013). STAT5 is well documented for its importance in regulating expression of genes essential for mammary development and lactogenesis (Watson, 2001). Naturally occurring dominant-negative isoforms of STAT5 have also been shown to suppress the transcriptional activity of the estrogen receptor



in MCF-7 cells (Yamashita *et al.*, 2003). Previous research has shown discordant results in the ability of the *ABCG2* promoter to be upregulated in MCF-7 cells when treated with 17 $\beta$ -estradiol (Imai *et al.*, 2005; Yasuda *et al.*, 2009). Therefore, it is possible that without stimulation of the STAT5 pathways, there is a suppressive factor bound between -499 and -312 of the *ABCG2* promoter in MCF-7 cells that inhibits promoter activity. This indicates a fragile and complex network of transcription factors that bind to the *ABCG2* promoter and regulate its expression in a cell/tissue contextual manner.

Two of the *ABCG2* promoter variants (rs76656413 and 59370292) decrease the hepatic activity of the *ABCG2* promoter *in vitro* and *in vivo*. A third variant, rs66664036, significantly decreased *in vitro* promoter activity in a hepatic cell line and showed an almost 50% decrease in *in vivo* hepatic promoter activity that did not reach significance. Similar to *in vitro* and *in vivo* analysis of enhancer variants in *ABCG2* and other pharmacogenes (Kim *et al.*, 2011; Eclov, Kim, Smith, *et al.*, 2017; Eclov, Kim, Chhibber, *et al.*, 2017) there was not a complete concordance between promoter assays in cell lines and results from the *in vivo* assay. Discordance between *in vitro* and *in vivo* results might reflect differences between human and murine transcription factors, and highlights one of the limitations of the tail vein assay. This assay is also restricted to analysis of only hepatic transcriptional activity, and additional studies are needed to determine if variants that alter *in vitro* *ABCG2* promoter activity in renal, intestinal and breast cell lines affect *in vivo* *ABCG2* expression in those tissues.

Three out of four variants in the *ABCG2* promoter that altered promoter activity *in vitro* have reported low minor allele frequencies. This is in concordance with a large analysis of ABC and SLC gene promoter variation that found the proximal promoters of these gene families had low nucleotide diversity (Hesselson *et al.*, 2009) and global genome analysis showing

enrichment in promoters for rare variants (X Li *et al.*, 2017). Due to the low frequency of these variants, it is difficult to correlate them with the expression of *ABCG2* and thus, whether they contribute significantly to population variability in *ABCG2* expression cannot be determined. Nonetheless, consistent with similar global rare variant observations, these rare variants could have large effects on expression within individuals (X Li *et al.*, 2017). Further studies are needed to examine in more detail the association of these variants with the expression of *ABCG2* and the function of the BCRP transporter in both the liver and extrahepatic tissues important for xenobiotic disposition.

The *ABCG2* promoter SNP rs76656413 had strong evidence for altering the transcriptional activity of the *ABCG2* promoter. It attenuated the relative luciferase activity of the *ABCG2* promoter by 50% in all four cells lines and decreased *ABCG2* liver promoter activity by 70% *in vivo*. Transcription factor binding site analysis predicted rs76656413 to have significant losses in USF-1, n-Myc, Max and Myc-Max binding (data not shown) that is consistent with its location in the middle of several USF-1 and c-Myc ChIP-seq peaks reported by ENCODE. Not only does the SNP fall in the middle of ChIP-Seq peaks, it is directly within a canonical motif for both Myc and Max. Additionally, c-Myc and Max have been reported to direct the transcriptional regulation of *ABC* genes, particularly the unmethylated *ABCG2* promoter, in human leukemic hematopoietic progenitor cells (Porro *et al.*, 2011). Furthermore, the expression of *ABCG2* is altered by the overexpression of c-Myc in human breast epithelial cells (Kang *et al.*, 2009). Competition assays and transcription factor supershift experiments based on the predicted binding of transcription factors did not reveal changes in the binding of specific transcription factors by the rs76656413 SNP. Further studies are needed to confirm that USF-1, Max and c-

Myc transcription factors bind to the *ABCG2* promoter and that rs76656413 changes USF-1, Max and/or Myc binding, thus altering the transcriptional activity of the *ABCG2* promoter.

The *ABCG2* promoter SNP rs59370292, located just upstream of the antioxidant response element, has the lowest reported MAF of the four variants that alter *in vitro* *ABCG2* promoter activity. It altered the relative luciferase activity of the *ABCG2* promoter in three of four cell lines and had the largest effect of any *ABCG2* promoter variant *in vivo*, decreasing the promoter activity by 80%. EMSA showed reduced binding of nuclear liver extract to the rs59370292 mutated probe. The transcription factor predicted to have the largest reduced binding due to rs59370292 is the vitamin D receptor (VDR, data not shown). Although VDR has not been directly linked to *ABCG2* expression, VDR is important in regulating bile acid transporters, and its ligands include bile acid derivatives and steroids (Germain *et al.*, 2006; Halilbasic *et al.*, 2013). Since *ABCG2* encodes a bile acid transporter (Blazquez *et al.*, 2012) and has been shown to be important for the pharmacokinetics and pharmacodynamics of statins (Generaux *et al.*, 2011), VDR could be the link for statin regulation of *ABCG2* expression. However, supershift or competition EMSA assays were not able to demonstrate a role for VDR and further studies are needed to determine if VDR binding to the proximal promoter of *ABCG2* is involved in *ABCG2* transcription.

In summary, the *ABCG2* promoter (-499 to +21) had strong activity in HCT116 and HEK293T cell lines and *in vivo* in the liver. The rs76656413 and rs59370292 SNPs within the basal promoter of *ABCG2* affect its function both *in vitro* and *in vivo*. We found these SNPs to have altered transcription factor binding through EMSAs. Although their low allele frequencies limit their impact on population level expression of *ABCG2*, these and other rare variants in *ABCG2* could be important for regulating expression in individual patients.

## **Authorship Contributions**

*Participated in research design:* Eclov, Kim, Smith, Ahituv, Kroetz

*Conducted experiments:* Eclov, Kim, Smith

*Performed data analysis:* Eclov, Kim, Ahituv, Kroetz

*Contributed to the writing of the manuscript:* Eclov, Kim, Smith, Ahituv, Kroetz

## References:

- 1000 Genomes Project Consortium, Abecasis GR, Altshuler D, Auton A, Brooks LD, Durbin RM, Gibbs RA, Hurles ME, and McVean GA (2010) A map of human genome variation from population-scale sequencing. *Nature* **467**:1061–1073.
- Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, and Dean M (1998) A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Research* **58**:5337–5339.
- Bailey-Dell KJ, Hassel B, Doyle LA, and Ross DD (2001) Promoter characterization and genomic organization of the human breast cancer resistance protein (ATP-binding cassette transporter G2) gene. *Biochim Biophys Acta* **1520**:234–241.
- Barrett JC, Fry B, Maller J, and Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21**:263–265.
- Blazquez AG, Briz O, Romero MR, Rosales R, Monte MJ, Vaquero J, Macias RIR, Cassio D, and Marin JJG (2012) Characterization of the role of ABCG2 as a bile acid transporter in liver and placenta. *Molecular Pharmacology* **81**:273–283.
- Chen Z, Shi T, Zhang L, Zhu P, Deng M, Huang C, Hu T, Jiang L, and Li J (2016) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family in multidrug resistance: A review of the past decade. *Cancer Letters* **370**:153–164.
- Eclov RJ, Kim MJ, Chhibber A, Smith RP, Ahituv N, and Kroetz DL (2017) ABCG2 regulatory single-nucleotide polymorphisms alter in vivo enhancer activity and expression. *Pharmacogenetics and Genomics* **27**:454–463.
- Eclov RJ, Kim MJ, Smith RP, Liang X, Ahituv N, and Kroetz DL (2017) In Vivo Hepatic Enhancer Elements in the Human ABCG2 Locus. *Drug Metabolism and Disposition*

45:208–215.

Ee PLR, Kamalakaran S, Tonetti D, He X, Ross DD, and Beck WT (2004) Identification of a novel estrogen response element in the breast cancer resistance protein (ABCG2) gene.

*Cancer Research* **64**:1247–1251.

ENCODE Project Consortium (2011) A user's guide to the encyclopedia of DNA elements (ENCODE). *Plos Biol* **9**:e1001046.

Generaux GT, Bonomo FM, Johnson M, and Mahar Doan KM (2011) Impact of SLCO1B1(OATP1B1) and ABCG2(BCRP) genetic polymorphisms and inhibition on LDL-C lowering and myopathy of statins. *Xenobiotica* **41**:639–651.

Germain P, Staels B, Dacquet C, Spedding M, and Laudet V (2006) Overview of Nomenclature of Nuclear Receptors. *Pharmacological Reviews* **58**:685–704.

Ha Choi J, Wah Yee S, Kim MJ, Nguyen L, Ho Lee J, Kang J-O, Hesselson S, Castro RA, Stryke D, Johns SJ, Kwok P-Y, Ferrin TE, Goo Lee M, Black BL, Ahituv N, and Giacomini KM (2009) Identification and characterization of novel polymorphisms in the basal promoter of the human transporter, MATE1. *Pharmacogenetics and Genomics* **19**:770–780.

Halilbasic E, Claudel T, and Trauner M (2013) Bile acid transporters and regulatory nuclear receptors in the liver and beyond. *J Hepatol* **58**:155–168.

Hesselson SE, Matsson P, Shima JE, Fukushima H, Yee SW, Kobayashi Y, Gow JM, Ha C, Ma B, Poon A, Johns SJ, Stryke D, Castro RA, Tahara H, Choi JH, Chen L, Picard N, Sjödin E, Roelofs MJE, Ferrin TE, Myers R, Kroetz DL, Kwok P-Y, and Giacomini KM (2009) Genetic variation in the proximal promoter of ABC and SLC superfamilies: liver and kidney specific expression and promoter activity predict variation. *PLoS ONE* **4**:e6942.

Imai Y, Ishikawa E, Asada S, and Sugimoto Y (2005) Estrogen-mediated post transcriptional

- down-regulation of breast cancer resistance protein/ABCG2. *Cancer Research* **65**:596–604.
- Innocenti F, Undevia SD, Iyer L, Chen PX, Das S, Kocherginsky M, Karrison T, Janisch L, Ramirez J, Rudin CM, Vokes EE, and Ratain MJ (2004) Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. *J Clin Oncol* **22**:1382–1388.
- International HapMap Consortium (2007) A second generation human haplotype map of over 3.1 million SNPs. *Nature* **449**:851–861.
- Kang KW, Im YB, Go W-J, and Han H-K (2009) c-Myc Amplification Altered the Gene Expression of ABC- and SLC-Transporters in Human Breast Epithelial Cells. *Mol Pharmaceutics* **6**:627–633.
- Kenna GA, Zywiak WH, McGeary JE, Leggio L, McGeary C, Wang S, Grenga A, and Swift RM (2009) A Within-Group Design of Nontreatment Seeking 5-HTTLPR Genotyped Alcohol-Dependent Subjects Receiving Ondansetron and Sertraline. *Alcoholism: Clinical and Experimental Research* **33**:315–323.
- Kim MJ, Skewes-Cox P, Fukushima H, Hesselson S, Yee SW, Ramsey LB, Nguyen L, Eshragh JL, Castro RA, Wen CC, Stryke D, Johns SJ, Ferrin TE, Kwok P-Y, Relling MV, Giacomini KM, Kroetz DL, and Ahituv N (2011) Functional characterization of liver enhancers that regulate drug-associated transporters. *Clinical Pharmacology & Therapeutics* **89**:571–578.
- Krishnamurthy P (2004) The Stem Cell Marker Bcrp/ABCG2 Enhances Hypoxic Cell Survival through Interactions with Heme. *Journal of Biological Chemistry* **279**:24218–24225.
- Kroetz DL, Yee SW, and Giacomini KM (2010) The pharmacogenomics of membrane transporters project: research at the interface of genomics and transporter pharmacology. *Clinical Pharmacology & Therapeutics* **87**:109–116.

- Leslie EM, Deeley RG, and Cole SPC (2005) Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicology and Applied Pharmacology* **204**:216–237.
- Li L, Koo SH, Hong IHK, and Lee EJD (2009) Identification of functional promoter haplotypes of human concentrative nucleoside transporter 2, hCNT2 (SLC28A2). *Drug Metabolism and Pharmacokinetics* **24**:161–166.
- Li X, Battle A, Karczewski KJ, Zappala Z, Knowles DA, Smith KS, Kukurba KR, Wu E, Simon N, and Montgomery SB (2014) Transcriptome sequencing of a large human family identifies the impact of rare noncoding variants. *Am J Hum Genet* **95**:245–256.
- Li X, Kim Y, Tsang EK, Davis JR, Damani FN, Chiang C, Hess GT, Zappala Z, Strober BJ, Scott AJ, Li A, Ganna A, Bassik MC, Merker JD, GTEx Consortium, Hall IM, Battle A, and Montgomery SB (2017) The impact of rare variation on gene expression across tissues. *Nature* **550**:239–243.
- Liu H, and Naismith JH (2008) An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. *BMC Biotechnol* **8**:91.
- Maliepaard M, Scheffer GL, Faneyte IF, van Gastelen MA, Pijnenborg AC, Schinkel AH, van De Vijver MJ, Scheper RJ, and Schellens JH (2001) Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Research* **61**:3458–3464.
- Mao Q, and Unadkat JD (2015) Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update. *AAPS J* **17**:65–82.
- McLeod HL, Sargent DJ, Marsh S, Green EM, King CR, Fuchs CS, Ramanathan RK, Williamson SK, Findlay BP, Thibodeau SN, Grothey A, Morton RF, and Goldberg RM



- (2010) Pharmacogenetic predictors of adverse events and response to chemotherapy in metastatic colorectal cancer: results from North American Gastrointestinal Intergroup Trial N9741. *Journal of Clinical Oncology* **28**:3227–3233.
- Ni Z, Bikadi Z, Rosenberg MF, and Mao Q (2010) Structure and function of the human breast cancer resistance protein (BCRP/ABCG2). *Curr Drug Metab* **11**:603–617.
- Poonkuzhali B, Lamba J, Strom S, Sparreboom A, Thummel K, Watkins P, and Schuetz E (2008) Association of breast cancer resistance protein/ABCG2 phenotypes and novel promoter and intron 1 single nucleotide polymorphisms. *Drug Metabolism and Disposition* **36**:780–795.
- Porro A, Iraci N, Soverini S, Diolaiti D, Gherardi S, Terragna C, Durante S, Valli E, Kalebic T, Bernardoni R, Perrod C, Haber M, Norris MD, Baccarani M, Martinelli G, and Perini G (2011) c-MYC Oncoprotein Dictates Transcriptional Profiles of ATP-Binding Cassette Transporter Genes in Chronic Myelogenous Leukemia CD34+ Hematopoietic Progenitor Cells. *Molecular Cancer Research* **9**:1054–1066.
- Pradhan M, Bembinster LA, Baumgarten SC, and Frasor J (2010) Proinflammatory cytokines enhance estrogen-dependent expression of the multidrug transporter gene ABCG2 through estrogen receptor and NF{ $\kappa$ }B cooperativity at adjacent response elements. *Journal of Biological Chemistry* **285**:31100–31106.
- Robey RW, To KKK, Polgar O, Dohse M, Fetsch P, Dean M, and Bates SE (2009) ABCG2: a perspective. *Advanced Drug Delivery Reviews* **61**:3–13.
- Ross DD, Karp JE, Chen TT, and Doyle LA (2000) Expression of breast cancer resistance protein in blast cells from patients with acute leukemia. *Blood* **96**:365–368.
- Simonet WS, Bucay N, Lauer SJ, and Taylor JM (1993) A far-downstream hepatocyte-specific

control region directs expression of the linked human apolipoprotein E and C-I genes in transgenic mice. *J Biol Chem* **268**:8221–8229.

Singh A, Wu H, Zhang P, Happel C, Ma J, and Biswal S (2010) Expression of ABCG2 (BCRP) is regulated by Nrf2 in cancer cells that confers side population and chemoresistance phenotype. *Molecular Cancer Therapeutics* **9**:2365–2376.

Tan KP, Wang B, Yang M, Boutros PC, MacAulay J, Xu H, Chuang AI, Kosuge K, Yamamoto M, Takahashi S, Wu AML, Ross DD, Harper PA, and Ito S (2010) Aryl Hydrocarbon Receptor Is a Transcriptional Activator of the Human Breast Cancer Resistance Protein (BCRP/ABCG2). *Molecular Pharmacology* **78**:175–185.

To KKW, Robey R, Zhan Z, Bangiolo L, and Bates SE (2011) Upregulation of ABCG2 by Romidepsin via the Aryl Hydrocarbon Receptor Pathway. *Molecular Cancer Research* **9**:516–527.

Toffoli G, Cecchin E, Gasparini G, D'Andrea M, Azzarello G, Basso U, Mini E, Pessa S, De Mattia E, Re Lo G, Buonadonna A, Nobili S, De Paoli P, and Innocenti F (2010) Genotype-driven phase I study of irinotecan administered in combination with fluorouracil/leucovorin in patients with metastatic colorectal cancer. *Journal of Clinical Oncology* **28**:866–871.

Urquhart BL, Ware JA, Tirona RG, Ho RH, Leake BF, Schwarz UI, Zaher H, Palandra J, Gregor JC, Dresser GK, and Kim RB (2008) Breast cancer resistance protein (ABCG2) and drug disposition: intestinal expression, polymorphisms and sulfasalazine as an in vivo probe. *Pharmacogenetics and Genomics* **18**:439–448.

Wang D, Chen H, Momary KM, Cavallari LH, Johnson JA, and Sadee W (2008) Regulatory polymorphism in vitamin K epoxide reductase complex subunit 1 (VKORC1) affects gene expression and warfarin dose requirement. *Blood* **112**:1013–1021.

- Wang H, Lee E-W, Zhou L, Leung PCK, Ross DD, Unadkat JD, and Mao Q (2008) Progesterone receptor (PR) isoforms PRA and PRB differentially regulate expression of the breast cancer resistance protein in human placental choriocarcinoma BeWo cells. *Molecular Pharmacology* **73**:845–854.
- Watson CJ (2001) Stat transcription factors in mammary gland development and tumorigenesis. *J Mammary Gland Biol Neoplasia* **6**:115–127.
- Wu AML, Dalvi P, Lu X, Yang M, Riddick DS, Matthews J, Clevenger CV, Ross DD, Harper PA, and Ito S (2013) Induction of multidrug resistance transporter ABCG2 by prolactin in human breast cancer cells. *Molecular Pharmacology* **83**:377–388.
- Yamashita H, Iwase H, Toyama T, and Fujii Y (2003) Naturally occurring dominant-negative Stat5 suppresses transcriptional activity of estrogen receptors and induces apoptosis in T47D breast cancer cells. *Oncogene* **22**:1638–1652.
- Yasuda S, Kobayashi M, Itagaki S, Hirano T, and Iseki K (2009) Response of the ABCG2 promoter in T47D cells and BeWo cells to sex hormone treatment. *Mol Biol Rep* **36**:1889–1896.
- Yee SW, Shima JE, Hesselson S, Nguyen L, De Val S, Lafond RJ, Kawamoto M, Johns SJ, Stryke D, Kwok P-Y, Ferrin TE, Black BL, Gurwitz D, Ahituv N, and Giacomini KM (2009) Identification and characterization of proximal promoter polymorphisms in the human concentrative nucleoside transporter 2 (SLC28A2). *J Pharmacol Exp Ther* **328**:699–707.
- Zamber CP, Lamba JK, Yasuda K, Farnum J, Thummel K, Schuetz JD, and Schuetz EG (2003) Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. *Pharmacogenetics* **13**:19–28.

## Footnotes

These studies were supported by the National Institute of Health [U01 GM61390] (NA and DLK), National Human Genome Research Institute [R01HG005058] (NA), National Institute of Child Health & Human Development [R01HD059862] (NA), National Institute of General Medical Sciences Predoctoral Training Grant [T32 GM007175] (RJE and MJK) and were part of the Pharmacogenetics of Membrane Transporters project in the Pharmacogenetics Research Network. R. Eclov was supported by an American Foundation for Pharmaceutical Education Predoctoral fellowship, M. Kim was supported in part by the UCSF Quantitative Biosciences Consortium fellowship for Interdisciplinary Research and the Amgen Research Excellence in Bioengineering and Therapeutic Sciences fellowship, and R. Smith was partially supported by a Canadian Institute of Health Research fellowship in Hepatology.

Send reprint requests to:

Deanna L. Kroetz

1550 4<sup>th</sup> Street RH584E

San Francisco, CA 94158-2911

Phone: (415) 476-1159

Fax: (415) 514-4361

deanna.kroetz@ucsf.edu

**Figure 1: Schematic of the *ABCG2* promoter region.** Genomic region (chr4:89079997-89080517; hg19) with red boxes indicating location of basal transcription factors and the transcriptional start site (TSS) and blue boxes indicating nuclear response elements for NF- $\kappa$ B (NFkBRE), hypoxia (HRE), aryl hydrocarbons (AhRE), estrogens (ERE), progesterones (PRE) and antioxidants (ARE). The promoter is also covered by a CpG island indicated by a dark green bar. Binding of transcription factors determined by ChIP-seq from ENCODE data with the length and shading of the bar indicating the breadth and strength of the peak. Within the bar, a light green box indicates the site of a canonical motif for the corresponding factor. Cell lines for peaks are indicated by letters: K, K562; H, HeLa-S3; L, HepG2; A/a, A549; p, PBDE; M/m, MCF-7; 1, H1-hESC; n, NB4. Finally, the location and rs number for SNPs reported in dbSNP 138 are indicated.

**Figure 2: Effect of promoter variants *in vitro*.** Luciferase assay of *ABCG2* reference and variant promoter sequences was measured in transiently transfected A) kidney (HEK293T), B) liver (HepG2), C) intestine (HCT116) and D) breast (MCF-7) cell lines. Promoter activity is expressed as the ratio of firefly to *Renilla* luciferase activity normalized to empty pGL4.11b. Data is expressed as box-whisker plots of mean values from multiple experiments (N = 4-8 biological replicates with 3-6 wells per construct). Differences between reference and variant promoter constructs were tested by an ANOVA followed by a post-hoc Bonferroni's multiple comparison *t*-test; \*  $P < 0.05$ .

**Figure 3: Effect of promoter variants *in vivo*.** The luciferase activity in mouse liver homogenates was measured 24 hr after plasmid injection. Promoter activity is expressed as the

ratio of firefly to *Renilla* luciferase activity normalized to empty pGL4.11b activity. An enhancer for *ApoE* was used as the positive control (Simonet *et al.*, 1993). Data is expressed as box-whisker plots for 4-5 mice. Differences between reference promoter or *ApoE* and pGL4.11b were tested by an unpaired Student's *t*-test,  $\dagger < 0.05$ ; reference and variant promoter sequences were compared using a one-way ANOVA followed by a Bonferroni's multiple comparison *t*-test,  $* P < 0.05$ .

**Figure 4: Effect of rs76656413 and rs59370292 on DNA-protein binding.** Representative electromobility shift assay using HepG2 nuclear extracts incubated with IRDye700 labeled probes for reference and (A) rs76656413 or (B) rs59370292 sequences. Competition assays were performed with 40-fold excess unlabeled oligonucleotides with arrows indicating specific DNA/protein bands. Reference and variant DNA sequences surrounding each nucleotide mutation (underlined) are shown below their respective gel.

**Table 1: *ABCG2* Promoter SNPs**

Variant	Position <sup>1</sup>	$\Delta$ NT <sup>2</sup>	MAF (%) <sup>3</sup>				
			AFR	AMR	EAS	EUR	SAS
rs61181041	-84	C>T	NR	NR	0.01	NR	NR
rs61535534	-151	G>C	0.00	0.00	0.00	0.03	0.00
rs76656413	-169	C>T	0.00	0.00	1.00	0.00	0.00
rs57327643	-266	AGTGTTT>-	1.20	3.70	0.00	7.60	5.00
rs66664036	-267	->G	NR	3.40	NR	NR	NR
rs139256004	-267	GTTA>-	2.80	0.10	0.00	0.00	0.00
rs2231134	-307	G>C	0.20	2.60	0.00	4.10	0.10
rs45604438	-340	G>T	6.80	0.60	0.00	0.20	0.00
rs58132660	-400	G>A	0.20	0.10	0.01	0.00	0.01
rs142365584	-424	C>G	0.00	0.10	0.00	0.00	0.0
rs59370292	-435	C>T	NR	NR	0.80	NR	NR
rs59172759	-483	T>A	5.50	0.30	0.00	0.20	3.60

<sup>1</sup>SNP position is noted relative to the transcription start site

<sup>2</sup>Nucleotide change of the reference allele to the variant allele on the anti-strand as obtained from the UCSC genome browser

<sup>3</sup>Minor allele frequency (MAF) for African American (AFR), Mixed American (AMR), Eastern Asian (EAS) European (EUR) and South Asian (SAS) reported in dbSNP release 37 by 1000 genomes or PMT.

Abbreviations: NT, nucleotide; NR, not reported

Window Position  
Scale

chr4:89,079,997-89,080,517 (521 bp)

200 bases

DMD Fast Forward. Published on February 21, 2018 as DOI: 10.1124/dmd.117.079541  
This article has not been copyedited and formatted. The final version may differ from this version.

TSS

Sp1

Sp1

Sp1

CCAAT

AP1

NFkBRE

HRE

AhRE

ERE

PRE

ARE

CpG Islands

E2F1

HHM

RBBP5

1

GABPA

A

POLR2A

EGR1

K

CCNT2

K

GATA1

>>>>

pp

NR2F2

K

MYC

>>

HKMM

TFAP2C

<<<

E2F6

>>>>

KK

MAX

>>

KAHLKn

SIN3A

SIN3AK20

1p

TAL1

K

TFAP2A

<<<

USF1

1LK

USF2

1

JUND

HA-E2F1

Hm

Pol2

HLaap

GATA-1

p

Egr-1

K

TAF1

L

CCNT2

K

TAL1\_(SC-12984)

K

E2F1

H

USF-1

1K

AP-2gamma

H

E2F6\_(H-50)

K

HEY1

L

rs61181041

rs61535534

rs377282230

rs139256004

rs45604438

rs59370292

rs76656413

rs57327643

rs2231134

rs36068118

rs59172759

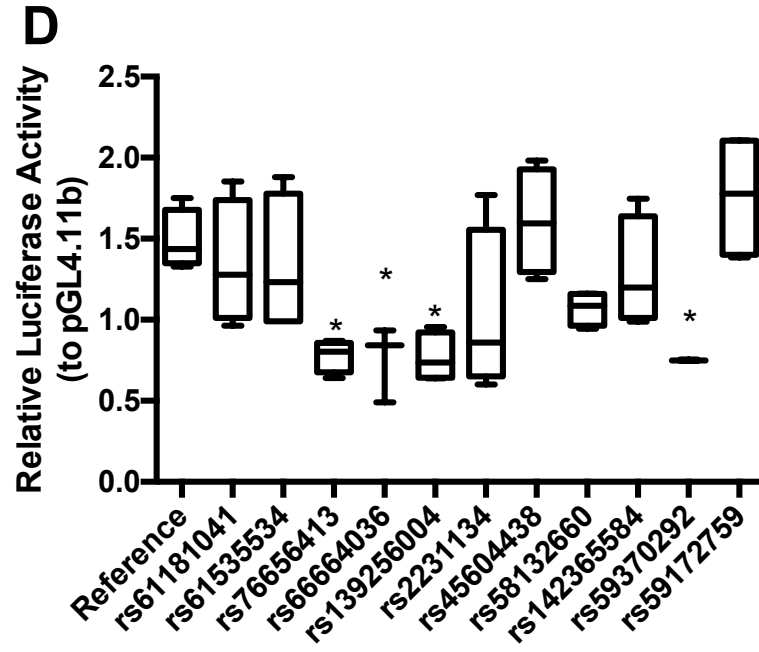
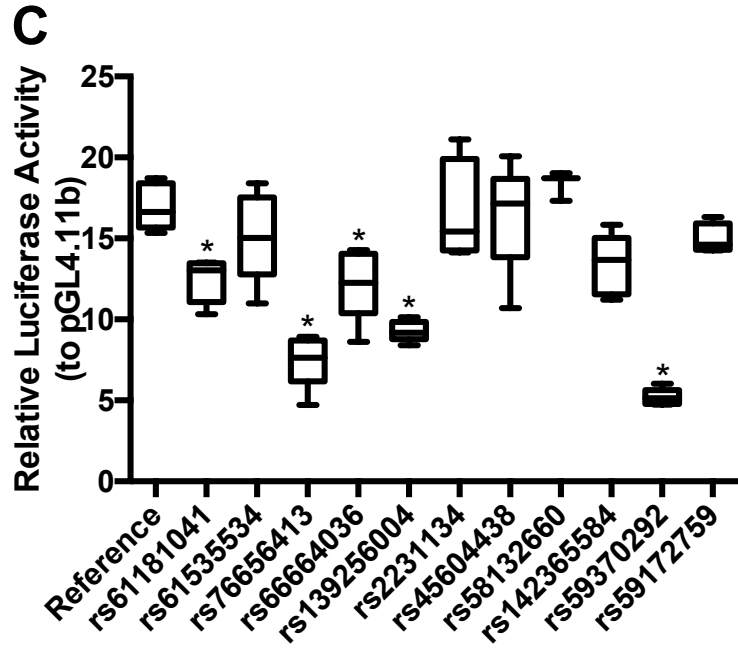
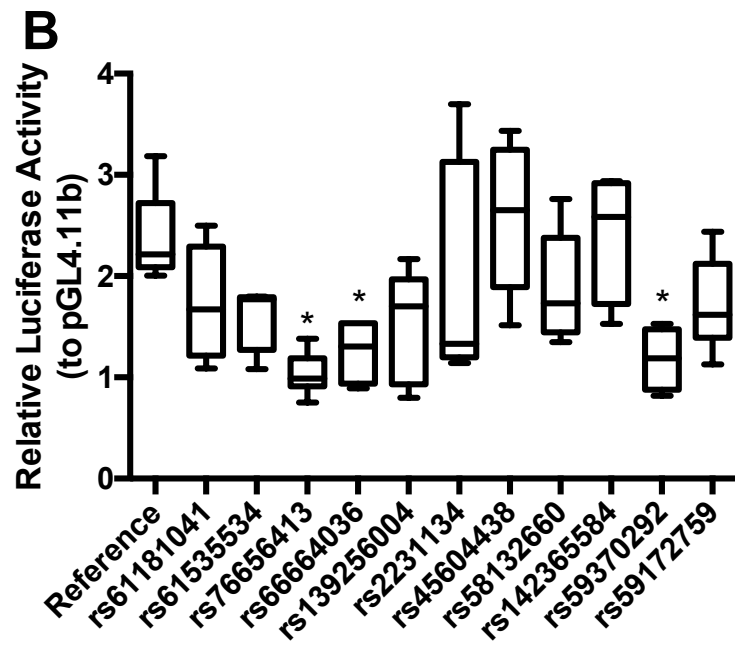
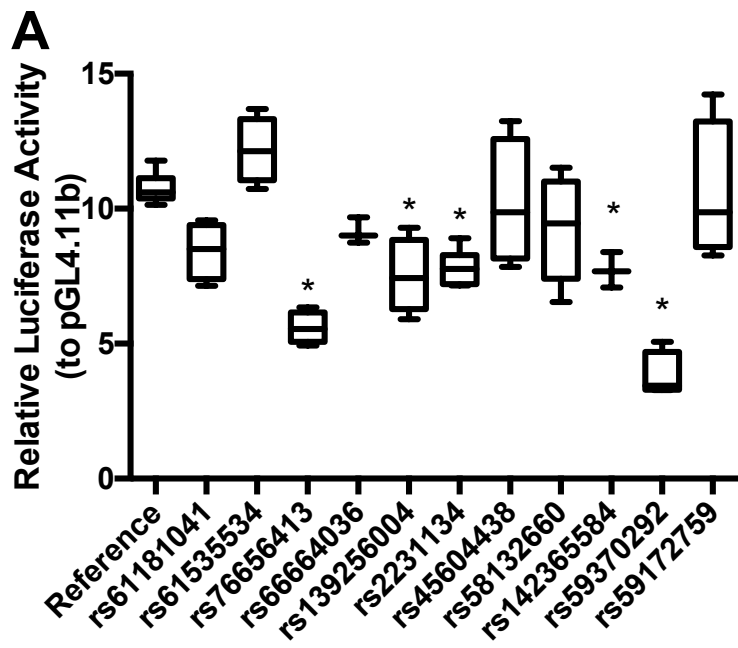
rs66664036

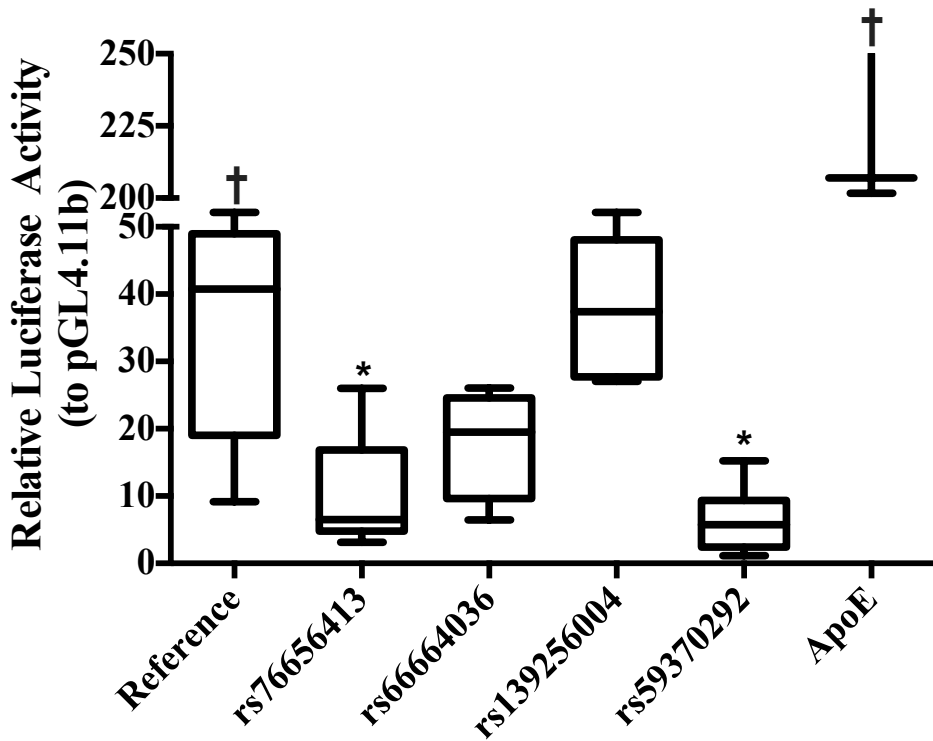
rs368986954

rs58132660

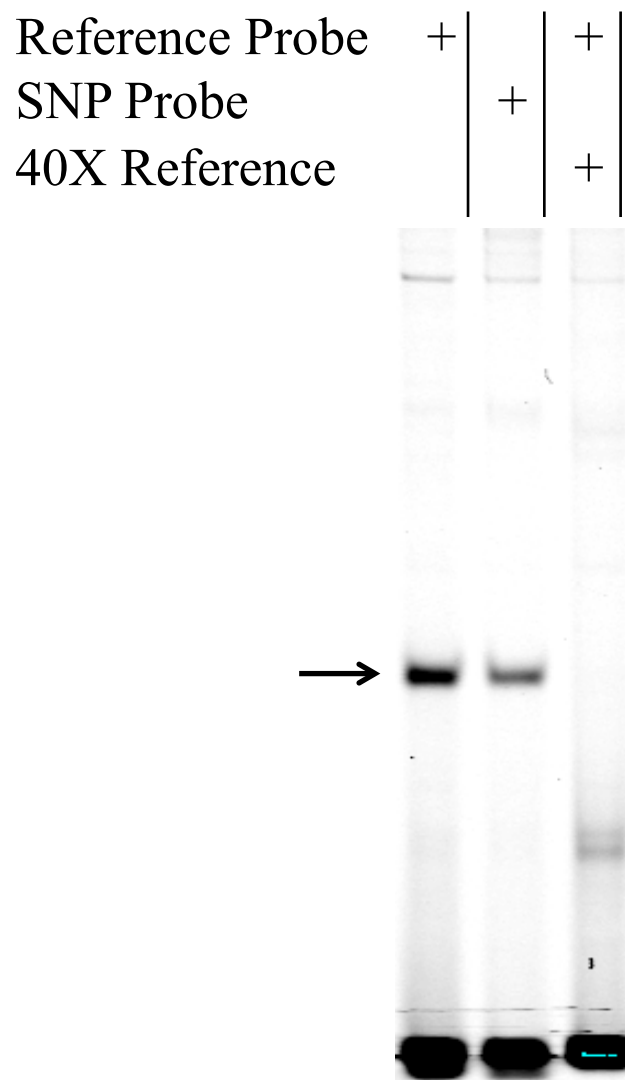
rs142365584





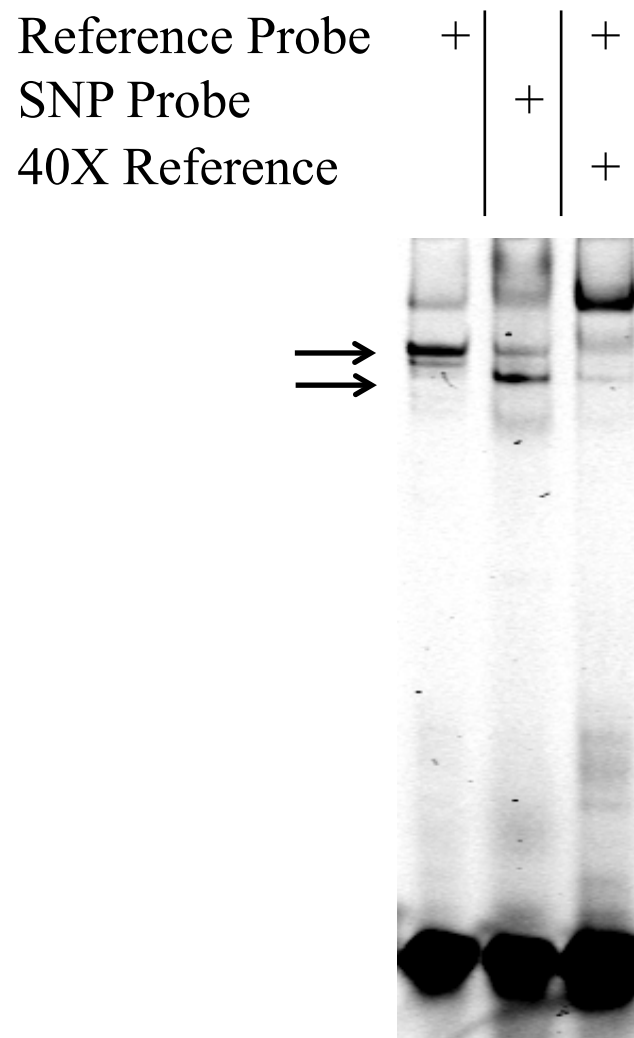


A



Promoter 5'-GAATGGGGATTCTG-3'  
 rs59370292 5'-GAATGGGAATTCTG-3'

B



Promoter 5'-GCACACGTGTCCT-3'  
 rs76656413 5'-GCACACATGTCCT-3'