New insights into the regulation of cytochrome P450 2C9 gene expression: 
the role of the transcription factor GATA-4

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Running Title: GATA-4 regulates CYP2C9 expression

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None-standard abbreviations:
AB, antibody; EMSA, electrophoretic mobility shift assay; ds oligonucleotide, double stranded oligonucleotide; NE, nuclear extract; PCR, polymerase chain reaction; fw, forward; rev, reverse; ChIP Chromatin Immunoprecipitation
Abstract

Cytochrome P4502C9 (CYP2C9) is an important drug metabolizing enzyme that metabolizes e.g. warfarin, antidiabetics and antiphlogistics. The endogenous regulation of this enzyme is however largely unknown. Here we examined the role of GATA transcription factors in the gene expression of CYP2C9. We investigated four putative GATA binding sites within the first 200 bp of CYP2C9 promoter at the positions I: -173/-170, II: -167/-164, III: -118/-115 and IV: -106/-103. Luciferase activity driven by a wild type CYP2C9 promoter construct was strongly upregulated in Huh-7 cells upon co-transfection with expression plasmids for GATA-2 and GATA-4, whereas mutations introduced into GATA binding site III or I and II reduced this induction to a significant extent. Electrophoretic mobility shift assays revealed specific binding of GATA-4 and GATA-6 to the oligonucleotides containing GATA binding sites I and II. Furthermore, the association of GATA-4 with CYP2C9 promoter was confirmed by chromatin immunoprecipitation assays in HepG2 cells. Taken together these data strongly suggest an involvement of liver-specific transcription factor GATA-4 in the transcriptional regulation of CYP2C9.
Introduction

Cytochrome P450 2C9 (CYP2C9) is an important enzyme involved in the metabolism of a large number of different drugs. It is the second most abundant cytochrome P450 enzyme in human liver (Miners and Birkett, 1998) and responsible for the transformation of about 16% of all used therapeutics including drugs like warfarin, losartan, phenytoin, tolbutamide, and different antiphlogistics (Urquhart et al., 2007). CYP2C9 is polymorphically expressed. The most common allelic variants in Caucasians are CYP2C9*2 and CYP2C9*3 which occur at a frequency of about 7% and 11%, respectively. Carriers of these variants show a slower metabolism towards CYP2C9 substrates and a considerably higher risk for adverse drug reactions (Kirchheiner and Brockmoller, 2005). An important example is the occurrence of bleeding complications upon treatment of CYP2C9 slow metabolizers with warfarin (Flockhart et al., 2008).

Interestingly, the CYP2C9 activity varies significantly within wild type carriers (Yasar et al., 2001; Scordo et al., 2002; Sandberg et al., 2004). Possible reasons for this phenomenon are the inducibility of CYP2C9 by different substrates, interindividual differences in the constitutive CYP2C9 expression (Peyvandi et al., 2004; Kirchheiner and Brockmoller, 2005), as well as polymorphic variations of the regulatory region (Kramer et al., 2008) (REFERENCE). Thus, it was demonstrated that CYP2C9 expression can be regulated by HNF4α and its co-regulators PGCα an SRC1 via Direct Repeat 1 (DR1) promoter elements (Kawashima et al., 2006; Martinez-Jimenez et al., 2006). CYP2C9 promoter activity is moreover influenced by HNF3γ (Bort et al., 2004) and by a crosstalk between Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR) with HNF4α upon induction by rifampicin (Chen et al., 2005).
In this study we focus on the zinc finger transcription factor family GATA, which is an important group of transcriptional regulators. The GATA family comprises six different members, GATA-1 to GATA-6, which recognize the consensus sequence (A/T)GATA(A/G).

GATA-1, -2 and -3 regulate the expression of genes involved in the development of blood cells (Harigae, 2006; Wu et al., 2007). GATA-4, -5 and -6 are specifically expressed in cardiac tissue (Peterkin et al., 2005) and play a key role in the transcriptional regulation of different genes involved in cardiac development and cardiomyocyte differentiation (Reiter et al., 1999; Crispino et al., 2001). In addition, GATA-4 is also expressed in liver and regulates here the expression of different liver detoxifying enzymes and transporters (Zhu et al., 2004; Kwintkiewicz et al., 2007; Sumi et al., 2007).

*In silico* analysis of the proximal CYP2C9 promoter indicates the presence of multiple GATA binding motifs, which prompted us to study the possible involvement of these factors in the regulation of CYP2C9 expression. Here we show by luciferase gene reporter assay, EMSA and ChIP analysis that CYP2C9 can be regulated by the transcription factor GATA-4.

Furthermore GATA-4 dependent activation of CYP2C9 is down regulated by an important co-regulator of GATA-4, Friend Of GATA-2 (FOG-2).
Materials and Methods

Plasmid constructs. Fragments of different length of CYP2C9 promoter were subcloned into the MluI/XhoI cloning sites of pGL3-Basic vector (Promega) upstream of the luciferase gene (constructs 2C9_-735, 2C9_-421, 2C9wt_ -331, Table 1, Figure 1B). Constructs with destructive mutations at all detected hypothetical GATA binding sites (positions (-173/-170, site I), (-167/-164, site II), (-118/-115, site III) and (-106/-103, site IV)) alone (constructs 2C9_-331_m1, 2C9_-331_m2, 2C9_-331_mut3, 2C9_-331_m4) or in combination (site I and site II, construct 2C19_-331_mut1+2) were generated using GeneTailor kit (Invitrogen) (Table 1, Figure 1B).

The human pCMV-FLAG2-GATA2 and mouse pcDNA1.1-GATA4 expression plasmids were kind gifts of Prof. Gokhan Hotamisligil (Harvard University, Boston) and Prof. Jeffery Molkentin (Children’s Hospital Medical Center, Cincinnati), respectively. The human pcDNA3-FOG-2 wild type construct as well as human pcDNA3-FOG-2_1-247 (expresses truncated protein missing the GATA-4 binding zinc finger domain) were kind gifts of Professor Erik Svensson (University of Chicago) (Svensson et al., 2000).

Transient transfections. Huh-7 human hepatoma cells were grown at 37°C in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). One day prior to transient transfection 2x10^5 Huh-7 cells were plated into 12-well plates. Two µg of the different pGL3-Basic expression vectors carrying CYP2C9 promoter fragments of different lengths as well as the constructs mutated at the different GATA sites were co-transfected with 0.5 µg of mouse pcDNA1.1-GATA4, human pCMV-FLAG2-GATA2 or 0.5 µg pcDNA3.1 empty vector (negative control). To investigate possible interactions between GATA and FOG proteins, GATA-2 or GATA-4
constructs were co-transfected with different amounts of a wild type (pcDNA3-FOG2) or 1µg of a mutated FOG construct (pcDNA3_FOG-2_1-247). Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's recommendations. Cells were harvested and analyzed for luciferase activity 24 hours (GATA co-transfections, Figure 2A and 2B) or 48 hours (co-transfections with GATA and FOG, Figure 5) after co-transfection. In each transfection mixture 2 ng of the plasmid harboring Renilla luciferase gene (pRL SV40, Promega) were included as an internal control for the transfection efficiency. Luciferase activity is expressed therefore as a ratio of Firefly luciferase activity (in arbitrary units) to the corresponding activity of the Renilla luciferase. All experiments were performed in triplicates and repeated three times.

**Electrophoretic Mobility Shift Assay (EMSA).** Nuclear protein extracts from Huh-7 cells were prepared according to the protocols of Dignam et al. (Dignam et al., 1983) and Nakabayashi et al. (Nakabayashi et al., 1991) with slight modifications. Eight different double stranded (ds) oligonucleotides comprising 50 bp of the CYP2C9 promoter were generated by annealing sense and antisense oligonucleotides (Table 1). The oligonucleotides were carrying the hypothetical GATA binding sites in wild type or in mutated form. Oligonucleotide labelling was carried out using $^{32}$P (Perkin Elmer) and T4 DNA polynucleotide kinase system (Invitrogen) in a final reaction volume of 25 µl. The mixes were incubated at 37°C for 15 min. The reaction was stopped with 5 µl of 0.2 M Na$_2$EDTA. For binding reactions 4% glycerol, 8 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma Chemical Co, St Louis, Mo) pH-7.9, 0.6 mmol/L MgCl$_2$, 50 mM NaCl, 1 µg of polydeoxyinosine:deoxycytosine (Poly[dI-dC].poly[dI-DC]), 12.8 fmol of $^{32}$P-labeled double-stranded probe (approx. 20000 cpm), and 4 µg of nuclear protein were mixed together in a total end volume of 25 µl. After 15 min of preincubation at 37°C for 15 min the different
labelled double stranded oligonucleotides were added to the mixes and the complete mixture was again incubated at 37°C for 15 min. For competition experiments, 1 and 10-fold excess of the respective unlabeled double stranded oligonucleotide was added to the probe prior to the addition of ^32^P-labelled ds oligonucleotides. Supershift experiments were carried out adding 6µg of GATA-2, -3, -4, or GATA-6 antibody (sc-1235x, sc-268x, sc 1237x, sc 9055x, Santa Cruz Biotechnologies) to the respective binding reaction before the addition of labelled oligonucleotides and samples were incubated on ice for 45 min. Finally 5µl of loading buffer were added to each sample. Protein-bound as well as unbound DNA was resolved on a 4% none denaturing polyacrylamide gel. Dried gels were subjected to autoradiography using phosphoimager (Fujifilm BAS-1800).

**Chromatin Immunoprecipitation (ChIP).** ChIP assay kit (Millipore) was used according to the manufacturer’s protocol. HepG2 cells were grown at 37°C in small dishes in Minimal Essential Medium (MEM), supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen) as well as 1% sodium pyruvate and 1% none essential amino acids. DNA-bound proteins were cross-linked to chromatin by adding 1% formaldehyde and incubated at 37°C. Harvested and lysed cells were sonicated to shear the DNA to fragments of around 500 bp. DNA was pre-cleared with salmon sperm DNA/protein A agarose slurry and afterwards incubated overnight with 2 µg of GATA-4 antibody (sc-1237X, Santa Cruz Biotechnology) or control IgG (rabbit normal IgG, sc-2027, Santa Cruz Biotechnology) at 4°C. The antibody/histone complexes were collected with salmon sperm DNA/protein A agarose slurry rotating the mixes for 2 hours at 4°C. The immunoprecipitate was pelleted and washed with low and high salt containing buffers and with TE buffer. The histone complex was eluted from the antibody and histone-DNA crosslinks were reversed at 65°C overnight. After treatment with proteinase K (Qiagen) and
puriﬁcation using QIAamp DNA Mini Kit (Qiagen), samples, including the input/sonicated DNA sample (positive control), were subjected to touch-down PCR using a primer pair, which generates a 225 bp fragment including all four GATA sites of interest.

**Statistical Analysis.** Statistical differences in reporter gene activity among *CYP2C9* promoter constructs were determined by one-way analysis of variance (ANOVA) followed by a Turkey's *post hoc* test using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA (www.graphpad.com). A p-value threshold of <0.05 was considered as statistically signiﬁcant in all analyses.
Results

**GATA factors activate the CYP2C9 promoter.** Analysis of the CYP2C9 promoter revealed four putative GATA binding sites at the positions (-173/-170), (-167/-164), (-118/-115) and (-106/-103) (Figure 1A). To investigate the influence of GATA transcription factors on CYP2C9 expression, 5’deletion fragments of CYP2C9 promoter were cloned upstream to firefly luciferase gene into the pGL3-Basic vector (Figure 1B) and transiently transfected into Huh-7 cells together with GATA-2, 4 and 6 expression vectors. The strongest effect was observed for GATA-2 and 4 transcription factors (GATA-6 results are not shown). As demonstrated in Figure 2A and 2B both factors strongly upregulate 331 bp long 5´deletion construct of the wild type CYP2C9 promoter. Identical upregulation was observed also for two other 5´deletion constructs, 435 and 735 bp long (results not shown), which would suggest localization of the potential GATA responsive site(s) in the proximal CYP2C9 promoter region.

**GATA dependent upregulation of CYP2C9 promoter is driven by GATA binding site I+II.** To investigate to which extent the *in silico* detected putative GATA binding sites are important for CYP2C9 regulation, the 331 bp long promoter constructs containing wild type or mutant GATA binding motifs (Figure 1B) were co-transfected with GATA-2 or GATA-4 expression vectors into Huh-7 cells. Artificial disruption of GATA sites I or II did not affect GATA-2 and GATA-4 dependent upregulation of luciferase activity (Fig. 2A, B). By contrast, disruption of the putative GATA site III caused a drastic drop of GATA-2 and GATA-4 effects down to pGL3basic level. Surprisingly, an equally strong loss in luciferase activity was observed for the CYP2C9 promoter construct carrying combined GATA I+II mutations (Fig. 2A, B).
Interestingly, the luciferase activity pattern in mock transfected cells always mimicked (though at a significantly lower level) the activity pattern observed with GATA-2 and GATA-4 overexpression. This phenomenon might be explained by the endogenous expression of GATA factors in the Huh7 cell line (data not shown).

**GATA-4 binds to two different GATA binding sites in proximal CYP2C9 promoter.** Next we investigated whether the putative GATA binding sites indeed interact with GATA transcription factor(s) using electrophoretic mobility shift assay. Different wild type and mutant $^{32}$P-labelled oligonucleotides comprising different combinations of wild type or disrupted forms of GATA binding sites (Fig. 3A and 3B) were incubated with nuclear extracts from Huh-7 cells. Protein-DNA complexes were formed with both 2C9wt1+2 (comprises GATA binding sites I and II) and 2C9wt3+4 (comprises GATA binding sites III and IV) oligonucleotides (Fig. 3A and 3B, lane 1). Mutation of site I and II (Fig. 3A, lanes 2, 3) did not affect the binding, whereas the oligonucleotide containing both mutations (mut1+2, fig. 3A, lane 4) is completely devoid of any binding activity. Similarly, disruption of the GATA site III (but not site IV) led to a significant loss of protein binding activity (Fig. 3B, lanes 2, 3). Taken together these results suggest binding of the nuclear factor to GATA site I+II as well as to the GATA site III, which is consistent with the gene reporter data.

Next we attempted to identify the protein that binds to these sites using antibodies against different members of the GATA family (GATA-2, GATA-3, GATA-4 and GATA-6). Among all antibodies used anti-GATA-4 and anti-GATA-6 were able to successfully supershift the formation of the protein/oligonucleotide complex observed with oligonucleotides 2C9wt1 and 2C9wt1+2 (Fig. 3A lanes 10, 11). Neither of these antibodies was found to shift the 2C9wt3 complex (Fig. 3B). These findings suggest that GATA-4 and GATA-6 can interact with CYP2C9 promoter.
GATA-4 is associated with CYP2C9 promoter. To investigate whether GATA-4 is indeed endogenously associated with the hypothetical GATA binding sites within CYP2C9 promoter we performed a chromatin immunoprecipitation assay using genomic DNA from HepG2 cells and antibodies against GATA-4. Promoter fragments were amplified by PCR using a primer set encompassing the putative GATA binding sites (Table 1). As shown on Figure 4 (lane 1) the primer set was able to generate a PCR product that matches exactly with the predicted promoter fragment containing the GATA sites in question. No PCR product was observed with immune complexes formed by control IgGs demonstrating the specificity of the assay (Figure 4, lane 2). This finding confirms that GATA-4 is associated with CYP2C9 promoter even in intact cells.

FOG-2 counters the GATA-4 dependent CYP2C9 promoter activation. GATA proteins are known to be often co-regulated by the transcription factor family FOG, including the members FOG-1 and FOG-2. In particular, FOG-2 interacts with GATA-4 and influences a GATA-4 dependent transcriptional regulation of different target genes. Several studies showed that a disruption of FOG-2 / GATA-4 interaction can lead to pathological forms of heart development during organogenesis (Tevosian et al., 1999; Svensson et al., 2000; Crispino et al., 2001). To investigate whether FOG-2 is also involved in GATA-4-dependent CYP2C9 promoter regulation we used the luciferase gene reporter approach co-transfecting GATA-4 and FOG-2 expression plasmids with the CYP2C9 promoter constructs used in previous experiments. Figure 5 shows the co-transfection results for promoter construct 2C19_-331_wt, GATA-4 and FOG-2. While the co-transfection only with GATA-4 led to an expected upregulation of CYP2C9 promoter (Figure 5, bar no. 2), the addition of FOG-2 attenuated this effect. The extent of inhibition correlated with the amount of transfected FOG-
2 plasmid (Figure 5, bar no. 3 to 6). In line with this result the inhibitory effect was clearly reduced when co-transfecting the mutant construct pcDNA3-FOG-2_1-247 that lacks the fragment coding for the GATA-binding zinc finger domain (Figure 5, bar no.7). These data strengthen the hypothesis that CYP2C9 is regulated by the GATA-4 transcription factor.
Discussion

Present study provides for the first time with the experimental evidence for the involvement of GATA transcription factors in the regulation of CYP2C9 expression. This assumption is supported by the following facts: i. GATA-2 and 4 significantly increase the activity of CYP2C9 promoter fragments containing potential GATA binding sites; ii. FOG-2, a known regulator of GATA proteins was found to modulate these effects; iii. Physical interaction of GATA factors (GATA-4 in particular) with the promoter was shown by ChIP and EMSA experiments indicating that such binding may occur both in vivo and in vitro.

Which of the GATA family transcription factors are actually important for CYP2C9 regulation and which of the four potential GATA cis-elements in the promoter are their immediate targets? Whereas the comprehensive answers to these questions are still not available, our data would support the following hypotheses:

1. GATA-2, 4, and 6 displayed CYP2C9 promoter regulating activity in various experimental approaches shown in this study. This is not surprising provided high degree of conservation between the DNA binding domains of different family members and their ability to bind to the conserved GATA response element. Therefore, it can be speculated that the selection of CYP2C9 specific GATA factor should be resolved by the tissue specific expression of the particular GATA factor. It has been previously shown that some liver expressed P450s and drug transporters are regulated by GATA-4. These include CYP19  (Cai et al., 2007) and epoxide hydroxylase (Zhu et al., 2004) and the ATP-binding cassette transporters ABCG5 and ABCG8 (Sumi et al., 2007). These data suggest that GATA-4, which has been previously associated mainly with the regulation of genes involved in heart development, seems to be also important for the expression of genes implicated in the drug metabolism or drug transport
in the liver. Our findings are furthermore supported by the fact that liver cells predominantly express only two members of GATA family, GATA-4 and to a much lesser extent GATA-6, two important regulatory factors of liver-specific gene expression (Molkentin, 2000).

2. To answer the question, which of the potential GATA binding sites is actually more involved in the interaction with GATA factors, a comparison of all results obtained for both putative double binding sites is necessary. The ChIP assay gives ambiguous information since the minimal length of the GATA site carrying promoter fragment, which is a target of PCR amplification, is about 200 bp and it comprises all four predicted GATA binding elements. The gene reporter assay indicates site I+II and III as binding candidates. Both sites form specific complexes in EMSA, however, only the site I+II complex was supershifted by GATA-4 and 6. In addition, this sequence is strongly conserved in the proximal promoter of the closely related CYP2C19. Therefore this site can be suggested as a most likely candidate for the interaction with GATA factor(s).

CYP2C9 has been shown also to be regulated by different other transcription factors including e.g. HNF4α and HNF3γ (Bort et al., 2004; Kawashima et al., 2006). Binding sites for these transcription factors are located in direct neighborhood of the newly detected GATA sites within CYP2C9 promoter. It remains to be investigated to which extent GATA factors are able to interact with these regulatory proteins.

Transcriptional regulation of CYP2C9 by the GATA transcription factor family might be partly responsible for interindividual differences in CYP2C9 activity seen in wild type carriers of CYP2C9. Further understanding of this variation is of importance as CYP2C9, a principal metabolizing enzyme of coumarins together with VKORC1, is strongly involved in the development of drug side effects seen in patients.
In addition to hepatocytes, GATA-mediated regulation of CYP2C9 might be of physiological relevance also in other tissues, in particular in endothelial cells. CYP2C9 is expressed in these cells where it metabolizes arachidonic acid into epoxyeicosatrienoic acids with a concomitant generation of reactive oxygen species (Chehal and Granville, 2006). Both factors are important vasoreactive regulators and are also involved in the pathogenesis of cardiovascular diseases. GATA-2 is the most abundantly expressed GATA factor in endothelial cells (Lee et al., 1991), and is the key regulator of endothelial-specific genes (Lugus et al., 2007). Further exploration of the GATA-2 involvement in the regulation of CYP2C9 in cardiovascular system will certainly shed more light on the role of CYP2C9 in vascular biology and cardiovascular diseases.
References


Footnotes

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# the authors share the last authorship
Figure legends

Figure 1. Detected GATA motifs in CYP2C9 promoter and design of luciferase reporter constructs encompassing four possible GATA binding sites.

A. The DNA sequence surrounding four possible GATA binding sites of CYP2C9 gene promoter. The A of the first codon ATG is numbered as +1. GATA motifs I to IV are shown in upper case and highlighted in bold. B. Schematic representation of 5´-truncated CYP2C9 promoter fragments cloned upstream to luciferase reporter gene. Numbers refer to fragment length. Mutated GATA binding sites are indicated in italic.

Figure 2. GATA-2 and GATA-4 upregulate CYP2C9 promoter in gene reporter assay

Relative luciferase activities of CYP2C9 promoter fragments subcloned into pGL3-Basic vector (Figure 1B) and of pGL3-Basic control vector (negative control) after co-transfection with pGATA2-CMV-FLAG2 (GATA-2) or pcDNA3.1 empty vector (mock) (A) and pGATA4-pcDNA1.1 (GATA-4) or pcDNA3.1 empty vector (mock) (B) in Huh-7 cells. All deletion constructs were in general highly upregulated upon co-transfection with GATA-2 or GATA-4. **p<0.01 for 2C9wt_-331, 2C9_mut1_-331, 2C9_mut2_-331 (A) and 2C9wt_-331 (B) co-transfected with pcDNA3.1 against co-transfection with GATA2 (A) or GATA -4 (B). ***p<0.001 for 2C9_mut1_-331, 2C9_mut2_-331 and 2C9_mut4_-331 (B) co-transfected with pcDNA3.1 against co-transfection with GATA -4 (B). ***p<0.001 for 2C9_-331_mut1+2 or 2C9_-331_mut3 versus 2C9_-331_wt (A), and ***p<0.001 for 2C9_-331_mut1+2 or 2C9_-331_mut3 (B) versus 2C9_-331_wt. Data are presented as mean values ± SD of three independent experiments. Each experiment was performed in triplicate.
Figure 3. GATA-4 binds to the oligonucleotides containing GATA binding motif I and II in EMSA analysis using nuclear extracts from Huh-7 cells.

EMSA was performed using double stranded oligonucleotides (Table 1) comprising GATA binding site I and II (A) or GATA binding site III and IV (B), respectively, in wild type (2C9 wt 1+2 or 2C9 wt 3+4) or mutated (2C9 mut 1, 2C9 mut 2, 2C9 mut 1+2, 2C9 mut 3, 2C9 mut 4) forms. Nuclear extracts were prepared from Huh-7 cells. The binding complexes are indicated by white arrows. Supershift experiments were performed using antibodies against GATA-2, -3, -4, and -6. A successful competition was observed with antibodies against GATA-4 and GATA-6 using oligonucleotide 2C19 wt 1+2 (Figure 3A, lane 10 and 11, indicated by black arrows); Comp, competition reactions with cold 2C9 wt 1+2 (A) and 2C9 wt 3+4 (B). An additional competition controls were performed using cold mutant oligonucleotides in combination with labeled wild types oligonucleotides (results not shown).

Figure 4. GATA-4 associates with CYP2C9 promoter in HepG2 cells.

GATA-4 binding to CYP2C9 promoter was analyzed using ChIP assay. Immune complexes precipitated with GATA-4 antibody and control IgG antibody were PCR amplified using a primer set encompassing a fragment around all four putative GATA binding sites. Successful amplification was seen in the agarose gel electrophoresis after immunoprecipitation with GATA-4 antibody. Additional negative control was performed with a primer set generating an amplicon not containing any GATA site (results not shown). Input is a positive PCR control, i.e. sonicated DNA (starting material before immunoprecipitation).

Figure 5. FOG-2 attenuates GATA-4 effects on CYP2C9 promoter.

Relative luciferase activities of promoter construct 2C9_-331_wt co-transfected with 0.5µg GATA-4 and varying amounts of wild type FOG-2 or FOG-2_1-247 in Huh7 cells.
pcDNA3.1 empty vector (mock) was used as a negative control and to adjust the total amount of transfected plasmid to a minimum level of 0.55µg or a maximum of 1.5µg.

*** p<0.001. Data are presented as mean values ± SEM of three independent experiments.
Table 1. Oligonucleotides used for cloning, EMSA and ChIP experiments.

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<th>Primer Name</th>
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<td><strong>Cloning primers</strong></td>
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<td>Primer set</td>
<td>5’ - CCAA CCAAGTACAGTGAAACT</td>
</tr>
<tr>
<td></td>
<td>5’ - TTAAGACAAACCATGAGCTGTCA</td>
</tr>
</tbody>
</table>

Primers for cloning were designed based on the CYP2C9 sequence (GenBank accession number **NT_030059**). Fragments for reporter constructs were PCR amplified from a CYP2C9 construct encompassing the first 1.8kb of CYP2C9 5’ - flanking region (kind gift of Dr. Mia Sandberg Lundblad, construct slightly modified). Nonsense mutations in GATA sites I to IV were introduced into construct 2C9_-331_wt resulting in the plasmids 2C9_-331_m1, 2C9_-331_m2, 2C9_-331_m3, 2C9_-331_m4, and 2C9_-331_m1+2, respectively. 2C9wt1+2 and
2C9wt3+4: EMSA oligonucleotides containing wild type GATA binding site I and II or GATA binding site III and IV. 2C9mut1, 2C9m2, 2C9m3, 2C9m4, 2C9m1+2: Oligonucleotides with destructive mutations in GATA binding site I, II, III, IV or I and II together. GATA binding sites are underlined. Mutations that destroy any GATA motifs are highlighted in bold. ChIP Primer set was used in ChIP analysis leading to a PCR product of 225bp of length. The PCR product includes all four possible GATA binding sites. fw, forward; rev, reverse primers.
1A

Site I  Site II
-173  attt tatattt  TATCtTATC  cagtgggtca  -144

Site III  Site IV
-118  ggacctaggt  GATTgttca  attTATC  catcaagag  -83

1B

2C9_-735
-735

2C9_-421
-421

2C9_-331

2C9_-331_m1

2C9_-331_m2

2C9_-331_m3

2C9_-331_m4

2C9_-331_m1+2