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**Regulation of N-Myc downstream regulated gene 2 by bile acids**

C dric Langhi<sup>1</sup>, Elena Pedraz-Cuesta, Yolanda Donate, Pedro F. Marrero, Diego Haro, and Joan C. Rodr guez\*

Department of Biochemistry and Molecular Biology, Faculty of Pharmacy and the Institute of Biomedicine of the University of Barcelona, E-08028 Barcelona, Spain

\*Corresponding author. Address: Departament de Bioqu mica i Biologia Molecular, Facultat de Farmàcia, Universitat de Barcelona, Joan XXIII s/n, E-08028 Barcelona, Spain. Tel. and Fax: 34-934024520.

*E-mail address:* jcrodriguez@ub.edu (J.C Rodr guez)

<sup>1</sup>Present address: Department of Biochemistry and Molecular Biology, Edward A. Doisy Research Center, Saint Louis University School of Medicine, 1100 South Grand Ave. St. Louis, MO 63140

**ABSTRACT**

Here we report that bile acid chenodeoxycholic acid (CDCA) and synthetic farnesoid X receptor (FXR) agonist GW4064 robustly induced tumor suppressor N-Myc downstream regulated gene 2 (*NDRG2*) expression in human hepatoma cells and primary hepatocytes. Knockdown of FXR abolished the induction by CDCA, whereas overexpression of a constitutively active form of FXR increased *NDRG2* expression. A FXR-response element was identified within intronic regions of human and murine genes. Moreover, mice given GW4064 exhibit an increase of *Ndr2* expression in liver and kidney, where both *NDRG2* and FXR are enriched. The identification of *NDRG2* as a bile acid regulated gene may provide novel knowledge towards the understanding of *NDRG2* physiological function and the link between metabolism and cancer.

**Keywords:** bile acids; *NDRG2*; nuclear receptor; FXR; cancer; metabolism; kidney.

**Abbreviations:** CDCA, chenodeoxycholic acid; EMSA, electrophoretic mobility shift assay; ENaC, epithelial sodium channel; FXR, farnesoid X receptor; HCC, hepatocellular carcinoma; I-BABP, ileal-bile acid-binding protein; IR1, inverted repeat separated by a nucleotide; MOI, multiplicity of infection; *NDRG2*, N-myc downstream-regulated gene 2; PLTP, phospholipid transfer protein; QPCR, real-time quantitative PCR; RXR, retinoid X receptor; siRNA, small interfering RNA; SHP, small heterodimer partner; TK, thymidine kinase; TSS, transcription start site.

## INTRODUCTION

N-myc downstream-regulated gene 2 (NDRG2) belongs to the *NDRG* family, comprised of four members, *NDRG1-4*, which are reportedly involved in cell proliferation and differentiation [1,2]. *NDRG2* is highly expressed in many normal tissues, especially in the brain, heart, skeletal muscle, liver, and kidney, whereas low expressed or undetectable in a variety of human cancer cell lines and carcinomas [1,2]. Recently, NDRG2 was identified as a novel tumor metastasis suppressor in hepatocellular carcinoma (HCC) [3]. Although the precise molecular function of NDRG2 is not yet known, accumulated data indicate that low expression of *NDRG2* may serve as a prognostic biomarker for malignant transformation in hepatocytes. In addition, it has been demonstrated that *NDRG2* overexpression diminishes the proliferative, invasive and migratory abilities of HCC cells [3]. Thus, the identification of factors capable of inducing hepatic *NDRG2* expression may provide new opportunities for therapeutic intervention in liver cancer. Interestingly, *Ndr2* was initially characterized as an early aldosterone-induced gene in rat kidneys [4]. Thereafter, it was proposed that NDRG2 may affect the function of the epithelial sodium channel (ENaC) [5], which is localized in the apical membranes of sodium absorbing epithelia like the distal nephron and hence plays a key role in the maintenance of blood pressure. Consequently, the physiological role of NDRG2 may extend beyond cell proliferation.

The farnesoid X receptor  $\alpha$  (FXR $\alpha$ , NR1H4, hereafter referred to as FXR), a member of the nuclear hormone receptor superfamily, is the chief sensor of intracellular levels of bile acids [6]. FXR is highly expressed in the liver, kidney, intestine and the adrenal gland. In response to bile acid binding, FXR transcriptionally regulates a variety of genes involved in bile acid, lipid and glucose metabolism [6]. Since two independent groups reported that the loss of FXR in *Fxr*-null mice resulted in spontaneous hepatocarcinogenesis [7,8], considerable research efforts have been

focused on the identification of the underlying mechanisms of liver cancer inhibition by FXR and its relevance in the pathogenesis of human HCC. Subsequently, it has shown that FXR expression in human HCC samples was decreased compared to normal liver tissues [9]. Notwithstanding, the specific nature of the FXR target genes involved in the inhibition of liver cancer is still unknown.

## MATERIALS and METHODS

### Plasmids

A 2.4-kb fragment spanning from the 5' flanking region to the second exon of *Ndr2* gene was amplified from genomic DNA using primers 5'-TATCATAAGCGGCGGGACTCGGCTGAGGAG-3' and 5'-AGTCGCTAGCCTCCTGAAGTTCTGCAATGGTG-3', *KpnI/NheI* digested, and subcloned into pGL3-basic vector (Promega) to obtain p-437/+1966NDRG2. Mutagenesis was performed using QuickChange<sup>TM</sup> site-directed mutagenesis kit (Stratagene) with primers 5'-GGGGAGATTAGAAAGAAATTAGTGATTTCTGGAATCCTGGGTCTTGGC-3' and 5'-GCCAAGACCCAGGATTCCAGAAATCACTAATTTCTTTCTAATCTCCCC-3'. Reporter plasmids p(NDRG2IR1)<sub>n</sub>-TK (*n* = 1, 2, 4) were generated by insertion of an oligonucleotide obtained by annealing 5'-GATCCTTAGAAAGGGTTAGTGACCCTGGAA-3' and 5'-GATCTTCCAGGGTCACTAACCCTTTCTAAG-3' into the *Bgl*III site of pGL3-TK [10]. Similarly, the four-copy mutant IR1 construct p(mutNDRG2IR1)<sub>4</sub>-TK was generated by annealing 5'-GATCCTTAGAAAGAAATTAGTGATTTCTGGAA-3' and 5'-GATCTTCCAGAAATCACTAATTTCTTTCTAAG-3'. Plasmids expressing human retinoid X receptor  $\alpha$  (RXR $\alpha$ , NR2B1) and FXR into pSG5 have been previously described [10].

### **Animal experiments**

Experimental protocols with mice were performed with the approval of the animal ethics committee of the University of Barcelona (Spain). C57BL/6J mice and Fxr-null mice [11] (FXR<sup>-/-</sup>, #007214) were obtained from The Jackson Laboratory (Bar Harbor, ME). 10-week-old male mice were injected i.p. with either vehicle (corn oil 5% DMSO) or GW4064 (GlaxoSmithKline, RTP, NC) dissolved in vehicle (10 mg/ml) at a dose of 50 mg/kg and then fasted for 8 h before sacrificed for tissue collection. Livers and kidney were excised, snap-frozen in liquid nitrogen and stored at -80 °C.

### **Cell culture and treatment conditions**

Human hepatoma HepG2 and Huh7 cells and mouse hepatoma Hepa 1-6 cells were cultured in DMEM supplemented with 10% FBS. Mouse immortalized hepatocytes AML12 were grown in DMEM/Ham's F-12 medium supplemented with 10% FBS, 100 nM dexamethasone, and ITS (Roche Applied Science). Human primary hepatocytes were obtained commercially (Ready Heps<sup>TM</sup> Fresh Hepatocytes, Lonza, Switzerland) and maintained in Hepatocyte Complete Medium (HCM<sup>TM</sup> bulletkit, Lonza). Cells were treated with ligands in the same medium supplemented with 10% charcoal stripped FBS (Biological Industries). For each experiment, duplicate or triplicate dishes were plated for each condition, as indicated in the figures.

### **siRNA transfection**

For siRNA-mediated FXR knockdown experiments, the human FXR siGENOME SMARTpool (M-003414-01) or siGENOME Non-Targeting siRNA #1 (D-001210-01)

(Dharmacon, Lafayette, CO) were used to transfect Huh7 cells in triplicate with the DharmaFECT 4 Transfection Reagent at a final concentration of 25 nM each for 48 h, followed by 24 h of treatments as described above.

### **Adenoviral infection**

Huh7 and Hepa 1-6 cells were infected at a multiplicity of infection (MOI) of 25 or 50 for 48h with adenoviruses expressing VP16 (AdVP16) or VP16FXR chimeras (AdVP16FXR) previously described [12].

### **Cell transfection and reporter assays**

HepG2 cells were transiently transfected as previously described [10]. After 6h, cells were treated for 24 h with the vehicle (DMSO), 100  $\mu$ M CDCA, or 1  $\mu$ M GW4064. All transfections were performed in triplicate, and similar results were obtained in at least three independent experiments.

### **Western blot analysis**

Whole protein cell extracts were obtained from cultured cells or liver and kidney of mice. Cells were homogenized in Nonidet P-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40) supplemented with a mixture of protease inhibitors and 0.1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Equal amounts of protein (30-50  $\mu$ g) were run in 10% polyacrylamide gels, transferred onto Immobilon-P membranes (Millipore, Bedford, MA) and probed with antibodies: anti-NDRG2 (1:200, Abnova, cat# H00057447-M03); anti-FXR (1:1000, Invitrogen, cat# A9033A); anti-actin (1:1000, Sigma-Aldrich, cat# A2066).

**RNA isolation and real-time quantitative PCR analysis (QPCR)**

Total RNA was isolated by using Tri-Reagent and was further treated with DNase I (Ambion). cDNA was synthesized from total RNA (1 µg) by murine leukemia virus reverse transcriptase (Invitrogen) and p(dN)6 random primers (Roche Diagnostics). QPCR was performed using Platinum<sup>®</sup> Quantitative PCR SuperMix-UDG with ROX (Invitrogen) and TaqMan<sup>®</sup> Gene Expression Assay probes (Applied Biosystems): *NDRG2*, Hs00212263\_m1; *PLTP*, Hs01067287\_m1; *NR0B2*, Hs00222677\_m1; *NR1H4*, Hs00231968\_m1; *Ndr2*, Mm00443483\_m1; *Nr0b2*, Mm00442278\_m1. Relative mRNA abundance was obtained by normalizing to 18S levels.

***In silico* analysis of FXR response elements (FXREs)**

The analysis of genomic sequences for the identification of putative FXREs was performed using NUBIScan computer algorithms ([www.nubiscan.unibas.ch/](http://www.nubiscan.unibas.ch/)). The alignment of sequences of different mammals was carried out using VISTA tools ([genome.lbl.gov/vista/index.shtml](http://genome.lbl.gov/vista/index.shtml)).

***In vitro* transcription/translation and electrophoretic mobility shift assay (EMSA)**

Oligonucleotides corresponding to the sequence spanning nt +887 to +911 of *Ndr2* (NDRG2IR1) were radiolabeled and used as probe as previously described [10]. For competition experiment, increasing fold molar excess of unlabeled probe NDRG2IR1, or a modified version harboring mutations in the IR1 hexamers (mutNDRG2IR1), were included during a 15 min preincubation on ice. A cold probe containing the FXRE of the human ileal-bile acid-binding protein (*I-BABP*) gene was used as a control as previously described [10].



**Chromatin immunoprecipitation (ChIP) assay**

HepG2 cells were treated with 1% formaldehyde for 10 min, and then disrupted in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8) with protease inhibitors for 10 min. Chromatin was sonicated to shear DNA, precleared using Protein A/G PLUS Agarose (sc-2003, Santa Cruz Biotechnology), and immunoprecipitated overnight with 5  $\mu$ g of anti-FXR (sc-1204 X, Santa Cruz Biotechnology) or goat IgG (I9140, Sigma-Aldrich). Precipitated DNA was extracted with phenol/chloroform and resuspended in 50  $\mu$ l of water. Input and immunoprecipitated DNA were subjected to PCR amplification using primers encompassing the FXRE in the proximal promoter of human *NR0B2* (5'-AGCTAGTGATAAGGCACTTCC-3' and 5'-GTGGCACTGATATCACCTCAG-3'), the putative FXRE in the second intron of human *NDRG2* (5'-CCTCTTACCTCCACATCAGC-3' and 5'-ACACAGCAA CGAGGTGAATG-3'), or an unrelated genomic region from human alcohol dehydrogenase gene cluster lacking any functional FXRE (C. Langhi and J. C. Rodríguez, unpublished results) (5'-AGGAGACATCGGATCTTCTGG-3' and 5'-CTGTCATGCTAGCATCTCTCC-3'). PCR products were electrophoresed in agarose gel, visualized with ethidium bromide, quantified by ImageJ and normalized by input amplification.

**Statistical Analysis**

Data are expressed as mean  $\pm$  SEM as determined by analysis of multiple independent samples, as indicated in figure legends. Significant differences compared with the corresponding controls were assessed using a two-tailed Student's *t* test. Values of  $p < 0.05$  were considered to be significant.

## RESULTS

### Human hepatic *NDRG2* mRNA and protein levels are increased by FXR ligands

Microarray analysis revealed that expression of *NDRG2* is up-regulated by FXR agonists (E. Pedraz and J. C. Rodriguez, unpublished data). To confirm this observation, human hepatocarcinoma Huh7 were treated with increasing concentrations of CDCA, a natural bile acid agonist of FXR, or GW4064, a synthetic FXR agonist, and the endogenous expression of *NDRG2* was determined by QPCR. Treatment with both FXR ligands resulted in a dose-dependent increase in the levels of *NDRG2* mRNAs (Fig. 1A). To ensure that the induction of *NDRG2* by FXR ligands is not limited to a single hepatoma cell line, we also analyzed RNA from HepG2 cells, obtaining similar results (Fig. 1B). In order to extend our results to a more physiologically relevant system, we treated human primary cultured hepatocytes. Both FXR agonists induced mRNA levels of *NDRG2* and phospholipid transfer protein (*PLTP*), an established FXR target gene [13], to a similar extent (Fig. 1C). Next, Western blot analyses performed on whole cell lysates from Huh7 incubated with CDCA, GW4064, or vehicle revealed that the quantity of *NDRG2* protein was robustly increased (15-fold) by both FXR ligands (Fig. 1D).

### Induction of *NDRG2* expression by CDCA and GW4064 requires FXR expression

Since bile acids may exert their actions through FXR-independent pathways, we silenced FXR by siRNA to determine whether the induction of *NDRG2* expression observed upon treatment with CDCA was only dependent on FXR expression. Huh7 cells were therefore transfected with non-targeting siRNA or siRNA complexes directed against FXR (siFXR) before

ligand treatments. siFXR-mediated knock-down of endogenous FXR levels (Fig. 2A and B) completely blocked the induction of *NDRG2* expression by CDCA (Fig. 2C). We also confirmed by siFXR the dependence on FXR in the response to GW4064. As a control, CDCA- and GW4064-dependent increase of the mRNA levels of *NR0B2* (hereafter referred to as *SHP*), a previously characterized FXR target gene [14], was similarly attenuated in siFXR-transfected cells (Fig. 2C and D).

### **Gain-of-function on FXR induces *NDRG2* expression**

We performed gain-of-function studies by ectopically expressing a chimera of FXR and transcriptional activator VP16 (VP16FXR) that is constitutively active. Infection of Huh7 cells with increasing MOIs of adenoviruses expressing VP16FXR resulted in a dose-dependent response induction of *NDRG2* expression compared to VP16 alone (Fig. 2E).

### **Mouse hepatic *NdrG2* expression is induced by activated FXR**

Activation of FXR in AML12 cells, a non tumorigenic established cell line from mouse normal liver, strongly induced *NdrG2* mRNA levels (Fig. 3A). On the other hand, no induction of *NdrG2* expression was observed in mouse hepatoma Hepa 1-6 cells that do not express FXR (Fig. 3B and C). However, a significant induction of *NdrG2* expression was observed in Hepa 1-6 when FXR was ectopically overexpressed (Fig. 3C and D). We next treated mice with either vehicle or 50 mg/kg of GW4064 for 8 h and determined liver NDRG2 protein levels by Western blot analysis. As shown in Fig. 3E, NDRG2 levels were significantly increased by GW4064 *in vivo*. Taken together, these expression data indicate that hepatic regulation of the NDRG2/*NdrG2* gene by FXR shares a conserved mechanism between human and mouse.

### ***Ndr*2 expression is induced by activated FXR in kidney**

Given the roles of NDRG2 in renal function [4,5] and since both FXR and NDRG2 are enriched in kidney, we investigated whether *Ndr*2 expression was regulated by FXR activation in this organ. The i.p. administration of GW4064 induced *Ndr*2 expression in kidneys of wild type, but not FXR<sup>-/-</sup> mice (Fig. 3F). As expected, activation of FXR *in vivo* also increased *Shp* mRNA levels in the kidneys. In addition, we determined that GW4064 treatment markedly increased renal NDRG2 protein levels of wild type mice (Fig. 3G).

### **Characterization of an IR1 element within intronic regions of *NDRG2/Ndr*2 genes as a functional FXRE**

Most known FXREs consist of an inverted repeat of the hexanucleotide motif RGGTCA with minor variants spaced by one nucleotide (IR1) [6]. Therefore, we scanned the 5' flanking regions of human and mouse *NDRG2/Ndr*2 genes for putative FXREs using the NUBIScan computer algorithm. However, no putative IR1 was identified in the region 10 kb upstream of the transcription start site (TSS) of any of these genes. Further *in silico* analysis of the intronic regions led to the identification, as the best hit, of a putative IR1 present within the second intron of human *NDRG2* gene (at +1560 from TSS) and the first intron of murine *Ndr*2 gene (at +894 from TSS) that matched the consensus for FXREs (Fig. 4A). Sequence alignment using VISTA tools revealed that this IR1 is conserved in several mammalian species (Fig. 4B).

Treatment of transfected cells with natural (CDCA) or synthetic (GW4064) FXR agonists significantly increased luciferase activity of a reporter construct under the control of a 2.4-kb fragment of the *Ndr*2 gene (p-437/+1966NDRG2) (Fig. 4C). Cotransfection of a FXR

expression plasmid robustly enhanced gene activation. Mutation of this IR1 element completely abolished the response to activated FXR (Fig. 4D). Next, this IR1 motif was tested in the context of heterologous promoter to evaluate whether it is sufficient to confer FXR-mediated regulation. Activated FXR enhanced the activity of *Ndr2* IR1-driven thymidine kinase (TK) promoter constructs in a copy-dependent manner, whereas a reporter construct with the TK promoter alone or with four copies of a mutated version of *Ndr2* IR1 was not stimulated at all (Fig. 4E).

EMSA were performed using *in vitro* translated FXR and RXR $\alpha$  and an oligonucleotide containing the *Ndr2* IR1 element. Neither FXR nor RXR $\alpha$  alone bound to the probe (Fig. 4F, lanes 2 and 3), but a complex with the probe was formed when both proteins were present, indicating that the IR1 element is bound by FXR/RXR $\alpha$  heterodimers (lane 4). The specificity of this retarded complex was demonstrated by competition analysis showing that it was competed away by increasing concentrations of either the unlabeled NDRG2IR1 probe (Fig. 4F, lanes 5-7) or a cold probe containing a well characterized IR1/FXRE from the *I-BABP* (Fig. 4F, lanes 11-13), whereas it was relatively unaffected by the presence of the unlabeled DNA containing the mutated IR1 sequence (mutNDRG2IR1) (Fig. 4F, lanes 8-10). Furthermore, ChIP experiments demonstrated that FXR binds the identified *NDRG2* IR1 in HepG2 cells (Fig. 4G).

## DISCUSSION

Our data show that *NDRG2* expression is induced by bile acids. We have ascertained that these responses depend on FXR and do not require the synthesis of intermediate proteins. As demonstrated by mutation analysis in transient transfection and binding experiments, induction by FXR may be attributed to an IR1 located within intronic regions of human and murine genes.

Taken together, our results demonstrate that *NDRG2* is a direct target of FXR. During the process of submitting our results for publication, Deuschle and colleagues have reported that FXR synthetic agonist PX20606 reduce liver tumor growth and metastasis in an orthotopic mouse xenograft model and proposed that *NDRG2* partly mediates these effects [15]. These results are coincident to our current report in showing the induction of *NDRG2* mRNA by FXR synthetic agonists in human hepatoma cell lines and livers of mice. Main data in the present study not addressed in that report include: a) results showing that natural bile acid CDCA induces *NDRG2* expression (Fig. 1A-D) and knockdown experiments demonstrating that the physiological regulation of *NDRG2* by bile acids absolutely requires FXR (Fig. 2C); b) induction of *NDRG2* by FXR agonists in primary cultures of human hepatocytes (Fig. 1C); and c) induction of *Ndr2* expression in kidneys of wild type, but not FXR<sup>-/-</sup> mice (Fig. 3G). Notably, in addition to tumor suppression, *NDRG2* has also been related to the regulation of sodium gradient in several reports. First, *NDRG2* was identified as a mineralocorticoid-induced gene in renal cortical collecting duct, the site of aldosterone-regulated sodium absorption [4]. Later, studies in heterologous expression systems showed that co-expression of *NDRG2* and ENaC resulted in a stimulation of ENaC-mediated Na<sup>+</sup> currents [5]. More recently, Yao and co-workers have shown that *NDRG2* directly binds and stabilizes the  $\beta$ 1-subunit of Na<sup>+</sup>/K<sup>+</sup>-ATPase, the motor of sodium reabsorption [16]. Based on our data showing regulation of *Ndr2* expression by FXR in kidney, we believe that characterization of a potential role of FXR in regulating sodium reabsorption and fluid volume homeostasis is an interesting area of future investigation.

In conclusion, our results have demonstrated a direct link between bile acids and *NDRG2*, which may help in the elucidation of its physiological role.

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## REFERENCES

- [1] L. Yao, J. Zhang, X. Liu, NDRG2: a Myc-repressed gene involved in cancer and cell stress, *Acta Biochim. Biophys. Sin. (Shanghai)*. 40 (2008) 625-635.
- [2] V. Melotte, X. Qu, M. Ongenaert, W. van Criekinge, A.P. de Bruine, H.S. Baldwin, M. van Engeland, The N-myc downstream regulated gene (NDRG) family: diverse functions, multiple applications, *FASEB J.* 24 (2010) 4153-4166.
- [3] D.C. Lee, Y.K. Kang, W.H. Kim, Y.J. Jang, D.J. Kim, I.Y. Park, B.H. Sohn, H.A. Sohn, H.G. Lee, J.S. Lim, J.W. Kim, E.Y. Song, D.M. Kim, M.N. Lee, G.T. Oh, S.J. Kim, K.C. Park, H.S. Yoo, J.Y. Choi, Y.I. Yeom, Functional and clinical evidence for NDRG2 as a candidate suppressor of liver cancer metastasis, *Cancer Res.* 68 (2008) 4210-4220.
- [4] S. Boulkroun, M. Fay, M.C. Zennaro, B. Escoubet, F. Jaisser, M. Blot-Chaubaud, N. Farman, N. Courtois-Coutry, Characterization of rat NDRG2 (N-Myc downstream regulated gene 2), a novel early mineralocorticoid-specific induced gene, *J. Biol. Chem.* 277 (2002) 31506-31515.
- [5] M.O. Wielputz, I.H. Lee, A. Dinudom, S. Boulkroun, N. Farman, D.I. Cook, C. Korbmacher, R. Rauh, (NDRG2) stimulates amiloride-sensitive Na<sup>+</sup> currents in *Xenopus laevis* oocytes and fisher rat thyroid cells, *J. Biol. Chem.* 282 (2007) 28264-28273.
- [6] Y. Zhang, P.A. Edwards, FXR signaling in metabolic disease, *FEBS Lett.* 582 (2008) 10-18.
- [7] I. Kim, K. Morimura, Y. Shah, Q. Yang, J.M. Ward, F.J. Gonzalez, Spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice, *Carcinogenesis.* 28 (2007) 940-946.

- [8] F. Yang, X. Huang, T. Yi, Y. Yen, D.D. Moore, W. Huang, Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor, *Cancer Res.* 67 (2007) 863-867.
- [9] N. Liu, Z. Meng, G. Lou, W. Zhou, X. Wang, Y. Zhang, L. Zhang, X. Liu, Y. Yen, L. Lai, B.M. Forman, Z. Xu, R. Xu, W. Huang, Hepatocarcinogenesis in FXR<sup>-/-</sup> mice mimics human HCC progression that operates through HNF1alpha regulation of FXR expression, *Mol. Endocrinol.* 26 (2012) 775-785.
- [10] X. Prieur, H. Coste, J.C. Rodriguez, The human apolipoprotein AV gene is regulated by peroxisome proliferator-activated receptor- $\alpha$  and contains a novel farnesoid X-activated receptor response element, *J. Biol. Chem.* 278 (2003) 25468-25480.
- [11] C.J. Sinal, M. Tohkin, M. Miyata, J.M. Ward, G. Lambert, F.J. Gonzalez, Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis, *Cell.* 102 (2000) 731-744.
- [12] S. Modica, S. Murzilli, L. Salvatore, D.R. Schmidt, A. Moschetta, Nuclear bile acid receptor FXR protects against intestinal tumorigenesis, *Cancer Res.* 68 (2008) 9589-9594.
- [13] N.L. Urizar, D.H. Dowhan, D.D. Moore, The farnesoid X-activated receptor mediates bile acid activation of phospholipid transfer protein gene expression, *J. Biol. Chem.* 275 (2000) 39313-39317.
- [14] T.T. Lu, M. Makishima, J.J. Repa, K. Schoonjans, T.A. Kerr, J. Auwerx, D.J. Mangelsdorf, Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors, *Mol. Cell.* 6 (2000) 507-515.
- [15] U. Deuschle, J. Schuler, A. Schulz, T. Schluter, O. Kinzel, U. Abel, C. Kremoser, FXR Controls the Tumor Suppressor NDRG2 and FXR Agonists Reduce Liver Tumor Growth and Metastasis in an Orthotopic Mouse Xenograft Model, *PLoS One.* 7 (2012) e43044.
- [16] Y. Li, J. Yang, S. Li, J. Zhang, J. Zheng, W. Hou, H. Zhao, Y. Guo, X. Liu, K. Dou, Z. Situ, L. Yao, N-myc downstream-regulated gene 2, a novel estrogen-targeted gene, is involved in the regulation of Na<sup>+</sup>/K<sup>+</sup>-ATPase, *J. Biol. Chem.* 286 (2011) 32289-32299.

## FIGURE LEGENDS

### **Fig. 1. Induction of *NDRG2* expression by bile acid CDCA and a synthetic FXR agonist.**

Huh7 (A) and HepG2 (B) cells were treated for 24 h with vehicle (Control), or increasing concentrations of CDCA or GW4064. (C) Primary hepatocytes isolated from human liver were



incubated with vehicle (Control), 50  $\mu$ M CDCA, or 5  $\mu$ M GW4064 for 24 h. Total RNA was extracted and reverse transcribed for analysis by QPCR. Specific *NDRG2* and *PLTP* mRNA levels normalized to 18S content are expressed relative to control set as 1 (mean  $\pm$  SEM). (D) Huh7 cells were treated in triplicate dishes with vehicle (Control), 50  $\mu$ M CDCA, or 5  $\mu$ M GW4064 for 24 h, and 30  $\mu$ g of total protein lysates prepared from each dish were loaded in each lane for analysis by Western blot. Protein signals were quantified and *NDRG2* levels were normalized to actin content. Significant differences compared with the corresponding controls are as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

**Fig. 2. Studies with FXR silencing and gain-of-function.**

(A) Huh7 cells were transfected in triplicate for 48 h with either control siRNA (siControl) or siRNA targeting FXR (siFXR), and *FXR* mRNA expression levels were determined using QPCR. (B) Western blot analysis were performed with 30  $\mu$ g of total protein lysates prepared from two of the replicate dishes in each transfection condition (one dish per lane). (C-D) siRNA transfected Huh7 cells were treated with vehicle (Control), 50  $\mu$ M CDCA (C), or 5  $\mu$ M GW4064 (D) for 24 h. (E) Huh7 cells were infected with increasing MOI of adenovirus expressing either VP16 (AdVP16) or a constitutively active chimera of VP16 and FXR (AdVP16FXR) for 48 h. Specific mRNA levels of *FXR* and *NDRG2* were determined by QPCR, normalized to 18S content and expressed relative to control set as 1 (mean  $\pm$  SEM). Significant differences compared with the corresponding controls are as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

**Fig. 3. Induction of mouse *Ndr2* expression by activated FXR.** AML12 (A) and Hepa 1-6 (B) cells were treated in triplicate dishes with vehicle (Control), 50  $\mu$ M CDCA, or 5  $\mu$ M GW4064 for 24 h. (C) Western blot analysis using an antibody against FXR and cell lysates from Huh7 and Hepa1-6 that were infected or not (No Ad) in duplicate with 50 MOI of adenovirus expressing either VP16 (AdVP16) or a chimera of VP16 and FXR (AdVP16FXR) for 48 h. Each lane was loaded with 30  $\mu$ g of total protein lysates prepared from individual dishes. (D) Relative mRNA levels of *Ndr2* in Hepa 1-6 infected in triplicate with 50 MOI of the indicated adenovirus. (E) Western blot analysis of cell lysates from livers of mice that were treated for 8 h with either vehicle (Control) (n=4) or 50 mg/kg GW4064 (n=5). Protein signals were quantified and NDRG2 levels were normalized to actin content. (F) Relative mRNA levels of *Ndr2* and *Shp* in kidneys of wild-type (WT) and FXR<sup>-/-</sup> mice that were treated as in (E) (n=5/group). (G) Renal protein levels of NDRG2 were determined by Western blot, and signals were quantified and normalized to actin. Significant differences compared with the corresponding controls are as follows: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

**Fig. 4. Characterization of an intronic IR1 in *NDRG2/Ndr2* genes as an FXRE.** (A) Schematic representation of exon-intron structure of human and mouse *NDRG2/Ndr2* genes and localization of the IR1 elements identified as FXREs. Empty boxes and black solid boxes indicate untranslated and translated regions of exons, respectively. (B) Comparison of sequences of different mammals. (C) HepG2 cells were transfected with a plasmid containing a luciferase reporter gene driven by a 2.4-kb fragment of the *Ndr2* gene or the empty pGL3-basic vector along with a plasmid expressing FXR or the empty pSG5 as control. Cells were treated with vehicle (Control), 100  $\mu$ M CDCA, or 1  $\mu$ M GW4064 for 24 h, and luciferase activities were

measured. (D) Experiments as in (C) using reporter construct containing wild-type or IR1 site-directed mutated sequence, or the empty pGL3-basic vector, together with a plasmid expressing FXR or pSG5, in the presence of vehicle or 1  $\mu$ M GW4064. (E) Experiments as in (C) with reporter constructs containing 1, 2, or 4 copies of the wild-type (NDRG2IR1), or 4 copies of mutant (mutNDRG2IR1) *Ndr2* IR1 site in front of TK promoter-driven luciferase gene. Results are expressed as -fold induction over control. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . (F) EMSAs were performed using in vitro transcribed/translated FXR, RXR $\alpha$ , or unprogrammed reticulocyte lysate (-), and a labeled oligonucleotide containing the *Ndr2* IR1 element (NDRG2IR1). The FXR-RXR $\alpha$ -FXRE complex is indicated by an arrow. Competition experiments for FXR-RXR $\alpha$  binding were performed by adding a 50-, 200-, and 500-fold molar excess of unlabeled probes. Mutations in the modified version of *wtNDRG2IR2* (mutNDRG2IR1) are shown in (D). *I-BABP*, FXRE of *I-BABP* gene [10]. (G) ChIP assays were performed with chromatin from HepG2 cells and either IgG or FXR-specific antibody and primers encompassing FXREs in human *NDRG2* and *SHP*, as positive control, or an unrelated genomic region with no FXRE (Control), as negative control.

Figure 1

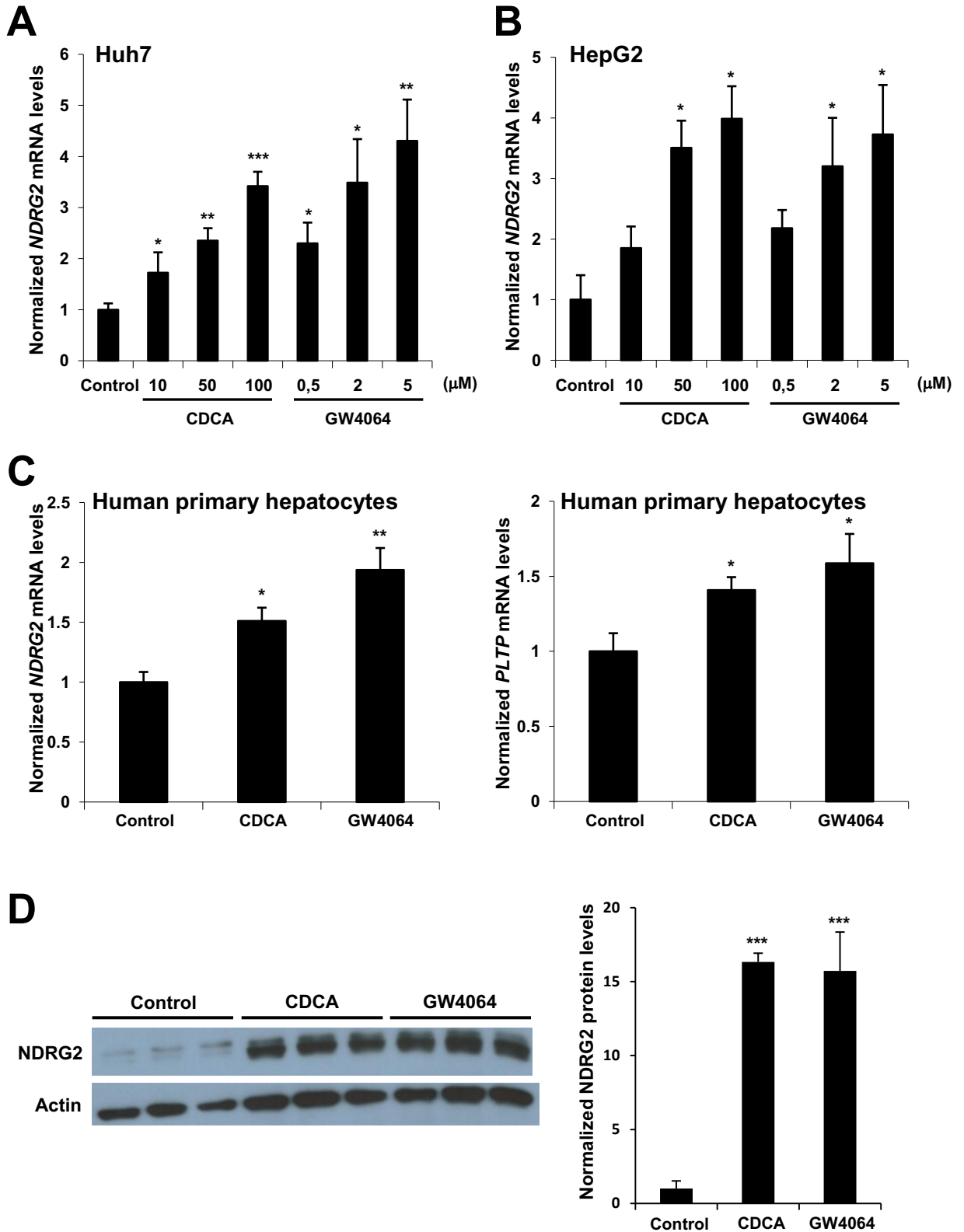


Figure 2

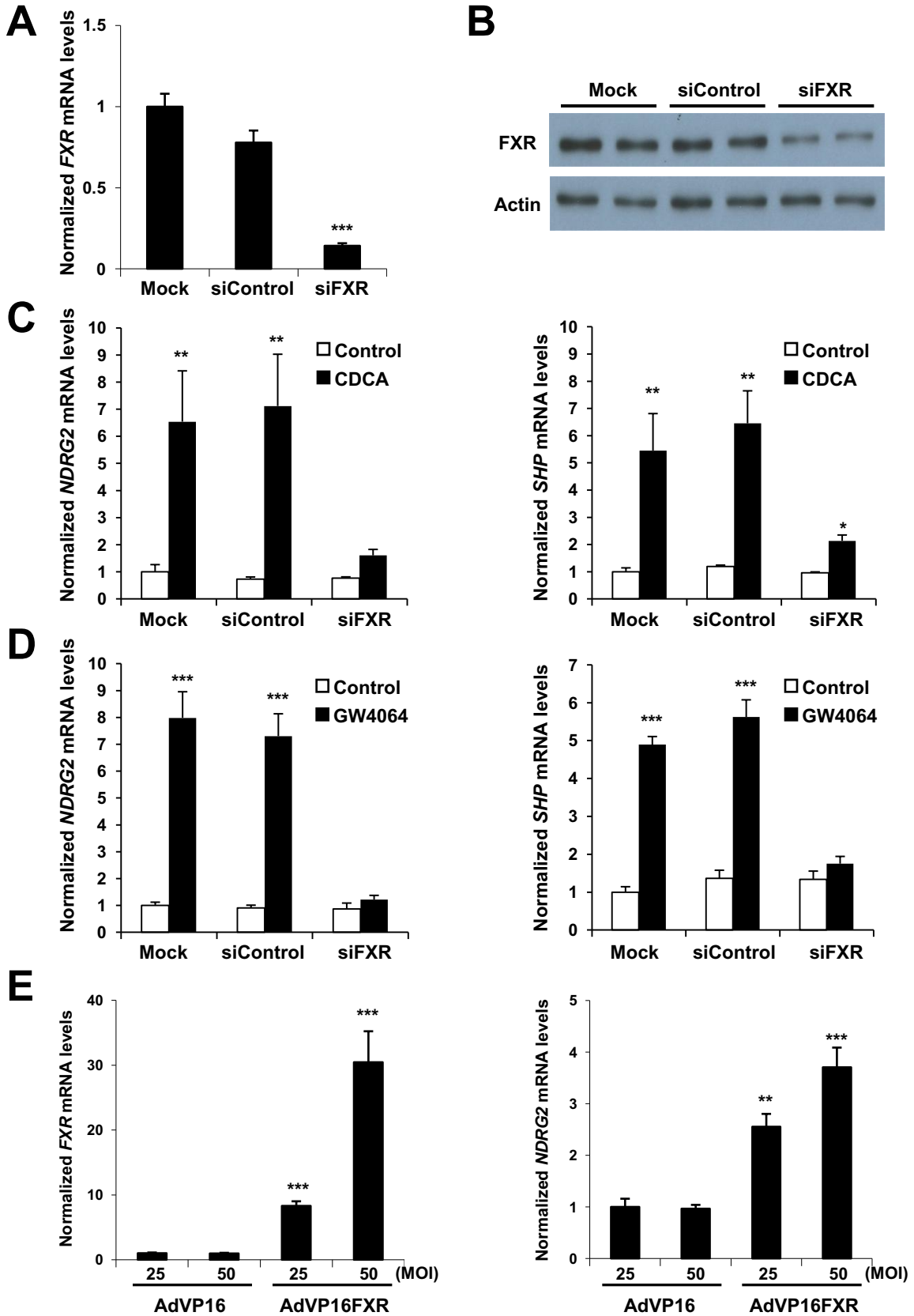
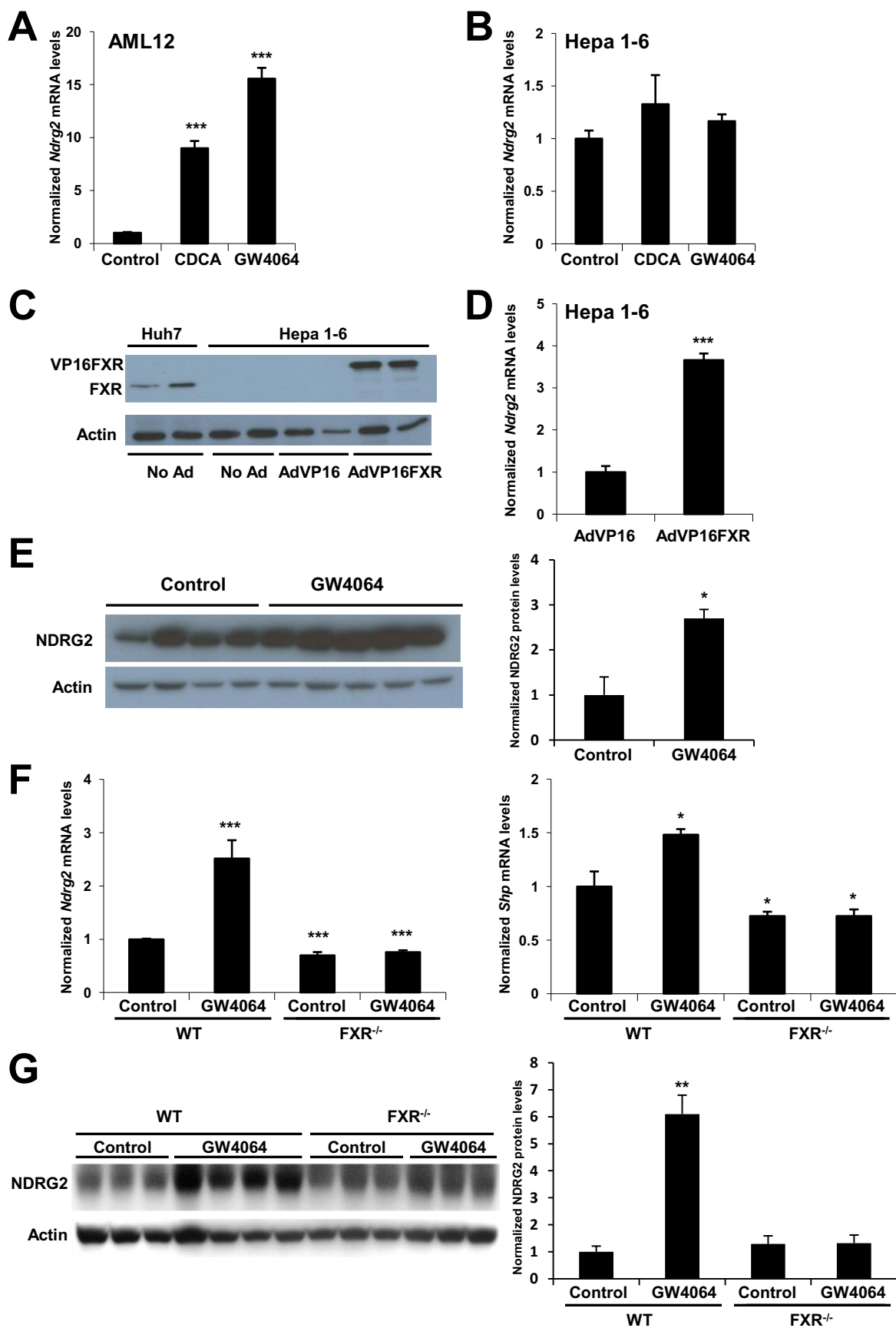
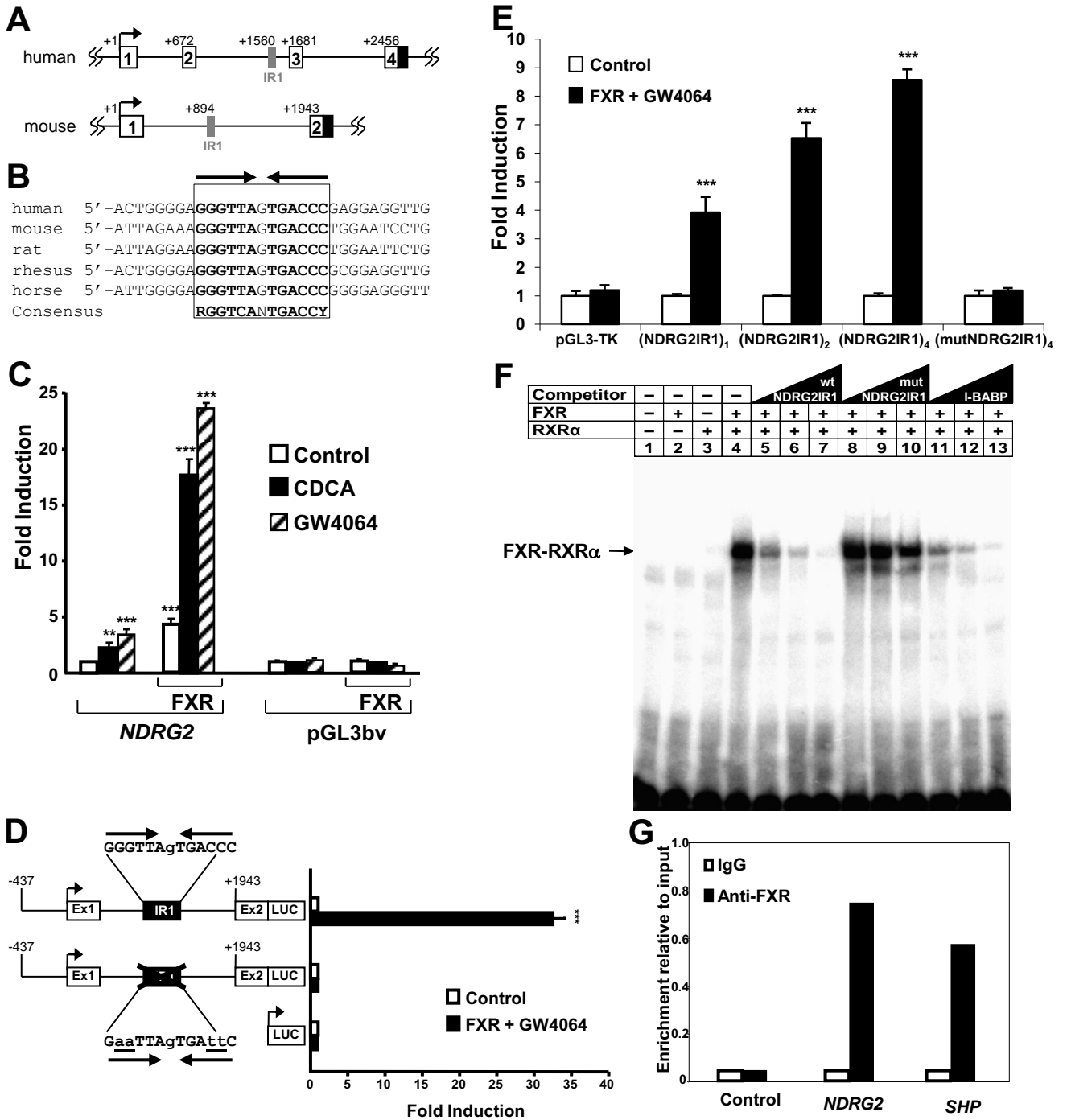


Figure 3



**Figure 4**



**Highlights**

- Bile acid CDCA induces tumor suppressor NDRG2 expression in hepatocytes.
- Induction of NDRG2 by bile acids require FXR expression.
- An intronic FXR-response element was identified in human and murine genes.
- FXR activation increases NDRG2 mRNA and protein levels in kidney.
- We provide knowledge towards the understanding of NDRG2 physiological function.

ACCEPTED MANUSCRIPT