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Title page

The human ADFP gene is a direct LXR target gene and differentially regulated by synthetic LXR ligands.

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Running title page

Running Title: hADFP regulated by LXR

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List of abbreviations

ADFP, adipocyte differentiation-related protein; PAT family, Perilipin, ADFP and TIP47 family; LXR, liver X receptor (gene symbols: LXRα, NR1H3 and LXRβ, NR1H2); GW3965, 3-[3-[[[2-Chloro-3-(trifluoromethyl)phenyl]methyl](2,2- diphenylethyl)amino]propoxy]benzeneacetic acid hydrochloride; T0901317,N-(2,2,2-Trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1(trifluoromethyl)ethyl]phenyl] benzenesulfonamide; RXR, retinoid X receptor (gene symbol NR2B); LXRE, LXR response element; ChIP, chromatin immunoprecipitation; UTR, untranslated region; TG, triglyceride; PAT, ; NR, nuclear receptor; SREBP1c, sterol regulatory element binding protein 1c; DR, direct repeat; IR, inverted repeat; PXR, pregnane X receptor (gene symbol NR1I2); FXR, farneosid X receptor (gene symbol NR1H4); 9c-RA, 9cis-retinoic acid; SR12813, [[3,5-Bis(1,1-dimethylethyl)-4-hydroxyphenyl]ethenylidene]bis-phosphonic acid tetraethyl ester; GW4064, 3-[2-[2-Chloro-4-[[3-(2,6-dichlorophenyl)-5-(1-methylethyl)-4-isoxazolyl]methoxy]phenyl]ethenyl]benzoic acid; PMSF, phenylmethylsulphonyl fluoride; bp, base pair; CBP/p300, CREB-binding protein/p300 ; Pol II, RNA polymerase II; qRT-PCR, quantitative real-time polymerase chain reaction.

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ABSTRACT

Expression of adipocyte differentiation-related protein (ADFP), residing on the surface of lipid droplets, correlates to hepatic fat storage. In the context of consequences and treatment of metabolic disorders, including hepatic steatosis, it is imperative to gain knowledge about the regulation of the human ADFP gene. The nuclear receptor liver-X-receptor (LXR) is a key regulator of hepatic fatty acid biosynthesis and cholesterol homeostasis, and a potential drug target. Here, we report that two synthetic LXR ligands differently regulate human ADFP expression. The partial LXR agonist GW3965 significantly induces ADFP expression in human primary hepatocytes whereas the full agonist T0901317 does not. Bioinformatics analysis revealed several potential LXREs response elements (LXREs) in the human ADFP gene. By using chromatin immunoprecipitation (ChIP) and luciferase reporter assays, we show that LXR, upon stimulation with GW3965, directly regulates human ADFP transcription by binding to LXREs located in the 3'UTR and the 5'-flanking regions. The ligand-stimulated LXR recruitment was associated with recruitment of RNA polymerase II and the coactivators CBP/p300 to the promoter region demonstrating that the identified LXREs are functional and able to induce transcription. Moreover, our results show that sequence identity of the hexamer repeats in DR4 elements is not sufficient to determine if the element binds or not binds LXR. The partial agonist GW3965 specifically regulates ADFP gene transcription and our data prove that the two synthetic LXR agonists, commonly used in experimental research, can differentially regulate gene expression. This has implications for pharmaceutical targeting of LXR.

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INTRODUCTION

Excessive accumulation of lipids, particularly in non-adipose tissues, is implicated in conditions associated with metabolic disorders; examples of the conditions are insulin resistance, beta cell dysfunction, atherosclerosis and hepatic steatosis. The structure of the storage site of neutral lipids such as triglycerides (TG) and cholesterol esters, the lipid droplet, resembles that of lipoprotein particles; lipid droplets consist of a core of neutral lipids, surrounded by a monolayer of phospholipids and cholesterol onto which lipid-droplet associated proteins are attached (reviewed in (Martin and Parton, 2006; Olofsson et al., 2009)). Adipocyte differentiation-related protein (ADFP), a member of the PAT family of proteins (Londos et al., 1999), is ubiquitously expressed and found on the surface of lipid droplets in most cells (Brasaemle et al., 1997). Mice deficient in the *Adfp* gene have reduced hepatic TG content, are resistant to diet-induced fatty liver and have increased hepatic insulin sensitivity (Chang et al., 2006; Varela et al., 2008). Furthermore, in hepatic cells in culture, overexpression of ADFP increases intracellular TG storage while inhibition of ADFP expression decreases the amount (Magnusson et al., 2006). Thus, the expression of ADFP correlates to fat storage in the liver.

In pharmaceutical strategies aimed at finding remedies for metabolic disorders members of the nuclear receptor (NR) family of proteins, including liver-X-receptors (LXRs), have emerged as potential drug targets (Makishima, 2005; Michael et al., 2005). A wealth of data demonstrates the importance of LXR in the context of cholesterol homeostasis and that pharmacological activation of LXRs has beneficial anti-atherogenic effects (Joseph and Tontonoz, 2003). However, a severe side effect is induction of hypertriglyceridemia and hepatic steatosis, largely due to LXR activation of SREBP1c, a key regulator of lipogenic genes in the liver (Grefhorst et al., 2002). There are two LXR isoforms, LXRα (NR1H3) and LXRβ (NR1H2), with LXRα being highly expressed in the liver and thus, suggested to be the mediator of the adverse effects.

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Endogenous LXR-ligands are oxidized cholesterol derivatives, oxysterols, and the LXRs form obligate heterodimers with the retinoid X receptors (RXRs) and bind LXR response elements (LXREs) in target genes containing direct repeats (DR) of the prototypical sequence AGGTCA separated by four nucleotides (DR4) or by one nucleotide (DR1); an inverted repeat separated by one nucleotide (IR1) has also been identified as an LXRE (Varga and Su, 2007). The synthetic dual LXRα/β agonists, T0901317 (Schultz et al., 2000) and GW3965 (Collins et al., 2002), have been widely utilized to explore LXR biology. It has however been found that T0901317 is also a very potent pregnane-X-receptor (PXR; NR1I2) ligand (Mitro et al., 2007). Activation of the bile acid sensor, the farnesoid-X-receptor (FXR; NR1H4) by T0901317 has also been demonstrated (Houck et al., 2004).

In a recent study on primary human hepatocytes we could, as expected, show that exposure to GW3965 increased TG accumulation and genome wide expression profiling suggested induction of the ADFP gene. Indeed, we could confirm increased ADFP mRNA expression in response to GW3965, which correlated to increased ADFP protein levels (Kotokorpi et al., 2007). In the present study we have shown that the human ADFP gene is a direct LXR target gene and differentially regulated by the synthetic LXR ligands, GW3965 and T0901317.

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MATERIALS AND METHODS

Cell cultures

Human primary hepatocytes were isolated from resected or unused donor liver tissue essentially as previously described (Strom et al., 1996). Primary cells were seeded onto biomatrix-coated dishes and cultured as previously described (Kotokorpi et al., 2007). Willams' E medium (Invitrogen, Paisely, Scotland, UK) supplemented with antibiotics and 3 nM insulin (Actrapid, NovoNordisk A/S, Denmark) was used. HepG2 and HeLa cells from ATCC (Manassas, VA) and Huh7 cells originating from JCRB Cell Bank (Osaka, Japan) were grown in DMEM (Invitrogen) supplemented with penicillin (100U/ml) and streptomycin (100 µg/ml), L-glutamine (2 mM) and 10% fetal bovine serum (FBS) under 5% CO_2 at 37°C. HepG2 cells were serum deprived for 5 hours prior to ligand treatment for 18 hours, unless otherwise indicated. Human primary hepatocytes were treated with ligands for 18 hours. Cycloheximide, GW3965, T0901317, 9c-RA and SR12813 were from Sigma-Aldrich. GW4064 was a generous gift from Dr Tim Willson GlaxoSmith Kline.

Plasmid constructs

A 279 bp region containing a putative LXRE in the human ADFP 3'UTR was cloned into pGL3promoter (Promega, Nacka, Sweden) using the SacI and XhoI sites, LXRE(279c). Likewise an 817 bp region in the 5'-flanking region containing two potential LXREs was cloned into pGL3promoter using the Xho1 and MluI sites, LXRE(817a+/a). Primers used were Fw-actagagagctcACCCAGTCTCTACTAAAAACATA and Rv-tagcagctcgagATGGCGCAATCTCAGCTCACT for LXRE(279c), and Fw-aacgatacgcgtTTGAGATGGAGTCTTGCACCTGTTG and Rv-atattcctcgagTGAGATGGAGTCGCACTCTGTTGC for LXRE(817a+/a). Lower case letters indicate nucleotides introduced for cloning purposes. The QuickChange® II XL Site directed Mutagenesis kit (Stratagene) was used to make the constructs LXRE(279c)M2 and LXRE(279c)M3 in which the putative

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LXRE in the LXRE(279c) was mutated (Fig. 8B). The 2x(DR4) control plasmid harbors two classical LXREs (AGGTCAtttcAGGTCA) spaced by 64 nucleotides cloned into the MluI and BglII sites in the pGL3promoter plasmid. Human full length LXRα, LXRβ and RXRα were cloned into the pSG5 vector (Stratagene) and were generous gifts from Tomas Jakobsson, Dept. of Biosciences and Nutrition, Karolinska Institutet, Huddinge, Sweden. All constructs were verified by sequencing.

RNA Analysis

Total RNA was isolated using the RNeasy kit (Qiagen). Approximately 500 ng RNA was reverse-transcribed using the Superscript II reverse transcriptase kit (Invitrogen). Quantitative real time-PCR (qRT-PCR) was performed using the Fast SYBR Green master mix (Applied Biosystems) and amplified in an ABI Prism 7500 Sequence detector. Primers were designed using Primer Express software (Applied Biosystems), and primer sequences are available on request. Relative changes were calculated by the comparative method using 18S as the reference gene.

Transfections

HeLa and Huh7 cells grown in 24-well plates in 5% FBS were transfected using FugeneHD (Roche Applied Science) at a ratio of 2:5 (DNA:FugeneHD). 100 ng of Luciferase reporter vector and 10 ng of each nuclear receptor DNA was added per well and transfections were continued for 5-6 hours. Cells were treated with 5 μM GW3965, 1 μM T0901317 or vehicle in serum free media for 24 hours. Cells were lysed with Passive Lysis Buffer (Promega) and Luciferase activity was measured using the Luciferase assay kit (BioThema).

Chromatin Immunoprecipitation (ChIP) assay

Human primary hepatocytes were treated for 1.5 hours with 5 μM GW3965 while HepG2 cells were treated 4 hours with the indicated ligands. Prior to ligand treatment, HepG2 cells were starved in serum free media over night. Approximately 40 x 10^6 primary hepatocytes and 20 x 10^6 HepG2 cells were cross

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linked for 15 min at room temperature by adding 1% formaldehyde-containing solution. In HepG2 cells cross linking was stopped by adding glycine to a final concentration of 125 mM for 5 min. Cells were then rinsed with phosphate-buffered saline (PBS), harvested and centrifuged at 1000 rpm for 5 min at 4°C. Pellets were resuspended in lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris, 1 mM phenylmethylsulfonyl (PMSF), leupeptin – pepstatin A - aprotinin at 5µg/ml, pH 8.1) and rotated for 10 min at 4°C. The nuclei were collected by centrifugation, resuspended in wash buffer and rotated again. Washed nuclei were centrifuged and resuspended in ChIP buffer (0.01 % SDS, 1.1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, 1 mM PMSF, leupeptin – pepstatin A - aprotinin at 5µg/ml, pH 8.1) and subsequently sonicated, leading to DNA fragment sizes of 0.2 to 0.8 bp. Samples were cleared by centrifugation at 14,000 rpm for 10 min at 4°C. Ten percent of the cleared supernatant was used as the input, and the remaining volume was immunoprecipitated with antibodies against RXR (sc-774), CBP/p300 (sc-369 and sc-584), Pol II (sc-9001) from Santa Cruz Biotechnology (Santa Cruz, CA) or a pan LXR antibody (Jakobsson et al., 2009).The immunoprecipitates were analyzed with qRT-PCR using the following primers: ADFP promoter Fw: GTGCCCGAGGGTGACACT, ADFP promoter Rv: CGCACTCACCGACGGACT; ADFP DR4 type c Fw: CTTGGTAGCTCACGGCCTG, ADFP DR4 type c Rv: GGCCTCTCCTGACCTCTTGAT; ADFP DR4 type b Fw:AATAGGCCAGGCGCTGTG, ADFP DR4 type b Rv:TTGTAGAGAAAGGGTTTCACGTTG; ADFP DR4 typ a Fw: GACTCACGCCTGTAATCCAA, ADFP DR4 typ a Rw: GAGTAGCTGGGATTACAGGAG; ADFP DR4 typ a (+) Fw: GACTCACGCCTGTAATCCA, ADFP DR4 typ a (+) Rw: GAGTAGCTGGGATTACAGGTG; ABCA1 Fw:TGCTTTCTGCTGAGTGACTGA, ABCA1 Rv: CAATTACGGGGTTTTTGCCG.

Statistics

Values are presented as the mean ± SD. The GraphPad Software (GraphPad Software Inc, CA) was used for statistical analyses. Comparisons between groups were made using Student's *t*-test or one-way

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ANOVA followed by Neuman-Keul's test when multiple comparisons were made. Samples were considered significantly different at $P<0.05$.

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RESULTS

GW3965 but not T0901317 regulates ADFP gene expression in human hepatocytes

Our previous study showed that exposure of primary human hepatocytes to 2 μM GW3965 induces the mRNA levels of ADFP and SREBP1c, and that increased ADFP mRNA correlated to increased ADFP protein. In this study we confirm that ADFP is induced by GW3965 (Fig. 1). Surprisingly, in several independent experiments using primary human hepatocytes we observed that the more potent LXR agonist T0901317, which, however, is less specific than GW3965, induced SREBP1c as expected but unexpectedly failed to induce ADFP (Fig. 1A and 1B). In dose-response experiments in primary human hepatocytes, both ADFP and SREBP1c were dose-dependently induced by GW3965 (Fig. 1C and 1D).

To further investigate this unexpected observation, and possibly taking advantage of this difference in exploring the regulation of the ADFP gene, we used the human hepatic cell line HepG2. Consistent with the observations in primary hepatocytes only GW3965 induced ADFP while both agonists dose-dependently and with similar efficacy induced SREBP1c in HepG2 cells (Fig. 1E and 1F).

The ADFP gene is a direct LXR target gene

As shown in Fig. 2, increased levels of ADFP and SREBP1c mRNA in HepG2 cells were observed after 3 and 6 h, respectively, of GW3965 exposure (5 μM). The induction of ADFP by GW3965 was maximal at 6 h and did not differ between 6 and 30 h (Fig. 2A). In contrast, SREBP1c levels were not significantly increased until 19 h of exposure and continued to increase up to 10-fold at 30 h of exposure (Fig. 2B). T0901317-exposure up to 30 h did not induce ADFP (data not shown).

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To examine whether the GW3965-induced expression of ADFP was sensitive to inhibition of ongoing protein synthesis, cells were co-treated with the protein synthesis inhibitor cycloheximide (CHX) and GW3965. In primary human hepatocytes CHX did not affect the GW3965 induction of ADFP or SREBP1c (Fig. 2C and 2D). This is consistent with a direct effect of GW3965 on both genes. Similar results were obtained in HepG2 cells (data not shown). Taken together, the data suggests that LXR regulates ADFP gene expression at the transcriptional level.

PXR, FXR and RXR activation do not modulate ADFP gene expression

That GW3965 but not T0901317 induced the ADFP gene raised the question whether the previously described non-LXR mediated effects of T0901317, *i.e.* activation of PXR and FXR (Houck et al., 2004; Mitro et al., 2007), was responsible for the difference. This made us investigate whether induction of ADFP in HepG2 cells by GW3965 was affected by simultaneous treatment with specific ligands to PXR or FXR. Treatment with the PXR agonist SR12813 (0.1-2 μM) or the FXR agonist GW4064 (0.1-1 μM) alone had no effect on SREBP1c expression (Fig. 3 and data not shown). However, the PXR ligand, SR12813, potentiated the inducing effect of GW3965 on SREBP1c (Fig. 3B). The PXR ligand had no effect on the expression of ADFP but in contrast the FXR-specific ligand, GW4064, induced the expression of ADFP; an effect that appeared additive to the effect of GW3965 (Fig. 3A). Both SR12813 and GW4064 affected the expression of known target genes; *Cyp7A1* was down regulated by GW4064 and *Cyp3A4* was induced by SR12813 (data not shown). These results show that the lack of effect of T0901317 on ADFP expression was not due to concurrent activation of LXR and PXR or FXR.

The LXR/RXR heterodimer, conveying a classical LXR-mediated response, is in certain genes activated by ligands for either receptor, oxysterols or 9-cis retinoic acid (9c-RA); synergistic and additive effects on target gene induction have been demonstrated (Antonio et al., 2003; Li et al., 2002). 9c-RA did not potentiate the effect of GW3965 on ADFP expression in HepG2 cells but potentiated the GW3965 effect on SREBP1c expression (Fig. 4).

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Putative LXRE sequences in the ADFP gene

In an attempt to find putative LXREs in the ADFP gene we used predictive response element modeling (Sandelin and Wasserman, 2005; Varga and Su, 2007). Thirty-one putative LXR binding sites, representing 21 DR4, 7 IR1 and 3 DR1 elements, were found with delta log scores ranging from 0.66 to 3.74 in the NHR scan (Sandelin and Wasserman, 2005). Five of the suggested DR4 elements had the sequence GGATCAn_4AGGTCA, denoted type a, with delta log scores 3.00-3.16. This type of DR4 has previously been identified as a low affinity non-responsive LXR-binding element (Laffitte et al., 2001; Li et al., 2002) or as a negative LXRE (Wang et al., 2008). Five additional putative binding elements with delta log scores >3 were revealed; four of these had the sequence AGATCAn_4AGGTCA, denoted type b, and one had the sequence AGGTCAn_4AGGCCA presenting the highest delta log score 3.74, denoted type c. In Fig. 5, showing a schematic presentation of the human ADFP gene, from -20 kb upstream of the first exon to 5 kb downstream of the last exon, the putative LXREs with delta log scores >3 (type a, b and c) are indicated. A peroxisome proliferator response element (PPRE) in the ADFP gene (Targett-Adams et al., 2005) at -2.3 kb is also indicated in Fig. 5. In the LXRα gene the low affinity non-responsive LXRE elements, here denoted type a, exist within *Alu* elements (Li et al., 2002); we found that nine out the ten putative LXREs (a, b and c type) in the human ADFP existed in regions with ≥78 percent homology to an *AluY* element (Batzer and Deininger, 2002).

LXR directly regulates the ADFP gene via LXREs in the 3'UTR and the 5'-flanking regions

To further characterize the binding of LXR to the putative LXREs in the human ADFP gene, ChIP assays were performed on samples from human primary hepatocytes. Upon GW3965 stimulation, LXR and RXR were enriched in the 3'-UTR containing the DR4 type c, but not in the adjacent region containing the DR4 type b (Fig. 6A and 6B). In addition, upon GW3965 stimulation, an interaction of LXR and RXR was detected at the promoter of the ADFP gene (Fig. 6C). Furthermore, Pol II and the nuclear receptor coactivators CBP and p300 were recruited to the promoter upon its interaction with the 3'-UTR DR4 type

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c, but not with the DR4 type b (Fig. 6A - C). We also analyzed the recruitment of LXR and RXR to the two most proximal DR4 type a elements in the 5'-flanking region (Fig. 6D and E). Albeit identical DR4 repeats in the two elements, recruitment was only to the most proximal element residing on the minus strand and not to the adjacent upstream element on the plus strand. Interaction of this most proximal DR4 type a element with the promoter region was evident by the recruitment of Pol II and CBP/p300. As a control experiment we performed ChIP assays on samples from HepG2 cells; upon GW3965 stimulation, LXR, RXR and Pol II were enriched at the ADFP promoter region and around the 3'-UTR DR4 type c, but in contrast, upon T0901317 stimulation, no enrichment of Pol II or the LXR/RXR heterodimeric partners was observed on the promoter (Fig. 7A). With both the synthetic ligands, GW3965 and T0901317, LXR and Pol II were similarly recruited to the promoter of the ABCA1 gene (Fig. 7B). Of note is that LXR to some extent seems to interact with the promoter region and the 3'-UTR DR4 type c also in unstimulated cells (Fig. 6F), but not to the same degree as in the ABCA1 promoter where the enrichment was 40-fold in unstimulated cells (Jakobsson et al., 2009).

DR4 mediated LXR responsiveness in transient transfections

To characterize the LXR responsiveness of the putative LXRE in the 3'UTR of the human ADFP gene, a luciferase construct containing a 279 bp fragment encompassing the DR4 type c (LXRE(279c)) was transiently transfected into HeLa cells together with expression plasmids for human RXRα and LXRα or LXRβ (Fig. 8A). Both LXR isoforms conveyed GW3965-activated induction of the luciferase activity. The 2x(DR4) plasmid, harboring two classical LXREs, was similarly induced. Mutation of the putative LXRE in LXRE(279c), LXRE(297c)M2 and LXRE(279c)M3 (Fig. 8B), mitigated the ligand mediated induction. In contrast to the response of the endogenous gene both GW3965 and T0901317 activated the LXRE(279c) construct when transfected into Huh7 cells together with LXRα and RXRα expression plasmids (Fig. 8C). Similarly a luciferase construct containing an 817 bp fragment encompassing the two DR4 type a elements (LXRE(817a+/a) was induced by both ligands in Huh7 cells (fig. 8C). These data

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show that the DR4 type c identified in the 3' UTR of the human ADFP gene is a *bona fide* LXR responsive element and that at least one DR4 element of type a also confers LXR responsiveness.

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DISCUSSION

LXRα is the predominant receptor subtype in the liver and also the subtype to which adverse effects of LXR activation, including increased TG synthesis, have been attributed; hypertriglyceridemia in mice is evident from numerous studies. In cultured primary human hepatocytes treated with GW3965, TG synthesis is indeed increased and so is the cellular accumulation of TG while the output of VLDL-TG from the cells is reduced (Kotokorpi et al., 2007). This is concordant with *in vivo* studies in monkeys in which pharmacological LXR activation with GW3965 does not result in hypertriglyceridemia (Groot et al., 2005). Excessive TG not being secreted is bound to accumulate within the cell. In this study, we have identified the human ADFP gene, shown to have a central role in the formation of lipid droplets (Imamura et al., 2002), as a direct LXR target gene in hepatocytes, supporting the notion that pharmacological LXR activation in humans might lead to hepatic steatosis.

Using published algorithms (Sandelin and Wasserman, 2005; Varga and Su, 2007), we found several putative LXREs in the human ADFP gene, in particular DR4 elements, which were denoted type a, b or c (Fig. 5). In previously experimentally verified positive LXREs the third position in the first repeat contains a G (30 out of 35) or a T (5 out of 35) (Varga and Su, 2007). The DR4 type a and the b elements have an A in the third position in the first repeat and the DR4 type a has previously been characterized as a low affinity non-responsive LXR-binding element in the human LXRα gene or as a negative LXRE in the human CYP51A1 gene (Laffitte et al., 2001; Li et al., 2002; Wang et al., 2008). The C in the fourth position of the second repeat of LXRE type c is atypical but has been found in at least two positive LXREs (Landis et al., 2002; Marathe et al., 2006). Using ChIP analysis on cross-linked chromatin from primary human hepatocytes, it became evident that sequence identity of the hexamer repeats in DR4 elements is not sufficient to determine if the element binds or not binds LXR; to the most proximal DR4 type a element but not to the more distal one in the 5'flanking region was LXR/RXR recruited upon

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GW3965 stimulation. The two type a elements that we analyzed reside on different strands and also the surrounding sequences differ which could play a role for this difference. Of the regions harboring the DR4 type b and type c that we analyzed, only the region with the type c was found to bind LXR/RXR and associated coactivators. The recruitment of LXR/RXR to the type a element and to the type c element was also associated with Pol II and cofactor recruitment to the promoter. Clearly, the natural context of response elements determines their ability to convey transcriptional regulation. The LXRE type a and type c identified in the ADFP gene extends the list of positive LXREs; when cloned upstream of a reporter gene these elements mediated LXR ligand-dependent activation.

Binding of LXR to the ADFP LXRE type c is not confined to hepatocytes; ChIP assays revealed that LXR was also enriched at the ADFP promoter and the type c element in differentiated THP-1 cells, a human macrophage cell line, upon GW3965 stimulation (data not shown). Thus, LXR-mediated regulation of the ADFP gene is not cell specific in humans. On the other hand, LXR-mediated regulation of the ADFP is likely to be species specific. It has been shown that LXR activation has no effect on ADFP expression in mice *in vivo* or in cultured rodent hepatocytes (Dalen et al., 2006), which is consistent with our previous comparative studies on primary human and rat hepatocytes (Kotokorpi et al., 2007). This is further corroborated by the absence of the LXRE type c in the 3'UTR of the mouse ADFP gene, *i.e*. the responsive element is not conserved. In this context it should be mentioned that the LXRE type c exists within an *Alu* element, which is highly represented in the human genome but not in rodent genomes.

The finding that T0901317 did not induce ADFP in primary human hepatocytes or in HepG2 cells was most surprising since GW3965 has been suggested to be a gene selective LXR modulator in the liver and also less potent than T0901317 (Miao et al., 2004). However, our data are consistent with recently published data showing that T0901317 has no effect on ADFP expression in a placental cell line of human origin (Tobin et al., 2006). We were concerned that this was due to the ability of T0901317 to bind

and activate also PXR and FXR (Houck et al., 2004; Mitro et al., 2007), which possibly could regulate ADFP negatively. However, by co-treatment of HepG2 cells with GW3965 and a specific PXR (SR12813) or FXR (GW4064) ligand we could exclude this possibility.

GW3965 is considered to be a partial LXR agonist and T0901317 a full agonist, which might be due to differential coactivator and corepressor recruitment (Albers et al., 2006; Miao et al., 2004). Cell type and promoter specific differences in coregulator recruitment have been described for other nuclear receptors with different ligands *e.g*. the estrogen receptors (ERs) and the ligands tamoxifen and raloxifen (Shang and Brown, 2002). While both GW3965 and T0901317 induce an agonist conformation of helix 12, differences in the ligand-binding pocket are observed (Farnegardh et al., 2003). Whether these differences translate into sequence specific DNA binding/recognition of the receptor complex is not known but a distinction like this could explain the difference seen in the regulation of ADFP. Several studies show both ligand- and gene specific effects conveyed by LXR activation. Studies by Kase et al. have revealed differential effects on lipid metabolism by the natural LXR agonist 22-hydroxycholesterol (22-R-HC) and T0901317 suggesting that LXR induction of certain genes is ligand specific (Kase et al., 2006). Furthermore, differential and gene specific displacement of the corepressor NCoR by T0901317 and GW3965 has been shown (Albers et al., 2006; Phelan et al., 2008). This indicates that the interplay between LXR and coregulators is ligand and gene specific.

It could be postulated that GW3965, but not T0901317, induces an LXR conformation that allows the receptor complex to bind to the identified LXREs in the 3' UTR and in the 5'-flanking region of the human ADFP gene and recruit coactivators such as CBP/p300. These distal regulatory elements may come in direct contact with the transcription start site and the general transcription machinery by forming a chromatin loop. The LXR/RXR recruitment in the promoter region and on the LXRE type c also in non-ligand stimulated cells (Fig. 6F) may indicate a pre-existing chromatin loop. Chromatin looping, such as

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physical interaction between distal enhancers and a promoter, has been described for the LXR regulated ABCG1 gene where an enhancer is located in the seventh intron of the gene (Jakobsson et al., 2009).

In contrast to the endogenous ADFP gene, the LXRE type a and type c luciferase reporter constructs, LXRE(817a+/a) and LXRE(279c), respectively, were activated by both GW3965 and T0901317. This further supports an LXR-mediated activation of the human ADFP gene and suggests that the chromatin structure is differently modified by the two ligands. In the human ADFP promoter, a functional PPRE has been identified (Targett-Adams et al., 2005), which is conserved in the murine ADFP gene (Chawla et al., 2003). A functional Ets/AP-1element has been recognized in the mouse ADFP promoter, and it is suggested that this site, in addition to the PPRE, is crucial for PPAR-mediated activation of the mouse ADFP promoter (Wei et al., 2005). Similarly, one could imagine that additional elements in the human ADFP gene could contribute to the divergent effects seen by the two synthetic LXR ligands. Also hitherto not characterized DR4 elements might be involved in the regulation.

Taken together, our results demonstrate that the human ADFP gene is a direct LXR target gene and that different LXR agonists differentially regulate the endogenous gene. The differential mechanisms are not yet clear but calls for refined strategies and the use of appropriate experimental models in developing selective LXR agonists for treatment of human metabolic disorders.

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FOOTNOTES

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Conflict of interest

J-Å.G. is a consultant and shareholder of KaroBio AB, Sweden. The other authors have nothing to declare.

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FIGURE LEGENDS

Figure 1. Induction of ADFP and SREBP1c in primary human hepatocytes and HepG2 cells in response to LXR agonists. Isolated hepatocytes (A-D) were cultured for 96 h with vehicle (open bars) or the indicated dose of GW3965 (GW, grey bars) or T0901317 (T, black bars) for the last 18 h. Cells from four donors were used. Data shown are the average of multiple dishes from two donors. HepG2 cells (E-F) were treated for 18 h with increasing concentrations of GW or T. Relative expression of ADFP and SREBP1c was analyzed by qRT-PCR. HepG2 data shown are the average ± SD of multiple dishes from three independent experiments. Asterisks indicate statistically significant differences versus vehicle treated cells (open bars); ** $P < 0.01$ and *** $P < 0.001$.

*Figure 2.*Time-course induction and the effect of cycloheximid on GW3965-induced expression of ADFP and SREBP1c. HepG2 cells (A and B) were treated for the indicated time points with vehicle (open bars) or 5 μM GW3965 (GW, grey bars). Relative expression of ADFP and SREBP1c was analyzed by qRT-PCR. Data shown are the average ± SD of multiple dishes from two independent experiments. Vehicle-treated cells (Ctrl) at the six hour time point was set to 1. Primary hepatocytes (C and D) were cultured for 96 h and treated with vehicle (open bars) or 2 μM GW3965 (GW, grey bars) ± the indicated concentrations of cykloheximide (CHX, hatched bars) for the last 18 h. Relative expression data are the average ± SD of multiple dishes from representative experiments. Statistically significant differences are indicated; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 3. Effect of activation of PXR or FXR on GW3965-induced expression of ADFP. HepG2 cells were treated with vehicle (open bars) or 5 μM GW3965 (grey bars) ± 1 μM SR12813 (PXR agonist; hatched left bars) or GW4064 (FXR agonist; hatched right bars) for 18 h. Relative expression of ADFP (A) and SREBP1c (B) was analyzed by qRT-PCR. Data shown are the average ± SD of multiple dishes from one representative experiment. Asterisks, ** $P < 0.01$ and *** $P < 0.001$, indicate significant

differences versus vehicle or SR12813 and GW4064 treated cells, respectively; ##, P <0.01, versus vehicle treated cells; §§, P <0.01 and §§§, P <0.001, versus GW3965 treated cells.

Figure 4. Effect of 9c-RA on GW3965-induced expression of ADFP. HepG2 cells were treated for 18 h with vehicle (open bars) or 5 µM GW3965 (GW; grey bars) ± 10 µM 9c-RA (hatched bars). Relative expression of ADFP (A) and SREBP1c (B) was analyzed by qRT-PCR. Data shown are the average ± SD of multiple dishes from two independent experiments. Significant differences are indicated; ** P <0.01 and *** P <0.001 versus vehicle or 9cRA treated cells; ### P <0.001 versus GW3965 treated cells.

Figure 5. Schematic presentation of the human ADFP gene structure with 5'- and 3'-flanking sequences. Putative LXREs of type a, b and c are indicated. Alu elements encompassing the putative LXREs are indicated by an A and + indicates that the element is present on the plus stand. Regions amplified by qRT-PCR in ChIP experiments are indicated by asterisks.

Figure 6. Effect of GW3965 on LXR binding to the human ADFP promoter and potential LXREs in human primary hepatocytes. Primary hepatocytes were treated for 1.5 h with vehicle (open bars) or 5 µM GW3965 (grey bars). Chromatin was crosslinked, sonicated and immunoprecipitated with LXR, RXR, Pol II, CBP and P300 antibodies. Enrichment of specific DNA fragments (see Fig. 5) was detected with qRT-PCR using primers at the DR4 type c (A), at the DR4 type b (B), around the transcription start site of ADFP (C), at the DR4 type a+ (D) and at the DR4 type a (E). Data are expressed as fold induction with the level in vehicle treated cells set to one. Data in (F) are expressed as enrichment of the LXR specific antibody over non-specific IgG, which was set to 1, in non-ligand stimulated cells and A-E refer to the elements in panels A-E. Data shown are the average ± SD from three independent dishes. Significant differences are indicated; ** P <0.01 and *** P <0.001 versus vehicle treated cells (panels A-E) or versus non-specific IgG (F).

Figure 7. Effect of GW3965 on LXR binding to the human ADFP start site and potential LXREs. HepG2 cells were treated for 4 h with vehicle (open bars) 5 μM GW3965 (Grey bars) or 1 μM T0901317 (black bars). Chromatin was crosslinked, sonicated and immunoprecipitated with LXR, RXR, Pol II and CBP/p300 antibodies. Enrichment of specific DNA fragments was detected with qRT-PCR using primers around the transcription start site of ADFP or at the DR4 type c (A) and around the promoter of the ABCA1 gene (B). Data are expressed as fold induction with the level in vehicle treated cells set to one.

Figure 8. Effect of LXR activation on the luciferase construct harbouring the putative LXRE type c from the human ADFP gene. (A), HeLa cells were transiently transfected with LXRE(279c), LXRE(279c)M2 or LXRE(279c)M3 and expression vectors for human RXRα, LXRα or LXRβ and treated for 24 h with vehicle (open bars) or GW3965 (2 μM; grey bars). (B) The DR4 element sequences in the different constructs. (C), Huh7 cells were transiently transfected with LXRE(279c) or LXRE(817a+/a), and expression vectors for human RXRα and LXRα and treated for 24 h with vehicle (open bars), GW3965 (grey bars) or T0901317 (T1317, black bars). Data shown are the average ± SD of three dishes from one representative experiment. Data are shown as fold induction relative to empty vector transfected cells treated with vehicle (A) or vehicle, GW3965 or T0901317 (C). Asterisks, ** P <0.01 and *** P <0.001, in (A) indicate statistically significant effects of the GW3965 or T0901317 treatment and in (C) statistically significant effects of LXR/RXR co-transfection in cells treated with vehicle (open bars), GW3965 (grey bars) or T0901317 (T1317, black bars). In (A), ##, P <0.01 and ###, P<0.001 indicate statistically significant differences between empty vector and LXR/RXR transfected cells and in (C) statistically significant differences of GW3965 and T09013 treatment versus vehicle treated cells.

Fig. 1

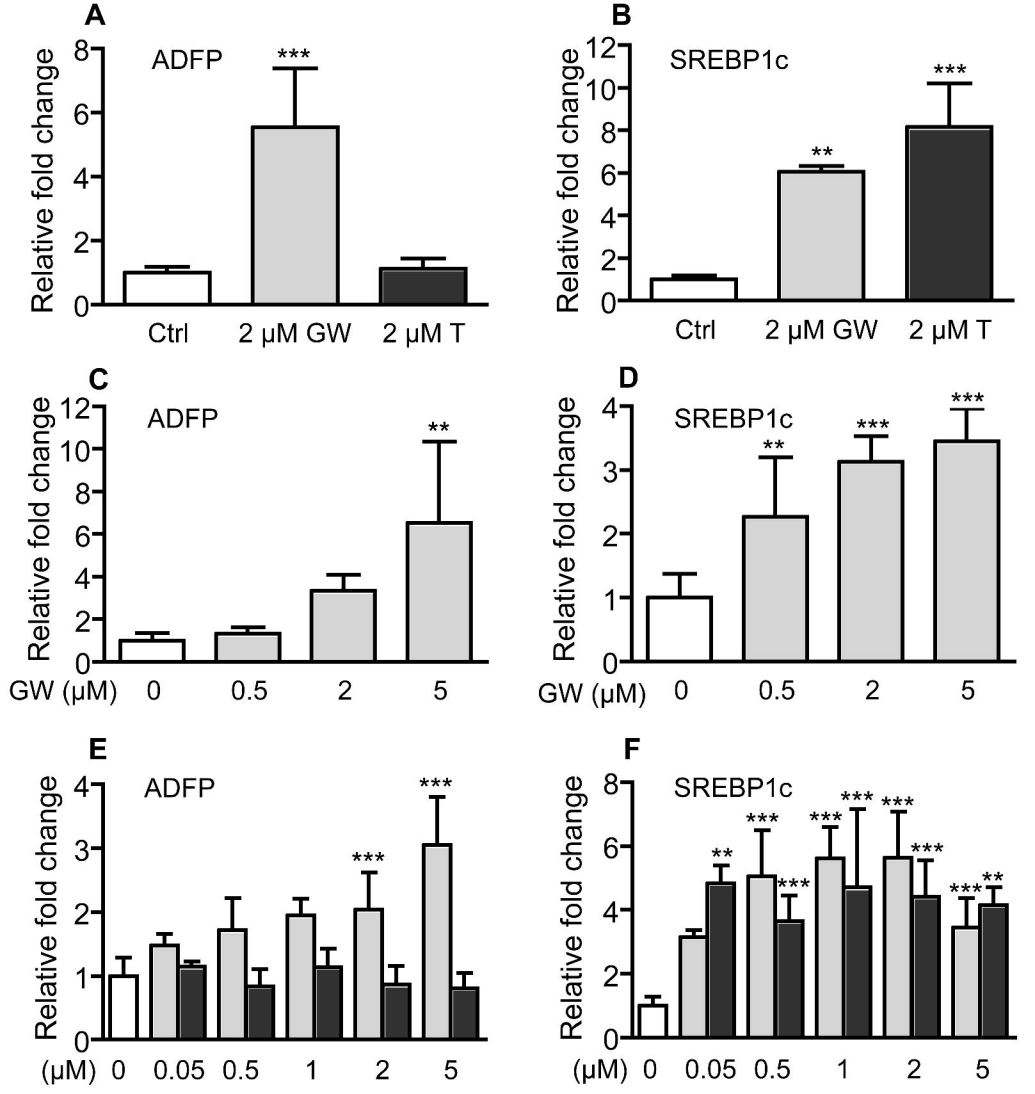

A
ADFP
Relative fold change
Ctrl
2 μM GW
2 μM T
B
SREBP1c
C
D
GW (μM)
E
F
(μM)

Fig. 2

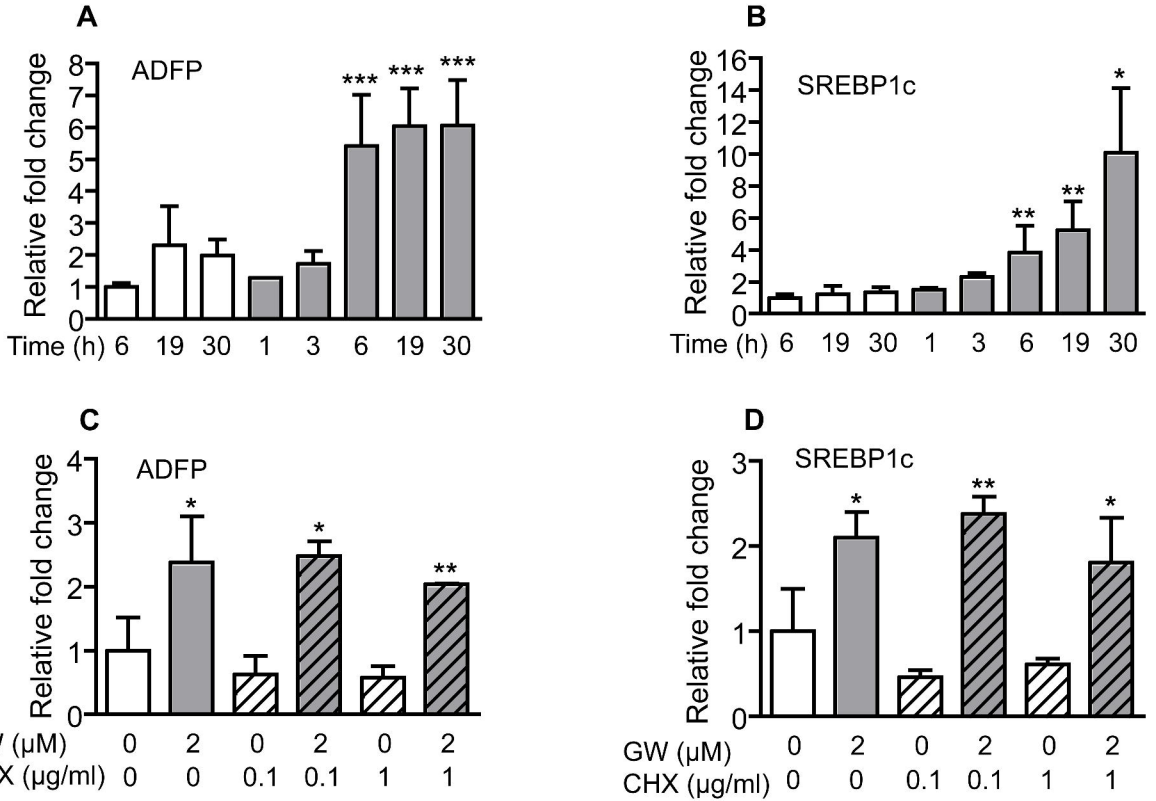
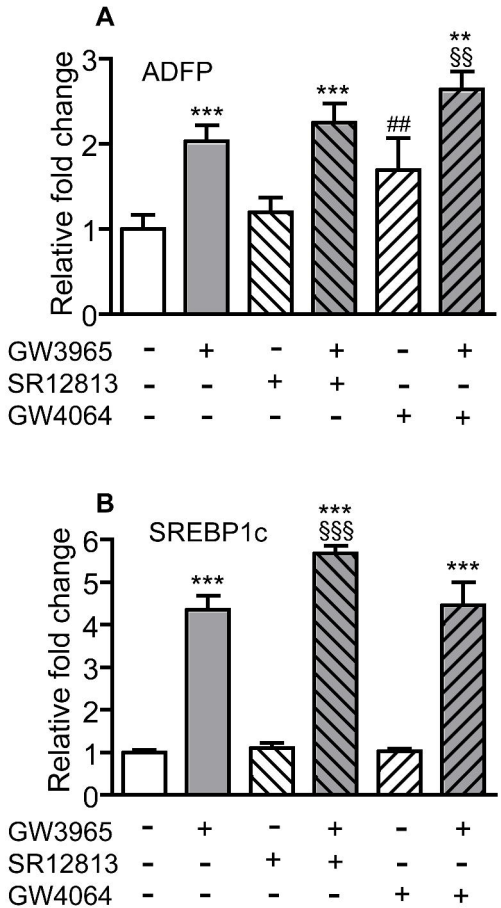

A
ADFP
Relative fold change
Time (h) 6 19 30 1 3 6 19 30
B
SREBP1c
Relative fold change
Time (h) 6 19 30 1 3 6 19 30
C
ADFP
Relative fold change
GW (μM) 0 2 0 2 0 2
CHX (μg/ml) 0 0 0.1 0.1 1 1
D
SREBP1c
Relative fold change
GW (μM) 0 2 0 2 0 2
CHX (μg/ml) 0 0 0.1 0.1 1 1

Fig. 3


A
ADFP
Relative fold change
0
1
2
3
***
***
##
**
§§
GW3965 - + - + - +
SR12813 - - + + - -
GW4064 - - - - + +
B
SREBP1c
Relative fold change
0
1
2
3
4
5
6
***
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§§§
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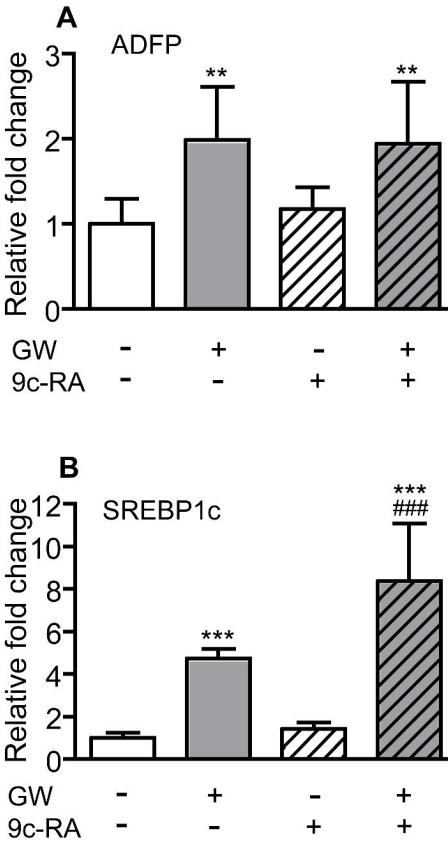
SR12813 - - + + - -
GW4064 - - - - + +

Fig. 4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ADFP
Relative fold change
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**
**
GW
9c-RA
-
+
-
+
-
-
+
+
B
SREBP1c
Relative fold change
12
10
8
6
4
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***
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###
GW
9c-RA
-
+
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+
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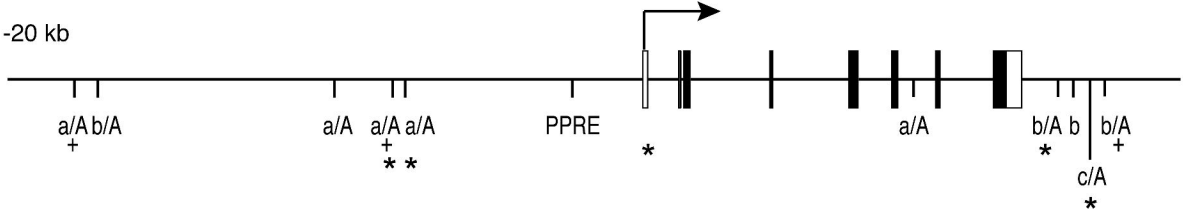
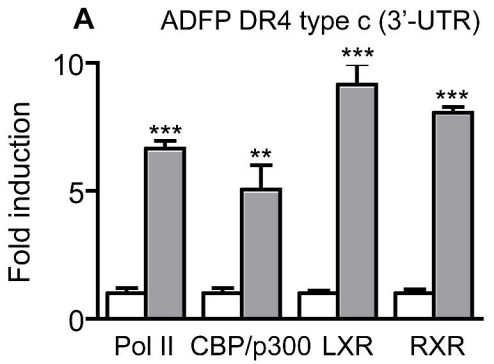
-
+
+

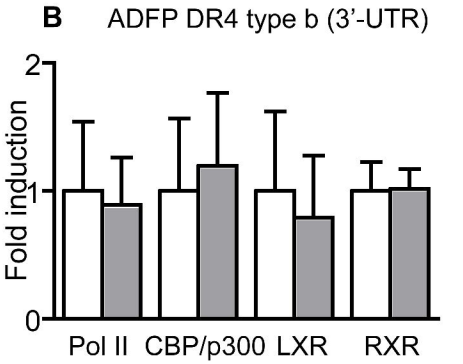
Fig 5

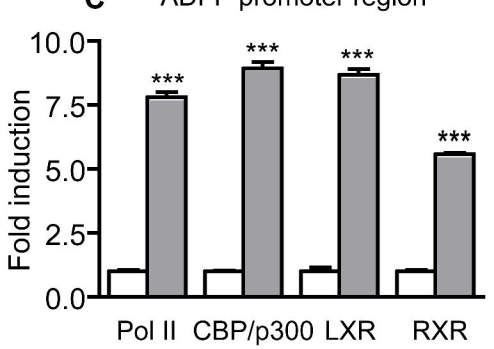


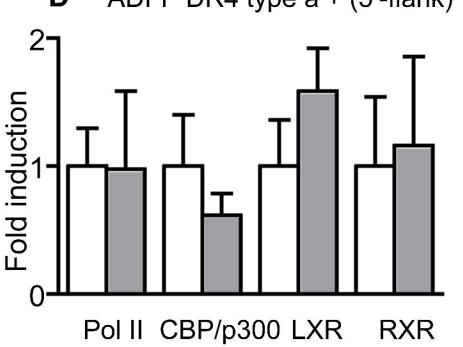
Type	Putative LXRE
a	GGATCAnnnnAGGTCA
b	AGATCAnnnnAGGTCA
c	AGGTCAnnnnAGGCCA

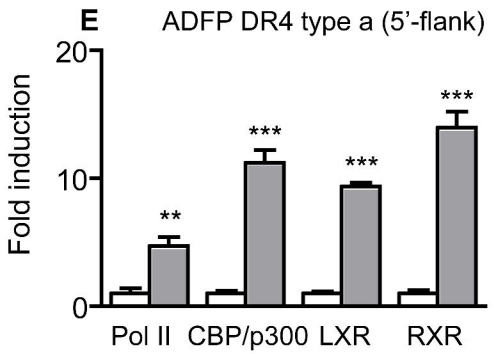
Fig. 6


A
ADFP DR4 type c (3'-UTR)
Fold induction
10
5
0
***
**
***
***
Pol II
CBP/p300
LXR
RXR


B
ADFP DR4 type b (3'-UTR)
Fold induction
2
1
0
Pol II
CBP/p300
LXR
RXR


C
ADFP promoter region
Fold induction
10.0
7.5
5.0
2.5
0.0
***
***
***
***
Pol II
CBP/p300
LXR
RXR


D
ADFP DR4 type a + (5'-flank)
Fold induction
2
1
0
Pol II
CBP/p300
LXR
RXR


E
ADFP DR4 type a (5'-flank)
Fold induction
20
10
0
**
***
***
***
Pol II
CBP/p300
LXR
RXR

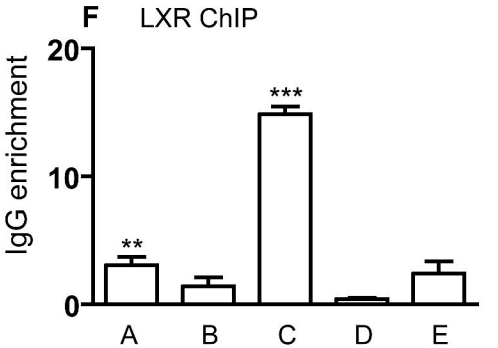

F
LXR ChIP
IgG enrichment
20
10
0
**
***
A
B
C
D
E

Fig. 7

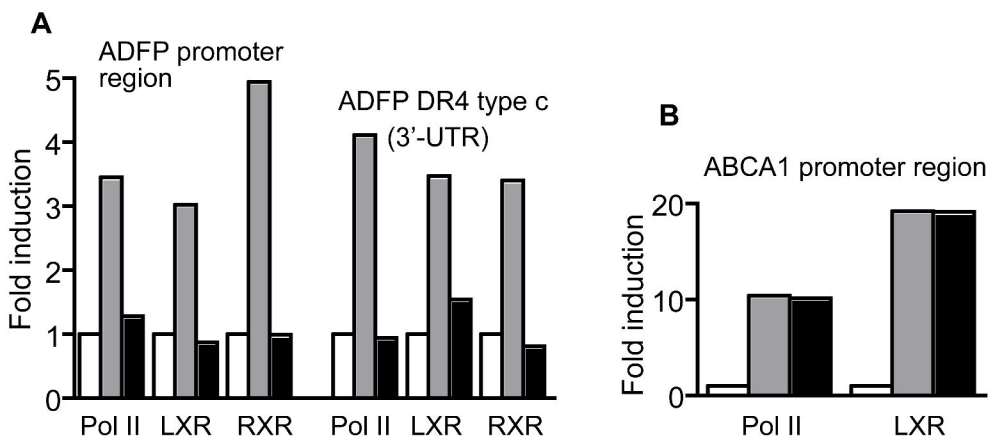
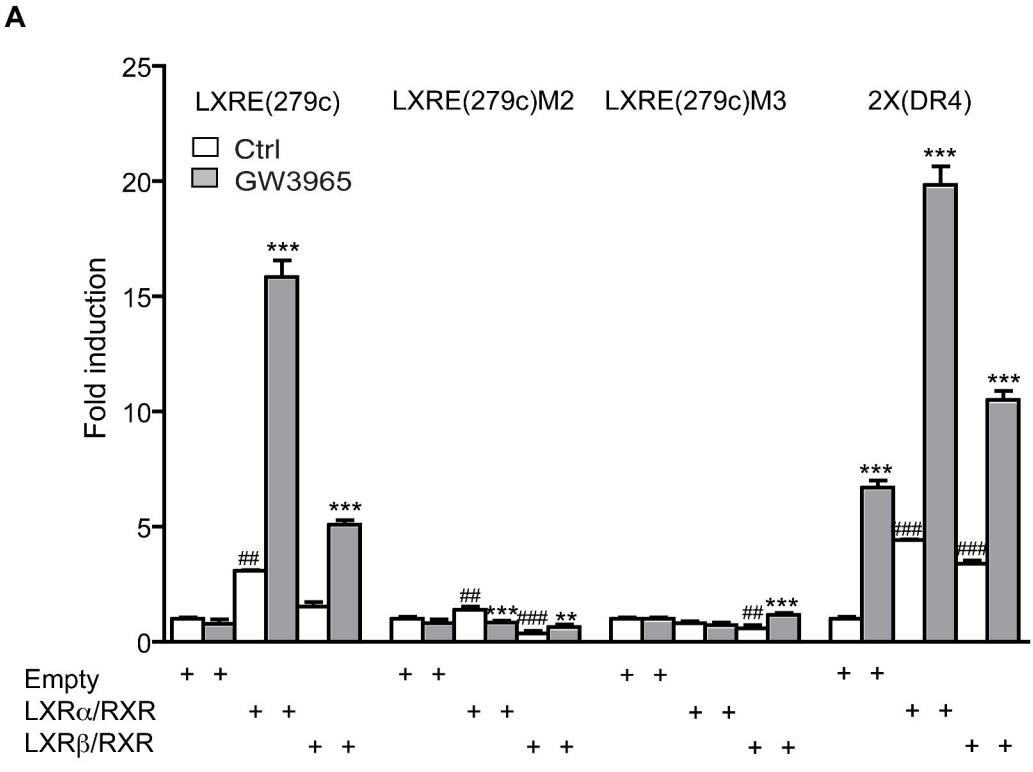

A
ADFP promoter region
ADFP DR4 type c (3'-UTR)
Fold induction
0
1
2
3
4
5
Pol II
LXR
RXR
Pol II
LXR
RXR
B
ABCA1 promoter region
Fold induction
0
10
20
Pol II
LXR

Fig. 8



B

LXRE(279c)	AGGTCAggagAGGCCA
LXRE(279c)M2	TTTTTTggagACCCCT
LXRE(279c)M3	AGATCAggagAGCCCA
DR4	AGGTCAtttcAGGTCA