Published in final edited form as:

J Proteome Res. 2015 November 06; 14(11): 4571–4580. doi:10.1021/acs.jproteome.5b00379.

SERBP1 is a component of the Liver Receptor Homolog-1 transcriptional complex

Yelenis Mari¹, Graham M. West², Catherina Scharager-Tapia², Bruce D. Pascal², Ruben Garcia-Ordenez¹, and Patrick R. Griffin^{1,*}

¹The Scripps Research Institute, Department of Molecular Therapeutics, Jupiter, FL 33458

²Mass Spectrometry & Proteomics Core, Jupiter, FL 33458

Abstract

Liver receptor homolog-1 (LRH1) is an orphan nuclear receptor that has been shown to play a role in the transcriptional regulation of pathways involved in cancer. Elucidating the components of the LRH1 transcriptional complex to better understand endogenous regulation of the receptor as well as its role in cancer remains a high priority. A sub-cellular enrichment strategy coupled with proteomic approaches was employed to identify putative LRH1 coregulators. Nuclear fractionation protocol was essential for detection of LRH1 peptides by mass spectrometry (MS), with most peptides being observed in the insoluble fraction (receptor bound to DNA). SERBP1 and ILF3 were identified as LRH1 interacting partners by both western blot and MS/MS analysis. Receptor knockdown by siRNA showed an increased in SERBP1 expression while ILF3 expression was unchanged. In contrast, receptor overexpression decreased only SERBP1 mRNA levels. Consistent with these data, in a promoter:reporter assay, binding of LRH1 to the promoter region of SERBP1 resulted in a decrease in the expression level of the reporter gene, and subsequently, inhibiting transcription. Given the receptor's role in cancer progression, the study here elucidates additional transcriptional machinery involved in LRH1 signaling and potentially provides new targets for therapeutics development.

Keywords

Liver receptor homolog-1 (LRH1, NR5A2); Nuclear receptor (NR); Interleukin enhancer binding factor-3 (ILF3); SERPINE1 mRNA binding protein-1 (SERBP1); Protein arginine methyltransferase 1 (PRMT1); Peroxisome proliferator-activated receptor γ co-activator-1 α (PGC1 α)

Introduction

Liver receptor homolog-1 (LRH1, NR5A2) is an orphan nuclear receptor and a member of the NR5A (Ftz-F1) subfamily of ligand regulated nuclear receptors (1). LRH1 binds as a monomer to identical DNA sequences (response elements) and has been implicated in binding phospholipids in the ligand binding pocket (2–4). LRH1 is highly expressed in

*Corresponding Author: Patrick R. Griffin, PhD, The Scripps Research Institute, Scripps Florida, 130 Scripps Way #2A2, Jupiter, FL 33458, Phone: (561) 228-2200, Fax: (561) 228-3081, pgriffin@scripps.edu.

tissues of endodermal origin as well as in ovary and adipose tissue. Receptor expression is also essential for normal liver, intestine, and pancreas function. Recently, LRH1 has been shown to play a role in the transcriptional regulation of pathways involved in cancer. The transcriptional activation of the aromatase cytochrome p450 gene (*CYP19*) in preadipocytes has been shown to be LRH1 dependent (5). Cell culture studies showed that cAMP stimulators dramatically induce LRH1-dependent *CYP19* expression and this increase can be abrogated by overexpression of SHP, a negative regulator of LRH1 (6, 7). LRH1 has also been implicated in cell proliferation leading to tumor progression in intestinal, pancreatic and ovarian cancer (8–10). For example, in pancreatic cancer, LRH1 was shown to induce cell proliferation by acting in synergy with β -catenin to activate CyclinD1 and CyclinE1 expression (11). Furthermore, these effects were reversed by knocking down LRH1 by siRNA or by overexpression of SHP, demonstrating a role for LRH1 in cell proliferation. This increasing evidence for the role of LRH1 in cancer has led to the development of synthetic LRH1 antagonists/inverse agonists (12–14). Specifically, treatment of pancreatic cancer cells with an LRH1 antagonist resulted in a decrease in LRH1 mRNA as well as a decrease in expression of LRH1 target genes (12). Combined, these studies highlight the importance of LRH1 function in cancer and the importance to further investigate LRH1 as a target for cancer therapeutics. Therefore there is a need for techniques to evaluate LRH1 regulation in cells.

While many nuclear receptors (NRs) require binding of a ligand to become transcriptionally active, LRH1 appears to be constitutively active when expressed in cells. A number of laboratories have identified the presence of phospholipids in the ligand-binding pocket (LBP) of LRH1 and their presence leads to the recruitment of coactivators *in vitro* (3, 4, 8). Whether phospholipids are endogenous ligands of LRH1 remains unclear. Recently, it was shown that the dietary phospholipid DLPC activates LRH1 activity in mice (15). These findings do confirm that LRH1 is capable of binding ligands (16) that can modulate its activity *in vivo*. NR function can also be controlled by posttranslational modifications (PTMs) such as phosphorylation, acetylation, and conjugation with ubiquitin and/or the small ubiquitin-related modifier (SUMO) (17). For example, the transcriptional activity of LRH1 can be stimulated by phosphorylation in response to phorbol myristate (PMA) (18) and the cellular localization of the receptor has been shown to be controlled by conjugation with SUMO (19). In the latter study, SUMOylation of LRH1 alters localization of the receptor in promyelocytic leukemia protein nuclear bodies resulting in repression of its activity and it was shown that SUMO-dependent translocation (nuclear export) of LRH1 was modulated by cAMP signaling (20). These studies suggest that alteration of the PTM status of LRH1 can lead to modulation of its function and cellular location.

To date, the components of the LRH1 transcriptional complex remain poorly understood. In addition to other NR coactivators/corepressors, interleukin enhancer binding factor-3 (ILF3) has been shown to be a novel LRH1 coactivator by acting in synergy with PRMT1 and PGC1 α , and thus, promoting LRH1-dependent gene expression (21). It has been established that PRMT1 methylation of other proteins like SERBP1 affects cytoplasmic/nuclear distribution (22). However, it remains to be determined if PRMT1 methylates or directly interacts with LRH1 and whether this interaction affects LRH1 nuclear translocation. SERBP1, also known as PAI-RBP1, is a plasminogen activator inhibitor 1 (PAI-1) mRNA

binding protein that regulates the stability of PAI-1 mRNA by binding to its cyclic nucleotide-responsive sequence (23). These cyclic nucleotides recruit SERBP1 to stabilize PAI-1 mRNA within the nucleus. In addition to stabilizing mRNA, SERBP1 also has known protein-protein interactions within the nucleus. SERBP1 interacts with CHD proteins, which are members of the chromodomain family of proteins that regulate chromatin remodeling and transcription (24). Furthermore, SERBP1 has been shown to interact with PGRMC1, to mediate the antiapoptotic actions of the progesterone receptor, another NR (25–27). Interestingly, SERBP1 overexpression has been correlated with a favorable prognosis in human breast cancer (28). It remains to be seen whether any protein interaction between SERBP1 and LRH1 exists.

Elucidating the components of the LRH1 transcriptional complex to better understand the receptor's endogenous control mechanism is relevant to understanding its role in cancer. Here a sub-cellular enrichment strategy coupled with a bottom-up proteomic approach was employed to identify putative coregulators of LRH1. This study demonstrated that LRH1 has a direct binding interaction with SERBP1 and that LRH1 is a transrepressor of SERBP1.

Experimental Procedures

Cell Culture and Plasmids

HEK293T cells were cultured in DMEM supplemented with 10% FBS (fetal bovine serum) and 1% PenStrep (penicillin/streptomycin) while Huh7 cells were in RPMI1640 medium supplemented with 10% FBS and 1% PenStrep. All cells were maintained at 37°C with 5% CO₂. AviTag and HaloTag plasmids, including human LRH1 isoforms 1 and 2 as well as empty vector, were purchased from Genecopoeia and Promega, respectively. Alternative splicing of the LRH1 gene produces several isoforms/variants; isoform1 (v1) is the longest (541 AAs) while isoform2 (v2) is missing exon 2, resulting in a shorter protein (22–67). Both of these LRH1 isoforms were studied in this manuscript.

Transient Transfection and Sub-Cellular Fractionation

HEK293T cells were transfected with HaloLRH1 and AviLRH1 plasmids at a 1:3 ratio of DNA:PEI (Polyethylenimine) following Promega's Technical Manual protocol Part # TM348. Post transfection (24hrs), cells were harvested and fractionated by using gentle lysis/buffer conditions (Qiagen Qproteome Nuclear Protein Kit) to isolate nucleic-acid binding proteins (nuclear fraction) from nuclear proteins that are intimately associated with DNA/histones (insoluble fraction).

Western Blot

For detection of overexpressed LRH1 protein from HaloLRH1 and AviLRH1 samples, cells were harvested 24hrs or 48hrs post transfection by adding iced-cold PBS and removing cells from culture plate by gentle scraping with cell-scraper. Cells were then transferred to a pre-chilled tube and the cell pellet was collected following centrifugation. Gentle lysis/buffer conditions were used to properly fractionate the cell to isolate the nuclear and insoluble fractions as discussed above (Qiagen Qproteome Nuclear Protein Kit). Protein concentration was determined by BCA protein assay (Pierce). Proteins were separated by SDS-PAGE and

membranes were probed with anti-HaloTag antibody (1:1000, Promega) for overexpressed HaloLRH1 samples or anti-Streptavidin-HRP (1:200000, Thermo Scientific) for AviLRH1 samples. Overexpressed LRH1 was also detected with anti-LRH1 antibody, which recognizes both LRH1 isoforms (1:500, R&D Systems). Similarly, endogenous LRH1 from Huh7 cells was harvested, lysed, and detected (as discussed above) with the same anti-LRH1 antibody (R&D Systems). SERBP1 antibody was purchased from Abnova (Taiwan) while the ILF3 antibody was from Sigma. Western blots were developed with the Pierce Fast Western Blot Kit, SuperSignal West Femto Substrate (Thermo Scientific). The AviLRH1 western blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Immunoprecipitation

Following cell harvest, sub-cellular fractionation, and protein quantification, LRH1 protein was captured on affinity beads (Halo Resin for HaloLRH1 and Avidin Resin for AviLRH1 samples) and eluted from resin for analysis (western blot or MS/MS) or digested on-bead for MS/MS. Pull down of HaloLRH1 from the Halo resin was conducted as indicated in Promega's Technical Manual protocol Part # TM348. On-bead trypsin digestion on the Avidin resin was performed as suggested by Weerapana et. al (29). Endogenous LRH1 and SERBP1 was IP from Huh7 cells using Pierce Crosslink Magnetic IP/Co-IP kit following manufacture's protocol. The IP antibodies used were anti-LRH1 (R&D Systems) and anti-SERBP1 (Abnova). Normal mouse IgG antibody was used as a control and purchased from Santa Cruz Biotechnology.

Protein Digestion and LC MS/MS Analysis

Following LRH1 IP from overexpressed samples, HaloLRH1 samples were digested with trypsin (Promega V5111) using the filter-aided sample preparation (FASP) while AviLRH1 samples were trypsin digested on-bead (29, 30). For detection of endogenous LRH1 or SERBP1 from Huh7 IP samples, protein was separated on SDS-PAGE and stained with Coomassie blue stain for 1 hour at room temperature with shaking followed by destaining in water overnight. The gel bands were excised, dehydrated using a 2:1 acetonitrile: 25mM ammonium bicarbonate solution, and washed using a 25mM ammonium bicarbonate solution twice. The existing disulfide bonds were reduced by incubating in 10 mM DTT at 56°C for 1 hour. The resulting thiols were alkylated with 55mM iodoacetamide (IDA) in the dark for 45 minutes. Proteins were digested using trypsin (Promega – V5111), at a 1:100 (w:w) ratio (trypsin:protein), overnight at 37°C. Dried peptides were brought up in 23 µL of 0.1% formic acid in water, sonicated for 5 min at RT in a Branson 5510 sonicator, and 10µL of sample was injected on column for LCMS/MS analysis. All samples were run on a quadrupole/orbitrap with a Q Exactive mass spectrometer (Thermo Scientific) coupled to an Easy-nLC 1000 with a Nanospray Flex ion source (Thermo Scientific). All columns were C12 selfpacked capillaries. Trap columns (IntegraFrit IF360-100-50-N-5) were packed with 2 cm stationary phase and separation columns (Cat# IntegraFrit IF360-75-50-N-5) were packed with 15 cm of stationary phase, Jupiter 4µ Proteo 90A (04A-4396). Peptides were separated using 120 min LC gradient, from 5 to 35% acetonitrile/0.1% formic acid. Data were acquired using a FullMS/dd-MS2(Top10) method at a resolution of 70,000, and in some cases an exclusion/inclusion list was incorporated into the instrument method. The raw

data were processed using Mascot Daemon v2.4.0 (Matrix Sciences, Ltd.) and searched against an in-house NR database sequences or human proteins in SwissProt. The search parameters were: carbamidomethyl (Cys), oxidation (Met) and phosphorylation (Ser & Thr) as variable modifications; trypsin was selected for enzyme specificity and 2 missed cleavages were allowed; parent ion tolerance of ± 10 ppm was selected; and a fragment ion mass tolerance of 0.02 Da. The results from the database searches were imported into Scaffold v4.3.0 and identification parameters were set to 20% Protein Threshold, 2 peptides minimum, and 90% Peptide Threshold. A false discovery rate was assessed using a search against a decoy database. Annotated spectra are provided at the link below: http://figshare.com/articles/SERBP1_is_a_component_of_the_Liver_Receptor_Homolog_1_transcriptional_complex/1383212

mRNA Level Measurements

RNA was isolated following the Qiagen RNeasy mini prep manufacturer's protocol. cDNA was prepared from 2 μ g total RNA using the Applied Biosystems high capacity cDNA kit following the manufacturer's protocol. Gene expression was analyzed with the Applied Biosystems 7900HT real-time PCR instrument using GAPDH as the housekeeping gene. Sequences of primers used are listed in Supplemental Table 1.

siRNA Transfection

Huh7 cells were transfected for 24hrs with ON-TARGETplus Human NR5A2 siRNA (GE Dharmacon, cat #: L-003430-00-0005) following instructions for DharmaFECT transfection reagent to efficiently knockdown LRH1 mRNA levels. Briefly, cells were seeded onto 6-well plates (2.5×10^5 cells/well) overnight in RPMI1640 media with 10% FBS. The following day, media was removed and replaced with media containing a final siRNA concentration of 25 nM and 2.5 μ L DharmaFECT transfection reagent per well in 2 ml of media. As a negative control, cells were also transfected with a Non-Targeting siRNA (Scramble siRNA Control) at the same concentration. Cells were harvested 24hrs post transfection and RNA was isolated following the Qiagen RNeasy mini prep manufacturer's protocol. cDNA was prepared from 2 μ g total RNA using the Applied Biosystems high capacity cDNA kit following the manufacturer's protocol. Gene expression was analyzed with the Applied Biosystems 7900HT real-time PCR instrument using GAPDH as the housekeeping gene.

Promoter:Reporter Assay of Overexpressed LRH1

Transient transfections were performed in bulk by plating 3×10^6 HEK293T cells per 10 cm plate with 9 μ g of total LRH1 DNA (1:1 ratio of receptor to reporter) and FuGene6 (Roche) in a 1:3 DNA to lipid ratio. After 24 hours, cells were re-plated in 384 well plates at a density of 10,000 cells/well. Luciferase levels were assayed following an additional 20hr incubation period by a one-step addition of BriteLite Plus (Perkin Elmer) and read using an Envision plate reader (Perkin Elmer). Data were normalized as fold change over vector.

SERBP1 Promoter Luciferase Assay

The NCBI target gene accession NM_001018067.1 reference promoter sequence (–1339 to + 101) was synthesized as doubled-stranded gBlock fragment by Integrated DNA Technologies. The vector pGL4.14[luc2/Hygro] (Promega) was digested with EcoRV-HF (New England Biolabs). Ligation of the promoter gBlock with the linear vector followed the Gibson Isothermal method (Gibson assembly kit – New England Biolabs). Transient transfections were then performed in bulk by plating 3×10^6 HEK293T cells per 10 cm plate with 9 μ g of total DNA (1:1 ratio of SERBP1 to Avi vector or AviLRH1) and FuGene6 (Roche) in a 1:3 DNA to lipid ratio. After 24 hours, cells were re-plated in 384 well plates at a density of 10,000 cells/well. Luciferase levels were assayed following an additional 20hr incubation period by a one-step addition of BriteLite Plus (Perkin Elmer) and read using an Envision plate reader (Perkin Elmer). Data were normalized as fold change over vector.

Immunocytochemistry

HEK293T cells were plated on 1-well chamber slides (3×10^5 cells/chamber) (BioExpress). The following day, cells were transfected with both HaloLRH1 isoforms and empty vector at a 1:3 DNA to PEI ratio. After 24hrs, cells were labeled with HaloTag TMRDirect Ligand (1:200) following Promega's protocol Part # TM260. Next day (48 hrs post transfection), cells were then fixed in 4% paraformaldehyde per manufacture's protocol and processed for immunocytochemistry. Briefly, the fixed cells were incubated with 0.01% TritonX-100 to permeabilize the cells and incubated with monoclonal anti-human LRH1 (R & D Systems) overnight at 4°C. The next day, cells were washed and Alexa488-anti mouse IgG was used as the secondary antibody and incubated for 30 minutes at room temperature. Cells were then washed and counterstained with DAPI (1:5000, AppliChem) for 20 minutes at room temperature. Images were analyzed by confocal microscopy and captured on an Olympus Fluoview 1000 with SIM scanner.

Statistical Analysis

Statistical differences were determined using unpaired t-tests to compare groups and were considered significant if $p < 0.05$. For multiple group comparisons a 1-way ANOVA was used. When significant differences were determined with an ANOVA, post-hoc analysis was conducted using a Tukey or Dunnett Test to compare all groups or to a control group, respectively.

Results

A sub-cellular enrichment strategy and the use of HaloTag and AviTag technologies were employed to elucidate LRH1 interacting proteins and to better understand receptor function. To establish the feasibility of the HaloLRH1 and AviLRH1 plasmids, cells were transiently transfected with LRH1 v1, v2 or empty vector. Following sub-cellular fractionation, LRH1 protein is present in both fractions of the LRH1 v1 (isoform 1) and v2 (isoform 2) lanes (Figure 1A). HaloLRH1 protein was also confirmed by the HaloTag TMRDirect ligand (Supplemental Figure 1). Moreover, immunocytochemistry studies of overexpressed HaloLRH1 confirmed LRH1 localizes primarily in the nucleus only when LRH1 is expressed since no LRH1 staining is seen in the vector-transfected cells (Supplemental

Figure 2). Similar results were observed with AviLRH1 transfection and detection of biotinylated LRH1 by anti-Streptavidin-HRP (Figure 1B). The expression of LRH1 was restricted to the nuclear and insoluble fractions only since no LRH1 protein was detected in the cytosolic fraction of either HaloLRH1 or AviLRH1 samples (data not shown). To confirm that both HaloLRH1 and AviLRH1 plasmids were functionally active, a luciferase reporter assays using these receptors and native DNA response element was performed. HEK293T cells were cotransfected with LRH1 and a luciferase reporter gene driven by the promoter for StAR (steroidogenic acute regulatory protein), a known downstream target of LRH1. Cotransfection of LRH1 receptor with either HaloTag or AviTag resulted in a significant increase in luminescence as compared to empty vector, suggesting these plasmids are functional (Supplemental Figure 3A and 3B). Similarly, there is a 10,000 fold increase in LRH1 mRNA levels after AviLRH1 overexpression as compared to empty vector (Supplemental Figure 3C). The expression of SHP, a known LRH1 target gene, was also increased (Supplemental Figure 3D). Combined these results suggest that LRH1 is primarily located within the nucleus and LRH1 protein from both LRH1 isoforms were present in the nuclear and insoluble fractions but not in the cytosol.

To gain insight into the LRH1 transcriptional complex, overexpressed HaloLRH1 from both nuclear and insoluble fractions were captured on Halo affinity beads and eluted for Western blot or LC MS/MS analysis. Similar to results shown in Figure 1, HaloLRH1 was detected in both fractions using anti-LRH1 antibody (Figure 2A). Sample enrichment with the nuclear fractionation protocol was crucial for identifying LRH1 peptides by MS, as most of the peptides identified were present in the insoluble fraction (LRH1 bound to DNA). In the nuclear fraction, two LRH1 peptides were identified, including a LRH1 v1-specific peptide. While in the insoluble fraction, overexpression of both LRH1 v1 and v2 resulted in the identification of seven LRH1 peptides (16 peptide spectral matches, 18% protein sequence coverage) and 5 peptides (10 peptide spectral matches, >9% protein sequence coverage), respectively. In addition, duplicate proteomic runs using an inclusion/exclusion list improved the number of LRH1 peptides identified including LRH1 isoform-specific and fusion-specific peptides. Moreover, results from these proteomics analysis identified ILF3 and SERBP1 as potential LRH1 interacting partners. A summary table of the peptide sequences identified as well as the mascot ion scores is shown in Figure 2B. These protein hits were then confirmed by western blot (Figure 2C). Both the nuclear and insoluble fractions of untransfected HEK293T cell lysate showed an intense band for SERBP1 and ILF3 expression, while no LRH1 protein was detected (data not shown). Thus, the presence of a band in the vector lanes suggests that endogenous ILF3 and SERBP1 expression is abundant in HEK293T cells (Figure 2C).

LRH1 has been shown to play a critical role in tumor development and cancer progression. To probe the endogenous LRH1 transcriptional complex, LRH1 was immunoprecipitated from Huh7 cells, a hepatocarcinoma cell line with high LRH1 expression level. Western blot analysis revealed an LRH1 band at ~55kDa in both the nuclear and the insoluble fractions that is not present at the molecular weight in the IgG lanes (Figure 3A and 3B). In addition, SERBP1 (Figure 3A and 3B) and ILF3 (data not shown) were detected in the LRH1 IP lane, but not in the IgG control, confirming this interaction is evident at endogenous levels and

was not just an effect of overexpression. Interestingly, SERBP1 was also detected in the unbound (wash) lanes, which suggest that additional free protein is present in the nucleus.

In order to localize LRH1 isoforms to specific nuclear fractions, the nuclear and insoluble fractions were ran on a gel and bands were excised around 55kDa from the coomassie stained gel. Tryptic digests of each of the gel bands were analyzed using a bottom-up LC MS/MS approach. An LRH1 v1-specific peptide was identified using tandem MS/MS in the nuclear fraction (Figure 4 and Supplemental Figure 4A). In contrast, an LRH1 v2-specific peptide was identified in the insoluble fraction (Figure 5 and Supplemental Figure 4B). In the insoluble fraction an additional 4 LRH1 peptides were detected. However, LRH1 peptides were not detected from whole cell lysate digests (data not shown), thus highlighting the importance of sample preparation with the sub-cellular enrichment strategy to successfully detect this low abundant transcription factor by MS.

To demonstrate that LRH1 and SERBP1 directly interact co-immunoprecipitation was used. Presence of LRH1 in the SERBP1-targeted immunoprecipitate was confirmed by western blot analysis (Figure 6). LC MS/MS of the SERBP1 pull down identified two LRH1 peptides in both the nuclear and insoluble fractions, including the LRH1 v1-specific peptide VETEALGLAR. ILF3 was also shown to immunoprecipitate with SERBP1 (data not shown). These results indicate that the transcriptional complex containing LRH1, SERBP1 and ILF3 is also relevant under physiological conditions.

SERBP1 has also been shown to play a crucial role in cancer (28, 31). Identifying the role of the LRH1-SERBP1-ILF3 transcriptional complex may shed light into novel function of these proteins and their involvement in cancer progression. To test this, Huh7 cells were transfected with siRNAs targeting LRH1 to efficiently decrease LRH1 mRNA expression. As seen in Figure 7, there is a significant inhibition of both LRH1 and SHP, a known LRH1 target gene (Figure 7A–B). Moreover, knockdown of LRH1 by siRNA showed an increased SERBP1 expression while ILF3 expression was unchanged (Figure 7C–D). Conversely, overexpression of LRH1 with AviLRH1 v1 and v2 in Huh7 cells, resulted in a significant increase in the mRNA expression level of LRH1 and SHP, while SERBP1 expression appeared to be diminished (Figure 8A–C). ILF3 expression was not significantly altered (Figure 8D). Consistent with these data, ILF3 has been shown to be an LRH1 coactivator by acting in synergy with PGC1 α and not an LRH1 target gene (21). Bioinformatics analysis reveals LRH1 response elements (SFRE) on the SERBP1 promoter (Supplemental Figure 5). The effects of LRH1 on SERBP1 expression and the bioinformatics analysis raise the possibility that SERBP1 may be a novel LRH1 target gene. To address this hypothesis, an SERBP1 promoter containing luciferase was cotransfected along with Avi vector, AviLRH1 v1 or v2, and luminescence was measured. As expected, SERBP1 was shown to be constitutively active (baseline luminescence ~10000) and the coexpression with AviLRH1 v1 resulted in ~50% decrease in luminescence, suggesting LRH1 binding to the SERBP1 promoter leads to SERBP1 transrepression (Figure 9A). This effect was more pronounced with cotransfection of LRH1 v1. Therefore, our data suggest SERBP1 may be a novel LRH1 target gene.

Discussion

Detection of transcription factors including NRs and determining their interacting proteins using mass spectrometry-based proteomics has been challenging. The main issues for the study of NRs by MS are low protein expression level and the fact that the complexes are tightly associated with chromatin. Proteins that bind at selected sites, such as transcription factors bound to specific promoters, represent only 0.01–0.001% of total cellular protein, making it challenging to study and identify protein interactions by MS. Therefore, there is a need for strategies which enable NR detection and identification by proteomic technologies. Employing an overexpression system and a sample enrichment strategy coupled with an inclusion/exclusion proteomic method we have identified novel coregulators of the nuclear receptor, LHR1. This approach may now open the door for future proteomic studies to evaluate other LHR1 interacting proteins, to study LHR1 post translational modifications (PTMs), as well as other NR functional mechanisms and protein-protein interactions.

The data presented in this study show that sample enrichment with the nuclear fractionation protocol turned out to be critical to identifying LHR1 peptides by MS, as many of the peptides identified were in the insoluble fraction. Presumably, the insoluble fraction contains LHR1 bound to DNA. Using the HaloLHR1 overexpression system along with duplicate proteomic runs using an inclusion/exclusion list, an improved number of LHR1 peptides were identified, including LHR1 isoform-specific peptides. However, even better LHR1 sequence coverage (36% as compared to 18% with HaloLHR1 transfection) and a greater number of proteins (375 as compared to 46 protein hits with HaloLHR1 transfection) were detected when doing on-bead trypsin digestion on the Avidin resin vs eluting the complex from the Halo beads. Thus, our data show that on-bead tryptic digests yielded more peptide spectral matches, better protein sequence coverage, and a greater number of protein hits.

LHR1 pull down under physiological conditions (Huh7 cells with no transfection) shows a very different result. In the nuclear fraction, only 1 peptide was identified while 5 LHR1 peptides were detected in the insoluble fraction. To gain insight into the relative abundance of LHR1 peptides between overexpressed and endogenous levels, a label-free quantification approach was used to measure the area under the curve of the MS1 spectra. It was determined that there is a 12.8 fold increase in the area of the LHR1 v1-specific peptide in the nuclear fraction of the overexpressed LHR1 as compared to the endogenous LHR1. This effect was not as pronounced in the insoluble fraction when comparing the LHR1 v2-specific peptide, since only a 6 fold change increase in the area was calculated. These data emphasize the importance of optimizing sample preparation and enrichment protocols to successfully detect endogenous levels of nuclear receptors by mass spectrometry-based proteomics approaches.

The overexpression studies identified 48 proteins as unique to LHR1 as compared to empty vector. ILF3 and SERBP1 were 2 of the proteins identified as unique LHR1 interacting partners in both LHR1 overexpression and endogenous studies. Western blot and LC MS/MS analysis revealed that ILF3, SERBP1 and LHR1 co-IP in Huh7 cells, suggesting these 2 proteins are part of the LHR1 transcriptional complex under physiological conditions. Knockdown of LHR1 by siRNA showed an increased SERBP1 expression while

ILF3 expression was unchanged. Conversely, overexpression of LRH1 tends to diminish SERBP1 mRNA levels. In conclusion, it was determined that LRH1 binding to the promoter region of SERBP1 resulted in a decrease in luminescence and thus, SERBP1 transrepression. This inversely proportional relationship between LRH1 and SERBP1 is of great significance.

SERBP1 is a plasminogen activator inhibitor 1 (PAI-1) mRNA binding protein that regulates the stability of PAI-1 mRNA by binding to its cyclic nucleotide-responsive sequence (23). PAI-1 is a major physiological inhibitor of fibrinolysis in the blood and its increased expression has been implicated as a risk factor for myocardial infarction (32, 33). In HepG2 cells, a hepatocarcinoma cell line similar to Huh7 cells used in this study, it was shown that SERBP1 upregulation lead to decreased expression of PAI-1 (34). Although PAI-1 levels were not measured in this study, our LRH1 siRNA experiment suggests that a similar effect on PAI-1 would have been seen since SERBP1 expression was significantly increased. Pertinent to cancer, SERBP1 overexpression has been correlated with a favorable prognosis in human breast cancer (28). LRH1 has been extensively studied in breast cancer. The transcriptional activation of the aromatase cytochrome p450 gene (*CYP19*) in preadipocytes has been shown to be LRH1 dependent (5). Cell culture studies also showed that cAMP stimulators dramatically induce LRH1-dependent *CYP19* expression and this increase can be abrogated by overexpression of SHP, a negative regulator of LRH1 (6, 7). LC MS/MS analysis of both LRH1 and SERBP1 pull downs, show catenins (A1, A2, B1, D1) and CDKs (11B, 13) proteins as LRH1- and SERBP1-specific interacting proteins. Consistent with these data, β -catenin is a well-established LRH1 target gene and in pancreatic cancer, LRH1 acts in synergy with β -catenin to activate CyclinD1 and CyclinE1 expression to increase cell proliferation (35) (11). These observations along with the study presented here showing the inverse relationship between LRH1 and SERBP1 (Figure 7 and 8), highlight the importance of targeting LRH1 for cancer therapeutics.

Our data also raises the possibility that LRH1 may be post translational modified by methylation since LRH1 and SERBP1 co-IP. SERBP1 has been shown to be methylated by PRMT1 and this affects nuclear/cytoplasmic distribution (22). ILF3 has also been identified as an LRH1 coactivator in synergy with PRMT1 and PGC1 α (21). Thus, it remains to be seen if PRMT1 only affects the PTM status of the receptor or whether it directly interacts with LRH1. The cellular localization of LRH1 has been shown to be controlled by conjugation with SUMO (19). It has been shown that SUMOylation of LRH1 alters localization of the receptor in promyelocytic leukemia protein nuclear bodies resulting in repression of its activity and that SUMO-dependent translocation (nuclear export) of LRH1 was modulated by cAMP signaling (20). In a yeast two-hybrid screen using SERBP1 as a bait, 8 interacting proteins were identified which have been described as permanent or transient components of promyelocytic leukemia protein nuclear bodies (36). These studies suggest that it is highly likely that the LRH1-SERBP1 interaction may be influenced by SUMOylation and affect the PTM status of LRH1 and/or its cellular localization. In the SERBP1 IP, several enzymes involved in the SUMO cascade were identified (PIAS3, SENP1, SENP3). A cartoon summarizing the components of the LRH1 transcriptional complex and post translational modifications of the receptor known to date is shown in Figure 10B.

It is essential to establish the role of each LRH1 isoform in cancer development and/or progression in order to develop LRH1 compounds that can successfully be moved to the clinics. The expression level, cellular localization and function of each LRH1 isoform across cancer cell lines remains to be elucidated. Alternative splicing of the LRH1 gene results in the production of several isoforms; isoform1 (v1) is the longest (541 AAs) and isoform2 (v2) results from removal of exon 2 leading in a shorter protein (22–67). Recently, a third isoform was identified (removal of AAs 1–67) but is exclusively expressed in the ovaries (37). It is not known whether the 45 amino acids that are missing in the N-terminus of v2 but present in v1 play a role in DNA binding or chromatin accessibility, receptor translocation, or ligand/coregulator accessibility to the receptor ligand binding domain. Data presented here suggest that at least in Huh7 cells, endogenous LRH1 v1 is the most abundant isoform present in the nuclear fraction while v2 is tightly associated with DNA/chromatin (insoluble fraction) (Figure 10A). It remains to be determined if this localization is cell line-specific. To the best of our knowledge, this is the first report to show distinct LRH1 isoform localization using a sub-cellular fractionation protocol and confirmed by mass spectrometry-based proteomic analysis. Now that this sample preparation and enrichment strategy has been established, quantitative proteomic studies are currently under way to determine the relative abundance of endogenous LRH1 isoforms in the nuclear and insoluble fractions of Huh7 cells and other cancer cell lines. In conclusion, using a sub-cellular enrichment strategy coupled with proteomic approaches, functionally relevant LRH1 coregulators were identified.

Conclusions

This study highlights the importance of optimizing sample preparation and enrichment protocols to successfully detect nuclear receptors by mass spectrometry-based proteomics approaches. Furthermore, the study also emphasizes the need to develop LRH1 inverse agonists for cancer therapeutics, and elucidates additional transcriptional machinery involved in LRH1 signaling that potentially provides new targets for therapeutics development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Fayard E, Auwerx J, Schoonjans K. LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. *Trends in Cell Biology*. 2004; 14:250–260. [PubMed: 15130581]
2. Li Y, Choi M, Cavey G, Daugherty J, Suino K, Kovach A, Bingham NC, Kliewer SA, Xu HE. Crystallographic identification and functional characterization of phospholipids as ligands for the orphan nuclear receptor steroidogenic factor-1. *Molecular cell*. 2005; 17:491–502. [PubMed: 15721253]
3. Solomon IH, Hager JM, Safi R, McDonnell DP, Redinbo MR, Ortlund EA. Crystal structure of the human LRH-1 DBD-DNA complex reveals Ftz-F1 domain positioning is required for receptor activity. *Journal of molecular biology*. 2005; 354:1091–1102. [PubMed: 16289203]
4. Krylova IN, Sablin EP, Moore J, Xu RX, Waitt GM, MacKay JA, Juzumiene D, Bynum JM, Madauss K, Montana V, Lebedeva L, Suzawa M, Williams JD, Williams SP, Guy RK, Thornton JW,

- Fletterick RJ, Willson TM, Ingraham HA. Structural Analyses Reveal Phosphatidyl Inositols as Ligands for the NR5 Orphan Receptors SF-1 and LRH-1. *Cell*. 2005; 120:343–355. [PubMed: 15707893]
5. Clyne CD, Speed CJ, Zhou J, Simpson ER. Liver Receptor Homologue-1 (LRH-1) Regulates Expression of Aromatase in Preadipocytes. *J Biol Chem*. 2002; 277:20591–20597. [PubMed: 11927588]
6. Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Willson TM, Kliewer SA. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Molecular cell*. 2000; 6:517–526. [PubMed: 11030332]
7. Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf DJ. Molecular Basis for Feedback Regulation of Bile Acid Synthesis by Nuclear Receptors. *Molecular cell*. 2000; 6:507–515. [PubMed: 11030331]
8. Schoonjans K, Dubuquoy L, Mebis J, Fayard E, Wendling O, Haby C, Geboes K, Auwerx J. Liver receptor homolog 1 contributes to intestinal tumor formation through effects on cell cycle and inflammation. *Proceedings of the National Academy of Sciences*. 2005; 102:2058–2062.
9. Benod C, Vinogradova MV, Jouravel N, Kim GE, Fletterick RJ, Sablin EP. Nuclear receptor liver receptor homologue 1 (LRH-1) regulates pancreatic cancer cell growth and proliferation. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:16927–16931. [PubMed: 21949357]
10. Chand AL, Pathirage N, Lazarus K, Chu S, Drummond AE, Fuller PJ, Clyne CD. Liver receptor homologue-1 expression in ovarian epithelial and granulosa cell tumours. *Steroids*. 2013; 78:700–706. [PubMed: 23537609]
11. Botrugno OA, Fayard E, Annicotte JS, Haby C, Brennan T, Wendling O, Tanaka T, Kodama T, Thomas W, Auwerx J, Schoonjans K. Synergy between LRH-1 and beta-catenin induces G1 cyclin-mediated cell proliferation. *Molecular cell*. 2004; 15:499–509. [PubMed: 15327767]
12. Benod C, Carlsson J, Uthayaruban R, Hwang P, Irwin JJ, Doak AK, Shoichet BK, Sablin EP, Fletterick RJ. Structure-based discovery of antagonists of nuclear receptor LRH-1. *The Journal of biological chemistry*. 2013; 288:19830–19844. [PubMed: 23667258]
13. Busby, S.; Nuhant, P.; Cameron, M.; Mercer, BA.; Hodder, P.; Roush, WR.; Griffin, PR. Probe Reports from the NIH Molecular Libraries Program. Bethesda (MD): 2010. Discovery of Inverse Agonists for the Liver Receptor Homologue-1 (LRH1; NR5A2).
14. Rey J, Hu H, Kyle F, Lai CF, Buluwela L, Coombes RC, Ortlund EA, Ali S, Snyder JP, Barrett AG. Discovery of a new class of liver receptor homolog-1 (LRH-1) antagonists: virtual screening, synthesis and biological evaluation. *ChemMedChem*. 2012; 7:1909–1914. [PubMed: 22961990]
15. Lee JM, Lee YK, Mamrosh JL, Busby SA, Griffin PR, Pathak MC, Ortlund EA, Moore DD. A nuclear-receptor-dependent phosphatidylcholine pathway with antidiabetic effects. *Nature*. 2011; 474:506–510. [PubMed: 21614002]
16. Musille PM, Pathak MC, Lauer JL, Hudson WH, Griffin PR, Ortlund EA. Antidiabetic phospholipid-nuclear receptor complex reveals the mechanism for phospholipid-driven gene regulation. *Nature structural & molecular biology*. 2012; 19:532–537. S531–532.
17. Marciano DP, Chang MR, Corzo CA, Goswami D, Lam VQ, Pascal BD, Griffin PR. The therapeutic potential of nuclear receptor modulators for treatment of metabolic disorders: PPARgamma, RORs, and Rev-erbs. *Cell metabolism*. 2014; 19:193–208. [PubMed: 24440037]
18. Lee YK, Choi YH, Chua S, Park YJ, Moore DD. Phosphorylation of the hinge domain of the nuclear hormone receptor LRH-1 stimulates transactivation. *The Journal of biological chemistry*. 2006; 281:7850–7855. [PubMed: 16439367]
19. Chalkiadaki A, Talianidis I. SUMO-dependent compartmentalization in promyelocytic leukemia protein nuclear bodies prevents the access of LRH-1 to chromatin. *Mol Cell Biol*. 2005; 25:5095–5105. [PubMed: 15923626]
20. Yang FM, Pan CT, Tsai HM, Chiu TW, Wu ML, Hu MC. Liver receptor homolog-1 localization in the nuclear body is regulated by sumoylation and cAMP signaling in rat granulosa cells. *The FEBS journal*. 2009; 276:425–436. [PubMed: 19125815]

21. Ohno M, Komakine J, Suzuki E, Nishizuka M, Osada S, Imagawa M. Interleukin enhancer-binding factor 3 functions as a liver receptor homologue-1 co-activator in synergy with the nuclear receptor co-activators PRMT1 and PGC-1 α . *The Biochemical journal*. 2011; 437:531–540. [PubMed: 21554248]
22. Lee YJ, Hsieh WY, Chen LY, Li C. Protein arginine methylation of SERBP1 by protein arginine methyltransferase 1 affects cytoplasmic/nuclear distribution. *Journal of cellular biochemistry*. 2012; 113:2721–2728. [PubMed: 22442049]
23. Heaton JH, Dlakic WM, Dlakic M, Gelehrter TD. Identification and cDNA cloning of a novel RNA-binding protein that interacts with the cyclic nucleotide-responsive sequence in the Type-1 plasminogen activator inhibitor mRNA. *The Journal of biological chemistry*. 2001; 276:3341–3347. [PubMed: 11001948]
24. Lemos TA, Passos DO, Nery FC, Kobarg J. Characterization of a new family of proteins that interact with the C-terminal region of the chromatin-remodeling factor CHD-3. *FEBS letters*. 2003; 533:14–20. [PubMed: 12505151]
25. Zhang L, Kanda Y, Roberts DJ, Ecker JL, Losel R, Wehling M, Peluso JJ, Pru JK. Expression of progesterone receptor membrane component 1 and its partner serpine 1 mRNA binding protein in uterine and placental tissues of the mouse and human. *Molecular and cellular endocrinology*. 2008; 287:81–89. [PubMed: 18440126]
26. Peluso JJ, Lodde V, Liu X. Progesterone regulation of progesterone receptor membrane component 1 (PGRMC1) sumoylation and transcriptional activity in spontaneously immortalized granulosa cells. *Endocrinology*. 2012; 153:3929–3939. [PubMed: 22719051]
27. Peluso JJ, Romak J, Liu X. Progesterone receptor membrane component-1 (PGRMC1) is the mediator of progesterone's antiapoptotic action in spontaneously immortalized granulosa cells as revealed by PGRMC1 small interfering ribonucleic acid treatment and functional analysis of PGRMC1 mutations. *Endocrinology*. 2008; 149:534–543. [PubMed: 17991724]
28. Serce NB, Boesl A, Klamann I, von Sereniy S, Noetzel E, Press MF, Dimmler A, Hartmann A, Sehoul J, Knuechel R, Beckmann MW, Fasching PA, Dahl E. Overexpression of SERBP1 (Plasminogen activator inhibitor 1 RNA binding protein) in human breast cancer is correlated with favourable prognosis. *BMC cancer*. 2012; 12:597. [PubMed: 23236990]
29. Weerapana E, Speers AE, Cravatt BF. Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP)--a general method for mapping sites of probe modification in proteomes. *Nature protocols*. 2007; 2:1414–1425. [PubMed: 17545978]
30. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nature methods*. 2009; 6:359–362. [PubMed: 19377485]
31. Koensgen D, Mustea A, Klamann I, Sun P, Zafrakas M, Lichtenegger W, Denkert C, Dahl E, Sehoul J. Expression analysis and RNA localization of PAI-RBP1 (SERBP1) in epithelial ovarian cancer: association with tumor progression. *Gynecologic oncology*. 2007; 107:266–273. [PubMed: 17698176]
32. Alessi MC, Juhan-Vague I. Metabolic syndrome, haemostasis and thrombosis. *Thrombosis and haemostasis*. 2008; 99:995–1000. [PubMed: 18521499]
33. Morange PE, Saut N, Alessi MC, Yudkin JS, Margaglione M, Di Minno G, Hamsten A, Humphries SE, Tregouet DA, Juhan-Vague I. Association of plasminogen activator inhibitor (PAI)-1 (SERPINE1) SNPs with myocardial infarction, plasma PAI-1, and metabolic parameters: the HIFMECH study. *Arteriosclerosis, thrombosis, and vascular biology*. 2007; 27:2250–2257.
34. Iwaki S, Yamamura S, Asai M, Sobel BE, Fujii S. Posttranscriptional regulation of expression of plasminogen activator inhibitor type-1 by sphingosine 1-phosphate in HepG2 liver cells. *Biochimica et biophysica acta*. 2012; 1819:1132–1141. [PubMed: 22819712]
35. Wagner RT, Xu X, Yi F, Merrill BJ, Cooney AJ. Canonical Wnt/beta-catenin regulation of liver receptor homolog-1 mediates pluripotency gene expression. *Stem cells*. 2010; 28:1794–1804. [PubMed: 20734354]
36. Lemos TA, Kobarg J. CGI-55 interacts with nuclear proteins and co-localizes to p80-coilin positive-coiled bodies in the nucleus. *Cell biochemistry and biophysics*. 2006; 44:463–474. [PubMed: 16679534]

37. Kawabe S, Yazawa T, Kanno M, Usami Y, Mizutani T, Imamichi Y, Ju Y, Matsumura T, Orisaka M, Miyamoto K. A novel isoform of liver receptor homolog-1 is regulated by steroidogenic factor-1 and the specificity protein family in ovarian granulosa cells. *Endocrinology*. 2013; 154:1648–1660. [PubMed: 23471216]

Figure 1.

Overexpression of LRH1 isoforms using HaloTag and AviTag. HEK293T cells were transfected with HaloLRH1 (24hrs) and AviLRH1 (48hrs) plasmids. Post transfection, cells were harvested and a sub-cellular enrichment strategy was used to separate the nuclear and insoluble fractions. A) Proteins were separated by SDS-PAGE, membranes were probed with anti-HaloTag antibody (1:1000, Promega), and developed with Pierce Fast Western Blot Kit, SuperSignal West Femto Substrate (Thermo Scientific). B) Proteins were separated by SDS-PAGE, membranes were probed with anti-Streptavidin-HRP (1:200000, Thermo Scientific), and developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Figure 2.

HaloLRH1 pull down identifies SERBP1 and ILF3 as LRH1 interacting partners. A) HEK293T cells were transfected with HaloLRH1. Post transfection (24hrs), cells were harvested and a sub-cellular enrichment strategy was used to separate the nuclear and insoluble fractions. Proteins were separated by SDS-PAGE, membranes were probed with anti-LRH1 antibody (1:500, R&D Systems), and developed with Pierce Fast Western Blot Kit, SuperSignal West Femto Substrate (Thermo Scientific). B) Table summarizing the results from the LCMS/MS analysis of samples prepared in A. C) Western blot analysis of samples prepared in A, probed with anti-SERBP1 antibody (1:500, Abnova) and anti-ILF3 (1:250, Sigma), and developed with Pierce Fast Western Blot Kit, SuperSignal West Femto Substrate (Thermo Scientific).

Figure 3. SERBP1 immunoprecipitates with endogenous LRH1 in Huh7 cells. Approximately 120×10^6 Huh7 cells were harvested and a sub-cellular enrichment strategy was used to separate the nuclear (A) and insoluble fractions (B). Using Pierce Crosslink Magnetic IP/Co-IP kit, LRH1 and normal mouse IgG were immunoprecipitated from the fractions. The same antibody was used for IP and detection (anti-LRH1, R&D Systems). Membranes were also probed with anti-SERBP1 antibody. The flow through fractions containing the unbound proteins is also shown.

Figure 4.

Detection of endogenous LRH1 isoform 1-specific peptide in the nuclear fraction by mass spectrometry-based proteomics analysis. LRH1 pull down samples were prepared as described in Figure 3 and the nuclear fraction was analyzed by LC MS/MS. Extracted ion chromatogram of the MS1(A and B) and MS2 (C) data of the indicated peptide. The delta ppm between the theoretical (panel A) and observed (panel B) monoisotopic mass was determined to be <1ppm.

Figure 5.

Detection of endogenous LRH1 isoform 2-specific peptide in the insoluble fraction by mass spectrometry-based proteomics analysis. LRH1 pull down samples were prepared as described in Figure 3 and the insoluble fraction was analyzed by LC MS/MS. Extracted ion chromatogram of the MS1 (A and B) and MS2 (C) data of the indicated peptide. The delta ppm between the theoretical (panel A) and observed (panel B) monoisotopic mass was determined to be <1ppm.

Figure 6. SERBP1 and LRH1 co-immunoprecipitate in Huh7 cells. Approximately 113×10^6 Huh7 cells were harvested and a sub-cellular enrichment strategy was used to separate the nuclear (A) and insoluble fractions (B). Using Pierce Crosslink Magnetic IP/Co-IP kit, SERBP1 and normal mouse IgG were immunoprecipitated from the fractions. The same antibody was used for IP and detection (anti-SERBP1, Abnova). Membranes were also probed with anti-LRH1 antibody. The flow through fractions containing the unbound proteins is also shown.

Figure 7.

LRH1 knockdown with siRNA increases SERBP1 expression in Huh7 cells. Huh7 cells were transfected for 24hrs with ON-TARGETplus Human NR5A2 siRNA. RNA was isolated, cDNA was prepared from 2 μ g total RNA, and gene expression was analyzed by QPCR using GAPDH as the housekeeping gene. The mRNA expression level of LRH1 (A), SHP (B), SERBP1 (C), and ILF3 (D) was determined. Data was normalized as fold change over Scramble siRNA. Statistically significant difference is depicted by asterisks ($p < 0.05$) as analyzed by t-test.

Figure 8.

LRH1 overexpression decreases SERBP1 expression in Huh7 cells. Huh7 cells were transfected with AviLRH1 for 24hrs. RNA was isolated, cDNA was prepared from 2 μ g total RNA, and gene expression was analyzed by QPCR using GAPDH as the housekeeping gene. The mRNA expression level of LRH1 (A), SHP (B), SERBP1 (C), and ILF3 (D) was determined. Data was normalized as fold change over vector. Statistically significant difference is depicted by asterisks ($p < 0.05$) as analyzed by one-way ANOVA.

Figure 9.

LRH1 binds to the SERBP1 promoter. A) Transient transfections were performed by plating 3×10^6 HEK293T cells per 10 cm plate with $9 \mu\text{g}$ of total DNA (1:1 ratio of SERBP1 to Avi vector or AviLRH1) and FuGene6 (Roche) in a 1:3 DNA to lipid ratio. Luciferase levels were assayed following an additional 20hr incubation period by a one-step addition of BriteLite Plus (Perkin Elmer) and read using an Envision plate reader (Perkin Elmer). Data was normalized as fold change over vector. Statistically significant difference is depicted by asterisks ($p < 0.05$) as analyzed by one-way ANOVA. B) Cartoon depicting LRH1 transrepression of SERBP1 (left picture). When LRH1 is knocked down by siRNA, then the de-repression is removed (right picture) and transcription is turned on.

Figure 10.

LRH1 isoforms in the nucleus of Huh7 cells and components of the LRH1 transcriptional complex. A) Cartoon depicting the LRH1 isoforms identified in the nucleus of Huh7 cells. B) Summary of the components of the LRH1 transcriptional complex known to date.