

Research Article

ROR α phosphorylation by casein kinase 1 α as glucose signal to regulate estrogen sulfation in human liver cells

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Estrogen sulfotransferase (SULT1E1) metabolically inactivates estrogen and SULT1E1 expression is tightly regulated by multiple nuclear receptors. Human fetal, but not adult, livers express appreciable amounts of SULT1E1 protein, which is mimicked in human hepatoma-derived HepG2 cells cultured in high glucose (450 mg/dl) medium. Here, we have investigated this glucose signal that leads to phosphorylation of nuclear receptor ROR α (NR1F1) at Ser100 and the transcription mechanism by which phosphorylated ROR α transduces this signal to nuclear receptor HNF4 α , activating the *SULT1E1* promoter. The promoter is repressed by non-phosphorylated ROR α which binds a distal enhancer (–943/–922 bp) and interacts with and represses HNF4 α -mediated transcription. In response to high glucose, ROR α becomes phosphorylated at Ser100 and reverses its repression of HNF4 α promoter activation. Moreover, the casein kinase CK1 α , which is identified in an enhancer-bound nuclear protein complex, phosphorylates Ser100 in *in vitro* kinase assays. During these dynamic processes, both ROR α and HNF4 α remain on the enhancer. Thus, ROR α utilizes phosphorylation to integrate HNF4 α and transduces the glucose signal to regulate the *SULT1E1* gene in HepG2 cells and this phosphorylation-mediated mechanism may also regulate SULT1E1 expressions in the human liver.

Introduction

Estrogen is metabolically inactivated by sulfation and estrogen sulfotransferase (SULT1E1) specifically and efficiently ($K_m \sim 4$ nM) catalyzes this reaction [1]. SULT1E1 is a member of the cytosolic sulfotransferase (SULT) family of enzymes that collectively sulfate numerous endobiotics and xenobiotics by transferring the sulfate group from 3'-phosphoadenosine 5'-phosphosulfate [2,3]. The expression of SULT1E1 is tightly regulated in tissues and organs differently to regulate metabolic balances of estrogen. In the liver, SULT1E1 is barely expressed in normal mice but highly induced in diabetic mice such as *db/db* mice [4]. SULT1E1 is also induced by treatments with therapeutics such as phenobarbital [5]. In human hepatoma-derived HepG2 cells, SULT1E1 is highly expressed when cells are cultured in medium containing high glucose concentrations [6]. Whereas these observations indicate that SULT1E1 may be involved in interplays between estrogen, glucose and diabetes, the cellular and molecular regulation of SULT1E1 expression is not understood well.

Sult1e1 cDNA first cloned from mouse testis was utilized to demonstrate induced expression of *Sult1e1* mRNA in the liver of diabetogenic *db/db* mice [4]. Genetic aberrations in *Sult1e1* improved anti-inflammatory responses and metabolic dysfunction in the liver of *ob/ob* female mice [7]. This suggests that SULT1E1 regulates estrogen's anti-inflammatory function by altering hepatic levels of active estrogen via sulfation. On the other hand, hepatic SULT1E1 is highly induced in inflammatory-related disease conditions such as sepsis, a deteriorating disease prognosis [8,9].

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Therefore, by regulating levels of active estrogen, *SULT1E1* can become either a beneficial or detrimental factor in disease developments and a target of clinical treatments. For these notions, it is fundamental for us to understand the molecular basis of *SULT1E1* expression.

SULT1E1 is expressed in fetal livers and repressed in adult livers of humans [10] as well as in human primary hepatocyte cultures [11]. The elevated *SULT1E1* expression phenotype is modeled in human hepatoma-derived HepG2 cells [6] when cells are cultured in a DMEM medium that contains high glucose concentration (450 mg/dl). Consistent with this high expression, the promoter of the human *SULT1E1* gene is highly activated in HepG2 cells. This promoter contains a DR1 motif within the 100 bp distal enhancer (−1000/−901 bp), to which nuclear receptor HNF4 α binds and loops the enhancer closely to a proximal region of the promoter to activate it [6]. Therefore, HepG2 cells may be a useful experimental model to investigate the glucose response mechanism of *SULT1E1* expression.

In addition to HNF4 α , various other nuclear receptors are independently reported to regulate hepatic *SULT1E1* expression, which include glucocorticoid receptor (GR), liver X receptor (LXR), constitutive active/androstane receptor (CAR), pregnane X receptor (PXR), farnesoid X receptor (FXR) and retinoid-related orphan receptor α (ROR α) [5,6,12–16]. Among these nuclear receptors, ROR α is unique in its function to co-activate CAR in mouse livers [5]. In response to phenobarbital, ROR α interacts with CAR on the *Sult1e1* promoter and undergoes phosphorylation at Ser100. Phosphorylated ROR α co-activates CAR-mediated transcription of the promoter. These observations suggest that this inducible phosphorylation enables ROR α to co-regulate other nuclear receptors. Therefore, we selected ROR α as a possible candidate for regulating HNF4 α on the 100 bp enhancer of the *SULT1E1* promoter in HepG2 cells and examined whether Ser100 is phosphorylated in response to glucose, allowing phosphorylated ROR α to co-activate HNF4 α .

In this manuscript, a phosphor-Ser100 peptide antibody was used to detect phosphorylated ROR α . A 22 bp sequence overlapping DR1 and RORE (−943/−922 bp) was delineated from the 100 bp enhancer of the *SULT1E1* promoter and analyzed by cell-based reporter and gel-shift assays. Co-immunoprecipitation assays examined interactions between ROR α and HNF4 α . Chromatin immunoprecipitation and chromatin conformation capture assays were employed to examine bindings of nuclear receptors in response to glucose concentrations. The 22 bp DNA oligonucleotide was used in affinity chromatography to purify nuclear proteins for subsequent mass spectroscopic analysis and identify candidate protein kinases that might phosphorylate ROR α . Experimental evidence will be presented to demonstrate that ROR α bound to RORE is phosphorylated at Ser100 by casein kinase 1 α in response to high glucose concentrations, interacting with HNF4 α and co-activating the *SULT1E1* promoter. Subsequently, a novel concept that nuclear receptors integrate their function through phosphorylation to regulate genes will be discussed.

Materials and methods

Chemicals, antibodies, plasmids and primers and siRNAs

Common chemicals, HRP conjugated anti-GFP, anti-M2 Flag, anti-V5 His antibodies and anti-Flag M2 affinity agarose gel were from Sigma–Aldrich; media, supplements, reagents and primers (Supplementary Table S2) were from Thermo Fisher Scientific; ATP [γ - 32 P] and [35 S] phosphor-adenosine phosphosulfate (PAPS) were from PerkinElmer; media and reagents were from Thermo Fisher Scientific; anti-phosphor Serine 100 of ROR α and anti-ROR α antibodies were from GenScript; antibodies against HNF4 α (H171 and H1), β -actin (C4) and goat anti-rabbit or mouse HRP conjugated IgGs were from Santa Cruz Biotechnology; anti-human *SULT1E1* antibody was from Proteintech; anti-CK1 α and anti-RACK7 antibodies were from Novus Biologicals and Bethyl Laboratories, respectively; normal mouse and rabbit IgGs were from Cell Signaling Technology; anti-GFP sepharose beads were kindly provided by the protein expression core at NIHES; the −1081 bp human *SULT1E1* promoter (Δ -1081-*SULT1E1*) and the *SULT1E1* construct containing a 175 bp *SULT1E1* enhancer and a 105 bp *SULT1E1* promoter (Δ -1081-*SULT1E1*) were previously described [6]. Plasmids Flag-tagged pcDNA3.1-HNF4 α WT; pEGFP-HNF4 α WT; Flag-tagged pcDNA3.1 ROR α WT, ROR α S100A and ROR α S100D; GST-tagged ROR α WT and ROR α S100A were previously described [17]. SMARTpool: ON-TARGETplus HNF4A siRNA (L-003406-00-0005) and RORA siRNA (L-003440-00-0005) were from Dharmacon.

Cell cultures, treatments and subcellular fractionations

Human liver hepatocellular carcinoma cells Huh7, HepG2 and monkey kidney Cos-1 cells were obtained from ATCC and cultured in standard DMEM medium. DMEM glucose-free media supplemented with different

concentrations of glucose were used for cell treatments. Cell viabilities of these treated cells were measured by using Cell Counting Kit-8 (Dojindo Laboratories). Cytosolic and nuclear proteins from cultured cells were prepared as described previously [6]. Buffer A (10 mM HEPES pH = 7.6, 10 mM KCl, 1.5 mM MgCl₂, 0.3% NP-40, protease inhibitors cocktail) was used to obtain cytosolic fractions. NE buffer (10% glycerol, 10 mM HEPES, 0.1 M KCl, 3 mM MgCl₂, 300 mM NaCl, 0.1 mM EDTA, 1 mM NaVO₄, protease inhibitors cocktail) was used to extract nuclear fractions.

siRNA knockdown and quantitative reverse transcription PCR

Lipofectamine RNAiMAX and siRNAs were used for knockdown experiments in Huh7 cells. Total cellular RNA was extracted from cells by using TRIzol reagent in accordance to the manufacturer's instructions. Extracted mRNAs were reverse transcribed to cDNAs by using High-Capacity cDNA Reverse Transcription Kit. TaqMan probes (Supplementary Table S2) and Taqman Master mix were applied to measure respective mRNA expressions. Relative mRNA expressions were compared with the β -actin housekeeping gene of the treated cells. Real-time PCR was performed by using CFX96™ Real-time system (Bio-Rad Laboratories).

Estrogen sulfating activity assays

An amount of 20 μ g of HepG2 cytosolic extract and [³⁵S] PAPS as a sulfate donor were mixed in the presence of 0.1 μ M or 100 μ M E2 in a final 25 μ l reaction containing 50 mM HEPES-NaOH, pH = 7.0. Reactions were first incubated at 37°C for 30 min, followed by 95°C for 5 min to inactivate reactions. The [³⁵S] sulfated E2 in supernatants was separated by using TLC procedures with ethyl acetate/n-butanol (2:1 by volume) on Whatman silica gel plate (PGC Scientific) and was visualized on X-ray film, which was subsequently counted in the Beckman LS6500 scintillation counter for radioactivity.

Electrophoretic mobility shift assays

The probes containing the respective DR1 and RORE spanning from -943 to -922 bp upstream of the *SULT1E1* translation start site and a consensus RORE are listed in Supplementary Table S2. These probes were labeled with ATP [γ -³²P] by using a T4 polynucleotide kinase (NEB) and purified using Illustra MicroSpin G-25 Columns (GE Healthcare). Each probe was incubated with respective proteins, and/or antibodies in binding buffer (10 mM Tris-HCl, pH = 7.5, 50 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.5 μ g poly (dI-dC), 0.05% NP-40). The samples were separated by electrophoresis on a non-denaturing 4% polyacrylamide gel in running buffer (7 mM Tris-HCl, pH = 7.5, 3 mM NaAc, 1 mM EDTA). The radioactive signal was subsequently developed onto autoradiography film (Denville Scientific).

Site direct mutagenesis and reporter gene assays

A series of *SULT1E1* promoter mutants were generated by using pGL3/ Δ -1081-*SULT1E1* as a template and respective primers (Supplementary Table S2) according to manufacturer's instructions (QuikChange Site-Directed Mutagenesis Kit, Agilent Technologies). Luciferase reporter gene assays were conducted by transfecting respective *SULT1E1* promoters together with HNF4 α or ROR α into Huh7 cells, respectively. Luciferase activities of the transfected cells were measured using the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation (ChIP) and co-immunoprecipitation (CoIP) assays

ChIP samples of the treated cells were prepared using ChIP-IT Express Chromatin Immunoprecipitation Kit (Active Motif) according to the manufacturer's instructions. Precipitated chromatin was then used as a template for quantitative and real-time PCRs with specific primers (Supplementary Table S2). GFP-tagged HNF4 α was cotransfected with Flag-tagged ROR α WT, ROR α S100A or ROR α S100D into Cos-1 cells, respectively. Nuclear fractions of the transfected cells were obtained, which were used in CoIP reactions. 50 μ g of nuclear extract was diluted in 100 μ l of IP buffer (20 mM Tris, pH = 7.4, 0.5 mM EDTA, 1% Triton-X100, 10% Glycerol, 500 mM NaCl, protease inhibitors cocktail), which was incubated with 10 μ l of anti-GFP antibody conjugated sepharose beads for precipitating GFP-tagged HNF4 α . The precipitated proteins and 10% of the total input samples were subjected to SDS-PAGE for western blotting with anti-Flag-M2 HRP conjugated or anti-GFP HRP conjugated antibody, respectively.

***In vitro* phosphorylation assays and western blotting**

Escherichia coli BL21 (DE3) (Agilent Technologies) bacteria expressed GST-hROR α WT and its alanine mutant GST-hROR α S100A which were then purified by glutathione sepharose 4B (GE Healthcare). An amount of 6 μ g of each purified protein was incubated with or without 200 ng of GST-CK1 α (Promega), respectively, in 30 μ l of kinase buffer (40 mM Tris pH = 7.5, 20 mM MgCl₂, 0.1 mg/ml BSA, 50 μ M DTT) and 200 μ M ATP at 30°C for 30 min. Samples were denatured and subjected to SDS-PAGE for western blotting analysis with antibodies against phosphorylated ROR α at Ser100 or ROR α , respectively. The membrane was blocked in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% non-fat milk for 1 h and probed with the relevant antibody overnight at 4°C. HRP-conjugated secondary antibody was reacted with the probed primary antibody after washing. Signal was developed by using Western Bright ECL or Sirius kit (Advansta).

Protein purification and mass spectrometry

HepG2 cells were seeded and exposed to low glucose or high glucose conditions for 48 h, respectively, followed by nuclear protein extractions. The 22 bp *SULT1E1* enhancer was repeated four times and was subcloned into pGL3-TK basic plasmid between KpnI and BglII. The 4 \times *SULT1E1* enhancer was amplified from the plasmid by using the primers (Supplementary Table S2) and purified by using a QIAquick gel extraction kit (QIAGEN). The purified DNAs were biotinylated by using DNA Polymerase I, Large (Klenow) Fragment (NEB), and immobilized to Dynabeads M-280 Streptavidin (Thermo Fisher Scientific) for enriching the associated gene regulatory complexes from the HepG2 cell nuclear extracts. Beads containing biotinylated DNAs were incubated with the nuclear extracts for 3 h. The DNA-protein complexes were washed by the NE buffer, then eluted by an elution buffer (5 mM Tris-HCl, pH = 7.5, 500 mM EDTA, 1 M NaCl), followed by mass spectroscopic analysis. A complete list of enriched factors was submitted to the ProteomeXchange Consortium via the PRIDE [18] partner repository with the dataset identifier PXD016385.

Chromosome conformation capture (3C) assays

3C assays were conducted as previously described with minor modifications [6]. 1.5×10^6 HepG2 cells were seeded and treated with low glucose or high glucose conditions for 48 h, respectively. These cells were fixed with formaldehyde [final 2% (v/v)] for 10 min. Cells were suspended in 2 ml lysis buffer (50 mM Tris pH = 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1% Triton X-100, protease inhibitors). Cell pellets were harvested and suspended in 600 μ l restriction enzyme buffer 2.1 containing 0.3% SDS (w/v), which were incubated at 37°C for 1 h. Thereafter, Triton X-100 was added in the reaction to a final concentration of 2.1% (v/v), followed by incubation at 37°C for 1 h. The treated cells were digested by 600 U restriction enzyme BstYI (NEB) for 15 min at 60°C, then overnight at 37°C. The digested chromatin was ligated by 20 μ l T4 DNA ligase (NEB) in a 7 ml reaction at 16°C overnight. Proteinase K, NaCl, and EDTA were added to the ligation mixtures to final concentrations of 40 μ g/ml, 0.2 M and 1 mM, respectively for reverse cross-linking, 16 h at 65°C. RNase A was added to the mixtures to a final concentration of 40 μ g/ml. The mixtures were incubated for 45 min at 37°C before purification by using a QIAquick PCR purification kit (QIAGEN). The purified DNAs were subjected to RT-PCR and quantitative PCR analysis of control and ligated fragments by respective primers (Supplementary Table S2).

Statistical analysis

Two group comparisons were analyzed by the student's *t*-test. Multiple group comparisons were analyzed with one-way or two-way analysis of variance (ANOVA) followed by the least significant difference post hoc test. Differences were considered significant when $P < 0.05$.

Results

***SULT1E1* is induced at high glucose cultured HepG2 cells**

DMEM media containing different concentrations of glucose were utilized to treat HepG2 cells for 48 h, respectively, followed by measuring *SULT1E1* and *STS* mRNA expression levels. Low glucose (40 mg/dl), normal glucose (140 mg/dl) and high glucose (450 mg/dl) were selected as they imitate hypoglycemia, normal and hyperglycemia conditions in humans, respectively [19,20]. *SULT1E1* mRNA expression in HepG2 cells was 114 times higher at high glucose condition than it was at low glucose condition (Figure 1A). No significant differences were observed between the normal glucose condition and high glucose condition. However, steroid sulfatase (*STS*), the enzyme that activates sulfated estrogen to estrogen, was significantly repressed in the high glucose condition (Figure 1B). Consistent with mRNA

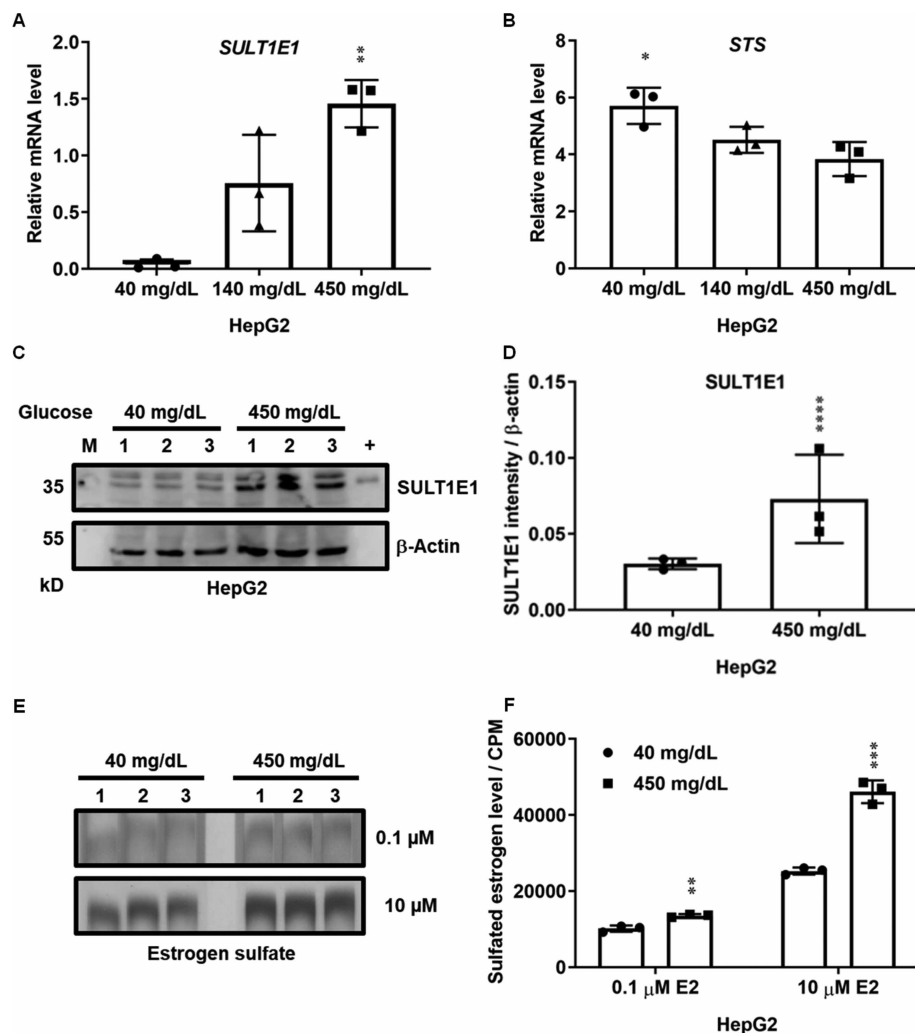


Figure 1. High glucose induces SULT1E1 expression in HepG2 cells.

Glucose-responsive (A) *SULT1E1* and (B) *STS* gene expressions were detected in HepG2 cells by qRT-PCR. The values ($N = 3$) represent means \pm S.D. Mean difference is significant from low glucose treated group at $** P < 0.005$; $* P < 0.05$ (One-way ANOVA). (C) SULT1E1 protein expressions in response to glucose exposures were measured in HepG2 cells by western blotting, followed by (D) densitometry analysis of the SULT1E1 band intensity. Experiments were conducted in triplicates. '+' indicates a bacterial expressed SULT1E1. (E) Estrogen sulfation was measured in both low glucose treated and high glucose treated HepG2 cells in the presence of 0.1 μM or 10 μM E2. (F) [^{35}S] sulfated E2 was separated by TLC procedures, followed by measuring their radioactivities. The values ($N = 3$) represent means \pm S.D. Mean difference is significant from low glucose treated group at $**** P < 0.0001$; $*** P < 0.0005$; $** P < 0.005$ (Student's t -test).

expression, SULT1E1 protein expression was significantly induced in high glucose cultured HepG2 cells (Figure 1C, D). Estrogen sulfation was significantly activated in high glucose treated HepG2 cells in the presence of both 0.1 μM and 10 μM E2 as substrate (Figure 1E,F). This induction of *SULT1E1* by high glucose was also observed in the HepG2 3D culture and Huh7 cells (Supplementary Figure S1A,B). Both low and high glucose exposures for HepG2 and Huh7 cells did not significantly affect their cell viabilities (Supplementary Figure S1C,D). These observations suggested that glucose exposures regulate *SULT1E1* gene expression in cells. The SULT1E1 activation and STS suppression at high glucose conditions may serve as a negative feedback loop to inactivate estrogens in human liver cells.

A HNF4 α and ROR α overlapping enhancer on the *SULT1E1* gene

To investigate how *SULT1E1* is regulated in cells, a detailed DNA sequence analysis of the *SULT1E1* promoter was performed by using the software MatInspector [21]. Intriguingly, a putative DR1 (HNF4 α binding) and a

putative RORE (ROR α binding) overlapping region was detected spanning from -943 to -922 bp upstream of *SULT1E1* translation start site (Figure 2A, boxed sequences). It was previously shown that HNF4 α binds to the DR1 element of the *SULT1E1* gene, looping a 100 bp *SULT1E1* enhancer (Figure 2A) to its proximal promoter thereby activating gene expression [6]. However, the role of the putative RORE within the *SULT1E1* enhancer in the regulation of gene expression remains unclear. To decipher the possible bindings of HNF4 α and ROR α to the *SULT1E1* enhancer, two probes that represent the DR1 containing RORE half-site (DR1, solid line) and the RORE containing DR1 half-site (RORE, dashed line) were utilized in gel shift assays. Incubating the DR1 probe with a HepG2 nuclear extract resulted in the formation of two complexes, which are indicated by a solid arrow (upper) and blank arrow (lower), respectively (Figure 2B, lane 2). However, the predominant complex was the upper complex. Both complexes were supershifted by an antibody against HNF4 α (Figure 2B, lane 3, dashed arrow), suggesting the binding of HNF4 α to the DR1 probe. The antibody against ROR α significantly weakened the binding for the upper complex and shifted the binding of the lower complex (Figure 2B, lane 4), indicating a possible interaction between HNF4 α and ROR α and a weak binding of ROR α to the DR1 probe. Similarly, the same complexes were formed when incubating the RORE probe with a HepG2 nuclear extract, whereas the major complex was the lower complex (Figure 2B, lane 7). The complexes were supershifted by both anti-HNF4 α antibody (Figure 2B, lane 8) and anti-ROR α antibody (Figure 2B, lane 9), respectively, which suggested ROR α binding and a possible interaction between ROR α and HNF4 α on the RORE probe. The consensus RORE which is known for ROR α binding was used as a positive control to validate the quality of the assays (Figure 2B, lanes 11–14). These observations indicated a HNF4 α and ROR α binding complex on the *SULT1E1* enhancer.

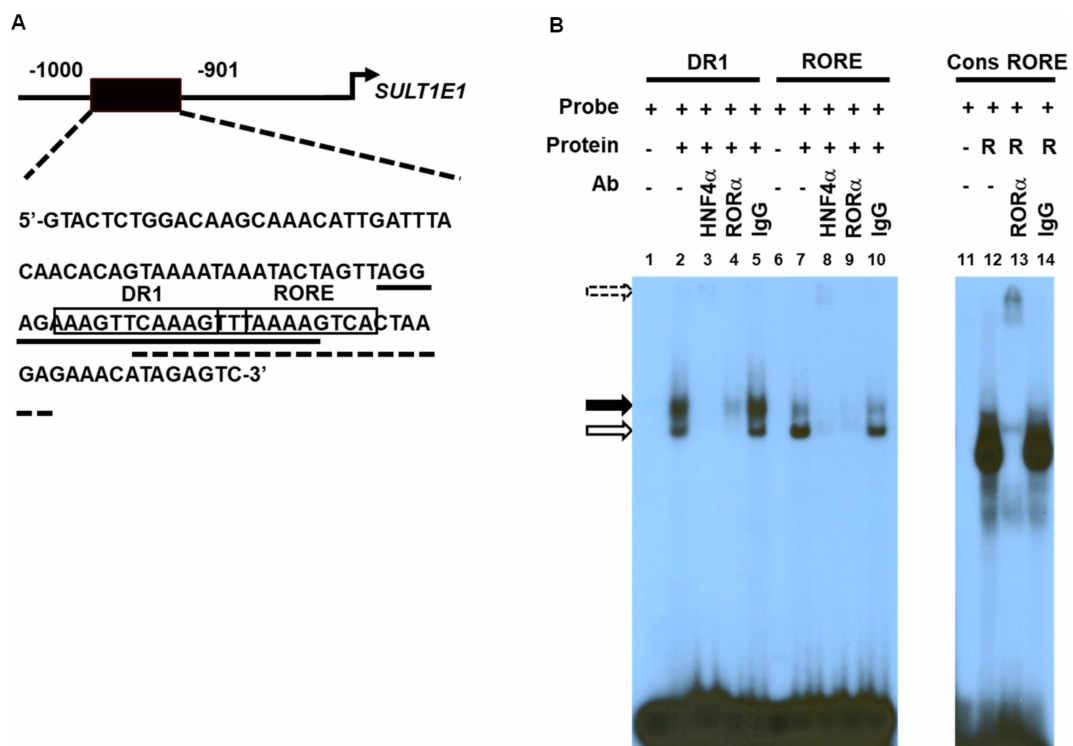


Figure 2. Nuclear receptors HNF4 α and ROR α bind to the *SULT1E1* gene.

(A) Schematic representation of the HNF4 α (DR1) and ROR α (RORE) binding elements on the *SULT1E1* gene. A DR1 and RORE overlapping region (boxed sequences) was detected ~1000 bp upstream of the *SULT1E1* promoter. The *SULT1E1* DR1 containing RORE half-site (solid line), and the *SULT1E1* RORE containing DR1 half-site (dashed line) were used as probes in the gel shift assays. (B) Bindings of HNF4 α and ROR α to the *SULT1E1* gene. The probes were radioactively labeled, which were incubated with a HepG2 nuclear extract or an *in vitro* translated ROR α (R), respectively. The antibodies against HNF4 α and ROR α were used to supershift (dashed arrow) the formed protein/DNA binding complexes (solid and blank arrows). A normal IgG was used as a negative control for the antibodies used in the study. The Cons RORE was served as a positive control of the assays. Experiments were repeated three times.

HNF4 α and ROR α cooperatively regulate *SULT1E1* promoter

To characterize the roles of HNF4 α and ROR α in regulating *SULT1E1* promoter activities, a -1081 bp *SULT1E1*-Luc reporter, an 800 bp internal deleted Δ -1081 bp *SULT1E1*-Luc reporter containing the 22 bp enhancer and the 105 bp proximal promoter, and their respective mutants on DR1 or/and RORE were constructed (Figure 3A). They were transfected into Huh7 cells for reporter gene assays. First, the internal deletion of the *SULT1E1* promoter increased its promoter activity about seven times compared with the full-length

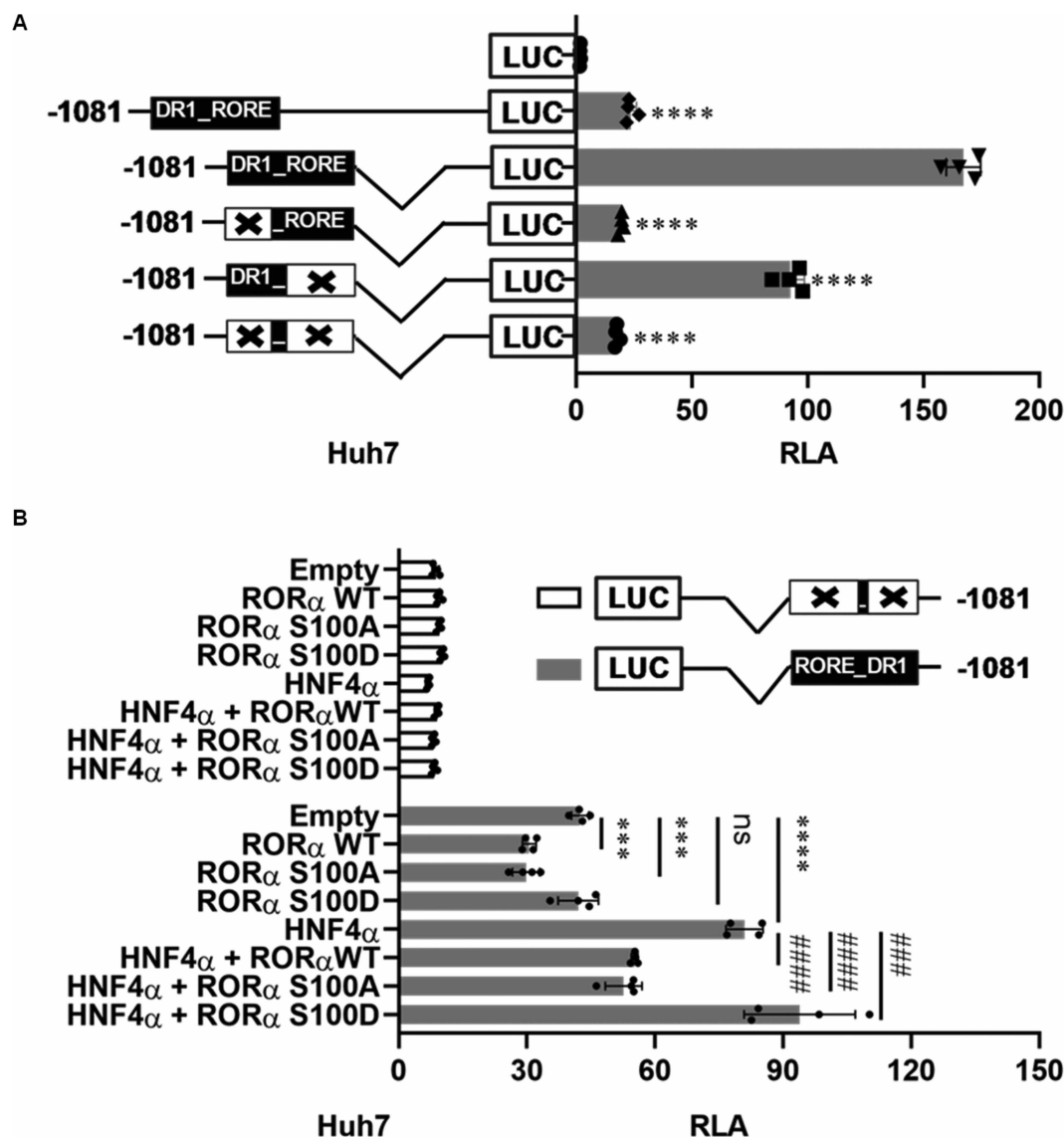


Figure 3. Nuclear receptors HNF4 α and ROR α cooperatively regulate *SULT1E1* via a 22 bp enhancer.

(A) Role of the 22 bp enhancer in regulating *SULT1E1* promoter. -1081 *SULT1E1*-Luc, Δ -1081 *SULT1E1*-Luc or its mutants was transfected into Huh7 cells, respectively for 24 h. Luciferase activities of the transfected cells were measured, which were normalized by Renilla luciferase (transfection control) activities. The values ($N = 4$) represent means \pm S.D. Mean difference is significant from Δ -1081 *SULT1E1*-Luc transfected group at **** $P < 0.0001$ (One-way ANOVA). (B) Roles of HNF4 α and ROR α in regulating *SULT1E1* promoter. HNF4 α and Δ -1081 *SULT1E1*-Luc or mutated Δ -1081 *SULT1E1*-Luc were cotransfected with ROR α WT, ROR α S100A or ROR α S100D into Huh7 cells, respectively for 24 h. Luciferase activities of the transfected cells were measured, which were normalized by Renilla luciferase activities. The values ($N = 4$) represent means \pm S.D. Mean difference is significant from empty vector transfected group at **** $P < 0.0001$; *** $P < 0.0005$; and from HNF4 α transfected group at #### $P < 0.0001$; ### $P < 0.0005$ (Two-way ANOVA).

promoter, confirming that the internal deletion facilitates the looping between the enhancer and the promoter thereby activating its promoter activity (Figure 3A). Mutating the DR1 and RORE individually or in combination dramatically decreased promoter activity, illustrating critical roles of the DR1 and RORE in supporting *SULT1E1* promoter activity (Figure 3A). To investigate whether HNF4 α , ROR α and its mutants regulate *SULT1E1* promoter via the 22 bp enhancer, HNF4 α and Δ -1081 *SULT1E1*-Luc or mutated Δ -1081 *SULT1E1*-Luc were cotransfected with ROR α WT, ROR α S100A (non-phosphorylatable mutant) or ROR α S100D (phosphomimetic mutant) into Huh7 cells, respectively. *SULT1E1* promoter activity was significantly repressed by ROR α WT and ROR α S100A, whereas ROR α S100D did not affect promoter activity, and HNF4 α greatly activated it (Figure 3B). Intriguingly, HNF4 α together with ROR α WT or ROR α S100A dramatically repressed *SULT1E1* promoter activity, while HNF4 α along with ROR α S100D significantly activated it (Figure 3B). The mutation of the 22 bp enhancer on the *SULT1E1* promoter remarkably lost its activity and abolished its responsiveness to both HNF4 α and ROR α in Huh7 cells (Figure 3B). These observations, together with the data shown in Figure 2, indicated that ROR α interacting with HNF4 α co-repress *SULT1E1* transcription, while phosphorylated ROR α at Ser100 interacts with HNF4 α to co-activate its expression via the enhancer. The roles of HNF4 α and ROR α in regulating endogenous *SULT1E1* transcription were further demonstrated by siRNA knockdown experiments in Huh7 cells (Supplementary Figure S2). Additionally, ROR α S100D overexpression activated the endogenous *SULT1E1* transcription in HepG2 cell, further demonstrating that phosphorylated ROR α and HNF4 α activate the promoter in human liver cells (Supplementary Figure S3).

Glucose responsive bindings of HNF4 α and ROR α to the *SULT1E1* gene

Subsequently, ChIP assays were conducted to examine whether HNF4 α and ROR α are responsive to glucose exposures and bind the *SULT1E1* enhancer in human liver cells. The anti-phosphor Ser100 ROR α antibody was applied to detect whether the conserved phosphorylation site becomes a regulatory factor for *SULT1E1* gene expression in response to glucose exposures. In HepG2 cells, binding of HNF4 α to the *SULT1E1* enhancer was slightly weaker under low glucose conditions than it was with high glucose conditions, suggesting that HNF4 α may interact with different factors binding to the enhancer at both low glucose and high glucose conditions (Figure 4A). In ROR α ectopically expressed HepG2 cells, ROR α weakly bound to the *SULT1E1* enhancer in low glucose conditions, whereas phosphorylated ROR α was significantly enriched on it at high glucose conditions (Figure 4B). Moreover, the phosphorylation of ROR α at Ser100 was induced in high glucose treated HepG2 cells (Figure 4C). In agreement with the results shown in Figures 2 and 3, ROR α together with HNF4 α suppresses the *SULT1E1* promoter while phosphorylated ROR α and HNF4 α activate its expression. We hypothesize that ROR α interacting with HNF4 α binds *SULT1E1* gene to co-repress its expression at low glucose conditions. In response to high glucose signal, ROR α is phosphorylated at Ser100, interacting with HNF4 α to co-activate *SULT1E1* transcription. The phosphorylation of Ser100 on ROR α may be a key factor to regulate *SULT1E1* in response to glucose exposures.

ROR α forms a complex with HNF4 α binding to the *SULT1E1* gene

A series of CoIP and gel shift assays were deployed to illustrate the interactions between ROR α and HNF4 α on the *SULT1E1* enhancer. First, CoIP assays showed that HNF4 α strongly interacted with ROR α regardless of its phosphorylation on Ser100 (Figure 5A), which is consistent with previous observations that HNF4 α interacts with ROR α to bind the DR1 and RORE overlapping enhancer on the *SULT1E1* gene (Figures 2–4). Incubating the DR1 and RORE probe with a HepG2 nuclear extract resulted in the formation of two equal complexes (Figure 5B, lane 1). An anti-HNF4 α antibody shifted the HNF4 α complex but not the ROR α complex (Figure 5B, lane 2), while both complexes were shifted by an anti-ROR α antibody (Figure 5B, lane 3). This confirmed that ROR α forms a complex with HNF4 α to bind the enhancer and ROR α itself binds to the enhancer as well. The interaction between ROR α and HNF4 α was further demonstrated by using *in vitro* translated proteins in gel shift assays (Supplementary Figure S4). Interestingly, the anti-phosphor Ser100 ROR α antibody specifically shifted the HNF4 α /ROR α complex but not the ROR α complex (Figure 5B, lane 7), suggesting that phosphorylated ROR α formed a complex with HNF4 α binding to *SULT1E1* enhancer. The specificity of this binding was demonstrated by incubating the consensus RORE probe with a HepG2 nuclear extract and respective antibodies (Figure 5B, lane 8–12). These observations are consistent with previous results that phosphorylated ROR α forms a complex with HNF4 α to bind the *SULT1E1* enhancer at high glucose conditions.

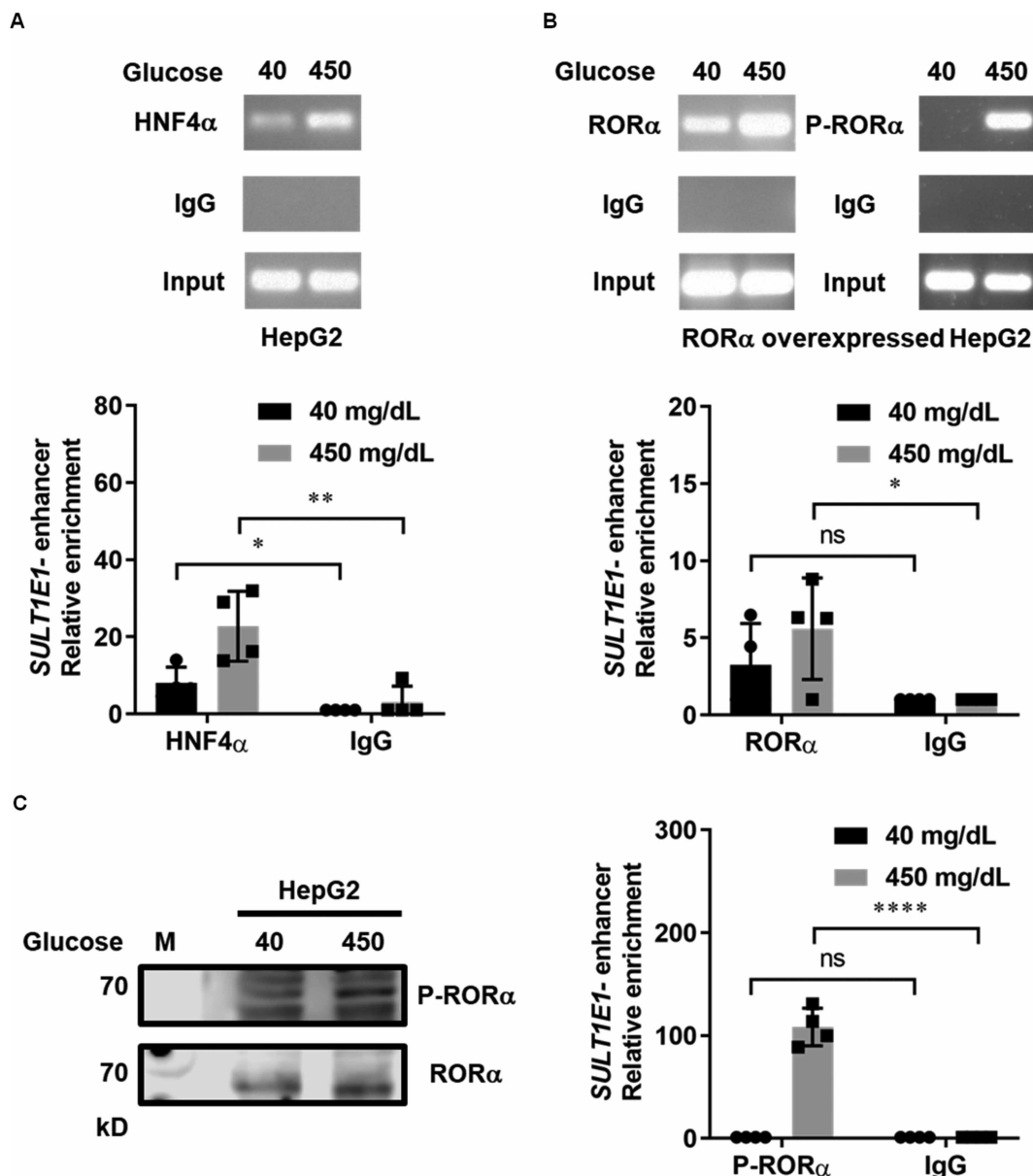


Figure 4. Glucose responsive RORα and HNF4α bindings to the SULT1E1 enhancer.

(A) HepG2 cells or (B) RORα overexpressed HepG2 cells were exposed to low glucose or high glucose medium, respectively for 48 h, followed by chromatin immunoprecipitations. The antibodies against HNF4α, RORα and phosphorylated RORα were used to precipitate protein/chromatin binding complexes from the treated cells. The precipitated chromatin was subjected to both real-time PCR and qRT-PCR analysis with a specific primer set (Supplementary Table S2) amplifying the SULT1E1 enhancer region. A normal IgG was served as a negative control in the assays. The values (N = 4) represent means ± S.D. Mean difference is significant from IgG group at **** P < 0.0001; ** P < 0.005 * P < 0.05; ns: non-significant (Student's t-test).

(C) Phosphorylated RORα expression in response to glucose exposures was detected in HepG2 cells by western blotting with anti-phosphor Ser100 RORα antibody. Non-phosphorylated RORα was served as a loading control.

Casein kinase 1α phosphorylates RORα at Ser100 on SULT1E1 enhancer

An emerging question as to how casein kinase may mediate RORα phosphorylation at Ser100 in response to high glucose signal was then raised. To address this question, four times repeated DR1 and RORE overlapping enhancer was used as bait to enrich the binding complexes from the low glucose cultured and high glucose

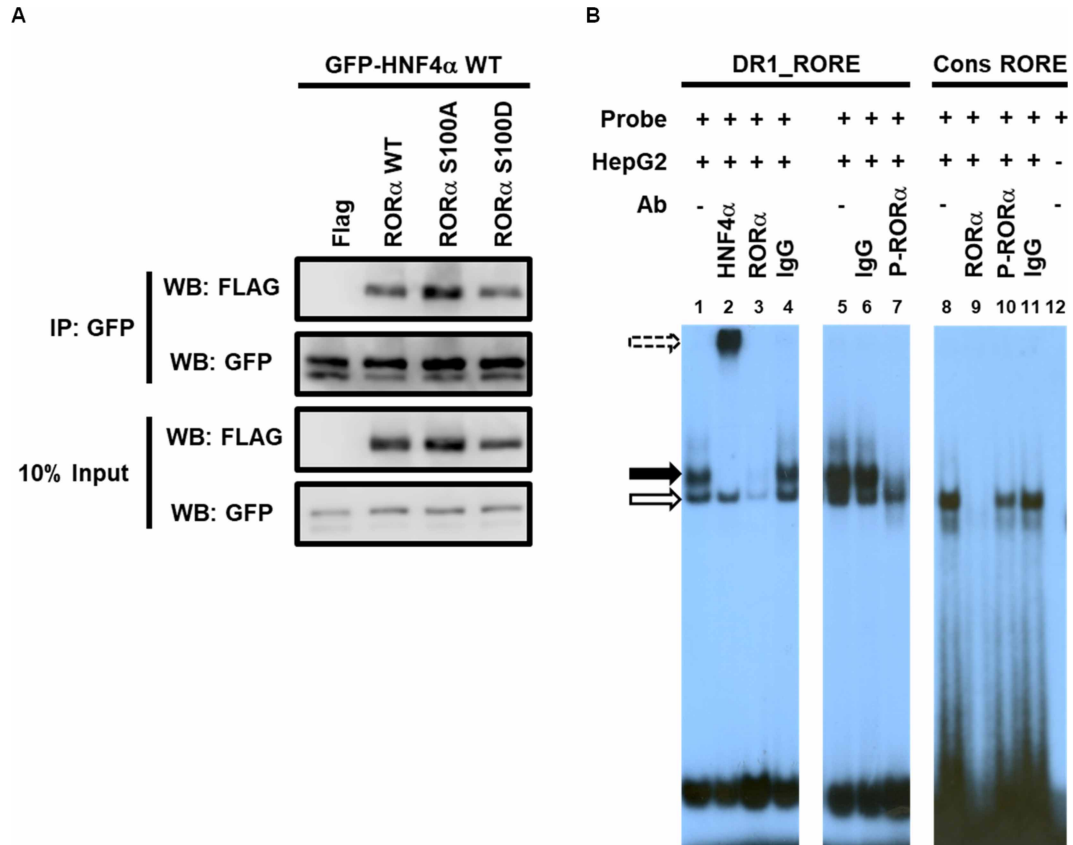


Figure 5. Characterization of ROR α /HNF4 α binding complexes on the *SULT1E1* enhancer.

(A) Physical interaction between HNF4 α and ROR α . GFP-tagged HNF4 α was cotransfected with Flag-tagged ROR α WT, ROR α S100A or ROR α S100D into Cos-1 cells, respectively for 24 h, followed by CoIP reactions with their nuclear extracts. An anti-GFP antibody was used to precipitate GFP-tagged proteins and an anti-Flag antibody was used to detect the proteins that interacted with GFP-tagged HNF4 α by western blotting. (B) EMSA assays detected the HNF4 α and ROR α binding complexes on the *SULT1E1* DR1_RORE was radioactively labeled, which was incubated with a HepG2 nuclear extract. The antibodies against HNF4 α , ROR α and phosphorylated ROR α at Ser100 were used to supershift (dashed arrow) the formed protein/DNA binding complexes (solid and blank arrows), respectively. A normal IgG was used as a negative control for the used antibodies. The Cons RORE was served as a control to indicate specific interaction between phosphorylated ROR α and HNF4 α on *SULT1E1* enhancer. Experiments were conducted in triplicates.

cultured HepG2 cell nuclear extracts, respectively. The enriched complexes on the DR1 and RORE overlapping region were then subjected to mass spectrometric analysis, from which the enriched kinases were listed in Figure 6A. A complete list of enriched factors was submitted to the ProteomeXchange with identifier PXD016385. A ratio (Low/High) between numbers of peptides detected at low glucose and numbers of peptides detected at high glucose was calculated and subjected to Log₂(Low/High) analysis. Of note, the casein kinase 1 α (CK1 α) and protein kinase C-binding protein 1 (RACK7) were significantly enriched at high glucose condition compared with low glucose condition (Figure 6A).

To test whether CK1 α and RACK7 affect the binding of ROR α to the *SULT1E1* RORE, gel shift assays were applied by incubating the RORE probe with a HepG2 nuclear extract and respective antibodies. The anti-CK1 α antibody significantly blocked the binding of ROR α to the RORE (Figure 6B, lane 3), whereas the anti-RACK7 and normal IgG did not affect binding (Figure 6B, lanes 4–5), suggesting an interaction between CK1 α and ROR α on the enhancer. The specificity of this interaction was illustrated by incubating the consensus RORE with a HepG2 nuclear extract and respective antibodies (Figure 6B, lane 6–10). To further investigate if CK1 α mediates ROR α phosphorylation at Ser100, *in vitro* phosphorylation assays were performed. First, sequence

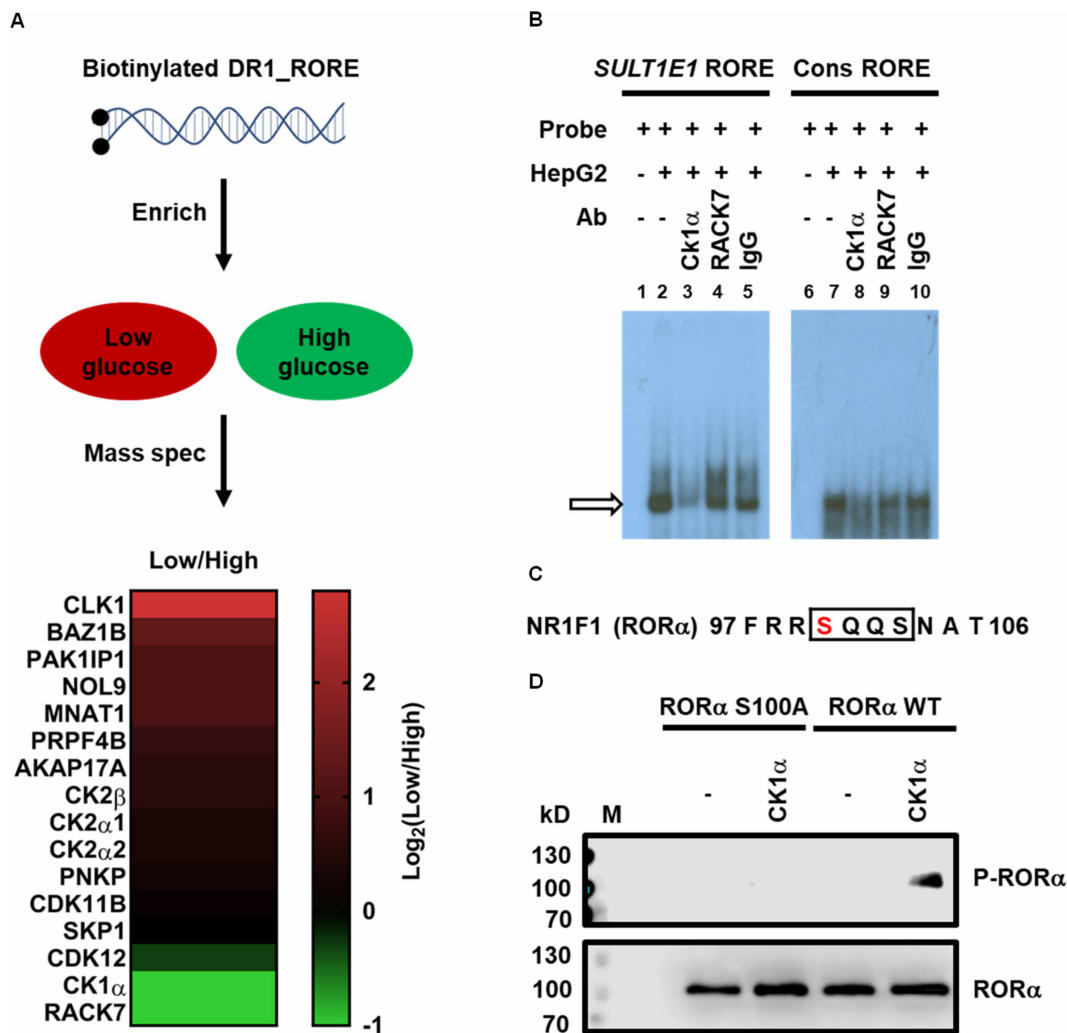


Figure 6. DNA enrichment mass spectrometry detects CK1α to phosphorylate RORα at Ser100.

(A) DNA enrichment mass spectrometry analysis. Biotinylated 4 × DR1_RORE of the *SULT1E1* enhancer was incubated with low glucose or high glucose medium treated HepG2 cell nuclear extract, respectively. The enriched binding complexes on the *SULT1E1* enhancer were subjected to quantitative mass spectrometry analysis. A ratio (Low/High) between numbers of peptides detected from low glucose group and numbers of peptides detected from high glucose group was calculated and subjected to $\text{Log}_2(\text{Low/High})$ analysis. The enriched kinases were listed on a heat map. (B) EMSA assays reveal the binding of CK1α to the RORα on *SULT1E1* enhancer. Radioactive labeled *SULT1E1* RORE was incubated with a HepG2 nuclear extract and respective antibodies. Consensus RORE was served as a positive control. The formed RORα/DNA binding complex was indicated by a blank arrow. (C) The Ser100 of RORα (red letter) is a conserved phosphorylation site within a perfect consensus CK1α phosphorylation motif (S/T-X-X-S/T, rectangle). (D) RORα was phosphorylated at Ser100 by CK1α. GST-hRORα S100A and GST-hRORα WT were incubated with or without GST-CK1α in kinase buffer containing ATP, respectively, followed by western blotting analysis with the antibodies against phosphorylated RORα and RORα, respectively. Experiments were repeated three times.

analysis of RORα unveiled a consensus CK1α phosphorylation motif on RORα (Figure 6C, rectangle) whose conserved phosphorylation site (Ser100 of RORα) is within the motif [22]. Incubating RORα WT or non-phosphorylatable RORα S100A mutant with or without CK1α, followed by western blotting with an antibody against phosphorylated RORα at Ser100 showed that the RORα WT but not RORα S100A was phosphorylated by CK1α (Figure 6D). These observations illustrate that CK1α phosphorylates RORα at Ser100 on *SULT1E1* enhancer at high glucose conditions.

***SULT1E1* enhancer loops its promoter to regulate gene expression**

Chromatin conformation capture (3C) assays were applied to detect the chromatin structure of the *SULT1E1* promoter in response to glucose exposures. HepG2 cells were treated by low glucose or high glucose DMEM for 48 h, respectively, followed by chromatin DNA cross-linking and BstYI restriction enzyme digestion. *SULT1E1* promoter was cut at two restriction sites, -1832 bp and +39 bp from the transcription start site, respectively (Figure 7A).

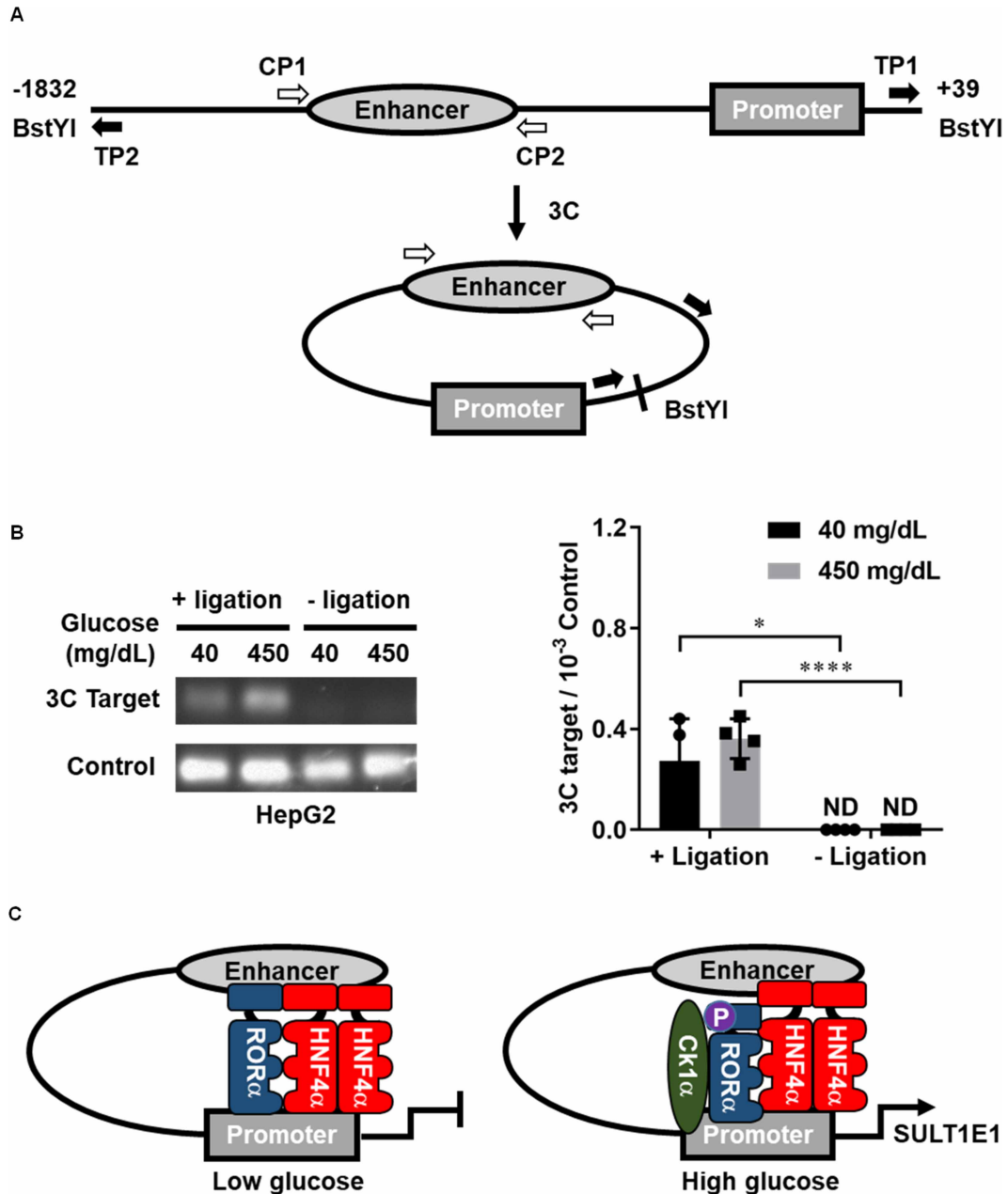


Figure 7. *SULT1E1* chromatin structures in response to glucose signals.

(A) Schematic diagram of 3C assay ligated position and primer positions on the *SULT1E1* gene. A 125 bp control product was amplified by using the primers CP1 and CP2 (blank arrows) and a 138 bp ligated product was amplified by using the primers TP1 and TP2 (solid arrows). (B) HepG2 cells were treated by low glucose or high glucose DMEM for 48 h, respectively, followed by fixations and BstYI restriction enzyme digestions. After + ligation or - ligation, the 3C ligated product and the control product were detected by real-time PCR and qRT-PCR, respectively. The values ($N = 4$) represent means \pm S.D. Mean difference is significant from - ligation group at **** $P < 0.0001$; * $P < 0.05$ (Student's t -test). (C) A model of glucose responsive *SULT1E1* gene regulation.

After ligation, the ligated point was checked by the primer set (TP1 and TP2, indicated by solid arrows) that amplifies a 138 bp DNA fragment by PCR. Another primer set (CP1 and CP2, indicated by blank arrows) that amplifies a 125 bp *SULT1E1* enhancer fragment was used as loading control primers (Figure 7A). As expected, the *SULT1E1* enhancer appeared to be looped to its promoter as a 138 bp DNA fragment was amplified by RT-PCR after ligation. However, the looped chromatin conformation of the *SULT1E1* gene in HepG2 cells was not affected by the glucose exposures (Figure 7B). The sequences of the ligated fragment were then confirmed by DNA sequencing, from which the joint point was shown in Supplementary Figure S5A. A non-coding region (from +7066 bp to +8570 bp) of the *SULT1E1* gene that has a similar size to the *SULT1E1* promoter was also cut by BstY1 and used as a negative control in the assays. The primer set (TP3 and TP4, indicated by solid arrows) did not amplify any fragments by RT-PCR before or after ligation (Supplementary Figure S5B). The efficacy of these primer sets was validated by RT-PCR using purified fragments (3C target and control) and synthetic DNA (non-3C target) as templates (Supplementary Figure S5C). These results suggested that ROR α and HNF4 α remained on *SULT1E1* promoter regardless of glucose exposures. High glucose signal stimulated ROR α phosphorylation at Ser100 by CK1 α , co-activating HNF4 α to activate *SULT1E1* transcription (Figure 7C).

Discussion

The liver produces and supplies glucose to peripheral tissues. Unregulated over-production causes diseases such as diabetes and the development of diabetes-related complications including chronic organ inflammation [23]. Various endobiotics modulate these disease states, one of which is estrogen. Postmenopausal women have significantly elevated blood glucose levels, insulin resistance as well as increased circulating inflammatory cytokines, compared with premenopausal women [24–26]. Moreover, E2 administrations improved those diagnoses of postmenopausal women with diabetes [27]. In both male and ovariectomized female mice, estrogen treatment restored insulin action and glucose tolerance [28,29]. In mice fed with high-fat diets, estrogen treatment decreased hepatic expression of glucose-6-phosphatase, lipogenic genes and fasting blood glucose [30]. For these estrogen regulations, *SULT1E1* may play a critical role in glucose signaling by metabolically inactivating estrogen in the liver. In fact, hepatic *SULT1E1* is induced in high plasma glucose conditions of various mouse models [4,7,31]. Our microarray data revealed that glucose exposures have significantly altered xenobiotic metabolism transcript levels in HepG2 cells (Supplementary Table S1). *Sult1e1* KO mice were utilized to demonstrate that its absence worsened diabetic inflammation to develop sepsis and hepatic ischemia-reperfusion injury [7–9,32].

High glucose induces *SULT1E1* expression by activating the *SULT1E1* gene in HepG2 cells. A 22 bp DNA sequence overlapping DR1 and RORE is characterized as the distal enhancer to which HNF4 α and ROR α bind, respectively, regulating the *SULT1E1* promoter. Phosphorylation of ROR α at Ser100 is found to be the glucose response regulator of the transcription. Non-phosphorylated ROR α bound RORE interacts with HNF4 α on the DR1, which co-represses the *SULT1E1* promoter in low glucose conditions. In response to high glucose, ROR α becomes phosphorylated at Ser100 by CK1 α , converting its function to co-activate the promoter. During these conversions and co-activation, both ROR α and HNF4 α remain on the enhancer and keep them in a looping structure with the proximal promoter (Figure 7C). This phosphorylation-mediated mechanism provides us with insights into understanding how two nuclear receptors communicate to regulate hepatic *SULT1E1* expression in prenatal livers as well as in diseases and drug treatments of humans.

SULT1E1 is repressed in the liver of adult mice and humans [4,10]. Up-regulation of hepatic *SULT1E1* expression in liver-specific HNF4 α KO mice suggests that the *Sult1e1* gene is repressed by HNF4 α [33]. The molecular mechanism that regulates this repression remains un-investigated. Consistent with previous observations in human fetal livers [11], the *SULT1E1* gene is activated in HepG2 cells in high glucose medium, but is repressed in low glucose medium. HNF4 α is found to function as both a transcription activator and repressor, and ROR α is characterized as a glucose response regulator of this HNF4 α function. Phosphorylated ROR α is a glucose response co-activator that binds HNF4 α to activate the enhancer. In low glucose medium, ROR α is dephosphorylated to bind and co-repress HNF4 α . Thus, ROR α converts its function from a high glucose response co-activator to a low-glucose response co-repressor through phosphorylation-dephosphorylation of Ser100. Previous studies also showed that an activation function of HNF4 α can be repressed. The antibiotic rifampicin activates nuclear receptor PXR which binds HNF4 α and dissociates HNF4 α from the *SULT1E1* promoter and represses its activity [6]. In other cases, ligand-activated nuclear receptor FXR suppressed the *SULT1E1* promoter by binding PGC1 α and preventing it to co-activate HNF4 α [12]. Bile acid activated FXR was also found to restrain HNF4 α driven *SULT1E1* gene expression through the competitive binding of protein

acetylase-CREB binding protein to the HNF4 α , decreasing HNF4 α acetylation and nuclear retention [16]. In these types of ligand-dependent repressions, PXR and FXR execute a single function which is to remove the activation activity of HNF4 α . On the other hand, ROR α plays the dual role of regulating HNF4 α to which ROR α remains bound, and to either co-activate or co-repress it through phosphorylation.

CK1 α is now purified by 22 bp DNA from the *SULT1E1* enhancer-affinity chromatography and is identified by mass spectrometry. ROR α 's Ser100 constitutes a perfect consensus motif (S/T-X-X-S/T), and is phosphorylated by CK1 α in *in vitro* kinase assays. CK1 α forms a complex with ROR α on the *SULT1E1* promoter, which results in the phosphorylation of Ser100 in response to high glucose. Apparently, CK1 α is the kinase that transduces this glucose signal to ROR α in HepG2 cells. Previously, yeast casein kinase 1 α (YCK1 α) was reported to be a down-stream target kinase of glucose signaling that regulates glucose transport and metabolism in yeast cells [34]. The function of casein kinases as glucose signal transducers appears to be conserved cross-species. CK1 α is a member of the casein kinase superfamily. Vaccinia virus-related kinase 1 (VRK1), which also belongs to this family, transduces the glucose signal to phosphorylate PXR at Ser350 within the ligand binding domain to regulate gluconeogenesis in HepG2 cells [35]. Recently, we have identified the amino acid residue phosphorylated to activate VRK1 down-stream kinases in response to high glucose in HepG2 cells and phosphorylated VRK1 (manuscript in preparation). VRK1 was also reported to phosphorylate FXR at Ser154 in response to a ligand activation in HepG2 cells [17]; Ser154 resides within the DBD and corresponds to Ser100 of ROR α . Casein kinases such as CK1 α and VRK1 may be positioned at critical steps of glucose signaling and conserved during evolution. Phenobarbital (PB), a sedative widely used to treat epileptic patients, activates the *Sult1e1* gene transcription in mouse liver [5]. As observed with ROR α in HepG2 cells, ROR α also repressed the *Sult1e1* gene in mouse liver and, following PB treatment, was phosphorylated at Ser100 to co-activate the CAR-mediated transcription of this gene [5]. Phosphorylation of Ser100 is utilized as a common target of both PB and glucose to transduce their signals to ROR α . Future investigations may establish CK1 α and/or VRK1 as signal transducers that respond to not only glucose and PB but also various other endogenous and exogenous stimuli.

ROR α phosphorylated at Ser100 interacted with HNF4 α and converted it to a co-activator. Ser100 is conserved as a phosphorylation motif in 41 out of the total 46 human nuclear receptors and their mouse counterparts [36]. Phosphorylation of this motif and its regulation and regulatory function were first confirmed with CAR at Thr38 in the liver [37]. Phosphorylation of Thr38 represses CAR's constitutive activity and retains CAR in the cytoplasm, conferring CAR with the capability to be activated via dephosphorylation. Subsequently, in addition to ROR α , FXR, RXR α and ER α were found to be phosphorylated at their motifs [17,38–40]. Phosphorylated ER α is expressed in neutrophils infiltrating into the mouse uterus and provides neutrophils with migration capability [38]. RXR α is phosphorylated at Thr167 in mouse fat tissues. Our study with RXR α T167A KI mice demonstrated that this phosphorylation regulates energy metabolism in fat tissue and blood glucose levels [39]. Based on these observations, it may be that this conserved phosphorylation functions as a common regulator for nuclear receptors to gain their functional specificities.

The *Sult1e1* was activated in response to phenobarbital-treated or diabetic mouse livers, in which ROR α was shown to be phosphorylated at Ser100 to regulate this activation [5,40]. In addition to these drug and pathophysiological responses, the *Sult1e1* gene undergoes multiple and complex expressions, one of which is sexually dimorphic regulation in the liver [41]. However, the lack of proper cell models that conserve this phenotype prevented us to investigate whether phosphorylated ROR α regulated sexually dimorphic expressions of the *Sult1e1* gene. HepG2 cells allowed us to specifically examine the glucose responsive regulation without any involvement of hormones such as insulin, glucagon and glucocorticoid [35]. On the other hand, obtained findings were not directly implicated to this regulation in the livers until proper animal models are developed in the future.

In conclusion, we determined the glucose response mechanism by which nuclear receptors ROR α and HNF4 α communicate to activate the *SULT1E1* gene transcription in HepG2 cells. In response to high glucose, CK1 α phosphorylates ROR α at Ser100 on the *SULT1E1* promoter. Subsequently, phosphorylated ROR α co-activates HNF4 α and, thereby, the promoter. In low glucose, ROR α was dephosphorylated and co-represses HNF4 α . Thus, this phosphorylation serves as a glucose response 'switch' to transduce a glucose signal. Ser100 of ROR α is conserved in the majority of mouse and human nuclear receptors. Future studies may characterize CK1 α , as a glucose sensor, targeting the corresponding residue of numerous nuclear receptors to integrate their functions in both physiology and pathophysiology, such as in fetal livers and diabetes.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions

H.H. and M.N. designed the experiments and wrote the article. H.H. conducted the experiments and analyzed the data.

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016385. The microarray data have been uploaded to the GEO [42] with accession number: GSE140867. All data set generated in the current study will be made to the research community upon requests.

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Abbreviations

ANOVA, analysis of variance; CAR, constitutive active/androstane receptor; FXR, farnesoid X receptor; PAPS, phosphor-adenosine phosphosulfate; PB, phenobarbital; PXR, pregnane X receptor; QIAGEN, QIAquick gel extraction kit; STS, steroid sulfatase; SULT1E1, sulfotransferase; VRK1, Vaccinia virus-related kinase 1.

References

- 1 Falany, C.N., Wheeler, J., Oh, T.S. and Falany, J.L. (1994) Steroid sulfation by expressed human cytosolic sulfotransferases. *J. Steroid Biochem. Mol. Biol.* **48**, 369–375 [https://doi.org/10.1016/0960-0760\(94\)90077-9](https://doi.org/10.1016/0960-0760(94)90077-9)
- 2 Negishi, M., Pedersen, L.G., Petrotchenko, E., Shevtsov, S., Gorokhov, A., Kakuta, Y. et al. (2001) Structure and function of sulfotransferases. *Arch. Biochem. Biophys.* **390**, 149–157 <https://doi.org/10.1006/abbi.2001.2368>
- 3 Kodama, S. and Negishi, M. (2013) Sulfotransferase genes: regulation by nuclear receptors in response to xeno/endo-biotics. *Drug Metab. Rev.* **45**, 441–449 <https://doi.org/10.3109/03602532.2013.8335630>
- 4 Song, W.C., Moore, R., McLachlan, J.A. and Negishi, M. (1995) Molecular characterization of a testis-specific estrogen sulfotransferase and aberrant liver expression in obese and diabetogenic C57BL/KsJ-db/db mice. *Endocrinology* **136**, 2477–2484 <https://doi.org/10.1210/endo.136.6.7750469>
- 5 Fashe, M., Hashiguchi, T., Yi, M., Moore, R. and Negishi, M. (2018) Phenobarbital induced phosphorylation converts nuclear receptor ROR α from a repressor to an activator of the estrogen sulfotransferase gene Sult1e1 in mouse livers. *FEBS Lett.* **592**, 2760–2768 <https://doi.org/10.1002/1873-3468.13199>
- 6 Kodama, S., Hosseinpour, F., Goldstein, J.A. and Negishi, M. (2011) Liganded pregnane X receptor represses the human sulfotransferase SULT1E1 promoter through disrupting its chromatin structure. *Nucleic Acids Res.* **39**, 8392–8403 <https://doi.org/10.1093/nar/gkr458>
- 7 Gao, J., He, J., Shi, X., Stefanovic-Racic, M., Xu, M., O'Doherty, R.M. et al. (2012) Sex-specific effect of estrogen sulfotransferase on mouse models of type 2 diabetes. *Diabetes* **61**, 1543–1551 <https://doi.org/10.2337/db11-1152>
- 8 Chai, X., Guo, Y., Jiang, M., Hu, B., Li, Z., Fan, J. et al. (2015) Oestrogen sulfotransferase ablation sensitizes mice to sepsis. *Nat. Commun.* **6**, 7979 <https://doi.org/10.1038/ncomms8979>
- 9 Guo, Y., Hu, B., Huang, H., Tsung, A., Gaikwad, N.W., Xu, M. et al. (2015) Estrogen sulfotransferase is an oxidative stress-responsive gene that gender-specifically affects liver ischemia/reperfusion injury. *J. Biol. Chem.* **290**, 14754–14764 <https://doi.org/10.1074/jbc.M115.642124>
- 10 Dubaisi, S., Caruso, J.A., Gaedigk, R., Vyhldal, C.A., Smith, P.C., Hines, R.N. et al. (2019) Developmental expression of the cytosolic sulfotransferases in human liver. *Drug Metab. Dispos.* **47**, 592–600 <https://doi.org/10.1124/dmd.119.086363>
- 11 Dubaisi, S., Barrett, K.G., Fang, H., Guzman-Lepe, J., Soto-Gutierrez, A., Kocarek, T.A. et al. (2018) Regulation of cytosolic sulfotransferases in models of human hepatocyte development. *Drug Metab. Dispos.* **46**, 1146–1156 <https://doi.org/10.1124/dmd.118.081398>
- 12 Wang, S., Yuan, X., Lu, D., Guo, L. and Wu, B. (2017) Farnesoid X receptor regulates SULT1E1 expression through inhibition of PGC1 α binding to HNF4 α . *Biochem. Pharmacol.* **145**, 202–209 <https://doi.org/10.1016/j.bcp.2017.08.023>
- 13 Gong, H., Jarzynka, M.J., Cole, T.J., Lee, J.H., Wada, T., Zhang, B. et al. (2008) Glucocorticoids antagonize estrogens by glucocorticoid receptor-mediated activation of estrogen sulfotransferase. *Cancer Res.* **68**, 7386–7393 <https://doi.org/10.1158/0008-5472.CAN-08-1545>
- 14 Gong, H., Guo, P., Zhai, Y., Zhou, J., Uppal, H., Jarzynka, M.J. et al. (2007) Estrogen deprivation and inhibition of breast cancer growth in vivo through activation of the orphan nuclear receptor liver X receptor. *Mol. Endocrinol.* **21**, 1781–1790 <https://doi.org/10.1210/me.2007-0187>
- 15 Sueyoshi, T., Green, W.D., Vinal, K., Woodrum, T.S., Moore, R. and Negishi, M. (2011) Garlic extract diallyl sulfide (DAS) activates nuclear receptor CAR to induce the Sult1e1 gene in mouse liver. *PLoS ONE* **6**, e21229 <https://doi.org/10.1371/journal.pone.0021229>

- 16 Liu, X., Xue, R., Yang, C., Gu, J., Chen, S. and Zhang, S. (2018) Cholestasis-induced bile acid elevates estrogen level via farnesoid X receptor-mediated suppression of the estrogen sulfotransferase SULT1E1. *J. Biol. Chem.* **293**, 12759–12769 <https://doi.org/10.1074/jbc.RA118.001789>
- 17 Hashiguchi, T., Arakawa, S., Takahashi, S., Gonzalez, F.J., Sueyoshi, T. and Negishi, M. (2016) Phosphorylation of Farnesoid X receptor at serine 154 links ligand activation With degradation. *Mol. Endocrinol.* **30**, 1070–1080 <https://doi.org/10.1210/me.2016-1105>
- 18 Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundu, D.J. et al. (2019) The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* **47**, D442–Dd50 <https://doi.org/10.1093/nar/gky1106>
- 19 Cryer, P.E. (2007) Hypoglycemia, functional brain failure, and brain death. *J. Clin. Invest.* **117**, 868–870 <https://doi.org/10.1172/JCI31669>
- 20 American Diabetes Association. (2012) Diagnosis and classification of diabetes mellitus. *Diabetes Care* **35**, S64–S71 <https://doi.org/10.2337/dc12-s064>
- 21 Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A. et al. (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* **21**, 2933–2942 <https://doi.org/10.1093/bioinformatics/bti473>
- 22 Miller, C.J. and Turk, B.E. (2016) Rapid identification of protein kinase phosphorylation site motifs using combinatorial peptide libraries. *Methods Mol. Biol.* **1360**, 203–216 https://doi.org/10.1007/978-1-4939-3073-9_15
- 23 Gerich, J.E. (1993) Control of glycaemia. *Baillieres Clin. Endocrinol. Metab.* **7**, 551–586 [https://doi.org/10.1016/S0950-351X\(05\)80207-1](https://doi.org/10.1016/S0950-351X(05)80207-1)
- 24 Carr, M.C. (2003) The emergence of the metabolic syndrome with menopause. *J. Clin. Endocrinol. Metab.* **88**, 2404–2411 <https://doi.org/10.1210/jc.2003-030242>
- 25 Pfeilschifter, J., Koditz, R., Pfohl, M. and Schatz, H. (2002) Changes in proinflammatory cytokine activity after menopause. *Endocr. Rev.* **23**, 90–119 <https://doi.org/10.1210/edrv.23.1.0456>
- 26 Sites, C.K., Toth, M.J., Cushman, M., L'Hommedieu, G.D., Tchernof, A., Tracy, R.P. et al. (2002) Menopause-related differences in inflammation markers and their relationship to body fat distribution and insulin-stimulated glucose disposal. *Fertil. Steril.* **77**, 128–135 [https://doi.org/10.1016/S0015-0282\(01\)02934-X](https://doi.org/10.1016/S0015-0282(01)02934-X)
- 27 Salpeter, S.R., Walsh, J.M., Ormiston, T.M., Greyber, E., Buckley, N.S. and Salpeter, E.E. (2006) Meta-analysis: effect of hormone-replacement therapy on components of the metabolic syndrome in postmenopausal women. *Diabetes Obes. Metab.* **8**, 538–554 <https://doi.org/10.1111/j.1463-1326.2005.00545.x>
- 28 Stubbins, R.E., Holcomb, V.B., Hong, J. and Nunez, N.P. (2012) Estrogen modulates abdominal adiposity and protects female mice from obesity and impaired glucose tolerance. *Eur. J Nutr.* **51**, 861–870 <https://doi.org/10.1007/s00394-011-0266-4>
- 29 Yan, H., Yang, W., Zhou, F., Li, X., Pan, Q., Shen, Z. et al. (2019) Estrogen improves insulin sensitivity and suppresses gluconeogenesis via the transcription factor Foxo1. *Diabetes* **68**, 291–304 <https://doi.org/10.2337/db18-0638>
- 30 Bryzgalova, G., Lundholm, L., Portwood, N., Gustafsson, J.A., Khan, A., Efendic, S. et al. (2008) Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice. *Am. J Physiol. Endocrinol. Metab.* **295**, E904–E912 <https://doi.org/10.1152/ajpendo.90248.2008>
- 31 Leiter, E.H. and Chapman, H.D. (1994) Obesity-induced diabetes (diabesity) in C57BL/KsJ mice produces aberrant trans-regulation of sex steroid sulfotransferase genes. *J. Clin. Invest.* **93**, 2007–2013 <https://doi.org/10.1172/JCI117194>
- 32 Garbacz, W.G., Jiang, M., Xu, M., Yamauchi, J., Dong, H.H. and Xie, W. (2017) Sex- and tissue-specific role of estrogen sulfotransferase in energy homeostasis and insulin sensitivity. *Endocrinology* **158**, 4093–4104 <https://doi.org/10.1210/en.2017-00571>
- 33 Lu, H., Gonzalez, F.J. and Klaassen, C. (2010) Alterations in hepatic mRNA expression of phase II enzymes and xenobiotic transporters after targeted disruption of hepatocyte nuclear factor 4 alpha. *Toxicol. Sci.* **118**, 380–390 <https://doi.org/10.1093/toxsci/ktq280>
- 34 Snowdon, C. and Johnston, M. (2016) A novel role for yeast casein kinases in glucose sensing and signaling. *Mol. Biol. Cell* **27**, 3369–3375 <https://doi.org/10.1091/mbc.E16-05-0342>
- 35 Gotoh, S., Miyauchi, Y., Moore, R. and Negishi, M. (2017) Glucose elicits serine/threonine kinase VRK1 to phosphorylate nuclear pregnane X receptor as a novel hepatic gluconeogenic signal. *Cell. Signal.* **40**, 200–209 <https://doi.org/10.1016/j.cellsig.2017.09.003>
- 36 Negishi, M. (2017) Phenobarbital meets phosphorylation of nuclear receptors. *Drug Metab. Dispos.* **45**, 532–539 <https://doi.org/10.1124/dmd.116.074872>
- 37 Mutoh, S., Osabe, M., Inoue, K., Moore, R., Pedersen, L., Perera, L. et al. (2009) Dephosphorylation of threonine 38 is required for nuclear translocation and activation of human xenobiotic receptor CAR (NR113). *J. Biol. Chem.* **284**, 34785–34792 <https://doi.org/10.1074/jbc.M109.048108>
- 38 Shindo, S., Moore, R., Flake, G. and Negishi, M. (2013) Serine 216 phosphorylation of estrogen receptor α in neutrophils: migration and infiltration into the mouse uterus. *PLoS ONE* **8**, e84462 <https://doi.org/10.1371/journal.pone.0084462>
- 39 Sueyoshi, T., Sakuma, T., Shindo, S., Fashe, M., Kanayama, T., Ray, M. et al. (2019) A phosphorylation-deficient mutant of retinoid X receptor α at Thr 167 alters fasting response and energy metabolism in mice. *Lab. Invest.* **99**, 1470–1483 <https://doi.org/10.1038/s41374-019-0266-1>
- 40 Yi, M., Fashe, M., Arakawa, S., Moore, R., Sueyoshi, T. and Negishi, M. (2020) Nuclear receptor CAR-ER α signaling regulates the estrogen sulfotransferase gene in the liver. *Sci. Rep.* **10**, 5001 <https://doi.org/10.1038/s41598-020-61767-9>
- 41 Matsushita, N., Hassanein, M.T., Martinez-Clemente, M., Lazarou, R., French, S.W., Xie, W. et al. (2017) Gender difference in NASH susceptibility: roles of hepatocyte ikk β and Sult1e1. *PLoS ONE* **12**, e0181052 <https://doi.org/10.1371/journal.pone.0181052>
- 42 Barrett, T., Troup, D.B., Wilhite, S.E., Ledoux, P., Evangelista, C., Kim, I.F. et al. (2011) NCBI GEO: archive for functional genomics data sets—10 years on. *Nucleic Acids Res.* **39**, D1005–D1D10 <https://doi.org/10.1093/nar/gkq1184>