

Fasting-induced transcription factors repress vitamin D bioactivation, a mechanism for vitamin D deficiency in diabetes

Running Title: Diabetes represses vitamin D activation

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Tweet: Researchers have found that molecular mechanisms activated physiologically during fasting and pathologically by diabetes could suppress the vitamin D bioactivation in the liver and cause vitamin D deficiency in diabetics.

Attach figure number 6

Abstract

Low 25-hydroxyvitamin D levels correlate with the prevalence of diabetes, however, the mechanisms remain uncertain. Here, we show that nutritional deprivation responsive mechanisms regulate vitamin D metabolism. Both fasting and diabetes suppressed hepatic cytochrome P450 (CYP) 2R1, the main vitamin D 25-hydroxylase, responsible for the first bioactivation step. Overexpression of coactivator peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1 α), induced physiologically by fasting and pathologically in diabetes, resulted in dramatic downregulation of CYP2R1 in mouse hepatocytes in an estrogen-related receptor α (ERR α)-dependent manner. However, PGC-1 α knockout did not prevent fasting-induced suppression of CYP2R1 in the liver indicating that additional factors contribute to the CYP2R1 repression. Furthermore, glucocorticoid receptor (GR) activation repressed the liver CYP2R1, suggesting GR involvement in the regulation of CYP2R1. GR antagonist mifepristone partially prevented CYP2R1 repression during fasting suggesting that glucocorticoids and GR contribute to the CYP2R1 repression during fasting. Moreover, fasting upregulated the vitamin D catabolizing CYP24A1 in the kidney through the PGC-1 α -ERR α pathway. Our study uncovers a molecular mechanism for vitamin D deficiency in diabetics and reveals a novel negative feedback mechanism controlling cross-talk between energy homeostasis and the vitamin D pathway.

Keywords: vitamin D metabolism, diabetes, fasting, CYP2R1, CYP24A1, PGC-1 α , 25-hydroxyvitamin D

Vitamin D is an endocrine regulator of calcium homeostasis, but emerging evidence indicates a role in regulation of energy homeostasis (1, 2). There is strong evidence that vitamin D affects adipogenesis and adipocyte lipid metabolism, however, the effects vary between species and cells models used (1, 3). Both *Vdr*^{-/-} and *Cyp27b1*^{-/-} mice display lean phenotype with decreased fat mass, probably due to increased energy expenditure (1, 4). In contrast, in humans low 25-hydroxyvitamin D (25-OH-D) associates with obesity (1, 2).

Vitamin D deficiency is a widespread medical health problem worldwide (5). Ample epidemiological evidence associates vitamin D deficiency with the prevalence of metabolic diseases, and low 25-OH-D levels have been reported to correlate with the incidence of type 1 and 2 diabetes (6-9). However, the causal relationship is uncertain, and the mechanisms involved unclear. Furthermore, the intervention studies aiming at diabetes prevention with vitamin D supplementation have been largely disappointing or inconclusive (6).

Vitamin D is a prohormone activated in two enzymatic steps, 25-hydroxylation in the liver and 1 α -hydroxylation in the kidney to produce the main active form 1 α ,25-dihydroxyvitamin D (1 α ,25-(OH)₂-D) (10). Vitamin D status is usually assessed by measuring the main circulating vitamin D metabolite, i.e. 25-OH-D (5). Cytochrome P450 2R1 (CYP2R1) is the predominant vitamin D 25-hydroxylase in the liver (11, 12). A genetic defect in the CYP2R1 gene has been shown to cause an inherited form of vitamin D deficiency and rickets in children (13-15). Large-scale studies have identified CYP2R1 gene variants as one of the major genetic determinants of low 25-OH-D levels (16-18). Additionally, polymorphism in the CYP2R1 gene has been associated with susceptibility to type 1 diabetes (19, 20). However, little is known about the regulation of CYP2R1, and vitamin D 25-hydroxylation has even been considered as an unregulated step (10).

Cytochrome P450 24A1 (CYP24A1) enzyme catalyzes 24-hydroxylation of 1 α ,25-(OH)₂-D and also 25-OH-D (10). 24-hydroxylation is the main inactivation step limiting the effect of the main vitamin D receptor ligand, 1 α ,25-(OH)₂-D, but also reducing the pool of 25-OH-D available for 1 α -

hydroxylation. CYP24A1 plays an important role in regulation of vitamin D action and is under stringent regulatory control (10).

In this report, we show that CYP2R1 and its catalytic activity, vitamin D 25-hydroxylation are suppressed in the liver during fasting and in both type 1 and type 2 diabetes mouse models. Mechanistically, we demonstrate involvement of at least two molecular pathways, the peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1 α)/estrogen-related receptor α (ERR α) axis and the glucocorticoid receptor (GR). Furthermore, CYP24A1 is induced during fasting under the control of PGC-1 α . Altogether, these results indicate that energy metabolism regulating factors control the vitamin D metabolism and establish repression of vitamin D bioactivation as an important, novel mechanism inducing vitamin D deficiency in diabetes.

Research Design and Methods

Animal experiments

All animal experiments were approved by the National Animal Experimental Board, Finland, or the local animal committees in the USA, Germany or Hungary. All animals were housed in standard conditions with 12-hour dark-light cycle. If not otherwise stated, at the end of the experiments, the mice were killed with CO₂ inhalation and neck dislocation and tissues were collected and frozen in liquid nitrogen. All animals were male.

Fasting experiments

DBA/2 male mice, obtained from the Laboratory Animal Center, University of Oulu, aged 8-12 weeks, were fasted for 12 or 24 h (control and 12 h fast n = 9, 24 h fast n= 8). The animals had free access to drinking water. At the end of the experiment, the mice were anesthetized with a solution containing fentanyl-fluanisone (Hypnorm®) and midazolam (Dormicum®) and sacrificed. Independent repetition of the experiment gave similar results.

Pgc-1 α ^{-/-} mice (21), age 3-4 months, and *Pgc-1 α* ^{+/+} littermates were fasted for 12 h after which the animals were sacrificed and the tissues were collected. Liver-specific *Pgc-1 β* ^{-/-} mice (22) or *Pgc-1 β* ^{+/+} littermates, 8 weeks of age, were housed individually and fasted for 24 h. Animals were sacrificed and tissues were harvested and snap frozen in liquid nitrogen. Both knockout lines were in the C57BL/6 background.

Wistar rats, aged 10 weeks, were fasted for 24 h as has been described previously (23).

High-fat diet treatment

C57BL/6 mice aged 8 weeks were fed high-fat diet (60% fat, Envigo TD.06414) or regular chow (5 % fat, Envigo TD.2018) for 16 weeks. The mice were fasted for 12 hours overnight and fasting blood glucose was determined. The mice were sacrificed by CO₂ inhalation, and blood was drawn into EDTA-primed syringe from vena cava and tissues collected. The chow diet and the HFD contained 39.39 ug/kg and 52.35 ug/kg of vitamin D, respectively. The estimated amount of the chow diet eaten

by a mouse was 4 g/day. However, the HFD-treated mice ate less i.e., 3,1 g/day. Therefore, the daily intake of the vitamin D was about 0,16 ug/day for both the chow and the HFD-treated mice.

Streptozotocin treatment

Experimental type 1 diabetes was induced by intraperitoneal injections of C57BL/6 mice with streptozotocin (STZ) as has been described previously (24, 25).

Treatment with nuclear receptor agonists and inhibitors

For estrogen-related receptor α (ERR α) inhibition in the kidney, the C57BL/6 mice, aged 8 weeks, were treated i.p. with an ERR α inverse agonist, XCT790, 0.48 mg/kg dissolved in DMSO/corn oil or vehicle once daily for 3 days. 12 h after the last XCT790 injection, mice were either fed or fasted overnight for additional 12h and the tissues were collected.

For the dexamethasone treatment, C57BL/6 mice aged 8 weeks were fasted 1 h before an i.p. injection of dexamethasone (3 mg/kg) dissolved in DMSO/corn oil or vehicle only. The mice were fasted 6 h and the tissues collected. In some experiments, simultaneously with the dexamethasone injections, the GR was inhibited with the GR antagonist mifepristone (i.p. 100 mg/kg) dissolved in DMSO/corn oil. In all the treatment groups, the vehicle amount was kept similar.

To inhibit GR in the liver of fasting mice, C57BL/6 mice, aged 8-9 weeks, were injected with mifepristone (i.p. 100 mg/kg) or vehicle (DMSO/corn oil) two times (the first injection at 9 am and the second injection at 9 pm) and subsequently the mice were either fed or fasted overnight for additional 12h and the tissues were collected.

Cell culture

Mouse primary hepatocytes were isolated from male DBA/2 (Fig 2A and 4A) or C57BL/6 mice (Laboratory Animal center, University of Oulu, Finland) aged 8-10 weeks as described (26) and cultured in William's E medium containing 2.0 g/L D-glucose and ITS (insulin 5 mg/L, transferrin 5 mg/L, sodium selenate 5 μ g/L, 10 % FBS, Sigma-Aldrich, St. Louis, MO, USA). The cultures were

maintained for additional 12-24 hours in serum free William's E medium before adenoviral infections or chemical treatments. HepG2-cells (ATCC, Manassas, Virginia; USA) were maintained in basic DMEM medium (4.5 g/L glucose) (Gibco, Invitrogen, Carlsbad, CA, USA) supplied with 10% FBS (Gibco, Invitrogen) and 1% penicillin-streptomycin (Gibco, Invitrogen).

RNA preparation and quantitative RT-PCR

From the fasted male DBA/2 mice livers, RNAs were extracted with cesium chloride (CsCl) centrifugation method (27). From all the other tissues and cell samples, total RNA extraction was performed using either TRI Reagent or RNazol reagent (Sigma-Aldrich) according to manufacturer's protocol. One μg of RNA was used for cDNA synthesis using p(dN)6 random primers (Roche Diagnostics, Mannheim, Germany) and M-MLV reverse transcriptase (Promega, Madison, WI, USA) or RevertAid reverse transcriptase (ThermoFisher scientific, Waltham, MA, USA). The quantitative PCR reactions were performed using SYBR Green chemistry or TaqMan chemistry (Applied Biosystems, Foster City, CA). The sequences for the primers and TaqMan probes are listed in Table 2. The fluorescence values of the qPCR products were corrected with the fluorescence signals of the passive reference dye (ROX). The mRNA levels of target genes were normalized against 18S, GAPDH or TBP control levels using the comparative C_T ($\Delta\Delta C_T$) method.

Adenoviruses and shRNA knockdown in cells

PGC-1 α -2x9 and PGC-1 α -L2L3M plasmids were kindly provided by Dr. Donald McDonnell (Duke University School of Medicine, USA). Recombinant adenoviruses expressing green fluorescent protein (GFP-Ad), LacZ (LacZ-Ad), PGC-1 α (PGC-1 α -Ad, PGC-1 α -2x9-Ad, PGC-1 α -L2L3M-Ad) were prepared as described previously (26). For overexpression of PGC-1 α , PGC-1 α -2x9, PGC-1 α -L2L3M, 0.5 MOI was used for each virus. ERR α -Ad was purchased from Vector Biolabs (Malvern, PA, USA). Mouse primary hepatocytes were infected with adenoviruses in William's E growth medium without serum for the indicated time periods before RNA or protein extractions.

Adenoviruses containing scramble shRNA (shScr-Ad) and $ERR\alpha$ targeting shRNA (sh $ERR\alpha$ -Ad) were purchased from Vector Biolabs (Malvern, PA, USA). For the shRNA experiments, mouse primary hepatocytes were first infected with either shScr or sh $ERR\alpha$ at MOI 30 in William's E medium for 24 hours after which cells were infected with either PGC-1 α -Ad or control virus LacZ-Ad at MOI 2. After 48 h the cells were collected and RNA isolation. The efficiency of the knockdown was tested by measuring $ERR\alpha$ mRNA by qPCR. For inhibition of the $ERR\alpha$ by XCT790 in combination with the PGC-1 α overexpression, PGC-1 α -Ad was used at MOI 2.

Western blot

CYP2R1 was detected from mouse liver microsomal fractions. Microsomal fractions were prepared by differential centrifugation (28) and protein content was measured by Bradford reagent (Bio-Rad, Hercules, CA, USA). CYP24A1 was detected from total protein fractions prepared as described previously (29). Protein fractions were subjected to precast SDS-polyacrylamide gel (10-12% in polyacrylamide, Bio-Rad) electrophoresis and transferred to polyvinylidene fluoride or nitrocellulose membrane (Millipore, Billerica, MA, USA). Membranes were incubated with appropriate primary antibody in 5% skimmed milk or Amersham ECL Prime Blocking Reagent in Tris buffered saline with 0.1% Tween, usually overnight, followed by secondary HRP-conjugated antibody incubation. The immunoreactive bands were visualized with Chemiluminescent Peroxidase Substrate 1 reaction (Sigma-Aldrich), and Amersham ECLTM start western blotting detection reagent (GE healthcare, UK) and quantified by Quantity One software and Image studio software.

Vitamin D 25-hydroxylase assay

Mouse liver microsomal samples (0.5 mg/mL protein) were subjected to incubation with 2 μ M cholecalciferol together with 0.1M PBS and preincubated for 5 minutes. Enzymatic reactions were initiated with addition of 0.5 mM NADPH and quenched using ice-cold acetonitrile at 1:1 volume

ratio after 40 minutes' shaking at 37°C. Samples were mixed well and kept at -20°C until analyzed using LC-MS/MS.

LC-MS/MS

Quantitative analysis of 25-hydroxyvitamin D was performed by reversed phase liquid chromatography (Waters Acquity UPLC instrument, Milford, MA, USA) combined with mass spectrometric detection (Waters XEVO T-QS triple quadrupole mass spectrometer, Milford MA, USA). The chromatographic separation was carried out with an Acquity BEH Shield RP18 column (Waters, 2.1 x 50mm, 1.7µm). The column temperature was set to 45°C and a gradient elution with mobile phase A (0.5% formic acid) and mobile phase B (15% isopropanol & 85% acetonitrile), at a flow rate of 500 µL min⁻¹ was used. The elution gradient consisted of raising the part of mobile phase B from 20% up to 90% in 3.5 min, followed by column equilibration until 4.5 min. The injection volume was 4 µL. The retention time of 25-hydroxyvitamin D was 2.82 min. The monitored fragmentation reaction was m/z 383 > m/z 211. Positive mode of electrospray ionization (ESI+) was used as ionization source. Data was processed with MassLynx V4.1. software (Milford, MA, USA).

Measurement of the plasma 25-hydroxyvitamin D

The plasma level of the 25-OH-D was measured using Vitamin D^s EIA kit (Immunodiagnostic system, Tyne & Wear, UK) by ValiFinn Company (Oulu, Finland) according to the manufacturer's protocol.

Bioinformatics analysis of the mouse *Cyp2r1* gene promoter and reporter gene assays

The mouse *Cyp2r1* gene promoter ERRα binding sites were predicted using the MatInspector software (30) (Genomatix, Munich, Germany). 1.2 kb (-10 to -1220 bp, relative to the TSS) promoter region of the mouse *Cyp2r1* gene was amplified with PCR from the C57BL/6 mouse DNA by using the following primers; FW (TTCTCGAG-CTTCAAGCCTTAAAATGATGTGAG; TT is extranucleotide for efficient binding of the restriction enzyme, CTCGAG is the XhoI restriction site.)

and RV (TTAAGCTT-CTACGAACCAGTCCGGAGC; TT is extranucleotide for efficient binding of the restriction enzyme, AAGCTT is the HindIII restriction site) and inserted upstream of the Firefly Luciferase gene reporter in the pGL3-basic vector. The pGL3 vector containing the wild type promoter construct was subjected to site-directed mutagenesis using Quickchange II site-directed mutagenesis kit to generate a construct containing the promoter sequence with mutated ERR α binding site (-1117 to -1122, relative to the TSS).

The *Cyp2r1*-promoter constructs were transiently transfected into HepG2 cells, using the FuGene transfection reagent (Promega, Madison, WI, USA), together with the pRL-TK Renilla luciferase reporter to normalize for transfection efficiency. Empty pGL3-basic vector was used as a negative control. 24 hours post transfection, PGC-1 α and ERR α were overexpressed using the adenovirus at MOI 2. The LacZ-Ad-infected cells were used as a negative control. Cells were incubated further 24 hours and the luciferase activities were measured (Varioskan Flash, ThermoFisher scientific, USA). The Firefly luciferase values were normalized with the Renilla luciferase values. The data are expressed as relative to the LacZ-Ad infected cells.

Microarray

The DNA microarray experiment to study the PGC-1 α regulated genes in mouse (C57BL/6) primary hepatocytes was done as described (23). Microarray data can be accessed at the NCBI Gene Expression Omnibus (GEO), with the accession number GSE114485.

Analysis of the published ChIP-seq data

The published ChIP-seq data (PPARGC1A and ESRRA ChIP-seq in HepG2, GSE31477) (31), and (NR3C1 (GR) ChIP-seq in mouse liver, GSE72084) (32) were retrieved and analyzed by using Cistrome database (33) and visualized using the UCSC genome browser.

Statistical analysis

The statistical data analysis was performed using GraphPad Prism Software (La Jolla, CA, USA). Unless otherwise stated, the comparison of means of two groups was done by Student's two-tailed *t*-

test, whereas multiple groups were compared by One-way ANOVA followed by Tukey's *post hoc* test. Differences were considered significant at $P < 0.05$ (* or # $P < 0.05$; ** or ## $P < 0.01$; *** or ### $P < 0.001$; **** or #### $P < 0.0001$).

Results

Fasting and diabetes repress CYP2R1, the vitamin D 25-hydroxylase, in the liver

We observed that fasting represses CYP2R1 expression in the mouse liver in vivo. Remarkably, the expression of CYP2R1 mRNA was strongly repressed, by 50%, already after 12 h fasting, and further suppressed by 80% after 24 h (Fig. 1A). CYP2R1 expression was regulated in negative correlation with the fasting-induced gluconeogenic gene phosphoenolpyruvate carboxykinase (PEPCK) ($r = -0.605$, $P = 0.0011$) (Fig. 1B, Fig. S1A). Furthermore, CYP2R1 protein was effectively decreased at both time points, to 45% after 12 h and to 33% after 24 h compared to the controls (Fig. 1C). Consistent with the mRNA and protein results, fasting strongly decreased the liver microsomal vitamin D 25-hydroxylase activity to 54% after 12 h and below the detection level after 24 h fasting (Fig. 1D). However, in accordance with the long half-life of the metabolite, the plasma level of 25-OH-D was not affected by short term, 12-hour fasting compared to the fed controls (Fig. S1B). In addition to mouse, fasting repressed CYP2R1 expression in rat liver after 24 h fasting (Fig. 1E).

Hepatic CYP2R1 was repressed also in the mouse diabetes models. In high-fat diet (HFD) induced mouse model of obesity and type 2 diabetes (Fig. S1C,D), hepatic CYP2R1 mRNA was repressed by 45% (Fig. 1F). Consistent with the CYP2R1 mRNA, the plasma level of 25-OH-D was significantly reduced in the HFD-treated mice compared to the chow-fed controls (Fig. 1G). Also in the type 1 diabetic mouse model (Fig. S1E), induced with streptozotocin (STZ), CYP2R1 mRNA was repressed by 43% and protein by 29% (Fig. 1H,I). Analysis of published microarray data (GSE39752) support the finding that CYP2R1 is repressed in the livers of STZ-treated mice (Fig. S1F). Altogether, these data show a clear modulation of vitamin D bioactivation by the metabolic state. CYP2R1 expression and the vitamin D 25-hydroxylation were markedly repressed in the livers of fasted as well as diabetic animals.

PGC-1 α -ERR α pathway represses CYP2R1 expression

We next investigated the mechanisms of CYP2R1 repression and hypothesized that nutrition responsive coactivator PGC-1 α would be involved in this process, since PGC-1 α plays a central role in the fasting response and in uncontrolled diabetes (34). PGC-1 α overexpression in mouse primary hepatocytes with PGC-1 α -adenovirus (Ad) downregulated CYP2R1 strongly and dose dependently, resulting in only 11% expression at multiplicity of infection (MOI) 1 compared to GFP-Ad control (Fig. 2A). To explore the mechanism in more detail, we transduced mutant PGC-1 α into hepatocytes (Fig. S2A). Gaillard et al. described PGC-1 α mutants selective for nuclear receptor interactions; PGC-1 α -L2L3M mutant is unable to bind any nuclear receptors while PGC-1 α -2 \times 9 mutant interacts selectively with nuclear receptors ERR α or HNF-4 α (35). Interestingly, PGC-1 α -2 \times 9 downregulated CYP2R1 expression almost similarly to the wild type (WT) (Fig. 2B), while the L2L3M mutation abolished the CYP2R1 repression and even resulted in weak induction (Fig. 2B). These results indicate that an interaction with a nuclear receptor, most probably ERR α , is indispensable for PGC-1 α -mediated CYP2R1 suppression. Supporting this hypothesis, several ERR α target genes (35) were upregulated by the WT and the 2 \times 9 mutant PGC-1 α (Fig. S2B-D). Moreover, the majority of the PGC-1 α -2 \times 9 mutant induced genes are dependent on ERR α (35).

Several approaches were used to explore the role of ERR α in the regulation of CYP2R1. First, we performed ERR α knockdown by shERR α -Ad virus combined with PGC-1 α -Ad treatment in mouse primary hepatocytes. PGC-1 α -Ad induced the expression of ERR α about 4-fold, as expected (36) (Fig. S2E,F). ERR α knockdown abolished CYP2R1 suppression by PGC-1 α -Ad (Fig. 2C). Furthermore, we confirmed the role of ERR α by using ERR α inverse agonist XCT790. Indeed, 2 μ M XCT790 prevented CYP2R1 repression by PGC-1 α (Fig. 2D) without any effect on PGC-1 α or ERR α expression (Fig. S2G,H). Analysis of public ENCODE data indicates that PGC-1 α and ERR α bind to two common regions within the human *CYP2R1* gene in HepG2 cells (31) (Fig. S2M). We performed a bioinformatics promoter analysis of the mouse *Cyp2r1* gene with MatInspector software and

identified a potential ERR α binding site in the proximal promoter. When -1.2 kb *Cyp2r1*-5'-luciferase-reporter construct was transfected into human hepatoma HepG2 cells and the cells were infected with PGC-1 α -Ad, luciferase activity was repressed; however, mutation of the ERR α binding site at position -1117 to -1122 (relative to the transcription start site) abolished the PGC-1 α response (Fig. 2E). Interestingly, ERR α -Ad did not have effect on the luciferase activity, indicating crucial need for PGC-1 α (Fig. 2E). Altogether, these data indicate that ERR α has a novel, indispensable role in PGC-1 α -mediated down-regulation of CYP2R1 expression in mouse hepatocytes.

Next, we investigated the CYP2R1 repression mechanism *in vivo* by utilizing PGC-1 α knockout (KO) mice fasted for 12 hours. Interestingly, PGC-1 α mRNA was not significantly induced by fasting in the livers of the *Pgc-1 α ^{+/+}* animals compared to the fed control animals (Fig. S2I). On the other hand, PEPCK expression was significantly induced 4.9-fold by fasting in the *Pgc-1 α ^{+/+}* mice, and 3.4-fold in the *Pgc-1 α ^{-/-}* mice indicating fasting response (Fig. S2J). CYP2R1 expression was repressed in the fasted *Pgc-1 α ^{+/+}* mice livers, down to 25% compared to the fed controls (Fig. 2F). Interestingly, PGC-1 α KO was not sufficient to abolish the CYP2R1 repression by fasting (Fig. 2F).

Curiously, the expression of PGC-1 β was induced more potently in the livers of fasted PGC-1 α KO mice (6.1-fold) compared to the WT mice (2.0-fold) (Fig. 2G). Also ERR α induction was significant only in the PGC-1 α KO mice (Fig. S2K). We therefore hypothesized that PGC-1 β , a close relative of PGC-1 α and also a fasting-inducible factor (37), could compensate for the chronic loss of PGC-1 α in the knockout mice, or could even play an independent role in the suppression of CYP2R1.

We therefore investigated the fasting effect on CYP2R1 also in the liver specific-PGC-1 β KO mice. In 24-hour fasted WT mice CYP2R1 was again repressed by 35% (Fig. 2H). However, PGC-1 β KO did not reverse the repression of CYP2R1 (Fig. 2H). PGC-1 β KO did not affect the PGC-1 α induction by fasting (Fig. S2L).

Activation of glucocorticoid receptor represses CYP2R1

GR activated by cortisol is another key pathway controlling the fasting response in the liver, and is also activated in diabetes (38). To investigate the role of GR in the regulation of CYP2R1, we treated mice with a GR agonist, dexamethasone, for 6 h. The treatment decreased liver CYP2R1 mRNA levels by 49%, along with changes in the expression of several known GR target genes, ANGPTL8, NR1D1 and TAT (38, 39), and increased PGC-1 α levels 3.3-fold (Fig. 3A), suggesting involvement of GR in the regulation of CYP2R1 *in vivo*. GR is also a known interaction partner for PGC-1 α (34). The CYP2R1 protein was decreased 26% by 6 h dexamethasone treatment (Fig. 3B). Furthermore, analysis of the published microarray data (GSE24256) (40) supports the effect of dexamethasone on CYP2R1 expression in mouse liver (Fig. S3A).

To verify the role of GR, we cotreated mice with dexamethasone and the GR antagonist mifepristone. Mifepristone completely prevented induction of PGC-1 α and TAT by dexamethasone and inhibited repression of CYP2R1 and NR1D1 (Fig. 3C-F). Furthermore, analysis of published ChIP-sequencing data indicates that GR binds to *Cyp2r1* gene proximal promoter in mouse liver (41) (Fig. S3B). To evaluate the role of GR in the fasting mediated repression of CYP2R1, we investigated the effect of pharmacological inhibition of GR by mifepristone during fasting. Mifepristone abolished the repression of a control gene ANGPTL8 (Fig. 3H). Furthermore, mifepristone partially, but significantly prevented the effect of fasting on the CYP2R1 in the liver (repression decreased from 56% to 18 %), however, the fasting effect remained still statistically significant (Fig. 3G).

Fasting induces CYP24A1 in the kidney through the PGC-1 α -ERR α pathway

DNA microarray analysis of PGC-1 α responsive genes in mouse hepatocytes indicated that CYP24A1, the vitamin D 24-hydroxylase and the main inactivator of active vitamin D (42, 43), was among the most upregulated genes (Table 1). This was further confirmed by PGC-1 α -Ad dose response experiments using quantitative PCR (Fig. 4A).

CYP24A1 is primarily regulated by VDR (44), and VDR is an interaction partner for PGC-1 α (45). We therefore explored the role of VDR in the PGC-1 α -mediated induction of CYP24A1. After treating mouse primary hepatocytes with the VDR ligand calcitriol, CYP24A1 was induced 120-fold (Fig. 4B-D). PGC-1 α -Ad induced CYP24A1 much more potently, up to 20,000-fold. However, combined treatment with calcitriol did not potentiate CYP24A1 induction by PGC-1 α (Fig. 4B). Furthermore, CYP24A1 protein could be detected after PGC-1 α -Ad transduction but not after calcitriol treatment (Fig. 4E). PGC-1 α -Ad treatment had no effect on VDR expression but ERR α was induced 11-fold (Fig. 4F,G). Altogether, these results suggest that VDR does not play a major role in the CYP24A1 induction by PGC-1 α .

We next investigated the putative role of ERR α in the CYP24A1 induction by PGC-1 α . PGC-1 α -2 \times 9 mutant induced CYP24A1 similarly to the wild type PGC-1 α , while PGC-1 α -L2L3M mutant had no effect, suggesting involvement of ERR α (Fig. 4H). Indeed, induction of CYP24A1 was abolished by ERR α knockdown, indicating a novel PGC-1 α -ERR α pathway mediated regulatory mechanism for CYP24A1 (Fig. 4I).

Although we detected CYP24A1 in hepatocytes after PGC-1 α overexpression, we could not detect CYP24A1 in the mouse liver *in vivo*. Indeed, the main site for CYP24A1 expression and function is the kidney (42). CYP24A1 was induced up to 2.7-fold in the kidney by fasting (Fig. 5A) in agreement with a previous study (46). However, the fasting effect was abolished in the PGC-1 α KO mice (Fig. 5B-D), indicating that PGC-1 α plays an indispensable role in the CYP24A1 induction by fasting in the kidney. Furthermore, ERR α antagonist XCT790 treatment diminished the fasting mediated induction of CYP24A1, suggesting that fasting induces CYP24A1 in the kidney through the PGC-1 α -ERR α pathway (Fig. 5E-H).

Discussion

Plasma 25-OH-D level is regularly used as the measure of the vitamin D status. The reason behind is the rather long half-life of 25-OH-D, of about two weeks (47). Furthermore, 25-hydroxylation has been considered not to be under efficient metabolic control and 25-OH-D should thus reflect the intake of vitamin D (48). Several cytochrome P450 enzymes, including CYP2R1, CYP27A1, CYP2D25, CYP2C11 and CYP3A4 have reported to be capable for vitamin D 25-hydroxylation *in vitro* (48). However, genetic studies, expression data and catalytic properties all suggest that CYP2R1 is the main vitamin D 25-hydroxylase enzyme in liver. Indeed, both the knockout studies in mouse and the genetic evidence in humans indicate that defect in CYP2R1 gene results in vitamin D deficiency i.e low plasma 25-OH-D level (13, 49, 50). In humans this has been shown to result in symptomatic rickets, vitamin D-dependent rickets type 1B (15). These patients respond poorly to ordinary vitamin D supplementation, but may benefit from 25-OH-D treatment (15).

We now show that the CYP2R1 enzyme may be repressed also functionally at the level of gene regulation. 12-hour fasting suppressed liver microsomal vitamin D 25-hydroxylation about 50% and after 24-hour fasting we were unable to detect any 25-OH-D formation. Thus, the first vitamin D bioactivation step is under the strict control of nutritional state. Although the acute food deprivation resulted in strong effect on vitamin D 25-hydroxylase activity, this was not reflected to the plasma 25-OH-D concentration, presumably because of the long half-life of 25-OH-D (47). Therefore, it seems unlikely that short term fasting would have significant effect on vitamin D functions at systemic level. This raises the question of the physiological purpose of the CYP2R1 repression during fasting. A likely explanation is that fasting launches physiological adjustment as precaution for possible longer-term food shortage. This may potentially be related to the role of vitamin D in energy homeostasis (1) and could have been evolutionary beneficial during periods of starvation. Alternatively, 25-OH-D could have some unknown local function in liver. Furthermore, we observed induction of CYP24A1 in the kidney during fasting. This is a mechanism limiting the level of $1\alpha,25-$

(OH)₂-D and consequently activation of vitamin D receptor (10). The CYP24A1 induction and the CYP2R1 repression are expected to suppress vitamin D signaling in a synergistic manner.

The suppression of vitamin D bioactivation by fasting-activated mechanisms has important implications in context of human metabolic diseases. Hepatic signaling pathways triggered physiologically during fasting display typically prolonged, constant activation in diabetes. The classical consequence is increased activation of gluconeogenesis resulting in fasting hyperglycemia (51). Since in diabetes, unlike in short term fasting, the activation of these molecular mechanisms is long-standing, the suppression of vitamin D 25-hydroxylation will eventually lead to lower plasma 25-OH-D level. In agreement with this theory, we observed reduced plasma 25-OH-D concentrations in the HFD-treated mice. Thus, we propose that repression of vitamin D bioactivation represents a novel mechanism playing a role in vitamin D deficiency in diabetes.

PGC-1 α is one of the major molecular factors regulating gluconeogenesis and other metabolic pathways activated in the diabetic liver (34, 51). We now show that PGC-1 α , ERR α dependently, also represses vitamin D bioactivation and thus establish regulation of vitamin D metabolism as a novel metabolic function under the control of PGC-1 α . Furthermore, also the CYP24A1 induction by fasting in the kidney was demonstrated to be under the control of PGC-1 α -ERR α . Thus, the PGC-1 α -ERR α pathway appears to play a major role in the cross-talk between energy homeostasis and vitamin D metabolism. Interestingly, a recent study showed that *Cyp2r1*-deficiency in zebrafish affected lipid metabolism through vitamin D regulated function of PGC-1 α (52). PGC-1 α and vitamin D metabolism could thus form a regulatory loop.

Although, the PGC-1 α -ERR α pathway was found to be an effective regulator of CYP2R1, the PGC-1 α knockout did not prevent CYP2R1 suppression during fasting. This indicates that additional molecular mechanisms play a role in the regulation of CYP2R1 during fasting. Activation of GR was found to be a second mechanism capable for CYP2R1 suppression. Indeed, cortisol levels are increased during fasting and in diabetes as well (38). By using pharmacological inhibition of GR we

could partially prevent fasting mediated repression of CYP2R1 suggesting that GR is involved CYP2R1 repression by fasting. However, we cannot exclude the possibility that additional regulatory mechanisms mediate CYP2R1 repression by fasting. From the point of view of drug therapy, the observed repression of CYP2R1 by pharmacological glucocorticoid treatment may explain the observed association between glucocorticoid use and vitamin D deficiency (53).

In summary, our results reveal a novel cross-talk between energy homeostasis and the vitamin D pathway suggesting a physiological need for suppression of vitamin D signaling during nutrient deprivation (Fig. 6). This may be related to the role of vitamin D in energy metabolism (1, 3). Altogether, our study provides a mechanism that may explain the lower vitamin D levels in diabetic patients and suggests that vitamin D deficiency is a consequence, not the cause, of diabetes.

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Author Contributions S.M.A., M.S.E and J.H designed the study. S.M.A. and M.S.E performed a majority of the experiments and measurements. O.K. and M.K. performed the HFD experiment and helped with the other animal experiments. M.B. and S.M.A. performed the PGC-1 α microarray experiment. P.V. performed the 12 and 24h fasting experiments in mice and helped with the hepatocyte primary cultures. V.R performed measurements of the 25-OH-D by LC-MS. M.M and P.T performed the PGC-1 α knockout mice experiments. A.F and R.J.W performed the STZ mice study. K.T.C and B.N.F performed the liver specific PGC-1 β knockout mice study. J.H. supervised the overall conduct of the study. S.M.A., M.S.E and J.H. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Germany, 18-20 September 2016; EMBO/EMBL Symposium: Metabolism in Time and Space: Emerging Links to Cellular and Developmental Programs, EMBL, Heidelberg, Germany, 11-13 May 2017.

Data and Resource Availability: 1) Data Sharing: The datasets generated during and/or analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository (Our microarray for PGC-1 α overexpression in mouse primary hepatocytes, GSE114485), (Published microarray for Dexamethasone treatment in mice livers, GSE24256), (Published PPARGC1A and ESRRA ChIP-seq in HepG2, GSE31477), and (Published NR3C1 (GR) ChIP-seq in mouse liver, GSE72084).

2) Resource Sharing: The Resources generated during and/or analyzed during the current study is available from the corresponding author on reasonable request.

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Table 1. The top ten up- and downregulated genes in mouse primary hepatocytes transduced with PGC-1 α -Ad compared to GFP-Ad treated control cells. Full data available at GEO with the accession number GSE114485.

Gene symbol	Gene name	Fold change
Upregulated		
G6pc	Glucose-6-Phosphatase Catalytic Subunit	388.9
Nr0b2	Nuclear Receptor Subfamily 0 Group B Member 2	290.7
Cyp24a1	Cytochrome P450 Family 24 Subfamily A Member 1	179.0
Slc16a5	Solute Carrier Family 16 Member 5	133.2
Ldhb	Lactate Dehydrogenase B	135.9
Cox7a1	Cytochrome C Oxidase Subunit 7A1	79.3
Cidec	Cell Death Inducing DFFA Like Effector C	108.9
Upp1	Uridine Phosphorylase 1	68.1
Usp2	Ubiquitin Specific Peptidase 2	46.0
Hpd1	4-Hydroxyphenylpyruvate Dioxygenase Like	46.8
Downregulated		
Fbxo5	F-Box Protein 5	-14.3
E2f7	E2F Transcription Factor 7	-12.6
Figl1	Fidgetin Like 1	-11.5
Uhrf1	Ubiquitin Like With PHD And Ring Finger Domains 1	-10.8
Ccne2	Cyclin E2	-10.1
4632417K18Rik	RIKEN cDNA 4632417K18 gene	-8.9
Cdc2a	Cyclin D1	-7.3
Ccnf	Cyclin F	-7.3
Shcbl1	SHC Binding And Spindle Associated 1	-7.2
Aurka	Aurora Kinase A	-6.9

Table 2. Sequences of the PCR primers

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
mPGC-1α	GCAGGTCGAACGAAACTGAC ^a TCCTCCTCATAAAGCCAACC ^b CTGCTCTGGTTGGTGAGGA ^c AGCCGTGACCACTGACAACGAG ^d	CTCAGCCTGGGAACACGTTA ^a GCCTTGGGTACCAGAACAAC ^b GCAGGCTCATTGTTGTAAG ^c GCTGCATGGTTCTGAGTGCTAAG ^d
PEPCK	GGTGTTTACTGGGAAGGCATC	CAATAATGGGGCACTGGCTG
mERRα	ATCTGCTGGTGGTTGAACCTG	AGAAGCCTGGGATGCTCTTG
mCYCS	CCAAATCTCCACGGTCTGTTC	ATCAGGGTATCCTCTCCCCAG
mATP5B	GGTTCATCCTGCCAGAGACTA	AATCCCTCATCGAACTGGACG
mVDR	GAATGTGCCTCGGATCTGTGG	GGTCATAGCGTTGAAGTGGA
rCYP2R1	AAACTACAACCAATGTGCTCCG	CTTCCCAAGAAGGCCTCCTGT
18S	CGCCGCTAGAGGTGAAATTC	CCAGTCGGCATCGTTTATGG
TBP	GAATATAATCCCAAGCGATTTG	CACACCATTTTTCCAGAACTG
mPGC-1β	CTCCAGGCAGGTTCAACCC	GGGCCAGAAGTTCCTTAGG
mGR	AGCTCCCCCTGGTAGAGAC	GGTGAAGACGCAGAAACCTTG
mNR1D1	TCCCAAGAGAGAGAAGCAA	CTGAGAGAAGCCCACCAAAG
mTAT	TGCTGGATGTTTCGCGTCAATA	CGGCTTCACCTTCATGTTGTC
mGAPDH	GGTCATCATCTCCGCCCC	TTCTCGTGGTTCACACCCATC
mG6PASE	CATCAATCTCCTCTGGGTGG	TGCTGTAGTAGTCGGGTGTC
mCYP2R1	Mm01159414_m1 (Life Technologies)	
mCYP24A1	Mm00487245_m1 (Life Technologies)	

^aUsed for measuring PGC-1 α mRNA in Fig. 4C, 5G.

^bUsed for measuring PGC-1 α mRNA in Fig. 3A, E, Fig. S3L

^cFor adenoviral PGC-1 α detection.

^dUsed for measuring PGC-1 α mRNA in Fig. 5C, Fig. S2I.

Figure legends

Figure 1-Fasting and diabetes repress CYP2R1 expression and function in liver. *A and B*: Fasting represses the CYP2R1 mRNA, but induces PEPCCK mRNA in the mouse liver (n= controls and 12h fasted 9, 24h fasted 8). *C and D*: Fasting decreases the CYP2R1 protein (n=4) and the vitamin D-25-hydroxylase activity in the liver microsomes (n= controls and 24h fasted 6, 12h fasted 5). *E*: 24h fasting reduces CYP2R1 mRNA in rat liver (n= controls 3, fasted 4). *F and G*: HFD-induced obesity and type 2 diabetes in mouse downregulates CYP2R1 mRNA in the liver and reduces the plasma 25-OH-D (n= chow 10, HFD 9). *H and I*: The CYP2R1 mRNA and protein were reduced in the liver of the type 1 diabetic mouse model (STZ-treated mice) (n= controls 8, STZ 4). The box-and-whisker plots indicate the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In addition, the mean is indicated with +. In the dot-plots the mean is indicated. In *E, F, G, and H*, two-tailed *t*-test and *A, B, D* One-way ANOVA (Tukey's *post hoc* test, 95 % confidence interval).

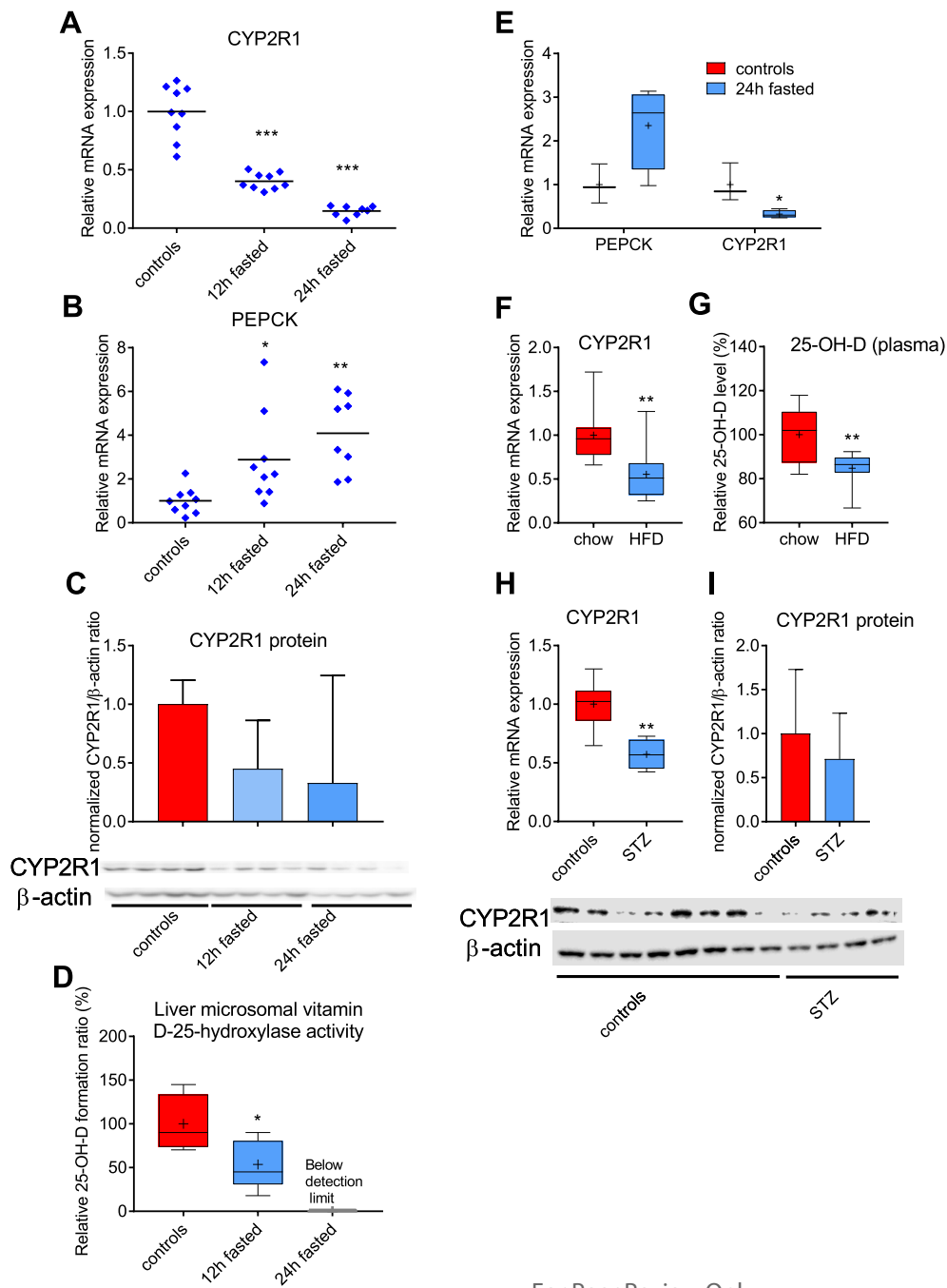
Figure 2-The PGC-1 α -ERR α pathway represses CYP2R1. *A*: PGC-1 α -Ad reduces the CYP2R1 mRNA in mouse primary hepatocytes (n=3). *B*: PGC-1 α mediated suppression of CYP2R1 requires interaction with ERR α (n=6). *C and D*: ERR α knockdown by shERR α -Ad (n=6) or ERR α inhibition by XCT970 (XCT) (n= DMSO 4, 1 μ M XCT 6, 2 μ M XCT 5) abolish the suppression of CYP2R1 by PGC-1 α . *E*: An ERR α binding site the *Cyp2r1* promoter mediates PGC-1 α -prompted reduction of the luciferase activity (n= 12 PGC-1 α -Ad experiment (left panel), 5 ERR α -Ad experiment (right panel)). *F*: PGC-1 α knockout does not abolish the CYP2R1 repression by fasting in mouse liver (n= PGC-1 α ^{+/+} 7, PGC-1 α ^{-/-} 6). *G*: PGC-1 α knockout potentiates PGC-1 β induction by fasting. *H*: Liver-specific PGC-1 β knockout (LS-PGC-1 β ^{-/-}) does not abolish the CYP2R1 repression by fasting in mouse liver (n=4). The box-and-whisker plots indicate the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In addition, the mean is indicated with +. *A-D*, One-way ANOVA (Tukey's *post hoc* test, 95 % confidence interval) and *E-H*, two-tailed *t*-test.

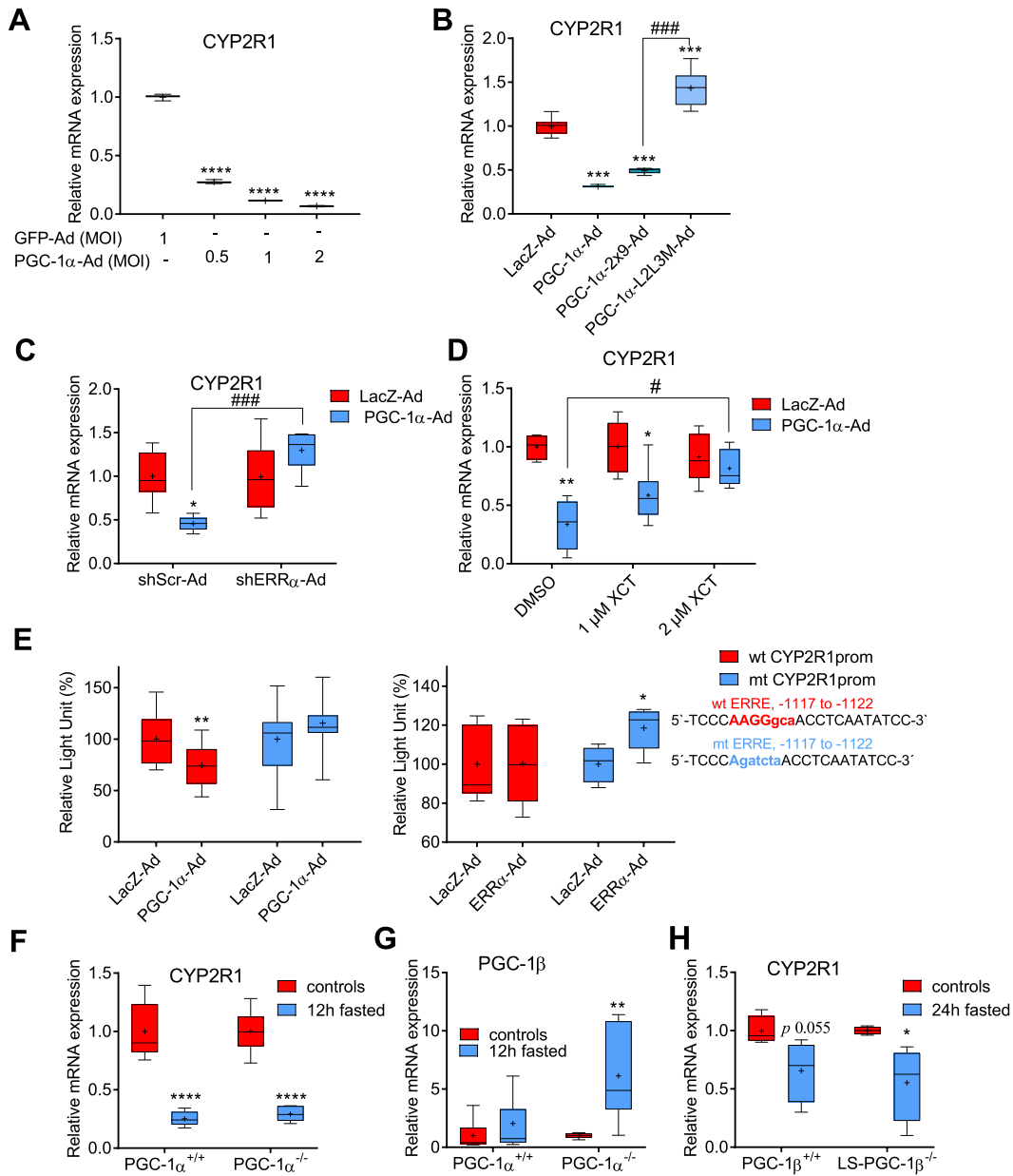
Figure 3-Activation of glucocorticoid receptor represses CYP2R1. *A and B:* Treatment with dexamethasone (DEXA) reduces the CYP2R1 mRNA and protein in mouse liver (n=7). *C-F:* The GR antagonist mifepristone (MIF) attenuates the repression of CYP2R1 and NR1D1, and the induction of PGC-1 α and TAT by DEXA in mouse liver (n=7). *G and H:* The effect of GR antagonist mifepristone (MIF) on fasting response of CYP2R1 and ANGPTL8 in mouse liver (n= vehicle 8, MIF(fed) 8, MIF(fast) 7). The box-and-whisker plots indicate the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In addition, the mean is indicated with +. *A*, two-tailed *t*-test and *C-H*, One-way ANOVA (Tukey's post hoc test, 95 % confidence interval).

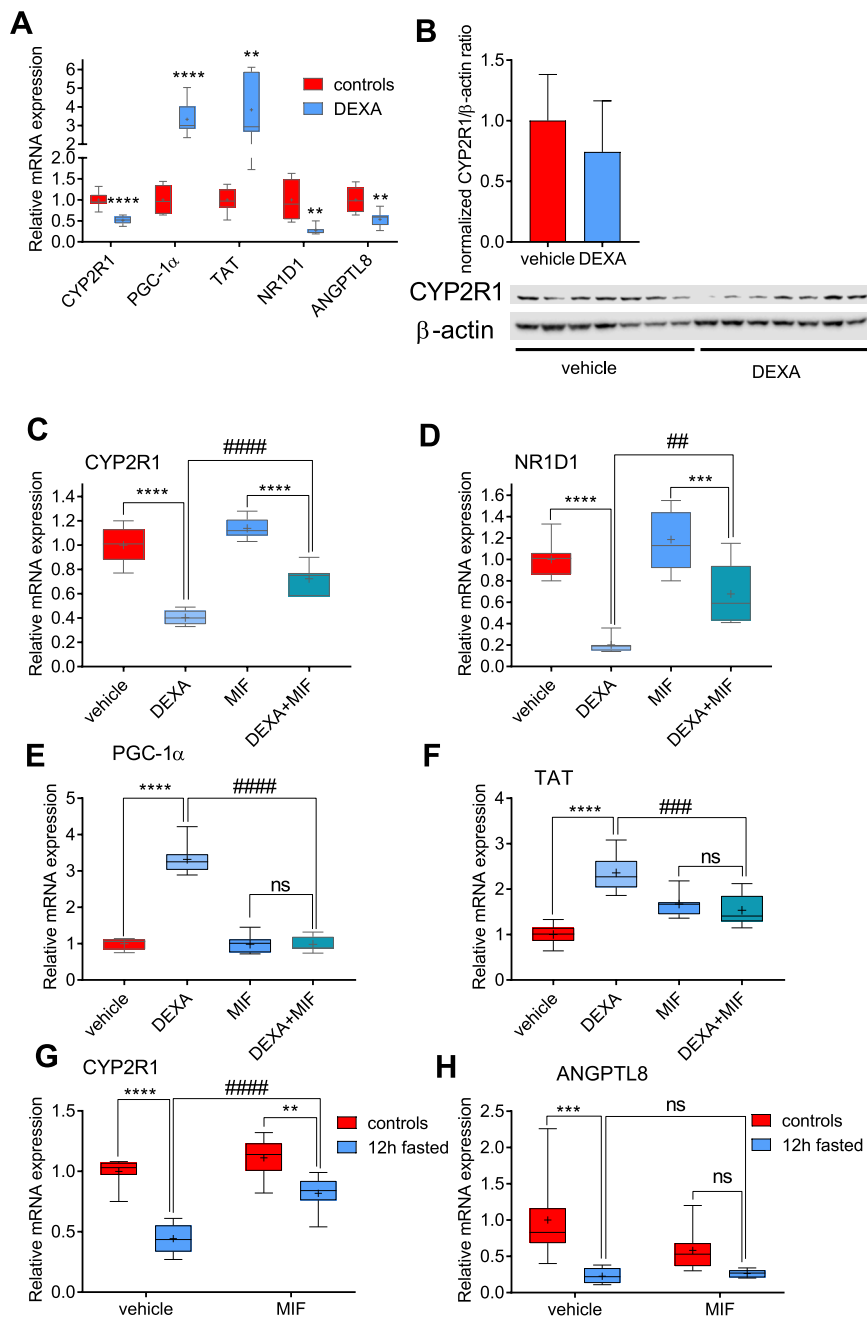
Figure 4-The PGC1 α -ERR α pathway mediates the CYP24A1 induction in hepatocytes. *A:* PGC-1 α induces the CYP24A1 expression in mouse primary hepatocytes (n=3). *B:* PGC-1 α -Ad induces the CYP24A1 expression much more potentially than calcitriol treatment in mouse primary hepatocytes (PGC-1 α -Ad and LacZ-Ad MOI 1, calcitriol 200 nM, n=3). *C:* PGC-1 α was upregulated as expected by the PGC-1 α -Ad (PGC-1 α -Ad and LacZ-Ad MOI 1, calcitriol 200 nM, n=3). *D:* PEPCCK was induced as expected by PGC-1 α induction (PGC-1 α -Ad and LacZ-Ad MOI 1, calcitriol 200 nM, n=3). *E:* CYP24A1 protein was detected by immunoblotting after the PGC-1 α induction but not after calcitriol treatment (PGC-1 α -Ad and LacZ-Ad MOI 1, calcitriol 200 nM, n=3). *F and G:* PGC-1 α did not affect the VDR expression, while ERR α was induced in mouse primary hepatocytes (PGC-1 α -Ad and LacZ-Ad MOI 1, calcitriol 200 nM, n=3). *H:* CYP24A1 induction by PGC-1 α requires interaction with ERR α in the mouse primary hepatocytes (n=6). *I:* The ERR α knockdown abolishes the induction of CYP24A1 by PGC-1 α in mouse primary hepatocytes (n=6). The bars indicate mean \pm SD. The box-and-whisker plots indicate the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In addition, the mean is indicated with +. The data was analyzed with One-way ANOVA (Tukey's post hoc test, 95 % confidence interval). In *A*, *H*, and *I* some control values without PGC-1 α induction were below the detection level and are not show.

Figure 5-The PGC1 α -ERR α pathway mediates the CYP24A1 induction in the kidney by fasting. *A*: 12h fasting induces the CYP24A1 in the mouse kidney (n=10). *B*: PGC-1 α knockout abolishes the induction of the CYP24A1 by fasting in the mouse kidney (n= PGC-1 $\alpha^{+/+}$ 7, PGC-1 $\alpha^{-/-}$ 6). *C*: PGC-1 α was not detected in the kidneys of either fed or fasted PGC-1 $\alpha^{-/-}$ mice. *D*: The ERR α mRNA in the kidneys of wild type and PGC-1 $\alpha^{-/-}$ mice. *E*: Inhibition of ERR α by XCT790 (XCT) attenuates the CYP24A1 induction by fasting in the mouse kidney (n= vehicle 10, XCT 8). *F-H*: PEPCK, PGC-1 α and ERR α were measured as control genes in the XCT790 experiment. The box-and-whisker plots indicate the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In addition, the mean is indicated with +. *A-D*, two-tailed t-test and *E-H* One-way ANOVA (Tukey's post hoc test, 95 % confidence interval).

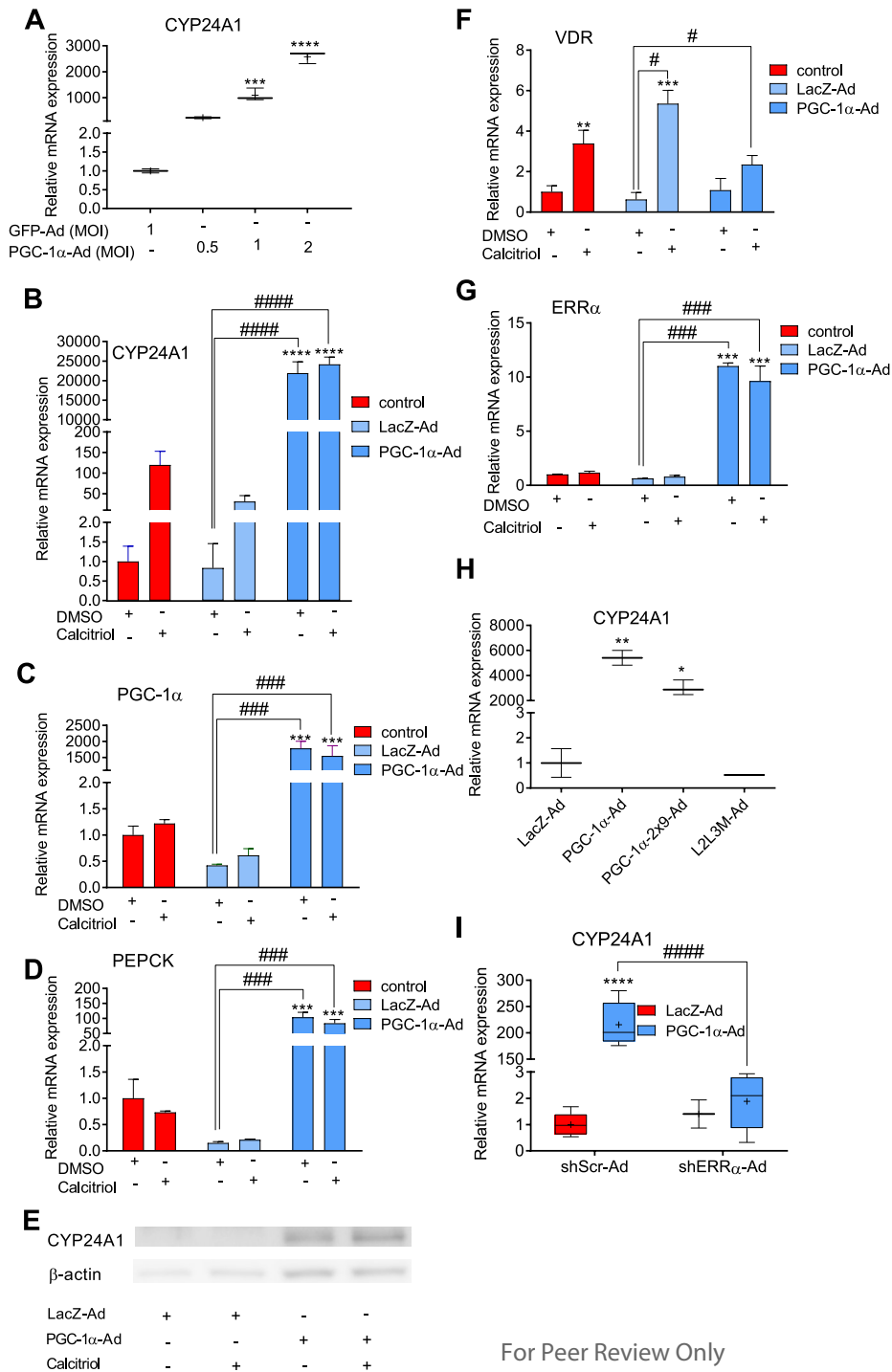
Figure 6-A proposed model how nutrition sensing factors regulate vitamin D metabolism in response to fasting and diabetes. We proposed that at least two molecular pathways are involved in the suppression of the CYP2R1 by fasting in the liver. These same metabolism-regulating pathways are activated in diabetes. The first one is mediated by the fasting inducible cofactor PGC-1 α through nuclear receptor ERR α . The second one is mediated through the cortisol/GR pathway. CYP2R1 repression results in suppression of vitamin D 25-hydroxylation, the first bioactivation step in the liver. On the other hand, fasting induces CYP24A1 in the kidney through a mechanism involving PGC-1 α /ERR α pathway. Induction of CYP24A1 is expected to induce catabolism of vitamin D. Suppression of the 25-hydroxylation in the liver, and induction of the deactivation step in the kidney may lead to lower plasma level of the 25-hydroxyvitamin D and in turn vitamin D deficiency.



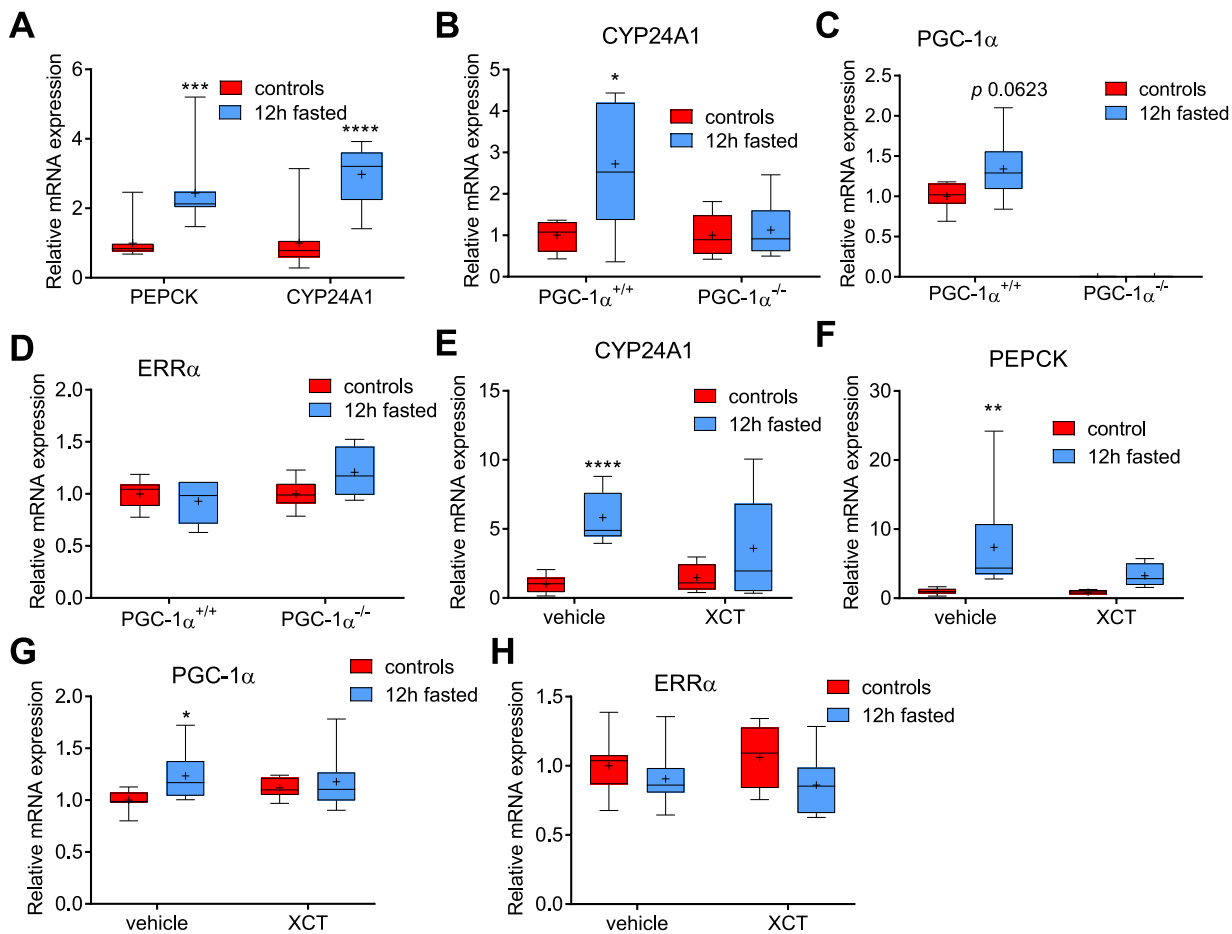




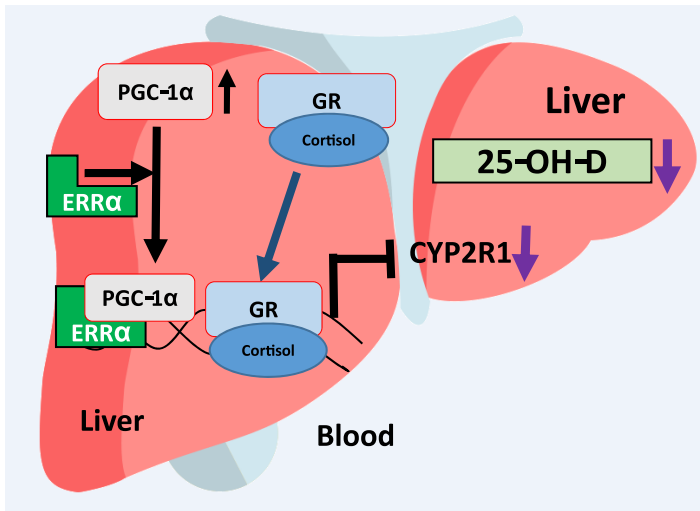
Diabetes



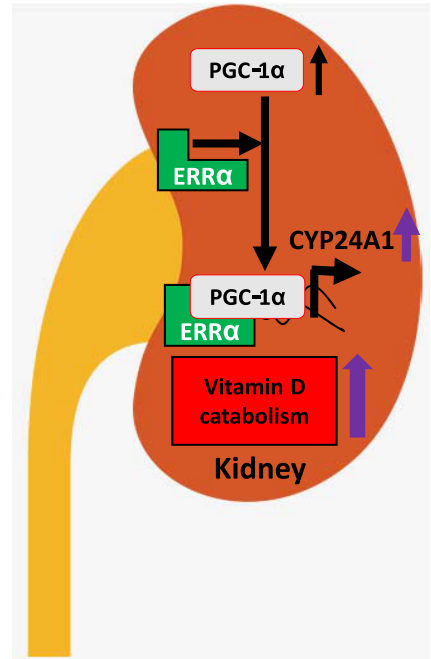
Diabetes



Physiologic stimulus: Fasting
 Pathologic stimulus: Diabetes



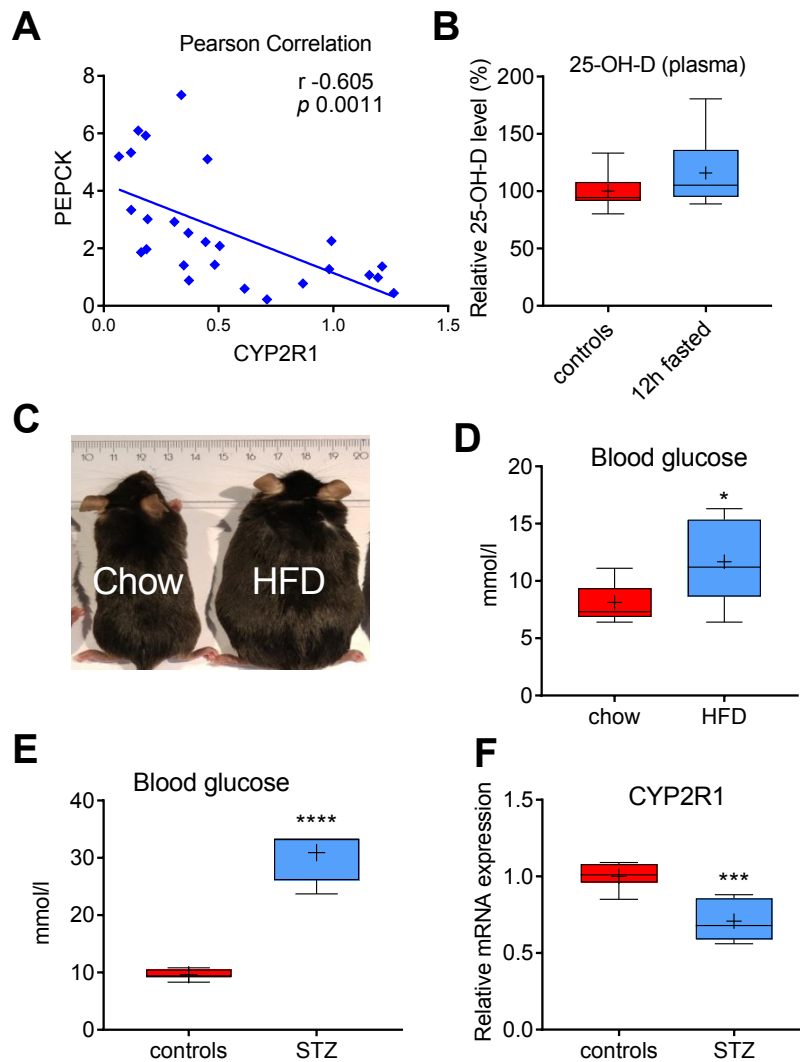
Physiologic stimulus: Fasting



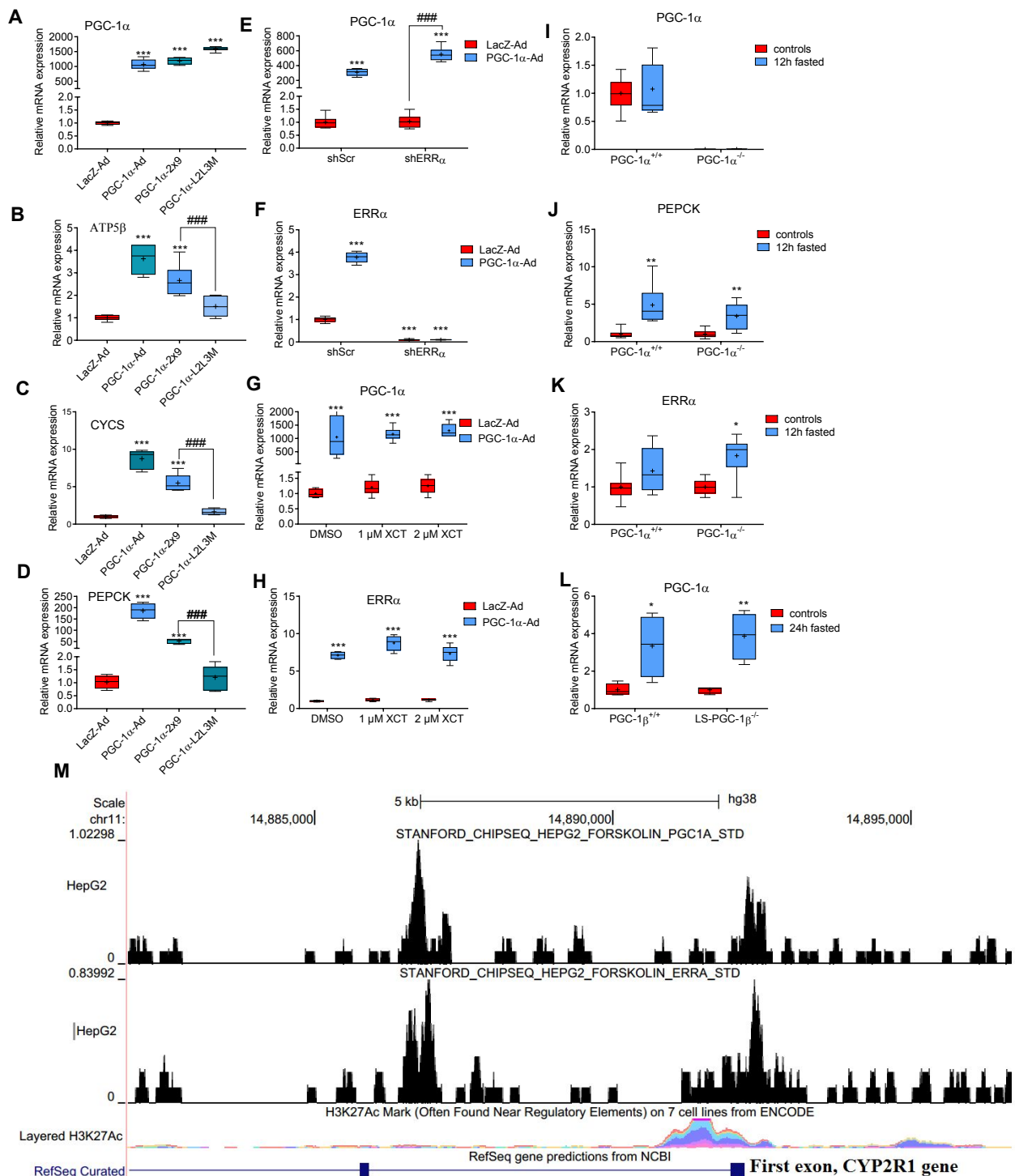
Supplementary Materials

Reagents and antibodies

Dexamethasone, mifepristone, streptozotocin, DMSO, calcitriol, TRI reagent, RNazol reagent, William's E medium, cholecalciferol and XCT790 were purchased from Sigma-Aldrich (St. Louis, MO, USA). High-fat diet (Envigo TD.06414, 60% of calories from fat) and regular chow (Envigo TD.2018) from Envigo Teklad Diets, USA. Rabbit polyclonal CYP2R1 antibody (#T2849, Figure 1c) was purchased from Epitomics (Burlingame, CA, USA), Rabbit polyclonal CYP2R1 antibody (Center, SAB1300955, Figure 1i, 2j), and mouse monoclonal β -actin antibody (A1978) were purchased from Sigma-Aldrich. Rabbit polyclonal CYP24A1 antibody (H-87, sc-66851) was from Santa-Cruz Biotechnology (Santa Cruz, CA, USA), Goat anti-rabbit IgG-HRP and anti-mouse IgG-HRP used as secondary antibodies were purchased from Santa Cruz Biotechnology and GE healthcare (Little Chalfont, UK), respectively. Amersham ECL Prime Blocking Reagent (RPN418) was purchased from GE Healthcare Bio-Sciences (USA). FuGENE® HD Transfection Reagent (E2311) and Dual-Glo(R) Luciferase Assay System (E2940) were purchased from Promega (Madison, WI, USA). Quickchange II site-directed mutagenesis kit (#200523) was purchased from Agilent, USA.

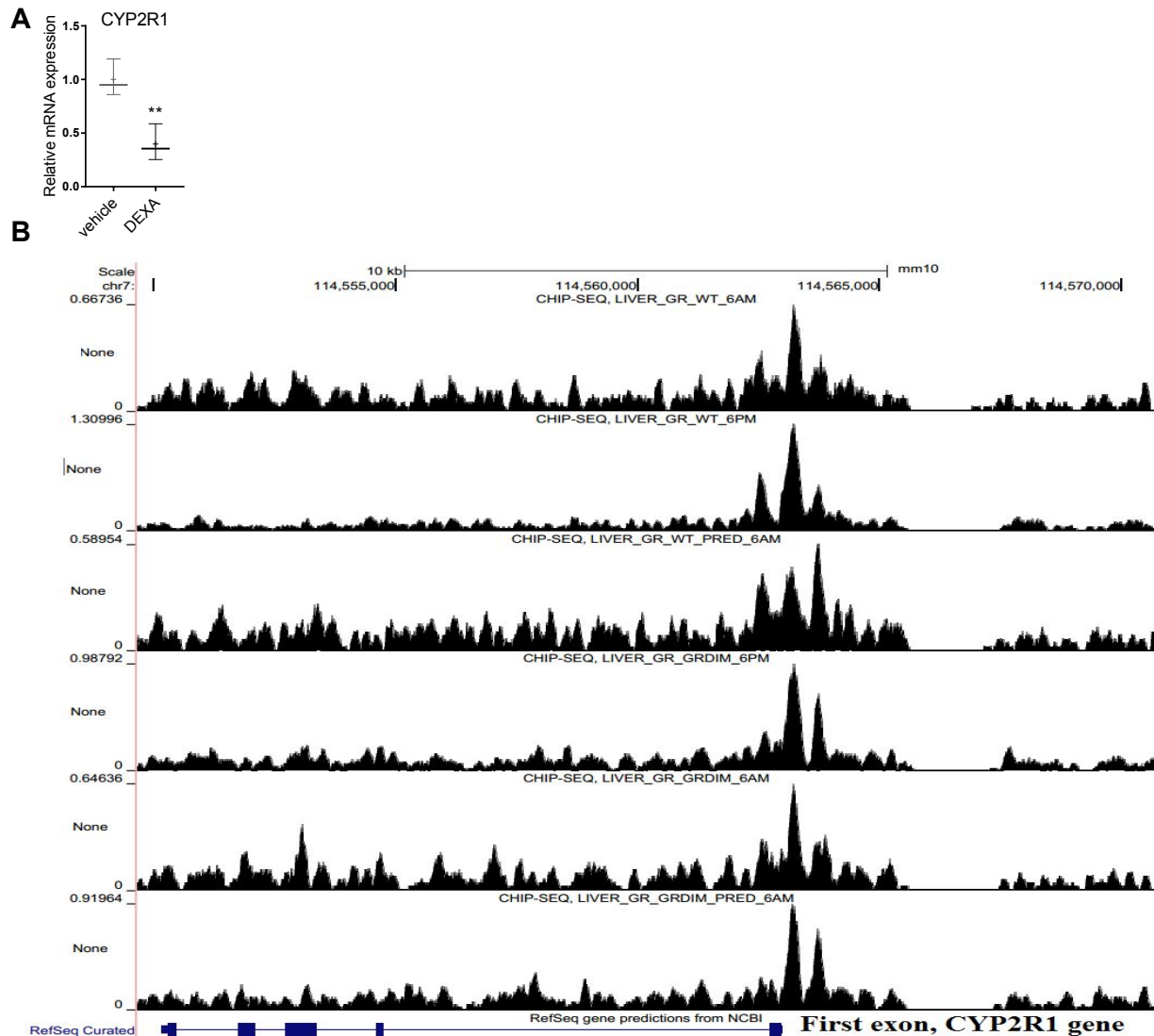


Supplementary Figure 1-CYP2R1 expression and 25-OH-D levels were studied in fasting and diabetes models. *A*: Fasting represses CYP2R1 in mouse liver in a negative correlation with the fasting induced gluconeogenic gene PEPCK. The line represents best-fit by the linear regression. Furthermore, the Pearson correlation was calculated. *B*: The plasma levels of the 25-OH-D in the fed and the 12h fasted mice ($n=10$). *C*: The HFD-treatment induced obesity and diabetes in mice. *D*: The fasted blood glucose was significantly higher in the HFD-treated mice compared to lean controls ($n=$ controls 10, HFD 9). *E*: The nonfasted blood glucose was higher in the STZ-treated mice compared to controls ($n=$ controls 8, STZ 4). *F*: Analysis of published microarray data (GSE39752) indicate that CYP2R1 was repressed in the livers of STZ-treated mice compared to vehicle-treated controls ($n=$ controls 7, STZ 6). The box-and-whisker plots indicate the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In addition, the mean is indicated with +. The data was analyzed by two-tailed *t*-test.



Supplementary Figure 2—Role of the PGC-1 α -ERR α axis in the CYP2R1 suppression. *A*: WT PGC-1 α and the PGC-1 α -2x9 and PGC-1 α -L2L3M mutants were similarly expressed in mouse primary hepatocytes (n=6). *B-D*: PGC-1 α /ERR α -target genes ATP5 β , CYCS, and PEPCK were induced with WT PGC-1 α and the PGC-1 α -2x9 mutant, but not with the PGC-1 α -L2L3M mutant (n=6). *E*: PGC-1 α is induced efficiently by adenovirus in both Scr-Ad and shERR α -Ad infected cells (n= 6). *F*: ERR α was knocked down by shERR α -Ad. *G and H*: XCT790 (XCT) does not affect PGC-1 α and ERR α expression induced by PGC-1 α -Ad (n= DMSO 4, 1 μ M XCT 6, 2 μ M XCT 5). *I*: PGC-1 α mRNA was not detected in the livers of the PGC-1 α knockout mice (n= PGC-1 α ^{+/+} 7, PGC-1 α ^{-/-} 6). *J and K*: PEPCK and ERR α were induced in the livers of fasted wild type and PGC-1 α ^{-/-} mice livers. *L*: PGC-1 α was induced similarly by fasting in the livers of liver-specific PGC-1 β knockout mice and the WT mice (n=4). *M*: Analysis of the published ChIP-seq data (GSE31477) indicate PGC-1 α and ERR α bind to two overlapping regions, in the proximal promoter and in the first intron of the human CYP2R1

gene in the HepG2 cells treated with forskolin. The box-and-whisker plots indicate the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In addition, the mean is indicated with +. The data was analyzed by One-way ANOVA (Tukey's *post hoc* test, 95 % confidence interval).



Supplementary Figure 3-Role of the GR in the CYP2R1 suppression *in vivo*. *A*: Analysis of the published microarray data (GSE24256) indicates that dexamethasone (DEXA) significantly represses the CYP2R1 in mouse liver compared to vehicle-treated mice (n=3). *B*: Analysis of the published ChIP-seq data (GSE72084) indicate that GR binds (both monomer (GRDIM) and homodimer (WT)) to the proximal promoter, close to TSS, of the mouse *Cyp2r1* gene in the mouse liver. The box-and-whisker plots indicate the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In addition, the mean is indicated with +. The data was analyzed by *A*, two-tailed *t*-test.