MOLECULAR AND CELLULAR BIOLOGY, Feb. 2010, p. 565–577 Vol. 30, No. 3
0270-7306/10/$12.00 doi:10.1128/MCB.00927-09

Hepatocyte Nuclear Factor 4α Coordinates a Transcription Factor Network Regulating Hepatic Fatty Acid Metabolism▽

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Received 16 July 2009/Returned for modification 31 August 2009/Accepted 12 November 2009

Adaptation of liver to nutritional signals is regulated by several transcription factors that are modulated by intracellular metabolites. Here, we demonstrate a transcription factor network under the control of hepatocyte nuclear factor 4α (HNF4α) that coordinates the reciprocal expression of fatty acid transport and metabolizing enzymes during fasting and feeding conditions. *Hes6* is identified as a novel HNF4α target, which in normally fed animals, together with HNF4α, maintains PPARγ expression at low levels and represses several PPARα-regulated genes. During fasting, Hes6 expression is diminished, and peroxisome proliferator-activated receptor α (PPARα) replaces the HNF4α/Hes6 complex on regulatory regions of target genes to activate transcription. Gene expression and promoter occupancy analyses confirmed that HNF4α is a direct activator of the *Pparα* gene *in vivo* and that its expression is subject to feedback regulation by PPARα and Hes6 proteins. These results establish the fundamental role of dynamic regulatory interactions between HNF4α, Hes6, PPARα, and PPARγ in the coordinated expression of genes involved in fatty acid transport and metabolism.

Hepatic fatty acid metabolism is a tightly controlled process that involves regulation at the levels of uptake, oxidation, de novo synthesis, and export to the circulation. Regulation is achieved by the action of hormones, like insulin, or intracellular metabolites, notably fatty acids and sterols, that can activate transcription factors, including nuclear hormone receptors (peroxisome proliferator-activated receptor [PPARα or NR1C1], PPARγ [NR1C3], liver X receptor α [LXRα or NR1H3]), the carbohydrate response element binding protein ChREBP, and the sterol regulated factor SREBP1c (5, 16, 20, 41). Activities of these transcription factors are subject to modulation by phosphorylation, by regulated shuttling between the cytoplasm and the nucleus, by exchange of coregulators on target promoters, and by intracellular metabolites that function as ligands. PPARα and PPARγ are key regulators of genes encoding proteins involved in fatty acid uptake, storage, and degradation (31). Various intracellular fatty acids, particularly unsaturated fatty acids and eicosanoids, derived from arachidonic acid, prostaglandin J2, or linoleic acid can bind to the ligand-binding domains of PPARα and PPARγ (5, 31). Fatty acid ligands promote heterodimerization of PPARα with retinoid X receptor (RXR) and their binding to the PPAR response elements (PPRE) at target promoters to initiate transcription activation (15).

Ligand-dependent activation of PPARα and PPARγ provides the principal mechanisms for sensing changes in the concentrations of intracellular metabolites during hormonal or nutrient signaling. Earlier observations, however, suggested that expression of these transcription factors is also subject to regulation in the liver. For example, PPARγ is expressed at low levels in hepatocytes, reduced during fasting, and activated during high-fat diet feeding (14, 30, 39). PPARγ mRNA levels are highly elevated in mouse models of diabetes and obesity (28, 30). Fasting also leads to a robust increase in PPARα expression in the liver (5, 21). Although the molecular mechanisms are poorly understood, these findings raise the possibility that regulation of these factors at the transcriptional level may also contribute to the adaptive response of hepatocytes to hormonal and nutritional signals. Control of the metabolic transcription factors should be a coordinated process since in most cases multiple factors are involved in the regulation of different sets of genes under specific metabolic states.

In this respect hepatocyte nuclear factor 4α (HNF4α or NR2A1) is of particular interest, given its crucial function in a regulatory network required for maintenance of the hepatocyte phenotype (24, 27) as well as its role in the regulation of several metabolic genes involved in gluconeogenesis, bile acid synthesis, conjugation, and transport (13, 18, 19, 35). Liver-specific inactivation of HNF4α leads to hepatomegaly and abnormal deposition of glycogen and lipid in the liver (13). Lipid accumulation in liver has been attributed to selective disruption of very-low-density lipoprotein (VLDL) secretion due to the downregulation of apolipoprotein B (ApoB) and microsomal triglyceride transfer protein (MTTP) expression (13).

The fatty liver phenotype of HNF4α liver-specific knockout (KO) mice raised the possibility that HNF4α may play a broader role in the regulation of fatty acid metabolism. In this study the transcriptional regulation of genes involved in fatty acid uptake, oxidation and ketogenesis, and triglyceride secretion were examined. *Hes6* is identified as a novel HNF4α target gene and found to have an important modulatory role in the

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▽ Published ahead of print on 23 November 2009.

expression of several fatty acid metabolism-associated genes. We show that in mice in the fed state, Hes6/HNF4α complex maintains repression of target genes that upon fasting are induced via the replacement of the HNF4/Hes6 repression complex by activated PPARα. In addition, gene expression and chromatin immunoprecipitation (ChIP) analyses revealed that the function of HNF4α is not limited to downstream genes encoding fatty acid-metabolizing enzymes but extends to the regulation of the transcription factors PPARα and PPARγ. These results identify a dynamic network between the main regulators of metabolic genes that provides an additional level of control for the gene expression program involved in the precise adaptation of the liver to fasting-feeding conditions.

MATERIALS AND METHODS

Animals and histological analysis. *Hnf4*^{loxP} and *Alb-Cre* (13) mice were backcrossed to a CBA-CA × C57BL/10 background, maintained in grouped cages in a temperature-controlled virus-free facility on a 12-h light/dark cycle, and fed by standard chow diet (Altromin 1324; 19% protein and 5% fat) and water *ad libitum*. Further breedings were performed to obtain *Hnf4*$^{lox/lox}$*/Alb-Cre* mice in which the exons 4 and 5 of the *Hnf4* gene were excised fully and specifically in the liver (Hnf4-LivKO) between postnatal day 35 (P35) and P40. To generate Hes6 transgenic mice (Hes6Tg), the Flag epitope containing Hes6 cDNA was inserted into the StuI site of the pTTR1-ExV3 plasmid (43). A 6.1-kb HindIII fragment containing the mouse transthyretin enhancer/promoter, intron 1, *Hes6* cDNA, and simian virus 40 (SV40) poly(A) site was used to microinject CBA-CA × C57BL/10 fertilized oocytes. Founder animals were identified by Southern blotting and crossed with F1 CBA-CA × C57BL/10 mice to generate transgenic lines. Transgene expression reached maximum levels at P30. For our studies we used a transgenic line that expressed Hes6 at 2-fold higher than endogenous levels. Lines with significantly higher expression of the transgene died between postnatal days 15 and 25, probably due to metabolic defects. The Hes6Tg mice were further crossed with *Hnf4*$^{lox/lox}$*/Alb-Cre* mice to obtain Hes6Tg/Hnf4KO mice. *Ppar*α null animals were described previously (25). *Hnf4a*/*Ppara* double-KO animals were obtained by crossing standard PPARα KO mice with *Hnf4*$^{lox/lox}$*/Alb-Cre* mice. These mice lack HNF4α in the hepatocytes and PPARα in all organs. All of our experiments were conducted in 45-day-old male animals, using wild-type mice as controls.

Serum and tissue chemistry. Serum samples were prepared from whole blood collected from the hearts of anesthetized animals. Extraction of lipids from whole livers was performed as described previously (4). The organic layer was dried under nitrogen gas and resolubilized in phosphate-buffered saline (PBS) containing 1% Triton X-100 before measurements. Triglycerides (Diasys), free fatty acid (FFA) (Wako), and β-hydroxybutyrate (Diasys) levels were assayed enzymatically by the respective commercial kits. Statistical comparisons between two groups were conducted using a nonparametric test, the Mann-Whitney U test, using commercial software (StatEl; ad Science).

RNA, protein, and chromatin analysis. Total RNA was prepared by Trizol extraction and, after digestion with DNase I, was further purified by using an RNeasy kit from Qiagen. Reverse transcription-PCR (RT-PCR) and real-time PCR assays were performed as described before (23, 38). The nucleotide sequences of primer sets are available in Table S2 at http://www.fleming.gr/en/investigators/Talianidis/MCB_00927-09_Supplement.pdf.

Chromatin immunoprecipitation, preparation of whole-cell extracts and nuclear extracts, and Western blot analysis were performed as described previously (22, 38). For coimmunoprecipitation assays nuclear extracts were adjusted to 25 mM HEPES (pH 7.9), 200 mM KCl, 1 mM EDTA, 0.5% NP-40, and 10% glycerol and incubated with 2 μg of antibody and 30 μl of protein G-Sepharose beads at 4°C for 6 h. The beads were extensively washed with the same buffer and subjected to SDS-PAGE. The genomic positions and nucleotide sequences of the primers used for chromatin immunoprecipitations are available in Table S1 at http://www.fleming.gr/en/investigators/Talianidis/MCB_00927-09_Supplement.pdf.

The antibodies used in this study were as follows: mouse polyclonal antibody against Hes6 was raised by immunization of BALB/c mice with recombinant full-length Hes6 protein purified from *Escherichia coli* under native conditions. After three boosts in 1-month intervals, serum was collected and tested in different applications (see Fig. S1 at http://www.fleming.gr/en/investigators/Talianidis/MCB_00927-09_Supplement.pdf). The antibody against HNF4α has been described previously (12). Antibodies against TFIIB (sc-225), PPARα (sc-9000x), and PPARγ1 (sc-7196x) were from Santa Cruz Biotechnology.

Cell culture, transfection, and mobility shift DNA binding assays. HepG2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum. The cells were seeded 24 h before transfection at 50 to 60% confluence. In experiments where Wy-14,643 induction was assayed, the medium was replaced by DMEM containing 10% dextran-charcoal-stripped serum at the day of transfection. Reporter plasmids and expression vectors along with 1 μg of CMV-β-Gal (where CMV is cytomegalovirus and β-Gal is β-galactosidase) plasmid were introduced to the cells by the calcium phosphate-DNA coprecipitation method as previously described (12). Thirty-six hours later the cells were harvested and lysed by three consecutive freeze-thaw cycles. Luciferase and β-galactosidase assays were performed as described previously (12) using constant amounts of proteins, and the values were used to normalize for variations in the transfection efficiency.

For electrophoretic mobility shift assays, double-stranded oligonucleotides were radiolabeled by filling in with Klenow enzyme in the presence of [α^{32}P] dCTP. Binding reactions were performed in a 15-μl volume containing 20 mM HEPES, pH 7.9, 50 mM KCl, 2 mM $MgCl_2$, 4 mM spermidine, 0.02 mM Zn-acetate, 0.1 μg/ml bovine serum albumin, 10% glycerol, 0.5 mM dithiothreitol (DTT), 2 μg of poly(dI-dC), and 5 to 10 μg of nuclear extracts derived from HEK-293 cells transfected with either CMV-HNF4α or CMV-PPARα and CMV-RXRα. In competition experiments various amounts of cold oligonucleotides were also included in the binding reaction mixtures. After incubation in ice for 30 min, the protein-DNA complexes were resolved in 5% native polyacrylamide gels and visualized by autoradiography. Quantitation of bound and free radiolabeled probes was performed using a STORM phosphorimager analyzer and multiple exposures.

RESULTS

HNF4α regulates the expression of Hes6, PPARγ, and PPARα. Oil red O staining of HNF4α-deficient (Hnf4-LivKO) mouse livers revealed massive lipid accumulation in hepatocytes (13). Direct measurements of triglycerides and free fatty acids (FFA) in liver extracts and sera of Hnf4-LivKO mice confirmed this observation: hepatic triglyceride and FFA levels were significantly increased, with a concomitant decrease of triglycerides in the sera of Hnf4-LivKO mice (Fig. 1A). Similar changes were observed during fasting of either wild-type or Hnf4-LivKO mice. Serum β-hydroxybutyrate levels were also increased in HNF4α-deficient mice, suggesting that lipid accumulation is accompanied by increased ketogenic activity (Fig. 1A). Cholesterol levels in the sera of Hnf4-LivKO mice were reduced, but hepatic cholesterol levels were not altered significantly (Fig. 1A).

To elucidate the mechanism of fatty liver formation, we analyzed the expression of PPARα and PPARγ, known regulators of genes involved in fatty acid uptake and catabolism. PPARα mRNA and protein levels were at about one-half in HNF4α-deficient livers compared to wild-type mouse livers (Fig. 1B and C). However, this reduced level is likely in the range sufficient to induce its target genes when activated by ligand (see below).

Surprisingly, HNF4α inactivation led to an increase of PPARγ mRNA and protein expression (Fig. 1B and C). Since HNF4α is an activator of transcription, this finding can be explained by an indirect mechanism. Analogous to this situation, a previous study demonstrated that the Hes1 (Hairy Enhancer of Split 1) protein could mediate CREB-dependent repression of the *Ppar*γ gene (14). Thus, we asked whether activation of the *Ppar*γ gene in Hnf4-LivKO mice is a result of derepression from Hes1 action. We did not observe changes in *Hes1* mRNA levels in Hnf4-LivKO mice (Fig. 1B), nor could we detect Hes1 recruitment to the *Ppar*γ promoter in wild-type or Hnf4-

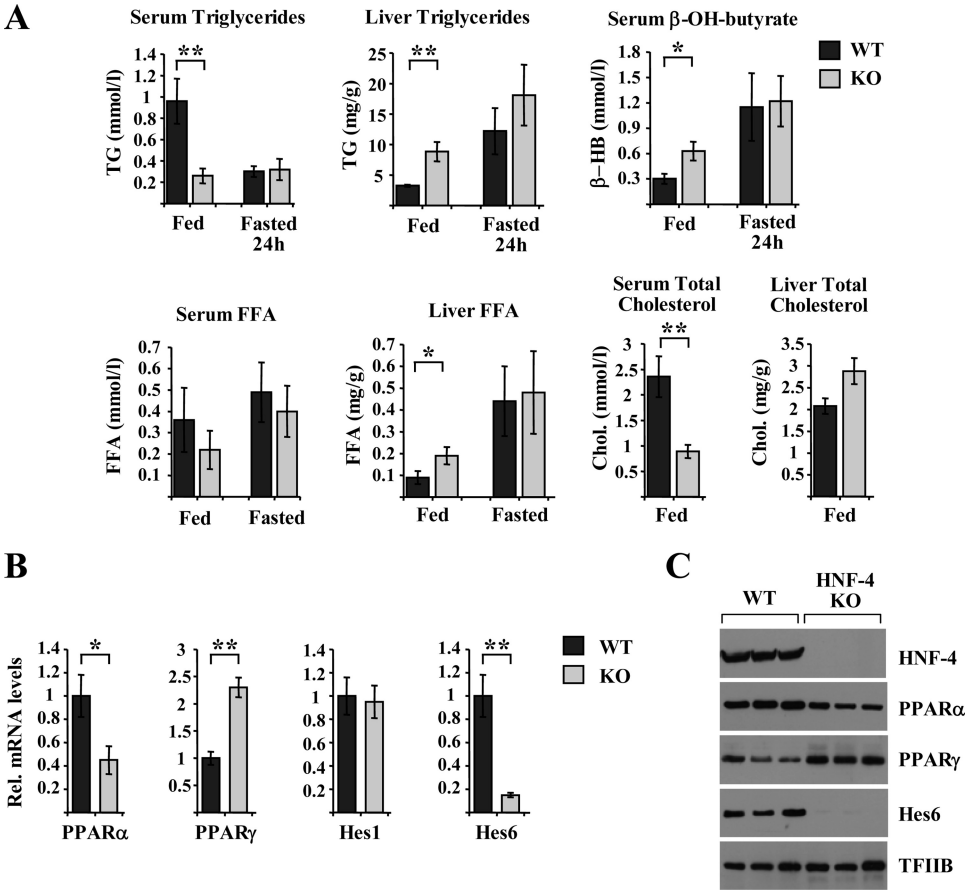

FIG. 1. Altered expression of PPARα, PPARγ, and Hes6 in Hnf4-LivKO mice, which display fatty-liver phenotype. (A) Metabolic parameters in fed and fasted Hnf4-LivKO mice. The concentrations of the indicated metabolites were measured in wild-type (WT) and Hnf4-LivKO (KO) mice. Bars represent means ± standard deviations from five male mice. Statistical analysis was performed by a Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.005$. (B) mRNA levels of PPARα, PPARγ, Hes1, and Hes6 in fed wild-type (WT) and Hnf4-LivKO (KO) mice. Bars represent mean values and standard deviations of mRNA levels normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, relative to wild-type values. Statistical analysis was performed by unpaired Student's *t* test ($n = 5$ mice per group) *, $P < 0.05$; **, $P < 0.005$. (C) Western blot analysis of HNF4α, PPARα, PPARγ, and Hes6 protein levels in fed wild-type (WT) and Hnf4-LivKO (HNF4KO) mice. Nuclear extracts (40 μg of protein per lane) from the livers of three individual animals were analyzed using antibodies recognizing proteins indicated at the right. TG, triglycerides; chol, cholesterol.

LivKO livers (data not shown). On the other hand, the expression of Hes6, another member of the Hairy Enhancer of Split family, was greatly affected by loss of HNF4α (Fig. 1B and C). Furthermore, HNF4α was recruited to the *Hes6* promoter in wild-type mouse liver (Fig. 2A) and also transactivated a *Hes6* promoter-driven reporter construct in transfection assays (Fig. 2B), demonstrating that Hes6 expression is directly regulated by HNF4α. In fasting mice, HNF4α dissociated from the *Hes6* promoter, which correlated with the dramatic reduction of Hes6 mRNA and protein levels (Fig. 2A, C, and F). Coimmunoprecipitation assays revealed a physical interaction between HNF4α and Hes6 proteins (Fig. 2D), indicating that HNF4α, in addition to inducing expression of Hes6, may also facilitate its recruitment to gene regulatory regions. Importantly, *in vitro* glutathione *S*-transferase (GST) pulldown assays revealed that association of Hes6 with HNF4α prevents binding of the coactivator protein CBP/p300, suggesting that Hes6 association may interfere with HNF4α transcriptional activity (Fig. 2E).

To study the role of Hes6 in PPARγ expression, we generated transgenic animals ectopically expressing Hes6 in the liver. The transgenic line used in this study expressed about 2-fold higher amounts of Hes6 in the liver than the wild-type mice (Fig. 2F and G). In fasting mice, when endogenous Hes6 expression is diminished, ectopic expression of the gene in these mice restored hepatic *Hes6* mRNA levels (Fig. 2F). When Hes6Tg mice were crossed into the Hnf4-LivKO background, transgene-derived expression compensated the loss of endogenous Hes6 mRNA and protein levels (Fig. 2F and G).

PPARγ mRNA levels decreased to about 50% in Hes6Tg mice, and Hes6 overexpression suppressed *Pparγ* promoter activity in transfection assays (Fig. 3A and B). ChIP assays revealed that in wild-type animals Hes6 was associated with the *Pparγ* promoter, together with HNF4α (Fig. 3C). Sequential ChIP-reChIP experiments with both combinations of anti-Hes6 and anti-HNF4α as first and second antibodies confirmed the cooccupancy of the *Pparγ* promoter by Hes6 and HNF4α (Fig. 3D). Stimulation of PPARγ transcription in Hnf4-LivKO mice correlated with the dissociation of both HNF4α and Hes6

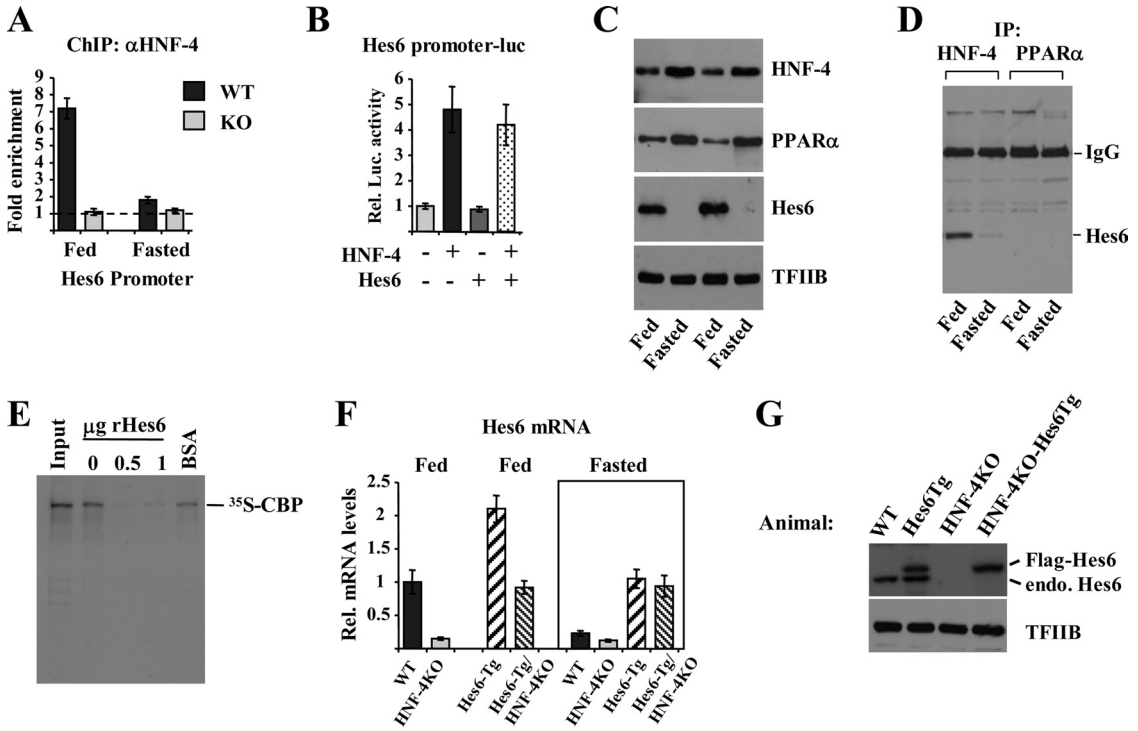

FIG. 2. HNF4α regulates Hes6 expression. (A) Chromatin immunoprecipitation assays in fed and fasted livers from wild-type (WT) and Hnf4-LivKO (KO) mice were performed with anti-HNF4α (αHNF4α) antibody. The data from quantitative PCR (qPCR) amplifications of the Hes6 promoter region were normalized to the input and expressed as fold enrichment over data obtained with control antibody (anti-hemagglutinin epitope), which was set at a value of 1 (dashed horizontal line). Bars represent mean values and standard errors from three independent experiments. (B) HepG2 cells were transfected with 1 μg of luciferase reporter constructs containing the bp +30 to −2700 region of the mouse Hes6 upstream regions and 0.25 μg of pCMV-HNF4α or pCMV-Hes6 expression vector. Bars show relative luciferase activities normalized to LacZ controls from four independent experiments. (C) Western blot analysis of HNF4α, PPARα, and Hes6 protein levels in the livers of fed or 24-h-fasted mice. (D) Coimmunoprecipitation of HNF4α and Hes6. Nuclear extracts from the livers of fed and 24-h-fasted mice were immunoprecipitated (IP) with HNF4α or PPARα antibodies as indicated. The presence of Hes6 protein in the immunopurified material was detected by Western blot assay using anti-Hes6 antibody. (E) Hes6 prevents the interaction of CBP/p300 with HNF4α *in vitro*. One microgram of GST-HNF4α fusion protein was immobilized to glutathione-Sepharose beads and preincubated with the indicated amounts of full-length His-tagged recombinant Hes6 (rHes6) protein or 1 μg of bovine serum albumin (BSA) as a control. After unbound proteins were washed away, the beads were incubated with *in vitro* translated ^{35}S-labeled CBP/p300. Input lane corresponds to 5% of the total ^{35}S-labeled CBP/p300 applied to the column. (F) RT-PCR analysis of Hes6 mRNA in the livers of fed and 24-h-fasted wild-type (WT), Hnf4-LivKO (HNF4KO), Hes6Tg, and Hes6Tg/Hnf4KO mice. The graphs show relative mRNA levels normalized to GAPDH mRNA, and data are presented as described in the legend to Fig. 1B (n = 5). (G) Liver-specific expression of Hes6 protein in TTR-Hes6 transgenic mice (Hes6Tg), Hnf4-LivKO (HNF4KO), and Hes6Tg/Hnf4KO mice was evaluated by Western blotting using anti-Hes6 antibody. Transgene-derived Hes6 protein exhibits slower mobility due to the presence of Flag epitope tag. endo, endogenous.

from its promoter (Fig. 3C). On the other hand, restoration of Hes6 expression in Hes6Tg/Hnf4KO mice did not result in the recruitment of Hes6 to the promoter or repression of the *Pparγ* gene (Fig. 3C). As expected, CBP occupancy was reduced in Hes6Tg mice, alongside with reduced ChIP signals of histone H3 acetylation. While H3K4 trimethylation (H3K4me3), which is also characteristic of activated genes, was not changed, H3K9 trimethylation, a modification associated with silent chromatin, was increased in Hes6Tg mice (Fig. 3E). These results suggest that recruitment of Hes6 is mediated by the interaction with HNF4α and that the HNF4α/Hes6 complex negatively modulates PPARγ transcription in wild-type livers. The repression mechanism involves displacement of the coactivator protein CBP and the generation of a repressive chromatin structure, possibly through facilitating the recruitment of other histone-modifying enzymes.

As expected, PPARα expression, which is suppressed in Hnf4-LivKO mice under both fed and fasted conditions (Fig. 3F), was not subject to Hes6-dependent regulation. The *Pparα* promoter was occupied by HNF4α but not by Hes6, and the levels of PPARα mRNA were not affected in Hes6Tg mice (Fig. 3F and G). In addition, we could not detect significant changes between wild-type and Hes6Tg mice in CBP occupancy or histone modification patterns on *Pparα* promoter (Fig. 3H).

Importantly, association of HNF4α with the promoter was increased during fasting, which correlates with increased PPARα mRNA and protein levels (Fig. 3F and G and 1C). These results indicate that PPARα is a canonical HNF4α-regulated gene not modulated by Hes6.

Hes6 and PPARα feedback regulates *Hnf4α* transcription. Previous studies established that HNF4α regulates its own expression through facilitating the interaction between the *Hnf4α* enhancer and promoter (10, 24). HNF4α is recruited to its upstream enhancer, and due to loop formation between the enhancer and promoter, it is also detected on the proximal regulatory region by

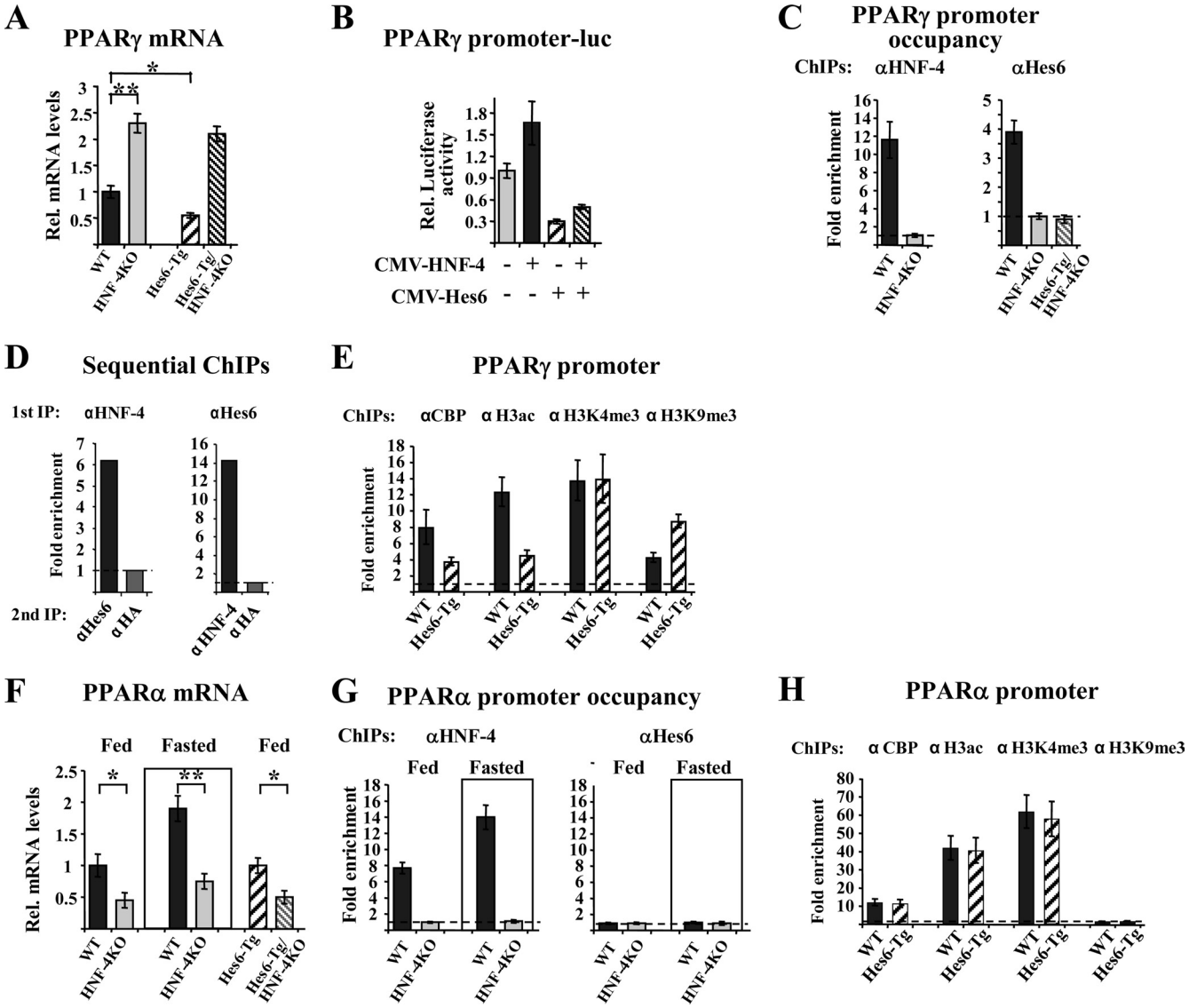

FIG. 3. Regulation of *Pparγ* and *Pparα* genes by HNF4α and Hes6. (A and F) mRNA levels of PPARγ and PPARα in different animal models. The graphs show relative PPARγ and PPARα mRNA levels normalized to GAPDH mRNA, in models of fed and 24-h-fasted animals, as indicated below the *x* axis. The data are presented as described in the legend to Fig. 1B ($n = 5$). (B) Hes6 suppresses *Pparγ* promoter activity. HepG2 cells were transfected with 1 μg of luciferase reporter constructs containing the bp +76 to −2525 region of the mouse *Pparγ* upstream region and 0.25 μg of pCMV-HNF4α or pCMV-Hes6 expression vector. Bars show relative luciferase activities normalized to LacZ controls from four independent experiments. (C and G) Occupancy of *Pparγ* and *Pparα* promoter by HNF4α and Hes6. ChIP assays were performed with antibodies indicated at the top in animal models indicated below the *x* axis. Graphs show qPCR amplifications of the *Pparγ* and *Pparα* promoter regions, and data are presented as described in the legend of Fig. 2A ($n = 3$). (D) Cooccupancy of *Pparγ* promoter by HNF4α and Hes6. After immunoprecipitations (IP) with the indicated first antibodies, the immunoprecipitated chromatin was eluted from the protein G-Sepharose beads by incubation with 10 mM DTT for 30 min at 37°C. After dilution the eluted material was subjected to immunoprecipitation with the indicated second antibodies. Graphs show qPCR amplifications of the *Pparγ* promoter region from one of two representative experiments, and data are presented as described in the legend of Fig. 2A. (E and H) CBP occupancy and histone modification patterns of *Pparγ* and *Pparα* promoter regions in wild-type and Hes6Tg mice. ChIP assays were performed with antibodies indicated at the top in animal models indicated below the *x* axis. Graphs show qPCR amplifications of the *Pparγ* and *Pparα* promoter regions, and data are presented as described in the legend of Fig. 2A. ($n = 3$). α, anti.

ChIP assays (Fig. 4D) (11, 24). HNF4α mRNA and protein levels decreased significantly in Hes6Tg animals (Fig. 4A and B). In contrast, increased HNF4α mRNA and protein were detected in fasted wild-type mice, where Hes6 protein is lost (Fig. 4A and 1C). In addition, overexpression of Hes6 repressed *Hnf4* promoter-driven transcription in transient transfection assays (Fig. 4C). This points to a feedback regulation between HNF4α and Hes6 in wild-type animals, which was further confirmed by the detection of Hes6 recruitment to the *Hnf4α* regulatory regions (Fig. 4D). As expected, Hes6 recruitment was not detected in fasted wild-type animals or in Hnf4-LivKO mice, where Hes6 expression is diminished. We also failed to detect Hes6 association with the *Hnf4α* regulatory regions in Hes6Tg/Hnf4KO mice, in which Hes6 expression is restored (Fig. 4D). These data sug-

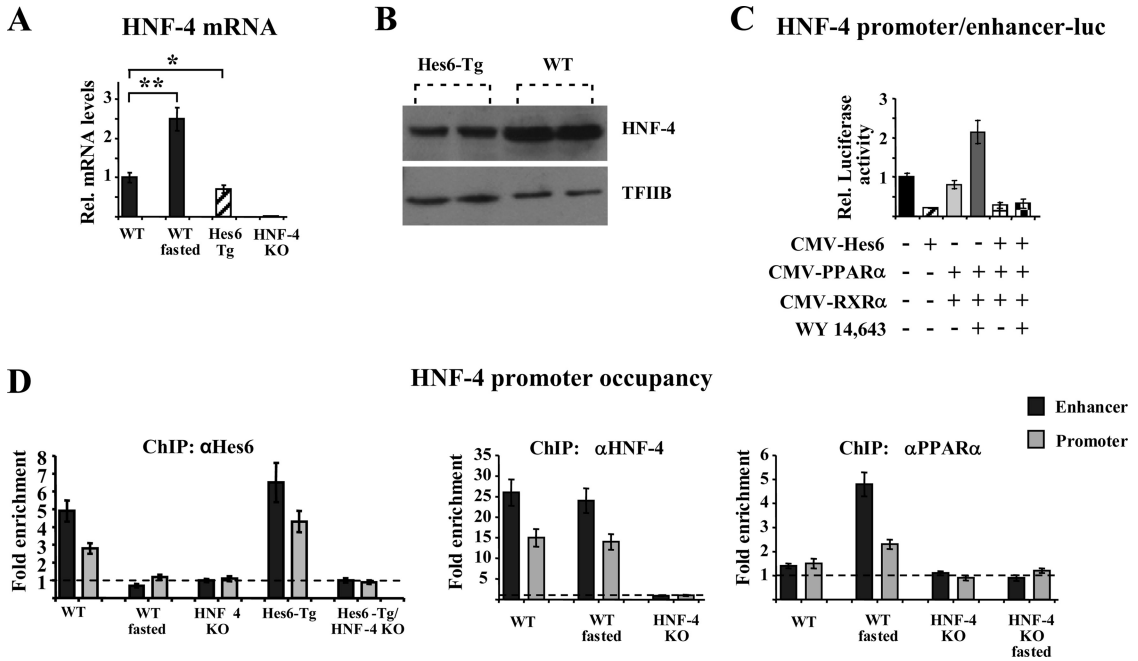

FIG. 4. Hes6 and PPARα feedback regulates HNF4α expression. (A) mRNA levels of HNF4α in different animal models. The graphs show relative HNF4α mRNA levels normalized to GAPDH mRNA, in animal models indicated below the *x* axis, and data are presented as described in the legend to Fig. 1B ($n = 5$). (B) Effects of Hes6 and PPARα overexpression on *Hnf4α* promoter activity. HepG2 cells were transfected with 1 μg of luciferase reporter constructs containing the bp +10 to −6700 region of the *Hnf4α* gene and 0.25 μg of pCMV-Hes6, pCMV-PPARα, and pCMV-RXRα expression vectors. Where indicated, Wy-14,643 was added at a 1 μM final concentration 24 h before harvest. Bars show relative luciferase activities normalized to LacZ controls from four independent experiments. (C) Occupancy of *Hnf4α* regulatory regions by Hes6, HNF4α, and PPARα. ChIP assays were performed with antibodies indicated at the top in the animal models indicated below the *x* axis. Graphs show qPCR amplifications of the *Hnf4α* promoter region, and data are presented as described in the legend of Fig. 2A. ($n = 3$).

gest that HNF4α is required for the recruitment of Hes6 to its own regulatory region.

To identify the potential direct role of PPARα in the mechanism of fasting-dependent induction of *Hnf4α* transcription, we analyzed the recruitment of PPARα to the *Hnf4α* promoter under feeding and fasting conditions. PPARα association could be detected only in fasted wild-type animals but not in fed or Hnf4-LivKO mice (Fig. 4D). This suggests that PPARα association requires the presence of HNF4α. Activation of *Hnf4α* transcription upon fasting can therefore be explained by replacement of the negative modulator Hes6 by activated PPARα in the *Hnf4α* regulatory region. In agreement with this, in reporter assays we could detect ligand-dependent transactivation of the *Hnf4α* promoter/enhancer by PPARα, which was abolished by Hes6 overexpression (Fig. 4C).

Critical role of the HNF4α/Hes6 regulatory axis in the modulation of PPARα target genes involved in fatty acid uptake, degradation, and ketogenesis. Analysis of the metabolic profiles of Hes6Tg mice revealed that doubling the amount of intracellular Hes6 protein leads to significantly reduced serum β-hydroxybutyrate and increased hepatic free fatty acid levels, while serum and hepatic triglyceride levels were not affected (Fig. 5A). This raised the possibility that Hes6, besides modulating HNF4α and PPARγ transcription, may also influence the expression of some genes involved in fatty acid metabolism in a direct manner. The hepatic mRNAs encoding CD36, a major fatty acid transport protein (7), and ACOT1, which hydrolyzes long-chain acyl coenzymes A (acyl-CoAs) to free fatty acids, were highly elevated in Hnf4-LivKO mice but decreased in Hes6Tg mice (Fig. 5B and C). These genes are activated during fasting and have previously been identified as PPARα target genes (1, 6). Therefore, we analyzed the association of transcription factors with their promoters in fed and fasted animals. HNF4α and Hes6 occupied both *Cd36* and *Acot1* promoters in fed wild-type animals when the genes were expressed at low levels (Fig. 5B and C). The lack of Hes6 recruitment to these promoters in Hes6Tg/Hnf4KO mice suggests that its association requires HNF4α. In these latter animals, induction of *Cd36* and *Acot1* genes was not affected. In Hnf4-LivKO mice, PPARα replaced HNF4α and Hes6 on both promoters, and PPARγ also associated with the *Cd36* promoter (Fig. 5B and C). Importantly, an exchange of HNF4α/Hes6 complex by PPARα was also observed in fasted animals. These results suggest that in wild-type mice, the *Cd36* and *Acot1* genes are repressed by the HNF4α/Hes6 complex, while in Hnf4-LivKO mice or in fasted animals, they are activated via a derepression-activation mechanism mediated by the exchange of HNF4α/Hes6 repressor complex with activated PPARα.

An analogous situation was evident in the regulation of two other PPARα target genes, *Fgf21* (fibroblast growth factor 21) and *Hmgcs2* (hydroxymethyl-glutaryl-CoA synthase 2). These correspond to a key regulator and the rate-limiting enzyme of the ketogenesis pathway, respectively (2, 17, 36). The mRNAs encoding FGF21 and HMGCS2 were significantly increased in Hnf4-LivKO mice but decreased in Hes6Tg mice (Fig. 6A and

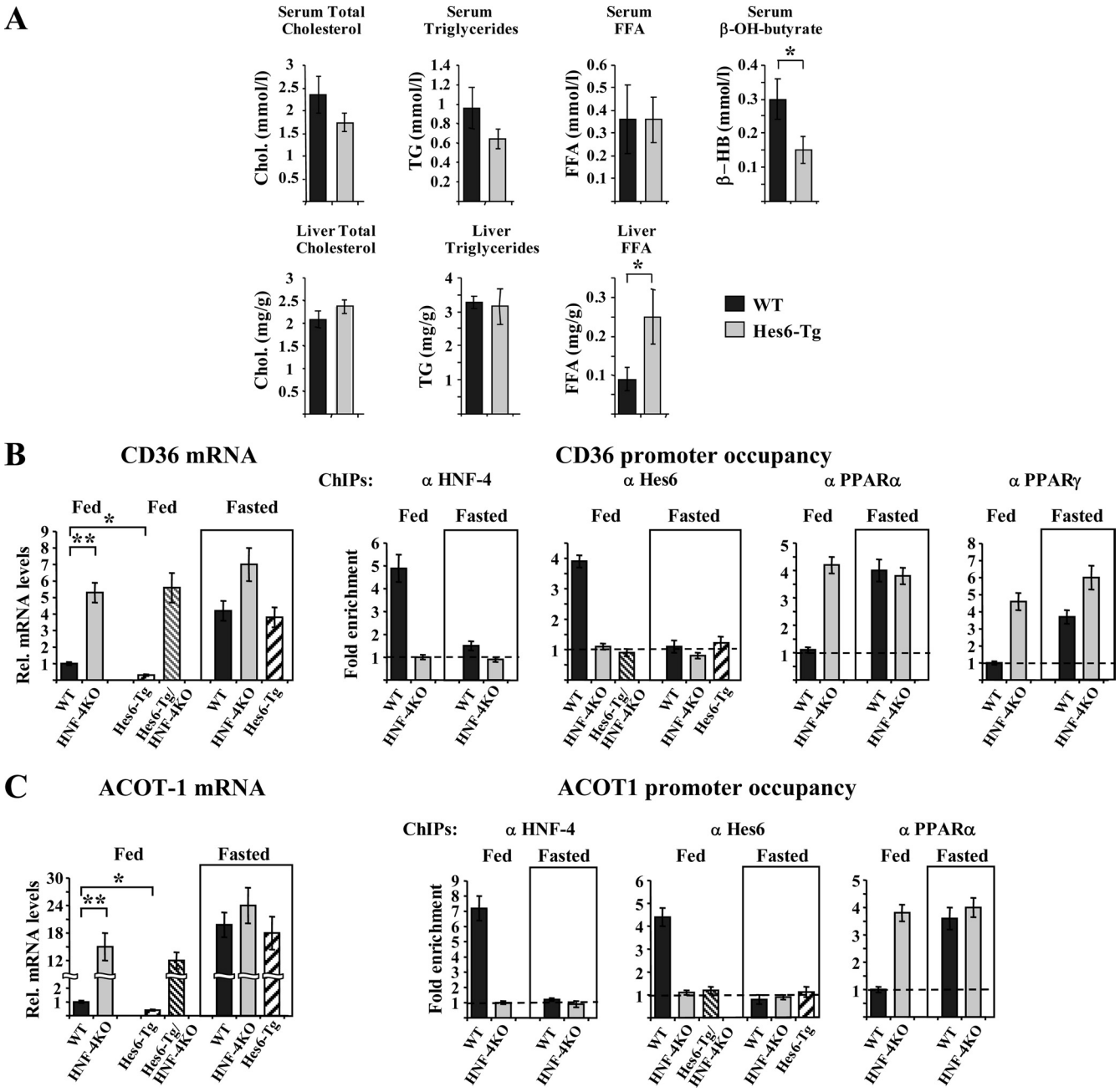

FIG. 5. Interplay between HNF4α, Hes6, PPARγ, and PPARα regulates the expression of *Cd36* and *Acot1* genes under feeding-fasting conditions. (A) Metabolic parameters in Hes6Tg mice. Bars represent means ± standard deviations from five male mice. Statistical analysis was performed by a Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.005$. (B) mRNA levels of CD36 ($n = 5$) and occupancy of its promoter by HNF4α, Hes6, PPARα, and PPARγ ($n = 3$) in fed and 24-h-fasted animals. (C) mRNA levels of ACOT1 ($n = 5$) and occupancy of its promoter by HNF4α, Hes6, and PPARα ($n = 3$) in fed and 24-h-fasted animals. Graphs are labeled as described in the legend to Fig. 4.

B). HNF4α and Hes6 occupied the *Fgf21* and *Hmgcs2* promoters in wild-type animals and dissociated from them during fasting when recruitment of PPARα was observed (Fig. 6A and B). In the case of the *Hmgcs2* promoter, PPARα association could also be detected in wild-type animals and in Hnf4-LivKO mice, which may explain the lower extent of induction of the gene during fasting.

Further evidence for the requirement of PPARα in the activation of *Cd36*, *Acot1*, *Hmgcs2*, and *Fgf21* genes in Hnf4-LivKO mice was provided by the analysis of their mRNA levels in *Hnf4α*/*Pparα* double KO mice. The mRNA levels of HMGCS2, ACOT1, and FGF21 were not significantly altered in these animals compared to wild-type counterparts (see Fig. S2 at http://www.fleming.gr/en/investigators/Talianidis/MCB_00927-09_Supplement.pdf). In *Hnf4α*/*Pparα* double KO mice *Cd36* mRNA was still increased, albeit to a lower extent than in Hnf4-LivKO mice. This can be explained by the action of PPARγ on the *Cd36* promoter since HNF4α deficiency-mediated

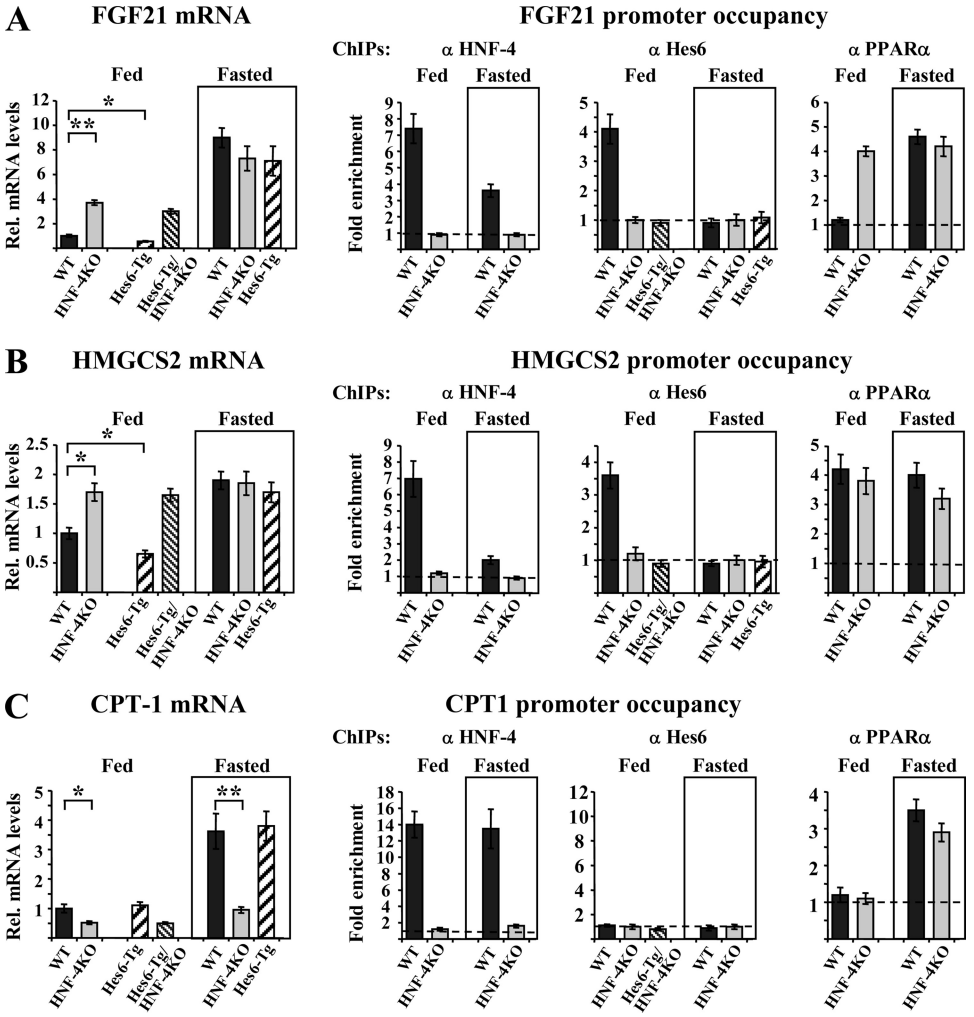

FIG. 6. Interplay between HNF4α, Hes6, and PPARα regulates the expression of *Fgf21*, *Hmgcs2*, and *Cpt1* genes under feeding-fasting conditions. (A) mRNA levels of FGF21 ($n = 5$) and occupancy of its promoter by HNF4α, Hes6, and PPARα ($n = 3$) in fed and 24-h-fasted animals. (B) mRNA levels of HMGCS2 ($n = 5$) and occupancy of its promoter by HNF4α, Hes6, and PPARα ($n = 3$) in fed and 24-h-fasted animals. (C) mRNA levels of CPT1 ($n = 5$) and occupancy of its promoter by HNF4α, Hes6, and PPARα ($n = 3$) in fed and 24-h-fasted animals. Graphs are labeled as described in the legend to Fig. 4.

induction of PPARγ was not affected by the simultaneous loss of PPARα (see Fig. S2 at the URL given above).

Carnitine palmitoyl transferase 1 (CPT1) catalyzes the transfer of long-chain fatty acids into mitochondria, the rate-controlling step of fatty acid oxidation pathway (29). The expression of CPT1 was reduced to about 50% in Hnf4-LivKO mice (Fig. 6C). A similar level of reduction was observed in *Hnf4α*/*Pparα* double KO mice, suggesting that in the fed state CPT1 expression is mainly regulated by HNF4α (see Fig. S2 at http://www.fleming.gr/en/investigators/Talianidis/MCB_00927-09_Supplement.pdf). During fasting CPT1 mRNA levels were significantly elevated in wild-type but not in Hnf4-LivKO animals (Fig. 6C). We did not observe changes of CPT1 expression in Hes6Tg mice, nor could we detect occupancy of the *Cpt1* promoter by Hes6 in the different animal models and under different conditions. Together with the finding that HNF4α occupied the *Cpt1* promoter in both fasted and fed state, these data suggest that *Cpt1* transcription is fully dependent on HNF4α and not modulated by Hes6 (Fig. 6C). PPARα was recruited to the *Cpt1* promoter in both wild-type and Hnf4-LivKO animals but only under fasting conditions. However, the lack of induction of CPT1 mRNA levels in fasted Hnf4-LivKO animals suggests that starvation-dependent stimulation of the *Cpt1* gene requires a synergism between HNF4α and activated PPARα (Fig. 6C).

Examination of the binding site sequences on the studied genes revealed simple direct repeat 1 (DR-1)-type motifs in the regulatory regions of *Acot1*, *Cd36*, *Fgf21*, and *Hmgcs2*, where PPARα replaces HNF4α during fasting and a composite motif in the *Cpt1* promoter, where PPARα is corecruited with HNF4α (see Table S1 at http://www.fleming.gr/en/investigators/Talianidis/MCB_00927-09_Supplement.pdf). This raised the question whether the nature of the binding site may influence the binding affinities of PPARα and HNF4α, which may explain the different *in vivo* recruitment patterns observed in the two groups of genes. To this end, we compared the *in vitro* DNA binding affinities of PPARα and HNF4α to promoter elements derived from the *Acot1* and *Cpt1* genes. Using a

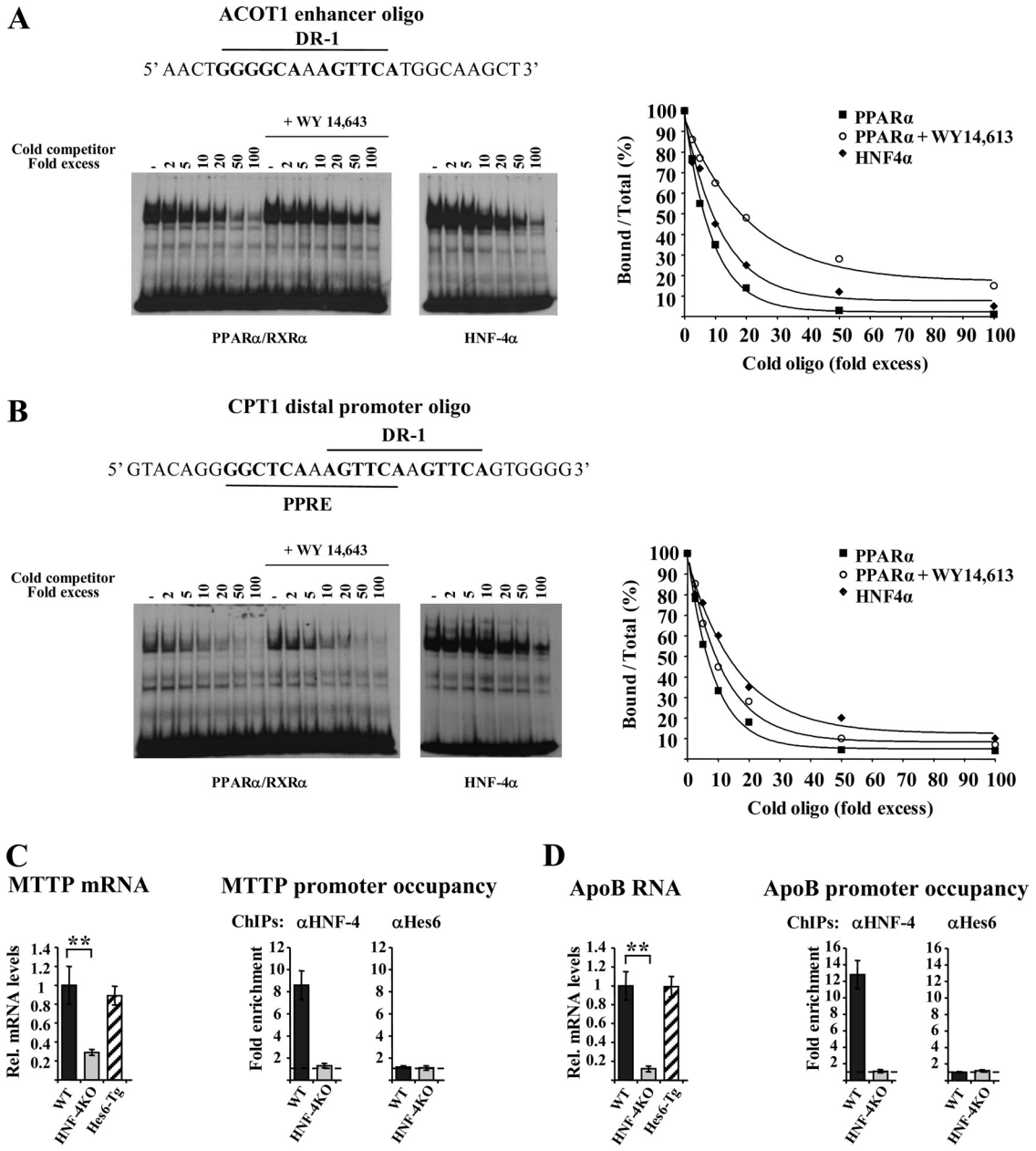

FIG. 7. *In vitro* binding affinities of HNF4α and PPARα and regulation of *Mttp* and *ApoB* genes by HNF4α. Double-stranded oligonucleotides from the *Acot1* enhancer (A) or *Cpt1* distal promoter (B) were used in mobility shift assays in the absence or presence of increasing amounts of cold competitor oligonucleotides. Where indicated, binding reaction mixtures were supplemented with 40 μM Wy-14,643 ligand. The DR-1 sequence from the binding site in *Acot1* enhancer and the composite PPAR response element (PPRE) and DR-1 in the *Cpt1* promoter are shown above the autoradiograms. Quantitation of bound and free probes was performed by phosphorimager analysis. Competition curves are presented at right. (C) mRNA levels of MTTP ($n = 5$) and occupancy of its promoter by HNF4α and Hes6 ($n = 3$) in Hnf4-LivKO and Hes6Tg mice. (D) mRNA levels of ApoB ($n = 5$) and occupancy of its promoter by HNF4α and Hes6 ($n = 3$) in Hnf4-LivKO and Hes6Tg mice. Graphs are labeled as described in the legend of Fig. 4.

probe from the *Acot1* enhancer, where fasting-mediated exchange between HNF4α and PPARα occurs *in vivo* (Fig. 5C), 50% competition of binding by unliganded PPARα or liganded PPARα or HNF4α was observed in the presence of 6-fold, 18-fold, and 9-fold molar excesses of cold probe, respectively (Fig. 7A). In agreement with the competition assays, ligand addition lowered the dissociation constant of PPARα binding to the ACOT1 probe (K_d of 1.60 nM) compared to K_d values of unliganded PPARα (K_d of 6.18 nM) or HNF4α (K_d of 2.70 nM) (see Fig. S3 at the URL given above). This suggests that ligand binding increases the affinity of PPARα association with the element to a level that exceeds the affinity of HNF4α binding. In the case of the *Cpt1* probe, the amounts of cold oligonucleotide required for 50% competition of binding were 6-fold (PPARα without ligand), 9-fold (PPARα with ligand), and 13-fold (HNF4α) (Fig. 7B). The dissociation constants for

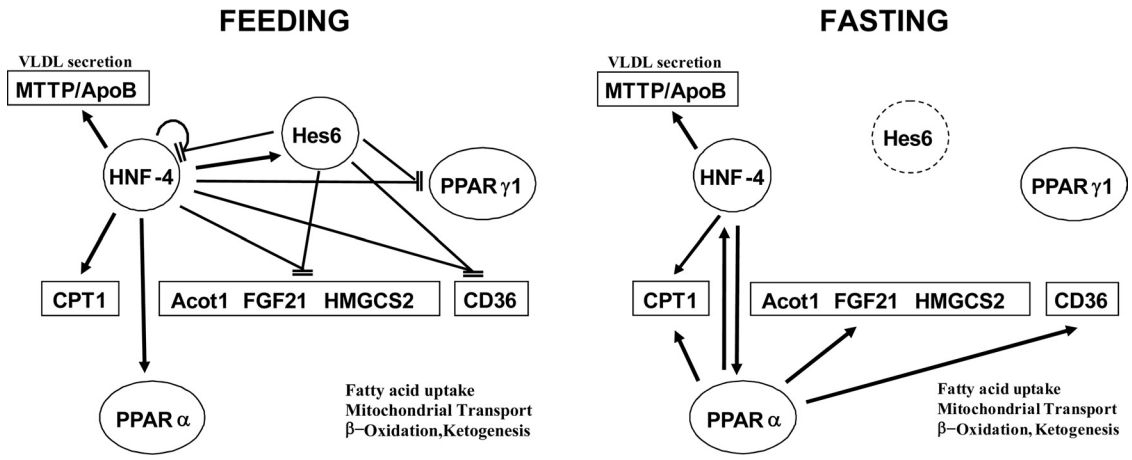

FIG. 8. Schematic presentation of cross-regulatory interactions in fed and fasted states. Transcriptional regulators and downstream genes are presented in circles and boxes, respectively. Arrows indicate positive regulations. Double bars indicate repressive effects.

HNF4α, unliganded PPARα, and liganded PPARα binding were 2.53 nM, 6.36 nM, and 3.32 nM, respectively, suggesting that ligand addition increases the affinity of PPARα to the CPT1 site, reaching a level that does not exceed the affinity of HNF4α binding (see Fig. S3 at the URL given above).

Finally, we analyzed the role of HNF4α and Hes6 in the regulation of genes involved in lipid secretion. Previous studies have demonstrated that the microsomal triglyceride transfer protein (MTTP) and apolipoprotein B (ApoB), which are essential for VLDL formation, are downregulated in Hnf4-LivKO animals (13). Our analysis confirmed this finding and also showed that these genes are occupied by HNF4α but not by Hes6 (Fig. 7C and D), indicating that *Mttp* and *ApoB* are bona fide direct HNF4α target genes.

DISCUSSION

The results of this study reveal that combinatorial regulation of the expression of the *Pparα*, *Pparγ*, *Hnf4α*, and *Hes6* genes represents an important control mechanism in fatty acid metabolism. These transcriptional regulators are acting in a coordinated fashion on their downstream target genes under different nutritional conditions. Coordination is governed in part by previously unanticipated upstream cross-regulatory pathways that play determinative roles in achieving proper expression and nuclear concentrations of transcription factors during fasting and feeding states. The network of regulatory interactions identified in this study is summarized in Fig. 8.

Hes6 and HNF4α maintain a repressed state of PPARα target genes in fed animals. Previous genome-wide studies have indicated that the expression of a considerable number of HNF4α-occupied genes is upregulated in Hnf4-LivKO mice (3). Among them is the *Acot1* gene, whose promoter is activated by overexpression of HNF4α in transfection assays, but, paradoxically, its mRNA is highly upregulated in HNF4α-liver-deficient mice (6). Taking into account that HNF4α generally acts as a transcriptional activator, potential repressive effects can be explained by indirect mechanisms. Such indirect pathways may involve a regulatory cascade where HNF4α transcriptionally activates a repressor, which, in turn, can modulate the expression of downstream genes.

In this study we identify Hes6 as a novel regulatory factor that acts as a corepressor in a subset of HNF4α-regulated genes. We identified a number of key genes in the fatty acid metabolism pathway, including *Pparγ*, *Acot1*, *Fgf21*, *Hmgcs2*, and *Cd36*, whose expression is elevated in HNF4α-deficient cells. Several lines of evidence suggest that the transcription of these genes in normally fed animals is repressed by Hes6, which is recruited to promoters through interaction with HNF4α. First, doubling the amounts of endogenous Hes6 in transgenic mice had a repressive effect on the expression of the *Pparγ*, *Acot1*, *Fgf21*, *Hmgcs2*, and *Cd36* genes. Second, in fed animals, where these genes are expressed at low levels, Hes6 together with HNF4α occupied their promoter regions. Upon fasting, when the genes are activated, Hes6 and HNF4α dissociate from the promoters. Third, experiments in Hes6Tg/Hnf4KO mice demonstrated that Hes6 recruitment to the regulatory regions was absolutely dependent on the presence of HNF4α.

Of particular interest is the finding that *Hes6* is a bona fide HNF4α-regulated gene and that, in turn, feedback regulates the expression of its own activator, HNF4α. These results unravel an intricate repression mechanism in which an activator (HNF4α) induces the expression of a repressor (Hes6) and subsequently recruits the repressor to other target genes of the activator, including its own regulatory region.

Regulation of PPARγ expression by Hes6 and Hes1. PPARγ is highly expressed in fat tissue, where it plays an essential role in the induction of adipocyte differentiation and fat storage (40). On the other hand, PPARγ is expressed at low levels in peripheral tissues including liver, which appears important for preventing hepatocytes from entering an abnormal adipogenic program (8). In line with this, results from studies of transgenic mice indicated that forced overexpression of PPARγ in liver leads to hepatic steatosis (44). Induction of PPARγ expression in adipocytes is achieved through the actions of the C/EBP family of proteins (40). Since C/EBPα is also abundantly expressed in the liver, the low hepatic levels of the PPARγ gene

point to the operation of an active repression mechanism. Our findings on Hes6-mediated repression of PPARγ in normal hepatocytes may provide a mechanistic explanation of how PPARγ mRNA and protein levels are kept low, despite the high levels of C/EBPα expression in these cells.

Hes1 and *Hes6* are members of the Hairy Enhancer of Split gene family, which has an antagonistic function in neurogenesis. Hes1 inhibits the expression of proneural genes while Hes6 relieves Hes1-mediated repression (9). In neural cells Hes6 is recruited to promoters via interaction with DNA-bound Hes1 and activates them by displacing the Groucho/TLE corepressor complex. Our results on fatty acid metabolism genes revealed a reverse function of Hes6 in the liver. It is recruited to target genes by an activator (HNF4α) and displaces the coactivator protein CBP/p300. These findings suggest that the effect of Hes6 on transcription can be either activation or repression, depending on the nature of its interacting partner.

In the liver, Hes1 was identified as a regulator of PPARγ expression (14). Hes1 directly inhibits PPARγ expression via binding to E-box motifs of the *Pparγ* promoter. Hes1 expression is induced by CREB and repressed by activated glucocorticoid receptor, which provides a molecular explanation for the opposite regulation of PPARγ transcription during cyclic AMP (cAMP) and glucocorticoid signaling (14, 26). A different type of mechanism is involved in Hes6-dependent repression. Hes6 also represses PPARγ expression, but its recruitment requires HNF4α. Due to this mode of recruitment, Hes6 action may be limited to HNF4α-occupied genes. Furthermore, Hes6 functions under conditions different from those of Hes1: unlike Hes1, the expression of Hes6 is downregulated during cAMP signaling, and it is not affected by glucocorticoids (data not shown). Hes6-mediated repression of the *Pparγ* promoter is restricted to normal fed states, where it is essential for keeping PPARγ expression at low levels.

Exchange between HNF4α/Hes6 and PPARα on promoter regions of fasting-induced genes. The HNF4/Hes6-repressed downstream target genes, *Acot1*, *Fgf21*, *Cd36*, and *Hmgcs2*, are activated during fasting. The activation mechanism involves the replacement of HNF4/Hes6 complex at the promoter regions by activated PPARα. This exchange also provides a molecular explanation for the phenotype of Hnf4-LivKO mice, which resembles that of fasting livers in several aspects: activation of several PPARα-regulated genes, accumulation of triglycerides, and increased ketogenesis.

A straightforward explanation for the molecular basis of fasting-mediated replacement of HNF4α with PPARα on target genes would be competition between the two factors for common binding sites. Such competition, however, is not driven by alterations in the nuclear concentrations of the proteins since levels of both HNF4α and PPARα increase during fasting. A more likely explanation considers potential differential affinities of the two factors to their chromatin-embedded binding sites: liganded PPARα may possess higher affinity to these regions than HNF4α, as opposed to unliganded PPARα, which may bind with a lower affinity and cannot displace HNF4α. At least *in vitro*, we could demonstrate such differences between the binding affinities of the two factors with the common binding site at the *Acot1* promoter, where exchange between HNF4α and PPARα takes place during fasting, but not with the binding site derived from *Cpt1* promoter, where fasting-mediated exchange was not observed.

Coactivator/corepressor proteins may also modulate the stability of transcription factor interaction with chromatin. For example CBP/p300-mediated acetylation of HNF4α increases its DNA binding potential (37). Since Hes6 displaces CBP/p300 from HNF4α, the HNF4α/Hes6 complex on specific chromatin regions may represent a relatively unstable protein-DNA configuration that can easily be displaced by ligand-activated PPARα.

Because HNF4α is also a positive regulator of PPARα expression (32, 34; also this study), the present findings indicate the operation of a multicomponent regulatory loop (Fig. 8). The biological importance of this loop is to provide cells a capacity for feedback control by producing bistable systems that can switch between two alternate states (e.g., under fed and fasted conditions).

Feed-forward regulatory loop between HNF4α and PPARα regulates fasting-dependent activation of the *Cpt1* gene. An additional level of complexity in the regulatory circuitry is indicated by the interplay of factors regulating fasting-dependent induction of the *Hnf4α* gene. Previous studies have shown that in hyperinsulinemic mice HNF4α expression is downregulated via the direct repressive effect of SREBP2 (42). Reduced SREBP2 protein levels during fasting could lead to further induction of the HNF4α via a derepression mechanism (42). Our results indicate that, in parallel to SREBP2, derepression from Hes6 and direct activation by PPARα also operate in the mechanism of fasting-mediated induction of HNF4α. In fed animals, Hes6 association with the HNF4α enhancer negatively modulates *Hnf4α* expression. Hes6 protein levels sharply decrease during fasting, which should result in the activation of the *Hnf4α* gene via derepression. Interestingly, however, besides dissociation of Hes6 repressor, we also observed the simultaneous association of activated PPARα with the regulatory region of the *Hnf4α* gene. Likewise, HNF4α is a positive regulator of the *Pparα* gene, and HNF4α is essential for its further induction during fasting. This positive feedback regulatory mechanism could provide for high levels of expression of the two factors during fasting when there is an increased demand for them to activate target genes.

A recent study in *Drosophila*, which lacks PPAR orthologs, demonstrated the essential role of *Drosophila* HNF4 in the regulation of *Cpt1* and other β-oxidation genes in the fat body under both fed and starvation conditions (33). Thus, it was suggested that, during the course of evolution, PPARα adopts the ancestral function of HNF4. Our results, however, demonstrate that part of HNF4 function is retained in higher organisms since *Cpt1* mRNA was reduced in Hnf4-LivKO mice, and HNF4α deficiency prevented its induction during fasting. Further activation of the *Cpt1* gene during starvation correlated with the recruitment of PPARα to its promoter. However, association of PPARα with the *Cpt1* promoter in fasted Hnf4-LivKO mice could readily be detected, without leading to the stimulation of *Cpt1* transcription. This suggests that synergism between PPARα and HNF4α is required for fasting-mediated induction of the *Cpt1* gene.

In light of these observations, we propose that in higher organisms, such as mammals, positive feedback regulation together with a synergism between HNF4α and PPARα on spe-

cific target genes creates an efficient feed-forward regulatory loop, which can generate two distinct active promoter configurations. In fed animals CPT1 transcription is driven by HNF4α while under fasting conditions it is further activated by the joint action of HNF4α and PPARα.

Deregulation of multiple pathways contributes to the fatty liver phenotype of HNF4α-deficient mice. Hnf4-LivKO mice exhibit hepatic steatosis. Alteration of hepatic fatty acid and triglyceride levels can be the result of disrupting the balances between fatty acid uptake, de novo synthesis, degradation, storage, and secretion pathways. In Hnf4-LivKO mice, the expression of ApoB and MTTP is decreased, which is expected to result in defects of VLDL-mediated secretion of triglycerides. Furthermore, activation of CD36 transporter and inhibition of CPT1 should lead to enhanced uptake and reduced degradation via β-oxidation of fatty acids, respectively. The expression of ACOT1, which converts long chain acyl-CoAs to free fatty acids, is also highly induced in Hnf4-LivKO mice. This should lead to the shuttling of fatty acids away from esterification in the cytosol, preventing their transport to the mitochondria for β-oxidation. As a result of the above, HNF4-deficient hepatocytes should exhibit increased import and reduced degradation of fatty acids as well as reduced export of triglycerides. The combined effects of the above could explain the massive deposition of lipids in Hnf4-LivKO mice.

ACKNOWLEDGMENTS

We thank P. Karagianni for helpful comments on the manuscript.

This work was supported by grants from the EU (MTKD-CT2005 029610, LSHM-CT2006 037498, and PITN-GA2009 238821).

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