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Coactivation of Foxa2 through Pgc-1β promotes liver fatty acid oxidation and triglyceride/VLDL secretion

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Summary

Forkhead transcription factor Foxa2 activates genes involved in hepatic lipid metabolism and is regulated by insulin. Activation of Foxa2 in the liver leads to increased oxidation and secretion of fatty acids in the form of triacylglycerols (TAGs), a process impaired in type 2 diabetes. Here, we demonstrate that Foxa2 is coactivated by PPARγ coactivator β (Pgc-1β). Adenoviral expression of Foxa2 and Pgc-1β in livers of *ob/ob* mice results in decreased hepatic TAG content and increased plasma TAG concentrations. In addition, the concerted action of Foxa2/Pgc-1β activates genes in mitochondrial β oxidation and enhances fatty acid metabolism. Furthermore, Foxa2/Pgc-1β induce the expression of microsomal transfer protein, thereby increasing apoB-containing VLDL secretion. This process is inhibited by insulin through a Foxa2-dependent mechanism. These data demonstrate that Foxa2/Pgc-1β regulate hepatic lipid homeostasis by affecting the clearance rate of fatty acids through oxidation and/or secretion of lipids in response to insulin.

Introduction

Triacylglycerols (TAGs) are the most concentrated biological fuels available, making them an ideal form of energy storage. However, because of their extreme hydrophobicity, regulated transport into the polar medium of circulating blood is only possible in the form of TAG-rich lipoproteins. Two types of these particles are known, namely chylomicrons that are secreted by enterocytes of the gut in response to dietary TAG, and very-low-density lipoprotein (VLDL) secreted by hepatocytes of the liver in response to de novo synthesized TAGs (Shelness and Sellers, 2001). Most of the TAGs transported in the plasma during the postabsorptive phase are associated with chylomicrons. Hypertriglyceridemia, which is characterized by an increase of plasma TAG concentration, has been shown to be a component of the metabolic syndrome and is an independent risk factor for coronary artery disease (Rubins, 2000; Ginsberg, 2002). Furthermore, increasing evidence suggests that postprandial hyperlipidemia also contributes to the development of atherosclerosis (Heine and Dekker, 2002).

The assembly and secretion of VLDL is a complex process that brings together the very large and highly amphipathic apoB polypeptide and different classes of lipids in a fixed sequence. In humans, the liver produces VLDL particles that are composed of full-length apoB-100, while the small intestine produces chylomicrons containing apoB-48 (amino terminal 48% of apoB-100; Shelness and Sellers, 2001; Gibbons et al., 2004). The secretion and assembly of VLDL-associated triglycerides is regulated at various levels. The key enzyme controlling VLDL synthesis is the microsomal triacylglycerol transfer protein (MTP). Genetic ablation of MTP prevents the secretion of VLDL into the plasma compartment by inhibiting the generation of apoB-containing VLDL precursors (Raabe et al., 1998). Furthermore, overexpression of MTP results in increased secretion of VLDL triglycerides and apoB (Horton, 2002). In addition to regulation by MTP, VLDL synthesis and secretion is regulated by substrate availability, which in turn is regulated by key lipogenic enzymes, i.e., fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC). Activities of these enzymes in turn are controlled by the sterol regulatory element binding protein-1c (SREBP-1c; Horton, 2002).

Insulin has been shown to inhibit VLDL output from the liver of rats and humans, and exogenous insulin administration also suppresses the secretion of VLDL triacylglycerol and apoB in humans (Durrington, et al., 1982; Patsch et al., 1983; Patsch et al., 1986). This inhibition of VLDL assembly/secretion contrasts the regulation of VLDL synthesis by SREBP-1c, as insulin is a known activator of SREBP-1c. Although the mechanism is not completely understood, it is believed that insulin interferes with the maturation phase of VLDL assembly but does not inhibit the overall lipolytic mobilization of hepatic cytosolic TAG. Instead, insulin signaling mediates the return of TAGs to the cytosolic pool, an effect similar to that of MTP inhibition, which is also required for the efficient production of the TAG-rich VLDL precursors but has no effect on TAG synthesis.

On a cellular level, the metabolic responses to changes in plasma insulin levels are mediated by the insulin/PI3-kinase/Akt signaling pathway, which regulates the activity of several forkhead transcription factors. We have recently shown that the forkhead transcription factor A2 (Foxa2) is phosphorylated in response to insulin signaling, resulting in inhibition of its transcriptional activity by nuclear exclusion (Wolfrum et al., 2003; Wolfrum et al., 2004). The forkhead box A family of transcription factor in mammals include three genes designated as Foxa1, Foxa2, and Foxa3 (Kaestner et al., 1994). All three members share a high homology and a conserved winged-helix DNA target site (Brennan, 1993); however, their biological functions are diverse. Foxa2 plays a central role in maintaining lipid and glucose homeostasis by regulating gene expression of rate-limiting enzymes in response to insulin inactivation (Wolfrum et al., 2004). Induction of Foxa2 activity at low-insulin concentrations leads to an increase in mitochondrial and peroxisomal

β oxidation, ketone body production, and plasma TAG concentration. These observed changes in hepatic lipid metabolism are mirrored by the effect of the coactivating factor peroxisome proliferator γ coactivator β (PGC-1β) on hepatic lipid metabolism (Lin et al., 2005).

PGC-1β is a recently identified transcriptional coactivator with high sequence similarity to PGC-1α. Both factors show similar tissue distribution; however, their capacity to regulate gene expression varies (Lin et al., 2002). While PGC-1α activates genes encoding enzymes of gluconeogenesis and mitochondrial biogenesis/fatty acid oxidation, PGC-1β solely activates the latter pathways (Lin et al., 2002). Thus, it was recently shown that overexpression of PGC-1β leads to an increase in mitochondrial β oxidation as well as to an increase in plasma TAG concentration (Lin et al., 2005). In this study, we demonstrate that Pgc-1β is a coactivator of Foxa2 and that the concerted action of these factors regulate fatty acid β oxidation and TAG-associated VLDL secretion from the liver.

Results

Coactivation of Foxa2 by Pgc-1β through Foxa2 domain aa 266–340

In spite of Foxa1 and 2's nearly identical DNA winged-helix domain and their ability to bind to the same Foxa binding sites in vitro, Foxa1 and Foxa2 have been shown to transactivate different target genes and regulate diverse biological pathways. In an attempt to identify the sequences that are responsible for conferring transcriptional activity and specificity, we generated hybrids of both factors (Figure 1A) and analyzed their ability to activate transcription by reporter gene assays. The Foxa hybrid constructs were cotransfected into HepG2 cells along with reporter constructs containing 5′ regulatory sequences of CptI and Mcad. Both promoters harbor conserved Foxa binding sites and are activated by Foxa2 (Wolfrum et al., 2004). To ensure equal transfection efficiencies of the hybrid vectors, all constructs were HA tagged, and the relative expression of Foxa1, 2, and hybrid proteins were assayed by Western blotting (data not shown). Figures 1B and 1C show that, when expressed at similar levels, Foxa2 is a stronger transcriptional activator than Foxa1, an observation that we reported previously (Duncan et al., 1998). The differences in the ability of the Foxa-hybrids to activate both CptI and Mcad promoters allowed us to identify a 74 amino acid residue region (266–340), hereafter referred to as domain A, that is responsible for the higher transactivation potential of Foxa2 (Figures 1A–1C). The exchange of this domain in Foxa1 with the corresponding coding sequence from Foxa2 (Hyb.) restored the transcriptional activity of Foxa1 to levels observed for Foxa2 (Figures 1B and 1C).

We speculated that the observed differences in transactivation capacity might be due to the recruitment of transcriptional coactivators. Candidate factors that may interact with Foxa2 included members of the PGC family because (1) they have previously been shown to regulate genes that are known targets of Foxa2, and (2) Pgc-1α interaction has been reported with Foxo1, a different member of the forkhead box transcription factor family. Interaction of Pgc-1α and β isoforms with either Foxa1 or Foxa2 were first assessed in a mammalian two-hybrid system in which Pgc-1α or Pgc-1β (baits) were fused with the Gal4 DNA binding domain, and Foxa1, 2, or domain A (target proteins) were expressed as a fusion protein with the VP16 transactivation

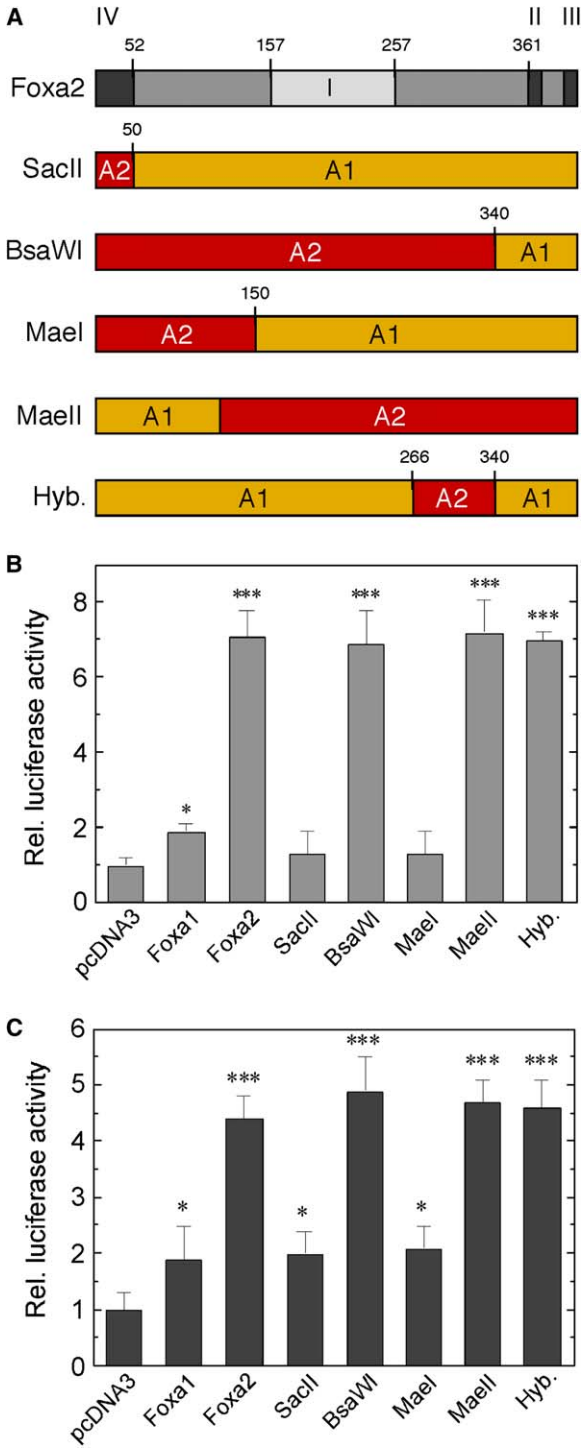


Figure 1. Generation of Foxa1/Foxa2 hybrids

A) Schematic representation of the Foxa1/Foxa2 hybrids. Sequence from Foxa2 is denoted in red, while sequence from Foxa1 is shown in yellow.

B) HepG2 cells were transfected with Foxa1, Foxa2, or Hybrid expression vectors, pCMV-β-Gal, and the luciferase reporter genes harboring 967 bp of the Mcad promoter. Luciferase activity was normalized to β-Gal activity. Each value represents mean of six independent experiments ±SD.

C) HepG2 cells were transfected with Foxa1, Foxa2, or Hybrid expression vectors, pCMV-β-Gal, and the luciferase reporter genes harboring 831 bp of the CptI promoter. Luciferase activity was normalized to β-Gal activity. Each value represents mean of six independent experiments ±SD. *$p \leq 0.05$; ***$p \leq 0.001$.

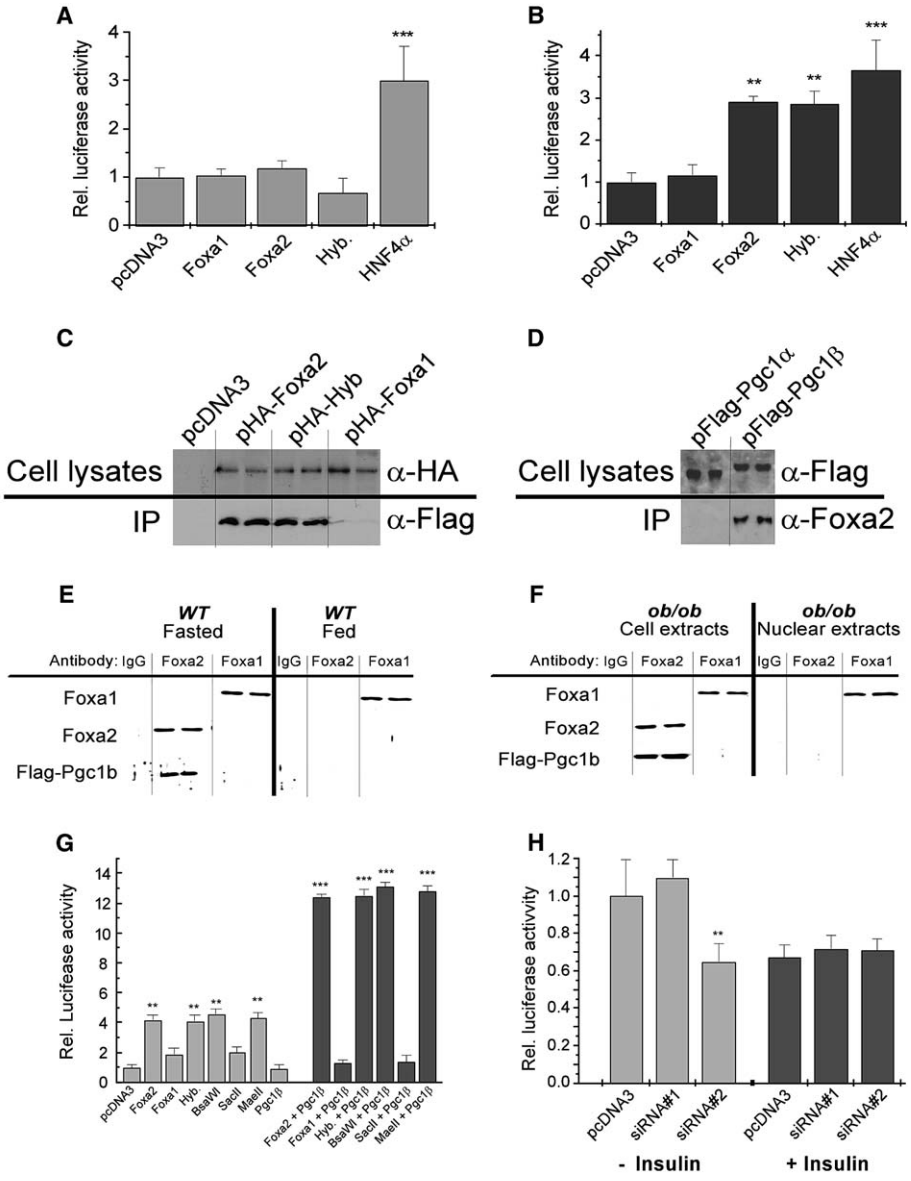


Figure 2. Foxa2 but not Foxa1 is an interaction partner of Pgc-1β

A) HepG2 cells were transfected with Foxa1, Foxa2, or Hybrid-Gal4 DNA binding domain expression vectors, an expression vector for a fusion protein of VP16AD and Pgc-1α, pCMV-β-Gal, and a Gal4-luciferase reporter gene. Luciferase activity was normalized to β-Gal activity. Each value represents mean of 6 independent experiments ±SD.

B) HepG2 cells were transfected with Foxa1, Foxa2, or Hybrid-Gal4 DNA binding domain expression vectors, an expression vector for a fusion protein of VP16AD and Pgc-1β, pCMV-β-Gal, and a Gal4-luciferase reporter gene. Luciferase activity was normalized to β-Gal activity. Each value represents mean of six independent experiments ±SD.

C) HEK293 cells were transfected with HA-Foxa1, HA-Foxa2, or HA-Hybrid expression vectors together with an expression vector for FLAG-Pgc-1β. Proteins from whole-cell lysates were precipitated with α-HA antibodies. Precipitates were immunoblotted and stained using α-HA or α-FLAG antibodies.

D) HepG2 cells were transfected with expression vectors for either FLAG-Pgc-1α or FLAG-Pgc-1β. Proteins from whole-cell lysates were precipitated with α-FLAG antibody. Precipitates were immunoblotted using α-FLAG or α-Foxa2 antibodies.

E) Foxa1, Foxa2, and FLAG-Pgc-1β were immunoprecipitated from nuclear extracts of livers from fasted and fed wt animals, which had been infected with Ad-Pgc-1β. Precipitates were immunoblotted using α-FLAG or α-Foxa2 and α-Foxa1 antibodies to detect interaction between Foxa1, Foxa2, and Pgc-1β.

F) Foxa1, Foxa2, and Flag-Pgc-1β were immunoprecipitated from whole-cell extracts and nuclear extracts of *ob/ob* mice, which had been infected with Ad-Pgc-1β. Precipitates were immunoblotted using α-FLAG or α-Foxa2 and α-Foxa1 antibodies to detect interactions between Foxa1, Foxa2, and Pgc-1β.

G) HEK293 cells were transfected with expression vectors for Foxa1, Foxa2, or Hybrids, alone or in combination with an expression vector of Pgc-1β, and pCMV-β-Gal and a Mcad-luciferase reporter gene. Each value represents the mean of six independent experiments ±SD.

H) HepG2 cells were infected with siRNAs targeting Pgc-1β in the presence and absence of insulin, together with pCMV-β-Gal and a MCAD-luciferase reporter plasmid. Luciferase activity was normalized to β-Gal activity. Each value represents mean of six independent experiments ±SD. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

domain. If the bait and target proteins interact, the activation domain is brought in close contact with the DNA binding domain, resulting in transcriptional activation of the luciferase reporter gene. While Pgc-1α did not interact with Foxa1, Foxa2, or domain A (Hnf4α served as positive control; Figure 2A), we found a significant increase in luciferase activity when assaying the interaction of Pgc-1β and Foxa2 (Figure 2B). This interaction was also observed with domain A alone, while Foxa1 did not show any interaction with Pgc-1β (Figure 2B). The interaction between Foxa2/domain A with Pgc-1β was similar to that observed for interaction between Pgc-1β and Hnf4α (Figure 2B). To confirm interaction of Foxa2 and Pgc-1β, we coimmunoprecipitated proteins from lysates of HEK293 cells that had been transfected with either pcDNA3, HA-Foxa1, HA-Foxa2, or HA-Hybrid-Foxa together with FLAG-Pgc-1β using an α-HA-antibody. Coprecipitated FLAG-Pgc-1β was detected with α-Flag-antibody (Figure 2C). Similar to the mammalian two-hybrid experiments, we found that Foxa2 and the Foxa-hybrid, but not Foxa1, interacted with Pgc-1β. Further conformation was obtained by overexpressing FLAG-Pgc-1α or FLAG-Pgc-1β in HepG2 cells. Both proteins were precipitated using α-FLAG antibodies, and interaction of endogenous Foxa2 was analyzed by immunostaining the coprecipitates with α-Foxa2 antibody. We observed an interaction of Foxa2 and Pgc-1β but not with Pgc-1α (Figure 2D). To measure interaction of Foxa2 and Pgc-1β in vivo, we precipitated endogenous Foxa1 and Foxa2 from (1) whole-cell extracts of *ob/ob* mice livers, (2) nuclear extracts of *ob/ob* mice livers, (3) nuclear extracts of fasted wt mice, and (4) nuclear extracts of wt fed mice, which were infected with Ad-Pgc-1β. We observed an interaction of Foxa2 with Pgc-1β in *ob/ob* whole-cell extracts

and in fasted wt mice nuclear extracts (Figures 2E and 2F). No interaction between Foxa1 and Pgc-1β was observed in any animal models studied (Figures 2E and 2F).

Since we could show that Pgc-1β could interact with Foxa2 and Hybrid-Foxa, we analyzed whether Pgc-1β could augment the transcriptional activation of a Foxa2 reporter gene. We transfected HEK293 cells (which do not express Foxa genes or Pgc-1β) with Foxa1, Foxa2, and the different hybrids, together or without Pgc-1β, and measured luciferase activity of different reporter genes (Mcad and CptI promoters). We observed a significant 120% increase in transcriptional activity when transfecting expression vectors for either Foxa2 or domain A containing Hybrid-Foxa2 (Hyb.) together with Pgc-1β and either Mcad or CptI reporter gene vector (Figure 2G and data not shown). No increase was observed when Foxa1 was coexpressed with Pgc-1β, and neither did Pgc-1β augment reporter gene activity when a Foxa1/2 hybrid expression vector was used that lacked domain A (Figure 2G). In contrast Pgc-1α was not able to increase the transactivation potential of either Foxa2 or a domain A containing Foxa2 hybrid (data not shown). To demonstrate that Foxa2/Pgc-1β transactivation is regulated by insulin signaling, we transfected HepG2 cells with different siRNAs targeted against Pgc-1β together with the Mcad reporter gene and analyzed the differences in transactivation in the presence or absence of insulin (Figure 2H). Transfection of siRNA#2, which reduces Pgc-1β expression ≈60%, decreased transactivation of the Mcad promoter by approximately 40%, similar to the effect observed by insulin. In contrast, an ineffective siRNA (siRNA#1) had no effect on luciferase activity. No further decrease of transactivation could be observed in siRNA-treated cells that were incubated in the presence of insulin.

Regulation of β oxidation by Foxa2/Pgc-1β in vivo

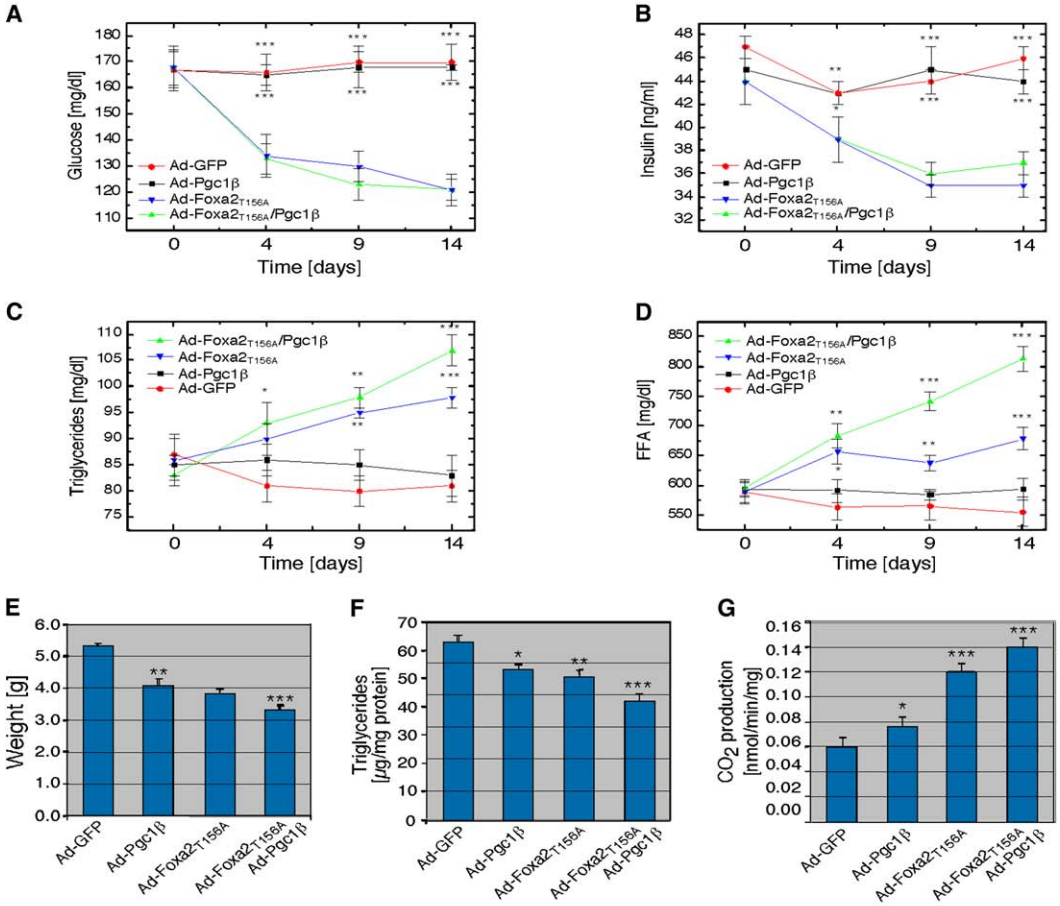
To address whether coactivation of Foxa2 by Pgc-1β has physiological relevance, we studied the metabolic effect of Foxa2 and Pgc-1β on hepatic lipid metabolism in *ob/ob* mice and fed wt littermates. We used these mice as a Foxa2 loss-of-function model since we have shown previously that Foxa2 is permanently inactivated in the liver of these animals by nuclear exclusion (Wolfrum et al., 2004). Mice were injected with an adenovirus expressing $Foxa2_{T156A}$ (Ad-$Foxa2_{T156A}$), which is resistant to Akt-mediated phosphorylation and therefore not inactivated through high levels of insulin (Wolfrum et al., 2004) and/or an adenovirus expressing Pgc-1β (Ad-Pgc-1β). We have shown previously that infection of mice with constitutive active $Foxa2_{T156A}$ at a titer of 10^9 pfu/mouse results in levels of nuclear Foxa2 that are similar to overnight-fasted mice (Wolfrum et al., 2004; see Figure S1 in the Supplemental Data available with this article online). A recombinant adenovirus expressing only EGFP (Ad-GFP) was used as a control. Blood was taken from the animals at day 0, 4, 9, and 14, and the animals were sacrificed on day 14. Infection of mice with $Foxa2_{T156A}$ led to a time-dependent decrease in plasma glucose (Figure 3A and data not shown), an effect that was not observed in mice infected with Ad-Pgc-1β alone. Coexpression of Pgc-1β and Ad-$Foxa2_{T156A}$ also did not lead to a further decrease of plasma glucose in these animals. Similar results were obtained for plasma insulin levels, which did not change in response to Ad-Pgc-1β expression alone and were only marginally reduced in Ad-$Foxa2_{T156A}$/Ad-Pgc-1β coinjected mice compared to Ad-$Foxa2_{T156A}$-treated animals (Figure 3B and data not shown). In contrast, we measured a modest increase in plasma TAG in mice that were infected with Pgc-1β alone (Figure 3C and data not shown). Overexpression of $Foxa2_{T156A}$ in livers of *ob/ob* mice led to a significantly higher increase in plasma TAG. Interestingly, even though there was only a modest effect of Pgc-1β on plasma TAG, coexpression of $Foxa2_{T156A}$ and Pgc-1β led to significant increase in plasma TAG concentration in comparison to plasma TAG levels in animals infected with Ad-$Foxa2_{T156A}$ alone (Figure 3C and data not shown). The effect of adenoviral infection was mirrored by plasma free fatty acids (FFA), which were increased in response to overexpression of $Foxa2_{T156A}$ alone as well as in combination with Pgc-1β (Figure 3D and data not shown). Liver weight and hepatic triglyceride content were decreased in mice that were injected with either Ad-$Foxa2_{T156A}$ or Ad-Pgc-1β. Hepatic coexpression of both factors led to a more pronounced decrease than that observed for the independent factors (Figures 3E and 3F and data not shown). No effect of adenoviral Foxa2 or Pgc-1β expression was observed on muscle triglyceride levels (data not shown). Mitochondrial β oxidation was increased slightly (≈25%) in Ad-Pgc-1β-infected mice and doubled in response to Ad- $Foxa2_{T156A}$ infection. Coexpression of both $Foxa2_{T156A}$ and Pgc-1β led to a further increase in mitochondrial β oxidation (Figure 3G and data not shown).

We next determined the mechanism underlying the increase in β oxidation in livers of these mice. Gene expression of several known Foxa2 and Pgc-1β target genes was analyzed by RT-PCR. Key enzymes of mitochondrial β oxidation, namely CptI, Mcad, and Vlcad were upregulated in animals that were infected with either Ad-$Foxa2_{T156A}$ or Ad-Pgc-1β, alone or in combination (Figures 3H and 3I). While Pgc-1β overexpression alone upregulated the expression of Mcad and Vlcad approximately 2-fold, $Foxa2_{T156A}$ increased the expression 3- to 4-fold. The highest expression levels were observed in animals that were infected with both constitutive active Foxa2 and Pgc-1β (5- to 6-fold), demonstrating that Foxa2 and Pgc-1β have a cooperative effect on the expression of target genes in lipid metabolism.

Foxa2/Pgc-1β regulate VLDL secretion

To elucidate the mechanism underlying the increase in liver triglyceride secretion, we first determined whether triglyceride synthesis was increased in these mice. The effect of Foxa2 and Pgc-1β expression led to a small but significant increase in triglyceride synthesis (Figure 4A and data not shown). Since this increase was too small to explain the increased triglyceride secretion, we next analyzed the localization of the secreted triglycerides in circulating plasma lipoproteins. VLDL particles constitute the major transport system of plasma TAGs; however, small amounts of TAGs are also found in LDL and HDL particles. To assess whether the increase in plasma concentration is due to increased VLDL levels, we separated plasma lipoproteins of the infected *ob/ob* mice by FPLC and analyzed the fractions for triglyceride content. Infection of the animals with Pgc-1β alone did not significantly change plasma VLDL levels compared to Ad-GFP-infected mice (Figure 4B), while infection of *ob/ob* animals with Ad-$Foxa2_{T156A}$ alone or in combination with Ad-Pgc-1β led to an increase in plasma triglyceride and VLDL concentrations (Figure 4B).

Several pathways control VLDL synthesis and secretion in the liver. To assess which enzymes might be involved in the upregulation of VLDL secretion in Ad-$Foxa2_{T156A}$/Pgc-1β-infected mice, we quantified mRNA expression of genes that are known



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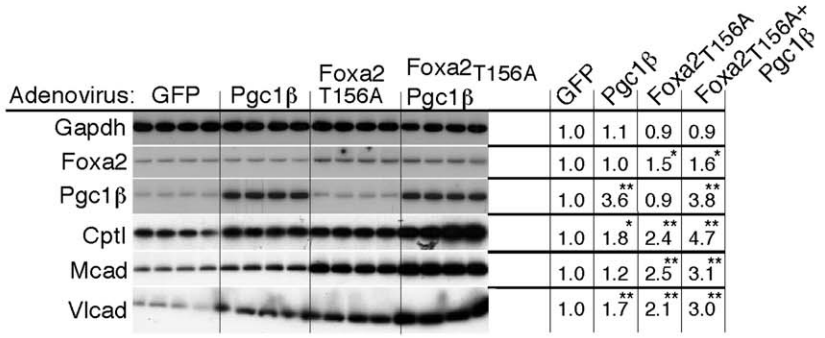

	GFP	Pgc1β	Foxa2T156A	Foxa2T156A+ Pgc1β
Gapdh	1.0	1.1	0.9	0.9
Foxa2	1.0	1.0	1.5*	1.6*
Pgc1β	1.0	3.6**	0.9	3.8**
Cptl	1.0	1.8*	2.4**	4.7**
Mcad	1.0	1.2	2.5**	3.1**
Vlcad	1.0	1.7**	2.1**	3.0**

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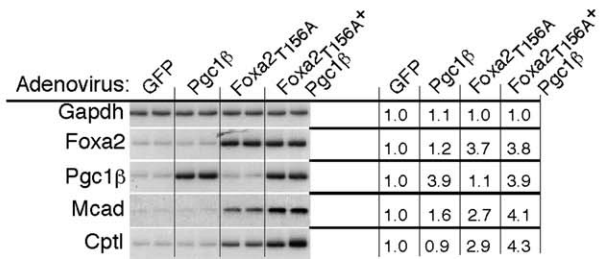

	GFP	Pgc1β	Foxa2T156A	Foxa2T156A+ Pgc1β
Gapdh	1.0	1.1	1.0	1.0
Foxa2	1.0	1.2	3.7	3.8
Pgc1β	1.0	3.9	1.1	3.9
Mcad	1.0	1.6	2.7	4.1
Cptl	1.0	0.9	2.9	4.3

Figure 3. Adenoviral expression of Foxa2$_{T156}$ and Pgc-1β in *ob/ob* mice alters lipid and glucose metabolism

Twelve-week-old *ob/ob* mice were injected with Ad-GFP, Ad-Foxa2$_{T156A}$, and/or Ad-Pgc-1β via the tail vein. Mice were sacrificed at day 14. All measurements were performed after a moderate 6 hr fasting period. We quantified (**A**) plasma glucose concentrations, (**B**) plasma insulin levels, (**C**) plasma triglyceride levels, (**D**) plasma FFA concentrations, (**E**) liver weight, (**F**) liver triglyceride content in μg/mg of liver protein, and (**G**) Mitochondrial β oxidation.

H) Foxa2 target-gene expression of genes involved in β oxidation was measured by RT-PCR; Gapdh served as control. n = 4 in each group.

I) Expression of genes involved in β oxidation in wt mice that were infected with Ad-GFP, Ad-Foxa2$_{T156}$, and/or Ad-Pgc-1β via the tail vein was measured by RT-PCR. Gapdh served as control. n = 4 in each group. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

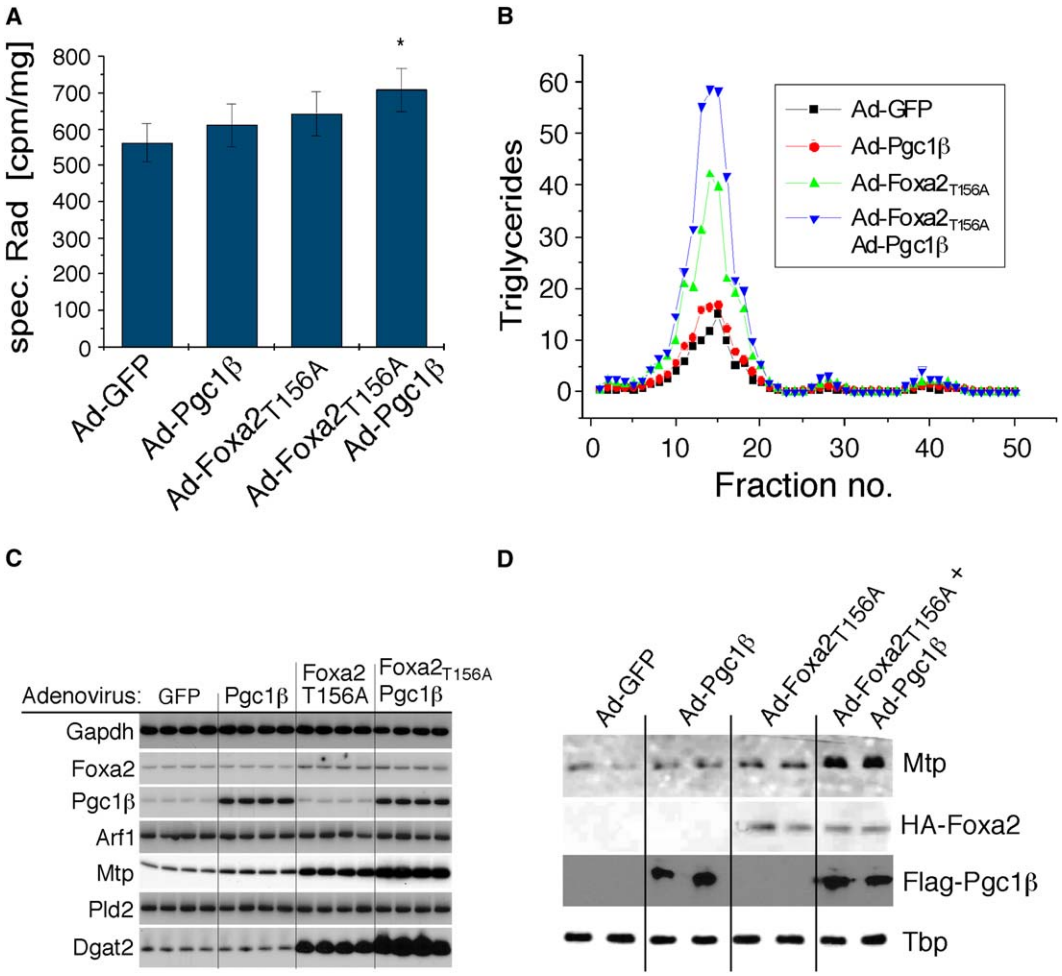

Figure 4. Foxa2 and Pgc-1β concertedly induce hepatic VLDL secretion in *ob/ob* mice

Twelve-week-old *ob/ob* mice were injected with Ad-GFP, Ad-Foxa2$_{T156A}$, and Ad-Pgc-1β via the tail vein. All measurements were performed after a moderate 6 hr fasting period.

A) Triglyceride synthesis was quantified by measuring ^{14}C-palmitic acid incorporation into triglycerides in whole-cell liver extracts.

B) Plasma of virus-treated animals was analyzed 14 days after tail-vein injection. Plasma samples (100 μl) were applied to a FPLC gel filtration column. The fractions of the eluate were assayed for total triglycerides.

C) Foxa2 target-gene expression of genes involved in hepatic VLDL secretion was measured by RT-PCR; Gapdh served as control.

D) Expression levels of HA-Foxa2 and FLAG-Pgc-1β in whole-cell extracts from liver of infected *ob/ob* mice were quantified by immunoblot analysis using α-HA and α-FLAG antibodies, TATA binding protein (Tbp) served as a loading control. n = 4 in each group. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

to affect VLDL secretion, including ADP ribosylation factor 1 (Arf1), phospholipase D2 (Pld2), diacylylglycerolacyl transferase 2 (Dgat2), and microsomal transfer protein (Mtp; Wiggins and Gibbons, 1996; Jamil et al., 1998; Stone et al., 2004; Asp et al., 2005). We found that the mRNA levels of both Mtp and Dgat2 were increased in response to Ad-Foxa2$_{T156A}$ infection alone or in combination with Ad-Pgc-1β, while Ad-Pgc-1β alone did not have any effect (Figure 4C). Mtp protein expression in adenovirus-treated *ob/ob* mice was upregulated only marginally in response to Pgc-1β overexpression, while Ad-Foxa2$_{T156A}$ infection led to a 2.6-fold increase (Figure 4D). Mice that were infected with both Ad-Pgc-1β and Ad-Foxa2$_{T156A}$ exhibited a 6.2-fold increase in Mtp, demonstrating that Pgc-1β and Foxa2 work in concert to regulate the expression of Mtp. Comparison of Mtp and Dgat2 expression between fasted and fed wt or *ob/ob* mice showed that both genes were decreased in the fed state and in *ob/ob* animals compared to fasted wt littermates (Figure S2).

Analysis of the upstream regulatory sequences of the MTP gene led to the identification of a conserved Foxa site at position −473 (relative to the transcription start site). To show that MTP is a direct target of Foxa2, we performed chromatin immunoprecipitation experiments (ChIP) and transactivation assays (Figure 5A). For the ChIP assay, primary hepatocytes were used, which had been infected with an adenovirus expressing Pgc-1β (Ad-Pgc-1β) in the presence or absence of insulin. Figure 5A shows that in the absence of insulin Foxa2 binds to the conserved Foxa site in the Mtp promoter, while neither Foxa1 nor Foxo1 could be shown to bind to this promoter. Pgc-1β bound to the same conserved site that Foxa2 occupies (Figure 5A). Interestingly, in the presence of insulin neither Foxa2 nor Pgc-1β were found to bind to the Foxa2 consensus site in the Mtp promoter. As a control, we analyzed binding of the same transcription factors and cofactors to conserved Foxa2 binding sites in the Cptl and the Mcad gene. Both ChIP assays produced similar

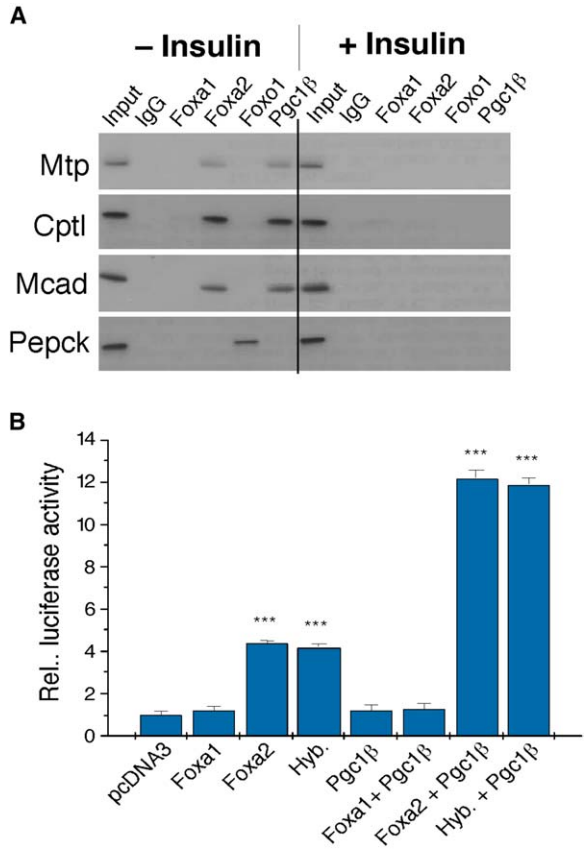

Figure 5. A functional and conserved Foxa2 binding site activates the Mtp promoter

A) Primary hepatocytes were infected with Ad-Pgc-1β and treated for 2 hr with insulin. The association of Foxa1, Foxa2, Foxo1, and Pgc-1β to the Mtp promoter was measured by ChIP assay. Chromatin/protein complexes of each group were precipitated with the pertinent antibody, following PCR amplification with primer pairs specific for the corresponding regions in the Mtp promoter. Chip assays of the Cptl, Mcad, and Pepck promoter served as control.

B) HEK293 cells were transfected with expression vectors for Foxa1, Foxa2, or Hybrids, alone or in combination with an expression vector of Pgc-1β, and pCMV-β-Gal and a Mtp promoter-luciferase reporter gene. Luciferase activity was normalized to β-Gal activity. Each value represents mean of six independent experiments ±SD. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

results as those observed with the Mtp promoter. We did not observe any binding of endogenous Foxa2 or Foxa1 to the Pepck promoter, while Foxo1 strongly bound this promoter region (Figure 5A). To quantify binding affinities, we studied binding of Foxa1 and Foxa2 to the respective binding sites in the Cptl, Mcad, and Mtp genes by electrophoretic mobility shift assay (EMSA) analysis. We found that in vitro Foxa2 bound to these sites with high affinity, whereas Foxa1 DNA binding activity could only be observed for the Mcad promoter (with ≈5-fold less affinity than Foxa2; data not shown). Together, these results demonstrate that the Foxa2 binding site in the Mtp promoter is specific and functional in vivo.

We also quantified the transactivation potential of Foxa2 by cloning a 1 kb promoter sequence of Mtp into a luciferase reporter gene vector and by determining the ability of Foxa2 and/or Pgc-1β to activate this promoter. Transactivation assays in HEK293 cells demonstrated that Foxa2 increased luciferase activity approximately 4-fold, while Foxa1 had no significant effect on Mtp promoter activity (Figure 5B). Coexpression of Pgc-1β and Foxa2 led to an additional 120% increase in transactivation (Figure 5B). To quantify the relative contribution of the conserved Foxa2 binding site in the Mtp promoter in comparison to the DR-1 element, which has been shown to mediate PPARα- and HNF4α-dependent activation of the Mtp gene, we generated a reporter gene construct with a mutated DR-1 motif (Ameen et al., 2005). Mutation of the DR-1 element (DR-1mut) completely abolished transactivation by PPARα and/or HNF4α (Figure 6A). The observed increase in transactivation of the Mtp promoter was similar for Foxa2 and PPARα, while HNF4α had a much lower transactivation activity (Figure 6A). To further study the effect of these transcription factors, we transfected the wild-type and mutated Mtp reporter constructs into HEK293 cells and analyzed the transactivations by PPARα, HNF4α, or Foxa2. Again, the transactivation of the Mtp promoter was similar for Foxa2 and PPARα (Figure 6B) with a significantly lower activity of HNF4α, suggesting that both sites regulate expression of the Mtp promoter with equal potency. To directly quantify the effect of Foxa2 on triglyceride secretion from hepatocytes, we infected mouse primary hepatocytes with either Ad-Foxa2 or Ad-Pgc-1β alone or in combination and measured triglyceride secretion into the medium in a pulse/chase experiment (Figure 7A). Cells were "pulsed" for 2 hr with ^{14}C-palmitic acid, and triglyceride secretion was "chased" for 4 hr after labeling of the cells. We observed a 4-fold induction in triglyceride secretion in cells that were transfected with Ad-Foxa2 and a 5.5-fold induction when cells were infected with Ad-Foxa2 and Pgc-1β in combination. To assess how much of this triglyceride secretion is linked to an increased VLDL production as evidenced by apoB100 secretion, we quantified the effect of Foxa2 on apoB secretion from mouse primary hepatocytes that were infected with either Ad-Foxa2 or Ad-Pgc-1β alone or in combination. Cells were "pulsed" for 2 hr with a ^{35}S-labeled methionine/cystein mixture, and apoB secretion was "chased" for 4 hr after labeling of the cells. Secreted apoB was immunoprecipitated and quantified by SDS PAGE/autoradiography. Figure 7B shows that Foxa2 overexpression led to a 3.5-fold increase in apoB secretion, while coexpression of Pgc-1β increased the secretion 5.5-fold. To analyze whether the effect on apoB secretion was insulin-dependent, we infected primary hepatocytes with either Ad-Foxa2 or Ad-Foxa2$_{T156A}$ in combination with Ad-Pgc-1β and treated the cells with insulin 2 hr prior to the experiment. Treatment of Ad-Foxa2-infected cells with insulin during the study led to a decrease in Foxa2-stimulated apoB secretion. In contrast, insulin had no effect on apoB secretion from cells that were infected with Ad-Foxa2$_{T156A}$ (Figure 7C). We also investigated whether silencing of Pgc-1β by RNAi leads to a decrease in VLDL secretion. Pulse/chase experiments (as described above) and apoB secretion studies were carried out in HepG2 cells following transfection with siRNAs against the endogenous Pgc-1β. Figure 7C shows that a ≈50% reduction of Pgc-1β mRNA by siRNA#2 led to a 2- to 3-fold decrease in apoB secretion compared to mock-transfected cells using an ineffective siRNA (siRNA#1). These results demonstrate that Foxa2 and Pgc-1β mediate the insulin-regulated inhibition of VLDL secretion in hepatocytes.

Discussion

In this study, we have identified a region in Foxa2, located between the winged helix and the C-terminal transactivation

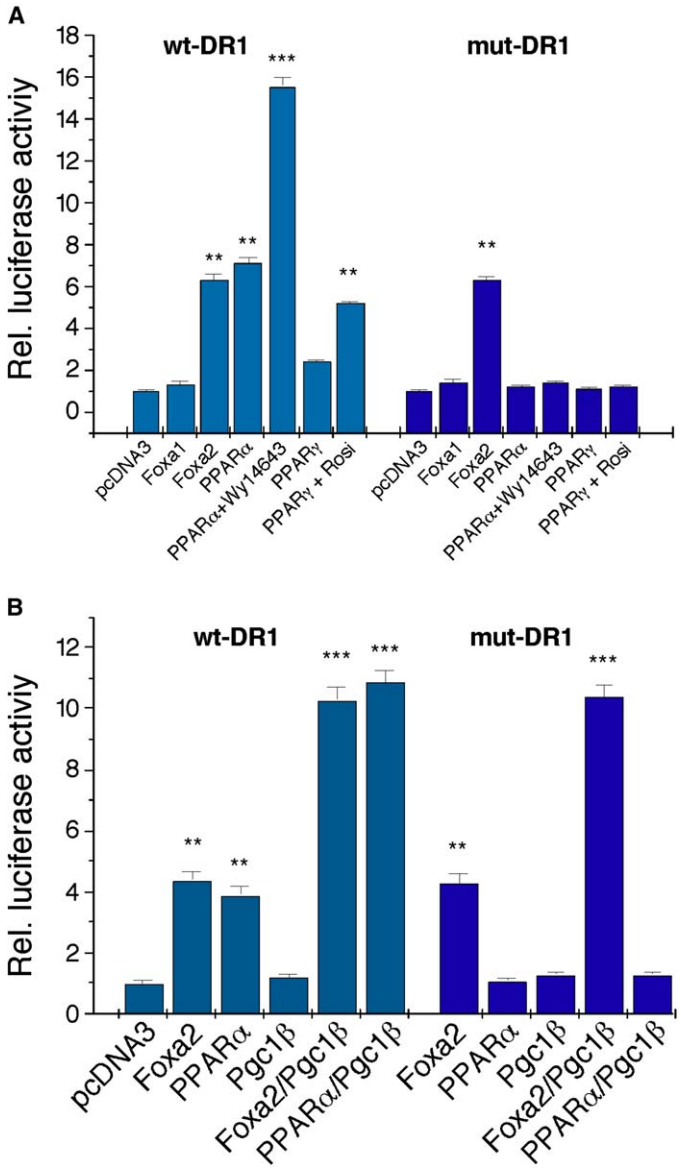

Figure 6. PPARα and Foxa2 are transcriptional activators of the Mtp promoter

A) HepG2 cells were transfected with expression vectors for Foxa1, Foxa2, PPARα, or PPARγ, alone or in combination with an expression vector for Pgc-1β, and pCMV-β-Gal, and a Mtp-luciferase reporter gene with or without a mutation in the DR-1 element (Ameen et al., 2005). PPARα and PPARγ activity was stimulated by addition of Wy14,643 and rosiglitazone, respectively. Luciferase activity was normalized to β-Gal activity.

B) HEK293 cells were transfected with expression vectors for Foxa1, Foxa2, or PPARα, alone or in combination with an expression vector for Pgc-1β, and pCMV-β-Gal and a Mtp-luciferase reporter gene with or without a mutation in the DR-1 element (Ameen et al., 2005). Luciferase activity was normalized to β-Gal activity. Each value represents mean of six independent experiments ±SD. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

domain, which is responsible for recruiting the transcriptional coactivator Pgc-1β to Foxa2. Together, these two factors regulate the expression of genes involved in lipid β oxidation and triglyceride synthesis/secretion, thereby controlling hepatic lipid content. Inhibition of the Foxa2/Pgc-1β complex by insulin leads to a decrease in apoB-dependent VLDL secretion, while constitutive active $Foxa2_{T156A}$ is resistant to this effect.

Even though Foxa1 and Foxa2 are highly homologous and can exhibit similar transactivation and DNA binding capabilities in vitro and display an overlapping tissue expression pattern (Besnard et al., 2004), it is still unclear whether these factors regulate gene expression via similar mechanisms or by independent pathways. It was recently suggested that Foxa1 and Foxa2 compensate for each other in regulating lung and liver gene expression using a tissue-specific double-knockout approach (Lee et al., 2005; Wan et al., 2005). It has to be noted, however, that these genetic studies cannot distinguish between a compensatory function and a synergistic function of both factors involving a set of target genes. In contrast, promoter analyses of liver-enriched genes (e.g., transthyretin) have been shown to selectively bind Foxa2 but not Foxa1 (Kaestner et al., 1999). In addition, several documented genetic models provide evidence that either factor cannot compensate for the loss of the other. For example, despite coexpression of Foxa1 and Foxa2 in pancreatic α cells and similar activation of the glucagon promoter in vitro, Foxa1 mice die shortly after birth due to impaired glucagon secretion (Kaestner et al., 1999; Shih et al., 1999), suggesting that Foxa2 cannot compensate for the loss of Foxa1 expression. Similarly, pancreatic β cell-specific ablation of Foxa2 results in unregulated insulin secretion, whereas insulin secretion can still be regulated in mice with Foxa1 deficiency (Shih et al., 1999; Lantz et al., 2004). Therefore, one explanation of how Foxa1 and Foxa2 may mediate differential gene expression is by specific posttranslational modifications and/or cofactor interactions that are not accounted for by in vitro assays and could therefore contribute to the observed in vivo differences between Foxa1 and Foxa2.

We have shown recently that Foxa2 is inactivated by insulin mediated phosphorylation and nuclear exclusion, while Foxa1 is not subject to regulation by insulin (Wolfrum et al., 2003). In this study, we have demonstrated that Foxa2, but not Foxa1, interacts with Pgc-1β to coactivate gene expression. Even though Foxa2 and Foxa1 show a high sequence homology (>90%) in the DNA winged helix and transactivation domains I–IV (Figure 1A), the 74 amino acid residue spanning domain A ($Foxa2_{266–340}$), which we identified to be responsible for the interaction between Foxa2 and Pgc-1β, is not conserved between Foxa1 and Foxa2 (homology <9%). Several studies have addressed interaction between members of the Pgc family and transcription factors, and the most common motif reported to facilitate interaction appears to be a leucine-rich region (LXXLL). Because this motif was shown to be responsible for interaction with different transcriptions factors, it was suggested that it was mandatory for the interaction between a Pgc family member and a transcription factor. Interestingly, the domain we identified, in itself sufficient to interact with Pgc-1β, does not harbor such a sequence motif. Recent studies, however, have shown that Pgc-1β can also interact with transcription factors via other motifs, as the interaction of Pgc-1β with Srebp and Lxr is not dependent on an LXXLL motif (Lin et al., 2005). Interestingly, a comparison of the interaction domain in Srebp and the 74 aa domain responsible for Foxa2 and Pgc-1β interaction identified by us shows sequence similarity (Figure S3). However, a more detailed analysis of the interaction is necessary to elucidate the exact structural basis for the interaction between Foxa2 and Pgc-1β.

It was previously shown that overexpression of Pgc-1β leads to an induction of genes involved in lipid β oxidation (Lin et al., 2002; Lin et al., 2005). We observe here a concomitant increase

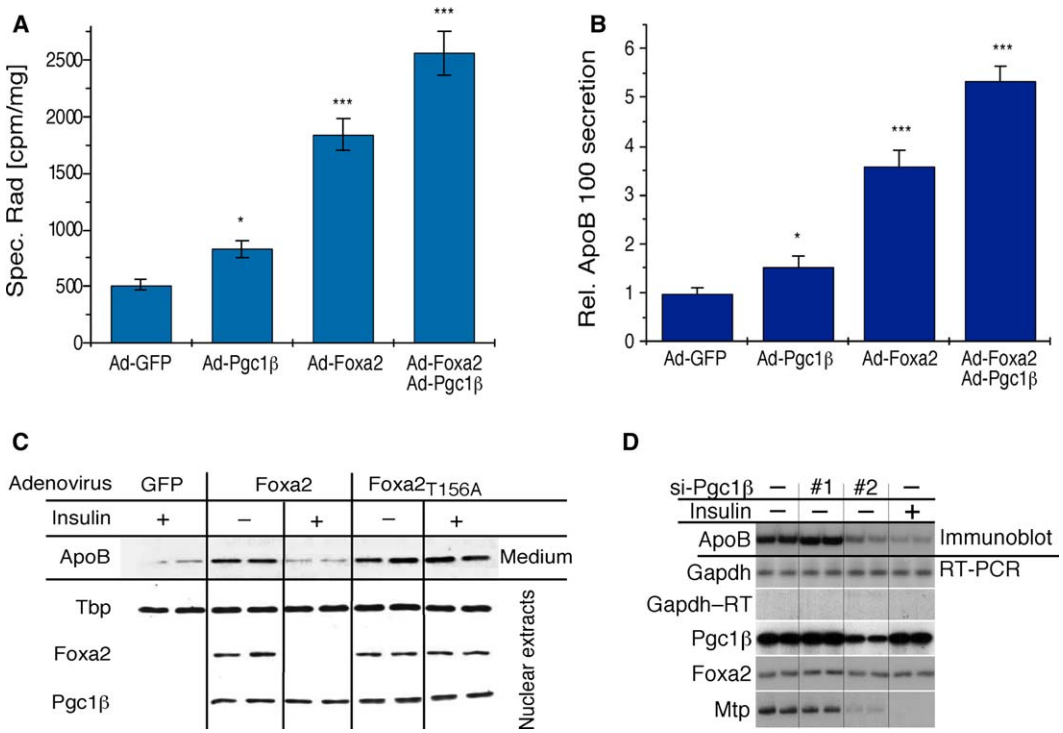

Figure 7. Foxa2 and Pgc-1β induce apoB secretion from primary hepatocytes

A) Primary mouse hepatocytes were infected with either Ad-Foxa2 or Ad-Pgc-1β. The cells were pulsed with ^{14}C-palmitic acid for 30 min and incorporation of the label into secreted triglycerides was measured by extraction of the lipid fraction followed by liquid scintillation counting. Each value represents the mean of nine independent experiments ±SD.

B) Primary mouse hepatocytes were infected with either Ad-Foxa2 or Ad-Pgc-1β. The cells were pulsed with ^{35}S-methionine for 2 hr, and secretion of radiolabel was chased for 4 hr in DMEM. ApoB was immunoprecipitated from the medium samples with a polyclonal α-apoB antibody. After immunoprecipitation the apoB protein secretion was quantified by SDS-PAGE, autoradiography, and densitometry. Each value represents the mean of six independent experiments ±SD.

C) Primary mouse hepatocytes were infected with either Ad-Foxa2, Ad-Foxa2$_{T156A}$, and cells were treated with insulin 4 hr prior to the start of the experiment. Pulse/chase experiments were performed as described above. The expression levels of HA-Foxa2 were quantified using α-HA antibodies. Tbp served as a loading control.

D) HepG2 cells were transfected with two different siRNAs against Pgc-1β (si-Pgc-1β #1, #2). Pulse/chase experiments were performed as described above. Expression levels of Foxa2, Pgc-1β, Dgat2, Mtp, and Mcad mRNA were quantified using RT-PCR. Gapdh served as loading control. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

in gene expression of key enzymes in mitochondrial β oxidation when both Foxa2 and Pgc-1β are coexpressed. However, our experiments suggest that even though both factors lead to induction of the same genes there is a degree of independency, as Pgc-1β alone is sufficient to activate gene expression of Mcad and CptI in livers of *ob/ob* and fed wt animals where Foxa2 is constitutively inactivated. This suggests that Pgc-1β regulates the expression of these genes not only via interaction with Foxa2 but also by interacting with other transcription factors. Since it was reported that PPARα is an interaction partner of Pgc-1β (Lin et al., 2002), it is to be expected that interaction of these factors will lead to an increase in target-gene expression of genes involved in mitochondrial β oxidation in the absence of active Foxa2. Interestingly, high-fatty acid concentrations induce Pgc-1β expression, while at the same time activating the transcriptional activity of PPARα, thereby leading to an increased metabolism of fatty acids. This could be part of a feedback loop that controls catabolism of fatty acids, which act both as signaling molecules and metabolites (Lin et al., 2005).

While the small increases in expression levels of β oxidation genes in hepatocytes infected with Ad-Pgc-1β alone suggest that Pgc-1β has a modest ability to activate β oxidation independently of Foxa2, probably by coactivating PPARα, the effect of Pgc-1β on triglyceride metabolism seems to be more or less exclusively mediated by Foxa2. This is evidenced by the fact that Pgc-1β fails to significantly induce plasma triglyceride levels or expression of either Mtp or Dgat2 in *ob/ob* or fed wt mice where Foxa2 is constantly inactivated, while infection of wt rats which could be expected to have active nuclear Foxa2 with Ad-Pgc-1β led to a significant increase in VLDL secretion (Lin et al., 2005). Furthermore, the induction of apoB-dependent VLDL secretion from primary hepatocytes by Pgc-1β can be blocked by the addition of insulin and can be reversed by transfecting the cells with an insulin resistant Foxa2$_{T156A}$, which demonstrates that the effect of Pgc-1β on triglyceride secretion is PPARα independent.

It has recently been shown that Pgc-1β interacts with Srebp and Lxr, and this interaction has been correlated to an increase in hepatic triglyceride secretion (Lin et al., 2005). Even though there is good evidence that Pgc-1β interacts with both transcription factors in vitro, the importance for the in vivo function of this interaction remains elusive for several reasons. First, transgenic overexpression of Srebp in the liver leads to lipid accumulation and reduced plasma triglyceride levels in comparison to wt animals (Shimano et al., 1996). In contrast, adenoviral-mediated Pgc-1β expression in liver lowers hepatic triglyceride content and increases plasma triglycerides (Lin et al., 2005). Thus, Pgc-1β has the opposite effect on hepatic triglyceride metabolism than one of its putative target genes. Second, induction of Lxr expression in the liver through its synthetic activator T0901317 leads to

hepatic lipid accumulation (Grefhorst et al., 2002). Even though these mice have increased VLDL secretion rates, no increase in plasma VLDL steady-state levels are observed after agonist treatment for 4 days. Therefore, the phenotype observed after activating a putative target of Pgc-1β is the opposite of what is observed when mice are infected with Ad-Pgc-1β. Third, Ldlr expression, which is increased in response to both Lxr and Srebp induction in the liver, is not regulated by Pgc-1β (Lin et al., 2005), suggesting that Pgc-1β is not the main activator for these transcription factors in the liver. Since Pgc-1β in addition to liver is highly expressed in muscle and fat, it is possible that tissue-specific differences exist and that both Srebp and Lxr coactivation through Pgc-1β is important for controlling gene regulation in other tissues beside liver.

The increase in serum triglycerides of mice injected with Ad-Foxa2$_{T156A}$/Pgc-1β is most likely the result of induction of Mtp and Dgat2 expression. Dgat2 catalyzes the esterification reaction of diacylglycerol to triglycerides and is highly expressed in the liver (Cases et al., 2001; Lardizabal et al., 2001). Mice lacking Dgat2 exhibit early lethality and are severely depleted of triglycerides in tissues and plasma, a defect that cannot be compensated by Dgat1, another member of the acyl-CoA:cholesterol acyltransferase gene family (Stone et al., 2004). This suggests that Dgat2 is the enzyme responsible for the majority of TAG synthesis in mice. Since the de novo triglyceride synthesis is increased only marginally in animals infected with Ad-Foxa2$_{T156A}$/Ad-Pgc-1β, it is most likely that this increase in triglyceride synthesis only plays a minor role in the observed increase in plasma triglycerides. Changes in Mtp expression or activity have also been linked to altered plasma triglyceride levels and VLDL secretion. Pharmacological inhibition of Mtp leads to a dose-dependent decrease of secretion of apoB-containing lipoproteins by HepG2 cells, WHHL rabbits, rats, and humans (Jamil et al., 1996; Benoist et al., 1996; Haghpassand et al., 1996; Chandler et al., 2003). Heterozygous Mtp-deficient mice have reduced rates of apoB secretion, and plasma apoB levels are 70% of those in wt mice (Raabe et al., 1998). More importantly for our study, augmented Mtp activity over basal levels has been reported to increase apoB secretion in HepG2 cells (Jamil et al., 1998). Furthermore, an ≈4-fold overexpression of Mtp by recombinant adenoviral infection in mice leads to ≈50% increase in plasma triglycerides and a 2- to 3-fold increase in apoB secretion (Liao et al., 1999). Interestingly, infection of mice with Ad-Foxa2$_{T156A}$/Ad-Pgc-1β in our study led to an ≈4-fold induction of Mtp expression and a similar increase in plasma triglycerides and apoB secretion. Together, these data suggest that the Ad-Foxa2$_{T156A}$/Pgc-1β-induced increase in apoB/VLDL secretion is mainly due to activation of Dgat2 and Mtp expression; however, we cannot rule out that other target genes of this complex are also involved in this regulation. Since we could observe a comparable increase in total triglyceride secretion and apoB secretion, we presume that Foxa2 regulates the VLDL particle numbers that are secreted but does not affect the size or composition of the VLDL particle.

Insulin has been shown to inhibit VLDL output from the liver of rats and humans and exogenous insulin administration also suppresses the secretion of VLDL in humans (Durrington et al., 1982; Patsch et al., 1983; Patsch et al., 1986). However, the molecular mechanism of this regulation has been elusive so far. In this study, we have provided evidence that Mtp, a major regulator of apoB secretion, is a direct transcriptional target of Foxa2/Pgc-1β and that the physiological regulation of apoB/VLDL secretion by hepatocytes is Foxa2 dependent. Since Foxa2 is a direct target of the insulin/PI3-kinase/Akt pathway leading to its phosphorylation and inactivation by nuclear exclusion, this pathway could explain the mechanism by which insulin controls VLDL secretion. Pharmacological intervention affecting either Foxa2 or Pgc-1β could be used to develop novel strategies to treat patients with lipodystrophy or hypertriglyceridemia.

Experimental procedures

Animal and metabolic studies

All animal models were maintained in *C57Bl/6J* background and maintained on a 12 hr light/dark cycle in a pathogen-free animal facility. Twelve-week-old *ob/ob* animals were injected via the tail vein with Ad-GFP, Ad-Foxa2$_{T156A}$, and Ad-Pgc-1β (gift from B. Spiegelman), alone or in combination at a final dose of 10^9 pfu/mice. Blood was taken by retroorbital bleeding at days 1, 4, and 9, and mice were sacrificed at day 14.

Adenovirus generation

Adenoviruses were generated using the Rapid Adenovirus Production System (Viraquest). Viruses were designed to express GFP from an independent promoter, in addition to Foxa2 or Foxa2$_{T156A}$ (Ad-Foxa2 and Ad-Foxa2$_{T156A}$, respectively). For in vivo experiments, mice were injected with 1×10^9 pfu of adenovirus. Empty virus expressing only GFP served as control (Ad-GFP).

Transfection and transactivation assays

Transfection of HepG2 cells and luciferase transactivation assays were carried out as described previously (Wolfrum et al., 2003).

Chromatin immunoprecipitation (ChIP)

ChIP analysis was carried out using insulin-treated HepG2 cells, which were infected with either Ad-Foxa2, Ad-Foxa2$_{T156A}$ and/or Ad-Pgc-1β and the ChIP Assay kit (Cell Signaling) according to the manufacturers protocol. All proteins/chromatin complexes were precipitated using α-FLAG antibody.

RNA interference

HepG2 cells were grown to 60%–70% confluence and transfected with two siRNAs against Pgc-1β (#1, NNTTGTACAGAACTACATAAG; #2, NNGACGT GCTCTGTGATGTCA), respectively (200 pmol/6-well plate) using Fugene6 (Roche). Silencing efficiency of Pgc-1β was determined by RT-PCR.

Immunoblotting

Cytosolic and nuclear protein extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell) by electroblotting. Foxa2 was detected with anti-Foxa2 antiserum (1:1000; Weinstein et al., 1994), FLAG and HA peptides were detected with α-FLAG and α-HA antibodies (Sigma), respectively. Membranes were incubated with primary antibodies overnight at 4ºC.

Laboratory measurements

Blood samples were taken from mice using nonheparinized capillary tubes. Insulin was quantified using a radioimmunoassay (Linco). Free fatty acids were measured using a colorimetric assay system (Wako Chemicals). Glucose was measured using a standard glucose sensor (Glucometer Elite, Bayer). Triglycerides were determined using a colorimetric assay system (Roche).

Mitochondrial β oxidation

Mitochondria from perfused livers of mice were isolated by differential centrifugation as described by Hoppel et al. (1979). An aliquot of freshly isolated mitochondria was used to determine mitochondrial protein. The β oxidation of [1-^{14}C]palmitic acid by liver mitochondria was assessed as described by Lang et al. (2001). CO_2 trapped on the filter papers was counted for 1-^{14}C activity using a scintillation counter.

Triglyceride synthesis

Two hundred milligrams of liver tissue were homogenized in oxygenized Krebs-Ringer buffer (pH 7.4). The solution was incubated with 5 mM of

albumin bound palmitate-1-^{14}C and incubated for 1 hr at 37ºC. After incubation, 10% trichloroacetic acid was added and the suspension was centrifuged for 10 min at 14,000 × g. The residue extracted with 2 ml of cold 5% trichloroacetic acid. The solid residue was extracted once with 2 ml of warm 80% ethanol, twice with 2 ml of 100% ethanol, and once with 2 ml of ether. The alcohol and ether extracts were combined, the solvents were removed by evaporation, and the residue was extracted three times with 2 ml portions of light petroleum. The residues were resuspended in 2 ml of acetone, and phospholipids were isolated by precipitation as their magnesium complexes. The lipids remaining in the acetone solution were saponified in 4 N aqueous potassium hydroxide for 3 hr at 80ºC. After extraction of the unsaponified fraction by light petroleum, the aqueous layer was acidified with 5 N HCL, and fatty acids were extracted with light $CHCl_3$/ethanol (3:1) and counted by liquid scintillation counting.

RT-PCR

Total RNA was extracted from livers and contaminating genomic DNA was removed by treating with 5 u of RNase-free DNase-I (Roche Molecular Biochemicals)/10 μg of RNA. cDNA was synthesized using moloney leukemia virus reverse transcriptase with dNTPs and random hexamer primers (Invitrogen). The cDNAs provided templates for polymerase chain reactions (PCRs) using specific primers at annealing temperatures ranging between 60ºC and 65ºC in the presence of dNTPs, [α-^{32}P]dCTP, and Taq DNA polymerase. The primer sequences used for PCR are available upon request.

apoB pulse/chase experiments

Two days prior to the start of the experiment, HepG2 cells were plated on 110 mm culture dishes. The medium was replaced by DMEM containing 10% FCS, 0.75 mM oleate, and 25 μM glycerol. After 20 hr, the medium was removed and the cells were washed three times with HBSS buffer. The cells were pulsed with methionine- and cysteine-free DMEM containing 150 μCi/dish of Express-^{35}S-labeling mix (NEN) for 2 hr. After the 2 hr pulse, radioactivity was chased for 4 hr in DMEM. The chase medium was collected, and the cells were washed several times with HBSS. apoB was immunoprecipitated from the medium samples with a polyclonal sheep-anti-human apoB antibody (Biodesign). After immunoprecipitation, the apoB proteins were separated by SDS-PAGE, and the gel was impregnated with Amplify for 15–30 min and dried. Autoradiography of the gel was performed at −80ºC for 6–8 days.

FPLC/triglycerides

Lipoproteins from plasma (10–100 μl) were separated by FPLC using two Superose-6 FPLC columns in series (HR10/30) in 0.15 M NaCl, 0.01 M Na_2HPO_4, 0.1 mM EDTA (pH 7.5) at 0.5 ml/min. Columns were calibrated using high and low molecular weight standards (Pharmacia). Triglycerides were quantified using the TG-detection kit (Roche).

Statistical analysis

Results are given as mean ±SD. Statistical analyses were performed by using a Student's t test, and the null hypothesis was rejected at the 0.05 level. Linear regression was calculated using Origin (Microcal).

Supplemental data

Supplemental Data include three figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/3/2/99/DC1/.

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References

Ameen, C., Edvardsson, U., Ljungberg, A., Asp, L., Akerblad, P., Tuneld, A., Olofsson, S.O., Linden, D., and Oscarsson, J. (2005). Activation of peroxisome proliferator-activated receptor alpha increases the expression and activity of microsomal triglyceride transfer protein in the liver. J. Biol. Chem. *280*, 1224–1229.

Asp, L., Magnusson, B., Rutberg, M., Li, L., Boren, J., and Olofsson, S.O. (2005). Role of ADP ribosylation factor 1 in the assembly and secretion of apoB-100-containing lipoproteins. Arterioscler. Thromb. Vasc. Biol. *25*, 566–570.

Benoist, F., Nicodeme, E., and Grand-Perret, T. (1996). Microsomal triacylglycerol transfer protein prevents presecretory degradation of apolipoprotein B-100. A dithiothreitol-sensitive protease is involved. Eur. J. Biochem. *240*, 713–720.

Besnard, V., Wert, S.E., Hull, W.M., and Whitsett, J.A. (2004). Immunohistochemical localization of Foxa1 and Foxa2 in mouse embryos and adult tissues. Gene Expr. Patterns *5*, 193–208.

Brennan, R.G. (1993). The winged-helix DNA binding motif: Another helix-turn-helix takeoff. Cell *74*, 773–776.

Cases, S., Stone, S.J., Zhou, P., Yen, E., Tow, B., Lardizabal, K.D., Voelker, T., and Farese, R.V., Jr. (2001). Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members. J. Biol. Chem. *276*, 38870–38876.

Chandler, C.E., Wilder, D.E., Pettini, J.L., Savoy, Y.E., Petras, S.F., Chang, G., Vincent, J., and Harwood, H.J., Jr. (2003). CP-346086: an MTP inhibitor that lowers plasma cholesterol and triglycerides in experimental animals and in humans. J. Lipid Res. *44*, 1887–1901.

Duncan, S.A., Navas, M.A., Dufort, D., Rossant, J., and Stoffel, M. (1998). Regulation of a transcription factor network required for differentiation and metabolism. Science *281*, 692–695.

Durrington, P.N., Newton, R.S., Weinstein, D.B., and Steinberg, D. (1982). Effects of insulin and glucose on very low density lipoprotein triglyceride secretion by cultured rat hepatocytes. J. Clin. Invest. *70*, 63–73.

Gibbons, G.F., Wiggins, D., Brown, A.M., and Hebbachi, A.M. (2004). Synthesis and function of hepatic very-low-density lipoprotein. Biochem. Soc. Trans. *32*, 59–64.

Ginsberg, H.N. (2002). New perspectives on atherogenesis: role of abnormal triglyceride-rich lipoprotein metabolism. Circulation *106*, 2137–2142.

Grefhorst, A., Elzinga, B.M., Voshol, P.J., Plosch, T., Kok, T., Bloks, V.W., van der Sluijs, F.H., Havekes, L.M., Romijn, J.A., Verkade, H.J., and Kuipers, F. (2002). Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low-density lipoprotein particles. J. Biol. Chem. *277*, 34182–34190.

Haghpassand, M., Wilder, D., and Moberly, J.B. (1996). Inhibition of apolipoprotein B and triglyceride secretion in human hepatoma cells (HepG2). J. Lipid Res. *37*, 1468–1480.

Heine, R.J., and Dekker, J.M. (2002). Beyond postprandial hyperglycaemia: metabolic factors associated with cardiovascular disease. Diabetologia *45*, 461–475.

Hoppel, C., DiMarco, J.P., and Tandler, B. (1979). Riboflavin and rat hepatic cell structure and function. Mitochondrial oxidative metabolism in deficiency states. J. Biol. Chem. *254*, 4164–4170.

Horton, J.D. (2002). Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. Biochem. Soc. Trans. *30*, 1091–1095.

Jamil, H., Gordon, D.A., Eustice, D.C., Brooks, C.M., Dickson, J.K., Jr., Chen, Y., Ricci, B., Chu, C.H., Harrity, T.W., Ciosek, C.P., Jr., et al. (1996). An inhibitor of the microsomal triglyceride transfer protein inhibits apoB secretion from HepG2 cells. Proc. Natl. Acad. Sci. USA *93*, 11991–11995.

Jamil, H., Chu, C.H., Dickson, J.K., Jr., Chen, Y., Yan, M., Biller, S.A., Gregg, R.E., Wetterau, J.R., and Gordon, D.A. (1998). Evidence that microsomal triglyceride transfer protein is limiting in the production of apolipoprotein B-containing lipoproteins in hepatic cells. J. Lipid Res. *39*, 1448–1454.

Kaestner, K.H., Hiemisch, H., Luckow, B., and Schutz, G. (1994). The HNF-3 gene family of transcription factors in mice: gene structure, cDNA sequence, and mRNA distribution. Genomics *20*, 377–385.

Kaestner, K.H., Katz, J., Liu, Y., Drucker, D.J., and Schutz, G. (1999). Inactivation of the winged helix transcription factor HNF3alpha affects glucose homeostasis and islet glucagon gene expression in vivo. Genes Dev. *13*, 495–504.

Lang, C., Schafer, M., Serra, D., Hegardt, F., Krahenbuhl, L., and Krahenbuhl, S. (2001). Impaired hepatic fatty acid oxidation in rats with short-term cholestasis: characterization and mechanism. J. Lipid Res. *42*, 22–30.

Lantz, K.A., Vatamaniuk, M.Z., Brestelli, J.E., Friedman, J.R., Matschinsky, F.M., and Kaestner, K.H. (2004). Foxa2 regulates multiple pathways of insulin secretion. J. Clin. Invest. *114*, 512–520.

Lardizabal, K.D., Mai, J.T., Wagner, N.W., Wyrick, A., Voelker, T., and Hawkins, D.J. (2001). DGAT2 is a new diacylglycerol acyltransferase gene family: purification, cloning, and expression in insect cells of two polypeptides from Mortierella ramanniana with diacylglycerol acyltransferase activity. J. Biol. Chem. *276*, 38862–38869.

Lee, C.S., Friedman, J.R., Fulmer, J.T., and Kaestner, K.H. (2005). The initiation of liver development is dependent on Foxa transcription factors. Nature *435*, 944–947.

Liao, W., Kobayashi, K., and Chan, L. (1999). Adenovirus-mediated overexpression of microsomal triglyceride transfer protein (MTP): mechanistic studies on the role of MTP in apolipoprotein B-100 biogenesis. Biochemistry *38*, 10215.

Lin, J., Puigserver, P., Donovan, J., Tarr, P., and Spiegelman, B.M. (2002). Peroxisome proliferator-activated receptor gamma coactivator 1beta (PGC-1beta), a novel PGC-1-related transcription coactivator associated with host cell factor. J. Biol. Chem. *277*, 1645–1648.

Lin, J., Yang, R., Tarr, P.T., Wu, P.H., Handschin, C., Li, S., Yang, W., Pei, L., Uldry, M., Tontonoz, P., et al. (2005). Hyperlipidemic effects of dietary saturated fats mediated through PGC-1β coactivation of SREBP. Cell *120*, 261–273.

Patsch, W., Franz, S., and Schonfeld, G. (1983). Role of insulin in lipoprotein secretion by cultured rat hepatocytes. J. Clin. Invest. *71*, 1161–1174.

Patsch, W., Gotto, A.M., Jr., and Patsch, J.R. (1986). Effects of insulin on lipoprotein secretion in rat hepatocyte cultures. The role of the insulin receptor. J. Biol. Chem. *261*, 9603–9606.

Raabe, M., Flynn, L.M., Zlot, C.H., Wong, J.S., Veniant, M.M., Hamilton, R.L., and Young, S.G. (1998). Knockout of the abetalipoproteinemia gene in mice: reduced lipoprotein secretion in heterozygotes and embryonic lethality in homozygotes. Proc. Natl. Acad. Sci. USA *95*, 8686–8691.

Rubins, H.B. (2000). Triglycerides and coronary heart disease: implications of recent clinical trials. J. Cardiovasc. Risk *7*, 339–345.

Shelness, G.S., and Sellers, J.A. (2001). Very-low-density lipoprotein assembly and secretion. Curr. Opin. Lipidol. *12*, 151–157.

Shih, D.Q., Navas, M.A., Kuwajima, S., Duncan, S.A., and Stoffel, M. (1999). Impaired glucose homeostasis and neonatal mortality in hepatocyte nuclear factor 3alpha-deficient mice. Proc. Natl. Acad. Sci. USA *96*, 10152–10157.

Shimano, H., Horton, J.D., Hammer, R.E., Shimomura, I., Brown, M.S., and Goldstein, J.L. (1996). Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. J. Clin. Invest. *98*, 1575–1584.

Stone, S.J., Myers, H.M., Watkins, S.M., Brown, B.E., Feingold, K.R., Elias, P.M., and Farese, R.V., Jr. (2004). Lipopenia and skin barrier abnormalities in DGAT2-deficient mice. J. Biol. Chem. *279*, 11767–11776.

Wan, H., Dingle, S., Xu, Y., Besnard, V., Kaestner, K.H., Ang, S.L., Wert, S., Stahlman, M.T., and Whitsett, J.A. (2005). Compensatory roles of Foxa1 and Foxa2 during lung morphogenesis. J. Biol. Chem. *280*, 13809–13816.

Weinstein, D.C., Ruiz i Altaba, A., Chen, W.S., Hoodless, P., Prezioso, V.R., Jessell, T.M., and Darnell, J.E., Jr. (1994). The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. Cell *78*, 575–588.

Wiggins, D., and Gibbons, G.F. (1996). Origin of hepatic very-low-density lipoprotein triacylglycerol: the contribution of cellular phospholipid. Biochem. J. *320*, 673–679.

Wolfrum, C., Besser, D., Luca, E., and Stoffel, M. (2003). Insulin regulates the activity of forkhead transcription factor Hnf-3beta/Foxa-2 by Akt-mediated phosphorylation and nuclear/cytosolic localization. Proc. Natl. Acad. Sci. USA *100*, 11624–11629.

Wolfrum, C., Asilmaz, E., Luca, E., Friedman, J.M., and Stoffel, M. (2004). Foxa2 regulates lipid metabolism and ketogenesis in the liver during fasting and in diabetes. Nature *432*, 1027–1032.