Foxa2 integrates the transcriptional response of the hepatocyte to fasting

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Summary

Survival during prolonged food deprivation depends on the activation of hepatic gluconeogenesis. Inappropriate regulation of this process is a hallmark of diabetes and other metabolic diseases. Activation of the genes encoding gluconeogenic enzymes is mediated by hormone-responsive transcription factors such as the cyclic AMP response element binding protein (CREB) and the glucocorticoid receptor (GR). Here we show using cell-type-specific gene ablation that the winged helix transcription factor Foxa2 is required for activation of the hepatic gluconeogenic program during fasting. Specifically, Foxa2 promotes gene activation both by cyclic AMP, the second messenger for glucagon, and glucocorticoids. Foxa2 mediates these effects by enabling recruitment of CREB and GR to their respective target sites in chromatin. We conclude that Foxa2 is required for execution of the hepatic gluconeogenic program by integrating the transcriptional response of the hepatocyte to hormonal stimulation.

Introduction

Mammals have evolved complex systems to maintain blood glucose levels within a narrow range even when food supply is limited. The liver is the major provider of glucose in times of food restriction, initially through mobilization of glycogen and in the long term by de novo synthesis of glucose, or gluconeogenesis. Red blood cells and the brain are dependent on glucose as metabolic fuel, and severe reductions in blood glucose may result in seizures or death. The enzymes required for hepatic gluconeogenesis are regulated at the transcriptional level, with multiple transcription factors cooperating to ensure the appropriate adaptations to the metabolic and hormonal status of the organism. In response to fasting, blood insulin levels drop, while glucagon and glucocorticoid levels rise, promoting hepatic gluconeogenesis through activation of the cAMP response element binding protein (CREB) and the glucocorticoid receptor (GR), respectively. A long-standing paradox has been the fact that both CREB and GR are expressed in multiple organs, yet the activation of the gluconeogenic program occurs primarily in the liver and to a lesser extent in the kidney (Hanson and Reshef, 1997). Analysis of *cis*-regulatory elements of genes encoding gluconeogenic enzymes has led to the finding that binding sites for CREB and GR are often located in close proximity to those of nuclear factors expressed predominantly in hepatocytes, including hepatocyte nuclear factor 4α (HNF-4α) and the Foxa (also known as HNF-3) proteins (Lucas and Granner, 1992; Nitsch et al., 1993; Nitsch and Schutz, 1993; Wang et al., 1996). However, the requirement for the Foxa factors in hormone-dependent activation of the hepatic gluconeogenic program has not been investigated by genetic means in vivo until now. Here we utilize tissue-specific gene ablation of Foxa2 in hepatocytes to demonstrate a requirement for this factor in the hepatic response to a prolonged fast.

Results

Derivation of mice deficient for Foxa2 in hepatocytes

As embryos lacking *Foxa2* die in midgestation due to an essential role for *Foxa2* in the formation of the node and notochord (Ang and Rossant, 1994; Weinstein et al., 1994), we employed cell-type-specific gene ablation to delete the gene only in hepatocytes. Using a loxP-flanked allele of *Foxa2* (*Foxa2*^{loxP}) and a transgene in which expression of Cre is dependent on the albumin promoter and the α-fetoprotein enhancer (AlfpCre), the *Foxa2* gene was deleted quantitatively in the liver (Figures 1A and 1B). To evaluate potential regulatory relationships among the *Foxa* genes, which might result in partial compensation, we determined the mRNA levels of the two closely related genes, *Foxa1* and *Foxa3*. While Foxa3 expression is not dependent on Foxa2, Foxa1 mRNA expression was increased by about 70% (Figure 1C), consistent with the presence of a Foxa binding site in the *Foxa1* promoter described previously (Peterson et al., 1997).

Foxa2 is required for the induction of hepatic gene expression in response to fasting

To investigate how Foxa2 regulates the hepatic response to fasting, we chose to evaluate the expression of three representative genes that are exquisitely sensitive to hormonal regulation (Hanson and Reshef, 1997; Lucas and Granner, 1992; Unterman et al., 1994): PEPCK (phosphoenolpyruvate carboxykinase), the rate-limiting enzyme in gluconeogenesis; TAT (tyrosine aminotransferase), and IGFBP-1 (insulin growth factor binding protein 1). Concordant with previous findings, expression of all three genes is robustly induced by a prolonged fast in wild-type mice (Figures 2A–2C). However, activation of these genes is blunted significantly in the absence of Foxa2. These

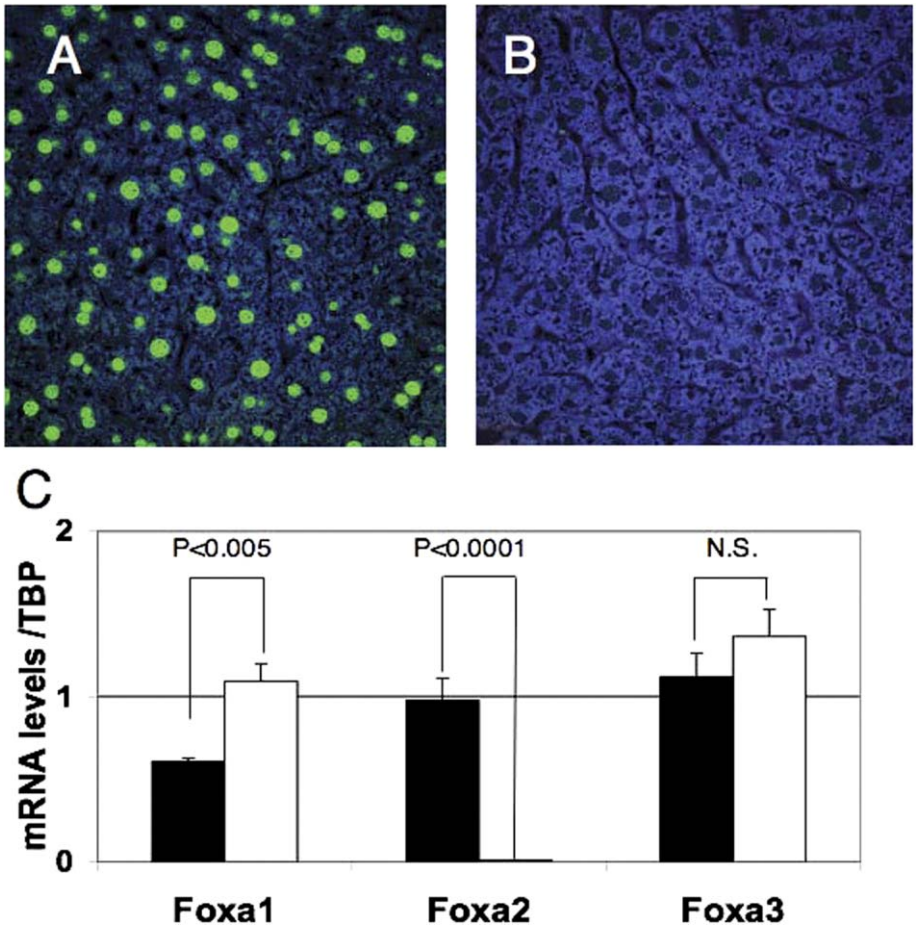

Figure 1. Foxa2 is deleted efficiently in *Foxa2*$^{loxP/loxP}$; AlfpCre mice

A) Immunofluorescence staining of wild-type liver with a monospecific antibody to Foxa2 demonstrates nuclear localization of Foxa2.

B) Foxa2 is quantitatively deleted in hepatocytes from *Foxa2*$^{loxP/loxP}$; AlfpCre mice.

C) Quantitative reverse transcription PCR analysis confirms absence of Foxa2 expression in livers from *Foxa2*$^{loxP/loxP}$; AlfpCre mice and shows a 70% increase in Foxa1 expression. Black bars, control mice. White bars, *Foxa2*$^{loxP/loxP}$; AlfpCre mice. Error bars are standard error of the mean (SEM).

results clearly demonstrate that Foxa2 is required for the full activation of the hepatic transcriptional response to fasting.

Foxa2 is required for full stimulatory activity of both glucocorticoid and cAMP signaling

The shift from the fed to the fasted state results in induction of complex hormonal responses, with the activation of the hypothalamic-pituitary-adrenal axis resulting in the elevation of glucocorticoid levels, and a switch in the endocrine pancreas from the release of insulin to glucagon. In order to define the specific contribution of Foxa2 in this complex in vivo process, we isolated primary hepatocytes and cultured them in a three-dimensional collagen matrix to assess responsiveness to individual hormones in defined conditions. As shown in Figure 2D, in cultured hepatocytes, the PEPCK gene is most responsive to cAMP, which mimics activation of the glucagon receptor signaling cascade, while both TAT and IGFBP-1 are induced more effectively by glucocorticoids (dexamethasone; Figures 2E and 2F). In the absence of Foxa2, however, both the induction of PEPCK by cAMP and the activation of TAT and IGFBP-1 by glucocorticoids are severely blunted, explaining the reduced activation of these genes during fasting (Figures 2D–2F). Thus, Foxa2 is required for both cAMP and glucocorticoid signaling in hepatocytes.

Foxa2 is nuclear in hepatocytes regardless of metabolic state and occupies its targets constitutively

By what mechanism does Foxa2 facilitate the hormonal response of the hepatocyte to fasting? Given the recent observations that Foxa2 can be subject to nuclear exclusion after activation of insulin signaling (Wolfrum et al., 2003, 2004), we hypothesized that, in the fed state, Foxa2 would be localized in the cytoplasm and therefore unable to activate its target genes. According to this model, Foxa2-deficient hepatocytes lack a facultative transcriptional activator, i.e., one that is only active in the fasted state when insulin is low.

We evaluated this model in two ways. First, we utilized immunohistochemistry with three independently derived Foxa2 antibodies to determine the localization of Foxa2 under various metabolic conditions. As is demonstrated in Figure 3A, Foxa2 staining is clearly detectable in all hepatocyte nuclei in livers from fasted mice. In contrast to previous findings (Wolfrum et al., 2003, 2004), we found no evidence for nuclear exclusion of Foxa2 immunostaining under two different conditions of elevated insulin levels. In fed mice (Figure 3B) and severely insulin-resistant *ob*/*ob* mice (Figure 3E), in which insulin levels were increased more than 10-fold to 30 ng/ml, Foxa2 immunostaining was detected solely in the nucleus. In order to ascertain the specificity of the antibody, we performed immunohistochemical staining of sections from Foxa2-deficient livers in

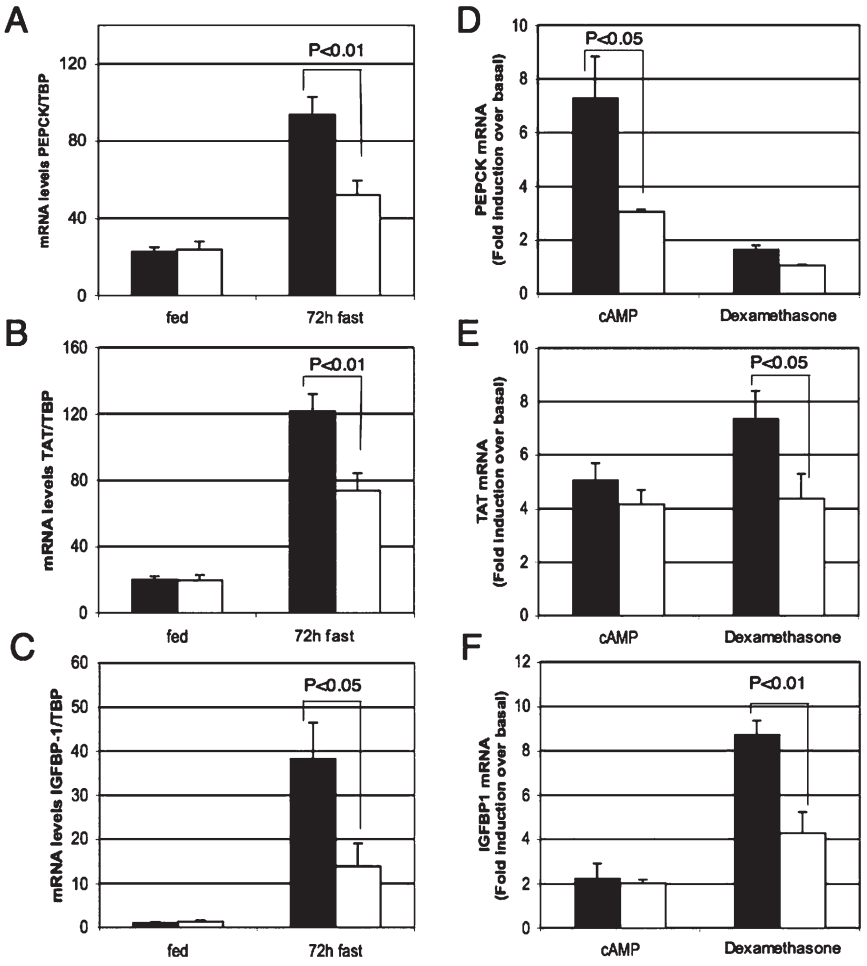

Figure 2. The transcriptional response of the hepatocyte to fasting is dependent on Foxa2

Liver RNA from fed and fasted wild-type or *Foxa2*$^{loxP/loxP}$; AlfpCre mice was analyzed for expression of PEPCK (**A**), TAT (**B**), or IGFBP1 (**C**) by quantitative RT-PCR normalized to TBP as internal control. The normal induction of all three genes in response to fasting is significantly blunted in the absence of Foxa2. Black bars, control mice. White bars, *Foxa2*$^{loxP/loxP}$; AlfpCre mice. The induction of TAT, PEPCK, and IGFBP1 by cAMP or glucocorticoids in primary hepatocytes depends on Foxa2. Primary hepatocytes were isolated from control and *Foxa2*$^{loxP/loxP}$; AlfpCre mice and exposed to cAMP (the second messenger for glucagon) or dexamethasone (a synthetic glucocorticoid) for 16 hr before RNA isolation and quantitative RT-PCR analysis. (**D**) PEPCK is strongly induced by cAMP and to a lesser extent by dexamethasone. The induction by cAMP is reduced by 60% in Foxa2-deficient hepatocytes. TAT (**E**) and IGFBP1 (**F**) mRNA levels are increased by exposure to dexamethasone, a response that is significantly blunted in the absence of Foxa2. Black bars, control mice. White bars, *Foxa2*$^{loxP/loxP}$; AlfpCre mice. Values are represented as means plus standard error. P values were determined by Student's t test.

comparison to those of wild-type mice. No signal was present in *Foxa2*$^{loxP/loxP}$; AlfpCre mice, confirming that the anti-Foxa2 antiserum employed was specific for Foxa2 (Figures 3C and 3D).

In our second approach, we determined promoter and enhancer occupancy of Foxa2-dependent genes in the liver in vivo, as this determines whether Foxa2 can exert its effects on the transcriptional regulation of its targets under varying metabolic conditions. Therefore, we isolated liver chromatin from fasted and fed animals and assessed Foxa2 occupancy of the TAT enhancer and PEPCK and IGFBP-1 promoters using chromatin immunoprecipitation. Foxa2 occupied its binding sites in all three target genes in the fasted state as expected; however, this binding was not altered when the mice were fed and insulin levels increased (Figure 3F). Therefore, the decreased activation of the PEPCK, TAT, and IGFBP-1 genes in Foxa2-deficient hepatocytes that we observed solely in the fasted state (Figure 2) indicates that Foxa2 is essential to the transcriptional response to food deprivation.

Foxa2 promotes binding of CREB and GR to their targets in chromatin in vivo

Next, we considered the possibility that Foxa2 is required for the expression of CREB and GR, or the potent coactivator and metabolic regulator PGC-1α (peroxisome proliferator-activated receptor-γ coactivator 1), which itself is a CREB target (Herzig et al., 2001). However, quantitative RT-PCR demonstrated equal mRNA levels in wild-type and mutant livers for all these factors (data not shown).

Finally, we investigated whether Foxa2 might be required to allow chromatin access for hormone-dependent transcription factors that are activated during the fasting response. This notion is based on the aforementioned proximity of Foxa2 binding sites to CREs and GREs in *cis*-regulatory elements of several gluconeogenic genes (Lucas and Granner, 1992; Nitsch et al., 1993; Nitsch and Schutz, 1993; Unterman et al., 1994; Wang et al., 1996, 1999, 2000) as well as the role of Foxa proteins as chromatin remodeling factors during fetal hepatic development (Cirillo et al., 2002). During differentiation of the hepatic primordium from the foregut endoderm, Foxa proteins bind to enhancers and promoters of hepatocyte-specific genes such as albumin even before the transcription of these target genes is activated (McPherson et al., 1993). By altering chromatin structure, the Foxa proteins facilitate the subsequent binding of other hepatic transcription factors (Gualdi et al., 1996). In addition, it was recently demonstrated that Foxa1 and Foxa2 cooperate to establish developmental competence of the foregut endoderm to initiate the hepatogenic program (Lee et al., 2005).

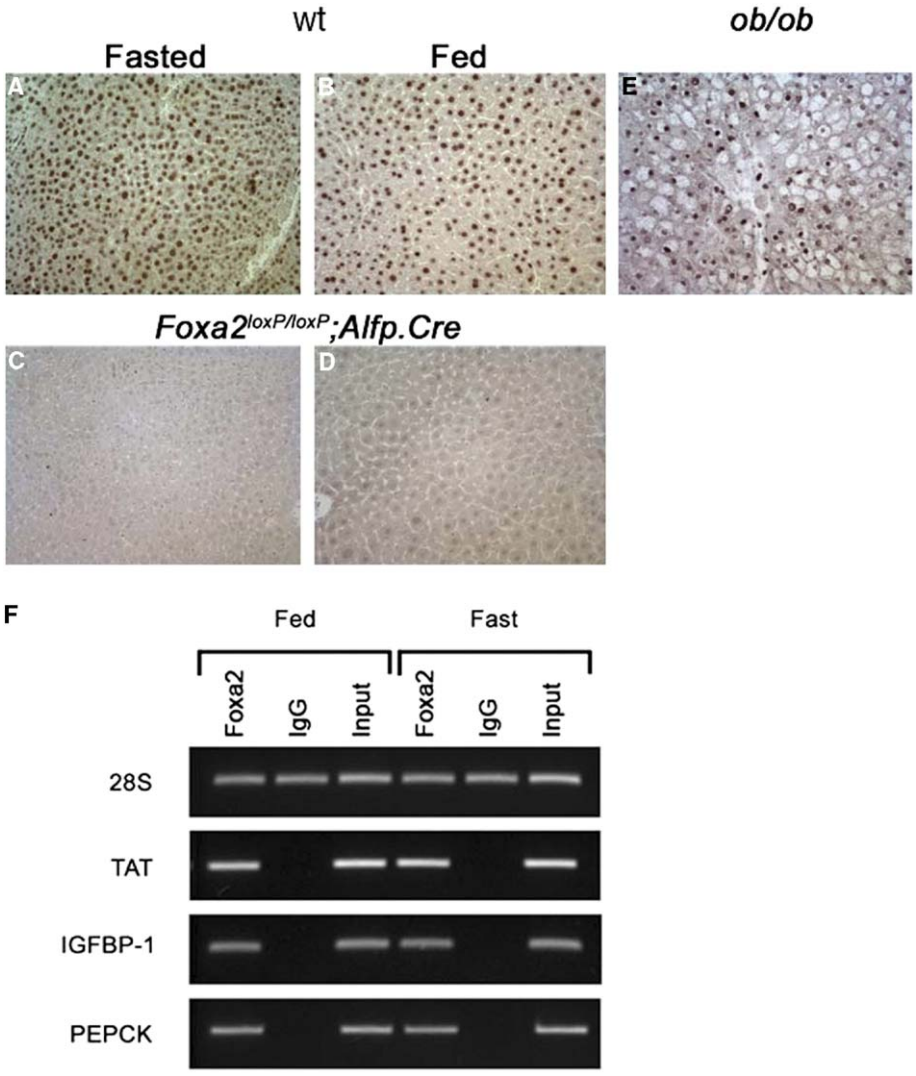

Figure 3. Hepatocyte Foxa2 protein is nuclear irrespective of metabolic state and occupies promoters and enhancers of target genes involved in the hormonal response to fasting

A–E) Immunohistochemical detection of Foxa2 protein using an antiserum monospecific for Foxa2. Sections from livers from fasted (**A** and **C**), fed (**B** and **D**), and Ob/Ob (**E**) mice were stained with a rabbit polyclonal antiserum directed against Foxa2 as described in Experimental Procedures. Regardless of metabolic state and insulin levels, Foxa2 is exclusively nuclear. Note that, in the Ob/Ob mouse, insulin levels were 30 ng/ml, or 30 times higher than in randomly fed mice. The specificity of the staining procedure is demonstrated by the absence of signal in Foxa2-deficient livers (**C** and **D**).

F) Immunoprecipitation of liver chromatin isolated from fed and fasted wild-type mice with anti-Foxa2 antibodies or control IgG. Occupancy of the Foxa2 sites in the TAT enhancer and the IGFBP1 and PEPCK promoters is not different between the fed and fasted state. Control immunoprecipitations with nonrelevant IgG demonstrate the specificity of the assay. Error bars are SEM.

To address the possibility that Foxa may also enable binding of hormone-dependent transcription factors during fasting, we performed chromatin immunoprecipitation with antibodies specific for CREB or GR on wild-type and Foxa2-deficient livers and compared the enrichment of transcription factor binding to the *cis*-regulatory elements of these gluconeogenic genes. We focused on binding of CREB to the PEPCK promoter and on the glucocorticoid receptor occupancy of the TAT enhancer and IGFBP1 promoter. This choice was the result of our hepatocyte experiments, in which we had observed that the absence of Foxa2 was associated with blunted induction of PEPCK in response to cAMP, and IGFBP1 and TAT by dexamethasone (see Figure 2). As expected, the glucocorticoid receptor occupies the promoter of the IGFBP-1 gene and the –2.5 kb enhancer of the TAT gene in wild-type liver, and this binding is enhanced more than 4-fold during fasting (Figures 4A, 4C, and 4D). This increased binding of GR to its target sites is significantly blunted in the absence of Foxa2, demonstrating that Foxa2 is required to allow the GR full access to its targets. We observed an equivalent requirement for Foxa2 in the case of CREB and its target site in the PEPCK promoter. CREB binding was augmented approximately 5-fold in fasted wild-type mice compared to fed controls, while this increase is less than 2-fold in Foxa2-deficient hepatocytes (Figures 4A and 4B), indicating that Foxa2 also increases the efficacy of CREB binding to selected target sites. Given the similar biochemical characteristics of the Foxa family of transcription factors and the upregulation of Foxa1 in hepatocytes in the absence of Foxa2 demonstrated above, it appears likely that partial compensation masks an even more striking effect of Foxa2 deficiency.

Discussion

The model of regulation of Foxa2 activity by nuclear exclusion in response to insulin signaling as proposed by Wolfrum and colleagues (Wolfrum et al., 2003, 2004) provides a plausible explanation to the question of how insulin exerts its well-known effects on transcription in the liver, especially the inhibition of expression of gluconeogenic enzymes. The model extends the paradigm first described for the related transcription factor

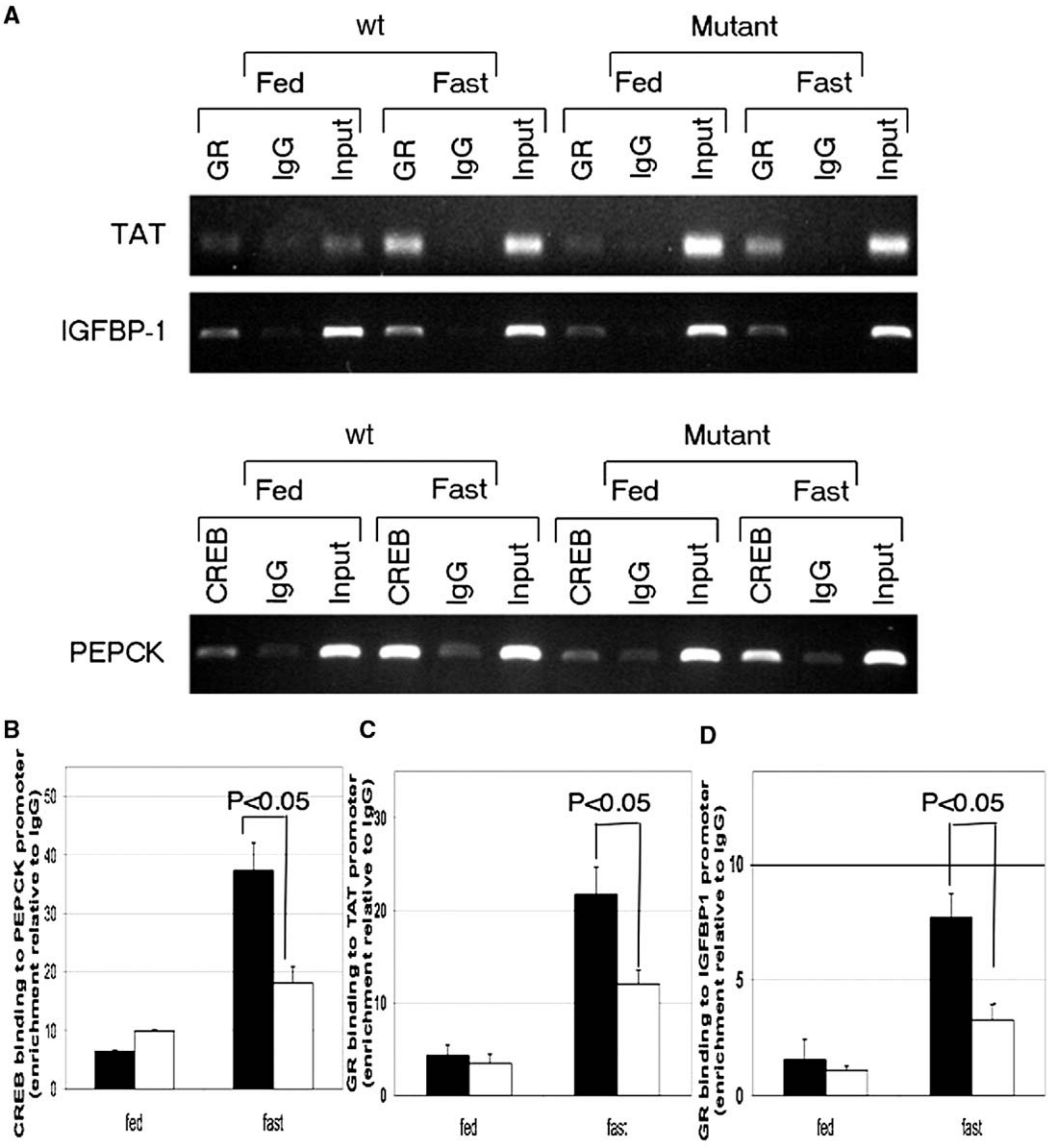

Figure 4. Foxa2 enables binding of CREB and GR to their binding sites in fasting responsive genes

A) Immunoprecipitation of liver chromatin isolated from fed and fasted wild-type or *Foxa2*$^{loxP/loxP}$; AlfpCre mice with anti-CREB (for PEPCK) and anti-GR (for TAT and IGFBP1) antibodies. Occupancy of the GREs in the TAT and IGFBP1 genes is increased in fasted wild-type mice compared to fed controls. This induction is significantly decreased in livers deficient for Foxa2. Similarly, binding of CREB to the promoter of the PEPCK gene is increased by fasting, a response that is dependent on Foxa2. Chromatin occupancy by CREB or GR was evaluated by quantitative PCR in chromatin from fed and fasted wild-type or *Foxa2*$^{loxP/loxP}$; AlfpCre mice fed and fasted wild-type or *Foxa2*$^{loxP/loxP}$; AlfpCre mice (n = 3 for each group). Binding of CREB to the CRE in the PEPCK promoter (**B**), as well as binding of GR to the GREs in the −2.5 kb enhancer in the TAT gene (**C**) and the IGFBP1 promoter (**D**), is decreased by approximately 50% in the absence of Foxa2. Black bars, control mice. White bars, *Foxa2*$^{loxP/loxP}$; AlfpCre mice. Values are represented as means plus standard error. P values were determined by Student's t test.

Foxo1, the mammalian ortholog of the *C. elegans* gene *Daf16*, which had been established by genetic means to act downstream of the insulin receptor and protein kinase B/AKT (Lee et al., 2001). This initial discovery was followed by others showing that the mammalian homolog of *Daf16*, Foxo1, could be phosphorylated by protein kinase B/AKT, resulting in nuclear exclusion and concomitant loss of transcriptional activation of its target genes (Biggs et al., 1999; Brunet et al., 1999; Guo et al., 1999; Rena et al., 1999). In addition, the functional significance of Foxo1 as an effector of the insulin signal transduction pathway in mammals was also explored by genetic means. Thus, heterozygocity for Foxo1 ameliorates the diabetic phenotype in insulin receptor deficient mice (Nakae et al., 2002).

However, the situation regarding Foxa2 is less clear. While phosphorylation of Foxa2 by protein kinase B/AKT in vitro and nuclear exclusion in hepatocytes in response to elevated insulin have been reported (Wolfrum et al., 2003, 2004), our results did not confirm these findings. Importantly, the latter study employed the same anitserum as ours (Wolfrum et al., 2004). This

antiserum, originally generated in the Jessell laboratory, detected nuclear staining for Foxa2 in mouse embryonic tissues (Ruiz i Altaba et al., 1993). A possible explanation for the discrepancy between our results and previous studies might be the concentration of the antiserum employed. While we utilized the Foxa2 antiserum at a 1:2000 dilution, similar to the original report (Ruiz i Altaba et al., 1993), Wolfrum et al. used a higher concentration of 1:100, potentially leading to crossreactivity with other antigens. Our results are consistent with another study showing localization of Foxa2 exclusively in the nucleus regardless of metabolic condition (Steneberg et al., 2005).

A further discrepancy between the data reported here and those published by Wolfrum et al. exists regarding the targets of Foxa2. Several genes encoding gluconeogenic enzymes have been shown by multiple in vitro assays to be targets of Foxa2, including the three investigated here in our in vivo model of Foxa2 deficiency. However, Wolfrum et al. reported that, in their chromatin immunoprecipitation assay, the promoter of the PEPCK gene was not occupied by Foxa2 (Wolfrum et al., 2004). In contrast, we detected a clear enrichment for the PEPCK promoter in immunoprecipitation with the Foxa2 antibody (see Figure 4F). Our results are consistent with the previous reports that identified a Foxa binding site in the PEPCK promoter (O'Brien et al., 1995; Wang et al., 1996, 1999, 2000).

The results described above have general implications for the computational identification of *cis*-regulatory elements commonly performed to identify transcription factor "targets" using positional weight matrices derived from a relatively small number of known targets. As shown here for the mammalian liver, the question of whether a CRE or GRE actually functions as a binding site for its cognate transcription factor depends on the context of the neighboring sequences, i.e., the presence of Foxa binding sites, and on the cellular environment in which these transcription factors are expressed.

In summary, we have shown that Foxa2 is required for the activation of the hepatic transcriptional program of gluconeogenesis, a major evolutionary adaptation to limiting and unpredictable food supply. This is achieved by integrating the response of the hepatocyte to glucagon and glucocorticoids. Binding of Foxa2 to its target sites allows the transcription factors CREB and GR and their potent coactivators access to *cis*-regulatory elements of genes involved in the hepatic response to fasting. The integration of the tissue-specific factor Foxa2 with the two ubiquitously expressed but hormone-dependent activators CREB and GR assures that the gluconeogenic response occurs only in the liver and only under the appropriate metabolic circumstances.

Experimental procedures

Derivation of *Foxa2*$^{loxP/loxP}$; AlfpCre mice

The derivation of the *Foxa2*^{loxP} allele has been described previously (Sund et al., 2000). To achieve hepatocyte-specific expression of Cre, we fused the Cre coding sequence to a 2 kb fragment of the mouse albumin promoter and a 4.6 kb fragment of the mouse α-feto protein enhancer (Hammer et al., 1987) and obtained transgenic lines by pronuclear injection into fertilized C57Bl/6; SJL F1 hybrid oocytes.

Immunofluorescence and immunohistochemistry

Livers were fixed in 4% PFA overnight at 4°C, embedded in paraffin, cut to 6 μm sections, and applied to Probe-on Plus slides (Fisher Scientific). Slides were deparaffinized in xylene and rehydrated through a series of ethanol washes. Slides were subjected to microwave antigen retrieval, washed in PBS, and blocked with protein blocking reagent (Immunotech) for 20 min at RT. Rabbit anti-Foxa2 (K2 1:2000; a gift from Dr. T. Jessel) was diluted in antibody diluent (Zymed) and incubated with the sections overnight at 4°C. Slides were washed in PBS and incubated with Cy2-conjugated donkey anti-rabbit IgG (1:400; Jackson) for 2 hr at RT. Slides were rinsed in PBS, mounted, and examined using confocal microscopy (Leica).

Slides subjected to immunohistochemistry were incubated in 2.25% H_2O_2 for 15 min, washed in PBS, and blocked with avidin and biotin blocking reagents (Vector) for 15 min each at RT. Following blocking with protein blocking reagent (Immunotech), slides were incubated overnight at 4°C with either rabbit anti-Foxa2 (K2 1:2000; a gift from Dr. T. Jessel) or rabbit anti-Foxa2 (1:500; a gift from Dr. J.A. Whitsett) or goat anti-Foxa2 antibody (1:50; Santa Cruz). Slides were washed in PBS, incubated with biotinylated goat anti-rabbit or donkey anti-goat antibody (1:200; Vector) diluted in PBT for 30 min at 37°C, washed in PBS, and incubated with HRP-conjugated ABC reagent (Vector Elite Kit) for 30 min at 37°C. Following PBS wash, signal was developed using DAB Substrate Kit for peroxides (Vector). Slides were washed in water, dehydrated, mounted, and viewed using a bright-field microscope (Nikon).

Primary hepatocyte isolation and culture

Hepatocytes were isolated from 12-week-old male mice via collagenase (0.05% collagenase type I, Worthington) digestion and Percoll (Amersham Biosciences AB) gradient purification and cultured in a three-dimensional collagen matrix to maintain the differentiated state of the cells (Gomez-Lechon et al., 1998). Freshly isolated hepatocytes were resuspended in Ham F-12/Leibovitz-15 medium supplemented with 2% newborn calf serum, 30 mM proline, 0.2% bovine serum albumin (BSA), and antibiotics. Cellular viability was assessed by trypan blue exclusion; only preparations with viability greater than 90% were used.

Hepatocytes were seeded in collagen type I (1.5 mg/ml) at a final density of 0.5×10^6 cells per well. One hour after cell attachment, hepatocytes were cultured in serum-free medium supplemented with or without 500 nM dexamethasone (DEX, Sigma), 500 μM 8-Br-cAMP (Sigma), or 100 μM insulin (Sigma) for 16 hr.

RNA reverse transcription and real-time PCR

Isolation of total cellular RNA from cultured hepatocytes or liver was performed using the RNeasy Kit (Qiagen). RNA was quantified using the RNA 6000 Nano Assay program of the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA (5 μg) was reverse transcribed using 1 μg oligo (dT) primer and Superscript II Reverse Transcriptase (Invitrogen) at 42°C for 1 hr. Primers for real-time PCR were designed using Primer3 software with a 3′ bias of NCBI mRNA sequences. PCR reaction mixes were assembled using the Brilliant SYBR Green QPCR Master Mix (Stratagene), 10 μM primers, and the included reference dye at a 1:200 dilution. Reactions were performed using the SYBR Green program on the Mx4000 PCR System (Stratagene). Cycling parameters were 95°C for 10 min and 40 cycles of 95°C (30 s), 60°C (1 min), and 72°C (30 s) followed by a melting curve analysis. All reactions were performed in triplicate with reference dye normalization, and the median cycle threshold (C_T) value was used for analysis. Expression levels were normalized to those of TBP (TATA box binding protein) as internal control. The specificity of the PCR amplification was confirmed by dissociation curve analysis. Primer sequences are available upon request.

Chromatin immunoprecipitation assay

The ChIP assay protocol was adapted from methods described previously (Friedman et al., 2004; Weinmann et al., 2001). Mouse liver was minced in cold PBS and passed through a 21 gauge needle. The minced tissue was crosslinked in 1% formaldehyde/PBS for 15 min with constant shaking. Crosslinking was quenched by the addition of glycine to a final concentration of 0.125 M. The tissue was rinsed in PBS and homogenized further with a Dounce homogenizer. After 5 min centrifugation at 13,000 × g, the nuclear pellet was resuspended in cell lysis buffer (5 mM PIPES [pH 8.0], 85 mM KCL, 0.5% NP40, 10 μM aprotinin, 10 μM leupeptin, 1 mM PMSF) and incubated on ice for 15 min. The lysate was divided into 500 μl aliquots and sonicated using the Fisher Scientific Sonic Dismembrator Model 100 with a microtip probe and set at a power output of 4–6 W for three cycles of 20 s each. Insoluble debris was removed by centrifugation and the super-

natant collected and flash frozen in liquid nitrogen. Quantitative PCR was performed on the input DNA fractions to ensure that equal amounts of chromatin DNA were used in all immunoprecipitations. Immunoprecipitations with anti-GR (Santa Cruz Antibody sc-1002), anti-CREB (Santa Cruz Antibody sc-186), and anti-Foxa2 (Khoor et al., 2004) (a kind gift from Dr. J.A. Whitsett) or control IgG were performed as described (Friedman et al., 2004). Immunoprecipitated DNA was then amplified by PCR with primers specific to the promoters of the IGFBP-1 and PEPCK genes and the –2.5 kb enhancer of the TAT gene. For quantification of the relative enrichment of the target DNA fragments in the immunoprecipitated DNA, real-time PCR was performed using the SYBR Green as described above. Primer sequences are available upon request.

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