A Mutant Allele Encoding DNA-Binding-Deficient Foxo1 Differentially Regulates Hepatic Glucose and Lipid Metabolism

(Running title: Cook et al., Foxo1 DNA binding-deficient mutant)

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ABSTRACT

Insulin signaling in liver blunts glucose production and stimulates triglyceride biosynthesis. FoxO1 is required for cAMP induction of hepatic glucose production, and is permissive for insulin’s ability to suppress this process. Moreover, FoxO1 ablation results in unchecked lipogenesis. In this study, we investigated the pleiotropic actions of FoxO1 on glucose and lipid metabolism. To this end, we reconstituted FoxO1 function in mice with a liver-specific deletion of Foxo1 using targeted knock-in of an allele encoding a DNA binding-deficient FoxO1 mutant (L-DBD). Chow-reared L-DBD mice showed defects in hepatic glucose production but normal liver triglyceride content despite increased rates of de novo lipogenesis and impaired fatty acid oxidation in isolated hepatocytes. Gene expression studies indicated that FoxO1 regulates expression of glucokinase via a cell-nonautonomous co-regulatory mechanism while its regulation of glucose-6-phosphatase proceeds via a cell-autonomous action as a direct transcriptional activator. These conclusions support a differential regulation of hepatic glucose and lipid metabolism by FoxO1 based on the mechanism by which it alters expression of key target genes involved in each process.
INTRODUCTION

Hepatic insulin resistance is a hallmark of type 2 diabetes (1). In addition to causing an increase in the rate of glucose production, hepatic insulin resistance is also associated with multiple abnormalities of lipid metabolism, including increased triglyceride (TG) synthesis, accumulation, and secretion as VLDL (2). This association represents an unmet challenge to our basic understanding of the pathophysiology of diabetes, as well as a conundrum for the design of clinically useful insulin sensitizers (3). Thus, the identification of signaling nodes regulating these conjoined processes has widespread implications.

The forkhead transcription factor FoxO1 is a lynchpin of insulin’s control of hepatic glucose production (HGP) (4-6). Liver-specific deletion of FoxO1 (L-FoxO1) impairs cAMP induction of glucose-6-phosphatase (G6pc), resulting in increased insulin sensitivity and fasting hypoglycemia (5;7). Conversely, a constitutively active FoxO1 prevents the ability of insulin to curtail HGP (4;8). In addition, FoxO1 regulates hepatic lipid metabolism in multiple ways (9-11), including via its control of bile acid pool composition (12).

FoxO1 can regulate gene expression either by direct DNA binding, or by acting as a transcriptional co-regulator (13-15). However, it remains unclear whether FoxO1 regulation of hepatic glucose and lipid metabolism requires DNA binding. Understanding the mechanism by which FoxO1 regulates these processes may therefore allow for novel therapeutic approaches to this well-established mediator of diabetes pathophysiology. We have applied a genetic approach to address this question. We reintroduced an allele encoding a DNA-binding deficient (DBD) FoxO1 mutant in mice with a liver-specific FoxO1 knockout (L-FoxO1), and investigated the resulting phenotype. We show that the DBD mutant fails to restore glucose production in vivo.
and is unable to suppress lipogenesis and activate lipid oxidation in primary hepatocytes. The data raise the possibility that FoxO1 controls glucose metabolism by functioning as a transcription factor, while regulating lipid metabolism both as a transcription factor and as a transcriptional co-regulator.
RESEARCH DESIGN & METHODS

Mice and Diets

Male mice aged 12-20 weeks were used for all experiments. L-FoxO1 and L-FoxO1,3,4 mice have been previously described (5;7). Heterozygous DBD knock-in mice were generated through homologous recombination by recombinase-mediated cassette exchange (16;17). Targeting vector and genotyping primer sequences are available upon request. Mice were weaned at 3 weeks of age to standard (chow) diet. Western-type diet (Harlan; 21% anhydrous milk fat, 34% sucrose, 0.2% cholesterol) was fed to animals as indicated beginning at 6 weeks of age for 10 weeks. The Columbia University Institutional Animal Care and Use Committee approved all animal procedures.

Metabolic Testing

Body composition of ad libitum-fed adult male mice was performed via MRI (Bruker Optics). Overnight fasts were conducted for 16 hr, from 17:00 to 09:00. Mice to be re-fed were then given ad libitum access to chow from 09:00 to 13:00. Blood glucose measurements were made from tail vein blood using OneTouch glucose monitor and strips, immediately before sacrifice for terminal procedures (Lifescan). Measurements of insulin and lipids were made by ELISA (Mercodia) and colorimetric assays (Wako for non-esterified fatty acids and cholesterol, Thermo for TG), respectively, using blood collected by cardiac puncture immediately following sacrifice. Intraperitoneal glucose and pyruvate tolerance tests were performed in overnight-fasted mice using a dose of 2 g/kg dextrose (aq) or sodium pyruvate (aq); intraperitoneal insulin tolerance tests were performed in 5-hr-fasted mice using a dose of 0.8 U/kg Novolog insulin (Novo
Nordisk). Oral lipid tolerance test (OLTT) and TG secretion experiments were performed in mice fasted for 5 h. OLTT was performed using olive oil administered orally at 10 µL/g body weight. TG secretion was measured following intraperitoneal injection of Poloxamer 407 (aq) at 10 µL/g. In both cases tail vein blood was collected at indicated time points and TG content measured by colorimetric assay. Hepatic lipids were extracted from ~50 mg snap-frozen tissue samples using the method of Folch as previously described (18). TG and cholesterol content were assayed colorimetrically and normalized to sample weight (12).

**Luciferase Assays**

HEK293 cells were transiently transfected with plasmids encoding either Foxo1<sup>wt</sup>, Foxo1<sup>dbd</sup>, or empty vector as well as 3X IRE-luc reporter plasmid or empty vector using Lipofectamine 2000 (Invitrogen) in DMEM supplemented with 10% FBS. 36 h after transfection of plasmids, media was changed to serum-free DMEM/PCSM. 12 h after serum starvation, cells were lysed and luciferase assay was performed using the Dual Luciferase Reporter Assay System (Promega) in a Monolight 310 luminometer (PharMingen).

**Primary Hepatocyte Studies**

Primary hepatocytes were isolated from male mice via collagenase perfusion as described (8). Following attachment to collagen-coated cultureware, cells were washed with PBS and incubated in serum-free medium (Medium 199 + 1% BSA [always w/v]) overnight except for β-oxidation experiments. For glucose production assay, serum-free medium was replaced with glucose production medium (glucose- and phenol red-free DMEM supplemented with 1% BSA, 3.3 g/L NaHCO<sub>3</sub>, 20 mM calcium lactate, and 2 mM sodium pyruvate). Cells were incubated with 100
µM 8-CPT-cAMP (Sigma) + 1 µM dexamethasone (Sigma) or vehicle for 6 hr. At indicated time points, aliquots of medium were sampled, centrifuged, and glucose content was measured via peroxidase-glucose oxidase assay (Sigma) and normalized to protein content. For gene expression data, following overnight serum starvation cells were incubated for 6 hr in serum-free medium containing either vehicle or 100 µM 8-CPT-cAMP + 1 µM dexamethasone with or without 100 nM insulin (Novolog) and lysed for RNA extraction. For de novo lipogenesis, following overnight serum starvation, medium was changed to serum-free medium with or without 10 nM insulin. After 2 hr, the medium was spiked with 0.6 µCi/mL [1,2-14C]-acetic acid (PerkinElmer Life Sciences) and incubated for an additional 3 hr. Lipids were extracted using 3:2 hexane:isopropanol dried in glass scintillation vials under N2 gas and resuspended in 2:1 chloroform:methanol. For total DNL, resuspended lipids were analyzed by liquid scintillation counting. For measurement of TG, resuspended samples were transferred onto TLC plates using a SpotOn TLC Sample Applicator (Analtech). TLC was performed using a mobile phase of 70:30:1 hexane:diethyl ether: acetic acid. Areas of silica containing TG, as identified by staining with iodine vapor, were scraped into glass scintillation vials and radiolabeled TG were then counted using a liquid scintillation counter (PerkinElmer). Counts were normalized to total cellular protein.

For fatty acid oxidation (FAO), 24h after plating, cells were washed three times with PBS, and incubated for 4 hr with Medium 199 supplemented with 1.5% fatty acid-free BSA, 0.2 mM unlabeled oleic acid, and 1 µCi/mL [1-14C]-oleic acid. Media from each well were transferred to glass Erlenmeyer flasks sealed with rubber plugs containing a suspended center well holding alkalinized filter paper. 70% perchloric acid was injected into each flask. Flasks were then agitated at room temperature for 1 hr. Radiolabeled CO2 content of each filter paper
was then assessed by scintillation counting and normalized to total cellular protein after correcting for specific activity of the original labeling medium in each well.

**mRNA Studies**

Samples of frozen liver (~10 mg) were homogenized in QiaZOL (Qiagen) using a dounce homogenizer. Primary hepatocytes were lysed in QiaZOL. Lysates were extracted with chloroform and the aqueous phase precipitated with 70% ethanol. Samples were processed using the RNeasy Lipid Tissue Mini Kit (Qiagen). 1 µg of RNA was reverse transcribed using the GoScript Reverse Transcription System (Promega). cDNAs were diluted 1:10 and RT-PCR was performed using a DNA Engine Opticon 2 System (Bio-Rad) with SYBR Green (Promega). Primer sequences are available upon request. Gene expression levels were normalized by TATA-binding protein (Tbp) using the $2^{\Delta\Delta Ct}$ method (18).

**Western Blotting**

Frozen livers (~50 mg) were homogenized in or primary hepatocytes directly lysed in ice-cold lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 2% NP-40, 1 mM EDTA, 20 mM NaF, 30 mM Na$_3$P$_2$O$_7$, 0.2% (w/v) SDS, 0.5% (w/v) sodium deoxycholate) supplemented with protease/phosphatase inhibitors (Cell Signaling). Protein concentration was assessed by bicinchoninic acid assay (Sigma). Antibodies used were all purchased from Cell Signaling: FoxO1 C29H4, Akt, pAkt Thr308, GSK3β, and pGSK3β Ser9. Densitometric analysis was performed using ImageJ software (National Institutes of Health).
RESULTS

Generation and Analysis of L-DBD Mice

We generated a Foxo1 allele (Foxo1<sup>dbd</sup>) bearing mutations of residues necessary for DNA binding (N208A, H212R, K219R) (Figure 1A) (19). These mutations abolish binding of FoxO1 to target promoters, but do not affect Akt-mediated nucleocytoplasmic partitioning of the protein (20;21). We confirmed that FoxO1-DBD, unlike wild-type FoxO1, is incapable of driving luciferase activity from a reporter-gene construct containing canonical FoxO1 consensus binding sites (Figure 1B).

Homozygosity for alleles encoding FoxO1-DBD results in embryonic lethality, effectively phenocopying complete FoxO1 loss-of-function (22). To dissect the role of the transcriptional vs. coregulatory functions of FoxO1 in liver, we introduced the Foxo1<sup>dbd</sup> allele in mice bearing a liver-specific Foxo1 knockout. We obtained mice that are heterozygous for the Foxo1<sup>dbd</sup> allele throughout the body, but express only Foxo1<sup>dbd</sup> in liver. Quantitative RT-PCR with allele-specific primers demonstrated the generation of the desired genotypes (Figure 1D). Western blotting analysis verified the absence of FoxO1 protein in liver extracts from L-FoxO1, but not L-DBD mice (Figure 1E), indicating that L-DBD mice express purely DNA binding-deficient FoxO1 in liver. The expression of Foxo3 and Foxo4 were not significantly different from control in either L-FoxO1 or L-DBD livers (Figure 1F), indicating that the loss of FoxO1 is not compensated for by upregulation of other FoxO isoforms (7).
Metabolic Features of Heterozygous Foxo1<sup>dbd</sup> Mice and Hepatocytes

To rule out extra-hepatic metabolic effects of Foxo1<sup>dbd</sup> heterozygosity per se, we compared adult male control (Foxo1<sup>fl/fl</sup>) and heterozygous Foxo1<sup>fl/dbd</sup> (henceforth, DBD-het) with mice heterozygous for a null allele of Foxo1 (FoxO1<sup>fl/–</sup>; henceforth, FoxO1-het) (Figure 1C-D). We found no differences in fasting or re-fed glucose or insulin levels, glucose, pyruvate, or insulin tolerance tests, body weight and composition (Figure 2A-D, Table 1), or in the expression of known hepatic FoxO1 target genes following an overnight fast (Figure 2E). These data are consistent with prior findings in FoxO1-het (4;23). Primary hepatocytes from control, FoxO1-het, and DBD-het mice showed no impairment of basal or cAMP- and dexamethasone-stimulated glucose production (Figure 2F-G). Thus, we conclude that Foxo1<sup>dbd</sup> heterozygosity per se does not result in a metabolic phenotype that might confound the interpretation of data from the L-DBD mouse.

Metabolic Characterization of L-DBD Mice

We analyzed the metabolic features of adult L-DBD male mice. They gained weight at the same rate as L-FoxO1 and control mice (Table 1 and data not shown), and showed no differences in body composition (Table 1). Likewise, there were no differences between L-DBD and control mice in glucose or insulin levels following an overnight fast or a 4-hr re-feed, whereas L-FoxO1 mice showed a modest decrease in re-fed insulin levels compared to controls (Figure 3A-B).

L-DBD mice exhibited an enhancement of glucose tolerance (GTT) identical to L-FoxO1 mice (Figure 3C) (5; 24), suggesting that FoxO1-DBD is effectively a null mutant with respect to glucose tolerance. These results were borne out by pyruvate tolerance tests (PTT), showing similar curves in L-FoxO1 and L-DBD mice (Figure 3D) (5). Intraperitoneal insulin tolerance
tests (ITT) on fasted animals failed to reveal differences between control and L-FoxO1, but showed a modest enhancement in L-DBD mice (Figure 3E). Quantitative analyses of the areas under the curve (AUC) from experiments on multiple cohorts confirmed these conclusions (Figure 3F). Moreover, RT-PCR analysis of RNA extracted from livers of overnight-fasted L-FoxO1 and L-DBD mice showed equally decreased *G6pc, Igfbp1*, and *Irs2* relative to control (Figure 3G). Consistent with our previous reports, we did not detect a significant decrease in *Pck1* in either L-FoxO1 or L-DBD livers [5]. These results indicate that deletion of hepatocellular FoxO1 results in decreased HGP.

**Impaired Glucose Production in Hepatocytes from L-DBD Mice**

Next, we isolated primary hepatocytes from control, L-FoxO1, or L-DBD mice and assessed their ability to generate glucose from pyruvate and lactate either basally or in the presence of CPT-cAMP and dexamethasone (cAMP/dex). Glucose production nearly doubled in control hepatocytes in a time-dependent manner following the addition of cAMP/dex (Figures 4A-B). In contrast, primary hepatocytes from L-DBD mice showed a nearly 30% decrease in basal and cAMP/dex-stimulated glucose production, similar to L-FoxO1 hepatocytes (Figures 4A-B). Consistent with these findings, L-FoxO1 and L-DBD primary hepatocytes showed a >80% decrease in the effect of cAMP/dex on *G6pc* and a ~40% decrease of *Pck1*, as a result of which the suppressive effect of insulin on both genes was virtually abolished (Figure 4C-D) (5).

**Hepatic Lipid Metabolism in L-DBD Mice**

Next, we examined features of hepatic lipid metabolism in L-DBD mice. We found no differences in circulating levels of non-esterified fatty acids, TG, or cholesterol among mice of
different genotypes (Table 1) (5; 12). Liver weight was modestly increased in re-fed, but not in overnight-fasted L-FoxO1 mice (Figure 5A). This difference was due at least in part to increased TG content (Figure 5B) and was not observed in L-DBD mice. There was no difference in liver cholesterol content among genotypes in the fasted or re-fed states (Table 1).

We analyzed different aspects of hepatic lipid handling in order to parse out the mechanism underlying differential liver triglyceride content. Oral lipid tolerance tests and hepatic TG secretion were normal (Figures 5C-E). In contrast, β-oxidation of radiolabeled oleic acid decreased by ~40% in L-FoxO1 hepatocytes, and by ~60% L-DBD hepatocytes (Figure 5F). Analysis of de novo lipogenesis (DNL) demonstrated a ~35% increase in TG synthesis in primary hepatocytes from L-FoxO1 mice under basal as well as insulin-stimulated conditions. Hepatocytes of L-DBD mice showed an even greater increase of ~75% (Figure 5G). The inability of L-DBD hepatocytes to restore lipid oxidation and lipogenesis to their control levels indicates that these effects require direct FoxO1 DNA binding.

To determine the mechanism of the alteration in DNL, we measured levels of several regulators of lipogenesis (Figures 6A-F). We observed significant elevations in fasting levels of stearoyl-CoA desaturase-1 (Scd1) in L-FoxO1, but not L-DBD, mice compared to controls; Srebf1c expression was significantly higher in L-DBD than control mice while there was no significant difference in L-FoxO1. On the other hand, fasting levels of pyruvate kinase (Pklr), a target of the lipogenic transcription factor ChREBP (25), were significantly lower, while those of acetyl-CoA carboxylase-1 (Acaca) were unchanged in L-FoxO1 and L-DBD livers compared to controls. We also sought to determine whether the significant increase of DNL in insulin-treated L-DBD hepatocytes was due to enhanced insulin signaling. However, phosphorylation of Akt
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(T308) and GSK3β (S9) in response to insulin was rather decreased in primary hepatocytes from L-FoxO1 and L-DBD mice (Figure 6G).

We recently showed that FoxO regulation of de novo lipogenesis in the transition to refeeding is partly based on modulation of carbon flux through coordinated activation of G6pc and inhibition of Gck expression during fasting (26). Consistent with these data, we found Gck expression to be significantly increased by over threefold in L-FoxO1 hepatocytes compared to control, while in L-DBD hepatocytes Gck expression was intermediate and not significantly different from control (Figure 6F). FoxO1 inhibition of Gck in vivo therefore likely proceeds in part by a co-regulatory mechanism, as has previously been suggested by reporter-gene studies (27;28). On the other hand, we found no significant differences in Gck expression between genotypes in isolated hepatocytes (Figure 6H). Thus, it appears that FoxO1 regulation of Gck expression is not cell autonomous. On the other hand, measurement of de novo lipogenesis in primary hepatocytes can necessarily reflect only processes that are cell autonomous, for example regulation of G6pc expression or of glucose production generally. This may therefore help us to reconcile the apparent discrepancy between measured in vitro de novo lipogenesis and liver TG levels. Indeed, in primary hepatocytes isolated from L-FoxO1,3,4 mice, which also lack the other two major FoxO isoforms FoxO3 and FoxO4 in liver, expression of Gck was increased by up to nearly 80-fold versus control (Figure 6I) (26). In keeping with the expectation of increased glycolytic flux in the presence of higher Gck expression, the rate of total de novo lipogenesis was increased by over 2.5-fold in L-FoxO1,3,4 hepatocytes (Figure 6J), consistent with previous studies (10).
These experiments indicate that loss of FoxO1 function increases lipogenesis and decreases FFA oxidation, and that FoxO1-DBD fails to restore these functions. We conclude that FoxO1 physiologically inhibits these processes in a DNA binding-dependent manner (Figure 7I).

**Lipid Metabolism in WTD-Fed L-DBD Mice**

We have previously demonstrated that FoxO1 ablation increases hepatic TG deposition in mice fed a Western-type diet (WTD) (12). We therefore placed L-DBD, L-FoxO1, and control mice on WTD for ten weeks and analyzed them in either the *ad libitum*-fed or 5-hr-fasted state. At the completion of the diet there were no significant differences among genotypes in body weight or circulating levels of glucose, insulin, FFA, TG, and cholesterol in either state (Table 1). Liver weight increased by ~25% in fed L-FoxO1 and L-DBD mice (Figure 7A), accompanied by a near doubling of liver TG, although this difference did not reach statistical significance owing to large individual variations (Figure 7B). Histologic examination of liver sections taken from these mice confirmed the presence of hepatic steatosis in L-FoxO1 and L-DBD mice (Figure 7C). These findings were complemented by coordinate increases in levels of mRNA encoding *Fasn*, *Gck*, and *Scd1* (Figures 7D-G).

Finally, we analyzed whether FoxO1-DBD modified the effects of WTD feeding on insulin signaling in liver and primary hepatocytes. Fasting levels of p-Akt and p-GSK3β were uniformly increased in WTD-fed mice of all genotypes, blunting the increase in response to feeding (Figure 7H). This is probably due to hyperinsulinemia (29). We investigated this process by pre-incubating primary hepatocytes with insulin as a surrogate of *in-vivo* hyperinsulinemia (Figure 6H) (30-32). Following this treatment, basal (i.e., “fasted”) phosphorylation levels of Akt and GSK3β increased relative to non-exposed cells, but were not further augmented by acute
insulin treatment ("fed" state). As in fed livers, L-FoxO1 and L-DBD hepatocytes exhibited a trend toward lower levels of Akt and GSK3β phosphorylation following acute insulin challenge. Thus, it appears that FoxO1-DBD does not exert independent effects on insulin signaling.
DISCUSSION

This study demonstrates a mechanistic dissociation of the pleiotropic effects of FoxO1 on hormone- and nutrient-dependent gene expression on the basis of DNA binding (Figure 7I). FoxO1 regulation of gene expression via binding to conserved *cis*-acting elements in target promoters is well characterized, and this study demonstrates that this action of FoxO1 is required for its regulation of hepatic glucose production. Another, less recognized mode of action exists whereby FoxO1 engages in non-DNA-based interactions with components of the transcriptional complex to regulate gene expression (14). The present study indicates that a co-regulatory mode of action is at least partly responsible for FoxO1 regulation of net hepatic TG content. Surprisingly, however, we show that reconstitution of a FoxO1 DNA-binding-deficient allele in mice that lack endogenous FoxO1 fails to restore lipogenesis in isolated hepatocytes. While the conclusion that FoxO1 controls HGP by binding to consensus sites on target promoters was predicted by previous work (8;33), the finding of increased lipogenesis in L-DBD hepatocytes is surprising in the face of normal hepatic TG levels, especially as the inhibition of this process by FoxO1 is more easily reconciled with a co-repressor function (15). Another important finding of the present study is the heretofore-unrecognized effect of FoxO1 ablation, mimicked by the DBD mutant, to reduce FFA oxidation (6;10;11;34).

The segregation of different functional outputs of a transcription factor on the basis of DNA binding-dependent vs. independent actions has been observed in other contexts. For instance, it appears to be a feature of bHLH transcription factors, including Hand2 and Scl (35;36). With regard to FoxO1, a DBD mutant can suppress myogenic differentiation of C2C12 myoblasts as efficiently as wild-type FoxO1 (14). Likewise, constitutively nuclear FoxO1-DBD retains the ability to enhance basal phosphorylation of Akt in liver (9). DNA binding-defective
FoxO1 does not merely represent a hypomorphic variant; expression profiling of cultured cells shows that DBD-mutant FoxO1 induces a distinct class of genes compared to the native protein (13). Our study provides a critical in vivo extension of these results.

Under what circumstances does this dual regulatory mechanism spring into action? At this point, we can only speculate. One possibility is that the multiple post-translational modifications of FoxO1 modulate its ability to bind to DNA without affecting its nuclear localization. In this regard, we and others have shown that, even when FoxO1 is restricted to the nucleus, it is still subject to regulation, either by targeting to sub-nuclear bodies or by modification of its stability (37;38). Supportive of this view is the little remarked-upon observation that nuclear exclusion of FoxO1 is a heterogeneous process (39). In response to insulin or growth factor treatment, it is not uncommon to see cells with cytoplasmic FoxO1 juxtaposed with cells with nuclear FoxO1, indicating that, aside from cellular heterogeneity, factors other than nuclear exclusion modulate FoxO1 function.

Moreover, the interaction of FoxO1 with any given promoter could entail transcriptional and co-regulatory functions. For example, chromatin immunoprecipitation studies reveal that FoxO1-DBD can be recruited to the G6pc promoter without activating expression of the gene (data not shown), likely through interactions with HNF-4α and PGC-1α (28;33). Ergo, the mechanism of FoxO1 regulation of gene expression must be assessed on a case-by-case basis by coupling promoter occupancy with gene expression data.

**Mechanism of FoxO1 Regulation of Lipogenesis and Liver TG Content**

Perhaps the most striking finding of our study is the ability of FoxO1 to regulate liver TG content as a transcriptional co-regulator. Our data suggest a model in which FoxO1 alters lipid
metabolism at multiple levels. First, in a cell-autonomous fashion, FoxO1 represses DNL (and activates FAO) via methods requiring direct binding to DNA. Work from our laboratory has shown that the ratio of $G6pc$ to $Gck$ expression is a reliable indicator of the direction of glucose flux (i.e., of gluconeogenesis/glycogenolysis $\rightarrow$ HGP vs. glycolysis $\rightarrow$ DNL) (26). FoxO1 inhibition of $Gck$, unlike its activation of $G6pc$, is non-cell-autonomous, in keeping with previous reports on neural modulation of insulin-induced $Gck$ expression in liver (40). Thus, in both L-FoxO1 and L-DBD primary hepatocytes, a defect in $G6pc$ expression in the absence of a significant change in $Gck$ would decrease the $G6pc:Gck$ ratio. This, in turn, would impede gluconeogenesis, as observed in this study, while increasing the availability of acetyl-CoA for use in DNL, especially in the presence of insulin (26;41). In L-FoxO1,3,4 primary hepatocytes, $Gck$ expression is frankly increased and $G6pc$ decreased, consistent with the dramatic elevation in DNL compared to control (10;26). Even if altered expression of $G6pc$ per se is not directly responsible, gluconeogenesis is decreased in L-FoxO1 and L-DBD hepatocytes and thus, as in the case of decreased G6Pase action, would be expected to promote lipogenesis (41).

Unlike in primary hepatocytes, fasting and feeding regulation of $Gck$ expression via FoxO1 can proceed as normal in whole liver. Thus, by the end of an overnight fast, L-FoxO1 livers have accumulated significantly more $Gck$ mRNA than controls. At the onset of re-feeding, these livers are better primed for efficient TG synthesis (42-44), hence the increase in re-fed liver TG in L-FoxO1 but not in L-DBD mice relative to controls. On the other hand, L-DBD livers retain a partial ability to suppress $Gck$ expression, thus not allowing them as much of a “head start” on DNL after re-feeding. That the expression of $Gck$ is elevated to the same extent in both L-FoxO1 and L-DBD livers in the WTD-fed state may explain the lack of difference in liver TG levels between these mice, especially given the heightened contribution of DNL to hepatic TG in
the steatotic liver (45;46). Evidently the ability of haplosufficient FoxO1-DBD to regulate \( Gck \) expression in the chow-fed state is lost in the WTD-fed state, thus altering the \( \text{G6pc}:\text{Gck} \) ratio similarly in these mice.

This model is not mutually exclusive with other hypotheses regarding FoxO1 control of hepatic TG, such as via modulation of bile acid metabolism (47). Indeed, this model alone is not sufficient to explain the augmentation in DNL observed in L-DBD primary hepatocytes even relative to L-FoxO1. Thus, it is likely that other mechanisms also come into play. One possibility is a partial dominant-negative effect of FoxO1-DBD on FoxO3a and FoxO4 through sequestration of co-regulatory proteins (48), especially as \( Gck \) expression trends slightly higher in L-DBD cells. In light of the decrease in Akt signaling in L-FoxO1 and L-DBD primary hepatocytes, another possible pathway through which FoxO1 affects lipogenesis is p38, which may mediate a feedback loop between FoxO1 and Akt (49) and thereby regulate DNL (50).

The decrease in Akt phosphorylation that we observe in primary hepatocytes from L-FoxO1 and especially L-DBD mice appears to be at odds with our observation of increased \textit{de novo} lipogenesis \textit{ex vivo} and preserved or even increased liver TG content \textit{in vivo}. We therefore performed intravenous insulin injections in mice of each genotype but did not detect any difference in phosphorylation of Akt or GSK3\( \beta \) between genotypes (data not shown). We also did not detect any differences in the phosphorylation of these signaling intermediaries in the more physiologic context of fasting/re-feeding of chow-reared mice (data not shown). Thus, the difference we see in the phosphorylation of Akt in Fig. 6G appears to be restricted to the setting of primary hepatocytes and may reflect on a greater contribution of a FoxO1 \( \rightarrow \) IRS2 homeostatic loop to the regulation of insulin responsiveness \textit{ex vivo} than \textit{in vivo} (9). In support of this hypothesis, we detected decreased levels of IRS2 at both the mRNA and protein levels in
primary hepatocytes lacking FoxO1 with no difference in phosphorylation or total levels of InsRβ (data not shown).

Similarly, our finding of decreased Akt phosphorylation in the livers of WTD-fed L-FoxO1 and L-DBD mice appears inconsistent with the increased liver TG content and lipogenic gene expression even relative to WTD-fed controls. Again, de novo lipogenesis – a process, again, stimulated by insulin – has been shown to be increased in hepatic steatosis, while we would expect a relative impairment in the face of decreased Akt activation (11;45;46). However, even though Akt and GSK3β phosphorylation are somewhat lower in L-FoxO1 and L-DBD livers, this may not translate into a functional impairment of lipogenic gene expression and lipid biosynthesis. For example, mRNA and protein levels of the lipogenic transcription factor SREBP-1c, the expression of which is stimulated by insulin (30), are not significantly decreased in L-FoxO1 or L-DBD livers, although it does trend lower in the latter. Thus, the decrement in Akt activation is not sufficient to significantly impair its action in this context.

Furthermore, insulin signaling is not absolutely necessary to drive lipogenesis, as carbohydrates per se can induce lipogenic gene expression and ramp up DNL through activation of the ChREBP pathway (25). Well-established ChREBP targets include Fasn and Scd1, both of which are increased in WTD-fed L-FoxO1 and L-DBD livers compared to controls (Fig. 7E,G). Another classic ChREBP target, Pklr, is expressed at equivalent levels in each genotype (data not shown), again consistent with intact ChREBP activity. In conclusion, this study provides new insight into the coordinated regulation of hepatic glucose and lipid metabolism by FoxO1.
AUTHOR CONTRIBUTIONS

J.R.C. designed and performed experiments, analyzed data, and wrote the manuscript. M.M. designed and performed experiments. A.S.B. and T.K. designed and generated Foxo1<sub>dbd</sub> transgenic mice. K.T. performed original breeding and establishment of transgenic mouse colony and provided technical guidance. D.A. designed experiments, oversaw research, and wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

We have no conflict of interest to disclose.

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GUARANTOR INFORMATION

Dr. Domenico Accili is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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FIGURE LEGENDS

Figure 1. Generation and Characterization of the Foxo1<sup>dbd</sup> Allele

(A) Schematic diagram of the FoxO1 primary sequence identifying the residues mutated in Foxo1<sup>dbd</sup>. (B) Reporter-gene assay in 293 cells transfected with either FoxO1<sup>wt</sup>, or FoxO1<sup>dbd</sup>, or empty vector as well as with either 3X IRE-Luc reporter construct or control. Data represent mean ± SEM. * p < 0.05 relative to control by Tukey’s post-hoc analysis following one-way ANOVA. (C) Schematic diagram of mouse models used in this study. (D) Liver RT-PCR using allele-specific primers for total Foxo1, Foxo1<sup>wt</sup> or Foxo1<sup>dbd</sup>. Data represent mean ± SEM. (E) Western blot of liver extracts from fasted mice.

Figure 2. Metabolic Characterization of FoxO1- and DBD-het Mice

(A-B) Glucose (A) and pyruvate tolerance tests (B) in overnight-fasted mice (N≥7 for all genotypes). (C) Insulin tolerance test in 5-hr-fasted mice (N = 5-6 for all genotypes). (D) Quantification of the area under the curve (AUC) for the results in A-C. (E) Gene expression levels in fasted livers assessed by RT-PCR. Data represent mean ± SEM. * p < 0.05 by Tukey’s post-hoc analysis following one-way ANOVA. (F) Glucose production assay performed in medium containing either vehicle (open circles) or cAMP/dex (closed circles). Data are normalized to vehicle-treated control at 2h. (G) Area under the curve quantified from the data in panel F. Data in F-G represent mean ± SEM of a representative experiment of three, each performed in triplicate. ** p < 0.01, *** p < 0.001 by Tukey’s post-hoc analysis following two-way ANOVA.
Figure 3. Glucose Metabolism in L-FoxO1 and L-DBD Mice

(A-B) Glucose (A) and insulin (B) in mice fasted overnight or re-fed for 4 hr. For (A)-(B), **p < 0.01 by Tukey’s post-hoc analysis following two-way ANOVA. (C-D) Glucose (C) and pyruvate (D) tolerance tests in overnight-fasted mice. (E) Insulin tolerance test in 5-hr-fasted mice. For (C)-(E), *p < 0.05, **p < 0.01, ***p < 0.001 for control vs. L-FoxO1; #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001 for control vs. L-DBD; and ¶p < 0.05 for L-FoxO1 vs. L-DBD by Tukey’s post-hoc analysis following one-way ANOVA. (F) Quantification of the area under the curve (AUC) for the results in C-E. (G) Gene expression levels in fasted livers assessed by RT-PCR. For (F)-(G), *p < 0.05, **p < 0.01, ***p < 0.001 by Tukey’s post-hoc analysis following one-way ANOVA. All mice were reared on a chow diet and studies were performed at 16-20 weeks of age, N ≥ 9 for all genotypes in all experiments. Data represent mean ± SEM.

Figure 4. Glucose Production in Primary Hepatocytes

(A) Glucose production assays in cells incubated with glucose-production medium supplemented with vehicle (circles) or with cAMP/dex (squares) for 6 hr. (B) Quantification of the AUC from the data in panel A. (C-D) RT-PCR of G6pc and Pck1 levels in the presence or absence of cAMP/dex and insulin. *p < 0.05 and ****p < 0.0001 by Tukey’s post-hoc analysis following two-way ANOVA. All data are presented as mean ± SEM of a representative experiment of three, each performed in triplicate.
Figure 5. Lipid Metabolism in Mice and Primary Hepatocytes

(A) Liver weight relative to body weight. Mice of each genotype were fasted overnight or fasted overnight and re-fed for 4 hr (N ≥ 10 for each genotype). (B) Liver TG content in fasted or 4-hr-re-fed mice, normalized to total liver weight (N ≥ 6 for each genotype). (C) Oral lipid tolerance test (OLTT) in 5-hr-fasted mice. (D) TG secretion assay in 5-hr-fasted mice (N ≥ 5 for each genotype). (E) Area under the curve (AUC) of OLTT and TG secretion data in (C-D). Data are normalized to a control sample for each procedure. All mice used in (A-D) were reared on chow diet and studies were performed at 16-20 weeks of age. (F) Fatty acid oxidation in primary hepatocytes from control, L-FoxO1, and L-DBD mice. Data shown as average of three independent experiments performed in triplicate. For (A)-(F), * p < 0.05 by Tukey’s post-hoc analysis following one-way ANOVA. (G) De novo lipogenesis of TG in primary hepatocytes isolated from control, L-FoxO1, and L-DBD mice and treated with vehicle or 10 nM insulin. Data shown are the mean ± Satterthwaite-corrected SEM of three independent experiments performed in triplicate. £ p < 0.05 for main effect as assessed by two-way ANOVA and * p < 0.05, ** p < 0.01 using Bonferroni’s post-hoc analysis.

Figure 6. Factors Affecting Lipogenesis in Mice and Primary Hepatocytes

(A-F) Expression of lipogenic genes in livers from either fasted or re-fed mice. N ≥ 7 for all conditions tested. ** p < 0.01 by Tukey’s post-hoc analysis following one-way ANOVA. (G) Insulin signaling in primary hepatocytes treated with saline or with 1 nM insulin for 30 min following treatment for 24 h with either saline or 100 nM insulin. (H-I) Expression of Gck in primary hepatocytes from mice of the indicated genotypes following 6 h of treatment either with 10 nM insulin or vehicle. Data in (H) are the mean of two (insulin) or three (vehicle) independent
experiments performed in triplicate ± Satterthwaite-corrected SEM and analyzed by Tukey’s post-hoc test following one-way ANOVA. (J) Total de novo lipogenesis in primary hepatocytes isolated from L-FoxO1,3,4 or Cre-control mice and treated with vehicle or 10 nM insulin. Data shown are normalized to vehicle-treated control cells and are representative of two independent experiments performed in triplicate. Data in (I-J) are mean ± SEM of a representative experiment of two, each performed in triplicate. # p < 0.05, ## p < 0.01 vs. corresponding control by unpaired, two-tailed student’s t test.

**Figure 7. Metabolic Characterization of Mice on Western-Type Diet**

(A-B) Liver weight relative to body weight (A) and liver TG content (B) in 5-hr-fasted or ad libitum-fed mice. (C) Hematoxylin and eosin staining of liver sections from WTD-fed mice. (D-G) RT-PCR measurements of lipogenic genes in livers from ad libitum-fed or 5-hr-fasted mice. Data represent means ± SEM (N ≥ 7 for each genotype). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. corresponding control by Tukey’s post-hoc analysis following one-way ANOVA. (H) Western blots of livers from ad libitum-fed or 5-hr-fasted mice. Each lane represents pooled liver homogenate from three mice of the same cohort. Relative phosphorylation of Akt and GSK3β are depicted above the respective blot and are calculated densitometrically as the ratio of phosphorylated to total protein. (I) Schematic diagram depicting the mechanism of FoxO1’s metabolic actions in the liver.
TABLE 1. Metabolic features of mice analyzed in this study.

|                | Control (n ≥ 9) | DBD-het (n ≥ 7) | L-FoxO1 (n ≥ 10) | L-DBD (n ≥ 7) |
|----------------|----------------|-----------------|------------------|--------------|
| Chow Body weight (g) Fed | 25.6 ± 0.6 | 25.2 ± 0.4 | 25.1 ± 0.8 | 24.2 ± 0.8 |
| Lean mass % Fed | 80.2 ± 0.6 | 78.8 ± 1.2 | 80.3 ± 1.2 | 81.4 ± 0.2 |
| Fat mass % Fed | 12.0 ± 0.6 | 13.2 ± 0.3 | 11.8 ± 1.2 | 10.5 ± 0.4 |
| Fluid mass % Fed | 7.7 ± 0.3 | 8.0 ± 0.1 | 7.8 ± 0.2 | 8.1 ± 0.3 |
| Free fatty acids (mEq/L) Fasted | 1.09 ± 0.19 | 0.88 ± 0.08 | 1.23 ± 0.09 | 1.14 ± 0.13 |
| Re-fed | 0.19 ± 0.02 | 0.21 ± 0.03 | 0.20 ± 0.03 | 0.21 ± 0.03 |
| TG (mg/dL) Fasted | 76 ± 6 | 67 ± 3 | 84 ± 4 | 72 ± 12 |
| Re-fed | 102 ± 11 | 106 ± 11 | 92 ± 7 | 121 ± 9 |
| Cholesterol (mg/dL) Fasted | 92 ± 4 | 98 ± 4 | 102 ± 5 | 96 ± 6 |
| Re-fed | 92 ± 3 | 84 ± 4 | 85 ± 4 | 90 ± 3 |
| Liver cholesterol (mg/g liver) Fasted | 1.66 ± 0.16 | 1.94 ± 0.17 | 1.56 ± 0.13 | 1.75 ± 0.11 |
| Re-fed | 1.17 ± 0.10 | n.d. | 1.10 ± 0.07 | 1.12 ± 0.13 |
| WTD Body weight (g) Fed | 33.2 ± 1.2 | 34.9 ± 1.8 | 38.0 ± 1.0 | 36.0 ± 2.8 |
| Glucose (mg/dL) Fed | 218 ± 3 | 223 ± 2 | 190 ± 4 | 203 ± 7 |
| Fasted | 241 ± 14 | 242 ± 10 | 214 ± 7 | 220 ± 9 |
| Insulin (ng/mL) Fed | 2.87 ± 0.22 | 2.43 ± 0.55 | 4.22 ± 0.95 | 6.66 ± 3.15 |
| Fasted | 2.58 ± 0.30 | 2.11 ± 0.27 | 2.68 ± 0.32 | 2.25 ± 0.42 |
| Free fatty acids (mEq/L) Fed | 0.76 ± 0.04 | 0.72 ± 0.08 | 0.71 ± 0.05 | 0.79 ± 0.06 |
| Fasted | 0.71 ± 0.06 | 0.70 ± 0.05 | 0.74 ± 0.05 | 0.71 ± 0.07 |
| TG (mg/dL) Fed | 113 ± 11 | 118 ± 12 | 104 ± 7 | 118 ± 7 |
| Fasted | 59 ± 6 | 67 ± 6 | 53 ± 3 | 63 ± 9 |
| Cholesterol (mg/dL) Fed | 296 ± 21 | 344 ± 27 | 364 ± 28 | 428 ± 42 |
| Fasted | 337 ± 34 | 342 ± 27 | 398 ± 31 | 376 ± 55 |

None of the differences between genotypes reach statistical significance by Tukey’s post-hoc test following one-way ANOVA.
**Diabetes**

**FoxO1**

N

- T24
- H212R
- N208A
- K219R
- S256
- S319

Forkhead (DNA-binding) domain (158-248)

Transactivation domain (249-652)

**Foxo1 alleles (Figs. 1B,C):**

- **Foxo1**<sup>wt</sup> (floxed)
- **Foxo1**<sup>dbd</sup> (not floxed)
- **Foxo1** (null)

**B**

![Graph showing relative luciferase activity](image)

**Control**

- 3X IRE-Luc

**DBD-het**

- AT-Cre<sup>+</sup> Foxo1<sup>fl/fl</sup>

**L-FoxO1**

- AT-Cre<sup>+</sup> Foxo1<sup>fl/dbd</sup>

**L-DBD**

- AT-Cre<sup>+</sup> Foxo1<sup>fl/dbd</sup>

**D**

![Graph showing mRNA levels](image)

**Control**

- Total Foxo1
- Foxo1-WT
- Foxo1-DBD

**E**

![Western blot analysis](image)

**Genotypes (Figs. 1D,F):**

- **Control**
- **L-FoxO1**
- **L-DBD**

**F**

![Graph showing mRNA levels](image)

**Foxo3**

- **Control**
- **DBD-het**
- **L-FoxO1**
- **L-DBD**

**Foxo4**

- **Control**
- **DBD-het**
- **L-FoxO1**
- **L-DBD**
**A**

Glucose production AUC (AU)

**B**

GTT

PTT

ITT

**C**

Relative AUC (AU)

**D**

GTT

PTT

ITT

**E**

mRNA (AU)

**F**

Glucose production (AU)

**G**

Glucose production AUC (AU)

Control (AT-CreFoxo1\(^{fl/fl}\))

FoxO1-het (AT-CreFoxo1\(^{fl/-}\))

DBD-het (AT-CreFoxo1\(^{fl/dbd}\))

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Diabetes
A

Glucose production (AU)

Time (h)

B

Glucose production AUC (AU)

Vehicle  cAMP/dex

C

mRNA (AU)

G6pc

D

mRNA (AU)

Pck1

-  +  +  cAMP/dex

-  -  +  Insulin
