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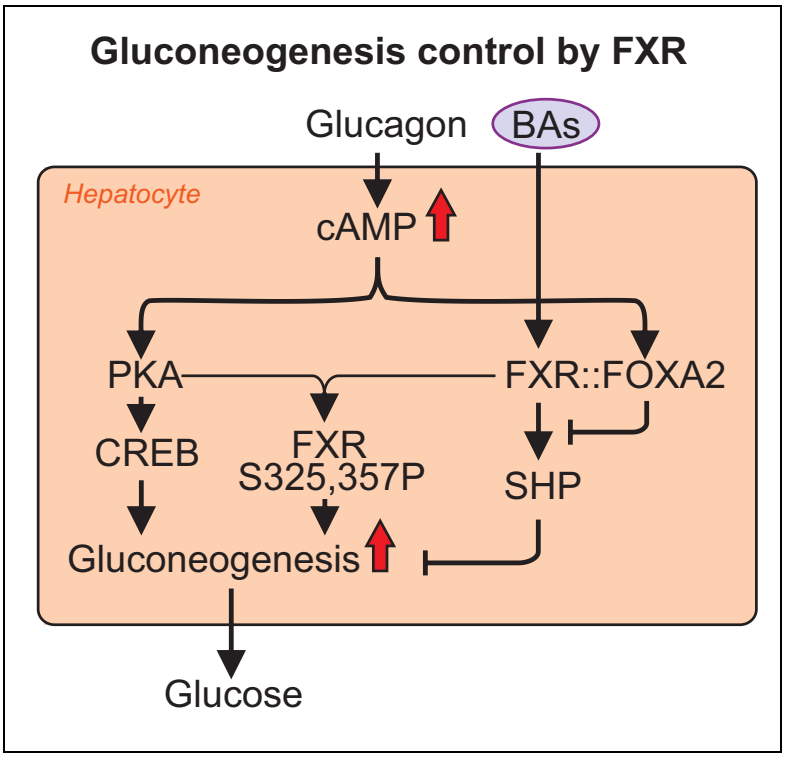
Graphical abstract



Highlights

- FXR synergizes with the glucagon signaling pathway to regulate hepatic gluconeogenesis *in vivo* and *in vitro*.
- FXR positively regulates gluconeogenic genes through PKA-catalyzed phosphorylation.
- The glucagon-regulated transcription factor FOXA2 negatively regulates FXR's ability to activate the expression of the SHP.

Authors

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Lay summary

Activation of the nuclear bile acid receptor FXR regulates gene expression networks, controlling lipid, cholesterol and glucose metabolism, which are mostly effective after eating. Whether FXR exerts critical functions during fasting is unknown. The results of this study show that FXR transcriptional activity is regulated by the glucagon/protein kinase A and the FOXA2 signaling pathways, which act on FXR through phosphorylation and protein-protein interactions, respectively, to increase hepatic glucose synthesis.

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The nuclear bile acid receptor FXR is a PKA- and FOXA2-sensitive activator of fasting hepatic gluconeogenesis

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Background & Aims: Embedded into a complex signaling network that coordinates glucose uptake, usage and production, the nuclear bile acid receptor FXR is expressed in several glucose-processing organs including the liver. Hepatic gluconeogenesis is controlled through allosteric regulation of gluconeogenic enzymes and by glucagon/cAMP-dependent transcriptional regulatory pathways. We aimed to elucidate the role of FXR in the regulation of fasting hepatic gluconeogenesis.

Methods: The role of FXR in hepatic gluconeogenesis was assessed *in vivo* and in mouse primary hepatocytes. Gene expression patterns in response to glucagon and FXR agonists were characterized by quantitative reverse transcription PCR and microarray analysis. FXR phosphorylation by protein kinase A was determined by mass spectrometry. The interaction of FOXA2 with FXR was identified by cistromic approaches and *in vitro* protein-protein interaction assays. The functional impact of the crosstalk between FXR, the PKA and FOXA2 signaling pathways was assessed by site-directed mutagenesis, transactivation assays and restoration of FXR expression in FXR-deficient hepatocytes in which gene expression and glucose production were assessed.

Results: FXR positively regulates hepatic glucose production through two regulatory arms, the first one involving protein kinase A-mediated phosphorylation of FXR, which allowed for the synergistic activation of gluconeogenic genes by glucagon, agonist-activated FXR and CREB. The second arm involves the inhibition of FXR's ability to induce the anti-gluconeogenic nuclear receptor SHP by the glucagon-activated FOXA2 transcription factor, which physically interacts with FXR. Additionally, knockdown of *Foxa2* did not alter glucagon-induced and FXR agonist enhanced expression of gluconeogenic genes, suggesting that the PKA and FOXA2 pathways regulate distinct subsets of FXR responsive genes.

Conclusions: Thus, hepatic glucose production is regulated during physiological fasting by FXR, which integrates the glucagon/cAMP signal and the FOXA2 signal, by being post-translationally modified, and by engaging in protein-protein interactions, respectively.

Lay summary: Activation of the nuclear bile acid receptor FXR regulates gene expression networks, controlling lipid, cholesterol and glucose metabolism, which are mostly effective after eating. Whether FXR exerts critical functions during fasting is unknown. The results of this study show that FXR transcriptional activity is regulated by the glucagon/protein kinase A and the FOXA2 signaling pathways, which act on FXR through phosphorylation and protein-protein interactions, respectively, to increase hepatic glucose synthesis.

Introduction

Glucose supply to tissues is maintained through a complex regulatory network mostly driven by the pancreatic hormones insulin and glucagon which control glucose use, storage and synthesis. Through glycogenolysis and gluconeogenesis, the liver contributes to ~70–80% of glucose production during an overnight fast,[1] the remaining 30% coming from intestinal and kidney gluconeogenesis in physiological conditions.[2,3]

Glucagon-induced gluconeogenesis is the only source of glucose when glycogen stores are exhausted during fasting. Gluconeogenic substrates (lactate, alanine, and pyruvate) are funneled to mitochondria to generate oxaloacetate (OAA) through biotin-dependent pyruvate carboxylase. Cytosolic OAA is decarboxylated and phosphorylated to yield phosphoenolpyruvate (PEP), the primary building block of glucose, through phosphoenolpyruvate carboxylase (PEPCK), a rate-limiting enzyme of gluconeogenesis. Glycerol from triglyceride breakdown also contributes by varying extents to gluconeogenesis, feeding into the gluconeogenic pathway as glyceraldehyde-3-phosphate to generate fructose 1,6-biphosphate, the substrate of fructose 1,6-bisphosphatase (FBP1) which irreversibly yields fructose 6-phosphate (F6P).[4] The regulation of hepatic glucose production (HGP) is achieved through a sophisticated signaling network involving post-translational protein modifications, allosteric regulation and transcription factor activation and

Keywords: Liver; Gluconeogenesis; Glucagon; PKA; Transcription; Nuclear receptor; Bile acid; FXR; FOXA2.
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repression[5,6] which essentially control the gene expression of three rate-limiting enzymes, glucose-6-phosphatase (*G6pc*), *Fbp1* and PEPCK (*Pck1*)[7] in a glucagon/cAMP-dependent manner.[8–11]

Multiple transcription factors orchestrate hepatic gluconeogenesis, such as PPARγ coactivator 1α (PGC1α), Forkhead box protein O1 (FOXO1), small heterodimer partner (SHP) and cAMP response element-binding protein (CREB), by regulating the expression of gluconeogenic genes.[12] The nuclear bile acid (BA) receptor farnesoid X receptor (FXR) is a key regulator of essential hepatic functions.[13] Besides acting on BA homeostasis and lipid metabolism, FXR participates in the regulation of glucose homeostasis. Sequestration of intestinal BAs decreases plasma glucose in patients with type 2 diabetes.[14] This effect correlates with increased GLP-1 expression and secretion which may be attributed at least in part to FXR-mediated ChREBP inhibition in intestinal L-cells,[15] a mechanism equally operative in the liver, where FXR represses glycolysis.[16,17] Liver FXR controls HGP through the regulation of *Pck1* and *G6pc* gene expression. Indeed, *in vivo* administration of natural or synthetic FXR agonists improves glucose tolerance, decreases *Pck1* and *G6pc* expression and accordingly diminishes HGP in rodent models of obesity or diabetes.[18–22] Gene deletion studies mostly support a repressive role of FXR on gluconeogenesis,[18,20] consistent with the reported inhibitory action of SHP, a direct FXR target gene, on gluconeogenic gene expression *in vivo*[20] and *in vitro*.[23,24] However, whereas FXR activation may improve glucose metabolism by downregulating HGP in pathological models of obesity and diabetes, its role in physiological fasting conditions appears different. $Fxr^{-/-}$ mice develop transient hypoglycemia and exhibit a delayed increase in HGP upon fasting.[25–28] In addition, the induction of hepatic *G6pc* and *Pck1* expression is significantly blunted in fasting $Fxr^{-/-}$ mice.[26,29] Taken together, these data point to pro-gluconeogenic properties of FXR during fasting. Since the molecular mechanisms of FXR action in fasting are unknown, we studied the role of FXR in the control of HGP and gluconeogenic gene expression in physiological fasting.

Materials and methods

Mice strains and experimentation

Male homozygous $Fxr^{-/-}$ and wild-type littermates ($Fxr^{+/+}$) mice[30] were bred on the C57BL/6J genetic background and housed in a specific-pathogen-free facility on a 12 h light/12 h dark cycle with free access to water and to a standard chow diet (UAR A04, Villemoison/Orge, France) unless stated otherwise. Mice were sacrificed by cervical dislocation after 6 h fasting and tissues were removed and immediately frozen in liquid nitrogen.

Mouse primary hepatocytes (MPHs) were isolated from 8–10-week-old C57Bl6/J male mice from Charles River (Saint Germain sur l'Arbresle, France) or from homozygous $Fxr^{-/-}$ and wild-type ($Fxr^{+/+}$) male mice housed on a 12 h light/12 h dark cycle with free access to water and to a standard chow diet unless stated otherwise.

Overnight fasted mice (5 pm–9 am) were injected intraperitoneally with sodium pyruvate (P4562, Sigma) (2 g/kg body mass). Blood glucose levels were measured from the tail vein at the indicated time points using an automatic glucose monitor (One Touch, Lifescan).

All experimental protocols conform to the ARRIVE guidelines and were approved by the Lille Pasteur Institute ethical committee (Agreement # 20152152254461) and carried out in agreement with European Union and French Ethical Guidelines.

Glucose production

Glucose production assessment: MPHs were maintained overnight (16 h) in DMEM medium containing 5.5 mM glucose (#21885, Gibco-Life Technologies) supplemented with 0.1% BSA, 100 nM dexamethasone, 1% glutamine and 1% penicillin/streptomycin and treated as indicated. MPHs were then cultured in DMEM without glucose (#11966, Gibco-Life Technologies) supplemented with 100 nM dexamethasone, 20 mM sodium DL-lactate (L1375, Sigma-Aldrich), 2 mM sodium pyruvate (#11360, Gibco-Life Technologies) and the indicated treatments for 8 h. Glucose secretion was measured using the Glucose (GO) assay kit (GAGO-20, Sigma-Aldrich).

Statistical analysis

Raw data were analyzed using Graph Pad Prism 7.0. Results are expressed as mean ± SEM and groups were compared using either a *t* test or a 2-way ANOVA as indicated in figure legends.

For further information regarding chemicals, cell culture, MPH preparation, plasmids and adenoviruses, protein extraction, purification and analysis, *in vitro* phosphorylation and mass spectrometry analysis, gene expression assays, ChIP-PCR and ChIP-seq protocols please refer to the supplementary information and CTAT table.

Results

FXR enhances cAMP-induced glucose production by mouse primary hepatocytes

To evaluate the contribution of FXR to fasting HGP, C57Bl6/J $Fxr^{+/+}$ or $Fxr^{-/-}$ mice (Fig. S1A) were subjected to fasting to deplete hepatic glycogen stores and an intraperitoneal pyruvate tolerance test was performed. The glycemic excursion in response to pyruvate was blunted in C57Bl6/J $Fxr^{-/-}$ mice when compared to $Fxr^{+/+}$ mice (Fig. 1A), suggesting a positive contribution of FXR to HGP. The ability of FXR to regulate glucose production by MPHs from $Fxr^{+/+}$ or $Fxr^{-/-}$ mice was assayed after an 8 h-treatment with the synthetic FXR agonist GW4064, with or without the protein kinase A (PKA) activator 8-CPT-cAMP (Fig. 1B), under experimental conditions defined as optimal for observing glucose production by isolated hepatocytes (Fig. S1B,C). GW4064 did not significantly affect GP, whereas 8-CPT-cAMP and glucagon increased glucose production (Fig. S2A). Simultaneous activation of PKA and FXR with GW4064 or the natural FXR agonist CDCA potentiated glucose production (Fig. 1B, S2B). While showing a lower basal glucose production, $Fxr^{-/-}$ MPHs were similarly sensitive to PKA stimulation as $Fxr^{+/+}$ MPHs [fold change (FC) ~2], but were insensitive to FXR agonism (Fig. 1B), like wild-type MPHs in which *Fxr* mRNA expression was knocked down (Fig. S3A,B). The expression of the gluconeogenic *Foxo1* transcription factor did not vary between the two genotypes and was mildly induced by glucagon (Fig. S3C,D). *Foxo1* knockdown, while affecting basal glucose production by MPHs, did not affect the potentiation of cAMP by the FXR agonist (Fig. S3E,F). We conclude that FXR activation enhances HGP under conditions mimicking fasting, independently of FOXO1.

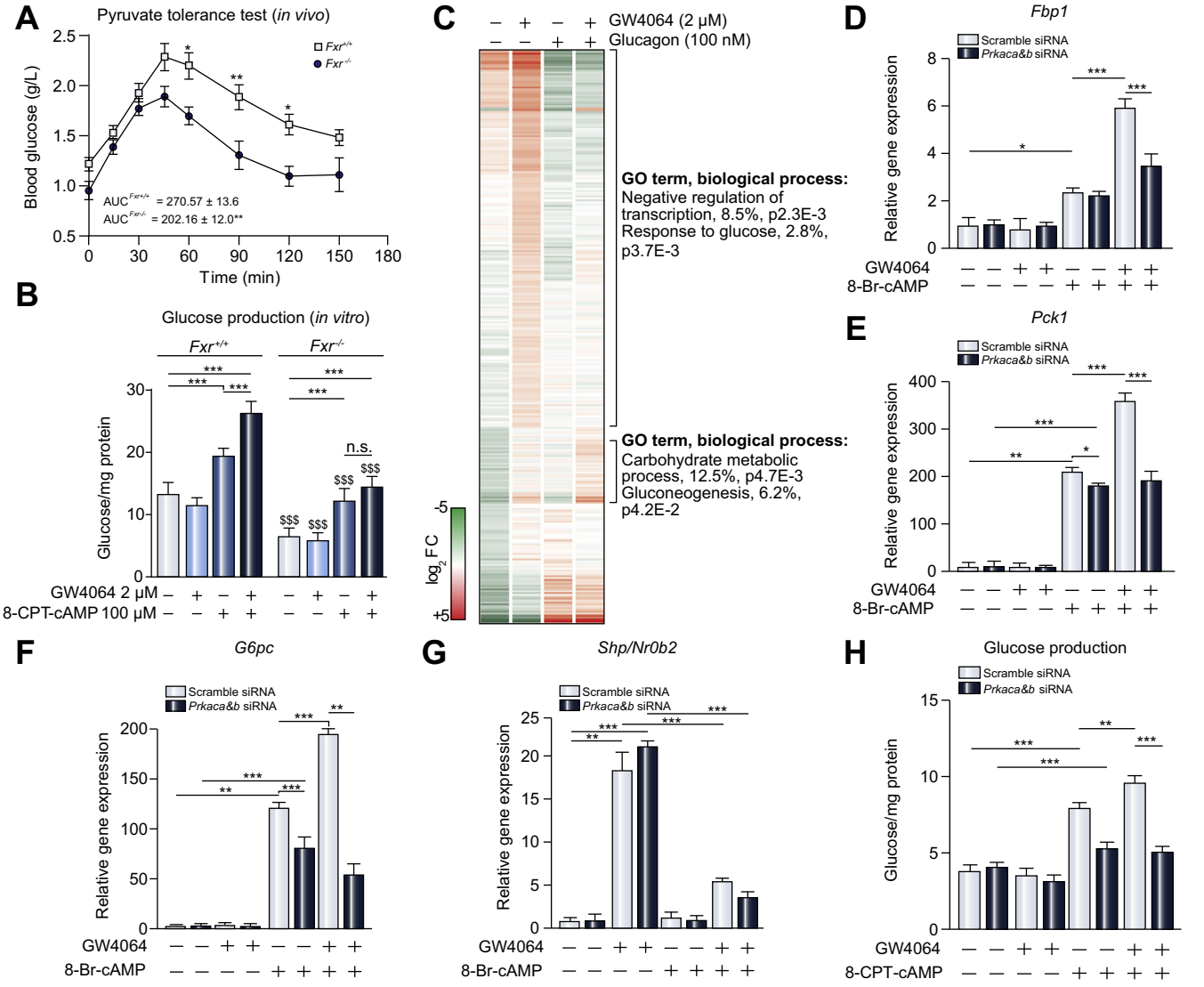

Fig. 1. FXR is a positive regulator of hepatic glucose production. (A) Pyruvate tolerance test in wild-type and FXR-deficient mice. Fasted mice were injected with sodium pyruvate. Blood glucose concentration was assayed at the indicated times. (B) GP in wild-type and FXR-deficient MPHs. GP by MPHs was assayed after an 8 h-treatment. (C) Gene expression pattern in MPHs. RNAs were extracted after a 6 h-treatment and analyzed on Affymetrix MoGene arrays. The heatmap was generated using Genespring. The most relevant gene ontology terms (biological process) are shown with the enrichment percentage and *p* value. D-G) Protein kinase A is selectively involved in the regulation of a subset of genes. MPHs transfected with the indicated siRNAs were treated as indicated and gene expression measured by RT-qPCR. (H) GP in PKA-depleted MPHs. Conditions and presentation of results as in (B). (A, B, H) Results are the mean +/− SEM (n = 3) and values were compared using a 2-way ANOVA with a Bonferroni *post hoc* test. (D-G) Results are the mean +/− SEM (n = 5) and are expressed relative to the basal level measured in untreated cells arbitrarily set to 1. Data were compared using a 2-way ANOVA and a Tukey *post hoc* test. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.005; for intragroup comparisons. $^{\$\$\$}p$ <0.005 for intergroup comparisons. GP, glucose production; MPH, mouse primary hepatocytes; RT-qPCR, quantitative reverse transcription PCR.

The glucagon/PKA pathway differentially regulates FXR-driven gene expression

The crosstalk between the glucagon/cAMP and FXR signaling pathways was investigated at the gene expression level. Gene expression patterns from naive or glucagon-treated MPHs, with or without GW4064 stimulation, were assayed at normal glucose concentrations to avoid interference with the energy- or glucose-sensitive AMP-activated protein kinase (AMPK) or hexosamine pathways, respectively.[31,32] A significant portion of upregulated genes became refractory to FXR agonism in the presence of glucagon (Fig. 1C). Conversely, a set of 71 transcripts either poorly modulated, or not modulated, by GW4064 became sensitive to FXR agonism in the presence of glucagon (Fig. 1C and Fig. S4A,B), with 45 out of 71 transcripts being induced in physiological or prolonged fasting conditions *in vivo* (Table S1). A gene ontology term enrichment analysis identified transcription- and glucose metabolism-related processes as significantly represented within these two gene clusters (Fig. 1C). Further examination using gene and protein assays pointed to known FXR-regulated genes and gluconeogenic genes, as becoming sensitive (*Fbp1, Pck1, G6pc, Slc51b/Ostβ*) or refractory (*Shp/Nr0b2, Abcb11/Bsep and Abcc4/Mdr3*) to FXR synthetic or natural agonists in the presence of glucagon in an FXR-dependent manner (Fig. S4B-D, S5, S6A-F).

The expression of catalytic PKA subunits α and β (*Prkaca* and *Prkacb*, Fig. S6G) was necessary for the synergistic induction of *Fbp1*, *Pck1* and *G6pc* by FXR and cAMP (Fig. 1D-F) but did not alter Shp/*Nr0b2* responsiveness to FXR agonism in the presence

of a cAMP analogue (Fig. 1G). Importantly, these transcriptional effects translated into an altered biological output since cAMP-induced and cAMP/FXR-regulated glucose production were blunted upon PKA knockdown (Fig. 1H). Thus, the potentiation of glucagon-stimulated glucose production by FXR agonism relates to a combinatorial upregulation of gluconeogenesis-promoting, glucagon/PKA-regulated genes (*Fbp1, Pck1, G6pc*) and downregulation of the gluconeogenesis-inhibiting, PKA-independent *Shp* gene.

Phosphorylation of FXR S325 and S357 by PKA regulates FXR activity

We then assessed whether FXR is a direct PKA target. Purified mouse FXRα1 or FXRα3 were used in an *in vitro* phosphorylation assay, in which an efficient PKA-dependent transfer of radiolabeled phosphate to both FXR isoforms was observed (Fig. S7A) and of similar magnitude to that observed with protein kinase C alpha, a previously identified FXR kinase (Fig. S7B).[33] Mass spectrometry identified phosphopeptides (Fig. 2A, Fig. S8) that corresponded to three bioinformatically-predicted and two *de novo* identified phosphoserines (S132, S151, S357 and S114, S325 respectively, Fig. 2A). GSK3β, which is regulated by PKA,[34] was unable to phosphorylate FXR in similar conditions (Fig. S7C).

FXR transcriptional activity in response to PKA activation was tested using a transactivation assay in which FXR is expressed as a Gal4-DNA binding domain fusion protein (one-hybrid assay, Fig. 2B). While FXR was insensitive to PKA stimulation in the absence of the FXR agonist GW4064 (Fig. 2B), activation of PKA by the adenylate cyclase activator forskolin increased agonist-induced FXR activity (Fig. 2B). A similar potentiating effect was observed when using an IR1-driven reporter gene, mFXRα1 (Fig. 2C). S325 and S357 were necessary and sufficient for PKA-enhanced FXR activity (Fig. S9A, Fig. 2C). As individual mutations of S325 and S357 to alanine affected neither the response to GW4064 (Fig. 2C) nor FXR protein stability (Fig. S9B), we concluded that these mutations did not introduce major structural changes.

The contribution of FXR phosphorylation by PKA to HGP was characterized in *Fxr*$^{-/-}$ cells, in which wild-type or S325,357A FXR was expressed (Fig. 2D). While wild-type FXR restored

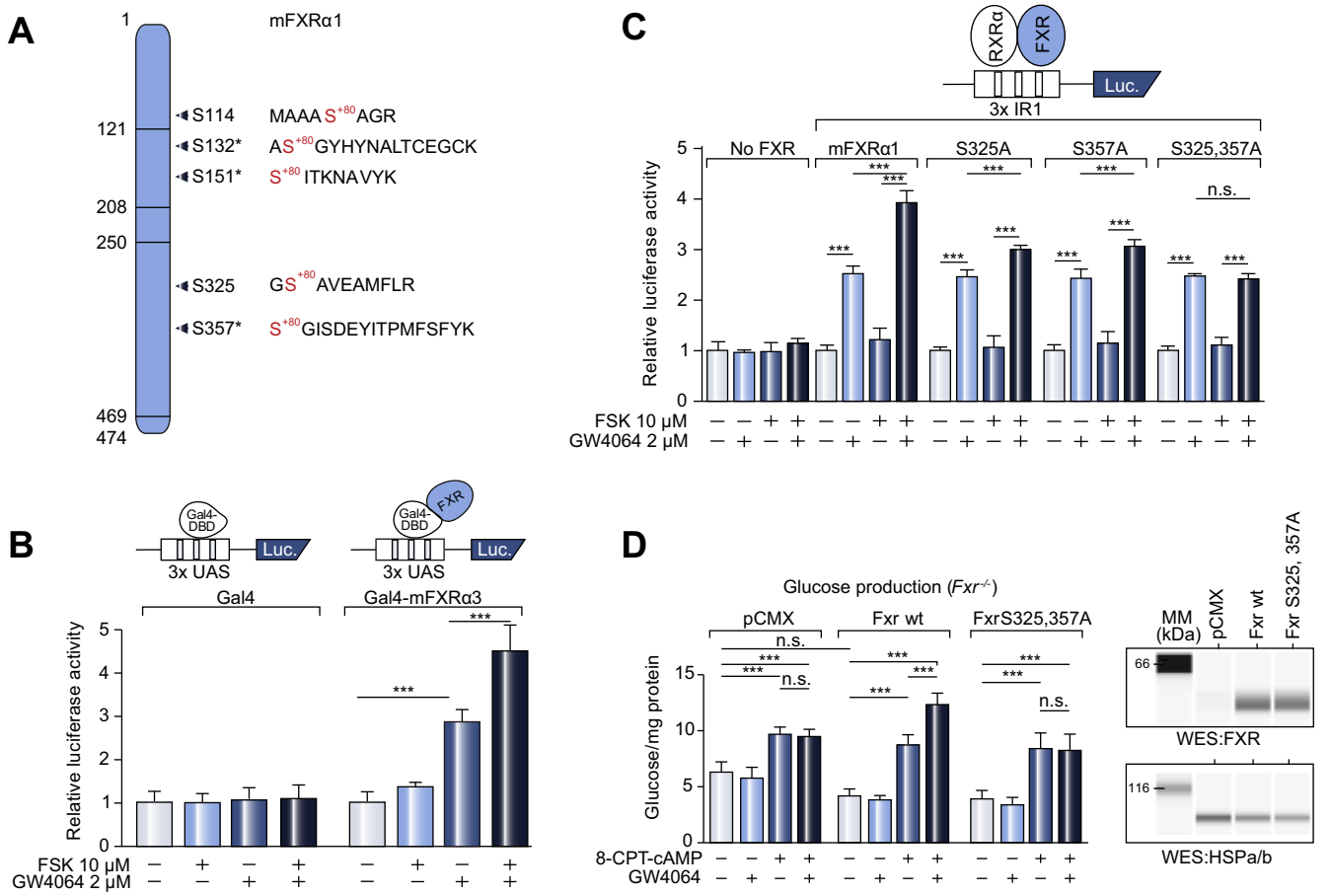

Fig. 2. Protein kinase A regulates FXR transcriptional activity and ability to regulate hepatic glucose production. (A) Identification of phosphopeptides by ETD mass spectrometry. Tryptic (phospho)peptides from *in vitro* phosphorylated recombinant, purified mFXRα1 were identified by LC-MS/MS. *Indicates phosphopeptides containing a PKA phosphorylation consensus site. (B) PKA activation potentiates FXR transcriptional activity in a one-hybrid assay. HEK293A cells were transfected as depicted and treated for 6 h. (C) FXR S325 and S357 are required for PKA-mediated potentiation of FXR transcriptional activity. HEK293A cells were transfected and treated as depicted. (D) GP in Fxr$^{-/-}$ MPHs overexpressing either the wild-type or S325,357A mFXRα1. MPHs were transfected with pCMX-based expression vectors. Upper panel: GP was assessed as in Fig. 1A 24 h after transfection. Results were expressed and values were compared as in Fig. 1A. Lower panel: FXR protein content in MPHs. pCMX: empty vector (control). (B, C) Results from luciferase assays were expressed as normalized relative light units relative to basal conditions (vehicle treatment only) arbitrarily set to 1. Values represent the mean +/− SEM (n = 3–6) which were compared using a 2-way ANOVA and a Tukey *post hoc* test. *p <0.05, **p <0.01, ***p <0.005. ETD, electron-transfer dissociation; GP, glucose production; LC-MS, liquid chromatography-mass spectrometry; MPH, mouse primary hepatocyte. (This figure appears in colour on the web.)

the GW4064-induced potentiation of glucose production triggered by PKA activation, the FXR mutant, which cannot be phosphorylated, did not convey this synergistic response. Importantly, *G6pc*, *Pck1* and *Fbp1* gene expression followed a similar pattern (Fig. S9C-E).

PKA potentiates FXR-induced gene transcription

How FXR and PKA cooperatively regulate gluconeogenesis was investigated for the two cAMP-regulated gluconeogenic genes *Fbp1* and *Pck1*. FXR binding site(s) coordinates were identified in the *Fbp1* and *Pck1* upstream regulatory regions (URRs) using ChIP-seq data from mouse livers after fasting[32,35] (Fig. 3A, S10A), which overlapped with CREB DNA binding sites.[36] The DNA sequences encompassing both FXR and CREB co-binding regions (CBRs) were cloned upstream of a luciferase reporter gene to assess their functionality. Transactivation assays (Fig. 3B, S10B,C) showed that the PKA-FXR cooperativity was maintained on these chimeric constructs when using the wild-type FXR. However, FXR S325,357 to A mutations ablated this cooperative effect in line with the observed effect on HGP. Endogenous gene regulation was measured in MPHs (Fig. 3C, S10D) in which either *Creb1* or *Fxr* expression was knocked down (Fig. 3D, S3B). In line with our previous results, FXR was required for the cooperative response with glucagon (Fig. 3C, S10D). CREB contributed to the cooperative induction, in agreement with FXR co-binding with CREB to both the *Fbp1* (Fig. 3E,F) and *Pck1* URRs (Fig. S10E,F). Strikingly, the FXR density in URRs was highest when MPHs were simultaneously treated with GW4064 and glucagon (Fig. 3E, S10E), suggesting that increased transcription may stem from increased or stabilized binding of FXR to DNA.

Glucagon abrogates FXR-mediated transcription of *Shp/Nr0b2* URR through FOXA2

FXR-induced expression of *Nr0b2/Shp* is counteracted by glucagon and cAMP analogues in a PKA-independent manner (Fig. 1G). Although many mechanisms may account for the observed inhibition of FXR transcriptional activity, we hypothesized that it results from a functional interaction with a glucagon/cAMP-sensitive transcriptional regulator (TR). Since a similar regulation pattern of *Nr0b2/Shp* was observed in both hepatoblastoma HepG2 cells and MPHs (Fig. 4A, 4C, S11A-C) and confirmed at the protein level (Fig. 4C), we tested this hypothesis by determining which TRs co-localize closely with FXR in the HepG2 cell genome by comparing the FXR cistrome[31] to that of 51 TRs generated by the ENCODE Consortium[37] (Fig. S12A).

FXR co-localizing TRs included the FXR obligate heterodimerization partner RXRα,[38] validating this approach to identify direct, functionally relevant, protein-protein interactions. Forkhead box A2 transcription factor (FOXA2) appeared as a potential candidate (Fig. S12A), as its activity is inhibited by insulin and enhanced by glucagon in mouse livers.[39,40] FOXA2 co-occupied FXR genomic binding sites, with 1,642 FOXA2 binding sites being detected within +/− 100 bp from the center of the detected 11,574 FXR peaks (Fig. 4B). These CBRs were enriched in consensus binding sequences for FXR and FOXA2 as previously reported,[41] Distances between FXR and FOXA2 binding sites diverged from the ones obtained by a random repartition of the same number of ATF3 binding regions (Fig. 4B). A similar analysis comparing the liver FXR and FOXA2 cistromes in the liver genomes of mice after fasting[35,42] yielded a figure similar to that extracted from HepG2 cell cistromes,

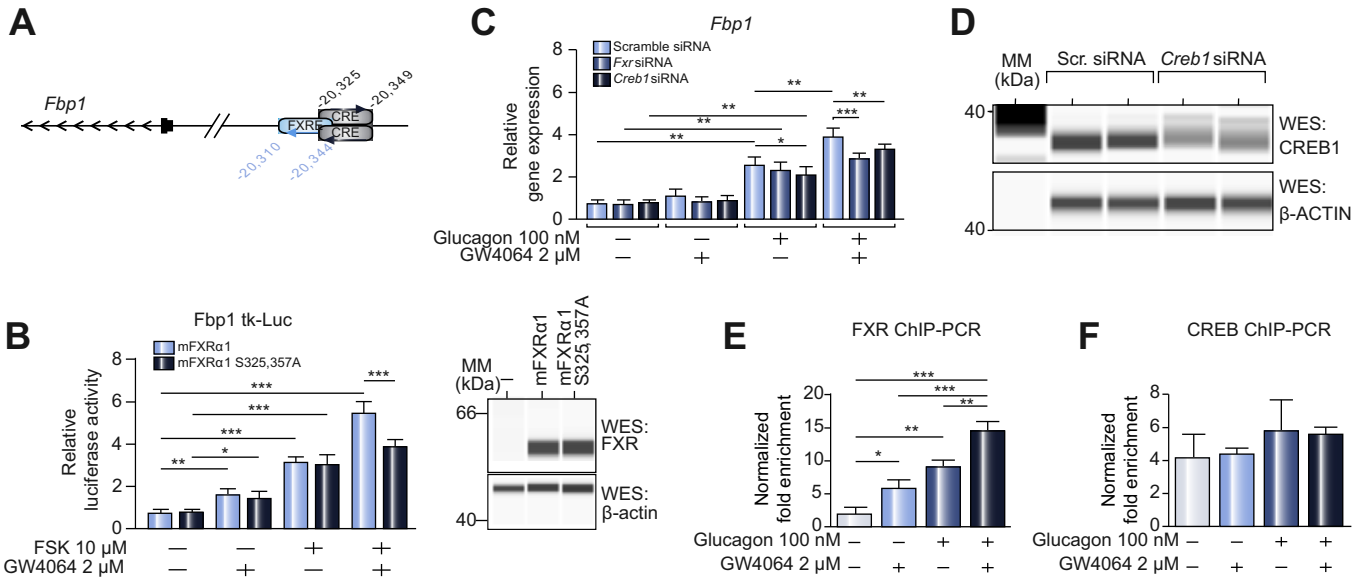

Fig. 3. The FXR and PKA signaling pathways co-activate target genes in an FXR phosphorylation dependent manner. (A) Identification of potentially active FXR response element in the vicinity of the *Fbp1* gene. FXR and CREB chromatin binding sites were identified from [32, 35, 36]. Consensus and degenerated DNA binding sequences for FXR and CREB were identified using MatInspector (Genomatix). Numbers indicate the position of each putative response element relative to the TSS. (B) Transcriptional activity of the identified response element. The activity of potentially active FXRE was assayed in a transactivation assay as in Fig. 2C (HEK293A cells). Right panel: FXR protein expression in transfected cells. (C) Endogenous gene expression. MPHs were transfected with the indicated siRNAs and *Fbp1* gene expression was monitored by RT-qPCR. (D) CREB1 protein expression levels, (E-F) Endogenous transcription factor loading at identified chromatin binding sites in MPHs. The density of FXR (E) or CREB (F) at the Fbp1 composite response element was assayed by ChIP-qPCR. Data are expressed as the mean +/− SEM (n = 5–6) and values were compared using a 2-way ANOVA followed by a Tukey *post hoc* test. *p <0.05, **p <0.01, ***p <0.005. ChIP-qPCR, chromatin immunoprecipitation quantitative PCR; FXRE, FXR-responsive element; MPH, mouse primary hepatocyte; RT-qPCR, quantitative reverse transcription PCR; TSS, transcription start site; WES, Simple Western™ immunoassay.

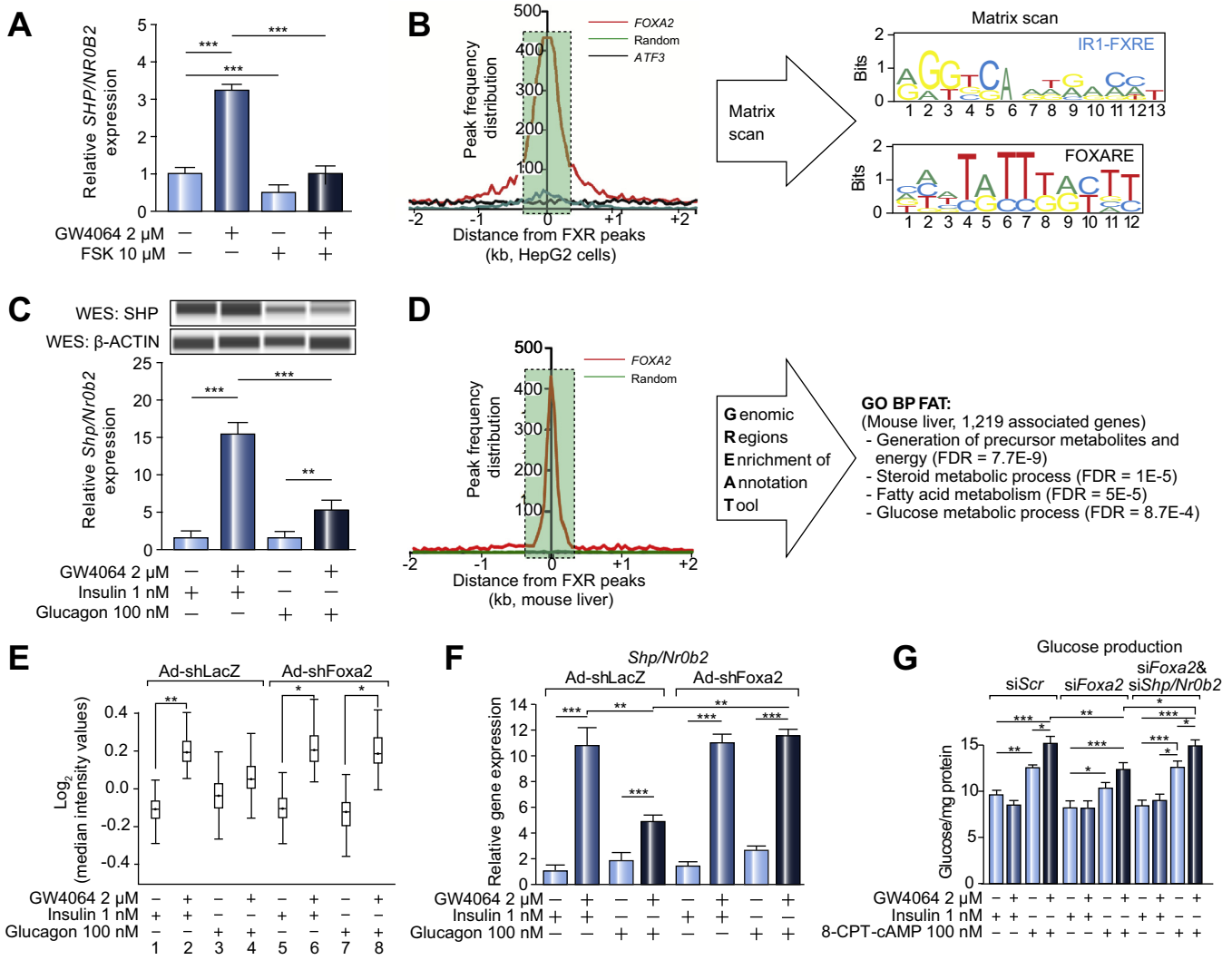

Fig. 4. The FXR-mediated induction of the *Nr0b2/Shp* gene is negatively regulated by Foxa2. (A) *NR0B2/SHP* gene expression in HepG2 cells. HepG2 cells were treated for 4 h, as indicated. *SHP/NR0B2* mRNA levels were assessed by RT-qPCR. mRNA levels in untreated HepG2 cells were arbitrarily set to 1. (B) Frequency distribution of the distance between FXR binding sites and the closest FOXA2 binding site, ATF3 binding site or a random distribution of the same number of sites than FOXA2. The green box indicates FXR/FOXA2 CBRs within 100 bp from the center of the FXR binding sites in the HepG2 cell genome. Inset: Consensus binding site analysis of FXR/FOXA2 CBRs. (C) *Shp/Nr0b2* gene and protein expression in MPH treated with insulin or glucagon. MPHs were treated as indicated for 4 h and *Shp/Nr0b2* mRNA levels were assessed by RT-qPCR. mRNA levels in insulin-treated MPHs were arbitrarily set to 1. Upper panel: protein expression was assayed by WES analysis. (D) Frequency distribution of the distance between mouse liver FXR binding sites and the closest FOXA2 binding sites. Right panel: Functional annotation of genes neighboring FXR-FOXA2 binding sites. Associated genes defined by GREAT were annotated with the Gene Ontology Biological Process database and the most significantly enriched terms are shown. (E) Foxa2-dependent repression of FXR-regulated genes. mRNA levels from MPHs were assessed by DNA microarray analysis. Hierarchical clustering defined ca. 200 transcripts whose expression was regulated by glucagon and sensitive to FOXA2 expression levels (FC >1.2, p <0.05). Box plots for these transcripts indicate the median, the 25th and 75th percentile. (F) The glucagon-mediated repression of *Nr0b2* expression is FOXA2-dependent. Gene expression was assayed by RT-qPCR. The *Shp/Nr0b2* mRNA level in shLacZ-transduced, insulin-treated MPH was arbitrarily set to 1. (G) GP by MPHs. MPH were treated as indicated after transfection with the indicated siRNAs. GP was assessed as above and was normalized to the protein content. (A,C,F,G) Results are the mean (n = 3–6) ± SEM and values were compared using a 2-way ANOVA with a Bonferroni *post hoc* test. *p <0.05, **p <0.01, ***p <0.005. CBR, co-binding region; FDR, false discovery rate; GP, glucose production; MPH, mouse primary hepatocyte; RT-qPCR, quantitative reverse transcription PCR; WES, Simple Western™ immunoassay. (This figure appears in colour on the web.)

with 1,219 FXR/FOXA2 CBRs out of 7,457 total FXR binding sites (Fig. 4D). Interestingly, several CBR-associated genes are involved in glucose metabolic processes (Fig. 4D). The contribution of FOXA2 to FXR-regulated transcription was then characterized by microarray analysis of RNAs from wild-type or FOXA2-depleted MPHs after glucagon or insulin stimulation (Fig. 4E and Fig. S12B, C, D). In control MPHs (Ad-shLacZ), a subset of genes (197) displayed a reduced inducibility by GW4064 in the presence of glucagon (Fig. 4E, lanes 3 *vs.* 4) when compared to GW4064 treatment in the presence of insulin (Fig. 4E, lanes 1 *vs.* 2). The ability of glucagon to blunt the expression of this subset of FXR-inducible genes was lost in *Foxa2*-depleted cells (Ad-sh*Foxa2*) (Fig. 4E, lanes 7 *vs.* 8, compare to 3 *vs.* 4), thereby identifying FOXA2 as a repressor of a subset of FXR-regulated genes. In particular, glucagon-mediated repression of *Shp/Nr0b2* was FOXA2-dependent (Fig. 4F), thereby identifying FOXA2 as a critical regulator of FXR activity at this locus. Finally, blunting FOXA2 expression in MPHs (Fig. S12E) decreased glucose production in response to cAMP alone or to cAMP and GW4064 in a *Shp/Nr0b2*-dependent manner

(Fig. 4G, S12E). This suggests that FOXA2 positively contributes to FXR-independent and FXR-induced gluconeogenesis by inhibiting *Shp* expression.

FOXA2 interacts with DNA-bound FXR at the *Shp/Nr0b2* locus

FXR and FOXA2 both bind to the *Shp* URR in mouse liver (Fig. 5A). FOXA2 DNA binding was dependent on FXR (Fig. 5B) and was increased by glucagon and GW4064 (Fig. 5C). A ChIP-reChIP analysis confirmed the co-localization of these two TRs at this genomic locus (Fig. 5D). GST pulldown and co-immunoprecipitation assays established that FXR specifically interacts with FOXA2 in HepG2 cells (Fig. 5E,F) and in mouse liver (Fig. S12F). FOXA2 binding to the *Shp* URR was also FXR-dependent in the immortalized mouse hepatocyte cell line AML12 (Fig. 6A), which displays transcriptional regulatory features similar to HepG2 cells and MPHs (Fig. S11A,B;S13). FOXA2 opposed FXR-mediated activation of *Shp/Nr0b2* (Fig. 6B), however FXR binding was not dependent on FOXA2 (Fig. 6C). FOXA2 inhibited the FXR-mediated induction of an IR1 FXRE-driven reporter gene by all four FXR isoforms (Fig. 6D,E). The DNA binding activity of FOXA2 relies on the integrity of a highly conserved winged-helix DNA binding domain. Mutation of S237 and W239 within this domain abolishes DNA binding[43] and FOXA2 transcriptional activity (Fig. 6F). Comparing the repressive activity of wild-type FOXA2 to that of the DNA binding-crippled FOXA2 in the IR1 FXRE-tk-Luc transactivation assay demonstrated that the repressive activity of FOXA2 is independent of its interaction with DNA (Fig. 6G). Thus, FOXA2 likely represses FXR transcriptional activity by a tethering mechanism.

FOXA2 and PKA regulate FXR target genes independently

Whether PKA-regulated FXR target genes are also sensitive to the insulin-inhibited, glucagon-activated FOXA2 transcription factor was investigated next. The FOXA2 target gene *Igfbp1* was induced by glucagon in a FOXA2-dependent manner and was insensitive to FXR agonism in both wild-type and *Foxa2*-depleted MPHs (Fig. 7A). The FXR target gene *Ostβ* was induced upon FXR agonism, but not by glucagon treatment. Simultaneous treatment with glucagon and GW4064 cooperatively induced *Ostβ* expression in a FOXA2-independent manner (Fig. 7B). Expression of the gluconeogenic genes *Fbp1*, *Pck1* and *G6pc* was induced by glucagon and further enhanced by FXR agonist treatment, a response which was not altered by *Foxa2* knockdown (Fig. 7C-E). This suggests that the FOXA2 and PKA signaling pathways regulate distinct subsets of FXR-responsive genes, cooperating to enhance HGP (Fig. 7F).

Discussion

Glucose homeostasis is regulated by a complex and intricate signaling network involving multiple organs. The BA nuclear

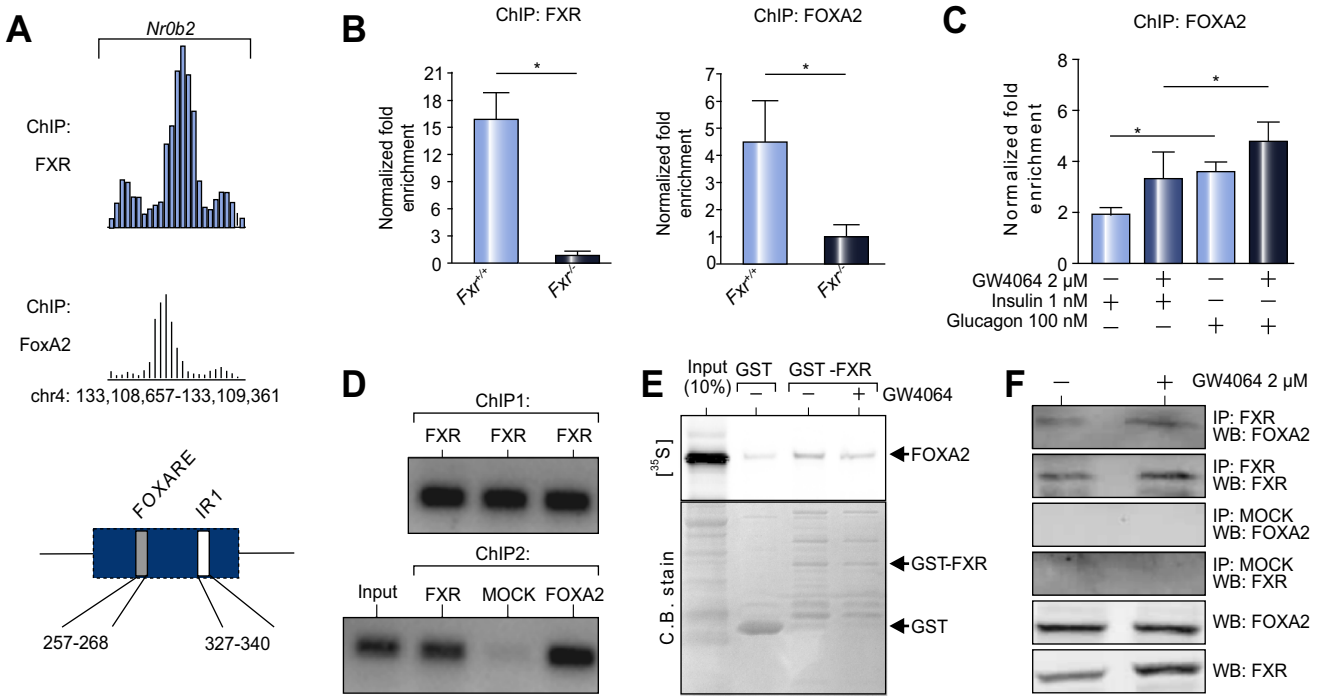

Fig. 5. FOXA2 interacts with FXR. (A) Characterization of the FXR-dependent regulatory region of the *Nr0b2/Shp* gene. The mouse liver ChIP-seq profile of FXR and FOXA2 binding to the *Shp/Nr0b2* upstream regulatory region is shown. Numbers indicate the limit of binding motifs for FXR and FOXA2 (lower panel). (B) FXR-deficient mice display decreased FOXA2 binding to the liver *Shp/Nr0b2* promoter. ChIP-qPCR analysis of FXR and FOXA2 binding to the *Shp/Nr0b2* promoter in the liver from $Fxr^{-/-}$ and $Fxr^{+/+}$ mice. (C) FOXA2 loading at the *Shp/Nr0b2* promoter. MPHs were treated as indicated and processed for ChIP-qPCR after chromatin immunoprecipitation with an anti-FOXA2 antibody. (D) FOXA2 and FXR co-binding to the *Shp/Nr0b2* promoter. A ChIP-reChIP/PCR assay was performed with C57Bl6 mouse liver chromatin. Amplicons were detected by agarose gel electrophoresis and BET staining. (E) Interaction between FXR and FOXA2 in solution. A GST-pulldown assay was carried out using GST-fused full-length FXR and $^{[35]}$S-labeled FOXA2 in the presence or absence of GW4064. (F) FOXA2 co-immunoprecipitates with FXR. Nuclear extract from HepG2 cells treated or not with GW4064 (24 h) were immunoprecipitated with agarose-conjugated control or anti-FXR IgG. Bead-bound material was analyzed by western blotting as indicated. (B,C) *p* values were calculated using a *t* test. (n = 6 Mean ± SEM, $^{*}p$ <0.05). C.B., Coomassie blue; ChIP-qPCR, chromatin immunoprecipitation quantitative PCR; ChIP-seq, chromatin immunoprecipitation sequencing; RT-qPCR, quantitative reverse transcription PCR.

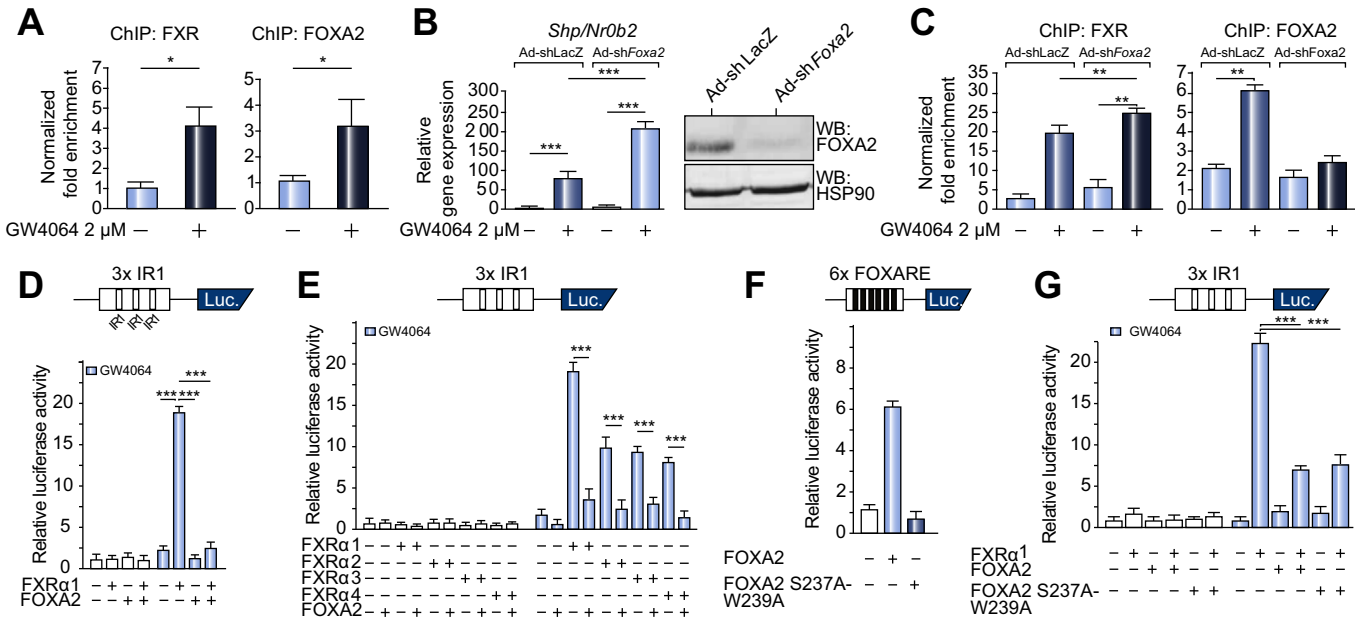

Fig. 6. FOXA2 represses FXR transcriptional activity by a tethering mechanism. (A) FXR and FOXA2 binding to the *Shp*/*Nr0b2* promoter in mouse AML12 cells. Cells were maintained in 5.5 mM glucose medium (16 h) and treated as indicated for 4 h. FXR and FOXA2 densities at the *Shp*/*Nr0b2* FXR-FOXA2 CBR were assayed by ChIP-qPCR. (B) FOXA2 represses FXR transcriptional activity in mouse AML12 cells. AML12 cells were transduced with an adenovirus coding for a shRNA targeting *Foxa2* or for a control shRNA. Shp/*Nr0b2* gene and FOXA2 protein expression were assessed by RT-qPCR (left panel) and western blotting (right panel) respectively. (C) FXR and FOXA2 binding at the *Shp*/*Nr0b2* CBR. AML12 cells were treated as in (B) and processed for ChIP-qPCR. (D) FOXA2 represses FXR-mediated activation. HepG2 cells were transfected with a pGL3 tk-Luc vector containing 3 FXR IR1 response elements and treated as indicated. The activity in the absence of transfected FXR or FOXA2 and of any treatment was arbitrarily set to 1. Luciferase assays were carried out 72 h after transfection. (E) FOXA2 represses the activity of all FXR isoforms. HepG2 cells were transfected as above with expression vectors containing mFXRα1, mFXRα2, mFXRα3 or mFXRα4. (F) The DNA binding-defective FOXA2 S237A-W239A is transcriptionally inactive. HepG2 cells were transfected as above using a pGL3tk Luc reporter gene driven by six FOXA2 response elements. (G) The FOXA2 S237A-W239A mutant represses FXR transcriptional activity. HepG2 cells were transfected with expression vectors encoding either wild-type FOXA2 or the FOXA2 mutant S237A-W239A and a 3 x IR1-driven luciferase reporter gene. (A-G) Data are expressed as the mean (n = 4–6) +/− SEM and *p* values were calculated using a *t* test (A) or a 2-way ANOVA with Bonferroni *post hoc* test (n = 3, ±SEM, $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.005). CBR, co-binding region; ChIP-qPCR, chromatin immunoprecipitation quantitative PCR; RT-qPCR, quantitative reverse transcription PCR.

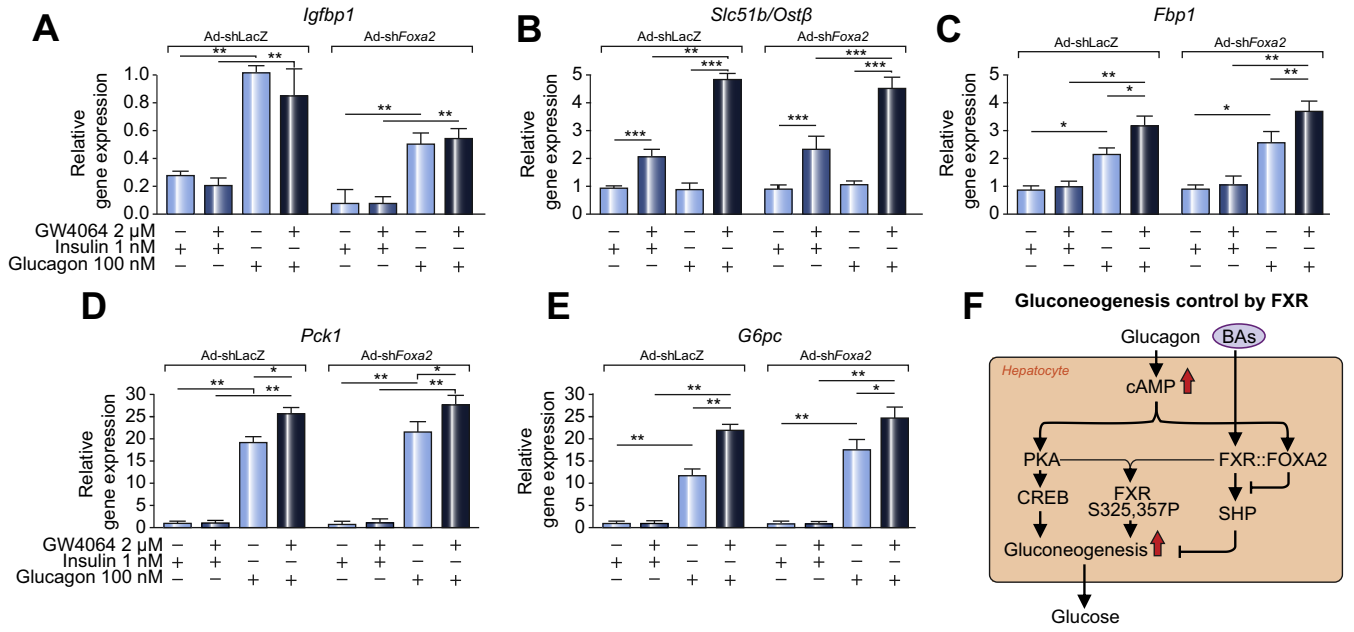

Fig. 7. Specific regulation of FXR target genes. (A-E) Expression levels of the FOXA2 target gene *Igfbp1*, of the FXR target gene *Slc51b/Ostb* and of the gluconeogenic genes *Fbp1*, *Pck1* and *G6pc* in response to GW4064, insulin, glucagon in naive or FOXA2-depleted MPHs. (F) Gluconeogenesis regulation by FXR. (A-E) Data are expressed as the mean (n = 4–6) +/− SEM and *p* values were calculated using a 2-way ANOVA with Bonferroni *post hoc* test (n = 3, ±SEM, $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.005). BA, bile acid; MPH, mouse primary hepatocytes. (This figure appears in colour on the web.)

receptor FXR is integrated into this regulatory network and participates in glucose handling and metabolism. Intestinal FXR favors glucose absorption and induces FGF15/19 secretion which, through signaling via the hepatic β-Klotho/FGFR4 membrane receptor, inhibits GSK3β, hence increasing glycogenesis. Liver FXR inhibits ChREBP activity, hence decreasing glycolysis and pancreatic FXR potentiates glucose-induced insulin secretion. All these effects contribute to maintain glucose homeostasis in the post-prandial state[44] and led to the prediction that FXR activation could favorably impact on glucose metabolism. Prolonged *in vivo* activation of FXR by natural or synthetic agonists led to unclear results. Contrasting with cholic acid treatment of C57BL/6J mice (five days), GW4064 treatment did not modulate gluconeogenic gene expression.[20] GW4064 treatment for seven days in C57Bl6 mice increased the expression of gluconeogenic genes without detectable increases in plasma glucose[45] Fasting plasma glucose was increased in high fat diet-fed C57BL/6J mice treated for three months with GW4064[46] but decreased after a six-week treatment.[22] Thus, long term interference of the FXR signaling pathway by either whole body gene knockout or prolonged agonist treatment did not provide information on a potential role of FXR in the highly dynamic physiological fasting response, which we investigated in this study.

In our study, FXR acts positively on the gluconeogenic pathways through two arms. The first positive arm is controlled through the novel glucagon/cAMP/PKA/FXR pathway, which potentiates gluconeogenic gene transcription. This synergy requires PKA-catalyzed phosphorylation of FXR on S325 and S357 and CREB, which co-localizes with FXR at the *Fbp1* and *Pck1* URRs. ChIP-PCR assays showed that PKA activation correlates with increased FXR DNA binding. Three-dimensional structures of the FXR ligand binding domain bound to natural or synthetic ligands show that S325 is located in helix 7 (H7) which constitutes part of the coactivator LXXLL binding groove and is poorly exposed to solvent in the agonist-bound, coactivator-FXR complex. S357 localizes on the β-loop connecting H7 and H8 and is more accessible to solvent than S325 in this configuration. In light of these structural data, it is still unclear how phosphorylation of S325 and S357 might increase/stabilize DNA binding.

Nevertheless, our data add PKA to the growing list of metabolism-sensitive FXR modifiers that includes O-GlcNAc transferase,[31] AMPK,[32] protein kinase C alpha,[33] sirtuin 1 (Sirt1) and p300.[47,48] A pending question is how these post-translational modifications (PTMs) vary according to the metabolic status. Prolonged energy shortage could lead to selective activation of AMPK and FXR inhibition, whereas PKA would predominantly specify FXR activity in normal fasting conditions. These PTMs have been studied independently and Sirt1-mediated deacetylation and activation of FXR is likely to directly superimpose its regulatory effect on FXR transcriptional activity. As daily variation of protein subcellular localization, phosphorylation and activities[49,50] are very likely to top on these metabolically-regulated FXR functional alterations, it becomes mandatory to decipher the PTM code of FXR during fasting/feeding periods to fully appreciate how this affects FXR-regulated biological output(s). These outputs might extend beyond metabolic control, as we recently showed that liver FXR may also regulate other specific gene sets and biological pathways as it interacts with other TRs.[51]

The prototypical FXR target gene *Shp/Nr0b2* controls BA synthesis and lipogenesis[52] and has been proposed to be a negative regulator of gluconeogenesis through interactions with the pro-gluconeogenic glucocorticoid receptor, HNF4α, Foxo-related transcription factors or C/EBPα.[53] This repressive activity provides a direct link between the observed plasma glucose lowering effect of prolonged FXR agonism in mice and gluconeogenic gene transcription. Our data however show that FoxA2 could serve as a repressor of FXR transcriptional activity on a limited number of genes, including *Shp/Nr0b2*, in short-term fasting conditions which represents another example of signal integration at specific genes. FOXA2-mediated repression of FXR activity proceeds from a DNA binding-independent mechanism, and affects a limited number of genes with no common function, as studied by gene set enrichment analysis or gene ontology term enrichment analysis (data not shown). The repression of *Shp* gene transcription by FOXA2 is intriguing in light of the ability of SHP to prevent FOXA2 DNA binding *in vitro*.[54] However, the physiological significance of these findings is unclear, as these investigations were carried out without including hormonal signals such as glucagon, which triggers FOXA2 acetylation and subsequent activation to control fatty acid oxidation and ketogenesis in a process involving Sirt1 and p300.[55] It is important to note here that our mechanistic investigations were carried out at normal glucose concentrations to avoid any confounding effects related to either energy depletion, hence activating Sirt1 and/or AMPK, or to glucose overload, hence activating the hexosamine biosynthetic pathway. A highly integrative approach combining biochemical, proteomic, epigenomic and transcriptomic approaches is required to fully understand PTM-dependent FXR activity variations in the physiologically-varying fasting and fed conditions, to which we now add the glucagon/cAMP pathway as an important regulator of FXR. Whether this physiological mechanism is dysregulated in type 2 diabetes remains to be explored. A decreased activity of both hepatic FOXA2 and FXR through phosphorylation and acetylation, respectively, has been reported in rodent models of T2D,[47,56] and we observed that exposure of MPHs to glucolipotoxic conditions abolished FXR's contribution to glucose production (data not shown). However, no correlation between FXR expression and that of its cognate target genes could be established with the diabetic status of human patients (Table S2), suggesting that this novel regulatory pathway is more likely at play in physiological conditions.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contribution

Conceptualization: MP, CM, BS, AHC, PL; Methodology: MP, CM, CG, AB, JE, JCD, HD, SC, JMS; Formal analysis and investigation: MP, CM, CG, XM, JE, JCD, VD, KBB, HD, SC, JMS; Writing - original draft preparation: MP, CM, BS, PL; Writing – review and editing: BS, PL; Funding acquisition: BS, PL; Resources: JCD, JE, HD, SC, JMS, BS, PL; Supervision: AHC, JE, BS, PL.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhep.2018.06.022.

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Author names in bold designate shared first co-authorship

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