HNF4α (hepatocyte nuclear factor 4α) is one of the master regulators of pancreatic β-cell development and function, and mutations in the HNF4α gene are well-known monogenic causes of diabetes. As a member of the nuclear receptor family, HNF4α exerts its gene regulatory function through various molecular interactions; however, there is a paucity of knowledge of the different functional complexes in which HNF4α participates. Here, to find HNF4α-binding proteins in pancreatic β-cells, we used yeast two-hybrid screening, a mammalian two-hybrid assay, and glutathione S-transferase pulldown approaches, which identified EBP1 (ErbB3-binding protein 1) as a factor that binds HNF4α in a LXXL motif-mediated manner. In the β-cells, EBP1 suppressed the expression of HNF4α target genes that are implicated in insulin secretion, which is impaired in HNF4α mutation-driven diabetes. The crystal structure of the HNF4α ligand-binding domain in complex with a peptide harboring the EBP1 LXXL motif at 3.15Å resolution hinted at the molecular basis of the repression. The details of the structure suggested that EBP1’s LXXL motif competes with HNF4α coactivators for the same binding pocket and thereby prevents recruitment of additional transcriptional coactivators. These findings provide further evidence that EBP1 plays multiple cellular roles and is involved in nuclear receptor-mediated gene regulation. Selective disruption of the HNF4α–EBP1 interaction or tissue-specific EBP1 inactivation can enhance HNF4α activities and thereby improve insulin secretion in β-cells, potentially representing a new strategy for managing diabetes and related metabolic disorders.

HNF4α (hepatocyte nuclear factor 4α) is a unique member of the nuclear receptor (NR) superfamily and plays a critical role in early vertebrate development and metabolic regulation (1). It is highly expressed in the liver, kidney, intestine, and pancreas, and its crucial role in these vital organs has been proven by a genome-wide expression profiling study (2) and conditional inactivation of its gene in mice (3–5). HNF4α regulates expression of a wide variety of essential genes, including those involved in the liver and pancreatic cell differentiation, glucose metabolism, lipid homeostasis, and amino acid metabolism (6–8). In pancreatic β-cells, it regulates the expression of numerous genes involved in the insulin secretion signaling pathway (3, 4, 9, 10). As such, mutations in HNF4α cause a dominantly inherited form of diabetes referred to as MODY1 (maturity onset diabetes of the young 1) (11), further underscoring its pivotal role in human pancreatic β-cell function and metabolic regulation (4, 9).

As a member of the NR superfamily, HNF4α is comprised of distinctive modular domains and exerts its function through various molecular interactions via combinatorial recruitment of multiprotein complexes, including transcriptional coregulators and mediators that in turn facilitate remodeling of the chromatin structure. Well-known transcriptional coregulators of HNF4α include p160/SRC coactivators such as SRC1 and GRIP1/NCoA2 (13), CBP (14), and PGC-1α (15) and NR corepressors such as NCoR, SMRT, and SMILE (16, 17). In addition, components of the mediator complex such as MED1 and MED25 have been hitherto identified as transcriptional binding partners of HNF4α (18–20). However, its entire protein recruiting network and their physiological roles have not been well-characterized, especially in β-cells, and many more key
EBP1 acts as a novel transcriptional repressor for HNF4α

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**Results**

**Initial identification of HNF4α–EBP1 interactions**

To search out additional key binding partners of HNF4α in pancreatic β-cells, we performed yeast two-hybrid screens (utilizing the service provided by the Yeast Model System Genomics facility at Duke University, which is now closed) and identified EBP1 as one of its binding partners. The bait vectors containing various constructs of HNF4α were made and screened against the pretransformed mouse pancreatic library as prey. When full-length HNF4α or the ligand-binding domain (LBD) only was used as bait, EBP1 was identified as one of the putative binding partners, together with the well-known HNF4α transcriptional coregulators such as PGC-1α, GRIP1/ NCoA2, and the mediator component MED25 of RNA polymerase II (the original figures of the plates with proper controls were not provided to us except the table of potential binding partners and their sequence information). DNA sequence analysis of the positive clones revealed four cDNAs derived from the same gene, encompassing the 197–373 or 286–367 region of EBP1 (NP_035249) containing the LXXLL motif (positions 354–358). This mouse sequence shares 99% sequence identity with its human counterpart (only 4 amino acid differences of 394).

**Further confirmation of the interaction between HNF4α and EBP1 and interaction domain mapping**

To confirm the initial yeast two-hybrid findings, mammalian two-hybrid assay was carried out with HeLa cells. As shown in Fig. 1A, HNF4α–EBP1 mutual interaction was evident even in the absence of additional external ligand, and this interaction was greatly reduced when the EBP1 mutant (EBP1–NR) in which the sole LXXLL sequence was mutated to LXXAA was used, suggesting its involvement in this interaction. A weak residual interaction by EBP1–NR suggests a possible involvement of other regions of EBP1 for HNF4α interactions, although the LXXLL motif plays a major role.

To map the critical domains of HNF4α and EBP1 for their mutual interactions, mammalian two-yeast assay and GST pull-down experiments were performed with a series of HNF4α truncation mutants and EBP1 full-length protein. The crystal structures of EBP1 and HNF4α are available, and they indicate that the entire structure of EBP1 is made of a single well-folded domain (34–35), whereas HNF4α is made of several functional modular domains (36–38). Therefore, several different constructs of HNF4α encompassing various regions of the protein...
were made and used for examination, whereas a single construct of EBP1 full-length was used. As shown in Fig. 1B and Fig. S1, all the truncated mutants containing the HNF4α–LBD (positions 151–377) made interactions, and the LBD alone proved to be sufficient for interaction with EBP1. These findings are in line with the initial yeast two-hybrid screening outcomes in which the protein products corresponding to the amino acid 197–373 or 286–367 region of EBP1 containing the LXXLL motif (positions 354–358) showed interactions with both HNF4α–LBD alone and the full-length HNF4α. Taken together, these binding analysis results prove that EBP1 physically interacts with HNF4α in living cells and present EBP1 as a potential transcriptional regulator.

EBP1 represses HNF4α-mediated transactivation in the LXXLL motif–dependent manner

Because EBP1 is a well-known transcriptional corepressor for another NR, AR-regulated genes (28, 29), we first tested its involvement in HNF4α-mediated transactivation by overexpressing both proteins and measuring the changes in the reporter gene expression level by HNF4α luciferase assays with HeLa cells. The reporter vector pGL3-HNF1α contained one copy of the HNF4α response element (−64 to −52) within the promoter of human HNF1α (−398 to the first AGT) (20). As shown in Fig. 2A, EBP1 substantially reduced the expression of the reporter gene (lanes 3 and 7), whereas this effect is completely masked by the EBP1 LXXAA mutant (EBP1–NR) and gene knockdown experiment with shRNA (lanes 4 and 5). For ERα and PPARγ, 10 nM estradiol, a potent ligand for ERα, and 1 μM troglitazone, an agonist for PPARγ, were added, respectively. EBP1 showed repressive effects on these NR-mediated transactivations except ERα (C).

Figure 2. Effects of EBP1 on HNF4α and other NR-mediated transcription.

A, overall transcriptional activity measured by standard luciferase-based transcriptional reporter assays on HNF4α-responsive elements. The LXXLL motif mutant (EBP1–NR) and gene knockdown experiment with shRNA were also performed to test the mutational and specific protein effects, respectively (lanes 4 and 5 and lanes 8 and 9). The left five lanes are without HNF4α transfection (thus empty vector, single transfection of EBP1-expressing vectors, or with shEBP1), and the right four lanes correspond to the ones with HNF4α transfection (thus single or double transfections of HNF4α and EBP1-expressing vectors or with shEBP1). The scatter plots with individual data points (n = 6) are shown. The midlines indicate the average (or mean) values, and the vertical lines indicate the S.E. * indicates a p value < 0.001 with respect to each other. ns, nonsignificant. All data have been normalized against firefly Renilla luciferase activity.

B–F, overall transcriptional activity measured by standard luciferase-based transcriptional reporter assays on other representative NR-responsive elements (PPARγ, ERα, PR, RARα, and RXRα). For ERα and PPARγ, 10 nM estradiol, a potent ligand for ERα, and 1 μM troglitazone, an agonist for PPARγ, were added, respectively. EBP1 showed repressive effects on these NR-mediated transactivations except ERα (C).
EBP1 acts as a novel transcriptional repressor for HNF4α

(Fig. 2, B–F). This is quite contrast to our previous findings in which HNF4α and ERα displayed the same positive response to the overexpression of MED25 (mediator complex subunit 25), whereas other NRs did not (20). Thus, it appears that coregulatory recruitments by NRs are member-specific, and each coregulator or mediator component works for its own cognate members of the NR superfamily (39, 40).

**EBP1 reduces HNF4α target gene expression involved in glucose-stimulated insulin secretion in β-cells**

MODY patients are mainly characterized by a severe impairment of insulin secretion, and the mutations in the MODY gene products, including HNF4α, are monogenic causes of an insulin secretion defect resulting in diabetes onset (41, 42). Thus, to probe the involvement of EBP1 in HNF4α-specific target gene expression and insulin secretion in β-cells, we next tested whether EBP1 is required for HNF4α transcriptional activation of previously known HNF4α target genes that are directly involved in β-cell insulin secretion such as PPARα (3), l-pyruvate kinase (l-PK) (9, 10), GLUT2 (9, 10), and Kir6.2 (3, 4). These proteins are involved in the insulin secretion signaling pathway at a respective critical point such as glucose sensing and transport (GLUT2), TCA, or Krebs cycle, i.e. ATP production by mitochondrial enzymes (l-PK), ATP-dependent potassium channel (Kir6.2), or transcriptional regulation of additional gene products along the insulin secretion pathway (PPARα).

Target gene expression levels were measured by means of transient transfection in MIN6 cells followed by quantification of reverse-transcribed and amplified DNA products by real time PCR and Q-PCR. Our results showed that EBP1 represses the activation of the majority of HNF4α-specific target genes involved in insulin secretion. As shown in Fig. 3 (A and B), expression of aforementioned HNF4α target genes were mostly reduced upon transfections of HNF4α and EBP1 (nearly 50%), whereas Kir6.2 showed no response. Although the ATP-dependent potassium channel is one of the central players in glucose-stimulated insulin secretion, and altered potassium channel activity is related to the impaired insulin secretion in MODY patients (43, 44), there are contradicting data on whether or not
EBP1 acts as a novel transcriptional repressor for HNF4α

We further pursued delineation of the molecular basis for EBP1 repression against HNF4α by determining the crystal structure of the complex. Although crystallization attempts with the full-length EBP1 were unsuccessful, its fragment containing the key interaction LXXL motif in complex with HNF4α–LBD produced the crystals for X-ray diffraction data analysis. Details of the crystallization and structure determination are provided under “Experimental procedures,” and the typical crystals/diffraction patterns and final refinement statistics are shown in Fig. S4 and Table 1, respectively.

Although this particular crystal form contains an unusually high number of molecules in the asymmetric unit (8 dimers or 16 monomers in the asymmetric unit), this space group assignment was correctly confirmed by the program Zanuda (45) provided by the CCP4 program suits (46). Multiple independent molecules in the asymmetric unit is not uncommon, and 8–16 monomers in the asymmetric unit have been observed in several previous structures (47). In our crystal, each dimer contains both open and closed conformations of HNF4α with a bound ligand (long-chain fatty acid, modeled as lauric acid) in each monomer and the EBP1 peptide only for the closed (active) conformation (Fig. 4A). Overall, the entire 16 monomers are packed in such a way that there are two sets of eight monomers (a set of two tetramers of functional dimer assembly following a local D2 symmetry) in different orientations. However, there are few structural variations among the eight dimers in the asymmetric unit (Table S1). One set of eight monomers are well-packed in the crystals, whereas the other set is loosely packed, resulting in higher B-factors and relatively weak electron density for the proteins as well as bound ligands and peptides. As a result, the ligands and peptides were not modeled for the second set of eight protein molecules with weak electron densities.

Crystal structure of the complex and the competitive nature of EBP1 repression against HNF4α

The overall structure of HNF4α and the binding mode of the EBP1 LXXL motif are nearly identical to those observed in the HNF4α–LBD–SRC1 peptide and HNF4α–LBD–PGC-1α peptide complexes that we previously reported (38, 48) (Fig. S5A). SRC1 and PGC-1α are well-known coactivators for HNF4α (13, 15), and the same binding mode used by EBP1, thus potentially blocking coactivator interactions, hinted at the competitive nature of repression. When the bound peptides are superimposed, the HNF4α proteins superimpose extremely well with root-mean-square deviations of Ca-atom positions 0.855 and 0.858 Å between the EBP1-bound protein and each of the coactivator-bound proteins (SRC1- and PGC-1α–bound proteins, respectively) (Fig. S5B). Their binding modes define the canonical NR/LXXL motif interactions that are comprised of leucine-mediated hydrophobic interactions within the hydrophobic groove and a “charge clamp” at both ends created by the hydrogen bonds between the backbone atoms of the peptides and the side chains of HNF4α (38, 48).

Table 1

| Statistics of HNF4α–EBP1 complex crystallographic analysis |  |
|-------------------------------------------------------------|---|
| Data collection                                             | 1.000 |
| Wavelength (Å)                                              | 100 |
| Space group P2                                              |  |
| Unit-cell parameters                                        |  |
| a, b, c (Å)                                                 | 49.63–3.15(3.20–3.15) |
| α, β, γ(°)                                                  | 90.00, 90.61, 90.00 |
| Resolution (Å)                                              | 92.5(76.2) |
| Completeness (%)                                            | 3.5(2.2) |
| Average multiplicity                                        | 9.3(2.7) |
| Residues (%)                                                | 7.5(40.2) |

*The values in parentheses are for the highest-resolution shell.

| Refinement |  |
|------------|---|
| Resolution (Å) | 49.63–3.17 |
| Number of reflections | 48,984 |
| Rmerge (%) | 0.262 |
| Rmerge (%) | 0.274 |
| Number of atoms Proteins | 26,226 |
| Lauric acids | 112 |
| Solvents | 44 |
| Average B-factor (Å²) | 124.4 |
| Protein atoms | 68.6 |
| Solvents | 45.3 |
| Root-mean-square deviation from ideal geometry Bond lengths (Å) | 0.004 |
| Bond angles (°) | 0.856 |
| Ramachandran plot Favored (%) | 92.3 |
| Allowed (%) | 7.5 |
| Outliers (%) | 0.2 |
| Rotamer outliers (%) | 0.0 |

* 5% of the reflection data were excluded from refinement.

Table S1

| Statistics of HNF4α–EBP1 complex crystallographic analysis |  |
|-------------------------------------------------------------|---|
| Data collection                                             | 1.000 |
| Wavelength (Å)                                              | 100 |
| Space group P2                                              |  |
| Unit-cell parameters                                        |  |
| a, b, c (Å)                                                 | 49.63–3.15(3.20–3.15) |
| α, β, γ(°)                                                  | 90.00, 90.61, 90.00 |
| Resolution (Å)                                              | 92.5(76.2) |
| Completeness (%)                                            | 3.5(2.2) |
| Average multiplicity                                        | 9.3(2.7) |
| Residues (%)                                                | 7.5(40.2) |

*The values in parentheses are for the highest-resolution shell.

* 5% of the reflection data were excluded from refinement.
EBP1 acts as a novel transcriptional repressor for HNF4α

Another prominent feature of the structure as previously mentioned is the mixed monomer conformations within the functional dimer of HNF4α–LBD wherein one monomer adopts a closed (active) conformation, thus occupied by the EBP1 peptide, and the other monomer displays an open (inactive) conformation with the final helix H12 extended despite the presence of a bound fatty acid (Fig. 4, A and B). The LXXLL motif binding is known to fully induce closed (active) conformations for both monomers (38, 48); however, despite the same binding mode in the crystal structures, EBP1 (or its LXXLL motif) was not able to lock both monomers into an active conformation. One possibility for this observation is that EBP1 peptide displays a relatively weak binding compared with that of well-known coactivators such as SRC1 and PGC-1α (Fig. 4C).

Thus, we tested their relative binding capabilities by measuring the degrees of protein stability elevation when peptides are bound. As shown in Fig. 4D, EBP1 peptide binding caused a smaller degree of shift in protein melting temperature compared with those caused by coactivator peptide bindings. Although not very quantitative, these data suggest that EBP1 binds HNF4α rather weakly yet effectively serves as a competitive repressor against the coactivators by preventing them from binding to the same pocket and recruiting the main transcriptional machinery. A similar means of repression utilizing the same LXXLL motif has been reported to other NR-repressors such as NCoR and RIP140 (49, 50).

Discussion

The current model of eukaryotic gene regulation is best described by the combinatorial recruitment involving multiple transcriptional regulators (51, 52); however, the full extent of tissue-specific and protein-specific recruitment has not been well-characterized. Transcription factors initiate transcription by recognizing their target genes and mediating additional interactions with various proteins as part of their combinatorial recruitment involving multiple transcriptional regulators and mediators to recruit the remainder of the main transcriptional machinery (51, 52). To gain additional molecular insights into HNF4α function, we performed yeast two-hybrid screens and identified EBP1 as a functional binding partner of HNF4α in pancreatic β-cells.

Our study was focused on β-cells because HNF4α is one of the culprit gene products for a dominantly inherited form of diabetes, MODY1, which is mainly characterized by the defect in insulin secretion from β-cells (41, 42). The interaction between HNF4α and EBP1 was initially identified by yeast two-hybrid analysis and further confirmed by mammalian two-hybrid assay and GST pulldown. Unlike other novel NR repressors that typically use the repressor-specific motifs such as LXX(I/L)XXX(I/L) (53), EBP1–HNF4α interaction utilizes the same LXXLL motif used by the activators, thus representing an atypical corepressor with binding properties of a coactivator and
Expression of EBP1 does not alter the level of ERα.

Regulatory effect by EBP1 appears to be NR-specific, and overexpression with many NR family members, this transcriptional repressor of HNF4α serves as a novel transcriptional repressor for HNF4α.

Recently, tumor suppressor or oncogenic activities of EBP1 have also been observed in various cancer types, and EBP1 can contribute these cancer developments. EBP1 mutations have also been reported to be associated with certain cancer types such as colorectal cancers.

Experimental procedures

Yeast two-hybrid

The initial screening using the lexA system was carried out through the Yeast Model System Genomics facility at Duke University. HNF4α-LBD (positions 142–368) was cloned into the bait vector pGBK7T7short (a modified version of pGBK7 (Clontech) with tags between Gal4BD and bait removed), and the mouse pancreatic library (Clontech) was used as prey. Self-activation was tested with an empty Gal4BD bait vector before the screening against the library. Two-hybrid screens were performed with a standard method using the yeast strain S. cerevisiae AH109. Primary isolates were restreaked on Trp−/Leu−/His−/3 mx 3-amino-1,2–4-triazol plates and grown several days for LacZ assays. Positive colonies that showed a color change in LacZ assays were picked for colony PCR or for isolation of DNA. Approximately 6 × 10⁶ independent transformations were screened, of which 27 clones were positive for LacZ assays. Each positive hit was retested for one to one interaction by examining the growth of the transformant and performing LacZ assays. Subsequently, the positive clones were sequenced using automated DNA sequence analysis (ABI), and homologies were identified using BLASTN/BLASTX (National Center for Biotechnology Information).

Construction of expression vectors and HNF4α-LBD protein purification

The luciferase reporter plasmids, pCMV Sport6 EBP1 harboring the full-length cDNA of human EBP1, pcDNA3.1 HNF4α FL containing the full length of human HNF4α, and firefly reporter vector pGL3-HNF1α, containing one copy of the HNF4α response element (−46 to −52) within the promoter of human Hnf1α (−298 to the first AGT), were constructed as described previously (48). The same vectors were used for transfection and insulin secretion assays. For in vitro binding studies, recombinant EBP1–FL (positions 1–394) proteins were cloned into pcDNA3.1 (Novagen) with a FLAG tag, whereas the HNF4α (1–474) (full-length), 1–377, 1–133, 46–474, 46–377, 46–133, 134–474, 134–377, 151–377, and 377–474 were also cloned into pcDNA3.1 (Novagen) vector with GST tag. For crystallization, DNA encoding the LBD of human HNF4α (residues 140–382) was subcloned into the pET41a vector (Novagen) by PCR. Protein was expressed in Escherichia coli BL21(DE3) cells (Invitrogen) and isolated from lysates using Talon cobalt affinity resin (Clontech). The His⁶ affinity tag was cleaved using tobacco etch virus protease, and the protein was further purified by ion-exchange chromatography (Mono Q FPLC).

EBP1–NR mutant generation

The QuikChange multisite-directed mutagenesis kit (Stratagene) was used to generate the EBP1–NR (LXXAA) mutant construct. The plasmid templates used in the mutagenesis protocol were pCMV Sport6 EBP1–FL. All of the generated constructs with the mutated sequences were verified with DNA sequencing.
EBP1 acts as a novel transcriptional repressor for HNF4α

Cell culture

For mammalian two-hybrid and transcriptional assays, HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin G, 50 μg/ml streptomycin, and 0.1% nonessential amino acids. For the remaining cell culture experiments, mouse insulinoma 6 (MIN6) cells of passage 28 through 30 were cultured in Dulbecco’s modified Eagle’s medium containing 5 mM glucose, 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin, 2 mM glutamine, and 100 μM β-mercaptoethanol (66). MIN6 cells were a kind gift from Dr. Sabire Özcan at the University of Kentucky (67).

Mammalian two-hybrid assays

The DNAs, pBIND-HNF4α FL, AF1, DNA-binding domain, LBD, FD, pACT-EBP1 WT, NR, and pG5-luciferase, were combined as indicated in each lane in the total amount of DNA not exceeding more than 100 ng/well. The transfection was performed with TurboFect (Fermentas, Glen Burnie, MD) and following the manufacturer’s recommended protocols (Promega E2440). The cells were disrupted by addition of 150 μl of cell lysis buffer directly into each well of the 48-well plate, and then aliquots of 70 μl were added to each well of a 96-well luminescence plate. The luminescence activity was measured automatically by microplate reader (SpectraMax M5; Molecular Devices). The relative luciferase activity was calculated and normalized based on the pG5-luciferase basal control. For assessment of transfection efficiency, the Renilla luciferase activity assay was used.

GST pulldown assays

MIN6 cells were transiently transfected with expression plasmids as indicated in the figures by using Metafectene Pro transfection reagent (Biontex). After 24 h, the cells were harvested and sonicated in 1 ml of the lysis buffer containing 50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 μg/ml BSA, 0.2 mM phenylmethylsulfonyl fluoride plus protease inhibitors (50 μg of aprotinin and 5 μg of leupeptin/ml). After centrifugation, 500 μg of a freshly solubilized post-nuclear supernatant was mixed with 25 μg of GST-fused HNF4α (full-length or LBD-only) immobilized onto GSH-Sepharose in buffer (100 mM Tris, pH 7.5, 10 mM EDTA, 300 mM NaCl, 2 mM DTT, 0.02% Nonidet P-40, 0.4 μg/ml BSA, 0.4 mM phenylmethylsulfonyl fluoride plus protease inhibitors). After incubation at 4 °C for 2 h, the beads were extensively washed, and bound proteins were eluted by adding 20 mM GSH. The elution samples and 10% of the homogenate (labeled input in Fig. S1) were applied to SDS-PAGE prior Western blotting detection with antibodies against EBP1 (Santa Cruz Biotechnology).

Transient transfection and transcription assays (luciferase reporter assays)

The full-length cDNA of human HNF4α WT or the mutant were subcloned into the pcDNA3(+)Neo vector (Invitrogen), and the reporter vector pGL3-HNF1α containing one copy of the HNF4α response element (-64 to −52) within the promoter of human Hnf1α (−298 to the first AGT) was constructed and used for luciferase assays in the absence or presence of EBP1. HeLa cells were transfected using Opti-MEM and Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer’s recommendations. Briefly, a total of 30 ng of pcDNA3 HNF4α and 150 ng of pCMV Sport6 PGC-1α, 50 ng of pCMV Sport6 EBP1, 50 ng of pGL3-HNF1α, and 10 ng of pRL-TK (control Renilla luciferase vector) were used for transfection of 1 × 10^5 cells seeded on 24-well plate 1 day before transfection. For gene silencing experiments, 20 pmol of shRNA of EBP1 and HNF4α were used for inhibition. For ERα and PPARγ luciferase assays, 6 h after transfection, the cells were treated with 10 nM of estradiol or 1 μM of troglitazone for 48 h. These additional luciferase vectors were kind gifts from Dr. Dan Noonan at the University of Kentucky. After transfection and incubation, the cells were washed with 1× PBS and lysed with luciferase lysis buffer supplied with the Luciferase assay kit (Promega). Luciferase activity was measured using the Dual Luciferase assay system (Promega) and Luminoskan (Thermo Fisher Scientific). All values were normalized by the relative ratio of firefly luciferase activity and Renilla luciferase activity. At least four independent transfections were performed in duplicate.

Gene knockdown (shRNA) for cellular studies

We used shRNA for EBP1 interference in the real time PCR/Q-PCR and insulin secretion assays. EBP1 shRNA was purchased from Origene with the following sequences: 5’-ATGTTGATGCGCTTCATCGCTAATGTAGCT-3’. Cells grown to 50% confluence were transfected using Metafectene pro transfection reagent (Biontex) with EBP1 or scrambled shRNA according to the manufacturer’s instructions.

Real-time PCR

MIN6 cells were transiently transfected with HNF4α and EBP1 using Metafectene protransfection reagent (Biontex) for 24 h. Total RNA from the treated cells was prepared with the Tri reagent (Sigma–Aldrich), and 0.5 μg of RNA was reverse transcribed using Onestep RT-PCR kit (Qiagen) and amplified by PCR whose product formation was monitored continuously during PCR using sequence detection system software (version 1.7; Applied Biosystems). Accumulated PCR products were detected directly by monitoring the increase of the reporter dye (SYBR). The expression levels of PPARα, t-PK, GLUT2, and Kir6.2 in the exposed cells were compared with those in control cells at each time point using the comparative cycle threshold (Ct) method. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of actin, a housekeeping gene.

Q-PCR

MIN6 cells were transfected with HNF4α and EBP1 plasmids. 24 h after transfection, total RNA was extracted using Tri reagent (Sigma–Aldrich) according to the manufacturer’s protocol. cDNA synthesis and semiquantitative PCR for PPARα, t-PK, Kir6.2, and GLUT2 mRNA were performed, and the PCRs were electrophoresed using a 2% agarose gel. The band
intensities of the amplified DNA products were visualized using the SYBR Green I DNA gel stain kit (Invitrogen).

**ChIP assay**

EBP1 and/or HNF4α plasmids-transfected MIN6 cells were cross-linked with formaldehyde in PBS at room temperature for 10 min then sonicated to shear DNA strands into the size of 200–500 bp. The sonicated chromatin–DNA complexes were precipitated with antibodies against HNF4α (Santa Cruz Biotechnology catalog no. sc-6556) or nonspecific mouse IgG. PCR analysis was performed using 2 µl of purified DNA, oligonucleotide primers, and Platinum Taq DNA polymerase. After 40 cycles, the products were analyzed using a SYBR green I DNA gel stain kit (Invitrogen).

**Insulin secretion assays**

This functional study was performed with the MIN6 cell line, which exhibits the characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets (68). To quantify the amount of insulin secreted, MIN6 cells were grown on a 6-well dish (about 1 × 10^5 cells) for 20–500 bp. The sonicated chromatin–DNA complexes were precipitated with antibodies against HNF4α (Santa Cruz Biotechnology catalog no. sc-6556) or nonspecific mouse IgG. PCR analysis was performed using 2 µl of purified DNA, oligonucleotide primers, and Platinum Taq DNA polymerase. After 40 cycles, the products were analyzed using a SYBR green I DNA gel stain kit (Invitrogen).

**Crystallization of the HNF4α–LBD–EBP1 peptide complex**

An over 20-fold molar excess of synthetic EBP1 peptide (VQDAELKALLQSSASRKTQK, residues 349–368) was incubated with the protein in the optimal buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 1 mM DTT, 5% glycerol) that maximizes the monodispersity of the samples (69) for initial crystallization screening for 1 h at 4 °C prior to concentration (15 mg/ml). The crystals were obtained at room temperature by the vapor diffusion method in 2-µl drops containing equal volumes of protein–peptide complex and crystallization buffer (0.1 M Bis-Tris, pH 6.25, 26–28% MPD, 3–4% PEG 8000, 20–50 mM glycine, and 10% glycerol). Crystals reached maximum dimensions of 0.3 × 0.2 × 0.05 mm within 5 days (Fig. S4A).

**Crystal data collection and structure determination**

The crystals belong to the space group P21 with the unit cell dimensions a = 139.727 Å, b = 104.947 Å, c = 139.573 Å, and β = 90.613°. The best crystals after optimizing freezing conditions diffracted to 3.15 Å resolution with the synchrotron radiation (collected at the beamline SER-CAT 22ID, Advanced Photon Source, Argonne, IL). There are 16 HNF4α–LBD–ligand–EBP-1–NR-box-fragment complexes (eight functional dimeric complexes) in the crystal asymmetric unit resulting in 54.5% solvent content. Oscillation images (every 1.0°) were collected at 100 K, and the data were processed using the HKL2000 software package (70) (Table 1).

Primary phasing was accomplished by molecular replacement using PHASER (McCoy, 2007). Our previous structure of human HNF4α–LBD (PDB accession code 1PZL) (38), after deleting a bound ligand and the SRC-1 peptide, was used as a search model. The initial R value was 0.48 with a correlation coefficient of 0.57. The subsequent σ-weighted 2Fo − Fc map after rigid body refinement clearly revealed density corresponding to the bound EBP1 peptide that was not present in the search model. Further refinement was carried out with Refmacs as run by PHENIX (71) alternating with manual fitting in COOT (72) until convergence. A twin inspection utilizing the Xtriage data analysis tool within PHENIX revealed that there is an apparent twin law (l, k, h operator) given the crystal symmetry that really stands out over the remainder of the possible twin laws (Robs 0.115 versus >0.450 for the rest of possible operators). Thus, this particular twin target function was used throughout the refinement. Individual atomic coordinates, group B-factors, and NCS (non-crystallographic symmetry) constraints (eight open and eight closed conformations of HNF4α and eight bound EBP1 peptides) were utilized for initial rounds of refinement while enforcing a twin law of the l, −k, h operator. Toward the end of refinement, individual B-factors, TLS refinement parameters, and NCS restraints were employed, with geometry and B-factor restraint weightings being optimized for each cycle. Solvent molecules were added manually in COOT. The final model was validated with AutoDeplInputTool (74) and MolProbity (73) prior to deposition in the PDB. Data and refinement statistics are provided in Table 1. The figures were prepared with PyMOL (Schrödinger, LLC).

**Fluorescence-based thermal shift assay**

Purified recombinant HNF4α–LBD protein was used to measure peptide interactions in a fluorescence-based thermal shift assay as described previously (12). HNF4α–LBD protein was aliquoted into PCR tubes in a buffer containing 20 mM Tris, pH 8.0, and 200 mM NaCl. The final protein concentration in a 20-µl reaction volume was 20 μM. Peptides to be tested were added at either 5× or 10× concentration such that the DMSO concentration never exceeded 2%. SYPRO Orange dye (Invitrogen) was added last at a 5× concentration. The PCR tubes were then sealed, centrifuged, and heated from 25 to 95 °C degrees at a rate of 1 °C/min on 7500 real-time PCR machine (Applied Biosystems). Raw data analysis and curve fitting to calculate Tm values were performed as described by Niesen et al. (12).

**Statistical analysis**

Presented data are expressed as means ± S.E. of at least three independent groups. Statistical significance was determined by one-way analysis of variance followed by Student–Newman–Keuls method using Sigma Stat 3.1 software (Systat Software, J. Biol. Chem. (2019) 294(38) 13983–13994 13991
EBP1 acts as a novel transcriptional repressor for HNF4α

San Jose, CA). A probability value $p < 0.05$ was considered statistically significant.

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