The glucose-sensing transcription factor ChREBP is targeted by proline hydroxylation

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Cellular energy demands are met by uptake and metabolism of nutrients like glucose. The principal transcriptional regulator for adapting glycolytic flux and downstream pathways like de novo lipogenesis to glucose availability in many cell types is carbohydrate response element–binding protein (ChREBP). ChREBP is activated by glucose metabolites and post-translational modifications, inducing nuclear accumulation and regulation of target genes. Here we report that ChREBP is modified by proline hydroxylation at several residues. Proline hydroxylation targets both ectopically expressed ChREBP in cells and endogenous ChREBP in mouse liver. Functionally, we found that specific hydroxylated prolines were dispensable for protein stability but required for the adequate activation of ChREBP upon exposure to high glucose. Accordingly, ChREBP target gene expression was rescued by re-expressing WT but not ChREBP that lacks hydroxylated prolines in ChREBP-deleted hepatocytes. Thus, proline hydroxylation of ChREBP is a novel post-translational modification that may allow for therapeutic interference in metabolic diseases.

Carbohydrate response element–binding protein (Mlxipl or MondoB, here referred to as ChREBP) is a glucose-sensitive transcription factor of the Mondo family (1). ChREBP is highly expressed in liver, white and brown adipose tissue, muscle, kidney, and small intestine (2). ChREBP-deficient mice are intolerant toward simple carbohydrates like glucose and fructose (2). Indeed, ChREBP is pivotal for adjusting glucose utilization to its availability by inducing the expression of genes involved in glycolysis and de novo lipogenesis (3). Moreover, ChREBP prevents toxicity of high glucose exposure by controlling genes that regulate cellular ATP homeostasis (4).

The mice that lack ChREBP globally (2, 5) or in adipose tissue (6) are glucose-intolerant and insulin-resistant. Adipose tissue–specific expression of a constitutive active ChREBP protects from metabolic deterioration upon feeding an obesogenic diet (7). Similarly, liver-specific expression of an active ChREBP mutant in mice improves insulin signaling and glucose tolerance, despite greater hepatic steatosis (8). Insights from human studies analyzing mRNA expression or SNPs of ChREBP support its central role in glucose and fatty acid metabolism (9). In contrast, whole body deletion (10) or liver-specific knockdown (11) of ChREBP in ob/ob mice restores glucose tolerance and insulin sensitivity. ChREBP also mediates fructose-induced hepatic glucose production and insulin resistance (12–14), indicating that ChREBP can cause maladaptive metabolic effects in a context-dependent manner.

ChREBP heterodimerizes with Max-like protein x (Mlx), a member of the Myc/Max family of basic HLH/leucine zipper transcription factors, and binds to genomic carbohydrate response elements (ChoREs) (15). ChoREs consist of two consensus CACGTG E-boxes separated by five bases (16, 17). In hepatocytes, ChREBP mediates most of the transcriptional responses induced by exposure to high glucose concentrations (18). Mechanistically, ChREBP is activated by glucose metabolites like glucose-6 phosphate, resulting in nuclear accumulation and enhanced transcriptional activity (19). Direct binding of glucose metabolites to ChREBP is a plausible hypothesis (20) but has not been tested yet. On the other hand, certain α-ketoacids, saturated and unsaturated fatty acids, adenosine monophosphate, and ketone bodies such as β-hydroxybutyrate inhibit nuclear localization (21). Moreover, Mlx-family transcription factors, including ChREBP, were shown to bind lipid droplets via C-terminal amphipathic helices in several cell types, which reduces their availability for transcriptional activity and attenuating the response to glucose (22). In adipocytes, its physical interaction with cytosolic hormone-sensitive lipase was also shown to limit ChREBP’s transcriptional activity (23).

In addition to metabolites and cytosolic partitioning, post-translational modifications (PTMs) like phosphorylation (24, 25), acetylation (26), and O-linked conjugation to GlcNAc (27–29) regulate stability, nuclear translocation, DNA recruitment, and transcriptional activity of ChREBP in a PTM-specific manner. These modifications can occur concomitantly to glucose exposure or are additional input signals that fine-tune ChREBP activity, for instance by regulating cytosolic retention via interaction with 14-3-3 proteins (30, 31).

N-terminal and in particular central domains of ChREBP are rich in proline. Whether these residues carry functions beyond their innate role in establishing protein structure is unknown. We therefore asked whether proline residues in ChREBP are

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post-translationally modified. Indeed, we found that several prolines are targeted by hydroxylation in ectopically expressed or endogenous ChREBP from mouse liver. We show that these sites are required for full activation of ChREBP and the induction of its target genes upon high glucose concentrations in hepatocytes. Moreover, we identify specific proline hydroxylases that physically interact with ChREBP and whose depletion impairs ChREBP-mediated glucose sensing in primary hepatocytes, rendering them potential candidates to hydroxylate ChREBP. Thus, proline hydroxylation is a novel and functionally relevant PTM of ChREBP and may represent a target for therapeutic interference in metabolic diseases.

Results

Ectopically expressed ChREBPα is hydroxylated at Pro\textsuperscript{536} and Pro\textsuperscript{141}

ChREBP exists in two isoforms, ChREBPα with generally higher tissue expression and the more active ChREBPβ that lacks part of the N-terminal low-glucose inhibitory domain (LID) (5, 32). ChREBPα prolines allocate primarily to N-terminal and central regions, in particular to a domain termed the proline-rich region. N-terminal prolines are more conserved across mammalian species (Fig. S1A) because of the generally higher conservation of LID and glucose-response activation conserved element domains of ChREBP (20). FLAG-tagged mouse ChREBPα was expressed in HEK293 cells and enriched by FLAG immunoprecipitation (IP) (Fig. 1A). Enriched proteins were subjected to carboxamidomethylation to protect the cysteine side chains and then separated by SDS-PAGE. The Coomassie-stained band corresponding to ChREBPα was excised and in-gel digested, and the resulting peptides were analyzed by LC−MS/MS. Sequence coverage was 73% (Fig. 1B), and peptides were analyzed for hydroxylation, which represents the most important proline modification. We found two peptides (Fig. 1C) whose mass shift of ~16 (oxygen) and fragmentation spectrum allowed for the unambiguous identification of hydroxylation sites, at Pro\textsuperscript{536} (Fig. 1, D and E) and Pro\textsuperscript{141} (Fig. S1, B and C), located in the proline-rich region and LID of ChREBPα, respectively. Four other peptides were found hydroxylated, but we were unable to decipher which of the multiple prolines caused their mass shift (Fig. 1C). Taken together, ectopically expressed ChREBPα protein in HEK293 cells is hydroxylated at several residues, including Pro\textsuperscript{536} and Pro\textsuperscript{141}.

Mouse liver ChREBPα is hydroxylated at Pro\textsuperscript{536} and Pro\textsuperscript{141}

To analyze whether proline hydroxylation of ChREBP occurs in a physiologic setting, we next enriched protein isolated from mouse liver by ChREBP-IP (Fig. 2A) and analyzed endogenous ChREBPα for proline hydroxylation. Sequence coverage of ChREBP was comparable (65%; Fig. 2B), and although it was detectable at lower intensities and ion scores, we detected the expected mass shift in four peptides, of which three had been found also in ectopically expressed ChREBPα protein in HEK293 cells. These included the peptides that harbor Pro\textsuperscript{536} and Pro\textsuperscript{141} (Fig. 2C and Fig. S2, A–D). In addition, more complete N-terminal coverage identified Pro\textsuperscript{15} as a third hydroxylation site in liver-derived ChREBPα protein. Thus, Pro\textsuperscript{536} within the proline-rich region and Pro\textsuperscript{141} of the LID are hydroxylated residues in both ectopically or endogenously expressed ChREBP (Fig. 2D). In accordance with the described conservation of ChREBP domains, Pro\textsuperscript{141} and surrounding amino acids are shared among mammalian species, birds, and amphibians, whereas Pro\textsuperscript{536} is less well-conserved (Fig. 2E).

Pro\textsuperscript{536} and Pro\textsuperscript{141} are required for high glucose-induced ChREBP activity

We then explored whether mutating either or both prolines to alanine affects ChREBP functionality in a heterologous cell reporter system. Equimolar amounts of plasmids encoding WT or mutated ChREBPα were transfected in HEK293 cells, yielding comparable mRNA levels (Fig. 3A). ChREBPα protein was detectable at similar amounts, suggesting that mutated prolines are dispensable for protein stability (Fig. 3B). ChREBP activity was analyzed in cells co-transfected with Mlx and a luciferase reporter driven by acetyl-CoA carboxylase 1 (Acc1) ChoREs (33). As expected, exposure to 25 mM glucose strongly induced ChREBP activity. Strikingly, mutating either or both prolines greatly reduced glucose-induced ChREBP activity, with the Pro\textsuperscript{141} mutation exhibiting the stronger impairment (Fig. 3C).

Pro\textsuperscript{536} and Pro\textsuperscript{141} are required for ChREBP target gene expression in hepatocytes

To test functionality of hydroxylated prolines in a physiologic context, we took advantage of AML12 cells, a nontransformed mouse hepatocyte cell line transgenic for transforming growth factor α (34). CRISPR/Cas9 was used to induce a guided mutation in the ChREBP gene to establish ChREBP-deficient hepatocytes. After selection for successful transfection, hepatocytes were reamplified from single-cell clones. Clone 2 lacked detectable ChREBPα protein when analyzed with an antibody raised against a C-terminal peptide (Fig. 4A). Sequencing of genomic DNA of this clone identified a heterozygous single-nucleotide insertion and frameshift in exon 6 that results in translating an amino acid 1–214 truncation of ChREBPα and 1–37 of ChREBPβ, both predicted to be inactive. The lack of detectable ChREBPα protein suggests that a single WT allele is not sufficient to express ChREBP protein in these hepatocytes or that another mutation, not covered by the genomic area sequenced, blunted expression from this allele. We then compared the induction of known ChREBP target genes in WT and clone2 (ChREBP loss of function) hepatocytes. Of all target genes tested, Txnip (thioredoxin-interacting protein) (35) and Hmgs2 (hydroxy-methylglutaryl-CoA synthase 2) (36) showed the strongest induction in WT hepatocytes upon the exposure to 25 mM glucose. However, ChREBP loss-of-function hepatocytes failed to induce either gene to a comparable extent as WT hepatocytes (Fig. 4B). This demonstrates the requirement of ChREBP for glucose responsiveness of Txnip and Hmgs2 in AML12 hepatocytes. Residual glucose responsiveness of these genes may be due to the presence of other glucose-sensing factors like MLX-interacting protein (Mlxip, or MondoA).

We next transfected equimolar amounts of WT or mutated ChREBPα plasmids in ChREBP loss-of-function hepatocytes. mRNA and protein levels of reintroduced WT and mutated...
ChREBPα were similar (Fig. S3A and Fig. 4C), indicating that also in hepatocytes neither mutation affects stability of the ChREBPα protein. Re-expressing WT ChREBPα rescued the induction of Txnip expression by 25 mM glucose; however, mutating either or both prolines greatly impaired this rescue (Fig. 4D, left panel). High glucose–induced Hmgcs2 expression showed a similar pattern, with the expected lower dynamics (Fig. 4D, right panel). These finding show that Pro536 and Pro141 are required for the glucose-induced up-regulation of canonical ChREBP target genes in hepatocytes. Mechanistically, we found that mutating either or both proline residues to alanine did not impair ChREBP binding to the 20.2 kb Txnip ChoRE (Fig. S3B).

Co-expression of proline hydroxylases and ChREBP in murine tissues

Proline hydroxylation is a covalent, irreversible PTM and is introduced by two different enzyme families: prolyl 4/3-hydroxylases (P4H/P3H) and proline hydroxylase domain proteins/egl-9 family hypoxia-inducible factor enzymes (Egln). Whereas P4H/P3H target primarily structural proteins like collagen and elastin (37–39), the three Egln enzyme isoforms (Egln1–3) are best known for hydroxylation hypoxia-inducible factors (HIFs) (40, 41). HIF proline hydroxylation leads to recognition by the E3-ubiquitin ligase von Hippel–Lindau tumor suppressor (VHL) and proteasomal degradation. P4H/P3H strictly require Xaa-Pro-Gly and Pro-Pro–4-OH-Gly for hydroxylation, whereas Egln enzymes target Leu-Xaa-Xaa-Leu-Ala-Pro in HIF but seem to act on diverse sequence motifs on a wide array of other target proteins in a hydroxylation-dependent or -independent manner (39, 42).

None of the identified ChREBPα prolines locate to motifs that could be recognized by P4H/P3H, suggesting that Egln family members are more likely candidates to hydroxylate ChREBP. To further narrow down the responsible isoform, we compared tissue expression patterns of Egln1–3 with that of ChREBP in mice. As expected, total ChREBP, determined by amplifying a transcript region that is common to both isoforms, was robustly expressed in liver, BAT, and intestine (Fig. 5A). Of the three isoforms, Egln3 expression mirrored ChREBP most closely (Fig. 5A). We next subjected mice to 24 h of fasting or fasting with subsequent 18 h of refeeding. Hepatic expression of ChREBPb, known to correlate well with ChREBP activity (5), was robustly induced in refed mice (Fig. 5B). Refeeding had no significant effect on the expression of Egln1 and Egln2, whereas Egln3 was up-regulated (Fig. 5B). These correlations may suggest a functional link between Egln3 and ChREBP.

ChREBP physically interacts with proline hydroxylases

We next tested whether ChREBP could physically interact with Egln proline hydroxylases. Indeed, ChREBP was co-IP'ed
Proline hydroxylation of ChREBP

Depletion of Egln3 impairs glucose-induced ChREBP activation in primary hepatocytes

Mutating hydroxylated prolines of ChREBP impaired its activity and target gene expression, in particular upon exposure to high glucose when ChREBP is most active (Figs. 3C and 4D). We therefore tested whether inhibiting all proline hydroxylases with CoCl2 interferes with the expression of ChREBP target genes. As expected, CoCl2 induced vascular endothelial growth factor A (Vegfa) by stabilizing HIF and, indeed, led to an overall down-regulation of ChREBP target genes in hepatocytes cultured in media containing high glucose concentrations (Fig. 7A). Moreover, primary hepatocytes induced the expression of canonical ChREBP targets after supplementing 25 mM glucose but showed lower basal and high glucose-induced expression when Egln3 was depleted by siRNA (Fig. 7B and Fig. 5A). This was likely mediated by impaired activation of endogenous ChREBP, because Egln3 depletion reduced ChoRE-driven luciferase activity in primary hepatocytes (Fig. 7C).

Finally, we tested whether ectopic ChREBP could rescue decreased target gene expression caused by Egln3 loss of function. Primary hepatocytes from mice homozygous for a floxed Egln3 allele (45) were isolated and infected with adenoviruses expressing GFP or Cre. Cre-induced Egln3 deletion (Fig. S5A) was rescued by siRNA (Fig. 7B and Fig. 5A). This was likely mediated by impaired activation of endogenous ChREBP, because Egln3 depletion reduced ChoRE-driven luciferase activity in primary hepatocytes (Fig. 7C).

Discussion

We discovered that ChREBPα is hydroxylated at several proline residues. Hydroxylation was detectable in ectopically expressed ChREBPα isolated from HEK293 cells and endogenous protein from mouse liver, suggesting that this PTM is physiologically relevant. Two of these sites, Pro141 and Pro536, were identified unambiguously in both ectopic and endogenous expressed ChREBPα. Egln3 deletion reduced ChoRE-driven luciferase activity in primary hepatocytes and that ectopic ChREBP rescues defects associated with loss of Egln3.

Figure 2. Endogenous ChREBPα protein in mouse liver is proline-hydroxylated. A, murine liver input, ChREBP-IP, and supernatant were analyzed for ChREBP and GAPDH protein by immunoblotting (IB). B, sequence coverage of detected ChREBP peptides. C, ChREBP peptides with hydroxylation of specified and nonspecified proline residues. D, protein domains of murine ChREBPα comprised of 864 amino acids (a.a.). Prolines are marked yellow, and red stars mark unambiguous hydroxilation sites identified in both ectopically and endogenously expressed ChREBPα. E, conservation of ChREBPα Pro141 and Pro536.

by FLAG-tagged EGLN1 and EGLN3, but not by EGLN2, when expressed ectopically in HEK293 cells (Fig. 6A). Egln enzymes differ in substrate specificity, protein stability, cellular localization, and tissue expression pattern (43). Because the tissue expression pattern and hepatic regulation of Egln3 resembled that of ChREBP, we focused on this proline hydroxylase. Performing the reciprocal IP of FLAG-tagged ChREBP pulled down Egln3, validating the interaction (Fig. 6B). A truncated ChREBP lacking the 239 N-terminal amino acids was pulled down by FLAG-tagged Egln3, suggesting that the shorter ChREBPβ also interacts with proline hydroxylases (Fig. 6C). The presence of either or both hydroxylated Pro536 and Pro141 was not required, because Pro → Ala ChREBP mutants still interacted with Egln3 (Fig. 5A), nor was the interaction dependent on high glucose concentrations (Fig. 5B). We next tested for interaction of endogenous proteins in primary hepatocytes, despite the known limitations of the available antibodies for both Egln3 and ChREBP. Hepatocytes were treated with CoCl2, stabilizing HIF and inducing expression of its known target gene Egln3 (44), by competing with ferrous iron in the active site of EGLN’s and by other mechanisms (43). This up-regulated Egln3 protein, facilitating the detection of pulled down endogenous Egln3 by ChREBP-IP (Fig. 6D). Thus, the interaction of ChREBP with certain Egln isoforms can be detected in ectopically or endogenously expressed proteins and renders them likely candidates to proline hydroxylate ChREBP.

Figure 2. Endogenous ChREBPα protein in mouse liver is proline-hydroxylated. A, murine liver input, ChREBP-IP, and supernatant were analyzed for ChREBP and GAPDH protein by immunoblotting (IB). B, sequence coverage of detected ChREBP peptides. C, ChREBP peptides with hydroxylation of specified and nonspecified proline residues. D, protein domains of murine ChREBPα comprised of 864 amino acids (a.a.). Prolines are marked yellow, and red stars mark unambiguous hydroxilation sites identified in both ectopically and endogenously expressed ChREBPα. E, conservation of ChREBPα Pro141 and Pro536.
required for its constitutive, high activity (5, 32). However, acquiring experimental proof for hydroxylation will be challenging because of low abundance/stability of ChREBPβ protein in most tissues, including the liver (5, 32). Accordingly, we failed to detect a discernable band migrating at ~80 kDa during immunoblotting that could represent this isoform in ChREBP-IP–enriched liver protein. In contrast, we show that ChREBPα, migrating at ~100 kDa, is readily detectable and strongly enriched by ChREBP-IP, allowing for subsequent MS analysis.

Strikingly, mutating hydroxylated Pro141 and Pro536 of ChREBPα to alanine strongly impairs its activity in a heterologous reporter cell system and in the physiologic setting of hepatocytes. Genomic binding to an established ChREα was not affected by these mutations, indicating that other mechanisms such as the recruitment of transcriptional co-factors may be responsible for reduced ChREBP activity. Based on their localization within the mondo conserved region III of the LID (Pro141) and the proline-rich region (Pro536) and their general conservation and functional implications (20), we would have hypothesized that mutating Pro141 would show more robust effects than mutating Pro536. Although true for ChoRE activity, induction of Tnxip expression in hepatocytes differed from that for currently unknown reasons. Notably, mutating Pro536 and Pro141 of ChREBPβ had no effect on protein levels, suggesting that these specific proline residues are not required for stability of ChREBPα protein. Because neither proline is part of highly conserved α-helices of ChREBP that are essential for proper secondary protein structure, we would argue that the most likely interpretation of these mutation studies supports the notion that hydroxylation of Pro141 and Pro536 is required for adequate ChREBP activity. However, there is the possibility that lower activity in the Pro→Ala ChREBP mutants is due to other effects, independently of preventing hydroxylation.

None of the ChREBP prolines targeted by hydroxylation share consensus motifs for P4H/P3H or Egln enzymes, the two families known to catalyze this reactions. Because the latter is suggested to hydroxylate prolines lacking the consensus Leu-Xaa-Xaa-Leu-Ala-Pro motif (42), Egln enzymes may carry out proline hydroxylation of ChREBP. Although our study is limited by missing direct evidence for Egln-mediated hydroxylation of ChREBP, in part because of the finding that in vitro, Egln-mediated proline hydroxylation of target prolines works well for HIF peptides but not for other putative substrates (46). To overcome this, we performed a variety of experiments to narrow down the most likely Egln isoform to do so. We found that among Egln isoforms, expression and regulation of Egln3 resembles that of ChREBP most closely. Moreover, we show that ectopically expressed or endogenous Egln3 physically interacts with ChREBP and that Egln3 loss of function interferes with glucose-induced expression of ChREBP target genes in primary hepatocytes. Although this is not proof for proline hydroxylation ChREBP, Egln3 presents itself as the most likely candidate.

Interestingly, Egln3 was implicated previously in the regulation of glucose and fatty acid metabolism. Acute deletion of Egln3 in livers of mice reduced expression of gluconeogenic

Figure 3. Pro141 and Pro536 are required for glucose-induced ChREBP activity. HEK293 cells were transfected with empty vector, WT, and single or double proline mutations of ChREBPα as indicated. A and B, expression of mRNA (A) and protein of ChREBP (B) were determined by qPCR and immunoblotting (IB), respectively. C, similarly treated cells co-transfected with Mlx and an Acc1-Chore luciferase reporter were incubated overnight with cell culture medium containing 25 mM glucose. Then the cells were switched to medium containing 2.5 mM glucose for 6 h before exposing cells to either 2.5 or 25 mM glucose for 24 h. The cells were harvested, and luciferase activity was analyzed. The data are presented as the means ± S.D.*, P < 0.05 versus empty vector; #, P < 0.05 versus WT ChREBP.

Figure 4. Pro141 and Pro536 are required for glucose-induced ChREBP target gene expression in hepatocytes. A, ChREBP deletion in murine AML12 hepatocytes by CRISPR/Cas9-guided mutations was validated by immunoblotting (IB; clone 2 = loss of function; clones 1 and 3 = WT). B, WT and ChREBP loss of function AML12 cells were incubated with medium containing 2.5 mM glucose for 6 h before exposing cells to either 2.5 or 25 mM glucose for 24 h. mRNA was analyzed for the expression of ChREBP target genes by qPCR. C, ChREBP loss-of-function AML12 cells were transfected with empty vector, WT, and single or double proline mutations of ChREBPα as indicated, and ChREBP protein expression was determined by immunoblotting. D, cells treated as in C were incubated with glucose as described in B, and mRNA expression of Tnxip and Hmgcs2 was analyzed by qPCR. The data are presented as the means ± S.D.*, P < 0.05 versus 2.5 mM glucose and #, P < 0.05 versus AML12 WT in B; *, P < 0.05 versus empty vector and #, P < 0.05 versus WT ChREBP in D.
and lipogenic genes, including glucose-6 phosphatase (G6pc) and fatty acid synthase (Fasn), which was attributed to an activation of HIF2α (47). Another study showed lower phosphoenolpyruvate carboxykinase (Pck1) and G6pc expression in Egln3-depleted primary hepatocytes, linked to an impairment of cAMP-induced peroxisome proliferator–activated receptor γ co-activator-1α activity. Pck1, G6pc, and Fasn are also direct targets of ChREBP (3, 12, 48, 49), suggesting that ChREBP may be involved in the observed regulations. Moreover, Egln3 was shown to bind to, hydroxylate, and activate acetyl-CoA carboxylase (ACC) 2 in states of nutrient abundance, thereby suppressing fatty acid oxidation (50). Hydroxylation of ACC2 was higher in cells exposed to high glucose, suggesting that glucose induces Egln3 activity (50). Thus, high glucose concentrations may increase proline hydroxylation of ChREBP. Also Acc2 expression is under transcriptional control of ChREBP (13, 17), which implies functional synergism via different Egln3-dependent mechanisms. Intriguingly, Egln proline hydroxylases serve as cellular oxygen sensors and, by their control of HIF degradation, are pivotal in initiating cellular adaptation to reduced oxygen tension. One could speculate that Egln3 integrates oxygen sensing with fine-tuning glucose metabolism via hydroxylating ChREBP. However, more research is needed to address this potential relationship.

Taken together, we discovered that several proline residues of ChREBP are hydroxylated and that mutation of these residues impairs its activation upon exposure to high glucose. We further identify Egln3 as potential candidate to mediate this hydroxylation. Targeting proline hydroxylation of ChREBP may allow for therapeutic interference in metabolic diseases.

**Experimental procedures**

**Materials and software**

Chemicals, if not stated otherwise, were purchased from Sigma–Aldrich. Cell culture reagents like antibiotics and fetal bone serum were purchased from Thermo Fisher Scientific. siRNA oligonucleotides were acquired from Eurogentec (Liège, Belgium). AML12 hepatocytes were purchased from ATCC. Amino acid annotation, homology, and proline visualization was performed by the browser-based web application STRAP (51). CRISPR guide RNA design was carried out using the online CRISPR Design Tool from the Zhang laboratory (52).

**Primary hepatocyte isolation and cell culture**

Hepatocytes were isolated as described previously (53). In short, livers of anesthetized male WT mice or male mice with floxed Egln3 alleles (45) were perfused with digestion buffer containing 5000 units of collagenase (Worthington). After filtration and separation by Percoll gradient centrifugation (GE Healthcare), the cells were seeded on collagen-coated 12-well plates in Dulbecco’s modified Eagle’s medium (DMEM) without pyruvate containing 25 mM glucose, 10% fetal bone serum, and 1% penicillin/streptomycin. HEK293 cells and AML12 hepatocytes were cultured according to the manufacturer’s instructions. Glucose concentrations for sensing experiments were used as indicated.

**FLAG and ChREBP-IP**

HEK293 cells, 2 days after transient transfection with murine ChREBPα, were lysed in RSB-100 (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM MgCl2, 0.1% Triton X-100, phosphatase, and protease inhibitor tablets (Roche)) directly on the cell culture plate. After 15 min of incubation on ice, the cells were scraped down and sonicated three times with 5 impulses at low intensity and 2 min of incubation on ice in between. Protein lysate was vortexed vigorously every 2 min during another 15-
min incubation on ice. After centrifugation at 13,000 rpm in a table-top centrifuge at 4 °C, the supernatant was collected, and protein concentration was determined. IP was performed with 4000 mg of protein in a total volume of 1 ml (RSB-100). FLAG-IP was performed with anti-FLAG M2 affinity gel (Sigma–Aldrich) for 2 h at 4 °C. The beads were collected by centrifugation and washed five times with 1 ml of RSB-100. ChREBP-IP was performed accordingly with RSB-100 protein extracts of mouse liver (8000 mg) for 4 h at 4 °C with 10 mg of ChREBP antibody (NB400-135) and subsequent binding to protein A-Sepharose beads (GE Healthcare) for 2 h at 4 °C and five cycles of washing with 1 ml of RSB-100.

**ChIP**

ChIP analyses were performed as described previously (54). Approximately 50 μg of cross-linked and sonicated chromatin extracts of AML12 hepatocytes were used for each IP and incubated with 4 μg of a ChREBP antibody (NB400-135, Novus Biologicals) overnight. Experiments were performed on at least three independent sets of transfected cells exposed to 25 mM glucose. qPCRs were performed using SYBR Green qPCR MasterMix (Eurogentec) and evaluated according to the standard curve method. mRNA expression data were normalized to murine 36B4 or human HPRT. The primer sequences are listed in Table S1.

**RNA isolation, cDNA synthesis, and qPCR**

RNA was purified by spin column kits (Macherey–Nagel). cDNA was synthesized using Moloney murine leukemia virus-RT (Promega). qPCRs were performed using SYBR Green qPCR MasterMix (Eurogentec) and evaluated according to the standard curve method. mRNA expression data were normalized to murine 36B4 or human HPRT. The primer sequences are listed in Table S1.

**Transient transfection of AML12 hepatocytes and HEK293 cells**

AML12 hepatocytes and HEK293 cells were transfected transiently with WT or proline-mutated pCMV4-mChREBP-FLAG using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s protocol.

**Gene silencing in primary hepatocytes by siRNA**

Depletion of gene expression in murine primary hepatocytes was performed as described previously (55). In short, primary hepatocytes were allowed to attach to the cell culture plate, and medium was replaced by 500 μl of DMEM without pyruvate containing 25 mM glucose and lacking supplements. The cells were transfected with 1 nmol of siRNA and 4 μl of Lipofectamine 2000 per 1 well of a 12 well cell culture plate overnight. The next morning, the medium was replaced by complete DMEM. siRNA sequences are listed in Table S2.

**Site-directed mutagenesis of ChREBP**

Expression plasmids for ChREBP Pro → Ala mutants were generated by using the QuikChange XL site-directed mutagenesis kit (Agilent) with the pCMV4-mChREBP-FLAG vector.
and specific primers for amplification. Mutations were validated by sequencing.

CRISPR/Cas9 mediated deletion of ChREBP in hepatocytes

Genome editing was performed according to a published protocol (56). In short, AML12 hepatocytes were transfected with the px458;SpCas9-2A-EGFP vector containing a guide sequence targeting ChREBP (Table S3) by Lipofectamine 2000. Then 3 days later, single-cell clones of GFP-positive cells were established by FACS and expanded. Whole cell extracts were analyzed for ChREBP protein by immunoblotting and genomic DNA sequenced.

Luciferase reporter assays

HEK293 cells were transfected with WT or proline-mutated pCMV4-mChREBP-FLAG, pCMV4-Mlx, and a luciferase reporter that contained two copies of the Acc1 ChoRE in its promoter driving firefly luciferase expression (pGL3-Acc1-ChoRE-luc) by Lipofectamine 2000 overnight. Then 3 days later, single-cell clones of GFP-positive cells were established by FACS and expanded. Whole cell extracts were analyzed for ChREBP protein by immunoblotting and genomic DNA sequenced.

LC–MS/MS

IP-enriched proteins were denatured for 7 min at 95°C, cooled down to room temperature, and incubated with 15 mM iodoacetamide for 30 min in the dark for alkylation of cysteine residues. After centrifugation, the proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The band corresponding to ChREBP was excised, and protein digestion was carried out using trypsin at an enzyme-to-protein ratio of 1:20 (w/w) at 37°C overnight as previously described (57). LC–MS/MS analysis was performed using an UltiMate 3000 RSLC nano LC system coupled on-line to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Reversed-phase separations were performed using a capillary column (Acclaim PepMap100, C18, 3 μm, 100 A, 75-μm inner diameter × 25 cm, Thermo Fisher Scientific) at an eluent flow rate of 300 nl/min using a linear gradient of 0–40% B in 85 min. Mobile phase A was 0.1% formic acid in water; mobile phase B was 0.1% formic acid in acetonitrile. Mass spectra were acquired in a data-dependent mode with one MS survey scan with a resolution of 60,000 (Orbitrap Elite) and MS/MS scans of the 15 most intense precursor ions in the linear trap quadrupole. The processed MS/MS spectra (Thermo Proteome Discoverer 2.3.0) and the search engine Mascot Daemon (version 2.5.1, Matrix
Proline hydroxylation of ChREBP

Science Ltd., London, UK) were used to search in-house against the UniProtKB/Swiss-Prot database MOUSE 2016 okt (57,951 sequences; 26,573,730 residues). A maximum of two missed cleavages was allowed, and the mass tolerance of precursor and sequence ions was set to 20 ppm and 0.35 Da, respectively. Cysteine, carbamidomethyl and propionamide modification of C, oxidation of H, W, M and hydroxylation of P were considered as possible variable modifications. For the protein level, a protein was accepted as unambiguously identified if the total MASCOT score was greater than the significance threshold and if at least two peptides appeared first in the time in the report and were the top ranking peptides. Based on decoy database searches, the false-positive rate was estimated to be <1%. To identify individual peptides, probability-based scoring (MASCOT score) was used. The threshold/expectation value for accepting peptide spectra were ions score >34, indicating identity or extensive homology (p < 0.05). Ions score is = \(-10^{\log(P)}\), where P is the probability that the observed match is a random event. Table S4 includes the precursor charge, m/z, modifications observed, sites of proline hydroxylation, and peptide ions scores for each assigned ChREBP peptide. Mass and ion designation labeled MS/MS fragment ion spectra of all proline-hydroxylated ChREBP peptide sequences derived from cells and mouse liver are shown in Figs. S6 and S7, respectively.

Adenovirus preparations and infections

An adenovirus expressing murine Egln3 was generated using the Adeno-X System 3 (Takara). Adenoviruses expressing GFP, mChREBPα, mChREBP amino acids 240–864 (32), Cre, or Egln3 were amplified in HEK293 cells, purified by CsCl gradient centrifugation, and dialyzed against 0.9% saline. Titers were determined by the Adeno-X Rapid Titer kit (Takara). For infections, the cells were incubated overnight with adenoviruses before washing cells once with PBS the next morning. Titers were chosen dependent on desired transgene expression level in the respective cell context. For Cre-mediated deletion, primary hepatocytes were incubated with \(3 \times 10^{5}\) infectious units in 500 μl of medium in 12 wells overnight.

Immunoblotting

Whole cell proteins were isolated and homogenized in radioimmune precipitation assay buffer by standard methods, sonicated, and separated by SDS-PAGE before blotting to polyvinylidene difluoride membranes by tank blot overnight. Protein concentrations were determined by the BCA method (Thermo Fisher Scientific). After incubation with antibodies for ChREBP (NB400-135, lot J1, M1, M6, or Q4; Novus Biologicals), anti-FLAG-HP (A8592; Sigma), EGLN3 (NB100-303 or NB100-139; Novus Biologicals), β-ACTIN (sc-47778, Santa Cruz), or GAPDH (2118; Cell Signaling), a secondary horseradish peroxidase-conjugated antibody was added (Pierce), and a chemiluminescent substrate kit (Thermo Fisher Scientific) was used for detection.

Mouse experiments

Animal procedures were in accordance with institutional guidelines and approved by the corresponding authorities. Gene expression in different mouse tissues was performed in 3-month-old (V1124-300 ssniff®Spezialdiäten GmbH, Soest, Germany) male C57BL/6j mice that were fed ad libitum, kept under a standard 12-h light/12-h dark cycle, and euthanized at ZT 2–3. The mice were fasted and refed for time periods as indicated.

Statistical analyses

For cell culture experiments, representative results of at least three independent experiments performed in triplicate are shown and presented as the means ± S.D. The animal data are presented as means ± S.E. Significance was determined by the two-tailed Student’s t test or analysis of variance, as appropriate, and *p < 0.05 was deemed significant (#, *, p < 0.05).

Data availability

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (58) with the data set identifier PXD019137.

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Abbreviations—The abbreviations used are: ChREBP, carbohydrate response element–binding protein; ChoRE, carbohydrate response element; PTM, post-translational modification; LID, low-glucose inhibitory domain; IP, immunoprecipitation or immunoprecipitate; HIF, hypoxia-inducible factor; DMEM, Dulbecco’s modified Eagle’s medium; qPCR, quantitative PCR.

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