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HGFAC is a ChREBP Regulated Hepatokine that Enhances Glucose and Lipid Homeostasis

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Abstract:

Carbohydrate Responsive Element-Binding Protein (ChREBP) is a carbohydrate sensing transcription factor that regulates both adaptive and maladaptive genomic responses in coordination of systemic fuel homeostasis. Genetic variants in the ChREBP locus associate with diverse metabolic traits in humans, including circulating lipids. To identify novel ChREBP-regulated hepatokines that contribute to its systemic metabolic effects, we integrated ChREBP ChIP-seq analysis in mouse liver with human genetic and genomic data for lipid traits and identified Hepatocyte Growth Factor Activator (HGFAC) as a promising ChREBP-regulated candidate in mice and humans. HGFAC is a protease that activates the pleiotropic hormone Hepatocyte Growth Factor (HGF). We demonstrate that HGFAC KO mice have phenotypes concordant with putative loss-of-function variants in human HGFAC. Moreover, in gain- and loss-of-function genetic mouse models, we demonstrate that HGFAC enhances lipid and glucose homeostasis, which may be mediated in part through actions to activate hepatic PPARγ activity. Together, our studies show that ChREBP mediates an adaptive response to overnutrition via activation of HGFAC in the liver to preserve glucose and lipid homeostasis.
Introduction

Carbohydrate Responsive Element Binding Protein (ChREBP, also known as Mlxipl) is a transcription factor expressed in key metabolic tissues including liver, adipose tissue, kidney, small intestine, and pancreatic islets (1, 2). It is activated by sugar metabolites, and in the liver and small intestine, it is robustly activated following fructose ingestion (3, 4). Activated ChREBP stimulates expression of genomic programs contributing to adaptive and maladaptive metabolic responses (1). Hepatic ChREBP activity is increased in human obesity and diabetes (5, 6). Knockdown or knockout of hepatic ChREBP protects against metabolic disease in diet and genetic forms of obesity (3, 7, 8).

ChREBP plays a significant role in human metabolic physiology. Common genetic variants in the ChREBP locus associate with pleotropic metabolic traits including circulating lipids and cholesterol, BMI, waist-hip ratio, height, diverse hematological parameters, serum urate, liver enzymes, and blood pressure (9). The complement of ChREBP transcriptional targets regulate these diverse traits is incompletely understood. To date, Chromatin Immunoprecipitation-sequencing (ChIP-seq) assays have implicated thousands of genes as ChREBP targets (10, 11). It is well established that ChREBP regulates glycolysis and fructolysis, hepatic and adipose lipogenesis, and hepatic glucose production via regulation of key enzymes involved in these metabolic pathways (4, 12-14). At the same time, most putative ChREBP transcriptional targets have unknown or poorly defined metabolic impact.

We performed ChIP-seq for ChREBP in mouse liver and integrated this with human genetic data to identify novel ChREBP-dependent hepatokines that might regulate systemic metabolism. Through this screen we identified Hepatocyte Growth Factor Activator (HGFAC) as a promising candidate. HGFAC is a liver-secreted, circulating protease that activates Hepatocyte Growth Factor (HGF) which regulates pleiotropic biological activities including morphogenesis, cell migration, cell state transition, and proliferation in epithelial and other cell types throughout the body (15-17). We demonstrate that HGFAC is indeed nutritionally regulated in a ChREBP-dependent
manner and participates in an adaptive response maintaining carbohydrate and lipid homeostasis.

**Results:**

*HGFAC is a ChREBP genomic target associating with metabolic traits in humans*

To identify ChREBP transcriptional targets that participate in the regulation of ChREBP associated metabolic programs and phenotypes, we performed ChIP-seq analysis for ChREBP in livers of two strains of male mice gavaged with either water or fructose. We identified 4,860 distinct genomic sites enriched for ChREBP binding (Supplementary Table 1) which include well-defined loci in canonical ChREBP targets involved in glycolysis, glucose production, fructolysis, and lipogenesis such as liver pyruvate kinase (PKLR), glucose-6-phosphatase (G6PC), fatty acid synthase (FASN), and ketohexokinase (KHK), respectively (Figure 1A). Although fructose gavage can acutely induce ChREBP-dependent changes in gene expression, ChREBP ChIP-seq peaks were readily detectable in fasted mice, and fructose gavage did not enhance ChREBP ChIP-seq peak height even at a liberal false discovery rate of 0.20. This indicates that increased chromatin occupancy is not essential for fructose to induce ChREBP-dependent gene transcription. Most ChREBP ChIP peaks occurred within 10 kb of transcriptional start sites (Figure 1B). Consistent with ChREBP’s known functions, Genomic Region Enrichment Analysis (GREAT) of putative ChREBP binding sites demonstrated enrichment for numerous metabolic processes including carbohydrate and lipid metabolism (Figure 1C) (18).

Variants in the *ChREBP* locus are strongly associated with hypertriglyceridemia in human populations (19, 20). However, the complement of ChREBP transcriptional targets that mediate its effects on circulating lipids is uncertain. We sought to determine whether genomic loci containing human homologues of mouse ChREBP target genes are enriched for variants that associate with hypertriglyceridemia in human populations. Via Meta-Analysis of Gene-set ENrichmenT of variant Associations (MAGENTA), we confirmed that loci in proximity to human homologues of mouse genes that are within 20
kb of ChREBP binding sites are enriched for SNPs that associate with hypertriglyceridemia in humans (Adjusted P-val = 0.003). 87 loci/genes contributed to this enrichment with an FDR of 0.05 (Table 1 and Supplementary Table 2) (21). This list includes known ChREBP transcriptional targets such as GCKR, TM6SF2, KHK, and ChREBP (MLXIPL) itself, all previously implicated in regulating carbohydrate and triglyceride metabolism. Of these 87 loci, seven encoded putative secretory proteins including several lipoproteins (APOC2, APOE, and APOA5), VEGFA which is most well-known for its role in angiogenesis, but also implicated in metabolic control, and HGFAC (Supplementary Table 2) (22). To our knowledge, HGFAC has not been identified as a ChREBP transcriptional target nor studied extensively in the context of systemic fuel metabolism.

HGFAC is a serine protease expressed predominately in hepatocytes and secreted as a zymogen into circulation where it is found in a single chain pro-HGFAC form (23, 24). In-vitro studies have identified thrombin and kallikrein-related peptidases KLK-4 and KLK-5 to be potent activators of pro-HGFAC (25, 26). Once activated, HGFAC cleaves and activates Hepatocyte Growth Factor (HGF) which can then bind and activate the c-Met receptor tyrosine kinase (c-MET) (23). HGF and c-MET have pleiotropic biological activities as mitogens and motogens in organogenesis, tissue repair, and cell migration, and also function as anti-inflammatory, apoptotic, and cytoprotective signals depending on the context (15). Variants in c-MET also associate with circulating triglycerides at genome wide significance in humans consistent with a potential role for HGFAC in regulating triglyceride levels through activation of HGF (27). Moreover, increased levels of circulating HGF in people associate with features of cardiometabolic disease including obesity, risk for type 2 diabetes, and risk for cardiovascular disease (28-31). Circulating HGF levels are influenced by variants in the HGFAC locus (32). A missense variant in HGFAC, rs3748034, that associates with increased circulating HGF also associates with increased circulating triglycerides in GWAS aggregate data at genome wide significance (beta = 0.0302, p < 5e-28) as well as other cardiometabolic risk factors and pleiotropic biological traits (33, 34). The Ala218Ser mutation encoded by rs3748034 is predicted to be “possibly damaging” by PolyPhen-2 (35). Furthermore, another missense variant in HGFAC, rs16844401, that associates with increased
circulating triglycerides also associates with increased coronary artery disease risk (36). These associations motivated further investigation to determine whether ChREBP regulates HGFAC expression and whether this interacts with nutritional status to regulate systemic fuel metabolism and cardiometabolic risk factors.

*Nutritional regulation of HGFAC is ChREBP-dependent.*

To confirm specific binding of ChREBP to the putative binding site in proximity to the *Hgfac* gene, we performed targeted ChIP-qPCR on livers from control and liver-specific ChREBP KO mice with anti-ChREBP and control IgG. The putative binding site was enriched when ChREBP immunoprecipitation was performed on control but not ChREBP LKO liver samples (Figure 2A). ChREBP activity in the liver is responsive to diets high in fructose. To test whether acute fructose feeding induces hepatic expression of *Hgfac*, we fed overnight-fasted Wistar rats with high fructose diet (HFrD) or control chow diet for 4 hours and measured hepatic *Chrebpβ* and *Hgfac* mRNA levels. Acute fructose feeding induced *Chrebpβ* expression by more than 20-fold (p<0.0001) while *Hgfac* mRNA levels increased by 25% (p<0.05) (Figure 2B). To examine the role of hepatic ChREBP in the regulation of HGFAC in rodents, we measured hepatic *Hgfac* mRNA and HFGAC protein in the liver and plasma of mice with liver specific deletion of ChREBP after 8 weeks on high fructose (HFrD) or control diet. High fructose feeding increased hepatic *Hgfac* mRNA expression 1.7-fold (p<0.0001) in control mice, and this induction was abrogated in liver-specific ChREBP KO mice (Figure 2C). Fructose-induced increases in hepatic *Hgfac* mRNA expression were accompanied by 4- and 2-fold increases in hepatic and circulating pro-HGFAC protein levels (Figure 2D, Supplementary Figure 1A). Basal liver and circulating HGFAC protein levels tended to be decreased in chow fed liver-specific ChREBP KO mice and were not induced with fructose feeding. Circulating HGFAC also increased in mice fed a high fat/high-sucrose (HF/HS) diet and in genetically obese Zucker fatty rats on chow diet (Supplementary Figure 1B and C), where hepatic ChREBP activity is also robustly increased independently of an obesogenic diet (37). Altogether, these data show that hepatic ChREBP mediates diet and obesity induced increases in circulating HGFAC.
Next, we examined whether the genomic region containing the ChREBP binding site in proximity to Hgfac in mice (chr5:35,029,873-35,030,157) is conserved in humans. Analysis using the UCSC genome browser demonstrated that the corresponding region in the human genome was highly homologous to the mouse region with 76.8% of nucleotides identity, while in rats this homology achieved 99.6% identity (38). Next, we sought to determine whether ChREBP-mediated regulation of HGFAC might be conserved in humans. To that end, we analyzed hepatic mRNA expression levels of HGFAC and other ChREBP transcriptional targets in the Genotype-Tissue Expression (GTEx) Biobank (39). Expression of the potent ChREBP-β isoform is an excellent surrogate marker of tissue ChREBP activity (14). However, it is expressed at low levels which are typically below the sequencing depth of most RNA-seq experiments. Consistent with this, GTEx RNA-seq data does not distinguish between ChREBP-β and -α isoforms. Due to the lack of ChREBP-β specific expression data, we used a composite expression vector comprised of 5 well-validated ChREBP target genes (FASN, PKLR, KHK, ALDOB, and SLC2A2) and found that this composite vector strongly correlates with the expression of HGFAC (Pearson correlation r²=0.44, p <0.0001) (Figure 2E). Transcription factor enrichment analysis of the 5% of hepatic genes that best correlated with hepatic HGFAC expression in the GTEx Biobank showed strong enrichment for genes co-expressed with ChREBP (Adjusted P-val = 1.75E-25) indicating conservation of the ChREBP-mediated regulation of HGFAC in humans (Figure 2F) (40). Additionally, hepatic HGFAC mRNA expression is upregulated in patients with obesity and uncontrolled diabetes (Figure 2G), conditions that are associated with increased hepatic ChREBP activity (5, 41). Collectively, these data support the hypothesis that hepatic HGFAC expression and circulating levels of HGFAC are regulated by ChREBP activity both in rodents and in humans, and hepatic HGFAC expression is increased in obesity and diabetes.

**Murine HGFAC KO recapitulates the phenotype of putative human LOF HGFAC variants.**

To study the roles of HGFAC in systemic metabolism, we generated whole body HGFAC KO mice that lack a portion of exon 1 and all of exon 2 (Figure 3A). The deletion was confirmed by genomic PCR, by the absence of detectable circulating HGFAC protein,
and by quantification of hepatic Hgfac mRNA (Figure 3B-D). HGFAC KO mice were born
at normal Mendelian ratios and did not appear to have any gross abnormalities when
compared to their littermate controls. Activated HGFAC activates HGF and c-MET
signaling. However, there is redundancy in this system and other proteases including
Hepsin (HPN) and coagulation factors XIa and XIIa are also capable of activating HGF
(42, 43). We sought to determine whether the ability to activate endogenous HGF is
impaired in HGFAC KO mice. c-MET signaling was assessed in HepG2 cells incubated
with thrombin treated sera obtained from control and HGFAC KO mice. Thrombin is one
of the proteases that is capable of activating HGFAC in-vitro (26). Serum from control
mice increased c-MET phosphorylation 1.9-fold when compared to controls
(DMEM+BSA), while this induction was attenuated with serum from KO mice (Figure 3E).
These results demonstrate that sera from HGFAC KO mice has reduced capacity to
activate HGF and c-MET signaling.

A putative loss of function variant in HGFAC (rs3748034) strongly associates with
increased circulating triglycerides, LDL-cholesterol, albumin, and platelets among other
traits (Figure 3F) (33). We determined whether HGFAC KO mice have similar
phenotypes. Male HGFAC KO mice had a 28% increase in circulating triglycerides (100
+/- 6.5 mg/dl vs 72+/- 4.5 mg/dl, p<0.001) and cholesterol (82+/- 11.5 mg/dl vs 69+/-14.8
mg/dl, p<0.05) (Figure 3G). Similarly, high levels of circulating triglycerides were present
in female HGFAC KO mice (89 +/- 5.4 mg/dl vs 65+/- 3 mg/dl, p<0.005) (Supplementary
Figure 2A). These differences were detected in adlib fed but not in overnight fasted
condition, while non-esterified fatty acids were similar between the groups in both ad
libitum fed and fasted states (Supplementary Figure 2B-C). Additionally, HGFAC KO
mice had a 15% increase in circulating albumin (4.8 +/- 0.19 vs 4.1 +/- 0.15 g/dl, p<0.01),
and a 15% increase in circulating platelets (1237 +/- 22 cells*10^3/ul vs 1048 +/- 57
cells*10^3/ul, p<0.05) (Figure 3H). No hematological parameter other than platelet count
was altered (Supplementary Figure 2D). Collectively, these data indicate that murine
HGFAC KO recapitulates phenotypes in putative loss-of-function human HGFAC
variants.

HGFAC KO mice develop impaired glucose homeostasis.
To examine the potential role of HGFAC in systemic metabolism, we challenged 8-week-old HGFAC KO mice and their littermate controls with high-fat/high-sucrose (HF/HS) diet for 18 weeks. We did not observe any differences in body weight or fat mass during the study (Figure 4A and B). However, a modest reduction in lean body mass was observed in HGFAC KO mice (Figure 4C). To assess glucose homeostasis, we performed glucose and glycerol tolerance tests in HGFAC KO mice and controls at time points throughout the study. Glycerol is a preferred gluconeogenic substrate and glycerol tolerance tests reflect hepatic glucose production capacity (44). After 4 weeks on HF/HS diet, HGFAC KO mice are glycerol intolerant with a 1.4-fold increase in glycemic excursion (p<0.05) (Figure 4D). At this time point, there was no difference between KO mice and controls with respect to glycemic excursion during a glucose tolerance test (Figure 4E), suggesting that young HGFAC KO animals may have dysregulated hepatic glucose production. However, after 13 weeks of HF/HS diet, HGFAC KO mice developed glucose intolerance with a 1.6-fold increase in incremental AUC (p<0.005) as well as insulin resistance with a 30% decrease in area above the curve (p<0.05), as measured by IP glucose and insulin tolerance tests, respectively (Figure 4F and G). HGF has been proposed to regulate pancreatic beta-cell development and insulin secretory capacity (45). To test insulin secretory capacity in HGFAC KO mice, we performed an oral mixed meal tolerance test which triggers more robust and sustained insulin secretion compared to IP glucose administration. Basal insulin and glucose levels were not different between HGFAC KO mice and controls (Figure 4H). At 10 min, insulin levels were 1.6-fold higher in HGFAC KO mice compared to controls (3.37+/− 0.48 ng/ml HGFAC KO vs 2.1+/− 0.4 ng/ml controls, p<0.05) with only a modest increase in glycemia at this time point. Altogether, these data indicate that HGFAC KO mice subjected to HF/HS diet develop early dysregulated hepatic glucose production followed by systemic insulin resistance with intact insulin secretory capacity.

We also examined whether HF/HS diet might exacerbate the increase in circulating triglyceride levels observed in chow-fed HGFAC KO mice. In contrast with the data in chow diet, we did not observe consistent differences in circulating triglycerides after 7-8 weeks of HF/HS diet but in most study cohorts. We only observed higher circulating triglycerides and a trend towards increased cholesterol on HF/HS diet in one additional
cohort (Supplementary Figure 3A-B). Similarly, triglyceride levels were not different between HGFAC KO and control after IP administration of poloxamer 407 which inhibits lipoprotein lipase and peripheral triglyceride clearance (Supplementary Figure 3C) indicating that VLDL production is similar between genotypes in this dietary context (46).

**HGFAC KO downregulates hepatic PPARγ expression.**

To define mechanisms that might contribute to altered triglyceride and carbohydrate metabolism in HGFAC KO mice, we performed RNA-seq analysis on liver from chow and HF/HS-fed HGFAC KO mice and littermate controls after 4 weeks on diet. *Hgfac* was the most significantly downregulated mRNA on both diets, confirming successful KO (Figure 5A). By pathway enrichment analysis (Figure 5B), genes involved in cell cycling were the most downregulated set in chow-fed HGFAC KO mice. This is consistent with HGF’s known effects to stimulate hepatocyte proliferation (47). Pathway analysis also suggested changes in lipid metabolism with reduced “PPAR signaling pathway” and “Fatty acid degradation” in KO mice on both diets. Upregulation of genes involved in ribosomal function were observed in the HGFAC KO mice potentially consistent with reduced cell cycling and enhanced differentiated function as a result of reduced HGF signaling. Gene sets associated with complement and coagulation pathways were also upregulated in HGFAC KO mice. Upregulation of complement and coagulation pathways is notable as putative loss of function variants in the HGFAC locus also associate with increased circulating fibrinogen levels (48).

Consistent with the pathway analysis, *Pparg* was in the top 10 most differentially expressed genes comparing chow-fed HGFAC KO mice and controls (Supplementary Table 3). To confirm this, we quantified hepatic mRNA gene expression by qPCR which revealed that *Pparg* but not *Ppara* is downregulated in livers of chow and HF/HS-fed HGFAC KO mice compared to controls (Figure 5C). Furthermore, PPARγ target genes were also downregulated. These results were replicated in a second cohort (Supplementary Figure A). Surprisingly, we found that downregulation of *Pparg* and its targets is liver-specific, as sub-cutaneous adipose tissue (inguinal) expression of *Pparg* and *Cd36* were similar between HGFAC KO and control mice (Supplementary Figure 4B). Hepatic PPARγ is reported to enhance liver fat accretion yet preserve hepatic and
systemic insulin sensitivity (49, 50). HF/HS-feeding increased the levels of hepatic triglycerides by 49% and 34% in HGFAC KO mice and controls, respectively (Figure 5D). However, hepatic triglyceride levels were reduced by 40% and 32% in HGFAC KO mice compared to controls on chow and HF/HS-diets, respectively. Recently, Shannon et al. reported that pioglitazone, a PPARγ agonist, inhibits the activity of catalytic subunit E1α of hepatic pyruvate dehydrogenase (PDHA) and diminishes hepatic glucose output but increases the level of hepatic triglycerides (51). Consistent with this mechanism, we observed reduced inhibitory S293-PDHA phosphorylation in HGFAC KO animals on chow and HF/HS-diets indicative of increased PDHA activity. Moreover, hepatic PPARγ protein levels were reduced in HGFAC KO mice both on chow and HF/HS-fed conditions, indicating diminished PPARγ activity (Figure 5E-H). Altogether, phenotypes in HGFAC KO mice are consistent with liver-specific deletion of PPARγ which results in reduced hepatic steatosis and impaired hepatic glucose homeostasis eventually leading to the development of peripheral insulin resistance (49, 50). This may be in part mediated by the effects of PPARγ on hepatic PDHA activity.

HGFAC overexpression enhances glucose homeostasis.

As HGFAC deficiency decreased expression of hepatic Pparg and its targets, we examined whether HGFAC overexpression has reciprocal molecular and metabolic effects. Adenoviral (ADV) mediated overexpression of HGFAC resulted in a robust increase of circulating HGFAC over a period of two weeks compared to ADV-GFP controls (Figure 6A). HGFAC overexpression had no effect on body weight or body composition (Figure 6B), but was associated with markedly improved glucose tolerance with a 30% reduction in incremental AUC (p<0.005) (Figure 6C) and a 50% reduction in glycemic excursion during a glycerol tolerance test performed in second cohort (p<0.0005) (Supplementary Figure 5A). Additionally, glucose levels were modestly but significantly lower in ADV-HGFAC mice in the ad libitum fed condition (p<0.05, t-test), as well as the overnight fasted and 3-hours refed conditions (p<0.05, Two-way ANOVA, main effect). However, peripheral insulin levels in fasted and refed ADV-HGFAC mice were not different from the levels of ADV-GFP mice (Figure 6D and Supplementary Figure 5B). The combination of reduced glycemia without changes in insulin following HGFAC
overexpression are suggestive of increased insulin sensitivity. Analysis of hepatic gene expression revealed that HGFAC overexpression induced expression of Pparg but not Ppara, as well as PPARγ target genes such as Cd36 and Fabp4 as well as Pdk4 which may participate in regulation of PDHA phosphorylation (Figure 6E). Furthermore, complementary to HGFAC KO mice, HGFAC overexpression increased hepatic PPARγ protein levels and phosphorylation of PDHA (S293), as well as Proliferating Cell Nuclear Antigen (PCNA) levels, indicating increased proliferation (Figure 6F). Whereas short-term overexpression of HGFAC was sufficient to produce glycemic and gene expression phenotypes reciprocal to HGFAC KO, we did not observe changes in hepatic or circulating triglyceride levels in this time frame (Figure 6G). Thus, HGFAC overexpression can induce changes in hepatic PPARγ expression and glucose homeostasis independently of its effects on hepatic lipids.

To assess whether HGFAC’s effect to induce Pparg expression is likely mediated through its ability to activate HGF and c-MET signaling, we treated murine AML12 hepatocyte-like cells with recombinant, active HGF. HGF treatment increased c-MET phosphorylation and increased Pparg mRNA expression by 30% (Figure 6H). These effects were inhibited by pre-treatment with PHA665752, a c-MET inhibitor (Figure 6I) (52). Altogether, these results support a model whereby overnutrition enhances ChREBP-dependent upregulation of HGFAC which activates an HGF-PPARγ signaling axis to preserve systemic glucose homeostasis.

Discussion:

ChREBP is a key transcription factor that is activated in major metabolic tissues by cellular carbohydrate metabolites and mediates genomic and physiological responses to overnutrition. The mechanisms by which carbohydrates activate ChREBP remain controversial (see (1)). Putative mechanisms include carbohydrate mediated translocation of ChREBP protein from the cytosol to the nucleus, alterations in ChREBP post-translational modifications, and/or allosteric effects of specific carbohydrate metabolites on ChREBP to enhance transactivation. We previously demonstrated that fructose gavage acutely and robustly activates ChREBP-dependent gene expression in mouse liver (4). Here, we performed ChIP-seq for ChREBP following fructose gavage
after a 5 hour fast to map ChREBP binding in mouse liver chromatin. We identified ~ 4000 ChREBP binding sites in livers from two mouse strains that are similar to previous efforts (10). To our surprise, while fructose acutely activates ChREBP-dependent gene transcription, chromatin-bound ChREBP was readily detectable in fasted animals and no marked increase in binding was observed following fructose gavage. These results suggest that carbohydrate-stimulated nuclear translocation and accumulation of nuclear ChREBP is not essential for the ability of carbohydrates to enhance ChREBP’s transcriptional activity. These results favor models suggesting that either carbohydrate-mediated post-translational modification or allosteric activation are the key mechanisms to stimulate ChREBP’s transcriptional activity.

Variants in the human ChREBP locus associate with pleiotropic biological traits with a particularly strong association with hypertriglyceridemia. The transcriptional targets that mediate ChREBP’s pleiotropic biological effects remain incompletely defined. By mapping ChREBP genomic binding sites in mouse liver and integrating this with human genetics data, we identified candidate contributors to ChREBP-mediated regulation of circulating lipids. While genes and loci in proximity to ChREBP binding sites were enriched for variants that associated with hypertriglyceridemia, of the thousands of hepatic ChREBP binding sites, only ~ 2% of such sites contributed to the enrichment. We anticipate that relatively small subsets of distinct ChREBP gene targets may contribute to its regulation of other metabolic traits.

A small minority of candidates were annotated as circulating factors or “hepatokines” that might regulate metabolism systemically. We elected to focus attention on HGFAC as a putative ChREBP-regulated hepatokine and demonstrated that circulating HGFAC is indeed nutritionally regulated in a ChREBP-dependent manner. Moreover, we showed that it participates in an adaptive metabolic response to obesogenic diets in part through its effects to stimulate hepatic Pparg expression and transcriptional activity (Figure 7).

To test the role of HGFAC in metabolism, we generated global HGFAC knockout mice. The ability of serum from HGFAC KO mice to activate HGF and facilitate c-MET signaling was impaired. The attenuation, but not fully abrogation, of this activity is
consistent with known redundancy in enzymes capable of HGF activation (43, 53). Alternative proteases including kallikreins, urokinases, matriptase, and hepsin may compensate for loss of HGF activity (53-56).

While *HGFAC* is highly expressed in the liver, it is also expressed at orders of magnitude lower levels in other tissues including the testes, intestines and possibly the pancreatic islets (23, 24, 57). While we cannot rule out the contribution of extra-hepatic HGFAC on the observed phenotypes, the majority of HGFAC found in circulation is likely originating from the liver. Indeed, liver-specific ChREBP KO mice have lower circulating levels of HGFAC, which failed to increase on high fructose diet. Additionally, adenoviral overexpression of hepatic HGFAC increased circulating HGFAC levels and produced systemic metabolic effects, further indicating that HGFAC is a ChREBP-regulated hepatokine.

We observed that HGFAC KO reduced and increased hepatic and circulating triglyceride, respectively. This was associated with impaired hepatic and systemic glucose tolerance. While higher circulating triglycerides were present in chow-fed cohorts, this phenotype was not consistently observed with HF/HS diet challenges. One potential explanation is that HF/HS diet through increased fat delivery and storage can overwhelm the subtle effects of HGFAC and hepatic PPARγ on circulating triglycerides in mice. Additional experiments assessing triglyceride secretion and/or clearance will be required to fully explain these observations. ADV-HGFAC overexpression produced a reciprocal phenotype with respect to glucose homeostasis but did not alter liver or circulating lipids in the short time frame of this experiment. Our results contrast with the reported effects of acute treatment with recombinant, active HGF in rodents to reduce steatosis and with inconsistent effects on circulating triglycerides (58, 59). Additionally, marked and sustained transgenic overexpression of HGF under a metallothionein promoter also reduced steatosis in contrast with our observations (60). The differences observed in these publications and our experiments may be due to differences in gain- versus loss-of-function experiments, differential effects in acute versus chronic paradigms, and the degree of changes in HGF activity and signaling.
The specific mechanism by which pro-HGF is activated, either by HGFAC versus other proteases, also appears to have marked impact on where HGF signaling may be enhanced and on the resultant systemic metabolic effects. As an example, hepsin (HPN) is a membrane bound protease expressed in multiple tissues that is also capable of HGF activation. Hepsin KO which also reduces HGF-c-MET signaling produces a vastly different metabolic phenotype compared with HGFAC KO mice. Global hepsin KO mice are resistant to diet induced obesity and this lean phenotype is associated with enhanced glucose and lipid homeostasis (61). Profound changes in energy homeostasis in hepsin KO mice and its lean phenotype appear to be due to extensive expansion of brown fat and increased thermogenesis, features which we did not observe in HGFAC KO mice.

Proteases such as HGFAC and HPN are promiscuous and may activate other peptide hormones which may also contribute to their differing biological effects. For instance, HGFAC can also cleave and activate pro-macrophage stimulating protein (pro-MSP, also known as MST1) which then activates the RON receptor tyrosine kinase (also known as MST1R) (62, 63). Although we determined that HGF activation and c-MET signaling is impaired in experiments conducted with serum from Hgfac KO mice, it remains possible that some of the HGFAC mediated changes that we observe are an effect of decreased signaling through MSP-RON cascade or other, unknown HGFAC proteolytic targets. Nevertheless, concordant associations in human HGFAC and c-MET variants with phenotype in HGFAC KO mice indicate that some of the key biological effects observed in HGFAC knockout mice are likely mediated through reduced HGF-c-MET signaling.

Our results show that the ChREBP-HGFAC axis regulates hepatic PPARγ signaling in mice. We further validated this observation by showing that HGF treatment can increase Pparg expression in hepatocyte-like AML12 cells and this can be blocked by a c-MET inhibitor. While the metabolic role of PPARγ is most well recognized with respect to adipogenesis, hepatic PPARγ also appears important in regulating systemic metabolism (64-66). Liver-specific deletion of Pparg reduces steatosis but leads to hypertriglyceridemia and glucose intolerance associated with muscle and adipose insulin resistance (49). While the beneficial effects of hepatic PPARγ have been attributed to its
effects on reducing circulating lipids, recent work demonstrated that the PPARγ agonist pioglitazone enhances hepatic insulin sensitivity independently of its effects on hepatic lipids and is instead dependent on PPARγ’s ability to inhibit hepatic pyruvate dehydrogenase activity (51). Data from HGFAC KO mice are consistent with this hypothesis in that decreased PPARγ activity is accompanied by a reduction in inhibitory phosphorylation of the PDH catalytic subunit on Ser293. Adenoviral overexpression of HGFAC led to marked improvement in glucose tolerance with increased hepatic PPARγ expression and increased phosphorylation of PDH consistent with this model. While HGFAC overexpression increased hepatic PDK4 mRNA levels, we did not detect a reciprocal decrease in PDK4 expression in HGFAC KO mice, suggesting that other kinases and/or phosphatases may mediate HGFAC-induced changes in PDHA phosphorylation. Interestingly, recent work by Huang et al suggests that the HGF receptor, c-MET, itself can phosphorylate and inactivate PDHA by direct interaction (66). While we have not tested the putative direct interaction between c-MET and PDHA in our models, this work supports our observations indicating that increased HGF signaling via HGFAC activity leads to inhibition of the PDH complex. Altogether, our results indicate that HGF and PPARγ may mediate some of HGFAC’s effects on glucose homeostasis through regulation of hepatic PDH phosphorylation.

Putative loss of function variants in human HGFAC associate with increased circulating triglycerides, albumin, and platelets and these phenotypes are recapitulated in HGFAC KO mice (34). This concordance supports the hypothesis that putative HGFAC loss of function variants likely impair its catalytic activity. Moreover, these results suggest that this molecular physiology is conserved from rodents to humans. Interestingly, the rs1801282 (Pro12Ala) PPARG variant associated with increased PPARG expression and reduced risk for diabetes and circulating triglycerides also associates with reduced albumin levels (67). These effects on albumin are directionally concordant with the changes in albumin that occur in HGFAC KO mice and the reduction in hepatic Pparg. Again, this suggests that an HGF-PPARγ signaling axis is conserved in humans and that some of the beneficial effects of PPARγ on systemic metabolism could be mediated through effects in the liver in addition to adipose tissue.
Our results suggest an integrated physiology whereby carbohydrate sensing via ChREBP impacts systemic growth factor signaling (HGFAC-HGF-c-MET) that may mediate both adaptive and maladaptive responses through paracrine and endocrine effects. In the context of obesogenic diets, this signaling axis enhances hepatic PPARG expression which may mediate a compensatory response to preserve systemic glucose homeostasis. HGF, the principal target for HGFAC has previously been implicated in other aspects of glucose homeostasis. For example, HGF may enhance pancreatic beta cell proliferation (45, 68-70). Increased ChREBP-mediated HGFAC secretion might be a potential mechanism to increase beta cell mass in the setting of increased dietary carbohydrate burden. Additionally, within the liver, HGF has been reported to enhance insulin signaling and hepatic glucose clearance via physical interactions between its receptor, c-MET, and the insulin receptor (71). HGF also is secreted by adipocytes and can promote angiogenesis in adipose tissue and adipose angiogenesis is an integral feature of adipose tissue expansion (72-74). Therefore, elevated ChREBP-HGFAC-HGF may promote healthy expansion of adipose tissue for efficient storage of fuel during overnutrition. These observations may support a role for ChREBP mediated upregulation of HGFAC and HGF signaling as an adaptive response to increased nutritional burden, and will require further investigation. ChREBP itself has been shown to regulate mouse hepatocyte and murine and human beta cell proliferation (75-77). The ChREBP-HGFAC axis may provide an important mitogenic signal through HGF when ChREBP senses abundant carbohydrates indicative of ample building blocks supporting proliferation. Our data supports this hypothesis, as HGFAC KO animals have decreased expression of hepatic cell cycle genes, and adenoviral overexpression of HGFAC leads to marked upregulation of proliferating cell nuclear antigen in the liver, a marker of proliferation.

Putative loss of function variants in HGFAC associate with increased circulating HGF in humans and also associate with increased cardiovascular risk factors (32, 34). Increased circulating HGF itself is increasingly recognized as a cardiometabolic risk factor that may be independent of other canonical cardiovascular risk factors (30, 32, 78-80). Further investigation into the relationship between ChREBP, HGFAC, and HGF signaling may define new mechanisms contributing to the pathogenesis of cardiometabolic disease in humans.
Methods:

Reagents. Glucose (8769), glycerol (G2025-1L), poloxamer407 (16758-250G) and dextran sulfate (D8906-5G): Sigma; Ensure Original Nutritional Shake: retail pharmacy; PHA-665752 (14703): Cayman Chemicals; mouse recombinant active HGF protein (2207-HG): R&D systems; mouse Ultra-Sensitive Insulin Elisa: Crystal Chem Inc (90080); Triglyceride LiquiColor test (2200225): StanBio Laboratories; total cholesterol (Wako 999-02601), NEFA-HR(2) (Wako), Thrombin (T4648-1KU): Sigma.

Animals and diets. Floxed ChREBP mice were generated at UT Southwestern Medical Center as previously described (12). Albumin-Cre mice (stock 003574) were purchased from Jackson Laboratory. Liver-specific ChREBP KO experiments were performed on a mixed C3H/HeJ and C57BL/6J background as previously described (3). HGFAC KO mice on a C57BL/6J background were generated at the Duke Transgenic and Knockout Mouse Core, by introducing an 857 bp spanning mid exon 1 and exon 2 by CRISPR/CAS9. Adenoviral overexpression of HGFAC was performed in wild type C57BL/6J male mice purchased from Jackson Laboratory. Mice were fed a chow diet (LabDiet 5008 or 5053), 60% fructose diet (TD.89247 Harlan Teklad) or 45% fat / 18% sucrose diet (D12451i, Research Diets) ad libitum for indicated times. Experimental mice were housed at 21°C–22°C on a 12-hour light-dark cycle in ventilated cages with 30 air exchanges per hour. All experiments were conducted with male mice, except where stated otherwise. Genotyping primers can be found in Supplemental table 4. Rat experiments were performed in double-housed 8-week-old male Wistar rats (Charles River) maintained on a standard chow diet (TD.7001, Harlan Teklad). Rats were fasted overnight and then fed either standard chow or 60% fructose diet (TD.89247, Harlan Teklad) ad-libitum. Rats were sacrificed 4-hours later and livers were snap-frozen for further analysis.

Cell lines. AML12 (CRL-2254) and HepG2 (HB-8065) cells were obtained from ATCC. AML12 cells were cultured in DMEM/F12 + 10% FBS supplemented with 1X Insulin-Transferrin-Selenium (ITS-G, 100X, Thermo-Fisher, 41400045) and 40 ng/ml dexamethasone (Sigma, D4902). HepG2 cells were cultured in DMEM + 10% FBS (Thermo-Fisher, 16000044).
**ChIP-seq and Analysis.** Wild-type, male 8-week-old C3H/HeJ and C57BL/6J mice were fasted for 5 hours and gavaged with fructose (4 g/kg BW) versus water control (n=6/group). Mice were euthanized 90 min after gavage and tissues were harvested and snap-frozen in liquid nitrogen for further analysis. Chromatin was prepared using truChIP Chromatin Shearing Tissue Kit (Covaris). 25-30 mg of frozen liver tissue were quickly minced with razor blades in PBS at room temperature. Tissue was crosslinked with 0.5 M disuccinimidyl glutarate in PBS for 45 min at room temperature, followed by fixation with 1% formaldehyde in Fixing Buffer A (Covaris) for 5 min at room temperature. Crosslinking was stopped by Quenching Buffer E (Covaris). After washing, nuclei were isolated by Dounce homogenization followed by centrifugation. The nuclear pellet was resuspended in cold 0.25% SDS Shearing Buffer (Covaris). Chromatin was sheared in 1 ml AFA milliTUBE(s) (Covaris) using Covaris S220X focused ultrasonicator with the following parameters: peak incident power 140W, duty factor 5%, cycles per burst 200 for 12 min. The sheared chromatin was centrifuged at 13,000rpm for 10min at 4°C to remove debris, and a 10 ul aliquot was de-crosslinked and used for quantification with Qubit (Thermo-Fisher Scientific). Sheared chromatin (1.5 – 3 μg) was diluted in ChIP dilution buffer (16.7 mM Tris [pH8], 1.2 mM EDTA, 25 mM NaCl, 1.1% Triton X-100, 0.01% SDS), and 1 μg of ChREBP antibody (Novus, NB400-135) or control rabbit IgG was added, followed by overnight incubation at 4°C. Reactions were then incubated for 1h at 4°C with protein A/G dynabeads (Invitrogen) pre-blocked in PBS/0.5% and BSA/0.5% Tween. Beads were then washed in low salt wash buffer (20 mM Tris [pH8], 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS), high salt wash buffer (20 mM Tris [pH8], 1 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS), LiCl wash buffer (10 mM Tris [pH8], 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, 250 mM LiCl) and TE buffer (10 mM Tris [pH8], 1 mM EDTA) and eluted and reverse cross-linked in elution buffer (10 mM Tris [pH8], 5 mM EDTA, 0.1% SDS, 300 mM NaCl, 0.8 mg/ml proteinase K, 10 μg/ml RNase A) by incubating at 65°C for 10 hours. DNA was extracted using AMPure XP beads following the manufacturer’s manual and quantified by Qubit (Thermo-Fisher Scientific). Immunoprecipitated chromatin was pooled by genotype and gavage condition for further analysis.
Library preparation, sequencing, and analysis were performed in the Boston Nutrition Obesity Research Functional Genomics and Bioinformatics Core. The sequencing ChIP-seq reads were demultiplexed using bcl2fastq and aligned to the GRCm38 mouse genome using Bowtie2 (81). PCR duplicates and low-quality reads were removed by Picard. Reads were processed using SAMtools and subjected to peak-calling with MACS2. SAMtools was also used to obtain 2 pseudoreplicates per sample (82, 83). Only the peaks present in both pseudoreplicates were included for further downstream analysis. The coverage for peaks was obtained using BEDtools multicov (84). Normalization and differential analysis were performed using edgeR between fructose and water gavage conditions (85). To visualize ChIP-seq signals, reads were converted to the BigWig file format using BEDtools and bedGraphToBigWig (86). Peaks were tied to genes based on the nearest gene and transcription start site (TSS) within a radius of 200kb distance. The gtf file from GENCODE version M24 was filtered to include only processed transcript and protein coding transcript types as well as filtered for well supported transcripts (using only transcript support levels 1 and 2).

For Meta-Analysis of Gene-set ENrichmenT of variant Associations (MAGENTA) analysis, genes included in the analysis were further filtered for transcriptional start sites which resided within 20 kb of a ChIP-seq peak. Human homologues of this set of mouse genes were analyzed using the MAGENTA algorithm in conjunction with joint Metabochip and GWAS triglyceride data from the global lipids genetics consortium (21, 27). Candidate genes were called secretory proteins based upon their annotation in the UniProt database (87).

**ChIP-PCR.** Male, liver-specific ChREBP KO mice on C3H background and littermate controls were fasted overnight and fed HFrD for 3 hours (n=3 / group). Mice were euthanized and tissues were harvested and snap-frozen in liquid nitrogen for further analysis. Chromatin immunoprecipitation was performed as above. qPCR was performed as described below.

**Metabolic testing.** Body composition was measured by Bruker Minispec LF 90II. Circulating triglycerides were measured from ad libitum fed mice at 1pm in blood collected from the tail vein. For glucose and glycerol tolerance tests, mice were fasted for 5 hours
starting at 7 am and glycerol or glucose (2g/kg body weight) were injected intraperitoneally. For Insulin tolerance tests, mice were fasted overnight and 1U insulin/kg (Humulin R, Lilly) was injected intraperitoneally. Glucose measurements were performed using a handheld glucometer (Bayer Contour). For mixed meal tolerance tests, mice were fasted overnight and gavaged with 10 ul/g of Ensure. Blood was collected from tail vein at 0- and 10-minute time points for insulin measurement. For VLDL secretion assay, mice were fasted for 3 hours and injected with 1 g/kg poloxamer 407.

**Hepatic Triglyceride measurements.** Liver neutral lipids were extracted with a modified Folch method. 100 mg of liver tissue was homogenized in 3 ml Chloroform:methanol (2:1) and incubated overnight with shaking. Next, 800 ul of 0.9% saline was added, vortexed, and centrifuged (2000g for 10 min). The chloroform phase was collected and dried overnight. Triglycerides were dissolved in butanol/Triton X-100/methanol (60/27/13 by volume) and measured using colorimetric triglyceride assay (StanBio).

**HGFAC/HGF activation assay.** Blood was collected from 3 control and 3 HGFAC KO mice and allowed to clot at room temperature for 1 hour, centrifuged at 7000g for 15 min and serum was collected. Serum was incubated with 10 ug/ml dextran sulfate and 1U of Thrombin for 3 hours at 37°C with 0.05M Tris, 0.05M NaCl and 0.05M CaCl2. HepG2 cells were treated with serum diluted with DMEM media (1:10) for 5 min and then harvested. Activation of c-MET was assessed by immunoblotting.

**Mouse Complete blood Count.** Mouse complete blood count was performed with K2 EDTA treated plasma obtained from tail veins via an Element HT5 veterinary hematology analyzer (Duke University Veterinary Diagnostic Laboratory).

**Immunoblotting.** Whole liver tissues were homogenized in lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, phosphatase (Pierce, A32957) and protease inhibitors (Sigma-Aldrich, P8340). Protein concentration was measured with the BCA method (Thermo-Fisher, 23225). Approximately 15-40ug of protein was used for liver immunoblots. For plasma samples, 1ul of plasma was mixed directly with 15 ul of Laemml buffer with reducing reagent added (NuPAGE™ Sample Reducing Agent, NP0004). Lysates were then subjected to immunoblotting with the indicated antibodies. Anti-HGFAC (R&D systems, AF1715), anti-β-Actin (Cell Signaling,
4970S), anti-phospho-c-MET (Cell Signaling 3077), anti-total c-MET (Cell Signaling 3127), anti-PPARγ (Cell Signaling 2435), anti-PDHA1 (phospho S293) antibody (Abcam, ab92696), Pyruvate Dehydrogenase (Cell Signaling 3205), anti-p85 (Upstate, 06-496) anti-PCNA (Cell Signaling, 2586). Quantification of blots was performed with a ChemiDoc XP (Bio-Rad) and Image Lab software v6.0. For loading normalization, whole lane protein was quantified using Bio-Rad Stain free technology.

qPCR. TRI reagent (Sigma, T9424) was used for RNA isolation from mouse liver and cell lines. RNA was reverse transcribed using a SuperScript VILO kit (Invitrogen). Gene expression was analyzed with the ABI Prism sequence detection system (SYBR Green; Applied Biosystems). Gene-specific primers were synthesized by Thermo-Fisher (Supplementary table 4). Each sample was run in duplicate and normalized to Tbp (CHREBP LKO cohorts), Ppib (HGFAC cohorts), or rplp0 for rat experiments.

Adenoviral overexpression of HGFAC in mice. Murine Hgfac cDNA (Sino Biological, Cat# MG50039-M) was cloned via Gateway recombination into the pAd/PL-DEST adenoviral vector with CMV promoter (Thermo-Fisher, Cat# V49420). The ADV-GFP control vector has been previously described (88). Adenoviral vectors were produced and purified as previously described (88). Anesthetized mice were injected with 5*10^10 adenoviral particles expressing HGFAC versus GFP control via retro-orbital injection. Expression of HGFAC was assessed by immunoblotting plasma for circulating HGFAC three days after adenoviral transduction.

RNA sequencing and analysis. RNA was isolated from mouse liver with TRI reagent (Sigma, T9424). RNA-seq was performed in Duke Center for Genomic and Computational Biology. RNA quality was assessed using a Fragment Analyzer (Advanced Analytical). mRNA capture, fragmentation, and cDNA library construction were conducted using a stranded mRNA-Seq Kit (Kapa Biosystems, KR096, v6.17). 50bp paired-end sequencing was performed on an Illumina NovaSeq 6000 and at least 35M reads were obtained per sample. Sequencing data were uploaded to https://usegalaxy.org/ and aligned with HISTAT2 (2.1.0) using mouse genome assembly GRCm38 (mm10). Transcript levels were quantified using FeatureCounts (89). Transcript level count was uploaded to the
BioJupies server and analyzed for differential gene expression and KEGG pathway enrichment (90-92).

**Human hepatic HGFAC gene expression and analysis.** HGFAC mRNA expression values for lean, obese, and obese/diabetic patients were extracted from data deposited in GEO (GSE15653) (41). Liver RNA-seq read counts were obtained from the GTEX project (version 8, 2017-06-05). Genes with average expression value > 20 were log transformed and transformed to z-scores. Pearson correlations were calculated for each gene with HGFAC. The top 5% of correlated genes were analyzed with enrichR against ARCS4 transcription factor co-expression database (40, 93). For correlation between HGFAC and ChREBP targets, a composite expression vector for validated ChREBP targets (PKLR, ALDOB, FASN, KHK and SLC2A2) was computed by averaging the log transformed, z-score expression values for each of these genes.

**Statistics.** All data are presented as the mean ± SEM. Data sets were analyzed for statistical significance with GraphPad Prism using two-tailed unpaired t-tests, and where indicated 2-way ANOVA and with post-hoc comparisons performed with Sidak’s test or 1-way ANOVA with Holm-Sidak’s multiple comparisons test between control and individual groups. Statistical significance was set at P < 0.05.

**Genomic Data.** Genomic data has been deposited in GEO (GSE217983).

**Study approval.** All rodent studies were approved by the Beth Israel Deaconess Medical Center or the Duke University Medical Center Institutional Animal Care and Research Advisory Committee.

**Author contributions:**

MAH, AS, LD, SAH, and IA designed, performed, and interpreted mouse experiments. PJW and SAH performed rat experiments. MAH, LD, AS, WT, HS, RI and LT designed, performed, and interpreted computational analyses. AS and MAH designed, performed, and interpreted in-vitro experiments. JMH designed and performed construction of adenoviral vectors, and MA and PJW prepared purified adenoviruses, PAG, WT, RM
and HHK assisted with performing and interpreting experiments. MAH conceived of, designed, and supervised the experimental plan, interpreted experiments. AS and MAH wrote the manuscript. All authors edited the manuscript.

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Figure 1. ChREBP is bound to liver genomic targets following water and fructose gavage after a 5 hour fast. A) ChREBP ChIP-seq signal tracks in liver of male C57 and C3H mice after a 5 hour fast and 90 minutes after water versus fructose gavage (4 g/kg body weight) in selected ChREBP transcriptional targets including Pklr, Gp6c, Fasn, and Khk. B) Heatmaps showing hepatic ChREBP peaks after fructose versus water gavage. The amplitude of each peak center is represented by the z score and shown in blue. C) “GO Biological Process” and “Mouse Phenotype” pathway analysis for ChREBP peaks.
Figure 2. ChREBP Links Nutritional Status to Circulating HGFAC. A) ChIP was performed from hepatic tissues of control and liver-specific ChREBP KO mice with anti–carbohydrate responsive element–binding protein (anti-ChREBP) antibody or control IgG. qPCR was performed on immunoprecipitated chromatin with primers spanning the E-box in the Pklr promoter and the putative ChREBP binding site in the proximity of HGFAC, and in nonspecific genomic control regions (neg) in proximity to both regions containing ChREBP response elements (n = 3/group). B) Hepatic Chrebpβ and Hgfac mRNA expression of overnight fasted and 4-hour control chow or HFrD fed Wistar rats. (n=7/group). C) Liver mRNA expression and circulating levels of HGFAC protein in control (wild-type, littermate control) and KO (liver specific ChREBP KO) mice after 8 weeks on chow versus high fructose diet (HFrD) with their quantification by densitometry (n=4-5/group). E) Correlation between HGFAC mRNA expression and a composite vector comprised of canonical ChREBP transcriptional targets in human livers from the GTEx project (Pearson correlation R2=0.44, p<0.0001, n=226). F) Factors ranked by odds ratio for enrichment of the 300 genes most highly co-expressed with the factor in the ARCHS4 project that are also present in the top 5% of genes that correlate with HGFAC expression in the GTEx project. Combined score = log(p)*z, where p is calculated by Fisher’s exact test and z is the z-score calculated by assessing the deviation from the expected rank. The size of the circle corresponds to the enrichment score and the color corresponds to the adjusted p-value. G) Expression of HGFAC mRNA in livers of healthy controls, obese non-diabetic subjects, obese subjects with well controlled diabetes, and obese subjects with poorly controlled diabetes, (n=4-5/group). Data represent means ± SEM. Statistics were assessed by 2-way ANOVA with Sidak’s multiple comparisons between individual groups, # p<0.05, for main effects, ^ p<0.05 for comparison across genotypes within diets; or one-way ANOVA with Holm-Sidak’s multiple comparisons test between control and other groups, & p<0.05.
Figure 3. The phenotype in HGFAC KO mice recapitulates the phenotype of putative loss of function variant in human HGFAC. A) Schematic depiction of Hgfac gene and the deleted region in red; FWD and REV indicate the positions of forward and reverse primers, respectively, used in genomic PCR shown in (B) confirming the deletion of an 857 bp region in the Hgfac gene. C) Representative immunoblot of circulating HGFAC in control (wild-type, littermate control) and KO (HGFAC KO) plasma. D) Hepatic Hgfac mRNA levels measured by qPCR in control and HGFAC KO mice, (n=7-9/group). E) Immunoblot and quantification of phospho-c-MET in HepG2 cells treated with activated sera of control and HGFAC KO mice, (n=3/condition). F) Forest plot of phenotypes associated with the rs3748034 putative loss of function coding variant in human HGFAC. G) Quantification of plasma triglyceride and cholesterol levels in ad libitum chow fed male control and HGFAC KO mice, (n=8-13 / group), and H) plasma albumin concentrations in male control and HGFAC KO mice, (n=11-17 / group), and plasma platelet levels in male control and HGFAC KO mice, (n=9-17 / group). Data represent means ± SEM, Statistics were assessed by two-tailed unpaired t-test, * p<0.05; or one-way ANOVA with Holm-Sidak’s multiple comparisons test between groups, & p<0.05.
Figure 4. HGFAC KO mice have impaired carbohydrate metabolism on HF/HS diet. A) Body weight of male control and HGFAC KO mice during 18 weeks of HF/HS feeding (n=11-12/group unless otherwise specified) B) Fat and C) lean mass by NMR at 18 weeks. Glucose homeostasis was assessed at intervals throughout the study including D) IP glycerol tolerance test at 4 weeks E) IP glucose tolerance at 5 weeks, F) IP glucose tolerance test at 13 weeks, G) IP insulin tolerance test at 14 weeks (n=10-11/group), and H) a mixed meal tolerance test to assess insulin secretion was performed at 16 weeks. Tail vein insulin levels were measured at 0 and 10 minutes. Data represent means ± SEM, Statistics were assessed by two-tailed unpaired t-test, *p<0.05; or two-way ANOVA with Sidak's multiple comparisons between individual groups, ^ p<0.05 for comparison across genotypes within time points, $ p<0.05 for comparison across time points within genotypes.
Figure 5. Hepatic PPARγ is down-regulated in HGFAC KO mice. A) Volcano plot depicting differentially expressed genes from livers of chow and HF/HS fed HGFAC KO mice versus controls. Named genes in red represent top 10 most differentially expressed genes ranked by p-value. B) Pathway analysis including the top 10 most downregulated and upregulated gene sets, respectively in chow and HF/HS fed HGFAC KO livers compared to controls. C) Hepatic mRNA levels of Pparg, Ppara, Cd36, Acox1, Pck1 and Cpt2 after 4 weeks of Chow or HF/HS diet (n=5-7/group). D) Hepatic triglyceride levels in control and HGFAC KO mice on chow and HF/HS diet after overnight fasting followed by 4-hour ad libitum refeeding (n=6-7/group). E) Immunoblot analysis and quantification of hepatic phospho-S293 PDHA, total PDHA and PPARγ and P85 loading control in chow or HF/HS fed HGFAC KO and controls with quantification of phospho-S293 PDHA normalized to (F) P85 or to total PDHA (n=6/group), and PPARG normalized to P85 (n=4/group). Data represent means ± SEM, Statistics were assessed by two-way ANOVA with Sidak’s multiple comparisons between individual groups, # p<0.05, for genotype main effects, ^ p<0.05 for comparison across genotypes within diets.
Figure 6. HGFAC overexpression enhances glucose homeostasis. A) Immunoblot and quantification by densitometry of plasma HGFAC collected 3 days after 8-week-old male mice were transduced with adenovirus expressing GFP (ADV-GFP) or HGFAC (ADV-HGFAC). B) Weights, lean and fat mass of ADV-GFP and ADV-HGFAC mice after 9 days of transduction (n=10/group). C) IP GTT and corresponding iAUC performed 5 days after viral transduction (n=8-9/group). D) Overnight fasted and 3-hour refed glycemia and peripheral insulin levels of GFP and HGFAC transduced mice (n=10). E) Hepatic mRNA levels of Hgfac, Pparg and -α and PPARγ targets measured by qPCR 14 days after viral transduction. F) Hepatic PPARγ, phospho-S293 PDHA, total PDHA, and PCNA immunoblots of liver from ADV-HGFAC and ADV-GFP transduced mice, and quantification of PPARγ normalized to P85, p-PDHA normalized to total PDHA and PCNA normalized to the total protein content, n=4-5 per group. E) Hepatic and circulating triglyceride levels 14 days after viral transduction in ad libitum fed mice. F) Pparg mRNA levels in AML12 cells after overnight treatment with 50 ng/ml HGF or BSA. G) c-MET phosphorylation by HGF in AML12 cells is inhibited by the c-MET inhibitor PHA 665752 (2.5uM) preventing induction of Pparg mRNA. Data represent means ± SEM. Statistics assessed by two-tailed unpaired t-test, * p<0.05; or by two-way ANOVA with Sidak’s multiple comparisons between individual groups, ^ p<0.05 for comparison of effects of inhibitor within HGF treatment condition, $ p<0.05 for effect of HGF within inhibitor or control treatment.
Figure 7. ChREBP mediated activation of a HGFAC-HGF-PPARγ signaling axis mediates an adaptive response to preserve glucose tolerance in the setting of diets high in sugar. Glucose and fructose from high sugar diets enhance production of sugar metabolites (hexose-phosphates) in the liver that activate hepatic ChREBP and lead to increased Hgfac transcription and translation. HGFAC is secreted into the circulation where, once activated, it can act in a paracrine or endocrine fashion to proteolytically cleave and activate HGF. HGF binds and activates the c-MET tyrosine kinase receptor on hepatocytes and other cell types. In liver, this leads to upregulation of PPARγ expression that in turn activates transcriptional programs to promote hepatic triglyceride storage and to decreased circulating triglycerides. Additionally, hepatic PPARγ activity decreases activation of the pyruvate dehydrogenase complex and this contributes to enhance systemic glucose tolerance.
Table 1. Top 25 gene candidates in proximity to ChREBP ChIP-seq binding sites and that contribute to enrichment for association with hypertriglyceridemia as assessed by MAGENTA.

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