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Materials and Methods

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Materials and methods:

Generation of mice and mouse samples: All mouse breeding and experiments were performed with protocols approved by the City of Hope Institutional Animal Care and Use Committee and Institutional Biosafety Committee. Sperm from a male *Slc25a13 -/- Gpd2 -/-* C57BL6/J mouse(*14*) were a kind gift of Dr. Saheki and the Citrin Foundation. In vitro fertilization was performed with C57BL6/J eggs and a pseudopregnant C57BL6/J female recipient by Walter Tsark in the City of Hope Transgenic Mouse Core. Resulting diheterozygous offspring were intercrossed to generate wild-type, *Slc25a13 -/-*, *Gpd2 -/-*, and *Slc25a13 -/- Gpd2 -/-* mice of both sexes. At weaning, genotypes were determined by PCR of tail tissues (Transnetyx, Cordova, TN), and mice were single-sex group-housed until experimental use. Mice were maintained at 21 °C under a standard 12:12-hour light-dark cycle and provided with *ad libitim* access to food and water. For analysis of FGF21 expression, hepatic gene expression and metabolite accumulation, 8-16 week old mice were single-housed with chow (LabDiet irradiated PicoLab Rodent Diet 20, 5053) and a single bottle of either water or 5% (w/v) glycerol for two days. Mice were then euthanized by decapitation via guillotine without sedation, exsanguinated with a funnel for blood collection into 1.5 ml microcentrifuge tubes on ice, and livers were freeze-clamped in liquid nitrogen immediately after harvest(*94, 95*). Sera were obtained by centrifuging blood at 8,000 *g* for 10 minutes at 4°C. Liver samples were pulverized with a mortar and pestle cooled by liquid nitrogen.

FGF21 quantification: Mouse/Rat FGF21 Quantikine ELISA Kit from R&D Systems (Cat. No. MF2100) was used for FGF21 quantification of mouse sera. 50 µl of assay diluent RD1-41 was added to each well followed by equal volumes of standard, control, and experimental samples. After incubation for 2 hr at room temperature, wells were aspirated and washed 5 times with 400 µl of wash buffer. 100 µl of horseradish peroxidase-conjugated FGF21 antibody solution was then added to each well and incubated at room temperature for 2 hr followed by five washes with 400 µl of wash buffer. After washing, 100 µl of freshly prepared substrate was added to each well and incubated for 30 minutes at room temperature. Assays were terminated with addition of 100 µl of stop solution followed by reading at 450 nm with wavelength correction at 570 nm.

RNAseq: 20 mg liver aliquots were used for RNA isolation (Qiagen, RNeasy Mini Kit, 74104) and DNase I treatment (Qiagen, RNase-Free DNase Set, 79254). RNA integrity was assessed using an Agilent 4200 Tapestation, ensuring RNA integrity number > 5.0 for all samples. Library preparation and sequencing was performed by 7 Traits of Watertown, MA. Libraries were prepared using the NEBNext UltraExpress RNA Library Prep Kit (New England Biolabs, Ipswich, MA), following the manufacturer's protocol using 150 ng RNA per sample. mRNA was enriched using poly(A) selection. Enriched mRNA was fragmented, reverse transcribed, and converted to cDNA. cDNA underwent end repair, adapter ligation, and size selection. PCR amplification was performed using 11 cycles to ensure sufficient library yield while minimizing amplification bias. Library quality and size distribution were assessed using a Tapestation

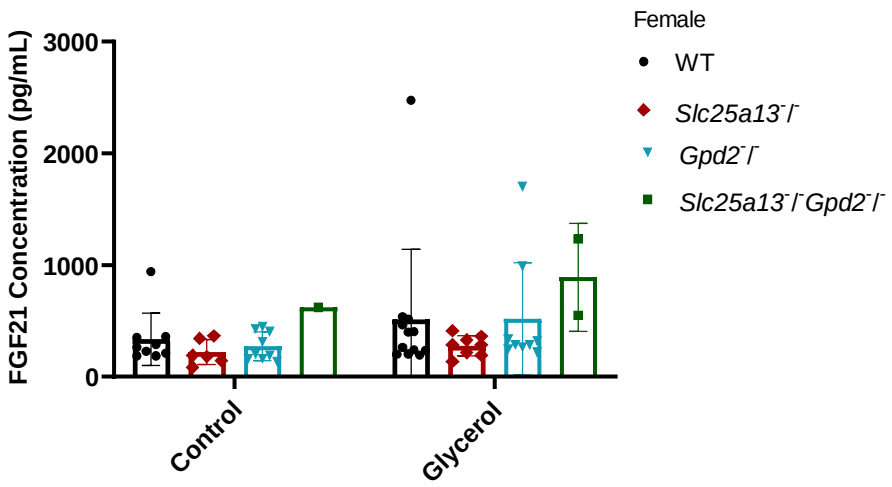
(Agilent) and quantified by Qubit (Thermo Fisher Scientific). Libraries were pooled on an equimolar basis and sequenced on an Illumina NovaSeq X Plus platform using a 25B lane configuration with paired-end 150 bp reads with a minimum of 20M reads per sample. Base calling and demultiplexing were performed using Illumina's DRAGEN Bio-IT platform. Raw sequencing reads were processed for quality control using FastQC and trimmed to remove adapters and low-quality bases using Trimmomatic(*96*)and polyA tails using FASTP(*97*). Processed reads were mapped back to the mouse genome (mm10) using STAR software (v. 2.6.0.a)(*98*). HTSeq software (v.0.11.1) was employed to generate the count matrix with default parameters(*99*). Differential expression analysis was performed by normalizing read counts to expression values using the TMM normalization method in edgeR(*100, 101*). Generalized linear models were applied to identify differentially expressed genes between glycerol and water-exposed liver samples of the same genotype or between liver samples of different genotypes. Normalized expression levels from TMM were used as the dependent variable, while sequencing batches were included as an independent variable to account for batch effects. Genes with an FDR-adjusted p-value below 0.05 and a fold change greater than 2 or less than 0.5 were classified as significantly upregulated or downregulated, respectively.

Metabolomic analysis of mouse livers: For G3P quantification, 2.0 mg samples of pulverized frozen liver were spiked with $^{13}C_3$-G3P (2.28 µM final concentration, Sigma Aldrich). Frozen samples were immediately processed with a boiling buffered ethanol extraction (600 µL of 25% 10 mM HEPES Buffer and 75% Ethanol) followed by vigorous vortexing. Samples were placed on a thermomixer for 5 min at 55 ˚C with shaking at 1,200 rpm, then cooled on ice for 30 seconds. Samples were then placed in a water bath sonicator for 1 minute followed by an additional 30 seconds on ice. Samples were clarified by centrifugation at 16,100 *g* in a prechilled centrifuge at 4 ˚C; the supernatant was transferred to a clean tube followed by a second round of centrifugation. Clarified supernatants were transferred to new tubes and dried for 5 hours in a vacuum centrifuge at 4 ˚C. Dried samples were resuspended in 80 µl of LC-MS water. Twenty-folded diluted samples were transferred to MS vials for analysis on LC-MS/MS. For analysis of hexose phosphates and pentose phosphates, 2.0 mg samples of pulverized frozen liver were spiked with a ^{13}C glucose-grown yeast extract (ISO101, Cambridge Isotope Laboratories) as an internal standard(*102*) and the same workup was performed. Samples were analyzed on a Vanquish Horizon UHPLC with a tandem Thermo Scientific Orbitrap Fusion mass spectrometer. Vials were maintained in autosampler at 4 ˚C. Instrument source parameters were held at 3 kV negative ion spray voltage, 300˚C ion transfer tube temperature, 250 ˚C vaporizer temperature, sheath gas of 20, auxiliary gas of 10, and sweep gas of 3. Liquid chromatography separation was carried out using an Acquity Premier HSS T3 column with VanGuard FIT, 1.8 µm, 2.1 x 150 mm with mobile phases A (5 mM tributylamine and 10 mM acetic acid with 5% v/v methanol in LC-MS grade water) and B (LC-MS grade methanol) at a constant flow rate of 0.5 ml/min. Separation was carried out with starting condition of 0% B; 0-10 min, 10.5% B; 10-18 min, 52.6% B; 18-19 min 52.6% B; 19-20 min, 0% B; 20-26 min, 0% B. Spectra for G3P were acquired using a targeted MS2 scan with parent ion of *m/z* 171.0058 with collision energy 25. Spectra for $^{13}C_3$-G3P were acquired with parent ion *m/z* of 174.0165 with collision energy 25 with primary fragment ion of *m/z* 78.9588. Hexose phosphate spectra were acquired using a targeted MS2 scan of parent ion *m/z* 259.0198. $^{13}C_6$-hexose phosphate spectra were acquired with a parent ion of *m/z* 265.0426 with collision energy of 20 and primary fragment ion *m/z* 96.9690. Pentose phosphate spectra were acquired using targeted MS2 scan for the parent ion *m/z* 229.0124 at collision energy 30 with a primary fragment ion *m/z* of 78.9588.

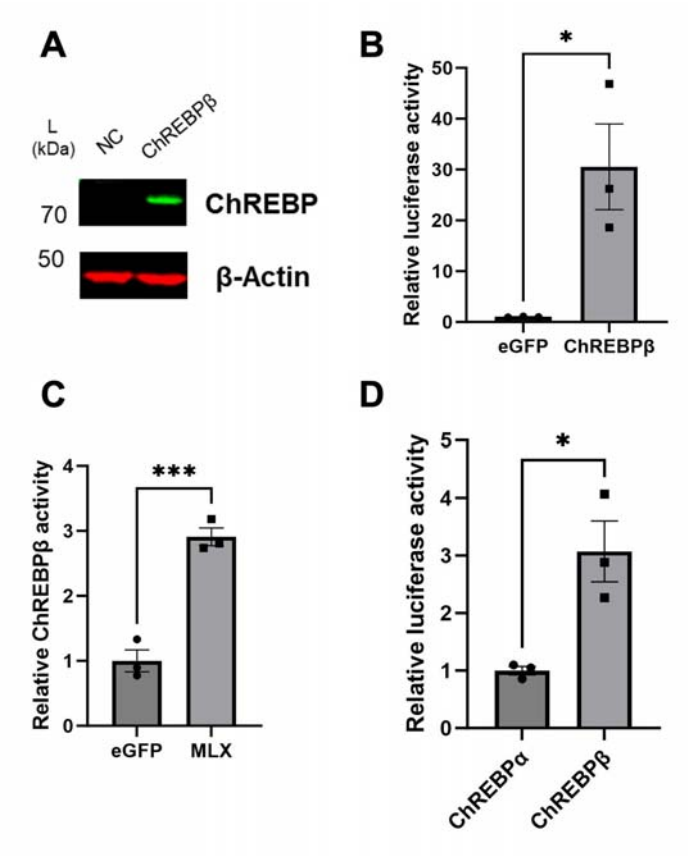
Cellular reconstitution of ChREBP-MLX-dependent ChORE-luciferase activity: Human ChREBPα (accession number NM_032951.3), human ChREBPβ (accession number XM_047420437.1), human MLX (accession number NM_170607.3), and eGFP coding sequences were synthesized by Genewiz (Suzhou, China) and cloned into pcDNA3.1 vectors (Invitrogen). GK was subcloned into pcDNA3.1 (Invitrogen) from pWZL-Neo-Myr-Flag-GK (Addgene plasmid 20493). pcDNA3.1-GPD1 and pcDNA3.1-GAPDH were purchased from GenScript (clone ID: OHu20325 and OHu20566). pcDNA3.1-*Lb*NOX, pcDNA3.1-*Ec*STH, pGL4.14 [luc2/Hygro]-ChoRE, and pGL4.75[hRluc/CMV] were as described(*59*). HEK293T cells were seeded into 24-well plates and maintained in DMEM medium (Gibco) with 10% FBS (Gibco) and 1% Pen-Strep (Gibco) at 37 °C and 5% CO_2 overnight. On the following day, media were replaced by OPTI-MEM (Gibco) 1 hour before plasmid co-transfection. Lipofectamine 3000 reagent (Invitrogen) was used for co-transfections for 5 hours, after which media were replaced with DMEM containing 10% FBS for 48 hours. Cells were then collected, and luciferase assays were performed using the Firefly and Renilla Single Tube Luciferase Assay Kit (Biotium) following the manufacturer's protocol. A Tecan Infinite M Plex plate reader was used to measure Firefly and Renilla luminescence. Firefly luciferase values were normalized to corresponding Renilla luciferase measurements to correct for cell quantity and transfection efficiency.

Metabolomic analysis of transfected HEK293T cells: HEK293T cells were seeded into 6-well plates overnight and then underwent plasmid co-transfection as described above for 48 hours. Cells were then washed with PBS, quenched with dry ice-cold 80% methanol, and transferred to conical tubes. Relative quantification of 137 metabolites was performed as described(*59*).

Biophysical characterization of the ChREBP GSM: The coding sequence for mouse ChREBPα (amino acids 43 to 307) were converted to *E. coli*-optimized codons and inserted into a pET vector carboxyl to $MetHis_6$. The plasmid, pVB240306, was transformed into in *E. coli* Arctic Express, and expression was induced at 12.5 °C with 1 mM IPTG for 18 hrs. Total protein from 1 liter culture was solubilized in 15 ml denaturing lysis buffer (8M urea, 100 mM NaH_2PO_4, 10 mM Tris Cl, pH 8.0, 0.05% Tween 20). Recombinant protein was captured by overnight incubation with 3 ml Ni-NTA resin (Qiagen) followed by washing with 30 ml denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris Cl, pH 6.3, 0.05% Tween 20). On-column protein refolding was performed by washing with 30 ml of 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 0.1% Triton X-100, followed 30 ml of 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 5 mM β-cyclodextrin, 0.05% Tween 20, and 30 ml of 50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 0.05% Tween 20. His-tagged ChREBP43-307 was recovered in 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 0.05% Tween 20, 350 mM imidazole at a yield of 1-2 mg/liter culture. All ligands were prepared in the same buffer. Ligand binding was determined by isothermal titration calorimetry using a Nano ITC (TA Instruments, Waters, USA) to measure ligand concentration-dependent heat changes. Ligands were delivered in a 50 µl syringe to 185 µl His-tagged ChREBP43-307 in the sample cell. After an initial 0.8 µl injection, 19 additional 2.5 µl injections were made with a time interval of 200 s between each injection. Measurements were made at 20 °C with stirring at 250 rpm. Informative ligand concentrations were determined empirically to observe saturable binding. Binding profiles were fitted to a blank and independent model with single-site binding using analysis software supplied with the instrument. Each experiment was performed in triplicate.



Supplementary Fig. 1. Glycerol and loss of both NADH shuttle systems tend to elevate circulating FGF21 in female mice.



Supplementary Fig. 2. Reconstitution of a ChoRE-dependent ChREBPβ-MLX transcription system in HEK293T cells. A) ChREBP expression depends on transfection of specific *MLXIPL* constructs. B) ChoRE-luciferase activity is powerfully induced by ChREBPβ. C) ChREBPβ-dependent ChoRE-luciferase activity is stimulated by *MLX* co-expression. D) In the presence of *MLX*, ChREBPβ is a stronger inducer of ChORE-luciferase than is ChREBPα.

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THE GLUCOSE FATTY-ACID CYCLE
ITS ROLE IN INSULIN SENSITIVITY AND THE METABOLIC DISTURBANCES OF DIABETES MELLITUS *

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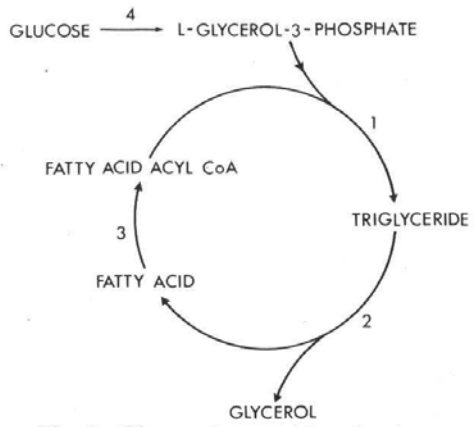

Fig. 1—Glucose fatty-acid cycle, tissue phase.

1. Esterification.
2. Lipolysis.
3. Fatty-acid activation.
4. Glycerol phosphate synthesis.

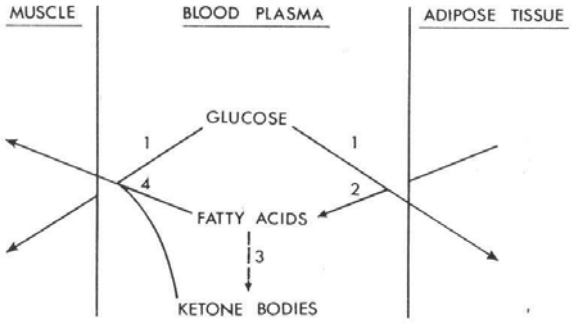

Fig. 2—Glucose fatty-acid cycle, blood phase.

1. Uptake of glucose by muscle and adipose tissue.
2. Release of fatty acids from adipose tissue to plasma albumin.
3. Formation of ketone bodies from fatty acids by liver.
4. Uptake of fatty acids and ketone bodies by muscle.

Supplementary Fig. 3. Original sketches of the Randle Cycle(*64*). According to our model, G3P is not only the backbone for triglyceride synthesis but also the transcriptional driver of triglyceride synthesis via activation of ChREBP. According to this model, fructose, ethanol and mitochondrial insufficiency, would be lipogenic by virtue of promoting G3P formation. Further, formation of malonyl-coA by G3P-ChREBP-dependent transcriptional induction of acetyl-coA carboxylase would be a key mechanism by which carbohydrate availability depresses fatty acid oxidation.

Supplementary Table 1. Genotype-dependent hepatic gene expression (mean FPKM) of ChREBPβ and FGF21 mRNAs

mRNA	WT	*Slc25a13 -/-*	*Gpd2 -/-*	*Slc25a13 -/- Gpd2 -/-*
ChREBPβ	1.29	7.70	7.94	26.22
Fgf21	1.19	1.50	1.84	5.03

Supplementary Table 2. Most frequent pathological variants of *SLC25A13* in gnomAD (March 12, 2024)

RSID	Allele count	Origin
rs80338720	185	Asian
rs80338722	102	Asian
rs780525233	84	European
rs80338716	73	European
rs80338729	58	South Asian
rs200237622	56	Ashkenazi
rs80338721	32	European
rs80338725	30	Asian
rs781452100	30	European
rs80338723	23	Asian
rs143181462	22	European

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