Selective Liver Estrogen Receptor α Modulation Prevents Steatosis, Diabetes, and Obesity Through the Anorectic Growth Differentiation Factor 15 Hepatokine in Mice

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Hepatocyte estrogen receptor α (ERα) was recently recognized as a relevant molecular target for nonalcoholic fatty liver disease (NAFLD) prevention. The present study defined to what extent hepatocyte ERα could be involved in preserving metabolic homeostasis in response to a full (17β-estradiol [E2]) or selective (selective estrogen receptor modulator [SERM]) activation. Ovariectomized mice harboring a hepatocyte-specific *ERα* deletion (*LERKO* mice) and their wild-type (WT) littermates were fed a high-fat diet (HFD) and concomitantly treated with E2, tamoxifen (TAM; the most used SERM), or vehicle. As expected, both E2 and TAM prevented all HFD-induced metabolic disorders in WT mice, and their protective effects against steatosis were abolished in *LERKO* mice. However, while E2 still prevented obesity and glucose intolerance in *LERKO* mice, hepatocyte *ERα* deletion also abrogated TAM-mediated control of food intake as well as its beneficial actions on adiposity, insulin sensitivity, and glucose homeostasis, suggesting a whole-body protective role for liver-derived circulating factors. Moreover, unlike E2, TAM induced a rise in plasma concentration of the anorectic hepatokine growth differentiation factor 15 (Gdf15) through a transcriptional mechanism dependent on hepatocyte ERα activation. Accordingly, ERα was associated with specific binding sites in the *Gdf15* regulatory region in hepatocytes from TAM-treated mice but not under E2 treatment due to specific epigenetic modifications. Finally, all the protective effects of TAM were abolished in HFD-fed *GDF15*-knockout mice. *Conclusion:* We identified the selective modulation of hepatocyte ERα as a pharmacologic strategy to induce sufficient anorectic hepatokine Gdf15 to prevent experimental obesity, type 2 diabetes, and NAFLD. (*Hepatology Communications* 2019;3:908-924).

As a key regulator of energy and glucose homeostasis, estrogen receptor α (ERα) is now considered a relevant target to develop new therapeutic approaches for obesity-related metabolic disorders, such as type 2 diabetes and nonalcoholic fatty liver disease (NAFLD).[1] Understanding the mechanisms involved in ERα-mediated metabolic protection thus remains a crucial challenge to

Abbreviations: AF, activation function; Angptl, angiopoietin-like; BS, binding site; CBP, cyclic adenosine monophosphate response element-binding protein; ChIP, chromatin immunoprecipitation; CoA, coenzyme A; Cyp17a1, cytochrome P450, family 17, subfamily a, polypeptide 1; Dgat2, diacylglycerol O-acyltransferase 2; E2, 17β-estradiol; Enho, energy homeostasis associated; ERα, estrogen receptor α; Fgf21, fibroblast growth factor 21; Gck, glucokinase; Gdf15, growth differentiation factor 15; GDF15KO, growth differentiation factor 15-deficient; H&E, hematoxylin and eosin; H3K27ac, twenty-seventh amino acid in histone H3, acetylated; HDL, high-density lipoprotein; HFD, high-fat diet; Igf, insulin-like growth factor; KO, knockout; LDL, low-density lipoprotein; LERKO, mice with hepatocyte-restricted ERα deletion; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; Nr1h, nuclear receptor subfamily 1, group H; Pltp, phospholipid transfer protein; Ppar, peroxisome proliferator activator receptor; SERM, selective estrogen receptor modulator; Srebf1, sterol regulatory element binding transcription factor 1; TAM, tamoxifen; VEH, vehicle; WT, wild type.

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optimize pharmacologic strategies for ERα selective modulation.[2]

Interestingly, recent studies highlighted the critical role of hepatic ERα signaling in the protective effects conferred by estrogens against high-fat diet (HFD)-induced steatosis and insulin resistance. Indeed, silencing ERα expression in the liver using adenoviral short hairpin RNA has been shown to markedly increase hepatic triglyceride accumulation in HFD-fed C57BL/6 female mice, while overexpression of human ERα reduced the level of steatosis in obese ob/ob female mice.[3] Zhu et al.[4] then reported that both female and male mice with hepatocyte-restricted *ERα* deletion (*LERKO*) are prone to steatosis and insulin resistance in response to HFD feeding. Noteworthy, 17β-estradiol (E2) administration failed to preserve ovariectomized *LERKO* female mice from fatty liver and insulin resistance, although this treatment still exerted a significant protection against adipose tissue accumulation and glucose intolerance.[4]

As a member of the nuclear receptor superfamily, ERα acts as a transcription factor that regulates gene transcription through two activation functions (AFs), namely ERα-AF1 and ERα-AF2.[5] ERα-AF1 and ERα-AF2 are both fully activated by E2, but their respective roles in gene expression regulation depend on ligands and cell types. Using mouse models with a selective ERα-AF1 or ERα-AF2 deletion, we demonstrated that the prevention of HFD-induced obesity and insulin resistance by E2 required ERα-AF2 activation, whereas ERα-AF1 appeared dispensable.[6] Besides natural estrogens, several drugs, known as selective estrogen receptor modulators (SERMs), acting as agonists or antagonists in a tissue-specific manner have been developed for clinical use. Nevertheless, their abilities to activate or repress ERα-AF1 or ERα-AF2 remain largely unknown, except for tamoxifen (TAM).[5] Indeed, ERα-AF2 is the direct target of TAM antagonism, whereas its agonistic activity, which depends on both cell types and target genes, results from ERα-AF1 activation.[7]

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We recently showed that chronic TAM administration prevents HFD-induced obesity, steatosis, insulin resistance, and glucose intolerance in ovariectomized female C57BL/6 mice, along with a significant reduction in food intake.[8] All the protective effects of TAM were abrogated in *ERα*$^{-/-}$ and *ERα-AF1*$^{-/-}$ mice,[8] thus attesting that TAM-induced metabolic protection is specifically mediated by ERα-AF1. However, the specific contribution of hepatocyte ERα to the protection conferred by SERMs, such as TAM, against HFD-induced metabolic disorders still remain to be investigated.

The present study aimed at defining to what extent hepatocyte ERα could be involved in the prevention of steatosis and in the preservation of whole-body metabolic homeostasis in response to a selective (SERM) mode of activation compared to a full (E2) activation. To this end, we combined the use of pharmacologic tools (E2 and TAM) and transgenic mouse models to demonstrate that, in contrast to the results obtained with E2, hepatocyte ERα not only mediates the protective effect of TAM against steatosis but is also absolutely and unexpectedly required to prevent HFD-induced obesity and glucose intolerance. Noteworthy, this overall metabolic protective action conferred by the selective modulation of hepatocyte ERα requires the anorectic hepatokine growth differentiation factor 15 (Gdf15), secretion of which is sharply enhanced by TAM.

Materials and Methods

ANIMALS, TREATMENT, AND DIET

Female mice with a hepatocyte-specific deletion of ERα (*LERKO*, n = 40)[9] and *Gdf15*-deficient mice (*GDF15KO*, n = 23)[10] (see Supporting Methods) as well as their respective wild-type (WT) littermates (*LER*$^{+/+}$, n = 40; *GDF15*$^{+/+}$, n = 14) underwent bilateral ovariectomy at 4 weeks of age to standardize the exposure to ERα ligands. All mice were then subcutaneously implanted with pellets releasing either TAM (1.2 mg/kg/day), E2 (80 μg/kg/day), or vehicle (VEH) for either 6 (*GDF15KO*) or 12 weeks (*LERKO)* and concomitantly fed an HFD (energy content: 45% fat, 20% protein, and 35% carbohydrate; 3.7 kcal/g; Research Diets, New Brunswick, NJ).

IN VIVO EXPERIMENTAL PROCEDURES

Mice were anesthetized with an intraperitoneal injection of ketamine (10 mg/kg; Merial, Lyon, France) and xylazine (1 mg/kg; Sigma-Aldrich, Isle d'Abeau Chesnes, France) for ovariectomy and with exposition to 2% isoflurane for pellet implantation. Food intake and body weight were recorded weekly. One week before being killed, body composition (whole body fat and lean masses) was analyzed by EchoMRI in live animals. Intraperitoneal glucose tolerance tests were performed on the following day in overnight-fasted mice. Blood glucose concentrations were monitored with a glucose meter (Roche Diagnostic, Meylan, France) –30, 0, 30, 60, and 90 minutes after glucose injection (1 g/kg body weight). In some experiments, basal metabolism was evaluated by indirect calorimetry 1 week before being killed and after 24 hours of acclimatization in individual cages. Oxygen consumption (VO_2), carbon dioxide production (VCO_2), and food and water intake were measured (Phenomaster; TSE-Systems) in individual mice at 10-minute intervals during a 24-hour period at constant temperature (20°C). We calculated the respiratory exchange ratio as VCO_2 /VO_2 and energy expenditure (kilocalories of heat produced). Ambulatory physical activity was monitored by an infrared photocell beam interruption method. All mice were killed at 11:00 AM, after 3 hours of fasting with free access to water. Blood samples were collected from the retro-orbital venous plexus and stored at –20°C. Mice were euthanized by cervical dislocation, and organs were carefully removed, weighed, frozen in liquid nitrogen, and stored at –80°C.

LIVER TISSUE HISTOLOGY

Liver tissues were quickly excised, fixed in 10% buffered formalin, and embedded in paraffin, and 3-μm sections were stained with hematoxylin and eosin (H&E). Additional fresh liver samples were immersed in Tissue-Tek optimal cutting temperature compound (Sakura, Japan) and then frozen in isopentane cooled by liquid nitrogen; cryosections (7 μm) were then stained with Oil Red O to assess neutral lipid accumulation, as described with minor modifications.[11] Images of each sample were obtained at original magnifications of ×150 and ×400 with an Eclipse Ci Nikon microscope and using a DS-FI camera driven by NIS-AR element

software (Nikon) (H&E sections) or scanned (Oil Red O sections) with a nanozoomer scanner (Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with NDP view software (Hamamatsu Photonics).

LIVER LIPID CONTENT ANALYSIS

Hepatic levels of triglycerides, free cholesterol, and cholesterol esters were determined using Bligh and Dyer methodology,[12] and lipid extracts were analyzed by gas–liquid chromatography as described.[13]

BIOCHEMICAL ANALYSES

Plasma samples were used to measure alanine aminotransferase and lipid profiles (triglycerides, total cholesterol, and high-density lipoprotein (HDL) cholesterol). Plasma insulin and adipokines (resistin, leptin, adiponectin) levels were determined using the Multiplex Immunoassay Technology Xmap (MADKMAG-71K-05 and MADPNMAG-70K-01, MILLIPEX; Millipore, Saint-Quentin-en-Yveline, France). Serum Gdf15 levels were determined with a commercial enzyme-linked immunosorbent assay kit (MGD150; R&D Systems Europe, Abingdon, United Kingdom) according to the manufacturer's instructions.

OTHER METHODS

Methods used for ERα protein determination by western blot on isolated hepatocytes, gene expression analysis, and chromatin immunoprecipitation (ChIP) assays are detailed in Supporting Methods.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism, version 5.00, for Windows (GraphPad Software, San Diego, CA; www.graphpad.com). A Student *t* test was used to compare the respective effects of TAM and E2 with the effect of VEH within each genotype (WT or knockout [KO] mice). To test the interaction between treatments and genotypes for body weight evolution and glycemic changes during intraperitoneal glucose tolerance tests, a two-way analysis of variance with repeated measures was carried out. In the case of interaction between treatment and genotype, Bonferroni post-hoc tests were subsequently performed. $P < 0.05$ was considered statistically significant.

Results

BOTH E2 AND TAM PREVENT HFD-INDUCED STEATOSIS THROUGH HEPATOCYTE ERα ACTIVATION

LERKO mice were first used to determine whether ERα signaling in hepatocytes contributes to the prevention of HFD-induced steatosis conferred by TAM and E2. Demonstrating the selective *ERα* deletion in this mouse model, ERα messenger RNA (mRNA) levels were decreased by 80% in total livers and primary hepatocytes from *LERKO* mice compared to their WT littermates, while no differences were observed in other tissues (Supporting Fig. S1A). Furthermore, ERα protein expression was not detected in the culture of primary hepatocytes from *LERKO* mice (Supporting Fig. S1B). Both E2 and TAM protected ovariectomized WT mice from HFD-induced steatosis, as illustrated by histologic staining (Fig. 1A) and confirmed by a significant decrease in intrahepatic concentrations of triglycerides (Fig. 1B). Prevention of steatosis development by either TAM or E2 was abolished in *LERKO* mice (Fig. 1A,B). Histologic analyses found no lobular inflammation, hepatocyte ballooning, or fibrosis, irrespective of the treatment and genotype (data not shown). Accordingly, no significant changes were observed in the hepatic expression of a set of genes involved in inflammation and fibrosis (Supporting Fig. S2; data not shown). Finally, in TAM-treated mice, we checked that hepatocyte *ERα* deletion does not alter the expression of genes encoding P450 enzymes that contribute to metabolize this SERM in the liver (Supporting Fig. S3). These data first confirm that hepatocyte ERα plays an important role in the hepatoprotective actions of E2[4] and then demonstrate that TAM-induced prevention of steatosis[8] also results from ERα activation in hepatocytes.

INVOLVEMENT OF HEPATOCYTE ERα IN THE REGULATION OF HEPATIC METABOLIC PATHWAYS BY TAM AND E2

To gain further insights into the contribution of hepatocyte ERα to the protective effects of E2 and TAM against HFD-induced steatosis, we explored the regulation of the main hepatic metabolic pathways,

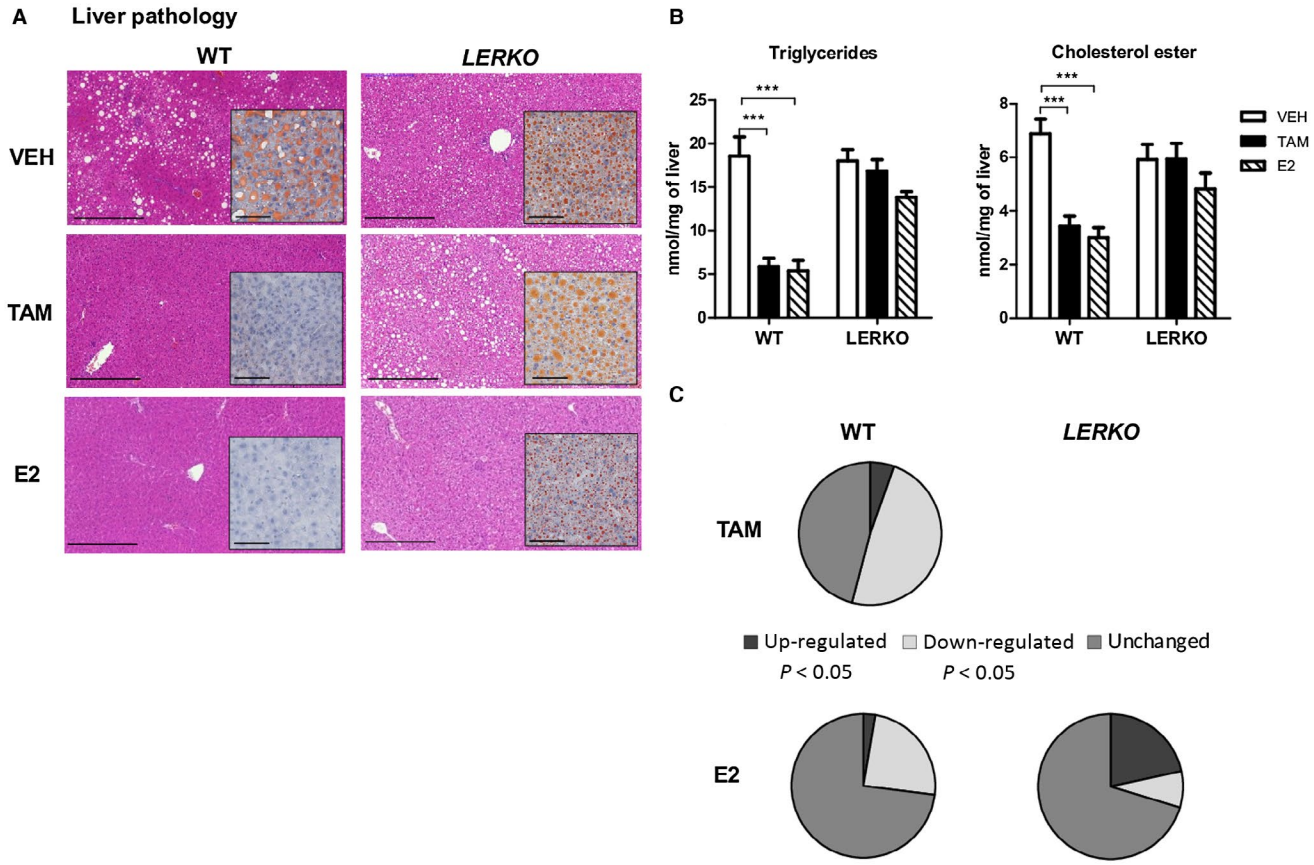

FIG. 1. Prevention of HFD-induced steatosis by either E2 or TAM requires hepatocyte ERα activation. *LERKO* mice (n = 40) and their respective WT littermates (n = 40) were fed an HFD and concomitantly treated with TAM, E2, or VEH for 12 weeks. (A) Liver histology: representative images of H&E (scale bars, 100 μm) and Oil Red O staining (insets; scale bars, 50 μm). (B) Intrahepatic lipid content. (C) Liver mRNA expression (37 genes involved in metabolic pathways). Genes were defined as up- or down-regulated if the fold change compared to control was statistically significant ($P < 0.05$, Student *t* test). Data are expressed as mean ± SEM. * indicates differences between VEH and treated (TAM or E2) mice within each genotype. ***$P < 0.001$.

focusing on a set of 37 selected genes involved in lipid and glucose metabolism. In WT-treated mice, 20/37 and 10/37 genes were significantly regulated by TAM and E2, respectively (summarized in Fig. 1C). In our experimental settings, no significant changes were observed in the mRNA expression levels of acetyl-coenzyme A (CoA) acyltransferase 2 (*Acaa2*), acyl-CoA dehydrogenase, long-chain (*Acadl*), apolipoprotein B (*Apob*), carnitine palmitoyltransferase 1A (*Cpt1a*), cytochrome P450, family 17, subfamily a, polypeptide 1 (*Cyp17a1*), fatty acid binding protein 4, adipocyte (*Fabp4*), glucose-6-phosphatase, catalytic (*G6pc*), 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), insulin receptor (*Insr*), insulin receptor substrate 2 (*Irs2*), lipoprotein lipase (*Lpl*), MLX interacting protein-like (*Mlxipl*), nuclear receptor subfamily 1, group H, member 4 (*Nr1h4*), patatin-like phospholipase domain containing 2 (*Pnpla2*), sirtuin 1 (*Sirt1*), and solute carrier family 2 member 4 (*Slc2a4*) following either TAM or E2 administration (data not shown). In TAM-treated WT mice, genes involved in *de novo* lipogenesis and lipid synthesis (acetyl-CoA carboxylase alpha [*Acaca*], CCAAT/enhancer binding protein, alpha [*Cebpa*], diacylglycerol O-acyltransferase 2 [*Dgat2*], fatty acid synthase [*Fasn*], *Nr1h3,* phosphatidylethanolamine N-methyltransferase [*Pemt*], peroxisome proliferator activator receptor delta [*Ppard*], stearoyl-CoA desaturase 1 [*Scd1*], and sterol regulatory element binding transcription factor 1 [*Srebf1*]), cholesterol metabolism and lipoprotein assembly (*Apoa1*, *Apoa4*, *Apoa5,* and phospholipid transfer protein [*Pltp*]), lipid transport (*Fabp1*) and catabolism (*Acacb*,

acyl-CoA oxidase 1, palmitoyl (*Acox1*), peroxisome proliferator activated receptor alpha [*Ppara*]), and glucose metabolism (glucokinase [*Gck*]) were down-regulated compared to VEH-treated mice (Supporting Fig. S4). Inversely, genes encoding for gluconeogenic enzyme (phosphoenolpyruvate carboxykinase 1, cytosolic [*Pck1*]*)* and hepatic insulin sensitivity (leptin receptor [*Lepr*]) were up-regulated by TAM. Noteworthy, TAM-sensitive genes were all dependent on hepatocyte ERα because their regulations were abolished in *LERKO* mice (Fig. 1C; Supporting Fig. S4). In E2-treated WT mice, some genes involved in *de novo* lipogenesis and lipid synthesis (*Acaca*, *Dgat2*, *Fasn*, *Scd1,* and *Srebf1*), cholesterol metabolism and lipoprotein assembly (*Pltp*), lipid catabolism (*Acacb*, *Ppara*), and glucose metabolism (*Gck*) were down-regulated, while *Cyp17a1,* a key gene for steroidogenesis, was up-regulated compared to VEH-treated mice (Supporting Fig. S4). The up-regulation of *Cyp17a1* and the down-regulation of *Acaca, Acacb, Dgat2, Srebf1, Pltp, Ppara,* and *Gck* by E2 were abrogated in *LERKO* mice, indicating that only 80% of E2-regulated genes were dependent on hepatocyte ERα activation (Fig. 1C; Supporting Fig. S4). Based on the protection conferred by TAM and E2 against HFD-induced steatosis, both molecules thus down-regulate the expression of key lipogenic genes. Although other cellular targets could contribute to E2-mediated control of these hepatic metabolic pathways, the present data demonstrate that their regulation by TAM entirely relies on the direct activation of hepatocyte ERα.

HEPATOCYTE ERα MEDIATES PROTECTIVE EFFECTS OF TAM ON WHOLE-BODY ENERGY AND GLUCOSE HOMEOSTASIS

We next considered the role of hepatocyte ERα in the preventive actions of TAM and E2 against HFD-induced systemic disorders, namely obesity, insulin resistance, and hyperglycemia. In agreement with other reports,[6,8] both molecules protected ovariectomized WT female mice from body weight gain (Fig. 2A), but only TAM administration significantly decreased food intake (Fig. 2B). E2 and TAM reduced fat mass accumulation to a similar extent (Fig. 2C), including subcutaneous and visceral sites (Fig. 2D), without any impact on lean mass (Fig. 2C). As expected, the two treatments also prevented HFD-induced glucose intolerance and insulin resistance in WT mice (Fig. 2E). E2 administration still protected *LERKO* female mice from HFD-induced obesity (Fig. 2A,C,D), as reported,[4] and from glucose intolerance, although to a lesser extent than in WT mice (Fig. 2E). In contrast, the preventive effects of TAM on body weight gain (Fig. 2A), fat mass accumulation (Fig. 2C,D), insulin resistance, and glucose intolerance (Fig. 2E) as well as its anorectic effect (Fig. 2B) were totally abrogated in *LERKO* mice. Plasma lipid profiles in WT mice revealed that TAM significantly reduced total and HDL-cholesterol levels together with an increase in low-density lipoprotein (LDL)-cholesterol concentrations, while E2 treatment was associated with a decrease in HDL-cholesterol and an increase in LDL-cholesterol levels (Table 1). Adiponectin and leptin plasma concentrations were significantly decreased by both treatments in WT mice, while resistin levels were only reduced by TAM administration (Table 1). In *LERKO* mice, all the effects of E2 on plasma lipid and adipokine profiles were preserved, whereas those of TAM were abolished except for adiponectin concentration that was still reduced (Table 1). These results indicate that specific activation of hepatocyte ERα is dispensable for E2-mediated prevention of obesity and associated metabolic disorders but is absolutely required for the systemic effects of TAM, including the control of food intake as well as the preservation of whole-body energy and glucose homeostasis.

HEPATOCYTE ERα IS REQUIRED FOR THE EFFECT OF TAM ON FOOD INTAKE AND ENERGY EXPENDITURE

To further characterize the involvement of hepatocyte ERα in the respective influence of TAM and E2 on energy homeostasis, HFD-fed WT and *LERKO* mice were assessed in metabolic cages, including indirect calorimetry measurements. No significant change in respiratory exchange ratio was observed with either TAM or E2 (Fig. 3A). E2 did not influence energy expenditure (Fig. 3B) but tended to increase nocturnal physical activity, both in WT and in *LERKO* mice (Fig. 3C), suggesting that metabolic effects induced by E2 involve central mechanisms independent of hepatocyte ERα activation. Conversely, TAM tended to decrease nocturnal

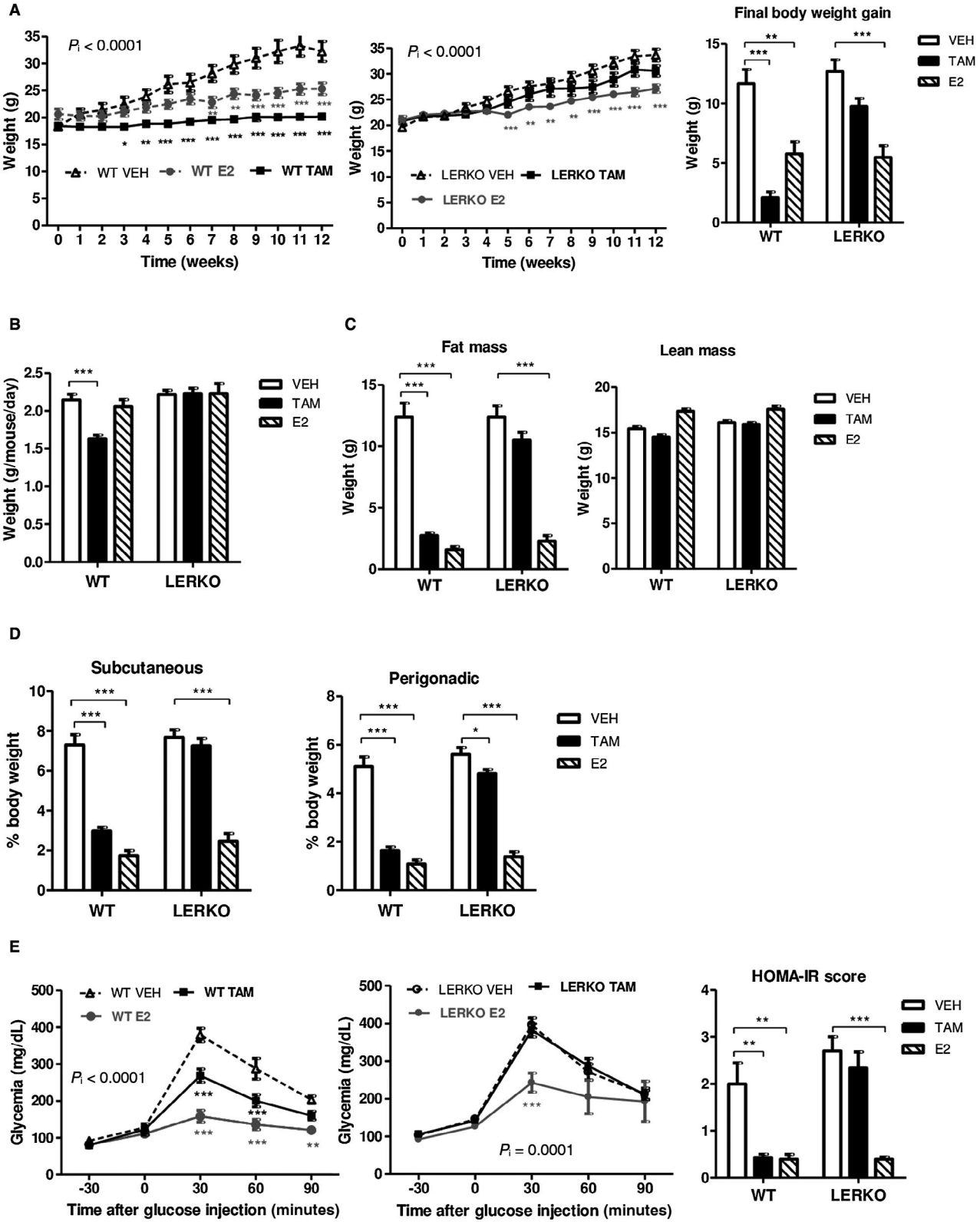
A
Pi < 0.0001
Weight (g)
Time (weeks)
WT VEH
WT E2
WT TAM
Pi < 0.0001
Weight (g)
Time (weeks)
LERKO VEH
LERKO TAM
LERKO E2
Final body weight gain
Weight (g)
VEH
TAM
E2
WT
LERKO
B
Weight (g/mouse/day)
VEH
TAM
E2
WT
LERKO
C
Fat mass
Weight (g)
WT
LERKO
Lean mass
Weight (g)
VEH
TAM
E2
WT
LERKO
D
Subcutaneous
% body weight
WT
LERKO
Perigonadic
% body weight
VEH
TAM
E2
WT
LERKO
E
Glycemia (mg/dL)
WT VEH
WT TAM
WT E2
Pi < 0.0001
Time after glucose injection (minutes)
Glycemia (mg/dL)
LERKO VEH
LERKO TAM
LERKO E2
Pi = 0.0001
Time after glucose injection (minutes)
HOMA-IR score
VEH
TAM
E2
WT
LERKO

FIG. 2. Hepatocyte ERα mediates the protective effects of TAM but not of E2 against HFD-induced obesity, insulin resistance, and glucose intolerance. *LERKO* mice (n = 40) and their respective WT littermates (n = 40) were fed an HFD and treated with TAM, E2, or VEH for 12 weeks. (A) Body weight evolution and final body weight gain; (B) mean food intake; (C) body composition; (D) adipose tissue distribution; (E) intraperitoneal glucose tolerance tests and HOMA-IR score. Data are expressed as mean ± SEM. $P_i < 0.0001$, interaction between treatments and genotypes; *indicates differences between VEH and treated (TAM or E2) mice within each genotype. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Abbreviation: HOMA-IR, homeostasis model assessment of insulin resistance.

TABLE 1. INFLUENCE OF TAM AND E2 ON PLASMA METABOLIC PARAMETERS IN HFD-FED *LERKO* MICE

	WT			*LERKO*		
	VEH	TAM	E2	VEH	TAM	E2
ALT, U/L	36 ± 8	23 ± 9	15 ± 10	28 ± 5	25 ± 20	28 ± 19
Chol, mmol/L	3.20 ± 0.20	2.07 ± 0.33†	3.23 ± 0.64	3.57 ± 0.35	3.55 ± 0.22	3.21 ± 0.23
HDL-C, mmol/L	1.67 ± 0.05	1.23 ± 0.08‡	1.32 ± 0.12*	1.86 ± 0.21	1.83± 0.14	1.34 ± 0.16†
LDL-C, mmol/L	0.35 ± 0.03	0.43 ± 0.04*	0.77 ± 0.25*	0.47 ± 0.05	0.39 ± 0.04*	0.64 ± 0.15*
TG, mmol/L	0.88 ± 0.05	0.82 ± 0.06	0.77 ± 0.16	0.87 ± 0.09	0.98 ± 0.20	0.71 ± 0.05*
FFA, mmol/L	0.77 ± 0.11	0.93 ± 0.14	0.63 ± 0.14	1.07 ± 0.14	0.87 ± 0.07*	0.78 ± 0.17*
Adiponectin, ng/mL	6.7 ± 1.0	4.7 ± 0.4†	2.8 ± 0.5†	7.1 ± 0.9	4.2 ± 0.3‡	3.1 ± 0.6‡
Leptin, ng/mL	20.3 ± 4.8	3.8 ± 1.1‡	2.1 ± 1.1‡	23.8 ± 4.7	19.1 ± 3.4	3.3 ± 2.3‡
Resistin, ng/mL	5.2 ± 0.9	3.1 ± 0.8†	5.0 ± 1.6	4.8 ± 1.7	3.6 ± 0.7	3.9 ± 0.6

Ovariectomized female *LERKO* mice and their respective WT littermates were fed an HFD and concomitantly treated with TAM, E2, or VEH for 12 weeks. Blood samples were collected at death in 3-hour-fasted mice. Data are expressed as mean ± SEM.
Differences between VEH-treated and either TAM- or E2-treated mice according to the Student *t* test; $^*P < 0.05$; $^\dagger P < 0.01$; $^\ddagger P < 0.001$.
Abbreviations: ALT, alanine aminotransferase; C, cholesterol; Chol, total cholesterol; FFA, free fatty acids; TG, triglyceride.

physical activity (Fig. 3C) in WT mice along with a significant reduction in energy expenditure during both day and night periods (Fig. 3B). TAM-treated WT mice were also characterized by a reduction in oxygen consumption and carbon dioxide production (Supporting Fig. S3) that was associated with a significant decrease in food intake (Fig. 2B), thus suggesting that the metabolic benefit conferred by TAM probably results in a large part from its anorectic effect. Finally, all the effects of TAM on food intake (Fig. 2B) and energy expenditure (Fig. 3B) were entirely abrogated in *LERKO* mice, demonstrating that they are mediated through ERα activation in hepatocytes.

TAM ENHANCES *Gdf15* EXPRESSION THROUGH HEPATOCYTE ERα ACTIVATION

To identify the link between hepatocyte ERα activation and whole-body metabolic protection conferred by TAM, we first explored whether TAM and E2 differently influence the expression of hepatokines known to exert specific actions on energy and glucose homeostasis. Neither E2 nor TAM altered angiopoietin-like 4 (*Angptl4*), *Angptl6*, insulin-like growth factor 1 (*Igf1*), and insulin-like growth factor binding protein 1 (*Igfbp1*) hepatic expression levels in WT mice, whereas *Angptl4 and Igfbp1* were significantly increased in E2-treated *LERKO* mice (Fig. 4A). However, E2 enhanced fibroblast growth factor 21 (*Fgf21*) mRNA expression level in the liver from WT mice (Fig. 4A), as reported,[14] and this positive regulation was still observed in *LERKO* mice (Fig. 4A). Similarly, E2 induced a significant upregulation of energy homeostasis associated (*Enho*) that codes for adropin, a hepatokine known to promote nocturnal physical activity and to prevent insulin resistance and adiposity, independently of hepatocyte ERα activation (Fig. 4A). TAM significantly increased the expression of *Igfbp2* in WT but not *LERKO* mice (Fig. 4A). The plasma level of this hepatokine has been demonstrated to be inversely correlated with incidence of type 2 diabetes. However, the strongest

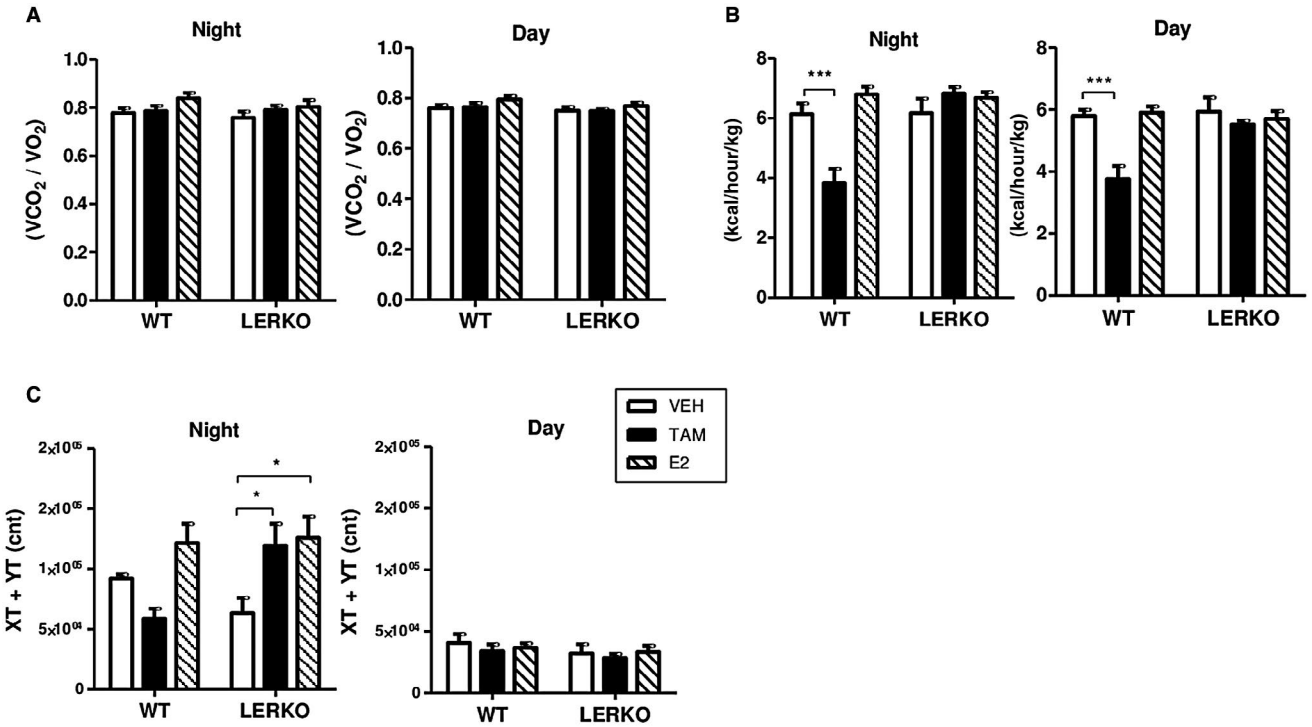

FIG. 3. Effects of TAM and E2 and role of hepatocyte ERα on energy homeostasis. Indirect calorimetry measurements were performed in *LERKO* mice (n = 19) and their respective WT littermates (n = 21) fed an HFD and treated with TAM, E2, or VEH for 12 weeks. (A) Respiratory exchange ratio calculated as carbon dioxide production/oxygen consumption, (B) energy expenditure (kcal heat produced). (C) Ambulatory physical activity as measured by the average number of beambreak counts in different dimensions (XT + YT). Data are expressed as mean ± SEM. *indicates differences between VEH and treated (TAM or E2) mice within each genotype. $^{*}P < 0.05$; $^{***}P < 0.001$. Abbreviations: VCO_2, carbon dioxide production; VO_2, oxygen consumption.

effect of TAM on gene expression concerned *Gdf15*, which codes for a molecule described as a powerful cytokine that inhibits food intake and decreases body weight through direct action on feeding centers in the hypothalamus and brainstem.[15,16] Indeed, an 8-fold increase in *Gdf15* mRNA hepatic expression was observed in TAM-treated WT mice, contrasting with the lack of E2 effect on this hepatokine expression (Fig. 4A). TAM-mediated up-regulation of *Gdf15* mRNA hepatic expression was totally abolished in *LERKO* mice (Fig. 4A) and, importantly, serum Gdf15 concentrations were perfectly correlated with its liver expression profile in WT as in *LERKO* mice (Fig. 4B). We also determined *Gdf15* mRNA levels in adipose tissue from WT and *LERKO* mice and found no influence of E2 and only a slight increase in TAM-treated mice from both genotypes (Fig. 4C), supporting the conclusion that serum Gdf15 concentration mainly reflects liver secretion. Our data thus demonstrate that unlike E2, TAM administration induces a marked increase in *Gdf15* hepatic expression and Gdf15 circulating concentration through the activation of ERα in hepatocytes.

DIFFERENTIAL REGULATION OF *Gdf15* EXPRESSION IN THE LIVER WITH TAM AND E2

To further explore the molecular mechanisms involved in the differential regulation of *Gdf15* expression by both ERα ligands, ovariectomized female mice were treated with E2 or TAM either acutely (single subcutaneous injection) or chronically (through subcutaneous pellet over 3 weeks). After acute treatment, E2 and TAM up-regulated *Gdf15* expression at the same level (Fig. 5A), in agreement with our previous study emphasizing *Gdf15* mRNA induction in the livers of female mice following oral

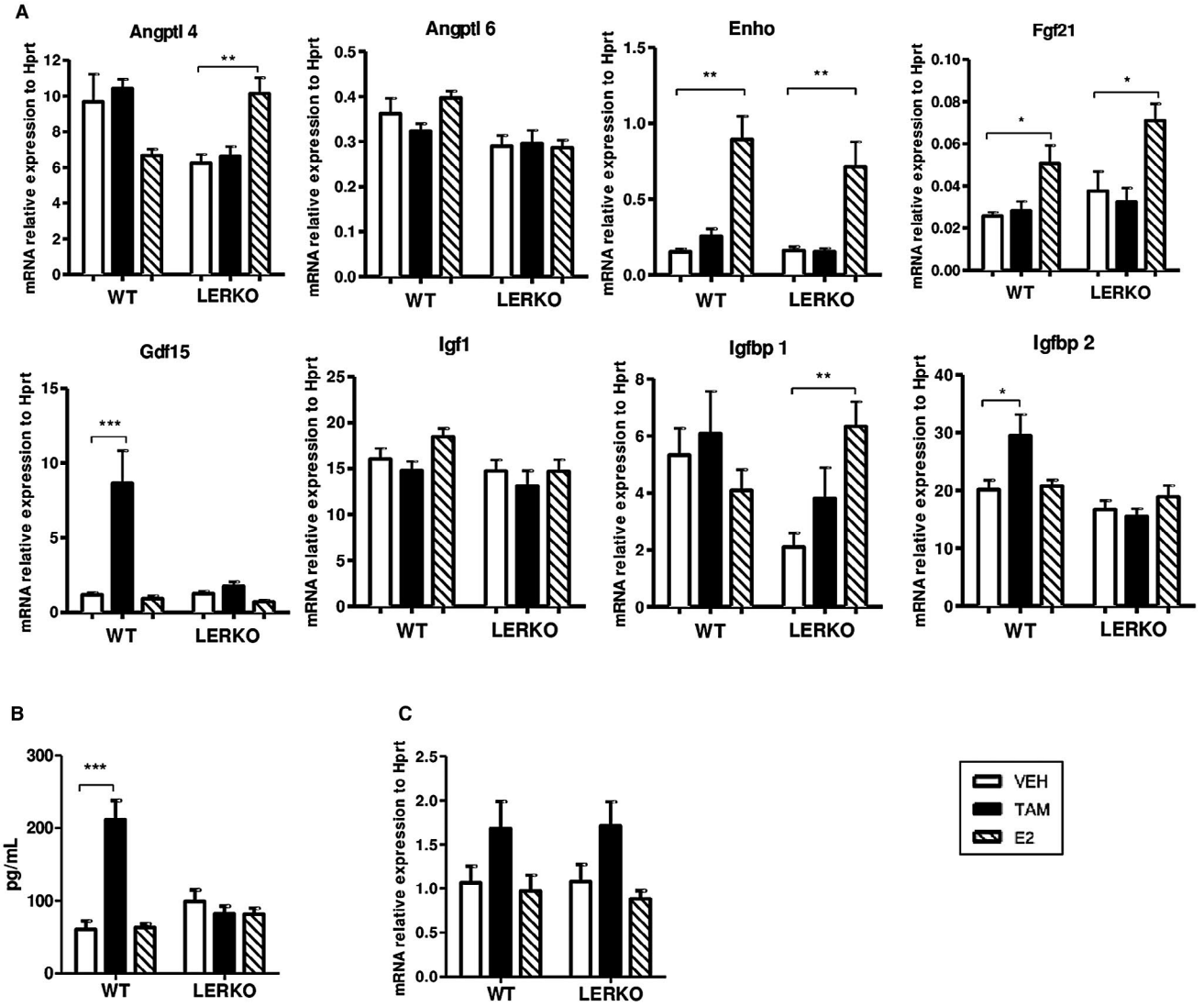

FIG. 4. Role of hepatocyte ERα to TAM or E2 regulation of hepatokines known to improve glucose and lipid homeostasis and/or to control food intake. *LERKO* mice (n = 18) and their respective WT littermates (n = 17) were fed an HFD and treated with TAM, E2, or VEH for 12 weeks. (A) Hepatic mRNA expression of hepatokines (*Angptl4, Angptl6, Enho, Fgf21, Gdf15, Igf1, Igfbp1,* and *Igfbp2*) in liver tissue samples. (B) Serum Gdf15 concentration in blood samples. (C) *Gdf15* mRNA expression in white adipose tissues. Data are expressed as mean ± SEM. *indicates differences between VEH and treated (TAM or E2) mice within each genotype. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Abbreviation: Hprt, hypoxanthine phosphoribosyl transferase.

E2 administration.[17] However, no *Gdf15* mRNA level induction was observed after E2 chronic treatment (Fig. 5A), as shown in HFD-fed mice (Fig. 4A). By contrast, TAM chronic treatment still increased *Gdf15* expression. This was not due to ERα protein degradation in response to E2 because neither E2 nor TAM chronic treatment altered ERα protein level (Fig. 5B). In ChIP-quantitative polymerase chain reaction (qPCR) experiments, ERα and its coactivator CREB binding protein (CBP) were found to be associated to their binding sites (BS1 and BS2) in the *Gdf15* regulatory region[17] in response to TAM but not to E2 chronic administration (Fig. 5C; Supporting Fig. S6A). In liver from E2-treated mice, the lack of ERα binding to the *Gdf15* regulatory region was associated with trimethylation of the twenty-seventh amino acid in histone H3 (H3K27) on BS1 (Fig. 5D; Supporting Fig. S6B), an epigenetic modification recently described to silence *Gdf15* expression.[18] These ChIP assays also demonstrated

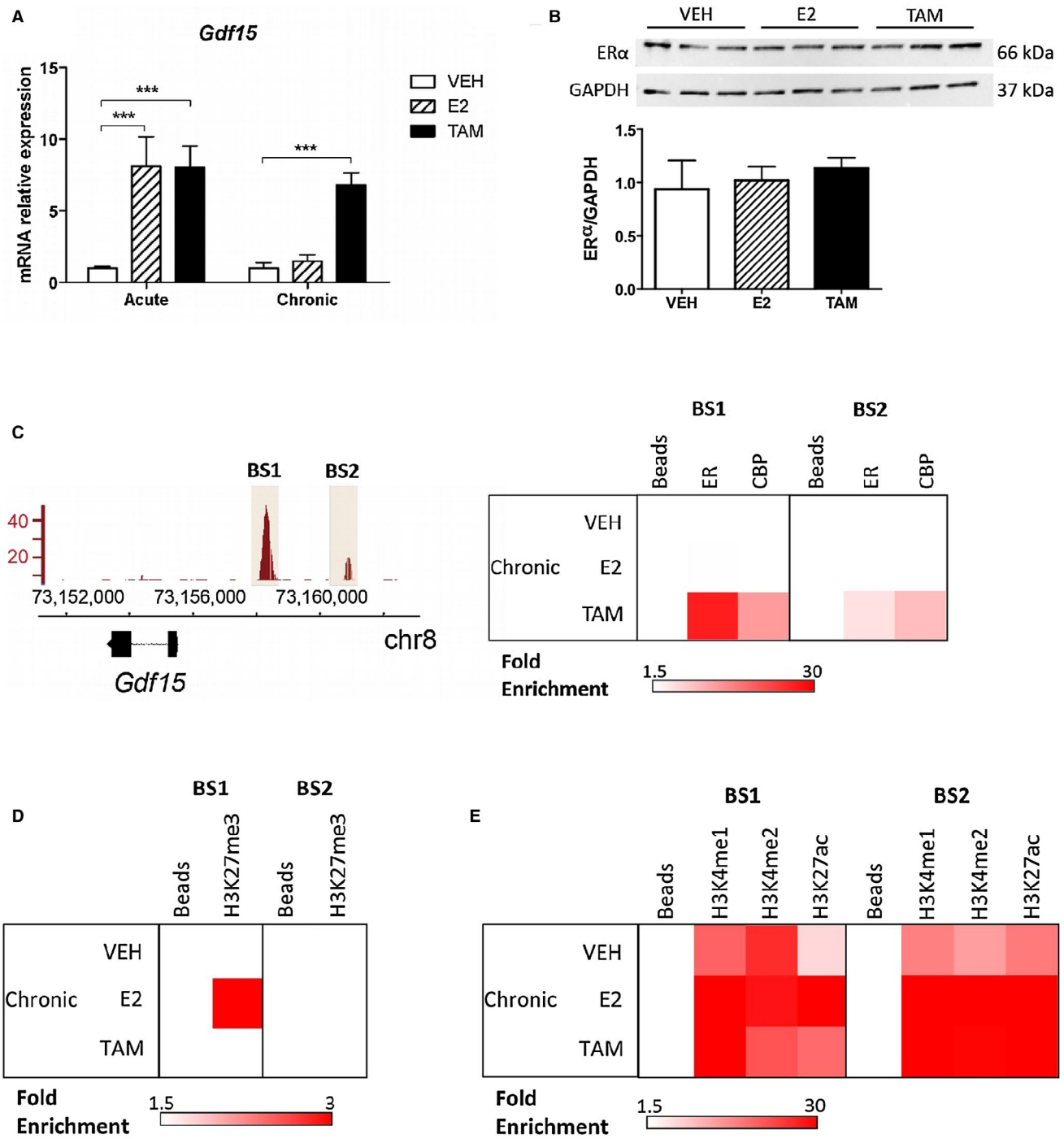

FIG. 5. Regulation of *Gdf15* expression in the liver with TAM and E2. Ovariectomized female *C57Bl/6J* mice (n = 12) were treated with TAM, E2, or VEH for 2 hours (acute) or for 3 weeks (chronic). (A) Hepatic *Gdf15* mRNA expression was determined by RT-qPCR in liver tissue samples. Data are expressed as mean ± SEM. ***$P < 0.001$. (B) Hepatic ERα protein level after chronic treatment was determined by western blot and normalized to Gapdh. (C) ERα enrichment at BS1 and BS2 on the *Gdf15* promoter region (left panel) was quantified by ChIP-qPCR experiments from livers treated as in panel B (right panel). The presence of histone marks (D) H3K27me3 and (E) H3K4me1, H3K4me2, and H3K27ac on BS1 and BS2 were assessed by ChIP-qPCR experiments on chromatin prepared from the same livers as those used in panel C. Abbreviation: chr, chromosome; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; RT, reverse transcription.

that the nucleosomes located around both BS retained acetylated H3K27 (H3K27ac) and H3K4 monomethylation (me1) and dimethylation chromatin marks (Fig. 5E). Interestingly, both BS1 and BS2 still exhibited an enhanced enrichment in H3K4me1 and H3K27ac marks in the presence of E2 compared to VEH (Supporting Fig. S6C). This is consistent with the hypothesis that these sequences were likely to have undergone activation at an earlier time point in accordance with the E2-mediated induction of *Gdf15* observed in the acute condition. Altogether, these data fit the hypothesis that at least BS1 begins to acquire features from "poised" enhancers, i.e., genomic sites that were active and underwent relative transient inactivation.[19] Finally, the chronic administration of TAM appears to be less efficient than E2 in inducing the enrichment of both BS1 and BS2 in H3K4me1 and H3K27ac (Supporting Fig. S6C). This may reflect the weaker ability of ERα to recruit cofactors in the presence of TAM compared to E2, which in this precise case does not prevent ERα from modulating the transcription rate of *Gdf15*.

HEPATIC AND WHOLE-BODY METABOLIC PROTECTIVE EFFECTS OF TAM ARE ABOLISHED IN HFD-FED *GDF15KO* MICE

To determine whether Gdf15 contributes to the overall metabolic protective actions of TAM, ovariectomized *GDF15KO* mice and their WT littermates were treated with TAM, E2, or VEH for 6 weeks and concomitantly fed an HFD. The protective actions of E2 observed in WT mice in terms of body weight gain, fat mass accumulation, glucose tolerance, and steatosis were not altered by *Gdf15* deficiency (Fig. 6A-E). In striking contrast, TAM failed to protect *GDF15KO* mice from HFD-induced obesity (Fig. 6A,B), glucose intolerance (Fig. 6D), and steatosis (Fig. 6E). In addition, the anorectic effect of TAM was abolished and an increase in food intake was even observed in TAM-treated *GDF15KO* mice (Fig. 6C). Altogether, our results demonstrate that the selective modulation of hepatocyte ERα with TAM preserves whole-body energy and glucose homeostasis, in a context of HFD feeding, through the up-regulation of *Gdf15* hepatic expression.

Discussion

The present study further emphasizes the protective role of hepatocyte-specific ERα signaling against diet-induced steatosis. Noteworthy, in our experimental settings, such a beneficial effect on the liver was not only observed with E2 administration but also and to a similar extent following TAM treatment. This latter observation is undoubtedly more surprising because numerous clinical observations reported that TAM is able to promote or to worsen fatty liver in women prescribed with this treatment for breast cancer, although recent studies concluded that the incidence of TAM-induced steatosis is very low.[20] Studies conducted in rodent models led to divergent results depending on the experimental settings, some of them reproducing TAM-induced steatosis[21,22] while others, in perfect agreement with our observations, reported protective effects of TAM on the liver[23,24] but also on glucose and lipid metabolism.[24-28] Accordingly, Ceasrine et al.[24] highlighted the need for caution in the interpretation of metabolic studies that require transient high-dose TAM administration protocols for creating conditional KO in tamoxifen-inducible estrogen receptor (*ERT2*)-*Cre* thyroglobulin (*Tg*) mice, demonstrating an improved glucose tolerance in both male and female mice 1 week after the last dose and even 3 weeks later in male mice.

However, our main finding is that hepatocyte ERα, following the peculiar mode of activation of TAM, is also able to preserve whole-body energy and glucose homeostasis from the deleterious effects of HFD feeding. Indeed, hepatocyte-restricted ERα deletion differently alters the metabolic protection conferred by E2 and TAM, two ERα ligands respectively leading to the full activation or to the AF1-selective modulation of the receptor.[6,8] Although E2-treated *LERKO* female mice were no longer protected from steatosis, as reported,[4] these mice were still preserved from HFD-induced adiposity, insulin resistance, and glucose intolerance, underlining the involvement of targets other than hepatocytes for these latter actions of E2. In contrast, similar extensive protective actions exerted by TAM are dependent on hepatocyte ERα activation as they were all abolished in *LERKO* mice. Consequently, although TAM and E2 similarly protect female mice from HFD-induced metabolic disturbances through ERα-dependent mechanisms, our results support the conclusion that their respective effects involve distinct cellular targets.

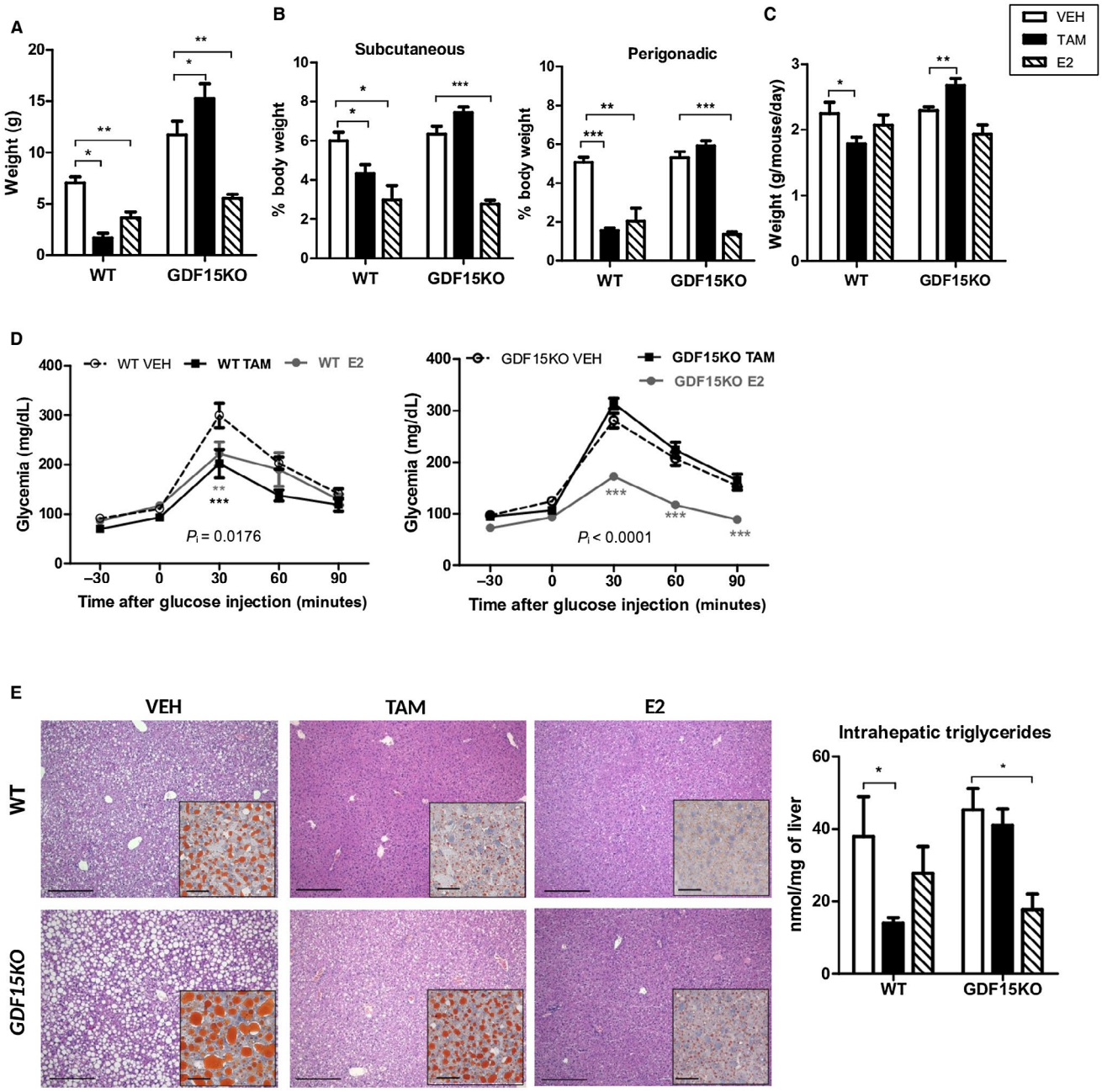

FIG. 6. Protective metabolic effects of TAM are abolished in *GDF15KO* mice. *GDF15KO* mice (n = 23) and their respective WT littermates (n = 14) were fed an HFD and treated with TAM, E2, or VEH for 6 weeks. (A) Body weight gain; (B) food intake; (C) adipose tissue distribution; (D) intraperitoneal glucose tolerance tests; (E) liver histology, representative images of H&E (scale bars, 100 μm) and Oil Red O (insets; scale bars, 50 μm) staining and intrahepatic triglyceride content. Data are expressed as mean ± SEM. P_i, interaction between treatments and genotypes. *indicates differences between VEH and treated (TAM or E2) mice. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

It is now well recognized that the overall beneficial metabolic action of E2 requires ERα activation in various tissues, including the central nervous system, adipose tissue, liver, and endocrine pancreas.[29] In particular, estrogens have been demonstrated to regulate different aspects of energy homeostasis through direct activation of ERα in specific types of neurons.[30] ERα deletion restricted to hypothalamic

steroidogenic factor-1 neurons induces hypometabolism and abdominal obesity without associated hyperphagia, whereas ERα activation in hypothalamic pro-opiomelanocortin neurons controls food intake without any direct influence on energy expenditure or fat distribution.[30]

In our experimental settings, E2 administration enhanced nocturnal physical activity level in both WT and *LERKO* female mice while TAM lowered energy expenditure in WT mice, probably resulting from a significant decrease in food intake as described in murine models.[25,31-33] Interestingly, although TAM anorectic effect has been attributed to Fasn inhibition in the ventromedial nucleus of the hypothalamus, leading to accumulation of malonyl-CoA,[31] no study has demonstrated that this action relies on the direct activation of ERα in hypothalamic neurons. Noteworthy, demonstrating that the influence of TAM on food intake and energy expenditure was totally abrogated in *LERKO* mice, the present data suggest that the control of these central regulations by TAM mainly results from peripheral signals induced by ERα selective modulation in hepatocytes.

For insight into the mechanisms leading to the discrepant metabolic consequences of hepatocyte ERα activation by E2 and TAM, we addressed their respective influence on hepatic gene expression, first focusing on a set of genes involved in the main metabolic pathways. In line with their similar protective effects against HFD-induced steatosis, both molecules down-regulated the transcription of genes involved in *de novo* lipogenesis and lipid synthesis, although with a wider influence of TAM that more specifically impacted some of them (*Cebpa*, *Nr1h3*, *Pemt*) in WT mice. E2 and TAM also differently alter the expression of some genes involved in lipid and/or glucose metabolism (*Apoa1*, *ApoA4*, *Cyp17a1*, *Lepr*). However, the most important observation is that TAM-induced gene regulations were all abolished in *LERKO* mice whereas E2 still influenced the expression of several liver genes in these animals, thus probably through indirect mechanisms mediated by other ERα-expressing cellular targets that remain to be identified.

We then hypothesized that the whole-body protection conferred by TAM through hepatocyte ERα activation could be mediated by liver-secreted circulating factors, and we decided to focus our analyses on the respective influence of E2 and TAM on the expression of hepatokines described to exert metabolic actions. It must be acknowledged that additional experiments would have been useful to definitely demonstrate that TAM acts through hepatocyte-initiated endocrine mechanisms. From our data, we cannot exclude that neurovegetative signals and other liver-derived factors, including other proteins or small molecules, could contribute to the systemic effects of TAM. Importantly, hepatocyte ERα is already known as a key mediator of systemic regulation as it controls *Igf1* expression.[9] In our experiments, E2 treatment led to a significant increase in hepatic expression of *Fgf21*, as reported,[14] and also of *Enho*, which codes for adropin, an hepatokine known to increase night physical activity and to prevent insulin resistance and adiposity.[34] These regulations are demonstrated to be totally independent from hepatocyte ERα and could thus have contributed, at least partially, to the preservation of energy and glucose homeostasis by E2 in HFD-fed WT and *LERKO* mice. Conversely, neither *Fgf21*, in line with the action of another SERM (bazedoxifene),[14] nor *Enho* expression was modified in TAM-treated mice livers. Among the genes coding for hepatokines tested in our experimental settings, we found that *Gdf15* and, to a much lesser extent *Igfbp2*, displays a gene expression profile strictly dependent on TAM-induced hepatocyte ERα activation.

Noteworthy, *Gdf15* gene expression in the liver perfectly correlates with plasma concentrations measured in WT and *LERKO* mice. Mainly produced by the liver,[35] Gdf15 is a divergent member of the transforming growth factor-β superfamily, which exerts anorectic effects and controls body weight through the activation of the glial cell line-derived neurotrophic factor family receptor α-like (GFRAL), recently identified as the specific receptor of Gdf15 in hindbrain neurons of the area postrema and nucleus of the solitary tract.[36-38] In rodent models, even a moderate increase in Gdf15 circulating levels reduces food intake, and a sustained exposition to this hepatokine leads to cachexia, both under normal diet and HFD.[15,39] Accordingly, *GDF15KO* mice are characterized by accelerated weight gain and increased fat mass, mainly localized in visceral abdominal sites, but their food intake and body weight returned to the WT level following infusion with recombinant human GDF15.[40] Moreover, *GDF15KO* mice are also prone to develop steatosis, while *GDF15*-transgenic mice are protected from steatosis and associated metabolic disorders.[41]

Finally, in agreement with our results, prevention of obesity in HFD-fed mice treated with recombinant GDF15 was not associated with an increase in energy expenditure, suggesting that the protective action of this hepatokine is likely driven by the reduction in food intake.[36] These considerations prompted us to directly address the role of Gdf15 in whole-body metabolic protective TAM effects. The effects of TAM were confirmed in *GDF15*$^{+/+}$ female mice but were all totally abrogated in *GDF15KO* mice.

Although hepatocytes have been recognized as the main cell sources of Gdf15 in the liver,[42] whether TAM-enhanced *Gdf15* expression is restricted to hepatocytes or concerns other cell types remains to be determined. However, this lack of demonstration of the cellular sources of Gdf15 in the liver of TAM-treated mice does not challenge our main conclusion, i.e., the specific regulation of liver *Gdf15* by TAM requires ERα activation in hepatocytes. *GDF15* expression levels are physiologically low but rapidly increase in pathophysiological conditions, such as tissue injury, inflammation, and malignancy, as well as during dysmetabolic status, including obesity, type 2 diabetes, and NAFLD.[41] Interestingly, our study demonstrates that hepatocyte ERα is also able to enhance *Gdf15* expression according to its mode of activation. Contrasting with the significant increase induced by TAM, *Gdf15* liver expression and Gdf15 circulating levels were not altered by E2 chronic treatment. This latter observation is surprising because we previously observed and confirmed in the present study that acute E2 administration significantly increases liver *Gdf15* gene transcription.[17] The discrepancy in Gdf15 transcription regulation following chronic treatments cannot be explained by ERα protein degradation in response to E2, but ChIP-qPCR revealed that ERα and its coactivator CBP were bound to BS1 and BS2 in the Gdf15 regulatory region only in TAM-treated mice. Accordingly, our data suggest that chronic E2 treatment induced epigenetic modifications that contribute to silent Gdf15 expression, as recently reported.[18]

In line with our recent work,[6,8] the present study further illustrates the redundancy between ERα-AF1 and ERα-AF2 in terms of metabolic protection. In contrast, both ERα-AFs are required to mediate proliferative effects on the mammary gland and contribute to the increased risk of breast cancer elicited by estrogens, contrasting with the spectacular protection conferred by ERα-AF2 antagonism of TAM.[43] However, this ERα-AF2 antagonism has no adverse impact on the main cellular target(s), mainly hepatocytes as demonstrated here, involved in TAM metabolic effects. This underlines the therapeutic potential of SERMs resulting in the specific activation of only ERα-AF1. Furthermore, as discussed above, the cellular targets mediating the metabolic protective actions are different according to the nature of the ligand, although leading to a similar overall benefit.

From a therapeutic perspective, it would be very interesting to design new strategies for a preferential delivery of ERα-AF1-selective modulators to the liver, as recently proposed for targeting other cellular types, such as the pancreas with a glucagon-like peptide-1-estrogen conjugate.[44]

The present work provides further evidence that hepatocyte ERα represents a promising target for the prevention of NAFLD and demonstrates for the first time that the selective modulation of ERα in hepatocytes is sufficient to preserve whole-body energy and glucose homeostasis. The mechanism accounting for this major widespread metabolic action relies on the induction of the hepatokine Gdf15. Selective modulation of hepatocyte ERα leading to sustained increase in circulating Gdf15 could thus be sufficient to fight obesity and its related complications, such as type 2 diabetes and NAFLD.

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Supporting Information

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