Received: 10 June 2021 | Revised: 15 July 2021 | Accepted: 26 July 2021

DOI: 10.1002/hep.32122

ORIGINAL ARTICLE

Up-regulation of thioesterase superfamily member 2 in skeletal muscle promotes hepatic steatosis and insulin resistance in mice

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Funding information
Supported by the National Institute of Diabetes and Digestive and Kidney Diseases (R37 DK048873 and R01 DK056626; to D.E.C. and R01 DK103046 to D.E.C and E.A.O.); the Georgia Clinical & Translational Science Alliance of the National Institutes of Health (UL1TR002378; to E.A.O.); an American Heart Association Postdoctoral Fellowship (19POST34380692, to N.I.; 18POST33990445, to M.A.-B.); the American Liver Foundation Irwin M. Arias, MD Postdoctoral Research Fellowship Award (to N.I.); and the Liver Foundation NASH Fatty Liver Disease Postdoctoral Research Fellowship Award (to M.A.-B.)

Background and Aims: Thioesterase superfamily member 2 (Them2) is highly expressed in liver and oxidative tissues, where it hydrolyzes long-chain fatty acyl-CoA esters to free fatty acids and CoA. Although mice globally lacking Them2 (*Them2*$^{-/-}$) are protected against diet-induced obesity, hepatic steatosis (HS), and insulin resistance (IR), liver-specific *Them2*$^{-/-}$ mice remain susceptible. The aim of this study was to test whether Them2 activity in extrahepatic oxidative tissues is a primary determinant of HS and IR.

Approach and Results: Upon observing IR and up-regulation of Them2 in skeletal, but not cardiac, muscle of high-fat-diet (HFD)-fed wild-type compared to *Them2*$^{-/-}$ mice, we created mice with Them2 specifically deleted in skeletal (*S-Them2*$^{-/-}$) and cardiac muscle (*C-Them2*$^{-/-}$), as well as in adipose tissue (*A-Them2*$^{-/-}$). When fed an HFD, *S-Them2*$^{-/-}$, but not *C-Them2*$^{-/-}$ or *A-Them2*$^{-/-}$, mice exhibited reduced weight gain and improved glucose homeostasis and insulin sensitivity. Reconstitution of Them2 expression in skeletal muscle of global *Them2*$^{-/-}$ mice, using adeno-associated virus, was sufficient to restore excess weight gain. Increased rates of fatty acid oxidation in skeletal muscle of *S-Them2*$^{-/-}$ mice contributed to protection from HFD-induced HS by increasing VLDL triglyceride secretion rates in response to greater demand. Increases in insulin sensitivity were further attributable to alterations in production of skeletal muscle metabolites, including short-chain fatty acids, branched-chain amino acids, and pentose phosphate pathway intermediates, as well as in expression of myokines that modulate insulin responsiveness.

Conclusions: These results reveal a key role for skeletal muscle Them2 in the pathogenesis of HS and IR and implicate it as a target in the management of NAFLD.

Abbreviations: AAV8, adeno-associated virus serotype 8; ACC, acetyl-CoA carboxylase; Acot, acyl-CoA thioesterase; Acsl, long-chain acyl-CoA synthetase; Adipoq, adiponectin, C1Q, and collagen domain containing; Akt, protein kinase B; AMPK, adenosine monophosphate-activated protein kinase; BAT, brown adipose tissue; BSA, bovine serum albumin; *Cidea*, cell death activator; CPT1, carnitine palmitoyltransferase I; CXCL1, chemokine ligand 1; FAO, fatty acid oxidation; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; HFD, high-fat diet; HS, hepatic steatosis; *Igf1*, insulin-like growth factor 1; IR, insulin resistance; Mck, muscle creatine kinase; Myf5, myogenic factor 5; Myh6, myosin-heavy chain 6; OCR, oxygen consumption rate; PC-TP, phosphatidylcholine transfer protein; PPP, pentose phosphate pathway; TG, triglyceride; Them2, thioesterase superfamily member 2; WAT, white adipose tissue.

Thioesterase superfamily member 2 (Them2; also known as acyl-CoA thioesterase [Acot] 13) catalyzes the hydrolysis of long-chain fatty acyl-CoA esters into free fatty acids and CoA.[1] Them2 is abundant in the liver and primarily localized to mitochondria, where it regulates trafficking of fatty acids to oxidative versus lipid biosynthetic pathways, depending upon metabolic conditions.[1] Them2 is also expressed at high levels in other oxidative tissues, including skeletal and cardiac muscle, as well as in adipose tissue. However, its function in these tissues is not understood.[2] Mice globally lacking Them2 (*Them2*$^{-/-}$) are protected against diet-induced obesity and hepatic steatosis (HS) and exhibit markedly improved glucose homeostasis.[3,4] The global absence of Them2 is also associated with reduced hepatic endoplasmic reticulum stress in the setting of overnutrition.[5] Studies in Them2 liver-specific knockout (*L-Them2*$^{-/-}$) mice have revealed a mechanistic role for Them2 in the hepatic trafficking of acyl-CoA molecules toward triglyceride (TG) synthesis and incorporation into VLDL particles.[6] However, high-fat diet (HFD)-fed *L-Them2*$^{-/-}$ mice exhibit similar body weights as controls and are not protected against HS or insulin resistance (IR).[6]

To explore the hypothesis that Them2 activity in extrahepatic tissues may be required for the development of IR and HS, we generated tissue-specific knockout mice lacking Them2 in skeletal muscle, cardiac muscle, and adipose tissue. Only skeletal-muscle–specific Them2 knockout mice recapitulated the protection from IR and HS that was observed in *Them2*$^{-/-}$ mice. Our results demonstrate that Them2 limits rates of fatty acid oxidation (FAO) in skeletal muscle, and its up-regulation in the setting of overnutrition promotes skeletal muscle IR and HS by complementary mechanisms. In addition to reducing the demand for hepatic VLDL secretion, the reduction in FAO in skeletal muscle decreases the production of short-chain fatty acids and pentose phosphate pathway (PPP) intermediates that maintain insulin sensitivity. In addition, Them2 suppresses the expression of myokines capable of modulating skeletal muscle physiology, as well as crosstalk with liver and adipose tissue. When taken together, these findings suggest that Them2 in skeletal muscle could be targeted as a therapeutic strategy in the management of NAFLD.

MATERIALS AND METHODS

Animals and diets

Mixed-background global Them2 knockout mice were backcrossed 20 generations with C56BL/6J.[3,4] Wild-type C56BL/6J mice were obtained from The Jackson Laboratory (Stock #000664; Bar Harbor, ME). Tissue-specific knockout mice were created by a LoxP/Cre system.[6] Mice with Them2 flanked by two LoxP sites (*Them2*$^{flox/flox}$) were generated[6] and backcrossed four times to the C57BL/6J genetic background. Them2 floxed mice were then crossed with mice expressing Cre recombinase driven by the myogenic factor 5 (Myf5) promoter to generate skeletal-muscle–specific deletion of *Them2*$^{-/-}$ (*S-Them2*$^{-/-}$), the myosin-heavy chain 6 (Myh6) promoter to generate cardiac-muscle–specific deletion of *Them2*$^{-/-}$ (*C-Them2*$^{-/-}$), and the adiponectin, C1Q, and collagen domain containing (Adipoq) promoter to generate adipose-tissue–specific deletion of *Them2*$^{-/-}$ (*A-Them2*$^{-/-}$), respectively (Myf5-cre, Stock #007893; Myh6-cre, Stock #011038; Adipoq-cre, Stock #010803; The Jackson Laboratory).[7–9] These mice were viable and displayed no apparent developmental abnormalities. Presence of Cre allele was determined by PCR analysis using the primers specified by The Jackson Laboratory. Mice were housed in a barrier facility on a 12-h light/dark cycle with free access to water and diet. Male mice were weaned at 4 weeks of age and fed chow (PicoLab Rodent Diet 20; LabDiet, St. Louis, MO). Alternatively, 5-week-old male mice were fed a HFD (D12492: protein 20% kcal, fat 60% kcal, carbohydrate 20% kcal, and energy density 5.21 kcal/g; Research Diets Inc., New Brunswick, NJ) for 12 weeks. Following 6-h fasting (9:00 AM to 3:00 PM) or 18-h fasting (6:00 PM to 12:00 PM), 17-week-old mice were euthanized and plasma was collected by cardiac puncture. Tissues were harvested for immediate use or snap-frozen in liquid nitrogen and stored at −80°C. Mice were monitored with daily health-status observations by technicians who were supported by veterinary staff. Housing and husbandry were conducted in facilities with a sentinel colony health-monitoring program and strict biosecurity measures to prevent, detect, and eradicate adventitious infections. Animal use and euthanasia protocols were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College (New York, NY).

Experimental methods

Details of experimental procedures are provided as Supplemental Materials and Methods in the Supporting Information.

Statistical analyses

Data are presented as mean values with error bars representing SEM. Statistical significance was determined by using two-tailed unpaired Student *t* tests when two groups were compared. Correlations were evaluated by Pearson's correlation coefficient analysis. Threshold values were determined by segmental linear regression. Multiple group comparisons were performed

using two-way ANOVA. Differences were considered significant for $p < 0.05$ (GraphPad Prism 8; GraphPad Software, La Jolla, CA).

RESULTS

Them2 suppresses insulin sensitivity in skeletal muscle and limits exercise capacity in HFD-fed mice

Because skeletal muscle and the heart extensively use fatty acids to produce ATP, we examined the potential contribution of Them2 to the regulation of insulin action in these tissues. We initially confirmed that *Them2*$^{-/-}$ mice on a C57BL/6J genetic background were protected against HFD-induced obesity (Figure 1A,B) and glucose intolerance (Figure 1C), as we have observed for a mixed genetic background.[3] In response to exogenous insulin administration (Figure 1D), HFD-fed *Them2*$^{-/-}$ mice exhibited a 62% reduction in blood glucose concentrations compared with a nonsignificant 24% decrease in *Them2*$^{+/+}$ mice, with an associated 2.6-fold increase in protein kinase B (Akt) phosphorylation in skeletal muscle, which together indicated improved insulin sensitivity in the absence of Them2 expression. There was a 1.7-fold increase in Them2 expression in skeletal muscle, but not in cardiac muscle, in HFD-fed compared to chow-fed mice (Figure 1E).

Notwithstanding reduced body weight of *Them2*$^{-/-}$ mice, both skeletal muscle and cardiac tissues from *Them2*$^{-/-}$ mice were proportionate in size and histologically normal (Figure S1A,B). Whereas skeletal muscle of *Them2*$^{-/-}$ mice exhibited no changes in mRNA expression levels of myosin heavy chains, there were modest decreases in proportions of type 1 muscle-fiber–type relative to type 2 fiber-type proteins (Figure S1C,D). *Them2*$^{-/-}$ mice also exhibited a similar expression of myosin-heavy chains and hypertrophy-related genes in cardiac muscle as control mice (Figure S1E,F). In chow-fed mice, Them2 expression did not influence exercise capacity, as assessed by treadmill testing (Figure 1F) and lactate production (Figure S1G). However, in the setting of HFD feeding, *Them2*$^{-/-}$ mice showed improved exercise capacity compared to *Them2*$^{+/+}$ controls (Figure 1F). We next investigated the impact of Them2 on cardiac function in response to chronic ionotropic stimulation. Isoproterenol infusion for 2 weeks was sufficient to induce cardiac hypertrophy in *Them2*$^{+/+}$ mice, as reflected by increases in both ejection fraction and heart wall thickness (Figure 1G). Whereas cardiac hypertrophy is accompanied by decreased rates of FAO and increased use of glucose,[10] Them2 expression did not influence its development (Figure 1G).

Metabolic monitoring is a comprehensive approach to the assessment of energy balance in the mouse, as determined by noninvasive measurements of physical activity, by food intake, as well as by energy expenditure calculated based on rates of oxygen consumption (VO_2) and carbon dioxide production (VCO_2). By this approach, there were no differences in voluntary (wheel) running (Figure 1H), spontaneous physical activity, or food intake (data not shown) attributable to Them2 expression. Similar to *Them2*$^{-/-}$ mice on a mixed genetic background,[3] chow-fed *Them2*$^{-/-}$ C57BL/6J mice exhibited increased energy expenditure (Figure 1I). Although no differences were found in energy expenditure during voluntary running (Figure 1I), respiratory exchange ratios (VCO_2/VO_2) of *Them2*$^{-/-}$ mice were elevated during the light cycle after acclimation to the running wheel (Figure 1J), indicative of greater use of glucose in skeletal muscle in the absence of Them2 expression during a period when mice were more sedentary. Taken together, these results suggested that Them2 regulates use of energy substrate in skeletal muscle.

Skeletal muscle expression of Them2 promotes obesity and IR in HFD-fed mice

To systematically explore contributions of extrahepatic Them2 to obesity, HS, and IR, we created mice lacking Them2 in skeletal muscle, cardiac muscle, and adipose tissues, each on a C57BL/6J genetic background (Figure 2A). Skeletal muscle deletion of Them2 with preserved cardiac expression (*S-Them2*$^{-/-}$ mice) was accomplished using mice expressing Cre recombinase driven by the *Myf5* promoter. Because *Myf5* is expressed in brown adipose tissue (BAT) in addition to skeletal muscle, mice lacking Them2 in skeletal muscle were also deficient in BAT. The *Myh6* promoter was used to generate cardiac muscle deletion of Them2 with preserved skeletal muscle expression (*C-Them2*$^{-/-}$ mice). *A-Them2*$^{-/-}$ mice lacking Them2 in BAT and white adipose tissue (WAT), which expresses Them2 at low levels, were generated using mice expressing Cre recombinase driven by the adiponectin promoter. Only *S-Them2*$^{-/-}$ mice were protected against diet-induced overweight (Figure 2B), excess fat accumulation (Figure 2C), glucose intolerance (Figure 2D), and IR (Figure 2E), as observed for global *Them2*$^{-/-}$ mice (Figure 1A–D). These differences were not associated with substantial alterations in expression of other skeletal muscle acyl-CoA thioesterase (*Acot*) or long-chain acyl-CoA synthetase (*Acsl*) isoforms that mediate the cleavage or addition, respectively, of a CoA molecule from or to a fatty acid (Figure S2A,B). The possibility that this phenotype was attributable to the absence of Them2 expression in BAT was excluded by demonstrating similar weight differences (Figure S3A) and body compositions (Figure S3B) for mice housed at 30°C, a thermoneutral temperature that suppresses BAT activity. This was further supported by the phenotypes

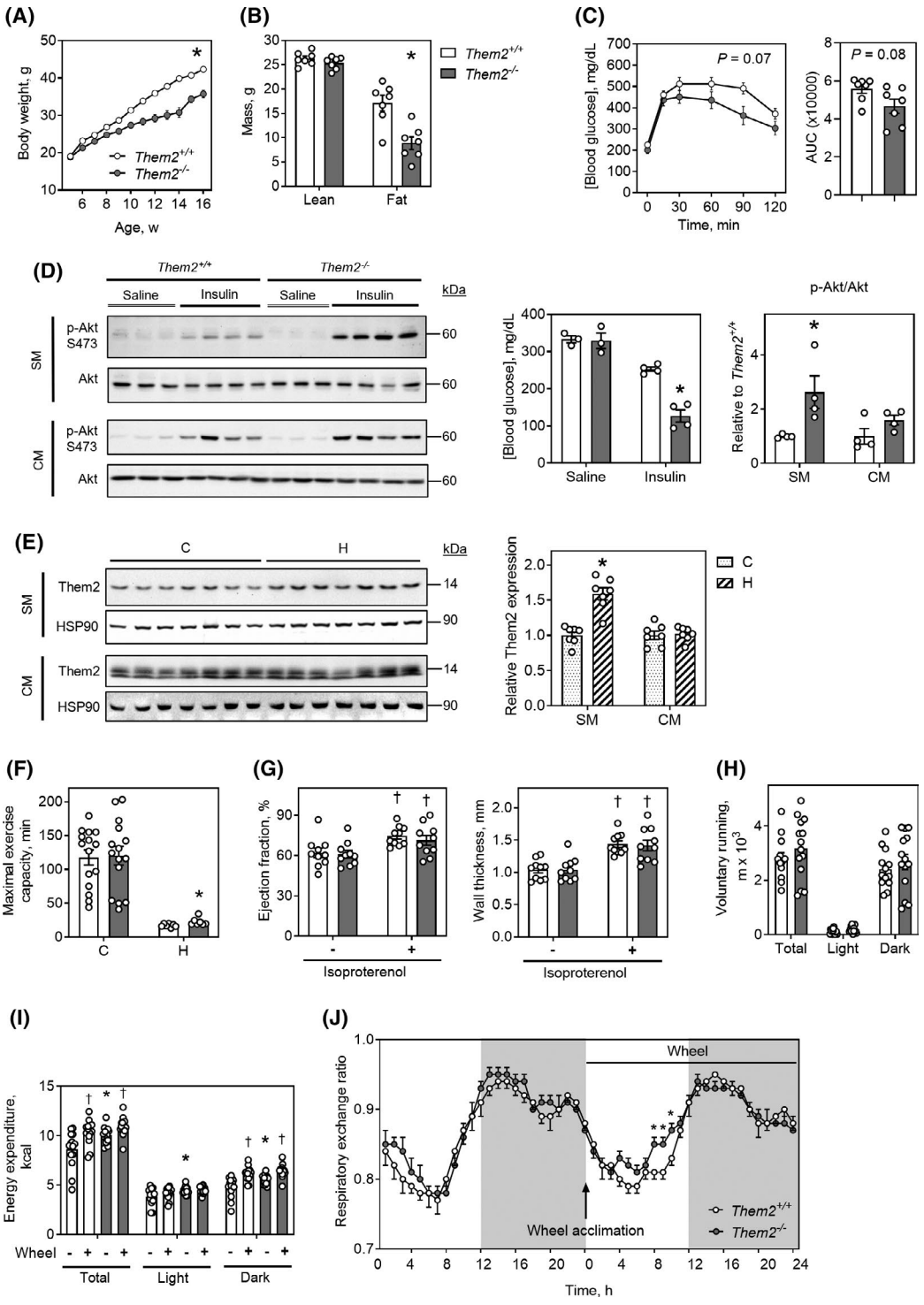

FIGURE 1 Them2 is up-regulated in skeletal muscle by HFD feeding, and its ablation improves skeletal muscle insulin sensitivity and exercise capacity in HFD-fed mice. Mice were fed a HFD (H) from 5 to 16 weeks. (A) Body weights and (B) lean and fat mass (determined at 16 weeks of age). Tolerance tests to glucose (C) were conducted between 16 and 17 weeks of age. Mice were injected IP with saline or insulin (10 mU/g) before (D) measurements of blood glucose concentrations, as well as immunoblotting analysis of Akt phosphorylation in cardiac and gastrocnemius muscle tissues. (E) Expression of Them2 in gastrocnemius muscle (skeletal muscle; SM) and heart (cardiac muscle; CM) for mice fed 12 weeks with chow (C) or a HFD (H). (F) Maximal exercise capacity was analyzed by a graded treadmill test (n = 7–15 per group). (G) Cardiac ejection fraction and left ventricle wall thickness were evaluated by echocardiography before and after isoproterenol osmotic minipump implantation ($^{\dagger}p < 0.05$ before vs. after treatment). Chow-fed mice were individually housed in metabolic cages for the measurement of (H) voluntary running on an exercise wheel, (I) energy expenditure, and (J) respiratory exchange ratio (n = 14 per group), $^{*}p < 0.05$, *Them2*$^{-/-}$ versus *Them2*$^{+/+}$; $^{\dagger}p < 0.05$ with running wheel versus without running wheel. Data are mean ± SEM. Statistical analyses were conducted using the Student t test or two-way ANOVA with repeated measures, where appropriate. HSP90, heat shock protein 90; p-Akt, phosphorylated Akt; S473, serine 473

of *A-Them2*$^{-/-}$ mice (Figure 2B,D), which also lack Them2 expression in BAT, but were not protected against HFD-induced obesity and IR. We used a gain-of-function approach to confirm the metabolic contribution of skeletal muscle Them2: Adeno-associated virus serotype 8 (AAV8)/muscle creatine kinase (Mck)–driven rescue of Them2 expression in skeletal muscle of global *Them2*$^{-/-}$ mice (Figure S3C) resulted in higher body weights than AAV8-Mck-LacZ–treated *Them2*$^{-/-}$ mice (Figure S3D) with increases in both lean and fat mass (Figure S3E). Taken together, these results indicate that skeletal muscle Them2 plays a central role in the development of obesity and IR in response to HFD feeding.

To investigate mechanisms of improved glucose homeostasis in HFD-fed *S-Them2*$^{-/-}$ mice, we performed hyperinsulinemic-euglycemic clamp studies. *S-Them2*$^{-/-}$ mice exhibited higher glucose infusion rates than controls (Figure 3A), as well as increased rates of glucose uptake in skeletal muscle (Figure 3B). By contrast, basal and clamped hepatic glucose production rates were not influenced by skeletal muscle expression of Them2 (Figure 3C). In keeping with these findings, there were marked increases in Akt phosphorylation by 2.5-fold in skeletal muscle of HFD-fed *S-Them2*$^{-/-}$ mice compared to controls in response to acute administration of insulin (Figure 3D). These results reveal that Them2 in skeletal muscle promotes whole-body glucose intolerance by reducing skeletal muscle insulin sensitivity.

Them2 controls rates of FAO in skeletal muscle

We next examined the role of Them2 in the regulation of FAO in skeletal muscle. Rates of FAO in skeletal muscle homogenates were increased in both *Them2*$^{-/-}$ (Figure 4A) and *S-Them2*$^{-/-}$ mice (Figure 4B). Muscle strips isolated from *Them2*$^{-/-}$ mice exhibited increased oxidation rates of palmitate from endogenous (pulsed) palmitate, but not from exogenous (chase) sources (Figure 4C).

To gain mechanistic insights, we performed immunoblotting analyses of gastrocnemius muscle samples to assess the expression of key proteins that control FAO. In contrast to chow-fed control mice, HFD-fed controls exhibited 2-fold increases in Them2 expression levels (Figure 4D,E). Suggestive of its role in regulating Them2 expression in skeletal muscle, the Them2-interacting protein, PC-TP (phosphatidylcholine transfer protein),[2,5] was reduced in chow-fed *S-Them2*$^{-/-}$ mice relative to controls (Figure 4D,E). Although phosphorylation of adenosine monophosphate-activated protein kinase (AMPK; threonine 172), which increases FAO rates in skeletal muscle, showed no genotype-dependent changes, chow-fed *S-Them2*$^{-/-}$ mice exhibited reduced phosphorylation of acetyl-CoA carboxylase (ACC) compared to control mice (Figure 4D,E). Phosphorylated ACC is inactive, whereas dephosphorylated ACC enhances conversion of acetyl-CoA to malonyl-CoA, and this, in turn, reduces FAO owing to inhibition of carnitine palmitoyltransferase I (CPT1).[11] Although CPT1 protein expression was not altered in chow-fed mice, relative mRNA expression of CPT1 was reduced in skeletal muscle of chow-fed *S-Them2*$^{-/-}$ mice (Figure S4A). Notwithstanding the absence of changes in ACC phosphorylation in HFD-fed mice, increased CPT1 protein expression in HFD-fed *S-Them2*$^{-/-}$ mice suggests increased use of mitochondrial fatty acid in skeletal muscle of *S-Them2*$^{-/-}$ mice. Consistent with a lack of effect of Them2 expression on oxidation rates of exogenous fatty acids, we observed no changes in skeletal muscle expression of CD36, which mediates myocellular fatty acid uptake.

To further ascertain mechanisms underlying increased skeletal muscle FAO, we surveyed the expression of genes that control lipid and glucose homeostasis. Expression of these genes was largely unchanged either in chow-fed (Figure S4A,B) or HFD-fed mice (Figure S4A,B), suggesting that increased FAO in *S-Them2*$^{-/-}$ mice was not compensated by fatty acid uptake or use of glucose.

In keeping with increased rates of FAO, total fatty acyl-CoAs in skeletal muscle of chow-fed *S-Them2*$^{-/-}$ mice tended to decrease (Figure 4F), but without changes in individual molecular species (Figure S5A). The absence of a change in fatty acyl-CoAs following HFD feeding is likely attributable to abundant cellular nutrients in the setting of overnutrition. Acetyl-carnitine forms when acetyl-CoA concentrations exceed what is required by the tricarboxylic acid cycle and is an essential molecule for maintaining metabolic flexibility and insulin sensitivity.[12] Skeletal muscle of *S-Them2*$^{-/-}$ mice exhibited increased acetyl-carnitine concentrations for mice fed an HFD (Figure 4G), without discernible changes in individual acyl-carnitine molecular species (Figure S5B). These results are consistent with increased FAO rates in skeletal muscle lacking Them2 even after HFD feeding. Free fatty acid concentrations were higher in skeletal muscle of *S-Them2*$^{-/-}$ mice (Figure 4H), with increases being most pronounced in short-chain molecular species fatty acids (Figures 4I and S5C,D), which can influence glucose homeostasis.[13]

To gain further insight into Them2 metabolic regulation in skeletal muscle, we conducted targeted metabolomics profiling for 216 predefined polar metabolites. Unsupervised clustering of differences identified both diets and genotypes (Figure 4J). Metabolic pathway analysis identified significant alterations in *S-Them2*$^{-/-}$ mice fed either chow (Figure S6A) or a HFD (Figure S6B). Of these, amino acid metabolism and the PPP were affected by skeletal muscle Them2 expression in mice fed both diets.

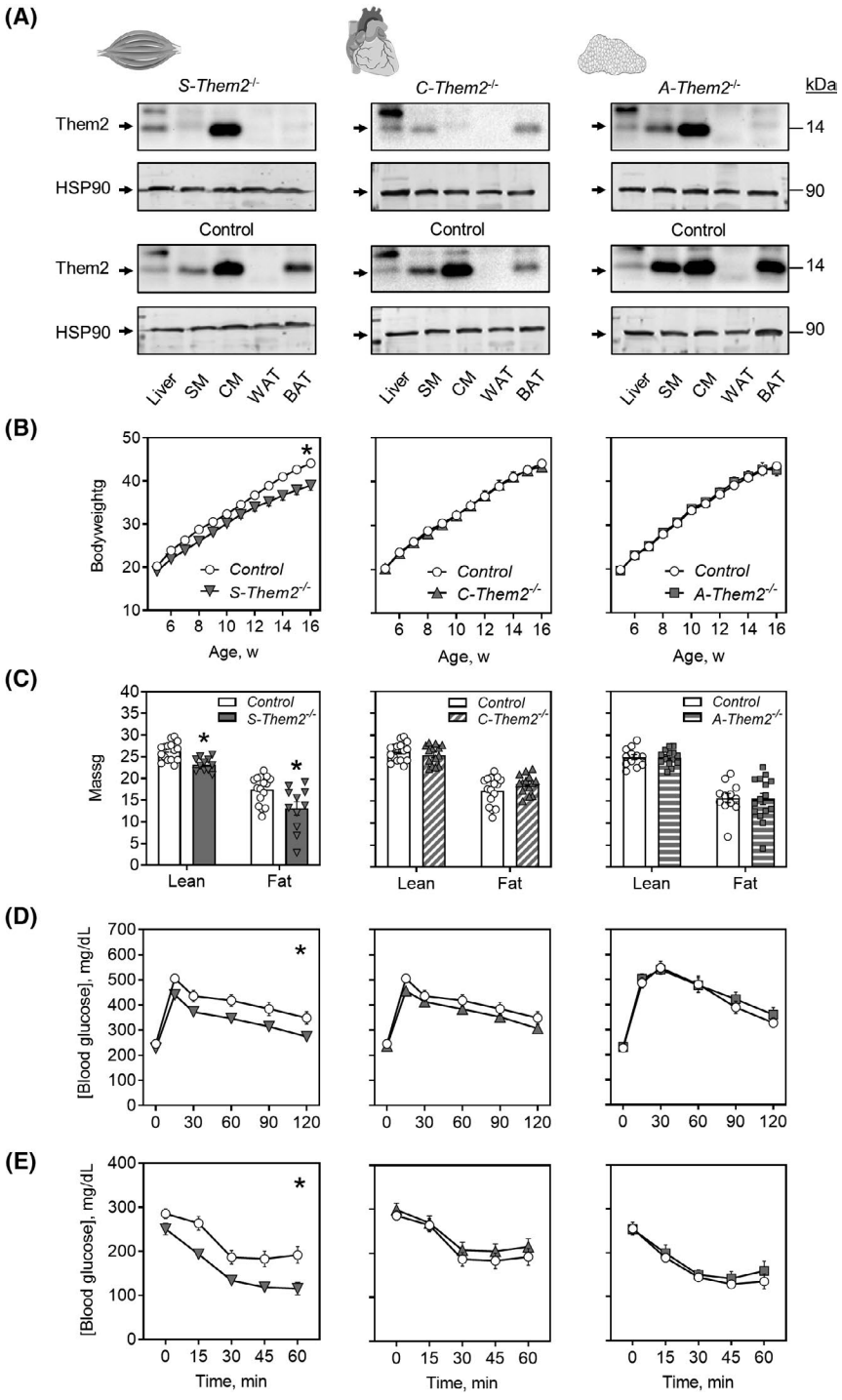

FIGURE 2 Selective deletion of Them2 in skeletal muscle reduces weight gain and improves glucose homeostasis and insulin sensitivity in HFD-fed mice. Male C57BL/6J mice with (left to right) skeletal muscle deletion of Them2 (*S-Them2*$^{-/-}$, *Them2*$^{Flox/Flox}$ × *Myf5-Cre*$^{Tg/0}$); cardiac muscle deletion of Them2 (*C-Them2*$^{-/-}$, *Them2*$^{Flox/Flox}$ × *Myh6-Cre*$^{Tg/0}$); and adipose tissue deletion of Them2 (*A-Them2*$^{-/-}$, *Them2*$^{Flox/Flox}$ × *Adipoq-Cre*$^{Tg/0}$). (A) Immunoblotting analysis of Them2 with HSP90 as a loading control. Mice were fed a HFD (H) from 5 to 17 weeks. (B) Body weights and (C) lean and fat mass (determined at 16 weeks). Tolerance tests to (D) glucose and (E) insulin were conducted between 16 and 17 weeks of age. (B-E) Data are mean ± SEM; n = 6–18 per group. Statistical analyses were conducted using two-way ANOVA with repeated measures (B,D,E) or the Student t test (C); *$p < 0.05$ compared to control. CM, cardiac muscle; HSP90, heat shock protein 90; SM, skeletal muscle

Absence of Them2 in skeletal muscle increased tissue concentrations of nine amino acids in chow-fed mice (Figure S7A), whereas it decreased concentrations of four amino acids in HFD-fed mice (Figure S7B). For both diets, ablation of Them2 mainly altered branched-chain amino acids. Branched-chain amino

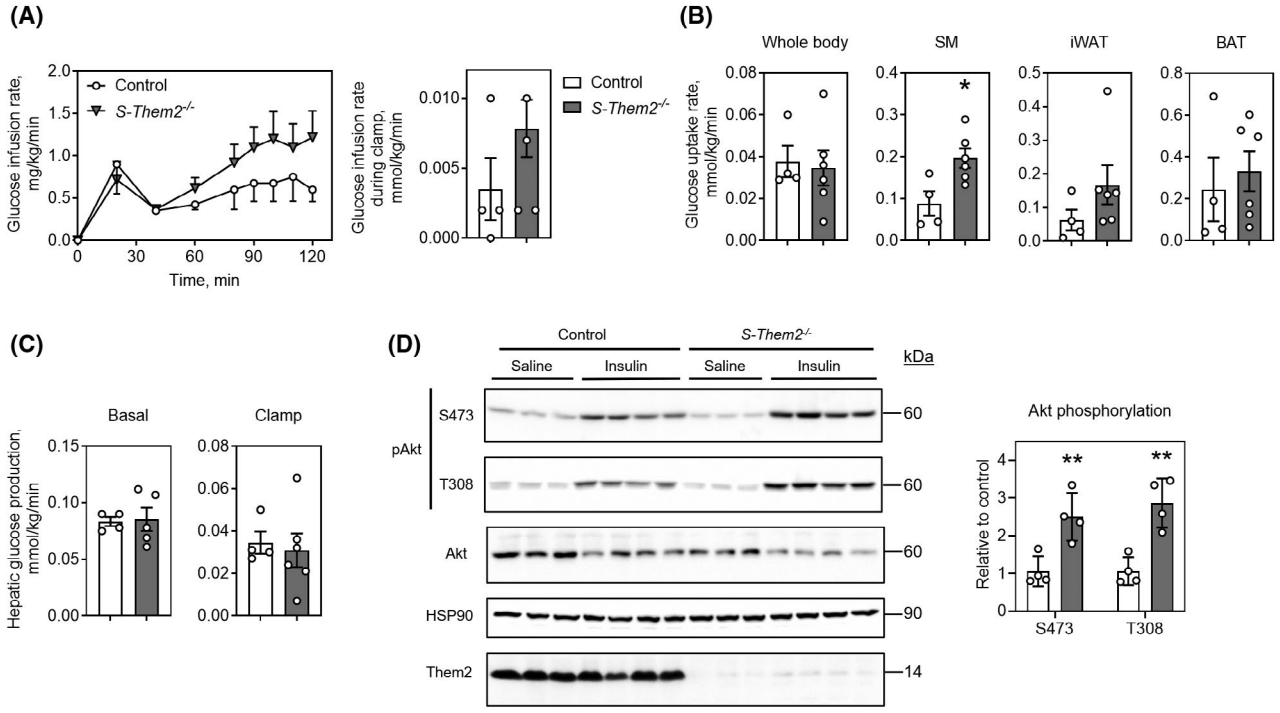

FIGURE 3 Increased glucose uptake and improved insulin sensitivity in skeletal muscle of HFD-fed *S-Them2*$^{-/-}$ mice. Hyperinsulinemic-euglycemic clamp studies were performed in mice fed a HFD for 12 weeks. (A) Time course of glucose infusion rates, with bar plot presenting glucose infusion rates during the clamp. (B) Glucose uptake rates were measured for whole-body, gastrocnemius muscle (skeletal muscle, SM), inguinal white adipose tissue (iWAT), and BAT. (C) Hepatic glucose production rates measured under basal and clamp conditions (*S-Them2*$^{-/-}$, $n = 6$; control, $n = 4$). (D) Gastrocnemius muscle samples were collected from mice fed a HFD for 5–17 weeks, 10 min after IP injection with saline or insulin (10 mU/g), and subjected to immunoblotting analysis ($n = 4$ mice per group). $^{**}p < 0.01$, *S-Them2*$^{-/-}$ versus control. Statistical analyses were conducted using the Student *t* test. Data are mean ± SEM. HSP90, heat shock protein 90; p-Akt, phosphorylated Akt; S473, serine 473; T308, threonine 308

acids promote protein and glutamine synthesis in skeletal muscle, and increased levels of branched-chain amino acids correlate with improvements in glucose metabolism.[14] Therefore, increased levels of branched-chain amino acids in muscle of chow-fed *S-Them2*$^{-/-}$ mice may have contributed to protection against diet-induced IR.

Whereas most metabolites in the PPP pathway were unchanged in both chow diet and in the HFD (Figure 4K), ribose 5-phosphate was significantly reduced in chow-fed mice and both ribulose 5-phosphate and ribose 5-phosphate were increased in skeletal muscle of HFD-fed *S-Them2*$^{-/-}$ mice, suggestive of up-regulation of this pathway by overnutrition in the absence of Them2. The PPP generates reducing equivalents to prevent oxidative stress,[15] and its up-regulation may have contributed to improved insulin sensitivity by clearing excess glucose-induced reactive oxygen species in skeletal muscle. Them2 expression in skeletal muscle did not influence the activity of superoxide dismutase (Figure S8A) or thiobarbituric acid reactive substances (Figure S8B), suggesting that ablation of Them2 did not promote oxidative stress in skeletal muscle. Taken together, these studies revealed that Them2 in skeletal muscle modulates tissue concentrations of short-chain fatty acids, branched-chain amino acids, and PPP metabolites, each of which may play a role in the regulation of glucose homeostasis.

Them2 limits use of fatty acid in cultured myotubes

To assess whether Them2 alters use of mitochondrial fatty acid in skeletal muscle, we used C2C12 myotubes that were transduced to overexpress mouse Them2. AAV8-Them2 transduction increased Them2 expression by 1.5-fold (Figure 5A). Although increased Them2 expression did not alter myotube diameter or fusion index,[16] AAV8-Them2–treated myotubes exhibited greater myotube areas, suggesting that Them2 expression enhances myotube elongation (Figure 5B,C). Mitochondrial respiratory function in myotubes was determined as a surrogate for FAO. Oxygen consumption rate (OCR) of myotubes was measured following additions of palmitate and the CPT1 inhibitor, etomoxir. Oligomycin, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), and antimycin A plus rotenone were sequentially added to each well during the monitoring of OCR. Oligomycin inhibits

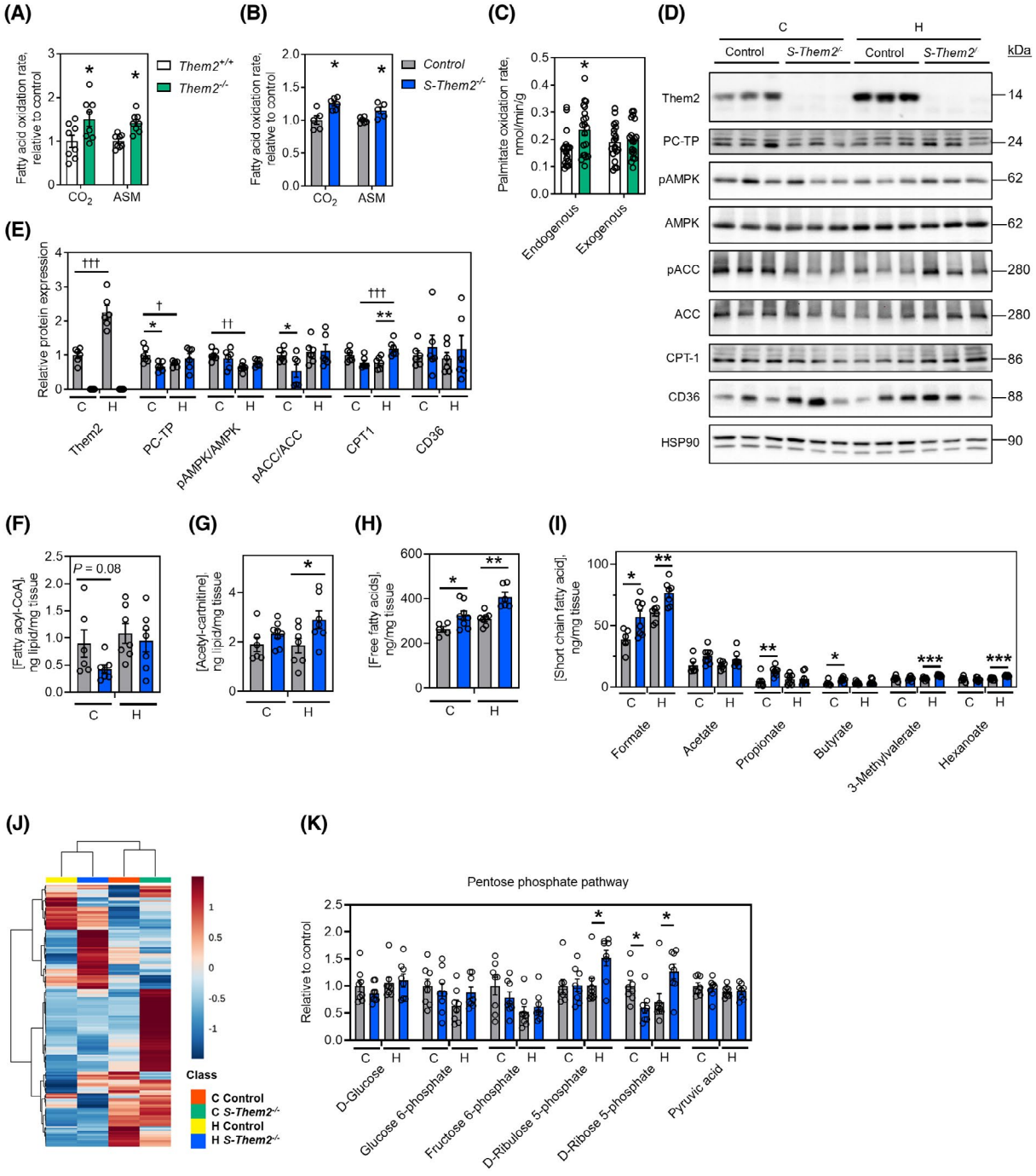

FIGURE 4 Increased oxidation of endogenous fatty acids and associated metabolic changes in skeletal muscle of *S-Them2*$^{-/-}$ mice. FAO rates were assessed according to degradation rates of [^{14}C]-palmitate into acid-soluble metabolites [^{14}C]-labeled (ASM) and CO_2 in homogenized gastrocnemius (skeletal) muscle from chow-fed (A) global *Them2*$^{-/-}$ and (B) *S-Them2*$^{-/-}$ mice (n = 8 per group). (C) FAO rates were measured in isolated soleus muscle strips from chow-fed mice using a using [^{3}H]-palmitate in a pulse-chase design that differentiated between endo- and exogenous fatty acids (n = 10 per group). (D,E) Gastrocnemius samples were collected from chow-fed mice (C) or HFD (H) for 12 weeks. (D) Immunoblotting analyses and (E) corresponding densitometry of proteins that regulate fatty acid metabolism. Skeletal muscle concentrations of (F) fatty acyl-CoAs, (G) acetyl-carnitine, (H) free fatty acids, and (I) short-chain fatty acids (n = 6–8 per group). (J) Unsupervised clustering of data obtained by targeted profiling of 216 polar metabolites in gastrocnemius muscle. (K) Intermediates of the PPP (n = 8 per group). Statistical analyses were conducted using the Student t test; *p < 0.05; **p < 0.01; ***p < 0.001, *S-Them2*$^{-/-}$ versus control. $^{\dagger}p$ < 0.05; $^{\dagger\dagger}p$ < 0.01; $^{\dagger\dagger\dagger}p$ < 0.001, chow versus HFD. Data are mean ± SEM. HSP90, heat shock protein 90; pACC, phosphorylated ACC; pAMPK, phosphorylated AMPK

ATP synthesis and suppresses respiratory chain function. FCCP is an ionophore that disrupts membrane potential, leading to rapid oxygen consumption. Antimycin A and rotenone are respiratory chain complex inhibitors. Myotubes that overexpressed Them2 also exhibited higher rates of OCR during both basal and maximal

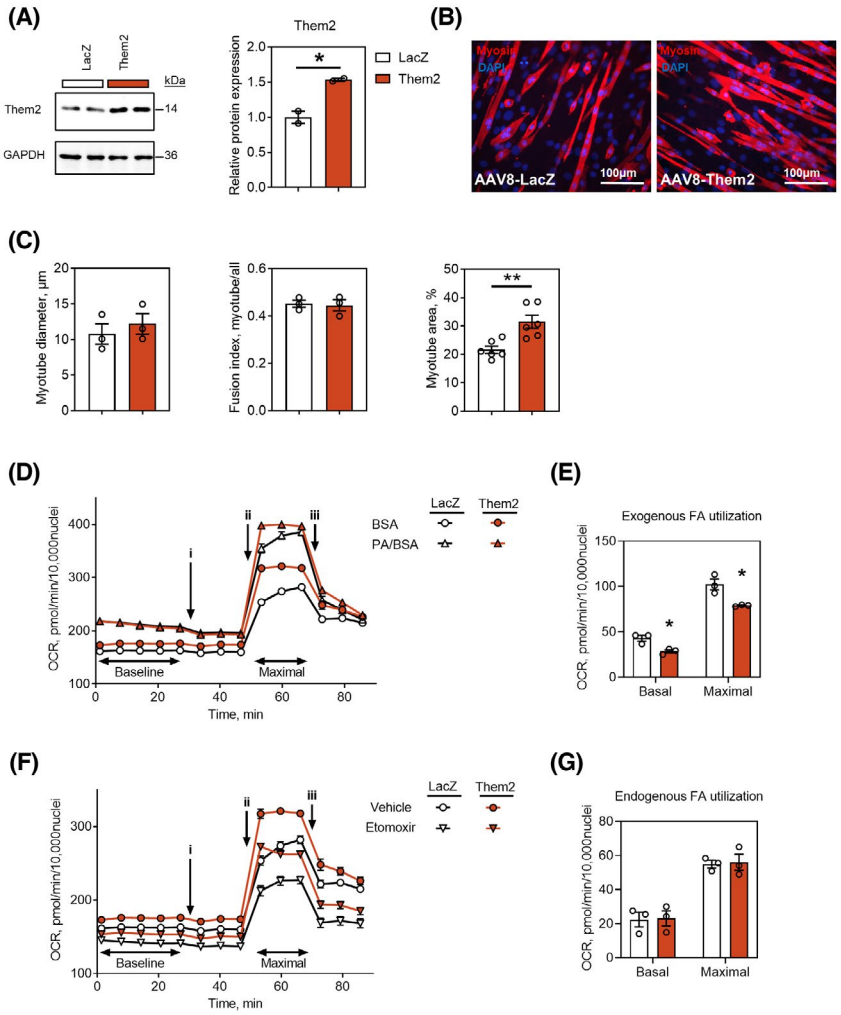

FIGURE 5 Them2 regulates use of myocellular fatty acid. Cultured C2C12 myotubes were transduced with 1×10^5 GC AAV8-Mck-LacZ or AAV8-Mck-Them2. (A) Immunoblotting analysis of Them2, with GAPDH used to control for unequal loading. (B) Representative images of transduced C2C12 myotubes. Cells were fixed and immunostained with antimyosin antibody (red) to reveal sarcomeres. Cell nuclei were stained with DAPI (blue). (C) Myotube area, diameter, and fusion index were evaluated using ImageJ software (National Institutes of Health, Bethesda, MD). OCRs of AAV8-transduced C2C12 myotubes were measured by Seahorse XFe96 following the sequential injections of (i) oligomycin, (ii) FCCP, and (iii) rotenone/antimycin A. (E) OCR values were in the presence of BSA-treated and palmitic acid (PA) plus BSA. (F) Use of exogenous fatty acids (FA) was determined by subtracting OCR values in the presence of BSA alone from PA/BSA. (F) OCR values were measured in the presence of the CPT1 inhibitor, etomoxir, or vehicle (DMSO). (G) Use of endogenous FA was determined by subtracting OCR in the presence of etomoxir from vehicle. Data represent combined results from three independent experiments. Bar plots present FA use during the baseline (basal) and the three time points following the addition of FCCP (maximal). Statistical analyses were conducted using the Student *t* test; $*p < 0.05$; $**p < 0.01$. Data are mean ± SEM. GAPDH, glyceraldehyde 3-phosphate dehydrogenase

respiration (Figure 5D). Of note, when palmitate was made available, there were no differences in basal or maximal respiration (Figure 5D), with differences between bovine serum albumin (BSA) treatment alone and palmitate plus BSA exposure signifying oxidation of extracellular palmitate. As evidenced by higher OCR values following treatment with BSA alone, AAV8-Them2–transduced myotubes used less extracellular palmitic acid (Figure 5E).

A reduction in OCR after etomoxir treatment under substrate-limited conditions provides a measure of intracellular FAO (Figure 5F), which was not influenced by Them2 expression (Figure 5G). Taken together, these results suggest that Them2 regulates use of extracellular fatty acid through its control of the intracellular fatty acid pool that is available for oxidation.

Skeletal muscle Them2 expression enables HFD-fed mice to exceed a body-weight threshold value for HS and metabolic dysfunction

In humans, HS is largely a function of adipose tissue IR and serves an important barometer of metabolic dysfunction, which becomes fully established at a threshold level of 5.5% liver fat.[17] Although HFD-fed mice are widely used in studies of NAFLD, a threshold

value for pathogenic intrahepatic TG accumulation and its relationship to body composition remains open for systematic exploration. To enable accurate weight-based comparisons between *S-Them2*$^{-/-}$ and control mice, 87 C57BL/6J male mice were fed a HFD for 12 weeks. Body weights exhibited a normal distribution, ranging from 31.1 to 52.2 g (mean ± SEM; 42.3 ± 0.5 g; median, 42.5; Figure 6A). Both lean and fat mass increased linearly as functions of body weight (Figure 6B), and fat mass was a linear function of lean mass (Figure 6C). By contrast, liver weight exhibited two very distinct linear relationships to body weight (Figure 6D): Up to a body weight threshold of 41.6 g (corresponding to fat mass of 15.6 g), there was a relatively flat slope of liver weight versus body weight (0.031 g liver/g body weight). In excess of 41.6 g, the slope was 4.3-fold steeper (0.134 g liver/g body weight). Additionally, there was a similar biphasic dependence of liver weight on fat (Figure 6E), but not lean, weight of mice (Figure 6F).

HS exhibited a biphasic relationship to body weight, with a threshold body weight of 41.6 g corresponding to 31% HS (Figure 6G). By contrast, both liver weights (Figure 6H) and hepatic TG concentrations (Figure 6I) were linearly correlated with histological steatosis. The body weight threshold of 41.6 g also discriminated plasma concentrations of free fatty acids, glucose, and cholesterol (Figure 6J), as well as hepatic mRNA expression levels of specific genes that govern the metabolism of fatty acids (alternative oxidase [*Aox*] and *CD36*) and lipid droplets (cell death activator [*Cidea*]; Figure 6K). Indicative that the 41.6-g weight threshold for HS reflected changes in skeletal muscle dysfunction, expression of *Acsls* and *Acots* was significantly altered in skeletal muscle of mice exceeding this body weight (Figure 6L). Additionally, five myokine genes were down-regulated above this weight threshold, as was peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1a*), which controls mitochondrial biogenesis in skeletal muscle (Figure 6M). Taken together, these findings reveal two distinct phases in the development of HS and metabolic dysfunction in HFD-fed C57BL/6J mice, with a threshold of 31% at a body weight of 41.6 g. After 12 weeks of HFD feeding, body weights of most (80%) *S-Them2*$^{-/-}$ mice fell below the 41.6-g threshold, compared with 36% of controls (Figure 6N).

In keeping with reduced weight and improved glucose homeostasis, HFD-fed *S-Them2*$^{-/-}$, but not *L-Them2*$^{-/-}$[6] or *C-Them2*$^{-/-}$, mice were also protected against HS, as evidenced by histopathology (Figure 7A) and hepatic TG concentrations (Figure 7B), as was previously observed in *Them2*$^{-/-}$ mice.[3] Pyruvate tolerance tests revealed reduced hepatic glucose production only in *S-Them2*$^{-/-}$ mice (Figure 7C), as was also observed in global *Them2*$^{-/-}$ mice.[3] There were no differences in plasma activities of aspartate aminotransferase, alanine aminotransferase, or creatine kinase or in insulin concentrations (Figure S9A,B). Of note, for mice under the 41.6-g body-weight threshold, hepatic TG concentrations were also reduced in HFD-fed *S-Them2*$^{-/-}$ mice compared with controls (Figure 7D). These findings suggested that metabolic improvements in *S-Them2*$^{-/-}$ mice were, in part, attributable to reduced body-weight gain because most mice lacking Them2 did not achieve a body-weight threshold for the development of more-severe HS, but, importantly, were also attributable, in part, to expression of skeletal muscle Them2 per se.

Skeletal muscle Them2 expression promotes HS by suppressing VLDL secretion

We next explored the mechanisms underlying skeletal muscle Them2-mediated development of HS. We initially examined evidence for increased rates of hepatic FAO. Fasting concentrations of ß-hydroxybutyrate were reduced in HFD-fed control mice compared with chow-fed controls, but this was not observed for *S-Them2*$^{-/-}$ mice, in which levels remained unchanged by HFD-feeding and were significantly higher than in HFD-fed control mice (Figure S9C). This suggested that the absence of Them2 in skeletal muscle might be associated with increase rates of FAO in liver. However, direct measurement of hepatic FAO in HFD-fed *S-Them2*$^{-/-}$ mice did not reveal increases (Figure 7E), suggesting that differences in ß-hydroxybutyrate may instead have reflected Them2-mediated changes in rates of ketone body use in skeletal muscle. Chow-fed, but not HFD-fed, *S-Them2*$^{-/-}$ mice exhibited increased fasting plasma concentrations of free fatty acids (Figure S9D), which argues against reduced hepatic uptake of fatty acids as a mechanism by which *S-Them2*$^{-/-}$ mice were protected against steatosis. Plasma concentrations of TGs, total and free cholesterol, as well as phospholipids were unaffected by skeletal muscle Them2 expression in either chow- or HFD-fed mice (Figure S9D), and despite dramatic reductions in HS, RNA-sequencing analysis of liver identified only a limited number of genes with altered expression (Figure S10A). Among these was the HS marker, *Cidea*, which was markedly down-regulated, suggesting reductions in TG storage. This was also implicated by pathway analysis, which identified increased degradation of hepatic TGs as the most up-regulated pathway (Figure S10B). In keeping with this possibility, *S-Them2*$^{-/-}$ mice exhibited increased rates of VLDL TG secretion (Figure 7F). Taken together, these findings suggest that increased demand by skeletal muscle for fatty acids in the form of secreted hepatic TGs may have explained the reduced steady-state concentrations in the liver.

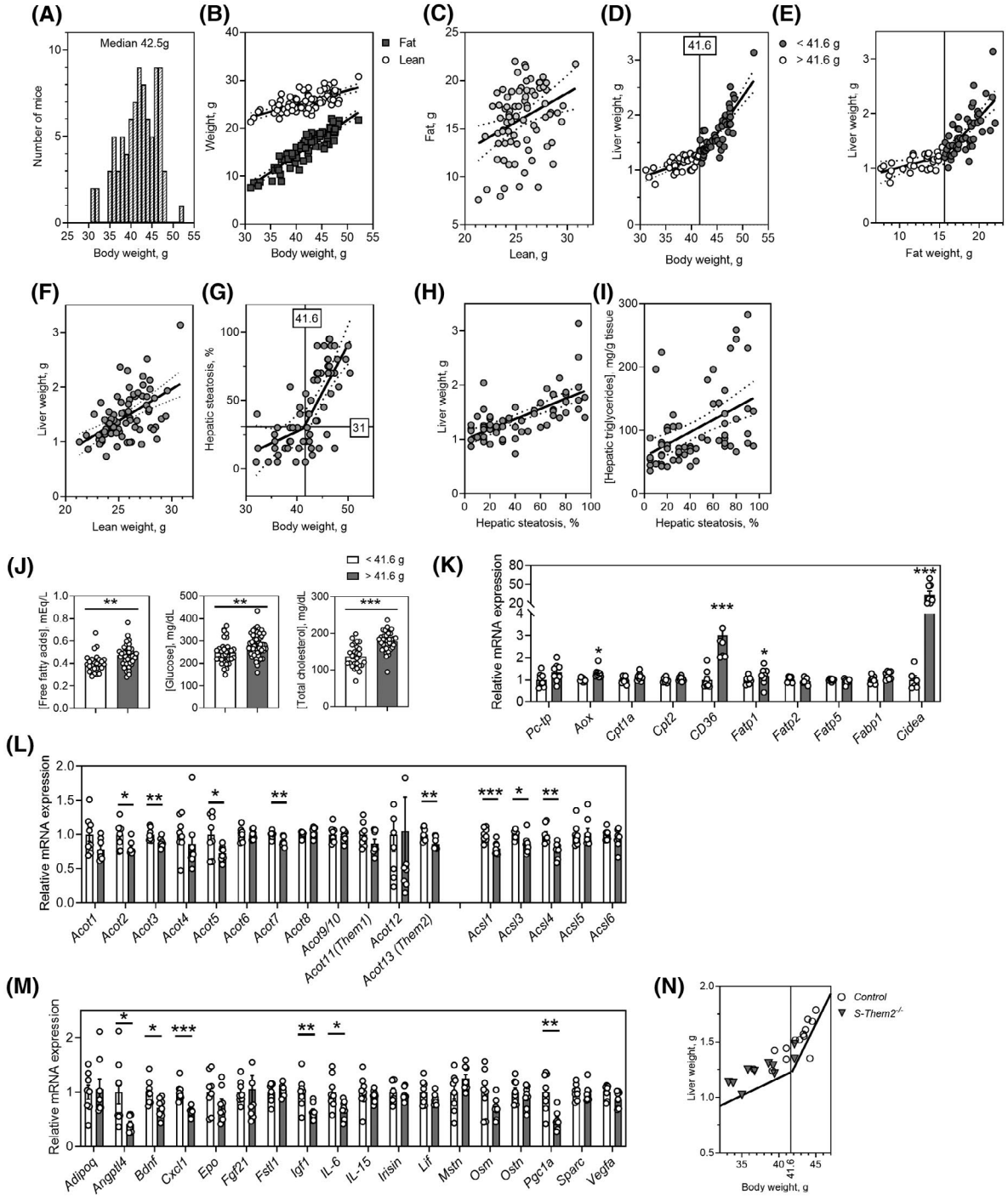

FIGURE 6 Skeletal muscle Them2 expression enables HFD-fed mice to exceed a threshold value for accelerated HS and metabolic dysfunction. C57BL/6J mice were fed a HFD for 12 weeks. (A) Body-weight distribution (n = 84) and (B) fat and lean weights. (C) Linear relationship between fat and lean weights. (D,E) Biphasic relationships between liver weight and (D) body weight and (E) fat weight (n = 64). Threshold value of 41.6 g of body weight was determined by segmental linear regression. (F) Linear relationship between liver weight and lean weight. (G) Biphasic relationship between HS determines histologically and body weight. (H) Linear relationship of liver weight to HS. (I) Linear relationship of hepatic TG concentrations to HS (n = 64). (J) Plasma concentrations of free fatty acids, glucose, and total cholesterol. (K) Hepatic mRNA expression levels of selected genes that govern the metabolism of fatty acids and lipid droplets (n = 8 per group). Relative mRNA expression in skeletal muscle (L) of genes that regulate use of fatty acids and (M) myokines. (N) Body and liver weights of HFD-fed *S-Them2*$^{-/-}$ and control mice (n = 10–14 per group) relative to the biphasic linear regression derived for HFD-fed C57BL/6J mice (D). Statistical analyses were conducted using the Student t test; *p < 0.05; **p < 0.01; ***p < 0.001, body weight <41.6 g versus body weight >41.6 g. Data are mean ± SEM. *Angptl4*, angiopoietin-like 4; *Bdnf*, brain-derived neurotrophic factor; *Epo*, erythropoietin; *Fabp*, fatty acid binding protein; *Fatp*, fatty acid transport protein; *Fgf21*, fibroblast growth factor 21; *Fstl1*, follistatin-like 1; *Lif*, leukemia inhibitory factor; *Mstn*, myostatin; *Osm*, oncostatin M; *Ostn*, osteocrin; *Pctp*, phosphatidylcholine transfer protein; *Sparc*, secreted protein acidic and rich in cysteine

Muscle Them2 mediates crosstalk between skeletal muscle and the liver

Last, we examined evidence of regulatory crosstalk between muscle and liver that may have also contributed to protection against HS and metabolic dysfunction in *S-Them2*$^{-/-}$ mice. Conditioned medium from C2C12 myotubes transduced to overexpress Them2 suppressed basal and maximal OCR values in Hepa1-6 cells (Figure 8A). We next assessed for Them2-dependent expression of myokines in skeletal muscle. Ablation of Them2 altered myokine gene expression, depending on dietary conditions (Figure 8B). Overnight fasting induced substantial changes in myokine gene expression in *S-Them2*$^{-/-}$ mice (Figure 8B). Skeletal muscle of chow-fed *S-Them2*$^{-/-}$ mice showed increased expression of insulin-like growth factor 1 (*Igf1*) and *IL-15*, both of which regulate muscle mass,[18,19] whereas *IL-15* also decreases plasma TGs and adipose tissue mass.[20] In HFD-fed mice, *S-Them2*$^{-/-}$ mice exhibited modest increases in chemokine ligand 1 (*CXCL1*), which regulates angiogenesis and fatty acid metabolism.[21] Independent of diet, there was consistent down-regulation in *osteoclin*, which is up-regulated by palmitic acid and promotes IR in muscle.[22–24] Because skeletal muscle of HFD-fed *S-Them2*$^{-/-}$ mice is protected against excess body-weight gain, these alterations in myokine genes might have been secondary to changes in body weight. However, differences in relative mRNA expression were still observed in weight-matched HFD-fed mice, as defined by body weights that fell below the threshold of 41.6 g (Figure 8C): *Myostatin* and *osteoclin* were down-regulated in skeletal muscle of *S-Them2*$^{-/-}$ mice compared with control mice. These results suggested that Them2-mediated control of skeletal muscle myokine gene expression may contribute to crosstalk among skeletal muscle, adipose tissue, and the liver (Figure 8D).

DISCUSSION

This study identified a key role for skeletal muscle Them2 in the regulation of skeletal muscle metabolism, as well as in the pathogenesis of HFD-induced IR and HS in mice. Them2 limits rates of FAO by skeletal muscle, which, in turn, reduces rates of insulin-mediated glucose disposal by reducing insulin-sensitizing metabolites and myokines. This also reduced demand for exogenous fatty acids, leading to the development of HS.

We previously demonstrated that *Them2*$^{-/-}$ mice exhibited reduced plasma free fatty acid concentrations, as well as reduced HS and glucose production.[4,25] However, these effects were not recapitulated in *L-Them2*$^{-/-}$ mice, suggesting that extrahepatic Them2 expression is required in order to reproduce the phenotype in the setting of overnutrition.[6]

Whereas Them2 is most highly expressed in the heart, its deletion from cardiac muscle did not result in discernable differences in organ function or whole-body metabolism. To preserve function, cardiac muscle has extraordinary metabolic flexibility that enables it to adapt to changes in substrate availability.[10] For this reason, we speculate that alterations in this specific enzyme of long-chain fatty acid metabolism would not cause obvious functional or metabolic consequences in *C-Them2*$^{-/-}$ mice. This notion is supported by our observation that Them2 expression in heart of control mice was unaffected by HFD feeding. By contrast, HFD feeding robustly up-regulated Them2 expression in skeletal muscle, suggesting a pathogenic role in skeletal muscle mechanical dysfunction and alterations in metabolism.

As evidenced by reduced rates of glucose uptake during clamp studies and diminished insulin-stimulated Akt phosphorylation, Them2 contributed to the development of skeletal muscle IR, presumably as a reflection of reduced rates of FAO.[26] Previous studies have demonstrated that the accumulation of excess free fatty acids and their derivatives, including long-chain acyl-CoAs, in skeletal muscle are key determinants of IR.[27] Although the absence of a fatty acyl-CoA thioesterase might have been expected to increase skeletal muscle concentrations of fatty acyl-CoAs and decrease concentrations of free fatty acids, we observed the opposite in *S-Them2*$^{-/-}$ mice. However, the preferred substrate of Them2 is long-chain fatty acyl-CoA, and the observed increase in free fatty acid concentrations was mainly attributable to increases in short-chain fatty acids. Short-chain fatty acids are predominantly generated by colonic bacteria and are metabolized by entero- and hepatocytes. Short-chain fatty acids are also formed in peroxisomes during shortening of very-long-chain fatty acyl-CoAs that can be hydrolyzed by peroxisomal *Acots* and released into the cytosol.[28] We speculate that increased rates of mitochondrial ß-oxidation in skeletal muscle of *S-Them2*$^{-/-}$ mice were also accompanied by increases in peroxisomal oxidation, which resulted in the production of more short-chain fatty acids. The reduction in skeletal muscle fatty acyl-CoA concentrations most likely reflects their increased rates of oxidation, as evidenced by the reciprocal rise in acetyl-carnitine concentrations.

Short-chain fatty acids influence muscle FAO, glucose uptake, and insulin sensitivity by both direct and indirect mechanisms.[13,29] Of note, acetate and butyrate improve skeletal muscle glucose metabolism and insulin sensitivity.[13] When taken together with observed increases in branched-chain amino acids and PPP metabolites,[14,15] these data suggest that elevated concentrations of short-chain fatty acids in skeletal muscle of *S-Them2*$^{-/-}$ mice contributed to the

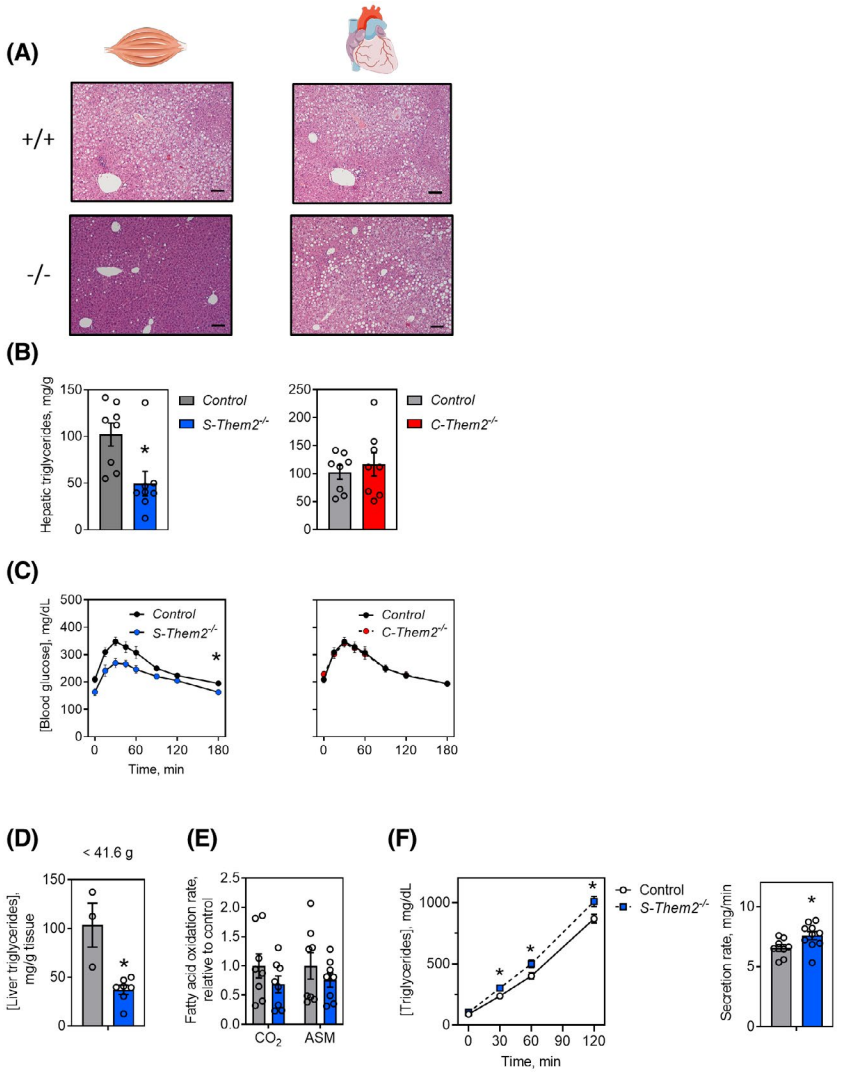

FIGURE 7 Them2 promotes HS by suppressing VLDL secretion. Left to right: Mice with skeletal muscle (*S-Them2*$^{-/-}$) and cardiac muscle (*C-Them2*$^{-/-}$) Them2 deletion were fed a HFD for 12 weeks, and then livers were analyzed for (A) HS by H&E staining (bars represent 100 μm) and (B) hepatic TG concentrations. (C) Pyruvate tolerance tests conducted in HFD-fed mice at 16 weeks of age. Data are mean ± SEM (n = 6–12 per group). Statistical analyses were conducted by the Student t test (B) or two-way ANOVA with repeated measures (C); *p < 0.05 versus control. Data are presented as mean ± SEM. (D) Hepatic TG concentrations of HFD-fed *S-Them2*$^{-/-}$ and control mice with body weights of <41.6 g (n = 3–7 per group). (E) FAO rates in livers of HFD-fed *S-Them2*$^{-/-}$ mice (n = 8 per group). (F) Rates of TG hepatic secretion in HFD-fed mice (n = 8–10 per group). Statistical analyses were conducted using the Student t test or two-way ANOVA with repeated measures, where appropriate. *p < 0.05, *S-Them2*$^{-/-}$ versus control. Data are mean ± SEM. ASM, acid-soluble metabolites [^{14}C]-labeled; H&E, hematoxylin and eosin

improved insulin sensitivity observed in the setting of overnutrition.

Although the reduction in lean mass of *S-Them2*$^{-/-}$ mice at the thermoneutrality was similar as at room temperature, there was less reduction in fat mass. This may have been because Them2 in BAT also plays a role in HFD-induced obesity by adaptive thermogenesis.[4] Because fat mass is tightly correlated with lean mass in HFD-fed mice, we further postulate that reduction in lean body mass attributable to loss of skeletal muscle Them2 also exerted metabolic control through associated decreases in adipose tissue.

Unlike humans,[17] C57BL/6J mice exhibited remarkable accelerations in hepatic TG accumulation at a discrete threshold of body weight (41.6 g). Because fat weight is tightly correlated with lean weight in HFD-fed mice, we inferred that reduced HS in *S-Them2*$^{-/-}$ mice was attributable, at least in part, to the reduced body-weight gain, such that these mice rarely exceeded body weights of 41.6 g. Nevertheless, hepatic TG concentrations were still reduced in HFD-fed *S-Them2*$^{-/-}$ mice compared with HFD-fed control mice weighing <41.6 g, revealing that Them2 expression per se is sufficient to regulate hepatic lipid metabolism.

A potential mechanism whereby skeletal muscle Them2 directly controls HS is organ crosstalk through the secretion of myokines, which are bioactive proteins that regulate organ function by autocrine, paracrine,

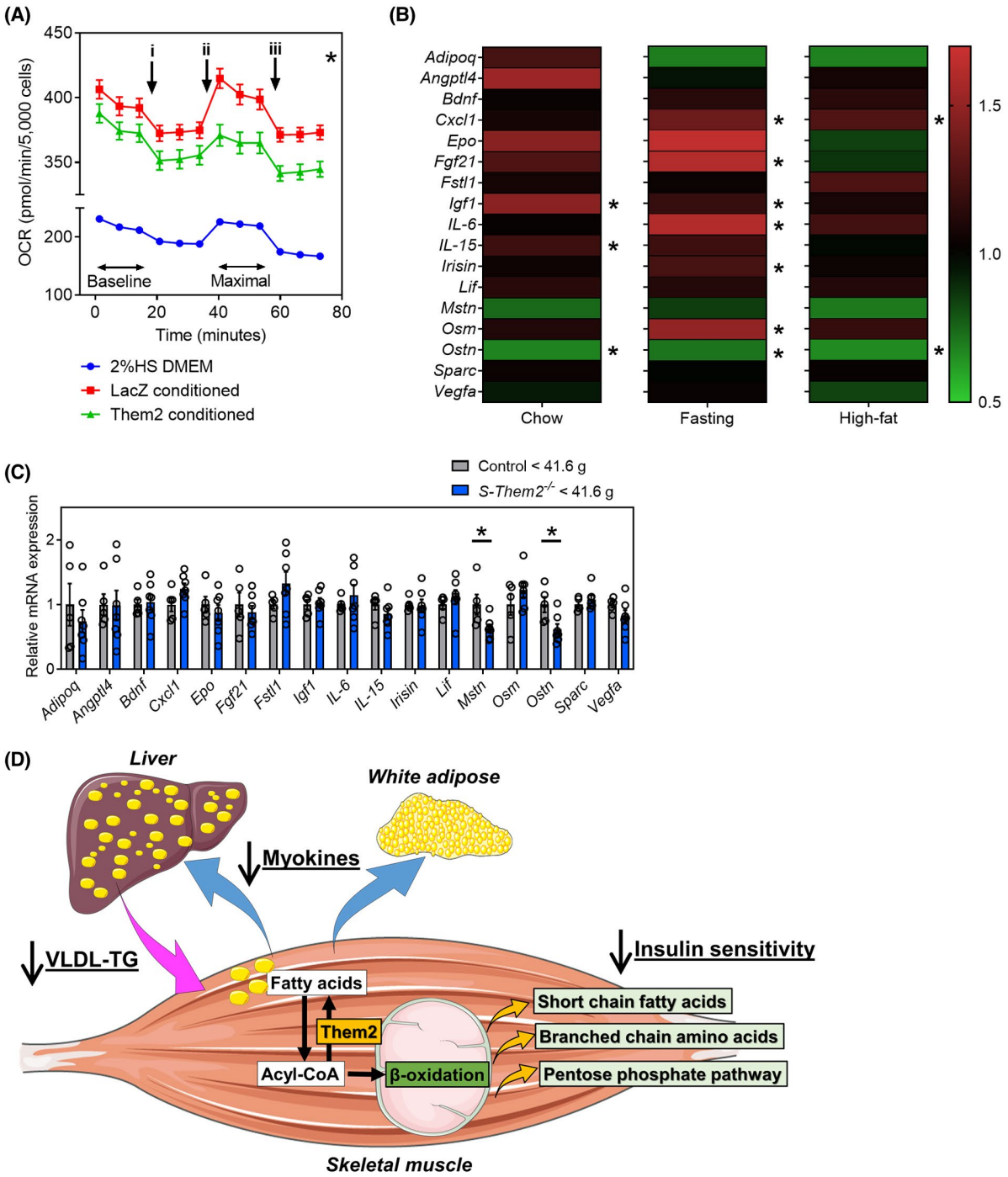

FIGURE 8 Skeletal muscle Them2 mediates crosstalk between muscle and the liver. (A) OCRs of Hepa1-6 cells cultured with conditioned medium. Sequential additions of oligomycin (i), FCCP (ii), and rotenone/antimycin A (iii) are indicated. Conditioned medium was collected from C2C12 myotubes reconstituted with AAV-LacZ or AAV-Them2. Data represent combined results from three independent experiments. (B) Relative mRNA expression of myokine genes in soleus (skeletal) muscle dissected from mice fed a chow diet or a HFD for 12 weeks. Myokine genes were also analyzed in chow-fed mice after 18-h fasting. Heatmap represents relative values to controls for the same diet. (C) Relative mRNA expression of myokine genes in body-weight–matched (<41.6 g) mice fed a HFD for 12 weeks (n = 5–7 per group). *p < 0.05, *S-Them2*$^{-/-}$ versus body-weight–matched (<41.6 g) control. (D) Schematic summary of metabolic role of Them2 in skeletal muscle and its influence on the development of HS. Them2 in skeletal muscle decreases dependence on FAO to produce ATP, therefore using less circulating free fatty acids. Them2 limits FAO in skeletal muscle, consequently altering the production of short-chain fatty acids, branched-chain amino acids, and the PPP. These alterations are most evident in the setting of overnutrition and limit insulin sensitivity in skeletal muscle. Them2 also regulates muscle-to-organ crosstalk by multiple myokine genes, depending on dietary conditions; therefore, Them2 may coordinate the metabolism of fatty acids in skeletal muscle, adipose tissue, and the liver. Statistical analyses were conducted using the Student *t* test (B,C) or two-way ANOVA with repeated measures (A). Data are mean ± SEM. 2%HS, 2% horse serum; *Angptl4*, angiopoietin-like 4; *Bdnf*, brain-derived neurotrophic factor; *Epo*, erythropoietin; *Fgf21*, fibroblast growth factor 21; *Fstl1*, follistatin-like 1; *Lif*, leukemia inhibitory factor; *Mstn*, myostatin; *Osm*, oncostatin M; *Ostn*, osteocrin; *Sparc*, secreted protein acidic and rich in cysteine

and endocrine actions.[30] Deletion of Them2 in skeletal muscle induced expression of several myokines. Skeletal muscle of chow-fed *S-Them2*$^{-/-}$ mice showed significantly higher gene expression of *Igf1* and *IL-15*, which regulate muscle hypertrophy.[18,19] *IL-15* also decreases lipid deposition in preadipocytes and reduces WAT mass.[20] In the setting of overnutrition, there was also a modest increase in skeletal muscle expression of *CXCL1* in *S-Them2*$^{-/-}$ mice. *CXCL1* is the functional homologue to human *IL-8*,[31] and it plays a role in angiogenesis and neutrophil chemoattractant activity.[32] Increased expression of *CXCL1* attenuates diet-induced obesity in mice through enhanced FAO in muscle.[21] *Osteoclin* (synonym: *musclin*), which was down-regulated in skeletal muscle of *S-Them2*$^{-/-}$ mice, is a myokine that is controlled by nutritional status and promotes IR. *Musclin* expression inhibits glucose uptake and glycogen synthesis,[2] and *Musclin* mRNA is regulated by the forkhead box O1 transcription factor downstream of the phosphoinositide 3-kinase/*Akt* pathway in response to insulin action.[24] Notwithstanding the suggestive data that control of hepatic nutrient metabolism and VLDL secretion by skeletal muscle Them2 expression occurs through myokines, other circulating factors, such as lipid mediators or exosomes, are alternative mechanisms of control. Further analyses, including plasma proteomics, will likely be required to ascertain the key factors responsible for Them2-mediated organ crosstalk.

Taken together, our findings demonstrate a central role for skeletal muscle Them2 in the control of nutrient metabolism and in the pathogenesis of diet-induced IR and HS. Inhibition of Them2 activity in muscle could offer an attractive strategy for the management of obesity and NAFLD.

ACKNOWLEDGMENT

We thank Dr. Scott Rodeo, Hospital for Special Surgery, for assistance with treadmill experiments. The Weill Cornell Medicine Metabolic Phenotyping Core and the Emory Integrated Lipidomics Core are subsidized by Weill Cornell Medicine and the Emory University School of Medicine, respectively.

CONFLICT OF INTEREST

Nothing to report.

AUTHOR CONTRIBUTIONS

Norihiro Imai designed research studies, conducted experiments, acquired data, analyzed data, and wrote the manuscript. Hayley T. Nicholls conducted experiments and analyzed data. Michele Alves-Bezerra designed research studies and conducted experiments. Yingxia Li acquired data. Anna A. Ivanova conducted experiments and analyzed data. Eric A. Ortlund analyzed data. David E. Cohen designed research studies, analyzed data, and wrote the manuscript.

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How to cite this article: Imai N, Nicholls HT, Alves-Bezerra M, Li Y, Ivanova AA, Ortlund EA, et al. Up-regulation of thioesterase superfamily member 2 in skeletal muscle promotes hepatic steatosis and insulin resistance in mice. Hepatology. 2022;75:154–169. https://doi.org/10.1002/hep.32122