Autophagy deficiency leads to protection from obesity and insulin resistance by inducing Fgf21 as a mitokine

Kook Hwan Kim1,2, Yeon Taek Jeong1,4, Hyunhee Oh3,14, Seong Hun Kim2, Jae Min Cho1, Yo-Na Kim3, Su Sung Kim3, Do Hoon Kim1, Kyu Yeon Hur1, Hyoung Kyu Kim4, TaeHee Ko4, Jin Han4, Hong Lim Kim5, Jin Kim5, Sung Hoon Back7, Masaaki Komatsu8, Hsiuchen Chen9, David C Chan9,10, Morichika Konishi11, Nobuyuki Itoh12, Cheol Soo Choi3,13 & Myung-Shik Lee1,2

Despite growing interest and a recent surge in papers, the role of autophagy in glucose and lipid metabolism is unclear. We produced mice with skeletal muscle–specific deletion of Atg7 (encoding autophagy-related 7). Unexpectedly, these mice showed decreased fat mass and were protected from diet-induced obesity and insulin resistance; this phenotype was accompanied by increased fatty acid oxidation and browning of white adipose tissue (WAT) owing to induction of fibroblast growth factor 21 (Fgf21). Mitochondrial dysfunction induced by autophagy deficiency increased Fgf21 expression through induction of Atf4, a master regulator of the integrated stress response. Mitochondrial respiratory chain inhibitors also induced Fgf21 in an Atf4-dependent manner. We also observed induction of Fgf21, resistance to diet-induced obesity and amelioration of insulin resistance in mice with autophagy deficiency in the liver, another insulin target tissue. These findings suggest that autophagy deficiency and subsequent mitochondrial dysfunction promote Fgf21 expression, a hormone we consequently term a ‘mitokine’, and together these processes promote protection from diet-induced obesity and insulin resistance.

Autophagy is an evolutionarily conserved process that delivers cytoplasmic constituents to lysosomes for degradation of aggregated proteins and recycling of organelles or nutrients1. Autophagy is tightly controlled by amino acid availability, and amino acids produced by autophagy are used for energy production or other purposes in nutrient deficiency. In addition to its role in amino acid metabolism, autophagy is important in whole-body glucose and lipid homeostasis. For instance, mice with a targeted disruption of an autophagy-related gene in pancreatic beta cells have defective insulin release2,3. Conditional knockout of autophagy genes in insulin target tissues has shown that autophagy participates in the differentiation of adipocytes and disposal of excess lipid in a process called ‘lipophagy’4–6. Notably, recent papers have suggested that autophagy deficiency could be an important element in the pathogenesis of insulin resistance and diabetes7–9.

Mitochondrial turnover is crucially controlled by autophagy in a process called mitophagy. Structural or functional abnormalities of mitochondria have been reported in several previous autophagy deficiency models8,10–12, and, similar to autophagy deficiency, mitochondrial dysfunction has been implicated in the pathogenesis of type 2 diabetes and insulin resistance13. Microarray analysis studies have shown that the expression of genes involved in mitochondrial oxidative metabolism is reduced in humans with insulin resistance and diabetes14,15. Furthermore, increased intracellular fat accumulation in the liver and skeletal muscle leads to impaired activity of mitochondrial oxidative phosphorylation (mtOxPhos) and insulin resistance, suggesting that obesity and mitochondrial dysfunction are inter-related. However, contradictory results have also been reported: mtOxPhos activity in skeletal muscle of subjects with diabetes or mice fed a high-fat diet (HFD) was normal or even increased16,17. Notably, it has been shown that mitochondrial flavoprotein apoptosis-inducing factor (Aif1m1)-knockout mice with impaired mtOxPhos activity are resistant to HFD-induced obesity and diabetes18, suggesting that the relationship between mitochondrial function and insulin sensitivity is not straightforward. Muscle-specific mitochondrial transcription factor A (Tfam)-knockout mice also show enhanced glucose tolerance, despite decreased mitochondrial DNA content and function19. Thus, it is not clear whether or not mtOxPhos deficiency contributes to insulin resistance.

1Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea. 2Samsung Advanced Institute for Health Sciences and Technology, Sungkyunkwan University School of Medicine, Seoul, Korea. 3Korea Mouse Metabolic Phenotyping Center, Lee Gil Ya Cancer and Diabetes Institute, Gachon University Graduate School of Medicine, Incheon, Korea. 4National Research Laboratory for Mitochondrial Signaling, Department of Physiology, College of Medicine, Cardiovascular and Metabolic Disease Center, FIRST Mitochondrial Research Group, Inje University, Busan, Korea. 5Integrative Research Support Center, College of Medicine, The Catholic University of Korea, Seoul, Korea. 6Department of Anatomy and Cell Death Disease Research Center, College of Medicine, The Catholic University of Korea, Seoul, Korea. 7School of Biological Sciences, University of Ulsan, Ulsan, Korea. 8Protein Metabolism Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan. 9Division of Biology, California Institute of Technology, Pasadena, California, USA. 10Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California, USA. 11Department of Microbial Chemistry, Kobe Pharmaceutical University, Kobe, Japan. 12Department of Genetic Biochemistry, Kyoto University Graduate School of Pharmaceutical Sciences, Kyoto, Japan. 13Department of Internal Medicine, Gil Medical Center, Gachon University Graduate School of Medicine, Incheon, Korea. 14These authors contributed equally to this work. Correspondence should be addressed to C.S.C. (cschoi@gachon.ac.kr) or M.-S.L. (mslee0923@skku.edu).

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Although several mice have been produced with targeted disruption of autophagy-related genes in beta cells or insulin target tissues, the metabolic impact of autophagy in skeletal muscle—accounting for 80% of whole-body insulin-mediated glucose utilization\(^{20}\)—has not been investigated. Here we have found that autophagy deficiency in skeletal muscle leads to impaired mitochondrial function and subsequent induction of Fgf21, a mitokine that exerts a strong effect on lipid mobilization or utilization and burning of WAT. Consequently, mice with skeletal muscle-specific autophagy deficiency showed a marked decrease in fat mass, resistance to obesity and improved insulin sensitivity. This is contrary to the current view that mitochondrial dysfunction leads to insulin resistance and that autophagy deficiency in insulin target tissues is associated with the development of obesity-induced diabetes.

RESULTS

**Atg\(^{7\text{sm}}\) mice have reduced muscle and fat mass**

To address the role of muscle autophagy in glucose and lipid homeostasis, we generated skeletal muscle-specific autophagy-knockout (Atg\(^{7\text{sm}}\)) mice by breeding Atg7\(^{-}\)floxed mice (Atg7\(^{-}\)) with myosin light chain 1 fast (Mlc1f\(^{-}\))Cre mice. The expression of Atg7 protein was markedly lower in gastrocnemius and soleus muscles of Atg\(^{7\text{sm}}\) mice compared to control mice (Fig. 1a). Immunoblotting showed that conversion of Lc3-I to Lc3-II, an indicator of autophagy, was reduced, and p62 or ubiquitinated proteins accumulated in muscles of Atg\(^{7\text{sm}}\) mice (Fig. 1a), suggesting skeletal muscle-specific autophagy deficiency. Moreover, confocal microscopy showed colocalization of p62 and ubiquitin (Fig. 1b), as reported previously\(^{11}\).

Male Atg\(^{7\text{sm}}\) mice had lower body weight than control male Atg7\(^{-}\) littermates on a chow diet (Fig. 1c). Magnetic resonance spectroscopic analysis of body composition showed lower lean body mass of Atg\(^{7\text{sm}}\) mice compared to control mice (Fig. 1d). Muscle weight and muscle fiber size were significantly less in Atg\(^{7\text{sm}}\) mice compared to control mice (P < 0.01) (Fig. 1e,f), owing to muscle atrophy resulting from autophagy deficiency\(^{11}\).

In contrast to the differences in muscle weight, there were no significant differences in the weights of heart, lung, kidney, spleen and liver between Atg\(^{7\text{sm}}\) and control mice (data not shown, P > 0.05 for all comparisons), indicating that lower muscle mass is responsible for the reduced lean body mass of Atg\(^{7\text{sm}}\) mice. Notably, fat mass was significantly lower in Atg\(^{7\text{sm}}\) mice compared to control mice (P < 0.05) (Fig. 1d), whereas autophagic activity was intact in WAT of Atg\(^{7\text{sm}}\) mice (Fig. 1a). Consistent with the decrease in fat mass, fat-pad weight and adipocyte size were lower in Atg\(^{7\text{sm}}\) mice than in control mice (Fig. 1g,h). Similarly to the results for male Atg\(^{7\text{sm}}\) mice, female Atg\(^{7\text{sm}}\) mice also had lower body weight and fat mass than female Atg7\(^{-}\) littermates (data not shown).

**Enhanced energy expenditure in Atg\(^{7\text{sm}}\) mice**

To determine the mechanism of reduced fat weight in Atg\(^{7\text{sm}}\) mice, we performed indirect calorimetry in chow-fed male mice. Analysis of covariance (ANCOVA), one of the most appropriate methods to compare parameters between groups with different body weights\(^{21}\), showed that food intake adjusted for body weight was not different between Atg\(^{7\text{sm}}\) and control mice (Supplementary Fig. 1a). Unadjusted food intake was also not different between them (Supplementary Fig. 1b). Atg\(^{7\text{sm}}\) mice, although not hyperphagic, showed significantly lower feed efficiency (gain of body weight per cumulated food intake) during the observation period compared to control mice (P < 0.01) (Supplementary Fig. 1c), suggesting that autophagy deficiency in skeletal muscle increases energy expenditure. Consistent with this, energy expenditure adjusted for body mass was higher in Atg\(^{7\text{sm}}\) mice compared to control mice (Supplementary Fig. 1d), although the difference was not statistically significant (P = 0.06). Unadjusted energy expenditure was not different between Atg\(^{7\text{sm}}\) and control mice (Supplementary Fig. 1e). Thus, increased energy expenditure is likely to be a cause of reduced fat mass in chow-fed Atg\(^{7\text{sm}}\) mice. Despite muscle atrophy observed in Atg\(^{7\text{sm}}\) mice, locomotor activity was not different between Atg\(^{7\text{sm}}\) and control mice (Supplementary Fig. 1f), suggesting that causes other than...
changes in locomotor activity lead to increased energy expenditure in \textit{Atg7}^{sm} mice. In contrast, in response to acute exercise until exhaustion, \textit{Atg7}^{sm} mice showed slightly shorter running distance compared to control mice, although the difference was not statistically significant \((P = 0.11)\) (Supplementary Fig. 1g).

We next studied whether muscle autophagy deficiency affects glucose homeostasis in addition to body mass. Nonfasting glucose concentration was not different between \textit{Atg7}^{sm} and control mice (data not shown). However, fasting glucose and insulin concentrations were significantly lower in \textit{Atg7}^{sm} mice compared to control mice \((P<0.05)\) (Supplementary Fig. 1h), which is contrary to our expectation that autophagy deficiency would worsen glucose homeostasis. In addition, an intraperitoneal glucose tolerance test (GTT) and insulin tolerance test (ITT) revealed that \textit{Atg7}^{sm} mice had enhanced glucose tolerance and insulin sensitivity compared to controls (Supplementary Fig. 1i). However, plasma triacylglycerol, free fatty acid (FFA) and cholesterol concentrations were not different between the two types of mice (data not shown). In contrast to male \textit{Atg7}^{sm} mice, glucose tolerance and concentrations of plasma glucose, triacylglycerol, cholesterol and FFA were not different between chow-fed female \textit{Atg7}^{sm} and control mice (data not shown).

**Improved insulin sensitivity in \textit{Atg7}^{sm} mice fed high-fat diet**

Given that \textit{Atg7}^{sm} mice had a lean phenotype and enhanced glucose tolerance probably secondary to decreased fat mass, we studied the impact of metabolic stress by feeding male \textit{Atg7}^{sm} mice an HFD. Body weight and weight gain in HFD-fed \textit{Atg7}^{sm} mice were significantly lower compared to control HFD-fed mice \((P<0.01–0.001)\) (Fig. 2a and Supplementary Fig. 2a). HFD-fed \textit{Atg7}^{sm} mice also had significantly lower weight and fat mass compared to control mice \((P<0.01–0.001)\) (Supplementary Fig. 2b,c).

We next conducted indirect calorimetry after a short-term HFD for 1 week to determine calorimetric parameters in the early dynamic phase of weight gain before the late static phase was reached after prolonged HFD. Energy expenditure adjusted for body mass by ANCOVA was significantly higher in HFD-fed \textit{Atg7}^{sm} mice compared to HFD-fed control mice \((P<0.001)\) (Fig. 2b), whereas food intake adjusted in the same way was not different between the two groups (Supplementary Fig. 2d). Unadjusted energy expenditure and food intake were not different between \textit{Atg7}^{sm} and control mice (Supplementary Fig. 2e).

In HFD-fed male \textit{Atg7}^{sm} mice, nonfasting glucose concentration was significantly lower compared to HFD-fed control mice \((P<0.01–0.001)\) (Fig. 2c). Moreover, fasting glucose and insulin concentrations were significantly lower in \textit{Atg7}^{sm} mice compared to control mice \((P<0.001)\) (Fig. 2d). As a result, the homeostasis model assessment of insulin resistance (HOMA-IR) was remarkably lower in \textit{Atg7}^{sm} mice compared to control mice (Fig. 2d). Because of ameliorated insulin resistance, HFD-fed \textit{Atg7}^{sm} mice had significantly lower insulinemia compared to HFD-fed control mice \((P<0.001)\) (data not shown). In parallel, a GTT and ITT showed that HFD-fed \textit{Atg7}^{sm} mice had significantly improved glucose tolerance and insulin sensitivity compared to HFD-fed control mice \((P<0.01–0.001)\) (Fig. 2e and Supplementary Fig. 2f).

To gain further insight into the whole-body and tissue-specific glucose metabolism in HFD-fed male \textit{Atg7}^{sm} mice, we performed a hyperinsulinemic-euglycemic clamp. Consistent with GTT and ITT results, whole-body insulin sensitivity was markedly greater in HFD-fed \textit{Atg7}^{sm} mice compared to HFD-fed control mice, as reflected by a marked increase in the glucose infusion rate during the clamp (Fig. 2f). Insulin-stimulated whole-body glucose uptake, glycolysis and glycogen synthesis were all significantly greater in HFD-fed \textit{Atg7}^{sm} mice compared to HFD-fed control mice \((P<0.05–0.01)\) (Fig. 2g), indicating improved peripheral insulin sensitivity. In \textit{Atg7}^{sm} mice, glucose uptake of skeletal muscle was greater than in control mice during the clamp study (Fig. 2h), suggesting that the increase in whole-body glucose uptake could largely be attributed to the increase of insulin-stimulated muscle glucose uptake despite autophagy deficiency in skeletal muscle. Additionally, hepatic glucose production was significantly suppressed in HFD-fed \textit{Atg7}^{sm} mice compared to HFD-fed control mice \((P<0.01)\) (Supplementary Fig. 2g). In parallel, HFD-fed \textit{Atg7}^{sm} mice showed improved pyruvate tolerance and lower expression of gluconeogenic genes than HFD-fed control mice (Supplementary Fig. 2h.i).
Similarly to male Atg7sm mice, HFD-fed female Atg7sm mice had an improved metabolic profile compared to HFD-fed female control mice (Supplementary Fig. 3a–f).

Enhanced lipid catabolism and WAT browning in Atg7sm mice

Because Atg7mice had decreased fat mass, we next assessed lipid metabolism in those mice. When we evaluated in vivo β-oxidation by measuring release of 14CO2 after administration of [1-14C]oleic acid, the β-oxidation rate was significantly higher in HFD-fed Atg7sm mice compared to HFD-fed control mice (P < 0.01) (Fig. 3a). To gain further insight into the increased in vivo β-oxidation of HFD-fed Atg7sm mice, we performed an ex vivo β-oxidation assay using major metabolic organs such as adipose, liver and skeletal muscle. The rate of β-oxidation in WAT of HFD-fed Atg7sm mice was significantly higher than that of HFD-fed control mice (P < 0.05) (Fig. 3b). β-Oxidation in brown adipose tissue (BAT) also seemed higher in HFD-fed Atg7sm mice, although this difference was marginal (P = 0.09; Fig. 3b). Consistent with these results, expression of genes related to β-oxidation was significantly upregulated in WAT (perirenal and inguinal) and BAT of Atg7sm mice compared to control mice (P = 0.05–0.001) (Fig. 3c,d and Supplementary Fig. 4a). Notably, despite autophagy deficiency, the rate and gene expression for β-oxidation in skeletal muscle of Atg7sm mice were not altered compared to control mice (Supplementary Fig. 4b,c), suggesting that increased β-oxidation in WAT and BAT but not in skeletal muscle contributes to the enhanced in vivo β-oxidation of Atg7sm mice. In the liver of HFD-fed Atg7sm mice, we observed markedly lower lipid accumulation and higher expression of β-oxidation–related genes compared to HFD-fed control mice (Fig. 3e,f and Supplementary Fig. 4d), although the rate of hepatic β-oxidation was not different between the two mice (Supplementary Fig. 4e). Furthermore, lymphoid infiltration in the liver and hepatic injury estimated by serum alanine aminotransferase or aspartate aminotransferase concentration were lower in HFD-fed Atg7sm mice compared to control mice (Fig. 3f and Supplementary Fig. 4f), as was expression of genes associated with hepatic inflammation (Fig. 3e).

We next measured parameters of lipolysis that might contribute to the decreased fat mass of Atg7sm mice in addition to β-oxidation. Fasting serum glycerol concentration was significantly higher in HFD-fed Atg7sm mice compared to HFD-fed control mice (P < 0.05) (Supplementary Fig. 4g), suggesting increased in vivo lipolysis. Similarly, lipolytic gene expression was upregulated in perirenal WAT and BAT of HFD-fed Atg7sm mice (Fig. 3c,d), although expression of these genes in inguinal WAT was not different between HFD-fed Atg7sm and control mice (Supplementary Fig. 4a). Serum FFA concentration was also slightly but significantly higher in HFD-fed Atg7sm mice compared to HFD-fed control mice (P < 0.05), probably resulting from enhanced lipolysis (Supplementary Fig. 4g), despite increased in vivo β-oxidation. Probably owing to the combined effect of elevated β-oxidation and lipolysis, HFD-fed Atg7sm mice had smaller adipocyte size in WAT and BAT compared to HFD-fed control mice (Fig. 3g).

In contrast, lipogenic gene expression in the liver was downregulated in HFD-fed Atg7sm mice (Fig. 3e); this might be a factor in the decreased lipid injury and triacylglycerol content in the liver. Notably, uncoupling protein 1 (Ucp1) gene expression was significantly higher in WAT (perirenal and inguinal) and BAT of HFD-fed Atg7sm mice compared to HFD-fed control mice (P = 0.05–0.001) (Fig. 3c,d and Supplementary Fig. 4a). Ucp1 expression in skeletal muscle was similar between the two groups (Supplementary Fig. 4b). Furthermore, the expression of other marker genes of brown-like adipocytes was also significantly upregulated in WAT (perirenal and inguinal) and BAT of HFD-fed Atg7sm mice (P < 0.05–0.001) (Fig. 3c,d and Supplementary Fig. 4a). Consistent with this, the protein abundance of peroxisome proliferator-activated receptor (Ppar) γ coactivator 1t (Pgc1t), a transcriptional coactivator that modulates Ucp1 expression and thermogenesis, was also greater in perirenal or inguinal WAT and BAT of HFD-fed Atg7sm mice compared to HFD-fed control mice (Supplementary Fig. 4i). Moreover, HFD-fed Atg7sm mice had higher body temperature than HFD-fed control mice (Supplementary Fig. 4j). Additionally, glucose uptake in BAT of HFD-fed Atg7sm mice, reflecting BAT activity, was significantly higher than in HFD-fed control mice (P < 0.05) (Fig. 3h).
Increased Fgf21 in Atg7Δsm mice

To investigate the molecular mechanism of enhanced energy expenditure and insulin sensitivity in Atg7Δsm mice, we measured the concentrations of free T3, adiponectin, leptin and cathelicidin, which could increase energy expenditure or insulin sensitivity.23–25 However, serum T3, adiponectin and cathelicidin concentrations were similar between HFD-fed Atg7Δsm and control mice (Supplementary Fig. 5a–c). Serum leptin concentration was lower in HFD-fed Atg7Δsm mice compared to control mice (Supplementary Fig. 5d), probably owing to decreased fat mass. Muscle fiber types that might affect metabolic parameters were not changed, as the expression patterns of myosin heavy chain (Myhc) isoforms in skeletal muscle were not different between Atg7Δsm and control mice (data not shown), as reported previously.26 Because changes in metabolic parameters of Atg7Δsm mice could not be explained by changes of hormones and fibroblasts, we conducted microarray analysis focusing on the expression of myokines that are released from skeletal muscle and could modulate energy homeostasis.26,27 Notably, Fgf21 gene expression was markedly upregulated in gastrocnemius and soleus muscles of male (Fig. 4a and Supplementary Fig. 5e) and female Atg7Δsm mice (data not shown), whereas the expression of other putative myokines was not different (Fig. 4a). Serum Fgf21 concentration was also significantly higher in male Atg7Δsm mice compared to control mice (P < 0.001) (Fig. 4a), suggesting that Fgf21 produced in autophagy-deficient muscle exerts a systemic effect as an endocrine factor. Because Fgf21 is a myokine-like molecule that is expressed in tissues other than muscle, we examined its expression in other potential sites of Fgf21 production. However, Fgf21 mRNA expression in the liver, WAT and BAT of male Atg7Δsm mice was not altered compared to control mice (Supplementary Fig. 5f). Next, to determine whether Fgf21 expression in autophagy deficiency is induced in a cell-autonomous manner, we used Ad7-null and Tet-off Ad3-null mouse embryonic fibroblasts (MEFs). Notably, Fgf21 gene expression was well induced in the two autophagy-deficient MEFs compared to respective control MEFs (Supplementary Fig. 5g). Consistent with this, adenovirus-mediated Atg7 knockdown in C2C12 myotubes increased Fgf21 expression (Supplementary Fig. 5h), showing cell-intrinsic Fgf21 induction. Consistent with previous reports that Fgf21 has beneficial metabolic effects,28–32 Fgf21 increased ex vivo lipolysis in adipose tissues (Supplementary Fig. 5i). Furthermore, we observed evidence of Fgf21...
downstream activation such as Frs2α or Erk phosphorylation and Egr1 induction in the liver of Atg7Δsm mice compared to control mice (Supplementary Fig. 5j). These results are consistent with a previous report showing Fgf21 action in the liver through the Frs2α-Erk-Egr1 pathway, although we cannot exclude the possibility that other factors not yet defined might activate this pathway, which is not unique to Fgf21.

To demonstrate that increased Fgf21 ameliorates diet-induced obesity and insulin resistance in Atg7Δsm mice, we generated Fgf21−/−; Atg7Δsm mice by crossing Fgf21−/− mice with Atg7Δsm mice. The lower body weight or higher degrees of glucose tolerance and insulin sensitivity in HFD-fed male Fgf21+/+; Atg7Δsm mice compared to HFD-fed male Fgf21+/+; Atg7+/+ mice were attenuated in HFD-fed male Fgf21−/−; Atg7Δsm mice by 50% (Fig. 4b-d). Additionally, HFD-fed male Fgf21−/−; Atg7Δsm mice had greater fat mass and more hepatic lipid droplets compared to HFD-fed male Fgf21+/+; Atg7Δsm mice (Fig. 4e and Supplementary Fig. 6a). The improved metabolic profile in HFD-fed female Fgf21+/+; Atg7Δsm mice compared to HFD-fed female Fgf21+/+; Atg7+/+ mice was also attenuated in HFD-fed female Fgf21−/−; Atg7Δsm mice (Supplementary Fig. 6b-e). However, metabolic parameters were not different between HFD-fed Fgf21+/+; Atg7+/+ and Fgf21−/−; Atg7Δsm mice (Fig. 4b-e and Supplementary Fig. 6a-e).

**Atf4-dependent Fgf21 induction in autophagy deficiency**

We next investigated the mechanism for the higher levels of Fgf21 expression in Atg7Δsm mice. The concentration of growth hormone that can upregulate Fgf21 expression was not different between Atg7Δsm and control mice (data not shown). Notably, we observed elevated expression of activating transcription factor 4 (Atf4) and Atf4 target genes in autophagy-deficient muscle by microarray analysis (data not shown) and real-time RT-PCR (Supplementary Fig. 7a). Immunoblotting also showed that Atf4 protein expression and phosphorylation of Eif2α, a protein upstream of Atf4, were higher in muscle of Atg7Δsm mice compared to control mice (Fig. 4f).

Because Atf4 is a master regulator of the integrated stress response and inhibition of autophagy may affect stress responses, we tested whether Fgf21 induction in autophagy deficiency is mediated by Atf4. As we hypothesized, adenosine-mediated overexpression of Atf4 in C2C12 myotubes induced Fgf21 gene expression (Fig. 4g). Further, in a transient transfection assay, Fgf21 reporter activity was increased by Atf4 overexpression in C2C12 myotubes (Fig. 4h). Reporter assays using deletion (ATF4ARE1 and ATF4ARE1/RE2) or point mutants (ATF4mutRE1 and ATF4mutRE1/2) showed the functional importance of two putative Atf4-responsive elements (ATF4REs) in the Fgf21 promoter (Fig. 4h). Consistent with Fgf21 mRNA induction in autophagy-deficient cells, reporter activity of wild-type Fgf21 promoter in Atg7-null MEFs was higher than that in wild-type MEFs (Fig. 4i). Meanwhile, reporter activity of the ATF4mutRE1/2 mutant in Atg7-null MEFs was lower than that of wild-type reporter (Fig. 4i). Furthermore, siRNA-mediated knockdown of Atf4 suppressed Fgf21 reporter activity in Atg7-null MEFs (Fig. 4j), confirming that Fgf21 induction in autophagy-deficient cells depends on Atf4. Accordingly, Atf4 target gene expression was higher in Atg7-null MEFs compared to wild-type MEFs (Supplementary Fig. 7b).

Because recent reports have shown that increased p62 in autophagy deficiency enhances transcriptional activity of Nrf2 (ref. 37), which may upregulate Atf4 expression48, we investigated whether p62 (encoded by Sgstm1) is involved in Atf4 or Fgf21 induction. Adenovirus-mediated Sgstm1 overexpression did not affect Atf4 expression (Supplementary Fig. 8a). Sgstm1 overexpression also had no effect on Atf4 target gene expression in C2C12 myotubes (Supplementary Fig. 8a) or Atf4-induced Fgf21 promoter activity (Supplementary Fig. 8b). Furthermore, Sgstm1 overexpression did not influence expression of mtOxPhos-related genes or glucose uptake (Supplementary Fig. 8a,c). When we examined effects on Ppara, which can positively influence Fgf21 expression32, we observed that Atf4 overexpression did not affect Ppara promoter activity or transcriptional activity (Supplementary Fig. 8d,e). Adenovirus-mediated Atf4 overexpression also did not affect Ppara expression (Supplementary Fig. 8f). Moreover, Ppara gene expression was not different in skeletal muscle of Atg7Δsm mice compared to control mice (Supplementary Fig. 8g), indicating that Pparα is not associated with Atf4-dependent Fgf21 expression in autophagy-deficient skeletal muscle.

**Impaired mtOxPhos is responsible for Fgf21 induction by Atf4**

We next investigated how Atf4 is activated in autophagy deficiency. Because mitochondrial dysfunction is observed in autophagy deficiency and mitochondrial stress can induce Atf4 expression39,40, we studied changes in the morphology and function of mitochondria in autophagy-deficient muscle. In agreement with previous reports8,10-12, electron microscopy showed pronounced accumulation of morphologically abnormal, swollen mitochondria in autophagy-deficient muscle (Fig. 5a). Additionally, mitochondrial O2 consumption, cytochrome c oxidase (Cox) activity, ATP content and expression of mtOxPhos-related genes were lower in skeletal muscle of Atg7Δsm mice compared to control mice (Fig. 5b,c and Supplementary Fig. 9a,b). In contrast, Tom20 protein expression and citrate synthase activity, reflecting mitochondrial mass, were higher, probably owing to deficient mitophagy (Supplementary Fig. 9c,d). This indicates that autophagy deficiency causes functional impairment of mitochondria. Moreover, protein carbonylation was greater in autophagy-deficient muscle compared to control (Supplementary Fig. 9e), implying that oxidative stress is increased in skeletal muscle with autophagy deficiency, presumably owing to mitochondrial dysfunction.

To study whether impaired mitochondrial function affects Fgf21 expression through Atf4 activation, we treated C2C12 myotubes with rotenone (a mitochondrial complex I inhibitor) or antimycin A (a mitochondrial complex III inhibitor). Notably, such mitochondrial stressors induced Fgf21 expression and activation of the Eif2α-Atf4 pathway (Fig. 5d,e), suggesting that Fgf21 is a mitokine released in response to mitochondrial stress. Moreover, mitochondrial stressor-induced Fgf21 mRNA expression was suppressed in C2C12 myotubes transfected with Atf4-targeted siRNA, and in Atf4-null or Eif2αA/A mutant MEFs, compared to the respective control cells (Fig. 5f-h). To further confirm our hypothesis, we used muscle-specific mitofusin 1 and mitofusin 2 double-knockout (Mfn1/2Δsm) mice that show profound mtOxPhos impairment owing to defective mitochondrial fusion and suppressed glucose concentration41. Induction of Fgf21 mRNA and protein expression and of Atf4 protein expression, as well as phosphorylation of Eif2α, were markedly greater in skeletal muscle of Mfn1/2Δsm mice compared to control mice (Fig. 5i,j). Atf4 target gene expression was also significantly upregulated in muscle of Mfn1/2Δsm mice (P < 0.05) (Supplementary Fig. 7c).

**Role of Fgf21 in Atg7Δhep and nutrient-deficient mice**

As autophagy deficiency in skeletal muscle protected mice from diet-induced obesity and insulin resistance, we produced mice with targeted disruption of Atg7 in another major insulin target tissue, liver (Atg7Δhep mice). The expression of Atg7 mRNA and protein was...
markedly downregulated in the liver of Atg7<sup>f/f</sup> mice; these mice showed irregular hepatic lobules and hepatomegaly, accompanied by reduced conversion of Lc3-I to Lc3-II and p62 accumulation (Supplementary Fig. 10a,b), as reported previously<sup>10</sup>. Similarly to Atg7<sup>Δsm</sup> mice, chow-fed Atg7<sup>Δhep</sup> mice had lower body and fat weights, and enhanced glucose tolerance, compared to control mice (Fig. 6a-c). We observed less fasting-induced hepatic lipid accumulation in autophagy-deficient liver compared to control (Fig. 6d).

Gene expression related to fatty acid and triacylglycerol synthesis was also downregulated in the liver of chow-fed Atg7<sup>Δhep</sup> mice compared to control mice (Supplementary Fig. 10c). This could contribute to the decreased lipid accumulation in autophagy-deficient liver by curtailing endogenous fatty acid synthesis, adding to the effect of diminished exogenous fatty acid supply from adipose tissue owing to reduced fat mass in Atg7<sup>Δhep</sup> mice. However, possible changes of lipid secretion from the liver could not explain the reduced lipid in

**Figure 5** Impairment of mtOxPhos in autophagy deficiency is responsible for Atf4-dependent Fgf21 induction. (a) Representative electron micrographs of gastrocnemius muscle from Atg7<sup>f/f</sup> and Atg7<sup>Δsm</sup> mice. Scale bars, 1 μm. (b) Mitochondrial state 3 and 4 respiration in skeletal muscle of Atg7<sup>f/f</sup> and Atg7<sup>Δsm</sup> mice (n = 5). (c) Representative Cox staining of gastrocnemius muscle from Atg7<sup>f/f</sup> and Atg7<sup>Δsm</sup> mice. Scale bars, 100 μm. (d) Relative Fgf21 mRNA expression in C2C12 myotubes after treatment with DMSO (Veh), rotenone (Rot; 0.5 μM) or antimycin A (AA; 4 μM) for 8 h. (e) Immunoblotting for Atf4, phosphorylated Eif2α (p-Eif2α), Eif2α and β-actin in C2C12 myotubes treated with DMSO or rotenone (0.5 μM) for 30 min or 1 h. (f) Relative Fgf21 mRNA expression in siCON- or siAtf4-transfected C2C12 myotubes after treatment with rotenone (0.5 μM) for 8 h. (g,h) Relative Fgf21 mRNA expression in Atg7<sup>Δhep</sup> or Atg7<sup>Δhep</sup> MEFs (g) and E12<sup>ΔA/ΔA</sup> (wild type) or E12<sup>ΔA/ΔS</sup> MEFs (mutant) (h) after treatment with DMSO, rotenone (0.5 μM) or antimycin A (4 μM) for 8 h. (i) Left, standard RT-PCR (upper two lanes) and immunoblotting for Fgf21 (lower two lanes) in muscle of 9-week-old Mfn1<sup>Δhep</sup> (control) and Mfn1<sup>Δhep</sup> mice. Right, real-time RT-PCR of Fgf21 in muscle of Mfn1<sup>Δhep</sup> and Mfn1<sup>ΔΔhep</sup> mice. (j) Immunoblotting for Atf4, phosphorylated Eif2α (p-Eif2α) and Eif2α in muscle of Mfn1<sup>Δhep</sup> and Mfn1<sup>ΔΔhep</sup> mice. Hsp90, a loading control. Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 6** Atg7<sup>Δhep</sup> mice are protected from HFD-induced obesity and insulin resistance. (a) Body weight of Atg7<sup>f/f</sup> and Atg7<sup>Δhep</sup> mice fed chow diet (n = 3 or 4). (b) Gross image and weight of epididymal fat from 20-week-old mice (n = 4). (c) GTT in 16-week-old Atg7<sup>f/f</sup> and Atg7<sup>Δhep</sup> mice fed chow diet (n = 6). (d) Representative Oil Red O staining of liver from fasted 16-week-old mice fed chow diet. Scale bars, 50 μm. (e) Representative Cox staining of liver from 16-week-old mice fed chow diet. Scale bars, 50 μm. (f) Left, relative Fgf21 mRNA expression in primary hepatocytes from chow-fed mice (n = 4). Right, serum Fgf21 concentration in fed mice on chow diet (n = 8). (g) Body weight of Atg7<sup>f/f</sup> and Atg7<sup>Δhep</sup> mice fed HFD (n = 6). (h) Fasting blood glucose and insulin concentrations and HOMA-IR in mice fed HFD for 13 weeks (n = 5). (i) GTT in mice fed HFD for 13 weeks (n = 5). (j) H&E staining of liver from mice fed HFD for 13 weeks. Scale bars, 100 μm. Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.
the liver of Atg7<sup>shp</sup> mice because triacylglycerol release measured after injection of tyloxapol, a lipoprotein lipase inhibitor that blocks triacylglycerol uptake, was not higher but lower in Atg7<sup>shp</sup> mice compared to control mice, probably as a result of diminished lipid content in the liver (Supplementary Fig. 10d). Changes in β-oxidation, another way to eliminate lipid from hepatocytes, also could not explain the decreased lipid content in the liver of Atg7<sup>shp</sup> mice, as β-oxidation–related gene expression was downregulated in the liver of Atg7<sup>shp</sup> mice (Supplementary Fig. 10c). Furthermore, Atg7<sup>shp</sup> mice had lower hepatic Cox activity and mOxPhos-related gene expression than control mice (Fig. 6e and Supplementary Fig. 10e). Probably owing to impaired mitochondrial function, Atf4 target gene expression was upregulated in autophagy-deficient liver compared to controls (Supplementary Fig. 10f). Consequently, hepatic Fgf21 mRNA expression and serum Fgf21 concentration were markedly higher in Atg7<sup>shp</sup> mice compared to control mice (Fig. 6f), suggesting that higher Fgf21 contributes to the lower fat mass and higher glucose tolerance in these mice.

When fed HFD, Atg7<sup>shp</sup> mice had significantly lower body weight and nonfasting glucose concentration than HFD-fed control mice (P < 0.05–0.001) (Fig. 6g and Supplementary Fig. 10g). Additionally, fasting glucose and insulin concentrations and the HOMA-IR index were lower in HFD-fed Atg7<sup>shp</sup> mice than in HFD-fed control mice (Fig. 6h). Consistent with the improved metabolic profile, HFD-fed Atg7<sup>shp</sup> mice showed greater glucose tolerance in GTT than HFD-fed control mice (Fig. 6i). Notably, hepatic steatosis was not observed in HFD-fed Atg7<sup>shp</sup> mice, in contrast to marked hepatic lipid accumulation in HFD-fed control mice (Fig. 6j), similar to the decrease of fasting-induced lipid accumulation in autophagy-deficient liver. Additionally, gene expression related to fatty acid and triacylglycerol synthesis was significantly downregulated in the liver of HFD-fed Atg7<sup>shp</sup> mice compared to HFD-fed control mice (P < 0.05–0.001) (Supplementary Fig. 10h), which might explain the considerably diminished hepatic lipid accumulation in these mice. To investigate whether autophagy deficiency can induce Fgf21 expression in tissues other than muscle or liver, we used β-cell–specific autophagy-knockout (Atg7<sup>βββ</sup>-cell) mice<sup>3</sup>. Fgf21 expression in islets of Atg7<sup>βββ</sup>-cell mice that showed deteriorated metabolic profiles was not altered compared to control mice, which was consistent with unchanged Fgf21 concentration in serum (data not shown).

Finally, we found that the Atf4-Fgf21 axis has a physiologically important role in a leucine deprivation model as well (Supplementary Results and Supplementary Fig. 11).

**DISCUSSION**

Although several studies have reported that autophagy regulates lipid metabolism, adipose differentiation, muscle mass and beta cell homeostasis<sup>2,7,11,42</sup>, the metabolic impact of autophagy deficiency in skeletal muscle has not been addressed. We observed that autophagy deficiency in skeletal muscle induces Fgf21 expression through Atf4 activation. Consequently, increased Fgf21 enhanced β-oxidation and energy expenditure through burning of WAT, leading to resistance to HFD-induced obesity. HFD-fed Atg7<sup>sm</sup> mice therefore showed improved glucose homeostasis and lower insulin concentration, which may be attributable to the lean phenotype. These results are contrary to suggestions by other investigators that autophagy disruption would lead to insulin resistance. Previously, autophagy has been considered to be crucial in maintaining intracellular homeostasis by modulating the cell-intrinsic metabolism of amino acids or other nutrients. Thus, our data provide the first evidence, to our knowledge, for endocrine metabolic effects of autophagy or its deficiency affecting the metabolism of distant organs or the whole body.

We found that Atf4-dependent Fgf21 induction in autophagy-deficient muscle is due to mitochondrial dysfunction. Mitochondrial stressors also induced Fgf21 through Atf4. Recently, Fgf21 induction has been observed in mice with mutations of a mitochondrial replicative helicase and in human subjects with muscle-manifesting mitochondrial respiratory chain deficiencies<sup>43,44</sup>. We also observed a strong induction of Atf4 and Fgf21 in Mfn1/2<sup>−/−</sup> mice that have suppressed glucose concentration. All these results suggest that mitochondrial stress or dysfunction caused by autophagy deficiency results in Atf4-mediated Fgf21 induction, which leads to fat loss and improved insulin sensitivity by increasing β-oxidation and burning of WAT. Induction of Fgf21 in autophagy-deficient muscle with mitochondrial dysfunction, and its action in distant metabolic tissues such as adipose tissues and liver in an endocrine manner, are consistent with the functional definition of ‘mitokine’ that has been postulated in a Caenorhabditis elegans model<sup>45</sup>. Recently, induction of adiponectin has been observed in transgenic mice that express mitoNEET in adipose tissue and show mitochondrial dysfunction; however, causality between adiponectin induction and mitochondrial dysfunction has not been studied in those mice<sup>46</sup>. Thus, Fgf21 may be one of the first mitokines identified in a mammalian system.

When we investigated the role of Fgf21 in the improved metabolic profile of Atg7<sup>sm</sup> mice, Fgf21<sup>−/−</sup>-Atg7<sup>sm</sup> mice showed considerable attenuation in the fat weight loss and the enhancement of glucose tolerance and insulin sensitivity seen in Fgf21<sup>−/−</sup>-Atg7<sup>sm</sup> mice. This indicates that increased Fgf21 is crucial in the metabolic improvement of Atg7<sup>sm</sup> mice. Fgf21-independent pathways may also contribute to the improved metabolic profile in these mice. Further study is needed to evaluate Fgf21-independent mechanisms in Atg7<sup>sm</sup> mice.

Recently, Fgf21 has been reported to ameliorate HFD-induced obesity and insulin resistance by increasing lipolysis and β-oxidation<sup>28–33</sup>. Here we observed that increased Fgf21 in autophagy-deficient muscle ameliorates HFD-induced deterioration of the metabolic profile by enhancing β-oxidation, lipolysis and burning of WAT. Although concentrations of serum FFA and glycerol, two potential markers of lipolysis, were higher in HFD-fed Atg7<sup>sm</sup> mice, a direct in vivo role of serum Fgf21 in the increased lipolytic activity of Atg7<sup>sm</sup> mice was not determined in this investigation. In contrast, we obtained strong evidence that β-oxidation in BAT and WAT of HFD-fed Atg7<sup>sm</sup> mice is greater than that in HFD-fed control mice. However, there was no difference in hepatic β-oxidation rate between Atg7<sup>sm</sup> and control mice despite higher expression of β-oxidation–related genes and less lipid accumulation in the liver of HFD-fed Atg7<sup>sm</sup> mice. Notably, the β-oxidation rate in skeletal muscle was not altered in HFD-fed Atg7<sup>sm</sup> mice compared to control mice despite impaired mitochondrial function. Possible changes of diverse factors potentially involved in the control of β-oxidation, such as fatty acid uptake or transport, might be affected by autophagy deficiency. Indeed, we found lower expression of Cd36, related to FFA uptake<sup>47</sup>, in the liver and higher expression of fatty acid–binding protein 3 (Fabp3), involved in FFA transport<sup>48</sup>, in skeletal muscle of HFD-fed Atg7<sup>sm</sup> mice compared to HFD-fed control mice (K.H.K., unpublished data); this may contribute to the lack of increased β-oxidation in the liver and the absence of reduced β-oxidation in skeletal muscle of HFD-fed Atg7<sup>sm</sup> mice. Thus, we speculate that the enhanced β-oxidation in BAT and WAT is the predominant contributor to the higher rate of in vivo β-oxidation of HFD-fed Atg7<sup>sm</sup> mice compared to control mice.
In addition, Ucp1 expression was upregulated in BAT and WAT of HFD-fed Atg7\textsuperscript{△sm} mice, whereas Ucp expression was not changed in skeletal muscle with autophagy deficiency. Furthermore, other thermogenic gene expression was upregulated in WAT of HFD-fed Atg7\textsuperscript{△sm} mice, together with greater Pgc1α protein abundance, indicating browning of WAT in Atg7\textsuperscript{△sm} mice with elevated Fgf21 concentration. These results are consistent with a previous finding that Fgf21 is important in browning of WAT and adaptive thermogenesis\textsuperscript{49}. Our data indicate that the enhanced thermogenic activity of adipose tissues, but not that of skeletal muscle, contributes to the increased energy expenditure in Atg7\textsuperscript{△sm} mice.

Our results showing ameliorated insulin resistance in Atg7\textsuperscript{△sm} mice with mitochondrial dysfunction contrast with previous suggestions that mitochondrial dysfunction induces glucose intolerance or insulin resistance\textsuperscript{13}, but they are consistent with findings in liver- or muscle-specific Atf5\textsuperscript{-}knockout mice showing enhanced glucose tolerance\textsuperscript{18}. Different metabolic impacts of mitochondrial dysfunction or stress might depend on the location of mitochondrial defects. For instance, beta cell–specific Tfam deletion causes impaired mitochondria function, deficient insulin release and glucose intolerance\textsuperscript{50}, whereas Tfam deletion in skeletal muscle leads to enhanced glucose tolerance\textsuperscript{19}. Similarly to mitochondrial dysfunction, metabolic impacts of autophagy loss might also depend on the location of autophagy deficiency. Autophagy disruption in beta cells leads to impaired insulin release and glucose intolerance. In contrast, autophagy deficiency in classical insulin target tissues may have different outcomes, as noted in previous studies using adipose tissue–specific autophagy-deficient mice\textsuperscript{5,6} and in this investigation. Metabolic outcomes of autophagy deficiency may also be affected by the duration of autophagy deficiency or the method of gene disruption, as mice show glucose intolerance upon acute autophagy deficiency in the liver induced by adenoviral delivery of shRNA targeting Atg7, in contrast to our data\textsuperscript{7}.

It has recently been reported that mice with Bcl2 knock-in mutations (T69A, S70A and S84A; Bcl2\textsuperscript{AAA}) have intact basal autophagy but defective stimulus-induced autophagy\textsuperscript{52}. The mice do not show exercise-mediated protection against HFD-induced glucose intolerance, supporting beneficial metabolic effects of stimulus-induced autophagy. In contrast to Atg7\textsuperscript{△sm} mice, Bcl2\textsuperscript{AAA} mice do not show metabolic improvement on HFD compared to control mice, probably owing to the absence of mitochondrial dysfunction. Thus, the mode of autophagy deficiency (basal or stimulus-induced) may also affect the metabolic outcome.

We observed lower hepatic lipid content in Atg7\textsuperscript{△hep} mice after HFD or overnight fasting, which is consistent with a previous report\textsuperscript{52}. How can hepatic lipid content be lowered while mtOxPhos activity is impaired in autophagy-deficient liver? Several possibilities can be considered. Three major sources of hepatic lipid are diet, adipose tissues and de novo fatty acid synthesis. Approximately 60% of hepatic fat is derived from adipose tissues, with lesser contributions from de novo fatty acid synthesis (25%) and diet (15%)\textsuperscript{53}. Because Atg7\textsuperscript{△hep} mice have a very low fat mass, probably owing to β-oxidation and browning of WAT induced by elevated Fgf21, reduced FFA supply to the liver from adipose tissues of Atg7\textsuperscript{△hep} mice may contribute to low lipid content in the liver of Atg7\textsuperscript{△hep} mice. Particularly under fasting conditions, hepatic FFAs originate predominantly from adipose tissues, which is consistent with our data showing reduced hepatic lipid content in fasted Atg7\textsuperscript{△hep} mice. In addition to decreased lipid supply from adipose tissue, hepatic expression of genes related to de novo fatty acid and triacylglycerol synthesis was downregulated in chow-fed Atg7\textsuperscript{△hep} mice. Moreover, the contribution of de novo fatty acid and triacylglycerol synthesis to hepatic fat accumulation is larger in HFD-fed mice compared to chow-fed mice\textsuperscript{54–57}. We also observed markedly lower expression of these genes in the liver of HFD-fed Atg7\textsuperscript{△hep} mice compared to control mice. Thus, both reduced fatty acid supply from adipose tissues and impaired endogenous production of lipids might contribute to low fat accumulation in the liver of Atg7\textsuperscript{△hep} mice.

In contrast to these results, increased hepatic lipid accumulation in the same Atg7\textsuperscript{△hep} mice has been reported\textsuperscript{4}. Differences in the genetic background of the mice or in experimental procedures might lead to different phenotypes. The effect of autophagy on lipid metabolism is complex, and further studies are required to evaluate the conflicting roles of autophagy in lipid metabolism.

Together, our results show that autophagy deficiency in skeletal muscle induces expression of Fgf21, a mitokine, through Atf4, which leads to increased β-oxidation and browning of WAT, and finally to protection from HFD-induced insulin resistance, probably owing to leaness. These results are in contrast to the previous suggestion that autophagy deficiency would worsen glucose and lipid metabolism. Thus, the role of autophagy in energy metabolism might be more complicated than expected. Our results suggest that the metabolic impact of autophagy disruption may involve non–cell-autonomous or endocrine effects, such as induction of mitokines, depending on the location, duration and mode of autophagy deficiency. These would add to previously reported cell-autonomous effects such as changes in amino acid concentrations by inhibition of long-lived protein degradation or changes in lipids by inhibition of ‘lipophagy’. Although further studies are required to address the conflicting effects of autophagy in glucose and lipid metabolism, our findings provide new insights regarding the role of autophagy in metabolic homeostasis and mitochondrial stress response, and suggest an innovative strategy to develop novel therapeutic agents against diabetes, obesity and metabolic syndrome.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available on the online version of the paper.

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AUTHOR CONTRIBUTIONS

K.H.K., C.S.C. and M.-S.L. designed the study, analyzed data and wrote the manuscript. K.H.K. conducted all experiments except the portions indicated below, assisted by S.H.K., J.M.C., D.H.K. and K.X.H. Y.T.J. analyzed the metabolic profiling of Atg7\textsuperscript{△hep} mice. H.O., Y.-N.K. and S.S.K. performed measurements of body composition, indirect calorimetry, the hyperinsulinemic-euglycemic clamp and the fatty acid oxidation experiments. H.K.K., C.S.C. and M.-S.L. designed the study, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
ONTINE METHODS

Mouse experiments. Atg7<sup>f/f</sup> mice were bred with Mlc1f-Cre (ref. 38) and Albumin-Cre mice (Jackson Laboratory) to generate Atg7<sup>β<sub>β</sub>/β</sup> and Atg7<sup>β<sub>β</sub>/β<sub>Ⅲ</sub></sup> mice, respectively. Atg7<sup>β<sub>β</sub>/β</sup> mice have been described<sup>3</sup>. Fgf21<sup>+</sup>/+ mice were produced by standard gene-targeting technology using C57BL/6 ES cells and maintained on a C57BL/6 background<sup>59</sup>. Fgf21<sup>+</sup>/+ mice were crossed with Atg7<sup>β</sup>β or Atg7<sup>7<sub>β<sub>β</sub>/β</sub><sub>Ⅲ</sub></sup> mice to generate Fgf21<sup>+</sup>/β<sub>Ⅲ</sub>β<sub>β</sub>, Fgf21<sup>+</sup>/β<sub>Ⅲ</sub>β<sub>β</sub>, Fgf21<sup>+</sup>/β<sub>β</sub>β<sub>β</sub> and Fgf21<sup>+</sup>/β<sub>β</sub>β<sub>Ⅲ</sub> mice. Both male and female mice were fed either HFD (60% of calories from fat, Research Diets no. D12492) or chow. For the calorie-restriction experiment, male mice were fed either a calorie-restricted diet (35% reduction of calorie intake, Research Diet no. D12491G) or control diet (Research Diets no. D12451). For the leucine-deficiency experiment, male mice were fed either a leucine-deficient diet (Research Diets no. A05080202) or control diet (Research Diets no. A10021B). To ensure a similar food or calorie intake in experiments using calorie-restricted and leucine-deficient diets, mice were pair-fed. All animal care and treatments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Sungkyunkwan University School of Medicine.

Glucose, insulin and pyruvate tolerance tests. Glucose and pyruvate tolerance tests were done in overnight-fasted mice by an intraperitoneal injection of glucose (1 g per kg body mass) or pyruvate (1 g per kg body mass), respectively. Insulin tolerance tests were done with an intraperitoneal injection of insulin (1 U per kg body mass) to mice after 6 h of fasting. Blood glucose concentrations were measured with an Accu-Check glucometer (Roche).

Blood chemistry and metabolite analysis. Serum leptin, adiponectin and Fgf21, insulin, free T3 and growth hormone concentrations were measured with ELISA kits (R&D Systems for leptin, adiponectin and Fgf21; Shibayagi for insulin; Alpha Diagnostic International for free T3; Millipore for growth hormone). Serum triacylglycerol, cholesterol, alanine aminotransferase or aspartate aminotransferase concentrations were measured with a Fuji Dri-Chem 3500 biochemistry analyzer (FujiFilm). Serum FFA concentration was determined with a SICDIA NEFAZYME Kit (Shinyang Chemical). Serum glycerol concentration was measured with a Glycerol Determination Kit (Sigma). Plasma catecholamine concentrations were measured with an Adrenaline/Noradrenaline ELISA Kit (IBL International). ATP content was measured in fresh isolated gastrocnemius muscle with a commercial kit (ATP Bioluminescence Assay Kit HS II, Roche). Triacylglycerol content in the liver was measured with a Triglyceride Kit (GPO-Trinder, Sigma). Glucose uptake in C2C12 myotubes was measured with a 2-Deoxyglucose Uptake Measurement Kit (Cosmo Bio). Mitochondrial citrate synthase activity was measured in gastrocnemius muscle with a Citrate Synthase Activity Assay Kit (Shima) was measured in gastrocnemius muscle with a Citrate Synthase Activity Assay Kit (Shima). HOMA-IR was calculated via the following formula: (fasting insulin x fasting glucose)/22.5.

Histology, staining and confocal microscopy. The liver, muscle, white fat, interscapular brown fat and pancreas were obtained and immediately fixed in 10% formalin and 4% paraformaldehyde to make paraffin- and OCT-embedded blocks, respectively. H&E staining, immunohistochemistry (for insulin) and immunofluorescent staining (for p62 and ubiquitin) were done with paraffin-embedded tissue sections. Hepatic lipid content was assessed by Oil Red O staining of OCT-embedded liver sections. Cox staining was done as described<sup>59</sup>. Briefly, unfixed cryosections of gastrocnemius muscle were incubated in a reaction buffer containing 10 mg cytochrome c, 5 mg dianmonobenzidine tetrahydrochloride (DAB), 9 ml sodium phosphate buffer (0.1 M, pH 7.4) and 1 ml catalase (20 µg ml<sup>-1</sup>). Cox activity was visualized with a standard DAB reaction. Images were acquired with a light microscope (Nikon Eclipse E80i) or a confocal microscope (Nikon E800).

Statistical analysis. All values are expressed as mean ± s.e.m. Statistical significance was tested by the unpaired two-tailed Student’s t test or ANCOVA. P values less than 0.05 were considered to represent statistically significant differences.

Additional methods. Detailed methodology is described in the Supplementary Methods.