筑　波　大　学

博 士 （医 学） 学 位 論 文

Ablation of *Elovl6* protects pancreatic islets from high-fat diet-induced impairment of insulin secretion.

（Elovl6 の欠損は膵臓ランゲルハンス氏島における高脂肪食誘導性インスリン分泌の障害を保護する）

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1. Abstract

ELOVL family member 6, elongation of very long chain fatty acids (Elovl6), is a microsomal enzyme that regulates the elongation of C12-16 saturated and monounsaturated fatty acids (FAs) and has been shown to be related to the development of obesity-induced insulin resistance via modifying FA composition. In this study, we investigated the role of Elovl6 in pancreatic islet and β-cell function. Elovl6 was expressed in islet and β-cell line. Compared to wild-type (WT) mice, islets from *Elovl6*$^{-/-}$ mice displayed a normal architecture and β-cell mass in a chow-fed state, but the islet hypertrophy by a high-fat, high-sucrose (HFHS) diet was attenuated. Glucose-stimulated insulin secretion (GSIS) was increased in the islet of the HFHS diet-fed *Elovl6*$^{-/-}$ mice than that of WT mice. Enhanced GSIS in the HFHS *Elovl6*$^{-/-}$ islets was associated with increased ATP/ADP ratio and suppression of ATF-3 expression. Our data suggest that Elovl6 could be involved in insulin secretory capacity and diabetes.

2. Introduction

Obesity is a major cause of type 2 diabetes (T2D) in humans because of the progressive deterioration of pancreatic β-cell insulin secretory function and its capacity to compensate for increased peripheral insulin resistance [1] [2]. Accumulation of lipids in non-adipose tissues has been implicated in both pathologies, a phenomenon known as lipotoxicity as a molecular link between obesity and glucose homeostasis dysregulation [3] [4]. Pancreatic β-cells are known to be highly susceptible to lipotoxicity, and both exogenous and endogenous sources of FAs are believed to be involved in the deterioration of β-cell function.

FA composition of the lipid species could be another determinant of the development of lipotoxicity. ELOVL family member 6, elongation of very long chain fatty acids (Elovl6) is a microsomal enzyme involved in the elongation of saturated and monounsaturated FAs with 12, 14, and 16 carbons [5] [6]. Loss of Elovl6 function reduces stearate (C18:0) and oleate (C18:1n-9) levels, and increases palmitate (C16:0) and palmitoleate (C16:1n-7) levels [7]. In our previous study, we have reported that mice with targeted disruption of Elovl6 (*Elovl6*$^{-/-}$) are protected against hepatic insulin resistance development when

fed a HFHS diet despite hepatosteatosis and obesity in these mice and the wild-type mice being similar, suggesting that FA composition, particularly C16:0 to C18:0 conversion, is crucial for insulin sensitivity rather than mere lipid accumulation [7]. Therefore, inhibition of Elovl6 could be a potential therapeutic target for T2D. A major unanswered question is whether the inhibition of this elongase will leads to reduced susceptibility to pancreatic β-cell failure. Here, we studied whether Elovl6 participates in the regulation of insulin secretion and β-cell function.

3. Materials and methods

3.1 Animals.

All animal husbandry and animal experiments were consistent with the University of Tsukuba's Regulation of Animal Experiments and were approved by the Animal Experiment Committee of University of Tsukuba. Homozygous *Elovl6*$^{-/-}$ and sex- and age-matched *Elovl6*$^{+/+}$ littermates housed in a pathogen-free barrier facility with 12 h light/12 h dark cycle, were maintained on a standard laboratory chow or HFHS diet [7].

3.2 Cell culture.

Mouse insulinoma MIN6 cells and mouse hepatoma hepa1c1c7 cells were cultured as described previously [8] [7].

3.3 Isolation of pancreatic islet.

The isolation of mouse pancreatic islets was carried out according to the collagenase digestion methods described previously [9].

3.4 Analysis of insulin secretion, insulin content, and DNA

content of islets.

Isolated islets were incubated for 2 h in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Ten islets were then preincubated for 30 min in 2.8 mM glucose in 0.5% BSA Krebs-Ringer Bicarbonate Hepes (KRBH) buffer. Finally, they were incubated in 2.8 mM glucose, 20 mM glucose, or 2.8 mM glucose with 30 mM KCl in 0.5% BSA KRBH buffer for 30 min, and insulin secretion was analyzed using mouse insulin ELISA kit (Shibayagi). Insulin secretion, insulin and DNA contents of the islets were measured as previously described [10].

3.5 Measurement of ATP and ADP content of islets.

ATP and ADP levels per 10 isolated islets were measured as described previously, using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) [10].

3.6 Measurement of triglycerides content of islets.

One hundred isolated islets were used for lipid extraction. Triglycerides were measured by extracting lipids using the method of Folch [11], followed by triglyceride determination with the GPO-trinder kit (Sigma) [10].

3.7 Fatty acid composition of islets.

Total lipid was extracted from isolated islets (400 islets/sample) according to Bligh-Dyer's procedure [12]. After saponification, the fatty acids in each sample were methyl-esterified and the relative abundance of each fatty acid was quantified by gas chromatography as described previously [7].

3.8 Gene expression.

Total RNA extraction with the NucleoSpin RNA (TaKaRa) was performed according to the manufacture's instruction. cDNA was synthesized with the PrimeScript RT Master kit (TaKaRa) and real-time PCR analysis was performed using SYBR Green Dye (GeneAce SYBR qPCR Mix α, NIPPON GENE) with an ABI 7300 instrument (Applied Biosystems) as previously described [10]. mRNA levels were normalized to cyclophilin expression.

3.9 Histology and quantification of islet mass.

Mice were euthanized, and the pancreases were excised, fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin & eosin.

3.10 Statistics.

Data were represented as means ± SEM. Unpaired Student's t-test was used for measuring the statistical significance of differences between two groups. $P < 0.05$ was considered of statistical significance.

4. Results

4.1 Elovl6 is expressed in pancreatic islet and β-cell lines

Real-time PCR analysis revealed that Elovl6 mRNA was detected in isolated mouse islets as well as MIN6 cells (Figure 1A and B). Elovl6 expression in isolated islets is comparable to that in brain, liver, and white adipose tissue, where Elovl6 plays a regulatory role.

4.2 Morphology of islets of Elovl6$^{-/-}$ mice

Chronic hyperinsulinemia due to insulin resistance is associated with hyperplasia and hypertrophy of islets caused by the adaptive proliferation of β-cells to maintain blood glucose level. To determine whether endogenous Elovl6 is involved in the regulation of β-cell function under conditions of obesity, WT and *Elovl6*$^{-/-}$ mice were fed a chow or HFHS diet for 12 weeks. Histology of pancreatic sections demonstrated that *Elovl6*$^{-/-}$ mice display no apparent morphological abnormalities of the islet on chow-fed state (Figure 2A). WT mice fed a HFHS diet showed a marked increase in size of islets, suggesting an adaptive enlargement of β-cell mass in response to insulin resistance (Figure 2A). In contrast, islet hypertrophy was

suppressed in the HFHS *Elovl6*$^{-/-}$ pancreas compared to WT mice (Figure 2A and B), presumably at least in part because Elovl6 deletion ameliorates the development of diet-induced insulin resistance [7]. Staining with antibodies against insulin and glucagon evidenced that the organization of β- and α-cells was preserved in *Elovl6*$^{-/-}$ islet (Figure 2C).

4.3 GSIS is improved in islets isolated from Elovl6$^{-/-}$ *mice*

Insulin secretion was estimated from islets isolated from WT and *Elovl6*$^{-/-}$ mice fed a chow or HFHS diet for 12 weeks (Figure 3A). Basal insulin secretion in low-glucose medium (2.8 mM) was similar between WT and *Elovl6*$^{-/-}$ mice in both nutritional states. Insulin secretion in high glucose medium (20 mM) was slightly decreased in the HFHS WT islets compared with the chow WT islets. Conversely, the HFHS *Elovl6*$^{-/-}$ islets exhibited significantly higher insulin secretion than the chow *Elovl6*$^{-/-}$ islets and HFHS WT islets. Insulin secretion stimulated by KCl was slightly, but significantly increased in the chow *Elovl6*$^{-/-}$ islets than that of WT islets.

Islet contents of insulin (Figure 3B) and triglycerides (TG) (Fig. 3c) of *Elovl6*$^{-/-}$ islets were indistinguishable from those of WT islets in the chow-fed state, and these contents in

islets were increased similar both WT and *Elovl6*$^{-/-}$ islets by HFHS diet. Consistent with enhanced GSIS, the ATP/ADP ratio of the HFHS *Elovl6*$^{-/-}$ islets was significantly increased than that of the HFHS chow islets (Figure 3D).

4.4 Elovl6 deficiency alters islet FA composition

To estimate the impact of *Elovl6* deficiency on FA composition in pancreatic islets, we analyzed FA composition of islets isolated from WT and *Elovl6*$^{-/-}$ mice fed the chow or HFHS diet for 12 weeks (Figure 4A). There were no significant differences in C16:0, C16:1, C18:0 or C18:1 FA composition between WT and *Elovl6*$^{-/-}$ mice on a chow diet. Islet FA composition of the HFHS *Elovl6*$^{-/-}$ mice showed decreased C18:0 and increased C18:1 compared with the HFHS WT mice. The ratio of C18:0/C16:0, the marker of the Elov6 activity, was consistently decreased in the islets of *Elovl6*$^{-/-}$ mice as compared to WT controls in both nutritional states. The ratio of C18:1/C18:0, the marker of the SCD activity, was increased the islets of the HFHS *Elovl6*$^{-/-}$ mice as compared to WT mice.

4.5 SREBP-1c and ATF-3 are downregulated in Elovl6$^{-/-}$ *islets.*

Expression of various genes in islets was estimated by

real-time PCR to elucidate the molecular mechanism for enhanced insulin secretion of the HFHS *Elovl6*$^{-/-}$ islets. Expression of lipogenic genes, including Elovl6 and sterol regulatory element binding protein (SREBP) -1c were not different but that of fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD)-1 were decreased in the HFHS WT mice compared with the chow WT mice (Figure 4B). Expression of SREBP-1c and its target genes FAS and SCD-1 were repressed in the islets of chow *Elovl6*$^{-/-}$ mice versus chow WT mice. Expression of SREBP-1c was decreased, but FAS and SCD-1 were increased in the islets of the HFHS *Elovl6*$^{-/-}$ mice versus the HFHS WT mice. Gene expression of uncoupling protein (UCP)-2, a SREBP target established to disrupt β-cell energy metabolism and insulin secretion [13] [14], was significantly increased in the islets of HFHS-fed WT mice, but not in the *Elovl6*$^{-/-}$ islets, explaining increased ATP/ADP ratio in the HFHS *Elovl6*$^{-/-}$ islets (Figure 4B). Expression of Pancreatic and duodenal homeobox factor-1 (PDX-1) and musculoaponeurotic fibrosarcoma oncogene family A (MafA), two master transcription factors regulating the insulin gene and GSIS in the mature β-cell[15], were decreased in islets of the chow *Elovl6*$^{-/-}$ mice versus chow WT mice, but coordinately repressed

by HFHS diet in both group of mice (Figure 4C). The expression levels of insulin receptor substrate (IRS)-2 and glucose transporter (GLUT)-2 were similar between WT and *Elovl6*$^{-/-}$ mice (Figure 4C). In WT mice, expression of activating transcription factor (ATF)-3, the integrated stress response gene involved in β-cell dysfunction and apoptosis [16,17,18,19,20], was induced significantly by the HFHS diet (Figure 4D). Strikingly, *Elovl6*$^{-/-}$ mice displayed markedly lower expression of ATF-3 by the HFHS diet.

Discussion

In the present study, we show that Elovl6 mRNA is expressed in mouse islets and β-cell line MIN6 cells. Real time-PCR analysis revealed that C57BL/6J mice significantly express Elovl6 in the islets. Elovl6 expression in isolated islets is comparable to that in brain, liver, and white adipose tissue (WAT) where Elovl6 plays a regulatory role [6] [7] [21]. The chow-fed *Elovl6*$^{-/-}$ mice do not display on altered islet morphology and β-/α-cell distribution, suggesting that Elovl6 is not required for the maintenance of islet architecture or β-cell mass in mice under normal conditions. However, analysis of the expression of several genes related to FA metabolism and islet function revealed decreased levels in the chow *Elovl6*$^{-/-}$ islets. SREBP-1c, FAS, and SCD-1 contribute to FA synthesis [22], and PDX-1 and MafA contribute to β-cell specific and glucose-responsive insulin gene transcription, suggesting that Elovl6 plays some roles in the islet function.

Our findings indicate that *Elovl6*$^{-/-}$ mice resist HFHS diet-induced islet hypertrophy at least in part as a result of enhanced insulin secretion. As shown previously, *Elovl6*$^{-/-}$ mice were protected from the HFHS diet-induced hepatic insulin

resistance[7]. In addition, IRS-2, reported to be important for growth and survival of β-cells, was sustained in islets of *Elovl6*$^{-/-}$ mice. Thus, the attenuated β-cell expansion in the HFHS *Elovl6*$^{-/-}$ mice could be due to the combined effects of the ameliorated insulin resistance and enhanced GSIS per β-cell.

Our results also indicated that the enhanced GSIS in the HFHS *Elovl6*$^{-/-}$ islets is not due to increased insulin contents or reduced islet triglyceride contents but mediated through the elevation of ATP/ADP ratio at high glucose concentration. UCP-2, which has the proton leak activity and negative effects on ATP synthesis, was markedly upregulated in the HFHS WT islets, whereas it was increased only slightly in the HFHS *Elovl6*$^{-/-}$ islets, indicating that the improved glucose metabolism in β-cells partially contributed to the increment of GSIS.

One important aspect of this study is the potential effect of cellular FA composition on β-cell function. Our results show that the ratio of stearate in pancreatic islet is higher than that of other tissues such as liver, adipose tissue, and muscle. The relative amounts of C18:0 and the ratio of C18:0/C16:0 were significantly reduced by Elovl6 deficiency after the HFHS diet. Furthermore, the increase in the ratio of C18:1 to C18:0, which is consistent with the increased expression of SCD1 in the islets

of HFHS *Elovl6*$^{-/-}$ mice, could rescues saturated FAs-induced islets dysfunction. Recently, Green *et al.* clearly demonstrated that knockdown of Elovl6 or overexpression of SCD2 in INS1 β-cells attenuated palmitate-induced ER stress and apoptosis [23]. These results suggest that the fatty acid composition of *Elovl6*$^{-/-}$ islets, namely the reduction of C18 FAs and the high ratio of C18:1/C18:0, may be protective against islet lipotoxicity in HFHS-fed mice.

Suppression of ATF-3 expression in islets of the HFHS *Elovl6*$^{-/-}$ mice could play an important role in molecular mechanism for enhanced GSIS. It has been demonstrated that ATF-3 is induced by signals relevant to the pancreatic β-cell dysfunction such as proinflammatory cytokines, ROS, ER stress, and high concentration of glucose and FAs [16]. In addition, ATF-3 is involved in the reduction of PDX-1, which leads to pancreatic β-cell dysfunction and apoptosis [18]. Considering that, suppression of this factor could explain the improved β-cell function that was observed in the HFHS *Elovl6*$^{-/-}$ mice, although the molecular link between Elovl6 and ATF-3 remains to be elucidated.

The phenotypes observed in *Elovl6*$^{-/-}$ mice leave unanswered the question of the role of Elovl6 in β-cell. In our

study, it was not possible to distinguish in which islet cell type Elovl6 mRNA is expressed. Since deficiency of Elovl6 raises numerable changes caused from phenotype of liver or the other tissues [7] [24] [25] [26], pancreatic β-cell-specific deletion will be required to further determine the precise role of Elovl6 on pancreatic β-cells.

In conclusion, our results show that Elovl6 is expressed in pancreatic islet and β-cell and that Elovl6 deficiency inhibits impaired GSIS in obese mice. These findings suggest that this FA elongase may be involved in the regulation of islet function and open promising new perspectives for the prevention of T2D. Further studies are needed to clarify the details and the precise mechanisms of a link between FAs and insulin secretion.

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Figure Legends

Fig. 1. Elovl6 is expressed in pancreatic islets and β-cells. (A) Elovl6 mRNA levels in the pancreatic islets and various tissues of 14-16 week-old male C57BL/6J mice (n = 3). (B) Elovl6 mRNA levels in Hepa1c1c7 hepatoma cells and MIN6 cells (n = 4-5).

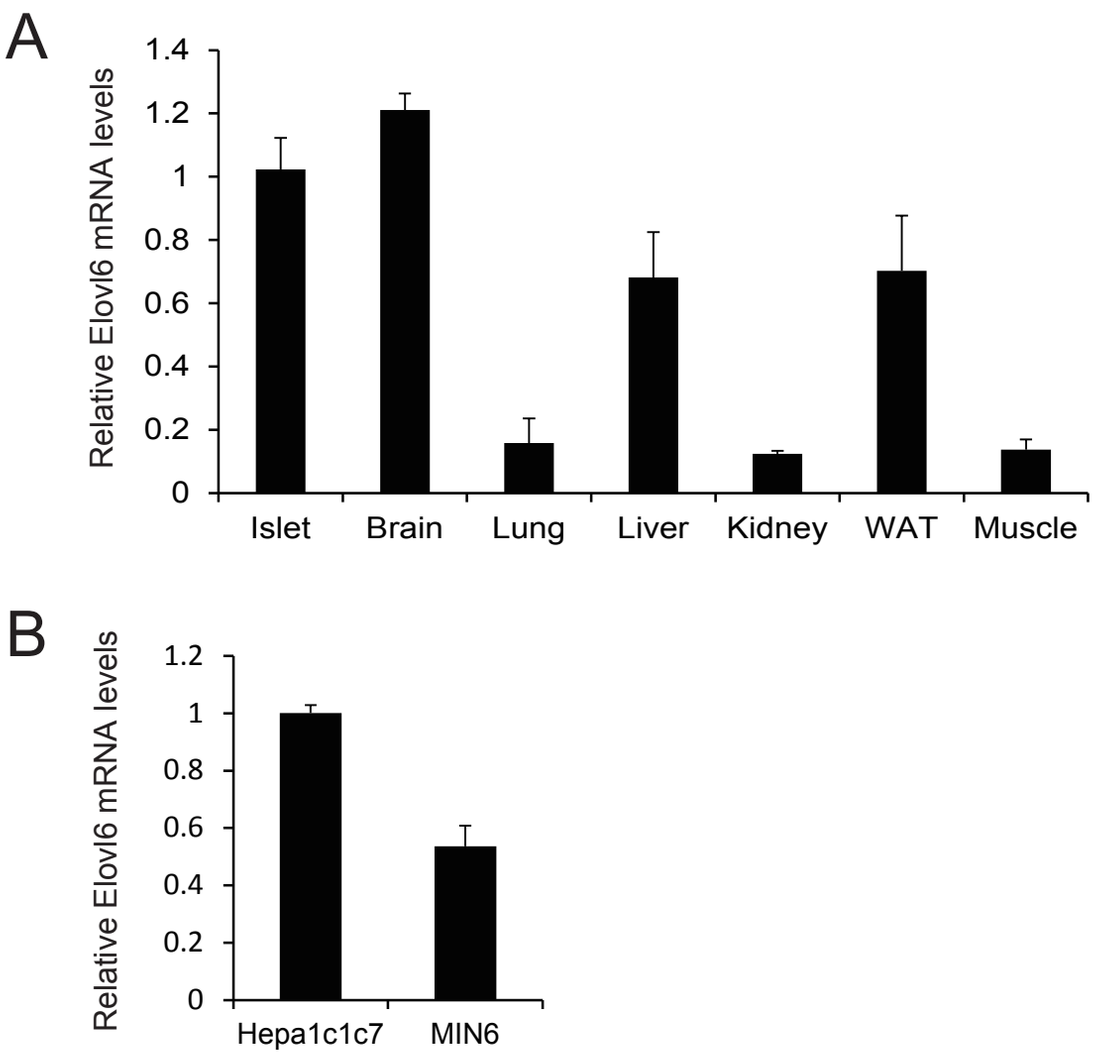
Fig. 2. Islet morphology of *Elovl6*$^{-/-}$ mice. (A) Representative pancreatic sections of wild-type (WT) and *Elovl6*$^{-/-}$ mice fed a chow or HFHS diet for 12 weeks stained with hematoxylin and eosin for histological analysis. (B) Islet mass under HFHS diet were quantified using NIH image. At least 30 islets from each pancreas were quantified and total number of islets scanned was 275-291 for each genotype (n = 7). ** P < 0.01. (C) Representative pancreatic sections of WT and *Elovl6*$^{-/-}$ mice fed a HFHS diet stained with insulin (red) and glucagon (green). Scale bar, 50μm.

Fig. 3. Elovl6 deficiency results in enhanced islet glucose-stimulated insulin secretion and increased ATP/ADP ratio. (A) Glucose- or KCl- stimulated insulin secretion from

isolated islets of WT and *Elovl6*$^{-/-}$ mice fed a chow or HFHS diet for 12 weeks (n = 6). (B-D) Insulin contents (n = 4) (B), triglyceride contents (n = 4) (C), and ATP/ADP ratio (n = 5-6) (D) in isolated islets of WT and *Elovl6*$^{-/-}$ mice fed a chow or HFHS diet for 12 weeks. * P < 0.05, ** P < 0.01.

Fig. 4. FA composition and gene expression profile in isolated islets from WT and *Elovl6*$^{-/-}$ mice fed a chow or HFHS diet. (A) Islet FA composition of WT and *Elovl6*$^{-/-}$ mice fed a chow or HFHS diet for 12 weeks (n = 3-4). * P < 0.05, # P < 0.01. (B-D) mRNA levels of genes involved in FA metabolism (B), important for β-cell function (C), and ATF3 (D) in isolated islets from WT and *Elovl6*$^{-/-}$ mice fed a chow or HFHS diet for 12 weeks (n = 4-8). * P < 0.05, ** P < 0.01.

Figure 1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1.2
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0.8
0.6
0.4
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0
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Brain
Lung
Liver
Kidney
WAT
Muscle
B
Relative Elovl6 mRNA levels
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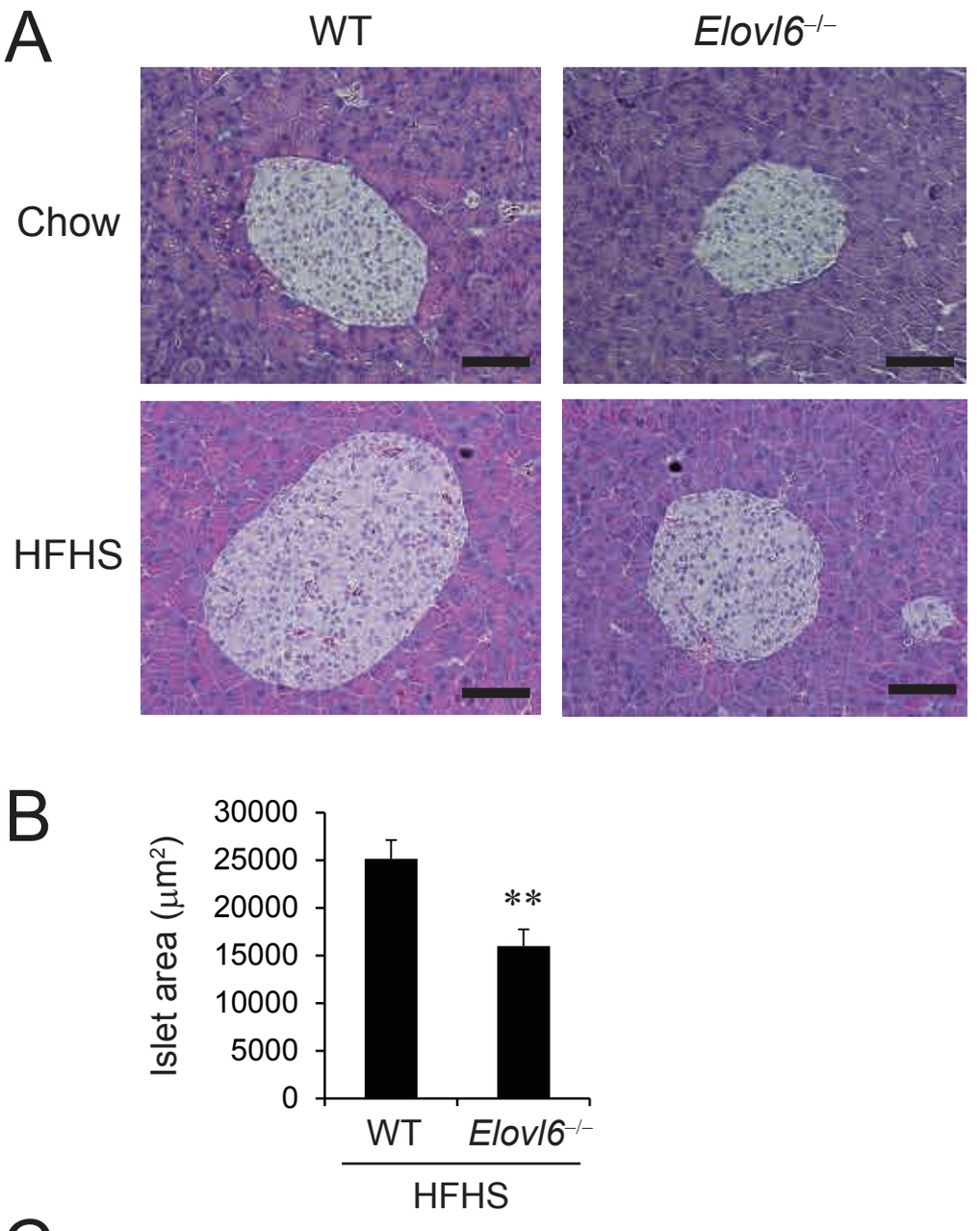
0.8
0.6
0.4
0.2
0
Hepa1c1c7
MIN6

Figure 2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WT
Elovl6−/−
Chow
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B
Islet area (μm2)
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25000
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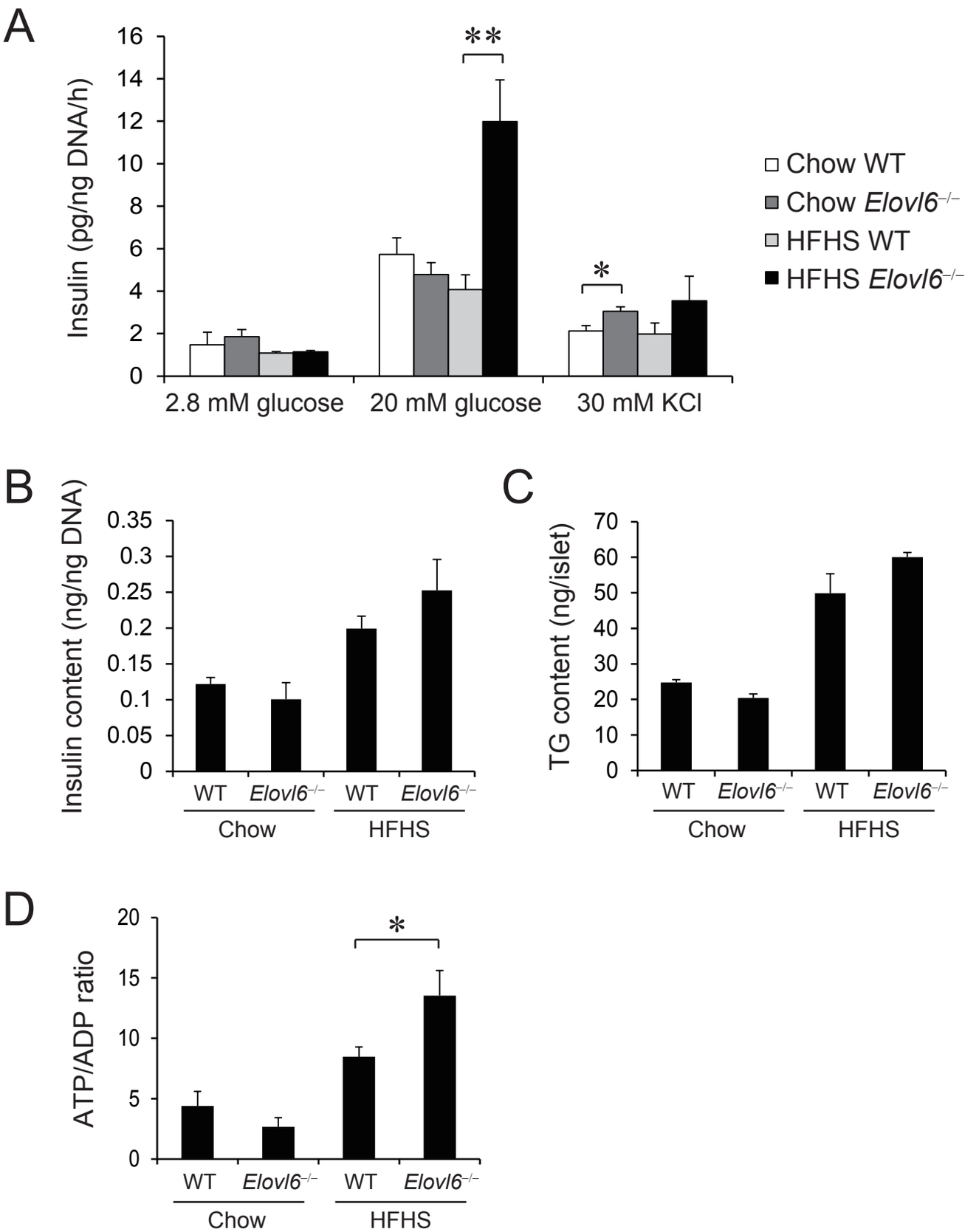
WT
Elovl6−/−
HFHS
C
WT
Elovl6−/−

Figure 3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14
12
10
8
6
4
2
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*
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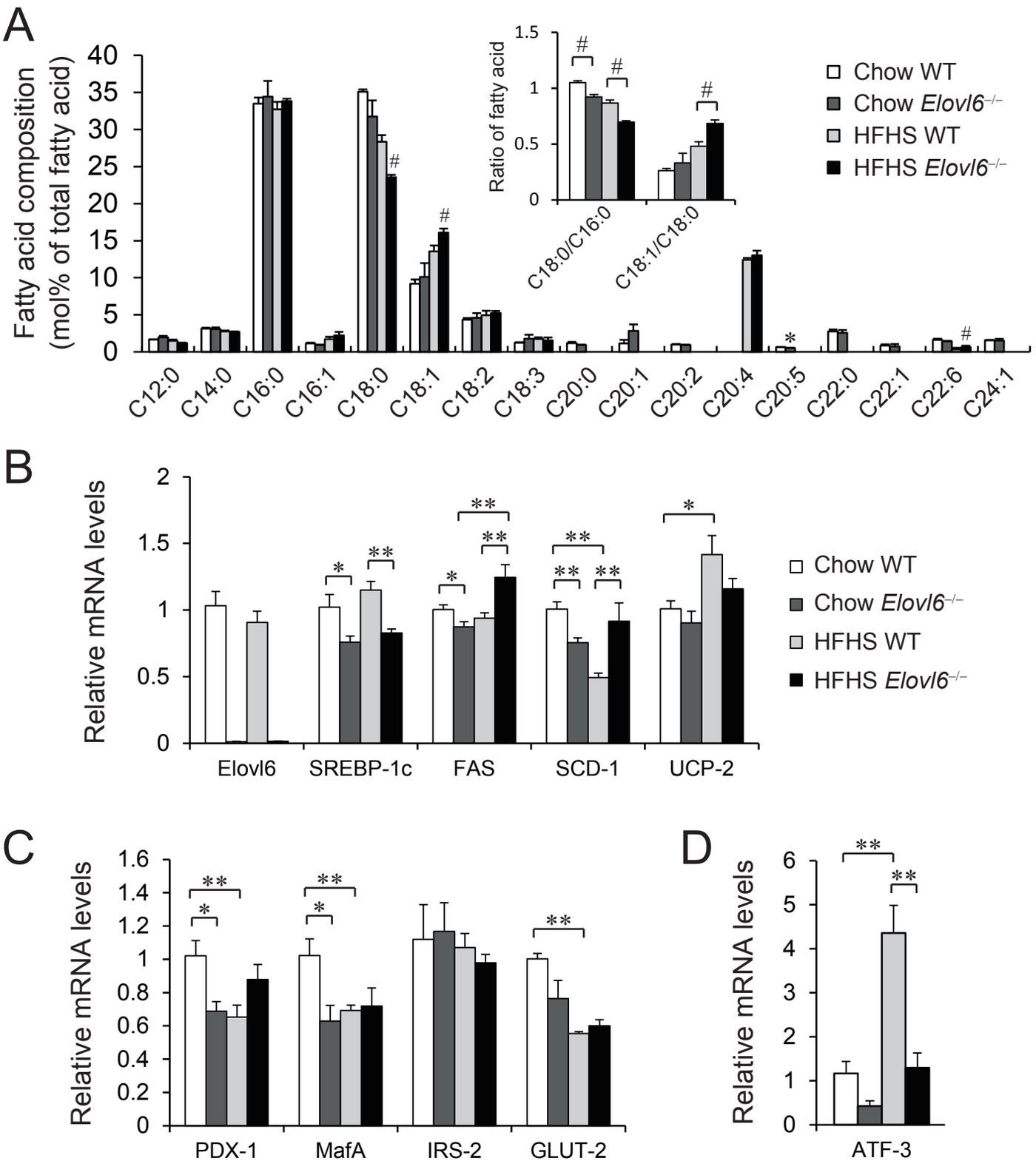
20 mM glucose
30 mM KCl
Chow WT
Chow Elovl6−/−
HFHS WT
HFHS Elovl6−/−
B
Insulin content (ng/ng DNA)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
WT
Elovl6−/−
WT
Elovl6−/−
Chow
HFHS
C
TG content (ng/islet)
70
60
50
40
30
20
10
0
WT
Elovl6−/−
WT
Elovl6−/−
Chow
HFHS
D
ATP/ADP ratio
20
15
10
5
0
*
WT
Elovl6−/−
WT
Elovl6−/−
Chow
HFHS

Figure 4


A
Fatty acid composition (mol% of total fatty acid)
Ratio of fatty acid
C18:0/C16:0
C18:1/C18:0
Chow WT
Chow Elovl6−/−
HFHS WT
HFHS Elovl6−/−
C12:0
C14:0
C16:0
C16:1
C18:0
C18:1
C18:2
C18:3
C20:0
C20:1
C20:2
C20:4
C20:5
C22:0
C22:1
C22:6
C24:1
B
Relative mRNA levels
Elovl6
SREBP-1c
FAS
SCD-1
UCP-2
C
Relative mRNA levels
PDX-1
MafA
IRS-2
GLUT-2
D
Relative mRNA levels
ATF-3