Cell Metabolism
Short Article

Autophagy Is Important in Islet Homeostasis and Compensatory Increase of Beta Cell Mass in Response to High-Fat Diet

Chie Ebato,[1,6] Toyoyoshi Uchida,[1,6] Masayuki Arakawa,[1] Masaaki Komatsu,[3,4,5] Takashi Ueno,[3] Koji Komiya,[1] Kosuke Azuma,[1] Takahisa Hirose,[1,2] Keiji Tanaka,[4] Eiki Kominami,[3] Ryuzo Kawamori,[1,2] Yoshio Fujitani,[1,2,*] and Hirotaka Watada[1,*]

[1]Department of Medicine, Endocrinology, and Metabolism
[2]Center for Therapeutic Innovations in Diabetes
[3]Department of Biochemistry
Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[4]Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613, Japan
[5]PRESTO, Japan Science and Technology Corporation, Kawaguchi 332-0012, Japan
[6]These authors contributed equally to this work
*Correspondence: fujitani@juntendo.ac.jp (Y.F.), hwatada@juntendo.ac.jp (H.W.)
DOI 10.1016/j.cmet.2008.08.009

SUMMARY

Autophagy is an evolutionarily conserved machinery for bulk degradation of cytoplasmic components. Here, we report upregulation of autophagosome formation in pancreatic beta cells in diabetic *db/db* and in nondiabetic high-fat-fed C57BL/6 mice. Free fatty acids (FFAs), which can cause peripheral insulin resistance associated with diabetes, induced autophagy in β cells. Genetic ablation of *atg7* in β cells resulted in degeneration of islets and impaired glucose tolerance with reduced insulin secretion. While high-fat diet stimulated β cell autophagy in control mice, it induced profound deterioration of glucose tolerance in autophagy-deficient mutants, partly because of the lack of compensatory increase in β cell mass. These findings suggest that basal autophagy is important for maintenance of normal islet architecture and function. The results also identified a unique role for inductive autophagy as an adaptive response of β cells in the presence of insulin resistance induced by high-fat diet.

INTRODUCTION

Recent studies have shown that progressively decreased pancreatic β cell function and β cell mass are common features of subjects with type 2 diabetes mellitus (Butler et al., 2003; Rhodes, 2005). Early in the disease, pancreatic β cells overproduce insulin to compensate for insulin resistance but eventually fail to do so, and this may result in cell death, with clinical manifestations of diabetes. Because of the high secretory activity, β cells are constantly exposed to various kinds of stresses, such as glucolipotoxicity and oxidative stress (DeFronzo, 2004; Unger and Zhou, 2001). However, little is known so far about the molecular mechanisms by which β cells cope with various stresses and cellular damage associated with the high capability of insulin secretion, which may be accelerated by increased insulin resistance.

Autophagy is a catabolic process that involves the degradation of cellular components through the lysosomal machinery. It is a tightly regulated process and plays an important role in cell growth, development, and homeostasis, where it helps to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular components (Levine and Klionsky, 2004). The principal role of autophagy is to reallocate nutrients from unnecessary processes to more pivotal processes required for survival (Kuma et al., 2004). In addition, a low level of constitutive autophagy is also important for maintaining the quality of proteins and organelles, in order to maintain cell functions, as recently reported in various organs, including the liver (Komatsu et al., 2005), brain (Hara et al., 2006; Komatsu et al., 2006), and heart (Nakai et al., 2007). Thus, autophagy functions as a cell-protective mechanism. Autophagy is upregulated when cells are preparing to rid themselves of damaging cytoplasmic components, for instance, during infection or protein aggregate accumulation (Levine and Kroemer, 2008). While it has been reported that significant number of autophagic vacuoles are found in β cells of ZDF rats, a rodent model of type 2 diabetes (Li et al., 2006), there is little information on the physiopathological roles of autophagy in β cells, and no causal link has been reported between autophagy and pathogenesis of diabetes. In the present study, we describe the generation of a conditional knockout mouse of *Atg7*. We then used these mice to examine the role of autophagy in adult pancreatic β cells. The results suggest that autophagy is important for maintaining normal islet homeostasis and compensatory β cell hyperplasia in response to high-fat (HF) diet intake.

RESULTS

Induction of Autophagy in Pancreatic β Cells

We first examined whether autophagic vacuoles are present in mouse pancreatic β cells under various pathophysiological conditions. Electron microscopic (EM) analysis indicated that

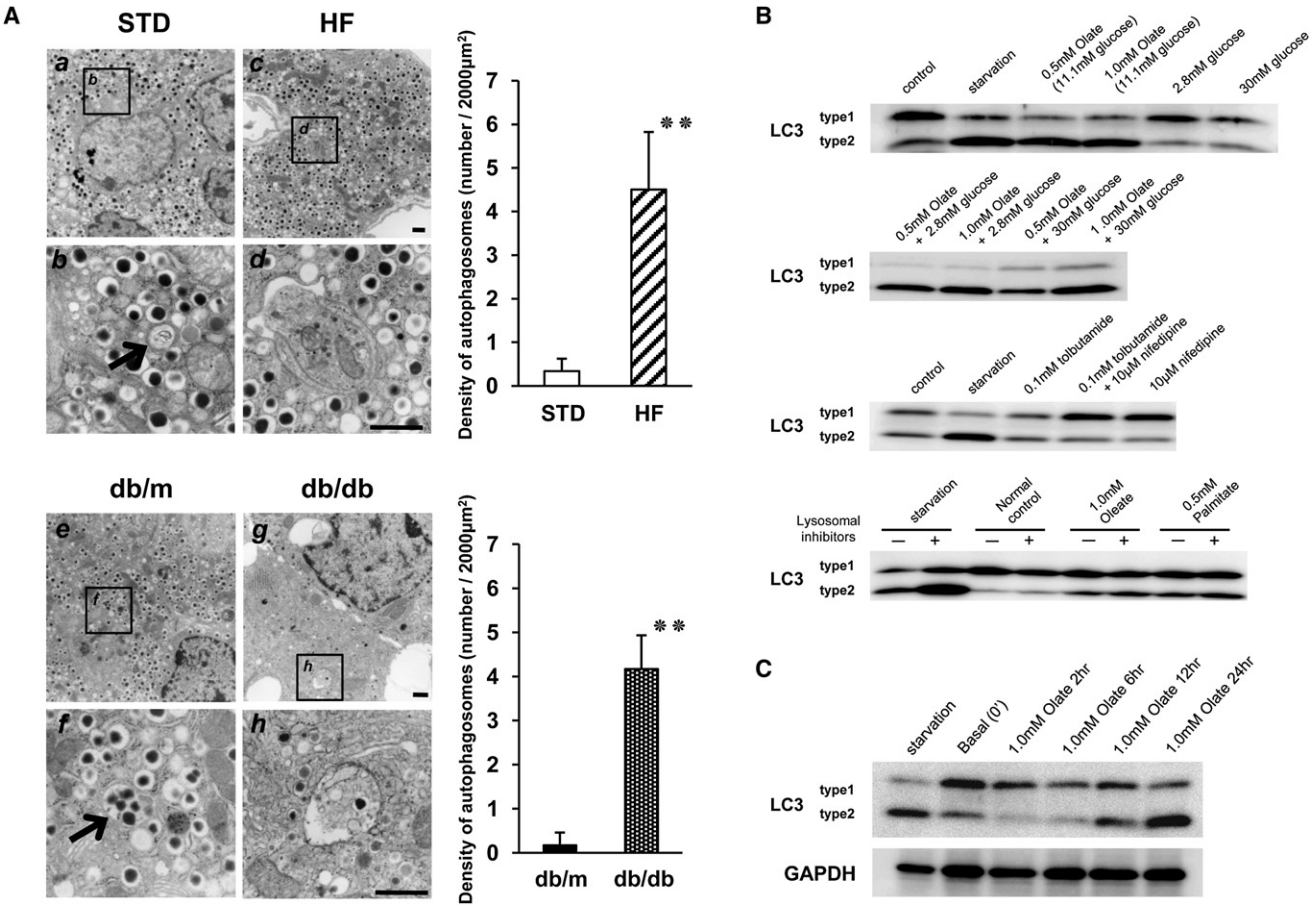

Figure 1. Upregulation of Autophagosome Formation in HF-Fed and in Diabetic Mice

(A) Electron microscopic (EM) analysis revealed autophagosome formation in β cells of 20-week-old mice fed standard (STD) diet (*a* and *b*) or HF diet for 12 weeks (from 8 to 20 week of age) (*c* and *d*). Beta cells in 10-week-old *db/misty* (*db/m*) (*e* and *f*) and *db/db* (*g* and *h*) mice were examined. Scale bars represent 1 μm. Density of autophagosomes was calculated, and data are presented as mean ± SEM of three mice for each group. **p < 0.01 versus controls.

(B) FFA but not high glucose can induce autophagy in INS-1 cells. For "control," cells were incubated in RPMI 1640 supplemented with FCS and amino acids. For "starvation," cells were incubated for 2 hr in Hank's buffer free of serum and amino acids.

(C) Time course of FFA-induced autophagy. So that accumulation of LC3-II could be visualized, the lysosome inhibitors pepstatin-A (10 μg/ml) and E-64-d (10 μg/ml) were added to the culture medium for the last 2 hr period unless otherwise indicated (B and C). Data for western blots are presented as a representative from more than five independent experiments.

autophagosomes were hardly observed in β cells of C57BL/6 mice and in *db/misty* mice, and those identified were of relatively small size (~0.5 μm) (Figure 1A, *b* and *f*, arrows). In contrast, analysis of β cells in *db/db* and in C57BL/6 mice that had been fed HF diet for 12 weeks showed active formation of large autophagosomes (0.5–2 μm in diameter), which were recognized as double-membrane structures containing multilamellar structures, mitochondria, and insulin granules (Figure 1A, *c*, *d*, *g*, and *h*). Different from what is seen in most other organs (Kuma et al., 2004), overnight starvation of C57BL/6 mice did not result in any increase in autophagic vacuole formation in β cells (data not shown). On the other hand, ex vivo starvation of islets isolated from C57BL/6 mice resulted in increased formation of small autophagic vacuoles, similar to those observed in C57BL/6 mice fed standard chow diet (data not shown).

In the next step, we investigated the signals that stimulate autophagy in β cells. Marked induction of autophagy, comparable to the levels induced by amino acid deprivation, was observed in INS-1 β cells exposed to free fatty acid (FFA) (0.5 and 1 mM olate) for 24 hr, assessed by conversion of microtubule-associated protein 1 light chain 3-I (LC3-I) to LC3-II (Figure 1B, top). Increase in LC3-II levels in the presence of lysosomal inhibitors (Figure 1B, bottom) suggested increase in autophagic flux by FFA stimulation. This finding is consistent with a previous report that cultured human islets exposed to FFAs showed autophagic vacuole formation (Lupi et al., 2002), although they did not observe increased autophagic flux. FFA exposure for more than 12 hr induced autophagy in INS-1 cells, whereas FFA exposure for a shorter time had no significant effects (Figure 1C). The FFA-related autophagosome induction was further confirmed by the observation of extensive induction of dot-like LC3-positive structures, representing autophagosomes, in INS-1 cells exposed to FFA (data not shown). In contrast, exposure of these cells to high glucose (from 2.8 to 30 mM) or to tolbutamide did not induce autophagy (Figure 1B, top and third panel). In addition, high glucose did not augment autophagy levels induced by FFA

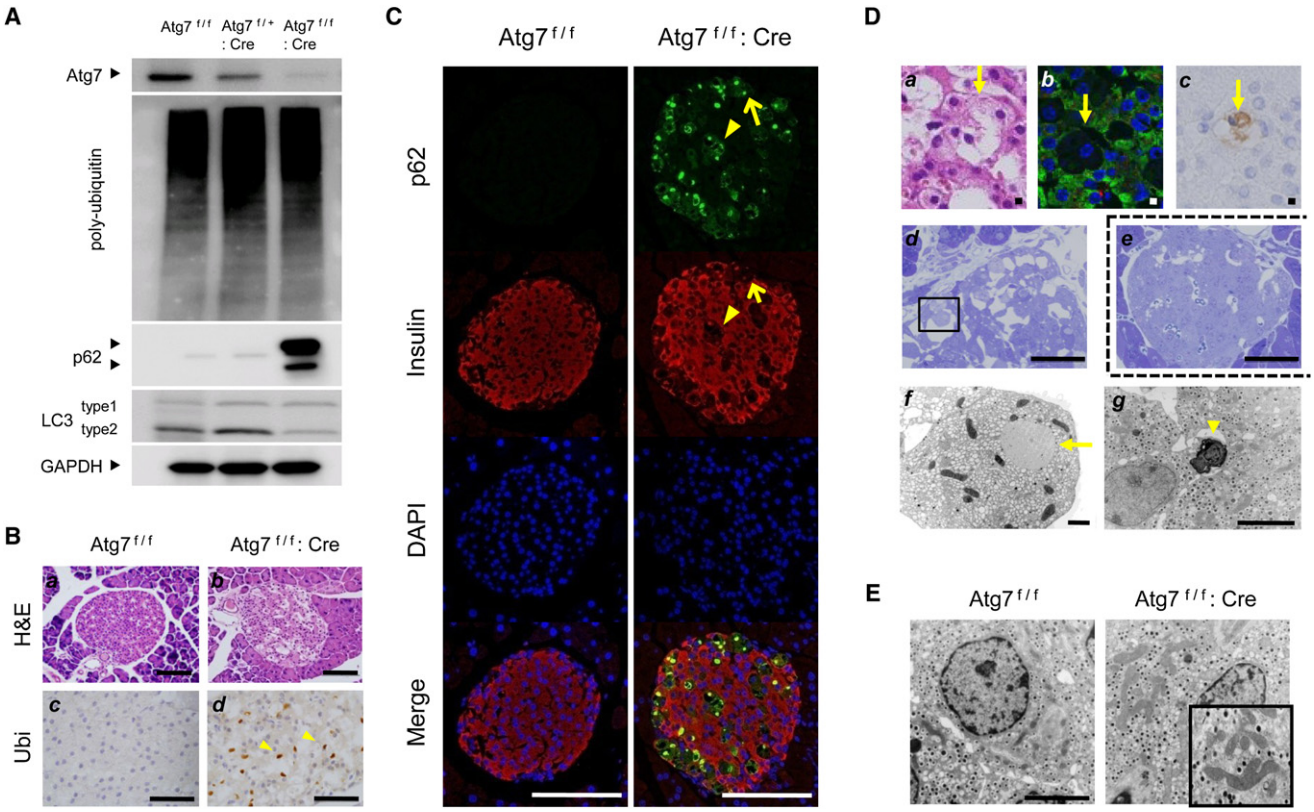

Figure 2. Beta Cell-Specific Inactivation of *Atg7* and Islet Morphological Abnormalities in Mutant Mice

(A) Pancreatic islets were isolated from 16-week-old *Atg7*$^{f/f}$, *Atg7*$^{f/+}$*:RIP-Cre*, and *Atg7*$^{f/f}$*:RIP-Cre* (*Atg7*$^{f/f}$*:Cre*) mice. Cell lysates were subjected to immunoblot analysis of Atg7, poly-ubiquitin, p62, and LC3.

(B) Representative images of H&E staining and immunostaining with ubiquitin antibodies of 20-week-old *Atg7*$^{f/f}$ and *Atg7*$^{f/f}$*:Cre* islets. Arrowheads in *d* indicate ubiquitin-positive aggregates. Scale bars represent 100 μm (*a* and *b*) and 50 μm (*c* and *d*).

(C) Double staining for insulin (red) and p62 (green) of 20-week-old islets. Nuclei were labeled by DAPI (blue). Colocalization of p62 and insulin-depleted β cells is indicated by arrowheads and arrows. Scale bars represent 100 μm.

(D) Magnified images of degenerative lesions in *Atg7*$^{f/f}$*:Cre* islets. H&E staining (*a*), insulin (green) and DAPI (blue) staining (*b*), caspase-3 staining (*c*), Toluidine-blue staining of *Atg7*$^{f/f}$*:Cre* (*d*), and *Atg7*$^{f/f}$ (*e*) islets. Scale bars represent 5 μm (*a*–*c*) and 50 μm (*d* and *e*). Electron microscopic analysis of a degenerative β cells indicated by the small square in *d* is shown (*f*, *g*). Note the presence of inclusion bodies (arrows in *f* and *g*) and concentric membranous structures in β cells of *Atg7*$^{f/f}$*:Cre* (arrowhead in *g*). Scale bars represent 5 μm.

(E) Electron micrographs of β cells. Deformed mitochondria were noted in *Atg7*-deficient β cells (small inset). Scale bars represent 5 μm.

(Figure 1B, second panel). Since FFA is a key mediator of peripheral insulin resistance in diabetes (DeFronzo, 2004; Unger and Zhou, 2001), upregulation of β cell autophagy by FFA suggests its potential role in the regulation of β cell function in diabetes or obesity-associated conditions.

Basal Autophagy Is Indispensable for the Maintenance of Normal Islet Architecture

To determine the physiological role of autophagy in pancreatic β cells, we generated mice deficient for *Atg7*, an essential gene for autophagosome formation, specifically in pancreatic β cells. We crossed mice carrying an *Atg7*^{flox} allele (Figure S1 available online) with transgenic mice that expressed the *Cre* recombinase in a β cell-specific manner (*Rip-Cre*) (Gannon et al., 2000). The resulting *Atg7*$^{flox/flox}$*:Rip-Cre* mice (referred hereafter to as *Atg7*$^{f/f}$*:Cre*) were indistinguishable in body weight and food intake (Figure S2) from age-matched control *Atg7*$^{f/f}$ littermates (from newborn until at least 16 weeks of age). In *Atg7*$^{f/f}$*:Cre* mice, we observed approximately 70% reduction in Atg7 protein level in isolated islet preparation (Figure 2A). Reduction of autophagy in *Atg7*$^{f/f}$*:Cre* mice was confirmed by suppression of Atg7-dependent conversion of LC3-I to LC3-II (a phosphatidylethanolamine conjugate) and accumulation of p62 and polyubiquitin. These changes were also confirmed by immunohistochemical staining of pancreata of 20-week-old *Atg7*$^{f/f}$*:Cre* mice for polyubiquitin, insulin, and p62 (Figures 2B and 2C).

Haematoxylin and eosin (H&E) staining of islets of 20-week-old *Atg7*$^{f/f}$ and *Atg7*$^{f/f}$*:Cre* mice revealed degenerative changes in islets. The mutant islets contained multiple cyst-like structures, measuring 15–20 μm in diameter, which were detectable as early as 4 weeks of age and increased in number in an age-dependent manner (Figure 2D, *a*, *b*, and *c*; arrows and Figure S3). These cyst-like structures, occasionally associated with caspase-3-positive apoptotic cells (Figure 2D, *c*), were not fat-laden adipocytes, judged by oil red O staining (data not shown). Toluidine-blue staining of thin-sliced sections showed that mutant islets, but

not control islets, contained enlarged "balloon-like" cells with a pale-stained cytoplasm located at the islet periphery (Figure 2D, small inset in *d*), which corresponded to the cyst-like structures in H&E sections. EM analysis indicated that the "balloon-like" cells were most likely degenerating β cells, on the basis of the observation that they were relatively rich in mitochondria and always associated with a small number of insulin vesicles (Figure 2D, *f* and *g*). These cells frequently contained inclusion bodies (Figure 2D, arrow in *f*) and aberrant concentric membranous structures (Figure 2D, arrowhead in g), similar to those observed in *Atg7*-deficient livers (Komatsu et al., 2005). The accumulation of inclusion bodies increased in an age-dependent manner (data not shown). Despite the presence of highly degenerated β cells, referred here as "balloon-like" cells, many normal-looking insulin-positive cells were also found in the mutant islets. Double staining for insulin and glucagon revealed that the normal mantle-like structure of adult islets in *Atg7$^{f/f}$:Cre* mice was generally maintained (data not shown).

Autophagy is responsible for constitutive protein turnover in quiescent hepatocytes even under nutrient-rich conditions, and defective autophagy is associated with accumulation of large, ubiquitin-containing inclusion bodies with overexpression of LC3-binding protein p62 in liver and brain (Komatsu et al., 2005, 2006, 2007). The present data suggest that ubiquitin and p62 are also involved in the formation of protein aggregates in β cells. Insulin and p62 double staining showed colocalization of insulin-depleted ballooning β cells with p62 (Figure 2C, arrows and arrowheads), suggesting that the expression of p62 reflects damaged β cells. The mutant β cells contained deformed and branched mitochondria (Figure 2E). Similar disorganization of mitochondria was reported in *Atg7*-deficient hepatocytes and neural cells and *Atg5*-deficient cardiomyocytes (Komatsu et al., 2005; Nakai et al., 2007). These results suggest that the turnover of mitochondria is commonly regulated by autophagy in these organs.

Impaired Insulin Secretion in *Atg7*-Deficient Mice

Next, we evaluated whether the changes in islet morphology were associated with changes in β cell function. The nonfasting blood glucose level of *Atg7$^{f/f}$:Cre* mice was significantly higher than that of control mice throughout the study (Figure 3A). Intraperitoneal glucose tolerance tests (IPGTT) showed glucose intolerance with reduced insulin secretion in *Atg7$^{f/f}$:Cre* mice (Figure 3B). Insulin tolerance test (ITT) revealed that insulin sensitivity of *Atg7$^{f/f}$:Cre* mice was comparable with that of the control group (Figure S4A). These results suggest that the impaired glucose tolerance in *Atg7$^{f/f}$:Cre* mice is primarily caused by insufficient glucose-stimulated insulin secretion. Glucose-stimulated insulin release in isolated islets was significantly reduced in *Atg7$^{f/f}$:Cre* mice compared to control mice (Figure 3C). Given that there was no difference in KCl-induced insulin secretion between the two groups, the deficiency of signaling events upstream of K_{ATP} channel closure appears to be responsible for the defects in glucose-stimulated insulin secretion. Since the mitochondrion is a key machinery involved in the regulation of glucose-induced insulin secretion in β cells (Silva et al., 2000), the defective insulin secretory capacity of mutant β cells could be due to accumulation of dysfunctional mitochondria in *Atg7$^{f/f}$:Cre* islets. To test this possibility, we measured glucose-stimulated ATP production in isolated islets with the ATP assay (Fujimoto et al., 2002). The significantly lower ATP production from *Atg7$^{f/f}$:Cre* islets (1.16-fold increase relative to unstimulated islets) relative to control islets (1.60-fold increase, $p < 0.05$) suggested a defective mitochondrial function under standard (STD) diet conditions (Figure 3D).

Inductive Beta Cell Autophagy as an Adaptive Response against Increased Insulin Resistance

Next, we investigated the physiopathological effect of induced autophagy in the presence of HF-diet-induced insulin resistance. Eight-week-old *Atg7$^{f/f}$:Cre* and *Atg7$^{f/f}$* mice were fed HF diet for 12 weeks. During the period, both mice groups consumed the same amount of food and displayed similar weight gain (Figures S5A and S5B). HF-fed *Atg7$^{f/f}$:Cre* mice had significantly higher nonfasting glucose levels and severely impaired glucose tolerance compared with HF-fed *Atg7 $^{f/f}$* mice (Figures 4A and 4B). These results indicate that under HF diet, the difference in glucose tolerance between *Atg7$^{f/f}$:Cre* and *Atg7$^{f/f}$* mice seems to become larger than under STD diet (Figures 3A and 3B versus Figures 4A and 4B).

Morphometric analysis showed that the islet cell area during the 12 week HF diet increased by 2-fold in *Atg7$^{f/f}$* mice but not in *Atg7$^{f/f}$:Cre* mice, indicating failure of compensatory hyperplasia of β cells in the latter group (Figure 4C). Furthermore, a smaller number of Ki67^{+} cells and accelerated accumulation of caspase-3^{+} apoptotic cells were noted in the islets of HF-laden *Atg7$^{f/f}$:Cre* mice, compared with *Atg7$^{f/f}$* mice. These findings may account for the lack of β cell hyperplasia (Figures 4D and 4E). However, the concentration of immunoreactive insulin (IRI) was higher in *Atg7$^{f/f}$:Cre* mice despite the lower β cell mass (Figure 4B). Since the insulin ELISA used in this study does not discriminate between insulin and proinsulin, it is possible that a significant amount of the detected IRI is secreted as biologically inactive proinsulin, as reported in subjects with type 2 diabetes (Roder et al., 1999).

In agreement with the increased apoptosis described above, degeneration of islets in *Atg7$^{f/f}$:Cre* mice became more pronounced when these mice were fed HF diet; the number of degenerative vacuoles and p62 immunoreactivity were increased (Figure 2C versus Figure 4G). These degenerative changes were associated with upregulation of polyubiquitinated proteins and p62, compared with STD-diet-fed mice (Figure S6).

Recently, we reported that liver injury caused by autophagy deficiency is highly dependent on accumulation of p62 and that this accumulation is associated with the activation of detoxifying enzymes, including glutathione 300 S-transferase (GST) families, cytochrome P450 families, and NAD(P)H dehydrogenase quinone 1 (Nqo1) (Komatsu et al., 2007). In the next step, we quantitated the expression levels of downstream targets for p62 accumulation, including *Nqo1*, *GST-mu*, and *GST-theta* in *Atg7$^{f/f}$:Cre* and *Atg7$^{f/f}$* mice and found that two of them were moderately increased (Figure 4H). These results suggest the possible involvement of p62 in β cell injury caused by autophagy deficiency, but this needs further investigation in future studies.

DISCUSSION

Our study showed that autophagic vacuole formation is markedly upregulated in the presence of insulin resistance associated

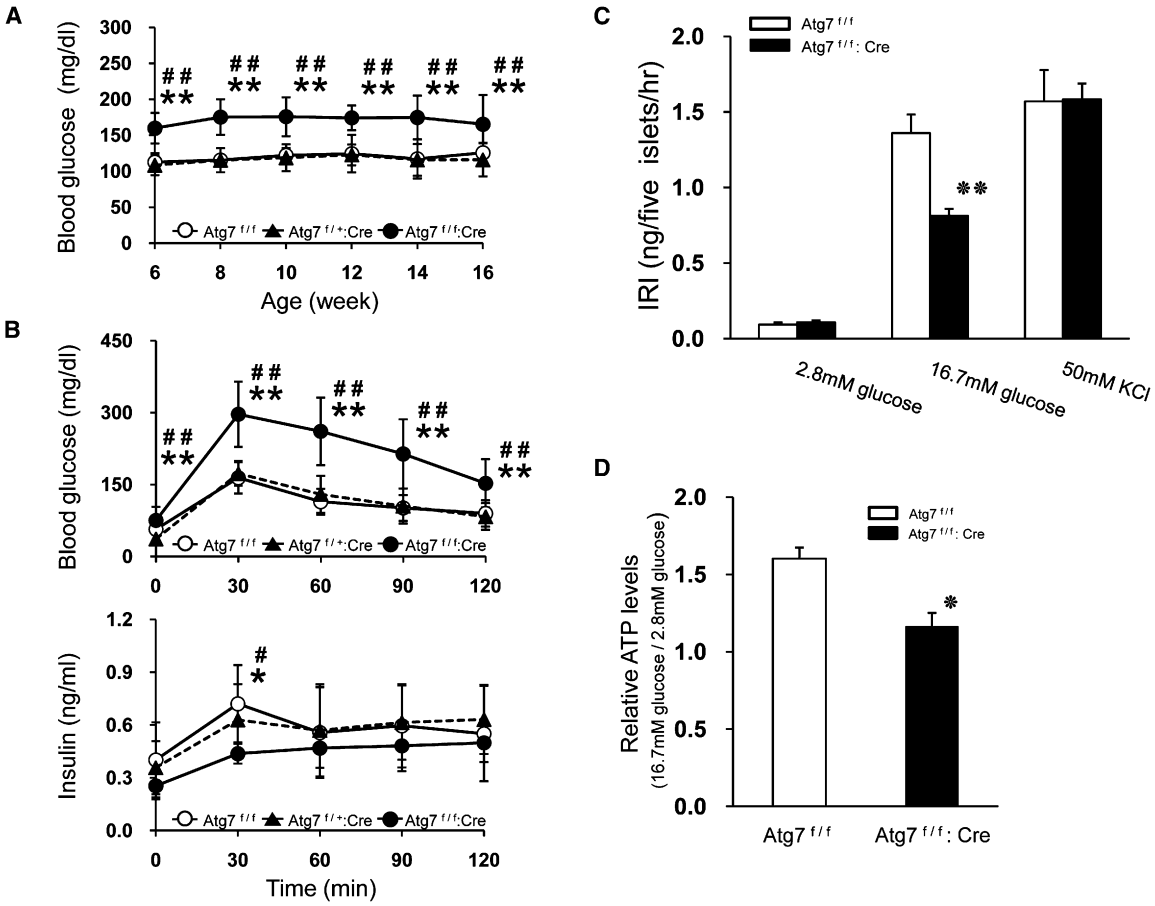

Figure 3. Impaired Glucose Tolerance and Defective Insulin Secretion in *Atg7$^{f/f}$:Cre* Mice

(A) Nonfasting glucose levels of STD-diet-fed *Atg7$^{f/f}$* (n = 10), *Atg7$^{f/+}$:Cre* (n = 9), and *Atg7$^{f/f}$:Cre* (n = 10) mice.

(B) Blood glucose concentrations and serum insulin levels measured during IPGTT at the age of 16 weeks. Open circles, *Atg7$^{f/f}$* mice (n = 9); solid triangles, *Atg7$^{f/+}$:Cre* mice (n = 9); solid circles, *Atg7$^{f/f}$:Cre* mice (n = 9). Data are mean ± SEM. *p < 0.05, **p < 0.01 (*Atg7$^{f/f}$* versus *Atg7$^{f/f}$:Cre*); $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 (*Atg7$^{f/+}$:Cre* versus *Atg7$^{f/f}$:Cre*).

(C) Insulin secretion induced by insulinotropic agents in batch-incubated pancreatic islets isolated from STD-diet-fed *Atg7$^{f/f}$* (open bars) and *Atg7$^{f/f}$:Cre* (solid bars) mice at 16 weeks of age. Data are mean ± SEM. **p < 0.01 (*Atg7$^{f/f}$* versus *Atg7$^{f/f}$:Cre*). Data were obtained from at least eight replicates for each group.

(D) Glucose-stimulated ATP production from isolated islets of STD-diet-fed *Atg7$^{f/f}$* and *Atg7$^{f/f}$:Cre* mice. Data are presented as fold increase of ATP levels induced by glucose stimulation (16.7 mM glucose) relative to the levels before stimulation (2.8 mM glucose). *p < 0.05 (*Atg7$^{f/f}$* versus *Atg7$^{f/f}$:Cre*). Data were obtained from at least eight replicates for each group.

with type 2 diabetes or induced by HF diet. To examine the role of autophagy in β cells, we inactivated *Atg7* selectively in β cells. Loss of autophagy in β cells resulted in age-dependent accumulation of ubiquitinated proteins and damaged organelles, which may cause dysfunction and degeneration of β cells. The results suggest that ubiquitinated proteins, which otherwise form toxic aggregates, are constantly cleared by autophagy, and that autophagy can act as a defense mechanism against various types of cellular damage in β cells. This notion is in agreement with a recent study showing that diabetes-induced oxidative stress can induce ubiquitination and storage of proteins into cytoplasmic aggregates of pancreatic β cells (Kaniuk et al., 2007). Recently, Marsh et al. reported that autophagy is markedly upregulated in secretory-deficient *Rab3*$^{-/-}$ β cells (Marsh et al., 2007). In this case, autophagy appears to be involved in maintaining intracellular insulin content by accelerating insulin degradation rate in β cells. Our in vivo study provides genetic evidence that autophagy is more globally involved in the maintenance of β cell homeostasis by constantly degrading *potentially toxic* cytoplasmic components, such as damaged organelles and p62, which is commonly observed in other pivotal organs such as liver and heart (Komatsu et al., 2005; Nakai et al., 2007). Hepatic injury in liver-specific *Atg7*-deficient mice largely depends on the intrahepatic accumulation of p62 (Komatsu et al., 2007). Similar mechanisms could account for the β cell failure in β cell-specific *Atg7*-deficient mice. This needs to be thoroughly investigated by the generation of β cell-specific *Atg7* and *p62* double-deficient mice.

Autophagy has long been characterized as a key strategy for maintaining energy homeostasis under nutrient-poor conditions. Indeed, autophagy is necessary for survival in the early neonatal starvation period in mice (Kuma et al., 2004). Interestingly, our study revealed that β cell-autophagy is markedly upregulated by high calorie intake, a common state in many developed

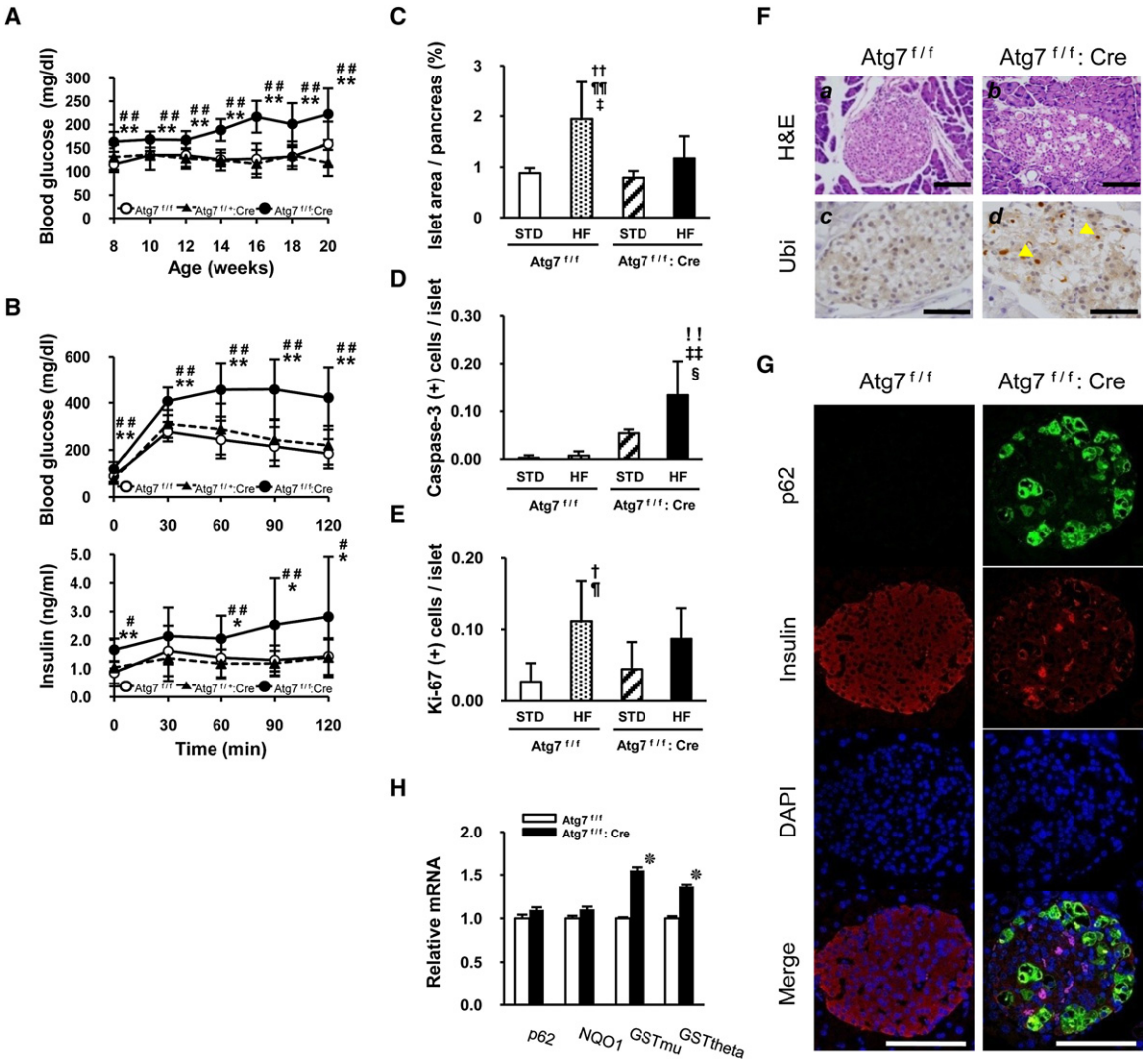

Figure 4. Deterioration of Glucose Tolerance in HF-Fed *Atg7$^{f/f}$:Cre* Mice

(A) Nonfasting glucose levels of *Atg7$^{f/f}$* (n = 9), *Atg7$^{f/+}$:Cre* (n = 8), and *Atg7$^{f/f}$:Cre* (n = 8) mice during HF-diet periods.

(B) Blood glucose concentrations and serum insulin levels measured during IPGTT at the age of 20 weeks after 12 weeks of HF diet. Open circles, *Atg7$^{f/f}$* mice (n = 10); closed triangles, *Atg7$^{f/+}$:Cre* mice (n = 9); closed circles, *Atg7$^{f/f}$:Cre* mice (n = 9). Data are mean ± SEM. *p < 0.05, **p < 0.01 (*Atg7$^{f/f}$* versus *Atg7$^{f/f}$:Cre*); $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 (*Atg7$^{f/+}$:Cre* versus *Atg7$^{f/f}$:Cre*).

(C) Percent islet area of *Atg7$^{f/f}$* and *Atg7$^{f/f}$:Cre* mice fed STD and HF diet.

(D and E) Number of cleaved caspase-3- and Ki-67-positive intraislet cells in *Atg$^{f/f}$* and *Atg7$^{f/f}$:Cre* mice fed STD and HF diet. Data are mean ± SEM. †p < 0.05, ††p < 0.01 (*Atg7$^{f/f}$* with STD versus *Atg7$^{f/f}$* with HF); ¶p < 0.05, ¶¶p < 0.01 (*Atg7$^{f/f}$* with HF versus *Atg7$^{f/f}$:Cre* with STD); ‡p < 0.05, ‡‡p < 0.01 (*Atg7$^{f/f}$* with HF versus *Atg7$^{f/f}$:Cre* with HF); $^{!!}$p < 0.01 (*Atg7$^{f/f}$* with STD versus *Atg7$^{f/f}$:Cre* with HF); §p < 0.05 (*Atg7$^{f/f}$:Cre* with STD versus *Atg7$^{f/f}$:Cre* with HF). For morphometric analysis, four mice were examined for each genotype (C–E).

(F) Representative images of H&E staining, immunostaining with ubiquitin antibody of HF-fed *Atg7$^{f/f}$* and *Atg7$^{f/f}$:Cre* islets (at 20 weeks of age). Arrowheads in *d* indicate ubiquitin-positive aggregates. Scale bars represent 100 μm (*a* and *b*) and 50 μm (*c* and *d*).

(G) Double staining for insulin (red) and p62 (green) of 20-week-old islets. Nuclei were labeled by DAPI (blue). Scale bars represent 100 μm.

(H) The mRNA expression levels of various genes in *Atg7*-deficient islets. The mRNA levels of the indicated genes were determined by real-time RT-PCR analysis with total islet RNA extracted from *Atg7$^{f/f}$* (open bars) and *Atg7$^{f/f}$:Cre* (black bars) mice fed STD diet. Values for *Atg7$^{f/f}$:Cre* islets were expressed relative to the mRNA level of each gene in *Atg7$^{f/f}$* islets. Data are mean ± SEM. *p < 0.05 (*Atg7$^{f/f}$* versus *Atg7$^{f/f}$:Cre*).

countries, in contrast to energy starvation. This inductive autophagy appears to be triggered by increased insulin resistance caused by high calorie intake or obesity. A candidate mediator is serum FFA that is increased in insulin-resistant states. Prolonged high serum FFA concentrations result in increased production of reactive oxygen species, β cell dysfunction, and apoptosis of β cells, a state termed "β cell lipotoxicity"(DeFronzo, 2004; Unger and Zhou, 2001). As shown in our study (Figure 4D), loss of autophagy resulted in substantial cell death of β cells when animals were fed HF diet. Thus, the concept of FFA-induced activation of autophagy in β cells is conceivable as an adaptive response, providing cells with a safety mechanism to eliminate

damaged mitochondria and/or other cell structures and to avoid apoptosis under insulin-resistant states and high FFA concentrations (Meijer and Codogno, 2007). Autophagy induction by FFA was not inhibited by Etomoxir, a CPT-1 inhibitor (K.K., unpublished data), suggesting that FFA influx into mitochondria is not essential for this process. Elucidation of molecular mechanisms underlying the FFA-induced autophagy awaits further investigation. Based on the possible implication of autophagy in the pathogenesis of diabetes, manipulation of autophagic activity in β cells could be useful therapeutically by preventing disease progression, particularly under insulin-resistant states, such as obesity and metabolic syndrome.

EXPERIMENTAL PROCEDURES

Animal Experiments

The study protocol was reviewed and approved by the Animal Care and Use Committee of Juntendo University. All mice were housed in specific pathogen-free barrier facilities, maintained under 12 hr light/dark cycle, and fed a standard rodent food (Oriental Yeast) for the STD study or rodent food containing 60% fat (Research Diet) for the 12 week HF study and provided with water ad libitum. We used the Rat Insulin Promotor-driven Cre recombinase (*RIP-Cre*)-Flox strategy to delete *ATG* gene in a pancreatic β cell-specific manner. Generation of $Atg7^{flox/+}$ mice was described previously (Komatsu et al., 2005). We crossed $RIP\text{-}Cre^{+/-}$:$Atg7^{flox/+}$ mice with $Atg7^{flox/+}$ mice to generate $Atg7^{flox/flox}$ ($Atg7^{f/f}$), $RIP\text{-}Cre^{+/-}$:$Atg7^{+/+}$, $RIP\text{-}Cre^{+/-}$:$Atg7^{flox/+}$ ($Atg7^{f/+}$:*Cre*), and $RIP\text{-}Cre^{+/-}$:$Atg7^{flox/flox}$ ($Atg7^{f/f}$:*Cre*) mice. We used 6- to 16-week-old male mice for the STD study and 8- to 20-week-old mice for the HF study. Then, these mice were used for further analyses. PCR analysis for determination of genotype was performed as described previously (Komatsu et al., 2005).

Cell Culture and Immunoblot Analysis

INS-1 cells were cultured as previously described (Asfari et al., 1992). Oleic acid (100 mM, Sigma) was dissolved in 0.1N NaOH, then adjusted to 1 mM oleic acid and 1% bovine serum albumin solution in RPMI 1640. Immunoblot analysis was performed as described previously (Uchida et al., 2007). Anti-LC3 and anti-ubiquitin antibodies (Abs) were described previously (Komatsu et al., 2005). Anti-p62 Ab (1:1,000) and anti-GAPDH Ab (1:1,000) were purchased from Progen and Cell Signaling, respectively. Immunoreactivity was visualized and quantitated with Fuji LAS 3000 (Fuji Film).

Assay of Insulin Secretion from Isolated Islets

Pancreatic islets were obtained from five to six mice at 16 weeks of age by collagenase digestion, as described previously (Uchida et al., 2007). In brief, five size-matched islets were incubated in 400 μl Krebs-Ringer solutions containing glucose or KCl for 60 min or 10 min, respectively. Insulin concentration of supernatants of isolated islets was analyzed by enzyme-linked immunosorbent assay (ELISA).

Data Analysis

Results are presented as mean ± SEM. Differences in serial data of fed glucose concentration, body weight, food intake, glucose concentrations, and insulin levels during IPGTT and ITT were examined for statistical significance by the nonrepeated ANOVA, followed by post hoc analysis (Bonferroni's test). Statistical differences between two groups were calculated by the unpaired Student's t test. A p value less than 0.05 denoted the presence of a statistically significant difference.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/8/4/325/DC1/.

ACKNOWLEDGMENTS

We thank Dr. C.B. Wollheim (Geneva) for the gift of INS-1 cells, Dr. K. Otsu (Osaka University) for the valuable suggestions, and Ms. N. Daimaru, Ms. E. Magoshi, and Mr. M. Kamimura for the excellent technical assistance. This work was supported by grants from the Ministry of Education, Sports and Culture of Japan (to R.K. [19659236], Y.F. [19591067], and H.Y. [19044039]) and by the Takeda Science Foundation, Astellas Foundation, and Suzuken Memorial Foundation (to Y.F.).

Received: March 30, 2008
Revised: June 28, 2008
Accepted: August 12, 2008
Published: October 7, 2008

REFERENCES

Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P.A., and Wollheim, C.B. (1992). Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. Endocrinology *130*, 167–178.

Butler, A.E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R.A., and Butler, P.C. (2003). Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes *52*, 102–110.

DeFronzo, R.A. (2004). Dysfunctional fat cells, lipotoxicity and type 2 diabetes. Int. J. Clin. Pract. Suppl., 9–21.

Fujimoto, S., Mukai, E., Hamamoto, Y., Takeda, T., Takehiro, M., Yamada, Y., and Seino, Y. (2002). Prior exposure to high glucose augments depolarization-induced insulin release by mitigating the decline of ATP level in rat islets. Endocrinology *143*, 213–221.

Gannon, M., Shiota, C., Postic, C., Wright, C.V., and Magnuson, M. (2000). Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. Genesis *26*, 139–142.

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., and Mizushima, N. (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature *441*, 885–889.

Kaniuk, N.A., Kiraly, M., Bates, H., Vranic, M., Volchuk, A., and Brumell, J.H. (2007). Ubiquitinated-protein aggregates form in pancreatic beta-cells during diabetes-induced oxidative stress and are regulated by autophagy. Diabetes *56*, 930–939.

Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., Ezaki, J., Mizushima, N., Ohsumi, Y., Uchiyama, Y., et al. (2005). Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J. Cell Biol. *169*, 425–434.

Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E., and Tanaka, K. (2006). Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature *441*, 880–884.

Komatsu, M., Waguri, S., Koike, M., Sou, Y.S., Ueno, T., Hara, T., Mizushima, N., Iwata, J., Ezaki, J., Murata, S., et al. (2007). Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell *131*, 1149–1163.

Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, H., Yoshimori, T., Ohsumi, Y., Tokuhisa, T., and Mizushima, N. (2004). The role of autophagy during the early neonatal starvation period. Nature *432*, 1032–1036.

Levine, B., and Klionsky, D.J. (2004). Development by self-digestion: Molecular mechanisms and biological functions of autophagy. Dev. Cell *6*, 463–477.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. Cell *132*, 27–42.

Li, X., Zhang, L., Meshinchi, S., Dias-Leme, C., Raffin, D., Johnson, J.D., Treutelaar, M.K., and Burant, C.F. (2006). Islet microvasculature in islet hyperplasia and failure in a model of type 2 diabetes. Diabetes *55*, 2965–2973.

Lupi, R., Dotta, F., Marselli, L., Del Guerra, S., Masini, M., Santangelo, C., Patane, G., Boggi, U., Piro, S., Anello, M., et al. (2002). Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic

islets: Evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. Diabetes *51*, 1437–1442.

Marsh, B.J., Soden, C., Alarcon, C., Wicksteed, B.L., Yaekura, K., Costin, A.J., Morgan, G.P., and Rhodes, C.J. (2007). Regulated autophagy controls hormone content in secretory-deficient pancreatic endocrine beta-cells. Mol. Endocrinol. *21*, 2255–2269.

Meijer, A.J., and Codogno, P. (2007). Macroautophagy: Protector in the diabetes drama? Autophagy *3*, 523–526.

Nakai, A., Yamaguchi, O., Takeda, T., Higuchi, Y., Hikoso, S., Taniike, M., Omiya, S., Mizote, I., Matsumura, Y., Asahi, M., et al. (2007). The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat. Med. *13*, 619–624.

Rhodes, C.J. (2005). Type 2 diabetes-a matter of beta-cell life and death? Science *307*, 380–384.

Roder, M.E., Dinesen, B., Hartling, S.G., Houssa, P., Vestergaard, H., Sodoyez-Goffaux, F., and Binder, C. (1999). Intact proinsulin and beta-cell function in lean and obese subjects with and without type 2 diabetes. Diabetes Care *22*, 609–614.

Silva, J.P., Kohler, M., Graff, C., Oldfors, A., Magnuson, M.A., Berggren, P.O., and Larsson, N.G. (2000). Impaired insulin secretion and beta-cell loss in tissue-specific knockout mice with mitochondrial diabetes. Nat. Genet. *26*, 336–340.

Uchida, T., Iwashita, N., Ohara-Imaizumi, M., Ogihara, T., Nagai, S., Choi, J.B., Tamura, Y., Tada, N., Kawamori, R., Nakayama, K.I., et al. (2007). Protein kinase Cdelta plays a non-redundant role in insulin secretion in pancreatic beta cells. J. Biol. Chem. *282*, 2707–2716.

Unger, R.H., and Zhou, Y.T. (2001). Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover. Diabetes *50* (*Suppl 1*), S118–S121.