Depletion of Adipocyte **Becn1** Leads to Lipodystrophy and Metabolic Dysregulation

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**Becn1/Beclin-1** is a core component of the class III phosphatidylinositol 3-kinase required for autophagosome formation and vesicular trafficking. Although **Becn1** has been implicated in numerous diseases such as cancer, aging, and neurodegenerative disease, the role of **Becn1** in white adipose tissue and related metabolic diseases remains elusive. In this study, we show that adipocyte-specific **Becn1** knockout mice develop severe lipodystrophy, leading to adipose tissue inflammation, hepatic steatosis, and insulin resistance. Ablation of **Becn1** in adipocytes stimulates programmed cell death in a cell-autonomous manner, accompanied by elevated endoplasmic reticulum (ER) stress gene expression. Furthermore, we observed that **Becn1** depletion sensitized mature adipocytes to ER stress, leading to accelerated cell death. Taken together, these data suggest that adipocyte **Becn1** would serve as a crucial player for adipocyte survival and adipose tissue homeostasis.

Adipose tissue plays a crucial role in the regulation of metabolic homeostasis through cross talk with other metabolic tissues. Adipose tissue is expanded by two processes, formation of new adipocytes (hyperplasia) and enlargement of existing adipocytes (hypertrophy) (1). In response to nutritional states, adipose tissue can dynamically increase its lipid storage capacity through hypertrophic expansion to accommodate excess nutrients. Besides, adipocyte hyperplasia is known to contribute to the healthy expansion of adipose tissue. In obesity, however, adipocyte hypertrophy often promotes adipocyte cell death, thereby leading to adipose tissue inflammation and systemic insulin resistance (2).

Adipose tissue dysregulation, as observed in obesity, is closely associated with metabolic complications, including insulin resistance, hepatic steatosis, and diabetes (3). In contrast, lipodystrophies, caused by genetic and environmental factors, are characterized by complete or partial loss of adipose tissue and can also lead to metabolic diseases. For example, congenital generalized lipodystrophy, which is caused by inherited **AGPAT2**, **BSCL2**, **CAV1**, or **PTRF** mutations, promotes metabolic alterations, such as insulin resistance, dyslipidemia, and hepatic steatosis (4). In addition, multiple nongenetic factors are often involved in acquired lipodystrophies caused by antiretroviral treatment of HIV, autoimmunity, or unknown reasons (5,6). Notably, the severity of lipodystrophy is often correlated with a loss of adipose mass caused by adipocyte death (1,7). Thus, it is imperative to understand the underlying mechanisms responsible for

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adipocyte survival and maintenance of adipose tissue homeostasis, which are critical for systemic energy metabolism.

Macroautophagy (referred to in this article as autophagy) is the catabolic process that delivers damaged and unnecessary cytosolic contents to lysosomes for degradation. Autophagic removal of defective subcellular organelles and long-lived proteins is of great importance for cell survival. Although appropriate autophagic machinery is required for survival, it can also mediate apoptosis in the absence of core apoptotic machinery (8). In mammals, several studies in knockout (KO) mice for autophagy-related (Atg) genes revealed that autophagy could regulate adipocyte differentiation and beiging/browning of adipose tissue (9–11). For example, adipocyte-specific Atg7 KO mice (aP2-Cre-Atg7fl/fl) exhibited reduced fat mass with attenuated white fat cell differentiation, but showed an increased number of beige adipocytes (9,10). Selective ablation of Atg12 or Atg5 driven by uncoupling protein 1 promoter also revealed that autophagy-mediated mitochondrial clearance is critical for beige adipocyte maintenance by inhibiting the beige-to-white fat transition (11). Consequently, it has been suggested that inhibition of autophagy could be a therapeutic strategy to counteract obesity via promoting beiging/browning of white adipose tissue (WAT) (11). As autophagy has been implicated in adipogenesis and beige-to-white fat transition, growing interest has emerged to identify its role in mature adipocytes. Moreover, recent studies have revealed that defective autophagy completion could cause failure in maintaining adipose tissue homeostasis and lipid metabolism. For instance, adipocyte-specific Atg3 and Atg16L1 deletion induces mitochondrial defects, accompanied by heterogeneous adipocyte size and decreased adipocyte number, resulting in dysregulated lipid metabolism (12). Nevertheless, underlying mechanisms by which autophagy-related genes could affect adipose tissue homeostasis remain elusive.

Becn1/Beclin-1, the ortholog of yeast Atg6, is a haploinsufficient tumor suppressor gene that acts as a regulator of the class III phosphatidylinositol 3-kinase complex, which is involved in autophagosome formation and membrane trafficking (13). It has been demonstrated that the cross talk between apoptosis and autophagy is regulated by Becn1. For instance, as the binding partner of Bcl-2, one of the well-known antiapoptotic mediators, Becn1 is a key determinant as to whether cells are resistant to apoptosis or autophagy under pathophysiological conditions (14). Becn1 is also involved in metabolic regulation as well as cell survival. Whereas Becn1+/− mice did not exhibit obvious metabolic defects compared with wild-type (WT) mice (15), a recent report with Becn1F121A knockin mice, in which Becn1 is hyperactivated, suggests that Becn1-mediated autophagy hyperactivation differentially regulates insulin sensitivity in a tissue-dependent manner (16). While dysregulation of Becn1-mediated autophagy would be involved in metabolic disorders, underlying physiological roles of Becn1 in adipocytes are not fully elucidated.

In this study, we generated and characterized adipocyte-specific Becn1 KO mice using the Adipoq-Cre line. Adipocyte-specific Becn1 KO mice showed severe lipodystrophy associated with adipose tissue inflammation, insulin resistance, hyperglycemia, fatty liver, and a higher risk of death. Further, the ablation of Becn1 in adipocytes resulted in adipocyte cell death in a cell-autonomous manner, leading to adipose tissue inflammation and insulin resistance. Also, we found that Becn1 KO adipocytes exhibited elevated endoplasmic reticulum (ER) stress–related gene expression to stimulate apoptotic signals, mediating that Becn1 would be important for adipocyte survival. These data suggest that Becn1 plays a key role in maintaining adipose tissue homeostasis and systemic metabolic regulation.

RESEARCH DESIGN AND METHODS

Animals Used in the Study
Adipoq-Cre (JAX stock number 010803) and β-actin–FLPe (JAX stock number 005703) were obtained from The Jackson Laboratory. Becn1tm1a(KOMP)Wtsi mice (project identification number CSD 38776) were obtained from the Knockout Mouse Project repository. Mice used in studies were maintained in a C57BL/6J BomTac genetic background and housed in a controlled environment with a 12-h light/dark cycle. Male mice were fed either a normal chow diet (NCD) or high-fat diet (HFD) for indicated times and provided with free access to food and water. Body composition was analyzed by nuclear magnetic resonance using a Bruker Minispec LF50 (Bruker, Hamburg, Germany). Animal care and experiments were performed in accordance with the guidelines of the Korean Food and Drug Administration and were approved by the Institutional Animal Care and Use Committees of the Laboratory Animal Research Center at Yonsei University (permit number 201702-157-02). Mice were maintained in the specific pathogen-free facility of the Yonsei Laboratory Animal Research Center.

Generation of KO Mice
The KO first allele (tm1a) of Becn1 contains a lacZ-trapping cassette and a neo cassette inserted into the intron 3 of Becn1 (Supplementary Fig. 1). Mice containing the floxed allele were generated by crossing Becn1tm1a(KOMP)Wtsi mice with β-actin–Flpe–transgenic mice to remove the gene trap cassette. The resulting excised alleles were validated by PCR analysis using genomic DNA extracted from mouse tail tips. Homozygous Becn1−/− mice were then crossed with Adipoq-Cre–transgenic mice to delete exons 4–7 of Becn1, triggering nonsense-mediated decay of the transcript by a frame-shift mutation.

Genomic DNA Extraction and Genotyping
Mouse tail biopsies were lysed overnight at 55°C in a buffer (50 mmol/L Tris, pH 8.0, 50 mmol/L EDTA, pH 8.0, 0.5% SDS, and protein kinase K). On the following day, genomic DNA was extracted from the lysates by the phenol-chloroform method. For routine genotyping, the following primers were used for detecting both WT and
flooed alleles: P1-forward (5'-GCTTAGGTCTGA CTGGGTGC-
3') and P2-reverse (5'-ACCCAGGCAAGTGTTTTC-3'). For
confirming deleted Beclin alleles, the following primers were
used: P3-forward (5'-TTGTACCAGTATTGGGCTTGTCG-
3') and P2-reverse (5'-ACCCAGGCAAGTGTTTTC-3').

Western Blotting and Antibodies

Western blotting was performed as described previ-
ously (17) with slight modifications. Mouse tissue was
homogenized in radioimmunoprecipitation assay buffer
(50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% Nonidet
P-40, 0.5% deoxycholate, 0.1% SDS, 1 mmol/L EDTA, pH
8.0, and protease inhibitor cocktail). The samples were
incubated with the following primary antibodies: anti-
Becn1 (#3738; Cell Signaling Technology), anti-AKT
(#9272; Cell Signaling Technology), anti-phospho-AKT
(#9271; Cell Signaling Technology), anti-FABP4 (#2120;
Cell Signaling Technology), anti-FASN (#3180; Cell Signal-
ing Technology), anti-ubiquitin (sc-166553; Santa Cruz Biotech-
nology), anti-BAX (sc-493; Santa Cruz Biotechnology),
anti-CHOP (#2895; Cell Signaling Technology), anti-BIM
(#2933; Cell Signaling Technology), anti-LC3 (L8918; Sigma-Aldrich), anti-p62 (H00008878-M01;
Abnova), and anti-GRP78 (ab21685; Abcam). After incubation
with the primary antibodies, the membranes were incubated
with the appropriate peroxidase-conjugated secondary anti-
body (GenDEPOT). Protein expression was detected by
enhanced chemiluminescence reagents and LAS-3000 image
analyzer (Fujifilm, Tokyo, Japan).

Glucose and Insulin Tolerance Tests

For glucose tolerance tests (GTTs) and insulin tolerance
tests (ITTs), mice were fasted for 6 h and then received an
intraperitoneal injection of 20% glucose (2 g/kg body
weight) or human insulin (0.75 units/kg body weight).
Blood samples were collected from the tail tips at the
indicated time points for glucose measurement. The
blood glucose was measured using a glucometer AGM-
3000 (GlucoDr Plus; Allmedicus, Gyeonggi-do, Republic
of Korea).

Histology

Tissue samples were fixed in 10% formalin (#HT501640;
Sigma-Aldrich) and then incubated overnight. Samples
were dehydrated and embedded in paraffin using an
automated tissue processor (Leica TP1020; Leica Biosystems,
Newcastle upon Tyne, U.K.). The sections (4–5-μm thick)
were deparaffinized and stained with hematoxylin and
eosin (H&E) or taken for immunohistochemistry analysis.
For immunohistochemistry analysis, the sections were
 incubated overnight with the primary antibody PLIN1
(20R-PP004; Fitzgerald Industries), CD11b (14-0112-81;
eBioscience), CD11c (14-0114-81; eBioscience), and BAX
(sc-23959; Santa Cruz Biotechnology). Secondary antibo-
dies conjugated to horseradish peroxidase or a fluores-
cent molecule were applied for detection. The TUNEL assay
was performed using the ApopTag Fluorescein In Situ Apop-
tosis Detection Kit (S7110; Millipore). To quantify the
adipocyte areas, >800 adipocytes/mouse were counted
from images of histological sections and then analyzed
using the Adiposoft software (18).

Metabolic Parameters

Blood was obtained from the retro-orbital sinus using
heparinized capillary tubes. Serum insulin levels were
measured by a mouse insulin ELISA kit (AKRIN-011T;
Shibayagi Corporation, Gunma, Japan). Serum triglyceride
(TG) and total cholesterol were measured by a Dri-Chem
4000i (Fujifilm). Serum free fatty acid (FFA) (K612; Bio-
Vision Inc.) and β-hydroxybutyrate (β-HB) (K632; BioVi-
sion Inc.) were measured according to the manufacturer’s
instructions.

RNA Extraction and Quantitative RT-PCR Analysis

Total RNA was extracted from the tissues and cells using
TRIzol Reagent (#15596026; Invitrogen) and RNaseasy Mini
Kit (#74104; Qiagen, Hilden, Germany). First-strand
cDNA was prepared from 500 ng of total RNA using
RevertAid First Strand cDNA Synthesis (#K1622; Thermo
Fisher Scientific) according to the manufacturer’s instruc-
tions. The quantitative RT-PCR (qRT-PCR) analysis was
performed using primers listed in Supplementary Table 1.
Samples were analyzed on a CFX Connect Real-Time PCR
Detection System (Bio-Rad) and normalized to Ppia or
Tbp1 expression.

Adipose Tissue Fractionation

Adipose tissue was fractionated as previously described
(19). Briefly, epididymal WAT (eWAT) and inguinal WAT
(iWAT) of mice were isolated, dissected out, chopped, and
incubated in collagenase buffer (0.1 mol/L HEPES, 0.125
mol/L NaCl, 5 mmol/L KCL, 1.3 mmol/L CaCl2, 5 mmol/L
glucose, 1.5% [w/v] glucose, and 0.1% [w/v] collagenase I)
for 20 min at 37°C with shaking. Cell suspensions were
centrifuged and then washed twice. Supernatants contain-
ing adipocytes were used for Western blot analysis. Pel-
leted stromal vascular cell (SVC) fractions were used for
flow cytometry and SVC-derived adipocyte differentiation.

SVC-Derived Adipocyte Differentiation

Preadipocytes were grown to confluence (day 0) in the
induction medium consisting of DMEM, 10% FBS, 167
mmol/L insulin, 1 μmol/L 3,3’,5-triiodo-L-thyronine
(T3), 2 μmol/L rosiglitazone, 520 μmol/L isobutylmethyl-
exanthine, and 1 μmol/L dexamethasone. After a 2-day
incubation in the induction medium, the cells were trans-
fected to a differentiation medium (DMEM, 10% FBS,
167 nmol/L insulin, 1 μmol/L T3, and 2 μmol/L rosiglitazone), which was changed every other day.

**SVC Immortalization and Becn1 Conditional KO**

Human embryonic kidney 293 (HEK293) cells were transfected with simian virus 40 large T antigen containing plasmid using Lipofectamine. Media was then harvested 40 h post-transfection. The virus-containing media in the presence of polybrene (5 μg/mL) was treated to SVCs extracted from mice mated with Becn1fl/fl and ROSA-CreERT2. Six to 8 h later, the viral supernatant was removed and reinfected for another day. SVCs immortalized using simian virus 40 T antigen (imSVCs) were selected based on adipocyte differentiation efficiency. Once terminal differentiation occurs (day 8), imSVCs were treated with tamoxifen (500 nmol/L, 72 h) to knock out Becn1.

**ER Stress Induction and Inhibition**

Tunicamycin (SML1287) was purchased from Sigma-Aldrich. Five μmol/L of tunicamycin was treated to mature adipocytes in culture for indicated days. Taurosodeoxycholic acid (TUDCA) (580549) was purchased from Millipore. A total of 400 μmol/L was treated to mature adipocytes in culture for the indicated days.

**Flow Cytometry**

Flow cytometric analysis was performed as previously described (20). SVC fractions were separated from red blood cells by adding lysis buffer (155 mmol/L NH₄Cl/0.1 mol/L Tris-HCl [pH 7.65] [9:1]). SVCs were stained with monoclonal antibodies against CD11b (27-0112-81; eBioscience), F4/80 (45-4801-80; eBioscience), CD11c (12-0114-82; eBioscience), and CD206 (123009; BioLegend) for macrophage analysis using an FACSCanto II (BD Biosciences).

**Luciferase Assay**

HEK293 cells were transiently transfected with various DNA plasmids (PPARγ, RXRα, β-galactosidase, and DR-1) by the calcium phosphate method, as previously described (21). Luciferase and β-galactosidase activities were measured according to the manufacturer’s instructions (Promega). Relative luciferase activity was normalized to β-galactosidase activity in each sample.

**Statistical Analysis**

Data are presented as mean ± SEM. Results were analyzed using GraphPad Prism 5 software. Unpaired Student t test was used to compare two groups, while two-way ANOVA was used to compare more than two groups. A repeated-measures ANOVA was used for analysis of body weight, GTT, and ITT data. A Bonferroni post hoc test was used to test for significant differences as determined by the ANOVA. A P value <0.05 was considered statistically significant.

**Data and Resource Availability**

The data sets generated during the current study are available from the corresponding author on reasonable request.

**RESULTS**

**Generation of Adipocyte-Specific Becn1 KO Mice**

To investigate the role of Becn1 in adipose tissue, we generated adipocyte-specific Becn1 KO mice by crossing floxed Becn1 mice with Adipoq-Cre mice (Supplementary Fig. 1A and B) (Adipoq-Crefl/+; Becn1fl/fl, hereafter referred to as AKO). Littermates of Adipoq-Crefl/+; Becn1fl/fl (hereafter referred to as WT) mice were used as controls. Western blot analyses revealed that BECN1 expression was decreased in the adipocytes of AKO mice, with impaired autophagic flux as indicated by autophagy markers such as p62, ubiquitin, and LC3 (Fig. 1A). However, there were no significant differences in Becn1 expression and autophagic flux in nonadipose tissues such as liver and skeletal muscle between WT and AKO mice (Fig. 1A), indicating that Becn1 is specifically depleted in adipose tissue of AKO mice.

**Loss of WAT Mass in Becn1 AKO Mice**

Even though there were no significant differences in body weights between WT and AKO mice (Fig. 1B), WAT mass of AKO mice was dramatically lower, while lean mass was significantly higher (Fig. 1C). As shown in Fig. 1D, iWAT and eWAT of AKO mice were reduced by 61.9% and 80.8%, respectively (Fig. 1D). The decrease in eWAT mass of AKO mice closely correlated with a high frequency of small adipocytes (Fig. 1E). This tendency was enhanced upon aging (Supplementary Fig. 1C). Nevertheless, there was no significant difference in food and water intake between the two genotypes (Supplementary Fig. 1D and E). Accordingly, a significant decrease in mRNA expression of key adipogenic markers including Pparγ2 (~2.6-fold), Cebpα (~1.8-fold), Plin1 (~3.1-fold), Fabp4 (~2.2-fold), and Adipoq (~2.6-fold) was observed in AKO mice (Fig. 1F). These results were further confirmed by Western blot analyses of the adipogenic markers (Fig. 1G). In contrast, the mass and size of the livers in AKO mice were significantly higher (Fig. 1H), which could contribute to an increase in the lean mass. As adipose tissues in AKO mice were unable to store lipid, there could be ectopic lipid deposition in the liver. Indeed, the livers of aged AKO mice were grossly enlarged and appeared pale yellow (Fig. 1I). Moreover, histological examination confirmed substantial lipid accumulation in the liver of AKO mice (Fig. 1J). These data reveal that Becn1 deficiency in adipose tissue promotes WAT reduction, accompanied by ectopic lipid accumulation in the liver.

**Increased Adipose Tissue Inflammation in Becn1 AKO Mice**

Compared with WT littermates, AKO mice exhibited smaller adipocytes in eWAT with a larger eosinophilic cytoplasm
**Figure 1**—Adipocyte Beclin1 deficiency reduces WAT mass. **A** (left panel): Western blot analysis of protein lysates isolated from adipocytes of eWAT from adipocyte-specific Beclin1 WT and AKO mice. **A** (right panel): Western blot analysis of Beclin1, p62, and LC3 expression in protein lysates from the liver and muscle of WT and AKO mice. **B**: Body weight changes in chow-fed WT and AKO mice from 4 to 16 weeks of age (n = 11). **C**: Fat and lean mass from WT and AKO mice (n = 3), measured by nuclear magnetic resonance–based body composition analysis. **D**: Tissue weight of iWAT and eWAT from 10-week-old WT and AKO mice (n = 10). **E**: The cell area distribution of adipocytes from 10-week-old Beclin1 WT and AKO mice (n = 8). The adipocyte area was calculated from eWAT of mice using the Adiposoft software. Scale bars, 100 μm. **F**: The mRNA expression levels of fat differentiation markers in eWAT from 10-week-old WT and AKO mice (n = 7). The qRT-PCR results were normalized to Ppia. **G**: Western blot analysis of protein lysates from eWAT of 12-week-old WT and AKO mice. Data are presented as mean ± SEM. **H**: Tissue weight of nonadipogenic tissues from 10-week-old WT and AKO mice (n = 10). **I** (top panel): Morphological analysis of the liver from 6-month-old WT and AKO mice. Scale bar, 1 cm. **I** (bottom panel): H&E staining of the liver from 6-month-old WT and AKO mice. Scale bar, 100 μm. Data are presented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. Br, brain; Ht, heart; Lu, lung; Kd, kidney; Lv, liver; Ms, muscle; Sp, spleen; Ub, ubiquitin.
(Fig. 2A). Moreover, crownlike structures (CLs) were frequently observed in AKO mice, indicating increased dead or dying adipocytes with macrophage infiltration (Fig. 2A and B). Consistently, the mRNA levels of macrophage genes such as F4/80, Cd11b, and Cd11c were elevated in eWAT of AKO mice (Fig. 2C). Enhanced infiltration of monocytes/macrophages in eWAT of AKO mice was assessed by staining with antibodies for CD11b and CD11c and measuring fluorescence intensity in the histological analysis (Fig. 2D). It is well known that HFD-induced obese mice show greatly elevated M1-like proinflammatory macrophage infiltration in eWAT (22). It is of interest to note that NCD-fed AKO mice showed higher mRNA expression of both M1 (Tnf-α and Mcp-1) and M2 markers (CD206 and Il-10) in eWAT (Fig. 2E), implying that eWAT of AKO mice appeared to be vulnerable to macrophage infiltration. Consistently, FACS of SVCs isolated from eWAT of WT and AKO mice showed that the number of F4/80 and CD11b double-positive macrophages was significantly higher in AKO mice (Fig. 2F). In eWAT of AKO mice, numbers of both M1-like (F4/80, CD11b, and CD11c triple-positive) and M2-like (F4/80, CD11b, and CD206 triple-positive and CD11c-negative) adipose tissue macrophages (ATMs) were elevated (Fig. 2G). These results suggest that the ablation of Beclin1 in adipocytes potently induces adipose tissue inflammation through infiltration of both M1-like and M2-like ATMs.

**Effects of HFD on Beclin1 AKO Mice**

Obesity is characterized by imbalanced expansion and chronic inflammation in adipose tissue. To determine the effects of adipocyte Beclin1 deficiency on the pathological remodeling of adipose tissue in obesity, an HFD challenge was performed. Compared with HFD-fed WT littermates, HFD-fed AKO mice exhibited much less body weight gain and severely restricted increase of eWAT and iWAT mass (Supplementary Fig. 2A and B). When HFD-fed WT and AKO mice were subjected to tests with metabolic cages, AKO mice did not show significant difference in respiratory exchange ratio, food intake, or energy expenditure (Supplementary Fig. 2C–E). In contrast, we observed severe inflammatory response in adipose tissue. As indicated in Supplementary Fig. 2H and I, eWAT of AKO mice contained more ATMs with either NCD or HFD. Although macrophage infiltration is more frequently observed in eWAT than iWAT upon HFD, significant infiltration of macrophages was also observed in iWAT of HFD-fed AKO mice (Supplementary Fig. 2J), accompanied by a higher percentage of M1-like macrophages and M1/M2 ratio in the SVCs (Supplementary Fig. 2K and L).

**Dysfunctional Adipose Tissue in Beclin1 AKO Mice**

We next examined the serum profiles of WT and AKO mice under different nutritional states, such as feeding and fasting conditions. While fed WT and AKO mice showed no significant changes in serum levels of TG, FFA, as well as β-HB, fasted AKO mice revealed significantly lower serum levels of TG (~68.3%), FFA (~73.6%), and β-HB (~53.3%) (Fig. 3A). As lipodystrophy is often accompanied by insulin resistance and glucose intolerance (23), we could also observe that compared with WT mice, AKO mice showed elevated blood glucose levels during a fasting state (Fig. 3B). Also, serum insulin levels of AKO mice were significantly higher than those of WT mice (Fig. 3C). Further, high levels of HOMA of insulin resistance indicated that AKO mice could develop severe insulin resistance. To confirm the effects of adipocyte Beclin1 deletion on glucose and insulin sensitivity, we performed a GTT and an ITT. Compared with WT mice, AKO mice showed glucose (Fig. 3D) and insulin intolerance (Fig. 3E). When we examined insulin signaling pathways, insulin-dependent AKT phosphorylation was found to be significantly reduced in eWAT, liver, and muscles of AKO mice (Fig. 3F). Together, these results propose that adipocyte Beclin1 ablation leads to lipodystrophy accompanied by defects in lipid and glucose metabolism as well as insulin sensitivity.

**No Defects in Adipocyte Differentiation in Beclin1 AKO Mice**

To determine the cause of WAT reduction in AKO, we next investigated whether Beclin1 ablation might affect adipocyte differentiation. As it has been reported that Beclin1 knockdown inhibits lipid droplet formation in 3T3-L1 cells (24), we hypothesized that decreased adiposity in AKO mice might result from defects in adipocyte differentiation. As shown in Fig. 4A, Beclin1 AKO showed ~70% reduction of Beclin1 mRNA only in adipocytes, but not in SVCs (Fig. 4A). Six days after induction of adipogenic stimuli, when Beclin1 expression was significantly diminished, there was no significant difference in the adipogenic potential of SVC-derived adipocytes from WT and AKO mice, at least in the aspect of lipid accumulation (Fig. 4B). Consistently, the mRNA levels of adipogenic genes were not altered by deletion of Beclin1 in adipocytes (Fig. 4C). To assess whether Beclin1 can directly modulate the activity of PPARγ, a key transcription factor for adipogenesis, we also conducted luciferase assays in HEK293 cells. As shown in Fig. 4D and E, there were no changes in PPARγ reporter activity by transient overexpression of BECN1 in the absence or presence of rosiglitazone, the synthetic ligand of PPARγ. Together, these data propose that reduced WAT in AKO mice did not result from impaired adipocyte differentiation.

**Cell-Autonomous Death of Adipocytes in Beclin1 AKO Mice**

As CLs were frequently observed in the WAT of AKO mice (Fig. 2A and B), we asked whether decreased adiposity in AKO mice might be due to enhanced adipocyte death. Since adipose tissue is comprised of various cell types, we assessed adipocyte death by staining the adipocyte-specific lipid droplet binding protein PLIN1/perilipin 1 (25). Compared with WT mice, PLIN1-negative dead adipocytes were abundant in AKO mice (Fig. 5A). Similarly, the levels of PLIN1 protein and mRNA were remarkably lower in eWAT.
Figure 2 — Adipocyte Beclin1 deficiency promotes adipose tissue inflammation. A: Histological analysis of eWAT from 10-week-old WT and AKO mice. WAT sections were examined by H&E staining. Black arrows indicate CLSs. Scale bars, 50 μm. B: Quantification of CLSs in WT and AKO mice (n = 8). C: The mRNA expression levels of F4/80, CD11b, and CD11c in eWAT from 10-week-old WT and AKO mice (n = 7). D: Representative immunofluorescence images of eWAT from 10-week-old WT and AKO mice. The eWAT sections were stained with CD11b (cyan) and CD11c (red) antibodies. Nucleus was stained with DAPI (blue). Scale bars, 75 μm. E: The mRNA expression of M1 and M2 macrophage-specific markers in eWAT from 10-week-old WT and AKO mice (n = 7). F: Total number of F4/80^+CD11b^+ SVCs per gram of eWAT was determined by flow cytometry in WT and AKO mice (n = 3). G: The percentages of CD11c^− and CD11c^+CD206^+ in F4/80^+CD11b^+ cells were measured by flow cytometry in the SVCs of WT and AKO mice (n = 3). The qRT-PCR results were normalized to Ppia. Data are presented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; #P = 0.12. exp., expression; FM, fat mass.
of AKO mice (Fig. 5B). In accordance with enhanced adipocyte death in AKO mice, there was a significant increase in the mRNA levels of proapoptotic genes, including Bax (~1.8-fold), Noxa (~1.9-fold), Bim (~1.8-fold), and Apaf1 (~1.7-fold) in AKO mice (Fig. 5C). In eWAT of AKO mice, the levels of BIM, an essential initiator of apoptosis, were upregulated, while BCL2, an antiapoptotic protein, was downregulated (Fig. 5D). Increased apoptotic cell death in AKO mice was further confirmed using TUNEL analysis that detects apoptotic DNA fragmentation and 6A7 antibody that recognizes the activated form of BAX, respectively (Fig. 5E). These data propose that Beclin1 deficiency in adipose tissue leads to apoptotic cell death via enhanced intrinsic apoptosis pathway. To exclude the possibility that adipocyte death may be caused by a nonautonomous manner (e.g., secretory factors from ATMs), we isolated SVCs from iWAT of WT and AKO mice and treated them to differentiate into adipocytes with adipogenic stimuli. Although there was no difference in the adipogenic potential of SVCs from both genotypes (Fig. 4), we observed ruptured adipocytes with debris in Beclin1-deficient adipocytes after day 6 (Supplementary Fig. 3), which seemed to be features of adipocyte death (26). In line with this, the number of primary adipocytes stained
with propidium iodide was higher in Beclin1-decient adipocytes (Fig. 5F). Consistent with in vivo results, PLIN1 staining and Plin1 mRNA levels were significantly decreased in Beclin1-deficient adipocytes, while the mRNA levels of proapoptotic markers Bim, Bax, and Apaf1 were increased (Fig. 5G and H). Collectively, these results suggest that Beclin1 deficiency in adipose tissue could cause apoptotic adipocyte death in a cell-autonomous manner.

**Activation of ER Stress in Beclin1 AKO Mice**

To understand the features of adipocyte cell death in AKO mice, we performed microarray analysis in eWAT of WT and AKO mice. Consistent with data above, differentially expressed genes were primarily enriched in gene ontology terms involved in the regulation of apoptotic cell death, cell cycle, autophagy, apoptosis, and immune responses (Fig. 6A). Among them, a heat map analysis revealed that ER stress might be related to apoptotic cell death in AKO mice (Fig. 6B). Accumulating evidence suggests that activation of autophagy relieves ER stress and prolongs cell survival by reducing misfolded proteins (27,28). Moreover, autophagy defects in pancreatic β-cells lead to extensive ER stress, loss of β-cell mass, and insulin resistance, suggesting an adaptive role of autophagy in the unfolded protein response (UPR) (29,30). Similarly, the mRNA levels of ER stress markers Grp78/Bip, Atf3, and Chop were increased in eWAT of AKO mice (Fig. 6C). Western blot analysis also confirmed an increase in the protein levels of p62, GRP78, and CHOP (Fig. 6D). Since ER stress markers can be upregulated by indirect pathways of UPR, a dilated ER lumen in eWAT of AKO mice was observed by electron microscopy (Fig. 6E), indicating that AKO mice would suffer
from elevated ER stress. As CHOP is a key transcription factor of ER stress, which promotes severe genes involved in apoptosis (31,32), the mRNA levels of CHOP-related target genes, including DR5, Trib3, Gadd34, and Ero1α, were upregulated in eWAT of AKO mice (Fig. 6F). To test whether UPR-mediated apoptosis might be involved in Becn1-depleted adipocytes, we performed an in vitro analysis of SVCs with a Becn1 conditional KO system using the ROSA-CreERT2

**Figure 5**—Adipocyte Becn1 deficiency promotes adipocyte apoptosis in a cell-autonomous manner. A: Representative immunofluorescence images of eWAT from 10-week-old WT and AKO mice. The eWAT sections were stained with an antibody to PLIN1 (green). Nucleus was stained with DAPI (blue). Scale bars, 75 μm. B (left panel): Western blot analysis of PLIN1 expression in protein lysates from eWAT of WT and KO mice. B (right panel): The mRNA expression (exp.) levels of Plin1 in eWAT from WT and AKO mice (n = 7). C: The mRNA expression levels of apoptosis markers in eWAT from 10-week-old WT and AKO mice (n = 7). D: Western blot analysis of BIM, BAX, and BCL2 expression in protein lysates from eWAT of 12-week-old WT and AKO mice. E: Detection of adipocyte apoptosis using TUNEL and BAX staining. The eWAT sections were stained with TUNEL and antibody to BAX. White arrowheads indicate TUNEL-positive staining in eWAT of AKO mice. Scale bars, 100 μm. F: Fluorescence microscopy of propidium iodide (PI)–stained SVCs derived from iWAT of WT and AKO mice after 12 days (D12) of differentiation. Scale bars, 100 μm. G: Immunostaining of PLIN1 in the adipose SVCs derived from WT and AKO mice after 12 days of differentiation. The qRT-PCR results were normalized to Ppia. Data are presented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. DIC, differential interference contrast.
Figure 6—Adipocyte Becn1 deficiency increases ER stress. A: Gene ontology pathways enriched in upregulated genes in eWAT from 10-week-old WT and AKO mice. B: Heat map showing expression of genes involved in ER stress response in eWAT from WT and AKO mice. C: The mRNA expression (exp.) levels of ER stress markers in eWAT from 10-week-old WT and AKO mice (n = 7). D: Western blot analysis of p62, GRP78/BIP, and CHOP expression in protein lysates from eWAT of 12-week-old WT and AKO mice. E: Transmission electron microscope analysis of eWAT from 10-week-old WT and AKO mice. Black arrows indicate dilated ER. F: The mRNA expression levels of Chop target genes in eWAT from WT and AKO mice (n = 7). G: Western blot analysis of ER stress–associated genes in imSVC lines after differentiation. Representative bands of the named proteins are marked with red arrows. H (top panel): TUNEL assay of ER stress–induced imSVCs. Tunicamycin (1 μmol/L) or serum-deprived (0%) media was treated to mature adipocytes and incubated 10 days, with media changed every 3 days. H (bottom panel): Proportion of TUNEL-positive cells from four distinct confocal microscopy images (original magnification ×40) is shown in bar graphs. Counting TUNEL–positive cells was withheld as a blinded experiment. I: Western blot analysis of ER stress–induced and inhibited adipocytes. Serum deprivation–mediated ER stress induction was mitigated by TUDCA (400 μmol/L, 3 days). Band intensity visualized in a bar graph using ImageJ. The qRT-PCR results were normalized to Ppia. Data are presented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. FC, fold change; L, lipid; N, nucleus.
promoter. SVCs isolated from a mouse mated with Becn1+/fl and ROSA-CreERT2 were immortalized using simian virus 40 T antigen to establish a cell line (imSVCs). Becn1 could then be conditionally knocked out upon tamoxifen treatment (Supplementary Fig. 4A). To examine the effect of Becn1 in mature adipocytes, we treated tamoxifen when adipocytes were fully differentiated (day 8). As shown in Fig. 6G, we obtained consistent data, as observed in vivo, that imSVCs exhibited enhanced ER stress–induced adipocyte death in the absence of Becn1. Among three UPR pathways, imSVCs underwent an elevated PERK-eIF2α-ATF4–CHOP axis as well as IRE1α-mediated Xbp1 mRNA splicing (Fig. 6G and Supplementary Fig. 4B). Furthermore, to ensure ER stress–mediated adipocyte death would be implicated in Becn1-depleted adipocytes, TUNEL assay was performed in ER stress–induced imSVCs after adipocyte differentiation. When imSVCs were cultured with serum-deprived or tunicamycin-containing media, Becn1 KO adipocytes were prone to apoptotic death through ER stress accumulation (Fig. 6H). In contrast, enhanced adipocyte apoptosis appeared to be downregulated by TUDCA, an inhibitor of ER stress (Fig. 6I and Supplementary Fig. 4C). Taken together, these data suggest that Becn1 deficiency in adipose tissue promotes the development of a lipodystrophic phenotype, accompanied by persistent ER stress–mediated adipocyte death.

**DISCUSSION**

Becn1 plays an important role in cell survival and death as a key regulator of the phosphatidylinositol 3-kinase and BCL-2 complex. Its direct interaction with BCL2 family members such as BIM and BCL2 makes it plausible to be involved in BAX-dependent apoptosis (33). Thus, apart from other autophagy-related proteins such as ATG7, Becn1-deficient mice exhibited more severe apoptotic cell death in neurons and embryos, supporting that Becn1 is associated with cell survival mechanisms distinct from Atg7–mediated autophagy (34,35). Nevertheless, as underlying mechanisms by which Becn1 could affect cell fate are quite complex, this remains controversial. For instance, while Becn1 contains a proapoptotic BH3 motif that interacts with BCL-2, overexpression of Becn1 does not induce apoptosis and fails to neutralize antiapoptotic BCL-2 family members (36). However, it has been also reported that Becn1 has an antiapoptotic role in the TNF-related apoptosis-inducing ligand pathway, chemotherapy, irradiation, immunotherapy, and nutrient deprivation (28). Given that adipocyte cell death is important for adipose tissue homeostasis, how Becn1 modulates adipocyte survival remains largely unknown.

In this study, we demonstrated that the deletion of adipocyte-specific Becn1 in AKO mice promoted programmed cell death, adipose tissue inflammation, and insulin resistance, which are common in patients with lipodystrophy (4). AKO mice also exhibited enhanced ER stress, which is observed in obesity-induced apoptosis of adipocytes (37). These features align with previous study showing that increased adipocyte death is observed with accelerated adipose tissue inflammation, accompanied by insulin resistance (38). Although it has not yet been reported that patients with lipodystrophy have BECN1 mutations, publicly available genome-wide association study data suggest that variants of BECN1 are strongly associated with type 2 diabetes and insulin sensitivity (https://www.type2diabetesgenetics.org). However, a direct link between Becn1 deficiency and adipocyte cell death remains to be investigated.

Given that genetic deletion of core autophagic genes such as Atg5, Atg7, and Becn1 results in impaired adipogenesis in mouse embryonic fibroblasts or 3T3-L1 cells (9,24,39), it is widely believed that autophagy is required for adipocyte differentiation by removing unwanted and redundant cellular contents (40). In this study, however, expression levels of the adipogenic markers were unaltered in AKO mice did not result from altered adipogenic potentials. Of course, our model is not sufficient to investigate how Becn1 regulates adipocyte differentiation since Becn1 gene expression in AKO mice would be diminished only when adiponectin expresses via adipoc-re. There are some discrepancies between the data obtained from 3T3-L1 cells with knockdown of Atg genes and floxed Atg mice driven by the Adipoq-Cre line. These inconsistencies may not be baffling, if we consider that adiponectin expression increases after adipocyte differentiation. As adipogenesis is a process that consists of a series of steps including lineage commitment, mitotic clonal expansion, growth arrest, and terminal differentiation (41), it is quite complex to discriminate at exactly which steps Becn1 directly or indirectly affects adipocyte differentiation.

Adipose tissue devoid of Becn1 showed increased expression of CHOP target genes (Fig. 6C). It is known that the accumulation of unfolded proteins in ER activates CHOP and mediates apoptosis (42). Recently, it has been suggested that Becn1-mediated hyperactivation of autophagy in insulin-responsive cells increases insulin sensitivity by suppressing ER stress (16), which led us to speculate how Becn1 deficiency–derived ER stress could initiate apoptosis in adipocytes. Indeed, we observed that Becn1-depleted adipocytes exhibited enhanced ER stress–mediated apoptosis (Fig. 6G and H), implying that persistent induction of ER stress could precede adipocyte death in AKO mice.

It has been reported that deletion of Atg3 or Atg16L1 in adipocytes leads to dysfunctional mitochondria, resulting in systemically elevated lipid peroxides (12). While these mice exhibited similar fat mass compared with WT mice, Becn1 AKO mice exhibited significant reduction of WAT mass regardless of NCD- or HFD-fed conditions (Fig. 1C and D and Supplementary Fig. 2B). Depleting Becn1 from fully differentiated adipocytes also revealed that they undergo both persistent ER stress and its accumulation. In this study, we can assume that Becn1 deletion contributes
in a greater magnitude to induce apoptotic response in mature adipocytes. In addition, depletion of Atg3 led to increased number of CLSs with enhanced F4/80 staining. Beclin1 AKO mice also exhibited similar phenotypes as shown in Fig. 2A–D, and further investigation of macrophage polarization found that the ratio of M1/M2 was increased in Beclin1 AKO mice (Fig. 2E–G and Supplementary Fig. 3K and L). These results provide an insight that inflammatory response in adipose tissue could be a common physiological outcome of inhibition of autophagy flux. However, how Beclin1 plays more a influential role in regulating death signals in response to stress compared with other autophagy-related genes remains to be further elucidated.

As the concurrence of ER stress and autophagy is common in several human pathologies, their response is tightly regulated (43). Further delineation of the relationship between ER stress and autophagy revealed a selective elimination of damaged mitochondria through enhanced expression of PARK2, p62, and NBR1, shedding light on the possibility that selective autophagy could emerge through ER stress induction (44,45). Beclin1 being the essential protein for autophagosome formation and lysosome fusion, Beclin1-deficient models may experience incompletion of selective autophagy induced by ER stress—especially, as shown from fluorescent reporter mito-Keima, when mitophagy was suppressed in Beclin1−deficient adipocytes (Supplementary Fig. 5A and B), which is consistent with the data from Gelmetti et al. (46) that Beclin1 is required for mitophagy completion. Additionally, we observed attenuated mitochondrial potential (Supplementary Fig. 5C and D) and morphologically abnormal mitochondria in AKO adipocytes (Supplementary Fig. 5E). It is likely that suppressed mitophagy by Beclin1 deficiency caused aberrant mitochondria quality control in AKO adipocytes. However, there was little difference in mitochondrial number between two genotypes (Supplementary Fig. 5F). Thus, it remains to be elucidated whether inability to alleviate stress due to inhibition of selective autophagy leads to adipocyte death in our mouse model (47–49).

In summary, we show that the deletion of Beclin1 in adipocytes leads to an increase in apoptosis accompanied by ER stress. In addition, AKO mice develop severe lipodystrophy with hepatic steatosis and show a dramatic decline in survival rates with fasting-induced hypothermia, indicating that adipocyte Beclin1 is a crucial regulator of adipose tissue homeostasis.

Author Contributions. Y. Jin wrote the manuscript and performed the majority of the experiments. Y. Ji wrote the manuscript and performed the majority of the experiments. Y.S. wrote the manuscript and performed designed experiments. S.S.C. helped designing the experiments. Y.G.J. helped with luciferase assay and microarray data analysis. H. Na helped with mouse management. T.W.N. helped with mouse management. H.J.K. helped with Western blot experiments. H.Nah. conducted FACS analysis. S.M.K. helped with adipocyte differentiation and mouse management. J.-w.K. oversaw the project. K.T.N. helped with immunohistochemistry experiments. J.K.S. helped with indirect calorimetric data analysis. D.H. helped with microarray data analysis. C.B.P. oversaw the project. I.H.L. oversaw the project. J.B.K. and H.-W.L. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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