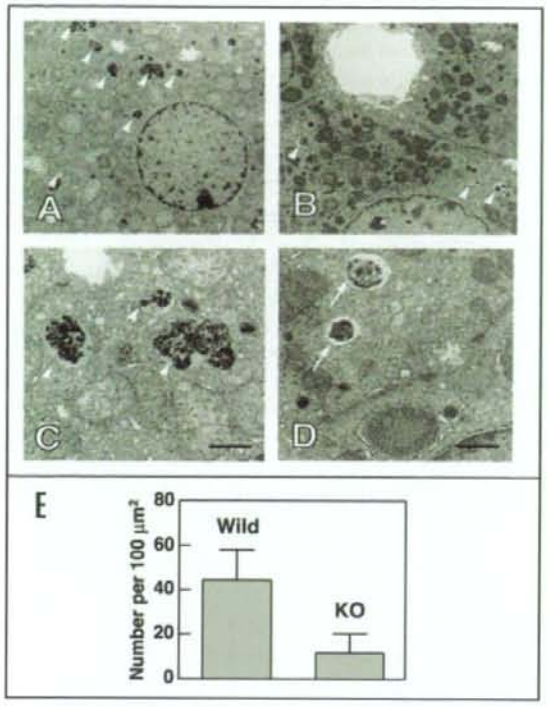


Figure 3. Electron microscopic examination of control and Pten-deficient livers. Normal control and liver-specific Pten-deficeint mice were starved for 48 hours and leupeptin (0.15 mg/10 g body weight) was injected intraperitoneally. One hour later, the livers were perfusion-fixed and subjected to electron microscopic analyses as described in Materials and Methods. Autophagic vacuoles accumulating in the cytoplasm of control hepatocytes (A and C) and mutant hepatocytes (B and D) are shown by arrowheads. Bar, 1 μm. E. Morphometric analysis of autophagic vacuoles was performed with 30 different areas of the cytoplasm of control and mutant hepatocytes. Numbers of the vacuoles per 100 μm² cytoplasm were expressed as the mean ± S.D.

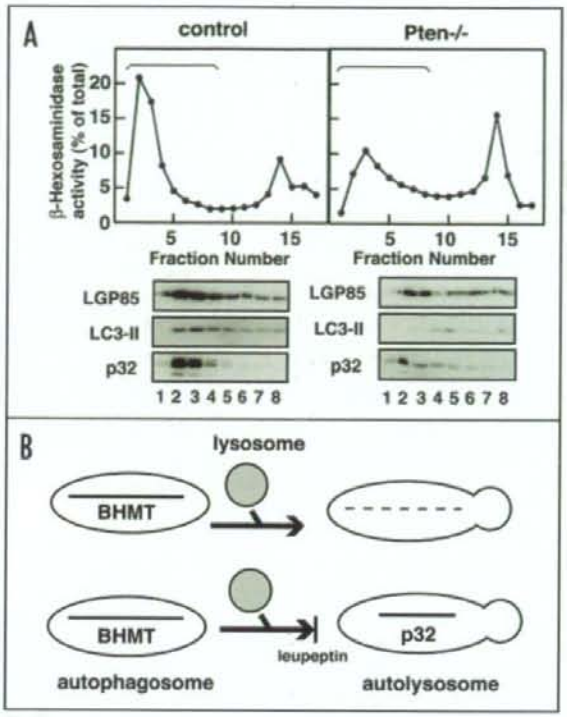


Figure 4. Density-shift of leupeptin-inhibited autolysosomes revealed b Percoll gradient centrifugation. (A) Liver mitochondrial/lysosomal fractio (35 mg protein) isolated from control (+/+) and Pten-deficient (-/-) mic starved for 48 hours was loaded onto 53% Percoll in 5 mM Tes-NaOH (pl 7.5)-0.3 M sucrose and centrifuged at 50,000 x g for 40 min. After centrifi gation, 1.5 ml fractions were collected from the bottom (No. 1) to the to (No. 17). Beta-hexosaminidase activity was assayed and plotted in uppe line graphs. Total activity recovered was 565 μmole/min (control) and 49 μmole/min (Pten-deficient), respectively. Distribution of LGP85, LC3-II, an the p32 fragment of BHMT (p32) in the denser autolysosome fractions (Nc 1–No. 8) were determined by immunoblotting analysis. (B) Schematic pr sentation showing how a p32 fragment of BHMT accumulates in leupeptir treated autolysosomes. In the absence of leupeptin, the BHMT sequestere in autophagosomes is completely degraded upon fusion of autophagosome with the lysosomes to form autolysosomes (upper route). In the presence c leupeptin, BHMT is degraded to form a 32 k-Da fragment, which remains i the autolysosomal lumen (lower route). For details, see reference 27.

order to examine this possibility further, we conducted a density-shift assay of autolysosomes on Percoll gradients.

When leupeptin was intraperitoneally administered to starved rats or mice, it was preferentially incorporated into hepatocyte lysosomes, eliciting considerable inhibition of autolysosomal proteolysis.[35-41] As a consequence, autolysosomal turnover was also inhibited, resulting in the accumulation of autolysosomes holding sequestered components in their lumen.[38-41] Due to the accumulated autophagic substrates in the lumen, the density of these autolysosomes was higher than other cell organelles and membranes, thus enabling them to be separated by Percoll gradient centrifugation.[39-41] We isolated mitochondrial/lysosomal fractions from leupeptin-administered control and mutant livers. The mitochondrial/lysosomal fraction, as isolated, was loaded onto isotonic 53% Percoll and centrifuged as described in the Materials and Methods section. As the distribution of lysosomal β-hexosaminidase activity as well as LGP85 and LC3-II shows, the denser autolysosomes were separated in fractions No. 1 to No.8 (Fig. 4A, left) in the control livers. More than 60% of the β-hexosaminidase activity was recovered in the lower autolysosomal fractions. In contrast, the accumulation of denser autolysosomes was significantly inhibited and ~40% of the β-hexosaminidase activit was recovered in autolysosomal fractions in the Pten-deficient liver (Fig. 4A, right) We previously found that betaine homocystein methyltransferase was sequestered in the autophagosomal lume together with other cytoplasmic proteins in the liver.[27] The enzym was immediately degraded by lysosomal proteinases upon maturatio of autophagosomes into autolysosomes. In the presence of leupeptir however, the degradation ceased to accumulate a 32 k-Da fragmen (p32) of the enzyme as a partially digested intermediate (Fig. 4B) As the production of p32 is dependent on the inhibition of cyctein proteinases by leupeptin, p32 can be used as a convenient marker fc autolysosomes accumulating in the presence of leupeptin. As show in Figure 4A, distribution of p32 coincides with those of LGP8 and LC3 in these fractions obtained from control livers. In contras

weaker signals of LC3-II and LGP85 as well as p32 were detected in these fractions of mutant specimens. These data indicate that autophagosome maturation into autolysosomes is also substantially inhibited in Pten deficient livers.

## Discussion

Hepatocyte-specific Pten-deficient mice have lower glucose levels than wild type mice and exhibit hyper insulin sensitivity,[23,24] indicating that a hepatic Pten deficiency exerts profound effects on the entire metabolism of the body. As expected, phosphorylated Akt and MAPK, two signaling molecules that are located downstream of class I PI3-kinase, were markedly increased in Pten-deficient hepatocytes, compared to control hepatocytes.[23,24] Thus, the loss of Pten function in the liver caused the constitutive activation of the class I PI3-kinase/Akt pathway.

The findings herein show that the autophagic protein degradation of long-lived proteins is strongly suppressed in Pten-deficient hepatocytes, compared to control hepatocytes. The magnitude of the suppression was almost equivalent to that of Atg7-deficient hepatocytes.[34] Our data are consistent with the previous observations of colon cancer HT29 cells, in which the class I PI3-kinase/Akt pathway was activated by incubating the cells with dipalmitoyl PtdIns(3,4,5)$P_3$, a class I PI3-kinase product, but not with dipalmitoyl PtdIns(4,5)$P_2$, leading to the inhibition of autophagic protein degradation.[18] Activation of Akt stimulates the mTor/P70S6-kinase pathway and the activation of mTor/P70S6-kinase pathway induces suppression of autophagy.[17] We confirmed that both ribosomal S6 and initiation factor 4E-binding protein, downstream components of the mTor/P70S6-kinase pathway, were phosphorylated in starved Pten-deficient livers. However, rapamycin, which induces autophagic protein degradation of control hepatocytes even under non-starved conditions by inhibiting mTor/P70S6-kinase pathway, did not stimulate protein degradation of Pten-deficient hepatocytes. Thus, autophagy suppression of Pten-deficient hepatocytes cannot be explained as a result of chronic activation of the mTor/P70S6-kinase pathway, but rather may be attributable to some mTor-independent mechanism that has yet to be understood. Transcription regulation by Foxo3 has been proposed recently as a potential mechanism for controlling the ubiquitin-proteasome system and autophagy in skeletal muscles.[42,43] Activated Akt phosphorylates Foxo3 and inhibits its transcriptional functions by keeping Foxo3 away from the nucleus. Dephosphorylated Foxo3 stimulates the transcription of autophagy-related genes—including some ATG genes. It has been also found that a trimeric GTP-binding protein $G_{i3}$ mediates insulin-dependent suppression of autophagy in the rat liver.[44] Connection between activated class I PI3-kinase and $G_{i3}$ may be also considered. Further investigations are necessary to clarify the mechanism by which hyper-activation of the class I PI3-kinase in Pten-deficient mouse livers causes suppression of autophagy.

RT-PCR analysis indicated that the transcription of some *ATG* genes that are involved in the *ATG* conjugation system was significantly decreased. We therefore speculated that chronic activation of Akt might cause the direct inhibition of the *ATG* conjugation reaction. However, the decreased expression of such messages was not connected to a decrease in translation: the cellular levels of these gene products were increased slightly in Pten-deficient livers compared to a normal control. Notably, the levels of Atg12-Atg5 conjugate are higher in mutant livers than in control livers, and LC3 is prese in both the free (LC3-I) and lipidated form (LC3-II) in bo livers. Furthermore, cell fractionation analysis revealed that there no significant difference in intracellular distribution of LC3-I a LC3-II between normal and Pten-deficient livers (Fig. 2C). The data clearly indicate that the loss of Pten does not affect *ATG* conj gation reactions per se. The slight increase in the levels of these A proteins may reflect a compensatory reaction to circumvent t autophagic defect under Pten-deficiency.

Morphological data obtained by electron microsopy demonstra that autophagic vacuoles (autophagosomes plus autolysosome accumulating in the presence of leupeptin decreased by ~70% Pten-deficient livers compared with control livers. This mark reduction in the number of autophagic vacuoles is likely due an impaired autophagosome formation and is considered to be major cause of the defect in autophagic protein degradation. addition, we were able to assess the reduction in autophagosor maturation into autolysosome in Pten-deficiency by biochemi techniques. The centrifugal analysis of denser autolysosomes isolat from leupeptin-administered livers clearly showed that the inhil tion of autophagosome maturation into autolysosomes contribu significantly to the defects in autophagic proteolysis. Leupepti inhibited autolysosomes from control livers were separated at t bottom fractions in Percoll density-gradient centrifugation a possessed lysosomal (β-hexosaminidase and LGP85), autophag somal (LC3-II), and autolysosomal (p32) markers. The distributi of these marker proteins in favor of the denser fractions was consid ably inhibited in the fractions obtained from Pten-deficient livers.

The inactivation and decreased expression of Pten has frequen been observed in many types of cancer cells, including hepatocellu carcinomas, glioblastomas, breast cancers and prostate cancer.[45,46] has been also noted that autophagy is suppressed in many cancer ce and the inactivation of Pten has been proposed as a potential mech nism for the suppressed autophagy in cancer cells.[17] In summa our data provides the first evidence showing that the loss of Pt function causes a strong inhibition of the formation and subseque maturation of autophagosomes, but does not affect *ATG* conjugati reactions. In the yeast *Saccharomyces cerevisiae*, all *ATG* gene produ cooperate with one another to form the pre-autophagosomal stru ture in starvation-induced autophagy.[47] The pre-autophagoson structure consists of an Atg12-Atg5 conjugate and the lipidat form of Atg8 (yeast counterpart of LC3-II) as essential componen and is thought to be an organizing center for autophagosomes. I the organization of the pre-autophagosomal structure, the fur tion of the class III PI3-kinase complex together with Atg16 a Atg9 is required. Meanwhile, some Atg proteins, including At protein kinase (class A Atg proteins), are required for the late stag of autophagosome formation. In other words, they play pivo roles as a bridge between the pre-autophagosomal structure a autophagosomes.[47] It is possible that the loss of Pten function hyper activation of class I PI3-kinase/Akt elicits the inhibition the mammalian counterparts of class A Atg proteins. The identifi tion and characterization of mammalian class A Atg proteins, whi awaits future investigation, will be important in developing a bet understanding of the mechanism of the autophagy defect in Pten-deficiency.

## Materials and Methods

**Control and hepatocyte specific Pten-deficient mice.** *Pten*$^{flox/flox}$ mice (129Ola x C57BL6/J F2) were mated to *AlbCre* transgenic mice (C57BL6/J) as described previously.[24] The expression of Cre is controlled by the promoter of the hepatocyte-specific *albumin* gene. Offspring carrying *AlbCre* plus two copies of the floxed *Pten* allele (*AlbCrePten*$^{flox/flox}$), *AlbCre* plus one copy of the floxed *Pten* allele (*AlbCrePten*$^{Flox/+}$), and *AlbCre* plus two copies of the wild type *Pten* allele (*AlbCrePten*$^{+/+}$) correspond to homozygous mutant, heterozygous mutant and wild-type mice, respectively. For all experiments described herein, homozygous mutant mice and wild type mice between 10 to 15 weeks after birth were used as Pten-deficient and control mice, respectively. All animal experiments were approved by the Institutional Review Board of the Akita University School of Medicine and the Review Board of the Center for Biomedical Research Resources of Juntendo University.

**DNA microarray.** RNAs purified from Pten-deficient and control hepatocytes were amplified, converted to complementary DNAs, and labeled with cyanin 3-CTP and cyanin 5-CTP using a LRIFLA kit (Agilent Technologies, Palo Alto, CA) following the manufacturer's recommended protocol. The resulting amplified complementary RNAs were fragmented and hybridized using the Agilent in situ hybridization plus kit (Agilent Technologies) and subjected to DNA microarray analysis as described by Sato et al.[25]

**RT-PCR.** RT-PCR was performed for the following seven genes: beclin (Atg6), MAP-LC3B, Atg3, Atg7, Atg10, Atg12, Atg16 and β actin. One microgram of RNA samples prepared as templates for the DNA microarray analysis was treated with DNase (Life Technologies, Gaithersburg, MD) and then reverse transcribed using the TaqMan SuperScript™ first strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was carried out according to the method described previously.[24] The following primer sequences were used: Beclin, forward primer (AGCTCAGTACCAGCGGGAGT) and reverse primer (TGGAAGGTGGCATTGAAGAC); LC3B, forward primer (AGATAATCAGACGGCGCTTG) and reverse primer (ATGTCTCCTGCGAGGCATAA); Atg3, forward primer (ATCCTCATCTCCCACCACCT) and reverse primer (TTGGAATGACAGCTTGAACAAA); Atg7, forward primer (GCTGTGGAGCTGATGGTCTC) and reverse primer (CCAGGCTGACAGGAAGAACA); Atg10, forward primer (CCCAGCAGGAACATCCAATA) and reverse primer (AGGCTCAGCCATGATGTGAT); Atg12, forward primer (AACAAAGAAATGGGCTGTGG) and reverse primer (TTGCAGTAATGCAGGACCAG); Atg16L, forward primer (GAATTCAAGGGCTCCCTGTC) and reverse primer (CCTGTGAGTGTGTGCCGTAA); β actin, forward primer (TCC ATG AAA TAA GTG GTT ACA GGA AGT C) and reverse primer (CAA AAA TGA AGT ATT AAG GCG GAA GAT T). The signal for β actin RNA was used as an internal control. All samples were run in triplicate and the final quantification was archived using a relative standard curve.

**Electron microscopy.** Mouse livers were perfused and fixed with 3% glutaraldehyde (TAAB Laboratory Equipment Ltd., Reading, UK) in 0.05 M sodium cacodylate buffer at pH 7.2, post-fixed with 2% osmic acid, epon-embedded, sectioned at a thickness of 1 μm, and stained with toluidine blue. Ultrathin sections were observed using a Hitachi H7100 electron microscope.

**Protein degradation assay.** Primary hepatocytes were isolate from control wild type and mutant mice according to a convention collagenase perfusion procedure[26] and cultured in Williams' mediur E containing 10% FCS (Williams E/10% FCS), as described.[2 Hepatocytes grown in 24-well microplates were incubated wit Williams E/10% FCS containing [$^{14}$C]-leucine (0.5 μCi/ml) fc 22 h to label long-lived proteins. The cells were then washed wit Williams E/10% FCS containing 2 mM unlabelled leucine an incubated with the medium for 2 h to allow degradation of shor lived proteins and to minimize the incorporation of labeled leucin released by proteolysis into protein. The cells were then washed wit 20 mM Na-phosphate, pH 7.4, 0.15 M NaCl (PBS) and incubate at 37°C with Krebs-Ringer bicarbonate buffer and Williams E/10 FCS in the presence or absence of protease inhibitors. During th next 4 h of the chase period, aliquots of the medium were taken an a one-tenth volume of 100% trichloroacetic acid was added to eac aliquot. The mixtures were centrifuged at 12,000 x g for 5 minute and acid-soluble radioactivity was determined by liquid scintillatio counting. At the end of the experiment, the cultures were washe with PBS, and 1 ml of cold trichloroacetic acid was added to fix th cell proteins. The fixed-cell monolayers were washed with trichlc roacetic acid and dissolved in 0.5 ml of 1N NaOH at 37°C. Th amount of radioactivity in an aliquot of 1 N NaOH was determine by liquid scintillation counting. Percent protein degradation wa calculated according to a previously published procedure.[28]

**Density-shift assay of leupeptin-induced autolysosomes usin percoll gradient centrifugation.** Leupeptin (0.4 mg) dissolved i 0.9% NaCl was injected intraperitoneally into control and mutan mice that had been starved for 48 h. The mice were killed after 1 and the livers were excised, dissected into small pieces with scissor and suspended in 4 volumes of ice-cold 5 mM Tes-NaOH, pH 7.4 0.3 M sucrose, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin (extractio buffer). The mixture was homogenized with a motor-driven, loose fitting glass/Teflon homogenizer (4 up-down strokes at 800 rpm) The homogenate was centrifuged at 500 x *g* for 5 minutes, and th postnuclear supernatant was carefully removed. The precipitate wa suspended in extraction buffer (5 ml/liver) and recentrifuged at 50 x *g* for 5 minutes. The combined postnuclear supernatants wer supplemented with 100 mM $CaCl_2$ to give a final concentration of mM. The resulting suspension was incubated at 30°C for 10 minute to allow the mitochondria to swell, and then it was centrifuged a 12,000 x *g* for 20 minutes. The pelleted mitochondrial/lysosom fraction was suspended in extraction buffer. A portion (2 ml) of thi suspension was loaded onto 23 ml of 53% Percoll containing 5 mM Tes-NaOH, pH 7.4, 0.3 M sucrose and centrifuged at 50,000 x g fc 45 minutes using a Beckman 50.2 Ti rotor. After the centrifugation fractions of 1.5 ml were collected from the bottom to the top.

In experiments shown in Figure 2C, mitochondrial/lysosoma microsomal and cytosolic fractions were prepared from starve normal and Pten-deficient livers without leupeptin administration Namely, the post-nuclear supernatants obtained as described abov were centrifuged at 12,000 x *g* for 20 minutes. The pelleted mitc chondrial/lysosomal fraction was suspended in extraction buffe The supernatants (post mitochondrial/lysosomal supernatant) wer further centrifuged at 100,000 x *g* for 1 hr. The resultant pellet and supernatants were used as microsomal and cytosolic fraction respectively.

**Reagents.** Leupeptin, pepstatin and E64d were obtained from the Peptide Institute, Inc., (Osaka, Japan). Percoll was purchased from GE Healthcare Biosceince Co., (Piscataway, NJ). Protein A-Agarose was obtained from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). Williams medium E was purchased from Invitrogen Corp., (Carlsbad, CA). Fetal calf serum albumin (FCS) was purchased from JRH Biosciences Inc., (Menesas, KS). Paraformaldehyde was obtained from Merck (Darmstadt, Germany). Nonidet P-40 (NP-40) and digitonin were obtained from Nacalai Tesque, Inc., (Kyoto, Japan). [$^{14}$C]-leucine (300 mCi/mmol) was purchased from Perkin Elmer Co., Ltd. (Boston, MA).

**Antibodies.** Rabbit antibodies against phosphorylated and unphosphorylated forms of Pten, Akt and ribosomal S6 subunit were obtained from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-labeled antibodies against rabbit and mouse IgG (heavy and light chains) were purchased from Jackson ImmunoResearch Laboratories, Inc., (West Grove, PA).

To prepare antibodies against Atg12, LC3, beclin and GABARAP, the maltose-binding protein (MalBP) fused with mouse Apg12 (mAtg12), and glutathione-S-transferase (GST) fused with human LC3 (GST-hLC3), human beclin 1 (GST-hbeclin) and human GABARAP (GST-hGABARAP) were overexpressed in *E. coli* grown in a 1 l culture at 37°C for 18 h. The fusion proteins were purified by affinity chromatography on an amylose or glutathione-Sepharose column, and 200 mg of each of the purified fusion proteins was emulsified in complete Freund's adjuvant and injected subcutaneously into Japanese white rabbits. Four, six and eight weeks later, each rabbit was injected with a booster of 100 mg of the appropriate antigen emulsified in incomplete Freund's adjuvant. Antisera were precipitated by ammonium sulfate (50% saturation) and dialyzed against PBS, and the antibodies were affinity-purified by adsorption to MalBP-mAtg12-, GST-hLC3- GST-hbeclin- or GST-hGABARAP-immobilized Sepharose columns, followed by elution with 0.2 M glycine-HCl (pH 2.8). Each eluate was concentrated using an Amicon Diaflo apparatus (Danvers, MA) and passed through a MalBP- or GST-immobilized Sepharose column to remove anti-MalBP and anti-GST antibodies. Antibodies to Atg7 and Atg5 were prepared as described previously.[29,30] An antibody that specifically recognizes a 32 k fragment of betaine homocysteine methyltransferase (BHMT) was prepared according to a previously published procedure.[27] An antibody to initiation factor 4E-binding protein was purchased from Zymed Laboratories, Inc., (South San Francisco, CA).

**Biochemical procedures.** Protein concentrations were determined using a BCA protein assay following the manufacturer's protocol (Pierce). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out according to a published procedure.[31] Immunoblot analyses were performed as described previously[32] using an ECL Western Blot Detection Kit (Amersham) as the substrate for the horseradish peroxidase conjugate of the second antibodies. Beta-hexosamidase activity was measured as described previously[33] using 4-methyl-umbelliferyl-b-D-glucosaminide as the substrate.

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