Malignant cells often display defects in autophagy, an evolutionarily conserved pathway for degrading long-lived proteins and cytoplasmic organelles. However, as yet, there is no genetic evidence for a role of autophagy genes in tumor suppression. The beclin 1 autophagy gene is monoallelically deleted in 40–75% of cases of human sporadic breast, ovarian, and prostate cancer. Therefore, we used a targeted mutant mouse model to test the hypothesis that monoallelic deletion of beclin 1 promotes tumorigenesis. Here we show that heterozygous disruption of beclin 1 increases the frequency of spontaneous malignancies and accelerates the development of hepatitis B virus–induced premalignant lesions. Molecular analyses of tumors in beclin 1 heterozygous mice show that the remaining wild-type allele is neither mutated nor silenced. Furthermore, beclin 1 heterozygous disruption results in increased cellular proliferation and reduced autophagy in vivo. These findings demonstrate that beclin 1 is a haplo-insufficient tumor-suppressor gene and provide genetic evidence that autophagy is a novel mechanism of cell-growth control and tumor suppression. Thus, mutation of beclin 1 or other autophagy genes may contribute to the pathogenesis of human cancers.
**Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene**

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Malignant cells often display defects in autophagy, an evolutionarily conserved pathway for degrading long-lived proteins and cytoplasmic organelles. However, as yet, there is no genetic evidence for a role of autophagy genes in tumor suppression. The beclin 1 autophagy gene is monoallelically deleted in 40–75% of cases of human sporadic breast, ovarian, and prostate cancer. Therefore, we used a targeted mutant mouse model to test the hypothesis that monoallelic deletion of beclin 1 promotes tumorigenesis. Here we show that heterozygous disruption of beclin 1 increases the frequency of spontaneous malignancies and accelerates the development of hepatitis B virus–induced premalignant lesions. Molecular analyses of tumors in beclin 1 heterozygous mice show that the remaining wild-type allele is neither mutated nor silenced. Furthermore, beclin 1 heterozygous disruption results in increased cellular proliferation and reduced autophagy in vivo. These findings demonstrate that beclin 1 is a haplo-insufficient tumor-suppressor gene and provide genetic evidence that autophagy is a novel mechanism of cell-growth control and tumor suppression. Thus, mutation of beclin 1 or other autophagy genes may contribute to the pathogenesis of human cancers.

diseases (reviewed in ref. 9). Moreover, there is often an inverse relationship between autophagic activity and malignant potential, raising the possibility that defects in cellular autophagy contribute to the development of cancer. Specifically, malignant or transformed cells often display lower basal autophagic activity than their normal counterparts and do not show increased autophagic protein degradation rates in response to serum deprivation or high cell density (10–13). In rat liver carcinogenesis models, autophagic activity is mildly reduced at a pre-neoplastic stage and becomes more substantially diminished in primary hepatocellular tumor cells (14). However, it is not known whether the decrease in autophagy observed in malignant cells is mechanistically important in tumorigenesis or merely an epiphenomenon associated with malignant transformation.

The identification of several genes required for autophagy provides the opportunity to use genetic approaches to investigate the role of autophagy defects in the development of cancer. Genetic screens in yeast have identified a set of evolutionarily conserved genes — known as the apg or aut genes — that are essential for the execution of autophagy (15, 16). Previously, we identified Beclin 1 (17), the mammalian homolog of yeast Apg6/Vps30p (18), and demonstrated that beclin 1 complements autophagy in yeast disrupted of APG6/VPS30 and promotes starvation-induced autophagy in MCF7 human breast carcinoma cells (19). As part of a class III PI3K complex, Beclin 1 is thought to be important in mediating the localization of other autophagy proteins to pre-autophagosomal structures (20).

The beclin 1 gene maps to a tumor-susceptibility locus on human chromosome 17q21 that is monoallelically deleted in up to 75% of ovarian cancers (21–24), 50% of breast cancers (25, 26), and 40% of prostate cancers (27), raising the possibility that it may be a tumor suppressor. However, biallelic inactivations of beclin 1 have not been demonstrated in human cancers, and therefore, as yet, the gene does not fulfill the Knudson two-hit hypothesis (28) for classification as a tumor suppressor. For example, in a mutational analysis of breast cancer cell lines with net beclin 1 deletions, no mutations were found in the coding regions or splice junctions of remaining alleles, and Northern blot analysis did not identify abnormalities in beclin 1 transcripts (29).

Even though biallelic mutations of beclin 1 have not been demonstrated in human cancer, three lines of evidence suggest that decreased Beclin 1 protein expression may contribute to mammary tumorigenesis. First, Beclin 1 protein is expressed in all normal breast epithelial cells, but the levels of the protein are frequently low or undetectable in malignant breast epithelial cells (19). Second, the autophagy-promoting effects of beclin 1 gene transfer in MCF7 human breast carcinoma cells are associated with tumor-suppressor function (19, 30). In these cells, inducible expression of wild-type Beclin 1, but not an autophagy-defective nuclear export signal mutant of Beclin 1, leads to the loss of malignant morphologic properties, decreased rate of cell proliferation, impaired clonogenicity in vitro, and reduced ability to form tumors in nude mice. Third, Beclin 1 expression and autophagy in MCF7 cells can be induced by tamoxifen (31), an antiestrogen compound that is widely used in the chemoprevention and treatment of breast cancer.

Based on these observations, coupled with the lack of documented biallelic mutations of beclin 1 in human cancer, we hypothesized that beclin 1 is a haplo-insufficient tumor-suppressor gene. In this study, we demonstrate that heterozygous disruption of beclin 1 results in increased spontaneous tumorigenesis, accelerated hepatitis B virus–induced (HBV-induced) neoplasia, increased cellular proliferation, and reduced autophagy in mice. These findings suggest a fundamental role for the beclin 1 autophagy gene in negative cell growth control and tumor suppression.

Methods
Mice. A 129Sv/J genomic bacterial artificial chromosome (BAC) library (Genome Systems Inc., St. Louis, Missouri, USA) was screened with a mouse beclin 1 cDNA fragment to isolate a BAC clone (no. 20463) containing the entire mouse beclin 1 gene. The targeting vector was generated by ligation of a 5′ arm (5.7-kb fragment upstream of exon 1 of beclin 1) and a 3′ arm (0.8-kb fragment within intron 2 of beclin 1) flanking the neomycin resistance cassette (pPGK neo) into the vector, pSP72 (Promega Corp., Madison, Wisconsin, USA). The targeting vector was linearized by NotI and electroporated into 129Sv/J ES cells. Correct targeting resulted in the replacement of beclin 1 exons 1 and 2 by the neomycin resistance gene and was verified by PCR and Southern blot analysis. Chimeric mice were produced by microinjection of correctly targeted ES cells into C57BL/6J blastocysts. Chimeric males were mated with C57BL/6J females, and germ-line transmission of the mutated allele was verified by PCR and Southern blot analysis of tail DNA from F1 offspring. All mice studied for spontaneous tumor development were the F2 generation of 129Sv/J × C57BL/6J crosses. Wild-type littermates were used as controls for beclin 1 heterozygous-deficient mice and sacrificed at identical time points for histopathologic analyses. Note that there is no reported increase in the incidence of any of the tumor types observed in our study in either 129Sv/J or C57BL/6J genetic strains (32), suggesting that strain-specific modifiers are unlikely to account for phenotypic differences observed between the beclin 1−/− and beclin 1+/− F2 littermates in this study. For studies of hepatocellular carcinogenesis, 129Sv/J × C57BL/6J F2 males were crossed with C57BL/6 female mice of the 50-4 lineage (33) that transgenically express the HBV large-envelope polypeptide under the transcriptional control of the mouse albumin promoter. For studies of autophagy in vivo, GFP-LC3 C57BL/6J mice were crossed with beclin 1−/− 129Sv/J × C57BL/6J mice. (A more detailed analysis of the development of the GFP-LC3 C57BL/6J mice for in vivo autophagy detection will be presented elsewhere; N. Mizushima and Y.
Ohsumi, unpublished data). For all analyses of cellular proliferation and autophagy in vivo, studies were performed using beclin 1+/– and beclin 1+/+ mice that had been backcrossed to C57BL/6J for six generations.

All procedures involving animals and their care were approved by the Institutional Animal Care and Use Committee of Columbia University according to guidelines that are in compliance with the NIH’s Guide for the Care and Use of Laboratory Animals.

Southern blot analysis, PCR genotyping, and mutation analysis. Genotyping of tail genomic DNA was performed to detect wild-type and beclin 1 knockout alleles by Southern blotting using EcoRI digestion and a 380-bp 3′ external genomic probe, and by PCR amplification. The sense primer 5′-CTGGACACGAGTTTCAAGATCCTG-3′ (primer 2 in Figure 1a) and the antisense primer 5′-GGGACATGGTGACACAGACCTC-3′ (primer 1 in Figure 1a) were used to detect the wild-type beclin 1 allele (yielding a PCR product of 1,130 bp), and the sense primer 5′-TGCGGGCCAGAGGCCACTGTGTAGC-3′ (primer 2 in Figure 1a) and the identical antisense primer were used to detect the knockout beclin 1 allele (yielding a PCR product of 1,130 bp). The presence of the HBV large-envelope gene was detected by PCR amplification of tail genomic DNA (sense primer, 5′-GAGTCTAGACTCGTGTTGGA-3′; antisense primer, 5′-CAACACTGAACAAA TGCC-3′) and by serum ELISA to detect hepatitis B surface antigen (Auszyme Monoclonal Diagnostic kit; Abbott Laboratories, Abbott Park, Illinois, USA). Genomic DNA from tumors arising in beclin 1+/– mice and from corresponding normal tissue was subjected to Southern blot analysis as described above to detect wild-type and beclin 1 knockout alleles. Mutation analysis of the 12 exons and promoter region of beclin 1 was performed on tumor genomic DNA by sequencing PCR products generated using intronic primer sets provided in Supplementary Table 1 (http://www.jci.org/cgi/content/full/112/12/1809/DC1).

Histologic analysis. For studies examining the effects of beclin 1 heterozygous deletion on spontaneous tumorigenesis, complete necropsies were performed on 104 beclin 1+/+ mice and 95 beclin 1+/– littermates, including 67 beclin 1+/+ and 49 beclin 1+/– mice aged 13–16 months and 37 beclin 1+/– and 46 beclin 1+/+ mice aged 16–18 months. Mice were randomly sacrificed during this age interval without any preselection for adverse clinical status or the presence of palpable tumors. Tumors greater than 1 mm that were present on gross examination were recorded. H&E-stained sections of all major organs were examined separately by two pathologists blinded to genotype (G. Bhagat and G. Cattoretti), and all malignant lesions noted on microscopic examination were recorded. Lymphoid lesions were classified using the Bethesda proposal for classification of lymphoid neoplasms in mice (34).

All neoplastic lesions were stained for Beclin 1 using the polyclonal rabbit anti–Beclin 1 antibody described by Kihara et al. (20) (provided by T. Yoshimori, National Institute of Genetics, Mishima, Japan). Routine immunohistochemistry protocols were used to detect thyroid transcription factor-1 (TTF-1) expression in lung carcinomas, and Pax5, B220, CD3, BCL-6, IRF4, and K light chains in lymphoproliferative lesions (35). For studies examining the effect of beclin 1 heterozygous deletion on HBV carcinogenesis, livers were
resected from 13-month-old offspring of HBV transgenic × beclin 1+/− crosses, including 27 HBV beclin 1+/− mice, 32 HBV beclin 1+/− mice, 35 HBV beclin 1+/− mice, and 29 HBV beclin 1+/− mice. H&E-stained liver sections were examined by a liver pathologist (G. Bhagat) blinded to genotype, and classified according to the extent of preneoplastic changes in a semiquantitative fashion.

Mammary glands from 6- to 9-month-old virgin beclin 1+/− and beclin 1+/− female littermates were examined by whole-mount preparations and H&E staining of whole-mount sections as described previously (36). Mouse mammary gland proliferative lesions were classified according to the Annapolis guidelines (37). For quantitation of mammary epithelial cell proliferation experiments, 5-week-old female virgin mice were injected with 100 µg BrdU/g body weight 2 hours prior to sacrifice. BrdU incorporation was detected in mammary gland sections using a commercially available kit (Roche Diagnostics Corp., Indianapolis, Indiana, USA), and apoptosis was detected by TUNEL staining of adjacent sections using ApopTag Peroxidase In Situ Apoptosis Detection Kit (Intergen Co., Purchase, New York, USA) according to the manufacturer’s instructions.

For studies of germinal center formation, 2-month-old female mice were immunized intraperitoneally with 5 × 10⁶ sheep rbc’s (Colorado Serum Co., Denver, Colorado, USA). Eight days after immunization, mice were sacrificed, and spleen sections were stained with the germinal center B lymphocyte marker peanut agglutinin (PNA; Sigma-Aldrich) (38) to identify germinal centers in immunohistochemical staining. For isolation of germinal B center lymphocytes for quantitative electron microscopy analysis, spleen cells were stained with the B220 B cell marker and PNA, and the B220-high, PNA-high cell population was isolated by FACS sorting as described previously (39) using FACStarPLUS (Becton Dickinson and Co., Franklin Lakes, New Jersey, USA) equipped with two lasers (488 nm and 680 nm).

**Autophagy analysis.** For analysis of autophagy by quantitation of punctate GFP-LC3 staining, 2-month-old male and female starved GFP-LC3 transgenic beclin 1−/−, GFP-LC3 transgenic beclin 1+/−, and GFP-LC3 negative control mice were perfused with 4% paraformaldehyde, and cryosections were prepared from selected organs. GFP fluorescence staining was visualized using an Invision Deconvolution Microscope, and ten images per organ were recorded for five mice per genotype (using a ×100 objective for muscle and a ×40 objective for lung). The number of GFP-LC3 punctate regions per 1,000 µm² of muscle and the number of GFP-LC3 punctate regions per linear millimeter of bronchial epithelium were quantitated by an observer blinded to genotype. For analysis of autophagy by quantitative electron microscopy, Epon-embedded cells prepared as described previously (40) were used to determine the number of autophagic vacuole profiles per square micrometer of cell area. Autophagic vacuole profiles were counted under the microscope using ×12,000 magnification, and cell area was estimated by point counting (41) from negatives taken at ×400 magnification by an observer blinded to genotype. The volume density of both early, immature autophagic vacuoles and late, degradative autophagic vacuoles was determined.

**Results**

Beclin 1 heterozygous disruption in mice results in increased spontaneous tumorigenesis. To evaluate the hypothesis that beclin 1 is a haplo-insufficient tumor-suppressor gene, we studied spontaneous tumorigenesis in mice with heterozygous deletion of beclin 1. First, we generated mice with a targeted disruption in the beclin 1 locus by homologous recombination. The targeting vector was designed to replace exons 1 and 2 of beclin 1 with the neomycin resistance gene (Figure 1a). ES cells and mice harboring the targeted allele were identified by Southern blotting and PCR analyses (Figure 1, b and c). Beclin 1 heterozygous mice were crossed against each other, and the viable offspring included 156 wild-type mice and 298 heterozygous-deficient mice, indicating embryonic lethality of homozygous-deficient mice. We confirmed that beclin 1 heterozygous disruption results in decreased Beclin 1 protein expression both in ES cells and in tissues of beclin 1 heterozygous-deficient (beclin 1+/−) mice (Figure 1d).

Next, we sacrificed approximately 100 beclin 1+/− and 100 beclin 1+/− control littermates aged 13- to 18-months (Figure 2) and examined all major organs macroscopically and microscopically. Beclin 1+/− mice had a significantly higher probability of having a malignancy detected upon gross examination (P < 0.0001, log-rank test; Figure 2a); approximately 15% of beclin 1+/− mice had palpable tumors visible at the time of autopsy that were histologically confirmed to represent malignancies, as compared with only 1% of beclin 1+/− littermates (P < 0.0001, Fisher’s exact test; Figure 2c). Upon microscopic examination, beclin 1+/− mice also had a significantly higher probability than beclin 1+/− mice of harboring a malignancy (P < 0.0001, log-rank test), although more microscopic tumors were observed at an increased frequency with advancing age in mice of both genotypes (Figure 2b). In total, approximately 30% of beclin 1+/− as compared with 14% of beclin 1+/− mice had malignancies (P < 0.0001, Fisher’s exact test; Figure 2d), indicating that beclin 1 heterozygous disruption significantly increases the chance of developing a spontaneous malignancy in mice. In addition to this large cohort of mice that we sacrificed between 13 and 18 months of age, we also observed some tumor-associated mortality in 6- to 13-month-old beclin 1+/− mice, but not in control beclin 1+/− littermates (data not shown).

Three types of malignancies were noted in the beclin 1+/− mice: lung cancers, liver cancers, and lymphomas. Beclin 1−/− mice developed a greater number of lung carcinomas (Figure 2e) than beclin 1+/− mice, and the lung carcinomas tended to be significantly larger, with 80% visible as macroscopic subpleural nodules, whereas the few lung carcinomas observed in the beclin 1+/− mice were only detectable microscopically. The lung cancers showed features of well-differentiated human papillary adenocarci-
nomas, including a bronchioalveolar growth pattern with papillary architecture, nuclear enlargement, prominent nucleoli, increased mitotic rate (Figure 3a), and nuclear immunoreactivity for TTF-1, a tissue-specific transcription factor expressed in bronchial and type II alveolar epithelial cells (42) (Figure 3c). In addition, all lung carcinomas had detectable Beclin 1 protein expression in the tumor cells (Figure 3b). 

Both beclin 1+/– and beclin 1+/+ control littermates developed lymphomas, but the lymphomas occurred more frequently, at an earlier age, and were more likely to present as palpable masses (versus only as a microscopic finding) in the beclin 1+/– mice (Figure 2g). The observed lymphomas were distributed into five morphologic types according to the Bethesda proposal for
classification of lymphoid neoplasms in mice (34), including diffuse large-cell lymphoma (DLCL), follicular lymphoma, the immunoblastic variant of DLCL (DLCL-IB), anaplastic plasmacytoma, and histiocytic sarcoma (see Figure 3, g–i, for representative images of DLCL in a beclin 1+/– mouse). Both beclin 1+/– and beclin 1+/+ mice had DLCLs and follicular lymphomas, whereas DLCL-IBs (n = 3) and histiocytic sarcomas (n = 2) were observed only in beclin 1+/– mice and anaplastic plasmacytoma was observed in one beclin 1+/– mouse. DLCLs were the most common type of observed lymphomas, and most of these were immunoreactive for BCL-6, a transcriptional repressor protein that controls germinal center formation and is implicated in the pathogenesis of human B cell lymphomas (reviewed in ref. 44) (Figure 3i). Using a PCR clonality assay (45), we confirmed clonal Ig gene rearrangements in two representative BCL-6–positive DLCLs from beclin 1+/– mice, and, using a PCR assay for analysis of Ig gene hypermutation (46), we confirmed that the V genes in the BCL-6–positive DLCLs had somatic hypermutation, consistent with a germinal center or post-germinal
center phenotype (data not shown). In addition to frank lymphomas, we also noted that beclin 1+/– mice had an increased frequency of extranodal, splenic, and nodal lymphoproliferative disease (Figures 2g and 3j). Malignancies in beclin 1+/– mice do not contain mutations in the wild-type beclin 1 allele. The increased frequency of spontaneous malignancies in multiple different organs in beclin 1+/– mice suggested that beclin 1 is a haplo-insufficient tumor-suppressor gene. To confirm this, we performed Southern blot and mutational analyses of genomic DNA from all macroscopic malignancies present in the beclin 1+/– mice (n = 15). Southern blot analysis did not reveal any deletions or rearrangements of the remaining wild-type beclin 1 allele (Figure 3k).

Similarly, we sequenced all 12 beclin 1 exons and the beclin 1 promoter region in genomic DNA from these malignancies and did not observe any mutations in the wild-type beclin 1 allele. In addition, as noted above, Beclin 1 protein was detected by immunohistochemistry in all lung carcinomas and all hepatocellular carcinomas in the beclin 1+/– mice (Figure 3, b and e). Furthermore, Beclin 1 protein expression was detected by Western blot analysis in the lymphomas in beclin 1+/– mice (data not shown). Thus, we could find no evidence for beclin 1 mutation or silencing of Beclin 1 protein expression in the tumors observed in beclin 1+/– mice. Together, these data demonstrate that functional inactivation of one beclin 1 allele is sufficient to promote tumorigenesis and that beclin 1 is a haplo-insufficient tumor-suppressor gene.

Beclin 1 heterozygous disruption in mice accelerates the development of HBV-induced premalignant lesions. To examine whether beclin 1 heterozygous deletion also accelerates the neoplastic process in an established mouse tumor model, we crossed beclin 1+/– and beclin 1+/+ mice with mice that transgenically express the HBV large-envelope polypeptide under the transcriptional control of the mouse albumin promoter (herein referred to as HBV transgensics) (33). The livers of HBV transgenic mice progress through a series of hyperplastic and preneoplastic stages that culminate in frank hepatocellular carcinoma (33). We chose this model because the liver is a major site of nutrient starvation–induced autophagy (reviewed in ref. 47); because decreased autophagic activity has been postulated to play a role in chemical-induced hepatocellular carcinogenesis in rats (14); and because protein caloric restriction, a trigger of autophagy, represses HBV-induced liver injury in HBV transgenic mice (48).

To detect differences in the progression of HBV-induced liver cell neoplasia, we sacrificed beclin 1+/– and beclin 1+/+ HBV transgenic mice at a time point prior to the predicted onset of hepatocellular carcinoma (33). At 13 months of age, we found that the severity of preneoplastic changes, characterized by the extent of liver with small-cell dysplasia—a important histopathologic predictor of malignant transformation (49–51)—was significantly increased in the beclin 1+/– compared with the beclin 1+/+ mice (P = 0.0289, Mantel-Haenszel χ² test; Figures 2h and 3f). Out small-cell dysplastic changes were observed in the livers of HBV–littermates that were either beclin 1+/– or beclin 1+/+. Thus, beclin 1 heterozygous deletion both increases susceptibility to spontaneous malignancies and accelerates HBV-induced hepatocellular carcinogenesis.

Beclin 1 heterozygous disruption results in increased cellular proliferation in vivo. These findings raised the question of whether beclin 1 heterozygous deletion affects cell growth control in vivo. Although mammary tumors were not observed in our large cohort of 13- to 18-month-old mice, we found proliferative lesions present in mammary gland whole-mount examinations from 10 of 15 beclin 1+/– compared with 0 of 15 beclin 1+/+ 6- to 9-month-old mice. Histologically, these lesions included mammary intraepithelial neoplasia (also known as ductal carcinoma in situ), adenomyoepithelioma, and acinar neoplasia (Figure 4, a–d). These observations, coupled with the frequent monoallelic deletions of beclin 1 in human breast cancer (29), suggested that mammary epithelial cell growth control may be altered by beclin 1 heterozygous disruption.

To evaluate this, we quantitated epithelial cell proliferation (using BrdU incorporation staining) and apoptosis (using TUNEL staining) in developing mammary glands from 5-week-old female mice. No differences were detected in the percentage of TUNEL-positive cells in terminal end buds (TEBs) and ducts in beclin 1+/– versus beclin 1+/+ mice (data not shown), indicating that beclin 1 heterozygous deletion does not alter mammary epithelial cell apoptosis. However, we observed significant differences both in the distribution of cellular proliferation in the TEBs and in the quantity of cellular proliferation in the TEBs and ductal epithelial cells. In the TEBs from mammary glands of wild-type littersmates, a normal pattern of cellular proliferation was observed and the majority of cells undergoing DNA synthesis were localized in the cap cell and body region (Figure 4f). In contrast, in the mammary glands of beclin 1+/– mice, there was a marked increase in BrdU staining in the neck region (Figure 4e) and a significant increase in the overall percentage of BrdU-positive cells in the TEBs (P = 0.025; Figure 4g), indicating that beclin 1 heterozygous deficiency results in abnormal cellular proliferation in the TEBs. In addition, the mammary ducts of beclin 1+/– mice showed a marked increase in DNA synthesis, with more than 35% of cells labeling positive with BrdU as compared with approximately 5% in the beclin 1+/+ mice (P < 0.0001; Figure 4j). Consistent with the hyperproliferation of the mammary ductal epithelial cells, the mammary ducts in the beclin 1+/– mice frequently were morphologically abnormal (Figure 4h), with multiple layers of epithelial cells (versus single layers in the wild-type mice; Figure 4i) and intraluminal collections of epithelial cells.

We further confirmed the pro-proliferative effect of beclin 1 heterozygous disruption in a cell type that gave rise to increased spontaneous malignancies in the...
beclin 1+/– mice, the germinal center splenic B cell. Following immunization of 2-month-old mice with sheep rbc's, we found a significant increase in both the number (P = 0.027, t test) and the size (P = 0.018, t test) of germinal centers in the spleens of beclin 1+/– mice (Figure 4, k–n). Together with the results in mammary epithelial cells, these results indicate that beclin 1 heterozygous disruption increases cellular proliferation in vivo, beginning at an early age. We speculate that the increased cellular proliferation in beclin 1+/– mice may increase the number of genetic mutations that occur over the lifetime of the animals, thereby contributing to the increased spontaneous tumorigenesis that occurs in older beclin 1+/– mice.

Beclin 1 heterozygous disruption decreases autophagy in vivo. As noted above, previous studies have indicated that mammalian Beclin 1, like its yeast (18) and Caenorhabditis elegans (52) orthologs, plays a role in cellular autophagy (19, 30). The yeast homolog Apg6/Vps30p is also involved in vacuolar protein sorting (53, 54), raising the possibility that mammalian Beclin 1, as part of a PI3K complex that may localize in the trans-Golgi network (20), is involved in other membrane-trafficking pathways. However, such other functions have not yet been identified in mammalian cells, and human beclin 1 and its orthologs in C. elegans and Arabidopsis thaliana rescue only autophagy, but not vacuolar protein sorting function in apg6/vps30–null yeast.
Therefore, we focused on determining whether heterozygous deletion of beclin 1 in mice affects its known function in autophagy. To accomplish this, we measured autophagy in tissues from beclin 1+/– and beclin 1+/+ mice that transgenically express an autophagy marker, GFP-LC3 (N. Mizushima and Y. Ohsumi, unpublished data). In the absence of autophagy, LC3 and its yeast homolog Apg8/Aut7p have a diffuse cytoplasmic distribution. Upon stimulation of autophagy, LC3 and Apg8/Aut7p localize to pre-autophagosomal and autophagosomal membranes (55–57). Previous studies have demonstrated that the visualization of GFP-positive punctate regions is a useful marker of autophagy in mammalian cells expressing GFP-LC3 (57) and in yeast cells expressing GFP–Apg8/Aut7p (58, 59). In GFP-LC3 transgenic mice, a punctate appearance of GFP staining also corresponds to pre-autophagosomal or autophagosomal structures identified at the ultrastructural level, and the muscle has been shown to be an important site of starvation-induced autophagy (N. Mizushima and Y. Ohsumi, unpublished data).

First, we quantitated the amount of autophagy in starved muscle from 2-month-old beclin 1+/+ and beclin 1+/– GFP-LC3 transgenic mice. We found that the number of GFP-LC3–positive dots was significantly decreased in the muscle of starved beclin 1+/– as compared with beclin 1+/+ mice (P < 0.0001, t test). Next, we asked whether beclin 1 heterozygous deletion affects autophagy in any of the tissues associated with increased spontaneous tumorigenesis. We found that the GFP-LC3 transgene was not expressed in lymphocytes, variably expressed in the liver, and strongly expressed in both muscle and bronchial epithelial tissue (Figure 5, e–h).

![Figure 5](image)

**Figure 5**

Beclin 1 heterozygous deletion decreases autophagy in muscle (a–d), bronchial epithelia (e–h), and germinal center B lymphocytes (i). (a–d) Representative images of GFP-LC3 staining in (a–c) and quantitation of GFP-LC3–positive dots (d) in muscle from 2-month-old mice subjected to 24-hour starvation. The x axis labels denote beclin 1 genotype. Results shown in d represent the mean ± SEM for approximately 50 images obtained from five mice per genotype (P < 0.0001, t test). (e–h) Representative images of GFP-LC3 staining (e–g) and quantitation of GFP-LC3–positive dots (h) in bronchial epithelial tissue from 2-month-old mice subjected to 24-hour starvation. The x axis labels denote beclin 1 genotype. Results shown in h represent the mean ± SEM for approximately 50 images obtained from five mice per genotype (P < 0.0001, t test). (i) Morphometric electron microscopic quantitation of autophagic vacuole (AV) profiles in germinal center B lymphocytes isolated from 2-month-old nonstarved mice. The x axis labels denote beclin 1 genotype. Avi denotes early, immature autophagic vacuoles, and Avd denotes late, degradative autophagic vacuoles. Results shown in i represent the mean ± SEM for parallel quantitations from seven to eight grid squares (P = 0.02, t test). Arrows in b and f denote representative GFP-LC3–positive punctate dots that are quantitated in d and h, respectively. Scale bars: 10 nm.
type II alveolar and bronchial epithelial cells in the lung. Since the well-differentiated papillary lung carcinomas observed in the beclin 1+/– mice in our study displayed morphologic features strongly suggestive of a bronchial cell origin, we quantitated autophagy in starved bronchial epithelial cells from 2-month-old mice. While the absolute amount of autophagy was significantly less in bronchial epithelial cells than in muscle, autophagy was significantly reduced in bronchial epithelial cells from beclin 1+/– as compared with beclin 1+/+ mice (P < 0.0001, t test) by a magnitude similar to that observed in muscle (Figure 5, e–h).

To further evaluate whether autophagy is reduced in young beclin 1+/– mice in cell types associated with increased spontaneous tumorigenesis in older mice, we performed quantitative electron microscopic morphometric analysis of autophagic vacuole accumulation in germinal center B lymphocytes (i.e., B220+, PNA+) isolated from beclin 1+/– and beclin 1+/+ mice 10 days after immunization with sheep rbc’s. Our results indicate that beclin 1+/– germinal center B lymphocytes have significantly fewer autophagic vacuoles than beclin 1+/+ germinal center B lymphocytes (P = 0.02, t test; Figure 5i). Together, these findings demonstrate that beclin 1 heterozygous deletion reduces autophagic activity in a tissue that undergoes starvation-induced increases in autophagy (i.e., muscle) and in at least two cell types that are associated with increased spontaneous tumorigenesis in older mice (i.e., bronchial epithelial cells and germinal center B lymphocytes).

Discussion

Our observations provide direct genetic evidence that beclin 1, a gene that is monoallelically deleted in a high percentage of human breast, ovarian, and prostate cancers, is a haplo-insufficient tumor-suppressor and autophagy gene. To date, a number of different tumor-suppressor genes have been identified that function in different cellular processes thought to be important in tumor suppression, including cell cycle regulation, DNA damage response, apoptosis regulation, and preservation of genomic stability (60). Our findings suggest that autophagy genes may represent a novel class of tumor-suppressor genes and that genetic disruption of autophagy may represent a novel mechanism of tumorigenesis.

While beclin 1 is the first downstream autophagy-execution gene linked to human cancer, it is important to note that certain oncogenes (e.g., PI3K and AKT) are already known to function in the negative regulation of autophagy (61, 62), and that the PTEN tumor-suppressor gene is known to function in the positive regulation of autophagy (62). Furthermore, rapamycin analogs that inhibit mTOR, a downstream target of class I PI3K and Akt, induce autophagy in yeast and mammalian cells (63); reduce neoplasia in tumors that arise in PTEN-deficient mice (64); and have yielded encouraging results in early clinical cancer trials (65, 66). It has been commonly assumed that the mechanisms by which the class I PI3K signaling pathway promotes oncogenesis involve the induction of cell growth in response to mitogenic signals, the inhibition of apoptosis, or the promotion of angiogenesis. Likewise, it is generally believed that the PTEN tumor-suppressor gene and the rapamycin analogs exert their antitumor effects by antagonizing these effects of class I PI3K signaling. However, based on our finding that genetic inactivation of beclin 1 (an autophagy gene known to be downstream of TOR in yeast; ref. 6) is sufficient to promote tumorigenesis, we propose that the downregulation of autophagy — by activating mutations in class I PI3K or AKT or inactivating mutations in PTEN — may contribute to oncogenesis.

Although the role of autophagy regulation by the class I PI3K signaling pathway in oncogenesis has not yet been established, recent data in C. elegans suggest that beclin 1 and other autophagy genes are necessary for a dauer developmental-arrest phenotype that is negatively regulated by the C. elegans class I PI3K signaling pathway and positively regulated by the C. elegans PTEN ortholog daf18 (52). Such findings raise the strong possibility that autophagy genes play an evolutionarily conserved role in growth-arrest phenotypes that are negatively regulated by the oncogenic class I PI3K signaling pathway and positively regulated by the PTEN tumor suppressor. This possibility has important implications for the role of autophagy as a downstream cellular pathway that contributes to tumor suppression.

The precise mechanisms by which the autophagy function of Beclin 1 contributes to tumor suppression are not known. In the late 1970s, several studies demonstrated that the rate of cell growth represents a balance between the amount of protein synthesized and the amount of autophagic protein degradation and established autophagy as a lysosomal pathway involved in cell-growth control (10, 67–70). Autophagy may also contribute to tumor suppression by degrading specific cellular organelles and long-lived proteins that are essential for regulating cell growth, thereby functioning as a brake on cell growth in response to mitogenic signals. In this manner, decreased autophagy would be predicted to result in increased cellular proliferation — which is what we observed in the mammary glands and germinal center B cells in younger mice in our study — and, consequently, to increase the likelihood of the accumulation of secondary oncogenic mutations. Alternatively, by removing damaged organelles that increase genotoxic stress, autophagy may serve a more direct role in protecting cells against genetic mutations. The transformation potential of mutations in other oncogenes or tumor-suppressor genes may also be enhanced by decreased cellular autophagy; in support of this concept, we have previously shown that the tumorigenicity of MCF7 human breast carcinoma cells can be inhibited when autophagic activity is restored by beclin 1 gene transfer (19, 30). This ability of beclin 1 gene transfer to inhibit tumorigenicity of breast carcinoma cells suggests that beclin 1 functions as a tumor suppressor in a cell-autonomous fashion; it therefore seems unlikely.
that the increased spontaneous tumorigenesis observed in beclin 1 heterozygous-deficient mice can be explained by possible effects of reduced Beclin 1 expression on immune surveillance. Although autophagy has been described as a form of nonapoptotic cell death (reviewed in ref. 31), we did not observe a decrease in cell death in tissues of the beclin 1+/- mice (Figure 4 and data not shown), and it seems unlikely that decreased cell death contributes to the increased tumorigenesis in mice with heterozygous disruption of beclin 1. However, we cannot rule out the possibility that as-yet undefined functions of mammalian Beclin 1 in vesicular transport (as part of a class III PI3K complex) contribute to its role in regulating cellular proliferation and tumorigenesis. Further studies in other autophagy-gene mouse knockout models will be important to confirm the role of the cellular autophagic machinery in tumor suppression.

In summary, our findings demonstrate that beclin 1 is a haplo-insufficient tumor-suppressor gene and raise the possibility that the genetic disruption of autophagy—either by mutation of downstream autophagy-execution genes or by mutations in upstream autophagy-regulatory signaling pathways—may be an important mechanism of oncogenesis. Although previous studies have reported an inverse association between levels of autophagy and malignant transformation (reviewed in refs. 31, 71), our findings directly demonstrate that genetic inactivation of an autophagy-execution gene promotes tumorigenesis. Furthermore, since beclin 1 is monoallelically deleted in a high percentage of human sporadic breast and ovarian cancers (reviewed in ref. 29), the demonstration of a haplo-insufficient tumor-suppressor phenotype of beclin 1 in mice may have direct implications for the role of such genetic alterations in the molecular pathogenesis of human cancer.

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