Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor

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Contributed by Arnold J. Levine, September 29, 2003

The biochemical properties of beclin 1 suggest a role in two fundamentally important cell biological pathways: autophagy and apoptosis. We show here that beclin 1$^{-/-}$ mutant mice die early in embryogenesis and beclin 1$^{+/-}$ mutant mice suffer from a high incidence of spontaneous tumors. These tumors continue to express wild-type beclin 1 mRNA and protein, establishing that beclin 1 is a haploinsufficient tumor suppressor gene. Beclin 1$^{-/-}$ embryonic stem cells have a severely altered autophagic response, whereas their apoptotic response to serum withdrawal or UV light is normal. These results demonstrate that beclin 1 is a critical component of mammalian autophagy and establish a role for autophagy in tumor suppression. They both provide a biological explanation for recent evidence implicating beclin 1 in human cancer and suggest that mutations in other genes operating in this pathway may contribute to tumor formation through deregulation of autophagy.

Autophagy is a complex catabolic program for lysosomal degradation of proteins and other subcellular constituents. It is often activated in response to nutrient deprivation, when it leads to recycling of organelles and other cytoplasmic substances to provide metabolic precursors. Genetic analysis in yeast has identified a large number of genes required for autophagy and has begun to place them into an ordered pathway that is required for the response to starvation (1–3). Failure to activate autophagy in response to nutrient deprivation, or its constitutive activation in response to stress, can lead to cell death. For this reason, autophagy is sometimes referred to as a second form of programmed cell death. Autophagy and apoptosis are often activated together in response to stress (4, 5). Although autophagy has been shown to be activated in response to starvation, and in many neurodegenerative conditions, its role in normal development and tissue homeostasis has not been determined.

Beclin 1 is the mammalian orthologue of the yeast Apg6/Vps30 gene. It can complement the defect in autophagy present in *apg6* yeast strains and stimulate autophagy when overexpressed in mammalian cells (6). Beclin 1 can also bind to Bcl-2, an important regulator of apoptosis (7). Beclin 1 is monoallelically deleted in human breast and ovarian cancers and is expressed at reduced levels in those tumors (6, 8). In the nervous system, beclin 1 is present in a complex bound to glutamate receptor δ2 (GluRδ2), whose constitutive activation in the lurcher mouse leads to the activation of autophagy and death of cerebellar Purkinje cells (9). To understand the role of beclin 1 and autophagy in development and tissue homeostasis, we have generated and analyzed beclin 1$^{-/-}$ mutant mice. Our results demonstrate that beclin 1 is essential for early embryonic development and is a haploinsufficient tumor suppressor gene, and they show that beclin 1 is not required for apoptosis but that it is necessary for autophagy. Taken together, these results demonstrate that beclin 1 and autophagy are critical for maintenance of tissue homeostasis *in vivo*. They also suggest that failures in the regulation or execution of autophagy as a result of mutations in other genes known to regulate this pathway may play an important role in human cancer.

Methods

Generation of Beclin 1-Deficient Embryonic Stem (ES) Cells and Targeted Deletion of Beclin 1 Mice. A 4.0-kb *Eco*RI fragment in the 5′ flanking region and a 2.8-kb genomic fragment spanning the second intron of the beclin 1 gene were isolated from a BAC clone containing the beclin 1 gene and subcloned into vector with PGK-Neo^r cassette. The resulting targeting vector was electroporated into ES cells. ES clones resistant to G418 (100 μg/ml) were screened, and targeted ES clone$^{+/-}$ was identified by Southern blot analysis. The homozygous ES mutant clones were selected by growing heterozygous ES clones in medium with elevated G418 concentration (2 mg/ml). Two independent homozygous mutants of ES clones$^{-/-}$ were obtained and confirmed by Southern blot and competitive PCR analyses. An oligo primer (TGGAGGGCAGTCCATACCCTGG) common to the beclin$^+$ and beclin$^-$ alleles and two primers specific to the beclin$^+$ (GAGCTGGCTCCTGTGAGTATG) or beclin$^-$ (CGCCTTCTATCGCCTTCTTGACGAGTTCT) allele were combined in the same PCR reaction. ES clone$^{+/-}$ was injected into C57BL/6 blastocysts to obtain chimeric mice. The transmitted heterozygous mice were intercrossed to generate beclin 1$^{-/-}$ embryos. For studies of tumors in the beclin 1$^{+/-}$ animals, littermates from hybrid background of C57BL/6 and 129/Ola were analyzed. The genotypes of the mice were determined by Southern blot or PCR analyses from tail genomic DNA.

Mouse Embryo Study. Embryonic stage (days postcoitum) was estimated by timed pregnancies. Embryos were dissected and imaged followed by PCR genotyping with genomic DNA extracted from embryo. Acridine orange staining of live embryos was done as described (10). For histology of embryos, pregnant females were perfused with 4% paraformaldehyde, embryos in decidua were postfixed overnight and embedded in paraffin, and 8-μm serial sections were prepared. Embryo sections were immunostained with anti-beclin 1 monoclonal antibody (10 μg/ml) according to the MOM protocol (Vector Laboratories), followed by hematoxylin staining.

Embryoid Body Analysis. Wild-type (wt) and beclin 1$^{-/-}$ ES clones were plated at the same cell density and cultured in the absence of leukemia inhibitory factor (LIF) and feeder cells as described (11). Embryoid bodies (EBs) at day 14 were collected and fixed either in 4% paraformaldehyde for standard histology [hema-

Abbreviations: EB, embryoid body; E*n*, embryonic day *n*; ES, embryonic stem; H&E, hematoxylin/eosin; VE, visceral endoderm; wt, wild type.

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toxylin/eosin (H&E) stainings] or in 2% glutaraldehyde followed by plastic embedding for semithin (1-μm) section. Some paraffin sections were immunostained with anti-amnionless antibody (1:400) and counterstained with hematoxylin. Semithin sections were stained with toluidene blue and imaged under light microscope.

ES Cell Apoptotic Cell Assay. ES cells were grown in medium containing 10% serum and leukemia inhibitory factor (LIF). Cell death was induced by serum withdrawal for 72-h culture or by UV shining at 20 J/m^2, followed by an additional 12-h incubation. Cell death was quantified by fluorescence-activated cell sorting (FACS) after propidium iodide (5 μg/ml) staining.

Electronic Microscopy for Autophagy Assay. ES cells were grown on gelatinized plates in normal medium or in Earle's balanced salt solution (EBSS) for 2 h. Cell samples were then processed for conventional electronic microscopy essentially as described (9). NIH IMAGE software was used to analyze the area of autophagy vacuoles (including AVi and AVd) (12) and the total area of cell in each section. Twenty-five sections were examined for either wt or beclin $1^{-/-}$ ES cells.

Tumor Histological Examination. Complete necropsies were performed at the first sign of morbidity or when tumors were of a visible size. The sections from the same tumors were immunostained with anti-beclin 1 antibody (10 μg/ml) according to the MOM procedure (Vector Laboratories). Tumor tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Sections (4–5 μm) were prepared and stained with H&E according to standard protocol. Representative tumors were selected for immunohistochemical analysis. The statistical analysis was performed by using Fisher's exact *t* test with a 2 × 2 table.

Results

Targeted Deletion of Beclin 1 in ES Cells. The murine beclin 1 gene was inactivated in ES cells by using a targeting vector that replaced exon 1, exon 2, and 1.6 kb of the 5′ flanking DNA with a neomycin resistance cassette (Fig. 1*a*). This resulted in deletion of both the transcription initiation site and the translation start codon. Both heterozygous and homozygous deletions were created in ES cells (Fig. 1 *b* and *c*). Two independent homozygous beclin $1^{-/-}$ ES cell clones were identified. Western blot analysis confirmed a decrease in beclin 1 protein in beclin $1^{+/-}$ ES cells and a complete lack of beclin 1 in beclin $1^{-/-}$ ES cells, demonstrating that the targeted allele is a null mutation (Fig. 1*d*).

Loss of Beclin 1 Contributes to Embryonic Lethality. Chimeric mice produced from injecting an ES$^{+/-}$ clone into C57BL/6 blastocysts transmitted the mutant allele to F_1 offspring. However, no homozygous mutant offspring were born from intercross of beclin $1^{+/-}$ mice in >100 pups genotyped at weaning by Southern blot or PCR analysis, indicating that beclin 1 homozygous mice die during embryonic development (Fig. 2 *a* and *b*). Genotyping of embryos isolated from this cross at embryonic day (E) 8.5 failed to reveal any null mutant embryos, whereas the typical Mendelian ratio of wt, heterozygous, and homozygous embryos was recovered at E7.5 (Fig. 2*a*). However, embryos devoid of beclin 1 exhibit a profound developmental delay that is evidenced by their severely reduced size at E7.5 (Fig. 2 *f–j*). Strong beclin 1 expression is detected in the extraembryonic visceral endoderm (VE) in wt or heterozygous embryos at E6.5 (Fig. 2*c*) and E7.5 (Fig. 2*d*). Although beclin 1 expression is not detected in the null mutant embryos, it is apparent that extraembryonic VE can form in these embryos despite the lack of beclin 1 expression and growth retardation (Fig. 2 *f* and *g*). Proamniotic cavities were clearly observed from both extraembryonic and

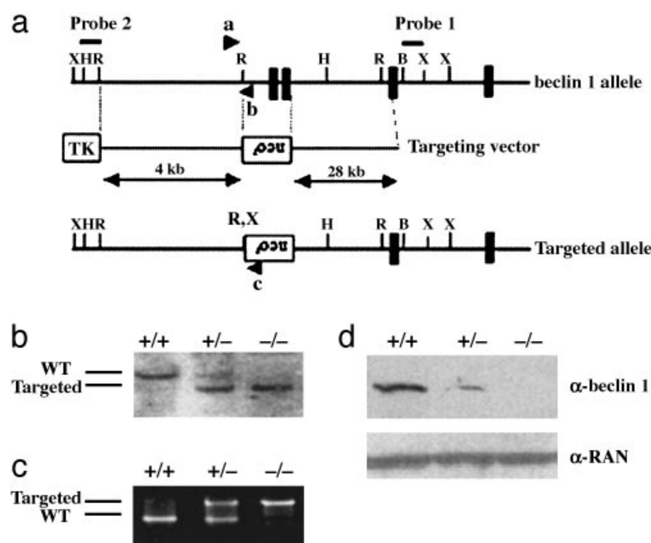
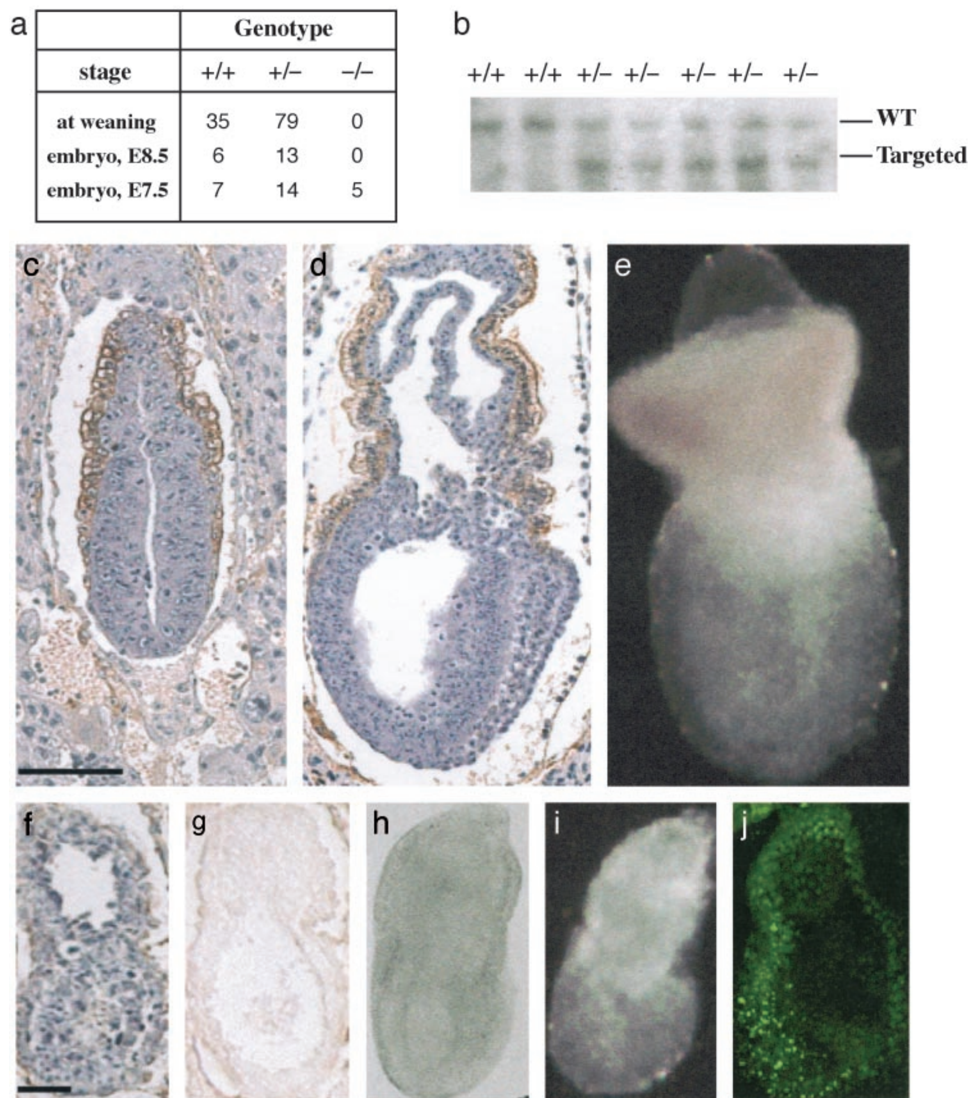


Fig. 1. Targeted disruption of beclin 1 in embryonic cells. (*a*) Genomic structure of beclin 1 (showing exons 1–4 only; solid box), targeting vector, and beclin 1 allele after targeted deletion. The start codon is indicated by an asterisk. Arrow "a" represents a common primer for both the wt and targeted beclin 1 alleles, arrow "b" represents a specific primer for the wt allele, and arrow "c" represents a specific primer for the targeted allele. X, *Xba*I; H, *Hin*dIII; R, *Eco*RI; B, *Bam*HI; neo^r, neomycin resistance cassette; TK, thymidine kinase marker. (*b*) Southern blot analysis of beclin 1 mutant ES clones after digestion with *Xba*I. The probe used to distinguish wt and targeted allele is indicated in *a*. wt allele is detected at a size of 11 kb, and mutant allele is detected at 7 kb. (*c*) Competitive PCR assay of three different genotypes of ES cells (+/+, +/−, and −/−) with primers a–c described in *a*. wt allele (250 bp) can be separated from mutated allele (500 bp) on a 1.5% agarose gel. (*d*) Western blot study of beclin 1 expression in ES cells from three different genotypes with anti-beclin 1 antibody. Anti-RAN antibody was used for protein loading control in each lane.

embryonic components in null mutant embryos, although there is no evidence of closure of the proamniotic canal, and the amniotic fold failed to develop in null mutant embryos (Fig. 2 *h* and *j*). In contrast to the restricted pattern of cell death reported in wt embryos at this age (9), acridine orange staining revealed widespread cell death in beclin $1^{-/-}$ embryos at E7.5 (Fig. 2*j*).

EB formation from ES cells cultured in the absence of leukemia inhibitory factor (LIF) and feeder cells has been used for *in vitro* studies of early embryonic development and developmental cell death (13, 14). To gain further insight into the consequences of the beclin 1 mutation, EB formation was assessed in wt and beclin $1^{-/-}$ ES cell cultures. EB cultured from wt ES cells form cavitated or cystic EB as a result of programmed cell death in the core of the EB. With continuous growth, the cavity of cystic EB increases and develops into expanded cystic EB (Fig. 3*a Left*) (12). At day 14 of culture, inspection of 250 EBs derived from wt ES cells revealed 12 expanded cystic EBs (≈5%). No expanded cystic EBs were observed among 250 EBs derived from beclin $1^{-/-}$ ES cells (Fig. 3*b Left*) ($P = 0.002$). The failure of beclin $1^{-/-}$ ES culture to form expanded cystic EBs is presumably due to a reduction of cell death or cell clearance at the core of the EB (13). Similar results were obtained from EBs derived from two independent beclin $1^{-/-}$ ES cell clones. Staining of EBs with antiserum against amnionless (15) revealed that EBs derived from both wt and null mutant ES cells can form VE, although in beclin $1^{-/-}$ EB, this cell layer was composed of larger, less well organized cells (Fig. 3 *a* and *b*, *Right*). Thus, the average cell area from beclin $1^{-/-}$ VE cells (87.75 ± 41.3 μm^2, $n = 150$) was approximately twice that of wt VE cells (43.44 ± 10.81 μm^2, $n = 150$). Despite this difference,



stage	Genotype +/+	+/–	–/–
at weaning	35	79	0
embryo, E8.5	6	13	0
embryo, E7.5	7	14	5

Fig. 2. Disruption of beclin 1 causes early embryonic lethality. (*a*) Genotype distribution of offspring and embryos from beclin $1^{+/-}$ intercrosses examined by Southern blotting or PCR. (*b*) Southern blot analysis of mouse genotype at age of weaning from a litter born by heterozygous parents. (*c* and *d*) Immunohistochemical analysis of beclin 1 expression in wt or heterozygous embryo at E6.5 and E7.5, respectively. Sections were counterstained with hematoxylin. (*e*) Image of wt whole embryo at E7.5. *c–e* are at the same magnification with a scale bar of 100 μm. (*f* and *g*) Sections from the same null mutant embryo (E7.5) were immunostained with anti-beclin 1 antibody and counterstained with (*f*) or without (*g*) hematoxylin. (*h*) Whole embryo of null mutant (E7.5) viewed under a Zeiss Axiovert confocal microscope with differential interference contrast microscopy. (*i*) The same embryo as in *h* viewed under serological microscope. (*j*) Whole embryo of null mutant stained with acridine orange and viewed with fluorescence under a Zeiss confocal microscope. *f–j* are at the same magnification with a scale bar of 20 μm.

the typical features of VE cells, including brush border microvilli and the presence of vacuoles, were observed in VEs from both wt and beclin $1^{-/-}$ EBs. The high expression of beclin 1 in VE of wt embryos (Fig. 2 *c* and *d*) and the abnormalities in VE formed from beclin $1^{-/-}$ EB (Fig. 3*b*) suggest that defects in VE contribute to the death of beclin $1^{-/-}$ embryos.

Beclin $1^{+/-}$ Mutant Mice Suffer from a High Incidence of Spontaneous Tumors. Beclin $1^{+/-}$ mice develop normally and are fertile. However, many adult beclin $1^{+/-}$ mice developed signs of morbidity and displayed visible tumors as they aged. To further investigate this phenotype, 18- to 22-month-old beclin $1^{+/-}$ mice and their wt littermates were killed and examined for tumors. We have found that 16 of 27 ($\approx$59%) autopsied beclin $1^{+/-}$ mice developed tumors, whereas only 3 of 21 ($\approx$14%) wt mice developed tumors (Fig. 4*a*). These included seven B cell lymphomas (Fig. 4 *b* and *c*), one lymphoblast cell lymphoma, seven heptocellular carcinomas (Fig. 4 *d–f*), and one mouse that developed both a B cell lymphoma and adenocarcinoma of the lung (Fig. 4*g*). The tumors present in wt mice were all lymphomas. Thus, beclin $1^{+/-}$ mice not only had a higher cancer rate ($P = 0.001$, Fig. 4*a*) but also a different spectrum of tumor types. Furthermore, the tumors found in beclin $1^{+/-}$ mice were larger than those found in wt mice, indicating that these tumors may have formed at an earlier age.

To determine whether the tumors present in beclin $1^{+/-}$ mice occur through loss of heterozygosity or because of a gene dosage effect, we analyzed the expression of beclin 1 protein from tumor tissues of beclin $1^{+/-}$ mice. Western blot analysis (Fig. 5*a*) and immunohistochemical study (Fig. 5 *b* and *c*) showed that beclin 1 protein was present in all tumors examined. Furthermore, sequence analysis of beclin 1 cDNAs isolated from six tumors

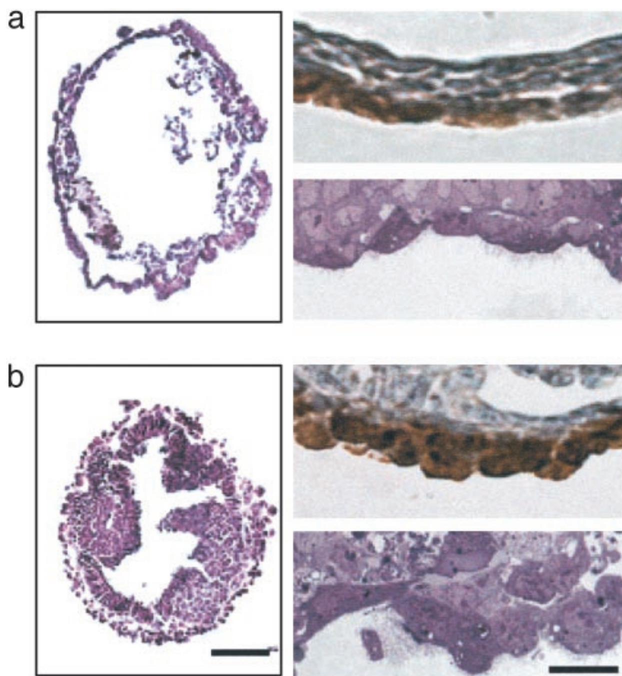
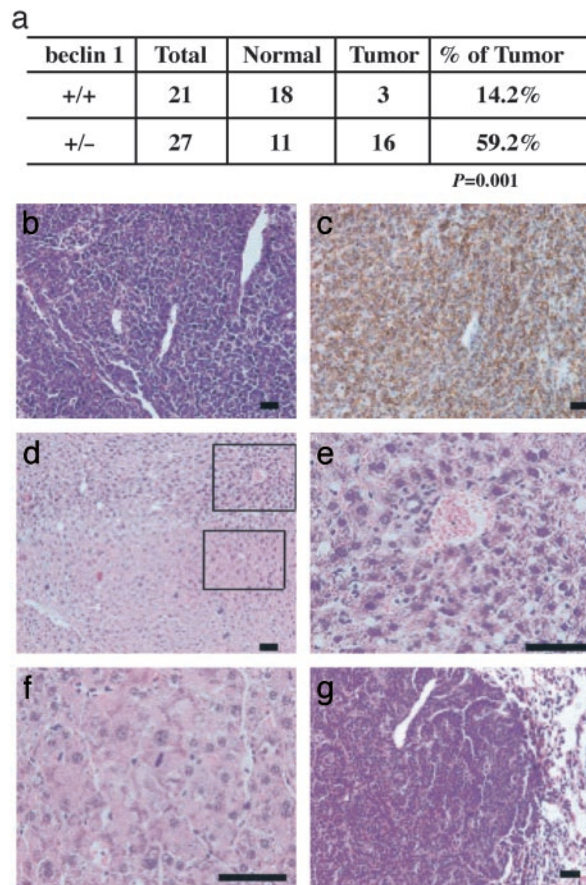


Fig. 3. Disruption of beclin 1 causes abnormal formation and cell growth in VE of EB. Analysis of EBs (day 14) derived from ES cells of wt (*a*) and beclin $1^{-/-}$ (*b*) is shown. (*a* and *b*, *Left*) H&E staining of paraffin sections. Note the expanded cystic form for wt and the cystic cavitated form for beclin $1^{-/-}$. (Scale bar, 100 μm.) (*a* and *b*, *Right*) Sections immunostained with anti-amnionless antibody (*Upper*) and toluidene blue-stained, 1-μm sections (*Lower*). (Scale bar, 10 μm.)

present in beclin $1^{+/-}$ mice demonstrated that they all encoded wt beclin 1 protein (data not shown). These studies exclude loss of heterozygosity (LOH) as an explanation for the increased occurrence of tumors in beclin $1^{+/-}$ mice. Therefore, increased tumor incidence in beclin $1^{+/-}$ mice results from decreased beclin 1 gene dosage, demonstrating that beclin 1 is a haploinsufficiency tumor suppressor gene.

Beclin $1^{-/-}$ Mutant ES Cells Are Defective in Autophagy but Undergo Apoptosis Normally. To understand the cellular basis for the lethal phenotype observed in beclin $1^{-/-}$ mutant mice and the increased tumor incidence found in beclin $1^{+/-}$ mice, null mutant ES cells were used to assay potential defects in apoptosis and autophagy. In response to UV light, ES cells undergo p53-dependent apoptotic cell death involving Bcl-2 family proteins and caspase activation (16–18). In contrast, on serum withdrawal a second form of programmed cell death is induced in ES cells that involves an apoptosis-inducing factor and can be uncoupled from caspase activation (13). No differences in cell death between wt ES cells and two independently derived beclin$^{-/-}$ ES cell lines were observed in response to either UV light or serum deprivation (Fig. 6*a*). We conclude that beclin 1 does not play an essential role in apoptosis in ES cells and that the phenotypes observed in beclin 1 mutant mice do not arise from defects in apoptotic cell death, consistent with the massive cell death evident in beclin$^{-/-}$ embryos (Fig. 2*j*).

To determine whether beclin$1^{-/-}$ ES cells undergo normal autophagy in response to starvation, they were cultured under conditions of nutrient deprivation for 2 h, fixed, embedded, and examined under an electron microscope (Fig. 6*b*). As previously demonstrated, activation of autophagy in wt ES cells in response to starvation resulted in the formation of numerous autophagic vacuoles (12) (Fig. 6*b Left*, arrows). In contrast, beclin $1^{-/-}$ ES



beclin 1	Total	Normal	Tumor	% of Tumor
+/+	21	18	3	14.2%
+/–	27	11	16	59.2%

P=0.001

Fig. 4. Increase of cancer rate in beclin $1^{+/-}$ mice. (*a*) Summary of cancer incidents and cancer rate in wt and beclin $1^{+/-}$ mice (age and gender matched). (*b* and *c*) B cell lymphoma. (*b*) H&E staining of tumor tissue. (*c*) Immunohistochemical staining of the same tumor in *b* with antibody against B220 (B cell marker). (*d*–*f*) Heptocellular carcinoma. (*d*) H&E staining of liver tissue containing heptocellular carcinoma. Note the upper portion showing normal tissue and the lower portion showing tumor. (*e* and *f*) High magnitude of normal and tumor tissue, respectively, in *d*. (*g*) Lung adenocarcinoma; H&E staining showing tumor (left portion) and normal (right portion) tissue of the lung. (Scale bar, 50 μm.)

cells displayed clear abnormalities in the induction of autophagy. Thus, fewer autophagic vacuoles were evident in beclin $1^{-/-}$ ES cells, and the vacuoles formed were smaller and appeared to be less developed (Fig. 6*b Right*, arrows). To quantify this result, the area covered by autophagic vacuoles in nutrient-deprived wt and beclin $1^{-/-}$ ES cells was determined. The fractional area of autophagic vacuoles in wt ES cells ($\approx$2.6%) was $\approx$4-fold greater than that in beclin $1^{-/-}$ ES cells ($\approx$0.6%), indicating that the autophagic response of ES cells to nutrient deprivation is strongly abrogated in the absence of beclin 1, consistent with previous studies demonstrating that beclin 1 can rescue defects in autophagy in yeast *apg6* mutants (6) and that it can induce autophagy in cultured mammalian cells (6, 9). We conclude that the major cellular defect in beclin $1^{-/-}$ mutants is a disruption in autophagy.

Discussion

The data presented here demonstrate that beclin 1 is required for autophagy, embryogenesis, and normal tissue homeostasis. Thus, the apoptotic response of beclin $1^{-/-}$ ES cells to UV light and serum withdrawal is normal, whereas their autophagic response to nutrient deprivation is severely disrupted. We show that the VE, which provides an exchange system responsible for

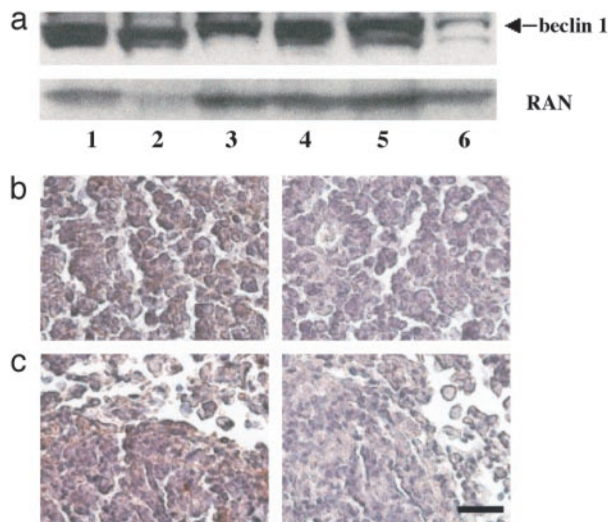
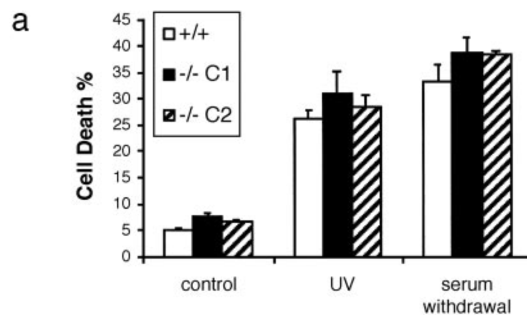


Fig. 5. Expression of beclin 1 protein in tumors from beclin $1^{+/-}$ mice. (*a*) Western blot analysis of cell extracts from tumors with anti-beclin 1 antibody or anti-Ran antibody (control). Lane 1, cell lysate from wt spleen; lanes 2–4, B cell lymphomas; lane 5, lung adenocarcinoma; lane 6, heptocellular carcinoma. (*Lower*) Ran protein level in each lane as loading control. (*b*) Section from B cell lymphoma stained with (*Left*) or without (*Right*) anti-beclin 1 antibody. (*c*) Section from lung adenocarcinoma stained with (*Left*) or without (*Right*) anti-beclin 1 antibody. (Scale bar, 20 μm.)



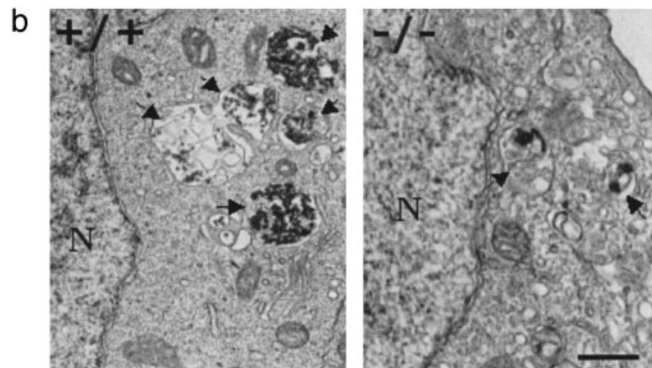


Fig. 6. Disruption of beclin 1 causes autophagy deficiency. (*a*) Apoptotic cell death of wt or beclin $1^{-/-}$ ES cells induced by UV irradiation or serum withdrawal. Mean values ± SEM are depicted ($n = 3$). C1 and C2 represent two independent beclin $1^{-/-}$ ES clones. (*b*) Ultrastructural examination of ES cells after nutrient deprivation from beclin 1 wt (*Left*) and null mutant (*Right*) by electronic microscopy. Arrows indicate autophagy vacuoles. N, nucleus. (Scale bar, 1 μm.)

nutrition and waste product detoxification in the developing embryo (19), is abnormal in beclin $1^{-/-}$ embryos and EBs. We further report that mice heterozygous for the beclin 1 mutation develop a high incidence of tumors that continue to express beclin 1 from the remaining wt allele, identifying beclin 1 as a haploinsufficient tumor suppressor gene.

The identification of beclin 1 as a dose-dependent tumor suppressor gene and the demonstration that beclin 1 mutant ES cells can undergo apoptosis normally but are severely abnormal for autophagy, taken together with previous data documenting monoallelic deletion of beclin 1 in human tumors (6), strongly suggest that autophagy plays an important role in human tumor suppression. Although a role for autophagy in tumor suppression has not been previously demonstrated, human tumor suppressor genes are known to interact with this pathway. For example, loss-of-function mutations of the PTEN gene (21), as well as increased expression of both phosphoinositol-3-kinase (PI3K) and Akt kinase (Akt) (22, 23), are frequent events in human tumors. It has recently been demonstrated that PTEN, an inhibitor of the PI3K/Akt pathway, can promote autophagy (24). Furthermore, the tuberous sclerosis tumor suppressor genes TSC1 and TSC2 (25) have been shown to directly inhibit the mammalian target of rapamycin (mTOR), a protein involved in sensing intracellular amino acid pools that plays a critical role in both cell size regulation and autophagy (26). Furthermore, beclin 1 has recently been identified as an E2F target gene (27). E2F is an important component of the Rb pathway, which is disrupted in most human cancers (28). Finally, analysis of diethylnitrosamine-induced rat liver carcinomas has previously demonstrated a decreased autophagic activity during tumor growth (20). These observations, taken together with the data presented in this study, provide strong support for the idea that autophagy plays an important role in the regulation of tumor development *in vivo* (6, 29) and that it is tightly regulated in coordination with cell growth, proliferation, and death. The detailed mechanisms by which beclin 1 and autophagy contribute to tumor suppression and the potential of this pathway as a new target for cancer chemotherapy are important issues that deserve a great deal of attention.

We thank Dr. E. Lacy for anti-amnionless antiserum; Dr. K. Monova for advice on embryo study; Drs. K. Onel and C. Cordon-Cardo for histopathological analysis of tumors; H. Shio for EM study; and J. Xing, W. Yang, J. Gao, and A. Teresky for technical support. This work was supported by the Howard Hughes Medical Institute (N.H. and Z.Y.).

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