Induction of autophagy and inhibition of tumorigenesis by *beclin 1*

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The process of autophagy, or bulk degradation of cellular proteins through an autophagosomic-lysosomal pathway[1], is important in normal growth control and may be defective in tumour cells[2]. However, little is known about the genetic mediators of autophagy in mammalian cells or their role in tumour development. The mammalian gene encoding Beclin 1 (ref. 3), a novel Bcl-2-interacting, coiled-coil protein, has structural similarity to the yeast autophagy gene, *apg6/vps30* (refs 4, 5), and is mono-allelically deleted in 40–75% of sporadic human breast cancers and ovarian cancers[6]. Here we show, using gene-transfer techniques, that *beclin 1* promotes autophagy in autophagy-defective yeast with a targeted disruption of *agp6/vps30*, and in human MCF7 breast carcinoma cells. The autophagy-promoting activity of *beclin 1* in MCF7 cells is associated with inhibition of MCF7 cellular proliferation, *in vitro* clonigenicity and tumorigenesis in nude mice. Furthermore, endogenous Beclin 1 protein expression is frequently low in human breast epithelial carcinoma cell lines and tissue, but is expressed ubiquitously at high levels in normal breast epithelia. Thus, *beclin 1* is a mammalian autophagy gene that can inhibit tumorigenesis and is expressed at decreased levels in human breast carcinoma. These findings suggest that decreased expression of autophagy proteins may contribute to the development or progression of breast and other human malignancies.

Fourteen genes have been identified in *Sarcharomyces cerevisiae* that are required for yeast autophagy[7]. Yeast that are lacking one of these genes, *apg6/vps30*, are defective in both their ability to undergo nitrogen deprivation-induced autophagy[4] and their ability to properly sort selective vacuolar proteins[5]. Human Beclin 1 shares 24.4% amino-acid identity (and 39.1% conservation) with Apg6/Vps30p. To determine whether Beclin 1 is a functional homologue of Apg6/Vps30, we investigated whether *beclin 1* expression could restore autophagic activity and vacuolar protein sorting in *apg6/vps30*-disrupted yeast. Four hours after transfer to nitrogen-starvation media, we compared the percentage of cells with autophagic bodies within the yeast vacuole in isogenic wild-type yeast (strain SEY620), *apg6/vps30*-disrupted yeast (strain JCY3000), JCY3000 yeast transformed with *apg6/vps30*, JCY3000 yeast transformed with *beclin 1* and JCY3000 yeast transformed with empty vector (Fig. 1). In nitrogen-deprivation conditions, wild-type yeast accumulate autophagic bodies within their central vacuole (the mammalian equivalent of lysosomes) and detection of autophagic bodies is facilitated by phenylmethylsulphonyl fluoride (PMSF), a protease inhibitor that blocks their degradation[8]. In the presence of PMSF, we observed numerous cells with autophagic bodies within the vacuole in wild-type SEY6210 yeast, and in *apg6/vps30* and *beclin-1*-transformed JCY3000 yeast (Fig. 1b, c). In contrast, significantly fewer cells were seen with autophagic bodies within the vacuole among nontransformed JCY3000 yeast or JCY3000 yeast transformed with empty vector ($P < 0.0001$, analysis of variance). These

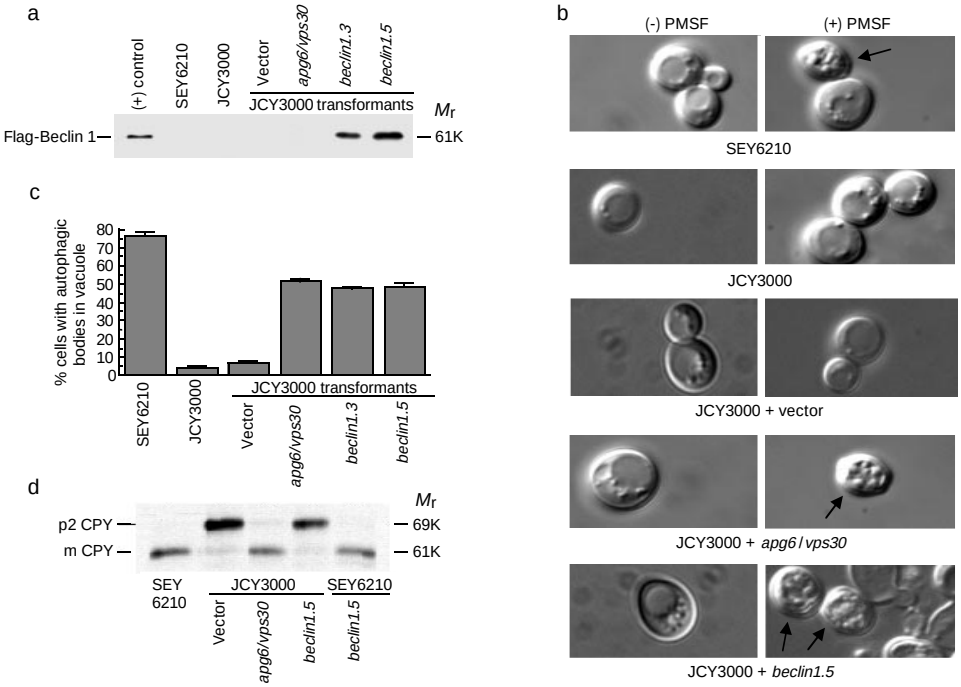

Figure 1 Complementation by *beclin 1* of the yeast autophagy but not vacuolar protein sorting function of *apg6/vps30*. **a**, Western blot analysis of yeast cell lysates with polyclonal anti-flag antibody to detect flag-tagged human Beclin 1 expression. Two-hundred micrograms of protein per lane was loaded. SEY6210, wild-type yeast; JCY3000, SEY6210 disrupted in *apg6/vps30*; (+) control, lysates of mammalian cells transfected with flag-*beclin 1*. **b**, Autophagicbody formation in yeast deprived of nitrogen in the presence (+) and absence (–) of 1 mM PMSF. Arrows denote cells that would be scored as positive in the experiment shown in **c**. **c**, Quantitative effects of *apg6/vps30* and *beclin 1* transformation on autophagicbody formation in *apg6/vps30*-disrupted yeast in the presence of PMSF. Cells with one or more autophagic bodies within the vacuole were scored as positive (see arrows in **b**). A minimum of 100 cells was counted for each sample. Results represent the mean (±s.e.m.) percentage of cells with autophagic bodies within the vacuole for triplicate samples. Similar results were obtained in five independent experiments. **d**, Sorting of vacuolar protein carboxypeptidase Y, (CPY). p2CPY, precursor form; mCPY, sorted mature form. M_r, relative molecular mass.

results indicate that *beclin 1* restores autophagy induced by nitrogen deprivation in autophagy-defective *apg6/vps30*-disrupted yeast. However, unlike JCY3000 yeast transformed with *apg6/vps30*, JCY3000 yeast transformed with *beclin 1* were unable to properly sort and mature the vacuolar protein, carboxypeptidase Y (CPY) (Fig. 1d). Thus, *beclin 1* complements the autophagy but not the vacuolar protein sorting function of *apg6/vps30* in *apg6/vps30*-disrupted yeast.

The *beclin 1* gene maps to a tumour susceptibility locus on human chromosome 17q21 (refs 9, 10) that is mono-allelically deleted in up to 40–75% of cases of sporadic ovarian and breast carcinomas[6]. This observation, coupled with the homology between *beclin 1* and *apg6/vps30*, led us to propose that *beclin 1* is a mammalian autophagy gene that negatively regulates tumorigenesis. Therefore, we investigated the effects of *beclin 1* gene transfer on the autophagic activity (Fig. 2) and growth properties (Fig. 3) of MCF7 human breast carcinoma cells. MCF7 cells were originally derived from a patient with 17q21 loss of heterozygosity[11] and do not express detectable levels of endogenous Beclin 1 (Fig. 4a). To permit conditional expression of a potential antiproliferative gene, we stably transfected MCF7 cells with a tetracycline-repressible vector, pTC (ref. 12), containing flag-epitope-tagged full-length human Beclin 1 (referred to as MCF7.*beclin1* clones) (Fig. 3a), flag epitope-tagged *beclin 1* containing a premature stop codon at nucleotide position 270 (MCF7.*beclin1*stop clones) (Fig. 3b) or no insert (MCF7.control clones).

Three MCF7.control clones, three MCF7.*beclin1* clones and one MCF7.*beclin1*stop clone, which were either maintained in normal growth conditions or subjected to serum and amino-acid deprivation for 4 hours were analysed by electron microscopy (Fig. 2). In normal cultured cells, serum and amino-acid deprivation is a potent inducer of autophagy[13], which can be recognized at the ultrastructural level as double-membrane vacuolar structures containing visible cytoplasmic contents[14–16]. We found that baseline numbers of autophagic vacuoles per cell were significantly higher in MCF7.*beclin1* compared with MCF7.control or MCF7.*beclin1*stop clones ($P < 0.0001$, analysis of variance; see Fig. 2a, c, g). Furthermore, serum and amino-acid deprivation did not increase the mean number of autophagic vacuoles per cell in MCF7.control or MCF7.*beclin1*stop clones (Fig. 2b, g), but did induce a significant increase in MCF7.*beclin1* clones ($P < 0.0001$, *t*-test; Fig. 2d, g). Although autophagic vacuoles were found in the cytoplasm, the nuclei of MCF7.*beclin1* clones appeared normal, without evidence of apoptotic morphologic changes (Fig. 2c, d; data not shown). The serum and amino-acid deprivation-induced increase in autophagic vacuole formation in MCF7.*beclin1* clones was inhibited by pre-treatment with 3-methyladenine (3-MA), a nucleotide derivative that inhibits the earliest stages of autophagosome formation[17] (Fig. 2g). These data indicate that MCF7 human breast carcinoma cells lack Beclin 1 expression and are unable to undergo nutrient deprivation-induced autophagy. In contrast, enforced *beclin 1* expression promotes nutrient deprivation-induced autophagy in MCF7 cells.

To confirm these morphologic observations using a biochemical approach, we measured the rates of intracellular degradation of long-lived proteins following nutrient deprivation of MCF7.*beclin1*, MCF7.control and MCF7.*beclin1*stop clones (Fig. 2h). In response to nutrient deprivation or high density culture conditions,

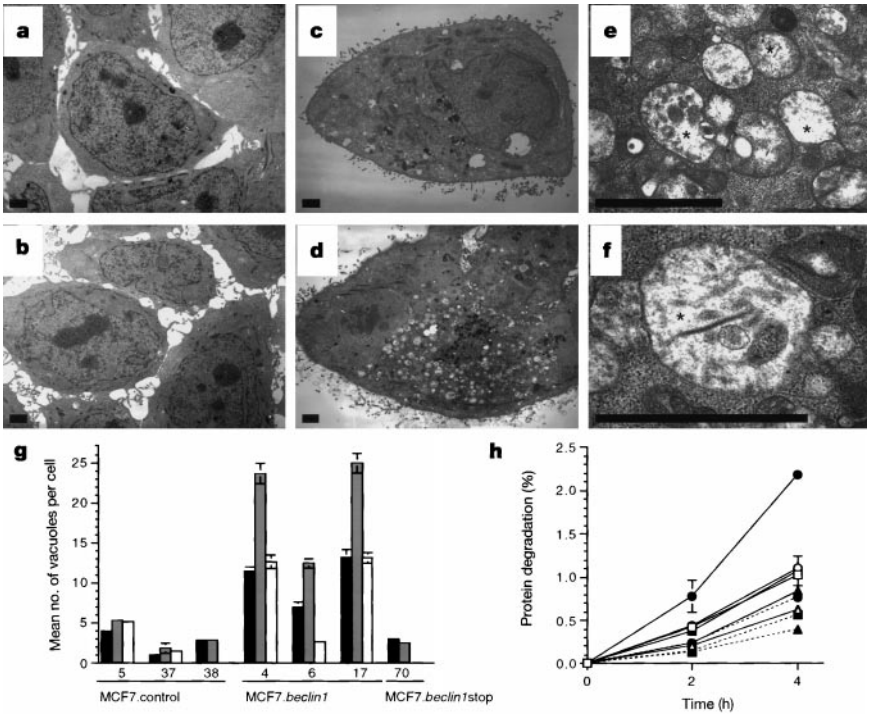

Figure 2 Promotion of autophagy by enforced *beclin 1* expression in human MCF7 breast carcinoma cells. **a**–**f**, Electron micrographs of MCF7.control (clone 38) (**a**, **b**) and MCF7.*beclin1* (clone 17) cells (**c**–**f**) grown in nutrient-rich media (**a**, **c**) or subjected to 4 h of serum and amino-acid deprivation (**b**, **d**–**f**). Asterisks in **e** and **f** denote autophagic vacuoles that would be counted in the experiment shown in **g**. Autophagic vacuoles were defined as double-membrane vacuolar structures containing recognizable cytoplasmic contents. Scale bars, 1 μm. **g**, Quantitative effects of *beclin 1* on basal and nutrient-deprivation-induced autophagy of MCF7 cells. Bars indicate mean (±s.e.m.) number of autophagic vacuoles per cell for cells growing in normal media (black), for cells subjected to 4 h of serum and amino-acid deprivation (grey), and for cells pre-treated for 30 min with 10 mM 3-MA and subjected to 4 h of serum and amino-acid deprivation in the presence of 3-MA (open). Mean was determined by counting the total number of autophagic vacuoles in each cell for 100 cells per clone per treatment. **h**, Comparison of the rates of degradation of long-lived proteins in MCF7.control cells (squares, clone 38), MCF7.*beclin1* cells (circles, clone 17) and MCF7.*beclin1*stop cells (triangles, clone 70), in EBSS + 10% serum and complete amino acids (open symbols, solid lines) in EBSS alone (closed symbols, solid lines), and in EBSS + 10 mM 3-MA (closed symbols, dotted lines). Results are mean (±s.e.m.) of triplicate wells. Similar results were obtained in three independent experiments. Results shown with these clones are representative of results obtained with other clones analysed by electron microscopy in **g**.

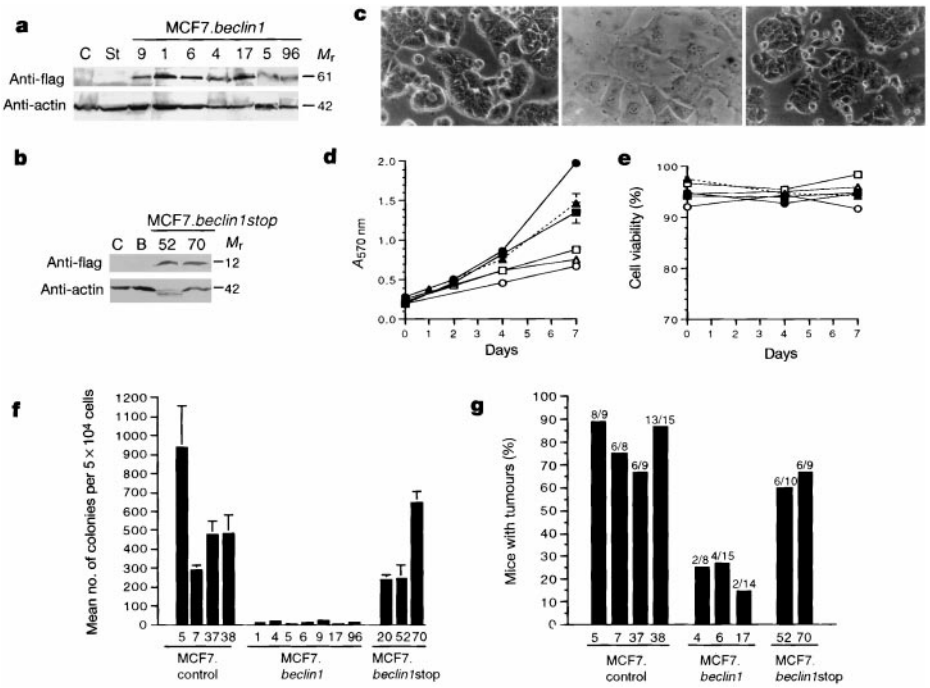

Figure 3 Effects of enforced *beclin 1* expression on the growth properties and tumorigenicity of MCF7 cells. **a**, Western blot analysis of flag-tagged *beclin-1*-transfected MCF7 clones. C, MCF7.control cells (clone 38); St, MCF7.*beclin1*stop cells (clone 70). M_r, relative molecular mass. **b**, Western blot analysis of flag-tagged *beclin1*stop-transfected MCF7 clones. C, MCF.control cells (clone 38); B, MCF7.*beclin1* cells (clone 1). **c**, Photomicrographs of MCF7.control cells (left panel, clone 38), MCF7.*beclin1* cells (middle panel, clone 17) and MCF7.*beclin1*stop cells (right panel, clone 70) 48 h after seeding at similar densities. **d**, Proliferation of MCF7.control cells (clones 37 and 38; closed squares and circles, respectively, solid lines), MCF7.*beclin1* cells (clones 1, 6, 17; open squares, circles, triangles, respectively, solid lines) and MCF7.*beclin1*stop cells (clone 70, closed triangles, dotted line). Results represent mean Absorbance (*A*) (± s.e.m.) for six wells; similar results were obtained in three independent experiments. **e**, Cell viability of MCF7 clones determined by trypan blue staining. Symbols for each clone are as in **d**. **f**, Clonigenicity in semisolid medium (soft agar) of MCF7 clones. Results are mean (±s.e.m.) for pooled triplicate wells from 3–4 independent experiments. **g**, Tumour formation in NCR nude mice injected subcutaneously with MCF7 clones. Numbers on top of bar indicate number of autopsy-confirmed tumours at 8 weeks per number of mice injected with each clone.

increased autophagy results in enhanced proteolysis of long-lived proteins, and this response is often impaired in transformed cells[2,18–23]. We found that MCF7.*beclin1* clones, but not MCF7.control or MCF7.*beclin1* stop clones, had a significant increase in rate of intracellular proteolysis following nutrient deprivation ($P < 0.0001$, *t*-test), and that this increase was completely blocked by the autophagy inhibitor 3-MA. Thus, during nutrient deprivation the effects of *beclin 1* on autophagic vacuole formation in MCF7 cells are associated with enhanced degradation of long-lived cellular proteins.

To evaluate the effect of *beclin 1* on MCF7 growth properties, we compared the morphology, cellular proliferation rates, *in vitro* clonigenicity and *in vivo* tumorigenicity of MCF7.*beclin1*, MCF7.control and MCF7.*beclin1*stop clones. MCF7.*beclin1* clones displayed several morphologic characteristics consistent with a less malignant phenotype, including flatter appearance, larger size, firmer attachment to tissue culture plate and increased contact inhibition (Figs 2a, c, 3c). Consistent with the autophagy-promoting effects of *beclin 1*, MCF7.*beclin1* clones proliferated more slowly than MCF7.control or MCF7.*beclin1*stop clones (Fig. 3d). This lower rate of proliferation was not explained by increased cell death, as cell viability determined by trypan blue staining was similar in MCF7.*beclin1*, MCF7.*beclin1*stop and MCF7.control cells (Fig. 3e). All MCF.*beclin1* clones were also severely impaired in their clonigenicity *in vitro*, when compared with MCF.control and MCF7.*beclin1*stop clones which formed colonies in soft agar with high efficiency ($P < 0.0001$, chi-squared test) (Fig. 3f). Moreover, the incidence of tumour formation of three MCF7.*beclin1* clones that were injected into nude mice (14–27%) was significantly lower than that of four MCF7.control clones (60–89%) and two MCF7.*beclin1*stop clones (60–67%) ($P < 0.0001$; chi-squared test) (Fig. 3g). Together, these data show that *beclin 1* can function as a negative regulator of mammary cell growth and tumorigenesis.

The frequent allelic deletions of *beclin 1* in human breast carcinoma, coupled with its autophagy-promoting and growth inhibitory effects, suggest that functional inactivation of Beclin 1 may be important in breast cancer development or progression. A previous study did not identify any coding region mutations in *beclin 1* genomic DNA or abnormalities in *beclin 1* messenger RNA expression in human breast carcinoma cell lines[6]. To evaluate whether levels of Beclin 1 protein expression are decreased in human breast carcinoma, we analysed by western blot human breast carcinoma-derived cell lines and matched normal breast and breast carcinoma tissue from patients with invasive sporadic breast carcinoma. Out of 11 human breast carcinoma cell lines examined, only three had detectable Beclin 1 protein expression (Fig. 4a). Out of 17 pairs of equivalent volumes of matched normal breast and breast carcinoma tissue, 15 samples had higher levels of Beclin 1 in normal than in tumour breast tissue (Fig. 4b). In contrast, 16 tumour samples had higher expression of control proteins (for example, actin, cytokeratin) than in the matched normal tissue samples. Therefore, despite higher overall levels of protein in breast carcinomas (which consist primarily of malignant epithelial cells) than in normal breast tissue (which consists primarily of fat cells), specific expression of Beclin 1 protein is usually decreased in breast carcinomas.

To determine whether the decreased levels of Beclin 1 protein expression in breast carcinoma samples reflected a loss of expression specifically in carcinoma cells rather than in stroma or non-neoplastic epithelium, we immunohistochemically stained tissue sections. Adjacent paraffin-embedded sections from matched normal breast and breast carcinoma samples, including 10 of the

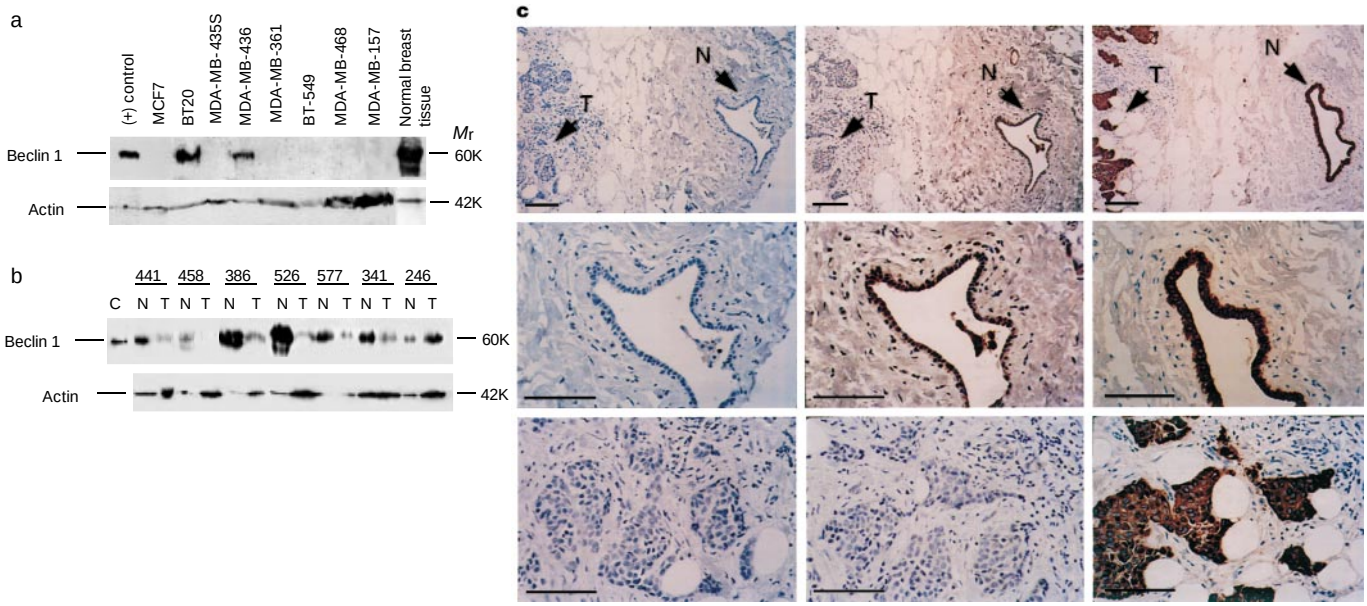

Figure 4 Decrease in Beclin 1 protein expression in human breast carcinoma cell lines and tissue. **a**, Western blot analysis of human breast carcinoma cell lines. Results are from 8 representative cell lines out of a total of 11 analysed. **b**, Western blot analysis of matched normal breast and breast carcinoma tissue from patients with sporadic invasive breast carcinoma. Results are from 7 representative cases out of a total of 17 analysed. N, normal breast tissue; T, tumour tissue; M_r, relative molecular mass. **c**, Immunohistochemical analysis of paraffin-embedded sections of breast tissue from patient 441 shown in **b**. Adjacent sections are stained with pre-immune 843 rabbit serum (left column), 843 anti-Beclin 1 serum (middle column) and anti-cytokeratin (right column). Top row shows low power view of region of breast containing tumour (T) and normal mammary epithelial duct (N). Middle row shows higher power view of normal mammary epithelial duct. Bottom row shows higher power view of tumour. Scale bars, 1 mm.

samples analysed by western blot and 22 additional samples, were stained with anti-Beclin 1 and anti-cytokeratin. We found that in all 32 cases, there was strong Beclin 1 immunoreactivity in all normal breast epithelial cells and strong cytokeratin immunoreactivity in all neoplastic as well as normal breast epithelial cells (see Fig. 4c). (Beclin 1 immunoreactivity was also seen in the media of vessels and in some inflammatory cells.) However, in 18 out of 32 cases, there was a significant decrease in the amount of Beclin 1 protein expression in breast carcinoma cells compared with normal breast lobular or ductal epithelial cells (Fig. 4c; Table 1). Thus, *beclin 1* is expressed ubiquitously in normal breast epithelial cells, but its expression is commonly decreased in breast carcinoma cells.

In summary, our data indicate that, to our knowledge, *beclin 1* is the first identified mammalian gene with a role in mediating autophagy. Earlier studies showed that signalling through the S6 kinase pathway exerts inhibitory effects on mammalian autophagy[24], but no genes were identified that induced autophagy in mammalian cells. Our findings, that enforced expression of an autophagy gene not only promotes nutrient deprivation-induced autophagy in human breast carcinoma cells but also inhibits their tumour-forming potential, indicate that autophagy may be a fundamental mechanism for preventing the deregulated growth of tumour cells. Further studies are necessary to determine whether induction of autophagy by *beclin 1* is, in fact, the mechanism by which it inhibits tumorigenesis. Nonetheless, the frequent monoallelic deletions and decreased expression of *beclin 1* in human breast carcinoma suggest that specific molecular alterations in autophagy pathways may contribute to tumorigenesis. □

Table 1 Level of Beclin 1 immunoreactivity in different regions of breast tissue samples from patients with invasive breast carcinoma

Beclin 1 immunoreactivity	No. of cases (%) Blood vessels	Luminal ducts	Luminal lobules	Tumour*
0 (absent)	0 (0.0)	0 (0.0)	0 (0.0)	9 (28.1)
1+ (mild)	1 (3.1)	11 (34.4)	9 (28.1)	16 (50.0)
2+ (moderate)	12 (37.5)	16 (50.0)	18 (56.3)	5 (15.6)
3+ (strong)	19 (59.4)	5 (15.6)	5 (15.6)	2 (6.3)

*$P < 0.0001$ for the comparison of tumour with luminal ducts/luminal lobules; chi-squared test.

Methods

Yeast strains, media and genetic methods

The *S. cerevisiae* strains used for cloning, immunochemical analysis and autophagy assays were SEY6210 (*MATα leu2-3, 112 ura3-52 his Δ200 trp1-Δ901 lys2-801 suc2-Δ9*) and JCY3000 (SEY6210 *Δvps30::HIS3*)[5]. JCY3000 yeast were transformed with pRS424.*vps30* (ref. 5) as-described[25], and flag-*beclin 1* was substituted for the open reading frame of *apg6/vps30* in pRS424.*vps30* using recombination-mediated PCR-directed plasmid construction *in vivo*[26] to generate the two clones JCY3000+*beclin1*.3 and JCY3000+*beclin1*.5. Yeast were grown in YEPD, in synthetic complete medium lacking tryptophan for transformation selection (SC − trp), or in SD(−N) for starvation experiments, consisting of 2% glucose with 1.7 g l^{-1} of yeast nitrogen base without amino acids and ammonium sulfate.

Mammalian cell lines and transfections

Human breast carcinoma cell lines were obtained from ATCC (American Type Culture Collection) and maintained according to ATCC instructions. MCF7 cells were transfected using liposome-mediated transfer with the plasmid, pTC (ref. 12), containing flag epitope-tagged human *beclin 1* cloned into the *Nhe*I site or no insert. Stable transfectants were selected for with 300 μg ml^{-1} hygromycin and maintained in 2 μg ml^{-1} tetracycline; tetracycline was withdrawn 5 days before all experiments.

Immunochemical procedures

Yeast and mammalian cell lysates were separated by SDS-PAGE and immunoblotted with anti-flag monoclonal (M2, VWR; 20 μg ml^{-1}), polyclonal anti-flag antibody (Zymed, 1:200 dilution), polyclonal anti-Beclin 1 antibody 843 (ref. 3) (1:200 dilution) or anti-actin antibody (Boehringer Mannheim, 1:400 dilution). For immunoperoxidase staining to detect endogenous Beclin 1 expression, we used 843 (1:200) and the ABC method (Vector Laboratories). Control staining was performed with pre-immune 843 serum (1:200) and anti-cytokeratin (1:100, Boehringer Mannheim).

Autophagy analysis

Yeast strains were grown overnight in YEPD or liquid SC – trp, transferred to SD(–N) at a concentration of 2×10^7 cells ml^{-1}, incubated for 4 h in the presence or absence of 1 mM PMSF, visualized using DIC optics with a Plan-Apochromat 100× 1.4 NA objective, and imaged with a cooled CCD camera using IP Lab software. MCF7 cells were grown in normal media or serum and amino-acid free media, fixed with 2.5% glutaraldehyde, postfixed with 1% OsO_4 and embedded in Lx-112 (Ladd Research Industries, Inc.) and Embed-812 (E.M.S.). Thin sections were cut on MT-700 RMC, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy using a JEOL JEM-1200 EXII.

Vacuolar protein sorting

Sorting of the vacuolar protein, CPY, was measured as described[5], with the exception that yeast cells were not separated into intracellular and extracellular fractions.

Measurement of degradation of long-lived proteins

Long-lived proteins were labelled by incubating cells for 72 h in media containing reduced concentrations of unlabelled leucine (0.065 mM) and [^{3}H] leucine (1 μCi ml^{-1}). Cells were washed, incubated for 24 h in medium containing 2 mM unlabelled leucine to allow degradation of short-lived proteins and removal of unincorporated labelled leucine, and then subjected to 4-h incubation in normal media or deprivation conditions. Excess unlabelled leucine (2 mM) was added to prevent reincorporation of labelled degraded proteins. At 0, 2 and 4 h, aliquots of the media were measured for acid-soluble radioactivity. At the end of the incubation, the acid-precipitable radioactivity of the cell monolayers was measured and the percentage of long-lived protein degradation was calculated using a described formula[21].

Proliferation, cell viability, clonigenicity and tumorigenicity assays

MCF7 clones were seeded in 96-well plates at a density of 5×10^3 cells per well, and cell proliferation was measured at serial time points using the MTT Cell Proliferation Kit (Boehringer Mannheim). At identical time points, triplicate wells were subjected to trypan blue staining to determine cell viability. MCF7 clones were plated in triplicate at a density of 5×10^4 cells per 35-mm well in semisolid medium (soft agar) as described[27] and colonies were counted at 21 days. Five week-old NCR nude mice (Taconic Farms) were implanted with slow release oestrogen pellets (1.7 mg per 60 days) and injected subcutaneously with 5×10^6 tumor cells. Animals were necropsied 8 weeks after injection for histologic confirmation of tumour.

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Binding of paxillin to α_4 integrins modifies integrin-dependent biological responses

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The α_4 integrins are indispensable for embryogenesis, haematopoiesis and immune responses[1,2], possibly because α_4 regulates cellular functions differently from other integrins through its cytoplasmic tail[3]. We used novel mimics[4] of the α_4 tail to identify molecules that could account for α_4-specific signalling. Here we report that the α_4 tail, but not several other α-subunit tails, binds tightly to the signalling adaptor paxillin. Paxillin physically associated with α_4 integrins in Jurkat T cells at high stoichiometry, and joining the α_4 tail to α_{IIb} resulted in a complex of integrin $\alpha_{IIb}\beta_3$ with paxillin. This association markedly enhanced the rates of $\alpha_{IIb}\beta_3$-dependent phosphorylation of focal adhesion kinase and cell migration. It also reduced cell spreading, focal adhesion and stress fibre formation. A point mutation within the α_4 tail that disrupts paxillin binding reversed all of these effects. Furthermore, $\alpha_4\beta_1$-dependent adhesion to VCAM-1 led to spreading of mouse embryonic fibroblasts derived from paxillin-null but not from wild-type mice. Thus, the tight association of paxillin with the α_4 tail leads to distinct biochemical and biological responses to integrin-mediated cell adhesion.

To investigate the unusual signalling properties of α_4 integrins, we analysed the binding of cellular proteins to model protein mimics of dimerized α_4 integrin cytoplasmic domains. We made the model proteins by fusing the cytoplasmic tail of the α_4 or β_{1A} subunits to amino-terminal modules that contained four heptad-repeat sequences. The heptad repeats direct assembly of coiled-coil dimers in which the tails are dimerized parallel to each other and held in a fixed vertical stagger[4], thus resembling the predicted topography of native integrin cytoplasmic domains[5]. Jurkat T-cell lysates were incubated with α_4-tail mimics and bound proteins were detected by immunoblotting for integrin-associated polypeptides. Paxillin was enriched more than 57-fold in the bound fraction (Fig. 1a) relative to the cell lysate. In contrast, the β_{1A} tail bound paxillin with no enrichment relative to the cell lysate. There was no

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