

Beclin 1 Contains a Leucine-rich Nuclear Export Signal That Is Required for Its Autophagy and Tumor Suppressor Function¹

Xiao Huan Liang, Jie Yu, Kristy Brown, and Beth Levine²

Departments of Medicine [X. L., J. Y., B. L.] and Pathology [K. B.], Columbia University College of Physicians & Surgeons, New York, New York 10032

ABSTRACT

Beclin 1 encodes a Bcl-2-interacting coiled-coil protein with autophagy and tumor suppressor function and is monoallelically deleted in 40–75% of sporadic human breast and ovarian cancers. Beclin 1 contains a leucine-rich nuclear export signal motif raising the possibility that its autophagy and/or tumor suppressor function may require regulated, CRM1-dependent, nucleocytoplasmic transport. In this study, we show that wild-type Beclin 1 colocalizes with both intracytoplasmic organelles and nuclei in COS7 monkey kidney and MCF7 human breast carcinoma cells. Inhibition of CRM1-dependent nuclear export with leptomycin B or mutation of the nuclear export signal motif of Beclin 1 results in predominantly nuclear localization. Unlike wild-type Beclin 1, the nuclear export mutant of Beclin 1 fails to promote nutrient deprivation-induced autophagy and fails to inhibit *in vitro* clonogenicity and *in vivo* tumorigenicity of MCF7 cells. Thus, *beclin 1* has a leptomycin B-sensitive leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. These findings suggest that the CRM1 nuclear export pathway may be important in the functional regulation of autophagic growth control.

INTRODUCTION

Leucine-rich short amino acid sequences responsible for efficient nuclear export have been identified in a number of different cellular and viral proteins (reviewed in Refs. 1–4). The first identified example of a leucine-rich NES³ was a minimal 11-amino acid peptide (LQLPLERLTL) in the HIV-1 Rev protein in which mutation of critical leucines abolished export activity (5, 6). Functional NESs that resemble the Rev NES “consensus” sequence (Lx_(2–3)Lx_(2–3)LxL) have since been detected in other viral and cellular proteins required for RNA export [*e.g.*, human T-cell lymphotropic virus 1 Rex (7, 8), herpes simplex virus ICP27 (9), ribosomal protein L5 (10)], cellular proteins involved in signal transduction [*e.g.*, PKI (11), c-ABL (12), IκBα (13), MAPKK (14)], transcription factors [*e.g.*, TFIIIA (15)], tumor suppressors [*e.g.*, p53 (16)], and oncogenes [*e.g.*, hdm2 (17)]. The Rev-type NES forms a complex with the nuclear export receptor, CRM1, and Ran GTP (18–23). Leptomycin B, a fungicide, inhibits the formation of this complex by targeting CRM1 and blocks nuclear export of NES-containing proteins (21, 24).

The use of NESs and the CRM1 export pathway has been most extensively studied in the context of viral and cellular RNA export (reviewed in Ref. 25). However, the increasing number of different types of identified proteins with Rev-type NESs suggests that the CRM1 export pathway is important in the regulation of other eukaryotic cellular functions, including kinase regulation, signal transduc-

tion, and growth control. For example, PKI, an inhibitor of the protein kinase protein kinase A, binds to the active catalytic [C] subunits of cyclic AMP in the nucleus, leading to the exposure of its nuclear export signal, rapid export of the C subunit-PKI complex, and termination of signaling (1). Similarly, the NES of IκBα is also postulated to play a role in a negative feedback loop, in which it terminates nuclear factor-κB signaling once in the nucleus (1). The NES of the oncogene *hdm2* is required for its ability to block p53-mediated transcriptional activation and to accelerate p53 export and ubiquitin-mediated degradation (17). p53 export may also be regulated by its own intrinsic NES, and a model has been proposed in which NES masking by tetramer formation regulates the subcellular localization and activity of the p53 tumor suppressor (16).

A novel potential use for the Rev-related nuclear export pathway is suggested by a structural analysis of human Beclin 1. Beclin 1 contains a short leucine-rich amino acid sequence that resembles the Rev-type NESs and it is the first identified mammalian protein to mediate autophagy (26), a process of bulk protein degradation that is required for differentiation, survival during nutrient deprivation, and normal growth control (reviewed in Refs. 27–29). Fourteen gene products have been identified in yeast that are required for autophagy (30), including a serine/threonine protein kinase (31) and two proteins involved in a ubiquitin-like protein conjugation system (32). However, far less is known about the molecular events controlling autophagy in mammalian cells. The presence of a Rev-type NES in a mammalian autophagy protein raises the interesting possibility that the Rev-related nuclear export pathway may be used for autophagy regulation.

Beclin 1 was originally isolated in a yeast two-hybrid screen for Bcl-2-interacting proteins and it encodes a *M_r* 60,000 predicted coiled-coil protein (33). Human Beclin 1 shares 24.4% amino acid identity (and 39.1% conservation) with a yeast gene product, *Apg6/Vps30p*, that is required for both nitrogen deprivation-induced autophagy (34) and for proper vacuolar protein sorting (35). In gene transfer studies, *beclin 1* promotes autophagy, but not vacuolar protein sorting, in yeast with a targeted disruption of *apg6/vps30* (26). Furthermore, *beclin 1* gene transfer increases basal levels of autophagy and nutrient deprivation-induced autophagy in human breast carcinoma cells that lack detectable levels of endogenous Beclin 1 protein. The mechanisms by which Beclin 1 promotes autophagy in yeast or mammalian cells are not yet understood.

The autophagy function of Beclin 1 may play a role in negative regulation of tumorigenesis. Beclin 1 maps to a tumor susceptibility locus on chromosome 17q21 and is frequently monoallelically deleted in human breast, ovarian, and prostate cancers (reviewed in Ref. 36), indicating that it may be a tumor suppressor. Although biallelic mutations of *beclin 1* have not as of yet been demonstrated in human cancer, two lines of evidence suggest that functional inactivation of *beclin 1* may contribute to tumorigenesis. First, Beclin 1 protein is expressed ubiquitously in all normal breast epithelial cells, but frequently has low or undetectable expression in malignant breast epithelial cells (26). Second, *beclin 1* gene transfer in human breast carcinoma cells results in the loss of malignant morphological properties, decreased rates of cell proliferation, impaired clonogenicity *in vitro*, and impaired ability to form tumors in nude mice (26). Thus,

Received 8/31/00; accepted 2/19/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by American Cancer Society Grant RPG-98-339-CCG, NIH Grant RO1 CA84254, and a Mallinckrodt Scholar Award (to B. L.).

² To whom requests for reprints should be addressed, at Department of Medicine, Columbia University College of Physicians & Surgeons, P & S 8-463, 630 West 168th Street, New York, NY 10032. Phone: (212) 305-7312; Fax: (212) 305-7290; E-mail: levine@cuccfa.ccc.columbia.edu.

³ The abbreviations used are: NES, nuclear export signal; mt NES, mutant nuclear export signal; 3-MA, 3-methyladenine; EBSS, Earle's balanced salt solution; ER, endoplasmic reticulum; Ab, antibody; PKI, protein kinase inhibitor; IκB, inhibitor κB.

Beclin 1, an autophagy protein, can also function as a negative regulator of mammary cell growth and tumorigenesis.

Nothing is known about structural domains of Beclin 1 that are important for autophagy and tumor suppressor function. Aside from limited homology within the central coiled-coil domain with myosin-like proteins, Beclin 1 shares no significant homology with any mammalian proteins of known function (33). However, amino acids 180–189 of human Beclin 1 contain a core of closely spaced leucine residues that conform to the consensus motif of the Rev family of NESs. Therefore, we evaluated whether Beclin 1 contains an NES and if so, whether the NES is required for its autophagy and tumor suppressor function.

MATERIALS AND METHODS

Plasmid Constructions. Using the previously described plasmid pSG5/flag-*beclin1* (33) as a template, two-step PCR mutagenesis was used to introduce two leucine → alanine substitutions at amino acid positions 184 and 187 of Beclin 1 to generate mtNES Beclin1. The cDNA encoding mtNES *beclin1* was cloned into PCR vector (Invitrogen, San Diego, CA), sequenced using the dideoxychain termination method, and subsequently cloned into pSG5 (Stratagene, La Jolla, CA) or pTRE (Clontech Laboratories, Palo Alto, CA).

Cell Lines. COS7 monkey kidney cells and MCF7 human breast carcinoma cells were obtained from American Type Culture Collection (Manassas, VA) and MCF7/tet-off cells were obtained from Clontech Laboratories; all cells were grown according to the suppliers' instructions.

Transfections. For transient expression, the plasmids pSG/flag-*beclin1* or pSG5/flag-mtNES*beclin1* were transfected into COS7 cells using SuperFect polycationic reagent (Qiagen, Valencia, CA) and the plasmids pTRE/flag-*beclin1* and pTRE/flag-mtNES*beclin1* were transfected into MCF7/tet-off cells using PerFect lipid (Invitrogen) according to the manufacturer's instructions. For stable expression, MCF7/tet-off cells were cotransfected with pTK-Hyg and pTRE/flag-*beclin1* or pTRE/flag-mtNES*beclin1* and maintained in 200 µg/ml hygromycin and 2 µg/ml tetracycline. Hygromycin-resistant clones were screened for inducible wild-type flag-Beclin 1 or flag-mtNES Beclin 1 protein expression following tetracycline withdrawal by immunoblot analysis using M2 anti-flag epitope monoclonal Ab (1:200; Kodak, Rochester, NY) and peroxidase-conjugated horse antimouse IgG (1:2000) with the enhanced chemiluminescent method of detection (Amersham, Arlington Heights, IL). A monoclonal antiactin Ab (1:400; Boehringer Mannheim, Indianapolis, IN) was used as a control for protein loading in immunoblot analysis.

Immunofluorescence Studies. COS7 and MCF7 cells were grown on glass chamber slides, transfected with plasmids (2 µg per 4.0-cm² well) expressing wild-type Beclin 1 or mtNES Beclin 1, and fixed 24 h after transfection with 100% ethanol. Alternatively, leptomycin B was added at a concentration of 2.5 ng/ml (gift from Minoru Yoshida, University of Tokyo, Tokyo, Japan) 24 h after transfection, and cells were fixed 8 h after leptomycin B treatment (32 h after transfection). To detect flag-Beclin 1 and flag-mtNES Beclin 1, cells were stained with M2 anti-flag Ab (1:20) and FITC-conjugated horse antimouse Ab (1:40; Vector Laboratories, Burlingame, CA). The nuclei of all cells were labeled with Hoechst 33258 (1 ng/ml). Double immunofluorescent staining was also performed using either a polyclonal Ab against peptide SSRα (1:50) (37) to detect the ER or a polyclonal anti-lamin B Ab (38) (1:100) to detect the perinuclear membrane (gifts from Howard Worman, Columbia University, New York, USA) followed by rhodamine-conjugated goat anti-rabbit Ab. To colabel mitochondria and Beclin 1, live transfected COS7 and MCF7 cells were incubated with a MitoTracker probe (400 nM; Molecular Probes, Eugene, OR) for 30 min and then fixed and stained with M2 anti-flag as described above. For dual-labeling analysis, cells were examined using a Zeiss LSM410 scanning confocal microscope equipped with an argon-krypton laser. The percentage of cells demonstrating nuclear staining was calculated by analyzing 200–300 transfected cells per transfection experiment; results represent the mean ± SE for six independent transfection experiments. Cells demonstrating FITC immunoreactivity that colocalized with Hoechst 33258 were scored as positive.

Autophagy Analysis. Five days following tetracycline removal from the media, MCF7/tet-off clones stably transfected with empty pTRE (MCF7.control

cells), pTRE/flag-*beclin1* (MCF7.*beclin1* cells), or pTRE/flag-mtNES*beclin1* (MCF7.mtNES*beclin1* cells) were grown for 4 h in either (a) EBSS + 10% serum and complete amino acids, (b) EBSS, or (c) EBSS following a 30-min pretreatment with 10 mM 3-MA. Cells were fixed with 2.5% glutaraldehyde in 0.1 M Sorenson's buffer (pH 7.2) for 1 h, postfixed with 1% OsO₄ in 0.1 M Sorenson's buffer, and embedded in Lx-112 (Ladd Research Industries, Burlington, VT) and Embed-812 (EM Science, Fort Washington, PA). Thin sections were cut on MT-7000 RMC, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy using a JEOL JEM-1200 EXII. The number of autophagic vacuoles per cell was determined for 100 cells/clone for each treatment group in a blinded fashion to calculate the mean number of autophagic vacuoles per cell. Autophagic vacuoles were defined as double-membrane vacuolar structures containing recognizable cytoplasmic contents using a magnification of ×50,000.

Clonogenicity and Tumorigenicity Assays. To measure *in vitro* clonogenicity, MCF7.control, MCF7.*beclin1*, and MCF7.mtNES*beclin1* clones were seeded at a density of 5 × 10⁴ cells/well in triplicate 35-mm wells in semi-solid media (0.3% agar in DMEM with 20% fetal bovine serum). The number of anchorage-independent colonies (with >20 cells/colony) per well was counted at 21 days.

To measure *in vivo* tumorigenicity, 5-week-old female NCR nude mice (Taconic Farms, Germantown, NY) were implanted with 1.7 mg of estrogen/60-day release pellets (Innovative Research of America, Toledo, OH) and injected s.c. with 5 × 10⁶ MCF7 tumor cells. Mice were monitored for the development of tumors and tumor size was measured in two dimensions [length (a) and width (b)]. Tumor volume was calculated according to $V = ab^2/2$. Mice were necropsied after 8 weeks, and mean tumor volumes were determined for the subgroup of mice with tumors present upon necropsy. All animal studies were performed in accordance with Institutional Animal Care and Use Committee guidelines.

RESULTS

Subcellular Localization of Beclin 1. We examined the subcellular distribution of wild-type Beclin 1 in transfected COS7 cells and MCF7 cells (Figs. 1 and 2). Since antibodies are not yet available that detect Beclin 1 by immunofluorescent staining, we used flag epitope-tagged constructs. [In previous studies, flag epitope-tagged Beclin 1 has been shown to have autophagy and tumor suppressor function (26) and demonstrates an immunofluorescent staining pattern in transfected cells similar to that observed with immunoperoxidase staining of endogenous Beclin 1 in human brain and breast tissue (26, 33)]. In *flag-beclin1*-transfected COS7 cells, the majority of cells demonstrated granular cytoplasmic staining suggestive of localization within intracytoplasmic organelles (Fig. 2A, top row) and very rare cells (<1%) demonstrated any nuclear staining. Dual-labeling experiments showed that the FITC-staining of flag-Beclin 1 colocalized with a MitoTracker, a rhodamine dye that targets to the mitochondria, and with antibodies to SSRα (37) and lamin B (38) that label the ER and perinuclear membrane, respectively (Fig. 1A). The flag-Beclin 1 staining was specific in that no immunostaining was observed in COS7 cells transfected with an empty vector and stained with anti-flag M2 Ab (data not shown). Thus, Beclin 1 is infrequently observed in the nucleus of COS7 cells and is predominantly associated with intracytoplasmic structures including mitochondria, ER, and the perinuclear membrane. The association of Beclin 1 with these organelles is similar to that previously reported for the Beclin 1-interacting proteins Bcl-2 and Bcl-x_L (39, 40).

In *flag-beclin1*-transfected MCF7 cells, a higher percentage (46.5 ± 4.2%) of cells demonstrated some nuclear staining (Fig. 2B, top row). In cells with exclusive cytoplasmic or nuclear and cytoplasmic staining, the cytoplasmic distribution of Beclin 1 was similar to that observed in COS7 cells (Fig. 1B). Flag-Beclin 1 colocalized with MitoTracker as well as with antibodies to the ER and perinuclear membrane. Thus, MCF7 cells differed from COS7 cells with respect

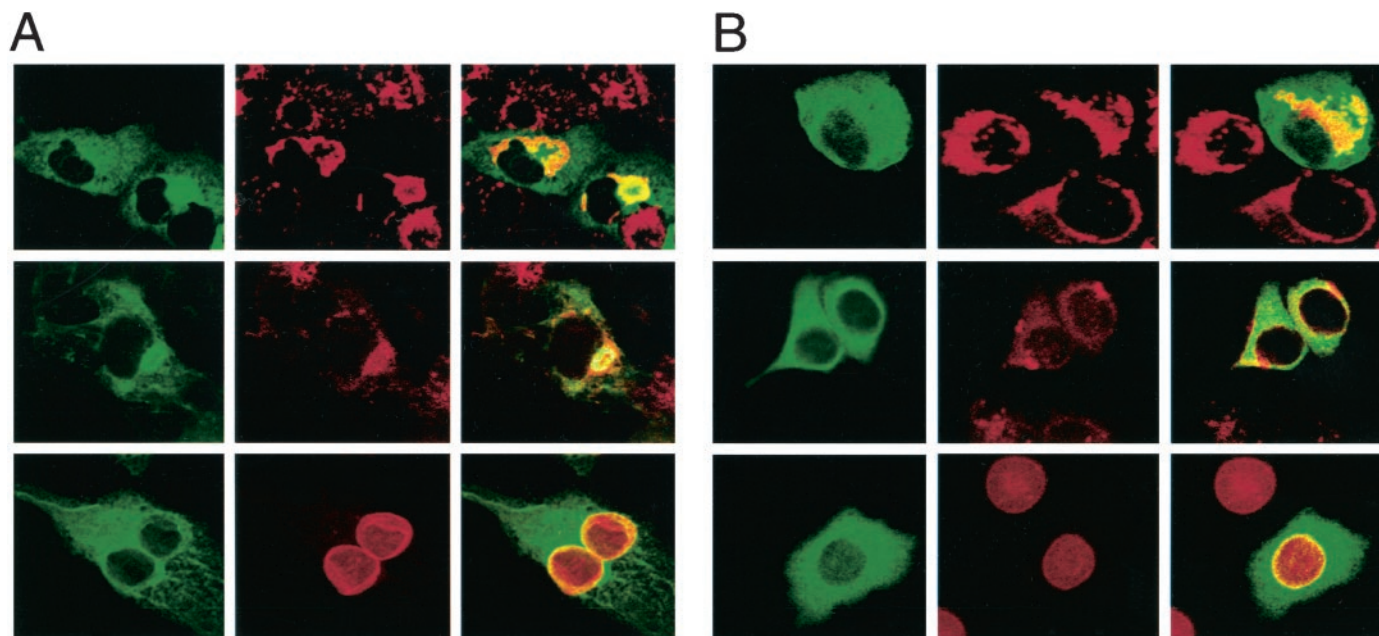


Fig. 1. Beclin 1 colocalizes with mitochondria, ER, and perinuclear membrane markers in COS7 and MCF7 cells. Confocal scanning laser microscopy images of COS7 cells (A) and MCF7 cells (B) transfected with flag-beclin1. Left column of each panel, FITC anti-flag immunostaining to detect flag-Beclin 1. Middle column of each panel, staining with rhodamine-labeled markers of cytoplasmic organelles, including MitoTracker to detect mitochondria (top), anti-SSR α to detect ER (middle), and anti-lamin B to detect perinuclear membrane (bottom). Right column of each panel, overlay of FITC and rhodamine staining; yellow, colocalization of flag-Beclin 1 and cytoplasmic organelle.

to the amount of Beclin 1 nuclear staining but not with respect to the subcellular distribution of cytoplasmic Beclin 1.

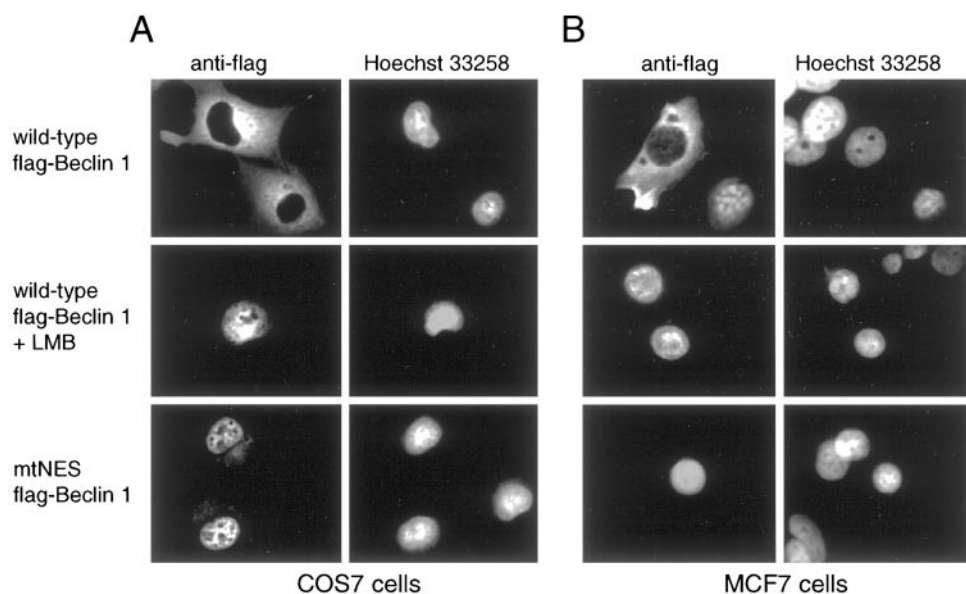
Taken together, these results indicate that Beclin 1 demonstrates both cytoplasmic and nuclear localization with cell type-specific differences in the frequency of steady-state nuclear localization.

Leptomycin B Blocks the Nuclear Export of Beclin 1. Given the localization of Beclin 1 in both nuclei as well as intracytoplasmic organelles, coupled with the presence of a leucine-rich NES motif spanning amino acids 180–189, we hypothesized that Beclin 1 may undergo CRM1-dependent nuclear export. To examine this hypothesis, we evaluated the effects of leptomycin B, an inhibitor of CRM1-dependent nuclear export (21, 24), on the subcellular localization of flag-Beclin 1 in COS7 and MCF7 cells (Fig. 2, middle row). In both cell types, treatment with leptomycin B resulted in the nuclear localization of Beclin 1 in the majority of transfected cells. At 8 h after

leptomycin B treatment, exclusive cytoplasmic staining was observed in only $4.0 \pm 0.7\%$ of leptomycin B-treated COS7 cells as compared to $98.3 \pm 0.8\%$ of untreated COS7 cells, and in $1.5 \pm 0.3\%$ of leptomycin B-treated MCF7 cells as compared to $53.5 \pm 5.9\%$ of untreated MCF7 cells. Consistent with previous reports indicating that leptomycin B results in nuclear retention of NES-containing proteins within a very short period of time (5–90 min) (21, 41–43), we did not observe any significant differences in the percentage of COS7 or MCF7 cells with nuclear Beclin 1 staining at 2, 4, 6, 8, 12, and 18 h after treatment (data not shown). The increased nuclear localization of Beclin 1 in both COS7 and MCF7 cells following leptomycin B treatment is consistent with a leptomycin B-sensitive, CRM1-dependent pathway of nuclear export for Beclin 1.

Mutation of the NES of Beclin 1 Blocks Its Nuclear Export. To determine whether the NES motif of human Beclin 1 has nuclear

Fig. 2. Leptomycin B treatment and mutation of the Beclin 1 NES alters the subcellular localization of Beclin 1 in COS7 and MCF7 cells. COS7 cells (A) and MCF7 cells (B) transfected with wild-type flag-Beclin 1 (top and middle rows of each panel) in the absence (top rows of each panel) or presence of leptomycin B (middle rows of each panel) or transfected with mtNES flag-Beclin 1 (lower rows of each panel). Images shown represent FITC anti-flag immunostaining to detect flag-Beclin 1 (left column of each panel) or Hoechst 33258 staining to detect nuclear DNA (right columns of each panel).



Human Beclin 1	L Q M E L - K E L A L E	aa 180-190
Human mtNES Beclin 1	L Q M E A - K E A A L E	aa 180-190
Mouse Beclin 1	L Q R E L - K E L A L E	aa 178-188
HIV-1 Rev	L - P P L - E R L T L D	aa 75-84
HTLV-1 Rex	L S A Q L Y S S L S L D	aa 82-93
PKI	L A L K L - A G L D I N	aa 37-47
MAPKK	L Q K K L - E E L E L D	aa 33-43
IκB-α	I Q Q Q L - G Q L T L E	aa 265-275
hdm-2	I S L S F D E S L A L C	aa 189-200
TFIIIA	L - P V L - E N L T L K	aa 330-339
p53	F - R E L N E A L E L K	aa 341-351
NES CONSENSUS	L X ₍₂₋₃₎ L X ₍₂₋₃₎ L X L	

Fig. 3. Alignment of the Beclin 1 NES with other known leucine-rich Rev-type NESs. Numbers indicate the position of the amino acids within the proteins. Conserved hydrophobic residues (primarily leucines) are shown in **boldface**. Underlined amino acids represent those mutated in human mtNES Beclin 1. In the NES consensus sequence, X is any amino acid and L (leucine) can be substituted by other large hydrophobic residues.

export function, we examined the subcellular localization of an NES mutant of Beclin 1. Fig. 3 shows an alignment of the NES of human Beclin 1 and mouse Beclin 1 with other well-characterized leucine-rich NESs. Based upon mutational analyses of NESs in HIV-1 Rev and PKI (5, 11), we predicted that mutation of the two leucine residues at 184 and 187 should block nuclear export of Beclin 1. Therefore, we mutated these two leucine residues to alanine residues (mtNES Beclin 1) and examined the subcellular distribution of flag-mtNES Beclin 1 (Fig. 2, *bottom row*). The subcellular localization of mtNES flag-Beclin 1 contrasted markedly with wild-type flag-Beclin 1 in untreated COS7 and MCF7 cells, but was similar to that observed following leptomycin B treatment of wild-type *beclin 1*-transfected cells. Nuclear staining of mtNES1 was observed in $99.1 \pm 0.9\%$ of transfected COS7 cells and in $97.2 \pm 5.3\%$ of transfected MCF7 cells, as compared to nuclear staining of wild-type Beclin 1 in $<1\%$ of transfected COS7 cells and in $46.5 \pm 4.2\%$ of transfected MCF7 cells. The nuclear localization of mtNES flag-Beclin 1 in almost all transfected cells indicate that the leucines at amino 184 and 187 in human Beclin 1 are critical for its nuclear export.

Mutation of the NES of Beclin 1 Blocks Its Autophagy Function. The above studies demonstrate that Beclin 1 has a leucine-rich NES that undergoes CRM1-dependent nuclear export. To determine whether the NES of Beclin 1 is required for its autophagy function, we compared the ability of wild-type Beclin 1 and mtNES Beclin 1 to promote nutrient deprivation-induced autophagy in MCF7 cells. Serum and amino acid deprivation is a potent inducer of autophagy in normal cells (reviewed in Ref. 27), but not in MCF7 cells that lack detectable endogenous Beclin 1 expression (26). However, enforced wild-type Beclin 1 expression increases basal levels of autophagy in MCF7 cells and promotes nutrient deprivation-induced autophagy that can be inhibited by the nucleotide derivative 3-MA (26).

To permit conditional expression of an antiproliferative gene, we stably transfected MCF7/tetoff cells with a tetracycline-repressible vector, pTRE, containing no insert (referred to as MCF7.control cells), wild-type flag-*beclin 1* (referred to as MCF7.*beclin1* cells), or flag-mtNES *beclin 1* (referred to as MCF7.mtNES*beclin1* cells). One MCF7.control clone, one MCF7.*beclin1* clone, and two MCF7.mtNES*beclin1* clones which expressed different levels of mtNES Beclin 1 protein were chosen for further analysis (Fig. 4A). After normalization using an actin control, densitometric analysis revealed comparable levels of mtNES Beclin 1 expression in MCF7.mtNES*beclin1* clone 17 cells and wild-type Beclin 1 expression in MCF7.*beclin1* clone 6 cells (data not shown).

MCF7 clones were either maintained in normal growth conditions or subjected to 4 h of serum and amino acid starvation and analyzed by electron microscopy for the presence of autophagic vacuoles (Fig.

4, B and C; see Fig. 4C for representative images of the ultrastructural appearance of MCF7 clones in normal growth or starvation conditions.) Ultrastructural analysis is the most definitive method to identify autophagic vacuoles, which are defined as double membrane-bound 0.3–2 μm vesicles with clearly recognizable cytoplasmic contents (44, 45). An example of an autophagic vacuole which would be counted in our quantitative electron microscopy analysis is shown in Fig. 4C, *lower right panel*. Using quantitative electron microscopy analysis, we found that the baseline numbers of autophagic vacuoles per cell were comparable in the MCF7.control clone and the two MCF7.mtNES*beclin1* clones, which was significantly less than that observed in the MCF7.*beclin1* clone (Fig. 4B). Furthermore, serum and amino acid deprivation increased the number of autophagic vacuoles in MCF7.*beclin1* cells, but not in MCF7.mtNES*beclin1* cells or in MCF7.control cells. The nutrient deprivation-induced autophagy in MCF7.*beclin1* cells was completely blocked by pretreatment with the autophagy inhibitor 3-MA. Thus, mtNES *beclin 1*, unlike wild-type *beclin 1*, failed to promote basal or nutrient deprivation-induced autophagy in MCF7 cells.

Mutation of the NES of Beclin 1 Blocks Its Tumor Suppressor Function. To evaluate the tumor suppressor activity of mtNES *beclin 1*, we compared MCF7.control, MCF7.*beclin1*, and MCF7.mtNES*beclin1* cells with respect to their *in vitro* clonogenicity and tumorigenicity in nude mice (Fig. 5). MCF7.mtNES*beclin1* cells formed colonies in soft agar as efficiently as MCF7.control cells, whereas MCF7.*beclin 1* cells were severely impaired in anchorage-independent growth (Fig. 5A). The inability of mtNES *beclin 1* to suppress clonogenicity in soft agar was associated with an inability to suppress MCF7 tumorigenicity in nude mice. After an 8-week observation period, only 10% of mice injected with MCF7.*beclin1* cells developed tumors, as compared with 78% of mice injected with MCF7.control cells or 71–86% of mice injected with MCF7.mtNES*beclin1* cells (Fig. 5B). In addition, the tumor volume was significantly less in the tumor that did develop in the mouse injected with MCF7.*beclin1* cells (32 mm³) as compared with the mean tumor volume in mice injected with MCF7.control (218 ± 65 mm³) or MCF7.mtNES*beclin1* cells (381 ± 170 mm³) for clone 17, 758 ± 282 mm³ for clone 23; ($P < 0.001$, *t* test). These data demonstrate that mtNES *beclin 1*, unlike wild-type *beclin 1*, lacks tumor suppressor function in MCF7 human breast carcinoma cells.

DISCUSSION

Our results demonstrate that Beclin 1 has a functionally important leucine-rich NES. Inhibition of the CRM1 nuclear export pathway by leptomycin B or mutation of the conserved leucine residues within the Beclin 1 NES blocks the nuclear export of Beclin 1. Mutation of the NES interferes with the two known functions of the protein, *e.g.*, the promotion of nutrient-deprivation induced autophagy and the suppression of mammary cell tumorigenesis. These results suggest a role for the Beclin I NES and the Rev-related nuclear export pathway in the regulation of autophagy and cell growth in mammalian cells.

The finding of leucine-rich NESs in signal transduction molecules (such as MAPKK, PKI, and IκB) suggests that nuclear export signals are important in coordinating the cell's response to stress and external stimuli. Since autophagy is tightly regulated in response to environmental stimuli (*e.g.*, amino acid availability, hormones, growth factors) (reviewed in Refs. 27 and 29), our findings lead to the hypothesis that nuclear cytoplasmic shuttling of autophagy proteins may be a cellular mechanism for the control of autophagy. Interestingly, we have noted that the yeast counterpart of Beclin 1, Apg6/Vps30p, has a sequence near its NH₂ terminus (LDPSLEGLSL) that conforms to the Rev-type NES consensus sequence (Lx₍₂₋₃₎Lx₍₂₋₃₎LxL). Although it is not yet known whether this sequence is an NES for

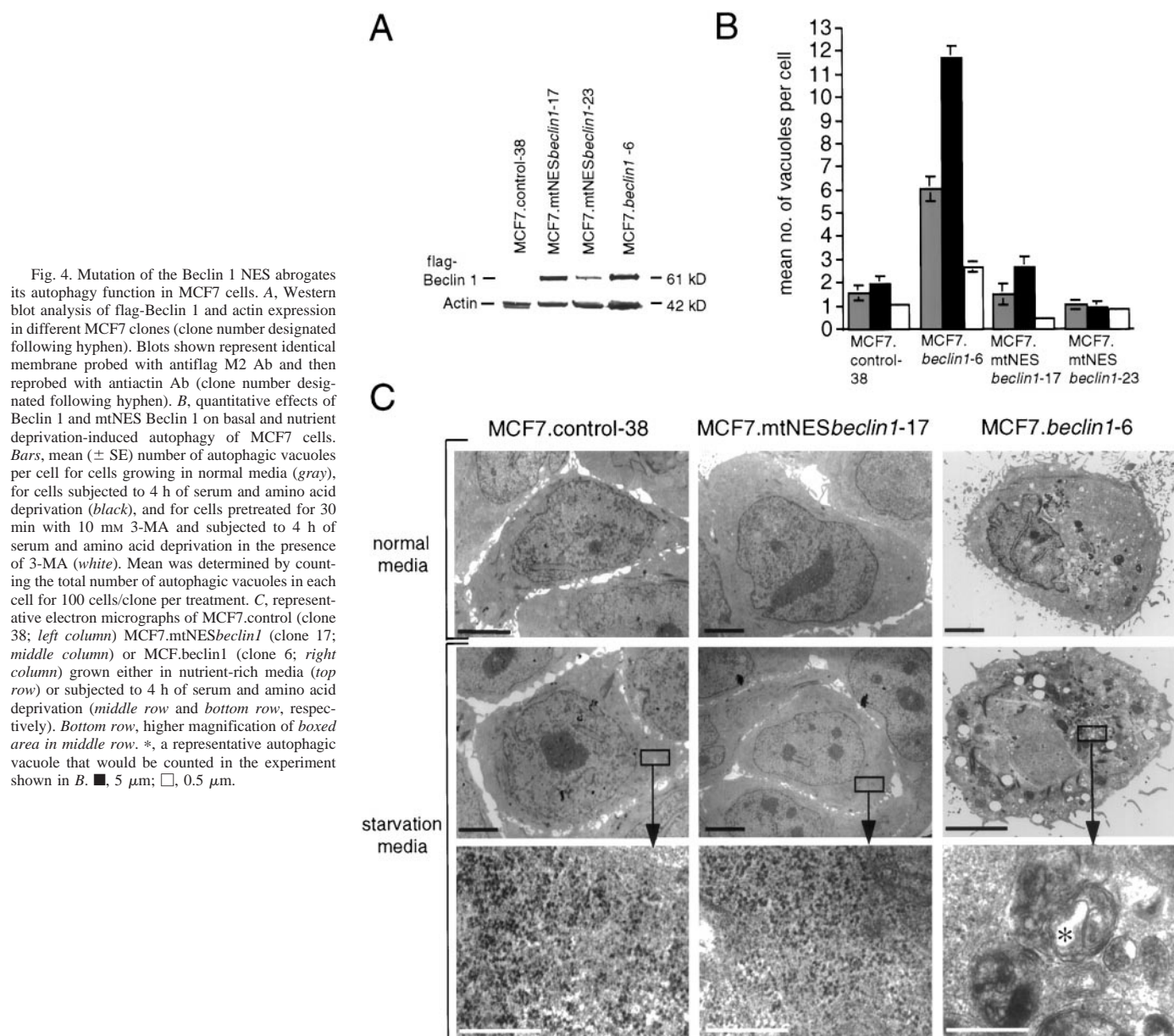


Fig. 4. Mutation of the Beclin 1 NES abrogates its autophagy function in MCF7 cells. **A**, Western blot analysis of flag-Beclin 1 and actin expression in different MCF7 clones (clone number designated following hyphen). Blots shown represent identical membrane probed with anti-flag M2 Ab and then reprobed with antiactin Ab (clone number designated following hyphen). **B**, quantitative effects of Beclin 1 and mtNES Beclin 1 on basal and nutrient deprivation-induced autophagy of MCF7 cells. Bars, mean (\pm SE) number of autophagic vacuoles per cell for cells growing in normal media (gray), for cells subjected to 4 h of serum and amino acid deprivation (black), and for cells pretreated 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 (white). Mean was determined by counting the total number of autophagic vacuoles in each cell for 100 cells/clone per treatment. **C**, representative electron micrographs of MCF7.control (clone 38; left column) MCF7.mtNESbeclin1 (clone 17; middle column) or MCF7.beclin1 (clone 6; right column) grown either in nutrient-rich media (top row) or subjected to 4 h of serum and amino acid deprivation (middle row and bottom row, respectively). Bottom row, higher magnification of boxed area in middle row. *, a representative autophagic vacuole that would be counted in the experiment shown in B. ■, 5 μ m; □, 0.5 μ m.

Apg6/Vps30p, the evolutionarily conservation of this motif in the yeast and mammalian counterparts of an autophagy gene suggest that it may play an essential functional role.

Our findings also support the hypothesis that nuclear cytoplasmic shuttling is an important mechanism for regulating cell growth. Previous studies have demonstrated that nuclear cytoplasmic shuttling may be critical for the regulation of p53 tumor suppressor function; the CRM1 nuclear export pathway is used by cellular [e.g., *hdm2* (17), *mdm2* (46)] and viral oncogenes [e.g., HPV E6 (46)] to target p53 for ubiquitin-mediated degradation. Recent evidence also suggests that a hydrophobic NES with loose similarity to the Rev-type NESs may play a role in the cell cycle-dependent nuclear localization of the mitotic cyclin (47–49). Before mitosis, cyclin B is rapidly exported from the nucleus resulting in a steady-state cytoplasmic localization and this export is blocked by leptomycin B. Phosphorylation of cyclin B1 at the beginning of mitosis blocks its interaction with CRM1 and prevents its return to the cytoplasm (49). The failure of a nuclear export-deficient mutant of Beclin 1 to suppress the tumor-forming potential of human breast carcinoma cells further suggests that nuclear

cytoplasmic shuttling is critical for the function of cell growth regulatory proteins. However, in contrast to the p53 tumor suppressor which is functionally inactivated by transport to the cytoplasm, nuclear export of Beclin 1 is required for its tumor suppressor activity.

This requirement for an intact nuclear export signal of Beclin 1 in tumor suppressor function is most likely interrelated with the requirement for an intact nuclear export signal in autophagy function. Based upon inverse correlations between cellular malignant transformation and autophagic capacity, it has been postulated that autophagy is an important mechanism for negative growth control (reviewed in Ref. 28). In addition, inactivation of the autophagy inhibitory TOR/RAFT1 signaling pathway results both in autophagy and a G_0 arrest (50, 51). Our earlier finding that enforced expression of Beclin 1 both promotes nutrient deprivation-induced autophagy and inhibits tumorigenicity of human breast carcinoma cells suggested that *beclin 1* may provide a genetic link between autophagy and tumor suppressor pathways (26). The identification of a common structural requirement in Beclin 1, an intact Rev-type NES, for both its autophagy and tumor suppressor function further suggests that these two functions may be interrelated.

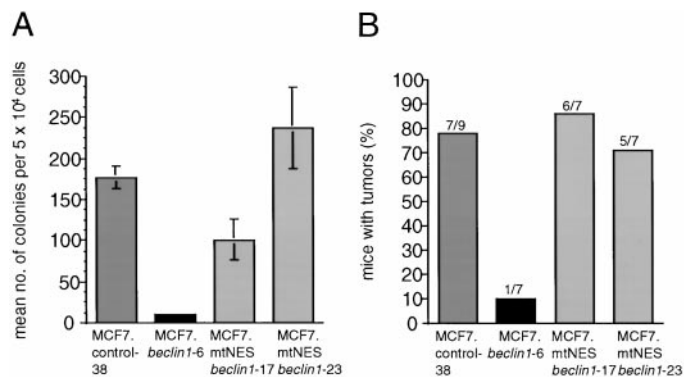


Fig. 5. Mutation of the Beclin 1 NES abrogates its tumor suppressor function in MCF7 cells. *A*, clonogenicity in semisolid medium (soft agar) of MCF7 clones. Results are mean for pooled triplicate wells from four to six independent experiments; bars, \pm SE. *B*, tumor formation in NCR nude mice injected s.c. with MCF7 clones. Numbers above bars, number of autopsy-confirmed tumors at 8 weeks per number of mice injected with each clone.

If this proves to be correct, the loss of autophagy function resulting from failure to undergo nuclear export could be directly responsible for the loss of tumor suppressor function of the nuclear export-deficient mutant of Beclin 1. However, we cannot exclude the possibility that Beclin 1 has two independent functions, both of which require the leucine-rich NES motif.

Although we speculate that the loss of autophagy and tumor suppressor function of mtNES Beclin 1 results from aberrant nucleocytoplasmic transport, it is also possible that this domain of Beclin 1 functions through independent mechanisms to regulate autophagy and tumor suppressor function. For example, the leucine-rich NES of p53 is located within its tetramerization domain, and a model has been proposed in which p53 tetramerization occludes its NES and ensures nuclear retention of the DNA-binding form (16). It is possible that the leucine-rich NES of Beclin 1, which is located within the coiled-coil domain of the protein, is important for homodimerization or for heterodimerization with other proteins and that such protein-protein interactions could modulate the function of Beclin 1. The yeast homologue of Beclin 1, Apg6p, has been shown to interact with another coiled-coil protein Apg14p (34). However, to date, there is no known mammalian homologue of Apg14p and the only known Beclin 1-interacting proteins are Bcl-2 and Bcl-x_L which bind to a region of Beclin 1 (amino acids 88–150) that is upstream of the NES motif (33).

To further understand the role of the NES of Beclin 1 in its autophagy and tumor suppressor functions, it will be necessary to identify the effects of stimuli that promote and inhibit autophagy on the subcellular localization of Beclin 1. The yeast homologue, Apg6/Vps30p, has been previously shown to associate with intracellular membranes (34), and, in this study, we found that Beclin 1 associates with intracytoplasmic organelles such as the ER, mitochondria, and perinuclear membrane. Since structural algorithmic analyses of Beclin 1 fail to reveal any hydrophobic transmembrane domains (33), the association of Beclin 1 with intracytoplasmic membranes presumably occurs through association with other integral membrane proteins. Given that the double membranes of autophagic vacuoles are primarily derived from invaginations of the ER membrane (44, 45), it is possible that Beclin 1 must be present in the ER for autophagy to proceed. According to this model, the nuclear compartmentalization of Beclin 1 would represent a mechanism for blocking autophagy from occurring in the cytoplasm. An alternative explanation is that the nuclear export of Beclin 1 is required to facilitate the degradation of another autophagy-inhibitory protein; this model would be analogous to the scenario observed with hdm2 and p53 (17).

Viewed in the context of these models, the cell type-specific dif-

ferences that we observed in the subcellular localization of wild-type Beclin 1 may have functional significance for the regulation of autophagy and tumorigenesis. MCF7 cells are a human breast carcinoma cell line that fails to undergo nutrient deprivation-induced autophagy, and we found that the percentage of cells with nuclear localization of Beclin 1 in MCF7 cells was significantly higher than in COS7 cells. Although COS7 cells are transformed by SV40T antigen, we have also observed a predominantly cytoplasmic pattern of staining of flag-Beclin 1 in nontransformed cells such as BHK cells (33) and NIH3T3 cells.⁴ The molecular basis for the increased nuclear localization of Beclin 1 in MCF7 as compared to other cell types is unknown; it could reflect cell type-specific differences in as of yet undefined modifications of Beclin 1 that influence nuclear export (e.g., binding to other proteins, phosphorylations) or cell type-specific differences in CRM1 or other components of the intrinsic nuclear export machinery. It is already known that the *beclin 1* gene is frequently monoallelically deleted (36) and that Beclin 1 protein expression is frequently decreased in human breast cancer (26). It is possible that the blockade of Beclin 1 nuclear export represents an additional mechanism to inactivate its tumor suppressor function in human breast carcinoma cells.

ACKNOWLEDGMENTS

We thank Howard Worman for helpful discussions and for providing antibodies and Minoru Yoshida for providing leptomycin B.

REFERENCES

- Nigg, E. Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature (Lond.)*, 386: 779–784, 1997.
- Ohno, M., Fornerod, M., and Mattaj, J. W. Nucleocytoplasmic transport: the last 200 nanometers. *Cell*, 92: 327–336, 1998.
- Gorlich, D. Transport into and out of the cell nucleus. *EMBO J.*, 17: 2721–2727, 1998.
- Gerace, L. Nuclear export signals and the fast track to the cytoplasm. *Cell*, 82: 341–344, 1995.
- Fischer, U., Huber, J., Boelens, W. C., Mattaj, J. W., and Luhrmann, R. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell*, 82: 475–483, 1995.
- Meyer, B. E., Meinkoth, J. L., Tsien, R. Y., and Taylor, S. S. Nuclear transport of human immunodeficiency virus type 1, visna virus, and equine infectious anemia virus Rev proteins: identification of a family of transferable nuclear export signals. *J. Virol.*, 70: 2350–2359, 1996.
- Bogerd, H. P., Fridell, R. A., Benson, R. E., Hua, J., and Cullen, B. R. Protein sequence requirements for function of the human T-cell leukemia virus type 1 rex nuclear export signal delineated by a novel *in vivo* randomization-selection assay. *Mol. Cell. Biol.*, 16: 4207–4214, 1996.
- Palermi, D., and Malim, M. H. The human T-cell leukemia virus type 1 posttranscriptional transactivator Rex contains a nuclear export signal. *J. Virol.*, 70: 6442–6445, 1996.
- Sandri-Goldin, R. M. ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. *Genes Dev.*, 12: 868–879, 1998.
- Rosorius, O., Fries, B., Stuaber, R. H., Hirschmann, N., Bevec, D., and Hauber, J. Human ribosomal protein L5 contains defined nuclear localization and export signals. *J. Biol. Chem.*, 275: 12061–12068, 2000.
- Wen, W., Meinkoth, J. L., Tsien, R. Y., and Taylor, S. S. Identification of a signal for rapid export of proteins from the nucleus. *Cell*, 82: 463–473, 1995.
- Taagepera, S., McDonald, D., Loeb, J. E., Whitaker, L. L., McElroy, A. K., Wang, J. Y. J., and Hope, T. J. Nuclear-cytoplasmic shuttling of c-ABL tyrosine kinase. *Proc. Natl. Acad. Sci. USA*, 95: 7457–7462, 1998.
- Arenzana-Seisdedos, F., Turpin, P., Rodriguez, M., Thomas, D., Hay, R. T., Virelizier, J.-L., and Dargemont, C. Nuclear localization of I κ B α promotes active transport of NF- κ B from the nucleus to the cytoplasm. *J. Cell Sci.*, 110: 369–378, 1997.
- Fukuda, M., Gotoh, I., Gotoh, Y., and Nishida, E. Cytoplasmic localisation of mitogen-activated protein kinase kinase directed by its NH₂-terminal, leucine-rich short amino acid sequence, which acts as a nuclear export signal. *J. Biol. Chem.*, 271: 20024–20028, 1996.
- Fridell, R. A., Fischer, U., Luhrmann, R., Meyer, B. E., Meinkoth, J. L., Malim, M. H., and Cullen, B. R. Amphibian transcription factor IIA proteins contain a sequence element functionally equivalent to the nuclear export signal of human

⁴ X-H. Liang and B. Levine, unpublished data.

- immunodeficiency virus type 1 Rev. Proc. Natl. Acad. Sci. USA, 93: 2936–2940, 1996.
16. Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., and Wahl, G. M. A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. EMBO J., 18: 1160–1672, 1999.
 17. Roth, J., Dobbstein, M., Freedman, D. A., Shenk, T., and Levine, A. J. Nucleocytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. EMBO J., 17: 554–564, 1998.
 18. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell, 90: 1051–1060, 1997.
 19. Stade, K., Ford, C. S., Guthrie, C., and Weis, K. Exportin 1 (Crm1p) is an essential nuclear export factor. Cell, 90: 1041–1050, 1997.
 20. Ossareh-Nazari, B., Bachelier, F., and Dargemont, C. Evidence for a role of CRM1 in signal-mediated nuclear protein export. Science (Wash DC), 278: 141–144, 1997.
 21. Fukuda, M., Nakamura, S. A. T., Adachi, M., Yoshida, M., Yanagid, M., and Nishida, E. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature (Lond.), 390: 308–311, 1997.
 22. Askjaer, P., Bachi, A., Wilm, M., Bischoff, F. R., Weeks, D. L., Ogniewski, V., Ohno, M., Niehrs, C., Kjems, J., Mattaj, I. W., and Fornerod, M. RanGTP-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-Box helicase. Mol. Cell. Biol., 19: 6276–6285, 1999.
 23. Ullman, K. S., Powers, M. A., and Forbes, D. J. Nuclear export receptors: from importin to exportin. Cell, 90: 967–970, 1997.
 24. Kudo, N., Wolff, B., Sekimoto, T., Shreiner, E. P., Yoneda, Y., Yanagid, M., Horinouchi, S., and Yoshida, M. Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. Exp. Cell Res., 242: 540–547, 1998.
 25. Nakiely, S., and Dreyfuss, G. Nuclear export of proteins and RNAs. Curr. Opin. Cell Biol., 11: 241–247, 1997.
 26. Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. Induction of autophagy and inhibition of tumorigenesis by *beclin 1*. Nature (Lond.), 402: 672–676, 1999.
 27. Dunn, W. A. J. Autophagy and related mechanisms of lysosomal-mediated protein degradation. Trends Cell Biol., 4: 139–143, 1994.
 28. Kisen, G. O., Tessitore, L., Costelli, P., Gordon, P. B., Schwarze, P. E., Baccino, F. M., and Seglen, P. O. Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. Carcinogenesis (Lond.), 14: 2501–2505, 1993.
 29. Klionsky, D., and Emr, S. D. Autophagy as a regulated pathway of cellular degradation. Science (Wash DC), 290: 1717–1721, 2000.
 30. Tsukada, M., and Ohsumi, Y. Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. FEBS Lett., 333: 169–174, 1993.
 31. Funakoshi, T., Matsuura, A., Noda, T., and Ohsumi, Y. Analyses of *APG13* gene involved in autophagy in yeast, *Saccharomyces cerevisiae*. Gene (Amst.), 192: 207–213, 1997.
 32. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M., and Ohsumi, Y. A protein conjugation system essential for autophagy. Nature (Lond.), 395: 395–398, 1998.
 33. Liang, X. H., Kleeman, L., Jiang, H. H., Gordon, G., Goldman, J. E., Berry, G., Herman, B., and Levine, B. Protection against fatal Sindbis virus encephalitis by Beclin, a novel Bcl-2-interacting protein. J. Virol., 72: 8586–8596, 1998.
 34. Kametaka, S., T. T. O., Ohsumi, M., and Ohsumi, Y. Apg14p and Apg6/Vps30p form a protein complex essential for autophagy in the yeast, *Saccharomyces cerevisiae*. J. Biol. Chem., 273: 22284–22291, 1998.
 35. Seaman, M. N. J., Marcusson, E. G., Cereghino, J. L., and Emr, S. D. Endosome to Golgi retrieval of the vacuolar protein sorting receptor, Vps10p, requires the function of the VPS29, VPS30, and VPS35 gene products. J. Cell Biol., 137: 79–92, 1997.
 36. Aita, V. M., Liang, X. H., Murty, V. V. S., Pincus, D. L., Yu, W., Cayanis, E., Gilliam, T. C., and Levine, B. Cloning and genomic structure of *beclin 1*, a candidate tumor suppressor gene on chromosome 17q21. Genomics, 59: 59–65, 1999.
 37. Migliaccio, G., Nicchitta, C. V., and Blobel, G. The signal sequence receptor, unlike the signal recognition particle receptor, is not essential for protein translocation. J. Cell Biol., 117: 15–25, 1992.
 38. Cance, W. G., Chaudhary, N., Worman, H. J., Blobel, G., and Cordon-Cardo, C. Expression of the nuclear lamins in normal and neoplastic human tissues. J. Exp. Clin. Cancer Res., 11: 233–246, 1992.
 39. Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W., and Reed, J. C. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res., 53: 4701–4714, 1993.
 40. Gonzalez-Garcia, M., Perez-Ballester, R., Ding, L., Duan, L., Boise, L. H., Thompson, C. B., and Nunez, G. Bcl-x_L is the major Bcl-x mRNA from expressed during murine development and its product localizes to mitochondria. Development (Camb.), 120: 3033–3042, 1994.
 41. Hoshino, H., Kobayashi, A., Yoshida, M., Oyake, N. K. T., Motohashi, H., Hayashi, N., Yamamoto, M., and Igarashi, K. Oxidative stress abolishes leptomycin B-sensitive nuclear export of transcription repressor Bach2 that counteracts activation of Maf recognition element. J. Biol. Chem., 275: 15370–15376, 2000.
 42. Adachi, M., Fukuda, M., and Nishida, E. Nuclear export of MAP kinase (ERK) involves a MAP kinase kinase (MEK)-dependent active transport mechanism. J. Cell Biol., 148: 849–900, 2000.
 43. Yao, Z., and Seger, R. Leptomycin B: a potent inhibitor of nuclear export. Cell Transmissions, 16: 9, 2000.
 44. Dunn, W. A. Studies on the mechanisms of autophagy; formation of the autophagic vacuole. J. Cell Biol., 110: 1923–1933, 1990.
 45. Dunn, W. A. Studies on the mechanisms of autophagy; maturation of the autophagic vacuole. J. Cell Biol., 110: 1935–1945, 1990.
 46. Freedman, D. A., and Levine, A. J. Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6. Mol. Cell. Biol., 18: 7288–7293, 1998.
 47. Hagting, A., Karlsson, C., Clute, P., Jackman, M., and Pines, J. MPF localization is controlled by nuclear export. EMBO J., 17: 4127–4138, 1998.
 48. Toyoshima, F., Moriguchi, T., Wada, A., Fukuda, M., and Nishida, E. Nuclear export of cyclin B1 and its possible role in the DNA damage-induced G₂ checkpoint. EMBO J., 17: 2728–2735, 1998.
 49. Yang, J., Bardes, E. S. G., Moore, J. D., Brennan, J., and Powers, M. Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. Genes Dev., 12: 2131–2143, 1998.
 50. Noda, T., and Ohsumi, Y. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J. Biol. Chem., 273: 3963–3966, 1998.
 51. Zaragoza, D., Ghavidel, A., Heitman, J., and Schultz, M. C. Rapamycin induces the G₀ program of transcriptional repression in yeast by interfering with the TOR signaling pathway. Mol. Cell. Biol., 18: 4463–4470, 1998.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Beclin 1 Contains a Leucine-rich Nuclear Export Signal That Is Required for Its Autophagy and Tumor Suppressor Function

Xiao Huan Liang, Jie Yu, Kristy Brown, et al.

Cancer Res 2001;61:3443-3449.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/61/8/3443>

Cited articles This article cites 50 articles, 30 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/61/8/3443.full.html#ref-list-1>

Citing articles This article has been cited by 28 HighWire-hosted articles. Access the articles at:
</content/61/8/3443.full.html#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.