Divergent roles of BECN1 in LC3 lipidation and autophagosomal function

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Abbreviations: ATG, autophagy related; BafA1, bafilomycin A1; BCL2L1/Bcl-xL, BCL2-like 1; BECN1, Beclin 1, autophagy related; BECN1P1/BECN2, Beclin 1, autophagy related, pseudogene 1; EM, electron microscopy; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; KO, knockout; MAP1LC3-I/LC3-I, soluble, proteolytically processed microtubule-associated protein 1 light chain 3; MAP1LC3-I/LC3-I, soluble, proteolytically processed microtubule-associated protein 1 light chain 3; MAP1LC3-II/LC3-II, proteolytically processed and lipid-modified microtubule-associated protein 1 light chain 3; MAP1LC3B/LC3B, microtubule-associated protein 1 light chain 3 β; PtdIns3K, phosphatidylinositol 3-kinase 3-kinase; PIK3C3/VPS34, phosphatidylinositol 3-kinase, catalytic subunit type 3; PIK3R4/VPS15, phosphoinositide-3-kinase, regulatory subunit 4; SQSTM1/p62, sequestosome 1; TUBB, tubulin, β class 1; TALEN, transcription activator-like effector nuclease; UVRAG, UV radiation resistance associated; ZFYVE1/DFCP1, zinc finger, FYVE domain containing 1.

BECN1/Beclin 1 is regarded as a critical component in the class III phosphatidylinositol 3-kinase (PtdIns3K) complex to trigger autophagy in mammalian cells. Despite its significant role in a number of cellular and physiological processes, the exact function of BECN1 in autophagy remains controversial. Here we created a BECN1 knockout human cell line using the TALEN technique. Surprisingly, the complete loss of BECN1 had little effect on LC3 (MAP1LC3B/LC3B) lipidation, and LC3B puncta resembling autophagosomes by fluorescence microscopy were still evident albeit significantly smaller than those in the wild-type cells. Electron microscopy (EM) analysis revealed that BECN1 deficiency led to malformed autophagosome-like structures containing multiple layers of membranes under amino acid starvation. We further confirmed that the PtdIns3K complex activity and autophagy flux were disrupted in BECN1−/− cells. Our results demonstrate the essential role of BECN1 in the functional formation of autophagosomes, but not in LC3B lipidation.

Introduction

Autophagy is responsible for the bulk degradation of cellular organelles and macromolecules to maintain cellular homeostasis. During this “self-consuming” process, a closed double-membrane vacuole called autophagosome forms to engulf the unused cellular constituents, and subsequently fuses with the lysosome to form an autolysosome for the degradation.

When autophagy is induced, MAP1LC3B/LC3B localizes to the site of autophagosome nucleation and phagophore assembly site from cytoplasm in mammalian cells, and then conjugates to the membrane lipid phosphatidylethanolamine (PE) and becomes membrane-associated to drive vacuolar elongation with other ATG proteins. Thus, the lipidation modification of LC3B from soluble (LC3B-I) to lipid-modified form (LC3B-II) and its aggregation in cytoplasm to autophagosomes have been widely used to indicate the occurrence of autophagy.

BECN1, the mammalian ortholog of Vps30/Atg6 in yeast, is evolutionarily conserved and regarded as an essential component for the initiation of conventional autophagy. BECN1 regulates the lipid kinase PIK3C3/VPS34 and forms a stable core of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex with PIK3C3 and PIK3R4/VPS15. This complex binds additional proteins such as UVRAG or ATG14 to regulate the autophagy process. ATG14 directs the PtdIns3K (BECN1-PIK3C3/VPS34-PIK3R4/VPS15) complex to form the phagophore assembly site and recruits other ATG proteins for membrane elongation and vesicle completion. The UVRAG-containing complex is involved in both autophagy initiation at early stage and maturation at the late stage. Although the suppression of BECN1 impairs the...
autophagy-associated post-translational processing of LC3-I to LC3-II.\textsuperscript{16-18} BECN1-independent autophagy has been reported, in which LC3B lipidation fails to respond to BECN1 level.\textsuperscript{19-33} Notably in yeast cells upon autophagy induction, \textit{Agg}8 can still be lipidated to form \textit{Agg}8-PE and exhibit a punctate structure in the absence of \textit{Vps30}/\textit{Atg}6, the BECN1 ortholog in yeast.\textsuperscript{13,34} It is important therefore to clarify the role of BECN1 in the LC3B lipidation and the overall autophagy function in mammalian cells.

To better understand the role of BECN1 in conventional autophagy pathway in mammalian cells, we created a \textit{BECN1}\textsuperscript{−/−} knockout HeLa cell line, and investigated the autophagy signaling under usual inducing conditions. To our surprise, BECN1 has little effect on LC3B lipidation, but plays a critical role for the functional formation of autophagosomes and macromolecule degradation through the autophagy pathway.

## Results

**BECN1 is not required for LC3/LC3B lipidation during autophagy induction**

To clarify the role of BECN1 in autophagy, we constructed \textit{BECN1}\textsuperscript{−/−} knockout HeLa cell lines through the TALEN technique (Fig. 1A). Among 45 randomly selected clones that harbor TALEN constructs targeting \textit{BECN1}, BECN1 expression completely disappeared in 11 clones as indicated by immunoblotting analysis (Fig. 1B). Sequencing analysis of 6 such clones showed that the loss of BECN1 expression was due to multiple indels occurring at the genome locus of \textit{BECN1}, all of which led to frame shifts and consequently the gene knockout (Fig. 1C). To evaluate the basic autophagy pathway in these \textit{BECN1}\textsuperscript{−/−} clones, we starved these cells by amino acid deprivation and then examined the dynamics of LC3B as indicated by the appearance of the lipidated LC3B (LC3B-II), the phagophore- and autophagosome-specific marker, converted from its unconjugated form (LC3B-I) (Fig. 1D and Fig. S1). To our surprise, the LC3B-II/LC3B-I ratio in these \textit{BECN1}\textsuperscript{−/−} clones in response to the amino acid starvation is comparable to that in the wild-type HeLa cells, while the total LC3B levels in \textit{BECN1}\textsuperscript{−/−} clones were generally higher than those in the wild type. We then selected one \textit{BECN1}\textsuperscript{−/−} clone, KO-28, for extensive study, designated as HeLa \textit{BECN1}\textsuperscript{−/−}. The complete loss of BECN1 expression in this very clone was further confirmed by the immunoblotting analysis using 2 antibodies targeting the N- and C-terminal parts of BECN1, respectively (Fig. 1E). Since it has been reported that UVRAG stability is dependent on BECN1,\textsuperscript{11} we examined the...
protein level of UVRAG under different conditions, and found
that its level was significantly decreased in HeLa BECN1−/−, and
the exogenous expression of BECN1 was able to restore the level
of UVRAG in HeLa BECN1−/− (Fig. 1F). Interestingly, the
level of BECN1 expression has no effect on BECN1p1/BECN2
expression (Fig. S2). These results demonstrate that this iso-
lated clone, HeLa BECN1−/−, is a genuine knockout cell line
with the complete loss of both BECN1 protein production and
its function.

Next, we examined the dynamics of LC3B in HeLa cells with
BECN1 knockout or ATG7 knockout when cells were exposed to
H2O2 (Fig. 1G). The absence of BECN1 has little effect on
LC3B turnover when autophagy is induced. In contrast, the
absence of ATG7 completely blocked LC3B lipidation. These
results indicate that, unlike ATG7, BECN1 is not required for
the conversion of LC3B-I to LC3B-II during the initiation of
autophagy.

BECN1 deficiency results in malformed autophagosomes
To investigate the exact role of BECN1 in autophagy, we
examined the morphology of autophagosomes under amino acid
starvation. The fluorescence microscopy indicated that amino
acid-starved HeLa cells showed an evident increase of the auto-
phagosomes indicated by the green fluorescent LC3B puncta.
Similar to those in the wild type, increased amount of LC3B
puncta was also observed in HeLa BECN1−/− cells (Fig. 2A).
The total numbers of LC3B puncta per cell were counted from
randomly selected cells under amino acid starvation, and the
average numbers of LC3B puncta in wild type were slightly
higher than those in BECN1 knockout cells (Fig. 2B), suggesting
that BECN1 only has marginal effect on the formation of auto-
phagosomes, a result consistent with our above observation
(Fig. 1D). However, the close-up examination revealed that the
sizes of autophagosomes in HeLa BECN1−/− were significantly
smaller than those in the wild type (Fig. 2C), which was further
confirmed by a statistical survey (Fig. 2D).

To investigate the potential difference of the autophagosome
structure between the wild type and the BECN1-deficient
mutant, we examined the subcellular images of cells under elec-
tron microscopy (EM). Unlike double-membrane vesicles in
the wild-type HeLa cells indicating autophagosomes under
amino acid starvation, a large amount of malformed vesicles
manifested by multilayers of membranes were observed in
BECN1−/− cells, and the exogenous expression of BECN1 was
able to reverse this phenotype (Fig. 2E). In addition, there were
hardly any autolysosomes in BECN1−/− cells compared to the
wild-type or HeLa BECN1−/−/BECN1 cells. The statistical
survey indicated that the amount of the defective vesicles in
BECN1−/− cells was significantly higher than that in the wild-
type or BECN1−/−/BECN1 cells (Fig. 2F). These results dem-
onstrate that BECN1 is important for the formation of the nor-
mal structure of autophagosomes.

Autophagy flux is blocked in the absence of BECN1
Although the deficiency of BECN1 has little effect on the
LC3B lipidation, the autophagosomes appeared abnormal and
much smaller than those in the wild-type cells. To determine
whether these malformed multimembrane autophagosome-like
structures retain their full function, we monitored the SQSTM1/
p62 degradation and LC3B turnover in the presence of a down-
stream inhibitor under amino acid starvation. The SQSTM1
degradation was observed in both HeLa and HeLa BECN1−/−/
BECN1, but not in HeLa BECN1−/−/BECN1 cells (Fig. 3A and B).
Furthermore, using bafilomycin A1 (BafA1), an inhibitor that
blocks the vacuolar-type ATPase and hence autophagosome-lyso-
some fusion, to monitor autophagy flux, we found that the fold-
crease of LC3B-II/LC3B-I was lower in the starved BECN1−/−
cells than in the wild-type cells (Fig. 3C and D and Fig. S3),
suggesting that BECN1 is essential for the overall autophagy flux
to degrade the autophagosome-enclosed material, such as long-
lived proteins.

BECN1 is required for PtdIns3K activity
Given the fact that BECN1 does not contribute to LC3B lipi-
dation, we wondered whether BECN1 affects the formation of
the PtdIns3K complex. The endogenous coimmunoprecipitation
(coIP) assay showed that the amount of UVRAG or ATG14 pre-
cipitated with PIK3C3 decreased dramatically in HeLa
BECN1−/− cells (Fig. 4A). Consistent with our previous observa-
tion from the whole cell lysate (Fig. 1F), the protein levels of
ATG14 and UVRAG in BECN1 mutants were much lower than
those in the wild type (Fig. 4A), also in agreement with the previ-
ous report that the stability of ATG14 or UVRAG is dependent
on BECN1. To further determine whether BECN1 specifically
affects the interaction of PIK3C3 to ATG14 or UVRAG, we
overexpressed PIK3C3, ATG14, and UVRAG with different
tags, and conducted the coIP analysis. We found that the interac-
tion of PIK3C3-UVRAG as well as PIK3C3-ATG14 was not
affected by the absence of BECN1 (Fig. S4A), suggesting that
BECN1 does not play a role for the direct interaction between
PtdIns3K components, and rather suggesting that it is important
to maintain the steady state level of UVRAG and ATG14 to
ensure the PtdIns3K complex formation. In a parallel experi-
ment, we found that the interaction of BCL2L1/Bcl-xL to either
UVRAG or ATG14 was significantly decreased in the absence
of BECN1 (Fig. S4B), suggesting that BCL2L1 interacts with
PtdIns3K complex through BECN1, as previously reported. We
then investigated whether BECN1 affects PIK3C3 activity
by monitoring ZFYVE1/DFCP1 localization, because it was
reported that ZFYVE1, a PtdIns3P-binding protein, is recruited
to the endoplasmic reticulum (ER) by PIK3C3-produced
PtdIns3P to initiate omegasome formation under starvation con-
ditions. Compared with the wild-type cells, ZFYVE1-GFP-
localized omegasomes were significantly reduced in HeLa
BECN1−/− cells under amino acid starvation, while exogenous
DNA-driven expression of BECN1 restored the reduced omega-
some formation in HeLa BECN1−/− (Fig. 4B and C). These
results demonstrated that BECN1 plays an important role in
PIK3C3 activity and omegasome formation, also suggesting that
the activation of PIK3C3 and omegasome formation are inde-
dependent of LC3B lipidation, but important for the functional
formation of autophagosomes.
Figure 2. For figure legend, see page 744.
Discussion

Our study, based on the BECN1 knockout in human cells, clarified the roles of BECN1 in autophagy pathway. First, the complete loss of BECN1 has little effect on LC3B lipidation when autophagy is triggered by starvation or H2O2 exposure. Second, the expression of BECN1 is important for the normal structure and function of autophagosomes to ensure autophagy flux.

BECN1 has been regarded as a critical component of PtdIns3K complex to initiate autophagy in mammalian cells. LC3B is a protein marker widely used to label autophagosomes since LC3B-II is reliably associated with phagophores and the completed autophagosomes. The effect of BECN1 on LC3B lipidation has been controversial, our biochemical and fluorescence microscopy data showed that there is little difference in LC3B lipidation between the wild-type and BECN1−/− cells. Similar observations have been reported previously.

Further study demonstrated that BECN1 expression is critical for the functional formation of autophagosomes. Although LC3B turnover could be detected in BECN1−/− cells, the autophagosomal structures were aberrant with multiple layers of membranes. These malformed autophagosomes were confirmed to lose their normal function manifested by the degradation of SQSTM1 under autophagy-inducing conditions. In addition, the PtdIns3K complex activity for omegasome formation was suppressed in the absence of BECN1. Therefore, BECN1, just like its ortholog Vps30/Atg6 in yeast, is required for the autophagy pathway in mammalian cells.

Interestingly, the total LC3B background appeared higher in BECN1−/− cells than in the wild type. Since we have observed the increase of both BECN1 and LC3B levels under autophagy-inducing conditions (data not shown), it is unlikely that BECN1 functions as an inhibitor of LC3B production. One plausible explanation is that LC3B, especially its lipidated form LC3B-II that is located on the membrane of autophagosomes and is degraded following the maturation of autophagosomes, is readily accumulated in BECN1−/− cells because the malformed autophagosomes lose their capability for macromolecule degradation.

Noticeably, BECN1 is not the only autophagy-essential molecule that has little effect on LC3B turnover. It has been reported that the LC3B-II band is detectable in rb1c1/ftp200- and atg14-knockout mouse embryonic fibroblasts, and in cells with RNA interference-mediated downregulation of ATG13, ATG14, and PIK3C3, where autophagosome formation and autophagic flux are profoundly or completely blocked. Similarly, Atg8 lipidation is unaffected in atg1, atg2, vps30/Atg6, atg9, atg13, atg14, atg16, and atg17 mutants in yeast. Taken together, it is noteworthy that these LC3 lipidation-independent autophagy-related proteins are mainly associated with 2 complexes, ULK1-ATG13-RB1CC1 and the PtdIns3K complexes, both involved in the very early stage of autophagy initiation.

Materials and Methods

Cell lines and medium

HeLa cells were cultured in Dulbecco’s Modified Eagle’s Medium (Gibco 12800-017) containing 10% fetal bovine serum (Hyclone SN30087.02).
Antibodies and reagents

Monoclonal antibodies against LC3B (M186-3), ATG7 (PM039) and SQSTM1/p62 (PM045) were from MBL. Monoclonal antibodies targeting N-terminal BECN1 (3495), UVRAG (5320), and MYC-Tag (71D10, rabbit, 2278s) were from Cell Signaling Technology. Anti-C-terminal BECN1 monoclonal antibody (612112) was from BD Transduction Laboratories. Polyclonal antibody against BECN1P1/BECN2 (NB110-60984) was ordered from Novus Biologicals. Flag-HRP monoclonal antibody (A8592) was ordered from Sigma. The secondary antibodies HRP-conjugated goat anti-mouse IgG (H\&L) (115-035-003) and HRP-conjugated goat anti-rabbit IgG (H\&L) (111-035-003) were purchased from Jackson ImmunoResearch.

Plasmids

The GFP-ZFYVE1 constructs were kindly provided by Li Yu (Tsinghua University). pcDNA4-BECN1-Flag (24388) and pcDNA4-PK3C3-Flag (24398) were purchased from Addgene. pEF-ATG14 and pEF-UVRAG were constructed in the pEF6-BSD-myc/his-B plasmid (Invitrogen, V962-20). BCL2L1-Flag plasmid was kindly provided by Min Fang (Peking University).
Fluorescence microscopy

HEK cells were grown on glass cover slips in 6-well plates. After starvation treatment, cells were washed with phosphate-buffered saline (Gibco, 10010023) twice, fixed with pre-cooled methanol for 10 min at −20 °C, blocked with 1% BSA (Roche, 735094) for one h at room temperature, and incubated in primary antibody (1:100 diluted in 1% BSA) for 1.5 h followed by secondary antibody for one h, all at room temperature. Finally, the coverslips were mounted onto slides using mounting medium containing DAPI solution (Vector Laboratories, Vectashield, H-1200), and the cells were examined by either LSM 510 laser-scanning confocal microscope (Zeiss, Germany) or a TCS SP2 spectral confocal system (Leica, Germany).

Immunoblotting analysis and coimmunoprecipitation (coIP)

For immunoblotting, cells were treated with H2O2 exposure or amino acid starvation (amino acid-free Earle’s Balanced Salt Solution, HyClone, SH30029.02) for different time periods, and then lysed for immunoblotting analysis according to standard protocol.48 For CoIP assay, plasmids were transfected into HEK cells prior to different treatment and the cells were lysed by NP40 lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris-Cl, pH 7.2). The cells were washed 3 times for 30 min and then dehydrated and embedded in resin (Sigma, SPI-812). Ultrathin sections (70 nm) were mounted on formvar/carbon coated copper grids and then stained with 2% uranyl and lead citrate and imaged on a transmission electron microscope under 80kV (H-7650B; Hitachi Limited, Japan). Images were acquired using a digital AMT V600 camera (AMT Corp, USA).

Electron microscopy

The cells were fixed using 2.5% glutaraldehyde and 100 mM phosphate buffer, pH 7.2, one to 1.5 h at room temperature. The cells were washed 3 times for 30 min each with 100 mM phosphate buffer, pH 7.2. After the final wash, the cell were washed 3 times for 30 min each with 100 mM phosphate buffer, pH 7.2, one to 1.5 h at room temperature. Finally, the coverslips were mounted onto slides using mounting medium containing DAPI solution (Vector Laboratories, Vectashield, H-1200), and the cells were examined by either LSM 510 laser-scanning confocal microscope (Zeiss, Germany) or a TCS SP2 spectral confocal system (Leica, Germany).

Statistical procedures

All data in the images were selected randomly using the Imaris software and presented as mean ± s.e.m., and P values were determined by the Student t test.

Construction of stable knockout cell lines using the TALEN technique

The design and assembly of the 2 pairs of TALENs constructs used for BECN1 and ATG7 gene-knockout were based on our own ULtIMATE protocol.35 More specifically, the 2 targeting sequences for BECN1 loci are 5′-CCATTACTTACCA3′ for TALEN1 and 5′-CCTCCTGGGTCTCTC-3′ for TALEN2, with a spacer sequence (5′-gccgagggagaccg-3′); and the 2 targeting sequences for ATG7 loci are 5′-CCTGGACTCTTAAA3′ for TALEN3 and 5′-CAAGGCACTAATA3′ for TALEN4, with a spacer sequence (5′-ctcgagttgccctc-3′). The identification and verification of gene knockout events were based on both sequencing analysis of genome PCR fragments of targeting loci (for BECN1) and immunoblotting analysis using antibodies specifically targeting BECN1 or ATG7.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

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