

BECN1s, a short splice variant of BECN1, functions in mitophagy

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Abbreviations: Atg, autophagy-related; bp, base pairs; BECN1s, BECN1 short isoform; CCCP, carbonyl cyanide m-chlorophenylhydrazide; EBSS, Earle's balanced salt solution; ECD, evolutionarily conserved domain; GFP, green fluorescent protein; LAMP1, lysosomal-associated membrane protein 1; MAP1LC3/LC3, microtubule-associated protein 1 light chain 3; PtdIns3K, class III phosphatidylinositol 3-kinase

Mitochondria selective autophagy, known as mitophagy, plays a pivotal role in several biological processes, such as elimination of the damaged mitochondria, removal of the mitochondria from immature red blood cells and sperm. The defects in mitophagy are associated with a wide spectrum of human diseases, including neurodegenerative disease, aging, cardiac disease and autoimmune disease. However, the mechanism underlying mitophagy remains largely unclear. Here, we report the characterization of a novel splice variant of BECN1/Beclin 1, BECN1s, which is produced by an alternative splicing mechanism. BECN1s is primarily associated with the outer-membrane of mitochondria. Unlike unspliced BECN1, which is essential for nonselective macroautophagy induction, BECN1s is indispensable for mitochondria-selective autophagy. Furthermore, BECN1s plays an important role in starvation- and membrane depolarization-induced mitophagy. Taken together, our findings broaden the view of BECN1 as an important regulator in autophagy, and implicate BECN1s as a specific mitophagy mediator.

Introduction

Mitochondrial dysfunction has severe cellular consequences and has been linked to many human diseases including neurodegenerative disease, aging, cardiac and autoimmune diseases.^{1–4} Thus, maintaining a healthy population of mitochondria is essential for cellular homeostasis. Selective autophagy of mitochondria, known as mitophagy, which mediates the selective removal of damaged mitochondria, plays an important role in mitochondrial quality control. Mitophagy also mediates clearance of mitochondria in developing erythrocytes^{5–7} and sperm-derived mitochondria as well.⁸

Mitophagy can be induced under many conditions including starvation,⁹ photodamage,⁹ hypoxia,^{10,11} ROS production,^{12,13} and mitochondrial uncoupler-induced mitochondrial depolarization.^{14,15} To date, many specific regulators of mitophagy have been identified. In yeast, the autophagy-related (Atg) protein Atg32 is required for mitochondrial autophagy. While in mammalian species, BNIP3L, a BH3-only member of the BCL2 family, is indispensable for autophagic removal of mitochondria during reticulocyte maturation.^{16–20} In addition, the PINK1-

PARK2 pathway is responsible for elimination of damaged mitochondria in mammals.^{14,15,21–23} Once mitochondrial damages occur, PINK1 accumulates and recruits PARK2/PARKIN specifically to the damaged mitochondria, thereafter initiating mitophagy.

BECN1/Beclin 1, the first mammalian autophagy protein to be described, plays a central role in autophagy.²⁴ BECN1 performs this function as part of a core complex that contains PIK3C3/VPS34 (the catalytic subunit of the class III phosphatidylinositol 3-kinase [PtdIns3K]) and PIK3R4/VPS15. BECN1 has a BCL2 binding domain (BBD), a central coiled-coil domain (CCD), and an evolutionarily conserved domain (ECD).²⁵ Recent structural data indicate that BECN1 has a β - α repeated, autophagy-specific (BARA) domain at the C terminus, and the ECD is a part of this domain.²⁶ Although noncanonical BECN1-independent autophagy and mitophagy contribute to MPP+ toxicity,²⁷ BECN1 is thought to be an essential protein for autophagy.²⁸ Many, if not all of gene products comprise either their transcriptional variants or structurally related isoforms, for BECN1, its isoforms have not yet been reported. In this study, we identify a novel splice variant of BECN1 that we

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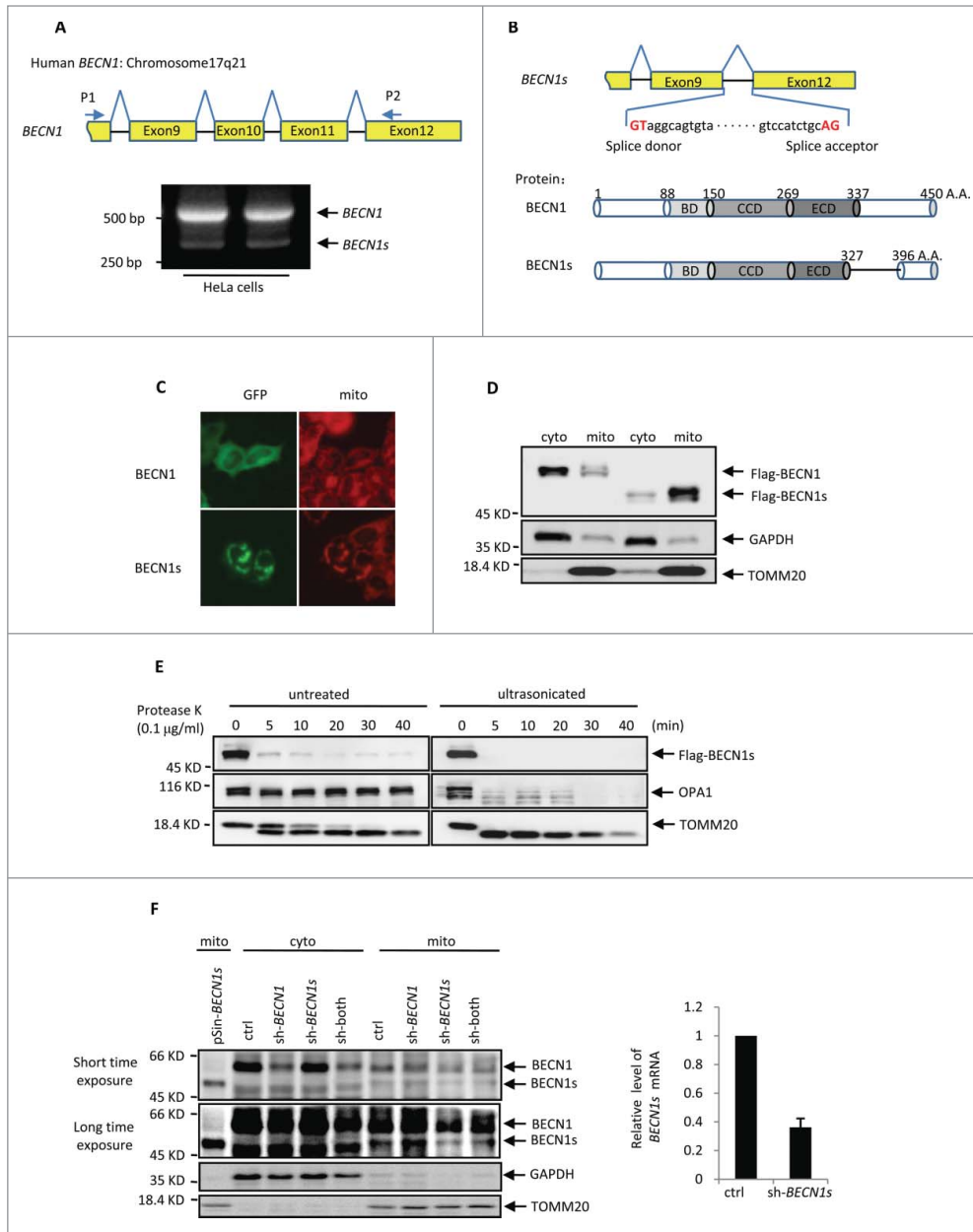


Figure 1. *BECN1s* is a splice variant of *BECN1* (A) RT-PCR was performed with total RNAs extracted from HeLa cells using primer P1 and P2. The locations of P1 and P2 on *BECN1* cDNA are indicated. (B) A schematic illustration of cDNA and protein sequences of the *BECN1* and *BECN1s*. BD, BCL2 binding domain; CCD, coiled-coil domain; ECD, evolutionarily conserved domain. (C) HeLa cells expressing either GFP-*BECN1* or GFP-*BECN1s* were stained with MitoTracker Red. The images were taken under a fluorescence microscope. (D) HEK 293T cells were transfected with either Flag-*BECN1* or Flag-*BECN1s*. Twenty-four h after transfection, cells were subjected to cytoplasm/mitochondria subcellular fractionation. Proteins from cytoplasmic and mitochondrial fractions were analyzed by western blot. GAPDH and TOMM20 were used as markers for cytoplasmic and mitochondrial fractions, respectively. (E) HEK 293T cells were transfected with Flag-*BECN1s*. Twenty-four h after transfection, cells were homogenized for mitochondria isolation. The isolated mitochondria were then treated with proteinase K for the indicated periods of time before or after supersonic treatment, followed by western blot analysis to detect Flag-*BECN1s* levels. (F) HeLa cells expressing the indicated shRNAs and *BECN1s* proteins were subjected to cytosolic and mitochondrial subcellular fractionation. Sh-both indicates the shRNA targeting both *BECN1* and *BECN1s*. Proteins from cytoplasmic and mitochondrial fractions were analyzed by western blot using anti-*BECN1* antibody. GAPDH and TOMM20 were used as markers for cytoplasmic and mitochondrial fractions, respectively. The *BECN1s* knockdown efficiency was also shown.

designated as *BECN1s* (for short isoform), which is a specific regulator of mitophagy. *BECN1s*, which lacks exon 10 and exon 11 of unspliced *BECN1*, is mainly associated with the outer-membrane of mitochondria. Although it retains the ability to activate PIK3C3, *BECN1s* is not essential for induction of nonselective macroautophagy. Intriguingly, *BECN1s* is indispensable for starvation- and mitochondrial depolarization-induced mitophagy.

Results

BECN1s is a splice variant of *BECN1*

When we amplified the *BECN1* cDNA from HeLa cells using primers P1 and P2 that were complementary to exon 8 and exon 12 of the *BECN1* gene, respectively, an additional 324 base pairs (bp) PCR product was obtained in addition to the predicted 546 bp band encoding the C terminus of *BECN1* (Fig. 1A). This 324 bp PCR product was also observed in other cell lines such as U2OS, HCT116, H1299, MCF7 and SH-SY5Y (Fig. S1A). The subsequent DNA sequencing analysis showed that the lower molecular weight band was identical to *BECN1* cDNA except that the entire exons 10 and 11 were missing (Fig. 1B). We named this short isoform as *BECN1s*. The ORF of *BECN1s* encoded a protein of 396 amino acids, which lacks the partial evolutionarily conserved domain (ECD) and C-terminal domains as compared with *BECN1* (Fig. 1B). Careful inspection of the *BECN1s* coding sequence revealed that GT and AG were indeed utilized as the donor and acceptor splice sites, respectively (Fig. 1B). These findings indicate that *BECN1s* is a novel splice variant of *BECN1*.

We next examined the cellular localization of *BECN1s*. The

immunofluorescence and the cytosolic and mitochondrial subcellular fractionation analyses revealed that unlike BECN1, which was evenly distributed in the cytoplasm, BECN1s predominantly colocalized with mitochondria (Fig. 1C-D). Similar results were found in U2OS and MEF cells (Fig. S1B and S1C). To further determine whether BECN1s is a membrane protein in mitochondria, isolated mitochondria were incubated with proteinase K before or after ultrasonication. As was expected, proteinase K did not affect the inner-membrane protein OPA1 when mitochondria were intact, but it destroyed OPA1 after mitochondria disruption by ultrasonication (Fig. 1E). Similar to the outer-membrane protein TOMM20, BECN1s was quickly degraded by proteinase K regardless of the mitochondrial integrity (Fig. 1E), suggesting that BECN1s is associated with the outer membrane of mitochondria.

To confirm further the authenticity of BECN1s, we designed 2 shRNAs. *BECN1s* shRNA, targeting the *BECN1* exon9-exon12 junction, was able to specifically knock down BECN1s but not BECN1. In contrast, *BECN1* shRNA, targeting *BECN1* exon 10, can only knock down BECN1. The cytosolic and mitochondrial fractions of HeLa cells expressing either *BECN1* shRNA or *BECN1s* shRNA were then analyzed by western blot with anti-BECN1 antibody recognizing the N terminus of BECN1. A specific 50-kDa band was detected in the mitochondrial fraction of the control cells, and the size of this band was comparable to that of untagged BECN1s (Fig. 1F). The intensity of this 50-kDa band was decreased by *BECN1s* shRNA but not by *BECN1* shRNA. Also, introduction of the shRNA targeting both *BECN1* and *BECN1s* was able to decrease the intensities of both this 50-kDa and upper BECN1 bands (Fig. 1F). Taken together, our data strongly suggest the cellular existence of BECN1s.

BECN1s is not essential for the initiation of macroautophagy

It has been well recognized that BECN1 plays a central role in the initiation of autophagy through interacting with and

regulating class III PtdIns3K activity. The evolutionarily conserved domain of BECN1 is required for PIK3C3 binding.²⁵ Since BECN1s lacks a partial ECD, we asked whether BECN1s could still bind to PIK3C3 and regulate its activity. By performing an immunoprecipitation assay, we showed that both BECN1 and BECN1s were able to interact with PIK3C3 (Fig. 2A), indicating that loss of the partial ECD in BECN1s does not affect its binding ability to PIK3C3. We next sought to investigate whether BECN1s could induce PIK3C3 activity like BECN1 does. We employed GFP-tagged double FYVE finger of HGS/Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) to detect the lipid phosphorylation activity of PIK3C3. Because the FYVE probe specifically binds to phosphatidylinositol 3-phosphate,²⁹ the product of activated PIK3C3, and forms the GFP-FYVE puncta, we could measure PIK3C3 activity by detecting FYVE fluorescence. The results showed that both BECN1 and BECN1s were able to stimulate GFP-FYVE puncta

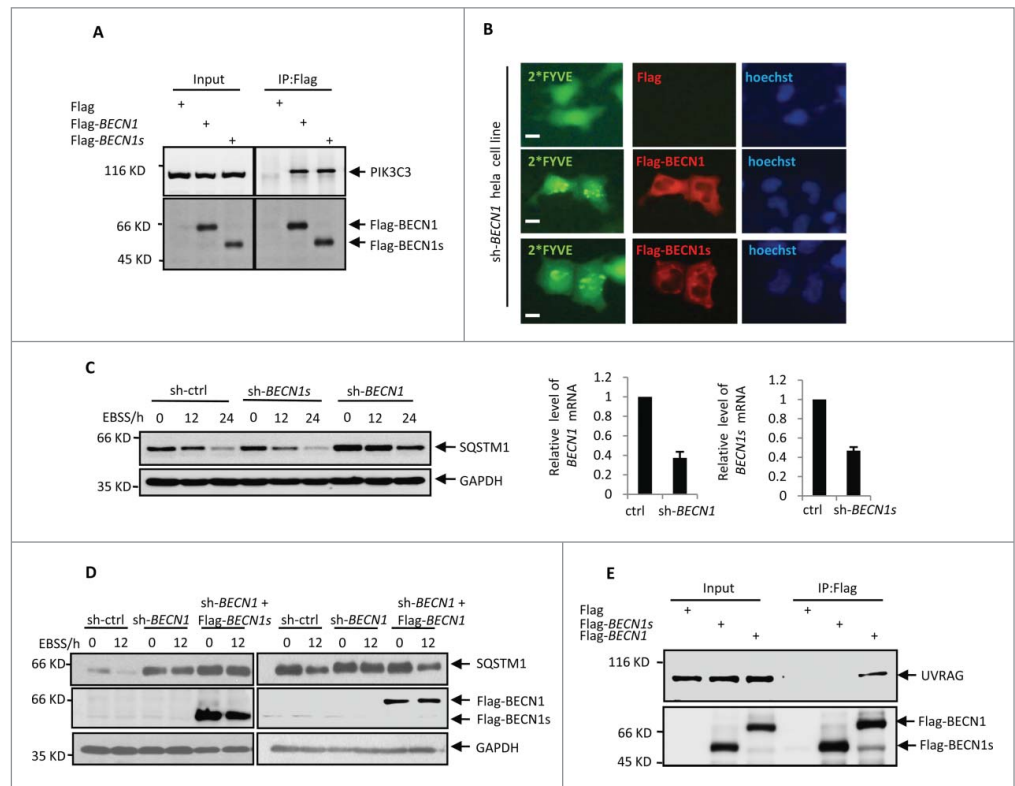


Figure 2. BECN1s is not involved in macroautophagy initiation. (A) HEK 293T cells were transfected with control vector, Flag-BECN1, or Flag-BECN1s as indicated. Twenty-four h later, cell lysates were immunoprecipitated with anti-Flag antibody, followed by western blot analysis with anti-PIK3C3 antibody. (B) HeLa cells with BECN1 stable knockdown were transfected with GFP-2*FYVE plus either control vector, Flag-BECN1, or Flag-BECN1s as indicated. Twenty-four h after transfection, cells were immunostained with anti-Flag antibody. The images were taken under a fluorescence microscope. Scale bar: 20 μm. (C) HeLa cells expressing control shRNA, *BECN1* shRNA, or *BECN1s* shRNA were treated with EBSS for the indicated periods of time. Cell lysates were then analyzed by western blot with anti-SQSTM1 and anti-GAPDH antibodies. The shRNA-mediated knockdown efficiency for *BECN1* and *BECN1s* was also shown. (D) HeLa cells expressing the indicated shRNAs and proteins were treated with EBSS for the indicated periods of time. Cell lysates were then analyzed by western blot with anti-SQSTM1, anti-Flag and anti-GAPDH antibodies. (E) HEK 293T cells were transfected with either control vector, Flag-BECN1 or Flag-BECN1s as indicated. Twenty-four h after transfection, cell lysates were immunoprecipitated with anti-Flag antibody, followed by western blot analysis with anti-UVRAG and anti-Flag antibodies.

formation in BECN1 knockdown HeLa cells (Fig. 2B). These combined data indicate that BECN1s still retains the ability to interact with PIK3C3 and regulate its activity.

To assess whether BECN1s is involved in starvation-induced autophagy, HeLa cells expressing either *BECN1* shRNA or *BECN1s* shRNA were treated with or without EBSS. Consistent with the previous reports,³⁰ BECN1 knockdown markedly impaired the EBSS-induced reduction of the autophagy marker

protein SQSTM1/p62 levels (Fig. 2C), reinforcing the critical role of BECN1 in the regulation of autophagy. However, the EBSS-initiated autophagy was not inhibited by BECN1s knockdown (Fig. 2C). In addition, unlike BECN1, BECN1s failed to restore autophagy under EBSS treatment condition when endogenous BECN1 was knocked down (Fig. 2D). These data suggest that BECN1s is not essential for starvation-initiated macroautophagy.

Many proteins participate in BECN1-mediated autophagy through interacting with BECN1-PtdIns3K complex. Among these proteins, UVRAG is an essential component of the BECN1-PtdIns3K lipid kinase complex and is an important signaling checkpoint for autophagy.^{31,32} We therefore evaluated the binding ability of BECN1s to UVRAG. Correlated with its function, which cannot induce nonselective autophagy, BECN1s did not interact with UVRAG (Fig. 2E). We also checked whether BECN1s could interact with other BECN1 regulator proteins such as ATG14 and BCL2. The results showed that similar to BECN1, BECN1s was also able to associate with ATG14 and BCL2 (Fig. S2). These results suggest that loss function of BECN1s in mediating starvation-induced autophagy may be due to the compromised ability of BECN1s to interact with UVRAG.

BECN1s induces mitochondrial autophagy

Since BECN1s was mainly associated with mitochondria, we asked whether BECN1s regulates selective mitochondrial autophagy (mitophagy). The constructs encoding either BECN1 or BECN1s were transfected into HeLa cells. With the increasing expression of BECN1s but not BECN1, levels of the mitochondrial protein TOMM20 were concurrently decreased (Fig. 3A). In addition, ectopic expression of BECN1s also led to the decreased expression of other mitochondrial proteins, such as CYCS

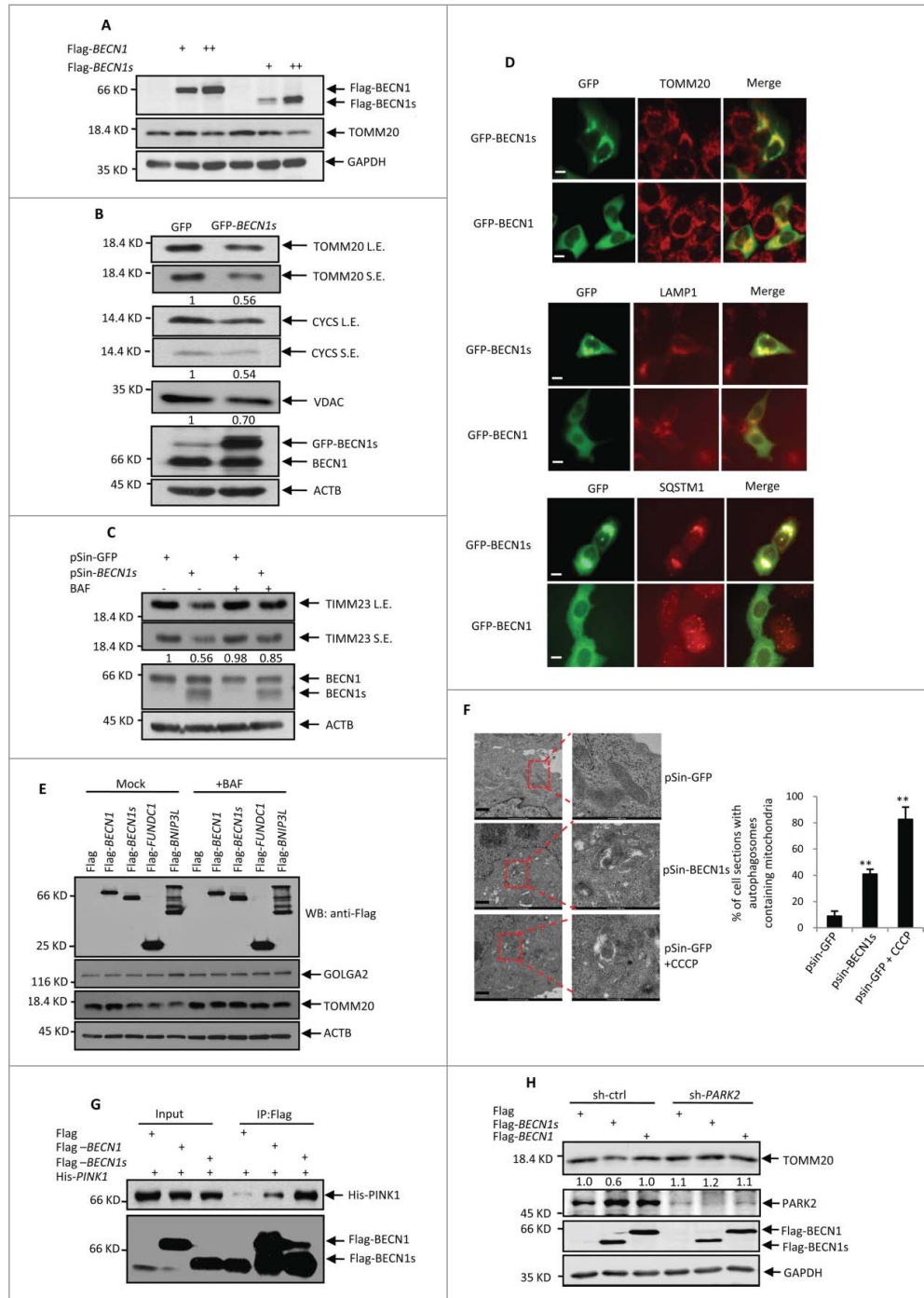


Figure 3. For figure legend, see page 2052.

(cytochrome c, somatic) and VDAC1 (Fig. 3B). To verify whether mitophagy plays a key role in BECN1s-mediated reduced expression of the mitochondria proteins, an inhibitor of autophagic vacuole maturation bafilomycin A₁ (BAF) was utilized. When autophagy was blocked by BAF, BECN1s-mediated decreased expression of the mitochondrial protein TIMM23 was greatly recovered (Fig. 3C), indicating that BECN1s induces mitophagy. In supporting of this, the immunofluorescence analysis showed that BECN1s was well colocalized with mitochondrial protein TOMM20, lysosomal-associated protein LAMP1 and autophagy-associated protein SQSTM1 (Fig. 3D). Like other well-known mitophagy-induced proteins such as BNIP3L and FUNDC1, BECN1s was able to reduce levels of the TOMM20 protein but not levels of endoplasmic reticulum protein GOLGA2. Again, BECN1s-induced reduction of TOMM20 levels can be reversed by BAF (Fig. 3E). To further prove that BECN1s induces mitophagy, we performed an electron microscopy assay. In this assay, carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used as a positive control. CCCP is a mitochondrial uncoupler, which can trigger mitophagy by severely reducing the mitochondrial membrane potential.³³ As was expected, treatment with CCCP dramatically increased the formation of the mitochondria-containing autophagic vesicles in HeLa cells (Fig. 3F). Intriguingly, ectopic expression of BECN1s also enhanced the mitochondria-containing autophagic vesicle formation (Fig. 3F). Taken together, these findings demonstrate that BECN1s specifically induces mitophagy.

It has been well recognized that the PINK1-PARK2 pathway is important for mitophagy initiation. We therefore sought to explore whether BECN1s induces mitophagy through the PINK1-PARK2 pathway. The immunoprecipitation analysis revealed that BECN1s is able to strongly interact with PINK1 (Fig. 3G), but not other mitophagy related proteins HSP90, FUNDC1 and BNIP3L (Fig. S3), indicating that BECN1s may work in concert with PINK1 to regulate mitophagy. We also showed PARK2 knockdown was able to reverse the inhibitory effect of BECN1s on TOMM20 protein levels (Fig. 3H),

indicating that BECN1s-induced mitophagy is PARK2-dependent.

BECN1s plays an important role in EBSS- and CCCP-induced mitophagy

To determine the physiological significance of BECN1s-induced mitophagy, we first investigated whether BECN1s is essential for starvation-initiated mitophagy. HeLa cells expressing control shRNA, *BECN1* shRNA or *BECN1s* shRNA were treated with EBSS, followed by western blot analysis to detect the mitochondria protein levels. In control cells, EBSS treatment dramatically decreased levels of the mitochondrial proteins TOMM20, cytochrome c and MFN1 (Fig. 4A). Knockdown of BECN1 failed to repress this decrease of mitochondrial proteins expression (Fig. 4A and S4A), indicating that BECN1 is dispensable for starvation-induced mitophagy. Nevertheless, knockdown of BECN1s greatly reversed EBSS-decreased mitochondrial proteins expression (Fig. 4A and S4A). Similar results were also obtained in U2OS cells (Fig. S4B). In line with these findings, treatment with *BECN1s* shRNA but not *BECN1* shRNA diminished the autophagic vesicles containing mitochondria in HCT116 cells (Fig. 4B). In addition, treatment with BAF reversed EBSS-reduced TOMM20 expression in control cells but not in BECN1s knockdown cells (Fig. 4C and S4C). Also, knockdown of BECN1s did not regulate the effect of EBSS on SQSTM1 and LC3 protein levels (Fig. 4C, and S4C and 4D). These data suggest that BECN1s is essential for EBSS-induced mitophagy.

We next evaluated whether BECN1s plays a pivotal role in mitochondrial depolarization-induced mitophagy. HCT116 cells expressing control shRNA, *BECN1* shRNA or *BECN1s* shRNA were treated with CCCP, followed by western blot and immunofluorescence analyses to examine TOMM20 expression. The results showed that BECN1s knockdown but not BECN1 knockdown recovered CCCP-decreased expression of TOMM20 (Fig. 4D to E and S4E). As a positive control, BNIP3L knockdown also dramatically inhibited CCCP-induced mitophagy (Fig. 4F). More importantly, ectopic expression of BECN1s was

Figure 3 (See previous page). BECN1s induces mitophagy. (A) HeLa cells were transfected with the increasing amounts of constructs encoding either Flag-*BECN1* or Flag-*BECN1s*. Twenty-four h after transfection, cell lysates were analyzed by western blot with anti-Flag, anti-TOMM20 and anti-GAPDH antibodies. (B) HeLa cells were transfected with either GFP control vector or GFP-*BECN1*. Twenty-four h after transfection, cell lysates were analyzed by western blot with the indicated antibodies. The blots were also qualified by using Gel-Pro analyzer software (Rockville, MD, USA). The value of each band indicates the relative expression levels of the indicated protein after normalizing to the loading control ACTIN. L.E. and S.E. indicate long time exposure and short time exposure, respectively. (C) HeLa cells were infected with lentiviruses expressing GFP or BECN1s. Forty-eight h after infection, cells were treated with or without BAF for another 12 h. Cell lysates were then analyzed by western blot with anti-TIMM23, anti-BECN1 and anti-ACTIN antibodies. The blot was also qualified by using Gel-Pro analyzer software (Rockville, MD, USA). The value of each band indicates the relative expression levels of TIMM23 after normalizing to the loading control ACTIN. L.E. and S.E. indicate long time exposure and short time exposure, respectively. (D) HeLa cells were transfected with either GFP control vector or GFP-*BECN1s*. Twenty-four h after transfection, cells were immunostained with anti-TOMM20, anti-LAMP1 and anti-SQSTM1 antibodies, respectively. Scale bar: 20 μm. (E) HeLa cells were transfected with the constructs encoding Flag-*BECN1*, Flag-*BECN1s*, Flag-BNIP3L and Flag-FUNDC1 as indicated. Twenty-four h after transfection, cells were treated with or without BAF for another 12 h. Cell lysates were analyzed by western blot with the indicated antibodies. (F) Cells expressing GFP or BECN1s were treated with or without Carbonyl cyanide m-chlorophenylhydrazone (CCCP) for 12 h. Cells were then subjected to electron microscopy analysis. The shown images are representatives of 3 independent experiments. Scale bar: 1 μm. The percentage of cells with autophagosomes containing mitochondria is also shown as mean±SD from 3 independent experiments. n>28 cells per experiments. **, P<0.01. (G) HEK 293T Cells were transfected with Flag-*BECN1*, Flag-*BECN1s* and His-PINK1 in the indicated combinations. Twenty-four h after transfection, cell lysates were immunoprecipitated with anti-Flag antibody, followed by western blot analysis with anti-His antibody. (H) SH-SY5Y cells expressing control shRNA or *PARK2* shRNA were transfected with Flag-*BECN1* or Flag-*BECN1s*. Forty-eight h after transfection, cell lysates were analyzed by western blot with anti-TOMM20 antibody. The blot was also qualified by using Gel-Pro analyzer software (Rockville, MD, USA). The value of each band indicates the relative expression levels of TOMM20 after normalizing to the loading control GAPDH.

able to restore the ability of CCCP to reduce TOMM20 levels in BECN1s knockdown HCT116 cells (Fig. 4G). Collectively, these results suggest that BECN1s is also indispensable for depolarization-induced mitophagy.

Discussion

Previous studies have discovered that many regulators participate in processes of mitophagy induced by different stress conditions, such as BNIP3L,^{18,34,35} PINK1,^{14,15,21,23} and FUNDC1.³⁶

In this study, we provide evidence demonstrating that BECN1s, a novel BECN1 splice variant, plays an important role in mitophagy induced by starvation and mitochondrial depolarization.

Compared to BECN1, BECN1s loses the function that initiates macroautophagy. This loss of function may be due to the inability of BECN1s to interact with autophagy regulating protein, such as UVRAG. Although BECN1s is not essential for macroautophagy initiation, BECN1s is able to specifically induce mitochondria-selective autophagy. Also, BECN1s retains the ability to activate PtdIns3K, which is important for initiation of autophagy,³⁷ despite the lack of a partial ECD and C-terminal

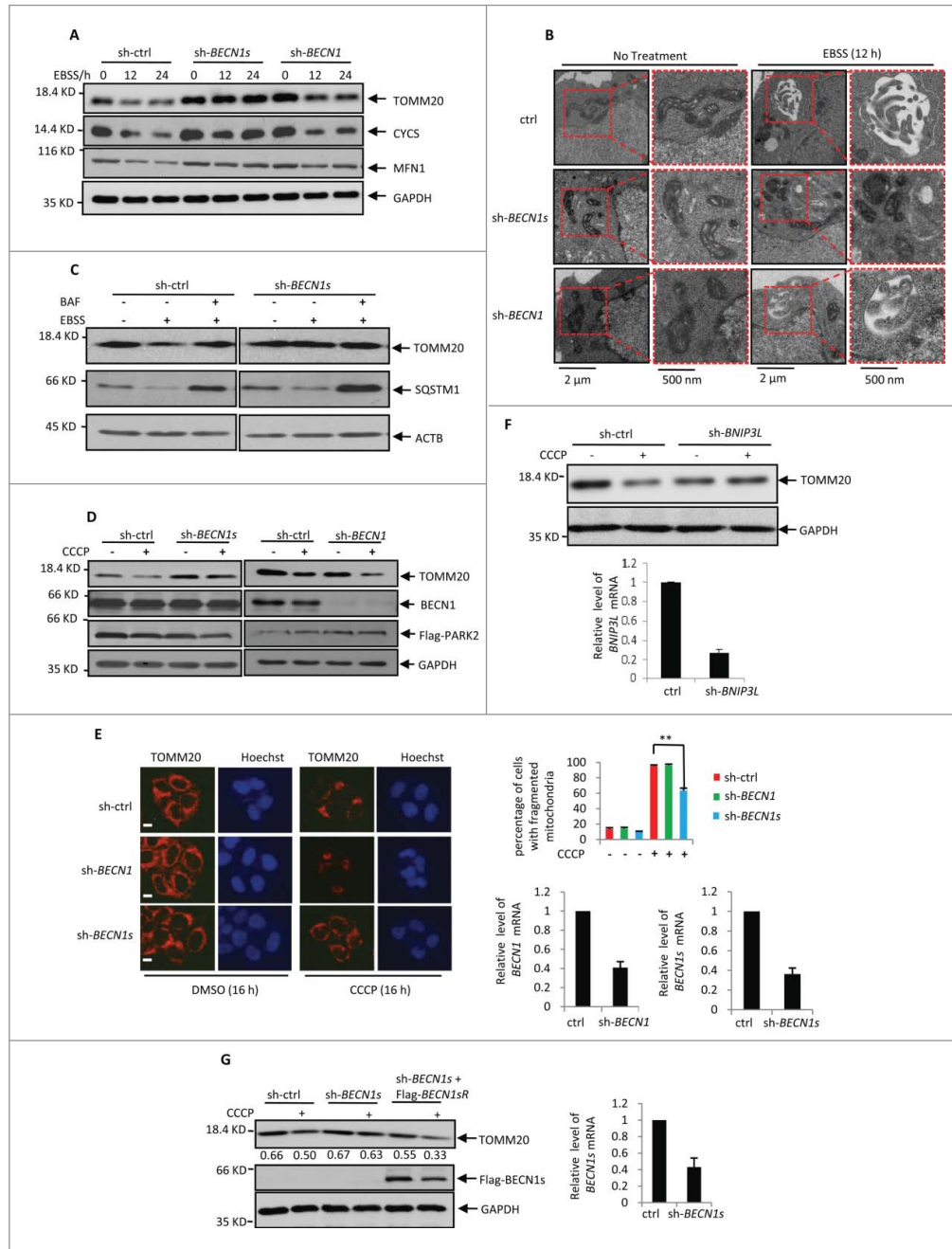


Figure 4. For figure legend, see page 2054.

would be important to address how cellular BECN1s expression is controlled, whether BECN1 and BECN1s expression needs to be balanced under physiological conditions, and whether BECN1s dysregulation is associated with any human diseases. In summary, our findings suggest that BECN1s is a novel mitophagy regulator, and open a new avenue for the study of BECN1-mediated autophagy where the splicing event of BECN1 should definitely be considered.

Materials and Methods

Cell culture and reagents

HeLa, U2OS, HCT116 H1299, MCF7, SH-SY5Y and HEK 293T cell lines were cultured in DMEM (Dulbecco's modified Eagle's medium) medium containing 10% fetal bovine serum. Antibodies against GAPDH (sc-365062), OPA1 (sc-393296), MFN1 (sc-166644), TOMM20 (sc-17764), TIMM23 (sc-514463), VDAC1 (sc-390996), LC3 (sc-134226) and SQSTM1/p62 (sc-28359) were purchased from Santa Cruz Biotechnology. Antibodies against BECN1 (ab92389) and PIK3C3 (ab124905) were obtained from Abcam. Anti-ACTB/ β -ACTIN (A5441) and anti-Flag (F1804) antibodies were ordered from Sigma Aldrich. Anti-GFP (632381) antibody was ordered from Clontech. Proteinase K (P2308), bafilomycin A₁/BAF (B1793), and CCCP (C2759) were purchased from Sigma.

Knockdown of BECN1 and BECN1s

To knock down BECN1 and BECN1s, shRNAs against *BECN1* and *BECN1s* were cloned into a pLKO.1 vector (Addgene, 8453). The target sequences are 5'-GAG GTT GAG AAA GGC GAG A-3' (*BECN1*) and 5'-TTT CAG AGG ATG GAT GTG G-3' (*BECN1s*). shRNA-mediated knockdown of BECN1 or BECN1s was performed as previously described.⁴⁰

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol (Invitrogen, 15596-02), followed by RT-PCR analysis by using a PrimeScriptTM RT-PCR Kit (TakaRa, Dalian, China) according to the manufacturer's instruction. The PCR primer sequences for RT-PCR are as follows: 5'- AAT GCA ACC TTC CAC ATC TGG and

5'- TTT CTC CAC ATC CAT CCT CTG (*BECN1s*); 5'-TTT AAT GCA ACC TTC C (P1) and 5'-TCA TTT GTT ATA AAA TTG TGA GG (P2).

Cytosolic and mitochondrial subcellular fractionation

Cytosolic and mitochondrial subcellular fractionation was performed as previously described.⁴ Briefly, cells were homogenized in homogenization buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose [BBI, SB0498] and protease inhibitor cocktail [Roche, 04693132001]). Membrane rupture of cells was verified by microscopy. The homogenates were subjected to centrifugation at 600×g for 5 min at 4°C. The resulting low-speed pellet fractions, which contained nuclei and unbroken cells, were discarded. The postnuclear supernatant fraction was further centrifuged at 7000×g for 10 min at 4°C to obtain cytoplasmic and mitochondrial fractions. The mitochondrial pellet was washed and solubilized in TNC buffer (10 mM Tris acetate, pH 8.0, 0.5% Nonidet P-40 [Sangon, NDB0385], 5 mM CaCl₂ and protease inhibitor cocktail).

Overexpression of BECN1s in HeLa cells

To generate lentiviruses expressing BECN1s, HEK293T cells grown on a 6-cm dish were transfected with 2 μ g of pSin-BECN1s construct, 1 μ g of pmd2.g and 2 μ g of pspax2. Twelve h after transfection, cells were cultured with DMEM medium containing 20% fetal bovine serum for an additional 24 h. The culture medium containing lentivirus particles was filtered through a 0.45-mm polyvinylidene difluoride filter and incubated with HeLa cells for 12 h, followed by selection with 5 μ g/ml puromycin for another 24 h.

Measurement of PIK3C3 lipid phosphorylation activity

To detect the lipid phosphorylation activity of PIK3C3, HeLa cells were transfected with GFP-2*FYVE construct (a kind gift from Dr. Harald Stenmark, The Norwegian Radium Hospital),⁴¹ which encodes GFP-tagged double FYVE fingers of HGS/Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate). The GFP-2*FYVE fingers bind specifically to the PIK3C3 product,

Figure 4 (See previous page). EBSS- and CCCP-induced mitophagy is BECN1s-dependent. **(A)** HeLa cells expressing control shRNA, *BECN1* shRNA or *BECN1s* shRNA were treated with EBSS for the indicated periods of time. Cell lysates were analyzed by western blot with the indicated antibodies. The shRNA-mediated knockdown efficiency for *BECN1* and *BECN1s* was shown in Fig. S4A. **(B)** HCT116 cells expressing the indicated shRNAs were incubated under normal or starvation conditions for 12 h, followed by electron microscopy analysis. **(C)** HCT116 cells transfected with indicated shRNA were cultured in normal growth medium or treated with EBSS in the absence or presence of BAF. Cell lysates were analyzed by western blot with indicated antibodies. The shRNA-mediated knockdown efficiency for *BECN1s* was shown in Fig. S4C. **(D)** HCT116 cells were transfected with Flag-*PARK2* plus the indicated shRNAs. Twenty-four h after transfection, cells were treated with 10 μ M CCCP for another 16 h. Cell lysates were analyzed by western blot with anti-TOMM20, anti-BECN1 and anti-GAPDH antibodies. The shRNA-mediated knockdown efficiency for *BECN1* and *BECN1s* was shown in Fig. S4E. **(E)** HeLa cells expressing the indicated shRNAs were treated with 10 μ M CCCP for 16 h. Cells were then immunostained with anti-TOMM20 antibody. Scale bar: 20 μ m. The percentage of cells with fragmented mitochondria was calculated and shown accordingly. **, $P < 0.01$. The shRNA-mediated knockdown efficiency for *BECN1* and *BECN1s* was also shown. **(F)** HCT116 cells expressing either control shRNA or *BNIP3L* shRNA were treated with or without 10 μ M CCCP for 16 h. Cell lysates were then analyzed by western blot with anti-TOMM20 antibody. The efficient knockdown of *BNIP3L* was also confirmed by real-time RT-PCR analysis. **(G)** Lysates from HCT116 cells expressing the indicated shRNAs and proteins were treated with 10 μ M CCCP for 16 h. Cell lysates were analyzed by western blot with antibodies against TOMM20, Flag and GAPDH. The blot was quantified using Gel-Pro analyzer software (Rockville, MD, USA). The value of each band indicates the relative expression levels of TOMM20 after normalizing to the loading control GAPDH. The shRNA-mediated knockdown efficiency for *BECN1s* was also shown.

phosphatidylinositol-3-phosphate, and form the puncta. Twenty-four h after transfection, the GFP-2*FYVE puncta were observed under a fluorescence microscope.

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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