

A small-molecule enhancer of autophagy decreases levels of A β and APP-CTF *via* Atg5-dependent autophagy pathway

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ABSTRACT The hallmarks of Alzheimer's disease are the aggregates of amyloid- β (A β) peptide and tau protein. Autophagy is one major cellular pathway leading to the removal of aggregated proteins. We examined the possibility of inducing autophagy to reduce A β peptide and the amyloid precursor protein (APP)-derived fragment APP-CTF levels in cell lines and primary neuronal cultures. We found that induction of autophagy either by small-molecule enhancers of rapamycin (SMER)28, a small-molecule enhancer of autophagy, or following starvation greatly decreased the levels of A β peptide (apparent EC₅₀ of ~10 μ M) and APP-CTF (apparent EC₅₀ of ~20 μ M) in a γ -secretase-independent manner. Pharmacological inhibition of autophagy led to a significant accumulation of A β peptide and APP-CTF and diminished the effect of SMER28. Three essential components of the autophagic pathway, autophagy-related protein (Atg)5, Beclin1, and Ulk1, were shown to be involved in the degradation of A β and APP-CTF, and Atg5 was necessary for the effect of SMER28. In addition, the autophagic marker light chain 3-II cocompartmentalized with APP-CTF. These results support the involvement of autophagy in the clearance of A β and APP-CTF. We therefore propose that small molecule enhancers of autophagy, such as SMER28, may have therapeutic potential for the treatment of Alzheimer's disease and other proteinopathies.—Tian, Y., Bustos, V., Flajolet, M., Greengard, P. A small-molecule enhancer of autophagy decreases levels of A β and APP-CTF *via* Atg5-dependent autophagy pathway. *FASEB J.* 25, 000–000 (2011). www.fasebj.org

Key Words: Alzheimer's disease • SMER28 • Beclin1 • Ulk1

ALZHEIMER'S DISEASE (AD) is the most common neurodegenerative disorder. The hallmarks of the disease are the tau tangles and amyloid- β (A β) plaques. A β is generated *via* sequential proteolysis of amyloid precursor protein (APP; refs. 1, 2). APP can be cleaved by β -secretase to produce a C-terminal fragment (β CTF), which is then further processed by γ -secretase to release A β peptides. Alternatively, APP can be successively cleaved by α -secretase and then γ -secretase to produce α CTF and P3 peptides. Although altered proteolytic

processing of APP plays a central role in the production and accumulation of A β , failure of A β clearance can contribute to the pathogenesis in sporadic AD (3). It has been reported that A β accumulates within autophagic vacuoles in swollen dystrophic neurites in human AD brain, suggesting the involvement of autophagy in AD pathogenesis (4).

Macroautophagy, hereafter referred to as autophagy, is the major cellular pathway for degradation of long-lived and aggregated proteins, as well as cytoplasmic organelles (5, 6). Since most aggregate-prone proteins are high-molecular-weight complexes and they are too large to enter the narrow pore of the proteasome barrel, such proteins or complexes can only be cleared by the autophagy pathway (7). Morphologically, autophagy is initiated when a cup-shaped "isolation" membrane (a phagophore) is formed. The membrane of phagophore undergoes elongation and sequesters cytosolic components and organelles into a double membrane-bound autophagic vacuole or autophagosome (8–10). Subsequently, autophagosomes fuse with lysosomes for content degradation (11).

Autophagy can be induced under physiological stress, such as starvation. Indeed, under nutrient-limiting conditions, the activity of the mammalian target of rapamycin (mTOR) kinase, a central sensor of nutrient signal, is inhibited. The inhibition of mTOR leads to dephosphorylation of autophagy-related protein 13 (Atg13) and Ulk1, resulting in the activation of the Ulk1-Atg13-FIP200 complex to trigger autophagy (12). During initiation of autophagosome formation, Vps34, a class III PI3K, can recruit other Atg proteins to form an autophagy-regulating macromolecular complex (13–15). This complex, together with the Ulk1-Atg13-FIP200 complex, plays an important role in the initiation of autophagosome formation. Furthermore, the activity of Vps34 is enhanced by binding to Beclin1

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(7, 14). Two ubiquitin-like conjugation reactions are essential for the elongation process of the phagophore membrane. These reactions consist of the conjugation of Atg12 to Atg5, which requires Atg7 as E1 and Atg10 as E2, and the conjugation of LC3 to phosphatidylethanolamine to form LC3-II, sharing the same E1 (Atg7) but a different E2 (Atg3) (16). Once the autophagosomes form, they fuse with lysosomes for content degradation. However, the mechanism of autophagosome-lysosome fusion in mammalian autophagy is not clear. In addition to mTOR-dependent autophagy, mTOR-independent autophagy was discovered when autophagy was found to be induced by lowering intracellular inositol or inositol 1,4,5-trisphosphate levels independently of mTOR (17).

Age-dependent decrease in autophagy was suggested to be responsible for the accumulation of abnormal proteins during aging (18). Impairment of the autophagy pathway is suggested to be involved in neurodegeneration and a variety of neurodegenerative diseases, including Parkinson disease, Huntington disease, and AD (7, 19–21). Enhancing autophagy may be a possible therapeutic strategy for neurodegenerative disorders. Therefore, considerable effort has been made to identify autophagy-inducing molecules (7, 22). Small-molecule enhancers of rapamycin (SMERs), identified by chemical screens, were discovered to induce mTOR-independent autophagy and reduce mutant huntingtin aggregates and A53T α -synuclein in Huntington and Parkinson disease cellular models (23). Nevertheless, it is not known if those compounds are able to remove aggregated proteins in other neurodegenerative diseases, such as AD.

It was recently reported that the Beclin1 complex regulates APP processing and plays an important role in AD pathology (24). In our study, we have demonstrated that basal A β and APP-CTF can be degraded by autophagy and that the autophagic proteins Atg5, Beclin1, and Ulk1 are all involved in the process. SMER28, a small-molecule enhancer of autophagy, can promote the Atg5-dependent degradation of A β and APP-CTF. Therefore, we propose that small molecule autophagy enhancers, such as SMER28, have significant therapeutic potential for the treatment of AD and possibly other proteinopathies.

MATERIALS AND METHODS

Reagents

The following antibodies were used at 1:1,000 dilutions: RU-369, a rabbit polyclonal antibody that recognizes the C-terminal of APP695 (25); Ab14 antiserum targeting residues 1–25 of presenilin 1 (PS1)-NTF (26); 6E10 antibody against A β 1–16 (Convance, Princeton, NJ, USA); anti-LC3 (Sigma, St. Louis, MO, USA); anti-APLP1 (Calbiochem, Gibbstown, NJ, USA); anti-Beclin1 (BD Biosciences, San Jose, CA, USA); anti-PS1-CTF (Millipore, Billerica, MA, USA); anti- γ -Adaptin (BD Biosciences); and anti-Bip (Abcam, Cambridge, MA, USA). Compound SMER28 was purchased from

EMD Chemicals (Gibbstown, NJ, USA). LDH assay kit was purchased from Roche (Nutley, NJ, USA).

Cell culture and siRNA

Mouse embryonic fibroblast (MEF) cells generated from WT and Atg5^{-/-} embryos (5) were obtained from the RIKEN BRC cell bank (Tsukuba, Ibaraki, Japan) and maintained in DMEM with 10% FBS. Starvation was carried out in DMEM without amino acids and FBS for 2 h. Mouse neuroblastoma (N2a) cells were maintained in medium containing 50% DMEM and 50% Opti-MEM, supplemented with 5% FBS (Invitrogen, Carlsbad, CA, USA). The siRNAs for Beclin1 and Ulk1 were purchased from Dharmacon (Lafayette, CO, USA; On-TARGETplus set of 4 siRNAs, J-055895-05; On-TARGETplus SMARTpool, L-040155-00-0005). The control siRNA was purchased from Dharmacon (On-TARGET plus GAPD Control siRNA, D-001830-02-05).

Primary neuronal cultures

Cerebral cortices or hippocampi were dissected from embryonic day 18 (E18) rat embryos. Cells were dissociated with trypsin and grown in Neurobasal medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with B27, N2, and 0.5 mM glutamine (Gibco-BRL). Cortical neurons were plated at a density of $8 \times 10^4/\text{cm}^2$ on 24-well cell culture plates coated with poly-L-lysine (Sigma).

Immunofluorescence and confocal microscopy

Cells were grown in 4-well slide chambers (Lab Tek; Nalge Nunc, Rochester, NY, USA) and fixed with 4% paraformaldehyde. Cells were then permeabilized in 0.1% Triton X-100 and stained with primary antibodies, followed by FITC-conjugated secondary antibodies. The coverslips were mounted by Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA). The images were acquired using a confocal microscope (LSM510 META; Carl Zeiss MicroImaging, Thornwood, NY, USA) and the LSM5 3.2 software.

Subcellular fractionation

For sucrose density gradient fractionation, cells were prepared as described previously (27, 28). Briefly, cells were homogenized by using a stainless steel ball-bearing homogenizer in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM MgAc₂, and a protease inhibitor cocktail. The homogenate was loaded on top of a step gradient comprised of 1 ml of 2 M sucrose, 4 ml of 1.3 M sucrose, 3.5 ml of 1.16 M sucrose, and 2.0 ml of 0.8 M sucrose. The gradients were centrifuged for 2.5 h at 390,000 *g* in a Beckman SW41 Ti rotor (Beckman Instruments, Fullerton, CA, USA). Fractions (1 ml) were collected from the top of each gradient and assayed by Western blot using γ -adaptin, BIP, LC3, Rab9, and Ru-369 antibodies.

RESULTS

SMER28 induces a marked decrease in A β and APP-CTF levels

Previous reports (7, 29) described the identification of small-molecule compounds capable of inducing autophagy. Based on safety profiles and therapeutic potentials,

we tested 4 of those compounds, pimozone, fluspirilene, SMER28, and spermidine, for their ability to reduce A β 40 peptide and APP- β CTF levels in cultured cells overexpressing β CTF. SMER28 induced a significant decrease in secreted A β 40 peptide, and it had a greater effect on reducing APP- β CTF levels (Fig. 1A). For these reasons, and the absence of cytotoxicity under the conditions tested, we chose to focus our efforts on SMER28. N2a cells stably expressing APP-695 (N2a-APP) were treated with various concentrations of SMER28 for 16 h (Fig. 1B) or with a fixed concentration (50 μ M) for various periods of time (Fig. 1C). SMER28 induced both a dose- and time-dependent reduction of APP-CTF (Fig. 1B, C) with apparent EC₅₀ of \sim 20 μ M. With the concentrations of SMER28 tested, no cytotoxicity by LDH assay was observed (Fig. 1D). N2a cells stably expressing β CTF were also treated with various concentrations of SMER28 for 16 h. The levels of β CTF, detected using the 6E10 antibody, were decreased by SMER28 in a dose-dependent manner (Supplemental Fig. S1). We next tested the effect of SMER28 on primary neuronal cultures. Mixed cultures of cortical-hippocampal neurons were prepared from rat E18 embryos, cultured for 8 d, and exposed to SMER28 for 16 h. The secreted conditioned media were assayed for A β 40 and A β 42 by ELISA, and cell lysates were

assayed for APP-CTF by Western blotting. SMER28 induced a significant decrease of secreted A β 40 and A β 42 (EC₅₀ \sim 10 μ M) and of endogenous APP-CTF (EC₅₀ of \sim 20 μ M) in a dose-dependent manner in the primary neuronal cultures (Fig. 1E, F).

SMER28 and starvation enhance the degradation of A β and APP-CTF *via* autophagy

We next investigated whether autophagy was involved in the mechanism by which SMER28 induces down-regulation of A β 40/A β 42 and APP-CTF. To this end, N2a-APP cells were treated with SMER28 for 6 h or starved (medium depleted in amino acids and serum) for 2 h. The increase in LC3-II, a classical autophagy marker, in the SMER28- and starvation-treated samples indicated the occurrence of autophagy (Fig. 2B). As with SMER28, starvation induced a marked decrease of both A β 40/A β 42 and APP-CTF levels but only slightly affected full-length APP expression level (Fig. 2A, B). APLP1 and Notch are two other proteins that are targeted by γ -secretase and undergo proteolysis. Therefore, we tested the specificity of the effects by investigating whether APLP1 and Notch signaling are regu-

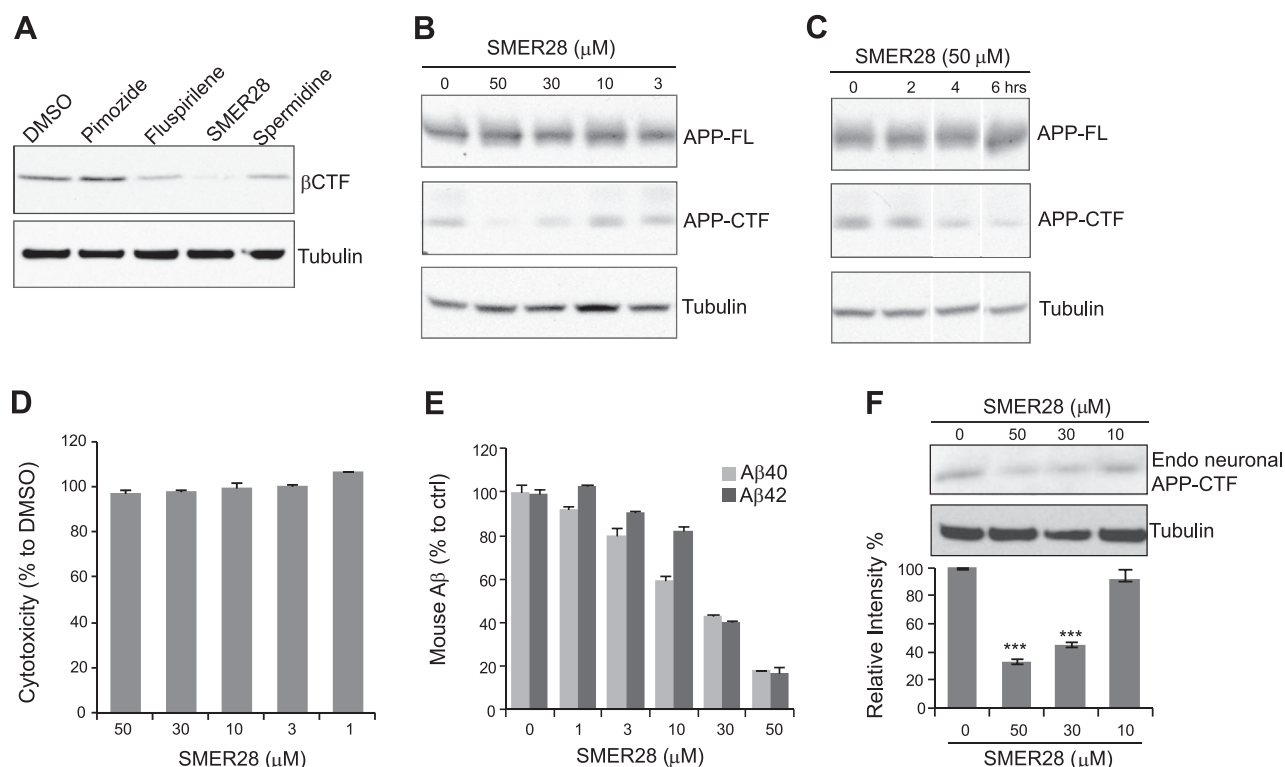


Figure 1. Effect of SMER28 on levels of A β peptide and APP-CTF. A) MEF cells overexpressing β CTF were treated with autophagy-enhancing compounds (10 μ M) for 16 h. Cell lysates were analyzed by SDS-PAGE and Western blotting using anti- β CTF (6E10) and anti-tubulin antibodies. B, C) N2a-APP cells were treated with increasing concentrations of SMER28 for 16 h (B) or with a fixed concentration of SMER28 (50 μ M) for various periods of time (C). Cell lysates were analyzed by SDS-PAGE and Western blotting for the presence of APP-FL and APP-CTF using RU-369 antibody. D) N2a-APP cells were treated with various concentrations of SMER28 for 16 h, and the cells were analyzed using an LDH assay (Roche) according to the manufacturer's protocol. E) Rat primary neuronal cultures were treated with various concentrations of SMER28 for 16 h. Conditioned media were analyzed for the presence of soluble A β 40 and A β 42 peptides by ELISA. F) Top panels: cell lysates were analyzed by SDS-PAGE and Western blotting for endogenous APP-CTF. Bottom panel: quantification of APP-CTF ($n \geq 3$). Error bars = SE. *** $P < 0.001$.

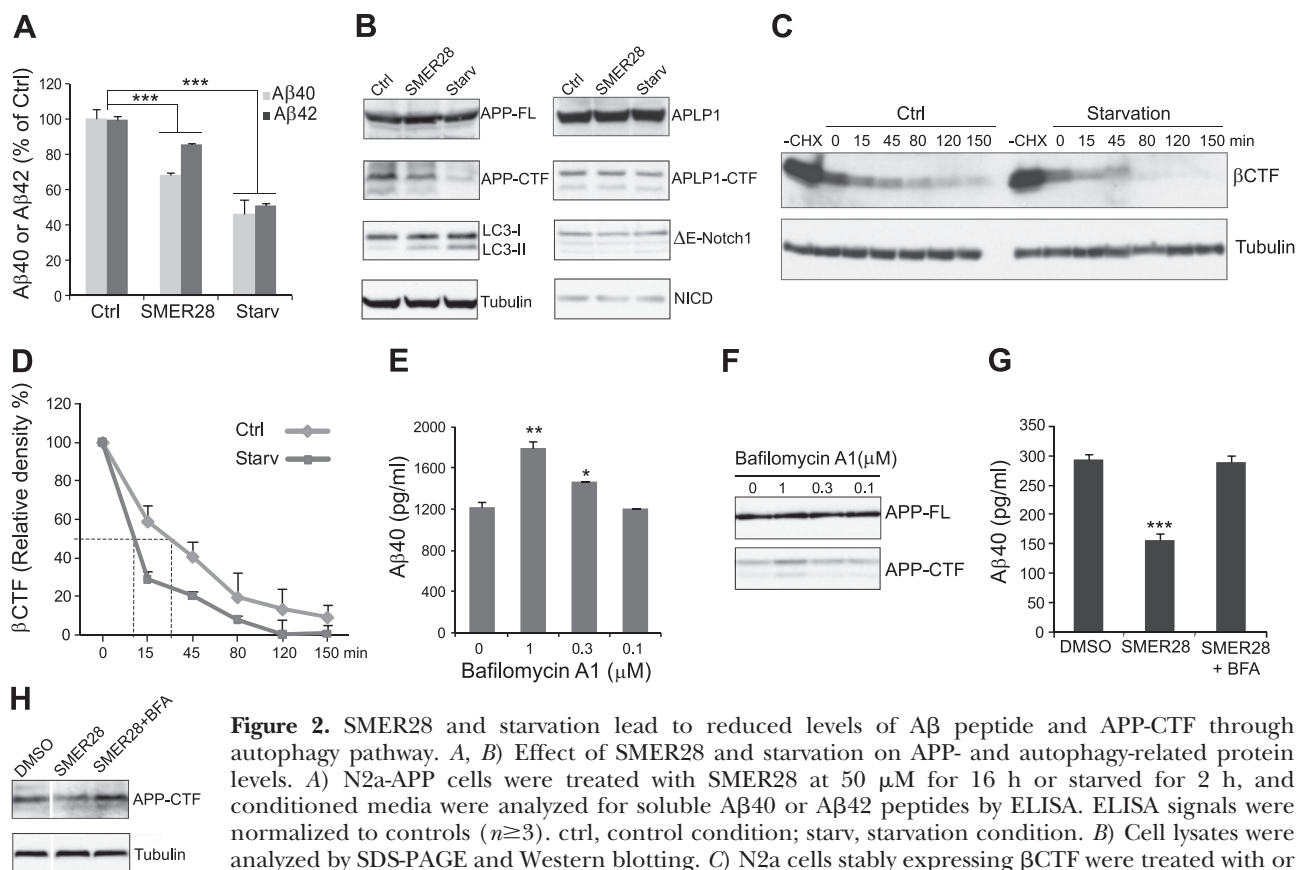


Figure 2. SMER28 and starvation lead to reduced levels of A β peptide and APP-CTF through autophagy pathway. *A, B*) Effect of SMER28 and starvation on APP- and autophagy-related protein levels. *A*) N2a-APP cells were treated with SMER28 at 50 μ M for 16 h or starved for 2 h, and conditioned media were analyzed for soluble A β 40 or A β 42 peptides by ELISA. ELISA signals were normalized to controls ($n \geq 3$). ctrl, control condition; starv, starvation condition. *B*) Cell lysates were analyzed by SDS-PAGE and Western blotting. *C*) N2a cells stably expressing β CTF were treated with or without cycloheximide (CHX; 50 μ g/ml) for 1 h in the presence of γ -secretase inhibitor L-685,458 (1 μ M). CHX-treated cells were then starved for different amounts of time (as indicated), and cell lysates were analyzed by SDS-PAGE and Western blotting for β CTF using 6E10 antibody. *D*) Kinetics of the disappearance of β CTF in control and starved cells. Dashed lines indicate half-time for disappearance. *E, F*) Effect of bafilomycin A (BFA), a blocker of the fusion of autophagosomes to lysosomes, on A β 40 peptide (*E*) and APP-CTF levels (*F*) in N2a-APP cells ($n \geq 3$). *G, H*) BFA (0.1 μ M) blocked the effect of SMER28 (50 μ M) on A β 40 (*G*) and APP-CTF (*H*) in N2a-APP cells ($n \geq 3$). Error bars = SE. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

lated by SMER28 or by starvation. As shown in Fig. 2*B*, there was no change in N2a cells in the levels of expression of either endogenous full-length APLP1 or, more important, the processed form APLP1-CTF. Furthermore, by introducing an engineered form of Notch capable of being cleaved directly by γ -secretase (Δ E-Notch1) into the same cellular system, we were able to show that Δ E-Notch1 and NICD, a fragment generated after γ -secretase cleavage, were not affected on SMER28 or starvation treatment. To further characterize the effect of autophagy on APP-CTF, we used cycloheximide to inhibit protein synthesis and measured the half-life of β CTF on starvation. The disappearance of β CTF in the starved cells was more rapid than in control cells (no starvation), with a half-time of 10 min compared with 32 min for the control cells (Fig. 2*C, D*). Taken together, our results demonstrate that SMER28 and starvation specifically stimulate degradation of A β 40/A β 42 and APP-CTF. Fusion to lysosomes is required for the degradation of proteins sequestered in autophagosomes, and this fusion can be blocked by bafilomycin A1 (BFA). When N2a-APP cells were treated with 1 μ M BFA, a significant accumulation of A β 40 peptide and APP-CTF fragment was observed (Fig. 2*E, F*). Although 0.1 μ M BFA had no effect on

A β 40 and APP-CTF basal levels (Fig. 2*E, F*), it was able to prevent the SMER28 induced decrease of A β 40 and APP-CTF (Fig. 2*G, H*). These results indicate that the autophagic-lysosomal degradation pathway is required for basal or SMER28-induced removal of A β and APP-CTF.

SMER28- and starvation-induced degradation of A β and APP-CTF are γ -secretase independent

To clarify whether the decrease of A β peptides is due to a decrease in γ -secretase activity on autophagy induction, we analyzed the levels of PS1-NTF and PS1-CTF, the catalytic core of γ -secretase (26, 30), (31), on SMER28 and starvation treatment. Their levels of expression were similar to the ones observed in control conditions (Supplemental Fig. S2*A*). Furthermore, we used compound L-685,458 to block the activity of γ -secretase. As expected, γ -secretase inhibitor treatment led to accumulation of APP-CTF (Supplemental Fig. S2*B, C*). Starvation and SMER28 treatment dramatically reduced the amount of accumulated APP-CTF in the presence of γ -secretase inhibitor (Supplemental Fig. S2*B, C*). Taken together, these results demon-

strated that the reductions of A β peptides and APP-CTF by starvation or SMER28 were not dependent on γ -secretase.

Atg5 plays an essential role in degradation of A β and APP-CTF, induced by starvation or SMER28

The biological functions of autophagy have been examined by generating mice lacking several critical genes such as: *Atg5* (5), *beclin 1* (32, 33), and *Atg7* (34). All showed severe defects in autophagy. To further investigate the involvement of autophagy in the degradation of A β 40 and APP-CTF, we used *Atg5*^{-/-} MEF and control *Atg5*^{+/+} MEF cells (5). In MEF cells overexpressing APP-695, A β 40 levels increased 3.7-fold in the *Atg5*^{-/-} cells compared with wild-type *Atg5*^{+/+} cells (Fig. 3A). APP-CTF levels were increased 9.4-fold in the *Atg5*^{-/-} control cells compared with *Atg5*^{+/+} controls, while APP695 was increased by 38% (Fig. 3B, top 3 panels). Furthermore, endogenous APP/APP-CTF levels were analyzed in *Atg5*^{-/-} and *Atg5*^{+/+} MEF cells. In this context, endogenous APP-CTF also showed a significant accumulation in *Atg5*^{-/-} cells (increase 4.4-fold) in comparison to *Atg5*^{+/+} cells, while full-length APP level increased only by 25% (Fig. 3C, top 2 panels). These results suggested that Atg5 was required for the housekeeping clearance of A β and APP-CTF, in addition to the partial removal of APP full length. Moreover, starvation of the cells reduced 74% of endogenous APP-CTF (Fig. 3C, 2nd panel) and 43% of the overexpressed APP-CTF (Fig. 3B, middle 2 panels) in *Atg5*^{+/+} cells, but it only induced a reduction of 20

and 4.6%, respectively, in *Atg5*^{-/-} cells. These results indicated that the degradation of APP-CTF triggered by starvation also required Atg5. It is worth noting that there was a modest reduction of APP-CTF in *Atg5*^{-/-} cells following starvation. To determine whether the effect of Atg5 deletion on A β 40 and APP-CTF degradation was specific, we analyzed the levels of PS1-CTF and tubulin. No significant difference was detected among *Atg5*^{+/+} and *Atg5*^{-/-} cells, with or without starvation (Fig. 3C). Taken together, these results suggested that Atg5 is required for the clearance of A β and APP-CTF *via* autophagy.

Next, we sought to determine whether Atg5 is necessary for the effect of SMER28 on A β and APP-CTF degradation. *Atg5*^{+/+} and *Atg5*^{-/-} MEF cells were treated with SMER28 for 6 h, and endogenous APP-CTF fragment was analyzed by SDS-PAGE and Western blotting. We observed a dose-dependent decrease of APP-CTF in *Atg5*^{+/+} MEF cells but not in *Atg5*^{-/-} MEF cells (Fig. 3D). These results suggest that SMER28 induced APP-CTF degradation is through an Atg5-dependent pathway.

Beclin1 and Ulk1 regulate basal, but not SMER28-induced, clearance of A β and APP-CTF

To further confirm the role of autophagy in clearing A β and APP-CTF, we examined whether Beclin1 or Ulk1, two other components of the autophagy pathway, were involved. siRNA was used to knock down either Beclin1 or Ulk1 in N2a-APP cells. As shown in Fig. 4B, D, E, we obtained high Beclin1 or Ulk1 silencing

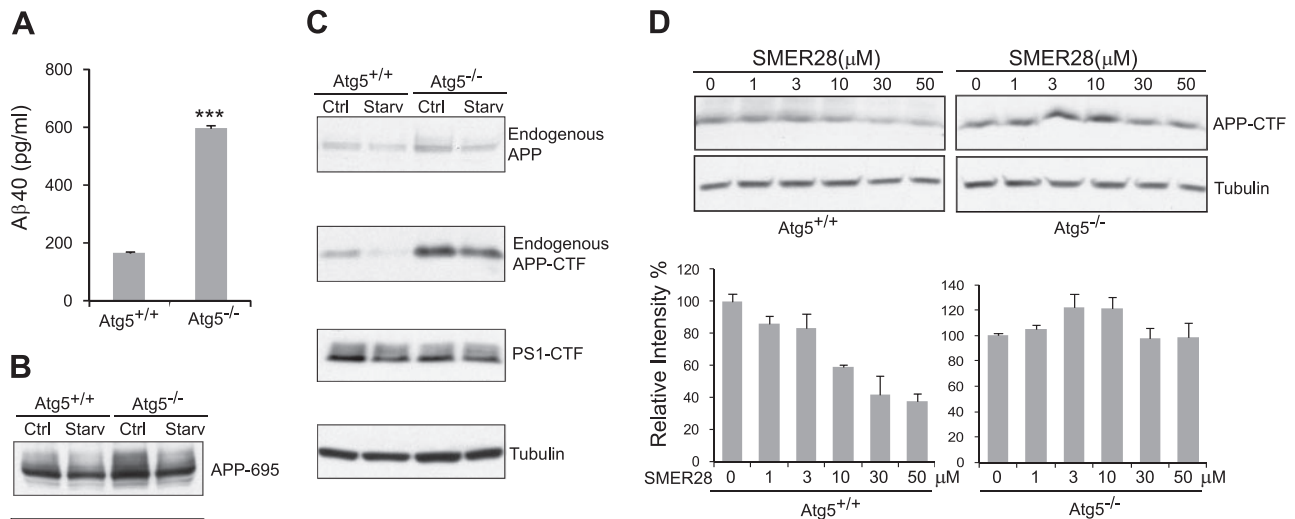


Figure 3. SMER28-induced clearance of A β 40 and APP-CTF is dependent on Atg5. *A, B*) *Atg5*^{+/+} and *Atg5*^{-/-} MEF cells were transfected with a plasmid encoding APP695. *A*) At 16 h post-transfection, conditioned media were collected and subjected to A β 40 peptide analysis by ELISA ($n \geq 3$). *** $P < 0.005$. *B*) Cell lysates were analyzed by SDS-PAGE and Western blotting. *C*) *Atg5*^{+/+} and *Atg5*^{-/-} MEF cells were starved for 2 h, lysed, and analyzed for endogenous APP-FL, APP-CTF, PS1-CTF, and tubulin using SDS-PAGE and Western blotting. *D*) Top panels: *Atg5*^{+/+} and *Atg5*^{-/-} MEF cells were treated with increasing concentrations of SMER28 for 6 h. Endogenous APP-CTF and tubulin expression levels were analyzed by SDS-PAGE and Western blotting. Bottom panels: results of APP-CTF quantifications from Western blots. Error bars = se.

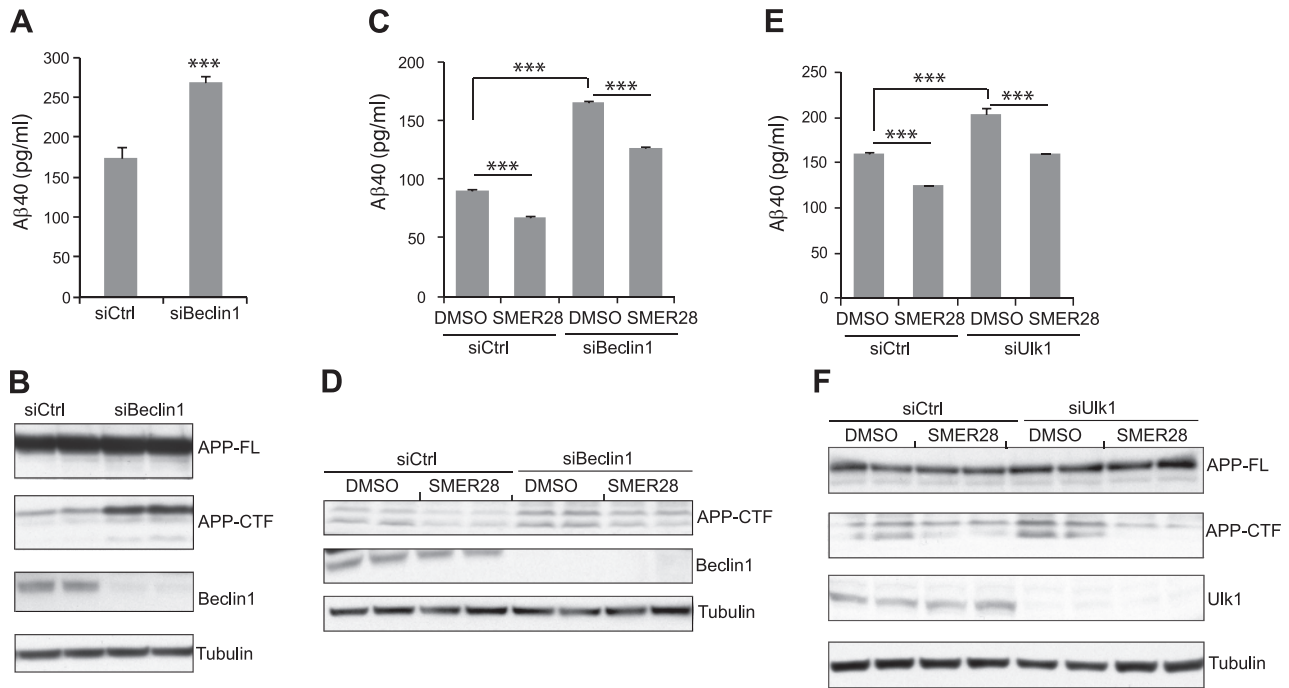


Figure 4. Beclin1 and Ulk1 regulate basal, but not SMER28-induced, clearance of A β 40 and APP-CTF. *A, B*) N2a-APP cells were transfected with control siRNAs or with a mixture of 4 Beclin1 siRNAs for 24 h. *A*) Conditioned media were recovered, and the presence of soluble A β 40 was measured by ELISA ($n \geq 3$). *B*) Cell lysates were prepared and analyzed by SDS-PAGE and Western blotting for APP-FL, APP-CTF, Beclin1, and tubulin. *C, D*) N2a-APP cells were transfected with control or Beclin1 siRNAs for 24 h and then treated with SMER28 (50 μ M) for 6 h. Conditioned media (*C*) and cell lysates (*D*) were harvested for analysis as described above ($n \geq 3$). *E, F*) N2a-APP cells were transfected with control siRNA or a mixture of Ulk1 siRNAs for 24 h and then treated with SMER28 (50 μ M) for 6 h. Conditioned media (*E*) and cell lysates (*F*) were harvested for analysis as described above ($n \geq 3$). Error bars = SE. *** $P < 0.005$.

efficiency. We found that A β 40 (Fig. 4A) and APP-CTF (Fig. 4B) significantly increased after Beclin1 silencing. Similarly, silencing of Ulk1 also caused significant accumulation of both A β 40 and APP-CTF (Fig. 4E, F). Subsequently, Beclin1- or Ulk1-silenced cells were treated with SMER28 for 6 h. We observed a 25% decrease of A β 40 and a 49% decrease of APP-CTF in both control and Beclin1-silenced cells (Fig. 4C, D), suggesting that knocking down of Beclin1 had no inhibitory effect on SMER28-induced A β 40 and APP-CTF degradation. Likewise, Ulk1 silencing had no obvious inhibitory effect on SMER28 treatment (Fig. 4E, F). These results suggested that Beclin1 and Ulk1 are required for the degradation of A β 40 and APP-CTF through autophagy and that SMER28 may act downstream of Beclin1 and Ulk1 for the reduction of A β 40 and APP-CTF levels. However, we can not rule out the possibility that residual levels of Beclin1 and Ulk1 after siRNA knockdown are sufficient to carry out its function in autophagy on SMER28 treatment.

APP-CTF and LC3-II are cocompartmentalized on SMER28 treatment

To investigate whether the trafficking pathway of APP-CTF overlaps with the autophagy pathway, we examined, first by subcellular fractionation, whether APP-CTF cocompartmentalizes with the autophagosome

marker LC3-II. N2a-APP cells were treated with SMER28 for 6 h, and cell lysates were fractionated by sucrose centrifugation. Twelve fractions from top to bottom were collected and analyzed by SDS-PAGE and Western blotting. The majority of APP-CTF was localized in fraction 6, where γ -adapin, a Golgi marker protein, is also localized (Fig. 5A). Interestingly, the autophagy marker LC3-II was also enriched in fraction 6. In contrast, LC3-I showed a more diffuse pattern, with enrichment in lower-density fractions. Bip, an ER protein marker, mostly localized to fractions 11 and 12. This result suggested APP-CTF cocompartmentalized with autophagosome. We further assessed the colocalization of APP-CTF and LC3 by immunofluorescence. N2a-APP cells were transfected with LC3-EGFP and treated with SMER28 for 6 h. APP and APP-CTF were stained with an antibody recognizing the C-terminal of APP. After SMER28 treatment, typical punctuate structures of LC3-EGFP were seen (Fig. 5B, top left panel) and APP/APP-CTF were localized at Golgi and trans-Golgi compartments (35) as expected (Fig. 5B, top middle panel). After merging LC3 and APP staining, a certain degree of colocalization of APP-CTF with the LC3-marked autophagosome (shown as green dots) was indeed observed (Fig. 5B, top right panel; Supplemental Fig. S3). We used APLP1 staining as a negative control and showed that it was mostly located at the cell surface (Fig. 5B, bottom middle panel), which is con-

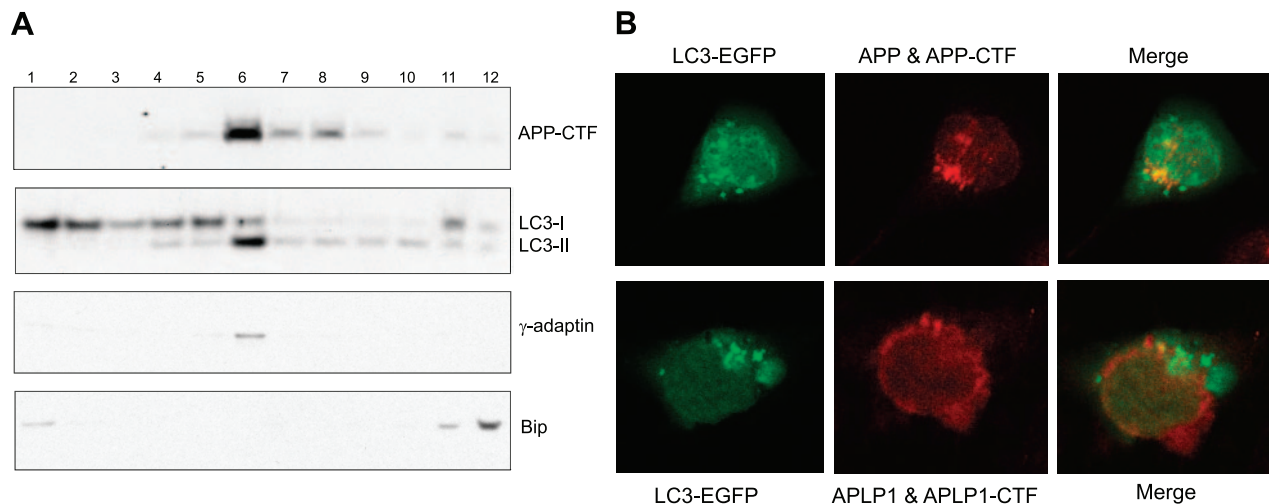


Figure 5. SMER28 induced cocompartmentalization of APP-CTF and LC3-II. *A*) N2a-APP cells were treated for 6 h with SMER28 (50 μ M), and whole-cell lysates were fractionated by sucrose gradient. An aliquot of each of the 12 fractions recovered was analyzed by SDS-PAGE and Western blotting for APP-CTF, LC3, γ -adaptin, and Bip. *B*) N2a-APP cells were transfected with an LC3-EGFP-containing plasmid; at 16 h post-transfection, cells were treated for 6 h with SMER28 (50 μ M). Cells were fixed and immunostained with RU-369, an APP C-terminal antibody, or APLP1 antibody and imaged by confocal microscopy.

sistent with a previous report (36), and that it does not colocalize with LC-3 puncta (Fig. 5*B*, bottom right panel). Taken together, the above results using both the sucrose fractionation and immunofluorescence staining methods support the concept that the trafficking pathway of APP/APP-CTF can merge, at least partially, with the autophagosome-lysosomal pathway.

DISCUSSION

The A β peptides, which aggregate and accumulate in the brains of patients with AD, are prime targets for AD therapies. In the present study, we have shown that the autophagic proteins Atg5, Beclin1, and Ulk1 are involved in the housekeeping clearance of A β and APP-CTF. Furthermore, a small molecule, SMER28, reduced the levels of A β and APP-CTF in an Atg5-dependent fashion. We propose that low-molecular-weight autophagy enhancers have significant therapeutic potential for AD treatment.

It has been thought that basal autophagic activity is low in the healthy brain. However, a recent study (37) has shown that autophagy in primary cortical neurons is highly efficient and that newly formed autophagosomes are rapidly removed by fusion with lysosomes. Consistent with those results, we observed that in Atg5-knockout MEF cells (Atg5^{-/-}), as well as in Beclin1 and Ulk1 knocked-down cells, A β and APP-CTF levels were dramatically increased, suggesting that under physiological conditions, A β and APP-CTF are rapidly cleared by autophagy. Therefore, autophagy might be a housekeeping mechanism for the removal of A β and APP-CTF. We think that in parallel to the decline of autophagy in aging, the efficiency of the housekeeping clearance decreases, so that A β , normally rapidly degraded, will gradually accumulate until reaching concentrations compatible with aggregate formation.

Therefore, enhancing the degradation of the A β and APP-CTF in aging brain might be beneficial to prevent and/or treat AD.

Recent studies (19, 20, 38), demonstrating that loss of basal autophagy in mouse neuronal cells resulted in neurodegeneration, suggest that autophagy may have a protective role against the development of various neurodegenerative diseases. In addition, it has been shown that pharmacological stimulation of autophagy can increase life span in yeast (39, 40), *C. elegans* (41, 42), *D. melanogaster* (43), and mice (44) and is beneficial for A β induced toxicity *in vivo* (45, 46) in AD. Furthermore, it was reported that in Parkinson disease and Huntington disease, misfolded proteins, α -synuclein and huntingtin, respectively, were targeted by autophagy (7, 23, 47). Therefore, the use of pharmacological boosters of autophagy could represent an effective therapeutic approach to prevent or treat age-related neurodegenerative symptoms and progression (48). Theoretically, one can reduce A β accumulation in AD either through inhibiting its production or through enhancing its degradation. Enormous effort has been made to inhibit its production, but progress has been slow due to lack of specificity and possibly the side-effects of inhibiting a multitasking protease. Therefore, activating autophagy to enhance the degradation of A β is an alternative approach for AD therapy. Our present studies showed that SMER28, a small-molecule enhancer of autophagy, can promote A β and APP-CTF degradation without affecting related proteins, such as Notch and APLP1. Since APP- β CTF was implicated in endosome dysfunction in Down syndrome (49), pharmacological removal of APP- β CTF and A β might be beneficial not only to patients with AD but also patients with Down syndrome. FJ

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