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Ubiquilin-1 Modulates γ-Secretase-Mediated ε-Site Cleavage in Neuronal Cells

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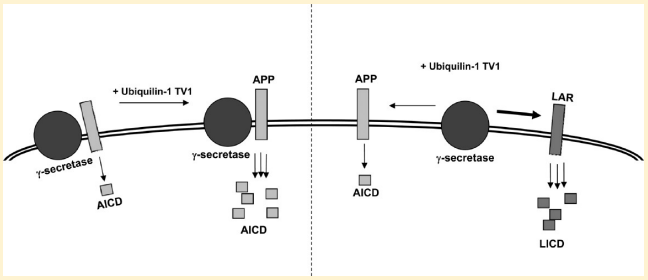
Supporting Information

ABSTRACT: Ubiquilin-1 is an Alzheimer's disease-associated protein, which is known to modulate amyloid precursor protein (APP) processing, amyloid-β (Aβ) secretion, and presenilin-1 (PS1) accumulation. Here, we aim to elucidate the molecular mechanisms by which full-length transcript variant 1 of ubiquilin-1 (TV1) affects APP processing and γ-secretase function in human neuroblastoma cells stably overexpressing APP (SH-SY5Y-APP751). We found that TV1 overexpression significantly increased the level of APP intracellular domain (AICD) generation. However, there was no increase in the levels of secreted $Aβ_{40}$, $Aβ_{42}$, or total Aβ, suggesting that ubiquilin-1 in particular enhances γ-secretase-mediated ε-site cleavage. This is supported by the finding that TV1 also significantly increased the level of intracellular domain generation of another γ-secretase substrate, leukocyte common antigen-related (LAR) phosphatase. However, in these cells, the increase in AICD levels was abolished, suggesting a preference of the γ-secretase for LAR over APP. TV2, another ubiquilin-1 variant that lacks the protein fragment encoded by exon 8, did not increase the level of AICD generation like TV1 did. The subcellular and plasma membrane localization of APP or γ-secretase complex components PS1 and nicastrin was not altered in TV1-overexpressing cells. Moreover, the effects of TV1 were not mediated by altered expression or APP binding of FE65, an adaptor protein thought to regulate AICD generation and stability. These data suggest that ubiquilin-1 modulates γ-secretase-mediated ε-site cleavage and thus may play a role in regulating γ-secretase cleavage of various substrates.



Alzheimer's disease (AD) is a neurodegenerative disease characterized by memory impairment and failing cognitive capacity. The neuropathological hallmarks of AD include the deposition of aggregated amyloid-β (Aβ) in the brain parenchyma, hyperphosphorylated tau deposits within axons in the form of neurofibrillary tangles, and the loss of synapses and neurons.[1] Amyloid precursor protein (APP) gives rise to Aβ through sequential enzymatic cleavages mediated by β- and γ-secretases.[2] The β-secretase or BACE1 (β-site APP-cleaving enzyme 1) is an aspartic acid protease, while the γ-secretase is an enzyme complex consisting of presenilin (PS), presenilin enhancer 2 (PEN-2), anterior pharynx-defective 1 (APH1), and nicastrin (NCT).[3,4] β-Secretase cleavage of APP results in the release of the APP ectodomain leaving the APP C-terminal fragment (CTF) C99 within the membrane. The CTF is cleaved by the γ-secretase at the ε-site, which releases the APP intracellular domain (AICD). This is followed by the γ-cleavage that releases the Aβ peptides. Mutations in PS1- and PS2-encoding genes *PSEN1* and *PSEN2*, respectively, cause the familial form of AD and increase the level of Aβ generation.[5] For these reasons, processing of APP by the secretases is of key importance in AD pathogenesis and is a potential target of therapeutic interventions against AD.

Many proteins interact with PSs and thereby possibly modulate γ-secretase-mediated APP processing and consequent Aβ production. One such protein, ubiquilin-1, was discovered in 2000 by Mah et al.[6] The authors showed that ubiquilin-1 interacts with and stabilizes PS proteins. Our recent data have confirmed these findings.[7] Ubiquilin-1 is a ubiquitin-like protein consisting of characteristic ubiquitin-associated (UBA) and ubiquitin-like (UBL) domains capable of binding poly-ubiquitinated proteins and the proteasome, respectively. Through these domains, ubiquilin-1 is suggested to mediate proteasomal degradation of proteins.[8,9] One of these proteins is PS, which implies that ubiquilin-1 is especially relevant in AD.[6,7,10]

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So far, four naturally occurring alternatively spliced ubiquilin-1 transcript variants (TVs) have been found in the brain, namely, TV1−TV4.[11,12] Ubiquilin-1 full-length protein (TV1) contains polypeptide fragments encoded by 11 exons. The other variants lack specific exons and are smaller in size. The potential involvement of the individual TVs in AD pathogenic mechanisms has not been studied extensively. We observed previously that an intronic single-nucleotide polymorphism (SNP) downstream of exon 8 in the *UBQLN1* gene was significantly associated with AD.[12] This risk allele in the *UBQLN1* gene led to an increase in the ratio of TV2 to TV1 mRNA levels in the brain tissue in a dose-dependent manner. Moreover, our data have demonstrated that particular ubiquilin-1 variants regulate PS1 accumulation and targeting to aggresomes and alleviate endoplasmic reticulum (ER) stress.[7,11] Both aggresome formation and ER stress are processes that have been implicated in AD pathogenesis.[11,13,14] Furthermore, we have previously shown that ubiquilin-1 downregulation accelerates APP maturation and processing and increases the level of Aβ secretion in human embryonic kidney 293 (HEK293) and human neuroglioma H4 cells.[15] Additionally, our recent studies showed that specific ubiquilin-1 variants significantly increased the level of secretion of Aβ_{40} and Aβ_{42} in cultured primary cortical cells from mice overexpressing human APP and PS1.[7] Taken together, the genetic and functional findings strongly suggest that ubiquilin-1 TVs may play an important role in APP processing and Aβ generation.

In this study, we wanted to elucidate the molecular mechanisms of the involvement of ubiquilin-1 in the altered APP processing and γ-secretase activity. Our results indicate that ubiquilin-1 modulates γ-secretase-mediated ε-site cleavage of APP and another γ-secretase substrate, leukocyte common antigen-related (LAR) phosphatase, suggesting that ubiquilin-1 may regulate the generation of the intracellular domain from various γ-secretase substrates.

EXPERIMENTAL PROCEDURES

Plasmids. Plasmids encoding ubiquilin-1 TV1 (full-length, containing polypeptide fragments encoded by all 11 exons), TV2 (lacking the protein fragment encoded by exon 8), FE65, and PS1 cDNAs were used for transfections. TV1 and TV2 cDNA constructs containing a 5′-end myc tag yielding N-terminally myc-tagged TV1 and TV2 (myc-TV1 and myc-TV2, respectively) were also used. The myc tag adds ∼3 kDa to the molecular mass of the protein. Additionally, full-length LAR (FL-LAR) cDNA tagged with a V5 His tag at the 3′-end, producing C-terminally tagged FL-LAR-V5-His, was used.[16] The tag adds ∼5 kDa to the molecular mass of the expressed LAR protein. pcDNA3.1 or HIV-pBOB plasmids were used as controls.

Cell Culture and Transfection. SH-SY5Y human neuroblastoma cells stably overexpressing the APP751 isoform (SH-SY5Y-APP751) were cultured in a humidified cell culture incubator in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (DMEM-C) and supplemented with 200 μg/mL Geneticin. The cells were plated on cell culture dishes (Nunc) and transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The medium was changed 24 h later, and the proteins were collected 48 h after the transfection.

Western Blot Analysis. The cells were scraped in an appropriate volume of tissue protein extraction buffer (TPER, Pierce) supplemented with EDTA-free protease inhibitor cocktail (Thermo Scientific). The protein lysates were then centrifuged at 10000*g* for 10 min at 4 °C. Protein concentrations were measured from the supernatant using the BCA protein assay kit (Pierce). Proteins (20−50 μg) were separated on 4 to 12% Bis-Tris gels (Invitrogen) under denaturating conditions and blotted onto polyvinylidene difluoride (PVDF) membranes (Amersham Hybond-P, GE Healthcare). The blots were probed with the following primary antibodies: mouse anti-ubiquilin-1 (34-4400, 1:1000, Zymed Laboratories Inc.), rabbit anti-ubiquilin-1 (U7258, 1:1000, Sigma-Aldrich), rabbit anti-APP C-terminus (A8717, 1:2000, Sigma), mouse anti-APP N-terminus (22C11, MAB348, 1:1000, Millipore), mouse anti-PS1 (MAB5232, detecting full-length PS1 and PS1-CTF, 1:1000, Chemicon), rabbit anti-PS1 (Ab14, detecting full-length PS1 and PS1-NTF, 1:1000, a gift from S. E. Gandy), rabbit anti-NCT (PA1-758, 1:1000, ABR), rabbit anti-PEN-2 (Ab18189, 1:150, Abcam), rabbit anti-APH1A (PC728, 1:1000, Calbiochem), and goat anti-FE65 (SC-19751, 1:1000, Santa Cruz Biotechnology). Mouse anti-myc (05-724, 1:1000, Millipore) was used to detect myc-TV1 and myc-TV2 and mouse anti-V5 (R960-25, 1:5000, Invitrogen) to detect LAR-V5-His and LICD-V5-His. Antibodies against mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab8245, 1:15000, Abcam) and mouse anti-transferrin receptor (TfR) (13-6800, 1:1000, Zymed Laboratories Inc.) were used for normalization of the protein levels. Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare) and enhanced chemiluminescence substrate (ECL, GE Healthcare) were used to detect the bands using ImageQuant RT ECL Imager (GE Healthcare). The bands were quantified using Quantity One (Bio-Rad). All protein levels were normalized to those of housekeeping genes in the same samples.

Measurements of Secreted Aβ. The Aβ_{40} and Aβ_{42} levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (The Genetics Co.) according to the manufacturer's instructions. Total Aβ levels were measured using the human Aβ 1-x ELISA kit (IBL) according to the manufacturer's instructions.

***In Vitro* AICD Generation Assay.** An *in vitro* AICD generation assay was performed according to the method of Hiltunen et al.[15] The cells were transfected with TV1, TV2, or control cDNA plasmids. The cells were scraped in buffer A [50 mM HEPES, 150 mM NaCl, and 5 mM 1,10-phenanthroline monohydrate (PNT) (pH 7.4)] and homogenized by being pushed through a 25 gauge needle 10 times and centrifuged at 10000*g* for 15 min at 4 °C. The supernatants containing the cytosolic proteins were used to confirm overexpression of ubiquilin-1 TVs. The pellet containing the membrane-associated proteins was resuspended in buffer A. Equal amounts of protein from TV1- or TV2-overexpressing and control samples were incubated at 37 °C for 2 h to allow AICD generation. A negative control sample was incubated at 4 °C, at which temperature AICD is not released. The samples were then centrifuged at 10000*g* for 15 min at 4 °C, and the resultant supernatant contained the generated AICD. The pellet was resuspended in TPER with protease inhibitors and centrifuged as described previously. The supernatant from this centrifugation step contained the remaining C-terminal fragments (CTFs). The AICD and CTF fractions were analyzed using

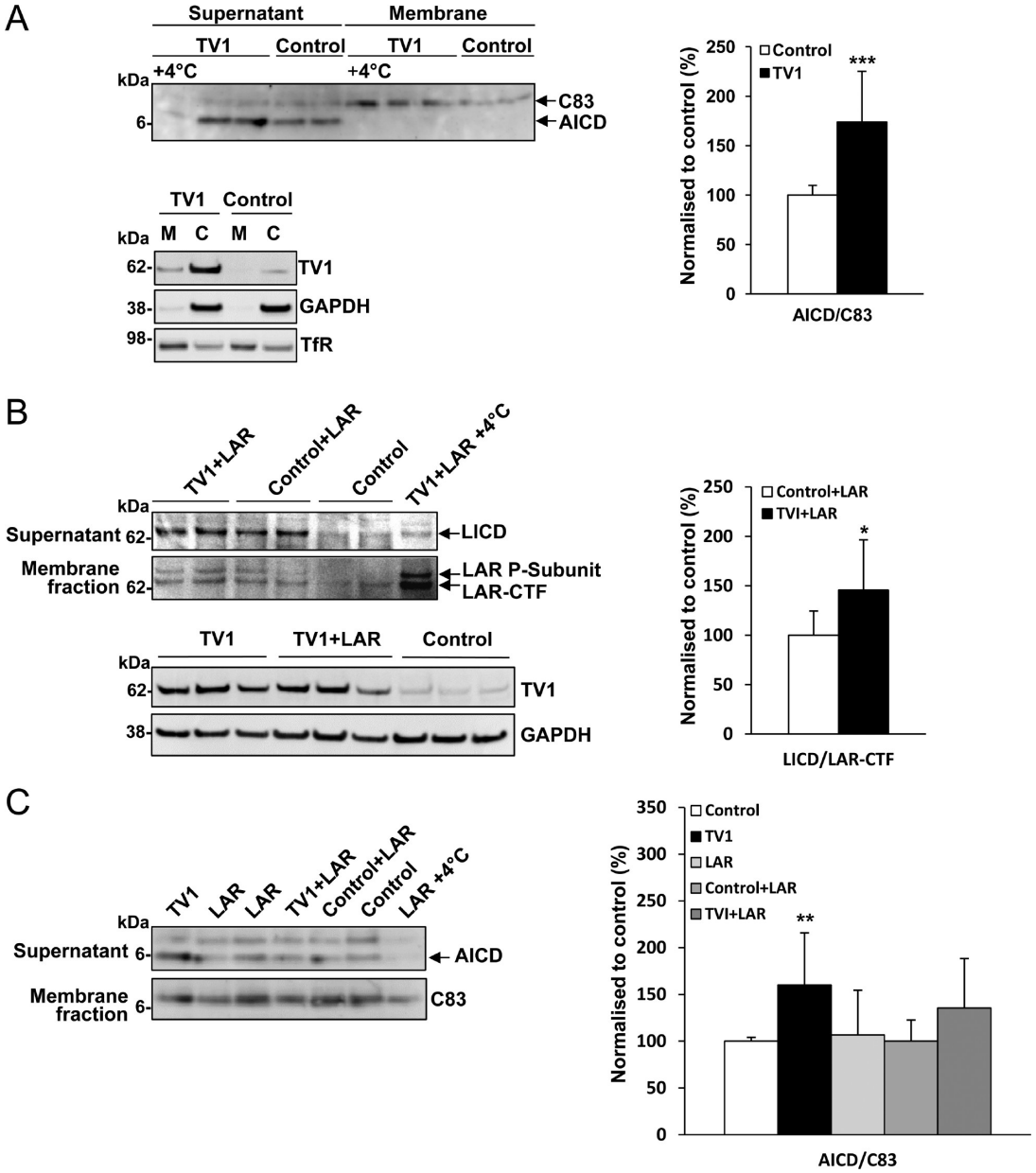

Figure 1. Overexpression of TV1 in SH-SY5Y-APP751 cells increases the level of AICD and LICD production. (A) Western blot showing the effects of TV1 overexpression on AICD production using an *in vitro* AICD generation assay. Quantification of the APP-CTF C83-normalized levels of AICD (AICD/C83) is shown at the right (n = 14−15). A reaction at 4 °C was used as a negative control showing no AICD generation and prominent C83 accumulation. The faint upper band detected in the supernatant samples is C83, which originates from the leftovers of membrane proteins after the separation of supernatant and membrane fractions by centrifugation. The bottom panel shows TV1 overexpression in the cytosolic fraction, but not in the membrane fraction. (B) Western blot showing the effects of TV1 and full-length LAR co-expression on LICD generation (LAR-CTF-normalized LICD levels; LICD/LAR-CTF). Quantification is shown at the right (n = 5−9). The bottom panel shows TV1 overexpression in the cytosolic fraction of SH-SY5Y-APP751 cells. (C) Western blot showing the effects of TV1 and full-length LAR co-expression on AICD generation. Using the same supernatant and membrane fractions as in panel B, the level of AICD generation is increased upon TV1 overexpression. Co-expression of TV1 and LAR significantly increased the level of LICD generation, while the level of AICD generation was not significantly increased. Quantification is shown at the right (n = 6−9). Abbreviations: C, cytosolic fraction; M, membrane fraction; TfR, transferrin receptor. $^{*}p < 0.05$; $^{***}p < 0.001$. Mean ± SD. Numbers on the left of the blots are molecular masses in kilodaltons.

Western blotting with an antibody recognizing the APP C-terminus (A8717). The AICD levels were normalized to the C83 levels in the CTF fraction from the same samples.

Biotinylation of Cell Surface Proteins. Cells were washed twice with PBS supplemented with 0.01 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-Ca-Mg) and preincubated in fresh PBS-Ca-Mg for 15 min at 4 °C. Sulfo-NHS-LC-Biotin (EZ Link, Pierce) in PBS-Ca-Mg was added to the cells and the mixture incubated for 30 min at 4 °C. Excess biotin was quenched by incubating cells in PBS-Ca-Mg supplemented with 0.1 mM glycine for 20 min. The cells were scraped in TPER containing protease inhibitors and centrifuged at 10000g for 10 min at 4 °C. Fifty micrograms of proteins was mixed with binding buffer [PBS and 1% Nonident P40 (NP40)] and incubated overnight with agarose beads cross-linked with streptavidin (Pierce). The samples were centrifuged at 5000g

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for 1 min to obtain a pellet containing the biotinylated fraction and supernatant containing the unbiotinylated fraction for Western blotting.

Co-Immunoprecipitation. Proteins were extracted from cells overexpressing TV1, FE65, or TV1 and FE65. Sixty micrograms of protein was added to RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, and 0.5% NP40] to a final volume of 500 μL. Protein A/G beads (Pierce) were added to the proteins and incubated for 1 h at 4 °C for sample preclearing to reduce the level of nonspecific protein binding in the actual immunoprecipitation reactions. The samples were centrifuged at 8000*g* for 1 min. Ten percent of the precleared proteins from each sample were used to confirm overexpression. The remainder was mixed with approximately 2 μg of anti-FE65 (SC-19751) or 2.5 μg of anti-myc (05-724) antibody and rotated for 1 h at 4 °C. Fresh protein A/G beads were added and the mixtures rotated at 4 °C overnight. The samples were centrifuged at 8000*g* for 1 min. The immunoprecipitated complexes were washed with RIPA buffer four times, and the precipitated proteins were detached from the beads by being heated at 95 °C for 10 min in 30 μL of 1× LDS loading buffer (Novex) containing 5% β-mercaptoethanol. The samples were centrifuged at 13000*g* for 15 min, and the supernatants were analyzed using Western blotting. The sample without an antibody (beads only) was used as a negative control. A sample with an antibody known not to interact with APP or LAR (anti-myc or anti-GAPDH, respectively) was used as an additional control for the specificity of the assay.

Confocal Microscopy. Cells were fixed in 4% paraformaldehyde (PFA) and incubated in PBS containing 0.1% Triton X-100 and 5% BSA for 30 min for permeabilization and to prevent unspecific antibody binding. Cells were then incubated with following primary antibodies for 1.5 h: mouse anti-myc (05-724, 1:100, Millipore), rabbit anti-ubiquilin-1 (ab3341, 1:500, Abcam), rabbit anti-APP C-terminus (A8717, 1:1000, Sigma), mouse anti-APP N-terminus (22C11, MAB348, 1:1000, Millipore), mouse anti-PS1 (MAB5232, 1:400, Chemicon), rabbit anti-LC3B (ab51520, 1:200, Abcam), and mouse anti-mono- and -poly-ubiquitinated proteins FK2 (BML-PW8810-0500, 1:100, Enzo). The following fluorescent secondary antibodies were used at a dilution of 1:500: anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 568 (Invitrogen). The nuclei were stained with Hoechst 33342 (1:500, Sigma). Staining without primary antibodies was used as a negative control. Confocal images were obtained with a Nikon Eclipse-TE300 microscope and an Ultra VIEW laser scanning confocal unit (Perkin-Elmer) at 60× magnification. The images were processed by using Adobe Photoshop (Adobe Systems, San Jose, CA).

Statistical Analyses. Statistical analyses were performed using SPSS, version 14.0. An independent sample *t* test, a Mann−Whitney U test, and one-way analysis of variance were used to test statistical significance. Values are means ± the standard deviation (SD). The level of statistical significance was set at $p < 0.05$.

RESULTS

Overexpression of TV1 Increases the Level of Production of the Intracellular Domain of APP and LAR in SH-SY5Y-APP751 Cells. Our previous studies showed that ubiquilin-1 downregulation affected APP holoprotein and CTF levels in H4 neuroglioma and HEK293 cells.[15] Here, we wanted to elucidate whether ubiquilin-1 affects γ-secretase-mediated cleavage of APP. For this purpose, we overexpressed ubiquilin-1 full-length transcript variant 1 (TV1) in SH-SY5Y human neuroblastoma cells stably overexpressing the APP751 isoform (SH-SY5Y-APP751). TV1 levels in the transfected SH-SY5Y-APP751 cells were approximately 10-fold greater than endogenous ubiquilin-1 levels [GAPDH-normalized TV1 levels = 1036 ± 471% ($p < 0.01$) compared to GAPDH-normalized endogenous ubiquilin-1 levels in control samples = 100 ± 12% (Figure 1A)]. We then assessed the ε-cleavage in these cells using an *in vitro* AICD generation assay, in which AICD is released from the extracted cell membranes to the supernatant after incubation at 37 °C for 2 h. After the separation of the supernatant and the remaining membrane pellet by centrifugation, the generated AICD fragments and the remaining C83 still embedded in the membranes were analyzed using Western blotting. The generated AICD levels in the supernatant were normalized to the C83 levels detected from the membrane fraction of the same sample to ensure that the differences in the starting substrate levels do not affect the results. Thus, the initial amount of full-length APP or APP CTFs does not affect the *in vitro* AICD generation assay results as the levels of the generated AICD are normalized to the remaining substrate levels.[17] Subsequently, we found that the C83-normalized AICD levels were significantly increased by ∼1.7-fold in TV1-overexpressing cells as compared to control cells (Figure 1A). In contrast, AICD was not generated at 4 °C, and as a result, APP-C83 accumulated in that sample (Figure 1A). This suggests that TV1 overexpression increased the level of γ-secretase-mediated ε-site cleavage of APP.

To investigate whether the observed effect on ε-site cleavage was specific for APP only, we tested whether generation of the ICD from another known γ-secretase substrate, LAR (leukocyte-common antigen-related) receptor tyrosine phosphatase,[16] was also augmented in TV1-overexpressing SH-SY5Y-APP751 cells. In cells co-overexpressing LAR along with TV1 [in samples overexpressing TV1 separately, GAPDH-normalized TV1 levels = 1551 ± 731% ($p < 0.01$), in samples overexpressing TV1 together with full-length LAR, GAPDH-normalized TV1 levels = 1464 ± 747% ($p < 0.01$), as compared to endogenous ubiquilin-1 levels in control samples = 100 ± 28% (Figure 1B)], we found that the LAR-ICD (LICD) levels normalized to the LAR-CTF levels were significantly increased (Figure 1B). The P-subunit of LAR, which is the C-terminal membrane-bound subunit of full-length LAR,[16] is also seen in the blot. Interestingly, in these samples, AICD levels were no longer significantly augmented by TV1 overexpression (Figure 1C). To address the possible mechanism behind the observed substrate preference, we next assessed whether ubiquilin-1 interacts directly with LAR and/or APP and whether there are differences in terms of these interactions when LAR is co-expressed with ubiquilin-1 as compared to the samples overexpressing ubiquilin-1 only. Co-immunoprecipitation indicated that TV1 did not co-immunoprecipitate with LAR or APP [in samples overexpressing myc-TV1 separately, GAPDH-normalized TV1 levels = 168 ± 59% ($p < 0.05$), in samples overexpressing myc-TV1 together with full-length LAR, GAPDH-normalized TV1 levels = 241 ± 84% ($p < 0.05$), as compared to endogenous ubiquilin-1 levels in control samples = 100% (Figure 1 of the Supporting Information)], suggesting that TV1 does not directly interact with LAR or APP or that these interactions may be transient or too weak to be detected under the conditions used here. However, these results suggest that when co-expressed, APP and LAR compete for γ-secretase-

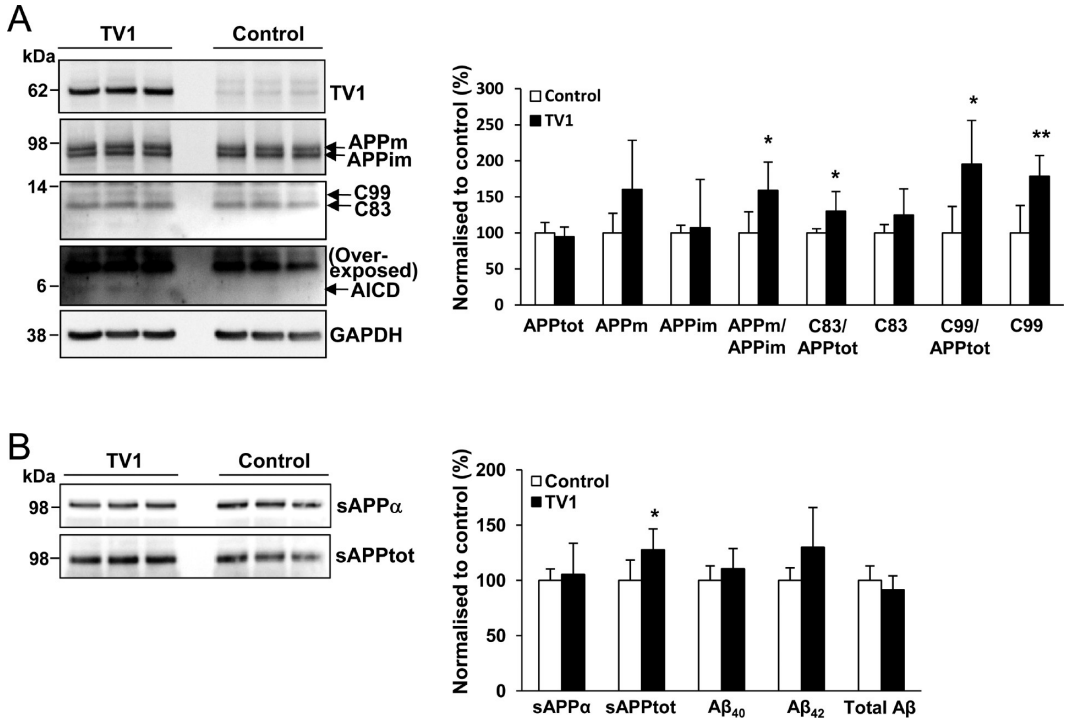

Figure 2. Overexpression of ubiquilin-1 TV1 in SH-SY5Y cells increases the level of maturation and/or processing of exogenous and endogenous APP. (A) Western blot showing the effects of TV1 overexpression in SH-SY5Y cells stably overexpressing APP751. Quantification is shown at the right (n = 4–6). (B) Secreted sAPPα and total sAPP (sAPPtot) levels analyzed by Western blotting and Aβ_{40}, Aβ_{42}, and total Aβ levels measured using an ELISA, from cell culture medium of TV1-overexpressing and control cells from panel A (n = 4–6). All parameters were assessed from cell culture medium and normalized to total protein levels. *p < 0.05; **p < 0.01. Mean ± SD. Numbers on the left of the blots indicate molecular masses in kilodaltons.

mediated ε-site cleavage and that TV1 overexpression may lead to a preference of γ-secretase for LAR over APP.

Because the overexpression of TV1 increased the level of *in vitro* generation of AICD, we next elucidated the effects of TV1 on APP processing and γ-secretase-mediated Aβ production in the SH-SY5Y-APP751 cells overexpressing TV1 (Figure 2). Our findings showed that overexpression of TV1 in SH-SY5Y-APP751 cells led to augmented levels of APP CTFs C83 and C99 (Figure 2A). Furthermore, the ratio of mature APP to immature APP was moderately increased in the TV1-overexpressing SH-SY5Y-APP751 cells as compared to control cells. Importantly, TV1-overexpressing SH-SY5Y-APP751 cell lysates, but not control lysates, revealed the appearance of a 6 kDa protein band, which matches the size of AICD (Figure 2A). Because increased APP CTF levels and AICD generation point toward an increased level of APP processing, we measured the total protein normalized soluble APP (sAPPtot and sAPPα) and Aβ levels in the cell culture medium of the TV1-overexpressing SH-SY5Y-APP751 cells. Although the sAPPtot levels were significantly increased, the total protein-normalized Aβ_{40}, Aβ_{42}, and total Aβ levels were unchanged in the culture medium of TV1-overexpressing cells (Figure 2B).

Collectively, our results show that overexpression of TV1 in SH-SY5Y-APP751 cells enhances APP processing and ICD production from two different γ-secretase substrate proteins (AICD and LICD), suggesting that specifically the level of γ-secretase-mediated ε-like cleavage is increased in TV1-overexpressing cells. Importantly, an increased level of AICD production did not coincide with augmented Aβ_{40}, Aβ_{42}, or total Aβ levels. Moreover, TV1 may affect the γ-secretase substrate preference and thus create a competition between APP and LAR for the γ-secretase cleavage, further suggesting that TV1 modulates γ-secretase function.

Overexpression of the Ubiquilin-1 TV2 Variant in SH-SY5Y-APP751 Cells Does Not Affect AICD Generation and APP Processing to an Extent Similar to That of TV1. We have previously shown that a risk variant in the *UBQLN1* gene increases the ratio of TV2 to TV1 mRNA levels in brain tissue.[12] We wanted to determine whether TV2 (lacking exon 8) affects AICD generation in a manner similar to that of TV1. For this purpose, we used myc-tagged TV1 (myc-TV1) and TV2 (myc-TV2) constructs. The *in vitro* AICD generation assay revealed that myc-TV2 failed to enhance C83-normalized AICD generation in SY5Y-APP751 cells [GAPDH-normalized myc-TV2 levels = 728 ± 216% (p < 0.01) compared to endogenous ubiquilin-1 levels in control samples = 100 ± 34% (Figure 3A)]. Conversely, TV1, which was used as a control, revealed an approximate ~1.5-fold increase in the level of C83-normalized AICD production similar to that shown in Figure 1A. These data suggest that TV2 does not modulate γ-secretase-mediated ε-site cleavage of APP in the same way as TV1.

Because there were differences between the two TVs with regard to AICD generation, we also wanted to assess the effects of myc-TV1 and myc-TV2 on APP processing upstream of AICD generation. We found that myc-TV1 overexpression, and to a lesser extent myc-TV2 overexpression, resulted in an increase in C83 and C99 levels [GAPDH-normalized myc-TV1 levels = 1720 ± 1454% (p < 0.05) and GAPDH-normalized myc-TV2 levels = 1014 ± 851% (p < 0.05) compared to endogenous ubiquilin-1 levels in control samples = 100 ± 38% (Figure 3B)]. The total protein-normalized Aβ_{40} and Aβ_{42}

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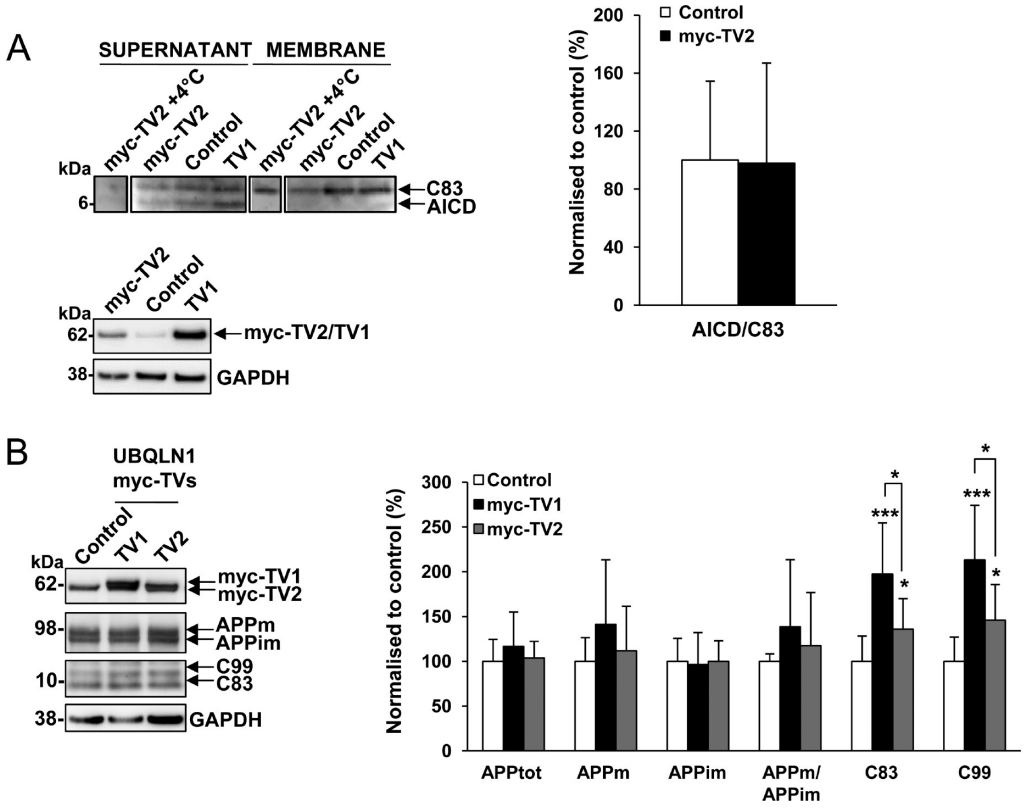

Figure 3. Overexpression of N-terminally myc-tagged TV1 and TV2 in SH-SY5Y-APP751 cells affects APP-CTF production. (A) Western blot showing the effects of myc-TV2 overexpression on AICD production using an *in vitro* AICD generation assay. Quantification of the amount of AICD generated normalized to C83 levels is shown at the right (n = 3). The reaction at 4 °C was used as a negative control. Samples overexpressing TV1 were used as a positive control showing an increased level of AICD generation. The ubiquilin-1-specific antibody (Zymed) was used to detect overexpressed TV1 and TV2. (B) Western blot showing the effects of myc-TV1 and myc-TV2 overexpression on APP levels and processing. Quantification is shown at the right (n = 8–9). *p < 0.05; ***p < 0.001. Mean ± SD. Numbers on the left of the blots indicate molecular masses in kilodaltons.

levels in the cell culture media of both myc-TV1- and myc-TV2-overexpressing cells did not reveal statistically significant changes (data not shown). These results suggest that both TVs exert similar effects on APP processing, but the effects of TV2 are more moderate compared to those of TV1.

Overexpression of TV1 Does Not Affect the Levels or Subcellular Localization of the γ-Secretase Complex Components in SH-SY5Y-APP751 Cells. To study whether the increase in the level of γ-secretase-mediated cleavage was due to increased levels of the γ-secretase complex components, we assessed the levels of PS1-CTF, PS1-NTF, PEN-2, NCT, and APH1A in SH-SY5Y-APP751 cells overexpressing TV1 and PS1 [GAPDH-normalized TV1 levels = 1581 ± 909% (p < 0.05) compared to endogenous ubiquilin-1 levels in control samples = 100 ± 36% (Figure 4A)]. There were no major changes in the levels of the components. We also investigated the endogenous levels of PS1-CTF, PS1-NTF, and NCT in SH-SY5Y-APP751 cells overexpressing only TV1, but not PS1, and found no differences after TV1 overexpression (data not shown). These data indicate that the increase in the ε-like cleavage activity of the γ-secretase is not due to augmented γ-secretase complex component levels in SH-SY5Y-APP751 cells overexpressing TV1.

Previous studies have shown that the ICDs of different γ-secretase substrates are predominantly produced in the plasma membrane and/or early endosomes.[18] We wanted to assess whether the increased level of ICD generation in SH-SY5Y-APP751 cells resulted from increased levels of APP or the γ-secretase complex on the cell surface. Cell surface biotinylation experiments revealed no significant differences in the levels of NCT on the cell surface between TV1-overexpressing and control cells [GAPDH-normalized TV1 levels = 263 ± 45% (p < 0.01) compared to endogenous ubiquilin-1 levels in control samples = 100 ± 40% (Figure 4B)]. Similarly, there were no differences in the levels of endogenously expressed APPm on the plasma membrane between TV1-overexpressing and control cells (Figure 4B). Because the fully active γ-secretase is formed by the four core components whose levels are tightly inter-regulated,[19] the unchanged cell surface NCT levels and PS1 subcellular localization suggest that the levels of the active γ-secretase complex remained unaltered at the plasma membrane. Together, these results suggest that TV1 overexpression does not lead to changes in the cell surface levels of APP or the γ-secretase complex.

Overexpression of TV1 Does Not Modulate FE65 Levels or Binding of FE65 to APP in SH-SY5Y-APP751 Cells. FE65 has previously been shown to stimulate the production of AICD.[20] Also, FE65 was demonstrated to interact with and stabilize AICD and to be involved in AICD-mediated gene transcription.[20] Therefore, we wanted to assess whether the increase in AICD levels in the TV1-overexpressing SH-SY5Y-APP751 cells takes place through the modulation of FE65 levels or through altered binding of FE65 to APP. We overexpressed TV1 and FE65 separately and together and

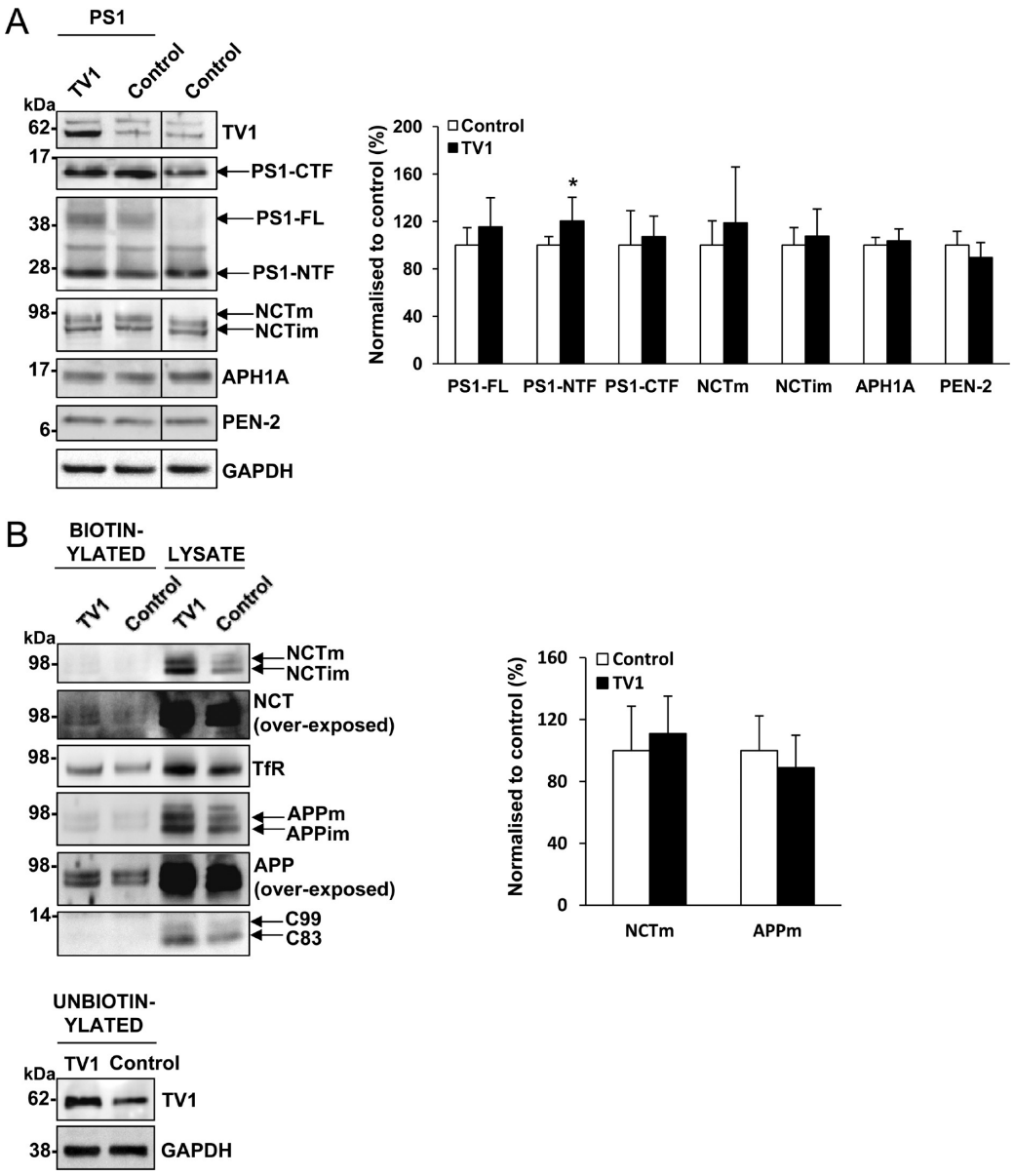

Figure 4. Overexpression of TV1 in SH-SY5Y-APP751 cells does not affect levels of γ-secretase complex components. (A) The levels of the γ-secretase complex components were assessed in the presence of PS1 overexpression using Western blotting. Borders between lanes indicate different wells from the same Western blot gel. Quantifications are shown at the right. (B) Western blot showing the effects of TV1 overexpression on the levels of mature nicastrin (NCT) and mature APP (APPm) on the plasma membrane using a cell surface biotinylation assay.The bottom panel shows the levels of TV1 in the unbiotinylated fraction of the same samples. Quantification of the cell surface levels of mature nicastrin (NCTm) and APP (APPm) is shown at the right (n = 3–6). Abbreviations: C, control; TfR, transferrin receptor. *p < 0.05. Mean ± SD. Numbers on the left of the blots indicate molecular masses in kilodaltons.

subsequently analyzed the effects on APP processing using Western blotting (Figure 5A). In samples overexpressing TV1 separately [GAPDH-normalized TV1 levels = 654 ± 394% (p = 0.001) (Figure 5A)] and in samples overexpressing TV1 together with FE65 [GAPDH-normalized TV1 levels = 691 ± 495% (p < 0.01) compared to endogenous ubiquilin-1 levels in control samples = 100 ± 32% (Figure 5A)], we found that the GAPDH-normalized endogenous and exogenous FE65 levels were increased on average 1.3-fold in the presence of TV1, but the increase was not statistically significant (Figure 5A,C). Overexpression of FE65 alone or in combination with TV1 significantly reduced the levels of APPim, leading to an increase in the APPm/APPim ratio. Co-expression of TV1 with FE65 did not further enhance the decrease in the APPim levels caused by FE65 overexpression. Importantly, the C83 and C99 levels were unaffected by FE65 overexpression (Figure 5A). In fact, co-expression of FE65 with TV1 appeared to prevent the increase in C83 and C99 levels caused by TV1 alone. Thus, these results indicate that the FE65 overexpression phenotype in terms of APP processing is different from that of TV1 overexpression alone.

Because the phosphorylation of threonine 668 in APP (APP-Thr668) is crucial for binding of FE65 to the C-terminus of APP,[21] we wanted to elucidate the phosphorylation status of this site upon TV1 and FE65 expression (Figure 5B). The phosphorylation status of APP-Thr668 was not affected upon expression of TV1 or FE65 in SH-SY5Y-APP751 cells. Interestingly, however, co-expression of TV1 and FE65 showed

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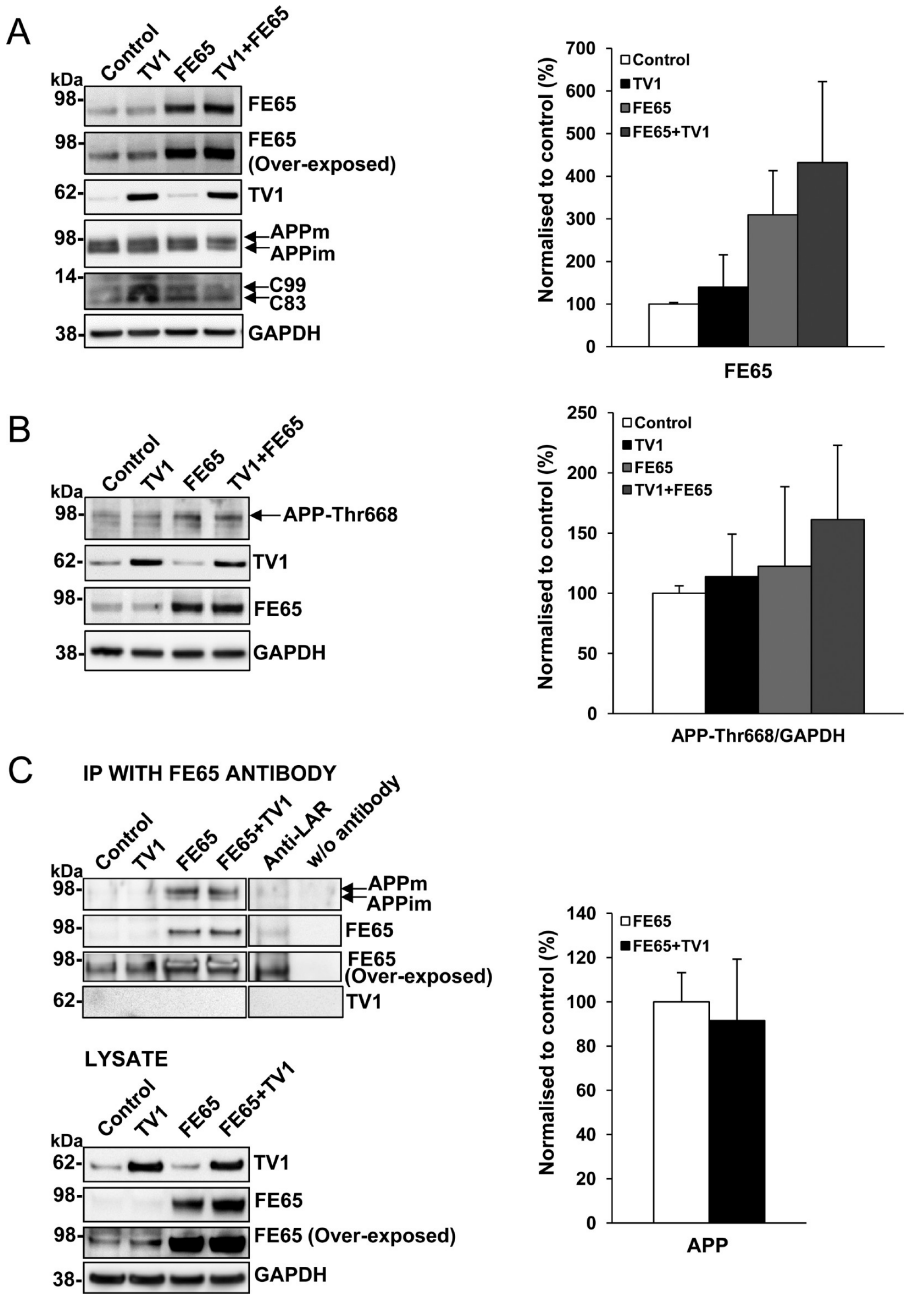

Figure 5. (A) Interaction of APP with FE65 is not affected by TV1 in SH-SY5Y-APP751 cells transiently transfected with TV1 and/or FE65. Western blot showing the effects of TV1 and FE65 overexpression separately and together on APP levels and processing. Overexpression of TV1 increases endogenous as well as exogenous levels of FE65. Quantifications are shown at the right (n = 7−11). (B) The effects of TV1 and FE65 separately and together on APP phosphorylation at threonine 668 (APP-P-Thr668) were assessed by Western blotting. Overexpression of TV1 slightly increases the level of phosphorylation of endogenous as well as exogenous APP at Thr668 (n = 5). (C) Western blot showing results of co-immunoprecipitation of APP with FE65. The FE65 antibody was used for pull down of the protein complexes, and the bands were detected with antibodies against APP, FE65, and ubiquilin-1 (Zymed and U7258). Quantifications show the levels of APP co-immunoprecipitated with FE65 and normalized to FE65 levels in the absence or presence of TV1. The anti-LAR antibody was used as a control, and it did not pull down any APP. The lysate panel shows TV1 and FE65 overexpression in the total protein lysates from the same samples (n = 3−5). Abbreviations: IP, co-immunoprecipitation. *p < 0.05; ***p < 0.001. Mean ± SD. Numbers on the left of the blots indicate molecular masses in kilodaltons.

a trend toward an increase in the level of phosphorylation of APP-Thr668. Because the binding sites for both TV1 and FE65 are at the C-terminus of APP, we hypothesized that co-expression of TV1 and FE65 would create a competition for APP binding between the two proteins. To test this, we performed co-immunoprecipitation experiments in SH-SY5Y-APP751 cells to determine whether there are differences in the amount of APP co-immunoprecipitated with FE65 in the presence and absence of TV1 overexpression. The amount of APP co-immunoprecipitating with endogenous FE65 was below the detection level and therefore could not be quantified (Figure 5C, first two lanes in the top blot). However, APP efficiently co-immunoprecipitated with FE65 in FE65-over-expressing cells, but there were no differences in the APP levels co-immunoprecipitating with FE65 in the presence or absence of TV1. Additionally, TV1 did not co-immunoprecipitate with

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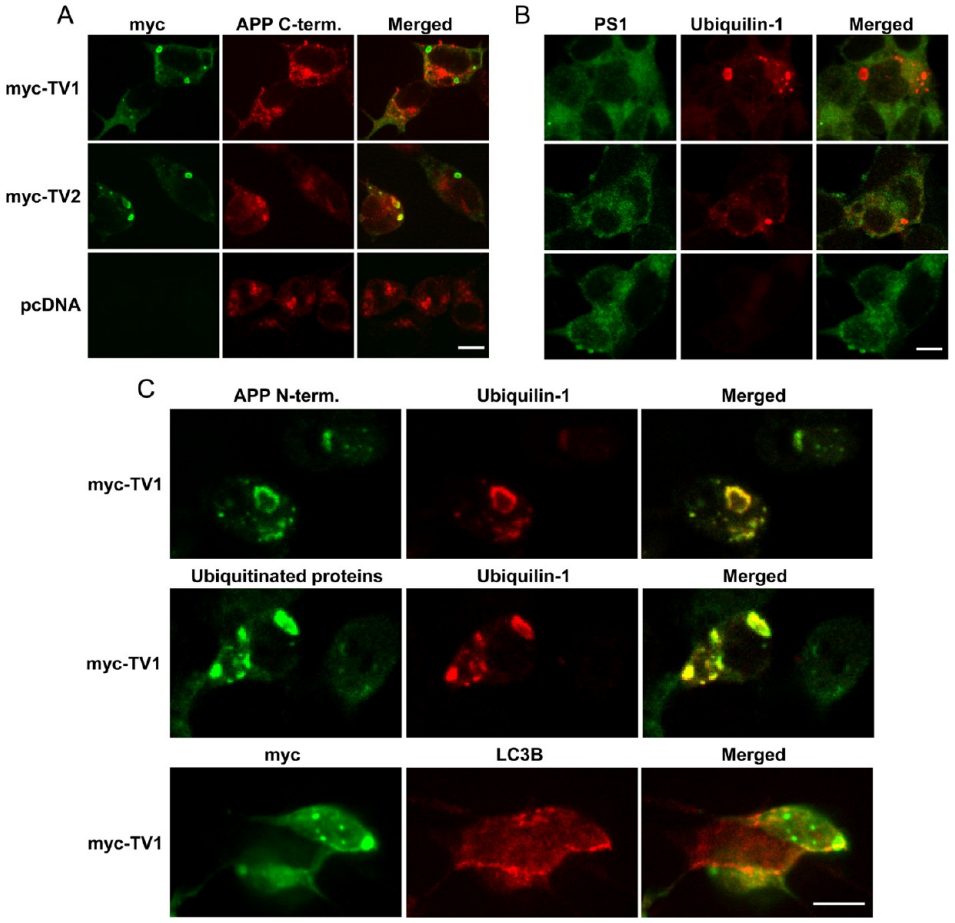

Figure 6. Subcellular localization of APP or PS1 is unchanged in SH-SY5Y-APP751 cells overexpressing TV1 or TV2. (A) Confocal microscope images of SH-SY5Y-APP751 cells overexpressing myc-TV1, myc-TV2, or the pcDNA control plasmid stained using the anti-myc antibody to detect ubiquilin-1 TV1 and ubiquilin-1 TV2 (green) and the anti-APP C-terminal antibody to detect APP (red). APP subcellular localization is similar in TV1, TV2, and control cells. Ubiquilin-1 partially accumulates in bright cytoplasmic structures in both TV1- and TV2-overexpressing cells. These structures are not positive for APP C-terminal antibody staining (merged image). (B) Confocal microscope images of SH-SY5Y-APP751 cells overexpressing myc-TV1, myc-TV2, or the pcDNA control plasmid stained with the anti-PS1 C-terminal antibody to detect PS1 (green) and the anti-ubiquilin-1 antibody (ab3341) to detect TV1 and TV2 (red). There are no changes in PS1 localization in TV1- or TV2-expressing cells as compared to control cells. Like the myc staining in panel A, anti-ubiquilin-1 staining (red) reveals the accumulation of ubiquilin-1 in cytoplasmic structures in TV1- and TV2-overexpressing cells. PS1 does not colocalize with these structures (merged image). (C) Characterization of the ubiquilin-1-positive cytoplasmic structures in cells overexpressing myc-TV1. The top row shows staining of the anti-APP N-terminus (22C11; green) and anti-ubiquilin-1 staining (ab3341; red) colocalized in the cytoplasmic structures (yellow in merged). The middle row shows the FK2 antibody against mono- and poly-ubiquitin (green) and anti-ubiquilin-1 (ab3341; red) costain the cytoplasmic structures (yellow in merged). The bottom row shows anti-myc staining indicating TV1 (green) and anti-LC3B staining (red). LC3B does not colocalize with TV1 in the cytoplasmic structures (merged). The scale bar is 5 μm for all images.

FE65 as determined by using two different antibodies against ubiquilin-1, suggesting that TV1 is not present in the complex of FE65 and APP (Figure 5C). Collectively, these data suggest that TV1 does not affect the phosphorylation status of APP-Thr668, FE65 levels, or binding of FE65 to the C-terminus of APP, excluding the possibility that TV1 augments AICD production through FE65-related mechanisms.

TV1 and TV2 Colocalize with N-Terminal APP in Ubiquitin-Positive Cytoplasmic Structures in SH-SY5Y-APP751 Cells. Our results suggest that TV1 overexpression affects APP maturation and processing and modulates γ-secretase-mediated ϵ-site-like cleavage of APP and LAR and that some differences exist between the effects of TV1 and TV2 on these events. Finally, we wanted to assess whether these effects resulted from altered subcellular localization of APP and/or PS1. To elucidate the possible effects of TV1 or TV2 overexpression on the subcellular localization of APP and PS1, we employed confocal laser scanning microscopy in SH-SY5Y-APP751 cells overexpressing myc-TV1 and myc-TV2 (Figure 6A,B). In the control transfected SH-SY5Y-APP751 cells, APP was localized in the intracellular compartments and to some extent on the plasma membrane. On the basis of our previous subcellular characterization studies in the SH-SY5Y-APP751 cells, APP mainly localizes in the late endosomes and lysosomes (LEL) and Golgi compartments, and these findings are in line with those data.[17,22] There were no differences in the APP subcellular localization among myc-TV1, myc-TV2, and control cells. PS1 was localized on the plasma membrane and within intracellular compartments in SH-SY5Y-APP751 cells where it partially colocalized with ubiquilin-1 in a manner similar to that shown previously by us and others.[6,7] We did not find major differences in PS1 localization between myc-TV1- and myc-TV2-overexpressing cells compared to control cells. These

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results demonstrate that TV1 and TV2 overexpression does not alter the subcellular localization of APP or PS1.

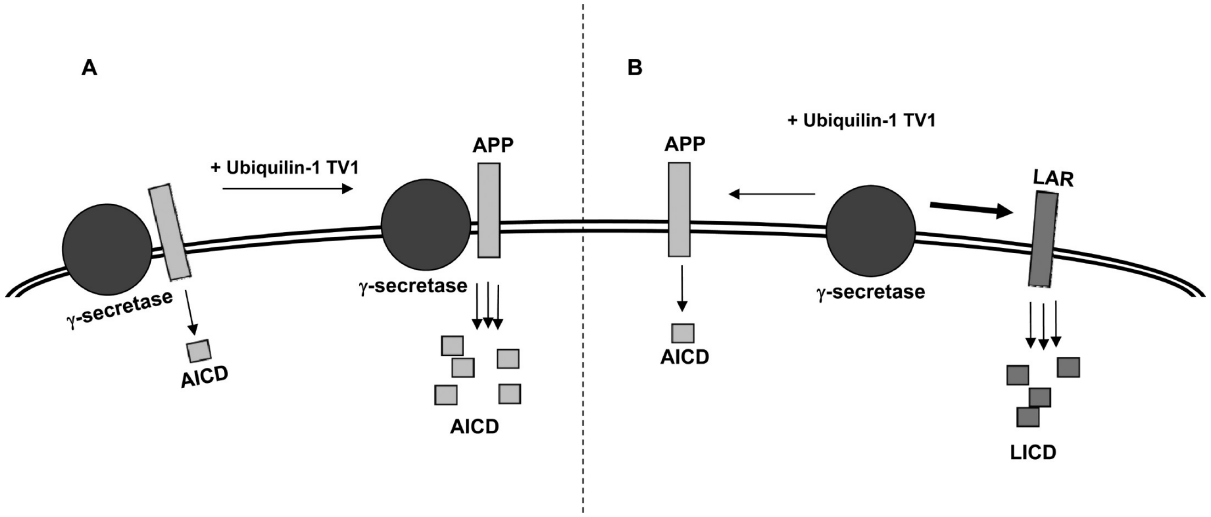
Interestingly, ubiquilin-1 staining revealed the presence of TV1- and TV2-positive cytoplasmic structures in SH-SY5Y-APP751 cells (Figure 6A,B), resembling those seen previously in HeLa cells overexpressing ubiquilin-1.[23] These structures were not positive for an antibody recognizing PS1 or the C-terminus of APP but were positively stained with an antibody against the N-terminus of APP (Figure 6C, top row). Ubiquilin-1 has previously been found to colocalize with ubiquitin-positive structures[24] and suggested to play a role in the ubiquitin proteasome system (UPS).[8,9] Moreover, ubiquilin-1 has been shown to localize in autophagosomes and mediate autophagy-dependent degradation of proteins, such as huntingtin.[14,23] Therefore, we stained the SH-SY5Y-APP751 cells for mono- and poly-ubiquitinated proteins and autophagosomal marker LC3B. Our results indicated that the ubiquilin-1- and APP N-terminus-positive structures also contained mono- and poly-ubiquitinated proteins (Figure 6C, middle row). However, they were not positive for LC3B, suggesting that these structures may not represent autophagosomes (Figure 6C, bottom row). To test whether TV1 induces autophagosome formation as shown previously,[23,25] we assessed the levels of autophagosomal marker LC3 upon TV1 overexpression in SH-SY5Y-APP751 cells. Maturation of LC3-I to LC3-II correlates with autophagosome formation.[26] We found no evidence of the maturation of LC3-I to LC3-II after TV1 overexpression [in samples overexpressing myc-TV1, GAPDH-normalized TV1 levels = 1439 ± 1197% ($p < 0.01$) compared to endogenous ubiquilin-1 levels in control samples = 100 ± 85% (Figure 2 of the Supporting Information)]. This suggests that TV1 overexpression per se does not induce autophagosome formation in the SH-SY5Y-APP751 cells. In summary, these results indicate that overexpressed TV1 and TV2 as well as N-terminal APP accumulate in SH-SY5Y-APP751 cells within ubiquitin-positive structures.

DISCUSSION

Ubiquilin-1 protein associates with AD both genetically and functionally. We have previously shown that ubiquilin-1 downregulation enhances APP maturation and trafficking to the plasma membrane and increases C83 levels and the level of Aβ secretion in HEK293 and H4 neuroglioma cells without affecting the γ-secretase activity.[15] In the study presented here, we found that transient overexpression of the full-length ubiquilin-1 variant (TV1) in SH-SY5Y-APP751 cells resulted in a significant increase in the level of AICD generation in the *in vitro* AICD generation assay, which was coupled with only a modest increase in Aβ levels. Our data are consistent with previous findings in a similar SH-SY5Y-Gal4 cell line.[27] One possible explanation for this finding could be that TV1 specifically modulates the ε-cleavage but to a lesser extent the γ-cleavage of APP, both mediated by γ-secretase activity. This possibility is supported by our finding that TV1 overexpression does not lead to changes in the $Aβ_{40}$, $Aβ_{42}$, or total Aβ levels. It is known that γ-secretase mediates at least three intramembranous cleavages, namely, γ-, ε-, and ζ-cleavages. The γ-cleavage(s) produces different forms of Aβ, such as $Aβ_{40}$ and $Aβ_{42}$, while the ε-cleavage occurs between residues 49 and 50 of the Aβ sequence, releasing the AICD fragment. These cleavages have been reported to take place sequentially, whereby ε-cleavage precedes γ-cleavage.[28−32] However, while both γ- and ε-cleavages are PS-dependent, they may be differentially regulated. For example, some PS1 mutations promote γ-cleavage while inhibiting ε-cleavage,[33] and certain γ-secretase inhibitors reduce the level of Aβ generation without affecting AICD production.[34] Additionally, proteins interacting with PS1 may differentially modulate γ-secretase-mediated cleavages. TMP21, a PS1-interacting protein, has been shown to regulate γ-cleavage but not ε-cleavage of APP[35] by a mechanism that thus far has been elusive. Ubiquilin-1 is another PS-interacting protein. Its C-terminal UBA domain interacts with PS loop and C-terminal domains[36] both *in vitro* and *in vivo*.[10] Because the increase in the AICD levels caused by TV1 was observed using the *in vitro* AICD generation assay, which is performed with extracted cell membranes, it is unlikely that the increased level of AICD generation results from AICD stabilization by cytosolic proteins. Instead, it is possible that ubiquilin-1 TV1 might specifically affect ε-cleavage through interacting with PS1. Additionally, total γ-secretase complex component levels, the levels of NCT on the plasma membrane, or PS1 subcellular localization was unchanged in this study, demonstrating that TV1 does not affect these factors, which could have been the cause of the increased AICD levels. Another modifier of γ-secretase activity is a class of drugs known as γ-secretase modulators (GSMs). GSM activity was first described among a subset of NSAIDs by Koo and colleagues in 2001.[37] These drugs selectively reduce the level of generation of the more aggregation prone $Aβ_{42}$ while increasing the levels of shorter fragments such as $Aβ_{40}$ and $Aβ_{38}$. However, because TV1 did not alter $Aβ_{40}$, $Aβ_{42}$, or total Aβ levels, it is unlikely that TV1 acts as a GSM.

The γ-secretase is known to cleave ∼90 different proteins in addition to APP.[38] These additional substrates, such as Notch, cadherins, or LAR, undergo an ε-site-like cleavage similar to that of APP to release their cognate ICDs, although the exact γ-secretase cleavage site in the case of many substrates has not yet been determined. We have shown that γ-secretase-mediated cleavage of the receptor-type protein tyrosine phosphatase LAR at or adjacent to the boundary of its transmembrane and cytoplasmic domains releases the LICD to the cytosol.[16] In the study presented here, our results indicate that when APP and LAR are co-expressed, TV1 overexpression leads to a significant increase in the level of LICD production at the expense of AICD generation. This suggests a competition between APP and LAR for γ-secretase-mediated cleavage in the presence of TV1, whereby γ-secretase prefers LAR over APP as a substrate. These findings are in accordance with previous studies showing a competition between APP and other γ-secretase substrate proteins, such as Notch 1 or LRP, for γ-secretase cleavage.[39−41] However, the factors regulating the γ-secretase substrate preference are currently not known. On the basis of unchanged total and plasma membrane APP levels, the possibility that the changes in APP trafficking are the cause for substrate competition between APP and Notch 1 was previously ruled out.[41] Instead, it was suggested that a direct modulation of the γ-secretase complex may lead to substrate competition. Similarly, we did not observe changes in the total or plasma membrane APP levels in TV1-overexpressing cells. Furthermore, TV1 did not alter the levels of γ-secretase complex components on the plasma membrane. Additionally, results from the co-immunoprecipitation experiments indicated that neither APP nor LAR co-immunoprecipitated with TV1, arguing against the possibility that the altered interaction(s) of TV1 with the substrates is the mechanism behind the substrate preference. However, it should be emphasized that

Scheme 1. Summary of the Effects of TV1 on γ-Secretase[a]



[a](A) TV1 overexpression leads to an increased level of AICD generation by the γ-secretase. (B) TV1 co-overexpression with APP and LAR leads to an increased level of LICD generation, while the increase in the level of AICD is abolished. This indicates substrate competition between APP and LAR over γ-secretase cleavage.

TV1 and APP have been reported to be in close proximity in intact cells when they were analyzed using fluorescence lifetime imaging microscopy (FLIM).[15] Moreover, recent studies by Stieren et al. indicated that the interaction of ubiquilin-1 and APP is transient and the level of co-immunoprecipitiation of the two proteins is greatly increased by using a cross-linking agent.[42] Thus, it is possible that the experimental conditions used here were not suitable for detecting direct interaction between APP and TV1. Collectively, these findings support the idea that substrate competition between APP and LAR does not result from altered TV1−substrate interaction(s) or TV1-mediated effects on the transport of protein to the plasma membrane but rather is a result of direct γ-secretase modulation.

Alternative splicing of the *UBQLN1* gene results in the generation of at least four different TVs.[11,12] The effects of the different TVs on the key molecular pathogenic events of AD, such as APP metabolism, have thus far been poorly characterized. In this context, ubiquilin-1 TV2, which lacks the protein fragment encoded by exon 8, is especially relevant because the risk variant in the *UBQLN1* gene has been shown to increase the ratio of TV2 to TV1 mRNA levels in brain tissue.[12] Our results here show that as opposed to overexpression of TV1, overexpression of TV2 did not increase the level of AICD generation. The differences between the effects of TV1 and TV2 on APP processing and AICD generation via ε-cleavage could be attributed to the structural differences between the two TVs. Ubiquilin-1 belongs to the family of ubiquitin-like proteins, which contain characteristic UBA and UBL domains. The C-terminal UBA domain has been suggested to interact with proteins such as PS1, while the N-terminal UBL domain is implicated in binding the proteasome complex.[43] TV2 lacks the protein fragment encoded by exon 8 adjacent to the UBA domain, which might induce conformational changes in ubiquilin-1 protein structure and thus modulate interactions with APP or PS1. Our results suggest that different ubiquilin-1 TVs may exert differential biological functions, although further studies are required to characterize these potential TV-specific effects on factors or pathways involved in AD molecular pathogenesis.

FE65 has been shown to contribute to AICD stability and translocation to the nucleus.[20] FE65 has also been suggested to form a complex with AICD that mediates gene transcription, even though the role of AICD in transcription is currently under debate.[44,45] We hypothesized that the TV1-induced increase in AICD levels could be mediated by FE65. However, the overexpression of TV1 in SH-SY5Y-APP751 cells did not significantly affect the levels of FE65. Additionally, FE65 and TV1 overexpression phenotypes were found to be dissimilar. Ando et al. have previously shown in HEK293 cells that FE65 increased the levels of immature APP, thereby delaying APP maturation.[21] This effect was mediated by interaction of FE65 with APP phosphorylated at Thr668. Our findings in SH-SY5Y-APP751 cells indicate that overexpression of FE65 significantly decreased levels of immature APP, which is the opposite of the previous findings.[21] It is possible that these differences result from cell type-specific differences in APP metabolism in non-neuronal and neuronal cells. We also hypothesized that because both FE65 and TV1 bind APP at the C-terminus, binding of TV1 to APP might prevent binding of FE65 to the C-terminus of APP. However, there were no differences in the amount of APP co-immunoprecipitated with FE65 in cells with or without TV1 overexpression. Also, TV1 did not co-immunoprecipitate with FE65 and APP, suggesting that TV1 does not directly bind the FE65−APP complex. Furthermore, we did not observe alterations in the phosphorylation status of APP-Thr668 upon TV1 overexpression, which is an important finding considering that the phosphorylation of APP at this site has been suggested to be indispensable for FE65 binding.[21] Together, these data argue against the possibility that FE65 mediates the increased level of AICD generation in TV1-overexpressing cells.

Confocal microscopy indicated that TV1 and TV2 colocalized together with N-terminal APP in ubiquitin-positive cytoplasmic structures. This is in line with the previous findings showing that ubiquilin-1 plays a role in the proteasomal degradation of ubiquitinated proteins and that ubiquilin-1 enhances K63-linked poly-ubiquitination of APP.[8,9,46] Ubiq-

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uilin-1 has also been shown to mediate protein accumulation, protein aggregation, and autophagy-mediated protein degradation and in this way enhance cytoprotection and cellular survival.[7,14,47,48] Furthermore, our previous data implicate ubiquilin-1 in the accumulation of PS1 and targeting of the accumulated PS1 to the proteasome or the aggresomes.[7] The findings presented here suggest that TV1 and TV2 mediate the accumulation of excess ubiquitinated N-terminal APP, leading to the formation of punctate cytoplasmic structures. The presence of ubiquilin-1 in punctate structures, possibly representing autophagosomes, has also been described previously.[14,25] Indeed, studies conducted in HeLa cells showed that reduction of the ubiquilin-1 levels resulted in a decreased number of autophagosomes because of failed maturation of LC3-I to LC3-II.[23] Moreover, a recent article described that another ubiquilin family member, ubiquilin-4, mediates the interaction of ubiquilin-1 with the autophagosomal machinery, highlighting the importance of ubiquilins in autophagy.[25] However, the punctate cytoplasmic structures we observed were not positive for autophagosomal marker LC3B, and TV1 overexpression did not affect the maturation of LC3, suggesting that TV1 overexpression per se does not induce the formation of autophagosomes in SH-SY5Y-APP751 cells. Alternatively, these observed ubiquitin-positive structures may represent intermediate aggregates containing APP that may later be targeted to the proteasome or autophagy for disposal. These observations are in accordance with a recent report showing that ubiquilin-1 regulates APP degradation by enhancing polyubiquitination of APP-Lys688.[46] Taken together, these results provide further support for the proposed role of ubiquilin-1 in the regulation of protein levels and degradation.

In this study, we have shown that ubiquilin-1 TV1 appears to specifically affect γ-secretase-mediated ε-cleavage of two different γ-secretase substrates, APP and LAR (see Scheme 1). Our observations suggest that the effects of TV1 on APP and γ-secretase are imposed by separate underlying mechanisms that do not involve FE65. We also demonstrate for the first time that the AD-associated TV2 does not modulate γ-secretase-mediated ε-cleavage in a manner similar to that of TV1, suggesting that different ubiquilin-1 TVs may display differential independent functions that can be attributed to their different domain structures. For example, TV2 lacks the protein fragment encoded by exon 8 adjacent to the UBA domain, which may affect the conformation or protein interactions of this domain. Because the ubiquilin-1 levels have been reported to be downregulated in the AD brain irrespective of *UBQLN1* genotype,[42] it is an important to elucidate the effects of ubiquilin-1 on the pathways relevant for AD as they might provide further insights related to the underlying molecular mechanisms. This study sheds light on the possible effects of altered ubiquilin-1 expression on AD-related molecular pathogenesis, such as APP processing and γ-secretase-mediated ε-site cleavage. While the ubiquilin-1-related effects reported here are fairly modest, it should be kept in mind that AD is a progressive disease manifesting slowly over decades. This allows even minor alterations to accumulate over a period of time and lead to profound consequences. Thus, these findings in conjunction with the already existing data suggest that ubiquilin-1 is a pleiotropic modulator, which affects several different molecular pathways linked to AD, and thus may be a potential target when novel therapeutic approaches against AD are considered. Although further *in vitro* and *in vivo* investigations are warranted to fully understand the role of ubiquilin-1 and its different variants in the context of AD molecular pathogenesis, the observations in this study suggest a specific novel role for ubiquilin-1 TV1 in the regulation of γ-secretase-mediated release of ICDs from various γ-secretase substrates.

■ ASSOCIATED CONTENT

Supporting Information

Additional supplemental figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Aβ, amyloid-β; AD, Alzheimer's disease; AICD, APP intracellular domain; APH1, anterior pharynx-defective 1; APP, amyloid precursor protein; APPim, immature APP; APPm, mature APP; APP-Thr668, APP phosphorylated at threonine 668; BACE1, β-site APP-cleaving enzyme 1; CTF, C-terminal fragment; ER, endoplasmic reticulum; GSM, γ-secretase modulator; HEK293, human embryonic kidney 293; HRP, horseradish peroxidase; LAR, leukocyte common antigen-related; LICD, LAR intracellular domain; NCT, nicastrin; NTF, N-terminal fragment; PEN-2, presenilin enhancer 2; PFA, paraformaldehyde; PNT, 1,10-phenanthroline monohydrate; PS, presenilin; PS1 and -2, presenilin 1 and 2, respectively (proteins); PSEN1 and -2, presenilin 1 and 2, respectively (genes); PVDF, polyvinylidene difluoride; sAPP, secreted APP; SD, standard deviation; SNP, single-nucleotide polymorphism; TV, transcript variant; UBA, ubiquitin-associated domain; UBL, ubiquitin-like domain.

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