Ubiquilin 1 (UBQLN1) is a ubiquitin-like protein, which has been shown to play a central role in regulating the proteasomal degradation of various proteins, including the presenilins. We recently reported that DNA variants in UBQLN1 increase the risk for Alzheimer disease, by influencing expression of this gene in brain. Here we present the first assessment of the effects of UBQLN1 on the metabolism of the amyloid precursor protein (APP). For this purpose, we employed RNA interference to down-regulate UBQLN1 in a variety of neuronal and non-neuronal cell lines. We demonstrate that down-regulation of UBQLN1 accelerates the maturation and intracellular trafficking of APP, while not interfering with α-, β-, or γ-secretase levels or activity. UBQLN1 knockdown increased the ratio of APP mature/immature, increased levels of full-length APP on the cell surface, and enhanced the secretion of sAPP (α- and β-forms). Moreover, UBQLN1 knockdown increased levels of secreted Aβ40 and Aβ42. Finally, employing a fluorescence resonance energy transfer-based assay, we show that UBQLN1 and APP come into close proximity in intact cells, independently of the presence of the presenilins. Collectively, our findings suggest that UBQLN1 may normally serve as a cytoplasmic “gatekeeper” that may control APP trafficking from intracellular compartments to the cell surface. These findings suggest that changes in UBQLN1 steady-state levels affect APP trafficking and processing, thereby influencing the generation of Aβ.

Alzheimer disease (AD) is the most common cause of progressive neurological disorder leading to dementia. It is neuropathologically characterized by extracellular deposits of amyloid beta (Aβ) peptide and by the generation of intracellular neurofibrillary tangles. Mutations in the amyloid precursor protein (APP), presenilin-1 (PSEN1), and presenilin-2 (PSEN2) genes are responsible for roughly half of the rare autosomal dominant, early-onset forms of the disease, which usually occur before the age of 60 (1–4). Meanwhile, apolipoprotein E (APOE) is the only commonly accepted susceptibility factor for late-onset AD (5, 6). Most mutations in APP, PSEN1, and PSEN2 genes lead to the increased production of Aβ42 (relative to Aβ40). Aβ is released from APP via sequential proteolytic cleavage by the β- and γ-secretases (7). In addition to APP, the presenilins, and APOE, it is evident that additional AD susceptibility genes exist; successfully identifying these novel risk genes is an extremely important task that will not only facilitate prediction and diagnosis of AD but can also elucidate novel therapeutic approaches to treating and preventing AD.

We have recently shown that genetic variants in the ubiquilin 1 (UBQLN1) gene, located on chromosome 9q22, increase the risk for AD, possibly by altering the expression and alternative splicing of this gene in brain (8). As is often the case with gene variants exerting modest effects on disease risk, subsequent genetic studies have both supported (9, 10) and not supported (11, 12) the initial genetic finding. Further support for the candidacy of UBQLN1 as an AD risk gene will require a comprehensive assessment of the functional role of UBQLN1 in the key pathways relevant to AD pathology. UBQLN1 has previously been shown to interact with presenilin 1 (PS1) and presenilin 2 (PS2) proteins; overexpression of UBQLN1 was reported...
to enhance the accumulation of presenilin holoproteins (13, 14). More recently, down-regulation of UBQLN1 was reported to modulate PS1 endoproteolysis along with protein levels of nicastrin and PEN-2 in non-neuronal cell lines (15). These findings are particularly interesting given that the presenilins, nicastrin, and PEN-2 are all essential components of the γ-secretase complex. Based on these data, it was anticipated that if the steady-state levels of these proteins are controlled by UBQLN1, APP processing and Aβ generation would be affected by changes in UBQLN1 expression. Immunohistochemical analyses have shown that anti-ubiquilin antibodies stain neurofibrillary tangles in AD brain as well as the Lewy bodies in Parkinson disease (13). Moreover, UBQLN1 was recently identified as part of purified polyglutamine aggregates and found to associate with the neuronal intranuclear inclusions in a mouse model of Huntington disease (16). These findings suggest that, beyond AD, UBQLN1 may play a more generalized role in neurodegenerative diseases characterized by abnormal protein accumulations, e.g. frontal lobe dementia, amyotrophic lateral sclerosis, and Parkinson disease.

UBQLN1 is an ubiquitin-like (UBL) protein that contains UBL and ubiquitin-associated (UBA) domains in its N and C termini, respectively. In addition to the presenilins, UBQLN1 plays a central role in regulating the proteasomal degradation of various proteins, including cyclin A, γ-aminobutyric acid receptor, and hepatitis C virus RNA-dependent RNA polymerase proteins (17–19). Interestingly, UBQLN1 has been reported to exert differential effects on these proteins. Although overexpression of UBQLN1 has been reported to enhance presenilin and γ-aminobutyric acid receptor expression, it has also been reported to promote the degradation of cyclin A and hepatitis C virus RNA-dependent RNA polymerase proteins. The UBL domain is known to be responsible for the binding of UBQLN1 to Rpn3, Rpn10a, and Rpn10e proteins in the 19 S subunit of the 26 S proteasome complex, whereas the UBA domain prefers to bind poly-, but not mono-ubiquitinated proteins (20). The UBL domain of UBQLN1 is also responsible for interaction with the ubiquitin-interacting motif of epidermal-growth factor receptor pathway substrate 15 (Esp15) (21). The UBL/ubiquitin-interacting motif-based interaction was proposed to be responsible for the sequestration of certain ubiquitin-interacting motif-containing endocytic proteins like epidermal-growth factor receptor pathway substrate 15 into cytoplasmic ubiquitin-rich protein aggregates. Collectively, these data suggest that UBQLN1 is a key regulatory protein linking the ubiquitination machinery and the proteasome in mammalian cells. Therefore, even minor changes in the expression and/or function of this protein could potentially affect the steady-state levels of multiple protein targets.

Given the association of UBQLN1 gene variants with risk for AD and altered expression in brain (8), we set out to determine the as of yet unknown effects of UBQLN1 on APP synthesis, maturation, and metabolism. For this purpose, UBQLN1 levels were down-regulated in human H4 neuroglioma and human embryonic kidney (HEK293) cells using RNA interference (RNAi), and effects on APP holoprotein levels, APP maturation and turnover, APP ectodomain shedding (sAPPα, -β, and total), and Aβ secretion were assessed. Down-regulation of UBQLN1 dramatically increased the rate of APP maturation and trafficking through the secretory pathway leading to increased secretion of sAPP and Aβ. Meanwhile, α-, β-, and γ-secretase levels and activity were not affected by UBQLN1 RNAi treatment. We also employed a fluorescence resonance energy transfer (FRET)-based assay to show that UBQLN1 and APP come into close proximity in intact cells, independently of the presence of PS1 or PS2.

**EXPERIMENTAL PROCEDURES**

**siRNAs and Plasmids**—Silencer® Pre-designed siRNA targets to exon 5 of the human UBQLN1 gene (GGGCGATGTA-CACAGATAT) was used to knock down UBQLN1 using RNAi (Ambion). Silencer® Negative control #1 siRNA was used as a control in RNAi experiments (Ambion). Human ADAM10 wild-type cDNA was cloned into peak 12 plasmid with HA tag fused at the C terminus. A human BACE1 wild-type cDNA was cloned into pcDNA4 with Myc-His tag fused at the C terminus of BACE1 (22).

**Cell Cultures and Transfections**—H4 human neuroglioma cells overexpressing APP751 or PS1 wild-type were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml G418. H4 naïve cells were grown in the same media, but without G418. HEK293-AP-APP cells overexpressing alkaline phosphatase (AP) and APP695 fusion protein and Bcl-XL/CrmA were grown as previously described (23). AP ectodomain was fused to the N terminus of full-length APP 695 lacking signal peptide (24). PS1/PS2 double knock-out mouse embryonic fibroblasts (PSDKO-MEF) were maintained in OPTI-MEM media containing 10% fetal bovine serum. Human wild-type PS1 overexpressing PSDKO-MEFs were maintained in OPTI-MEM media containing 10% fetal bovine serum and 2.5 μg/ml puromycin. All cells were grown in an incubator at 37°C containing 5% CO2.

Both the UBQLN1 and control siRNA were transfected into H4 cells (APP751/PS1 wild-type overexpressing and naïve) according to the manufacturer’s instructions using a Nucleofector device (Amaxa). Transfection efficiency in each H4 cell line was found to be ~80–90% based on counting of enhanced green fluorescent protein-positive cells. HEK293-AP-APP cells were transfected as previously described (23) using Lipofectamine 2000 (Invitrogen). Transfection efficiency was determined to be ~70–80% according to enhanced green fluorescent protein.

**Western Blotting**—For Western blotting analysis, total protein lysates (30–50 μg/lane) were separated on 4–12% BisTris-polyacrylamide gel electrophoresis and blotted to Immobilon-Blot polyvinylidene difluoride membranes (Bio-Rad). After primary and secondary antibody incubations, proteins were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Primary antibodies against PS1 NTF/full-length (Ab14, a gift from Dr. S. Gandy), CTF/full-length (Ab5232, Chemicon International), NCT (PA1–758, Affinity BioReagents), PEN-2 (PNT1, a gift from Dr. S. Sisodia), and APH1aL (Oncogene) were used in Western blotting for detecting γ-secretase complex components. Mouse anti-Ubiquilin (Zymed Laboratories Inc.) or PA1–759 (Affinity BioReagents)
were used to detect human UBQLN1 levels. Anti-HA (Cell Signaling) and anti-Myc (Cell Signaling) antibodies were used to detect ADAM10 wild-type and BACE1 levels, respectively, from transfected HEK293-AP-APP cells. Anti-ADAM10 (2051, ProSci), anti-BACE1 (PA1–757, Affinity BioReagents) anti-FE65 (E20, Santa Cruz Biotechnology, Santa Cruz, CA), anti-APLP2 (A gift from Dr. W. Wasco) (25), and anti-β-tubulin (Sigma) antibodies were used to detect endogenous levels of these proteins. Western blot images were quantified using Quantity One software (Bio-Rad).

**RNA Extraction and Real-time PCR**—Total RNA was extracted from H4-APP751 cells transfected with UBQLN1 and control siRNAs after 48 h of transfection using RNeasy Mini Kit (Qiagen). Equal quantities of DNase-treated RNA samples were subjected to cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen). Subsequently, SYBR Green Master PCR Mix (Applied Biosystems) and target-specific PCR primers for APP (5'-TGACCCGATGACTCACTCCTCTGACTC-3' and 5'-CCAGGCTGAACACTCTCATT-3'), UBQLN1 (5'-GATCTCCAGTCAGCAAAACA-3' and 5'-GTATTCCACCAAGCTCACTGAGA-3'), and GAPDH (5'-GGTCACCTCTAGTTCACAA-3' and 5'-GTGAGGAGTCTTCTCTTCTT-3') were used for amplification of cDNA samples with iCycler real-time PCR machine (Bio-Rad). PCR primers were designed to amplify a region flank-
Ubiquilin 1 Regulates APP Secretion

**RESULTS**

Down-regulation of UBQLN1 Decreases Levels of APP Holoprotein—Effects of UBQLN1 knockdown on APP holoprotein and APP CTF (C83/C99) levels were assessed in naïve human H4 neuroglioma cells as well as H4 cells overexpressing APP751 (H4-APP751 cells) and HEK293 cells overexpressing APP695 fused to alkaline phosphatase (HEK293-APP695 cells) (Fig. 1). β-Tubulin was used to normalize UBQLN1 levels in all cell lines. UBQLN1 levels were specifically down-regulated an average 60–70% in UBQLN1 siRNA (siUBQLN1) samples compared with control siRNA (scControl)-transfected samples. Levels of the C-terminal fragments, APP-C83 and APP-C99, were unchanged in the H4-APP751 cells, however, both APP immature (APPim) and APP mature (APPm) levels were decreased on average 30% after down-regulation of UBQLN1 (Fig. 1A). Similarly, levels of endogenous APP holoprotein were decreased in H4 naïve cells (Fig. 1B). In the HEK293-APP cells, both overexpressed (APP-APP695) and endogenous APP holoprotein levels were decreased to a similar extent as that observed in the H4 cells (Fig. 1C). Because endogenous APP holoprotein levels were decreased to a similar extent as overexpressed APP in both cell lines, we could rule out artifactual effects of UBQLN1 knockdown on APP owing to overexpression of APP constructs. Interestingly, whereas we did not observe any differences in C83 or C99 levels in the APP overexpressing cells lines, in H4 naïve cells, C83 levels were significantly increased by an average 1.3-fold (p < 0.05). This result suggested that the observed effects of UBQLN1 knockdown effects on APP holoprotein were likely the result of altered APP processing.

4 P. J. Jones, L. Herl, O. Berezovska, A. N. Kumar, B. J. Bacska, and B. T. Hyman, manuscript in preparation.
To further investigate the observed effects of UBQLN1 knockdown on APP holoprotein levels, we assessed effects on APP mRNA levels. For this purpose, APP, UBQLN1, and GAPDH mRNA levels were determined in UBQLN1 siRNA-transfected and control siRNA-transfected H4-APP751 cells using real-time PCR. PCR primers used for APP mRNA quantification were designed to amplify the cDNA region from exons 16 and 17, thus capturing all three major mRNA variants of APP. In the UBQLN1 siRNA-transfected cells, GAPDH-normalized UBQLN1 mRNA levels were reduced to a similar extent (~65%) as UBQLN1 protein in cell lysates. GAPDH-normalized APP mRNA levels did not differ between UBQLN1 siRNA- and control siRNA-transfected samples (Fig. 1D).

**Down-regulation of UBQLN1 Increases Secretion of APP**—We next assessed the effects of UBQLN1 knockdown on the levels of secreted APP (sAPPα, sAPPβ, and total sAPP) in the conditioned media of H4 and HEK293 cells. Western blot analysis revealed that sAPPα/sAP-APPα and sAPPtotal/sAP-APPtotal levels (normalized to total protein) were significantly increased 1.6- to 2.7-fold following UBQLN1 knockdown in conditioned media of H4-APP751 and HEK293-AP-APP cells (Fig. 2, A and B). sAPPβ and sAP-APPβ levels were unchanged, although a slight increase was observed in the conditioned media of H4-APP751 cells following UBQLN1 knockdown. sAPPα levels in conditioned media of H4 naïve cells were significantly increased to that observed for the APP-overexpressing cells following UBQLN1 knockdown (*p* < 0.05, *n* = 3, data not shown). The alkaline phosphatase moiety N-terminally fused to the APP construct in the HEK293-AP-APP cells allowed us to measure sAPPtotal levels using an AP protein assay (Fig. 2C). Consistent with the Western blotting results with the APP N-terminal antibody, sAPPtotal levels (normalized to total protein) were consistently increased an average 2.0-fold (*p* < 0.001, *n* = 8). Thus, UBQLN1 knockdown also increased APP secretion in the HEK293-AP-APP cells. No differences in sAPPtotal levels were observed between control siRNA and mock transfected samples (Fig. 2C). UBQLN1 levels in protein lysates (normalized to β-tubu-
Down-regulation of UBQLN1 were decreased an average 60–70% after UBQLN1 RNAi in H4 and HEK293 cells. Down-regulation of UBQLN1 Increases Aβ Secretion and Does Not Affect Steady-state Levels of ADAM10 or BACE1—We next set out to determine UBQLN1 knockdown affects Aβ secretion or α- and β-secretase levels. First, we transfected HEK293-AP-APP cells with either ADAM10 or BACE1 cDNA constructs together with UBQLN1 siRNA or control siRNA. BACE1 levels were not affected by UBQLN1 knockdown (Fig. 3A). Consistent with previous studies (32, 33), we observed an average 2.5-fold increase in APP-C99 (C89) levels and a decrease in C83 levels in the BACE1-transfected samples compared with mock samples (Fig. 3A). Both pro- and mature levels of ADAM10 were unchanged following UBQLN1 knockdown (Fig. 3B). In addition, endogenous ADAM10 (pro/mature) and BACE1 levels were not affected after UBQLN1 knockdown in HEK-293 AP-APP cells (Fig. 3C).

Next, we measured sAPP total levels from ADAM10- and BACE1-transfected samples using the AP protein assay. In agreement with previous study (23), sAPP total levels in ADAM10- and BACE1-transfected samples were increased an average of 2.5- and 12.1-fold, respectively, when compared with samples transfected with control siRNA alone (Fig. 3D). In samples co-transfected with ADAM10 plus UBQLN1 siRNA, sAPP total levels were increased to a similar extent (2.4-fold) as in samples transfected with UBQLN1 siRNA alone. Similar increases in sAPP-APPα/sAPPα levels were observed in samples co-transfected with ADAM10 plus UBQLN1 siRNA using 6E10 for Western blot analysis of conditioned media (Fig. 3B). In BACE1 plus UBQLN1 siRNA-transfected samples, the increase in sAPP total levels (1.5-fold) was not as pronounced as in the ADAM10 plus UBQLN1 siRNA-transfected samples (2.4-fold). However, Western blot analysis of conditioned media from BACE1 plus UBQLN1 siRNA-transfected samples revealed an average of 3.0-fold increase (p < 0.001, n = 4) in sAPP-APPβ levels (Fig. 3A). These data suggest that UBQLN1 knockdown affects both sAPPα and sAPPα secretion to a similar extent.
untransfected samples. Interestingly, BACE1 overexpression also elevated $\alpha\beta \times 42$ levels (4.0-fold increase) more profoundly than $\alpha\beta \times 40$ levels (2.0-fold increase). Co-transfection of BACE1 and UBQLN1 siRNA significantly increased $\alpha\beta \times 40$ levels (1.3-fold, $p < 0.05$), whereas $\alpha\beta \times 42$ levels were also increased, but not as significantly as those of $\alpha\beta \times 40$.

Down-regulation of UBQLN1 Does Not Affect Steady-state Levels of $\gamma$-Secretase Components or in Vitro AICD Genera-
We next asked whether increased Aβ secretion following UBQLN1 down-regulation was linked to effects on the γ-secretase complex. For this purpose, we assessed steady-state levels of the γ-secretase components PS1, nicastrin, PEN-2, and APH1αL in H4 cells stably overexpressing wild-type PS1. Levels of γ-secretase components were not affected following 60–70% siRNA-mediated down-regulation of UBQLN1 protein levels (Fig. 4A). UBQLN2 was almost undetectable in this cell line, and no differences in UBQLN2 protein levels were observed between the UBQLN1 siRNA- and control siRNA-transfected samples. Since down-regulation of UBQLN1 was previously reported to modulate PS1 endoproteolysis along with the protein levels of nicastrin and PEN-2 in HEK-293 cells (15), we measured the levels of γ-secretase components in HEK-293 AP-APP cell line not overexpressing PS1 (Fig. 4B). Despite the 70% down-regulation of UBQLN1, endogenous PS1 CTF/NTF, nicastrin, PEN-2, and APH1αL levels were unaffected.

To determine the effects of UBQLN1 knockdown on γ-secretase activity, an in vitro APP intracellular domain (AICD) generation assay was performed in the H4-APP751 cells. Membrane fractions of transfected samples were incubated at +4°C and +37°C for 2 h, after which both the membrane fractions and the supernatants were analyzed using APP C-terminal antibody in Western blots to detect C83 and AICD (Fig. 4C). Down-regulation of UBQLN1 was confirmed in the cytosolic protein fraction; as expected UBQLN1 was not detectable in the membrane fraction. Consistent with unchanged γ-secretase component levels, down-regulation of UBQLN1 did not affect in vitro generation of AICD (normalized to APP C83 fragment).

**Down-regulation of UBQLN1 Does Not Affect the Turnover of APP**

To further investigate the mechanism responsible for altered secretion of sAPP and Aβ following knockdown of UBQLN1, we performed a series of experiments, including a cycloheximide degradation time course to test whether UBQLN1 knockdown affects the turnover/degradation of APPim in H4-APP751 cells. We observed no significant differences in the mean half-lives of APPim between UBQLN1 knockdown (39 ± 3 min) and control (42 ± 4 min) samples (p = 0.23, n = 3) during the 3-h time course (Fig. 5A). In accordance with previous results (Fig. 1A), UBQLN1 knockdown significantly decreased both APPim levels and APPm levels (−30%, normalized to β-tubulin) at time point 0 h (p < 0.05, n = 3), whereas APP C83 and C99 levels (normalized to β-tubulin) were comparable to control levels (Fig. 5A). After 30 min of cycloheximide treatment, however, APPim levels (normalized to β-tubulin) were significantly more affected (~39%) by UBQLN1 knockdown than were APPm levels (~2%) (p < 0.05, n = 3) (Fig. 5B). Consistent with this observation, comparison of the 0- and 30-min time points revealed that the APPm/APPim ratio was significantly increased an average of 1.5-fold after 30 min of cycloheximide treatment (Fig. 5C). Similar increases were also observed at later time points (Fig. 5A). Although C83 and C99 levels (normalized to β-tubulin) were unchanged at both 0- and 30-min time points (Fig. 5A), levels of both CTFs were significantly increased, relative to APPtot following UBQLN1 knockdown at the 30-min time point (Fig. 5D). These data indicate that APP-CTF levels were not decreased simultaneously with APP holoprotein levels following down-regulation of UBQLN1. UBQLN1 levels were not affected by 3-h cycloheximide treatment, in agreement with previous study (13). Taken together, these data suggest that, although UBQLN1 knockdown does not affect APPim half-life, it does serve to accelerate APP maturation, consistent with increased APP secretion.

**Down-regulation of UBQLN Increases the APPm/APPim Ratio**

The cycloheximide degradation time course experiments suggested that knockdown of UBQLN1 may increase the APPm/APPim ratio owing to accelerated maturation of APP. However, to rule out the possibility that the effect of UBQLN1 down-regulation on APP maturation was not simply a consequence of cycloheximide blocking APP translation, we carried out a pulse-chase assays with [35S]Met in H4-APP751 cells (Fig. 6A). Similar to the results of the cycloheximide time course, the APPm/APPim ratio was significantly increased an average 1.6-fold (p < 0.01, n = 4) after a 30-min chase in the UBQLN1 knockdown samples (Fig. 6B). Simultaneously, C83 levels were increased relative to APPtot in the UBQLN1 knockdown samples, in agreement with the cycloheximide experiments (Fig. 5C). Meanwhile, total APP levels were decreased an average 21%, consistent with the notion that UBQLN1 knockdown leads to faster maturation and secretion of APP. The ratio of APPim between 0 and 30 min (30-min APPm/0-min APPim) was not significantly different between UBQLN1 and control siRNA samples. This is also consistent with the cycloheximide time-course results in which the APPim half-life was not affected by UBQLN1 knockdown. UBQLN1 protein levels (normalized to β-tubulin) were decreased an average 70% following UBQLN1 RNAi treatment.

**Down-regulation of UBQLN Increases Cell Surface Levels of APP**

To further investigate the ability of UBQLN1 knockdown to enhance APP maturation, APP secretion, and Aβ generation, we next set out to test whether levels of cell

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**FIGURE 3. Down-regulation of UBQLN1 increases Aβ secretion but does not affect steady-state levels of ADAM10 or BACE1.** A, co-transfection of HEK293-AP-APP cells with Myc-tagged BACE1 and UBQLN1 siRNA (siUBQLN1) did not affect β-tubulin-normalized BACE1 levels (n = 4). Overexpression of BACE1 increased APP-C99 (C89) levels and decreased APP-C38 levels. sAPPβ levels (normalized to total protein) were increased in conditioned media on average 3-fold after co-transfection of BACE1 and UBQLN1 siRNA as compared with samples transfected with BACE1 and control (siControl) siRNA. B, co-transfection of HEK293-AP-APP cells with HA-tagged ADAM10 and UBQLN1 siRNA did not affect β-tubulin-normalized levels of pro- or mature ADAM10 (n = 4). In addition, APP-C83 and APP-C99 levels were not affected in samples co-transfected with ADAM10 plus UBQLN1 siRNA compared with ADAM10 plus siControl samples. C, endogenous ADAM10 (pro/mature) and BACE1 levels (β-tubulin normalized) were not affected by UBQLN1 knockdown in HEK293-AP-APP cells (n = 4). D, sAPPtot levels (normalized to total protein) were determined in conditioned media from HEK293-AP-APP cells using the APP assay after co-transfection with ADAM10 or BACE1 constructs together UBQLN1 (siUBQLN1) or control (siControl) siRNAs (n = 4). sAPPtot levels of co-transfected samples were calculated relative to control siRNA sample (normalized to 1). E, Aβ×40 and Aβ×42 levels (normalized to total protein) were measured using a sandwich enzyme-linked immunosorbent assay (n = 4) in conditioned media of HEK293-AP-APP cells transfected with UBQLN1 (siUBQLN1) siRNA or control (siControl) siRNAs, alone or together with BACE1. UBQLN1 knockdown increased levels of both Aβ×40 and Aβ×42. **p < 0.05; ***p < 0.01; ****p < 0.001.
Ubiquilin 1 Regulates APP Secretion

(A) Western blot analysis showing the expression levels of various proteins under different conditions. The figure includes a comparison of UBQLN1 and control groups, with normalized protein bands illustrating the regulation of APP secretion.

(B) Similar analysis to (A) but with an additional condition (+37°C) for UBQLN1 and control groups. The graphs indicate the effect of temperature on protein expression.

(C) Western blot with temperature conditions (+4°C and +37°C) applied to different samples. The blot shows the expression of AICD and C83 proteins with a focus on UBQLN1 and control groups. The bar graphs illustrate the normalized expression levels of these proteins.

These results suggest that ubiquilin 1 plays a critical role in the regulation of APP secretion, possibly through temperature-dependent mechanisms.
surface holo-APP are increased following down-regulation of UBQLN1. For this purpose, H4-APP751 cell surface proteins were biotinylated with sulfo-NHS-LC-Biotin at +4 °C, immunoprecipitated with streptavidin-agarose beads, and then analyzed by Western blotting with an APP C-terminal antibody. APP<sub>m</sub> levels (normalized to transferrin receptor (TFR)) were significantly increased an average 1.5-fold in the UBQLN1 knockdown samples as compared with control samples (Fig. 7, A and B). Also, comparison of raw values for levels of APP<sub>m</sub> and TFR in the UBQLN1 siRNA and control siRNA samples revealed that APP<sub>m</sub> levels were increased while TFR levels were unchanged. A faint APP<sub>m</sub> band was observed in the biotinylated samples suggesting that a minor portion of unbiotinylated APP<sub>m</sub> may have been pulled down together with biotinylated APP<sub>m</sub>. However, as expected, we did not observe any APP-CTFs or UBQLN1 in the biotinylated protein fraction. UBQLN1 was detected in the unbiotinylated protein supernatant obtained after streptavidin immunoprecipitation. To test whether greater amounts of newly synthesized APP is localized to the cell surface following down-regulation of UBQLN1, H4-APP751 cells were pulsed for 20 min with [35S]Met and chased for 20 min. Subsequently, cell surface proteins were biotinylated, and the levels of radiolabeled APP molecules on the cell surface were determined using sequential immunoprecipitation with APP C-terminal antibody followed by streptavidin. Consistent with the non-radioactive biotinylation results (Fig. 7, A and B), more 35S-Met-labeled/biotinylated APP<sub>m</sub> was detected at the cell surface (Fig. 7C) following knockdown of UBQLN1. TFR normalized AP-APP levels were also significantly increased (p < 0.05, n = 4, data not shown) an average 1.3-fold in HEK293-AP-APP cells following UBQLN1 knockdown.

**UBQLN1 and APP Are in Close Proximity in Intact Cells**—To determine whether close proximity between UBQLN1 and APP could be a determinant in APP maturation and trafficking, we developed a high throughput FRET-based assay using MEFs from double knock-out mice for PS1 and PS2 (PSDKO-MEF) that overexpress wild-type human PS1. In these cells, UBQLN1 and APP are expressed at endogenous levels that are sufficient for immunostaining and for the lifetime analysis. To assess changes in donor fluorophore lifetime, the UBQLN1 N terminus (UBQLN1-NT) was labeled with the donor fluorophore Alexa 430, and the APP C terminus (APP-CT) was labeled with the acceptor fluorophore Cy3. As is shown in Table 1, the lifetime of Alexa 430 (labeling the UBQLN1-NT) in the absence of an acceptor fluorophore was ~3800 ps. A second lifetime of ~1100 ps was observed when APP-CT was labeled with an acceptor fluorophore, indicating FRET, i.e. close proximity between a significant percentage of the two epitopes. The measured FRET strength varied between 11 and 27%, suggesting that approximately one-fifth of the donor-fluorophore-labeled UBQLN1 molecules were in close proximity to a Cy3-labeled APP molecule. However, several attempts to co-immunoprecipitate APP and UBQLN1 from overexpressing cells or brain homogenates were unsuccessful. This could be due to the transient state of the interaction or might result from the small fraction of interacting molecules, as indicated by the low FRET strength in the high throughput FRET assay.

To test whether close proximity between UBQLN1 and APP occurs independently of the reported interaction of UBQLN1 with PS1 (13), the same FRET-based assay was performed in the PSDKO-MEF cells with no PS1 or PS2 present. As shown in Table 2, a second lifetime representing a similar FRET efficiency was observed with a FRET strength of between 14 and 25%, when the UBQLN1-NT and the APP-CT were both labeled with donor and acceptor fluorophores, respectively. Taken together, these data indicate that UBQLN1-NT and APP-CT are in close proximity to each other independently of the presence of PS1 and PS2 in the cell.

**Down-regulation of UBQLN1 Decreases APLP2 Holoprotein Levels but Does Not Affect Steady-state Levels of FE65**—To assess whether the effects of UBQLN1 knockdown are specific for APP, we next measured levels of the APP homologue amyloid β (A4) precursor-like protein 2 (APLP2) following UBQLN1 RNAi treatment (Fig. 8A). APLP2 immature levels (normalized to β-tubulin) were decreased an average 50% following UBQLN1 knockdown in H4-APP751 cells, whereas APLP2 mature levels were unchanged (Fig. 8B). As observed with APP, the ratio of APLP2 mature relative to APLP2 immature was increased an average 2.2-fold following UBQLN1 knockdown in H4-APP751 cells, whereas APLP2 mature levels were unchanged (Fig. 8B). As observed with APP, the ratio of APLP2 mature relative to APLP2 immature was increased an average 2.2-fold following UBQLN1 knockdown (Fig. 8B), ruling out the possibility that UBQLN1 knockdown directly affects FE65 levels.

**DISCUSSION**

We have shown that down-regulation of UBQLN1 increases APP maturation and secretion resembling the phenotype previously seen in FE65/FE65L-overexpressing cells (34, 35), we next determined whether UBQLN1 knockdown affects FE65 levels in H4-APP751 cells (Fig. 8A). Steady-state levels of FE65 remained unchanged following UBQLN1 knockdown (Fig. 8B), ruling out the possibility that UBQLN1 knockdown directly affects FE65 levels.
UBQLN1 Regulates APP Secretion

A

|          | siControl | siUBQLN1 |
|----------|-----------|----------|
| Time (h) |           |          |
| 0        | 0.5       | 1.5      | 3.0      |
| APPIm    | 0         | 0.5      | 1.5      | 3.0      |
| C99      | 10        | 10       | 10       | 10       |
| C83      | 98        | 98       | 98       | 98       |
| UBQLN1   | 50        | 50       | 50       | 50       |
| β-TUBULIN| 62        | 62       | 62       | 62       |

B

C

D

FIGURE 5. UBQLN1 knockdown does not affect the half-life of APPim in a cycloheximide degradation time course. Cycloheximide degradation time-course experiments were conducted on H4-APP751 cells transfected with UBQLN1 (siUBQLN1) or control (siControl) siRNAs. A, after 48 h of transfection, cells were treated with 30 µg/ml cycloheximide for 0.5, 1.5, and 3.0 h and subsequently analyzed by Western blotting with anti-APP, anti-UBQLN1, and anti-β-tubulin antibodies (n = 3). Down-regulation of UBQLN1 did not affect immature APP (APPim) turnover during this time course. B, UBQLN1 knockdown decreased levels of immature APP (APPim) and mature APP (APPm) significantly at time point 0 (normalized to β-tubulin). However, after 30 min of cycloheximide treatment, APPm levels were decreased to similar extent as in the 0-time point, whereas APPim levels were still comparable to the levels with control siRNA. C, the APPm/APPim ratio between 0 and 30 min was significantly increased on average 1.5-fold. D, UBQLN1 knockdown significantly increased levels of APP-C83 and APP-C99 relative to APPtotal in 30-min time point. *, p < 0.05, ±S.D.

amounts of newly synthesized, radiolabeled holo-APP accumulate at the cell surface. Additionally, biotinylation of cell surface proteins revealed that more holo-APP was located on the cell surface following knockdown of UBQLN1. UBQLN1 knockdown also significantly increased the APPm/APPim ratio as assayed by cycloheximide degradation time-course and pulse-chase experiments. These findings indicate that down-regulation of UBQLN1 affects the APP maturation process in the early secretory pathway. The effects of UBQLN1 knockdown on APP maturation and trafficking appear to be independent of γ-secretase, because steady-state levels of γ-secretase complex components and γ-secretase activity (based on in vitro generation of AICD) were not affected. In addition, UBQLN1 knockdown did not affect the levels of BACE1 and α-secretase candidate, ADAM10, or their functions. It is still possible that UBQLN1 knockdown may affect other α-secretase candidates such as TACE (tumour necrosis factor α-converting enzyme) and ADAM9. However, this possibility seems unlikely in light of the combined effects of UBQLN1 knockdown on APP maturation, trafficking, cell surface localization, and sAPP/Aβ secretion. Consistent with the notion of accelerated trafficking and processing of APP, UBQLN1 knockdown also significantly decreased steady-state levels of APP holoprotein, particularly immature APP. In contrast, levels of APP-CTFs were either unchanged (in APP-overexpressing cell lines) or increased (C83 levels in H4 naïve cells). UBQLN1 knockdown increased both C83 and C99 levels relative to APPim in H4-APP751 cells after 30 min of cycloheximide treatment or pulse chase. Collectively, our data suggest that UBQLN1 knockdown enhances the flux of APP through the secretory pathway leading to an increased portion of mature APP reaching the cell surface and increased secretion of sAPP and Aβ. Because we also observed that UBQLN1 knockdown also increased levels of secreted Aβ in HEK293 cells, it is unlikely that accumulation of APP on the cell surface is a consequence of the decreased rate of endocytosis. In support of this, it has recently been shown that the overexpression of dynamin I dominant negative mutant (K44A) increases shedding of the APP ectodomain (sAPPα) while significantly reducing the release of Aβ in HEK293 cells consistent with the...
premise that APP internalization is necessary for Aβ generation in HEK293 cells (36). In this context, however, it should be noted that, in HeLa cells, the dynamin I mutant (K44A) not only increased sAPP levels, but also increased endogenous Aβ secretion suggesting that Aβ can be produced directly at the plasma membrane (37).

UBQLN1 has previously been shown to interact with PS1 and PS2, and overexpression of UBQLN1 enhanced the accumulation of PS holoprotein in non-neuronal cells (13). More recently, it was reported that UBQLN1 knockdown increases levels of the presenilin N-terminal and C-terminal endoproteolytic fragments, along with levels of nicastrin and PEN-2 in HEK293 cells (15). In the present study, down-regulation of UBQLN1 did not affect levels of PS1 holoprotein or its endoproteolytic fragment in H4 cells line stably overexpressing wild-type PS1. In agreement with Massey et al. (15), we did observe a slight, but statistically insignificant increase in steady-state levels of PEN-2 (1.1-fold) and nicastrin (1.2-fold) following down-regulation of UBQLN1; APH1aL levels were unchanged. Similar to results obtained from H4 cells, we did not detect any changes in γ-secretase component levels in both neuronal and non-neuronal cell lines, UBQLN1 knockdown did not affect in vitro generation of AICD, indicating that down-regulation of UBQLN1 does not interfere with γ-secretase activity in human H4 neuroglioma cells. Thus, the discrepancy between our study and that of Massey et al. (15) regarding the effects of UBQLN1 knockdown on γ-secretase complex component levels cannot be explained simply by use of different cell lines (non-neuronal versus neuronal). Because down-regulation of UBQLN1 altered levels of PS1, nicastrin, and PEN-2 by ~1.2- to 1.4-fold in normal HEK-293 cells (15), it is possible that such subtle changes may have been undetectable in the present study.

Although UBQLN1 knockdown did not affect PS1 levels in the current study, it is interesting to note that UBQLN1 knockdown and PS1 deficiency or the expression of PS1 loss-of-function mutants (38, 39) exerted similar effects on at least the maturation and trafficking of APP. In contrast to our study, Aβ
Ubiquilin 1 Regulates APP Secretion

TABLE 1

| FRET donor, Alexa 430 | FRET acceptor, Cy3 | Alexa 430 lifetime, mean ± S.D. | FRET strength, mean ± S.D. | p value, compared to Alexa 430 alone |
|----------------------|------------------|-------------------------------|--------------------------|-----------------------------------|
| UBQLN1 NT (n = 48)   | Cy3 anti-Alexa 430 | 3753 ± 36                     | 39 ± 7                   | p < 0.0001                        |
| UBQLN1 NT (n = 47)   | APP CT            | 1135 ± 46                     | 15 ± 7                   | p < 0.0001                        |
|                     |                  |                               |                          |                                   |

TABLE 2

| FRET donor, Alexa 430 | FRET acceptor, Cy3 | Alexa 430 lifetime, mean ± S.D. | FRET strength, mean ± S.D. | p value, compared to Alexa 430 alone |
|----------------------|------------------|-------------------------------|--------------------------|-----------------------------------|
| UBQLN1 NT (n = 56)   | Cy3 anti-Alexa 430 | 3756 ± 100                  | 78 ± 15                  | p < 0.0001                        |
| UBQLN1 NT (n = 54)   | APP CT            | 1095 ± 167                   | 19 ± 6                   | p < 0.0001                        |

**FIGURE 8.** Down-regulation of UBQLN1 decreases APLP2 holoprotein levels but does not affect steady-state levels of FE65.

**A**. H4-APP751 cells were transfected with UBQLN1 (siUBQLN1) or control (siControl) siRNAs for 48 h, and the APLP2 and FE65 levels were determined using Western blot analysis (n = 4). B, quantification of APLP immature (m) and mature (m) levels (β-tubulin normalized) revealed that APLP2m levels were significantly decreased similar to APP while APLP2m levels were unchanged. The APLP2m/APLP2m ratio was significantly increased on average 2.2-fold. Steady-state levels of FE65 remained unchanged following UBQLN1 knockdown. **, p < 0.01, ± S.D.

levels were not increased in these two PS1 loss-of-function studies owing to lack of γ-secretease activity. It has previously been suggested that PS1 regulates the recruitment or association of trafficking factors with cytoplasmic sorting signals within APP, thus controlling the sorting of APP to the cell surface (39). In this context, it is tempting to speculate whether UBQLN1 is one of those suggested trafficking factors, a “gatekeeper,” which together with PS1 and other trafficking factors co-operatively modulates APP trafficking to the cell surface in the secretory pathway. In this scenario, down-regulation of UBQLN1 might be expected to potentiate APP trafficking from the trans-Golgi network to the cell surface. On the other hand, it was recently shown that APP and PS1 are packed differently into COPII vesicles early in the secretory pathway suggesting that APP and PS1 trafficking from the ER are normally uncoupled (40).

UBQLN1 has been reported to regulate nicotine-induced up-regulation of neuronal nicotinic acetylcholine receptors (41). Overexpression of UBQLN1, together with neuronal nicotinic acetylcholine receptors, dramatically reduced the expression of receptors on the cell surface. Consistent with the hypothesis that UBQLN1 and PS1 co-operatively regulate trafficking of proteins to the cell surface, Leem et al. (38) showed that a PS1 deletion mutant increased assembly and surface expression of nicotinic acetylcholine receptors, in addition to APP. These data suggest that other cell surface proteins, beyond APP, may also be affected by UBQLN1 knockdown. Because UBQLN1 knockdown did not affect trafficking of TFR to the cell surface, protein trafficking was not globally affected. However, the fact that APLP2 holoprotein levels and maturation were changed in a similar manner to that observed for APP suggests that UBQLN1 down-regulation also affects trafficking of other APP family members.

Because UBQLN1 has previously been shown to interact with the cytoplasmic loop and C-terminal domain of PS1 (13), we considered the possibility that UBQLN1 modulates APP trafficking via PS1. Given that we could observe FRET between fluorescently labeled APP and UBQLN1 proteins in intact cells independently of the presence of PS1 and PS2, the presenilins do not seem to be required for bringing UBQLN1 and APP into close proximity. It has been previously shown that overexpression of FE65/FE65L leads to similar effects on APP maturation/secretion as those observed here following knockdown of UBQLN1 (34, 35). Based on these data, we speculated that UBQLN1 might regulate the binding of adapter proteins such as FE65 to APP or, alternatively, that down-regulation of UBQLN1 might modulate FE65 levels through an unknown mechanism. In the current study, we did not observe any changes in FE65 levels following UBQLN1 knockdown, ruling out the possibility that UBQLN1 directly regulates FE65 levels. However, keeping in mind that UBQLN1 is in close proximity to APP, it remains to be determined whether the down-regulation of UBQLN1 modulates binding of other adapter proteins to APP, e.g. FE65L, X11, and others.
In summary, we have observed that down-regulation of UBQLN1 accelerates APP maturation and secretion while not interfering with α-, β-, or γ-secretase levels or function in both neuronal and non-neuronal cell lines. We also show that knockdown of UBQLN1 increases cell surface levels of APP, and secretion of sAPP, Aβ40, and Aβ42. Finally, we showed that UBQLN1 comes into close proximity to APP in the absence of PS1 in intact cells. Collectively, our findings suggest that UBQLN1 may normally serve as a cytoplasmic gatekeeper, which together with PS and other factors may control APP trafficking from intracellular compartments to the cell surface. These findings also suggest that changes in UBQLN1 steady-state levels in the brain may affect APP trafficking and processing, thereby influencing the generation of sAPP and Aβ, and thus risk for AD. These data, together with previous data implicating DNA variants in UBQLN1 gene as minor risk factors conferring for late-onset AD, warrant further investigation of the potential role of UBQLN1 in the etiology and pathogenesis of AD.

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Ubiquilin 1 Regulates APP Secretion

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