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New Implications for the Role for Ubiquilin-1 in Molecular Mechanisms of Alzheimer's Disease: Interrelationship with BACE1

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Abstract

Ample evidence links ubiquilins to the pathogenesis of various neurodegenerative disorders. Ubiquilin-1 (also called PLIC-1) is associated to the pathogenesis of Alzheimer's disease (AD) both genetically and functionally as indicated by investigations in different *in vitro* and *in vivo* models and human brain. Previous studies by us and others have identified ubiquilin-1 as a central regulator of the metabolism, subcellular localization, trafficking, as well as accumulation and degradation of various neurodegenerative disease-linked proteins, including the AD-associated β-amyloid precursor protein (APP) and presenilins. Our recent report reveals a previously uncharacterized relationship between ubiquilin-1 and AD-associated β-site cleaving enzyme 1 (BACE1), the rate-limiting enzyme in the generation of the β-amyloid (Aβ) peptides, in cell-based model systems *in vitro* as well as in the brains of AD model mice *in vivo* and human patients. Our data suggest that ubiquilin-1 controls BACE1 levels and localization to the late endosomal compartment, the preferred cellular site for Aβ generation. Therefore, the observed decreased levels of ubiquilin-1 in AD brain may result in altered APP processing and Aβ accumulation. Here, we provide a short review on the links between ubiquilin-1 and mechanisms of AD and some other neurodegenerative diseases and then summarize the data in our recent report regarding the newly observed interrelationship between ubiquilin-1 and BACE1.

Keywords: Alzheimer's disease; Ubiquilin-1; BACE1; Beta-amyloid; Ubiquitin-proteasome system; Autophagosome-lysosome pathway; Neurodegeneration

Introduction

Cell stress and impairments in cellular functions, such as protein folding, trafficking and quality control systems, have been suggested to play a role in the pathogenesis of neurodegenerative disorders, including Alzheimer's disease (AD) [1,2]. Indeed, decreased activity and dysfunction of the ubiquitin-proteasome system (UPS) or autophagosome-lysosome pathway (ALP), the two main protein degradation machineries in the cells, are known to associate with neurodegenerative diseases and aging [3-8].

Different molecular chaperones, such as heat shock proteins (Hsps, including Hsp70), play an essential role in protein refolding and targeting unnecessary, misfolded, damaged, or aggregated proteins to disposal via UPS or ALP [9]. UPS manages the degradation of soluble proteins, but is usually not capable of degrading protein aggregates. Instead, these can be targeted to the ALP for disposal [10]. The signal for targeting a protein for UPS- or ALP-mediated degradation is covalent attachment of ubiquitin chains to the protein by the coordinated action of different ubiquitin ligases, i.e., poly-ubiquitination. Lysine 48-linked poly-ubiquitin chains are the classical signal for degradation by the barrel-shaped 26S proteasome complex, which contains a channel where the proteins are enzymatically degraded when they pass through [11]. Lysine 63-linked poly-ubiquitination functions as a signal for targeting the proteins or protein aggregates for autophagy [12]. In the ALP, the proteins or protein aggregates are engulfed within a double-membrane, which forms the autophagosome. Different autophagy receptors, such as p62/SQSTM1, are essential in the recruitment of lysine 63-ubiquitin-linked proteins to autophagic degradation [13,14]. The autophagosomes may fuse with late endosomes or multivesicular bodies to form amphisomes. The autophagosomes or amphisomes finally fuse with lysosomes, leading to the degradation of their contents [8,15].

Neurons, as postmitotic and highly compartmentalized cells, are especially dependent on the efficient function of the UPS and ALP and microtubule-based protein transport in order to handle accumulated, misfolded and aggregated proteins. Protein degradation via the UPS can take place locally at different subcellular sites in neurons, such as pre- or postsynaptic compartments [16]. Autophagosomes are generated in distal axons, while mature lysosomes, which operate at the late stages of ALP, mainly localize in the soma of neurons [17,18]. Therefore, microtubule-based retrograde transport of autophagosomes from the axons to the soma is crucial for delivering the autophagosomal cargoes for lysosomal degradation in neurons [19]. A typical feature in AD is accumulation of autophagic vesicles in dystrophic neurites, implicating impaired microtubule-based transport of autophagic vesicles as a central feature in AD pathology [20]. A recent study by Feng et al. showed that activation of autophagy in the neurons of AD model mice resulted in aberrant accumulation of β-site cleaving enzyme 1 (BACE1) in autophagic vesicles in the distal axons and decreased localization of BACE1 in the lysosomes in the neuronal soma [21]. BACE1 is the initial and rate-limiting enzyme in the generation of β-amyloid peptides (Aβ) from β-amyloid precursor protein (APP). BACE1 mainly resides in the endosomes, which harbor an acidic pH providing optimal conditions for BACE1 enzymatic activity and Aβ generation, and its levels are regulated by lysosomal degradation [22,23]. The study by Feng et al. [21]

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further showed that impaired axonal transport of BACE1-containing autophagic vesicles to the soma subsequently led to increased levels of the BACE1-cleaved APP C-terminal fragments (β-CTFs) C99 and C89 from which Aβ peptides are generated by γ-secretase-mediated cleavage. These phenomena were rescued by enhancement of retrograde transport by overexpression of snapin-1, a component of the dynein-mediated motor protein complex [21]. In addition to BACE1, APP and APP β-CTFs have previously been shown to undergo autophagic degradation and blockade of autophagosomal and/or lysosomal degradation leads to their accumulation and increased levels of Aβ [24-27]. Therefore, APP and BACE1 intracellular trafficking are essential in the regulation of their levels, function, and generation of Aβ.

Ubiquilins are a protein family implicated in the alleviation of different cellular stress conditions and targeting of specific proteins to degradation through the UPS or ALP, thus functioning as molecular chaperones [28]. Importantly, previous studies have shown that ubiquilins regulate the subcellular targeting and degradation of several neurodegenerative disease-associated proteins, including presenilin-1 and -2 (PS1 and PS2), mutant huntingtin proteins, or TAR DNA binding protein 43 (TDP-43), through these pathways [29-33]. The facts that polymorphisms in *UBQLN1* gene underlie the increased risk of AD [34] and that mutation in *UBQLN2* gene lead to the pathogenesis of amyotrophic lateral sclerosis (ALS) or ALS/dementia [35] also provide a strong link between the function of ubiquilin family members and pathogenic processes in neurodegenerative diseases. Particularly one of the ubiquilin protein family members, ubiquilin-1, is implicated in the regulation of the trafficking, accumulation, or clearance of several AD-associated proteins as well as in alleviating ER and oxidative stress [36-41].

Ubiquilin-1 Structure and Function

Ubiquilin-1, also known as PLIC-1 (protein linking integrin-associated protein with cytoskeleton-1) [37], belongs to a highly conserved group of ubiquitin-like proteins. These proteins are suggested to function as a chaperones or shuttle proteins within the UPS delivering poly-ubiquitinated proteins to the proteasome or the autophagosomes for degradation [30,39,42-44]. In humans, ubiquilin-1 is encoded by the *UBQLN1* gene, which consists of eleven exons. Ubiquilin-1 protein contains two characteristic domains directly associated to its shuttle function in the UPS. The N-terminal ubiquitin-like domain (UBL) mediates the interaction with the 26S proteasome by directly binding to S5a-component of the 19S proteasomal subunit. The C-terminal ubiquitin-associated domain (UBA) binds to poly-ubiquitin chains attached to e.g. misfolded or accumulated proteins destined for degradation [30,39,44]. The central region of ubiquilin-1 consists of conserved asparagine- and proline-rich (Asn-Pro-rich) repeats. These repeats interact with specific domains of other proteins, such as epidermal growth factor substrate 15 homology (EH) – domain present in a number of proteins that regulate endocytosis and vesicle sorting, suggesting involvement of ubiquilin-1 in intracellular vesicular trafficking [30].

Ubiquilin-1 is ubiquitously expressed in most, if not all, tissues [42]. In the human brain, ubiquilin-1 is present in neurons. Ubiquilin-1 localizes in the cytoplasm, endoplasmic reticulum (ER), and to a lesser extent in the nucleus and peripheral parts of the cell [30]. Four alternatively spliced *UBQLN1* transcript variants (TVs) have been identified in human brain [34,41]. These TVs encode four protein isoforms, which differ in their domain composition. However, whether the different isoforms display differential cellular functions is not clear [45].

Functional studies in different *in vitro* and *in vivo* models have proven that ubiquilin-1 regulates the trafficking, function, levels and degradation of numerous proteins. The diversity of the ubiquilin-1 interactome suggests that it is involved in a variety of physiological and pathophysiological functions [29] (Figure 1). In addition to its function as a chaperone in the UPS, ubiquilin-1 may also mediate the clearance of aggregated proteins, cellular waste and pathogens via the ALP [33,46-49]. Ubiquilin-1 is suggested to interact with autophagosomes through its UBA domain and it itself is a substrate for ALP [33,48]. The chaperone function of ubiquilin-1 in the UPS or ALP is especially important in situations when the capacity of the UPS or ALP to degrade accumulatesd proteins or protein aggregates becomes overwhelmed. Under excessive protein accumulation, ubiquilin-1 has been shown to target proteins into intracellular inclusion bodies, termed aggresomes [31,33,50-52]. Structurally, these juxtanuclear inclusion bodies closely resemble the characteristic intracellular inclusions containing aggregated proteins in the brain of patients with AD and other neurodegenerative diseases, such as tau in tauopathies, including AD, α-synyclein in PD, or TDP-43 in frontotemporal lobar degeneration (FTLD) or ALS [2]. It is clear that formation of these inclusion bodies is associated with disease pathogenesis and neurodegeneration. Yet, accumulating evidence implies that they in fact may represent a cytoprotective mechanism in diseased cells, since they sequester potentially harmful proteins into restricted compartments, which may later be safely disposed of through the ALP [37,45].

Ubiquilin-1 and Cell Stress

Several studies indicate that ubiquilin-1 plays a role during different cellular stress conditions [32,40,41,49,53]. Ubiquilin-1 protects cells from starvation-induced apoptosis in an autophagy-dependent mechanism [49]. Moreover, ubiquilin-1 levels are up-regulated during the unfolded protein response (UPR) and it has been shown to protect cells from ER-stress-associated apoptotic cell death [39,41,53]. All ubiquilin-1 TVs, except the shortest TV4, alleviate the induction of UPR-inducible stress genes and subsequently provide cytoprotection during ER-stress and hypoxia [41]. The beneficial effect of ubiquilin-1 during acute stress is suggested to take place by enhancing the proteasomal disposal of ER-associated degradation (ERAD) substrates [53,54]. Supporting this idea, ubiquilin-1 down-regulation *in vivo* in *Caenorhabditis elegans* or mice was reported to result in the accumulation of misfolded and poly-ubiquitinated proteins during induced ER-stress, oxidative stress and ischemia [40,53]. On the contrary, ubiquilin-1 overexpression protected mice from oxidative stress, neuronal injury, and motor defects following ischemic stroke [40].These studies suggest a role for ubiquilin-1 in relieving stress by inhibiting the accumulation of damaged proteins.

Ubiquilin-1 and Protein Aggregation in Neurodegeneration

A number of studies link ubiquilin-1 to AD at both genetic and functional level. Specific genetic variants in *UBQLN1* are associated with increased AD risk [34,55-58], although this association could not be replicated in all studies [59-64]. However, the risk allele of UBQ-8i single nucleotide polymorphism (SNP) has been reported to alter the *UBQLN1* mRNA ratio of TV2 to TV1 similarly to human brain and cause a pathological phenotype in *Drosophila melanogaster* [34,65,66]. Stieren et al. have reported that ubiquilin-1 protein levels are significantly decreased in the brain of AD patients as compared to control subjects [67], implying decreased ubiquilin-1 function in AD. Furthermore, emphasizing the association of *UBQLN* gene family

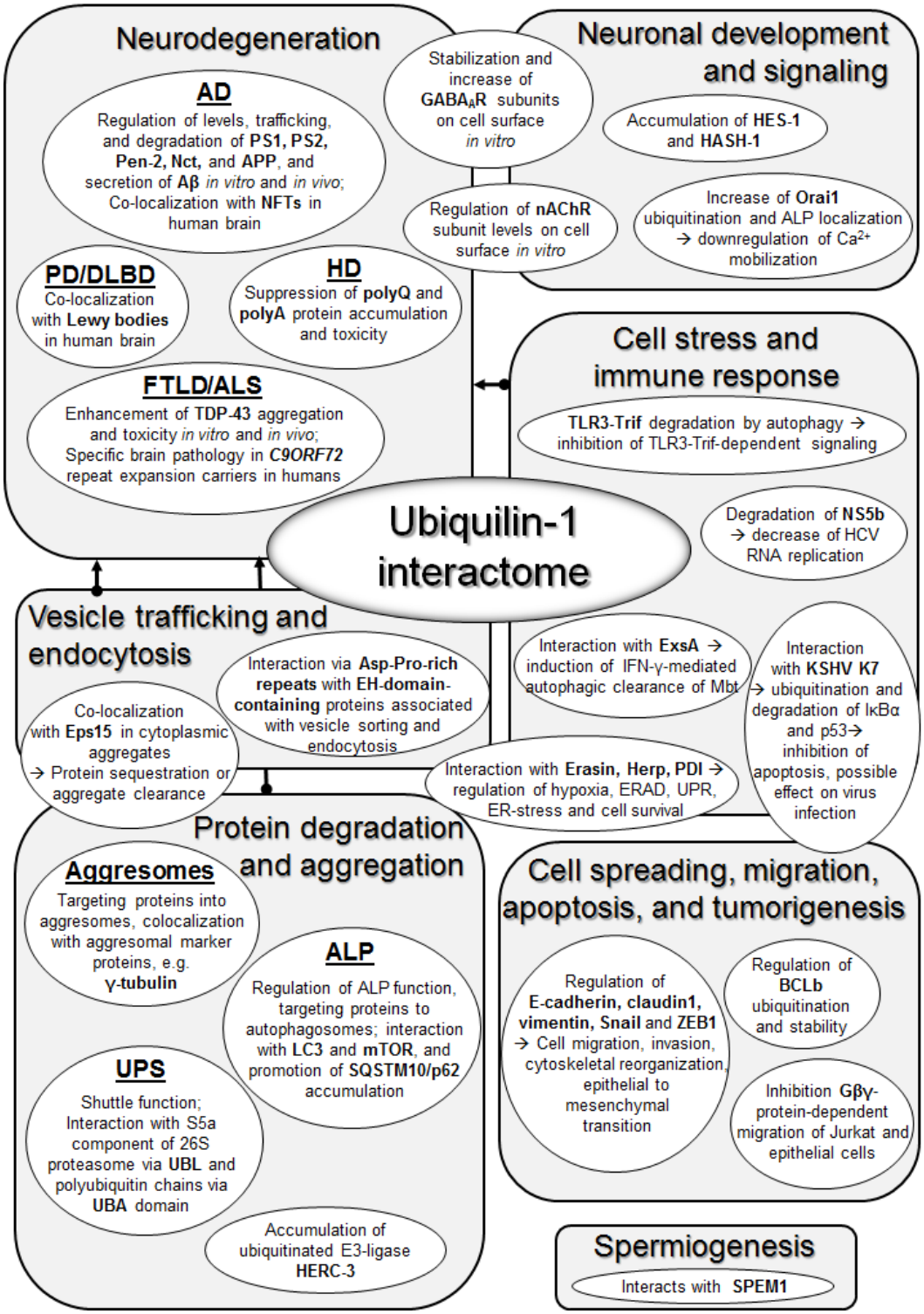

Figure 1: Ubiquilin-1 protein interactome.

Abbreviations: AD: Alzheimer's Disease; ALP: Autophagosome-Lysosome Pathway; ALS: Amyotrophic Lateral Sclerosis; DBLD: Diffuse Lewy Body Disease; FTLD: Frontotemporal Lobar Degeneration; HD: Huntington's Disease; NFT: Neurofibrillary Tangle; PD: Parkinson's Disease; UBA: Ubiquitin-Associated Domain; UBL: Ubiquitin-Like Domain; UPS: Ubiquitin-Proteasome System

with neurodegenerative diseases and protein aggregation, mutations in *UBQLN2* encoding a ubiquilin-1 homolog, ubiquilin-2, have been shown to cause rare familial ALS with dementia and lead to defects in the protein degradation pathway, abnormal protein aggregation and neurodegeneration [35].

Ubiquilin-1 was originally identified as a PS1- and PS2-interacting protein [30]. PSs are essential components of the γ-secretase complex, which cleaves APP to generate the Aβ peptides [68]. Subsequent studies have demonstrated that ubiquilin-1 specifically increases the accumulation and aggresomal targeting of ubiquitinated high-molecular weight (HMW) PS-complexes [30,31,33,65,69-72]. Interestingly, our previous study indicated that this function may partially be isoform-dependent, since especially ubiquilin-1 TV3, which lacks the proteasome-interacting UBL domain, enhanced PS1 accumulation and localization to aggresomes [33]. Even though the fact that ubiquilin-1 regulates PS levels and accumulation suggests that ubiquilin-1 may affect the processing of PS-dependent γ-secretase substrates, including APP, the functional consequences of the interrelationship between ubiquilin-1 and the PSs as well as the other γ-secretase complex components Pen-2 and Nicastrin have thus far remained poorly understood [33,70,73]. Another link between ubiquilin-1 and AD molecular pathogenesis is provided by studies reporting that modulation of ubiquilin-1 levels *in vitro* in cells or *in vivo* in *Drosophila melanogaster* leads to altered APP processing, maturation, trafficking and proteolysis, consequently affecting Aβ production and secretion in a PS/γ-secretase-independent mechanism [36,38,66,67, 69,73,74]. However, these data have been partly conflicting in different cell types and need to be confirmed in further studies.

Increasing evidence suggests the involvement of ubiquilin-1 in the pathogenesis of other neurodegenerative disorders beyond AD. Ubiquilin-1 co-localizes with neurofibrillary tangles (NFTs), dystrophic neurites, and Hirano bodies in the AD brain and Lewy bodies in Parkinson's disease (PD) and diffuse Lewy body disease (DLBD) [30,75,76]. A recent study suggested that early NFT changes are associated with upregulation or nuclear translocation of ubiquilin-1 in hippocampal neurons [75], but the possible relationship between tau and ubiquilin-1 has not been studied in more detail. Ubiquilin-1 has also been reported to localize in the ubiquitin/p62/SQSTM1- and TDP-43-immunopositive intracellular inclusions in FTLD and ALS brain and mediate the stability and toxicity of these aggregates *in vitro* and *in vivo* [32,77,78]. Interestingly, ubiquilin-1 has also been linked to repeat expansion disorders, such as Huntington's disease (HD), ataxias, and FTLD and ALS related to the *C9ORF72* hexanucleotide repeat expansion. Ubiquilin-1 was found to regulate the accumulation and toxicity of expanded polyQ repeat-containing proteins, such as huntingtin and polyA-containing proteins associated predominantly with congenital malformation syndromes [79-83]. Moreover, the brains of *C9ORF72* hexanucleotide repeat expansion-carrying FTLD and ALS patients show distinct ubiquilin pathology. Even though ubiquilin-1 appears to associate with several different neurodegenerative diseases, it remains to be determined whether ubiquilin-1 aggravates or alleviates their pathogenesis.

New Evidence on the Interrelationship between Ubiquilin-1 and AD-Associated BACE1

Information regarding ubiquilin-1 expression and function in diseased human brain and in mammalian animal models is thus far limited. In our recent study by Natunen et al. [84], we examined ubiquilin-1 expression in human brain in relation to AD-related neurofibrillary pathology at different stages of the disease as indicated by Braak staging [85]. Furthermore, we characterized the effects of ubiquilin-1 overexpression on the regulation of BACE1, neuroinflammation and neuronal viability in different *in vitro* and *in vivo* mammalian model systems, including neuronal cell lines, co-cultures of mouse embryonic primary cortical neurons and microglial cells under acute neuroinflammation and the brain of APdE9 transgenic mice at the early phase of the development of Aβ pathology.

We first analyzed *UBQLN1* mRNA expression in *post mortem* human brain samples. These investigations using probes against different *UBQLN1* exons revealed a global decrease in *UBQLN1* mRNA expression in relation to advancing neurofibrillary pathology (Braak stages 0-VI). Overall, the highest *UBQLN1* mRNA expression was observed at Braak stage 0 and the expression significantly decreased along with the disease progression. We also observed a similar decreasing trend in the ubiquilin-1 protein levels along with advancing neurofibrillary pathology. These data are in accordance with the previous report by Stieren et al. [67], showing decreased ubiquilin-1 levels in AD brain as compared to age-matched controls. Interestingly, further screening of the levels of ubiquilin-1 and key proteins involved in APP processing in human brain revealed a positive correlation between the levels of ubiquilin-1 and BACE1 proteins. There was no association between ubiquilin-1 levels and β-secretase activity.

Previous studies have linked ubiquilin-1 to different cellular stress conditions and suggested that ubiquilin-1 may alleviate oxidative and ER stress *in vivo* and *in vitro* in cultured cells [41,53,54,86]. The role of ubiquilin-1 in neuroinflammation, which is centrally involved in AD pathogenesis [87], had not been previously investigated. Using lentivirus-mediated overexpression of human ubiquilin-1 in mouse primary cortical neurons in co-cultures with mouse microglial BV2 cells, we detected an upregulation of the levels of overexpressed ubiquilin-1 under lipopolysaccharide (LPS) and interferon-γ (IFN-γ)-induced acute neuroinflammation. Moreover, ubiquilin-1 overexpression resulted in enhanced neuroinflammatory stress as indicated by an increase in tumor necrosis factor-α (TNF-α) levels in the co-culture medium. Furthermore, ubiquilin-1 overexpression led to decreased neuronal cell viability not only under neuroinflammation but also under normal culture conditions. Thus, in contrast to previous studies showing alleviation of ER or oxidative stress by ubiquilin-1 overexpression, our data suggest that ubiquilin-1 is not able to alleviate neuroinflammatory stress and that ubiquilin-1 overexpression itself could be detrimental in neurons. Additionally, ubiquilin-1 overexpression led to significantly upregulated levels of BACE1 in the co-cultures under both normal conditions and neuroinflammation, providing corroboration to our initially observed link between ubiquilin-1 and BACE1 levels in the human brain. In line with previous studies showing that BACE1 expression is promoted under inflammation after treatment with proinflammatory compounds, such as LPS and IFN-γ [88,89], a slight increase in BACE1 protein levels after LPS and IFN-γ-treatment in the co-cultures was also detected in our study.

Lentivirus-mediated overexpression of ubiquilin-1 *in vivo,* starting at four months of age when the Aβ pathology starts to develop [90], in the hippocampus of APdE9 AD model mice resulted in a mild increase of BACE1 protein levels and β-secretase after five months. Strong BACE1 staining was observed around Aβ plaques in these mice, but this was unaffected by ubiquilin-1 overexpression. On the other hand, β-secretase activity significantly correlated with soluble $A\beta_{40}$ and $A\beta_{42}$ levels in the hippocampi of APdE9 mice. Despite the previously reported effects of ubiquilin-1 on APP maturation, intracellular trafficking, proteolytic processing, and degradation *in vitro* in cultured cells [33,36,38,67,73,74], we did not observe significant changes in APP maturation, the levels of

APP metabolites (APP CTFs or Aβ) or APP ubiquitination status *in vivo* in the hippocampi of APdE9 mice overexpressing ubiquilin-1. However, Aβ plaque load was modestly decreased, while soluble and insoluble $A\beta_{40}$ and soluble $A\beta_{42}$ levels were increased in the hippocampi of these mice overexpressing ubiquilin-1. These observations agree with the idea of the dynamic nature of Aβ aggregates, which have been described to undergo constant aggregation and dissolution of Aβ molecules to and from the plaques [91]. They also are in line with the recent report by Adegoke et al. [92], which shows that ubiquilin-1 overexpression alleviates AD-like cognitive and motor deficits and reduces Aβ accumulation in APdE9/ubiquilin-1 transgenic mice.

We did not detect significant alterations in tau phosphorylation at the AD-related AT8 epitopes or total tau protein levels in the hippocampi of APdE9 mice upon ubiquilin-1 overexpression. In the *in vitro* co-cultures of ubiquilin-1 overexpressing neurons and microglial cells, we noticed a significant increase in total protein levels of the 0N3R-tau isoform upon induction of neuroinflammation, but no effects on tau phosphorylation (AT8), in contrast to previous *in vivo* studies indicating an increase in tau phosphorylation after LPS treatment [93,94]. Even though further studies are warranted to elucidate the effects of ubiquilin-1 on tau levels and phosphorylation, our findings implicate that ubiquilin-1 may modulate tau levels under inflammatory conditions. It appears that ubiquilin-1 overexpression itself does not result in neuroinflammation *in vivo* in the mouse brain, as we did not detect alterations in the levels of glial fibrillary acidic protein (GFAP) or the CD45 immunopositive area.

To shed light into the molecular mechanisms of the observed interrelationship between ubiquilin-1 and BACE1, we then utilized different human neuronal cell lines either stably or transiently overexpressing ubiquilin-1. Ubiquilin-1 overexpression led to significantly increased BACE1 expression levels in all the studied cell lines and the increase was reversed by siRNA-mediated ubiquilin-1 downregulation. The protein levels of ubiquilin-1 and BACE1, both before and after ubiquilin-1 downregulation, strongly correlated with each other, confirming the interrelationship of these two proteins observed in the human and APdE9 mouse brain. Using cycloheximide time-course assay, in which *de novo* protein synthesis is prevented and degradation rate of the protein of interest can be followed over time, indicated that ubiquilin-1 overexpression led to a significantly prolonged half-life of BACE1 protein. This result implicated that overexpression of ubiquilin-1 increases BACE1 levels by slowing down its degradation, which mainly takes place in lysosomes [22]. Thus, we next studied by co-immunofluorescence the co-localization of BACE1 with markers of endosomes and lysosomes, the subcellular compartments where BACE1 has previously been reported to mainly reside in [95]. Accordingly, we also observed a prominent co-localization of BACE1 with Rab7, a marker for late endosomes and lysosomes (LEL), transferrin receptor (TfR), a marker for early and recycling endosomes, and EEA1, a marker for early endosomes. In ubiquilin-1 overexpressing cells, the co-localization of BACE1 with Rab7 in the LEL was significantly decreased concomitantly with increased co-localization with TfR in the earlier endosomal compartments. These data indicated that overexpression of ubiquilin-1 leads to decreased lysosomal degradation of BACE1. We failed to find evidence that the decreased lysosomal targeting of BACE1 to lysosomes resulted from alterations of the levels of proteins previously reported to regulate BACE1 subcellular trafficking or sorting, such as GGA1, GGA3, ARF6 or seladin-1, or changed lysine 48 or lysine 63-linked BACE1 ubiquitination in the ubiquilin-1 overexpressing cells. Therefore, whether ubiquilin-1 controls BACE1 intracellular trafficking directly or indirectly via another protein(s) still remains to be clarified in future studies. In conclusion, our study by Natunen et al. [84] suggests a previously unknown interrelationship between BACE1 and ubiquilin-1, which may influence Aβ accumulation and development of Aβ pathology in mouse and human brain. However, further investigations will provide additional insights into the underlying mechanisms of the relationship between ubiquilin-1 and these pathogenic events.

Conclusion

Together with the previous reports, the data in our report by Natunen et al. [84] suggest that ubiquilin-1 specifically interacts with several AD-associated proteins and plays a multifaceted role in different stress conditions. Thus, it is obvious that ubiquilin-1 is a complex factor that affects multiple phenotypic traits and cellular processes, which may hamper its use as a straightforward therapeutic target in the context of neurodegenerative diseases, such as AD. Finally, pre-clinical studies *in vivo,* such as those recently reported by Adegoke et al. [92], will shed light on whether the targeting of ubiquilin-1 has the potential for subsequent clinical intervention studies in AD.

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