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FULL LENGTH ARTICLE

Ubiquitination status does not affect Vps34 degradation

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Abstract Vps34 (vacuolar protein-sorting 34) plays important role in autophagy and endosomal trafficking. These processes are closely associated protein ubiquitination and degradation. We have hypothesized that Vps34 ubiquitination status would also control its degradation. Here, we report that our results did not support this assumption. In cells transiently transfected with ubiquitin (UB) constructs contained different lysine residues (Ks), Vps34 ubiquitination could occur regardless of the presence of any Ks in UB. However, Vps34 protein levels were not significantly altered in cells transiently transfected with these UB mutants. We further found that Vps34 protein was altered by pharmacological manipulation of E2/E3 activity; yet this effect was not significantly affected by UB overexpression. In vivo experiments revealed that in APP/PS1 mice, an animal model of Alzheimer's disease (AD), although ubiquitination of Vps34 was significantly reduced, Vps34 protein levels remained unchanged. Vps34 indeed was subjected to proteasomal or lysosomal degradation, as prolonged treatment of proteasomal inhibitor MG132 or lysosomal inhibitor chloroquine elevated Vps34 protein levels. We conclude that unlike most of other proteins, Vps34 ubiquitination is not closely associated with its degradation.

Abbreviations: Vps34, vacuolar protein-sorting 34; PI3K, class III phosphatidylinositol 3-kinase; UB, ubiquitination; Ks, lysine residues; UPS, ubiquitin proteasome system; AD, Alzheimer's disease; APP, β-amyloid precursor protein; APP/PS1, expressing Swedish APP and Presenilin1 delta exon 9 mutations; WT, wild-type.

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Introduction

Vps34 (vacuolar protein-sorting 34) is the catalytic unit of class III phosphatidylinositol 3-kinase (PI3K) complex that regulates vesicular trafficking, autophagy, and nutrient sensing.[1] In the central nervous system, Vps34 is involved in brain development and neuronal excitation.[2,3] Conditional knockout of Vps34 leads to rapid but differential neurodegenerations, with a disruption in endosomal pathway.[4] Irregular PI3K activity is associated with numerous human pathological conditions, such as diabetes, cancer and inflammation.[5] Interestingly, Vps34 also plays an important role in Alzheimer's disease, as Vps34 deficiency enhances the amyloidogenic processing of amyloid precursor protein.[6] Therefore, maintaining the adequate protein level of Vps34 is essential for normal brain function.

Protein ubiquitination is a post-translational modification in nearly all aspects of cell biology.[7] The attachment of ubiquitin (UB) to its substrate proteins is catalyzed by the sequential actions of ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligating (E3) enzymes.[8] UB protein contains seven lysine (K) residues at positions 6, 11, 27, 29, 33, 48 and 63, respectively.[9] This feature allows UB to be attached as a single moiety or in the form of polymeric chains.[10] It has been suggested that the UB recognition code plays important role in variety of cellular processes including ubiquitin proteasome system (UPS)- and autophagy-mediated protein degradation.[11,12]

Previous studies have demonstrated that Vps34 protein stability is controlled by E3 enzymes, suggesting that Vps34 protein level is affected by ubiquitination.[13,14] In our study, we found that Vps34 protein levels were not altered in cells transiently transfected with Ub mutants WT, K0, K6, K11, K29, K33, K48 and K63, respectively. In APP/PS1 mice, an animal model of AD, although Vps34 ubiquitination was significantly decreased, its protein levels remained unaltered. However, long-term treatment of UPS or autophagy inhibitors did increase Vps34 proteins. We suggest that Vps34 degradation process is not affected by ubiquitination status.

Materials and methods

Animals

APPswe/PS1E9 transgenic mice (APP/PS1) were obtained from Nanjing University (China). Mice were generated and group housed under specific pathogen-free (SPF) conditions at a constant room temperature of 22—24 °C with a 12 h-12 h light/dark cycle and with unrestricted access to food and water. All animal experiments were approved by the Institutional Animal Ethics Committee of Chongqing Medical University.

Antibodies and reagents

Antibodies used in this study were as follows: rabbit anti-VPS34 (CST, 4263), rabbit anti-Flag tag (PTG, 24147), mouse anti-HA tag (PTG, 66006-1), rabbit anti-ubiquitin (PTG, 10201-2-AP), rabbit anti-TAU (Abcam, ab80579), anti-GAPDH (PTG, 60004-1), HRP-conjugated Goat Anti-Mouse IgG (PTG, SA00001-1), HRP-conjugated Goat anti-rabbit IgG (PTG, SA00001-2). Reagent information was as follows: protease inhibitor MG132 (MCE, HY-13259), protein biosynthesis inhibitor cycloheximide (MCE, HY-12320), DNA enzyme topoisomerase I inhibitor campathecin (MCE, HY-16560), lysosomal inhibitor chloroquine (MCE, HY-17589) and UBE2N (Ubiquitin Conjugating Enzyme E2 N) inhibitor NSC697923 (Selleck, S7142).

Cell culture, plasmid and transfection

HEK293 (human embryonic kidney) and HT22 (mouse hippocampal neuron) cell lines were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Biological Industries). Both cell lines were cultured at 37 °C in a humidified 5% CO_2 atm.

pcDNA4-Vps34-Flag was purchased from Addgene (#24398). All ubiquitin mutants were made from pRK5-HA-ubiquitin plasmid (Addgene) by mutagenesis. pRK5-HA-Ubiquitin-K0 (no lysine), pRK5-HA-Ubiquitin-K6 (keep lysine at position 6), pRK5-HA-Ubiquitin-K11, pRK5-HA-Ubiquitin-K27, pRK5-HA-Ubiquitin-K29, pRK5-HA-Ubiquitin-K33, pRK5-HA-Ubiquitin-K48, pRK5-HA-Ubiquitin-K63. 24 h before transfection, cells were seeded to obtain a final confluence of 60—70%. Transfections were carried out using Lipofectamine™ 2000 (Thermo Fisher Scientific, #11668019) according to manufacturer's protocols. PcDNA3.1 was used as an expression control. siRNA sequence for UBE2N was: GAACCAGTTCCTGGCATCA (RiboBio silencer, ID#stB000527A).[15]

Immunoprecipitation

Immunoprecipitation (IP) was performed as described previously.[16] Cells were harvested in lysis buffer (50 mM Tris—HCl, pH 7.4, 250 mM NaCl, 0.5% NP-40, supplemented with complete protease inhibitor mixture and 10uM N-Ethylmaleimide (Sigma—Aldrich, E3876)). Protein A/G magnetic beads (Bimake, B23201) were used to pre-clear the lysate for 2 h. Cell lysates were incubated with Flag tag antibody for 6 h at 4 °C. The resultant mixture was then incubated with protein A/G magnetic beads overnight at 4 °C and heated at 95 °C for 5min before sampling.

Western blotting

Proteins were extracted using RIPA buffer (50 mM Tris,1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with protease inhibitors. Protein concentrations were measured using a BCA Protein Assay Kit (Beyotime, P0010). Protein samples were separated on 8—10% SDS-PAGE and were transferred onto 0.22-μm PVDF membranes (Millipore, ISEQ00010). The membranes were blocked for 1.5 h in Tris-buffered saline containing 0.1% Tween-20 and in 5% (w/v) non-fat milk powder, and then probed with the respective primary antibodies against the target protein overnight at 4 °C. The blots were then washed and incubated for 1 h at room temperature with horseradish peroxidase-

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conjugated anti-rabbit or anti-mouse secondary antibodies. The membranes were visualized using an ECL reagent (Thermo, Marina, CA, USA) and a Fusion FX5 image analysis system (Vilber Lourmat, Marne-la-Vallée, France).

Statistical analysis

Data are shown as the mean ± SEM from three independent experiments. All data were analyzed by GraphPad Prism6 software. The statistical comparisons between two groups were tested using Student's *t*-test; and the comparisons among groups were tested using one-way or two-way analysis of variance (ANOVA).

Results

Ubiquitination of VPS34 did not alter VPS34 protein levels

It is reported that distinct UB-chains are differentially involved in Vps34 protein stability.[13,17] To examine which UB linkage may be associated with Vps34 protein levels, we first assessed Vps34 ubiquitination and protein levels in cells transiently transfected with empty vector or UB constructs WT (wild-type), K0, K6, K11, K29, K33, K48 and K63, respectively. In WT and K0 all 7 Ks were included or mutated to arginine (R), respectively, while in K6—K63, only the numbered K residue was present. Surprisingly, we did not found significant variations of ubiquitin levels in these cells (Fig. 1A). And the endogenous Vps34 protein levels were not significantly altered among these groups (Fig. 1B). To further confirm these findings, we assessed the exogenous Vps34 ubiquitination and protein levels in cells co-transfected with HA-tagged UB mutants and Flag-tagged Vps34. As shown in Fig. 1C&D, although ubiquitination of total protein (Fig. 1C) and immunoprecipitated Vps34 (Fig. 1D) varied among different UB mutants including K0 and WT, the exogenously introduced Vps34 protein level was not significantly changed. These results indicated that there was no clear relationship between ubiquitination and protein level of Vps34, suggesting that UB status did not affect Vps34 protein levels.

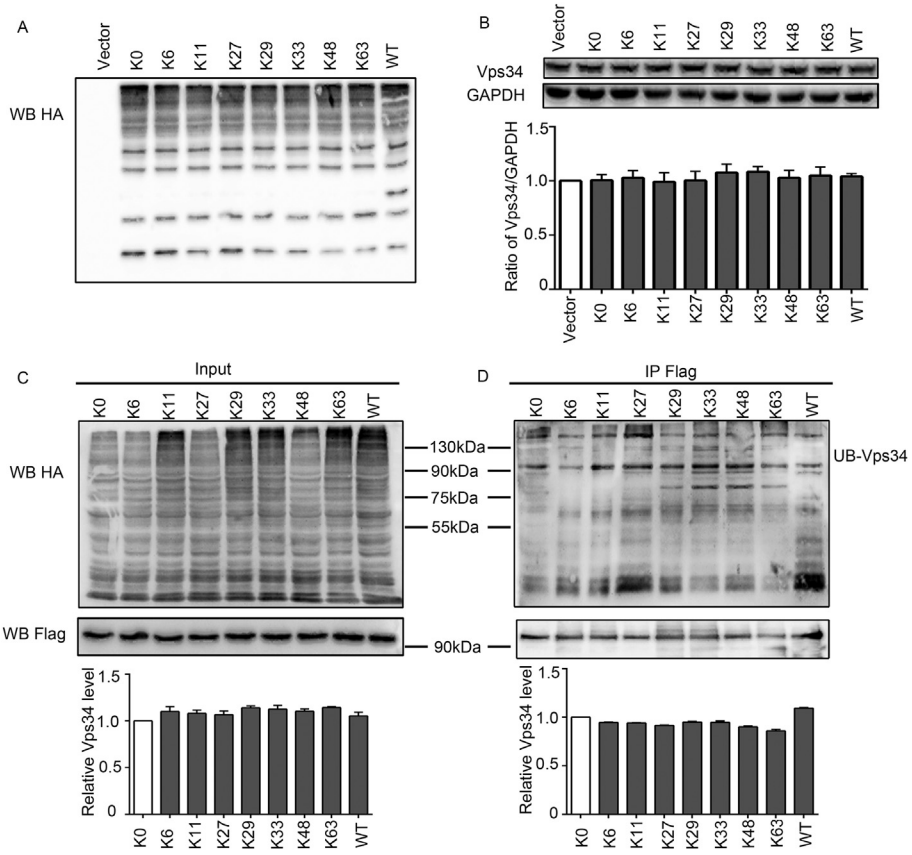

Figure 1 Ubiquitnation does not alter Vps34 protein levels. **(A)** Representative Western blots of ubiquitin (HA-tag) in HEK293 cells transiently expressing vector, or ubiquitin constructs WT, K0, K6, K11, K29, K27,K33, K48 and K63, respectively. Cells were treated with MG132 (20 μM) for 4 h before sample was harvested (n = 3). **(B)** Representative Western blots (top) and quantification (bottom) of endogenous Vps34 in samples taking from A. **(C)** Representative Western blots of HA-tagged ubiquitin (top) and Flag-tagged VPS34 (middle) in inputs from HEK293 cells co-transfected with VPS34 and ubiquitin constructs WT, K0, K6, K11,K27, K29, K33, K48 and K63, respectively. Quantification of relative levels of Vps34 protein is shown on the bottom (n = 3). After 44 h of transfection, cells were treated with MG132 (20 μM) for 4 h before harvest. **(D)** Representative Western blots of HA-tagged ubiquitin (top) and Flag-tagged VPS34 (middle) in cell extracts immunoprecipitated by Flag-Vps34 antibody. Samples were taken from C. Quantification of relative levels of Vps34 protein is shown on the bottom (n = 3).

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In *C elegans*, the E2 UB-conjugating enzyme UBC13 (human homolog of UBE2N) is known to regulate Vps34 protein levels through K63-linked poly UB.[17] Consistently, UBE2N siRNA significantly reduced Vps34 protein levels in HEK293 cells (Fig. 2A), and its specific inhibitor NSC697923 (NCS)[18] mimicked the effect of UBE2N knockdown (Fig. 2B), indicating that UBE2N also controls Vps34 degradation in mammalian cells. It is reported that camptothecin (CPT), a cytotoxic quinolone alkaloid, can activate DNA damage response and promote the interaction of Vps34 with UB ligase FBXL20 (F-box and leucine rich repeat protein 20), which in turn facilitates Vps34 ubiquitination and degradation.[14] As shown in Fig. 2C, CPT treatment indeed reduced Vps34 protein in HEK293 cells. To further test whether ubiquitination might be involved in UBE2N- and FBXL20-mediated degradation of Vps34, we assessed the effect of NCS and CPT in cells transfected with wild-type UB construct. As shown in Fig. 2D, overexpression of UB did not prevent NSC- and CPT-induced reduction of Vps34 protein levels. Similarly, in K63 overexpressing cells, NSC-mediated reduction of Vps34 protein remained existed (Fig. 2D). These results suggest that UBE2N- and FBXL20-mediated regulation of Vps34 is not affected by ubiquitination status.

Alterations of ubiquitination and protein level of Vps34 did not coincide in APP/PS1 mice

To further determine whether VPS34 protein level was associated with ubiquitination in vivo, we first assessed VPS34 protein levels in APP/PS1 mice (APP/PS1) in the cortex and hippocampus at 6 mon and 12 mon, respectively. As shown in Fig. 3A&B, VPS34 protein levels were not significantly changed in these brain areas tested at both 6 and 12 mon, compared with age-matched wild-type mice (WT). We then selected the cortex at 12 mon to assess Vps34 ubiquitination. As shown in Fig. 3C, a relatively weak signal of ubiquitination was found in the total homogenates of APP/PS1 mice compared with control, whereas the total Vps34 protein level was not changed. Further immunoprecipitation experiments revealed that although ubiquitination of Vps34 was

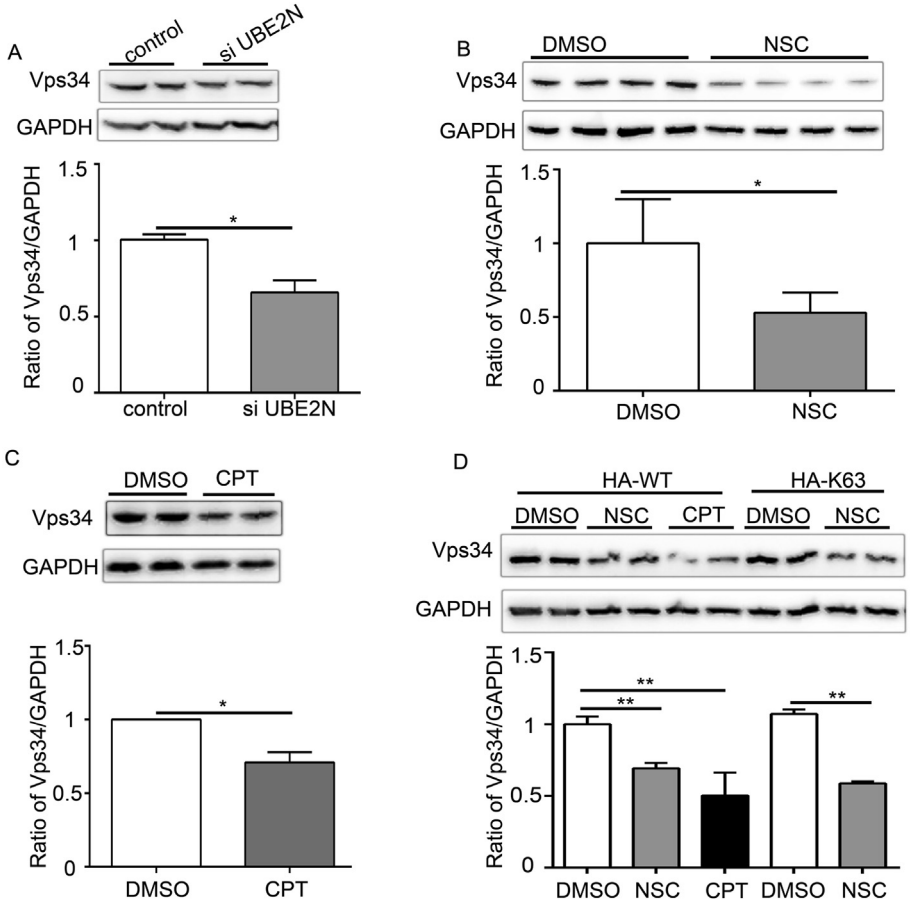

Figure 2 UBE2N- or FBXL20-mediated regulation of Vps34 is not affected by ubiquitination status. (A) Representative Western blots (top) and quantification (bottom) of Vps34 in HEK293 cells transfected with UBE2N siRNA (n = 3). **(B)** Representative Western blots (top) and quantification (bottom) of Vps34 in HEK293 cells treated with vehicle (DMSO) or NSC697923 (NSC, 5 μM) for 24 h (n = 3). **(C)** Representative Western blots (top) and quantification (bottom) of Vps34 in HT-22 cells treated with vehicle (DMSO) or camptothecin (CPT, 2 μM) for 24 h (n = 3). **(D)** Representative Western blots (left) and quantification (right) of Vps34 in HEK293 cells transiently transfected with ubiquitin construct WT or K63. After 24 h of transfection, cells were treated with NSC697923 or CPT for 24 h (n = 3). *P < 0.05; **P < 0.01.

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significantly decreased, the protein levels of Vps34 remained unaltered (Fig. 3D). These results suggested that ubiquitination and protein level of Vps34 were not closely linked in APP/PS1 mice.

Long-term treatment of proteasomal or lysosomal inhibitor elevated Vps34 protein levels

We next argued whether Vps34 protein is subjected to proteasomal or lysosomal degradation. Thus, we first assessed Vps34 protein level in HT-22 cells (hippocampal neuron) treated with short-term (4 h) proteasomal inhibitor MG132, which is effective for inhibiting protein degradation.[19] As shown in Fig. 4A, 4 h treatment of MG132 at different concentrations did not alter Vps34 protein levels. In contrast, MG132 treatment elevated TAU protein levels (Fig. 4B) as previously reported.[20] To further test whether lysosomal function may be involved in Vps34 degradation, we assessed Vps34 protein in HT-22 cells treated with lysosomal inhibitor chloroquine (CQ) at different concentrations for 12 h.[21] As shown in Fig. 4C, Vps34 protein levels were not altered in the presence of CQ relative to vehicle. These results indicated that short-term MG132 or CQ did not change Vps34 protein levels.

To further determine whether Vps34 would be degraded after all, we assessed Vps34 protein cells treated with MG132 or CQ for up to 24 h in HT-22 cells. A protein biosynthesis inhibitor cycloheximide (CHX, 20ug/ml) was added to prevent the potential effect of protein biosynthesis on Vps34 protein levels.[22] As shown in Fig. 4D, Vps34 protein levels were not elevated at 12 and 18 h, but were increased at 24 h in the presence of MG132 or CQ. These results indicated that Vps34 was relatively stable within 18 h, and that both UPS and lysosome played a role in Vps34 degradation.

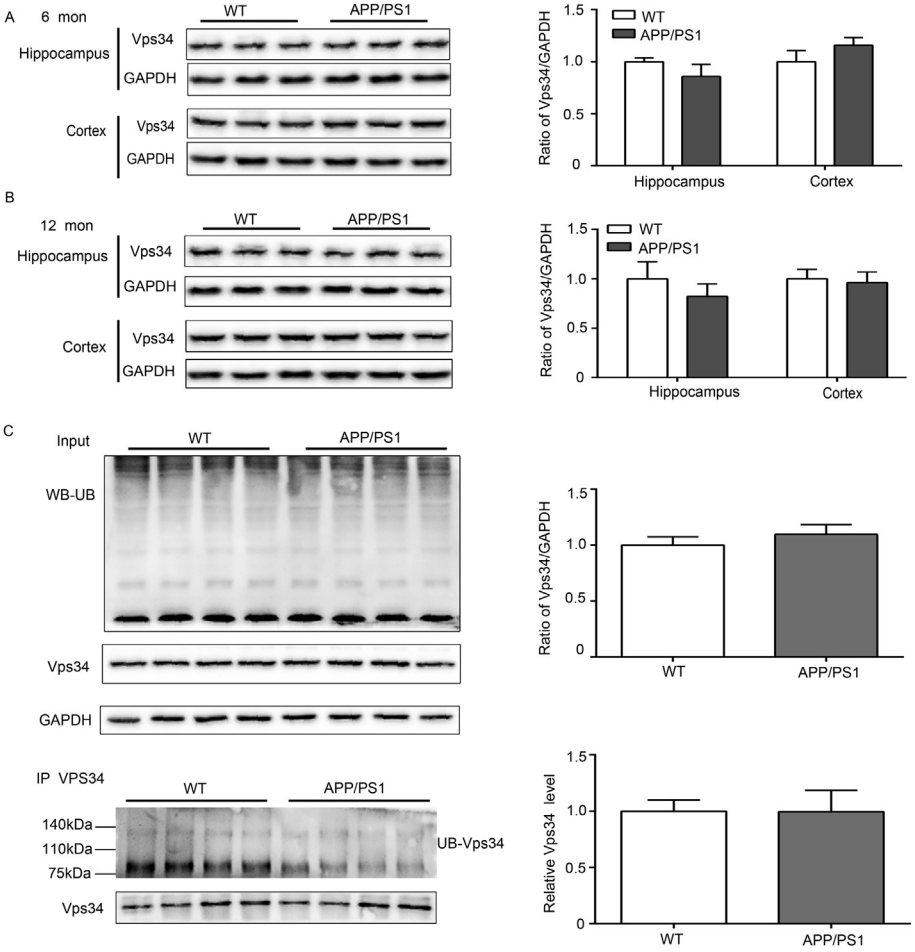

Figure 3 Vps34 protein and ubiquitination levels are not consistently altered in the brain of APP/PS1 mice. (A) Western blots (left) and quantification (right) of Vps34 in the hippocampus and cortex of wild-type (WT) and APP/PS1 mice at 6 mon (n = 6 in each). **(B)** Western blots (left) and quantification (right) of Vps34 in the hippocampus and cortex of WT and APP/PS1 mice at 12 mon (n = 8 in each). **(C)** Representative Western blots of ubiquitin and Vps34 in the input from the cortex of WT and APP/PS1 mice at 12 mon are shown on the left panel, whereas quantification of Vps34 protein levels is shown on the right (n = 8). (D) Representative Western blots of ubiquitin (top) and VPS34 (bottom) in cell extracts from the cortex of WT and APP/PS1 mice at 12 mon immunoprecipitated by Vps34 antibody are shown on the left panel, whereas quantification of Vps34 relative protein levels is shown on the right (n = 8).

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Discussion

In our study, Vps34 ubiquitination seems to occur in all UB mutants including K0 (Fig. 1), suggesting that UB can attach to Vps34 without any K residues. A previous study has demonstrated that replacing all K residues does not affect 3D structure and stability of UB.[23] The K-less UB is able to bind substrate protein through an isopeptide bond, although further formation of poly-UB chains are prevented.[24] Several studies have demonstrated the involvement of ubiquitination in Vps34 regulation. For instance, pharmacological inhibition of UB-specific peptidases USP10 and USP13 promotes Vps34 degradation.[25] CPT that enhances FBXL20 association with Vps34 promotes Vps34 degradation.[14] During prolonged starvation, KLHL20 (kelch like family member 20), a key regulator of autophagy termination, facilitates Vps34 degradation with the concomitant alteration of K48 binding to Vps34 [13]. Moreover, dominant negative UBC13 reducesVps34 protein levels in *C elegans*, which is associated with K63-linked chains.[17] However, these studies did not provide evidence showing any K residues that directly controls Vps34 protein. In our study, Vps34 protein levels are not altered in cells transfected with all UB mutants including K0 and WT, with respect to both exogenous and intrinsic Vps34 (Fig. 1). Despite that CPT and NSC treatment reduces Vps34 protein levels, these effects are not prevented by overexpression of UB constructs including K63 (Fig. 2). We speculate that although the UB status does not directly affect Vps34 degradation, the E2/E3 or autophagy regulators mentioned above might control Vps34 degradation through unknown mechanisms.

Vps34 plays important role in AD. Specific knockout of VPS34 in neurons leads to progressive synaptic loss followed by neurodegeneration.[26,27] Deletion of PIK3C3 gene that encodes Vps34 causes specific learning disorders.[3] The potential mechanisms are associated with endosomal and lysosomal dysfunction and the altered amyloidogenic processing of amyloid precursor protein.[6,28] It has also been reported that the PI3P levels are decreased in the brain of patients with AD and mouse models,[6] suggesting that Vps34 is dysfunctional. However, the Vps34 protein is not altered in APP/PS1 regardless of age in our study, which leads to the speculation that the altered PI3P levels in AD might be caused by enzymes other than Vps34 that are involved in the phosphorylation of phosphatidylinositol 3, such as phosphatase PIKfyve and myotubularin phosphatases.[29] Meanwhile, in contrast to the unaltered Vps34 protein levels, Vps34 ubiquitination is significantly reduced in APP/PS1 mice (Fig. 3). These results further support that Vps34 ubiquitination is not necessarily linked to the altered protein levels of Vps34.

Ubiquitination marks proteins for proteasomal or lysosomal degradation.[30] It is predicted that VPS34 is relatively stable with a long half life (https://web.expasy.org/protparam/). In line with this, we found that short-term (4 h) treatment of either UPS inhibitor or lysosomal inhibitor does not change VPS34 protein levels (Fig. 4). The effect of the inhibitors does not occur until at 24 h of treatment. In addition, both proteasomal and lysosomal

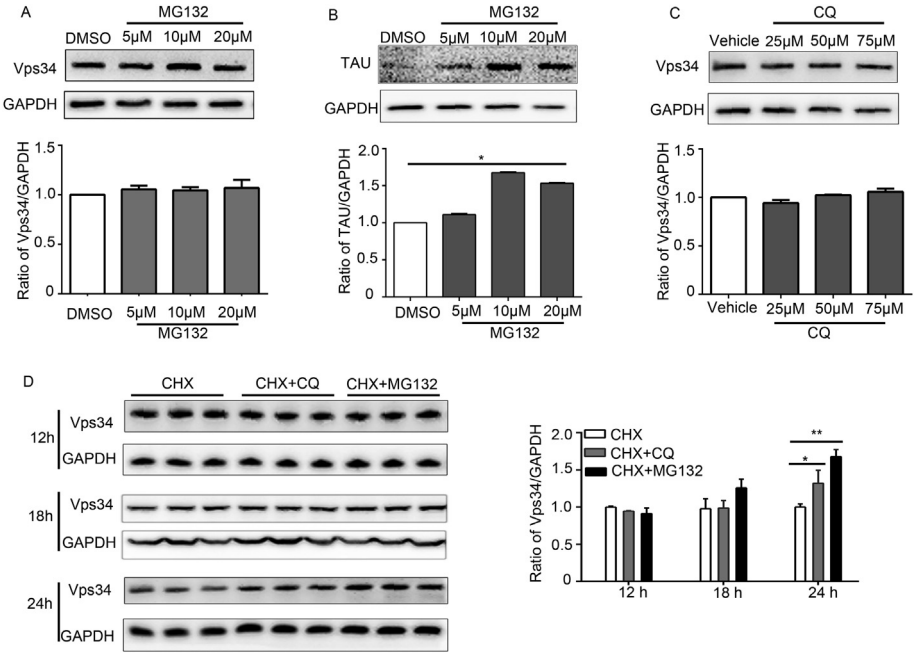

Figure 4 Vps34 protein level is elevated by long-term treatment of proteasomal or lysosomal inhibitor. (A) Representative Western blots (top) and quantification (bottom) of Vps34 in HT-22 cells treated with vehicle (DMSO) or MG132 at 5, 10 and 20 μM for 4 h, respectively. **(B)** Representative Western blots (top) and quantification (bottom) of Tau protein in HT-22 cells treated with DMSO or MG132 at 5, 10 and 20 μM for 4 h, respectively (n = 3). **(C)** Representative Western blots (top) and quantification (bottom) of Vps34 in HT-22 cells treated with vehicle or chloroquine (CQ) at 25, 50 and 75 μM for 12 h (n = 3). **(D)** Representative Western blots (left) and quantification (right) of Vps34 in HT-22 cells treated with CHX (20 μg/ml) alone, CHX (20 μg/ml) and MG132 (5 μM), CHX (20 μg/ml) and CQ (30 μM) for 12, 18 and 24 h (n = 3). *P < 0.05; **P < 0.01.

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inhibitor are able to increase Vps34 protein levels, suggesting that UPS and lysosome are involved in Vps34 degradation.

In conclusion, our study fails to show that ubiquitination status may affect Vps34 protein levels, which is supported by the finding that the decreased ubiquitination levels coincide with the unaltered Vps34 protein levels in APP/PS1 mice. However, Vps34 does subject to degradation, as proteasomal and lysosomal inhibitors result in increased Vps34 protein levels. Our study highlights that although Vps34 ubiquitination may finally affect its degradation through autophagy- or UPS-mediated mechanisms, caution should be paid in the explanation of Vps34 degradation through the direct involvement of ubiquitination.

Conflict of interest

There is no conflict of interest.

Acknowledgements

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