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BACE1 SUMOylation increases its stability and escalates the protease activity in Alzheimer’s disease

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Amyloid beta (Aβ) is a major pathological marker in Alzheimer’s disease (AD), which is principally regulated by the rate-limiting β-secretase (i.e., BACE1) cleavage of amyloid precursor protein (APP). However, how BACE1 activity is posttranslationally regulated remains incompletely understood. Here, we show that BACE1 is predominantly SUMOylated at K501 residue, which escalates its protease activity and stability and subsequently increases Aβ production, leading to cognitive defect seen in the AD mouse model. Compared with a non-SUMOylated K501R mutant, injection of wild-type BACE1 significantly increases Aβ production and triggers cognitive dysfunction. Furthermore, overexpression of wild-type BACE1, but not non-SUMOylated K501R mutant, facilitates senile plaque formation and aggravates the cognitive deficit seen in the APP/PS1 AD mouse model. Together, our data strongly suggest that K501 SUMOylation on BACE1 plays a critical role in mediating its stability and enzymatic activity.

BACE1 SUMOylation | enzymatic activity | cognitive deficit | Alzheimer’s disease

Alzheimer’s disease (AD) is the most common form of dementia, and age is a major risk factor for this devastating neurodegenerative disease (1). The major pathological hallmarks of AD are the accumulation of senile plaque in the extracellular space and intraneuronal neurofibrillary tangle formation associated with excessive neuronal cell loss (1–3). Senile plaque is predominantly composed of amyloid beta (Aβ) generated from amyloid precursor protein (APP) via the amyloidogenic pathway (4, 5). Aβ is generated by sequential cleavage of APP by β-secretase (i.e., BACE1) and γ-secretase, with BACE1 as the rate-limiting enzyme in this process (6–10). Accumulated evidence demonstrates that BACE1 enzymatic activity increases in AD (11–13), while BACE1 mRNA and protein levels in AD remain unchanged (14–16), though there are contradictory reports indicating that BACE1 protein levels might increase (17–19).

APP cleavage by BACE1 preferentially occurs in the early or late endosomes that provide the acidic environment necessary for optimal BACE1 activity (20–22). After translation, BACE1 is modified by acetylation and N-glycosylation that might mediate BACE1 folding and enzymatic activity (23, 24). BACE1 is phosphorylated on S498, which mediates its intracellular trafficking (7, 25, 26). It has previously been documented that BACE1 can be ubiquitinated at K501 residue, regulating its half-life (27). The half-life of BACE1 is not greater than 12 h, allowing for substantial trafficking and activation-inactivation cycling between different subcellular compartments (28–30).

In the current study, we provide extensive in vitro and in vivo evidence demonstrating that BACE1 is SUMOylated on the same residue (K501), mediating its stability. Moreover, we show that the SUMOylation of BACE1 escalates its protease activity, resulting in augmentation of Aβ production. Accordingly, injection of wild-type BACE1, but not non-SUMOylated K501R mutant, facilitates Aβ production and accelerates senile plaque deposit and the cognitive defect associated with extensive synaptic degeneration.

Results

**BACE1 Is SUMOylated at K275 and K501 Residues.** Our previous data demonstrate that SUMO-1 (small ubiquitinlike modifier protein) 1 is highly increased in AD patients; we also reported that tau is SUMOylated in response to Aβ treatment (31). Hence, we explored whether or not BACE1 is also SUMOylated. We transfected HEK293 cells with FLAG-tagged BACE1, Myc-SUMO-1, and UBC9. Immunoprecipitation with anti-FLAG revealed that BACE1 was highly SUMOylated (Fig. 1A). Moreover, we made the similar observation in the neuronal cell line N2A, which is APP stably transfected. We found that endogenous BACE1 was also highly SUMOylated in the presence of UBC9 (Fig. 1B). One month after injection of FLAG-tagged BACE1 into wild-type C57BL/6J mice, we conducted immunoprecipitation with anti-FLAG, and found that BACE1 once again was strongly SUMOylated (Fig. 1C). Thus, these data suggest that BACE1 is greatly SUMOylated in the presence of UBC9.

To map the potential SUMOylation sites on BACE1, we performed SUMOylation analysis using a computer program, and found that K275 and K501 residues are potential SUMOylation sites (Fig. 1D). Protein alignment demonstrates that these two sites are well conserved among different species (Fig. 1E).

**Significance**

BACE1 is a rate-limiting enzyme for amyloid beta polypeptide production, which plays a crucial role in Alzheimer’s disease (AD) pathogenesis. However, how this essential protease is posttranslationally regulated remains incompletely understood. In the current study, we show that K501 residue on BACE1, a ubiquitin modification site, is also competitively SUMOylated. We discovered that SUMOylation of BACE1 augments its stability and enzymatic activity, resulting in senile plaque formation and cognitive defect. Identification of the posttranslational modification on BACE1 provides insight into the molecular mechanism in AD.


The authors declare no conflict of interest.

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SUMO-1 and UBC9. When we employed the double K275R/501R mutant, the SUMOylation was completely abolished, indicating that K275 and K501 residues are the major SUMOylation sites on BACE1 (Fig. 1F). The observation was similar in N2A/APP stable cell lines in the presence of UBC9 (Fig. 1G). Therefore, these findings imply that BACE1 can be SUMOylated at K275 and K501 residues.

**SUMOylation of BACE1 at K501 Increases Its Stability.** To compare the contribution of these two potential sites in BACE1 SUMOylation, we employed different amounts of protein lysates from the individual mutant. We found that K275R mutation substantially decreased BACE1 SUMOylation, and the non-SUMOylated BACE1 amount was also reduced (Fig. 2A). Strikingly, K501R SUMOylation was almost completely abolished. Noticeably, non-SUMOylated BACE1 was evidently diminished. Interestingly, similar results were observed when the same membranes were stripped and reprobed with anti–SUMO-1 antibody (Fig. 2A). To quantitatively analyze whether SUMOylation affects the half-life of BACE1, we treated FLAG-BACE1 plus UBC9–transfected cells with the translation inhibitor cycloheximide (CHX) for different time points. Immunoblotting analyses indicated that non-SUMOylated K501R half-life was clearly reduced compared with wild-type BACE1 (Fig. 2B–D). Again, the SUMOylated version of BACE1 was temporally attenuated in the presence of CHX (Fig. 2B and C). By contrast, the mRNA levels between wild-type BACE1 and K501R remained comparable (Fig. 2E), indicating that the time-dependent reduction of BACE1 and K501R mutant is not due to different mRNA levels. Presumably, SUMOylation might delay BACE1 degradation.

To address that non-SUMOylated K501R BACE1 becomes more prone to degradation compared with wild-type BACE1, we monitored their distribution in different types of subcellular compartments. The lysosome (LAMP1) and the endosomal compartment (Epsin1) were analyzed by immunofluorescence (Fig. 2F). In BACE1 WT, SUMOylated BACE1 was localized in both the lysosome and endosomal compartment (Fig. 2F, white arrow). In K501R BACE1, the SUMOylation was almost completely abolished (Fig. 2F, red arrow). The lysosomal compartment was also reduced in K501R BACE1 (Fig. 2F, blue arrow).

SUMOylation makes BACE1 more stable and decreases its degradation in the presence of translation inhibitor CHX. This suggests that SUMOylation might delay BACE1 degradation.
organelles. Immunofluorescence staining showed that both wild-type BACE1 and K501R occurred in the trans-Golgi network (TGN) stained with GM130 (Fig. S1A). Notably, wild-type BACE1 mostly localized in the endosomes stained with the biomarker EEA1 (Fig. S1B) and in plasma membrane stained with APP (Fig. S1C). By contrast, non-SUMOylated K501R BACE1 resided in the lysosomes stained with LAMPI for degradation (Fig. 2F). Together, these findings suggest that SUMOylation of BACE1 at K501 may mediate its intracellular trafficking and increase its stability.

**BACE1 K501 SUMOylation Increases Its Enzymatic Activity.** BACE1 cleaves APP and releases a secreted fragment of sAPPβ. To quantitatively compare the effect of SUMOylation on BACE1 enzymatic activity, we monitored sAPPβ production in the cells transfected with wild-type BACE1 or non-SUMOylated mutant K501R. We found that sAPPβ was significantly reduced in K501R-transfected cells compared with wild-type (Fig. 3A and B). However, it somehow increased the sAPPβ amount compared with vector control. Subsequently, we monitored the secretase Aβ40 and Aβ42 levels by ELISA in the medium. As expected, overexpressing wild-type BACE1 highly increased the levels of both Aβ40 and Aβ42. By contrast, K501R only slightly increased Aβ compared with vector control (Fig. 3C and D). Together, these data strongly suggest that SUMOylation of BACE1 at K501 enhances its protease activity and produces more Aβ.

**BACE1 SUMOylation Induces Cognitive Impairment and Aβ Toxicity.** Overexpression of BACE1 increases its SUMOylation and protease activity in cells. To investigate whether SUMOylation plays any role in mediating BACE1 enzymatic activity in animals, we injected wild-type BACE1 or non-SUMOylated K501R AAV2 into the hippocampus of wild-type C57BL/6J mice. In a month, we performed the Morris water maze behavior test and found that overexpressed wild-type BACE1 initiated evident cognitive dysfunction compared with vector control or non-SUMOylated K501R (Fig. 4 A–C). Nonetheless, there is no significant difference between vector control and K501R mice (Fig. 4A–C). The swimming speed remained comparable among the groups, indicating that BACE1 or its non-SUMOylated mutant does not affect motor function (Fig. 4D). Immunohistochemistry (IHC) staining with MAP2 in the CA1 region (AAV injection site) of the hippocampus demonstrated that the dendrites were greatly reduced in wild-type BACE1-injected mice, but not non-SUMOylated K501R, while non-SUMOylated BACE1 remained comparable to the control (Fig. 4 E and F). By contrast, the dendrites remained similar among the groups in CA3 and dentate gyrus regions (Fig. S2A–C). The synapses were remarkably decreased in wild-type BACE1-injected mice compared with vector control or non-SUMOylated K501R mutant mice. Immunoblotting analysis revealed that the synaptic proteins, including PSD95, GluR1, synaptophysin, synapsin-1, and synaptotagmin, were all reduced in the hippocampus injected with wild-type BACE1; in contrast, non-SUMOylated K501R-injected mice remained comparable to vector control (Fig. S2D–J). These observations were in alignment with Golgi staining (Fig. 4G–I) and dendrite staining results, indicating that the synapses are extensively lost in wild-type BACE1–overexpressed mice.

Next, we compared the expression levels of injected wild-type BACE1 and K501R. Surprisingly, we found wild-type BACE1 levels at around 55 kDa and 72 kDa, which were highly increased compared with K501R, indicating that its SUMOylation might regulate its stability (Fig. 4J and K). Accordingly, sAPPβ was substantially increased in wild-type BACE1–overexpressed mice compared with the control and non-SUMOylated K501R samples (Fig. 4J and L). It is worth noting that insulin-degrading enzyme, which cleaves Aβ, remained constant among all of these samples, suggesting that Aβ accumulation is not due to its degradative enzyme alteration in response to BACE1 expression (Fig. 4J). To validate the injected BACE1 K501R expression level, we performed IHC staining. As expected, the FLAG-tagged K501R signal was markedly reduced compared with wild-type BACE1 (Fig. S2J). IHC study with the anti-Aβ antibody 6E10 also supported that Aβ was highly aggregated in wild-type BACE1-injected mice compared with vector control or non-SUMOylated K501R (Fig. 4M). To further study the alteration of Aβ production under wild-type BACE1 and K501R AAV transection, we quantitatively analyzed Aβ levels, and found that Aβ40 and Aβ42 were prominently increased in wild-type BACE1–overexpressed mice compared with the control and non-SUMOylated K501R samples (Fig. 4N and O). Thus, these data strongly support that SUMOylation increases its stability and augments Aβ production compared with non-SUMOylated mutant, and that BACE1 SUMOylation aggravates Aβ toxicity and escalates cognitive impairment.

**Non-SUMOylated BACE1 Mutant Decreases Aβ Deposition and Cognitive Impairment in APP/PS1 Mice.** To assess whether BACE1 SUMOylation plays any role in AD pathologies, we employed an APP/PS1 AD mouse model. Firstly, to investigate whether BACE1 SUMOylation is age-dependently regulated in the AD mouse model, we collected brain samples from different ages of APP/PS1 mice and incubated them with the deSUMOylation inhibitor N-ethylmaleimide-crystalline (NEM). We found that BACE1 (with ~55-kDa bands) and SUMOylated BACE1 (with ~72-kDa bands) were up-regulated in an age-dependent manner; by contrast, in the absence of deSUMOylation inhibitor, BACE1 SUMOylation was obviously reduced (Fig. 5A and B). To further validate this observation, we performed immunoprecipitation with anti-SUMO-1 or anti-BACE1 antibody, and found that BACE1 SUMOylation was escalated in an age-dependent way (Fig. 5C and D). IHC study also supported that BACE1 SUMOylation was age-dependently up-regulated. Interestingly, BACE1 and SUMO-1 are tightly colocalized in CA1 hippocampal neurons in 12-mo-old APP/PS1 mice (Fig. 5E). As expected, Aβ deposit with thiophavin S staining was also age-dependently escalated in the

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*Fig. 3.* SUMOylation of BACE1 at K501 increases its enzymatic activity. (A and B) FLAG-BACE1 WT plus UBC9 or different lysine residue mutant (K275R and K501R) plasmids plus UBC9 were cotransfected to N2A/APP cells for 48 h; next, the secreted fragment APP-β cleaved by BACE1 and the total APP in lysate were examined by Western blotting and quantitative analysis. (C and D) The Aβ40 and Aβ42 levels in cell medium were measured by ELISA. ***p < 0.01 vs. vector; **P < 0.01 vs. BACE1 WT.
APP/PS1 mouse hippocampal region (Fig. 5F). In accordance with these observations, MAP2 IHC study indicated the dendrites were also temporally reduced in the hippocampus (Fig. S3).

To explore whether BACE1 SUMOylation regulates the cognitive functions in the APP/PS1 AD mouse model, we injected AAV2-vector, AAV2-BACE1 WT, or AAV2-BACE1 K501R into the hippocampus and performed the fear-conditioning experiment to monitor its effect in the cognitive function. Wild-type BACE1–injected mice revealed greatly reduced memory compared with the control or non-SUMOylated mutant (Fig. 5G and H). In 4-mo-old APP/PS1 mice, overexpression of wild-type BACE1 facilitated amyloid deposit compared with vector control; however, non-SUMOylated BACE1 mutant mice barely increased senile plaque formation in the hippocampus (Fig. 5I and J). Together, these data strongly suggest that BACE1 SUMOylation escalates Aβ deposition and cognitive impairment in APP/PS1 mice.

Discussion

In the current study, we provide extensive evidence supporting that BACE1 is SUMOylated in neuronal cell lines and in an AD mouse model. We found that K275 and K501 residues are the major SUMOylation sites on BACE1. Interestingly, K501 SUMOylation is required for BACE1 stability. It is worth noting that SUMOylation of BACE1 at K501 highly increases its enzymatic activity, resulting in Aβ-production escalation in the AD brain. Overexpression of wild-type BACE1, but not non-SUMOylated K501R, in wild-type mice and in the APP/PS1 AD mouse model highly increases Aβ production. Moreover, the synapses and dendrites are decreased in wild-type BACE1–injected mice, but not in non-SUMOylated mutant. In alignment with these observations, the behavior studies demonstrate that overexpression of wild-type BACE1 elicits cognitive dysfunction in wild-type mice, and facilitates amyloid deposit and cognitive impairment in APP/PS1 mice.

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BACE1 and mediate its enzymatic activity and biological functions (46–49). Moreover, they could intermediate BACE1 trafficking and lipid microdomain distribution (50). It remains unknown at this moment whether SUMOylation at K501 regulates BACE1 binding activity with different binding partners, including APP. We previously reported that SUMOylation is enhanced in AD patients’ brains (31) and that Aβ treatment increases SUMOylation and augments tau SUMOylation, which subsequently elevates its half-life and escalates its aggregation, leading to its neurotoxicity enhancement (31). Here, we demonstrate that BACE1 SUMOylation augments its stability and enzymatic activity, resulting in Aβ production up-regulation. Conceivably, SUMOylation abnormality in AD might regulate numerous major pathological players, including tau and BACE1, and mediate its pathogenesis.

Materials and Methods

Plasmids, Viruses, Chemicals, and Antibodies. The plasmids encoding FLAG-tagged full-length BACE1 or His-tagged full-length SUMO-1 were generated in our laboratory. Mutation of BACE1 lysine 275 or 501 into arginine (K275R or K501R) was carried out using the QuikChange site-directed mutagenesis kit by following the manufacturer’s instructions (GeneChem). All plasmids were sequenced and prepared using an endotoxin-free plasmid extraction kit (Qiagen). AAV-pCAG-FLAG-BACE1WT and AAV-pCAG-FLAG-BACE1K501R were constructed and packaged by Obio Technology Co., Ltd. Lipofectamine 2000 (Invitrogen) transfection reagents were from Invitrogen. NEM was purchased from Sigma. CHX was from Merck KGaA. Bicinchoninic acid protein detection kit was from Pierce. Reagents for cell culture were from Gibco BRL. Antibodies employed in this study are listed in Table S1.

Cell Culture, Transfection, and Drug Treatment. The human embryonic kidney 293 (HEK293T) cells were cultured in DMEM supplemented with 10% FBS (Gibco BRL), and mouse neuroblastoma N2A cells with stable expression of APP (N2A/APP) were cultured in DMEM supplemented with 10% FBS and 200 mg/mL G418. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were seeded in a 6- or 12-well culture plate overnight and cotransfected with the mixture containing a total of 1.8 μg of plasmids and 4 μL of Lipofectamine 2000 according to the manufacturer’s protocols. To explore the effect of BACE1 SUMOylation on degradation, the cells were treated with 100 μg/mL CHX for different times.

Other Methods. SI Materials and Methods includes details regarding RNA extraction, reverse transcription, quantitative real-time PCR, Aβ plaque

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was performed using either Student’s t test (two groups) or one-way analysis of variance (more than two groups), followed by post hoc comparison; differences with \( P < 0.05 \) were considered as statistically significant.

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