14-3-3 ζ Captures SET in the Cytoplasm, Mediating Tau Pathology and Cognitive Impairments

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14-3-3ζ captures SET in the cytoplasm, mediating tau pathology and cognitive impairments

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Abstract:

Background: 14-3-3 ζ is overexpressed in brain regions affected by tau pathology in Alzheimer's disease (AD), and its expression correlates with disease progression. The nuclear protein SET is a specific and efficient endogenous inhibitor of PP2A, which acts as a major protein phosphatase regulating tau phosphorylation and is compromised in AD. However, whether 14-3-3 ζ mediates the cytoplasmic retention of SET and its related downstream events in AD remains elusive.

Methods: We performed Spearman correlation analysis between 14-3-3 and SET in normal control brains and AD brains in a large-scale proteomic study of AD, and observed the distribution of SET and 14-3-3 in AD brain neurons, thus finding the cytoplasmic retention event of SET. We synthesized the peptides that prevented SET from binding to 14-3-3, and evaluated the effects of these peptides in blocking tau pathology.

Results: Here, we reported that there is a significant increase in 14-3-3 ζ interaction with SET during aging in 3 $\times$ Tg AD mice and the human AD brain. 14-3-3 ζ captures and sequesters SET in the cytoplasm and subsequently inhibits PP2A activity, induces tau hyperphosphorylation and triggers neurodegeneration, leading to behavioral impairments in mice infected with adeno-associated virus encoding 14-3-3 ζ . Interestingly, as a dimer and scaffold protein, 14-3-3 ζ was found to bind to SET while simultaneously recruiting CKII, which induced SET phosphorylation and cytoplasmic retention. Conversely, silencing 14-3-3 ζ impeded CKII phosphorylation of SET. Moreover, blocking the 14-3-3 ζ interaction with SET with the peptide NESGDPSSKST substantially induced SET translocation back to the nucleus, augmented PP2A activity and subsequently led to reduced tau hyperphosphorylation, decreased synapse loss and rescue of cognitive deficits in 14-3-3 ζ mice. Furthermore, the peptide NESGDPSSKST but not the scramble peptide attenuated tau pathology and cognitive deficits in a 3 $\times$ Tg AD mouse model.

Conclusions: Our data strongly indicate that 14-3-3 ζ captures SET in the cytoplasm and simultaneously recruits CKII, and this interaction plays a critical role in mediating tau-related clinical and neuropathological alterations. Inhibition of the

14-3-3 ζ interaction with SET may be therapeutically useful for treating AD.

Keywords: Alzheimer's disease (AD), 14-3-3 ζ , SET, PP2A, tau, CKII, cognitive impairment.

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Background

Abnormal hyperphosphorylation of tau is a common and critical pathway for some neurodegenerative diseases, including Alzheimer's disease (AD). Pathological hyperphosphorylation severely interferes with tau's ability to regulate microtubule (MT) dynamics and induces the formation of intracellular neurofibrillary tangles (NFTs), which positively correlate with the severity of clinical dementia in AD, implicating hyperphosphorylation as a potent inducer of tau pathology. The expression of protein phosphatase-2A (PP2A), a major tau phosphorylation regulator [1], is downregulated in the AD brain [2], while the level of its endogenous inhibitor 2 (I_2^{PP2A}), known as SET, is increased in AD [3].

The SET protein, also called template-activating factor 1 β (TAF1 β) or the PHAPII protein, was identified as a 39-kDa phosphoprotein [4] and is normally found predominantly in the nucleus [5], where it has been reported to block both DNase activity and acetylation of histones [6]. In the AD brain, SET is principally mislocated in the cytoplasm and translocated from the nucleus, where it colocalizes with PP2A and hyperphosphorylated tau [3,7]. Furthermore, overexpression of SET in PC12 cells causes abnormal hyperphosphorylation of tau [8]. However, little is known about the exact regulatory mechanism by which SET is retained in the cytoplasm or the pathological consequences of its translocation in AD.

14-3-3 participates in the regulation of diverse biological processes, including neuronal development, cell growth control, apoptosis, signal transduction, etc. Moreover, some studies point to an important role of 14-3-3 in the nuclear transport of some cargoes, such as Cdc25, RSG and HDAC4 [9-11]. 14-3-3 β/ζ is present in tangles in AD and associates with tau proteins [12-14]. 14-3-3 ζ is positively stained in AD brain regions affected by tau pathology and correlates with disease progression [15]. As a dimer, 14-3-3 ζ can simultaneously bind both tau and GSK-3 β , enhancing tau phosphorylation [16]. These findings imply that 14-3-3 β/ζ has a significant impact on tau phosphorylation in AD. Whether 14-3-3 β/ζ also interacts with SET and participates in tau pathology during the development of AD is unknown.

We noticed coincidentally that the ¹⁴⁶NESGDPSSKST¹⁵⁶ sequence of SET fits with the

putative 14-3-3 binding motif *RXI-2SX2-3S* [17]. Therefore, we aimed to investigate whether ¹⁴⁶NESGDPSSKST¹⁵⁶ affects the interaction of SET with 14-3-3 and its downstream events in the current study. Our recent studies have shown that casein kinase II (CKII) phosphorylates Ser9 on SET, leading to its cytoplasmic translocation and tau phosphorylation [18, 19]. Whether 14-3-3 mediates CKII phosphorylation of SET and induces SET cytoplasmic retention remains unknown.

In this report, we show that 14-3-3 ζ captures and retains SET in the cytoplasm, which subsequently inhibits PP2A activity, resulting in tau hyperphosphorylation. Furthermore, the 14-3-3 ζ interaction with SET is upregulated in 3 $\times$ Tg AD mice. Consistent with this finding, 14-3-3 ζ colocalization with SET in the neuronal cytoplasm is detected in human AD brains. 14-3-3 ζ is required for CKII phosphorylation of SET and its cytoplasmic retention. The synthetic peptide NESGDPSSKST substantially blocks the interaction of 14-3-3 ζ with SET and attenuates tau hyperphosphorylation, protecting against cognitive defects in 14-3-3 ζ mice and the 3 $\times$ Tg AD mouse model. Hence, our findings imply that the capture of SET by 14-3-3 ζ and the simultaneous recruitment of CKII to the cytoplasm might be a mechanism-based therapeutic target for treating tau-related diseases, including AD.

Materials and Methods

Reagents

The primary antibodies employed in this study and their properties are listed in Table 1. Secondary antibodies for Western blotting were from Amersham Pharmacia Biotech (Little Chalfort, Buckinghamshire, UK).

Plasmids 14-3-3 β and 14-3-3 ζ were obtained from Prof. Xiaoqian Chen, and pCDNA3.1+ and his-SET were sequenced and prepared using an endotoxin-free plasmid extraction kit (Tiangen). AAV viral packaging was from Neuron Biotech Co. Ltd.: pAOV.CMV.bGlobin. EGFP AAV2 (EGFP, titer=6.34 $\times 10^{12}$ v.g./ml), pAOV.CMV.bGlobin.3 $\times$ FLAG AAV2 (Ctr, titer=4.91 $\times 10^{12}$ v.g./ml), and pAOV.CMV.bGlobin.14-3-3 ζ AAV2 (14-3-3 ζ , titer=6.83 $\times 10^{12}$ v.g./ml).

Cell culture and transfection

HEK293 cells were stably transfected with the longest human tau (tau441) (HEK293/tau). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, Bleiswijk, Netherlands) in the presence of 200 µg/ml G418 containing 10% fetal bovine serum (FBS) and kept in a humidified incubator aerated with 95% air and 5% CO₂ at 37 °C. HEK293/tau cells were seeded into 6-well plates 1 day prior to transfection, which was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Primary embryonic hippocampal neurons from the mice (18 d) were collected and incubated with 4 ml of 0.125% trypsin in calcium- and magnesium-free Hanks' balanced salt solution for 15 min and then centrifuged at 80 × g for 5 min after the addition of 4 ml of neuronal plating medium containing DMEM/F12 with 10% FBS. The cells in the pellet were triturated and plated onto a 60 mm plastic culture dish (400-500 neurons/mm²) and incubated in a humidified incubator with 5% CO₂ at 37 °C for 2-4 h. Then, the plating medium was replaced with neurobasal medium supplemented with 2% B27 (maintenance medium), and the cells were cultured for 14 days in vitro (DIV). The medium was changed every 3 days, with replacement of half the volume with fresh maintenance medium each time.

Animals

Male C57/BL mice (4 months old, 20±2 g) supplied by the Experimental Animal Central of Tongji Medical College were housed with ad libitum access to food and water. 3×Tg AD mice (PS1m146v/APPswe/TauP301L) were purchased from the Jackson Laboratory. All animal experiments were approved by the Animal Care and Use Committee of Huazhong University of Science and Technology and performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were kept in cages under a 12:12 light-dark cycle with the light on from 7:00 AM to 7:00 PM.

Tat-peptide treatment

HEK293/tau cells or primary embryonic hippocampal neurons were treated with 50 μ M PBS, dissolved Peptide 1 (P1, Tat-NESGDPSSKST) or Peptide 2 (P2, Tat-NSSTEDGPKSS) as a negative scrambled control. Tat (YGRKKRRQRRR), from an HIV-1-encoded potent trans-activator protein, is a cell membrane transduction domain and is able to permeate the fusing peptide through the cell membrane [20-22]. To test whether the Tat peptide could be delivered into the cells, Tat-fluorescein isothiocyanate (FITC, 50 μ M) was added. Mice were intravenously administered 10 mg/kg Peptide 1 or Peptide 2 for 7 days. Peptides with 99% purity were synthesized by ChinaPeptides (Shanghai, China). The peptides were numbered, and the experimenters were unaware of which peptides were applied in all experiments.

Human tissue samples

Postmortem brain samples were dissected from frozen brains of four AD patients (age 74.5 ± 11.2 years, mean $\pm$ S.D.) and four nondemented control subjects (age 73.9 ± 12.7 years) from the Emory Alzheimer's Disease Research Center. Informed consent was obtained for all subjects. The study was approved by the Emory University CND Tissue Committee. AD was diagnosed according to the criteria of the Consortium to Establish a Registry for AD and the National Institute on Aging. Diagnoses were confirmed by the presence of amyloid plaques and neurofibrillary tangles in formalin-fixed tissue. The postmortem interval was similar between the AD group and the control group.

Immunofluorescence and image analysis

The immunofluorescence protocol was as described previously [18]. In brief, transfected/treated cells or mouse brain slices were fixed and incubated for 24-48 h at 4 °C with primary antibodies followed by 1 h at 37 °C with Alexa Fluor® 568- or Alexa Fluor® 488-conjugated secondary antibodies (Invitrogen). Hoechst 33258 (1 μ g/ml) (Sigma) was used for nuclear staining. The subcellular localization of SET in HEK293/tau cells was visualized with a confocal laser microscope (LSM710 Carl

Zeiss). Image analysis to quantify relative subcellular localization was performed as previously described [23]. The nuclear (Fn) and cytoplasmic (Fc) fluorescence ratios were determined from single-cell measurements using ImageJ 1.41o public domain software (National Institutes of Health (NIH), Bethesda, MD, USA). Images of EGFP-labeled dendritic processes at the outer molecular layer were acquired at 0.5- μ m intervals with the Bio-Rad R2100 confocal system with a plan apochromatic 60 $\times$ oil lens (numerical aperture (NA), 1.4; Nikon) and a digital zoom of 5. The length of each dendritic segment was determined by tracing the center of the dendritic shaft, and the number of spines was counted manually from the two-dimensional projections. Classification of mushroom spines was performed as described previously [24], subsequent to the subtraction of background fluorescence due to autofluorescence.

Hippocampal stereotactic injection

The bilateral hippocampal DG zone of mice was injected with pAOV.CMV.bGlobin. EGFP AAV2 (EGFP, titer= 6.34×10^{12} v.g./ml) or pAOV.CMV.bGlobin.14-3-3 ζ AAV2 (14-3-3 ζ , titer= 6.83×10^{12} v.g./ml, total volume=2 μ l) as described previously [21]. The injection site for adult mice was calculated as follows by using the position of the bregma as a reference: anteroposterior, 2 mm; lateral, +/-1.6 mm; ventral, -2.1 mm.

Subcellular fractionation

Cytoplasmic and nuclear extracts were prepared according to the instructions of the nuclear and cytoplasmic extraction kit (BestBio, Shanghai, China). The quantitative changes in SET in the nucleus or cytoplasm were detected by Western blotting. LaminB-1 was used as a nuclear envelope marker. Raf-1 was used as a cytosolic protein marker. The amount of protein present was normalized to either Raf-1 or LaminB-1.

Immunoprecipitation and Western blotting

His-tagged SET was immunoprecipitated using an anti-His antibody, according to the recommendations of the manufacturer. Briefly, cells were harvested 48 h

post-transfection, lysed in 300 μ l of lysis buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with complete protease inhibitors and then incubated on ice for 15 min. Cell debris was removed by centrifugation at 14,000 rpm for 20 min at 4 °C. The protein extract was diluted, and the optimal concentration was 1-5 mg/ml. The sample was incubated overnight at 4 °C with the recommended amount of anti-His antibody. Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) (1/10 of the volume of each sample) was prewashed with lysis buffer, added to the lysate/antibody mix, and incubated for 4 h at 4 °C. The beads were pelleted by centrifugation at 1000 rpm for 1 min, and the beads were then washed 3 times in 1 ml of TBS; the samples were centrifuged, and the supernatant was discarded after each wash. Then, the supernatant from the last wash was removed, and 2 $\times$ loading buffer (the same volume as Protein A/G PLUS-Agarose) was added. Proteins were eluted from the beads using SDS lysis buffer (0.2% bromophenol blue, 10% glycerol, 200 mM DTT, 2% SDS, and 100 mM Tris; pH 8.3), 3/4 of the eluate was subjected to polyacrylamide gel electrophoresis (10% gel), and the separated proteins were then transferred to a nitrocellulose membrane. The membrane was blocked in 5% skim milk powder (w/v) in 1 $\times$ TBS for 30 min at RT. Detection of 14-3-3 β / ζ or importin α binding to SET was performed by incubating the membranes with 14-3-3 antibody (ab9063) or importin α (yD-18) antibody (sc-32681) overnight at 4 °C. After three washes with TBS-T (3 $\times$ 10 min), the samples were incubated with fluorescently labeled secondary antibody (1:10,000; Odyssey) for 60 min at RT, followed by another three washes with TBS-T (5 min $\times$ 3). The membrane was scanned in the appropriate channels (700 nm for the Cy5.5 antibody, 800 nm for the IRDye800TM antibody), and protein bands were quantitatively analyzed with the OdysseyTM System following the manufacturer's instructions. The lanes marked "input" were loaded with 10% of the starting material used for immunoprecipitation. Similarly, 14-3-3 β / ζ or importin α was immunoprecipitated. Forty-eight hours (48 h) after transfection, HEK293/tau cells were rinsed twice in ice-cold PBS (pH 7.5) and lysed with buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.02%

sodium azide, 100 µg/ml phenylmethanesulfonylfluoride, and 10 µg/ml protease inhibitors (leupeptin, aprotinin and pepstatin), followed by sonication for 5 s on ice. After centrifugation at 12,000 g for 5 min at 4 °C, the supernatants were removed, and an equal volume of 2× Laemmle sample buffer was added. Samples were boiled for 10 min before electrophoresis. Protein concentrations were estimated with a BCA kit (Pierce, Rockford, IL, USA).

For brain samples, mouse hippocampal tissues were quickly isolated and homogenized on ice to generate 12% (w/v) homogenate in buffer containing 50 mM Tris·HCl (pH 7.4), 8.5% sucrose, 2 mM EDTA, 10 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/ml leupeptin, and 2 µg/ml each of aprotinin and pepstatin A. The tissue homogenate used for Western blots also contained 20 mM β-glycerophosphate, 50 mM NaF, and 1 mM Na₃VO₄ to inhibit phosphatase activity. The tissue samples were then boiled in Laemmli buffer in a water bath for 5 min and subjected to 10% SDS-PAGE and Western blotting.

PP2A activity assay

PP2A activity in the cell extracts was measured using the V2460 phosphatase kit according to the manufacturer's instructions (Promega), as previously described [25].

Contextual fear conditioning

This test was performed as described previously [26]. Briefly, two foot shocks of 0.5 mA for 2 s separated by a 60 s lap time were administered after the 2-min period of context exploration. One hour and 24 h after training, the mice were tested for contextual fear memory by placing the animals in the conditioning context for a 5-min period. The incidence of freezing was scored in 5-s intervals as either “freezing” or “not freezing”. The percentage of freezing indicates the number of intervals in which freezing was observed divided by the total number of 5-s intervals.

Morris water maze (MWM) test

The standard MWM procedure was used [27]. Briefly, mice were handled daily for 6

days before the experiment and trained with 3 trials per day protocol. Each mouse swam until it found the hidden platform. If the mouse had not found the platform after 60 s, it was gently guided to the platform and stayed there for 10 s before being returned to the cage. For the probe test, the platform was removed, and each mouse was allowed to swim for 60 s. The swimming trajectory was monitored with a Noldus video tracking system (Ethovision, Noldus Information Technology, Holland).

Long-term potentiation (LTP) measurements

LTP was measured using the MED64 multielectrode array (Alpha Med Sciences, Kadoma, Japan) as described previously [28]. Briefly, mouse brain slices (300 μm) were transferred to the recording chamber, which consisted of an 8×8 electrode array. Correct placement of the electrodes at the DG–CA1 region was done manually and monitored with a microscope (MIC-D, Olympus Ltd., Japan). The section was constantly perfused with warmed (32 °C) aCSF at a flow rate of 2 ml/min and supplied with humidified 95% O₂ and 5% CO₂. A stimulation channel and a recording channel were selected out of the 64 possible electrodes. Single-pulse stimuli were delivered from the stimulation channel, and the slope of the line from the resulting EPSPs was measured from the defined recording channel. Stimulus amplitude was selected by determining the maximum response slope and then selecting the stimulus amplitude that produced a response that was ~40% of the maximal response. Baseline recordings were collected for a minimum of 30 min. Following baseline, an induction protocol that evoked LTP was applied. The LTP induction protocol consisted of 1 train of 100 Hz stimulus that lasted for 1 s, and the field potential response for 1 h after the tetanus was recorded. LTP was quantified as the % change in the average amplitude of the fEPSPs taken from 50 to 60 min intervals after E-LTP induction.

Statistical analysis

Data were analyzed using SPSS 18.0 statistical software. All results are expressed as the mean $\pm$ SEM. One-way ANOVA followed by LSD post hoc tests was used to determine the statistical significance of differences in means. For a single comparison,

the significance of differences between means was determined by a t-test. $P < 0.05$ was accepted as the significance criterion.

Proteomic data analysis

The Mount Sinai Brain Bank (MSBB) cohort dataset includes proteomics data from samples derived from the parahippocampal gyrus (PHG) of 185 healthy control and AD subjects [29]. The age at the time of death (AOD) of this population ranges from 61 to 108 years, with a mean and standard deviation of 84.7 ± 9.7 . Among 185 subjects, there were 116 females and 69 males. We further evaluated each donor and corresponding brain sample for multiple cognitive and neuropathological features, such as the mean plaque density, Braak staging for neurofibrillary tangles (NFTs), clinical dementia rating (CDR), and neuropathology scale, as determined by the consortium to establish a registry for Alzheimer's disease (CERAD) protocol. We applied the multiplexed TMT method and two-dimensional LC/LC-MS/MS for protein identification. We conducted protein quantification via the JUMP software suite following the methodology of Bai *et al.* In total, we identified 12,147 distinct proteins. Quantified and normalized protein levels were log₂ transformed and further adjusted by PMI, AOD, race, sex, and batch using a linear model. The residuals from the regression model were used for the correlation analysis.

Results

14-3-3 ζ interacts with SET in the cytoplasm in the human AD brain and 3 $\times$ Tg AD mice

Upregulation of 14-3-3 is observed in AD brain regions affected by neurofibrillary degeneration [12, 15, 30], and we have previously shown that SET translocation from the nucleus to the cytoplasm is tightly associated with tau pathology [18, 19]. However, whether 14-3-3 associates with SET and whether this event is involved in AD remain unclear. To investigate this possibility, we first examined how 14-3-3 and SET are correlated in normal control brains and AD brains in a large-scale proteomic study of AD and then performed immunofluorescence staining analysis of AD brains.

The Mount Sinai Brain Bank (MSBB) cohort includes proteomics data for samples from the parahippocampal gyrus (PHG) of 185 healthy control and AD subjects. In this cohort, we identified two protein isoforms for both YWHAZ (14-3-3 ζ) and SET. We performed Spearman correlation analysis between YWHAZ and SET protein isoforms in the normal control group and the AD group. As shown in Fig. 1A-B and Table 2. No correlation was found between YWHAZ and SET in the normal controls ($r=0.1$, $p=0.63$), but a highly significant correlation was observed in the AD group ($r=-0.41$, $p<2.2E-16$), suggesting that 14-3-3 ζ interacts with SET only in AD brains but not in healthy control brains. These data are consistent with the immunofluorescence results, in which SET was observed to be translocated from the nucleus to the cytoplasm and colocalized with 14-3-3 ζ in AD brains, while SET resided in the nucleus in normal control brains (Fig. 1C-D). To further support this observation, we also performed co-immunoprecipitation (co-IP) with antibodies against 14-3-3 ζ and SET and found that the 14-3-3 interaction with SET in the AD brain was markedly increased relative to that in the normal control brain (Fig. 1E-F and Fig. S1A-B). Next, we extended our study to an AD animal model. We observed that the level of 14-3-3 ζ was significantly increased in 6-month-old 3 $\times$ Tg AD mice compared to 2- and 4-month-old mice (Fig. 1G-H). Although there was no significant difference in 14-3-3 ζ levels between 2- and 4-month-old 3 $\times$ Tg AD mice, co-IP analysis showed that the nominal association of 14-3-3 ζ /SET in 4-month-old 3 $\times$ Tg AD mice was significantly increased compared to that in 2-month-old mice (Fig. 1I-J and Fig. S1C-D), suggesting that the interaction of 14-3-3 ζ with SET increases in an age-dependent manner. Immunofluorescence assays showed similar results, with a tight interaction between 14-3-3 ζ and SET in the neuronal cytoplasm of the hippocampus (Fig. 1K-L) and cortex (Fig. S1E-F) in 4-month-old 3 $\times$ Tg AD mice. Together, our data strongly suggest that SET gradually and increasingly binds to 14-3-3 ζ in the cytoplasm as AD progresses.

Overexpression of 14-3-3 induces cytoplasmic accumulation of SET

As an endogenous inhibitor of PP2A, the nuclear protein SET translocates to the cytoplasm, where it colocalizes with PP2A and hyperphosphorylated tau in AD [3,7]. However, the molecular mechanism underlying the retention of SET in the cytoplasm remains unclear. To investigate whether 14-3-3 mediates SET cytoplasmic retention,

we transfected HEK293/tau cells with pcDNA3.1+, 14-3-3 β , and 14-3-3 ζ for 48 h and then detected the SET distribution by immunofluorescence. We observed that SET translocated from the nucleus to the cytoplasm in 14-3-3 β/ζ -transfected HEK293/tau cells (Fig. 2A). Quantitative analysis showed that overexpression of 14-3-3 β/ζ markedly increased SET cytoplasmic fluorescence (Fc), with a simultaneous decrease in nuclear fluorescence intensity (Fn), especially following 14-3-3 ζ overexpression, revealing a more obvious effect of 14-3-3 ζ than 14-3-3 β (Fig. 2B). Subcellular fractionation and immunoblotting showed that coexpression of 14-3-3 β/ζ and SET remarkably increased the cytosolic level of SET, with concomitant reduction of SET levels in the nuclear fraction, whereas SET mainly remained in the nuclear fraction in cells transfected with SET alone (Fig. 2C-E). Taken together, these data demonstrate that overexpression of 14-3-3 β/ζ promotes cytoplasmic SET accumulation.

To further confirm this observation, we performed immunoprecipitation (IP) with a 14-3-3 antibody and found that SET tightly interacted with 14-3-3 (upper panel of Fig. 2F). Normal SET nuclear import is mediated by a classical NLS-dependent pathway through the formation of the ternary complex containing SET, importin α and importin β [19, 30]. We hypothesize that overexpressed 14-3-3 competes with importin α for binding with SET, interferes with the formation of the SET/importin α complex, and subsequently induces SET retention in the cytoplasm. To address this hypothesis, we performed IP with anti-his (SET) (middle panel of Fig. 2F) or anti-importin α (lower panel of Fig. 2F) antibodies and found that 14-3-3 β/ζ significantly decreased the SET interaction with importin α compared to that in the pcDNA3.1+ control (Fig. 2F and G). Notably, 14-3-3 ζ displayed a much stronger affinity for SET than did 14-3-3 β . Thus, these data strongly suggest that 14-3-3 β and ζ , especially 14-3-3 ζ , capture SET and impede the formation of the SET nuclear import complex, resulting in SET cytoplasmic retention.

PP2A activity is inhibited and tau is highly phosphorylated in 14-3-3 β/ζ -transfected HEK293/tau cells

A previous study demonstrated that SET inhibits PP2A activity in cells [31]. However, whether 14-3-3 β/ζ capture of SET in the cytoplasm induces PP2A inactivation is unknown. To address this question, we transiently transfected HEK293/tau cells with the same amount of pcDNA3.1+ vector, 14-3-3 β , 14-3-3 ζ , his-SET, 14-3-3 β +his-SET and 14-3-3 ζ +his-SET and then measured PP2A activity at 48 h following transfection. Compared with that in the vector control group, PP2A activity in each treated cell was significantly decreased. Interestingly, PP2A activity in 14-3-3 β/ζ +his-SET cells was significantly decreased compared to that in his-SET cells (Fig. 3A). PP2A is a major phosphatase that regulates tau phosphorylation [1]; thus, inhibition of PP2A would be expected to induce tau hyperphosphorylation. Next, we monitored tau phosphorylation status with different antibodies via immunoblot analysis. As expected, tau phosphorylation at the Ser199, Ser202/Thr205 (AT8), Thr231, Ser262, Ser396 and Ser404 sites was tightly correlated with PP2A phosphatase activity (Fig. 3B), and the quantitative data are summarized in Fig. 3C. These data suggest that 14-3-3 β/ζ -mediated retention of SET in the cytoplasm results in PP2A inhibition and consequent tau hyperphosphorylation.

14-3-3 ζ is required for CKII phosphorylation of SET

Our recent studies showed that CKII phosphorylates SET, leading to inhibition of PP2A, which results in tau pathology in AD [18,19]. Whether 14-3-3 ζ is associated with CKII phosphorylation of SET remains unknown. To address this point, we first investigated the interaction among 14-3-3 ζ , CKII and SET in the brains of AAV2-14-3-3 ζ -transfected C57/BL6 mice. Immunofluorescence assays showed that 14-3-3 ζ and CKII colocalized with SET in the cytoplasm in the hippocampus and cortex of 14-3-3 ζ mice, while SET was mostly localized in the nucleus in the hippocampus and cortex of control mice (Fig. 4A), suggesting that these three proteins might interact with each other. To confirm this point, co-IP with antibodies against 14-3-3 ζ , CKII and SET was performed. We found that 14-3-3 ζ , CKII and SET were bound to each other (Fig. 4B-D). Based on these results, we speculate that as a dimer, 14-3-3 ζ simultaneously recruits CKII and SET, thus making it easier for SET to become phosphorylated by CKII. To test this possibility, we used TBB, an inhibitor

of CKII, to treat HEK293/tau cells transfected with 14-3-3 ζ . We found that overexpression of 14-3-3 ζ led to SET hyperphosphorylation (Fig. 4E and F) and a decrease in PP2A activity (Fig. 4G). However, inhibition of CKII by TBB rescued SET hyperphosphorylation and partially increased PP2A activity (Fig. 4E-G) in 14-3-3 ζ -transfected HEK293/tau cells. Next, to confirm that 14-3-3 ζ is a key factor for CKII phosphorylation of SET, we knocked down 14-3-3 ζ in HEK293/tau cells transfected with CKII. We found that CKII overexpression induced an increase in SET phosphorylation (Fig. 4H and I) and a decrease in PP2A activity (Fig. 4J). Surprisingly, knockdown of 14-3-3 ζ rescued CKII-induced SET phosphorylation and PP2A deficiency (Fig. 4H-J). Taken together, these findings underscore that 14-3-3 ζ is required for CKII-induced phosphorylation of SET and subsequent PP2A inhibition.

Overexpression of 14-3-3 ζ induces cognitive deficits

To determine whether 14-3-3 ζ capture of SET in the cytoplasm induces any cognitive defects, we injected AAV2 virus overexpressing 14-3-3 ζ into the dentate gyrus of 4-month-old C57/BL6 mice (Fig. 5A). One month after treatment, we performed a fear conditioning assay and found that the ratios of freezing time in 14-3-3 ζ mice were markedly decreased compared to those in control mice at 24 h but not 1 h after foot shocks, revealing a long-term hippocampal-dependent memory deficit (Fig. 5B). Next, we used a Morris water maze (MWM) test to further measure the effects of 14-3-3 ζ on hippocampus-dependent spatial learning and memory. The escape latencies to find the hidden platform in 14-3-3 ζ mice were significantly increased compared to those in control mice on the last day of a 6-day learning trial (Fig. 5C). Moreover, compared to the control, overexpression of 14-3-3 ζ induced remarkable reductions in the mean number of annulus crossings and time spent in the target quadrant on the 7th and 9th days, when the platform was removed (Fig. 5D and E). However, overexpression of 14-3-3 ζ did not affect motor activity because swimming speed remained comparable in control and 14-3-3 ζ -overexpressing mice (Fig. 5F). Thus, our data indicate that 14-3-3 ζ overexpression results in cognitive defects in C57/BL6 mice.

The ¹⁴⁶NESGDPSSKST¹⁵⁶ peptide blocks the 14-3-3ζ interaction with SET and rescues PP2A activity, alleviating tau hyperphosphorylation

To further investigate the involvement of 14-3-3ζ in SET cytoplasmic translocation and phosphorylation, we asked whether blocking the 14-3-3ζ interaction with SET would release SET from the cytoplasm back to the nucleus and recover PP2A activity and its downstream events, including cognitive impairments. To this aim, we first sequenced full-length SET and found that the sequence ¹⁴⁶NESGDPSSKST¹⁵⁶ is consistent with a consensus recognition motif of 14-3-3, RX₁₋₂SX₂₋₃S [17]. Therefore, we synthesized two peptides: Peptide 1 (P1) (Tat-NESGDPSSKST) and Peptide 2 (P2) (Tat-NSSTEDGPKSS) as a negative scrambled control. To test whether Tat-peptide could penetrate the cells, we treated HEK293/tau cells with Tat-FITC (50 μM). Immunofluorescence showed that Tat-peptide penetrated the cells, indicating that Tat permeates the cell membrane (Fig. S2A). Next, we treated 14-3-3ζ-transfected HEK293/tau cells with the peptides for 48 h and then measured the subcellular localization of SET. We observed that peptide 1-treated cells showed nuclear localization of SET, while peptide 2-treated cells displayed cytoplasmic SET localization similar to that of PBS-treated control cells, indicating that Peptide 1 but not scramble Peptide 2 blocked SET cytoplasmic retention (Fig. 6A). Quantitative analysis showed that nuclear SET (Fn) levels were significantly increased and cytosolic SET (Fc) levels were strikingly decreased compared to those of the PBS control in the presence of Peptide 1, while Peptide 2 treatment did not change the Fn and Fc levels of SET (Fig. 6B), suggesting that Peptide 1 induces the return of SET back to the nucleus in 14-3-3ζ-transfected cells. We also isolated the cytoplasmic and nuclear fractions from 14-3-3ζ-transfected HEK293/tau cell lysates and carried out Western blot analysis with anti-SET, LaminB-1 and Raf-1 antibodies (Fig. 6C). Quantitative data showed that overexpression of 14-3-3ζ induced marked cytosolic retention of SET with a reduced nuclear distribution, which could be abrogated by Peptide 1, but not Peptide 2 (Fig. 6D-E).

SET nuclear import depends on the formation of a ternary complex containing SET,

importin α and importin β . We hypothesized that Peptide 1 competes with SET to interact with 14-3-3 ζ and thus allows SET to escape from being retained in the cytoplasm by 14-3-3 ζ ; therefore, more 14-3-3 ζ -free SET becomes available in the cytoplasm, which then binds to importin α and forms the nuclear import complex, ultimately inducing translocation of SET back to the nucleus. To test this hypothesis, co-IP was used to detect the binding efficiency between 14-3-3 ζ and SET or importin α and SET under peptide treatment at low, medium and high concentrations (Fig. 6F). Quantitative data showed that Peptide 1 remarkably inhibited the association of SET with 14-3-3 ζ in a dose-dependent manner (Fig. 6G) and boosted the association of SET with importin α (Fig. 6H), while Peptide 2 treatment did not change the binding efficiency between these proteins (Fig. 6G-H). These findings suggest that 14-3-3 ζ binding with SET interferes with the formation of the SET nuclear import complex.

14-3-3 ζ -mediated retention of SET in the cytoplasm leads to inhibition of PP2A and hyperphosphorylation of tau (Fig. 3). To assess the effect of Peptide 1 on PP2A activity and tau phosphorylation, we treated 14-3-3 ζ -transfected HEK293/tau cells with these peptides. We found that overexpression of 14-3-3 ζ led to a significant reduction in PP2A phosphatase activity, which was abrogated by Peptide 1 but not the control Peptide 2 treatment (Fig. 6I). Next, we monitored tau phosphorylation status with different antibodies by immunoblot analysis. Tau phosphorylation at the Ser199, Ser202/Thr205 (AT8), Thr231, Ser396 and Ser404 sites tightly correlated with PP2A phosphatase activity (Fig. 6J), and the quantitative data are summarized in Fig. 6K. These data demonstrate that the ¹⁴⁶NESGDPSSKST¹⁵⁶ peptide blocks the 14-3-3 ζ interaction with SET and favors SET cytoplasmic-to-nuclear translocation, therefore relieving repression of PP2A activity and downregulating tau phosphorylation.

Blocking the 14-3-3 ζ interaction with SET ameliorates memory deficits in 14-3-3 ζ mice

To examine whether Peptide 1 can rescue 14-3-3 ζ -induced cognitive defects, we injected AAV2-14-3-3 ζ into C57/BL6 mice. First, we evaluated the efficiency with

which peptides permeated the blood-brain barrier (BBB) using Tat-FITC treatment for 7 consecutive days via the vena caudalis. Immunofluorescence showed that Tat-peptide penetrated brain cells, indicating that these peptides can cross the BBB (Fig. S2B). One month later, we evaluated the sublocalization of SET under peptide treatment (see methods) in 14-3-3 ζ mice. We found that 14-3-3 ζ colocalized with SET in the cytoplasm in the cortex of 14-3-3 ζ mice, while SET was mostly localized to the nucleus in wild-type control mice (Fig. S3A-H). Peptide 1 treatment decreased cytoplasmic 14-3-3 ζ -SET colocalization and induced SET nuclear localization (Fig. S3I-L), but scrambled peptide 2 did not affect cytoplasmic 14-3-3 ζ colocalization with SET in 14-3-3 ζ mice (Fig. S3M-P), suggesting that peptide 1 blocked the 14-3-3 ζ interaction with SET in 14-3-3 ζ mice and led to SET nuclear translocation. Next, we performed contextual fear conditioning and MWM tests to investigate cognitive alterations and found out that Peptide 1 but not Peptide 2 completely reversed 14-3-3 ζ -induced cognitive defects in both fear conditioning (Fig. 7A) and MWM assays (Fig. 7B-E). Together, these data suggest that blocking the 14-3-3 ζ interaction with SET via Peptide 1 treatment ameliorates memory deficits in 14-3-3 ζ mice.

Electrophysiological analysis of hippocampal slices from 14-3-3 ζ mice (Fig. S4A) showed that Peptide 1 significantly increased the fEPSP slope to the normal control level. In contrast, Peptide 2 displayed no effect (Fig. S4B-C and Fig. 7F), suggesting that peptide 1 attenuates the loss of synaptic plasticity, which might be induced by 14-3-3 ζ in mice. To further investigate whether 14-3-3 ζ overexpression affects synapses, we infected primary hippocampal neurons with AAV2-EGFP + AAV2-14-3-3 ζ and treated them with Peptide 1 or 2. Thirteen days later, we conducted immunofluorescence staining and found that overexpression of 14-3-3 ζ decreased the total dendritic length, average number of dendritic branches, spine density and number of mushroom-type spines. All of these synaptic changes were abrogated by peptide 1 but not peptide 2 treatment (Fig. 7G-L). To support this observation, we also performed immunoblot analysis of a panel of synaptic proteins in the hippocampus of 14-3-3 ζ mice. We found that numerous synaptic proteins,

including synaptotagmin, synaptophysin, synapsin1 and NR2B, were reduced after 14-3-3 ζ infection, an effect that was rescued by Peptide 1 but not Peptide 2 (Fig. 7M). The quantitative data are summarized in Fig. 7N-Q. Thus, our data strongly suggest that 14-3-3 ζ retention of SET in the cytoplasm mediates cognitive defects by reducing synaptic plasticity and synaptogenesis. These deficits are largely reversed by treatment with Peptide 1.

Downregulating the 14-3-3 ζ /SET interaction with peptide 1 rescues tau pathology and cognitive impairment in AD model mice

To test whether the 14-3-3 ζ interaction with SET is indeed responsible for cognitive deficits, we treated 3 $\times$ Tg AD mice with inhibitory peptides and performed behavioral tests. Again, as in 14-3-3 ζ mice, Peptide 1 but not scrambled Peptide 2 completely reversed cognitive defects in both fear conditioning (Fig. 8A) and MWM (Fig. 8B-E) assays. Then, we performed PP2A phosphatase activity analysis. As expected, Peptide 1 markedly augmented PP2A activity in 3 $\times$ Tg AD mice compared to control mice. In contrast, scrambled peptide 2 displayed no effect (Fig. 8F). Tau phosphorylation at the Ser202/Thr205 (AT8) and Thr231 sites tightly correlated with PP2A phosphatase activity, while total tau levels remained the same among the groups (Fig. 8G). The quantitative data are summarized in Fig. 8H. Next, electrophysiology analysis of hippocampal slices from 14-3-3 ζ mice showed that Peptide 1 and not Peptide 2 treatment significantly increased the fEPSP slope relative to that of control mice (Fig. 8I-J), supporting the notion that Peptide 1 treatment might attenuate the loss of synaptic plasticity. Notably, Golgi staining indicated that Peptide 1 treatment induced substantially more dendritic spines and mushroom-type spines than the control (Fig. 8K-M). Moreover, immunoblotting also supported this observation since synapsin and NR2B levels were clearly increased after Peptide 1 but not scrambled Peptide 2 treatment (Fig. 8N-O), supporting the hypothesis that Peptide 1 treatment rescues cognitive impairments through recovery of synaptic plasticity. To further confirm that Peptide 1 blocks the 14-3-3 ζ interaction with SET in 3 $\times$ Tg AD mice, we performed co-IP and found that Peptide 1 but not scrambled Peptide 2 treatment markedly decreased 14-3-3 ζ binding to SET compared to that of the control (Fig. 8P-R). We also prepared cytoplasmic and nuclear fractions from the hippocampus of 3 $\times$ Tg AD

mice and found that Peptide 1 treatment significantly reduced the cytosolic levels of SET compared to those of the control. In contrast, scrambled Peptide 2 displayed no effect (Fig. 8S-U). Together, these data strongly suggest that decreasing the 14-3-3 ζ /SET interaction with Peptide 1 might attenuate tau pathology and cognitive impairment in AD model mice.

Discussion

Downregulation of PP2A activity is reported to be a key point for the abnormal hyperphosphorylation of tau protein in the pathogenesis of AD [1]. SET is a naturally potent inhibitor of PP2A [31, 32]. In the AD brain, the level of SET is increased significantly [8], and SET is principally located in the cytoplasm, where it colocalizes with and inhibits PP2A, subsequently inducing tau hyperphosphorylation [7]. However, as a nuclear protein, how SET is retained in the cytoplasm to inhibit PP2A in the AD brain and the downstream events in neurons are not fully understood. Previous studies suggest that the cleavage of SET into I₂N^{TF} and I₂C^{TF} may allow these smaller molecules to freely translocate from the neuronal nucleus to the cytoplasm in the affected areas of the AD brain [3]. However, full-length SET is also observed in the cytoplasm in the AD brain [5], implying that an unknown mechanism mediates SET retention in the cytoplasm. In the current study, we provide extensive evidence demonstrating that 14-3-3 ζ binds the NESGDPSSKST sequence of SET, leading to its cytoplasmic retention, where it blocks PP2A activity, resulting in tau hyperphosphorylation and synaptic dysfunction in an AD mouse model. Overexpression of 14-3-3 ζ led to SET cytoplasmic accumulation, tau hyperphosphorylation and cognitive defects, supporting the hypothesis that SET cytoplasmic retention is a consequence of 14-3-3 ζ upregulation in AD. On the other hand, we provide additional evidence that blocking the 14-3-3 ζ interaction with SET using the Tat-NESGDPSSKST peptide reverses tau pathology and cognitive defects in 14-3-3 ζ -transfected cells, 14-3-3 ζ mice and 3 $\times$ Tg AD mice. Together, our data strongly support the notion that 14-3-3 ζ binding to and cytoplasmic sequestration of SET mediates tau pathology and cognitive defects in AD.

14-3-3 β/ζ is present in tangles in AD and associates with tau protein [13, 14, 33]. Moreover, 14-3-3 ζ is overexpressed in AD brain regions affected by tau pathology, and its level correlates with disease progression [15], implying that 14-3-3 ζ is involved in AD pathogenesis. Previous studies suggested that 14-3-3 proteins play an important role in opposing the nuclear transport of some cargoes [9,11]. Here, we showed that SET binds to 14-3-3 ζ in human AD brains and in 14-3-3 ζ -transfected cells. Consequently, SET becomes retained in the cytoplasm, where it inhibits PP2A activity and subsequently leads to tau hyperphosphorylation. Similar findings were observed in 14-3-3 ζ mice and 3 $\times$ Tg AD mice. Combined with data from previous studies, these data strongly suggest that 14-3-3 ζ acts as a major player in the etiopathogenesis of AD by capturing and sequestering SET in the cytoplasm and thus increasing its cytoplasmic level, with downstream consequences.

We have provided evidence that 14-3-3 ζ binds to SET in the cytoplasm of neurons from AD patients and animal models; however, it is not clear how this binding occurs. To address how 14-3-3 ζ captures SET, we sequenced full-length SET and found that the ¹⁴⁶NESGDPSSKST¹⁵⁶ sequence of SET is consistent with the putative 14-3-3 recognition motif *RX1-2SX2-3S* [17]. We thus synthesized two peptides: Peptide 1 (P1) (Tat-NESGDPSSKST) and Peptide 2 (P2) (Tat-NSSTEDGPKSS) as a negative scrambled control. Tat, a potent trans-activator protein encoded by HIV, is a cell membrane transduction domain that allows the fusing peptide to penetrate the cell membrane [20-22]. We found that Peptide 1 blocked the SET interaction with 14-3-3 ζ and induced SET translocation to the nucleus, thus improving PP2A activity in 14-3-3 ζ -overexpressing cells and mice. Peptide 1 also substantially induced SET nuclear translocation, increased PP2A activity and subsequently led to reduced tau hyperphosphorylation, decreased synapse loss and rescue of cognitive deficits in 3 $\times$ Tg AD mice. Therefore, all of these observations support the idea that 14-3-3 ζ binding of SET at ¹⁴⁶NESGDPSSKST¹⁵⁶ induces tau-related pathological features similar to those found in human AD and animal models.

Conclusions

Our previous study showed that CKII phosphorylates SET at Ser9 and induces its cytoplasmic retention [18, 19]. Here, we show that 14-3-3 ζ , as a dimer, can bind to SET and simultaneously recruit CKII, which brings SET closer to CKII, favoring CKII-mediated SET phosphorylation and further resulting in SET cytoplasmic retention. In summary, our data strongly suggest that 14-3-3 binds SET and simultaneously recruits CKII; together, these events mediate SET phosphorylation and cytoplasmic sequestration, with SET inhibiting PP2A phosphatase activity, leading to tau pathology and cognitive defects. Given the deleterious effects of cytoplasmic SET retention on numerous pathological events in AD, we investigated the hypothesis and provided evidence that blockage of 14-3-3 binding to SET by small molecules might provide pharmacological interference for AD.

Abbreviations

AD: Alzheimer's disease; AOD: the age at the time of death; AT8: tau phosphorylation at the Ser202/Thr205; BBB: blood-brain barrier; CDR: clinical dementia rating; CERAD: the consortium to establish a registry for Alzheimer's disease; co-IP: co-immunoprecipitation; CKII: casein kinase II; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; Fc: the cytoplasmic fluorescence; FITC: Tat-fluorescein isothiocyanate; Fn: the nuclear fluorescence; HEK293/tau: HEK293 cells were stably transfected with the longest human tau (tau441); HFS: high-frequency stimulation; I_2^{PP2A} : the endogenous inhibitor 2 of protein phosphatase-2A; IF: immunofluorescence; IP: immunoprecipitation; LTP: Long-term potentiation; Mono-: monoclonal; MSBB: The Mount Sinai Brain Bank; MT: microtubule; MSBB-AD: The Mount Sinai Brain Bank AD; MWM: Morris water maze; NA: numerical aperture; NIH: National Institutes of Health; NFTs: neurofibrillary tangles; P1: Peptide 1; P2: Peptide 1; PHG: the parahippocampal gyrus; poly-: polyclonal; PP2A: protein phosphatase-2A; SDS: sodium dodecyl sulfate; TAF1 β : template-activating factor 1 β ; WB: Western blotting

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Authors' contributions

XCW designed and planned all experiments and organized all results, including the writing of the manuscript. YX and QZ planned and performed all experiments and participated in the writing of the manuscript. FH and KZ performed in vitro cell culture and biochemical analysis. DK assisted with the manuscript preparation. QW assisted with the manuscript preparation. RL analyzed and interpreted the data. JZW and XG analyzed and interpreted the data. BZ developed the concept and designed the proteomic analysis. EW, RN, KY, YL, LG, SV and MW processed and analyzed the proteomic data. BZ, VH and JP developed the proteomic cohort. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Post-mortem brain samples were from the Emory Alzheimer's Disease Research Center. Informed consent was obtained from all subjects. The study was approved by the Emory University CND Tissue Committee.

All animal experiments were approved by the Animal Care and Use Committee of Huazhong University of Science and Technology, and performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

All authors consented to publication of this paper.

Competing Interests

The authors declare that they have no competing interests.

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Figure Legends

Fig. 1. 14-3-3 ζ is associated with SET in Alzheimer's disease. (A) YWHAZ and SET levels were not correlated in the PHG in the normal control subjects. Correlation coefficient and p-value are provided. (B) YWHAZ and SET levels were significantly correlated in the PHG of AD subjects. (C) Immunofluorescence showed the colocalization of 14-3-3 ζ and SET in the cerebral cortex of AD patients. Scale bar, 50 μ m; in insets, 10 μ m. (D) The relative ratio of cells with cytosolic 14-3-3 ζ -bound SET was quantified in the cerebral cortex of AD patients, n=3. (E) Lysates from AD patients were collected for co-IP using anti-SET and anti-14-3-3 antibodies and (F) quantitative analysis of the 14-3-3 interaction with SET, n=3. (G-H) 14-3-3 ζ expression in the brains of 2-, 4- and 6-month-old 3 $\times$ Tg AD mice was measured (male, n=3 each group). (I) Hippocampal lysates from 2- and 4-month-old 3 $\times$ Tg AD mice were collected for co-IP and (J) quantitative analysis of 14-3-3 bound SET (male, n=3 each group). (K) Immunofluorescence showed subcellular localization of 14-3-3 ζ and SET in the hippocampus of 2- and 4-month-old 3 $\times$ Tg AD mice. Scale bar, 50 μ m; in insets, 10 μ m. (L) The relative ratio of cells with cytosolic 14-3-3 ζ -bound SET was quantified in the hippocampus of 2- and 4-month-old 3 $\times$ Tg AD mice (male, n=3 each group). All data represent the mean $\pm$ SEM, *P < 0.05, **P < 0.01 vs. control (Ctr) or 2-month-old mice.

Fig. 2. Overexpression of 14-3-3 augments SET accumulation in the cytoplasm.

(A) Confocal microscopy images of HEK293/tau cells transfected with pCDNA3.1+, 14-3-3 β , and 14-3-3 ζ for 48 h. Channel images: 14-3-3 (green), SET (red), nuclear dye Hoechst 33342 (blue). Bottom chart bar=50 μ m. Top chart bar=10 μ m. The hollow arrow shows SET accumulation in the cytoplasm with overexpression of 14-3-3 β / ζ . (B) Quantitative analyses of immunofluorescence images presented in A by ImageJ. Average Fn (nucleus) and Fc (cytoplasm) values are expressed as percentages relative to SET (n>50). **P<0.01 vs. cells transfected with PCDNA3.1+, ##P<0.01 vs. 14-3-3 β . (C) Cytoplasmic and nuclear fractions were prepared from HEK293/tau cells transfected with PCDNA3.1+, 14-3-3 β , 14-3-3 ζ , his-SET, 14-3-3 β +his-SET, and

14-3-3 ζ +his-SET for 48 h, and the protein levels of SET in each fraction were analyzed by Western blotting. Anti-LaminB-1 and anti-Raf-1 were used as markers of nuclear and cytoplasmic proteins, respectively. **(D-E)** Quantitative Western blot data showed that overexpression of 14-3-3 β/ζ markedly increased the cytosolic retention of SET. **(F)** Then, the cell lysates were immunoprecipitated using anti-14-3-3, anti-His, and anti-importin α antibodies. Anti-His (SET)-associated importin α and 14-3-3 β/ζ were analyzed by Western blotting. **(G)** Quantitative analyses of Western blots showed that overexpression of 14-3-3 β/ζ markedly reduced SET binding with importin α . *P<0.05, **P<0.01 vs. cells transfected with PCDNA3.1+, ###P<0.01 vs. His-SET, n=3.

Fig. 3. Overexpression of 14-3-3 β/ζ aggravates PP2A inhibition and leads to tau hyperphosphorylation. **(A)** HEK293/tau cells were transfected with PCDNA3.1+, 14-3-3 β , 14-3-3 ζ , his-SET, 14-3-3 β +his-SET, and 14-3-3 ζ +his-SET for 48 h, and then the PP2A activity in cell lysates was measured by a chemical assay. **P<0.01 vs. cells transfected with PCDNA3.1+, ###P<0.01 vs. His-SET. **(B)** Levels of tau phosphorylation in the cells were measured by Western blotting using a panel of phosphorylation site-specific antibodies, as labeled, normalized against total tau probed by tau-5, and β -actin was used as a loading control. **(C)** Quantitative data showed that tau phosphorylation levels at Ser199, Ser202/Thr205 (AT-8), Thr231, Ser262, Ser396 and Ser404 were remarkably increased at 48 h after transfection of 14-3-3 β , 14-3-3 ζ , his-SET, 14-3-3 β +his-SET, and 14-3-3 ζ +his-SET. *P<0.05, **P<0.01 vs. cells transfected with PCDNA3.1+, #P<0.05, ###P<0.01 vs. His-SET, n=3.

Fig. 4. CKII recruited by 14-3-3 ζ promotes SET Ser9 phosphorylation. **(A)** The bilateral hippocampal DG zone of 14 male mice was injected with pAOV.CMV.bGlobin.3 $\times$ FLAG AAV2 (Ctr, titer=4.91 $\times$ 10¹² v.g./ml, total volume=2 μ l, 7 male mice) or pAOV.CMV.bGlobin.14-3-3 ζ AAV2 (14-3-3 ζ , titer=6.83 $\times$ 10¹² v.g./ml, total volume=2 μ l, 7 male mice). Four weeks later, confocal microscopy was

employed to study the protein localization in the mouse brain. Channel images: 14-3-3 ζ (green), CKII (gray), SET (red), and nuclear dye Hoechst 33342 (blue). Immunofluorescence showed that 14-3-3 ζ and CKII colocalized with SET in the cytoplasm in the hippocampus and cortex of 14-3-3 ζ -overexpressing mice, while SET was mostly localized in the nucleus in the hippocampus and cortex of control mice. **(B-D)** Then, mouse brain lysates were immunoprecipitated using anti-14-3-3 ζ , anti-CKII, and anti-SET antibodies. The 14-3-3 ζ , CKII and SET levels were analyzed by Western blotting. **(E)** HEK293/tau cells were transfected with PCDNA3.1+ or 14-3-3 ζ . Twenty-four hours later, the cells were treated with DMSO (0.25%) or TBB (50 μ M). Then, anti-SET pSer-9, SET, 14-3-3 ζ , CKII α , and β -actin were used for WB. **(F)** Quantitative data showed that inhibition of CKII by TBB rescued SET hyperphosphorylation *P<0.05, **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3 ζ , n=3. **(G)** HEK293/tau cells treated as in **E**. PP2A activity in cell lysates was measured by a chemical assay. *P<0.05, **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3 ζ , n=3. **(H-I)** HEK293/tau cells transfected with PCDNA3.1+ and CKII α and then treated with – (Ctr), Ssi (CKII+Ssi), or si14-3-3 ζ (CKII+ si14-3-3 ζ). Anti-SET pSer-9, SET, 14-3-3 ζ , CKII α , and β -actin were used for Western blotting. Lower panel, quantitative analyses of Western blots. **P<0.01 vs. Ctr, ##P<0.01 vs. CKII+Ssi, n=3. **(J)** HEK293/tau cells treated as in **H**. PP2A activity in cell lysates was measured by a chemical assay. **P<0.01 vs. Ctr, ##P<0.01 vs. CKII, n=3.

Fig. 5. Overexpression of 14-3-3 ζ induces cognitive deficits. **(A)** The bilateral hippocampal DG zone of 14 male C57/BL6 mice was injected with pAOV.CMV.bGlobin.3 $\times$ FLAG AAV2 (Ctr, titer=4.91 $\times$ 10¹² v.g./ml, total volume=2 μ l) or pAOV.CMV.bGlobin.14-3-3 ζ AAV2 (14-3-3 ζ , titer=6.83 $\times$ 10¹² v.g./ml, total volume=2 μ l). Immunofluorescence assays showed the expression of 14-3-3 ζ 4 weeks after infection. Channel images: nuclear dye Hoechst 33342 (blue), 14-3-3 ζ (green), Bar=200 μ m. **(B)** Four weeks after infection, the contextual fear conditioning test showed the percent of freezing for native, 1 h, and 24 h. **(C)** In the Morris water maze

(MWM) test, the escape latency to find the hidden platform from day 1 to day 6. **(D)** The number of mean annulus crossings in the target quadrant on day 7 and day 9 after removing the platform. **(E)** The swimming time in the target quadrant. **(F)** The mean swimming speeds. Male, n=7 each group, *P<0.05, **P<0.01 vs. Ctr.

Fig. 6. Tat-NESGDPSSKST promotes the return of SET to the nucleus from the cytoplasm and rescues PP2A activity in 14-3-3 ζ -transfected HEK293/tau cells. **(A)** Confocal microscopy images of HEK293/tau cells transfected with 14-3-3 ζ for 48 h with simultaneous treatment with PBS, Peptide 1 (Tat-NESGDPSSKST, P1, 50 μ M), and Peptide 2 (Tat-NSSTEDGPKSS, as a negative scrambled control, P2, 50 μ M). Channel images: 14-3-3 (green), SET (red), nuclear dye Hoechst 33342 (blue). Bottom chart bar=50 μ m. Top chart bar=10 μ m. The hollow arrow shows SET accumulation in the cytoplasm with 14-3-3 ζ , and the solid arrow shows SET accumulation in the nucleus. **(B)** Quantitative analyses of immunofluorescence images presented in A by ImageJ. Average Fn (nucleus) and Fc (cytoplasm) values are expressed as percentages relative to SET (n>50). **P<0.01 vs. PBS control. **(C)** Cytoplasmic and nuclear fractions were prepared in transfected HEK293/tau cells, and the protein levels of SET in each fraction were analyzed by Western blotting. Anti-LaminB-1 and anti-Raf-1 were used as markers of nuclear and cytoplasmic proteins, respectively. **(D-E)** Quantitative Western blot data showed that Peptide 1 markedly decreased the cytosolic retention of SET. The data were from 3 separate experiments. **P<0.01 vs. cells transfected with pCDNA3.1+. **(F)** HEK293/tau cells were transfected with pCDNA3.1+, his-SET, or 14-3-3 ζ +his-SET for 48 h and simultaneously treated with PBS, Peptide 1 (low dose=1 μ M, L1; medium dose= 10 μ M, M1; high dose=100 μ M, H1), or Peptide 2 (low dose=1 μ M, L2; medium dose= 10 μ M, M2; high dose=100 μ M, H2). Then, the cell lysates were immunoprecipitated using anti-His, anti-14-3-3, and anti-importin α antibodies. Anti-His (SET)-associated importin α and 14-3-3 ζ were analyzed by Western blotting. **(G)** Quantitative analyses of Western blots showed that Peptide 1 markedly reduced SET binding with 14-3-3 ζ , **P<0.01 vs. cells transfected with 14-3-3 ζ +his-SET. **(H)** Quantitative analyses of

Western blots showed that Peptide 1 markedly rescued SET binding with importin α , **P<0.01 vs. cells transfected with 14-3-3 ζ +his-SET. **(I)** HEK293/tau cells were transfected with 14-3-3 ζ for 48 h and simultaneously treated with PBS, Peptide 1, or Peptide 2. The PP2A activity was measured by a chemical assay. **P<0.01 vs. cells transfected with pCDNA3.1+, ##P<0.01 vs. 14-3-3 ζ . **(J)** Then, the cell lysates were collected for Western blotting. Levels of tau phosphorylation in the cells were measured using a panel of phosphorylation site-specific antibodies, as labeled, normalized against total tau probed by tau-5, and β -actin was used as a loading control. **(K)** Quantitative analyses of Western blots, *P<0.05, **P<0.01, ***P<0.001 vs. cells transfected with pCDNA3.1+, n=3.

Fig. 7. Peptide 1 ameliorates memory deficits induced by overexpression of 14-3-3 ζ . **(A)** The bilateral hippocampal DG zone of 28 male mice was injected with pAOV.CMV.bGlobin.3 $\times$ FLAG AAV2 (Ctr, titer=4.91 $\times$ 10¹² v.g./ml, total volume=2 μ l, 7 male mice) or pAOV.CMV.bGlobin.14-3-3 ζ AAV2 (14-3-3 ζ , titer=6.83 $\times$ 10¹² v.g./ml, total volume=2 μ l, 21 male mice). Four weeks later, virus-transfected mice were treated with Peptide 1 and Peptide 2 via the vena caudalis at 10 mg/kg/day for 7 days. Contextual fear conditioning test showed the percent of freezing for native, 1 h, and 24 h. Male, n=7 each group, **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3 ζ . In the MWM test, the escape latency **(B)** to find the hidden platform from day 1 to day 6; the number of mean annulus crossings **(C)**, the swimming time **(D)** and the mean swimming speed **(E)** in the target quadrant on day 7 and day 9 were determined by removing the platform. Male, n=7 each group, *P<0.05, **P<0.01 vs. Ctr, #P<0.05, ##P<0.01 vs. 14-3-3 ζ . **(F)** Electrophysiological analysis of hippocampal slices from 14-3-3 ζ mice showed the fEPSP slope. Male, n=3 each group, **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3 ζ . **(G-H)** Primary neurons were treated with pAOV.CMV.bGlobin. EGFP AAV2 (titer=6.34 $\times$ 10¹² v.g./ml, total volume=1 μ l) on day 3, pAOV.CMV.bGlobin.14-3-3 ζ AAV2 (14-3-3 ζ , titer=6.83 $\times$ 10¹² v.g./ml, total volume=1 μ l) on day 7. Peptide 1 or Peptide 2 was supplied for one week (50 μ M/day). Neurons with EGFP were visualized by confocal scanning microscopy on

day 14. Bar in G=100 μ m, bar in H=200 μ m. **(I-L)** Quantitative analysis showed the total dendritic length, the number of branches, the spine density and the percentage of mushroom-type spines. **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3 ζ , n=7-8 each group. **(M-Q)** Lysates from treated primary neurons were collected for Western blotting and quantitative analyses of the blots. **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3 ζ , n=3 each group.

Fig. 8. Peptide 1 rescues tau pathology and cognitive impairment in AD model mice. **(A)** Three-month-old 3 $\times$ Tg mice were injected with Peptide 1 and Peptide 2 via the vena caudalis at 10 mg/kg/day for 7 days, and saline served as a control (Ctr), male, n=9 in each group. **(B-E)** One month later, fear conditioning and Morris water maze tests were performed to assess mouse behavior. Male, n=9 each group, *p<0.05, **P < 0.01 vs. Ctr. **(F)** Hippocampal homogenates were collected for the PP2A activity assay. Male, n=3 each group, **P < 0.01 vs. Ctr. **(G)** Western blots using AT8, pT231 and tau5 showed the levels of tau phosphorylation and total tau in the hippocampus, and **(H)** quantitative analysis was performed. Male, n=3 each group, **P < 0.01 vs. Ctr. **(I)** The slope of fEPSPs in DG-CA1 was normalized to the baseline after HFS. **(J)** Quantitative analyses of fEPSPs relative to baseline after high-frequency stimulation (HFS; 100 Hz, 1-s duration). Male, n=3 each group, *p<0.05 vs. Ctr. **(K)** Representative Golgi staining of hippocampal neurons, scale bar=2 μ m. **(L-M)** Quantification of the spine density and the mushroom-type spine densities from randomly selected dendritic segments of randomly selected hippocampal neurons. Male, n=3 each group, **P < 0.01 vs. Ctr. **(N)** The levels of synapsin1, NR2B and β -actin in the hippocampus were detected, and **(O)** quantitative analysis was performed. Male, n=3 each group. *p<0.05 vs. Ctr. **(S)** The lysates were immunoprecipitated using anti-14-3-3 and anti-SET antibodies. **(Q-R)** Quantitative analyses of blots in **P**. Males, n=3 per group. **P < 0.01, ***P < 0.001 vs. Ctr. **(S-U)** Cytoplasmic and nuclear fractions were prepared from mouse samples, and the protein levels of SET in each fraction were analyzed by Western blotting.

Anti-LaminB-1 and anti-Raf-1 were used as markers of nuclear and cytoplasmic proteins, respectively. Male, n=3 each group. *p<0.05, **P < 0.01 vs. Ctr.

Supplementary Figure Legends

Fig. S1 Lysates from 3 AD patients (A) and 2- and 4-month-old 3 × Tg AD mice (C) were collected for co-IP using anti-SET and anti-14-3-3 antibodies, respectively. Quantitative analysis of the blots in A and C (B, D). (E) Immunofluorescence showed the subcellular localization of 14-3-3 ζ and SET in 2- and 4-month-old 3 × Tg AD mice (male, n=3 each group). Scale bar, 50 μ m; in insets, 10 μ m. (F) Quantitative analysis of the immunofluorescence images presented in E by ImageJ. All data represent the mean $\pm$ SEM, *P < 0.05, **P < 0.01 vs. control (Ctr) or 2-month-old mice.

Fig. S2 (A) Immunofluorescence analysis of Tat-FITC-treated HEK293/tau cells. PBS or 50 μ M Tat-FITC was added to the medium of HEK/293 cells. Two hours later, the cells were incubated with Hoechst for 20 minutes and then subjected to confocal scanning microscopy. Scale bar, 50 μ m. (B) Immunofluorescence staining analysis of Tat-FITC-injected mice. Male C57/BL mice (4 months old, 20 $\pm$ 2 g, n=3) were injected with Tat-FITC via the vena caudalis at 10 mg/kg/day for 7 days. Immunofluorescence showed that Tat-peptide arrived at the cortex, CA1, CA3 and dentate gyrus (DG) of the hippocampus and penetrated the cells, suggesting that Tat permeates the cell membrane. Scale bar, 100 μ m.

Fig. S3 Peptide 1 blocked the 14-3-3 ζ interaction with SET in 14-3-3 ζ mice and led to SET translocation back to the nucleus from the cytoplasm. The bilateral hippocampal DG zone of 28 male mice was injected with pAOV.CMV.bGlobin.3 $\times$ FLAG AAV2 (Ctr, titer=4.91 $\times$ 10¹² v.g./ml, total volume=2 μ l, 7 male mice) or pAOV.CMV.bGlobin.14-3-3 ζ AAV2 (14-3-3 ζ , titer=6.83 $\times$ 10¹² v.g./ml, total volume=2 μ l, 21 male mice). Four weeks later, virus-transfected mice were treated with Peptide

1 and Peptide 2 via the vena caudalis at 10 mg/kg/day for 7 days. Confocal microscopy images of mice. Channel images: 14-3-3 (green), SET (red), nuclear dye Hoechst 33342 (blue). Immunofluorescence showed that 14-3-3 ζ colocalized with SET in the cytoplasm in the cortex of 14-3-3 ζ mice, while SET was mostly localized to the nucleus in wild-type mice (**A-H**). Peptide 1 treatment induced SET nuclear residency (**I-L**), while scrambled Peptide 2 did not affect 14-3-3 ζ colocalization with SET in the cytoplasm in 14-3-3 ζ mice (**M-P**). Male, n=3 each group. Scale bar=50 μ m.

Fig. S4 (A) Brain slice with a MED64 array chamber in warm artificial cerebral spinal fluid (ACSF). LTP was induced by applying three trains of high-frequency stimulation (HFS; 100 Hz, 1-s duration). (B) Representative analog traces of evoked potential before (blue line) and after (red line) HFS. (C) The slope of fEPSPs in DG-CA1 was normalized to the baseline after HFS. Male, n=3 each group.

Table 1. Antibodies employed in this study

Antibody	Specific	Type	Dilution	Source
his	Histidine tagged, clone HIS.H8	Mono-	1:1000 for WB	Millipore, Temecula, CA, USA
I ₂ PP2A(SET)	Amino acids 1-120 of I ₂ PP2A of human origin	Mono-	1:1000 for WB 1:500 for IF	Santa Cruz, Dallas, TX, USA
SET pS9	Phosphorylated SET at Ser9	Poly-	1:200 for WB	Abmart, Shanghai, China
Importina	N-terminus of Importina of <i>Saccharomyces cerevisiae</i> origin	Poly-	1:1000 for WB	Santa Cruz, Dallas, TX, USA
LaminB-1	Residues 400-500 of Mouse Lamin B1.	Poly-	1:1000 for WB	Abcam, Cambridge, MA, USA
Raf-1	C-terminus (amino acids 637-648 (CTLTSPRLPVF)) of human Raf-1	Mono-	1:1000 for WB	Millipore, Temecula, CA, USA
14-3-3	Total isoforms of 14-3-3	Poly-	1:1000 for WB 1:500 for IF	Abcam, Cambridge, MA, USA
14-3-3 β	C-terminus of 14-3-3 β of human origin	Poly-	1:1000 for WB	Santa Cruz, Dallas, TX, USA
14-3-3 ζ	14-3-3 ζ of human origin	Poly-	1:1000 for WB 1:500 for IF	Santa Cruz, Dallas, TX, USA
AT-8	Phosphorylated PHF tau at Ser202+Ser205	Mono-	1:1000 for WB	Thermo Rockford, IL, USA
pS199	Phosphorylated tau at Ser199	Poly-	1:1000 for WB	Invitrogen California, CA, USA
pT231	Phosphorylated tau at Thr231	Poly-	1:1000 for WB	Signalway Antibody College Park, MD, USA
pS262	Phosphorylated tau at Ser262	Poly-	1:1000 for WB	Signalway Antibody College Park, MD, USA
pS396	Phosphorylated tau at Ser396	Poly-	1:1000 for WB	Signalway Antibody College Park, MD, USA
pS404	Phosphorylated tau at Ser404	Poly-	1:1000 for WB	Signalway Antibody College Park, MD, USA
Tau-1	Nonphosphorylated tau at Ser198/199/202	Mono-	1:1000 for WB	Millipore, Temecula, CA, USA
Tau-5	Total tau	Mono-	1:1000 for WB	Millipore, Temecula, CA, USA
synaptotagmin	Total synaptotagmin	Mono-	1:2000 for WB	Abcam, Cambridge, MA, USA
Synaptophysin	SYN 245-258	Poly-	1:1000 for WB	Abcam, Cambridge, MA, USA
p-Synapsin-1	Phosphorylated SYN at Ser62,67	Mono-	1:1000 for WB	Abcam, Cambridge, MA, USA
NR2B	NMDAR2B C-term	Poly-	1:1000 for WB	Abcam, Cambridge, MA, USA
β -actin	Total actin	Mono-	1:1000 for WB	Abcam, Cambridge, MA, USA

Mono-, monoclonal; poly-, polyclonal; WB, Western blotting; p, phosphorylated; IF, immunofluorescence

Table 2. Spearman correlations between YWHAZ and SET proteins in the AD and control groups.

Cohort	Dataset	Protein1.ID	GN1	Protein2.ID	GN2	rho	p.value	p.bonferroni	p.BH	AD or NL
MSBB	proteomics	P63104	YWHAZ	Q01105	SET	-0.1440	0.4632	0.9264	0.62853	control
MSBB	proteomics	E7EX29	YWHAZ	Q01105	SET	-0.0952	0.6285	1.0000	0.62853	control
MSBB	proteomics	P63104	YWHAZ	Q01105-2	SET	-0.2512	0.1965	0.3929	0.284291	control
MSBB	proteomics	E7EX29	YWHAZ	Q01105-2	SET	-0.2091	0.2843	0.5686	0.284291	control
MSBB	proteomics	P63104	YWHAZ	Q01105	SET	-0.3983	0.0000	0.0000	3.92E-06	AD
MSBB	proteomics	E7EX29	YWHAZ	Q01105	SET	-0.4081	0.0000	0.0000	3.92E-06	AD
MSBB	proteomics	P63104	YWHAZ	Q01105-2	SET	-0.3974	0.0000	0.0000	4.13E-06	AD
MSBB	proteomics	E7EX29	YWHAZ	Q01105-2	SET	-0.4186	0.0000	0.0000	2.24E-06	AD

Figures

Figure1

Figure 1

14-3-3ζ is associated with SET in Alzheimer's disease. (A) YWHAZ and SET levels were not correlated in the PHG in the normal control subjects. Correlation coefficient and p-value are provided. (B) YWHAZ and SET levels were significantly correlated in the PHG of AD subjects. (C) Immunofluorescence showed the

colocalization of 14-3-3 ζ and SET in the cerebral cortex of AD patients. Scale bar, 50 μ m; in insets, 10 μ m. (D) The relative ratio of cells with cytosolic 14-3-3 ζ -bound SET was quantified in the cerebral cortex of AD patients, n=3. (E) Lysates from AD patients were collected for co-IP using anti-SET and anti-14-3-3 antibodies and (F) quantitative analysis of the 14-3-3 interaction with SET, n=3. (G-H) 14-3-3 ζ expression in the brains of 2-, 4- and 6-month-old 3 $\times$ Tg AD mice was measured (male, n=3 each group). (I) Hippocampal lysates from 2- and 4-month-old 3 $\times$ Tg AD mice were collected for co-IP and (J) quantitative analysis of 14-3-3 bound SET (male, n=3 each group). (K) Immunofluorescence showed subcellular localization of 14-3-3 ζ and SET in the hippocampus of 2- and 4-month-old 3 $\times$ Tg AD mice. Scale bar, 50 μ m; in insets, 10 μ m. (L) The relative ratio of cells with cytosolic 14-3-3 ζ -bound SET was quantified in the hippocampus of 2- and 4-month-old 3 $\times$ Tg AD mice (male, n=3 each group). All data represent the mean $\pm$ SEM, *P < 0.05, **P < 0.01 vs. control (Ctr) or 2-month-old mice.

Figure 2

Figure 2

sfectected with PCDNA3.1+, 14-3-3β, 14-3-3ζ, his-SET, 14-3-3β+his-SET, and 29 14-3-3ζ+his-SET for 48 h, and the protein levels of SET in each fraction were analyzed by Western blotting. Anti-LaminB-1 and anti-Raf-1 were used as markers of nuclear and cytoplasmic proteins, respectively. (D-E) Quantitative Western blot data showed that overexpression of 14-3-3β/ζ markedly increased the cytosolic retention of SET. (F) Then, the cell lysates were immunoprecipitated using anti-14-3-3, anti-His, and anti-importina antibodies.

Anti-His (SET)-associated importina and 14-3-3 β/ζ were analyzed by Western blotting. (G) Quantitative analyses of Western blots showed that overexpression of 14-3-3 β/ζ markedly reduced SET binding with importina. *P<0.05, **P<0.01 vs. cells transfected with PCDNA3.1+, ##P<0.01 vs. His-SET, n=3.

Figure 3

Figure 3

Overexpression of 14-3-3 β/ζ aggravates PP2A inhibition and leads to tau hyperphosphorylation. (A) HEK293/tau cells were transfected with PCDNA3.1+, 14-3-3 β , 14-3-3 ζ , his-SET, 14-3-3 β +his-SET, and 14-3-3 ζ +his-SET for 48 h, and then the PP2A activity in cell lysates was measured by a chemical assay. **P<0.01 vs. cells transfected with PCDNA3.1+, ##P<0.01 vs. His-SET. (B) Levels of tau phosphorylation in the cells were measured by Western blotting using a panel of phosphorylation site-specific antibodies, as labeled, normalized against total tau probed by tau-5, and β -actin was used as a loading control. (C)

Quantitative data showed that tau phosphorylation levels at Ser199, Ser202/Thr205 (AT-8), Thr231, Ser262, Ser396 and Ser404 were remarkably increased at 48 h after transfection of 14-3-3 β , 14-3-3 ζ , his-SET, 14-3-3 β +his-SET, and 14-3-3 ζ +his-SET. *P<0.05, **P<0.01 vs. cells transfected with PCDNA3.1+, #P<0.05, ##P<0.01 vs. His-SET, n=3.

Figure 4

Figure 4

CKII recruited by 14-3-3 ζ promotes SET Ser9 phosphorylation. (A) The bilateral hippocampal DG zone of 14 male mice was injected with pAOV.CMV.bGlobin.3 $\times$ FLAG AAV2 (Ctr, titer=4.91 $\times$ 10¹² v.g./ml, total volume=2 μ l, 7 male mice) or pAOV.CMV.bGlobin.14-3-3 ζ AAV2 (14-3-3 ζ , titer=6.83 $\times$ 10¹² v.g./ml, total volume=2 μ l, 7 male mice). Four weeks later, confocal microscopy was employed to study the protein localization in the mouse brain. Channel images: 14-3-3 ζ (green), CKII (gray), SET (red), and nuclear dye Hoechst 33342 (blue). Immunofluorescence showed that 14-3-3 ζ and CKII colocalized with SET in the cytoplasm in the hippocampus and cortex of 14-3-3 ζ -overexpressing mice, while SET was mostly localized in the nucleus in the hippocampus and cortex of control mice. (B-D) Then, mouse brain lysates were immunoprecipitated using anti-14-3-3 ζ , anti-CKII, and anti-SET antibodies. The 14-3-3 ζ , CKII and SET levels were analyzed by Western blotting. (E) HEK293/tau cells were transfected with PCDNA3.1+ or 14-3-3 ζ . Twenty-four hours later, the cells were treated with DMSO (0.25%) or TBB (50 μ M). Then, anti-SET pSer-9, SET, 14-3-3 ζ , CKII α , and β -actin were used for WB. (F) Quantitative data showed that inhibition of CKII by TBB rescued SET hyperphosphorylation *P<0.05, **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3 ζ , n=3. (G) HEK293/tau cells treated as in E. PP2A activity in cell lysates was measured by a chemical assay. *P<0.05, **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3 ζ , n=3. (H-I) HEK293/tau cells transfected with PCDNA3.1+ and CKII α and then treated with – (Ctr), Ssi (CKII+Ssi), or si14-3-3 ζ (CKII+ si14-3-3 ζ). Anti-SET pSer-9, SET, 14-3-3 ζ , CKII α , and β -actin were used for Western blotting. Lower panel, quantitative analyses of Western blots. **P<0.01 vs. Ctr, ##P<0.01 vs. CKII+Ssi, n=3. (J) HEK293/tau cells treated as in H. PP2A activity in cell lysates was measured by a chemical assay. **P<0.01 vs. Ctr, ##P<0.01 vs. CKII, n=3.

Figure 5

Figure 5

Overexpression of 14-3-3ζ induces cognitive deficits. (A) The bilateral hippocampal DG zone of 14 male C57/BL6 mice was injected with pAOV.CMV.bGlobin.3×FLAG AAV2 (Ctr, titer=4.91×10¹² v.g./ml, total volume=2 μl) or pAOV.CMV.bGlobin.14-3-3ζ AAV2 (14-3-3ζ, titer=6.83×10¹² v.g./ml, total volume=2 μl). Immunofluorescence assays showed the expression of 14-3-3ζ 4 weeks after infection. Channel images: nuclear dye Hoechst 33342 (blue), 14-3-3ζ (green), Bar=200 μm. (B) Four weeks after infection, the

contextual fear conditioning test showed the percent of freezing for native, 1 h, and 24 h. (C) In the Morris water maze (MWM) test, the escape latency to find the hidden platform from day 1 to day 6. (D) The number of mean annulus crossings in the target quadrant on day 7 and day 9 after removing the platform. (E) The swimming time in the target quadrant. (F) The mean swimming speeds. Male, n=7 each group, *P<0.05, **P<0.01 vs. Ctr.

Figure 6

Tat-NESGDPSSKST promotes the return of SET to the nucleus from the cytoplasm and rescues PP2A activity in 14-3-3 ζ -transfected HEK293/tau cells. (A) Confocal microscopy images of HEK293/tau cells transfected with 14-3-3 ζ for 48 h with simultaneous treatment with PBS, Peptide 1 (Tat-NESGDPSSKST, P1, 50 μ M), and Peptide 2 (Tat-NSSTEDGPKSS, as a negative scrambled control, P2, 50 μ M). Channel images: 14-3-3 (green), SET (red), nuclear dye Hoechst 33342 (blue). Bottom chart bar=50 μ m. Top chart bar=10 μ m. The hollow arrow shows SET accumulation in the cytoplasm with 14-3-3 ζ , and the solid arrow shows SET accumulation in the nucleus. (B) Quantitative analyses of immunofluorescence images presented in A by ImageJ. Average Fn (nucleus) and Fc (cytoplasm) values are expressed as percentages relative to SET (n>50). **P<0.01 vs. PBS control. (C) Cytoplasmic and nuclear fractions were prepared in transfected HEK293/tau cells, and the protein levels of SET in each fraction were analyzed by Western blotting. Anti-LaminB-1 and anti-Raf-1 were used as markers of nuclear and cytoplasmic proteins, respectively. (D-E) Quantitative Western blot data showed that Peptide 1 markedly decreased the cytosolic retention of SET. The data were from 3 separate experiments. **P<0.01 vs. cells transfected with pCDNA3.1+. (F) HEK293/tau cells were transfected with pCDNA3.1+, his-SET, or 14-3-3 ζ +his-SET for 48 h and simultaneously treated with PBS, Peptide 1 (low dose=1 μ M, L1; medium dose= 10 μ M, M1; high dose=100 μ M, H1), or Peptide 2 (low dose=1 μ M, L2; medium dose= 10 μ M, M2; high dose=100 μ M, H2). Then, the cell lysates were immunoprecipitated using anti-His, anti-14-3-3, and anti-importin α antibodies. Anti-His (SET)-associated importin α and 14-3-3 ζ were analyzed by Western blotting. (G) Quantitative analyses of Western blots showed that Peptide 1 markedly reduced SET binding with 14-3-3 ζ , **P<0.01 vs. cells transfected with 14-3-3 ζ +his-SET. (H) Quantitative analyses of Western blots showed that Peptide 1 markedly rescued SET binding with importin α , **P<0.01 vs. cells transfected with 14-3-3 ζ +his-SET. (I) HEK293/tau cells were transfected with 14-3-3 ζ for 48 h and simultaneously treated with PBS, Peptide 1, or Peptide 2. The PP2A activity was measured by a chemical assay. **P<0.01 vs. cells transfected with pCDNA3.1+, ##P<0.01 vs. 14-3-3 ζ . (J) Then, the cell lysates were collected for Western blotting. Levels of tau phosphorylation in the cells were measured using a panel of phosphorylation site-specific antibodies, as labeled, normalized against total tau probed by tau-5, and β -actin was used as a loading control. (K) Quantitative analyses of Western blots, *P<0.05, **P<0.01, ***P<0.001 vs. cells transfected with pCDNA3.1+, n=3.

Figure 7

Figure 7

Peptide 1 ameliorates memory deficits induced by overexpression of 14-3-3ζ. (A) The bilateral hippocampal DG zone of 28 male mice was injected with pAOV.CMV.bGlobin.3×FLAG AAV2 (Ctr, titer=4.91×10¹² v.g./ml, total volume=2 μl, 7 male mice) or pAOV.CMV.bGlobin.14-3-3ζ AAV2 (14-3-3ζ, titer=6.83×10¹² v.g./ml, total volume=2 μl, 21 male mice). Four weeks later, virus-transfected mice were treated with Peptide 1 and Peptide 2 via the vena caudalis at 10 mg/kg/day for 7 days. Contextual fear

conditioning test showed the percent of freezing for native, 1 h, and 24 h. Male, n=7 each group, **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3ζ. In the MWM test, the escape latency (B) to find the hidden platform from day 1 to day 6; the number of mean annulus crossings (C), the swimming time (D) and the mean swimming speed (E) in the target quadrant on day 7 and day 9 were determined by removing the platform. Male, n=7 each group, *P<0.05, **P<0.01 vs. Ctr, #P<0.05, ##P<0.01 vs. 14-3-3ζ. (F) Electrophysiological analysis of hippocampal slices from 14-3-3ζ mice showed the fEPSP slope. Male, n=3 each group, **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3ζ. (G-H) Primary neurons were treated with pAOV.CMV.bGlobin. EGFP AAV2 (titer=6.34×10¹² v.g./ml, total volume=1 μl) on day 3, pAOV.CMV.bGlobin.14-3-3ζ. AAV2 (14-3-3ζ, titer=6.83×10¹² v.g./ml, total volume=1 μl) on day 7. Peptide 1 or Peptide 2 was supplied for one week (50 μM/day). Neurons with EGFP were visualized by confocal scanning microscopy on day 14. Bar in G=100 μm, bar in H=200 μm. (I-L) Quantitative analysis showed the total dendritic length, the number of branches, the spine density and the percentage of mushroom-type spines. **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3ζ, n=7-8 each group. (M-Q) Lysates from treated primary neurons were collected for Western blotting and quantitative analyses of the blots. **P<0.01 vs. Ctr, ##P<0.01 vs. 14-3-3ζ, n=3 each group.

Figure 8

Figure 8

Peptide 1 rescues tau pathology and cognitive impairment in AD model mice. (A) Three-month-old 3xTg mice were injected with Peptide 1 and Peptide 2 via the vena caudalis at 10 mg/kg/day for 7 days, and saline served as a control (Ctr), male, n=9 in each group. (B-E) One month later, fear conditioning and Morris water maze tests were performed to assess mouse behavior. Male, n=9 each group, *p<0.05, **P < 0.01 vs. Ctr. (F) Hippocampal homogenates were collected for the PP2A activity assay. Male, n=3 each

group, $**P < 0.01$ vs. Ctr. (G) Western blots using AT8, pT231 and tau5 showed the levels of tau phosphorylation and total tau in the hippocampus, and (H) quantitative analysis was performed. Male, $n=3$ each group, $**P < 0.01$ vs. Ctr. (I) The slope of fEPSPs in DG-CA1 was normalized to the baseline after HFS. (J) Quantitative analyses of fEPSPs relative to baseline after high-frequency stimulation (HFS; 100 Hz, 1-s duration). Male, $n=3$ each group, $*p < 0.05$ vs. Ctr. (K) Representative Golgi staining of hippocampal neurons, scale bar=2 μm . (L-M) Quantification of the spine density and the mushroom-type spine densities from randomly selected dendritic segments of randomly selected hippocampal neurons. Male, $n=3$ each group, $**P < 0.01$ vs. Ctr. (N) The levels of synapsin1, NR2B and β -actin in the hippocampus were detected, and (O) quantitative analysis was performed. Male, $n=3$ each group. $*p < 0.05$ vs. Ctr. (S) The lysates were immunoprecipitated using anti-14-3-3 and anti-SET antibodies. (Q-R) Quantitative analyses of blots in P. Males, $n=3$ per group. $**P < 0.01$, $***P < 0.001$ vs. Ctr. (S-U) Cytoplasmic and nuclear fractions were prepared from mouse samples, and the protein levels of SET in each fraction were analyzed by Western blotting. Anti-LaminB-1 and anti-Raf-1 were used as markers of nuclear and cytoplasmic proteins, respectively. Male, $n=3$ each group. $*p < 0.05$, $**P < 0.01$ vs. Ctr.

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