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Cdk5 phosphorylation of EFhd2 at S74 affects its calcium binding activity

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Abstract: EFhd2 is a calcium binding protein, which is highly expressed in the central nervous system and associated with pathological forms of tau proteins in tauopathies. Previous phosphoproteomics studies and bioinformatics analysis suggest that EFhd2 may be phosphorylated. Here, we determine whether Cdk5, a hyperactivated kinase in tauopathies, phosphorylates EFhd2 and influence its known molecular activities. The results indicated that EFhd2 is phosphorylated by brain extract of the transgenic mouse CK-p25, which overexpresses the Cdk5 constitutive activator p25. Consistently, *in vitro* kinase assays demonstrated that Cdk5, but not GSK3β, directly phosphorylates EFhd2. Biomass, tandem mass spectrometry, and mutagenesis analyses indicated that Cdk5 monophosphorylates EFhd2 at S74, but not the adjacent S76. Furthermore, Cdk5-mediated phosphorylation of EFhd2 affected its calcium binding activity. Finally, a phospho-specific antibody was generated against EFhd2 phosphorylated at S74 and was used to detect this phosphorylation event in postmortem brain tissue from Alzheimer's disease and normal-aging control cases. Results demonstrated that EFhd2 is phosphorylated *in vivo* at S74. These results imply that EFhd2's physiological and/or pathological function could be regulated by its phosphorylation state.

Keywords: phosphorylation; calcium binding; Cdk5; EFhd2; tau; tauopathy

Introduction

EFhd2 is a highly conserved protein (from human to nematodes) expressed in different tissues, but it is more abundant in the central nervous system.[1–4] The physiological function of EFhd2 is poorly understood; previous studies have shown that EFhd2 modulates the apoptotic signaling pathway induced by the activation of the B-cell receptor (BCR) in B cells and WEHI231.[1,2,5] Recent work demonstrated that overexpression of EFhd2 enhances the amount of free calcium detected in WEHI231 cells after stimulation with IgM antibodies, which suggest that EFhd2 functions as a calcium sensor.[5,6] It has also been indicated that EFhd2 is a scaffolding protein that brings together spleen tyrosine kinase, SLP-65 and PLCγ2 to mediate BCR-induced calcium flux.[5] In contrast, EFhd2 is found in cross proximity to F-actin in epithelial cells, where may mediate cell spreading and migration.[7,8] However, the role that EFhd2's calcium binding activity plays in the central

Abbreviations: AD, Alzheimer's disease; CB, coomassie blue; ΔCC, coiled-coil truncated mutant; Cdk5, cyclin-dependent kinase 5; GSK3β, glycogen synthase kinase 3 beta; FL, full length; HIS, histidine-tag; ΔNT, N-terminal truncated mutant.

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nervous system, where is predominantly expressed, has not been determined.

Identification of potential posttranslational modifications and, subsequent, characterization could provide insights into EFhd2's physiological function. Previous studies identified EFhd2 as two protein spots in two-dimensional gel electrophoresis (MW: 30–26 kDa; pI 4.6–5.3) or two protein bands recognized by western blot analysis.[1,3] Posttranslational modifications, such as phosphorylation, could explain the presence of these two EFhd2 protein species. Consistently, several phosphoproteome analyses on mitotic growing cells and cancerous cells identified EFhd2 as a phosphoprotein. The EFhd2's phosphorylation sites identified on these studies were S11, S74, S76, Y83, and S204.[9–17] Dephoure *et al*. identified EFhd2 phosphorylated at S74 and S76 in cell arrested at G1 and M phase of the cell cycle.[11] In contrast, Olsen *et al*. identified EFhd2 phosphorylated at S11 and S204 in S phase, which may represent cell cycle specific phosphorylation events on EFhd2 proteins.[16] These global phosphoproteome analyses suggest that EFhd2 phosphorylation may be associated to the activation of proline-directed kinases such as Cdk1, Cdk2, and MAPK, but the specific kinase that mediated EFhd2 phosphorylation is still unknown. Furthermore, Nagano *et al*. identified EFhd2 as a phosphoprotein in response to nocodazole treatment, a microtubule destabilizing drug, often used to synchronize cell cycle signaling.[17] Additionally, Huh *et al*. showed that EGF treatment of HEK293T cells induces the phosphorylation of EFhd2 at S183.[7] Thus, it is possible that EFhd2 may become phosphorylated in response to cellular cues or stress rather than being regulated through the cell cycle.

EFhd2 is found associated with the microtubule associated protein tau in the tauopathy mouse model JNPL3 and human tauopathy cases, such as Alzheimer's disease (AD) and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP17).[3,18] The association between EFhd2 and tau was not detected in 3 months old JNPL3 mice, where tau pathology is under detectable levels and no neurodegeneration phenotype is observed.[3] Furthermore, EFhd2 co-purified with sarkosyl-insoluble tau and was found associated to tau filamentous structures in AD brain.[3,18] We also demonstrated that EFhd2 is a novel amyloid protein that self-oligomerize *in vitro*, which is mediated by its coiled-coil domain.[18] However, it is still unknown if calcium binding or posttranslational modifications regulate the association of EFhd2 with tau in AD and other tauopathies.

Deregulation of protein kinases is known as a molecular mechanism associated with the pathobiology of tau-mediated neurodegeneration. Hyperphosphorylation of tau proteins induced its dissociation from microtubules, leading to the formation of tau filaments that aggregate in an ultrastructure known as neurofibrillary tangles. GSK3β and Cdk5 have been identified as the two principal kinases involved in tau hyperphorphorylation.[19–29] *In vitro* studies indicated that GSK3β prime tau proteins for subsequent phosphorylation by Cdk5/p35 or Cdk5/p25.[22] Bioinformatics analysis of EFhd2's protein sequence indicates that it has several regions with the consensus P-(S/T) sequence that is phosphorylated by proline-directed kinases, such as Cdk5/p35 and GSK3β. Therefore, based on the association of EFhd2 to tau-mediated neurodegeneration, it is plausible to hypothesize that EFhd2 could be a substrate of either or both of these kinases. In this study, this hypothesis was directly tested using brain extract from a transgenic mouse that overexpresses p25 (CK-p25) and *in vitro* phosphorylation assays. Additionally, it was determined the effect that EFhd2 phosphorylation exerts on its calcium binding. Finally, a phospho-specific antibody was generated and evaluated to determine the *in vivo* phosphorylation of EFhd2 in AD and normal aging control.

Results

CK-p25 brain extract phosphorylates EFhd2 protein

CK-p25 is a transgenic mouse that inducibly overexpresses human p25 protein under the control of the CamKII alpha promoter, restraining the expression to the forebrain.[30] Neurodegeneration was detected after only 2 weeks of p25 overexpression in the forebrain of transgenic mice.[30] Concurrently with the overexpression of p25, phosphorylation of known Cdk5 substrates, such as tau, neurofilament H, and Amyloid precursor protein, was detected.[30] Thus, subcortical and cortical brain regions from non-transgenic and CK-p25 mice after 2 (2W) or 4 (4W) weeks of induction were homogenized. No change in the level of Cdk5 was detected [Fig. 1(A)]. As expected, however, overexpression of p25 was detected in the cortical region of CK-p25 mice after 2W and 4W of induction [Fig. 1(A)]. After 4W of induction, p25 was also detected in the subcortical brain region [Fig. 1(A)].

The brain extracts were incubated with purified recombinant HIS-EFhd2 for *in vitro* kinase assay [Fig. 1(B)]. After incubation with brain extract, the purified recombinant proteins were resolved in SDS-PAGE and visualized using coomassie blue [Fig. 1(B)]. ProQ-diamond phospho-specific dye was used to detect phosphorylated proteins. The results indicated that HIS-EFhd2 is phosphorylated after incubation with brain extract, especially by the protein extract derived from cortical region where p25 is predominantly overexpressed [Fig. 1(B)].

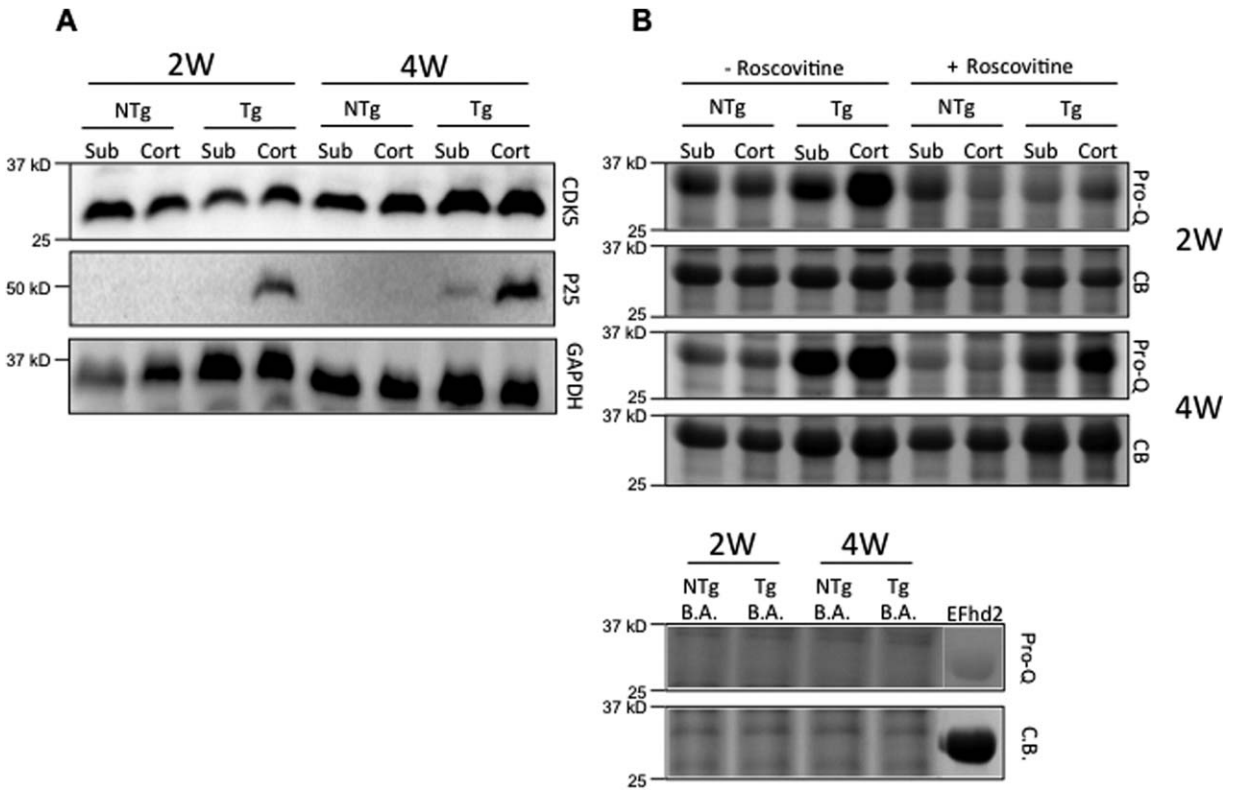

Figure 1. CK-p25 brain extract phosphorylates EFhd2. (A) Western blot analysis of brain extract derived from CK-p25 mice using anti-Cdk5 and anti-p25 confirmed the induction of p25 at 2 weeks (2W) and 4 weeks (4W), preferentially in the cortical region. The level of the Cdk5 protein did not change. (B) HIS-EFhd2 full length (FL) was exposed to cortical (cort) and subcortical (sub) brain extract after 2W or 4W of induction in absence (−) or presence (+) of 100 μ*M* roscovitine. The level of phosphorylated HIS-EFhd2 was detected using Pro-Q diamond phospho-protein staining and coomassie blue (CB) staining was used to detect total protein used. Beads alone (beads) were used as negative control for non-specific binding and to detect background levels of the Pro-Q diamond staining.

Cdk5 or a kinase activated by it could mediate the detected phosphorylation of EFhd2. To corroborate that Cdk5 mediates EFhd2 phosphorylation, roscovitine, a potent Cdk5 inhibitor,[31,32] was added to the reaction. Roscovitine blocked the phosphorylation of the recombinant HIS-EFhd2 when the 2W induced brain extract was used [Fig. 1(B)]. However, roscovitine only reduced the phosphorylation of HIS-EFhd2 detected upon incubation with the 4W induced brain extract [Fig. 1(B)]. It is possible that after 4 weeks of p25 induction, the hyperactivity of Cdk5 could not be completely inhibited by roscovitine. Alternatively, the hyperactivity Cdk5 may activate other kinases that also mediate the phosphorylation of EFhd2 proteins. Nevertheless, the results demonstrate that EFhd2 can be phosphorylated *in vitro*.

Cdk5 phosphorylates EFhd2 in vitro

To determine if Cdk5 directly phosphorylates EFhd2, purified recombinant HIS-EFhd2 proteins were incubated with recombinant Cdk5/p35, Cdk5/p25, or GSK3β [Fig. 2(A)]. *In vitro* kinase assays were performed and the level of EFhd2 phosphorylation was determined using the phospho-specific ProQ-diamond dye. Recombinant EFhd2 protein was incubated without kinase as negative control [Fig. 2(A)]. The results demonstrated that GSK3β did not phosphorylate EFhd2, but it did phosphorylate recombinant tau proteins [Fig. 2(A)]. In contrast, both Cdk5/p35 and Cdk5/p25 phosphorylated recombinant EFhd2 after just 5 min of incubation, reaching the maximum detected level at 60 min [Fig. 2(A)]. No significant difference was detected in the kinetics of EFhd2 phosphorylation by both Cdk5/p35 and Cdk5/p25.[33] Therefore, EFhd2 is a novel substrate of the proline-directed Cdk5 kinase, but not GSK3β.

To gain insights into the protein region that bears the phosphorylation site(s) where Cdk5 phosphorylates EFhd2, recombinant Cdk5/p25 was incubated with different EFhd2 (FL) truncation mutants [Fig. 2(B)]. EFhd2 N-terminal truncated mutant (ΔNT) lacks the first 50 amino acids of the protein sequence, whereas the C-terminal truncation (ΔCC) lacks the coiled-coil domain that expands from amino acid 199–240 [Fig. 2(B)]. *In vitro* kinase assays were performed using radioactive ATPγP^{32} in presence or absence of Cdk5/p25. Recombinant histone proteins were used as positive control [Fig.

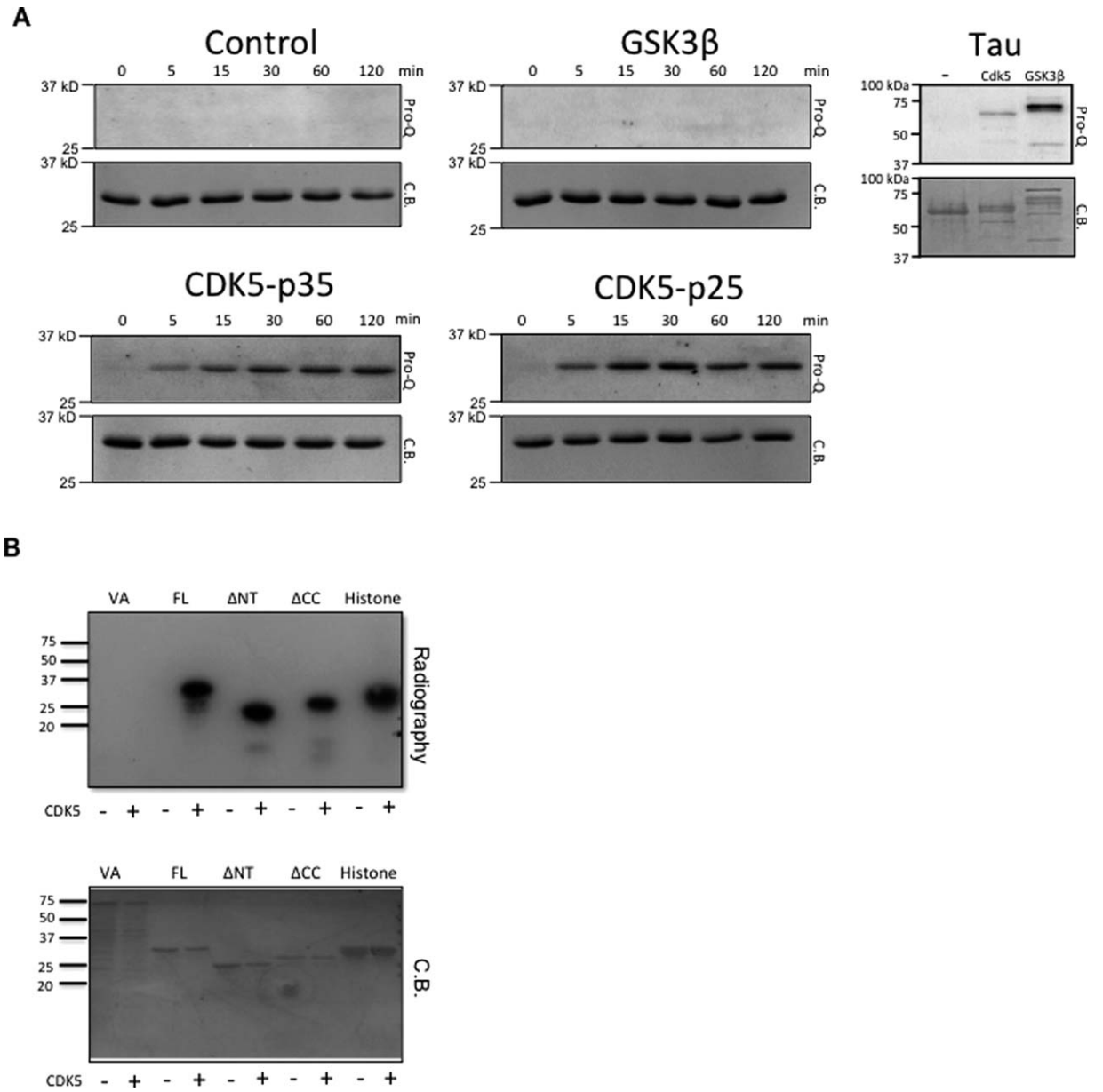

Figure 2. Cdk5, but not GSK3β, phosphorylates EFhd2 *in vitro*. (A) Time course of *in vitro* phosphorylation was done incubating HIS-EFhd2-FL with recombinant Cdk5 (with either p35 or p25) or GSK3β. Time points were collected at 0, 5, 15, 30, 60, and 120 min. Phosphorylation was detected using Pro-Q diamond phospho-protein staining and equal protein loading was detected using coomassie blue staining. HIS-EFhd2-FL exposed to the same conditions of phosphorylation but without kinase was used as negative control (Control). The activity of both Cdk5 and GSK3b was also determined using recombinant 4R0N tau as substrate. (B) *In vitro* kinase assay using radioactive ATPγP^{32} in presence or absence of Cdk5/p25 was done using recombinant HIS-EFhd2 (FL), N-terminus (ΔNT), and C-terminus (ΔCC) truncation mutants. After *in vitro* phosphorylation, the recombinant EFhd2 proteins were resolved on a SDS-PAGE and the gel exposed to an X-ray film. Recombinant histone 3 was used as positive control. (C) Schematic representation of the EFhd2 full length (FL), the N-terminus truncation (ΔNT; lacks the first 50 amino acids of the protein), and coiled-coil truncation (ΔCC; lacks the coiled-coil domain) used in the *in vitro* phosphorylation assay. The EFhd2 FL contains the poly-alanine region (PA), two EF-hand domains (EF1 and EF2), and the coiled-coil domain (CC).

2(B)]. After *in vitro* phosphorylation, the purified recombinant EFhd2 proteins were resolved on SDS-PAGE and exposed to X-ray film. The radioactive γP^{32} signal conjugated to EFhd2 was detected in the samples containing Cdk5/p25, consistent with the ProQ diamond phospho-specific dye signal [cf. Fig. 2(A) and Fig. 2(B)]. Although it, the C-terminal truncation mutant, showed a lower signal, the results suggest that Cdk5 phosphorylates EFhd2 between amino acids 51 and 199 [Fig. 2(B)].

Cdk5 monophosphorylates EFhd2 at S74

To determine the extent of EFhd2 phosphorylation, we subjected recombinant HIS-EFhd2 to biomass analyses after Cdk5/p25-mediated phosphorylation. After deconvolution, the mass obtained for non-

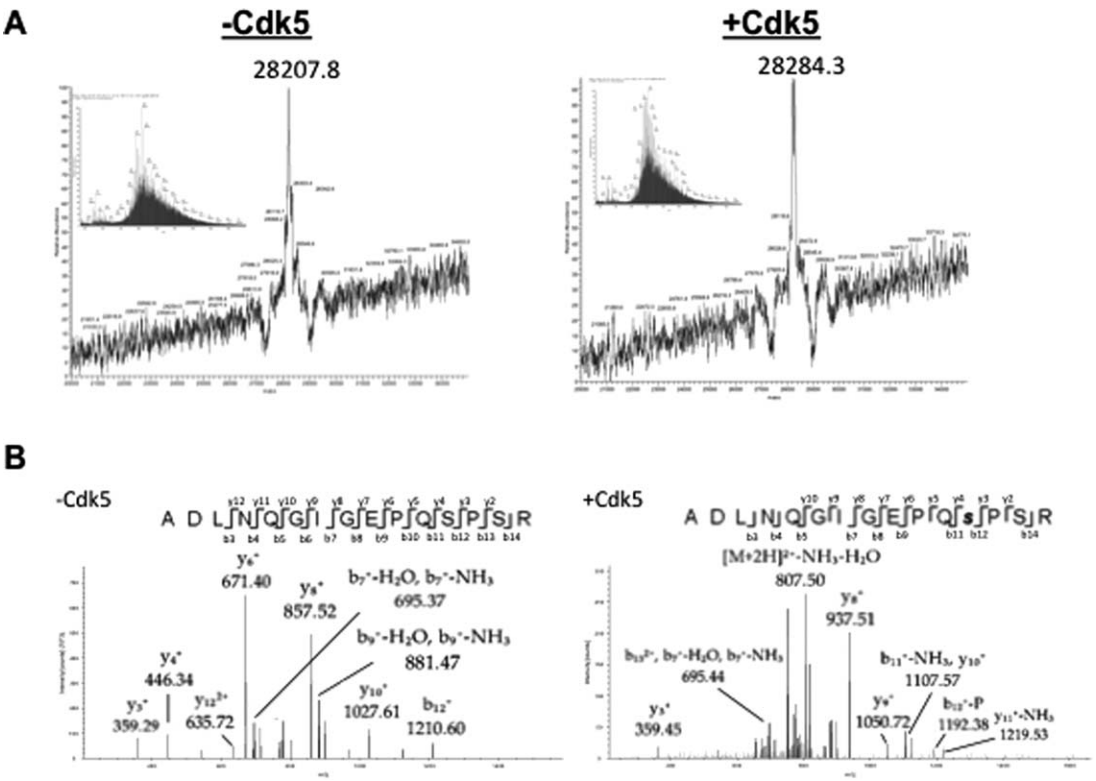

Figure 3. Cdk5 phosphorylates EFhd2 at S74. (A) Biomass analysis of Cdk5-phosphorylated (+Cdk5) HIS-EFhd2 demonstrated a mass increase of 77, in comparison to non-phosphorylated recombinant HIS-EFhd2 (−Cdk5). This shift in mass suggests the conjugation of one phosphate group. (B) Tandem mass spectrometry analysis of Cdk5-phosphorylated HIS-EFhd2 identified a phosphorylated peptide corresponding to the EFhd2's sequence from amino acid 63 to 77 (+Cdk5). No phosphorylated peptides were detected in the non-phosphorylated EFhd2 sample (−Cdk5). The MS/MS spectra illustrate the b and y ions used to determine the sequence of the identified peptides.

phosphorylated EFhd2 was 28207.8 [Fig. 3(A), −Cdk5]. In contrast, EFhd2 incubated with Cdk5/p25 showed a mass increase of 77 Da, suggesting the addition of one phosphate group (theoretical mass is 79.99 Da) [Fig. 3(A), +Cdk5). This result indicates that Cdk5 monophosphorylates recombinant EFhd2.

To identify the phosphorylation site, phosphorylated EFhd2 protein was subjected to trypsin digestion and the tryptic peptides subjected to tandem mass spectrometry. Non-phosphorylated EFhd2 was used as negative control [Fig. 3(B), −Cdk5]. In the sample where EFhd2 was incubated with Cdk5, a phosphorylated peptide was identified corresponding to amino acids 63–77; Cdk5 phosphorylated EFhd2 at a serine residue in position 74 (S74) [Fig. 3(B), +Cdk5]. However, the same peptide was also found phosphorylated at S76 instead of S74 (data not shown). It is important to mention that this second phosphorylated site barely passed the filters applied to avert false positives. The identification of two phosphorylation sites (S74 and S76) and the biomass analysis that indicated the conjugation of one phosphate group is not incongruous. Because of the proximity of these two Serines, EFhd2 could be indiscriminately phosphorylated at either S74 or S76. Alternatively, the identification of phosphorylated S74 or S76 could be an algorithmic error during the assignment of the phosphorylated Serine residue.

To confirm the phosphorylation site on EFhd2, S74 and S76 were mutated to codify the amino acid alanine. The wild-type EFhd2 and the point mutants EFhd2^{S74A} and EFhd2^{S76A} were purified and subjected to *in vitro* kinase assay using ATPγP^{32}. These recombinant proteins were incubated with either Cdk5/p25 (p25) or GSK3β (G) [Fig. 4(A)]. As negative control, the recombinant proteins were incubated in kinase buffer with ATPγP^{32} but without recombinant kinase [Fig. 4(A)]. The result indicated that Cdk5/p25, but not GSK3β, phosphorylated both wild-type EFhd2 and EFhd2^{S76A} mutant proteins [Fig. 4(A)]. However, the EFhd2^{S74A} mutant was not phosphorylated by Cdk5/p25, indicating that Cdk5 specifically phosphorylates S74 [Fig. 4(A)]. To further confirm these results, both EFhd2^{S74A} and EFhd2^{S76A} were subjected to *in vitro* phosphorylation using CK-p25 brain extract [Fig. 4(B)]. Consistently with the use of recombinant Cdk5/p25, EFhd2^{S74A} was not phosphorylated after incubation with brain extract from 2W and 4W induced CK-p25 mice. In contrast, 4W-induced CK-p25 brain extract readily phosphorylated EFhd2^{S76A} mutant [Fig.

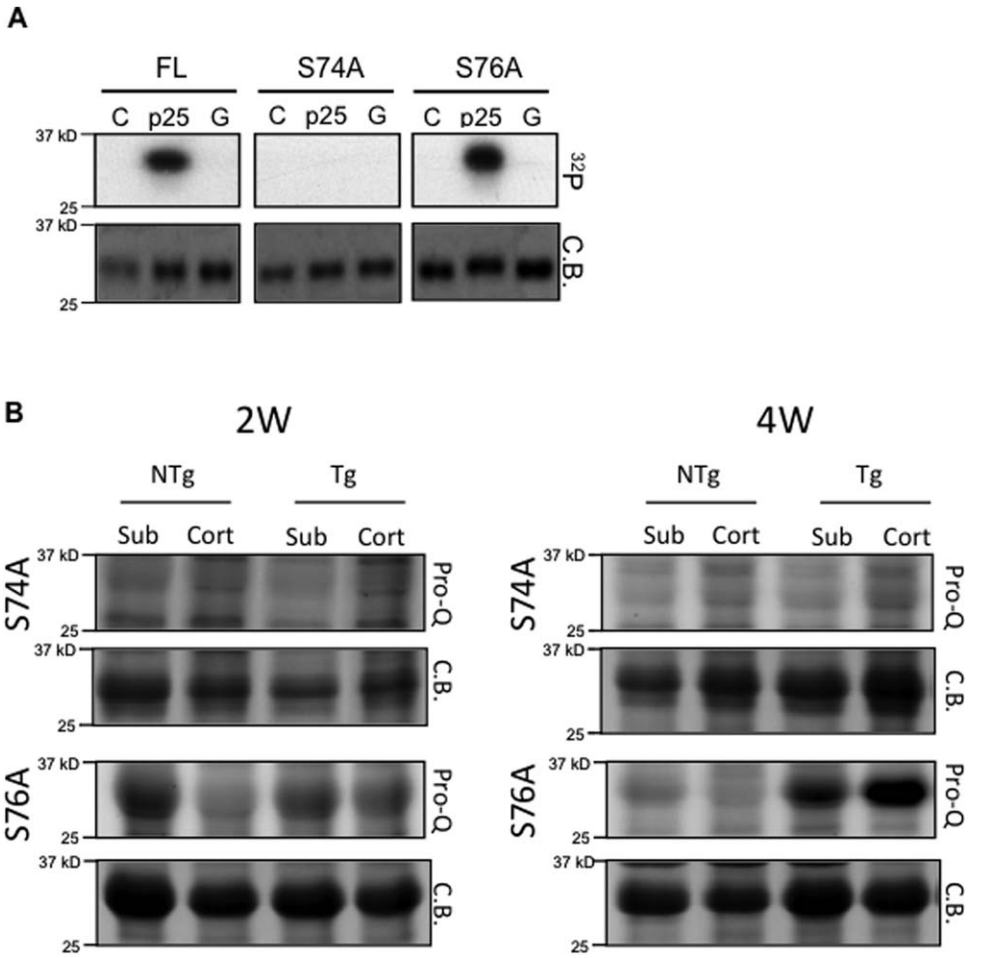

Figure 4. CK-p25 brain extract phosphorylated EFhd2 at S74. (A) HIS-EFhd2 point mutations were done to alter the codification of amino acid serine to alanine (S74A or S76A). HIS-EFhd2^{S74A}, HIS-EFhd2^{S76A}, and wild-type HIS-EFhd2 (FL) were subjected to *in vitro* kinase assay in presence of recombinant Cdk5/p25 (p25) or GSK3β (G) using radioactive ATP-γP^{32}. As negative control (C) the reaction was performed without adding the recombinant kinase. The purified proteins were resolved on a SDS-PAGE and exposed to X-ray film. As loading control, the gel was stained with coomassie blue (CB). (B) HIS-EFhd2^{S74A} and HIS-EFhd2^{S76A} point mutants were exposed to cortical (cort) and sub-cortical (sub) brain extract derived from CK-p25 mouse model after 2W or 4W of induction. Phosphorylation of HIS-EFhd2 mutants was detected using Pro-Q diamond phospho-protein staining and coomassie blue (CB) staining was used to evaluate protein loading.

4(B)]. EFhd2 incubated with 2W-induced CK-p25 brain extract showed a weaker signal than when wild type was used [cf. Fig. 1(B) and Fig. 4(B)]. There are several ways we can explain this result. One possibility could be the sensitivity of the Pro-Q dye, which may contribute to a lower signal especially after 2W of p25 induction. Conversely, S76A mutant protein may be less efficiently phosphorylated at S74 (or other sites) requiring higher induction of p25 before phosphorylated EFhd2^{S76A} is above detection. Nevertheless, the results demonstrated that Cdk5 directly phosphorylates EFhd2 specifically at S74.

Effect of Cdk5 phosphorylation on EFhd2's calcium binding activity

EFhd2 calcium binding activity has been shown associated with putative physiological functions and to influence the stabilization of its structure.[5,6,34] Thus, *in vitro* calcium binding assays were performed to determine the effect of Cdk5-mediated EFhd2 phosphorylation [Fig. 5(A)]. Recombinant EFhd2 protein was incubated with or without Cdk5/p25. As expected, EFhd2 (FL) binds calcium effectively in comparison to the negative control (beads) [Fig. 5(A)]. However, the Cdk5 phosphorylated EFhd2 protein (FL/CDK5) showed a significant reduction on its calcium binding capacity [Fig. 5(A)]. This result suggests that EFhd2 phosphorylation may regulate its calcium binding activity *in vivo*.

EFhd2 is phosphorylated at S74 in AD and normal aging control brain extracts

To confirm that EFhd2 can be phosphorylated *in vivo* at S74, a phospho-specific antibody against phospho-serine 74 (anti-pSer74) was generated. It is

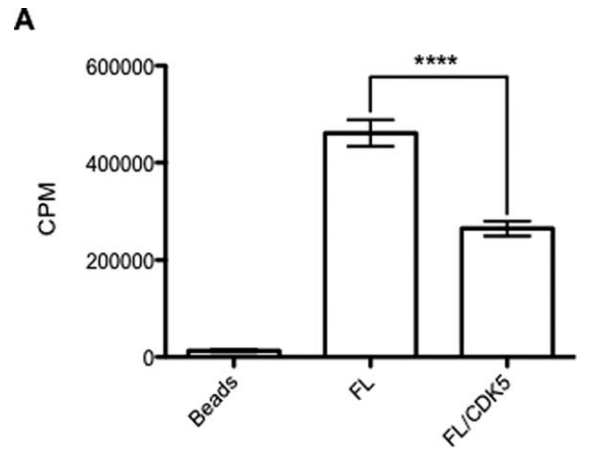

Figure 5. Effect of CDK5 phosphorylation on EFhd2 calcium binding activity. (A) *In vitro* calcium binding assay was performed using phosphorylated (FL/CDK5) and non-phosphorylated (FL) HIS-EFhd2 incubated with radioactive $^{45}CaCl_2$. The radioactivity associated to the recombinant proteins was determine using a scintillation counter and illustrated in the graph as counts per minute (CPM). Beads alone (beads) were used as negative control. For this experiment $n = 6$ and $^{****}P < 0.0001$ and the error bar represents the standard error of the mean (SEM).

important to mention that the sequence of the phosphorylated peptide at S74 used to generate the phospho-specific antibody is 100% conserved from mouse to human; it is expected that this antibody will recognize both mouse and human phosphorylated EFhd2 at S74. The generated monoclonal phospho-specific antibody was tested against Cdk5 phosphorylated wild-type EFhd2 (FL) and the point mutants EFhd2^{S74A} (S74A) and EFhd2^{S76A} (S76A) [Fig. 6(A)]. Samples without Cdk5 were used as negative control. Western blot analysis demonstrated the specificity of the new EFhd2 phospho-specific pSer74 antibody and confirms Cdk5-specific phosphorylation of S74 [Fig. 6(A)].

The anti-pSer74 antibody was used to detect phosphorylated EFhd2 at S74 *in vivo*. As EFhd2 is associated to the pathobiology of AD, we conducted western blot analyses of protein extracts derived from postmortem frontal cortexes of normal-aging controls and AD cases. The results indicate that EFhd2 is phosphorylated at S74 in frontal cortexes of AD and normal aging patients [Fig. 6(B)], suggesting that phosphorylation of EFhd2 is a molecular event that takes place *in vivo*. Curiously, a significant reduction of EFhd2 phosphorylation at serine 74 (pSer74) was observed in AD brain compared with normal aging controls [Fig. 6(B,C)]. No reduction on total tau protein was detected and increased PHF1-tau was detected in AD brain, as expected [Fig. 6(B)]. Further studies are required to determine the physiological and pathological relevance of EFhd2 phosphorylation at S74.

Discussion

Both, brain extract from CK-p25 transgenic mice and recombinant Cdk5/p35 and Cdk5/p25, but not GSK3β, readily phosphorylated wild-type EFhd2 and EFhd2^{S76A}. These results demonstrated that Cdk5 phosphorylates EFhd2 at S74. In contrast, a previous phosphoproteomics study found that EFhd2 could be phosphorylated at either S74 or S76.[11] These putative phosphorylation sites were not found simultaneously phosphorylated, consistent with what was observed in this study. It is important to mention that proteins identified, and their corresponding phosphorylated peptides, by phosphoproteomics approaches are often not validated. Peptide fragmentation differences that affect sequence coverage contribute to errors in the assignment of post-translational modifications on adjacent amino acids. In this study, the results obtained from tandem mass spectrometry analysis and their validation using mutagenesis demonstrated that Cdk5 phosphorylates EFhd2 specifically at S74 and not at S76.

The physiological and pathological roles that EFhd2 may play in the central nervous system are not understood. We showed that EFhd2 is a calcium binding protein.[3] Calcium stabilizes the secondary structure of EFhd2 and reduces its ability to self-oligomerize *in vitro*, which is dependent of its coiled-coil domain.[18,34] Recently, Kwon *et al*. studies confirmed our previously published results indicating that the coiled-coil domain mediates EFhd2 dimerization.[8] In contrast, they showed that calcium facilitates EFhd2 dimer formation.[8] Although it may be perceived as contradicting results, it is important to consider that these studies measured different aspects of EFhd2's structural dynamics. In our studies, we showed a reduction in Thio-S signaling in the presence of calcium, indicating that calcium-induced structural stabilization affects the transition from alpha confirmation to beta-amyloid structure, but did not abolish it.[18] Kwon *et al*. used co-immunoprecipitation assays to determine the dimerization of EFhd2.[8] Therefore, EFhd2 dimerization may require calcium whereas amyloid oligomerization does not.

In addition, we showed here that EFhd2 phosphorylation significantly reduces its calcium binding activity. These results suggest that EFhd2's phosphorylation may serve as a negative signal for the regulation of calcium binding. The S74 is located in a predicted random coil in the modeled structure of EFhd2.[34] Previously, we showed that deletion of the first 50 amino acids N terminus enhanced calcium binding activity of EFhd2 proteins.[34] The structural analyses performed on this study suggested that the N terminus may constrained the access of calcium ions to the EF-hand motif.[34] Taken together, these results indicate that alterations in the N terminus have an intrinsic effect on EFhd2's calcium binding

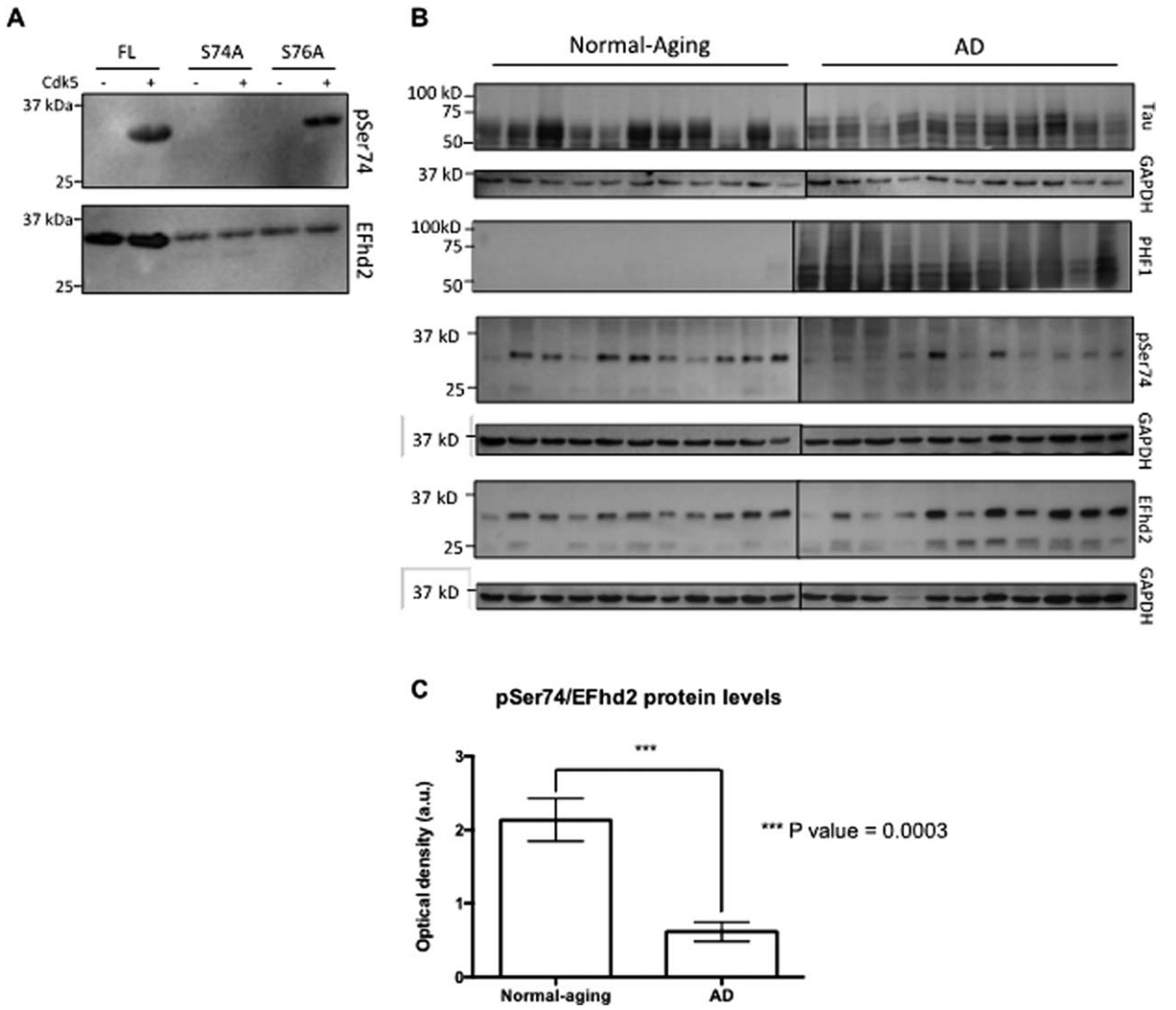

Figure 6. EFhd2 phosphorylation at S74 detected in AD and normal-aging postmortem brain. (A) Evaluation of phospho-specific antibody (anti-pSer74) against EFhd2 phosphorylated at S74 was performed by western blot, using Cdk5-phosphorylated EFhd2 (FL) and point mutants EFhd2^{S74A} (S74A) and EFhd2^{S76A} (S76A). Samples without Cdk5 were used as negative control (−). The loading of phosphorylated and unphosphorylated EFhd2 was confirmed using anti-EFhd2 antibodies. (B) Western blot analysis was performed to confirm that the phosphorylation of EFhd2 at S74 occurs *in vivo*. Frontal cortexes of AD and normal aging controls were evaluated using antibodies against total tau, PHF-1, EFhd2 phosphorylated at S74 (pSer74), and GAPDH. For total tau was load 5 μg of total protein and for all others was load 50 μg. GAPDH was detected in the same membrane of as loading control. (C) The graph shows densitometry analysis of the amount of pSer74 EFhd2 corrected by the amount of total of EFhd2. For these experiments $n = 11$, $^{***}P = 0.0003$ and the error bar represents the standard error of the mean (SEM).

activity. However, the biological relevance of EFhd2 phosphorylation needs to be further studied.

The biological relevance of EFhd2 is still unknown. Here, however, we showed that EFhd2 protein is phosphorylated at S74 in normal-aging controls and AD brain extract, indicating that this phosphorylation event takes place *in vivo*. These results suggest that phosphorylation may work as a negative regulator of EFhd2's calcium binding activity, promoting its oligomerization. This hypothesis is consistent with the fact that EFhd2 is found co-aggregated with pathological tau in AD brain, where Cdk5 is known to be hyper-activated and there is increased intracellular calcium level. Therefore, the Cdk5-mediated EFhd2 phosphorylation may be associated to a pathological role. In contrast, the abundance of phosphorylated EFhd2 in normal aging control brains, but significant reduction in AD brain, suggests that this posttranslational modification may have an effect on its physiological function. Cdk5-mediated phosphorylation may play an important regulatory role on the physiological function of EFhd2, whereas in neurodegeneration there may be other yet to be identified molecular events that regulate the formation of EFhd2 amyloid structures. A significant reduction of phosphorylated EFhd2 in

AD samples in comparison with normal-aging suggests that the phosphorylation event may be related to its physiological function rather than its putative pathological role. However, further studies are required to determine the physiological or pathological role of EFhd2 phosphorylation at S74 and the direct role of Cdk5 in the regulation of EFhd2 function.

Materials and Methods

Recombinant EFhd2 and point mutant generation

6x-His-tagged full length (FL), N terminus (ΔNT), and the coiled-coil (ΔCC) domain truncated isoforms of EFhd2 were generated as previously reported.[34] Generation of point mutants $EFhd2^{S74A}$ and $EFhd2^{S76A}$ was performed using site-directed mutagenesis (Stratagene Agilent technologies, Santa Clara, CA) following the manufacturer's recommendations. The primers used in the point mutants generation were: serine 74 to alanine (Rev. 5′-GAA GAC GCG GCG GCT GGG CGC CTG TGG CTC GCC GAT GCC-3′ and Fwd. 5′-GGC ATC GGC GAG CCA CAG GCG CCC AGC CGC CGC GTC TTC-3′) and serine 76 to alanine (Rev. 5′-GGG GTT GAA GAC GCG GCG GGC GGG CGA CTG TGG CTC GCC-3′ and Fwd. 5′-GGC GAG CCA CAG TCG CCC GCC CGC CGC GTC TTC AAC CCC-3′).

Antibodies used in western blot

Antibody to p35/25 (1:500) was purchased from Santa Cruz (Santa Cruz, CA), GAPDH (1:2000) and Tau13 (1:50,000) were purchased from Abcam (Cambridge, MA), anti-Cdk5 (1:10) was kindly provided by Dr. Li-Huei Tsai and PHF-1 (1:500) was kindly provided by Dr. Peter Davies. Phospho-specific antibody that detects EFhd2 phosphorylated at S74 was generated by CDI Laboratories (Mayaguez, PR). A mouse was immunized with the synthetic phosphopeptide GEPQpSPSRRVC (Tufts University Core Facility), hybridoma cells were generated and culture medium from the hybridoma was used to perform the western blot experiments.

Human brain lysis

AD and normal aging post-mortem frontal cortex tissues were obtained from UPENN Pathology Core Facility. All AD patients were pathologically confirmed with abundant senile plaques and Braak and Braak stage IV or more for tau pathology. Brain tissue was homogenized in four volumen of lysis buffer (20 m*M* Tris-Base, 150 m*M* NaCl, 50 m*M* Sodium Phosphate, 5 m*M* Sodium Pyrophosphate, 30 m*M* β-Glycerolphosphate, 30 m*M* Sodium fluoride, and pH 8.0). The protein extract was centrifuged at 14,000*g* for 10 min. The concentration of the supernatant was measured using Bradford assay and 50 μg of total protein was analyzed by western blot.

Phosphorylation assay

6xHis-tagged EFhd2 recombinant full length and mutant proteins were purified from bacterial lysates using nickel column beads (Sigma, Atlanta, GA). Beads with bound protein were equilibrated in lysis buffer (20 m*M* Tris-Base, 150 m*M* NaCl, 50 m*M* Sodium Phosphate, 5 m*M* Sodium Pyrophosphate, 30 m*M* β-Glycerolphosphate, 30 m*M* Sodium fluoride, and pH 8.0). Recombinant proteins were eluted with 250 m*M* imidazole and the amount of recombinant protein purified was estimated by densitometry analysis of coomassie blue stained proteins bands. CK-p25 tissue samples were kindly provided by Drs. Tsai and Seo. Subcortical and cortical brain regions from non-transgenic and CK-p25 mice, after 2 (2W) or 4 (4W) weeks of induction, were homogenized in lysis buffer supplemented with protease and phosphatase inhibitors followed by a centrifugation at 21,913*g*. The reaction was prepared adding 1 mg of brain protein extract (or 500 ng of Cdk5/p35, Cdk5/p25 [both from Sigma, Atlanta, GA], or GSK3β [New England Biolabs, Ipswich, MA]), 0.5 μg/μL of EFhd2 protein in reaction buffer (25 m*M* $MgCl_2$ and 300 μ*M* ATP [or 6 μCi of $ATP\gamma P^{32}$]). The reaction was placed at room temperature overnight. The recombinant EFhd2 protein was purified using nickel column and, after several washes with reaction buffer, resolved in a SDS-PAGE. Protein phosphorylation was detected using Pro-Q Diamond in-gel staining (Invitrogen, Carlsbad, CA) following manufacturer's recommendations and coomassie blue staining for loading control. When $ATP\gamma P^{32}$ was used, 2 μL of the beads-bound proteins were resolved in a SDS-PAGE and the gel exposed to X-ray film. For inhibition of Cdk5, brain protein extracts were pre-treated with 100 μ*M* of roscovitine (EMD Millipore, Darmstadt, Germany) for 1 h at room temperature.

Mass spectrometry analysis

Phosphorylation assay and protein purification were performed as described above. For biomass analysis, the protein buffer was exchanged to acetonitrile:-water (20:80, v/v). The sample was diluted in a solution containing acetronitrile:water (50:50, v/v) and 0.2% formic acid. The diluted protein was directly infused into the nano-electrospray source. The ionization source was set at a spray voltage of 1.5 kV and capillary temperature 250°C. The samples were analyzed in a LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) recording mass spectra for 1 min, using positive mode. The data acquired were analyzed using BioMass calculation and deconvolution tool of BioWorks 3.1 software. The parameters for BioMass calculation and deconvolution were set as follow: enable averaging, enable

smoothing (Gaussian; 3), deconvoluted spectrum (20,000–35,000), adduct ion mass (+ proton [+1.008]), and mass step size 0.25 units. For tandem mass spectrometer analysis, 1 μg of trypsin was added to the sample and incubated 18 h at 37°C. The tryptic-peptides were loaded onto the HPLC and separated using a PicoFrit™ column packed with ProteoPep™ C18 (New Objective, Woburn, MA) and eluted using a linear gradient (55 min) of water:acetonitrile (2%:80%) in 0.2% formic acid. The tryptic-peptides were identified using Thermo Proteome Discoverer version 1.2.0.208 (Thermo Fisher Scientific, Waltham, MA) that correlates MS–MS spectra with sequences from the NCBI EFhd2 mouse protein database using SEQUEST database search algorithms. Search parameters used were: two trypsin missed cleavage sites allowed, the precursor and fragment tolerance were set to 2 Da and 1 Da, respectively, and phosphorylation as dynamic modification to serine, threonine, and tyrosine. The decoy database search was set to target false discovery rate strict and relaxed to 0.01 and 0.05, respectively. Only peptides with Xcorr higher than 1.5 (+1), 2.0 (+2), or 2.5 (+3) and high confidence were considered in the analysis.

Calcium binding assay

Calcium binding assay was performed as previously reported.[3,34] After *in vitro* phosphorylation, the nickel-beads bound recombinant proteins, incubated with or without kinase (as described above), were equilibrated in calcium binding buffer to eliminate the $MgCl_2$ that could interfere with Ca^{2+} binding.

Statistical analysis

All *P*-values for column graphs were obtained using one-tail Student's *t*-test. Column graphs were prepared using GraphPad PRISM (Prism Mac Serial number(s): GPM4-034396-RJD-2023). Error bars represent average ±SEM. Statistical analyses were performed using GraphPad PRISM.

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