Chapter 11

The Study of Posttranslational Modifications of Tau Protein by Nuclear Magnetic Resonance Spectroscopy: Phosphorylation of Tau Protein by ERK2 Recombinant Kinase and Rat Brain Extract, and Acetylation by Recombinant Creb-Binding Protein

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Abstract

Nuclear magnetic resonance (NMR) spectroscopy can be used as an analytical tool to investigate posttranslational modifications of protein. NMR is a valuable tool to map the interaction regions of protein partners. Here, we present protocols that have been developed in the course of our studies of the neuronal Tau protein. Tau is found aggregated in the neurons of Alzheimer's disease patients. Development of the disease is accompanied by increased, abnormal phosphorylation and acetylation of Tau. We have used NMR to investigate how these posttranslational modifications of Tau affect the interactions with its partners. We present here detailed protocols of *in vitro* phosphorylation of Tau by recombinant kinase, ERK2, or kinase activity of rat brain extracts, and acetylation by recombinant Creb-binding protein (CBP) acetyltransferase. The analytical characterization of the modified Tau by NMR spectroscopy is additionally described.

Key words Phosphorylation, Acetylation, ERK kinase, Creb-binding protein, Acetyltransferase, NMR spectroscopy, Recombinant proteins

1 Introduction

Tau protein is hyperphosphorylated in the paired helical filaments (PHF) found in neurons in brain affected by Alzheimer's disease. The longest Tau protein isoform (441-residue) has 80 Threonine (T) and Serine (S) residues exposed since Tau is an intrinsically disordered protein with all these sites prone to modification by numerous kinases [1]. In the PHF-Tau, up to about 45

Caroline Smet-Nocca (ed.), *Tau Protein: Methods and Protocols*, Methods in Molecular Biology, vol. 1523,
DOI 10.1007/978-1-4939-6598-4_11,

phosphorylation sites can be modified [2, 3] while 15–30 Tau phosphorylation sites were identified by mass spectrometry in Tau extracted from both normal mice and a mouse AD disease model [4] or cognitively normal human brain. Most of them correspond to proline-directed sites located mainly in the proline-rich region of Tau. Given the number and the proximity of these phosphorylation sites, to identify the phosphorylation sites in a phosphorylated Tau sample and to evaluate the level of modification for each of these sites are difficult [4]. Recently, acetylation of Tau protein was shown to be associated with Tauopathies [5, 6]. In this case, the lysine residues are modified by an acetyl group on the NH3 moiety of the side chain, neutralizing the positive charge of the lysine and modifying the steric characteristics of the side chain. Mass spectrometry analysis revealed 14 lysine residues were acetylated in Tau samples purified from mice, and these were mainly located in the microtubule binding region of Tau [4].

In addition, these posttranslational modifications affect the binding of Tau to several molecular partners, an aspect of interest to study by NMR spectroscopy [7]. The need for *in vitro* modified Tau for these NMR experiments has led us to develop protocols to generate the required amount of modified protein. In addition, we have used NMR spectroscopy to characterize enzymatically modified Tau samples with success [8–12]. Given the improvement of the probe sensitivity, we can now work with samples in the 10 μM range, in volumes that can be limited to 200 μl. A typical good sample of Tau would be 1 mg in 200 μl or 100 μM of Tau protein. This still is a lot of material compared to most of the applications of biochemistry, such as the immunodetection for the phosphorylation characterization or the GST pull-down for the interaction investigations. Yet compared to these methods we can obtain information at the molecular level and define the interaction sites up to the residue. Hence, the drawback of the material quantity is compensated by the resolution of the results.

We will here first describe the preparation of isotopically labeled Tau for NMR investigation. The in vitro phosphorylated samples of Tau by rat brain extract have been used for years as a model of hyperphosphorylated Tau [13–15]. Phosphorylation by rat brain extract has been described in Goedert et al. 1993 [14]. The protocol here presented is an adaptation to the need of biophysical analysis requiring the preparation of sufficient quantity of protein. In the course of our investigation, we also observed that a Tau protein phosphorylated by ERK2 is modified on numerous sites described as pathological sites of phosphorylation, and thus represents an interesting model of hyperphosphorylated Tau. ERK2 is activated by phosphorylation by MEK [16–18]. The acetylation of Tau is obtained in vitro by a fragment of the CBP protein, a histone acetyltransferase [12]. In addition to the modified, isotopically labeled Tau protein preparation, we describe the NMR strategy used for the identification of the PTMs.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Media and stock solutions for bacterial culture, and all buffers are sterilized.

2.1 Preparation of Recombinant ^{15}N-Tau, ^{15}N, ^{13}C-Tau and $^{15}N_2$-Lysine/^{13}C-Tau

1. TaupET15b recombinant T7 expression plasmid (Novagen).
2. BL21(DE3) transformation competent *E. coli* bacteria (New England Biolabs).
3. Autoclaved LB Broth, Lennox (DIFCO).
4. M9 buffer: 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl per liter; dissolve in water and autoclave.
5. 1 M $MgSO_4$: prepare in ultrapure water and autoclave.
6. 100 mM $CaCl_2$: prepare in ultrapure water and autoclave.
7. MEM vitamin complements 100× (Sigma; *see* **Note 1**).
8. 100 mg/ml stock solution of ampicillin antibiotics: prepare in ultrapure water and store 1 ml aliquots at −20 °C.
9. 1 M stock solution of IPTG (Isopropyl β-D-1-thiogalactopyranoside): prepare in ultrapure water, 0.2 μM filter-sterilize and store 1 ml-aliquots at −20 °C.
10. ^{15}N-NH_4Cl (Isotec) or ^{14}N-NH_4Cl.
11. $^{13}C_6$-Glucose (Isotec).
12. $^{12}C_6$-Glucose.
13. $^{15}N_2/^{13}C_6$-L-Lysine hydrochloride (Isotec).
14. ^{15}N-ISOGRO complete medium powder (Isotec) (*see* **Note 1**).
15. ^{15}N, ^{13}C-ISOGRO complete medium powder (Isotec) (*see* **Note 1**).
16. The M9 medium is reconstituted by addition of 1 ml 1 M $MgSO_4$, 1 ml 100 mM $CaCl_2$ (*see* **Note 2**), 10 ml 100× MEM vitamin complement (Sigma), 1 ml 100 mg/ml Ampicillin to 1 L of M9 buffer. In addition, depending on the isotope labeling scheme, the components 17, 18, and 19 are dissolved in 10 ml of M9 buffer and directly 0.2 μM filter-sterilized into the M9 medium (*see* **Note 3**).
17. For the ^{15}N uniform labeling: 300 mg ^{15}N-ISOGRO complete medium, 1 g ^{15}N-NH_4Cl, and 4 g $^{12}C_6$-glucose.
18. For the ^{15}N, ^{13}C uniform labeling: 300 mg ^{15}N, ^{13}C-ISOGRO complete medium, 1 g ^{15}N-NH_4Cl, and 2 g $^{13}C_6$-glucose (*see* **Note 4**).
19. For the selective ^{15}N-Lysine and uniform ^{13}C-labeling: 1 g ^{14}N-NH_4Cl, 2 g $^{13}C_6$-glucose and 20 min before induction 150 mg of $^{15}N_2/^{13}C_6$-L-Lysine hydrochloride.

20. Cation-exchange chromatography (CEX) A buffer (*see* **Note 5**): 50 mM phosphate buffer pH6.5, 1 mM EDTA; autoclave.
21. Extraction buffer: CEX A buffer supplemented with Protease inhibitors 1× (1 pellet for 50 ml, Roche) and 2000 U DNaseI for 50 ml (20,000 U/ml, EUROMEDEX).
22. Cation-exchange chromatography CEX B buffer: CEX A buffer with 1 M NaCl; autoclave.
23. SP sepharose FF 5 ml column (GE Healthcare).
24. HiPrep 26/10 desalting column (GE healthcare).
25. Lyophilization buffer: 50 mM Ammonium Bicarbonate.
26. Emulsiflex-C3 homogenizer (Avestin).
27. Lyophilizer.

2.2 Preparation of Recombinant ERK2

1. His-ERK recombinant T7 expression plasmid.
2. BL21(DE3) transformation competent *E. coli* bacteria (New England Biolabs).
3. Autoclaved LB broth Lennox (Difco).
4. 100 mg/ml stock solution of ampicillin antibiotics.
5. 1 M stock solution of IPTG.
6. PBS: phosphate buffer saline.
7. Extraction buffer: 50 mM phosphate buffer pH7.6, 300 mM NaCl, 10 mM Imidazole, Protease inhibitor 1× (1 tablet for 50 ml, EDTA-free; Roche), 2000 U DNaseI for 50 ml (20,000 U/ml, EUROMEDEX).
8. Ni-NTA chromatography cartridge 1 ml (Thermo Science).
9. Wash buffer: 50 mM phosphate buffer pH7.6, 300 mM NaCl, 10 mM Imidazole; autoclave.
10. Elution buffer: 50 mM phosphate buffer pH7.6, 300 mM NaCl, 250 mM Imidazole (*see* **Note 6**).
11. Dialysis bags (12–14 kDa cutoff, 0.32 ml/cm; Spectrum Laboratories).
12. Centrifugal concentration devices (10 kDa cutoff, 2 ml; Sartorius).
13. 12% SDS Polyacrylamide precast gels (Pierce).
14. Activation buffer: 10 mM Hepes.KOH, pH7.3, 1 mM DTT, 5 mM $MgCl_2$, 100 mM NaCl, and 10% Glycerol.
15. Conservation buffer: 50 mM Tris–HCl, pH7.5, 150 mM NaCl, 0.1 mM EGTA, 20% Glycerol.
16. Emulsiflex-C3 homogenizer (Avestin).

2.3 Preparation of Recombinant GST-MEK1 R4F

1. GST-MEK1 R4F recombinant pGEX expression plasmid [19].
2. BL21(DE3) transformation competent *E. coli* bacteria (New England Biolabs).

3. Autoclaved LB broth Lennox (Difco).
4. 100 mg/ml stock solution of ampicillin antibiotics.
5. 1 M stock solution of IPTG.
6. PBS: phosphate buffer saline.
7. Extraction buffer: 50 mM Tris–HCl, pH7.5, 1 mM EDTA, 300 mM NaCl, Protease inhibitor 1× (1 tablet for 50 ml, EDTA-free; Roche), 2000 U DNaseI for 50 ml (20,000 U/ml, EUROMEDEX).
8. Glutathione Agarose 4B chromatography cartridge 5 ml (Protino, Macherey Nagel).
9. Wash buffer: 50 mM Tris–HCl, pH7.5, 1 mM EDTA, 300 mM NaCl.
10. Elution buffer: 50 mM Tris–HCl, 10 mM Glutathione reduced, pH7.5 (*see* **Note 6**).
11. Dialysis bags (12–14 kDa cutoff, 0.32 ml/cm; Spectrum Laboratories).
12. Centrifugal concentration devices (10 kDa cutoff, 2 ml; Sartorius).
13. Activation buffer: 10 mM Hepes.KOH, pH7.3, 1 mM DTT, 5 mM $MgCl_2$, 100 mM NaCl and 10% Glycerol.
14. Conservation buffer: 50 mM Tris–HCl, pH7.5, 150 mM NaCl, 0.1 mM EGTA, 20% Glycerol.
15. Emulsiflex-C3 homogenizer (Avestin).

2.4 Recombinant ERK2 Activation

1. GST-MEK1 R4F recombinant protein (Subheadings 3.5 and 3.6) or commercial activated-MEK1 (Millipore 14-429).
2. Recombinant His-ERK2 protein in activation buffer or conservation buffer (Subheadings 3.3 and 3.4).
3. Activation buffer: 10 mM Hepes.KOH, pH7.3, 1 mM DTT, 5 mM $MgCl_2$, 100 mM NaCl.
4. PD-10 G25 resin desalting column (GE-Healthcare).
5. Centrifugal concentration devices (10 kDa cutoff, 2 ml, Sartorius).
6. Glutathione Agarose 4B beads (Protino).

2.5 Phosphorylation of Tau by Activated ERK2

1. 1 mg of ^{15}N and 5 mg of ^{15}N, ^{13}C lyophilized recombinant full-length Tau (Subheadings 3.1 and 3.2).
2. Activated His-ERK2 in activation buffer (Subheading 3.7).
3. Phosphorylation buffer 5×: 250 mM Hepes.KOH, pH8.0, 62.5 mM $MgCl_2$, 5 mM EDTA, 250 mM NaCl.
4. Protease inhibitor 40× (1 tablet in 1 ml Phosphorylation buffer, EDTA-free; Roche).
5. PD MidiTrap G25 resin desalting column (GE-Healthcare).

6. Lyophilization buffer: 50 mM Ammonium Bicarbonate.
7. NMR buffer: 50 mM deuterated *d11*-Tris-HCl (Isotec), pH6.5, 30 mM NaCl, 2.5 mM EDTA.
8. 12% SDS Polyacrylamide precast gels (Pierce).
9. Lyophilizer.

2.6 Phosphorylation of Tau by Rat Brain Extract

1. 1 mg of ^{15}N-labeled recombinant full-length Tau per phosphorylation assay (Subheadings 3.1 and 3.2).
2. 100 μM okadaic acid (Sigma).
3. Homogenizing buffer H: 10 mM Tris–HCl pH7.4, 5 mM EGTA, 2 mM DTT, 1 μM okadaic acid (Sigma) supplemented with 20 μg/ml Leupeptin and 40 mM Pefabloc.
4. HMED buffer 5×: 200 mM Hepes.KOH, pH7.3, 10 mM $MgCl_2$, 25 mM EGTA, 10 mM DTT.
5. PD-10 G25 resin desalting column (GE-Healthcare).
6. Lyophilization buffer: 50 mM Ammonium Bicarbonate.
7. 12% SDS Polyacrylamide precast gels (Pierce).
8. NMR buffer: 50 mM deuterated *d11*-Tris–HCl (Isotec), pH6.5, 30 mM NaCl, 2.5 mM EDTA, 1 mM DTT, 5% D_2O, and 1 mM TMSP.
9. Lyophilizer.
10. Ultracentrifuge and type 50 Ti rotor (Beckman Coulter).

2.7 Preparation of Recombinant CBP

1. GST-CBP[1202-1848] recombinant T7 expression plasmid (pGEX-6P-1).
2. BL21(DE3) transformation competent *E. coli* bacteria (New England Biolabs).
3. Autoclaved LB Broth, Lennox (DIFCO).
4. 100 mg/ml stock solution of ampicillin antibiotics.
5. 1 M stock solution of IPTG.
6. PBS 10×: phosphate buffer saline.
7. Extraction buffer: PBS 1× pH7.6, 10% glycerol, 1% Triton X-100, 10 mM EDTA, 2 mM DTT, Protease inhibitor 1× (1 tablet for 50 ml; Roche), 2000 U DNaseI for 50 ml (20,000 U/ml, EUROMEDEX).
8. Glutathione sepharose resin beads (GE Healthcare): 20 μl of resin beads/mL of soluble extract.
9. Wash buffer: PBS 1× pH7.6, 10% glycerol, 1% Triton X100, 10 mM EDTA, 2 mM DTT.
10. Conservation buffer: 25 mM Hepes.KOH pH7.8, 0.1 mM EDTA, 1 mM THP (Tris (3-hydroxypropyl) phosphine), 50% glycerol (i.e., acetylation buffer with 50% glycerol).

11. 10% SDS Polyacrylamide gels.
12. Acetylation buffer: 25 mM Hepes.KOH pH7.8, 0.1 mM EDTA, 1 mM THP.
13. Stock solution of acetyl-coenzyme A (AcCoA) at 20 mM: 25 mg of AcCoA trisodium salt (FW 875; Sigma) are dissolved in 1.423 ml acetylation buffer and stored at −20 °C.
14. Stock solution of CBP peptide substrate (Ac-QPVEPKKPVES KKSGKSAKSKEKQ-NH_2, 8 TFA) at 10 mM: 18 mg of peptide was dissolved in 0.5 ml of deionized water. The pH was adjusted to 7.0 with a solution of NaOH 10 M.
15. C18 Zorbax 300SB analytical column 4.6 × 150 mm (Agilent).
16. 10% TFA (v/v).
17. Ultrasonic bath.
18. Buffers for reverse-phase chromatography: Buffer A (equilibration): 0.1% TFA (10 ml of the 10% TFA solution per liter), 2% acetonitrile in water; Buffer B (elution): 0.1% TFA: 80% acetonitrile in water; both buffers are degassed in an ultrasonic bath for 15 min at room temperature.
19. Emulsiflex-C3 homogenizer (Avestin).

2.8 Acetylation of Tau by CBP

1. Acetylation buffer: 25 mM Hepes.KOH pH7.8, 0.1 mM EDTA, 1 mM THP.
2. Stock solution of acetyl-coenzyme A (AcCoA) at 20 mM: 25 mg of AcCoA trisodium salt (Sigma) is dissolved in 1.423 ml acetylation buffer and stored at −20 °C.
3. Recombinant GST-CBP[1202-1848] fragment (Subheadings 3.11 and 3.12).
4. ^{15}N-labeled or ^{15}N-Lysine-labeled recombinant Tau protein (Subheadings 3.1 and 3.2).
5. C8 Zorbax 300SB semi-preparative column 9.4 × 250 mm (Agilent).
6. 10% TFA (v/v).
7. Ultrasonic bath.
8. Buffers for reverse-phase chromatography: Buffer A (equilibration): 0.1% TFA (10 ml of the 10% TFA solution per liter), 2% acetonitrile in water; Buffer B (elution): 0.1% TFA: 80% acetonitrile in water; both buffers are degassed in an ultrasonic bath for 15 min at room temperature.
9. HiPrep 26/10 desalting column (GE Healthcare).
10. Lyophilization buffer: 50 mM Ammonium Bicarbonate.
11. NMR buffer: 50 mM phosphate buffer pH6.4, 25 mM NaCl, 2.5 mM EDTA.
12. Lyophilizer.

3 Methods

3.1 Production of Recombinant ^{15}N-Tau, ^{15}N-Lysine,$^{13}C_u$-Tau or ^{15}N, ^{13}C-Tau

Bacterial fermentations have to be performed following the best practices of sterile manipulations.

3.1.1 Day 1: Bacterial Transformation

1. Transform Tau pET15b recombinant T7 expression plasmid (Novagen) into BL21(DE3) competent bacterial cells.
2. Mix 50 µl of competent BL21(DE3) bacteria with 100 ng of the plasmid DNA in a 1.5 ml plastic tube.
3. Incubate the mixture on ice for 30 min, followed by heat shock for 30 s at 42 °C. Place back the tube on ice for 5 min.
4. Add 1 ml of LB medium at room temperature and incubate the bacterial suspension at 37 °C for 30 min.
5. Spread 100 µl of the suspension at the surface of a LB plate containing 100 µg/ml of ampicillin antibiotics to select for the colonies having integrated the plasmid.
6. Incubate the plate overnight at 37 °C.

3.1.2 Day 2: Bacterial Growth

1. Start a small scale bacterial culture of 20 ml by using few colonies from the selection plate as inoculum.
2. Grow the Tau pET15b bacteria at 37 °C for 6 h in LB medium supplemented with 100 µg/ml of ampicillin, to reach saturation corresponding to an approximate OD600 of 4.0.
3. Meanwhile, reconstitute 1 L of M9 medium by the addition of the supplements and isotopes following one of the labeling schemes indicated in Subheading 2.1.
4. Start a large scale culture of 1 L by inoculating 20 ml of the LB saturated culture into 1 L of M9 medium supplemented with 100 µg/ml of ampicillin. Perform the culture in 2 L Erlenmeyer culture plastic baffled-flasks placed in an incubator programmed at 10 °C and 50 rpm for 14 h for the uniform ^{15}N or ^{15}N, ^{13}C-labeling or for 12 h for the ^{15}N-Lysine, ^{13}C-labeling considering that the growth phase will be slower due to the absence of complete medium for the later (*see* **Notes** 7 and **8**).
5. Program the incubator to switch to 200 rpm and 37 °C after this period.

3.1.3 Day 3: Induction Phase

1. Check the bacterial growth by measuring the OD at 600 nm of an aliquot of 1 ml of the culture.
2. For the uniform ^{15}N or ^{15}N, ^{13}C-labeling, induce the protein production by the addition of 400 µM IPTG when bacterial culture arrives at an OD600 of 0.8–1.0. Alternatively, for the ^{15}N-Lysine, ^{13}C-labeling, add 150 mg of $^{15}N_2/^{13}C_6$-L-Lysine hydrochloride when bacterial culture reaches an OD600 of

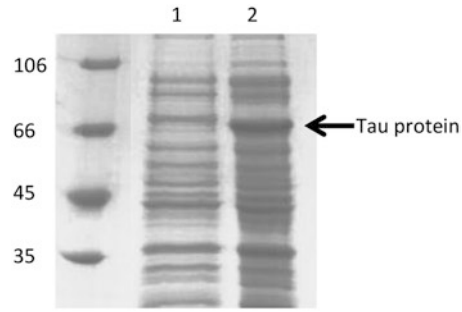


Fig. 1 Induction of recombinant Tau expression in BL21(DE3) bacteria. Lane 1: BL21(DE3) bacteria before the expression induction by IPTG. Lane 2: BL21(DE3) bacteria 4 h after induction. The band of recombinant Tau protein is shown by *black arrow* around 66 kDa

0.6–0.7, and continue the culture for 20 min. Then, induce the protein production by the addition of 400 μM IPTG.

3. Continue the bacterial culture for 4 h at 37 °C and 200 rpm.
4. Collect bacterial pellet by centrifugation for 20 min at 5500 × *g* in 1 L bottles.
5. Suspend the bacterial pellet in 50 ml of PBS buffer and centrifuge in 50 ml plastic tubes at 4000 × g.
6. Remove the supernatant solution and freeze the pellet at −20 °C until further use (Fig. 1).

3.2 Purification of ^{15}N-Tau, ^{15}N-Lysine,$^{13}C_u$-Tau or ^{15}N, ^{13}C-Tau

1. Unfreeze the bacterial pellet at room temperature and thoroughly suspend in 45 ml of extraction buffer.
2. Homogenize and lysate bacterial suspension with a high-pressure homogenizer.
3. Remove the insoluble material by centrifugation at 20,000 × *g* for 30 min.
4. Heat the bacterial protein extract for 15 min at 75 °C as a first purification step. The Tau protein is recovered in the soluble fraction after centrifugation at 15,000 × *g* for 30 min.
5. Perform purification of the ^{15}N-Tau, ^{15}N, ^{13}C-Tau, ^{15}N-Lysine,^{13}C-Tau protein by cation exchange chromatography on a 5 ml Hitrap SP sepharose FF column. After loading the sample on the column, wash the resin with CEX A buffer. Elute the protein by a three-step NaCl gradient with CEX B buffer. The first step corresponds to ten volumes of column to reach 250 mM NaCl, followed by a second step with five volumes of column to reach 500 mM NaCl, and the third step to two volumes of the column to reach 1 M NaCl (Fig. 2).

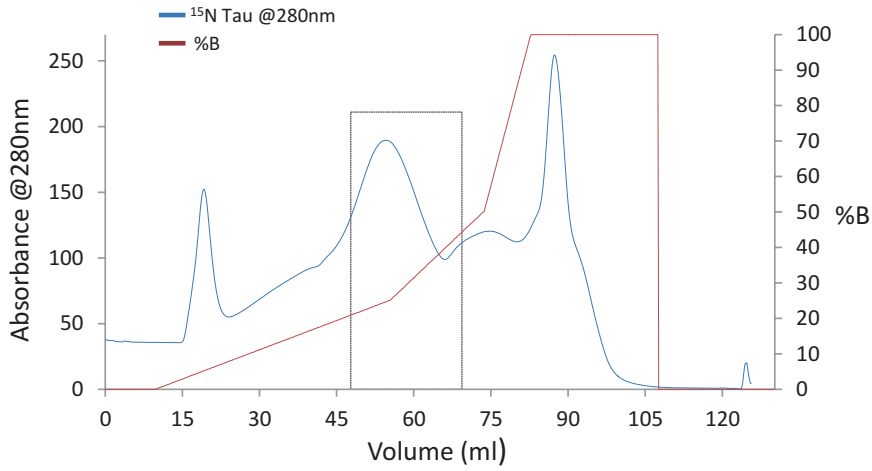


Fig. 2 Cation exchange chromatography purification step of the ^{15}N-Tau. The chromatogram shows the absorbance at 280 nm in milli-absorption units (graph in *blue*) and the percentage of CEX buffer B (graph in *marron*). The peak corresponding to ^{15}N-Tau is *boxed* in *black*

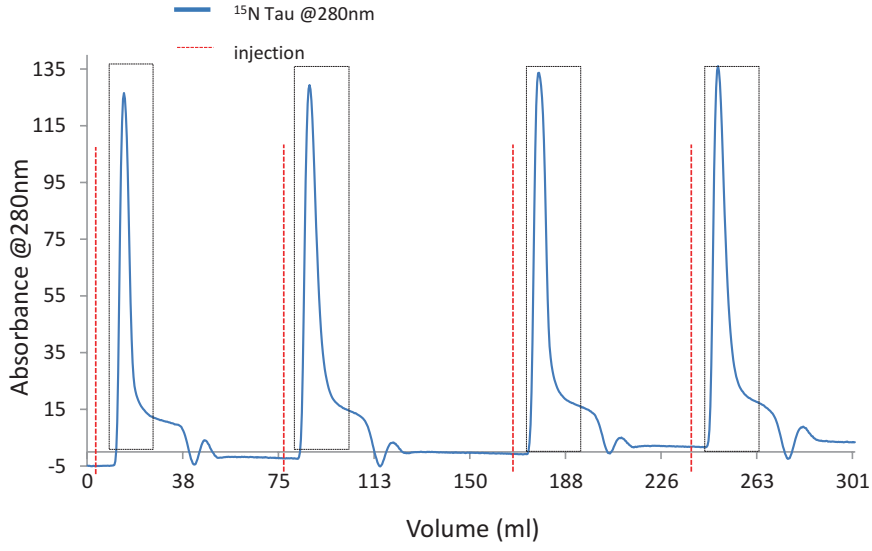


Fig. 3 Desalting of ^{15}N-Tau into ammonium bicarbonate buffer from the previous purification step of cation exchange chromatography (Fig. 2). The protein elution is monitored at 280 nm. Four injections of 5 ml ^{15}N-Tau are performed for one CEX chromatography. *Red lines* correspond to injections (5 ml loop). Collected ^{15}N-Tau fractions are shown in *black dotted box*

6. Transfer the pooled fractions from the chromatography purification step (corresponding to the box in Fig. 2, between 45 and 65 ml) on a 26/10 HiPrep Desalting column equilibrated in lyophilization buffer. Proteins elute in the void volume of about 12 ml. Collect the eluate and lyophilize the desalted fractions (*see* **Notes 9** and **10**). Injections of 5 ml are repeated four to five times, depending on the volume of the cation exchange pool (Fig. 3).

7. Suspend the lyophilized protein in a buffer suitable for further protein modification (phosphorylation or acetylation).

3.3 Production of Recombinant ERK2

Bacterial fermentations have to be performed following the best practices of sterile manipulations.

3.3.1 Day 1: Bacterial Transformation

1. Transform His6-tagged p42 MAP kinase from *Xenopus laevis* (His-ERK2) recombinant T7 expression plasmid into BL21(DE3) competent bacterial cells based on chemical transformation following the same procedure as described in Subheading 3.1.1.

3.3.2 Day 2: Bacterial Growth

1. Start a small scale bacterial culture of 10 ml with few colonies from the selection plate as inoculum.
2. Grow the His-ERK pET BL21(DE3) bacteria overnight in LB medium supplemented with 100 μg/ml of ampicillin to reach saturation corresponding to an approximate OD600 of 4.0.

3.3.3 Day 3: Induction Phase

1. Start a large scale culture of 1 L by inoculating 10 ml of the overnight culture into 1 L of LB medium supplemented with 100 μg/ml of ampicillin. Perform the culture in 2 L Erlenmeyer culture plastic baffled flasks placed in an incubator at 37 °C and shake at 200 rpm.
2. Check growth by measuring the OD at 600 nm of an aliquot of 1 ml of the culture.
3. When bacterial culture arrives at 0.8–1.0 of OD600, induce the protein production by the addition of 700 μM IPTG. Continue the bacterial culture during 4 h, at 30 °C and 200 rpm.
4. Collect bacterial pellet by centrifugation for 20 min at 5500 × *g* in 1 L bottles.
5. Suspend the bacterial pellet in 50 ml of PBS buffer and centrifuge in 50 ml plastic tubes at 4000 × *g*.
6. Remove the supernatant solution and freeze the pellet at −20 °C until further use.

3.4 Purification of Recombinant ERK2

1. Unfreeze the bacterial pellet at room temperature.
2. Suspend thoroughly in 45 ml extraction buffer.
3. Homogenize and lysate bacterial suspension with a high-pressure homogenizer.
4. Remove the insoluble material by centrifugation at 20,000 × *g* for 30 min.
5. Load the soluble bacterial lysate containing the His-ERK2 recombinant kinase (Fig. 4a) on a Ni-NTA resin equilibrated in wash buffer.
6. Wash the resin with wash buffer followed by 5 % elution buffer to remove residual lysate.

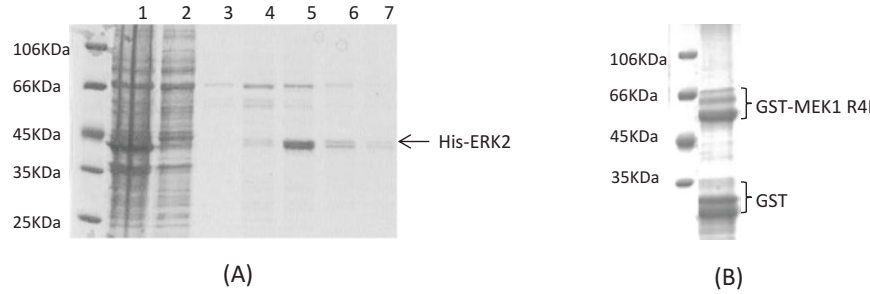


Fig. 4 (**a**) SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is run to monitor the preparation steps of His-ERK2. 5–10 μl of samples collected from the purification steps are run through 12% SDS-Polyacrylamide Gel. Line 1: bacterial lysate; Line 2: soluble extract; Lines 3 and 4: washing fractions collected with 5% elution buffer. Lines 5–7: purified sample fractions. Eluted His-ERK2 corresponds to the band appearing at 42 kDa in Line 5. A number of contaminating proteins are removed by washing the resin with 5% elution buffer (*see* Lines 3 and 4). (**b**) Analysis of purified GST-MEK1 R4F by SDS-PAGE. 5 μl collected from the elution step is run through 12% SDS-Polyacrylamide Gel. GST-MEK1 R4F fusion protein (*up*) and GST (*bottom*) are labeled. The GST tag is expected at 26 kDa and the GST-MEK1 R4F at 68 kDa. The multiple bands correspond to degradation products of the fusion protein

7. Elute His-ERK2 by equilibration of the resin into 100% elution buffer. Collect the eluted fractions by 1 ml aliquots which are analyzed by SDS-PAGE before pooling homogeneous fractions. His-ERK2 is obtained in about 5 ml of pooled fractions at 1.0–1.2 mg/ml concentration. The yield is approximately 5 mg of His-ERK2 per liter of LB broth.
8. Dialyze His-ERK2 against the activation buffer or the conservation buffer.
9. Concentrate His-ERK2 in the activation buffer up to 3 mg/ml using centrifugal devices. At this step, His-ERK2 (*see* **Note 11**) is ready for the activation step (Subheading 3.7). Freeze His-ERK2 in conservation buffer in liquid nitrogen and keep at −80 °C until further use.

3.5 Production of Recombinant GST-MEK1 R4F

Bacterial fermentations have to be performed following the best practices of sterile manipulations.

3.5.1 Day 1: Bacterial Transformation

1. Transform the pGEX plasmid containing the cDNA sequence of recombinant GST-MEK1 R4F MAPK kinase into BL21(DE3) competent bacterial cells based on chemical transformation following the same procedure as described in Subheading 3.1.1.

3.5.2 Day 2: Bacterial Growth

1. Start a small scale bacterial culture of 20 ml with few colonies from the selection plate as inoculum in LB medium supplemented with 100 μg/ml of ampicillin to reach saturation corresponding to an approximate OD600 of 4.0.

3.5.3 Day 3: Induction Phase

1. Start 2 L of BL21(DE3) bacterial culture in LB medium supplemented by 100 μg/ml ampicillin antibiotics by adding the 20 ml overnight saturated bacterial culture to the fresh medium. Perform the culture in a 2 L Erlenmeyer culture plastic baffled flask placed in an incubator at 37 °C and shake at 200 rpm.
2. Check growth by measuring the OD at 600 nm of an aliquot of 1 ml of the culture.
3. When bacterial culture arrives at 0.8–1.0 of OD600, induce the protein production by the addition of 700 μM IPTG.
4. Continue the bacterial culture during 4 h at 30 °C and 200 rpm.
5. Collect bacterial pellet by centrifugation for 20 min at 5500 × *g* in 1 L bottles.
6. Suspend the bacterial pellet in 50 ml of PBS buffer and centrifuge in 50 ml plastic tubes at 4000 × *g*.
7. Remove the supernatant solution and freeze the pellet at −20 °C until further use.

3.6 Purification of Recombinant GST-MEK1 R4F

1. Unfreeze the bacterial pellets from 2 L bacterial culture at room temperature and then suspend bacteria thoroughly in extraction buffer.
2. Homogenize and lysate the suspension using a high-pressure homogenizer.
3. Remove the insoluble material by centrifugation at 20,000 × *g* for 30 min.
4. Load the soluble bacterial lysate containing the recombinant GST-MEK1 R4F kinase on a Glutathione Agarose 4B chromatography column equilibrated with wash buffer at a slow flow rate of 0.3–0.5 ml/min.
5. Rinse the loaded resin with wash buffer until the baseline at 280 nm is stabilized.
6. Collect the GST-MEK1 R4F protein into elution buffer by 1 ml aliquots.
7. Analyze aliquots by SDS-PAGE to choose the fractions to pool. The pooled fractions (Fig. 4b) correspond to about 6 ml at 1 mg/ml.
8. Dialyze GST-MEK1 R4F protein against the activation buffer or the conservation buffer.
9. Concentrate GST-MEK1 R4F protein in activation buffer up to 3 mg/ml using centrifugal devices to be used in His-ERK2 activation step (*see* Subheading 3.7).
10. Freeze dialyzed GST-MEK1 R4F in conservation buffer in liquid nitrogen and keep at −80 °C until further use.

3.7 Activation of Recombinant ERK2

1. Mix 1 ml of His-ERK (3 mg/ml, Subheading 3.4) with 1 ml of GST-MEK1 R4F (3 mg/ml, Subheading 3.6) in activation buffer in the presence of 5 mM ATP and protease inhibitors cocktail 1× in a 5 ml reaction volume, in 15 ml plastic tubes [20].
2. Perform the activation at 30 °C during 15 h.
3. If His-ERK or GST-MEK1 R4F is frozen in conservation buffer, dialyze the kinase solution against activation buffer. Concentrate the kinases with a centrifugal device for further reaction if needed.
4. Remove 5 µl from the reaction mixture at various time points for SDS-PAGE analysis to control the progress of the reaction. A slight delay of His-ERK2 migration is observed from 30 min of incubation. The amount of soluble His-ERK2 is decreasing during the incubation due to protein precipitation (Fig. 5a). Remove the insoluble material by centrifugation.
5. Remove GST-MEK1 R4F from the reaction mixture by using 500 µl Glutathione Agarose 4B beads equilibrated in activation buffer. Incubate the beads on a roller mixer with slow agitation (rotation of 20 rpm/min) for 1 h at 4 °C with the activation mix and finally eliminate resin beads by centrifugation at 1000×*g* for 10 min. The final activated His-ERK is obtained at 0.1–0.15 mg/ml (2.5–4.0 µM) in 5 ml of reaction volume.
6. Add 10% Glycerol to the activated His-ERK2 in activation buffer.
7. Freeze aliquots of 100 µl in liquid nitrogen and conserve at −80 °C until further use.

3.8 Phosphorylation of Tau Protein by ERK2 Recombinant Kinase

1. Mix 100 µM recombinant ^{15}N-Tau protein (1 mg lyophilized protein, Subheading 3.2) with 50 µl activated ERK2 (4 µM in activation buffer, Subheading 3.7, final concentration 1 µM, *see* **Note 12**) in the presence of 2.5 mM ATP, 1 mM DTT, and

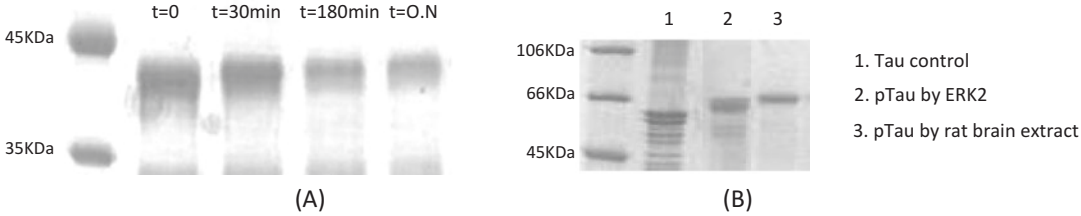


Fig. 5 (**a**) Time course of His-ERK2 *in vitro* activation by GST-MEK1R4F. 5 µl of samples is removed from the reaction mix at initial time, 30 min, 180 min, and at 16 h (*overnight*) incubation time. From 30 min of incubation, a slight migration delay of the band corresponding to His-ERK2 is observed compared to that at the initial time. The band upper shift increases until the end of reaction. The amount of soluble His-ERK2 decreases with the incubation time due to precipitation. (**b**) SDS-PAGE analysis of Tau phosphorylation. About 1.2 µg samples is loaded in 12% acrylamide gel. Compared to Tau control (*see* line 1), ERK2-phosphorylated Tau has a lower mobility resulting in a shift of the band from 52 kDa to 60 kDa (line 2) and phosphorylated Tau by rat brain extract a shift to 66 kDa (line 3)

1 mM EGTA complemented with protease inhibitors in a total sample volume of 200 µl in the phosphorylation buffer 1×.

2. Incubate the sample at 37 °C for 3 h. For the ^{15}N, ^{13}C-Tau protein, the reaction is scaled up to 5 mg lyophilized protein in 1 ml reaction mix.
3. After incubation, heat the samples at 75 °C for 15 min and centrifuge at 20,000 × *g* for 15 min to remove the ERK kinase.
4. Desalt the phosphorylated ^{15}N-Tau or ^{15}N, ^{13}C-Tau (Fig. 5b) into NMR buffer and conserve at −20 °C.
5. For NMR spectroscopy analysis, complement the samples with 1 mM trimethylsilyl propionate (TMSP, which serves as internal reference), 2 mM DTT, and 10% D_2O for the purpose of NMR spectrometer field locking.

3.9 Preparation of Rat Brain Extract

1. Wash the rat brain (about 1.8 g) immediately after collection in an ice-cold homogenizing buffer.
2. Cut the brain crudely into pieces with dissection scissors and place in a homogenizing buffer (2.5 ml/g of brain).
3. Prepare the rat brain extract by homogenizing the brain using a Potter pestle fitted on a tissue grinder and a 10 ml glass tube.
4. Perform ultracentrifugation at 100,000 × *g* for 1 h in a type 50 Ti rotor. The supernatant corresponds to the rat brain extract (*see* **Note 13**).

3.10 Phosphorylation of Tau Protein with Rat Brain Extract

1. Mix 4 µM recombinant ^{15}N-labeled Tau protein (around 1 mg protein in 5 ml of total volume) with 500 µl fresh rat brain extract in a final 5 ml reaction volume of HMED 1× buffer. Add 1 µM okadaic acid (phosphatase inhibitor), 2 mM ATP, and protease inhibitors 1×.
2. Incubate the phosphorylation mix for 24 h at 37 °C.
3. Inactivate enzymes in the sample by heating at 75 °C for 15 min.
4. Centrifuge the sample at 16,000 × *g* for 15 min to remove out precipitated proteins.
5. Perform buffer exchange into the lyophilization buffer using a PD-10 column (G25 resin). Load 2.5 ml of the inactivated reaction mix on the column. Repeat operation to desalt the total reaction volume. The desalted sample is obtained in a final volume of 7 ml.
6. Lyophilize the phosphorylated Tau (Fig. 5b) and conserve at −20 °C.
7. To analyze by NMR spectroscopy, dissolve the lyophilized sample in 200 µl NMR buffer complemented with 2 mM DTT, 1 mM TMSP, and 10% D_2O.

3.11 Production of Recombinant GST-CBP Acetyltransferase

Bacterial fermentations have to be performed following the best practices of sterile manipulations.

3.11.1 Day 1: Bacterial Transformation

1. Transform the pGEX plasmid containing the cDNA sequence of recombinant CBP[1202-1848] fragment of mouse CBP acetyltransferase into BL21(DE3) competent bacterial cells based on chemical transformation following the same procedure as described in Subheading 3.1.1 except that plates used for colony selection are additionally supplemented with 1% glucose.

3.11.2 Day 2: Bacterial Growth

1. Start a small scale bacterial culture of 20 ml with few colonies from the selection plate as inoculum in LB medium supplemented with 1% glucose and 100 μg/ml of ampicillin to reach saturation corresponding to an approximate OD600 of 4.0.

3.11.3 Day 3: Induction Phase

1. Centrifuge the bacterial culture at 4000 × *g* for 15 min and discard supernatant.
2. Start 2 L of BL21(DE3) bacterial culture in LB medium supplemented by 1% glucose and 100 μg/ml ampicillin antibiotics by suspending the bacterial pellet from the 20 ml culture to the fresh medium. Perform the culture in 2 L Erlenmeyer culture plastic baffled flask placed in an incubator at 37 °C and shake at 200 rpm.
3. Check growth by measuring the OD at 600 nm of an aliquot of 1 ml of the culture.
4. When bacterial culture arrives at 0.8–1.0 of OD600, induce the protein production by the addition of 200 μM IPTG.
5. Continue the bacterial culture for 4 h at 18 °C and 200 rpm.
6. Collect bacterial pellet by centrifugation for 20 min at 6000 × *g* in 1 L-bottles.
7. Suspend the bacterial pellet in 100 ml of PBS buffer, aliquot into four 50 ml-plastic tubes, and centrifuge at 4000 × *g*.
8. Remove the supernatant and freeze the pellets at −20 °C until further use. For prolonged storage (superior to 6 months), freeze the pellet at −80 °C.

3.12 Purification of Recombinant GST-CBP Acetyltransferase

1. Unfreeze one bacterial pellet on ice (equivalent to a 0.5 L bacterial culture), then suspend thoroughly in a 20 ml extraction buffer.
2. Homogenize and lysate the suspension using a high-pressure homogenizer.
3. Remove the insoluble material by centrifugation at 25,000 × *g* for 30 min at 4 °C.

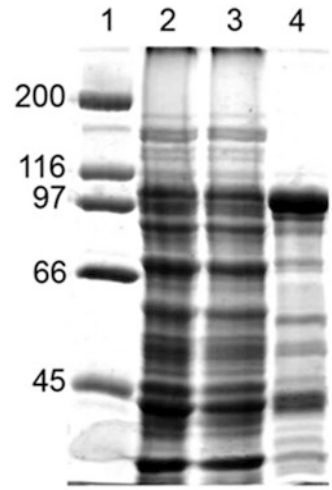


Fig. 6 SDS-10 % PAGE of the purification of GST-CBP[1202-1848] acetyltransferase on glutathione sepharose beads. Lane 1: molecular weight markers; lane 2: 5 µl of the soluble bacterial extract; lane 3: 7.5 µl of the pooled flow through and first wash supernatant; lane 4: 15 µl of the final glutathione beads slurry

4. Incubate the soluble bacterial lysate containing the recombinant GST-CBP[1202-1848] acetyltransferase (Fig. 6) for 3 h at 4 °C on a roller mixer with Glutathione Sepharose resin beads pre-equilibrated in the extraction buffer.
5. Rinse the loaded resin five times with 12 ml of wash buffer then five times with 12 ml of conservation buffer. The beads are kept as 50 % slurry in conservation buffer at –20 °C until further use (*see* **Note 14**). An aliquot of 15 µl is analyzed by SDS-PAGE.

3.13 Standard Analysis of CBP Acetyltransferase Activity on a Peptide Substrate

A peptide from the human Thymine DNA Glycosylase (TDG) is used as a substrate to check the activity of every new batch of CBP enzyme. The peptide sequence is Ac-QPVEPKKPVESKKSGKSA KSKEKQ-NH_2 (Ac and NH_2 mean that the N- and C-terminus are acetylated and amidated, respectively). The underlined residues in the peptide sequence correspond to three acetylation sites that have been previously identified [21].

1. For the acetylation reaction, mix 2.5 µl of the peptide solution at 10 mM (final concentration of 100 µM) with 25 µl of the stock solution of AcCoA at 20 mM (final concentration of 2 mM) or 25 µl of acetylation buffer as a control in 212.5 µl of acetylation buffer. Add 10 µl of the slurry of GST-CBP on glutathione beads (for a final volume of 250 µl).
2. Incubate the reactions overnight either at 20 °C or at 30 °C under rotative agitation.

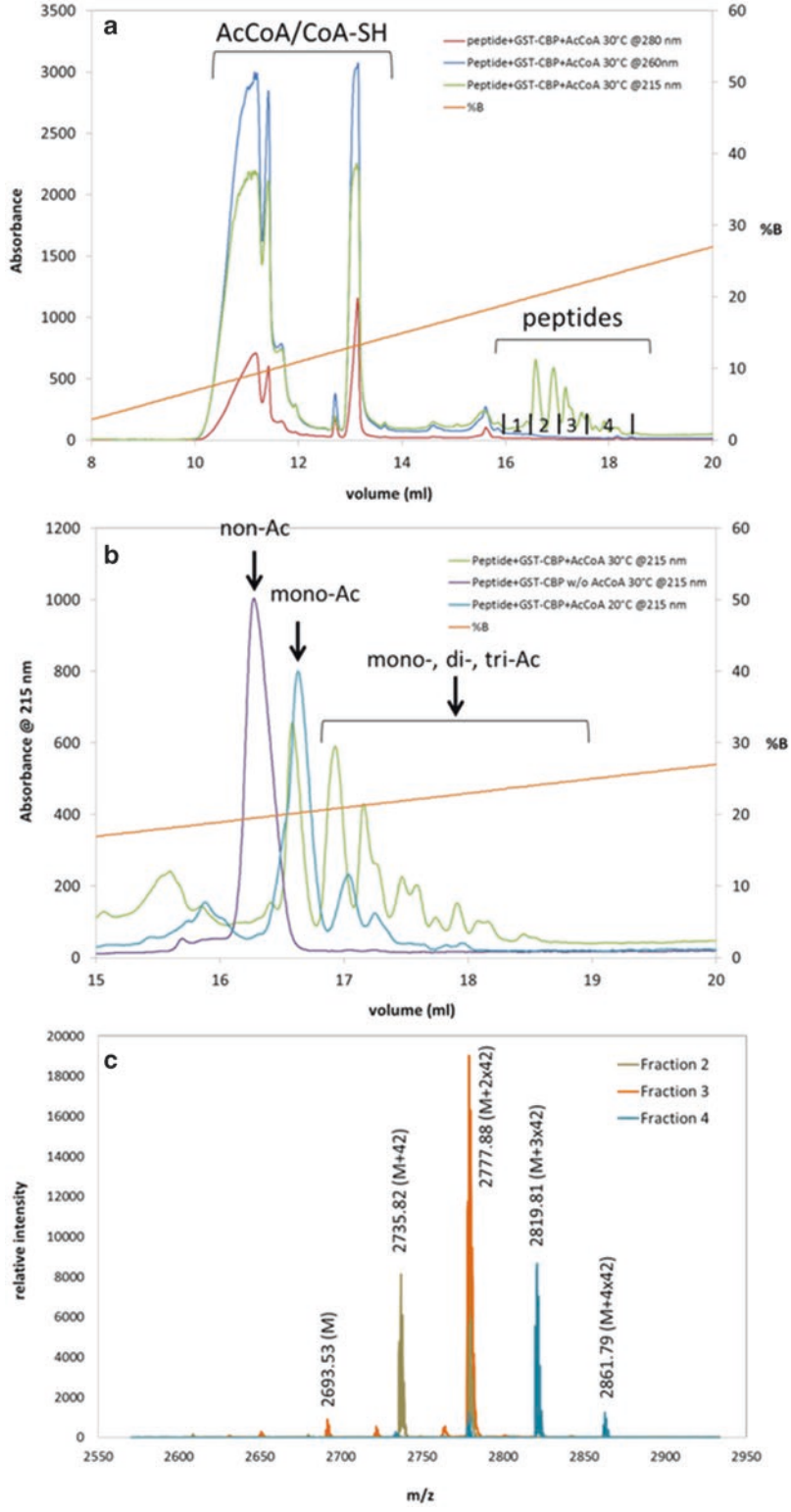
3. Centrifuge the reaction mixtures at 16,000 × *g* for 10 min at 4 °C and analyze each supernatant by reverse-phase chromatography on a C18 column at room temperature. Equilibrate the column at 1 ml/min in buffer A until the absorbance and conductivity are stable. Inject the total reaction volume (250 μl) in a 1 ml injection loop for 4 ml. Then, wash the column at 1 ml/min with 2.5 ml of Buffer A. Elute peptides with a linear gradient of acetonitrile from 0% to 60% buffer B in 30 min at 1 ml/min (Fig. 7).
4. Collect fractions of 0.5 ml for MALDI-TOF mass spectrometry analysis. Mass increments of +42 Da ($[M+H]^+$ 2735.82 Da), +84 Da ($[M+H]^+$ 2777.88 Da), and +126 Da ($[M+H]^+$ 2819.81 Da) as compared to the mass of the unmodified peptide ($[M+H]^+$ 2693.53 Da) confirm the incorporation of one, two, or three acetyl moieties, respectively (Fig. 7, *see* **Note 15**).

3.14 Acetylation of Tau Protein by CBP

Perform acetylation of ^{15}N- or ^{15}N-Lysine,^{13}C-labeled Tau protein with minor modifications of the conditions described for the TDG peptide (Subheading 3.13).

1. Weigh 17.6 mg AcCoA and dissolve in 5 ml acetylation buffer to obtain a final concentration of 4 mM.
2. Filter the solution on a 0.22 μ-filter; this solution is sufficient for four acetylation reactions.
3. Dissolve 5 mg of Tau protein in 1.09 ml of AcCoA-containing acetylation buffer (final concentration of 100 μM). For the negative control, omit AcCoA and dissolve directly Tau protein in acetylation buffer. Add 100 μl of GST-CBP glutathione beads slurry per ml of reaction volume.
4. Incubate the acetylation reaction at 25 °C overnight (16 h) under rotative agitation.
5. Centrifuge the reaction mixture at 4000 × *g* for 5 min at 4 °C.

Fig. 7 Reverse-phase analysis on a C18 column of the acetylation reactions on a peptide substrate with recombinant GST-CBP[1202-1848]. Due to the presence of at least three acetylation sites, a complex pattern of elution was detected at 215 nm at both 20 °C and 30 °C. (**a**) Absorbance at 280 nm (*red*), 260 nm (*blue*), and 215 nm (*green*) of the acetylation reaction performed with 2 mM AcCoA at 30 °C overnight. (**b**) Comparison of the absorbance detected at 215 nm of the acetylation reactions performed with (*green*) or without AcCoA (*violet*) at 30 °C overnight or with AcCoA at 20 °C overnight (*blue*). The linear gradient of buffer B (%B) is indicated by *orange line*. (**c**) MALDI-TOF mass spectra of the fractions 2, 3, and 4 of the analytical C18 chromatography (*see* the fractions on panel **a**); *m*/*z* values of $[M+H]^+$ species are annotated. The "M" character indicates the mass of the unmodified peptide


a
AcCoA/CoA-SH
peptide+GST-CBP+AcCoA 30°C @280 nm
Peptide+GST-CBP+AcCoA 30°C @260nm
Peptide+GST-CBP+AcCoA 30°C @215 nm
%B
peptides
1 2 3 4
Absorbance
%B
volume (ml)
b
non-Ac
mono-Ac
mono-, di-, tri-Ac
Peptide+GST-CBP+AcCoA 30°C @215 nm
Peptide+GST-CBP w/o AcCoA 30°C @215 nm
Peptide+GST-CBP+AcCoA 20°C @215 nm
%B
Absorbance @ 215 nm
%B
volume (ml)
c
Fraction 2
Fraction 3
Fraction 4
2693.53 (M)
2735.82 (M+42)
2777.88 (M+2x42)
2819.81 (M+3x42)
2861.79 (M+4x42)
relative intensity
m/z

6. Withdraw the supernatant cautiously and wash resin beads twice with 0.2 ml acetylation buffer.
7. Pool the supernatants and heat at 75 °C for 10 min followed by a centrifugation at 4000 × *g* for 15 min at 4 °C to remove potential contaminant proteins that might have leaked from glutathione beads.
8. Purify the acetylation and control reactions by reverse-phase chromatography on a C8 semi-preparative column at room temperature. Equilibrate the column at 4 ml/min in 20% buffer B until the absorbance and conductivity are stable. Inject the protein solution (1.5 ml) in a 2 ml injection loop for 20 ml, elute proteins with a linear gradient of acetonitrile from 20% to 60% buffer B in 24 min at 4 ml/min (Fig. 8). Collect 4 ml-fractions.
9. Lyophilize the collected fractions and dissolve in 200 µl of lyophilization buffer for SDS PAGE analysis on 10% acrylamide gels.
10. Desalt homogeneous fractions containing a high concentration of full-length protein in lyophilization buffer on a HiPrep 26/10 desalting column (Fig. 9), as described in Subheading 3.2, **step 6**.
11. Lyophilize the protein fraction and store at −20 °C until further use.

3.15 Assignment of Resonances of the Phosphorylated Residues: Identification of the Phosphorylation Pattern

A [^{1}H,^{15}N] HSQC 2D spectrum is recorded to detect the resonances of the phosphorylated S and T residues (*see* **Note 16**), because they are easily visualized, located around 8.5–9.5 ppm of amide proton and 117–125 ppm of ^{15}N-amide, outside the bulk of the ^{1}H, ^{15}N correlations (Fig. 10). As shown in Fig. 11, phospho-residue resonance assignment is based on [^{1}H,^{15}N,^{13}C] HNCACB 3D experiment that is recorded to link the resonances of the phosphorylated residues observed in the 2D [^{1}H,^{15}N] HSQC spectrum to a specific pS or pT residue in the Tau sequence. CA and CB chemical shift values of pS and pT are typical [22] and allow the identification of the nature of the phosphorylated i residue. pS and pT followed by a proline residue also in addition have a typical +2 ppm shift of the CA chemical shift value [23]. The nature of the residue at the i-1 position is defined by the chemical shift values of the CA and CB resonances corresponding to the i-1 residue (weaker set of signals compared to those of the i residue). A few identical phosphorylated patterns possess the same residue at position i-1 and cannot be uniquely identified from the HNCACB experiment alone, as is the case for K-pT175-P and K-pT181-P. Another 3D experiment lifts the redundancy by providing, in addition, the chemical shift value of ^{15}N of the i-1 residue. For example, the resonances corresponding to pT175 and pT181 can be successfully assigned due to the

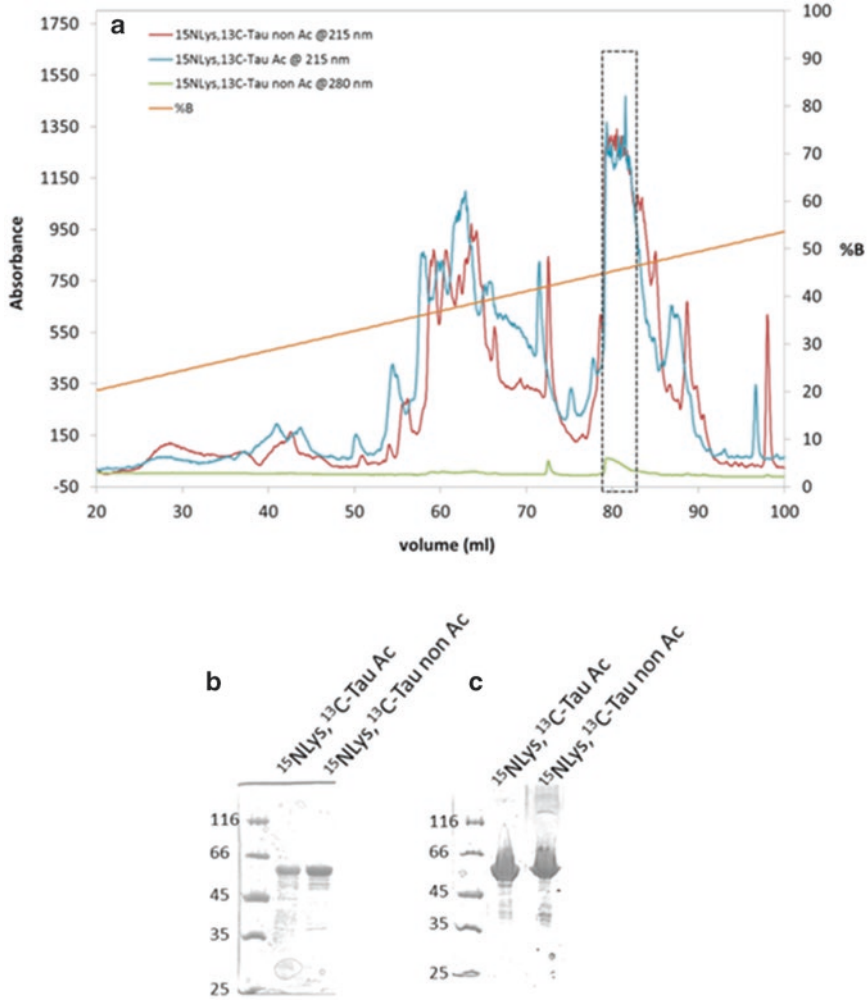


Fig. 8 Reverse-phase purification on a C8 column of the ^{15}N-Lys,^{13}C-labeled Tau protein before and after acetylation reaction with recombinant GST-CBP[1202-1848]. (**a**) Chromatogram of the purification of control and acetylated ^{15}N-Lys,^{13}C-labeled-Tau on a C8 column with a linear gradient of acetonitrile (*orange line*) as monitored by the absorbance at 215 nm for control ^{15}N-Lys,^{13}C-Tau (*red curve*) and acetylated ^{15}N-Lys,^{13}C-Tau (*blue curve*) and at 280 nm (*green curve*, control ^{15}N-Lys,^{13}C-Tau). (**b**, **c**) 12 % acrylamide SDS gel electrophoresis of the ^{15}N-Lys,^{13}C-Tau protein before and after the acetylation reaction (**b**) Fractions of non-acetylated and acetylated ^{15}N-Lys,^{13}C-Tau proteins purified by cation exchange chromatography. 2 μl of a 50 μM solution of protein are loaded on the gel. (**c**) Fractions of non-acetylated and acetylated ^{15}N-Lys,^{13}C-Tau proteins purified by reverse-phase chromatography. Only the fractions corresponding to the boxed region on the chromatograms in (**a**) are shown. 2.5 μl of the lyophilized fractions dissolved in 200 μl water is loaded on the gel

different values of ^{15}N chemical shift observed for A-K174 and L-K180 (Figs. 11 and 12).

1. Dissolve 0.5–1 mg of ^{15}N-Tau in 200 μl NMR buffer (50–100 μM) to fill a 3 mm tube.

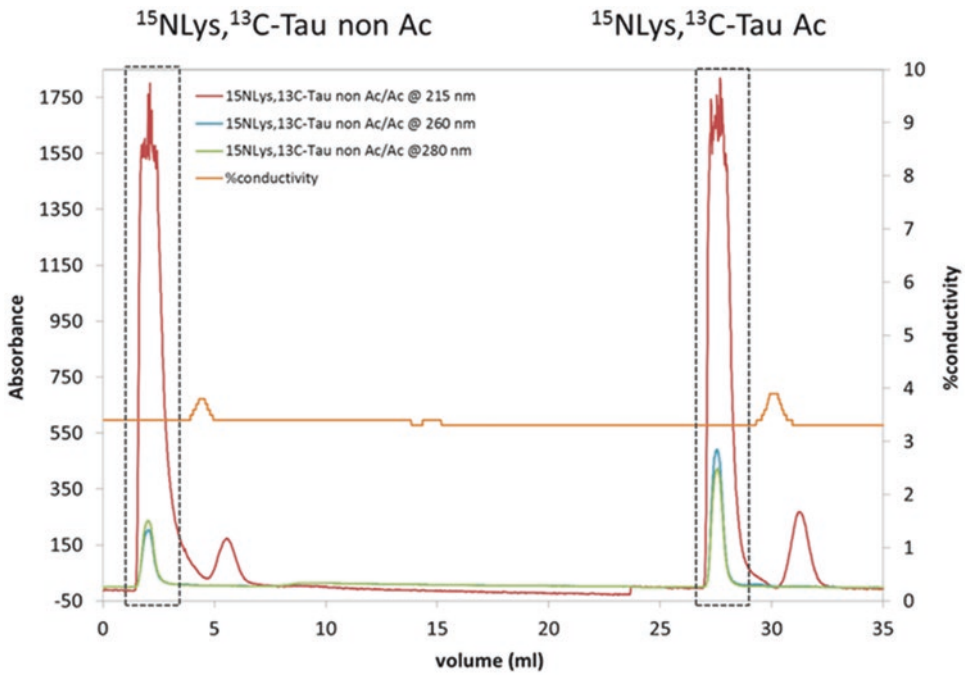


Fig. 9 Chromatogram of desalting in 50 mM ammonium bicarbonate of the ^{15}N-lysine,^{13}C-Tau protein in its non-acetylated and acetylated forms from fractions of the purification step by C8 reverse-phase chromatography

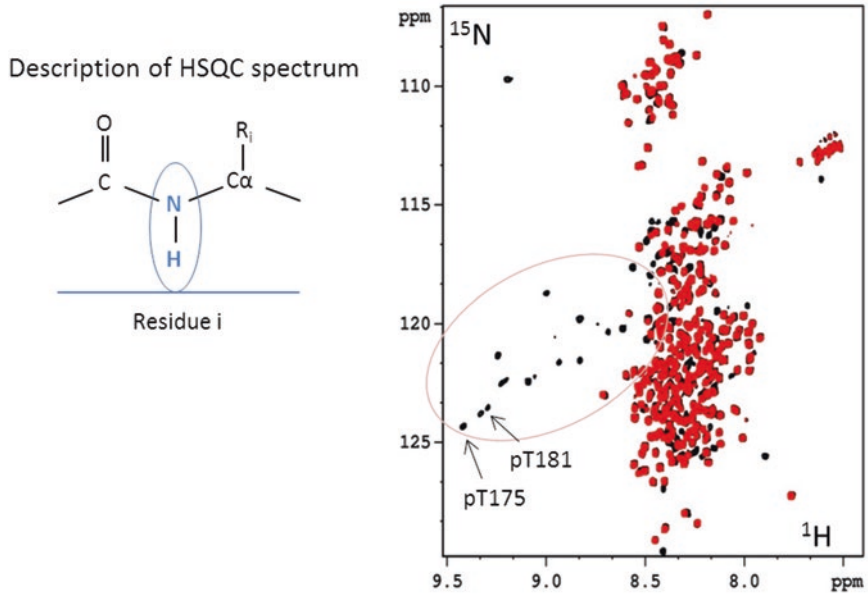


Fig. 10 Overlaid 2D [^{1}H,^{15}N] HSQC spectra of ^{15}N phosphorylated Tau in *black* with ^{15}N-Tau in *red*. HSQC spectrum records the J-coupling (through-bound magnetization transfer) correlation between ^{1}H and ^{15}N-nuclei. Each resonance thus corresponds to an amino acid of the protein backbone. The encircled multiple additional resonances in the black spectrum, compared to the red control spectrum of ^{15}N-Tau, correspond to phosphorylated residues of the Tau protein according to previous NMR studies of Tau protein [8] review in [11]

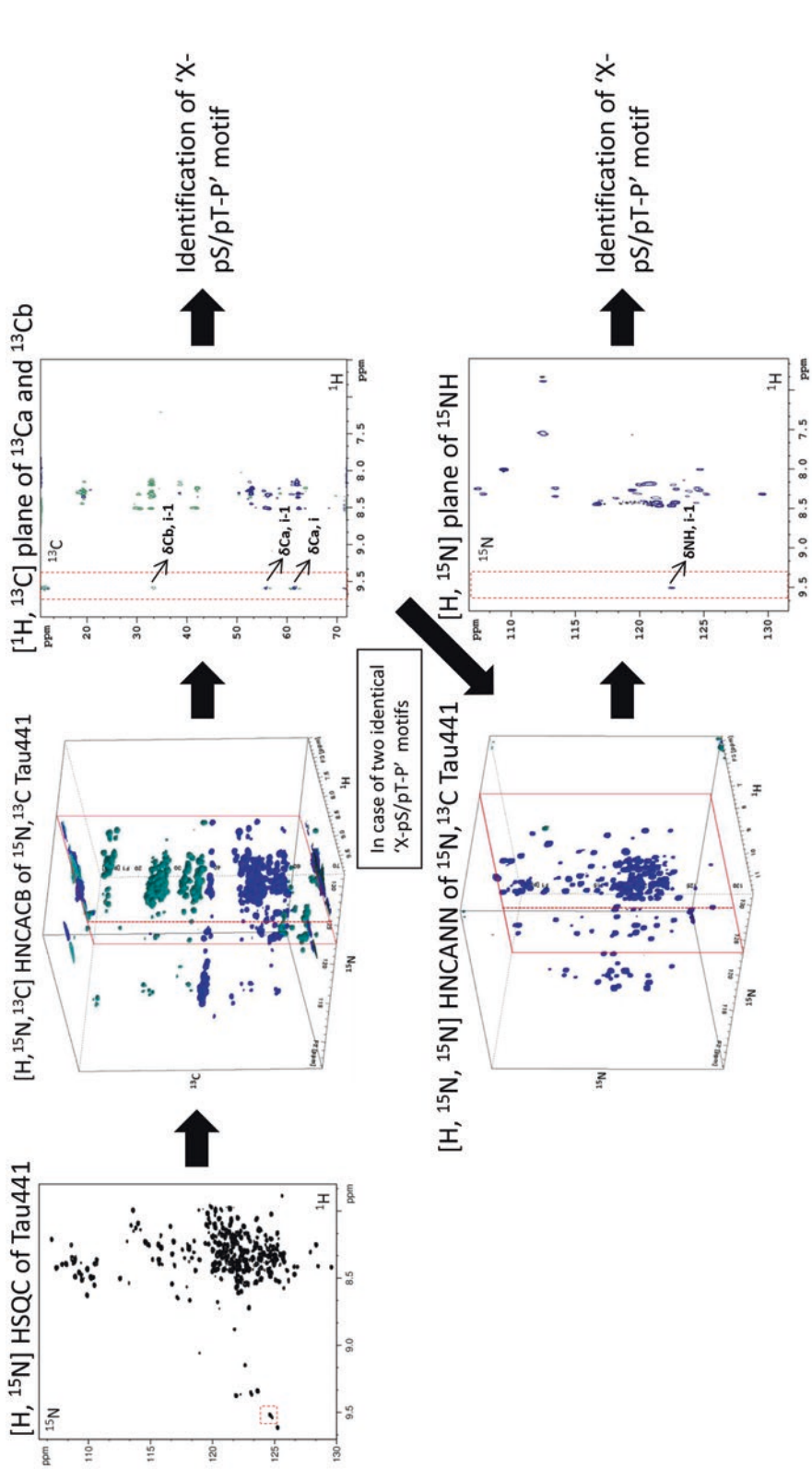


Fig. 11 Strategy of identification of X-pS/pT-P motifs in Tau protein. Chemical shifts of CA and CB of i and i-1 residues in ['H, ^{15}N, ^{13}C] HNCACB 3D experiment and chemical shift of ^{15}N of the i-1 residues in ['H, ^{15}N, ^{15}N] HNCANN 3D experiment are used to assign a resonance in the ['H, ^{15}N] 2D spectrum to a specific pS-P or pT-P motif in Tau sequence

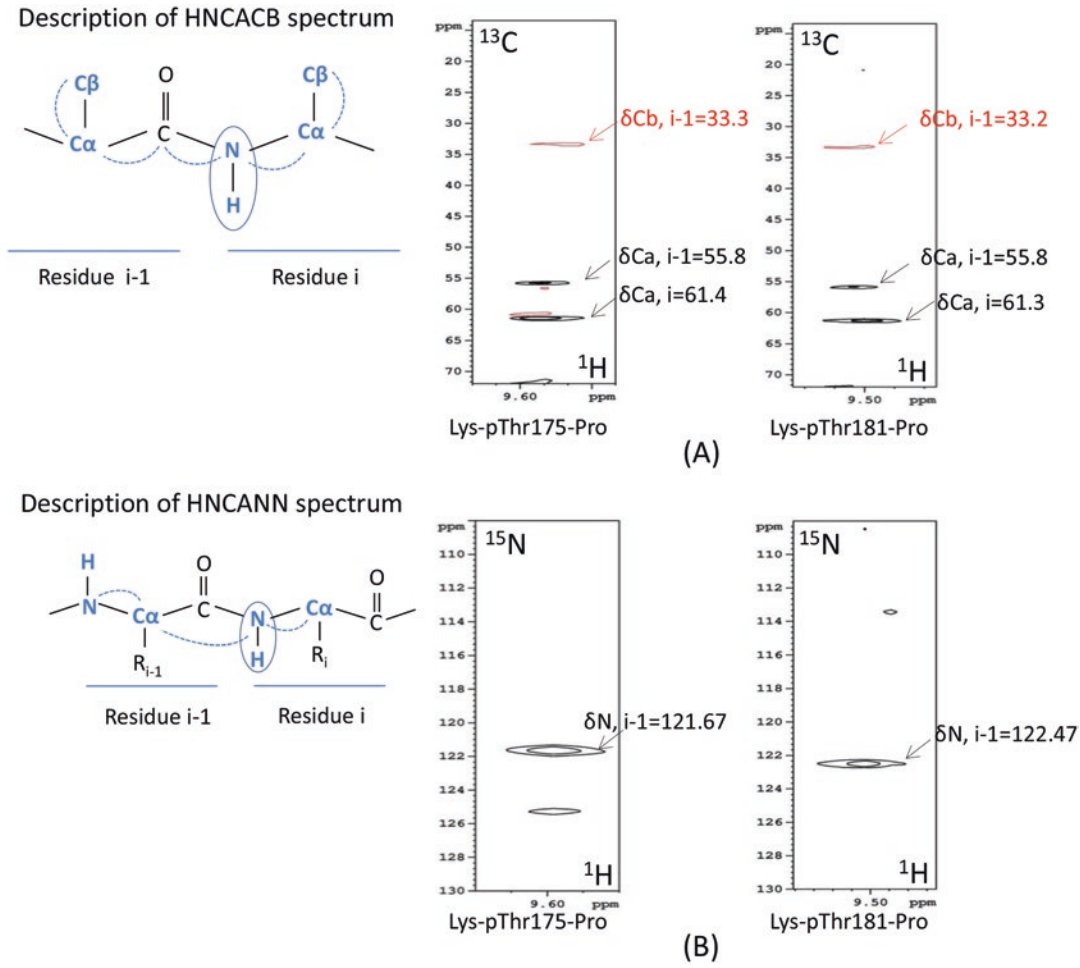


Fig. 12 (**a**) [^{1}H, ^{13}C] 2D planes extracted from the [^{1}H,^{15}N,^{13}C] HNCACB 3D spectrum, at ^{15}N-values of 125.24 ppm (*left*) and 124.76 ppm (*right*). The ^{13}C-CA resonances (in *black*) in side chain of pT175 (*left panel*) and pT181 (*right panel*) are indicated. ^{13}C-CB resonances for these residues are outside the spectrum window (74 ppm). This combination of CA and CB chemical shift values is typical of a pT-P dipeptide. The weaker set of signals (indicated by *arrows*) corresponds to ^{13}C-CA (in *black*) and ^{13}C-CB resonances (in *red*) of the prior residue. Because for both pT-P dipeptide the i-1 residue shows chemical shift values typical of a lysine residue (CA is 55.8 ppm, CB is 33.3 ppm), these resonances cannot be uniquely identified among the 9 pT-P motifs of Tau. (**b**) [^{1}H,^{15}N] 2D planes extracted from the [^{1}H,^{15}N,^{15}N] HNCANN 3D spectrum, at ^{15}N-values of 125.24 ppm (*left*) and 124.76 ppm (*right*). The ^{15}N-resonance in the third dimension represents the chemical shifts of the ^{15}N of the amide group of the K residue at position i-1. From the previously determined assignment of Tau [26–28], we could distinguish δN(i-1)K174 = 121.67 ppm and δN(i-1)K180 = 122.47 ppm, which allow discriminating the resonance of pT175 of pT181

2. Record a [^{1}H,^{15}N] HSQC 2D spectrum (hsqcetfpf3gpsi pulse sequence from Bruker, *see* **Note 17**) [24] (Fig. 10) at 293 K or 298 K on a Bruker900MHz Avance III NMR spectrometer equipped with a triple resonance cryogenic probehead (Bruker, Karlsruhe, Germany) or equivalent spectrometer,

with 3072 and 416 points for 14 and 25 ppm in the ^{1}H and ^{15}N dimensions, respectively. The relaxation delay is 1 s. The duration of the acquisition with 64 scans is 9 h.

3. Process the spectra with the Bruker TopSpin 3.1 software.
4. Dissolve 4 mg of ^{15}N, ^{13}C-Tau in 400 µl NMR buffer (200 µM) to fill a NMR Shigemi tube.
5. Record a [^{1}H,^{15}N,^{13}C] HNCACB 3D spectrum (pulse sequence hncacbgpwg3d from Bruker, *see* **Note 18**) at 298 K on a Bruker900MHz Avance III NMR spectrometer equipped with a triple resonance cryogenic probehead (Bruker, Karlsruhe, Germany) or equivalent spectrometer, with 2048, 256, 72 points for 14, 25, 61 ppm centered on 4.7, 119, 41 ppm in the ^{1}H, ^{15}N, and ^{13}C dimensions, respectively. The relaxation delay is 1 s. The duration of the acquisition with 16 scans is 4 days and 6 h. This experiment allows for recording the chemical shift values of CA and CB ^{13}C nuclei of the i and i-1 residues for each [^{1}H, ^{15}N] resonance.
6. Process the spectra with the Bruker TopSpin 3.1 software.
7. Extract [^{1}H,^{13}C] 2D planes from the [^{1}H,^{15}N,^{13}C] HNCACB 3D experiment (Fig. 12a) for each peak in the [^{1}H,^{15}N] HSQC. Make data analysis, peak picking and calculation of peak volumes with Sparky 3.114 software (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).
8. Record an additional [^{1}H,^{15}N,^{15}N] HNCANN 3D experiment (hncannhgpwg3d pulse sequence from Bruker, *see* **Note 19**) [25] to distinguish redundant phosphorylation patterns. Perform data acquisition on the same ^{15}N, ^{13}C-Tau sample in the same conditions as described in Subheading 3.15, **step 5** with 3072, 100, 100 points for 14, 21, 21 ppm centered on 4.7, 118.5, 118.5 ppm in the ^{1}H, ^{15}N, and ^{15}N dimensions, respectively. The duration of the acquisition with 16 scans is 1 day and 22 h.
9. Extract [^{1}H,^{15}N] 2D planes from the [^{1}H,^{15}N,^{15}N] HNCANN 3D experiment (Fig. 12b) for each ambiguous peak in the [^{1}H,^{15}N] HSQC (i.e., peaks that cannot be assigned with the HNCACB experiment only).^{15}N-chemical shift values in the third dimension of the recorded spectrum correspond to the ^{15}N chemical shift of the residue at the i, i+1, and i-1 positions.

3.16 Assignment of Resonances of Acetylated Residues: Identification of the Acetylation Pattern

The identification of acetylation sites involves the use of selective ^{15}N-lysine labeling of Tau samples to decrease the spectral complexity. The assignment of lysine residues in the non-acetylated Tau sample is made using the previously described [^{1}H,^{15}N] HSQC, HNCACB, and HNCANN experiments (*see* Subheading 3.15) recorded on uniformly ^{15}N-labeled Tau. These experiments are combined with a [^{1}H,^{15}N] HSQC recorded on a

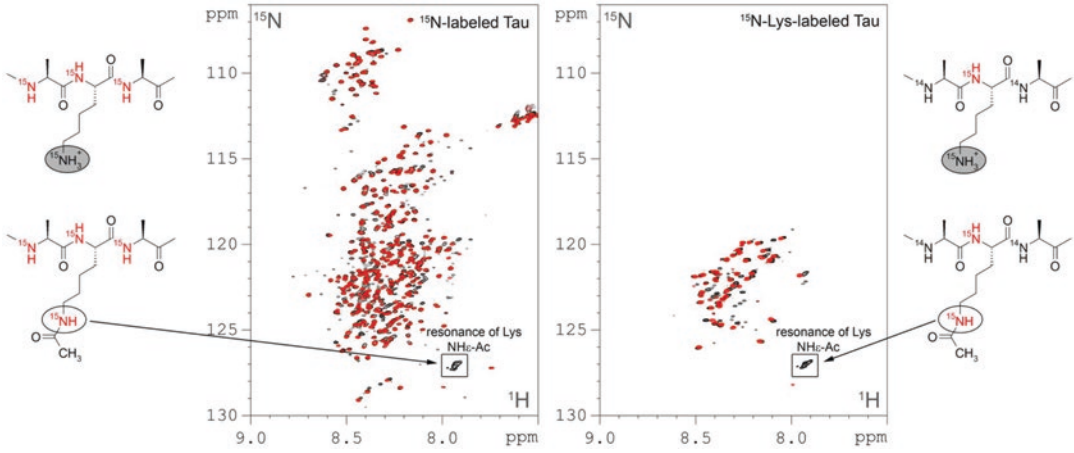


Fig. 13 Comparison of the [^{1}H,^{15}N] HSQC spectra of the non-acetylated (*red*) and acetylated (*black*) forms of uniformly ^{15}N-labeled Tau (*left spectra*) and selectively ^{15}N-lysine labeled Tau (*right spectra*)

selectively ^{15}N-lysine-labeled Tau sample for an easy identification of lysine resonances.

On the [^{1}H,^{15}N] HSQC of the ^{15}N-lysine labeled, acetylated Tau sample, one can detect some additional backbone amide resonances as compared to the control, non-acetylated Tau together with non-resolved peaks corresponding to the NHε acetamide functions of lysine side chains that are referred to as the indicator signal (Fig. 13, *see* **Note 20**) [11, 12]. Hence, identification of resonances corresponding to acetylated lysine residues is first required before their assignment. The resonances of acetylated lysine residues are identified in a selectively ^{15}N-lysine, uniformly ^{13}C labeled, acetylated Tau sample using the [^{1}H,^{15}N] HSQC-TOCSY experiment that enables the detection of proton resonances of the lysine side chain from either the backbone amide resonance or those of side chain acetamide (NHε-Ac, Fig. 14). Lysine assignment is then performed with the [^{1}H,^{15}N,^{13}C] HN(CO)CACB experiment for the identification of the i-1 residue (Fig. 15).

1. Dissolve 1–2 mg of either uniformly ^{15}N-labeled or selectively ^{15}N-lysine-labeled Tau in 200 µl NMR buffer (100–200 µM) to fill a 3 mm tube.
2. Record a [^{1}H,^{15}N] HSQC 2D spectrum (dipsihsqcf3gpsi3d pulse sequence from Bruker, *see* **Note 17**) [24] (Fig. 13) at 293 K on a Bruker900MHz Avance III NMR spectrometer equipped with a triple resonance cryogenic probehead (Bruker, Karlsruhe, Germany) or equivalent spectrometer.
3. For the uniformly labeled sample, acquire data with 2048 and 512 points for spectral widths of 14 and 30 ppm in the ^{1}H and ^{15}N-dimensions, respectively, centered on 4.7 ppm and

118.4 ppm. The duration of the acquisition with 32 scans is 5 h 15 min. For the selectively ^{15}N-lysine-labeled sample, acquire data with 2048 and 128 points for spectral widths of 14 and 9 ppm centered on 4.7 ppm and 123.5 ppm in the ^{1}H and ^{15}N-dimensions, respectively. The relaxation delay is 1 s. The duration of the acquisition with 32 scans is 80 min.

4. Process the spectra with the Bruker TopSpin 3.1 software.
5. Dissolve 11 mg of a ^{15}N-lysine selectively, ^{13}C uniformly labeled, acetylated Tau sample in 400 µl NMR buffer (600 µM) to fill a NMR Shigemi tube.
6. Acquire the three-dimensional spectra under a nonuniform sampling mode on a Bruker900MHz Avance III NMR spectrometer equipped with a triple resonance cryogenic probehead (Bruker, Karlsruhe, Germany) at 293 K.
7. Perform data processing, peak picking and calculation of peak intensities with the Bruker TopSpin 3.1 software.
8. Record a [^{1}H,^{15}N] HSQC-TOCSY spectrum (dipsihsqcf3gpsi3d pulse sequence from Bruker, *see* **Note 21**) with 32 scans per increment, a TOCSY mixing time of 120 ms and spectral widths of 16.0, 9.0, and 11.0 ppm in ^{1}H (F3), ^{15}N (F2), and ^{1}H (F1) dimensions that are sampled with 3072, 78, and 416 points, respectively. The proton and nitrogen dimensions are centered on 4.7 and 123.5 ppm, respectively. The relaxation delay is 1 s. The nonuniform sampling is done with 25 % sparse sampling, 2028 hypercomplex points in indirect dimension, T2 relaxation times of 0.3 s in each ^{1}H dimension, and 0.25 s in ^{15}N dimension. The duration of the acquisition is about 3 days and 21 h.
9. Extract [^{1}H, ^{1}H] 2D planes from the [^{1}H,^{15}N] HSQC-TOCSY 3days experiment for each peak in the [^{1}H,^{15}N] HSQC.
10. Measure the chemical shift values of ^{1}H nuclei of lysine side chain, especially the Hε chemical shifts. This latter value allows for discrimination of acetylated lysines from the non-acetylated ones (Fig. 14).
11. Extract [^{1}H,^{15}N] 2D planes from the [^{1}H,^{15}N] HSQC-TOCSY 3D experiment at 2.98 ppm and 3.14 ppm in the F1 dimension which corresponds to the Hε chemical shifts of a non-acetylated and acetylated lysine residue, respectively.
12. Superimpose each of this sub-spectrum to the [^{1}H,^{15}N] HSQC of acetylated Tau to discriminate between resonances of non-acetylated and acetylated lysine residues.
13. Record a [^{1}H,^{15}N,^{13}C] HN(CO)CACB 3D spectrum (hncocacbgpwg3d pulse sequence from Bruker, *see* **Note 22**) at 900 MHz and 293 K with 3072, 40, and 272 points for spectral widths of 14, 9, and 55 ppm in the ^{1}H, ^{15}N, and ^{13}C-dimensions, respectively, centered on 4.7, 123.5, and 39.0 ppm, respec-

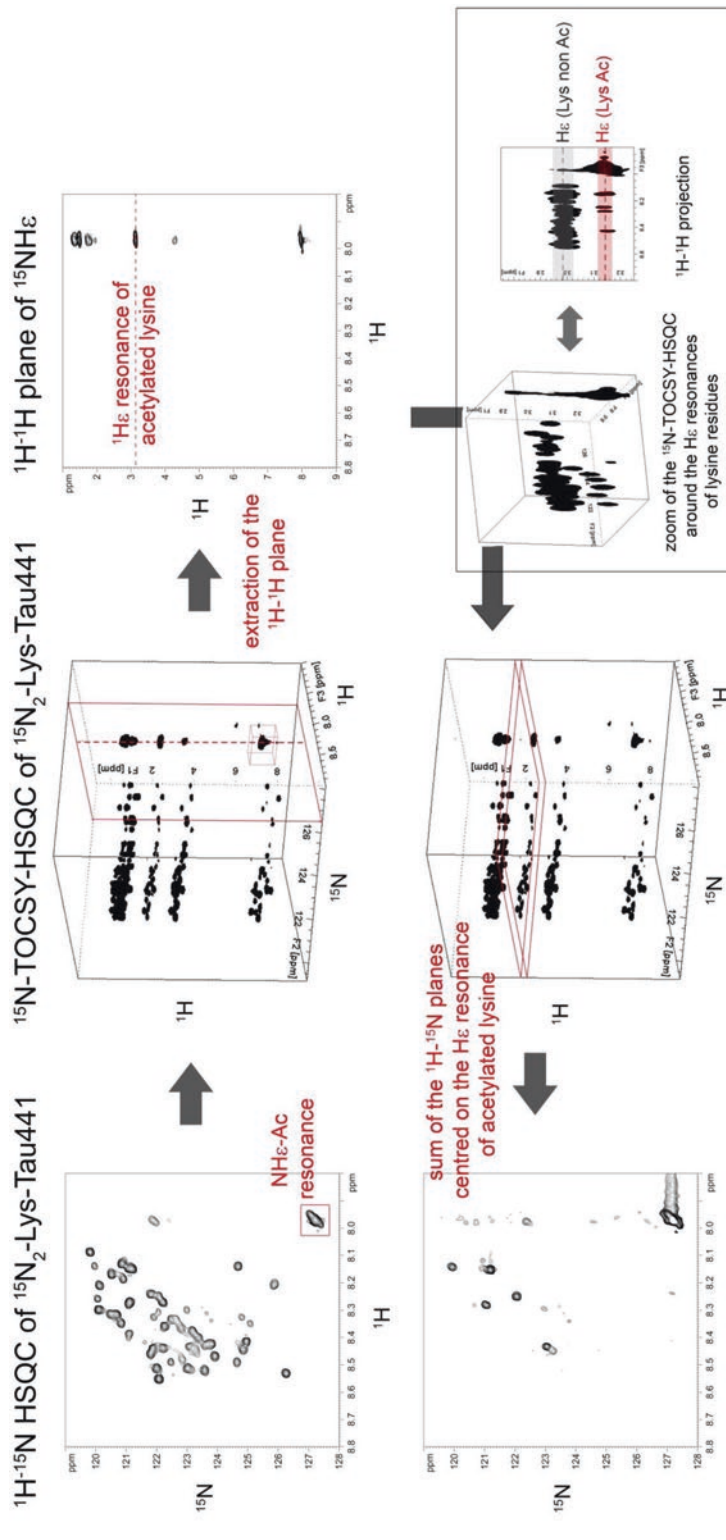


Fig. 14 Strategy of acetylated lysine identification based on the Hε chemical shift of lysine side chain from the [^{1}H,^{15}N] HSQC-TOCSY experiment acquired on ^{15}N-lysine, ^{13}C-labeled Tau protein

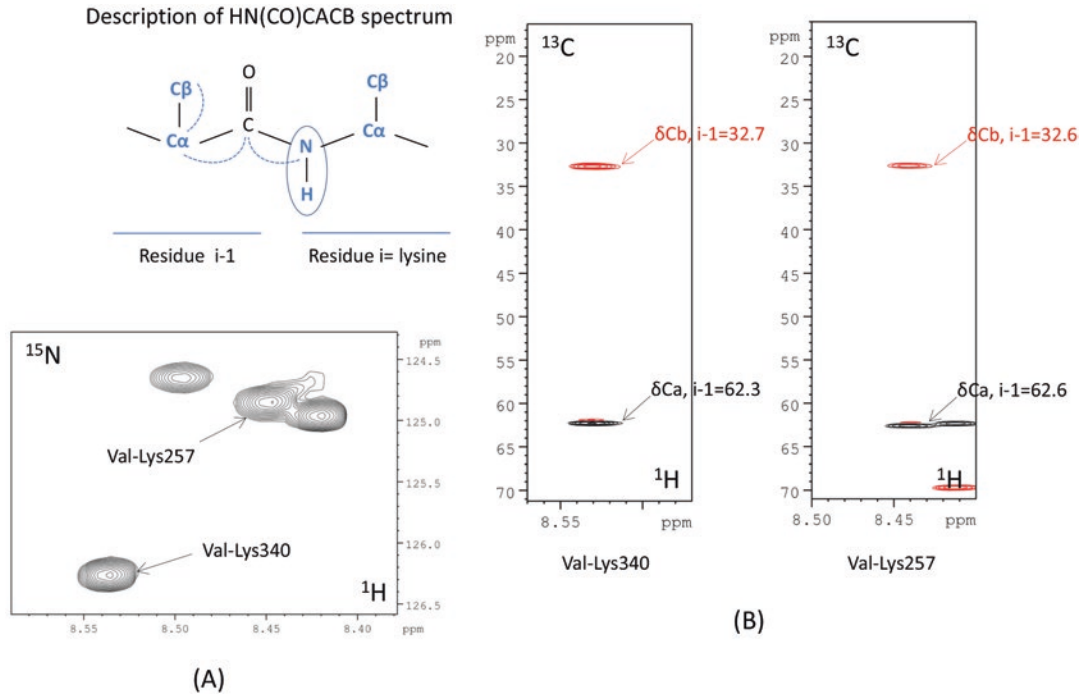


Fig. 15 Identification of lysine resonances using the [^{1}H,^{15}N,^{13}C] HN(CO)CACB experiment. (**a**) Zoom of the [^{1}H,^{15}N] HSQC experiment acquired on selectively ^{15}N-lysine-labeled, acetylated Tau. (**b**) [^{1}H, ^{13}C] 2D planes extracted from the [^{1}H,^{15}N,^{13}C] HN(CO)CACB 3D spectrum, at ^{15}N-values of 126.25 ppm (*left*) and 124.85 ppm (*right*). The ^{13}C-CA (*black*) and ^{13}C-CB resonances (*red*) of i-1 residue side chain of K340 (*left panel*) and K257 (*right panel*) are indicated. This combination of CA and CB chemical shift values is typical of a V residue. The unambiguous assignment of both V-K dipeptide has been made with a non-acetylated Tau sample and uniform ^{15}N-labeling using the previously described [^{1}H,^{15}N,^{13}C] HNCACB and [^{1}H,^{15}N,^{15}N] HNCANN three-dimensional experiments (Subheading 3.15)

tively. The relaxation delay is 1 s. The nonuniform sampling is done with 25% sparse sampling, 680 hypercomplex points in indirect dimension, T2 relaxation times of 0.3 s, 1 s, and 0.1 s in ^{1}H, ^{15}N, and ^{13}C dimensions, respectively. The duration of the acquisition with 48 scans is 1 day and 23 h.

14. Extract [^{1}H, ^{13}C] 2D planes from the [^{1}H,^{15}N,^{13}C] HN(CO)CACB 3D experiment for each peak in the [^{1}H,^{15}N] HSQC.
15. Measure the chemical shift values of "CA" and "CB" ^{13}C nuclei of the i-1 residues for each [^{1}H, ^{15}N] lysine resonance enabling residue identification (Figs. 15 and 16).
16. To avoid ambiguity related to redundant X-K dipeptides (where X is any amino acid) as illustrated in Fig. 15 with the V-K340/257 dipeptides, this experiment is combined with HNCACB and HNCANN experiments acquired on ^{15}N-Tau with uniform ^{15}N-labeling (*see* Subheading 3.15) for full assignment of lysine residues in the control, non-acetylated Tau sample. According to this latter strategy, a full sequential

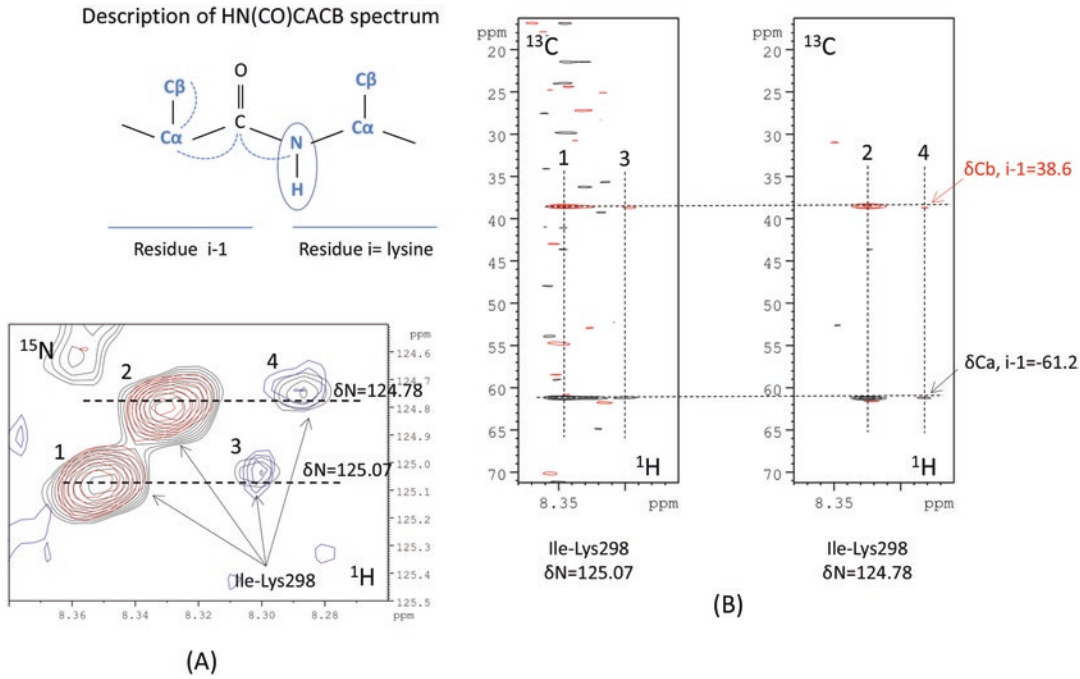


Fig. 16 Increasing spectral complexity in the case of two close acetylation sites showing four resonances for the single I-K298 dipeptide (numbered from 1 to 4). (**a**) Zoom of the [^{1}H,^{15}N] HSQC experiment acquired on selectively ^{15}N-lysine-labeled, acetylated Tau (*black*). Sub-spectra of non-acetylated (*red*) and acetylated (*blue*) lysine (Subheading 3.16) are overlaid and indicate that resonances 1 and 2 correspond to non-acetylated lysine while resonances 3 and 4 correspond to acetylated lysine. (**b**) [^{1}H,^{13}C] 2D planes extracted from the [^{1}H,^{15}N,^{13}C] HN(CO)CACB 3D spectrum at ^{15}N-values of 125.07 ppm (for resonances 1 and 3) and 124.78 ppm (for resonances 2 and 4). According to the ^{13}C-CA (*black*) and ^{13}C-CB values (*red*) of the i-1 residue, resonances numbered one to four are all identified as I-K dipeptide that is represented by the sole K298 along the Tau sequence. Hence, the splitting of each of non-acetylated and acetylated K298 resonance is due to the proximity of another acetylation site in the Tau sequence that has been identified as the i-4 residue according to the same assignment strategy

assignment enables the identification of the i-1 residue of X in each X-K dipeptide which is either E in the case of V-K340 or N in the case of V-K257.

17. Spectral complexity due to the presence of close acetylation sites is illustrated in Fig. 16 for the K298 residue. In this case, four resonances are detected for the single I-K dipeptide. Two of them come from a non-acetylated form of K298 and the two remaining from an acetylated K298 based on their respective Hε value (Fig. 16a), indicating the proximity of another acetylation site (identified as the K294 residue).

4 Notes

1. Complete medium and MEM vitamins addition are not compulsory but help to stimulate the growth and improve the

yield. MEM vitamins 100× (SIGMA) are aliquoted by 10 ml and aliquots are stored at –20 °C.

2. Upon addition of the 100 mM $CaCl_2$ stock a white precipitate will form.
3. The isotopes are added freshly at the time the bacterial culture is started. They are solubilized in M9 buffer and filtered sterilized at 0.2 μm.
4. To produce ^{15}N, ^{13}C-Tau, 300 mg of ^{15}N, ^{13}C-complete medium, 1 g of ^{15}N-NH_4Cl, and 2 g of ^{13}C-glucose are suspended in 10 ml of M9 buffer and are 0.2 μM filter-sterilized directly into the M9 medium. Glucose is the limiting factor for the bacterial growth and an OD600 of 1.4–1.6 is usually observed at the end of the fermentation.
5. Tau protein is a disordered protein sensitive to proteases. We find that sterilization (120 °C for 20 min) of buffers limits Tau degradation. The buffers are 0.22 μm-filtered or autoclaved and stored at 4 °C.
6. pH is adjusted after addition of 250 mM imidazole in phosphate buffer and 10 mM reduced Glutathione in Tris buffer, in the elution buffers.
7. We do not reconstitute a complete medium from the Isogro® powder, given the cost of the product, but use it as a culture supplement. Generally, bacterial cultures using M9 media supplemented with 0.05% Isogro® (w/v) reach the exponential growth phase (as measured by O.D. at 600 nm) after about 4 h of incubation at 37 °C under the conditions described to inoculate the culture. Without Isogro®, the exponential phase of growth is reached after 6 h at 37 °C.
8. The bacterial growth is slow in M9 medium. We use programmable incubators to start the culture at an early morning time, so that the protein production, collection of the bacterial pellet, and analytical control of the protein production can be conveniently scheduled during the work day hours.
9. The lyophilized Tau proteins are kept at –20 °C.
10. Tau protein concentration is difficult to define because its absorption coefficient at 280 nm is low (A280 nm = 0.14 for a solution at 1 mg/ml). To define Tau concentration, we use the surface of the peak of Tau protein absorption at 280 nm in the chromatogram in the desalting step in Ammonium Bicarbonate. This is the most reliable method we have found. This method was validated by analysis of one Tau sample by total amino acid quantification.
11. The His-ERK2 preparation is contaminated with a protein of 66 kDa (Fig. 4a).

12. The activated ERK kinase in activation buffer with 10% Glycerol is directly mixed with Tau protein in phosphorylation buffer, without prior buffer exchange. In an alternative protocol, His-ERK2, GST-MEK1 R4 and ^{15}N-Tau can be all mixed, to combine **steps 3.7** and **3.8**.
13. Rat brain extract can be stored at 4 °C for a few days only without loss of kinase activity.
14. Resin beads loaded with the GST-CBP[1202-1848] fragment can be stored without glycerol at 4 °C for few days. However, we have noticed a rapid decrease of acetyltransferase activity (within 2 or 3 days) in these conditions.
15. A peak with a mass increment of +168 Da ($[M+H]^+$ 2861.79 Da) corresponding to the incorporation of four acetyl moieties in the CBP standard substrate from TDG protein can be detected.
16. In contrast to S/T phosphorylation, Y phosphorylation does not induce a down field backbone-amide chemical shift of the modified Y residue resonance [22], probably because the phosphorylatable Y hydroxyl group is at a more distal position compared to the one of S and T residues.
17. [^{1}H,^{15}N] HSQC pulse sequence from Bruker is hsqcetfpf3gpsi avance-version: HSQC 2D H-1/X correlation via double INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) using sensitivity improvement, phase sensitive using Echo/Antiecho-TPPI gradient selection with decoupling during acquisition using f3—channel and flip-back pulse.
18. [^{1}H,^{15}N,^{13}C] HNCACB pulse sequence from Bruker is hncacbgpwg3d avance-version: HNCACB 3D sequence with inverse correlation for triple resonance using multiple INEPT transfer steps (F1(H)→F3(N)→F2(Ca→Cb,t1)→F3(N,t2)→F1(H,t3)), on/off resonance CA and CO pulses using shaped pulse, phase sensitive (t1), phase sensitive (t2), using constant time in t2 and water suppression using watergate sequence.
19. [^{1}H,^{15}N,^{15}N] HNCANN pulse sequence from Bruker is hncannhgpwg3d avance-version: (H)N(CA)NNH 3D sequence with inverse correlation for triple resonance using multiple INEPT transfer steps (F1(H)→F3(N,t1)→F2(Ca)→F3(N,t2)→F1(H,t3)), on/off resonance CA and CO pulses using shaped pulse, phase sensitive (t1), phase sensitive (t2), using semi-constant time in t1 and t2 and water suppression using watergate sequence.
20. In contrast to the phosphorylation sites that are easily identifiable due to the low field chemical shift of their backbone amide resonances (Fig. 10), the identification of acetylated lysine is not straightforward just by looking to the 2D [^{1}H,^{15}N] HSQC spectrum of acetylated ^{15}N-labeled Tau, or even in the

^{15}N-Lys-labeled Tau samples (Fig. 13). Furthermore, the CA and CB chemical shifts of lysine residues as extracted from the HNCACB experiment (Subheading 3.15, **step 7**) fail to inform about the lysine acetylation state. Only the extremity of the lysine side chain (from Cγ to Cε and their protons) is sensitive to the presence of an acetyl moiety [10].

21. [^{1}H,^{15}N] HSQC-TOCSY pulse sequence from Bruker is dipsihsqcf3gpsi3d avance-version: TOCSY-HSQC 3D sequence with homonuclear Hartman-Hahn transfer using DIPSI2 sequence for mixing, H-1/X correlation via double inept transfer using sensitivity improvement, phase sensitive (t1), phase sensitive using Echo/Antiecho-TPPI gradient selection (t2), using trim pulses in INEPT transfer, using f3—channel.
22. [^{1}H,^{15}N,^{13}C] HN(CO)CACB pulse sequence from Bruker is hncocacbgpwg3d avance-version: HNCOCACB 3D sequence with inverse correlation for triple resonance using multiple INEPT transfer steps (F1(H) → F3(N) → F2(C=O) → F2(Ca → Cb,t1) → F2(C=O) → F3(N,t2) → F1(H,t3)), on/off resonance CA and CO pulses using shaped pulse, phase sensitive (t1), phase sensitive (t2) using constant time in t2 and water suppression using watergate sequence.

Acknowledgment

The NMR facilities were funded by the Région Nord, CNRS, Pasteur Institute of Lille, European Community (FEDER), French Research Ministry and the University of Sciences and Technologies of Lille I. We acknowledge support from the TGE RMN THC (FR-3050, France) and the Research Federation FRABio (Univ. Lille, CNRS, FR 3688, FRABio, "Structural & Functional Biochemistry of Biomolecular Assemblies") for providing the scientific and technical environment. This study was supported by a grant from the LabEx (Laboratory of Excellence) DISTALZ (Development of Innovative Strategies for a Transdisciplinary approach to Alzheimer's disease), and in part by the French government funding agency Agence Nationale de la Recherche TAF. S.P. and J.G. were partially supported on NIH R01-GM08157.

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