Protein semisynthesis provides access to Tau disease-associated post-translational modifications (PTMs) and paves the way to deciphering the Tau PTM code in health and diseased states

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Supporting information

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Expression of Tau (2-245)-SR (1):

The gene encoding Tau (2-245) Mxe-His_6 was amplified by PCR, from pTau2-Mxe-His_6 and pMxe-his-tag. Tau (2-245) was amplified using the following primers: forward 5` GAG ATA TAC ATA TGG CTG AGC CCC GCC A 3` and reverse 5` CAG AAT TCC TGC TCT TCG GCA TGT CTG CAG GCG GCT CTT G 3`. Mxe-His_6 was amplified using the following primers: forward 5` GGC AAG AAT GGC TCT TCA TGC ATC ACG 3` and reverse 5` CGA TAA GCT TGG ATG GAA CAT CTG TCA GC 3`. The resulting PCR products were digested using NdeI / SapI for Tau(2-245), and with SapI / HindIII for MxeHis, and were subsequently ligated into the pT7-7 vector, which was predigested using NdeI / HindIII.

E.coli BL21 (DE3) cells were transformed with the corresponding plasmid and grown in 6 liters of LB medium at 37 °C until OD = 0.5-0.6, which was followed by induction with 1 mM IPTG for 5 h at 37 °C. After harvesting the cells by centrifugation at 5000 rpm, the cell pellet was lysed by ultrasonication (10 min: pulse on 59 sec: pulse off 59 sec: 70% Amp) in a buffer containing 50 mM Hepes, 500 mM NaCl, 5 mM EDTA, and 0.3 mM PMSF. The insoluble material was removed by centrifugation at 18000 rpm for 1 h at 4 °C. The supernatant was injected into a 20-ml Histrap column equilibrated with same buffer (flow: 3 ml/min). The protein was eluted using lysis buffer containing 500 mM imidazole (Figure S1A).

Fractions containing the fusion protein were pooled, and splicing was induced by adding of 0.5 M 2-mercaptoethanesulfonic acid (MES-Na) at pH 7.0 for 12 h at room temperature (Figure S1B & C). The spliced thioester was purified by RP-HPLC using a preparative C4 column with a linear gradient of 20-40% B (buffer A = H_2O +0.1% TFA, and buffer B = acetonitrile + 0.1% TFA) over 40 min to afford the corresponding thioester **(1)** in 4.7 mg/liter of bacteria yield (Figure S1D).

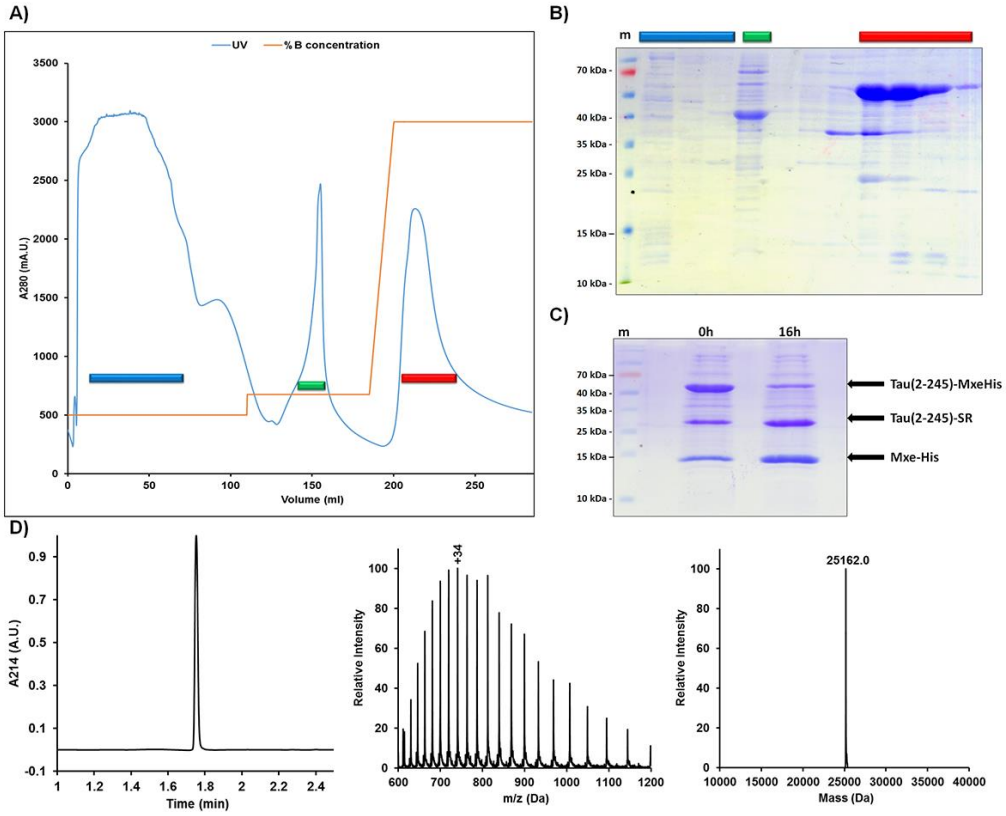

Figure S1: Expression and purification of the Tau (2-245)-SR fragment (1). A) FPLC chromatogram and elution of the fusion protein using a Ni^{+2}-IMAC column. B) SDS-PAGE analysis of the eluted fractions. C) SDS-PAGE analysis of the splicing reaction at time zero and after 16hr. D) Analytical RP-HUPLC / ESI-MS and SDS-PAGE of the purified peptide **1** (observed mass, 25,162 Da; calculated mass, 25,159 Da).

Synthesis of Tau (A246C-290)-$NHNH_2$ (2) and Tau (A246C-290, AcK280)-$NHNH_2$ (3):

Peptide **Tau (A246C-290)-$NHNH_2$ (2)** was prepared using Rink amide-MBHA resin (0.27 mmol/g, 0.1 mmol scale) and synthesized according to the following procedure. The first unnatural amino acid (AA), 3-(Fmoc-amino)-4-(methylamino) benzoic acid (prepared as described by Dawson et al. [1]), was double-coupled manually for 1 h using 4 eq of 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) and 8 eq of N,N-diisopropylethylamine (DIEA). The remaining amino acids were double-coupled using an automated CS 336X peptide synthesizer from CS Bio and the reaction was conducted in the presence of 4 eq of AA, 8 eq of DIEA and 4 eq of N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate/1-hydroxybenzotriazole hydrate

(HBTU/HOBt). Each coupling was kept for 45 min and Fmoc deprotection was achieved using 20 % piperidine and 50 mM HOBT for 3/5/3 min cycles. Then 2.5 eq of Fmoc LeuSer(ψMe,Me pro)-OH was manually coupled for 2 h at position Leu284-Ser285. The last AA was introduced with N-terminal Boc protection. After peptide elongation, the resin was re-swelled with dichloromethane (DCM) for 15 min, and reacted with a solution of p-nitrophenyl chloroformate (5 eq, in 2 ml DCM) for 1 h at room temperature. Then, the resin was washed with DCM and re-swelled with dimethylformamide (DMF) for 15min. A solution of 0.5 M DIEA in 1 ml DMF was thenadded for 15 min (x2).

Cleavage from the resin: After washing with DMF, methanol, and DCM the dried peptidyl-resin was treated with a cleavage cocktail that containing 95 % trifluoroacetic acid (TFA), 2.5 % water and 2.5% triisopropylsilane (TIPS) for 2.5 h at room temperature. The crude peptide was then precipitated by dropwise addition to a 10-fold volume of cold ether, redisolved in 50 % aqueous acetonitrile and lyophilized. The lyophilized crude peptide was dissolved in 6.0 M guanidine hydrochloride (GdnHCl) to a final concentration of ~6 mM. Hydrazine hydrate (100 eq) was added and the mixture was stored at room temperature for 1 h. The peptide was purified by RP-HPLC using a preparative C4 column with a linear gradient of 5-40 % B over 50 min to afford the corresponding hydrazine in 7.8 % yield (Figure S2).

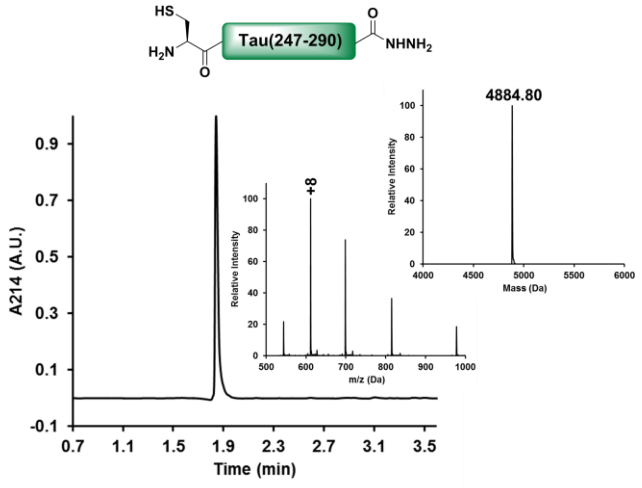

Figure S2: SPPS of Tau (A246C-290)-NHNH2. Analytical RP-HUPLC and ESI-MS of the purified peptide **2** (observed mass, 4,884.80 Da; calculated mass, 4,883.76 Da).

The peptide **Tau (A246C-290, AcK280)-NHNH$_2$ (3)** was synthesized in a similar manner to that described above for the wild type peptide **(2)** with the following changes: 2.5 eq of Fmoc-Lys(Ac)-OH was introduced at position Lys280 junction by manually coupling for 2 h (with HATU). The peptide was purified by RP-HPLC using a preparative C4 column with a linear gradient of 5-40 % B over 40 min to afford the corresponding thioester in 9.13 % yield (Figure S3).

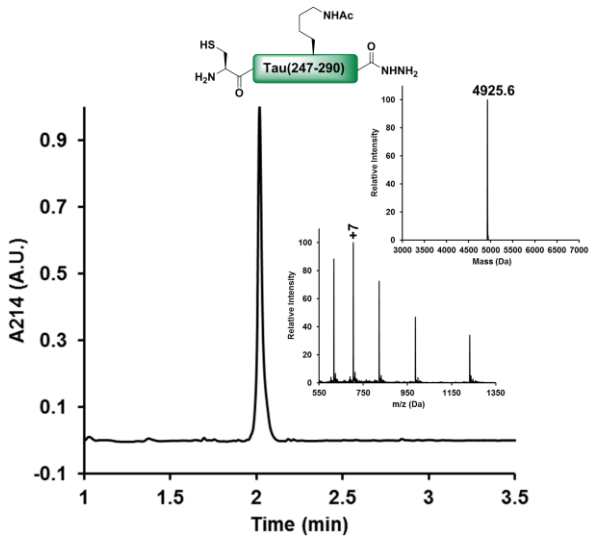

Figure S3: SPPS of Tau (A246C-290, AcK280)-NHNH2. Analytical RP-HUPLC and ESI-MS of the purified peptide **3** (observed mass, 4,925.60 Da; calculated 4,925.79 Da).

Preparation of Tau (2-290)-NHNH$_2$ (4):

Ligation: **Tau (2-245)-SR (1)** (15.0 mg, 1.3 eq) and **Tau (A246C-290)-NHNH$_2$ (2)** (2.24 mg, 1 eq) were dissolved in native chemical ligation (NCL) buffer (GdnHCl, 0.2 M sodium phosphate, 50 mM tris(2-carboxyethyl)phosphine (TCEP), 100 mM lysine, and 2 % trifluoroethanethiol (TFET), pH 7.4) that had been purged with argon. The mixture was incubated at 37 ºC for 5 h with orbital agitation at 600 rpm and was regularly monitored by RP-UHPLC and mass spectrometry analyses.

In situ desulfurization: Upon completion of the reaction, the mixture was purged with argon for 15 min to remove the TFET thiol. To the ligation mixture, a solution of 175 mM TCEP in argon purged 6.0 M GdnHCl, 0.2 M sodium phosphate pH 7.4, 40 mM glutathione and 40 mM 2,2'-

azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA–044) was added and incubated at 37 °C for 12 h with orbital agitation at 600 rpm. The progress of the reaction was monitored by RP-UHPLC and LCMS-ESI analyses. The ligated product was purified by RP-HPLC using a preparative C8 column with a linear gradient of 15-35 %B over 50 min to afford the corresponding product **4** in 41% yield over two steps (Figure S4 & S5).

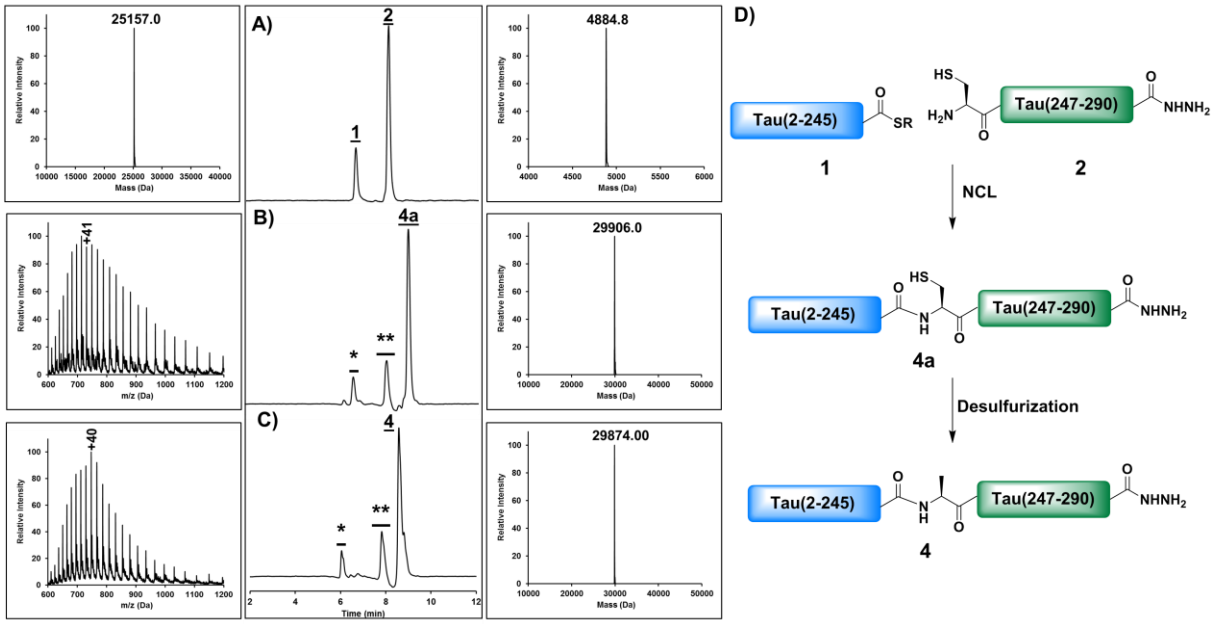

Figure S4: A, B) Analytical RP-HUPLC traces/ (ESI-MS) of the ligation reaction between **Tau (2-245)-SR**, and **Tau (A246C-290)-$NHNH_2$** at 0 h and after 5 h. Peak 4a corresponds to the ligation product (observed mass, 29,906 Da; calculated mass, 29,901 Da). The peak labeled with * corresponds to the hydrolyzed thioester, and the peak labeled with ** corresponds to the remaining traces of the starting material C) Analytical RP-HUPLC traces/ (ESI-MS) of the in-situ desulfurization reaction after 12 h. Peak 4 corresponds to the desulfurized product (observed mass, 29,874 Da; calculated mass, 29,869 Da). D) Schematic representation of the semi-synthesis of **Tau (2-290)-$NHNH_2$ (4)** fragment.

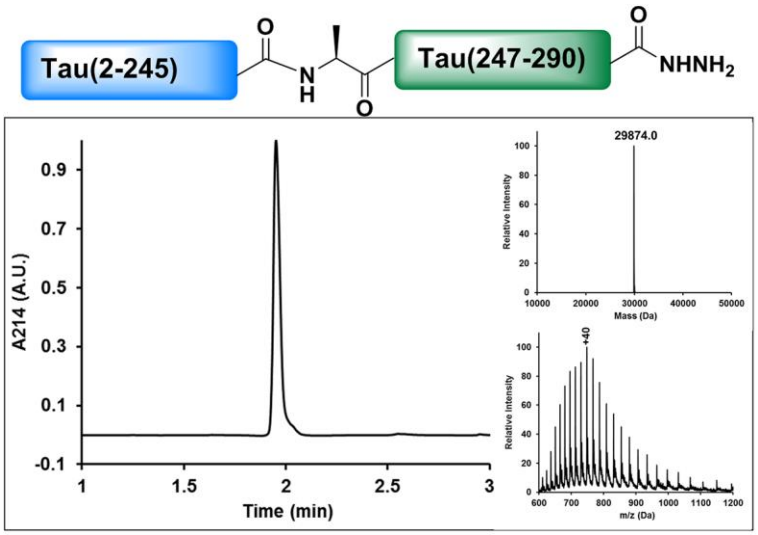

Figure S5: Analytical RP-HUPLC and ESI-MS of the purified **Tau (2-290)-$NHNH_2$ (4)** (observed mass, 29874.0 Da; calculated mass, 29868.8 Da).

Preparation of Tau (2-290, AcK280)-$NHNH_2$ (5):

Ligation: **Tau (A246C-290, AcK280)-$NHNH_2$ (3)** (5.76 mg, 2 eq) and **Tau (2-245)-SR (1),** (14.72 mg, 1.3 eq), were dissolved in NCL buffer (6.0 M GdnHCl, 0.2 M sodium phosphate, 50 mM TCEP, 100 mM lysine, 2 %TFET, pH 7.40) that had been purged with argon. The mixture was allowed to react at 37 ºC for 2 h with orbital agitation at 600 rpm and it was regularly monitored by RP-UHPLC and LCMS-ESI analyses.

In situ desulfurization: Upon completion of the reaction, the mixture was purged with argon for 15 min to remove the TFET thiol. To the ligation mixture, a solution of 175 mM TCEP argon purged 6.0 M GdnHCl, 0.2 M sodium phosphate pH 7.40, 40 mM glutathione and 40 mM VA–044 was added and incubated at 37 °C for 12 h with orbital agitation at 600 rpm. The progress of the reaction was monitored by RP-UHPLC and LCMS-ESI analyses. The ligated product was purified by RP-HPLC using a preparative C8 column with a linear gradient of 10-40% B over 40 min to afford the corresponding product **5** in 25% yield over two steps (Figure S6 and S7).

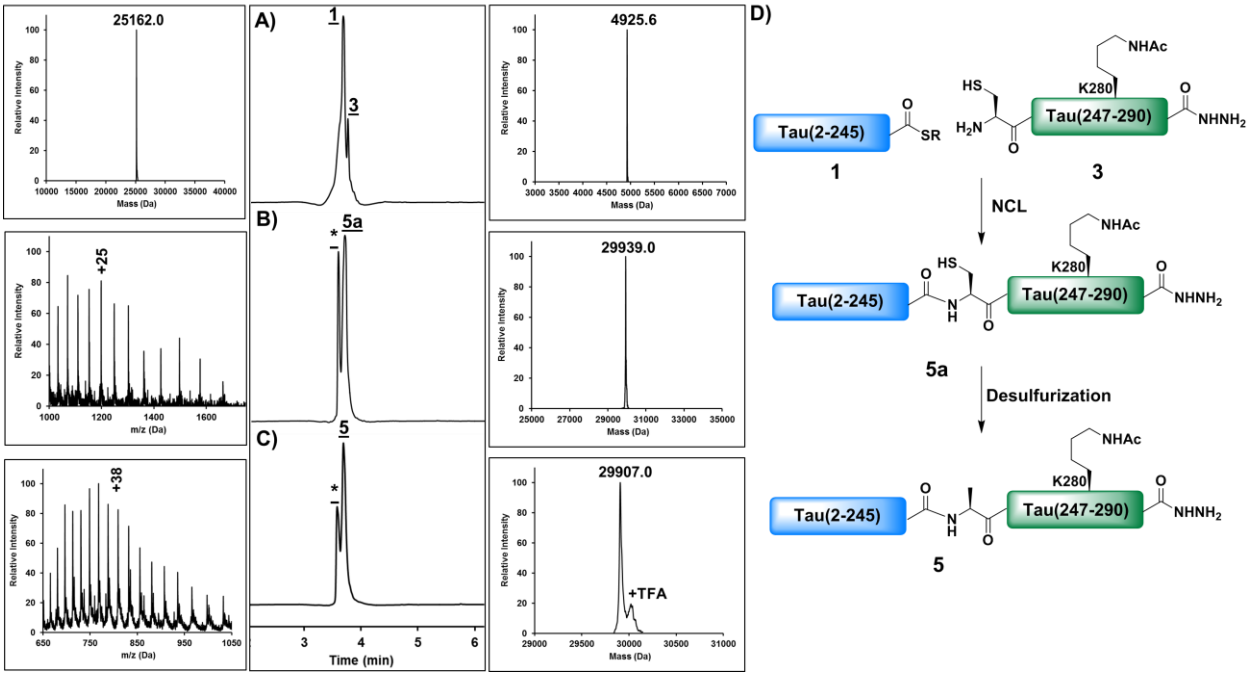

Figure S6: A, B) Analytical RP-HUPLC traces/ (ESI-MS) of the ligation reaction between **Tau (2-245)-SR**, and **Tau (A246C-290, AcK280)-$NHNH_2$** at 0 h and after 2 h. Peak 5a corresponds to the ligation product (observed mass, 29,939 Da; calculated mass, 29,943 Da). The peak labeled with * corresponds to the hydrolyzed thioester. C) Analytical RP-HUPLC traces/ (ESI-MS) of the in-situ desulfurization reaction after 12 h. Peak 5 corresponds to the desulfurized product (observed mass, 29,907 Da; calculated mass, 29,911 Da). D) Schematic representation of the semi-synthesis of **Tau (2-290, AcK280)-$NHNH_2$ (5)** fragment.

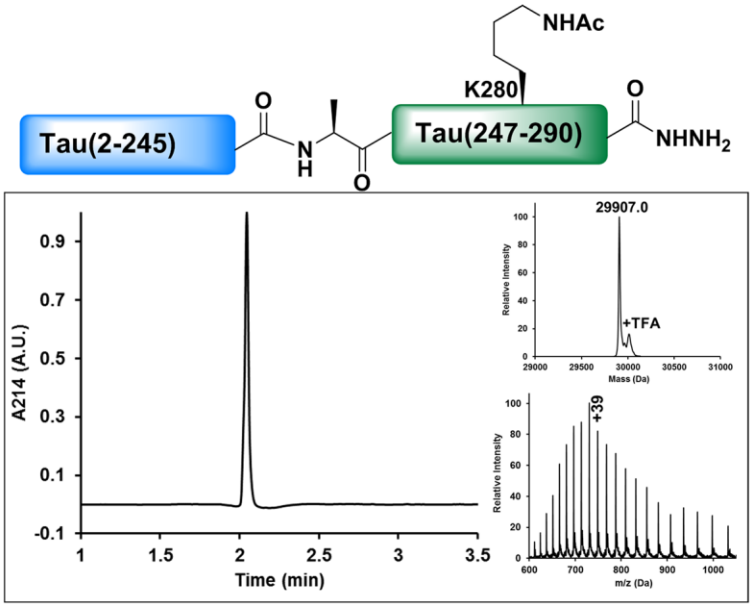

Figure S7: Analytical RP-HUPLC and ESI-MS of the purified **Tau (2-290, AcK280)-$NHNH_2$ (5)** (observed mass, 29907.0 Da; calculated mass, 29910.8 Da).

Synthesis of Tau (C322Thz-389)-SR (6):

Tau (C322Thz-389)-SR (6) was prepared using a similar strategy to that used for peptide **(2)** with the following changes: 2.5 eq of pseudoproline dipeptides of Lys-Thr, Ile-Thr and Lys-Ser were manually coupled (for 2 h) at positions Lys385-Thr386, Ile360-Thr361 and Lys340-Ser341, respectively. The last AA was introduced with N-terminal Boc protection.

After peptide elongation, the resin was re-swelled with dichloromethane (DCM) for 15 min and then reacted with a solution of p-nitrophenyl chloroformate (5 eq, in 2 ml DCM) for 1 h at room temperature. Then resin was then washed with DCM and re-swelled with DMF for 15 min. To the mixture, a solution of 0.5 M DIEA in 1 ml DMF was added for 15 min (x2).

Cleavage from the resin: After washing with DMF, methanol, and DCM the dried peptidyl-resin was treated with a cleavage cocktail that containing 95 % TFA, 2.5 % water and 2.5 % TIPS for 2.5 h at room temperature. The crude peptide was then precipitated by dropwise addition to a 10-fold volume of cold ether, redissolved in 50 % aqueous acetonitrile and lyophilized.The lyophilized peptide was purified by RP-HPLC using a preparative C4 column with a linear gradient of 15-40% B over 50 min to afford the corresponding thioester in 7.3% yield (Figure S8).

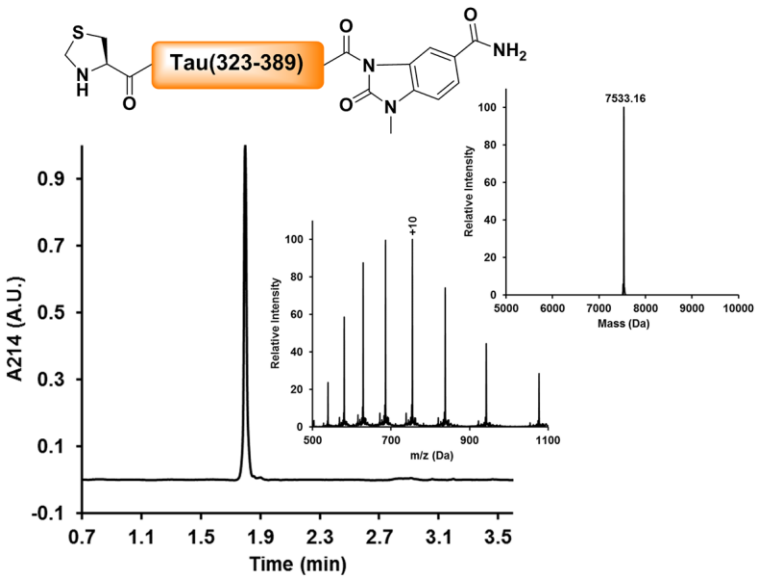

Figure S8: SPPS of Tau (C322Thz-389)-SR. Analytical RP-HUPLC and ESI-MS of the purified thioester **6** (observed mass, 7,533.16 Da; calculated mass, 7,532.34 Da).

Synthesis of Tau (A390C-441) (7):

Tau (A390C-441) (7) was prepared by CS Bio using an automated peptide synthesizer and standard Fmoc protocols. Wang resin (on which the first Leu residue was pre-loaded) was used. After peptide elongation, the resin was washed with DMF, methanol, and DCM, and the dried peptidyl-resin was then treated with reagent K containing 81.5% TFA, 5% phenol, 5% thioanisole 5% water, 2.5% 1,2-ethanedithiol 1 % TIPS for 3 h at room temperature. The crude peptide was then precipitated by dropwise addition to a 10-fold volume of cold ether. The peptide was purified twice by RP-HPLC using a preparative 1/2" C18 column with 0.1 % TFA in acetonitrile, and a final purity of 95% was obtained (Figure S9).

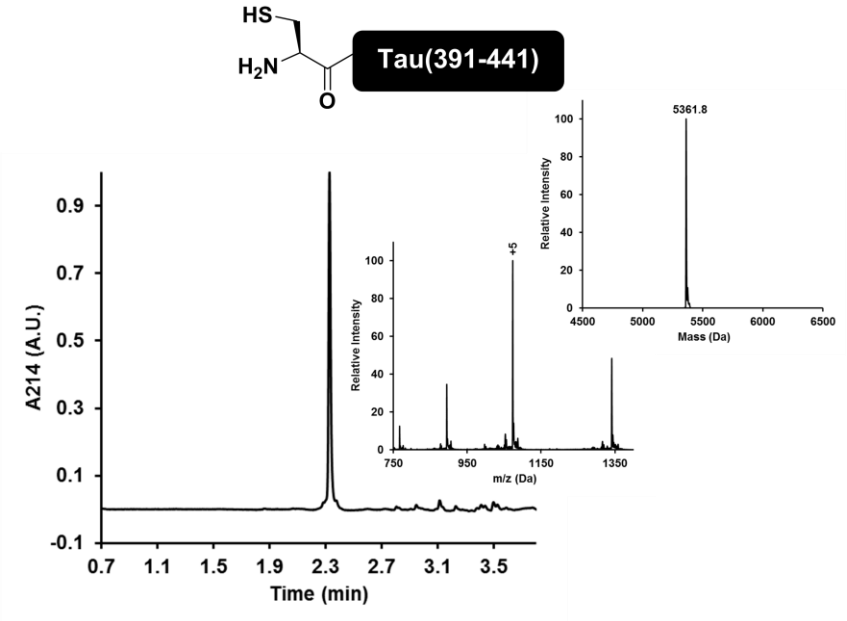

Figure S9: SPPS of Tau (A390C-441). Analytical RP-HUPLC and ESI-MS of the purified peptide **7** (observed mass of 5361.80 Da; calculated mass, 5361.99 Da).

Preparation of Tau (C322Thz-441) (8):

Ligation: **Tau (C322Thz-389)-SR (6)** (15.1 mg, 1.3 eq) and **Tau (A390C-441) (7)** (8.4 mg, 1 eq), were dissolved in NCL buffer (8.0 M urea, 50 mM TCEP, 2 %TFET, pH 7.0) that had been purged with argon. The mixture was left at 37 ºC for 1 h with orbital agitation at 600 rpm and was regularly monitored by RP-UHPLC and LCMS-ESI analyses.

In situ desulfurization: Upon completion of the reaction, the mixture was purged with argon for 15 min to remove the TFET thiol. A solution of 175 mM TCEP in an argon purged 8.0 M urea, 40 mM glutathione, and 40 mM VA–044 was added to the ligation mixture, then incubated at 37 °C for 1 h with orbital agitation at 600 rpm. The progress of the reaction was monitored by RP-UHPLC and LCMS-ESI analyses. The ligated product was purified by RP-HPLC using a preparative C8 column with a linear gradient of 20-35% B over 50 min to afford the corresponding product **(8)** in 30% yield over two steps (Figure S10 & S11).

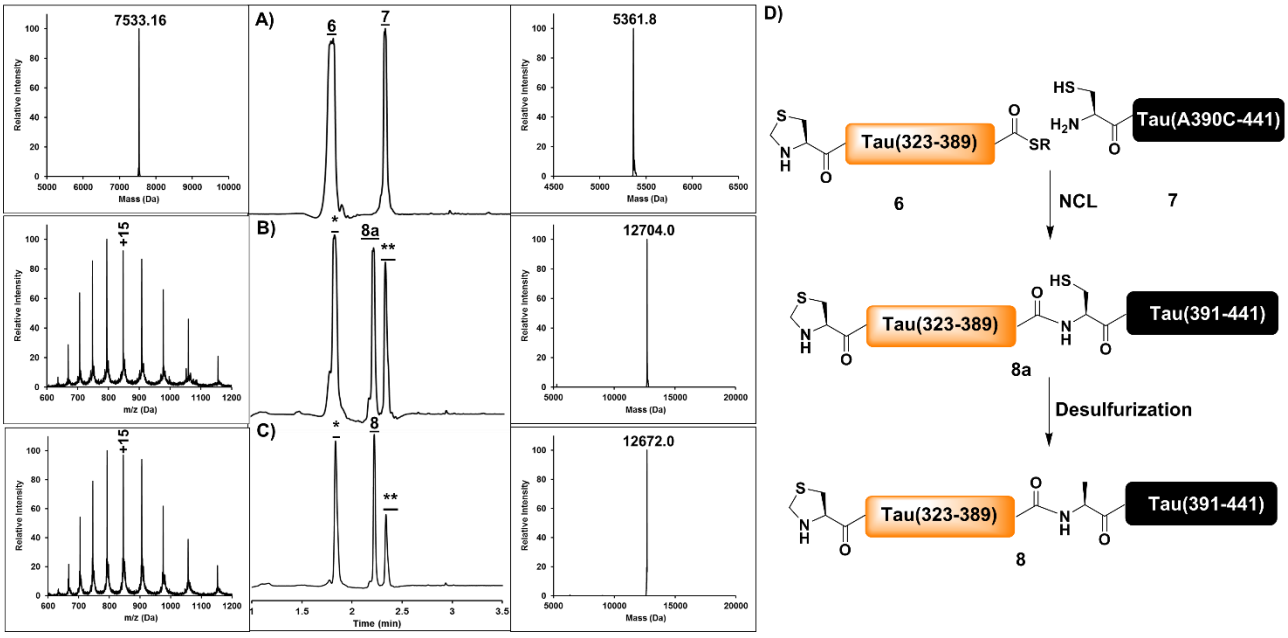

Figure S10: A, B) Analytical RP-HUPLC traces/ (ESI-MS) of the ligation reaction between **Tau (C322Thz-389)-SR (6)**, and **Tau (A390C-441) (7)** at 0 h (A) and after 4 h (B). Peak 8a corresponds to the ligation product (observed mass,12,704 Da; calculated mass 12,702 Da). The peak labeled with * corresponds to the hydrolyzed thioester, and the peak labeled with ** corresponds to the remaining traces of the starting material. C) Analytical RP-HUPLC traces/ (ESI-MS) of the in-situ desulfurization reaction after 1 h. Peak 8 corresponds to the desulfurized product (observed mass, 12,672 Da; calculated 12,670 Da). D) Schematic representation of the semisynthesis of **Tau (C322Thz-441) (8)** fragment.

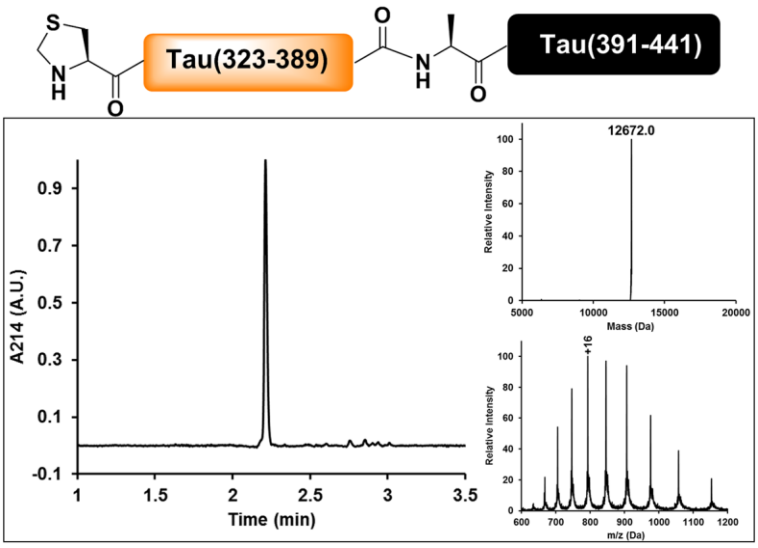

Figure S11: Analytical RP-HUPLC and ESI-MS of the purified **Tau (C322Thz-441) (8)** (observed mass, 12672.0 Da; calculated mass, 12670.2 Da).

Synthesis of Tau (C291Thz-321)-SR (9):

Peptide **Tau (C291Thz-321)-SR (9)** was prepared using a similar strategy to that used to prepare peptide **(6)**. The peptide was purified by RP-HPLC using a preparative C18 column with a linear gradient of 10-40% B over 40 min to afford the corresponding thioester in 22.5% yield (Figure S12).

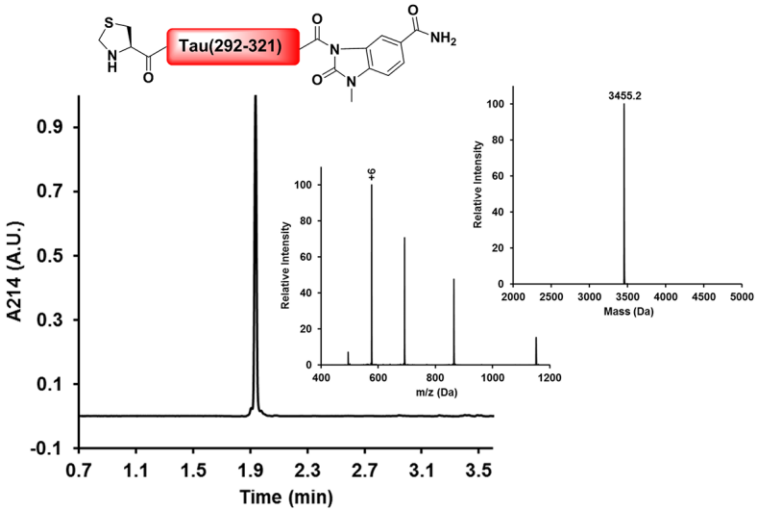

Figure S12: SPPS of Tau (C291Thz-321)-SR. Analytical RP-HUPLC and ESI-MS of the purified thioester **9** (observed mass, 3,455.20 Da; calculated mass, 3,455.50 Da).

Preparation of Tau (C291Thz-441) (11):

Ligation: **Tau (C322Thz-441) (8)** (6.0 mg, 1 eq) was dissolved in purged buffer that contains 8.0 M urea, TCEP (30 eq) and 0.1 M methoxylamine hydrochloride. The pH was adjusted to 4.0, and the reaction mixture was kept at 37 °C with orbital agitation at 600 rpm. The reaction was monitored by RP-UHPLC and mass spectrometry until complete deprotection of the Thz adducts. After 2 h the pH was adjusted to 7.0, and a solution of **Tau (C291Thz-321)-SR (9)** (2.3 mg, 1.4 eq) dissolved in NCL purged buffer (8.0 M urea, 50 mM TCEP, 2 % TFET, pH 7.0) was added. The mixture was left at 37 ºC for 5 h with orbital agitation at 600 rpm, and it was monitored by RP-UHPLC and mass spectrometry analyses. The ligated product was purified by RP-HPLC using a preparative C8 column with a linear gradient of 25-50% B over 50 min to afford the corresponding product **(11)** in 16% yield (Figure S13).

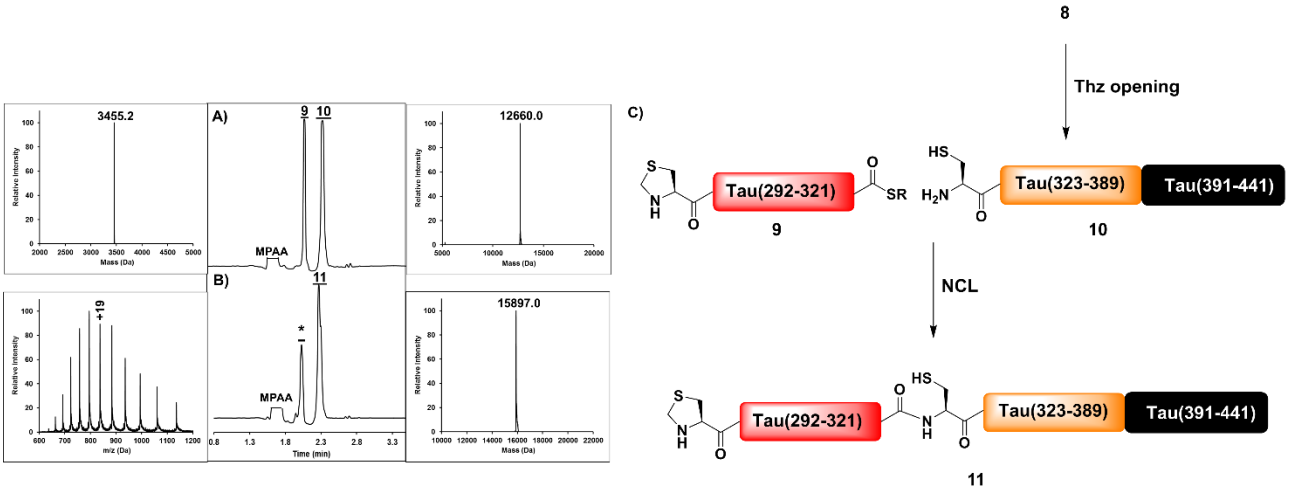

Figure S13: A, B) Analytical RP-HUPLC traces/ (ESI-MS) of the ligation reaction between **Tau (322-389)-SR**, and **Tau (C291Thz-321)-SR** at 0 h (A) and after 5 h (B). Peak 11 corresponds to the ligation product (observed mass of 15,897 Da; calculated mass, 15,894 Da). The peak labeled with * corresponds to the hydrolyzed thioester. C) Schematic representation of the semisynthesis of the **Tau (C291Thz-441) (11)** fragment.

Thz opening of Tau (C291Thz-441) (12):

Peptide **(11)** (1 mg, 1 eq) was dissolved in 100 µL of 6.0 M GdnHCl, 0.2 M sodium phosphate, TCEP (30 eq) and 0.1 M methoxylamine hydrochloride. The pH was adjusted to 4.0, and the reaction mixture was kept at 37 °C with orbital agitation at 600 rpm. The reaction was monitored by RP-UHPLC and mass spectrometry until complete deprotection of the Thz adducts. After 1 h the mixture was desalted using a PD-10 column (GE Healthcare) equilibrated with the elution

buffer (20% acetonitrile, 5% acetic acid). Fractions containing the desalted protein were diluted with water to 1% acetic acid and lyophilized to afford peptide **(12)** in 80% yield (Figure S14).

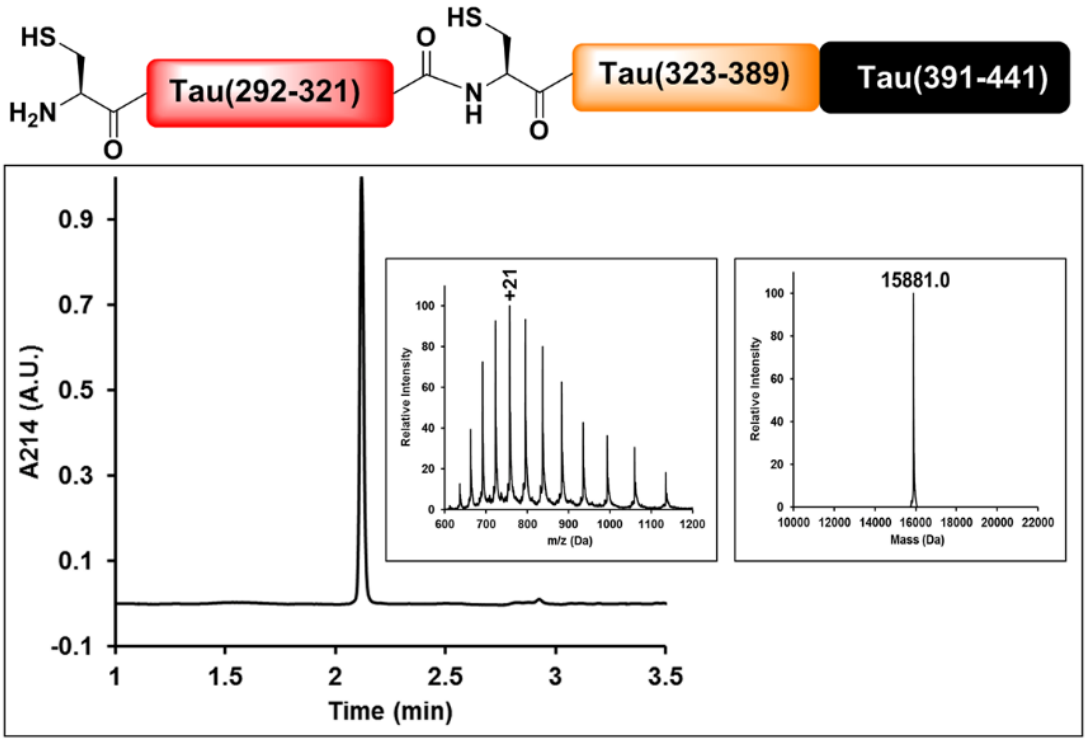

Figure S14: Analytical RP-HUPLC and ESI-MS of the purified peptide **12** (observed mass, 15,881 Da; calculated mass, 15,882 Da).

Switching Tau (2-290)-$NHNH_2$ (4) to Tau(2-290)-MPAA (13):

Peptide **4** (5.5 mg, 1 eq) was dissolved in 250 µL of 6.0 M GdnHCl and 0.2 M sodium phosphate pH 3.0, and treated with 100 eq of aqueous $NaNO_2$ (680 mM) at -15 °C for 20 min. The reaction was monitored by RP-UHPLC and LCMS-ESI until complete conversion of hydrazide to azide. Next, a solution of 4-mercaptophenylacetic acid (MPAA, 100 eq) was added for 30 min at 25 °C (pH 3.0). Then, the mixture was desalted using a PD-10 column (GE Healthcare) equilibrated with the elution buffer (20% acetonitrile, 5% acetic acid). Fractions containing the desalted protein were diluted with water to 1% acetic acid and lyophilized to afford peptide Tau(2-290)-MPAA **(13)** in ~80% yield (Figure S15).

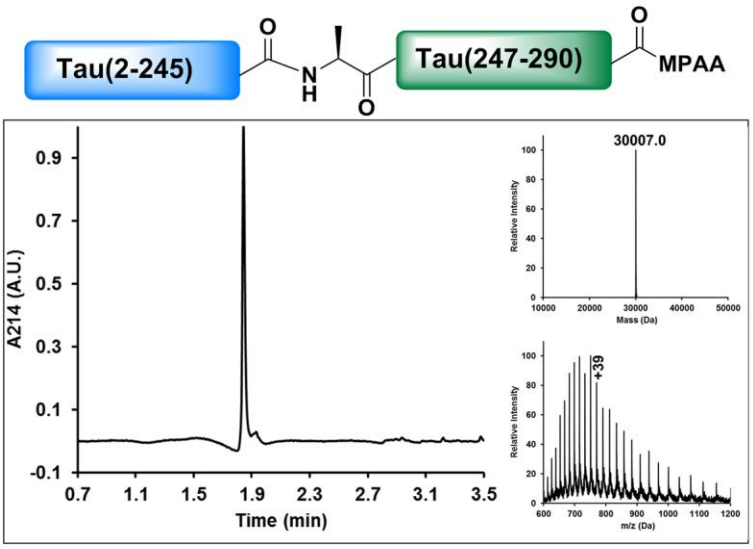

Figure S15: Analytical RP-HUPLC and ESI-MS of the purified peptide **13** (observed mass, 30,007 Da; calculated mass 30,005 Da).

Peptide Tau (2-290, AcK280)-MPAA **(14),** was prepared in the same way (Figure S16).

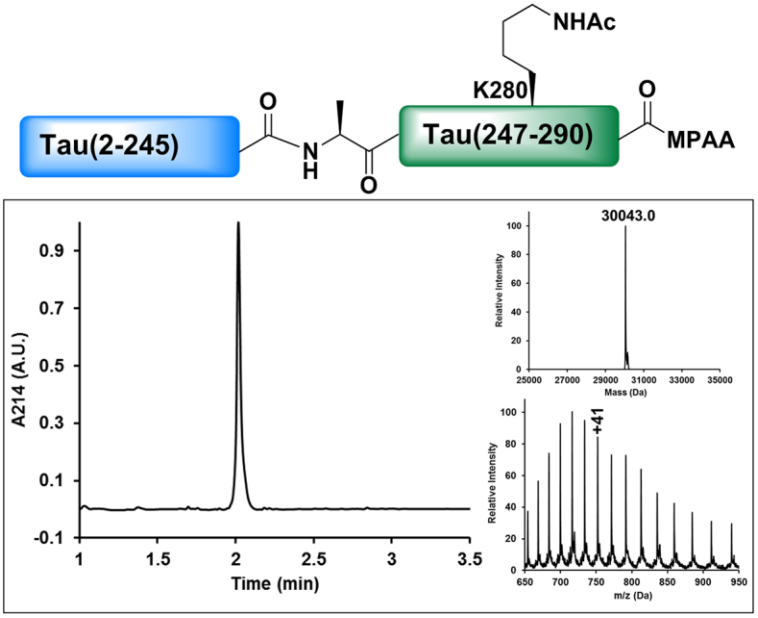

Figure S16: Analytical RP-HUPLC and ESI-MS of the purified peptide **14** (observed mass, 30,043 Da; calculated mass 30,048 Da).

Ligation of Tau(2-290)-MPAA (13) with Tau(291-441) (12):

Peptide **Tau(291-441) (12)** (0.8 mg, 1 eq) and **Tau (2-290)-MPAA (13)** (2.11 mg, 1.4 eq), were dissolved in NCL buffer (6.0 M GdnHCl, 0.2 M sodium phosphate, 35 mM TCEP, 30 mM

MPAA, pH 6.90) that had been purged with argon. The mixture was kept at 30 ºC for 6.5 h with orbital agitation at 300 rpm and it was regularly monitored by RP-UHPLC and LCMS-ESI analyses. The ligated product was purified by RP-HPLC using a preparative C4 column with a linear gradient of 20-40% B over 50 min to afford the corresponding wild-type full length Tau **15** in 20% yield (Figure 1).

Ligation of Tau(2-290, AcK280)-MPAA (14) with Tau(291-441) (12):

The procedure used for peptide **15** was followed, except that acetylated peptide **(14)** was used instead of the wild-type peptide **(13)**. The ligated product was purified by RP-HPLC using a preparative C4 column with a linear gradient of 20-40% B over 50 min to afford the corresponding Tau (2-441, AcK280) **(16)** in 24% yield (Figure S17).

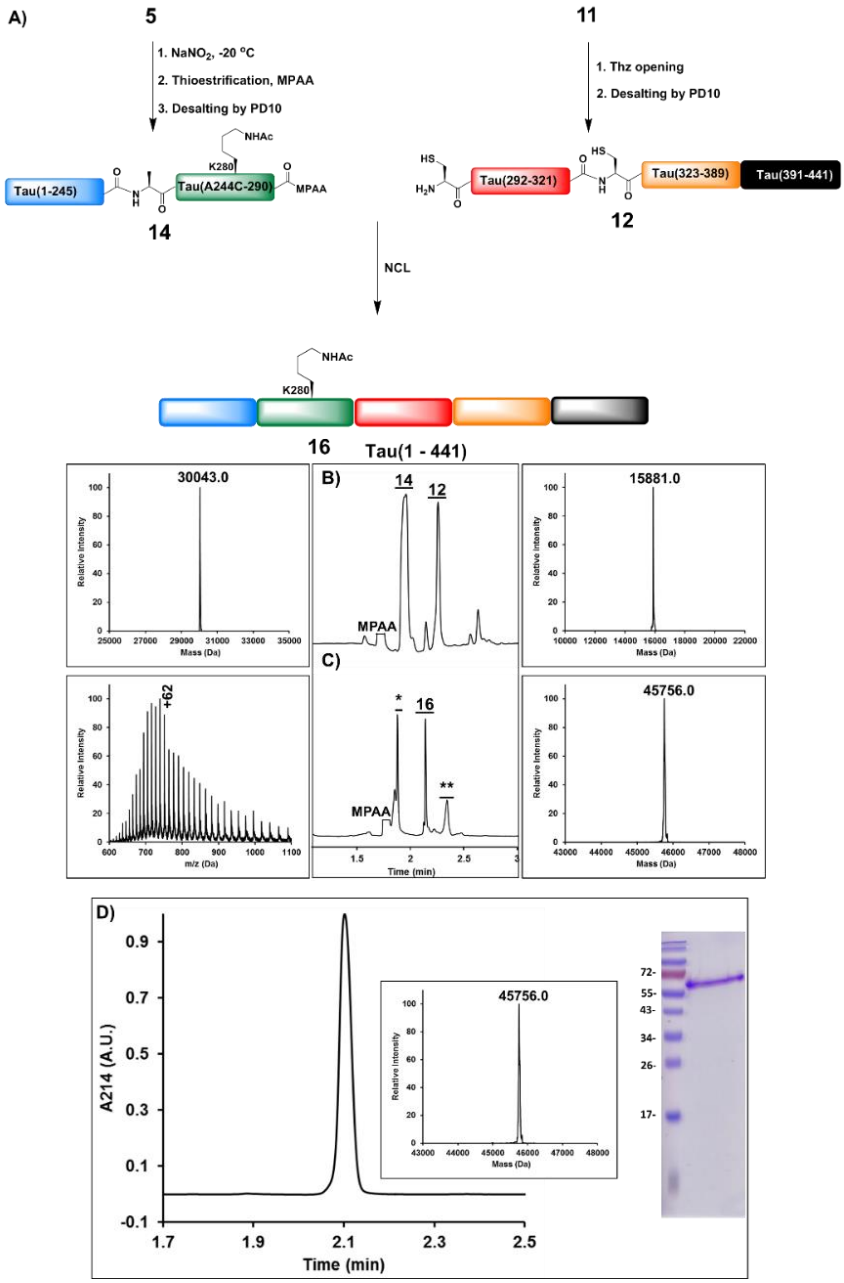

Figure S17: Characterization of the semisynthesized AcK280 Tau. A) Schematic representation of the last ligation step. B, C) Analytical UPLC traces/ (ESI-MS) of the ligation reaction between Tau (2-290, AcK280)-MPAA, and Tau (291-441) at 0 h (B) and after 6 h (C). Peak 16 corresponds to the ligation product. The peak ** corresponds to the remaining traces of the starting material, while the peak * corresponds to the hydrolyzed thioester. D) Analytical UPLC, ESI-MS and SDS-PAGE of the purified AcK280 Tau (observed mass, 45,756 Da; calculated mass 45,760 Da).

Expression and purification of Tau (2-441):

Tau (2-441) cloned into the pET-15b expression vector (a kind gift from Dr. Eliezer, Weill Cornell Medical College). *E.coli* BL21 (DE3) cells were transformed with the corresponding plasmid and grown in 4 liters of LB medium at 37 ^{o}C until OD = 0.5-0.6, followed by induction with 1 mM IPTG for 5 h at 37 °C. After harvesting the cells by centrifugation at 5,000 rpm, the cells were lysed by ultrasonication (10 min: pulse on 59 sec: pulse off 59 sec: 70% Amp) in a buffer containing 3 M urea in 10 mM MES pH 6.5, 1 mM DTT, 1 mM EDTA and 1 mM PMSF. The insoluble material was removed by centrifugation at 18,000 rpm for 1 h at 4 °C. The supernatant was mixed with streptomycin sulfate (1% (w/v)), and the solution was stirred for 1.5 h at 4 °C. After centrifugation at 18,000 rpm for 20 min at 4 °C the supernatant was dialyzed against 4 liters of IEX Buffer A (10 mM MES pH 6.5, 20 mM NaCl, 1 mM DTT, 1 mM EDTA) overnight using 7,000 MWCO tubing at 4 °C. After dialysis, a further centrifugation (18,000 rpm, 20 min, 4 °C), and filtration (0.22 µm PVDA) steps were performed. The supernatant was loaded onto a cation exchange column (Mono S 5/50 GL, GE Healthcare) that had been equilibrated with IEX Buffer A. The protein was eluted using IEX Buffer B: 10 mM MES pH 6.5, 1 M NaCl, and 1 mM DTT, 1 mM EDTA (Figures S18A & B). Fractions containing the protein were pooled and dialyzed overnight against 5% acetic acid and subsequently purified by RP-HPLC using a preparative C4 column with a linear gradient of 30-40% B over 40 min to afford the longest isoform Tau (yield, 18.8 mg / liter of bacteria yield, Figure S18C).

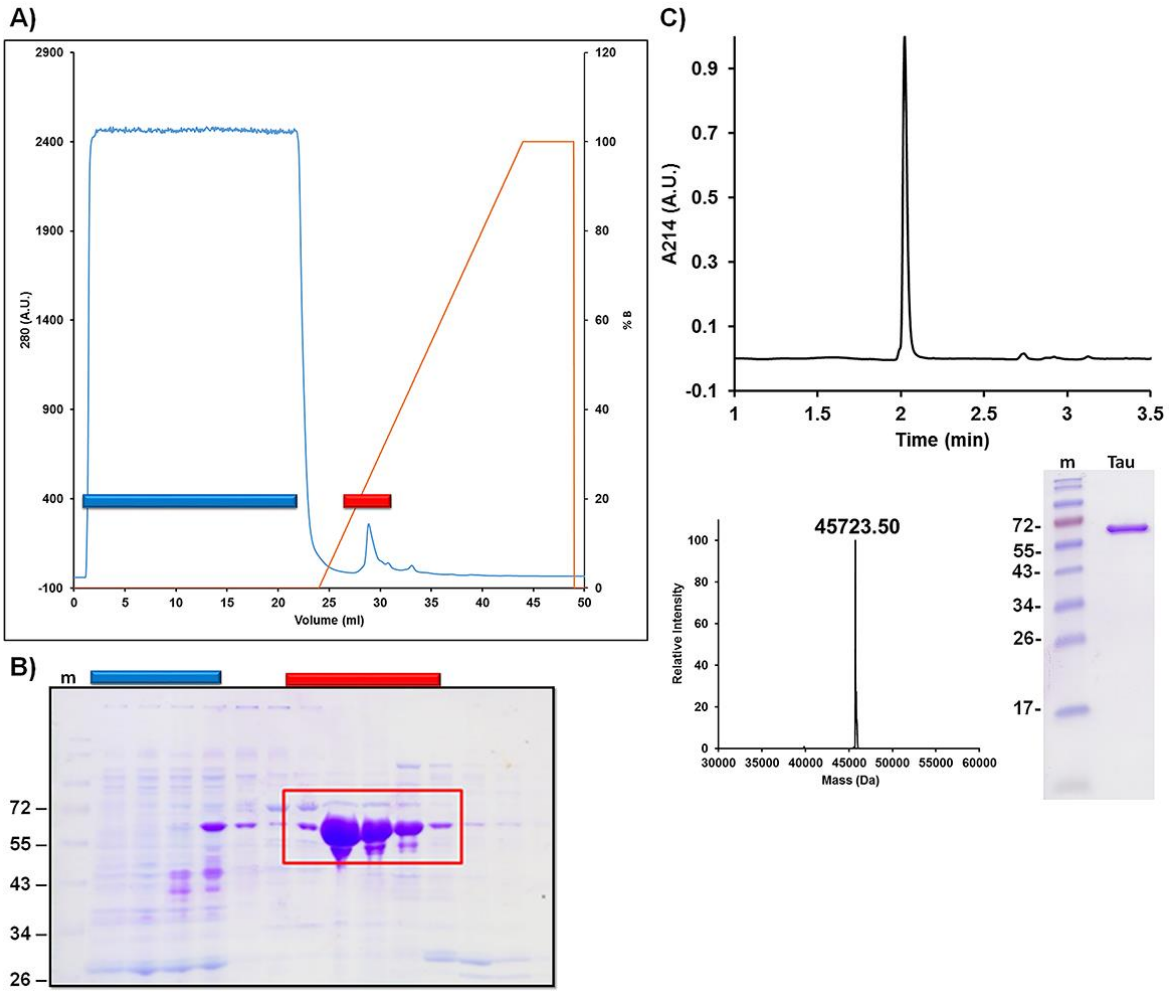

Figure S18: Expression and purification of Tau (2-441) fragment. A) FPLC chromatogram and elution of the fusion protein using a Mono S 5/50 GL column. B) SDS-PAGE analysis of the eluted fractions. C) Analytical RP-HUPLC / ESI-MS and SDS-PAGE of the purified Tau protein (observed mass, 45,723 Da; calculated mass 45,719 Da).

Expression and purification of Tau (2-441, K280Q):

His-SUMO-Tau (2-441, K280Q) cloned into the pD451-SR: T7-sRBS-ORF expression vector (ATUM). *E.coli* BL21 (DE3) cells were transformed with the corresponding plasmid and grown in 6 liters of LB medium at 37 ^{o}C until OD = 0.5-0.6, followed by induction with 1 mM IPTG for overnight at 20 °C. After harvesting the cells by centrifugation at 5,000 rpm, the cells were lysed by ultrasonication (10 min: pulse on 59 sec: pulse off 59 sec: 70% Amp) in a buffer containing 50 mM Hepes, 500 mM NaCl, 5 mM EDTA, and 0.3 mM PMSF. The insoluble material was removed by centrifugation at 18000 rpm for 1 h at 4 °C. The supernatant was applied to a 20-ml

Histrap column equilibrated with same buffer (flow: 3 ml/min). The protein was eluted using lysis buffer containing 500 mM imidazole (Figure S19A).

Fractions containing the fusion protein were pooled (Figure S19B), and cleavage of the His-SUMO tag was carried out by the addition of the SUMO protease ULP1 (0.5 mg/ml, 1 ml of ULP1/40 ml of protein solution) for 15 min at room temperature (Figure S19C). Upon completion of the reaction, the protein was purified by RP-HPLC using a preparative C4 column with a linear gradient of 20-45% B over 40 min to afford the desired protein in 6.7 mg/liter of bacteria yield (Figure S19D).

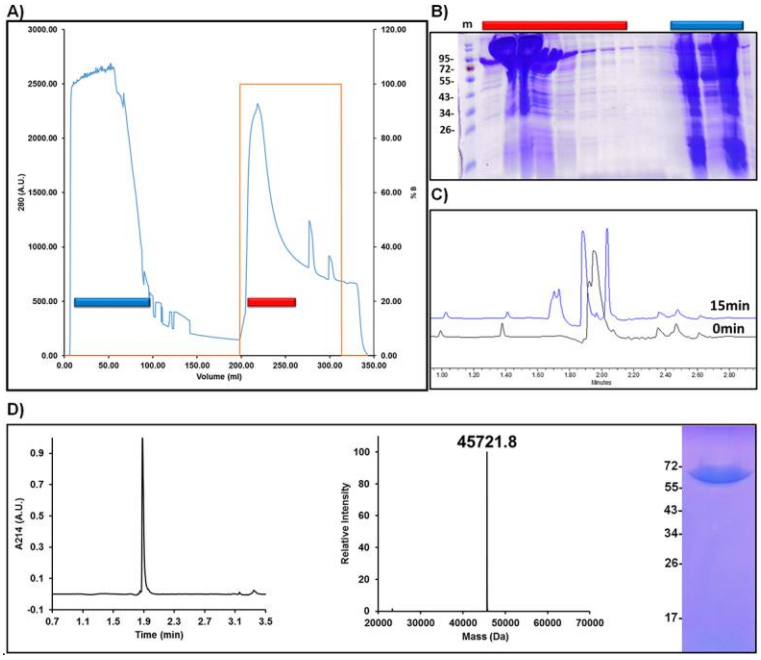

Figure S19: Expression and purification of Tau (2-441, K280Q). A) FPLC chromatogram and elution of the fusion protein using a Ni+2-IMAC column. B) SDS-PAGE analysis of the eluted fractions. C) Analytical RP-HUPLC of the cleavage reaction at time zero and after 15 min. D) Analytical RP-HUPLC / ESI-MS and SDS-PAGE of the purified Tau K280Q protein (observed mass, 45,722 Da; calculated mass 45,719 Da).

Preparation of the full length Tau (2-441, pS396, pS404) (22)

Synthesis of Tau (A390C-441, pS396, pS404) (17):

The peptide **Tau (A390C-441, pS396, pS404) (17)** was synthesized in a similar manner to that described above for the wild type peptide **(7)** with the following changes: 2.5 eq of Fmoc-Ser(HPO3Bzl)-OH was introduced at position Ser396 and Ser404 by manually coupling for 2 h

each. The peptide was purified by RP-HPLC using a preparative C4 column with a linear gradient of 5-40 % B over 40 min to afford the corresponding thioester in ~6 % yield (Figure S20).

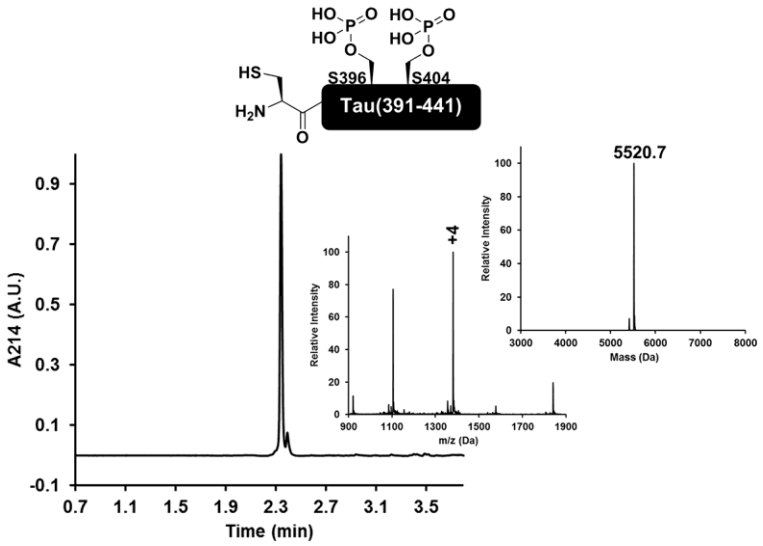

Figure S20: SPPS of Tau (A390C-441, pS396, pS404). Analytical RP-HUPLC and ESI-MS of the purified peptide **17** (observed mass, 5520.7 Da; calculated 5521.9 Da).

Preparation of Tau (C322Thz-441, pS396, pS404) (8):

Ligation: **Tau (C322Thz-389)-SR (6)** (17.0 mg, 1.4 eq) and **Tau (A390C-441,pS396, pS404) (17)** (9.0 mg, 1 eq), were dissolved in NCL buffer (8.0 M urea, 50 mM TCEP, 2 % TFET, pH 7.0) that had been purged with argon. The mixture was left at 37 ºC for 1 h with orbital agitation at 600 rpm and was regularly monitored by RP-UHPLC and LCMS-ESI analyses (Figure S21).

In situ desulfurization: Upon completion of the reaction, the mixture was purged with argon for 15 min to remove the TFET thiol. A solution of 175 mM TCEP in an argon purged 8.0 M urea, 40 mM glutathione, and 40 mM VA–044 was added to the ligation mixture, then incubated at 37 °C for 6 h with orbital agitation at 600 rpm. The progress of the reaction was monitored by RP-UHPLC and LCMS-ESI analyses. The ligated product was purified by RP-HPLC using a preparative C8 column with a linear gradient of 20-35% B over 50 min to afford the corresponding product **(18)** in 28% yield over two steps (Figure S21C & D).

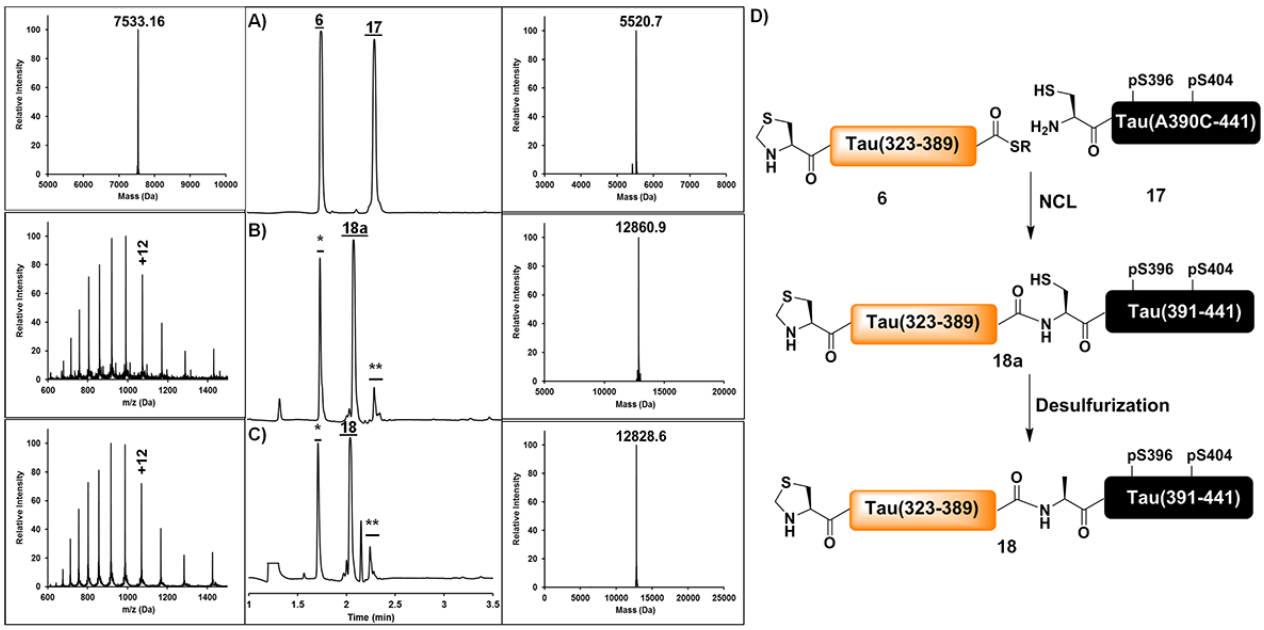

Figure S21: A, B) Analytical RP-HUPLC traces/ (ESI-MS) of the ligation reaction between **Tau (C322Thz-389)-SR**, and **Tau (A390C-441, pS396, pS404)** at 0 h (A) and after 1 h (B). Peak 8a corresponds to the ligation product (observed mass, 12,861 Da; calculated mass 12,861 Da). The peak labeled with * corresponds to the hydrolyzed thioester, and the peak labeled with ** corresponds to the remaining traces of the starting material. C) Analytical RP-HUPLC traces/ (ESI-MS) of the in-situ desulfurization reaction after 6 h. Peak 8 corresponds to the desulfurized product (observed mass, 12,829 Da; calculated 12,829 Da). D) Schematic representation of the semisynthesis of **Tau (C322Thz-441, pS396, pS404) (18)** fragment.

Preparation of Tau (C291Thz-441, pS396, pS404) (20):

Ligation: **Tau (C322Thz-441, pS396, pS404) (18)** (4.5 mg, 1 eq) was dissolved in purged buffer that contains 8.0 M urea, TCEP (30 eq) and 0.1 M methoxylamine hydrochloride. The pH was adjusted to 4.0, and the reaction mixture was kept at 37 °C with orbital agitation at 600 rpm. The reaction was monitored by RP-UHPLC and mass spectrometry until complete deprotection of the Thz adducts. After 1 h the pH was adjusted to 7.0, and a solution of **Tau (C291Thz-321)-SR (9)** (2.4 mg, 2 eq) dissolved in NCL purged buffer (8.0 M urea, 50 mM TCEP, 2 % TFET, pH 7.0) was added. The mixture was left at 37 ºC for 1.5 h with orbital agitation at 600 rpm, and it was monitored by RP-UHPLC and mass spectrometry analyses. The ligated product was purified by RP-HPLC using a preparative C8 column with a linear gradient of 25-45% B over 50 min to afford the corresponding product **(20)** in 35% yield (Figure S22).

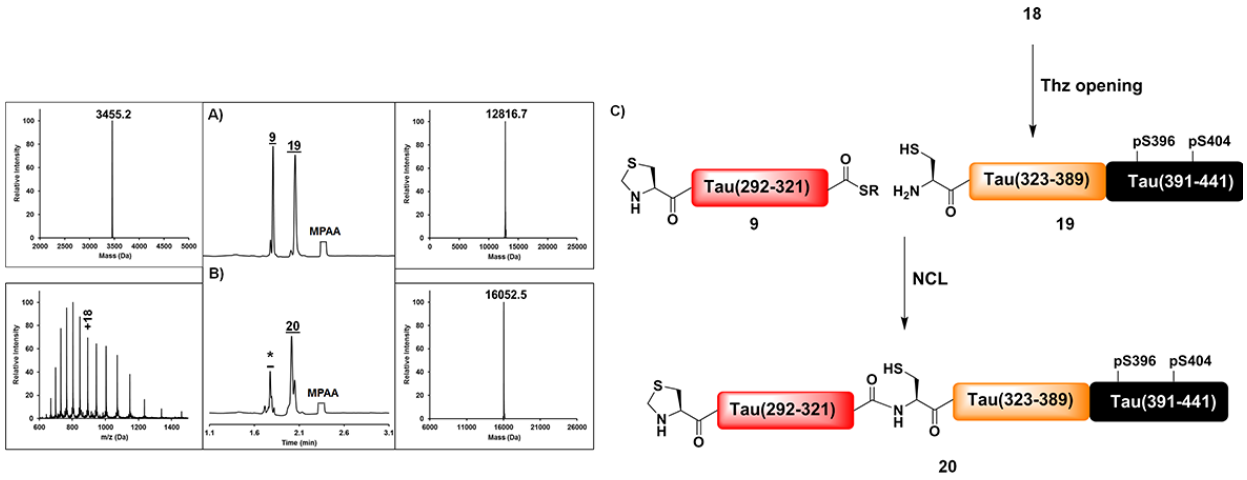

Figure S22: A, B) Analytical RP-HUPLC traces/ (ESI-MS) of the ligation reaction between **Tau (322-441, pS396, pS404)**, and **Tau (C291Thz-321)-SR** at 0 h (A) and after 1.5 h (B). Peak 20 corresponds to the ligation product (observed mass of 16,052 Da; calculated mass, 16,053 Da). The peak labeled with * corresponds to the hydrolyzed thioester. C) Schematic representation of the semisynthesis of the **Tau (C291Thz-441, pS396, pS404) (20)** fragment.

Ligation of Tau(2-290)-MPAA (13) with Tau(291-441, pS396, pS404) (20):

Thz opening of Tau (C291Thz-441, pS396, pS404): Peptide **(20)** (0.9 mg, 1 eq) was treated with 0.1 M methoxylamine hydrochloride as described for the wiled type peptide **(11)**. The pH was adjusted to 4.0, and the reaction mixture was kept at 37 °C with orbital agitation at 600 rpm. The reaction was monitored by RP-UHPLC and mass spectrometry until complete deprotection of the Thz adducts. After 1 h the mixture was desalted using a PD-10 and lyophilized.

Ligation: The procedure used for peptide **15** was followed, except that diphosphorylated peptide **(21)** was used instead of the wild-type peptide **(13)**. The ligated product was purified by RP-HPLC using a preparative C4 column with a linear gradient of 20-40% B over 50 min to afford the corresponding product **(22)** in 16% yield (Figure S23).

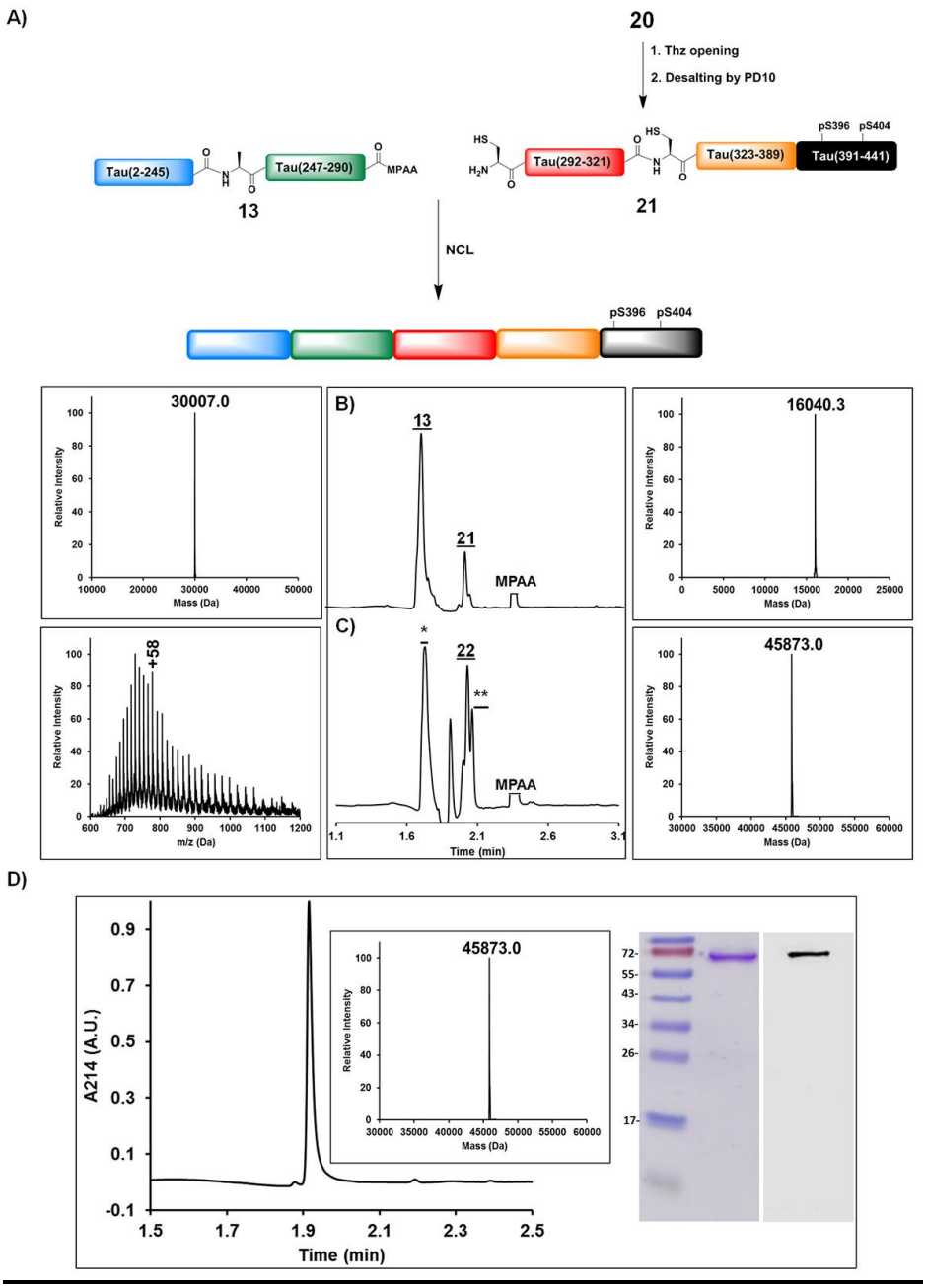

Figure S23: Characterization of the semisynthesized Tau (pS396, pS404). A) Schematic representation of the last ligation step. B, C) Analytical UPLC traces/ (ESI-MS) of the ligation reaction between Tau (2-290)-MPAA, and Tau (291-441, pS396, pS404) at 0 h (B) and after 1.5 h (C). Peak 22 corresponds to the ligation product. The peak ** corresponds to the remaining traces of the starting material, while the peak * corresponds to the hydrolyzed thioester. D) Characterization of the purified pS396, pS404 Tau by analytical UPLC, ESI-MS, SDS-PAGE, and immunoblotting with antibody that specifically recognize Tau phosphorylated at Ser396 & Ser404. (observed mass, 45,873 Da; calculated mass 45,878 Da).

Preparation of the full length Tau (2-441, pY310) (26)

Synthesis of Tau (C291Thz-321, pY310)-SR (23):

Peptide **Tau (C291Thz-321, pY310)-SR (23)** was synthesized in a similar manner to that described above for the wild type peptide **(9)** with the following changes: 2.5 eq of Fmoc-Tyr (HPO3Bzl)-OH was introduced at position Tyr310 by manually coupling for 2 h. The peptide was purified by RP-HPLC using a preparative C18 column with a linear gradient of 5-40 % B over 40 min to afford the corresponding thioester in ~6 % yield (Figure S24).

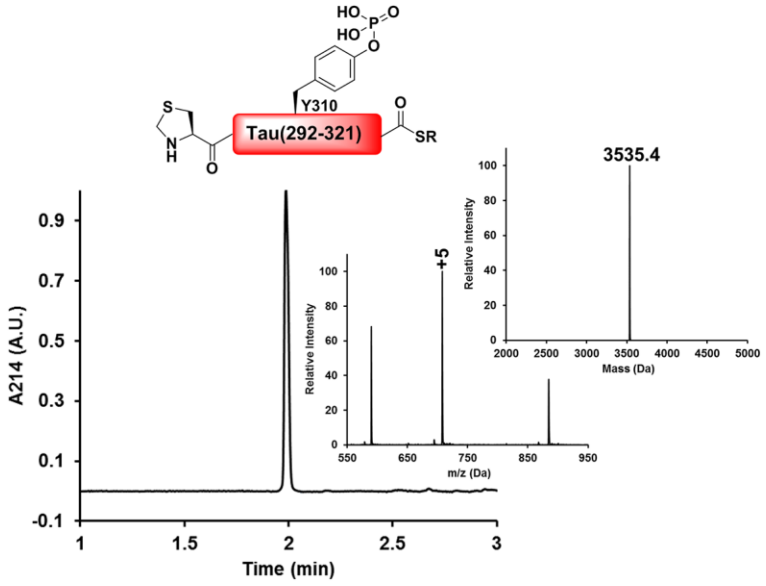

Figure S24: SPPS of Tau (C291Thz-321, pY310)-SR. Analytical RP-HUPLC and ESI-MS of the purified thioester **23** (observed mass, 3,535.4 Da; calculated mass, 3,536.7 Da).

Preparation of Tau (C291Thz-441, pY310) (24):

The procedure used for peptide **(11)** was followed, except that phosphorylated peptide **(23)** was used instead of the wild-type peptide **(9)**. The ligated product was purified by RP-HPLC using a preparative C8 column with a linear gradient of 25-50% B over 50 min to afford the corresponding product **(24)** in 18% yield (Figure S25).

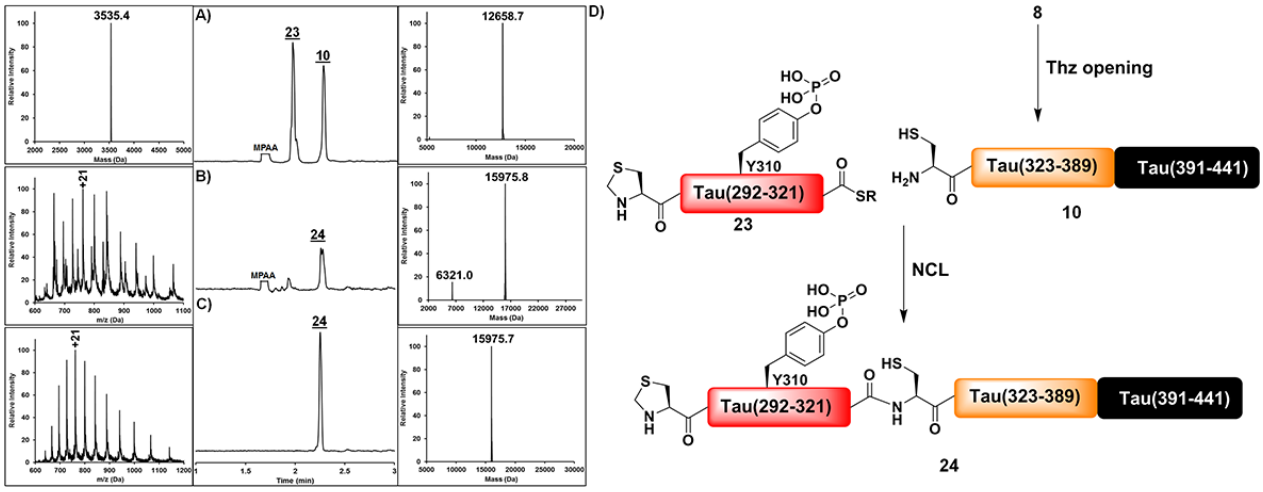

Figure S25: A-C) Analytical RP-HUPLC traces/ (ESI-MS) of the ligation reaction between **Tau (322-441, pY310)**, and **Tau (C291Thz-321)-SR** at 0 h (A), after 4 h (B) and after purification by HPLC (C). Peak 24 corresponds to the ligation product (observed mass of 15,976 Da; calculated mass, 15,973 Da). D) Schematic representation of the semisynthesis of the **Tau (C291Thz-441, pY310) (24)** fragment.

Ligation of Tau(2-290)-MPAA (13) with Tau(291-441, pY310) (24):

The procedure used for preparation for wild-type Tau **(15)** was followed, except that phosphorylated peptide **(25)** was used instead of the wild-type peptide **(12)**. The ligated product was purified by RP-HPLC using a preparative C4 column with a linear gradient of 20-40% B over 50 min to afford the corresponding product **(26)** in 13% yield (Figure S26).

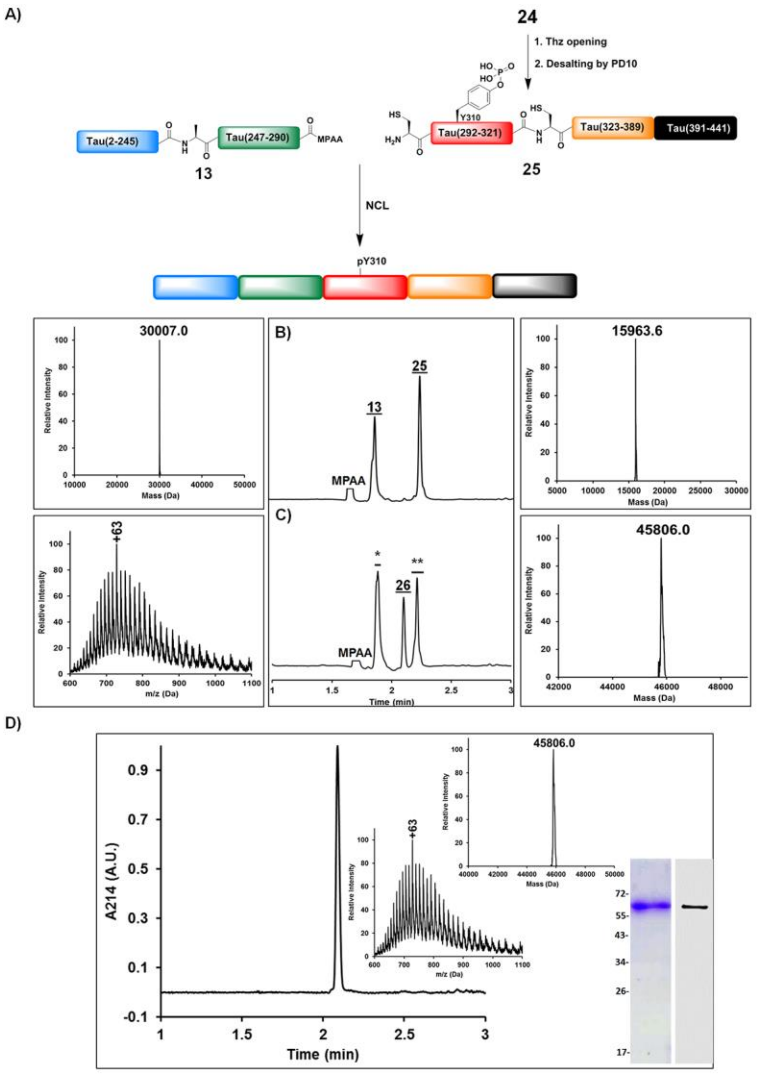

Figure S26: Characterization of the semisynthesized Tau (pY310). A) Schematic representation of the last ligation step. B, C) Analytical UPLC traces/ (ESI-MS) of the ligation reaction between Tau (2-290)-MPAA, and Tau (291-441, pY310) at 0 h (B) and after 5 h (C). Peak 26 corresponds to the ligation product **(26)**. The peak ** corresponds to the remaining traces of the starting material, while the peak * corresponds to the hydrolyzed thioester. D) Characterization of the purified pY310 Tau by analytical UPLC, ESI-MS, SDS-PAGE, and immunoblotting with antibody that specifically recognize Tau phosphorylated at Tyr310. (observed mass, 45,806 Da; calculated mass 45,799 Da).

Biophysical and biochemical analysis

Circular Dichroism (CD) measurements:

At each time point 120 µL of the sample was collected for the CD measurement. The CD spectra were obtained on a Jasco J-815 CD spectrometer using a 1-mm quartz cuvette. Spectra were recorded from 250 to 195 nm with a step size of 0.2 nm, a bandwidth of 1 nm, and a response time of 8 sec at 20 °C. For each spectrum, 5 scans were obtained and averaged. The averaged spectrum was smoothed using a binomial filter with a convolution width of 99 data points and the resulting spectrum was plotted as the mean residue molar ellipticity.

In vitro aggregation studies:

Lyophilized protein samples were dissolved in the aggregation buffer (10 mM phosphate, 50 mM NaF, pH 7.4) to obtain a final concentration of 5 µM. Protein concentrations were determined using using a Pierce® BCA Protein Assay Kit (Thermo Scientific) with bovine serum albumin as the standard. The pH was adjusted to 7.4 using 0.1 M NaOH, followed by filtration for 30 min, 14000 rpm, at 4 °C.

Aggregation was performed by incubating 5 µM Tau protein with heparin sodium salt (Applichem GmbH, Germany) at a 1:4 molar ratio (heparin:protein) and supplementation with 10 µM of ThS (T1892 SIGMA). The extent of fibrillization was measured in triplicate by recording the ThS fluorescence using an excitation filter at a wavelength of 440 nm and an emission filter wavelength of 480 nm in cycles of 10 min over-time, without shaking at 37°C using a Fluostar Omega® plate reader. The resulting curves were fitted to one-phase exponential association according to the following equation:

$$Y = y_{max}*(1-\exp(-k*t))$$

where Y is defined as the fluorescence at time t, y_{max} corresponds to the maximum signal of the curve, k is the growth rate constant, and $t_{1/2}$ is defined as $t_{1/2} = 0.69\ k$ (Table S1). Each experiment was conducted in triplicate, and repeated at least three times. The fitted curves were

determined from the mean of all experiments. The values presented in the table are the means ± SEM (Table S1).

Sedimentation assay:

Twenty microliter samples of the aggregation reactions were collected and pelleted by ultracentrifugation at 100,000 rpm for 2 h at 4 °C. Ten microliter samples of the supernatant (S) were then mixed with 2x Laemmli buffer and resolved using on a 15 % SDS-PAGE. The pellet s were re-suspend in 20 µl of the same buffer, and the 10 µl of the pellet (P) was mixed with 2x Laemmli buffer and resolved using 15 % SDS-PAGE.

Length measurements of aggregates:

A TEM image analysis was performed using Photoshop and Image J software to measure the length and diameter of the aggregates. Photoshop was used to label the aggregates and categorize the aggregates to two main categories; fibrils and oligomers. Image J software was used to measure the fibril length or oligomers diameter in units of nm.

Tubulin polymerization assay:

Microtubules activities assays of WT Tau or ACK280 Tau were conducted by mixing 15 µM of samples with tubulin (3 mg/ml in 100 µl total volume) in MT assembly buffer (80 mM PIPES pH 6.9, 2 mM $MgCl_2$ and 0.5 mM EGTA) supplemented with 1 mM GTP. Polymerization was started by incubation at 37 °C and assessed by monitoring the absorbance every 30 sec at 340 nm using a TECAN infinite M200PRO plate reader.

The resulting curves were fitted to Boltzmann sigmoidal according to the following equation:

$$y= y_0 + (y_{max} - y_0) / 1 + \exp((k(t_{1/2} –t))$$

where y is defined as the florescence at time t, y_0 and y_{max}, correspond to the minimum and the maximum signal of the curve respectively, $t_{1/2}$ corresponds to the reflection point (the value of time at the midpoint) k is the growth rate constant, and the lag time is defined as $t_{lag}= t_{1/2} – 1/2\ k$ (Table S2). Each experiment was performed in duplicate or triplicate, and repeated at least two

times. The fitted curves were determined from the mean of all experiments. The values in the presented in the table are the means ± SEM (Table S2).

Western blot analysis:

For western blot, Tau proteins were transferred to nitrocellulose membranes using the semidry transfer system from Bio-Rad. The membranes were incubated in Odyssey blocking solution (Li-COR Biosciences), for overnight at 4 ^{0}C, and latter incubated with the following primary antibodies for 1h at room temperature: monoclonal mouse anti-Tau (Tau-5, 1:1000, from ThermoFisher), monoclonal mouse anti-human Tau (Tau13, 1:1000, from Abcam), polyclonal rabbit anti-AcK280-Tau (Acetyl K280, 1:1000, from Anaspec), polyclonal rabbit anti-pY310-Tau (pY310, 1:5000, generated by Eurogentec S.A. (Belgium)), Monoclonal mouse anti-pS396,pS404 Tau (PHF-1, 1:1000, from Peter Davies).

Transmission Electron Microscopy (TEM):

5 µL of samples were deposited on onto glow-discharged Formvar-coated 200-mesh copper grids (Electron Microscopy Sciences) for 60-90 sec. The grids were then washed twice with dd H_2O, and stained with 0.7 % (w/v) uranyl formate for 30 sec and dried. A Tecnai Spirit BioTWIN electron microscope operated at 80 kV and equipped with a LaB_6 gun and a 4K x 4K FEI Eagle CCD camera was used to observe the samples.

In vitro acetylation:

Full-lengh WT Tau protein was dissolved in acetylation buffer (50 mM Tris-HCl pH 8, 10% glycerol, 1 mM DTT, 100 mM EDTA, and 1 mM PMSF) at a final concentration of 0.5 mg/ml. The acetylation reaction was initiated by incubating Tau with acetyl-CoA (0.4 mM) and p300 or the CBP catalytic domain (aa 1319-1710) (200 ng per 1 µg of Tau protein), for 1-4 h at 37 °C with continuous shaking at 900 rpm. At each time point, 5 µl of the reaction mixture was diluted with 25 µl of 50 % acetonitrile-water and analyzed by LCMS-ESI.

Supplemental results

Aggregation kinetics of the semisynthetic WT Tau and AcK280 Tau

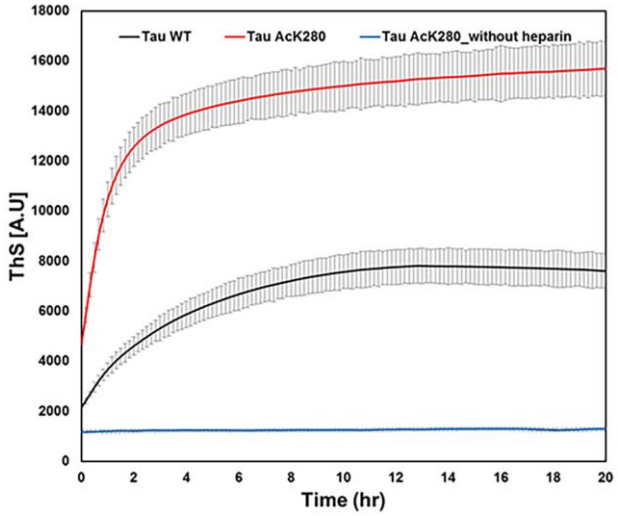

Figure S27: Thioflavin S fluorescence assay of the semisynthetic WT Tau and AcK280 Tau measured at 490nm (mean ± SEM, n=3).

Sedimentation assay of the WT and AcK280 Tau using ultracentrifugation

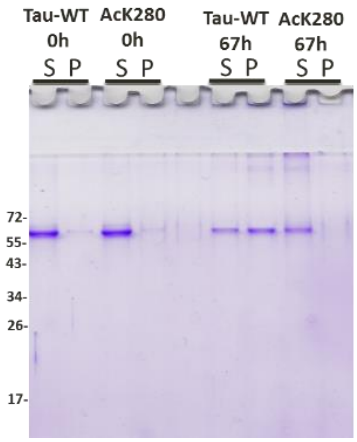

Figure S28: WT and AcK280 Tau proteins at the indicated time points after aggregation were centrifuged at 100,000 rpm for 2 h. 10 µl of the supernatant (S) and the pellet (P) were mixed with 2x Laemmli buffer and run on a 15 % SDS-PAGE.

Table S1. Summary of the aggregation kinetic parameters

	Tau AcK280	Tau K280Q	Tau WT
y_{max} (%)	63.1 ± 0.2	100.5 ± 0.1	56.2 ± 0.1
k (h^{-1})	0.65 ± 0.02	0.53 ± 0.01	0.236 ± 0.003
$t_{1/2}$ (h)	1.1	1.3	2.9

Table S2. Summary of the MTs polymerization kinetic parameters

	WT Tau	AcK280 Tau
y_0 (%)	= 0	= 0
y_{max} (%)	~100	82.6 ± 1.2
k (min^{-1})	1.434 ± 0.258	1.385 ± 0.187
$t_{1/2}$ (min)	5.064 ± 0.175	6.021 ± 0.129
t_{lag} (min)	4.347	5.328

In vitro acetylation of the WT Tau by p300 and CREB

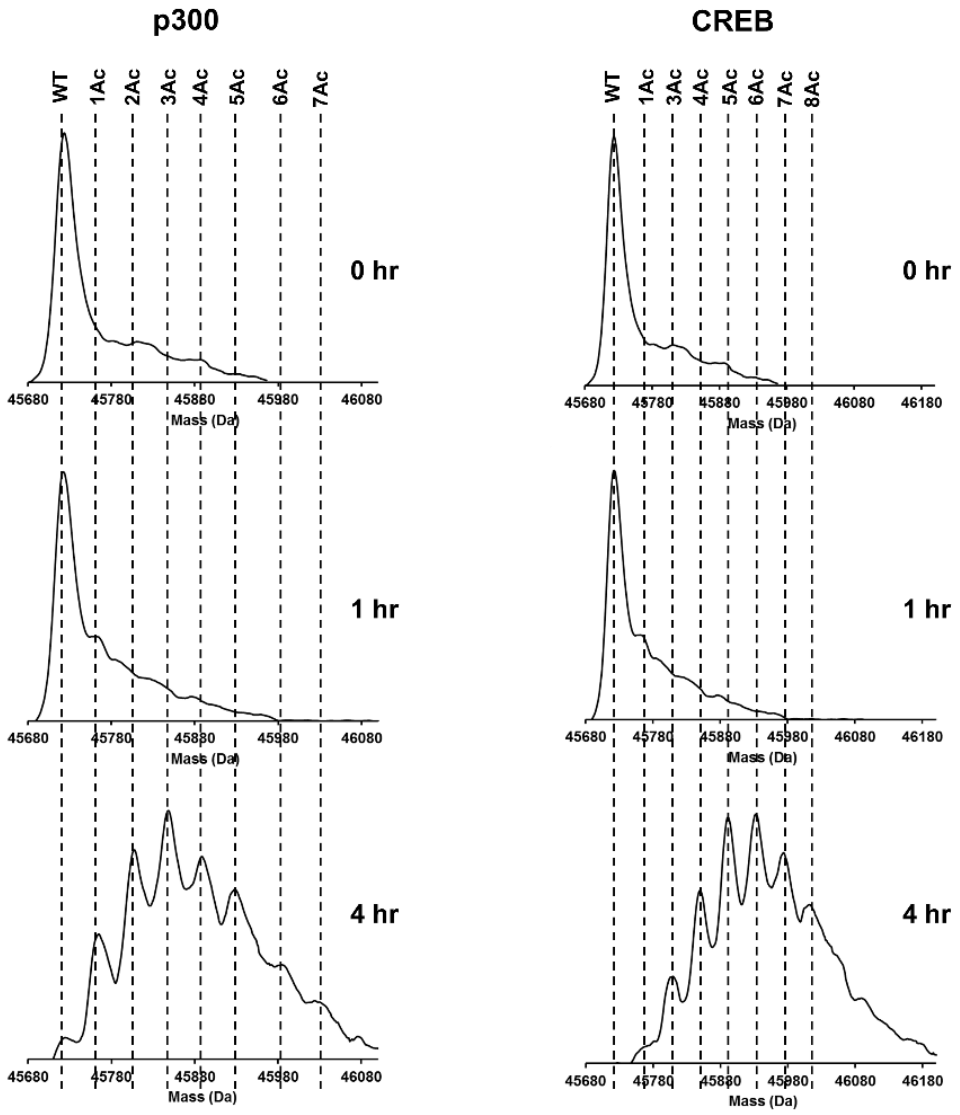

Figure S29: LCMS-ESI analysis after in-vitro acetylation of full-length Tau with p300 (left) or the CBP catalytic domain (right), demonstrating the lack of specificity of these enzymes.

Western blot analysis of the semisynthetic Tau variants

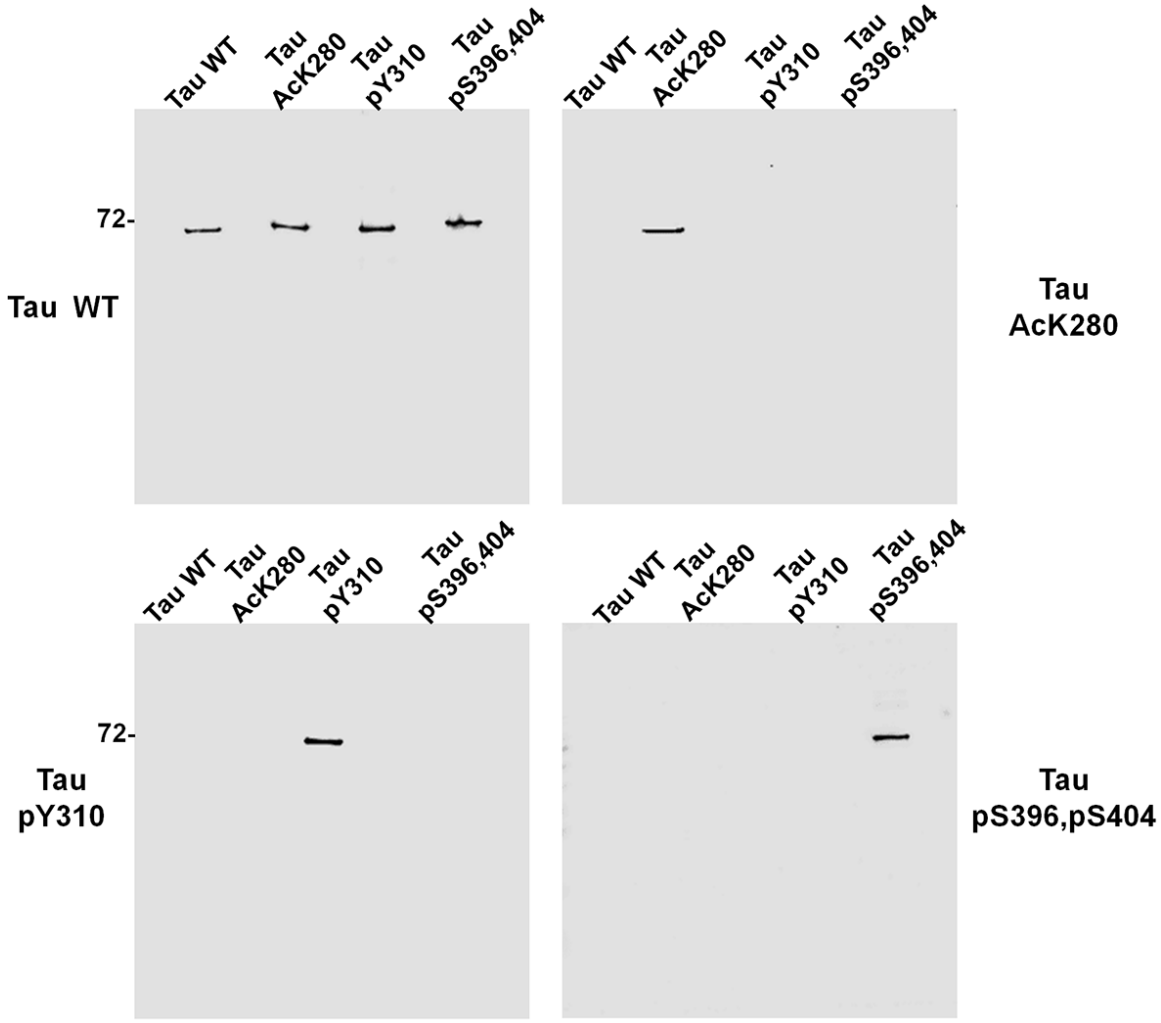

Figure S30: Western blot analysis of the purified semisynthetic Tau proteins using different antibodies (monoclonal mouse anti-human Tau (Tau13, 1:1000, from Abcam), polyclonal rabbit anti-AcK280-Tau (Acetyl K280, 1:1000, from Anaspec), polyclonal rabbit anti-pY310-Tau (pY310, 1:5000, generated by Eurogentec S.A. (Belgium)), Monoclonal mouse anti-pS396,pS404 Tau (PHF-1, 1:1000, from Peter Davies).