Identification of O-GlcNAc sites within peptides of the Tau protein and their impact on phosphorylation.

Caroline Smet-Nocca,[*a] *Malgorzata Broncel,*[b] *Jean-Michel Wieruszeski,*[a] *Caroline Tokarski,*[c] *Xavier Hanoulle,*[a] *Arnaud Leroy,*[a] *Isabelle Landrieu,*[a] *Christian Rolando,*[c] *Guy Lippens,*[a] *and Christian P. R. Hackenberger,*[*b]

[a] Unité de Glycobiologie Structurale et Fonctionnelle, CNRS UMR8576, IFR 147, Université de Lille1, 596655 Villeneuve d'Ascq Cedex, France.
Fax: +33 (0)3 2043 6555 ; Tel: +33 (0)3 2043 4997; E-mail: caroline.smet@univ-lille1.fr
[b] Institut für Chemie und Biochemie, Freie Universität Berlin, Takustrasse 3, 14195 Berlin, Germany.
Fax: +49 (0)3 08385 2551; Tel: +49 (0)3083852451; E-mail: hackenbe@chemie.fu-berlin.de
[c] Miniaturisation pour l'Analyse, la Synthèse & la Protéomique, USR CNRS 3290, IFR 147, Université de Lille1, 59655 Villeneuve d'Ascq Cedex, France.

1 General information

Materials: All reagents, amino acids, and solvents were purchased from commercial suppliers and used without further purification if not further mentioned. Solvents (CH_3CN, CH_2Cl_2, DMF) were purchased from ACROS ORGANICS.

Abbreviations: AD, Alzheimer's disease; CDK: cyclin-dependent kinase; ESI-TOF MS: ElectroSpray Ionization Time-Of-Flight mass spectrometry; ETD/ECD MS: Electron Transfer Dissociation/Electron Capture Dissociation mass spectrometry; FT-ICR: Fourier Transform Ion Cyclotron Resonance; GSK3β glycogen synthase kinase 3β; HSQC, heteronuclear single quantum correlation spectroscopy; IPTG: isopropyl-β-D-1-thiogalactopyranoside; MALDI-TOF MS: Matrix-Assisted Laser Desorption-Ionization Time-Of-Flight mass spectrometry; RP-HPLC: reverse phase high performance liquid chromatography; SPPS : solid phase peptide synthesis.

2 Supplemental Tables and Figures

2.1 Table S1

Summary of ESI-TOF MS data of the synthesized peptides used in this study. All the peptides were purified to more than 95% as checked by RP-HPLC.

peptide	ESI-TOF-MS
RSGYSS$_{199}$PGS$_{202}$PGT$_{205}$PGSR	m/z: 775.3748 $[M+2H]^{2+}$ (calcd.: m/z: 775.3718)
RSGYSSPGSPGpT$_{205}$PGSR	m/z: 815.3458 $[M+2H]^{2+}$ (calcd.: m/z: 815.3550)
RSGYSSPGpS$_{202}$PGTPGSR	m/z: 815.3471 $[M+2H]^{2+}$ (calcd.: m/z: 815.3550)
SSPGSPGTPGS$_{208}$RSRTPSLPT	m/z: 963.4867 $[M+2H]^{2+}$ (calcd.: m/z: 963.4879)
TPGSRSRTPS$_{214}$LPTPPT	m/z: 826.4409 $[M+2H]^{2+}$ (calcd.: m/z: 826.4422)
KKVAVVRT$_{231}$PPKS$_{235}$PSSA	m/z: 826.4966 $[M+2H]^{2+}$ (calcd.: m/z: 826.4968)
VRTPPKSPSS$_{238}$AKSRLQ	m/z: 869.9994 $[M+2H]^{2+}$ (calcd.: m/z: 870.0002)
KNVKSKIGS$_{262}$TENLKHQ	m/z: 906.0128 $[M+2H]^{2+}$ (calcd.: m/z: 906.0108)
IVYKS$_{396}$PVVS$_{400}$GDTS$_{404}$PRHLSNV	m/z: 1078.0814 $[M+2H]^{2+}$ (calcd.: m/z: 1078.0794)
IVYKSPVVSGDTpS$_{404}$PRHLSNV	m/z: 1118.0626 $[M+2H]^{2+}$ (calcd.: m/z: 1118.0626)
IVYKSPVVpS$_{400}$GDTSPRHLSNV	m/z: 1118.0634 $[M+2H]^{2+}$ (calcd.: m/z: 1118.0626)
IVYKpS$_{396}$PVVSGDTSPRHLSNV	m/z: 1118.0652 $[M+2H]^{2+}$ (calcd.: m/z: 1118.0626)

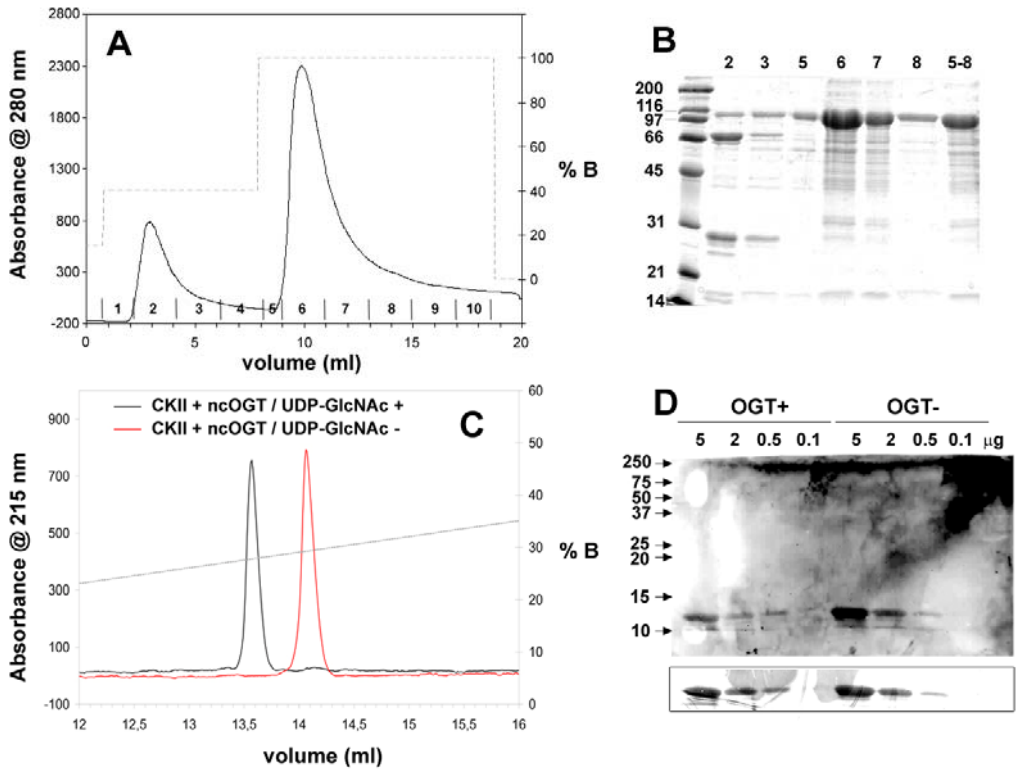
2.2 Table S2

Screening of the O-N-acetylglucosaminyltransferase activity on Tau peptides. Position of the first and the last residues in the Tau primary sequence (Tau441 isoform numbering) and phosphorylation sites are given in the first column. The peptide sequence is described in the second column. The O-GlcNAc sites as identified by ECD MS and NMR are indicated by an asterisk and mentioned in the last column. m/z values are given for the unmodified and the O-GlcNAcylated peptides when detected. N.d., no detectable activity.

peptide name	peptide sequence	m/z (unmodified peptide)	m/z (O-GlcNAc peptide)	O-GlcNAc site
Tau[194-209]	RSGYSSPGSPGTPGS*R	1550.7496	1753.4508	S208
Tau[194-209]pS202	RSGYSSPGpSPGTPGSR	1652.0514 (Na adduct)	1855.1332 (Na adduct)	
Tau[194-209]pT205	RSGYSSPGSPGpTPGSR	1629.1894	1832.1993	
Tau[198-217]	SSPGSPGTPGSRSRTPSLPT	1925.2733	2128.2889	S208
Tau[205-220]	TPGSRSRTPSLPTPPT	1652.8818	N.d.	
Tau[224-239]	KKVAVVRTPPKSPSS*A	1652.9932	1855.1463	S238
Tau[229-244]	VRTPPKSPSSAKSRLQ	1738.5494	1942.5808	S238
Tau[354-369]	KNVKSKIGSTENLKHQ	1812.0256	N.d.	
Tau[392-411]	IVYKSPVVS*GDTSPRHLSNV	2156.1588	2359.0423	S400
Tau[392-411]pS396	IVYKpSPVVSGDTSPRHLSNV	2236.1304	2439.3807	
Tau[392-411]pS400	IVYKSPVVpSGDTSPRHLSNV	2236.1268	N.d.	
Tau[392-411]pS404	IVYKSPVVSGDTpSPRHLSNV	2236.1252	2439.4535	

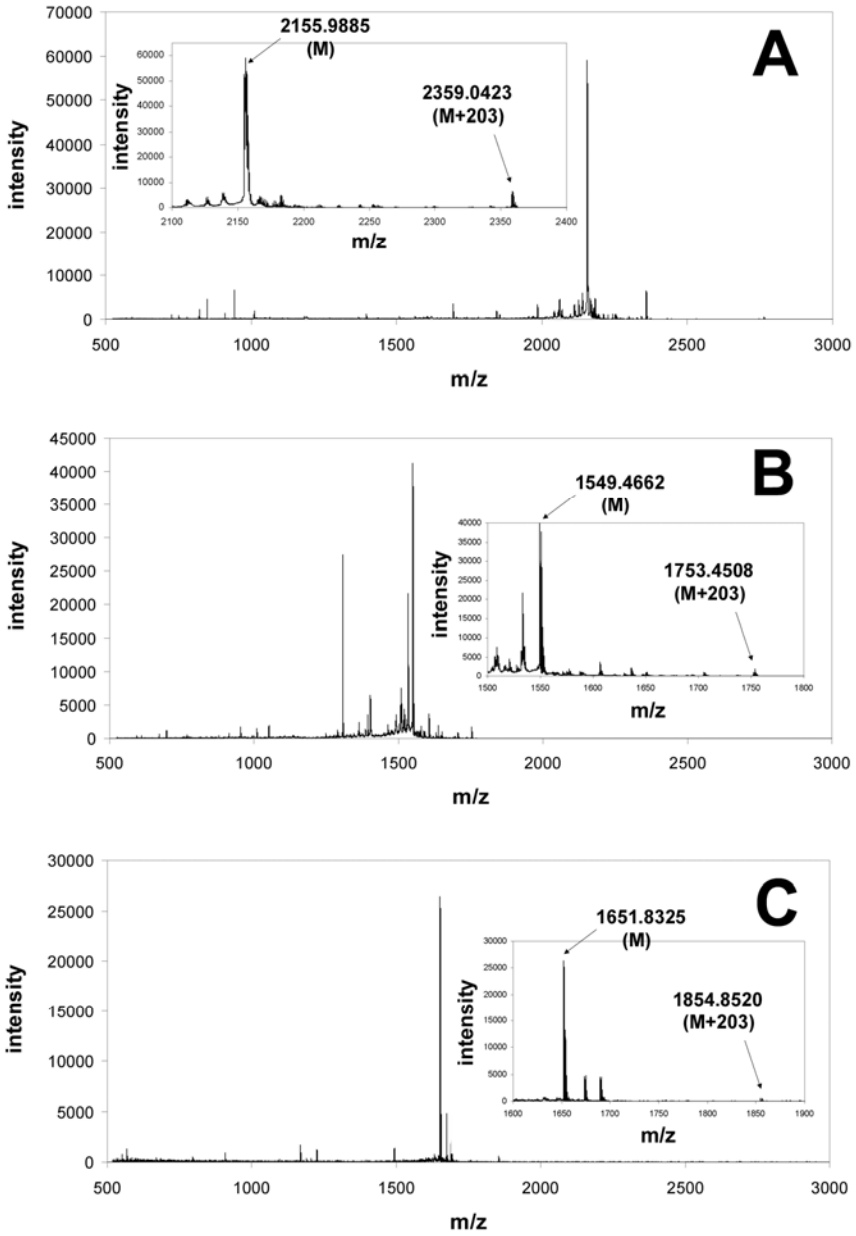
2.3 Figure S1

Purification of the recombinant ncOGT and O-N-acetylglucosaminyltransferase activity on a CKII peptide or 15N-labeled Tau[185-245] fragment. (A) ncOGT purification by affinity chromatography on a Ni2+ nitrilotriacetic column. The absorbance at 280 nm is depicted by the black line and the percentage of elution buffer (50 mM KH2PO4/K2HPO4 pH 7.60, 250 mM NaCl, 250 mM imidazole) by the dotted line (%B). A truncated form as shown in SDS PAGE of fractions 2 and 3 elutes at 40% B while the full-length ncOGT elutes at 100% B. Pooled fractions 5 to 8 were used for in vitro glycosylation experiments. (B) 12% polyacrylamide SDS gel electrophoresis of eluted fractions. (C) Reverse phase chromatography (absorbance at 215 nm) of the CKII peptide incubated with purified ncOGT and 1 mM UDP-GlcNAc (black chromatogram) or without UDP-GlcNAc (red chromatogram) as a control. The content of elution buffer (0.1% TFA in 80% acetonitrile) during the gradient is depicted by the grey line (%B). (D) HRP-WGA immunodetection of the 15N-labeled Tau[185-245] [1] NMR samples incubated (OGT+) or not (OGT-) with ncOGT. In the boxed area, equal protein loading as checked by coloration of the membrane by Ponceau red.



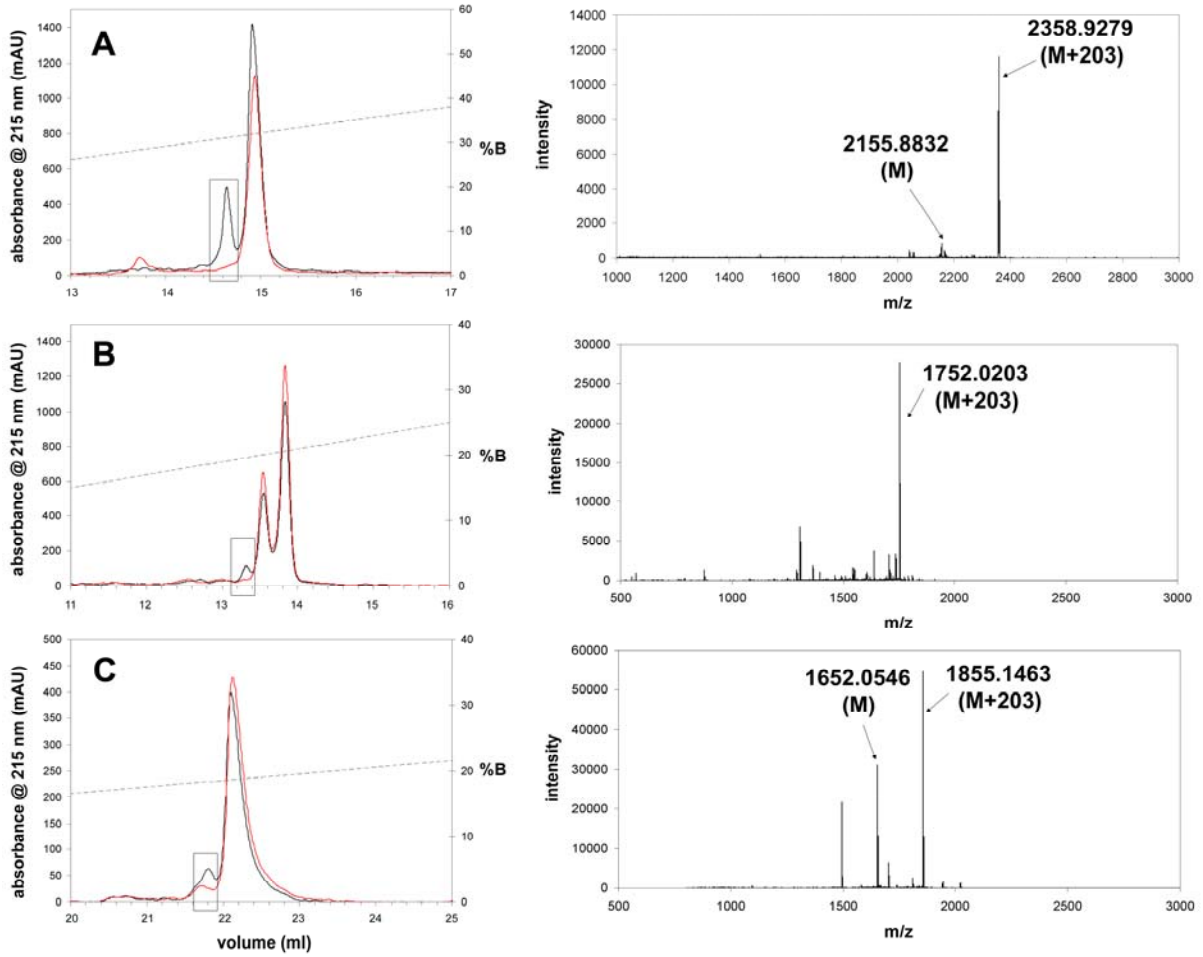
2.4 Figure S2

MALDI-TOF MS screening of Tau peptides for O-GlcNAc transferase activity of ncOGT. Analyses of crude reaction mixtures of Tau[392-411] (A), Tau[194-209] (B) and Tau[224-239] (C) peptides incubated at 37°C for 2 days with recombinant ncOGT.



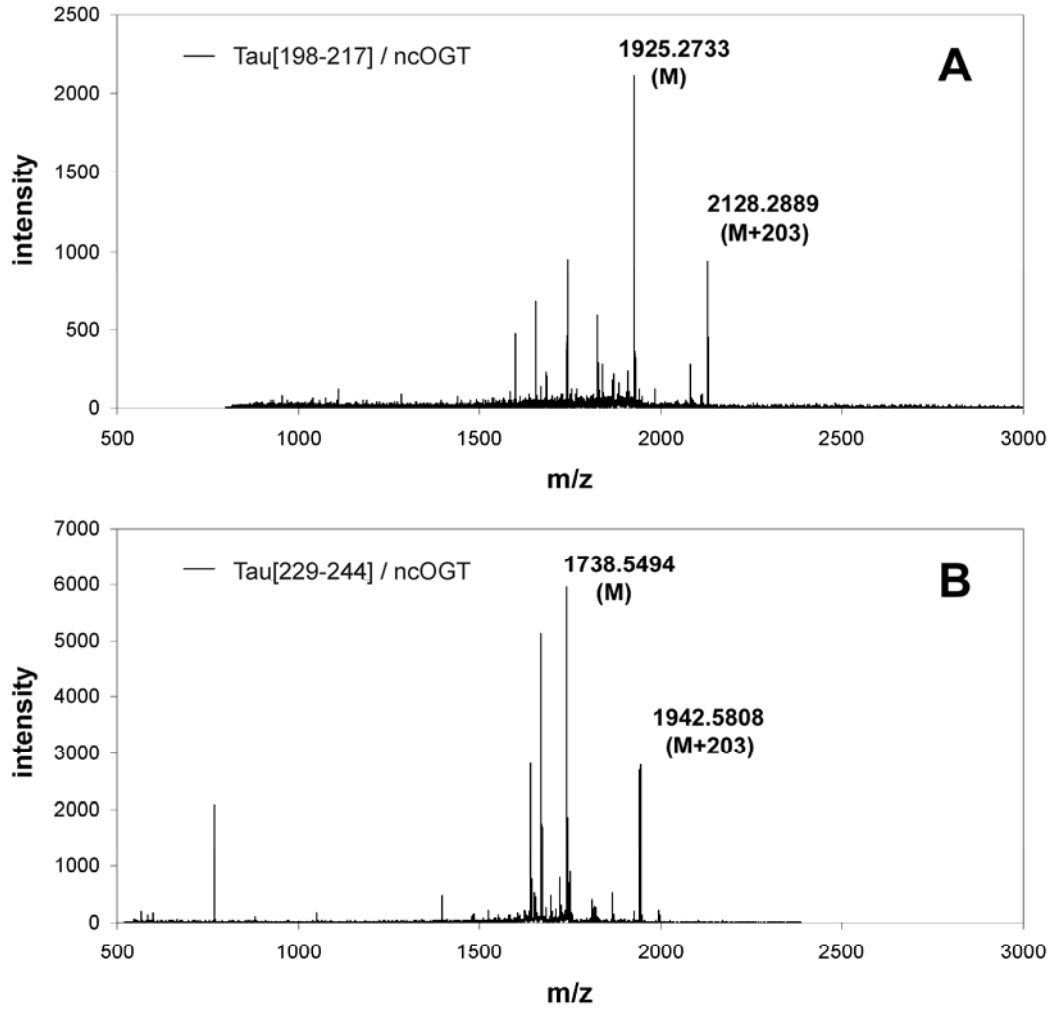
2.5 Figure S3

Enrichment of O-GlcNAc conjugated Tau peptides by reverse phase chromatography for determination of O-GlcNAc sites. Left panel, chromatograms obtained by reverse phase chromatography of crude reaction mixtures of Tau[392-411] (A), Tau[194-209] (B) and Tau[224-239] (C) peptides. Peptides were incubated at 1 mM with ncOGT and 10 mM UDP-GlcNAc at 37°C for 2 days (black curves) or without UDP-GlcNAc as a control (red curves). Right panel, MALDI-TOF MS analyses of O-GlcNAc-enriched fractions of Tau peptides corresponding to the boxed region in the chromatograms. 'M' indicates the mass of the unmodified peptide and 'M+203' the mass of the O-GlcNAc conjugated peptide.



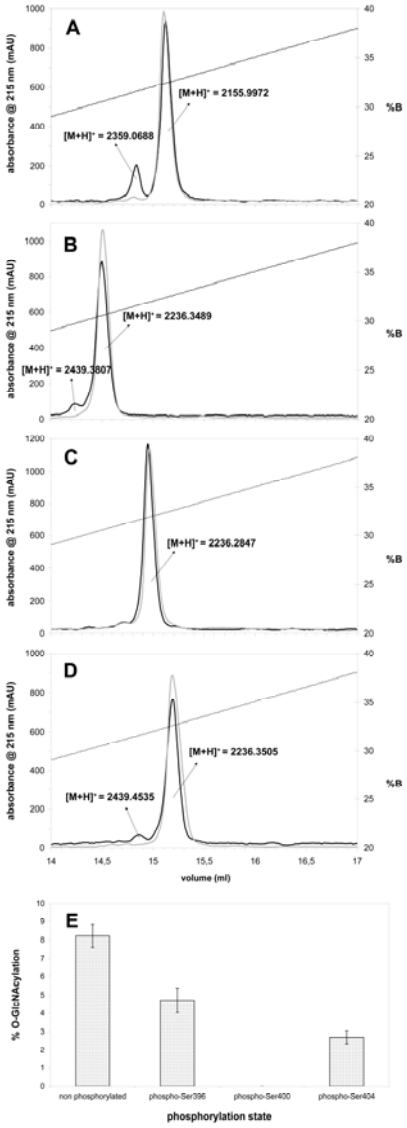
2.6 Figure S4

MALDI-TOF MS analyses of the O-GlcNAc enriched fractions of Tau[198-217] (A) and Tau[229-244] (B) peptides after ncOGT incubation for 2 days at 37°C with 10 mM UDP-GlcNAc and RP-HPLC purification, confirming the O-GlcNAc modification of the newly identified S208 and S238 sites. 'M' indicates the mass of the unmodified peptide and 'M+203' the mass of the O-GlcNAc conjugated peptide.



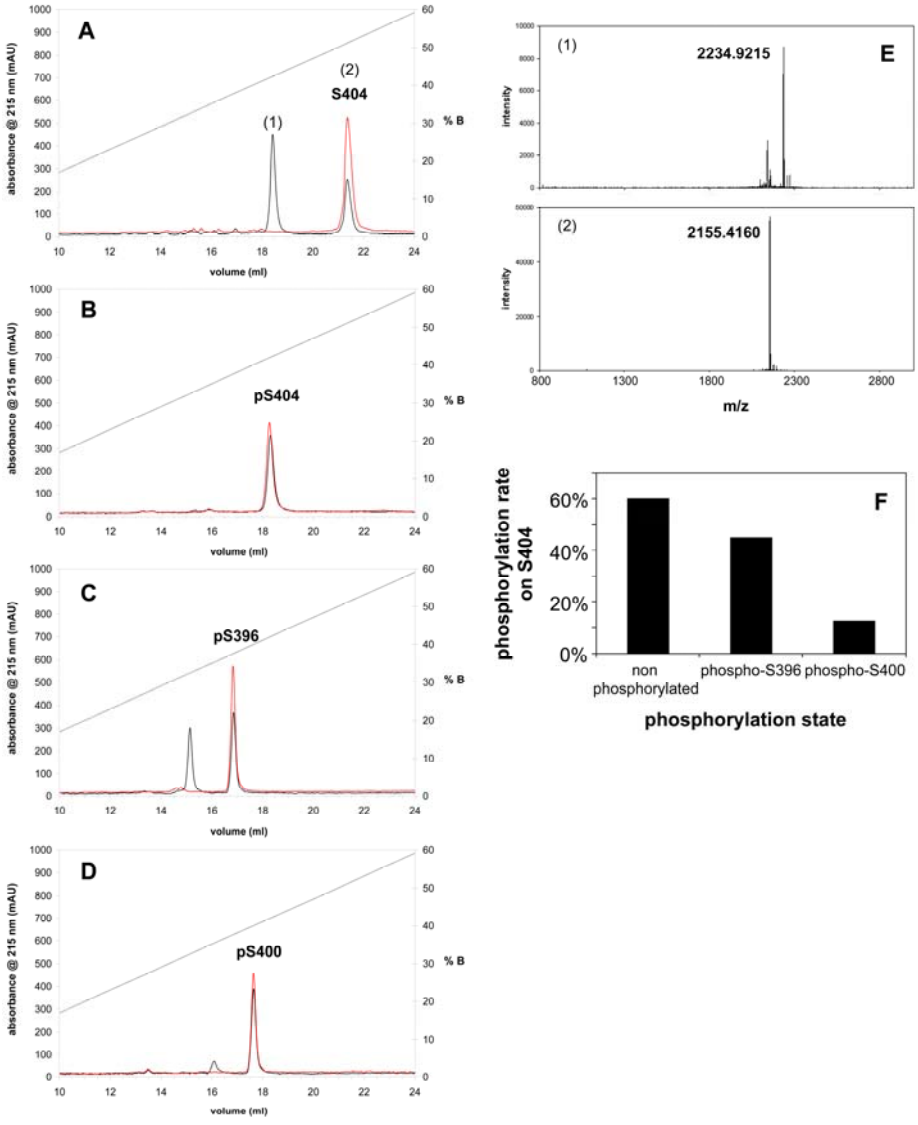
2.7 Figure S5

Down-regulation of S400 O-GlcNAcylation by S396 or S404 phosphorylation. (A-D) Reverse phase chromatography of crude O-GlcNAcylation mixtures in the presence of 1 mM UDP-GlcNAc (black chromatograms) of Tau[392-411] peptide in its non phosphorylated (A), phospho-S396 (B), phospho-S400 (C) and phospho-S404 (D) forms. Control experiments without UDP-GlcNAc were represented as grey chromatograms. $[M+H]^+$ represents the mass of ions determined by MALDI-TOF MS of HPLC fractions are indicated. (E) O-N-acetyltransferase activity of ncOGT on S400 residue is represented as a function of the Tau[392-411] phosphorylation state.



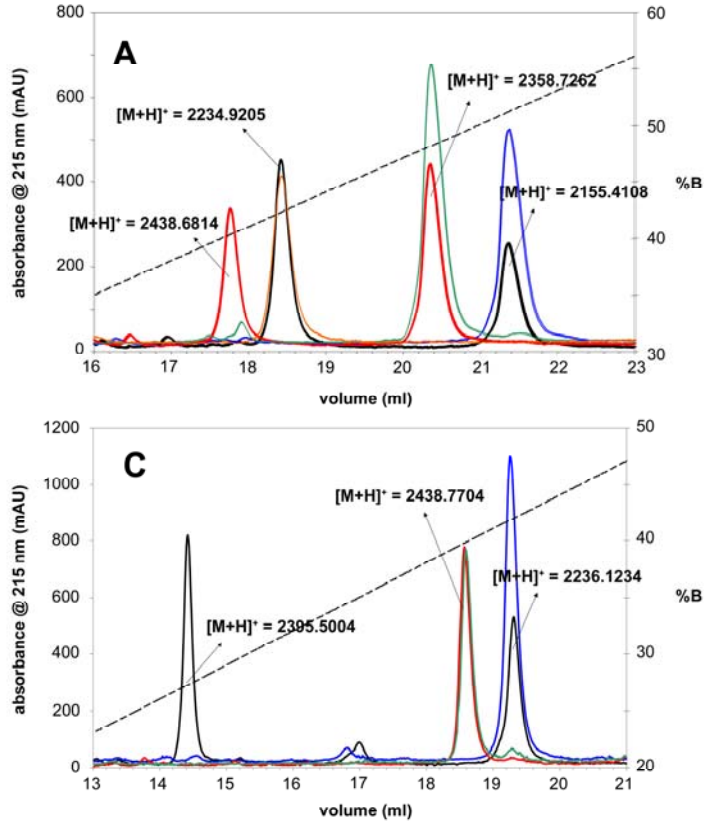
2.8 Figure S6

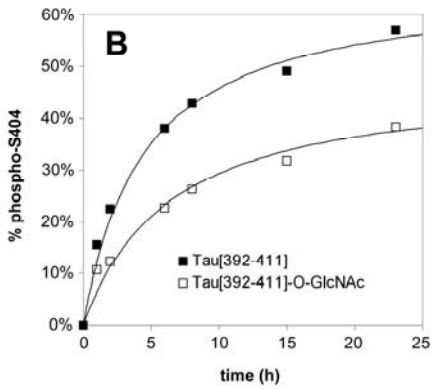
CDK2/cyclinA3-mediated phosphorylation of the Tau[392-411] series. (A-D) Chromatogram curves at 215 nm of crude phosphorylation mixtures. Tau[392-411] peptides either in its non phosphorylated (A), phospho-S404 (B), phospho-S396 (C) or phospho-S400 (D) forms are incubated with the CDK2/cyclinA3 complex in the presence of 1 mM ATP (black) or in the absence of ATP (red). The linear gradient of acetonitrile (%B) is represented by grey lines. (E) Mass spectra of the fractions (1) and (2) isolated from the Tau[392-411] phosphorylation reaction corresponding to the peak at 18.43 min and 21.36 min, respectively. (F) S404 phosphorylation levels as a function of the phosphorylation state of the Tau[392-411] peptide.



2.9 Figure S7

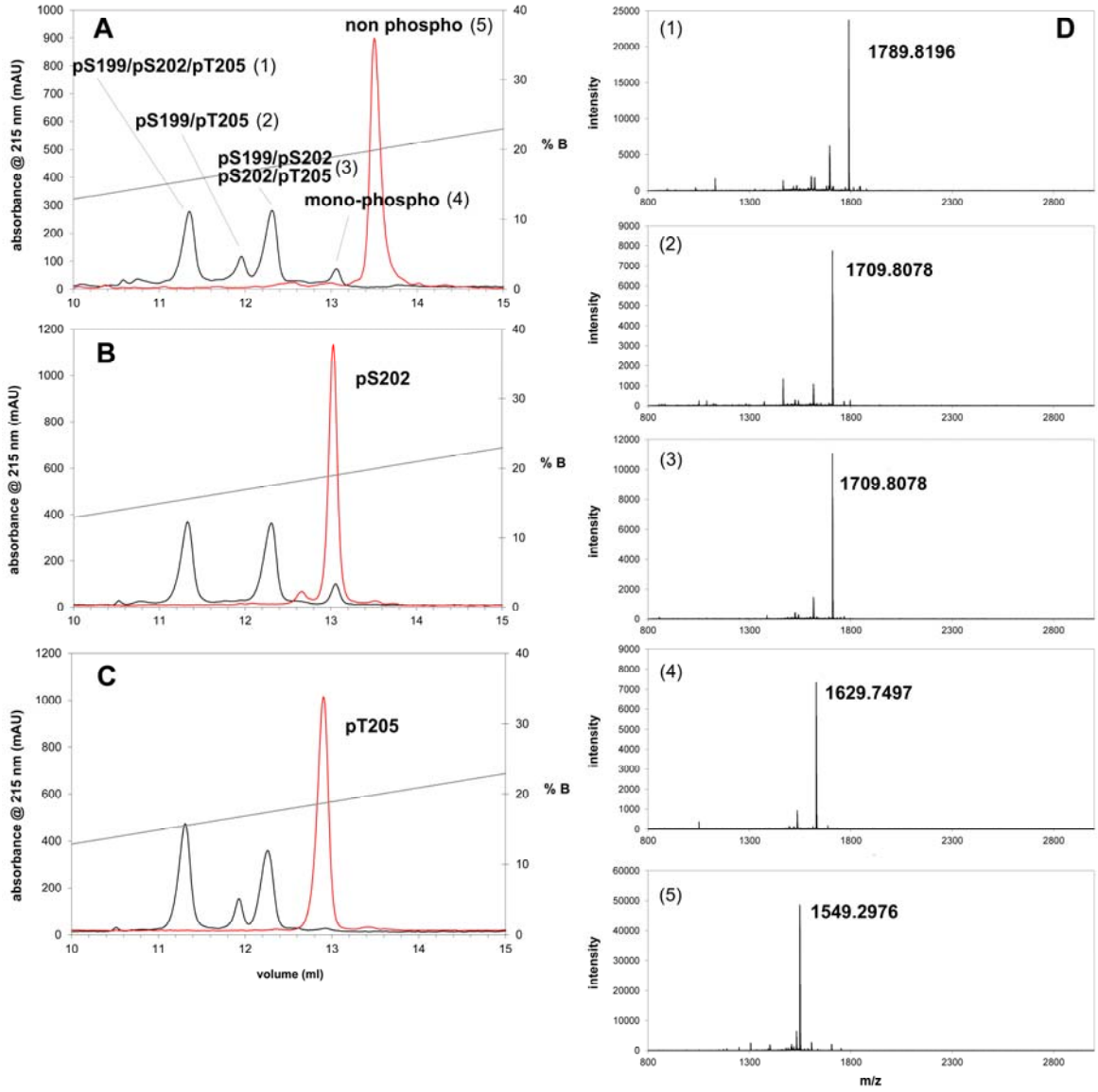
(A,B) Regulation of Tau[392-411] CDK-mediated phosphorylation on S404 by S400 O-GlcNAcylation. (A) Reverse phase chromatography of crude CDK2/cyclinA3 phosphorylation mixtures of Tau[392-411] (black) and Tau[392-411]-S400-O-GlcNAc (red) with 1 mM ATP. Control experiments without ATP on Tau[392-411] (blue), Tau[392-411]-S400-O-GlcNAc (green) were represented. The Tau[392-411]-pS404 peptide obtained by solid phase synthesis is represented as a indicator of the retention time (orange). (B) Graphical representation of CDK2/cyclinA3 phosphorylation kinetics on S404. (C) Disruption of GSK3β-mediated phosphorylation by S400 O-GlcNAcylation on Tau[392-411] peptide. RP-HPLC of crude phosphorylation mixtures of Tau[392-411]-pS404 (black) and Tau[392-411]-S400-O-GlcNAc/pS404 (red) with 4 mM ATP. Control experiments without ATP on the same peptides are shown (in blue and green, respectively). $[M+H]^+$ represents the mass of ions determined by MALDI-TOF MS of HPLC fractions. The linear gradient of acetonitrile (%B) is represented by a dotted line.





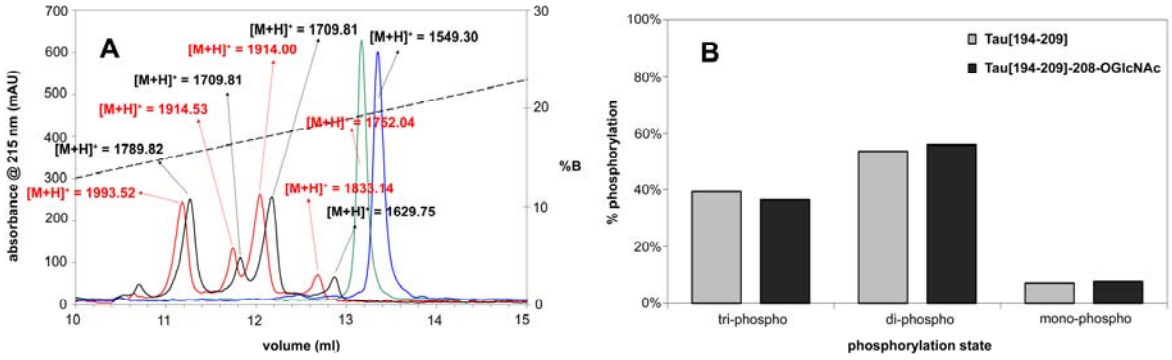
2.10 Figure S8

CDK2/cyclinA3-mediated phosphorylation of the Tau[194-209] series. (A-C) Chromatogram curves at 215 nm of crude phosphorylation mixtures. Tau[194-209] peptides either in its non phosphorylated (A), phospho-S202 (B) or phospho-T205 (C) forms are incubated with the CDK2/cyclinA3 complex in the presence of 2 mM ATP (black) or in the absence of ATP (red). The linear gradient of acetonitrile (%B) is represented by grey lines. (D) Mass spectra of the fractions (1) to (4) isolated from the Tau[194-209] phosphorylation reaction and the non phosphorylated control peptide (5) corresponding to the peaks at 11.33 min, 11.93 min, 12.29 min, 13.02 min and 13.49 min, respectively.



2.11 Figure S9

Regulation of Tau[194-209] CDK-mediated phosphorylation on S199/S202/T205 by S208 O-GlcNAcylation. (A) Reverse phase chromatography of crude CDK2/cyclinA3 phosphorylation mixtures of Tau[194-209] (black) and Tau[392-411]-S208-O-GlcNAc (red) with 2 mM ATP. Control experiments without ATP on the same peptides are shown (in blue and green, respectively). $[M+H]^+$ represents the mass of ions determined by MALDI-TOF MS of HPLC fractions. The linear gradient of acetonitrile (%B) is represented by a dotted line. (B) Graphical representation of CDK2/cyclinA3 phosphorylation pattern.



3 References

1. T. Lefebvre, S. Ferreira, L. Dupont-Wallois, T. Bussiere, M. J. Dupire, A. Delacourte, J. C. Michalski and M. L. Caillet-Boudin, *Biochim. Biophys. Acta*, 2003, **1619**, 167.