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Supporting Information

Disease-Associated Tau Phosphorylation Hinders Tubulin Assembly within Tau Condensates

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Supplementary Figures

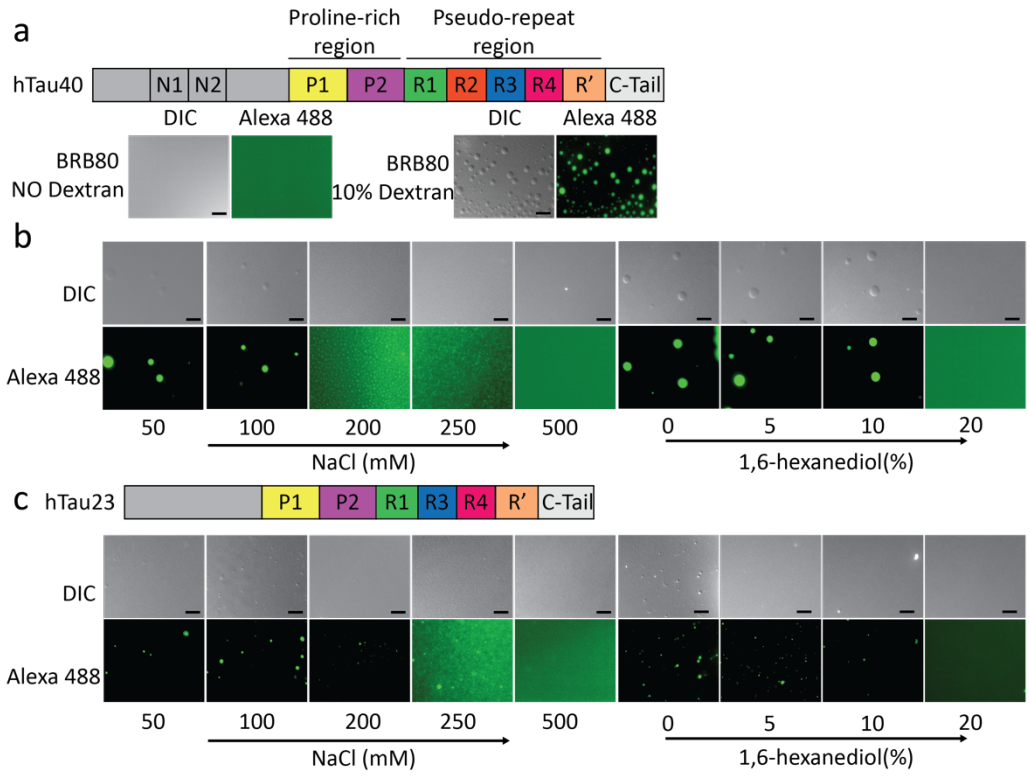

Figure S1. LLPS of hTau40 and the fetal isoform hTau23. **a**) DIC and fluorescence microscopy of liquid droplet formation of hTau40 (25 µM) in BRB80 buffer in the presence of 10 % dextran (right). In the absence of dextran, LLPS was not observed (left). Top, domain organization of hTau40. **b,c**) Sensitivity of hTau40 (50 µM) (b) and hTau23 (50 µM) (c) droplets to increasing ionic strength (left), or the addition of 1,6-hexanediol (right) monitored by DIC and fluorescence microscopy. Scale bars, 10 µm. In (c), the domain organization of hTau23 is shown.

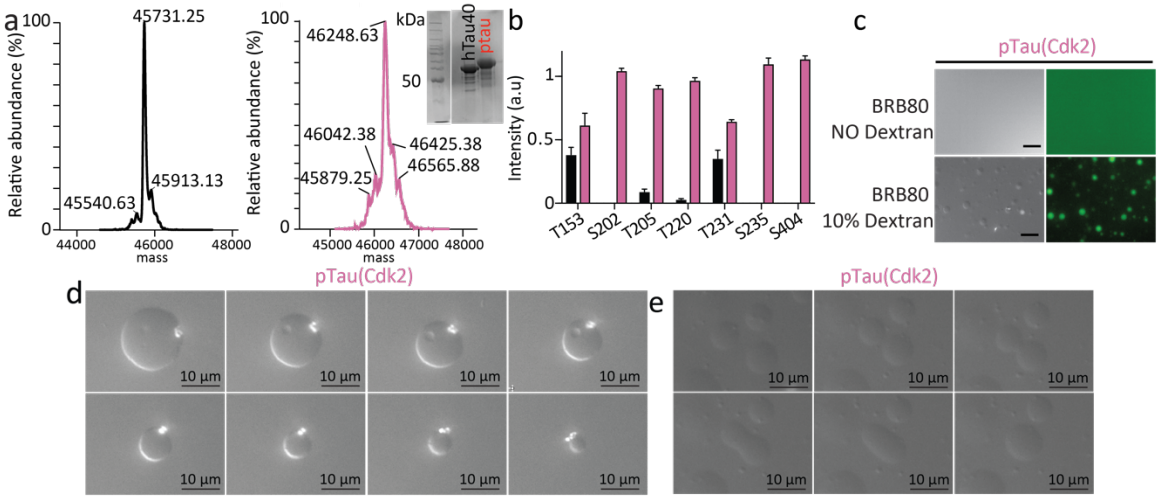

Figure S2. Liquid-like properties of pTau(Cdk2) droplets. **a**) Mass spectrometry of hTau40 unmodified (left; black) and phosphorylated by Cdk2 (right, pink) together with SDS PAGE gel demonstrating a phosphorylation-induced band shift. **b**) Residue-specific quantification of phosphorylation on the basis of NMR signal intensities. Normalized intensities of ^{1}H-^{15}N cross peaks of phosphorylated residues at the chemical shift in the phosphorylated (pink) and unphosphorylated (black) state. Error bars where derived on the basis of spectral signal-to-noise ratios. **c**) LLPS of 25 µM pTau(Cdk2) in BRB80 buffer in the presence of 10 % dextran. Scale bars, 10 µM. **d**) Time series of DIC images of a droplet of pTau(Cdk2) (in BRB80 buffer, 10 % dextran) shrinking upon addition of 20 % 1,6-hexanediol. Images are 30 seconds apart. **e**) Fusion of pTau(Cdk2) droplets displayed by sequential DIC images.

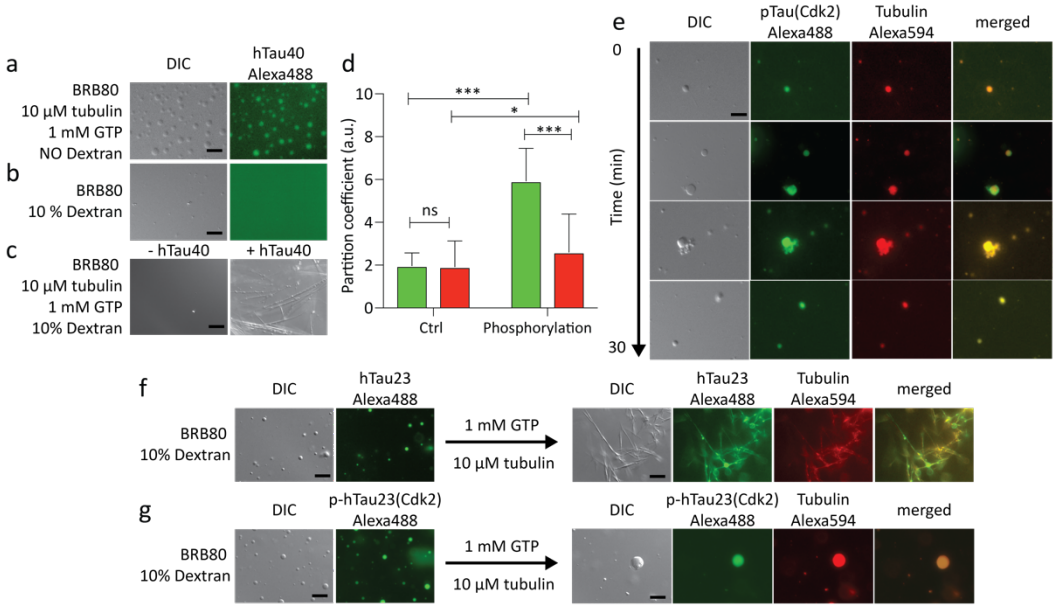

Figure S3. LLPS-based tubulin assembly assay of pTau(Cdk2). **a**) DIC and fluorescence microscopy of hTau40 (25 µM) in non-LLPS conditions, i.e. at room temperature in the absence of the molecular crowding agent dextran. Despite the presence of 1 mM GTP, tubulin (10 µM) did not polymerize in these conditions. Scale bar, 10 µm. **b**) Partition coefficient of tau (green) and tubulin (red) into droplets of unmodified or Cdk2-phosphorylated hTau40. Two-way ANOVA test, multiple comparison tests using Tukey model: ns, non-significant, * < 0.33, ** < 0.002, *** < 0.001. **c**) DIC micrographs of samples with 10 µM tubulin, 1 mM GTP in BRB80 buffer with 10 % dextran in either the absence (left) or presence of hTau40 (right). Samples were incubated for 30 minutes prior to imaging. Scale bar, 10 µm. **d**) LLPS of 50 µM pTau(Cdk2) in BRB80 buffer in the presence of 10 % dextran. Scale bar, 10 µm. **e**) DIC and fluorescence microscopy of pTau(Cdk2) (50 µM) droplets in the presence of 10 µM tubulin and 1 mM GTP. No tubulin polymerization was observed during a 30 minute incubation period. Scale bars, 10 µm. **f,g,** DIC and fluorescence microscopy of the growth of microtubules from performed droplets of unmodified hTau23 (100 µM; f), but not Cdk2-phosphorylated hTau23 (100 µM; g), upon addition of tubulin (10 µM) and GTP; scale bars, 10 µm.

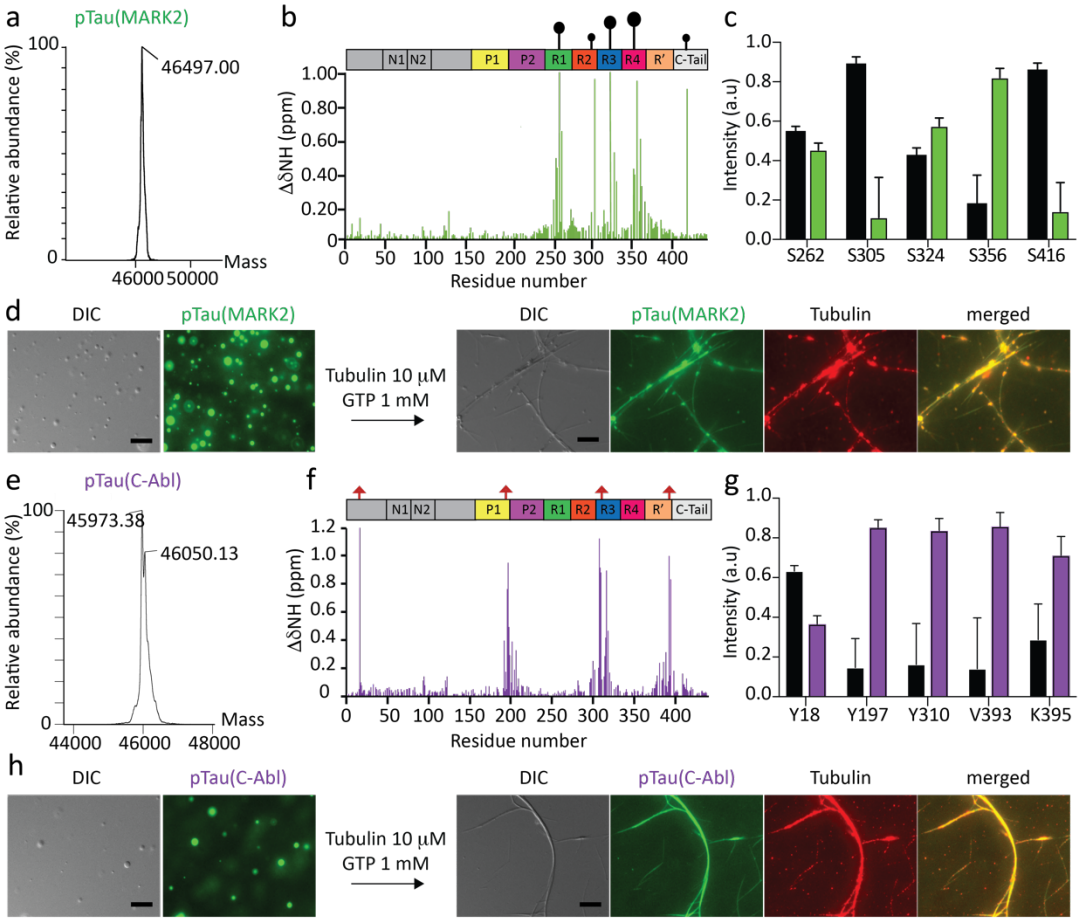

Figure S4. C-Abl- and MARK2-phosphorylated tau condensates allow tubulin polymerization. **a,e**) Mass spectrometry of tau phosphorylated by the MARK2 (a) and C-Abl (e). The mass spectrum of unmodified hTau40 is shown in Fig. S2a. **b,f**) ^{1}H-^{15}N chemical shift perturbation of hTau40 upon phosphorylation by MARK2 (b) and c-Abl (f) identifying sites of phosphorylation. Top, domain organization of hTau40 indicating the phosphorylated serines (in b, the size of the marks is proportional to the degree of phosphorylation). **c,g**) Residue-specific quantification of phosphorylation by MARK2 (c) and c-Abl (g) on the basis of NMR signal intensities. Normalized intensities of ^{1}H-^{15}N cross peaks of phosphorylated residues at the chemical shift in the phosphorylated (pink) and unphosphorylated (black) state. Error bars where derived on the basis of spectral signal-to-noise ratios. **d,h**) DIC and fluorescence microscopy of phase separated hTau40 phosphorylated by MARK2 (d) and c-Abl (h); scale bar, 10 μm. Addition of tubulin and GTP causes partitioning of tubulin into the droplets followed by rapid tubulin polymerization.

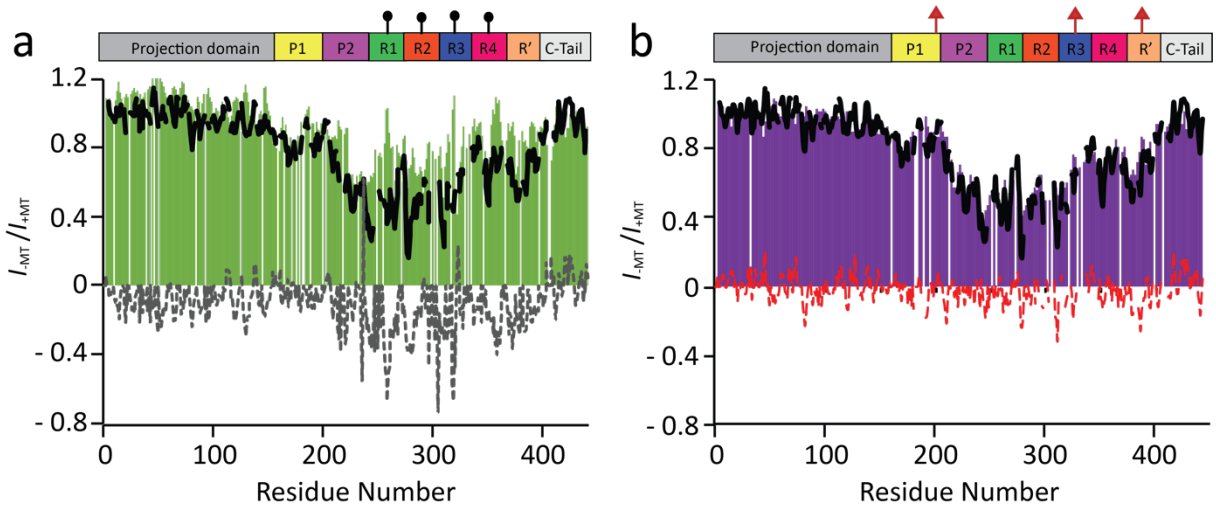

Figure S5. NMR-based characterization of the interaction of pTau(MARK2) and pTau(C-Abl) with microtubules. **a,b**) Residue-specific signal attenuation of ^{1}H-^{15}N cross peaks of 10 µM pTau(MARK2) (a) and 10 µM pTau(C-Abl) (b) upon addition of 5 µM paclitaxel-stabilized microtubules. The microtubule-induced intensity attenuation of hTau40 is shown for comparison (black line). Residue-specific differences between unmodified and phosphorylated hTau40 ´are shown as dashed line. Phosphorylated residues are marked in the domain diagram (top).

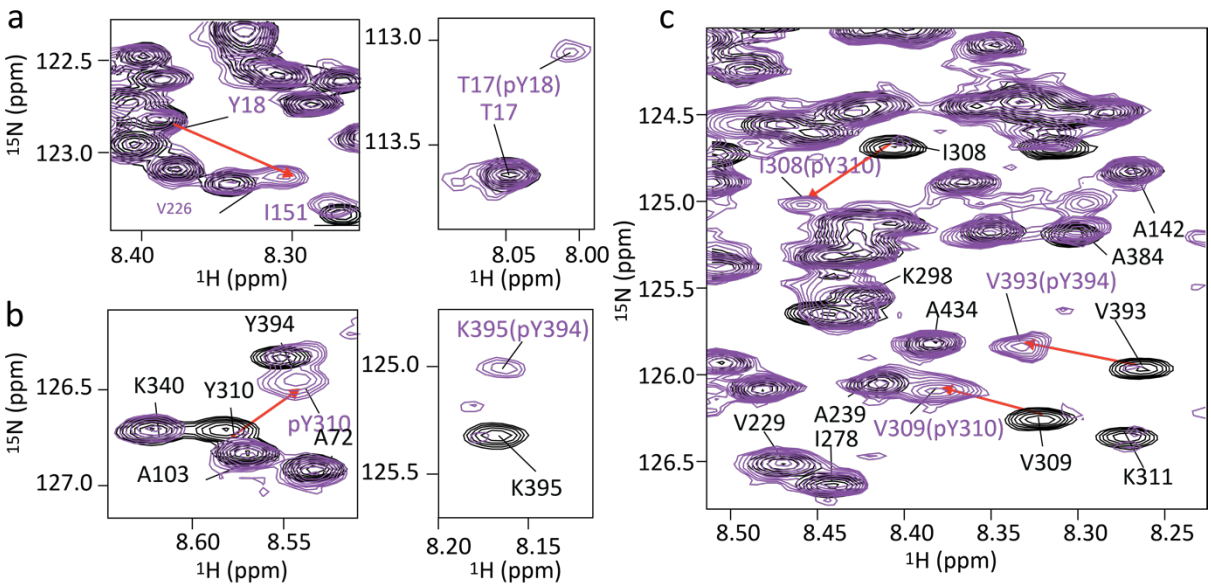

Figure S6. Selected regions from ^{15}N-^{1}H HSQC spectra of hTau40 phosphorylated by C-Abl (purple). Spectra of unmodified hTau40 are shown in black. **a**) Y18 and the nearby residue T17. **b**) Y310 and Y394. In the case of Y394, the chemical shift change was small, but a strong shift of the cross peak of the neighboring K395 was observed. **c**) Chemical shift perturbation of the cross peaks of residues next to Y310 (i.e. V309 and I308), and of Y394 (i.e. V393).

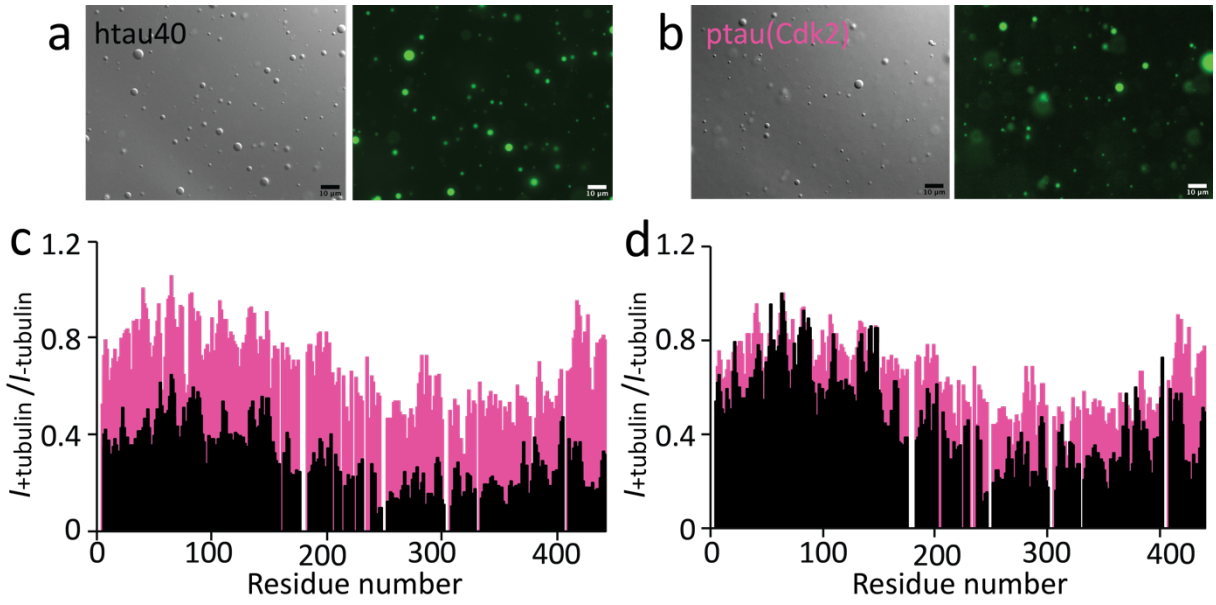

Figure S7. NMR spectroscopy of the tau/tubulin-interaction in crowded conditions. **a,b**) DIC and fluorescence microscopy of 25 µM hTau40 (a) and pTau(Cdk2) (b) in BRB80 buffer in the presence of 10 % dextran at 5 °C. **c,d**) Signal attenuation of ^{1}H-^{15}N signals of hTau40 (black) and pTau(Cdk2) (pink) (each 25 µM), in the presence of 10 % dextran at 5 °C, upon addition of 10 µM unpolymerized tubulin. In (d), the signal intensity ratios were normalized to the largest intensity ratio value. $I_{+tubulin}$ and $I_{-tubulin}$ are the intensities of tau cross peaks in two-dimensional ^{1}H-^{15}N SOFAST-HMQC experiments in the presence and absence of tubulin.

Supplementary Methods

Protein preparation

Human hTau40 (2N4R tau; 441 residues), hTau23 (0N3R; 352 residues) and the T231E/S235E-mutant of hTau23 were expressed in *Escherichia coli* strain BL21(DE3)[18a] from a pNG2 vector (a derivative of pET-3a, Merck-Novagen, Darmstadt) in presence of an antibiotic. The bacterial pellet was resuspended in lysis buffer (50 mM MES, 500 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM DTT, pH 6.8) and supplemented with a protease inhibitor mixture. Cells were disrupted using a French pressure cell and subsequently boiled for 20 min. The soluble extract was isolated by centrifugation and the supernatant was dialyzed twice against FPLC buffer (20 mM MES, 50 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 2 mM DTT, 0.1 mM PMSF, pH 6.8), and loaded onto a FPLC SP-Sepharose column. Proteins were eluted using a linear gradient of elution buffer (20 mM MES, 1 M NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 2 mM DTT, 0.1 mM PMSF, pH 6.8). Tau breakdown products were separated in a second chromatography step by using a Superdex G200 column (GE Healthcare) with the separation buffer (150 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4, 1 mM DTT). Protein samples uniformly enriched in ^{15}N were prepared by growing *E. coli* bacteria in minimal medium containing 1 g l^{-1} of $^{15}NH_4Cl$.

Protein phosphorylation

hTau40 was phosphorylated *in vitro* through incubation of 50-100 µM of protein with 0.4 µM of Cdk2/CycA2 (0.4 µM of MARK2 or 0.15 µM of C-Abl) in a buffer containing 50 mM sodium phosphate, pH 6.8, 2 mM DTT, 4 mM ATP, 2 mM EGTA, 0.5 mM PMSF, 5 mM $MgCl_2$. The mixtures were incubated in a thermo block at 25 °C, shaking at 300 rpm for 24 hours. The kinases were inactivated by boiling the sample for 20 minutes at 65 °C followed by centrifugation at 13000 rpm for 30 minutes. Prior to LLPS experiments, phosphorylated hTau40 samples were dialyzed overnight at 4 °C against a BRB80 buffer containing 100 mM PIPES, pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, 1 mM DTT. Phosphorylation of hTau40 was confirmed by MS/MS analysis of 50 µL (1 mg/ml) protein using a nanospray source fitted to a Quattro quadrupole mass spectrometer.

Microtubule assembly

Microtubule assembly assays in the presence of pre-formed tau (25 µM) droplets were performed in BRB80 buffer at room temperature. Tubulin and GTP were added to a final concentration of 10 µM and 1 mM, respectively. Microtubules used for NMR spectroscopy

were polymerized by incubation of fixed concentrations of tubulin (25 μM) in BRB80 buffer in the presence of 1 mM GTP and 25 μM paclitaxel. Polymerization was performed in a thermo block at 37 °C for 20-30 min. The suspensions of the samples were fractionated by ultracentrifugation at 40,000 × g for 20 min. The microtubule pellet was resuspended in 50 mM phosphate buffer, pH 6.8, 2 mM DTT and 10% D_2O for NMR measurements.

Fluorescence microscopy

HTau40, hTau23, hTau23(T231E/S235E), pTau(Cdk2), p-hTau23(Cdk2), pTau(MARK2) and pTau(C-Abl) proteins were fluorescently labelled using the Alexa 488 Microscale Protein Labeling Kit (Thermo Fisher Scientific, #A30006). Porcine brain tubulin was fluorescently labeled using the Alexa 594 Microscale Protein Labeling Kit (Thermo Fisher Scientific, #A30008). Subsequently, 5 μl of unlabelled hTau40 (or pTau(Cdk2), pTau(MARK2), pTau(C-Abl), hTau23, p-hTau23(Cdk2)) were mixed with 0.5 μl of the Alexa 488-fluorescently labelled protein in BRB80 buffer. LLPS was induced by addition of 10 % dextran (Dextran T500, Cat. No. 40030, Pharmacosmos) at room temperature. 10 μL of samples were loaded onto glass slides, covered with a 18 mm coverslip. Differential interference contrast (DIC) and fluorescent images were acquired on a Leica DM6000B microscope with a 63× objective (water immersion) and processed using Fiji software (NIH). Partition coefficients for hTau40 and tubulin into unmodified and Cdk2-phosphorylated Tau droplets were calculated as the ratio between the mean fluorescence intensities inside and outside of the droplets. For each group, 50-60 droplets were quantified and a two-way ANOVA test was used to compare the different groups. FRAP experiments were performed on a Leica TCS SP8 confocal microscope using a 63 x oil immersion objective at 4 x digital zoom, at room temperature. A 560-argon laser line at a power setting of 50 % was used for bleaching the fluorophore Alexa 488. The confocal pinhole was set to 2 Airy units. An acquisition speed of 400 Hz was combined with an image format of 128 x 128 pixels. For FRAP experiments of hTau40 and pTau(Cdk2) droplets, the samples were prepared by mixing each protein with BRB80 buffer and 10 % dextran to reach a final concentration of 50 μM. Circular ROIs of 5-8 μm in diameter were selected. A measurement on a single droplet consisted of 5 pre-bleaching scans, 20 bleaching and 100 post-bleaching scans. FRAP curves were analysed and fitted using an exponential recovery fitting equation according to the FRAP guide provided by Alexandre Webster (Aravin Lab, Caltech) and Malte Wachsmuth (EMBL). Three independent measurements were averaged to give a FRAP curve.

FRAP recovery curves were obtained by measuring the fluorescence intensities of prebleaching, bleached and reference ROI. Where the prebleaching ROI is a selected region in the droplet before bleaching; the bleached ROI corresponds to the bleached area while the reference ROI corresponds to an area which did not experience bleaching. For each of the described ROIs, background substraction was used to correct the measured fluorescence intensity. Thus, the FRAP recovery was calculated as:

$$FRAP = (I_{Bleached} - I_{background})/I_{(Av.Prebleaching)}$$

The value obtained was then corrected by multiplication with the acquisition bleaching correction factor (ABCF), which was calculated according to:

$$ABCF = (I_{reference} - I_{background})/I_{(Av.Reference)}$$

Finally, the curves were normalized according to:

$$Normalization = \frac{I_{(t)} - min.Intensity\,Value}{1 - min.Intensity\,Value}$$

NMR spectroscopy

2D ^{1}H-^{15}N HSQC experiments were acquired on 10-80 μM tau in 50 mM sodium phosphate buffer, pH 6.8, 10 % D_2O, using a 700 MHz spectrometer (Bruker, Germany) with Topspin 3.2 software. Spectra were processed using Topspin and analysed using NMRFAM-SPARKY 1.4. Identification of the phosphorylated residues was achieved by chemical shift perturbation analysis. ^{1}H and ^{15}N normalized weighted average chemical shifts were calculated using $\Delta\delta NH = [((\Delta H)^2 + (\Delta N)^2/5)/2]^{1/2}$, where ΔH and ΔN correspond to the ^{1}H and ^{15}N chemical shift differences between hTau40 and pTau(Cdk2)/pTau(MARK2)/pTau(C-Abl). Resonance assignments of hTau40 and phosphorylated residues were reported previously.[11c, 17a] Quantification of the degree of phosphorylation at each site was achieved by analysis of the cross peaks for the unphosphorylated and phosphorylated forms of each selected residue as described previously.[15] Errors were derived using error propagation on the basis of the estimated mean error of each spectrum.

Binding of hTau40 (and hTau23, hTau23(T231E/S235E), pTau(Cdk2); each 10 μM) to soluble tubulin was investigated using 2D ^{1}H-^{15}N HSQC experiments. The tau:tubulin molar ratio was 2:1. Experiments were recorded at 5 °C on a Bruker 800 MHz spectrometer equipped with a cryoprobe. NMR measurements of the guanidium protons of arginine side chains were performed at 5 °C on a Bruker 900 MHz spectrometer. In order to decrease the exchange of the labile protons with bulk solvent, a sodium phosphate buffer at pH 6.0 was used. In case of hTau40 and pTau(Cdk2), spectral widths of 7653.1 Hz and 182.4 Hz in the ^{1}H and ^{15}N dimension, respectively, were recorded. The number of increments was 128 in the ^{15}N dimension. In case of Tau(225-246)-AT180, spectral widths of 6009.6 Hz and 973.3 Hz in the ^{1}H and ^{15}N dimension, respectively, were recorded; the number of increments was 256 in the ^{15}N dimension.[19]

To study the binding of hTau40 and pTau(Cdk2) to soluble tubulin in crowded conditions (i.e. in the presence of 10 % dextran), SOFAST-HMQC experiments (Bruker standard pulse sequence: sfhmqc3gpph) were acquired at 5 °C on a 700 MHz spectrometer equipped with a cryoprobe (Bruker, Germany). SOFAST-HMQC experiments were recorded using 32 scans and 256 increments in the ^{15}N dimension for a total experiment time of 37 minutes.