The protein Tau aggregates into tangles in the brain of patients with Alzheimer’s disease. In solution, however, Tau is intrinsically disordered, highly soluble, and binds to microtubules. It is still unclear what initiates the conversion from an innocuous phase of high solubility and functionality to solid-like neurotoxic deposits. Here, we show that the microtubule-binding repeats of Tau, which are lysine-rich, undergo liquid–liquid phase separation in solution. Liquid–liquid demixing causes molecular crowding of amyloid-promoting elements of Tau and drives electrostatic coacervation. Furthermore, we demonstrate that three-repeat and four-repeat isoforms of Tau differ in their ability for demixing. Alternative splicing of Tau can thus regulate the formation of Tau-containing membrane-less compartments. In addition, phosphorylation of Tau repeats promotes liquid–liquid phase separation at cellular protein conditions. The combined data propose a mechanism in which liquid droplets formed by the positively charged microtubule-binding domain of Tau undergo coacervation with negatively charged molecules to promote amyloid formation.
Here, we show that the microtubule-binding repeats of Tau form liquid droplets in a phosphorylation-specific manner. Nuclear magnetic resonance (NMR) spectroscopy provides a residue-specific view into the structural properties of the phase-separated state and reveals crowding of amyloid-promoting elements upon liquid demixing. We further show that binding of negatively charged factors to Tau is not sufficient to trigger Tau amyloid formation, but requires coacervation into liquid droplets. In addition, three-repeat and four-repeat isoforms of Tau differ in their ability for LLPS, indicating that alternative splicing of pre-mRNA can influence the formation of Tau-containing membrane-less compartments in the reducing environment of a neuronal cell body.

Results
The propensity of Tau for granule formation. The amino acid sequence of human Tau contains only a small number of hydrophobic residues and is of low complexity. To investigate if the primary structure of Tau encodes the ability for liquid–liquid demixing, we subjected the sequence of hTau40, the longest isoform in the human central nervous system, hTau23, which lacks the N-terminal inserts and repeat R2, as well as K18 (the repeat region of 4R-Tau; Fig. 1a) to the program catGranule [42]. catGranule predicts on the basis of structural disorder, nucleic acid-binding propensities, and sequence composition whether a protein is prone to granule formation. Figure 1b shows the catGranule-propensity score along Tau’s amino acid sequence. Propensity scores below zero were predicted for the two N-terminal inserts, part of the proline-rich region P2 and Tau’s

A lzheimer’s disease (AD) and several other neurodegenerative diseases are characterized by the misfolding and pathological accumulation of the microtubule-associated protein Tau [4, 5]. The level of aggregation of Tau into neurofibrillary tangles (NFTs) correlates with the progressive destruction of nerve cells and the degree of cognitive decline in AD [6, 7]. Mutations in the Tau sequence modulate Tau’s ability to form tangles and cause frontotemporal dementia and parkinsonism linked to chromosome 17 [8, 9]. Aggregated Tau is unable to bind to microtubules, which changes the dynamic instability of microtubules [8, 10, 11]. Despite the pathological importance of misfolding and aggregation of Tau, the mechanisms underlying aberrant Tau aggregation and the pathways leading to tangle formation and neurotoxicity in AD have remained enigmatic.

Alternative splicing of exons 2, 3, and 10 of the Tau encoding MAPT-gene on chromosome 17 generates six different isoforms of Tau in the human central nervous system [12]. Tau isoforms differ in the number of N-terminal inserts and contain either three or four 31-residue to 32-residue-long imperfect repeat sequences (R1–R4). The repeat sequences are important for binding and assembly of microtubules and influence Tau’s ability to form tangles [13, 14]. In agreement with the importance of Tau repeats for pathogenic aggregation, the composition of tangles by 3R-Tau and 4R-Tau is connected to distinct clinical manifestations [1]. NFTs in AD contain a mixture of 4R-Tau and 3R-Tau [1], while deposits in progressive supranuclear palsy predominantly contain 4R-Tau [15], and Pick’s disease inclusions mostly have 3R-Tau [16].

When Tau was purified as a microtubule-associated protein in 1975, it was recognized that it is highly soluble. Because of the small number of hydrophobic residues and the low complexity of its amino acid sequence, Tau shows very little tendency to form liquid-like behavior such as P granules, nucleoli, and membrane-less compartments in the reducing environment of a neuronal cell body [17]. The Tau sequence encodes a strong propensity for lower-critical solution transition and granule formation. A Domain organization of the Alzheimer-related protein Tau. The longest Tau isoform (2N4R, htau40) in the human central nervous system contains four imperfect repeats (R1–R4) and two N-terminal inserts (NI, N2). htau23 (GN3R) is the shortest isoform and lacks the two N-terminal inserts as well as repeat R2. The Tau repeats form the core of NFTs [8]. The protein K25 contains only the N-terminal half of Tau, which is called the projection region. B Residue-specific propensity score for granule formation predicted by catGranule for full-length 4R-Tau (htau40), the repeat domain of 4R-Tau (K18), and K25. C Total catGranule-scores of different Tau proteins and α-synuclein (α-syn). D Amino-acid sequence of repeat domain of Tau. It contains 19 lysine residues (highlighted in blue) and four PGGG motifs (red)
The microtubule-binding domain of Tau undergoes LLPS. The computational analysis suggests that the Tau repeats, which are liberated from the full-length protein by proteases and endoproteases, have a strong propensity for liquid demixing. We therefore subjected a solution containing the 4R-protein K18 to a wide range of temperatures, pH values, and protein concentrations (Fig. 2). This screen is important because protein-LLPS depends on the properties of the aqueous solution. To exclude the influence of intramolecular and intermolecular cross-linking through Tau’s two native cysteine residues, C291 and C322, all experiments were performed in the presence of tris(2-carboxyethyl)phosphine (TCEP), mimicking the reducing environment inside neurons. We then used a standard assay to monitor liquid–liquid demixing of proteins, which is based on turbidity measurements at 350 nm. Indeed for pH values from 4.8 to 8.8 and a temperature of 37 °C, the K18 solution became more turbid with increasing protein concentration (Fig. 2a). Higher turbidity values were measured at more basic pH values approaching the protein’s pI of 9.8.

Changes in solution turbidity can arise from liquid–liquid demixing/LLPS, but also from formation of other types of aggregates. To support the presence of a liquid phase separated state of the repeat domain of Tau, we performed differential interference contrast (DIC) microscopy. Phase-contrast microscopy is particularly powerful for characterization of LLPS, because it reveals micrometer-sized structures, which are not visible with simple bright-field microscopes. Figure 2c shows DIC micrographs of a 100 μM solution of K18 at 37 °C, i.e., solution conditions that are optimal for K18 LLPS according to turbidity measurements (Fig. 2a), over a time period of 72 h. At the start of the imaging process, the solution is clear, indicating that K18 is present in solution as dispersed monomer. After 24 h, small droplets were observed. The number and diameter of droplets increased with time and after 72 h both large droplets with a diameter of approximately 15 μm and smaller (1 μm and below) droplets were observed. To mimic conditions of intracellular crowding, DIC micrographs were also recorded in the presence of 7.5% polyethylene glycol (PEG), supporting the time-dependent formation of K18 liquid droplets (Fig. 2c). The K18 liquid–demixed phase exhibited only little Thioflavin-T (ThT) fluorescence, indicating the absence of rigid cross-β-structure (Fig. 2d). The absence of amyloid-like structure is in agreement with the high solubility of K18, which requires an aggregation enhancer such as heparin to form amyloid fibrils at 37 °C, 100 μM protein concentration, on a time scale of 1–3 days. Detailed inspection of DIC micrographs showed that the droplets were able to fuse (Fig. 2f), in agreement with their liquid-like nature. Liquid droplets were also observed by DIC microscopy for solutions containing lower protein concentrations, e.g., at 10 μM K18 (Fig. 3b).

To demonstrate the presence of K18 in the liquid droplets, we performed confocal microscopy of fluorescently labeled protein. To this end, we labeled K18 with the fluorescent dye Alexa-488. The fluorescently labeled protein was then mixed with unlabeled K18 in a molar ratio of 1:20, to reach a final concentration of 100 μM, pH 8.8. The solution was subsequently incubated for 12 h at both 5 and 37 °C and analyzed by microscopy. Only when across the yeast proteome. The 4R-region (K18) even reaches a granule propensity of more than 2.0, i.e., well above values of proteins that were experimentally shown to undergo liquid–liquid demixing. Notably, the protein α-synuclein, which aggregates into Lewy bodies in Parkinson’s disease, has a lower predicted propensity for granule formation when compared to Tau, but could still undergo liquid–liquid demixing according to the catGranule score (Fig. 1c).

**Fig. 2** The microtubule-binding domain of Tau undergoes LLPS. **a** Influence of protein concentration and pH on turbidity of a K18 solution (50 mM sodium phosphate) at 37 °C. Turbidity values are reported as average absorbance across 350 nm from triplicate measurements for each sample. Normalization was done with respect to A(max) at pH 8.8, 100 μM K18 concentration. Errors were propagated as normalized standard error of mean (SEM). Increasing pH values are colored from blue to red. Note that all experiments were done in the presence of 0.5 mM TCEP, to avoid oxidation of Tau’s native cysteine residues. **b** K18 (100 μM in 50 mM sodium phosphate) undergoes liquid–liquid demixing above a critical temperature (~15 °C), consistent with a LCST. Increasing pH values are colored from blue to red. **c** Differential interference microscopy reveals the time-dependent formation of liquid droplets in a 100 μM solution of K18 (50 mM sodium phosphate, pH 8.8) at 37 °C, in both the absence (top row) and presence (bottom) of the molecular crowding agent PEG (7.5%). Scale bars correspond to 10 μm. **d** ThT fluorescence intensities for the samples imaged in **c** (no PEG) by DIC microscopy. Average intensities from three independent measurements are shown. **e** Fluorescence microscopy demonstrates the presence of K18 in liquid droplets formed at 37 °C (100 μM K18 in 50 mM sodium phosphate, pH 8.8). At 5 °C, K18 LLPS did not occur (upper row). Alexa-488-labeled protein was mixed with unlabeled protein in a molar ratio of 1:20. Scale bars correspond to 10 μm. **f** Fusion of K18 liquid droplets. Droplets, which were undergoing fusion when imaged, are marked by black arrows. The sample was identical to the one used in **c** (72 h; with PEG). Scale bars correspond to 10 μm.
incubated at 37 °C, but not at 5 °C, fluorescent droplets were observed (Fig. 2e). Taken together, the data show that the microtubule-binding domain of Tau undergoes LLPS at physiological conditions.

**Driving a lower critical solution transition (LCST).** Next, we fixed the protein concentration and varied the temperature from 5 to 50 °C (Fig. 2b). Below 15 °C we detected little solution turbidity. In addition, no evidence for liquid demixing was found at 5 °C by DIC microscopy (Fig. 2e). Thus, at high concentrations but low temperatures, K18 is present as dispersed monomer in solution. Above 15 °C, turbidity values rose, reached a maximum, and decreased at even higher temperatures. The maximum in turbidity was dependent on the pH of the solution and shifted from ~37 °C at pH 4.8 to ~42 °C at pH 8.8. Thus, the repeat region of Tau undergoes a so-called LCST.

The dependence of LLPS on temperature is governed by the protein’s amino acid composition. While a high content of arginine residues favours phase separation below a critical temperature, as observed for FUS, the presence of lysine residues has been associated with LCST. In addition, P–X₉₋₁–G motifs in intrinsically disordered peptides and proteins encode LCST behavior. In agreement with these sequence determinants for phase separation at increasing temperature, K18 contains only a single arginine residue, but 19 lysine residues, as well as an evolutionary-conserved PGGG motif at the N-terminal end of each Tau repeat (Fig. 1d). In contrast to K18, domains of proteins, which were previously shown to undergo LLPS (e.g., FUS), are glutamine/asparagine-rich and undergo liquid demixing only at low temperature (below 20 °C).

**LLPS promotes reversible β-structure in Tau repeats.** To gain insight into structural properties of Tau repeats induced by aqueous phase separation, we performed circular dichroism (CD) spectroscopy. In agreement with the intrinsically disordered nature of Tau, CD spectra of K18, recorded at 5 °C, were characteristic for random coil behavior (Fig. 4a). Upon increasing the temperature to 37 °C, the minimum of the CD signal became less pronounced and shifted to longer wavelengths. Besides spectral changes at ~197 nm, the CD signal at 218 nm increased at 37 °C, indicative of increased β-structure. The structural changes were largely reversible after returning to 5 °C (Fig. 4a), in agreement with the appearance of liquid droplets (Fig. 4b). Thus, the amount of β-structure in the Tau repeats is increased in solution conditions that promote LLPS. The increase in β-structure, however, is little when compared to K18 amyloid fibrils, in agreement with very low ThT fluorescence in the liquid demixed state (Fig. 2d).

**Crowding of Tau amyloid hot spots upon liquid demixing.** A critical aspect of liquid–liquid demixing is that the resulting membrane-less compartments have distinct physicochemical properties. Quantitative studies of liquid droplets, however, are complicated by their liquid nature and the rapid exchange with the surrounding environment. To dissect the consequences of LLPS on the molecular properties of the repeat domain of Tau, we used NMR spectroscopy. NMR spectroscopy not only provides residue-resolution, but is also able to characterize molecules that exchange between multiple states. A two-dimensional (H,15)N correlation spectrum, recorded for the repeat domain of Tau at 5 °C, 100 μM protein concentration, had small signal dispersion (Fig. 5a), in agreement with a dispersed monomeric protein (Fig. 2e). At 37 °C, the temperature at which DIC microscopy demonstrated the presence of phase separation (Fig. 2c, e), the
Due to increased solvent exchange, many $^1$H-$^1$5N cross-peaks of K18 are attenuated beyond detection at 37 °C. In addition, an overall shift of the backbone amide proton resonances was observed (Fig. 5a), because the NMR data were obtained on a phase-separated structure observed by CD spectroscopy (Fig. 4a). However, agreement with the overall low content of regular secondary structure observed by CD spectroscopy (Fig. 4a) or (ii) the fast exchange of K18 molecules from the interior of the liquid droplets into the surrounding environment (in agreement with the basic properties of liquid droplets) or (iii) a combination of the two. Because of the fast exchange in and out of liquid droplets, the NMR properties of K18 in conditions of LLPS might be similar to those of ligands that weakly bind to receptors.

To probe this hypothesis, we attached the paramagnetic nitroxide tag (1-oxy-2,2,5,5-tetramethyl-1-pyrroline-3-methyl)-methanethiosulfonate (MTSL) to the two native cysteines, C291 and C322, of K18 (in agreement with CD spectroscopy; Fig. 4a) or (ii) the fast exchange of K18 molecules from the interior of the liquid droplets into the surrounding environment (in agreement with the basic properties of liquid droplets) or (iii) a combination of the two. Because of the fast exchange in and out of liquid droplets, the NMR properties of K18 in conditions of LLPS might be similar to those of ligands that weakly bind to receptors.

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correlation spectra, because they are less affected by solvent exchange, in particular at 37 °C. The NMR analysis showed that at 5 °C, when the protein is present primarily as dispersed monomer (Fig. 2e), only residues in the immediate vicinity of the two cysteine residues such as T319 were broadened (Fig. 5b-e). In contrast, most K18 residues felt the presence of the paramagnetic center in the liquid–liquid demixed state at 37 °C (Figs. 2c, 5b). Indeed, all four threonine residues, which were well separated in 1H,13C correlation spectra (Fig. 5c), were strongly broadened (Fig. 5c, d).

In the monomeric dispersed state, only weak intramolecular and no intermolecular interactions are present in both full-length Tau and K18. The observation of severe resonance broadening in K18 at 37 °C, 100 μM protein concentration (Fig. 5c) thus demonstrates that Tau’s four repeats tightly interact with each other in the conditions, in which phase separation in solution occurs (Fig. 2c). The contacts can be within a single protein, as well as between different molecules. Importantly, the observed molecular contacts provide direct evidence for crowding of the repeat domain of Tau and its aggregation-prone hexapeptides. In contrast, we did not observe such strong molecular interactions in K18—even in the presence of the aggregation enhancer polyglutamic acid—when the temperature was kept at 5 °C, i.e., below the critical temperature for LLPS of K18 (Fig. 2). The data demonstrate that LLPS of Tau repeats results in a tight molecular mesh of amyloid-promoting elements (Fig. 5e).

LLPS promotes Tau fibrillation in the presence of heparin. Polyanions such as glycosaminoglycans and ribonucleic acids promote the aggregation of Tau and are found in NFTs isolated from the brains of patients with AD. A well-studied polyanion is heparin, which binds to the repeat domain of Tau. To investigate if LLPS is related to formation of insoluble protein deposits, we added heparin to a selected region of a K18 sample containing preformed liquid droplets. Contrast microscopy showed that 5 min after addition of heparin liquid droplets were connected by tube-like structures (Fig. 6a, upper panel). The phase-separated mesh was only observed in the region of the specimen where heparin had been added, while separated droplets remained in a distant region (Fig. 6a, upper panel). Twenty-five minutes later, the mesh-like network had spread over the whole sample (Fig. 6a, middle panel). After 1 day of incubation in the presence of heparin, larger liquid droplets were observed by contrast microscopy (Fig. 6a, lower panel). After 2 more days of incubation, abundant amyloid fibrils of K18 were observed by electron microscopy (Fig. 6h).

In order to provide further support for the importance of LLPS for polyanion-induced Tau aggregation, we compared in a very systematic manner the influence of temperature and ionic strength on heparin-free LLPS (Fig. 6b-e) with heparin-induced K18 fibrillation (Fig. 6f, g). Liquid demixing in the absence of heparin was strongly dependent on temperature (Fig. 6b). Little turbidity was observed at low temperature, in agreement with a LCST (Fig. 2b). The absence of LLPS at 5 °C was also supported...
by DIC microscopy (Fig. 2e). In addition, no evidence for liquid–liquid demixing was found at very high temperatures (65°C and above; Fig. 6b), demonstrating that the absence of LLPS at 5°C is not related to a very slow kinetic process. The detailed temperature scan shown in Fig. 6b revealed a maximum in solution turbidity at ~42°C, suggesting that close to physiological temperatures are optimal for liquid demixing of K18. At all tested temperatures, very little ThT fluorescence was detected (Fig. 6d), demonstrating the absence of amyloid fibrils in heparin-free conditions.

We then incubated K18 solutions with heparin for 3 days at different temperatures. At the end of the incubation period, the presence of fibrils was probed using the amyloid-specific dye ThT. With increasing incubation temperature the ThT intensity of the K18/heparin solution rose (Fig. 6f). Electron microscopy confirmed the presence of amyloid fibrils (Fig. 6h). Although ThT intensity is not a quantitative measure for protein fibrillation, the data suggest that K18 fibrillation in the presence of a K18/heparin molar ratio of 4:1 is most efficient at ~50°C (Fig. 6f), in agreement with previous results. When the temperature was increased beyond 50°C, however, very little ThT fluorescence was detected. Thus, despite the presence of heparin, the repeat domain of Tau was not able to aggregate into amyloid fibrils at high temperatures. The data show that heparin-free LLPS and heparin-induced fibrillation of K18 show a similar temperature dependence: Only at intermediate temperatures close to 37°C, but not a very low (<15°C) or very high (>65°C) temperatures K18 undergoes LLPS in the absence of heparin. Addition of heparin to K18 at temperatures, where no liquid demixing occurs, did not result in K18 fibrillation (Fig. 6f).

Notably, the inability of K18 to form amyloid fibrils in the presence of heparin at 5°C is not due to an impaired K18/heparin interaction, because NMR spectroscopy demonstrated that heparin still binds to K18 at 5°C (Fig. 7).

The inability of K18 to aggregate into amyloid fibrils at very low (<15°C) or very high (>65°C) temperatures in the presence of one of its most potent aggregation enhancers might be caused by temperature-dependent changes in hydrophobicity or entropy. To further tighten the connection between Tau LLPS and amyloid formation, we therefore systematically varied a second physicochemical parameter, ionic strength. Figure 6c shows that with increasing NaCl concentration the turbidity of a 100 μM solution of K18, pH 7.4, 37°C, decreased. The data suggest that at a NaCl concentration of 200 mM, the protein was only present as dispersed monomer. We then assayed the influence of the NaCl concentration on heparin-induced K18 fibrillation. Similar to heparin-free LLPS, heparin-induced K18 fibrillation showed a strong dependence on ionic strength: at NaCl concentrations of 200 mM and above no amyloid fibrils were formed (Fig. 6g). The data indicate that electrostatic interactions are important for both heparin-free LLPS and heparin-induced fibrillation.

The combined data show that heparin-free LLPS and heparin-induced fibrillation depend in a similar way on temperature and ionic strength of K18 solutions. In addition, heparin-induced fibrillation of K18 is more efficient at basic pH values, just as heparin-free LLPS (Fig. 2a). Thus, heparin-free LLPS and heparin-induced fibrillation of K18 have similar dependences on three distinct physicochemical solution properties: temperature, ionic strength, and pH. In addition, DIC microscopy revealed immediate changes in the liquid demixed state upon addition of heparin (Fig. 6a), which resulted in amyloid fibril formation upon continued incubation (Fig. 6h). Taken together, the data indicate that amyloid formation does not occur—in reducing conditions and in the presence of the aggregation enhancer—when the repeat domain of Tau is present as dispersed monomer. The data suggest a model, in which liquid demixing results in increased concentrations of the repeat domain in liquid droplets. Because the repeat domain is highly positively charged, polyanions are recruited to K18 liquid droplets, thereby increasing the local concentration of Tau/polyanion-complexes and promoting amyloid formation.

**Valency of homotypic Tau interactions.** Due to alternative RNA splicing, Tau occurs in six different isoforms in the human central nervous system. The isoforms differ by the number of inserts in the N-terminal half and the number of repeats in the C-terminal half of the protein. We therefore asked if the number of repeats influences the ability for Tau LLPS. To this end, we prepared the protein K19. K19 comprises the repeat region of 3R-Tau (Fig. 1a). With increasing temperature, a rise in the turbidity at 350 nm of a K19 solution was observed (Fig. 3a), indicating that K19 undergoes aqueous phase separation. Observed turbidity values, however, were lower than for K18 over a wide temperature range. In agreement with a lower propensity of K19 (repeat region of 3R-Tau) for LLPS, a smaller number of...
liquid droplets were observed by contrast microscopy (Fig. 3b). Even lower turbidity values were detected for K25 (Fig. 3a), the Tau fragment, which comprises the projection domain but no repeats (Fig. 1a). In agreement with turbidity values and DIC microscopy, a smaller amount of β-structure was stabilized in K19 than K18 upon LLPS (Fig. 3c). The data suggest that each imperfect repeat in Tau can be regarded as a single interaction motif, with the number of Tau repeats governing the multivalency of homotypic interactions in liquid demixing.

**MARK2 phosphorylation promotes K18 LLPS.** Phosphorylation of Tau precedes formation of insoluble Tau deposits. Because microtubule-associated protein/microtubule affinity-regulating kinases (MARKs) phosphorylate serine residues in the repeat domain of Tau, MARK kinases influence both Tau aggregation and microtubule binding. We therefore asked if phosphorylation by MARK2 modulates the ability of the repeat domain of Tau to undergo LLPS. To this end, we phosphorylated K18 by MARK2 (Fig. 8a). This resulted in complete phosphorylation of the serine residues S262, S324, and S356 (Fig. 8b). In addition, S293, S305, and S352 were phosphorylated by about 10–20% (Fig. 8a, c).

Initially, we again started with a low-protein concentration, at which non-phosphorylated K18 did not show any sign of phase separation by either DIC microscopy or turbidity measurements (Fig. 2). Strikingly, even at 2 μM, MARK2-phosphorylated K18 readily formed liquid droplets after incubation for 1 day at 37 °C (Fig. 8d, left panel). Because 2 μM is the estimated concentration of Tau in neurons, the data demonstrate that MARK2-phosphorylated K18 undergoes LLPS at physiological protein concentrations.

When the concentration was raised to 10 μM, liquid droplet formation of MARK2-phosphorylated K18 was further promoted. After 30 min of incubation at 37 °C, micrometer-sized droplets were already observed (Fig. 8d). Continued incubation of MARK2-phosphorylated K18 at 37 °C further increased the diameter of droplets, now reaching up to 20 μm (Fig. 8d). Taken together, the data demonstrate that MARK2-phosphorylation promotes K18 LLPS.

We then asked whether non-phosphorylated K18 is recruited to liquid droplets formed by MARK2-phosphorylated K18. To this end, we labeled the non-phosphorylated protein with Alexa-488, while the MARK2-phosphorylated K18 was not fluorescently labeled. In agreement with previous results, MARK2-phosphorylated K18 formed micrometer-sized liquid droplets at 37 °C in a time-dependent manner (Fig. 8e). Because MARK2-phosphorylated K18 was not labeled with a fluorescent dye, the droplets did not show any fluorescence (Fig. 8e, top panel). As a further control, the Alexa-488 labeled non-phosphorylated protein did not form droplets over a time period of 6 h when incubated at 5 °C (Fig. 8e, middle panel). To test recruitment of non-phosphorylated K18 to liquid droplets of MARK2-phosphorylated K18, we prepared a fresh sample containing a 4:1 mixture of MARK2-phosphorylated K18 and non-phosphorylated Alexa-488-labeled K18, and incubated the sample at 37 °C for 30 min. DIC microscopy showed that droplets had formed (Fig. 8e, lower panel). Notably, the droplets were fluorescent suggesting that non-phosphorylated K18 was...
incorporated into liquid droplets (Fig. 8e, lower panel), which are primarily formed by MARK2-phosphorylated K18 (Fig. 8d).

Discussion
Pathological inclusions of the microtubule-associated protein Tau are present in several neurodegenerative diseases, collectively referred to as taulopathies1–3. Because the appearance of insoluble Tau deposits correlates with loss of neurons in patients with AD, Tau inclusions are believed to be important diagnostic markers and targets for therapeutic intervention41, 62. The pathological importance of Tau aggregation stands in strong contrast to our knowledge about the underlying mechanisms and pathways that lead from the innocuous phase of high solubility to aberrant disease-relevant Tau states in neurodegeneration. Here, we showed that the microtubule-binding repeats of Tau undergo LLPS in reducing condition at physiological temperature (Fig. 9). The droplets formed by the microtubule-binding domain of Tau are liquid-like (Fig. 2f) and do not contain amyloid fibrils (Fig. 2d), in agreement with the very high solubility of the repeat domain of Tau in reducing conditions48. Because of their large size (up to several micrometers), their spherical/circular shape (Fig. 2c, e), their reversibility (Fig. 4), and their molecular properties (Fig. 5), K18 liquid droplets are distinct from soluble aggregates of Tau including soluble oligomers. In the absence of phosphorylation, rather high protein concentrations (>~10 μM; Fig. 2b) were required to observe phase separation. Phosphorylation by the kinase MARK2, however, lowered the critical concentration for phase separation, resulting in liquid demixing already at a concentration of 2 μM (Fig. 8d), the estimated concentration of Tau in neurons60. Importantly, droplets formed by the microtubule-binding domain of Tau vary in size (Fig. 2), suggesting that also in the confined environment of a neuronal cell body, liquid droplets are feasible. Through the process of liquid–liquid demixing, the local concentration of the Tau repeat domain is strongly increased, which causes molecular crowding of amyloid-promoting Tau elements (Figs. 5, 9). In addition, liquid demixing establishes a Tau reaction chamber with a high positive charge density (the pI of the repeat domain of Tau is 9.8), which can recruit polyanionic aggregation enhancers such as heparin. The microtubule-binding domain of Tau is lysine-rich, while the domains of prior examples driving protein de-mixing (e.g., FUS, hnRNPA2B1) are glutamine/asparagine-rich. Thus, Tau demixing suggests new/different biochemical principles that can drive LLPS.

Supersaturation of proteins, proteins whose cellular concentration is high relative to their solubility, is an important mechanism for aberrant aggregation33. In contrast, the protein Tau is well below supersaturation according to known estimated concentrations of Tau in neurons (~2 μM)49 and the protein’s very low intrinsic fibrillization propensity50. This analysis, however, does not include variations in concentration due to compartmentalization. We showed that liquid droplets formed by the Tau repeat region concentrate the most aggregation-prone Tau residues, the two hexapeptides 275VQINK ~300 and 306VQIVYK311 at the beginning of repeats R2 and R3. Compartment-specific concentration results in the formation of a tight network of molecular interactions (Fig. 5). Within this protein-dense mesh, Tau’s hexapeptides are brought into spatial proximity. Liquid droplets formed by the repeat domain of Tau thus represent a supersaturated, metastable state of the protein that is distinct from the kinetically stable, monomeric innocuous Tau.

Protein inclusions such as NFTs in AD are solid-like. Within NFTs, Tau and its more aggregation-prone fragments are locked into cross-β structure, resulting in long-term storage and inactivation64. In contrast, a characteristic property of liquid-like compartments, which are not bounded by a membrane, is the rapid exchange of molecules between the interior of these compartments and the surrounding cytoplasm39, 65. The observation of strong paramagnetic broadening of NMR signals, which however do not have unique chemical shifts but those of the monomeric dispersed protein (Fig. 5), indicates that the exchange between the interior of liquid droplets and the surrounding environment is fast on the NMR time scale. Indeed, the NMR properties of K18 in conditions of LLPS are radically different, when compared to oligomeric/higher order aggregates of K18. Over the many years that we have studied the Tau protein and K18, oligomer preparations always resulted in strong loss of signal intensities in the Cα-Hx region of 1H-13C heteronuclear single-quantum coherence (HSQC) spectra even in the absence of any paramagnetic tag. This NMR signal decrease in K18/Tau oligomers is due the slow (on the NMR time scale) exchange between the oligomeric/aggregated state and the monomeric protein, precluding up to this very day a more detailed structural analysis of Tau oligomers by NMR spectroscopy. Similar observations have been made for soluble oligomers of the Parkinson-related protein α-synuclein56. The slow exchange of K18/Tau molecules between the monomeric state and the oligomeric form is in strong contrast to our finding for K18 in liquid droplets, where signal broadening is only observed in the paramagnetic state, but not the diamagnetic protein (Fig. 5b), in agreement with the liquid-like nature of droplets formed by the microtubule-binding domain of Tau. At the same time, it cannot be excluded that for example a small fraction of soluble oligomers, which are not visible to NMR, might contribute to changes in the CD signal observed at 37 °C (Fig. 4a).

A variety of molecules have been demonstrated to promote fibrilization of Tau. Particularly efficient are polyanionic factors such as heparin and ribonucelic acids23, 24. Probing a large number of physicochemical conditions in a systematic manner, we showed that conditions, in which K18 undergoes LLPS, promote amyloid formation of the protein upon addition of heparin (Fig. 6). For example, in a solution of 100 μM K18 liquid droplets were formed when incubated at 37 °C in reducing conditions for 24 h (Fig. 2c, e). When then heparin is added in the optimal molar ratio of 4:1 (K18:heparin)48, amyloid fibrils are formed...
(Fig. 6a, f, g). In contrast, K18 did not form amyloid fibrils in conditions, in which we did not observe liquid demixing. At 5 °C, for example, the microtubule-binding domain of Tau did not phase separate according to DIC and fluorescence microscopy (Fig. 2e), in agreement with the nature of a LCST\(^{33}\). Although NMR spectroscopy demonstrated that heparin binds to K18 in this condition (i.e., at 5 °C; Fig. 7), the interaction was not able to convert the protein into amyloid fibrils (Fig. 6f). The data suggest that—in reducing conditions and at physiological temperature—LLPS is necessary to increase the local concentration of Tau repeats and recruit heparin, in order for the protein to misfold into amyloid fibrils. K18-heparin coacervation changes the phase properties of the solution, resulting in a phase-separated mesh (Fig. 6a) and aggregation into amyloid fibrils (Fig. 6h). A coacervation mechanism of Tau pathogenicity is in line with the concentration of RNA in liquid droplets formed by cationic peptides and proteins\(^{67}\).

In vivo additional factors, such as molecular chaperone\(^{68,69}\), are likely to influence liquid–liquid demixing of the Tau repeats. In addition, membrane-less structures with liquid-like behavior, such as P granules, nucleoli, and stress granules contain not a single, but several different proteins, with some of them recruiting RNA and potentially undergoing liquid–liquid demixing\(^{30,32–35}\). Thus, heterotypic multivalent interactions will further influence the presence of Tau in liquid-like compartments. Consistent with this hypothesis, the Tau protein is present as speckles in the nuclei of SH-SY5Y human neuroblastoma cells\(^{70}\) and together with other proteins in stress granules of patients with AD\(^{71,72}\). Further studies will have to clarify how these different proteins work together to form Tau-containing membrane-less compartments. Notably, laser capture microdissection found 72 different proteins in NFTs\(^{73}\), indicating that protein co-aggregation is important for aberrant accumulation of Tau.

In the brains of patients with AD, the protein Tau is hyper-phosphorylated\(^{4–76}\). Tau hyperphosphorylation precedes tangle formation and at least 39 phosphorylation sites have been identified in NFTs\(^{77}\). MARK kinases efficiently phosphorylate serine residues in the repeat domain of Tau\(^{78,79}\) and inhibition of MARK kinases abrogates Aβ-induced cellular toxicity\(^{80}\). Contrast microscopy showed that phosphorylation by MARK2 changes the kinetics and dimensions of liquid droplets and promotes LLPS at lower protein concentrations (Fig. 8). In agreement with the importance of post-translational modifications for LLPS\(^{67}\), phosphorylation of the soluble tail of neurofilament modulates liquid droplet formation of the N-WASP/NCK/nephin system\(^{36}\). The influence of MARK-phosphorylation on liquid–liquid demixing of the Tau repeat domain suggests that recruitment of Tau into liquid-like cellular bodies can be controlled by kinases.

Alternative splicing of pre-mRNA generates multiple isoforms of Tau, which contain either three (3R) or four (4R) Tau repeats\(^{12}\). 3R-Tau and 4R-Tau differ in their ability to assemble microtubules and have different aggregation propensities\(^{18,81}\). Consistent with the pathological importance of alternative splicing for Tau-related neurodegeneration, Tau inclusions in tauopathies with distinct clinical manifestations contain 3R-Tau and 4R-Tau at different molar ratios\(^{5,12}\). Our study shows that the number of Tau repeats influences aqueous phase separation. In the absence of cysteine oxidation, K19 (3R-Tau) has a lower propensity for LLPS than K18 (4R-Tau) and a repeat-less Tau fragment (K25) did not undergo LLPS (Fig. 3). In agreement with attenuated phase separation, K19 contained less β-structure and repeat-less Tau (K25) experienced no time-dependent changes in structure. Moreover, aggregation of Tau into amyloid fibrils requires at least two repeats, while the repeat order and connection in sequence are less important\(^{82}\). The data suggest that each Tau repeat can be regarded as a single interaction motif. The number of Tau repeats controls the valency of interactions that drive LLPS, similar to multiple SH3 domains in the protein LAT\(^{56}\). Cellular bodies thus not only contain splicing factors\(^{83}\) and pre-mRNA splicing can occur in subnuclear speckles\(^{84}\), but alternative splicing might regulate the physicochemical properties of liquid-like compartments through controlling the multivalency of intermolecular interactions.

The wild-type Tau sequence contains two cysteine residues, C291 and C322\(^{85}\). C291 and C322 are located in the Tau repeats R2 and R3, respectively. Oxidation of the two cysteine residues has a strong influence on the ability of Tau to form amyloid fibrils\(^{86}\). Importantly, the oxidation state of the cysteines and the number of Tau repeats is strongly coupled, because C291 is only present in 4R-isomers of Tau. Indeed, while oxidation promotes fibrilization of 3R-Tau through intermolecular disulfide bond formation, cysteine oxidation in 4R-Tau delays aggregation, because of the formation of intramolecular S–S bridges between C291 and C322\(^{86}\). Thus, at first sight the finding that K19 has a much lower propensity for LLPS when compared to K18 (Fig. 6) seems to be in disagreement with a widely published aggregational data, which consistently reported more efficient fibrillation of K19/3R-Tau when compared to K18/4R-Tau\(^{23,46,48}\). However, this conflict is resolved when the oxidation/reduction state in the experiments is considered. In a reducing environment both 3R-Tau and 4R-Tau have a very low propensity to form amyloid fibrils and both require co-factors (e.g., heparin) to aggregate into amyloid fibrils. Because in living cells a highly reducing environment is found due to the presence of glutathione, we performed all experiments for K18-LLPS in reducing conditions. Nevertheless, it will be interesting to investigate in future studies how oxidation of Tau’s native cysteine residues influences its ability to undergo LLPS. In addition, studies of Tau and other proteins are required to investigate a possible connection between LLPS and cell to cell transmission of Tau aggregates\(^{86}\).

### Methods

**Protein preparation.** K18 comprises all four repeats of the largest Tau isoform (2N4R; residues Q244–E372 plus initial M243), K19 is similar but lacks the second repeat, corresponding to fetal Tau (residues M244–Q254; E365–V397; without C291), K25 comprises the projection domain of 2N4R Tau (L1–E273). K18/4R-Tau (according to 0N4R notation; corresponding to L243 in 2N4R Tau). All constructs were expressed overnight at 20 °C in Escherichia coli strain BL21(DE3)\(^{34}\) from a pNPG2 vector (a derivative of pET-3a, Merck-Novagen, Darmstadt) in 1–10 L LB for unlabeled and M9 minimal medium for labeled protein, induction with 0.5 mM IPTG for 4 h. The expressed proteins were purified by extracting bacterial extracts by making use of the heat stability of the protein and by FPLC SP-Sepharose chromatography (Amersham Biosciences). The cell pellets were resuspended in boiling extraction buffer (50 mM MES, 50 mM NaCl, 1 mM MgCl\(_2\), 1 mM ethylene glycol tetraacetic acid (EGTA), 5 mM diithiothreitol (DTT), pH 6.8) complemented with a protease inhibitor mixture. The cells were disrupted with a French pressure cell and subsequently boiled for 20 min. The soluble extract was isolated by centrifugation, the supernatant was dialyzed against two changes of 10 mM sodium phosphate, pH 8.0, followed by equilibration in 50 mM sodium phosphate, pH 8.0. Free sulphydryl groups were reacted with a five-fold molar excess of MTSL solubilized in ethyl acetate, at 4 °C for 12 h. Unreacted spin label was removed by dialysis (as...
Phosphorylation of K18 by MARK2. With the aim of achieving a high degree of phosphorylation, K18 was incubated at 25 °C with MARK2:CaT-208E at a Tau:MARK2 ratio of 100:1 for 16 h. The buffer contained 25 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (pH 8.0), 100 mM NaCl, 5 mM MgCl₂, 2 mM EGTA, 1 mM DTT, 0.5 mM phenylmethanesulfonyl fluoride, and 1 mM ATP. After phosphorylation, Tau samples were buffered exchanged with 50 mM NaH₂PO₄/Na₂HPO₄ (pH 6.8) and 10% (v/v) D₂O. The sites of phosphorylation, as well as the degree of phosphorylation at each specific site was quantified using commercially published procedures using 2D 1H-15N HSQC spectra of the phosphorylated K18 sample and a non-phosphorylated K18 sample, both recorded at 5 °C on a Bruker 700 MHz NMR spectrometer.

Far-UV CD spectroscopy. Far-UV CD measurements were performed on a Jasco J-815 spectropolarimeter equipped with a Peltier cell temperature controller (±0.2 °C) using 0.1 cm path length cuvette. Typical protein concentrations were 0.1–0.2 mg ml⁻¹ in 25 mM sodium phosphate (pH 8.8). CD spectra were recorded from 260 to 190 nm with a scanning speed of 20 nm min⁻¹ and 10 accumulations per scan. Spectra were baseline corrected using buffer. All measurements were recorded in duplicate.

Turbidity measurements. Turbidity of protein samples was estimated from the optical density at 350 nm, recorded on an Infinite M1000Pro plate reader (Tecan) using flat bottom–white wells (Greiner). For temperature-dependent measurements, heating or cooling was performed at a rate of 1 °C min⁻¹. Protein concentrations and buffer conditions are specified in the Fig. legends (Figs. 2a,b, 3a, 6b,c).

Transmitting electron microscopy. To study the interaction of K18 with heparin, two-dimensional 1H-15N HSQC spectra were recorded on a Bruker 700 MHz spectrometer, equipped with a triple pulse cryogenic probe, at 5 °C either for the protein (50 μM K18 in 50 mM sodium phosphate, pH 8.8, 0.5 mM TCEP, 0.1 mM DSS, and 10% D₂O) alone or using a 4:1 K18:heparin molar ratio. Spectra were acquired with 1024 and 256 complex data points in the direct and indirect dimension, respectively, and were processed using Bruker Topspin 2.1. 1H chemical shifts were calibrated relative to DSS and 15N chemical shifts were referenced indirectly. Paramagnetic relaxation enhancement (PRE) effects of NMR signals were extracted from the peak intensity ratios between 2D 1H-15N HSQC NMR spectra acquired for MTSL-labeled K18 (100 μM K18 in sodium phosphate, pH 8.8, paramagnetic state) and after addition of 1 mM DTT (heated to 42 °C for 30 min before measurement) to the same sample. Addition of DTT cleaves the MTSL tag from the cysteine residue such that the spin label is no longer attached to the protein and the protein is in the diamagnetic state. We previously showed that oxidation of the MTSL tag by ascorbic acid gives very similar results in the case of Tau.38 For PRE profiles, the peak intensity of every residue in the 1H-15N HSQC, which was recorded for the sample with the MTSL-tag attached to the two native cysteine residues (C291 and C322), was divided by the peak intensity in the 1H-15N HSQC with the cleaved tags (PRE = I_protein/I_control). Measurements were performed at 5 °C where K18 is present as dispersed monomeric protein, and at 37 °C where DTE micelles show that the K18 solution has undergone LPS. Resonance assignments of K18 were previously determined55 and used for the identification of the Co-Hx cross-peaks of the four threonine residues in K18.

Data availability. All relevant data are available from the corresponding author upon reasonable request.

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DIC and confocal microscopy. Droplet formation of protein samples was monitored by DIC microscopy. 15 μl of sample were loaded onto glass coverslips and DIC images were acquired on a DM6000B (Leica) microscope with a 63×-objective (water immersion) using a 488 nm argon laser. Images were acquired on a DM6000B (Leica) microscope with a 63×-objective (water immersion) using a 488 nm argon laser. Images were processed using ImageJ.

Transmission electron microscopy. For electron microscopy, the aggregated K18 sample was bound to a glow discharged carbon foil covered grid. After staining with 1% uranyl acetate, samples were examined at room temperature with a CM 120 transmission electron microscope (FEI, Eindhoven, the Netherlands) using a Tecnam F416 CMOS camera (TVIPS, Germany).

NMR spectroscopy. To study the interaction of K18 with heparin, two-dimensional 1H-15N HSQC spectra were recorded on a Bruker 700 MHz spectrometer, equipped with a triple pulse cryogenic probe, at 5 °C either for the protein (50 μM K18 in 50 mM sodium phosphate, pH 8.8, 0.5 mM TCEP, 0.1 mM DSS, and 10% D₂O) alone or using a 4:1 K18:heparin molar ratio. Spectra were acquired with 1024 and 256 complex data points in the direct and indirect dimension, respectively, and were processed using Bruker Topspin 2.1. 1H chemical shifts were calibrated relative to DSS and 15N chemical shifts were referenced indirectly. Paramagnetic relaxation enhancement (PRE) effects of NMR signals were extracted from the peak intensity ratios between 2D 1H-15N HSQC NMR spectra acquired for MTSL-labeled K18 (100 μM K18 in sodium phosphate, pH 8.8, paramagnetic state) and after addition of 1 mM DTT (heated to 42 °C for 30 min before measurement) to the same sample. Addition of DTT cleaves the MTSL tag from the cysteine residue such that the spin label is no longer attached to the protein and the protein is in the diamagnetic state. We previously showed that oxidation of the MTSL tag by ascorbic acid gives very similar results in the case of Tau.38 For PRE profiles, the peak intensity of every residue in the 1H-15N HSQC, which was recorded for the sample with the MTSL-tag attached to the two native cysteine residues (C291 and C322), was divided by the peak intensity in the 1H-15N HSQC with the cleaved tags (PRE = I_protein/I_control). Measurements were performed at 5 °C where K18 is present as dispersed monomeric protein, and at 37 °C where DTE micelles show that the K18 solution has undergone LPS. Resonance assignments of K18 were previously determined55 and used for the identification of the Co-Hx cross-peaks of the four threonine residues in K18.
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Author contributions
S.A. performed experiments for Tau phase separation. J.B. prepared Tau proteins. D.R. recorded electron micrographs. E.M. supervised preparation of Tau proteins and wrote the paper. M.Z. designed the project and wrote the paper.

Additional information
Competing interests: The authors declare no competing financial interests.