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Review Article

Phase separation of the microtubule-associated protein tau

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The aggregation and misfolding of the neuronal microtubule-associated protein tau is closely linked to the pathology of Alzheimer's disease and several other neurodegenerative diseases. Recent evidence suggest that tau undergoes liquid–liquid phase separation *in vitro* and forms or associates with membrane-less organelles in cells. Biomolecular condensation driven by phase separation can influence the biological activities of tau including its ability to polymerize tubulin into microtubules. In addition, the high concentrations that tau can reach in biomolecular condensates provide a mechanism to promote its aggregation and the formation of amyloid fibrils potentially contributing to the pathology of different tauopathies. Here, the authors discuss the role of tau phase separation in physiology and disease.

Introduction

The intrinsically disordered protein (IDP) tau is the major constituent of the neurofibrillary tangles (NFTs) that are the pathological hallmark of a wide range of neurodegenerative diseases, collectively referred to as 'tauopathies' [1–3]. Physiologically, tau is predominantly bound to microtubules and regulates their dynamic instability [4–6]. However, under pathological conditions, hyperphosphorylation and other post-translational modifications (PTMs) of tau lead to its detachment from microtubules, mislocalization of tau to the somatodendritic compartment, tau oligomerization and the formation of insoluble amyloid fibrils that might spread throughout the brain during the course of Alzheimer's disease [4,7–10]. The pathways leading to pathological tau aggregation and its spread in the brain are thus intensively investigated and might provide novel strategies for targeting these devastating neurodegenerative diseases.

Liquid–liquid phase separation (LLPS) is referred to the de-mixing of a solution into two distinct liquid phases consisting of different solute concentrations. This is a thermodynamically driven phenomenon that is generally reversible. A large number of studies in the past decade suggest that cellular compartmentalization via LLPS regulates diverse biochemical processes [11–15]. In addition, a growing body of experimental data indicate that several IDPs associated with neurodegenerative diseases not only form amyloid fibrils but also undergo LLPS indicating a plausible connection between LLPS and protein aggregation [16–20].

In this mini-review, we will focus on the phase separation of the Alzheimer's disease-related protein tau. In 2017, three independent studies were published within 2 months demonstrating that tau readily undergoes LLPS in solution [21–23]. The studies showed that both full-length tau and shorter tau fragments comprising the highly aggregation-prone repeat region form liquid-like droplets in solution. Subsequently, many studies investigated the regulation of tau phase separation, its possible relevance for the cellular activities of tau and its association with Alzheimer's disease. Here, we will give a brief overview of the mechanism of tau phase separation *in vitro*, the effect of different PTMs and mutations on tau LLPS, as well as the cellular context of tau condensation.

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Homotypic LLPS of tau

Full-length 4-repeat tau (2N4R tau), either unmodified and expressed recombinantly in *Escherichia coli* or phosphorylated and purified from insect cells, undergoes LLPS in the presence of molecular crowding agents [21,24,25]. In addition, 4-repeat tau efficiently phase separates in the absence of crowding agents in conditions of low ionic strength [26]. The droplets formed by the full-length tau protein are sensitive to the ionic strength of the buffer with a lower propensity to undergo LLPS at higher salt concentrations indicating an important contribution of electrostatic interactions to tau phase separation [24].

The tau protein is amphipathic with a non-uniform distribution of charges throughout the sequence. Based on the cluster of charges, the sequence of 2N4R tau can be divided into a negatively charged N-terminus region, a positively charged proline-rich domain and repeat domain (middle region), and a slightly negatively charged C-terminal region [4,27,28,29] (Figure 1A). Attractive electrostatic interactions between the negatively charged N-terminal region and the positively charged middle region of tau were suggested to be important for tau phase separation [12,27] (Figure 1B). Electrostatic interactions between these regions are also present in monomeric tau in the dilute phase [30], as well as in tau amyloid fibrils [31,32]. The importance of electrostatic interactions on tau LLPS was further supported through the deletion of the N-terminal 117 residues, which attenuated the ability of tau to undergo LLPS [24]. Moreover, removal of the two highly negatively charged N-terminal inserts (corresponding to the tau isoform 0N4R) reduces tau's propensity to form liquid-like droplets [24].

Hydrophobic interactions between tau molecules are also likely to influence the homotypic phase separation of tau, i.e., tau phase separation in the absence of co-factors which directly bind to tau. For example, the repeat domain of tau (K18), which is most hydrophobic and aggregation-prone, phase separates through hydrophobic interactions [23]. Notably, NMR studies showed that the KXGS motifs present in the four pseudo-repeats of K18 adopt a β-hairpin-like structure upon LLPS [33], while the overall dimensions of K18 increase upon LLPS [34]. The hydrophobic interactions will always be present; however, additional neutralization of positive charges in the repeat domain and the proline-rich region might be required to decrease repulsion between tau molecules and thus promote homotypic LLPS [23,35]. Hydrophobic interactions are also important for tau phase separation at 4–5 M salt concentration [36].

Heterotypic LLPS of tau

The tau protein efficiently phase separates in the presence of negatively charged molecules such as RNAs, which neutralize the positive charges of tau in the proline-rich region and the repeat domain [22,26,37,38] (Figure 1B). Consistent with the importance of electrostatic interaction for complex coacervation, RNA-induced tau condensates are sensitive to changes in ionic strength but less sensitive to the addition of 1,6-hexanediol [22,39] (Figure 1B). RNA-induced phase separation of Tau increases with increasing temperature suggesting that it is entropically driven [22]. The increase in entropy can be rationalized by the release of hydration water upon condensation. Entropically driven condensation of polyelectrolytes and proteins is a widely occurring process [40–43]. In the presence of 100 mM salt, crowding agents and RNA, full-length tau can phase separate into liquid-like droplets at 2.5 μM, a concentration approaching the concentration of tau in neurons [22]. Because different tau species (monomer/oligomers/aggregates) are associated with RNAs *in vivo* [22,44,45], further studies are required to better understand RNA-driven condensation of tau and its link to tau pathology in neurodegenerative diseases.

Besides RNA, the sulphated glycosaminoglycan heparin enhances phase separation of tau [37,38]. Heparin is highly negatively charged and has been extensively used in the past to induce the aggregation of tau *in vitro* [46,47]. However, recent studies questioned the relevance of using heparin to induce the aggregation of tau into amyloid fibrils [31,48].

Tau LLPS can also be regulated by metal ions [49]. For example, zinc promotes the condensation of tau *in vitro* by lowering the critical concentration to undergo LLPS [49]. In addition, small molecules can modulate the LLPS propensity of tau [50,51], and thus might provide a novel strategy to interfere with tau misfolding and aggregation.

Post-translational modifications influence tau LLPS

PTMs change the electrostatic properties of a protein, thereby influencing its phase separation and aggregation propensity. Phosphorylation is the most abundant PTM in tau with 85 potential sites for modification in 2N4R tau [52]. Phosphorylation lowers the protein's isoelectric point through addition of negative charges, thereby favoring chain compactness [27]. 2N4R tau phosphorylated *in vitro* by the kinases MARK2, Cdk2, and C-abl can undergo LLPS [53], and phosphorylated tau produced in SF9 insect cells shows a high LLPS propensity [21]. In addition, phosphorylation of the tau repeat domain K18 by the kinase MARK2 lowers the critical concentration to undergo

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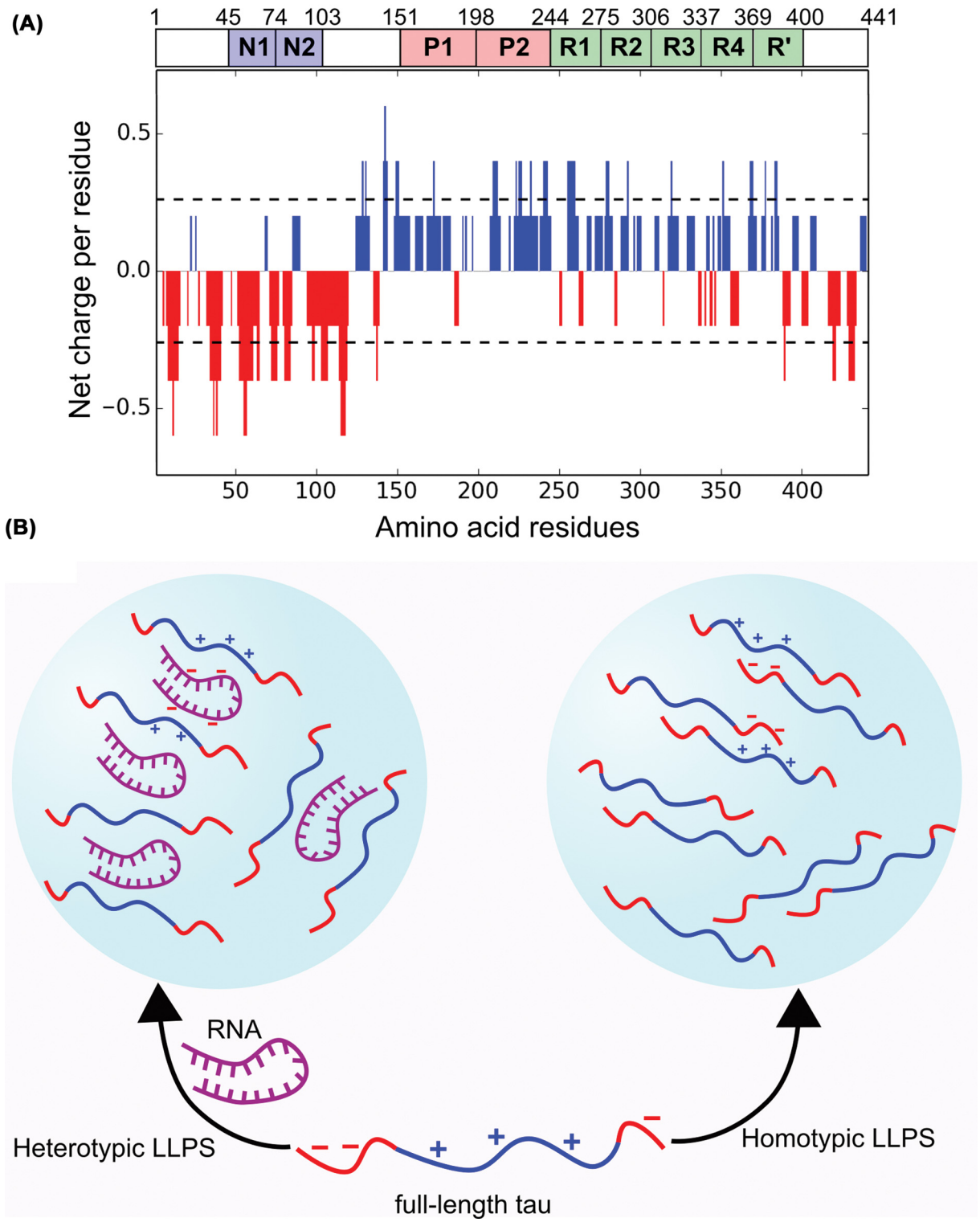

Figure 1. Homotypic and heterotypic phase separation of tau

(**A**) Net charge per residue plot of full-length tau (2N4R) protein calculated using CIDER v1.7 [28,29]. The negatively charged residues are colored in red and the positively charged residues are colored in blue. The domain diagram of full-length tau is shown above. (**B**) Homotypic and heterotypic phase separation of the microtubule-associated protein tau. The electrostatic interaction between the negatively charged N-terminus and the positively charged middle domain is important for the homotypic LLPS of tau. Attractive electrostatic interactions between RNA and tau drive heterotypic phase separation of tau.

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LLPS [23,35]. Notably, distinct patterns of phosphorylation are observed when tau is phosphorylated by different serine/threonine kinases or different tyrosine kinases. These distinct phosphorylation patterns can influence the structural properties of tau and thus modulate its LLPS propensity and aggregation.

Acetylation has emerged as another important PTM of tau associated with different tauopathies [54]. Acetylation removes the positive charge on lysine residues and thus might attenuate electrostatic interactions and heterotypic LLPS of tau. Consistent with this hypothesis, acetylation decreases the propensity of K18 to undergo LLPS in the presence of RNA resulting in droplet dissolution [26]. In addition, acetylation inhibits the homotypic condensation of full-length tau in the presence of crowding agents, supporting the importance of electrostatic interactions for tau phase separation [26,55]. We further note that tau fibrils purified from the brain of patients with Alzheimer's disease, Pick's disease, corticobasal degeneration, progressive supranuclear palsy, and globular glial tauopathy have been found to be acetylated [56,57]. It is therefore currently unknown how the phase separation behavior of acetylated tau is connected to its pathogenic aggregation.

The effect of disease-associated mutations on tau phase separation

Point mutations in the tau gene are found in patients diagnosed with the neurodegenerative disease Frontotemporal dementia with parkinsonism-17 (FTDP-17) [35]. Several of these mutations result in more rapid aggregation of tau *in vitro* [58]. Tau mutant proteins containing one of the four FTD-associated mutations P301L, P301S, ΔK280, or A152T efficiently phase separate into droplets at a concentration of 2 μM in the presence of crowding agents [35]. In contrast, unmodified tau, as well as the anti-aggregation mutant ΔK280/I277P/I308P-tau did not phase separate under the same conditions [35]. However, this behavior might be sensitive to the chosen conditions, because a later study reported that unmodified full-length tau and three FTD-associated mutants (P301L, ΔK280, and G272V) have identical saturation concentrations [38]. Thus, further studies are required to understand how disease-associated mutations in the tau gene influence its ability to phase separate.

Tau LLPS and microtubule formation

Inside of phase-separated liquid-like droplets, tau reaches high concentrations. Recruitment of tubulin into tau droplets might thus promote the polymerization of tubulin into microtubules [21,53,59,60]. Consistent with this hypothesis, the addition of GTP to tau droplets in the presence of tubulin heterodimers leads to the gradual deformation of tau drops and the growth of microtubule bundles from the drops [21] (Figure 2). Notably, however, the high concentrations of tau and tubulin reached inside the droplets was not sufficient to nucleate the formation of microtubules, but the droplet environment was crucial to observe efficient microtubule formation [21]. Moreover, full-length tau phosphorylated by the kinase Cdk2 efficiently forms droplets, but addition of tubulin and GTP to the phospho-tau droplets does not lead to the polymerization of tubulin into microtubules [53] (Figure 2). The inhibition of tau-promoted polymerization of tubulin is due to the phosphorylation of tau at the AT180-epitope (T231, S235) by Cdk2 [61]. NMR spectroscopy showed that upon phosphorylation of T231, the phosphate group forms an intramolecular salt bridge with the preceding R230 thereby attenuating the interaction of tau with tubulin [53,62]. Apart from phosphorylation, acetylation of tau attenuates the binding of tau to tubulin and its ability to promote microtubule formation from tau droplets [55].

Tau can also phase separate on the surface of microtubules [59,60]. Recruitment of tau to the microtubule surface promotes condensate-like structures, sometimes called tau islands, at extremely low tau concentrations (0.25 nM) [59,60]. Condensate formation and clustering of tau on the microtubule surface might be modulated by phosphorylation [60]. In addition, the disease-associated mutation R5L, which is located at the N-terminus far away from the microtubule-binding region of tau, modulates tau condensation/clustering on the surface of microtubules [63].

Liquid to solid phase transition of tau

The molecular factors that trigger the aggregation of tau, which is a highly soluble protein in the absence of co-factors, into insoluble deposits in tauopathies are still enigmatic. An important role of phase separation in this process is supported by a number of studies. For example, the physicochemical properties that promote LLPS of K18 also promote amyloid formation of K18 [23], and K18 phase separation weakly enhances the β-content of the protein [23]. In addition, droplets of full-length tau, which have been incubated for one day at room temperature, can be separated by centrifugation indicating maturation of the droplets into a gel-like phase [35]. After incubation for 10 days, most of the droplets transitioned into amorphous aggregates which upon binding to the amyloid dye Thioflavin-S displayed

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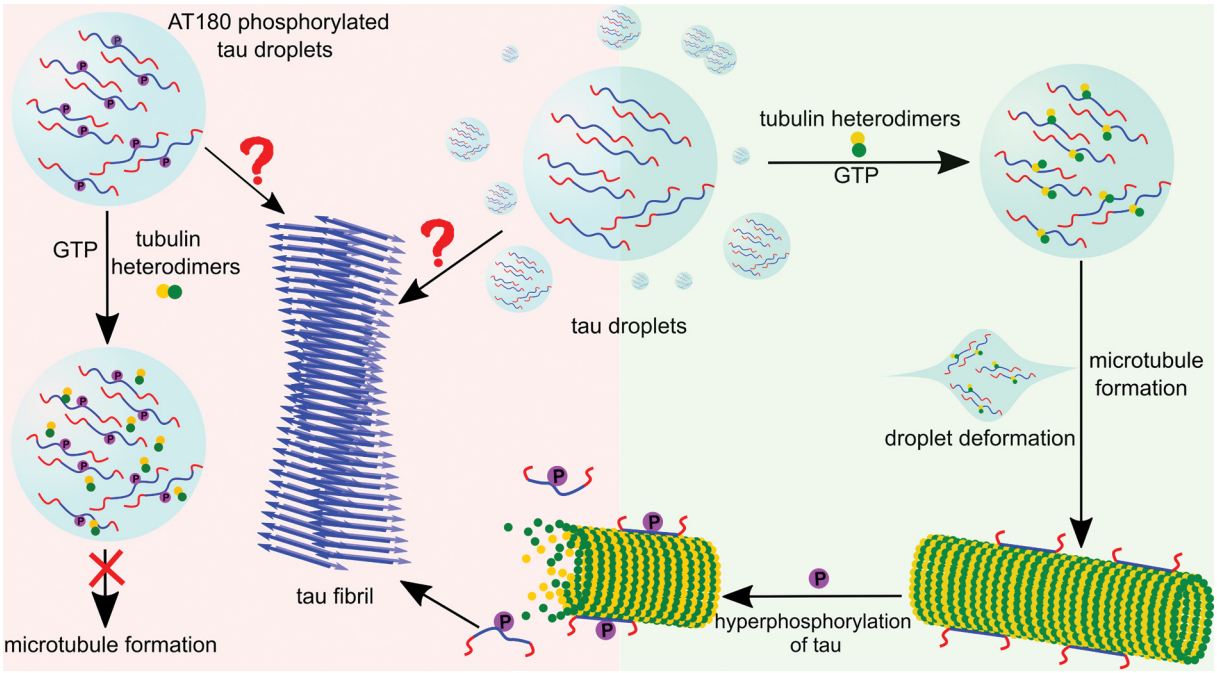

Figure 2. Tau LLPS, microtubule formation, and aggregation

In physiological conditions, tau condensates can recruit tubulin heterodimers and form stable microtubules from the droplets. Tau is bound to microtubules and stabilizes microtubule assembly. Under pathological conditions, tau is hyperphosphorylated, detached from the microtubule surface, and forms amyloid fibrils in the diseased brain. In addition, tau is phosphorylated at the AT180 epitope which blocks the formation of microtubules from tau condensates.

enhanced fluorescence [35]. In another study, prolonged incubation of droplets formed by unmodified full-length tau or P301L tau resulted in the formation of gel-like tau phases which were Thioflavin-S negative and contained tau oligomers [25]. Disease-associated mutations in tau might also not directly affect the LLPS propensity of tau directly, but enhance the rate of fibrillization within the droplets [38]. This would suggest that LLPS and fibrillization of disease-associated tau mutants are potentially driven by different types of interactions.

We recently reported that full-length tau can efficiently form amyloid fibrils *in vitro* that have structural similarities to brain-derived tau fibrils [31]. To induce the aggregation of tau, we used low ionic-strength conditions. Notably, full-length tau undergoes LLPS under similar conditions in the absence of crowding agents [26]. However, which role phase separation plays in the formation of co-factor-free tau fibrils is not yet known.

Phase separation of tau in cells

While we increasingly understand the factors that modulate tau phase separation *in vitro*, much less is known about tau condensation in cells. When GFP-tagged full-length tau is expressed in primary cortical mouse neurons, mobile droplet-like accumulations can be observed in the cytosol [35]. The droplet-like accumulations rapidly recover fluorescence after photobleaching with an immobile tau fraction of ∼30%. In addition, the droplets formed by full-length tau are less mobile when compared with droplets formed by the N-terminal projection domain (residues 1–256), likely because of the binding of full-length tau to microtubules through its repeat domain. Another study reported that the expression of GFP-tagged full-length tau in HT22 murine hippocampal neuronal cells leads to the formation of condensates in which tau proteins were distributed only at the shell of the drops [64]. In the present study, the tau isoform with two N-terminal inserts (2N4R) formed condensates more efficiently in the HT22 cells than the isoforms having one (1N4R) or no N-terminal insert (0N4R), suggesting an important contribution of the inserts to tau condensation in cells [64].

Tau also associates with stress granules [65], which are membrane-less compartments in the cytosol containing several RNA-binding proteins (RBPs) that assemble in response to stress conditions to assist cell survival. Under stressful conditions, tau undergoes hyperphosphorylation and is mislocalized to the somatodendritic compartment where the concentration of stress-granule-associated RNAs is high [65]. The abundance of RNAs in the stress granules

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might thus contribute to the interaction of tau with other RBPs. Different RBPs that appear in the interactome of both tau and stress-granules are hnRNPs, the RNA-binding protein EWS and the RNA-dependent RNA helicase DDX [65]. Mutations in the genes encoding these RBPs are associated with amyotrophic lateral sclerosis (ALS) in which the mutations increase the tendency of the protein to aggregate and accumulate in stress-granules. The association of tau with these RBPs might thus enable tau to regulate translational stress response [65]. The connection between tau, stress-granules and translational stress response is further supported by the observation that the expression of tau in primary neuronal cultures and neuronal cell lines leads to the formation of stress-granules [66]. However, reducing the level of the RBP TIA-1 prevents tau-positive stress-granules and tau-mediated toxicity [66,67]. Notably, different disease-associated mutations in the MAPT gene promote bigger and more stable stress granules [67]. Phosphorylation of tau also plays an important role in this pathway by promoting the formation of larger stress-granules [67]. On the other hand, acetylation of tau decreases its association with stress-granules [26]. Because acetylation is associated with enhanced neurotoxicity in different tauopathies [68,69], stress-granules might also have a protective role in the context of tau pathologies.

Summary

- Tau phase separates in solution in particular with RNA.
- Tau phase separation promotes tau aggregation.
- Phosphorylation enhances tau LLPS, but acetylation reduces tau LLPS.
- Tau condensates recruit tubulin and promote microtubule formation.
- Tau can undergo LLPS in cells and is associated with stress granules.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

P.C. – Writing original draft, review, and editing. **M.Z.** – Supervision; writing original draft, review, and editing.

Abbreviations

FTDP-17, frontotemporal dementia with parkinsonism-17; GFP, green fluorescent protein; GTP, guanosine triphosphate; IDP, intrinsically disordered protein; K18, repeat-domain of tau; LLPS, liquid–liquid phase separation; MAPT, microtubule-associated protein tau; NFT, neurofibrillary tangles; NMR, nuclear magnetic resonance; PTM, post-translational modification; RBP, RNA-binding protein; TIA-1, T-cell intracellular antigen 1.

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