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Review

Phase separation on microtubules: from droplet formation to cellular function?

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Microtubules are cytoskeletal polymers that play important roles in numerous cellular processes, ranging from the control of cell shape and polarity to cell division and intracellular transport. Many of these roles rely on proteins that bind to microtubule ends and shafts, carry intrinsically disordered regions, and form complex multivalent interaction networks. A flurry of recent studies demonstrated that these properties allow diverse microtubule-binding proteins to undergo liquid–liquid phase separation (LLPS) *in vitro*. It is proposed that LLPS could potentially affect multiple microtubule-related processes, such as microtubule nucleation, control of microtubule dynamics and organization, and microtubule-based transport. Here, we discuss the evidence in favor and against the occurrence of LLPS and its functional significance for microtubule-based processes in cells.

Highlights

Liquid–liquid phase separation (LLPS) is a common phenomenon observed for microtubule-binding proteins expressed recombinantly *in vitro*, or overexpressed in cells.

LLPS of microtubule-binding proteins *in vitro* can be driven by very different types of interactions, involving intrinsically disordered regions and folded domains.

Binding to microtubules can promote formation of protein condensates.

Condensates of tubulin-binding proteins can potentially promote microtubule nucleation and accelerate microtubule elongation.

Condensate formation by the same or homologous proteins strongly depends on the species, cell type, or cell cycle phase.

Conclusive evidence that LLPS occurs at physiological conditions in cells is often missing.

LLPS and formation of protein condensates *in vitro* and in cells

LLPS (see Glossary) is a process of demixing of two immiscible or semi-miscible liquids, often illustrated through the 'vinegar in oil' analogy. LLPS has been proposed to be the mechanism behind the formation of membraneless cell compartments, often termed 'biomolecular condensates', including nucleoli, P-bodies, and stress granules, where specific macromolecules such as proteins or nucleic acids are concentrated (reviewed in [1,2]). In this review, we will focus on microtubule-binding proteins and protein complexes and discuss the evidence that they form non-stoichiometric condensates through LLPS. We will use the term LLPS broadly, as is currently common in cell biology literature, although it is clear that the term is often used to describe phases that are not simple liquids [3].

What interactions drive proteins into the condensed phase? Homo- and heterotypic protein–protein interactions can depend on the binding between folded domains, between a folded domain and a linear motif, or the interactions between **intrinsically disordered protein regions (IDRs)** [4,5] (Figure 1A). Many proteins that exhibit LLPS *in vitro* carry IDRs; presence of IDRs correlates with the ability of these proteins to phase-separate [6]. IDRs often display poor sequence conservation, leading to the idea that formation of mesoscale compartments by LLPS is driven by low-affinity interactions between IDRs and relies on physical properties of amino acids rather than sequence-encoded specific, high-affinity interactions [7,8]. However, in principle, sequence-specific, high affinity interactions involving folded domains might also promote formation of protein condensates [4].

An important consequence of LLPS is 'concentration buffering': changing the amount of a phase-separating protein leads to the change in the volume of the condensed phase, while the protein's concentration in the 'dilute phase' remains constant (Figure 1A). A simple way to induce protein condensation is therefore to increase its concentration. *In vivo*, this can be achieved by overexpressing the protein of interest and observing formation of droplets that can fuse over time.

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When working with purified proteins *in vitro,* inert 'crowding agents' such as polyethylene glycol (PEG) can be added to deplete the solvent volume accessible to proteins and increase their local concentration to promote LLPS. However, the relevance of specific crowding agents in mimicking cellular environment is limited, because cellular components can both promote and inhibit condensate formation and overall can have major effects on phase separation of a particular protein [9].

These approaches have been routinely used to observe LLPS of numerous proteins, but in fact, studying LLPS of a given protein is only relevant if it happens at the physiological concentration. Given how easily overexpression can result in condensate formation, tight control of expression levels is therefore essential, but, unfortunately, often omitted in studies reporting LLPS (reviewed in [10]). Furthermore, as the name implies, for the LLPS mechanism to hold true, the condensed phase should remain liquid and exchanging with the dilute phase. Evidence to this is provided either qualitatively, by demonstrating 'fusion' of protein condensates, or quantitatively, by measuring **fluorescence recovery after photobleaching (FRAP)**. However, for microscopic condensates, fusion can be easily confused with co-localization of sub-diffraction protein foci and fluorescence recovery can be influenced by interactions distinct from LLPS, such as transient binding to a scaffold. In many cases, protein droplets are not exchanging with the solution and are described as 'hardening', or 'gelating'. In these conditions, it may be difficult to determine whether their formation occurs through LLPS or aggregation; alternatively, the concentration of molecules in the dilute phase may be simply very low to observe exchange with the condensed phase. Reagents widely used to distinguish LLPS from aggregation are derivatives of hexanediol, which disrupt certain types of hydrophobic interactions [11] and can affect LLPS of various proteins both *in vitro* and *in vivo*, while having little effect on some non-LLPS interactions; however, these reagents are cytotoxic [12] and thus performing appropriate controls is very difficult [13] (Box 1).

Microtubules as a platform for LLPS

Microtubules are cytoskeletal filaments with lattice-like walls built from globular tubulin subunits, which have negatively charged disordered C-terminal tails extending into solution (Figure 1B). Highly ordered polymeric structures of microtubule lattices can concentrate microtubule-binding proteins through specific interactions that depend on folded domains and/or IDRs. Additionally, some microtubule-binding proteins have increased affinity for microtubule ends, leading to their accumulation in even smaller volumes. This increased affinity can result from preferential binding to bent tubulin protofilaments, to specific tubulin conformations associated with certain states of GTP hydrolysis, or to tubulin surfaces or interfaces that are only exposed at microtubule ends [14,15]. Furthermore, many microtubule-binding proteins can associate with each other, forming complex multivalent interaction networks [14]. Together, all these interactions can trigger formation of microtubule surface-bound protein condensates or liquid droplets, held together by low-affinity interactions that cannot be observed in cytosol but require local enrichment provided by microtubule lattices and ends (Figure 1C; discussed in [16]). Condensates of microtubule-binding proteins can potentially help to locally control microtubule stability and dynamics, promote interactions with other cellular structures, or generate functionally different microtubule subsets that can be recognized by motors responsible for intracellular transport. Furthermore, the reverse process of concentrating tubulin by a droplet of a microtubule-binding protein can trigger microtubule nucleation or accelerate microtubule polymerization. Recent publications propose involvement of LLPS in the formation and function of various microtubule-based cellular structures (Figure 1D). Next, we consider different examples of microtubule-binding proteins with demonstrated ability to undergo LLPS *in vitro* and/or in cells and discuss the potential functional relevance of these observations.

Glossary

End binding (EB) proteins: EBs and their homologs form comet-like structures that follow the ends of growing microtubules.
Fluorescence recovery after photobleaching (FRAP): a technique used to measure exchange rates and/or mobility of fluorescently tagged molecules.
γ-TuRC: γ-tubulin ring complex, a protein complex serving as the template for microtubule nucleation.
Intrinsically disordered regions (IDR): regions of proteins, amino acid sequences without defined secondary structural features.
Liquid–liquid phase separation (LLPS): the process of demixing of two liquids with impaired miscibility.
Microtubule-organizing center (MTOC): sources of microtubule nucleation in the cell.
Ostwald ripening: tendency of phase-separated liquid droplets to fuse over time, driven by thermodynamic processes aimed at minimizing the surface area between two phases.
Plateau-Rayleigh instability: beading up of liquids that wet a particular surface poorly.
+TIPs: microtubule plus end-tracking proteins

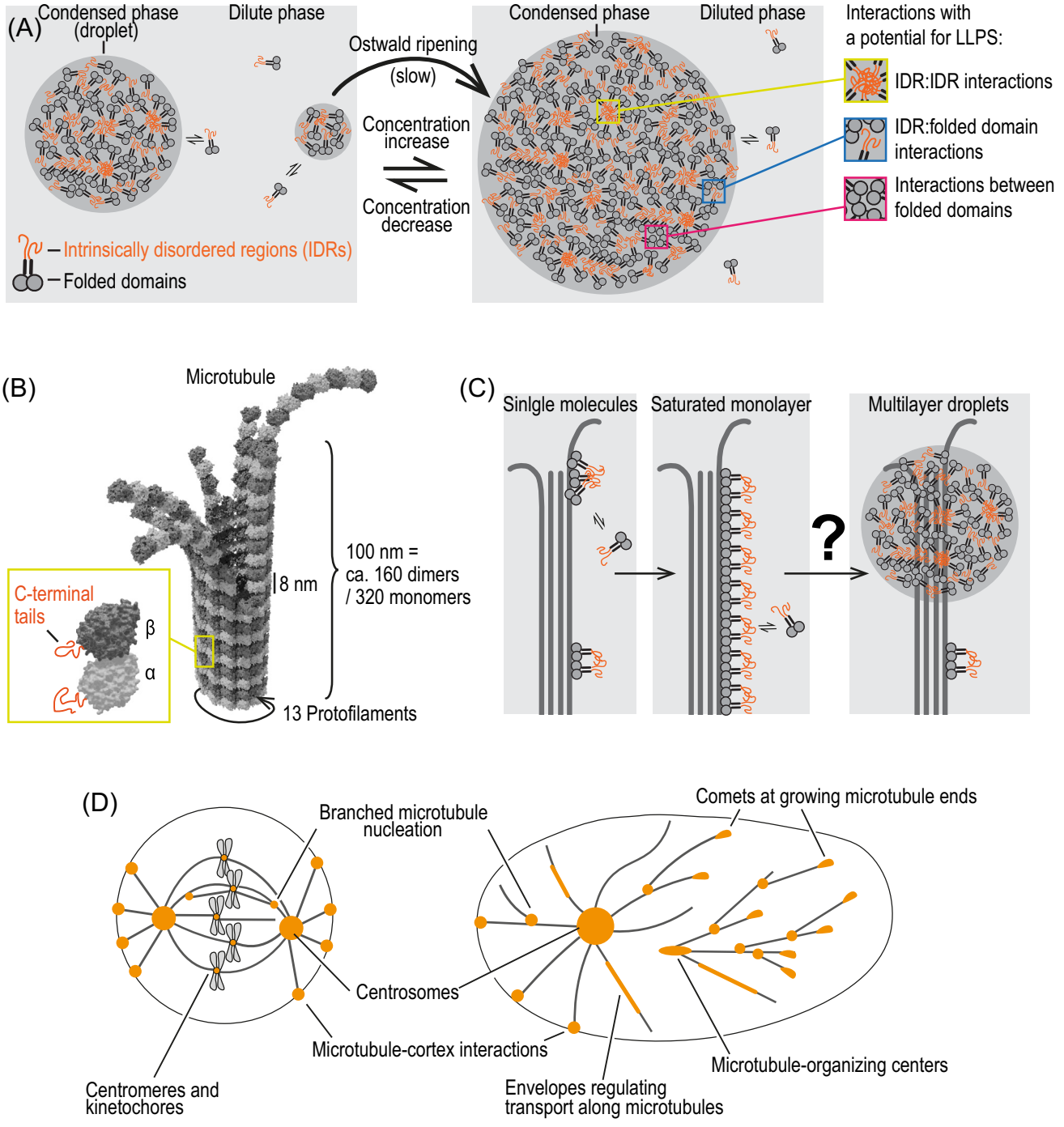

Figure 1. Mechanism and proposed significance of liquid–liquid phase separation (LLPS) on microtubules. (A) LLPS of a dimeric protein that contains folded domains (black) and intrinsically disordered regions (IDRs, orange). Increase in concentration leads to an increase in the volume of the condensed phase, with a minor effect on the protein concentration in the dilute phase. In addition, liquid droplets can fuse over time and material from smaller droplets can redistribute into larger ones, a phenomenon known as Ostwald ripening. (B) Microtubule structure. A hundred nanometers of microtubule lattice provides about 160 binding sites for a protein binding to every tubulin dimer. (C) Cooperative binding of a microtubule-associated protein can lead to formation of a saturated monolayer, where all binding sites on the

(Figure legend continued at the bottom of the next page.)

Box 1. Challenges in linking results of *in vitro* reconstitution to cellular function

Several common strategies are used to link formation of protein condensates *in vitro* to the existence of LLPS-mediated structures *in vivo* and to the conclusion that LLPS is functionally important:

(i) Deletion/mutation approach. Formation (or lack thereof) of spherical droplets or condensates of full-length and mutant proteins *in vitro* is correlated to localization of the same protein constructs in cells. This approach is limited by the fact that deleted/mutated protein regions might mediate interactions that are independent of LLPS. This approach can be potentially improved by generating protein constructs that lack LLPS properties, which may show some redundancy, but retain other relevant interactions (e.g., folding properties and binding to partners), and vice versa [4].

(ii) Protein overexpression with or without additional oligomerization domains such as Cry2 [100]. This approach is technically less challenging than studying endogenously tagged proteins and can be very useful for studying biophysical aspects of phase separation in cells, but it strongly promotes condensate formation and is by itself not informative about the behavior of endogenous, non-tagged proteins (reviewed in [10]).

(iii) Observation of 'liquid-like behavior' and 'fusion events' between protein condensates in cells. This approach can provide direct evidence for the liquid-like nature of protein oligomers, but distinguishing fusion from co-localization can be challenging for diffraction-limited foci, even using super-resolution microscopy [72–74].

(iv) FRAP to test the exchange between condensed and dilute phases. Low recovery rates could be interpreted as formation of stable condensates [18,20,21,48,72–74], while high recovery rates, as formation of liquid droplets [18,20,22,38,72–74,85,94], often within the same study. However, LLPS-independent interactions, such as binding between proteins, or interactions with a scaffold can also influence mobility of proteins [10]. Altogether, FRAP provides no information on the material properties of a particular cellular structure and may not be informative when the protein concentration in the dilute phase is very low.

(v) Use of hexanediol to dissolve condensates. Hexanediol has been proposed to selectively dissolve membraneless organelles formed by LLPS while leaving protein aggregates intact [12]. However, recent evidence points to side effects of this reagent on various cellular processes [13,101]. Furthermore, hexanediol preferentially disrupts hydrophobic interactions, whereas LLPS can also be driven by, for example, electrostatic interactions that are hexanediol-insensitive.

(vi) Analysis by electron microscopy and tomography. This technique can provide insight into the internal organization and dimensions of protein droplets and oligomers at the scale inaccessible to light microscopy [18,39,65,86]. However, interpreting flexible protein structures in crowded cellular environment is challenging and, even in the case of *in vitro* reconstituted oligomerization, these observations remain qualitative and do not allow to clearly distinguish LLPS from other protein–protein interactions [65].

LLPS in microtubule nucleation and spindle organization

The rate-limiting step in the initiation of microtubule polymerization is the formation of the primary nucleus or template (reviewed in [17]). Therefore, local concentration of tubulin in a small volume due to a high density of tubulin-binding molecules should facilitate nucleation of microtubules. With this mechanism in mind, the centrosome, a major **microtubule-organizing center (MTOC)** in animal cells, has been proposed to be a phase-separated condensate that concentrates tubulin-binding molecules and tubulin [18]. This idea is supported by the ability of a key player in centrosome formation in *Caenorhabditis elegans*, a coiled coil scaffold SPD-5, to undergo LLPS and subsequently harden into gel-like condensates that can concentrate worm homologs of microtubule and tubulin-binding proteins TPX2 and XMAP215, accumulate tubulin, and nucleate microtubules *in vitro* [18]. It should be noted, however, that microtubule-nucleating properties were also observed *in vitro* for condensates of proteins that are not known to nucleate microtubules on their own in cells [19–22]. Furthermore, if it is easy to nucleate microtubules through LLPS, how does the cell prevent formation of spurious MTOCs by every condensate of a tubulin-binding molecule? And finally, condensates of a plus-end specific microtubule polymerase such as XMAP215 might be expected to trigger formation of microtubule asters with the plus ends facing inward, and not outward, like in cellular MTOCs (Figure 2A). Cells seem to circumvent these problems by fine-tuning

microtubule are occupied. Further increase in the protein concentration can potentially result in formation of multiple layers (reviewed in [16]). Addressing the question whether LLPS properties can then lead to nucleation of liquid droplets at the microtubules requires further evidence. (D) Intracellular locations and functions where LLPS of microtubule-binding proteins has been proposed to result in a biological effect.

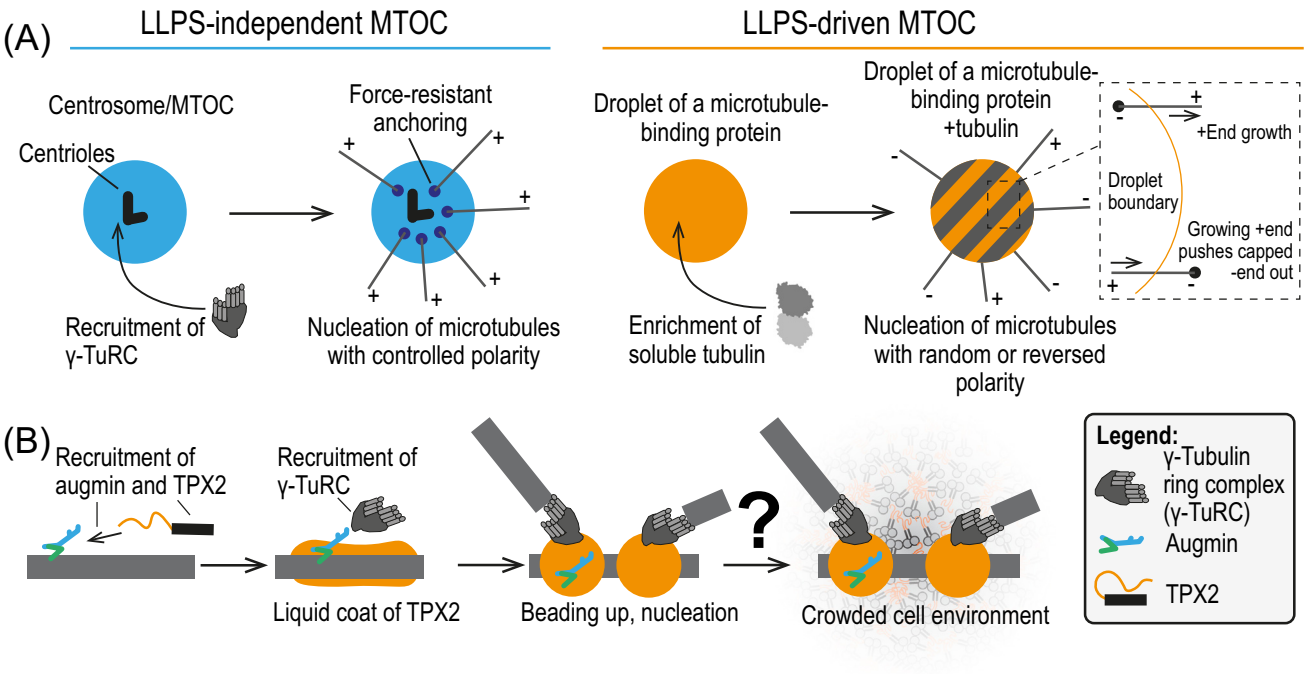

Figure 2. Liquid-liquid phase separation (LLPS)-dependent and -independent mechanisms of microtubule nucleation and branching. (A) Tubulin enrichment in a droplet of a microtubule-binding protein can result in nucleation of microtubules with mixed or reversed polarity (minus ends pointing outwards). Resulting polarity will depend on the properties of the droplet-forming protein. For example, plus-end binding proteins, such as XMAP215, will likely anchor plus ends of the nucleated microtubule inside the droplet, letting minus ends extend outwards, even if the minus ends are capped by the γ-tubulin ring complex (γ-TuRC). (B) Branched microtubule nucleation requires recruitment of γ-TuRC, with the help of augmin, TPX2, and XMAP215. Formation of liquid droplets of TPX2 can enhance this process and an important question is whether this occurs in cells and whether beading of TPX2 determines the spacing between microtubule 'branches' in spindles. Abbreviation: MTOC, microtubule-organizing center.

microtubule nucleation such that it predominantly occurs from a specialized template, the γ-tubulin ring complex (**γ-TuRC**), which caps and tethers microtubule minus ends. Isolated centrosomes that contain phase-separating components are unable to nucleate microtubules in the absence of γ-TuRC [23]. While γ-TuRC-independent microtubule nucleation can occur in some conditions [24,25], whether it is driven by protein condensates such as those that can form *in vitro* by the minus-end-decorating protein CAMSAP2 [26], remains to be investigated.

Overall, it appears that in cells, centrosomes (as well as other microtubule-nucleating structures) predominantly form by concentrating molecules that bind and activate γ-TuRC. The assembly of such proteins into centrosomes depends on their mutual interactions, binding to the major centrosomal scaffold, the centriole, and the minus-end-directed transport along centrosome-attached microtubules by dynein [27]. In interphase, centrosomal proteins appear to be organized in a highly ordered fashion around centriole walls [28]. When centrosomal proteins self-organize in interphase in the absence of centrioles, they form a linearly shaped compact cluster of particles that do not exchange with the cytoplasm and are brought together by dynein-based transport [29], features arguing against LLPS involvement. However, at the onset of mitosis, centrosomes strongly increase in size, because self-association of centrosome components is increased due to phosphorylation by mitotic kinases (reviewed in [27,30,31]). This leads to formation of condensate-like foci, which can be observed with endogenously tagged proteins, such as mammalian pericentrin [32]. In order to prevent ectopic microtubule nucleation by

condensates of centrosomal proteins, their formation is tightly regulated by proteolytic degradation involving ubiquitin ligase TRIM37 [33–35].

The concept of LLPS can thus help to explain important features of mitotic centrosome assembly, as well as the self-assembly of spindle pole components organizing the minus ends, such as NuMA [36]. However, not all observations are in line with this mechanism. For example, an essential centrosome component in dividing fly cells, Spd-2, is recruited to the centriole wall and then fluxes outwards, an orderly dynamic behavior distinct from formation of homogeneous droplets [30,37]. Centriole-dependent recruitment of centrosome proteins helps to explain how dividing cells form two centrosomes of equal size, whereas a mechanism based on LLPS alone predicts that fluctuations in centrosome size could be amplified through a thermodynamically driven process known as **Ostwald ripening** (Figure 1A), whereby material from smaller particles redistributes into larger ones. Altogether, LLPS alone is not sufficient to explain centrosome formation and the dynamics of its components.

LLPS was also proposed to be involved in branching microtubule nucleation, a process dependent on γ-TuRC, the γ-TuRC-binding complex augmin, XMAP215, and TPX2 [38,39] (Figure 2B). *In vitro* experiments with purified proteins in absence of crowding agents, or with *Xenopus* egg extracts showed that TPX2 at endogenous concentration can condense on microtubules through an IDR-based mechanism and form droplets that can accumulate tubulin and promote augmin-dependent microtubule nucleation [38,39]. Beading up of TPX2 can be interpreted by the process known as **Plateau-Rayleigh instability** (Figure 2B) and can define the density of new branching nucleation sites during initial spindle formation [39]. However, at present, it is unknown whether the spacing between new microtubule 'branches' within spindles in cells corresponds to the spacing between TPX2 condensates observed in cell extracts and whether it is driven by phase separation of TPX2 on spindle microtubules or by other numerous factors present within the spindle.

This question seems relevant as spindle microtubules were suggested to be decorated with other condensates, such as those formed by the potential spindle matrix component BuGZ, a protein that forms droplets *in vitro* in absence of crowding agents through hydrophobic interactions between low complexity regions [40]. During meiosis in mammalian oocytes, numerous centrosomal and microtubule-binding proteins associate with each other and form large dynamic droplets that permeate the spindle and accumulate at the poles [41]. No such structures are found in other dividing cells: TACC3 and its binding partner clathrin, which form the basis of droplets in oocyte spindles, crosslink kinetochore microtubules and do not appear as droplets in mammalian mitosis [42]. This indicates that condensate formation of certain proteins can be cell type-specific.

LLPS of proteins along microtubule shafts and control of microtubule-based transport

Condensation on microtubules can increase the density and decrease the mobility of proteins. An interesting example of this phenomenon is provided by tau, a microtubule-associated protein (MAP), which is involved in stabilizing microtubules in neuronal axons but is absent from dendrites, where another neural MAP, MAP2, predominates [43,44]. LLPS of tau has been linked to tau aggregation observed in several neurodegenerative diseases (reviewed in [45,46]). *In vitro*, microtubule-bound tau in absence of crowding agents can form dynamic regions of increased concentration and reduced mobility, termed 'islands' or 'envelopes' [47,48]. Tau envelopes are sensitive to hexanediol and form due to hydrophobic interactions between adjacent copies of the so-called pseudo-repeat domain, which does not represent the primary microtubule-binding region of tau but contributes to microtubule affinity; tau within the envelopes

does not bead up and seems to consist of a single layer, which depends on interactions with tubulin tails [48]. Analysis of tau-related MAPs showed that the dendritic protein MAP2c, but not ubiquitously expressed MAP4, can form envelopes and mix with tau [49]. This indicates that microtubule-driven condensation of these proteins shows some specificity but does not contribute to differential partitioning of tau and MAP2c to axons and dendrites.

The potential function of cohesive tau and MAP2c envelopes was demonstrated by their ability to locally inhibit microtubule severing by spastin and katanin; furthermore, tau envelopes were impenetrable to kinesin-1 and both tau and MAP2c inhibited but not blocked motility of dynein-dynactin-adaptor complexes [47–49] (Figure 3A). These inhibitory effects could be due to a different degree of steric hindrance, but in case of kinesin-1 could also be caused by the fact that tau (as well as MAP2c) envelopes prefer and induce compacted, GDP-like, microtubule lattice [49], whereas kinesin-1 prefers expanded microtubule lattice [50–52] (Figure 3B). Existence of tau envelopes in cells is potentially supported by the observation of tau puncta in developing neurons [48]. However, counting of tau molecules in microtubule-bound oligomers in cells revealed that the majority of tau exists as monomers, with less than half in dimers and trimers [53]. These measurements were, however, mostly performed in non-neuronal cells and more thorough analyses of the oligomerization status of endogenous tau in primary neurons would be needed. Since tau is abundantly localized in axons, and kinesin-1 is a major motor in this neuronal compartment [54], it is hard to imagine that extended tau envelopes would be present on all axonal microtubules, as they would impede axonal transport. However, there is increasing evidence that microtubules in neurons are organized as differentially modified subsets [55,56] and it is likely that MAPs also show similar inhomogeneous distribution. MAPs such as MAP7 [57] and MAP4 [49] can compete with tau and may thus potentially restrict its binding and envelope formation on specific microtubule subsets to promote transport. MAP7 homologs are particularly attractive candidates for this function as they recruit kinesin-1 to microtubules [57–60] and one of MAP7 isoforms was reported to be enriched in the proximal axon, where cargos enter this neuronal compartment [61]. Overall, the next step in this research area is to obtain detailed information about MAP compartmentalization on individual neuronal microtubules.

LLPS at growing microtubule plus ends

Whereas it is unclear whether MAPs are indeed compartmentalized along microtubule shafts, it is firmly established that growing microtubule ends are specifically decorated with the so-called plus-end tracking proteins (**+TIPs**) [14]. **End binding (EB) proteins** are among the most conserved +TIPs; through their N-terminal globular calponin homology (CH) domains, EBs recognize the GTP cap at the microtubule ends [62]; although CH domains are sufficient for plus-end tracking, additional affinity is provided by the adjacent positively charged IDRs [63–65]. EBs recruit to

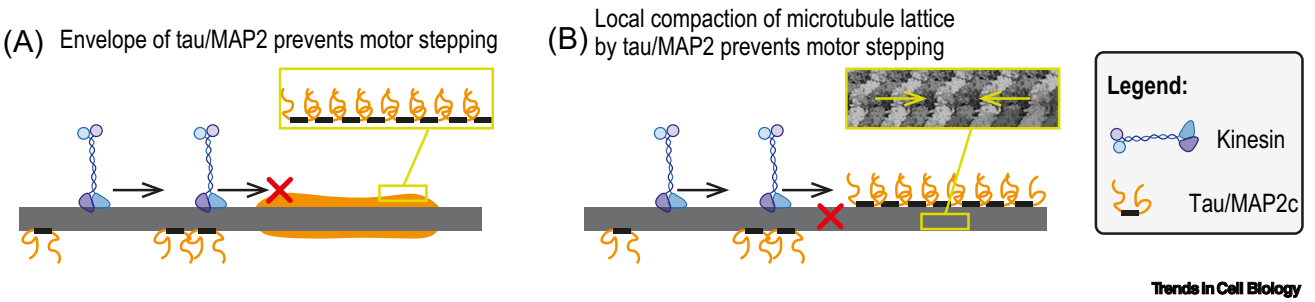

Figure 3. Liquid-liquid phase separation (LLPS)-dependent and -independent mechanisms regulating kinesin motility within 'envelopes' produced by tau and microtubule-associated protein 2 (MAP2). (A) Physical properties of tau/MAP2 'envelopes' prevent kinesin from passing through. (B) Tau-driven compaction of microtubule lattice inhibits binding of kinesin, which prefers expanded microtubule lattice.

microtubule ends a large variety of other +TIPs, which can be broadly divided into two classes: proteins containing globular CAP-Gly domains that bind to C-terminal acidic-aromatic motifs in disordered EB tails and α-tubulin, and proteins with positively charged IDRs containing linear SxIP or LxxPTPh motif(s) that bind to the dimeric α-helical EB homology domains [14,66,67] (Figure 4). Both types of interactions are highly specific and can be easily perturbed by single point mutations [68–70]. Furthermore, EB-binding +TIPs also specifically interact with each other, forming multivalent interaction networks [67], and this makes them potentially prone to LLPS.

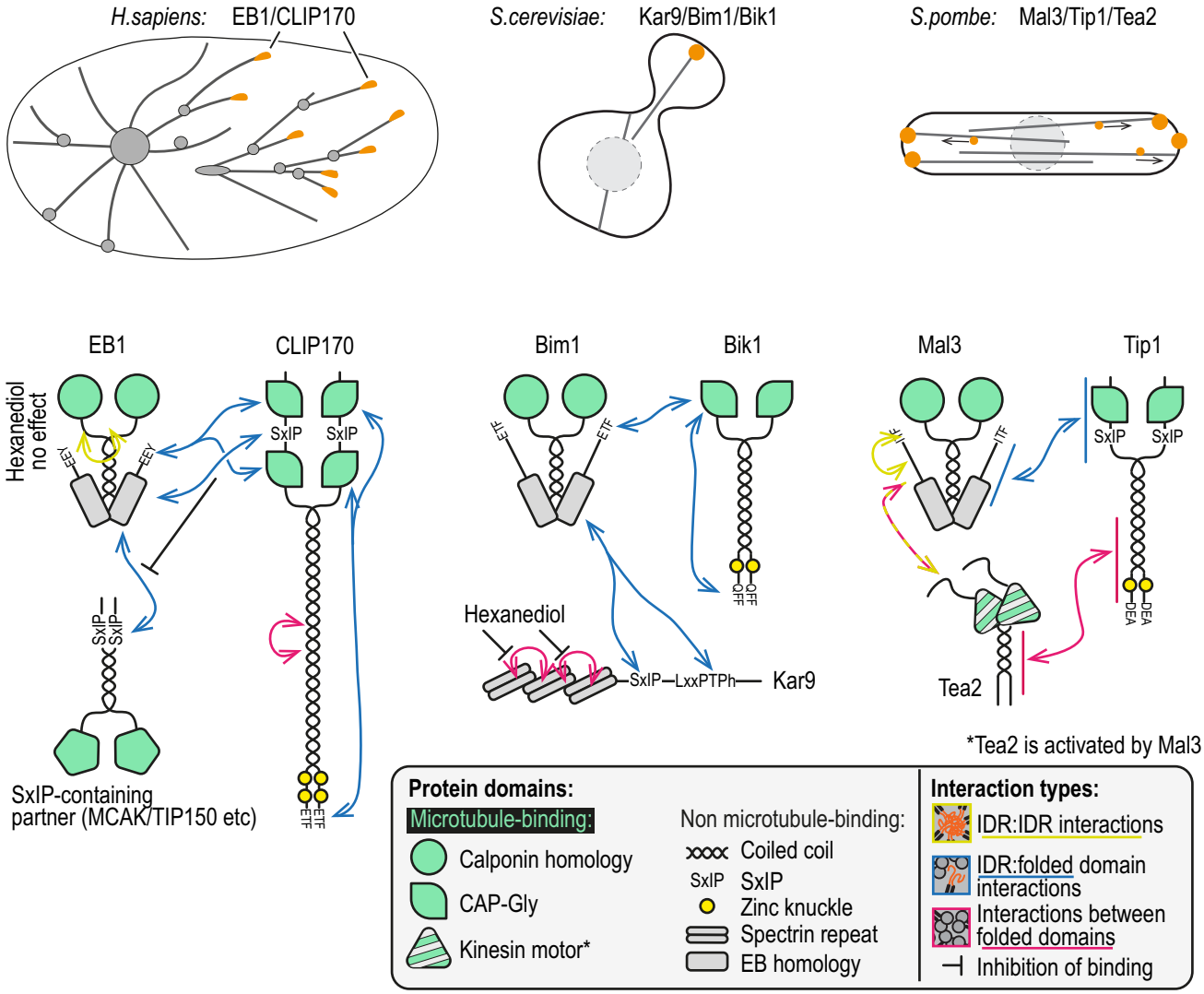

Figure 4. Comparison between domain organization and protein–protein interactions between +TIPs in humans and budding and fission yeasts. Microtubule-binding domains are shown in green. Interaction arrows are color-coded according to three interaction types between intrinsically disordered regions (IDR) and folded domains (IDR:IDR, yellow, IDR:folded, blue, and folded:folded, red, see Figure 1A). Formation of EB1:CLIP-170 complex prevents EB1 interaction with other SxIP-containing proteins [102,103], whereas Bim1 can form ternary complexes with the CLIP-170 homolog Bik1 and the EB1-binding motifs SxIP and LxxPTPh [104]. Self-interaction of EB1 and Mal3 depends on two different IDRs (yellow arrows). Bim1, however, solubilizes Bik1 self-interaction (not shown, see [72]). The interactions between Mal3 and Tip1 were mapped to the N-terminus of Tip1 and coiled-coil and EB-homology domain of Mal3 [105]. Kinesin domain of Tea2 is shown striped because it does not interact with microtubules in isolation and requires Mal3 to activate its ATPase [106,107]. In contrast with CLIP-170 and Bik1, the C terminus of Tip1 does not contain aromatic residues that are typically interacting with CAP-Gly domains. It is therefore unclear if Tip1 shows any self-interaction in absence of Mal3 or Tea2. Abbreviations: EB, end binding; *H. sapiens, Homo sapiens; S. cerevisiae, Saccharomyces cerevisiae; S. pombe, Schizosaccharomyces pombe.*

Recent reports showed that +TIPs can indeed undergo LLPS *in vitro* [65,71–74] (Figure 4), prompting a question whether microtubule end-bound comets form by LLPS-dependent or -independent mechanisms. Moreover, given that EBs can accelerate microtubule polymerization *in vitro* [63,75,76], it was proposed that +TIPs can concentrate tubulin to promote its addition to the microtubule tip and thus increase the rate of microtubule polymerization [73]. *In vitro,* mammalian EB1 and EB3, and their fission yeast homolog Mal3, formed condensates when their concentrations were sufficiently high [73,74] and/or in the presence of a crowding agent [65]. Robust droplet formation required presence of all parts of EB1 and Mal3, including their IDRs [65,74]; LLPS of EB1 was hexanediol-insensitive and depended on the positive charges in the IDR [74]. Another mammalian +TIP, CLIP-170, was also shown to readily form droplets as a purified protein *in vitro* with added crowding agents [73] or in crowding agent-free cell extracts [71], confirming the early demonstration of droplet-like structures at microtubule tips of overexpressing cells [77]. Formation of CLIP-170 condensates depended on the presence of the C-terminal domains [73] and was likely driven by the specific binding of the N-terminal CAP-Gly domains and the C-terminal zinc finger domain with the adjacent acidic-aromatic motif [78], an interaction previously implicated in the autoinhibition of CLIP-170 [79]. *In vitro,* addition of full-length CLIP-170 to EB3 enhanced EB3-dependent acceleration of microtubule growth [73]. However, in cells, the absence of all three EB isoforms [80,81] or inhibition of CLIP-170 [81] had no effect on microtubule growth rate. This argues against the functional importance of EB and CLIP-170 LLPS in accelerating polymerization of tubulin by concentrating it around microtubule tips.

In vitro experiments further showed that different combinations of mammalian and *Schizosaccharomyces pombe* +TIPs could co-condense *in vitro* [65,73,74] (Figure 4). The same was true for the +TIPs from budding yeast: EB1 homolog Bim1, its SxIP-containing partner Kar9, and CLIP-170 homolog Bik1 [72]. Interestingly, in this latter case, LLPS in absence of crowding agents strongly depended on several redundant site-specific interactions between folded spectrin repeat domains of Kar9 [72]. In contrast to mammalian EB1 [71,74], LLPS of budding yeast +TIPs was sensitive to hexanediol and Bim1 tended to dissolve condensates rather than promote their formation [72]. An argument in favor of the mechanistic importance of LLPS for +TIP function is that mutations perturbing LLPS *in vitro* inhibited protein accumulation at the ends of dynamic microtubules, either *in vitro* or in cells [65,73,74]. However, some of the studied mutations, such as those in the positively charged IDR of EB1 [74], could also diminish the interactions with tubulins (e.g., with their negatively charged tails). Other mutations, which affected site-specific interactions between proteins, could disrupt protein recruitment to microtubule ends in an LLPS-independent manner.

Another argument in favor of LLPS is formation of cohesive structures. In budding yeast, +TIPs form a single cohesive, possibly gel-like body that contains at least ~70 Kar9 molecules, tracks not only growing but also shrinking microtubule ends, and persists when microtubules are depolymerized [72]. It was proposed that the formation of this single +TIP body could be driven by Ostwald ripening [72]. Kar9, which forms the core of this +TIP body, does not exchange with the cytoplasm on a short time scale, because very few soluble Kar9 molecules seem to be present in cells [82]. Assembly of virtually all Kar9 in the cell into this single structure, which is localized at tip of one of the two astral microtubule bundles, ensures asymmetric interaction with actin and proper alignment of the spindle along the mother-bud axis (Figure 4).

Cohesive droplets were also observed for *S. pombe* +TIPs *in vitro* in presence of crowding agents; these droplets were transported by kinesin Tea2 [65]. In this system, cohesiveness could help protein delivery to the cell cortex, a process needed to control cell polarity and shape [83,84]. Imaged using electron microscopy/tomography, *S. pombe* +TIP droplets formed *in vitro* looked similar to other protein droplets studied *in vitro* [18,39,85] or *in situ* [86]: they had a

clear surface boundary and grainy internal organization [65]. However, these crowding agent-dependent droplets were distinct in their internal appearance from microtubule end-bound comets of the same +TIPs in the absence of a crowding agent [65]. +TIP accumulations formed on microtubules in the presence of a crowding agent appeared to consist of two layers: a clearly distinguishable comet-shaped density surrounded by more loosely structured material [65]. One interpretation of this observation is that crowding agent-assisted LLPS and +TIP comet formation might be two independent processes. Whether multi-layered cohesive +TIP droplets form around microtubule plus ends in *S. pombe* cells needs to be tested.

It is also unclear whether +TIPs form cohesive droplets in mammalian cells: unlike budding yeast Kar9, mammalian +TIPs exchange very rapidly with the cytoplasmic pool [87] and therefore do not form long-lived cohesive 'bodies'. Still, rapid exchange between the concentrated and dilute phase can be compatible with the formation of +TIP droplets at microtubule ends, as it is observed in other phase-separated systems [10]. However, +TIP distribution has a clear comet-like shape, apparently dominated by the density of the binding sites (e.g., GTP-tubulin) at the microtubule end and not by homotypic interactions between +TIPs and the surface tension of the droplet. +TIP comets in animal cells are up to ~1 μm long and thus potentially contain more than 1000 binding sites for +TIPs, which do not appear saturated: average copy number of EB1 molecules in a comet is estimated between 70 in *Drosophila melanogaster* [88] and 270 in LLC-PK1 cells [89]. It is unknown whether endogenous mammalian +TIPs form multiple layers at the outermost plus ends or tau-like, single-layer envelopes. Low saturation of the binding sites will disfavor homotypic interactions and phase separation. It remains to be established whether +TIPs in systems such as mammalian cells are present at microtubule ends in sufficient density to form phase-separated droplets.

LLPS in controlling microtubule interactions with cellular structures

Microtubule plus ends interact with specific cellular structures such as kinetochores of mitotic chromosomes, or the cell cortex. These interactions often involve proteins that contain IDRs and rely on multivalency in order to hold on to dynamic microtubule ends and transmit forces (reviewed in [90]), suggesting that LLPS could be involved in the assembly of MAP complexes at these locations. For example, the inner centromere was proposed to concentrate the chromosome passenger complex (CPC) to create a concentration gradient of Aurora B kinase, which corrects errors in chromosome attachment [91]. A recent paper linked CPC localization to the inner centromere with its ability to undergo LLPS *in vitro* in presence of a crowding agent, or *in vivo* after overexpression [21]. Mutations disrupting LLPS of the CPC *in vitro* resulted in reduction of CPC localization at the centromere; however, the authors could not rule out that the protein region involved in LLPS could also directly function in controlling CPC localization [21].

Correctly formed kinetochore–microtubule attachments are stabilized by the recruitment of the Astrin-SKAP complex, components of which have intrinsic microtubule-binding affinities [92,93]. SKAP was recently shown to undergo LLPS in presence of a crowding agent *in vitro* and when overexpressed in cells [94]. However, it is unclear whether endogenous SKAP or any other kinetochore proteins at endogenous expression levels undergo LLPS. Furthermore, IDRs of some kinetochore proteins involved in microtubule binding, such as Ndc80 and Dam1, can form ordered/partially folded structures upon binding to their partners [95,96]. Therefore, it would be premature to conclude that LLPS rather than site-specific multivalent interactions play a role in kinetochore function (reviewed in [4]).

Similarly, the complexes controlling microtubule stabilization at the cell cortex are composed of proteins that readily undergo phase separation when overexpressed in cells, such as a coiled coil protein ELKS [97]. In contrast, the dynamics of ELKS expressed at the endogenous level,

and its presence in cortical foci containing on average only ~2 dimers, is incompatible with the LLPS model, at least in non-neuronal cells [98]. Interestingly, in worm neurons, endogenously labelled ELKS homolog is part of the complexes that appear to phase separate and then form solid structures at the synapses [99]. These data support the general trend that for a given protein family, the ability to form condensates can be highly cell- and species-specific and does not need to be evolutionarily conserved.

Concluding remarks

The hypothesis that LLPS can help to explain formation of different types of cell compartments had a huge impact on cell biology and led to numerous investigations of the ability of different proteins to undergo phase separation. These studies demonstrated that it is not at all difficult to find more or less artificial conditions allowing condensate formation *in vitro* or in cells. The current challenge, therefore, is to demonstrate whether the physiological behavior of a given protein at the endogenous concentration in cells is explained by LLPS, or by other mechanisms (see Outstanding questions). Currently, there appear to be no well-defined and convincing tests to prove that phase separation indeed drives the cellular behavior and physiology of a particular protein [3].

The ability of a protein to display a certain type of dynamics and form condensates does not seem to necessarily be an evolutionarily conserved feature. Condensate formation by a given protein can be influenced by its expression level, post-translational modifications, and the abundance of soluble partners or structured scaffolds to which it can bind. Therefore, orthologs of a specific protein in different species (e.g., EB proteins in mammalian cells and budding yeast, or ELKS in mammalian cells and in worms) or the same protein in different cell types (e.g., TACC3 in oocytes and fibroblasts) may display slow or fast exchange with the cytoplasmic pool and may or may not form cohesive droplets. Similarly, a cellular structure may display features compatible with LLPS in some conditions and lack them in other conditions (e.g., the centrosome in mitosis and interphase). Therefore, we believe that the research field should move beyond reporting that LLPS of a protein can happen and making assumptions about LLPS based on *in vitro* studies and instead focus on investigating circumstances under which LLPS is necessary for the function of this protein, taking into account physiologically relevant expression levels, post-translational modifications, and cell types. This rigorous approach will require detailed analysis of homo- and heterotypic protein–protein interactions as well as a critical and balanced consideration of all alternative models that can explain the organization, stoichiometry, dynamics, and function of particular macromolecular assemblies.

Outstanding questions

Can binding to microtubules alone, in the absence of crowding agents or forced oligomerization, promote the formation of liquid droplets of microtubule-binding proteins at their endogenous concentrations?

How many protein molecules are present in microtubule-associated structures that were proposed to depend on LLPS (+TIP comets, MAP foci)? Are these numbers and protein stoichiometry in agreement with the existence of phase-separated condensates?

Does LLPS contribute to formation of MTOCs and, if it does, can such structures anchor microtubule minus ends in a manner resistant to pulling forces like *bona fide* MTOCs do?

Do MAPs form envelopes along microtubule shafts in cells in order to locally regulate the motility of motor proteins and the properties of microtubule lattice, such as its compaction state or resistance to depolymerization?

Does free tubulin concentrated in liquid droplets around growing microtubule ends promote microtubule polymerization?

Is the formation of some multivalent structures interacting with microtubule plus ends on chromosomes or membranes driven by LLPS?

Which functional aspects of microtubule regulation can be explained by LLPS better than by conventional protein–protein interactions?

Acknowledgments

V.A.V. is supported by a QMUL Start-up grant (SBC8VOL2). We thank A. Musacchio, Y. Barral, M.O. Steinmetz, and S. Meier for the thoughtful comments on the manuscript.

Declaration of interests

The authors declare no competing interests.

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