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Membraneless organelles in eukaryotic cells

Bezmembránové organely eukaryot

Bachelor's thesis

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Poděkování

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla využita k získání jiného nebo stejného akademického titulu.

Declaration

I honestly declare that I wrote this thesis on my own and that I stated all used literature and other information sources. This work or its significant part was not used to acquire any other or the same academic title.

V Praze / In Prague, 6. 6. 2020

Abstract

Membraneless organelles (MLOs) are a newly described type of cellular compartments. They consist of protein and nucleic acid molecules that undergo liquid-liquid phase separation (LLPS). MLOs are able to fulfill unique biological roles, because they are highly dynamic and their composition can be effectively regulated. Composition and function of these formations are swiftly being elucidated. The work summarizes the basic principles of LLPS in living organisms and further focuses on several types of MLOs functionally connected to microtubules (MTs). Their recurrent feature is the ability to nucleate MTs. This eventual role corresponds well with their high temporal and spatial dynamics.

Keywords

membraneless organelles, liquid droplets, liquid-liquid phase separation, microtubules, tau protein, tau droplets, SPD-5, Plk4, BuGZ, TPX2

Abstrakt

Bezmembránové organely jsou nedávno popsaným typem buněčných kompartmentů. Jsou tvořeny molekulami proteinů a nukleových kyselin, které podstupují fázovou separaci kapalina-kapalina. Jsou schopné plnit jedinečné biologické úlohy, protože jsou vysoce dynamické a jejich složení může být efektivně regulováno. Poznatků o charakteru a funkci těchto útvarů v poslední době rychle přibývá. Práce shrnuje základní principy fázové separace kapalina-kapalina v živých organismech a dále se zaměřuje na několik typů bezmembránových organel funkčně spojených s mikrotubuly. Jejich opakující se vlastností je schopnost nukleace mikrotubulů. Tato role je zároveň ve shodě s jejich vysokou časovou a prostorovou dynamikou.

Klíčová slova

bezmembránové organely, tekuté kapénky, fázová separace kapalina-kapalina, mikrotubuly, tau protein, tau kapénky, SPD-5, Plk4, BuGZ, TPX2

List of abbreviations

BuGZ	Bub3-interacting and GLE-2–binding sequence containing ZNF207
CDK5RAP2	CDK5 regulatory subunit-associated protein 2
Cep152	Centrosomal protein of 152 kDa
CPB	cryptic Polo-box
D-PLP	Drosophila Pericentrin-like protein
FUS	fused in sarcoma protein
GFP	green fluorescent protein
GTP	guanosine triphosphate
IDR	intrinsically disordered region
Kip3	kinesin-like protein 3
LCR	low complexity region
LLPS	liquid-liquid phase separation, to liquid-liquid phase separate
MAP	microtubule-associated protein
MARK2	microtubule affinity regulating kinase 2
MCAK	mitotic centromere-associated kinesin
MLO	membraneless organelle
MT	microtubule
MTOC	microtubule-organizing centre
PCM	pericentriolar material
PEG	polyethylene glycol
PLK, Plk	Polo-like kinase
SPD	spindle-defective protein
STIL	SCL-interrupting locus protein
TPX2	targeting protein for Xklp2
TPXL-1	TPX2-like protein 1
YFP	yellow fluorescent protein
ZYG9	zygote defective protein 9

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1 Introduction

Historically, cytoplasmic organelles emerged as membrane-bound compartments within the eukaryotic cell, which performed a specific function. However, it was soon made clear that even non-membranous organelles exist, such as the ribosome or the centrosome. Lately, a new group of non-membranous organelles arose, termed membraneless organelles (MLOs). MLOs separate from the surrounding cytosol on the basis of spontaneous phase separation, analogously to oil separating from water. They typically remain highly dynamic, with molecules rapidly mixing up inside the compartment and also interchanging with their cytosolic bulk (Brangwynne et al. 2009; Banjade and Rosen 2014). Some MLOs form readily upon activation signal and are able to break up quickly as well (Brangwynne et al. 2009). Thus, MLOs make up a separate group of organelles with distinct features and capability to perform distinct tasks within the cell.

However, the span of the field is still to be explored. The first organelles discovered to behave as "liquid droplets" and consequently termed MLOs were P-bodies in *Caenorhabditis elegans*, just a decade ago (Brangwynne et al. 2009). Consequently, additional MLOs were identified in the bulk of already known organelles; the nucleolus, stress granules and Cajal bodies, among others (Brangwynne, Mitchison, and Hyman 2011; Wippich et al. 2013; Strzelecka et al. 2010), and new systems based on phase separation are emerging each year. Some of them are specific only to plants (Ouyang et al. 2020), or occur in prokaryotes (Hondele et al. 2019).

This work is focused on MLOs associated with microtubules (MTs). Many of these MLOs gather MT subunit tubulin and consequently facilitate MT nucleation (Hernández-Vega et al. 2017; Montenegro Gouveia et al. 2018), possibly revealing a general role of MLOs relevant to all components of cytoskeleton.

2 Characteristics of MLOs

MLOs are believed to serve many functions with very specific requirements. Foremost, they are highly dynamic in recruiting other molecules and in their assembly and disassembly. Membrane-bound bodies can achieve similar effects, but they need to involve the vesicular transport machinery, which is generally slower. Thus, liquid droplets participate for instance in T-cell receptor signal transduction (Su et al. 2016) or form readily in response to stress factors (Kedersha et al. 2000). Additionally, MLOs can create a specific environment capable of accelerating catalysis of a reaction or preserving a molecular species (Hernández-Vega et al. 2017). This set of abilities is achieved in an interesting manner described below.

2.1 Behaviour of liquid droplets

MLOs typically sequester from the surroundings spontaneously on the basis of liquid-liquid phase separation (LLPS), posing a situation where two liquids with different properties do not mix together and form a precise boundary between each other instead. Thus, formations comparable to liquid droplets with up to a few micrometers in diameter are to be observed under a microscope and the solution turns opalescent (P. Li et al. 2012). The droplets are more viscous than the surrounding solution (Brangwynne et al. 2009). They generally have a higher density and therefore can be separated from the bulk solution by centrifugation (P. Li et al. 2012).

Liquid droplets are capable of fusion, fission and surface wetting (adhesive contact with a solid surface)(Figure 1), these characteristics are therefore commonly used for proving LLPS origin of observed objects (Brangwynne et al. 2009; Boeynaems et al. 2018). Liquid droplets of the same type tend to fuse (or coalesce) spontaneously together, as it decreases the surface tension. In contrast, fission is not a favourable process and is typically demonstrated by applying external shear force (Hernández-Vega et al. 2017).

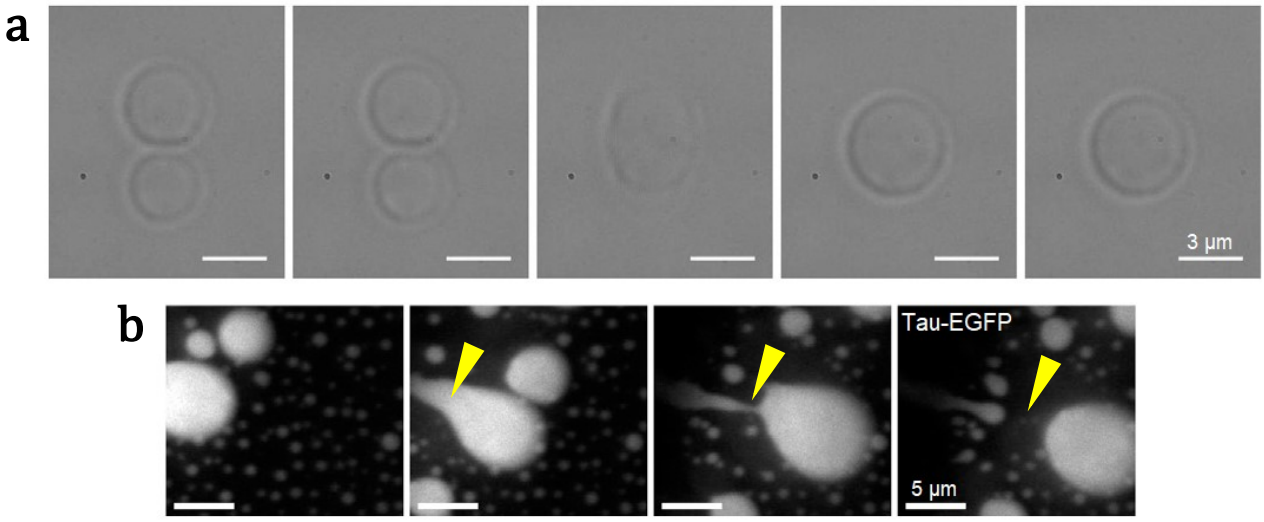

Figure 1: Fusion and fission of liquid droplets. ***(a)*** *Sequence of images taken during a fusion event of two tau protein liquid droplets, bright-field illumination.* ***(b)*** *Sequence of images of tau protein liquid droplets taken after shear flow application. A fission event is indicated by an arrow. Surface wetting behaviour can be noticed. Fluorescence microscopy image. (Hernández-Vega et al. 2017), modified.*

In addition to fusion, a phenomenon called Ostwald ripening can be observed. Ostwald ripening causes redeposition of individual molecules from smaller droplets to larger ones, ultimately resulting into a single large droplet (Y. Li et al. 2012). This process also involves surface tension, as molecules inside a droplet are more stabilized by bonding to each other than the ones on the liquid-liquid boundary. They easily get loose from the surface of small droplets and attach to larger ones, which is more energetically favourable. Therefore, as a result of both fusion and

Ostwald ripening, when a solution of liquid droplets sits still for long enough (hours or days), the number of liquid droplets tends to decrease, and their volume grows (Park et al. 2019).

Some liquid droplets are able to harden over time, becoming gel-like or solid-like (Yuan Lin et al. 2015; Patel et al. 2015). This process is called maturation. During maturation, molecules inside a droplet get generally less dynamic and more organized. The resulting structure can even have properties of fibrous lattice (Yuan Lin et al. 2015). In cells, maturation of MLOs can have either desirable (for instance in yeast stress granules)(Kroschwald et al. 2015) or pathological implication (for instance aggregation of protein FUS (fused in sarcoma), which is connected to amyotrophic lateral sclerosis)(Patel et al. 2015).

Apart from simple droplets, additional morphologies of MLOs have been observed. For example, they can form a layer of phase-separated molecules on membranes (Banjade and Rosen 2014), or even possess a droplet inside a droplet architecture, which is the case of the nucleolus (Feric et al. 2016).

2.2 Composition and interactions

MLOs consist of biopolymers, proteins and nucleic acids, that can be divided into two general types; the structural components and the clients (Banani et al. 2016). Structural components are responsible for liquid droplet formation. A few or even just one molecular species possess this role in individual types of MLOs. They bind to each other using multitude of weak interactions. In contrast to aggregates or complexes, the links in MLOs are only temporary and rapidly shift from one molecule to another, creating the liquid-like environment (Brangwynne et al. 2009; Brangwynne, Tompa, and Pappu 2015). Clients partition into liquid droplets on the basis of favourable interactions. Their properties characterize the function of the MLO, as they are often biologically active. Individual molecules, both structural components and clients, can freely rearrange within a MLO, and also pass into and from the surrounding solution.

Looking at the structural components, three physical parameters come into play during liquid droplet formation; the thermodynamic temperature, the mixing entropy (which favours evenly mixed solution of solvent and polymer) and the variations of interaction energy between polymer-polymer, polymer-solvent and solvent-solvent interactions. MLOs form spontaneously, without any energy input. Hence, the difference in free energy of the process needs to be negative.

$$\Delta F = \Delta E - T\Delta S^{mix}$$

F – free energy, E – interaction energy, T – thermodynamic temperature, S^{mix} – mixing entropy

The polymer-polymer interactions have to be stronger than between polymer and solvent. This state is termed poor solvent conditions and is a general driver of low molecule solubility. Liquid droplets form when the supportive interactions overcome the negative effect of macromolecule accumulation on entropy (Flory 1942; Nott et al. 2015)(Figure 2).

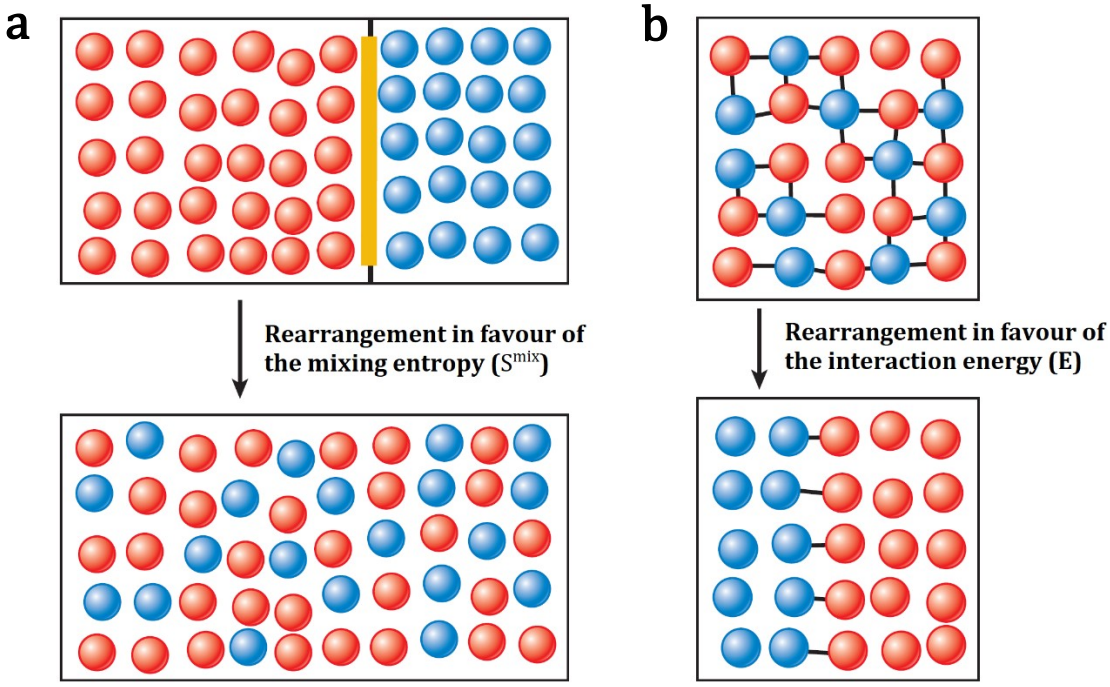

Figure 2: The interaction energy needs to overcome the mixing entropy to form liquid droplets. ***(a)*** *The mixing entropy favours mixed state. The colours represent two distinct molecular species.* ***(b)*** *A proposed situation when interactions between molecules of the same colour are favourable. The interaction energy leads the system into a demixed state. Black lines represent unfavourable interactions. (Hyman, Weber, and Jülicher 2014), modified.*

The effect of supportive polymer interactions can be depicted as a concave region in a free energy diagram (Figure 3a)(Flory 1942). Here, two distinct minima exist, each with a different solution composition. Thus, the system tends to split into two portions with different polymer concentrations, also termed dense and dilute phase (the dense phase corresponding to a MLO).

By observing phase behaviour of a solution in changing conditions such as salt concentration or temperature, a phase diagram can be extracted (Figure 4). Phase diagrams standardly reflect the existence of the mixed and demixed state as a function of a chosen condition and solution composition. The tip of the curve, termed the critical point, depicts the most extreme conditions for phase separation, in which only one exact composition of the solution is allowed. Horizontal lines inside the curve, or tie lines, correspond to a certain value of the chosen condition. All points on a single tie line result in one composition of the dense and dilute phase, with only their ratios

changing. Phase diagrams can expose valuable information such as impact of amino acid composition of the participating proteins or whether phase separation occurs in biologically relevant conditions (Su et al. 2016; Yanxian Lin et al. 2019).

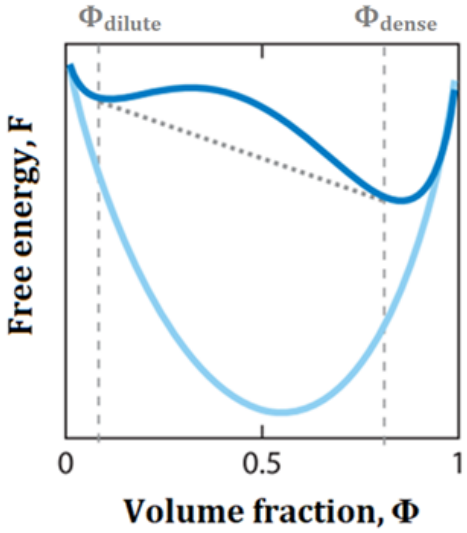

Figure 3: Free energy (F) as a function of volume fraction of polymer (Φ). Light blue curve corresponds to a system with no interactions between molecules. Dark blue curve depicts a state with supportive polymer-polymer interactions. (Φ_{dilute}, Φ_{dense}), compositions of dilute and dense phase. Dashed lines indicate free energy of a phase-separated system. (Hyman, Weber, and Jülicher 2014), modified.

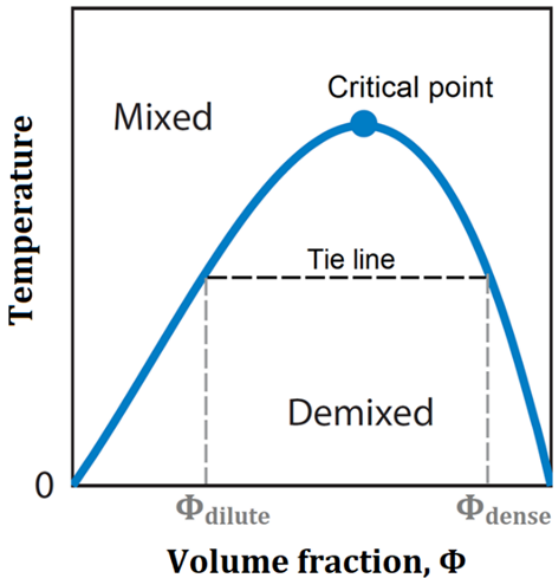

Figure 4: Phase diagram depicting state of a solution as the temperature and volume fraction (Φ) change. The critical point and one tie line are marked. (Φ_{dilute}, Φ_{dense}), compositions of dilute and dense phase. (Hyman, Weber, and Jülicher 2014), modified.

Phase behaviour of MLOs can be assumed using complex coacervation models developed for use in polymer chemistry. The Voorn and Overbeek model is the most common to use (Overbeek and Voorn 1957). It hypothesizes a lattice with individual sites, each being occupied by either polymer, simple ion or solvent. However, it puts a strong emphasis on electrostatic interactions and does not consider heteropolymers (and consequently the inhomogeneous charge distribu-

tion of proteins). Therefore, the model fits poorly to experimental phase diagrams in some cases. The random phase approximation is an extension of the Voorn and Overbeek model. It is, among others, able to consider various charge patterns. The random phase approximation was shown to give very accurate results on several occasions (Y.-H. Lin, Forman-Kay, and Chan 2016). The field theory simulations and the complexation approach are two examples of more sophisticated simulation models, beginning to appear in the field (Sing 2017; Feric et al. 2016; Yanxian Lin et al. 2019).

Interactions participating in phase separation can have very different basis. Among others, ionic interactions, π-π stacking and hydrophobic contacts occur commonly. However, the key attribute of liquid droplet components is multivalency, the ability of a molecule to bind multiple substrates at once (P. Li et al. 2012; Banjade and Rosen 2014; Wu and Fuxreiter 2016) (Figure 5). For instance, numerous RNA-binding domains are common in proteins forming MLOs (K.-H. Lee et al. 2016).

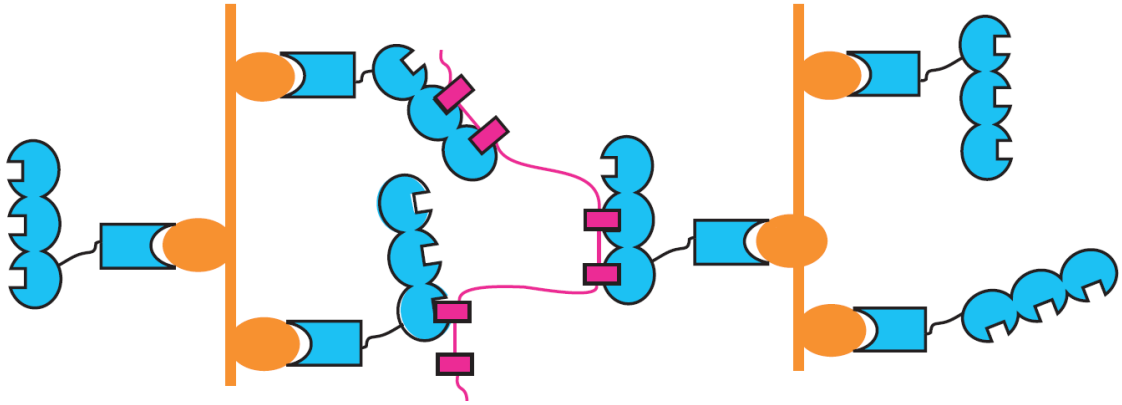

Figure 5: An illustration of multivalent interactions between three protein species, fuelling their LLPS. The repeating binding sites allow for formation of a dynamic mesh. Note that even one protein, interacting multivalently with itself, could make for similar mesh. This scheme is postulated for three components of a signalling pathway leading to actin polymerization. (P. Li et al. 2012), modified.

However, protein multivalency can be achieved both through folded binding domains and linear binding motifs. Repeating linear binding motifs are a common feature of intrinsically disordered regions (IDRs). These amino acid sequences do not adopt a stable folded structure and instead employ a multitude of conformations. IDRs often contain low complexity regions (LCRs), described as repetitive sequences, often rich in polar and charged amino acids (Huntley and Golding 2002). IDRs, and more importantly LCRs, were shown to have a crucial role in many phase separation systems (Kato et al. 2012; Frey and Görlich 2007; Martin and Mittag 2018). The postulated reason is that in an unwrapped arrangement, every amino acid has the opportunity to bind to other species and hence to increase the interaction energy (Zhou et al. 2018). Indeed, the composition and sequence of these regions is a cardinal parameter. Due to the biased

amino acid content, they are often able to multivalently bind oppositely charged protein sequences and nucleic acids (Pak et al. 2016). Moreover, a subtle change in LCR sequence can change properties of liquid droplets drastically, giving space to derivation of new phase separation arrangements and possibly clarifying occurrence of certain disorders (Statt et al. 2020).

The presence of ionic links can be demonstrated *in vitro* by gradually increasing salt concentration in solution of MLOs (Nott et al. 2015; Vieregg et al. 2018). In high salt concentration (and hence high permittivity of the milieu), ionic interactions lose their power and liquid droplets do not form. Analogically, 1,6-hexanediol is capable of weakening hydrophobic contacts and is used as evidence for their involvement (Updike et al. 2011; Shulga and Goldfarb 2003).

2.3 Crowding agents

The small number of liquid droplet structural components is convenient in *in vitro* studies, where a lot of MLOs can be recreated by mixing up only several ingredients (Su et al. 2016; Hernández-Vega et al. 2017). However, when considering a liquid droplet formation, the molecular environment must be taken into account as well. The cytosol is extremely crowded with other macromolecules that occupy space, which would otherwise be filled with water. This limits the number of possible ways in which the macromolecules could be placed, thus lowering the system's entropy and increasing its free energy (Ralston 1990). This effect, termed volume exclusion, was shown to increase the effective concentration of macromolecules and support molecule oligomerization, both of which underlie the liquid droplet formation (Minton 1992; Hancock 2004). Hence, artificial crowding agents are used in *in vitro* studies of MLOs to mimic the molecular crowding in the cell.

Typically, polar polymers such as polyethylene glycol (PEG), Ficoll or dextran are used. Purified proteins such as lysozyme or bovine serum albumin were shown to carry out similar results (Woodruff et al. 2017; Yuan Lin et al. 2015). Used concentration varies between individual experiments, but usually fluctuates between 1 – 10% w/v.

However, molecular crowders should be used with caution, as they do not fully recapitulate the complex environment present in cells (Alberti, Gladfelter, and Mittag 2019; Wang, Li, and Pielak 2010). The lowest concentrations possible are usually used and several different crowders are compared to prevent artifacts. Some phase separation systems do not need the support of a crowding agent at all.

3 Microtubules

MTs are prolonged protein assemblies present in all eukaryotic cells. In contrast to other components of cytoskeleton, MTs are less resilient and rather serve to transport material throughout the cell and organize organelles. Thus, many molecular motors have evolved, pacing along MTs in different directions and bearing different cargoes (Schliwa and Woehlke 2003).

MTs consist of repeating dimers of α and β-tubulin subunits, arranged into hollow cylinders. Upon binding GTP (guanosine triphosphate), they assemble as single protofilaments, chains of alternating α and β-tubulins (Erickson 1975; Muroyama and Lechler 2017). Protofilaments arrange into a circle, generating a long tubule. Onset of the assembly process is termed nucleation and occurs only in specific conditions, either under supercritical concentration of α and β-tubulin, or upon assistance of MT nucleating factors as γ-tubulin (Oakley et al. 1990; Zheng et al. 1995). MT nucleating capacity is mostly possessed only by microtubule-organising centres (MTOCs) of the cell. MTOCs are non-membranous organelles with various functions, resulting into their diverse morphology. In animals, for instance, two main types of MTOCs emerge, the centrosome and the basal body. Centrosomes are often located near the nucleus and organize the main bulk of cytosolic MTs, whereas basal bodies control ciliary and flagellar MTs. However, in majority of plants, the centrosome did not evolve and its function is fulfilled by association of MTs to plasma membrane and nuclear envelope (Schmit 2002).

Animal centrosomes were recently identified to contain proteins which undergo LLPS (Woodruff et al. 2017; Yamamoto and Kitagawa 2019). They are composed of two orthogonal centrioles surrounded by mostly unstructured mass of pericentriolar material (PCM). Centrioles typically consist of MT triplets arranged into cylinders. Although centrioles are able to nucleate MTs on their own, the main MT mass is nucleated and organized by the PCM (Berns and Richardson 1977; Gould and Borisy 1977), particularly by ring complexes of γ-tubulin (Zheng et al. 1995). MTs protrude radially out of the centrosome, creating an aster-like shape. Centrosomes are typically located near the cell nucleus. Another important role for centrosomes arises during cell division, when centrosomes divide and move to opposite poles of the cell, in order to form a mitotic spindle. The mitotic spindle organizes and separates chromosomes, facilitating cell division.

During cell division, the centrosome has to double as well. This can be achieved either more commonly by duplication of preexisting centrioles, which is termed the canonical pathway, or by centriole *de novo* genesis (Marshall, Vucica, and Rosenbaum 2001; Rodrigues-Martins et al.

2007). For example, centrioles can be created *de novo*, if the mother centriole is absent or damaged (La Terra et al. 2005). In both cases, they originate as immature procentrioles and develop into centrioles afterwards. Centrioles duplicate semiconservatively, which means that each of the older, mother centrioles gives rise to one daughter centriole and then each mother-daughter centriole pair establishes one centrosome. PCM is thereafter evenly distributed between both centrosomes.

Many proteins bind to the outside of MTs and are therefore termed microtubule-associated proteins (MAPs). MAPs have various roles; some are able to change properties of the MT such as its dynamicity or rigidity, others serve as molecular motors to transport cargo or during chromosome segregation in mitosis (Vale 2003; Wieczorek et al. 2015). Several MAPs were shown to undergo phase separation, involving the tau protein.

4 Tau phase separation

4.1 Tau protein

Tau is a MAP specific to vertebrates, preferentially localizing to neuronal axons (Binder, Frankfurter, and Rebhun 1985). It binds to the outside of MTs and is generally thought to support MT polymerization and stability (Weingarten et al. 1975; Cleveland, Hwo, and Kirschner 1977b), and to protect MT surface from MT-corrupting enzymes as katanin (Qiang et al. 2006).

Another role for tau is bundling of MTs (Chen et al. 1992; Chung et al. 2016). Typically, MTs in axons are organized in bundles, arrays of multiple parallel MTs which provide for more efficient transport across the axon. They are put together with an external protein, which also keeps spacing between individual MTs, necessary for molecular motor motion. Tau is one of the main bundlers in neurons and is able to promote bundle formation even to such extent that mere tau expression in fibroblasts leads to emergence of bundles and formation of cell protrusions (Chen et al. 1992).

Tau is commonly divided into four distinct parts; N-terminal region, proline-rich region, MT binding domain and C-terminal region (Figure 6a). MT binding domain shows only weak affinity to MTs in absence of the rest of the protein (Gustke et al. 1994). The additional power partaking in MT binding is the overall positive charge of tau (Cleveland, Hwo, and Kirschner 1977a), which pairs with negative amino acid residues on MT surface (Mukrasch et al. 2009; Gustke et al. 1994). Recently, the proline-rich region has been shown to bind MTs firmly (McKibben and Rhoades 2019).

The protein is highly disordered with more than 75% amino acid residues in nonperiodic conformation (Mukrasch et al. 2009)(Figure 6b). The unstructured N-terminal segment projects from the MT surface and is therefore sometimes termed the projection domain (Hirokawa, Shiomura, and Okabe 1988; Chen et al. 1992). It is important for maintenance of spacing between MTs in bundles and interacts with other molecules in the cytosol (Chen et al. 1992; Reynolds et al. 2008). In contrast, the MT binding domain shows substantial secondary structure.

Tau exists in six main isoforms ranging from 352 to 441 amino acids in length (Goedert et al. 1989). These isoforms vary in the number of MT binding sequences (either 3 or 4 repeats) and in the length of N-terminal region (one or two exons can be included)(Himmler et al. 1989; Goedert et al. 1989). Most studies use only the longest human isoform termed htau40, htau441 or 2N4R (Goedert et al. 1989; Hernández-Vega et al. 2017).

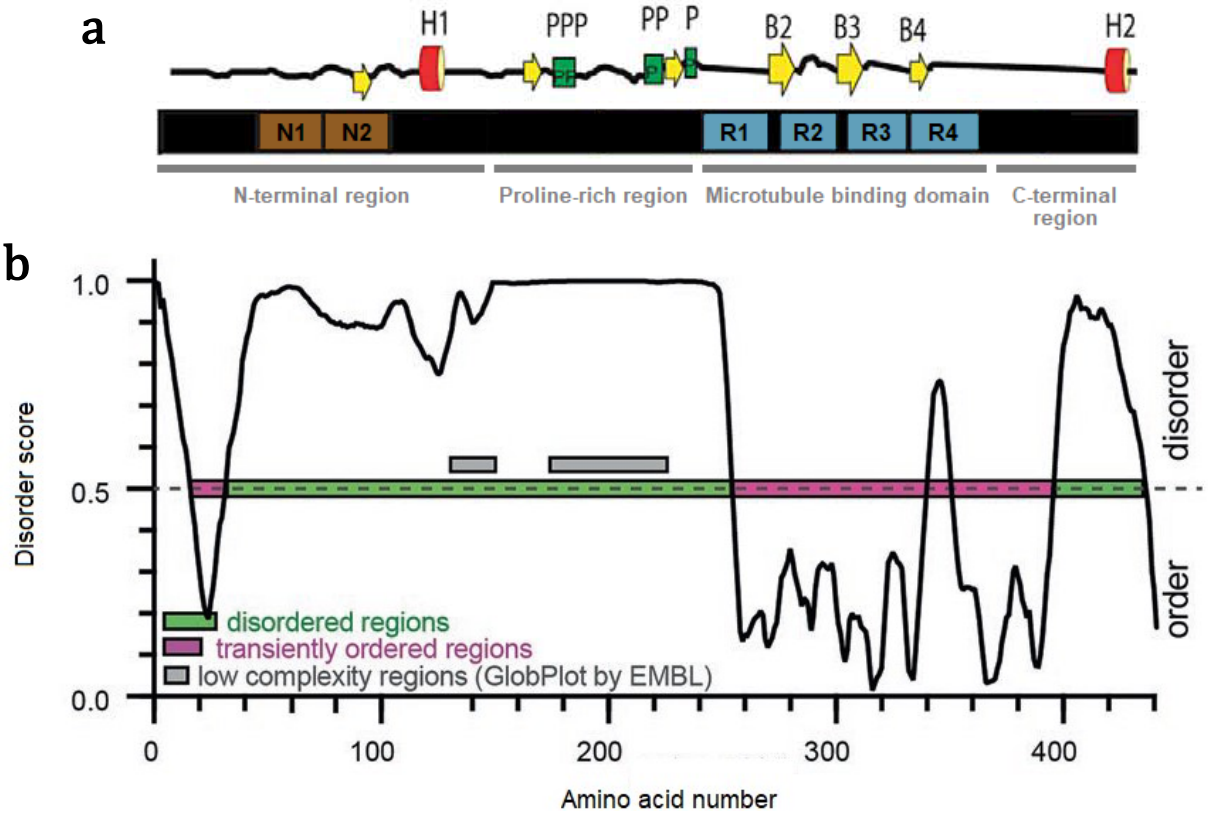

Figure 6: Structure of the longest human tau isoform, htau40. ***(a)*** *A scheme of a secondary structure prediction and localization of the four main segments of tau protein. N1, N2 and R2 sections can be omitted in other tau isoforms. Yellow arrow, β-sheet; red cylinder, α-helix; green rectangle, polyproline II helix (left-handed helix). (Mukrasch et al. 2009), modified.* ***(b)*** *PONDR prediction of structural disorder (http://www.pondr.com). Two low complexity regions (LCRs) are predicted by (http://www.globplot.embl.de). The N-terminus (or projection domain) is mostly disordered, whereas the microtubule binding domain shows secondary structure. (Wegmann 2019), modified.*

4.2 Tauopathies

Cells rely on MTs as pathways for long-distance transport. In neuronal axons, which can reach tens of centimeters in length, every transport defect is amplified. For this reason, tau gene mu-

tations or defects in post-translational modification manifest as neurodegenerative disorders, termed tauopathies. Altogether, there are more than 20 different tauopathies characterized so far (V. M.-Y. Lee, Goedert, and Trojanowski 2001). Above all, tau aggregation is involved in Alzheimer's disease, along with amyloid beta aggregation (Grundke-Iqbal et al. 1986). In Alzheimer's disease, abnormally phosphorylated tau changes its conformation from high intrinsic disorder to β-sheet structure and forms insoluble fibrous aggregates (von Bergen et al. 2005). Tau deposition causes its depletion on MTs and consequent MT degradation. Formation of these aggregates can be accelerated *in vitro* by addition of heparin, a negatively charged polysaccharide, which is thought to neutralize the positive charge of tau (Goedert et al. 1996). Existence of numerous tauopathies makes tau a hot topic for further research and a convenient protein to study phase separation.

4.3 Tau droplets

Although tau's aggregation in Alzheimer's disease has been known and studied for decades, its capability to LLPS was discovered just recently. Zhang et al. addressed the protein's capability to bind RNA along with its intrinsic disorder and multivalency (Xuemei Zhang et al. 2017). They showed that tau forms liquid droplets when in solution with several types of RNA (tRNA, poly(A) RNA and poly(U) RNA), although preferentially with tRNA. This was observed under wide range of tau:RNA mass ratios, but droplets only contained both polymers in approximately 1:1 charge ratios. Accordingly, Hernández-Vega et al. observed liquid droplet formation in solutions of tau and a crowding agent (25μM tau and 10% w/v dextran, PEG or Ficoll were used) which was further stimulated by addition of tubulin dimers (Hernández-Vega et al. 2017). Lastly, Wegmann et al. showed similar results overexpressing GFP (green fluorescent protein)-tagged tau *in vivo* in mouse neurons (Wegmann et al. 2018). This LLPS behaviour was proved to be of different nature than the previously observed aggregation with no ordered secondary structure and dynamic switching of molecules between droplet and solution (Xuemei Zhang et al. 2017; Hernández-Vega et al. 2017; Wegmann et al. 2018). Tau droplets were up to several μm in diameter, underwent fusion and fission, recovered rapidly after photobleaching and were sensitive to temperature and salt concentration, which confirmed their liquid-like nature and complex coacervation origin. Tau liquid droplet solution could be easily separated into the two phases by centrifugation (Ambadipudi et al. 2017).

The driving forces to tau liquid droplet formation are primarily the electrostatic interactions between negatively charged N-terminal region and positively charged C-terminal region,

whereas the role of hydrophobic interactions seems rather minor (Boyko et al. 2019; Ambadipudi et al. 2017)(Figure 7). In contrast, Wegmann et al. hypothesized larger role of hydrophobic interactions in a preceding study (Wegmann et al. 2018). It is also remarkable that tau MT binding domain can form liquid droplets on its own (Xuemei Zhang et al. 2017).

Lately, it was revealed that zinc cations strongly promote tau LLPS (Singh et al. 2020). Tau has several zinc-binding sites that presumably participate in this behaviour.

Tau can be phosphorylated on various sites, which could be a convenient way of regulating these interactions (or MT binding) as phosphorylation adds negative charge to the protein (Schwalbe et al. 2013). A study demonstrates that isolated repeat region of tau, phosphorylated by kinase MARK2 (microtubule affinity regulating kinase 2) forms liquid droplets more readily than the same construct without the modification (Ambadipudi et al. 2017). In contrast, Boyko et al. showed that even fully non-phosphorylated protein expressed in bacteria is capable of LLPS (Boyko et al. 2019). Another way of LLPS regulation could be the alternative splicing of tau leading to isoforms with either 3 or 4 MT binding repeats, as they interact with each other inter- and intramolecularly and the alteration of valency changes the ability of tau to demix from solution (Ambadipudi et al. 2017).

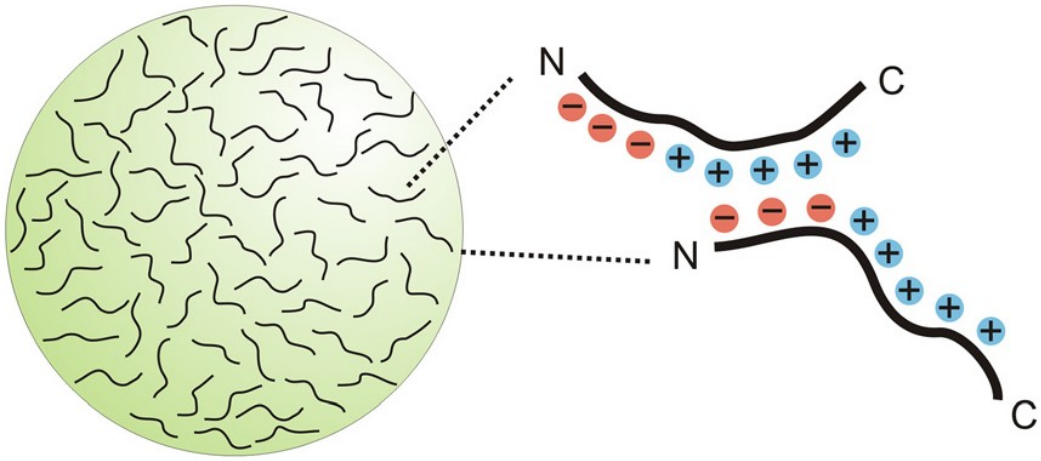

Figure 7: Proposed driving force behind tau liquid droplet formation; electrostatic interactions between negatively charged N-terminus and positively charged C-terminus of the protein. (Margittai 2019), modified.

It was reported on several occasions that tau droplets can mature in time into more gel-like consistency and thereafter even initiate tau aggregation in cells (Wegmann et al. 2018; Ambadipudi et al. 2017). However, a recent study from Lin et al. questions this observation, stating that tau aggregation and LLPS are two fully unrelated processes (Yanxian Lin et al. 2020).

Nonetheless, tau droplets were suggested to serve a physiological function as well. Primarily, they are able to uptake tubulin as a client protein. Hernández-Vega et al. demonstrated that ad-

dition of α/β-tubulin and GTP into tau liquid droplet emulsion results into tubulin sequestration into the droplets and its nucleation in overall solution concentration of tubulin more than 10× lower than the critical concentration needed for spontaneous nucleation (Hernández-Vega et al. 2017). This was probably a result of the local increase of tubulin concentration. Further, tau liquid droplets elongated and formed rod-like structures similar to tubulin bundles in neuronal axons. This rod formation could not be mimicked by simulation of environment inside droplets (corresponding concentrations of tau and tubulin, but without crowding agent), suggesting that not only tubulin concentration enrichment, but also the very existence of tau droplets is important.

4.4 Tau islands

Other reports propose that on solitary MTs, tau is able to form a monolayer of cooperating molecules. These "islands" of densely packed molecules with very little dynamics were observed when adding supercritical concentration of fluorescently labelled tau into solution of *in vitro* polymerized MTs (Siahaan et al. 2019; Tan et al. 2019). Tau molecules could be observed also between islands, but these were much more dynamic with rapid diffusion along MT lattice and an order of magnitude faster dissociation rate (Siahaan et al. 2019). The islands grew gradually in size by recruiting new tau molecules on their edges and diminished in similar way after removing tau from solution (Siahaan et al. 2019; Tan et al. 2019). The density of tau molecules inside islands was stable with 0.26 ± 0.05 tau molecules per tubulin dimer (possibly consistent with the 4 MT binding repeats in htau441). The collective data suggest cooperativity-based oligomerization of tau molecules, supposedly based on similar interactions as in tau droplets, but with no tau association in solution as tau molecules join islands individually and not in bulk. Structures reminiscent of tau islands have been observed *in vivo* as well (Tan et al. 2019).

Both studies agree on tau islands' inhibitive effect on MT-severing enzymes, katanin or spastin. Their association with MTs was reduced within tau islands, slowing down degradation of these regions significantly. Association outside islands was not dependent on tau density, demonstrating the importance of tau cooperation.

Tau islands also interact diversely with other MAPs; molecular motor kinesin-1 stops and dissociates when encountering an island boundary (Siahaan et al. 2019), whereas dynein-dynactin-cargo-adapter passes through them (Tan et al. 2019) and Kip3 (kinesin-like protein 3; a superprocessive motor from kinesin-8 family) even facilitates their disassembly (Siahaan et al. 2019).

Tan et al. noticed that the islands localize preferentially to curved areas of MTs, presumably implying that tau is perceptive to the disposition of the MT lattice (Tan et al. 2019). Accordingly, tau islands form more readily at GDP-tubulin regions of MTs, whose conformation is different from GTP regions. These findings were not interpreted yet, however, they collide with the routine MT stabilization by taxol, because taxol changes the properties of MT lattice as well and makes it more reminiscent of GTP-tubulin (Elie-Caille et al. 2007).

At the present time, it may still be too soon to resolve if tau islands form on the basis of LLPS and therefore belong to MLOs. Indeed, they form through similar interactions as tau droplets, on the other hand, tau islands have been demonstrated to be much more rigid than one would expect a liquid phase to be.

4.5 Phase separation of other MAPs

MAP2 is another MT bundling protein in neurons, localizing to dendrites. It is closely related to tau, with a proline-rich region and multiple MT binding repeats (Lewis, Wang, and Cowan 1988). MAP2 was even shown to partially compensate for tau depletion (Q.-L. Ma et al. 2014). Therefore, it is possible that these two proteins share also the ability to create MLOs. However, there are no studies reporting this yet. Moreover, MAP2 was shown not to bind RNA, which is a significant feature of tau (Xuemei Zhang et al. 2017). Generally, there are many MAPs with high structural disorder that could be interesting in further research. For example, studies on MAP7 and MAP9 revealed that these two proteins form coats on the surface of MTs, strongly resembling tau islands (Monroy et al. 2018, 2020).

5 Phase separation at the centrosome

5.1 Centrosome maturation

The interphase centrosome is small and strictly structured, consisting of only a thin layer of proteins tightly associated with the centrioles (Sonnen et al. 2012; Fu and Glover 2012). However, it undergoes vast changes during transition to mitosis. This process, called centrosome maturation, enables more MTs to be organised by the centrosome by supporting MT nucleation and eventually leads to the mitotic spindle formation (Gould and Borisy 1977). Upon centriole division, the amount of PCM grows significantly by taking up new protein components and it shows a lesser degree of order (Kuriyama and Borisy 1981; Sonnen et al. 2012; Khodjakov and Rieder 1999). Major MT nucleator, γ-tubulin, is enriched in centrosome more than 3× at the onset of mitosis (Khodjakov and Rieder 1999). Protein kinases Polo-like kinase 1 (PLK1) and Aurora A

are important regulators of centrosome maturation. Both of them have multiple substrates, whose phosphorylation leads to the uptake of γ-tubulin and other PCM members (Haren, Stearns, and Lüders 2009; Casenghi et al. 2003; Kinoshita et al. 2005).

5.2 PCM scaffold

Although there is no membrane separating centrosome from the cytosol, the organelle stays very coherent and even endures the tearing forces applied by spindle MTs. This physical resilience is a property of a rigid protein structure, termed PCM scaffold or centromatrix (Schnackenberg et al. 1998).

Pure centromatrix can be extracted from some cells with high concentration of KI (Moritz et al. 1998). It has a solid structure with very little inner dynamicity (Laos, Cabral, and Dammermann 2015). Centromatrix alone does not possess MT nucleation potential, but when incubated with cell extract, it recruits other PCM members, most importantly γ-tubulin, and recovers its MT nucleation ability (Schnackenberg et al. 1998; Moritz et al. 1998).

The two main models for centrosome dynamics are embryonic stages of the nematode *Ceanorhabditis elegans* and the fly *Drosophila melanogaster* (O'Connell 2000; Jana et al. 2016). Human centrosomes are the second choice in studying the topic and roles of certain human proteins are rather hypothesised based on similarities with these animals.

SPD-5 (spindle-defective protein 5) was identified as the main component of PCM scaffold in *C. elegans* (Hamill et al. 2002). It is also newly hypothesised to pursue phase separation of the centrosome (Woodruff et al. 2017). In *D. melanogaster*, Centrosomin (Cnn) is a functional homologue of SPD-5 (Conduit et al. 2014; Feng et al. 2017). Vertebrate protein CDK5RAP2 (CDK5 regulatory subunit-associated protein 2) shares conserved regions with Centrosomin and was proposed to be its homologue (Fong et al. 2008; Barr, Kilmartin, and Gergely 2010). Another centromatrix protein in vertebrates is Pericentrin, with a *D. melanogaster* homologue D-PLP (Drosophila Pericentrin-like protein)(Doxsey et al. 1994; Dictenberg et al. 1998; Martinez-Campos et al. 2004). Pericentrin does not have any known homologues in *C. elegans* so far. Table 1 offers a detailed look into the nomenclature and homologues of several principal PCM proteins.

C. elegans	*D. melanogaster*	vertebrates	function
SPD-5 [1]	Centrosomin (Cnn) [2]	CDK5RAP2 [3, 4]	PCM scaffold protein
	Pericentrin-like protein (D-PLP) [5]	Pericentrin [6]	PCM scaffold protein
SPD-2 [7, 8]	DSpd-2 [9]	CEP192 [10, 11]	PCM scaffold formation
PLK-1 [12]	Polo [13, 14]	Plk1 (Plx1) [15]	Kinase, PCM scaffold formation
γ-tubulin [16]	γ-tubulin [17, 18]	γ-tubulin [17, 19]	MT nucleation

Table 1: Proposed homologues of chosen PCM proteins in Caenorhabditis elegans, Drosophila melanogaster and vertebrates (studies made on Homo sapiens or Xenopus laevis). [1] (Hamill et al. 2002); [2] (Megraw et al. 1999); [3] (Fong et al. 2008); [4] (Barr, Kilmartin, and Gergely 2010); [5] (Martinez-Campos et al. 2004); [6] (Doxsey et al. 1994); [7] (O'Connell, Leys, and White 1998); [8] (O'Connell, Maxwell, and White 2000); [9] (Dix and Raff 2007); [10] (Pelletier et al. 2004); [11] (Gomez-Ferreria et al. 2007); [12] (Chase et al. 2000); [13] (Sunkel and Glover 1988); [14] (Llamazares et al. 1991); [15] (Golsteyn et al. 1995); [16] (Hannak et al. 2002); [17] (Zheng, Jung, and Oakley 1991); [18] (Sunkel et al. 1995); [19] (Shu and Joshi 1995).

5.3 Phase separation of PCM scaffold

The *C. elegans* PCM scaffold protein SPD-5 contains several coiled-coil regions which are thought to interact with each other and facilitate oligomerization (Hamill et al. 2002; Woodruff et al. 2015)(Figure 8). In a primary study, Woodruff et al. observed SPD-5 oligomers forming an irregular rigid structure, or a network (Woodruff et al. 2015)(Figure 9a). However, additional research revealed that SPD-5 is able to form micrometer-scale droplets *in vitro* in presence of crowding agents (PEG, Ficoll, dextran, lysozyme)(Woodruff et al. 2017)(Figure 9a). These assemblies showed clearly different behaviour from networks which form without crowding agents; they fuse, exchange molecules with solution and mix internally, suggesting their liquid-like properties. Assemblies older than 10 minutes are gradually becoming more rigid and their components more static, which is in better correspondence with features of *in vivo* centrosomes. Interestingly, the size and shape of these assemblies are also reminiscent of centrosomes, in contrast to SPD-5 networks (Figure 9b).

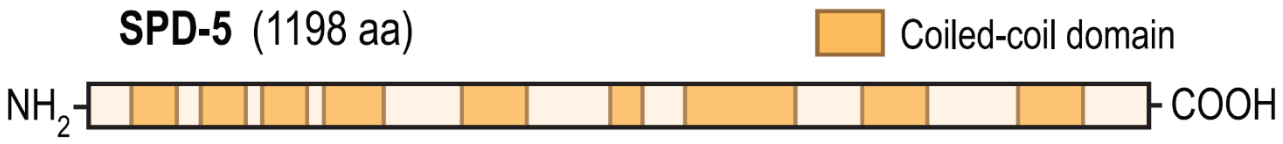

Figure 8: The 9 coiled-coil domains of SPD-5, as predicted by MARCOIL (Delorenzi and Speed 2002). The domains are thought to facilitate SPD-5 oligomerization and LLPS. (Woodruff et al. 2015), modified.

Droplets share common features with actual centrosomes; their formation is more efficient in solution containing SPD-2 seeds (created by incubation of SPD-2 with PEG) and is supported by

PLK-1 phosphorylation. Recruited PLK-1 is highly mobile both in SPD-5 droplets and in *in vivo* centrosomes.

Further, Woodruff et al. explored a MT nucleation pathway alternative to γ-tubulin. SPD-5 droplets were able to accumulate two core proteins of this pathway, ZYG-9 (zygote defective protein 9) and TPXL-1 (TPX2-like protein 1)(Woodruff et al. 2017; Roostalu, Cade, and Surrey 2015). This in turn led to α/β-tubulin recruitment, increasing its concentration inside droplets 5-fold. Similar scale enrichment was observed in *in vivo* PCM. In order of minutes, tubulin began to polymerize and protrude out of droplets, leading to formation of aster-like structures highly reminiscent of mitotic centrosomes. ZYG-9 and TPXL-1 alone associated together and nucleated MTs but did not exhibit the aster formation. Interestingly, the study relied only on the γ-tubulin-independent nucleators and did not report any uptake of γ-tubulin into SPD-5 liquid droplets, a fundamental feature of centrosomes.

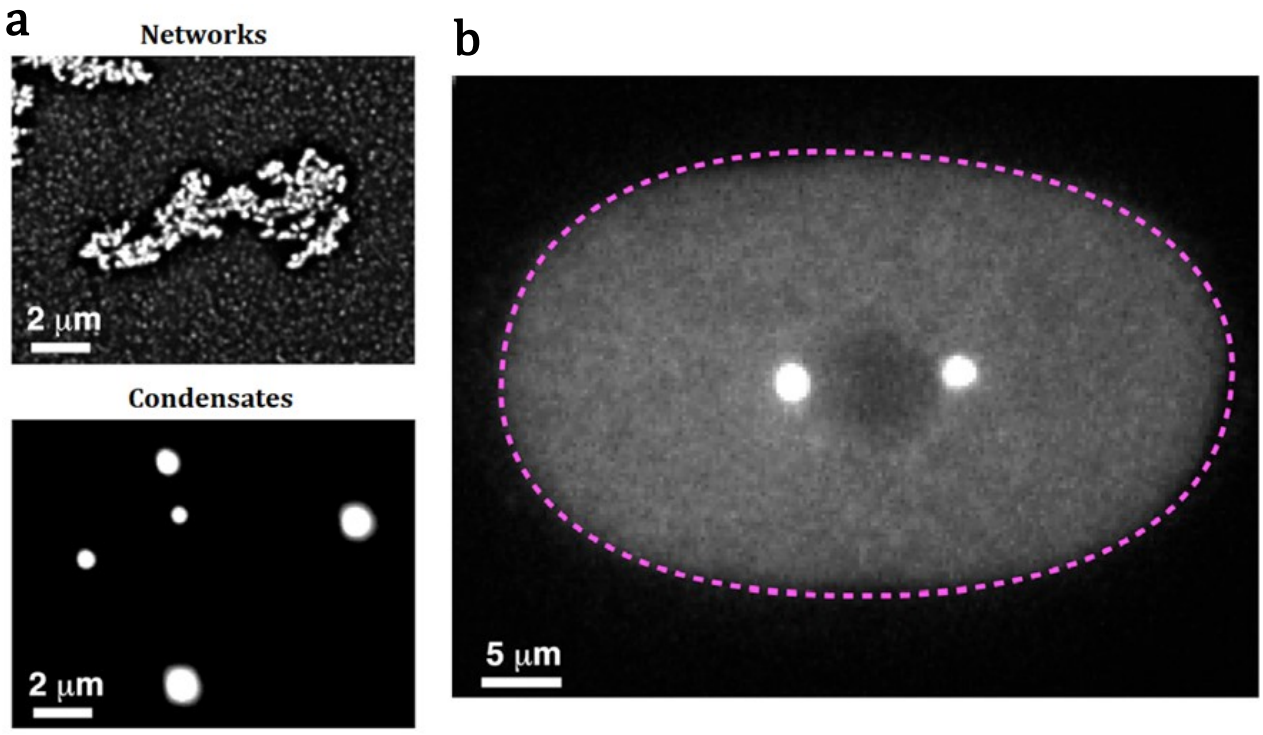

Figure 9: ***(a)*** *Comparison between previously observed SPD-5 networks and the novel SPD-5 condensates. GFP-labeled SPD-5 forms a porous structure, or a network, in the absence of PEG. In > 4% w/v PEG, droplet-shaped condensates can be observed.* ***(b)*** *GFP-labeled SPD-5 indicating two centrosomes in a C. elegans embryo. The SPD-5 condensates on the previous figure are remarkably similar. Dashed line, cell outline. (Woodruff et al. 2017), modified.*

In a system where two equal-sized condensates need to form every cell cycle, the question of Ostwald ripening seems essential. If one centriole was even slightly smaller at the beginning of mitosis, Ostwald ripening would lead to deepening of this difference if no regulatory measure was taken. Zwicker et al. proposed that centriole may have exclusive nucleation activity towards mitotic PCM (Zwicker et al. 2014). After nucleation, PCM grows autocatalytically so that the final

size is only reliant on the total amount of PCM material in the cell. Woodruff et al. proved that PCM nucleation is dependent on SPD-2, which forms a layer on the surface of mother centriole at mitosis onset (Woodruff et al. 2017). Thus, local concentration of SPD-2 may be the key regulator of centrosome size equality.

To sum up, SPD-5 is able to phase separate into droplets in the presence of crowding agents. Aged droplets correspond better with the static, less dynamic nature of centromatrix, showing a possible physiological role for liquid droplet maturation. SPD-5 droplets share important features with centromatrix and are able to concentrate proteins that lead to MT nucleation and aster formation, revealing a possibility that the PCM could be another type of MLO. Of course, high similarity to PCM is not a sufficient proof. It is still to be revealed if centrosomes truly originate on the basis of LLPS and observations *in vivo* are much needed.

In fly and vertebrate models, neither Centrosomin/CDK5RAP2 nor Pericentrin were shown to undergo LLPS. This may mean that no one has investigated the matter yet, or that centrosome maturation and PCM scaffold formation operates differently in this system.

5.4 Plk4 phase separation

Plk4 (Sak, Plx4) is another member of Polo-like kinase family found in vertebrates and *D. melanogaster* (Fode et al. 1994; Lowery, Lim, and Yaffe 2005). These kinases share repeated Polo-box motifs in their C-terminal domains, with Plk4 having one regular Polo-box and another two repeats forming a unique tandem called cryptic Polo-box (CPB) (Slevin et al. 2012; Leung et al. 2002)(Figure 10a). Through Polo-box motifs, Plk4 can form dimers and localize to mitotic centrosomes (Hudson et al. 2001; Park et al. 2019).

Its kinase activity is a critical driver of centriole duplication. During the canonical centriole duplication, Plk4 forms a ring encircling the mother centriole and accumulating in one spot of the circle more prominently (Kim et al. 2013; Ohta et al. 2014). Consequently, it undergoes a change in localization, becoming a distinct dot. This change is called ring-to-dot transition. The dot thereafter recruits other proteins and becomes the site of the daughter centriole biogenesis (Ohta et al. 2014). Plk4 overexpression leads to multiple daughter centriole assembly (Habedanck et al. 2005) and *de novo* centriole biogenesis (Lopes et al. 2015), denoting Plk4 as the main factor for centriole origination.

Plk4 is able to autophosphorylate on two sites; in kinase domain, phosphorylation increases Plk4 activity (Lopes et al. 2015), and on phosphodegron site, it promotes ubiquitination and consequent proteasomal degradation (Holland et al. 2010). The phosphodegron motif is located in

a flexible region, Linker 1, and can be phosphorylated on multiple sites (Klebba et al. 2013). Importantly, Linker 1 contains a disordered LCR, which is a common feature of proteins undergoing LLPS (Yamamoto and Kitagawa 2019) (Figure 10a,b). Plk4 activation is regulated in a concentration-dependent manner, therefore it is locally bound to the centriole where Plk4 levels are the highest (Lopes et al. 2015). Plk4 levels also increase in time as the cell cycle proceeds (Fode, Binkert, and Dennis 1996). In other instances, the degradation pathway outweighs and Plk4 is diminished. This mechanism is thought to ensure that centriole overduplication does not occur, as this state often leads to chromosomal aberrations and inability to complete cell division.

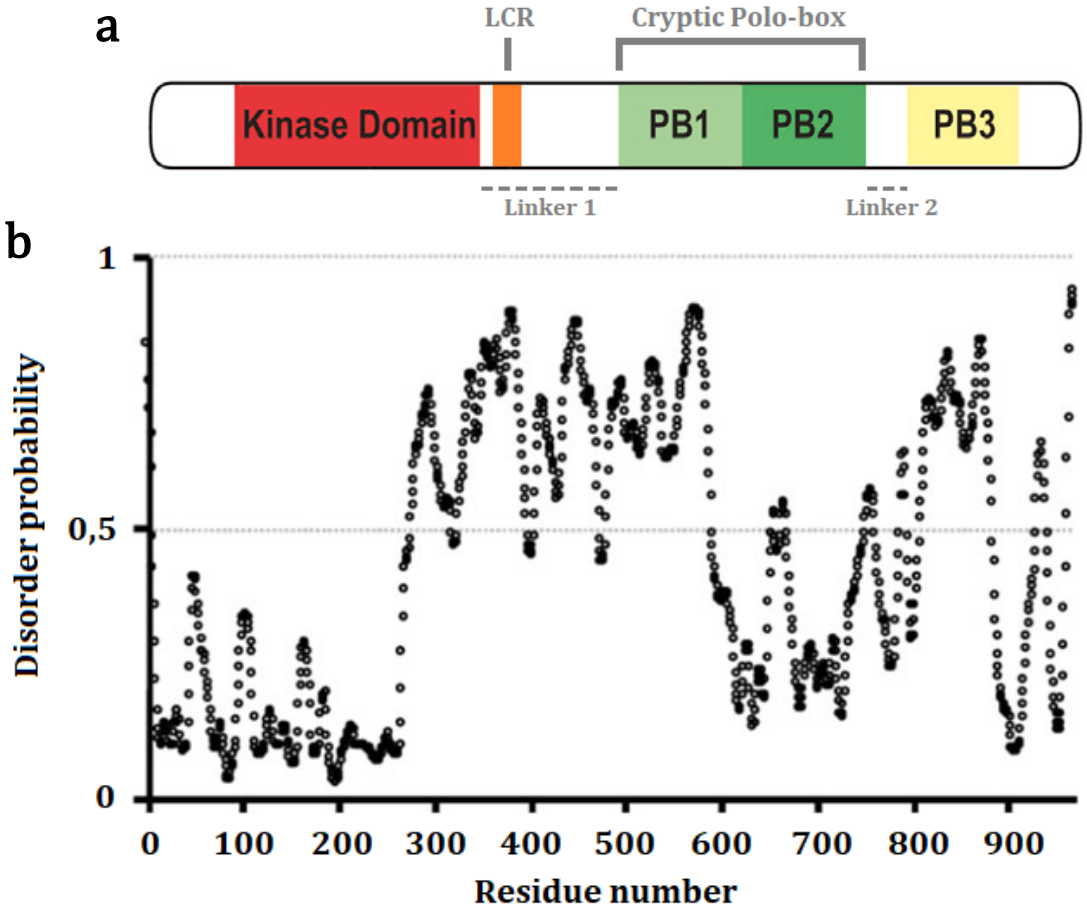

Figure 10: Structure of Plk4 protein. ***(a)*** *Scheme showing the kinase domain, low complexity region (LCR), and the three Polo-boxes (PB1-3). The cryptic Polo-box (CPB) comprises of PB1 and PB2.* (Lopes et al. 2015)*, modified with respect to* (Yamamoto and Kitagawa 2019)*. The LCR was predicted by (http://smart.embl-heidelberg.de).* ***(b)*** *Disorder prediction by the tool PrDOS (http://prdos.hgc.jp). The linker regions show very little secondary structure.* (Yamamoto and Kitagawa 2019)*, modified.*

Lately, studies reported that purified activated Plk4 forms spherical condensates in buffer with lowered NaCl concentration or in 1-4% w/v PEG (Montenegro Gouveia et al. 2018; Yamamoto and Kitagawa 2019). It is also possible to induce them *in vivo* by Plk4 overexpression. These formations' consistency is rather gel-like, with very limited dynamics, but they still show fluid-like qualities such as fusion. Interestingly, phosphorylation of Plk4 at Linker 1 lowers its ability

to condense and phosphorylated Plk4 condensates are more dynamic (Yamamoto and Kitagawa 2019). Because of the rigidity, hesitation about describing this behaviour as phase separation can be noticed.

A different study examined a phosphocluster in CPB, consisting of 4 phosphorylated amino acids clumped at 698-707 bp for human Plk4 (Park et al. 2019). Phosphorylation causes remodelling of CPB and increases its hydrophobic surface and structural disorder. This enables the molecule to phase separate, forming condensates of irregular, spherical shape. The condensates could be disrupted by 1,6-hexanediol, indicating their hydrophobic nature. They fused over time and recovered rapidly after photobleaching. These results suggest that even true LLPS can be achieved by phosphorylation of Plk4.

Opinion on the exact function of this behaviour remains disunited. One study demonstrated that Plk4 is able to directly bind tubulin as well as assembled MTs and thus acts as a stabilising MAP (Montenegro Gouveia et al. 2018). When *Xenopus* egg extract was added to Plk4 condensates, α/β-tubulin and other factors as γ-tubulin were recruited. This assembly nucleated tubulin and arranged MTs into asters, resembling a *de novo* formed acentriolar MTOC.

Another observation focused on the role of Plk4 during centriole duplication (Yamamoto and Kitagawa 2019). While unphosphorylated Plk4 assembled into ring-like structure and then remained as a dot on a side of the centriole, Plk4 phosphorylated at Linker 1 was only situated into one focus, colocalizing with the dot of unphosphorylated Plk4. After phosphorylation, the assembly recruited proteins downstream in the pathway and continued in procentriole formation. This suggests distinct roles of differentially phosphorylated – and thus differentially dynamic – Plk4 in centriole biogenesis. Phosphorylation, a product of high Plk4 accumulation at the centriole, may be the trigger to procentriole formation, making Plk4 condensation the chief regulator of the process.

The last study revealed that CPB phosphocluster, and thus Plk4 LLPS, is needed for Plk4 to proceed to dot-like structure during centriole duplication (Park et al. 2019). Overexpression of a gain-of-function mutant increased the rate of Plk4 condensation and led to elongated procentriole formation. At the same time, phosphorylation at phosphocluster site changed the protein's ability to bind upstream and downstream elements of centriole biogenesis pathway STIL (SCL-interrupting locus protein) and Cep152 (Centrosomal protein of 152 kDa), indicating its importance in the process.

Thus, the dot-like structure formed by Plk4 during centriole duplication could be a new type of MLO responsible for procentriole formation and MT nucleation. Phosphorylation unquestionably plays an important role in Plk4 condensation. Inconveniently, the amount of possible phosphorylation sites makes the subject hard to grasp and much more is needed to be known about their effects.

6 Phase separation at the mitotic spindle

6.1 Spindle matrix components and function

Similarly to the PCM, the mitotic spindle has its own protein meshwork as well, termed the spindle matrix (Leslie et al. 1987; Yao et al. 2012). It creates a dynamic, selective environment which contains and promotes the growth of the spindle MTs, but does not permit the admission of other objects as the endoplasmic reticulum, for instance. As a result, the spindle matrix accounts for correct development of the spindle apparatus during cell division.

The spindle matrix is temporarily preserved even after MT depolymerization. Based on this criterion, some of the identified spindle matrix components are BuGZ, lamin-B and a molecular motor dynein (Tsai et al. 2006; L. Ma et al. 2009; Jiang et al. 2014). A lot of the proteins are MAPs, corresponding to the proposed role of spindle matrix. However, the exact structure of spindle matrix remains elusive. It is also unclear whether it exists in all cell types as it was predominantly observed in early embryos (as *Xenopus* egg extracts and *Drosophila* embryos).

One of the key promoters of mitotic spindle assembly is the Aurora A kinase (Giet et al. 2002; Tsai and Zheng 2005). Its kinase activity activates further spindle assembly factors and inhibits contradictory proteins as the MT depolymerase MCAK (Xin Zhang, Ems-McClung, and Walczak 2008), leading to overall support of MT growth. Aurora A can be activated by other spindle proteins (Eyers et al. 2003). It is also able to autophosphorylate and thus activate itself, however, spontaneous autophosphorylation is a rare event at physiological concentrations of Aurora A (Zorba et al. 2014).

Intriguingly, the whole spindle possesses some liquid-like properties. For example, two separate spindles can fuse when brought together (Gatlin et al. 2009). Recent findings suggest that the whole spindle matrix may form on the basis of LLPS (Jiang et al. 2015; Huang et al. 2018). Another study proposes the existence of liquid spindle domains at the poles of the meiotic spindle in mammalian oocytes (So et al. 2019).

6.2 Phase separation of the spindle matrix

Bub3-interacting and GLE-2–binding sequence containing ZNF207 (BuGZ) is one of the established spindle matrix proteins (Jiang et al. 2014). It is a protein with multiple functions during interphase and cell division, highly evolutionarily conserved throughout many animal species. It localizes into the nucleus in interphase and acts as a splicing factor (Wan et al. 2015). During cell division, BuGZ becomes a component of the spindle matrix, driving correct assemblage of the spindle and activating Aurora A (Huang et al. 2018). Its second role during cell division is at the kinetochores, where BuGZ safeguards proper connection of spindle MTs (Jiang et al. 2014).

BuGZ is a highly unstructured protein (Figure 11). However, it has a structured region at its N-terminus, comprising two zinc finger domains. The N-terminal region binds Aurora A as well as MTs (and individual tubulin dimers) (Jiang et al. 2014; Huang et al. 2018).

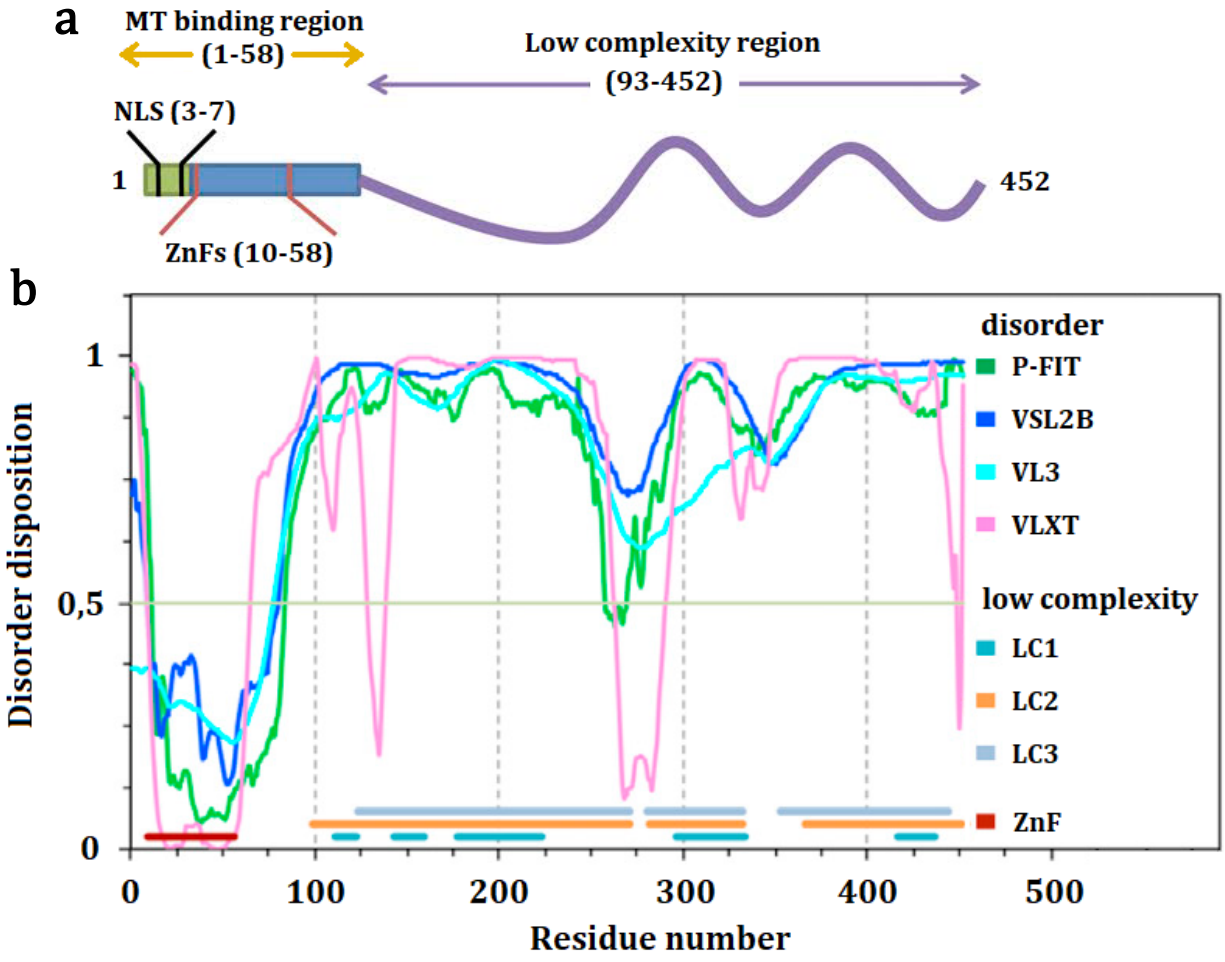

Figure 11: Protein structure of Xenopus BuGZ (xBuGZ). ***(a)*** *Scheme depicting localization of nuclear localization signal (NLS), zinc finger domains (ZnFs), the region responsible for binding of MTs and low complexity region. Amino acid numbers are displayed. Rectangles represent structured regions. (Huang et al. 2018), modified.* ***(b)*** *Prediction of structural disorder and low complexity of xBuGZ, determined using PONDR and SEG algorithms (http://www.pondr.com and* (Wootton 1994)*, respectively). Several different settings were used, delineated in different colours. (Jiang et al. 2015), modified.*

Jiang et al. addressed the high structural disorder of BuGZ and demonstrated that it is capable of LLPS (Jiang et al. 2015). BuGZ liquid droplets were observable *in vitro* in supercritical concentration of BuGZ or in the presence of >30% w/v PEG when in physiological concentration. Similar structures could be seen in an insect cell culture with overexpressed YFP (yellow fluorescent protein)-tagged *Xenopus* BuGZ.

The phase transition is sensitive to temperature as incubation on ice diminishes the liquid droplets. This can be rescued by incubation in room temperature.

The liquid droplet formation is driven exclusively by the low complexity region of the protein, with a key role of several conserved tyrosine and phenylalanine residues. When several of these residues were mutated into serines, the ability of YFP-BuGZ to form liquid droplets was lost. Another experiment revealed that a small fragment of the low complexity region cancelled out the capability of full-length BuGZ to LLPS, probably by disrupting the multivalent interactions.

Jiang et al. further showed that LLPS of BuGZ is a critical condition for assembly and maintenance of the spindle matrix. Importantly, the phase separation mutants also did not incorporate into extracted spindle matrices, in contrast to wild type BuGZ. This may be a convincing proof that the spindle matrix forms via LLPS.

BuGZ liquid droplets are able to concentrate tubulin as a client protein and stimulate MT polymerization *in vitro* (Jiang et al. 2015). LLPS of BuGZ was also shown to promote Aurora A activation (Huang et al. 2018). Interestingly, this is not a result of local increase of Aurora A concentration. The exact mechanism has not been discovered yet. Possibly, stabilization of Aurora A dimers (and its consequent autophosphorylation) may play a role.

There are several other proteins with low complexity within the spindle matrix components (Jiang et al. 2015). One of them, TPX2 (targeting protein for Xklp2), was recently shown to undergo LLPS as well (King and Petry 2020). TPX2 was previously described as a driver of MT nucleation along pre-existing MTs within the mitotic spindle (Petry et al. 2013). However, the exact mechanism was not known. King and Petry demonstrated that *Xenopus* TPX2 forms liquid droplets sensitive to salt concentration (King and Petry 2020). They localize preferentially on the surface of pre-existing MTs, in the form of uniform coats or separate beads. The TPX2 droplets are able to concentrate tubulin and supposedly also other TPX2 binding partners known to support MT nucleation. As a result, TPX2 liquid droplets increase the MT nucleation rate approximately 100× when in TPX2 physiological concentrations in *Xenopus* egg extract.

Collectively, these data offer a convincing proof of high importance of LLPS at the mitotic spindle. Thus, the spindle matrix could be a MLO encompassing the spindle apparatus and driving its development. However, it is necessary to note that majority of these results were obtained from *Xenopus laevis* eggs and egg extracts, which have many specificities in comparison to adult somatic tissues. A parallel work on cell cultures is needed at this point.

7 Conclusions

MLOs and LLPS in living organisms are two very recent topics in cell biology. Currently, we can observe the first steps in understanding their genesis and role. The published studies mostly rely on plain description of the phenomenon, but very little is known so far about MLOs' exact function, or their regulation. The *in vitro* experiments provide a minimalist system, enabling researchers to sequester the crucial molecules and driving forces from the noise of the complex cellular environment. However, this can also lead to oversimplification of the system and artifact creation (as in (Gallego et al. 2020), where Lge1 liquid droplets concentrate dextran with no reason). Therefore, caution is desirable when interpreting these experiments. Proper *in vivo* studies will be needed to complement the preceding data and characterize MLOs in their innate environment.

The structures associated with MTs and described in this work are still to be explicitly recognized as MLOs, mainly because there is only discreet evidence of individual proteins undergoing LLPS and sequestering their clients. A comprehensive description of the components and their liquid-like behaviour is awaited, as well as a complete information about the structures' function. Nevertheless, the initial results are promising in many cases.

MLOs are indeed changing our conception of organelles. They are generally simpler than canonical organelles, both in composition and function. This led to an idea that MLOs could have arisen as primitive organelles, or even as whole protocells (Stroberg and Schnell 2017). However, this hypothesis is not new, as it is based on the Oparin's coacervation theory (Oparin 1957). Some early experiments support the hypothesis (Koga et al. 2011; Keating 2012; Zwicker et al. 2017).

As much as tempting this idea is, any conclusions have not been drawn yet. Further research will be needed to understand both individual MLOs and the whole phenomenon origin. Until then, phase separation in cells is definitely a subject to keep an eye on.

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