PERSPECTIVE

Protein phase separation in plant membrane biology: more than just a compartmentalization strategy

Jonathan Michael Dragwidge[1,2*] and Daniël Van Damme[1,2]

[1] Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 71, 9052 Ghent, Belgium

[2] VIB Center for Plant Systems Biology, Technologiepark 71, 9052 Ghent, Belgium

*Corresponding author; email: jodra@psb.vib-ugent.be

Running title: Phase separation in membrane biology

One-sentence summary: This perspective discusses recent work on phase separation in membrane biology and provides practical advice for researchers looking to investigate membrane-associated condensates in plants.

Abstract

The formation of biomolecular condensates through phase separation is an important strategy to compartmentalize cellular functions. While it is now well established that condensates exist throughout eukaryotic cells, how condensates assemble and function on lipid membranes is only beginning to be understood. In this perspective, we highlight work from plant, animal, and yeast model systems showing that condensates assemble on many endomembrane surfaces to carry out diverse functions. In vesicle trafficking, condensation has reported roles in the formation of endocytic vesicles and autophagosomes, and in the inactivation of secretory COPII vesicles. We briefly discuss how membranes and membrane lipids regulate the formation and function of membrane-associated condensates. This includes how membranes act as surfaces for condensate assembly, with lipids mediating the nucleation of condensates during endocytosis and other processes. Additionally, membrane-condensate interactions give rise to the biophysical property of 'wetting', which has functional importance in shaping autophagosomal and vacuolar membranes. We also speculate on the existence of membrane-associated condensates during cell polarity in plants, and discuss how condensation may help to establish functional plasma membrane domains. Lastly, we provide advice on relevant in vitro and in vivo approaches and techniques to study membrane-associated phase separation.

Introduction

A fundamental question in biology is how cells separate different biochemical reactions. One way this is achieved in eukaryotic cells is by compartmentalizing biomolecules into membrane-enclosed structures (e.g.: endoplasmic reticulum, Golgi, vacuole), or membraneless organelles (e.g.: nucleolus, stress granules, P bodies). Recently the process of phase separation has been used to explain the formation and properties of many membraneless organelles (also called biomolecular condensates) (Banani et al., 2017; Emenecker et al., 2020; Alberti and Hyman, 2021). A surge of research on biomolecular condensates in plants has found that condensates in the nucleus and cytoplasm play important roles in many biological processes, including flowering time (Fang et al., 2019; Zhu et al., 2021), immunity (Huang et al., 2021; Zavaliev et al., 2020), hormone response (Powers et al., 2019), stress responses (Gutierrez-Beltran et al., 2021; Zhu et al., 2022), embryo development (Zhang et al., 2020), and seed germination (Dorone et al., 2021). It is becoming increasingly clear from work in animals, and more recently in plants, that condensates also assemble on membrane surfaces, including the plasma membrane and membrane-bound organelles (Snead and Gladfelter, 2019; Zhao and Zhang, 2020). While phase separation has also been used to describe the ordering of lipids at membrane domains (Sezgin et al., 2017), in this perspective piece we focus on phase separation driven by proteins.

In a simplified system, phase separation is the process where a solute (e.g.: a biomolecule) demixes into two distinct phases, one containing a high concentration of protein, and one with a low concentration of protein. This process occurs spontaneously when the bulk protein concentration exceeds a certain concentration (termed the saturation concentration) (reviewed in Brangwynne et al., 2015; Emenecker et al., 2021). In general, the capacity of a biomolecule to phase separate is dependent on its ability to participate in multivalent interactions, as well as environmental parameters including salt, pH, and temperature (Choi et al., 2020). In a complex cellular system, biomolecular condensates are not composed of a single biomolecule, but a network of interacting biomolecules (e.g. protein, RNA). The term ‘biomolecular condensate’ has been used to define the ability of these compartments to locally concentrate certain biomolecules, while excluding others, thereby allowing cells to compartmentalize biochemical reactions (Banani et al., 2017). Importantly, while phase separation may underly the formation of many cellular condensates, ‘biomolecular condensate’ is a general term that describes a diverse array of membraneless compartments, and does not define how they are formed. Regardless, condensates generally have distinctive liquid-like characteristics, such as the ability to undergo fusion. Furthermore, condensates have viscoelastic material properties that allow them to generate mechanical forces, for example, to bend and reshape membranes (Gouveia et al., 2022). Condensates can also readily allow the exchange of molecules with their environment, creating dynamic structures which accelerate molecular re-arrangement. It should be noted that condensates can exist with a broad range of material properties, with gel- and solid-like condensates having reduced dynamics (Boeynaems et al., 2018). These characteristics are relevant for the function of many membrane-associated condensates, discussed in detail below.

In this perspective, we highlight some studies involving interactions between membranes and condensates, primarily from work performed in animal model systems and in vitro reconstitution approaches. Although research on membrane-associated condensates in plants is limited, recent studies have shown a role for condensates during endocytosis (Dragwidge et al., 2022), actin nucleation (Liu et al., 2023a), regulation of lipid transferase

wetting (Liu et al., 2023b) and vacuole shaping (Kusumaatmaja et al., 2021a) discussed in more detail below. Condensation is likely involved in many more biological processes in plants, and we provide some practical advice for researchers to identify condensate-forming proteins, and study their function through in vivo and in vitro approaches.

Condensate assembly and properties are controlled by membrane surfaces

While there is ample evidence that condensates form on membranes (Zhao and Zhang, 2020), the mechanisms by which they are formed and regulated are less clear. Because phase separation is influenced by protein concentration, the localized enrichment of proteins within a region of the cell will lower the threshold required for phase separation. Importantly, membrane surfaces can reduce the energy barrier to form condensates, by increasing local protein concentration and restricting molecular diffusion (Figure 1A-B) (Snead and Gladfelter, 2019). Consistently, multivalent proteins attached to synthetic membranes phase separate at lower concentrations than unbound proteins in vitro (Zeng et al., 2018; Su et al., 2016; Liu et al., 2023b). Membranes can also alter the material properties of condensates. In an in vitro study using protein attached to supported lipid bilayers, condensates formed gel-like networks which displayed limited fusion and molecular diffusion (Figure 1B) (Snead et al., 2022). These in vitro studies highlight general principles of membrane condensation that are likely applicable to plant model systems.

Membranes also regulate the time and location that condensates are formed. Membrane surfaces are organized into ordered domains with distinct lipid and protein compositions (Sezgin et al., 2017; Gronnier et al., 2018). It was recently shown that human transmembrane adaptor protein Linker of Activation of T-cells (LAT) condensates are nucleated on ordered lipid domains (Wang et al., 2023) (Figure 1B). Interestingly, protein condensation on ordered lipid domains has been shown to be coupled to both surfaces of the membrane in vitro (Lee et al., 2022). Therefore, the ordering of lipids on the membrane is an essential factor for condensate formation. In plants, lipid-mediated condensate nucleation has been shown in vivo for the endocytic TPLATE complex subunit AtEH1 in Arabidopsis (*A. thaliana*) (Dragwidge et al., 2022). In this study, mutation of residues in AtEH1 implicated in phospholipid binding abolished condensate formation, indicating that lipids are required for nucleation. Thus, the composition of lipids on the plasma membrane controls when and where condensates are formed, and consequently the timing of endocytosis initiation.

However, lipids are not the only molecules that could assist the nucleation of condensates on membranes. In the more complex system of a living cell, the formation of ordered lipid domains depends on peripheral and integral membrane proteins, as well as interactions with the cytoskeleton and the cell wall in plants (Martiniere et al., 2012; Gronnier et al., 2018). For example, proteins driving condensation have been associated with membranes through interactions with transmembrane receptors in animals (Beutel et al., 2019), or by attaching to membrane-associated tethers in yeast (Fujioka et al., 2020). Interestingly, the TPLATE complex has been associated with both P bodies and stress granules in plants (Liu et al., 2023a; Dubiel et al., 2020), and may tether these membraneless organelles to the plasma membrane surface. Alternatively, P bodies or stress granules could promote condensation of the TPLATE complex. Receptor-mediated interactions or tethering of condensates may also be particularly relevant during membrane contact sites, such as interactions between two organelles or between organelles and the plasma membrane. For

example, condensates forming between the ER and plasma membrane could function in membrane shaping or recruitment of cytosolic components, relevant for many processes including endocytosis, autophagy, and plasmodesmata formation. Further understanding of the interplay between condensates, membrane proteins, the cytoskeleton, and the cell wall will be necessary to build a functional model describing membrane-condensate interactions.

Emerging biological functions of condensates formed at membranes

A growing body of evidence in animal, yeast, and plant systems has reported that many membrane-associated proteins assemble into condensates to carry out diverse biological functions (Zhao and Zhang, 2020). Besides endocytosis, plasma membrane-associated condensates have been identified to have functional roles at the neuronal synapse (Milovanovic et al., 2018; Zeng et al., 2018), and in immune cell activation (Su et al., 2016; Huang et al., 2019; Ditlev et al., 2019; Case et al., 2019b). Condensates have also been observed at the endoplasmic reticulum (Zacharogianni et al., 2014), the Golgi (Parchure et al., 2022; Rebane et al., 2019), and at lysosomes/vacuoles (Liao et al., 2019; Kusumaatmaja et al., 2021a). Many of these condensates have functions during cell signaling (reviewed in Case et al., 2019a; Jaillais and Ott, 2020), or vesicle trafficking, including the assembly, tethering, and clustering of vesicles. Here we provide some examples of interesting functions which have been shown for membrane-associated condensates.

Initiation of membrane events

Membrane-associated condensates have been linked to the initiation of vesicle trafficking and the formation of intracellular barriers. Clathrin-mediated endocytosis is an essential cellular internalization process involving the dynamic recruitment of cytosolic accessory proteins, the coat protein clathrin, and cargo into membrane-enclosed vesicles (Kaksonen and Roux, 2018). Notably, plants retained the evolutionarily ancient TPLATE/TSET complex which was lost from metazoan lineages (Gadeyne et al., 2014; Hirst et al., 2014). Recent work has shown that the highly disordered TPLATE complex subunits AtEH1 and AtEH2 promote biomolecular condensation of the complex during endocytosis (Dragwidge et al., 2022). Condensation of AtEH1 is sufficient to selectively recruit both early- and late-stage accessory proteins, as well as clathrin, suggesting that condensation may promote not just endocytosis initiation, but the sequential recruitment of proteins throughout endocytosis (Figure 2a) (Dragwidge et al., 2022). This differs from endocytosis in yeast and animal systems, where Ede1 (in yeast) and Eps15 (in humans) selectively promote condensation during endocytosis initiation. Furthermore, Dragwidge et al. (2022) show that the material properties of endocytic condensates are important, as the modification of condensate liquidity alters endocytosis progression. Condensation is also implicated in the initiation of autophagy. It was recently shown that the ‘pre-autophagosome structure’ in yeast is a condensate that recruits downstream components and vesicles, thereby specifying the site of autophagosome initiation (Fujioka et al., 2020). Since core components of autophagy machinery are shared throughout eukaryotes, this mechanism is likely conserved in plants.

Membrane-associated condensation has also been found to be required for the formation of tight junctions in animal epithelial cells (Beutel et al., 2019). Tight junctions are polarized cell-cell adhesion complexes that seal tissues and are formed through the polymerization of claudin receptors on the plasma membrane. Beutel et al. (2019), found that

zona occludens (ZO) proteins drive membrane condensation that selectively recruits and enriches tight-junction proteins, thereby promoting the assembly of a continuous tight junction 'belt' along the membrane. It is possible that similar processes in plants may underly the formation of polar membrane domains (discussed below) or polarized membrane structures such as the Casparian strip. Collectively, these examples illustrate how condensation can act to recruit specific assembly factors required to build complex structures on the membrane.

Inactivation/storage

Condensates can also function to inactivate cellular processes by sequestering active biomolecules, for example during transcriptional repression in Arabidopsis (Zhu et al., 2021). Inactivation by condensation has also been found to be a mechanism to inhibit vesicle trafficking from the endoplasmic reticulum in Drosophila (*D. melanogaster*) (Zacharogianni et al., 2014). Protein secretion at the endoplasmic reticulum to the Golgi occurs via the formation of COPII-coated vesicles at specialized sites, called ER exit sites (ERES). In response to nutrient starvation in Drosophila, certain COPII coat proteins assemble into condensates which sequester and inactivate other COPII components, thereby inhibiting secretory traffic (Figure 2b) (Zacharogianni et al., 2014). These condensates are readily dissolved when nutrient stress is relieved, allowing cells to rapidly resume secretory protein transport. The inactivation and storage of proteins through condensation is a powerful cellular strategy as it avoids potentially energetically inefficient protein degradation and de novo translation pathways and can be readily reversed by cells (i.e., via post-translational modifications). Condensate-mediated protein inactivation could be a useful cellular strategy in plants to temporarily de-activate membrane processes, for example during transient environmental stresses.

Membrane shaping/bending

One of the most interesting functions of condensates involves the generation of mechanical forces that can reshape membranes. A property of condensates is the ability to contact flat, rigid surfaces and physically deform or 'wet' on the membrane surface (Kusumaatmaja et al., 2021b). These wetting interactions can deform lipid bilayers, dependent on the membrane tension (i.e. the physical forces acting on the membrane) of the specific membrane (Kusumaatmaja et al., 2021b). Consequently, membranes under high tension (e.g. the plant plasma membrane) are not readily deformable by droplet wetting, while membranes with lower tension (e.g. the tonoplast) are more easily shaped (Figure 2c). This wetting phenomenon has recently been demonstrated to promote membrane reshaping in biological systems, including the wetting of autophagosome membranes on liquid-droplets during droplet autophagy (Agudo-Canalejo et al., 2021), and the shaping of protein storage vacuoles in plant cells (Kusumaatmaja et al., 2021a). Specifically, Kusumaatmaja et al. (2021a) found that liquid droplets containing seed storage proteins in the vacuole lumen can physically 'wet' the tonoplast membrane. The distinct droplet-membrane morphologies observed in the vacuole could be explained by a biophysical model which depends on the contact angle of the droplet and the membrane, and the membrane rigidity. This work illustrates how membrane wetting organizes liquids in cells.

Wetting as a biophysical model has important implications for understanding membrane-condensate organization and function, and should be carefully considered in future studies. For example, the AtEH subunits of the TPLATE complex form condensates which can

be degraded through autophagy (Wang et al., 2019; Dragwidge et al., 2022), and wetting of the autophagosome membrane on these condensates could assist their sequestration and degradation, similar to what has been previously shown for p62 condensates in mouse and human cells (Agudo-Canalejo et al., 2021). Furthermore, given that osmotic shock reduces plasma membrane tension in plants (Michels et al., 2020), condensate-membrane wetting interactions may be impaired upon drought and salt stress in plants.

A related, but independent membrane deformation strategy has also been described, related to phase separation induced by membrane-bound proteins. By anchoring a protein that phase separates on a membrane surface, Yuan et al. (2021) show that this produces mechanical forces sufficient to deform and tubulate synthetic giant unilamellar vesicle (GUV) membranes. Modeling approaches suggest that similar mechanical forces could provide sufficient energy to promote membrane invagination during endocytosis in yeast (Bergeron-Sandoval et al., 2021). Phase separation-induced membrane bending may be especially important during endocytosis in plants, as bending the plasma membrane inwards against the high turgor pressure inside plant cells is energetically demanding (Dragwidge and van Damme, 2020; Gradmann and Robinson, 1989), and plants do not use actin to aid membrane bending as in other systems (Narasimhan et al., 2020). As the TPLATE complex is required for membrane bending during endocytosis (Johnson et al., 2021), condensation of AtEH proteins could assist in generating sufficient mechanical force to bend the plasma membrane inwards (Dragwidge et al., 2022). Phase separation-induced membrane bending could be an important membrane remodeling strategy relevant for other processes in plants, for example during the formation of intraluminal vesicles within multi-vesicular bodies.

Condensation may underly the formation of polar membrane domains

One important membrane process where phase separation may play a role is during the establishment and/or maintenance of cell polarity. In plants, cell polarity is driven by many evolutionarily distinct polarity complexes (Muroyama and Bergmann, 2019), including the recently identified SOSEKI family of proteins (Yoshida et al., 2019). Interestingly, SOSEKI proteins form higher-order assemblies through oligomerization of their DIX domains, analogous to the polymerization of the Drosophila polarity protein Dishevelled (van Dop et al., 2020). Dishevelled contains a cross-linking domain that enables it to form higher-order assemblies, sufficient to drive the formation of cytoplasmic condensates when overexpressed in cells (Schwarz-Romond et al., 2007; Gammons et al., 2016). Consequently, it has been proposed that polar SOSEKI puncta are membrane-associated condensates (van Dop et al., 2020), although experimental evidence validating this hypothesis remains elusive. SOSEKIs are predicted to interface with the membrane through palmitoylation, as mutation of the predicted palmitoylation site disrupts membrane association (van Dop et al., 2020). It remains to be established whether these putative condensates are driven by polymerization of SOSEKI proteins on the membrane, or through a multivalent interaction network that includes other condensate-forming proteins, such as the lipid-like transferase SFH8 (Liu et al., 2023b).

Although these studies implicate a role for condensation in membrane polarity, a mechanistic link connecting these putative condensates to a specific function in polarity establishment has not been determined. What could be a biological advantage for membrane polarity complexes to be organized into higher-order assemblies with liquid-like properties? A potential clue comes from work on tight junctions, where condensation and wetting of zonula

occludens (ZO) proteins on the plasma membrane is required for the formation of continuous membrane assemblies (Beutel et al., 2019; Pombo-García et al., 2022). Given that polar membrane domains need to be established along a continuous cell axis, it is plausible that condensation and wetting of plant polarity complexes could function in a similar way to ensure the complete formation of membrane polarity domains in plant cells. Directly testing whether polar domains are organized through condensation remains experimentally challenging in vivo, and will likely require a combination of in vitro and in vivo experimental systems discussed in more detail below.

A short practical guide to studying membrane-associated condensates

How can researchers identify membrane proteins which form condensates, and subsequently investigate their molecular function? Protein phase separation is generally driven by 'scaffold' proteins which participate in multivalent protein interactions. This includes interactions mediated by folded domains, repetitive short linear motifs or domains, and intrinsically disordered regions (Choi et al., 2020). For example, a general phase separation mechanism during cell signaling in animals involves interactions between repeated SRC homology 3 (SH3) domains with proline-rich motifs (PRM) (Li et al., 2012). Furthermore, disordered amino acid stretches can participate in weak, non-specific interactions to drive phase separation (Boeynaems et al., 2018; Banani et al., 2017). Therefore, the prediction of repetitive motifs or domains and intrinsically disordered regions (IDRs) through sequence analysis tools could be useful to identify potential protein sequence regions which drive phase separation (reviewed by Alberti et al., 2019; Emenecker et al., 2021). Moreover, identifying membrane interface regions, such as phospholipid binding domains (Noack and Jaillais, 2017; Jong and Munnik, 2021), could give insight into how membrane-associated condensates are nucleated and regulated. Interestingly, many vesicle trafficking pathways contain intrinsically disordered proteins which could act as potential scaffold molecules to drive condensate formation (Pietrosemoli et al., 2013).

In practice, it is difficult to predict whether a protein undergoes phase separation through sequence analysis alone. An important first experiment is to test whether a protein has properties consistent with biomolecular condensates (i.e. concentration, salt and/or temperature-dependent droplet formation, droplet fusion, ability to exchange molecules). This can be achieved by in vitro experiments using purified protein, or in cells by overexpressing a fluorescently tagged reporter construct (e.g. by transient expression in *Nicotiana benthamiana* epidermal cells). Following this, researchers can use a broad range of biochemistry and light microscopy techniques to characterize condensate morphology, material properties, structure, and dynamics (detailed in the next section). Determining the material properties of condensates (e.g. visco-elasticity, interfacial tension) is challenging, and may require sophisticated biophysical techniques (reviewed in Wang et al., 2022b). While FRAP is a commonly used technique to assess whether a protein can undergo phase separation, it does not exclude other alternative mechanisms, such as protein clustering on a scaffold layer. Half-FRAP analysis of internal mixing has been shown to differentiate between these two scenarios (Muzzopappa et al., 2022). However, this technique is currently restricted to relatively large condensates in vivo, and further methods need to be developed to address this problem.

Determining whether the concentration and solution conditions used to assess phase separation are physiologically relevant is a complex issue (Alberti et al., 2019). Ideally,

researchers should examine protein behavior at physiological concentrations in vivo, for example by generating fluorescently tagged reporter lines expressed at endogenous levels in complemented mutant plant backgrounds. However, this can be experimentally challenging due to the dynamic nature and small (i.e. diffraction-limited) size of most membrane-bound structures. Consequently, researchers have turned to in vitro reconstitution approaches on artificial membranes including supported lipid bilayers (SLBs) and giant unilamellar vesicles (GUVs) to investigate phase separation in a simplified system (Ditlev, 2021; Stachowiak and Kirchhausen, 2022) (detailed in the next section**)**. These approaches have been used to reconstitute condensates at the postsynaptic density (Zeng et al., 2018), study lipid-mediated condensate formation during endocytosis in animals (Day et al., 2021), and examine phase separation-induced membrane deformation (Yuan et al., 2021).

Methods used to study biomolecular condensates

Protein phase separation can be studied using a variety of in vivo and in vitro approaches (reviewed in detail in Ganser and Myong, 2020; Mitrea et al., 2018; Emenecker et al., 2021; Wang et al., 2022b). In this section, we highlight some general approaches for studying condensates that are applicable to membrane-associated condensates. These techniques are not exhaustive, and new methods need to be developed, especially for studying condensates in vivo.

Fluorescence-based live cell imaging assays

Fluorescence microscopy assays (e.g. FRAP, FCS) can be performed in seedlings or *N. benthamiana* epidermal cells overexpressing fluorescently tagged proteins, or ideally in *A. thaliana* by using fluorescently tagged proteins expressed under native promoters in a complemented mutant background. While transient expression in *N. benthamiana* is relatively simple, caution should be taken as agroinfiltration can induce defense and immune responses (Pruss et al., 2008), and experiments generally involve expressing proteins at very high levels which may lead to artificial condensate formation. The main advantage of live cell assays is that they are generally more accessible than in vitro experiments, and can allow researchers to link phase separation directly to biological functions, for example, to determine changes in condensate formation or properties upon stress. Despite this, examining the properties of small or transient membrane-associated condensates may be technically challenging. This is especially a challenge in plant cells due to cell wall-induced light scattering and autofluorescence of metabolic compounds, especially in deeper tissues (reviewed in Grossmann et al., 2018). Researchers may use in vitro experiments as an alternative approach when in vivo studies are impractical (see below).

Fluorescence recovery after photobleaching (FRAP)

FRAP is a relatively simple and widely used method to qualitatively assess condensate fluidity and determine the mobility of molecules within condensates (Ishikawa-Ankerhold et al., 2012). Although FRAP is a relatively simply way to estimate the material properties of condensates, it is important to note that the relative diffusivity of different components within a single condensate can be dramatically different (Sun et al., 2018), and therefore should be used with caution. Furthermore, FRAP only provides as estimation of protein diffusion, and calculations can differ greatly depending on the model used to fit the data (Taylor et al., 2019). Because of the variation in protein levels in *N. benthamiana* infiltrated leaves, it may be useful to perform

a ratio-metric FRAP approach by expressing two reporters from a single T-DNA locus (Dragwidge et al., 2022). Additional in vivo approaches, including transmission electron microscopy of condensate structure (Dorone et al., 2021), or analysis of condensate fusion dynamics (Bose et al., 2022) should be carried out to make robust conclusions about material properties.

Fluorescence correlation spectroscopy (FCS)

FCS is a powerful technique involving the measurement of intensity fluctuations of a fluorescently labelled molecule, allowing the quantification of protein molecular diffusion and stoichiometry (Yu et al., 2021). FCS has been applied to study the oligomerization state of cytosolic ARF condensates in plants (Powers et al., 2019), and of tight-junction proteins in the cytoplasm (Beutel et al., 2019). A disadvantage of FCS is that it is only effective within a limited protein concentration range and protein diffusion rate (depending on the focal volume observed). Furthermore, FCS requires advanced confocal microscopy setup including high-sensitive detectors, and is prone to photobleaching artifacts. FCS-based variants such as scanning FCS could be used to improve accuracy and reduce photobleaching effects.

In vitro phase separation assays

In vitro assays allow researchers to assess phase separation in a simplified system, without the complexity of living cells. Purified proteins can be assessed for their ability to phase separate under different conditions, and quantified by sedimentation into pellets (Ouyang et al., 2020), or through fluorescence microscopy with labelled proteins. FRAP and analysis of fusion dynamics can be performed with labelled proteins, to provide information of protein dynamics and material properties in vitro. The purification of proteins that phase separate can be challenging as they are generally aggregation prone, however there are strategies to assist their purification (Alberti et al., 2018). Given that membrane surfaces greatly reduce the concentration threshold required for phase separation (Snead and Gladfelter, 2019), proteins which form condensates on the membrane may require very high concentrations to phase separate in 3D in vitro assays.

Artificial lipid bilayers

Artificial membranes are a powerful reconstitution approach to imitate the cell membrane to study the formation and properties of membrane-bound condensates. These include supported lipid bilayers (SLBs), small unilamellar vesicles (SUVs), and giant unilamellar vesicles (GUVs) (Ditlev, 2021; Stachowiak and Kirchhausen, 2022; Cheng et al., 2022). His-tagged purified proteins can be attached to artificial membranes composed of NTa-Ni2+ modified lipids (Case et al., 2019b; Snead et al., 2022; Zeng et al., 2018; Su et al., 2016), or covalently attached to membranes via maleimide chemistry (Huang et al., 2019). These membranes can be imaged through total internal reflection fluorescence (TIRF) microscopy which involves the selective imaging of the membrane surface. These methods are useful to study proteins that do not directly bind lipids, but may function in membrane condensation. GUVs can be made containing specific lipid compositions which form ordered and disordered phases (Sezgin et al., 2017). Proteins that bind specific lipids can partition into these ordered domains (Day et al., 2021). GUVs also can be used to study membrane bending (Yuan et al., 2021). A limitation of these techniques is that they require purified proteins and significant technical expertise to establish. Plant researchers should note that plant lipids differ from other

systems, and the lipid composition to generate artificial membranes should be considered accordingly (Grosjean et al., 2015).

Proximity biotinylation proteomics

Condensates can be thought of as a network of interacting proteins, with scaffold proteins acting to specifically recruit proteins. These protein networks can be resolved by using TurboID proximity proteomic techniques with scaffold proteins as bait in *A. thaliana* (Dragwidge et al., 2022; Liu et al., 2023a; Arora et al., 2020), allowing for the identification of interactors and regulators (i.e. client proteins) within the network. Client proteins identified using this technique can be validated by client partitioning assays in vivo (e.g., in *A. thaliana* or *N. benthamiana*) or in vitro. Potential drawbacks of TurboID include the presence of endogenous biotin in plants which reduces the ability to perform time-resolved experiments. A limitation for studying condensates is that the bait protein used may not always reside in the condensate, and produce background signal from cytoplasmic protein outside the condensate. Split-TurboID based approaches could remedy this issue (Cho et al., 2020).

Conclusions

Despite the technical challenges of in vivo studies of membrane-associated condensates in plants, there are also advantages when compared to simplified biological systems such as yeast or cultured human cells. As multi-cellular organisms, plants allow researchers to place their work in the broader context of the development of a complex organism containing specialized organs and cell types. For example, the P-body protein DCP1 is increasingly polarized to cell edges throughout root development, suggesting the function of P bodies differs depending on the tissue and cell type (Liu et al., 2023a).

Plants are also excellent models to study physiological responses of condensates to environmental cues. This is perfectly illustrated by the identification of FLOE1, a highly seed-enriched protein that undergoes biomolecular condensation in the cytosol in a hydration-dependent manner, thereby allowing embryos to sense water (Dorone et al., 2021). Through elegant work combining molecular, organismal, and ecological approaches, Dorone et al. (2021) identified that the material properties of FLOE1 condensates are regulated through a disordered aspartic acid- and serine-rich ‘DS-rich’ domain, which acts to modulate its hydration sensing capacity during germination. Strikingly, variation in the expression of FLOE1 isoforms (which have different DS domain lengths) was associated with variation in environmental precipitation levels in natural populations. Therefore, variation in liquid-to-solid transitions of FLOE1 condensates facilitates adaptive germination strategies to respond to fluctuating environmental conditions in natural populations.

Similar environmentally responsive strategies may be employed by plants for membrane-associated condensates, including for the TPLATE complex during endocytosis. As previously mentioned, phase separation and membrane tension are sensitive to osmotic changes which could potentially disrupt the function of condensation during clathrin-mediated endocytosis. Given that IDRs in plants have been shown to directly sense osmotic stress via cytosolic crowding (Wang et al., 2022a; Cuevas-Velazquez et al., 2021), it is interesting to speculate that the TPLATE complex or other endocytic disordered proteins could act as crowding sensors to adapt the material properties of endocytic condensates in a stress-responsive manner. This would allow endocytosis to function efficiently despite changes in

potential membrane-condensate wetting interactions or cytosolic osmolarity changes, which may otherwise impair condensate formation or function.

In this perspective, we have briefly discussed how protein condensation on membranes can function as more than a compartmentalization strategy, but can shape membranes, initiate/inactivate membrane trafficking, and potentially influence cell polarity. We expect more membrane-associated condensates to be identified in the coming years in plant model systems. To properly characterize these condensates and understand their function will likely require a combination of cell biology, biophysics, and biochemical approaches. While in vivo plant studies take considerable time, they remain a powerful approach to determine the physiological relevance and an environmental or developmental context for biomolecular condensation.

Acknowledgments

We thank members of the Van Damme lab for helpful comments on the manuscript.

Funding

J.M.D is supported by a Research Foundation–Flanders (FWO) postdoctoral fellowship (12S7222N).

Conflict of interest statement: None declared.

References

Agudo-Canalejo, J., Schultz, S.W., Chino, H., Migliano, S.M., Saito, C., Koyama-Honda, I., Stenmark, H., Brech, A., May, A.I., Mizushima, N., and Knorr, R.L. (2021). Wetting regulates autophagy of phase-separated compartments and the cytosol. Nature **591**: 142–146.

Alberti, S., Gladfelter, A., and Mittag, T. (2019). Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates. Cell **176**: 419–434.

Alberti, S. and Hyman, A.A. (2021). Biomolecular condensates at the nexus of cellular stress, protein aggregation disease and ageing. Nat. Rev. Mol. Cell Biol. **22**: 196–213.

Alberti, S., Saha, S., Woodruff, J.B., Franzmann, T.M., Wang, J., and Hyman, A.A. (2018). A User's Guide for Phase Separation Assays with Purified Proteins. J. Mol. Biol. **430**: 4806–4820.

Arora, D., Abel, N.B., Liu, C., Van Damme, P., Yperman, K., Eeckhout, D., Vu, L.D., Wang, J., Tornkvist, A., Impens, F., Korbei, B., Van Leene, J., Goossens, A., De Jaeger, G., Ott, T., Moschou, P.N., and Van Damme, D. (2020). Establishment of Proximity-dependent Biotinylation Approaches in Different Plant Model Systems. Plant Cell **32**: tpc.00235.2020.

Banani, S.F., Lee, H.O., Hyman, A.A., and Rosen, M.K. (2017). Biomolecular condensates: Organizers of cellular biochemistry. Nat. Rev. Mol. Cell Biol. **18**: 285–298.

Bergeron-Sandoval, L.P., Kumar, S., Heris, H.K., Chang, C.L.A., Cornell, C.E., Keller, S.L., François, P., Hendricks, A.G., Ehrlicher, A.J., Pappu, R. V, and Michnick, S.W. (2021). Endocytic proteins with prion-like domains form viscoelastic condensates that enable membrane remodeling. Proc. Natl. Acad. Sci. U. S. A. **118**: 145664.

Beutel, O., Maraspini, R., Pombo-García, K., Martin-Lemaitre, C., and Honigmann, A. (2019). Phase Separation of Zonula Occludens Proteins Drives Formation of Tight Junctions. Cell **179**: 923-936.e11.

Boeynaems, S., Alberti, S., Fawzi, N.L., Mittag, T., Polymenidou, M., Rousseau, F., Schymkowitz, J., Shorter, J., Wolozin, B., Van Den Bosch, L., Tompa, P., and Fuxreiter, M. (2018). Protein Phase Separation: A New Phase in Cell Biology. Trends Cell Biol. **28**: 420–435.

Bose, M., Lampe, M., Mahamid, J., and Ephrussi, A. (2022). Liquid-to-solid phase transition of oskar ribonucleoprotein granules is essential for their function in Drosophila embryonic

development. Cell **185**: 1308-1324.e23.

Brangwynne, C.P., Tompa, P., and Pappu, R. V (2015). Polymer physics of intracellular phase transitions. Nat. Phys. **11**: 899–904.

Case, L.B., Ditlev, J.A., and Rosen, M.K. (2019a). Regulation of Transmembrane Signaling by Phase Separation. Annu. Rev. Biophys. **48**: 465–494.

Case, L.B., Zhang, X., Ditlev, J.A., and Rosen, M.K. (2019b). Stoichiometry controls activity of phase-separated clusters of actin signaling proteins. Science (80-.). **363**: 1093–1097.

Cheng, X., Ullo, M.F., and Case, L.B. (2022). Reconstitution of Phase-Separated Signaling Clusters and Actin Polymerization on Supported Lipid Bilayers. Front. Cell Dev. Biol. **10**: 1–16.

Cho, K.F., Branon, T.C., Udeshi, N.D., Myers, S.A., Carr, S.A., and Ting, A.Y. (2020). Proximity labeling in mammalian cells with TurboID and split-TurboID. Nat. Protoc.: 1–29.

Choi, J.-M., Holehouse, A.S., and Pappu, R. V. (2020). Physical Principles Underlying the Complex Biology of Intracellular Phase Transitions. Annu. Rev. Biophys. **49**: 107–133.

Cuevas-Velazquez, C.L., Vellosillo, T., Guadalupe, K., Schmidt, H.B., Yu, F., Moses, D., Brophy, J.A.N., Cosio-Acosta, D., Das, A., Wang, L., Jones, A.M., Covarrubias, A.A., Sukenik, S., and Dinneny, J.R. (2021). Intrinsically disordered protein biosensor tracks the physical-chemical effects of osmotic stress on cells. Nat. Commun. **12**.

Day, K.J., Kago, G., Wang, L., Richter, J.B., Hayden, C.C., Lafer, E.M., and Stachowiak, J.C. (2021). Liquid-like protein interactions catalyse assembly of endocytic vesicles. Nat. Cell Biol. **23**: 366–376.

Ditlev, J.A. (2021). Membrane-associated phase separation: organization and function emerge from a two-dimensional milieu. J. Mol. Cell Biol. **13**: 319–324.

Ditlev, J.A., Vega, A.R., Köster, D.V., Su, X., Tani, T., Lakoduk, A.M., Vale, R.D., Mayor, S., Jaqaman, K., and Rosen, M.K. (2019). A composition-dependent molecular clutch between T cell signaling condensates and actin. Elife **8**: 1–44.

van Dop, M., Fiedler, M., Mutte, S., de Keijzer, J., Olijslager, L., Albrecht, C., Liao, C.Y., Janson, M.E., Bienz, M., and Weijers, D. (2020). DIX Domain Polymerization Drives Assembly of Plant Cell Polarity Complexes. Cell **180**: 427-439.e12.

Dorone, Y., Boeynaems, S., Flores, E., Jin, B., Hateley, S., Bossi, F., Lazarus, E., Pennington, J.G., Michiels, E., De Decker, M., Vints, K., Baatsen, P., Bassel, G.W., Otegui, M.S., Holehouse, A.S., Exposito-Alonso, M., Sukenik, S., Gitler, A.D., and Rhee, S.Y. (2021). A prion-like protein regulator of seed germination undergoes hydration-dependent phase separation. Cell **184**: 4284-4298.e27.

Dragwidge, J.M. and van Damme, D. (2020). Visualising endocytosis in plants: past, present, and future. J. Microsc. **0**: 1–7.

Dragwidge, J.M., Wang, Y., Brocard, L., Meyer, A. De, Hudeček, R., Eeckhout, D., Chambaud, C., Pejchar, P., Potocký, M., Vandorpe, M., Bernard, A., Jaeger, G. De, Pleskot, R., Fang, X., and Damme, D. Van (2022). AtEH/Pan1 proteins drive phase separation of the TPLATE complex and clathrin polymerisation during plant endocytosis. bioRxiv: 2022.03.17.484738.

Dubiel, M., De Coninck, T., Osterne, V.J.S., Verbeke, I., Van Damme, D., Smagghe, G., and Van Damme, E.J.M. (2020). The ArathEULS3 lectin ends up in stress granules and can follow an unconventional route for secretion. Int. J. Mol. Sci. **21**: 1659.

Emenecker, R.J., Holehouse, A.S., and Strader, L.C. (2021). Biological Phase Separation and Biomolecular Condensates in Plants. Annu. Rev. Plant Biol. **72**: 1–30.

Emenecker, R.J., Holehouse, A.S., and Strader, L.C. (2020). Emerging Roles for Phase Separation in Plants. Dev. Cell **55**: 69–83.

Fang, X., Wang, L., Ishikawa, R., Li, Y., Fiedler, M., Liu, F., Calder, G., Rowan, B., Weigel, D., Li, P., and Dean, C. (2019). Arabidopsis FLL2 promotes liquid–liquid phase separation of polyadenylation complexes. Nature **569**: 265–269.

Fujioka, Y., Alam, J.M., Noshiro, D., Mouri, K., Ando, T., Okada, Y., May, A.I., Knorr, R.L.,

Suzuki, K., Ohsumi, Y., and Noda, N.N. (2020). Phase separation organizes the site of autophagosome formation. Nature **578**: 301–305.

Gadeyne, A., Sánchez-Rodríguez, C., Vanneste, S., Di Rubbo, S., Zauber, H., Vanneste, K., Van Leene, J., De Winne, N., Eeckhout, D., Persiau, G., Van De Slijke, E., Cannoot, B., Vercruysse, L., Mayers, J.R., Adamowski, M., Kania, U., Ehrlich, M., Schweighofer, A., Ketelaar, T., Maere, S., Bednarek, S.Y., Friml, J., Gevaert, K., Witters, E., Russinova, E., Persson, S., De Jaeger, G., and Van Damme, D. (2014). The TPLATE adaptor complex drives clathrin-mediated endocytosis in plants. Cell **156**: 691–704.

Gammons, M. V, Renko, M., Johnson, C.M., Rutherford, T.J., and Bienz, M. (2016). Wnt signalosome assembly by DEP domain swapping of dishevelled. Mol. Cell **64**: 92–104.

Ganser, L.R. and Myong, S. (2020). Methods to Study Phase-Separated Condensates and the Underlying Molecular Interactions. Trends Biochem. Sci. **45**: 1004–1005.

Gouveia, B., Kim, Y., Shaevitz, J.W., Petry, S., Stone, H.A., and Brangwynne, C.P. (2022). Capillary forces generated by biomolecular condensates. Nature **609**: 255–264.

Gradmann, D. and Robinson, D.G. (1989). Does turgor prevent endocytosis in plant cells? Plant. Cell Environ. **12**: 151–154.

Gronnier, J., Gerbeau-Pissot, P., Germain, V., Mongrand, S., and Simon-Plas, F. (2018). Divide and Rule: Plant Plasma Membrane Organization. Trends Plant Sci. **23**: 899–917.

Grosjean, K., Mongrand, S., Beney, L., Simon-Plas, F., and Gerbeau-Pissot, P. (2015). Differential effect of plant lipids on membrane organization specificities of phytosphingolipids and phytosterols. J. Biol. Chem. **290**: 5810–5825.

Grossmann, G., Krebs, M., Maizel, A., Stahl, Y., Vermeer, J.E.M., and Ott, T. (2018). Green light for quantitative live-cell imaging in plants. J. Cell Sci. **131**: jcs.209270.

Gutierrez-Beltran, E., Elander, P.H., Dalman, K., Dayhoff, G.W., Moschou, P.N., Uversky, V.N., Crespo, J.L., and Bozhkov, P. V (2021). Tudor staphylococcal nuclease is a docking platform for stress granule components and is essential for SnRK1 activation in Arabidopsis. EMBO J. **40**: e105043.

Hirst, J., Schlacht, A., Norcott, J.P., Traynor, D., Bloomfield, G., Antrobus, R., Kay, R.R., Dacks, J.B., and Robinson, M.S. (2014). Characterization of TSET, an ancient and widespread membrane trafficking complex. Elife **3**: 1–18.

Huang, S., Zhu, S., Kumar, P., and MacMicking, J.D. (2021). A phase-separated nuclear GBPL circuit controls immunity in plants. Nature.

Huang, W.Y.C., Alvarez, S., Kondo, Y., Kwang Lee, Y., Chung, J.K., Monatrice Lam, H.Y., Biswas, K.H., Kuriyan, J., and Groves, J.T. (2019). A molecular assembly phase transition and kinetic proofreading modulate Ras activation by SOS. Science (80-.). **363**: 1098–1103.

Ishikawa-Ankerhold, H.C., Ankerhold, R., and Drummen, G.P.C. (2012). Advanced fluorescence microscopy techniques—Frap, Flip, Flap, Fret and flim. Molecules **17**: 4047–4132.

Jaillais, Y. and Ott, T. (2020). The nanoscale organization of the plasma membrane and its importance in signaling - a proteolipid perspective. Plant Physiol. **182**: 1682–1696.

Johnson, A., Dahhan, D.A., Gnyliukh, N., Kaufmann, W.A., Zheden, V., Costanzo, T., Mahou, P., Hrtyan, M., Wang, J., Aguilera-Servin, J., van Damme, D., Beaurepaire, E., Loose, M., Bednarek, S.Y., and Friml, J. (2021). The TPLATE complex mediates membrane bending during plant clathrin–mediated endocytosis. Proc. Natl. Acad. Sci. U. S. A. **118**: 2021.04.26.441441.

Jong, F. De and Munnik, T. (2021). Attracted to membranes: Lipid-binding domains in plants. Plant Physiol. **185**: 707–723.

Kaksonen, M. and Roux, A. (2018). Mechanisms of clathrin-mediated endocytosis. Nat. Rev. Mol. Cell Biol. **19**: 313–326.

Kusumaatmaja, H., May, A.I., Feeney, M., McKenna, J.F., Mizushima, N., Frigerio, L., and Knorr, R.L. (2021a). Wetting of phase-separated droplets on plant vacuole membranes leads to a competition between tonoplast budding and nanotube formation. Proc. Natl. Acad. Sci. U. S. A.

118: 3–5.

Kusumaatmaja, H., May, A.I., and Knorr, R.L. (2021b). Intracellular wetting mediates contacts between liquid compartments and membrane-bound organelles. J. Cell Biol. **220**: 1–10.

Lee, Y., Park, S., Yuan, F., Hayden, C.C., Choi, S.Q., and Stachowiak, J.C. (2022). Lateral compression of lipids drives transbilayer coupling of liquid-like protein condensates. bioRxiv.

Li, P., Banjade, S., Cheng, H.C., Kim, S., Chen, B., Guo, L., Llaguno, M., Hollingsworth, J. V., King, D.S., Banani, S.F., Russo, P.S., Jiang, Q.X., Nixon, B.T., and Rosen, M.K. (2012). Phase transitions in the assembly of multivalent signalling proteins. Nature **483**: 336–340.

Liao, Y.C., Fernandopulle, M.S., Wang, G., Choi, H., Hao, L., Drerup, C.M., Patel, R., Qamar, S., Nixon-Abell, J., Shen, Y., Meadows, W., Vendruscolo, M., Knowles, T.P.J., Nelson, M., Czekalska, M.A., Musteikyte, G., Gachechiladze, M.A., Stephens, C.A., Pasolli, H.A., Forrest, L.R., St George-Hyslop, P., Lippincott-Schwartz, J., and Ward, M.E. (2019). RNA Granules Hitchhike on Lysosomes for Long-Distance Transport, Using Annexin A11 as a Molecular Tether. Cell **179**: 147-164.e20.

Liu, C., Mentzelopoulou, A., Muhammad, A., Volkov, A., Weijers, D., Gutierrez-Beltran, E., and Moschou, P.N. (2023a). An actin remodeling role for Arabidopsis processing bodies revealed by their proximity interactome. EMBO J.: e111885.

Liu, C., Mentzelopoulou, A., Papagavriil, F., Ramachandran, P., Perraki, A., Claus, L., Barg, S., Dörmann, P., Jaillais, Y., Johnen, P., Russinova, E., Gizeli, E., Schaaf, G., and Moschou, P.N. (2023b). Phase transitions of a SEC14-like condensate at Arabidopsis plasma membranes regulate root growth. Biorxiv.

Martiniere, A., Lavagi, I., Nageswaran, G., Rolfe, D.J., Maneta-Peyret, L., Luu, D.-T., Botchway, S.W., Webb, S.E.D., Mongrand, S., Maurel, C., Martin-Fernandez, M.L., Kleine-Vehn, J., Friml, J., Moreau, P., and Runions, J. (2012). Cell wall constrains lateral diffusion of plant plasma-membrane proteins. Proc. Natl. Acad. Sci. **109**: 12805–12810.

Michels, L., Gorelova, V., Harnvanichvech, Y., Borst, J.W., Albada, B., Weijers, D., and Sprakel, J. (2020). Complete microviscosity maps of living plant cells and tissues with a toolbox of targeting mechanoprobes. Proc. Natl. Acad. Sci. U. S. A. **117**: 18110–18118.

Milovanovic, D., Wu, Y., Bian, X., and De Camilli, P. (2018). A liquid phase of synapsin and lipid vesicles. Science (80-.). **361**: 604–607.

Mitrea, D.M., Chandra, B., Ferrolino, M.C., Gibbs, E.B., Tolbert, M., White, M.R., and Kriwacki, R.W. (2018). Methods for Physical Characterization of Phase-Separated Bodies and Membrane-less Organelles. J. Mol. Biol. **430**: 4773–4805.

Muroyama, A. and Bergmann, D. (2019). Plant cell polarity: Creating diversity from inside the box. Annu. Rev. Cell Dev. Biol. **35**: 309–336.

Muzzopappa, F., Hummert, J., Anfossi, M., Tashev, S.A., Herten, D.P., and Erdel, F. (2022). Detecting and quantifying liquid–liquid phase separation in living cells by model-free calibrated half-bleaching. Nat. Commun. **13**: 1–15.

Narasimhan, M., Johnson, A., Prizak, R., Kaufmann, W.A., Tan, S., Casillas-Pérez, B., and Friml, J. (2020). Evolutionarily unique mechanistic framework of clathrin-mediated endocytosis in plants. Elife **9**.

Noack, L.C. and Jaillais, Y. (2017). Precision targeting by phosphoinositides: how PIs direct endomembrane trafficking in plants. Curr. Opin. Plant Biol. **40**: 22–33.

Ouyang, M., Li, X., Zhang, J., Feng, P., Pu, H., Kong, L., Bai, Z., Rong, L., Xu, X., Chi, W., Wang, Q., Chen, F., Lu, C., Shen, J., and Zhang, L. (2020). Liquid-Liquid Phase Transition Drives Intra-chloroplast Cargo Sorting. Cell **180**: 1144-1159.e20.

Parchure, A., Tian, M., Stalder, D., Boyer, C.K., Bearrows, S.C., Rohli, K.E., Zhang, J., Rivera-Molina, F., Ramazanov, B.R., and Mahata, S.K. (2022). Liquid–liquid phase separation facilitates the biogenesis of secretory storage granules. J. Cell Biol. **221**: e202206132.

Pietrosemoli, N., Pancsa, R., and Tompa, P. (2013). Structural Disorder Provides Increased Adaptability for Vesicle Trafficking Pathways. PLoS Comput. Biol. **9**.

Pombo-García, K., Martin-Lemaitre, C., and Honigmann, A. (2022). Wetting of junctional condensates along the apical interface promotes tight junction belt formation. bioRxiv: 2022.12.16.520750.

Powers, S.K., Holehouse, A.S., Korasick, D.A., Schreiber, K.H., Clark, N.M., Jing, H., Emenecker, R., Han, S., Tycksen, E., Hwang, I., Sozzani, R., Jez, J.M., Pappu, R. V., and Strader, L.C. (2019). Nucleo-cytoplasmic Partitioning of ARF Proteins Controls Auxin Responses in Arabidopsis thaliana. Mol. Cell **76**: 177-190.e5.

Pruss, G.J., Nester, E.W., and Vance, V. (2008). Infiltration with Agrobacterium tumefaciens induces host defense and development-dependent responses in the infiltrated zone. Mol. plant-microbe Interact. **21**: 1528–1538.

Rebane, A.A., Ziltener, P., LaMonica, L.C., Bauer, A.H., Zheng, H., López-Montero, I., Pincet, F., Rothman, J.E., and Ernst, A.M. (2019). Liquid–liquid phase separation of the Golgi matrix protein GM130. FEBS Lett.: 1–13.

Schwarz-Romond, T., Metcalfe, C., and Bienz, M. (2007). Dynamic recruitment of axin by Dishevelled protein assemblies. J. Cell Sci. **120**: 2402–2412.

Sezgin, E., Levental, I., Mayor, S., and Eggeling, C. (2017). The mystery of membrane organization: Composition, regulation and roles of lipid rafts. Nat. Rev. Mol. Cell Biol. **18**: 361–374.

Snead, W.T. and Gladfelter, A.S. (2019). The Control Centers of Biomolecular Phase Separation: How Membrane Surfaces, PTMs, and Active Processes Regulate Condensation. Mol. Cell **76**: 295–305.

Snead, W.T., Jalihal, A.P., Gerbich, T.M., Seim, I., Hu, Z., and Gladfelter, A.S. (2022). Membrane surfaces regulate assembly of ribonucleoprotein condensates. Nat. Cell Biol.

Stachowiak, J.C. and Kirchhausen, T. (2022). The beauty of simplicity in membrane biology. Nat. Cell Biol.

Su, X., Ditlev, J.A., Hui, E., Xing, W., Banjade, S., Okrut, J., King, D.S., Taunton, J., Rosen, M.K., and Vale, R.D. (2016). Phase separation of signaling molecules promotes T cell receptor signal transduction. Science (80-.). **352**: 595–599.

Sun, D., Wu, R., Zheng, J., Li, P., and Yu, L. (2018). Polyubiquitin chain-induced p62 phase separation drives autophagic cargo segregation. Cell Res. **28**: 405–415.

Taylor, N.O., Wei, M.T., Stone, H.A., and Brangwynne, C.P. (2019). Quantifying Dynamics in Phase-Separated Condensates Using Fluorescence Recovery after Photobleaching. Biophys. J. **117**: 1285–1300.

Wang, B., Zhang, H., Huai, J., Peng, F., Wu, J., Lin, R., and Fang, X. (2022a). Condensation of SEUSS promotes hyperosmotic stress tolerance in Arabidopsis. Nat. Chem. Biol. **18**: 1361–1369.

Wang, H.-Y., Chan, S.H., Dey, S., Castello-Serrano, I., Rosen, M.K., Ditlev, J.A., Levental, K.R., and Levental, I. (2023). Coupling of protein condensates to ordered lipid domains determines functional membrane organization. Sci. Adv. **9**: eadf6205.

Wang, P., Pleskot, R., Zang, J., Winkler, J., Wang, J., Yperman, K., Zhang, T., Wang, K., Gong, J., Guan, Y., Richardson, C., Duckney, P., Vandorpe, M., Mylle, E., Fiserova, J., Van Damme, D., and Hussey, P.J. (2019). Plant AtEH/Pan1 proteins drive autophagosome formation at ER-PM contact sites with actin and endocytic machinery. Nat. Commun. **10**: 1–16.

Wang, Z., Lou, J., and Zhang, H. (2022b). Essence determines phenomenon: Assaying the material properties of biological condensates. J. Biol. Chem. **298**: 101782.

Yoshida, S., van der Schuren, A., van Dop, M., van Galen, L., Saiga, S., Adibi, M., Möller, B., ten Hove, C.A., Marhavy, P., Smith, R., Friml, J., and Weijers, D. (2019). A SOSEKI-based coordinate system interprets global polarity cues in Arabidopsis. Nat. Plants **5**: 160–166.

Yu, L., Lei, Y., Ma, Y., Liu, M., Zheng, J., Dan, D., and Gao, P. (2021). A comprehensive review of fluorescence correlation spectroscopy. Front. Phys. **9**: 644450.

Yuan, F., Alimohamadi, H., Bakka, B., Trementozzi, A.N., Day, K.J., Fawzi, N.L., Rangamani, P.,

and Stachowiak, J.C. (2021). Membrane bending by protein phase separation. Proc. Natl. Acad. Sci. U. S. A. **118**.

Zacharogianni, M., Gomez, A.A., Veenendaal, T., Smout, J., and Rabouille, C. (2014). A stress assembly that confers cell viability by preserving ERES components during amino-acid starvation. Elife **3**: 1–25.

Zavaliev, R., Mohan, R., Chen, T., and Dong, X. (2020). Formation of NPR1 Condensates Promotes Cell Survival during the Plant Immune Response. Cell **182**: 1093-1108.e18.

Zeng, M., Chen, X., Guan, D., Xu, J., Wu, H., Tong, P., and Zhang, M. (2018). Reconstituted Postsynaptic Density as a Molecular Platform for Understanding Synapse Formation and Plasticity. Cell **174**: 1172-1187.e16.

Zhang, Y., Li, Z., Chen, N., Huang, Y., and Huang, S. (2020). Phase separation of Arabidopsis EMB1579 controls transcription, mRNA splicing, and development. PLoS Biol. **18**: e3000782.

Zhao, Y.G. and Zhang, H. (2020). Phase Separation in Membrane Biology: The Interplay between Membrane-Bound Organelles and Membraneless Condensates. Dev. Cell: 1–15.

Zhu, P., Lister, C., and Dean, C. (2021). Cold-induced Arabidopsis FRIGIDA nuclear condensates for FLC repression. Nature **599**: 657–661.

Zhu, S., Gu, J., Yao, J., Li, Y., Zhang, Z., Xia, W., Wang, Z., Gui, X., Li, L., and Li, D. (2022). Liquid-liquid phase separation of RBGD2/4 is required for heat stress resistance in Arabidopsis. Dev. Cell **57**: 583–597.

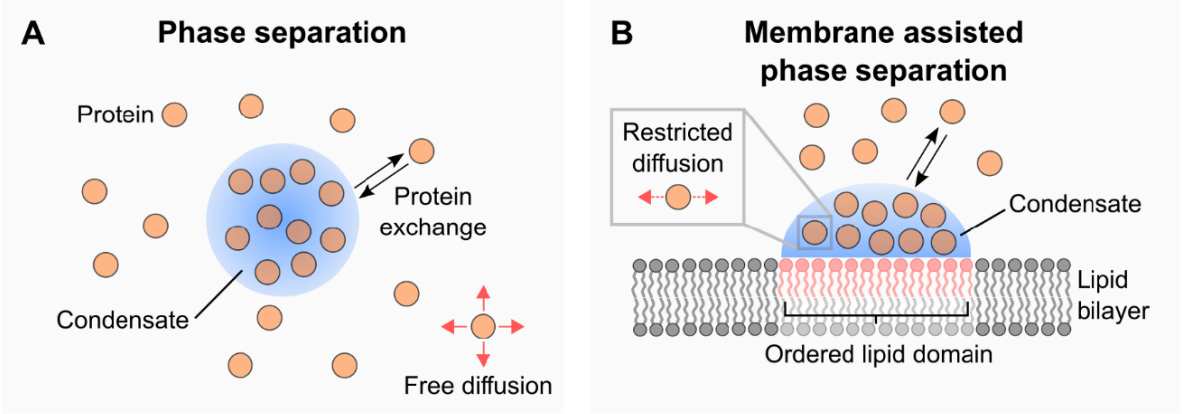

Figure 1. The assembly and properties of condensates are influenced by their surrounding environment. **A)** Condensates can assemble in the cytosol where protein diffusion is not limited. **B)** The binding of proteins to membrane surfaces can limit their diffusion, providing an energetically favorable environment for condensate nucleation. Condensates may preferentially form at ordered membrane domains enriched with specific lipids and/or proteins. The ability of condensates to exchange molecules with their environment is dependent on their material properties, with gel-like or solid-like condensates having reduced exchange compared to liquid-like condensates.

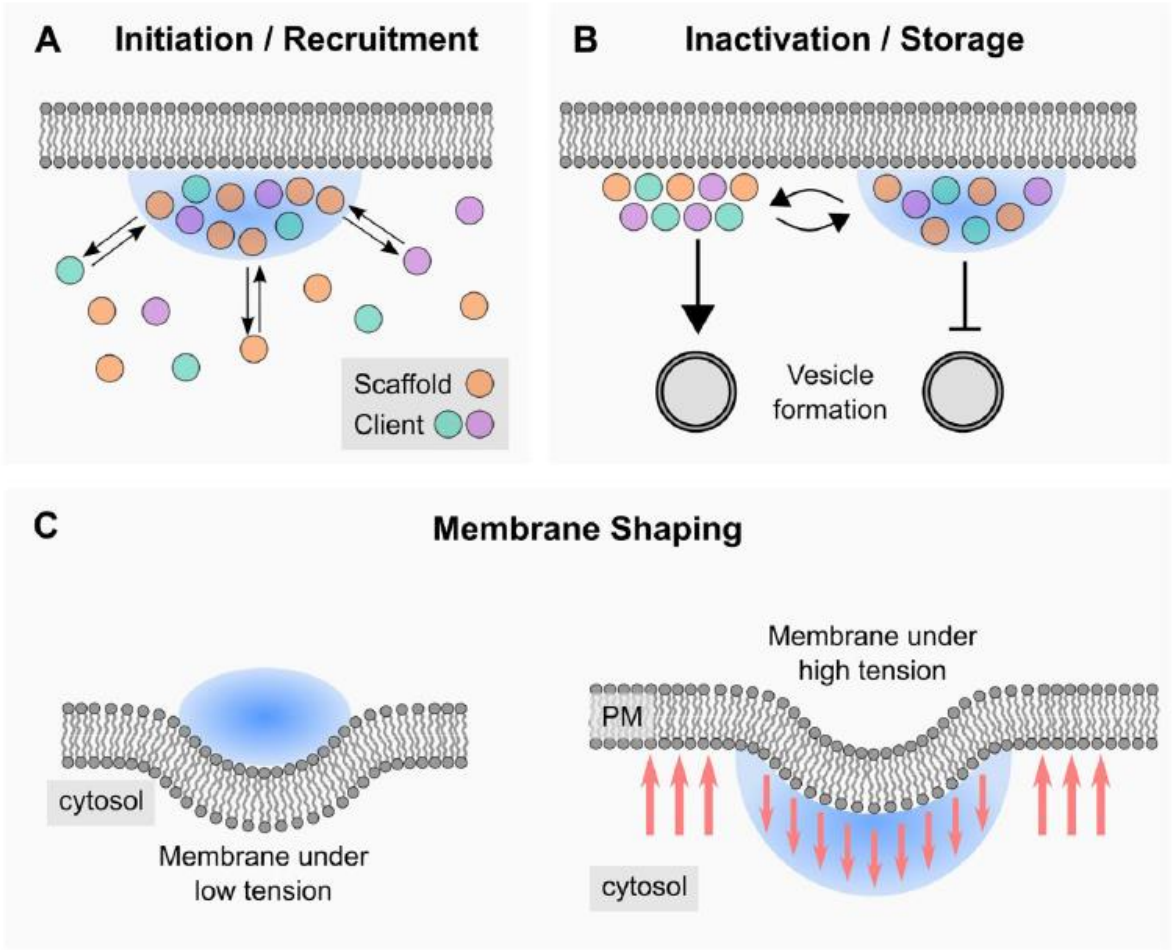

Figure 2. Biological functions of condensates on membranes. **A)** Scaffold proteins actively recruit client proteins from the surrounding environment into condensates, driving the initiation of cellular processes including endocytosis, autophagosome assembly, and tight junction formation. **B)** The assembly of cellular reactions such as vesicle formation can be inhibited by the sequestration of proteins into condensates, inhibiting their function (Zacharogianni et al. 2014). This process is readily reversible (i.e., by phosphorylation). **C)** (Left) Condensates can deform membranes through wetting, for example during the sequestration of membraneless organelles during autophagy (Agudo-Canalejo et al. 2021), and shaping of vacuole membranes (Kusumaatmaja et al. 2021a). (Right) Modeling approaches suggest protein condensates attached to the membrane can induce mechanical stress to deform membranes under high tension such as the plasma membrane (Bergeron-Sandoval et al. 2021). Note that gel-like and solid-like condensates are unlikely to form spherical caps (Kusumaatmaja et al. 2021a).