HHS Public Access

Author manuscript

Curr Opin Cell Biol. Author manuscript; available in PMC 2022 April 01.

Published in final edited form as:

Curr Opin Cell Biol. 2021 April ; 69: 48–54. doi:10.1016/j.ceb.2020.12.006.

Biomolecular condensates in membrane receptor signaling

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Abstract

Clustering is a prominent feature of receptors at the plasma membrane (PM). It plays an important role in signaling. Liquid-liquid phase separation (LLPS) of proteins is emerging as a novel mechanism underlying the observed clustering. Receptors/transmembrane signaling proteins can be core components essential for LLPS (such as LAT or nephrin) or clients enriched at the phase separated condensates (for example at the postsynaptic density or tight junctions). Condensate formation has been shown to regulate signaling in multiple ways, including by increasing protein binding avidity and by modulating the local biochemical environment. Moving forward, it is important to study protein LLPS at the PM of living cells, its interplay with other factors underlying receptor clustering, and its signaling and functional consequences.

Graphical Abstract

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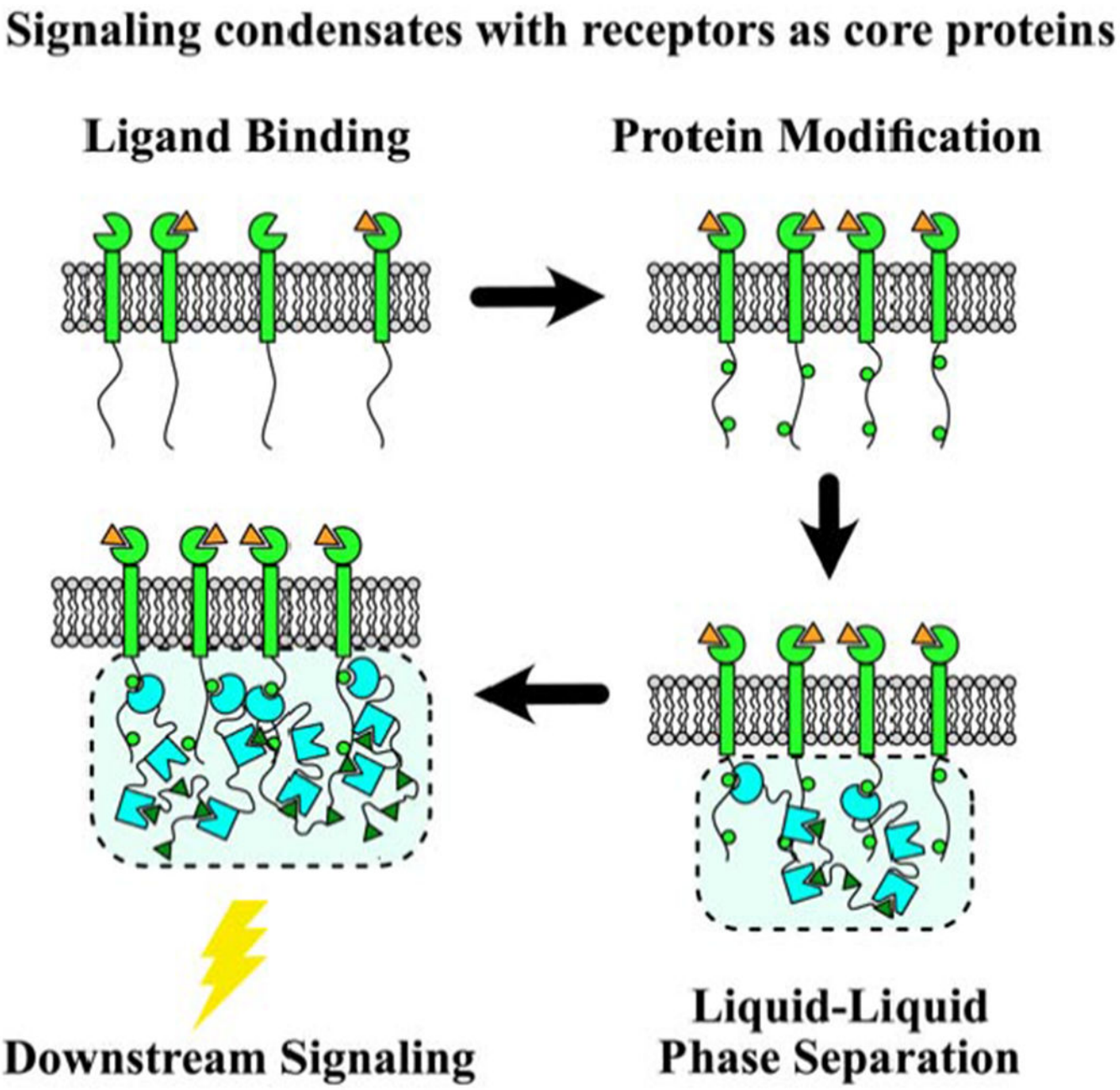
Declaration of interest

None.



Introduction

Clustering is a prominent feature of receptors at the plasma membrane (PM) [1,2]. Multiple mechanisms may lead to receptor clustering, such as dimerization, oligomerization and complex formation, co-compartmentalization within nanodomains, and clustering mediated by the cortical cytoskeleton [3,4]. Recently, mounting evidence indicates that another mechanism is also at play, namely liquid-liquid phase separation (LLPS) of proteins at the PM, where weak multivalent interactions between proteins drive the formation of biomolecular condensates [5]. For many systems shown to undergo LLPS in vitro, similar

mechanisms have been shown to promote cluster formation in cells [6–8]. This indicates that the molecular elements responsible for LLPS play an important role in protein clustering in cells. In this review, we discuss the different classes of condensates at the PM, and what has been discovered thus far of their signaling and functional consequences. We also highlight major open questions in the field, especially in terms of understanding the nature, regulation and functional consequences of LLPS in cells.

Condensates of transmembrane proteins and signaling partners

In one class of PM-associated condensates, LLPS is initiated by phosphorylation (i.e. activation) of transmembrane proteins (Fig. 1A). Two such systems are signaling complexes of the T cell adaptor protein LAT [6,9–11] and the kidney slit diaphragm protein nephrin [8,12–14]. LLPS is driven by multivalent phospho-tyrosine-SH2 domain interactions on the transmembrane proteins and their cytosolic binding partners (Grb2 and Gads for LAT; Nck for nephrin), followed by multivalent SH3 domain-Proline Rich Motif (PRM) interactions with downstream binding partners (Sos1 and SLP-76 for Grb2 and Gads; N-WASP for Nck). LAT condensates comprise a second layer of LLPS, involving phospho-SLP-76, Nck and WASP. In a somewhat analogous manner, the ABC transporter Rv1747 in M. tuberculosis undergoes LLPS, mainly driven by multivalent phospho-threonine-Forkhead Associated (FHA) domain interactions, although in this case both are on the cytosolic tail of Rv1747 [15].

For these systems, stimulation induces phosphorylation of specific residues to generate multiple binding sites to promote LLPS of signaling proteins. This couples upstream signaling to LLPS, which in turn regulates downstream signaling. The dependence on phosphorylation implies that LLPS will be greatly reduced or abolished in the absence of stimulation. Some molecules, e.g. LAT, exhibit low levels of basal phosphorylation [16]; however it is unclear if the phospho-tyrosines that are basally phosphorylated drive LLPS. Therefore, this class of condensates most likely does not underlie the clustering observed for many receptors, potentially with their downstream effectors, prior to stimulation [1,17]. However, such pre-clustering increases the local concentration of the proteins involved in LLPS, and it might enable LLPS upon stimulation, even if the average concentration at the PM is below what is required. For example, both LAT and nephrin (through its binding partner podocin) have an affinity for cholesterol-enriched membrane nanodomains [18–20]. Thus it will be important to investigate how protein and lipid phase separation may cooperatively promote the formation of membrane-associated condensates, where both lipids and proteins promote signaling [21,22].

Many transmembrane receptors, such as receptor tyrosine kinases (RTKs), are phosphorylated in their intracellular domains upon stimulation. This enables binding of multidomain scaffolding proteins, which may promote LLPS. Thus, LLPS might be a widespread feature of receptor activation and signaling [23]. Indeed, for the RTK EphB2, there is evidence that, upon stimulation, it forms small oligomers that then condensate into larger clusters [24]. In this case, cluster condensation serves to terminate signaling. Also, a recent study of FGFR2 suggests that it phase separates into signaling-competent condensates together with Shp2 and PLCγ1, by a process analogous to LAT and nephrin LLPS [25,26].

Because many RTKs consist of analogous domains and motifs, it will be important to determine the role of LLPS in the regulation of RTK signaling and cellular responses to numerous extracellular stimuli.

Condensates of scaffolding proteins that help organize receptors

In another class of PM-associated condensates, the phase separating proteins are scaffolding proteins (in the signaling sense) that organize receptors at the PM, and are thus critical for downstream signaling (Fig. 1B). One such system are proteins involved in the formation of the postsynaptic density (PSD), in particular through MAGUK family proteins (SAP-97, PSD-93, SAP-102 and PSD-95), which then help localize receptors such as AMPAR and NMDAR [27,28]. The receptors in this case are clients in the condensates [29]. With this, the condensates control cellular responses to neurotransmitter binding to receptors, such as actin polymerization [27,28,30] (Fig. 2). Another system are the Zona Occludens (ZO) 1 and 2 proteins, also of the MAGUK family, which drive the formation of tight junction complexes [7,31]. In both systems, LLPS requires a combination of PDZ, SH3 and GuK domains. ZO protein LLPS is reduced by intrinsically disordered region (IDR) phosphorylation [7]. It would be interesting to know whether phosphorylation of MAGUK family proteins in the PSD also inhibits LLPS.

Of note, the concentration needed for spontaneous LLPS of ZO proteins is higher than the concentration of ZO proteins in cells, suggesting that an active process triggers phase separation [7]. To better understand the role of LLPS in tight junction formation, it will be important to determine what processes trigger ZO protein LLPS, and to also elucidate the interplay between ZO protein LLPS and other protein clustering mechanisms at the PM, such as lipid phase separation, as several tight junction proteins have an affinity for cholesterol enriched nanodomains [32].

LLPS also plays an important organizational role in presynaptic boutons, where synapsin LLPS organizes synaptic vesicles [33], and RIM/RIM-BP LLPS organizes Ca^{2+}-voltage gated channels at an active zone condensate [34]. Both Synapsin and RIM/RIM-BP phase separate through IDR-SH3 domain interactions. Recently, the phase separation of *C. elegans* active zone core proteins SYD-2 and ELKS-1 was shown to be essential for proper active zone mixing and composition [35]. Specific IDRs within both SYD-2 and ELKS-1 drove phase separation of these proteins. Because multiple components of presynaptic boutons undergo LLPS and use LLPS to regulate active zone composition, more comprehensive investigations into the functional importance of LLPS in regulating neurotransmitter release will be essential for understanding the mechanisms that regulate neuronal signaling.

Furthermore, LLPS appears to play a role in cell polarity and asymmetry, for example through Par3/Par6 and Numb/Pon condensates at the cell cortex of Drosophila neuroblasts [36,37]. aPKC is a client in Par3/Par6 condensates, yet when it phosphorylates Par3, this leads to condensate dispersal [36]. Numb/Pon LLPS controls Notch degradation to antagonize Notch signaling and promote neuroblast differentiation [37]. The specific mechanisms by which LLPS of these condensates contributes to cell polarity and receptor signaling requires further investigation.

Modular condensates that assist with signaling at the PM

While not at the PM, the small GTPases GIT and PIX, which mediate signaling just downstream of proteins affiliated with focal adhesions, cell junctions and the PSD (Fig. 2), have been shown to phase separate [38]. GIT phase separates on its own, but β-Pix enhances LLPS by binding tightly to GIT and by forming a trimer itself. β-Pix alone does not phase separate. In cells, disruption of GIT/PIX LLPS results in reduced cell migration [38], suggesting that LLPS of these proteins disrupts cellular signal transduction.

Condensates enhance avidity at the molecular level to promote signaling

One prominent consequence of LLPS is that the concentration and proximity of proteins within condensates increases the dwell time (or residence time) of proteins involved in signaling events (Fig. 3). This consequence is consistent with previous predictions from modeling and experimental observations in the context of receptor clustering [3,39]. One consequence of increased dwell time is that it increases the likelihood of activation and signaling. For example, increased dwell time of WASP-family proteins at PM-associated condensates increases their assembly with the Arp2/3 complex. This increases the Arp2/3 complex's actin nucleation activity at nephrin-based condensates (Fig. 3), which is a rate-limiting step in actin assembly [8,40]. This mechanism is likely responsible for enhanced actin polymerization at LAT condensates as well [11]. Importantly, the activity of the Arp2/3 complex is regulated by condensate stoichiometry [8], thus providing a route for the regulation of signaling leading to actin polymerization. These findings explain previous observations made through quantitative live-cell microscopy and computational modeling [41], highlighting the novel biophysical understanding afforded by studying protein clustering in the context of LLPS. As another example, increased dwell time of SOS1 at LAT condensates increases the probability of autoinhibition release to catalyze nucleotide exchange in Ras [9] (Fig. 3). This mechanism enables kinetic proofreading, where long-dwelling SOS1 is activated to a greater degree than short-dwelling SOS1.

Another consequence of increased avidity is that it increases the collective binding affinity of condensate components to cytoskeletal structures, most notably actin filaments (Fig. 3). For example, while Nck and WASP family proteins bind actin only weakly as individual molecules, their collective binding affinity within condensates increases significantly, allowing LAT or nephrin cluster movement driven by actin filament movement [6,13]. This increased collective binding affinity might explain also PSD association with actin [27] and enable the translocation of ZO clusters to tight junctions via actin [7,31]. Further investigations into the molecular-level consequences of LLPS are necessary to determine the mechanisms that enable actin association with these condensates.

Other signaling-related functions of condensates at the PM

Condensates also regulate signaling through their composition. For example, LAT condensates actively segregate proteins in a charge-dependent manner (Fig. 3); positively charged proteins, e.g. the zeta-chain TCR tail and kinase ZAP-70, which promotes LAT phosphorylation and LLPS, are enriched, while negatively charged proteins, e.g. the

phosphatase CD45, which dephosphorylates LAT to prevent LLPS, are excluded [11]. Likewise, reconstituted PSDs can be enriched with CaMKII, a kinase that promotes signaling within the PSD, while excluding gephyrin, a protein that inhibits excitatory synapse formation [42,43]. These data strongly suggest that LLPS of signaling proteins can promote specific signaling pathways by creating unique biochemical environments for these pathways.

Condensates can also change composition, through which they might regulate signaling. For example, LAT condensates lose Nck (and consequently WASP) as they traverse the immunological synapse (IS) [6]. This weakens their binding to the actin cytoskeleton, which aids the condensates in continuing their movement toward the IS center despite the changing nature of the actin cortex [6]. Loss of Nck and WASP is also expected to reduce actin polymerization at LAT condensates [8,40]. Because actin polymerization at LAT condensates promotes PLCγ function [44], it will be interesting to determine whether this signaling function of LAT condensates diminishes with the loss of Nck and WASP.

Condensates may also imbue cell signaling with spatial memory. Using elegant optogenetic manipulations of the RTK FGFR1, Dine et al. [45] showed that, if cells are exposed to a localized stimulus that triggers LLPS, PM-associated condensates will form at the stimulus location, thus depleting monomers from other regions. Because of Ostwald Ripening, larger condensates will grow bigger while smaller condensates lose molecules. In the context of a cell, this leads to asymmetry that can persist even after the asymmetric stimulus is gone. In the case of FGFR1, the authors showed that this leads to asymmetric cytoskeletal responses [45].

Conclusions/outlook

Recent studies have revealed that LLPS of membrane-associated signaling complexes can spatially and biochemically control signaling pathways. Some are triggered by PM protein activation, some contribute to receptor organization as needed for signaling, while others act downstream of PM-associated components. In vitro reconstitution of membrane-associated condensates provides key insights into condensate formation and function. However, in the cellular environment, protein LLPS is more complex [46], occurring in the presence of many other factors that regulate PM-associated protein dynamics and organization. This includes lipid phase separation, as discussed above, as well as the cortical cytoskeleton. The cortical cytoskeleton has a wide range of effects on the PM, from confining molecular movement, to promoting protein clustering, to influencing lipid phase separation [3,47–49]. Therefore, it will be important to determine the interdependence between PM-associated protein LLPS, lipid phase separation, and the cortical cytoskeleton. For this, and for gaining a deeper understanding of the functional and signaling consequences of condensate formation and regulation, quantitative light microscopy experiments combined with mathematical modeling (Box 1) and controlled system manipulation [45,50,51] will be key. Investigating cellular signaling through the lens of phase separation will likely result in many exciting discoveries and provide novel biophysical insight into the mechanisms that control cellular signaling and function.

Acknowledgments

Funding: This work was supported by the National Institutes of Health [grant R35 GM119619] and the UT Southwestern Endowed Scholars program to KJ, and by the Hospital for Sick Children Research Institute to JAD.

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Box 1:

Characterizing PM protein LLPS and signaling in cells

Condensates formed by LLPS are highly dynamic structures. Quantitative light microscopy is ideally suited to probe their properties, regulation, and signaling consequences in cells. Singlemolecule imaging and fluorescence correlation spectroscopy are powerful approaches to probe the behavior and consequences of condensates at the molecular level, whether it be formation properties, movement properties, dwell times or activation times [9–11,52]. While most of the existing quantitative studies of condensates have been in vitro, similar studies should be feasible in cells. However, condensate/cluster behavior in cells is generally more complex than condensate behavior in vitro. For example, LAT condensates can be relatively stationary on a supported lipid bilayer, but they are highly mobile in cells [6]. Therefore, advances in computer vision and computational data analysis will be critical for addressing these questions in cells. Comparing single-molecule behaviors in the context of condensates/clusters in cells to those in vitro will shed light on additional factors that may regulate condensate/cluster formation in cells. In addition, because LLPS is well understood in a polymer chemistry context [53], it is possible in many situations to predict – through analytical means or through mathematical modeling and simulations – the expected behavior of condensates and their constituent molecules [8–10,54,55]. Thus, comparing the expected behaviors to those in cells will reveal the extent to which the behavior of clusters in cells is truly reflective of LLPS [56]. Such comparisons are not limited to the single-molecule level. For example, fluorescence recovery after photobleaching (FRAP) experiments have shown that ZO dynamics at tight junctions are slower than expected for a liquid phase, indicating that additional interactions constrain ZO proteins [7]. As for investigating the signaling consequences of LLPS, activity biosensors that allow the monitoring of molecular activity in living cells with high spatial and temporal resolution will be critical moving forward [57].

- Protein phase separation is a mechanism for plasma membrane receptor clustering
- Membrane proteins can be core phase separating components or enriched clients
- Increased binding avidity at condensates promotes signaling molecule activation
- Phase separation regulates actin nucleation, enzyme activity and RNA processing
- Signaling complexes and pathways may consist of multiple phase separating modules

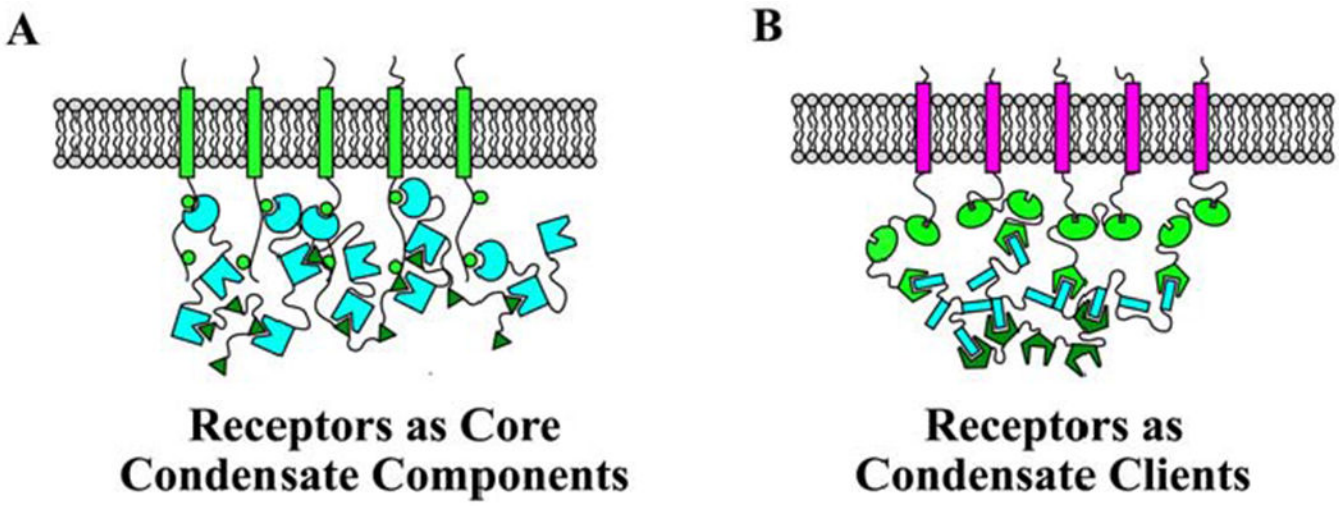

Figure 1. Two classes of phase separated condensates at the PM.

Green to Cyan colors indicate core proteins that are required for LLPS. Magenta colors indicate client proteins that localize to the condensate but are not required for LLPS. **A)** Schematic of a condensate in which receptors/transmembrane signaling proteins are a core component required for LLPS. Examples of such condensates are those initiated by LAT or nephrin phosphorylation. **B)** Schematic of a condensate formed by multivalent interactions between scaffolding proteins, which then organize receptors. In this condensate, receptors are clients that localize to the condensate at the PM, but are not required for condensate formation.

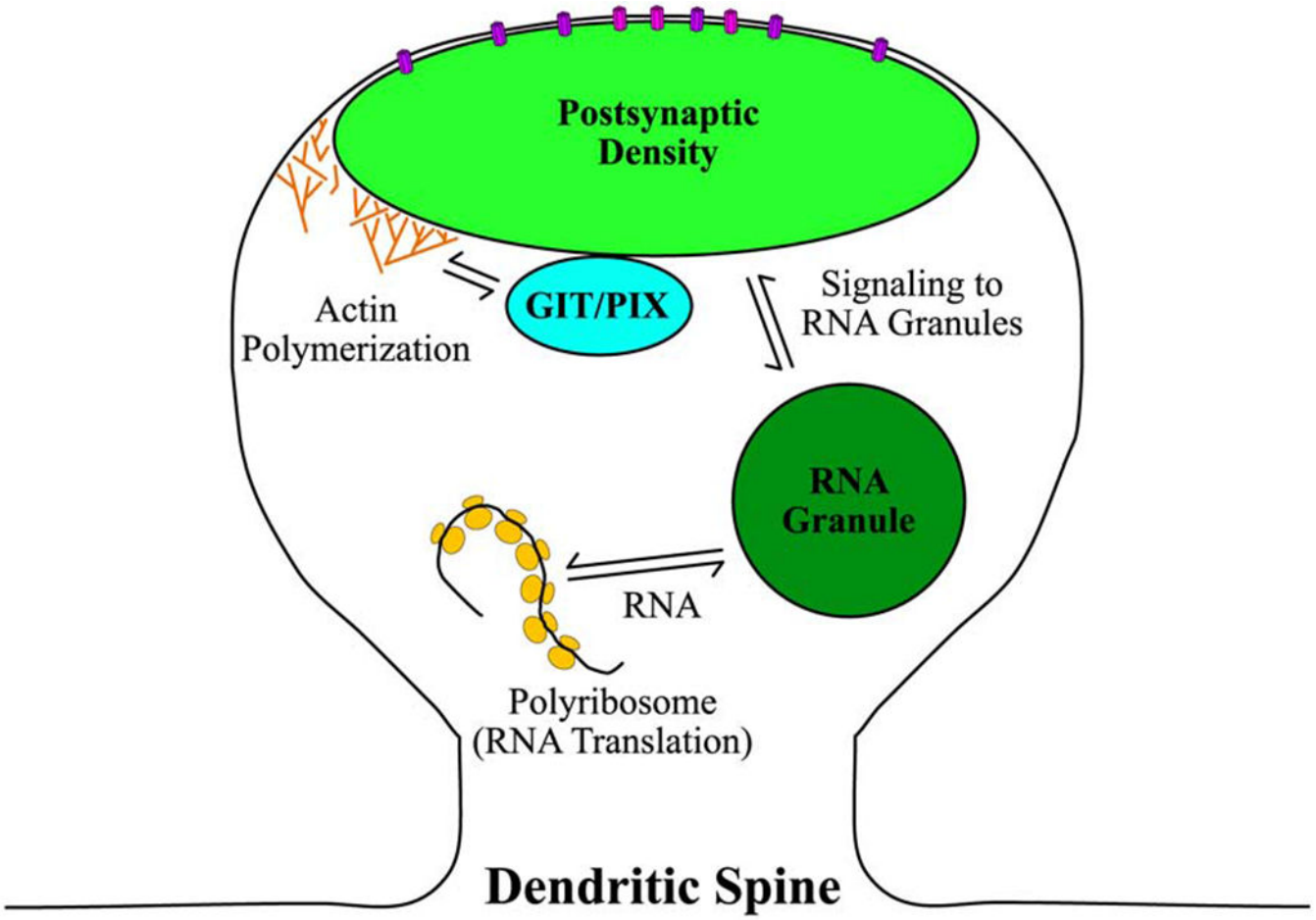

Figure 2. Multiple LLPS modules play a role in signaling in dendritic spines.

The PSD is a protein-rich condensate that resides at the plasma membrane in dendritic spines. Phase separation of core PSD proteins controls NMDAR (magenta) and AMPAR (violet) localization on the dendritic spine membrane. Binding of neurotransmitters to these receptors initiates signaling pathways to control local actin polymerization and RNA translation. Phase separated GIT/PIX condensates localize in dendritic spines and coordinate with the PSD to control actin polymerization by regulating Rho GTPases [38]. Both GIT/PIX and PSD condensates contain actin regulatory proteins, suggesting that they can simultaneously contribute to controlling local actin polymerization. Signaling in dendritic spines also triggers RNA granules, another condensate that localizes to dendritic spines, to process translationally repressed RNA [58]. The processed RNA can then be translated by polyribosomes in dendritic spines.

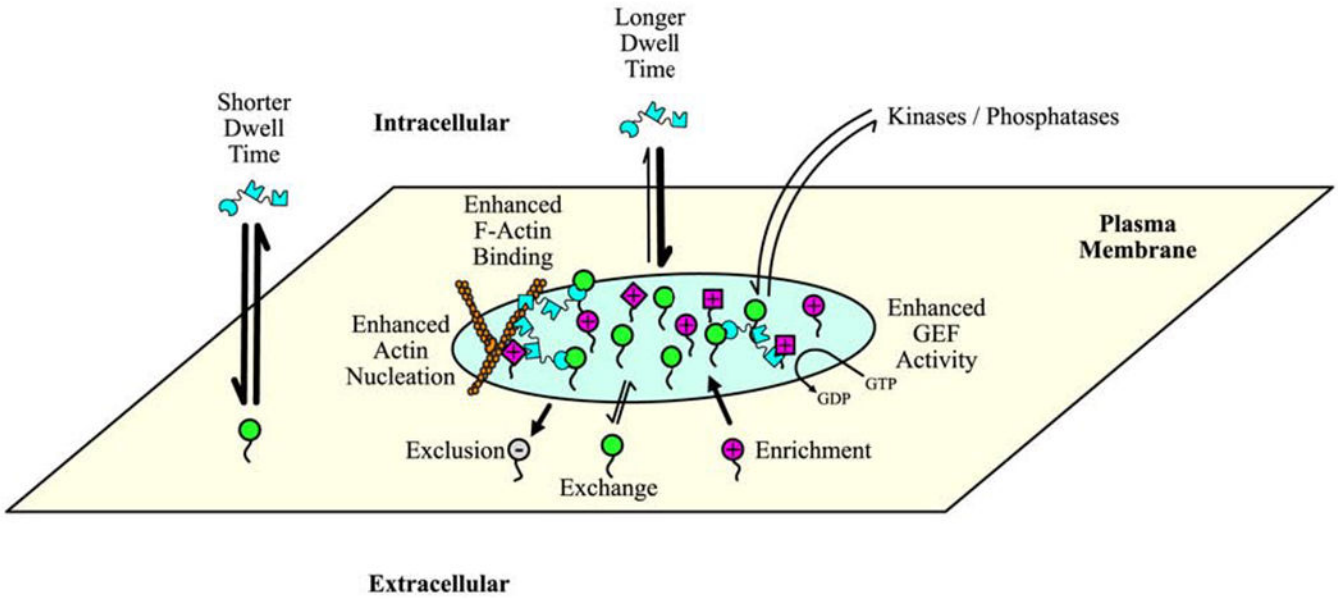

Figure 3. Composition and molecular-level functions of PM-associated condensates.

In this illustration, the condensate (light green) is composed of membrane-associated (green) and soluble (cyan) core proteins, as well as several client proteins (magenta, different shapes represent different proteins) [8,9]. Core proteins can freely exchange with the surrounding membrane or solution. Because of enhanced rebinding, exchange of soluble components is slower in the condensate, which results in increased dwell times when compared to exchange outside of the condensate (rate represented by arrow width). Increased dwell time enhances various functions, such as actin polymerization (illustrated on left side of condensate) and exchange of GTP for GDP on GTPases (illustrated on right side of condensate) [8,9]. Enhanced rebinding also increases the collective binding affinity of condensate components to cytoskeletal structures, such as actin filaments (illustrated on top-left side of condensate) [6]. Condensates can also exclude molecules (gray, (−) in circle on membrane protein) or enrich molecules (magenta, (+) in circle on membrane protein). In some instances, exclusion and enrichment are based on electrostatic repulsion and attraction, respectively [11], while in other instances the mechanism is yet to defined [27]. Other potential drivers of exclusion or enrichment include size and hydrophobicity.