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Phase separation in chemical and mechanical signal transduction

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Abstract

Signal transduction enables cells to sense and respond to chemical and mechanical information in the extracellular environment. Recently, phase separation has emerged as a physical mechanism that can influence the spatial organization of signaling molecules and regulate downstream signaling. Although many molecular components of signaling pathways, including receptors, kinases, and transcription factors, have been observed to undergo phase separation, understanding the functional consequences of their phase separation in signal transduction remains an ongoing area of research. In this review, we will discuss recent studies investigating how cells potentially use phase separation to regulate different signaling pathways by initiating signaling, amplifying signaling, or inhibiting signaling. We will also discuss recent observations that suggest a role for phase separation in mechanosensing in the Hippo pathway and at focal adhesions.

Introduction

Signal transduction enables cells to sense and respond to information in the extracellular environment. Cells sense chemical information, such as secreted peptides, hormones, ions, and growth factors, as well as mechanical information, such as tissue stretch, shear force, surface topology, and substrate stiffness[1]. Recently, phase separation has emerged as a physical mechanism that can influence the spatial organization of signaling molecules and regulate downstream signaling[2,3]. Phase separation occurs when a homogenous mixture of molecules spontaneously de-mixes to form two or more distinct phases[4]. Many biological molecules, including proteins and nucleic acids, can undergo phase separation to form liquid-like droplets that concentrate specific collections of molecules[3,4]. These compartments, termed biomolecular condensates, can be found throughout the kingdoms of life and function to organize cells and potentially regulate diverse cellular processes[3]. Proteins that undergo phase separation often contain multivalent folded domains and/or intrinsically disordered regions (IDRs) that can mediate multivalent, intermolecular

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Xiaohang Cheng: Conceptualization (equal); writing – original draft (equal); writing – review and editing (equal). Lindsay B. Case: Conceptualization (equal); funding acquisition (lead); supervision (lead); writing – original draft (equal); writing – review and editing (equal).

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

interactions[2,3]. Phase separation is thermodynamically driven, and whether a solution undergoes spontaneous phase separation depends on the concentration and identities of the macromolecules as well as environmental conditions such as temperature, salt concentration, and pH[3,5].

Understanding the functional consequences of phase separation in signal transduction remains an ongoing area of research, and there are challenges to rigorously studying the impact of phase separation on signaling *in vivo*[4,6]. For example, phase separation is sensitive to concentration, so experiments caried out with endogenous protein levels are ideal. Recent reviews discuss some challenges and best practices for phase separation experiments in more detail[4,6]. In this review, we will discuss recent studies investigating how cells use phase separation to regulate signaling pathways (Figure 1). We will also discuss recent observations that suggest a role for phase separation in mechanosensing in the Hippo pathway and at focal adhesions.

Phase separation can initiate signaling.

In many cases, fluctuations in environmental conditions can be directly sensed by the phase separation of intracellular molecules[7]. Since phase separation is exquisitely sensitive to factors such as pH, temperature, and crowding[6], the formation or dissolution of phase separated condensates can be a sensitive switch to initiate signaling in diverse physiological contexts.

Plant growth is sensitive to many environmental variables including hydration, temperature, and light, and plant cells use phase separation to initiate signaling and adapt to environmental fluctuations. A prion-like protein FLOE1 found in *Arabidopsis thaliana* embryos phase separates upon hydration, resulting in seed germination[**8]. Mutations that impair FLOE1 phase separation cause inappropriate seed germination in dehydration conditions. The evening complex protein ELF3, a component of the plant circadian clock, undergoes phase separation at high temperatures, leading to a decrease in its activity as a transcriptional repressor[9]. ELF3 phase separation is mediated by its prion-like domain and this domain is required for thermal responsive growth. Photoreceptor phytochrome B (PhyB), the major red/far-red light receptor in plants, undergoes phase separation to form condensates that selectively incorporate signaling components to activate signaling[*10]. Since formation of PhyB condensates is sensitive to both light and temperature, PhyB phase separation enables the integration of light and temperature signals.

Mammalian cells can also use phase separation to initiate signaling. Hypertonic stress causes a rapid decrease in cell volume, leading to an increase in intracellular crowding. In response, cells regulate ion transporters to drive net solute influx, leading to a reclamation of water and regulatory volume increase[**11]. This signaling pathway is initiated by with-no-lysine (WNK) kinases, which form cytoplasmic condensates within seconds of hypertonic stress. WNK kinase phase separation requires the C-terminal IDR and occurs in response to increased crowding, leading to an increase in WNK kinase activity to initiate the regulatory volume increase in response to hyperosmotic stress[11].

In the presence of cytosolic double stranded DNA (dsDNA), cyclic GMP-AMP synthase (cGAS) produces the second messenger cGAMP to activate the ER membrane localized STING protein, culminating in the activation of the innate immune response[12–14]. cGAS-STING signaling is initiated by the presence of double stranded DNA in the cytoplasm. Binding to dsDNA triggers cGAS phase separation, forming condensates that promotes cGAS activation and downstream signaling[12,13]. Thus, phase separation of cGAS may be used to sense viral infection and initiate signaling.

Phase separation can amplify signaling.

In many signaling pathways, an upstream signal leads to the phosphorylation of the cytosolic domain of a transmembrane receptor on multiple tyrosine residues. These phosphorylated receptors can then interact with collections of cytosolic adaptor proteins containing SH2 domains, SH3 domains, and proline rich motifs (PRMs). Multivalency in these molecules can drive their phase separation, leading to the formation of liquid-like signaling condensates on the plasma membrane[3]. For example, the cell-cell adhesion receptor nephrin undergoes multivalent interactions with the adaptor protein Nck (which contains one SH2 and three SH3 domains) and the actin regulatory protein N-WASP (which contains multiple PRMs), leading to the formation of phase separated condensates[15]. Similarly, the linker for activation of T-cells (LAT) receptor undergoes multivalent interactions with the adaptor protein Grb2 (which contains one SH2 and two SH3 domains) and the Ras GEF SOS (which contains multiple PRMs)[16]. More recently, several receptor tyrosine kinases (RTKs), including EGFR, FGFR, and VEGFR, have been observed to undergo phase separation when combined with cytosolic proteins containing multiple SH2 domains[17,18].

In these examples, phase separation requires phosphorylation on multiple tyrosine residues[15,19] and is likely regulated by competition between kinase and phosphatase activities. Specific protein interactions and the emergent chemical properties of condensates have been observed to protect receptors from dephosphorylation. Binding of the SH2 domain of phospholipase PLCγ1 to LAT favors phase separation by protecting a specific tyrosine residue from dephosphorylation[20]. LAT condensates are enriched with negative charge and can exclude negatively charged phosphatases through electrostatic repulsion[19]. Phosphorylated FGFR forms liquid-like condensates in cells and *in vitro* that concentrate both the phosphatase SHP2 and PLCγ1. However, the phosphatase activity of SHP2 is significantly reduced by the formation of condensates *in vitro*. Additionally, FGFR condensates are protected from the activity of nonspecific phosphatases, such as CIP[**18]. Reducing phosphatase activity through these mechanisms may help to stabilize multivalent phosphotyrosine condensates and sustain downstream signaling.

For Nephrin, LAT, EGFR, and FGFR2, phase separation has been observed to upregulate downstream signaling. Phase separation of nephrin promotes downstream actin polymerization by increasing the membrane dwell time of N-WASP and Arp2/3 complex[21]. Phase separation of the LAT receptor and EGFR can promote downstream Ras activation by increasing the membrane dwell time of SOS[16,17,19]. Phase separation of FGFR2 increases FGFR2 kinase activity and the lipolytic activity of PLCγ1[18]. When both kinases and their substrates are localized within engineered synthetic condensates, substrate phosphorylation

significantly increases both *in vitro* and in yeast cells[22]. Additionally, localization to condensates increased phosphorylation of unfavorable substrates that lacked docking motifs or contained non-consensus phospho-acceptor sequences. These results suggest that concentration of kinases and substrates within condensates can promote phosphorylation of sub-optimal substrates and thus expand kinase specificity[22]. In these examples, phase separation of signaling molecules propagates and amplifies the initial signal. In cancer, aberrant phase separation may lead to activation or amplification of signaling pathways to promote tumor growth or metastasis[23–28].

Phase separation can inhibit signaling.

In some pathways, phase separation has been observed to attenuate or inhibit signaling. During c-Gas-STING signaling, excessively produced cGAMP can trigger the formation of STING condensates on the ER that downregulate innate immunity by sequestering STING away from downstream signaling components[*29]. Thus, phase separation of STING dampens signaling, providing a mechanism to prevent overactivation of the innate immune response when pathogenic stimulus is too high. The Wnt/β-catenin signaling pathway regulates tissue homeostasis and cell fate decisions during animal development. In the absence of Wnt ligand, signaling is kept off through the formation of the destruction complex, which targets the transcription factor β-catenin for proteasomal degradation. The destruction complex is a condensate that contains the proteins Axin, APC, GSK3β and CK1α, and formation of the destruction complex requires Axin to undergo phase separation via its IDR. β-catenin is recruited to the destruction complex, where it is phosphorylated[30]. Phosphorylated β-catenin is then recognized by an E3 ligase and rapidly degraded by the proteosome. Ultimately, phase separation of Axin inhibits Wnt signaling by promoting β-catenin degradation in the cytoplasm. Upon Wnt ligand binding to the transmembrane receptor Frizzled, signaling is turned on by the formation of the signalosome at the plasma membrane. This requires recruitment of the cytosolic protein Dishevelled 2 (Dvl2) to the membrane. Additionally, Dvl2 has been observed to undergo phase separation, and mutations in the Dvl2 IDR that impair phase separation also reduce signalosome formation and Wnt signaling[31]. Axin is slowly recruited to the signalosome condensate at the plasma membrane, which disrupts the phase separation of the destruction complex, leading to an increase in cytosolic β-catenin concentrations[31]. The activation of Wnt signaling enables β-catenin to enter the nucleus and regulate transcription.

Phase separation can regulate mechanosensitive signaling.

In addition to sensing chemical signals, cells also sense and respond to mechanical signals in their environment. Recent studies suggest that phase separation may contribute to the regulation of certain cellular mechanosensitive signaling pathways. The Hippo pathway is a kinase cascade that negatively regulates YAP/TAZ, a homologous pair of transcriptional coactivators that promote cell proliferation, survival, and maintenance of stem cell fate. The Hippo pathway enables cells to sense and respond to diverse mechanical stimuli, including cell density, cell area, tissue stretch, shear forces, and substrate stiffness[32,33]. Activation of the Hippo pathway leads to phosphorylation and activation of MST1/2 kinases, which then phosphorylate and activate LATS1/2 kinases, which then phosphorylate YAP/TAZ. Phosphorylated YAP/TAZ is inactive and sequestered in the cytoplasm. Recently, many

components of the Hippo pathway have been observed to undergo phase separation[34,35]. For example, the positive upstream regulators AMOT and KIBRA form condensates that activate Hippo signaling, while the negative upstream regulator SLMAP forms condensates that inhibit Hippo signaling by recruiting MST and its phosphatase. However, these compositionally distinct condensates can coalesce to activate signaling by enriching the kinase cascade and excluding the phosphatase[**34]. In cancer, several non-protein molecules can dysregulate Hippo pathway phase separation and signaling. Excess glycogen in tumors can undergo phase separation, forming condensates that sequester and inhibit MST1/2[**24]. The tumor promoting long non-coding (lnc) RNA SNHG9 can bind to LATS1, which promotes LATS1 phase separation and reduces YAP phosphorylation[*36]. YAP/TAZ can also form liquid-like condensates in the nucleus in direct response to osmotic shock-induced crowding[37]. Together, these recent studies suggest that phase separation may provide a mechanism for cells to sense and integrate numerous signals that converge on Hippo signaling and YAP/TAZ regulated transcription.

Integrin-dependent signaling is another important mechanosensitive pathway in animal cells. Integrins are heterodimeric receptors that mediate adhesion to the extracellular matrix. Integrins cluster and assemble with numerous cytosolic adaptor proteins, signaling molecules, and actin regulatory proteins to form multiprotein adhesion complexes[38]. Mechanical forces are transmitted across integrin receptors, and integrin adhesion complexes play a central role in integrating biochemical and mechanical information within cells[39,40]. Several recent studies provide evidence that phase separation may contribute to the formation, maturation, and turnover of integrin adhesion complexes.

Integrins initially cluster with a subset of cytosolic proteins to form small, diffraction-limited puncta termed nascent adhesions. Several of the cytosolic proteins that localize within nascent adhesions, including phosphorylated p130Cas and focal adhesion kinase (FAK) undergo phase separation at physiological concentrations *in vitro*[**41]. Moreover, the p130Cas- and FAK-dependent pathways act synergistically to promote phase separation, integrin clustering, nascent adhesion formation and partitioning of key components *in vitro* and in cells. Thus, phase separation may provide an intracellular trigger for integrin clustering and nascent adhesion formation. After initial formation, a subset of nascent adhesions are stabilized and undergo a process of force-dependent growth and compositional maturation to form mature focal adhesions that attach to actin stress fibers[38,42]. The adaptor protein LIMD1 is recruited to maturing focal adhesions in a force-dependent manner, likely by a direct interaction with the protein vinculin. Additionally, LIMD1 also undergoes phase separation both *in vitro* and in cells to form droplets that enrich additional adaptor proteins such as Zyxin[**43]. Mutations that disrupt LIMD1 phase separation, such as phosphomimetic point mutations in the IDR, lead to impaired FA dynamics, reduced force transduction, and impaired mechanosensative cell migration. Thus, the force-dependent recruitment of LIMD1 to focal adhesions could trigger its subsequent phase separation to enable the enrichment of specific adaptor proteins, promote focal adhesion maturation, and regulate cellular mechanotransduction. The turnover and disassembly of focal adhesions is regulated by phosphorylation, protease activity, and endocytosis[40]. Recently, the focal adhesion protein tensin was observed to undergo phase separation as focal adhesions disassemble[44].

However, how tensin phase separation is regulated and the functional consequences of tensin condensates remain unclear.

Perspectives

Progress in understanding biological phase separation has been rapid, but many open questions remain. Although we have cited many examples of signaling molecules that can undergo phase separation, in some cases the functional consequences of this phase separation remain unclear, and more rigorous studies would help provide deeper insight into whether phase separation specifically impacts signaling[6]. One exciting approach to assess the functional relevance of phase separation is to explore the selection of phase separation in evolution[8,9]. While the *Arabidopsis* protein ELF3 undergoes temperature sensitive phase separation to regulate growth, plants from hotter climates contain an ELF3 protein that does not exhibit temperature sensitive phase separation and these plants lack thermal responsive growth[9]. Another question is the extent to which the emergent chemical and material properties of condensates have been selected for in evolution[6,8,45,46]. The material properties of condensates could be particularly important in mechanosensitive signaling pathways where mechanical forces are potentially sensed or transmitted at condensates. At membranes, lipids often play an important role in regulating molecular organization and activity. Recent studies have shown that membrane surfaces can promote protein and RNA driven phase separation[47] and that lipid phase separation can be coupled to multivalent protein driven phase separation[**48,49]. Lipid membranes are likely to regulate protein phase separation in diverse signaling pathways, although the feedback between lipid membranes and protein condensates remains under studied[50]. In conclusion, growing evidence suggests that diverse signaling molecules undergo phase separation, and future studies should focus on investigating the functional consequences of phase separation in signal transduction.

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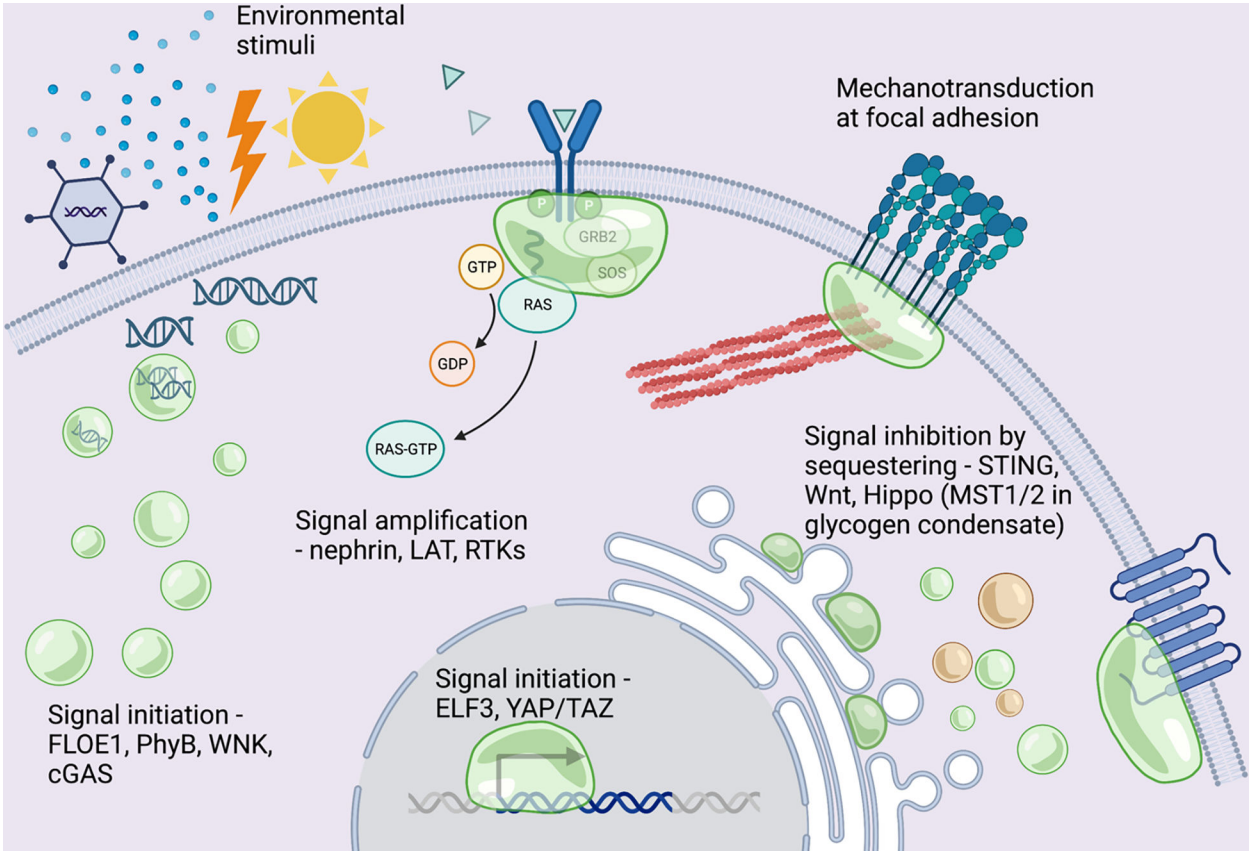

Figure 1.
Potential roles for phase separation in chemical and mechanical signaling pathways. Created with BioRender.