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Zhang, Jason
Mehta, Sohum
Zhang, Jin

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Liquid-liquid phase separation: a principal organizer of the cell's biochemical activity architecture

Jason Z. Zhang[1,2,4], **Sohum Mehta**[1], **Jin Zhang**[1,2,3,*]

[1]Department of Pharmacology, University of California San Diego, La Jolla, CA 92093, USA

[2]Department of Bioengineering, University of California San Diego, La Jolla, CA 92093, USA

[3]Department of Chemistry and Biochemistry, University of California San Diego, La Jolla, CA 92093, USA

[4]Current Address: Department of Biochemistry, University of Washington, Seattle, WA 98195, USA

Abstract

Numerous processes occur simultaneously within the cell for both normal function and in response to changes in the environment. The ability of cells to segregate biochemical reactions into separate compartments is essential for ensuring specificity and efficiency in cellular processes. The discovery of liquid-liquid phase separation as a mechanism of compartmentalization has revised our thinking regarding the intracellular organization of molecular pathways such as signal transduction. Here, we highlight recent studies that advance our understanding of how phase separation impacts the organization of biochemical processes, with a particular focus on the tools used to study the functional impact of phase separation. In addition, we offer some of our perspectives on the pathological consequences of dysregulated phase separation within biochemical pathways.

Compartmentalizing cellular processes

Our environment is ever changing and the cells in our body must dynamically adapt to these changes. A network of dynamic molecules is responsible for orchestrating the appropriate responses by decoding the input signals and passing along the cellular information. Mechanistically, each molecule relays the signaling information downstream by altering the next molecule in the pathway through a cascade of biochemical reactions, a process termed signal transduction. A pressing question in signal transduction research **is one of specificity. Many of the cell's** critical processes are regulated by a few key signaling nodes; thus, how is specificity achieved? One solution, proposed nearly 40 years ago, lies in the idea of compartmentation [1,2]: More than being just a random collection of molecular interactions and biochemical reactions, intracellular signaling networks are organized into distinct compartments embedded in the framework of a living cell and intricately regulated

*Correspondence: jzhang32@ucsd.edu.

Declaration of Interest

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in cellular space and time. Segregating the biochemical machinery into separate cellular domains through compartmentation allows cells to precisely control both the location and timing of biochemical activities being turned on and off, creating a sophisticated and dynamic biochemical activity architecture [3,4]. Such spatiotemporal regulation is essential for achieving specific control of cellular processes.

The typical intracellular compartments that often first come to mind are membrane-bound organelles, which enclose specific biochemical pathways within lipid membranes. In the past decade though [5], the discovery of liquid-liquid phase separation occurring in cells has shifted our understanding of how compartmentation can be achieved via membraneless compartments [6,7]. Liquid-liquid phase separation occurs when a given molecule that is uniformly dispersed in solution spontaneously de-mixes into two phases - a highly concentrated, condensed phase and a dilute phase - upon reaching a critical threshold concentration (Box 1 describes the molecular forces driving phase separation). Excitingly, there has been a recent acceleration in the discovery of phase-separated systems within cells, many of which are involved in signal transduction [8–14]. These phase-separated systems go by many names, including membraneless organelles [15], biomolecular condensates [16], phase-separated bodies [17], liquid droplets [6], granules [18], or assemblies [19], and these are used interchangeably in this review. Given the specialized, liquid-like properties of phase-separated condensates, it is possible that unique biochemical activities can arise by virtue of phase separating key components involved in signaling. Thus, this review will discuss how phase separation contributes to signaling activity architectures and explore the biochemical, physiological, and pathological consequences of such phase separation.

Box 1: Formation and regulation of phase-separated bodies

Phase-separated systems in biology are generally composed of “scaffolds” and “clients” [17]. Scaffold molecules drive phase separation, as they are necessary and sufficient for spontaneous droplet formation *in vitro* and in cells. Client molecules partition into scaffold-driven condensates and can influence the properties of the phase-separated system. Biomolecular phase separation is driven by networks of interactions between polymeric biomolecules, such as proteins and nucleic acids [20–23], and these multivalent interactions regulate the dynamics of phase separation by the scaffold molecule and determine which clients are sequestered into the condensate [16,24,25]. The stable multivalent interactions necessary for phase separation can be mediated by interactions between multiple folded domains or short linear motifs, such as Src homology domain 3 (SH3) modules or proline-rich motifs (PRMs), respectively [16,26]. In addition, phase separation can be driven by weak multivalent interactions from intrinsically disordered regions (IDRs) with multiple interaction motifs, a classic example being interactions between RNA and RNA-binding proteins [27,28]. Borrowing terminology from the polymer field, both folded domains and disordered interaction motifs can be viewed as “stickers” that mediate valency and are separated by “spacers” for flexibility [29].

While it is still hard to predict which proteins can phase separate, finding common features of scaffold proteins and mutagenesis experiments have unveiled several amino acid interactions that contribute to the phase separation of scaffold proteins [27,30–32]. Cation-

TT interactions from positively charged and aromatic residues and cation-cation interactions are necessary and sufficient for phase separation of certain systems [27,33]. Electrostatic forces also regulate phase separation by altering interdomain connections [34,35], and this may explain why some post-translational modifications (PTMs) such as phosphorylation can regulate condensation [36,37]. In addition, IDRs are enriched with glycine (Gly), serine (Ser), and Gln (Gln) residues [27]. While the cation-TT and cation-cation interactions can be considered as stickers in determining the propensity for condensation, the spacer regions within IDRs influence the phase behavior of droplets. Gly residues enhance liquidity of condensates possibly due to Gly enhancing backbone flexibility or altering hydrophobicity, whereas Gln and Ser residues promote hardening [27].

Condensates shape the biochemical activity landscape

The concentration of biomolecules throughout the cell is rarely uniform, in part due to membrane-bound organelles. Phase-separated bodies can also cause inhomogeneous distribution of biomolecules by concentrating these molecules into membraneless compartments. If the biomolecules sequestered into condensates were components of a biochemical pathway, such as an enzyme and its effector/substrate, unique activity dynamics and thus functions may arise due to these compartments. Condensation may enhance molecular interactions/collisions, which in turn will affect the kinetics of biochemical activities within and also influence processes ocurring outside the droplet. Here, we present three examples that highlight the functional impact of phase separation. Along with these examples, we also highlight the innovative tools and methods developed and used to characterize the activity and function of biomolecular condensates.

Dynamic sequestration of key effector molecules in condensates

By sequestering clients, condensates can act as reservoirs. Our recent discovery that a regulatory subunit of the cyclic AMP (cAMP)-dependent protein kinase (PKA), Rlα, undergoes liquid-liquid phase separation and dynamically buffers cAMP demonstrates this principle [38]. Spatial compartmentation of the ubiquitous second messenger cAMP has been central to our understanding of cAMP signaling specificity for more than three decades [2]. However, plausible mechanisms of cAMP compartmentation have been lacking given that cAMP-degrading phosphodiesterases (PDEs) have modest catalytic properties [39,40] and that cAMP diffusion can be fast [41–43], thus raising the question of whether and how cAMP is compartmentalized. Recent studies address these questions by showing that most cAMP is not freely diffusible within cells and is instead dynamically sequestered in Rlα phase-separated bodies [38,40].

Specifically, we used a split-GFP tagging system [44,45] to label endogenously expressed Rlα and observed the formation of dynamic, liquid-like Rlα puncta in live cells, while we also found that purified Rlα formed liquid droplets *in vitro* [38]. The formation of phase-separated Rlα bodies was driven by multivalent interactions through its dimerization/docking domain and intrinsically disordered linker region and closely mirrored intracellular cAMP elevations, with acute activation of cAMP signaling rapidly inducing Rlα phase separation in cells. Interestingly, overexpressing fluorescent protein (FP)-tagged PKA

catalytic subunit (PKAcat) together with Rlα revealed PKAcat co-phase separation into Rlα condensates [38]. These findings prompted us to explore the dynamics of cAMP and PKA signaling within Rlα bodies. To directly measure the cAMP and PKA dynamics inside native condensates in cells, we developed fluorescent sensors targeted to endogenous proteins (FluoSTEPs), a novel platform that combines genetically encoded biosensors with genome editing for straightforward monitoring of signaling activities at endogenously expressed proteins [38,46]. Like other FRET-based biosensors [47], FluoSTEPs utilize a molecular switch to sense a specific biochemical input and control the distance and orientation, and thus FRET, between a pair of FPs. Crucially, however, FluoSTEPs are split into two components: a small fragment of GFP tagged to a protein of interest (POI) via CRISPR/Cas technology, plus the remaining biosensor containing the other GFP fragment, RFP, and molecular switch (Figure 1) [38,46]. When both sensor components are expressed, the GFP fragments spontaneously reconstitute to yield an intact biosensor with a functional, fluorescent FRET donor localized to the POI. This scheme ensures that only the properly targeted biosensor will produce a FRET signal, allowing native signaling dynamics to be monitored around the POI without affecting its endogenous expression. FluoSTEPs are especially useful for investigating POIs that undergo phase separation, as they do not perturb endogenous stoichiometry and expression levels, which dictate droplet formation.

To monitor cAMP levels near endogenously tagged Rlα, we generated a FluoSTEP version of our FRET-based cAMP sensor Indicator of cAMP using Epac (FluoSTEP-ICUE), in which a fragment of the cAMP effector Epac1 is sandwiched between a pair of FPs [48]. Binding of cAMP induces the Epac domain to adopt a more open, extended conformation that translates increases in cAMP levels to changes in FRET efficiency. Stimulating cells expressing FluoSTEP-ICUE tagged to endogenously expressed Rlα revealed a clear increase in cAMP levels detected in diffuse Rlα regions, whereas the puncta-localized sensor showed no FRET change. Further investigation suggested that the FluoSTEP-ICUE FRET response was already fully saturated inside existing Rlα puncta and that newly forming Rlα puncta showed larger increases in cAMP levels compared with diffuse Rlα regions (Figure 1), suggesting that Rlα condensates are cAMP-rich structures that actively sequester cAMP [38]. Strikingly, we observed a similar level of cAMP enrichment *in vitro* when we treated Rlα liquid droplets with a fluorescently labeled cAMP analogue, with 99% of labeled cAMP being sequestered into Rlα droplets [38].

A key feature of the current model of cAMP compartmentation is the ability of PDEs to function as “sinks” that locally deplete cAMP levels. While these PDE-mediated cAMP sinks can be observed experimentally [39,40], mathematical modeling suggests that PDE catalytic activity alone is insufficient to produce these compartments [39]. Given the strong enrichment of cAMP that we observed within Rlα phase-separated bodies, we hypothesized that this behavior limits cAMP levels outside Rlα bodies, thus enabling the formation of nanometer-sized PDE-mediated cAMP sinks [38–40]. We tested this hypothesis by using a cAMP compartmentation assay based on an established cAMP “nanoruler” design [39,40] in which a FRET-based cAMP biosensor is fused directly to a PDE catalytic domain to detect the presence of cAMP within the immediate vicinity of the PDE. Specifically, we fused our cAMP reporter ICUE [48] to the catalytic domain of PDE4 (Figure 1), predicting that cAMP levels around PDE4 should be low when Rlα phase separation is present and

high when Rlα bodies are disrupted, which will be reflected in the PDE-tethered ICUE response. Indeed, when Rlα bodies were either pharmacologically or genetically disrupted, we observed that PDE4 could not keep pace with stimulated cAMP production and lost the ability to locally deplete cAMP to form sinks (Figure 1). Our results suggested that Rlα bodies act as a collective sponge, soaking up cAMP and thus restricting cAMP levels outside condensates to facilitate compartmentalized signaling. This study highlights the capabilities of biomolecular condensates to regulate signaling outside their borders.

Phase transitions mediate non-linear amplification of signaling cascades

Bringing together key components of the same pathway can augment pathway output. This has been seen in various signaling systems such as the Erk pathway, where the Kinase Suppressor of Ras acts as a scaffold in binding to Erk and its direct upstream effectors Raf and MEK [49]. Signaling condensates can also concentrate pathway components, potentially allowing for more diverse signaling dynamics such as non-linear signal amplification, as seen through the clustering behavior of Linker for activation of T-cells (LAT) [50].

The transmembrane protein LAT is an intermediate component of the T-cell receptor (TCR) signaling cascade, connecting upstream TCR activation - such as through antigen presentation - to downstream effects including actin polymerization and Erk signaling, two processes necessary for proper T-cell responses. TCR activation triggers phosphorylation of the LAT cytoplasmic tail, which serves as a scaffold for assembly of the adaptor protein Grb2 and subsequent recruitment and activation of the Ras guanine exchange factor Son of sevenless homolog 1 (Sos1) to signal downstream to the Ras/Raf/MEK/Erk pathway [51]. Assembly of these components into submicron-scale plasma membrane clusters has been known for over a decade to be required for proper TCR signaling, yet the precise mechanistic and functional implications of LAT clustering were unclear [50]. In 2016, Su and colleagues showed using *in vitro* reconstitution that LAT signaling clusters arise through liquid-liquid phase separation driven by multivalent interactions between phosphorylated LAT (pLAT), Grb2, and Sos1 [50]. These liquid-like structures selectively recruit pathway activators and exclude repressors (e.g. phosphatases), yielding a distinct signaling compartment that greatly enhances TCR downstream signaling. Although this phase transition has important biological roles, the mechanisms by which LAT phase separation regulates TCR signaling are unclear. For example, LAT clustering increases Ras activation by 8-fold compared to no clustering, despite increasing Sos1 recruitment by only 2-fold [52]. Therefore, concentration increases alone are not sufficient to explain the non-linear amplification of downstream events.

In a recent study, Huang *et al.* combined biophysical modeling with single-molecule imaging on supported lipid membranes (SLMs) to understand the amplification of downstream TCR signaling by LAT condensation [52]. SLMs are simplified membrane models containing various user-inputted biomolecules such as lipids, carbohydrates, and proteins, whose concentration, stoichiometry, and membrane environment can be precisely controlled [53], making them useful for deciphering the biochemical influence of phase transitions. For their study, Huang *et al.* constructed SLMs containing pLAT, Grb2, Sos1, and GDP-loaded Ras, with recruitment of green-fluorophore-labeled Sos1 by Grb2 and red-fluorophore-labeled

Ras binding domain (RBD) by GTP-bound (i.e. active) Ras serving as readouts for activation of this system (Figure 2). By performing single-particle tracking of Sos1 and RBD fluorescence via total internal reflection fluorescence microscopy, Huang *et al.* observed a temporal distribution of Sos1 recruitment that concomitantly led to different outcomes for RBD binding. Prolonged Sos1 recruitment (> 1 min) led to successful Ras activation and RBD translocation, whereas transient Sos recruitment (< 1 min) was not sufficient for Ras activation.

These kinetic requirements results in delayed, switch-like Sos1 activation, which the authors recapitulated computationally by modeling Sos1 activation as a 2-step process: 1) Sos1 recruitment by Grb2 bound to pLAT, and 2) PIP_2-facilitated release of Sos1 autoinhibition. Further investigation suggested that Sos1 deinhibition is rate limiting and acts as a kinetic-proofreading mechanism to filter out noise from transient Sos1 membrane recruitment [54,55]. Huang *et al.* further tested the role of LAT phase separation in their system by inputting the Sos1 proline-rich domain (PRD), which not only mediates Grb2 binding but also tunes LAT phase separation. PRD addition would be expected to decrease Sos1 activity through competition with full-length Sos1 if Sos1 sequestration in LAT clusters were purely stoichiometric. Instead, inputting the PRD increased Sos1 recruitment by 2-fold and Ras activation by 8-fold, an effect lost at low LAT densities, indicating a striking supra-stoichiometric enhancement caused by LAT phase separation. Interestingly, Sos1 membrane retention was increased 4-fold by LAT phase transitions [52], consistent with multivalent interactions greatly enhancing Sos1 membrane dwell times (Figure 2) [56]. Modeling the probability of Sos1 activation as a function of dwell time (Figure 2) further suggested that Sos1 activation exhibits switch-like behavior past a dwell-time threshold [52], whereby small increases in Sos1 dwell times, such as that achieved via co-phase separation into LAT condensates, promote sharp increases in Sos1 activation. Thus, Huang *et al.* were able to reveal a role for phase-separation in both confining and enhancing signaling activities.

Substrate channeling within liquid-like multi-enzyme assemblies

Controlled condensation of the various substrates and enzymes involved in a complex pathway can dictate the kinetics of biochemical activities, as seen in the previous examples. Moreover, localization of these complex structures with other cellular compartments that play a role in the same pathway can further fine-tune the flux of reactions by directing the flow of molecules between and within compartments. This process is exemplified by the formation of higher-order assemblies, called metabolons, containing enzymes responsible for catalyzing sequential steps in a metabolic pathway, which recently have been revealed to be essential for coordinating multi-step metabolic pathways [57].

The existence of metabolons was first hypothesized over 30 years ago [58,59], and several examples have been identified in connection to various metabolic pathways [57,59,60]. Among the more famous examples is the purinosome, which contains the 6 enzymes that catalyze the 10-step *de novo* purine biosynthesis pathway in eukaryotes (Figure 3) [59,61]. Purinosomes rapidly and reversibly form under purine-depleted conditions [62], depend on multivalent interactions through enzyme oligomerization [63,64], and exhibit dynamic, liquid-like properties [57,65]. These behaviors are strongly suggestive of phase-separated

condensates; however, additional studies are needed to determine whether LLPS indeed drives purinosome formation. These macromolecular granules are essential for dynamically regulating metabolic flux [66] by promoting substrate channeling, whereby clustering enhances transfer of metabolic intermediates between sequential enzymes [57]. Location is a key factor in this process: Purinosomes are often found near the mitochondrial surface [67], and these are thought to represent the enzymatically active pool of purinosomes [68]. Mitochondrial dysregulation can alter purinosome numbers in cells [67], highlighting a clear functional link, and indeed, mitochondrial metabolism generates several key factors required for *de novo* purine biosynthesis, such as Gly and formate (Figure 3) [59]. Thus, spatially linking purinosomes with mitochondria likely promotes substrate channeling by facilitating influx of these necessary materials.

In a recent effort to test the hypothesis of spatiotemporal coordination of enzymatic activity by metabolons, Pareek and colleagues utilized mathematical modeling, isotopic labeling experiments, and mass spectrometry imaging (MSI) to investigate purinosome activity and directly probe metabolon function [68]. The authors cultured HeLa cells in purine-depleted media supplemented with [$^{13}C_3$,^{15}N] Ser, such that mitochondrial conversion of Ser to Gly and formate would introduce isotopic labels into purine biosynthetic intermediates. They further modeled *de novo* purine biosynthesis as a completely diffusive process where each step occurs independently and all intermediates are fully equilibrated in the cytosol. Using this model, they then calculated the expected distribution of isotope-labeled species (isotopomers) that should form in the absence of purinosomes and compared these values with the actual distribution measured using high-resolution LC-MS of HeLa cell extracts. Remarkably, isotope-labeling experiments revealed that the actual isotopomer distribution, as well as the specific enrichment of ^{13}C and ^{15}N isotopes derived from formate and Gly, respectively, differed significantly from what would be expected in the absence of purinosomes and was instead consistent with highly channeled synthesis directed by an active metabolon [68]. Through further testing, the authors ultimately concluded that fully active purinosomes comprise all 9 enzymes responsible for catalyzing the 14-step synthesis of AMP and GMP near the mitochondrial surface, efficiently channeling mitochondrially derived substrates through *de novo* purine biosynthesis to increase pathway flux 7-fold.

Seeking to directly visualize metabolon function *in situ,* Pareek *et al.* then used gas cluster ion beam secondary ion mass spectrometry (GCIB-SIMS) to image purinosome biochemical activity within intact HeLa cells (Figure 3) [68]. GCIB-SIMS is an MSI approach that allows label-free 3D spatial profiling of chemical species within biological specimens [69,70]. In particular, this approach utilizes a primary ion beam composed of clusters of highly charged gas molecules accelerated to high velocity, enabling high-yield molecular desorption with relatively minimal chemical damage (e.g., molecular fragmentation) [69,70], with the resulting secondary ions extracted and analyzed via SIMS (Figure 3). Using a $(CO_2)_n^+$ (n>10000) GCIB focused to a 1-μm spot, the authors scanned frozen, dehydrated HeLa cells to collect mass spectra corresponding to individual 1 μm × 1 μm × 400 nm voxels, close to the estimated spatial dimensions of individual purinosomes [59]. Isotopic labeling with [^{15}N]Ser was used to promote enrichment of labeled metabolites within mitochondrially associated purinosomes, and the MS peak corresponding to the purine biosynthetic intermediate 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) was

used as a specific reporter for these active metabolons. GCIB-SIMS imaging revealed a striking heterogeneity in the distribution of ^{15}N-labeled AICAR, with specific voxels showing 300- to 1000fold AICAR enrichment versus that predicted for a uniform cytosolic distribution [68]. Further analysis showed that these AICAR hotspots were also enriched in labeled ATP, a pathway end product. These results highlight the utility of GCIB-SIMS to map out the molecular components of micron-sized compartments and provide evidence that purinosomes are active, substrate-channeling metabolons [68].

By selectively recruiting and localizing pathway components to functionally related compartments, these liquid-like multi-enzyme assemblies can thus coordinate various molecules and enzymes to organize complex, multi-step processes.

Concluding remarks and future perspectives

It is clear now that membraneless, liquid-like condensates play a major role in driving cellular compartmentation. Concentrating specific biomolecules into these phase-separated bodies allows for unique chemistries and reaction kinetics within these microdomains. When applied to biochemical machinery, condensation not only allows for unique pathway dynamics but also enables specific cellular processes that are critical, as their disruption leads to pathological consequences [8,38,71]. This shift in our paradigm of cellular compartmentation encourages us to think about new possibilities for spatiotemporal regulation of biochemical activity, which is fundamental to our basic understanding of how pathway specificity is achieved.

What happens, then, when phase separation behavior becomes dysfunctional? Emerging evidence suggests that protein aggregates found in various neurodegenerative diseases are phase-separated bodies [72] that become more gel-like during disease progression [35,73], thus providing new insights into decades-old questions regarding the origins of these pathogenic structures in patients [74,75]. Dysregulation of phase separation is also seen in other diseases such as cancer [7,76]. In the case of prostate cancer, mutations in the tumor suppressor and component of an E3 ubiquitin ligase, speckle-type POZ protein (SPOP) inhibit its interaction with substrates, thus blocking proper ubiquitination of various signaling cascade effectors, disrupting SPOP nuclear condensation and enhancing cell proliferation [77,78]. In breast cancer tissues, increased TAZ expression and more nuclear TAZ condensates compared to healthy breast tissue may serve as a potential biomarker [79]. In another example, the fusion of DnaJB1 exon 1 with the last 9 exons of PKAcat (DnaJB1-PKAcat) is exclusively detected in patients with fibrolamellar carcinoma (FLC) [80], a rare liver cancer that has little similarity with other liver cancers [81]. While it is clear that this chimeric fusion enzyme drives FLC, as it is sufficient to induce FLC-like tumors in mice [82], biochemical and structural studies show little difference between DnaJB1-PKAcat and wildtype PKAcat in terms of either activity or regulation [83–85]. Recent work has offered new insight into the oncogenic mechanisms of this fusion oncoprotein by revealing that DnaJB1-PKAcat disrupts RIα phase separation by recruiting Hsp70 and disrupting PKAcat myristoylation [38]. Furthermore, DnaJB1-PKAcat expression induces loss of cAMP compartmentation, while the loss of RIα phase separation

alone is sufficient to promote increased cell proliferation and transformation, suggesting that Rlα phase separation has tumor-suppressive roles.

In this review, we have highlighted three modes by which phase separation can shape the biochemical landscape, but there are many other ways that condensation can play a role in signal transduction. Uncovering more phase-separating systems and dissecting their functional effects of via new tools and methods [28,86–88] will be crucial for advancing our general understanding of how these membraneless compartments help construct biochemical architectures (see Outstanding Questions). Furthermore, the functional specificity imparted by these non-membrane-bound compartments presents a pharmacologically attractive opportunity [89—91] to therapeutically target specific cellular processes. Indeed, recent efforts have sought pharmacological agents that can disrupt phase-separated bodies [92,93], with the ultimate goal of developing small molecules, peptides, or antibodies that can influence the behavior of select phase-separated bodies [94]. Advancing our understanding of phase separation and developing new biomolecular tools and methods to target these compartments will push the boundaries for therapeutics and drug delivery in treating diseases.

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Outstanding Questions:

- What are the guiding principles for determining what is included and excluded from phase-separated bodies?
- How general is phase separation as an organizer for proper signal transduction?
- Are there other disease-related mutations that can alter the behavior of phase-separated systems?
- To characterize the functional impact of condensation, can tools be generated to induce, inhibit, or alter the material properties of specific phase-separated systems?
- Is specific targeting of pathological phase separation a viable therapeutic avenue?

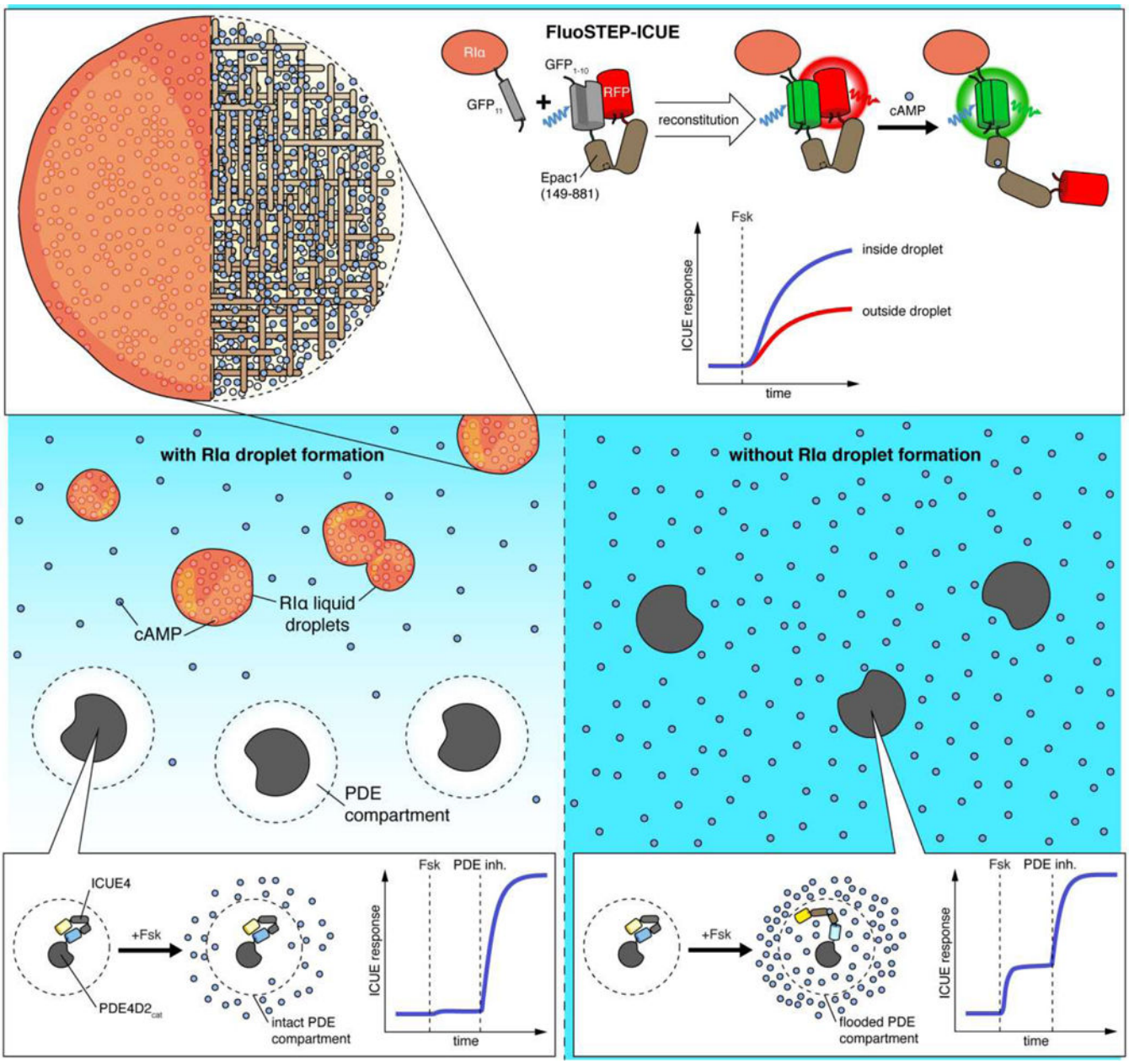

Figure 1. Fluorescent biosensors illuminate cAMP buffering and compartmentation by phase-separated PKA RIα.

(Upper panel) Liquid-like condensates of the PKA regulatory subunit RIα function like sponges that dynamically sequester cAMP. Zhang *et al.* were able to monitor this phenomenon directly in living cells using FluoSTEP-ICUE, a FRET-based cAMP indicator targeted to endogenously expressed RIα [38]. FluoSTEP-ICUE uses the first 10 β-strands of GFP ($GFP_{1–10}$) as a partial FRET donor, along with the 11^{th} β-strand (GFP_{11}) inserted into the RIα genomic locus via CRISPR/Cas9. When co-expressed, these two fragments spontaneously reconstitute and yield an intact sensor fused to RIα, enabling visualization of local cAMP dynamics both within and outside RIα condensates. (Middle panel) cAMP sequestration by RIα condensates is essential to reduce the levels of free cAMP in the

cytosol and thus allow cAMP phosphodiesterases (PDEs) to function as cAMP sinks and control cAMP compartmentation. (Lower panel) Zhang *et al.* were able to measure this effect by fusing the cAMP indicator ICUE4 directly to the PDE4D2 catalytic subunit ($PDE4D2_{cat}$).

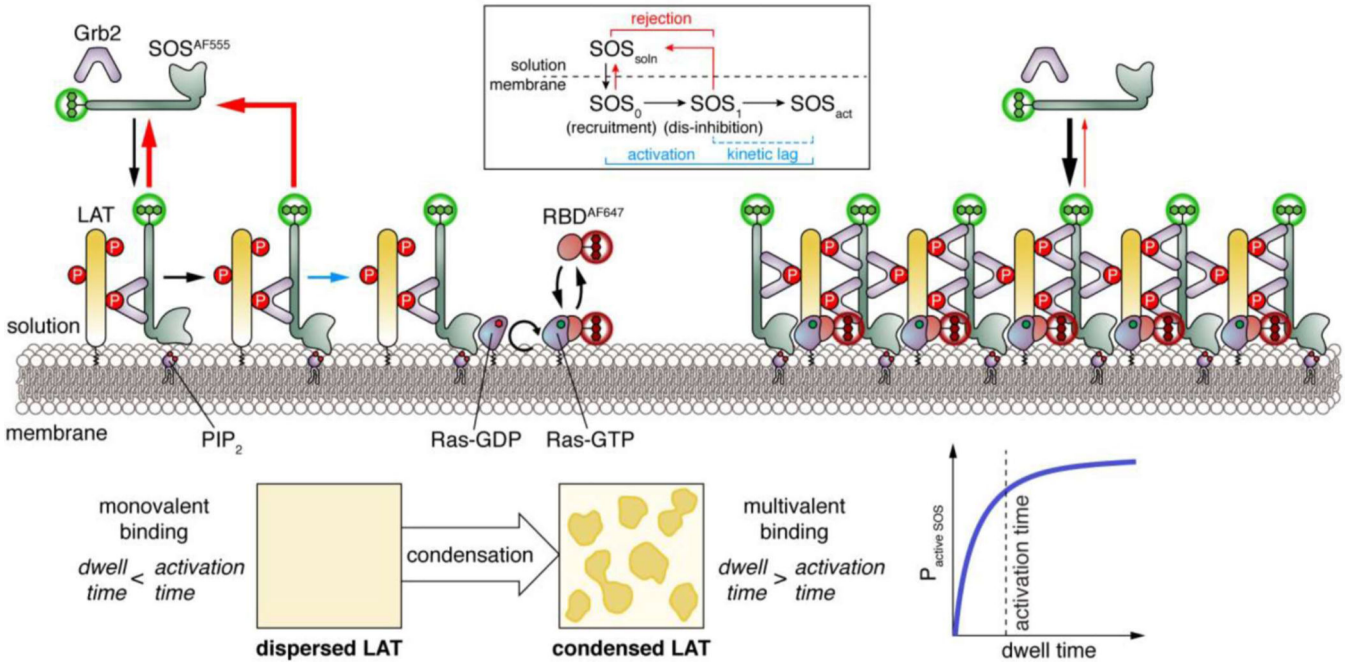

Figure 2. Recapitulating non-linear signal amplification by phase-separated LAT on supported lipid membranes.

Efficient activation of Ras by the guanine exchange factor Son of sevenless (SOS) is required to stimulate ERK signaling downstream of T-cell receptors. To investigate the role of membrane clustering by linker of activated T-cells (LAT), which recruits SOS to the membrane, in SOS activation, Huang *et al.* incubated synthetic membranes with phosphorylated LAT cytoplasmic tail (pLAT), the adaptor protein Grb2, and SOS labeled with AlexaFluor 555 (SOS^{AF555}) and monitored SOS membrane recruitment, as well as Ras activation via recruitment of AlexaFluor 647-labeled Ras binding domain (RBD^{AF647}) [52]. Combined with mathematical modeling, this approach revealed that SOS activation is subject to a kinetic proofreading step such that downstream Ras activation is largely restricted to pLAT phase-separated clusters. Specifically, full SOS activation required both pLAT-mediated recruitment (fast) and PIP_2-mediated dis-inhibition (slow). In the absence of LLPS, monovalent binding yields very short SOS membrane dwell times, favoring dissociation before dis-inhibition can occur (rejection, red arrows). Conversely, LAT condensation promotes multivalent binding that dramatically extends SOS dwell times above the threshold required for activation.

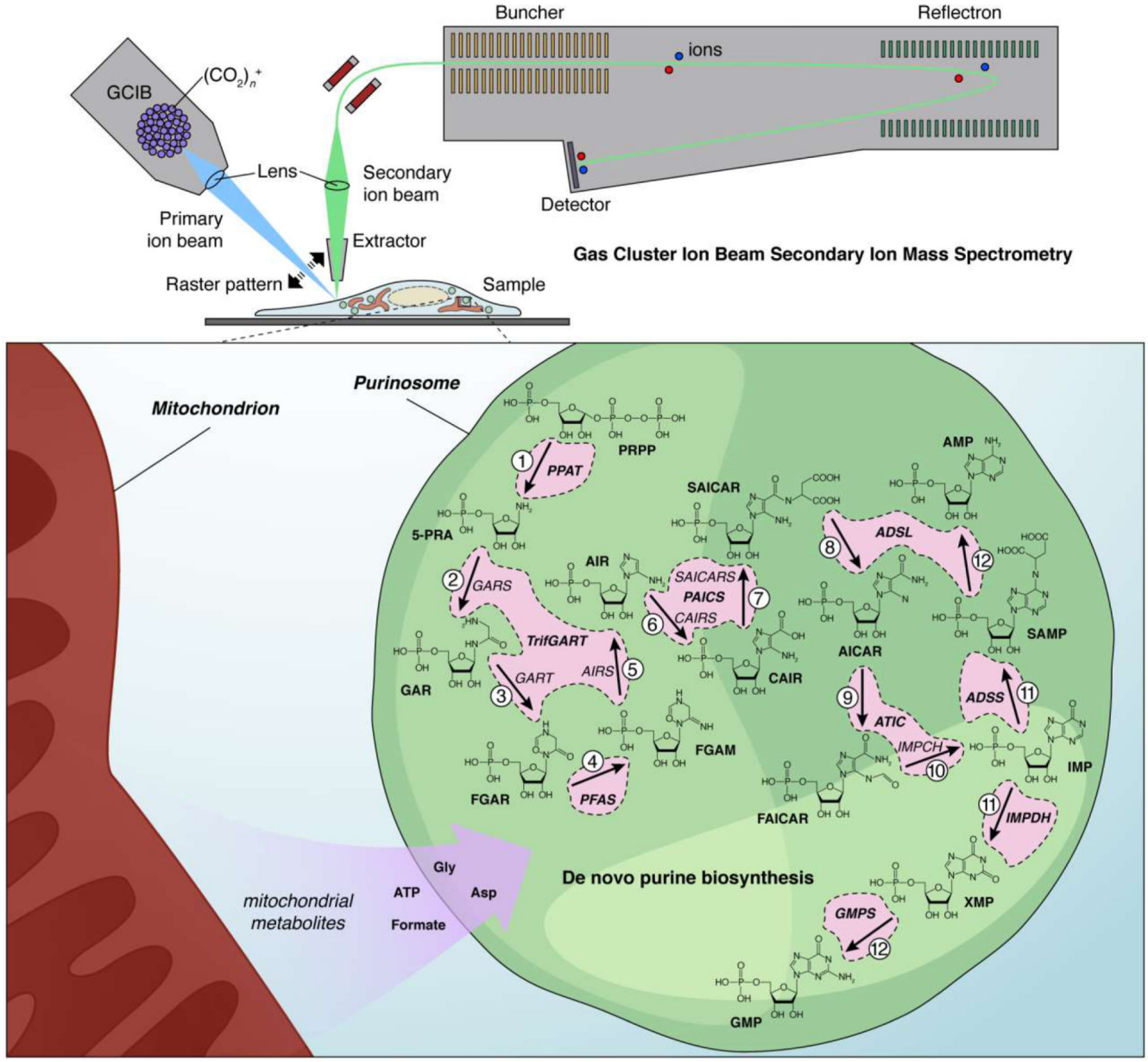

Figure 3. Mass spectrometry imaging reveals substrate channeling by the purinosome, a liquid-like multi-enzyme assembly.

(Upper panel) In gas cluster ion beam secondary ion mass spectrometry (GCIB-SIMS), clusters containing many thousands of ionized gas molecules (e.g., $[CO_2]_n^+$, where n>10,000) are a fired at high velocity (primary ion beam) to liberate molecular ions from the surface of a frozen sample (e.g., a cell). These molecular ions are then extracted and focused into a secondary ion beam that travels into the detector. The primary ion beam is focused into a small spot (e.g., 1 μm × 1 μm × 400 nm) and raster scanned at different depths across the sample to create a 3D "image" where each voxel corresponds to a mass spectrum. (Lower panel) Pareek *et al.* used this technology to directly visualize the substrate-channeling behavior of the purinosome, a multi-enzyme condensate that controls *de novo* purine biosynthesis, in HeLa cells [68]. Based on their studies, the authors were ultimately

able to conclude that active purinosomes comprise all 9 enzymes responsible for catalyzing the 14-step synthesis of AMP and GMP from PRPP in a highly channeled process, insulated from the bulk cytosol, and that localization near mitochondria further allows purinosomes to effectively channel necessary cofactors, produced via mitochondrial metabolism, directly into the *de novo* purine biosynthesis pathway.