Annual Review of Biophysics

Regulation of Transmembrane Signaling by Phase Separation

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Annu. Rev. Biophys. 2019. 48:465–94

First published as a Review in Advance on April 5, 2019

The *Annual Review of Biophysics* is online at biophys.annualreviews.org

https://doi.org/10.1146/annurev-biophys-052118-115534

Keywords

phase separation, receptor organization, cell signaling, receptor clusters, biomolecular condensates

Abstract

Cell surface transmembrane receptors often form nanometer- to micrometer-scale clusters to initiate signal transduction in response to environmental cues. Extracellular ligand oligomerization, domain-domain interactions, and binding to multivalent proteins all contribute to cluster formation. Here we review the current understanding of mechanisms driving cluster formation in a series of representative receptor systems: glycosylated receptors, immune receptors, cell adhesion receptors, Wnt receptors, and receptor tyrosine kinases. We suggest that these clusters share properties of systems that undergo liquid–liquid phase separation and could be investigated in this light.

Contents

INTRODUCTION

Cells are continually exposed to a variety of external signals that are sensed by diverse classes of transmembrane receptors. In response, these receptors initiate intracellular signaling cascades resulting in processes such as cell movement, differentiation, division, and apoptosis. Understanding mechanisms that propagate signals through transmembrane receptors is an important area of research that spans biophysics, biochemistry, cell biology, and organismic biology.

Many transmembrane receptors form dynamic assemblies that promote downstream signal transduction in cells (13, 134, 167). Diverse families of receptors including, but not limited to, receptor tyrosine kinases, cell adhesion receptors, and immune cell receptors undergo transitions from freely diffusing monomers or discrete oligomers to higher-order oligomers of indefinite stoichiometry following exposure to extracellular stimuli (3, 39, 122). In many cases, receptor organization into mesoscale (hundreds of nanometers) clusters, driven by higher-order oligomerization, appears to play an important role in downstream signaling (112, 115, 142, 146).

Recently, liquid–liquid phase separation (LLPS) driven by weak interactions between multivalent molecules was shown to be an important mechanism by which mesoscale structures can form in cells (5, 137). Such structures, termed biomolecular condensates, concentrate specific collections of proteins and nucleic acids without a surrounding membrane. In the cytoplasm and nucleoplasm, LLPS contributes to formation of condensates such as stress granules and the nucleolus. At membranes, LLPS promotes assembly of transmembrane proteins with their cytoplasmic binding partners into clusters (6, 36, 142, 173). LLPS has been observed in signaling pathways emanating from the intracellular domain of nephrin, a transmembrane adhesion molecule required to form the glomerular filtration barrier in kidneys, and from linker for the activation of T cells (LAT), a transmembrane protein required for T cell activation. Both proteins undergo LLPS following phosphorylation of multiple tyrosine residues and subsequent binding by cytoplasmic, multivalent adaptor proteins. Similar to nephrin and LAT, many other transmembrane receptors become

phosphorylated on multiple residues and bind multivalent adaptor proteins following activation (101). Many also interact extracellularly with multivalent ligands. Therefore, LLPS may represent a general cellular mechanism for clustering transmembrane receptors.

In this review, we briefly outline current thinking on the assembly and concomitant LLPS of multivalent proteins. We then discuss the properties and molecular interactions of a series of representative receptors that form clusters upon stimulation: glycosylated receptors, immune receptors, cell adhesion receptors, Wnt receptors, and receptor tyrosine kinases. We suggest that LLPS is potentially an important mechanism driving formation of transmembrane receptor clusters. Phase separation would result in receptor clusters that are organized in dynamic, but discrete, condensed phases that are orders of magnitude larger than the individual molecules and could have distinct biochemical and material properties that facilitate downstream signaling. We conclude with a discussion of future directions that will be useful for understanding how LLPS of both lipids and proteins on membranes might regulate signal transduction and cellular function.

COUPLED OLIGOMERIZATION AND PHASE SEPARATION

Multivalent molecules have long been understood to play an important role in regulating receptor oligomerization (54, 55, 62, 83, 109). Drawing from ideas in polymer chemistry, numerous groups have shown that interactions between multivalent proteins can lead to formation of oligomeric assemblies whose size depends on the concentration of species and whose physical properties depend on the affinity and kinetics of binding and valency of the molecules (55, 83, 98, 106, 165). At high concentrations, multivalent systems can undergo sol–gel transitions when the fractional connectivity between units exceeds a critical threshold, forming infinitely linked, macroscopic polymers (29, 47). To the best of our knowledge, however, until recently, studies of membrane proteins had not considered macroscopic phase separation, producing a density transition that is coupled to the sol–gel connectivity transition (or more generally, to oligomerization) (6, 59, 93, 132, 142). In poor solvent conditions, where molecules interact more strongly with themselves than with solvent, oligomerization decreases the inherent solubility of molecules and promotes macroscopic phase separation (5, 47). Additionally, the notion of a phase-separated compartment leads to the idea that interactions with the phase-separating components can recruit additional molecules across the phase boundary (i.e., cause them to concentrate into the second phase) and generate a local chemical environment that is distinct from the surroundings. Below we describe recent considerations of oligomerization coupled to phase separation, as a prelude to discussion of membrane protein clustering.

A Brief Introduction to Phase Separation

Phase separation occurs as a result of the intrinsic chemical properties of a macromolecule dissolved in a solvent (47). All macromolecules have varying degrees of weak nonspecific interactions with each other and with the surrounding solvent. If interactions between macromolecules are weaker than those with solvent, macromolecules will remain homogenously dissolved in solution. However, if interactions between macromolecules are stronger than those with solvent, a separate dense phase can form (35, 46). At a given temperature, phase separation occurs at the concentration where the favorable energetics of macromolecule–macromolecule interactions are stronger than the unfavorable entropy of demixing. Above this threshold concentration, the macromolecular solution separates into two distinct phases, one dilute and a second more concentrated and denser. For flexible molecules, both phases are typically liquids, and hence the process is termed

LLPS. The relative volumes of the two phases depend on the total macromolecule concentration. Just above the threshold, the dilute phase is much larger. As total macromolecule concentration increases, the concentrations of the dilute and dense phases remain constant, but the volume of the latter grows. When the total macromolecule concentration reaches (or exceeds) that of the dense phase, the solution again returns to homogeneity. The low- and high-concentration thresholds change smoothly as a function of temperature and map out the so-called binodal curve of the system (**Figure 1*a***). Importantly, the phase-separated state has the minimum free energy between the two threshold concentrations, and thus the condensed phase will be maintained indefinitely with no input of energy.

Oligomerization Can Be Coupled to Phase Separation in Multivalent Proteins

A key feature of polymer systems is that interactions between molecules become stronger with increasing size, so the balance between self–self and self–solvent interactions changes, favoring the former. Thus, polymers become less soluble (have lower threshold concentration for phase separation) as they become larger (**Figure 1*a***). The origins of this behavior are entropic, as the entropic penalty for segregating monomers into a second phase (demixing) is lower when those monomers are chemically connected into polymer chains. Analogous behavior has also been shown for folded proteins, including γ-crystallin (2), lysozyme, and albumin (159). These proteins all phase separate at high concentrations as monomers. But phase separation occurs at ∼10-fold to 100-fold lower concentrations when the proteins are crosslinked to dimers, trimers, and higher oligomers.

Oligomerization of multivalent proteins can be viewed in the same light. Multivalency enables formation of oligomeric assemblies, whose size increases with concentration (or, for a given concentration of monomeric units, with valency and affinity) (29, 47). As the size of such an assembly increases, its solubility decreases and phase separation becomes energetically favored (48). Thus, multivalency provides a mechanism to promote LLPS at much lower concentrations than would be possible with monomeric interaction elements. Within the condensed phase, the high concentration promotes further binding between molecules and can produce sol–gel transitions (**Figure 1*b***). In this way, oligomerization and phase separation are energetically coupled, with each favoring the other.

Although oligomerization (and sol–gel transitions) and phase separation are often coupled, it is important to note that they represent distinct physical processes that can be experimentally separated (106). Thus, each can occur without the other. For example, in polymerization of acrylamide gels for SDS-PAGE, molecules undergo a sol–gel transition (i.e., form a system-spanning network) but remain a homogeneous single phase. As described below, sol–gel transitions are often observed between multivalent carbohydrates and proteins, but this does not always coincide with phase separation (106, 165). Conversely, phase separation is often observed for proteins in the absence of a sol–gel transition, for example, with lysozyme (107) and numerous antibodies (120).

Computational and theoretical studies have suggested additional features of assembly and LLPS of cognate pairs of multivalent proteins. Using simulations, Harmon and colleagues predicted that the linkers between modular binding domains strongly influence whether proteins assemble with or without concomitant phase separation (59). Very short linkers favor formation of dimers over large oligomers and therefore inhibit phase separation. But for longer linkers, the chemical properties of the linker determine whether sol–gel transitions occur with or without phase separation. Linkers with favorable interactions with solvent inhibit phase separation, because they are highly extended (corresponding to a large excluded volume), thus leading to

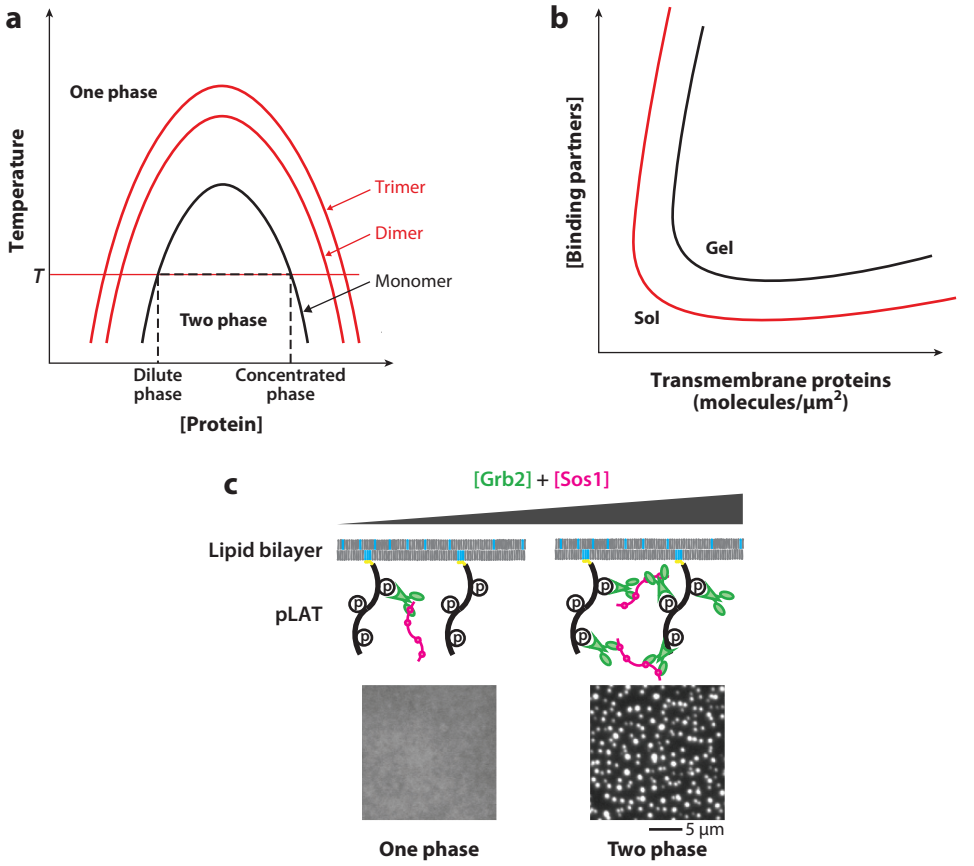


Figure 1

Regulation of sol–gel transitions and phase transitions. (*a*) Oligomerization promotes phase separation. Phase diagram as a function of protein concentration and temperature (binodal curve) for monomeric proteins (*black line*) or oligomers (*red lines*). At low concentrations, molecules exist in a single dilute phase (one-phase regime). For constant temperature below the critical temperature ($T < T_{crit}$), when concentration increases to the solubility threshold, molecules separate into a dilute phase and a concentrated phase (two-phase regime). Within the two-phase regime, as total concentration is increased further, the concentrated phase increases relative to the dilute phase. When total concentration reaches that of the concentrated phase, the system again exists as a single phase. Oligomerization, which can be achieved by chemical crosslinking (2, 159), or analogously by binding of multivalent ligands, shifts the binodal curve upward and outward, potentiating phase separation (*red curves*). (*b*) Sol–gel transitions of highly soluble (*black*) and less soluble (*red*) molecules, as a function of transmembrane protein density and cytosolic binding partner concentration. When molecules are highly soluble, sol–gel transitions require relatively high concentrations. When oligomers have limited solubility, sol–gel transitions can occur at lower concentrations, concomitant with phase separation. (*c*) Representative images showing in vitro phase separation of pLAT, Grb2, and Sos1 on membranes. At low Grb2 and Sos1 concentrations, the proteins exist in one dilute phase, but at higher concentrations, phase separation occurs. The figure shows only the known high-affinity modular interactions among the molecules that promote formation of oligomers. The very-low-affinity interactions of domains and/or linkers that govern solubility, and thus liquid–liquid phase separation, of the molecules and oligomers are not understood and are not shown. Abbreviation: pLAT, phospho-linker for the activation of T cells.

sol–gel transitions in homogeneous solution. In contrast, linkers with unfavorable interactions with solvent will promote phase separation, because they tend to be compact (small excluded volume), thus leading to sol–gel transitions concomitant with LLPS.

Computational modeling has also shown that the relative stoichiometries of component modules can affect the propensity for phase separation (49). The pyrenoid is a carbon-fixing organelle in algae that undergoes LLPS owing to multivalent interactions between Rubisco and EPYC1. Rubisco has eight binding sites for EPYC1, while EPYC1 has four binding sites for Rubisco. Modeling suggests that such systems will exhibit a magic number effect where certain numbers of particles form an unusually stable state. The magic number effect manifests when the valency of one partner is an integral multiple of the valency of the second and the binding sites of the two partners can be saturated. In this situation, LLPS is inhibited when the molecules are at the same module concentration owing to formation of closed oligomers that do not further polymerize. This magic number effect could impact the phase diagram in many biological contexts and is predicted to give rise to unexpectedly sharp phase transitions.

Dimensionality Considerations

In addition to concentration, affinity, valency, pH, and temperature, the dimensionality of a system is an important parameter that determines how a set of macromolecules will phase separate (125). Because the cytoplasm and nucleoplasm are three-dimensional (3D) environments (i.e., all molecules can diffuse in three dimensions), LLPS there produces liquid-like compartments that dynamically exchange components with their surroundings, can undergo fusion and fission, and maintain a spherical shape due to surface tension (75). Because the plasma membrane is a two-dimensional (2D) system (i.e., membrane-associated proteins diffuse in only two dimensions), LLPS of transmembrane receptors forms dynamic liquid-like clusters in the membrane plane (**Figure 1*c***). It remains unknown the degree to which soluble interaction partners assemble in a third dimension (for a given concentration) when they localize to these membrane-associated clusters. Analogous to their 3D counterparts, membrane-associated clusters formed through LLPS are dynamic; can undergo fusion and fission; and are dependent on the concentration, affinity, and valency of the interacting components (6).

In a 2D fluid bilayer, transmembrane receptors exhibit density-dependent multivalency, which is a clear distinction from 3D phase-separated droplets. At low density, the valency of a receptor is dictated only by its molecular structure. But at high densities, the valency of a receptor effectively increases as neighboring molecules can act together in engaging ligands. For example, at low membrane densities, diffusing membrane-attached monovalent proteins have a low affinity for soluble bivalent antibodies, because the probability of two binding events is low (169). However, as the monovalent protein density increases, the apparent affinity for the bivalent antibodies increases. The ability of increased receptor density to effectively increase valency is likely important in LLPS of membrane-associated systems.

Experimentally, we have observed that reducing the dimensionality of in vitro systems from three dimensions to two dimensions reduces the threshold concentration for phase separation as much as 30-fold (6, 93). As a result, if a multivalent cytoplasmic adaptor protein is at a concentration below the threshold for 3D phase separation but above the threshold for membrane-associated phase separation, the latter could be specifically triggered while constitutive phase separation in the cytoplasm is avoided.

In the following sections, we describe specific classes of transmembrane receptors that either undergo LLPS or are strong candidates for LLPS given their known interactions, physical properties, and cellular behaviors.

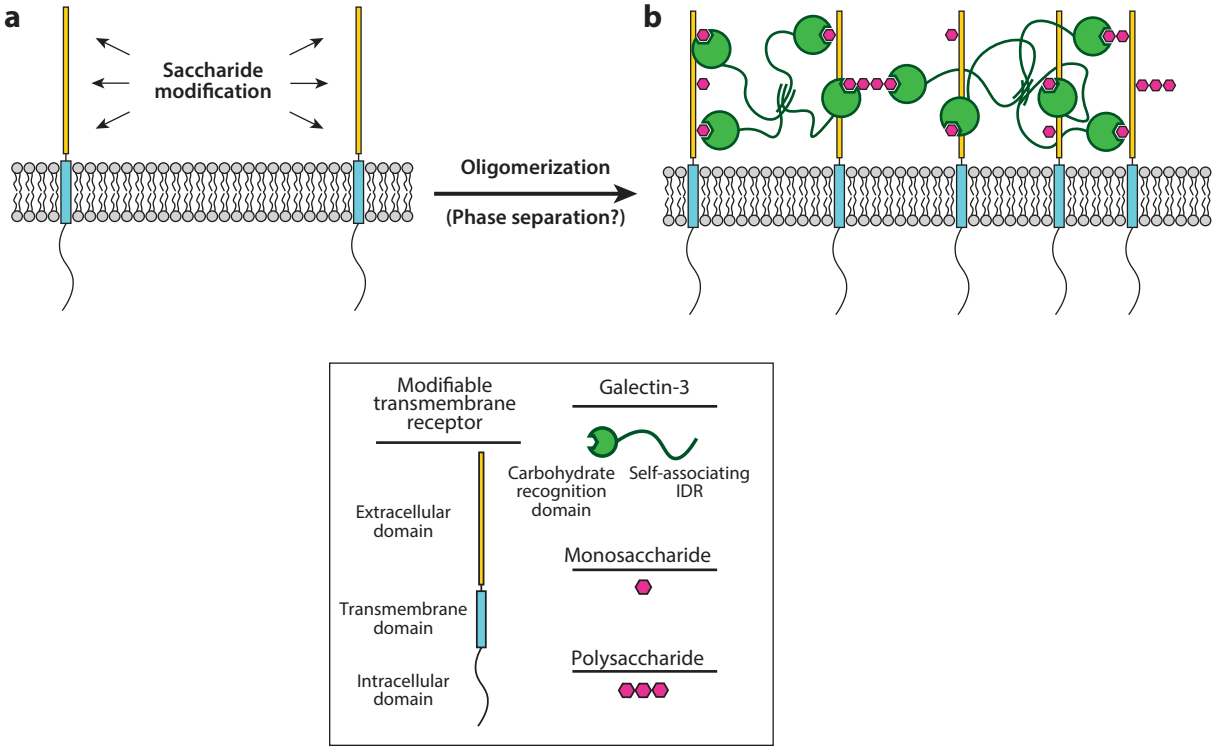


Figure 2

Carbohydrate–lectin interactions in receptor oligomerization. (*a*) Extracellular domain of transmembrane receptors (*yellow*) can be modified with monosaccharides and polysaccharides (*magenta*) to create binding sites for the carbohydrate recognition domain of galectin-3 (*green*). (*b*) Saccharide-modified transmembrane receptors are bound by the carbohydrate recognition domain of galectin-3. The IDR of galectin-3 self-associates with other IDRs of neighboring galectin-3 molecules to form a multivalent network of modified transmembrane receptors and galectin-3. Other lectins are constitutive oligomers and could act analogously to oligomerize glycosylated receptors. The figure shows only the known high-affinity modular interactions among the molecules that promote formation of oligomers. The very-low-affinity interactions of domains and/or linkers that govern solubility, and thus liquid–liquid phase separation, of the molecules and oligomers are not understood and are not shown. Abbreviation: IDR, intrinsically disordered region.

CARBOHYDRATE–LECTIN INTERACTIONS IN RECEPTOR OLIGOMERIZATION

Multivalent interactions between glycosylated cell surface receptors and different classes of lectins cause receptor oligomerization and initiation of downstream signaling (**Figure 2**) (16, 116). Glycoproteins are often modified with multiple saccharides, either as repeating units on a single oligosaccharide or as clustered repeats of the saccharide on the protein backbone (16, 108). Additionally, lectins often contain multiple carbohydrate binding domains or function as constitutive oligomers. Thus, carbohydrate–lectin interactions have the molecular features needed for oligomerization, sol–gel transitions, and potentially LLPS of molecules on the cell surface.

High-valency interactions between polysaccharides and members of the galectin family of lectins often produce ordered cross-linked lattices visible by electron microscopy (16). Galectin-3 is unique in its ability to form heterogeneous disorganized complexes with multivalent carbohydrates (1). When mixed with multivalent carbohydrates in vitro, galectin-3 rapidly precipitates into micrometer-sized irregularly shaped clusters, as determined by solution-based turbidity

assays and visualized by electron microscopy. This behavior is seen over a narrow range of carbohydrate concentrations and may represent a solid phase (1). In cells, galectin-3 organizes transmembrane receptors in clusters and slows their diffusion, consistent with more solid-like material properties (15). Recently, the disordered N-terminal domain of galectin-3 was shown to undergo LLPS through multivalent interactions with other galectin-3 carbohydrate recognition domains (94). Whether other galectins undergo similar phase transitions via multivalent self-association has not, to our knowledge, been investigated.

Concanavalin A (Con A), a tetrameric plant lectin, also oligomerizes and precipitates when mixed with multivalent carbohydrates in vitro (31, 55). Engineered ligands have been used to determine features that promote Con A oligomerization. Linear oligomeric ligands most effectively promote clustering of Con A, and the shape of the ligands is an important factor for determining the rate of oligomerization and density of Con A in clusters (55). Furthermore, properties of the linkers between the saccharides, such as flexibility and length, also affect oligomerization (31, 40).

These multivalent saccharide–lectin interactions share many parallels with the multivalent protein–protein interactions described below. Valency-dependent oligomerization and crystalline organization of lectin–saccharide complexes suggest that glycosylated receptors could undergo sol–gel transitions at the membrane to form a uniform solid-like gel. Indeed, lectin clustering often reduces the local mobility of membrane receptors (15, 108). With the recent discovery that galectin-3 can undergo LLPS, more research is needed to determine whether phase separation plays a role in saccharide–lectin oligomer formation.

IMMUNE CELL RECEPTORS

Clusters of transmembrane receptors on immune cells have been observed for decades. Early studies showed that the receptors in both T cells (162, 170) and B cells (32) formed clusters on membranes in response to external stimuli. More recently, the transmembrane adaptor protein, LAT, was also observed to assemble into clusters following T cell receptor (TCR) activation (18). Below, we discuss how LLPS of LAT promotes downstream signaling and end with a brief discussion about the potential role of LLPS in other immune receptor clusters (**Figure 3**).

LAT is a transmembrane adaptor protein that coordinates most of the proximal signals transmitted downstream of the TCR at the immune synapse [the interface between an immune cell bound to a target cell, e.g., a T cell bound to an antigen presenting cell (APC)] (38). LAT is composed of a short extracellular element, a transmembrane helix, and a disordered intracellular region that contains nine tyrosine residues. Upon activation of the TCR, these tyrosines are phosphorylated by the Syk family kinase ZAP-70 (177). Phosphorylation of the four distal tyrosine residues of LAT is necessary for downstream signaling (4) and LAT cluster formation (**Figure 3**) (19, 142). These phosphotyrosine residues are docking sites for multivalent Src homology 2 (SH2)/SH3 domain-containing proteins, including PLC-γ, Grb2, and Gads (124). The proline-rich motifs (PRMs) of Sos1, a guanine nucleotide exchange factor for Ras GTPase, bind to Grb2 resulting in Ras activation (72, 77). The PRMs of SLP-76, an adaptor protein, bind to Gads, and SLP-76 phosphorylation by Zap-70 results in recruitment of the actin effectors Nck, WASP, and the Arp2/3 complex to polymerize actin filaments (9, 88, 142). Together, these molecules generate the main proximal outputs of TCR activation, stimulation of the MAP kinase cascade (from Ras), calcium release and protein kinase C activation (from PLC-γ), and actin assembly (from the Arp2/3 complex).

Multivalency of the molecules enriched in LAT clusters suggested that their interactions could lead to LLPS and that this process might contribute to TCR signaling. Consistent with this idea,

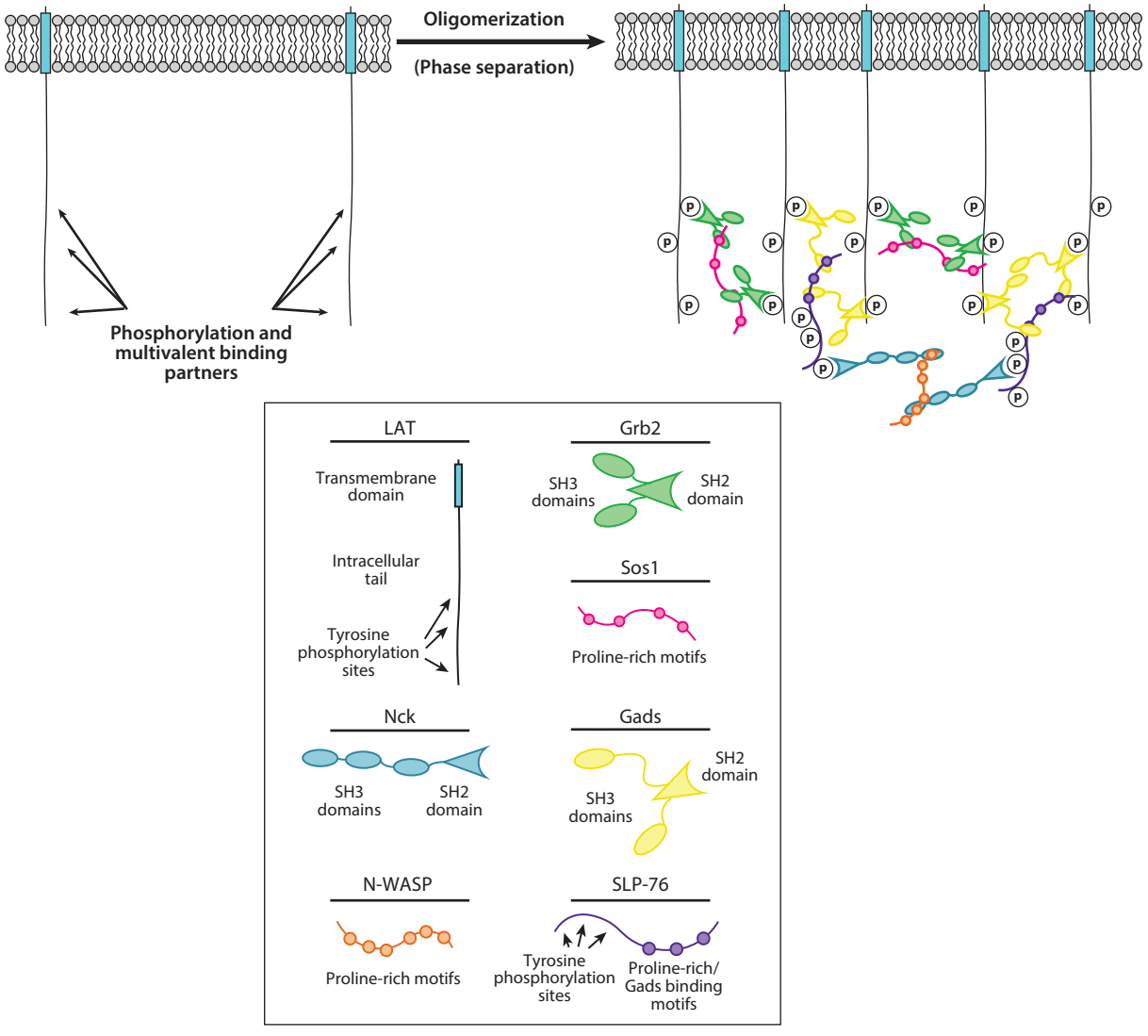


Figure 3

Phase separation of LAT in T cell signaling. (*Left*) LAT transmembrane adaptor proteins can be phosphorylated at three tyrosine residues in the disordered tail to created binding sites for the multivalent adaptor proteins Grb2 (*green*) and Gads (*yellow*). (*Right*) Phosphorylated tyrosine residues on LAT can be bound by the SH2 domain of Grb2. SH3 domains of Grb2 bind to proline-rich motifs of Sos1 (*magenta*) to form phase-separated clusters of LAT, Grb2, and Sos1. Similarly, the SH2 domain of Gads can bind phosphorylated tyrosine residues on LAT. SH3 domains of Gads bind to proline-rich motifs and an RxxK motif in SLP-76 (*purple*) to form a phase-separated cluster of LAT, Gads, and SLP-76. Phosphorylated tyrosine residues on SLP-76 can be bound by the SH2 domain of Nck (*cyan*). SH3 domains of Nck bind to the proline-rich motifs of N-WASP (*orange*) to create another level of multivalent interactions that can contribute to the phase separation of LAT on the membrane of T cells. The figure shows only the known high-affinity modular interactions among the molecules that promote formation of oligomers. The very-low-affinity interactions of domains and/or linkers that govern solubility, and thus liquid–liquid phase separation, of the molecules and oligomers are not understood and are not shown. Abbreviations: LAT, linker for the activation of T cells; SH, Src homology.

clustering of LAT at the immune synapse requires multivalent interactions among LAT, Grb2, and Sos1 (69). Clustering is driven by interactions among phospho-LAT (pLAT), Grb2, and the PRMs of Sos1, independent of Sos1 Ras guanine nucleotide exchange factor activity (85). Recent in vitro reconstitutions have demonstrated that, indeed, multivalent interactions among pLAT, Grb2, and Sos1 promote their LLPS, resulting in the formation of micrometer-scale clusters on membranes (**Figure 1*c***) (72, 142). Multivalent interactions of pLAT either with Grb2 and Sos1 or with Gads and SLP-76 are sufficient to promote this effect.

Su and colleagues biochemically reconstituted the signaling pathway from the ζ-chain of the TCR to the Arp2/3 complex, including formation of phase-separated clusters, and found that the clusters potently assembled actin filaments on the bilayer, similar to observations in activated T cells (88, 142). The authors showed clustering has three functional consequences. First, it increased the specific actin assembly activity of molecules in the pathway, demonstrating that phase separation not only produces changes in spatial organization, but also can alter biochemical activities. Second, the physical properties of phase-separated LAT clusters resulted in the sorting of molecules through electrostatic attraction or repulsion; positively charged proteins concentrated in LAT clusters while negatively charged proteins were excluded, producing a distinct chemical environment from the surrounding membrane. Finally, in vitro phase separation of LAT and its binding partners was correlated with in vivo MAPK signaling in T cells, suggesting that clustering enhances MAPK activation.

A very recent study examined how composition regulates the interaction of LAT clusters with dynamic actomyosin networks at the T cell plasma membrane (36). During activation of Jurkat T cells by α-TCR antibodies attached to a fluid lipid bilayer (mimicking an APC), LAT clusters form at the periphery of the immune synapse and move radially toward its center through the actions of cortical actomyosin. Nck, and presumably its ligand WASP, dissociates from clusters approximately halfway through this trajectory. At this same position, the actin architecture changes from a peripheral branched network to a central network of actin arcs. In biochemical reconstitutions it was shown that Nck and the WASP homolog, N-WASP, contain basic regions that, at high density, act as clutches that couple LAT cluster movement to actomyosin movement. Thus, in cells, the dissociation of Nck/WASP likely weakens the association of LAT clusters with cortical actomyosin and may be necessary to maintain radial movement through the two different actin networks. Consistent with this idea, clusters that constitutively contain a basic clutch deviate from their normal trajectory from periphery to center of the immune synapse. These observations show that cluster composition can be specifically tuned to control interactions with the local environment (in this case, cortical actomyosin), a principle that likely applies to many signaling pathways.

The TCR also forms clusters following engagement with major histocompatibility complex–peptide complex on an APC (37). Super-resolution microscopy shows that the TCR localizes at LAT clusters (42), suggesting TCR clusters associate mostly with LAT clusters. Yet, no concrete evidence exists for a direct, stable molecular connection between the TCR and LAT. However, a recent in vitro study showed that TCR-bound ZAP-70 can semiprocessively phosphorylate LAT on membranes (71). As LAT is phosphorylated, Grb2 may be able to bind its phosphotyrosine residues and drive LLPS before LAT can laterally diffuse away from the TCR–ZAP-70 complex. In this way, semiprocessive phosphorylation of LAT in the vicinity of activated TCRs could explain the colocalization of activated TCR clusters with LAT clusters that is observed in the absence of direct binding of the two proteins. TCR clustering appears to be important for intracellular signaling, as stable ZAP-70 recruitment to the TCR requires TCR clustering; ZAP-70 transiently associates with a single TCR molecule but persistently localizes with TCR clusters (146). Thus, clustering of TCRs might enable signal amplification by kinetic proofreading, a mechanism used

by the T cell to discriminate between agonist and endogenous ligands to ensure that the T cell mounts a specific response to an APC (26, 102).

In addition to LAT and the TCR, many other immune receptors form clusters, some of which can colocalize. For example, during T cell activation, PD-1 and CD28 clusters are mostly colocalized, whereas PD-1 and TCR clusters are only partially colocalized (73). Colocalization of receptor clusters likely has functional consequences. For example, PD-1 clusters recruit the phosphatase Shp2, which preferentially dephosphorylates CD28 over the TCR (73). Thus, T cells can sort clusters to regulate downstream signaling. However, the mechanisms by which PD-1 and CD28 receptors form clusters and how the clusters colocalize are currently unknown. It is likely that other clusters of immune receptors on T cells, B cells, and other types of immune cells form with the same general principles described above for LAT and the TCR. Many of these receptors bind to multivalent adaptor proteins including Grb2, Gads, and Nck (17, 25, 154, 160). Further investigations are needed to understand the role of LLPS in clustering, interactions, and functions of immune cell receptors.

CELL ADHESION RECEPTORS

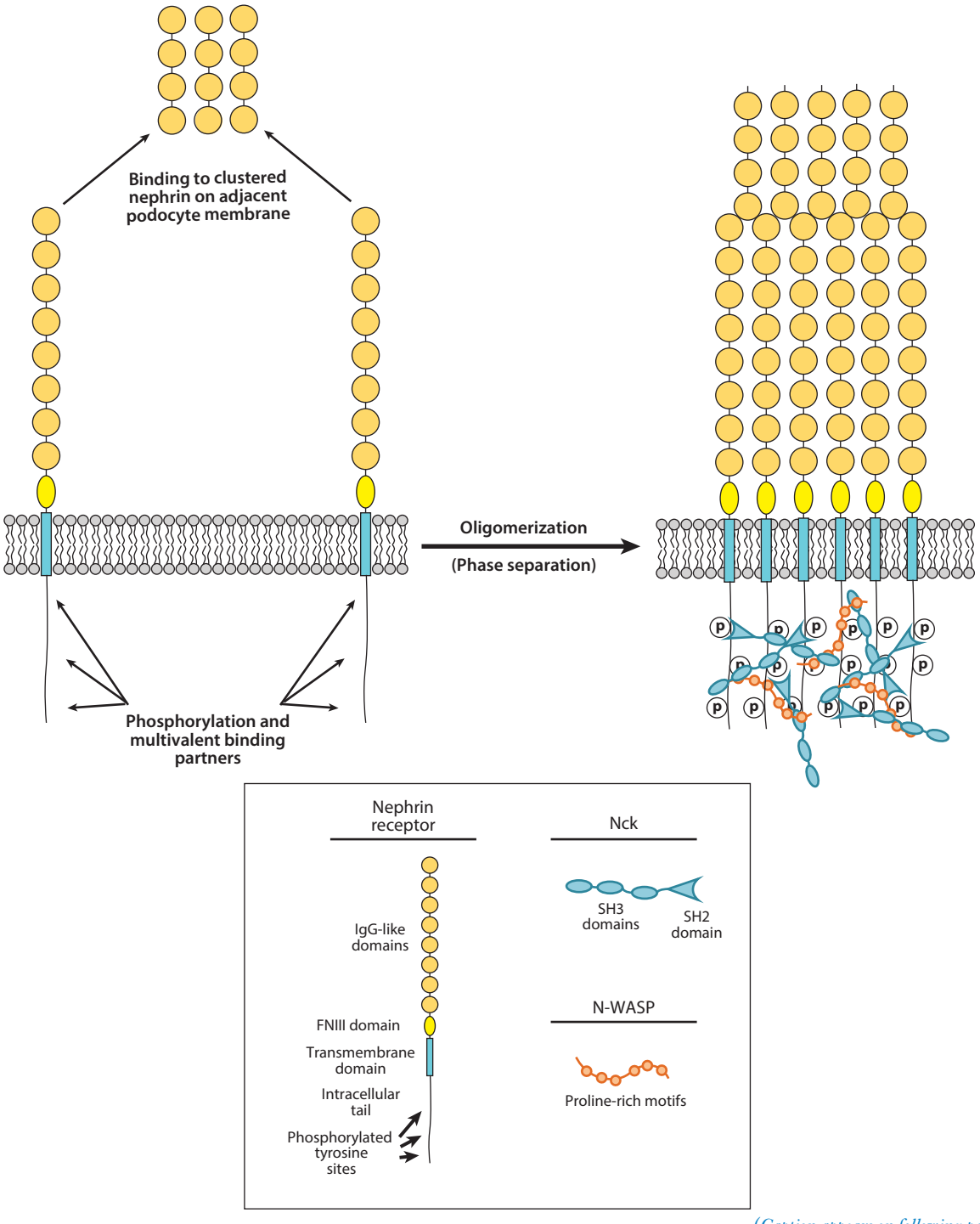
In multicellular organisms, adhesion receptors allow cells to interact with and acquire information from their environments. Receptors mediate adhesion both to other cells [e.g., by immunoglobulin (Ig) domain-containing proteins] and to the extracellular matrix (ECM) (by integrins). Cell adhesion molecules often form micrometer-sized clusters on the plasma membrane (23) with many cytoplasmic adaptor proteins, physically and functionally coupling to diverse downstream signaling pathways including MAPK, JNK, Rac, and the actin cytoskeleton (87). A growing body of evidence indicates that many of these cell adhesion clusters may form through LLPS.

Nephrin and the Glomerular Filtration Barrier

Nephrin is a transmembrane receptor that regulates cell–cell adhesion in podocyte cells of the kidney to form the glomerular filtration barrier (100). It is a member of a large family of cell adhesion molecules that participate in diverse processes including muscle formation, tissue patterning, and synaptogenesis (118). The extracellular domain of nephrin is composed of eight IgG-like motifs and a fibronectin type III domain (100). Nephrin extracellular domains on adjacent cells interact in homotypic fashion to form the slit diaphragm, a size-selective intercellular barrier that is the final element of the kidney filter (100).

Upon stimuli including engagement of the nephrin extracellular domains, crosslinking of nephrin extracellular domains by antibodies, and activation of nearby integrins, the nephrin cytoplasmic domain can be phosphorylated by Src family kinases on up to five tyrosine residues (156, 157). Three of these tyrosine residues are consensus binding motifs for the SH2 domain of the adaptor protein Nck (pYDXV) (6, 14, 156). Recruitment of Nck to nephrin is required for local remodeling of the actin cytoskeleton and proper maintenance of the kidney filtration barrier (80). In addition to its SH2 domain, Nck has three SH3 domains, which bind the numerous (six to nine) PRMs of N-WASP, leading to actin assembly at sites of nephrin adhesion (14), which appears to be necessary for maintenance of the slit diaphragm (80). Nephrin phosphorylation increases following kidney injury (81, 110, 156), and mutation of the phosphorylation sites causes progressive kidney disease in mice (110), demonstrating the physiologic importance of these modifications.

The multivalency of these interactions suggests the potential for assembly and concomitant phase separation (**Figure 4**). Indeed, both in 3D solution and when the phosphorylated intracellular tail of nephrin is attached to supported lipid bilayers, addition of Nck and N-WASP leads



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Phase separation of nephrin in kidney podocytes. (*Left*) The distal-most IgG-like extracellular domain (*gold*) of nephrin receptors can bind the distal-most IgG-like extracellular domain of nephrin receptors on the apposing podocyte membrane. The intracellular tail of nephrin is phosphorylated at three tyrosine residues. These phosphorylated residues can be bound by Nck (*cyan*). (*Right*) Phosphorylated tyrosine residues on nephrin can bind the SH2 domain of Nck. Nck SH3 domains bind to proline-rich motifs on N-WASP (*orange chains*) to form a phase-separated cluster of nephrin, Nck, and N-WASP. Extracellular interactions between the distal-most IgG-like domains of nephrin likely also contribute to cluster formation. The figure shows only the known high-affinity modular interactions among the molecules that promote formation of oligomers. The very-low-affinity interactions of domains and/or linkers that govern solubility, and thus liquid–liquid phase separation, of the molecules and oligomers are not understood and are not shown. Abbreviations: FNIII, fibronectin type III; Ig, immunoglobulin; SH, Src homology.

to LLPS, forming liquid droplets in solution and micrometer-scale clusters on membranes, respectively (6, 7, 93). Induced phosphorylation of transmembrane nephrin in cells also leads to formation of micrometer-scale clusters at the plasma membrane and larger dense clusters at the cell periphery (84).

In both biochemical and cellular settings, nephrin clusters are consistent with phase-separated structures. In vitro, for a fixed density/concentration of p-nephrin/N-WASP, clustering occurs sharply above a threshold concentration of Nck. Decreasing the number of phosphotyrosine sites on nephrin or SH3 domains of Nck raises the threshold. Paralleling these behaviors, the probability of observing nephrin clusters in cells increases with expression level of Nck, in a manner dependent on SH3 valency (84). In both settings, clusters exhibit liquid-like dynamics including the ability to fuse, rapid rearrangement of molecules within clusters, and rapid exchange of molecules between clusters and the surrounding solution or membrane (6, 84).

Local assembly of the actin cytoskeleton at nephrin clusters is important for proper maintenance of the filtration barrier in podocytes (80, 100). In vitro reconstitution experiments suggest that LLPS of nephrin, Nck, and N-WASP may play an important role in stimulating this Arp2/3 complex–dependent actin polymerization (6). Recent quantitative analyses of this activity have revealed that actin assembles preferentially on clusters not only because N-WASP is concentrated there, but also because the specific activities (actin polymerization rate per molecule) of N-WASP and the Arp2/3 complex are higher there than in surrounding regions of the membrane (24). This increased specific activity is due to increased membrane dwell time of N-WASP and the Arp2/3 complex, which results from the highly crosslinked nature of the phospho-nephrin/Nck/N-WASP assembly (24). A similar dependence of activity on membrane dwell time has also been observed for clusters formed by pLAT, Grb2, and full-length Sos1, which activate Ras (70, 72). In both systems, activity is mediated by slow, multistep nonequilibrium processes, suggesting that the dwell-time dependence is due to effects analogous to kinetic proofreading (68, 72, 102). Because many transmembrane receptors signal through analogous multistep nonequilibrium pathways and assemble into clusters through multivalent crosslinking, this enhancement of signaling by LLPS may be quite general. It remains an interesting question whether, as in classical kinetic proofreading during protein and DNA synthesis, the dwell-time dependence in signaling systems could also be used to generate specificity for one downstream output over another.

Tight Junctions

Epithelial monolayers are maintained by several types of cell–cell junctions including adherens junctions, desmosomes, and tight junctions (53). Tight junctions are the apical-most structure and are responsible for establishing the paracellular seal that creates a selectively permeable barrier (53). Tight junctions visualized with freeze-fracture electron microscopy appear as rows of punctate membrane contacts (152). By light microscopy, tight junctions appear as a discrete band containing numerous transmembrane and multivalent cytoplasmic adaptor proteins, many of which

rapidly exchange with the cytoplasmic pool (136). The visible appearance, rapid dynamics, and nature of the molecular components suggest that LLPS could play a role in forming tight junctions.

Tight junctions contain three classes of integral membrane proteins: claudins, tight junction–associated marvel domain-containing proteins, and junctional adhesion molecules (JAMs) (152). Claudins are the main mediators of the paracellular seal, whereas JAMs, such as JAM-A, and tight junction–associated marvel domain-containing proteins, such as occludin, seem to have more redundant or overlapping functions in regulating permeability. Crosslinking and native PAGE show that claudin-2 forms homodimers, likely through interactions between transmembrane domains, whereas claudin-4 and occludin are monomers (153). The short C-terminal intracellular tails of claudins and JAMs bind to PDZ domains in cytoplasmic ligands, whereas the tail of occludin binds to positively charged surfaces of its ligands (113).

Most of the cytoplasmic ligands of these receptors contain multiple modular interaction domains. ZO-1 and ZO-2 contain three N-terminal PDZ domains, a central SH3 domain, a region with homology to guanylate kinase, and a C-terminal actin-binding region (43). Deletion of ZO-1 and ZO-2 abolishes tight junction formation, suggesting these multivalent binding partners are essential for tight junction assembly (151). Relatedly, PSD-95, a postsynaptic density protein with similar domain structure to ZO-1, undergoes LLPS in vitro when mixed with SynGAP, a coiled-coil trimer with PDZ binding motifs (173, 174). These studies provide the first evidence that multivalent interactions between PDZ domains and PDZ binding motifs can be sufficient to drive LLPS.

In addition to ZO-1 and ZO-2, there are many other multivalent binding partners at tight junctions including MUPP-1 (13 PDZ domains), MAGI-1 (2 WW domains and 6 PDZ domains), PAT-J (10 PDZ domains), AF-6 (2 Ras-binding domains, 1 PDZ domain, and an actin-binding domain), cingulin (a homodimer with a globular head domain that mediates protein interactions), and Amotl1 [glutamine-rich domain similar to those driving LLPS in other proteins (176), coiled-coil domain, and a PDZ binding motif] (135). We speculate that LLPS of these scaffolds with their transmembrane binding partners could promote tight junction formation. FRAP data suggest that claudins are stably localized at tight junctions, whereas occludins freely diffuse in the membrane and ZO-1 dynamically exchanges with the cytoplasmic pool (136). Such heterogeneity in dynamic behavior has been observed in 3D biomolecular condensates (161) and may occur in membrane-associated clusters as well. Additionally, ZO-1 dynamics are actively regulated by the actin cytoskeleton. When myosin contractility is inhibited or the ZO-1 actin-binding domain is deleted, ZO-1 becomes much more stably associated with tight junctions (171). Analogous behavior has been observed in other contexts. For example, uncoupling N-WASP from the Arp2/3 complex decreases the rate of N-WASP turnover in signaling clusters generated by vaccinia virus (163), and abrogating ATP hydrolysis by the RNA helicase Dhh1 greatly slows its exchange between P-body condensates and the cytoplasm (21). The control of dynamics by active processes is another theme likely to span 3D and membrane-associated clusters (5).

During the final proof stage of this review, Alf Honigmann and colleagues at the Max Planck Institute of Molecular Cell Biology and Genetics reported that phase separation of ZO proteins drives formation of tight junctions, consistent with the ideas described above (12a).

Integrins and Focal Adhesions

Integrins are transmembrane receptors that mediate cell adhesion to multivalent ECM ligands such as fibronectin and collagen (76). Integrins are obligate heterodimers composed of an α and a β subunit. Integrins have large extracellular domains that impart ligand specificity and small (20–50 amino acid) cytoplasmic tails that mediate intracellular signaling. Integrins form clusters, termed focal adhesions, with numerous cytoplasmic adaptor proteins, kinases, and actin-binding

proteins that connect cells with their surroundings. Focal adhesions serve as sites of force transmission between the intracellular actin cytoskeleton and the ECM to drive tissue morphogenesis, cell movement, and ECM remodeling. They also act as signaling hubs to control the cell cycle, cell differentiation, and cell death. Thus, focal adhesions mediate an array of functions involving biochemical and physical interactions between the cell and its environment.

Integrins lack catalytic activity and do not directly bind actin. Rather, they signal by directly or indirectly recruiting cytoplasmic proteins such as kindlin, talin, vinculin, FAK, paxillin, α-actinin, and p130Cas (23). Focal adhesions initially form as small (<200-nm-diameter) nascent clusters that disassemble within ~1 min at the cell edge. A subset of these clusters becomes stabilized and matures through force-dependent growth and compositional changes. FRAP experiments reveal that kindlin, paxillin, FAK, and α-actinin rapidly exchange with the cytoplasm, whereas talin, vinculin, and tensin are less dynamic when cells are plated on stiff substrates (141). Neighboring focal adhesions occasionally fuse (143, 148). The cellular properties and molecular construction of focal adhesions suggest that LLPS may contribute to their formation and function (**Figure 5**).

There is evidence that multivalent interactions with extracellular ligands and intracellular adaptor proteins are required for integrin clustering and focal adhesion formation (28). Soluble monovalent ligands are unable to induce integrin cluster formation, whereas multivalent ligands or monovalent ligands added in tandem with multivalent antibodies promote integrin clustering (28, 104). Knocking out the cytoplasmic adaptors talin and kindlin inhibits integrin cluster formation, even in the presence of multivalent ligands (147). Thus, multivalent extracellular ligands are necessary, but not sufficient, for integrin cluster formation. Focal adhesions are enriched with a variety of intracellular multivalent proteins, many of which interact with each other (166). For example, talin and paxillin bind to different regions of vinculin, and each contains numerous vinculin binding sites (22). Talin also contains a single binding site for paxillin (172). These interactions provide a possible mechanism for vinculin to build higher-order talin/paxillin assemblies. Most of talin's 10 putative vinculin binding sites are exposed only when talin is stretched by force (33), and, thus, talin valency is mechanosensitive. Paxillin, FAK, and p130Cas also form complexes (65). FAK and paxillin have multiple binding sites for each other (126), the FAK C terminus also contains multiple PRMs that bind the SH3 domain of p130Cas (60), and paxillin can bind the p130Cas C terminus (175). p130Cas contains 15 tyrosine residues that, when phosphorylated, are binding sites for the SH2 domain of either Nck or Crk (11). As described above, these adaptors can bind to multivalent PRM proteins such as N-WASP, C3G, and EPS15, resulting in LLPS (127). These molecular interactions and the physical properties of focal adhesions suggest that LLPS may be a significant contributor to these cellular structures.

Super-resolution imaging indicates that focal adhesions do not behave as simple fluids—they are not isotropic in all dimensions. Rather, they are spatially organized to form a stratified structure (82). Some proteins such as paxillin and FAK localize with integrins near the plasma membrane, whereas others such as actin, VASP, and zyxin localize ~30 nm above the membrane. Talin, which has a long rod-like structure, is oriented with its N terminus colocalized with integrin and its C terminus colocalized with actin. The length of talin regulates the distance between actin and the membrane and may control the thickness of focal adhesions perpendicular to the membrane (96). Thus, focal adhesions may behave more akin to liquid crystals, with disorder in two dimensions and order in the third (note, however, as this order likely arises because of directionality imparted by the membrane rather than spontaneously, focal adhesions are not liquid crystals in a formal sense). Recent work has shown that the extracellular domains of integrins also orient parallel to the membrane in response to mechanical forces (144), suggesting focal adhesions may also become anisotropic in this direction. Super-resolution imaging of other membrane-associated clusters will be needed to determine whether anisotropic organization is generally observed.

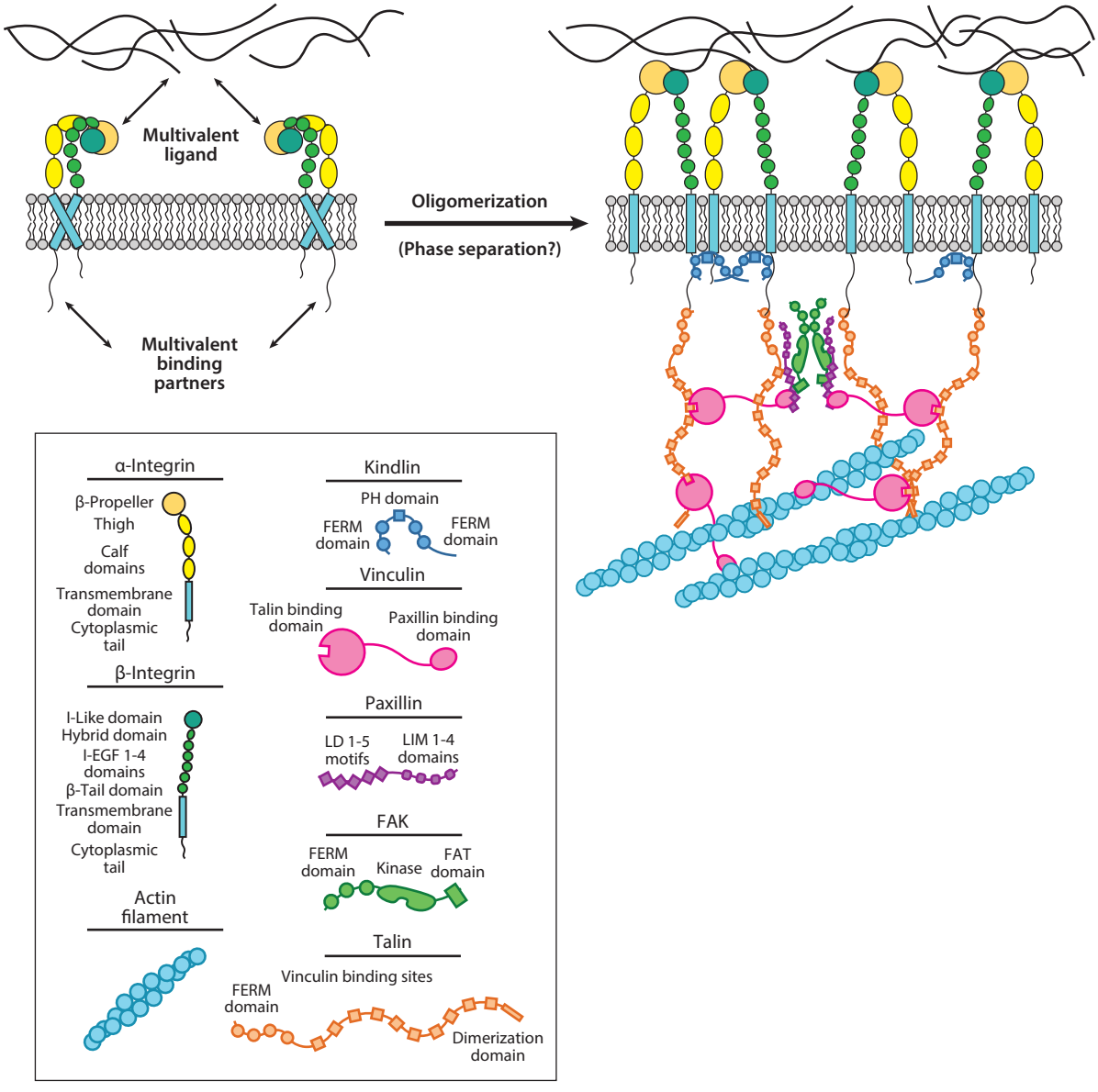


Figure 5

Multivalent interactions at focal adhesions. (*Left*) Integrin receptors in the inactive conformation. (*Right*) Integrin receptors can be activated when the extracellular domain binds multivalent components of the extracellular matrix, and the intracellular domain of β-integrin binds kindlin (*blue*) and/or talin (*orange chains*). Kindlin dimerizes by interactions in the FERM domain. Talin is composed of 10 putative vinculin binding sites and can bind actin filaments through a site in its C-terminal dimerization domain. Vinculin (*magenta*) can bind talin (via its N-terminal talin binding domain) and paxillin (*purple*) LD motifs and actin filaments through a site in its C terminus. Paxillin LD motifs can bind the vinculin C terminus and FAK FAT domains. FAK (*green*) can dimerize by interactions between its FERM domains. Each of these unique interactions between multiple proteins results in the formation of a highly interconnected oligomeric protein network in focal adhesions. The figure shows only the known high-affinity modular interactions among the molecules that promote formation of oligomers. The very-low-affinity interactions of domains and/or linkers that govern solubility, and thus liquid–liquid phase separation, of the molecules and oligomers are not understood and are not shown. Abbreviations: FAK, focal adhesion kinase; FAT, focal adhesion targeting; FERM, 4.1 protein, ezrin, radixin, moesin; I-EGF, integrin-epidermal growth factor; I-like, integrin-like; LIM, Lin11, Isl-1, and Mec-3; PH, pleckstrin homology.

CANONICAL WNT SIGNALING

Wnts are secreted proteins that bind to the extracellular domain of Frizzled (Fz) and LRP5/6 transmembrane receptors to regulate many aspects of development and adult homeostasis (139). In canonical Wnt signaling, binding of Wnt to Fz and LRP5/6 induces receptor heterodimerization and triggers clustering of the receptors into a macromolecular assembly that inhibits the degradation of β-catenin (30, 139). In the absence of Wnt, β-catenin is constantly targeted for proteasome-mediated degradation by the cytoplasmic β-catenin destruction complex. The Fz–LRP5/6 assembly on the membrane shares many molecular components with the destruction complex, including Axin and GSK3. Although the exact mechanism by which Wnt signaling inhibits β-catenin degradation remains unclear, evidence suggests that activated Fz–LRP5/6 clusters either titrate components away from the destruction complex or directly inhibit the activity of the destruction complex (139). Following Wnt binding, Fz and LRP5/6 bind to multivalent cytoplasmic adaptors Disheveled (Dvl) and Axin as well as many other effectors such as GSK3 and Cdk14 to form clusters (139).

When Dvl is overexpressed in tissue culture cells or is at high levels in cancer, liquid-like Dvl condensates are observed in the cytoplasm (129, 138). These condensates can undergo fusion and their components rapidly exchange with the cytoplasm (129). Both Dvl and Axin contain DIX domains, which interact in a weak, head to tail fashion that promotes assembly of either homotypic or heterotypic polymers (45, 128). Dvl also contains a DEP domain that mediates high-affinity homodimerization, effectively crosslinking the DIX domain polymers into higher-order structures (52). The formation of cytoplasmic Dvl condensates and subsequent inhibition of β-catenin degradation require assembly of Dvl and Axin DIX domains (45, 128) as well as crosslinking by the Dvl DEP domain (52). Thus, crosslinking of heterotypic Dvl–Axin polymers allows for higher-order oligomerization and subsequent LLPS (52).

Although Dvl forms cytoplasmic condensates when expressed at high levels, the protein localizes predominantly to 2D clusters containing Wnt, Fz, LRP, Axin, and GSK3 on the plasma membrane when expressed at endogenous levels in healthy cells (58, 138). Membrane association is mediated by interactions of the DEP domain and C terminus of Dvl with the intracellular domain of Fz as well as by potential interactions between Axin and GSK3 and the intracellular domain of LRP5/6 (30, 99, 103, 145). Thus, in normal canonical Wnt signaling, LLPS of Dvl and Axin polymers promotes formation of 2D clusters on the plasma membrane. Mechanistic lessons learned from the 3D condensates likely apply to the 2D membrane system as well (**Figure 6**).

RECEPTOR TYROSINE KINASES

Many receptor tyrosine kinases form clusters in response to extracellular stimuli (101). Here, we focus on the most abundant class, ephrin (Eph) receptors (79, 131), and suggest that principles governing cluster formation of Eph receptors can be applied to other receptor tyrosine kinases (**Figure 7**).

Eph receptors regulate cell–cell recognition in the nervous, vascular, immune, and skeletal systems (89). Eph receptors bind to glycosylphosphatidylinositol (GPI)-anchored Eph A or transmembrane Eph B on neighboring cells (79). Upon ligand binding, Eph receptors oligomerize into clusters that initiate and sustain downstream signal transduction (112). Both extracellular and intracellular multivalent interactions promote assembly of Eph receptors (**Figure 7**).

Eph receptors contain four extracellular domains (the ligand binding domain, a cysteine-rich domain, and two fibronectin repeats), a single-pass transmembrane helix, and up to four intracellular domains (the juxtamembrane region, a tyrosine kinase domain, a sterile alpha motif, and a phosphotyrosine binding motif) (79). Crystal structures have been determined for complexes of

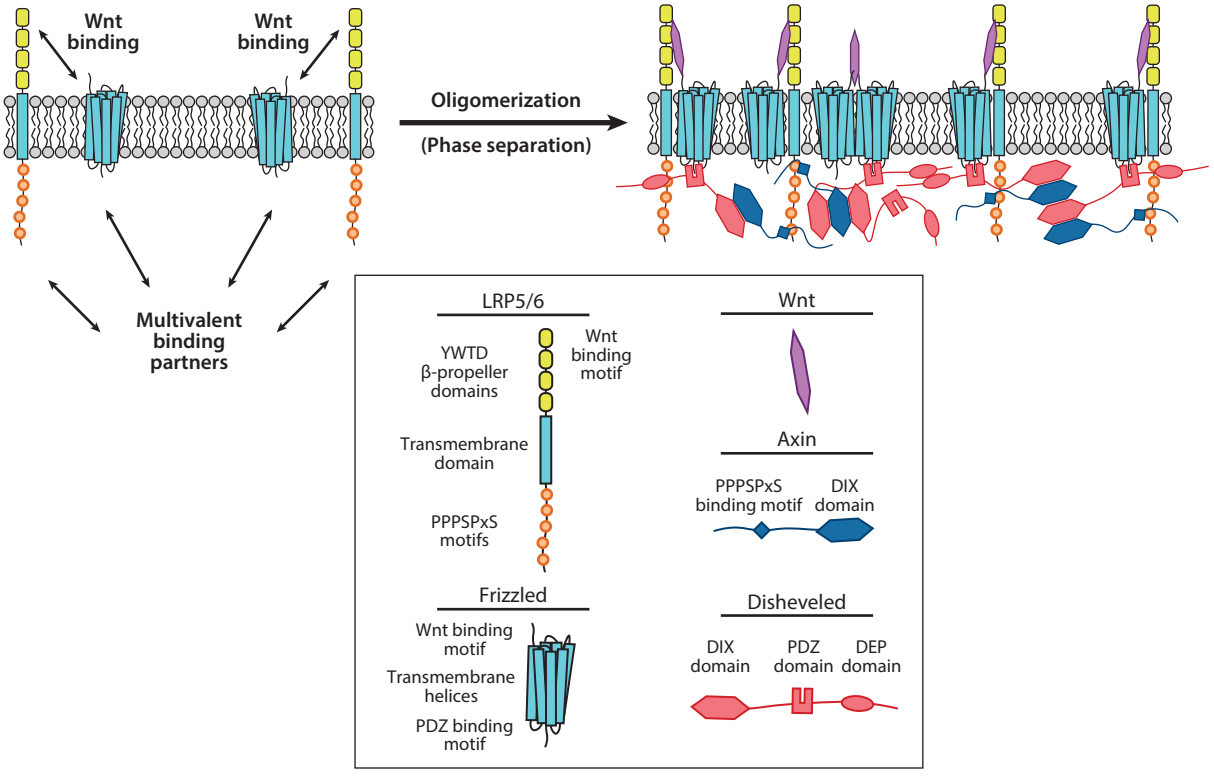


Figure 6

Phase separation in Wnt signaling. Wnt (*purple*) can bind to LRP5/6 (single-pass transmembrane receptor) and Fz receptors (multipass transmembrane receptor) to initiate Wnt signaling. LRP5/6 can bind Axin (*blue*), whereas Fz can bind Dvl (*red*). These interactions induce the formation of phase-separated clusters on the cell membrane. Following Wnt binding, phosphorylation of PPPSPxS motifs on LRP5/6 enables binding of Axin via an undefined region of its C terminus. Dvl can bind the Fz receptor intracellular tail through its PDZ domain. Polymerization of DIX domains in both Axin and Dvl promote clustering of membrane-associated proteins at Fz and LRP5/6 receptors. Dvl also contains a C-terminal dimerization (DEP) domain that enhances multivalent interactions within the Wnt signalosome. The figure shows only the known high-affinity modular interactions among the molecules that promote formation of oligomers. The very-low-affinity interactions of domains and/or linkers that govern solubility, and thus liquid–liquid phase separation, of the molecules and oligomers are not understood and are not shown. Abbreviations: DEP, disheveled, Egl-10, pleckstrin; Dvl, disheveled; Fz, frizzled; LRP5/6, low-density lipoprotein receptor-related protein 5/6; PDZ, post synaptic density protein, drosophila disc large tumor suppressor, zonula-occludens-1 protein; YWTD, tyrosine, tryptophan, threonine, aspartic acid.

the full extracellular domains bound to Eph ligands (64, 131). These revealed interactions between the ligand binding domain and Eph previously observed in complexes of the isolated domains (63). However, extensive additional contacts were observed in the crystal lattice between neighboring receptors and/or ligands. These involved conserved surfaces of the receptor ligand binding domain, cysteine-rich domain, and fibronectin domains. These contacts generate a complex network of both parallel and antiparallel receptor arrays. As organized on the cell surface, these interactions would occur both in *trans* (between cells) and in *cis* (on a given cell membrane). Mutation of either the *trans* or *cis* interfaces prevented the formation of Eph receptor oligomers in cells, leading to a model wherein extracellular domain interactions between receptors on the same cellular membrane could seed Eph receptor oligomer formation and macroscopic assembly (**Figure 7**).

Intracellular regions of the Eph receptors likely also contribute to assembly and, potentially, to LLPS. Upon extracellular ligand binding, the intracellular domains are phosphorylated on several

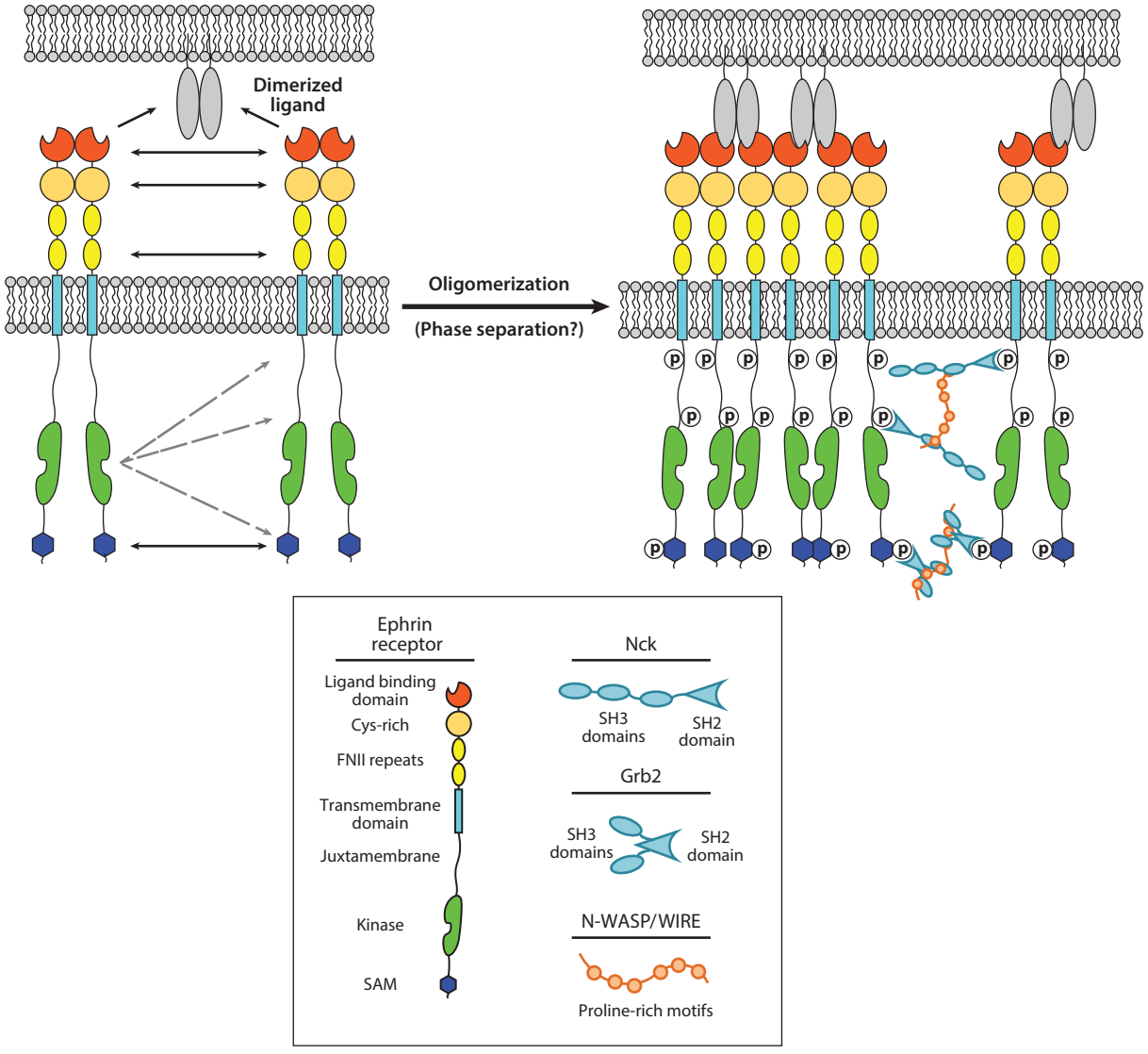


Figure 7

Multiple interactions drive dimerization and cluster formation of Eph receptors. (*Left*) Interactions among two Eph receptor extracellular ligand binding domains, Cys-rich domains, and FN-like domains can result in Eph receptor dimerization in the absence of ligands. Dimerized ligands expressed on the surface of an apposing cell can seed cluster formation. (*Right*) Upon extracellular ligand–induced cluster formation, the kinase domain of one Eph receptor can transphosphorylate intracellular tyrosine residues on surrounding receptors. These residues serve as docking sites for SH2 or SH3 adaptor proteins, such as Grb2 (*cyan*) and Nck (*cyan chains*), which can oligomerize through multivalent interactions with proteins containing proline-rich motifs such as N-WASP (*orange chains*). Intracellular SAMs also self-associate to promote receptor oligomerization. The figure shows only the known high-affinity modular interactions among the molecules that promote formation of oligomers. The very-low-affinity interactions of domains and/or linkers that govern solubility, and thus liquid–liquid phase separation, of the molecules and oligomers are not understood and are not shown. Abbreviations: Eph, ephrin; FNII, fibronectin type II; SAM, sterile alpha motif; SH, Src homology.

tyrosine residues by the kinase domain to create docking sites for the multivalent SH2/SH3 adaptor proteins Grb2, CrkII, and Nck (105, 111). These in turn bind to PRMs within the proteins WIRE and N-WASP (105). As described above for nephrin, such interactions have a strong propensity to drive LLPS (6). In addition, the sterile alpha motif of Eph receptors may dimerize or oligomerize (10), further contributing to receptor assembly (**Figure 7**). These domains and interactions raise the possibility that Eph receptor clusters are phase-separated compartments on cell membranes. In support of this, a recent study used computational modeling and quantitative microscopy to demonstrate that Eph receptor clusters initially form through the addition of individual Eph receptors. However, these smaller clusters then merge to form larger clusters (115), consistent with LLPS (6). Further investigation is needed to determine whether Eph receptor clusters are formed through phase separation and how LLPS may regulate functional consequences of Eph receptor cluster formation in human health and disease.

CONCLUSIONS AND PERSPECTIVES

Cell biological studies have demonstrated that many receptors assemble into large clusters upon activation, and in some cases, this higher-order assembly is important for downstream signaling. Several themes emerge from consideration of such systems. First, clustering is often mediated by multivalent interactions capable of generating networks consistent with ideas from classical polymer chemistry. Second, these multivalent interactions occur in both the extracellular and intracellular regions of the receptors, often through heterotypic interactions and sometimes also through homotypic interactions. Third, a frequently observed molecular mechanism for clustering involves a multiply tyrosine-phosphorylated disordered protein (either a receptor cytoplasmic tail or a proximal adaptor protein). This protein interacts directly with proteins containing an SH2 domain and multiple SH3 domains, which in turn bind proteins with large proline-rich regions containing multiple SH3 binding motifs. For some receptor–ligand combinations, experimental evidence demonstrates that these interactions promote macroscopic phase separation in vitro, with correlated behavior in cells. For others, the behavior of the cellular clusters and the features of the molecules that form them suggest that phase separation likely contributes to clustering.

Two interesting topics that have not yet been extensively explored in this area are (*a*) the relationship between protein phase separation and lipid phase separation and (*b*) the regulation and functional importance of receptor cluster size. We discuss these below in the context of future directions for studies of the biophysics, biochemistry, and cell biology of receptor signaling.

Phase Separation of Lipids in the Plasma Membrane

Like many macromolecules, lipids phase separate under certain conditions (61). The mechanisms that regulate the self-organization of lipids have been intensely studied via cellular and in vitro experimental approaches and computational modeling (20, 67, 92, 95). In vitro experimentation using planar supported lipid bilayers and giant unilamellar vesicles has revealed that lipid mixtures containing combinations of sterols as well as saturated and unsaturated lipids will spontaneously undergo LLPS to form macroscopic phase-separated domains (66). Sterols and saturated lipids partition into liquid-ordered domains, whereas unsaturated lipids partition into liquid-disordered domains (34, 164). Similar phenomena have been observed using giant plasma membrane vesicles that are blebbed from cells and are composed of representative cellular lipid and membrane-associated protein mixtures (12, 91, 155). Thus, the plasma membrane of cells contains components that are capable of demixing into separate lipid domains (133). However, long-lived, micrometer-scale domains like those observed in supported lipid bilayers, giant

unilamellar vesicles, or giant plasma membrane vesicles have not been observed in the plasma membranes of mammalian cells (although they have been observed in some specialized membranes, including the yeast vacuole and erythrocyte plasma membrane) (90, 121, 150, 158). Rather, super-resolution light microscopy and environmentally sensitive membrane dyes have revealed the presence of short-lived, nanoscopic (<100 nm) domains in the plasma membrane of mammalian cells (41, 117), suggesting that active processes prevent spontaneous formation of large membrane domains. In fact, several studies have demonstrated that the cortical actin cytoskeleton plays an active role in modulating membrane organization of lipids and proteins (56, 57, 119). These data indicate that ATP-dependent actomyosin contraction, rather than passive lipid phase separation, may drive lipid clustering and define the size and dynamics of lipid domains on the plasma membrane.

In addition to interactions with extracellular ligands and intracellular adaptor proteins, transmembrane receptors also interact with lipids in the plasma membrane. Thus, lipid phase separation and protein phase separation should be coupled—phase separation of membrane lipids should influence phase separation of transmembrane receptors, and vice versa. Indeed, self-organization of lipids within membranes modulates protein localization within lipid domains (97, 119). Lorent and colleagues (97) reported that both the surface area and length of the transmembrane domain and the palmitoylation state of membrane-associated proteins contribute to the targeting of proteins to either liquid-ordered or -disordered membrane regions. Using a combination of computational modeling, in vitro experimentation with cell-derived giant plasma membrane vesicles, and live-cell experiments, these authors predicted and described how proteins that are palmitoylated and have long, narrow transmembrane domains are more likely to associate with liquid-ordered domains that are tightly packed with saturated lipids, sterols, and glycosylated lipids. Concentrating similar proteins to specific lipids should prime receptors for phase separation prior to receiving any extracellular signals, essentially decreasing the degree of protein-mediated crosslinking needed to induce LLPS. Similarly, phase separation of transmembrane proteins through protein interactions should locally favor the formation of a lipid domain. In both aspects, the coupling of protein and lipid phase separation will enhance formation of membrane compartments with distinct physical properties and chemical compositions from the surroundings. Moreover, because both lipids and proteins can be linked to the actin cytoskeleton, actomyosin contraction could further modulate (in some cases, even dominate) the formation and dynamics of these compartments.

With recent technological advances in in vitro biochemical reconstitutions and in vivo microscopic imaging, we can now begin to address experimentally how the propensities of lipids and proteins to phase separate and the ability of each to interact with the actin cytoskeleton can act together to organize the plasma membrane. Such work will be essential for understanding the physical mechanisms of transmembrane signal transduction.

Receptor Cluster Size

In vitro membrane-associated protein LLPS produces clusters that have decaying size distributions, consistent with thermodynamic control (6). Distribution at any given time is dependent on multiple factors including the rates of nucleation, molecular association, and molecular dissociation as well as cluster fusion and lateral mobility (8). However, many integral membrane proteins form clusters in cells with peaked distributions (i.e., with a preferred size) (e.g., 27, 123, 148). This could be explained by so-called equilibrium cluster phases, which produce clusters of discrete sizes through a combination of short-range attraction and long-range repulsion (130, 140). Alternatively, active processes may control size distributions. The actin cytoskeleton and proteins

adhered to it can form barriers that restrain molecular movement, which could modulate cluster sizes (44, 50, 51). In vitro, actomyosin dynamics can alter cluster size distributions and drive their motion (36, 84), behaviors that could also be relevant in cells. For GPI-anchored proteins, and likely some transmembrane receptors as well, actomyosin contraction provides an active mechanism, distinct from phase separation, to produce clusters (57, 86, 119), which would also produce different size distributions. Finally, a recent theoretical model suggests that competing enzymes (e.g., a kinase and a phosphatase), if they favor and disfavor phase separation, respectively, could give peaked distributions of cluster sizes, whose means are defined by the relative rates of the two enzymes (168). Active processes are an important area of investigation for biomolecular condensates in general and should also be examined within membrane systems.

How might size impact the biochemical and cellular activities of clusters? Molecules in the center of a cluster are in a different chemical environment than those at the periphery (as manifest, for example, in macroscopic surface tension). This could impart different activities to molecules given their location (note that the size scale of center and periphery depends on the specific system and perhaps on the particular activity being measured). In small clusters, the average specific activity of the whole cluster changes as size increases, because the fraction of central molecules increases with size. But for larger clusters, the fraction of central molecules becomes asymptotic with size. In this regime, the average specific activity of the cluster does not change appreciably with size for reactions without diffusible intermediates. However, for reactions with diffusible intermediates, size is likely to be important in this larger range. Examples include signaling systems where intermediates dissociate from the membrane and then rebind before diffusing away (78, 114). In such cases, a larger cluster increases the probability that the intermediate will encounter other cluster molecules before diffusing away, thus increasing specific activity. Finally, compared with smaller clusters, larger clusters could transmit greater amounts of force, which may be relevant for systems that respond to and transmit cytoskeletal forces, such as focal adhesions and LAT clusters in T cells and B cells (23, 36, 69, 74, 122, 149). Further analyses of the size distributions of clusters and the relationship between size and activity will be necessary to explore these various possibilities.

In conclusion, recent investigations demonstrate that LLPS could be a general mechanism by which cell membrane receptors assemble into clusters. Although LLPS has been demonstrated only for a small number of signaling systems, many cell surface receptors share molecular attributes that could promote phase separation, such as binding to multivalent extracellular and intracellular ligands. Additionally, many receptors have cellular behaviors consistent with phase separation, such as forming dynamic, nanometer- to micrometer-scale structures. Future investigations of transmembrane signaling should thus consider phase separation as a potential mechanism underlying the formation, regulation, and function of clusters of cell surface receptors.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Work in the Rosen lab is supported by the Howard Hughes Medical Institute. L.B.C. is a Robert Black Fellow of the Damon Runyon Cancer Research Foundation (DRG-2249-16). This work was additionally supported by a National Research Service Award from NIDDK (F32 DK101188 to J.A.D.) and a Howard Hughes Medical Institute Collaborative Innovation Award (to M.K.R.).

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