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Biophysics of Protein Condensates on Supported Lipid Membranes

by

Nugent Lew

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Chemistry

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Jay T. Groves, Chair
Professor Ke Xu
Professor Kranthi K. Mandadapu

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by

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Abstract

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Professor Jay T. Groves, Chair

Biomolecular condensation has been recognized for the last decade-and-a-half as a ubiquitous modality of high-order molecular organization in living cells. This allows for compartmentalized, altered biochemistry that enables otherwise rare biochemistry. Condensates have been observed with both cytosolic (solution), and membrane-bound (surface) components. Linker for Activation of T Cells (LAT) and Epidermal Growth Factor Receptor (EGFR) are examples of transmembrane proteins that can undergo the latter type of condensation. The phase transition of the cytosol-facing phosphotyrosine tails of both of these proteins has been previously reconstituted on supported lipid bilayers (SLB). This type of phase transition is enabled by multivalent binding with downstream adaptors and signaling enzymes to form an extended, two-dimensional bond network. For LAT and EGFR, the adaptor protein Grb2 and the proline-rich scaffolding domain of the guanine exchange factor SOS, together, can reconstitute condensation. Previous work on these signaling condensates have shown that the condensed phase can directly impact downstream signaling kinetics. Additionally, the valency of crosslinking bonds and the bond association and dissociation kinetics within the condensate critically determine the phase transition kinetics and material properties of the resulting condensate. This dissertation details reconstitution of these condensates on SLBs along with fluorescence microscopy to further explore these facets of signaling condensates. We find that condensation can be driven solely by binding to Grb2 which can dimerize to crosslink signaling proteins without SOS. This dimer interface can in turn be controlled through a phosphotyrosine on Grb2 itself to regulate condensation. This Grb2-mediated condensate retains its enhancement of Ras activation kinetics through SOS which can now be modulated through phosphorylation of Grb2. We also observe that we can reconstitute simultaneous condensates in solution and on the membrane with the same components. The solution condensates, on contact with the membrane surface decorated with a membrane condensate, can undergo a partial transition from a bulk three dimensional condensate to a surface-bound two dimensional condensate.

To all the Loved Ones who believed I could come this far.
I hope to learn from your example.

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Chapter 1

Introduction

In the chemistry lecture hall, it is often said that enzymatic catalysis in cells exceeds the capabilities of any man-made catalysts for stereospecificity. Similarly, a cell's capabilities for physical chemistry should not be underestimated either. Biomolecules that form intracellular compartments have been subject to great interest and intense study for their progressively ubiquitous presence in all forms of biochemistry [1–10]. These so-called membraneless organelles are enriched in subsets of biomolecules without an encapsulating barrier like a membrane. In the last decade-and-a-half, the driving principle behind their formation has been understood to be the formation of condensed phases coexisting with the rest of the intracellular milieu [1, 11, 12]. This has inspired and enabled extensive physical characterization of membraneless organelle components from the lens of phase transitions and polymer physics [8, 12–16]. As such, these types of systems are also known broadly as biomolecular condensates.

As of today, biomolecular condensates have been shown or proposed to be involved in a vast array of biological processes. These including gene regulation [5, 17], stress response [18], signal transduction [11, 19], and cellular adhesion [20]. Additionally, condensates and condensate regulation have also been implicated in a number of diseases [4, 9, 21, 22]. Studies have been performed to identify condensates within biochemical pathways, to characterize their importance therein, and to understand how the principles of phase coexistence are leveraged. Some ideas have already been demonstrated previously: Control of saturation concentrations can be used to rapidly generate asymmetric distributions of certain molecules within liquid-like condensates [1], sharp transitions in local biomolecule concentrations can cause sharp increases in biochemical processes [5, 17], the unique condensate environment can alter biochemical parameters [23], and altered dynamics can enhance otherwise low-level enzymatic activity [24–26].

Studies on model systems have deeply broadened the physical theories used to understand biomolecular condensates [8, 14, 16]. In particular, reconstituting purified con-

condensate components enables precise tuning of the experimental parameters around the phase transitions and, in turn, the resultant condensates. This manner of experimentation allows for direct and unambiguous testing of hypotheses and models about the phase transition physics. Through such experiments, it was confirmed that some such systems exhibit liquid droplet-like behaviours [1, 5, 27] and form due to liquid phase demixing [5, 28–30]. It was also shown how such a phase transition is enabled by the amino acid sequence and structure of the component proteins [14, 31, 32]. Both early and later work also showed that condensates do not remain in a liquid state. Instead, it was observed that condensates can further evolve into a viscoelastic state in a process broadly termed aging [33], or transition to a fibrillated, solid aggregate [18, 34, 35]. Furthermore, common trends emerged in systems with similar molecular characteristics that defied classification as a conventional liquid. For example, in signal transduction systems, the formation of a dynamically arrested bond network enables potent activation of signaling proteins that are otherwise suppressed by enhancing a slow activation pathway. [25, 26]

A common feature of signal transduction pathways are transmembrane proteins bearing an intrinsically disordered domain with tyrosine residues that are subject to phosphorylation downstream of cellular receptor ligation. Two such systems are Linker for Activation of T cells (LAT) whose cytosolic domain is phosphorylated downstream of T Cell Receptor (TCR) ligation by intermediary kinases [36], and the cytosolic tail of Extracellular Growth Factor Receptor (EGFR, also known as ErbB1 and HER1) which is a receptor tyrosine kinase (RTK) capable of *trans*-autophosphorylation [37]. Biochemically, LAT and EGFR are positioned very similarly within their respective signal transduction networks: Downstream of their phosphorylation, the signal which originated from receptor ligation is transduced to effector enzymes which propagate the signal to further, cytosolic signaling molecules and, subsequently, transcription factors in the nucleus to effect cellular response through gene expression [38–41]. In fact, LAT and EGFR share a downstream pathway that results in the activation of extracellular signal-regulated kinase (ERK) which effects cell cycle entry. This downstream pathway is one of multiple branches for both LAT and EGFR, and diverges off from them through the binding of the proteins Grb2 and Son of Sevenless (SOS) [42, 43].

The cytosolic domain of LAT (herein referred to simply as LAT) and the cytosolic tail of EGFR (EGFR^{TAIL}), upon tyrosine phosphorylation, are able to be bound by Grb2 which then binds SOS. These proteins can only associate through their well-defined binding sites: Grb2 binds to phosphotyrosines (pY) through its Src-homology 2 (SH2) domain, and in turn binds to proline rich (PR) motifs in the C-terminal proline rich domain of SOS through its Src-homology 3 (SH3) domain [44, 45]. LAT and EGFR possess multiple pYs that bind Grb2, and SOS possess multiple PR motifs that bind Grb2 [46, 47]. Furthermore, two Grb2 can also dimerize while both bound to pY [48]. This multivalency in crosslinking interactions enable phosphotyrosyl protein domains like LAT and EGFR^{TAIL} to condense via an extended binding network decorating the cytosolic surface of the plasma membrane

[19, 49]. An important distinction is that this sort of condensate, both in-cell and in cell-free reconstitution assays, is effectively two-dimensional. Only a single-molecule layer of the protein is present on the membrane surface, and the molecules only condense laterally on the lipid membrane surface. This form of condensate has been amenable to reconstitution on supported lipid bilayers (SLB) [15, 19, 24, 25, 48, 50, 51].

SLBs are a powerful tool for reconstituting lipid membranes and membrane proximal biomolecules [52, 53]. The substrate, or support, gives the lipid membrane structure, rigidity, and mimics the presence of an actin cytoskeleton. The result is a laterally fluid lipid bilayer with diffusivity roughly equivalent to that observed in the plasma membrane [54–56]. Furthermore, lipids with useful headgroup chemistry can be utilized. It is common to incorporate lipids that exhibit either non-covalent or covalent binding with a reactive site or fusion tag on a protein, thereby tethering said protein to the exposed leaflet of the bilayer and mimicking either a cytosolic or extracellular domain [53, 57]. When contained within a flow cell, the solution adjoining the membrane can be exchanged freely without disrupting the bilayer integrity or the tethered proteins. Furthermore, the choice of substrate material allows for characterization. A common substrate is a glass coverslip. Glass is very amenable to SLB formation, and allows for subsequent use of optical microscopy, such as fluorescence microscopy, to directly visualize the bilayer and attached proteins. [58, 59]

The LAT and EGFR^{TAIL} condensates (herein referred to collectively as signaling condensates) have previously been reconstituted and studied using SLBs [19, 24, 25, 48, 50, 51]. In these experiments, the proteins are tethered to the SLB using fused histidine tags which attach non-covalently to lipids with nickel chelating headgroups [57]. They are then tyrosine phosphorylated in-situ with a purified kinase [24, 25, 48, 51]. The crosslinking factors, namely SOS (commonly SOS^{PR}) and Grb2, are further added in the adjoining solution to initiate condensation. The transition of these condensates can be directly visualized if the signaling proteins are labeled with a fluorophore. This type of experiment has been used to confirm the necessary conditions for a phase transition to occur. For example it was understood that the valency of Grb2-binding phosphotyrosines is a critical parameter [19]. Later high-resolution imaging also demonstrated that the state of such condensates lies out-of-equilibrium for an extended period of time [51]. Alternatively, downstream signaling molecules, such as Ras-GTPase or a catalytically active variant of SOS (which is Ras guanine exchange factor [GEF]), can be included and visualized for their catalytic activity [24, 25, 60]. This manner of experiment has been used to show that a single SOS protein requires extended contact time, or dwell time, with the lipid membrane before reaching full catalytic potency and that signaling condensates enhance the likelihood that SOS experiences long dwell times [25].

Here I, together with my colleagues, have aimed to further the work of understanding both the signaling condensates and their effects on downstream signaling molecules. The

work herein draws on foundational knowledge of signaling biochemistry, and the flexibility and control of cell-free reconstitution to gain structural and mechanistic insight on the functions of signaling condensates. Given that LAT and EGFR condensates occur central to crucial signaling pathways, we explore how the phase behaviour of these condensates is affected by its crosslinking factors, and how this in turn affects downstream signaling proteins. To do this, we use SLBs on glass substrates for reconstitution. This platform allows us to use total internal reflection configuration fluorescence (TIRF) configuration for microscopy (TIRFM).

Our questions are well suited for investigation through imaging with TIRFM. TIRF illumination in one of three wavelengths, 488 nm, 561 nm, or 640 nm generates an evanescent wave at the interface between the glass coverslip and solution inside the chamber. The illumination intensity decays exponentially away from the interface. This effectively selects for fluorophores positioned proximal to the SLB via tethering, binding to tethered proteins, binding to lipids, or by chance through diffusion [61]. Through judicious labeling, we are able to visualize either SLB-tethered LAT or EGFR^{TAIL} [24, 48, 51], or downstream SOS and Ras [25]. We reconstitute condensation as described above and either directly watch the signaling condensates evolve in time, or how the activation of Ras (swapping from guanine diphosphate [GDP] to guanine triphosphate [GTP] bound form) progresses via the catalytic activity of SOS. As a note, I will herein distinguish the truncations of SOS used for crosslinking, SOS^{PR}, from the purified full-length and catalytically active protein, SOS^{FL}, for clarity. These imaging modalities allow us to collect both high content and high resolution images pertaining to the proteins in question and their dynamics in real-time.

While LAT is natively a transmembrane protein. We also explore how its condensate behaves in solution as well. A similar condensate has been studied in this manner before [11, 62]. However, in chapter 2, we make a novel observation that the LAT condensate in solution can join with the LAT condensate on the membrane in an unexpected way. Ordinarily, solution condensates, like a liquid droplet, are expected to wet a surface on contact. While wetting entails involves macroscopic spreading of a droplet along the incident surface, usually it is a bulk phenomenon where a totally three-dimensional layer is adhered to the surface. Instead, we observe that LAT droplets (condensates formed in solution) partially transition into and spread as a two-dimensional condensate indistinguishable from the condensate formed from SLB-tethered LAT. We conclude that LAT, and similar signaling condensates possessing two-dimensional and three-dimensional condensed forms, are inherently able to transition from three- to two- dimensional on contact with a surface presenting an analogous condensate. This appears to be enabled by a combination of the fact that LAT is condensed through discrete, low-valency crosslinks (through its phosphotyrosines) and that the reconstituted LAT carries a histidine tag capable of non-covalent, reversible tethering through nickel affinity interactions on the SLB surface.

In chapter 3, we dig deeper into the structural elements of the signaling condensate by

removing the component SOS^{PR} . In this study we focus on the $\text{EGFR}^{\text{TAIL}}$ condensate, but also find that the same results apply to LAT, that SOS^{PR} (and, analogously, SOS in cells) is not among the minimal requirements to form a signaling condensate. This challenged previous assumptions that Grb2 required the presence of a multivalent PR domain for crosslinking. We find that Grb2 crosslinks through its homotypic interaction interface that allows it to dimerize while its SH2 domain remains bound to a pY [63–65](PDB:1GRI). Thus only $\text{EGFR}^{\text{TAIL}}$ and Grb2 are necessary to form a condensate. Furthermore, we uncover an avenue by which this condensation can be regulated: Grb2 residue Y160 is included in the interaction interface, and is a target for kinase activity [64, 66]. We observe that phosphorylation or phosphomimetic mutation of Y160 abolishes condensation without any alteration to $\text{EGFR}^{\text{TAIL}}$ itself. This presents a viable mechanism by which condensation can be modulated through the phosphorylation of Grb2 or similar crosslinking factors, and not just of signaling protein. Finally, we show that modulation of condensation, again using SOS^{PR} , strongly affects the kinetics of Ras activation by SOS^{FL} . While previously established for LAT, we now show that $\text{EGFR}^{\text{TAIL}}$ exhibits a very similar effect on Ras activation kinetics.

We further expand on condensed phase modulation in chapter 4 where we tease part the effects of Grb2 phosphorylation on Ras activation. Where previously we titrated SOS^{PR} to modulate condensation [48], we now titrate kinase activity towards the same effect. For this study, we focus on changes in downstream signaling, so we emphasize tracing Ras activation under different condensation conditions. Specifically, we increase kinase activity by increasing kinase concentration, and then counter that effect by including a tyrosine kinase inhibitor [67, 68]. We observe that increased kinase concentration reduces Ras activation kinetics and that this effect is indeed reversed by suppressing kinase activity with an inhibitor. We attribute these observations to modulation of phase condensation through Grb2 phosphorylation at Y160. As previously established, pY160 prevents the Grb2 dimerization crosslinking interaction (we do not use SOS^{PR} in these experiments) [48]. Not only do these results corroborate the avenue of Grb2 phosphorylation as a potential means of condensate regulation, they also illustrate the complexity of kinase activity towards signaling condensates. While phosphorylation of signaling phosphotyrosines is what enables condensation in the first place, further phosphorylation affecting Grb2 Y160 will oppose and even undo condensation. In other words, phosphorylation in general is a non-monotonic modulator of condensation.

Lastly in chapter 5, we hone in on the mechanisms by which condensation enhances the GEF activity of SOS. SOS^{FL} is a highly complex protein with auto-inhibition under multiple layers of regulation [69–71]. It has proven insightful to revisit SOS^{FL} regulation in light of the realization that a condensate plays an important upstream role. Previously, it was found that signaling condensates enable SOS^{FL} to reach an otherwise rare, processive catalytic state by extending the dwell time of SOS recruited to the membrane [25, 69]. This is a recruitment based regulatory mechanism. Another form of SOS regula-

tion which has been studied previously is positive feedback[72, 73]. SOS^{FL} possesses both an active and an allosteric binding site for Ras which has been suggested to facilitate positive feedback by preferentially binding RasGTP (the product of SOS^{FL} catalysis) over RasGDP (the substrate of SOS^{FL} catalysis) [74, 75]. By reconstituting Ras activation starting from the LAT condensate, and in combination with stochastic kinetic modeling, we find that the LAT condensate is the dominant driver of SOS^{FL} recruitment which is unaffected by Ras nucleotide state. Instead, the positive feedback in SOS^{FL} activity is due to a shift in activation probability. Allosterically binding RasGTP favors the entry of SOS^{FL} into its processive state. We observe this as an increasing apparent kinetic rate constant in SOS^{FL} catalysis on the membrane as the reaction proceeds, and recapitulate it with our stochastic kinetic model. Thus we delineate the role of condensation as enhancing recruitment and retention of SOS^{FL} at the membrane which enables its activation, while allosteric RasGTP shifts the population of SOS^{FL} toward the processive active state.

An interesting chronological progression underlies the work introduced above: The progression of knowledge about the physical nature of biomolecular condensates. For upwards of a decade, the field on biomolecular condensates made critical assumptions about the phase transition physics of these condensates all of which hinged on the phase coexistence being at thermodynamic equilibrium [1, 6, 11, 13, 17, 19, 62, 76, 77]. While some cited a parallel occurrence of the mechanism of gelation, this was reported as driving an equilibrium state and not as an alternative [14]. Gradually emergent evidence, both from our group and in literature, has shown that the assumption that condensates reach even a local equilibrium is not generally applicable [33, 51, 78].

In reaching equilibrium, a system evolves into a state of minimized free-energy or, equivalently, equalized chemical potential of all components. These are state functions that are affected by a limited set of thermodynamic parameters. For example, temperature and component concentration as in a classical phase diagram. For protein systems, conditions such as salt concentration and pH are also expected to have a significant effect on phase equilibria. An important corollary of this nature is that the experiment is expected to yield similar results so long as these conditions are well reproduced in each iteration. For example, coexisting liquid phases driven by immiscibility of solvent and solute (i.e. liquid-liquid phase separation) theoretically yields two phases of a well-defined concentration representing free-energy minima. These concentrations are thus not expected to change with respect to the global solute concentration. This principle is sometimes known as the lever rule [13, 79]. Recent studies have shown, both experimentally and theoretically, that a condensate driven by discrete, singly-saturated, low valency bonds like a signaling condensate does not reach an observable thermodynamic equilibrium [51, 78].

Before definitive proof was shown however, certain observations already suggested that our assumption of membrane protein LLPS may be flawed. Firstly that the emergence and evolution of the incipient protein-rich phase exhibited changing kinetics over time. [24] The

first observations from our lab already showed that a condensed phase would slowly and continually emerge from an initially mixed state. Conversely, the reverse process, dissolution of the condensed phase would occur with drastically faster kinetics. Condensed liquid phases at equilibrium expected to evolve towards a final state where the coexisting phases coalesce into two macroscopic phases. In our system, the condensed phase would gradually arrest over the course of one to two hours of observation time, without complete coalescence [15, 24, 51]. Furthermore, these condensed domains would rarely adopt circular morphologies as expected by a liquid system which is dominated by interfacial tension.

More recent work on the nature of biomolecular condensate shed new light on the physical nature of signaling condensates. A publication from our group showed that signaling condensates do not obey the lever rule and thus cannot be at the free-energy minima, or equalized chemical potentials, that characterize an equilibrium state [51]. In fact, the key factor is how these condensates are formed. Computational modelling showed that the condensation and subsequent material properties of systems similar to LAT are dominated by the kinetics of bond formation between constituent molecules [78]. In other words, Grb2 and SOS binding kinetics are the key parameter. This is further overlaid with the extent of tyrosine phosphorylation on the signaling proteins. Effectively no crosslinks can form without phosphorylation. This reliance on a small number of discrete and specific binding sites contrasts with non-specific, polarity driven phase separation that underlies liquid immiscibility.

Experimentally, our early assumptions about the nature of the phase transition was belied by continual inconsistencies in the outcome of the condensation reaction. Between iterations of the same experiment, and different experimenters, and despite careful efforts to control relevant experimental conditions, membrane condensation experiments frequently yielded inconsistent outcomes. This was particularly evident in the observations of condensation kinetics, condensed phase morphology, and condensed phase density. New evidence and progressive understanding about biomolecular condensates would begin to reconcile these observations. The kinetically limited nature of this system suggests that the condensation is sensitive to microscopic variations in the initial conditions likely beyond what can be controlled by the experimenter. In other words, since the condensates are arrested part way along the path towards a putative equilibrium state, the observable condition of the system need not be uniquely defined by thermodynamic variables. Going forward, further work on such a system should always take this fundamental nature into account.

The combined works of this dissertation show two broad faucets. Firstly, phase condensation of signaling proteins is broadly modulated by the crosslinking by and of its components. In turn, the precise crosslinking condition of a condensate affects biological function of the molecules acting downstream. In other words, modulating the conden-

sate through its components impacts downstream signal transduction pathways. Second, such novel effects are most accessible to reconstitution study. The intracellular milieu is crowded and active. Only by eliminating confounding factors and placing the reaction environment under experimental control were we able to confidently examine new physicochemical behaviours. The versatility of reconstitution on SLBs allowed us to discover novel phase behaviour, and previously unknown avenues of condensation modulation.

Chapter 2

Signaling protein condensates undergo a transition from 3D to 2D when wetting a lipid membrane

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2.1 Abstract

Biomolecular condensation underlies the formation of membraneless organelles that contribute to important spatial compartmentalization in cells. Condensates composed of proteins and nucleic acids have been described in many settings such as gene regulation, signal transduction, germline cell differentiation, and more. Typically, condensates formed by transmembrane proteins are described as two-dimensional, molecularly thin condensed domains while condensates of cytoplasmic proteins are three dimensional, liquid-like droplets. Reconstituting the disordered scaffold protein Linker for Activation of T Cells, along with its crosslinking factors Grb2 and Son of Sevenless, can result in both two-dimensional and three-dimensional condensates of the same components. Moreover, when formed in the same volume, the three-dimensional droplet can contact the two-dimensional condensate and undergo a novel rearrangement from a three-dimensional phase to a completely two-dimensional condensed phase without an observable wetting stage.

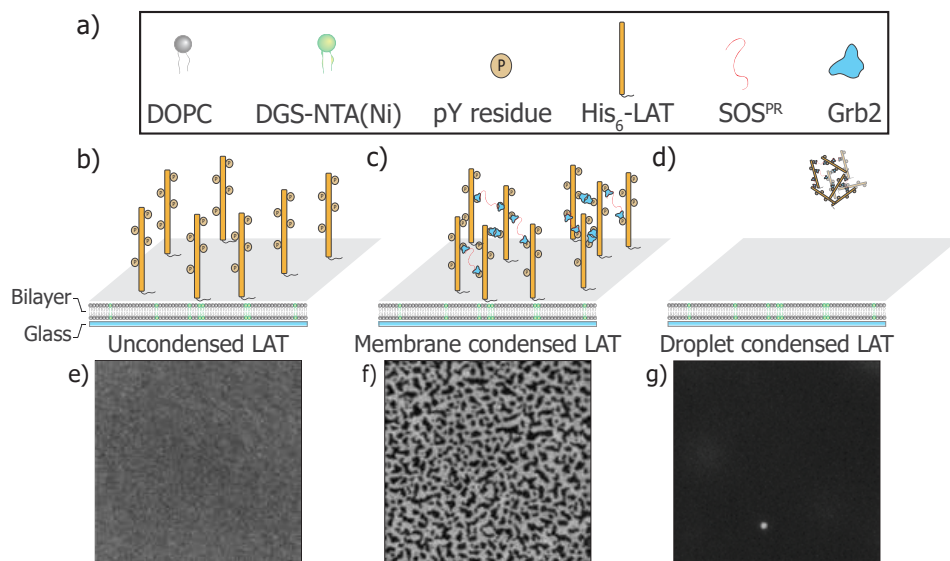


Figure 2.1: Images and schematics of LAT condensate reconstitution. **a)** Key of molecules represented by the graphical elements in schematics **b)-d)**. Schematics of **b)** tyrosine phosphorylated LAT tethered to the SLB (the degree of phosphorylation varies) before condensation, **c)** LAT condensed on the SLB through linkages formed by Grb2 and SOS^{PR}, and **d)** LAT condensed as a droplet in solution through linkages formed by Grb2 and SOS^{PR}. Representative fluorescent images of LAT, labelled with AF-555 channel (see materials and methods), corresponding to the scenario **e)** depicted by **a)**, **f)** depicted by **c)** where the bright domains are condensed LAT, and **g)** depicted by **d)**. Scale bars represent 5 μ m. **e)** and **f)** are TIRF images while **g)** is epifluorescence in the same wavelength

2.2 Introduction

Cellular cytoplasm is known to be spatiotemporally organized by two broad modalities: Membrane encapsulated organelles, and membraneless organelles. Examples of the latter have been subject to intense study in the last two decades with a focus on the underlying phase transition and coexistence physics as the driving principles [2, 3, 8, 13, 16, 80, 81]. Membraneless organelle, or more generally biomolecular condensates have been increasingly appreciated for their demonstrated and putative roles in facilitating biochemistry, signal transduction, membrane organization, and membrane remodeling [3, 26, 76, 82].

Lipid membranes are a ubiquitous surface in biology and are able to regulate both cytoplasmic and membrane-associated protein condensates [83, 84]. Transmembrane proteins with disordered scaffolding domains are a recurrent element in membrane-associated protein condensates [11, 19, 20, 24, 60, 62]. These systems exhibit multivalency in specific binding domains that often rely on additional cytoplasmic proteins to bind and crosslink [26]. In-vitro reconstitution and theoretical modeling of these system has been used to extract the underlying physics of these condensates in the absence of

confounding factors from the cellular milieu [14, 15, 50, 51, 85–87]. Reconstitution of purified components demonstrated that these systems generally do not require membrane attachment to condense, only sufficient protein-protein valency [11, 19, 51, 62]. It has also been shown that the condensation of membrane-attach proteins affects the phase boundary of the underlying lipids [87]. Conversely, membrane surfaces are also able to associate and regulate condensate formed from cytosolic proteins [88]. Evidently, the interaction between protein condensates and lipid membranes has a significant impact on their respective physical properties.

As a model for protein condensates, polymer coacervates have been studied in aqueous two-phase systems (ATPS) for their effect on free-standing lipid membranes. A series of studies has demonstrated multiple coacervate-membrane interaction behaviours including budding, tubulation, ruffling, encapsulation, and wetting [82, 89–93]. Wetting is a notable property in biological systems, and was used to deduce the liquid-like nature of early protein condensates [1]. Wetting of lipid membranes by biomolecular condensates is practically unavoidable within the cellular milieu, and in reconstitution has been noted to deform membranes through interfacial forces [93]. The contribution of interfacial energies is especially interesting considering that many proteins contain domains that bind to specific lipid headgroups. This has been reconstituted by fusing proteins to affinity tags that bind to synthetic lipids or by non-specific charge interactions [88, 93, 94].

Here we describe a novel behaviour of condensates composed of the transmembrane scaffold protein, Linker for Activation of T cells (LAT herein) on supported lipid bilayers (SLBs) when interacting with condensed droplets of the same protein components from the adjoining solution. LAT has been shown previously to condense laterally while attached to SLBs (Figure 1b,e,c,f), and natively in the plasma membrane, as a result of multivalent binding with downstream adaptor proteins and signaling enzymes [19, 24, 25, 50]. We observe that LAT, Grb2, and the proline-rich tail of Son of Sevenless (SOS^{PR} herein) are able to form analogous condensed droplets in solution as when LAT is attached to an SLB (Figure 2.1a,d,g). However, when a three-dimensional and two-dimensional condensate are formed in the same experimental volume, the three-dimensional droplet, on contact with the two dimensional condensates, partially transitions and spreads into a surface-bound, two-dimensional condensate at its outer edges. The result is a patch of condensed protein on the SLB which is indistinguishable from the condensed phase that originated from membrane-bound protein (Figure 2.2). In other words, the combination of condensation and surface binding causes the three-dimensional droplet to rearrange into a mono-layer condensate on the timescale of seconds. This behaviour has been theorized recently [95], but to our knowledge this is the first documented experimental demonstration of such behaviour by a protein system.

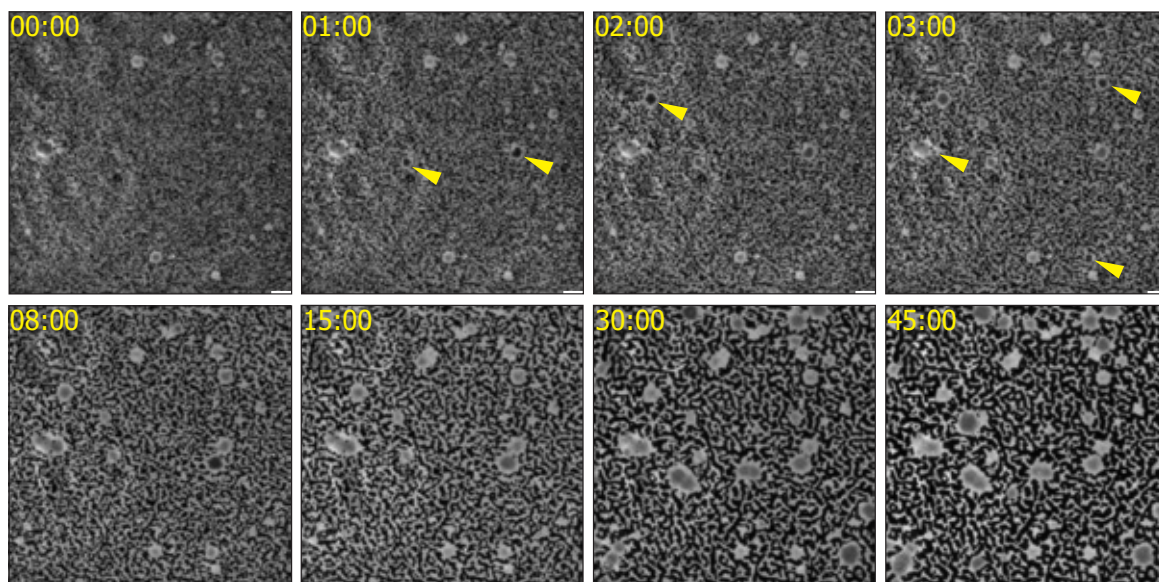


Figure 2.2: TIRF images of the simultaneous early lateral condensation of membrane tethered LAT and deposition of solution LAT droplets onto the bilayer. LAT, labelled with AF-555 channel, is imaged after initiated simultaneous solution and membrane condensation (see materials and methods). Lateral condensation of membrane tethered LAT, as well as depositions of solution condensed LAT are visualized in a time-lapse in this imaging configuration. Early timepoints are shown in the upper row. Time 0 is the start of the image acquisition. Indicators mark new droplets which have deposited from the adjoining solution. Later and long term time points are shown in the bottom row. After deposition, the droplets settle into the membrane condensate and continue to evolve as a surface layer throughout the entire acquisition. Scale bars represent 5 μ m

2.3 Results

We reconstitute our proteins on SLBs produced using the vesicle fusion method on a piranha etched glass coverslip, and enclosed into six individual reaction volumes using a flow cell (ibidi sticky-Slide VI 0.4). We purified the cytoplasmic domain of LAT (residues 27-233) fused to a 6x histidine tag (His-tag) and labeled at cysteine 29 with a C₂-maleimide modified AlexaFluor555 dye (invitrogen) (His₆-LAT555, referred to as LAT herein) on SLBs composed of 96 mol% DOPC (Avanti Polar Lipids, 18:1 (Δ 9-Cis) PC) and 4 mol% 18:1 NTA-DGS(Ni) (Avanti Polar Lipids) lipids (See Materials and Methods for details). When charged with nickel salt, the NTA-DGS lipids will bind to the histidine tails of LAT (and Hck) and further washes will remove all but the proteins that are directly tethered through this affinity interaction which dissociates slowly over the course of hours [57]. Imaging is done primarily in inverted total internal reflection fluorescence (TIRF) configuration with 561 nm illumination through a 1.49 numerical aperture, 100x oil immersion objective. Images of the adjoining solution were taken in epifluorescence configuration at the same wavelength. The average molecular density of tethered LAT was

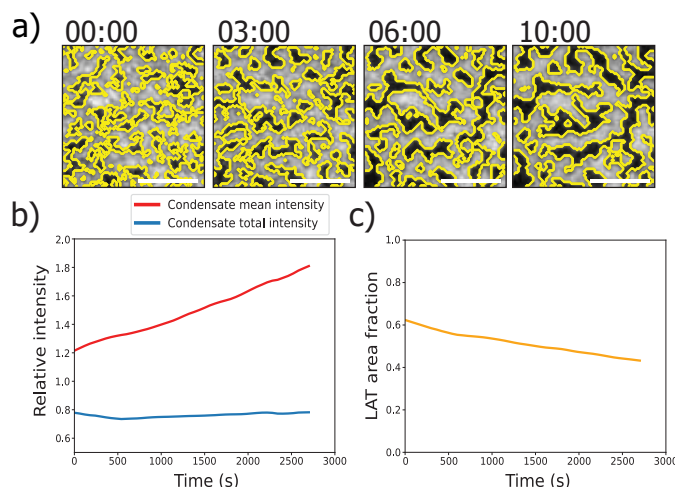


Figure 2.3: Time-lapse and quantification of image ROIs containing only laterally condensing LAT.

LAT imaged as it condenses on the membrane, and segmented through image processing to quantify only the regions containing condensed LAT. **a)** shows a time-lapse of a ROI that is free of any droplet depositions. Only the lateral condensation of membrane-tethered LAT contributes to the time evolution of the images and these boundaries are marked in yellow. **b)** The time trace of the mean (red) and integrated (blue) fluorescence intensity of only the bright regions of the images (bounded by the yellow lines in a), see materials and methods). The scale bars represent $5\mu\text{m}$. Mean condensate intensity corresponds to mean LAT density within the condensate, and increases over time due to the shrinkage of area occupied by the LAT. Conversely, the integrated intensity corresponding to the total number of LAT changes little over time. This indicates that the condensate contains the same amount of LAT which was present at the start of the acquisition and is thus independent of LAT being delivered from elsewhere (i.e. from solution droplets). **c)** The time trace of the number of bright pixels over time reflecting the area occupied by the condensate. As LAT density increases as a result of condensation, the area occupied by the condensate shrinks.

$2000\text{-}3000\mu\text{m}^{-2}$ before condensation from mapping fluorescent image intensity to fluorescence correlation spectroscopy measurements. We simultaneously include a His-tagged truncation of the Src family kinase Hck (residues 78-526) for in-situ phosphorylation of the phosphotyrosine residues of LAT [24, 25, 48, 51]. Surface (two-dimensional) condensation is initiated by exchanging the adjoining solution with buffer containing excess purified Grb2 (full length) and SOS^{PR} (hSOS1 residues 1051-1333) (typically $6\mu\text{M}$ and $2\mu\text{M}$, respectively) [19, 24, 25, 51]. To condense LAT in solution, LAT is preincubated with Hck and ATP in buffer containing Mg^{2+} for at least 2 hours before being diluted with solution containing Grb2 and SOS^{PR} to be injected for imaging. Final concentrations for this reaction are $1\mu\text{M}$ LAT, $45\mu\text{M}$ Grb2, and $3\text{-}15\mu\text{M}$ SOS^{PR} (See materials and methods for detailed protocols). This mixture is injected over an uncondensed LAT-tethered bilayer for simultaneous surface and solution condensation.

Simultaneous reconstitution of LAT condensation in solution and on the bilayer

On initiating the simultaneous condensation, two-dimensional phase transition into the laterally condensed state is visually observed after a delay [51]. The condensation phase transition progressed throughout the course of the experiment (20-30 minutes of observation). We imaged the experiment entirely in TIRF configuration at a slow capture rate of one image every minute to mitigate photodamage to the fluorophores and proteins over time. Slow dynamics enabled the capture of the evolution of the condensation even with a slow frame rate.

In parallel with the two-dimensional condensation, we observed that droplets which have condensed from the components in solution will impact the bilayer. This type of event is visualized through TIRF as the emergence of a rapidly spreading fluorescent patch (Figure 2.2). After a brief equilibration period, usually 1-2 frames, the patch which originated from solution reaches a stable morphology on the membrane and continues to behave as part of the two-dimensional condensate on the bilayer. Individual droplets spread in a roughly elliptical shape, and seem to combine with the coincident surface condensate. The resulting patches range from a couple microns to upwards six microns in diameter. New droplet depositions occur throughout the course of the observation time, and can intrude on the space of a previously spread droplet. Deposited droplets exhibit a dark central region which we believe indicates the presence of remaining bulk condensate adhered to the membrane surface and contributes material to the expanding surface condensate.

Concurrent membrane-bound condensation of LAT on the bilayer

We focus on a region of interest (ROI) in which the condensate originates and evolves only from laterally condensing LAT and no droplet depositions occur within (Figure 2.3). This is analogous to the form of LAT condensation observed previously [19, 24, 25, 50, 51]. We followed the evolution of the condensate by segmenting each frame using a local thresholding algorithm (Fiji ImageJ, see materials and methods). Briefly, a binary mask is produced and used to reduce the fluorescence intensity in the LAT-sparse phase to strictly zero. Thus all fluorescence intensity in the resultant images were collected from the LAT-dense phase. We plotted the time traces of the mean and integrated fluorescence intensities in each frame (Figure 2.3b) which corresponds to the mean LAT density and total LAT molecules, respectively, in each frame. This correspondence is roughly linear as previously observed through calibration against two-dimensional fluorescence correlation spectroscopy [51]. However, when condensed the density of fluorophore may be high enough to exceed the theoretical limit of linearity of the measurement. The area occupied by the LAT condensates is reflected by the number of non-zero pixels in the binarized image (Figure 2.3c). The mean intensity trace is plotted relative to the mean intensity of an image taken before any condensing molecules are added. This value should be

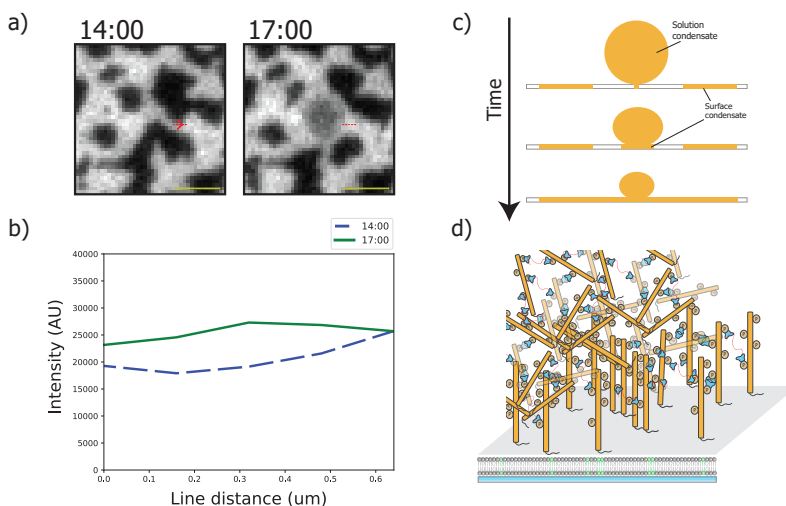


Figure 2.4: Images, quantification, and schematics of the LAT droplet deposition process. Analyses of an ROI centered around a single LAT deposition as visualized in the images. **a)** Images long before (left) and immediately after (right) the deposition of a LAT droplet occurring as the surface condensate evolves. Scale bar corresponds to 2 μm . Red line shows the coordinate of the linescan. The arrow in the left images shows the direction of the scan coordinate. **b)** are the line-scans of the images as indicated in the legend. **c)** a graphical representation of droplet deposition as imaged in a) but in the side view including bulk solution. The explicit details of the SLB are not shown both due to the scale of the images and for clarity. Note that despite being represented as a rectangular bar, the 2D LAT condensate is a single-molecule layer. **d)** a molecular graphic of how the droplet transitions into a surface condensate as it contacts the bilayer and decorated condensate. This represents a theoretical zoom-in on the corner of the droplet shown in the lowest panel in c) where the droplet, surface, and bulk surroundings all meet.

independent of the size of the ROI. Conversely, the integrated intensity is plotted relative to the integrated intensity of an area-matched ROI of an image before condensation.

The mean condensate density increased over time corresponding to the decrease in condensate area (Figure 2.3b (red), c). The integral LAT content did not change appreciably over time. This confirms that the amount of LAT being incorporated into the condensate is the same as visualized in the first frame (Figure 2.3a). Importantly, no LAT has been added to this region from any droplet depositions occurring elsewhere on the bilayer. This confirms that the two forms of condensates can evolve independently of each other when spatially separated.

LAT from a three dimensional droplet transitions into a two dimensional condensed layer on contact with the bilayer

To examine the nature of the droplet depositions, we examined the TIRF intensity of the boundary of growing LAT patches (Figure 2.4). TIRF selectively illuminates fluorophores proximal to the interface of water (below the SLB) well below a micron away from the

interface axially. This excludes the majority of a three-dimensional droplet in contact with the bilayer, but would illuminate a wetting layer of significant thickness composed of LAT molecules. In the images, such a wetting layer would present as an extremely bright patch. We would expect this intensity to rapidly decrease at the boundary between the spreading droplet and the laterally condensed LAT which is composed of a single-molecule layer of LAT (and attached fluorophore) only. Instead, taking an intensity line-scan of the expanding edge of the deposited patch, we see no such boundary. Instead the intensity of the growing LAT patch matched that of the laterally condensed LAT.

Since no sharp changes in TIRF intensity were observed around a droplet deposition (Figure 2.4b), this indicated that the growing patch had already rearranged into a layer with similar LAT density as the surrounding lateral condensate as sketched in Figure 2.4c. Furthermore, since the lateral condensate is composed only of a single layer of LAT molecules, the patch likely is also molecularly thin (Figure 2.4d). This change in dimensionality of the condensate occurred faster than the time lapse resolution of our acquisition, on the timescale of seconds. These aspects together suggest an unusual form of wetting: The three-dimensional condensed phase droplet of LAT, on contact with the bilayer presenting an analogous two-dimensional condensed phase and along with the lipid affinity His-tags, rapidly rearranged into a fully two-dimensional phase and incorporated into the surrounding phase. Based on the images, we surmise that the LAT droplet partially wets the bilayer as a thick bulk layer, but also progressively transfers molecules onto the membrane surface as a growing two dimensional condensate. The transition from three to two dimensions is indicated by the flush fluorescence intensity at the expanding edge of the droplet, while a central dark spot remains due to self-quenching of a high density of fluorophores as the point of contact between droplet and surface.

2.4 Discussion

In this work we present novel condensed phase behaviour exhibited by the LAT condensate system reconstituted on SLBs: When condensed both in solution and while attached to the membrane within the same volume, the solution droplets can deposit and rearrange into a membrane condensate. In other words, the three-dimensional condensate changes into a two dimensional condensate. This occurs on the seconds timescale, and without going through an observable intermediate spreading phase which would still be a three-dimensional phase.

Components of a natively transmembrane signaling condensate similar to LAT have been shown to form droplets also when reconstituted in solution [11, 62, 96]. Furthermore the effect of a nearby SLB membrane on the phase coexistence of otherwise-solution droplets has been reported [88]. Conversely, the effect of protein condensates on the morphology of unsupported membranes (giant vesicles) has separately been reported for solution

condensates without lipid binding motifs [82] and membrane condensates reconstituted similar to our system [94]. In our work we show how a condensate can behave when it is facilitated of a small and discrete number of crosslinking sites, and reconstituted with a lipid binding motif. The presence of and affinity interaction with the membrane, and a surface which is already decorated with a two dimensional condensate results in a three dimensional condensate transitioning into a two dimensional condensate in the process of wetting.

Our observations have a clear connection to the phenomenon of wetting, where a bulk, liquid droplet suspended in a medium contacts, adheres, and spreads on a solid surface. Wetting is characterized by a contact angle of the droplet boundary to the surface. Typical wetting layers formed by a spreading droplet have macroscopic thickness [93, 97, 98]. As mentioned above, if this were the case for our system, we would observe a stark change in fluorescence intensity where the LAT droplet interfaces with the surrounding surface condensate but remains macroscopically (or mesoscopically) thick. The observation that a molecularly thin layer instead emerges from the droplet bears resemblance to the phenomena of precursor films [97, 99], prewetting [100], or partial wetting with surface binding [95]. Further experimentation is needed to confirm whether our system conforms to either of these concepts. At the moment, no molecular description of wetting yet accounts for the limited-valency, non-covalent crosslinks that enable signaling condensates like LAT to condense. We have seen that the binding and unbinding kinetics of these crosslinks is the determining factor of the condensed phase behaviour [51].

2.5 Materials and Methods

Reagents

His-tagged LAT and Hck, along with Grb2 and SOS^{PR} were cloned and purified in-house (see "DNA cloning and protein purification"). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)-succinyl] (nickel salt) (DGS-NTA(Ni²⁺)) were purchased from Avanti Polar Lipids. AlexaFluor555 C₂-maleimide, 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, Tris-(2-carboxyethyl)phosphine (TCEP), and EDTA-free Pierce Protease inhibitor tablets were purchased from Thermo Fisher Scientific. Bovine serum albumin (BSA), β -mercaptoethanol, NiCl₂, H₂SO₄, ATP, DNase, and Kanamycin sulfate were from Sigma-Aldrich. MgCl₂ was from EMD Millipore. Tris buffered saline (TBS) was purchased from Corning. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was from Gold Biotechnology. 30 % H₂O₂, MCB grade glycerol and imidazole were from Fisher Scientific.

DNA cloning and protein purification

BL21-CodonPlus strain E.coli were purchased from Agilent. All DNA constructs utilized pET28a (Novagen) or pETM (similar to Addgene #108943) backbones under the T7 promoter for expression in E. Coli. Plasmids were amplified in XL1-Blues strain E.coli (QB3-Macrolab). All constructs were transformed, heat shocked, plated, and grown under Kanamycin selection. All purifications began with lysis at 15000 psi through an EmulsiFlex-C3 high pressure homogenizer (Avestin) in lysis buffer typically containing 75 mM NaH₂PO₄ pH 8.0, 300-700 mM NaCl, 2 mM β -mercaptoethanol, DNase, 5-10 mM imidazole, and 1-2 protease inhibitor cocktail tablets. Proteins were typically stored at -80 °C in 20 mM Tris-HCl (pH 8 at 4 °C, 200 mM NaCl, 1 mM TCEP) and 10% glycerol after a final size-exclusion chromatography polishing step (Superdex 75 or 200 column, 120 mL bed volume, Cytiva) and flash-freezing in liquid N₂.

Grb2 Full length human Grb2 was expressed with an N-terminal 6x histidine tail and cleavable tag (TEV protease, QB3-Macrolab). Purification was as specified previously [48] with the exception of a lack of heat treatment post-purification. Purified Grb2 was particularly susceptible to aggregation at high concentrations and was store at no higher than 100 μ M.

LAT Human LAT residues 27-233 with a C117F mutation (for site specific labeling) was expressed with an N-terminal 6x histidine tail and cleavable tag (TEV). No purifications of this construct included a TEV protease cleavage step. This construct was otherwise purified as described previously [101].

SOS^{PR} Human Son of Sevenless (residues 1051-1333) was expressed with a cleavable (TEV) N-terminal 6 histidine tag and fused Maltose Binding Protein. Of special note, it is essential this construct be purified by an ion exchange step (MonoS column, 1 mL bed volume, Cytiva) with high amounts of reducing agent (>10 mM BME for example, or a more potent agent like DTT). SOS^{PR} seems to form strong adducts with MBP and bacterial contaminants which can strongly affect results downstream, but these adducts can be disrupted by ionic differences and reducing agents. SOS^{PR} was cleaved with TEV to remove the N-terminal tags before use. Purification was otherwise as described previously [24].

Hck The Src module (SH2-SH3-catalytic domain), residues 78-526, of the kinase Hck was expressed with a cleavable (TEV) N-terminal 6x histidine tag. This purification utilizes a strategy for high-yield kinase expression in bacteria described previously [102]. Briefly, the Hck plasmid is expressed in e.coli previously transformed with a construct containing the phosphatase YopH under simultaneous Streptomycin selection. Of note, the YopH construct exhibits an unspecified, stubborn affinity for the Hck protein as well as nickel and cobalt affinity moieties found in affinity column matrices. Together with its similar molecular weight to Hck, this makes trace contaminant YopH a common occurrence

in such a purification. This is problematic as YopH is a potent tyrosine phosphatase, and stable tyrosine phosphorylation is a pre-requisite for LAT condensation [51]. YopH contamination can be mitigated by using high salt washes (650 mM NaCl) while Hck is bound to an affinity column. This is also preferably done while attached to a purification system, such as a Cytiva AKTA system, to monitor the 280nm absorbance profile of the flow through. Slow ion-exchange gradient separation is also helpful for this purpose. Fresh β -mercaptoethanol is necessary during SDS-PAGE analysis of the purified protein as Hck exhibits a concentration dependent dimer band.

Fluorescent labeling of LAT

LAT was labeled at cysteine 29 with thiol-reactive AlexaFluor555 following a variation of the manufacturer protocol. Briefly, LAT was diluted to 200 μ M with 5 mM TCEP, then allowed to react with the maleimide dye (2-3x molar excess) for 2 hrs at room temperature before quenching with 10 mM β -mercaptoethanol. Excess free dye was initially removed by gravity desalting columns (PD10, Cytiva) and further by size-exclusion (Superdex 75 increase, 24 mL bed volume, Cytiva). Of special note, we have observed LAT to appear as multiple SEC peaks (up to 3 or more), but these do not seem to be distinct populations for our purposes (if isolated, they all behave nominally the same on SLBs). We suggest simply pooling all SEC peaks of LAT (given they are sufficiently pure). Labeled fraction was determined by comparison of absorption at the dye's λ_{max} to the protein's UV absorption at 280 nm (after correction for the dye's contribution at 280 nm). This protocol can be found on the manufacturer manual.

SLB preparation

SUV preparation SLBs were produced using the vesicle fusion method similar as described previously [53, 57, 103]. Briefly, mixtures of 96:4 molar ratio DOPC and DGS-NTA(Ni^{2+}) were prepared from fresh ampules in a piranha etched 25 mL round bottom flask. If direct visualization of the bilayer was desired, a small amount of DOPC (0.005%) was exchanged for TR-DHPE. This mixture is dried under rotary evaporation (Buchi R-200) until the external condensation clears indicating no further evaporative cooling. If fluorescent lipids were included, aluminum foil was used to mitigate ambient photobleaching. In some cases, the lipid film is further immersed in a 37 °C water bath. For this composition, the bath made no systematic difference in the results. The dried lipid film is further dessicated under a stream of N_2 gas for 20 minutes minimum. Lipid films were rehydrated through vortexing in milliQ water (IQ 7000, EMD Millipore) to create a 2mL suspension at 1 mg/mL concentration. This suspension was transferred to a 5 mL round-bottom polystyrene tube (Falcon) for tip sonication using a polished 3mm stepped tip at 4 °C (Sonics VCX750 - 33% amplitude, 20 s sonication, 50 s rest, 5 cycles) to yield small unilamellar vesicles (SUVs). Clarification of the suspension indicated success. This sus-

pension is centrifuged at 20,000 g and 4 °C for 20 minutes before being stored airtight. 96:4 SUVs showed no quality deterioration for up to a week.

Precision Schott D 263 M glass coverslips (#1.5H, 75 x 25 mm, custom ordered from ThorLabs, similar to Part# CG15CH) were bath sonicated for 15 minutes while immersed in a 50/50 mixture of milliQ water and isopropanol before being thoroughly rinsed with fresh milliQ water. This removes solid and inorganic contaminants while ensuring no organic isopropanol remains adhered to the coverslips for the following step. Fresh piranha solution was prepared from a 3:1 mixture of concentrated H_2SO_4 and 30 % H_2O_2 with slow mixing of the latter into the former to prevent violent exothermic boiling. The coverslips were immersed in this solution for at least three minutes and no more than five. Insufficient clearance of isopropanol in the previous step can result in a violent reaction. Immediately after immersion, the coverslips are drained of piranha and continuously refreshed with a stream of milliQ water for several minutes. The dual-purpose etch step eliminates all organic contaminants and alters the surface chemistry of the glass to promote hydrophilicity. The coverslips can be stored in milliQ water for upwards of one week. Spontaneous degradation of the hydrophilic moieties is the limiting process in the storage life.

Chambers were prepared by adhering a 6-channel flow cell (ibidi sticky-slide VI 0.4) to a coverslip dried with N_2 gas and cured over a 37 °C hotplate for 20 minutes under a weight. Too long a curing can prematurely degrade the hydrophilic surface. SUVs were diluted to 0.5 mg/mL in buffer (see "protein tethering and imaging conditions" below) before being flowed over each flow cell channel and allowed to incubate for 30 minutes for SUV rupture. Unruptured SUVs were washed and 1 mg/mL BSA was applied and allowed to incubate for a maximum of 10 minutes to block defects (open glass) in the now-formed SLB. Too long of an incubation will begin to dissociate the SLB. In some instances, 100 mM NiCl_2 was used to ensure the NTA moieties were charged with nickel ions. Skipping this step did not affect the results of our experiments appreciably. The bilayer was washed between all steps.

Protein tethering and imaging conditions

Herein, individual channels of each flow cell containing an SLB will be referred to collectively as "the bilayer". SUVs were deposited after being diluted 1:1 with a 2x TBS solution. The bilayer was washed with excess buffer (1xTBS with 1mM TCEP) between each incubation step. His-tagged LAT and Hck were partially diluted in wash buffer before being centrifuged at 20,000 g and 4 °C for 20 minutes to remove aggregates. The supernatant was further diluted to working concentration and flowed over the bilayer and allowed to incubate for 45 minutes. Density can be semi-quantitatively controlled via the incubation concentration or time [57]. Typical working concentrations were 250 nM of LAT and 30 nM of Hck. After a wash, a dual purpose injection of 1 mM ATP (in TBS, 1 mM TCEP, 5 mM

MgCl₂) served to allow desorption of weakly bound protein and initial tyrosine phosphorylation of LAT by Hck [57]. The inclusion of 1 mM activated sodium orthovanadate can be used to inhibit tyrosine phosphatase activity. After an additional wash, the remaining tethered proteins are stable for upwards of an hour and ready to image immediately.

Imaging injections were prepared of 1x TBS with 5 mM MgCl₂, 10 mM β -mercaptoethanol, 1mM ATP, and 0.1 mg/mL BSA along with the desired concentration of solution protein (Grb2, SOS^{PR}, LAT) and sodium orthovanadate if necessary. Injections were typically 100 μ L per channel with concentration adjustments made as needed. For this work, a glucose-oxidase/catalase oxygen scavenger system was not used due to its acidification of the solution over long observation periods [24, 104].

Imaging hardware and acquisition

Hardware TIRF imaging experiments were performed on an inverted Nikon Eclipse Ti microscope using a 100X Nikon oil-immersion TIRF objective (1.49 NA). Micro-positioning was done using a motor-controlled stage (MS-2000, ASI). Images were acquired with an iXon Ultra 897 EMCCD camera (Andor Technology). Fluorescent LAT was excited (0.25 mW at the objective) with a 561 nm diode laser (OBIS laser diode; Coherent) controlled with a custom laser driver (Solamere Technology) using TTL signals from the camera. The laser was collimated with the objective position at the approximate sample z-focus before every experiment. A single quad-band dichroic cube was used for all acquisitions (ZT405/488/561/ 640rpcv2, Chroma). Fluorescence emission was additionally filtered by an ET600/50M filter (Semrock). Microscope and accessory hardware were controlled using Micro-Manager, version 4.0 [105]. Epifluorescence illumination is achieved using a mercury arc lamp, and wavelengths were selected by a 480/40-nm filter cube (FITC HYQ filter; Nikon) for a 488-nm channel and by a 560/55-nm filter cube (Texas Red HYQ filter; Nikon) for a 568-nm channel. Epifluorescence images were not used for quantification.

Imaging conditions and settings Experiments were conducted at ambient temperature, 21-23 °C. Unless indicated otherwise, images were acquired with a 30 ms camera exposure and 100 election multiplier gain, with 1 minute delays between time points for time-lapse measurements. Background images (5-25) were collected before the addition of condensing agents to be used for field illumination (“shade”) correction of non-uniform illumination and optical aberration. Dark images (with no LAT tethered) taken with identical settings and conditions were used for background subtraction. Corrections and quantitative image analyses were done with Fiji ImageJ [106] and custom Python scripts.

Image processing and analysis

Image processing and segmentation Image processing was done primarily in the Fiji distribution of ImageJ [106]. In particular, the *auto local threshold* function was used

with the Phansaulker algorithm with a 10 pixel radius to generate binary masks of the condensate images. Segmentation was further performed by multiplying each frame of raw data with its mask. All images were analyzed after shade correction and background subtraction. No images were filtered or scaled before analysis.

Image feature analysis The spatial scale of the images was set by the pixel size of our detector at 16 microns/pixel or 6.25 pixel/micron with 100x magnification. The measurement functions in ImageJ were used to generate time traces where practical, and further operations and plotting were done with custom Python scripts. Notably, line-scans were generated using the *measure.profile_line* function in the *scikit-image* package with 1 linewidth and no interpolation. No line-scans were performed diagonally across pixels.

Image presentation and data plotting All contrast adjustment was done for visual presentation only. Normalization was done as follows: Mean condensate intensity was normalized against the mean intensity of an uncropped, uncondensed LAT image after correction; integrated condensate intensity was normalized against the integrated intensity of an ROI of uncondensed LAT, with identical dimensions, after correction; finally, condensate area was normalized against the theoretical area of the full ROI (i.e. the area of the XY plane). Plots were smoothed using local regression smoothing via the *lowess* function in the *statsmodels.nonparametric.smoothers_lowess* module.

Chapter 3

A two-component protein condensate of the EGFR cytoplasmic tail and Grb2 regulates Ras activation by SOS at the membrane

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3.1 Abstract

We reconstitute a phosphotyrosine-mediated protein condensation phase transition of the ~200 residue cytoplasmic tail of the epidermal growth factor receptor (EGFR) and the adaptor protein, Grb2, on a membrane surface. The phase transition depends on phosphorylation of the EGFR tail, which recruits Grb2, and crosslinking through a Grb2-Grb2 binding interface. The Grb2 Y160 residue plays a structurally critical role in the Grb2-Grb2 interaction, and phosphorylation or mutation of Y160 prevents EGFR:Grb2 condensation. By extending the reconstitution experiment to include the guanine nucleotide exchange factor, SOS, and its substrate Ras, we further find that the condensation state of the EGFR tail controls the ability of SOS, recruited via Grb2, to activate Ras. These results identify an EGFR:Grb2 protein condensation phase transition as a regulator of signal propagation

from EGFR to the MAPK pathway.

3.2 Introduction

Recently, a class of phenomena known as protein condensation phase transitions has begun to emerge in biology. Originally identified in the context of nuclear organization [4] and gene expression [17], a distinct two-dimensional protein condensation on the cell membrane has now been discovered in the T cell receptor (TCR) signaling system involving the scaffold protein LAT [19, 24, 107]. TCR activation results in phosphorylation of LAT on at least four distinct tyrosine sites, which subsequently recruit the adaptor protein Grb2 and the signaling molecule PLC γ via selective binding interactions with their SH2 domains. Additional scaffold and signaling molecules, including SOS, GADS, and SLP76, are recruited to Grb2 and PLC γ through further specific protein–protein interactions [108, 109]. Multivalency among some of these binding interactions can crosslink LAT molecules in a two-dimensional bond percolation network on the membrane surface. The resulting LAT protein condensate resembles the nephrin:NCK:N-WASP condensate [62] in that both form on the membrane surface under control of tyrosine phosphorylation and exert at least one aspect of functional control over signaling output via a distinct type of kinetic regulatory mechanism [25, 60, 110]. The basic molecular features controlling the LAT and nephrin protein condensates are common among biological signaling machinery, and other similar condensates continue to be discovered [20, 111]. The LAT condensation shares downstream signaling molecules with the EGF-receptor (EGFR) signaling system, raising the question if EGFR may participate in a signaling-mediated protein condensation itself.

EGFR signals to the mitogen-activated protein kinase (MAPK) pathway and controls key cellular functions, including growth and proliferation [112–114]. EGFR is a paradigmatic model system in studies of signal transduction, and immense, collective scientific effort has revealed the inner workings of its signaling mechanism down to the atomic level [115]. EGFR is autoinhibited in its monomeric form. Ligand-driven activation is achieved through formation of an asymmetric receptor dimer in which one kinase activates the other to phosphorylate the nine tyrosine sites in the C-terminal tails [115, 116]. There is an obvious conceptual connection between EGFR and the LAT signaling system in T cells. The $\sim$ 200-residue-long cytoplasmic tail of EGFR resembles LAT in that both are intrinsically disordered and contain multiple sites of tyrosine phosphorylation that recruit adaptor proteins, including Grb2, upon receptor activation [117]. Phosphorylation at tyrosine residues Y1068, Y1086, Y1148, and Y1173 in the EGFR tail creates sites to which Grb2 can bind via its SH2 domain. EGFR-associated Grb2 subsequently recruits SOS, through binding of its SH3 domains to the proline-rich domain of SOS. Once at the membrane, SOS undergoes a multistep autoinhibition-release process and begins to catalyze nucleotide exchange of RasGDP to RasGTP, activating Ras and the MAPK pathway [73].

While these most basic elements of the EGFR activation mechanism are widely accepted, larger-scale features of the signaling complex remain enigmatic. A number of studies have reported higher-ordered multimers of EGFR during activation, including early observations by Förster Resonance Energy Transfer and fluorescence lifetime studies [118–120], as have more recent studies using single molecule [121, 122] and computational methods [123]. Structural analyses and point mutation studies on EGFR have identified a binding interface enabling EGFR asymmetric dimers to associate [124], but the role of these higher-order assemblies remains unclear. At the same time, many functional properties of the signaling system remain unexplained as well. For example, EGFR is a frequently altered oncogene in human cancers, and drugs (including tyrosine kinase inhibitors) targeting EGFR signaling have produced impressive initial patient responses [125]. All too often, however, these drugs fail to offer sustained patient benefits, in large part because of poorly understood resistance mechanisms [126]. Physical aspects of the cellular microenvironment have been implicated as possible contributors to resistance development [127], and there is a growing realization that EGFR possesses kinase-independent (e.g., signaling independent) prosurvival functions in cancer cells [128]. These points fuel speculation that additional layers of regulation over the EGFR signaling mechanism exist, including at the level of the receptor signaling complex itself.

Here we report that EGFR undergoes a protein condensation-phase transition upon activation. We reconstituted the cytoplasmic tails of EGFR on supported bilayers and characterized the system behavior upon interaction with Grb2 and SOS, using total internal reflection fluorescence (TIRF) imaging. This experimental platform has been highly effective for revealing both phase-transition characteristics and functional signaling aspects of LAT protein condensates [15, 19, 24, 25, 50, 51]. Published reports on the LAT system to date have emphasized SOS (or the SOS proline-rich [SOS^{PR}] domain) as a critical crosslinking element. Titrating the SOS^{PR} domain into an initially homogeneous mixture of phosphorylated LAT and Grb2 revealed a sharp transition to the condensed phase, which we have also observed with the EGFR:Grb2:SOS system. Under slightly different conditions, however, we report observations of an EGFR:Grb2 condensation-phase transition without any SOS or other crosslinking molecule. We show that crosslinking is achieved through a Grb2–Grb2 binding interface. Phosphorylation on Grb2 at Y160 as well as a Y160E mutation (both reported to disrupt Grb2–Grb2 binding [64, 129]) were observed to prevent formation of EGFR condensates. We note that the evidence of Grb2–Grb2 binding we observed occurred in the context of EGFR-associated Grb2, which is localized to the membrane surface; free Grb2 dimers are not necessary.

The consequence of EGFR condensation on downstream signaling is characterized by mapping the catalytic efficiency of SOS to activate Ras as a function of the EGFR condensation state. SOS is the primary Ras guanine nucleotide exchange factor (GEF) responsible for activating Ras in the EGFR-to-MAPK signaling pathway [130–133]. At the membrane, SOS undergoes a multistep process of autoinhibition release before begin-

ning to activate Ras. Once fully activated, SOS is highly processive, and a single SOS molecule can activate hundreds of Ras molecules before disengaging from the membrane [71, 74, 75]. Autoinhibition release in SOS is a slow process, which necessitates that SOS be retained at the membrane for an extended time in order for Ras activation to begin [24, 25]. This delay between initial recruitment of SOS and subsequent initiation of its Ras GEF activity provides a kinetic proofreading mechanism that essentially requires SOS to achieve multivalent engagement with the membrane (e.g., through multiple Grb2 or other interactions) in order for it to activate any Ras molecules.

Experimental results described here reveal that Ras activation by SOS is strongly enhanced by EGFR condensation. Calibrated measurements of both SOS recruitment and Ras activation confirmed enhanced SOS catalytic activity on a per-molecule basis, in addition to enhanced recruitment to the condensates. These results suggest that a Grb2-mediated EGFR protein condensation-phase transition is a functional element controlling signal propagation from EGFR downstream to the MAPK signaling pathway.

3.3 Results

Reconstitution of a membrane-surface EGFR:Grb2 protein condensate.

We reconstituted an EGFR:Grb2 protein condensation-phase transition on supported membranes using the C-terminal region of EGFR (residues 991 to 1186, where the signal peptide of EGFR is not included in numbering), which is expressed along with a His6 tag and a SUMO tag at the N-terminal end of the construct. Throughout this article, we refer to this construct of the cytoplasmic tail of EGFR as the EGFR^{TAIL}. EGFR^{TAIL} was anchored on the supported lipid bilayer through binding between its His tag and nickel-nitrilotriacetic acid (Ni-NTA) lipid (incorporated into the supported bilayer at a 4% molar ratio with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipid as the primary component), following previously published methods [24, 25]. The small ubiquitin-related modifier (SUMO) fusion tag was used to increase the expression yield of the disordered EGFR^{TAIL} and acted as the spacer between the surface of the bilayer and the tail of EGFR, where the native kinase domain of EGFR would normally be positioned. The EGFR^{TAIL} was fluorescently labeled with Alexa Fluor 488 through maleimide chemistry, and we achieved 66% labeling efficiency. EGFR^{TAIL} constructs anchored to the supported membrane underwent simple Brownian motion ($D = 1.76 \mu\text{m}^2/\text{s}$, measured by the fluorescence recovery after photobleaching experiments) and appeared to be uniformly distributed on the membrane surface under TIRF microscopy. The lateral density of the EGFR^{TAIL} on the membrane can be precisely measured by fluorescence correlation spectroscopy (FCS) [24, 134] and was controlled to between 50 and 3000 molecules/ μm^2 in the experiments presented here. To maintain the EGFR^{TAIL} in its active, phosphorylated state, a separate Src-family kinase

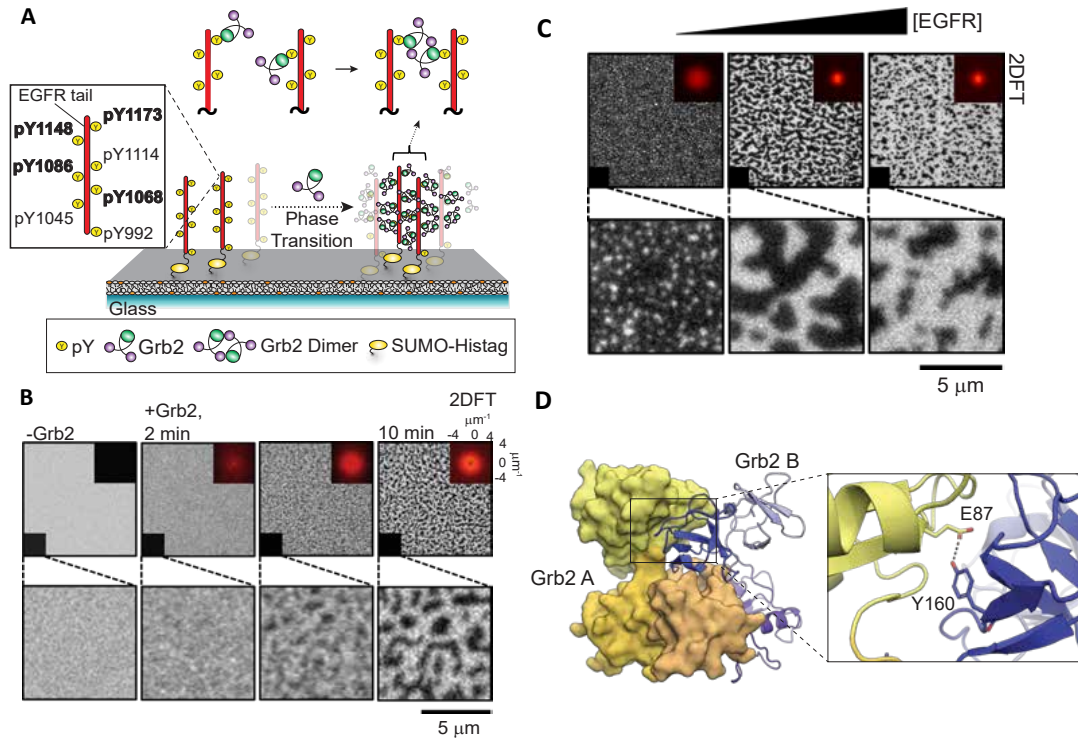


Figure 3.1: EGFR^{TAIL} phase transition induced by the dimerization of Grb2. All images are of EGFR^{TAIL} labelled in the 488 nm channel in TIRF configuration. (A) The schematic of the reconstitution experiment. The phosphorylated EGFR^{TAIL} is tethered on the supported membrane. EGFR^{TAIL} has several tyrosine sites that can be phosphorylated, including Tyr-1068, Tyr-1086, Tyr-1148, and Tyr-1173, related to Grb2 recruitment. Grb2 is injected into the chamber and recruited by EGFR^{TAIL}. Grb2 is shown to dimerize on the supported membrane. The dimerization of Grb2 drives the assembly of EGFR^{TAIL}:Grb2 into phase transition. EGFR^{TAIL} is initially in the state where it is mobile on the supported bilayer (Left). EGFR^{TAIL} becomes part of the protein condensate after the phase transition is triggered (Right). (B) TIRF images of Alexa Fluor 488-labeled EGFR^{TAIL} on the supported bilayer, revealing a phase transition within 2 min after the addition of Grb2 to form a condensed phase of 950 molecules/ μm^2 density. The protein condensate of EGFR^{TAIL}:Grb2 continued to mature over 10 min to achieve densities of $\sim 3,760$ molecules/ μm^2 . (Bottom) Magnified images of the lower-left corner in the images in the top row. (Top) The inserts in the upper right corners are the two-dimensional Fourier transform (2DFT) of the TIRF images, which confirm that the structure of the condensate is isotropic. (C) The macroscopic fractional area coverage of the EGFR^{TAIL}:Grb2 condensate, as well as its morphological features, scale with the density of EGFR^{TAIL}. Image data here, spanning from 50 to 3,000 molecules/ μm^2 , illustrate EGFR^{TAIL}:Grb2 condensate variation from small clusters (Left) to large interconnected domains (Right). (D) The crystal structure of Grb2 shows the asymmetric dimer (PDB:1GRI). The tyrosine 160 at the dimeric interface is illustrated in the magnified picture.

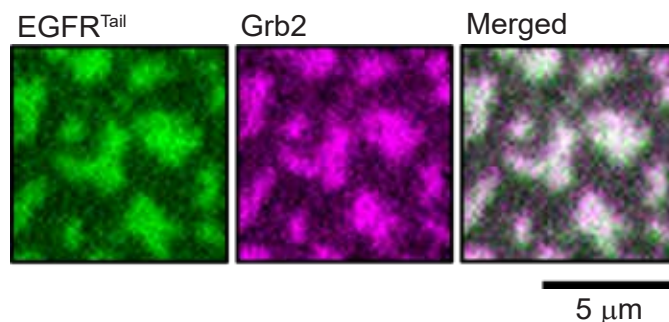


Figure 3.2: The colocalization between EGFR^{TAIL} and Grb2 after EGFR^{TAIL}:Grb2 phase transition is achieved. Unlabeled Grb2 mixed with Alexa Fluor 647-labeled Grb2 (0.2 μ M; labeling efficiency, 63%) was added to phosphorylated Alexa Fluor 488-labeled EGFR^{TAIL} to induce the phase transition of EGFR^{TAIL}:Grb2. The TIRF images were taken 10 min after the addition of Grb2. The first image (green) is from EGFR^{TAIL}. The second image (magenta) is from Grb2. The third image is the merged image from EGFR^{TAIL} and Grb2.

protein, Hck, was tethered to the membrane (also through His tag chemistry) and adenosine triphosphate (ATP) was provided in solution. Src kinases do not normally phosphorylate the EGFR^{TAIL} efficiently [117], but in this system, the preincubation with Hck sufficed to ensure robust phosphorylation of the tail. A similar strategy was used previously to maintain LAT in its phosphorylated state for condensation phase transition studies [15, 24, 86]. Grb2 and, in later experiments, SOS were added to the prephosphorylated EGFR on supported membranes to initiate the condensation phase transition. Upon addition of Grb2, the EGFR^{TAIL} underwent a robust condensation phase transition, as illustrated schematically in Figure 3.1A. Images tracking the phase transition process (Figure 3.1B) show an initially homogeneous distribution of the EGFR^{TAIL}, which underwent a coarsening process after injection of Grb2 (6 μ M) to form a dense phase (bright in these images) interspersed with a sparse phase containing a very low density of monomeric EGFR^{TAIL}. The domains exhibited undulating shapes and a sharp discontinuity in EGFR^{TAIL} density at the boundaries of the two phases. The Grb2-driven condensation of EGFR^{TAIL} occurred over a range of initial densities of the tails to form condensed phases of similar density but different overall area coverage. At lower EGFR^{TAIL} initial density, isolated circular EGFR^{TAIL}:Grb2 condensates were observed, while fully percolating domain patterns were observed at higher densities (Figure 3.1C). The presence of both Grb2 and EGFR^{TAIL} in the condensates was confirmed by separately labeling them with Alexa Fluor 488 (EGFR^{TAIL}) and Alexa Fluor 647 (Grb2), respectively, and performing two-color imaging (Figure 3.2).

The EGFR^{TAIL}:Grb2 condensation is reversible and can be dispersed by addition of phosphatase. Addition of the phosphatase YopH (1 μ M YopH and 6 μ M Grb2 in bulk solution) rapidly disrupts the EGFR^{TAIL}:Grb2 condensate, returning the system to a homogeneous

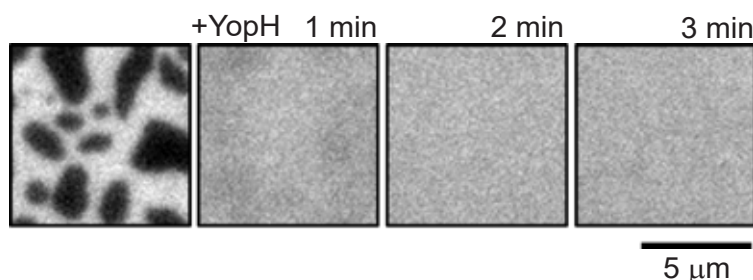


Figure 3.3: EGFR^{TAIL}:Grb2 phase transition is reversed by phosphatase. (Left) TIRF image of EGFR^{TAIL} condensation taken at 15 min after the addition of Grb2 to phosphorylated EGFR^{TAIL} on the supported bilayer. (Middle) Image taken after the addition of 1 μ M phosphatase, YopH. EGFR^{TAIL}:Grb2 condensate is rapidly dissolved in 1 min. (Right) Image taken at 3 min after the addition of YopH shows uniformly distributed EGFR^{TAIL}

and mobile distribution of EGFR^{TAIL} within 1 min (see Figure 3.3). This confirms that even in the condensed state, sufficient dynamic unbinding and rebinding between Grb2 and the phosphorylated tyrosine sites on EGFR enables ready access by phosphatases (similar dynamic behavior has been observed in the LAT protein condensates [24]).

Formation of the EGFR^{TAIL}:Grb2 protein condensates requires a multivalent crosslinking interaction. In previous studies of the LAT:Grb2:SOS protein condensate, SOS was presumed to be the primary crosslinking element. In the experiments described here, with no SOS present, we hypothesized that crosslinking must be occurring through Grb2 directly. The crystal structure of Grb2 reveals a dimeric assembly (PDB 1GRI) and Grb2-Grb2 interaction has been reported to play a functional role in FGFR signaling [129]. In the case of FGFR signaling, phosphorylation at Y160 on Grb2 breaks the Grb2-Grb2 interaction and enables downstream signaling. The Grb2 Y160 residue is located in the Grb2 dimer interface and a Y160E Grb2 mutant (Grb2^{Y160E}) has been reported to block dimerization [64]. To test if a Grb2 dimer interaction is responsible for the EGFR^{TAIL}:Grb2 condensation, we characterized the condensation with the Grb2^{Y160E} as well as phosphorylated wild-type Grb2 (pGrb2). Experimentally, pGrb2 was phosphorylated by Hck, and further analysis by mass spectrometry confirmed phosphorylation at Y160. In both cases, EGFR^{TAIL}:Grb2 condensation was fully disrupted. A Grb2 dimer interface serves as the crosslinking element in the EGFR^{TAIL}:Grb2 condensate and this interaction can be negatively regulated by phosphorylation of Grb2 at Y160. Control experiments without ATP confirmed that the EGFR^{TAIL}:Grb2 condensate is dependent on EGFR phosphorylation (Figure 3.4D). Additionally, a Grb2^{R86K} mutant (which has a disrupted SH2 domain [135]) could not induce condensation (Figure 3.4E), further affirming the essential role of Grb2-SH2 binding to phosphorylated tyrosine residues on EGFR^{TAIL}. Finally, we confirmed the Grb2^{Y160E} mutant is recruited to phosphorylated EGFR^{TAIL}, even though it is incapable of driving condensation, confirming Grb2^{Y160E} retains a functional SH2 domain.

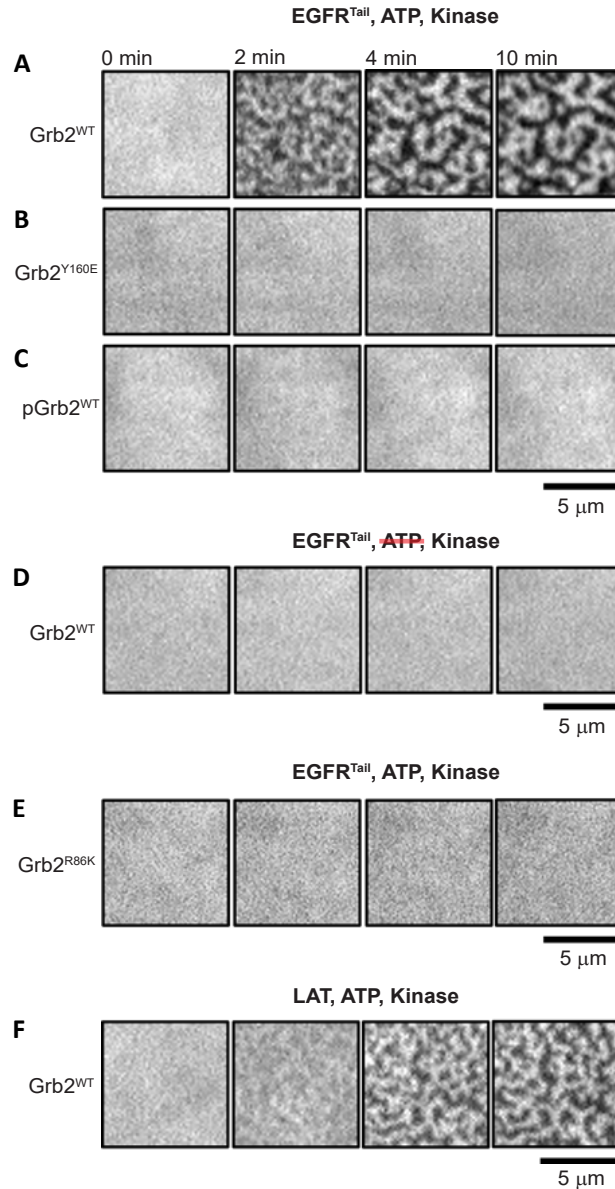


Figure 3.4: Grb2 dimerization as the key driving force to induce phase transition. TIRF images of EGFR showing the reconstitutions of (A) wild-type (WT) Grb2 and EGFR^{TAIL}. Phase transition is observed at 3 min. (B) Grb2^{Y160E} and EGFR^{TAIL}. EGFR^{TAIL} remains uniformly distributed. Phase transition is not detected. (C) pGrb2 and EGFR^{TAIL}. Phase transition is not detected. (D) WT Grb2 and EGFR^{TAIL} without ATP. Phase transition is not detected. (E) Grb2^{R86K}, SH2-impaired Grb2 mutant, and EGFR^{TAIL}. No phase transition is observed. (F) WT Grb2 and LAT. Phase transition of LAT:Grb2 is observed in 6 min. (A-F) Hck was the kinase used to phosphorylate EGFR^{TAIL}.

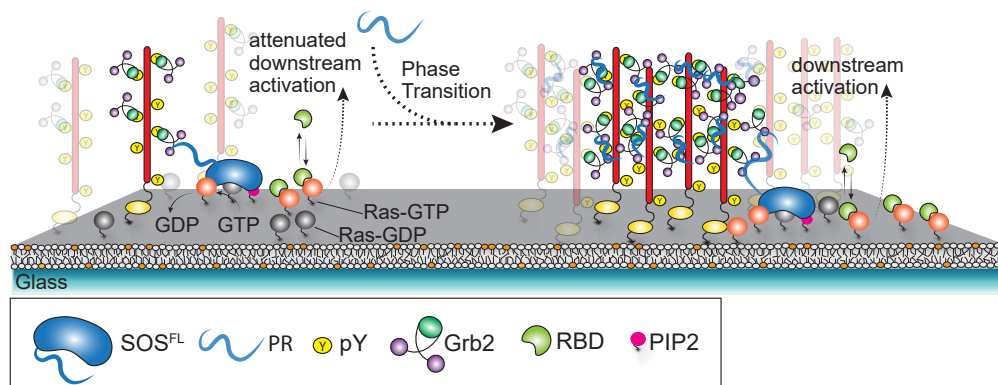


Figure 3.5: The schematic of downstream signaling modulated by SOS^{PR} -titrated $\text{EGFR}^{\text{TAIL}}:\text{Grb2}$ phase transition. SOS^{PR} was used as the strong crosslinker to induce the phase transition of the molecule assembly, $\text{EGFR}^{\text{TAIL}}:\text{Grb2}:\text{SOS}$, at different levels. To initiate the downstream signaling, Grb2, SOS^{PR} , GTP (1 mM), Alexa Fluor 555-labeled SOS^{FL} , and Alexa Fluor 647-labeled RBD (50 nM) were added together to the bilayer. Once SOS^{FL} was activated, the activated SOS^{FL} would stay on the membrane and processively activate Ras. The activated Ras (Ras-GTP) is detected by RBD. The downstream signaling at the activation of Ras was read by the fluorescence intensity of RBD on the bilayer. Also see Ras activation in Materials and Methods.

The condensate formed by Grb2 and the $\text{EGFR}^{\text{TAIL}}$ is analogous to that formed by Grb2 and LAT, and we speculated that the same Grb2-mediated effects are also present in LAT condensates. Experiments with LAT and Grb2 confirmed that a LAT:Grb2 condensate also forms without the addition of SOS or other crosslinking elements, in a manner very similar to what we have observed with the $\text{EGFR}^{\text{TAIL}}:\text{Grb2}$ system. (Figure 3.4F).

EGFR condensation enhances Ras activation by SOS

To measure the effects of EGFR condensation on downstream signaling, we reconstituted the signaling pathway from EGFR to Ras. This reconstitution is depicted schematically in Figure 3.5 and includes the $\text{EGFR}^{\text{TAIL}}$, Grb2, phosphatidylinositol-4,5 bisphosphate (PIP_2) lipid (which participates in SOS autoinhibition release), full-length SOS (SOS^{FL}), Ras, and a fluorescently labeled construct of the Ras binding domain (RBD) of Raf, which provides a final readout of Ras activation by its selective recruitment to RasGTP [25]. Ras is anchored to the membrane via coupling the C181 residue in the Ras hypervariable region to maleimide lipids (PE MCC), following well-established methods [75, 136, 137]. Prior to any experiments, membrane-associated Ras was loaded with guanosine diphosphate (GDP) to establish a starting condition of fully inactive Ras (Materials and Methods). In these experiments, Grb2 and the SOS^{FL} protein were maintained at sufficiently low concentrations that their presence alone was insufficient to trigger a condensation phase transition. To establish facile experimental control of the phase transition, we titrated in the PR domain of SOS (SOS^{PR} ; residues 1051 to 1333). SOS^{PR} is a strong crosslinker

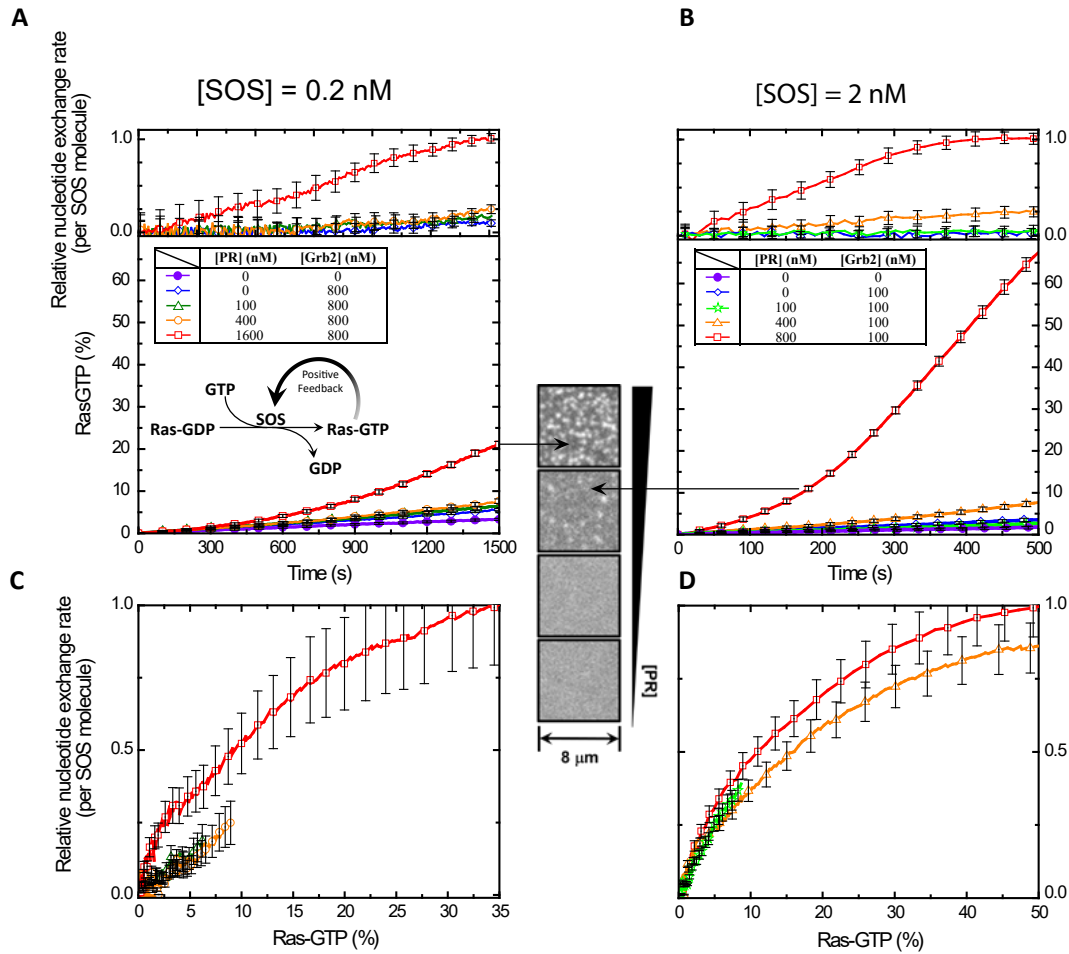


Figure 3.6: Ras activation modulated by EGFR^{TAIL}:Grb2 phase transition. (A) The kinetic traces of Ras activation and the relative nucleotide exchange as the function of time at an SOS concentration [SOS] of 0.2 nM. (B) The kinetic traces of Ras activation and the relative nucleotide exchange as the function of time at [SOS] of 2 nM. (C) The plot of the relative nucleotide exchange rate against the activated Ras showing the positive feedback of SOS at [SOS] of 0.2 nM. (D) The plot of the relative nucleotide exchange rate against the activated Ras showing the positive feedback of SOS at [SOS] of 2 nM. [Grb2], concentration of Grb2; [PR], concentration of SOS^{PR}. The TIRF images shown of EGFR^{TAIL} correspond to the condition of [SOS^{PR}] used to initiate the condensation that resulted in the indicated Ras activation traces.

of Grb2, which has been used to control the LAT:Grb2:SOS condensation [25], and behaves similarly with the EGFR^{TAIL}. Unlike SOS^{FL}, SOS^{PR} lacks the catalytic domains and thus cannot contribute to Ras activation. To initiate the Ras activation reaction, SOS^{FL} (Alexa Fluor 555 labeled), Grb2, and SOS^{PR} were added in various ratios together with guanosine triphosphate (GTP; 1 mM) and Alexa Fluor 647-labeled RBD (50 nM) to the prephosphorylated EGFR^{TAIL} and RasGDP on the supported membrane. Subsequently, Grb2 bound to the phosphotyrosine sites on the EGFR^{TAIL}, recruiting both SOS^{FL} and SOS^{PR}, and a lateral protein condensation phase transition proceeded to various degrees depending on the specific protein concentrations in each experiment. At the membrane, full-length SOS further engaged PIP₂ through its pleckstrin homology domain and Ras (in the GDP or GTP state) through its allosteric binding site [72, 138], ultimately releasing autoinhibition and beginning to catalyze the nucleotide exchange reaction of RasGDP to RasGTP. RasGTP levels were read out in real time through the selective binding of Alexa Fluor 647-labeled RBD to RasGTP. We use a modified RBD construct that exhibits fast and reversible binding kinetics [25] and achieves ~5-s time resolution for tracking RasGTP.

Ras activation trajectories were tracked as a function of the EGFR^{TAIL} condensation state for two different, overall SOS^{FL} concentrations (0.2 and 2 nM). Data plotted in Figure 3.6 A and B illustrates the overall Ras activation trajectories as well as the calibrated, per-molecule SOS activities for each condition. Representative images of the corresponding EGFR^{TAIL} condensation state are presented in Figure 3.6E. For both SOS^{FL} concentrations, titrating in SOS^{PR} led to an abrupt enhancement in Ras activation after macroscopic condensation was achieved, as seen by the sharp divergence of the fastest trajectory (red) from trajectories for the other conditions in each case. This enhancement of Ras activation in EGFR^{TAIL} condensates was observed in the calibrated, per-molecule, SOS activities (Figure 3.6 A and B, Top) as well as in overall reaction trajectories (Bottom). Thus, in addition to recruitment of more SOS to the condensates (e.g., through multivalent avidity effects), each SOS^{FL} molecule also activated more Ras over the same time compared with uncondensed state of the EGFR^{TAIL}. Also prominent in the fastest overall reaction trajectories from each SOS^{FL} concentration was RasGTP-driven positive feedback, which is evidenced by the positive curvature in the trajectories [73, 74, 139]. In these reactions, a constant catalytic rate from SOS^{FL} would have resulted in a Ras activation trajectory with strictly negative curvature as progressively less RasGDP would have been available to be activated. The use of SOS^{PR} to titrate the EGFR^{TAIL} condensation was for experimental convenience only. The EGFR^{TAIL} condensation, controlled exclusively through Grb2, was sufficient to modulate Ras activation by SOS^{FL} in a similar manner as observed with SOS^{PR}. We have also experimentally demonstrated that the small amounts of SOS^{PR} used here were insufficient to drive condensation if the Grb2^{Y160E} mutant was used—thus a Grb2-Grb2 interaction appears to be essential under these conditions.

Further insight into how the EGFR condensate may be facilitating SOS activation of Ras

can be obtained by examining the reaction rate as a function of RasGTP density. Plots of the relative overall nucleotide exchange rate as a function of RasGTP density for each condition in Figure 3.6 A and B are presented in Figure 3.6 C and D. Strong rate enhancements with increasing RasGTP are evident in all cases, again revealing RasGTP-driven positive feedback. The EGFR condensate-driven enhancement in Ras activation, which is dominant in the time trajectories of these reactions, is much less obvious when rates are mapped to RasGTP levels. At the higher SOS^{FL} concentration (Figure 3.6D), the EGFR condensate effect is essentially undetectable, with all trajectories nearly overlaying each other. At the lower SOS^{FL} concentration (Figure 3.6C), a modest increase (roughly double) in reaction rate is evident for the condensed state (red) compared with the other conditions. In the related LAT condensate system, Ras activation by SOS has been shown to follow a first-passage time mechanism, in which the overall system kinetics are predominantly set by the time it takes to get the first few SOS molecules activated [140] (although effects of LAT condensation were not addressed in that study). Ras activation measurements presented here suggest EGFR condensation is impacting the initiation of Ras activation more so than it is modulating steady-state aspects of the reaction. More efficient activation of the first few SOS molecules allows the RasGTP-driven positive feedback to kick in earlier, leading to a substantial difference in overall Ras activation.

3.4 Discussion

The protein condensate of the $\text{EGFR}^{\text{TAIL}}$ described here exhibits striking similarities with the LAT protein condensate with respect to its phase transition properties as well as the way it impacts Ras activation. However, the context of the LAT protein condensation in response to TCR activation differs dramatically from EGFR signaling with respect to the copy numbers of molecules involved. T cells have near single-molecule antigen-sensing capabilities [141–144] and can activate with only few triggered TCRs. In contrast, EGFR stimulation often involves thousands or even millions of activated receptors per cell [145–147]. Among molecules in the EGFR-MAPK pathway, SOS is expressed at very low levels and is likely a limiting component [147]. Thus, unlike the LAT:Grb2:SOS condensate, an EGFR:Grb2:SOS condensate would seem implausible if SOS was a key crosslinking element that needed to be present in stoichiometric ratios with EGFR. Observations of a two-component EGFR:Grb2 condensate that we report here, with a Grb2-Grb2 interaction serving as the primary crosslinker, relieve SOS of its requisite crosslinking role. We are then free to envision physiological EGFR condensates, primarily scaffolded by Grb2-Grb2 interactions, which contain substantially substoichiometric copy numbers of SOS relative to the numbers of EGFR molecules. EGFR condensation, nonetheless, exhibits significant control over the ability of SOS to activate Ras.

More broadly, the EGFR condensate establishes a layer of the EGFR signal transduction mechanism, proximal to ligand-mediated EGFR activation itself, that offers regulatory

control over the efficiency of downstream signal propagation. EGFR condensation may be influenced by other physical and chemical parameters, such as local mobility of EGFR, membrane topographical features, or other molecules participating in the condensation [58, 87, 148]. Additionally, the fact that Grb2-Grb2 binding is disrupted by Y160 phosphorylation reveals a potential active regulatory mechanism by which EGFR condensation could be curtailed. Since Grb2 in condensates is in constant exchange with cytosolic Grb2 [24], such a mechanism would require a cell-wide shift in Grb2 phosphorylation levels; it remains an open question if such regulation is utilized under physiological conditions. An intriguing side effect of Grb2 phosphorylation as a potential negative regulator is that tyrosine kinase inhibitors, such as those widely used as cancer therapeutics in EGFR-driven cancers [149], could also inhibit this negative regulatory mechanism—conceivably interfering with their desired effects.

3.5 Materials and methods

Chemicals

DOPC and 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid) - succinyl] (Ni^{2+} -NTA-DOGS; nickel salt) were purchased from Avanti Polar Lipids. Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647 maleimide dyes were purchased from Thermo Fisher. Bovine serum albumin (BSA), ($\pm$)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, catalase, 2-mercapto- ethanol (BME), NiCl_2 , H_2SO_4 , and ATP were purchased from Sigma-Aldrich. Glucose oxidase was purchased from Serva. Tris(2-carboxyethyl)phosphine (TCEP), glucose and H_2O_2 were purchased from Thermo Fisher Scientific. MgCl_2 was purchased from EMD Chemicals. Tris-buffered saline (TBS) was purchased from Corning.

Protein purification

EGFR^{TAIL} The C-terminal region of EGFR^{TAIL} (residues 1015 to 1210, where the signal peptide of EGFR is included in numbering) fused to a N-terminal His₆ and a SUMO tag with a tobacco etch virus (TEV) cleaving site was cloned into pProEX-HTb. The plasmid was transformed into BL21(DE3) *Escherichia coli*. The transformed cells were grown at 37 °C in 1 L of Terrific Broth medium until an optical density at 600 nm (OD₆₀₀) reached 0.7. The culture was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and allowed to grow overnight at 18 °C. After overnight expression, the culture was spun down for 15 min at 4,000 rpm and resuspended in 15 to 30 mL of Ni-NTA buffer (500 mM NaCl, 20 mM Tris·HCl at pH 8.5, 20 mM imidazole). The EGFR^{TAIL}-expressing cells were lysed by sonication. The insoluble fraction of the lysate was separated by ultracentrifugation. The supernatant was applied to a HisTrap FF column (GE Healthcare). The EGFR^{TAIL} was eluted by eluting buffer (500 mM NaCl, 20 mM Tris·HCl at pH 8.5, 500 mM imidazole).

The elution fractions were concentrated by an Amicon Ultra Centrifugal Filter Unit (30 kDa molecular weight cutoff [MWCO]; Millipore). The concentrated Grb2 was loaded onto an S75 30/300 column (GE Healthcare) equilibrated in Buffer A (150 mM NaCl, 20 mM Tris·HCl at pH 8.0, 1 mM TCEP). Finally, purified EGFR^{TAIL} was aliquoted and stored at -80 °C after flash freezing for further labeling reactions.

Grb2 pET-28 plasmid containing full-length Grb2 fused to N-terminal His6 was transformed into BL21(DE3) E. coli. The transformed cells were grown at 37 °C in 1L Terrific Broth medium until OD600 reached 0.7. The culture was induced with 1 mM IPTG and allowed to grow overnight at 18 °C. After overnight expression, the culture was spun down for 15 min at 4,000 rpm and resuspended in 15 to 30 mL of Ni-NTA buffer (500 mM NaCl, 20 mM Tris·HCl at pH 8.5, 20 mM imidazole, 5% glycerol). The Grb2-expressing cells were lysed by sonication with the addition of 1 mM phenylmethylsulfonyl fluoride and 0.1 mM BME. The insoluble fraction of the lysate was spun down at 16,500 rpm for 45 min at 4 °C. The supernatant was applied to a HisTrap FF column (GE Healthcare). Grb2 was eluted by eluting buffer (500 mM NaCl, 20 mM Tris·HCl at pH 8.5, 500 mM imidazole, 5% glycerol). The elution fraction was loaded onto a desalting column equilibrated in Buffer A (150 mM NaCl, 20 mM Tris·HCl at pH 8.0, 1 mM TCEP, 5% glycerol). The fraction of the Grb2 peak was collected. Grb2 was incubated with TEV protease overnight to cleave the His tag. The cleaved Grb2 was reapplied to a HisTrap column. The flow-through was collected and concentrated by an Amicon Ultra Centrifugal Filter Unit (10 kDa MWCO). The concentrated Grb2 was loaded onto an S75 30/300 column (GE Healthcare) equilibrated in Buffer A. The fractions of Grb2 monomer were collected and concentrated. Grb2 was further equilibrated at 37 °C for 10 min to reestablish the equilibrium between the monomer and dimer. Finally, Grb2 was aliquoted and stored at -80 °C after flash freezing.

HRas H-Ras S118C containing residue 1 to 181 fused to N-terminal His6 was purified. The N-terminal His6 tag was removed by TEV protease. The procedures were described in the previous work [69].

SOS^{FL} Full-length SOS protein was prepared via a split-intein approach. The N- and the C-terminal fragments of human SOS1 were expressed in BL21 (DE3) bacteria and purified separately. The two fragments with high purity were ligated by the intein reaction to generate SOS^{FL}. The details of the design for the intein reaction, expression, and purification are described in [25].

SOS^{PR} pETM vector containing the open reading frame (ORF) His₆-MBP-(Asn)₁₀-TEV-SOS1 (residue 1051 to 1333, human) PR domain was transformed into BL21 (DE3) bacteria. The expression and purification are reported in [24].

RBD-K656E pETM11 vector containing the ORF His₆-GST-PreScission-SNAPtag-Raf1

RBD (residue 56 to 131, K65E) derived from the Raf-1 human gene was transformed into BL21 (DE3) bacteria. RBD was expressed and purified as described previously [25].

Protein labeling

Proteins were first diluted to 100 μ M. Alexa Fluor 488, 555, or 647 maleimide dye was dissolved in anhydrous dimethyl sulfoxide to make a 10 to 20 mM working stock. The dye was added to the protein within three-fold molar excess over protein concentration to avoid overlabeling. The reaction mixture was incubated at room temperature for 1 h. To quench the labeling reaction, dithiothreitol was added to the reaction mixture at the final concentration of 10 mM. Amicon Ultra Centrifugal Filter Unit was used to wash the free dye away by adding more buffer while concentrating the protein. The concentrated protein was loaded to an analytical-grade size exclusion column (S75; GE Healthcare) for further purification. The fractions of the purified protein were collected and concentrated. Finally, the labeled protein was aliquoted and stored at -80 °C after flash freezing.

Supported lipid bilayers

Supported lipid bilayers (SLBs) were formed by rupturing small unilamellar vesicles (SUVs). SUVs were made by mixing DOPC, Ni²⁺-NTA-DOGS, PE MCC, and PIP₂ lipids (92:4:2:2 by molar percent) in chloroform. Chloroform of solution was evaporated using a rotary evaporator for 10 min at 40 °C to obtain the dried lipid films, which were further dried by purging N₂ for 10 min. The vesicle solution was formed from the hydration of the dried lipids by H₂O with the concentration of 1 mg/mL, using vortexing. SUVs were made by sonicating the vesicle solution for 90 s in an ice-water bath. SLBs were prepared on the glass substrate cleaned by piranha etch (glass coverslips, bottom thickness 170 μ m $\pm$ 5 μ m; Ibidi) combined with the flow chamber (sticky-Slide VI 0.4; Ibidi). The SUVs were mixed with 10 mM TBS buffer at pH 7.4 (1:1 ratio by volume) and injected into the chamber for 30 min of incubation to make SLBs. The incubation of 1 mg/mL BSA in TBS buffer for 10 min was used to block the defects in SLBs. H-Ras was anchored on PE MCC lipids through the maleimide chemistry by incubating 0.5 mg/mL H-Ras with SLBs for 3 h. The free PE MCC lipids were quenched by 5 mM BME. EGFR^{TAIL} and Hck were anchored on SLBs via his-tag-Ni²⁺-NTA chemistry by incubating EGFR^{TAIL} and Hck at 150 and 10 nM for 30 min. During this step, 1 mM GDP was included to ensure that Ras was loaded with GDP. To phosphorylate the tyrosine sites of EGFR^{TAIL}, 1 mM ATP and 5 mM MgCl₂ in TBS were added to SLBs and incubated for 10 min. The mobility of the membrane-bound proteins was examined by fluorescent recovery after photobleaching. The densities of the membrane proteins were determined by the averaged intensities of the epifluorescence images via the calibration curve between the intensity of the image and the density of the protein measured by the FCS, described previously [136].

TIRF microscopy

An Eclipse Ti inverted microscope (Nikon) with a TIRF system and iXon electron-multiplying charged-coupled device camera (Andor Technology) was used for imaging. TIRF microscopy was performed with a Nikon 100x 1.49 numerical aperture oil-immersion TIRF objective, a TIRF illuminator, a Perfect Focus system, a motorized stage, and a U-N4S four-laser unit as a laser source (Nikon). The laser unit has the solid-state lasers for the 488 nm, 561 nm, and 640 nm channels and was controlled using a built-in acousto-optic tunable filter. The laser powers of 488 nm laser, 561 nm laser, and 640 nm laser were set to 5.2, 6.9, and 7.8 mW respectively measured with the field aperture fully opened. The 405/488/561/638 nm Quad TIRF filter set (Chroma Technology Corp.), along with supplementary emission filters of 525/50 m, 600/50 m, 700/75 m for 488 nm, 561 nm, 640 nm channels, respectively, were used. Images are acquired using the Nikon NIS-Elements software with the exposure time of 50 ms.

Ras activation and imaging analysis

SOS^{PR} was used as the strong crosslinker to induce the phase transition of the molecular assembly, EGFR^{TAIL}:Grb2:SOS, at different levels while the concentrations of Grb2 were kept constant, avoiding the bias in signal outputs due to the competition for Grb2. SOS^{PR} without the catalytic domain for the nucleotide exchange of Ras would not contribute to the Ras activation done by SOS^{FL}. To initiate the downstream signaling, Grb2, SOS^{PR}, GTP (1 mM), Alexa Fluor 555-labeled SOS^{FL}, and Alexa Fluor 647-labeled RBD (50 nM) were added together to the bilayer. While the phase transition of EGFR^{TAIL} was occurring, SOS was recruited by Grb2. Once SOS^{FL} was activated, the activated SOS^{FL} would stay on the membrane and start to processively activate Ras. The activated Ras (Ras-GTP) on the membrane was further detected by RBD in the bulk solution via the interaction between Raf and activated Ras. The downstream signaling at the activation of Ras was read by the fluorescence intensity of RBD on the bilayer, using TIRF images. At the end of each Ras activation trace, 20 nM of the catalytic domain of SOS^{FL} was used to fully activate Ras on SLBs. The averaged intensity of the RBD TIRF image corresponding to total Ras molecules on SLBs was used as the normalization factor to calibrate different Ras densities over different traces. SOS^{FL} on the membrane was tracked at the same time as the fluorescence intensity of RBD on the bilayer was being monitored. The Ras activation by SOS^{FL} was allowed to interact directly with the phase transition of EGFR^{TAIL}. The concentrations of Grb2 (800 nM and 100 nM) were chosen to modulate the recruitment of SOS^{FL} by Grb2, making the time scale of Ras activation comparable within 30 min between the two data sets of low and high concentrations of SOS. Single-molecule images of SOS^{FL} were tracked by an ImageJ plugin, TrackMate [150], to obtain the number of activated SOS^{FL} molecules on the membrane. SOS^{FL} molecules were localized with the detector of difference of Gaussian. The initial diameter was set to 6 pixels. Typically, 50 to 3,000 molecules were detected per image. In this study, the Ras activation modu-

lated by EGFR^{TAIL} phase transition at both low and high concentrations of SOS (0.2 and 2 nM) were interrogated separately. Dividing the instantaneous increase of activated Ras by the number of SOS molecules on the bilayer gave the relative nucleotide exchange rate of SOS as a function of time. To study the positive feedback of SOS^{FL}, the relative nucleotide exchange rates of SOS^{FL} were plotted against activated Ras (percentage of Ras-GTP).

Chapter 4

Tyrosine Phosphorylation in EGFR Activation both Promotes and Inhibits Ras Activation through Competing Effects on the EGFR Protein Condensate

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4.1 Abstract

Upon ligand binding, epidermal growth factor receptor (EGFR) is activated and condenses into an extended network through recruitment and dimerization of growth factor receptor-bound protein 2 (Grb2). EGFR condensation promotes the ability of guanine nucleotide exchange factor Son of Sevenless (SOS) to activate Ras. However, Grb2 phosphorylation at residue Y160 breaks the Grb2-Grb2 interaction. Here, we show in a reconstituted system that kinase activity negatively regulates EGFR protein condensation by modulating the phosphorylation state of Grb2. Kinase inhibitors rescue EGFR condensation by inhibiting this negative regulatory step. When Ras is included, we find kinase activity inhibits Ras activation, but kinase inhibitors promote Ras activation through a double negative effect. These results identify Grb2 phosphorylation as a novel mechanism regulating EGFR protein condensation and subsequent downstream Ras signaling.

4.2 Introduction

Cells spatially and temporally organize biomolecular reactions by sequestering reaction components into membrane-bound compartments or, as recently discovered, into membraneless biomolecular condensates [2, 3, 151]. Of interest are protein condensates on the cellular membrane which form when membrane-bound receptors bind to extracellular ligands [152]. Epidermal growth factor receptor (EGFR) is one such receptor tyrosine kinase. EGFR consists of an extracellular ligand-binding module, a single transmembrane helix, a cytoplasmic tyrosine kinase domain, and an unstructured C-terminal tail [153]. Under non-stimulating conditions, EGFR is autoinhibited and primarily monomeric. However, upon the addition of a ligand, EGFR dimerizes and autophosphorylates tyrosine residues on its C-terminal tail [115]. In particular, phosphorylation of residues Y1068, Y1086, Y1148, and Y1173 recruits growth factor receptor-bound protein 2 (Grb2) through the Grb2 Src Homology 2 (SH2) domain [116]. In the MAPK pathway, Grb2 then recruits guanine nucleotide exchange factor Son of Sevenless (SOS) to the membrane [25, 154] through interaction between the SOS proline-rich (SOS^{PR}) domain and Grb2 Src Homology 3 (SH3) domains [49]. Multivalency in EGFR:Grb2 binding [116] and in Grb2:SOS binding [49] allows EGFR to condense into a three-component bond percolation network similar to the LAT condensate observed in T cell signaling [107, 155].

Once full-length SOS (SOS^{FL}) is recruited to the membrane, it can activate Ras GTPase. Usually under basal conditions, Ras is bound to GDP and is inactive. However, when SOS^{FL} is activated, it processively converts many molecules of RasGDP to RasGTP [71, 74, 75]. In addition, RasGTP binds to the SOS^{FL} allosteric site, resulting in positive feedback in Ras activation [Margaritis2003]. RasGTP then leads activation of the MAPK pathway and subsequent cellular responses such as cellular proliferation and differentiation [156]. In the cytoplasm, SOS^{FL} is autoinhibited, but recruitment to the membrane by Grb2 releases SOS^{PR} domain autoinhibition [71]. Furthermore, interaction with phosphatidylinositol-4,5 biphosphate (PIP_2) lipids removes autoinhibition from its N-terminal histone folds, Dbl-homology domain, and Pleckstrin-homology domains [70, 131]. These processes for SOS^{FL} autoinhibition release are multi-step and require a long dwell time on the membrane [70]. The three-component protein condensate between proteins with phosphorylated tyrosine residues, Grb2, and SOS regulates SOS^{FL} activity by extending SOS^{FL} dwell time on the membrane, which allows SOS^{FL} to escape autoinhibition and activate Ras GTPase [24].

While the three-component protein-condensation and its effect on downstream signaling is being studied [19, 24, 25], little is known about the two-component protein-condensation [48]. In the two-component protein condensate, Grb2 dimerizes on the membrane while retaining its ability to bind cellular ligands [157], allowing Grb2 to be the sole driver for protein condensation. While Grb2 may exist in multiple dimeric states [63, 157–160], one dimer conformation contains an interaction interface between the C-SH3 domain of one

Grb2 molecule and the SH2 domain of another Grb2 molecule [63]. In this conformation, residue Y160 on one Grb2 molecule forms a hydrogen bond with residue E87 of the other Grb2 molecule [157]. Phosphorylation of Y160 breaks the Grb2-Grb2 interaction [64] and consequently inhibits EGFR condensation [48]. This brings into question the role of kinases and their impact on downstream signaling.

Kinases are required for phosphorylation of the EGFR C-terminal tail, recruitment of Grb2, and subsequent activation of the MAPK pathway [116]. Furthermore, many cancers over-express EGFR, which suggests high kinase activity results in overactivation of the MAPK pathway and in increased cellular proliferation [161, 162]. Consequently, tyrosine kinase inhibitors (TKIs) are regularly used as cancer therapeutics intended to block EGFR-driven Ras/MAPK signaling [163, 164]. Nonetheless, patients treated with TKIs inevitably develop TKI resistance primarily thought to be due to secondary mutations or amplification of bypass signaling pathways [165, 166]. However, since Grb2 phosphorylation inhibits EGFR condensation, kinases may represent an important negative regulatory mechanism. Additionally, little is known how inhibition of this possible regulatory mechanism affects downstream Ras activation.

Here, we reconstitute the C-terminal tail of EGFR onto supported lipid bilayers (SLBs) and performed detailed microscopic imaging studies of EGFR condensation. By utilizing total internal reflection fluorescence (TIRF) microscopy, we visualize the distribution of EGFR upon injection of Grb2 and SOS. Once added, Grb2 and SOS induce an EGFR condensation, segregating EGFR into high- and low-density phases. However, high kinase concentrations prevent Grb2 dimerization, which inhibits the formation of EGFR condensates. Furthermore, kinase inhibitors disrupt this negative regulatory mechanism and promote EGFR condensation. Measurements of Ras activation reveal that dissolution of the EGFR condensate by Grb2 phosphorylation reduces downstream Ras activation. Tyrosine kinase inhibitors promote Ras activation through a double negative effect. The results demonstrate a novel mechanism by which the phosphorylation state of Grb2 modulates EGFR condensation and subsequent downstream signaling through Ras. Considering the low copy number of SOS in cells [147], Grb2 dimerization may be an essential mechanism by which EGFR phase transitions and propagates signals in living systems.

4.3 Results

Reconstitution of EGFR protein condensate on supported membranes

The EGFR protein-condensation phase transition can be reconstituted on SLBs [48] from purified proteins much like the LAT condensation phase transition [19, 24, 25, 51] (Figure 4.1A). Briefly, the C-terminal tail of EGFR (residues 1015 to 1210, including the 24-

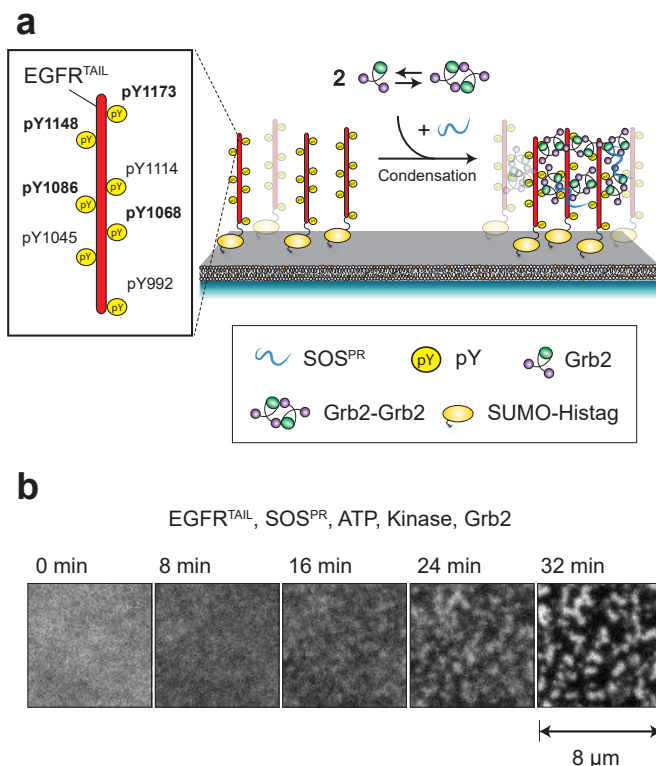


Figure 4.1: EGFR^{TAIL}, reconstituted on supported lipid bilayers (SLBs) and labelled with AF488, undergoes a protein condensation upon addition of Grb2 and SOS^{PR}. (A) Schematic of the reconstitution experiment. EGFR^{TAIL} can be phosphorylated on several tyrosine sites, of which Y1068, Y1086, Y1148, and Y1173 are implicated in Grb2 binding. Grb2 dimerizes through a Grb2-Grb2 interface and additionally interacts with the PR domain of SOS (SOS^{PR}). Grb2 and SOS^{PR} are injected into the chamber and are recruited to EGFR^{TAIL}. The crosslinking between EGFR^{TAIL}:Grb2:SOS^{PR} drives the assembly of a protein condensate. EGFR^{TAIL} is initially in a dispersed phase (Left), but the addition of Grb2 and SOS^{PR} drives EGFR^{TAIL} into a condensed phase (Right). (B) TIRF images of EGFR^{TAIL}-AF488 on SLBs. After the addition of Grb2 and SOS^{PR}, EGFR^{TAIL} condenses over the course of 32 min.

residue signaling peptide) is expressed with a His₆ tag and a small ubiquitin-related modifier (SUMO) fusion tag at the N-terminus of the construct. This EGFR^{TAIL} construct is labeled with Alexa Fluor 488 at 66% efficiency (22) and anchored onto SLBs through Ni²⁺-histidine chelation through its His₆ tag and 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (Ni²⁺-NTA-DOGS) lipids. SLBs are prepared with 4% Ni²⁺-NTA-DOGS by mole percent with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as the main constituent. The SUMO tag acts both as a solubility tag for increased purification yield and as a spacer that mimics the area of the EGFR kinase domain. To phosphorylate EGFR^{TAIL}, Hck, which is a Src-family kinase, was attached to SLBs also through a His₆ tag and Ni²⁺ chelation reaction. Since EGFR is not efficiently phosphorylated by Src-family kinases [117], a prephosphorylation strategy was

employed in which EGFR^{TAIL}, Hck, and adenosine triphosphate (ATP) are incubated together before the addition of any adaptor proteins. Before the addition of Grb2 or SOS^{PR}, EGFR^{TAIL} begins as a macroscopically homogenous distribution, but upon injection of adaptor proteins, EGFR^{TAIL} undergoes a protein-condensation phase transition and segregates into highly dense (bright) and disperse (dark) regions of EGFR^{TAIL} as observed by TIRF imaging (Figure 4.1B).

Kinase-driven phosphorylation of Grb2 can interfere with EGFR condensation

Due to the importance of the Grb2 phosphorylation state in driving EGFR condensation [48], we next investigated the role of kinases in an EGFR protein-condensation phase transition. In our model, we note kinase activity is required to phosphorylate EGFR^{TAIL} and recruit downstream molecules (Figure 4.2A). On the other hand, Grb2 interacts intermolecularly to form dimers, but kinase activity drives the formation of phosphorylated Grb2 monomers (Figure 4.2B, Supplemental Table 1). Initially, EGFR is in a dispersed state (Figure 4.2C, Left), but the addition of Grb2 and SOS induces a condensed state (Figure 4.2C, Middle). However, as kinases continuously phosphorylate Grb2, EGFR crosslinking decreases and returns to a dispersed state (Figure 4.2C, Right). To examine how kinase activity modulates condensation, EGFR was incubated with increasing concentrations of Hck. Since protein surface density increases monotonically with incubation concentration [57], increasing incubation concentrations of Hck represent increasing levels of kinase activity. After the addition of Grb2 and SOS^{PR}, at low levels of kinase activity, EGFR condenses (Figure 4.2D, Top). However, at high levels of kinase activity, EGFR remains macroscopically homogenous (Figure 4.2D, Bottom). Experiments with LAT demonstrate the same kinase-driven negative regulatory mechanism. Also, although phosphorylation of Grb2 may inhibit binding to SOS^{PR} [167, 168], full length Grb2 and prephosphorylated full length Grb2 recruit SOS^{FL} at similar rates, suggesting the dissolution of the EGFR protein condensate with increasing kinase concentration is due to decreased Grb2 dimer formation, not inhibited Grb2:SOS^{PR} crosslinking.

Grb2 mutants control EGFR condensation in a Grb2 phosphorylation independent manner

To further validate the crosslinking role of the Grb2 SH2/SH3 dimer, several mutations were introduced. In the SH2/SH3 dimer, residue Y160 lies on the dimer interface and forms a hydrogen bond with residue E87 of the other Grb2 molecule (Figure 4.3A). However, a Grb2 Y160E mutation (Grb2^{Y160E}) breaks the interface to promote monomeric Grb2 [64]. When Grb2^{Y160E} and SOS^{PR} are added to EGFR^{TAIL} with increasing concentration of kinase, EGFR^{TAIL} remains in a dispersed state regardless of the kinase activity (Figure 4.3B). Thus, formation of the SH2/SH3 dimer is critical for EGFR^{TAIL} condensation.

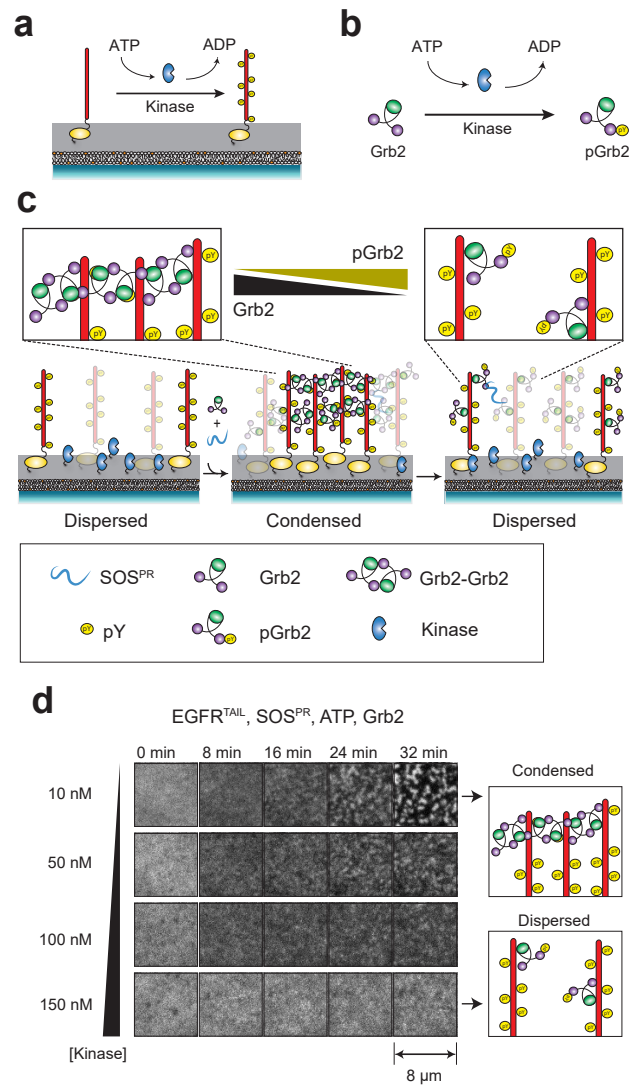


Figure 4.2: Phosphorylation of Grb2 inhibits EGFR condensation. All images are of EGFR^{TAIL} labelled with AF-488 (A) Schematic of the role of kinases in EGFR^{TAIL} phosphorylation. Kinases phosphorylate EGFR^{TAIL} on several tyrosine residues which recruit Grb2. (B) Schematic of the role of kinases in Grb2 phosphorylation. Kinases phosphorylate Grb2 to drive monomer formation. (C) Schematic of the role of kinases in EGFR^{TAIL} condensation. Before the addition of Grb2 and SOS^{PR}, EGFR^{TAIL} is in a dispersed state (Left). After the addition of Grb2 and SOS^{PR}, EGFR^{TAIL} condenses with Grb2 and SOS^{PR} acting as crosslinkers (Middle). At later time points, kinases phosphorylate Grb2, breaking dimer formation. EGFR^{TAIL} condensation is dissolved by the lack of Grb2 dimers because crosslinking through Grb2:SOS^{PR} is insufficient (Right). (D) TIRF images of EGFR^{TAIL}-AF488 on SLBs functionalized with increasing kinase concentrations. After the addition of Grb2 and SOS^{PR}, EGFR^{TAIL} condenses at low kinase conditions (Top) but remains homogenous at high kinase conditions (Bottom). Hck was utilized to phosphorylate both EGFR^{TAIL} and Grb2.

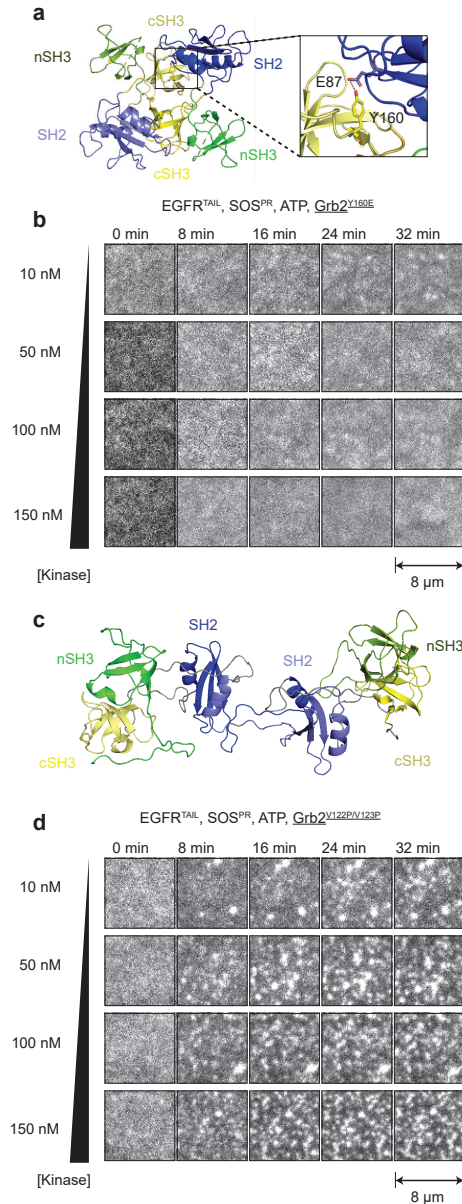


Figure 4.3: Kinases do not change EGFR condensation with mutant Grb2. (A) TIRF images of EGFR^{TAIL}-AF488 on SLBs functionalized with increasing kinase concentrations. After the addition of Grb2^{Y160E} and SOS^{PR}, EGFR^{TAIL} remains dispersed at all kinase concentrations. (B). TIRF images of EGFR^{TAIL}-AF488 on SLBs functionalized with increasing kinase concentrations. After the addition of Grb2^{V122P/V123P} and SOS^{PR}, EGFR^{TAIL} condenses at all kinase concentrations. Hck was utilized to phosphorylate both EGFR^{TAIL} and Grb2.

Breakage of this dimer interface by either phosphorylation or Y160E mutation is sufficient to block EGFR^{TAIL} condensation. However, Grb2 may exist in another dimeric conformation in which the α -helix of one SH2 domain swaps with the α -helix of the SH2 domain of the other Grb2 molecule [158–160]. Residues V122 and V123 lie on the hinge region, and Grb2 mutations to V122P and V123P (Grb2^{V122P/V123P}) promote formation of the SH2/SH2 domain-swapped dimer [158] (Figure 4.3C). To study the effect of the SH2/SH2 domain-swapped dimer on EGFR^{TAIL} condensation, EGFR^{TAIL} was incubated with increasing concentrations of Hck. After the addition of Grb2^{V122P/V123P} and SOS^{PR}, EGFR^{TAIL} condensed at all kinase concentrations (Figure 4.3D), which demonstrates Grb2^{V122P/V123P} promotes EGFR^{TAIL} condensation regardless of Grb2 phosphorylation state. Thus, EGFR^{TAIL} condensation driven by the Grb2 SH2/SH2 domain-swapped dimer is kinase-independent. This data in addition with the Grb2^{Y160E} data suggests that Grb2 primarily forms the SH2/SH3 dimer in reconstitution and that the SH2/SH2 domain-swapped dimer is not relevant in our system.

Tyrosine kinase inhibition can promote EGFR condensation

Kinase activity negatively regulates EGFR condensation through phosphorylation of Grb2, but tyrosine kinase inhibitors may negatively regulate this kinase-dependent mechanism. To elucidate the effect of kinase inhibitors, EGFR was reconstituted with increasing levels of kinase and injected with kinase inhibitor Dasatinib, a Src-family specific kinase inhibitor [67, 169], along with Grb2 and SOS^{PR}. Like before, EGFR forms a protein condensate with low concentrations of kinase. However, at high concentrations of Hck, kinase inhibitor promotes the EGFR protein-condensation phase transition as demonstrated by the segregation of EGFR into high and low density phases (Figure 4.4). Dasatinib also promotes the reconstituted LAT phase condensate even under high concentrations of Hck, demonstrating the generality of the effect of kinase inhibitors. While kinase activity negatively regulates EGFR condensation, kinase inhibitors can negatively regulate this inhibitory mechanism and promote a protein-condensation phase transition.

Tyrosine kinase inhibition can promote Ras activation through a double-negative effect

While the extent of an EGFR protein-condensate phase transition can enhance downstream Ras activation [48], the role of kinase activity in Ras activation was unknown. To study the effect of kinase activity, we reconstitute the EGFR:Grb2:SOS:Ras signaling pathway (Figure 5A) by attaching EGFR^{TAIL} and Hck onto SLBs containing PIP₂. We then attach Ras to SLBs through cysteine-maleimide chemistry with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide] (sodium salt) (PE MCC) lipids [136], and preload Ras with GDP [25, 74]. To measure Ras activation, we introduce a fluorescently labeled Ras binding domain (RBD-647), derived from Raf [25] with fast binding kinetics [170], along with Grb2, SOS^{PR}, fluorescently labeled full length

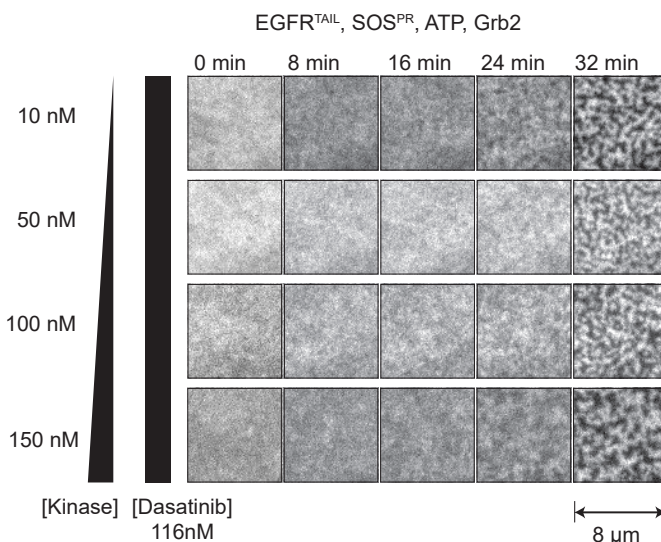


Figure 4.4: Tyrosine kinase inhibition promotes EGFR condensation. TIRF images of EGFR^{TAIL}-AF488 reconstituted on SLBs with increasing kinase concentrations. Tyrosine kinase inhibitor is added along with Grb2 and SOS^{PR}. EGFR^{TAIL} with kinase inhibitor condenses at all kinase concentrations.

SOS (SOS^{FL}-AF555), and GTP as in injection. Grb2 binds to the phosphorylated tyrosine residues of EGFR^{TAIL} and subsequently recruits SOS^{PR} and SOS^{FL}-AF555. The formation of Grb2 dimers crosslinks EGFR^{TAIL} and drives an EGFR protein-condensate phase transition. We then measure the fluorescence intensity of RBD-AF647 through TIRF imaging to measure the rate of Ras activation at different kinase concentrations.

At low kinase concentration conditions, there is a high level of Ras activation. Increasing the kinase concentration does not change the rate of Ras activation until the highest kinase concentration of 150 nM Hck, at which the rate dramatically drops, demonstrating kinase activity can modulate Ras activation (Figure 5B). By phosphorylating Grb2, kinases inhibit EGFR^{TAIL} condensation, which prevents SOS from dwelling on the membrane for long periods of time and from being released from its autoinhibited state. Subsequently, Ras activation will decrease in non-condensed conditions. Interestingly, moderate levels of kinase concentration do not noticeably alter the rate of Ras activation, but these conditions demonstrate small EGFR^{TAIL} inhomogeneities while only the highest concentration of kinase shows a macroscopically homogeneous distribution of EGFR^{TAIL} (Figure 5C). It appears these small condensates within the moderate kinase concentration conditions are sufficient to activate SOS^{FL}-AF555 and drive Ras activation.

Since modulation of the extent of the EGFR phase condensate through kinase activity affects Ras activation, tyrosine kinase inhibitors may remove this kinase dependence. To measure the effect of kinase inhibitor on Ras activation, Dasatinib is added to the injec-

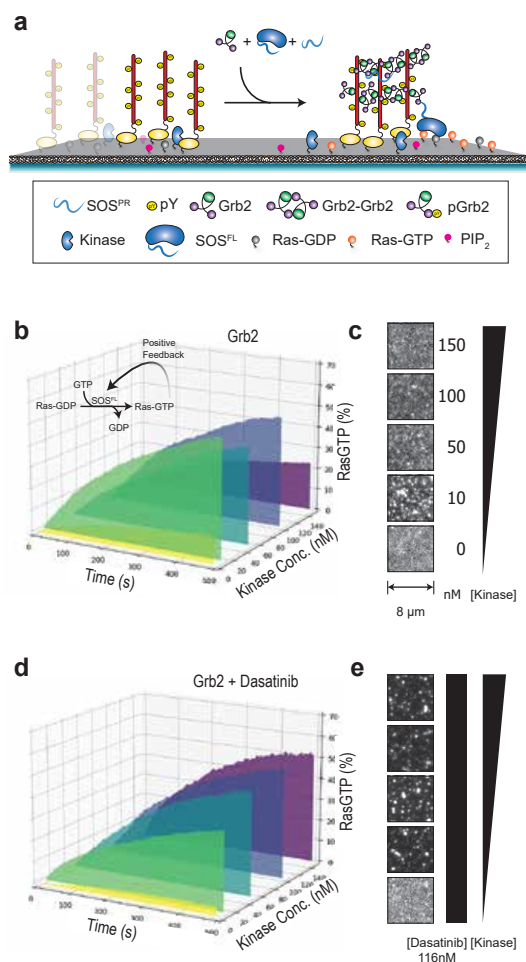


Figure 4.5: Tyrosine kinase inhibition can promote Ras activation through a double negative effect. (A) Schematic of Ras activation by EGFR^{TAIL}, Grb2, SOS^{PR}, and SOS^{FL} reconstituted on SLBs. Grb2, SOS^{PR}, SOS^{FL}-AF555, RBD-AF647, and GTP (1 mM) were injected onto SLBs functionalized with EGFR^{TAIL}-AF488, Hck, and Ras-GDP. Grb2 recruits SOS^{FL} to the bilayer which activates Ras by converting Ras-GDP to Ras-GTP. Activated Ras is measured by the fluorescence intensity of RBD-AF647, which specifically binds to Ras-GTP. Images shown are of the condensation of EGFR^{TAIL}-AF488 (B) Kinetic traces of Ras activation at different kinase concentrations. Conditions correspond to the images shown in (C) High kinase concentrations inhibit Ras activation. Error bars represent the standard error of the mean (SEM) of ≥ 2 replicates. (C) Representative TIRF images of EGFR^{TAIL}-AF488 on Ras-functionalized bilayers after the addition of Grb2, SOS^{PR}, and SOS^{FL}-AF555, and titrating kinase from 0-150 nM as indicated. (D) Kinetic traces of Ras activation at different kinase concentrations and with the addition of kinase inhibitor. Conditions correspond to the images shown in (E). Kinase inhibition rescues Ras activation at high kinase concentrations. Error bars represent the SEM of ≥ 2 replicates. (E) Representative TIRF images of EGFR^{TAIL} on Ras-functionalized bilayers after the addition of Grb2, SOS^{PR}, SOS^{FL}-AF555, kinase inhibitor, titrating kinase from 0-150 nM as indicated.

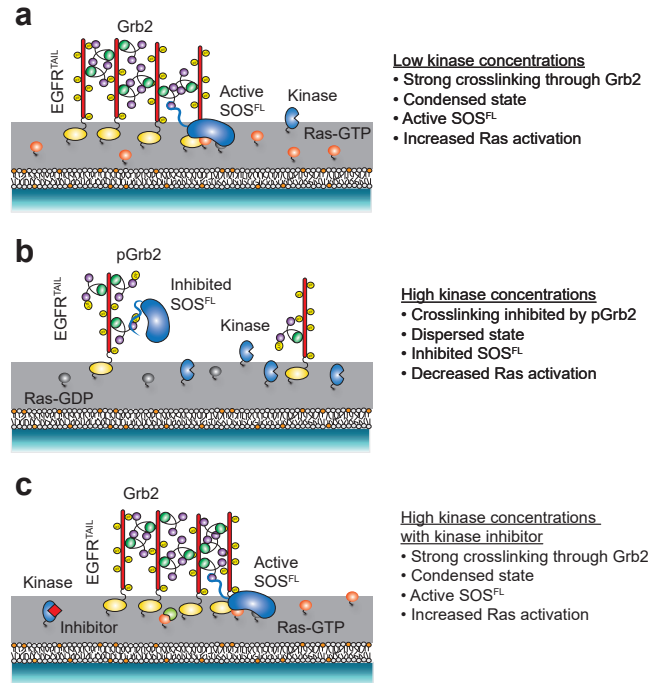


Figure 4.6: Model of the role of kinases in EGFR condensation. (A) At low kinase concentrations, Grb2 dimerizes and drives EGFR condensation. EGFR condensation promotes SOS^{FL} autoinhibition release and subsequent Ras activation. (B) At high kinase concentrations, Grb2 is phosphorylated and cannot drive EGFR condensation. SOS^{FL} remains autoinhibited, and Ras activation is inhibited. (C) At high kinase concentrations, kinase inhibitors prevent phosphorylation of Grb2. Grb2 can then dimerize to drive EGFR condensation and thereby promote Ras activation.

tion, and Ras activation is measured as a function of time. With kinase inhibitor and at low or moderate kinase concentrations, Ras is activated at similar rates as without kinase inhibitor. However, with the addition of kinase inhibitor, there is a high level of Ras activation at high kinase conditions (Figure 5D), demonstrating kinase inhibitor promotes Ras activation. Since the corresponding EGFR^{TAIL} condensation state exhibits highly condensed regions (Figure 5E), the mechanism by which kinase inhibitors promote Ras activation is by enhancing phase condensation by inhibiting the phosphorylation of Grb2. This would allow Grb2 to dimerize and crosslink EGFR^{TAIL}. Indeed, mutant Grb2^{Y160E}, which is a mutant form of Grb2 that cannot dimerize [64, 171], demonstrates low levels of Ras activation and no dependence on kinase activity. The kinase independent behavior of Ras activation and homogenous distribution of EGFR^{TAIL} at all kinase concentrations with Grb2^{Y160E} underscore the importance of Grb2 dimerization in the EGFR protein-condensation phase transition.

4.4 Discussion

Although kinase activity is required for the phosphorylation of the cytoplasmic tail of EGFR to recruit Grb2 and other proteins for MAPK activation [172], we have demonstrated that kinase activity modulates the extent of EGFR condensation. By interfering with Grb2 dimerization, kinases reduce its crosslinking capabilities and limit the size of a EGFR protein-condensation phase transition. This reduction downregulates Ras activation, although only at high concentrations of kinase. Moderate levels of kinase retain high Ras activation and show small EGFR^{TAIL} clusters (Figure 4.4D). However, these small EGFR^{TAIL} condensates may be the biologically relevant condensates because in a cell, membrane signaling condensates are within the size of microns [173, 174]. Since SOS signaling is controlled by a first-passage time process, in which rare, early activation events determine cellular outcomes [140], these small condensates are sufficient to trigger the activation of small numbers of SOS, which can processively activate hundreds of Ras molecules and result in macroscopic outcomes.

Interestingly, kinase activity is not the only mechanism by which EGFR can both positively and negatively regulate downstream MAPK activation. RasGRP1 is a RasGEF [175] similarly to SOS and is activated by EGFR upon stimulation [176]. RasGRP1 promotes SOS signals by priming SOS for activation with initial production of RasGTP [177, 178]. However, counterintuitively, RasGRP1 suppresses ERK/MAPK signaling by opposing EGFR-Grb2-SOS signals [176, 179]. Also, while RasGRP1 is overexpressed in many types of cancers [180, 181], RasGRP1 expression level can act as a predictive marker for anti-EGFR therapy in which low RasGRP1 expression correlates with increased anti-EGFR therapy effectiveness [182]. Together, our results suggest tyrosine kinase inhibitors may negatively regulate inhibitory mechanisms and thereby upregulate downstream activation. Although tyrosine kinase inhibitor resistance is thought to occur from secondary mutations or amplification of other pathways [165, 166, 183], these mechanisms elucidate different possible pathways in which resistance may develop. In combination with kinase-independent cancer-inducing mechanisms of EGFR [128, 184], it appears tyrosine kinase inhibitors alone cannot prevent EGFR-driven cancers.

While our results demonstrate kinase activity can modulate downstream Ras activation in a reconstitution setting, the effects in a live-cell context remain unknown. In our experiments, we use Src family kinase Hck to phosphorylate Grb2 and EGFR, not EGFR kinase domain. There is some evidence that EGFR does not phosphorylate Grb2 [154, 185, 186], but there is other data that EGFR may phosphorylate Grb2 [168, 187, 188]. We hypothesize Grb2 is not an efficient substrate for EGFR and is only phosphorylated under very high EGFR expression. Consistent with this hypothesis, A431 cells, which demonstrated Grb2 phosphorylation by EGFR, have about 5-10 times higher EGFR expression than A549 cells [145], and Her14 cells, which have some evidence of Grb2 phosphorylation by EGFR, also highly express EGFR [189, 190]. Also, it is interesting to note that H3255

cells, which have comparable EGFR expression levels to A431 cells [191], demonstrates Grb2 phosphorylation at residue Y160 [192]. although direct evidence of EGFR phosphorylating Grb2 in H3255 cells has not been demonstrated yet. Other kinases known to phosphorylate Grb2 are NPM-ALK fusion [193], FGFR2 [129], PRLR [185], and c-Src [194], and they may also play a role in negatively regulating EGFR protein condensation in a live cell context.

4.5 Materials and Methods

Chemicals

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (Ni^{2+} -NTA-DOGS), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexanecarboxamide](sodium salt) (PE MCC), and L- α -phosphatidylinositol-4,5-bisphosphate (Brain, Porcine) (ammonium salt) (Brain PIP_2) were purchased from Avanti Polar Lipids. Alexa Fluor 488 and Alexa Fluor 555 dyes were purchased from ThermoFisher. SNAP-Surface Alexa Fluor 647 was purchased from New England Biolabs. Bovine Serum Albumin (BSA), 2-Mercaptoethanol (BME), H_2SO_4 , NiCl_2 , ATP, GDP, GTP, phosphatase inhibitor cocktail 2 and 3, and Dasatinib were purchased from Sigma-Aldrich. H_2O_2 and Tris buffer saline (TBS) were purchased from Fisher Scientific. MgCl_2 was from EMD Chemicals.

Protein purification

EGFR^{TAIL} EGFR C-terminal tail (residues 1015-1210 including the 24 residue signaling peptide) was purified as described previously [48] using an N-terminal His₆ tag and SUMO tag with a Tobacco Etch Virus (TEV) cleaving site. The His₆ tag was left intact for membrane tethering.

LAT Cytoplasmic LAT (residues 27-233, human) with mutation C117F was purified as described previously [101] using an N-terminal His₆ tag with a TEV cleaving site. The His₆ tag was left intact for membrane tethering.

Grb2 Full length and mutant Grb2 were purified as described previously [48] using an N-terminal His₆ tag. The N-terminal His₆ tag was removed with TEV protease. Grb2 was further equilibrated at 37 °C for 10 minutes to reestablish equilibrium between monomer and dimer.

Hck Hck kinase domain (residues 83-526, human) was purified as described previously for Src-family kinases [102] using an N-terminal His₆ tag and TEV cleaving site. The His₆ tag was left intact for membrane tethering.

SOS^{PR} The proline rich domain of SOS (residues 1051-1333, human) was expressed with an N-terminal His₆-MBP-fusion tag with a TEV cleaving site. The protein was expressed and purified as described previously [24].

SOS^{FL} Full-length SOS protein was prepared via a split intein approach. The N- and the C-terminal fragments of human SOS1 were expressed in BL21 (DE3) bacteria and purified separately. The two fragments with high purity were ligated by the intein reaction to generate SOS^{FL}. The protein was purified as described previously [25].

RBD-K65E pETM11 vector containing the ORF, His₆-GST-PreScission-SNAPtag-Raf1 RBD (residue 56-131, K65E) derived from the Raf-1 human gene was transformed into BL21 (DE3) bacteria. The N-terminal His₆-GST tag was removed with PreScission before use. The protein was purified as described previously [25].

Protein labeling

EGFR^{TAIL}, LAT, and SOS^{FL} were diluted to 100 μ M in 5 mM TCEP. Alexa Fluor 488 and 555 maleimide dyes (ThermoFisher) were dissolved to make 10-20 mM stock. The dyes were added to protein solutions and allowed to react for 1 hr at room temperature. The reaction was quenched with 10 mM dithiothreitol (DTT) for 10 min. Amicon Ultra centrifugal filters were used to wash away free dye and to concentrate protein. Protein was loaded into an analytical grade size exclusion column (SE, GE Healthcare) for further purification. Labeled fraction was determined by comparison of absorption of the dyes λ_{max} to the protein's UV absorption at 280 nm corrected for the dye's absorption at 280 nm.

RBD-K65E was labelled with SNAP-Surface Alexa Fluor 647 (S9136S, New England Biolabs) according to the manufacturer protocol. Excess dye was removed with gravity desalting columns (PD10, Cytiva) and by SEC via FPLC (Superdex 75 increase, 24 mL bed volume, Cytiva).

SUVs and supported lipid bilayers

Small unilamellar vesicles (SUVs) were prepared using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)-succinyl] (nickel salt) (Ni²⁺-NTA-DOGS), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide] (sodium salt) (PE MCC), and L- α -phosphatidylinositol-4,5-bisphosphate (Brain, Porcine) (ammonium salt) (Brain PIP₂) (Avanti Polar Lipids). Purchased lipids were mixed into a piranha etched round bottom flask to 2 mg of total lipid in a ratio of 92:2:2:4 (by molar percent) of DOPC:PIP₂:PE MCC:DGS-NTA(Ni). After mixing, lipids were dried using a rotary evaporator for 10 min at room temperature and an additional 3 min at 42 °C. Lipid films were further dried under continuous N₂ flow for 15 min. Lipid films were rehydrated

with 2 mL of milli-Q water, resulting in a lipid concentration of 1 mg/mL and vigorously vortexed to ensure lipid film liftoff. SUVs were made by sonicating the lipid mixture with a 3 mm stepped tip (Sonics VCX750 33% amplitude, 20 s sonication, 50 s rest, and 5 complete cycles). SUVs were stored at 4 °C and used within 1 week of preparation.

Precision Schott D 263 M glass coverslips (#1.5H, 75 x 25 mm, custom ordered from Thor-Labs) were cleaned in 50% IPA:milli-Q water with bath sonication for 30 min. Glass substrates were then piranha etched with a 1:3 ratio of 30% H₂O₂ and concentrated H₂SO₄ for 7 min. Coverslips were washed excessively with milli-Q water after etching, stored in milli-Q water at room temperature, and used within 1 week. For use, etched coverslips were blow-dried under N₂ and attached to a 6-channel slide (sticky-Slide VI 0.4, Ibidi). Chambers were cured at 37 °C for 30 min under heavy weight.

SLBs were formed by rupture of SUVs. 1 mg/mL SUV solution was mixed with 10 mM TBS (1:1 ratio by volume), and 200 µL of the diluted solution was injected into the chamber with a 30 min incubation time. Channels were washed with 10 mM TBS, and 100 mM NiCl₂ was added to each channel to reload Ni²⁺-NTA-DOGS for 5 min. Channels were washed and then blocked with 1 mg/mL BSA in TBS buffer for 10 min. After washing, H-Ras was anchored on PE MCC through maleimide chemistry by incubating 0.5 mg/mL H-Ras in the chambers for 2.5 hr. Free PE MCC was quenched with 5 mM BME for 20 min. EGFR^{TAIL} and Hck were anchored on Ni²⁺-NTA-DOGS through His-tag and Ni²⁺ NTA chemistry and through incubation at 50 nM EGFR^{TAIL} for condensation experiments (Figure 4.1, Figure 4.2, Figure 4.3, Figure 4.4) or 20 nM EGFR^{TAIL} for Ras activation experiments (Figure 5) for 45 min. LAT experiments were performed at 150 nM LAT. During this step, 100 µM GDP was included to ensure Ras was loaded with GDP. After washing, EGFR^{TAIL} was phosphorylated by incubating 1 mM ATP and 5 mM MgCl₂ in TBS for 20 min. The incubation period also allows for weakly chelated proteins to dissociate. The mobility of the proteins was examined using fluorescence recovery after photobleaching (FRAP).

Total internal reflection fluorescence (TIRF) microscopy

Imaging experiments utilizing TIRF were performed on an inverted Nikon Eclipse Ti microscope with an 100x Nikon oil-immersion TIRF objective (1.49 NA). A 1.5x lens tube (Nikon) was used for a final magnification of 150x. A motor-controlled stage (MS-2000, ASI) was used for positioning, and images were acquired using an iXon Ultra 897 EM-CCD camera (Andor Technology). The laser unit has diode lasers of 488 nm, 561 nm, and 637 nm (OBIS laser diode, Coherent) controlled with a custom laser driver (Solamere Technology). The laser was collimated with the objective at the same approximate z-focus between experiments. A single quad-band dichroic cube was used for all acquisitions (ZT405/488/561/640rpcv, Chroma) and additionally filtered with an ET600/50M filter (Semrock). The microscopy and accessory hardware were controlled with Micro-Manager version 4.0 [105]. All experiments were conducted at 21-23 °C.

Condensation Imaging

After EGFR^{TAIL} phosphorylation, 1.5 μ M Grb2, 188 nM SOS^{PR}, 0.1 mg/mL BSA in TBS, and 1 mM ATP were added into the chamber as a 100 μ L injection. 232 μ M Dasatinib (Sigma-Aldrich) was added in the kinase inhibitor studies (Figure 4.4). Images were acquired with a 20 ms exposure time and 250 EM gain. EGFR^{TAIL} was excited with a 488 nm diode laser (OBIS laser diode, Coherent) with 1 mW (0.25 mW at objective) of power. Images were collected at 1 min intervals with acquisition durations as long as 1 hr. Image analysis and contrast adjustments were made in Fiji [106].

Ras Activation Imaging

Ras activation studies were performed similarly as described previously [48]. Briefly, to initiate downstream signaling of Ras, 1.5 μ M Grb2, 186 nM SOS^{PR}, 2 nM SOS^{FL}-AF555, 50 nM RBD-AF647, 0.1 mg/mL BSA in TBS, 1 mM GTP, and 1 mM ATP were added into the chamber as a 100 μ L injection. Concentrations between 1.16 nM and 232 μ M of Dasatinib (Sigma-Aldrich) (Figure 5) were added in the kinase inhibitor studies. SOS^{FL} is recruited to the membrane by Grb2 and begins to activate. Once activated, SOS^{FL} will processively convert Ras-GDP to Ras-GTP. RBD-647 binds specifically to Ras-GTP [25], and activated Ras was measured by the fluorescence intensity of RBD-647 on TIRF images. At the end of the Ras trace, 30 nM SOSCAT, which is the catalytic domain of SOS^{FL}, was used to fully activate Ras in the SLB. After 20 min, the average RBD intensity was used as a normalization factor for comparison of different chambers with various Ras densities due to random variations from different experiment days. The concentrations of EGFR, Grb2, and SOS^{FL} were determined to make the time scale of Ras activation about 10 min. Images were acquired with a 100 ms, 100 ms, and 20 ms exposure time and 100 EM gain, 500 EM gain, and 500 EM gain for 488 nm, 561 nm, and 637 nm channels respectively. 488 nm, 561 nm, and 637 nm lasers were at 1 mW, 2 mW, and 3 mW power respectively. Images were collected at 5 sec intervals for 25 min. Quantitative image analysis was done in Python.

Imaging of SOS Recruitment

After phosphorylation of EGFR^{TAIL}, 750 nM Grb2, 200 pM SOS^{FL}-AF555, 0.1 mg/mL BSA, and 1 mM ATP were added into the chamber as a 100 μ L injection. After 10 min to establish equilibrium, Images of SOS^{FL}-AF555 were acquired with the 561 nm diode laser at 50 mW power and 500 EM gain. Images were collected at a streaming acquisition (shortest possible time between frames) for 1500 frames. Single-molecule images of SOS^{FL} were analyzed using TrackMate [150], an ImageJ plugin, to obtain number of SOS^{FL} recruitment events. SOS^{FL} molecules were localized with Laplacian of Gaussian (LoG) segmentation, and the initial diameter was set to 3 μ m. Tracks were built with a max gap of 2 frames between particle tracks. Quantitative image analysis was done in Python.

Chapter 5

Positive feedback in Ras activation by full-length SOS arises from autoinhibition release mechanism

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5.1 Abstract

Signaling through the Ras-MAPK pathway can exhibit switch-like activation, which has been attributed to the underlying positive feedback and bimodality in the activation of RasGDP to RasGTP by SOS. SOS contains both catalytic and allosteric Ras binding sites, and a common assumption is that allosteric activation selectively by RasGTP provides the mechanism of positive feedback. However, recent single-molecule studies have revealed that SOS catalytic rates are independent of the nucleotide state of Ras in the allosteric binding site, raising doubt about this as a positive feedback mechanism. Here, we perform detailed kinetic analyses of receptor-mediated recruitment of full-length SOS to the membrane while simultaneously monitoring its catalytic activation of Ras. These results, along with stochastic kinetic modeling, expose the autoinhibition release step in SOS, rather than either recruitment or allosteric activation, as the underlying mechanism giving rise to positive feedback in Ras activation.

5.2 Introduction

Ras is a membrane-bound small GTPase that cycles between a GTP-bound on-state and a GDP-bound off-state [195]. In the GTP-bound state, Ras can recruit downstream effector proteins to the membrane, leading to activation of the MAPK and PI3K pathways, which ultimately leads to cell growth and proliferation [196]. Under non-activating conditions, Ras resides in the GDP-bound state. Ras activation to the GTP-bound state is controlled by guanine nucleotide exchange factors (GEFs) that catalyze exchange of Ras-bound nucleotide with free nucleotide from the cytosol, which is primarily GTP [175]. In the cellular setting, bimodality, or switch-like activity, in the Ras-MAPK signaling pathway has been shown to be important in lymphocytes [178] and contributes to the sharp boundary that discriminates positive and negative selection of thymocytes [197]. Ras misregulation is a common cause of cancers, including lung cancer, colorectal cancer, and pancreatic cancer [198–200].

Son of Sevenless (SOS) is a key RasGEF, which operates downstream of both the growth factor receptor and T-cell receptor pathways. The amine terminus of SOS consists of three regulatory domains: a histone fold (HF) domain [201], a Dbl homology (DH) domain, and a Pleckstrin homology (PH) domain [202]. The REM-Cdc25 catalytic core sits in the middle [72] and is followed by a carboxy-terminal, structurally disordered proline-rich (PR) tail [203]. Upon receptor activation, SOS is recruited via Grb2 to the cytosolic tail of epidermal growth factor receptor (EGFR) or, in the case of T cell receptor (TCR) signaling, to Linker for activation of T-cells (LAT). In both cases, SH3 domains on the adaptor protein Grb2 bind to the PR domain of SOS, while the Grb2 SH2 domain binds to phospho-tyrosine sites on the active receptor or scaffold [154, 204]. Once on the membrane, SOS can initiate the nucleotide exchange reaction after a multistep autoinhibition release process (Figure 5.1) [133].

An unusual feature of SOS, compared with other RasGEFs, is that it has two Ras binding sites in its REM-Cdc25 catalytic core [205]. There is one catalytic site at the Cdc25 domain and one non-catalytic allosteric site positioned between the REM and Cdc25 domains [72, 133]. Structural studies have shown that allosteric Ras binding is essential for SOS catalytic activity [131, 206]. Additionally, both solution and liposome nucleotide exchange assays using the catalytic core of SOS have shown positive feedback in the activation of Ras by SOS. The higher binding affinity for RasGTP relative to RasGDP at the SOS allosteric site as well as a presumption of nucleotide-specific enhancement of catalytic activity have generally been accepted as the mechanisms of positive feedback [131, 178, 207–209]. However, these observations were made with truncated SOS constructs lacking autoinhibition and membrane recruitment through accessory domains. Recent studies have started to provide detailed characterizations of the full-length SOS (SOS^{FL}) molecule [25, 71], uncovering behaviors that were not observed in earlier research.

Recent single-molecule membrane microarray studies of SOS have revealed several facets of its functional mechanism that were not evident in earlier work. First, SOS is highly processive and can activate hundreds of Ras molecules in a single membrane binding event [71, 74, 75]. Second, the catalytic rate of Ras activation by an individual SOS molecule is independent of the nucleotide state of Ras in the allosteric binding site [74]. Third, SOS^{FL} exhibits an autoinhibition release process on the membrane that introduces a long delay (tens of seconds) between initial recruitment of SOS to the membrane and onset of its catalytic activity. Analyses of the delay time indicates that this slow activation process consists of multiple steps occurring far from equilibrium [25]. This slow autoinhibition release feature of SOS enables a novel type of kinetic proofreading regulation of SOS activity, which is under the control of a protein condensation phase transition of the recruiting EGFR or LAT molecules [24, 25, 48]. Collectively, this body of work experimentally demonstrates that SOS^{FL} lacks the classically presumed positive feedback in allosteric enhancement of its catalytic rate. SOS^{FL} does, however, exhibit marked positive feedback in Ras activation [73], raising questions of what the actual underlying mechanism is.

In this study, we performed detailed kinetic analyses of receptor-mediated recruitment and activity of SOS^{FL} in a supported membrane platform. Using total internal reflection fluorescence (TIRF) microscopy, we monitor the recruitment (via Grb2) of SOS^{FL} to phosphorylated LAT scaffold molecules on the membrane while simultaneously tracking the Ras activity state using a modified Ras binding domain from Raf-1 (residues 56-131, K65E; referred to as RBD hereafter). This RBD construct exhibits substantially faster binding kinetics compared to wildtype and provides faithful real-time readout of RasGTP densities on the membrane [25]. The results confirm that receptor-mediated recruitment of SOS^{FL} is independent of the Ras-nucleotide state. Further, we obtain quantitative measurements of the effective catalytic rate of a defined ensemble of membrane-recruited SOS^{FL} molecules as a function of RasGTP density, which confirms the existence of positive feedback at the ensemble level that is not observed in the individual molecules [74]. Finally, we developed a stochastic kinetic model that incorporates details of the SOS^{FL} autoinhibition release mechanism, which successfully recapitulates the experimental observations. From these results, we conclude that the observed positive feedback in Ras activation by receptor-recruited SOS^{FL} is an ensemble effect stemming from RasGTP-mediated enhancement in the rate of autoinhibition release. Recently, several small molecule inhibitors that target the SOS:Ras interaction have emerged as candidates for pan-KRas cancer therapy [210, 211]. Developing a comprehensive understanding of the mechanism underlying Ras activation by SOS may be useful for understanding the behavior SOS:Ras targeting drugs.

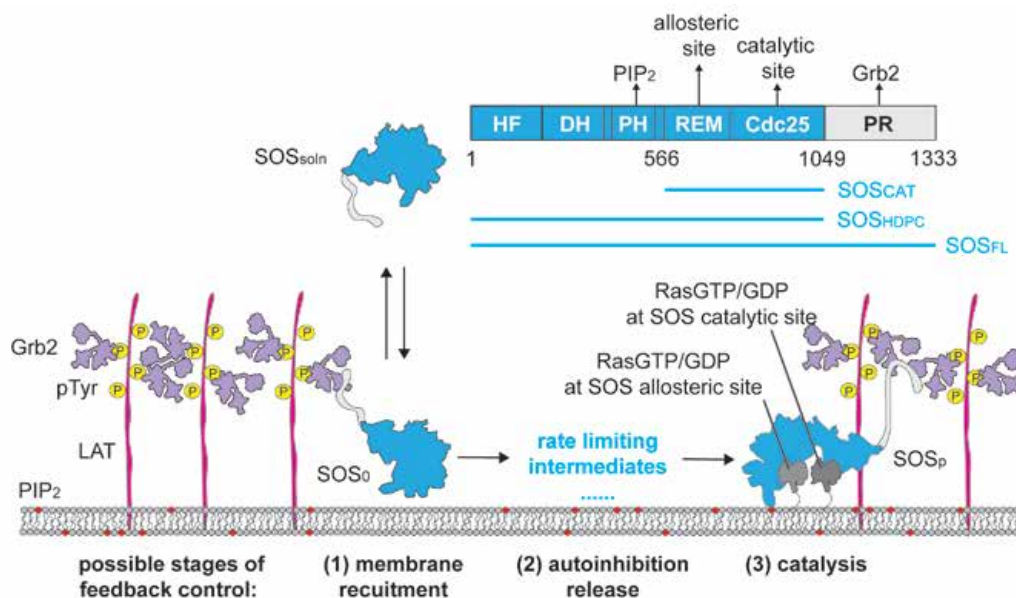


Figure 5.1: The activation and domain organization of SOS. SOS^{FL} stays in an autoinhibited conformation in solution (SOS_{soln}). After TCR stimulation and LAT phosphorylation, SOS^{FL} activation follows initial membrane recruitment in an autoinhibited conformation (SOS_0), slow autoinhibition release on the membrane, and processive Ras nucleotide exchange after activation (SOS_p). These three steps of activation provide potential mechanisms for feedback control in SOS.

5.3 Results

The receptor-mediated recruitment of SOS^{FL} is not sensitive to Ras nucleotide state

Although catalytic activity of SOS does not exhibit Ras-nucleotide state sensitivity at the single molecule level [74], enhanced membrane recruitment provides an alternative mechanism for positive feedback. The SOS catalytic core (SOS^{CAT}) and SOS^{HDPC} constructs, both of which lack the C-terminal PR domain responsible for receptor-mediated membrane recruitment (see SOS domain structure in Figure 5.1) are primarily recruited to the membrane through interaction with Ras and exhibit preference for RasGTP. Recruitment of enzymes that act on membrane substrates to the membrane via their own product is a direct form of positive feedback [139, 212]. Increased recruitment to RasGTP containing membrane is a likely source for the observed positive feedback in SOS^{CAT} and SOS^{HDPC} . The native SOS^{FL} , however, is robustly autoinhibited—including with respect to Ras binding [70, 131, 206]—and is primarily recruited to the membrane via Grb2 mediated binding to phosphorylated receptor or scaffold proteins on the membrane (LAT in the experiments described here, as shown in Figure 5.1). Thus, the native autoinhibition in SOS^{FL} is expected to prevent the recruitment based positive feedback observed in truncated SOS

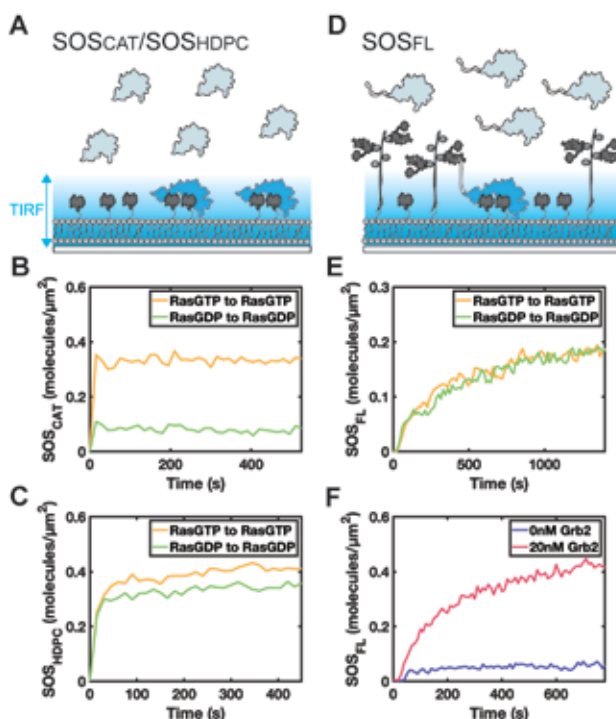


Figure 5.2: The receptor-mediated recruitment of SOS^{FL} is not sensitive to Ras nucleotide state. (A) Schematic of SOS^{CAT} or SOS^{HDPC} recruitment to Ras functionalized supported membrane. TIRF microscopy allows the quantification of SOS membrane recruitment under different membrane composition. Ras nucleotide-dependent membrane recruitment of (B) SOS^{CAT} and (C) SOS^{HDPC} . (D) Schematic of SOS^{FL} recruitment to pLAT and Ras functionalized supported membrane via Grb2. (E) Ras nucleotide-independent membrane recruitment of SOS^{FL} . (F) SOS^{FL} recruitment depends on Grb2 concentration.

constructs. To test this hypothesis, we perform detailed kinetic analysis of SOS recruitment in a supported bilayer configuration [25, 71, 74, 75, 213].

Purified Ras was tethered to the supported lipid bilayer through cysteine-maleimide chemistry at a density ~ 1500 molecule per μm^2 , following established protocols [74]. Local Ras densities in cells are inhomogeneous, and the density used here mimics the higher density estimates from Ras clusters [214]. Ras was preincubated with unlabeled SOS^{CAT} to control the initial nucleotide state. Residual SOS^{CAT} was thoroughly washed away before initiating the recruitment assay. To ensure similar densities of proteins incubated on the membrane, flow chambers for RasGDP and RasGTP were prepared side by side. SOS constructs were labeled with Alexa Fluor 555 or Alexa Fluor 647 and the recruitment of SOS to the membrane was quantified using single molecule counting via TIRF microscopy. Excess nucleotide was included in each flow chamber (GDP in the RasGDP chamber, and vice-versa for the RasGTP chamber) so that the nucleotide state of Ras would not change over the course of the measurement. In SOS^{FL} recruitment experi-

ment, the cytosolic domain of scaffold protein LAT (with N-terminal His₁₀ tag [57]) was linked to the bilayer through nickel-histidine interaction to achieve experimental densities of ~ 300 -1000 molecules per μm^2 . LAT was maintained in a fully phosphorylated state by the promiscuous Src family kinase, Hck (with His₆ tag), which was also on the bilayer [24]. In solution, Grb2 was included in the system at 20 nM to enable recruitment of SOS^{FL} to the membrane through a LAT:Grb2:SOS^{FL} complex, which we refer to as receptor-mediated recruitment. Under the conditions of these experiments, LAT does not form extended protein condensates but likely does form locally multivalent structures [25, 48].

Measurements of Ras-mediated recruitment of Alexa647-labeled SOS^{CAT} and Alexa647-labeled SOS^{HDPC} to RasGTP or RasGDP functionalized membranes confirm a strong preference by SOS^{CAT} for RasGTP and a mild preference by SOS^{HDPC} (Figure 5.2A-C). Some of this observed Ras-nucleotide preference likely stems from the binding affinity differences between SOS-RasGTP and SOS-RasGDP at equilibrium [131, 207]. However, single molecule SOS studies have revealed that SOS has several internal states, whereby a subset of membrane recruited molecules become stably associated with the membrane and enter a processive, catalytically-active state while others exhibit only transient membrane interactions [71, 213]. Thus, the ratio between processive and transient states of SOS will also affect saturation density differentially between different SOS constructs [213]. The discrepancy in the extent of Ras-nucleotide preference between SOS^{CAT} and SOS^{HDPC} may also stem from the accessibility of Ras binding site, as the regulatory domains keep SOS^{HDPC} in a more autoinhibited conformation than SOS^{CAT} [131]. Additionally, PI(4,5)p₂ and PH domain interaction [70, 215] may enhance SOS^{HDPC} membrane association in a Ras-independent manner.

In contrast to SOS^{CAT} and SOS^{HDPC}, SOS^{FL} clearly exhibits Ras nucleotide-independent recruitment to the membrane (Figure 5.2D and E). SOS^{FL} is primarily recruited via Grb2 to phosphorylated LAT on the membrane and exhibits minimal membrane binding in the absence of Grb2 (Figure 5.2F). From the SOS^{FL} membrane recruitment data in Figure 5.2E, it is evident that both the rise time (reflective of the kinetic on-rate for membrane recruitment) and the plateau density (reflective of equilibrium) are independent of Ras nucleotide state. This also directly shows that the dissociation rate, or kinetic off-rate, of SOS^{FL} is insensitive to the Ras nucleotide state as well. Therefore, even though the SOS^{FL} allosteric site has a higher binding affinity for RasGTP, this experimental evidence indicates that such preference does not contribute significantly to SOS^{FL} membrane association. Thus, SOS^{FL} does not exhibit positive feedback in receptor-mediated membrane recruitment.

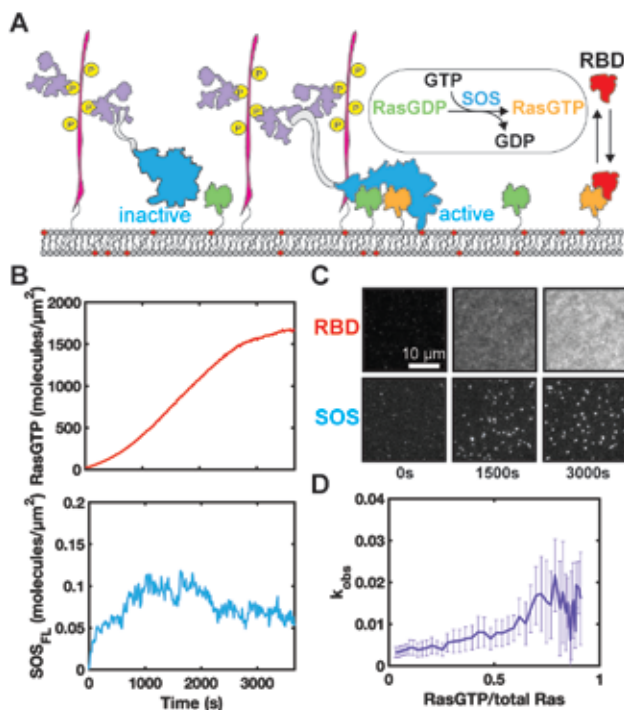


Figure 5.3: SOS^{FL} autoinhibition release depends on RasGTP levels. (A) Schematic of the activity assay for determining the fraction of active SOS on the membrane. Recruited SOS^{FL} is either in the process of autoinhibition release or in the active conformation. RBD sensor acts as a real-time RasGTP level readout. (B) Ras activation inferred from RBD recruitment and SOS membrane density were measured simultaneously. (C) Example time lapse images for RBD and SOS recruitment. (D) Average catalytic rate (k_{obs}) for all SOS^{FL} recruited to the membrane at different RasGTP level. The apparent unit for k_{obs} is molecule·s⁻¹·μm⁻¹.

Ensemble catalytic activity of SOS^{FL} exhibits RasGTP-driven positive feedback

Ras activation studies by SOS^{FL} were performed similarly as the recruitment assays discussed above, except that we simultaneously measured SOS^{FL} catalyzed Ras nucleotide exchange from RasGDP to RasGTP (Figure 5.3A). RasGTP density was measured by the membrane recruitment of a fluorescently labeled Ras binding domain (RBD) sensor, adapted from Raf with K65E point mutation to ensure fast binding and unbinding kinetics to Ras [25]. The RBD mean fluorescence intensity was calibrated to directly reflect RasGTP density. RasGTP levels and SOS^{FL} membrane density (Figure 5.3B) were simultaneously measured. Example time lapse images for RBD and SOS^{FL} membrane recruitment are shown in Figure 5.3C. Taking the ratio of the observed Ras nucleotide exchange rate to the measured momentary density of SOS^{FL} molecules on the membrane yields a clearly increasing apparent per-molecule catalytic rate (Figure 5.3D, see Materi-

als and Methods for details). Thus, SOS^{FL} exhibits RasGTP-dependent positive feedback, but evidently not by the same mechanism as SOS^{CAT} and SOS^{HDPC} .

With membrane recruitment and molecular catalytic activity already eliminated as possible mechanism for SOS^{FL} positive feedback, the only remaining mechanistic possibility is the autoinhibition release process. SOS^{FL} is recruited to the membrane in an autoinhibited state (here referred to as SOS_0 for stochastic kinetic modeling purposes). After initial membrane recruitment, the simplest model for the autoinhibition release process involves one dominant rate-limiting kinetic intermediate (SOS_1) before entering its active state (SOS_p), during which SOS begins to processively catalyze nucleotide exchange in Ras [25]. If RasGTP levels on the membrane alter the kinetics of SOS progression to its activated state, then this provides an alternative mechanism for RasGTP-driven positive feedback. We examine this possibility quantitatively with stochastic kinetic modeling.

The kinetic scheme for stochastic modeling is depicted in Figure 5.4A. SOS^{FL} recruitment and progression to its kinetic intermediate (SOS_1) state are represented as Ras-nucleotide state independent. SOS_1 then binds RasGTP or RasGDP at the allosteric binding site and the final transition to SOS_p has different rates depending on the nucleotide state of allosterically bound Ras. This is the presumed sequence since PI(4,5) P_2 binding releases the DH-PH domain and exposes the Ras allosteric binding pocket [69, 70]. All membrane-bound SOS molecules may desorb back into the solution with rates that depend on their activity state. The relaxation time back to the autoinhibited state once a SOS molecule has desorbed is short compared with the recycling time of SOS^{FL} back to the membrane. Therefore, all the newly bound SOS molecules start in an autoinhibited conformation. Stochastic simulations were performed following the Gillespie algorithm [216].

Based on the proposed model, there are three potential steps giving rise to positive feedback: These include Ras binding to the SOS allosteric site (k_5 and k_6), Ras-bound SOS entering the processively active state (k_7 and k_8), and Ras-bound SOS desorbing from the membrane (k_9 , k_{10} , k_{11} , and k_{12}). Since we inferred from the SOS^{FL} receptor-mediated recruitment assay that the membrane dissociation rate of SOS^{FL} is insensitive to the Ras nucleotide state (Figure 5.2E), we focused on the first two mechanisms in the simulation.

Ras binding to the SOS allosteric site at equilibrium has been the subject of numerous studies investigating the details of positive feedback in Ras activation by SOS [131, 207, 208]. However, the direct measurement of the kinetics of this binding process (k_5 and k_6) is lacking. We utilized the kinetics of SOS^{CAT} membrane recruitment obtained in the previous section to approximate Ras allosteric site binding kinetics. On both RasGTP and RasGDP bilayers, SOS^{CAT} reached a steady state in less than 15 seconds (Figure 5.2B). Assuming that the binding primarily involves simple reversible binding to the Ras allosteric site, the sum of k_{on} and k_{off} was estimated to be 0.6 s^{-1} [217]. Although membrane adsorption

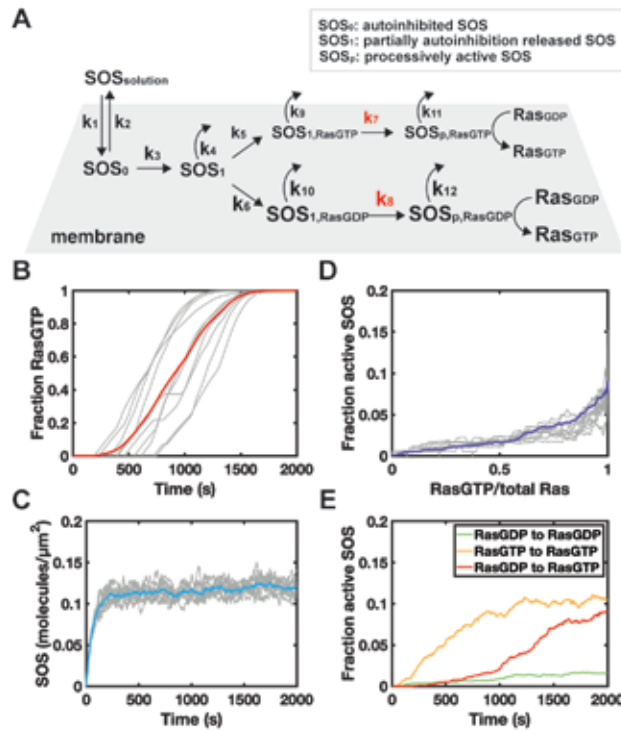


Figure 5.4: Stochastic simulations show accelerated autoinhibition release as a mechanism for positive feedback in SOS activation. (A) Schematic for the stochastic model. (B) Plots of RasGTP level in RasGDP to RasGTP nucleotide exchange reaction over time. Fraction RasGTP is the ratio between RasGTP and total Ras. The red trace corresponds to mean activation of 10 individual trajectories labeled in grey. (C) Plots of SOS membrane recruitment over time, obtained simultaneously with Ras activity (grey - individual trace, blue - mean trace). (D) Plots of fraction of active SOS over time, obtained from the ratio between processively active SOS and total SOS on the membrane (grey - individual trace, purple - mean trace). (E) Plots of Ras nucleotide-dependent activation of SOS.

kinetics are not directly transferable to collision rates on the membrane, this estimation reveals that the Ras binding step is a rapid process, significantly shorter than the time scale required for SOS activation [25]. Consequently, we concluded that Ras binding is not a rate-limiting step in SOS activation.

The transition rates of Ras-bound SOS into the processively active state (k_7 and k_8) have also not been experimentally measured before. Nevertheless, the constraints arising from the Ras nucleotide-independent recruitment of SOS^{FL} to the membrane offered insights into parameter selection for the model. To achieve similar overall recruitment levels along both the RasGTP and RasGDP pathways, we needed the average time SOS molecules spent on the membrane to be roughly equal. One way to achieve this parity was by ensuring that only a tiny fraction of all recruited SOS molecules entered the processive state. This was accomplished by introducing two slow steps (SOS_0 to SOS_1 and $SOS_{1,RasGTP}$ to

$\text{SOS}_{\text{p,RasGXP}}$) with similar rates. These steps ensured that, despite nucleotide preference, the average dwell time along the RasGTP pathway closely matched that of the RasGDP pathway, as inferred from the SOS^{FL} receptor-mediated recruitment assay experimental data (Figure 5.2E).

With these conditions, the model parameters were adjusted to replicate experimental observations. When comparing the effects of introducing nucleotide preference to either k_5 and k_6 ($k_5=10k_6$) or k_7 and k_8 ($k_7=10k_8$), we found that the slower pair (k_7 and k_8) had a more pronounced impact on the system. This confirms that accelerated autoinhibition release can indeed serve as a mechanism for positive feedback in activation of Ras by SOS. Firstly, SOS recruitment in the simulations was independent of the Ras nucleotide state. Secondly, the overall recruitment and nucleotide exchange reactions followed a time scale similar to that observed in experiments (Figure 5.4B-C). Thirdly, the fraction of active SOS, which is directly proportional to the apparent average catalytic rate per SOS molecule, increased with higher RasGTP levels (Figure 5.4D). Moreover, the simulation allowed us to calculate the fraction of SOS activation during homonucleotide exchange (RasGTP to RasGTP and RasGDP to RasGDP; Figure 5.4E), which could not be experimentally measured. The simulation results revealed that the system activated most rapidly when starting with all RasGTP, as all SOS activations occurred through the accelerated pathway. In contrast, in the RasGDP homonucleotide exchange condition, the fraction of activated SOS remained low throughout the simulation. A shift from slow to fast activation was observed in the RasGDP to RasGTP simulation as more RasGTP was produced from the nucleotide exchange reaction, resembling the positive feedback seen in the Ras activation by SOS^{FL} experiments.

5.4 Discussion and conclusion

In this study, we have resolved the mechanistic source of positive feedback in the native SOS^{FL} protein. SOS has long been known to play an important role in the bimodality behavior of signaling through Ras and the MAPK pathway [178, 197]. Although SOS has a preference towards RasGTP binding at the allosteric site [131], which would seem to be the most obvious source of positive feedback in its Ras activation kinetics, this preference becomes inconsequential in the native SOS^{FL} due to its autoinhibition. Additionally, single molecule experiments have demonstrated that SOS catalytic activity is independent of Ras-nucleotide state at the molecular level—ruling this out as a positive feedback mechanism. Here we have shown, through both experiments and stochastic kinetic modeling, that it is the autoinhibition release process in SOS^{FL} that provides the dominant source of positive feedback.

A few other recent studies have also revisited the origin of the positive feedback in Ras activation by SOS. The studies by Vo et al. [207] and Liao et al. [208] focused on the

interaction between Ras and SOS catalytic site and allosteric site, suggesting RasGDP is a weaker binder than RasGTP towards the allosteric site, but a stronger binder towards the catalytic site. However preferential binding of RasGDP to the catalytic site is not likely a driver of the positive feedback observed in the experiments described here, which were all performed at high Ras density where the catalytic nucleotide exchange step—not the RasGDP binding to the catalytic site—is rate limiting.

Another paper from our own group [213] has examined reconstituted bimodality in a competitive Ras activation-deactivation assay. In that work, processivity of SOS was further identified as a key driver of bimodality in the competitive reaction. Taken together, all these recent studies and the present work reveal a multifaceted mechanism of positive feedback in the activation of Ras by SOS. Though simple effects of allosteric activation by preferential binding of RasGTP to the allosteric binding site clearly leads to positive feedback in truncated SOS variants, the SOS^{FL} protein is much more complex and relies heavily on autoinhibition release and processivity to achieve its macroscopic behavior.

5.5 Materials and Methods

Protein purification

LAT-His₁₀ Human LAT cytosolic domain (27-233) was purified with N-terminal 10-histidine tag [24]. The histidine tag was uncleaved for lipid bilayer functionalization.

Hck-His₆ Human LAT cytosolic domain (27-233) was purified with N-terminal 10-Histidine tag [102]. The histidine tag was uncleaved for lipid bilayer functionalization.

SOS^{FL} Human SOS1 was purified though split intein as described in [25].

SOSHDP The purification of human SOS1 HDPC (1-1049) was described in [69].

SOS^{CAT} The purification of human SOS1 catalytic core (566-1049) was described in [69].

Grb2 Human full length Grb2 was purified with N-terminal 6-histidine tag and a TEV site as described in [48].

Ras Human H-Ras (1-181, C118S) was purified with N-terminal 6-histidine tag and a TEV site as described in [25].

RBD RBD-K65E was derived from human Raf1 Ras binding domain with one point mutation (56-131, K65E). It was purified with N-terminal 6-histidine tag, GST, a PreScission site, and a SNAP tag as described in [25].

Protein labeling

LAT, SOS^{FL}, and SOS^{HDPC} were labeled with Alexa-555 or Alexa-647 C2 Maleimide (Invitrogen). Proteins were diluted to 100 μ M or less with 5mM TCEP (Sigma-Aldrich), then react with 1 mM maleimide dye for 2 hr in room temperature. The reaction was then quenched with 5 mM 2-mercaptoethanol (BME, Sigma-Aldrich) for 10 min. Excessive dyes were removed by size exclusion chromatography (SEC) via FPLC (Superdex 75, Cytiva).

RBD sensor was labeled with SNAP-Surface Alexa-488 or Alexa-647 (New England Biolabs). Proteins were mixed with the SNAP-Surface dye at 1:1.5 reaction in PBS and 10 mM DTT at 18 °C overnight. The reaction mixture was buffer exchanged to 20 mM Tris, 200 mM NaCl, 10% glycerol, and 1 mM TCEP before running through SEC to remove excess dye.

Supported lipid bilayer preparation and assay

SUVs Small unilamellar vesicles were made using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), L- α -phosphatidylinositol-4,5-bisphosphate (Brain, Porcine) (ammonium salt) (PI(4,5)P₂), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)-cyclohexane-carboxamide] (sodium salt) (MCC), and 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (Ni-NTA) from Avanti Polar Lipids. Lipids were mixed in an etched and dried round bottom flask to yield 2 mmol total lipid at 94:2:2:2 (mol%) DOPC:PI(4,5)P₂:MCC:Ni-NTA. After mixing, lipids were dried by rotovap in a 37 °C water bath for 2 min then in room temperature for 15 min. Later, the round bottom flask was flushed with N₂ for 15 min. The lipid film was hydrated with 2 mL of milli-Q water for a final concentration of 1 mM total lipids. The cloudy lipid solution was added to a 5 mL polypropylene tube and treated by 3 mm stepped tip sonication at 33% amplitude, 20 s sonication, 50 s rest, 5 cycles (Sonics VCX750) while submerged in ice. The lipid solution was clarified after sonication.

Reaction chamber Precision Schott D 263 M glass coverslips (#1.5H, 75 x 25 mm) was rinsed with milli-Q. Coverslips were then etched with piranha made with 1:3 ratio of concentrated H₂SO₄ (Fisher Scientific) and 30% H₂O₂ (Fisher Scientific) for 5 min and rinse extensively with milli-Q water. To make the experiment chamber, etched coverslips were blow-dried with N₂ and attached to 6-channel flow cells (sticky-Slide VI 0.4, ibidi).

SLB Supported lipid bilayer were made through SUV vesicle fusion. The 1mM lipid solution was diluted to 0.25 mM in PBS (Fisher Scientific). Then, 150 μ L of 0.25 mM lipid solution was added to each channel and incubate for 30 min at room temperature. After incubation, the channel was washed with 1mL PBS and blocked with 1mg/mL blocker casein (Thermo Fisher Scientific) in PBS for 10 min. Each channel was washed with 1mL PBS.

Ras tethering To attach H-Ras on the bilayer, 100 μ L of 0.3-0.5 mg/ml H-Ras in reaction buffer (40 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM MgCl_2) was added to each channel at room temperature for 2 hr 30 min. Each channel was washed with 1 mL PBS and flowed with 100 μ L of 5 mM BME in reaction buffer to quench the remaining free maleimide. Each channel was washed with 1 mL PBS again before the Ras bilayer being stored in 100 μ L of 100 μ M guanosine-5'-diphosphate disodium salt (Chem-Impex) in reaction buffer overnight at 4 $^{\circ}\text{C}$.

LAT tethering To attach LAT and phosphorylate LAT on the bilayer, the chamber was first taken out of 4 $^{\circ}\text{C}$, equilibrated to room temperature, and washed with 1 mL PBS. Then, 100 μ L of 14 nM of LAT and 12.5 nM of HCK were added to each channel for 40 min. After washing the channel with 1 mL PBS, 100 μ L of 1 mM of ATP and 100 μ M of GTP were added to the channel for LAT phosphorylation and ensure nucleotide loading.

RasGxP recruitment assay Due to the intrinsic activity of Ras and supplied GDP in the solution, Ras started as RasGDP on the bilayer. To make RasGTP bilayer, the channel was washed with 1 mL PBS, then 100 μ L of 2 nM SOS^{CAT} , 1 μ M of GTP (Guanosine-5'-Triphosphate Trisodium Salt, Fisher Scientific), and 1 mM of ATP in reaction buffer was flowed into each channel for 30 min. To control for the perturbation brought by SOS^{CAT} to the experiment, a RasGDP bilayer was prepared side by side with 100 μ L of 2nM SOS^{CAT} , 1 μ M of GDP, and 1mM of ATP in reaction buffer for 30 min. To wash away SOS^{CAT} in the system, 3 mL of PBS with 100 μ M GTP or 100 μ M GDP was flow through the RasGTP bilayer or RasGDP bilayer respectively. The channel was stored in 1 mM of ATP and 100 μ M of GTP or GDP in reaction buffer before any assay. 1 nM of Alexa647-labelled SOS^{CAT} , SOS^{HDPC} , or SOS^{FL} (and 20nM Grb2 for SOS^{FL} exp) was added with 20 nM of Alexa488-labelled RBD, and 1 mM GXP (GTP for RasGTP membrane, GDP for RasGDP membrane) were injected in the flow cell.

Imaging buffer All imaging experiments were ran in imaging buffer with 40 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM MgCl_2 , 10 mM BME, 100 μ M ATP, and 2 mM UV-treated Trolox.

Microscope hardware and image acquisition

TIRF imaging experiments were performed on an inverted Nikon Eclipse Ti microscope using a 100X Nikon oil-immersion TIRF objective (1.49 NA), and the 1.5X lens tube were also used in the experiment. 488/561/647 nm diode laser (OBIS laser diode, Coherent) were controlled by a custom built Solemere laser driver to excite fluorophores, and the images were acquired with an iXon Ultra 897 EMCCD camera (Andor Technology). All microscope hardware was controlled using Micro-Manager v4.0. Images were acquired with 15 s time lapse, 500 EM gain, at 100 ms exposure time. High laser intensity (20 mW out of box) was used for single molecule density experiments, and lower laser intensity

(2mW) was used for bulk density measurements. For all kinetic traces, middle 250*250 pixels were used to extract data. RBD fluorescence signal were corrected for uneven illumination using shading corrector (Fiji). Single particles were identified with TrackMate (Fiji).

Ras and LAT density calibration

Surface density of Ras and LAT were measured using fluorescence correlation spectroscopy (FCS). The setup was previously described in (6). The FCS imaging experiments were performed on a home-built confocal system on a Nikon TE2000 inverted microscope using a 100x Nikon oil-immersion PlanFluor objective (1.30 NA). A pulsed supercontinuum laser (SuperK Extreme; NKT Photonics) with 100 ps pulse duration at 19.5 MHz repetition rate was used. The signals were recorded by an avalanche photodiode detector (Hamamatsu) and then processed by a hardware correlator (Correlator.com). Then, the autocorrelation function was fitted to a 2D diffusion model to extract surface density and diffusion coefficient. Ras density was measured with Ras nucleotide exchange to EDA-GDP-ATTO-488 (Jena Bioscience), as described in (6). LAT density was measured with Alexa-555 labeled LAT and corrected for labeling efficiency. A calibration was developed from Ras density measured from FCS and Ras density measured by RBD TIRF signal. Similar LAT density calibration was developed from FCS and TIRF fluorescently labeled LAT signal.

k_{obs} calculation and interpretation

Since Ras density is much higher than SOS^{FL} density, SOS^{FL} is assumed to work under substrate saturating regime. Therefore, the nucleotide exchanged reaction was approximated to be a second order reaction:

$$\frac{d[\text{RasGTP}]}{dt} = k_{\text{obs}} \cdot \sigma[\text{SOS}] \cdot \sigma[\text{RasGDP}] \quad (5.1)$$

Where σ denotes surface density, k_{obs} is the ensemble average catalytic activity per SOS^{FL} molecule. Due to intrinsic fluctuations in RBD and SOS recruitment, the raw k_{obs} calculations were scattered. To obtain the underlying trend, we grouped every 5 time points and used the mean RasGTP level and SOS^{FL} membrane density to calculate the k_{obs} . The standard deviation was carried to the error bar shown in Figure 5.3D.

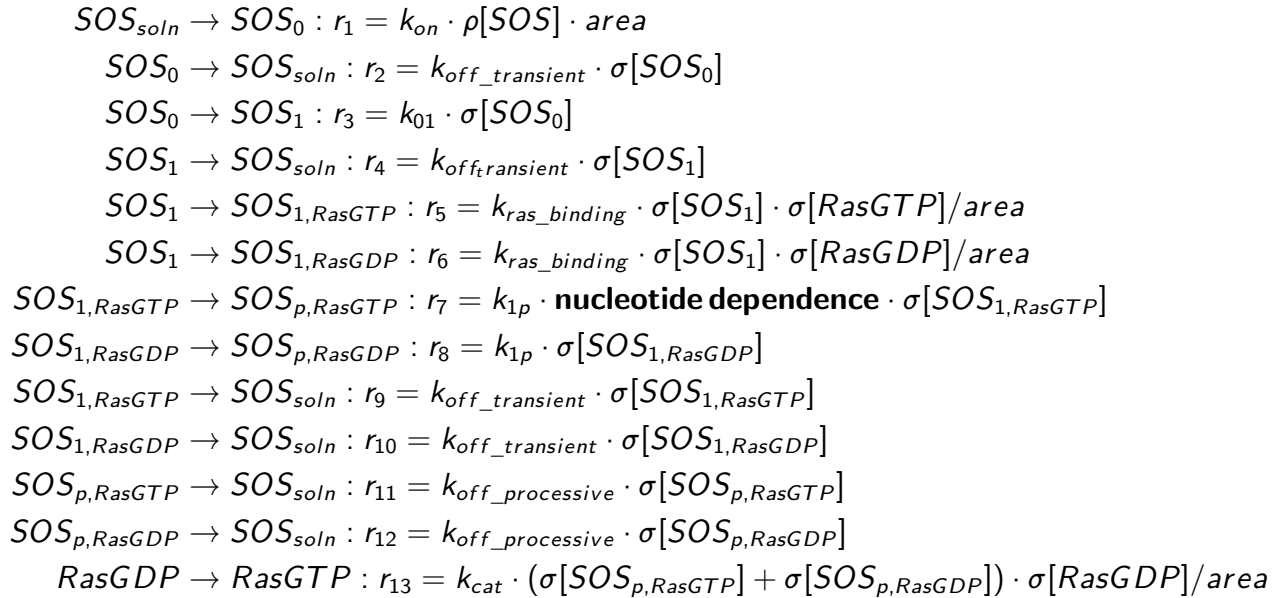
Stochastic simulation

The reaction pathway was listed in Fig. 4A. Since the nucleotide exchange rate (k_{SOS}) of processive SOS^{FL} is nucleotide independent, the fraction of active SOS^{FL} ($f(\text{act})$) is

proportional to the ensemble average catalytic rate:

$$k_{\text{obs}} = \frac{\sigma[\text{SOS}_{\text{active}}] \cdot k_{\text{SOS}} + \sigma[\text{SOS}_{\text{inactive}}] \cdot 0}{\sigma[\text{SOS}_{\text{active}}] + \sigma[\text{SOS}_{\text{inactive}}]} \cdot k_{\text{SOS}} = f(\text{act}) \cdot k_{\text{SOS}} \quad (5.2)$$

The time evolution for the number of each species follows the Gillespie Algorithm (7) and was conducted in MATLAB_R2020B. ρ denotes the solution concentration, and σ denotes the particle number on the surface.



Each simulation lasts 2000s, and 10 trials of simulation were conducted for each condition. The Ras density, on rate, and SOS concentration were chosen to be comparable to experiments. The area was chosen to be big enough to not be in the stochastic regime. The following kinetic parameters were used:

$$\begin{aligned} \text{area} &= 1600 \mu\text{m}^2 \\ \sigma[\text{Ras}] &= 500 \text{molecule} / \mu\text{m}^2 \\ k_{\text{on}} &= 0.0018 \text{s}^{-1} \text{nM}^{-1} \\ \text{nucleotide dependence} &= 10 \\ k_{\text{off_transient}} &= 0.016 \text{s}^{-1} \\ k_{\text{off_processive}} &= 0.001 \text{s}^{-1} \\ k_{01} &= 0.001 \text{s}^{-1} \\ k_{\text{ras_binding}} &= 0.1 \text{s}^{-1} \\ k_{1p} &= 0.0003 \text{s}^{-1} \\ k_{\text{cat}} &= 1.5 \text{s}^{-1} \end{aligned}$$

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