

UC Berkeley

UC Berkeley Electronic Theses and Dissertations

Title

Investigating protein condensation in ligand mediated cell signaling

Permalink

<https://escholarship.org/uc/item/5z87t11w>

Author

Stinger, Brittany

Publication Date

2024

Peer reviewed|Thesis/dissertation

Investigating Protein Condensation in Ligand Mediated Cell Signaling

by
Brittany Stinger

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 Andreas Martin

Summer 2024

Investigating Protein Condensation in Ligand Mediated Cell Signaling

Copyright 2024

By

Brittany Stinger

Abstract

Investigating Protein Condensation in Ligand Mediated Cell Signaling

by

Brittany Stinger

in Chemistry

University of California, Berkeley

Professor Jay T. Groves

T cells discriminate between foreign agonist- and self-antigen peptides presented on Major Histocompatibility Complexes (MHC) proteins that bind to T-cell Receptors (TCRs). Mimicking this process is difficult in vivo due to the monoclinal activity of pMHC. To overcome this, antibodies are commonly used to activate T-cells via conserved regions of the TCR. However, antibodies bind TCRs strongly and activate T-cells via a different mechanism than pMHC binding. We previously reported the binding capabilities of a Fab'-DNA ligand that mimics strong TCR agonists and only activates T-cells when tethered to a supported lipid bilayer. To mimic native pMHC, we created a panel of a human antiCD3 -fab' (OKT3-Fab') that more closely mimic native pMHC binding kinetics, but in a polyclonal manner. Single molecule characterization via total internal reflection fluorescence (TIRF) is used to determine dwell times and measure diffusion characteristics. Potential uses for these ligands include measuring the activation of human primary T-cells.

Following activation, adapter proteins including growth factor receptor bound protein 2 (Grb2) and son of sevenless (SOS) are recruited to the lipid bilayer. Two-dimensional protein condensation phase transitions on the membrane driven by tyrosine phosphorylation have been identified in several receptor signaling systems, including T cell receptor via the Linker for the Activation of T-cells (LAT) that contains a tyrosine sites and Epidermal Growth Factor Receptor (EGFR) that contains tyrosine sites on the cytoplasmic tail. In the cases of both LAT and EGFR, we have found that the receptor scaffold and Grb2 are sufficient to form a membrane surface condensate in reconstituted systems using supported bilayer membranes. Furthermore, phosphorylation at the Y160 residue on Grb2 is sufficient to block this condensation. To follow up on these reconstitution results in live cells, we investigated Grb2 mediated phase condensation in epithelial cells. CHO cells, a type of epithelial cell with a low expression of Grb2 and no endogenous EGFR, were transfected with various Grb2 mutants and EGFR-eGFP. Clustering of EGFR was observed with all Grb2 dimer mutants, suggesting EGFR clustering is not mediated solely through the Grb2:Grb2 dimer interaction. However, EGFR clustering was prevented with a mutant that blocks the Grb2:SOS interaction. EGFR clustering was recovered with larger amounts of this mutant, suggesting that the Grb2:Grb2 dimer plays a role in EGFR clustering under certain conditions.

Contents

Contents	i
List of Figures.....	iii
List of Tables	iv
Chapter 1 Introduction.....	1
Chapter 2 A two-component protein condensate of EGFR and Grb2 regulates Ras activation at the membrane.....	4
2.1 Abstract	4
2.2 Introduction	4
2.3 Results	6
2.4 Discussion and Conclusion	14
2.5 Materials and Methods	15
2.6 Supporting Information	19
Chapter 3 The role of Grb2 in the regulation of EGFR Clustering in CHO Cells.....	23
3.1 Introduction	23
3.2 Results	24
3.3 Discussion	30
3.4 Materials and Methods	33
3.5 Appendix	36
Chapter 4 A Synthetic Panel of OKT3-Fab' Ligands Modulates Binding Kinetics to TCR39	
4.1 Introduction	39
4.2 Results	41
4.3 Discussion	45
4.4 Materials and Methods	46
4.5 Appendix	51
Chapter 5 Conclusions and Future Directions.....	52
5.1 Conclusions	52
5.2 Investigation of EGFR Signaling in Live Cells	53
5.3 Probing Human Primary T-cells with OKT3-Fab' Ligands	54

References.....	55
------------------------	-----------

List of Figures

2.1 EGFR Reconstitution and Phase condensation with Grb2	8
2.2 Colocalization of EGFR ^{Tail} and Grb2.....	9
2.3 Reversible Phase Condensation of EGFR ^{Tail} and Grb2 with Phosphatase	10
2.4 Modulation of EGFR Phase Condensation via Grb2 Phosphorylation.....	11
2.5 Schematic of EGFR Phase Condensation.	12
2.6 Ras activation modulation by Grb2 Phase Condensation.	13
2.7 Supplementary Figure 1 FRAP measurements of EGFR pre and post Phase condensation...	19
2.8 Supplementary Figure 2 Recruitment of Grb2Y ^{160E} to EGFR ^{Tail}	20
2.9 Supplementary Figure 3 Ras Activation Comparison between Grb2 ^{WT} and Grb2 ^{Y160E}	22
3.1 EGFR Clusters in CHO Cells are a Result of EGFR Phase Condensation.....	26
3.2 EGFR Clusters Observed with Different Grb2 Mutants that prohibit Grb2 Dimerization	28
3.3 EGFR Clusters in the presence of Increasing Concentration of Grb2 ^{P49L}	29
3.4 Schematic of the Impact of Increasing Grb2 on the Phase Condensation of EGFR.....	31
3.5 Supplementary Figure 1 Time Series of EGFR Clustering.....	36
3.6 Supplementary Figure 2 Western Blot of Grb2 from increasing Grb2 Transfection Times. ..	37
3.7 Supplementary Figure 3 Western Blot of Grb2 from different Grb2 Constructs.....	37
3.8 Supplementary Figure 4 Recruitment of Grb2WT-mScarlet and Grb2P49L-mScarlet.....	38
3.9 Supplementary Figure 5 Average Intensity Increase of Grb2 after activation.....	38
4.1 Schematic for the Purification of OKT3-Fab' Ligands and labeling	41
4.2 OKT3-Fab' Ligands are mobile on the bilayer and exhibit 2-D Brownian Motion	43
4.3 Dwell Times of OKT3-Fab' Ligands.	44
4.4 Supplementary Figure 1 Density Calculations for OKT3-Fab'-DNA Ligands	51

List of Tables

2.1 Mass Spectra of Grb2 Phosphorylation Sites by Hck	21
---	----

Acknowledgements

I would also like to thank all the many mentors, teachers, and friends who have supported me throughout the years.

First, thank you to my dad and mom for supporting me throughout my PhD and for listening to me talk about my project. To my sisters, Arianna, Samantha, and Courtney, thank you for keeping my spirits high and making me laugh. To my grandmothers, Joan and Theresa, and my aunts, Sherry and Carol, thank you for the many words of support. Thank you to the rest of my family for your love and support.

My advisor, Jay Groves, for taking me on as a graduate student and helping me understand the complexities of T-cell signaling and allowing me to work on a variety of projects. Jay's insight was always helpful for bringing all the experiments into the bigger picture.

I would like to thank all the Groves lab members who taught me so many techniques in the lab, from how to make a lipid bilayer without defects to taking care of cells. To all the lab socials that reminded me to take a break from research and provide a distraction so I could come back to lab refreshed. A special shout out to camping trips, ski trips, hikes, bouldering and axe throwing adventures. I would like to extend a special thanks to Chun-Wei Lin, Nugent Lew, Henry Phan, Anand Vissa, Joey DeGrandchamp, Kiera Wilhelm, Saehyun Choi, He Ren, Shumpei Morita, Hyuneil Kim, Darren McAfee, Mark O'Dair, Sungi Kim, Timothy Eisen, Serena Huang, Hun-Ro Lee, Sarah Hoyle, Cindy Lu, and other past Groves lab members.

To all the friends I made in Berkeley, thank you for the camaraderie as we braved classes and quads together. I wouldn't have made it through without you. A special thanks to my friends, Desiree, Alana, Josh, Katie, Leah, and Eric, thank you for the many zoom calls, the words of encouragement, and visits that made adjusting to life in graduate school and life in Berkeley easier. To all my mentors and friends from high school and undergraduate that encouraged me and made me realize that I wanted to have a career in science.

Finally, to James Tyler, thank you for all your love, for your support through many stressful days, and for the many adventures so far and many more to come!

Chapter 1

Introduction

T-cells are an essential part of the immune system, responsible for identifying the presence of foreign pathogens and inducing a cellular response to kill these pathogens.^{1,2} The T-cells differ from other lymphocytes due largely to the presence of T-cell receptors (TCRs) at the membrane.² When a T-cell probes cells to identify foreign pathogens, the TCRs bind to proteins on the surface of cells called major histocompatibility complex (MHC).^{3-6,2} After an antigen presenting cell (APC) is identified, the T-cell releases cytokines and leads to the production of cells capable of killing the APC.^{1,2} To initiate signal transduction, T-cells must discriminate between self and agonist MHC to identify antigen presenting cells in an abundance of self-antigen of a ratio of about 1000:1 self-MHC to agonist MHC.⁷ T-cells that do not distinguish self and agonist MHC result in autoimmune diseases.^{8,9} This sophisticated mechanism of T-cell activation can be explained with a kinetic proofreading model in which the T-cell Receptor (TCR):ligand binding initiates a series of intermediates that eventually lead to an activated TCR.¹⁰ If the ligand unbinds during an intermediate step, the TCR does not activate and initiate downstream signaling.¹⁰ The consequence is that although many different ligands can bind to the TCR, only the longer interacting TCR:Ligand events lead to activation of the T-cell.^{7,11-14} This model allows for single molecule sensitivity of the T-cell and T-cells have been shown to activate for <10 ligands.¹¹

Upon activation of the TCR, the phosphorylation of tyrosine residues on the Linker for the Activation of T-cells (LAT) leads to the recruitment of adapter proteins like Growth Factor Receptor Bound Protein (Grb2) and Son of Sevenless (SOS) that allow for the activation of Ras and lead to the phosphorylation of ERK in addition to other proteins like phospholipase (PLC γ 1), and Grb2-related adaptor downstream of Shc (GADS) and SH2 domain-containing leukocyte phosphoprotein of 76kDa (SLP-76).¹⁵⁻²² The recruitment of Grb2 and SOS to LAT results in a phase condensation necessary to localize SOS to the membrane, resulting in SOS being released from its autoinhibited state and enabling the activation of Ras via guanine nucleotide exchange.^{17,20,23} The LAT phase condensation has been observed in T-cells as clustering.^{15,16}

Phase condensation of proteins and RNA is a well characterized phenomenon in cells.^{24,25} The organization of proteins into phase condensates compartmentalizes components in response to stress and allows cells to enact cellular functions like signal transduction.²⁶ These condensates are governed by multivalent interactions where proteins have multiple domains capable of interacting with specific domains on another protein.^{24,27} For example, in the LAT system, the SH2 domain of Grb2 recognizes specific phosphotyrosine residues and Grb2 has two SH3 domains capable of binding multiple proline rich domains on SOS. The ability of the phase condensation to form is a result of enthalpic and entropic contributions.²⁴ The protein-protein interactions or Protein-RNA interactions are governed by attractive forces like oppositely charged amino acid pockets.^{24,26} When these macromolecules order into a phase condensate, they must overcome the entropic barriers that result from demixing.²⁸ However, phase condensates mimic polymer physics where the creation of long chains minimizes the entropic loss from demixing.²⁹ The LAT phase

condensation is a phase condensation that occurs on the lipid bilayer. Although it is restricted to the lipid bilayer, the multivalent interactions and local copy number of proteins in the cell allow for a phase condensation to occur.

The MAPK/ERK is ubiquitous among many different cell types including cells expressing phosphotyrosine kinases like the ErbB family of receptor tyrosine kinase; two of receptors in the erbB family are epidermal growth factor receptor (EGFR) and Her2.³⁰ This receptor tyrosine kinase is analogous to LAT since it contains a cytoplasmic tail with phosphotyrosines capable of binding to adapter proteins like Grb2.¹⁹ Ligand binding of EGFR to the extracellular region leads to a conformational change in the juxtamembrane region and a dimerization of the transmembrane domain near the N termini leading to activation of the kinase domain and autophosphorylation of the cytoplasmic tail.^{31–33} EGFR is implicated in lung, brain, and breast cancers.³⁰ EGFR overexpression and deletion or point mutations cause a constitutively active conformation of EGFR leading to the overactivation of MAPK/ERK and related pathways.^{34–36} Current treatments for the EGFR related cancers include treatment with EGFR-tyrosine kinase inhibitors (TKIs), like gefitinib, and work by preventing the EGFR tyrosine kinase domain from autophosphorylation.^{37,38} Although TKI treatment is widespread, mutations in the kinase domain raise questions about how to target EGFR related cancers without patients developing resistance.^{38–42} To better understand EGFR related cancers, there needs to be a better understanding of the EGFR signaling in relation to the ability of EGFR to interact with a number of proteins. In addition to interacting with adapter proteins, EGFR is also known to form homo and hetero-dimers with itself or other members of the ErbB family of tyrosine kinase receptors.⁴³ EGFR is reported to form higher order oligomers and this phenomenon has been characterized by diffusion and FRET assays.^{44–47} Investigating EGFR interaction with other proteins could provide insight into EGFR overactivation in cancer cells.

We can use a supported lipid bilayer system (SLB) in which single unimolecular vesicles (SUVs) are deposited on a hydrophilic glass surface.^{48–50} This allows for the free diffusion of lipids on the glass that mimic the mobility of the cellular lipid bilayer. The lipids used for SLBs include Ni-NTA and MCC-PE, which allow for the conjugation of proteins to the supported lipid bilayer via Nickel-Histidine chemistry or cysteine maleimide chemistry. Fluorescently labelled proteins conjugated to the supported lipid bilayer are probed via Total internal fluorescence (TIRF) microscopy; this type of microscopy hits the glass at a specific angle allowing that creates an evanescent wave that decays exponentially as it travels away from the glass surface, thus allowing for selective activation of fluorophores on the supported lipid bilayer.⁵¹ This system is ideal for investigating different aspects of the MAPK/ERK and other signaling pathways that occur at the lipid bilayer. In previous studies, fluorescently labeled LAT is reconstituted on a supported lipid bilayer via a histidine tag capable of binding multiple nickel lipids.⁵² To the fluorescently labeled LAT, purified proteins Grb2 and SOS can be added to induce phase condensation observed as the fluorescence changes from a homogenous layer to areas with high and low intensities.^{17,18}

In Chapter 2, I report a EGFR phase condensation mediated by Grb2:Grb2 interactions that affect the activation of Ras.¹⁹ Here we reconstitute purified and fluorescently labeled EGFR tail on a supported lipid bilayer with adapter proteins Grb2 and SOS. Although the phase condensation between SOS, Grb2, and proteins with phosphotyrosine tails like LAT and EGFR has been well characterized, we observe phase condensation between EGFR tails and Grb2 only.^{17,18} This can be explained by the ability of Grb2 to interact with itself and form dimers.^{53,54} By phosphorylating

Grb2 or introducing a glutamate mutation at tyrosine residue 160 (Grb2^{Y160E}) there is a dissolution in the phase condensation. Further experiments involving the Grb2^{Y160E} mutant and SOS show a difference in Ras activation compared to Grb2^{WT} and SOS.

In Chapter 3, I expand upon the observations made in the reconstitution experiments to investigate the EGFR:Grb2 phase condensation in live cells. In these experiments, we transfect CHO cells with EGFR and various Grb2 mutants that disrupt the ability of Grb2 to interact with itself. Stimulation with EGF reveals EGFR clustering mediated in a phosphotyrosine dependent manner instead of EGFR oligomerization. The Grb2 mutants including Grb2^{Y160E} as well as Grb2^{V123D}, which is a mutant reported to disrupt the Grb2 SH2:SH2 domain swap dimer, do not prevent EGFR clustering in CHO cells. However, a Grb2 mutant that prevents interaction with SOS (Grb2^{P49L}) can prevent EGFR clustering. Increasing expression of Grb2^{P49L} recovers EGFR clustering, suggesting a more complex role of the Grb2:Grb2 interaction in the phase condensation in live cells.

In Chapter 4, I investigate a synthetic panel of TCR ligands with various potencies. T-cell activation usually consists of bulk activation and measurement of IL2 or cytokines, however, this does not allow for the single molecule sensitivity exhibited by T-cells.⁵⁵ Additionally, activating a polyclonal population of T-cells with pMHC is difficult due to TCR variability that would require the identity of each TCR and the complementary pMHC for each T-cell.^{6,56,57} Antibodies bypass this problem by binding the constant region of the TCR, however, their large binding constants can result in the overactivation of the T-cells. Additionally, antibodies can bind multiple TCRs, which artificially bring two TCRs together and resulting in overstimulation of the T-cells. Using the supported lipid bilayer system we can conjugate the Fragment of antigen binding (Fab') to a DNA-dye tether that interacts with a complimentary fragment of DNA on supported lipid bilayer.⁵⁸⁻⁶⁰ The modulation of the epitope and the length of the DNA tether allows us to probe mechanistic questions of the TCR triggering; however, due to the strong binding of the epitope activation of T-cells, these antibody fab' do not mimic native MHC conditions.⁵⁸ The development of a α -TCR β H57 Fab' with a mutation at residue R97 to leucine solves this issue by decreasing ligand potency in a way that mimics native pMHC binding.⁶⁰ H57 is a murine T-cell antibody and most research thus far has used isolated murine T-cells to study the mechanisms of T-cell activation.⁶⁰ Ligands capable of activating human T-cells in a similar manner to APCs would allow us to study differences in T-cells from different patients and explore quantitative studies of T-cell activation which may differ slightly from murine T-cells. In this chapter I characterize a panel of anti CD3 OKT3-Fab' ligands with different mutations suggested from a paper characterizing bulk T-cell activation.⁶¹ Initial results indicate that the dwell time of these ligands can be modulated via these mutations. These mutants will be used to probe quantitative questions of primary T-cells and mimic native pMHC binding to illicit an environment that mimics native T-cell activation.

Chapter 2

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

Copyright Notice

The following chapter and its figures were adapted and/or reprinted from “A two-component protein condensate of the EGFR cytoplasmic tail and Grb2 regulates Ras activation by SOS at the membrane” Chun-Wei Lin^{1,2,3}, Laura Nocka^{1,2,3}, Brittany Stinger¹, Steven Alvarez¹, Joey DeGrandchamp¹, Nugent Lew¹, Henry Phan¹, Yasushi Kondo^{1,2,3}, John Kuriyan^{1,2,3,4,*}, Jay T. Groves^{1,3,5,*} *Proc. Natl. Acad. Sci. U. S. A.* **119**, e2122531119 (2022).

¹Department of Chemistry

²Department of Molecular and Cell Biology

³California Institute for Quantitative Biosciences

⁴The Howard Hughes Medical Institute

University of California, Berkeley, Berkeley, CA 94720, USA

⁵Division of Molecular Biophysics and Integrated Bioimaging, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

2.1 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 the dimerization of Grb2, which provides the crosslinking element for condensation with EGFR. The Grb2 Y160 residue plays a structurally critical role in dimer formation, 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 EGFR condensation controls the ability of SOS 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.

2.2 Introduction

Recently, a class of phenomena known as protein condensation phase transitions have begun to emerge in biology. Originally identified in the context of nuclear organization²⁶ and gene expression²⁵, 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.^{16,18,23} 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 1 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 1 through further specific protein-protein interactions.^{21,22} 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 condensate²⁷ 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.^{17,24,62} 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.^{63,64} 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.^{33,65,66} 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.⁶⁷ 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.^{30,67} There is an obvious conceptual connection between EGFR and the LAT signaling system in T cells. The ~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 upon receptor activation.⁶⁸ 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 multi-step autoinhibition release process and begins to catalyze nucleotide exchange of RasGDP to RasGTP, activating Ras and the MAPK pathway.⁶⁹

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.^{43,70–72} Structural analyses and point mutations on EGFR have identified a binding interface enabling EGFR asymmetric dimers to associate,⁴³ 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.³⁴ All too often, however, these drugs fail to offer sustained patient benefits, in large part because of poorly understood resistance mechanisms.⁷³ Physical aspects of the cellular microenvironment have been implicated as possible contributors to resistance development⁷⁴ and there is a growing realization that EGFR possesses kinase-independent (e.g. signaling independent) pro-survival functions in cancer cells.⁷⁵ 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 reconstitute the cytoplasmic tails of EGFR on supported bilayers and characterize the system behavior upon interaction with Grb2 and SOS using total internal reflection fluorescence (TIRF) imaging. This experimental platform has been highly effective revealing both phase transition characteristics and functional signaling aspects of LAT protein condensates.^{16–18,76–78} Published reports on the LAT system to date have emphasized SOS (or the SOS PR domain) as a critical crosslinking element. Titrating the SOS PR domain into an initially homogeneous mixture of phosphorylated LAT and Grb2 reveals 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 dimer interface. Phosphorylation on Grb2 at Y160 as well as a Y160E mutation (both known to disrupt the Grb2 dimer^{53,54}) are observed to prevent formation of EGFR condensates.

The consequence of EGFR condensation on downstream signaling is characterized by mapping the catalytic efficiency of SOS to activate Ras as a function of EGFR condensation state. SOS is the primary Ras guanine nucleotide exchange factor (GEF) responsible for activating Ras in the EGFR to MAPK signaling pathway.^{79–82} At the membrane, SOS undergoes a multi-step process of autoinhibition release before beginning 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.^{83–85} 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.^{17,18} 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 confirm 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.

2.3 Results

Reconstitution of a membrane-surface EGFR:Grb2 protein condensate

We reconstitute an EGFR:Grb2 protein condensation phase transition on supported membranes using the C-terminal region of EGFR (residues 991–1186, 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 the text, we refer to this construct of the cytoplasmic tail of EGFR as the “EGFR^{TAIL}”. EGFR^{TAIL} is anchored on the supported lipid bilayer through binding between its His tag and Ni-NTA lipid (incorporated into the supported bilayer at 4% molar ratio with DOPC lipid as the primary component) following previously published methods.^{17,18} The

SUMO fusion tag is used to increase the expression yield of the disordered EGFR^{TAIL} and acts 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} is fluorescently labeled with Alexa Fluor 488 through the maleimide chemistry and we achieve 66% labeling efficiency. EGFR^{TAIL} constructs anchored to the supported membrane undergo simple Brownian motion ($D = 1.76 \mu\text{m}^2/\text{s}$, measured by the fluorescence recovery after photobleaching experiments, Supporting Information Figure 2.7) and appear uniformly distributed on the membrane surface under total internal reflection fluorescence (TIRF) microscopy. The lateral density of the EGFR^{TAIL} on the membrane can be precisely measured by fluorescence correlation spectroscopy (FCS)^{18,86} and is controlled between 50 to 3000 molecules/ μm^2 in the experiments presented here. To maintain the EGFR^{TAIL} in its active, phosphorylated state, a separate Src-family kinase protein, Hck, is tethered to the membrane (also through His tag chemistry) and ATP is provided in solution. Src kinases do not normally phosphorylate the EGFR^{TAIL} efficiently⁶⁸, but in this system the pre-incubation with Hck suffices 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.^{18,77,87} Grb2 and, in later experiments, SOS are added to the pre-phosphorylated EGFR on supported membranes to initiate the condensation phase transition.

Upon addition of Grb2, the EGFR^{TAIL} is observed to undergo a robust condensation phase transition, as illustrated schematically in Figure 2.1A. Images tracking the phase transition process (Figure 2.1B) show an initially homogeneous distribution of the EGFR^{TAIL}, which undergoes a coarsening process after injection of Grb2 (6 μM) 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 exhibit undulating shapes and a sharp discontinuity in EGFR^{TAIL} density at the boundaries of the two phases. The Grb2-driven condensation of EGFR^{TAIL} occurs 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 are observed while fully percolating domain patterns are observed at higher densities (Figure 2.1C). The presence of both Grb2 and EGFR^{TAIL} in the condensates is confirmed by separately labeling them with Alexa Fluor 488 (EGFR^{TAIL}) and Alexa Fluor 647 (Grb2), respectively and performing two-color imaging (Figure 2.2).

The EGFR^{TAIL}:Grb2 condensation is reversible and can be dispersed by addition of phosphatase. Addition of the phosphatase YopH (1 μM YopH and 6 μM Grb2 in bulk solution) rapidly disrupts the EGFR^{TAIL}:Grb2 condensate, returning the system to a homogeneous and mobile distribution of EGFR^{TAIL} within 1 minute (see Figure 2.3). This confirms that even in the condensed state, sufficient dynamic unbinding and rebinding between Grb2 and the phosphorylated tyrosine sites on EGFR occurs to enable ready access by phosphatases (similar dynamic behavior has been observed in the LAT protein condensates¹⁸).

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 dimerization plays a functional role in FGFR signaling.⁵³

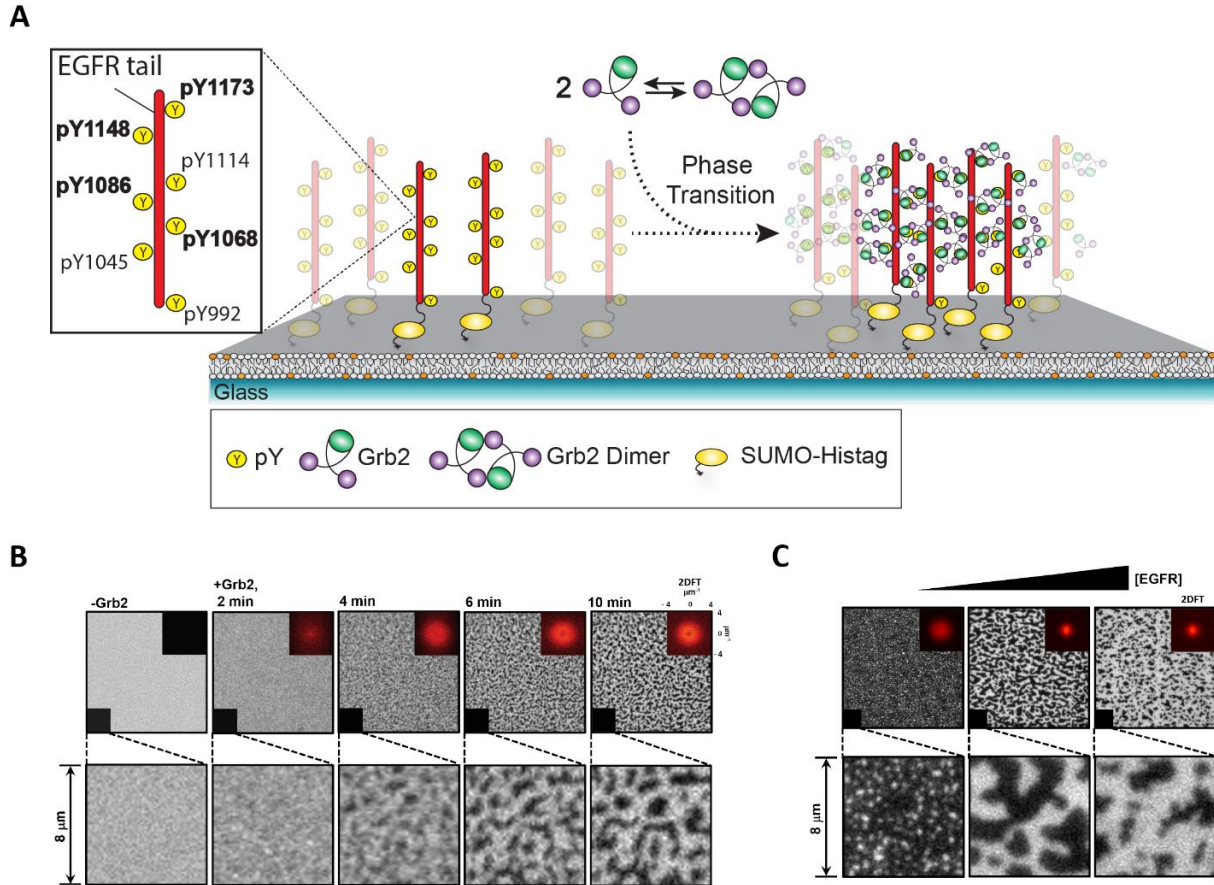


Figure 2.1: EGFR Reconstitution and Phase condensation with Grb2. EGFR^{TAIL} phase transition induced by the dimerization of Grb2. Figure 1. EGFR^{TAIL} phase transition induced by the dimerization of Grb2. (A) The schematic of the reconstitution experiment. The phosphorylated EGFR^{TAIL} is tethered on the supported membrane. EGFR^{TAIL} has several tyrosine sites that can be phosphorylated including Tyr 1068, Tyr 1086, Tyr 1148 and Try 1173 related to Grb2 recruitment. The monomeric Grb2 is shown to be in equilibrium with the Grb2 dimer. Grb2 is injected to the bilayer and recruited to the bilayer by EGFR^{TAIL}. 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. (B) The TIRF images of Alexa Fluor 488 labeled EGFR^{TAIL} on the supported bilayer. EGFR^{TAIL} undergoes phase transition in 2 minutes after the addition of Grb2. The protein condensate of EGFR^{TAIL}:Grb2 stabilizes in 10 minutes. The bottom figures are 8- μm by 8- μm zoom-in images of the lower-left corner in the upper images. The inserted figures at the upper right corner are the 2D Fourier Transform of the TIRF images. The phase transition shows the contribution at the low spatial frequencies. (C) The size of EGFR^{TAIL}:GRB2 condensate is modulated by the density of EGFR^{TAIL} from 50 to 3000 molecules/ μm^2 . EGFR^{TAIL}:Grb2 condensate varies from small clusters (left) to huge domains (right).

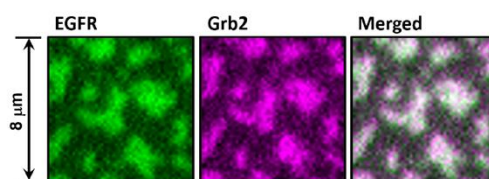


Figure 2.2: Colocalization of EGFR^{TAIL} and Grb2. The colocalization between EGFR^{TAIL} and Grb2 after EGFR^{TAIL}:Grb2 phase transition is achieved. Unlabeled Grb2 mixed with Alexa Fluor 647 labeled Grb2 (0.2 nM, labeling efficiency of 63%) was added to phosphorylated Alexa Fluor 488 labeled EGFR^{TAIL} to induce the phase transition of EGFR^{TAIL}:Grb2. The TIRF images were taken 10 minutes after the addition of Grb2. The first image in green is from EGFR^{TAIL}. The second image in magenta is from Grb2. The third image is the merged image from EGFR^{TAIL} and Grb2.

In the case of FGFR signaling, phosphorylation at Y160 on Grb2 breaks the dimer and enables downstream signaling. The Grb2 Y160 residue is located in the Grb2 dimer interface and a Y160E Grb2 mutant (referred to as Grb2^{Y160E}) has been reported to block dimerization.⁵⁴ To test if a Grb2 dimer interaction is responsible for the EGFR^{TAIL}:Grb2 condensation, we characterize the condensation with the Grb2^{Y160E} as well as phosphorylated wildtype Grb2 (pGrb2). Experimentally, wildtype Grb2 was phosphorylated by Hck and further analysis by mass spectrometry confirmed phosphorylation at Y160 (see Supporting Information Table 2.1). In both cases, EGFR^{TAIL}:Grb2 condensation is fully disrupted (Figure 2.4, A, B, and C, and Supporting Information Figure 2.8). 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. The condensate formed by Grb2 and the EGFR^{TAIL} is analogous to that formed by Grb2 and LAT, and we speculate that the same Grb2-mediated effects are also present in LAT condensates. Experiments with LAT and Grb2 confirm that a LAT:Grb2 condensate also forms in a manner very similar to what we have observed with the EGFR^{TAIL}, that is, Grb2 alone can drive the condensation, without the addition of SOS or other crosslinking elements (Figure 2.4D). Control experiments without ATP confirm that the EGFR^{TAIL}:Grb2 condensate is dependent on EGFR phosphorylation (Figure 2.4E).

EGFR condensation enhances Ras activation by SOS

To measure the effects of EGFR condensation on downstream signaling, we reconstitute the signaling pathway from EGFR to Ras. This reconstitution is depicted schematically in Figure 2.5 and includes the EGFR^{TAIL}, Grb2, phosphatidylinositol-4,5 biphosphate (PIP₂) (which participates in SOS autoinhibition release), full-length SOS (SOS^{FL}), Ras, and the Ras binding domain of Raf (referred as RBD), which provides a final readout of Ras activation by its selective recruitment to RasGTP^{43,44,17}. Ras is anchored to the membrane via MCC-PE lipids using maleimide chemistry following well-established methods.⁸⁸⁻⁹⁰ Prior to any experiments, membrane-associated Ras is loaded with GDP to establish a starting condition of fully inactive Ras (see 2.5 Material and Methods). In these experiments, Grb2 and the SOS^{FL} protein are maintained at sufficiently low concentrations that their presence alone is insufficient to trigger a condensation phase transition.

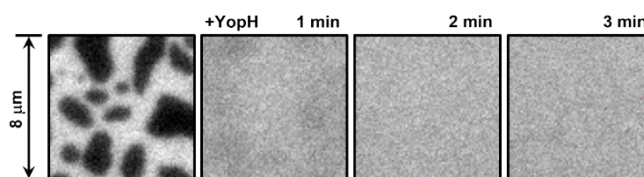


Figure 2.3: Reversible Phase Condensation of EGFR^{TAIL} and Grb2 with Phosphatase. EGFR^{TAIL}:Grb2 phase transition is reversed by phosphatase. The first image is the TIRF image of EGFR^{TAIL} condensation taken at 15 minutes after the addition of Grb2 to phosphorylated EGFR^{TAIL} on the supported bilayer. The second image is taken after the addition of 1 mM phosphatase, YopH. EGFR^{TAIL}:Grb2 condensate is rapidly dissolved in 1 minute. The last image, taken at 3 minutes after the addition of YopH, shows uniformly distributed EGFR^{TAIL} (also see Supporting Information Figure 2.7).

To control the phase transition, we titrate in the proline-rich domain of SOS (residues 1051-1333; referred as SOS^{PR}). SOS^{PR} is a strong crosslinker of Grb2 and has been previously used to control the LAT:Grb2:SOS condensation¹⁷, 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} are added in various ratios together with GTP (1 mM) and Alexa Fluor 647-labeled RBD (50 μM) to the pre-phosphorylated EGFR^{TAIL} and RasGDP on the supported membrane. Subsequently, Grb2 binds to the phosphotyrosine sites on the EGFR^{TAIL}, recruiting both SOS^{FL} and SOS^{PR}, and a lateral protein condensation phase transition proceeds to various degrees depending on the specific protein concentrations in each experiment. At the membrane, full-length SOS further engages PIP₂ through its PH domain and Ras (GDP or GTP state) through its allosteric binding site^{91,92}, ultimately releasing autoinhibition and beginning to catalyze the nucleotide exchange reaction of RasGDP to RasGTP. RasGTP levels are 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,¹⁷ and achieves ~5s time resolution for tracking RasGTP.

Ras activation trajectories are tracked as a function of EGFR^{TAIL} condensation state for two different overall SOS^{FL} concentrations (0.2 and 2 nM). Data plotted in Figures 2.6A and 2.6B illustrate 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 illustrated in Figure 2.6C. For both SOS^{FL} concentrations, titrating in SOS^{PR} leads to an abrupt enhancement in Ras activation after macroscopic condensation is 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 is observed in the calibrated, per-molecule, SOS activities (Figures 2.6A and 2.6B upper panels) as well as in overall reaction trajectories (lower panels). Thus, in addition to recruitment of more SOS to the condensates (e.g. through multivalent avidity effects), each SOS^{FL} molecule also activates more Ras over the same time period compared with uncondensed state of the EGFR^{TAIL}. Also prominent in the fastest overall reaction trajectories from each SOS^{FL} concentration is RasGTP-driven positive feedback, which is evidenced by the positive curvature in the trajectories.^{69,83,93}

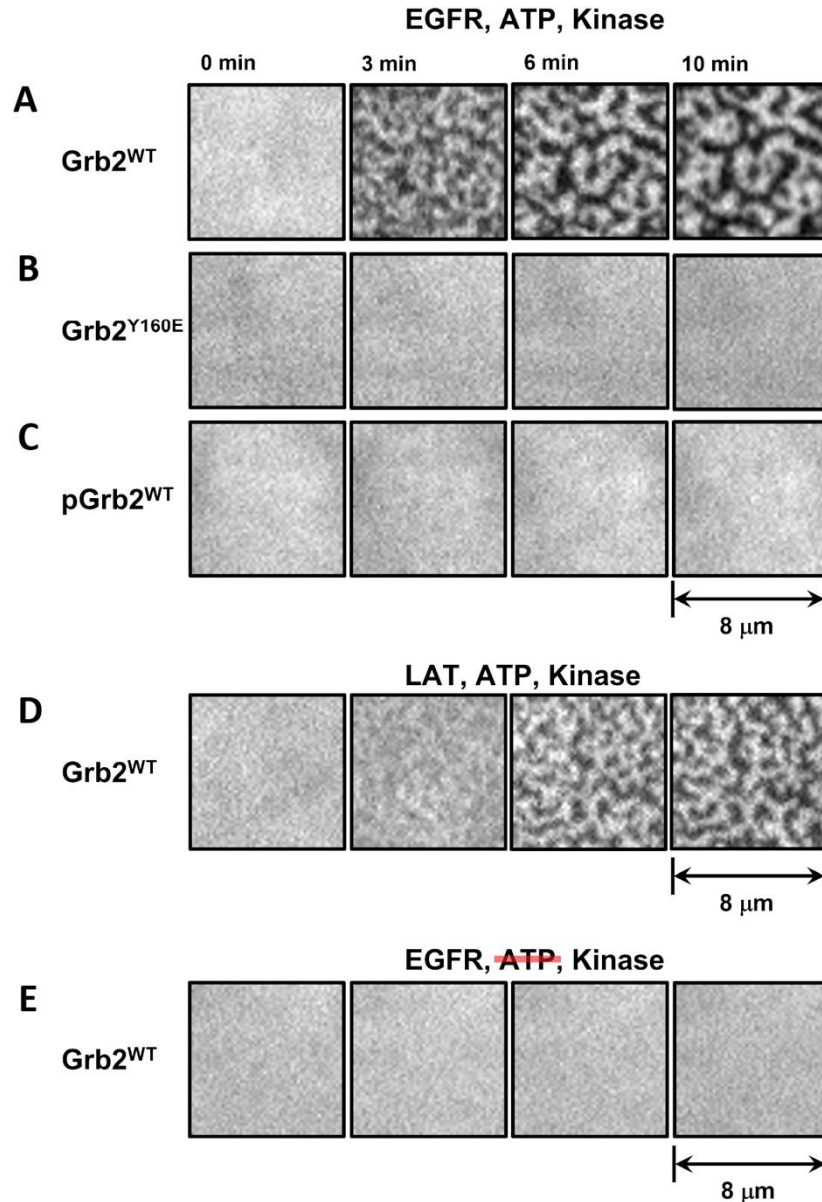


Figure 2.4: Modulation of EGFR Phase Condensation via Grb2 Phosphorylation. Grb2 dimerization as the key driving force to induce phase transition. TIRF images of EGFR showing the reconstitutions of (A) wildtype Grb2 and EGFR^{TAIL}. Phase transition is observed at 3 minutes. (B) Grb2^{Y160E} and EGFR^{TAIL}. EGFR^{TAIL} remains uniformly distributed. Phase transition is not detected. (C) pGrb2 and EGFR^{TAIL}. Phase transition is not detected. (D) wildtype Grb2 and LAT. Phase transition of LAT:Grb2 is observed in 6 minutes. (E) wildtype Grb2 and EGFR^{TAIL} without ATP. Phase transition is not detected. In (A-E), Hck is the kinase used to phosphorylate EGFR^{TAIL}.

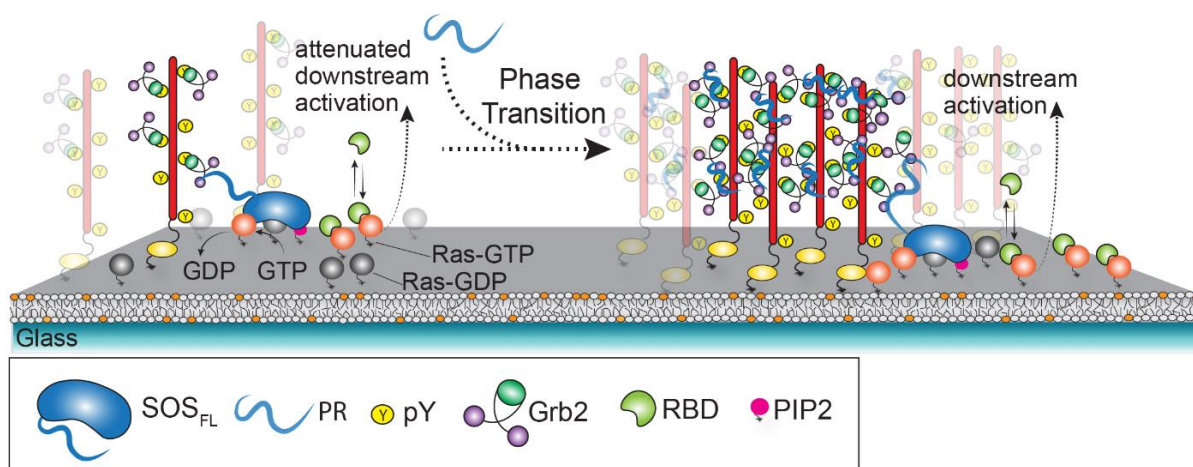


Figure 2.5: Schematic of EGFR Phase Condensation. The schematic of downstream signaling modulated by SOS^{Pr} titrated $\text{EGFR}^{\text{TAIL}}:\text{Grb2}$ phase transition. SOS^{Pr} was used as the strong crosslinker to induce the phase transition of the molecule assembly, $\text{EGFR}^{\text{TAIL}}:\text{Grb2}:\text{SOS}$, 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 mM) are 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 is read by the fluorescence intensity of RBD on the bilayer. Also, see Ras activation in Materials and Methods.

In these reactions, a constant catalytic rate from SOS^{FL} will result in a Ras activation trajectory with strictly negative curvature as progressively less RasGDP is available to be activated.

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 Figures 2.6A and 2.6B are presented in Figures 2.6C and 2.6D. 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 2.6E), the EGFR condensate effect is essentially undetectable, with all trajectories nearly overlaying each other. At the lower SOS^{FL} concentration (Figure 2.6D), a modest increase (roughly double) in reaction rate is evident for the condensed state (red) vs. 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 activated⁹⁴ (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.

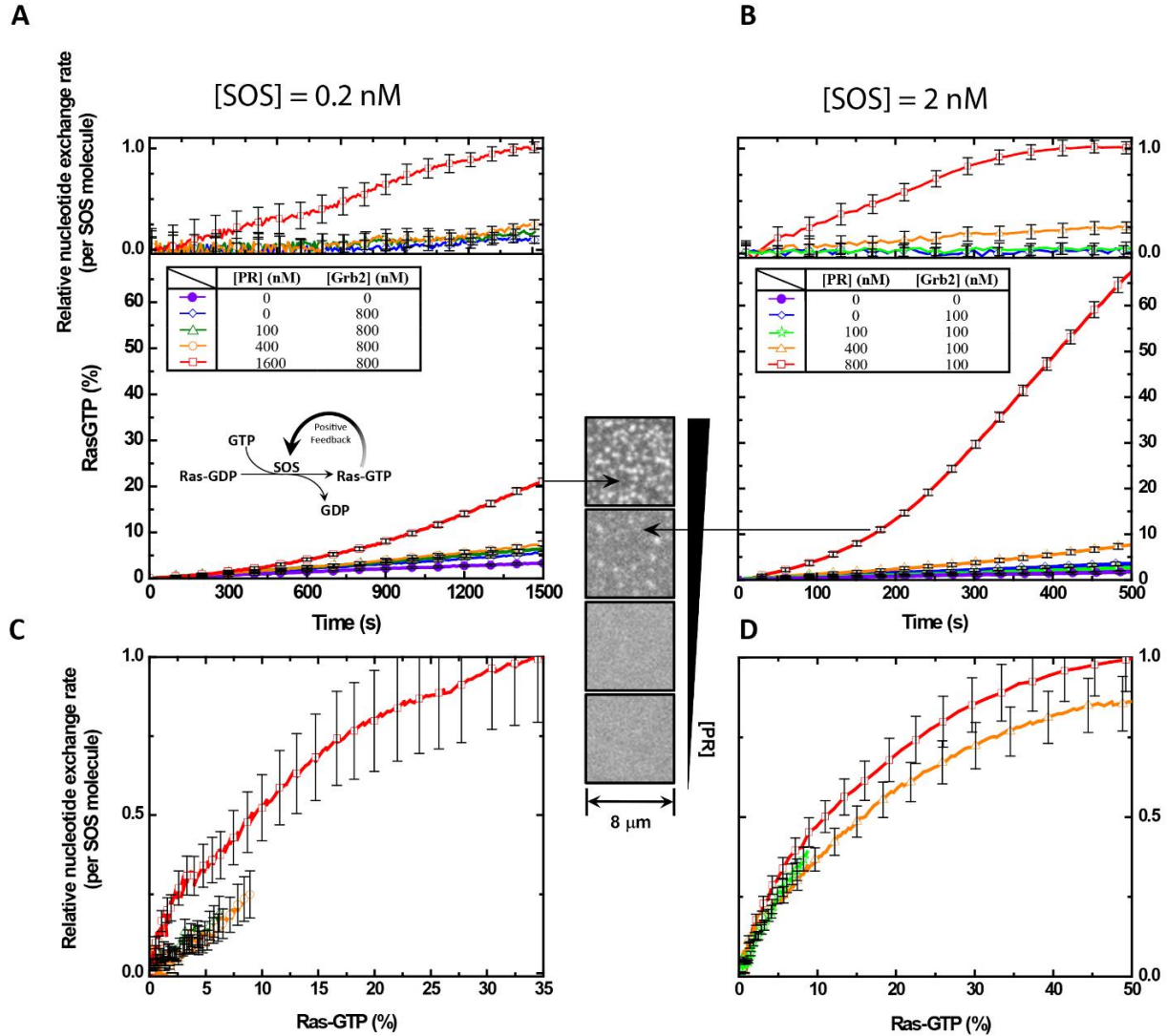


Figure 2.6: Ras activation modulation by Grb2 Phase Condensation. Ras activation modulated by EGFR^{TAIL}:Grb2 phase transition. (A) The kinetic traces of Ras activation and the relative nucleotide exchange as the function at $[SOS] = 0.2 \text{ nM}$. (B) The kinetic traces of Ras activation and the relative nucleotide exchange as the function of time at $[SOS] = 2 \text{ nM}$. (C) The plot of the relative nucleotide exchange rate against the activated Ras showing the positive feedback of SOS at $[SOS] = 0.2 \text{ nM}$. (D) The plot of the relative nucleotide exchange rate against the activated Ras showing the positive feedback of SOS at $[SOS] = 2 \text{ nM}$.

2.4 Discussion and Conclusion

The protein condensate of the EGFR^{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,^{95–98} and can activate with only few triggered TCRs. In contrast, EGFR stimulation often involves thousands or even millions of activated receptors per cell.^{99–101} Among molecules in the EGFR-MAPK pathway, SOS is expressed at very low levels and is likely a limiting component.¹⁰¹ 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 Grb2 dimerization 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 dimer interactions, which contain substantially sub-stoichiometric 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.^{102–104} Additionally, the fact that Grb2 dimerization 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 Grb2,¹⁸ 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 are widely used as cancer therapeutics in EGFR-driven cancers,¹⁰⁵ could also inhibit this negative regulatory mechanism—conceivably interfering with their desired effects.

2.5 Materials and Methods

Chemicals

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) (Ni^{2+} -NTADOGS) were purchased from Avanti Polar Lipids. Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647 maleimide dyes were purchased from ThermoFisher. Bovine Serum Albumin (BSA), ($\pm$)-6-Hydroxy-2,5,7,8-

tetramethylchromane-2-carboxylic acid (Trolox), Catalase, 2-Mercaptoethanol (BME), NiCl_2 , H_2SO_4 and ATP were purchased from Sigma-Aldrich. Glucose Oxidase was purchased from Serva. Tris(2-carboxyethyl)phosphine (TCEP) was brought from Thermo Scientific. Glucose and H_2O_2 were from Fisher Scientific. MgCl_2 was from EMD Chemicals. Tris buffer saline (TBS) was purchased from Corning.

Protein purification

EGFR^{TAIL} C-terminal region of EGFR^{TAIL} (residues 1015-1210, where the signal peptide of EGFR is included in numbering) fused to a N-terminal His₆ and a SUMO tag with a 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 1L Terrific Broth media until OD₆₀₀ reached 0.7. The culture was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and let grow overnight at 18 °C. After overnight expression, the culture is spun down for 15 minutes at 4000 rpm and resuspended in 15-30 mL of NiNTA buffer (500 mM NaCl, 20 mM Tris-HCl 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 (GE) column. EGFR^{TAIL} was eluted by eluting buffer (500 mM NaCl, 20 mM Tris-HCl pH 8.5, 500 mM Imidazole). The elution fractions was concentrated by an Amicon Ultra centrifugal filter unit (30k Da MWCO, Millipore). The concentrated Grb2 was loaded onto an S75 30/300 (GE Healthcare) column equilibrated in Buffer A (150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM TCEP). Finally, purified EGFR^{TAIL} was then 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) *Escherichia coli*. The transformed cells were grown at 37°C in 1L Terrific Broth media until OD₆₀₀ reached 0.7. The culture was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and let grow overnight at 18 °C. After overnight expression, the culture is spun down for 15 minutes at 4000 rpm and resuspended in 15-30 mL of NiNTA buffer (500 mM NaCl, 20 mM Tris-HCl pH 8.5, 20 mM Imidazole, 5% Glycerol). The Grb2 expressing cells were lysed by sonication with the addition of 1mM PMSF and 0.1 mM BME. The insoluble fraction of the lysate was spun down at 16500 rpm for 45 minutes at 4 °C. The supernatant was applied to a HisTrap FF (GE) column. Grb2 was eluted by eluting buffer (500 mM NaCl, 20 mM Tris-HCl 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 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 off. The cleaved Grb2 was reapplied to a HisTrap column. The flow-through was collected and concentrated by an Amicon Ultra centrifugal filter unit (10k Da MWCO). The concentrated Grb2 was loaded onto an S75 30/300 (GE Healthcare) column 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 then aliquoted and stored at -80 °C after flash freezing.

Ras H-Ras S118C containing residue 1-181 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.¹⁰⁶

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 the previous work..¹⁷

SOS^{PR} pETM vector containing the ORF, His6-MBP-(Asn)10-TEV-SOS1 (residue 1051–1333, human) proline-rich domain was transformed into BL21 (DE3) bacteria. The expression and purification can be found in the previous work.¹⁸

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. RBD was expressed and purified as described previously.¹⁷

Protein labeling

Proteins were first diluted to 100 μM. Alexa Fluor 488, 555 or 647 maleimide dye was dissolved in anhydrous DMSO to make a working stock that is 10-20mM. The dye was added to the protein within 3-fold molar excess over protein concentration to avoid overlabeling. The reaction mixture is incubated at the room temperature for 1 hr. To quench the labeling reaction, Dithiothreitol (DTT) 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 the 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 (SLB)

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 (Ibidi glass coverslips, bottom thickness 170 µm ± 5 µm) combined with the flow chamber (sticky-Slide VI 0.4, Ibidi, Planegg, Germany). 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 hr. 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 is 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 (FRAP). 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 fluorescence correlation spectroscopy (FCS) described previously.⁸⁸

Total internal reflection fluorescence (TIRF) microscopy

An Eclipse Ti inverted microscope (Nikon) with a TIRF system and iXon EMCCD (Electron Multiplying Charged-Coupled Device) camera (Andor Technology) was used for imaging. TIRF microscopy was performed with a Nikon 100 × 1.49 numerical aperture oil-immersion TIRF objective, a TIRF illuminator, a Perfect Focus system, a motorized stage and a U-N4S 4-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 (AOTF). 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 measured with the field aperture fully opened. The 405/488/561/638 nm Quad TIRF filter set (Chroma Technology Corp., Rockingham, Vermont) along with supplementary emission filters of 525/50 m, 600/50 m, 700/75 m for 488 nm, 561 nm, 640 nm channel were used respectively. Images are acquired using the Nikon NIS-Elements software with the exposure time of 50 milliseconds.

Ras Activation and Imaging Analysis

SOS^{PR} was used as the strong crosslinker to induce the phase transition of the molecule assembly, EGFR^{TAIL}:Grb2:SOS, at different levels while the concentrations of Grb2 were maintained 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 mM) were added together to the bilayer. While the phase transition of $\text{EGFR}^{\text{TAIL}}$ 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 SOS^{cat} , the catalytic domain of SOS^{FL} , was used to fully activate Ras on SLB. The averaged intensity of the RBD TIRF image corresponding to total Ras molecules on SLB was used as the normalization factor to calibrate different Ras densities over different traces. SOS on the membrane can be tracked at the same time while the fluorescence intensity of RBD on the bilayer was being monitored. The Ras activation by SOS was allowed to interact directly with the phase transition of $\text{EGFR}^{\text{TAIL}}$. The concentrations of Grb2 (800 nM and 100 nM) were chosen to modulate the recruitment of SOS by Grb2 making the time scale of Ras activation comparable within 30 min between the two data sets of low and high [SOS].

Single-molecule images of SOS^{FL} were tracked by an ImageJ plugin, TrackMate,¹⁰⁷ to obtain the number of activated SOS^{FL} on the membrane. SOS^{FL} molecules were localized with the detector of Difference of Gaussian (DoG). The initial diameter was set to six pixels. Typically, 50–3000 molecules were detected per image. In this study, the Ras activation modulated by $\text{EGFR}^{\text{TAIL}}$ phase transition at both low and high [SOS] (0.2 and 2 nM) were interrogated separately. Dividing the instantaneous increase of activated Ras by the number of SOS 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 (Ras-GTP %).

2.6 Supporting Information

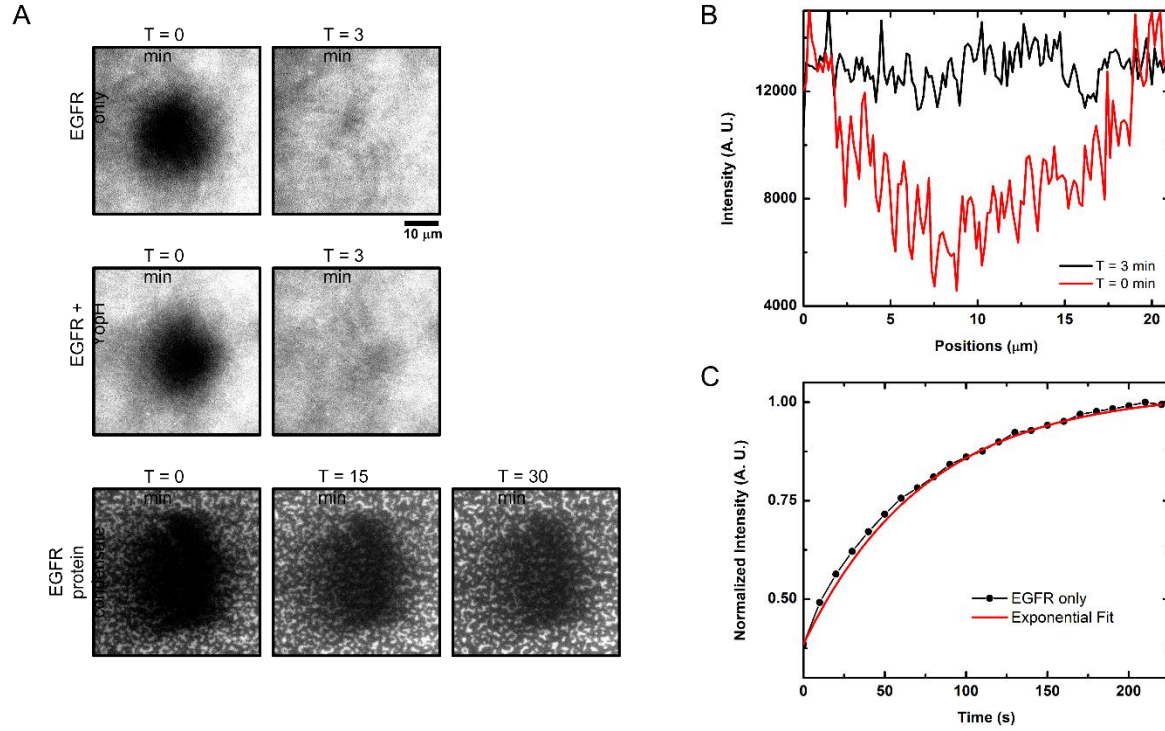


Figure 2.7: Supplementary Figure 1 FRAP measurements of EGFR pre and post Phase condensation. FRAP experiments of EGFR. (A) FRAP images. Top: EGFR on the bilayer, middle: EGFR^{TAIL} after YopH treatment, bottom: EGFR^{TAIL}:Grb2 protein condensate. (B) Intensity profile of FRAP image from EGFR^{TAIL} only at 0 and 3 min. (C) The recovery trace of EGFR^{TAIL} only. The red solid line is the exponential fit where $t_D = 73$ s.

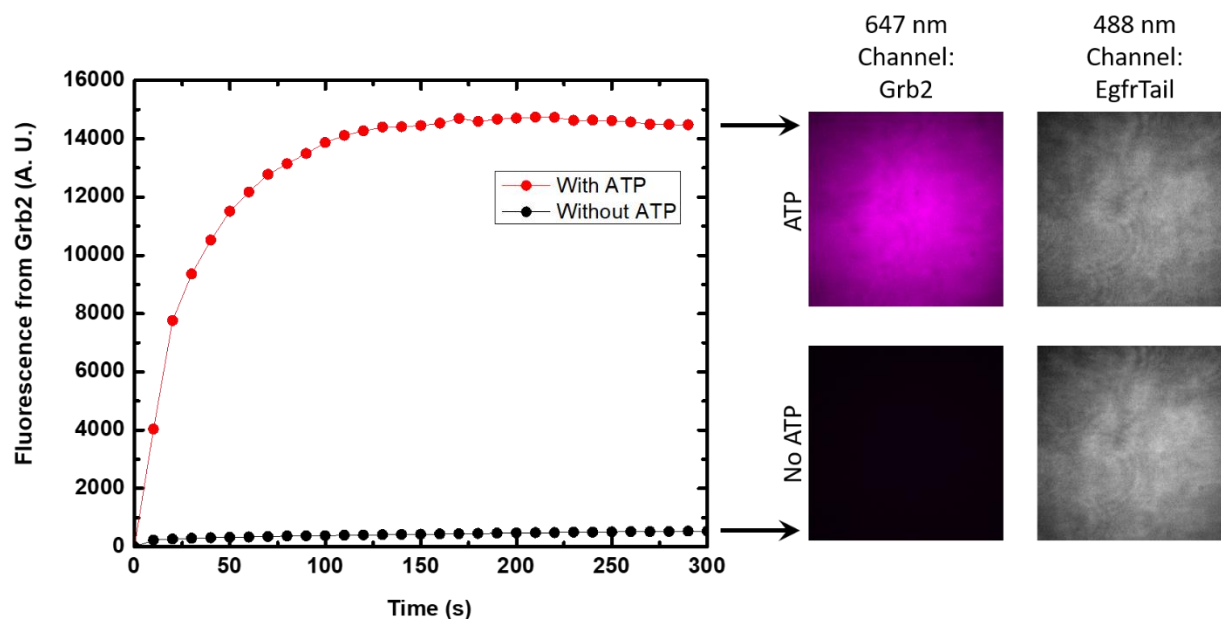


Figure 2.8: Supplementary Figure 2 Recruitment of Grb2^{Y160E} to EGFR^{Tail}. Grb2^{Y160E} responds to the phosphorylation of EGFR^{Tail}. Alexa Fluor 647 labeled Grb2^{Y160E} (6 mM, labeling efficiency of 48%) is added to phosphorylated EGFR^{Tail} on the bilayer. The averaged fluorescence intensity from the TIRF images of Grb2^{Y160E} on the supported bilayer increases rapidly corresponding to the recruitment by EGFR^{Tail} (the red curve in the left graph). The control experiment is shown by the black curve where EGFR^{Tail} is not phosphorylated. The TIRF images from Grb2^{Y160E} (in magenta) and EGFR^{Tail} (in gray) taken 10 minutes after the addition are shown at the right side of the figure. No phase transition is observed using Grb2^{Y160E}.

Sequence	Modifications	Charge	MH+ [Da]	m/z [Da]	Note
VLNEEcDQNWYK	C6(Carbamidomethyl)	2	1597.69658	799.35193	Tyr 37
VLNEEcDQNWyKAELNGK	C6(Carbamidomethyl); Y11(Phospho)	2	2289.98516	1145.49622	Tyr 37
VLNEEcDQNWYKAELNGK	C6(Carbamidomethyl)	3	2210.01670	737.34375	Tyr 37
NyIEMKPHPWFFGK	Y2(Phospho)	3	1873.84989	625.28815	Tyr 52
NYIEMKPHPWFFGK		2	1793.88445	897.44586	Tyr 52
DIEQVPQQPTYVQALFDFDPQEDGELGFR	Y11(Phospho)	3	3461.55979	1154.52478	Tyr 160
DIEQVPQQPTYVQALFDFDPQEDGELGFRR		3	3537.69577	1179.90344	Tyr 160
DIEQVPQQPTYVQALFDFDPQEDGELGFR		3	3381.59543	1127.87000	Tyr 160

Table 2.1: Mass Spectra of Grb2 Phosphorylation Sites by Hck. Three tyrosines of Grb2 show the phosphorylation by Hck. Grb2 has seven tyrosines. The peptide analysis of mass spectrum covers 85% of Grb2 sequence which includes six tyrosines in Grb2. The phosphorylated Grb2 sample was digested by trypsin overnight before the analysis by the mass spectrometer.

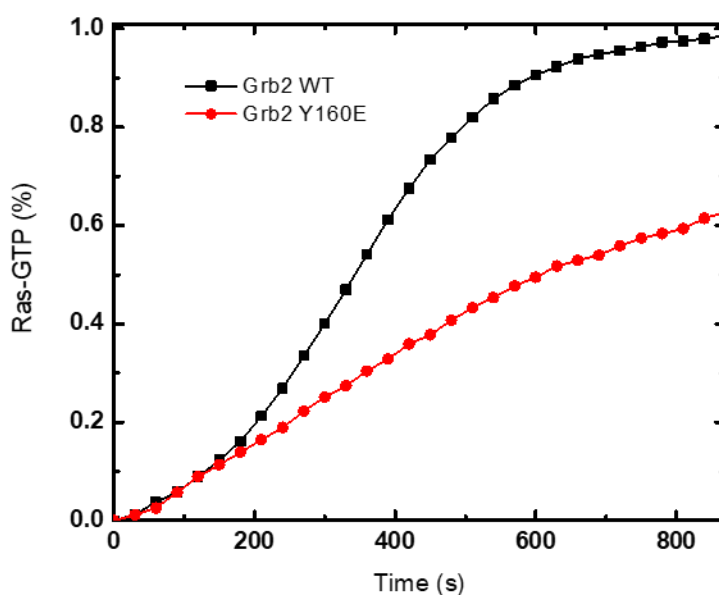


Figure 2.9: Supplementary Figure 3 Ras Activation Comparison between Grb2^{WT} and Grb2^{Y160E}. Ras activation between wildtype Grb2 and Grb2^{Y160E}. In this Ras activation assay, the crosslinker, SOS^{PR} is not used. To initiate the downstream signaling, Grb2 (200 nM), GTP (1 mM), Alexa Fluor 555 labeled SOS^{FL} (4 nM) and Alexa Fluor 647 labeled RBD (50 mM) are added together to phosphorylated EGFR^{TAIL} on the supported bilayer. The activated Ras (Ras-GTP) is detected by RBD. Also see Ras activation in Materials and Methods.

Chapter 3

The role of Grb2 in the regulation of EGFR Clustering in CHO Cells

Brittany Stinger¹, Henry Phan¹, Chun-Wei Lin², Serena Huang¹, Jay. T. Groves¹

¹Department of Chemistry, University of California, Berkeley, Berkeley, United States

²Department of Chemistry, National Tsing Hua University, Hsinchu City, Taiwan

3.1 Introduction

Epidermal Growth Factor Receptor (EGFR) is part of the erbB family receptor tyrosine kinases responsible for several cellular processes including cellular proliferation and differentiation.³⁷ In its inactive state, EGFR exists in an autoinhibited conformation. Upon ligand binding, EGFR undergoes a conformational change leading to the allosteric binding of the extracellular region of two EGF receptors, dimerization of the transmembrane (residues 622-644) near the N termini and activation of the kinase domain.³¹⁻³³ This leads to transphosphorylation of the tyrosine residues on the cytoplasmic tail leading to the recruitment of adaptor proteins with SH2 morphologies, like Grb2 and SHC. Signaling of the ERK-MAPK pathway from EGFR activation occurs from the recruitment of SOS and allosteric activation of RAS at the plasma membrane.^{108,109} EGFR is involved in other signaling pathways like the PI3K/Akt pathway and NF- κ B pathway through the recruitment of PLC γ 1.³⁶ Mutations to EGFR resulting in EGFR overexpression and signal amplification are well documented in cancers like breast, lung, and brain cancer.³⁴⁻³⁶ To attenuate the abundance of EGFR in cancers and inhibit overactivation of signaling pathways, EGFR-tyrosine kinase inhibitors (TKIs) are administered to prevent autophosphorylation, thereby decreasing the ability of EGFR to interact with downstream signaling molecules.^{37,38} Despite their widespread use and effectiveness, patients can acquire resistance to these inhibitors through various mutations to the kinase domain.³⁸⁻⁴² This raises the question if drugs targeting other proteins in the signaling pathways could prevent EGFR overactivation of these pathways.

Understanding the mechanisms and consequences of EGFR overactivation is an increasing area of study.¹¹⁰ In addition to interactions with adapter proteins, higher order EGFR oligomers are reported. A molecular basis exists for their formation through the interaction of the kinase and transmembrane domains from multiple EGFR dimers.⁴³ These higher order oligomers were previously observed as clusters in live cells.⁴⁴⁻⁴⁷ Additionally, experiments were reported to form higher order oligomers that can recruit Grb2 to varying degrees.^{46,111} Thus indicating that higher order oligomerization of EGFR contributes to the recruitment of adaptor proteins like Grb2 and the amplification of signaling pathways.

We previously report an EGFR phase condensation mediated by the Grb2 dimer interface in a reconstituted system.¹⁹ Fluorescently labeled EGFR-tail, anchored to the supported lipid bilayer via Nickel-Histidine chemistry, exhibited phase condensation with the addition of Grb2 and

Grb2/SOS.¹⁹ The phase condensation was dissolved by the phosphorylation of Grb2 and phosphomimetic Grb2 mutant, Y160E.¹⁹ Additionally, we showed Ras activation by Son of Sevenless (SOS) was enhanced by EGFR phase condensation.¹⁹ This is similar to other experiments conducted with the linker for the activation of t-cells (LAT), that is similar to EGFR as they share a similar phosphotyrosine morphology. These experiments with LAT show that phase condensation is essential for the recruitment of SOS and activation of Ras. Modulating the extent of the phase condensation by varying the stoichiometry of Grb2 to SOS showed that Ras activation is modulated by the EGFR phase condensation. Since SOS has low copy number in cells, it is possible that other adapter proteins could have a greater effect on EGFR condensation in live cells. The effect of Grb2 condensation in a reconstituted system raises questions about ability of EGFR condensation to be modulated via the Grb2:Grb2 dimer interface in a live cell context.

Here we report that EGFR clustering in CHO cells is mediated in a phosphotyrosine driven process by binding adapter proteins like Grb2. We transfect CHO cells with EGFR-eGFP to monitor EGFR clustering after EGF stimulation using total internal reflection fluorescence (TIRF) microscopy. EGFR clustering was revealed to be modulated in a phosphotyrosine dependent manner due to an EGFR mutant with all the tyrosine residues mutated to alanine (EGFR^{NoY}-eGFP) showing no clustering upon stimulation with EGF. This suggests that EGFR clustering is mediated through the phase condensation of EGFR and adapter proteins like Grb2 instead of EGFR oligomerization. Based on reconstitution experiments showing we can dissolve the phase condensation of EGFR by introducing mutations to Grb2 that break phase condensation, we investigated the impact of disrupting the Grb2:Grb2 interaction on EGFR clustering. By utilizing CHO cells, which have a relatively low amount of endogenous Grb2, we probed the effect on EGFR clustering by disrupting the Grb2 dimer interface with Grb2 mutants that abolish dimerization. Grb2 mutants, Y160E and N188D/N214D, which were previously reported to disrupt the Grb2 interaction at the SH2:SH3⁵⁴ domain swap interface were insufficient to block the EGFR clustering.¹⁹ Similarly, Grb2 mutant, V123D, previously reported to disrupt the Grb2:Grb2 interaction at the SH2:SH2 domain swap interface was insufficient to disrupt EGFR clustering.¹¹² This suggests the Grb2:Grb2 interaction is not the predominant driving force in EGFR clustering and, by extension, EGFR:Grb2 phase condensation in a live cell context.

To further probe the stoichiometry availability of Grb2 to bind to other adapter proteins, we investigated mutants that prevent binding of Grb2 to other adapter proteins. Under normal transfection conditions, the Grb2 P49L mutant was able to block EGFR clustering. However, when Grb2 P49L concentration was increased, EGFR clustering was recovered. This suggests that Grb2:Grb2 mediated phase condensation could play a role in cases where there is an increase of Grb2 stoichiometry to other adapter proteins.

3.2 Results

EGFR Clustering is Modulated in a Phosphotyrosine Dependent Manner

Previous studies report EGFR clustering in live cells like Cos-7 cells.⁴⁴ However, Chinese Hamster Ovarian (CHO) cells were chosen due to the CHO cell lack of EGFR expression and low endogenous Grb2 concentration. We transfect CHO Cells with full length Epidermal Growth

Factor Receptor (EGFR) – Enhanced Green Fluorescent Protein (eGFP), which we refer to as EGFR-eGFP for the rest of this paper. CHO cells were transfected with EGFR-eGFP and unlabeled Grb2. A western blot of CHO cells was performed for 0-21 hours Grb2 transfection time to ensure an increase of Grb2 in the cells (Appendix Figure 3.6). For imaging, cells were transfected using LTX Lipofectamine with the plasmids EGFR-eGFP and wildtype Grb2 (Grb2^{WT}) and starved in FBS free media for 4-5 hours prior to imaging. Immediately before imaging, transfection media was replaced with FBS free media. Cells were visualized via TIRF microscopy. Cells were identified and imaged with 488nm channel to visualize the EGFR-eGFP distribution prior to activation. Cells were activated with 100ng/mL EGF and analyzed by eye to determine clustering. Cells were designated no clustering if there was no change between the pre and post EGF image. Cells were designated weak clustering if there were clusters around the periphery of the cell, but not in the center of the cell. Cells were designated strong clustering if there were clusters throughout the entire cell. For cells transfected with EGFR and Grb2^{WT}, EGFR clusters were observed throughout the entire cell (Figure 3.1A). Over two experiments, 34 cells transfected with Grb2^{WT} and EGFR-eGFP were imaged with 31 cells clustering. Of those 31 cells that clustered, 81% exhibited strong clustering and 19% exhibited weak clustering. EGFR and Grb2^{WT} CHO cells contain low levels of endogenous Grb2 (see Appendix Figure 3.6). This basal level of Grb2 could account for some of the clustering we see. To see if cells cluster without the addition of Grb2, CHO cells were transfected with EGFR-eGFP only. Upon stimulation with EGF, a minimal amount of clustering is observed around the outside of the cells (Figure 3.1B). Of 28 cells imaged over two different experiments, only 9 cells exhibited clustering and the 9 cells that clustered all showed weak clustering. In subsequent measurements, if cells exhibit clustering only around the periphery of the cell, the clustering is mainly attributed to the endogenous Grb2 in the cell. The basal level of EGFR clustering was considered when evaluating the effect of Grb2 mutants on EGFR clustering.

Although EGFR is known to undergo phase condensation via Grb2 through interaction of the Grb2 SH3 domain with EGFR phosphotyrosines, EGFR is also reported to form higher order oligomers.^{45,47} To verify the EGFR clustering we observe is mediated via phosphotyrosine interactions, a EGFR-eGFP mutant was made with all tyrosine residues mutated to alanine residues. This mutant will be referred to throughout the rest of the text as EGFR^{NoY}-eGFP. CHO cells were transfected with EGFR^{NoY}-eGFP and unlabeled Grb2 and starved in FBS free media for 4-5 hours. After the FBS media was refreshed, the cells were visualized on the microscope prior to EGF addition. The 488nm channel revealed a homogenous distribution of EGFR throughout the cell. After EGF addition, the EGFR distribution remained homogenous and exhibited no clustering (Figure 3.1C). Of 17 cells imaged, none of these cells show clustering. The lack of strong and weak clustering suggests that the EGFR clustering we observe is due to the phase condensation between EGFR and adapter proteins.

Grb2 Dimer Mutants do not Affect EGFR Clustering

We previously reported the effect of Grb2 dimer on EGFR phase condensation on a supported lipid bilayer system.¹⁹ By anchoring the C-terminal region of EGFR containing the phosphotyrosine tail with a His₆ tag (EGFR^{Tail}) to a supported lipid bilayer via nickel – histidine chemistry, we observed

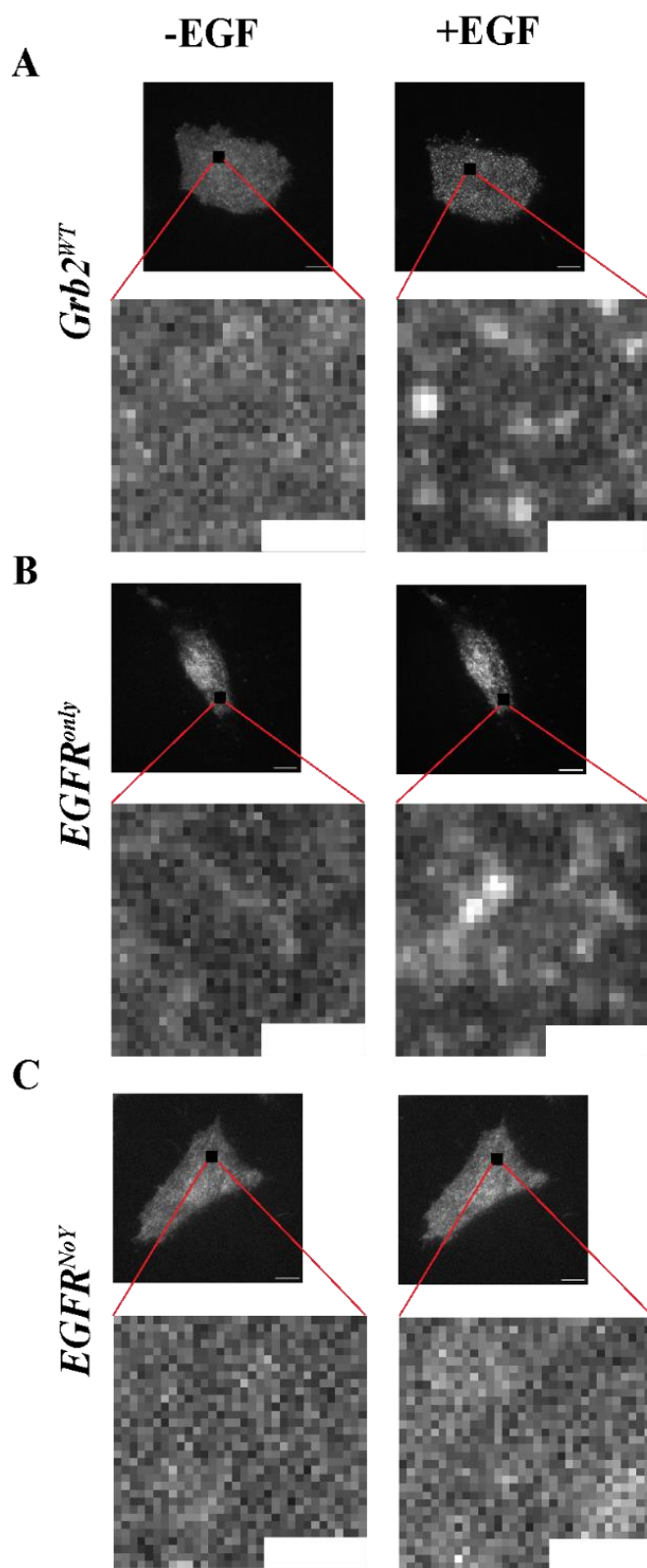


Figure 3.1: EGFR Clusters in CHO Cells are a Result of EGFR Phase Condensation.

EGFR in CHO cells before and after EGF addition with a final concentration of 100ng/mL shows EGFR clustering in (A) EGFR-eGFP WT and Grb2^{WT}. Strong clustering is observed. (B) EGFR-eGFP only. Minimal Clustering is observed. (C). EGFR-noY-eGFP and Grb2^{WT}. No Clustering is observed. Images taken are with -EGF and +EGF are taken with 488nm, 1-2mW power and 100ms exposure. +EGF conditions images taken ~5-10 minutes after excitation. Enlarged images are 25 μm^2 with 2 μm scale bars. The scale bars for the images showing the entire cell are 10 μm .

phase condensation upon the addition of Grb2 alone.¹⁹ More importantly, by mutating or phosphorylating Grb2 at Y160, a key residue at the Grb2 dimer interface, we observe a disruption of the EGFR phase condensation.¹⁹

The phosphorylation of Grb2 residue Y160 was previously reported to break Grb2 dimerization and enable downstream signaling in the FGFR signaling pathway.^{53,54} Additionally, the mutant, Grb2 Y160E (referred to as Grb2^{Y160E}) was previously reported to break Grb2 dimerization at the C-terminal SH3:SH2 interface.⁵⁴ To determine the effect of this mutant in a live cell system, we transfect CHO cells with EGFR-eGFP and Grb2^{Y160E}. Upon EGF stimulation, strong EGFR clustering is observed throughout the cell (Figure 3.1B) similar to the CHO cells transfected with Grb2^{WT} (Figure 3.2A). Of the 22 cells imaged for this condition, 21 of them exhibited clustering, with 71% of these cells showing strong clustering and 29% of these cells showing weak clustering. This strong clustering suggests that the Grb2 dimer mutant does not have the same effect on EGFR phase condensation and clustering in live cells. This clustering could also be explained by the ability of Grb2 to interact with other adapter proteins like SOS to promote EGFR phase condensation and clustering. Other mutants were reported to break dimerization at the Grb2 C-terminal SH3:SH2 interface dimer interface, including the double mutant N188D/N214D (referred to as Grb2^{N188D/N214D}). We similarly transfected CHO cells with EGFR-eGFP and Grb2^{N188D/N214D} to observe the effect on EGFR clustering.⁵⁴ Upon stimulation with EGF, we observe a change from a homogenous distribution of EGFR to strong EGFR clustering throughout the cells (Figure 3.2B). 22 cells were imaged, and 12 cells exhibited clustering, with 58% of cells showing strong clustering and 42% of cells showing weak clustering. If these mutants disrupted the formation of the EGFR phase condensation, then weak clustering would be observed for all cells like in the EGFR only condition (Figure 3.1B). The strong clustering observed for both mutants suggests that breaking the SH3:SH2 Grb2 dimer interface does not have the same effect on EGFR phase condensation and, by extension, clustering in live cells.

The Grb2^{Y160E} and Grb2^{N188D/N214D} mutants disrupt the Grb2-Grb2 interactions at the C-terminal SH3/SH2 interface, however, Grb2 is also reported to interact via its SH2 domains in a reported SH2-SH2 domain swap dimer interface. The Grb2 SH2-SH2 domain swap dimer interacts via the swapping of adjacent α -Helix residues located on the hinge loop between residues 121-123.¹¹² This dimer is reported to abolish dimerization via a mutation at V123 to a aspartate (referred to as Grb2^{V123D}).¹¹² CHO cells were transfected with EGFR-eGFP and Grb2^{V123D} to observe the effect of abolishing this dimer on EGFR signaling. Cells were transfected with EGFR-eGFP and Grb2^{V123D} and then starved in FBS free media for 4-5 hours prior to imaging. Upon stimulation with 100ng/mL of EGF, we observe strong EGFR clustering throughout the cell (Figure 3.2C). For 27 cells imaged over 2 experiments, 16 exhibited clustering, with 44% of the clustering cells showing strong clustering and 56% of the clustered cells showing weak clustering. Given the strong clustering observed for all the Grb2 dimer mutants meant to abolish interaction at the Grb2 dimer interfaces, it is likely that the Grb2:Grb2 interaction is offset by the ability of other adaptor proteins like SOS and PLC γ 1.

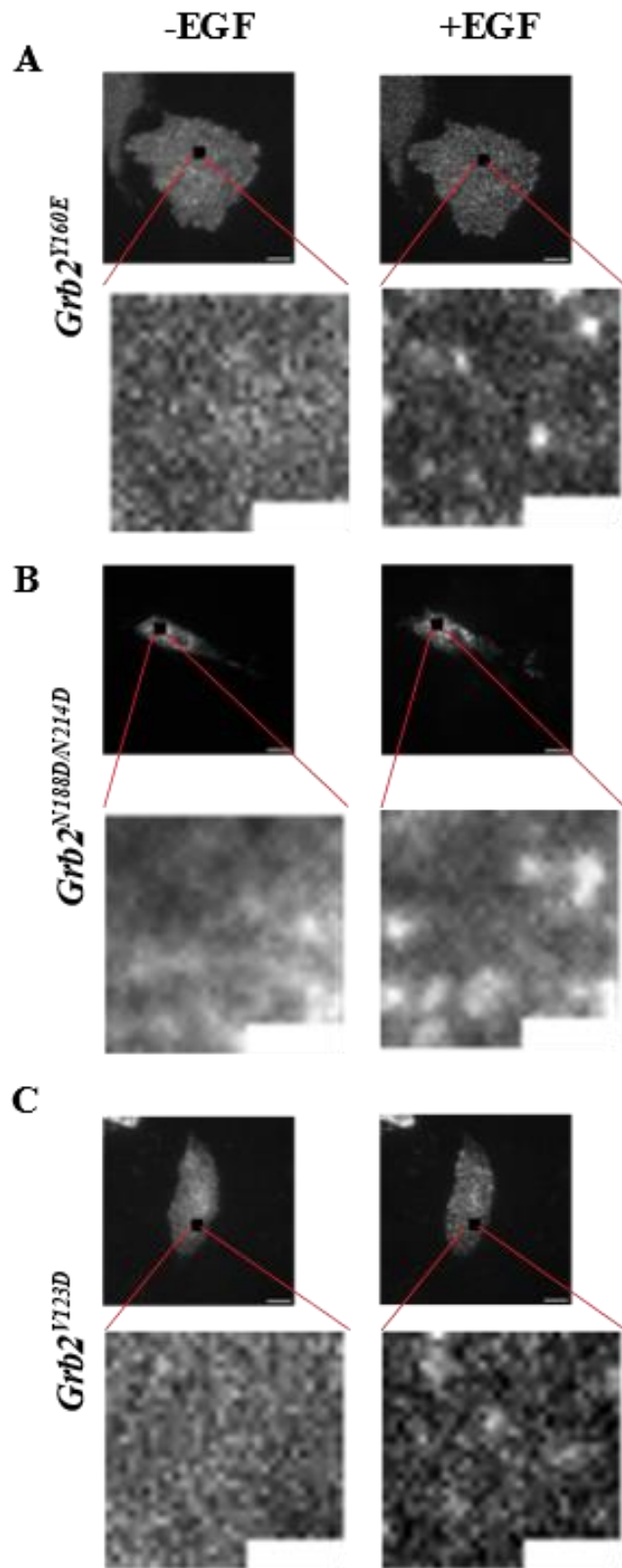


Figure 3.2: EGFR Clusters Observed with Different Grb2 Mutants the prohibit Grb2 Dimerization. EGFR-eGFP in CHO cells before and after EGF addition with various Grb2 dimer mutants. Images taken are with -EGF and +EGF are taken with 488nm, 1-2mW power and 100ms exposure. +EGF conditions images taken ~5-10 minutes after excitation. (A) Grb2^{Y160E}. (B) Grb2^{N188D/N214D}. (C) Grb2^{V123D}. All the Grb2 dimer mutants show strong EGFR clustering throughout the entire cell. The scale bars for the images showing the entire cell are 10μm and the scale bars for the enlarged images are 2μm.

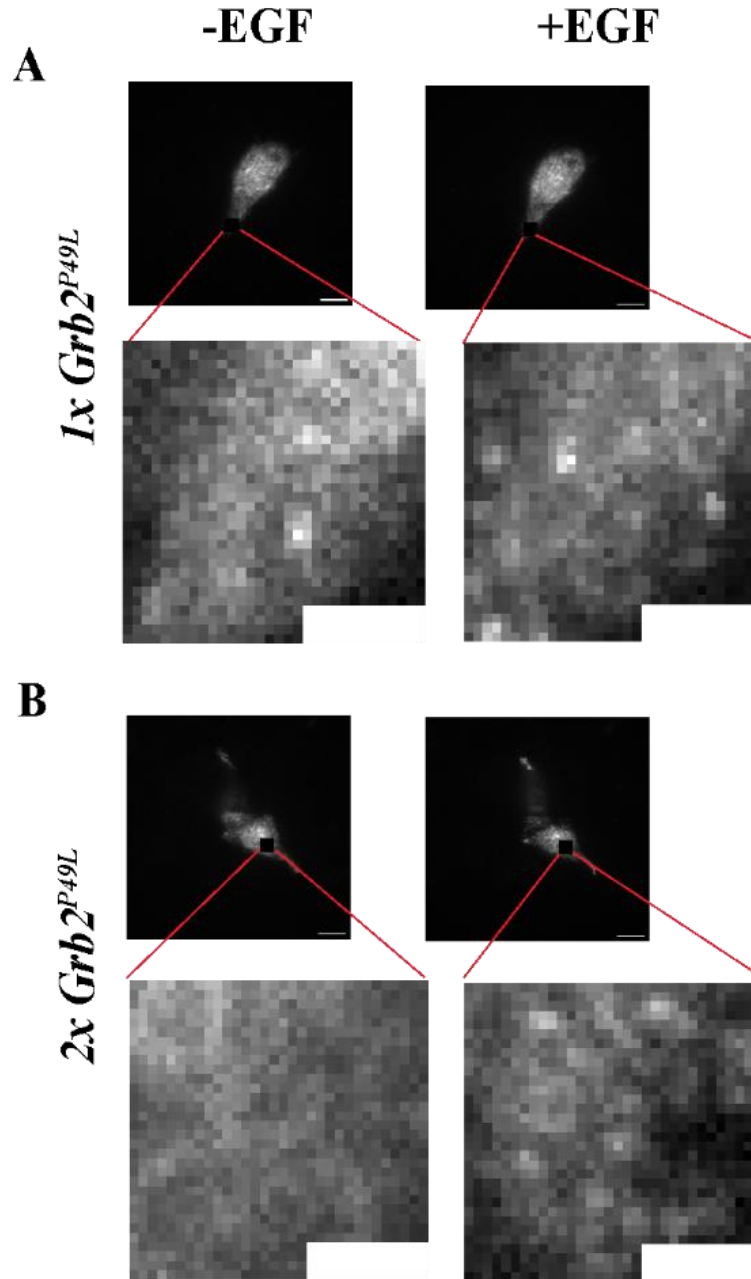


Figure 3.3: EGFR Clusters in the presence of Increasing Concentration of Grb2^{P49L}. EGFR-eGFP in CHO cells before and after EGF stimulation with Grb2-SH3 mutant. (A) 1x Grb2^{P49L}. Minimal EGFR clustering is observed in the 488nm channel. (B) 2x Grb2^{P49L}. Strong EGFR clustering observed in the 488nm channel. Images taken are with -EGF and +EGF are taken with 488nm, 1-2mW power and 100ms exposure. +EGF conditions images taken ~5-10 minutes after excitation. The scale bars for the images showing the entire cell are 10μm and the scale bars for the zoomed in images are 2μm.

Copy Number of Grb2 Effects EGFR Clustering

To evaluate other Grb2 interactions on EGFR clustering in live cells, we made a Grb2 mutant (Grb2^{P49L}) reported to block interaction with the SOS-PR domain while still maintaining the ability to bind to phosphotyrosines.¹¹³ This Grb2^{P49L} mutant is extensively reported in the literature as a functional mutant with the ability to abolish downstream Ras activation by preventing recruitment of SOS to phase condensate.¹¹⁴ To ensure that the Grb2 dimer can functionally bind this Grb2 mutant, a Grb2^{P49L}-mCherry construct was made to measure the recruitment of Grb2 to the membrane upon activation (see Appendix Figure 3.8 and Figure 3.9). EGFR clustering with this construct was observed by incubating CHO cells with EGFR-eGFP and Grb2^{P49L}. Upon EGF addition, minimal EGFR clustering was observed around the periphery of the cell (Figure 3.3A). 28 cells were measured for this condition, with 10 cells exhibiting clustering, with 80% of cells showing weak clustering and 20% showing strong clustering. This weak clustering is similar to the clustering observed for the EGFR-eGFP only condition and could be explained by the ability of SOS to bind endogenous Grb2. However, many of the cells do not exhibit strong clustering as observed with the Grb2 dimer mutants. One explanation for the strong clustering we observe in 2 cells transfected with Grb2^{P49L} could be explained by varying degrees of Grb2 transfection efficiency in the cell. As previously mentioned, the Grb2 transfected into the cells is unlabeled Grb2. Grb2 labelled with fluorescent proteins could artificially crosslink and lead to an artificial increase in clustering that would prevent us from accurately assessing the data, however, this also prevents us from measuring the Grb2 transfection efficiency on a cell to cell basis.

In the recruitment studies, we observed that cells expressed low amounts of the Grb2^{P49L}-mCherry compared to cells expressing Grb2^{WT}-mCherry, suggesting that expression level of the Grb2^{P49L} construct is relatively low in cells compared to the Grb2^{WT} construct. To increase expression level, the amount of Grb2^{P49L} plasmid added during transfection was doubled. The increase in Grb2^{P49L} expression level was verified via western blot (see Appendix figure 3.7). CHO cells transfected with EGFR-eGFP and 2x Grb2^{P49L} were stimulated with EGF. Upon stimulation, strong clustering was observed throughout the entire cell (Figure 3.3B). Of 47 cells imaged, 27 cells clustered with 41% of cells showing strong clustering and 58% of cells showing weak clustering. This marked increase in EGFR clustering can be attributed to the increase in Grb2^{P49L} expression. Increasing the amount of Grb2^{P49L} in the cell increased the amount of clustering despite the inability of Grb2 to bind adapter proteins like SOS, suggesting that the EGFR phase condensation we observe could be due to Grb2-Grb2 interactions as a result of the increased expression level of Grb2 in the cells. This increased expression level of Grb2 may allow for increased EGFR condensation through the Grb2-Grb2 interaction.

3.3 Discussion

The EGFR clustering we report here is mediated by EGFR phosphotyrosine driven phase condensation and not by EGFR oligomerization. The EGFR phosphotyrosine driven phase condensation differs from reconstitution experiments in that breaking Grb2 dimerization did not prevent phase condensation. One explanation for this is that there is some equilibrium between the two Grb2 dimers (SH2-CSH3 domain swap dimer and SH2-SH2 domain swap dimer). The Grb2

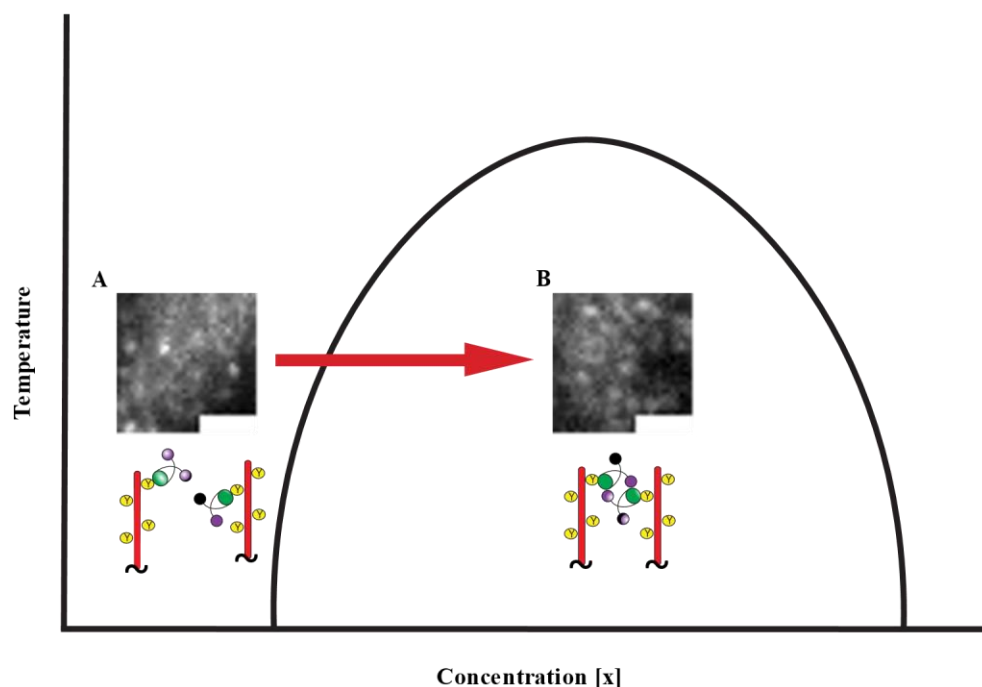


Figure 3.4: Schematic of the Impact of Increasing Grb2 on the Phase Condensation of EGFR. Phase Diagram of the EGFR-Grb2 and EGFR-Grb2 phase condensation in CHO cells. Increasing the expression of Grb2 in cells moves the phase condensate from the non-condensed phase to the condensed phase of the phase diagram. (A) EGFR-eGFP and 1x Grb2^{P49L} shows little to no clustering. (B) EGFR-eGFP and 2x Grb2^{P49L} with the overexpression of Grb2 shows strong clustering even without the ability of the Grb2 to bind SOS. Images are the zoomed in images from cells in figure 3.3 taken with a 488nm laser at 2mW power and 100ms. Scale bars are 2 μ m.

mutants described, Grb2^{160E}, Grb2^{N188D/N214D}, and Grb2^{V123D}, break only one of the dimer interfaces. We theorize a hypothetical Grb2 dimer with both Y160E and V123D mutations (Grb2^{Y160E/V123D}) could entirely prevent Grb2 dimerization, but more work would need to be conducted to ensure functionality of this mutant. Other Grb2 mutants that completely abolish Grb2 dimerization could yield further insight into the role of Grb2 dimerization in scaffolding the EGFR phase condensation.

Another explanation for the phase condensation is that the Grb2:Grb2 interaction is not the primary driver of EGFR clustering in live cells and that other adapter proteins like PLC γ 1 and SOS play a larger scaffolding role than previously described in reconstitution experiments.

Despite the Grb2 dimer mutants not abolishing EGFR clustering, the Grb2^{P49L} experiments suggest the Grb2 dimer may play a larger scaffolding role in certain stoichiometric conditions. Increasing the expression of Grb2^{P49L} resulted in a recovery of strong EGFR clustering comparable to clustering observed for Grb2^{WT} conditions. Due to the inability of Grb2 to interact with SOS, it is likely that increasing the copy number of Grb2 resulted in more Grb2 interactions via the Grb2

dimer interface and allowed for EGFR:Grb2 phase condensation (Figure 3.4). Grb2 overexpression is well documented in various types of ErbB related cancers and recently, inhibition of Grb2 has been explored as a target against cancers.^{115–121} Overexpression of Grb2 in these scenarios may lead to increased levels of phase condensation by increasing the phase condensates scaffolded by predominately EGFR:Grb2 interactions as opposed to EGFR:Grb2:SOS interactions. The potential to modulate EGFR phase condensation, and by extension downstream signaling like ERK phosphorylation, makes the Grb2:Grb2 interface an interesting therapeutic target. When designing and evaluating therapeutic targets for the EGFR signaling pathway, it may be necessary to consider the effect of those therapeutic targets on the Grb2:Grb2 dimer interface.

3.4 Materials and Methods

Cell Culture

Chinese Hamster Ovary (CHO) Cells obtained from the University of California, Berkeley Barker Hall Cell Culture facility were cultured in Duplecco's Modified Eagle Medium (DMEM), high glucose, GlutaMAXTM supplement Media with Phenol Red (Cat # 10566024, ThermoFisher Scientific, Waltham, MA) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) and 1% MEM Non-Essential Amino Acids Solution (Cat #11140-050, Fisher Scientific, Hampton, NH).

For Grb2 titration conditions, CHO cells at 80% confluency in a 6-well plate were transfected with Grb2 at the same concentration as imaging conditions (See Supplementary Information Figure 3.6). At 0, 1, 3, 5, 7, and 21 hours, cells were removed from the well using cell stripper and lysed with RIPA lysis buffer according to the manufacturer's protocol. For the 0 hour condition, after transfection media was added to the chamber, cells were immediately lysed.

On day 0, CHO cells were seeded at 20% confluency onto 35mm dish collagen coated coverslips (Cat # P35GCOL-1.5-10-C, Mattek, Ashland, MA). Cells were allowed to grow for 2 days until ~80% confluency. On day 2, cells were transfected with 2.75µg EGFR-eGFP and either 11µg or 22 µg Grb2 or Grb2-mScarlet depending on experiment conditions. The DNA constructs were diluted in 1.375mL of Opti-MEM (Cat # 31985062, ThermoFisher Scientific, Waltham, MA) with 8.8µL PLUS reagent (Cat # 15338100, Fisher Scientific, Hampton, NH) and allowed to sit at room temperature for 5 minutes. Meanwhile, 33µL of Lipofectamine LTX (Cat # 15338100, Fisher Scientific, Hampton, NH) was diluted in 1.375mL of Opti-MEM. After 5 minutes, the DNA/PLUS reaction mixture was added to the Lipofectamine LTX reaction mixture and allowed to incubate at room temperature for at least 30 minutes. After 30 minutes, the entire DNA mixture (2.75mL) was added to the chambers. 1.25 mL of DMEM, GlutaMAXTM supplement Media without phenol red and 1% NEAA was added to each chamber. The cells were incubated under these conditions for ~4-5 hours prior to imaging. DNA transfection was staggered for the chambers to ensure consistent transfection and starving conditions. Immediately prior to imaging, the Opti-MEM/DMEM media was removed from the chambers, washed with 1mL of DMEM, GlutaMAXTM supplement Media without phenol red and 1% NEAA, and replaced with 1.6mL of DMEM, GlutaMAXTM supplement Media without phenol red and 1% NEAA.

Microscopy

All imaging was carried out on a Nikon Ti Eclipse inverted microscope (Technical Instruments, Burlingame, CA) with a TIRF system. A laser launch with 405nm, 488nm, 560nm, and 640nm diode laser (Coherent OBIS, Santa Clara, CA) was aligned into a custom-built fiber launch (Solamere Technology Group Inc., Salt Lake City, UT). The filter set for TIRF illumination included a 405/488/561/647nm Quad TIRF filter set (Chroma Technology Corp. Rockingham,

Vermont) and emission filters (ET 525/50M, ET600/50M, and ET700/75) were used respectively. RICM images were filtered through a 50/50 beam splitter. The images were captured on an EM-CCD(iXon 897DU; Andor Inc., South Windsor, CT) and acquired with Micromanager software.

Prior to EGF addition, cells were located via the 488nm channel imaging EGFR-eGFP and recorded using the multi-D acquisition and spot locator. Approximately 15-30 cells were identified per imaging chamber. Cells were imaged with RICM (100ms) and 488nm (1-2mW, 50-100ms exposure time, 100-500 gain) prior to EGF addition. One cell was selected for time series imaging.

The selected cell for time series imaging was imaged using the 488nm channel (1-2mW, 50-100ms exposure time, 100-500gain). If cells were transfected with Grb2-mCherry, the 561nm channel (1-2mW, 50-100ms, 100-500gain) was imaged as well. The cell was imaged every 5s for a total of 6 minutes. At 1 minute, a prepared 400μL injection of EGF recombinant protein (cat # PHG0311, Fisher Scientific, Hampton, NH) was added for a final effective EGF concentration of 100ng/mL. After time series imaging, all cells were imaged using the RICM (100ms exposure time) and 488nm channel (1-2mW, 50-100ms exposure time, 100-500gain). If cells were transfected with Grb2-mCherry or other Grb2-mCherry mutants, the 561nm channel (1-2mW, 50-100ms, 100-500gain) was imaged as well.

Imaging Analysis

All images and videos were analyzed using ImageJ.¹⁰⁷ For EGFR clustering analysis, images from -EGF and +EGF conditions were compared in the 488nm channel. Cells were assigned the label of no clustering if the images before and after EGF addition remained unchanged. Cells were assigned the label of weak clustering if a small amount of clustering was observed at the edges of the cell. Cells were assigned the label of strong clustering if EGFR clustering was observed at the edges and in the middle of the cell.

For Grb2 recruitment, mean intensity traces of the 561nm channel were plotted for the cell measured during the videos of EGF addition. For all cells, Grb2 intensity at the bilayer was compared for EGF- and EGF+ conditions. Percent increase of Grb2-mCherry on the bilayer was calculated using the following equation:

$$\text{Percent Intensity Increase} = \frac{\text{Mean Intensity}_{EGF+} - \text{Mean Intensity}_{EGF-}}{\text{Mean Intensity}_{EGF-}} \times 100$$

Clustering Analysis

Cells were compared to preEGF and postEGF conditions to determine if the cell clustered. Clustering was determined by eye and separated into 3 categories: No Clustering, weak clustering,

strong clustering. Cells were designated no clustering if no change was observed before and after EGF addition. Cells were designated weak clustering if some clustering occurred around the periphery of the cell but did not extend throughout the middle of the cell nor throughout the entire cell. In some cases, weak clustering was observed around the entire cell and in other cases, weak clustering was observed on one side of the cell. Cells were designated strong clustering if clustering was observed throughout the entire cell.

Western Blot

Cells from imaging experiments were saved for western blot by stripping the cells ~20 minutes after EGF addition. Due to some CHO cell detachment after activation, imaging media was removed and saved. The chamber was incubated with 1mL of cell stripper for 10 minutes or TrypLE Express for 5 minutes. The imaging media and cell stripper media were combined and spun down for 5 minutes at 500g to collect a cell pellet. Cells were resuspended in 500 μ L of RIPA lysis buffer and frozen at -20C.

For the western blot, 8 μ L of lysis buffer was added to 2 μ L of 5x SDS-Page Loading buffer. Samples were loaded and run for an SDS-page gel. The gel was washed in NuPage™ Transfer Buffer (Cat # NP00061, fisher scientific) prior to membrane transfer. Proteins were transferred to the membrane for 1hr at 100volts. After transfer, the membrane was washed in TBST buffer before blocking with 25mL of 5% BSA in TBST for 1hour at 60rpm. The membrane was washed with TBST and incubated overnight with 5mL of 5% BSA in TBST with primary antibody, Grb2 (D2M8M) Rabbit mAb (Cat # 36344, Cell Signaling Technology). The next day, the membrane was washed 3 times with TBST buffer. The membrane was then incubated at room temperature for 1 hour in 5% BSA in TBST with secondary antibody like anti-rabbit Alexa647. After 1 hour, the membrane was washed 5 times with TBST buffer before imaging.

3.5 Appendix

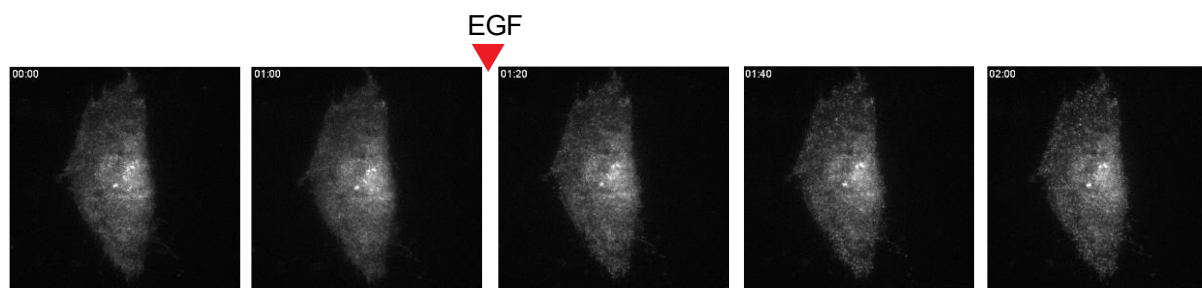


Figure 3.5: Supplementary Figure 1 Time Series of EGFR Clustering. Time series of cell transfected with EGFR and Grb2^{WT} for the first minute post activation. Almost immediately after EGF addition, clusters begin to form on the periphery of the cell and 1 minute after EGF addition, strong clustering is observed throughout the entire cell. EGF is added after 1 minute of imaging and images are taken every 5 seconds with a 488nm laser with 2mW power and 100ms exposure. Scale bars are 10 μ m.

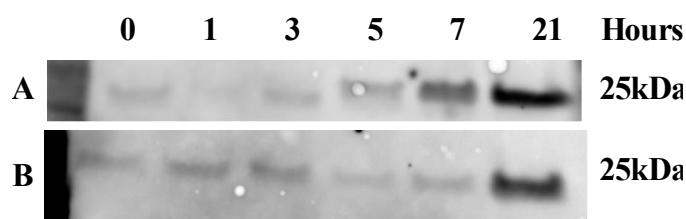


Figure 3.6: Supplementary Figure 2 Western Blot of Grb2 from increasing Grb2 Transfection Times. Western blots of CHO cells with Grb2 transfection times of 0, 1, 3, 5, 7, and 21 hours. (A) Grb2^{WT} (B) Grb2^{Y160E}. Western blots were taken using Typhoon Laser Imaging System at 640nm and 700V.

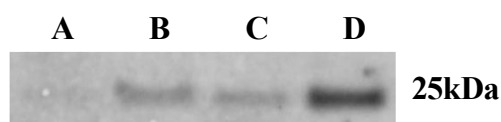


Figure 3.7: Supplementary Figure 3 Western Blot of Grb2 from different Grb2 Constructs. Western blots show the Grb2 amount in CHO cells from different transfection conditions. Cells were lysed 20 minutes post EGF activation. Western blot was taken using Typhoon Laser Imaging System at 640nm 1000V. Conditions are (A) EGFR only (B) EGFR + Grb2^{WT} (C) EGFR + 1x Grb2^{P49L} (D) EGFR + 2x Grb2^{P49L}.

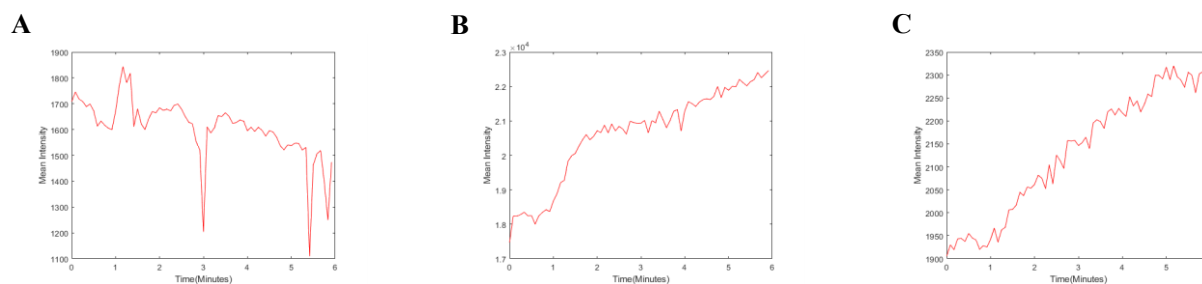


Figure 3.8: Supplementary Figure 4 Recruitment of Grb2^{WT}-mScarlet and Grb2^{P49L}-mScarlet. Mean Intensity traces (561nm laser 2mW and 100ms exposure) over time measured with to measure Grb2 recruitment with the following conditions (A) EGFR-eGFP only (B) EGFR-eGFP and Grb2^{WT}-mCherry and (C) EGFR-eGFP and Grb2^{P49L}-mCherry.

Transfection Type	Average Mean Percent Increase
EGFR Only	-12.4
EGFR + Grb2 WT	16.1
EGFR + Grb2 P49L	0.8
EGFR + Grb2 WT mScarlet	28.2
EGFR + Grb2 P49L mScarlet	34.9

Figure 3.9: Supplementary Figure 5 Average Intensity Increase of Grb2 after activation. Average Mean Percent Increase of 561nm channel (2mW power and 100ms exposure) after EGF addition.

Chapter 4

A Synthetic Panel of OKT3-Fab' Ligands Modulates Binding Kinetics to TCR

Brittany Stinger¹, Anand Vissa¹, Kiera Wilhelm¹, Shumpei Morita¹, Jay T. Groves¹

¹Department of Chemistry, University of California, Berkeley, Berkeley, California, USA

4.1 Introduction

T-cells are a critical part of the adaptive immune system; they are responsible for the recognition of foreign material in the body.^{1,2} The T-cell is a lymphocyte that differs from other lymphocytes due to the presence of the T-Cell receptor (TCR).² The TCR enables the T-cell to recognize antigen presenting cells (APCs) by probing the surface of the cell to detect agonist major histocompatibility complexes (MHCs).²⁻⁶ T-cells must detect agonist MHCs in an abundance of self MHC present on the surface of the hosts own cells, which are often in a ratio of 1:1000 agonist to native MHC.⁷ This selectivity is important since autoimmune diseases will result from a failure of the T-cell to distinguish these properly, resulting in the T-cell attacking its own cells.¹ The machinery of the T-cell is well tuned and can detect the presence of an APC with <10 ligands.¹¹ Previous experiments show that long TCR:ligand binding events lead to activation, while shorter binding events do not result in TCR activation. This sophisticated machinery is not well understood but can be explained by a kinetic proofreading model.¹⁰ In this model, ligand:TCR binding results in a series of kinetic mediated steps that eventually results in TCR activation.^{7,11-14} If at any point the ligand unbinds from the TCR before activation, the TCR moves from one of these intermediate states to the unbound state.^{11,12} To learn more about this process, selective control of TCR activation is necessary to tune activation.

Common methods of T-cell activation include bulk activation via antibody stimulation or other methods that measure bulk readouts like IL2 and cytokine release.⁵⁵ These methods are not tuned to measure the specific outcomes associated with TCR activation. Other papers report the existence of long binding events that lead to Linker for the Activation of T-cell (LAT) clustering.^{15,98} This clustering is representative of a well characterized phase condensation occurring between LAT and adapter proteins like Grb2, SOS, PLC γ 1, Gads, and SLP-76.¹⁵⁻²² In the case of Grb2 and SOS, the phase condensation serves to localize SOS to the lipid membrane so that it can readily activate Ras.^{1,5,20,84} This eventually leads to the phosphorylation of downstream signaling molecules ERK and the subsequent release of cytokines. The cell interprets information from the spatial and temporal recognition of these LAT clusters (condensates) to enact cellular processes.^{15,98} To measure the effect of the TCR:ligand binding events, tools like super resolution microscopy must be used to measure the recognition of these events and their cellular effects.¹²² One benefit of these super resolution techniques is that specific readouts like NFAT translocation and calcium fluctuations can be measured.^{15,58-60}

The TCR:ligand specificity hinders the ability to use a single MHC to activate a polyclonal T-cell population.^{6,56,57} This can be solved by the use of antibodies that bind the conserved region of the TCR, however, their strong affinity for the TCR could result in overactivation by bypassing the kinetic proofreading model by binding strongly for a long period of time.¹²³ Additionally, antibodies activate T-cells from solution and can bind multiple TCRs, potentially artificially coupling the TCRs and causing activation that does not mimic native conditions.⁹⁷ Thus, a different method of activation must be used to mimic native pMHC binding in which the ligands activate from a supported surface, have similar kinetics, and do not artificially nucleate TCR clusters.⁹⁷

We previously report on a supported lipid bilayer (SLB) system to which we can mimic an antigen presenting cell by attaching the fragment of antigen binding (Fab') of the antibody to activate the TCR.⁵⁹ The Fab' region of the antibody binds the conserved region of the TCR and allows us to activate a polyclonal T-cell population.⁵⁸⁻⁶⁰ Previously, the Fab' fragment was isolated via antibody digestion and then attached to the SLB via a DNA-Dye tether.^{58,59} By conjugating a complimentary strand of DNA to the supported lipid bilayer via cysteine-maleimide chemistry, the Fab'-DNA-dye can be added to the bilayer via DNA hybridization.^{58,59} The SLB is mobile, allowing the Fab' to move freely in 2-dimensions until it binds to a T-cell via the TCR. These discrete events can be measured and used to study the early activation of T-cells. Like pMHC, the Fab' does not activate T-cells and exhibits free diffusing Brownian motion on the SLB.^{59,124} This conjugation system allows for greater tunability of the system as the Fab' epitope and DNA-tether length can be tuned to probe questions on TCR activation.⁵⁸ We previously report that the epitope has no effect on cell activation, but longer DNA-tethers lead to decreased potency for Fab'-DNA ligands.⁵⁸ One drawback of this system is that the Fab' regions bind strongly to the TCR, preventing quantitative mechanistic studies of TCR activation.^{58,59} We recently report on an α -TCR β H57 Fab' fragment with a mutation at of residue R97 to Leucine that decreases the binding potency of the Fab' fragment and resembles native pMHC binding.⁶⁰ Although H57 is a murine anti-TCR antibody, but analogous mutations to human anti-TCR antibodies would allow us to develop an affinity panel for human primary T-cells. This could allow us to probe quantitative questions of early T-cell activation and conduct mechanistic studies on T-cells from human patients.

Anti-CD3- monoclonal antibody (OKT3) is an anti-TCR ligand that has been used as a T-cell therapy to block the development of cytotoxic T-cells.¹²⁵ Mutations at OKT3 residues R55 and Y57 to M and A (referred to as OKT3^{MA}) or L and T (referred to as OKT3^{LT}), respectively, were reported to decrease binding affinity to Jurkat T-cells.⁶¹ The study reported the bulk binding affinity as a decrease in mean calcium intensity from T-cells bound to the plasma membrane tethered OKT3-Fab' when antibody at increasing concentrations was added.⁶¹ In this study, we report an affinity panel of synthetic OKT3-Fab' ligands with varying binding affinities. The OKT3 ligands purified were an OKT3-Fab' isolated via antibody digestion (referred to as OKT3^{Antibody}), OKT3-Fab' WT purified from protein expression in XL1-Blue cells (referred to as OKT3^{Parental}), OKT3^{MA}, and OKT3^{LT}. After isolation, these OKT3-Fab' ligands were conjugated to a DNA-dye tether to allow for addition to our supported lipid bilayer system. Characterization of these ligands reveal 2-D Brownian motion on our supported lipid bilayer system and single photo-bleaching events. Dwell times measured with Jurkat cells reveal a decreased dwell time between OKT3^{Antibody} and OKT3^{Parental}, suggesting a modification like glycosylation to the OKT3^{Antibody} not possible for the OKT3^{Parental}. The dwell times of the OKT3^{MA} and OKT3^{LT} are significantly reduced compared to

the OKT3^{Antibody} and closely resemble pMHC dwell time kinetics.⁹⁷ The decreased binding kinetics of these ligands will allow us to probe human primary T-cells in future studies.

4.2 Results

Synthesis and Purification of OKT3-Fab' Ligands

OKT3-Fab' ligands were synthesized either via OKT3 antibody digestion or via purification from Ecoli. The isolated OKT3-Fab was conjugated to a DNA-Dye linker. DNA-dye linker is made using DNA sequence 1 (see Material and Methods) that has an amino modification at the 5' end and a thiol modification at the 5' end. Sequence 1 is first reacted with dye Atto647 maleimide where the maleimide on the dye reacts with the thiol modification on the DNA to produce the DNA-Dye. The DNA-dye is then reacted with succinimidyl-dPEG-maleimide (SM-(PEG)₆-maleimide) in which the amine on sequence 1 is reacted with the succinimidyl ester on the SM-(PEG)₆-maleimide. This DNA-dye-linker can then be reacted with a free cysteine on the OKT3-Fab' to form a fluorescently labelled OKT3-Fab' with a membrane tether. A schematic of these reactions is shown in Figure 4.1.

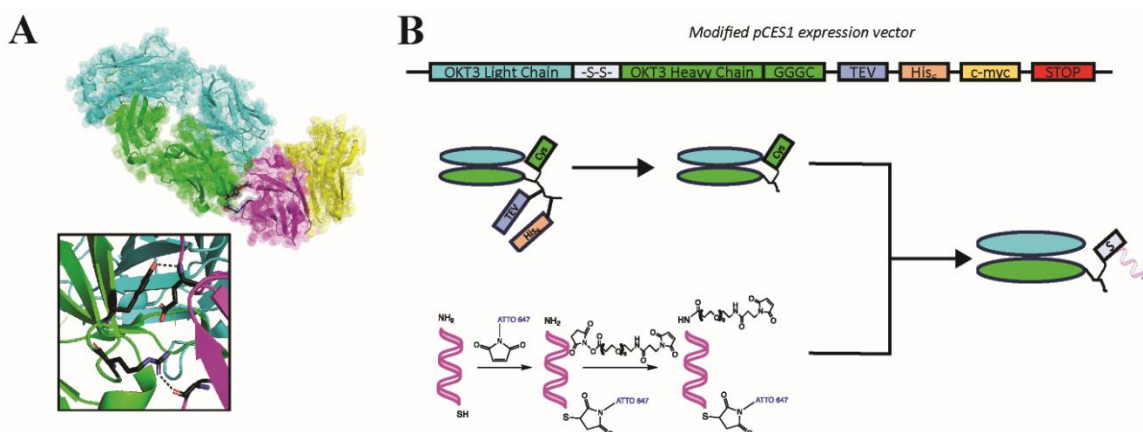


Figure 4.1: Schematic for the Purification of OKT3-Fab' Ligands and labeling. Expressing OKT3 fab' via E. Coli allows for point mutations. (A) Crystal Structure of OKT3 fab' bound to TCR displays hydrogen bonding residues R55 and Y57 on OKT3 Fab' heavy chain with G150 and E142 on TCR β , respectively. (B) OKT3 light and heavy chains were cloned into pCES1 expression vector. GGGC linker at the C-terminus of the heavy chain allows for OKT3 Fab' to be covalently bonded to a dye labeled DNA oligonucleotide.

Characterization of OKT3-Fab' Ligands

Diffusion and bleaching of OKT3-Fab' ligands on the supported lipid bilayer were conducted to ensure Brownian motion and ensure labeling efficiency. Supported lipid bilayers composed of 95% DOPC, 3% MCC, and 2% Ni-NTA were made as described in 4.4 Materials and Methods. To the supported lipid bilayer, DNA sequence 2 (4.4 Materials and Methods) was added through thiol maleimide chemistry via a thiol modification at the 5' end of the DNA sequence that reacted with the MCC lipid. To these supported lipid bilayers, a fixed amount of intercellular adhesion molecule (ICAM) with a histidine tag was added to mimic experimental conditions later in this report in which an immortalized cell line, Jurkat cells, are added to the system to monitor cell:ligand interactions. A varying amount of OKT3-Fab' ligands were added to the supported lipid bilayer via DNA hybridization of DNA sequence 2 on the supported lipid bilayer and DNA sequence 2 that is conjugated to the OKT3-Fab' ligands to generate an appropriate density. The OKT3-Fab' was mobile on the supported lipid bilayer and lower densities enabled tracking of these ligands (Figure 4.2B and 4.2C). Streaming videos were collected on the order of 10ms-20ms depending on the collected image size (see 4.4 Materials and Methods for more details). By plotting the step size distribution of the spots identified via trackmate, the diffusion constants were extracted (Figure 4.2D and 4.2E). The diffusion constants could vary depending on bilayer mobility. Bleaching data was collected for OKT3-Fab' ligands immobilized on a supported lipid bilayer. Single step photobleaching events were observed (Figure 4.2F) indicating the reaction of 1 OKT3-Fab' ligand with 1 DNA-dye-linker.

Modulation of Binding Affinity to the TCR

OKT3-Fab' was reconstituted on the supported lipid bilayer at densities that allow for a large number of distinguishable ligand:TCR interactions. An example of a ligand binding event is shown in figure 4.3A. The cell lands on the supported lipid bilayer and spreads as visualized in RICM with OKT3-Fab' ligands freely diffusing in the background measure with a 640nm laser with 2mW power and 500ms exposure with an interval of 500ms-2s between frames. When an OKT3' ligand engages a TCR the OKT3-Fab' stops diffusing and is shown as a bright spot in the cell area. The OKT3-Fab' ligand remains bound to the TCR for a fixed amount of time (Figure 4.3A). The amount of time the ligand remains bound to the TCR is defined as a binding event dwell time. A histogram for the probability distribution function of each of these binding events was made for each of the ligands (Figure 4.3B). The observed dwell time was calculated by fitting the distribution to a single exponential. To find the ligand k_{off} , a fast k_{on} rate was assumed. Bleaching data was collected for OKT3-Fab' ligands immobilized on the supported lipid bilayer using the same imaging conditions for the binding events. The k_{off} rates were calculated using the following equation.

$$f_{obs}(t) = (\langle \tau_{bleaching} \rangle^{-1} + \langle \tau_{off} \rangle^{-1}) e^{-(\langle \tau_{bleaching} \rangle^{-1} + \langle \tau_{off} \rangle^{-1})t}$$

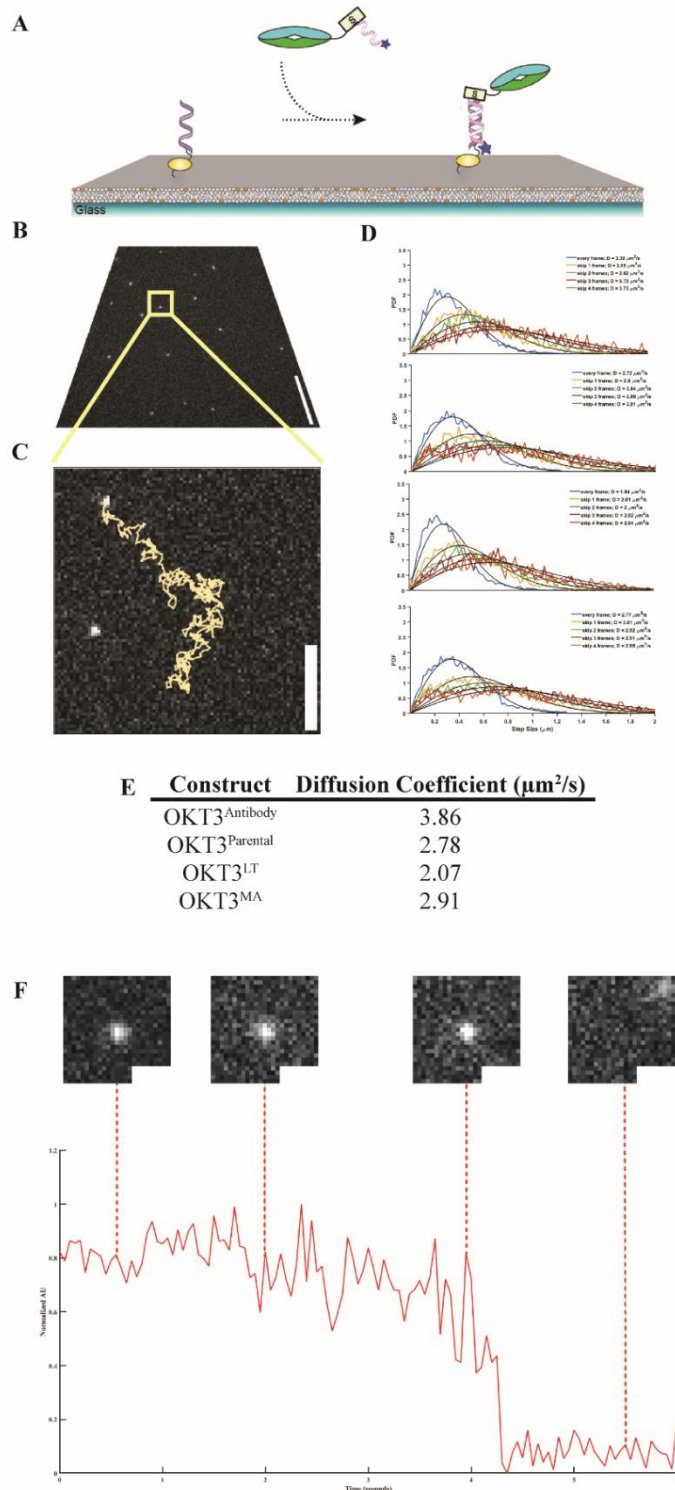


Figure 4.2: OKT3-Fab' Ligands are mobile on the bilayer and exhibit 2-D Brownian Motion. (A) Schematic of OKT3-Fab' conjugated to the supported lipid bilayer. (B) Flattened image of OKT3-Fab' parental on supported lipid bilayer taken with 640nm laser at 30mW power and 20ms exposure. (C) Track of OKT3 Fab' parental showing single particle motion in 2D Brownian motion. (D) Step-wise distribution of OKT3^{Antibody}, OKT3^{parental}, OKT3^{MA}, and OKT3^{LT}. (E) Corresponding diffusion coefficients extrapolated from step-wise distributions. (F) Intensity diagram showing bleaching of a single OKT3 Fab' parental particle. Image taken using a 640nm excitation at ~20ms exposure, scale bar is 1.0 μm .

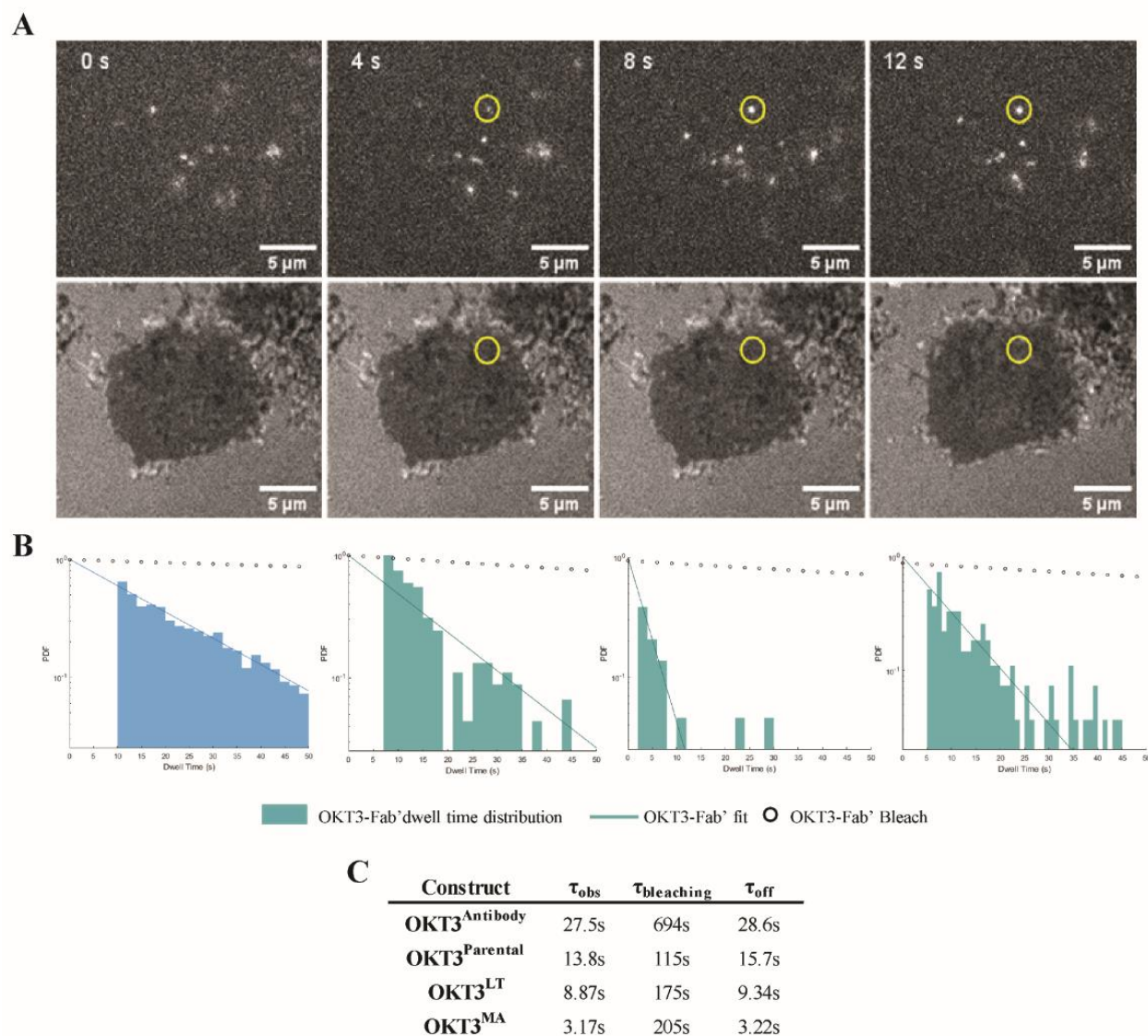


Figure 4.3: Dwell Times of OKT3-Fab' Ligands. (A) 640nm (2mW power and 500ms exposure) and RICM (500ms exposure) images showing binding event of OKT3-Fab' parental particles. (B) Dwell time distribution graphs for OKT3^{Antibody}, OKT3^{parental}, OKT3^{LT}, and OKT3^{MA} with bleaching curves to correct for photobleaching. (C) Observed rates, bleaching rates, and calculated off rates for OKT3-Fab' mutants.

A more detailed description of for the calculation of the k_{off} is described in 4.4 materials and methods section. The k_{off} decreased for the OKT3^{MA}-Fab' and OKT3^{LT}-Fab' compared to both the OKT3^{Antibody}-Fab' and the OKT3^{Parental}-Fab'. These k_{off} values have similar dwell times to pMHC, indicating that they could be used in place of pMHC to activate T-cells and in a similar manner as well. Notably, there was a decrease in the OKT3^{parental}-Fab' dwell time compared to the OKT3^{Antibody}-Fab' dwell time. This could be explained by modifications to the OKT3^{Antibody}-Fab' that are not possible during the Ecoli purification.

4.3 Discussion

The results of this study verify experiments conducted in bulk that show a decrease in the binding affinity for the OKT3^{MA} and OKT3^{LT} mutants compared to the OKT3^{WT} mutants. The single molecule sensitivity the supported lipid bilayer scaffold provides allows us to observe precise binding events on the supported lipid bilayer. There is a difference between the OKT3^{LT} and the OKT3^{MA} that differs slightly from a bulk activation study in that OKT3^{MA} has a shorter dwell time than OKT3^{LT} compared to a bulk activation study that shows OKT3^{LT} has less of a binding affinity. However, the bulk activation reveals similar that the binding affinities between OKT3^{MA} and OKT3^{LT} differ slightly. The slight difference between the bulk and single molecule study could be explained by some stochastic variation.

These ligands could bind to a polyclonal T-cell population isolated from a human patient. Additionally, the shorter dwell times of OKT3^{MA} and OKT3^{LT} are similar to the dwell time of agonist pMHC. These two properties make these ligands ideal candidates for the activation of human primary T-cells. Furthermore, these ligands could be used to probe the difference in T-cells from people with a variety of diseases to measure subtle differences in T-cell activation.

4.4 Materials and Methods

LB Plate, 2xYT Media, and Buffer Recipe

Below is a list of buffer composition for OKT3 Fab' purification.

LB Agar Plates: 5g tryptone, 2.5g yeast extract, 5g NaCl, 7.5g agar adjusted to pH 7

2x Yeast Extract Tryptone (YT) media: 16g Tryptone, 10g Yeast extract, 5g NaCl per 1L adjusted to pH 7

Buffers

TES Buffer: 0.2M Tris, pH 8, 0.5mM EDTA, 0.5 molar sucrose

Wash Buffer 1: 50mM Tris pH 8, 250mM NaCl

Wash Buffer 1.5: 50mM Tris pH 8, 250mM NaCl, 10mM imidazole

Wash Buffer 2: 50mM Tris pH 8, 500mM NaCl, 20mM imidazole

Elution Buffer: 50mM Tris pH 8, 500mM NaCl, 500mM imidazole

Dialysis Buffer: 1x PBS* pH 7, 2mM EDTA

SEC Buffer: 1x PBS*

MonoQ Buffer A: 20mM Tris pH 8, 150mM NaCl

MonoQ Buffer B: 20mM Tris pH 8, 1M NaCl

*PBS made from 10x Phosphate Buffered Saline (PBS) stock

DNA oligonucleotides

Sequence 1: 5'-/5AmMC6/GGT GTG ATG TAT GTG GA/3ThioMC3-D/-3'

Sequence 2: 5'-/5ThioMC6-D/CCA CAT ACA TCA CAC C-3'

DNA-Dye-Linker Synthesis

DNA sequence 1 was dissolved in H₂O and 2-3 μ L aliquot was saved. The DNA was reduced using 10 molar equivalents of TCEP in 100mM HEPES pH 8 for 1 hour. After 1 hour, unreduced DNA was removed using a NAP-5 column equilibrated in 1x PBS and 1mM EDTA. After desalting, 5 equivalents of ATTO-647N-Maleimide dye dissolved in DMSO was added to the DNA and the DNA-dye mixture shook at 60rpm and room temperature for 1 hour. Excess dye was removed using a NAP-5 column equilibrated in 1xPBS and 1mM EDTA. The sample was ethanol precipitated, dried, and kept overnight at -20C. The DNA-dye was resuspended in ~50-100 μ L H₂O and a small aliquot of 2-3 μ L was saved. SM-PEG₆-maleimide and DNA-dye were mixed in PBS to a final concentration of 0.5mM DNA Dye, 25mM SM-PEG₆-maleimide (50 equivalents), and

1x PBS. The SM-PEG₆-maleimide was added in two increments to reach 25mM; half was added to the DNA-dye and after a 30 minute incubation, the other half was added to the DNA-dye and the sample was incubated for an additional 30 minutes at room temperature. The sample was added to a NAP-5 column equilibrated in milli-Q H₂O and eluted in 0.5mL fractions. 2-3μL of the fractions were saved. The samples were ethanol precipitated, dried, and stored in the -20 C. Synthesis of the DNA-dye-linker was confirmed via MALDI-TOF.

OKT3 Fab'-DNA Synthesis

OKT3 Fab' Antibody was purified by antibody digestion as described in references 58-60.

OKT3 Fab' purification for OKT3^{Parental}, OKT3^{MA}, and OKT3^{LT} were purified from plasmid as previously described in 27. On day 0, OKT3 Plasmid was plated onto a Carbenicillin LB Agar plate and kept at 37°C overnight. On Day 1, 4 colonies were selected for amplification in 50mL 2xYT media with 2% glucose and 100μg/mL Carbenicillin. Cultures were grown overnight at 30C and 250 rpm. On Day 2, the 50mL culture was diluted to a starting OD₆₀₀ of ~0.05 in 4x 1L of 2xYT with 0.1% glucose and 100μg/mL Carbenicillin. Cultures were grown at 37C and 200rpm until an OD₆₀₀ of 0.6-0.8 was reached (approximately 4-6 hours). The cultures were induced with 1mM IPTG and allowed to grow overnight at 20°C and 200 rpm. Day 3, approximately 16-18 hours after IPTG induction, cells were spun down at 3500g for 30 minutes. Each pellet was resuspended in 20mL of TES buffer and shook at 4C for 30 minutes. Meanwhile, EDTA protease inhibitor was dissolved in ice cold water according to the manufacturer's instructions. After 30 minutes, 15mL of ice-cold water with protease inhibitor was added to each TES-cell pellet resuspension. The mixture shook slowly at 4C for an additional 30 minutes and then spun down at 7000g for 30minutes at 4C. The supernatant (periplasm extract), a thick, yellow mixture, was removed and saved. Optionally, the process was repeated (TES resuspension, protease inhibitor addition, centrifugation), although the periplasm extract was not as thick as the first extraction. The supernatant was a thick, yellow mixture. To each periplasm extract the following was added: 1mL of Ni-NTA Beads equilibrated in wash buffer 1 with MgCl to a final concentration of 10mM, and imidazole to a final concentration of 10mM. The periplasm extract was slowly rotated at 4C for ~1 hour. Periplasm extracts were spun down at 2000g for 10 minutes and the supernatant was removed and discarded. The Ni-NTA beads were packed into a column and washed with 20 volumes of wash buffer 2 (~80-160mL). After washing, the OKT3 Fab' was eluted with 3 column volumes of elution buffer. Following elution, the sample was dialyzed overnight in dialysis buffer.

Day 4, the dialyzed protein was concentrated to ~0.5mg/mL using 30kDA spin columns. TEV (2mg/mL) was added in a ratio of 1:50 TEV to OKT3 Fab'. The OKT3 fab' was allowed to shake slowly at room temperature for 2 hours. Following TEV cleavage and His-6-Tag removal, the sample was added to a Nap-10 column equilibrated in wash buffer 1. The sample was eluted in 1mL fractions using 10mL of wash buffer 1.5. Protein elution was verified using a nanodrop. To the elution, 80μL of Ni-NTA beads were added to each 1mL fraction and slowly rotated at 4C for 1 hour. Following rotation, samples were spun at 2000g for 10 minutes and the supernatant was saved. The Ni-NTA beads were washed with wash buffer 2 and immediately spun down for 2.5

minutes at 2000g. The supernatant was saved and combined with other supernatants. The sample was dialyzed overnight in dialysis buffer. Following OKT3-Fab' purification, samples were conjugated to the DNA-dye linker.

OKT3 Fab' Conjugation to DNA-Dye-Linker

Following OKT3-Fab' purification via antibody digestion or protein purification, the samples were conjugated to the DNA-Dye-Linker. Protein was concentrated to ~0.5mg/mL using a 30kDa spin column and incubated with 4x molar excess of the DNA-Dye-linker and rotated at room temperature for 2 hours. To remove excess dye, the sample was diluted to 4mL and run over a 30kDa spin concentrator. This was repeated. Following dye removal, the sample was loaded on to an SEC and eluted using SEC buffer. We ran a gel and stained it with Sypro Ruby to identify fractions with OKT3 Fab'. The fractions were concentrated using a 30kDa spin filter and the concentration was measured using a nanodrop. The sample was then run over an ion exchange column (MONO-Q). The sample was eluted in 20mM Tris pH 8 with a salt gradient of 150mM to 1M NaCl for 40 minutes at a rate of 1mL/minute. Fractions were collected and a gel was run to verify the collection and purity of OKT3-Fab'.

Supported Lipid Bilayer Preparation

Supported lipid bilayers were prepared in a similar manner to that previously described in references 58-60. Briefly, 25mm coverslips (Cat # 1217N82, Thomas Scientific) were placed in a coverslip mini-Rack (Cat #C14784, ThermoFisher Scientific) and sonicated in 1:1 Isopropanol/Water for 30 minutes. Similarly, a 50mL round bottom flask was sonicated in 1:1 isopropanol/water mixture for 30 minutes. Following sonication, the round bottom flasks and the coverslips were washed thoroughly with milliQ water. The coverslips and round bottom flasks were etched for 5 minutes in a piranha etching solution made from 3 parts sulfuric acid and 1 part hydrogen peroxide. After etching, the coverslips and round bottom flasks were washed thoroughly in milliQ water. The round bottom flask was allowed to dry in an oven at 50C for at least an hour to at most overnight. The coverslips were stored in DI water until chamber preparation.

For chamber preparation, DNA was reduced for 90 minutes at 37°C in 10mM Hepes, 2mM TCEP, and 197µM DNA. After reduction, the DNA was cleaned in 2 biospin-6 columns equilibrated in PBS. During the DNA reduction, chambers with supported lipid bilayers were prepared. Attofluor cell chambers were (Cat # A7816, Invitrogen) sonicated in IPA/H₂O at a ratio of 1:1. After sonication, the Attofluor cell chambers were washed with DI water and dried with Nitrogen gas. For some chambers, an insert was used to divide the chamber into smaller sections. To make the SUV, an etched round bottom flask was removed from the oven and allowed to cool before washing with chloroform. To the flask, lipids were added to make a composition of 95% DOPC, 2% NTA-DOGS, and 3% MCC-DOPE. The chloroform was removed from the lipids using a rotovacuum until all visible traces of the chloroform were gone. The lipids were dried under nitrogen gas for 8 minutes. The lipids were resuspended with an appropriate volume of water to reach a final

concentration of 0.5mg/mL. The lipids were sonicated using tip sonication with total pulse time of 1 minute 30 seconds with 15 second pulses separated by intervals of 45 seconds. The SUV solution was diluted with PBS in a ratio of 1:1 and 500 μ L of this solution was added to each chamber. The SUV was incubated for 30 minutes and washed with 1x PBS. 400 μ L of the reduced DNA diluted to 1 μ M in PBS was added to each chamber and incubated for 80-100minutes. Following incubation, the chambers were washed with 5mL of 1x PBS and stored overnight at 4°C.

To prepare the chambers for imaging, the chambers were incubated with 100mM NiSO₄ for 5 minutes. The chambers were washed with imaging buffer (20mM HEPES pH 7, 137mM NaCl, 5 mM KCl, 0.7mM Na₂HPO₄, 6mM D-Glucose, 1mM CaCl₂, 1mM MgCl, and 1% w/v BSA) and incubated for 10 minutes. A 500 μ L injection of ICAM and OKT3-Fab' in Imaging buffer were added to the chamber and incubated for 30 minutes. ICAM was incubated at a concentration of 15 μ M, but OKT3-Fab' constructs were titrated depending on the desired density. After a 30-minute incubation, chambers were washed with imaging buffer and placed on the microscope for 10 minutes prior to the cell addition.

Cell Culture

Jurkat cells obtained from the University of California, Berkeley Barker Hall Cell Culture facility were cultured in RPMI 160 medium, GlutaMaxTM supplement (Cat # 61870127, Fisher Scientific) with 10% FBS and 1% Penicillin-Streptomycin.

Microscopy

All imaging was carried out on a Nikon Ti Eclipse inverted microscope (Technical Instruments, Burlingame, CA) with a TIRF system. A laser launch with 405nm, 488nm, 560nm, and 640nm diode laser (Coherent OBIS, Santa Clara, CA) was aligned into a custom-built fiber launch (Solamere Technology Group Inc., Salt Lake City, UT). The filter set for TIRF illumination included a 405/488/561/647nm Quad TIRF filter set (Chroma Technology Corp. Rockingham, Vermont) and emission filters (ET 525/50M, ET600/50M, and ET700/75) were used respectively. RCM images were filtered through a 50/50 beam splitter. Epifluorescent excitation was carried out with band-pass filters, ET470/40x, D546/10x, ET545/30x, and ET620/60x, respectively for excitations with 488nm, 561nm, and 640nm excitations. The images were captured on an EM-CCD(iXon 897DU; Andor Inc., South Windsor, CT) and acquired with Micromanager software.

Image Analysis

Fab' Density

Fab' densities were calculated with 15-20 images taken at 640nm with a laser power of 30mW and exposure time of 20ms. Fab' were identified with the tracking software trackmate using a radius of 0.4 μ m. Other filters including a quality filter set between 800-1000 and a minimum intensity threshold were used to filter out noise.

Diffusion

The diffusion of single molecule particles was collected by streaming 640nm laser at a 20mW or 30mW power and 7-20ms exposure time depending on the size of the image being collected. The exposure time was limited by the ability of the camera to collect images. By decreasing the dimensions to be collected from 512x512 to smaller dimensions like 256x256 and 128x128, allowed for a shorter interval between frames. The density of the Fab' on the bilayer was at a low enough density to allow for single particle tracking with minimal overlap of the particles. Fab' was identified using trackmate with previously defined radius and threshold settings. Tracks were linked with a 0.4μm distance between two particles in adjacent images with a max closing distance of 0 frames. The diffusion constant, D, was calculated based on two-dimensional Brownian motion. To calculate the diffusion constant, a custom MATLAB script made by Kiera Wilhelm was used to calculate the step size distribution based off trackmate tracking data corrected for overlap events. This stepwise distribution was fit to a single-component diffusion model,

$$\rho(r, \tau, D) = \frac{r}{2D\tau} e^{\frac{-r^2}{4D\tau}}$$

Where r is the molecular displacement, τ is the time interval, and D is the diffusion constant.

Dwell time and k_{off} Rates

Cell binding events were collected with a 640nm laser with 2mW power and 500ms exposure with intervals of 500ms – 2 seconds between images depending on the OKT3-Fab' construct. RCM images were taken every 15-30 frames to measure the perimeter of the cell. A spot was identified as a binding event if the tracked spot resulted in a binding event that was within the cell outline and lasted for longer than 5 frames. The spots were identified using trackmate with previously defined radius and threshold settings. Tracks were linked with a 0.4μm distance between two particles in adjacent images with a max closing distance of 1 frame. This methodology is like those used in other reports.⁶⁰ Bleaching curves were calculated by imaging fixed particles on a supported lipid bilayer in the absence of cells. Bleaching conditions were the same as the imaging conditions for the tracked binding events (640nm, 2mW power, 500ms exposure, with 500ms-2second between images).

To determine K_{off} rates, the observed dwell times were corrected for photobleaching using the following formula.

$$f_{obs}(t) = (\langle \tau_{bleaching} \rangle^{-1} + \langle \tau_{off} \rangle^{-1}) e^{-(\langle \tau_{bleaching} \rangle^{-1} + \langle \tau_{off} \rangle^{-1})t}$$

Where $f_{obs}(t)$ is the probability of ligand binding as a function of the time, $\langle \tau_{bleaching} \rangle^{-1}$ is the fluorophore bleaching rate and $\langle \tau_{off} \rangle^{-1}$ is the ligand-off rate.

4.5 Appendix

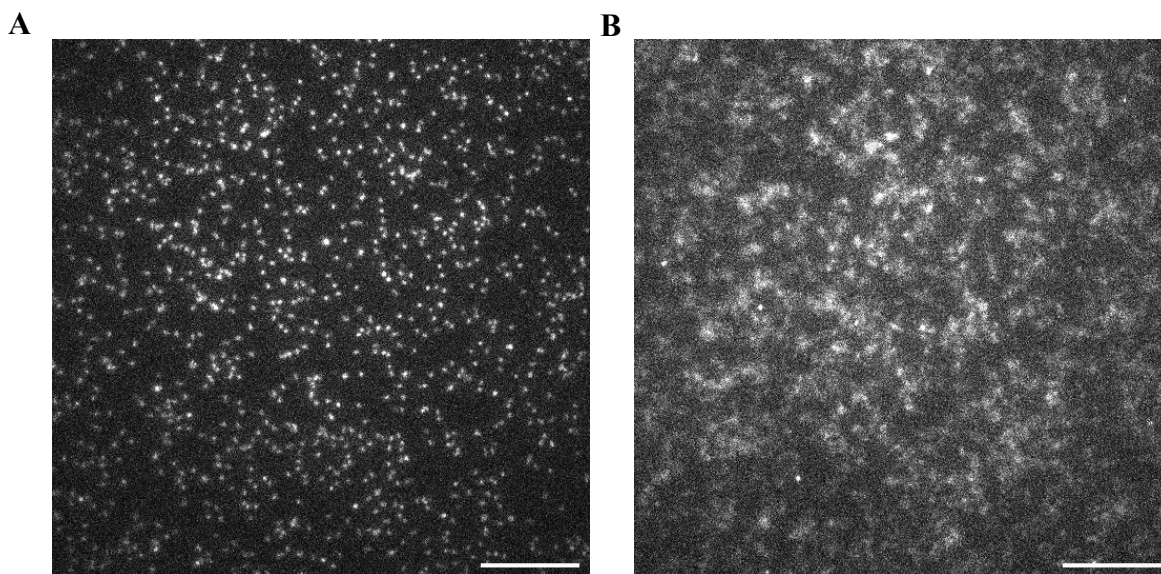


Figure 4.4: Supplementary Figure 1 Density Calculations for OKT3-Fab'-DNA Ligands. (A) Density and (B) diffusion images for OKT3^{Antibody}, taken before cells were added to the supported lipid bilayer. Images were taken at 640nm with 30mW power and 20ms exposure for and 640nm with 2mW power and 500ms exposure, respectively. Scale bars are 10 μ m.

Chapter 5

Conclusions and Future Directions

5.1 Conclusions

These investigations into the MAPK/ERK signaling pathway and the early activation of T-cell signaling reveal the effect of protein interactions on signaling in reconstitution and live cells. In Chapter 2, I show that Grb2 dimer interactions influence the phase condensation of EGFR. A Grb2 mutant that mimics Grb2 phosphorylation prevents the phase condensation of EGFR phosphotyrosine tail on a supported lipid bilayer. Prephosphorylation of Grb2 with kinase HCK has a similar effect in preventing EGFR^{Tail} phase condensation. Additionally, similar to the LAT condensate, the EGFR:Grb2:SOS condensate has a higher impact on the initial activation of Ras similar to the LAT condensate. Since EGFR, Grb2, and SOS do not exist in an equivalent ratio in cells with SOS often present at lower levels than EGFR and Grb2, it is likely that SOS is a limiting component of the condensate. If this is the case, Grb2:Grb2 interactions could be more important for phase condensation than previously considered.

I investigated the effect of the Grb2 dimer on EGFR phase condensation in CHO cells. The results show that EGFR clustering is a consequence of the phase condensation of EGFR and not EGFR oligomerization. Grb2 dimer mutants, Grb2^{Y160E} and Grb2^{V123D}, showed strong EGFR clustering similar to Grb2^{WT} condition, suggesting that the Grb2 dimer has a more complex role in EGFR phase condensation in live cells. Weak EGFR clustering was observed for a Grb2 mutant not capable of binding SOS (Grb2^{P49L}), however, increasing the amount of Grb2^{P49L} resulted in strong EGFR clustering throughout the cell. The increase in clustering despite the inability of Grb2^{P49L} to bind SOS suggests that Grb2 has a more complex role in the EGFR phase condensation. A phase diagram could explain the shift from uncondensed to condensed phase; increasing Grb2 concentration results in EGFR:Grb2 phase condensation. High levels of Grb2 are found in cancerous cells also containing high levels of EGFR. The high concentration of these proteins could allow for more Grb2:Grb2 interactions, resulting in the phase condensation of Grb2 and subsequent activation of the MAPK/ERK downstream signaling pathway.

The phase condensation is an essential part of cell signaling in both T-cells and epithelial cells, however, T-cell activation begins long before adapter proteins condense to LAT. I explore T-cell activation in chapter 4, where I detail a panel of synthetic T-cell ligands with different CD3 binding affinities. The strongest anti-CD3 OKT3-Fab' ligand, OKT3-Fab'^{Antibody} binds with a strong binding affinity and lower off rate, while the lowest binding affinity ligands (OKT3-Fab'^{MA} and OKT3-Fab'^{LT}) closely mimic the binding kinetics of pMHC. The lower k_{off} rate and ability of the Fab' to bind the constant region of the TCR will allow for these ligands to be used in mechanistic studies of Human Primary T-cells.

5.2 Investigation of EGFR Signaling in Live Cells

The exploration of the Grb2:Grb2 interaction in the EGFR signaling pathway is only scratching the surface in understanding the nuances of the EGFR signaling pathway. As discussed, one issue we faced is that there is an endogenous concentration of Grb2 in CHO cells. The endogenous Grb2 could be interfering with the readout from the Grb2 mutants since the endogenous Grb2 does not have the mutations that interfere with either the Grb2:Grb2 interactions or the Grb2:SOS interactions. In future experiments, a Grb2 knockdown or knockout experiment could be conducted to remove this background interference from the endogenous Grb2. Concerning the Grb2:Grb2 interaction, another possibility is that in the cell, Grb2 oscillates between both domain swap dimers. Potentially making a Grb2 mutant that has the mutations Y160E and V123D that eliminate both Grb2 interactions would prevent CHO cells from strongly clustering. More characterization of this mutant would need to be conducted to ensure that the Grb2 mutant retains its structure and function. Critically, the interactions between Grb2 and EGFR phosphotyrosines as well as Grb2 and SOS would need to be maintained to evaluate whether disrupting the Grb2:Grb2 interaction prevents EGFR clustering.

To better understand the effect of EGFR activation on the EGFR condensate and subsequent downstream signaling, we can study the effect of different ligands on the activation of EGFR. There are 7 ligands known to bind EGFR with varying K_d values ranging from 1 – 100nM for the stronger affinity ligands like EGF and TGF- α and greater than 100nM for the weaker ligands like epigen.¹²⁶ Different ligand binding can lead to different cellular functions like cellular proliferation or differentiation resulting from the stabilization of the EGFR dimer and lifetime of the active EGFR.^{127–130} The ligands accomplish this through the stabilization of the juxtamembrane region of EGFR that affects differences between even the stronger ligands.¹³¹ A cryo-EM study revealed ability of EGF to stabilize the EGFR dimer more effectively than TGF- α , resulting in greater EGFR activation in HEK293T cells.¹³¹ This could explain why EGF and TGF- α have different cellular effects, even though they have similar binding affinities, however, more research is necessary to determine downstream effects.¹²⁷ Stabilization of the EGFR dimer is known to affect signaling, where stronger dimers resulted in transient signaling and cellular proliferation, while weak dimers resulted in sustained signaling and cellular differentiation.¹³² The differences could potentially be explained by the activation of a negative feedback loop that is not achieved by the weakly bound ligands.¹³³ Additionally, EGFR ligands are known to cause different amounts of tyrosine phosphorylation, which could affect the potential of EGFR to interact with other proteins.^{132,134–136} The different amounts of phosphorylation could affect the ability of EGFR to recruit adapter proteins like Grb2 and SOS, therefore affecting EGFR clustering and subsequent downstream signaling. Differences in EGFR condensation and clustering could be measured using the experiments described in chapter 3, where CHO cells are activated with EGF ligand. Additionally, effects on downstream signaling would have to be conducted to find the impact, if any, EGFR clustering has on downstream signaling. Measuring the effect of these ligands on downstream activation would require a readout of downstream signaling. Western blots to measure ERK phosphorylation could be conducted, however, this does not allow for real time measurements, cell to cell variability, nor real time measurement of the kinetics of activation. The downstream readout could be analogous to the current readouts we have for T-cells, namely NFAT translocation and calcium flux.

To gain a better understanding of the EGFR signaling pathway using our platform, we need to have a readout of downstream signaling in these cells. An ideal readout of downstream signaling would be Ras activation. Typical readouts of Ras activation in live cells is completed Ras pulldown assays and western blot. However, this method does not account for cell to cell variability nor is the kinetics of Ras activation well characterized using this method. Ras has a low copy number in the cell and a high variability between cell types. For this reason, transfecting cells with fluorescently labelled Ras would result in an increase of Ras concentration in the cell, potentially affecting the natural signaling process in the cell. Ras biosensors could be used to measure the Ras activation in cells. A wide variety of biosensors have been developed including Förster resonance energy transfer (FRET)-biosensors that often contain a modified Ras that can act as a GEF biosensor.¹³⁷ This results in the biosensor relying on the amount and activity of GEF/GAP, therefore, they may not provide an accurate readout of Ras activation.¹³⁷ Other downstream processes could be used to find the effect the phase condensation has on downstream signaling. Specifically, recent advances in ERK and MEK cell imaging could provide a readout for downstream activation of EGFR signaling.¹³⁸ A FRET study measuring ERK and MEK binding in HEK293T cells showed from FRET the translocation of ERK to the nucleus.¹³⁹ This translocation could be akin to NFAT translocation in T-cells. More experiments are needed to determine the feasibility in our system, but could provide a way to conduct single cell studies and measure the kinetics of cellular activation, possibly leading to insights in EGFR differential signaling.

5.3 Probing Human Primary T-cells with OKT3-Fab' Ligands

Previous experiments have focused primarily on Antibody-Fab' that binds to murine T-cells.⁵⁸⁻⁶⁰ The ability of the panel of synthetic T-cell ligands to bind human TCRs in a polyclonal manner allows us to study human primary T-cells. After establishing a baseline of T-cell activation in human T-cells, these ligands could be used to measure differences in T-cell activation from patients with different diseases including cancer and immunodeficiencies. For example, these ligands could be used to better understand T-cell therapy, with T-cells that contain an engineered single-chain variable fragment (scFv) of an antibody.¹⁴⁰ However, these chimeric antigen receptors (CARs) are designed to bypass pMHC recognition and can often lead to toxic off-target effects.¹⁴⁰ Recently, TCR-mimic CARS have been developed to recognize tumor-associated antigens (TAAs).¹⁴⁰ By using this platform, we could better understand the ligand-receptor binding interactions and how they affect downstream signaling. Beyond the signaling platform, these ligands could be useful as a diagnostic tool, since they activate in a similar manner to native conditions and could be used to measure differences in biological readouts (IL2 secretion, NFAT translocation, etc.).

References

- (1) Shah, K.; Al-Haidari, A.; Sun, J.; Kazi, J. U. T Cell Receptor (TCR) Signaling in Health and Disease. *Signal Transduct. Target. Ther.* **2021**, *6* (1), 412. <https://doi.org/10.1038/s41392-021-00823-w>.
- (2) Bonilla, F. A.; Oettgen, H. C. Adaptive Immunity. *2010 Primer Allerg. Immunol. Dis.* **2010**, *125* (2, Supplement 2), S33–S40. <https://doi.org/10.1016/j.jaci.2009.09.017>.
- (3) Sundberg, E. J.; Deng, L.; Mariuzza, R. A. TCR Recognition of Peptide/MHC Class II Complexes and Superantigens. *Struct. Funct. Antigen Recept.* **2007**, *19* (4), 262–271. <https://doi.org/10.1016/j.smim.2007.04.006>.
- (4) Lanzavecchia, A.; Iezzi, G.; Viola, A. From TCR Engagement to T Cell Activation: A Kinetic View of T Cell Behavior. *Cell* **1999**, *96* (1), 1–4. [https://doi.org/10.1016/S0092-8674\(00\)80952-6](https://doi.org/10.1016/S0092-8674(00)80952-6).
- (5) Courtney, A. H.; Lo, W.-L.; Weiss, A. TCR Signaling: Mechanisms of Initiation and Propagation. *Trends Biochem. Sci.* **2018**, *43* (2), 108–123. <https://doi.org/10.1016/j.tibs.2017.11.008>.
- (6) Morris, G. P.; Allen, P. M. How the TCR Balances Sensitivity and Specificity for the Recognition of Self and Pathogens. *Nat. Immunol.* **2012**, *13* (2), 121–128. <https://doi.org/10.1038/ni.2190>.
- (7) Britain, D. M.; Town, J. P.; Weiner, O. D. Progressive Enhancement of Kinetic Proofreading in T Cell Antigen Discrimination from Receptor Activation to DAG Generation. *eLife* **2022**, *11*, e75263. <https://doi.org/10.7554/eLife.75263>.
- (8) Fernando, M. M. A.; Stevens, C. R.; Walsh, E. C.; De Jager, P. L.; Goyette, P.; Plenge, R. M.; Vyse, T. J.; Rioux, J. D. Defining the Role of the MHC in Autoimmunity: A Review and Pooled Analysis. *PLOS Genet.* **2008**, *4* (4), e1000024. <https://doi.org/10.1371/journal.pgen.1000024>.
- (9) Nepom, G. T.; Erlich, H. MHC Class-II Molecules and Autoimmunity. *Annu. Rev. Immunol.* **1991**, *9* (1), 493–525.
- (10) Goldstein, B.; Coombs, D.; Faeder, J. R.; Hlavacek, W. S. Kinetic Proofreading Model. In *Multichain Immune Recognition Receptor Signaling: From Spatiotemporal Organization to Human Disease*; Sigalov, A. B., Ed.; Springer New York: New York, NY, 2008; pp 82–94. https://doi.org/10.1007/978-0-387-09789-3_8.
- (11) Chakraborty, A. K.; Weiss, A. Insights into the Initiation of TCR Signaling. *Nat. Immunol.* **2014**, *15* (9), 798–807. <https://doi.org/10.1038/ni.2940>.
- (12) McKeithan, T. W. Kinetic Proofreading in T-Cell Receptor Signal Transduction. *Proc. Natl. Acad. Sci.* **1995**, *92* (11), 5042–5046. <https://doi.org/10.1073/pnas.92.11.5042>.
- (13) Rosette, C.; Werlen, G.; Daniels, M. A.; Holman, P. O.; Alam, S. M.; Travers, P. J.; Gascoigne, N. R.; Palmer, E.; Jameson, S. C. The Impact of Duration versus Extent of

- TCR Occupancy on T Cell Activation: A Revision of the Kinetic Proofreading Model. *Immunity* **2001**, 15 (1), 59–70. [https://doi.org/10.1016/s1074-7613\(01\)00173-x](https://doi.org/10.1016/s1074-7613(01)00173-x).
- (14) Pielak, R. M.; O'Donoghue, G. P.; Lin, J. J.; Alfieri, K. N.; Fay, N. C.; Low-Nam, S. T.; Groves, J. T. Early T Cell Receptor Signals Globally Modulate Ligand:Receptor Affinities during Antigen Discrimination. *Proc. Natl. Acad. Sci.* **2017**, 114 (46), 12190–12195. <https://doi.org/10.1073/pnas.1613140114>.
 - (15) McAfee, D. B.; O'Dair, M. K.; Lin, J. J.; Low-Nam, S. T.; Wilhelm, K. B.; Kim, S.; Morita, S.; Groves, J. T. Discrete LAT Condensates Encode Antigen Information from Single pMHC:TCR Binding Events. *Nat. Commun.* **2022**, 13 (1), 7446. <https://doi.org/10.1038/s41467-022-35093-9>.
 - (16) Su, X.; Ditlev, J. A.; Hui, E.; Xing, W.; Banjade, S.; Okrut, J.; King, D. S.; Taunton, J.; Rosen, M. K.; Vale, R. D. Phase Separation of Signaling Molecules Promotes T Cell Receptor Signal Transduction. *Science* **2016**, 352 (6285), 595–599. <https://doi.org/10.1126/science.aad9964>.
 - (17) Huang, W. Y. C.; Alvarez, S.; Kondo, Y.; Lee, Y. K.; Chung, J. K.; Lam, H. Y. M.; Biswas, K. H.; Kuriyan, J.; Groves, J. T. A Molecular Assembly Phase Transition and Kinetic Proofreading Modulate Ras Activation by SOS. *Science* **2019**, 363 (6431), 1098–1103. <https://doi.org/10.1126/science.aau5721>.
 - (18) Huang, W. Y. C.; Yan, Q.; Lin, W. C.; Chung, J. K.; Hansen, S. D.; Christensen, S. M.; Tu, H. L.; Kuriyan, J.; Groves, J. T. Phosphotyrosine-Mediated LAT Assembly on Membranes Drives Kinetic Bifurcation in Recruitment Dynamics of the Ras Activator SOS. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, 113 (29). <https://doi.org/10.1073/pnas.1602602113>.
 - (19) Lin, C.-W.; Nocka, L. M.; Stinger, B. L.; DeGrandchamp, J. B.; Lew, L. J. N.; Alvarez, S.; Phan, H. T.; Kondo, Y.; Kuriyan, J.; Groves, J. T. A Two-Component Protein Condensate of the EGFR Cytoplasmic Tail and Grb2 Regulates Ras Activation by SOS at the Membrane. *Proc. Natl. Acad. Sci. U. S. A.* **2022**, 119 (19), e2122531119. <https://doi.org/10.1073/pnas.2122531119>.
 - (20) Vidal, M.; Goudreau, N.; Cornille, F.; Cussac, D.; Gincel, E.; Garbay, C. Molecular and Cellular Analysis of Grb2 SH3 Domain Mutants: Interaction with Sos and dynamin11 Edited by A. R. Fersht. *J. Mol. Biol.* **1999**, 290 (3), 717–730. <https://doi.org/10.1006/jmbi.1999.2899>.
 - (21) Balagopalan, L.; Kortum, R. L.; Coussens, N. P.; Barr, V. A.; Samelson, L. E. The Linker for Activation of T Cells (LAT) Signaling Hub: From Signaling Complexes to Microclusters. *J. Biol. Chem.* **2015**, 290 (44), 26422–26429. <https://doi.org/10.1074/jbc.R115.665869>.
 - (22) Sherman, E.; Barr, V.; Samelson, L. E. Super-Resolution Characterization of TCR-Dependent Signaling Clusters. *Immunol. Rev.* **2013**, 251 (1), 21–35. <https://doi.org/10.1111/imr.12010>.

- (23) Kortum, R. L.; Balagopalan, L.; Alexander, C. P.; Garcia, J.; Pinski, J. M.; Merrill, R. K.; Nguyen, P. H.; Li, W.; Agarwal, I.; Akpan, I. O.; Sommers, C. L.; Samelson, L. E. The Ability of Sos1 to Oligomerize the Adaptor Protein LAT Is Separable from Its Guanine Nucleotide Exchange Activity in Vivo. *Sci. Signal.* **2013**, 6 (301), ra99. <https://doi.org/10.1126/scisignal.2004494>.
- (24) Zhang, Y.; Kutateladze, T. G. Liquid–Liquid Phase Separation Is an Intrinsic Physicochemical Property of Chromatin. *Nat. Struct. Mol. Biol.* **2019**, 26 (12), 1085–1086. <https://doi.org/10.1038/s41594-019-0333-8>.
- (25) Hnisz, D.; Shrinivas, K.; Young, R. A.; Chakraborty, A. K.; Sharp, P. A. A Phase Separation Model for Transcriptional Control. *Cell* **2017**, 169 (1), 13–23. <https://doi.org/10.1016/j.cell.2017.02.007>.
- (26) Shin, Y.; Brangwynne, C. P. Liquid Phase Condensation in Cell Physiology and Disease. *Science* **2017**, 357 (6357), eaaf4382. <https://doi.org/10.1126/science.aaf4382>.
- (27) Banjade, S.; Rosen, M. K. Phase Transitions of Multivalent Proteins Can Promote Clustering of Membrane Receptors. *eLife* **2014**, 3. <https://doi.org/10.7554/eLife.04123>.
- (28) Zhu, S.; Shen, Z.; Wu, X.; Han, W.; Jia, B.; Lu, W.; Zhang, M. Demixing Is a Default Process for Biological Condensates Formed via Phase Separation. *Science* **2024**, 384 (6698), 920–928. <https://doi.org/10.1126/science.adj7066>.
- (29) Perry, S. L. Phase Separation: Bridging Polymer Physics and Biology. *Spec. Top. Sect. Outst. Young Res. Colloid Interface Sci.* **2019**, 39, 86–97. <https://doi.org/10.1016/j.cocis.2019.01.007>.
- (30) Wang, Z. ErbB Receptors and Cancer. *Methods Mol. Biol. Clifton NJ* **2017**, 1652, 3–35. https://doi.org/10.1007/978-1-4939-7219-7_1.
- (31) Arkhipov, A.; Shan, Y.; Das, R.; Endres, N. F.; Eastwood, M. P.; Wemmer, D. E.; Kuriyan, J.; Shaw, D. E. Architecture and Membrane Interactions of the EGF Receptor. *Cell* **2013**, 152 (3), 557–569. <https://doi.org/10.1016/j.cell.2012.12.030>.
- (32) Zhang, X.; Gureasko, J.; Shen, K.; Cole, P. A.; Kuriyan, J. An Allosteric Mechanism for Activation of the Kinase Domain of Epidermal Growth Factor Receptor. *Cell* **2006**, 125 (6), 1137–1149. <https://doi.org/10.1016/j.cell.2006.05.013>.
- (33) Endres, N. F.; Engel, K.; Das, R.; Kovacs, E.; Kuriyan, J. Regulation of the Catalytic Activity of the EGF Receptor. *Proteins Catalysis Regul.* **2011**, 21 (6), 777–784. <https://doi.org/10.1016/j.sbi.2011.07.007>.
- (34) Sigismund, S.; Avanzato, D.; Lanzetti, L. Emerging Functions of the EGFR in Cancer. *Mol. Oncol.* **2018**, 12 (1), 3–20. <https://doi.org/10.1002/1878-0261.12155>.
- (35) da Cunha Santos, G.; Shepherd, F. A.; Tsao, M. S. EGFR Mutations and Lung Cancer. *Annual Review of Pathology: Mechanisms of Disease*, 2011, 6, 49–69. <https://doi.org/10.1146/annurev-pathol-011110-130206>.

- (36) Zandi, R.; Larsen, A. B.; Andersen, P.; Stockhausen, M.-T.; Poulsen, H. S. Mechanisms for Oncogenic Activation of the Epidermal Growth Factor Receptor. *Cell. Signal.* **2007**, *19* (10), 2013–2023. <https://doi.org/10.1016/j.cellsig.2007.06.023>.
- (37) Ono, M.; Kuwano, M. Molecular Mechanisms of Epidermal Growth Factor Receptor (EGFR) Activation and Response to Gefitinib and Other EGFR-Targeting Drugs. *Clin. Cancer Res.* **2006**, *12* (24), 7242–7251. <https://doi.org/10.1158/1078-0432.CCR-06-0646>.
- (38) Arora, A.; Scholar, E. M. Role of Tyrosine Kinase Inhibitors in Cancer Therapy. *J. Pharmacol. Exp. Ther.* **2005**, *315* (3), 971. <https://doi.org/10.1124/jpet.105.084145>.
- (39) Liu, Q.; Yu, S.; Zhao, W.; Qin, S.; Chu, Q.; Wu, K. EGFR-TKIs Resistance via EGFR-Independent Signaling Pathways. *Mol. Cancer* **2018**, *17* (1), 53. <https://doi.org/10.1186/s12943-018-0793-1>.
- (40) Uribe, M. L.; Marrocco, I.; Yarden, Y. EGFR in Cancer: Signaling Mechanisms, Drugs, and Acquired Resistance. *Cancers* **2021**, *13* (11). <https://doi.org/10.3390/cancers13112748>.
- (41) He, J.; Huang, Z.; Han, L.; Gong, Y.; Xie, C. Mechanisms and Management of 3rd-generation EGFR-TKI Resistance in Advanced Non-small Cell Lung Cancer (Review). *Int. J. Oncol.* **2021**, *59* (5), 90. <https://doi.org/10.3892/ijo.2021.5270>.
- (42) Tumbrink, H. L.; Heimsoeth, A.; Sos, M. L. The next Tier of EGFR Resistance Mutations in Lung Cancer. *Oncogene* **2021**, *40* (1), 1–11. <https://doi.org/10.1038/s41388-020-01510-w>.
- (43) Huang, Y.; Bharill, S.; Karandur, D.; Peterson, S. M.; Marita, M.; Shi, X.; Kaliszewski, M. J.; Smith, A. W.; Isacoff, E. Y.; Kuriyan, J. Molecular Basis for Multimerization in the Activation of the Epidermal Growth Factor Receptor. *eLife* **2016**, *5*, e14107. <https://doi.org/10.7554/eLife.14107>.
- (44) Gao, J.; Wang, Y.; Cai, M.; Pan, Y.; Xu, H.; Jiang, J.; Ji, H.; Wang, H. Mechanistic Insights into EGFR Membrane Clustering Revealed by Super-Resolution Imaging. *Nanoscale* **2015**, *7* (6), 2511–2519. <https://doi.org/10.1039/C4NR04962D>.
- (45) Kozer, N.; Barua, D.; Orchard, S.; Nice, E. C.; Burgess, A. W.; Hlavacek, W. S.; Clayton, A. H. A. Exploring Higher-Order EGFR Oligomerisation and Phosphorylation—a Combined Experimental and Theoretical Approach. *Mol. Biosyst.* **2013**, *9* (7), 1849–1863. <https://doi.org/10.1039/C3MB70073A>.
- (46) Hiroshima, M.; Pack, C.; Kaizu, K.; Takahashi, K.; Ueda, M.; Sako, Y. Transient Acceleration of Epidermal Growth Factor Receptor Dynamics Produces Higher-Order Signaling Clusters. *J. Mol. Biol.* **2018**, *430* (9), 1386–1401. <https://doi.org/10.1016/j.jmb.2018.02.018>.
- (47) Hofman, E. G.; Bader, A. N.; Voortman, J.; van den Heuvel, D. J.; Sigismund, S.; Verkley, A. J.; Gerritsen, H. C.; van Bergen en Henegouwen, P. M. P. Ligand-Induced

- EGF Receptor Oligomerization Is Kinase-Dependent and Enhances Internalization *. *J. Biol. Chem.* **2010**, 285 (50), 39481–39489. <https://doi.org/10.1074/jbc.M110.164731>.
- (48) Anderson, T. H.; Min, Y.; Weirich, K. L.; Zeng, H.; Fygenson, D.; Israelachvili, J. N. Formation of Supported Bilayers on Silica Substrates. *Langmuir* **2009**, 25 (12). <https://doi.org/10.1021/la900181c>.
- (49) Castellana, E. T.; Cremer, P. S. Solid Supported Lipid Bilayers: From Biophysical Studies to Sensor Design. *Surf. Sci. Rep.* **2006**, 61 (10). <https://doi.org/10.1016/j.surfrep.2006.06.001>.
- (50) Kiessling, V.; Domanska, M. K.; Murray, D.; Wan, C.; Tamm, L. K. Supported Lipid Bilayers. In *Wiley Encyclopedia of Chemical Biology*; 2008; pp 1–12. <https://doi.org/10.1002/9780470048672.webc663>.
- (51) Fish, K. N. Total Internal Reflection Fluorescence (TIRF) Microscopy. *Curr. Protoc. Cytom.* **2009**, 50 (1), 12.18.1–12.18.13. <https://doi.org/10.1002/0471142956.cy1218s50>.
- (52) Nye, J. A.; Groves, J. T. Kinetic Control of Histidine-Tagged Protein Surface Density on Supported Lipid Bilayers. *Langmuir* **2008**, 24 (8), 4145–4149. <https://doi.org/10.1021/la703788h>.
- (53) Lin, C.-C.; Melo, F. A.; Ghosh, R.; Suen, K. M.; Stagg, L. J.; Kirkpatrick, J.; Arold, S. T.; Ahmed, Z.; Ladbury, J. E. Inhibition of Basal FGF Receptor Signaling by Dimeric Grb2. *Cell* **2012**, 149 (7), 1514–1524. <https://doi.org/10.1016/j.cell.2012.04.033>.
- (54) Ahmed, Z.; Timsah, Z.; Suen, K. M.; Cook, N. P.; Lee, G. R.; Lin, C.-C.; Gagea, M.; Marti, A. A.; Ladbury, J. E. Grb2 Monomer–Dimer Equilibrium Determines Normal versus Oncogenic Function. *Nat. Commun.* **2015**, 6 (1), 7354. <https://doi.org/10.1038/ncomms8354>.
- (55) Boyman, O.; Kovar, M.; Rubinstein, M. P.; Surh, C. D.; Sprent, J. Selective Stimulation of T Cell Subsets with Antibody-Cytokine Immune Complexes. *Science* **2006**, 311 (5769), 1924–1927. <https://doi.org/10.1126/science.1122927>.
- (56) Rudolph, M. G.; Wilson, I. A. The Specificity of TCR/pMHC Interaction. *Curr. Opin. Immunol.* **2002**, 14 (1), 52–65. [https://doi.org/10.1016/S0952-7915\(01\)00298-9](https://doi.org/10.1016/S0952-7915(01)00298-9).
- (57) Stone, J. D.; Chervin, A. S.; Kranz, D. M. T-Cell Receptor Binding Affinities and Kinetics: Impact on T-Cell Activity and Specificity. *Immunology* **2009**, 126 (2), 165–176. <https://doi.org/10.1111/j.1365-2567.2008.03015.x>.
- (58) Wilhelm, K. B.; Shumpei Morita; Darren B. McAfee; Sungi Kim; Mark K. O’Dair; Jay T. Groves. Height, but Not Binding Epitope, Affects the Potency of Synthetic TCR Agonists. *Biophys. J.* **2021**, 120 (18), P3869–3880.
- (59) Jenny J. Lin; Geoff P. O’Donoghue; Kiera B. Wilhelm; Michael P. Coyle; Shalini T. Low-Nam; Nicole C. Fay; Katherine N. Alfieri; Jay T. Groves. Membrane Association Transforms an Inert Anti-TCR β Fab’ Ligand into a Potent T Cell Receptor Agonist. *Biophys. J.* **2020**, 118 (12), P2879–2893.

- (60) Wilhelm, K. B.; Vissa, A.; Groves, J. T. Differential Roles of Kinetic On- and Off-Rates in T-Cell Receptor Signal Integration Revealed with a Modified Fab'-DNA Ligand. *bioRxiv : the preprint server for biology*. United States April 2, 2024, p 2024.04.01.587588. <https://doi.org/10.1101/2024.04.01.587588>.
- (61) Chen, B.-M.; Al-Aghbar, M. A.; Lee, C.-H.; Chang, T.-C.; Su, Y.-C.; Li, Y.-C.; Chang, S.-E.; Chen, C.-C.; Chung, T.-H.; Liao, Y.-C.; Lee, C.-H.; Roffler, S. R. The Affinity of Elongated Membrane-Tethered Ligands Determines Potency of T Cell Receptor Triggering. *Front. Immunol.* **2017**, *8*.
- (62) Case, L. B.; Zhang, X.; Ditlev, J. A.; Rosen, M. K. Stoichiometry Controls Activity of Phase-Separated Clusters of Actin Signaling Proteins. *Science* **2019**, *363* (6431), 1093–1097. <https://doi.org/10.1126/science.aau6313>.
- (63) Beutel, O.; Maraschini, R.; Pombo-García, K.; Martin-Lemaitre, C.; Honigsmann, A. Phase Separation of Zonula Occludens Proteins Drives Formation of Tight Junctions. *Cell* **2019**, *179* (4), 923-936.e11. <https://doi.org/10.1016/j.cell.2019.10.011>.
- (64) Xie, Y.; Sun, J.; Han, X.; Turšić-Wunder, A.; Toh, J. D. W.; Hong, W.; Gao, Y.-G.; Miao, Y. Polarisome Scaffolder Spa2-Mediated Macromolecular Condensation of Aip5 for Actin Polymerization. *Nat. Commun.* **2019**, *10* (1), 5078. <https://doi.org/10.1038/s41467-019-13125-1>.
- (65) Avraham, R.; Yarden, Y. Feedback Regulation of EGFR Signalling: Decision Making by Early and Delayed Loops. *Nat. Rev. Mol. Cell Biol.* **2011**, *12* (2), 104–117. <https://doi.org/10.1038/nrm3048>.
- (66) Lemmon, M. A.; Schlessinger, J.; Ferguson, K. M. The EGFR Family: Not so Prototypical Receptor Tyrosine Kinases. *Cold Spring Harb. Perspect. Biol.* **2014**, *6* (4), a020768. <https://doi.org/10.1101/cshperspect.a020768>.
- (67) Kovacs, E.; Zorn, J. A.; Huang, Y.; Barros, T.; Kuriyan, J. A Structural Perspective on the Regulation of the Epidermal Growth Factor Receptor. *Annu. Rev. Biochem.* **2015**, *84*, 739–764. <https://doi.org/10.1146/annurev-biochem-060614-034402>.
- (68) Cantor, A. J.; Shah, N. H.; Kuriyan, J. Deep Mutational Analysis Reveals Functional Trade-Offs in the Sequences of EGFR Autophosphorylation Sites. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115* (31), E7303–E7312. <https://doi.org/10.1073/pnas.1803598115>.
- (69) Boykevisch, S.; Zhao, C.; Sondermann, H.; Philippidou, P.; Halegoua, S.; Kuriyan, J.; Bar-Sagi, D. Regulation of Ras Signaling Dynamics by Sos-Mediated Positive Feedback. *Curr. Biol.* **2006**, *16* (21), 2173–2179. <https://doi.org/10.1016/j.cub.2006.09.033>.
- (70) Chung, I.; Akita, R.; Vandlen, R.; Toomre, D.; Schlessinger, J.; Mellman, I. Spatial Control of EGF Receptor Activation by Reversible Dimerization on Living Cells. *Nature* **2010**, *464* (7289), 783–787. <https://doi.org/10.1038/nature08827>.
- (71) Nagy, P.; Claus, J.; Jovin, T. M.; Arndt-Jovin, D. J. Distribution of Resting and Ligand-Bound ErbB1 and ErbB2 Receptor Tyrosine Kinases in Living Cells Using Number and

- Brightness Analysis. *Proc. Natl. Acad. Sci.* **2010**, *107* (38), 16524–16529. <https://doi.org/10.1073/pnas.1002642107>.
- (72) Saffarian, S.; Li, Y.; Elson, E. L.; Pike, L. J. Oligomerization of the EGF Receptor Investigated by Live Cell Fluorescence Intensity Distribution Analysis. *Biophys. J.* **2007**, *93* (3), 1021–1031. <https://doi.org/10.1529/biophysj.107.105494>.
 - (73) Westover, D.; Zugazagoitia, J.; Cho, B. C.; Lovly, C. M.; Paz-Ares, L. Mechanisms of Acquired Resistance to First- and Second-Generation EGFR Tyrosine Kinase Inhibitors. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* **2018**, *29* (suppl_1), i10–i19. <https://doi.org/10.1093/annonc/mdx703>.
 - (74) Kai, F.; Drain, A. P.; Weaver, V. M. The Extracellular Matrix Modulates the Metastatic Journey. *Dev. Cell* **2019**, *49* (3), 332–346. <https://doi.org/10.1016/j.devcel.2019.03.026>.
 - (75) Thomas, R.; Weihua, Z. Rethink of EGFR in Cancer With Its Kinase Independent Function on Board. *Front. Oncol.* **2019**, *9*, 800. <https://doi.org/10.3389/fonc.2019.00800>.
 - (76) Zeng, L.; Palaia, I.; Šarić, A.; Su, X. PLC γ 1 Promotes Phase Separation of T Cell Signaling Components. *J. Cell Biol.* **2021**, *220* (6). <https://doi.org/10.1083/jcb.202009154>.
 - (77) Huang, W. Y. C.; Chiang, H.-K.; Groves, J. T. Dynamic Scaling Analysis of Molecular Motion within the LAT:Grb2:SOS Protein Network on Membranes. *Biophys. J.* **2017**, *113* (8), 1807–1813. <https://doi.org/10.1016/j.bpj.2017.08.024>.
 - (78) Sun, S.; GrandPre, T.; Limmer, D. T.; Groves, J. T. Kinetic Frustration by Limited Bond Availability Controls the LAT Protein Condensation Phase Transition on Membranes. *Sci. Adv.* **8** (44), eabo5295. <https://doi.org/10.1126/sciadv.abo5295>.
 - (79) Gale, N. W.; Kaplan, S.; Lowenstein, E. J.; Schlessinger, J.; Bar-Sagi, D. Grb2 Mediates the EGF-Dependent Activation of Guanine Nucleotide Exchange on Ras. *Nature* **1993**, *363* (6424), 88–92. <https://doi.org/10.1038/363088a0>.
 - (80) Sondermann, H.; Soisson, S. M.; Boykevich, S.; Yang, S.-S.; Bar-Sagi, D.; Kuriyan, J. Structural Analysis of Autoinhibition in the Ras Activator Son of Sevenless. *Cell* **2004**, *119* (3), 393–405. <https://doi.org/10.1016/j.cell.2004.10.005>.
 - (81) Lemmon, M. A.; Schlessinger, J. Cell Signaling by Receptor Tyrosine Kinases. *Cell* **2010**, *141* (7), 1117–1134. <https://doi.org/10.1016/j.cell.2010.06.011>.
 - (82) Bandaru, P.; Kondo, Y.; Kuriyan, J. The Interdependent Activation of Son-of-Sevenless and Ras. *Cold Spring Harb. Perspect. Med.* **2019**, *9* (2). <https://doi.org/10.1101/cshperspect.a031534>.
 - (83) Iversen, L.; Tu, H.-L.; Lin, W.-C.; Christensen, S. M.; Abel, S. M.; Iwig, J.; Wu, H.-J.; Gureasko, J.; Rhodes, C.; Petit, R. S.; Hansen, S. D.; Thill, P.; Yu, C.-H.; Stamou, D.; Chakraborty, A. K.; Kuriyan, J.; Groves, J. T. Molecular Kinetics. Ras Activation by SOS: Allosteric Regulation by Altered Fluctuation Dynamics. *Science* **2014**, *345* (6192), 50–54. <https://doi.org/10.1126/science.1250373>.

- (84) Christensen, S. M.; Tu, H.-L.; Jun, J. E.; Alvarez, S.; Triplet, M. G.; Iwig, J. S.; Yadav, K. K.; Bar-Sagi, D.; Roose, J. P.; Groves, J. T. One-Way Membrane Trafficking of SOS in Receptor-Triggered Ras Activation. *Nat. Struct. Mol. Biol.* **2016**, *23* (9), 838–846. <https://doi.org/10.1038/nsmb.3275>.
- (85) Lee, Y. K.; Low-Nam, S. T.; Chung, J. K.; Hansen, S. D.; Lam, H. Y. M.; Alvarez, S.; Groves, J. T. Mechanism of SOS PR-Domain Autoinhibition Revealed by Single-Molecule Assays on Native Protein from Lysate. *Nat. Commun.* **2017**, *8* (1), 15061. <https://doi.org/10.1038/ncomms15061>.
- (86) Chung, J. K.; Lee, Y. K.; Denson, J.-P.; Gillette, W. K.; Alvarez, S.; Stephen, A. G.; Groves, J. T. K-Ras4B Remains Monomeric on Membranes over a Wide Range of Surface Densities and Lipid Compositions. *Biophys. J.* **2018**, *114* (1), 137–145. <https://doi.org/10.1016/j.bpj.2017.10.042>.
- (87) Huang, W. Y. C.; Ditlev, J. A.; Chiang, H.-K.; Rosen, M. K.; Groves, J. T. Allosteric Modulation of Grb2 Recruitment to the Intrinsically Disordered Scaffold Protein, LAT, by Remote Site Phosphorylation. *J. Am. Chem. Soc.* **2017**, *139* (49), 18009–18015. <https://doi.org/10.1021/jacs.7b09387>.
- (88) Lin, W.-C.; Iversen, L.; Tu, H.-L.; Rhodes, C.; Christensen, S. M.; Iwig, J. S.; Hansen, S. D.; Huang, W. Y. C.; Groves, J. T. H-Ras Forms Dimers on Membrane Surfaces via a Protein–Protein Interface. *Proc. Natl. Acad. Sci.* **2014**, *111* (8), 2996–3001. <https://doi.org/10.1073/pnas.1321155111>.
- (89) Chung, J. K.; Lee, Y. K.; Lam, H. Y. M.; Groves, J. T. Covalent Ras Dimerization on Membrane Surfaces through Photosensitized Oxidation. *J. Am. Chem. Soc.* **2016**, *138* (6), 1800–1803. <https://doi.org/10.1021/jacs.5b12648>.
- (90) Christensen, S. M.; Triplet, M. G.; Rhodes, C.; Iwig, J. S.; Tu, H.-L.; Stamou, D.; Groves, J. T. Monitoring the Waiting Time Sequence of Single Ras GTPase Activation Events Using Liposome Functionalized Zero-Mode Waveguides. *Nano Lett.* **2016**, *16* (4), 2890–2895. <https://doi.org/10.1021/acs.nanolett.6b00969>.
- (91) Zhao, C.; Du, G.; Skowronek, K.; Frohman, M. A.; Bar-Sagi, D. Phospholipase D2-Generated Phosphatidic Acid Couples EGFR Stimulation to Ras Activation by Sos. *Nat. Cell Biol.* **2007**, *9* (6), 707–712. <https://doi.org/10.1038/ncb1594>.
- (92) Margarit, S. M.; Sondermann, H.; Hall, B. E.; Nagar, B.; Hoelz, A.; Pirruccello, M.; Bar-Sagi, D.; Kuriyan, J. Structural Evidence for Feedback Activation by Ras.GTP of the Ras-Specific Nucleotide Exchange Factor SOS. *Cell* **2003**, *112* (5), 685–695. [https://doi.org/10.1016/s0092-8674\(03\)00149-1](https://doi.org/10.1016/s0092-8674(03)00149-1).
- (93) Lee, A. A.; Huang, W. Y. C.; Hansen, S. D.; Kim, N. H.; Alvarez, S.; Groves, J. T. Stochasticity and Positive Feedback Enable Enzyme Kinetics at the Membrane to Sense Reaction Size. *Proc. Natl. Acad. Sci.* **2021**, *118* (47), e2103626118. <https://doi.org/10.1073/pnas.2103626118>.

- (94) Huang, W. Y. C.; Alvarez, S.; Kondo, Y.; Kuriyan, J.; Groves, J. T. Relating Cellular Signaling Timescales to Single-Molecule Kinetics: A First-Passage Time Analysis of Ras Activation by SOS. *Proc. Natl. Acad. Sci.* **2021**, *118* (45), e2103598118. <https://doi.org/10.1073/pnas.2103598118>.
- (95) Sykulev, Y.; Joo, M.; Vturina, I.; Tsomides, T. J.; Eisen, H. N. Evidence That a Single Peptide–MHC Complex on a Target Cell Can Elicit a Cytolytic T Cell Response. *Immunity* **1996**, *4* (6), 565–571. [https://doi.org/10.1016/S1074-7613\(00\)80483-5](https://doi.org/10.1016/S1074-7613(00)80483-5).
- (96) Irvine, D. J.; Purbhoo, M. A.; Krogsgaard, M.; Davis, M. M. Direct Observation of Ligand Recognition by T Cells. *Nature* **2002**, *419* (6909), 845–849. <https://doi.org/10.1038/nature01076>.
- (97) O'Donoghue, G. P.; Pielak, R. M.; Smoligovets, A. A.; Lin, J. J.; Groves, J. T. Direct Single Molecule Measurement of TCR Triggering by Agonist pMHC in Living Primary T Cells. *eLife* **2013**, *2*, e00778. <https://doi.org/10.7554/eLife.00778>.
- (98) Lin, J. J. Y.; Low-Nam, S. T.; Alfieri, K. N.; McAfee, D. B.; Fay, N. C.; Groves, J. T. Mapping the Stochastic Sequence of Individual Ligand-Receptor Binding Events to Cellular Activation: T Cells Act on the Rare Events. *Sci. Signal.* **2019**, *12* (564). <https://doi.org/10.1126/scisignal.aat8715>.
- (99) Zhang, F.; Wang, S.; Yin, L.; Yang, Y.; Guan, Y.; Wang, W.; Xu, H.; Tao, N. Quantification of Epidermal Growth Factor Receptor Expression Level and Binding Kinetics on Cell Surfaces by Surface Plasmon Resonance Imaging. *Anal. Chem.* **2015**, *87* (19), 9960–9965. <https://doi.org/10.1021/acs.analchem.5b02572>.
- (100) Endres, N. F.; Das, R.; Smith, A. W.; Arkhipov, A.; Kovacs, E.; Huang, Y.; Pelton, J. G.; Shan, Y.; Shaw, D. E.; Wemmer, D. E.; Groves, J. T.; Kuriyan, J. Conformational Coupling across the Plasma Membrane in Activation of the EGF Receptor. *Cell* **2013**, *152* (3), 543–556. <https://doi.org/10.1016/j.cell.2012.12.032>.
- (101) Shi, T.; Niepel, M.; McDermott, J. E.; Gao, Y.; Nicora, C. D.; Chrisler, W. B.; Markillie, L. M.; Petyuk, V. A.; Smith, R. D.; Rodland, K. D.; Sorger, P. K.; Qian, W.-J.; Wiley, H. S. Conservation of Protein Abundance Patterns Reveals the Regulatory Architecture of the EGFR-MAPK Pathway. *Sci. Signal.* **2016**, *9* (436), rs6–rs6. <https://doi.org/10.1126/scisignal.aaf0891>.
- (102) Parthasarathy, R.; Yu, C.; Groves, J. T. Curvature-Modulated Phase Separation in Lipid Bilayer Membranes. *Langmuir* **2006**, *22* (11), 5095–5099. <https://doi.org/10.1021/la060390o>.
- (103) Kaizuka, Y.; Groves, J. T. Bending-Mediated Superstructural Organizations in Phase-Separated Lipid Membranes. *New J. Phys.* **2010**, *12* (9), 095001. <https://doi.org/10.1088/1367-2630/12/9/095001>.
- (104) Chung, J. K.; Huang, W. Y. C.; Carbone, C. B.; Nocka, L. M.; Parikh, A. N.; Vale, R. D.; Groves, J. T. Coupled Membrane Lipid Miscibility and Phosphotyrosine-Driven Protein

- Condensation Phase Transitions. *Biophys. J.* **2021**, *120* (7), 1257–1265. <https://doi.org/10.1016/j.bpj.2020.09.017>.
- (105) Ayati, A.; Moghimi, S.; Salarinejad, S.; Safavi, M.; Pouramiri, B.; Foroumadi, A. A Review on Progression of Epidermal Growth Factor Receptor (EGFR) Inhibitors as an Efficient Approach in Cancer Targeted Therapy. *Bioorganic Chem.* **2020**, *99*, 103811. <https://doi.org/10.1016/j.bioorg.2020.103811>.
- (106) Gureasko, J.; Galush, W. J.; Boykevisch, S.; Sondermann, H.; Bar-Sagi, D.; Groves, J. T.; Kuriyan, J. Membrane-Dependent Signal Integration by the Ras Activator Son of Sevenless. *Nat. Struct. Mol. Biol.* **2008**, *15* (5), 452–461. <https://doi.org/10.1038/nsmb.1418>.
- (107) Tinevez, J.-Y.; Perry, N.; Schindelin, J.; Hoopes, G. M.; Reynolds, G. D.; Laplantine, E.; Bednarek, S. Y.; Shorte, S. L.; Eliceiri, K. W. TrackMate: An Open and Extensible Platform for Single-Particle Tracking. *Image Process. Biol.* **2017**, *115*, 80–90. <https://doi.org/10.1016/j.ymeth.2016.09.016>.
- (108) Herbst, R. S. Review of Epidermal Growth Factor Receptor Biology. *Int. J. Radiat. Oncol.* **2004**, *59* (2, Supplement), S21–S26. <https://doi.org/10.1016/j.ijrobp.2003.11.041>.
- (109) Wilson, K. J.; Gilmore, J. L.; Foley, J.; Lemmon, M. A.; Riese, D. J. 2nd. Functional Selectivity of EGF Family Peptide Growth Factors: Implications for Cancer. *Pharmacol. Ther.* **2009**, *122* (1), 1–8. <https://doi.org/10.1016/j.pharmthera.2008.11.008>.
- (110) Ferguson, K. M. Structure-Based View of Epidermal Growth Factor Receptor Regulation. *Annu. Rev. Biophys.* **2008**, *37*, 353–373. <https://doi.org/10.1146/annurev.biophys.37.032807.125829>.
- (111) Kozer, N.; Barua, D.; Henderson, C.; Nice, E. C.; Burgess, A. W.; Hlavacek, W. S.; Clayton, A. H. A. Recruitment of the Adaptor Protein Grb2 to EGFR Tetramers. *Biochemistry* **2014**, *53* (16), 2594–2604. <https://doi.org/10.1021/bi500182x>.
- (112) Sandouk, A.; Xu, Z.; Baruah, S.; Tremblay, M.; Hopkins, J. B.; Chakravarthy, S.; Gakhar, L.; Schnicker, N. J.; Houtman, J. C. D. GRB2 Dimerization Mediated by SH2 Domain-Swapping Is Critical for T Cell Signaling and Cytokine Production. *Sci. Rep.* **2023**, *13* (1), 3505. <https://doi.org/10.1038/s41598-023-30562-7>.
- (113) Lowenstein, E. J.; Daly, R. J.; Batzer, A. G.; Li, W.; Margolis, B.; Lammers, R.; Ullrich, A.; Skolnik, E. Y.; Bar-Sagi, D.; Schlessinger, J. The SH2 and SH3 Domain-Containing Protein GRB2 Links Receptor Tyrosine Kinases to Ras Signaling. *Cell* **1992**, *70* (3), 431–442.
- (114) Rozakis-Adcock, M.; Fernley, R.; Wade, J.; Pawson, T.; Bowtell, D. The SH2 and SH3 Domains of Mammalian Grb2 Couple the EGF Receptor to the Ras Activator mSos1. *Nature* **1993**, *363* (6424), 83–85. <https://doi.org/10.1038/363083a0>.
- (115) Watanabe, T.; Shinohara, N.; Moriya, K.; Sazawa, A.; Kobayashi, Y.; Ogiso, Y.; Takiguchi, M.; Yasuda, J.; Koyanagi, T.; Kuzumaki, N.; Hashimoto, A. Significance of

- the Grb2 and Son of Sevenless (Sos) Proteins in Human Bladder Cancer Cell Lines. *IUBMB Life* **2000**, 49 (4), 317–320. <https://doi.org/10.1080/15216540050033195>.
- (116) Giubellino, A.; Burke, T. R.; Bottaro, D. P. Grb2 Signaling in Cell Motility and Cancer. *Expert Opin. Ther. Targets* **2008**, 12 (8), 1021–1033. <https://doi.org/10.1517/14728222.12.8.1021>.
- (117) Lim, S.-J.; Lopez-Berestein, G.; Hung, M.-C.; Lupu, R.; Tari, A. M. Grb2 Downregulation Leads to Akt Inactivation in Heregulin-Stimulated and ErbB2-Overexpressing Breast Cancer Cells. *Oncogene* **2000**, 19 (54), 6271–6276. <https://doi.org/10.1038/sj.onc.1204014>.
- (118) Iwata, T.; Sedukhina, A. S.; Kubota, M.; Oonuma, S.; Maeda, I.; Yoshiike, M.; Usuba, W.; Minagawa, K.; Hames, E.; Meguro, R.; Cho, S.; Chien, S. H. H.; Urabe, S.; Pae, S.; Palanisamy, K.; Kumai, T.; Yudo, K.; Kikuchi, E.; Sato, K. A New Bioinformatics Approach Identifies Overexpression of GRB2 as a Poor Prognostic Biomarker for Prostate Cancer. *Sci. Rep.* **2021**, 11 (1), 5696. <https://doi.org/10.1038/s41598-021-85086-9>.
- (119) Yu, G. Z.; Chen, Y.; Wang, J. J. Overexpression of Grb2/HER2 Signaling in Chinese Gastric Cancer: Their Relationship with Clinicopathological Parameters and Prognostic Significance. *J. Cancer Res. Clin. Oncol.* **2009**, 135 (10), 1331–1339. <https://doi.org/10.1007/s00432-009-0574-8>.
- (120) Gril, B.; Vidal, M.; Assayag, F.; Poupon, M.-F.; Liu, W.-Q.; Garbay, C. Grb2-SH3 Ligand Inhibits the Growth of HER2+ Cancer Cells and Has Antitumor Effects in Human Cancer Xenografts Alone and in Combination with Docetaxel. *Int. J. Cancer* **2007**, 121 (2), 407–415. <https://doi.org/10.1002/ijc.22674>.
- (121) Thomas, X.; Paubelle, E. Grb2 Inhibition: A New Potential Targeted Therapy for Myeloid Malignancies? *Lancet Haematol.* **2018**, 5 (4), e128–e129. [https://doi.org/10.1016/S2352-3026\(18\)30031-0](https://doi.org/10.1016/S2352-3026(18)30031-0).
- (122) Rochussen, A. M.; Lippert, A. H.; Griffiths, G. M. Imaging the T-Cell Receptor: New Approaches, New Insights. *Curr. Opin. Immunol.* **2023**, 82, 102309. <https://doi.org/10.1016/j.coi.2023.102309>.
- (123) Herman, A.; Kappler, J. W.; Marrack, P.; Pullen, A. M. Superantigens: Mechanism of T-Cell Stimulation and Role in Immune Responses. *Annual Review of Immunology*, 1991, 9, 745–772. <https://doi.org/10.1146/annurev.iy.09.040191.003525>.
- (124) Boniface, J. J.; Rabinowitz, J. D.; Wülfing, C.; Hampl, J.; Reich, Z.; Altman, J. D.; Kantor, R. M.; Beeson, C.; McConnell, H. M.; Davis, M. M. Initiation of Signal Transduction through the T Cell Receptor Requires the Multivalent Engagement of Peptide/MHC Ligands. *Immunity* **1998**, 9 (4), 459–466. [https://doi.org/10.1016/S1074-7613\(00\)80629-9](https://doi.org/10.1016/S1074-7613(00)80629-9).
- (125) Norman, D. J. Mechanisms of Action and Overview of OKT3. *Ther. Drug Monit.* **1995**, 17 (6), 615–620. <https://doi.org/10.1097/00007691-199512000-00012>.

- (126) Sanders, J. M.; Wampole, M. E.; Thakur, M. L.; Wickstrom, E. Molecular Determinants of Epidermal Growth Factor Binding: A Molecular Dynamics Study. *PloS One* **2013**, *8* (1), e54136. <https://doi.org/10.1371/journal.pone.0054136>.
- (127) Trenker, R.; Jura, N. Receptor Tyrosine Kinase Activation: From the Ligand Perspective. *Cell Signal. 2020* **2020**, *63*, 174–185. <https://doi.org/10.1016/j.ceb.2020.01.016>.
- (128) Singh, B.; Carpenter, G.; Coffey, R. J. EGF Receptor Ligands: Recent Advances. *F1000Research* **2016**, *5*, F1000 Faculty Rev-2270. <https://doi.org/10.12688/f1000research.9025.1>.
- (129) Karl, K.; Paul, M. D.; Pasquale, E. B.; Hristova, K. Ligand Bias in Receptor Tyrosine Kinase Signaling. *J. Biol. Chem.* **2020**, *295* (52), 18494–18507. <https://doi.org/10.1074/jbc.REV120.015190>.
- (130) Hubbard, S. R.; Miller, W. T. Receptor Tyrosine Kinases: Mechanisms of Activation and Signaling. *Cell Regul.* **2007**, *19* (2), 117–123. <https://doi.org/10.1016/j.ceb.2007.02.010>.
- (131) Huang, Y.; Ognjenovic, J.; Karandur, D.; Miller, K.; Merk, A.; Subramaniam, S.; Kuriyan, J. A Molecular Mechanism for the Generation of Ligand-Dependent Differential Outputs by the Epidermal Growth Factor Receptor. *eLife* **2021**, *10*, e73218. <https://doi.org/10.7554/eLife.73218>.
- (132) Freed, D. M.; Bessman, N. J.; Kiyatkin, A.; Salazar-Cavazos, E.; Byrne, P. O.; Moore, J. O.; Valley, C. C.; Ferguson, K. M.; Leahy, D. J.; Lidke, D. S.; Lemmon, M. A. EGFR Ligands Differentially Stabilize Receptor Dimers to Specify Signaling Kinetics. *Cell* **2017**, *171* (3), 683–695.e18. <https://doi.org/10.1016/j.cell.2017.09.017>.
- (133) Lemmon, M. A.; Freed, D. M.; Schlessinger, J.; Kiyatkin, A. The Dark Side of Cell Signaling: Positive Roles for Negative Regulators. *Cell* **2016**, *164* (6), 1172–1184. <https://doi.org/10.1016/j.cell.2016.02.047>.
- (134) Swain, P. S.; Siggia, E. D. The Role of Proofreading in Signal Transduction Specificity. *Biophys. J.* **2002**, *82* (6), 2928–2933. [https://doi.org/10.1016/S0006-3495\(02\)75633-6](https://doi.org/10.1016/S0006-3495(02)75633-6).
- (135) Yoshida, T.; Okamoto, I.; Okabe, T.; Iwasa, T.; Satoh, T.; Nishio, K.; Fukuoka, M.; Nakagawa, K. Matuzumab and Cetuximab Activate the Epidermal Growth Factor Receptor but Fail to Trigger Downstream Signaling by Akt or Erk. *Int. J. Cancer* **2008**, *122* (7), 1530–1538. <https://doi.org/10.1002/ijc.23253>.
- (136) Guo, L.; Kozlosky, C. J.; Ericsson, L. H.; Daniel, T. O.; Cerretti, D. P.; Johnson, R. S. Studies of Ligand-Induced Site-Specific Phosphorylation of Epidermal Growth Factor Receptor. *J. Am. Soc. Mass Spectrom.* **2003**, *14* (9), 1022–1031. [https://doi.org/10.1016/S1044-0305\(03\)00206-X](https://doi.org/10.1016/S1044-0305(03)00206-X).
- (137) Weeks, R.; Zhou, X.; Yuan, T. L.; Zhang, J. Fluorescent Biosensor for Measuring Ras Activity in Living Cells. *J. Am. Chem. Soc.* **2022**, *144* (38), 17432–17440. <https://doi.org/10.1021/jacs.2c05203>.

- (138) Live-Cell Microscopy Reveals Small Molecule Inhibitor Effects on MAPK Pathway Dynamics. *PLoS ONE* **2011**, 6 (8). <https://doi.org/10.1371/journal.pone.0022607>.
- (139) Burack, W. R.; Shaw, A. S. Live Cell Imaging of ERK and MEK: SIMPLE BINDING EQUILIBRIUM EXPLAINS THE REGULATED NUCLEOCYTOPLASMIC DISTRIBUTION OF ERK*. *J. Biol. Chem.* **2005**, 280 (5), 3832–3837. <https://doi.org/10.1074/jbc.M410031200>.
- (140) Rafiq, S.; Hackett, C. S.; Brentjens, R. J. Engineering Strategies to Overcome the Current Roadblocks in CAR T Cell Therapy. *Nat. Rev. Clin. Oncol.* **2020**, 17 (3), 147–167. <https://doi.org/10.1038/s41571-019-0297-y>.