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Ras-ERK signaling Dynamics during B cell activation

by

Justin Donte McLaurin

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Molecular Biology

in the

GRADUATE DIVISION

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by

Justin D. McLaurin

For Ms. Moore.

Thank you.

Acknowledgements

As I've drafted this thesis and reflected on these past six years at UCSF, on who I was as I began the projects described here, and who I am now, I am surprised to find that I've grown more optimistic as these years have gone by. This is unusual, as my time here has been mostly challenging and just occasionally rewarding. Nevertheless, graduate school has shown me that even the most intractable problems have solutions should the right amount of time be invested, the right perturbations made, and outputs measured, and the right sacrifices be made to the correct suite of (scientific) gods. For that, I have many people to acknowledge:

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Statement Regarding Author Contributions

All procedures and experiments described in this dissertation were conceived by Justin Mclaurin and Orion Weiner. All experiments were performed by Justin Mclaurin. All panels, figures, tables and graphs were generated by Justin Mclaurin. Chapter two is a draft of manuscript in preparation for submission for publication. Doug Tischer cloned the GRB-BFP construct used in chapter two.

Live cell analysis of Ras-ERK signaling during B Cell Activation

Justin Donte McLaurin

In mammalian cells, the Ras-ERK module is deployed to couple external stimuli to cell growth, differentiation and division. Feedback circuits impinging on this signaling module sculpt the amplitude, duration and frequency response dynamics of the terminal kinase in the cascade, ERK. In B cells, Ras activation is proposed to be digital or switch-like, and this switch-like activation is thought to propagate downstream to ERK and other effectors. Here we use live imaging of endogenous Ras and ERK activity to investigate the input-output relationship between these nodes in the context of B cell activation. We find that Ras activation is switch-like ($n_H = 3$) and requires less receptor stimuli to maintain to initiate, consistent with hysteresis in the dose response curve. Surprisingly, we found that a specific threshold level of Ras activity corresponded to ERK activation at the single cell level, and that digital ERK responses persisted in the absence of positive feedback-mediated Ras activation. Collectively, these findings suggest that multiple sources of signal amplification exist within the Ras-ERK module of the BCR pathway.

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CHAPTER ONE

INTRODUCTION

“It’s a confocal [microscope]; you plug it in and press go”
- Ron Vale

Intracellular signaling networks allow cells to respond to the world around them. However, a paradox exists in that the range (from heat-stress, to nucleic acids; from small molecules to growth factors) of external cues capable of eliciting a distinct response vastly outnumbers the signaling molecules known to mediate said responses. While tissue-specific expression of signaling proteins accounts for some cell-type specific responses, many signaling pathways are widely expressed, and therefore repurposed to suit the context of their expression.

The MAPK cascade is one such pathway and has been implicated in a diverse set of cellular decisions across eukaryotes, ranging from the yeast mating response to axon-choice in neural precursors. How does one pathway drive such differing responses? One reason is due to the unique biochemical properties of the three-tiered MapK cascade (Figure 1A). In mammalian cells, Raf (or MAPKKK) is activated by GTP-loaded Ras on the inner-leaflet of the plasma membrane and proceeds to phosphorylate MEK (or MAPKK), leading to its activation. Active MEK molecules phosphorylate ERK (or MAPK), thereby triggering its translocation to the nucleus and allowing for the phosphorylation of dozens of downstream effectors (Carlson et al., 2011). However, the MAPK cascade is not simply linear (Figure 1B). Instead each kinase can phosphorylate its activating kinase, leading to its inhibition--MEK phosphorylates Raf, for example, leading to a reduction in Raf kinase activity (Lake et al., 2016; Kochańczyk et al., 2017). Moreover, scaffold proteins, phosphatases and other molecules can fine tune the activity and activation of each of these kinases, leading to a wide array of signaling dynamics (Kholodenko and Birtwistle, 2009). Negative feedback within the MAPK

cascade, for example, drives oscillations in ERK kinase activity and nuclear localization in endothelial cells (Shankaran et al., 2009; Cirit et al., 2010). In *Xenopus* Oocytes, positive feedback between p42 MAPK and Mos (the *Xenopus* Raf homolog) mediates the switch-like p42 MAPK activation known to control Oocyte maturation (Petty et al., 1998). Thus, feedback interactions between core MAPK components and other signaling proteins can diversify MAPK pathway output.

While feedback between and impinging on the core MAPK machinery is important for sculpting ultimate pathway output, Ras activation also plays an key role in determining MAPK signaling dynamics. Ras activity is binary on a per-molecule basis (GDP-loaded Ras can be considered “OFF,” while GTP-loaded Ras can be considered “ON”).

However, complex regulation of Ras activators (Guanine nucleotide exchange factors or GEFs) and inactivators (GTPase activating proteins or GAPs) can specify a wide array of Ras activity profiles (Boykevisch et al., 2006; Bos et al., 2007 Iwig et al., 2013; Iversen et al., 2014; Vercoulen et al., 2017). Cell-type specific expression of GEFs and GAPs, then, potentially plays a key role in determining the amplitude, duration and spatial distribution of active Ras.

How are Ras and ERK activity coupled? Given the complex web of feedbacks coalescing on these two signaling nodes, it is difficult to define a prevailing regulatory logic to this otherwise linear signaling pathway. Rather, the answer to this question is dependent on the suite of co-expressed Ras and MAPK regulators and effectors. In

short, context matters. However, some general principles may be gleaned by considering studies on Ras-ERK signaling from otherwise disparate pieces of biology.

Computational modeling of Ras-ERK signaling has provided a framework for understanding how subtle changes in activity or expression of pathway components can have profound effects of pathway output and dynamics. Simulations of this module from a pair of disparate biological systems (namely, the yeast mating response pathway and the T Cell receptor pathway) have contributed to my thinking in this regard.

During yeast mating, MapK components are corralled by the scaffold protein Ste5, allowing for sequential phosphorylation of pathway components (Choi et al., 1994; Bhattacharyya et al., 2006). There, phosphorylation of the terminal kinase, Fus3 (the yeast MAPK homolog) is graded, however yeast mating response (or ‘schmooing’) is highly switch-like (Conlon et al., 2016; Malleshaiah et al., 2010). While it was formally possible that the biochemical switch mediating switch-like mating response occurred downstream of Fus3 activation, computational models of the core MAPK components resolved this mystery by predicting that switch-like disassociation from Ste5 occurs via a zero-order ultrasensitivity mechanism (Malleshaiah et al., 2010).

In T cells, ERK phosphorylation is bimodal, meaning that cells either phosphorylate all (or most) of the ERK expressed in a cell following TCR triggering, or none of it. This pattern of phosphorylation implies the existence of nonlinear signaling underlying ERK activation in T cells. While positive feedback (i.e. in *Xenopus* Oocyte maturation) had

been previously established as a mechanism capable of generating this sort of response, the unusual means in which Ras is activated downstream of the TCR suggested that this scenario may be distinct from *Xenopus* (Petty et al., 1998). There, a combination of GEFs, RasGRP and SOS play important, but distinct roles in initiating TCR-triggered Ras responses. RasGRP plays the role of initiator, and in its absence, RasGTP production is abolished; SOS, on the other hand, plays the role of amplifier, and while not strictly required for Ras activation, is capable of enhancing RasGRP generated RasGTP levels (Roose et al., 2007). Modeling this minimal RasGTP producing circuit predicted that feedback at the level of Ras activation was sufficient to explain the bimodal ERK responses previously observed in antigen exposed lymphocytes (Das et al., 2009). Moreover, this theoretical study predicted that this circuit may produce bistable RasGTP responses—a condition in which RasGTP levels is either stably “on” or “off” at the single cell level. Ensemble cell-based assays broadly support the findings outlined in this model.

While many other theoretical studies of MapK signaling exist (in the context of PC12 cell differentiation and proliferation, *Xenopus* Oocyte maturation, and in *Drosophila* tracheal placode invagination, to name a few), these two referenced above highlight that there are myriad mechanisms capable of generating non-linear MAPK signaling responses. It is therefore important to choose experimental systems carefully so as to explicitly test the predications of these models.

Studies in cancer cell lines expressing constitutively active Ras and BRAF alleles provide a test case, in a sense, for the predications of these theoretical studies. Ras proteins are mutated in ~16-30% of human cancers, and yet ERK components is not always constitutively active as a result of activating Ras mutations (Prior et al., 2012). How might this be so? A linear pathway would no doubt propagate signal emanating from active Ras to downstream MAPK machinery, and so feedback within the MAPK module must monitor flux through the pathway. Indeed, in some tumor-derived cell lines, dual specificity phosphatases (DUSPs) are upregulated (via ERK signaling), thereby adjusting ERK activity in the face of activating Ras or BRAF signals (Haigis et al., 2008; Vartanian et al., 2013).

A seminal human study in BRAFV600E (+) melanoma patients treated with the BRAF inhibitor, vemurafenib, detailed a period of rapid post-treatment tumor recession, followed by tumor regrowth at the same positions (Wagle et al. 2011). While this study highlighted these patient's tumor's ability to acquire resistance mutations, it also shows how subtle re-wiring of the MapK pathway can drive diverse signaling and physiological outputs—tumorigenesis in this case. For example, subsequent studies showed that while these tumors appeared resistant to vemurafenib, they were actually addicted to the BRAF inhibition provided by this drug (Thakur et al., 2013). This suggests that the input-output response relationship between MAPK pathway may be fundamentally altered in the context of BRAFV600E expression, and vemurafenib inhibition (Bugaj et al., 2016).

Lastly, previous work from my thesis lab and others, using inducible systems for controlling MAPK signaling have played a key role in deconvolving the context-specific logic of the Ras-ERK pathway (Toettcher et al., 2013; Wilson et al., 2017). Using a light controlled SOS expressed in 3T3 fibroblasts, our group showed that relatively little signal amplification occurs between Ras activation and ERK nuclear localization in the absence of parallel pathways (Toettcher et al., 2013). Moreover, this work showed that the pathway was essentially ‘memoryless’ in this context, that is that previous stimulus had no discernable effect on future stimulus. Thus, a cell which was maximally stimulated with this optogenetic system responded the same to a second round of stimulation, indicating the absence of negative feedback. This is in contrast to other studies using inducible RAF and MEK methods that detail negative feedback between ERK and upstream MAPK pathway components that constrains ERK activity (Chatelle et al., 2016; Kamioka et al., 2010; Zhou et al., 2017). These and other studies highlight the importance of making careful measurements of RAS-MAPK input-output relationships, due to the diversity of network architectures setting the impulse-response functions of this signaling module.

This thesis is comprised of efforts to understand the signaling dynamics occurring within the Ras-ERK signaling module during B cell activation. B cells, like other cells of the lymphocyte lineage (including NK cells and T cells) are notable for their “all-or-none” activation following antigen encounter. B cell activation is characterized by sequences of proliferation, cytokine and chemokine secretion, upregulation of cell surface markers, and cell growth and differentiation. While antigen encounter alone is insufficient to

induce all of the aforementioned events, it can play an important permissive role (Xu and Bancherau., 2014; Yam-Puc et al., 2018). Downstream of the antigen receptor lies a complex web of signaling nodes, and the Ras-ERK pathway is just one of module in the network. However, loss-of-function studies in rodent models and cell lines have painted a clear picture of the importance of this module in B cell activation. For example, RasGRP1/3 double-deficient mice show isotype-specific (IgG1 and IgG2a) reductions in BCR-driven antibody responses (Coughlin et al., 2005). RasGRP1/3 deficiency in DT40 (chicken) B cells dramatically reduces BCR-driven ERK phosphorylation (Oh-hora et al., 2003). In mouse, ERK activity plays a key role in inducing Blimp-1 expression and subsequent plasma cell differentiation (Yasuda et al., 2011). These and other studies position the Ras-ERK module as a key regulator of humoral immunity and raise the possibility that signal flux through this pathway could serve as a rheostat on intensity of B cell immune responses.

My work here is principally focused on using live cell imaging to study and control signaling in context of B cell activation. In the second chapter, I employ pairwise imaging of Ras and ERK activity in Ramos B cells to investigate the source(s) of the digital signal amplification which underpins the bimodal ERK responses characteristic of active lymphocytes. There, I uncover a threshold change above basal Ras activity required to initiate bimodal ERK responses and provide evidence for an analog-to-digital converter downstream of Ras. In the third, I use an optogenetic system capable of inducible, reversible and titratable control of Ras activity (Opto-SOS) to study B cell cytokine responses mediated by this pathway and provide perspective on follow up

studies assessing the validity of hits from this screen. In the final chapter (chapter four) I detail efforts to develop a cryptochrome-based optogenetic system for controlling either B or T-cell responses via inducible localization of Syk-family kinases (Opto-ZAP) to the plasma membrane. I detail challenges and success with using this system to induce proximal and distal signaling responses in Jurkat T cells. I conclude this section with a perspective on future studies.

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FIGURE 1.1

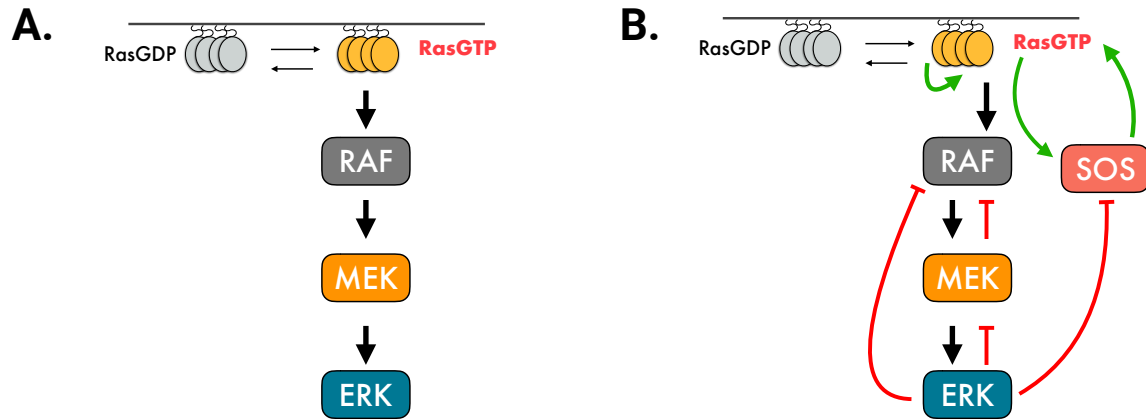


Figure 1.1. A paired down view on ERK activation in mammalian cells. (A)

Schematic depicting linear signal relay between active Ras (RasGTP), Raf, MEK and ERK. (B) A non-exhaustive schematic of feedback between individual components apart of the core MAPK machinery. These feedbacks likely occur in all cells where these components are co-expressed due to the conservation of residues mediating these feedbacks.

CHAPTER TWO

LIVE CELL ANALYSIS OF RAS-ERK SIGNALING

“...just a little careful input-out... as one does”
- Oliver Hoeller

SUMMARY

The Ras-MAP kinase (MAPK) cascade underlies functional cellular decisions in a variety of cell types and organisms. In B cells, positive feedback-driven Ras activation is a proposed source of the digital (all-or-none) MAPK responses characteristic of antigen exposed cells. However, an inability to measure endogenous Ras activity in living cells has hampered our ability to test this model directly. Here we leverage biosensors of endogenous Ras and ERK activity to quantify BCR-driven MAPK pathway dynamics with high temporal precision. We find that BCR ligation drives switch-like Ras activation responses and show that lower BCR signaling output is required to maintain Ras signaling than to initiate it in the first place. Interestingly, we found that a specific threshold level of Ras activity corresponded to ERK activation at the single cell level, and that digital ERK responses persisted in the absence of positive feedback-mediated Ras activation. Collectively, these findings suggest that multiple sources of signal amplification exist within the Ras-ERK module of the BCR pathway.

INTRODUCTION

Digital or switch-like biochemical responses enable cells to convert gradual changes in external stimuli into binary cellular decisions such as cell cycle entry, differentiation and programmed cell death (Spencer and Sorger, 2011; Tay et al., 2010; Huang et al., 2013). Positive feedback-driven protein activation is a common mechanism used to generate digital signaling responses. Classic studies in *Xenopus* oocytes, for example, described how positive feedback within the MAPK cascade results in digital activation of the terminal kinase, p42 MAPK (Ferrell Jr. and Machleder, 1998). Subsequent studies have implicated digital MAPK responses in coordinating processes ranging from yeast mating responses to *Drosophila* tracheal placode invagination (Malleshaiah et al., 2010; Ogura et al., 2018). While the specific molecular details may vary across these organismal contexts, the result is the same: each makes use of positive feedback impinging on the MAPK machinery to drive switch-like activation of the terminal kinase in the cascade, known as ERK in mammalian cells.

Cells of the B cell lineage present a unique scenario in that feedback at the level of Ras activation (rather within the MAPK cascade) is thought to underpin digital ERK responses (Das et al., 2009). Here, the interplay of two Ras GEFs, RasGRP and SOS form the basis of a positive feedback loop thought to generate switch-like activation of Ras. Following antigen receptor triggering, RasGRP generates a small amount of initiating RasGTP, which is then amplified via RasGTP-driven activation of SOS (Figure 1A) (Margarit et al., 2003; Boykevisch et al., 2006). Computational models of this minimal Ras activating circuit posit a sharp transition (from off-to-on) in the Ras

activation dose response curve, characteristic of switch-like activation (Das et al., 2009; Iversen et al., 2014; Jun et al., 2013). A potential result of this switch-like activation is a phenomenon known as hysteresis, a type of molecular memory, in which Ras activity persists in the absence of continuous antigen receptor engagement. However, Ras activation dynamics in these cells has never been directly observed.

Here we take a live imaging approach to analyze Ras-ERK signaling in individual living Ramos B cells. We find that BCR engagement drives switch-like RasGTP responses at the single cell level, giving rise to bimodal Ras activation at the population level. We show that less receptor-based stimulation is required for the maintenance than for the initiation of a Ras response, providing evidence for hysteresis in the BCR-RasGTP dose response curve. Surprisingly we found that ERK responses remain binary even in the absence of positive feedback-driven Ras activation. This work supports a model in which active Ras permits but does not instruct digital ERK response and suggests the existence of multiple sources of signal amplification within the B cell Ras/MapK pathway.

RESULTS

Visualizing Ras activity during B cell activation

Several groups have leveraged the high-affinity ($\sim 20\text{nM}$) interaction between the Raf-1 Ras binding domain (Raf1⁵¹⁻¹³¹, hence forward referred to as RBD) and RasGTP to generate FRET and membrane translocation-based reporters to quantify Ras activity in living cells (Chiu et al., 2002; Mochizuki et al., 2001; Oliveira and Yasuda, 2013).

However, these approaches often lack the sensitivity to detect endogenous Ras and require overexpression of Ras proteins to produce detectable signal, potentially altering Ras regulation. In other approaches, endogenous RasGTP has been detected in live cells using a reporter in which RBD is multimerized (to increase avidity) and mutagenized (to decrease affinity), but this approach has the potential difficulty of responding to Ras density or sequestering endogenous Ras due to the high avidity of the multimeric reporter (Augsten et al., 2006; Rubio et al. 2010). To circumvent these issues, we make use of an extended fragment of cRaf/Raf1 that includes a second Ras binding site, the cysteine rich domain (CRD) (Thapar et al., 2004; Williams et al., 2000). This monovalent probe, Raf1⁵¹⁻²²⁰ (hence forward referred to as RBD²²⁰), was previously shown to detect endogenous levels of RasGTP in a variety of cell types with 1:1 stoichiometry (Anderson et al. 2011; Bondeva et al., 2002; Hibino et al., 2009). When expressed in Ramos B Cells, RBD²²⁰ tagged with eGFP (RBD²²⁰-GFP) rapidly translocated to the plasma membrane following stimulation with BCR crosslinking F(ab')₂ fragments (αIgM) We observed an increase (37% on average) of RBD²²⁰ membrane association following BCR crosslinking with saturating amounts (10 $\mu\text{g/mL}$) of

α IgM (Figure 1D). GFP-tagged RBD, by contrast, showed no discernable membrane translocation or measurable cytoplasmic depletion to the same stimulus.

Previous work in which RBD²²⁰ was transfected into adherent cell lines suggested that this probe could inducibly, but possibly irreversibly associate with Ras molecules on the membrane, thereby impairing downstream ERK activation (Bondeva et al., 2002). In contrast, in Ramos B cells stably expressing this probe, we find that RBD²²⁰ membrane association is responsive to both initiation and termination of Ras activation, as BCR ligand removal induces cytosolic accumulation of membrane bound reporter (Figure S4B). Furthermore, flow cytometry analysis of RBD²²⁰ expressing cells showed no alteration in the levels or kinetics of ERK phosphorylation compared to control cells, indicating that this Ras reporter does not perturb downstream signaling (Figure S2A-C) for the level of reporter expression used in our experiments. Pretreating Ramos cells with Syk (BAY-61-3606) inhibitor abolished α IgM induced RBD²²⁰ translocation to the membrane, while stimulating cells with the PKC/RasGRP agonist, PDBu, drove rapid reporter translocation (Figure S3). When RBD²²⁰ reporter cells were incubated with α IgM-coated beads, RBD²²⁰ localized to the region of contact between the cells and beads, reminiscent of an immunological synapse (Figure 1E. Supplemental Movie 2). Together, these data indicate that RBD²²⁰ can be used as a sensitive dynamic reporter of BCR-stimulated Ras activation.

Analog Ras activation driven by PKC/RasGRP

In B cells, Phospholipase C gamma 2 (PLC γ 2) is recruited to the membrane following BCR ligation, where it catalyzes the cleavage of PI(4,5)P₂ into Diacylglycerol (DAG) and Inositol trisphosphate (IP₃). This DAG recruits and activates RasGRP and its activator Protein Kinase C (PKC) to initiate RasGTP production. Transfection studies performed with full-length RasGRP1 showed that overexpression of this protein produces linear increases in expression of the distal Ras-ERK signaling effector, CD69 as a function of RasGRP1 expression level (Das et al., 2009). Similar experiments performed with the catalytic domain of SOS (SOS_{cat}) showed that this protein induced exponential increases of CD69 expression as a function of SOS_{cat} expression level (Das et al, 2009.). While these and other experiments suggest that RasGRP proteins drive analog increases in RasGTP, the expression dynamics distal Ras-ERK effectors can serve as poor indicators for upstream Ras signaling dynamics (Wilson et al., 2017). Moreover, the activity of RasGRP proteins is subject to complex regulation by DAG, Ca²⁺ levels and phosphorylation, raising uncertainty about how RasGTP responses are regulated by RasGRP activity alone (Iwig et al., 2013; Teixeira et al., 2003; Zheng et al., 2005).

To probe how Ras activation responds in the absence of SOS-driven feedback, we performed dose-response experiments with phorbol 12,13-dibutyrate (PDBu), a chemical mimetic of diacyl glycerol (DAG) that acts stimulates the Ras GEF RasGRP but not SOS (Lorenzo et al., 2000). Western blot analysis revealed titratable induction of RasGRP3 T133 phosphorylation (a PKC phosphorylation site) upon PDBu addition,

validating this approach as a means of titrating RasGRP activity. Importantly, PDBu stimulation did not induce an increase in global phosphotyrosine or recruitment of GRB2 membrane recruitment. both of which are prerequisites for SOS membrane recruitment and activation (Figure S4A, B). Moreover, pretreating cells with the PKC inhibitor Gö6983 abolished PDBu driven RBD²²⁰ membrane recruitment and ERK phosphorylation, confirming the necessity of this enzyme in sustaining PDBu driven RasGTP levels (Figure S4C).

To analyze how Ras signaling changes as a function of RasGRP activity at the single cell level, we stimulated RBD²²⁰ reporter Ramos cells with different concentrations of PDBu and quantified RBD²²⁰ cytoplasmic depletion via time lapse microscopy. These dose-response experiments showed a step-like increase in RBD²²⁰ membrane recruitment that varied in mean amplitude as a function of PDBu dose (2B, C). While similar fractions of cells responded to all doses of PDBu (ranging from 83% at 0.01 μ M to 96% at 10 μ M) (Figure S5B), at the population level we observed a gradual increase in the distribution of mean RBD²²⁰ membrane intensity as PDBu concentration increased (Figure 2D). Consistent with this, we found that all doses of PDBu drove unimodal increases in RBD²²⁰ membrane intensity (Figure 2D) as assessed by Hartigans' dip test, a statistical test that distinguishes between bimodal and unimodal population distributions (Hartigan and Hartigan, 1985). Fitting these data with a Hill function produced an estimated Hill coefficient(nH) of 0.5 (Figure 2E). This suggests that PKC/RasGRP drive analog (non-ultrasensitive) RasGTP production when triggered in the absence of SOS, consistent with previous reports (Das et al, 2009).

Switch-like induction in BCR-driven Ras Responses

We next analyzed RasGTP signaling dynamics in the context of full BCR signaling. BCR ligation activates both RasGRP and SOS, and we reasoned that titrating BCR output could serve as a means tuning the activity of these proteins. α IgM stimulation drove a dose-dependent increases in global phosphotyrosine production, validating our ability to titrate receptor output with this approach (Figure 3A, S5A). Low-level (0.25 μ g/mL and below) stimulation with BCR agonist elicited dose-dependent increases in the rate and mean amplitude of Ras activity, while high level (0.5 μ g/mL and above) stimulation yielded saturating response rates and amplitudes (Figure 3B, C). In addition, Ras responses were, on average, persistent (>50 minutes) at all α IgM doses applied.

At the population level, the fraction of cells responding to α IgM stimulation ranged from 35% at 0.01 μ g/mL to 92% at 10 μ g/mL, increasing as a function of α IgM dose (Figure S5D). Hartigans' dip test analysis level showed unimodal steady state Ras responses at the two lowest (0.01 μ g/mL and 0.1 μ g/mL) and highest (1 μ g/mL and 10 μ g/mL) doses of α IgM stimulation, but bimodal distributions at intermediate doses (0.25 μ g/mL and 0.5 μ g/mL, $p < 0.05$, respectively) (Figure 3D). Consistent with these observations, steady-state Ras responses showed a sharp transition with an estimated Hill coefficient (nH) of 3, consistent with switch-like activation of Ras following BCR triggering (Figure 3E).

Hysteresis in BCR driven Ras activation

SOS-driven positive feedback is predicted to transiently maintain RasGTP levels in cells in which signal flux through the BCR pathway is halted via antigen removal (Das et al, 2009.; Chakraborty et al., 2009). This hysteresis in Ras activity is presumably mitigated via stable association of SOS with the plasma membrane, as has been observed in several cell types (Christensen et al, 2016). As a consequence of persistent SOS activity on the membrane, lower doses of receptor activity should be required to maintain Ras activity than to initiate a Ras response.

We used our live imaging setup to test this possibility using a previously-developed means for reversibly regulating antigen receptor triggering (Weiss et al., 1987). The lectin Concanavalin A binds and crosslinks the BCR and has been previously used as a surrogate BCR ligand. BCR crosslinking by ConA can be rapidly attenuated via the addition of the high affinity ConA-binder Methyl -D-mannopyranoside (α MM), providing a means to induce and revert BCR signaling (Figure 4A). Indeed, we find that ConA rapidly induces RBD²²⁰ membrane association, and addition of α MM terminates Ras activation (Figure 4B). Importantly, BCR surface expression is required for ConA-induced signaling, as ConA does not drive RBD²²⁰ membrane association in IgM-deficient cells despite retaining its ability to associate with the cell surface (Figure S6B). These experiments validate ConA/ α MM system as a reversible receptor triggering system compatible with our live imaging system.

To determine whether less receptor signaling is required to maintain Ras activity than to initiate it, we crosslinked surface BCR with a high dose of ConA (50 μ g/mL), relieved those crosslinks with α MM (100mM), and re-crosslinked receptors with a low dose of α IgM (0.25 μ g/mL) while monitoring RBD²²⁰ translocation dynamics. Importantly, we found that pretreating cells with 100mM α MM reduced the fraction of cells responding to saturating α IgM (10 μ g/mL) stimulus (Figure **S7A**), and 0.25 μ g/mL α IgM was the minimum stimulus required to induce a Ras response in this context (Figure **S7B**). Ras activity was maintained in 59% of cells in response to iterative addition of ConA, α MM and α IgM (Figure 4B-D). We termed these cells “hysteretic” and defined them as those whose Ras activity returns to within the 90th percentile of their maximum post-ConA level following the ConA/ α MM/ α IgM stimulus regimen (Figure 4C). Interestingly, we found that the delay between primary stimulus termination (α MM addition) and minimal stimulus application (α IgM) played a significant role in determining the fraction of hysteretic cells. Statistically similar, but, nevertheless declining fractions of hysteretic cells were found in instances where α IgM was added within ~1 minute of α MM addition (Figure 4D). In contrast, addition of α IgM at 5 and 20 minutes post- α MM addition drove a statistically significant decrease in the fraction of hysteretic cells (Figure 4D), suggesting a temporal window in which Ras activity may be maintained before committing to decay.

Signal Amplification downstream of Ras

How are switch-like Ras signals decoded by the downstream MapK machinery (Figure 5A)? SOS-mediated feedback is widely thought to drive the bimodal pattern of ERK

phosphorylation seen in active lymphocytes (Das et al, 2009). Consistent with this, we found that Ramos B cells exhibit a bimodal pattern of ERK phosphorylation following BCR crosslinking with α IgM (Figure 5B, left). To test whether this binarization of ERK responses originates at the level of Ras activation, we supplemented our cell line co-expressing RBD²²⁰ with the recently described live cell ERK kinase translocation reporter (ERK-KTR) (Regot et al., 2014). Co-expression of these reporters in the same cell allowed us to track Ras and ERK signal activity with high temporal precision within individual cells (Figure 5C, Supplemental Movie 3). Time-course imaging experiments revealed a mean lag time of ~4 mins between peak RBD²²⁰ membrane association and peak ERK-KTR activity in cells stimulated with a saturating dose (10 μ g/mL) of α IgM (Figure S8A). Low doses of α IgM (0.01 μ g/mL and 0.1 μ g/mL) rarely increased ERK-KTR response despite driving detectable increases in RBD²²⁰ membrane association (Figure 5D). Interestingly, these low doses of α IgM stimulus generally drove a <20% increase in RBD²²⁰ membrane association, suggesting that a threshold level of Ras activity may be required to trigger an ERK response in these cells. Stimulating cells with a higher dose (1 μ g/mL) of α IgM by contrast drove a >20% increase in RBD²²⁰ membrane association, and also induced potent ERK-KTR responses (Figure 5D, bottom right). At the single cell level, we found that an approximate 20% increase in Ras activity corresponded to induction of an ERK-KTR response (Figure 5E). Kernel density estimation (bandwidth = 0.5) of these data showed that >20% increases in Ras activity corresponded >4-fold increase in median ERK kinase activity, with the population density exhibiting a bimodal shape transition (Figure 5F).

Bimodal responses are generated by switch-like systems responding in an all-or-none manner to stimuli above a threshold, while ignoring sub-threshold stimuli (Ferrell Jr. and Ha, 2014). While our analysis supports the model that positive feedback at the level of Ras activation is highly switch-like, it is formally possible that bimodal ERK responses are generated downstream of Ras via independent feedback modules (Bhalla et al., 2002; Ferrell Jr. and Machleder, 1998; Shin et al., 2009; Shindo et al., 2016). To test this idea, we stimulated cells with PDBu (a condition in which Ras positive feedback is not engaged and Ras activation is graded, not bimodal) and analyzed the resulting ERK responses in single cells (Figure 6A). Surprisingly, we found that in PDBu stimulated Ramos cells, bimodal ERK phosphorylation is retained and is kinetically similar to BCR-activated cells (Figure 6B, 5B). Importantly, we found that this bimodal activation is preserved across a wide range of PDBu doses in both Ramos and Jurkat T cells, suggesting, conservation of the underlying signaling network architecture leading to bimodal ERK downstream of unimodal Ras (Figure S8B right panel, C). This led us to hypothesize that ERK may respond in a digital manner to changes in Ras activity. To test this, we performed two-step experiments in which we pulsed cells sequentially with low (0.1 μ M) and high (1 μ M) doses of PDBu, reasoning that analog increases in Ras activity driven by PDBu would allow for analysis of ERK responses in the absence of upstream feedback (Figure 6C). Live imaging of RBD²²⁰ showed two step-like increases of plasma membrane translocation in response to sequential pulses of low and high PDBu stimulus, consistent with analog Ras activation (Figure 6D, 6E bottom). In contrast, ERK-KTR responded to either low or high pulses of PDBu, with each cell exhibiting a single step-like response despite experiencing two-pulses of stimulus

(Figure 6D and 6E, top). Taken together, these data suggest that a threshold change in Ras activity is necessary to trigger downstream ERK signaling. Furthermore, the Ras/MAPK cascade contains two independent modules for generating bimodal responses-- one within and one downstream of Ras activation.

DISCUSSION

In this work we provide evidence for discrete signal amplification steps occurring at the level of Ras and ERK during B cell activation. In particular we find that BCR-driven Ras activation is highly switch-like ($n_H=3$), while PDBu driven Ras activation is graded ($n_H=0.3$), supporting the model that cooperation between RasGRP and SOS gives rise to nonlinear Ras pathway activation (Das et al, 2009.). We show that ERK activation is bimodal in BCR-triggered Ramos cells, and that these bimodal responses proceed following Ras activation beyond a strict threshold. Lastly, we demonstrate that ERK activation is digital in its own right, showing that all-or-none ERK responses occur in single cells in which graded increases of active Ras are driven by PDBu. While these findings broadly support the predominant model for digital Ras activation in active lymphocytes, they also raise important questions concerning the mechanistic basis for digitization of Ras-ERK signaling.

Feedback activation of SOS by RasGTP is the predominant mechanism invoked to explain bimodal nature of activation of Ras pathway effectors (i.e. ERK, CD69). However positive feedback is just one mechanism capable of mitigating these sorts of bimodal responses. Signaling protein oligomerization and phase separation, for example, provide additional mechanisms for initiating non-linear biochemical responses (Li, Banjade, Chen et al., 2009). Indeed, recent studies highlight the ability of GRB2 and SOS to form phase separated aggregates in activated T-Cells (Su et al., 2016; Huang et al., 2016). Moreover, active Ras molecules have been shown to form homotypic nanoclusters capable of digitizing downstream ERK signaling in EGF stimulated Baby

Hamster Kidney cells (Tian et al., 2007). It will be interesting to evaluate how each of these mechanisms contribute to the Ras pathway signaling dynamics we describe here.

We previously used a set of optogenetic tools to characterize the signal transmission between active Ras and downstream ERK in 3T3 fibroblast cells (Toettcher et al., 2013). There we found near-linear transmission of Ras signals persisting longer than an approximate 4-minute temporal threshold downstream to ERK. In contrast our data here, support a model in which an independent digital feedback circuit engages ERK activation once active Ras exceeds a threshold level. A parsimonious solution to this discrepancy would posit the existence of uncharacterized feedback circuits impinging on ERK activation operating in lymphocytes but not fibroblasts. Pairing optogenetics with genomic and proteomic approaches will provide a framework for uncovering these differences in Ras pathway network architecture (Toettcher et al., 2013; Wilson et al., 2017).

Lastly, our work provides an initial framework for investigating how the Ras pathway mitigates some of the physiological responses (i.e. differentiation, proliferation) induced by BCR binding to cognate antigen *in vivo*. For example, ERK activity has been implicated in antigen-mediated plasma cell differentiation and plays a key role in translating the affinity of antigen-BCR interactions into the amount of antibody produced (Yasuda et al. 2011, 2012; Wheeler et al. 2013). It is tempting to speculate that cytokine and co-receptor signals known to modulate these differentiation and antibody responses might augment or suppress the Ras pathway dynamics described here. Future work

pairing intravital imaging with Ras and ERK activity reporters will be key in testing this hypothesis.

Materials and Methods

Cell Culture

Ramos cells were obtained from the American Type Culture Center (ATCC). Jurkat cells were a kind gift from Art Weiss (UCSF). Both Jurkat and Ramos cells were maintained in 10G-RPMI (RPMI 1640 supplemented with L-Glutamine and 25 mM HEPES (Mediatech) and containing 1% (vol/vol) GlutaMAX (Gibco) and 10% (vol/vol) Fetal Bovine Serum (Gibco)). Cultures were maintained in a 37 °C/5% CO₂ incubator at a density ranging from 0.2-1 million cells/mL. HEK-293T cells (used to generate lentivirus for transduction) were maintained in DMEM (Mediatech) supplemented with 10% (vol/vol) Fetal Bovine Serum (Gibco). Cells were imaged in imaging media (RPMI 1640 without phenol red supplemented with 25mM HEPES, 1% L-glutamine and 0.1% fetal bovine serum).

Plasmids

All constructs were cloned into the pHR lentiviral backbone (kindly provided by Ron D. Vale, UCSF containing the spleen focus forming virus (SFFV) promoter via standard Gibson assembly. Human cRaf/Raf1 cDNA was a gift from William Loomis (UCSD). RBD⁵¹⁻¹³¹ and RBD⁵¹⁻²²⁰ coding sequences were amplified from this cDNA and subcloned into a GFP containing pHR vector to generate pHR-RBD-GFP and pHR-RBD²²⁰-GFP plasmids, respectively. The ERK^{KTR} sequence was amplified from pLentiCMV-Puro-ERK^{KTR}-Clover (Addgene # 59150) and subcloned into a pHR vector containing TagRFP-T to generate pHR- ERK^{KTR}-TagRFP-T. Murine GRB2 cDNA (kindly

provided by Mark Davis) was amplified and subcloned into a pHR plasmid containing TagBFP to generate pHR-GRB2-BFP.

Cell line generation and stimulation

Cell lines were generated via lentiviral transduction. Lentivirus was produced by co-transfecting vectors encoding lentiviral packaging proteins (pMD2.G and p8.91) along with a pHR vector containing a gene of interest into Hek-293T cells plated in 6-well plates (Thermo Fisher Scientific) grown to ~70% confluence. Transit-IT-293 (Mirrus Bio) was used for all transfections. Viral supernatants were harvested 2 days post-transfection, filtered through a 0.45µm syringe filter (Millex), and concentrated ~40 fold using Lenti-X Concentrator (Takara Bio Inc.). Viral supernatants were either used immediately, stored at 4 °C for up to one week, or stored at -80 °C for long-term storage. 0.5×10^6 Ramos cells resuspended in 250 µL 10G-RPMI were mixed at a 1:1 ratio with concentrated supernatant and incubated overnight. Following incubation, viral supernatants were removed by centrifugation, and transduced cells were cultured in 10G-RPMI for one week to recover. Transgene expressing cells were isolated by FACS. Clonal RBD²²⁰-eGFP/ERK^{KTR}-Tag-RFP-T co-expressing cells were isolated by limiting dilution. IGM-deficient Ramos cells were isolated by iterative rounds of sorting Ramos cells negative for cell surface IGM expression (assayed by cell surface staining with APC-conjugated anti-IgM antibody, Biolegend #314509).

Cells were stimulated as indicated in the text and figure legends. αIGM (Biolegend # 314502), PDBu (CST #12808S) and Concanavalin A (Sigma # C5275) were used at the

indicated concentrations and were diluted in imaging media. Methyl α -D-mannopyranoside (α MM) (Sigma #M6882) was used at final concentration of 100mM and a fresh stock diluted fresh in imaging media for every experiment (to guard against contamination).

Microscopy and Image analysis

Cells were plated in a well of a glass bottom 96-well glass plate (corning). Wells were coated with 1 mg/mL poly-L-lysine (Sigma) for ~30 mins, and washed 3X with PBS prior to cell seeding (at a concentration of 0.5 million cells/mL). Images were acquired on a Nikon Eclipse Ti inverted microscope equipped with a Yokogawa CSU-X1 spinning disk confocal, a 60x 1.4 Plan Fluor objective (Nikon) and a Prime95B cMOS camera (Photometrics). 405nm, 488nm, 561nm and 640nm (LMM5, Spectral Applied Research) laser lines were used for excitation. CellProfiler (Broad Institute) and Fiji (National Institutes of Health) were used for cell segmentation and image analysis. Excel (Microsoft), Pandas (Wes McKinney), PRISM 7 (GraphPad), and Seaborn (Michael Waskom) were used for data wrangling and visualization.

Raw microscopy images were background subtracted and corrected for stage drift prior to cell segmentation (to identify cells) and erosion (to remove peripheral pixels and identify the cytoplasm). Membrane intensity of signaling activity reporters (Ras, GRB2) was calculated by taking the inverse of the cytoplasmic intensity. Single cell intensity traces were normalized by dividing traces by a cell's integrated mean fluorescence prior to stimulation.

The cytoplasmic-to-nuclear ratio of KTR channel intensity was used to quantify KTR activity. The fluorescence intensity of KTR reporter was quantified in the nucleus (identified by nucblue signal) and the cytoplasm (identified by subtracting nuclear region of the cell from the cell mask). Due to the scarcity of cytoplasmic pixels in Ramos cells, the consistency of cell masking from frame to frame was evaluated and adjusted manually for KTR quantitation. As nuclei occupy a large fraction of the cytoplasmic area in Ramos cells, for experiments in which both Ras reporter and KTR signal was measured, cells were imaged at both a coverslip proximal focal plane (to exclude the nucleus, and improve Ras reporter quantitation, **Figure S1**) and equatorial focal plane (to include the nucleus and facilitate KTR quantitation).

Statistical analysis

Two-tailed students' t-tests were performed using PRISM 7 software (GraphPad). Tests with a p value <0.05 were considered statistically significant. In figures, ns indicates not significant, while * represents $p < 0.05$, ** represent $p < 0.01$ and *** represents $p < 0.001$. Hartigans' Dip Test was performed in R (diptest package, Martin Maechler). Tests with a p value <0.05 indicate significant bimodality, while tests with p values >0.05 but 0.1 indicated bimodality with marginal significance. The dip statistic (D) indicates the point in a population distribution in which the maximal difference between peaks in the distribution is achieved. For our purposes, it highlights a plausible separation between populations of cells responding to stimulation.

Western Blots and Fluorescence activated cell sorting (FACS)

For western blot experiments, cell lines were serum starved for 0.5-1 hour in imaging media at concentration of 0.5×10^6 cells/mL. Following stimulation, cells were immediately transferred to ice, pelleted via centrifugation and resuspended in 200 μ L ice-cold lysis buffer (1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na_3VO_4 , 10% glycerol, freshly supplemented with protease and phosphatase inhibitor tablets (Roche Applied Sciences). Lysates were incubated on ice for 10 minutes and centrifuged at 15,000g for 10 minutes. Protein concentration was measured by BCA Assay (Thermo Fisher Scientific), after which samples were solubilized in 4X Sample Buffer (Bio-Rad Laboratories) and boiled at 100°C for 10 minutes. Samples were either immediately loaded onto gels for Western blot analysis or flash frozen. Bolt 17-well precast 4-12% Bis-Tris Plus gels (Thermo Fisher Scientific) were run at 200V for 25 minutes to resolve proteins. Proteins were transferred from gels to nitrocellulose membranes using a Trans-Blot semi-dry transfer apparatus (Bio-Rad) run at 20V for 1 hour. Membranes were blocked in Odyssey Blocking buffer (Li-Cor) for 1 hour, exposed to primary antibodies diluted according to manufacturer instructions in Odyssey blocking buffer for 4°C overnight, washed in TBST, and incubated in IRDye (Li-Cor) anti-rabbit and anti-mouse secondary antibodies for 30 minutes at room temperature. Following a final wash in TBST, membranes were imaged using a Li-Cor imaging station.

Cell surface staining experiments (IgM, CD22) was performed in FACS buffer (PBS, 5% FBS, 1mM EDTA, 0.1% NAN_3) for 30 mins at 4 °C. Cells were washed 3X in ice-cold

FACs buffer, fixed at room temperature in Cytofix (BD Biosciences), washed three additional times in FACs buffer, and run immediately on a BD LSR II (BD Biosciences). For FACs staining of ERK phosphorylation (ppERK), cells were serum-starved as above in 96 well plates (Corning). A 2X stock of stimulus (I α GM, PDBu, etc...) was used to stimulate cells for the appropriate time. Cells were then fixed at room temperature with Cytofix (BD Biosciences), pelleted by centrifugation (500G for 5 mins), and resuspended in ice-cold methanol to permeabilize cell membranes overnight. Cells were washed 3X in ice-cold FACS buffer and stained with anti-ppERK antibody (CST. #4370) diluted 1:1000 in FACs buffer for 1 hour at room temperature. Cells were washed 3X in FACs buffer and stained with 405 conjugated anti-rabbit secondary antibodies (Abcam # ab175672) for 30 mins. Cells were washed 3 additional times in ice-cold FACS buffer and run immediately on BD LSR II (BD Biosciences). Analysis of flow cytometry data was performed in FlowJo (FlowJo LLC).

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FIGURE 2.1

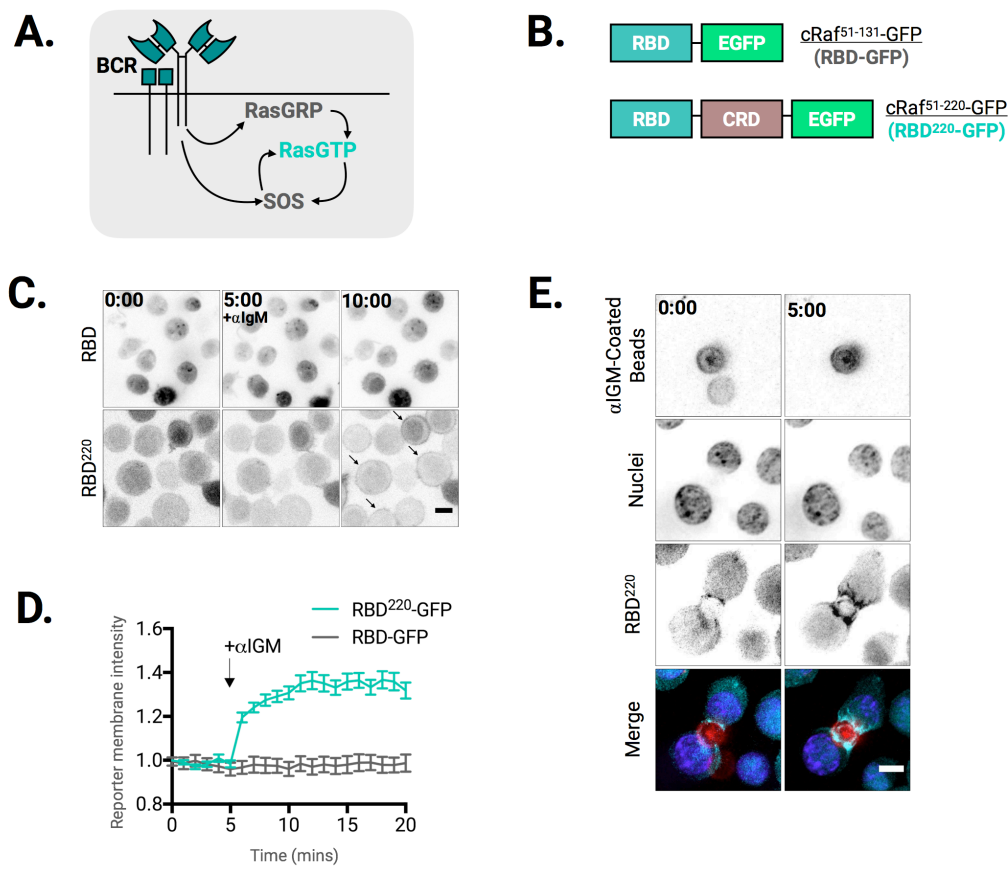


Figure 2.1. Visualizing Ras activation dynamics during B cell activation in living cells. **(A)** Schematic of the Ras GEFs that drive Ras activation downstream of B-Cell receptor triggering. **(B)** Domain structure of RasGTP biosensors. **(C)** and **(D)** Single cell image analysis of RasGTP biosensor localization following B cell activation. **(C)** Representative images of RBD-GFP (top) and RBD²²⁰-GFP (bottom) before and after addition of BCR agonist (α IgM, 10 μ g/mL). Scale bar is 5 μ m. Arrows highlight plasma membrane translocation of Ras biosensors. **(D)** Quantitation of normalized Ras biosensor intensity on the plasma membrane. Arrow indicates time α IgM addition (t = 5 mins) See supplemental methods for details on data collection and quantitation. **(E)** Representative images of RBD²²⁰-GFP localization in cells initially contacting BCR agonist α IgM-coated beads at the moment of contact (first column), and five minutes after contact (second column) Scale bar 5 μ m.

FIGURE 2.2

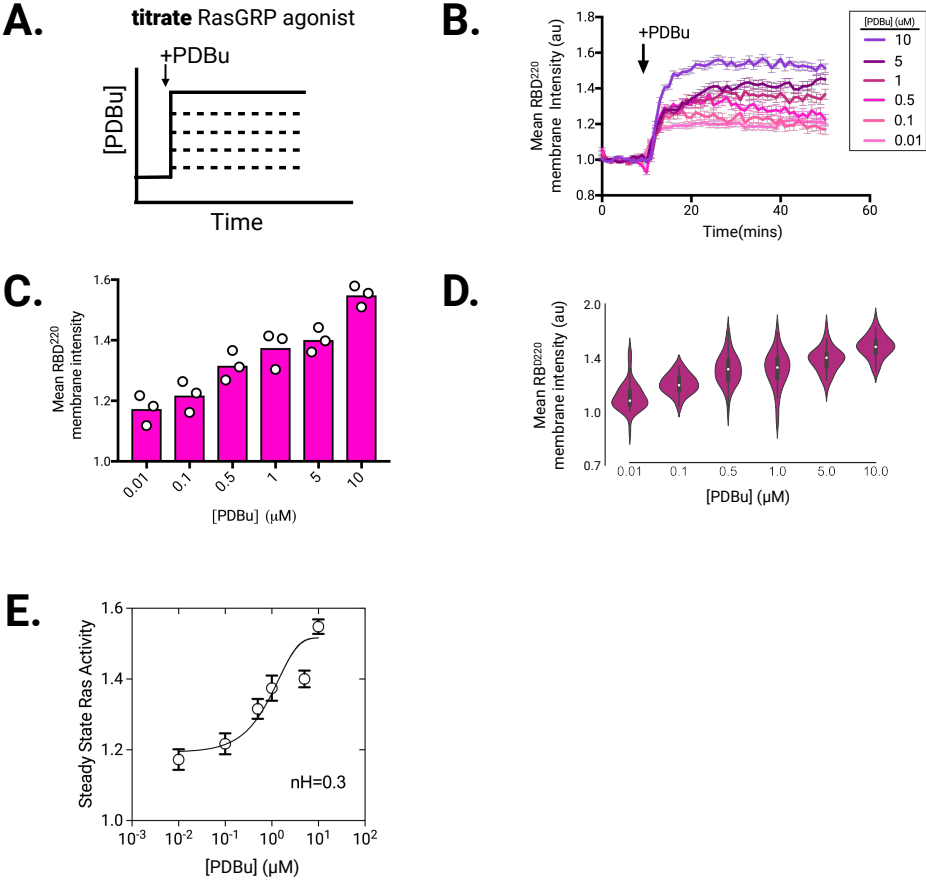


Figure 2.2. A water-soluble DAG mimetic (PDBu) drives graded, unimodal Ras activation responses **(A)** Schematic of dose response experiment for cells are exposed to step function increases of PDBu (RasGRP agonist) of different amplitudes. **(B-E)** Live cell analysis of RBD²²⁰-GFP membrane association (henceforth referred to as Ras activation) in response to PDBu. **(B)** Dose Response. Median intensity traces of cells stimulated with indicated doses of PDBu (see inset) over time. Arrowhead indicates time of PDBu addition (~t=10mins). Error bars are SEM. Median intensity traces are generated from at least n=50 cells per experiment. Traces are representative of three independent experiments. **(C)** Mean RBD²²⁰ membrane intensity (calculated from t = 20-40mins) from cells stimulated as indicated in (B). **(D)** Violin plots of cells stimulated as indicated in (B), showing unimodal Ras activation responses at all PDBu doses. Mean response to indicated dose of PDBu (x-axis) was calculated as the mean membrane intensity on a per-cell basis between t=20-40mins (n=50 cells displayed per PDBu dose). **(E)** Steady state Ras activity (see methods) across PDBu dose response experiments. nH (inset) indicates Hill coefficient, showing lack of ultrasensitivity for PDBu input.

FIGURE 2.3

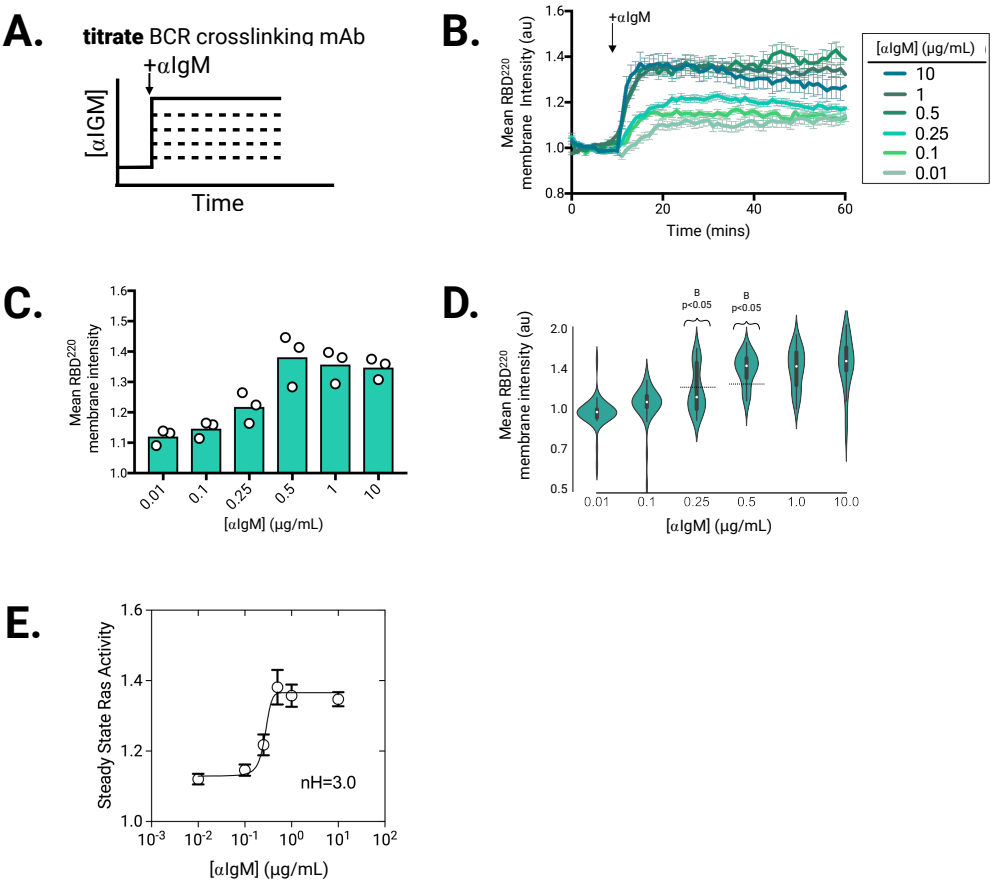


Figure 2.3. BCR agonist drives switch-like, bimodal Ras activation **(A)** Schematic of dose-response experiment for cells exposed to step increases of BCR agonist (α IgM) of different amplitudes. **(B-E)** Live cell analysis of RBD²²⁰-GFP membrane association. **(B)** Mean intensity traces of cells stimulated with indicated doses of α IgM (see inset) over time. Arrowhead indicates time of α IgM addition ($\sim t=10$ mins). Error bars are SEM. Mean intensity traces are generated from at least $n=50$ cells per experiment. Traces are representative of three independent experiments. **(C)** Mean RBD²²⁰ membrane intensity ($t = 20-40$ mins) from cells stimulated as indicated in (B). **(D)** Violin plots of cells stimulated as indicated in (B) showing bimodal Ras activation at intermediate doses of α IgM. Steady-state response to indicated dose of α IgM (x-axis) calculated as the mean membrane intensity between $t=20-40$ mins ($n=50$ cells displayed per α IgM dose). Bimodality (B) and p-values were computed by Hartigans' dip test (see methods). Dashed line indicates the dip statistic of cells stimulated with $0.25\mu\text{g/mL}$ and $0.5\mu\text{g/mL}$ α IgM (see methods). **(E)** Steady-state Ras activity (see methods) across α IgM dose response experiments. nH (inset) indicates Hill coefficient.

FIGURE 2.4

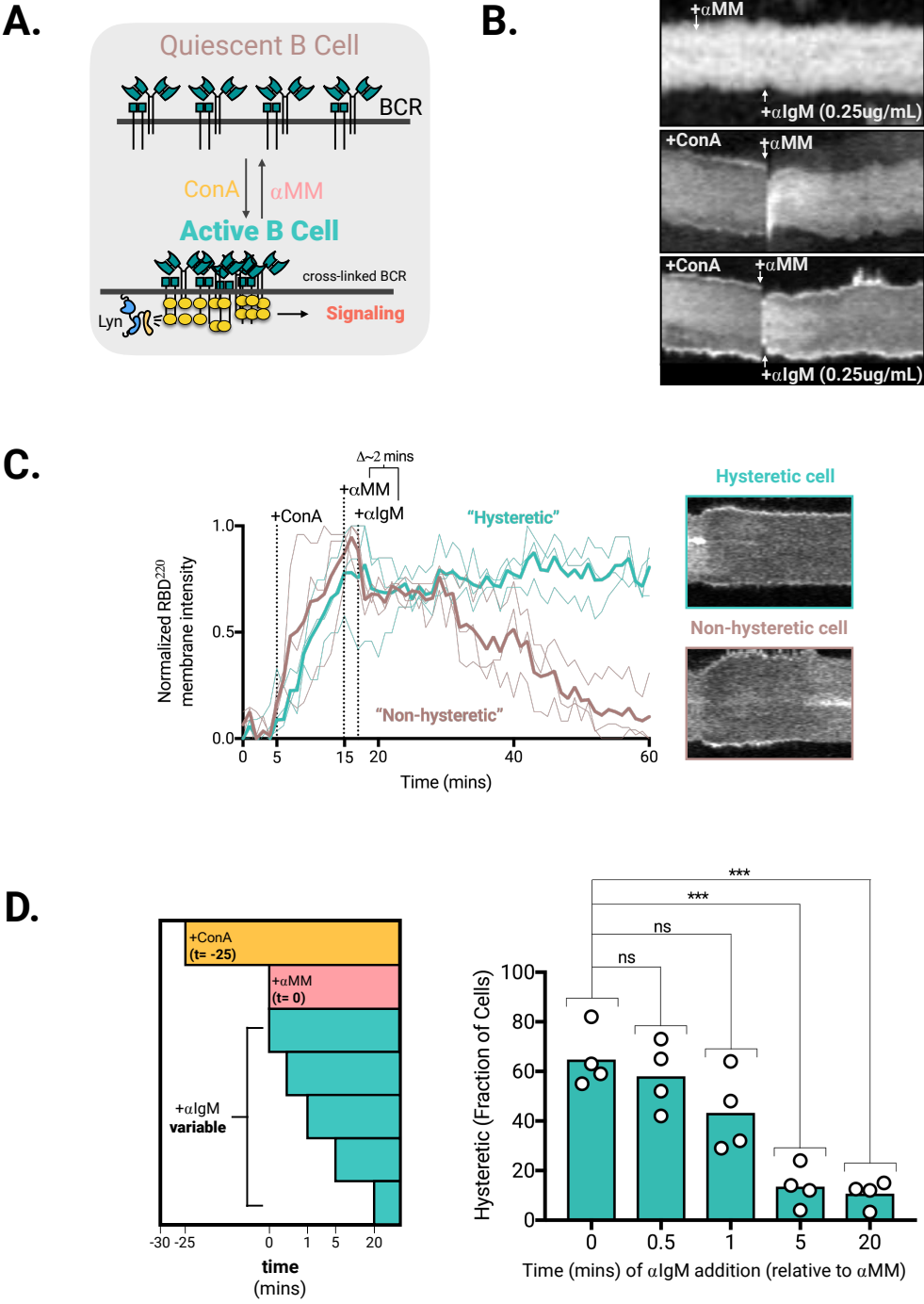


Figure 2.4. Hysteresis in BCR-driven Ras Activity. **(A)** Schematic of the reversible BCR triggering assay relying on ConA-mediated receptor crosslinking, and α MM-mediated reversal of B-cell receptor crosslinking. **(B)** Single cell kymographs of RBD²²⁰-GFP over time. **(Top)** Cells stimulated with 100mM α MM (t=2 mins), followed by 0.25 μ g/mL α IgM (t=12mins) **(Middle)** Cells stimulated with 50 μ g/mL ConA (t =2mins) followed by 100mM α MM (t=12mins). **(Bottom)** Cell stimulated with 50 μ g/mL ConA (t=2mins), 100mM α MM (t=12mins), and 0.25 μ g/mL α IgM (t=13 mins). Experiment run length is 45 mins. **(C)** (Right) Single-cell traces showing examples of cells responding in a “hysteretic” (turquoise) or “non-hysteretic” (brown) manner following stimulation with 50 μ g/mL ConA (t = 5 mins), 100mM α MM (t = 15 mins) and 0.25 μ g/mL α IgM (t=17 mins). (Left) Example kymographs of a hysteretic (top, turquoise border) and non-hysteretic (bottom, brown border) are displayed. **(D)** (Top) Schematic depicts relative time of addition of ConA (t = 2 mins), α MM (t = 27 mins), and α IgM (variable time points). (Bottom) Quantitation of the fraction of hysteretic cells as a function of time of α IgM addition relative to α MM addition. Hysteretic cells are defined as those whose Ras activity returns to within the 80th percentile of their maximum post-ConA level following addition of α IgM. Each circle represents a single experiment. At least 20 cells were quantified per experiment. *** denotes statistical significance (p< 0.001, two-tailed unpaired student’s t-test).

FIGURE 2.5

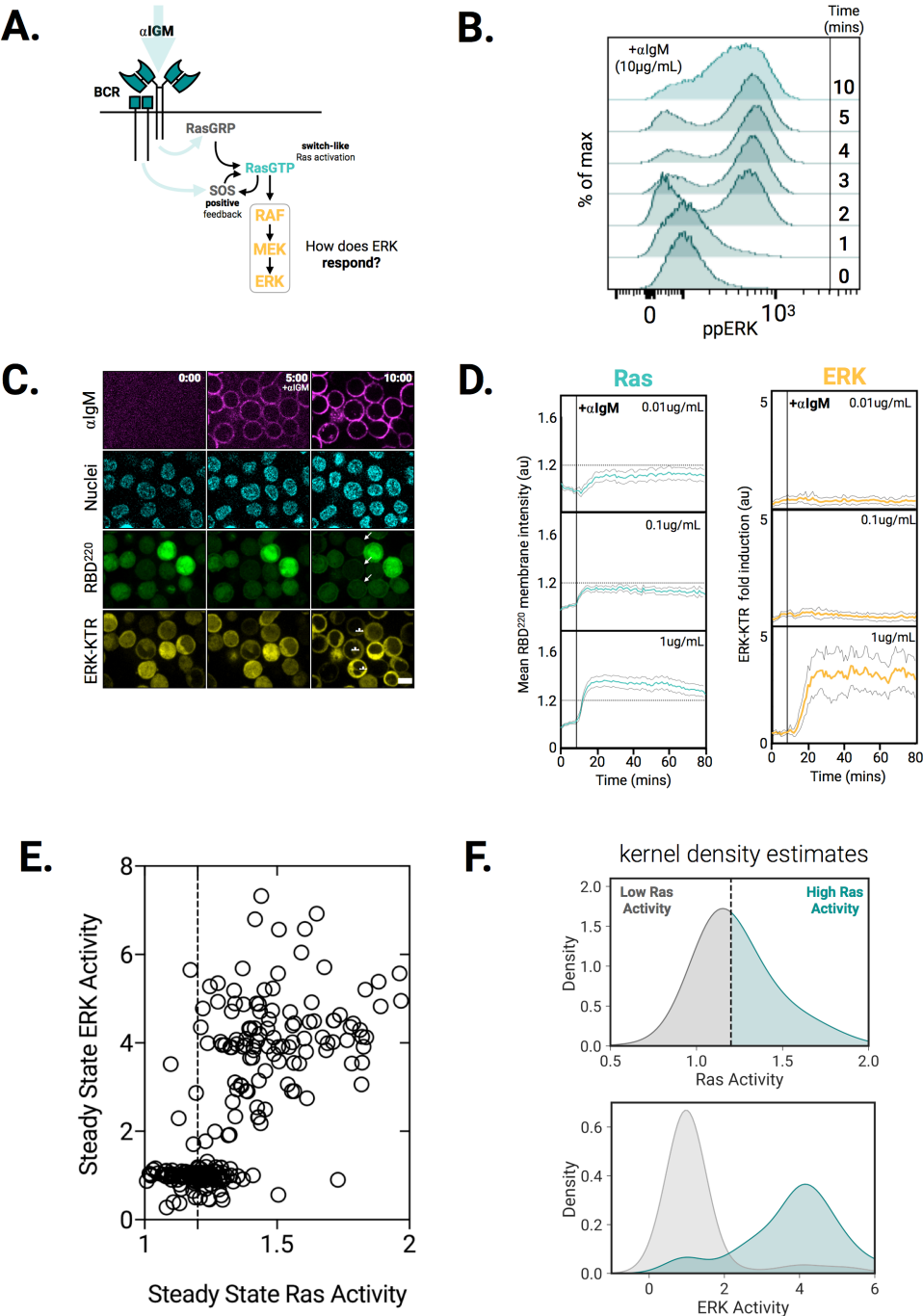


Figure 2.5. Bimodal ERK activation beyond a threshold level of Ras Activation (A)

Schematic depicting switch-like Ras activation arising from BCR-generated Ras positive feedback. **(B)** ERK phosphorylation time course from wildtype Ramos B cells stimulated α IgM (10 μ g/mL, right) (representative of three independent experiments). **(C)** Representative (n = 2) time course images of α IgM (top, magenta), nuclei (second row, teal), RBD²²⁰-GFP (third row, green) and ERK-KTR-TagRFP-T (yellow, bottom) following APC- α IgM addition (10 μ g/mL). Nuclei are labeled with nucblue (see methods). 5 μ m scale bar. **(D)** Cells co-expressing RBD²²⁰-GFP (left, purple) and ERK-KTR-TagRFP-T (right, brown) were stimulated with indicated doses (inset) of α IgM. Mean response (n= at least 50 cells per trace) plotted over time. 95% confidence interval (black lines) envelope mean traces. Dashed lines (left column) indicate apparent threshold of Ras activity required to trigger ERK response (~20% above basal). **(E)** Dose-response experiments. Representative single-cell mean steady-state ERK activity plotted as a function of mean steady-state Ras activity in individual cells. Each circle is data from an individual cell. Data are pooled from α IgM dose response experiments where cells were stimulated with 0.01, 0.1, 0.25, 1 or 10 μ g/mL α IgM. 50 cells per α IgM dose are plotted (250 cells total). Dashed line indicates 20% increase above basal Ras activity. **(F)** Kernel density estimations of Ras and ERK activity plotted in (E). Ras activity (top) is segmented into low and high by line (vertical, dashed) demarcating a 20% increase above basal activity. ERK activity (bottom) is plotted as a function of low (grey) and high (teal) Ras activity.

FIGURE 2.6

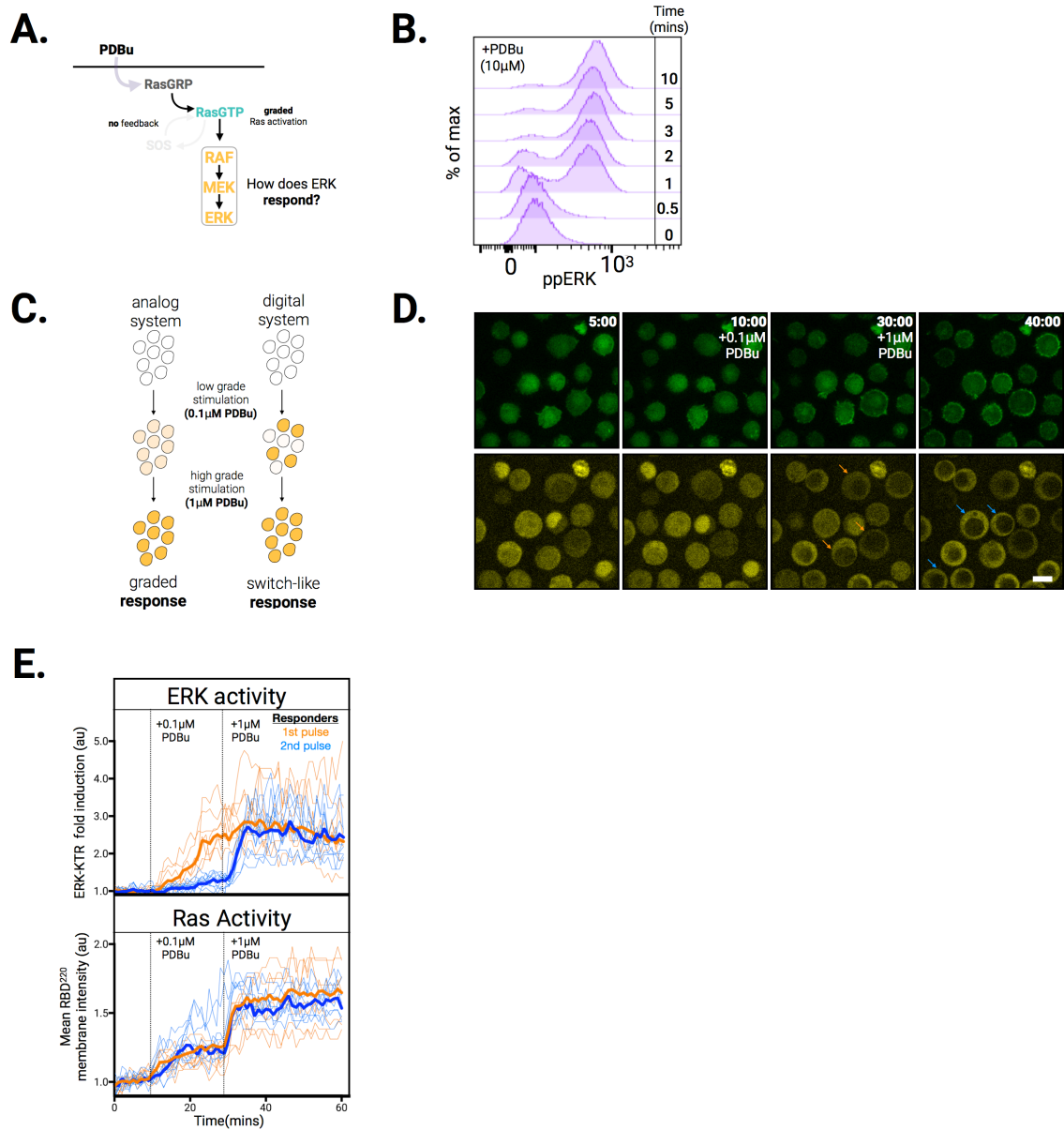


Figure 2.6. Digital ERK activation downstream of Ras. **(A)** Schematic depicting graded Ras activation driven by PDBu-mediated activation of RasGRP (this agonist does not engage the Ras positive feedback loop). **(B)** ERK phosphorylation time course from wildtype Ramos B Cells stimulated PDBu (10 μ M, right) (representative of three independent experiments). **(C)** Schematic depicting analog (left) and digital (right) cellular systems responding to two pulses of an external stimulus. Graded increases are characterized by gradual increases in cell responses (left, yellow coloration). Switch-like responses are characterized by all-or-none responses on a per-cell basis (right, yellow coloration). **(D)** Representative (n=3) time course images of RBD²²⁰-eGFP and ERK-KTR-TagRFP-T Ramos cells stimulated with 0.1 μ M (t=10mins) and 1 μ M PDBu (t=30mins). Arrow heads indicate ERK-KTR expressing cells responding to the first (orange) or second (blue) pulse of PDBu. **(E)** Single cell traces of cells stimulated as in (D) depicting Ras (top) and ERK (bottom) responses. Traces are color coded to indicate cells whose ERK-KTR responses are to the first (orange) or second (blue) pulse of PDBu.

FIGURE S2.1

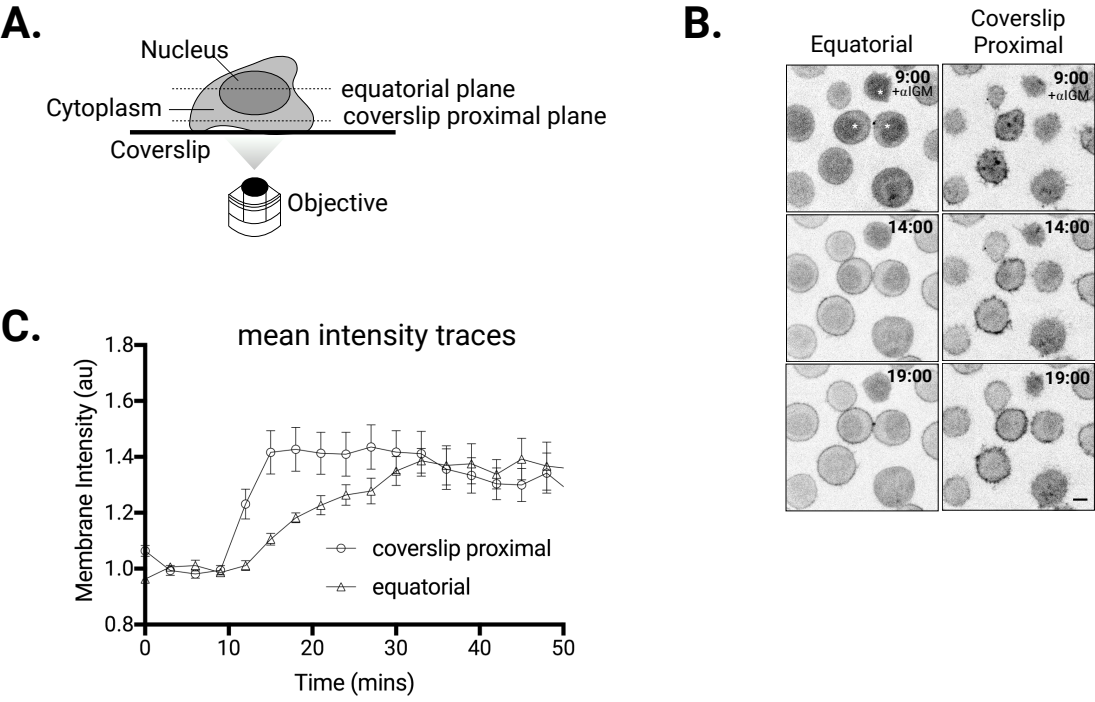


Figure S2.1. Quantitating Ras signaling dynamics in live cells (A) Schematic depicting imaging set up in which cells are imaged in two focal planes: 1. The equatorial plane, which often includes both the nucleus and cytoplasm, and 2. The coverslip proximal plane, which includes the cytoplasm and generally excludes the nucleus. **(B)** Representative ($n > 5$) time lapse images of RBD²²⁰-eGFP Ramos cells stimulated with 10 $\mu\text{g/mL}$ αIgM ($t = 9$ mins). Left column depicts the equatorial focal plane. Nuclei in top left panel are indicated with an asterisk (white). Right column depicts the coverslip proximal focal plane. **(C)** Mean intensity traces of cells ($n = 20$ cells per focal plane) stimulated as in (B).

FIGURE S2.2

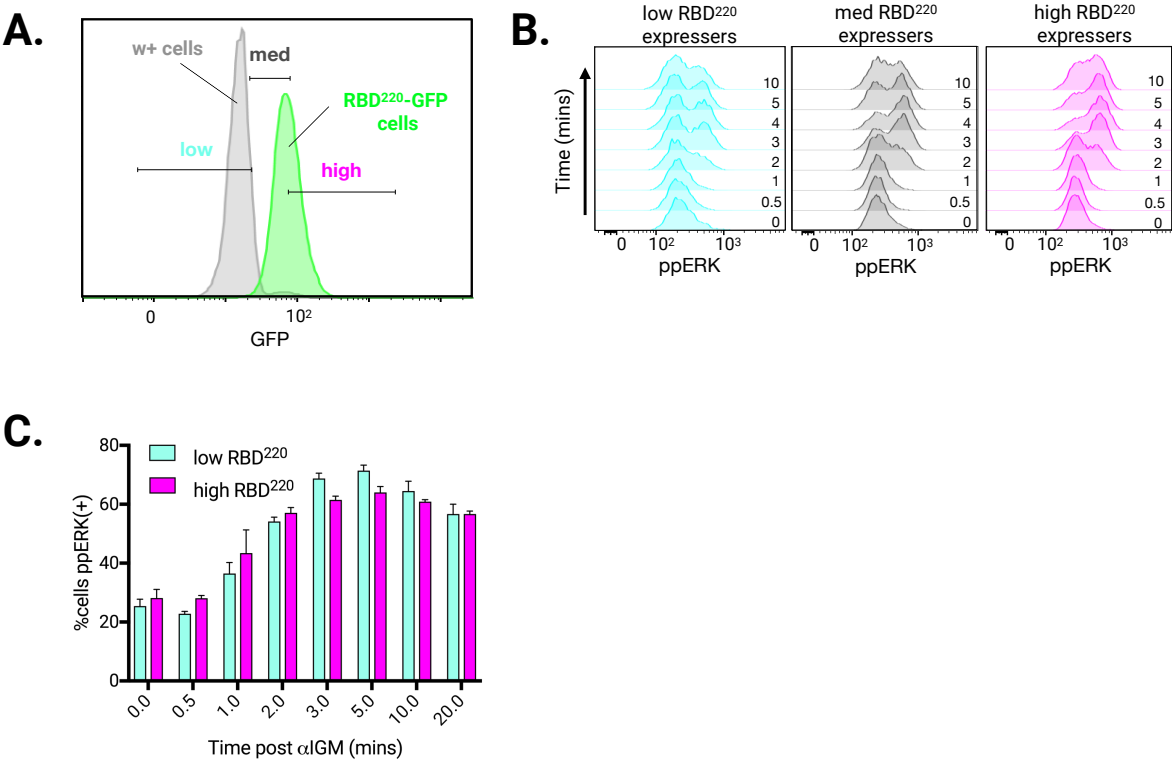


Figure S2.2. Similar ppERK responses across a wide RBD²²⁰ expression range in a stable cell line **(A)** GFP signal in wildtype and RBD²²⁰-eGFP Ramos cells (100,000 cells each) binned according to GFP signal. **(B)** ERK phosphorylation (ppERK) time course from RBD²²⁰-eGFP Ramos cells binned based on GFP expression level as in (A), stimulated with 10µg/mL αIgM (t=0 mins). **(C)** Fraction of RBD²²⁰low and RBD²²⁰hi cells staining positive for ppERK from experiment detailed in (B). Data were pooled from two independent experiments.

FIGURE S2.3

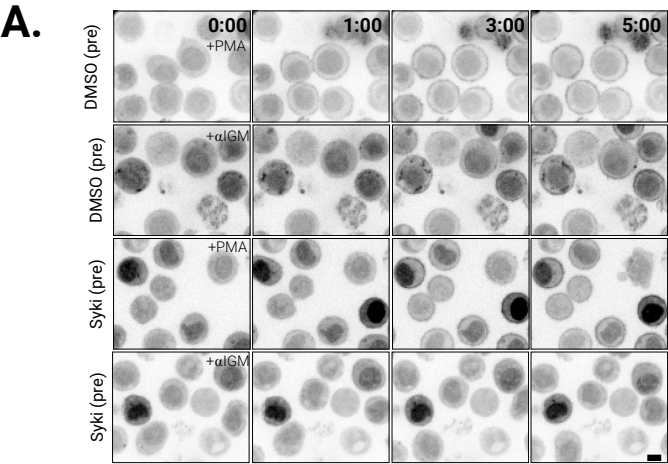


Figure S2.3. Syk activity is required for α IgM but not PMA induced RBD²²⁰

membrane translocation (A) Representative time course images of RBD²²⁰-eGFP

Ramos cells pretreated with either 0.1% DMSO (top two rows) or the Syk inhibitor BAY-61-3606 (10nM) and stimulated with 10 μ M PMA (1st, 3rd rows) or 10 μ g/mL α IgM (2nd, 4th rows) at t=0mins. Scale bar (black, bottom right panel) is 5 μ m.

FIGURE S2.4

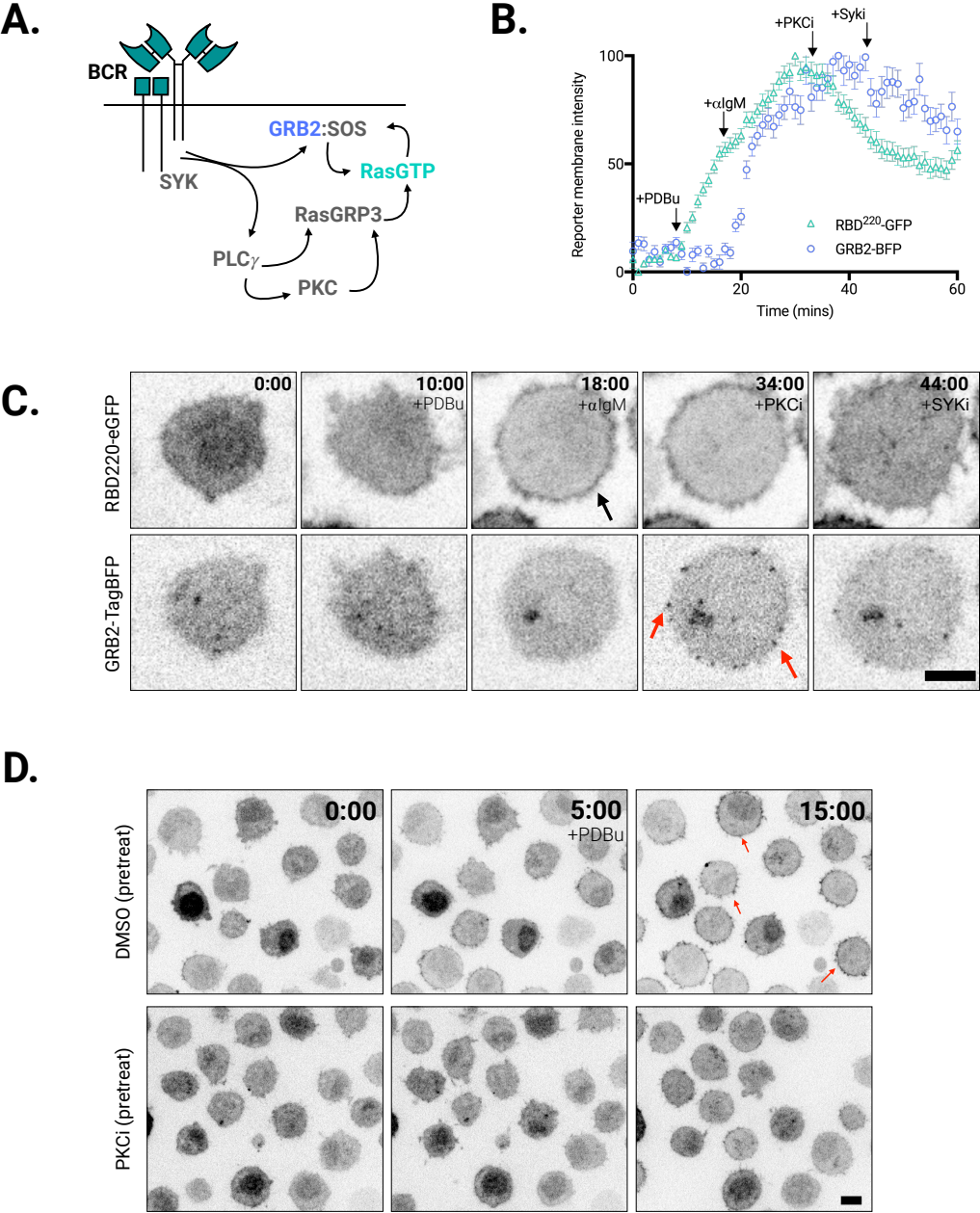


Figure S2.4. PDBu does not induce GRB2 membrane localization and requires PKC activity for inducing Ras-ERK signaling (A) Schematic depicting BCR triggered Ras activation. While α IgM stimulates both the RasGRP and SOS arm of the pathway, PDBu mimics the PLC γ product, DAG, thereby solely triggering the RasGRP arm. **(B)** Mean (n=50 cells) time course of GRB2-BFP (blue circles) and RBD²²⁰-eGFP (green triangles) co-expressing cells stimulated sequentially with 10 μ M PDBu (t=10mins), 10 μ g/mL α IgM (t=18mins), 50nM Gö 6938 (PKCi, t=34 mins) and 10nM BAY-61-3606 (Syki, t=44mins). Traces are representative of two independent experiments. Error bars are SEM. **(C)** Single cell stills from cells stimulated as in (B). Arrowheads indicate RBD²²⁰-eGFP membrane associate (black) and formation of peripheral GRB2-BFP clusters (red) following PDBu and α IgM stimulation, respectively. **(D)** Time course analysis of RBD²²⁰-eGFP Ramos cells pre-incubated in 0.1% DMSO (top row) or 50nM Gö 6938 (PKCi bottom row), treated with 10 μ M PDBu (t = 5 mins). Arrowheads (red) highlight RBD²²⁰ membrane translocation.

FIGURE S2.5

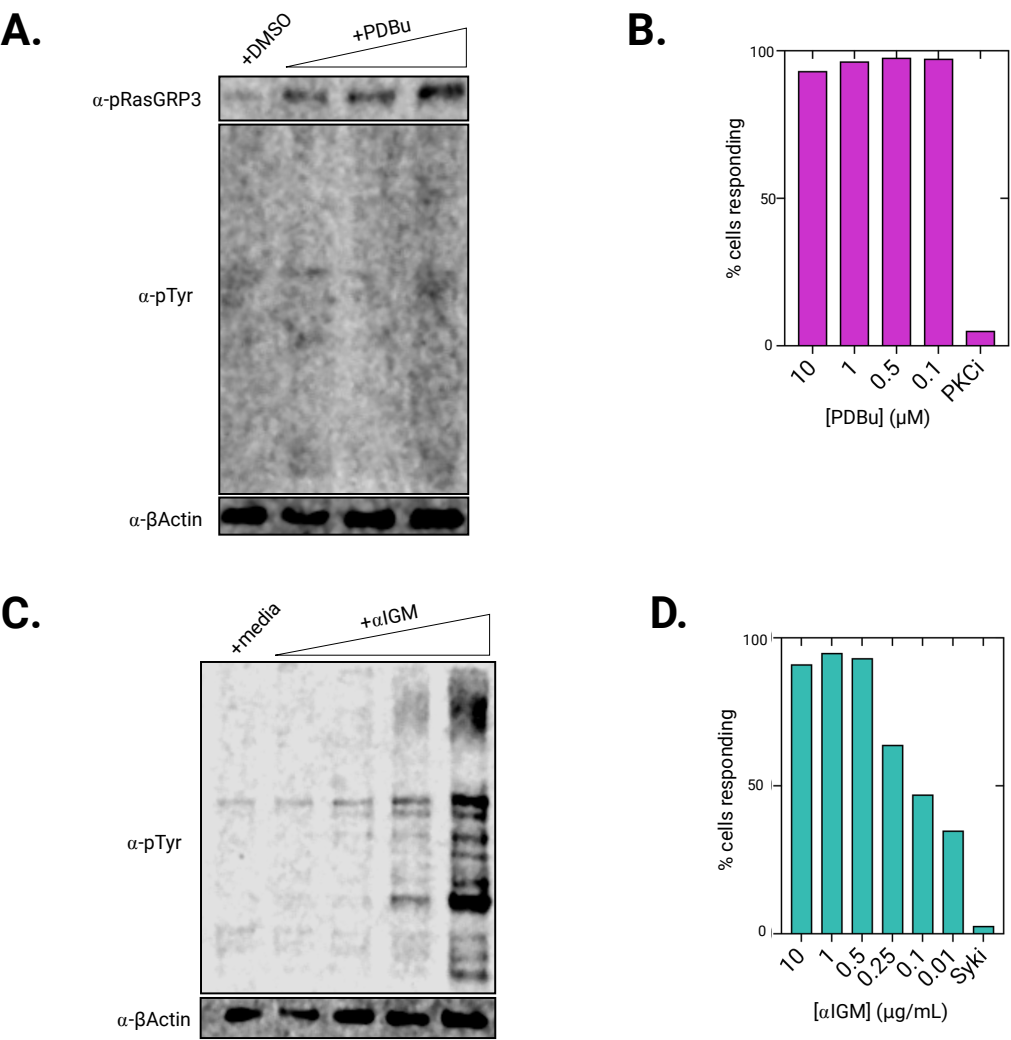


Figure S2.5. Tuning RasGRP and BCR signaling output by titrating PDBu and α IgM **(A)** Western blot analysis of RasGRP phosphorylation (T133, a PKC site), global phosphotyrosine (pTyr) and β -Actin (loading control) in wildtype Ramos cells stimulated with DMSO (0.1%) or an ascending concentration of PDBu (0.1 μ M, 1 μ M and 10 μ M, left to right). **(B)** RBD²²⁰-eGFP Ramos cells stimulated with the indicated concentration of PDBu or 50nM Gö 6938 (PKCi). Bars depict the fraction of cells responding. **(C)** Western blot analysis of RasGRP phosphorylation (T133, a PKC site), global phosphotyrosine (pTyr) and β -Actin (loading control) in wildtype Ramos cells stimulated with imaging media or an ascending concentration of α IgM (0.01 μ g/mL, 0.1 μ g/mL, 1 μ g/mL and 10 μ g/mL, left to right). **(D)** RBD²²⁰-eGFP Ramos cells stimulated with the indicated concentration of α IgM or 10nM Bay-61-3606 (Syki). Bars depict the fraction of cells responding.

FIGURE S2.6

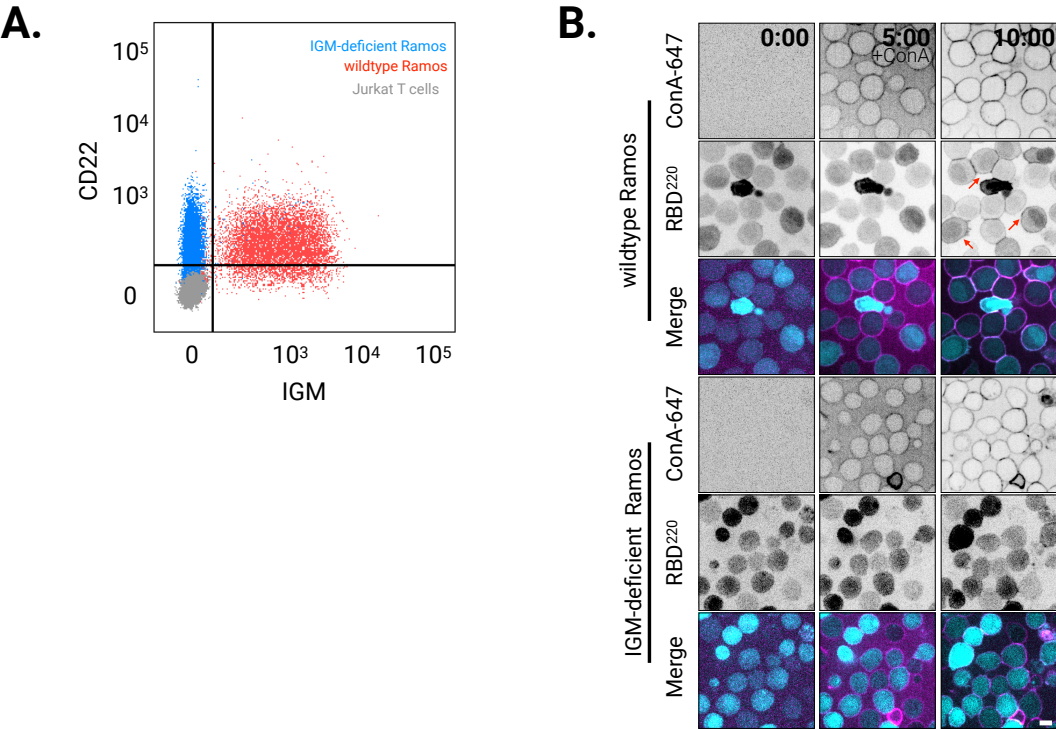
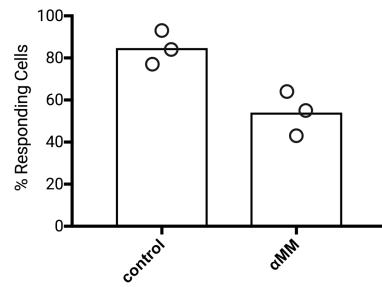


Figure S2.6. Cell Surface BCR expression is required for ConA-induced Ras activation (A) Cell surface staining profiles of IgM and CD22 in Jurkat T cells (grey, negative control), wildtype Ramos cells (red) and IgM-deficient Ramos cells (blue). **(B)** Time course stills of RBD²²⁰-eGFP expressing wildtype (top three rows) and IgM-deficient (bottom three rows) Ramos cells stimulated with 50µg/mL of 647-labeled ConA (t=5 mins). Arrows (red) indicate RBD²²⁰ membrane association. Scale bar (white, bottom right panel) is 5µm.

FIGURE S2.7

A.



B.

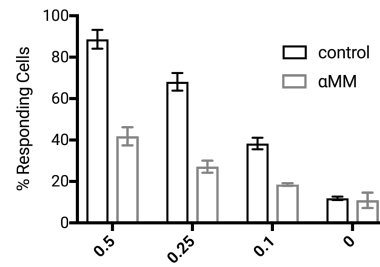


Figure S2.7. Pretreating cells with α MM partially attenuate α IgM-stimulated Ras responses **(A)** RBD²²⁰-GFP expressing Ramos cells were pretreated with either imaging media (control) or 100mM α MM (for 20 mins) prior to stimulation with 10 μ g/mL α IgM. The fraction of cells with membrane localized reporter 10 mins after α IgM stimulation is quantified. Each circle represents an independent experiment. At least 20 cells are counted per experiment. **(B)** The fraction of RBD²²⁰-GFP expressing Ramos cells responding to the indicated doses of α IgM stimulus following pretreatment with either imaging media (control) or 100mM α MM (for 20 mins) is plotted. Error bars are SEM (n=2). 0.25 μ g/mL was selected as the minimal stimulus eliciting an RBD²²⁰-GFP in cells pretreated with α MM for subsequent experiments (figure 4).

FIGURE S2.8

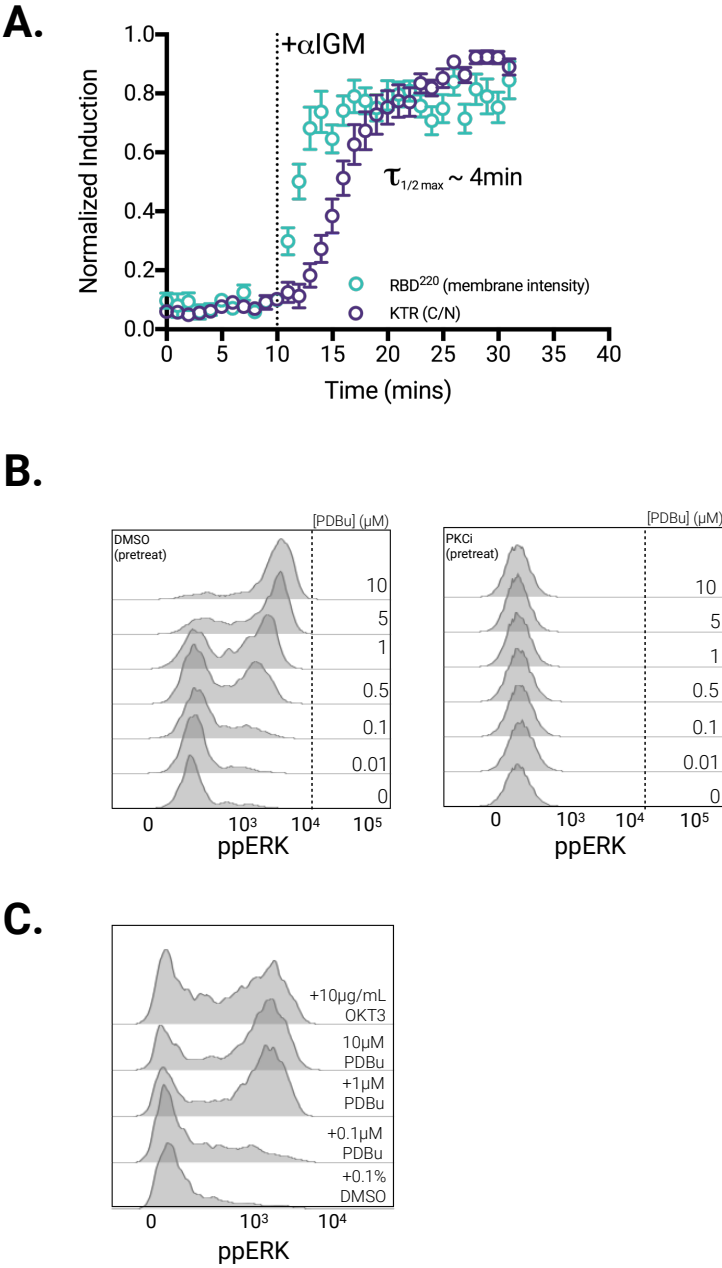


Figure S2.8. Live and fixed cell analysis of ERK activation (A) Time course analysis of RBD²²⁰membrane association and ERK-KTR activity from cells co-expressing RBD²²⁰-eGFP and ERK-KTR-TagRFP-T treated with 10µg/mL αIgM (t = 10mins). The lag time (τ) between half maximal Ras and ERK activity is indicated. **(B)** ERK phosphorylation analysis of wildtype Ramos cells pretreated with DMSO (0.1%, left) or 50nM Gö 6938 (PKCi, right), and treated with the indicated doses of PDBu for 5 mins prior to fixation. **(C)** ERK phosphorylation analysis of wildtype Jurkat T cells treated with the indicated concentrations of DMSO, PDBu or T-Cell receptor cross-linking antibody, OKT3, for 5 mins prior to fixation.

CHAPTER THREE

RAS/ERK PATHWAY MEDIATED B CELL CYTOKINE RESPONSES

SUMMARY

Cytokines allow immune cells to communicate and coordinate responses to an infection. These molecules are potent modulators of immune cell function and play a key role in both health and disease. Thus, the production of these molecules is under tight regulation. How individual signaling nodes contribute to cytokine production is unclear.

Here I take an optogenetic approach to analyze the suite of cytokines produced in a Ras signaling dependent manner in Ramos B cells. I identify several chemokines whose production are either enhanced or suppressed by Ras signaling, and speculate on the mechanistic underpinnings of these findings.

INTRODUCTION

Cytokines and chemokines play an important role in determining cell migration, differentiation and proliferation during an immune response (Dinarello., 2007). Following antigen receptor triggering, B cells produce a large number of cytokines that results in recruitment and activation of T cells and other cell types (Lund., 2008). However, the regulation of cytokine production is complex. For example, the promoters of many cytokine genes possess binding sites for multiple transcription factors (Shannon et al., 2001; Barski et al., 2017). The nuclear localization and DNA-binding capability of these transcription factors can be subject to their own regulation by any number of signaling modules (Carlson et al., 2011; Yissachar et al., 2013; Inoue et al., 2016). Once expressed, cytokine mRNAs may be degraded, stabilized or translated, subject to signaling protein activity (Palanisamy et al., 2012). Lastly, sequestration, degradation and secretion provide plausible regulatory steps for ultimate production of stimulation-induced cytokines.

While the suite of cytokines produced following BCR triggering is no doubt influenced by the affinity and dynamics of BCR-antigen interactions, it is also likely influenced by the suite of signaling modules engaged following antigen contact. We previously took an optogenetic approach to study how Ras pathway dynamics influenced the secretory response of 3T3 fibroblasts (Toettcher et al., 2013). Here I revisit this question in Ramos B cells.

RESULTS

Ramos B cells are a well-established model system for studying proximal signaling downstream of the B cell receptor. However, some groups have also leveraged this cell line to study distal gene expression events occurring following BCR triggering (Oilla et al., 2003; Van Belle et al., 2016). To study cytokine responses in these cells, I used cytokine arrays, an ELISA based assay which allows for analysis of the production of dozens of cytokines in tandem. As I was interested in Ras signaling in these cells in particular, I generated a stable Ramos cell line co-expressing a membrane targeted Phytochrome B (sPhyB-CAAX) in tandem with a PIF-tagged version of catalytic domain of the Ras activator SOS (Opto-SOS) (Figure 1). Previous work from our group showed that this combination of constructs is capable of driving fast, reversible and user-defined Ras-ERK signaling dynamics (Toettcher et al., 2013). In Ramos B cells, red-light (650nm) mediated recruitment of Opto-SOS to the membrane drove rapid induction of the ERK phosphorylation responses that was attenuated by the MEK inhibitor U0126 (Figure 1).

To identify Ras pathway induced cytokines in Ramos cells, I stimulated Opto-SOS/sPhyB-CAAX co-expressing cells with either α IgM (10 μ g/mL), α IgM+ERKi (10 μ g/mL, 10nM, respectively) or with 650nm light alone (to induce Opto-SOS membrane association). Interestingly, very few cytokines analyzed appeared to require ERK activity, as similar fold induction and reductions in cytokine production were observed in both α IgM and α IgM+ERKi samples (Figure 2).

Opto-SOS, on the hand, drove production of a range of cytokines. Notably, MDC, MCP-2 and IL1 β were produced at higher levels than either α IgM or α IgM+ERKi samples. Interestingly Opto-SOS stimulation induced a reduction of several cytokines below basal levels, including MIP1D, IL-10 and ENA-78 (Figure 2).

DISCUSSION

In this chapter, I took an optogenetic approach to identify Ras-ERK sufficient cytokines in Ramos B cells. Interestingly, I was also able to identify cytokines whose production was potentially inhibited by Ras-ERK signaling in isolation below both basal and BCR induced levels. Surprisingly, I found just one cytokine whose production was inhibited by inhibition of Ras-ERK signaling via the ERK inhibitor SCH772984.

MDC, MCP-2 and IL1 β were each produced at higher levels with Opto-SOS stimulation than with BCR crosslinking via α IgM. One hypothesis explaining this result is that Ras-ERK signaling is inhibited in some way in the context of BCR signaling that attenuates expression, mRNA stabilization, translation or secretion of these cytokines.

Interestingly, each of these cytokines contain binding sites for the hematopoietic-specific ETS-related transcription factor ELF-1. Members of the ETS family of transcription factors are phosphorylated by MAPK family members, and at least in some cases abolishes DNA binding activity (Carlson et al., 2011). Interestingly, ELF1 has several MAPK interaction motifs (Selvaraj et al., 2015). Future work may uncover whether a relationship exists between ERK, ELF1 and expression of these cytokines.

MIP1D, IL-10 and ENA78 were each repressed by Opto-SOS stimulation to a level below both basal and α IgM-induced levels. IL-10 production notably requires the MAPK effector AP-1, as well as other transcription factors (Wang et al., 2005). Interestingly, AP-1 often partners with other transcription factors at the promoter of several antigen-receptor induced genes, such as NFAT. The transcriptional program of activated lymphocytes may be dramatically rewired by loss of occupancy of one or more of these

transcription factors (Martinez et al., 2015). It will be interesting to analyze how IL-10 regulation is affected by combining Opto-SOS inputs with CA^{2+} ionophores as a means of restoring AP-1/NFAT partnership in this context.

MATERIALS AND METHODS

Cell line generation and Cell culture

Cell lines were generated by lentiviral transduction followed by cell sorting for high level sPhyB-mCherry-CAAX expression and intermediate level PIF-YFP-SOScat. Ramos B cells were cultured as described above (chapter 2).

Western Blots

Western blots were performed as described above (chapter 2). For optogenetic experiments, cells were incubated in PCB (10 μ M) in the dark for ~30 minutes prior and bathed in uniform 750nm light at 37°C prior to experiment. Cells were then stimulated with uniform 650nm light prior to lysis on ice and processing for western blot as described above.

Cytokine Arrays

Human cytokine antibody arrays (Abcam) were processed according to manufacturer direction. Cells were stimulated for ~12 hours in imaging media (see chapter 2), and media, and following cell removal by centrifugation, media was applied directly to cytokine arrays. Arrays were imaged in a LiCor imaging station, and data was processed using excel software.

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FIGURE 3.1

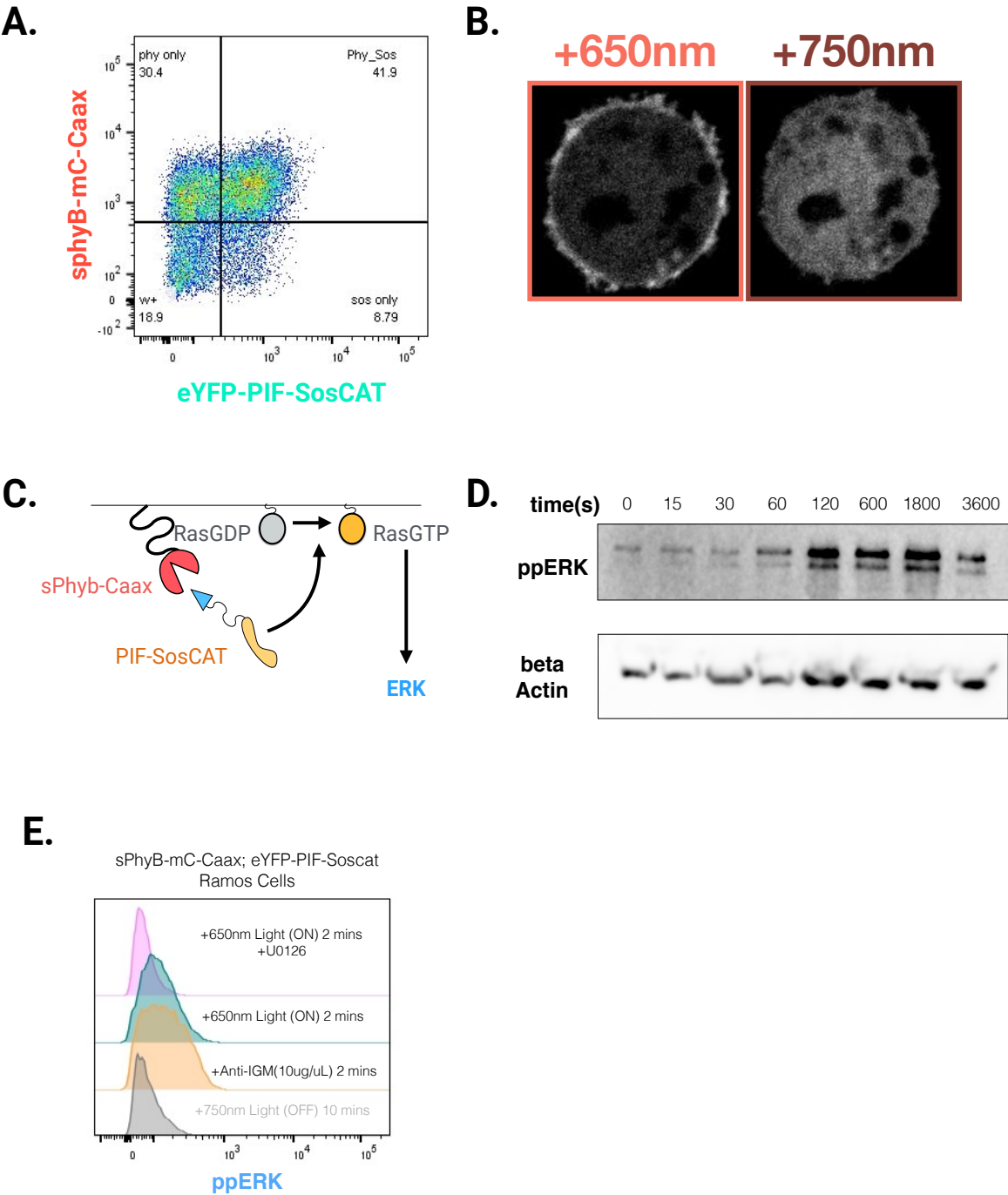


Figure 3.1. Light-Gated Ras-ERK signaling in Ramos B cells **(A)** Expression profile of a Ramos B cell line stably expressing sPhyB-mCherry-CAAX, and eYFP-PIF6-SOS2CAT (Phy/SOS Ramos). **(B)** Images depicting 650nm induced membrane recruitment in PCB (10 μ M)-treated Phy-SOS Ramos (left), and 750nm inhibition of membrane association. **(C)** Schematic depicting how light-gated recruitment of PIF-SOS2CAT to the plasma membrane drives ERK activation. **(D)** Time course western blot analysis of ERK phosphorylation in Phy/SOS Ramos cells driven by 650nm light. **(E)** Flow cytometry analysis of ERK phosphorylation in Phy/SOS Ramos cells.

FIGURE 3.2

A.

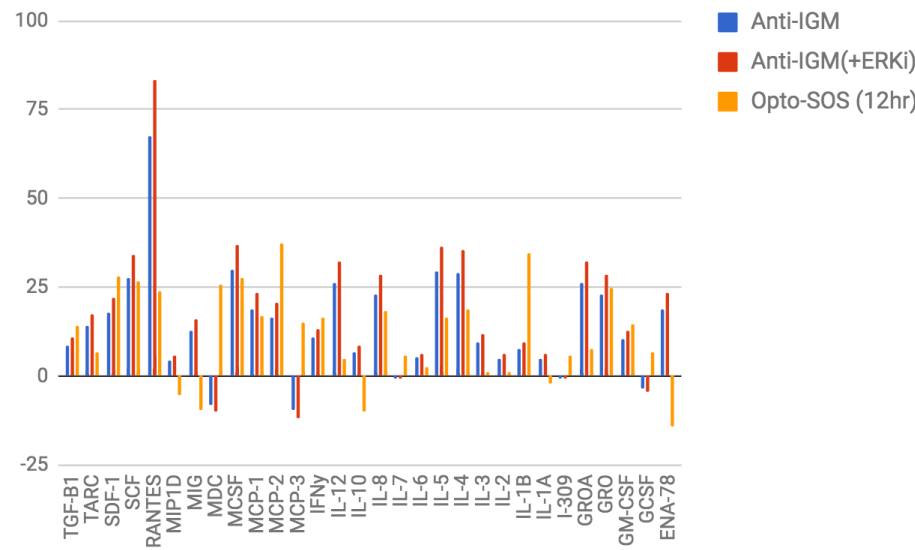


Figure 3.2. Opto-SOS induced cytokine production in Ramos B cells (A) Cytokine array analysis of a range of cytokines stimulated with α IgM (10 μ g/mL, blue), α IgM+ERKi (SCH772984, 10 μ M, red), or 650nm light (orange).

CHAPTER FOUR

OPTOGENETIC TOOLS FOR STUDYING BCR/TCR SIGNALING

"I used to say that it's like GFP...now I say it's like Cryo-EM."
- Orion Weiner (on optogenetics)

SUMMARY

Signal integration of across space and time is an important mechanism that cells use to respond appropriately to external cues. While step-function increases of cell surface ligands has been useful for studying signal transduction downstream of a number of receptors, this approach is fails to capture the dynamics of cell-cell and receptor-ligand contacts that occurs *in vivo*.

Optogenetics provides one means for recapitulating these dynamics, as light-gated proteins can be tuned by light intensity and spatial distribution. This project sought to develop a suite of optogenetic tools for controlling signaling antigen receptors signaling in lymphocytes. Using HEK-293Ts and Jurkat T cells as screening and model systems respectively, I tested and validated a Cry2 fusion proteins for their ability to induce proximal and distal T cell signaling responses in a blue-light mediated manner. One construct, a fusion between Cry2 and the kinase domain of Zap70 (Opto-Zap) was able to generate blue light inducible LAT phosphorylation in a screening assay, as well as ERK phosphorylation, CD69 upregulation and IL-2 production in Jurkat T cells. Prospects for future studies are discussed.

INTRODUCTION

Optogenetics has become an important tool for dissecting signaling pathways and networks both within and between cells. While initial uses of the technique focused on using light-gated ion-channels (i.e. rhodopsin) to control neural activity, the optogenetic tool kit has undergone a vast expansion in the last decade to include a number of different signaling currencies (Boyden et al., 2005; Li et al., 2005; Levskaya et al., 2009; Kennedy et al., 2010; Toettcher et al., 2013; Tischer and Weiner., 2014; Guntas et al., 2014; Wang et al., 2016).

In cell signaling applications, many optogenetic tools make use of light-gated homo or hetero-dimerization of components to relocalized a signaling protein (or catalytic domain) to a site of action—typically to the inner leaflet of the plasma membrane (Tischer and Weiner., 2014). This strategy is similar to one used previously by groups employing chemical dimerization systems to control cell signaling (Graef et al., 1997; Castellano et al., 2000; Suh et al., 2006; Varnai et al., 2006; Fivaz et al., 2008). One of the first pathways placed under chemical dimerization control was the T-cell receptor signaling pathway (Spencer et al., 1993; Grad et al., 1997). There, the Crabtree group used oligomerization of the CD3-zeta chains and membrane localization of the kinase Zap70 to control T cell signaling and downstream responses. It is surprising then, that to date no group has reported (in the primary literature) a set of optogenetic tools capable of light-gated induction of T-Cell (or for that matter, B cell or NK cell) signaling.

The ability to inducibly and reversibly control lymphocyte signaling would allow us to make inroads into long standing questions in lymphocyte biology. For example, integration of discontinuous TCR-pMHC contacts is proposed to play a role in cytokine secretion and T-Cell differentiation (Gett and Hodgkin., 2000; Marangoni et al., 2013; Au-Yeung et al., 2014; Khailaie et al., 2014; Textor et al., 2014; Zikerman and Au-Yeung., 2015). Antigen affinity and signal strength is a putative determinant of the amount of antibody secreted by activated B cells (Batista and Neuberger., 1998; Wheeler et al. 2013). Integration of signal strength across the cell surface is proposed to determine the polarity of lytic granule secretion in NK cells and cytotoxic T cells. In each case modulation of light intensity and spatial positioning across the cell surface as a means of controlling signal initiation would provide a novel means of tackling these problems.

The work outlined in this chapter details efforts and challenges in developing a suite of optogenetic tools for controlling lymphocyte antigen receptor signaling. It focuses primarily on using the Cryptochrome 2 (Cry2)-Cib system—a blue light induced heterodimerization system to do this (Kennedy et al., 2010). Here, I develop a tool termed Opto-ZAP—a fusion of the kinase domain of the T-Cell kinase Zap70 with Cry2 capable of inducible control of T-Cell signaling (read out via ERK phosphorylation and IL-2 production). I also generate a 293T reconstitution system for screening optogenetic tools likely capable of controlling proximal T-Cell signaling.

RESULTS

The PhyB-PIF optogenetic system is unique among light-based heterodimerization systems in that two wave lengths of light (650nm and 750nm) can be used to control the association and disassociation of the systems' components, respectively (Levskaya et al., 2009). This feature allows for fine titration of binding between PhyB and PIF by projecting ratios of 650nm and 750nm light of varying intensities onto a sample. It also allows for very fine spatial control of recruitment of PIF to a membrane anchored PhyB by projecting spatially patterned light onto a sample (Levskaya et al., 2009; Toettcher et al., 2011). However, the PhyB-PIF system is difficult to express—likely due to the size (>900 amino acids for PhyB) and complex structure of components—PhyB, for example is one of just a few proteins in the PDB that contains a structural motif called a 'knot'. These features are likely exacerbated in lymphocytes that have not yet experience antigen, as they are generally metabolically quiescent and have limited protein folding capacity.

Several strategies have been developed as work-arounds to these undesirable features of the system. Truncation of PhyB (using amino acids 1-651) has been useful for generating stable expression in organismal and cell culture systems (Buckley et al., 2016; Graziano et al., 2017). Indeed, this strategy works well in lymphocyte cell lines, as sPhyB can be readily expressed in both Jurkat T cells and Ramos B cells. However, in my hands I could never observe light gated recruitment of PIF-tagged proteins to membrane anchored PhyB in Jurkats—while, as chapter 3 outlines, I could in Ramos cells. One hypothesis is that PhyB may be post-translationally modified in Jurkat cells in a manner that renders it unable to bind PIF. While one would want to test this formally

by performing Mass spectrometry experiments with PhyB isolated from Jurkats (a system where the Phy-PIF system does not work) and 3T3 cells (a system where the Phy-PIF system works well), I was never invested enough in this project to push my experiments to that point. Phostag analysis with anti-PhyB antibodies from lysates from PhyB expressing Jurkats, 3T3 cells and purified proteins, however showed species of similar molecular weights between the two cell lines, indicating similar phospho-species in both (Figure 1). Moreover, mutation of all serines, tyrosines and threonines in PhyB (to alanine) rendered the protein poorly expressed precluding analysis of the role of phosphorylation in PIF binding in cell culture systems. Thus, a new strategy was needed in order to generate a system capable of light control of antigen receptor signaling.

The Cry2-CIB system is a blue light activated heterodimerization system that reverts in the dark (Kennedy et al., 2010). Cry2 monomers also homo-oligomerize under blue light stimulus, likely aiding signaling responses making use of this system (Bugaj et al., 2013; Duan et al., 2017). After spending a year trouble shooting PhyB based signaling systems in Jurkats, I resolved to conceive a systematic method for screening optogenetic systems with a high probability of controlling signaling responses. To do this I generated a 293T cell line stably expressing membrane targeted CIB (CIB-GFP-CAAX) the T cell scaffold protein LAT (Figure 2A). LAT is an important adapter protein involved in recruiting and activating myriad signaling proteins downstream of the TCR such as PLC γ , SOS, VAV and others. (Finco et al., 1998; Zhang et al., 1999). While LAT is normally phosphorylated by Zap70 following TCR triggering, it can also be phosphorylated by kinases of the SRC family such as LCK and LYN, and by Syk, the B

cell homolog of ZAP70 (Mukherjee et al., 2013). I generated constructs in which I fused Cry2 (and mCherry) to the kinase domain of a range of kinase domains and introduced them into CIB/LAT-293Ts by transient transfection. To test activity of these probes, I either stimulated 293T transfectants with blue light or left them in the dark for 5 minutes, lysed the cells, and analyzed LAT phosphorylation (on tyrosine 191) by western blot (Figure 2B). One challenge with this approach is that many kinase domains have high levels of activity in the absence of surrounding domains. This is especially true of SRC family kinases, and I had trouble expressing Cry2 fusions to many SRC family kinase domains at a level that did not saturate LAT phosphorylation responses in the dark (data not shown). However, using this approach I isolated three fusions (Cry2-Fyn^{KD}, Syk^{KD} and Zap70^{KD}) that had negligible LAT phosphorylation in the dark, and inducible LAT phosphorylation under blue light (Figure 2B).

When expressed in Raji B cells stably expressing a membrane targeted CIB (CIB-CAAX), Cry2-Syk^{KD} (Opto-Syk) could be rapidly relocalized and disassociated from the plasma-membrane using cycles of blue light (488nm) and darkness ($t_{1/2}$ ~5mins) (Figure 3A,B). However, Raji B cells do not have BCR-induced signaling (likely due to a mutation or failure to express a key proximal signaling component), and so were not used for follow-up studies with this construct. Instead, I stably coexpressed Cry2-Zap70^{KD} with CIB-CAAX in Jurkat cells via lentiviral transduction and tested this constructs ability to control proximal and distal antigen receptor signaling in this system. In Opto-Zap Jurkats, two minutes of blue-light stimulation induced ~3-fold increase ($p<0.05$) in ERK phosphorylation in comparison to cells left in the dark and stimulated

with a control IgG (Figure 4A). TCR crosslinking, by contrast generated ~4-fold increase in ERK phosphorylation in this cell line. Four hours of blue light stimulation induced ~3-fold median increase in CD69 cell surface expression, while OKT3 generated an ~5-fold median increase in expression of this cell surface marker (Figure 4B). Six hours of blue light-stimulation plus CD28 crosslinking with anti-CD28 antibodies generated potent (~10 fold) and seemingly bimodal increases in IL-2 production in this cell line (read out by intracellular cytokine staining, above staining levels in cells stimulated with blue light alone or left in the dark and stimulated with anti-CD28 antibodies alone (Figure 4C). These results establish the ability of Opto-Zap to serve as a for inducibly controlling both proximal and distal T-Cell responses.

DISCUSSION

Several issues to using the Opto-Zap system limit its utility for studying signaling dynamics and signal integration in T cells. First, while the kinetics of association between these proteins is fast ($t_{1/2} \sim 1\text{min}$), the kinetics of disassociation between Cry2 and CIB in the dark in cells is slow ($t_{1/2} \sim 5\text{mins}$). While this timescale may be sufficient for studying the effect of discontinuous signaling on distal T-Cell signaling readouts (i.e. cytokines, cell surface markers, etc...), it is insufficient for studying proximal signaling responses which may reach saturation in seconds post-receptor triggering. Second, while it is possible to stably express CIB-CAAX at the high levels necessary for membrane recruitment of Cry2-tagged constructs in Jurkats, it is difficult to stably express Cry2 in this manner. While I was able to isolate Jurkat cell lines expressing Opto-Zap by iterative rounds of drug selection, cell sorting and transduction, I found that these cells would lose expression of this construct over a time scale of a 2-3 weeks, likely due cell competition or gene silencing. Generating isogeneic clonal cell lines is one putative way to eliminate cell competition, and while I did not pursue this approach in this situation, I would note that I had tried this previously while testing the PhyB-PIF in Jurkats to no avail. I was therefore dissuaded from attempting this approach here. Moreover, as experiments in primary cells would require robust expression of both components, the need to generate single cell clones would sorely limit the utility of this system for studying physiological T cell responses. Lastly, since I began this particular project (in 2014), several other blue light heterodimerization systems with better technical performance than the Cry2-CIB system have been reported (Guntas et al., 2014; Wang et al., 2016). For example, the LOV2-based iLID system is smaller (and

more easily expressed) than the Cry2-CIB system, and mutagenesis to LOV2 has yielded variants with both fast and slow association and disassociation kinetics (Guntas et al., 2014; Zimmerman et al., 2016). However, it is important to note that these technical improvements may not be sufficient to solve the gene silencing or cell competition issues observed with the Cry2-based Opto-Zap system.

One potential mechanism by which cells expressing Opto-Zap could be weeded from the population is via antigen-induced cell death (Green et al., 2003). Jurkats (and other lymphocyte cell lines and primary cells) are well known for undergoing antigen exposure-induced apoptosis, and the lack of regulatory domains surrounding the Zap70 kinase domain in the Opto-ZAP construct may potentiate signaling levels sufficient to trigger this apoptotic pathway. Importantly, the rapamycin-based dimerization system used previously made use of full length (and therefore autoinhibited) Zap70 constructs (Graef et al., 1997; Yan et al., 2013). However, Cry2 fused to full length Zap70 showed no blue light-induced activity in my 293T assay, and so was not pursued in Jurkats for further testing.

In recent years, several groups have pursued a protein engineering approach in which light-controlled protein allostery is used to induce or inhibit activity of an enzyme (Dagliyan et al., 2016; Rose et al., 2017; Zhou et al., 2017). This approach has been used successfully to engineer light activation and inhibition of the MAPK pathway kinase, MEK and the Ras GEF SOS, among other proteins (Rose et al., 2017; Zhou et al., 2017). In this approach, a light or chemically controllable domain or domains (such

as LOV2 or BCL-XL/BAD) is inserted in a loop within a signaling protein in a manner that distorts or cages the active site. Then, upon light or chemical stimulus, the active site of the enzyme is allowed access to substrate via reconstitution or uncaging. Using this sort of approach for Zap70 in a manner that would allow for light gated induction or inhibition of activity, then would provide a solution to all of the problems encountered in my experiments with Opto-ZAP. Moreover, pairing this approach with CRISPR-mediated homologous recombination in cell lines or primary cells would allow for control of endogenous Zap70 (Leonetti et al., 2016; Roth et al., 2018).

MATERIALS AND METHODS

Cell Culture and Cell Lines

Jurkat T cells (a kind gift from Art Weiss) were maintained in 10G-RPMI (RPMI 1640 supplemented with L-Glutamine and 25 mM HEPES (Mediatech) and containing 1% (vol/vol) GlutaMAX (Gibco) and 10% (vol/vol) Fetal Bovine Serum (Gibco)). Cultures were maintained in a 37 °C/5% CO₂ incubator at a density ranging from 0.2-1 million cells/mL. HEK-293T cells (used to generate lentivirus for transduction) were maintained in DMEM (Mediatech) supplemented with 10% (vol/vol) Fetal Bovine Serum (Gibco). Cells were imaged in imaging media (RPMI 1640 without phenol red supplemented with 25mM HEPES, 1% L-glutamine and 0.1% fetal bovine serum).

Cloning and Constructs

All constructs were cloned into the pHR lentiviral backbone (A kind gift from Ron Vale). Cry2 constructs were tagged on the c-terminus with mCherry and a protein domain of interest via standard Gibson assembly.

Microscopy

Imaging experiments were performed in a well of a glass bottom 96 well plate (Corning) using a Nikon Eclipse Ti inverted microscope equipped with a Yokogawa CSU-X1 spinning disk confocal, a 60x 1.4 Plan Fluor objective (Nikon) and a Prime95B cMOS camera (Photometrics). 405nm, 488nm, 561nm and 640nm (LMM5, Spectral Applied

Research) laser lines were used for excitation. Blue light stimulus was provided by either 488nm excitation or illumination with a 450nm LED.

Western Blots and Flow Cytometry

Following stimulation, Jurkats and 293Ts were lysed in equivalent volumes of 2X lysis buffer (Cell Signaling Technologies) on ice for 10 mins prior to centrifugation at max speed at 4°C. Protein concentration was measured by BCA Assay (Thermo Fisher Scientific), after which samples were solubilized in 4X Sample Buffer (Bio-Rad Laboratories) and boiled at 100°C for 10 minutes. Samples were either immediately loaded onto gels for Western blot analysis or flash frozen. Bolt 17-well precast 4-12% Bis-Tris Plus gels (Thermo Fisher Scientific) were run at 200V for 25 minutes to resolve proteins. Proteins were transferred from gels to nitrocellulose membranes using a Trans-Blot semi-dry transfer apparatus (Bio-Rad) run at 20V for 1 hour. Membranes were blocked in Odyssey Blocking buffer (Li-Cor) for 1 hour, exposed to primary antibodies diluted according to manufacturer instructions in Odyssey blocking buffer for 4°C overnight, washed in TBST, and incubated in IRDye (Li-Cor) anti-rabbit and anti-mouse secondary antibodies for 30 minutes at room temperature. Following a final wash in TBST, membranes were imaged using a Li-Cor imaging station.

For flow cytometry experiments, Jurkats were stimulated for the indicated times, and either immediately stained with labeled primary antibody (i.e. CD69) at 4°C for ~30 minutes or treated with Golgistop (BD Biosciences) for ~4 hours, followed by fixation in Cytofix (BD Biosciences) at room temperature. For CD69 experiments, cells were

washed 3X in FACs buffer and run immediately on an LSR2. For IL-2 experiments, cells were permeabilized with 1X intracellular staining perm buffer (Biolegend) according to the manufacturer specifications. Following permeabilization, cells were resuspended in staining buffer (Biolegend) supplemented with 1:1000 labeled anti-human IL-2 antibody (Biolegend) for ~30 minutes. Following staining, cells were washed 3X in FACs buffer and run immediately on the LSR2.

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FIGURE 4.1

A.

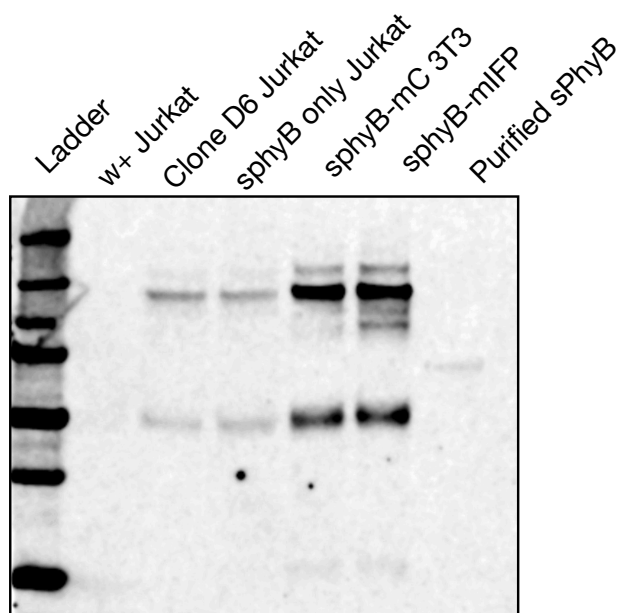
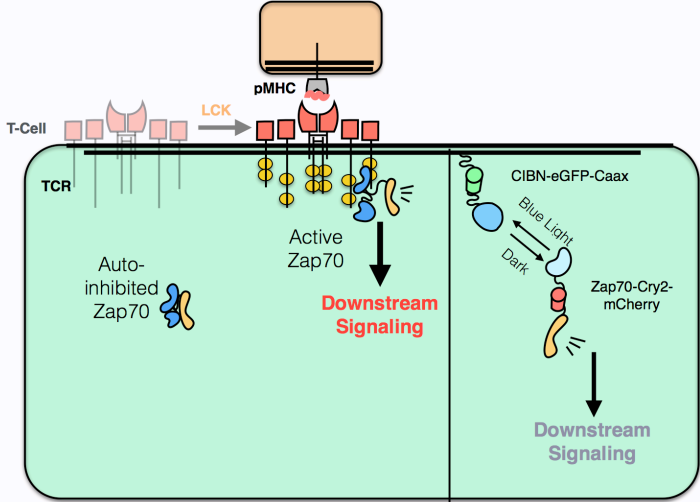


Figure 4.1. Phostag analysis of PhyB Expressing cell lines for differential phosphorylation (A) Lysates from wildtype Jurkats, a clonal Jurkat line co-expressing sPhyB-mC-CAAX and eYFP-PIF6-SOS2CAT (Clone E6), a heterogeneous Jurkat line expressing sPhyB,-mC-CAAX, sPhyB-mC-CAAX expressing 3T3 cells, sPhyB-mIFP expressing 3T3 cells, and purified sPhyB-10XHIS protein. Ladder is all-blue protein ladder.

FIGURE 4.2

A.



B.

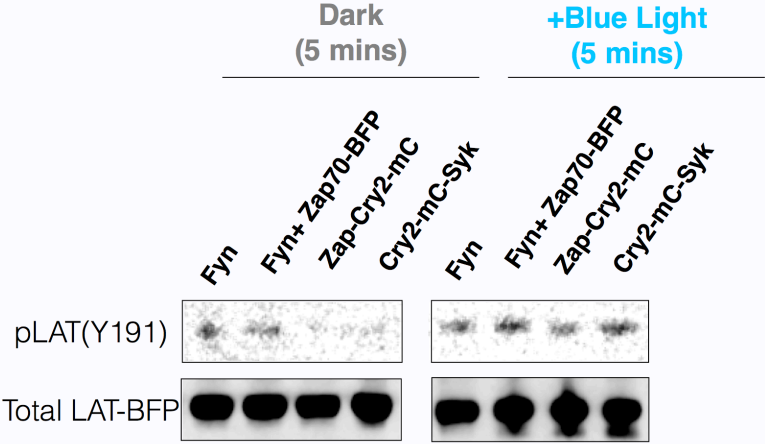


Figure 4.2. Screening CRY2-kinase domain fusion constructs for inducible LAT phosphorylation **(A)** Schematic depicting pMHC inducible recruitment and activation of ZAP70 (right) and inducible recruitment of the kinase domain of ZAP70 to the membrane as a potential means of inducing TCR signaling. **(B)** Western blot analysis of LAT phosphorylation in a 293T cell line co-expressing LAT-BFP and CIB-GFP-CAAX. Cells transfected with indicated kinases were stimulated with dark or blue light for 5 mins (to induce CRY-CIB interaction on the membrane). LAT phosphorylation is measured with mAb specific to phosphotyrosine 191 (a Zap70 phosphorylation site)

FIGURE 4.3

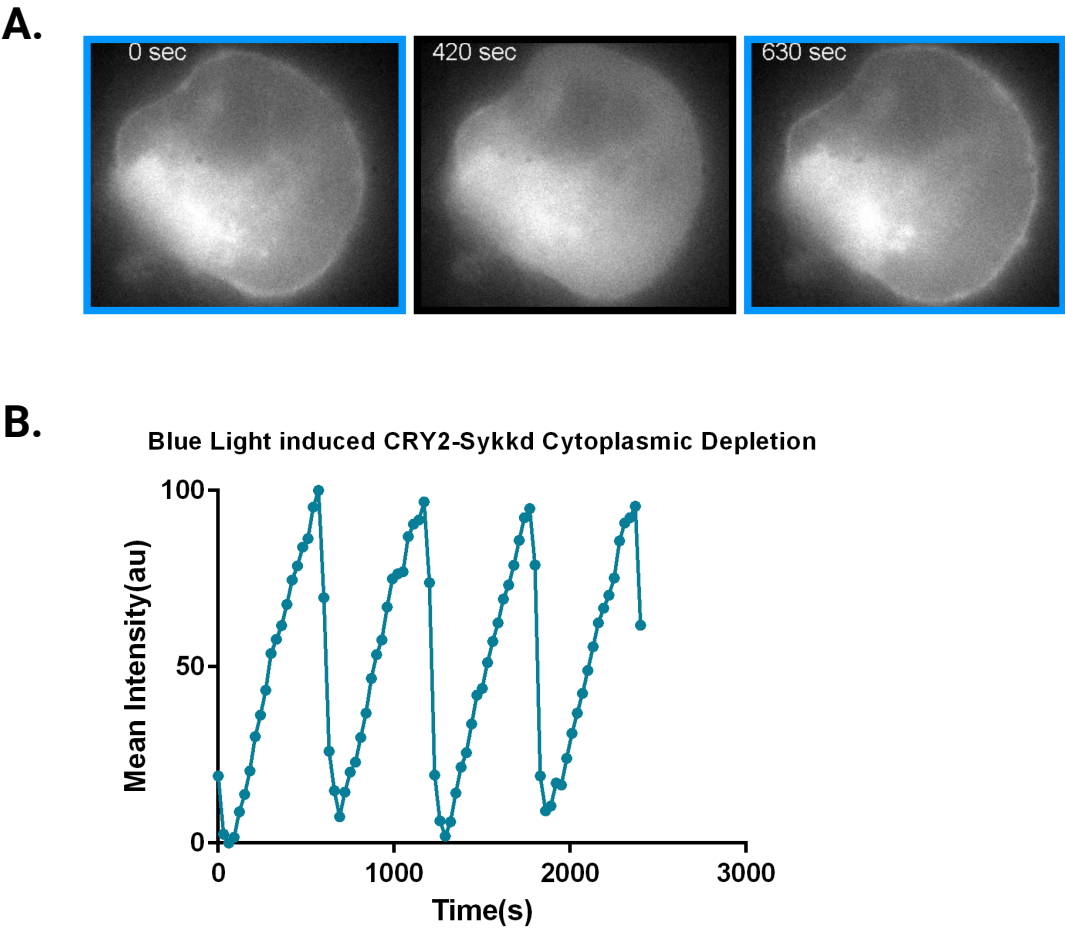


Figure 4.3. Reversible recruitment of Opto-SYK to the plasma membrane in Raji B cells (A) Time lapse stills of Cry2-mC-Syk^{KD} in cells treated with blue light (0 sec and 630 sec) and darkness (420 sec). **(B)** Quantitation of Cry2-mC-Syk^{KD} cytoplasmic intensity in cells stimulated with blue light at t= 9 mins, 19 mins and 29 mins)

FIGURE 4.4

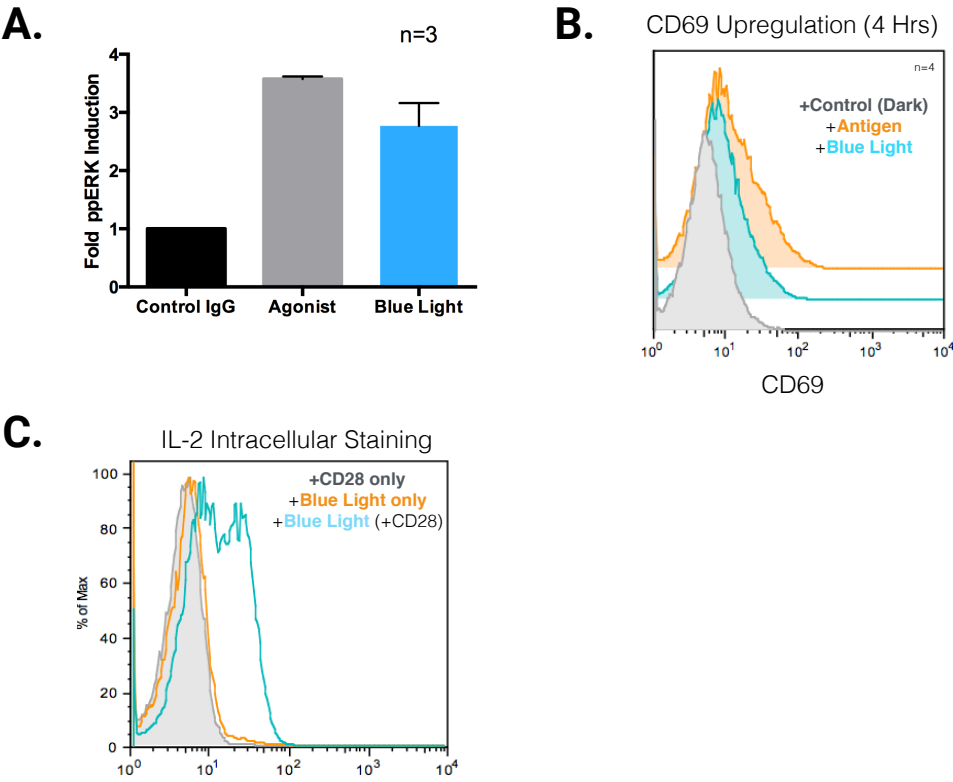


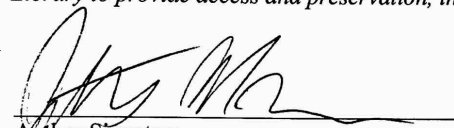
Figure 4.4. Optogenetic control of T-Cell signaling in Jurkat T cells **(A)** Quantitation of ERK phosphorylation in Cry2-mC-ZAP70^{KD} Jurkat T cells treated with IgG antibody (control, 10µg/mL), OKT3 (Agonist, 10µg/mL) or stimulated with 488nm light (blue light, 2 mins) **(B)** FACS analysis of CD69 surface expression in Cry2-mC-ZAP70^{KD} Jurkat T cells treated with darkness (grey), 488nm light (blue) or OKT3 (Agonist, 10µg/mL). **(C)** FACS analysis of intracellular IL-2 levels in Cry2-mC-ZAP70^{KD} Jurkat T cells treated with anti-CD28 mAb (10µg/mL , control, grey), 450nm LED (blue light, orange) or anti-CD28 mAb (10µg/mL) + blue light.

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