ENCODING INFORMATION AND
DIRECTING CELL FATE USING RAS/ERK SIGNALING DYNAMICS

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Abstract

The cells of developing and adult mammalian tissues are tasked with taking in information from their environment (e.g., growth cues, nutrients) and then using that information to make decisions about when and where to execute specific behaviors (e.g., proliferation, migration, differentiation). Hundreds of distinct cell surface receptors sense environmental inputs but, surprisingly, cells transmit this complex information from input to output using fewer than ten signal transduction pathways. This highlights an important question: how do cells accurately transmit complex information using so few ‘wires’? In this thesis, we examine one potential answer by studying cells’ ability to encode information in a language of time-varying or dynamic signaling activity. We focus on the Ras/Erk signaling network, an ideal system because of its pulsatile dynamics, its role as a central driver of proliferation, and the well-established suite of experimental tools for monitoring and controlling its dynamics.

Can we define the types of ‘signals’ generated by the Ras/Erk pathway and causally map their effects on cellular outputs? Here we examine this question at three levels. First, in Chapter 2, we dissect one of the Ras/Erk-regulated processes that allows it to control proliferation using optogenetic and metabolomic approaches. We discovered that extended Ras activation enhances the rate of glucose metabolism by upregulating four key nodes in the glycolytic pathway, one or more of which is consistently upregulated in human cancers. Chapter 3 focuses on the signal transduction process, as we explore how dynamic Ras/Erk signals are regulated and interpreted into proliferative decisions. We performed a drug screen in primary mouse keratinocytes and found multiple distinct classes of drug-altered Erk dynamics. We then used optogenetic manipulation to show that cells make different proliferative decisions in response to each class of drug-altered
dynamics. Finally, Chapter 4 zooms in further to focus on the Erk pulse generator itself, showing that pulses arise downstream of receptor-level processes. Our results provide fundamental insights into how pleiotropic signaling networks like Ras/Erk can use the dynamic activity of a single ‘wire’ to transmit information from upstream inputs into distinct cell fate outputs.

Collectively, this thesis draws in equal parts from classic molecular biology, engineering, biochemistry, and quantitative/systems biology to address fundamental scientific questions, from signal processing to emergent properties to cell fate control. We hope this work will inform and provide a framework for future quantitative studies of the ‘language’ of cell signaling.
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Chapter 1

Introduction

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Modern biologists and bio-engineers often draw an analogy between the cell and a computer. After all, cells and computers can both exhibit a complex mapping between inputs and outputs. In one variant of this analogy, genes are ‘programs’ that can be executed, and the machinery of transcription and translation serves as the computer’s hardware to execute the program of interest. Classic synthetic biology experiments, such as the construction of a gene oscillator or toggle switch, would then be studies in the art of cellular, rather than computer, programming\(^1\)–\(^3\).

But cells are more than digital logic machines that express genes in a precisely-defined sequence. They are equally analog robots, with a broad array of complex sensors and actuators that allow them to actively gain information about their environment and respond accordingly. And if the cell is a robot, then cell signaling – the biomolecular circuitry that operates between the cell’s exterior and its nucleus – comprises the robot’s
senses of sight, touch, and smell. This idea of signaling ‘modules’ as complex, analog circuits that enable cellular perception was explored in a seminal perspective twenty years ago. It and subsequent work ushered in an era in which we describe the functional performance of cell signaling systems using terms loaned from engineering disciplines, such as robustness, sensitivity, and gain. (Indeed, use of the word ‘robustness’ in PubMed manuscripts has increased by 20-fold in as many years, from 179 in 1997 to 3,279 in 2017.)

If anything, these early perspectives may have actually underestimated the extent of complex information processing carried out by signaling networks. Recently-developed fluorescent biosensors have revealed that individual cells naturally exhibit rich dynamics and transitions between signaling states that would have been impossible to predict from the single-timepoint, cell-averaged studies of the 20th century. Virtually every core eukaryotic signaling pathway has now been observed to undergo complex, non-monotonic dynamics in living cells, including pulses, oscillations, and even traveling waves. These dynamics are no artifact of tissue culture, as they persist in vivo and at the tissue scale: for instance, the mitogen-activated protein kinase (MAPK) Erk exhibits waves of activity that propagate across the skin of living mice. Complexity is also apparent in the spatial assembly of protein modules. A growing number of cell surface receptors, intracellular kinases, scaffold proteins, and even metabolic enzymes have been observed to assemble into higher-order complexes, rather than simple protein complexes with defined stoichiometry, and with material properties that range from liquid-like droplets to solid aggregates. Yet despite their ubiquity, the roles played by inducible protein clustering are still poorly defined.

Both the temporal behaviors of diffuse protein networks and the spatial architectures of signaling complexes are evidence of an intracellular signaling ‘code’ that we have not yet cracked. What collections of molecular interactions generate these
emergent behaviors? What features carry essential information about the cell’s sensory experiences? To achieve the ultimate goals of systems and synthetic biology – accurately predicting and programming cell behavior – we must first define the instruction set that is accessible through cell signaling, and then develop new methods to deliver these instructions to a particular cell and at a particular time (Figure 1.1).

Although there has long been considerable interest in questions of signal transmission, the field has largely lacked experimental approaches to directly control individual signal features (e.g., the timing or duration of a pulse of signaling, or the specific collection of proteins in an aggregate or droplet) and to assess their effect on downstream processes. Cellular optogenetics is ideally suited to meet this challenge, offering a modular and generalizable set of tools for controlling specific biochemical reactions in space and time. In this introductory chapter, we outline how a new generation of optogenetic techniques is enabling scientists to address this frontier of cell signaling. Much like the pioneering biochemistry that allowed scientists to define the biomolecular parts list
necessary to generate a cell response, optogenetics is poised to decode the minimum instruction set required to direct cell behaviors.

The subsequent chapters of this thesis are assembled in ascending order of granularity, moving from how dynamic signaling pathways control cell behavior, to how dynamic signals are processed, to how dynamic signals are generated. Chapter 2 begins at the systems-level, as we show that Ras/Erk signaling can direct cell behavioral outputs (i.e., proliferation) by controlling the activity of entire intracellular biochemical networks (i.e., glycolysis).

Chapter 3 of this thesis shifts the focus to how cells process dynamic Ras/Erk pathway signals. We perform a high-throughput live-imaging-based drug screen for altered Erk dynamics in primary mouse epidermal stem cells, identify previously uncharacterized inputs that regulate Erk dynamics, and use optogenetic control to demonstrate how cells interpret distinct dynamic inputs into distinct proliferative outputs.

Finally, in Chapter 4, we use live-cell imaging and optogenetic manipulation to define the molecular organization of the Erk pulse generator, showing that its oscillatory behavior does not require receptor-level inputs and instead arises via negative feedback loops acting on downstream pathway components.

By examining information transmission through three distinct levels of the Ras/Erk cascade (i.e., control of downstream processes, interpretation of signaling inputs into cellular outputs, and generation of dynamic signals), this work provides a detailed quantitative and molecular description of the language Ras/Erk signaling uses to control cell proliferation.
1.1 Optogenetic approaches for multiscale control of protein function

While light-controlled ion channels have matured into a tool in the arsenal of nearly every neurobiologist, light-gated protein tools for cell biology (the tools of so-called ‘cellular optogenetics’) are still under active development, and the field is undergoing advances in both the degree and kind of control that is achievable. It is convenient to divide the cellular optogenetic toolbox into three classes: single-protein tools, which employ individual chimeric proteins whose biological function has been made light-sensitive; dual-protein tools, where light-gated dimerization can be used to alter protein localization or protein-protein interaction; and multi-protein tools, where higher-order complexes are assembled or disassembled in response to a light stimulus. Here, we focus on a select set of recent approaches that we believe will be of particular importance for the next generation of precise perturbative experiments exploring the language of cell signaling.

1.1.1 Single-protein approaches: reversible uncaging of arbitrary linear motifs

Linear motifs play a dominant role in eukaryotic protein organization\(^\text{12}\). In contrast to well-folded protein domains, linear motifs obey a simpler set of rules, as a given motif’s function is defined solely by its primary amino acid sequence. These functions can be exceedingly diverse, from binding interactions with specific protein domains (e.g., MAPK-docking peptides or proline-rich motifs)\(^\text{13,14}\), degradation tags\(^\text{15}\), or signals that alter subcellular localization (e.g., nuclear export or localization sequences aka NESes and NLSes, respectively)\(^\text{16}\). A series of recent studies have established optogenetic systems that allow light-dependent presentation of a wide range of linear motifs, making this class of
protein-based switches arguably the first to be generally photoswitchable in living cells.

Linear motif photo-switching was enabled by engineering the LOV2 domain from *Avena sativa* Phototropin 1 (AsLOV2). Blue light stimulation of AsLOV2 causes its 20 amino acid C-terminal helix (termed the ‘Jα helix’) to become unstructured and un-dock from the photosensitive LOV domain, a process that reverses within minutes in the dark. Strickland *et al.* reasoned that, by altering Jα helix residues that are not required for docking, a novel linear motif could be encoded in the sequence and then exposed in a light-

![Diagram](image)

**Figure 1.2. Optogenetic techniques to control protein behavior.**
(a) Three prominent approaches have been developed for engineering optogenetic control over single protein activity: (left) an optogenetic switch can be used to establish light-dependent caging/uncaging of a functional linear motif; (middle) light-gated binding partners can be placed on either side of a protein’s active site to establish ‘clamshell’-based occlusion/exposure of the active site; and (right) a light-switchable hairpin domain can be engineered into a protein of interest such that light-dependent opening/closing of the hairpin allosterically alters the target protein’s active site.
(b) Two-protein optogenetic systems: (left) light-dependent heterodimerization partners, shown here controlling the subcellular localization of a target signaling domain; (right) light-dependent homodimerization partners, shown here controlling the interaction between two membrane-bound signaling domains.
(c) Multi-protein optogenetics: recent developments have enabled light-dependent assembly of protein clusters and higher-order structures by attaching intrinsically disordered protein regions (which self-aggregate) to optogenetic multimerization domains.
switchable manner\(^1^8\) (**Figure 1.2A**), right). This discovery led to an explosion of innovative LOV-based tools, including photoswitchable nuclear import\(^1^9,^2^0\), nuclear export\(^2^1,^2^2\), protein-protein binding\(^1^8,^2^3\), protein degradation\(^2^4\), and presentation of MAPK docking peptides\(^2^5\). These studies demonstrate this approach’s high degree of generality: simply fusing an engineered LOV domain to a protein of interest enables light-gated control over its subcellular localization, stability, and even interactions with specific targets.

### 1.1.2 Single-protein approaches: steric and allosteric modulation of protein activity

If LOV domains enable control over short linear motifs, what about direct optogenetic regulation of folded domains? Two recent intramolecular approaches aim to address this longstanding challenge, each using a distinct optogenetic strategy. For concision, we refer to these two approaches as photoswitchable clamshells and hairpins.

The first of these approaches is based on the idea of a ‘clamshell’ protein, where a domain of interest is flanked on both sides by sequences that can bind one another, sterically preventing the domain from associating with downstream targets (**Figure 1.2A**, middle). In such a system, chemically regulating the extent of association between these N- and C-terminal regulatory domains proved sufficient to regulate its activity\(^2^6\). To adapt this design to light-based control, Lin and colleagues engineered variants of the fluorescent protein Dronpa, whose homo-dimerization can be dissociated with light. By fusing copies of Dronpa to both the N and C termini of various target proteins, they established photoswitchable control over multiple guanine nucleotide exchange factors (GEFs), a viral protease, and a series of mammalian kinases\(^2^7\).

A second method to generate photoswitchable protein variants relies on allosteric control, rather than direct steric occlusion of an active site. Engineering allosteric
regulation is enabled by a particular feature shared by select ‘hairpin-like’ protein domains used as optogenetic and chemical biology tools: a short physical distance between the N and C termini of the domain that can be increased upon stimulation (Figure 1.2A, left). This short distance makes it possible to insert the hairpin domain into a solvent-exposed loop of a target protein without disrupting its structure. Upon stimulation, the increased distance between the hairpin termini can distort the normal conformation of the loop and induce a conformational change in the protein of interest. Such a system thus acts as a stimulus-switchable hairpin, opening and closing to alter a target protein’s binding or catalytic activity, resulting in a novel form of light-controlled allostery.

Hahn and colleagues realized that the same AsLOV2 domain described earlier has N and C termini that lie roughly 10 Å apart in the dark and that this distance is increased by light-induced unfolding of the Jα helix. In this seminal study, the authors used structural analyses to identify insertion positions whose motion is coupled to the protein’s active site, enabling them to design light-controlled allosteric hairpin switches into a number of kinases, Rho family GTPases, and GEFs. Similar approaches have now been extended to additional protein families, including the apoptotic protease caspase-3.

1.1.3 Two-protein approaches: light-gated hetero- and homodimerization

Many optogenetic tools come as a two-protein package, where a photoswitchable domain only binds to a target protein in one illumination condition (e.g., either in the dark or lit state). Such systems were among the first non-neuronal optogenetic tools to be developed and have emerged as the most widely-used optogenetic strategy in signaling biology (Figure 1.2B). These tools are primarily used to either control the localization of a single protein (i.e., by tethering one optogenetic domain to a defined
cellular location and linking its binding partner to an effector protein); or to drive the association between two proteins of interest. These are particularly powerful strategies for probing cell signaling, where altering protein localization (e.g., dimerization of cell surface receptors; membrane translocation of signaling effectors) is often sufficient to control biological function (Figure 1.2B).

At this point, many highly-efficient strategies are available for controlling homo- and hetero-dimerization with light. Notable recent advances include a series of ‘iLID’ proteins with lit-state affinities ranging from nanomolar to millimolar23-35 and the ‘ZDark’ protein which inverts the usual polarity and binds LOV domains only in the dark36. Optogenetic heterodimerizers have been used to create conditional activators of many intracellular signaling proteins, including the GTPases Ras, Rac, Rho, and Cdc42, the lipid kinase PI3K, and the Raf and Akt kinases, among others (Figure 1.2B, left)32,37,38. Similarly, optical homo-dimerization represents a powerful approach for triggering receptor activation and gene expression, primarily because self-association is often a prerequisite for activation of both receptors and transcription factors. By taking advantage of various proteins that homodimerize or aggregate upon light stimulation, it has been possible to create light-switchable receptor tyrosine kinases (RTKs)39, TGFβ receptors40, and transcription factors41,42 (Figure 1.2B, right).

1.1.4 ‘N’-protein approaches: light-dependent oligomerization and mesoscale protein clustering

Recent studies have revealed the widespread organization of proteins into so-called ‘membraneless organelles’ on a previously-unappreciated scale. Higher-order protein complexes are now appreciated to regulate diverse intracellular processes ranging from metabolic flux43 to receptor activation44,45, intracellular signaling9, and gene expression46.
Across these examples, the properties of mesoscale protein assemblies can be highly variable, ranging from gel-like aggregates to liquid-like protein droplets, but the field has lacked tools to drive transitions between these states on demand. A new suite of optogenetic tools has emerged to meet this challenge by providing precise control over the timing, location, and material properties of light-dependent protein clusters.

Photoswitchable protein clustering was first achieved using the Cryptochrome 2 (Cry2) protein from *Arabidopsis thaliana*, which forms ‘photobodies’ in plant nuclei in response to blue light\(^47\). This light-induced protein clustering was preserved when Cry2 was expressed in animal cells, and could be further enhanced by mutations that increase its oligomerization affinity\(^48,49\). Cry2 clustering was quickly put to use to control receptor clustering and activation in Wnt and RTK signaling\(^48,50\). However, the extent and kinetics of protein clustering were found to be highly variable, as clustering occurs more readily at certain intracellular locations, particularly in the nucleus or on the plasma membrane. To move beyond these subcellular idiosyncrasies, there was a clear need for better experimental techniques.

A great deal of attention has been paid to a different sort of protein cluster: those mediated by intrinsically disordered protein regions (IDRs). These flexible, unstructured domains are known to naturally phase-separate in vitro and at high intracellular concentrations\(^51\) but often fail to replicate this behavior under normal cellular conditions, likely because phase separation is itself a highly regulated cellular process. In work with the Brangwynne laboratory, we reasoned that we could engineer a form of this regulation using optogenetic clustering as a molecular ‘switch’ to regulate IDR-dependent phase separation (Figure 1.2C). Indeed, we found that a fusion of Cry2 and various IDRs could be made to cluster into light-dependent liquid-like droplets or more rigid gel-like aggregates depending on the IDR and Cry2 variant used\(^52\). This technique has since proven
to be highly general, and can be used by programming IDR to associate with ordered, multivalent scaffolds\textsuperscript{53} and to engineer light-dissociable droplets\textsuperscript{54}.

The emerging story of photoswitchable aggregation is not yet finished, and other exciting approaches continue to emerge. For instance, Inoue and colleagues recently reported that repeated arrays of light-gated heterodimerizers can form large-scale polymer networks\textsuperscript{55}. These and other innovations will continue to expand the toolbox for forming mesoscale assemblies of proteins and nucleic acids.

1.2 Connecting biological form with function using optogenetic stimuli

The tools of cellular optogenetics are opening the door to address the ‘minimum instruction set’ of cell signaling by enabling investigators to orchestrate the movement and activity of proteins inside living cells. A growing number of studies are now beginning to approach signaling biology as an engineering discipline, helping to redefine cell signaling in the language of network design and biological circuits. Here, we outline recent work that has pioneered the use of these approaches to better understand both the programming language and molecular circuitry of cell behavior.

1.2.1 Optogenetics for testing sufficiency of a pathway, protein, or molecular interaction

Can a full signaling response be activated by a single protein-protein interaction? And, in turn, is activating that signaling pathway enough to trigger a particular cellular response? Questions of biological cause and effect are fundamental to nearly every area of study, but
our understanding of these relationships has been shaped by the limits of our experimental technologies.

Typically, we consider a pathway to be ‘necessary’ if its inhibition causes an effect to vanish, and we consider it to be ‘sufficient’ if the effect can be induced by activating the pathway in isolation. But it is often difficult to truly isolate a particular pathway to test its sufficiency. For instance, many essential nodes (e.g., Erk, p53, NFκB) display rich dynamic signaling behaviors, but it has been challenging to determine whether the dynamics themselves are alone sufficient to drive cell fate because we cannot isolate the effects of a given dynamic behavior from the natural input that drives it (e.g., receptor ligands can alter dynamics but typically activate more than one pathway, and mutations or inhibitors tend to destroy dynamics altogether).

One classic example of this challenge is found in studies of dynamic cell fate control governed by the Ras/Erk MAPK pathway. Pioneering work on Erk-driven cell decision making in rat PC12 pheochromocytoma tumor cells suggested that these cells could be selectively driven to make one of two cell fate decisions simply by varying the dynamics of Ras/Erk pathway activation. Transient, pulsatile inputs were linked to cell division, while sustained signaling led to differentiation. Subsequent studies offered a possible explanation for how cells might decode persistent inputs, as it was found that the duration of nuclear Erk activity could be interpreted by the transcriptional profiles of a group of immediate early genes (IEGs). Further insight into persistence detection was gleaned from experiments showing that switching the dynamics caused by a given input was sufficient to switch the resulting cell fate. Santos et al. were able to initiate PC12 differentiation by rewiring MAPK feedback such that sustained Erk activity was generated in response to a growth factor that typically produced transient activation, suggesting that differentiating cells sense the nature of the stimulus – not its identity. Coming years before the introduction of live-cell biosensors or precise control of pathway inputs, this approach
set a conceptual standard for the field: using experimental perturbations to control a particular pathway/protein/property and assessing its sufficiency to alter a downstream cell fate response.

The story of dynamically-gated PC12 differentiation is far from complete, as statements about the sufficiency of Erk dynamics are complicated by our inability to truly isolate Ras/Erk activity from the many parallel signaling pathways co-activated by the classic growth factors used in these studies. Indeed, subsequent work has suggested that pathway combinations may play a central role in shaping PC12 cell fate choice. It seems that even framing this as a binary question of ‘transient’ versus ‘sustained’ Erk activity may be an oversimplification, as multiple reports have used live-cell biosensors to show that Erk exhibits ornate, pulsatile signaling dynamics in diverse cellular settings, ranging from cultured cells to whole living animals.

To properly address questions like these, in vitro biochemical reconstitution is widely considered the gold standard approach for demonstrating that a minimal set of molecular components are sufficient to perform a given emergent function (e.g., DNA replication from a purified polymerase and dNTPs or mitotic spindle formation from purified microtubules and synthetic chromosomes). But in many cases, all of the possible components involved in a given cellular process are not known, and the function cannot be reconstituted. In such cases, a direct solution could be to develop a form of ‘in vivo’ biochemical reconstitution’ by using intact cells as living test tubes that come pre-packaged with all necessary components. This type of approach is largely not possible using the tools of classic genetics, which are permanent and cannot be triggered at time zero. Instead, we must be able to noninvasively apply an input to a single biochemical reaction inside our living test tubes to assess whether controlling an individual node within a cell is sufficient to recreate a particular process.
An ideal experimental setup would thus need to combine real-time control of signaling inputs with dynamic live-cell readouts of downstream output (i.e., pathway activity, gene expression/protein accumulation, and subsequent cell fate). Signaling inputs can then be designed to mimic endogenous dynamics over their natural range and the resulting cellular responses can be traced, step-by-step, all the way from input to output. This approach can be used to effectively deconstruct an emergent cellular process and thus can directly test its sufficiency to drive cell physiologic outcomes. In this way, researchers can truly isolate a particular dynamic feature to dissect the cause and effect relationship between the feature itself (e.g., transient vs. sustained signaling) and a particular cell behavior (e.g., proliferation vs. differentiation).

A series of studies on dynamically controlled transcription in the yeast stress response beautifully encapsulated the first half of this approach: the authors experimentally manipulated the timing of a single input stimulus to show that its dynamics were sufficient to orchestrate distinct gene expression programs. They first established an experimental system that employed small molecule-mediated control over nuclear translocation of the stress-responsive Msn2 transcription factor using an automated microfluidic device. They then used live-cell imaging to measure how target gene reporters responded to a diverse range of input regimes that mirrored endogenous stress-induced Msn2 activity. This revealed that yeasts can encode specific information about extracellular stressors using the dynamic features of Msn2 translocation (e.g., duration, frequency, amplitude), suggesting that different regimes of signaling dynamics can be sufficient to direct cell behavior and that dynamic encoding may be a common strategy used by cells to transmit multiple types of information via a single pathway.

Optogenetics is ideally suited to tease apart questions of how signaling dynamics are interpreted into cellular responses; because light intensity can be tuned over time, we can directly alter the dynamics of pathway activation while monitoring live-cell reporters.
of cellular responses (e.g., gene expression or acquisition of a differentiated morphology).

Recent work from our group demonstrates exactly this sort of all-optical input/output analysis, providing an initial toehold into how dynamic Erk signaling is interpreted by mammalian cells. In this study, we traced the flow of information from Ras inputs to Erk activation, target gene transcription, and target protein accumulation by coupling optogenetic Ras control with live-cell fluorescent biosensors at each of these nodes\textsuperscript{75} (Figure 1.3A). We found that repeated, optimally-spaced pulses of light-driven Erk activity could be used to maximize target gene expression output, and that the optimal frequency for gene expression coincides with that observed from cells grown in highly proliferative conditions\textsuperscript{62}. Our findings thus suggest that the frequency, but not the level, of Erk activity can be the determining factor in target gene expression.

**Figure 1.3.** Testing sufficiency using optogenetic control to mimic endogenous dynamics.

(a) Transcriptional dynamics of Erk target genes in response to time-varying Ras/Erk inputs. For a set of five immediate early genes, maximum transcriptional output can be achieved by stimulating cells with an optimal, intermediate pulse frequency.

(b) Dynamics of the naturally oscillatory transcription factor Ascl1 during neural progenitor cell (NPC) fate decision making. Using optogenetic control over Ascl1, NPCs can be driven either to differentiate with sustained signaling input or to maintain a multipotent progenitor state with oscillatory inputs.
Although the above study links signaling dynamics to gene expression, there are still a scarcity of examples where such dynamics have been shown to control actual all-or-none cell fate decisions. However, in a landmark study, Imayoshi et al. used precise optogenetic control of temporal signaling activity to demonstrate that cell fate determination in neural progenitor cells (NPCs) is dynamically gated by the naturally oscillatory transcription factor Ascl1 \(^{76}\) (**Figure 1.3B**). NPCs are known to differentiate into multiple neuronal cell types in the developing mammalian brain, and while these distinct cell fate choices were shown to correlate with the oscillatory expression of Ascl1 and other transcription factors, their sufficiency had not previously been examined \(^{77,78}\). To address this question, the authors developed a system in which experimental inputs were the only source of Ascl1 dynamics: they inserted a light-dependent Ascl1 transgene into primary NPCs in which both endogenous copies of Ascl1 had been knocked out (removing the complication of underlying endogenous signals). They then exposed cells to either oscillatory or sustained light inputs and found that, while oscillatory Ascl1 dynamics maintained a multipotent progenitor state, sustained Ascl1 expression promoted neuronal differentiation. This approach represents an important experimental paradigm: one can directly test the sufficiency of a specific dynamic signaling feature (e.g., oscillations) by creating a feedback-less ‘open-loop’ system in which user-defined inputs are the only source of dynamics. In doing so, the authors definitively showed that different dynamics at a single node are, by themselves, sufficient to direct divergent cell fate choices.

### 1.2.2 Non-natural inputs to dissect network design in large signal processing circuits

Taking even more direct experimental cues from engineering disciplines, biologists have also used precise input control to define how protein networks are designed by measuring
cell responses over a non-natural range of precisely applied, time-varying inputs. Rather than simply recreating endogenous dynamic patterns, this type of engineering-style science can be used to simplify and understand the wiring of complex signaling networks. For instance, we can characterize the input/output relationships of linear systems using frequency-response experiments, or we can infer the properties of an unmeasurable “System 1” by precisely applying upstream inputs and measuring how the combined circuit of “System 1 + System 2” responds. In doing so, precise non-natural stimuli can be used to learn about and define the functions of a particular pathway even if the details of its molecular circuitry are unknown.

There is a rich history of leveraging precise temporal control of signaling networks in systems biology, as Hodgkin and Huxley were famously able to understand how neurons transmit signals long before ion channels had even been discovered\cite{hodgkin1952}. Over the past decade, researchers have taken similar cues from systems engineering to map the function of cell signaling pathways. Notably, studies of the yeast hyper-osmolarity glycerol (HOG) MAPK signaling pathway were able to outline its network architecture by applying non-natural time-varying inputs using modern microfluidic tools\cite{ashburner2006, ashburner2007}. Similar input/output analyses enabled Howard Berg’s group to characterize how signals are processed during bacterial chemotaxis\cite{berg1972, berg1973}. The authors were able to define the nature of feedback coupling between the chemotaxis receptor module (i.e., pathway activation) and the adaptation module (i.e., receptor inhibition) by applying sophisticated time-varying stimuli and measuring downstream signaling response using only a live-cell biosensor. Much like Hodgkin and Huxley a half century before, these studies thoroughly characterized the functions of complex biochemical signaling networks without ever having to measure or define their individual components.

However, most molecular/chemical inputs cannot be applied in this way, and thus this type of input/output analysis has historically only been possible in systems that can
be manipulated with mechanical stimuli (e.g., electric currents or ion flux leading to osmotic pressure changes) or with chemical stimuli where binding interactions are very weak and quickly reversible (e.g., bacterial chemotaxis). As optogenetic tools continue to improve and gain traction, great opportunities have emerged for engineering-style analyses to uncover the coordinated behaviors and design principles of large protein networks across all fields of biology.

For instance, mammalian cells are known to respond to various extracellular cues by mobilizing oscillations of intracellular $Ca^{2+}$ to activate $Ca^{2+}$-responsive transcription factors, and decades of research had linked the frequency of these oscillations to subsequent cell fate decisions.$^{87,88}$ Recently, Hannanta-anan and Chow were able to directly test these assumptions for the first time by building an optogenetic system to control intracellular $Ca^{2+}$ and using it to independently vary oscillations’ frequency, amplitude, and duty cycle (i.e., the “cumulative load” of $Ca^{2+}$ input)$^{89}$ (Figure 1.4A). By varying each of these features in isolation and measuring the resulting $Ca^{2+}$-dependent transcription, they showed that gene expression output of this pathway is specifically

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**Figure 1.4. Applying non-natural inputs to map network design features.**

(a) The frequency- and dose-responsiveness of calcium-dependent gene activation was mapped by holding one parameter constant and varying the other. Applying these non-natural input regimes showed that calcium-responsive transcriptional output depends on total calcium input received, rather than frequency of calcium oscillations.

(b) Applying non-physiological oscillatory osmotic inputs reveals an Achilles’ heel in the yeast hyperosmolarity signaling response, as maximum growth inhibition is achieved at an input ‘resonance’ frequency that achieves maximum pathway activation.
determined by the duty cycle rather than frequency of oscillations. Notably, mapping input-output relationships in this way does not define specific molecular mechanisms; rather, it can tell us what types of signals a protein network is designed to respond to and can thus be used to define where and what we look for in future mechanistic studies. As an example, a possible first order experiment to identify the signal integrators in this Ca\textsuperscript{2+}\,-responsive circuit might be to systematically increase input duty cycle and screen for downstream pathway components that slowly accumulate in cells over time.

In addition to exploring the types of inputs to which cells can respond, non-natural stimuli can by definition push signaling networks beyond their natural extremes to produce phenotypes that are not seen in nature. This allows us to explore the limits of cell signaling, potentially uncovering hidden pathway vulnerabilities by applying classes of inputs that, while never encountered in a cell’s natural environment, are detrimental to cell health. For instance, Mitchell et al. were able to identify an Achilles’ heel in the yeast hyperosmolarity response by applying non-physiologic oscillatory inputs over a wide range of experimental frequencies\textsuperscript{90} (Figure 1.4B). In nature, yeasts most often experience gradual, evaporation-induced increases in osmolarity and have thus evolved a stress response that ‘resets’ quickly after a step increase in osmolarity, as it readies itself to respond to subsequent step increases. However, because of this rapid adaptation, the authors discovered that a particular resonance frequency of non-natural osmolarity stimuli causes repeated hyperactivation of the yeast stress response, which had deleterious effects on cell health.

These findings are particularly relevant in light of a recent study in which Bugaj et al. performed so-called ‘optogenetic profiling’ of cancer cell signaling using non-natural inputs\textsuperscript{91}. This revealed that a particular oncogenic mutation (i.e., B-Raf G469A) rewire the Ras/Erk signaling network such that cells ‘misinterpret’ subthreshold inputs as proliferative signals. This type of approach holds promise not only for understanding the
functional consequences of a given mutation or pathway alteration, but also for potentially identifying similar network vulnerabilities and Achilles’ heels in rewired cancer cells that could be exploited for future generations of dynamically-targeted therapeutics.

1.2.3 Precise control over biochemical and biophysical parameters to test models of signal transmission

Many processes in biology undergo an abrupt transition above a critical parameter value. This can be the irreversible commitment to a new cell cycle phase that occurs above a critical cyclin/Cdk activity level, the formation of phase-separated protein/RNA droplets above a saturated concentration, or, in the case of T cell kinetic proofreading, the activation of a signaling response above a critical duration of receptor-ligand binding. Recent efforts to understand these phenomena have uncovered a third unique use for precise input controls, showing that they can be applied at a much more microscopic, granular level to dissect biochemical mechanisms. By rapidly varying individual ‘control parameters’ that govern specific all-or-none transition processes in living cells, investigators are able to map exactly how small variations in input can drive qualitative changes in a cell’s response.

Until recently, this level of experimental precision was largely unachievable, as the most common technique used to alter the function of a biological system involved engineering point mutations into a protein sequence. This is an effective but imperfect approach, because mutations are inherently discontinuous (features like binding affinity ‘jump’ from one value to another, with limited access to intermediate values); can cause multiple parameters to vary simultaneously (e.g., a tight-binding mutation can alter both the fraction of bound complexes and the dissociation kinetics of the complex); and are essentially permanent modifications, preventing the researcher from accessing distinct
parameter values within a single cell over time or at distinct spatial locations. Optogenetic manipulations present a potential solution, as visible light is a continuous parameter whose spatial range, intensity, and duration can be precisely controlled, thereby allowing us to study transitions in biology at previously unachievable levels of resolution.

To motivate consideration of how precise stimuli can be used at this level, we will first consider the case of receptor plasticity. Much like the signaling plasticity commonly observed at the level of whole signaling pathways (e.g., activation of the Ras/Erk pathway can lead to a number of distinct downstream cell behaviors), we see a similar degree of internal variation when considering how a single receptor transduces signals across the cell membrane.

An extreme case is found in the behavior of the T cell receptor (TCR), which is capable of binding to two broad classes of peptide-MHC complexes (pMHCs) – those containing self-antigens and foreign peptides – but only triggers a T cell response in the latter case\(^92\). Such signal transduction plasticity is thought to be a general phenomenon across different receptor families: EGFRs are capable of binding a wide variety of different ligands that can each trigger a distinct downstream response\(^93,94\), and multiple different signaling responses can be generated when a single interferon receptor heterodimer binds one of its nearly 25 possible ligands\(^95\).

In each case, the response initiated by a single type of receptor has long been thought to be governed by two parameters: the duration of ligand binding (allowing the cell to decouple the number of bound receptors from the affinity of a particular ligand-receptor complex), and the formation of mesoscale clusters of receptors (allowing the cell to alter or adapt its instantaneous ligand sensitivity).
Techniques enabling precise optogenetic control over these input parameters have now opened the door to studying how transitions in biology are governed by both. In two recent studies, optogenetic control over receptor-ligand binding was used to address the contribution of binding duration to T cell kinetic proofreading. The idea: replace the TCR’s extracellular ligand binding domain with an optogenetically-controllable binding interaction, enabling user-defined control over the exact duration of individual TCR-ligand signaling complex interactions\textsuperscript{96,97} (Figure 1.5A). Both studies use light to impose a wide range of receptor-ligand dwell times and then monitor the magnitude of subsequent TCR activation, and both converge on the same key finding: the duration of signaling complex formation is all-important for triggering downstream responses, not the number.

Figure 1.5. Optogenetic tuning of biochemical and biophysical parameters to test modes of signal transduction.
(a) Placing the TCR/pMHC interaction under light-gated control allows precise manipulation of binding parameters, providing experimental proof for the kinetic proofreading model of T cell activation.
(b) Protein clusters can maintain spatial asymmetry within cells. Optogenetic control over the dissolution of phase-separated protein clusters can be used to establish light-defined spatial asymmetries that are retained long after the removal of stimulus.
of complexes that are instantaneously present. Thus, the authors’ ability to isolate time of binding from all other associated parameters allowed them to define duration as the key control parameter that governs TCR kinetic proofreading.

Although dynamic regulation is widespread, spatial cues can be equally important in driving key all-or-none transitions in biology. Classical examples of spatial regulation include the interpretation of continuous morphogen gradients to discrete domains of gene expression during embryogenesis, the asymmetric condensation of P granules in the C. elegans embryo, and the front-back polarization of migrating cells. Traditional chemical-biology approaches are ill-suited to alter these complex spatiotemporal patterns, largely because slow binding kinetics and fast diffusion limit their precision. Because light can be patterned with high spatial precision, optogenetics is ideal to probe how spatially-restricted signals are sensed and interpreted by cells. This precision has enabled innovative studies in which spatially-defined optogenetic inputs were used to interrogate polarized collective epithelial cell movement, the local effects of cytoskeletal transport on axon outgrowth, and the impact of local cell contractility on global tissue organization during embryogenesis.

Another illustrative example of spatial control can be found in a recent study from our group, where we took advantage of precise input control to dissect a process by which spatial transitions may arise. We developed an optogenetic platform that we call ‘PixELLS’ where dark state proteins form phase-separated liquid droplets that can be instantly dissolved with light. We found that illuminating one region of the cell resulted in local dissolution of droplets and diffusion of monomeric protein to the dark side of the cell, setting up an asymmetric pattern of liquid droplets within minutes. Strikingly, cells retained this pattern for hours after light was removed, demonstrating that phase-separated bodies possess a form of long-term spatial memory that does not require any additional biochemical positive or negative feedback loops (Figure 1.5B). The future
appears bright for studies of biological transitions, as one can imagine how this or similar techniques might be applied to dissect the interpretation of spatial patterns in directed cell migration or the establishment, maintenance, and interpretation of morphogen gradients in embryogenesis or regenerating tissues.

1.3 Future challenges

Cellular optogenetics has accomplished a great deal in its nearly ten years of existence, but a number of challenges still lie ahead as this field matures into adolescence. With a few notable exceptions, we still lack the tools to plug in light as the sole source of protein activity at particular signaling nodes. Light-induced activity is often summed with activity from the endogenous pathway, and gain-of-function optogenetic systems cannot ‘carve into’ or disrupt this endogenous pattern. For instance, optogenetic control over Ras/Erk pathway activity in fly embryos can add additional signaling at the middle of an embryo but cannot decrease the endogenous Erk signaling found at the embryo’s poles\textsuperscript{105}. A possible solution is to combine optogenetic control with genetic replacement by exchanging endogenous proteins for light-controllable variants or expressing optogenetic variants in a genetically null background\textsuperscript{76}. With the increasing ease of CRISPR-based gene modification, such ‘knock-sideways’ techniques may be more broadly applied in the coming years.

Multi-color optogenetics represents a second emerging frontier, as it would enable real-time control over stimulus combinations, not just dynamics. For instance, endogenous receptor-level growth factor stimulation typically results in the combinatorial activation of multiple signaling cascades, meaning these pathways are rarely activated in isolation. Thus,
an important remaining challenge in cellular optogenetics is to establish the ability to mimic receptor-level inputs using orthogonal control over multiple distinct signaling pathways in the same cell. However, such applications have been challenging because most of the tools of cellular optogenetics (including all Cry-, LOV-, and BLUF domain-containing proteins) respond to blue light with broad, overlapping excitation spectra.

One solution may come from a different family of photosensitive proteins: the phytochromes. Many phytochromes are red-light-sensitive (thus immediately providing a second stimulus wavelength); moreover, recent studies have identified phytochrome family members that respond to many additional wavelengths, suggesting that it may be possible to engineer novel ‘colors’ of optogenetic tools. A second reason for optimism is that, because phytochromes are ‘photochromic’ (i.e., switched ON and OFF with two different wavelengths of light), they can be multiplexed with any number of fluorescent biosensors without altering the input received by the cell. This can be achieved because, even when imaging with wavelengths that activate Phy, a brief pulse of inactivating 750 nm light can instantly revert Phy back to an OFF state before a cellular response is initiated. Notably, phytochrome-based systems have earned a reputation as difficult to use because they typically require addition of the small-molecule chromophores phytochromobilin or phycocyanobilin, which can be cumbersome to purify and add, especially in vivo where delivery and clearance may be limiting. Recent methodologies for chromophore production in mammalian cells, as well as the development of phytochrome-based tools using biliverdin as a chromophore, suggest that these difficulties may soon be overcome.

The generalization of cellular optogenetics also faces challenges of practicality for systems in which optogenetic manipulation is either not possible or requires significant
departures from cell physiology. *In vivo* optogenetic studies also face added obstacles, as they typically necessitate the arduous process of breeding an entirely new strain of one’s model organism of choice. As such, there remains an unmet need for tools that remove the ‘genetics’ from optogenetics, enabling optical control over a given process without requiring genetic alteration. Efforts to address this by developing non-genetically-encoded light-based tools to manipulate endogenous proteins will require techniques to control the activity of molecules that bind to or otherwise regulate a protein of interest. This could conceivably be done by sequestering/releasing endogenous ligands, or by engineering photoswitchable agonists/antagonists\textsuperscript{110,111}. Such tools represent an important frontier for light-based cellular control that would greatly expand the scale of questions signaling biologists are able to ask.

In sum, optogenetic inputs coupled with downstream live-cell reporters are now enabling a new generation of engineering-informed signaling biology studies that test how specific spatiotemporal signals dictate information flow and cell decision-making. The studies and emerging scientific frameworks outlined in this chapter have helped reshape how we view cell signaling, but we are only just beginning to understand the design principles that underlie cells’ ability to encode information and program intricate behaviors. As the field of cellular optogenetics enters its second decade, we look forward to continued innovations that will provide fundamental insights into how cells encode/decode information, how this information is integrated to inform cell fate decisions, and how pathologic alterations in these dynamic networks contribute to disease.
Chapter 2

Ras/Erk signaling regulates glycolytic flux via four key nodes

Altered glycolysis is a hallmark of diseases including diabetes and cancer, in which activation of oncogenes such as Ras drive enhanced aerobic glycolysis, a phenomenon known as the Warburg effect. Despite intensive study of the contributions of individual glycolytic enzymes, systematic analyses of how cells control the rate (flux) of glycolysis remain limited. Here, in two mammalian cell lines, we overexpress enzymes catalyzing each of the 12 steps linking extracellular glucose to excreted lactate, and find substantial flux control at four steps: glucose import, hexokinase, phosphofructokinase, and lactate export (and notably, at no steps of lower glycolysis). These four steps are specifically upregulated by Ras/Erk signaling: optogenetic Ras activation rapidly induces the transcription of isozymes catalyzing these four steps and enhances glycolysis. Moreover, we show that at >1 isozyme catalyzing each of these four steps is consistently elevated in human tumors. Thus, flux control in glycolysis is concentrated in four key enzymatic steps, whose up-regulation in tumors likely underlies increased glycolysis linked to the Warburg effect. The work in this chapter was done in collaboration with Lukas Tanner, a post-doc
in the lab of Joshua Rabinowitz at Princeton University who led the project and is lead author on the final publication\textsuperscript{112}.

2.1 Glycolysis, signaling, and cancer

Glycolysis provides cellular energy and metabolic precursors for biomass production. Seminal studies performed over the past hundred years have established the mechanism and regulation of the ten enzymatic steps of glycolysis, which together catalyze the breakdown of glucose into two molecules of pyruvate. These ten steps can be thought of in combination with the lactate dehydrogenase reaction and two transport events – the uptake of glucose via glucose transporters and the excretion of lactate via monocarboxylate transporters – as together forming 13 potential nodes of regulation that directly control flux through glycolysis.

Control of glycolytic rate plays an important role in mammalian physiology, contributing to circulating glucose homeostasis and providing ATP and/or biomass building blocks in contexts such as cell proliferation, immune activation, and angiogenesis\textsuperscript{113–116}. Conversely, dysregulated glucose metabolism is a hallmark of diseases including diabetes and cancer.

Cancer cells extensively ferment glucose even in the presence of adequate oxygen\textsuperscript{117}. While initially attributed by Warburg to defective mitochondria, it is now clear that most cancer cells have functional mitochondria that account for much of their ATP production\textsuperscript{118–120}. Accordingly, we use the term ‘Warburg effect’ to refer to rapid aerobic glycolysis in cancer cells, irrespective of their oxidative phosphorylation usage. It has been argued that the Warburg effect promotes tumor growth by satisfying cancer cells’ high demand for both energy and central carbon metabolites for biosynthesis\textsuperscript{121}. The Warburg effect can be triggered by oncogenic mutations (e.g., in Ras, PI3K, c-Myc) and by environmental cues (e.g., growth factors)\textsuperscript{122–126}. 


Consistent with their high use of glycolysis, cancer cells can exhibit increased expression of most glycolytic enzymes\textsuperscript{124}. High expression levels of the glucose transporters GLUT1 and GLUT3 is associated with augmented glucose uptake and oncogenic growth\textsuperscript{127–129}. Elevated activities of hexokinase and phosphofructokinase favor tumor initiation, immune cell activation, and angiogenesis\textsuperscript{114–116, 130–134}. Aldolase A (ALDOA) has been shown to boost glycolysis upon phosphoinositide 3-kinase (PI3K/Akt) signaling\textsuperscript{135}. When upper glycolysis is activated in cancer cells, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been reported to become a rate-limiting pathway step\textsuperscript{136}. The importance of the final enzyme involved in pyruvate production, pyruvate kinase, to glycolytic flux control remains controversial. Earlier studies advocated for the PKM2 isoform as a key driver of the Warburg effect, but recent evidence suggests that the situation is more complex\textsuperscript{137–139}. Finally, lactate dehydrogenase A (LDHA) has been implicated as essential for c-Myc mediated transformation\textsuperscript{126}. Thus, nearly every enzyme linking glucose to lactate has been associated in some study with enhancing glycolytic flux.

Despite this extensive literature on glycolysis and the Warburg effect, a unified view of how cells control glycolytic flux is lacking. In particular, the key glycolytic enzymes whose upregulation is required for flux enhancement remains unclear. This in part likely reflects variation in flux control across cell lines and environmental conditions. It might also, however, reflect a failure to comprehensively experimentally probe the pathway. To obtain a thorough understanding of flux control in any given biochemical pathway, it is valuable to systematically perturb each component to assess its contribution to overall pathway control. However, previous studies have largely focused on the role of specific glycolytic enzymes in isolation, and efforts to systematically interrogate the entire pathway have primarily been theoretical and computational\textsuperscript{136, 140}.

Here we examine, in two mouse cell lines, the degree of flux control residing in each step of glycolysis by systematically overexpressing enzymes that catalyze every
glycolytic step, and by subsequently analyzing pathway flux. We find that across all enzymes tested, only four steps show significant flux control: the two committed steps of upper glycolysis (i.e., the phosphorylation of 6-carbon sugars by hexokinase and phosphofructokinase) strongly regulate flux in both cell lines, while we see that glucose import and lactate export regulate flux in one cell line but not the other. Notably, in both cell lines, the enzymes of lower glycolysis do not control glycolytic flux.

We further show that acute optogenetic stimulation of Ras is sufficient to enhance glycolytic flux and that Ras/Erk signaling specifically activates the transcription of genes encoding the same four steps revealed in our overexpression experiments. Finally, analysis of expression data from solid tumors and paired normal tissue samples reveals a transcriptional signature in which gene isoforms from each of these four steps are strongly and consistently upregulated across tumor types. While other enzymes may play additional regulatory roles in specific contexts, our findings show that glycolytic flux is controlled at a small number of pathway steps whose upregulation underlies increased glycolysis linked to the Warburg effect.

2.2 Systematic enzyme overexpression can reveal glycolytic flux control

To systematically identify flux-controlling steps in glycolysis, we transiently overexpressed at least one human isozyme catalyzing every pathway step from import of extracellular glucose to secretion of lactate. The specific isozymes were chosen based on their high expression in glycolytic cells and human cancers. We tested multiple isozymes of glucose transporters, phosphofructokinases (PFKs), and pyruvate kinases. In addition, we included fructose-1,6-bisphosphatase (FBP1), pyruvate dehydrogenase kinase 1 (PDK1), and the four isozymes of the phosphofructokinase 2 (PFK2) family, which phosphorylate
fructose-6-phosphate to make fructose-2,6-bisphosphate, a key activator of the glycolytic enzyme phosphofructokinase 1 (PFK1). PFK2 isozymes, which are known as PFKFB1-4, are bifunctional and also contain phosphatase domains that can consume fructose-2,6-bisphosphate\(^{131}\). In total, we tested a set of 24 glycolysis-related genes. Induced expression of GFP was used as a negative control.

Our initial experiments were performed in immortalized baby mouse kidney cells (iBMK), which were selected for their extensive metabolic characterization\(^{141}\) and high transfection efficiency. To minimize possible metabolic alterations induced by adaptation, we measured glycolytic flux within the first 30 h after transfection (Figure 2.1A, B.1A-B). Experiments were performed in both 25 mM glucose (common tissue culture concentration) and 5 mM glucose (typical human circulating concentration).

Each of the tested constructs was well-expressed, with substantial accumulation of the recombinant protein by 18 h and corresponding 5-fold or greater enhancement of the enzyme’s catalytic activity as measured in lysates (Figure 2.1B, B.1C-E). Overexpression of some individual glycolytic enzymes altered both glucose consumption and lactate secretion, but none significantly changed the ratio of glucose uptake to lactate secretion: cells excreted about 82% of incoming glucose carbon as lactate (Figure 2.1C, B.1F-G). Even greater conversion of glucose to lactate was observed in cells expressing oncogenic mutant Ras (H-Ras\(^{V12G}\); Figure 2.1C), consistent with previous reports that oncogenic Ras inhibits pyruvate oxidation and enhances glycolytic flux\(^{122,141}\).

Based on the nearly constant ratio of glucose uptake to lactate secretion across the cell lines, use of either metric leads to the same conclusions about glycolytic flux. Yet measurements of lactate secretion are more precise (Figure B.1F), as the fractional drop in extracellular glucose over 6 h is small and thus difficult to measure. Accordingly, in all subsequent analyses we used lactate secretion as a proxy for glycolytic flux.
Figure 2.1. Glycolytic flux is controlled by glucose import, fructose-1,6-bisphosphate production, and lactate export.

(A) Experimental setup used to determine glycolytic flux control. Enzymes were individually overexpressed in iBMK cells and change in lactate secretion (glycolytic flux) was assessed 24 h post-transfection; $f_E$ is the ratio between lactate secretion in enzyme-overexpressing cells versus vector-control cells.

(B) Western blot showing that the expression dynamics of different glycolytic enzymes is similar (see Figure B.1 for additional enzymes).

(C) Single glycolytic enzyme expression did not change the ratio of lactate produced to glucose consumed. Means of two biological replicates per condition are plotted. Parental, Akt, and Ras over-expressing iBMK cells are colored in red.  

(legend cont’d on next page)
The uncertainty in the glucose uptake measurements was too large for us to determine whether the absolute flux of glucose carbon not being excreted as lactate tracked with glycolytic flux.

The extent to which a particular pathway step impacts glycolytic flux can be expressed as a flux control coefficient ($C_E^J$): the fractional change in glycolytic flux ($J$) induced by a given fractional change in enzyme catalytic activity ($E$)\textsuperscript{142-144},

$$C_E^J = (\partial J / J) / (\partial E / E)$$  \hspace{1cm} (Equation 1)

Experimentally, measurement of $C_E^J$ based on Equation 1 is difficult. It requires quantitating small changes in both fluxes and enzyme activities. Fortunately, $C_E^J$ can be approximated by the fold-change in flux ($f_E^J$) (measured as the flux ratio between cells with enhanced versus basal enzyme activity) that occurs upon massively increasing the activity of a given enzymatic step\textsuperscript{145},

$$C_E^J \approx (f_E^J - 1) / f_E^J$$  \hspace{1cm} (Equation 2)
Note that dramatically increasing the activity of a given step does not correspondingly increase pathway flux unless that is the sole rate-limiting pathway step, in which case\( C_E^I \approx 1 \). For steps that are not rate controlling, the pathway may not respond, and\( C_E^I \approx 0 \).

2.3 Flux is controlled by glucose import, PFK, and lactate export

Of the 24 individually overexpressed genes, only seven significantly increased glycolytic flux in iBMK cells cultured in both high and low glucose media (Figures 2.1D-E): GLUT1, GLUT3, and GLUT5 (glucose transporters); MCT4 (lactate transporter); PFKP (phosphofructokinase; PFK1); and PFKFB1 and PFKFB3 (phosphofructo-2-kinase/fructose-2,6-bisphosphatases; PFK2). The flux control coefficient for PFKP was comparable to previous literature values determined in other mammalian cells\(^{145,146}\). Our results also agree with previous literature pointing to the importance of glucose uptake and FBP production in controlling glycolytic flux\(^{127–129,131–134}\).

In contrast, no enzymes in lower glycolysis significantly altered flux, including enzymes suggested in prior literature as inducers of the Warburg effect: ALDOA, GAPDH, PGK (phosphoglycerate kinase), PKM1 and PKM2 (pyruvate kinases), and LDHA (lactate hydrogenase). Nevertheless, we did observe significant flux control in the lactate export step, as overexpression of MCT4 led to a substantial increase in glycolytic flux (Figures 2.1C-E).

Certain genes, such as PKM2 and LDHA, have been classically associated with the Warburg effect but did not show any impact on glycolysis here. To rule out the possibility that these genes promote Warburg metabolism by suppressing oxidative phosphorylation or impairing glucose entry into the TCA cycle, we also examined oxygen consumption rate
and contribution of glucose carbon to TCA intermediates. Although there was a trend toward higher oxygen consumption with GLUT3 overexpression, the oxygen consumption rate did not vary significantly in response to overexpression of GLUT3, PFKP, GAPDH, PKM2, LDHA, or MCT4 (Figure B.1H). Glucose contribution to TCA was slightly but significantly decreased by PFKP overexpression but not affected by any other of these genes (Figure B.1I). Thus, the GLUT, PFK, and MCT steps promote glycolytic flux, while most other steps neither drive glycolytic flux nor modulate glucose oxidation.

The summation theorem of metabolic control analysis states that the sum of the flux-control coefficients in a linear pathway must equal 1\(^4\). This theorem refers to the sum of flux control across the pathway steps, not across isozymes. In our case, we observe flux control of roughly 30% for glucose uptake, 30% for FBP production (either via expression of PFK or its activators PFKFB1 or PFKBP3), and 40% for lactate export, together summing to approximately 100% (Figure 2.1F). Thus, our data are roughly consistent with the summation theorem.

We then tested the potential to further activate glycolytic flux in iBMK cells grown in 25 mM glucose by co-expressing GLUT3, PFKP, and MCT4 (Figure 2.1G). The ATP synthase inhibitor oligomycin was used as a positive control\(^{147,148}\). Combined overexpression of GLUT3, PFKP, and MCT4 triggered a 2-fold increase in glycolytic flux, which is comparable to oligomycin and significantly greater than any individual gene on its own (Figure 2.1G). Similar increases in glycolytic flux were obtained with co-expression of either transporter with PFKP, but not the two transporters together. The roles for glucose import, phosphofructokinase, and lactate export in controlling iBMK cell glycolysis are highlighted in Figure 2.1H.
2.4 PFK2 isoforms exhibit divergent effects on flux

Overexpression of 5 of the 24 tested genes significantly decreased glycolytic flux in both glucose conditions (Figure 2.1D). The reduction in glycolytic flux observed upon overexpression of fructose-1,6-bisphosphate phosphatase (FBP1) is consistent with its role in gluconeogenesis and inhibition of tumor progression\textsuperscript{149,150}. We also captured the well-documented anti-glycolytic function of the TP53-induced glycolysis and apoptosis regulator (TIGAR)\textsuperscript{151}. In contrast, we were surprised to find that hexokinase 2 (HK2) overexpression decreased flux, an observation that results from toxicity based on its excessive overexpression, as described below.

Another anti-glycolytic gene was the phosphofructokinase-2 isozyme PFKFB2. This contrasted with PFKFB1 and PFKFB3, which as expected increased glycolysis, and PFKFB4, which had no discernible effect (Figure 2.1D). While PFK2 isoforms are generally considered pro-glycolytic, their impact on glycolysis depends on their balance of kinase and phosphatase activity. Recent in vitro biochemistry studies have assigned the highest kinase-to-phosphatase ratio to the PFKFB3 isoform (>700:1)\textsuperscript{131}, which we found most strongly induced glycolytic flux. Existing biochemical data on the other three PFK2 isoforms, however, does not explain their divergent glycolytic flux phenotypes, as their in vitro kinase and phosphatase activities are similar. Our results highlight the functional diversity of PFK2 isoforms in cells, with the PFKFB2 isozyme acting to slow glycolysis.

2.5 Intracellular FBP concentration mirrors glycolytic flux

To explore potential connections between enzyme induction, flux, and metabolite levels, we measured changes in glycolytic intermediate levels by liquid chromatography-mass
spectrometry. These metabolomics data confirmed the functional activities of the overexpressed enzymes (Figure 2.2A). For example, overexpression of PFKP led to a decrease in fructose-6-phosphate (F6P) and to a concomitant increase in FBP, whereas the opposite was observed for fructose bisphosphatase (FBP1). Increased ALDOA activity resulted in lower FBP but elevated dihydroxyacetone phosphate (DHAP). We observed 2,3-bisphosphoglycerate (2,3BPG) accumulation in BPGM-overexpressing cells, which likely explains their decreased glycolytic flux (Figures 2.1D, 2.2A). Our analysis also confirmed recently-reported side products of GAPDH and pyruvate kinase\textsuperscript{152}: we found that GAPDH overexpression induced erythronate, and PKM1 and especially PKM2 generated 2-phospholactate (Figures B.2A-B). TIGAR-overexpressing cells were characterized by a marked reduction of lower glycolytic intermediates, especially 2,3BPG and phosphoenolpyruvate (PEP), but not FBP, and we did not detect any increase in hexose-phosphate species. These observations are consistent with recent evidence showing greater TIGAR phosphatase activity toward 2,3BPG and PEP than fructose-2,6-bisphosphate\textsuperscript{153}. Collectively, these metabolomics data support the successful induction of the desired enzymatic activities via protein overexpression, including for enzymes that did not significantly impact glycolytic flux.

In addition, our metabolite concentration data support a picture in which upper glycolytic enzymes control both glycolytic flux and metabolite levels. Overexpression of upper glycolytic enzymes caused strong metabolite changes across the entire pathway. In contrast, lower glycolytic enzymes tended to marginally increase lower glycolytic intermediates (with strong effects only on 2,3BPG) and to weakly deplete upper glycolytic intermediates (Figure 2.2A). Although overexpressed glucose and lactate transporters generally had only weak effects on metabolite concentrations, they did elevate FBP and to a lesser extent DHAP, which is linked to FBP via the reversible aldolase reaction.
Figure 2.2. Intracellular FBP levels mirror glycolytic flux.

(A) Heatmap summarizing changes in intracellular metabolite levels upon overexpression of glycolytic enzymes. Red and blue indicate increased and decreased levels, respectively. Light-yellow boxes highlight the identified flux-controlling steps from Figure 2.1. Glycolytic enzymes with positive glycolytic flux control are colored red.

(B) Quality of Michaelis-Menten fit of lactate secretion rate to individual metabolite concentration. Lighter bars show $R^2$ across all overexpressed constructs and darker bars represent $R^2$ excluding HK2. $R^2$ for fits were calculated by the residual sum of squares (RSS) and total sum of squares (TSS) with $R^2 = 1 - \text{RSS/TSS}$.

(C) Absolute glycolytic flux as a function of FBP concentration follows Michaelis-Menten kinetics ($p < 10^{-4}$). Dotted lines indicate fitted KM (1.5 mM; $p = 0.002$) and $V_{\text{max}}$ (0.3 µmol hr$^{-1}$ µL cell$^{-1}$; $p < 10^{-8}$) values. Gray represents the 95% confidence intervals of the fitted function. Data from HK2-overexpressing cells are shown as the lighter color dot and was omitted from the fitting.

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Consistent with prior literature in both microbes and mammalian cells\textsuperscript{154–157}, among the glycolytic intermediates FBP and DHAP concentrations correlated most strongly with glycolytic flux (\textbf{Figure 2.2B}). AMP, ADP, ATP, and citrate are textbook regulators of glycolysis\textsuperscript{158–160}, but none showed a comparable correlation with glycolytic flux (\textbf{Figure B.2C}). With the exception of cells overexpressing toxic levels of hexokinase (see below), flux across the cell lines was generally well-explained by a Michaelis-Menten function of FBP concentration (p < 10\textsuperscript{-4}) (\textbf{Figure 2.2C}). The fitted $K_m$ value of 1.5 mM (p = 0.002) is in the range of the basal FBP level in iBMK cells (1.8 mM), and the $V_{max}$ value of 0.3 µmol h\textsuperscript{-1} µl cell\textsuperscript{-1} (p < 10\textsuperscript{-8}) is two-fold higher than the basal glycolytic flux (0.15 µmol h\textsuperscript{-1} µl cell\textsuperscript{-1}) and equal to the maximum glycolytic flux obtained upon oligomycin treatment or combined GLUT3, PFKP, and MCT4 overexpression. Thus, glycolytic flux appears to be a saturable function of FBP levels.

## 2.6 Hexokinase is pro-glycolytic but toxic in excess

Hexokinase catalyzes an effectively irreversible step of glycolysis that has been shown to have significant flux control\textsuperscript{161}. HK2 overexpression caused accumulation of most glycolytic intermediates (\textbf{Figure 2.2A}). Accordingly, we were perplexed by its negative effect on glycolytic flux (\textbf{Figure 2.1D}). To address this discrepancy, we measured the dynamics of glycolytic flux in HK2-overexpressing cells as well as in cells overexpressing GLUT1, GLUT3, PFKP, PFKFB3, and PKM2 (\textbf{Figure 2.2D, B.2D}). This revealed that
HK2 initially accelerates glycolytic flux, but suppresses it at later time points. No other enzyme showed a similar biphasic response. Thus, HK2 exerts positive glycolytic flux control, but over time also impairs glycolysis.

To obtain a better understanding of the underlying mechanism, we measured the temporal evolution of metabolite concentrations in HK2-overexpressing cells (Figure 2.2E, B.2E). We found that glycolytic intermediates accumulated monotonically. AMP, and to a lesser extent ADP, also built up, suggestive of energy stress. ATP levels and adenylate energy charge dropped at the same time as glycolytic flux decreased (Figures 2.2D, 2.2F). A lower energy charge also manifested in depletion of creatine phosphate (Figure B.2F). These metabolic alterations likely result from excessive HK2 enzyme activity detrimentally consuming ATP through overactive hexose phosphorylation, with downstream glycolysis unable to maintain pace. Cell growth and viability were, however, largely maintained over this time interval (Figure B.2G). This HK2-induced energy stress is evocative of other instances where excessive ATP-driven phosphorylation leads to energy stress, e.g., glucose-induced ATP depletion in yeast lacking the enzyme trehalose-6-phosphate synthase (TPS1), which acts in part as a brake on hexokinase\textsuperscript{162–164} and fructose-induced drainage of ATP in hepatocytes via ketohexokinase\textsuperscript{165,166}.

To prove that HK2 is fundamentally pro-glycolytic but that excessive HK2 activity causes energy stress, we transfected iBMK cells with serial dilutions of plasmid DNA encoding HK2 or, for comparison, PFKP (Figure 2.2G). Both low and high copy numbers of PFKP had similar effects, increasing flux by \(~40\%\). In contrast, we found that, at the 30 h time point used in Figure 1, while concentrated HK2 decreased glycolytic flux, dilute HK2 increased glycolytic flux by \(~40\%\). Glycolytic flux began to decrease as the introduced HK activity exceeded endogenous activity by \(>5\) -fold (Figure B.2H). Thus, hexokinase exerts positive flux control in iBMK cells, but this was masked in our initial experiments involving massive overexpression because excessive HK2 activity causes ATP depletion.
2.7 Hexokinase and PFK also control flux in NIH3T3 fibroblasts

To examine glycolytic flux control in an additional mouse cell line, we conducted single enzyme overexpression experiments in NIH3T3 mouse fibroblasts (3T3). Parental 3T3 cells were transduced by lentiviruses carrying a glycolytic gene of interest and lactate secretion was measured after 48 h. Tested genes included a single isozyme for each core step of glycolysis through enolase, two pyruvate kinase isozymes (PKM1 and PKM2), four transporters (GLUT1, 3, 5 and MCT4), and two potential regulators (PFKFB3 and PDK1). Overall, the results closely mirrored those observed in iBMK cells (Figures 2.3A, B.3A). Of the eight glycolytic reactions that did not substantially control flux in iBMK cells, none had a significant effect in 3T3 cells. Similar to iBMK cells, the HK2 and PFK steps exerted substantial flux control, with expression of the PFK1-activator PFKFB3 yielding a similar effect to that of PFKP itself. In contrast to iBMK cells, we did not observe increased flux in response to single overexpression of glucose or lactate transporters. Overall, our results suggest that hexokinase and phosphofructokinase are common flux-controlling enzymes in glycolysis, with other core glycolytic enzymes exerting limited flux control.

2.8 Optogenetic Ras stimulation activates all four flux-controlling steps

The Ras/Erk pathway is a well-studied growth-promoting signal transduction pathway whose constituents are among the most frequently mutated genes in human cancers107. Oncogenic mutations in the Ras/Erk pathway have been shown to enhance both glucose uptake and use129,134,168,169. We have previously observed that acute optogenetic stimulation of wild-type Ras is sufficient to drive 3T3 cell transcription and proliferation38,75.
**Figure 2.3. Increase in glycolytic flux upon Ras activation is controlled by glucose import, G6P production, and lactate export.**

(A) Change in glycolytic flux in 3T3 cells upon individual overexpression of enzymes catalyzing every step in glycolysis. Means and 95% CI (n=3 biological replicates) are plotted. (B) Experimental setup to investigate acute metabolic perturbation in 3T3 cells using optogenetic control of Ras activation. (C–D) Changes after red light exposure in (C) lactate secretion at 6 h (n=12 biological replicates from two independent experiments; p < 10^-7) and (D) cell growth at 24 h (n=3 biological replicates; p < 0.02). Means and 95% confidence intervals are plotted.

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We wondered if this acute Ras-driven proliferation was associated with glycolytic enhancement, and, if so, whether the enhanced flux is driven by the flux-controlling reactions identified by our overexpression analysis.

To address these questions, we employed an optogenetic system (OptoSOS) to precisely control Ras signaling activity in 3T3 cells. Serum-starved 3T3 cells expressing the light-activatable OptoSOS system were stimulated with red light and glycolytic flux was measured at 6 h and cell growth after 24 h (Figure 2.3B). As expected, cell growth was increased by acute Ras signaling, and impressively, glycolytic flux was already enhanced after 6 h (Figures 2.3C-D, B.3B).

To obtain a more thorough understanding of early Ras-driven metabolic changes, we measured glycolytic intermediates after 4 h and 24 h of activating light (Figure 2.3E). We observed a general increase in glycolytic intermediates and adenosine nucleotide levels at 4 h, similar to changes observed upon HK2 overexpression in iBMK cells (Figures 2.2A, 2.3E). FBP and DHAP were the only metabolites that remained elevated after 24 h of Ras activation. The 2-fold elevation of FBP at 4 h was associated with a 1.5-fold increase in glycolytic flux, consistent with the relationship between FBP and glycolytic flux in iBMK cells (Figures 2.2B-C, 2.3C, 2.3E).

To explore potential mechanisms by which acute Ras activation might enhance glycolysis, we analyzed published RNA-sequencing data collected from OptoSOS 3T3 cells.
stimulated with activating red light over a 2 h timecourse. Most gene expression changes were, as expected, in signaling pathways (Figure B.3C). Among metabolic pathways, nucleotide biosynthesis was substantially increased, likely to support cell proliferation. In central carbon metabolism, four glycolytic genes were upregulated more than 2-fold at some time point. In contrast, upregulation was observed for only a single gene in the pentose phosphate pathway (ribose-5-phosphate isomerase A) and none in the TCA cycle (Figure B.3D). The glycolytic genes that exhibited >2-fold upregulation were GLUT1, HK2, PFKFB3, and MCT1, corresponding to our four identified flux-controlling steps. PFKP expression increased to a lesser extent (Figure 2.3F). Induction of GLUT1 and HK2 was particularly strong and rapid. These acute changes differ from those caused by chronic Ras activation, which also induces multiple lower glycolytic enzymes. In addition, we observed Ras-dependent decreases in expression of enzymes (BPGM and PFKFB2) that we identified as ‘anti-glycolytic’ in single overexpression experiments (Figure 2.1D). We did not observe altered expression of any glycolytic genes with low flux-control coefficients. Taken together, our results suggest that Ras controls a coordinated expression program to up- and downregulate glycolytic nodes in a manner that increases glycolytic flux. Figure 2.3G summarizes the flux-controlling steps identified in iBMK and 3T3 cells together with the transcriptional targets induced by acute Ras signaling.

2.9 Solid tumors overexpress flux-controlling genes

Enhanced glycolysis was the first molecular phenotype assigned to cancer and occurs in tumors originating from many different tissues and harboring different oncogenes and genomic alterations. We wondered whether the same four glycolytic flux-controlling steps identified in our cell culture data would also emerge in gene expression data across a variety of human tumors. To this end, we analyzed expression of glycolytic
genes in 666 paired solid tumor tissue samples from The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/). Based on KEGG database annotations, we curated a set of 40 glycolytic genes, covering all isozymes of glycolytic enzymes, including PFK2 isoforms and glucose and lactate transporters\(^{171-173}\). Gene expression of tumor samples was normalized to the corresponding benign adjacent tissue. Unsupervised clustering of solely this limited set of glycolytic genes was sufficient to group tumor samples based on their tissue of origin (Figures 2.4A-B).

At least one isoform of every glycolytic step was generally upregulated in each patient tumor (Figure 2.4A). In most cancer types, strongest increases were seen in at least one isoform of GLUT, of PFK (including PFK2), and of MCT, representing three of the four key flux-controlling nodes (Figures 2.4C, B.4A). While no hexokinase isozyme was particularly strongly overexpressed in lung, liver, or breast cancer, HK2 was the most strongly upregulated glycolytic enzyme in renal clear cell carcinoma and kidney renal papillary cell carcinoma tumors, HK3 in thyroid tumors. The less consistent upregulation of HK expression may reflect our observed toxicity of excess HK activity.

To more systematically evaluate overexpression at different glycolytic steps, we ranked glycolytic gene isoforms in each individual tumor specimen based on the extent to which each gene is upregulated relative to adjacent benign tissue. Figure 2.4D shows the frequency with which gene isoforms catalyzing different glycolytic steps ranked among the top four most upregulated glycolytic genes in a given patient. Consistent with our findings in cultured cells, we find that the most overexpressed glycolytic genes in human cancer encode for glucose import (GLUT1, GLUT5), glucose phosphorylation (HK2, HK3), FBP production (PFKFB4), PEP production (ENO2), and lactate export (MCT1, MCT4). Except for enolase, which catalyzes the dehydration of 2-PPG to PEP, these are exactly the four steps that we identified as flux-controlling in our cell culture studies.
Figure 2.4. Increased glucose uptake, FBP production, and lactate export is a general hallmark of tumor metabolism.

(A) Expression levels of glycolytic enzymes in solid tumor tissue samples compared with paired healthy tissue samples analyzed from TCGA data.

(B) Clustering of tumor types based on glycolytic gene expression data only: bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD & LUSC), lung squamous cell carcinoma (LUOS), pancreas adenocarcinoma (PAAD), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA), and uterine corpus endometrial carcinoma (UCEC).

(legend con’t on next page)
Analysis of published gene and protein expression data in lymphocyte, macrophage, and endothelial cell activation, which are physiological contexts in which glycolysis is upregulated, also reveals consistent upregulation of at least one isozyme catalyzing glucose uptake, glucose phosphorylation, FBP production, and lactate secretion\textsuperscript{174-177} (Figure B.4B). Thus, activation of glycolysis in mammals, both in physiological and disease contexts, is associated with induction of our four identified flux-controlling steps.

\textbf{2.10 Discussion}

Glycolysis is the earliest-described biochemical pathway. Our current understanding reflects the combined efforts of generations of scientists, with seminal contributions including Louis Pasteur’s input/output characterization in the 1850s and Gustav Embden’s reaction step outline in the 1930s. Motivated by Otto Warburg’s discovery of increased glycolytic rate in cancer in the 1920s, there is widespread and longstanding
interest in understanding how flux through glycolysis is controlled. This interest is mirrored by the many thousands of studies on the topic. Nevertheless, the resulting body of primary literature is mainly composed of papers focusing on individual enzymes, with every glycolytic enzyme proposed to be rate limiting in some study, and PFK emerging as the consensus main rate-limiting step in textbook descriptions of the pathway.

Systematic assessment of pathway regulation by metabolic control analysis, however, has revealed that rate-limiting steps are an oversimplification\(^\text{142,144}\). Indeed, as much as every pathway step must be thermodynamically forward-driven and thus consume some of the available pathway free energy, every step also exerts at least some modicum of flux control\(^\text{142,178,179}\). Therefore, a pragmatic understanding of metabolic control requires determining which steps substantially control flux

Here we assessed flux control in mammalian glycolysis using three approaches: glycolytic enzyme overexpression, optogenetic activation of Ras, and analysis of gene expression data from human tumors. The first of these approaches is particularly informative, as it directly probes the relationship between enzyme levels and flux, with our data covering each step of glycolysis. All three approaches consistently point to flux through glycolysis being controlled by glucose uptake and phosphorylation, FBP production, and lactate export, but not by lower glycolytic enzymes.

Our measurements of flux control apply to the context in which the enzyme overexpression was carried out (i.e., iBMK and 3T3 cells). This reflects flux-control coefficients depending on relative enzyme concentrations. As the level or activity of an enzyme goes down (compared with pathway flux), its flux control goes up. Thus, a relatively high basal capacity for glucose transport and lactate secretion, relative to glucose phosphorylation and FBP production, likely explains why GLUT and MCT do not exert flux control in 3T3 cells. Moreover, while we do not find evidence for substantial flux control in any of the lower glycolytic enzymes, these enzymes may yet exert flux control in
cellular contexts where their relative activities are lower. For example, ALDOA may gain flux control in contexts where it is substantially inactivated by binding to the actin cytoskeleton\textsuperscript{135}. GAPDH, whose catalytic cysteine can be oxidized, may exert flux control in the context of oxidative stress\textsuperscript{136,180–183}. More generally, any enzyme of glycolysis can gain flux control if sufficiently inhibited, e.g., by genetic or pharmacological manipulation. For this reason, while studies examining the effects of enzyme depletion are valuable to understand gene essentiality, they do not shed light on intrinsic flux control\textsuperscript{145}. In contrast, for the specific cell line in which they are conducted, our overexpression studies yield quantitative estimates of flux-control coefficients.

In considering how overexpression of a single enzyme increases glycolysis, it is important to keep in mind that increased pathway flux requires enhancement of flux through every enzymatic step of the pathway\textsuperscript{184}. A straightforward way to achieve this is to increase the expression of each pathway enzyme. Overexpression of a single enzyme may, however, drive flux through upstream and downstream pathway steps by changing metabolite levels. This appears to be the case for hexokinase and phosphofructokinase. The former increases all glycolytic intermediates. The latter depletes hexose phosphates (thereby relieving product inhibition of glucose import and phosphorylation) and increases FBP and all downstream metabolites (increasing substrate to drive these reactions). It remains unclear how GLUT overexpression drives downstream glycolytic reactions (or MCT upstream ones), as neither transporter markedly alters glycolytic intermediate concentrations.

The lag between GLUT expression and flux response raises the possibility that adaptation may be involved in inducing the flux increase. Consistent with this, once we include the positive flux control coefficient for hexokinase that was masked by energy stress in our initial measurements, we observe a surplus of flux control in iBMK cells (i.e., sum of flux-control coefficients >1), suggesting that overexpression of certain enzymes
may itself cause activation of other enzymes beyond simply providing substrate or consuming product. Notably, a near maximal glycolytic rate in iBMK cells is achieved by simultaneous upregulation of both FBP production and a transport step (but not by concomitant increase of both flux-controlling transport steps). The reasons for these non-linear interactions between different steps in glycolysis are not evident from the individual flux-control coefficients and are an important topic for future research.

As glycolytic rate is enhanced by overexpression of any particular flux-controlling enzyme, flux control will tend to transfer to other pathway steps. This may explain why HIF induces a broad spectrum of glycolytic enzymes including phosphoglycerate kinase and LDH, why tumors overexpress many glycolytic enzymes including aldolase and enolase\textsuperscript{185–187}, and why tumor types associated with particularly high glycolytic rates owing to mitochondrial defects (e.g., renal and thyroid cancer) overexpress hexokinase, even though excessive hexokinase activity can be toxic\textsuperscript{188–190}.

While our study does not address the extent of mitochondrial respiration in cancer cells, our finding that overexpression of certain glycolytic enzymes is sufficient to drive glycolysis without altering glucose entry into the TCA cycle is consistent with emerging evidence that control of glycolytic flux and TCA cycle flux are substantially independent\textsuperscript{191,192}. This reinforces the appropriateness of defining the Warburg effect as aerobic glycolysis in cancer, a robust clinical phenotype proved by fluorodeoxyglucose positron emission tomography imaging.

In summary, we provide a thorough experimental assessment of glycolytic flux control in two mammalian cell lines. We find that flux control is partially distributed, with 4 of the 13 steps collectively dominant. The four flux-controlling steps reside at the top (glucose import), at the two committed phosphorylation steps (hexokinase and phosphofructokinase) and at the bottom (lactate export) of glycolysis. In contrast, at least in these cells, lower glycolytic enzymes do not substantially control glycolytic flux. The
identified flux-controlling enzymes stand out for being transcriptionally induced by acute Ras signaling and for being strongly upregulated in human tumors, immune cell activation, and angiogenesis, arguing for the generality of their biological importance. By providing a systematic assessment of glycolytic flux control in mammalian cells, we hope that this work helps both to contextualize research on individual glycolytic enzymes and to motivate comprehensive studies of flux control in additional pathways.
Chapter 3

A screen for altered Ras/Erk signaling dynamics reveals principles of proliferative control

Complex dynamics are pervasive in mammalian cell signaling, but both their causes and consequences remain largely unknown. One obstacle to our understanding has been the difficulty of probing dynamics using the high-throughput screens that were previously so successful at identifying essential pathway components. Here, we describe a pipeline to screen for altered Erk signaling dynamics in primary mouse keratinocytes. Screening a library of 429 kinase inhibitors revealed both known and uncharacterized modulators of Erk dynamics, including inhibitors of the Met and VEGFR receptor tyrosine kinases (RTKs) that paradoxically increase Erk’s pulse frequency and overall activity. Direct optogenetic control further reveals how drug-induced dynamics are interpreted into cell proliferation. Slow, gradual Erk pulses drive proliferation in a broad range of media whereas fast, frequent pulses are only proliferative under more restrictive conditions. Our work opens the door to sensitive, high-throughput analysis of single-cell dynamics and reveals that Erk’s sufficiency to trigger proliferation depends on both its environmental context and its activity over time. The primary drug screen component of this chapter was
carried out in collaboration with Maxwell Wilson (a former post-doc in the Toettcher lab at Princeton and current Professor at UCSB) and Jillian Silbert (a former undergraduate thesis student in the Toettcher lab and current Fulbright scholar).

3.1 Encoding information using signaling dynamics

Animal cells must respond to a large number of external cues to function appropriately during development and adult tissue homeostasis. To that end, a typical mammalian cell is endowed with hundreds of distinct receptors. Yet only a few signaling pathways downstream of these receptors are tasked with responding to these many inputs. For instance, the 56 human receptor tyrosine kinases (RTKs) activate fewer than 10 overlapping intracellular pathways (e.g., Ras/Erk, PI3K/Akt, Src, PLCγ, calcium), yet can trigger diverse downstream cellular responses in developing and adult tissues. Cells are thus faced with the challenge of accurately transmitting information from a large number of upstream inputs using only a few ‘wires’ or signal transduction pathways.

One resolution to this paradox comes in the form of dynamic regulation. Two receptors may trigger different time-varying responses from a single pathway, which can then be interpreted into distinct fates. Indeed, many core mammalian signaling pathways have been observed to generate complex, time-varying signaling behaviors in response to input stimuli, suggesting that dynamic regulation is quite widespread. Advances in biosensors and in vivo imaging have revealed that Erk and p53 dynamics are also present in cells’ natural tissue context and that disease-associated Ras/Erk pathway mutations can alter dynamics in a manner that affects cell proliferation.

Yet it largely remains an open question how signaling dynamics are generated, regulated, and interpreted. We reasoned that a powerful tool to address this question would be to conduct a screen using dynamics as a phenotypic readout, though this has historically been challenging for many reasons. To capture time-varying activity and
overcome cell-to-cell variability, dynamics must be measured at high temporal resolution in single live cells over extended timecourses, which can severely limit experimental throughput. Such a screen could potentially generate many useful insights. Perturbations to certain signaling nodes might eliminate dynamics altogether (Figure 3.1A, top),

Figure 3.1. High-throughput screening for altered Erk dynamics.
(a) By screening small molecule libraries for inhibitors that alter cell signaling dynamics, it is possible to identify key nodes whose inhibition contributes to dynamic signaling. This approach can be used to understand the generation, regulation, and interpretation of dynamic signaling behaviors.
(b) Primary keratinocytes as a model system for studying Erk signaling dynamics. Basal keratinocytes can be isolated from P0 mouse pups, transduced with lentiviruses encoding live-cell biosensors, induced to form a uniform epithelial sheet, and imaged for dynamic activity via confocal microscopy.
(c) Imaging the H2B-tagRFP nuclear marker allows us to segment individual nuclei using TrackMate, from which we create a binary mask that we use to track changes in nuclear ErkKTR fluorescence over time. We can then apply our custom single-cell peak-finding MATLAB scripts to extract quantitative information about the features of Erk signaling dynamics.
identifying essential components for producing time-varying responses. Others might switch dynamics between normal and diseased states (Figure 3.1A, middle), suggesting intervention points to correct aberrant signaling. Finally, some perturbations might result in hitherto-unobserved signaling dynamics (Figure 3.1A, bottom); these might be analogous to classical mutant phenotypes, which can be highly informative despite (or because of) their differences from what is normally observed.

Motivated by these possibilities, we set out to establish a screening platform for signaling dynamics in primary mammalian cells. We focused on the mammalian Ras/Erk pathway, an ideal model system both because of its stimulus-dependent dynamics and the excellent tools available for monitoring and controlling pathway dynamics in individual cells. We screened a panel of 429 kinase inhibitors for altered Erk signaling dynamics in more than 100,000 individual primary mouse keratinocytes. Our screen recovered known modulators of signaling dynamics, including classic EGFR/Ras/Erk pathway inhibitors and B-Raf-targeting compounds that extend the duration of endogenous Erk pulses.

The screen also identified previously-unknown regulatory links that alter Erk dynamics: inhibition of the non-EGFR receptor tyrosine kinases (RTKs) Met and VEGFR2 drives a dramatic increase in Erk pulse frequency, with successive pulses firing as often as once every 20 min. Finally, we used precise optogenetic control over Ras/Erk to demonstrate that the dynamics elicited by different classes of drugs are also interpreted into distinct proliferative responses. Our work reveals new input connections into the Ras/Erk pathway, provides a compendium of drug-induced changes to signaling dynamics, and establishes an extensible experimental and computational platform for future live-cell screens.
3.2 Primary keratinocytes are a robust model system for Erk dynamics

To develop our dynamics screening platform, we first needed to identify a suitable cellular model of Erk dynamics. Previous work using a FRET-based Erk biosensor reported highly dynamic Erk activity in the basal epithelium of live adult mice\(^8\). The keratinocytes that make up this tissue can be cultured \textit{ex vivo} without transformation or immortalization and are readily accessible to imaging and transduction\(^{200,201}\). We thus hypothesized that they might serve as an ideal, tissue-relevant cellular context in which to further dissect Erk signaling dynamics.

We harvested basal keratinocytes from newborn CD1 mouse pups by soaking whole-skin isolates in dispase to separate the epidermis from the dermis, then digesting the epidermal sheet with trypsin to obtain single cells that we then plated on fibroblast feeder cells\(^{201}\). We transduced the resulting primary keratinocyte cultures with a lentiviral vector encoding a live-cell biosensor of Erk kinase activity (ErkKTR-BFP) and a retroviral vector encoding a fluorescent histone (H2B-RFP) for automated cell segmentation and tracking\(^{199}\) (Figure 3.1B-C). We then performed fluorescent cell sorting to obtain a homogenous population of dual-expressing, low-passage-number keratinocytes (termed KTR-H2B keratinocytes) that were used for all subsequent experiments.

We were surprised to find that even in the absence of any external mitogens, keratinocytes exhibited highly dynamic and robust Erk signaling activity. In KTR-H2B keratinocytes that were plated to form an epithelial sheet on fibronectin-coated glass and starved for 8 h prior to imaging (Figure 3.2A), we observed frequent Erk pulses in a majority of cells, even in the absence of any added serum, growth factors, or other additives (Figure 3.2B). To quantify dynamic activity we developed a computational pipeline to track cells using their H2B-tagRFP nuclear marker\(^{202}\) and automatically extract
Figure 3.2. Primary keratinocytes exhibit a ‘ground state’ of pulsatile Erk dynamics.

(a) Upper panel shows representative Erk activity traces from single primary keratinocytes in growth factor-free (GF-free) media and imaged every 3 min for 24 h. The avg. frequency of Erk activity pulses over 24 h for all imaged cells is plotted below. (b) Single-cell Erk activity was imaged for >4 h in primary keratinocytes (right) and immortalized MCF10A breast epithelial cells (left) treated with various doses of epidermal growth factor (EGF). Upper panels depict representative single-cell traces and lower plots depict the percentage of cells in each condition that exhibited ≥ 2 Erk pulses over the timecourse. Each point represents avg. data from > 100 single cells. (c-d) Representative single-cell traces of Erk activity in keratinocytes showing adaptation after cells are either (c) shifted from GF-free to complete media or (d) shifted from complete to GF-free media. (e) Schematic representation of the growth factor-dependent regime in which classical cell line models (e.g., MCF10As) exhibit Erk dynamics (left) versus the adaptable ‘ground state’ of pulsatile Erk dynamics observed in primary keratinocytes (right).
the timing, amplitude, and width of each Erk pulse over time (see Methods; Figure 3.1). We found that the amplitude, pulse width, pulse frequency, and overall activity were stable over at least 24 h of live-cell imaging in growth factor-free (GF-free) media, with one pulse of Erk activity occurring on average every 2 h (Figures 3.2A, C.1).

Prior studies have observed Erk activity pulses in a variety of cell lines, but in each case the pulses were strongly dependent on the levels of external growth factors and culture conditions (e.g., cell density). These features are suboptimal for conducting a screen, which will require a model system with rapid dynamics that are both consistent over time and between experiments. We thus sought to compare the conditions under which keratinocytes exhibit Erk activity pulses to those of a classic model system for studying Erk dynamics, MCF10A cells, an immortalized human breast epithelial cell line.

We treated KTR-H2B MCF10As and keratinocytes with varying doses of epidermal growth factor (EGF) and monitored Erk dynamics in individual cells for 4 h after stimulation (Figures 3.2B, C.1). In agreement with prior work, we found that MCF10A cells exhibited three distinct regimes of Erk dynamics: constant, low Erk activity in the absence of EGF, occasional pulses of Erk activity at intermediate concentrations (50-200 pg/mL) of EGF, and a constant, high state in the presence of saturating EGF doses (Figure 3.2B, left). MCF10A responses were highly heterogeneous: the maximum percentage of pulsing cells never exceeded 30%, even at the most permissive concentrations of EGF (Figure 3.2B, left). In contrast, keratinocytes exhibited robust, frequent Erk pulses over a broad range of EGF concentrations (from mitogen-free conditions up to 1 ng/mL EGF), with 80-100% of cells pulsing ≥2 times in all conditions (Figure 3.2B, right). Taken together, we find that Erk signaling is highly dynamic in primary epidermal cell cultures even in the absence of externally-supplied growth factors.

Multiple lines of evidence suggest that pulses of Erk activity represent a robust, baseline signaling state for keratinocytes. Pulse characteristics are highly stable over time.
(Figure 3.2A-B) and occur in a broad range of culture conditions and media formulations, including skin-like organotypic culture at an air-liquid interface (Figure C.2A-C). Immunostaining in embryonic mouse epidermis also revealed a salt-and-pepper pattern of Erk phosphorylation, consistent with sporadic bouts of activity in single cells in vivo (Figure C.2D). Finally, we found that keratinocytes adapt back to a pulsatile state in response to perturbations in growth signaling: both after an acute switch from GF-free to complete media (Figure 3.2C), and from complete media to GF-free media (Figure 3.2D). In both cases, following an initial phase of either constant Erk activation or inactivation immediately after the switch, all cells adapted back to a baseline state of pulsatile Erk signaling. Taken together, these findings suggest that primary mouse keratinocytes exhibit a pulsatile ‘idling motor’ of Erk signaling dynamics to which they return even in the face of strong physiological perturbations, in contrast to many classic epithelial cell lines (Figure 3.2E). The robustness and reproducibility of Erk activity in primary keratinocytes thus made them an ideal model system for quantitative studies of signaling dynamics.

3.3 An imaging-based drug screen for altered Erk dynamics

We next leveraged the potential of our primary keratinocyte cellular system to perform an imaging-based screen for compounds that alter Erk signaling dynamics. We used a small molecule library consisting of 429 validated kinase inhibitors. This library was ideal for three reasons. First, because kinase activity is a primary currency of intracellular signaling and regulates the EGFR/Ras/Erk signaling pathway at multiple nodes, we predicted that a kinase inhibitor library would be enriched for dynamics-altering compounds. Second, EGFR/Ras/Erk signaling is a major target of drug development, so many kinase inhibitors
Figure 3.3. A single-cell imaging-based drug screen reveals nodes that alter Erk signaling dynamics.

(a) Schematic overview of our signaling dynamics screening platform showing the experimental design (left) and the computational pipeline for extracting individual ‘features’ of signaling dynamics from single cells (right).

(b) Drug screen results are hierarchically clustered by all dynamic features.

(c) Plotting freq. vs. offset shows three main classes of drug-altered Erk dynamics.

(d) Representative single-cell traces from each class.

(e) IC50 values from LINCS database were used to examine target enrichment within each class of drug-altered Erk dynamics.
in the library serve as *bona fide* hits that we expect our screen to capture. Finally, extensive data on kinase inhibitor specificity\textsuperscript{205,206} enables us to identify the likely molecular targets for many drugs in this library, allowing us to move quickly from an initial hit to a potential molecular mechanism.

We developed an experimental pipeline to rapidly prepare and stimulate KTR-H2B keratinocytes in 50 wells of a 384-well plate per imaging session (Figure 3.3A). Precise volumes of each drug were delivered to a clean plate using an acoustic liquid handler. Drugs were then diluted to working concentrations in GF-free media and transferred to plates of keratinocytes to a final concentration of 2.5 μM. Each plate of 50 wells included multiple vehicle-only (DMSO) controls to monitor batch-to-batch variability, leading to a total of 450 wells (9 plates with 50 wells per plate) imaged in total during the screen.

After drug treatment, we performed two-color confocal microscopy on treated keratinocytes, imaging each well every 3 min over 5 h. From the resulting movies, we extracted four single-cell parameters or ‘features’ of Erk activity dynamics (the number of pulses, pulse amplitude, pulse width, inter-pulse interval) and one population-averaged parameter: the overall Erk activity state, which we measured by computing the mean cytosolic-to-nuclear ratio of the KTR across all cells (Figure 3.3A, right). We also extracted other cell state parameters (e.g., overall cell density; cell motility) to exclude correlations between Erk dynamics and variables that may not be related to specific drugs. Together, these parameters defined Erk dynamics within each well (Figure 3.3B).

To identify groups of inhibitors that had similar effects on each of our five Erk dynamic features, we clustered responses by all dynamic features (Figure 3.3B). We found that 76% (326/429) of drugs clustered together with the DMSO vehicle controls, with the remainder clustering into three easily-identifiable classes of perturbed dynamics (discussed below). Our hit rate of 24% is substantially higher than the 1-2% hit rate that is typical for unbiased cell-based screens\textsuperscript{207}. However, we expected a substantially higher hit
rate because our kinase inhibitor library includes many known Erk pathway regulators and hits were identified using multiple dynamic criteria. For subsequent analyses, we focused specifically on hits in three distinct classes: those that blocked Erk activity, those that increased Erk activity but slowed its pulsatile dynamics, and those that both increased Erk activity and increased pulsatile dynamics.

The first class of 48 Erk-altering inhibitors (referred to here as ‘Class 1’) consisted of drugs that suppressed dynamics by reducing Erk activity to a lower, constant level; this class accounted for roughly 11% of the compounds screened (Figure 3.3C-D). Nearly every Class 1 compound is a known inhibitor of a kinase along the core EGFR/Ras/Erk pathway, with multiple inhibitors targeting each of EGFR, Raf, MEK, and Erk (Figure 3.3E). This result suggests that Erk dynamics do not require permissive inputs from other canonical intracellular pathways, as off-pathway kinase inhibitors were largely unable to suppress dynamic Erk activity. Conversely, we only found one ‘missed’ inhibitor that was predicted to fall into Class 1 but in whose presence Erk dynamics were unaffected: the MEK inhibitor PD98059. This result can be explained by the high minimum inhibitory dose of PD98059, which is typically used above 10 μM. We thus conclude that our screening pipeline is both sensitive and specific for identifying inhibitors of Erk signaling.

A second class of 5 inhibitors (referred to here as ‘Class 2’) increased Erk activity while also profoundly altering its dynamics (Figure 3.3C-D). Treatment with these drugs led to slow, infrequent pulses of Erk activity on top of an elevated baseline. Similar slow Erk dynamics were reported in two recent studies after treatment with so-called ‘paradox-activating’ B-Raf inhibitors, compounds that potently inhibit certain mutant B-Raf alleles but can increase activity from other Raf isoforms by stabilizing active conformations. Indeed, our Class 2 compounds included two paradox B-Raf inhibitors, SB590885 and GDC0879 (Figure 3.3E), but 7 additional paradox-activating B-Raf inhibitors did not dramatically alter Erk dynamics. One of these, vemurafenib, was
also seen to have a weaker effect on Erk dynamics in a prior study. These data suggest that there may be a spectrum of paradoxical B-Raf activation, where the magnitude of the shift in Erk dynamics may reflect the degree to which Raf dimers are stabilized.

Our third class of inhibitors (termed ‘Class 3’) consisted of drugs that amplified Erk activity by increasing the frequency and/or set point of Erk pulses (Figure 3.3C-D). This was the second-largest class of inhibitors identified by our screen, consisting of 50/429 drugs. Although all drugs in this category enhanced Erk activity, we observed effects ranging from fast, large-amplitude pulses to small-amplitude pulses on a high baseline. The fast, frequent Erk pulses elicited by many Class 3 drugs are distinct from those produced at any EGF concentration (Figure 3.2B) or any previously-reported drug treatment, suggesting a novel mode of perturbation to EGFR/Ras/Erk signaling.

The drug screen results provide some immediate insights into the regulation of Erk signaling dynamics. Few kinase inhibitors suppressed Erk dynamics except those targeting the core EGFR/Ras/Erk cascade (Class 1), suggesting that this core cascade does not require permissive signaling inputs from other kinases to generate its dynamic activity. In contrast, many kinase inhibitors enhanced Erk activity (Classes 2 and 3), suggesting that the predominant crosstalk from other pathways is in the form of negative regulation. Additionally, some drug-treated cells exhibited Erk pulses as frequently as once every 20 min, a timescale that is unlikely to allow for complete cycles of protein synthesis/ degradation, receptor internalization/recycling, or ligand release/resynthesis. This observation suggests that the core Erk pulse generator operates through fast cycles of protein binding and post-translational modification, as has been demonstrated for other intracellular pulse generators. Taken together, these results demonstrate the power of high-throughput screening for probing dynamic signaling networks, and the screening pipeline we describe here could open the door to future large-scale investigations of dynamic cellular processes.
3.4 Non-EGFR RTK inhibition drives hyperactive Erk dynamics

The Erk dynamics-altering effects of our first two drug classes were primarily explained by modulation of known EGFR/Ras/Erk signaling components: Class 1 drugs are canonical pathway inhibitors, whereas Class 2 drugs target B-Raf through paradoxical activation. In contrast, how Class 3 drugs altered Erk dynamics remained a mystery. To gain insight into the mechanism of action of Class 3 inhibitors, we examined the compounds in this category for enrichment in various cellular functions.

We found that many Class 3 drugs (32 / 54) were developed as inhibitors against receptor tyrosine kinases (RTKs) other than EGFR (Figure 3.3E). In particular, multiple drugs targeting the c-Met and VEGF receptors potently increased Erk pulse frequency, baseline activity, or both. Prior reports indicate that Met and VEGFR are each expressed in keratinocytes and play functional roles in epidermal tissue, suggesting these receptors could act as bona fide modulators of Erk signaling in our screen. We thus set out to test whether inhibition of non-EGFR RTKs, particularly Met and VEGFR, can drive hyperactive Erk signaling dynamics.

As a first test, we asked whether an independent class of inhibitors against Met and VEGFR were able to drive similar changes in Erk dynamics. We focused on neutralizing antibodies against these RTKs because they target a distinct molecular surface than small-molecule kinase inhibitors (the extracellular ligand-binding domains vs. the intracellular kinase domain) and are highly specific for their cognate receptor. We treated KTR-H2B keratinocytes with neutralizing antibodies, kinase inhibitors, or vehicle controls and monitored Erk dynamics over time (Figure 3.4A-B). We found that both the antibodies and small-molecule inhibitors triggered similar changes in Erk dynamics; this effect was observed for both the Met and VEGFR receptors. These data strongly suggest that at least
Figure 3.4. Frequency-modulating RTK inhibitors enhance Erk dynamics by relieving Met- and VEGFR2-mediated negative feedback.

(a) Avg. keratinocyte pulse frequencies induced by either a Class 2 B-Raf inhibitor, Class 3 Met and/or VEGFR inhibitors, or neutralizing antibodies targeting Met or VEGFR2. Each replicate represents >300 single cells imaged every 3 min over 12 h.

(b) Average total Erk activity from drug-treated cells in (a).

(c) Distribution of drug-induced Erk activity levels in keratinocytes that had been pre-treated with either a vehicle control (labeled “-“) or with the EGFR inhibitor afatinib (labeled “+“). Points represent average ErkKTR cytoplasmic/nuclear ratios of >100 single cells from two replicates imaged over an 8 h timecourse.

(legend con’t on next page)
some Class 3 compounds increase Erk activity and pulse frequency by modulating the activity of the Met and VEGF receptors.

How might inhibition of non-EGFR RTKs alter activity within the EGFR/Ras/Erk network? We reasoned that one possible nexus of crosstalk is EGFR activity itself: prior studies of cancer therapeutics indicate that RTK inhibition can induce activation of parallel ligand-receptor pairs\textsuperscript{218,219}, and the identification of EGFR inhibitors as Class 1 drugs suggests that EGFR activity is essential for pulsatile Erk dynamics in keratinocytes (Figure 3.3B-D). To test whether Met and VEGFR inhibition activates EGFR, we used immunoblotting to measure EGFR and Erk phosphorylation at various timepoints after addition of the VEGFR inhibitor tivozanib and Met inhibitor golvatinib (Figure 3.4C). We observed high levels of Erk phosphorylation in response to both drugs, matching the increase in baseline Erk activity that we previously observed in the drug screen. In contrast, we found that EGFR phosphorylation was unaffected in these cells. We thus conclude that Class 3 RTK inhibitors are able to amplify Erk activity without altering the activity state of EGFR.

Although EGFR phosphorylation was not affected by VEGFR/Met inhibition, it may still serve as an essential permissive input for Erk dynamics. To test this hypothesis, we first treated KTR-H2B keratinocytes with the EGFR inhibitor lapatinib to extinguish EGFR-driven Erk dynamics and assessed whether Erk activity was restored by subsequent addition of Class 3 RTK inhibitors (Figure 3.4D). Indeed, we found that Class 3
compounds were unable to activate Erk in EGFR-inhibited cells. These data indicate that Class 3 drugs are not sufficient to activate Erk, and that active EGFR signaling is a necessary permissive input for their Erk-stimulatory effects. Taken together, our findings support a model in which Class 3 compounds can stimulate Erk activity by inhibiting the Met or VEGF receptors, relieving negative regulation on the Ras/Erk pathway at a node downstream of the EGF receptor (Figure 3.4E).

### 3.5 Drug-altered Erk dynamics have distinct effects on cell proliferation

Thus far we have described three classes of drug-induced changes to Erk dynamics: complete inhibition of Erk activity by Class 1 compounds; slow, wide Erk pulses in response to Class 2 compounds; and frequent, short pulses in response to Class 3 drugs. We next set out to test whether these different classes of Erk dynamics are also interpreted into distinct cellular responses. Prior work suggests that Ras/Erk dynamics are tightly coupled to cell proliferation\textsuperscript{61,62,91}, leading us to ask whether – and to what extent – proliferation is affected by compounds that alter Erk dynamics (Figure 3.5A).

Mammalian cells commit to cell cycle entry based on mitogenic inputs delivered over the course of an 8 h period during G1 phase\textsuperscript{220,221}. We thus first tested whether drug-induced changes to Erk dynamics were persistent over a similar time period such that they might impact cells’ proliferative decision making. We prepared KTR-H2B keratinocytes in multi-well plates as before, treated them with top drug hits from Classes 2 and 3, and imaged the resulting Erk dynamics over 14 h (Figure 3.5B). In all conditions, altered Erk dynamics were persistent over time: Class 2 drugs depressed pulse frequency and amplified total Erk activity equally at early and late time points, while Class 3 drugs caused stable increases in both pulse frequency and total Erk activity (Figure 3.5C). Thus, we
Figure 3.5. Kinase inhibitors that alter Erk dynamics also alter proliferative decision-making.

(a) Proliferation is assessed by flow cytometry after growing keratinocytes in the presence of inhibitors from each dynamics-altering class for 22 h, thus providing a readout of how different dynamic Erk signals are interpreted at the level of cell fate.

(b) Heatmaps of Erk activity in 40 randomly-selected cells imaged over 14 h after addition of DMSO vehicle control, a Class 2 B-Raf inhibitor (GDC0879), or a Class 3 frequency-modulating RTK inhibitor (pazopanib). Each row represents a separate single cell.

(c) Avg. Erk pulse frequencies for all cells calculated over the first and second halves of a 14 h timecourse. Each replicate represents >200 single cells imaged every 3 min.

(d-e) Keratinocytes were treated as indicated and allowed to grow for 22 h in either (d) complete or (e) GF-free media. The proliferative fractions, measured as the percentage of cells in S phase, are shown for keratinocytes treated with Class 1 (purple), Class 2 (green), and Class 3 drugs (blue). Each point represents a separate 20,000-cell biological replicate.
see that although keratinocytes are able to adapt and remodel their Erk signaling dynamics in response to changes in external mitogenic inputs (Figure 3.2C-D), they do not exhibit an equivalent adaptive response to pharmacological perturbations.

We next tested whether inhibitors of each class also triggered distinct effects on cell proliferation. Keratinocytes cultured in complete or GF-free media were treated with compounds from each of the three drug classes and assessed for a proliferative response by DNA content staining after 22 h (Figure 3.5A). As expected, Class 1 drugs that completely abolished Erk activity also blocked proliferation in all media conditions (Figure 3.5D-E; purple bars). Conversely, Class 2 compounds that trigger slow Erk pulses induced cell proliferation comparable to maximal EGF treatment in all conditions (Figure 3.5D-E; green bars). Surprisingly, treatment with Class 3 compounds increased proliferation in a context-dependent manner. Drug-treated cells grown in complete media exhibited increases in proliferation, with several inhibitors eliciting levels of proliferation comparable to EGF treatment (Figure 3.5D; blue bars). However, these same inhibitors failed to impact cell decision-making under GF-free conditions (Figure 3.5E; blue bars), as proliferation remained at baseline levels in all cases. These data reveal that drugs with different effects on Erk dynamics also elicit distinct proliferative outcomes, and suggest that the link between Erk activity and cell proliferation may depend on Erk’s temporal profile and additional external cues.

3.6 Optogenetic stimuli reveal causal relationships between Erk dynamics and cell proliferation

We have shown that certain classes of kinase inhibitors alter both Erk dynamics and cell proliferation, but the relationship between Erk dynamics and proliferation must be interpreted with caution. First, it is correlative, not causal; second, each drug alters
multiple features of Erk dynamics (e.g., pulse frequency and cumulative dose) and may also affect multiple additional signaling pathways. We thus sought to directly perturb Erk dynamics — simulating the changes to pulse frequency induced by different drug classes — to assess their effects on cell proliferation.

Optogenetic control is ideal for perturbing signaling dynamics and mapping their cell fate consequences\textsuperscript{222}, because light-induced activity can be targeted to a single intracellular node and controlled over time. We previously developed the OptoSOS system for reversibly controlling Ras activity on a minutes timescale\textsuperscript{38,75}. Here, we transduced keratinocytes with a lentivirally-encoded variant of the OptoSOS system that uses the blue-light-sensitive iLID/SspB heterodimer pair\textsuperscript{23,105} instead of the red-light-sensitive Phy/PIF pair\textsuperscript{32}. This iLID-based OptoSOS system is preferable for the current study as it is small enough to be encoded in a single lentiviral vector, does not require the addition of a small-molecule chromophore, and is well-expressed by keratinocytes.

Our goal is to completely control Erk dynamics with light, yet keratinocytes exhibit endogenous Erk pulses that may play a confounding role. We reasoned that pre-treatment with the EGFR inhibitor lapatinib could abolish endogenous dynamics without affecting direct Ras activation by light (Figure 3.6A). To validate this approach, we prepared KTR-H2B keratinocytes expressing the OptoSOS system that we imaged for 60 min, treated with lapatinib, and then applied 15 min pulses of 450 nm blue light. We observed stable and consistent toggling of Erk activity in these cells for $>8$ h (Figure 3.6B). These data demonstrate that combining EGFR inhibition and OptoSOS stimulation can be used to precisely control Erk signaling dynamics over extended periods of time in keratinocytes.

We next set out to mimic the dynamic alterations of Class 2 and Class 3 drugs with light. We previously observed that while both of these drug classes increase Erk activity, they do so with opposing effects on pulse frequency: Class 2 drugs induce long, slow pulses while Class 3 drugs elicit short, frequent pulses. We thus designed light stimuli with the
Figure 3.6. Optogenetic reconstitution of each class of drug-altered Erk dynamics induces identical proliferative responses.
(a) Keratinocyte Ras/Erk dynamics are placed under optogenetic control by first eliminating endogenous Erk pulses with an EGFR inhibitor, then using the OptoSOS system to apply light-dependent inputs directly to Ras.
(b) A test for the sufficiency of drug-induced dynamics: measuring whether cells that receive 1 h of total Ras/Erk input over 2 h will make different proliferative decisions if the input is delivered in one 60 min pulse, two 30 min pulses, or six 10 min pulses.
(c) Representative single-cell Erk activity traces from keratinocytes stimulated as in (a). Cells’ endogenous dynamics were imaged for 1 h, then the EGFR inhibitor lapatinib was added to extinguish this endogenous Erk signaling, after which blue light inputs to OptoSOS were toggled ON/OFF every 15 min for > 4.5 h.
(d-e) OptoSOS keratinocytes in (d) complete media or (e) GF-free media were first treated with either DMSO/EGF controls (gray) or the EGFR inhibitor lapatinib. Cells were then either kept in the dark (grey and purple) or placed under time-varying light inputs (green and blue) for 22 h. Proliferation is shown for cells that received no light inputs (grey and purple), long/slow light input (green), and fast/frequent light inputs (blue). Each point represents a separate 20,000-cell biological replicate.
total time on but different pulse frequencies, delivering repeated pulses on a 10'/10', 30'/30', or 60'/60' cycle (10'/10' indicates a loop of a 10 min light pulse followed by 10 min in the dark) using an Arduino microcontroller in a tissue-culture incubator (Figure 3.6C). Lapatinib-treated OptoSOS keratinocytes were exposed to either these pulse sequences or constant darkness in both complete and GF-free media conditions and were assayed for DNA content after 22 h.

The proliferative responses triggered by optogenetic Ras/Erk pulses broadly matched those obtained from Class 1, 2, and 3 compounds. Lapatinib-treated, dark-incubated cells exhibited low levels of proliferation in both media conditions (Figure 3.6D-E; purple bars), as expected from our earlier experiments (Figure 3.5D-E). Low-frequency (60'/60') optogenetic stimuli triggered comparable results to Class 2 compounds, resulting in high levels of proliferation in both complete and GF-free media (Figure 3.6D-E; green bars). Finally, higher-frequency stimuli (10'/10' and 30'/30') selectively increased proliferation in complete media (Figure 3.6D) but had no effect on cells grown in GF-free media (Figure 3.6E), matching the context-dependent control of proliferation that we observed in response to Class 3 drugs (Figure 3.5D-E).

Our small-molecule and optogenetic data can be summarized in a conceptual model of how alterations to Erk dynamics are interpreted into proliferative decision making (drug data: Figure 3.7A; optogenetic data: Figure 3.7B). A proliferative response can be considered as a point in a 2-dimensional landscape where the X and Y axes represent growth in GF-free media or complete media, respectively. Resting cells proliferate in complete media but not GF-free media (Figure 3.7, gray point), and cells with inhibited EGFR/Ras/Erk signaling exhibit low proliferation in both conditions (Figure 3.7, purple points). We find that different dynamics ‘move’ cellular responses in distinct directions, with slow, gradual Erk pulses associated with enhanced proliferation in all conditions (Figure 3.7, green points) and fast, frequent Erk pulses associated with
enhanced proliferation only in the presence of external growth factors (Figure 3.7, blue points). Movements in this two-dimensional landscape are consistent between distinct methods of altering Erk dynamics. We thus conclude that cell proliferation response combines independent cues from the cell’s external environment (e.g., complete vs. GF-free media) as well as the dynamics of Ras/Erk signaling activity.

3.7 Discussion

3.7.1 Primary epidermal stem cells as a platform to screen for dynamic perturbations
While most core mammalian signaling pathways exhibit complex signaling dynamics\textsuperscript{[195]}, the processes that underlie their generation, regulation, and interpretation remain largely uncharacterized. High-throughput screening is a logical approach to better understand these processes, as screens have provided fundamental insights into our understanding of biology for over a century\textsuperscript{[198,223,224]}. However, though \textit{in silico} approaches have demonstrated that signaling dynamics could serve as a valuable screening phenotype\textsuperscript{[197]}, the design and execution of a dynamics-focused screen in living cells has faced substantial experimental and computational obstacles.

One of the most notable obstacles has been the scarcity and inconsistency of dynamic behaviors in most classical model systems. Individual pulses of dynamic signaling activity are typically rapid (a single Erk pulse takes place over 10-15 min) but occur infrequently (once per multiple hours) and are highly variable between cells. This necessitates high time-resolution studies in hundreds of single cells over potentially multi-day-long time periods, which can preclude simultaneous analysis of the large numbers of stimulus conditions required for screens.

In this study, we report the establishment of an experimental and computational pipeline to enable screens for altered signaling dynamics. A crucial component of this pipeline is the use of primary mouse epidermal stem cells (keratinocytes) as a model system, which we report to undergo robust and highly dynamic Erk pulses even in the absence of externally-supplied growth factors (Figure 3.2A-B). The potency of starved keratinocytes’ endogenous Erk activity was a particularly surprising finding and underscores the extent to which epithelial cells autonomously generate their own external stimuli (e.g., EGFR activators etc.). The cell physiological purpose of these autocrine/paracrine signaling behaviors is still poorly understood but has been shown to be quite widespread, even in cultured cells\textsuperscript{[8,62,64,225]}, and is a phenomenon that merits further consideration in future studies.
3.7.2 A dynamics drug screen provides mechanistic insights into the Erk pulse generator

In this study, we used a small molecule kinase inhibitor screen to identify regulatory networks that control Ras/Erk signaling dynamics, and we identified a number of perturbations that can dramatically alter these dynamics (Figure 3.3B). One of our most striking findings was a subset of inhibitors that enhanced the frequency of Erk activity pulses (Figures 3.4A, 3.5B). Even before we consider how these compounds alter Erk dynamics, careful analyses of the high-frequency pulses themselves can shed light on the underlying network architecture. The rapid pulse frequencies achieved by our top hits suggest that we can largely rule out slower timescale processes (e.g., transcription/translation, ligand release, receptor internalization) as potential sources of pulsatile Erk signaling, which further implies that Erk pulses are likely generated by the more rapid processes of post-translational modification and protein-protein interactions.

We found that, for untreated keratinocytes imaged in GF-free media over 14 h, the distribution of times between consecutive pulses resembled an exponentially-modified Gaussian (EMG) (Figure C.3A). The composite EMG distribution has previously been shown to be relevant to the study of cell cycle times between consecutive divisions, and is broadly applicable to any process in which the time required for an object to pass from an initial state to a final state can be defined by two independent random variables: (1) the time it takes to move from an initial to an intermediate state, and (2) the time it takes to exit from that intermediate to the final state. The shape of the EMG distribution tells us about two important design features of the Erk pulse generator: first, it tells us that cells appear to move from an initial (post-pulse) state to an intermediate (ready-to-pulse) state via a deterministic process. Second, it tells us that cells then exit this intermediate state to reach a final (pulsing) state by a first-order process. This suggests that the Erk pulse
generator consists of two parts, a post-pulse refractory period of relatively fixed length during which a new pulse cannot arise, followed by a ‘permissive’ period in which pulses arise sporadically and during which the likelihood of pulsing increases with time.

These conclusions are further supported by data from our kinase inhibitor screen. Notably, we identified two drug classes that appear to push the distribution of inter-pulse timing in one direction or the other. First, we found that Class 2 drugs stabilize upstream inputs from Raf, which lengthens the duration and decreases the frequency of Erk pulses, thus shifting the distribution of inter-pulse times into a regime that resembles a pure Gaussian (Figure C.3B). By comparison, we showed that Class 3 drugs cause high-frequency Erk pulses by relieving pathway inhibition coming from non-EGFR RTKs, thereby pushing the inter-pulse distribution into a much more exponential regime (Figure C.3C).

3.7.3 A new class of ‘Erk-frequency-modulating’ kinase inhibitors enhance Erk signaling by targeting non-EGFR receptor tyrosine kinases

Few, if any, kinases are thought to actively suppress MAPK signaling such that their inhibition would result in increased Erk activity, which made the finding of any Erk activators in our kinase inhibitor library particularly unexpected. One notable exception is B-Raf, where the recent discovery of so-called ‘paradox-activating’ inhibitors shed light both on the clinical phenomenon of secondary cancers induced in patients treated with these drugs and also on the fundamental biology underlying B-Raf activation and signal transmission. Accordingly, the set of Erk-frequency-modulating receptor-tyrosine kinase inhibitors we describe in the current study may represent another such paradox, as RTKs are canonically thought to regulate Erk in the opposite direction.
Certain mechanistic similarities exist between these Erk-frequency-modulating RTK inhibitors and the paradox-activating B-Raf inhibitors. Most notably, in both cases, inhibiting a classic upstream Ras/Erk activator causes unexpected and acute Erk activation. More importantly, the Erk activation achieved by both of these drug classes does not rely on compensatory gene expression or rewiring caused by long-term exposure to a drug\textsuperscript{218,219,228,229}, and thus likely represents a design feature of the Erk pulse generator.

However, further investigation of these Class 3 frequency-modulating RTK inhibitors also revealed a number of surprises. Notably, the high-frequency phenotype produced by these drugs exceeds the Erk pulse frequencies obtained even by the most optimal dose of EGF (Figure 3.2B) and does not alter levels of EGFR phosphorylation (Figure 3.4D). This raised the possibility that perhaps Class 3 drugs could be modulating Ras/Erk dynamics simply by activating other receptors via autocrine/paracrine signaling loops, as upregulation of these processes has been implicated in RTK inhibitor-resistant cancers\textsuperscript{230,231}. However, because of the acute nature of these drugs’ effects (observed < 1 h after addition) and because treatment with Class 3 compounds had no effect on Erk signaling in EGFR-inhibited cells (Figure 3.4C), their frequency-modulating effects are unlikely to be acting by activating alternative receptors. Taken together with the finding that their effects on Erk dynamics are stable and long-lived (Figure 3.5B), these results suggest that Class 3 drugs amplify the frequency of Erk activity pulses via acute intracellular remodeling of the signaling network by relieving inhibitory connections downstream of EGFR (Figure 3.4E).

We identified Met and VEGFR2 as two of the non-EGFR RTKs whose inhibition underlies the effects of Class 3 drugs on keratinocyte Erk dynamics (Figure 3.4A), though one might logically question whether receptors that bind to ligands named “hepatocyte growth factor” (HGF, the ligand of Met) and “vascular endothelial growth factor” (the ligand family of VEGFRs) actually play functional roles in the basal epidermis. However,
not only is Met well-expressed by epidermal keratinocytes\textsuperscript{232}, but HGF is known to promote their migration, proliferation, and survival\textsuperscript{233,234} and Met signaling in keratinocytes is critical to the wound healing process\textsuperscript{216}. Similarly, all VEGFR isoforms are expressed by epidermal keratinocytes\textsuperscript{235}, where their role closely resembles that of Met, as VEGFRs have also been shown to be drivers of keratinocyte proliferation, migration, and wound healing\textsuperscript{217,236,237}. The broad overlapping roles that the Met and VEGF receptors play in keratinocyte physiology, combined with the similarity of their effects on Erk dynamics (Figure 3.4A), suggest that some of these similarities may be explained by one or more regulatory connections shared by both receptors that serve to tune the frequency of Ras/Erk signaling dynamics.

3.7.4 Differences in Erk dynamics can drive proliferative decision-making

Are dynamics alone sufficient to change cell fate decisions? Our findings reveal that proliferative decision-making is indeed gated to filter out high-frequency dynamic signals in the absence of external mitogens, as only long-duration Erk dynamics were able to uniformly elicit cell growth in all media conditions (Figure 3.7A-B). Additionally, these data also suggest that cells employ combinatorial logic when making proliferative decisions in certain contexts, as we found that favorable environmental conditions appeared to override this gating and allowed each of our slow, medium, and fast Erk dynamic regimes to enhance proliferation (Figure 3.7A-B).

Just as our previous analysis showed that Class 3 drugs hyperactivate Erk dynamics through a mechanism that depends on EGFR signaling (Figure 3.4C) but does not directly alter its biochemical activation (Figure 3.4D), the context-dependence of their proliferative effects is consistent with a model in which Class 3 inhibitors alter Erk
dynamics by relieving pathway inhibition (Figure 3.4E). Further, these results also suggest that cells interpret certain Erk dynamics using a type of logic-gate where high-frequency Erk signals require a second logical ‘AND’ signal from the environment (e.g., factors in complete media) to initiate proliferation, whereas slow/long-duration Erk dynamics can bypass this safeguard and initiate proliferation independent of cells’ environmental setting.

These experiments demonstrate the utility of optogenetics for testing the sufficiency of emergent properties to drive cell physiological outcomes. Because we could isolate and independently vary the timing of Erk activity, we were able to establish the cause-and-effect relationship between an individual dynamic feature (i.e., pulse frequency) and a downstream cell behavior, and thus were able to show that differences in Erk dynamics are alone sufficient to drive proliferation.
Chapter 4

Current work: Receptor-independent generation of pulsatile Erk dynamics

The Ras/Erk pathway regulates diverse processes including proliferation, migration, and survival. Receptor-level input signals to Ras are transmitted in single cells as short pulses of Erk kinase activity, and the dynamics of Erk activity are thought to allow this pathway to control multiple divergent cell fate decisions. Though the network topology of Ras/Erk signaling has been the subject of intense study, how complex Erk dynamics arise remains unclear. Here, we combine optogenetic control of Ras/Erk signaling with highly sensitive live-cell reporters to investigate this question. We show that single cells exhibit dynamic pulses of Erk kinase activity but not of Erk nuclear localization. However, turning the Ras/Erk pathway ON and OFF with our optogenetic system results in immediate localization changes of both reporters, suggesting that the regulation of Erk activity is uncoupled from the dynamics of upstream pathway activity. Supporting this hypothesis, we find that, while EGFR inhibitor treatment abolishes cellular Erk activity, the addition of constant, low-amplitude optogenetic stimulation is sufficient to restore Erk activity pulses. Finally, we find that while Erk pulses are unaffected by transcription inhibition,
pulsing is eliminated when the phosphatases DUSP1 and DUSP6 (but not other DUSP family phosphatases) are either overexpressed or chemically inhibited. These findings indicate that Erk is the direct target of transcription-independent negative feedback, which regulates its pulsatile activity.

4.1 Temporal Erk regulation

In Chapter 1, we compared cells to analog robots, each equipped with a broad set of sensors that allow it to gather information from its environment and actuators that allow it to respond to changes as necessary. However, a central puzzle in signaling biology is how cells transmit information from hundreds of inputs using fewer than ten distinct cell signaling pathways. Chapter 1 introduced the idea that cells can encode information in the time-varying activity of their core signaling pathways, thus allowing a single ‘wire’ to transmit more than one type of information. In Chapters 2 and 3, we investigated how Ras/Erk dynamics transmit complex information and found that long-duration pulses of Erk activity drive cell proliferation in part by enhancing glycolysis at four flux-controlling nodes. These findings support the idea that information can be encoded in the dynamics of Ras/Erk activity, but provide little insight into how such dynamics are generated.

Where in the Ras/Erk cascade are activity pulses generated? While previous studies have suggested that perhaps receptor-level mechanisms, negative feedback loops between MAPK pathway components, or mechanical forces and cell protrusions are involved in the generation Erk activity pulses, we still lack a definitive description of the pulse generator. This is driven in part by the fact that, although the major Ras/Erk pathway components are relatively few in number, tracing the origin of an emergent property like signaling dynamics remains a tremendous challenge because altering any node along the interconnected Ras/Erk kinase cascade using traditional knockdown or
mutagenesis approaches is likely to abolish dynamics regardless of whether or not said node is involved in pulse generation.

In this chapter, we take a systems approach to examine pulsatile signaling activity using the highly dynamic primary mouse keratinocyte model system introduced in Chapter 3. We first examine signaling dynamics at two distinct nodes in the Ras/Erk cascade and find that, while Erk kinase activity consistently exhibits robust pulsatile behavior, the nuclear/cytoplasmic localization of Erk itself (which depends on upstream MEK activity) remains static. Additionally, we find that although Erk activity pulses represent high-amplitude signaling events, using optogenetic control to apply a range of low- and high-amplitude inputs to Ras shows that Erk kinase activity and Erk nuclear localization both respond with similar sensitivities. We next show that, while EGFR inhibitors abolish Erk dynamics, pulsatile Erk activity can be rescued in EGFR-inhibited cells by applying constant, low-intensity optogenetic inputs. Finally, we examine possible downstream regulatory mechanisms, showing that Erk dynamics are regulated by a transcription-independent negative feedback loop that involves the phosphatases DUSP1 and DUSP6. Our work uncovers fundamental new insights into both the Erk pulse generator and the network architecture of the Ras/Erk MAPK pathway, while also demonstrating the power of optogenetics for probing the source of emergent properties.

4.2 Erk activity pulses are uncoupled from its nuclear/cytoplasmic localization

Work from our group and others has shown that upstream signaling from the EGF receptor is necessary for the generation of Erk activity pulses (See Chapter 3). However, while EGFR activity may be necessary for subsequent Erk activity, it remains unclear whether pulsatile signals arise at the level of the receptor. We reasoned that, if
upstream receptor-level inputs are indeed driving downstream pulsatile Erk signaling, we should be able to observe coordinated pulsatile behavior at each node as the dynamics are faithfully transmitted from EGFR to Erk.

To interrogate the pathway node at which pulsatile Erk dynamics arise, we took advantage of two live-cell biosensors that report on distinct enzymatic events in the Ras/Erk kinase cascade. First, we used the Erk kinase translocation reporter (ErkKTR), whose nuclear/cytoplasmic localization reports specifically on the instantaneous kinase activity of Erk\(^{99}\) (**Figure 4.1A**). In the absence of Erk activity, the ErkKTR localizes to the nucleus until Erk-mediated KTR phosphorylation exposes an NES and enhances the reporter’s cytoplasmic shuttling. Second, we used a FusionRed-tagged Erk2 (FR-Erk2), whose nuclear/cytoplasmic distribution is thought to report on MEK kinase activity\(^{239}\) (**Figure 4.1B**). This is because MEK actively binds non-phosphorylated Erk until an upstream signal causes it to phosphorylate and release Erk, which then rapidly translocates to the nucleus\(^{5,38,203,240}\).

To examine signaling dynamics at two adjacent Ras/Erk pathway nodes, we plated primary mouse keratinocytes on glass multi-well plates and co-transduced with lentiviral vectors encoding ErkKTR-YFP and FR-Erk2. Live imaging revealed that, while the ErkKTR revealed consistent and highly pulsatile Erk kinase activity in a majority of cells, cells did not exhibit observable changes in the nuclear/cytoplasmic ratio of FP-Erk2 (**Figure 4.1C**). These findings suggest that the pulsatile behavior we see at the level of Erk kinase activity may not be regulated by upstream pathway activity, as dynamics are not observable just one node upstream. This raises the possibility that Erk activity pulses may be generated via processes acting downstream of MEK.

Alternatively, these results could also support a scenario in which Erk activity pulses simply represent small fluctuations that are not of sufficient magnitude to activate the MEK-dependent nuclear localization of Erk2. To determine whether these Erk pulses
Figure 4.1. Endogenous Erk dynamics consist of high-amplitude activity pulses decoupled from its nuclear localization.

(a-b) Schematics of two live-cell biosensors that can be used to track activity at successive nodes in the Ras/Erk MAPK signaling cascade. The cytoplasmic/nuclear ratio of (a) ErkKTR reports on instantaneous Erk kinase activity, while the nuclear/cytoplasmic ratio of (b) fluorescently-tagged Erk2 reports on upstream pathway signals.

(c) Representative ErkKTR and FR-Erk2 traces from a single cell imaged every 2 min over 1.5 h. Changes in KTR localization over time show spontaneous pulses of Erk kinase activity, while FR-Erk2 nuclear/cytoplasmic localization remains constant.

(d) Immunofluorescence staining for ppErk in keratinocytes after treatment with DMSO vehicle control, fetal bovine serum, or the MEK inhibitor U0126 (8 µM). Images show fluorescent ErkKTR-iRFP (left) and antibody staining for ppErk (right).

(e) Average phospho-Erk levels quantified for cells that exhibited either an endogenous or FBS-induced KTR peak at the time of fixation (left) or an endogenous or U0126-induced KTR trough at the time of fixation (right). n=50 cells per condition.
represent low- or high-amplitude signaling events, we used quantitative immunostaining to measure the levels of biochemical activation achieved during a spontaneous pulse of activity. We plated keratinocytes stably expressing the ErkKTR on glass as before, treated them with either fetal bovine serum (FBS), the MEK inhibitor U0126, or DMSO vehicle control, and then fixed cells and stained for phospho-Erk (Figure 4.1D). The FBS- and U0126-treated conditions were respectively used to determine the upper and lower limits of achievable phospho-Erk levels, which allowed us to establish the dynamic range of the assay and provided reference values to which we could compare the levels of phospho-Erk achieved by spontaneous Erk activity pulses.

We identified unstimulated cells in which an endogenous pulse of Erk activity had, at the time of fixation, just caused the KTR to reach either a ‘peak’ (i.e., maximally active) or a ‘trough’ (i.e., maximally inactive) and compared their corresponding ppErk levels to the FBS- or U0126-treated controls (Figure 4.1E). Notably, we found that the cellular phospho-Erk levels at a KTR ‘peak’ were indistinguishable from the maximum values achieved by FBS treatment (p=0.27), while cells at a KTR ‘trough’ actually exhibited lower average phospho-Erk levels than the minimum values achieved by U0126 MEK inhibitor treatment (p=0.04) (Figure 4.1E). These findings demonstrate that Erk activity pulses represent genuine high-amplitude signaling events that drive levels phospho-Erk levels between the minimum and maximum achievable values. Together with our observation that Erk2 localization does not appear to exhibit pulsatile behavior (Figure 4.1C), these data support a model in which Erk activity pulses are independent of upstream dynamics.

4.3 Erk activity and nuclear localization respond to the same range of inputs

We have shown that Erk activity pulses represent high-amplitude events that are not visualized at the level of FR-Erk nuclear translocation, but it remained possible that FR-
Erk2 simply possessed too poor a dynamic range to report even on high-amplitude signaling events. An ideal approach to test this hypothesis would be to directly compare the input sensitivities of each reporter by stimulating single cells with uniform upstream inputs over a broad range of precisely-controlled amplitudes. Cellular optogenetics approaches have recently emerged as powerful tools for these types of questions; because the intensity and duration of light can be tuned in real time, they allow us to perform full amplitude scans in the same single cells. We thus employed the ‘OptoSOS’ system developed by our group to directly control Ras activity in single cells with 450 nm blue light\textsuperscript{38,105} (Figure 4.2A).

To validate this approach, we plated keratinocytes on glass, co-transduced them with lentiviral vectors encoding ErkKTR-iRFP, FusionRed-Erk2, and OptoSOS, and prepared them as before. We then added TAPI-1, a TNF-α converting enzyme and matrix metalloproteinase inhibitor, to minimize endogenous signaling activity and imaged KTR and Erk2 dynamic responses across a range of OptoSOS input amplitudes. Blue light was
held at a given amplitude for 15 min and was cycled through successive 15 min intervals of 0%, 50%, 5%, 100%, 10%, 20%, and 0% LED power (Figure 4.2B, right axis). We found that both reporters exhibited rapid, synchronous localization changes in response to all applied inputs, including stimuli as low as 5-10% (Figure 4.2B, left axis). As expected, the KTR and Erk2 nuclear/cytoplasmic shuttling responses were perfectly out of phase: in unstimulated cells Erk2 is predominantly cytoplasmic and KTR predominantly nuclear, while upstream Ras/MAPK pathway stimulation elicits opposing movement of both proteins (Figure 4.1A-B).

These data show that, while the ErkKTR possesses a larger dynamic range than FP-Erk2 (i.e., greater fold-change), both reporters display rapid responses to inputs across the full amplitude scan, indicating that both are indeed capable of reporting on dynamic changes in upstream inputs over a similar equally-broad range of amplitudes. And, given the observation that endogenous Erk pulses represent bona fide high-amplitude signaling events (Figure 4.1D-E), it is likely that our optogenetic amplitude scan achieved comparable levels of Ras/Erk activation.

Notably, despite differences in the overall fold-change of their responses, both reporters proved to be similarly sensitive, as even low-amplitude light inputs that elicited sub-maximal KTR responses were able to trigger appreciable MEK-dependent nuclear localization of Erk2. Together, these findings support a model in which Erk activity pulses are generated by a mechanism that does not drive similar pulses in upstream signaling activity and suggest that dynamic regulation of Erk activity is uncoupled from the dynamics of upstream pathway activity.
4.4 Unvarying Ras input reconstitutes Erk pulses in EGFR-inhibited cells

How can we test for the presence of a receptor-independent Erk pulse generator? The lack of observable changes in MEK activity during Erk pulses suggests that it may be possible to generate pulsatile Erk signaling by simply applying invariant upstream pathway inputs. To test this hypothesis, we returned to the OptoSOS system, which can be used to apply a constant, low-amplitude activating input that bypasses the receptor and plugs in directly to Ras38,107. However, keratinocytes naturally exhibit highly-active pulsatile Erk signaling dynamics (as outlined in Chapter 3) that could confound interpretation of these experiments. To establish complete optogenetic control over Ras/Erk activity, we pre-treated keratinocytes with the EGFR inhibitor lapatinib, which eliminates endogenous Erk pulses without altering the transmission of light-dependent OptoSOS inputs to Ras (See Chapter 3, Figure 3.6A-B).

We then set out to test whether time-invariant, low-amplitude OptoSOS inputs could reconstitute Erk activity pulses in EGFR-inhibited cells. We plated keratinocytes stably co-expressing ErkKTR-iRFP and a histone H2B-RFP-based nuclear marker, transduced with OptoSOS lentivirus, and prepared cells for imaging as before. Note that transiently transducing cells on the plate provided us with built-in negative controls, as it ensured that each imaging position contained a mixture of OptoSOS-expressing and non-expressing cells. If Erk pulses are indeed driven by receptor-independent mechanisms, we should expect to see that the addition of blue light restores pulsatile behavior only in cells that express OptoSOS (Figure 4.3A).

We first imaged endogenous Erk activity for 60 min, added lapatinib for an additional 60 min, and then applied low, constant light stimulation for > 4 h (Figure 4.3B). In the 60 minutes preceding addition of lapatinib, approximately 70 % of all cells
displayed one or more Erk activity pulses (Figure 4.3C, left), while lapatinib addition completely abolished pulsatile Erk dynamics in all cells over the following 60 min (Figure 4.3).
4.3C, middle). Subsequent introduction of a low, constant blue light stimulus was sufficient to restore Erk activity pulses in >30% of OptoSOS-expressing cells and had no effect on non-expressing cells, all of which remained inhibited (Figure 4.3C, right). Notably, the dynamic trajectories of light-reconstituted Erk pulses resembled those produced by endogenous signaling, both in frequency and in magnitude, suggesting that light inputs may be triggering the same feedback interactions and network architecture that underlie endogenous dynamics.

These data demonstrate that the emergent property of pulsatile Erk signaling does not require receptor-level input, as we show that similar time-varying Erk behavior can be driven by time-invariant, receptor-independent Ras activation. This matches our previous observation that spontaneous Erk pulses appear to take place in the presence of time-invariant FR-Erk2 movement (Figure 4.1C) and further suggests that the mechanisms driving pulsatile Erk signaling may lie, at least in part, downstream of Erk itself.

4.5 The Erk phosphatases DUSP1 and DUSP6 regulate pulsatile Erk signaling

The dynamic, time-varying output of a given signaling pathway is determined by the push-and-pull balancing act that takes place between upstream inputs that activate and negative regulators that inactivate. Our findings have thus far demonstrated that pulsatile Erk outputs can be generated by unvarying upstream activating inputs, which raised the possibility that negative feedback mechanisms acting directly on Erk itself may play a role in regulating its dynamic activity.

What negative regulators exist downstream of Erk? While a variety of interactions are known to tune Erk activity241, the dual-specificity MAPK phosphatases (known as MKPs or DUSPs) are the largest family of Erk-specific negative regulators242. Of the ten
catalytically active mammalian DUSPs, seven are thought to regulate Erk activity: the three Erk-selective cytoplasmic DUSPs (i.e., DUSPs 6, 7, and 9) and the four nuclear-inducible DUSPs (i.e., DUSPs 1, 2, 4, and 5)²⁴³⁻²⁴⁵.

Much of the literature regarding interactions between Erk and DUSPs suggests that activated Erk directly upregulates DUSP gene expression in a negative feedback loop that inactivates Erk by increasing overall DUSP protein levels²⁴⁵. However, we observed a discrepancy between the timing of a spontaneous Erk activity pulse (roughly 10-15 min from beginning-to-end) and the minimum ~45 min required to complete the processes of signal transduction, gene transcription, mRNA processing/export, translation, and protein folding/accumulation to appreciable levels⁷⁵. Moreover, the Erk frequency-modulating RTK inhibitors identified in Chapter 3 caused cells to pulse often as once every 20 mins (Figures 3.3, 3.4), far outstripping even the fastest possible gene expression circuit. We thus asked whether treating keratinocytes with the transcription inhibitor actinomycin D (ActD) would affect endogenous pulsatile Erk dynamics. Live imaging of Erk activity in keratinocytes revealed that pulsatile signaling was largely unaffected by the presence of ActD at 2 µg/mL, a concentration known to rapidly inhibit transcriptional elongation²⁴⁶ (Figure 4.4A). Importantly, a general upward trend in overall Erk activity after ActD addition can be clearly seen in Figure 4.4A, which supports the idea that expression of DUSPs and other negative regulators does indeed regulate Erk activity but that these effects are likely more relevant over longer timescales. However, we found that the dynamic features of pulsatile Erk signaling in ActD-treated cells were indistinguishable from those of vehicle control-treated cells over the 6 h following drug treatment (Figure 4.4B), demonstrating that Erk activity pulses do not require new transcript synthesis.

In light of these findings, we next set out to establish which, if any, DUSPs take part in the regulation of Erk activity pulses. We selected one or more candidate members
Figure 4.4. Erk activity pulses are transcription independent and regulated by DUSPs 1 and 6.

(a) Representative single cell traces of Erk activity after treatment with actinomycin D (red) or DMSO vehicle control (gray). Cells were imaged every 2 min for 6 h.
(b) Quantification of average Erk pulse frequency (upper) and average total Erk activity (lower) in cells treated with ActD (red) or DMSO (gray) as in (a). Points represent average values from n > 100 single cells per replicate.
(c) Single cell traces of Erk activity in cells overexpressing DUSPs 1, 4, 5, or 6. At 28 h after lentiviral transduction, cells were imaged every two minutes for 6 h. Two representative traces are shown per condition.
(d) Images showing expression and proper localization of each DUSP-BFP vector (left) along with corresponding KTR-iRFP expression (right).
(e) Single cell Erk activity trajectories before (left) and after (right, in red) treatment with the allosteric DUSP1/6 inhibitor BCI-215. Cells were imaged every 2 min for >5 h.
of each DUSP subfamily and generated lentiviral overexpression vectors encoding a BFP-tagged DUSP1, DUSP4, DUSP5, or DUSP6 transgene. Because each of these phosphatases has been shown to dephosphorylate Erk both in vitro and in full cells, the traditional Erk-activity-to-DUSP-transcription model of Erk activity regulation would predict that individual overexpression of each selected DUSP should be sufficient to inactivate Erk. Conversely, our ActD data suggest that DUSPs are either uninvolved in regulating pulsatile Erk dynamics or that their involvement cannot be explained by the Erk-activity-to-DUSP-transcription model.

We transduced KTR-/H2B-expressing keratinocytes with each of the individual DUSP constructs and performed live-cell imaging to monitor their effects on pulsatile Erk signaling dynamics. All DUSP-BFP constructs were well-expressed and properly localized at 48 h after lentiviral transduction (Figure 4.4D). Cells that overexpressed DUSP4 or DUSP5 displayed unperturbed Erk signaling dynamics over 6 h (Figure 4.4C), suggesting that although each of these phosphatases is capable of dephosphorylating Erk, none appear to be involved in regulating pulsatile Erk signaling. Conversely, cells that overexpressed DUSPs 1 or 6 exhibited stably inactive Erk signaling and produced no pulses over the course of 6 h (Figure 4.4C). These findings demonstrate that expression levels alone are insufficient to explain DUSPs’ role in regulating pulsatile Erk signaling, as only overexpression of DUSPs 1 and 6 altered spontaneous Erk activity pulses.

To further investigate how DUSPs 1 and 6 interact with Erk signaling, we next asked whether inhibiting these two regulatory nodes would also perturb pulsatile Erk dynamics. A series of recent studies identified and developed the small molecule ‘BCI’ that allosterically inhibits DUSP1 and DUSP6. Because the first-generation BCI compound proved to be fairly toxic, the same group recently reported a new chemical iteration termed ‘BCI-215’ that displayed minimal toxicity in vivo during zebrafish development and in paired cell culture assays. If DUSPs 1 and 6 indeed play a role in
regulating Erk activity pulses, we reasoned that inhibiting their phosphatase activity should disrupt Erk dynamics and result in rapid and sustained activation of Erk kinase activity.

We imaged endogenous Erk signaling behavior in keratinocytes for 2 h at 2 min intervals, then treated cells with the DUSP1/6 inhibitor BCI-215 and monitored the effects on Erk dynamics (Figure 4.4E). It is important to note that, while BCI-215 was substantially less toxic to our primary keratinocytes than the original BCI, we also observed negative effects on cell health following BCI-215 treatment, most notably a marked reduction in cell mobility (data not shown), and thus these findings must be interpreted with caution. With that caveat in mind, we did find that BCI-215 addition caused immediate, population-wide Erk activation that diminished its pulsatile signaling (Figure 4.4E), in line with what one might predict to happen upon the disruption of prominent negative regulatory interactions. Unexpectedly, cells subsequently underwent a period of gradual inactivation, which was followed by an extended steady state of intermediate largely non-pulsatile Erk activity. Thus, although inhibiting DUSPs 1 and 6 diminished dynamics and acutely increased Erk activity, these findings imply that some phosphatases retained the ability to dephosphorylate Erk and appear to have gradually reduced its activity to an intermediate level. We conclude that DUSPs 1 and 6 – but not DUSPs 4 or 5 – are key negative regulators that are involved in the establishment and maintenance of Erk signaling dynamics.

4.6 Discussion

Here we present strong new evidence that the mechanisms underlying the Erk pulse generator lie downstream of Erk itself and do not impose similar dynamics on upstream signaling activity. We have shown that unvarying, receptor-independent inputs to Ras can
re-generate pulsatile Erk dynamics in EGFR-inhibited cells (Figure 4.3) and that endogenous Erk activity pulses take place in the presence of apparent unvarying MEK activity (Figure 4.1C). We further present data suggesting that Erk activity pulses are high-amplitude events (Figures 4.1D-E) driven by constant upstream input and that they are modulated in a transcription-independent fashion by the phosphatases DUSP1 and DUSP6 (Figures 4.4A-E).

Efforts are ongoing in the lab to better characterize the proposed roles DUSPs 1 and 6 play in regulating Erk activity pulses. First, we are interested in using OptoSOS inputs to probe the Erk signaling networks of cells that either overexpress DUSPs 1/6 or have been treated with BCI-215. For DUSP-overexpressing cells, if we match the extreme levels of DUSP-mediated negative feedback in these cells with sufficiently high-amplitude light inputs, we should expect to be able to restore pulsing. For BCI-treated cells, if inhibition of DUSPs 1 and 6 truly eliminates the Erk pulse generator without affecting its ability to respond to upstream pathway activity, we anticipate that applying sequential OptoSOS step inputs to BCI-treated cells should tell us whether the system has become a linear signal integrator rather than an oscillator/pulse generator.

We are also currently exploring the possibility of manipulating the dynamic Ras/Erk signaling network using lentiviral overexpression vectors encoding either catalytically dead or dominant negative versions of DUSPs 1/6. The presence or absence of Erk pulses in cells overexpressing catalytically dead DUSPs 1/6 will indicate whether their phosphatase activities are required for their Erk-inhibitory function. If catalytically dead DUSPs can elicit the same effect as their wild-type counterparts, it would suggest that binding interactions between DUSPs and Erk (possibly via substrate sequestration or competition) could be underlying their inhibitory effects. Similarly, we hope to use dominant negative versions of DUSPs 1/6 as genetic versions of BCI-215 that will be less toxic while also conferring greater specificity for each individual target DUSP^{250–254}. While
genetic knockdowns are typically the preferred approach, attempts to use shRNA vectors for DUSP knockdown have in our hands been both inefficient and have appeared to induce the upregulation of compensatory DUSPs. Thus, we hope to use dominant negative mutations to monitor the effects of removing the activity of each candidate DUSP both individually and in pairs.

Finally, we also hope to further test the capacity of unvarying upstream signals to reconstitute Erk activity pulses by leveraging the recent discovery of new optogenetic tools that confer light-gated control over additional nodes in the Ras/Erk pathway. These tools consist of engineered light-switchable versions of either C-Raf/Raf1, MEK1, or MEK2 (See Chapter 1.1.2 and Figure 1.2A, middle). Using these approaches, we hope to test the ability of low-amplitude inputs (from nodes downstream of Ras) to re-activate pulsatile Erk signaling in EGFR-inhibited cells.

Taken together, our ongoing work is focused on defining the central Erk-directed feedback mechanisms that give rise to its pulsatile signaling behavior. In doing so, we hope to carefully describe the origins of the Ras/Erk pathway’s dynamic signaling language, allowing us to better understand its role as a central driver of cell proliferation not only in development and tissue homeostasis, but also in the context of diseases like cancer.
Chapter 5

Concluding remarks

Biological insight often begins with a simple observation that demands further investigation. When we first began this thesis project in the fall of 2015 (a bit more than 3.5 years ago), we had set our sights on understanding how oncogenic alterations to Ras/Erk signaling drive proliferation in triple-negative breast cancer (TNBC). However, close examination of Erk dynamics in >20 TNBC and untransformed control cell lines revealed an incredibly heterogeneous range of signaling behaviors, with no apparent distinction between cells of different origins. This observation made it clear that, if we hope to explain how a biological process goes awry, we first must obtain a thorough understanding of how the process works normally. And although Ras/Erk components are both highly-mutated and common therapeutic targets in human cancers167, we quickly learned that many attempts to understand (or treat) pathologies of Ras/Erk signaling are complicated by our lack of a clear definition of how it transmits information. We hope that this thesis will be a step towards such a definition.

Can we define the signaling ‘language’ that allows Ras/Erk dynamics to transmit information from multiple distinct upstream inputs? And can we map how those signals relate to subsequent downstream cellular outputs? One of the key elements that allowed us to address these questions in this thesis was our serendipitous observation of stable,
highly-dynamic Erk signaling activity in cultured primary keratinocytes, the result of a fantastic collaboration with the lab of Danelle Devenport (whose contribution to this thesis cannot be understated). This observation stood in stark contrast to the extensive heterogeneity we had observed in TNBC and untransformed human cell lines and thus provided us with a robust model system to systematically interrogate the Ras/Erk signal processing machinery.

Together, the work presented here demonstrates that, by examining signal processing at three processive levels, we can gain both a more quantitative and a better molecular understanding of how signals propagate through the Ras/Erk pathway: from the generation of dynamic signals, to the decoding of dynamic signaling inputs into cell fate outputs, to the orchestration of cell fate outputs by direct modulation of cellular biochemistry. Notably, our work emphasizes that both observational and perturbative studies are required for understanding the regulation and signal processing functions of complex signaling networks like Ras/Erk. We propose that untransformed primary cells – as well as quantitative metabolomics, high-throughput imaging-based screens, and temporally-precise optogenetic control – can provide a fertile platform for studies of complex dynamic signaling pathways, thus allowing researchers to assemble discrete signaling components into functional networks whose activity and regulation defines the language of cell signaling.
Appendix A

Optogenetic interrogation of the Ras/Erk signaling pathway

The Ras/Erk signaling pathway plays a central role in diverse cellular processes ranging from development to immune cell activation to neural plasticity to cancer. In recent years, this pathway has been widely studied using live-cell fluorescent biosensors, revealing complex Erk dynamics that arise in many cellular contexts. Yet despite these high-resolution tools for measurement, the field has lacked analogous tools for control over Ras/Erk signaling in live cells. In this chapter, we provide detailed methods for one such tool based on the optical control of Ras activity, which we call ‘OptoSOS.’ Expression of the OptoSOS constructs can be coupled with a live-cell reporter of Erk activity to reveal highly quantitative input-to-output maps of the pathway. Detailed herein are protocols for expressing the OptoSOS system in cultured cells, purifying the small molecule cofactor necessary for optical stimulation, imaging Erk responses using live-cell microscopy, and processing the imaging data to quantify Ras/Erk signaling dynamics.

A.1 The Ras/Erk signal transduction pathway

The Ras-to-Erk signaling cascade is one of the most intensely studied protein kinase networks in cell biology, responding to a variety of external stimuli by modulating cell
growth, survival, and differentiation. In recent years, advances in live-cell biosensors have revolutionized our ability to measure Erk activity in single cells, both *in vitro* and *in vivo*. These studies have uncovered a wealth of dynamic behaviors, including Erk responses that exhibit switchlike activation, oscillations, and traveling waves propagating from cell to cell across tissues.

The discovery of this rich world of Erk dynamics invites a question: what patterns of Erk activity determine a cell’s response to pathway input? We might imagine that certain genes respond selectively to a specific frequency of Erk oscillations or to the total integrated ‘area under the curve’ of Erk activity. Erk dynamics may also vary significantly between identically-treated cells. Yet some dynamic features vary more between cells than others, hinting that these noise-resistant features could be relied upon by the cell to accurately sense the environment.

Knowledge of the role played by such signaling dynamics has been limited because scientists have lacked the tools to selectively control Ras-Erk pathway activity. Extracellular ligands often bind tightly (and therefore cannot be washed on and off with high temporal resolution), and receptor internalization can desensitize cells to subsequent stimuli. Cellular optogenetics offers one solution to this challenge by engineering light-responsive proteins that can be expressed in cells to control specific pathways of interest, such as Ras/Erk. These light-inducible systems possess two unique features: (1) they are specific to only the pathway under optogenetic control, and (2) they allow for true dynamic regulation because input intensity can be precisely tuned in real time. Recent advances by our group and others have yielded a number of engineered signaling modules that can be controlled with spatially and temporally precise beams of visible light. While many excellent optogenetic systems have been developed, this chapter will focus on the light-inducible ‘Phy/PIF’ interaction system and its important advantages for use in cell signaling. Of currently available optogenetic tools, the Phy/PIF
system provides the fastest dynamic control, the broadest dynamic range, and the highest light-sensitivity (thereby limiting phototoxicity)\textsuperscript{264}.

In addition to studying how Erk dynamics control cellular responses, optogenetic inputs could also be used to dissect how Erk dynamics are generated. Ras/Erk signaling is subject to complex feedback regulation\textsuperscript{265–268}, crosstalk from other signaling pathways\textsuperscript{269}, and frequent mutation (pathway components are among the most commonly mutated nodes in human cancers)\textsuperscript{270,271}. By simultaneously stimulating Ras activity and measuring Erk responses in the presence of different extracellular cues, drugs, or mutations, we might learn how each condition affects transmission of signals through the pathway. In this chapter, we will describe how to use the Phy/PIF optogenetic system to control Ras-to-Erk signaling in cultured cell lines.

A.2 Controlling Erk activity with the Phy/PIF system

The Phy/PIF system involves light-dependent association of fragments of two \textit{Arabidopsis thaliana} proteins normally involved in stem elongation: phytochrome B (Phy) and phytochrome interaction factor (PIF)\textsuperscript{6,272,273}. Photoactivity of Phy depends on its ligation to the small molecule chromophore phycocyanobilin (PCB), which must be provided exogenously to cells of non-photosynthetic organisms. Red (650 nm) light induces a conformational change in PCB-bound Phy that causes PIF to bind in a matter of seconds (\textbf{Figure A.1}). In the absence of further light input, this interaction persists for hours, while administration of infrared (750 nm) light reverts Phy to its inactive conformation and promotes rapid dissociation of the Phy-PIF heterodimer\textsuperscript{32}. One of the great strengths of the Phy/PIF system is the speed with which Phy changes activation states in response to light input, inducing or terminating the Phy-PIF binding interaction within seconds of receiving the appropriate light input. Similarly, this system is highly robust and can be
switched ON/OFF hundreds of times with no detectable loss of signal and no cellular toxicity. The tremendous light sensitivity of this system minimizes the likelihood of phototoxicity, as it is activated/inactivated using dilute red (650 nm, 20 μmol/m²/s) and infrared (>750 nm, 300 μmol/m²/s) light, respectively.32

Here, we describe a Phy/PIF-based optogenetic system that can be used for the light-dependent control of Ras-to-Erk signaling. The Phy/PIF interaction is used to control Ras pathway activation by expressing the constituent Phy and PIF protein domains as fusions with components of MAPK signaling38 (Figure A.1). In our system, the PIF component (residues 1-100) is expressed as a fusion with a variant of the catalytic domain of SOS2 (referred to as SOScat), which is a Ras GTP exchange factor274. Two key features of the SOScat variant are that it is constitutently active but defective in membrane localization, as the ability of full-length SOS2 to activate Ras depends on transient plasma membrane recruitment38. Next, the Phy component (residues 1-621) is targeted to the
plasma membrane using a C-terminal linker followed by the CAAX membrane localization sequence from KRas\textsuperscript{260}. Thus, Ras/Erk signaling is placed under fine spatiotemporal control based on light-dependent recruitment of cytoplasmic PIF-SOScat to membrane-bound Phy-CAAX. This pair of Phy/PIF fusion constructs is referred to as ‘OptoSOS’\textsuperscript{38}.

To provide a parallel readout of Ras/Erk pathway activity in live cells, the light-activatable OptoSOS system can be coupled with a fluorescently-tagged reporter of downstream kinase activity. Such reporters are designed to alternate nucleocytoplasmic localization based on pathway activity, allowing downstream signaling to be monitored over time with live-cell confocal microscopy. We commonly use one of the following two reporters: FP-Erk, which is a fluorescent protein-bound Erk fusion protein, or ErkKTR-FP, which is an Erk kinase translocation reporter (KTR) bound to a fluorescent protein. In the case of the former, pathway activation causes FP-Erk to be transported into the

Figure A.2. NIH\textsubscript{3}T\textsubscript{3} cells expressing the Opto-SOS system.
Representative images of YFP-PIF-SOScat (upper) and BFP-Erk (lower) from OptoSOS NIH\textsubscript{3}T\textsubscript{3} cells, showing light-inducible cytoplasmic depletion of SOScat and nuclear import of BFP-Erk. Red-outlined cell is further analyzed in Figure A.4.
nucleus based on MEK-dependent phosphorylation\textsuperscript{5,203,240}. Alternatively, the ErkKTR is an Erk-specific substrate that undergoes nuclear export upon Erk-mediated phosphorylation\textsuperscript{199}. Combining either reporter individually with OptoSOS in a single cell line enables complete dynamic control and characterization of Ras/Erk signal processing in live cells (Figure A.2). Furthermore, these data represent signal processing in single cells, allowing assessment of both population-level and individual cell-to-cell variability. Because the activity of many cell signaling processes can be controlled based on the spatial and physical proximity of pathway components (e.g., membrane recruitment of SOScat), the Phy/PIF optogenetic system has a great variety of potential applications. Indeed, there is a growing literature demonstrating its use for light-based control of phosphoinositide 3-kinase (PI3K)\textsuperscript{37}, Ras-related C3 botulinum toxin substrate 1 (RAC1)\textsuperscript{32}, Ras homolog gene family member A (RhoA)\textsuperscript{32}, cell division control protein 42 homolog (Cdc42)\textsuperscript{32,275}, actin assembly\textsuperscript{275}, and organelle targeting\textsuperscript{276}.

A.3 Establishing OptoSOS + Erk reporter cell lines

Establishing stable cell lines with our Phy/PIF-based optogenetic Ras/Erk system has proven to be a highly general approach, as we have had success in a wide variety of cell lines, including everything from primary cells including chick neurons and mouse epidermal stem cells, to human cancer-derived cell lines, to NIH-3T3 fibroblasts. Transient co-transfection of OptoSOS and a downstream reporter may be sufficient for simpler experiments that require a short timescale, but we have found that generating stable cell lines increases the proportion of cells co-expressing both constructs and has a high rate of success with minimal additional work required.

A.3.1 Preparing reagents
1. Prepare complete growth medium for HEK 293T LX cells by supplementing standard DMEM with 10% FBS (v/v) and 10,000 u/mL of penicillin/streptomycin.

2. Prepare the necessary quantities of plasmid DNA for each required plasmid using a standard miniprep protocol and measure the resulting DNA concentrations. For each desired virus, the following quantities of plasmid DNA are required: pCMV-dR8.91 packaging plasmid: 1.33 μg; pMD2.G envelope plasmid: 0.17 μg; desired transfer vector (e.g., OptoSOS, FP-Erk, KTR-FP): 1.5 μg.

3.Immediately prior to transfection, warm the Opti-MEM reduced serum medium to 37ºC, and bring the FuGENE HD transfection reagent to room temperature.

A.3.2 Lentivirus production

1. 12 h prior to transfection, seed one well of a 6-well tissue culture dish with HEK 293T LX cells such that the cells reach a confluency of 50-60% at the time of transfection. Cells should be grown at 37ºC, 5% CO2 in 2 mL/well of complete growth medium (DMEM, 10% FBS, 1% P/S).

2. Prepare a sterile 1.5 mL tube containing 150 μL of warm Opti-MEM, which will serve as the base of the transfection mixture.

3. Begin assembling the transfection mixture by adding appropriate volumes of the two helper plasmids (i.e., pCMV-dR8.91 and pMD2.G) and of your transfer vector (containing either OptoSOS or the desired downstream reporter): pCMV-dR8.91: 1.33 μg, pMD2.G: 0.17 μg, transfer vector: 1.5 μg. Briefly pipette up and down to mix the reagents.

4. To complete the transfection mixture, carefully add 9 μL of the FuGENE HD transfection reagent by immersing the pipette tip to the bottom of the tube and then slowly
dispensing the FuGENE directly into the center of the Opti-MEM/plasmid solution. Mix the final transfection solution by gently pipetting the liquid up and down three times.

5. Allow FuGENE:DNA complexes to form by incubating the transfection mixture at room temperature for 15 min.

6. Gently draw up the entire volume of transfection mixture using a pipette and add it dropwise over the entire surface area of a single well of 293T LX cells without bringing the pipette tip into contact with the media. Gently swirl the plate to mix and return the plate to the 37°C incubator.

7. Harvest lentivirus at 48-52 h post-transfection. There is no need to change media or disturb the cells until lentivirus is collected.

8. For each well containing transfected 293T cells, use a sterile syringe to draw up all growth medium in the well (which contains the newly-produced lentivirus), then attach a 0.45 μm filter to the tip of the syringe and expel the growth medium through the filter into a sterile collection tube. This filtration step ensures that no floating cells or debris will contaminate future cells infected with the newly-formed lentivirus.

9. Tubes containing lentivirus can be stored at 4°C for the short-term (i.e., time measured in days) but must be frozen at -20°C or -80°C for long-term storage (see Note 1).

A.3.3 Lentiviral transduction of target cell line

1. Seed one well of a 6-well tissue culture dish with the desired recipient cell line. Cells should be plated at a density such that they will not overgrow the well in 48 h. Culture the cells in 2 mL of complete growth medium.

2. To each lentiviral stock, add 5 μg/mL Polybrene and 50 μM HEPES buffer (see Note 2).
3. Typically, 100 μL of lentiviral stock is required to transduce one well of a 6-well dish containing 50% confluent adherent cells. However, this volume will depend on many conditions, including viral titers obtained and the cell line of interest. Lentiviral stock should be added dropwise over the whole surface area of the well, and the plate should be gently rocked to mix.

4. Incubate the cells in the presence of lentivirus at 37ºC for a minimum of 8 h and maximum of 48 h. After this point, the cells can be transferred to new tissue culture dishes and the media can be changed, though this is only necessary if they have outgrown their current culture conditions.

5. To confirm lentiviral transduction, at 48 hours post infection (hpi), cells can be assessed for expression of YFP or BFP using a confocal or fluorescence microscope. Cells are unlikely to be expressing an appreciable amount of fluorescent protein prior to 48 hpi.

6. For cell lines exhibiting low transduction efficiency (i.e., those with a low percentage of total cells expressing desired constructs), fluorescence-activated cell sorting (FACS) can be used to enrich for cells that co-express both constructs.

A.4 Purifying phycocyanobilin

In many cases, phycocyanobilin (PCB) purchased from commercial vendors is suitable for experimental protocols using the Phy/PIF system. However, at high levels of Phy expression, we have encountered problems with contaminants in this commercial prep that lead to constitutive Phy activity (even in the inactive, 750 nm exposed state), as well as high levels of fluorescence in red and near-infrared wavelengths. Fortunately, the contaminants that cause both effects can be removed using high performance liquid chromatography (HPLC), thereby generating a highly active, pure PCB product. The
following steps detail an HPLC protocol that can be used to purify commercial PCB for use with the Phy/PIF-based OptoSOS system.

A.4.1 HPLC purification of PCB

*Warning: PCB is highly light sensitive. Ensure that all purified samples are kept in the dark and that all purification steps take place in low-light conditions (see Note 3).*

1. Dissolve 50 mg of phycocyanobilin in 100% HPLC grade methanol.

2. Load the entire PCB/methanol solution onto a 5 µm phenyl-hexyl 100 Å column.

3. Run the loaded column on HPLC with a constant flow rate of 12 mL/min at 400 bar, with the following gradient:
   a. At t = 0 min: 70% H2O / 30% acetonitrile with 0.1% formic acid
   b. At t = 5 min: 70% H2O / 30% acetonitrile with 0.1% formic acid
   c. At t = 45 min: 40% H2O / 60% acetonitrile with 0.1% formic acid
   d. At t = 50 min: 100% acetonitrile with 0.1% formic acid
   e. At t = 55 min: 100% acetonitrile with 0.1% formic acid

4. Collect the fractions corresponding to the phycocyanobilin peaks, as shown in Figure A.3. An initial early solvent peak will appear first, followed by a double peak that contains both our desired PCB product and a second molecule that is difficult to separate but does not affect the quality of Phy/PIF translocation. Next will be a small peak containing a molecule that leaves Phy in a constitutively active (i.e., PIF-bound) conformation even in the absence of light. It is important to remove this molecule. Finally, the last peak contains a compound that is extremely autofluorescent in red and near-infrared wavelengths.
6. Remove organic solvent from fractions through rotary evaporation, with evaporation flask pressure vacuumed to 40 torr and heated to 8-10°C. Cover the entire rotary evaporation apparatus with foil, to avoid exposing the sample to light during this step.

7. (Optional) Lyophilize any remaining material to obtain pure phycocyanobilin powder.

8. Weigh and resuspend the remaining material in 100% DMSO to a final concentration of 10 mM. This will serve as a 1000X working stock of pure PCB.

Figure A.3. HPLC purification of PCB.
A trace of the absorbance at 380 nm during HPLC fractionation of commercial PCB. Four sets of peaks represent functionally distinct chemical moieties: the solvent fraction, two peaks containing functional PCB (blue peaks), a peak that upon Phy ligation induces constitutive Phy/PIF binding, and a peak that is highly autofluorescent in the red/far red wavelengths.
A.5 Imaging OptoSOS cells

A.5.1 Preparing cells for microscopy

1. Dilute stock fibronectin 100-fold (from 1 mg/mL to 10 μg/mL) in D-PBS.

2. Prepare imaging medium by supplementing phenol red-free medium with 20 mM HEPES buffer (see Note 4).

3. In low-light conditions, add purified PCB to imaging medium and dilute to 1X. Recall that PCB and PCB-containing reagents should always be carefully protected from ambient light to prevent photo-degradation.

A.5.2 Fluorescence microscopy of OptoSOS cells

1. Coat individual wells of a 96-well glass-bottomed, black-walled plate with 10 μg/mL fibronectin and incubate the plate at room temperature for >30 min.

2. Remove fibronectin from the wells, ensuring that the coated surface does not remain dry for long (i.e., be prepared to add cells to the well <1 min after removing fibronectin).

3. Seed ~40,000 cells/well of a desired OptoSOS cell line in 100 μL of complete growth medium. The exact ideal number of cells per well will vary depending on the cell line, but roughly 50% confluence is typically ideal for imaging.

4. Spin the plate in a benchtop centrifuge at $210 \times g$ for 1 min to ensure that the cells adhere to the fibronectin-coated glass with an even plating density. Allow the cells to adhere by incubating at 37°C for a minimum of 4-8 h, though overnight incubation is preferred for most cell lines.

5. Carefully remove the growth medium and wash the cells with either PBS or plain imaging medium (i.e., serum-free medium without PCB added). This wash step removes
residual growth factors and serum proteins that may be left over from the original growth medium, the removal of which will allow cells to reach a truly inactive state of Ras signaling (see Note 5).

6. In low light conditions, add 100 μL per well of imaging medium containing 1X PCB and incubate the cells in the dark at 37ºC for >1 h. Perform this and all subsequent steps in dark or low light conditions (see Notes 6 and 7).

7. Prior to imaging, allow ≥30 min for the cells to acclimate to the microscope incubator conditions (37ºC, 5% CO₂), as temperature alterations can impact signaling responses.

8. For longer (>1 h) imaging protocols, add ~50 μL per well of mineral oil to prevent evaporation of imaging medium. Mineral oil should be added carefully such that the oil layer sits on top and does not mix with the underlying imaging medium.

9. Place a 750 nm bandpass filter directly in the microscope’s brightfield illumination path, which is typically located on top of the microscope condenser. This is to ensure that constant 750 nm light is applied to cells while imaging fields are first located by the user, thus keeping the OptoSOS system in an inactivated state.

10. Use a 40X or 60X oil objective to identify locations on the 96-well plate that contain cells with strong expression of both fluorescent components (i.e., OptoSOS and downstream reporter).

11. Select an imaging plane in the center of the nucleus so that nuclear translocation of the reporter will be maximally visible. Such a plane can be found by moving upward from the glass surface in the z-axis until the nucleus first becomes clearly visible. Turn on the autofocus function if it is available on your microscope to prevent focal drift (see Note 8).

12. Activate the OptoSOS system by replacing the 750 nm filter with a 650 nm bandpass filter. YFP-PIF-SOScat recruitment should be visible within seconds. We have found that
the timescale of reporter translocation can vary slightly between cell lines, but both BFP-Erk and KTR-BFP typically reach steady-state activation in 5-8 min. Use the microscope’s YFP imaging mode to capture images of YFP-PIF-SOScat membrane recruitment, and the BFP imaging mode to record reporter translocation.

13. Replace the 650 nm filter with the 750 nm filter to deactivate the Opto-SOS system, and again image cells in the YFP and BFP channels. Steps 12 and 13 can be repeated as frequently as required based on individual experimental protocols (see Note 9).

A.6  Data processing to measure Erk dynamics

In every cell line we have tested, optogenetic Ras activation induces Erk nuclear translocation that persists as long as SOScat membrane translocation is induced, and can be applied repeatedly over time. The ability to restimulate the same cells using different intensities of light thus makes it possible for the first time to carry out measurements of Ras-to-Erk signal processing, such as single cell dose-response curves (stimulating a cell with different light intensities and measuring nuclear Erk) or dynamic signal transmission (stimulating a cell with a time-varying light stimulus and measuring Erk’s response over time). In this section, we describe some useful approaches for quantifying SOScat membrane translocation and nuclear Erk dynamics.

A.6.1  Quantifying Erk dynamics in response to light

1. Open the BFP channel time-lapse movie in ImageJ. ImageJ supports a variety of formats used by both open- and closed-source microscope software packages. Draw an ellipsoidal region in the nucleus of a cell of interest expressing BFP-Erk, and press ‘M’ to measure its mean intensity. New regions can be drawn as the cell moves or changes shape. Advance
through the movie collecting mean intensities at each timepoint. The list of cytoplasmic intensities at each timepoint will be called \( B_{\text{nuc}}(t) \).

2. Rewind the movie to the first timepoint and draw a polygon or freehand region in the cytoplasm of the same cell measured in step 1. Again, press ‘M’ to measure its mean intensity at each timepoint. New regions can be drawn as the cell moves or changes shape. The list of cytoplasmic intensities at each timepoint will be called \( B_{\text{cyt}}(t) \).

3. Rewind the movie once more and draw an ellipsoidal region nearby, but in a region with no cells. This will measure the background intensity (i.e., autofluorescence of the medium and other sources of light). Again, press ‘M’ to measure its mean intensity at each timepoint. New regions can be drawn as cells move over the original background region. The list of background intensities at each timepoint will be called \( B_{\text{bkgd}}(t) \).

A.6.2 Quantifying SOScat dynamics in response to light

1. Open the YFP channel timelapse movie in ImageJ. Draw a polygonal or freehand region in the cytoplasm of the same cell of interest (as was measured in A.6.1) expressing YFP-PIF-SOScat. Press ‘M’ to measure its mean intensity at each timepoint. New regions can be drawn as the cell moves or changes shape. The list of cytoplasmic intensities at each timepoint will be called \( Y_{\text{cyt}}(t) \).

2. Rewind the movie and draw an ellipsoidal region nearby but in a region with no cells. This will measure the background intensity (i.e., autofluorescence of the medium and other sources of light). Again, press ‘M’ to measure its mean intensity at each timepoint. New regions can be drawn as cells move over the original background region. The list of background intensities at each timepoint will be called \( Y_{\text{bkgd}}(t) \).
Data processing to measure Erk dynamics

1. Subtract the intensity of the background from the measured nuclear and cytoplasmic BFP intensities at each timepoint to generate subtracted intensity values. For example, to calculate background-subtracted nuclear Erk (denoted $\bar{B}_{nuc}$), use the formula: $\bar{B}_{nuc}(t) = B_{nuc}(t) - B_{bkgd}(t)$. Repeat this calculation for all BFP images (representative traces are shown in Figure A.4).

2. We have found an easy and robust way to measure Erk nuclear-cytoplasmic dynamics that accounts for photobleaching and the movement of Erk protein between nucleus and cytoplasm over time are shown.

**Figure A.4. Analyzing SOScat and Erk levels over time in individual cells.**
All data shown are for the representative cell indicated in Figure A.2, which was stimulated with alternating 1 h cycles of red and infrared (IR) light for 5 h.
(a–c) Analysis of cytoplasmic SOScat levels. (a) Raw cytoplasmic SOScat, (b) bleaching-corrected SOScat, and (c) calculated membrane accumulation of SOScat over time.
(d–f) Analysis of nuclear and cytoplasmic BFP-Erk levels. (d) The raw nuclear and cytoplasmic Erk, (e) bleaching-corrected Erk, and (f) the calculated distribution Erk protein between the nucleus and cytoplasm over time are shown.

A.6.3 Data processing to measure Erk dynamics

1. Subtract the intensity of the background from the measured nuclear and cytoplasmic BFP intensities at each timepoint to generate subtracted intensity values. For example, to calculate background-subtracted nuclear Erk (denoted $\bar{B}_{nuc}$), use the formula: $\bar{B}_{nuc}(t) = B_{nuc}(t) - B_{bkgd}(t)$. Repeat this calculation for all BFP images (representative traces are shown in Figure A.4D).

2. We have found an easy and robust way to measure Erk nuclear-cytoplasmic dynamics that accounts for photobleaching and the movement of Erk protein between nucleus and cytoplasm over time are shown.
cytoplasm over time. This measurement takes advantage of the observation that cell size, nuclear size, and BFP-Erk levels are roughly constant on the timescales of our experiments (i.e., a few hours in starvation media) and that by taking care to limit laser intensity during imaging, photobleaching is roughly linear over the course of an experiment. We can thus represent this mass conservation with the following equation,

\[ V_{nuc} \bar{B}_{nuc}(t) + V_{cyt} \bar{B}_{cyt}(t) = E_T \times (1 - kt) \]

where \( V_{nuc} \) is the nuclear volume, \( V_{cyt} \) is the cytoplasmic volume, \( k \) is the photobleaching rate, and \( E_T \) is the total fluorescent Erk per cell (which is bleached at a rate \( kt \)). Solving this equation for \( \bar{B}_{nuc}(t) \) we can write:

\[ \bar{B}_{nuc}(t) = a + b \times \bar{B}_{cyt}(t) + c \times t. \]

This simple linear equation is of the form \( y = ax + bx + ct \) that can quickly be solved for the coefficients \( a, b, \) and \( c \) that best fit the nuclear and cytoplasmic data, for example by using the following two lines of MATLAB code for data vectors \( x \) (background-subtracted cytoplasmic Erk), \( y \) (background-subtracted nuclear Erk), and \( t \) (the timepoint number):

```matlab
coeff = [ones(N,1) x(:) t(:)] \ y(:);
```

```
a = coeff(1); b = coeff(2); c = coeff(3);
```

3. After finding the coefficients in Step 2, we can determine the photobleaching-corrected levels of nuclear and cytoplasmic Erk and the nuclear-cytoplasmic volume ratio for each cell. To do so, we note that \( V_{cyt}/V_{nuc} = -b \), and the photobleaching rate \( k = -b/a \). Thus,

\[ Erk_{nuc}(t)/V_{nuc} = \frac{\bar{B}_{nuc}(t)}{1 + (b/a)t}; \ Erk_{cyt}(t)/V_{cyt} = \frac{\bar{B}_{cyt}(t)}{1 + (b/a)t} \]

**Figure A.4E** shows representative traces of photobleaching-corrected Erk. After these calculations, one can readily calculate quantities like nuclear-cytoplasmic ratio, nuclear fold-change, or the fraction of Erk in the nucleus and cytoplasm (e.g., **Figure A.4F**).
A.6.4 Data processing to measure SOScat dynamics

1. Subtract the intensity of the background from the measured cytoplasmic YFP intensities at each timepoint to generate a subtracted intensity. For instance, to calculate background-subtracted nuclear Erk (denoted $Y_{\text{nuc}}$), use the formula: $Y_{\text{nuc}}(t) = Y_{\text{nuc}}(t) - Y_{\text{bgd}}(t)$. Repeat this calculation for all YFP images (a representative trace is shown in Figure A.4A).

2. For timepoints where 750 nm light is exclusively applied and during which SOS is exclusively localized to the cytoplasm, the following equation holds:

$$
\overline{Y}_{\text{cyt}}(t_{750}) = \frac{SOS_{T}}{V_{\text{cyt}}} \ast (1 - kt_{750})
$$

Again, this is of the form $y = a + bt$, where we can solve using the following two lines of MATLAB code for data vectors $y_{750}$ (background-subtracted cytoplasmic SOS at only those timepoints where 750 nm light is applied), and $t_{750}$ (the times for only those timepoints where 750 nm light is applied):

```matlab
coeff = [ones(N,1) t750(:)] \ y750(:);
a = coeff(1); b = coeff(2);
```

3. After finding the coefficients in Step 2, we can correct for SOScat photobleaching at all timepoints by computing

$$
\frac{SOS_{\text{cyt}}(t)}{V_{\text{cyt}}} = \frac{\overline{Y}_{\text{cyt}}(t)}{1 + \left(\frac{b}{a}\right)t}
$$

(a representative trace is shown in Figure A.4B).

4. At each timepoint we assume that SOS lost from the cytoplasm is gained on the membrane, where it is able to activate Ras. Thus, we can approximate the SOS gained on the membrane as the SOS lost in the cytoplasm by subtracting the cytoplasmic intensity at each timepoint from the maximum cytoplasmic pool of SOS measured at times when 750
nm light is applied (a representative trace is shown in Fig A.4C):

\[ SOS_{membrane}(t) \sim \frac{SOS_{cyt}(t_{750})}{V_{cyt}} - \frac{SOS_{cyt}(t)}{V_{cyt}} \]

Using the above calculations, it is possible to obtain high quality, bleaching-corrected measurements of both membrane SOS and nuclear Erk dynamics in response to time-varying light inputs, thereby enabling the study of the Ras-to-Erk transfer function in single cells.

A.7 Notes

1. It is thought that a reasonable proportion of lentivirus transduction efficiency is lost with each freeze/thaw cycle. While this is not universally true in our experience, what is true is that it is better to use lentivirus to infect cells shortly after harvest or to store lentiviral stocks in small single-use aliquots to prevent subjecting virus to multiple freeze/thaw cycles.

2. Polybrene is used to increase lentiviral transduction efficiency by reducing electrostatic repulsion between the plasma membrane and lentiviral capsid\(^{277}\). HEPES is used to buffer the pH, as certain viral components are highly sensitive to changes in pH.

3. It is vitally important that PCB be protected from prolonged exposure to ambient light during the entire purification process and thereafter when storing the purified product. Prolonged ambient light exposure results in photo degradation of the chromophore, which compromises the ability of Phy to respond to light stimuli. Thus, high quality PCB is crucial to ensuring the success of your experiments. Though it is not always visible to the naked eye, one sign of compromised quality is if a sample of PCB begins to change color from a rich, dark, royal blue to a more pale, purple hue.
4. When preparing imaging media for live cell confocal microscopy experiments, it is ideal to use formulations that do not include phenol red, as this will maximize signal-to-noise when imaging.

5. Incubation in serum-free imaging medium allows the cells to equilibrate to growth factor-free conditions with the Ras/Erk pathway turned ‘OFF.’

6. Cells are sensitive to light after the addition of PCB, and all subsequent procedures should thus be performed in the dark or very low-light conditions (e.g., turn off overhead lights in the lab, wrap all PCB-containing reagents in aluminum foil, etc.). These precautions are required because, in the presence of PCB, the OptoSOS system is easily activated by ambient light.

7. When setting up sensitive experiments in which cells must be in a truly ‘OFF’ Ras/Erk signaling state, it can be difficult to see what one is doing while also ensuring that ambient light does not aberrantly activate the Phy/PIF system. We have found that infrared LED lights (~750 nm) can be used to provide a degree of visibility while preparing reagents in a dark lab.

8. A confocal microscope is required for imaging the OptoSOS system, as epi-fluorescence microscopy cannot accurately resolve PIF membrane recruitment or nucleocytoplasmic shuttling of the downstream reporter.

9. BFP can be imaged *ad libitum* without harming Phy/PIF performance or perturbing translocation. However, imaging of YFP will partially activate Phy so it is prudent to limit frequency and duration of exposures.
Appendix B

Supporting materials and methods for Chapter 2

B.1 Supplementary figures

**Figure B.1**, related to **Figure 2.1**: (A) Cell growth of GFP overexpressing cells. (B) Measured lactate secretion rates between 24 and 30 h normalized to the packed cell volume (PCV) at 30 h matches previously determined secretion rates by \(^{141}\). Means and standard deviations (n=3 biological replicates) are plotted. (C) Western blot showing that
the expression dynamics of different glycolytic enzymes is similar (see Figure 2.1B). (D) Ponceau red staining of the Western blots in C. Box indicates overexpressed proteins. (E) Change in enzyme activity for HK2, GAPDH, PKM2, and LDHA overexpressing cells compared to GFP control. Means and standard deviations (n=3 biol. replicates) are plotted. (F) Standard deviations of glucose and lactate measurements from Figure 2.1C and (G) calculated lactate to glucose ratios. (H) Respiration rates (mean and standard errors from n=3 biol. replicates across 3 independent experiments) and (I) 13C-glucose labeling of malate in vector-, GFP-, GLUT3-, PFKP-, GAPDH-, PKM2-, LDHA-, and MCT4-overexpressing cells (mean and standard deviations from n=3 biol. replicates).

**Figure B.2**, related to **Figure 2.2**: Erythronate (A) and phospholactate (B) levels upon GAPDH and PKM overexpression. (C) Log-linear correlation of AMP, ADP, ATP, and citrate with lactate secretion compared to FBP. (D) Temporal dynamics of lactate secretion in PKM2-overexpressing cells. Temporal changes in metabolite (E) and creatine phosphate levels (F) upon HK2 overexpression. (G) Number of viable cells (determined by trypan blue exclusion assays) of HK2-overexpressing cells (orange) compared to GFP-expressing cells (blue). (H) HK activity (normalized to total protein content) as a function of transfected HK2 plasmid DNA concentration. For all panels, mean and standard deviations from n=3 biological replicates are plotted.
Figure B.3, related to Figure 2.3: (A) Confocal images showing similar expression levels of lentiviral constructs in NIH 3T3 cells at 48 h post transduction. (B) Kinetics of glycolytic intermediate labeling from U-13C-glucose in NIH 3T3 cells with or without OptoSOS stimulation. Mean and standard deviations from n=3 biological replicates are plotted. (C) Gene Set Enrichment analysis (GSEA) of genes that were upregulated >2-fold on at least one time point. Only pathways with a statistically significant enrichment score are shown (q<0.05). (D) Metabolic genes that changed >2-fold on at least one time point.

Figure B.4, related to Figure 2.4: (A) Modified version of Figure 2.4C. Rank order of glycolytic genes from most- to least-expressed relative to benign adjacent tissue, on
average, for different tumor types. Red filled circles represent top-ranked gene isoforms that catalyze glucose transport, FBP production, and lactate export for each tumor type. Black filled circles indicate the highest-expressed hexokinase isoform. Unfilled circles are the other isoforms (not in the top four most upregulated genes) belonging to glucose transport, FBP production, G6P production, and lactate export. (B) Gene expression changes in endothelial cells during angiogenesis (retinas at P6 compared to P15)\textsuperscript{174}, in activated M1 macrophages (activated compared to resting macrophages)\textsuperscript{175}, in activated T cells (stimulated compared to resting cells)\textsuperscript{177}, and changes in protein levels in activated CD4 T Cell (stimulated compared to naïve cells)\textsuperscript{176}.

B.2 Methods

B.2.1 Experimental model and subject details

All cell lines used in this study were cultured in standard Dulbecco’s Modified Eagle Medium (DMEM; with 4.5 g/L glucose and 0.584 g/L L-glutamine, but without sodium pyruvate) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO2. For passaging, cells were washed once with 1x phosphate buffered saline (PBS) and detached by trypsin/EDTA (0.05%). For glycolytic flux measurements and metabolomics analysis, DMEM was supplemented with 10% dialyzed FBS (dFBS).

B.2.2 Cloning of transient overexpression and lentivirus constructs

All purchased human cDNAs of the 24 glycolytic enzymes are listed above and corresponding primers are listed in Table B.1. For cloning into the pCMV-6-A-puro mammalian expression vector, the vector was digested by HindIII (5’end) and NotI (3’end) restriction enzymes for 4.5 h at 37°C. The digested vector was run on a 1% agarose gel (with ethidium bromide) and the cut band was gel purified using the Wizard® SV Gel
and PCR Clean-Up System. The open reading frame (ORF) of a given gene was amplified by polymerase chain reaction (PCR) using corresponding primers and the 2x PfuUltra II Hotstart PCR Master Mix according to manufacturer’s instructions. PCR products were cleaned up with the Wizard® SV Gel and PCR Clean-Up System before Gibson Assembly. For all Gibson Assembly reactions, a molar ratio of 1:3 (vector:insert) was used and the reaction was performed at 50°C for 1 h as per manufacturer’s instructions. Assembled constructs were transformed into NEB 5-alpha competent *E. coli* cells and grown on agar plates (100 µg/mL ampicillin) at 37°C overnight. Single colonies were inoculated in Luria broth (100 µg/mL ampicillin) overnight and plasmid DNA was extracted using the QIAprep Miniprep Kit. Constructs were sequence verified by Sanger sequencing (Genewiz, NJ, USA) and stocks for all experiments were prepared using the QIAGEN Maxi Kit.

For lentivirus transductions, ORFs were cloned into a pHR’ SFFVp lentiviral vector, upstream of IRES CIBN-BFP-CAAX, using the In-Fusion® HD Cloning Kit according to the manufacturer’s instructions. Briefly, the vector was linearized by backbone PCR and the ORFs were amplified by PCR as described above. The linearized vector and PCR products were run on a 1% agarose gel (with ethidium bromide) and the cut bands were cleaned up with the Wizard® SV Gel and PCR Clean-Up System. Using a molar ratio of 1:3 (vector:insert), all In-Fusion reactions were performed at 50 °C for 15 min as per manufacturer’s instructions. Assembled constructs were transformed into Clontech Stellar competent *E. coli* cells and grown on agar plates (50 µg/mL carbenicillin) at 37 °C overnight. Single colonies were inoculated in Luria broth (50 µg/mL carbenicillin) overnight. Plasmid DNA was prepared and sequenced as before.

### B.2.3 Transient overexpression of glycolytic genes in iBMK cells
iBMK cells were transfected using Lipofectamine 2000 as described in the manufacturer’s protocol. 24 h before transfection, 0.5 µl packed cell volume (PCV) iBMK cells were plated per well in a 6-well plate in DMEM (10% dFBS). For each well, 2.5 µg of plasmid DNA, 10 µl of transfection reagent, and 200 µl of OptiMEM were used. The DNA to lipofectamine ratio was kept equal for all experiments. For co-expression experiments, the total amount of plasmid DNA was 2.5 µg and the amount of the different constructs was kept equivalent. The single expression controls were adjusted to the lowest concentration and mixed with vector plasmid DNA to keep the total amount of plasmid DNA at 2.5 µg. Correspondingly, serial dilutions of a given construct were supplemented with vector plasmid DNA to keep the 2.5 µg total plasmid DNA amount. iBMK cells were incubated with the plasmid DNA-lipofectamine complexes in 2 ml DMEM (10% dFBS) for 6 h before switching to fresh DMEM (10% dFBS). Metabolomics experiments were performed in 6 cm dishes. For transfection in 6 cm dishes, 1 µl PCV iBMK cells were plated and grown overnight before transfection with 8 µg of plasmid DNA, 20 µl of lipofectamine as well as 500 µl of OptiMEM incubated in 5 ml of DMEM (10% dFBS) for 6 h.

Number of viable cells was counted at the indicated time points. Cells were transfected in 6-well plates as described above and viable cells were counted using Trypan blue exclusion assays and a Countess™ II FL Automated Cell Counter (ThermoFisher).

B.2.4 Western blotting

iBMK cells were collected at the indicated time points after transfection. Cells were washed once with ice cold 1x PBS and scraped into 200 µl RIPA buffer supplemented with phosphatase and protease inhibitors. Collected iBMK cells were vortexed, incubated for 10 min on ice and centrifuged at 17,000 g at 4 °C. Supernatant was transferred to a fresh tube and protein content was determined by Pierce™ BCA Protein Assay Kit. 10 µg of
protein per sample was analyzed by SDS-PAGE and western blotting. Primary and secondary antibodies (horse-radish peroxidase conjugated) were used at 1:1000 and 1:10,000 respectively. The SuperSignal™ ELISA Pico Chemiluminescent Substrate was used for protein detection.

### B.2.5 Glycolytic flux measurements

Glucose and lactate measurements were performed in 6-well plates between 24 h and 30 h post-transfection unless stated otherwise. Cells were washed twice with 2 mL of 1x PBS before 1 ml of fresh DMEM (10% dFBS) was added. After 6 h of incubation, 300 µl of supernatant was collected and analyzed by a YSI 2900D Biochemistry Analyzer (YSI, Yellow Springs, OH, USA). Measured glucose consumption and lactate secretion were normalized to the average PCV during the 6 h measurement interval as determined based on the terminal PCV measurement at 30 h and the known growth rate ($0.028 \text{ h}^{-1}$) of GFP expressing cells (Figure B.1A):

$$PCV_{Average} = \frac{1}{0.028 h^{-1}} \left( PCV_{End} - \frac{PCV_{End}}{e^{0.028 h^{-1} \times 6h}} \right) \frac{6h}{6h}$$

(Equation 3)

Measurement of the secreted lactate to consumed glucose ratio was done in 5 mM glucose conditions. To make growth medium with 5 mM glucose, DMEM powder without glucose, glutamine, or phenol red was dissolved in deionized water and supplemented with L-glutamine (0.584 g/L), phenol red (0.015 g/L), sodium bicarbonate (3.7 g/L), glucose (0.9 g/L) and 10% dFBS. Data were presented either as absolute values or the percentage change normalized to the vector control as indicated in the corresponding figure panels.

### B.2.6 Measurement of oxygen consumption rates
Oxygen consumption rates were measured using a Seahorse XF24 extracellular flux analyzer (Agilent, Santa Clara, CA). Briefly, 100 µL of a 0.5 µL cells ml⁻¹ solution were plated per well in a XF24 cell culture microplate. The next day, cells were transfected with Vector, GFP, GLUT3, PFKP, GAPDH, PKM2, LDHA, and MCT4 constructs as described above. 24 h post transfection, cells were equilibrated for 1 h in DMEM supplemented with 25 mM glucose and L-glutamine (0.584 g/L) in a non-CO₂ incubator. Oxygen consumption rates were monitored at baseline (5 measurements) and were normalized to cell number as determined by the CyQUANT Cell Proliferation Assay Kit. Experiments were performed with four replicates per overexpression condition and the average of three independent experiments from different days were reported with standard errors.

B.2.7 Calculation of flux control coefficients

Flux control coefficients $C_E^J$ for every glycolytic step were calculated using the relationship described by Small and Kacser accounting for large changes in enzymatic activity:\cite{145}

$$f_E^l = \frac{1}{1 - \frac{r_E}{r_E^l}} C_E^J$$

(Equation 1.1)

where $f_E^l$ is the fold-change in glycolytic flux $J$ as a function of change in enzyme activity $r_E$. The derivation of this equation assumes an unbranched pathway with linear reaction kinetics, neither of which applies to glycolysis. The equation is, nevertheless, a valid approximation, as long as flux is measured through the branch in which the enzyme variation occurred\cite{145,278}. This is true in our case as we are modulating glycolytic enzymes and measuring glycolytic flux. Moreover, based on numerical simulations, the approximation is robust to enzymes that follow Michaelis-Menten as opposed to linear
reaction kinetics, with typical deviations < 10%. Thus, we used this equation to approximate flux control coefficients.

Solving Equation (1.1) for $C_E^I$ results in the following equation:

$$C_E^I = \left( \frac{f_E^I - 1}{f_E^I} \right) \left( \frac{r_E}{r_E - 1} \right)$$

(Equation 1.2)

With the assumption that changes in protein levels of a given enzyme $E$ are directly proportional to changes in enzyme activities $r_E$, and since ectopic overexpression generally results in large changes in protein levels, the term $\frac{r_E}{r_E - 1}$ approximates 1 and Equation 1.2 can be simplified:

$$C_E^I \approx \left( \frac{f_E^I - 1}{f_E^I} \right)$$

(Equation 2)

Equation 2 is shown in Figure 2.1A. $C_E^I$ for a given overexpressed enzyme $E$ were calculated using Equation 2 based on the determined fold-change in lactate secretion $f_E^I = \frac{J_E}{J_{vector}}$ from experiments performed in 25 mM glucose conditions. Results were presented as the mean with 95% confidence intervals from $n \geq 6$ biological replicates of at least two independent experiments. For the calculation of the sum of determined $C_E^I$ in iBMK cells, only constructs exhibiting positive flux control in both 5 mM and 25 mM glucose conditions were considered:

$$\sum_{AHE} C_E^I = C_{GLUT}^I + C_{PFK}^I + C_{MCT}^I$$

(Equation 4.1)

whereby $C_E^I$ for different gene isoforms acting on the same glycolytic step were averaged:

$$C_{GLUT}^I = \frac{C_{GLUT1}^I + C_{GLUT2}^I + C_{GLUT5}^I}{3}$$

(Equation 4.2)
\[ C_{PK}^J = \frac{C_{PKP}^J + C_{PKFB1}^J + C_{PKFB3}^J}{3} \]  
(Equation 4.3)

\[ C_{MCT}^J = C_{MCT4}^J \]  
(Equation 4.4)

### B.2.8 Oligomycin treatment

Analogous to the transient overexpression experiments, 0.5 µL PCV iBMK cells were plated per well of a 6-well plate in DMEM (10% dFBS) the day before the experiment. The next day, iBMK cells were washed twice with 1x PBS and incubated with 1 mL of fresh DMEM (10% dFBS) supplemented with 1 µM oligomycin. Supernatant was analyzed after 6 h of incubation and measured lactate was normalized to PCV. Data was presented as the percentage change in lactate secretion compared to vector control.

### B.2.9 Metabolomic analysis using mass spectrometry

Metabolomics experiments were performed in 6 cm dishes. 24 h post transfection, iBMK cells were switched to fresh DMEM (10% dFBS) and incubated for another 2 h before extraction. Supernatant was subsequently aspirated and metabolism was directly quenched on dry ice using -20°C methanol:water (80:20 v/v %). Quenched cells were left on dry ice for at least 10 min before scraping and collecting them into fresh Eppendorf tubes. Samples were vortexed and centrifuged at 17,000 g at 4°C for 10 min. Supernatant was transferred to a fresh Eppendorf tube and dried under a stream of nitrogen. Freshly extracted samples were subjected to liquid chromatography mass spectrometry (LC-MS) analysis on the same day.
For labeling experiments using U-13C-glucose, cells were washed twice with 1x PBS and switched into the glucose labeled cell culture medium 24 h post transfection. The cells were kept in the labeled medium for 6 h before quenching. To make U-13C-glucose-containing cell growth medium, DMEM powder was dissolved in deionized water and supplemented with L-glutamine (0.584 g/L), phenol red (0.015 g/L), sodium bicarbonate (3.7 g/L), U-13C-glucose (4.5 g/L) and 10% dFBS.

For LC-MS, dried samples were reconstituted in HPLC grade water at 40 µL water per 1 µL PCV cells. At least 50 µL of sample were transferred to a fresh MS vial and 10 µL of sample were injected. Negatively charged metabolites were analyzed by reverse-phase ion-pairing chromatography coupled to an Exactive Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA). For LC separation, a previously described method was modified and an Atlantis T3 column (150mm × 2.1mm, 3µm particle size, Waters, Milford, MA) was used. Solvent A consisted of 97:3 water:methanol (v/v %) with 10 mM tributylamine and 15 mM acetic acid, and solvent B was 100% methanol. The LC gradient was run as following: 0 min, 0% B, 200 µL/min; 2 min, 0% B, 200 µL/min; 4 min, 20% B, 200 µL/min; 13 min, 80% B, 200 µL/min; 17 min, 100% B, 200 µL/min; 17.5 min, 100% B, 300 µL/min; 20 min, 100% B, 300 µL/min; 20.5 min, 0% B, 300 µL/min; 24 min, 0% B, 300 µL/min; 25 min, 0% B, 200 µL/min. The column and autosampler temperatures were kept at 25 °C and 5 °C, respectively. The mass spectrometer was operated in negative ion mode with resolving power of 100,000 at m/z 200 and scanning range of m/z 75-1000.

Data were analyzed using the open source software MAVEN and presented as log2 fold-changes compared to vector control unless stated otherwise. Previously determined absolute metabolite concentrations from iBMK cells were used to convert the measured metabolite intensities into absolute concentrations (assuming any effect of
the empty vector expression on absolute metabolite concentrations is small). Adenylate energy charge was calculated using the following equation:

\[
\text{Adenylate Energy Charge (EC)} = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}
\]

(Equation 5)

**B.2.10 Enzyme assays for HK, GAPDH, PKM, and LDH activities**

Cell culture for enzyme activity assays was performed in 6-well plates and iBMK cells were collected 24 h post transfection. Briefly, cells were washed once with ice-cold 1x PBS and directly scraped in 200 µL ice-cold assay buffer supplemented with phosphatase and protease inhibitors. Cells were lysed by sonication and whole cell lysates were centrifuged at 17,000 g at 4°C for 10 min. The supernatant was transferred to a fresh Eppendorf tube and 10 µL of whole cell lysate were used for enzyme assays. For GAPDH, PKM, and LDH activity assays, cell lysates were diluted 50-fold. Colorometric assay kits from Sigma (HK), ScienCell (GAPDH) and Abcam (PKM and LDH) were used to measure enzyme activities according to manufacturer’s instructions. Enzyme activities were normalized to the protein content as determined by the Pierce™ BCA Protein Assay Kit.

**B.2.11 Overexpression of glycolytic genes in 3T3 cells by lentiviral transduction**

Lentivirus production was performed in 6-well plates, with one plate (or 6 wells) used per individual construct. 50% confluent HEK293T LX cells were transfected with 1.33 µg of pCMVdR8.91 (packaging), 0.17 µg pMD2.G (envelope) and 1.5 µg of construct DNA using 9 µL of FuGENE. Supernatant was collected between 48 h and 52 h after transfection and
filtered using a 0.45 μm filter before transduction. Freshly-produced virus was directly used to transduce 3T3 cells on the same day.

0.3 μL PCV of 3T3 cells were plated per well of a 6-well plate in DMEM (10% dFBS) the day prior to transduction. Cell growth medium was aspirated and 700 μL of freshly-produced virus supplemented with polybrene (5 μg/mL) and HEPES (10mM) was added directly to each well. Cells were incubated for 4-6 h before adding 2 mL of fresh DMEM (10% dFBS; 5μg/mL polybrene) to the inoculum. Lactate secretion was measured between 42 h and 48 h after transduction as described above. Transduction efficiency was assessed using confocal imaging of blue fluorescent protein (BFP), which was expressed on the same lentiviral vector downstream of the target glycolytic gene using an IRES and GFP. Briefly, transduced 3T3 cells were harvested via trypsinization, seeded on black glass-bottom 96 well plates (Cellvis, Mountain View, CA, USA), and allowed to adhere for at least 6 h in normal growth medium. Subsequently, live-cell imaging was carried out with a Nikon Eclipse Ti Spinning Disk Confocal Microscope (Nikon Instruments, Melville, NY, USA), using a 20x objective and 405 nm (BFP) and 488 nm (GFP) laser lines.

B.2.12 Optogenetic stimulation of Ras signaling in NIH 3T3 OptoSOS cells

Optogenetics experiments were performed as previously described \textsuperscript{107}. Briefly, clonal NIH 3T3 cells expressing the light-activatable OptoSOS system were plated in DMEM (10% dFBS) and grown overnight. The next day, 4 h to 6 h prior to stimulation, the cells were washed twice with 1x PBS and serum-starved in fresh DMEM (without FBS) supplemented with 10 μM phycocyanobilin (PCB). For all steps following PCB addition, the cells were carefully protected from ambient light (e.g., experiments performed in low-light conditions and plates wrapped in aluminum foil) to prevent activation of the OptoSOS
system. Subsequently, the cells were either exposed to 650 nm red light (stimulated) or were left in the dark (unstimulated) until the end of the experiment. Glycolytic flux measurements were performed in 6-well plates and change in lactate secretion was assessed after 6 h of stimulation as described above. For metabolomics experiments, cells were grown in 6 cm dishes and intracellular metabolites were extracted and analyzed after 4 h and 24 h of stimulation as described earlier. Cell growth was determined by measuring change in PCV at 24 h post-stimulation.

Kinetic glycolytic flux profiling of stimulated and unstimulated cells using U-13C-glucose was performed in 6 cm dishes. Serum starved, PCB-treated cells were either pre-exposed to activating red light or left in the dark for 30 min before washing twice with 1x PBS and switching to 2 mL of U-13C-glucose-containing DMEM. Cells were subsequently collected at the indicated time points and subjected to LC-MS analysis as described above.

B.2.13 RNAseq analysis of 3T3 OptoSOS cells

Changes in gene expression of NIH 3T3 OptoSOS cells upon Ras activation were re-analyzed from a previously published dataset (GEO accession # GSE100816)\(^75\). The reads for the light activated samples (0, 30, 60, and 120 min) were analyzed using the Galaxy Workflow system\(^281\) and custom scripts (https://bitbucket.org/princeton_genomics/ltanner_glycolosis_flux_rnaseq/) implemented in R\(^282\). Briefly, adapters were removed and reads were quality trimmed using Trim Galore! V0.4.3.1 with default parameters\(^283\). The trimmed reads were mapped to the GRCm38/mm10 genome using Tophat 2.1.1\(^284\). The GTF option was used with the genes.gtf file from Illumina’s iGenomes (https://support.illumina.com/sequencing/sequencing_software/igenome.html) and the no-novel-juncs option was specified to ensure all samples used the same set of splice junctions. FeatureCounts 1.4.6.p5\(^285\) was used to determine the read counts for each gene.
specified in the iGenomes gtf file. Finally, DESeq2 1.16.1 was used to calculate normalization factors for each sample. Technical replicates were combined and genes with counts lower than 200 were excluded from further analysis. Differential expression was calculated at each time point compared to 0 min and the data was presented as log₂ fold-ratios. Gene set enrichment analysis (GSEA) was performed using the GSEA software. Only genes exhibiting a two-fold change on at least one time point were chosen and enrichment for hallmark- and KEGG-annotated gene sets was calculated.

### B.2.14 Gene expression analysis of TCGA data

RNAseq data from human solid tumor tissue samples were downloaded from The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov/) using the open-source software TCGA-assembler executed in R. Only primary tumor samples (TCGA barcode identifier: 01) with paired adjacent normal tissue samples (TCGA barcode identifier: 11) were considered for further analysis. This resulted in a final set of 666 paired human solid tumor tissue samples from 19 different tumor types (Table B.2).

A list of 40 glycolytic enzymes and transporters was manually curated based on annotation in KEGG pathways. Gene expression of primary tumor tissue samples was normalized to the corresponding adjacent healthy tissue samples and the normalized values were presented as a log₂ fold ratio. Gene and samples were clustered by Pearson correlation distances (centered) with average-linkage implemented in Cluster3.0. Calculated dendrograms and heat maps were subsequently visualized using Java TreeView. The clustered dendrogram heat map was generated using aheatmap command in the R package NMF.

For rank order analysis of the 40 glycolytic genes, the average fold-expression change relative to benign adjacent tissue of different glycolytic genes across all samples of
a given tumor type was ranked from highest to lowest. The topmost up-regulated GLUT, PFK, and MCT isoforms were highlighted until at least one gene isoform of each of these three glycolytic steps was covered.

To determine the four most up-regulated glycolytic steps in each tumor sample, gene isoforms were ranked based on their extent of up-regulation relative to adjacent benign tissue. Subsequently, the frequency with which gene isoforms were among the four topmost up-regulated glycolytic genes was tabulated. Gluconeogenic and anti-glycolytic (FBP1, PFKFB2, &TIGAR), poorly annotated (HKDC1), and pyruvate dehydrogenase (PDHA1 & PDHB) gene isoforms were excluded from this analysis (Table B.3). A Fisher’s exact t-test (with Bonferroni correction for multiple hypothesis testing) was performed to probe the statistical significance of whether a given isoform is more likely to rank in the four top most up-regulated genes than expected.

B.2.15 Quantification and statistical analyses

All statistical analyses were done in R\textsuperscript{282}. The following packages were additionally used for general data preparation: dplyr\textsuperscript{293}, plotrix\textsuperscript{294}, reshape/reshape2\textsuperscript{295}, stringr\textsuperscript{296} and tidy\textsuperscript{297}. For all experiments, mean and 95\% confidence intervals of at least n=3 biological replicates were plotted unless stated otherwise. Statistically significant differences (p<0.05) were calculated by two-tailed unpaired Student’s t test and multiple hypothesis correction (q<0.05) was performed using the \textit{qvalue} package\textsuperscript{298}. Michaelis-Menten curves were fitted using the \textit{nls} function and R\textsuperscript{2} for fits were calculated by the residual sum of squares (RSS) and total sum of squares (TSS) using the following formula:

\[
R^2 = 1 - \frac{RSS}{TSS}
\]

(Equation 6)
95% confidence intervals of the Michaelis-Menten fit were calculated using the \textit{drc} package\textsuperscript{299}. P values for Michaelis-Menten fits were calculated by linear regression of the predicted versus measured values. Metabolomics heat maps were generated with the \textit{pheatmap} package\textsuperscript{300}. All other plots were generated using \textit{ggplot2}\textsuperscript{301} and all figures were formatted in Illustrator (Adobe, San Jose, CA, USA).
Appendix C

Supporting materials and methods for Chapters 3 and 4

C.1 Supplementary figures

**Figure C.1**, related to **Figure 3.2**: (a) Schematic depicting the ‘inter-pulse interval’ on a
representative single-cell Erk activity trace. Inter-pulse interval is defined as the time between successive pulses of Erk activity, calculated at the full-width half-max of each pulse. (b) Erk activity was imaged every 2 min for 24 h in keratinocytes cultured in GF-free media (See Figure 3.2A). The distribution of intervals between each Erk pulse that occurred over the entire 24 h timecourse is depicted here (gray bars), fit to an exponentially-modified Gaussian distribution (dotted black line). Parameters of the fit are outlined above. (c-d) Average pulse frequency, plotted as number of pulses per cell (c), and average total Erk activity, plotted as the ErkKTR cytosolic/nuclear ratio (d) are plotted for the keratinocytes (left) and MCF10A cells (right) depicted in Figure 3.2B.

Figure C.2: (a) Schematic of air-liquid interface (ALI) keratinocyte culture. Primary keratinocytes are seeded on a 0.4 µm filter and grown in media for 3 d, after which the filter is inverted and ALI is established by removing media from the above the cells to induce epithelial stratification and differentiation over the following 3-4 d. (b) Image of ErkKTR localization in the basal layer of a 4 d ALI. (c) Erk activity trajectories for primary
KTR/H2B keratinocytes grown in ALI (see C.2b). Cells were cultured in ALI for 4 days and Erk activity was imaged in the basal layer for >6 h. (d) Images of whole-mount epidermis taken from E15.5 mouse embryos and immunostained for phospho-Erk1/2.

![Histograms](image)

**Figure C.3:** (a-c) Distribution of inter-pulse intervals for keratinocytes treated with (a) DMSO vehicle control, (b) the Class 2 B-Raf inhibitor GDC0879, or (c) the Class 3 frequency-modulating RTK inhibitor pazopanib. KTR-H2B keratinocytes were imaged every 3 min for 14 h, n>200 cells each. Cell trajectories and dynamic features are plotted in Figure 3.5B-C.

C.2 Methods

C.2.1 Cell culture

Dorsal epidermal keratinocytes derived from CD1 mice and stably expressing a retrovirally-delivered histone H2B-RFP were obtained from the Devenport lab and were cultured as described in Nowak & Fuchs\(^{201}\). Briefly, keratinocytes were given low calcium (50 µM) complete growth media (E media supplemented with 15% serum and 0.05 mM Ca\(^{2+}\)) in Nunc Cell Culture Treated Flasks with filter caps (Thermo) and were maintained in a humidified incubator at 37° C with 5% CO2. Cell passage number was kept below 30.
C.2.2 Plasmids

The pHR lentiviral backbone (302; Addgene #79121) was used for all DNA expression constructs. All constructs were cloned using PCR amplification of complementary fragments and In-Fusion HD-based assembly (Takara). ErkKTR-BFP was cloned by PCR amplification of TagBFP (75; Addgene #102350) and insertion into the pHR ErkKTR-iRFP construct used previously (54; Addgene #111510). BFP-SSPB-SOScat-2A-PuroR-2A-iLID-CAAX was cloned from the pTiger-OptoSOS construct used previously (105; Addgene #86439), followed by PCR amplification and insertion of 2A-PuroR (a gift from Brett Stringer; Addgene #98290) and TagBFP. ErkKTR-iRFP-2A-H2B-tRFP was cloned by PCR amplification of the 2A cleavage site from pTiger-OptoSOS and amplification of the H2B-tagRFP coding sequence (a gift from Philip Keller; Addgene #99271), followed by insertion into pHR ErkKTR-iRFP.

C.2.3 Lentivirus production and stable transduction

Lentivirus was generated as described in Goglia et al.107. Briefly, Lenti-X HEK 293T cells were seeded at roughly 50% confluency in 6-well dishes. Cells were allowed to settle for 12 h, after which FuGENE HD transfection reagent was used to co-transfect cells with the desired pHR expression vector and the two necessary lentivirus helper plasmids (pCMV-dR8.91 and pMD2.G – gifts from the Trono lab). Viral supernatants were collected 48 h after transfection, cell debris was removed using a 0.45 μm filter, and viruses were either used immediately or stored at -80º C.

For viral transduction, keratinocytes were plated at <50% confluency in 6-well dishes, allowed to adhere overnight, and then treated with 100 µL of an appropriate lentivirus. Viral supernatants were supplemented with 5 µg/mL Polybrene and 50 µM HEPES buffer. Virus-containing media was removed after 24 h and cells were transferred.
into new tissue culture flasks. Fluorescence-activated cell sorting (FACS) was used to isolate keratinocytes that stably expressed both high levels of the H2B-RFP nuclear marker and low levels of the Erk-KTR-BFP reporter. Sorting was performed using a FACSAniaIII Fusion system with 355, 405, 488, 561, and 637 nm laser excitation sources (BD Biosciences). For optogenetic experiments, a separate line of OptoSOS keratinocytes was generated using the same lentiviral transduction procedures outlined above and then, 3d after transduction, selecting for expression by exposing cells to puromycin (1.5 μg/mL) for > 3d. Sorted/selected cells were then expanded into multiple large culture flasks and 20 vials of each sorted line were frozen down and stored in liquid nitrogen.

C.2.4 Preparing cells for microscopy

Imaging experiments were performed in either 384- or 96-well black-walled, 0.17mm high performance glass-bottom plates (Cellvis). Before plating, the bottom of each well was pre-treated with a solution of 10 μg/ml bovine plasma fibronectin (Thermo Fisher) in phosphate buffered saline (PBS) to support cell adherence. Two days before imaging, keratinocytes were seeded at 16,000 cells/well in 50 μL of low-calcium E media (in a 384-well plate), plates were briefly centrifuged at 100 × g to ensure even plating distribution, and cells were allowed to adhere overnight. 24 h before imaging, wells were washed 2-3X with PBS and cells were shifted to high-calcium (1.5 mM) E media to promote epithelial monolayer formation. For starvation experiments, cells were washed once with PBS and shifted to high-calcium P media (i.e., DMEM/F12 containing only pH buffer, penicillin/streptomycin, and 1.5 mM CaCl₂) eight hours before imaging. To prevent evaporation during time-lapse imaging, 50 μL of mineral oil was added to the top of each well immediately before imaging.
The kinase inhibitor library was screened in rounds of 48 drugs plus two controls per plate for a total of 50 wells, as this was the maximum number of conditions that could be imaged every 3 min in two fluorescent channels. Thus, nine initial rounds of imaging were required to screen all 429 drugs in the library. Cell plating was staggered such that a fresh plate of 50 wells was ready to be imaged every 8 h. An additional two rounds were added to re-screen drugs from wells with insufficient cell density. After downstream analyses of the primary screen, new aliquots of all top drug hits were purchased, and drug effects were confirmed via extended time-lapse imaging.

C.2.5 Microscopy

Imaging was performed on a Nikon Eclipse Ti confocal microscope, with a Yokogawa CSU-X1 spinning disk, a Prior Proscan III motorized stage, an Agilent MLC 400B laser launch containing 405, 488, 561, and 650 nm lasers, a cooled iXon DU897 EMCCD camera, and fitted with an environmental chamber to ensure cells were kept at a 37° C and 5% CO₂ during imaging. All images were captured with either a 10X or 20X air objective and were collected at intervals of 2-3 min.

C.2.6 Optogenetics experiments

For microscopy experiments involving optogenetic stimuli, an X-Cite XLED1 light source coupled to a Polygon400 Mightex Systems digital micromirror device (DMD) was used to stimulate cells with patterns of 450 nm blue light, applied for 500 ms every 1 min. For proliferation experiments, light was delivered using custom-printed circuit boards of blue 450 nm light-emitting diodes (LEDs). During light stimulation, cells were maintained in an incubator at 37° C in separate foil-wrapped boxes covered with separate blue LED boards delivering different patterns of light inputs. Each LED board was connected to a
separate constant-current LED driver, all of which were controlled using an Arduino MEGA 2560 microcontroller board. The Arduino was programmed with open-source IDE software to deliver different dynamic light input regimes to each circuit board. To minimize phototoxicity, light inputs were delivered in cycles of 10 sec ON and 20 sec OFF – this allowed us to minimize light exposure while still delivering a constant stimulus to cells by taking advantage of the slow (~ 80 sec) dark decay rate of iLID activation.

C.2.7 Drug treatments

An Echo® acoustic liquid handler (Labcyte) was used to precisely spot plastic 96-well plates with 75 nL of either DMSO vehicle control or of individual drugs from the kinase inhibitor library (Selleck Chem).100 μL of P media was then added to each well that had been spotted by the Echo to create 3X stock solutions that could be rapidly added to plates of cells by multi-channel pipetting. Keratinocytes were plated on glass 384-well dishes in 50 μL of media and a final drug concentration of 2.5 μM was achieved by adding 25 μL of each 3X stock solution to individual wells of keratinocytes. For the drug screen, imaging was carried out 30 min after drug additions. For subsequent experiments (e.g., individual drug follow-ups, proliferation assays) drug additions were performed by first creating a 10X working stock by diluting drug/growth factor/antibody in culture medium and then adding an appropriate volume of this stock to cultured cells.

C.2.8 Cell lysate collection and Western blotting

Two days before lysate collection, cells were seeded at $3 \times 10^6$ in 6-well tissue culture dishes and allowed to adhere overnight. 24 h before collection, cells were washed 3X with PBS and shifted to high-calcium media to promote epithelial monolayer formation. Drugs/growth factors were added to cells at the indicated times before lysate collection.
(all drugs added at 2.5 μM; EGF added at 10 ng/mL). At indicated time points, media was aspirated, cells were washed with PBS and were lysed with 100 μL of ice-cold RPPA buffer (1% Triton X-100, 50 mM HEPES buffer, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na₃VO₄, 10% glycerol, freshly-prepared protease/phosphatase inhibitors). Cell scrapers were used to remove cells from the surface of each well, then each lysate was transferred to an ice-cold Eppendorf tube and centrifuged at 17,000 × g for 10 min at 4º C. Supernatants were transferred to new tubes, 25 μL of 4X NuPAGE LDS Sample Buffer (Thermo Fisher) was added to each, and samples were boiled at 98º C for 5 min. Samples were then stored at -80º C.

For Western blotting, lysates were run in 17-well 4-12% Bis-Tris gels (Thermo Fisher) at 120V for 1.5 h. A Trans-Blot SD semi-dry transfer cell (Bio-Rad) was used to transfer protein samples from gels to nitrocellulose membranes. Single-gel transfers were run at constant current of 70mA and max voltage of 25V for 1 h. Nitrocellulose membranes were then blocked for 1 h at room temperature in Odyssey blocking buffer (Li-Cor), then were incubated overnight at 4º C on a plate rocker in a 1:1000 dilution of primary antibody. The following antibodies were used: anti-phospho-Erk1/2 rabbit monoclonal antibody (Cell Signaling #4370), anti-phospho-EGFR rabbit monoclonal antibody (Cell Signaling #3777), and anti-total-Erk1/2 mouse monoclonal antibody (Cell Signaling #4696). The next day, blots were washed 5 × 5 min with TBST and then incubated for 1 h at room temperature in a 1:10,000 dilution of IRDye 680RD goat anti-mouse and 800CW goat anti-rabbit fluorescent secondary antibodies (Li-Cor). Blots were then washed 5 × 5 min with TBST, imaged on a Li-Cor Odyssey CLx imaging system, and images were analyzed using Image Studio software (Li-Cor).

C.2.9 Immunofluorescence staining
After appropriate cell preparation and growth on glass 96-well plates as described for imaging above, cells were fixed with Cytofix (BD Biosciences) for 10 min at room temperature and then permeabilized with ice-cold 90% methanol for 10 min at -20º C. Cells were then blocked in IF buffer (10% FBS, 2 mM EDTA in PBS) for 1 h at room temperature and then incubated overnight on a plate rocker at 4º C in a 1:200 dilution of anti-phospho-Erk1/2 (Thr202/Tyr204) rabbit monoclonal antibody (Cell Signaling #4370). The next morning, cells were washed 5 × 5 min with IF buffer containing 0.3% v/v Triton 100X (IF-Triton) and then incubated for 1 h at room temperature in a 1:750 dilution of Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (Thermo Fisher A21206). Cells were again washed 5 × 5 min with IF-Triton, DAPI was added to each well at 1 μg/mL, and cells were immediately imaged on a confocal microscope.

C.2.10 Proliferation and cell cycle analysis

Cells were plated as described for western blotting above and were incubated with appropriate drug/growth factor/optogenetic stimuli for 22 h before analysis. Cells were then trypsinized, spun down, and resuspended in PBS with 0.1% Triton X-100, 200 μg/mL propidium iodide (Sigma), and 200 μg/mL RNAse A (Sigma) to lightly permeabilize and stain for DNA content. Samples were incubated in the dark for 20-30 min and analyzed by flow cytometry as per Darzynkiewicz et al.303. 20,000 single cells were analyzed for each experimental replicate. All flow cytometry was performed on a BD LRSII Multi-Laser Analyzer using a 561 nm laser. Cell cycle fractions were determined using FlowJo software.

C.2.11 Quantification and data analysis

All image quantification was performed using ImageJ/FIJI304 or MATLAB. The TrackMate plugin was used to achieve accurate single-cell tracking and segmentation202,305. Western
blot data were quantified using Li-COR Image Studio™ software. All statistical analyses were performed in MATLAB.
Appendix D

List of publications

Several sections of this thesis appear in the following journal publications:


Appendix E

List of conference presentations

Several sections of this thesis have been presented at the following conferences:


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