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14. ABSTRACT The goal of this project was to characterize differences in Ras pathway signaling, using live cell activity measurements in cell types and genetic contexts relevant to NF1. This final report includes three publications arising from our study, along with a summary of our findings and research products. The results presented herein provide a rationale, based on the characteristics of ERK activity at the single-cell level, for treatment of patients carrying <i>NF1</i> mutations by sustained, long-term inhibition of ERK pathway activity.					
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INTRODUCTION: Many of the pathological effects of *NF1* mutations result from failure to regulate the activity of the RAS signaling pathway. Inhibition of this pathway is one of the major therapeutic strategies currently being explored for NF1 patients. The effectors of the RAS pathway include the RAF/MEK/ERK signaling cascade, which stimulates proliferation and controls the expression of many downstream genes. Recent cell biology studies have shown that the kinetics of ERK activation, including the intensity, duration, and probability of response, are critical for deciding how the cell responds to RAS activation and which genes are expressed. We have also found that different inhibitors of the RAS pathway have unique effects on these kinetic features and on cellular proliferation and gene expression. Therefore, an understanding of kinetic changes in kinase activity and their effects at the cellular level is important in designing effective inhibition strategies. Our results have demonstrated that these kinetic differences can be resolved at high temporal resolution using high-throughput live-cell imaging of ERK and quantified by rigorous statistical approaches. We executed the main objectives of this project by developing reporter cells and performing real-time signaling activity measurements in cell lines and genetic configurations relevant to NF1, to determine whether *NF1* mutant cells differ in signaling intensity, duration, threshold, or basal activity. We also investigated how ERK kinetics respond to a panel of pathway inhibitors to determine which dose levels and inhibitors represent the best strategy for normalizing mutant signaling activity and cell responses.

KEYWORDS: *Provide a brief list of keywords (limit to 20 words).* RAS, ERK, live-cell imaging, FRET reporter, inhibitor, kinetics.

ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

The major goal of this research project was to characterize the kinetic differences in RAS-driven signaling between wild type and NF1-deficient cells. The tasks and objectives are as follows:

Specific Aim 1 - Identify the kinetic differences in Ras effector activity resulting from NF1 mutations	Timeline	Site 1
Major Task 1 <i>Establish and validate isogenic reporter cell line models carrying NF1 mutants</i>	Months	
Obtain CRISPR reagents, generate NF1+/- and -/- astrocyte, Schwann cells, and C2C12s	1-3	100%
Transfect/transduce C2C12 and MPNST cell lines, select, and test by live-cell microscopy.	4-6	100%
Transfect immortalized astrocyte and Schwann cells, test by live-cell microscopy.	6-9	100%
Milestone(s) Achieved		
Stable reporter cell lines for initial test, NF1	3	100%
Optimized imaging protocol and analysis pipeline	6	100%
All stable reporter cell lines ready	9	100%

Major Task 2 <i>Measure and compare kinetic modes of ERK and Akt activity in NF1 wild type and mutant cell lines.</i>	Months	Completion
Collect data for different growth factor stimulation conditions	6-12	100%
Extract data and perform quality control on dataset.	6-14	100%
Perform statistical comparisons	12-16	100%
Milestone(s) Achieved:	4	
Full dataset collected	15	100%
Grid of statistical comparisons complete	16	100%
Major Task 3 <i>Measure ERK and Akt kinetics in patient-derived cells</i>	Months	Completion
Develop stable reporter cells and optimize imaging protocol	12-15	100%
Collect full dataset, extract data, perform quality control	14-18	100%
Perform statistical comparisons	16-18	80%
Milestone(s) Achieved:		
Dataset collected	18	100%
Statistical data available	19	80%
Major Task 4 <i>Correlate signaling kinetics with cellular behaviors</i>		
Perform live-cell and immunofluorescence experiments	16-20	100%
Data analysis	20-24	100%
Milestone(s) Achieved:		
Data set collected	20	100%
Table of similarity measurements completed	24	100%

Specific Aim 2 - Identify the Ras pathway inhibitors that best normalize signaling kinetics, gene expression, and cell proliferation rates for NF1 mutant cells.	Timeline	Site 1
Major Task 1 <i>Identify Ras-pathway inhibitors that reverse single-cell NF1 kinetic modes and responses in cell lines</i>	Months	Completion

Perform live-cell inhibitor experiments	20-24	100%
Image data extraction and data set assembly	20-26	100%
Statistical analysis and comparisons	26-30	100%
Milestone(s) Achieved		
Dataset collected	24	100%
Statistical data available	30	100%
Major Task 2 <i>Compare drug responses between different patient-derived cells</i>		
Collect inhibitor data on patient cell lines	18-24	100%
Image data extraction and data set assembly	22-25	100%
Statistical analysis and comparisons	28-32	100%
Milestone(s) Achieved:		
Dataset collected	26	100%
Statistical data available	32	100%
Major Task 3 <i>Link gene expression in response to Ras pathway inhibitors to cellular responses</i>		
Perform inhibitor treatment and proliferation/differentiation assays	24-30	100%
Collect mRNA samples and perform mRNAseq.	24-30	0%
Data analysis	28-32	0%
Milestone(s) Achieved		
Cell behavior data available	30	0%
mRNAseq data available	30	0%
Comparison of inhibitor data complete	32	0%
Major Task 4 <i>Characterize individual cell adaptive responses to Ras pathway inhibitors in MPNST cells</i>		
Perform live-cell imaging of inhibitor treatment in MPNST cells	30-32	20%
Extract image data and perform analysis	32-36	0%
Milestone(s) completed		
Image data set available	34	0%
Statistical comparisons complete	36	0%

What was accomplished under these goals?

Overview

Following the approaches outlined in the research strategy, we performed an extensive quantitative analysis of ERK signaling in cells with both normal and mutant RAS pathway configurations, including NF1 mutations. To perform this analysis, we developed a platform of statistical methods and computational tools for quantifying time-resolved signaling activity in single cells. These methods have now been published as a set of detailed protocols (publication #2 under Products). Our quantitative analysis of signaling has been published in two research papers (publications #1 and #3 under Products). In addition to the published papers, the results specific to NF1-deficient cells shown below will be included in a manuscript that is currently in preparation. The synopsis presented below emphasizes the key experiments that address the primary research questions of the project; additional experiments that support and validate the key findings are detailed in the attached manuscripts.

Major Task 1 *Establish and validate isogenic reporter cell line models carrying NF1 mutants*

The goal of this task was to create reporter cell lines for live-cell analysis of the effect of NF1 mutation on signaling kinetics and drug responses. Some unanticipated difficulties in culturing and in reporter gene delivery were encountered, and were addressed, leading to a streamlined protocol that is detailed in publication #2. After identifying appropriate protocols, we stably integrated ERK and Akt reporter genes into astrocyte cells, isolated these cells by flow sorting, and performed image analysis by live cell microscopy. Similar procedures were used to insert reporters into MPNST and C2C12 cell lines, as well as into a panel of mouse embryonic fibroblasts (MEFs) with targeted genetic changes in *NF1* and *RAS* genes. This panel of cell lines, which we investigated collaboratively with Frank McCormick's group at UCSF, proved to be particularly useful by allowing direct comparisons between NF1 deficiency and other RAS pathway mutations. The advantage of this system is that differences in signaling due to RAS pathway mutations can be definitively attributed to the genetic configuration of the RAS pathway, without ambiguities arising from other mutations, such as the ones typically found in cancer cell lines. This panel includes both KRas4b^{WT}, *NF1*^{+/+} and KRas4b^{WT}, *NF1*^{-/-} cells, enabling a direct comparison between NF1-deficient and wild type cells. Also included were a number of mutant isoforms of KRas (including G12V, G12C, and Q61R), which enabled us to compare the effects of loss-of-function mutants in NF1 (a RAS GTPase-activating protein, or GAP) to the effects of cancer-driving mutations that prevent RAS from responding to GAPs. Together, the data collected from these multiple cell types enabled us to find a consistent answer to the question of how NF1-deficient cells differ from wild type in RAS signaling kinetics, as described in further detail below.

Major Task 2 *Measure and compare kinetic modes of ERK and Akt activity in NF1 wild type and mutant cell lines*

Using our reporter cell lines (C8-D1A-EA), we performed a series of experiments to characterize growth factor and drug responses. During live imaging, cells were exposed to EGF, FGF, HGF, IGF, PDGF, or Heregulin. Inhibitors for EGFR (gefitinib), RAF (TAK-632), MEK (trametinib), ERK (SCH-772984), and mTORC1/2 were applied, either preceding or following growth factor treatment. Images were acquired for 100-200 cells per condition, every 6 minutes for experiments ranging in duration from 6-24 hours. We applied our image processing algorithm to all images, extracting data on fluorescence intensity for each cell over the time course of the experiment, resulting in datasets of hundreds of individual cell responses for each condition. Several findings resulted from this data. First, we noted that ERK activity in C8-D1A-EA cells responds robustly to FGF, weakly to HGF, IGF-1, EGF and Heregulin, and not to PDGF. Second, we found that, as expected, inhibitors of the RAF, MEK, and ERK kinases downstream of Ras potentially blocked ERK activation within 5 minutes following addition. Surprisingly however, we found that application of these inhibitors even in the absence of growth factors and serum sharply reduced ERK activity levels. This observation contrasts with data we have collected from other cell types, including both epithelial and fibroblast cells, where ERK activity is already near basal levels in the absence of growth factors and cannot be further suppressed even with high doses of inhibitors. This finding indicates that these immortalized astrocytes maintain a high basal level of ERK signaling. Because they were

in the absence of stimulus, and in the mean derivative of the baseline, which indicates that there is substantial fluctuation in the baseline level of each cell. Significant differences were not detected in growth factor-stimulated metrics such as amplitude or duration of ERK activity following EGF treatment; these were similar between wild type Ras, mutant Ras, and NF1-/- cells.

A second line of evidence for differences in Ras activity in NF1-deficient cells comes from the C8-D1A astrocyte cell line, in which we have also expressed ERK activity reporters. After isolation of a stable population of reporter cells, we introduced shRNA hairpins targeting NF1 mRNA using a lentiviral expression system. After selection, we confirmed reduction of NF1 protein by immunoblot. Extensive live-cell analysis of these NF1-kd cells showed that they responded to growth factor stimulation with similar magnitude and duration of ERK activation (Figure 2). However, when in an unstimulated state, NF1-kd displayed higher level of ERK activity than the control cells, and treatment with MEK inhibitor under these conditions resulted in a larger drop in reporter signal than in the control cells. We conclude that NF1 deficiency in these model astrocyte cells results in a higher level of ERK activity in the absence of stimulation. This result is consistent with the data obtained from the genetically engineered fibroblast experiments described above. We conclude that NF1 deficiency primarily influences the baseline level of Ras/ERK activity rather than altering the kinetics of activation upon stimulation.

Major Task 3 Measure ERK and Akt kinetics in patient-derived cells

To examine how Ras signaling kinetics are altered in NF1-affected relative to healthy individuals, we used our single-

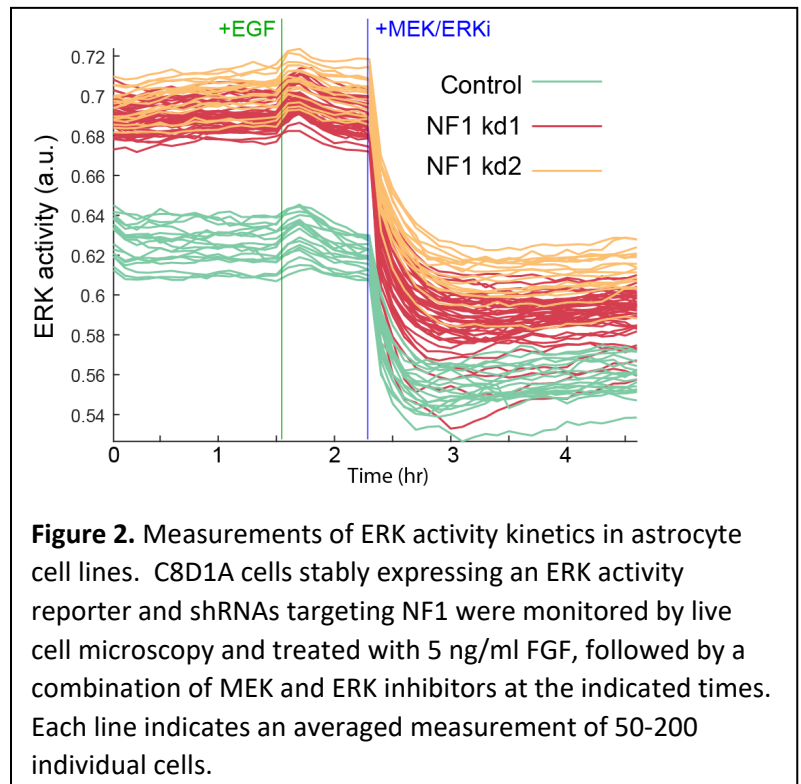


Figure 2. Measurements of ERK activity kinetics in astrocyte cell lines. C8D1A cells stably expressing an ERK activity reporter and shRNAs targeting NF1 were monitored by live cell microscopy and treated with 5 ng/ml FGF, followed by a combination of MEK and ERK inhibitors at the indicated times. Each line indicates an averaged measurement of 50-200 individual cells.

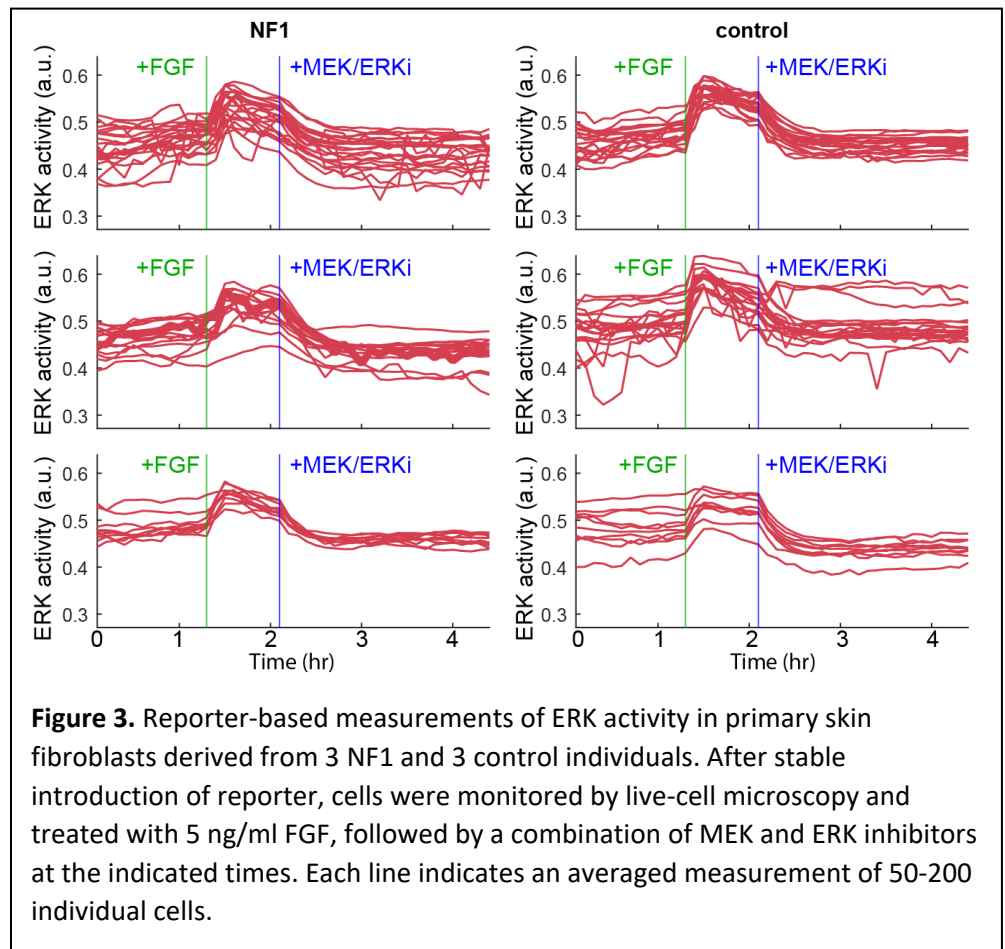
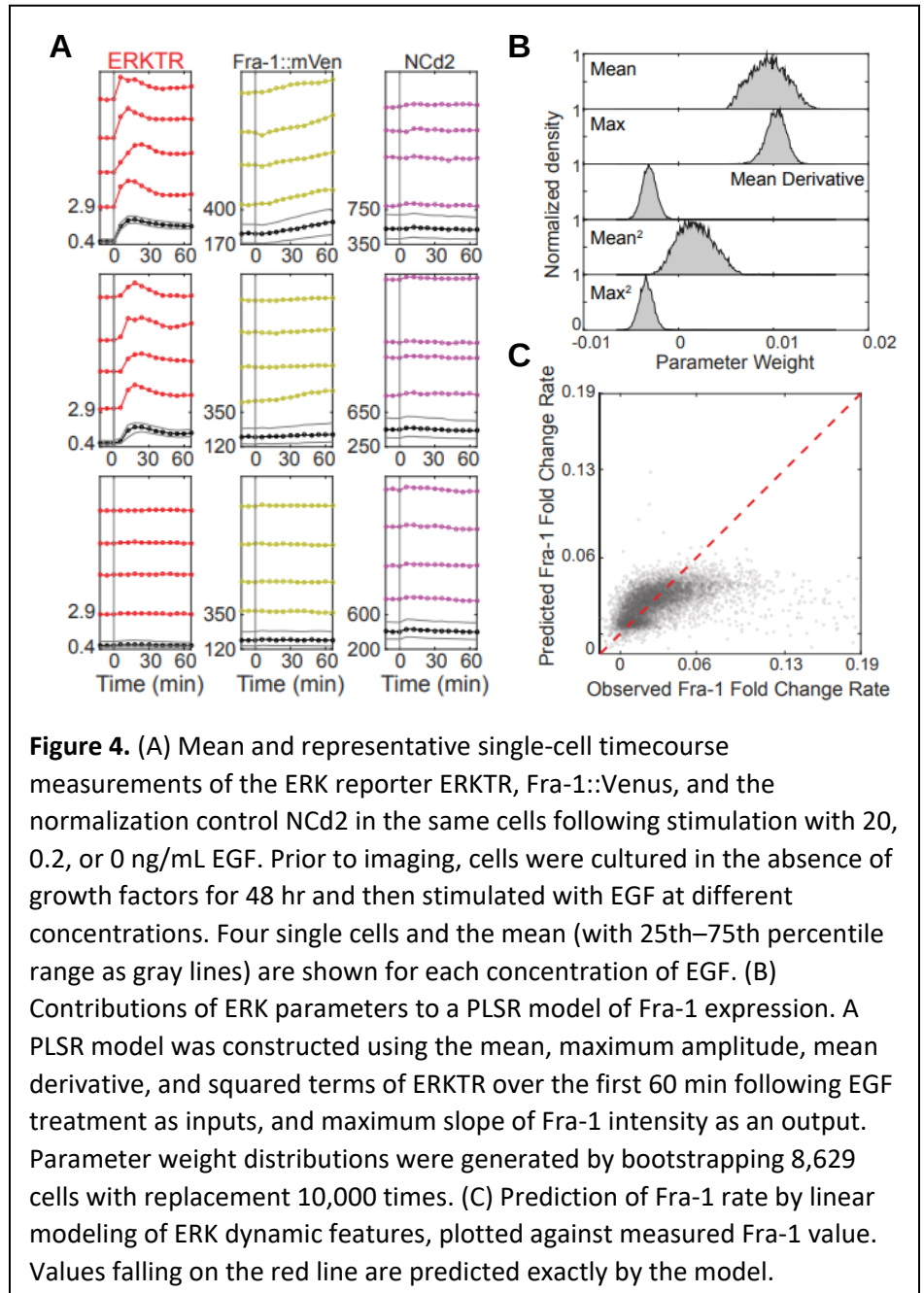


Figure 3. Reporter-based measurements of ERK activity in primary skin fibroblasts derived from 3 NF1 and 3 control individuals. After stable introduction of reporter, cells were monitored by live-cell microscopy and treated with 5 ng/ml FGF, followed by a combination of MEK and ERK inhibitors at the indicated times. Each line indicates an averaged measurement of 50-200 individual cells.

cell activity measurement to compare ERK signals in skin fibroblasts derived from NF1 patients and unaffected family members (Fig. 3). Using gene delivery methods refined in the previous reporting period, we generated populations of primary fibroblasts expressing a FRET-based ERK reporter. These cells were obtained from our collaborators Katherine Rauen (UC Davis MIND Institute) and Lauren Weiss (UCSF) and included both NF1-affected individuals (*NF1* +/-) and healthy individuals (*NF1*+/+). We collected a full dataset on ERK activity from these cells. Our observations indicate that there are only subtle differences between NF1-affected and control individuals at the level of signaling. A potential explanation for this similarity is that fibroblasts from NF1 individuals are heterozygous for *NF1*, and detectable changes in ERK activity may only manifest upon loss of the second allele. Evidence from mouse models has suggested that *NF1*^{+/-} cells are not equivalent to *NF1*^{+/+} cells in supporting fibroma development, but this difference may be too small to detect against the background of genetic variability between different individuals. These results further support the concept that the changes in RAS activity in cells with reduced NF1 activity are relatively subtle at the level of ERK activity. Our detailed investigation of the resistance of the RAS/ERK pathway to changes in activity is presented in publication #3.

Major Task 4 Correlate signaling kinetics with cellular behaviors

Our preliminary experiments showed that at the single-cell level, cell proliferation was poorly correlated with ERK activity levels. We therefore focused on a more proximal cell-fate endpoint, expression of the gene *FOSL1*, which encodes Fra-1, an AP-1 transcription factor that is known to play a role in cellular differentiation state in multiple cell types and which has previously been linked to invasive behavior in malignant cells. We utilized a model cell system, MCF10A, in which expression of *FOSL1* can be directly visualized using a CRISPR-mediated fluorescent protein knockout (Fig. 4). Using the methodology developed to quantify Ras/ERK signaling kinetics, we performed an extensive analysis of the relationship between ERK activity measurements and expression of Fra-1. MATLAB routines based on peak identification methods were used to extract all potentially relevant kinetic parameters from single-cell ERK activity trajectories, including peak



amplitude, average and maximum slope, average pulse width, inter-peak interval, and average peak frequency. While it is straightforward to compare each of these extracted parameters individually between different populations of cells, this approach ignores the possibility that combinations of multiple parameters may drive differences in the output of the pathway. We therefore developed a methodology to use partial least squares regression (PLSR) to develop multivariate models of the contribution of each kinetic parameter to the expression of a target gene (monitored using a knock-in fluorescent protein sequence in the ERK target gene FOSL1). As detailed in the attached publication #1, we found that Fra-1 expression increases proportionally to the integrated area of ERK activity, regardless of the amplitude or duration of activity. We also found evidence that other ERK-activated genes such as c-Fos and Egr-1 are less sensitive to cumulative activity and more responsive to sharp changes in activity. This finding suggests a model for how NF1 deficiency selectively activates genes involved in directing invasive cellular behavior. Because we found only moderate differences in baseline signaling of NF1-deficient cells relative to wild type cells, the question remains of how these relatively small changes result in changes in cell behavior that ultimately have a large impact on tissue physiology. Our analysis of Fra-1 suggests a possible explanation, which is that small changes in signaling activity over time lead to a large cumulative change at the level of gene expression. Such a response profile would be most sensitive to prolonged, low-dose inhibition of ERK activity, a possibility that we explored in more depth in Specific Aim 2 below.

Specific Aim 2 - Identify the Ras pathway inhibitors that best normalize signaling kinetics, gene expression, and cell proliferation rates for NF1 mutant cells.

Major Task 1 *Identify Ras-pathway inhibitors that reverse single-cell NF1 kinetic modes and responses in cell lines*

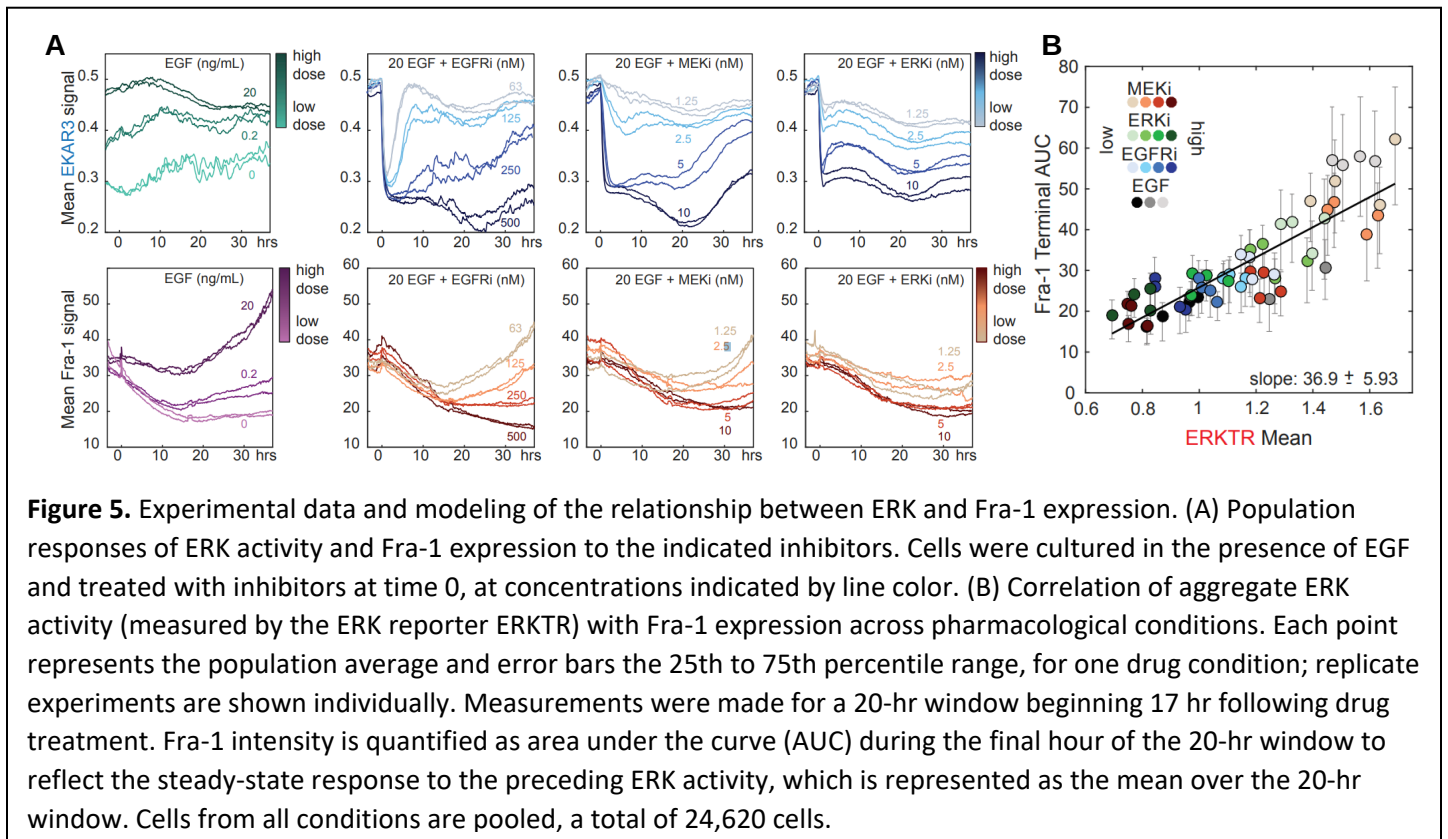
A priori, we anticipated that this task could pose substantial difficulties, depending on the complexity of the changes observed in ERK signaling kinetics as a result of *NF1* deficiency and the various pharmacokinetic properties of the available pathway inhibitors. However, as our analysis of signaling kinetics in Aim 1 demonstrated, NF1 mutants differed significantly only in showing a higher level of baseline RAS-mediated signaling (Fig. 1). Reversing this form of signaling defect turned out to be relatively straightforward; either MEK or ERK inhibitors applied continually were able to reduce the baseline level of signaling in NF1-deficient cells to the level of wild type cells (Figs. 2 and 3). These results mirror current clinical findings that prolonged treatment with the MEK inhibitor selumetinib can suppress tumor progression in NF1 patients (Gross et al., 2020a; Gross et al., 2020b).

Major Task 2 *Compare drug responses between different patient-derived cells*

As with major task 1 above, we anticipated the possibility that patient-derived cells could differ dramatically in their response to ERK pathway inhibitors due to the unique genetic background of each patient. However, our analysis found that patient-to-patient differences in RAS signaling kinetics were subtle, and there were no differences detected in the efficiency with which MEK inhibitor treatment reduced ERK activity (Fig. 3). Thus, we did not find support for the hypothesis that patient-specific differences in the RAS pathway response to inhibition can be detected by live-cell activity measurements and used to identify the best pathway-targeting drug for a given patient.

Major Task 3 *Link gene expression in response to Ras pathway inhibitors to cellular responses*

Because the target gene FOSL1 (Fra-1) is especially sensitive to accumulation during long-term activation of RAS signaling, we again used our MCF10A system as a model in which the effects of RAS/ERK pathway inhibition on downstream cellular responses could be quantified. We performed a thorough analysis of the correspondence between ERK activity suppression, and the expression of Fra-1 (Fig. 5A). As above, we modeled this relationship using PLSR. We found a highly linear correlation between aggregate ERK activity and FOSL1 expression across all drug treatments, along with an overall low predictivity at the single cell level (Fig. 5B). This result confirms that continual inhibition of ERK activity by multiple pathway inhibitors can reverse the



cumulative gene expression effects of elevated baseline ERK signaling.

While we initially planned to conduct gene expression profiling experiments to complement the live-cell findings in the final months funded by this grant, our ability to complete these experiments was precluded by the shutdown of our university due to COVID-19. While the official performance period of this grant has now ended, we nonetheless plan to complete these experiments when it becomes possible given current limitations on personnel and funding.

Major Task 4 Characterize individual cell adaptive responses to Ras pathway inhibitors in MPNST cells

This task proposed to examine signaling kinetics and inhibitor responses in MPNST cells. However, we encountered consistent problems in conducting these experiments due to the characteristics of MPNST cell lines. We found that in our hands, these cells lines showed poor expression of reporters, highly irregular morphologies, and inconsistent growth kinetics. These technical difficulties prevented us from collecting the datasets as planned. We do have plans to continue to develop our technology for difficult glial-type cells, which include FLIM-based microscopy that is less sensitive to irregular cellular morphology, and culture in more realistic media on 3D substrates, which may allow for more consistent cellular growth and viability.

Conclusions

While the technical aspects of this project proceeded largely as planned, a number of the results obtained were unexpected. *NF1* mutant cells showed only moderate quantitative differences in ERK activity relative to wild type cells. Given the known function of NF1 protein as a GTPase-activating protein for RAS proteins, we expected a quantitatively larger increase in RAS activity. Exploration of this effect in the context of various controls, including RAS mutant cells, revealed that this limited difference in ERK activity was not unique to NF1 mutant cells, but was in fact a general principle governing ERK activity across many cell types and contexts. This

discovery led to the conclusions discussed in the Year 3 report, and finally to our recently accepted publication #3, which we consider to be a significant conceptual advance in the RAS signaling field.

To summarize briefly, two major concepts have emerged from this work:

- 1) The dynamic range of ERK signaling is strongly restricted, even in cells carrying mutations such as *NF1* deletion or activating *RAS* mutations. Mutant cells do not have higher peak ERK activity or growth factor-stimulated responses than wild type cells. Instead, mutant cells have a moderately elevated baseline ERK signaling activity that allows for persistent stimulation of downstream gene expression.
- 2) Certain ERK-responsive genes respond as linear integrators of ERK activity. Protein expression of these genes can accumulate over time in proportion to continual ERK activation. Genes with this property include *FRA1*, which is known to promote malignant cell behavior.

Considered together, these concepts imply that the continuous, elevated ERK activity found in *NF1*-mutant cells is the primary form of ERK activity that promotes the development and progression of neurofibromas. This model provides a rationale for continuous treatment with pathway inhibitors that reduce ERK activity, which is an approach that is currently being explored clinically (Gross et al., 2020). Furthermore, our results suggest that there is considerable latitude in the types of RAS pathway inhibitors that can be used to achieve similar effects on pathway activity in *NF1*-deficient cells, which may be important because patients may tolerate MEK or ERK inhibitors differently

What opportunities for training and professional development has the project provided?

This research project provided training opportunities in experimental and computational analysis for a Ph.D. student, Taryn Gillies, who is now a postdoctoral scholar at Stanford University, and for a junior research specialist, Heather Blizard, who is now employed by BioMarin, a biotechnology company developing therapies for rare genetic diseases.

How were the results disseminated to communities of interest?

The results and methods developed by this project have been described extensively in the 3 publications listed. Results from this project were presented at conferences and oral presentations as listed in the Products section.

What do you plan to do during the next reporting period to accomplish the goals?

Not applicable (final report).

IMPACT: *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

Within the cancer signaling field, work from this project has led to an increasing recognition of the importance of signaling dynamics in controlling gene expression. Citations of our work in this context:

Köhler M, Ehrenfeld S, Halbach S, Lauinger M, Burk U, Reischmann N, Cheng S, Spohr C, Uhl FM, Köhler N, Ringwald K, Braun S, Peters C, Zeiser R, Reinheckel T, Brummer T. (2019) B-Raf deficiency impairs tumor initiation and progression in a murine breast cancer model. *Oncogene* 38(8):1324-1339. doi: 10.1038/s41388-018-0663-8.

Reischmann, N., Andrieux, G., Griffin, R., Reinheckel, T., Boerries, M., and Brummer, T. (2020). BRAF(V600E) drives dedifferentiation in small intestinal and colonic organoids and cooperates with mutant p53 and Apc loss in transformation. *Oncogene* 39, 6053-6070.

Our results have also informed efforts to develop and test pharmacological inhibitors of the RAS/ERK signaling pathway:

Beneker, C.M., Rovoli, M., Kontopidis, G., Roring, M., Galda, S., Braun, S., Brummer, T., and McInnes, C. (2019). Design and Synthesis of Type-IV Inhibitors of BRAF Kinase That Block Dimerization and Overcome Paradoxical MEK/ERK Activation. *J Med Chem* 62, 3886-3897.

Goglia, A.G., Wilson, M.Z., Jena, S.G., Silbert, J., Basta, L.P., Devenport, D., and Toettcher, J.E. (2020). A Live-Cell Screen for Altered Erk Dynamics Reveals Principles of Proliferative Control. *Cell Syst* 10, 240-253 e246.

What was the impact on other disciplines?

The methods for ERK activity recording that we have developed are used increasingly in developmental biology. Examples of citations of our work in this context:

Deathridge J, Antolović V, Parsons M, Chubb JR. (2019) Live imaging of ERK signalling dynamics in differentiating mouse embryonic stem cells. *Development* 146(12). pii: dev172940. doi: 10.1242/dev.172940.

Johnson HE, Toettcher JE (2019) Signaling Dynamics Control Cell Fate in the Early Drosophila Embryo. *Dev Cell* 48(3):361-370.e3. doi: 10.1016/j.devcel.2019.01.009.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Due to technical problems in growing available MPNST cell lines in our lab, we were unable to complete Aims 2.3 and 2.4. The morphology of the cells in culture and their high rate of death suggested that they were undergoing significant stress under the culture conditions that we tested. Because of these issues, we concluded that pursuing these aims would not provide easily interpretable data within the time frame of the project period, and we chose to focus on the quantitative analysis and publication of data collected in the other Aims.

Actual or anticipated problems or delays and actions or plans to resolve them

Because of the difficulty of reliably culturing MPNST lines without evidence of excessive cell stress, it became infeasible to complete certain experiments. We hope to return to this work at some point if more amenable cell lines become available.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

PRODUCTS:

Publications, conference papers, and presentations

Work funded by this grant was presented orally at the following conferences:

Advanced Imaging Methods Workshop, University of California, Berkeley, CA, February, 2020

Biotechnology for human and planetary health, Osaka University, Japan, November 2019

International Meeting on Optical Biosensors, Belgium, November 2018

Max Planck Institute (IMPRS-CMB) International Student Symposium, Germany, November 2018

qBio Summer School, Houston, TX, June 2018

Endocrine Society Annual Meeting, Chicago, IL March 2018

NCI Ras Initiative Symposium, Frederick, MD, December 2017

International Rasopathies Symposium, Orlando, FL, July 2017

Journal publications.

Overall publication record from the project:

1. Gillies, T.E., Pargett, M., Minguet, M., Davies, A.E., and Albeck, J.G. (2017). Linear Integration of ERK Activity Predominates over Persistence Detection in Fra-1 Regulation. *Cell Syst*.
2. Pargett, M., and Albeck, J.G. (2018). Live-Cell Imaging and Analysis with Multiple Genetically Encoded Reporters. *Curr Protoc Cell Biol* 78, 4.36.31-34.36.19.
3. Gillies, T.E., Pargett, M., Silva, J.M., Teragawa, C., McCormick, F., Albeck, J.G. (2020). Oncogenic mutant RAS signaling activity is rescaled by the ERK/MAPK pathway. *Mol. Syst. Biol.* (accepted)

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

In addition to the conference presentations listed above, results from this project were presented orally in the following venues:

Academia Sinica, Taipei, November 2019, Invited Seminar
University of Texas, Austin, TX, September 2019, Invited Seminar
University of Groningen, Netherlands, November 2018, Invited seminar
VIB Ghent, November 2018, Invited seminar
University of Wisconsin, Madison, WI, September 2017, Invited seminar
University of Colorado, Boulder, CO, August 2017, Invited seminar
Lawrence Livermore National Lab, Livermore, CA, June 2017, Invited seminar
National Cancer Institute, March 2017, Invited seminar
Johns Hopkins Medical School, March 2017, Invited seminar

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Our detailed methodology for image and reporter signal analysis has been published in detail in Publications #2 and #3.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Reporter cell lines were developed as described above, and are made available upon publication. Signal analysis software has been developed as described in our publications and is made available upon request.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**What individuals have worked on the project?**

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	<i>John Albeck</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>1234567</i>
Nearest person month worked:	<i>14</i>
Contribution to Project:	<i>Supervised all areas of research on the project, participated in data generation and analysis.</i>
Funding Support:	<i>N/A</i>

Name:	<i>Taryn Gillies</i>
Project Role:	<i>Graduate Research Assistant</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>15</i>
Contribution to Project:	<i>Developed signal analysis methods for application to ERK and Akt signals; constructed cell lines and performed microscopy experiments</i>
Funding Support:	<i>This award</i>

Name:	<i>Heather Blizzard</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>27</i>
Contribution to Project:	<i>Maintained cell cultures, generated DNA constructs, developed reporter cells and cell lines; prepared frozen cell stocks</i>
Funding Support:	<i>This award</i>

Name:	<i>Michael Pargett</i>
Project Role:	<i>Postdoctoral Associate</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>18</i>

Contribution to Project:	<i>Developed, maintained, and debugged image analysis software; developed signal analysis techniques; performed data analysis</i>
Funding Support:	<i>This award and National Institutes of Health</i>

Name:	<i>Nicholaus DeCuzzi</i>
Project Role:	<i>Graduate Research Assistant</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	<i>Performed cell culture and microscopy experiments</i>
Funding Support:	<i>This award and National Institutes of Health</i>

Name:	<i>Carolyn Teragawa</i>
Project Role:	<i>Research Scientist</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	<i>Performed cell culture and microscopy experiments</i>
Funding Support:	<i>This award and National Institutes of Health</i>

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Since the previous report, two funding sources have initiated:

IA-628171 (Albeck) 07/01/2019 – 06/30/2021 1.20 calendar
American Lung Association
Balancing metabolic-signaling homeostasis to treat and prevent diseases of the lung epithelium

A194373 (Albeck) 07/01/2019 – 06/30/2020 3.00 calendar
Kirin Corporation
High-content live cell-based analysis of candidate GF biological activity

What other organizations were involved as partners?

- **Organization Name:** University of California, San Francisco

- **Location of Organization:** San Francisco, CA
- **Partner's contribution to the project:**

Collaboration Wild type H, K, or N-Ras, mutant K-Ras, and NF1-deficient mouse fibroblasts were obtained from Dr. Frank McCormick. Dr. McCormick and Dr. Jillian Silva contributed to discussions of experimental planning, data analysis, and manuscript preparation.

SPECIAL REPORTING REQUIREMENTS

Nothing to report.

APPENDICES

Three published manuscripts are attached to this report.

LITERATURE CITED

Gross, A.M., Dombi, E., and Widemann, B.C. (2020a). Current status of MEK inhibitors in the treatment of plexiform neurofibromas. *Childs Nerv Syst* 36, 2443-2452.

Gross, A.M., Wolters, P.L., Dombi, E., Baldwin, A., Whitcomb, P., Fisher, M.J., Weiss, B., Kim, A., Bornhorst, M., Shah, A.C., *et al.* (2020b). Selumetinib in Children with Inoperable Plexiform Neurofibromas. *N Engl J Med* 382, 1430-1442.

Courtois-Cox, S., Genther Williams, S.M., Reczek, E.E., Johnson, B.W., McGillicuddy, L.T., Johannessen, C.M., Hollstein, P.E., MacCollin, M., and Cichowski, K. (2006). A negative feedback signaling network underlies oncogene-induced senescence. *Cancer Cell* 10, 459-472.

Gremer, L., Merbitz-Zahradnik, T., Dvorsky, R., Cirstea, I.C., Kratz, C.P., Zenker, M., Wittinghofer, A., and Ahmadian, M.R. (2011). Germline KRAS mutations cause aberrant biochemical and physical properties leading to developmental disorders. *Hum Mutat* 32, 33-43.

Hunter, J.C., Manandhar, A., Carrasco, M.A., Gurbani, D., Gondi, S., and Westover, K.D. (2015). Biochemical and Structural Analysis of Common Cancer-Associated KRAS Mutations. *Mol Cancer Res* 13, 1325-1335.

Konishi, H., Karakas, B., Abukhdeir, A.M., Luring, J., Gustin, J.P., Garay, J.P., Konishi, Y., Gallmeier, E., Bachman, K.E., and Park, B.H. (2007). Knock-in of mutant K-ras in nontumorigenic human epithelial cells as a new model for studying K-ras mediated transformation. *Cancer Res* 67, 8460-8467.

Omerovic, J., Hammond, D.E., Clague, M.J., and Prior, I.A. (2008). Ras isoform abundance and signalling in human cancer cell lines. *Oncogene* 27, 2754-2762.

Park, K.S., Jeon, S.H., Kim, S.E., Bahk, Y.Y., Holmen, S.L., Williams, B.O., Chung, K.C., Surh, Y.J., and Choi, K.Y. (2006). APC inhibits ERK pathway activation and cellular proliferation induced by RAS. *J Cell Sci* 119, 819-827.

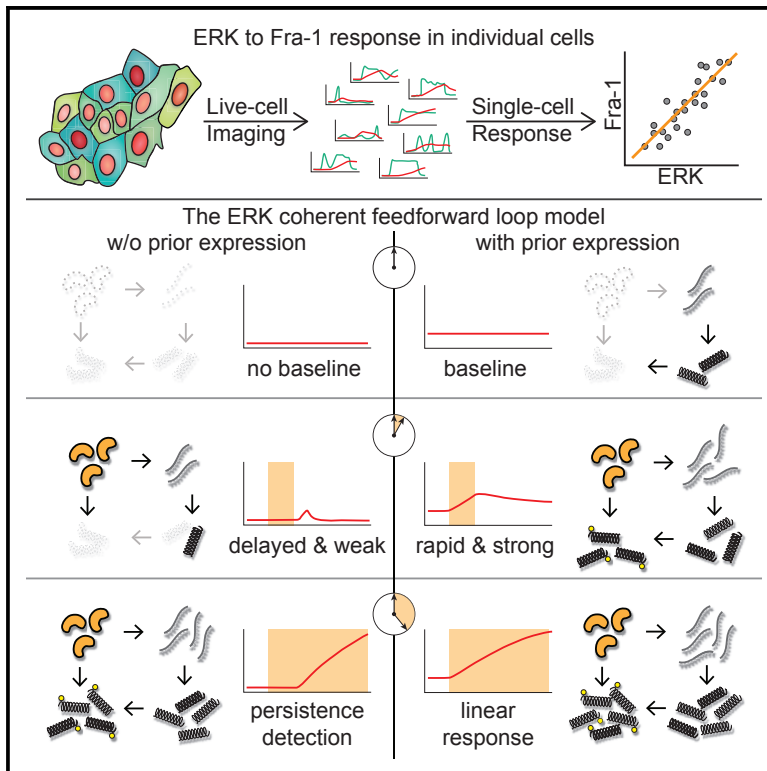
Smith, M.J., Neel, B.G., and Ikura, M. (2013). NMR-based functional profiling of RASopathies and oncogenic RAS mutations. *Proc Natl Acad Sci U S A* 110, 4574-4579.

Tuveson, D.A., Shaw, A.T., Willis, N.A., Silver, D.P., Jackson, E.L., Chang, S., Mercer, K.L., Grochow, R., Hock, H., Crowley, D., *et al.* (2004). Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell* 5, 375-387.

Yeh, J.J., Routh, E.D., Rubinas, T., Peacock, J., Martin, T.D., Shen, X.J., Sandler, R.S., Kim, H.J., Keku, T.O., and Der, C.J. (2009). KRAS/BRAF mutation status and ERK1/2 activation as biomarkers for MEK1/2 inhibitor therapy in colorectal cancer. *Mol Cancer Ther* 8, 834-843.

Linear Integration of ERK Activity Predominates over Persistence Detection in Fra-1 Regulation

Graphical Abstract



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In Brief

Using a combination of live-cell ERK activity reporters and mathematical analysis, Gillies et al. determine that the immediate-early gene, Fra-1, is produced in linear relation to total ERK activity. This demonstrates that the network functions as a linear integrator, not a selective filter, of ERK activity patterns.

Highlights

- Multiple reporters are needed to capture the full dynamic range of ERK activity
- Fra-1 induction is related linearly to the integrated activity of ERK
- Basal transcription rate determines whether a gene may act as a persistence detector
- ERK-independent factors contribute substantially to Fra-1 variance in single cells

Linear Integration of ERK Activity Predominates over Persistence Detection in Fra-1 Regulation

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SUMMARY

ERK signaling regulates the expression of target genes, but it is unclear how ERK activity dynamics are interpreted. Here, we investigate this question using simultaneous, live, single-cell imaging of two ERK activity reporters and expression of Fra-1, a target gene controlling epithelial cell identity. We find that Fra-1 is expressed in proportion to the amplitude and duration of ERK activity. In contrast to previous “persistence detector” and “selective filter” models in which Fra-1 expression only occurs when ERK activity persists beyond a threshold duration, our observations demonstrate that the network regulating Fra-1 expression integrates total ERK activity and responds to it linearly. However, exploration of a generalized mathematical model of the Fra-1 coherent feedforward loop demonstrates that it can perform either linear integration or persistence detection, depending on the basal mRNA production rate and protein production delays. Our data indicate that significant basal expression and short delays cause Fra-1 to respond linearly to integrated ERK activity.

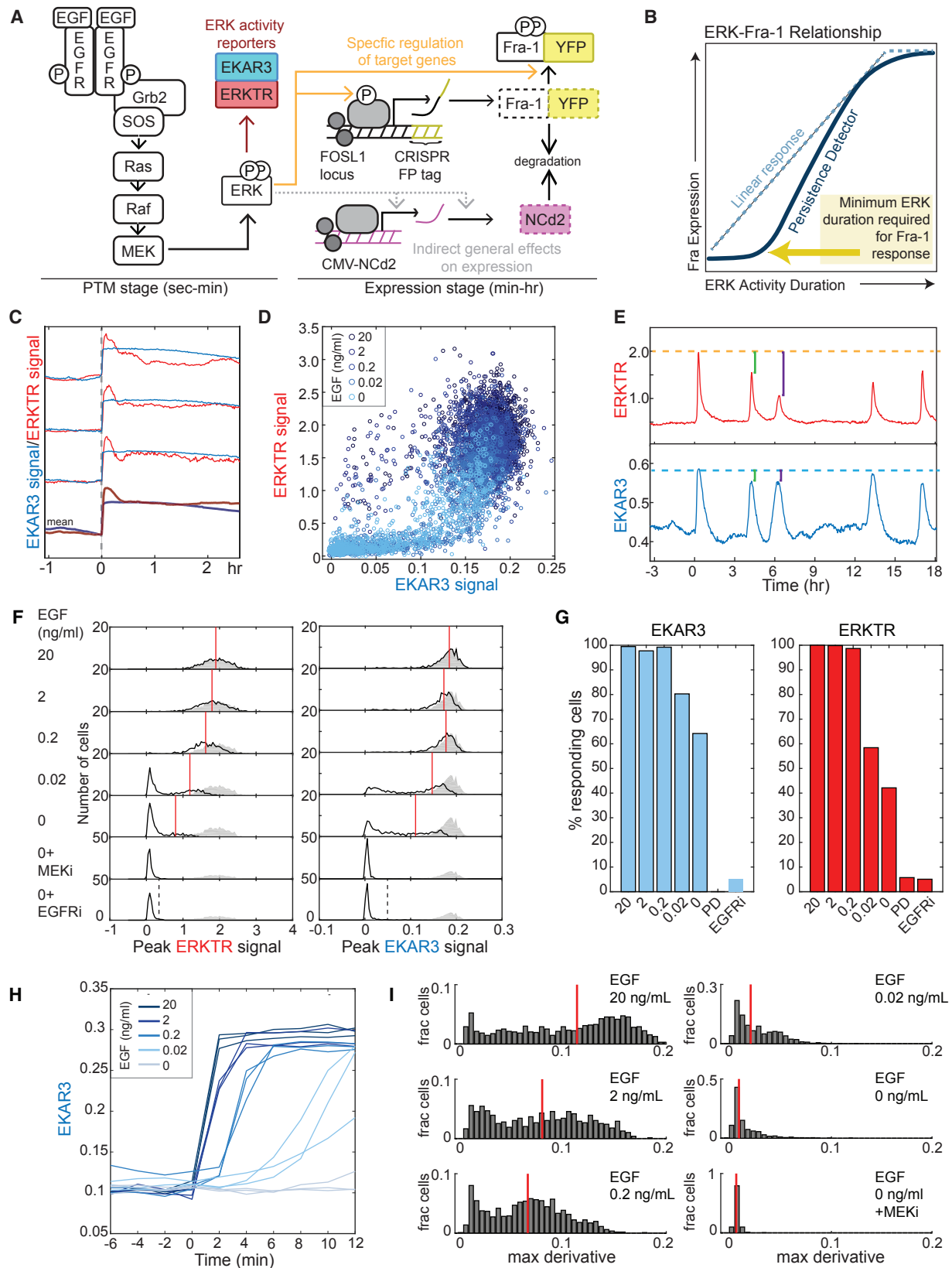
INTRODUCTION

Most mammalian signaling pathways induce post-translational protein modifications (PTMs) within minutes following stimulation, followed by changes in gene expression over the course of hours or days. Because gene expression ultimately causes changes in cellular phenotype, the transduction of immediate PTM events to transcriptional machinery is critical and has been the subject of a number of quantitative studies (Hansen et al., 2015; Hao and O’Shea, 2011; Lee et al., 2014; Tay et al., 2010). However, testing models of these systems is difficult because of the very different time scales of these processes. In principle, if all cells within a population responded homogeneously to stimuli, then traditional techniques, which report the average protein activity or transcript number across many cells, would be sufficient. However, recent studies have revealed that signaling responses at both the PTM and gene expression levels display a remarkable degree of cell-to-cell variability (Albeck et al., 2013; Aoki et al., 2013), and it is now clear that popula-

tion-level time-course measurements distort the underlying quantitative relationship. For example, averaging across a population containing both responding and non-responding cells may give the appearance of an analog response, when the underlying cellular behavior is in fact binary (Purvis and Lahav, 2013). Furthermore, “snapshot” approaches that collect single-cell data at fixed time points can fail to reveal dynamic behaviors or artificially indicate a bimodal response (Birtwistle et al., 2012). A more suitable dataset would provide time-course data for both PTM and gene expression events within the same individual cell, so that the dynamics of each can be cross-correlated without assumption or inference.

The Ras/MAPK pathway plays a central role in many cell types in controlling cellular proliferation and differentiation. Within this pathway, transduction begins with activation of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), and culminates with activation of the kinase ERK (Figure 1A). ERK activation has been observed as early as 30 s following exposure to growth factors and typically peaks 10–15 min later (Nakakuki et al., 2010; Saito et al., 2013). Depending on the cell type or the specific growth factor ligand, ERK activity may return rapidly to baseline within 30 min or remain elevated for hours (Niepel et al., 2014). ERK phosphorylates hundreds of substrates, including transcription factors (TFs) such as SRF and Elk-1 (Gille et al., 1995), which increase transcription rates for ERK target genes (ETGs) including c-Fos, c-Myc, Egr-1, and Fra-1 (Amit et al., 2007; Treisman, 1985). The resulting increases in mRNA and protein numbers are detectable within minutes, but typically take >30 min to reach their peak level. Several ETGs, including c-Fos, c-Myc, and Fra-1, are subject to an additional layer of regulation: the protein products of these genes are initially unstable, with a half-life of ~30 min, but are protected from degradation upon phosphorylation by ERK and RSK (a kinase also activated by ERK) (Okazaki and Sagata, 1995). Of these genes, Fra-1 is of particular importance within mammary epithelial cells, where it controls epithelial-to-mesenchymal transitions (Bakiri et al., 2015) and plays a role in the development of invasive characteristics during breast cancer progression (Tam et al., 2013).

Because ERK acts to increase the abundance of ETG proteins at the levels of both synthesis and turnover, the system forms a coherent feedforward loop (cFFL) (Mangan et al., 2003). This motif can potentially filter ERK inputs by responding to pulses of long, but not short, duration and driving approximately binary (all-or-none) expression of ETG proteins (Mackeigan et al., 2005; Murphy et al., 2002, 2004; Nakakuki et al., 2010), a behavior termed “persistence detection” (Purvis and Lahav, 2013). Other



(legend on next page)

studies have concluded that the amplitude (or peak response) of ERK activity qualitatively changes gene induction levels (Kwong et al., 2012; Woods et al., 1997). Thus, the nature of ETG regulation remains unclear, in part because studies to date have only tested a small number of conditions and used population-average or fixed-cell methods. While a common interpretation of the existing data is that ETG expression is preferentially (that is, non-linearly) sensitive to the duration of ERK activity (Legewie et al., 2005), it remains possible that ETGs respond more consistently to other patterns or to the integrated or cumulative ERK activity profile (Figure 1B). A more exact understanding of the input-output behavior of ETG expression is needed because these characteristics determine whether modulation of ETG expression by growth factors or drugs is sufficient to alter cell function.

Here, we describe a multiplexed combination of live-cell reporters to simultaneously track dynamic activity of ERK signaling and induction of Fra-1. This combination allows us to directly measure the correspondence between the kinetics of ERK activity and the rate of Fra-1 protein production in the same cell, which was not possible with previous methods. We describe an extensive analysis of how the activation of Fra-1 is affected by both the amplitude and duration of ERK activity, as controlled by both growth factor availability and small-molecule inhibitors. At both the population and the single-cell level, ERK amplitude and duration both contribute to the induction of Fra-1, such that the expression level of Fra-1 protein linearly reflects the integral of ERK activity. We incorporate this behavior into a generalized mathematical model that explains how the same gene expression motif can generate either rapid, high-gain linear responses or non-linear persistence detection of ERK activity duration.

RESULTS

ERK Response to Growth Factors Combines Digital and Analog Features

To assess how downstream effectors respond to ERK signals, it is first necessary to accurately measure ERK kinase activity. We

used two genetically encoded live-cell reporters, a Förster resonance energy transfer (FRET)-based Erk activity reporter (EKAR3) and a translocation-based reporter (ERKTR) (Regot et al., 2014) (Figure 1A). Both reporters produce a ratiometric signal that is related to ERK activity (see STAR Methods for details of ratio calculations). EKAR3 and ERKTR detect sporadic pulses of ERK activity with similar but distinct kinetics; EKAR3 rises faster and attenuates more slowly than ERKTR (Sparta et al., 2015). To reliably detect ERK activity, we measured the ERK response to increasing EGF concentrations following a period of growth factor withdrawal in cells expressing both EKAR3 and ERKTR (Figures 1C and S1). Both reporters responded rapidly to stimulation (rising by 2–4 min), but ERKTR typically peaked at 6–20 min and then rapidly declined to 50%–75% of its peak value, while EKAR3 peaked immediately and maintained a plateau for 3 or more hours, suggesting it may have reached saturation. Comparing the initial response amplitudes across various doses of EGF revealed that EKAR3 and ERKTR respond to different segments of the ERK dynamic range, with EKAR3 sensitive at lower activity levels and ERKTR at higher levels (Figure 1D). Moreover, at lower EGF doses ERKTR, but not EKAR3, measured sporadic pulses as lower in amplitude than the first pulse (Figure 1E, cf. green and purple bars). While sporadic pulses of ERK activity were previously reported to be quantal in size (Albeck et al., 2013), this uniformity is likely an artifact of the saturation of FRET-based reporters. Alternatively, it is possible that nuclear phosphatases induced by ERK activity attenuate ERKTR preferentially, due to its differential localization (Shah et al., 2017).

It has remained ambiguous whether ERK responses are digital/bimodal (Altan-Bonnet and Germain, 2005; Das et al., 2009; Ferrell and Machleder, 1998) or analog (O’Shaughnessy et al., 2011; Sturm et al., 2010) with respect to EGF dose. To address this question using the advantages of both reporters, we built dose-response distributions by resolving the peak signal in each cell. At doses of EGF >2 ng/mL, both reporters indicate that essentially 100% of cells respond with a uniformly high

Figure 1. Different ERK Reporters Measure Activity in Different Ranges

- (A) Schematic of the pathway and reporters used in this study. EKAR3 and ERKTR indicate ERK activity with minute-scale resolution, providing a readout of the PTM stage of EGF response. Genomic knockin of a YFP fusion tag at the *FOSL1* locus results in production of a fluorescent signal that responds to ERK activity at the level of both transcription and protein stabilization (orange arrows). NCd2 expressed from a viral promoter provides a control for general changes in gene expression capacity.
- (B) Expected behavior of a persistence detector. As the duration of ERK activity increases, Fra-1 expression responds selectively to longer periods of stimulation. In the absence of mechanisms imposing this selectivity, a saturating linear response would be expected (blue dotted line).
- (C) Sample EKAR3 and ERKTR measurements in single cells starved of growth factors and then stimulated at time 0 with 20 ng/mL EGF. Paired measurements from three individual cells are shown, and the mean for 489 cells below.
- (D) Scatterplot of initial peak amplitude on EGF stimulation, for 5,935 single cells, stimulated with different EGF concentrations (indicated by color). Each point represents the peak EKAR3 and ERKTR signals for an individual cell within a 1.5-hr window after stimulation.
- (E) Spontaneous ERK activity pulses occurring in a single cell, stimulated at time 0 with 0.02 ng/mL EGF, reported by both ERKTR and EKAR3. Dotted orange and blue lines indicate the peak signal immediately following stimulation for ERKTR and EKAR3, respectively. Green and purple bars indicate the differences of subsequent peaks relative to the initial response.
- (F) Histograms of the peak ERKTR (left) and EKAR3 (right) initial pulse amplitude reached in single cells, when stimulated with various concentrations of EGF. For comparison, shaded gray regions show the fully stimulated distribution (20 ng/mL EGF) in all panels. Red lines indicate the mean values of the responding population in each condition; responders are defined as cells whose peak ERK activity is above the 95th percentile of the EGFRi (gefitinib)-treated population (dashed lines). See also Figure S1.
- (G) Quantification of responding cells as detected by EKAR3 and ERKTR for multiple doses of EGF (as defined in F).
- (H) Individual cell traces of EKAR3 following EGF stimulation, imaged at higher time resolution (2 min), demonstrating the increase in initial slope of EKAR3 with EGF concentration.
- (I) Distributions of initial slope (maximum derivative within 30 min following stimulation) in EKAR3 following stimulation by EGF at different concentrations. Red bars indicate medians.

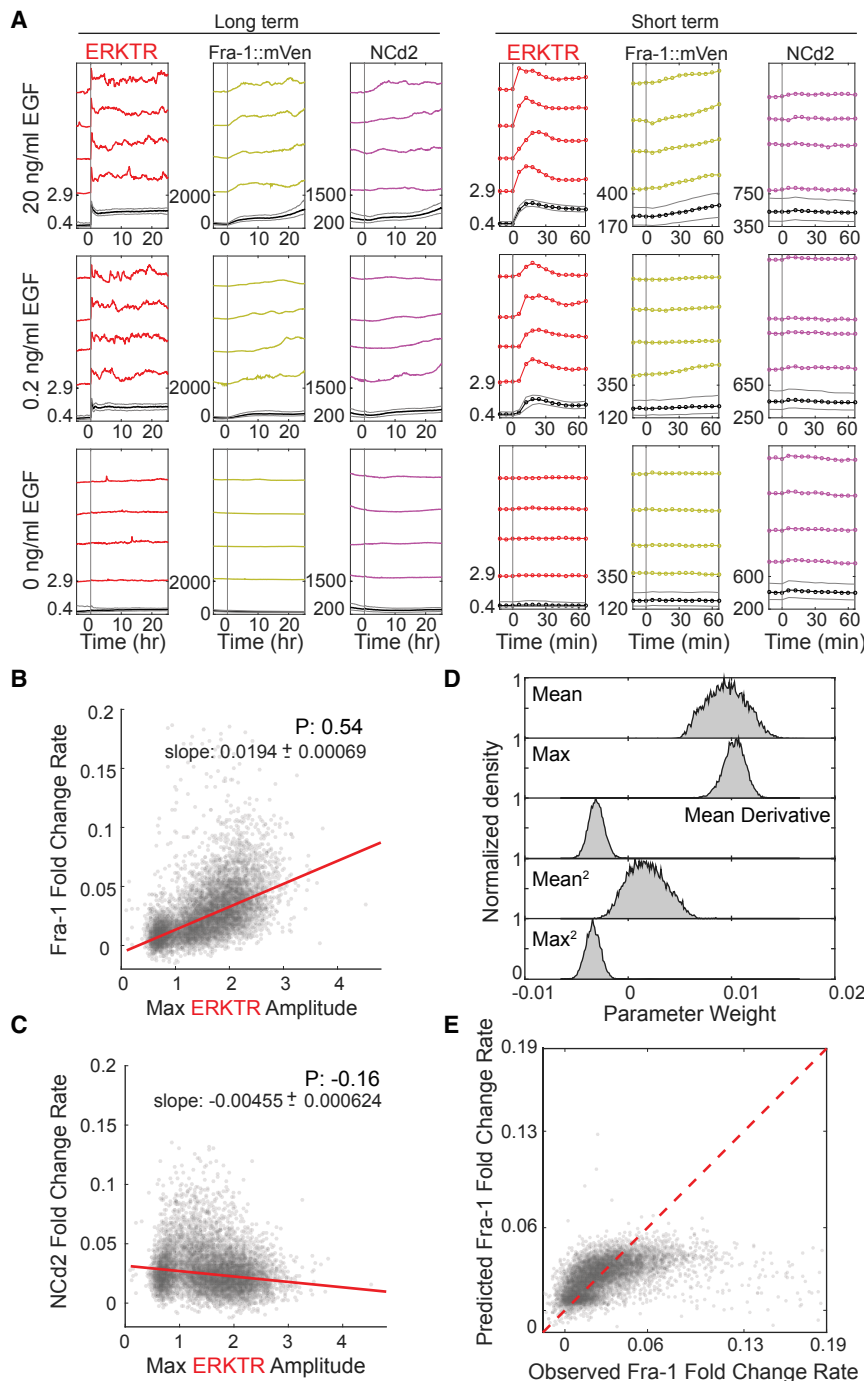


Figure 2. Measuring Fra-1 Production as a Function of ERK Activity

(A) Mean and representative single-cell time-course measurements of ERKTR, Fra-1::Venus, and NCd2 in the same cells following stimulation with 20, 0.2, or 0 ng/mL EGF. Prior to imaging, cells were cultured in the absence of growth factors for 48 hr and then stimulated with EGF at different concentrations. Four single cells and the mean (with 25th–75th percentile range as gray lines) are shown for each concentration of EGF, on both long and short time scales.

(B) Correlation between stimulated ERK activity and Fra-1 production rate. The maximum amplitude of ERKTR is plotted against the maximum slope of Fra-1::mVenus fold change within the first 60 min following EGF stimulation for each cell. Cells from multiple conditions are combined on a single plot. Red line represents a linear regression fit. P, Pearson's correlation; slope is indicated with 95% confidence intervals.

(C) Correlation between stimulated ERK activity and NCd2 production rate. Plot was constructed as in (B), using NCd2 instead of Fra-1::mVenus.

(D) Contributions of ERK parameters to a PLSR model of Fra-1 expression. A PLSR model was constructed using the mean, maximum amplitude, mean derivative, and squared terms of ERKTR over the first 60 min following EGF treatment as inputs, and maximum slope of Fra-1 intensity as an output. Parameter weight distributions were generated by bootstrapping 8,629 cells with replacement 10,000 times. ERKTR was used because of its broader amplitude response.

(E) Prediction of Fra-1 rate by linear modeling of ERK signal terms, plotted against measured Fra-1 value. Values falling on the red line are predicted exactly by the model.

amplitude signal (Figures 1F and 1G). Conversely, treatment with inhibitors of EGFR or MEK reduced all cells to a uniformly low ERK reporter signal, allowing us to set threshold criteria above which signals were considered specific ERK responses (Figure 1F). At 0.02 ng/mL EGF the fraction of responding cells fell to 80%, assessed by the more sensitive EKAR3 (58% when using ERKTR; Figure 1G). For the same EGF stimulus, we used ERKTR to distinguish peak ERK activity and found that the median amplitude for responding cells was 38% lower at 0.02 compared with 20 ng/mL EGF (20% lower for EKAR3; Figure 1F).

Instead, ERK activity reflects the stimulus strength in its amplitude and rate of activation, but with an increasing fraction of refractory cells at lower concentrations of EGF.

Fra-1 Production Rate Responds Linearly to ERK Activity

To enable dynamic measurements of ERK-responsive gene expression, we used CRISPR-mediated homologous recombination to insert monomeric, fast-folding fluorescent protein tags (mVenus or mCherry) as in-frame fusions at the C

When stimulated only with unsupplemented medium, 65% of cells displayed a detectable ERK response, with an even lower median (58% lower than with 20 ng/mL EGF). The rate of reporter signal increase also rose with increasing EGF dose (measured by EKAR3; Figures 1H and 1I). Another EGFR ligand, amphiregulin, displayed essentially identical dose-response characteristics (Figure S1). These data indicate that ERK activity in individual cells does not respond in a strictly digital manner to EGF and similar ligands.

terminus of the *FOSL1* locus (Fos-related antigen 1, encoding Fra-1; we refer to this genomic reporter as Fra-1::mVenus or Fra-1::mCherry). Correct integration was confirmed via western blot and genomic PCR; in cells, the nuclear Fra-1::mVenus signal was clearly distinguishable from autofluorescence (Figure S2). Integration of fluorescent protein tags into the genome is a reliable method to track gene expression from endogenous loci (Ghaemmamghami et al., 2003), and allows the net effect of transcriptional induction and stabilization by ERK to be monitored. We also introduced a destabilized, nuclear-localized fluorescent protein (NLS-mCerulean-d2, or NCd2) under a generic viral promoter/enhancer, to serve as a control for variation in the global rates of transcription and translation (Figure 1A). Constitutive degradation of NCd2 is controlled by an ornithine decarboxylase PEST sequence that is distinct from the Fra-1 PEST and is not regulated by phosphorylation.

To establish baseline ERK and Fra-1 levels, we withdrew growth factors for 48 hr. Under these conditions, baseline ERK activity was undetectable by EKAR3 (Figure S2), while Fra-1 decreased to low but detectable levels (Figures 2A and S2). We then exposed cells to different concentrations of EGF to produce initial ERK responses of varying intensity. Upon stimulation, Fra-1 began to rise within 12–24 min and continued to increase gradually over the course of 24 hr, while NCd2 began to rise after 2 hr (Figure 2A and Movie S1). To evaluate the direct relationship between ERK activity and Fra-1 expression, we focused on the initial rate of Fra-1 increase following stimulation, as this response is less likely to be affected by global cellular changes induced by active ERK. ERKTR was used to measure ERK activity, as it is most responsive to the higher amplitudes initially achieved upon stimulation by different EGF doses (see Figures 1C–1E). Collating experimental data across multiple EGF concentrations to span the full range of ERK activity levels, we found that the rate of Fra-1 increase rose linearly as a function of the amplitude of ERKTR (Figure 2B). In contrast, the rate of NCd2 accumulation was approximately constant across all ERKTR intensities (Figure 2C), indicating that the early relationship between ERK and Fra-1 induction was specific and not the result of global changes in protein production.

To comprehensively evaluate possible relationships between ERK and Fra-1 within each cell, we considered the maximum rate, peak amplitude, or mean amplitude of ERKTR during the first hour, as well as the squares of these metrics. We performed partial least-squares regression (PLSR) analysis, which reduces the complexity of the data and identifies the aspects of the input dataset most predictive of the output (Fra-1 expression). We found that mean and maximum amplitude were the most predictive of the rate of Fra-1 accumulation (Figure 2D). When comparing the predictions of the resulting PLSR linear model with the observed Fra-1 output, we find the expected linear 1:1 relationship with considerable variance, but also a discrepancy where the model fails to predict the highest levels of Fra-1 expression (Figure 2E). This discrepancy could arise because ERKTR (like EKAR) may eventually reach saturation and fail to provide an accurate measurement at very high ERK activity; alternatively, ERK-independent factors may drive high expression in some cells. Together, these analyses support a mass-action model in which, within minutes of EGF stimulation, the rate of Fra-1 accumulation is linearly regulated by the intensity of ERK activity.

Total Fra-1 Expression Responds Linearly to ERK Activity Duration

To assess the effects of the feedforward stabilization motif, we performed longer-term experiments inducing varied durations of ERK activity, directly testing the hypothesis that ETG responses are refractory to short-duration ERK activity. To generate ERK activity pulses of uniform amplitude, we used a high concentration of EGF to maximally stimulate ERK, followed by an MEK inhibitor at varying times ranging in duration from the shortest achievable pulse (approximately 24 min) to 10 hr (Figures 3A and S3). We found that even the shortest ERK activity pulses induced detectable Fra-1 expression (Figure 3B) as well as an increase in NCd2 (Figure 3C). While the response of Fra-1 depended strongly on the duration of stimulation, NCd2 displayed a more uniform increase regardless of ERK activity duration (Figures 3C and 3D), indicating that Fra-1 is specifically responsive to ERK while NCd2 reflects a general increase in expression capacity. Pretreatment with a MEK inhibitor reduced the amplitude of Fra-1 and delayed it to a similar time scale as NCd2 (Figures 3B and 3C), confirming that NCd2 reflects a non-specific component that likely results from EGF stimulation of other mitogenic pathways. Comparing the mean Fra-1 area under the curve (AUC) or peak intensity with the duration of ERK stimulation, we found a linear relationship, regardless of normalization (Figures 3E and S3). At longer durations of ERK activity (>6 hr), the expression of Fra-1 approached a saturation level, but we did not observe a minimum duration of ERK activity for Fra-1 induction (Figures 3E and S3) or a bimodal response with an increasing proportion of Fra-1-expressing cells (Figure 3F). Together, these data suggest that Fra-1 responds proportionally to cumulative ERK activity, rather than requiring a minimum duration of stimulation, across the practical range of durations of ERK activity. This analysis complements the above findings (Figure 2) that establish a linear response to ERK activity intensity after a 12- to 24-min delay, indicating that Fra-1 induction is controlled primarily by the cumulative integral of ERK activity.

Fra-1 Expression Integrates ERK Activity across Pharmacological Conditions

Pharmacological inhibitors that act at different parts of the pathway (denoted EGFRi, MEKi, and ERKi) have distinct effects on the kinetics of ERK activity (Figures 4A and S4) (Albeck et al., 2013). We therefore investigated whether Fra-1 production would still respond proportionally when ERK activity was suppressed by each of these compounds. As indicated by both EKAR3 and ERKTR reporters, ERK activity following EGFRi (gefitinib) treatment was initially suppressed, but then rebounded to a pulsatile state after a period of hours, consistent with the suppression of negative feedback loops (Duncan et al., 2012). With MEKi, a similar rebound occurred, but ERK activity reverted instead to an intermediate level with a moderate degree of pulsatility (with trametinib showing a slightly higher degree of pulsatility than PD325901). ERKi (SCH772984) induced a sharp drop in ERK activity that was maintained for 10–30 min, followed by a rapid rebound to a very stable level of ERK activity. These differences provide a means to test the response of Fra-1 to widely varying ERK kinetics.

When mean Fra-1 trajectories were examined for each drug, Fra-1 levels decreased upon the initial inhibitor treatment but

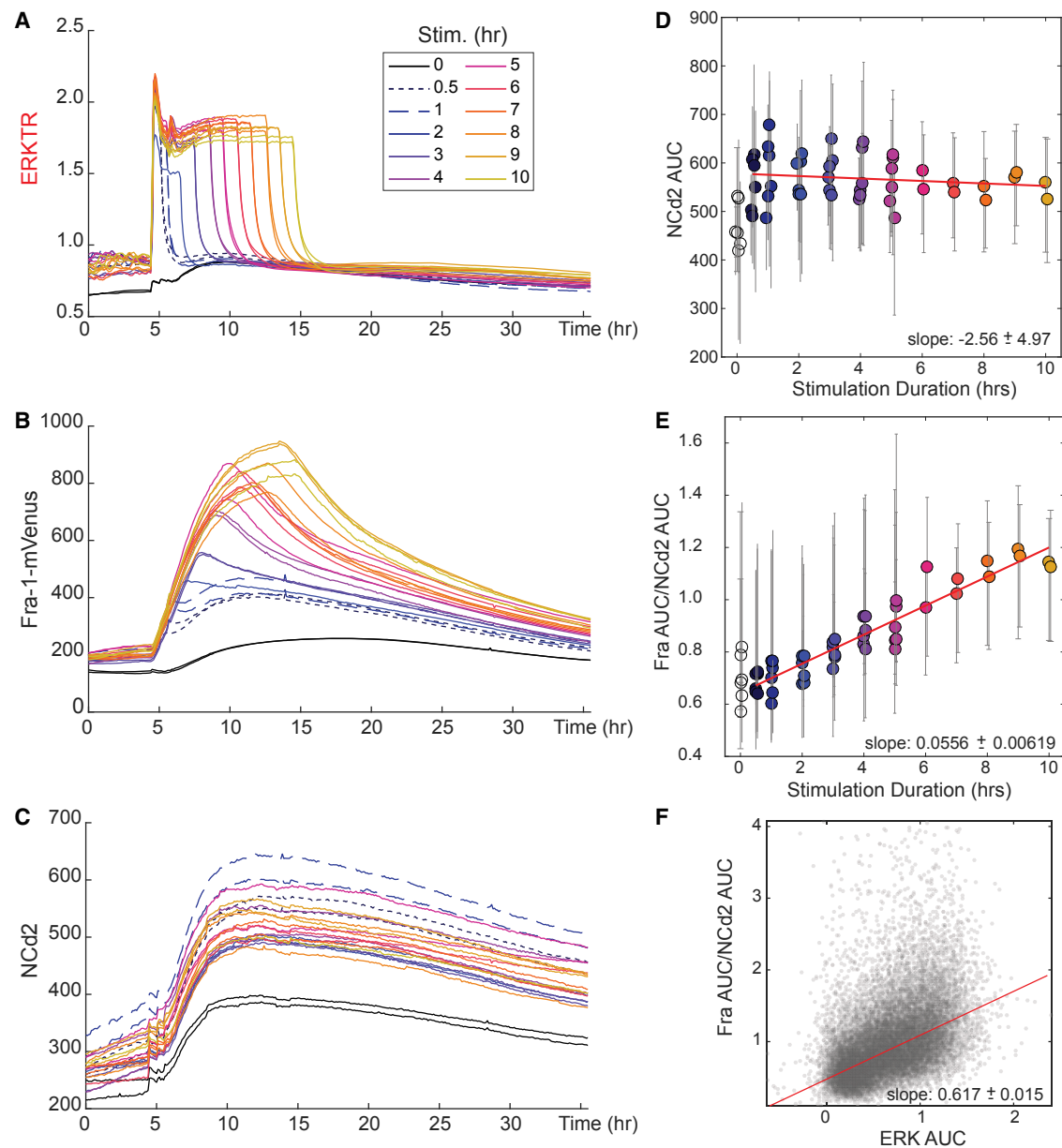


Figure 3. Fra-1 Induction as a Function of ERK Activity Duration

(A–C) Response of Fra-1 to ERK pulses of varying duration. MCF10A cells expressing Fra-1::mVenus, ERKTR, and NCd2 were cultured in the absence of growth factors for 48 hr and then stimulated by 20 ng/mL EGF. At various times following EGF treatment, ranging from 30 min to 10 hr, 100 nM PD325901 was added to acutely inhibit ERK activity. Stimulation duration 0 received PD 15 hr prior to EGF stimulation. Plots indicate the population mean values for ERKTR cytoplasmic/nuclear ratio (A), Fra-1::mVenus intensity (B), and NCd2 intensity (C); lines are color coded according to the interval between stimulation and inhibition.

(D and E) Plot of population-averaged AUC of NCd2 (D) or Fra-1::mVenus normalized to NCd2 (E) as a function of the interval between EGF addition and PD325901 addition. Error bars show 25th–75th percentile range. Red line indicates linear fit. Data were also fit to a library of non-linear models, and the linear model was chosen as the most parsimonious by Akaike's Information Criterion. Area under the curve (AUC) was used to reflect the net Fra-1 output for the single defined ERK pulse. Regressed slopes are indicated with 95% confidence intervals. See also Figure S3.

(F) Scatterplot of single-cell measurements of Fra-1::mVenus (normalized as in E) plotted against ERKTR AUC. Red line indicates linear fit.

gradually recovered after ~24 hr, correlating approximately with the time of ERK recovery (Figure 4B). At the level of cell populations, when the integrated ERK activity was compared with final level of Fra-1 expression during a 20-hr period spanning the recovery phase, we found a correlation across all drug conditions (Figures 4C and S4), in accord with the linearity

described above in the relationship between ERK activity and Fra-1 expression. The variation in ERK waveform between drugs did not appear to have a strong influence on Fra-1 recovery. At the single-cell level, we observed a high degree of variability within populations (Figure 4D). Potentially, this variability could reflect either non-ERK contributions or a failure to identify the

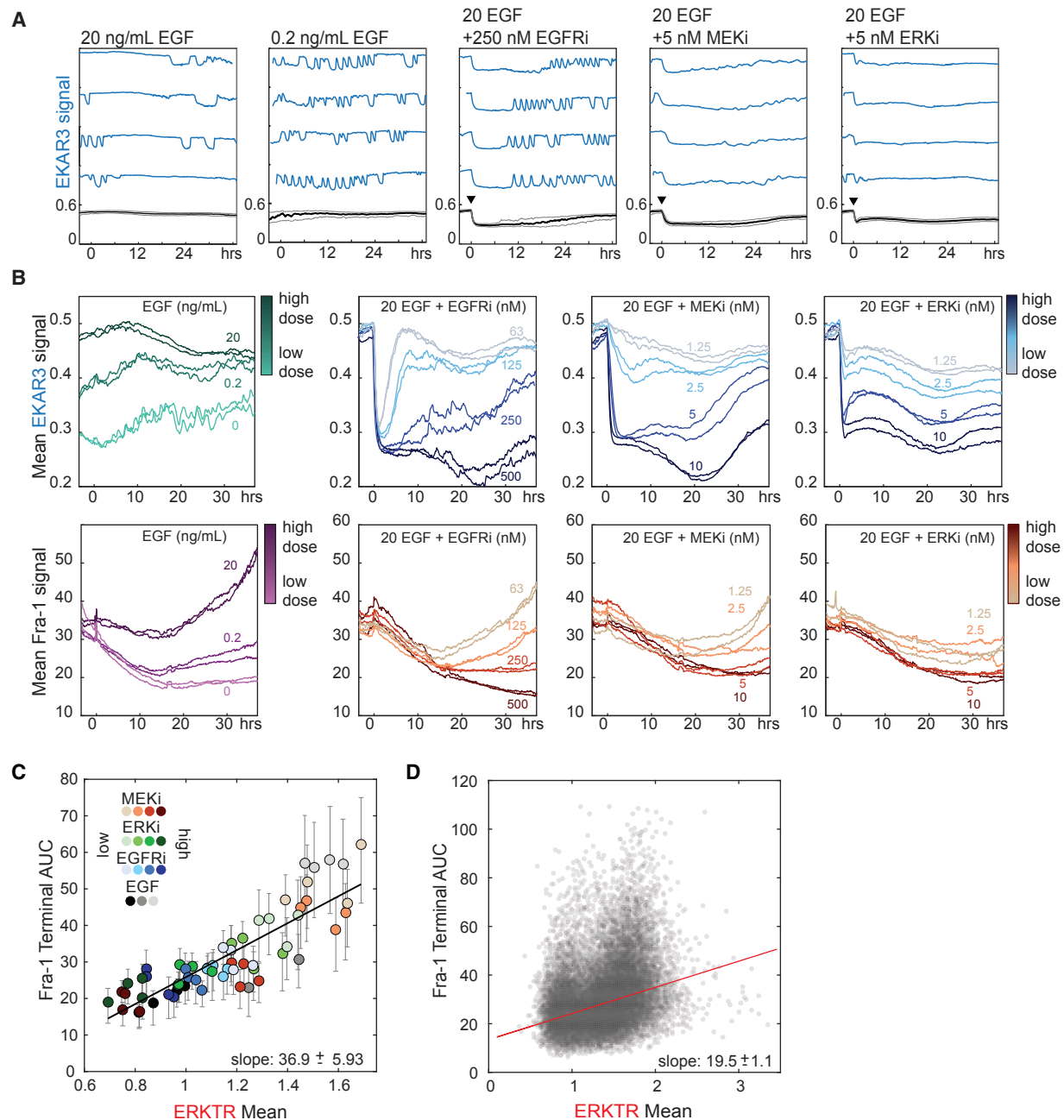


Figure 4. Fra-1 Expression in Response to Pharmacological Modulation of ERK Activity Patterns

(A) Single-cell responses of ERK activity to inhibitors. MCF10A cells expressing EKAR3 were cultured in imaging medium containing 20 or 0.2 ng/mL EGF and treated with inhibitors of EGFR (gefitinib), MEK (trametinib), or ERK (SCH727984) at time 0 (arrows). Each plot shows the mean value in black at the bottom, with lighter gray lines representing the 25th and 75th percentile. Colored lines above the mean depict four randomly selected single cells for each condition, plotted on the same scale as the mean. The higher-sensitivity EKAR3 is used here to show low ERK activity under inhibition; see Figure S4 for corresponding ERKTR data.

(B) Population responses of ERK activity and Fra-1 expression to the indicated inhibitors. Cells were cultured in the presence of EGF and treated at time 0, at concentrations indicated by line color.

(C and D) Correlation of integrated ERK activity with Fra-1 expression across pharmacological conditions. In (C), each point represents the population average and error bars for the 25th to 75th percentile range, for one drug condition, of Fra-1 intensity plotted against integrated ERKTR cytoplasmic/nuclear ratio; replicate experiments are shown individually. Measurements were made for a 20-hr window beginning 17 hr following drug treatment. Fra-1 intensity is quantified as AUC during the final hour of the 20-hr window to reflect the steady-state response to the preceding ERK activity, which is represented as AUC over the 20-hr window. In (D) each point represents a single-cell measurement of ERKTR and Fra-1, calculated over the same interval as in (C). Cells from all conditions are pooled, a total of 24,620 cells. ERKTR is used here to capture the wide dynamic range across all conditions; corresponding EKAR3 data are shown in Figure S4.

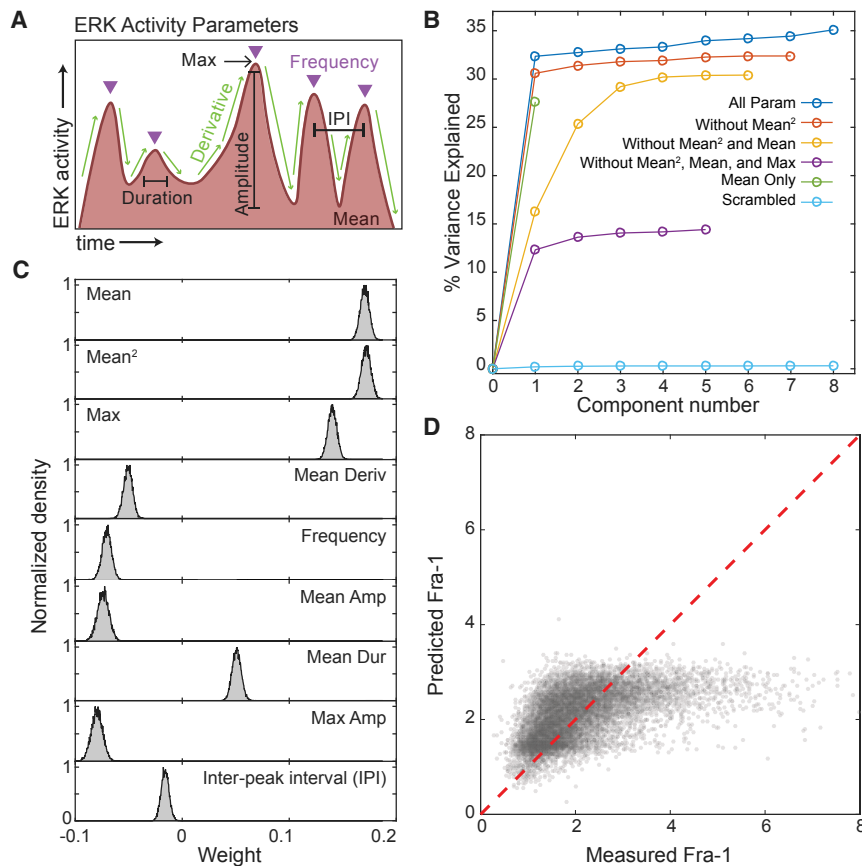


Figure 5. PLSR Modeling of the ERK-Fra-1 Relationship across All Conditions

(A) ERK activity metrics for single cells. Triangles indicate peak position; IPI, inter-pulse interval. For subsequent PLSR analysis, per-peak metrics were summarized on an individual cell basis, using the average or maximum as indicated in (C), over all measured peaks for that cell.

(B) Predictive capabilities of PLSR models for Fra-1 expression (measured by percentage of total variance explained) using different combinations of ERK activity measurements (by EKAR3). For the “scrambled” model, pairings between input and output measurements for each cell were randomly reassigned. Models generated using ERKTR data (Figure S5) showed similar trends but lower predictive capacity, possibly due to sampling bias within the dataset that included large numbers of low-ERK conditions.

(C) Contributions of ERK parameters to the PLSR model. Distributions of parameter coefficients generated by bootstrapping with replacement 10,000 times.

(D) Prediction of Fra-1 maximum value by linear modeling of ERK signal terms, plotted against measured maximum Fra-1 value. The PLSR model was constructed using the EKAR3 features in (A) over the 20-hr window, with the endpoint value of Fra-1 intensity as the output. Non-linear dependence was assessed by including mean-squared in the PLSR. Red line represents perfect prediction of the data by the model.

correct attributes of the ERK signal responsible for controlling Fra-1 expression. Therefore, to investigate the relationship of ERK to Fra-1 we applied PLSR modeling to a collated dataset containing a wide range of ERK activity patterns assembled from EGF and inhibitor concentrations over multiple independent experimental replicates, using both mVenus- and mCherry-tagged versions of Fra-1.

To create the input vector for PLSR, we reduced each single cell’s ERK activity trace to a set of eight scalar values, including mean activity, pulse frequency, and mean pulse duration (Figure 5A and Table S1). The predictive capacity of these models varied between 20% and 60% depending on the conditions included in the dataset. As a control, models in which ERK and Fra-1 signals were randomly paired predicted less than 1% of Fra variance, indicating that a common trend in both ERK activity and Fra-1 induction did not artificially elevate estimates of prediction capacity. When using the broadest set of conditions and the EKAR/Fra1:mCherry configuration (as shown in Figure 4C), the model improved only negligibly beyond the first component, which explained 32% of variance in Fra-1 (Figure 5B). We examined the PLSR weights in this component to determine the relative importance of the eight dynamic features on the response variable (Fra-1 AUC over the last hour of the modeled interval). Mean and mean-squared ERK activities were weighted most heavily, with a more moderate contribution from maximum ERK activity; similar weightings were observed across all of the datasets considered (Figures 5C and S5;

Table S2). These weightings again suggest that integrated (or mean) ERK activity is the primary determinant of Fra-1 expression within single cells. The weighting of the mean-squared term potentially indicates a non-linearity in the induction process, but models constructed without the mean-squared term were still able to capture 31% of the variance in Fra-1, while omitting both mean and mean-squared reduced predictive capacity to 16% (Figure 5B). Notably, the mean and mean-squared alone were capable of explaining 28% and 30% of variance, respectively. When model predictions of Fra-1 expression were compared with observed values on a cell-by-cell basis (Figure 5D), the variance not explained by the models was accounted for by both a spread around the central relationship, and by a subpopulation of 17% of cells for which the model underpredicted Fra-1 expression. Altogether, this comprehensive analysis of the dependence of Fra-1 expression on single-cell measurements of ERK indicates that (1) the relationship between ERK activity and Fra-1 expression is approximately linear across many conditions, (2) the specific dynamic form of ERK activity has little influence on the expression of Fra-1, and (3) other factors beyond ERK are likely to exert a strong influence on the Fra-1 expression level in a given cell.

Stabilization of Pre-existing Fra-1 Enables Linear Expression Responses

Given that cFFLs, such as the one connecting ERK and Fra-1, can in principle produce non-linear responses to ERK duration,

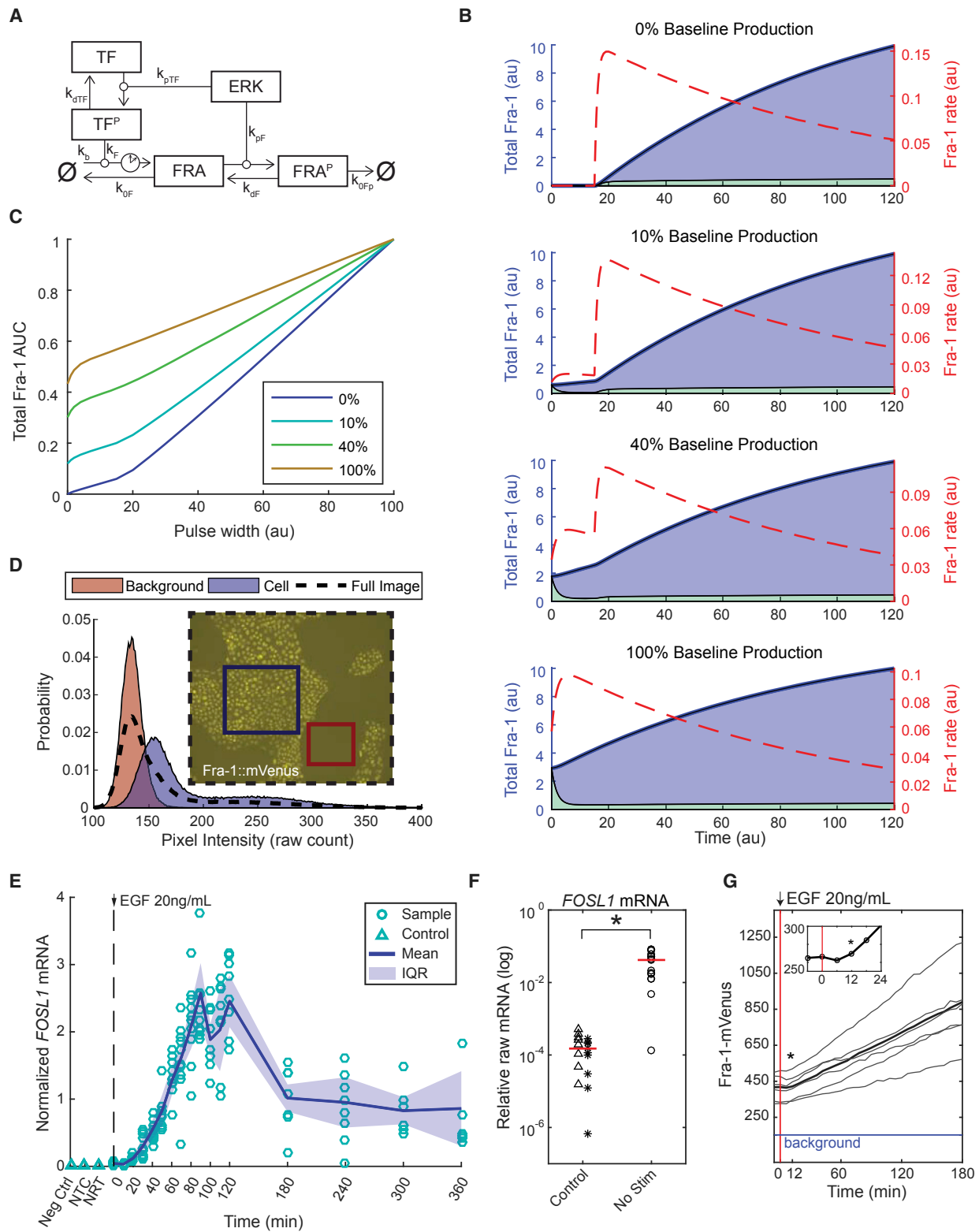


Figure 6. Model Analysis Predicts Linear Response with Baseline Fra-1

(A) Network diagram of the delay differential equation analyzed and simulated. Delay (clock symbol) is included for the stimulated production of Fra-1 by activated transcription factor (TF^P). See [STAR Methods](#) for a full description of the model.

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we sought to understand why Fra-1 accumulation is essentially linear with respect to ERK activity duration. We constructed a minimal mathematical model comprising ERK phosphorylation of a transcription factor that promotes Fra-1 mRNA production and ERK-mediated stabilization of Fra-1 protein (Figure 6A). The minimal model retains the structure of the known system and includes an explicit delay on the production of new protein, but avoids the additional complexity of cooperativity or modeling every intermediate; it is thus situated to predict conditions giving rise to persistence detection but conservatively represent their magnitude. We analyzed model conditions necessary for persistence detection by numerically simulating the time-dependent accumulation of Fra-1 over a screen of model parameters (Figure S6 and Table S3; STAR Methods).

The model indicates that persistence detection depends critically on two parameter values in the production of Fra-1: delay and baseline. Delay arises from the synthesis time of mRNA and protein, and sets the minimal input signal duration needed to allow both arms of the cFFL to be simultaneously active. Baseline refers to mRNA (and protein) production either in the absence of stimulus (ERK) or due to pre-existing stimulus. If baseline Fra-1 mRNA production is significant, ERK stimulation immediately drives accumulation of the stabilized protein (Figure 6B). Thus, the presence of baseline expression in any cFFL motif may prime the system to respond to stimuli shorter than its processing delay. The model therefore predicts that the linear response of Fra-1 could result from a short delay, a high baseline, or both. To evaluate the feasible range of experimental outcomes in light of the theoretical analysis, we simulated ERK pulse experiments, calculating the total Fra-1 AUC as a function of pulse duration (Figure 6C). The simulated AUC varies in initial slope and curvature according to baseline levels, and is strictly linear in the long term. Comparing these curves with Figures 3E, S3C, and S3E, the long-term linearity is well corroborated and the apparent short-term linearity of the data implies an intermediate mixture of baseline and induced expression.

We further tested the applicability of the model by evaluating whether experimental measurements of Fra-1 (*FOSL1*) mRNA and protein were consistent with the prediction of significant baseline expression as well as transcriptional induction, and/or relatively short delays. Fra-1 protein expression in the prolonged absence of ERK activity, evidenced by non-zero intensity in

serum-starved and inhibitor-treated cells (Figures 3B and 6D), suggests that baseline production is indeed substantial in this cell line. To evaluate transcription and processing delays, we measured *FOSL1* mRNA at the population level over a time course and observed a statistically detectable increase within 20 min of EGF stimulation ($p < 0.05$), followed by accumulation reaching a maximum at ~ 1.5 –2 hr (Figure 6E), confirming transcriptional induction. Without stimulus, *FOSL1* mRNA is significantly higher than RT controls, confirming non-zero baseline transcription (Figure 6F, $p < 0.05$). In comparison, increases in Fra-1::mVenus or Fra-1::mCherry fluorescence occurred on an equivalent or faster time scale, detectable within 12 min following EGF stimulation (Figure 6G, $p < 0.05$), consistent with Fra-1 protein already existing at the time of initial stimulus. Furthermore, the shortest spontaneous ERK activity pulses, or those achievable by timed stimulation-inhibition, are approximately 20 min long. That these are equivalent with the time required to produce *FOSL1* mRNA and protein leaves little room for any practical ERK pulses to be effectively rejected even in the absence of any baseline *FOSL1* mRNA. Together, these measurements indicate that the baseline expression and relatively short production delay of Fra-1 bias it against selectively excluding typical ERK pulses.

To evaluate the functional impact of transcriptional versus stabilization control on Fra-1, we performed an experiment to measure the stabilization response independently. We used a reporter termed FIRE (Fra-1-based integrative reporter of ERK), in which the regulated PEST domain of Fra-1 is fused to a fluorescent protein and expressed under the control of a constitutive viral enhancer (Albeck et al., 2013), thereby reproducing only the degradation effects of Fra-1 without specific transcriptional control (Figure 7A). We measured both the full feedforward system (Fra-1) and the non-feedforward, stabilization-only system (FIRE) in individual cells following stimulation with various concentrations of EGF. Strikingly, the time series traces of Fra-1::mVenus closely resembled those of FIRE (Figure 7B), with median correlation of 0.78–0.88 versus 0.27–0.55 against total RFP as a non-specific expression control (across multiple EGF doses, Figure 7C). Directly comparing Fra-1 and FIRE fold-change responses over time (Figure 7D) the distributions of the two reporters overlap extensively, and at early times the median signals are indistinguishable. After 3 hr, the median

(B) Minimal model simulations with various relative levels of baseline Fra-1 production (percentage given by $100 \times k_b/(k_b + k_r)$). Total Fra-1 (shaded regions) is plotted against left axes; lower (green) and upper (blue) regions correspond to naive and stabilized Fra-1, respectively. Fra-1 accumulation rate (dashed line) is plotted against right axes. Models were simulated with ERK activity beginning at time 0 and a 15 time-point delay for Fra-1 production; the effect of baseline production is most apparent in this window at the start of each simulation. See also Figure S6.

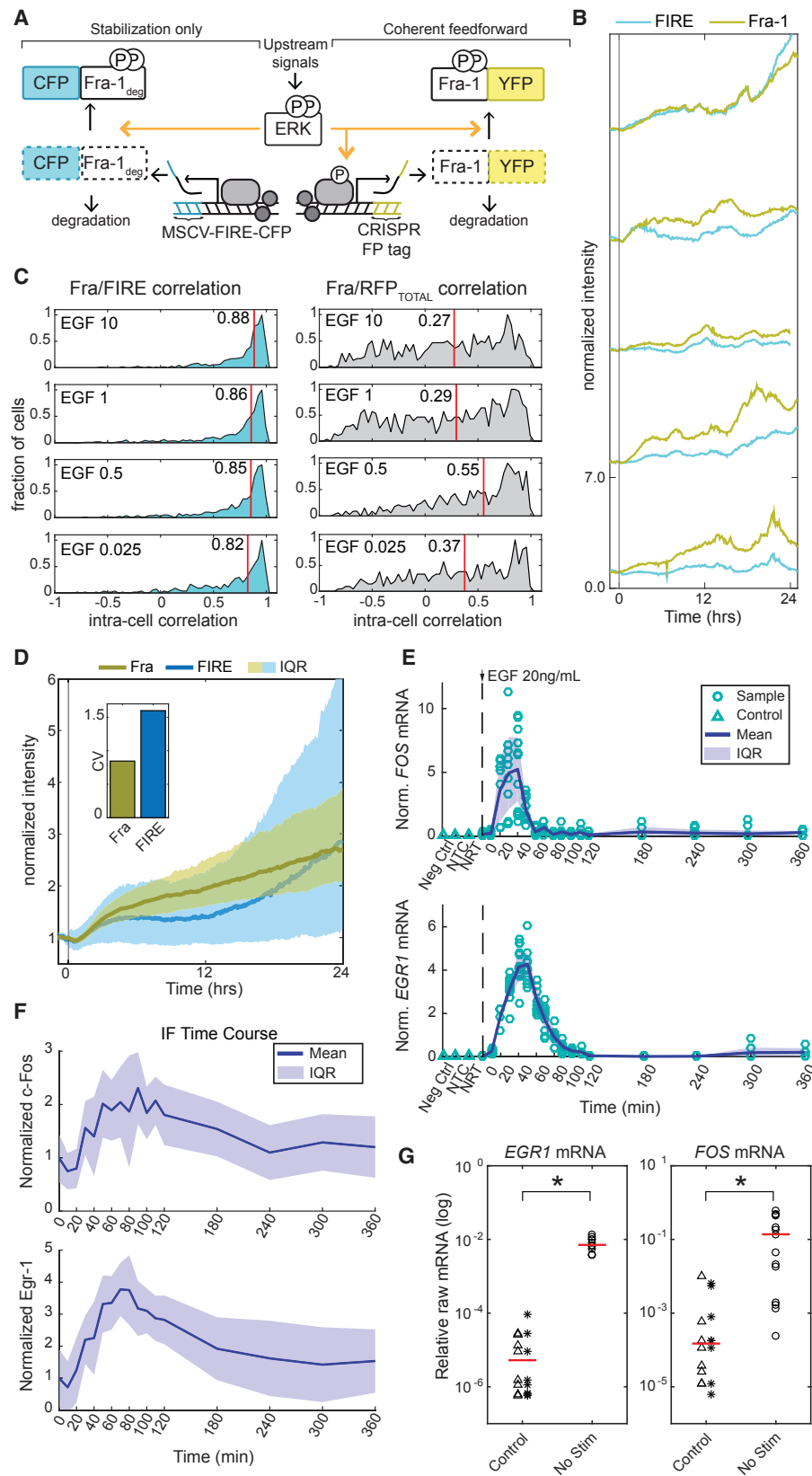
(C) Simulated experiments with varied ERK pulse width, using the same parameter values as plots in (B). The total Fra-1 AUC was calculated for square waves of ERK activity, performed with different relative baseline production levels (indicated in legend). Fra-1 AUC is plotted against the duration of ERK activity (as in Figure 3E).

(D) Sample image and pixel intensity histograms from cells expressing the Fra-1::mVenus fusion, after growth factor withdrawal for 48 hr and treatment with MEK inhibitor for another 15 hr. Histograms shown for regions indicated in the image: background only (red), cells (blue), whole frame (dashed line).

(E) Time-course measurement of Fra-1 mRNA in MCF-10A cells, by qRT-PCR. Cells were cultured without growth factors for 48 hr, stimulated with 20 ng/mL EGF, and lysed for RNA extraction at 10-min intervals up to 2 hr, followed by 1-hr intervals up to 6 hr. Data were compiled from three replicate RNA extractions, with at least two replicate qPCR measurements per sample. NTC, no target control; NRT, no reverse transcription; IQR, inter-quartile range from 25th to 75th percentile.

(F) Comparison of qRT-PCR measurements of Fra-1 mRNA in unstimulated cells (48 hr growth factor withdrawal) to NTC (stars) and NRT (triangles), shown on a log scale. These data are not normalized to the control, HINT1. Red line indicates median. * indicates significance at the $p < 0.05$ level by t test.

(G) Early Fra-1 response following EGF stimulation (red line). Data for Fra-1::mVenus in cells cultured in the absence of growth factors for 48 hr and then stimulated with 20 ng/mL EGF are replotted from Figure 3 to clarify early dynamics. Bold line indicates the population mean, and lighter lines represent six individual cells. Asterisk indicates first time point significantly greater than baseline (* $p < 0.05$). Blue line indicates the mean intensity of background (non-cell) pixels. Inset shows further magnification of the mean signal on a shorter time scale.



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Fra-1 signal continues to rise while the stabilization-only FIRE attenuates, consistent with a model in which the immediate increase following stimulation is due to stabilization of Fra-1 protein produced from pre-existing mRNA, while stimulated production of mRNA contributes to total Fra-1 levels at later times. Additionally, Fra-1 exhibits a median coefficient of variation 50% lower than that of FIRE, potentially resulting from the additional regulation acting at the endogenous promoter.

Finally, we investigated the potential for variance in mRNA production parameters governing persistence detection among ETGs. We sampled the mRNA and protein kinetics of c-Fos and Egr-1 via qRT-PCR and immunofluorescence. In comparison with Fra-1 measurements, c-Fos and Egr-1 showed an earlier peak in both mRNA and protein abundance and a return to baseline levels within 2 hr of stimulation, suggesting additional negative regulation (Figures 7E and 7F). *EGR1* mRNA appears even faster (within 10 min, versus 20 min for *FOSL1* and *FOS*), although protein levels only become detectably higher at 20 min. *EGR1* and *FOS* mRNA were also detectable even in the absence of stimulus (Figure 7G, $p < 0.05$). Thus, all three ETGs analyzed share kinetic characteristics that make them weak discriminators of characteristic ERK pulses.

DISCUSSION

Model Analysis Reconciles a Linear Fra-1 Response with Past Studies

At the center of quantitative signaling biology is the question of how the time-varying activation of an upstream regulator is converted into the time-varying outputs of downstream molecules, which ultimately alter cell fate (Kholodenko et al., 2010; Rhee et al., 2012). This process of information transfer can take many forms in principle, and remains poorly understood in many central mammalian signaling systems because of the difficulty of making adequate experimental measurements over time in the same cell. In this study, we developed a method to accurately track, in the same cell, both input and output for the ERK-ETG system, which has been heavily studied for both its relevance to human disease and its importance as a canonical model signaling system. Previous studies have highlighted the potential for this system to selectively transform the kinetics of ERK activity into different gene expression profiles. We show here that across many different conditions, the ETG Fra-1 is produced in essentially linear proportion to the integrated

magnitude of ERK activity, and that the related ETGs c-Fos and Egr-1 are similar in their response characteristics.

Under some interpretations of previous data, a linear response appears to contrast with the concept of the ERK-ETG feedforward motif as a persistence detector discriminating between short and long pulses of ERK activity. This apparent conflict can be resolved by careful consideration of previous studies, in which ERK activity was manipulated using two different growth factors (e.g., EGF and nerve growth factor) (Murphy et al., 2002; Nakakuki et al., 2010), or several concentrations of a growth factor (Murphy et al., 2004) to generate either short- or long-lived average ERK activity in the cell lines examined. Because only a small range of ERK activity profiles were compared in each case, these studies were able to establish that greater duration of ERK activity increases the amount of ETG protein produced, but could not directly test the idea that longer pulses would preferentially induce the ETG. Similarly, in later studies that compared more conditions, head-to-head comparisons in which only ERK duration varied were not performed (Saito et al., 2013). Methodological differences also play a role in the apparent inconsistency, as the average responses previously measured by immunoblot have lower time resolution and more potential for non-linear readouts (Janes, 2015). Taken together, the existing body of data is neutral on the question of if (and when) ETGs respond linearly or non-linearly to ERK activity.

Our data agree with previous studies showing that the duration of an ERK activity period can be converted into differences in the peak and in the AUC (or mean) of ETG protein abundance. However, these properties are not specific to the feedforward motif of the ERK-ETG system. Intuition suggests, and modeling confirms, that creating a selective duration-sensitive response requires a delay between the initiation of ETG transcription and stabilization of the protein product. For the ETGs we examine here this delay is on the order of 10–20 min, and it is reasonable that we observe little evidence of selective persistence detection when growth factor-stimulated ERK responses typically last >20 min. We emphasize that our analysis does not exclude the ability of ETGs (or their active forms) to conform to a non-linear persistence detector model, depending on their particular parameter values (i.e., production and degradation rates) (Uhlig et al., 2017). Even within an individual cell, response profiles for different genes may vary, or the same gene could be reconfigured by changes in chromatin state or general transcription factor availability. While

Figure 7. Comparative Analysis of ETG Responses between Configurations and Genes

(A) Schematic of FIRE/Fra-1 comparison experiment. FIRE consists of the Fra-1 degradation domain fused to mCerulean and expressed from a stably integrated constitutive viral promoter; relative to endogenous Fra-1 it lacks stimulated mRNA production.

(B) Simultaneous measurements of Fra-1 and FIRE signals in five individual cells treated with 100 ng/mL EGF at time 0.

(C) Distributions of correlations between Fra-1 and FIRE within individual cells. Pairwise Pearson's correlation coefficients were computed between Fra-1:mVenus and FIRE signals for 4,383 cells over a period of 60 hr following treatment with different EGF concentrations; mean values are shown as red lines and numbers. To control for the correlation of FIRE with general expression increases, we made the same calculations between FIRE and total (nuclear + cytosolic) ERKTR-RFP signal, which is not directly controlled by ERK activity (right panels).

(D) Median intensities of Fra-1 and FIRE in cells treated with 1 ng/mL EGF. Shaded region indicates inter-quartile range across all cells. Inset plot shows median (across all time points) coefficient of variation between cells.

(E) Measurements of c-Fos and Egr-1 mRNA by qRT-PCR. Cells were stimulated, analyzed, and annotated as in Figure 6E.

(F) Measurements of c-Fos and Egr-1 protein abundance by immunofluorescence following growth factor withdrawal for 48 hr and stimulation with 20 ng/mL EGF at 10-min intervals up to 2 hr, followed by 1-hr intervals up to 6 hr. c-Fos data are compiled from three replicates. Egr-1 data are compiled from two replicates.

(G) Comparison of qRT-PCR measurements of Egr-1 and c-Fos mRNA in unstimulated cells (48 hr growth factor withdrawal) to NTC (stars) and NRT (triangles), shown on a log scale. These data are not normalized to the control, HINT1. Red line indicates median. * indicates significance at the $p < 0.05$ level by t test.

the topological motif of a gene circuit is often interpreted as an indicator of its behavior and function, we demonstrate that in this case, topology can be misleading in predicting the output of a key gene regulatory event; this is consistent with theoretical studies that have emphasized the importance of parameter values over topology (Gutenkunst et al., 2007; Ingram et al., 2006). This importance is also evident at the level of EKAR3 and ERKTR, whose different parameters of response to phosphorylation yield distinct readouts to the same ERK activity pulse within the same cell. These data underscore that diversity in signaling responses is generated in part by differential tuning of the kinetic parameters specific to each effector (Pargett and Albeck, 2016).

Implications of Linear Fra-1 Response for Tissue and Cancer Biology

The lack of persistence detection in Fra-1 regulation raises the question of what function the cFFL motif plays in cellular regulation. Among ETGs, Fra-1 is prominent in the homeostasis of epithelial tissues, and expression of Fra-1 induces epithelial-to-mesenchymal transition and long-term cell motility (Vial et al., 2003). The capacity of the Fra-1 regulatory motif to integrate transient ERK signals may enable a cell to “remember” past ERK activity over hours or days, rather than reacting only to the immediate activity level. Such memory may increase the information transmission capacity of the pathway, preventing chance events from inducing inappropriate and costly cell-fate choices. Our comparison of the native cFFL configuration of Fra-1 regulation with stabilization-only control indicates that the cFFL enables both a rapid high-gain response and a more linear accumulation over time than stabilization alone, which are useful attributes for integrative memory. Our data also suggest that the cFFL motif reduces variance of Fra-1 protein abundance relative to stabilization alone, consistent with the concept that accurate information transmission may require multiple parallel pathways (Hansen and O’Shea, 2015). Notably, we also observe a high degree of variability in population-level *FOSL1* mRNA measurements (Figure 6E, >2 hr), which may suggest that the motif can perform dosage compensation. Together, the characteristics of Fra-1 regulation are consistent with the ability to reliably express this transcription factor in proportion to frequently fluctuating ERK inputs, which may be important for maintaining epithelial tissue integrity.

A precise kinetic understanding of ETG responses and their variance could allow more effective therapeutic use of ERK pathway inhibitors (Ryan et al., 2015). Overexpression of Fra-1 has been linked to metastasis in multiple cancer types (Chiappetta et al., 2007; Zhong et al., 2016), and in breast cancer drives a cancer stem cell-like gene expression program (Tam et al., 2013). Conversely, in liver pathology, loss of Fra-1 expression and its replacement by c-Fos result in fatty liver disease (Hasenfuss et al., 2014). Ideally, predictive models of multiple ETGs would identify pharmacological conditions that best manipulate the kinetic profile of ERK to restore healthy gene expression profiles. The small subset of cells (<20%) that we observe falling outside the linear trend with higher than expected Fra-1 levels may also be of particular importance under pathological conditions. Identifying parallel mechanisms of regulation, such as local histone modifications that alter the range of Fra-1 mRNA copies produced during an ERK activation event, may explain

the remaining variance and provide additional strategies to manipulate Fra-1 expression.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Cell Culture and Media
- METHOD DETAILS
 - Reporter Cell Line Construction
 - Live Cell Microscopy and Data Processing
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 - Normalization and Statistical Analysis
 - Dynamic Mathematical Modeling
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, three tables, and one movie and can be found with this article online at <https://doi.org/10.1016/j.cels.2017.10.019>.

AUTHOR CONTRIBUTIONS

T.E.G., M.P., M.M., A.E.D., and J.G.A. contributed to experimental data collection. T.E.G., M.P., and J.G.A. analyzed data and wrote the manuscript. M.P. performed mathematical modeling and analysis.

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REFERENCES

- Albeck, J.G., Mills, G.B., and Brugge, J.S. (2013). Frequency-modulated pulses of ERK activity transmit quantitative proliferation signals. *Mol. Cell* 49, 249–261.
- Altan-Bonnet, G., and Germain, R.N. (2005). Modeling T cell antigen discrimination based on feedback control of digital ERK responses. *PLoS Biol.* 3, e356.

- Amit, I., Citri, A., Shay, T., Lu, Y., Katz, M., Zhang, F., Tarcic, G., Siwak, D., Lahad, J., Jacob-Hirsch, J., et al. (2007). A module of negative feedback regulators defines growth factor signaling. *Nat. Genet.* 39, 503–512.
- Aoki, K., Kumagai, Y., Sakurai, A., Komatsu, N., Fujita, Y., Shionyu, C., and Matsuda, M. (2013). Stochastic ERK activation induced by noise and cell-to-cell propagation regulates cell density-dependent proliferation. *Mol. Cell* 52, 529–540.
- Bakiri, L., Macho-Maschler, S., Custic, I., Niemiec, J., Guio-Carrion, A., Hasenfuss, S.C., Eger, A., Muller, M., Beug, H., and Wagner, E.F. (2015). Fra-1/AP-1 induces EMT in mammary epithelial cells by modulating Zeb1/2 and TGFbeta expression. *Cell Death Differ.* 22, 336–350.
- Birtwistle, M.R., Rauch, J., Kiyatkin, A., Aksamitiene, E., Dobrzynski, M., Hoek, J.B., Kolch, W., Ogunnaike, B.A., and Kholodenko, B.N. (2012). Emergence of bimodal cell population responses from the interplay between analog single-cell signaling and protein expression noise. *BMC Syst. Biol.* 6, 109.
- Chiappetta, G., Ferraro, A., Botti, G., Monaco, M., Pasquinelli, R., Vuttariello, E., Arnaldi, L., Di Bonito, M., D'Aiuto, G., Pierantoni, G.M., et al. (2007). FRA-1 protein overexpression is a feature of hyperplastic and neoplastic breast disorders. *BMC Cancer* 7, 17.
- Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823.
- Das, J., Ho, M., Zikherman, J., Govern, C., Yang, M., Weiss, A., Chakraborty, A.K., and Roose, J.P. (2009). Digital signaling and hysteresis characterize ras activation in lymphoid cells. *Cell* 136, 337–351.
- Duncan, J.S., Whittle, M.C., Nakamura, K., Abell, A.N., Midland, A.A., Zawistowski, J.S., Johnson, N.L., Granger, D.A., Jordan, N.V., Darr, D.B., et al. (2012). Dynamic reprogramming of the kinome in response to targeted MEK inhibition in triple-negative breast cancer. *Cell* 149, 307–321.
- Ferrell, J.E., Jr., and Machleder, E.M. (1998). The biochemical basis of an all-or-none cell fate switch in *Xenopus* oocytes. *Science* 280, 895–898.
- Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. *Nature* 425, 737–741.
- Gille, H., Kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb, M.H., and Shaw, P.E. (1995). ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J.* 14, 951–962.
- Gutenkunst, R.N., Waterfall, J.J., Casey, F.P., Brown, K.S., Myers, C.R., and Sethna, J.P. (2007). Universally sloppy parameter sensitivities in systems biology models. *PLoS Comput. Biol.* 3, 1871–1878.
- Hansen, A.S., Hao, N., and O'Shea, E.K. (2015). High-throughput microfluidics to control and measure signaling dynamics in single yeast cells. *Nat. Protoc.* 10, 1181–1197.
- Hansen, A.S., and O'Shea, E.K. (2015). Limits on information transduction through amplitude and frequency regulation of transcription factor activity. *Elife* 4, <https://doi.org/10.7554/eLife.06559>.
- Hao, N., and O'Shea, E.K. (2011). Signal-dependent dynamics of transcription factor translocation controls gene expression. *Nat. Struct. Mol. Biol.* 19, 31–39.
- Hasenfuss, S.C., Bakiri, L., Thomsen, M.K., Williams, E.G., Auwerx, J., and Wagner, E.F. (2014). Regulation of steatohepatitis and PPARgamma signaling by distinct AP-1 dimers. *Cell Metab.* 19, 84–95.
- Ingram, P.J., Stumpf, M.P., and Stark, J. (2006). Network motifs: structure does not determine function. *BMC Genomics* 7, 108.
- Janes, K.A. (2015). An analysis of critical factors for quantitative immunoblotting. *Sci. Signal.* 8, rs2.
- Janes, K.A., Wang, C.C., Holmberg, K.J., Cabral, K., and Brugge, J.S. (2010). Identifying single-cell molecular programs by stochastic profiling. *Nat. Methods* 7, 311–317.
- Jaqaman, K., Loerke, D., Mettlen, M., Kuwata, H., Grinstein, S., Schmid, S.L., and Danuser, G. (2008). Robust single-particle tracking in live-cell time-lapse sequences. *Nat. Methods* 5, 695–702.
- Kholodenko, B.N., Hancock, J.F., and Kolch, W. (2010). Signalling ballet in space and time. *Nat. Rev. Mol. Cell Biol.* 11, 414–426.
- Kwong, L.N., Costello, J.C., Liu, H., Jiang, S., Helms, T.L., Langsdorf, A.E., Jakubosky, D., Genovese, G., Muller, F.L., Jeong, J.H., et al. (2012). Oncogenic NRAS signaling differentially regulates survival and proliferation in melanoma. *Nat. Med.* 18, 1503–1510.
- Lee, R.E., Walker, S.R., Savery, K., Frank, D.A., and Gaudet, S. (2014). Fold change of nuclear NF-kappaB determines TNF-induced transcription in single cells. *Mol. Cell* 53, 867–879.
- Legewie, S., Bluthgen, N., and Herzog, H. (2005). Quantitative analysis of ultra-sensitive responses. *FEBS J.* 272, 4071–4079.
- Little, S.C., Tikhonov, M., and Gregor, T. (2013). Precise developmental gene expression arises from globally stochastic transcriptional activity. *Cell* 154, 789–800.
- Mackeigan, J.P., Murphy, L.O., Dimitri, C.A., and Blenis, J. (2005). Graded mitogen-activated protein kinase activity precedes switch-like c-Fos induction in mammalian cells. *Mol. Cell. Biol.* 25, 4676–4682.
- Mangan, S., Zaslaver, A., and Alon, U. (2003). The coherent feedforward loop serves as a sign-sensitive delay element in transcription networks. *J. Mol. Biol.* 334, 197–204.
- Murphy, L.O., MacKeigan, J.P., and Blenis, J. (2004). A network of immediate early gene products propagates subtle differences in mitogen-activated protein kinase signal amplitude and duration. *Mol. Cell. Biol.* 24, 144–153.
- Murphy, L.O., Smith, S., Chen, R.H., Fingar, D.C., and Blenis, J. (2002). Molecular interpretation of ERK signal duration by immediate early gene products. *Nat. Cell Biol.* 4, 556–564.
- Nakakuki, T., Birtwistle, M.R., Saeki, Y., Yumoto, N., Ide, K., Nagashima, T., Brusch, L., Ogunnaike, B.A., Okada-Hatakeyama, M., and Kholodenko, B.N. (2010). Ligand-specific c-Fos expression emerges from the spatiotemporal control of ErbB network dynamics. *Cell* 141, 884–896.
- Niepel, M., Hafner, M., Pace, E.A., Chung, M., Chai, D.H., Zhou, L., Muhlich, J.L., Schoeberl, B., and Sorger, P.K. (2014). Analysis of growth factor signaling in genetically diverse breast cancer lines. *BMC Biol.* 12, 20.
- O'Shaughnessy, E.C., Palani, S., Collins, J.J., and Sarkar, C.A. (2011). Tunable signal processing in synthetic MAP kinase cascades. *Cell* 144, 119–131.
- Okazaki, K., and Sagata, N. (1995). The Mos/MAP kinase pathway stabilizes c-Fos by phosphorylation and augments its transforming activity in NIH 3T3 cells. *EMBO J.* 14, 5048–5059.
- Pargett, M., and Albeck, J.G. (2016). Mapping the spectrum of gene expression responses. *Cell Syst.* 2, 221–222.
- Pargett, M., Gillies, T.E., Teragawa, C.K., Sparta, B., and Albeck, J.G. (2017). Single-cell imaging of ERK signaling using fluorescent biosensors. *Methods Mol. Biol.* 1636, 35–59.
- Purvis, J.E., and Lahav, G. (2013). Encoding and decoding cellular information through signaling dynamics. *Cell* 152, 945–956.
- Regot, S., Hughey, J.J., Bajar, B.T., Carrasco, S., and Covert, M.W. (2014). High-sensitivity measurements of multiple kinase activities in live single cells. *Cell* 157, 1724–1734.
- Rhee, A., Cheong, R., and Levchenko, A. (2012). The application of information theory to biochemical signaling systems. *Phys. Biol.* 9, 045011.
- Ryan, M.B., Der, C.J., Wang-Gillam, A., and Cox, A.D. (2015). Targeting RAS-mutant cancers: is ERK the key? *Trends Cancer* 1, 183–198.
- Saito, T.H., Uda, S., Tsuchiya, T., Ozaki, Y., and Kuroda, S. (2013). Temporal decoding of MAP kinase and CREB phosphorylation by selective immediate early gene expression. *PLoS One* 8, e57037.
- Shah, M., Smolko, C.M., Kinicki, S., Chapman, Z.D., Brautigan, D.L., and Janes, K.A. (2017). Profiling subcellular protein phosphatase responses to coxsackievirus B3 infection of cardiomyocytes. *Mol. Cell. Proteomics* 16, S244–S262.
- Sparta, B., Pargett, M., Minguet, M., Distor, K., Bell, G., and Albeck, J.G. (2015). Receptor level mechanisms are required for epidermal growth factor (EGF)-stimulated extracellular signal-regulated kinase (ERK) activity pulses. *J. Biol. Chem.* 290, 24784–24792.
- Sturm, O.E., Orton, R., Grindlay, J., Birtwistle, M., Vyshemirsky, V., Gilbert, D., Calder, M., Pitt, A., Kholodenko, B., and Kolch, W. (2010). The mammalian

- MAPK/ERK pathway exhibits properties of a negative feedback amplifier. *Sci. Signal.* **3**, ra90.
- Tam, W.L., Lu, H., Buikhuisen, J., Soh, B.S., Lim, E., Reinhardt, F., Wu, Z.J., Krall, J.A., Bieri, B., Guo, W., et al. (2013). Protein kinase C α is a central signaling node and therapeutic target for breast cancer stem cells. *Cancer Cell* **24**, 347–364.
- Tay, S., Hughey, J.J., Lee, T.K., Lipniacki, T., Quake, S.R., and Covert, M.W. (2010). Single-cell NF- κ B dynamics reveal digital activation and analogue information processing. *Nature* **466**, 267–271.
- Treisman, R. (1985). Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences. *Cell* **42**, 889–902.
- Uhlitz, F., Sieber, A., Wyler, E., Fritsche-Guenther, R., Meisig, J., Landthaler, M., Klinger, B., and Bluthgen, N. (2017). An immediate-late gene expression module decodes ERK signal duration. *Mol. Syst. Biol.* **13**, 928.
- Vial, E., Sahai, E., and Marshall, C.J. (2003). ERK-MAPK signaling coordinately regulates activity of Rac1 and RhoA for tumor cell motility. *Cancer Cell* **4**, 67–79.
- Wang, L., Brugge, J.S., and Janes, K.A. (2011). Intersection of FOXO- and RUNX1-mediated gene expression programs in single breast epithelial cells during morphogenesis and tumor progression. *Proc. Natl. Acad. Sci. USA* **108**, E803–E812.
- Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. (1997). Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. *Mol. Cell. Biol.* **17**, 5598–5611.
- Zhong, G., Chen, X., Fang, X., Wang, D., Xie, M., and Chen, Q. (2016). Fra-1 is upregulated in lung cancer tissues and inhibits the apoptosis of lung cancer cells by the P53 signaling pathway. *Oncol. Rep.* **35**, 447–453.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Fra-1, clone C-12	Santa Cruz Biotechnology	Cat#sc28310; RRID: AB_627632
Anti-Egr-1, clone 15F7	Cell signaling	Cat#4153; RRID: AB_2097038
Anti-cFos, clone 9F6	Cell signaling	Cat#2250; RRID: AB_2247211
Chemicals, Peptides, and Recombinant Proteins		
Epidermal growth factor	Peptotech	Cat#AF-100-15
Amphiregulin	Peptotech	Cat# 100-55B
Gefitinib (ZD1839)	Selleck Biochemicals	Cat#S1025
PD0325901	Selleck Biochemicals	Cat#S1036
SCH772984	Selleck Biochemicals	Cat#S7101
Trametinib (GSK1120212)	Selleck Biochemicals	Cat#S2673
Cholera Toxin	Sigma-Aldrich	Cat#C8052
Hydrocortisone	Sigma-Aldrich	Cat#H0888
Insulin	Sigma-Aldrich	Cat#I9278
Bovine Serum Albumin	Sigma-Aldrich	Cat#A7906
Heat Inactivated Horse Serum	Life Technologies	Cat#26050
Puromycin	Life Technologies	Cat#A113803
Fugene HD	Promega	Cat#E2311
DMEM/F-12 1:1	Life Technologies	Cat#11320
Critical Commercial Assays		
RNAaqueous Phenol-free total RNA Isolation Kit	Invitrogen	Cat#AM1912
Nucleospin RNA	Machery-Nagel	Cat#740955.50
Experimental Models: Cell Lines		
Human: MCF-10A, clone 5E	Joan Brugge, Harvard Medical School (Janes et al., 2010)	RRID:CVCL_0598
Human: 5E/Fra1::mVenus/ERKTR-mCherry/NCd2	This paper	Available on request
Human: 5E/Fra1::mCherry/EKAR3	This paper	Available on request
Human: 5E/EKAR3/ERKTR-mCherry	This paper	Available on request
Human: 5E/Fra1::mVenus/FIRE-mCerulean/ERKTR-mCherry	This paper	Available on request
Recombinant DNA		
Plasmid: pPBJ-EKAR3nes-puro	(Sparta et al., 2015)	Addgene # forthcoming
Plasmid: pMSCV-ERKTR-mCherry-puro	(Sparta et al., 2015)	Addgene # forthcoming
Plasmid: pAAV-Fra1-mVenus	This paper	Addgene # forthcoming
Plasmid: pAAV-Fra1-mCherry	This paper	Addgene # forthcoming
pX330-U6-Chimeric_BB-CBh-hSpCas9	(Cong et al., 2013)	Addgene # 42230
Plasmid: pX330-FOSL1	This paper	Addgene # forthcoming
Plasmid: pMSCV-NCd2-puro	This paper	Addgene # forthcoming
Plasmid: pMSCV-FIRE-mCerulean-puro	This paper	Addgene # forthcoming
Oligonucleotides		
Fra1-forward sequencing primer: TCAGCTCATCGCAAGAGTAGC	This paper	N/A
Fra-1 reverse sequencing primer: AGCTCAAGCAGCTCAACCG	This paper	N/A
FOSL1 forward qPCR primer: TCCACCCAACCCTATCTCTCTG	This paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
FOSL1 reverse qPCR primer: TCTCAAAGCTGAGATCCGCAG	This paper	N/A
EGR1 forward qPCR primer: TCAGGAGTTGGAATGTTGTAGTTAC	This paper	N/A
EGR1 reverse qPCR primer: TGGGCAATAAAGCGCATTCAATG	This paper	N/A
FOS forward qPCR primer: GGGGCAAGGTGGAACAGTTA	This paper	N/A
FOS reverse qPCR primer: AGGTTGGCAATCTCGGTCTG	This paper	N/A
HINT1 forward qPCR primer: GCCTTGCTTTCCATGACATT	(Wang et al., 2011)	N/A
HINT1 reverse qPCR primer: GCCTTGCTTTCCATGACATT	(Wang et al., 2011)	N/A
Software and Algorithms		
NIS-Elements AR ver. 4.20	Nikon	RRID:SCR_014329
Bio-Formats ver. 5.1.1 (May 2015)	OME	RRID:SCR_000450
uTrack 2.0	(Jaqaman et al., 2008)	http://www.utsouthwestern.edu/labs/danuser/software/
MATLAB	Mathworks	SCR_001622
Other		
Glass Bottom Plates, #1.5 cover glass	In Vitro Scientific	Cat#P24-1.5H-N, P96-1.5H-N

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, John Albeck (jgalbeck@ucdavis.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture and Media

MCF-10A 5E cells (Janes et al., 2010) were used for all experiments. Cells were cultured in DMEM/F12 supplemented with 5% horse serum, 10 ng/mL insulin, 500 ng/mL hydrocortisone, 100 ng/mL Cholera Toxin, 20 ng/mL EGF, and 50 μ g/mL Penicillin/Streptomycin. Cells were maintained in 10 cm plates and passaged approximately every 4 days. For imaging, cells were cultured in DMEM/F12 lacking phenol red, folate, and riboflavin, supplemented with 0.1% BSA, hydrocortisone, and cholera toxin; EGF and inhibitors were added at the indicated concentrations.

METHOD DETAILS

Reporter Cell Line Construction

MCF10A cells with FP tags inserted at the endogenous *FOSL1* locus were generated by transfecting cells with a pX330 plasmid (Cong et al., 2013) carrying the Cas9 gene and an sgRNA guide specific for *FOSL1* together with a plasmid homology repair template carrying either mCherry or mVenus, flanked on either side by 1.4 kB of *FOSL1* genomic sequence. Positive integrants were selected by fluorescence-based cell sorting, and clonal populations were isolated. Correct integration was confirmed by sequencing of PCR products amplified from genomic DNA and by immunoblotting to confirm a 26 kDa shift in the molecular weight of Fra-1 corresponding to the FP tag. NCd2, ERKTR, and FIRE reporters were introduced using retroviral gene transfer, and the EKAR3 reporter was stably integrated using the piggyBAC transposase system (Pargett et al., 2017). For each reporter configuration shown in the paper, clonal cell lines were derived and used for analysis to eliminate heterogeneity arising from different integration sites.

Live Cell Microscopy and Data Processing

Multi-well plates with #1.5 glass bottoms were coated with collagen and seeded with reporter cell lines. Prior to imaging, growth medium was replaced with a custom imaging medium, iGM-base (DMEM/F12 lacking phenol red, riboflavin, and folic acid and supplemented with 0.1% BSA, hydrocortisone, and cholera toxin). Prepared culture plates were imaged on a Nikon Ti-E inverted microscope with a stage-top incubator to maintain the culture at 37°C and 5% CO₂ throughout the experiment. Microscopy and

image processing were performed as described previously (Pargett et al., 2017). Briefly, imaging sites within each well were selected and imaged sequentially at each acquisition time, automated via the NIS-Elements AR software. Images were captured using a 20x/0.75 NA objective and an Andor Zyla 5.5 sCMOS camera.

mRNA Isolation and RT-qPCR

MCF-10a cells were cultured in 10 cm dishes to approximately 50% confluence, and growth factors were withdrawn for 48 prior to the start of a time course. Cells were stimulated with 20 ng/ml EGF, followed by lysis for RNA isolation at 17 time points: 0 (no stimulation), 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 180, 240, 300, and 360 minutes. Lysis and isolation employed the Macherey-Nagel NucleoSpin RNA kit or the Invitrogen RNAqueous total RNA Isolation kit. RNA isolates were reverse transcribed using a poly-T primer to select for mRNA, and amplified under conditions preserving linearity (Janes et al., 2010), accompanied by No-Target and No-Reverse-Transcription controls. qPCR of the resulting cDNA was performed with Taq polymerase and the SYBR Green dye on a Bio-Rad CFX-96 Touch, using the primers listed under Key Resources. Three replicate time courses and RNA isolations were performed. For each, two replicate qPCR plates were prepared per target gene, with some replicate wells per plate (typically for early time points). All qPCR measurements were fit to a standard curve made by serially diluting purified amplicon on each plate. All qPCR measurements were normalized to HINT1, which was, on average, invariant across the time course. Control data (negative, NRT and NTC) were normalized by the mean of the HINT1 samples. Comparisons against control values were made without normalization to HINT1. Data were excluded from the analysis only for experimental error (e.g. missed wells or contamination indicated by grossly outlier values).

Immunofluorescence Microscopy

After growth and treatment as indicated on glass-bottom 96-well plates, cells were fixed for 20 min at room temperature with a freshly prepared solution of 4% paraformaldehyde in PBS and permeabilized with 1% Triton X-100. Samples were then stained with primary and secondary antibodies in PBS+0.1% Triton X-100+2% bovine serum albumin, and images were captured on a Nikon Ti-E inverted microscope with a 20x/0.75 NA objective with an Andor Zyla 5.5 sCMOS camera.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image Processing

Imaging data were processed to segment and average pixels within each identified cell's nucleus and cytoplasm, using a custom procedure written for MATLAB (Pargett et al., 2017). The procedure accessed image data from ND2 files generated by NIS Elements, using the Bio-Formats MATLAB toolbox, and tracked single cell positions over time using uTrack 2.0 (Jaqaman et al., 2008). The resulting single cell time series traces were filtered for quality (minimum length of trace, maximum number of contiguous missing or corrupt data points), and ratiometric reporter levels calculated. EKAR3 level was calculated as $1 - \frac{CFP/YFP}{R_p}$, where CFP and YFP are the pixel intensities of the cyan and yellow channels, respectively, and R_p is the ratio of total power collected in cyan over that of yellow (each computed as the spectral products of relative excitation intensity, exposure time, molar extinction coefficient, quantum yield, light source spectrum, filter transmissivities, and fluorophore absorption and emission spectra). ERKTR level was calculated as I_{Cyto}/I_{Nuc} , where I_{Cyto} and I_{Nuc} are the average fluorescent intensities in the cytoplasm and nucleus, respectively.

Linear Fitting

Linear regression analysis was preformed using a MATLAB implementation of the Theil-Sen estimator, which is insensitive to outliers and robust to heteroskedastic data.

Partial Least Squares Regression

To parameterize dynamic data, each single cell ERK time trace was processed to calculate features (see Table S1). Distinct pulses in dynamic data were identified by a customized procedure implemented in MATLAB. To be robust to noise and baseline drift, pulses were defined by where the intensity rises/falls across both a minimum peak threshold and maximum valley threshold, defined as 33% and 60% of the local signal (35 time points). Mean derivative was defined as the average absolute value point-to-point difference over the entire time window. Mean duration, mean amplitude, and max amplitude calculated from all peaks within the window. Inter-peak-interval (IPI) calculated as the distance between the centers of sequential peaks. The MATLAB implementation of the SIMPLS partial least squares regression algorithm was employed to fit paired data sets to a linear model. Data were first centered by subtracting the mean from each parameter and input parameters were scaled to unit variance.

Normalization and Statistical Analysis

For all experiments shown, a minimum of 100 cells were imaged and tracked for each condition. Single-cell data points were excluded as outliers if greater than six standard deviations from the dataset mean. For all analyses, at least two independent experimental replicates were performed. Where feasible, replicate datasets were merged by normalizing for experimental differences by minimizing the mean variance over a scaling factor attributed per experiment (not per replicate well in a multi-well plate). For visual clarity, some figures show individual replicate experiments, and we verified that similar trends were observable in every replicate.

A p value of 0.05 was considered significant for all hypothesis testing. Multiple comparison statistics were controlled for false discovery rate (at 5%) via the step-up procedure of Benjamini and Hochberg. All statistical and computational tasks were performed using MATLAB. Data dispersion and precision measures are provided in the figure legends.

Dynamic Mathematical Modeling

Mathematical modeling of ERK-to-Fra-1 regulation was designed as a system of delay differential equations (DDE). Simulation of the model and calculation of the associated metrics were performed using MATLAB, and its built-in DDE solver dde23. The purpose of this theoretical treatment was to evaluate the conditions required for a “persistence detector” in this production-stabilization context. More formally, a persistence detector requires a super-linear response to increasing input duration, meaning that the longer the input lasts, the faster the stable product is produced. We begin from the putative theoretical formulation around ERK and Fra-1. This basic model of Fra-1 regulation by ERK is analyzed to identify conditions (i.e. parameter ranges) corresponding with duration sensitivity that could give rise to a persistence detector system. From these parameter ranges, we infer qualitative conditions necessary for a persistence detector.

A basic model of the Fra-1 response to ERK activity is constructed (Figure 6A), considering ERK acting to upregulate Fra-1 via two mechanisms: activation of a transcription factor, and inhibition of Fra-1 protein degradation by phosphorylation. To facilitate analysis of the basic regulatory effects, we restrict the model to summary mass action kinetics without detailed accounting for the processes of transcription and translation. Although for some ETGs, such as c-Fos, multiple phosphorylation events appear to be involved (Murphy et al., 2002), we implicitly make the simplifying assumption that a single phosphorylation of Fra-1 protein is sufficient to confer stabilization, or that subsequent modifications are relatively rapid. We include the delay of protein production via the term τ (Equation 2) to account for latency in processes such as transcriptional initiation and mRNA splicing and transport. The resulting 3 differential equations are linear in their states, and describe the dynamics of an active transcription factor TF^P , Fra-1 protein FRA , and stabilized Fra-1 protein FRA^P (parameters are described in Table S3).

$$\frac{dTF^P}{dt} = k_{pTF} \cdot ERK \cdot (TF^T - TF^P) - k_{dTF} \cdot TF^P \quad (\text{Equation 1})$$

$$\frac{dFRA}{dt} = k_b + k_F \cdot TF^P(t - \tau) + k_{dF} \cdot FRA^P - k_{dF} \cdot FRA - k_{pF} \cdot ERK \cdot FRA \quad (\text{Equation 2})$$

$$\frac{dFRA^P}{dt} = k_{pF} \cdot ERK \cdot FRA - k_{dF} \cdot FRA^P - k_{dFP} \cdot FRA^P \quad (\text{Equation 3})$$

To evaluate super-linearity with respect to duration, we first establish a suitable measure of FRA accumulation, given that some portion of FRA may degrade rapidly (recall that the two forms of FRA may decay at differing rates, which depend on the parameter values for any particular model run). We seek a metric suitable to describe the overall FRA concentration remaining in the future (after ERK is deactivated) based only on current levels of FRA and FRA^P . This is possible if we assume a rapid deactivation of ERK and TF^P , as the subsequent decay of FRA species is completely defined by the model parameters. Without ERK and TF^P , modeled FRA and FRA^P levels decay according to Equations 4 and 5, i.e. exponentially depending only on their respective degradation and de-phosphorylation rates.

$$\frac{dFRA}{dt} = k_b + k_{dF} \cdot FRA^P - k_{dF} \cdot FRA \quad (\text{Equation 4})$$

$$\frac{dFRA^P}{dt} = -(k_{dF} + k_{dFP}) \cdot FRA^P \quad (\text{Equation 5})$$

We define and numerically evaluate this “future Fra” metric, derived from the explicit solution of the system after ERK deactivation (Equations 4 and 5). Solving first the homogenous equation for FRA^P , then the non-homogenous for FRA (using the FRA^P solution), results in the following (Equations 6 and 7). The sum of the two represents the total FRA, as a function of time after ERK deactivation, i.e. $FRA^{\text{TOTAL}}(t) = FRA(t) + FRA^P(t)$.

$$FRA = \left(FRA_0 - \frac{k_b}{k_{dF}} \right) e^{-k_{dF}t} + \frac{k_{dF} \cdot FRA_0^P}{k_{dF} - k_{dFP}} \cdot (e^{-(k_{dF} + k_{dFP})t} - e^{-k_{dF}t}) + \frac{k_b}{k_{dF}} \quad (\text{Equation 6})$$

$$FRA^P = FRA_0^P e^{-(k_{dF} + k_{dFP})t} \quad (\text{Equation 7})$$

To arrive at a scalar metric that avoids the dependence on time since ERK deactivation (while retaining the differences in degradation rate), we evaluate (Equations 6 and 7) at a non-zero value of t. We choose $t = 1$ for convenience, as any value of $t > 0$ will retain the qualitative relationships between different parameter values or starting FRA concentrations. The resulting metric, termed fF for

“future FRA”, depends on the model parameters and the concentrations of FRA and FRA^P when ERK is deactivated. The question of persistence detection (super-linear accumulation) regards the rate of *fF* accumulation, i.e. its time derivative (*dfF/dt*). A rise in this derivative (Equation 8, Figure S6A, red line) indicates super-linearity, while a constant value is perfectly linear and a falling value sub-linear.

$$\begin{aligned} \frac{dfF}{dt} = & (k_b + k_F \cdot TF^P + k_{dF} \cdot FRA^P - (k_{\emptyset F} - k_{pF} \cdot ERK) \cdot FRA) \cdot e^{-k_{\emptyset F}} \\ & + (k_{pF} \cdot ERK \cdot FRA - (k_{dF} + k_{\emptyset FP}) \cdot FRA^P) \cdot \\ & \left(e^{-(k_{dF} + k_{\emptyset FP})} + \frac{k_{dF}}{k_{\emptyset F} - k_{dF} - k_{\emptyset FP}} \cdot (e^{-(k_{dF} + k_{\emptyset FP})} - e^{-k_{\emptyset F}}) \right) \end{aligned} \quad (\text{Equation 8})$$

In a super-linear response, this value will rise to a peak significantly after the stimulation time, while an approximately linear response will start high. In both cases, peak rates cannot feasibly be sustained indefinitely and will decrease as the reactions saturate. We use the time value at which *dfF/dt* peaks as a scalar measure of this super-linear duration dependence. To evaluate a single parameter set, we simulate the system (Equations 1, 2, and 3), evaluate *dfF/dt* at each time point and identify the peak time. We numerically sample over the parameter space, evaluating the peak *dfF/dt* time, to map the requirements for duration sensitivity. All sampling is log-spaced and relative, with nominal values being 1 (10⁰), and ranging 2 orders of magnitude in each direction (10⁻² to 10²). Due to system stiffness under certain parameters with the delay term, numerical solution is intractable with the readily available dde23 algorithm in MATLAB (a Runge-Kutta order 2,3 procedure). For these parameter screens, we neglect the explicit delay term for the convenience of employing MATLAB's ode15s, a variable step solver designed for stiff systems. In this case, feasible persistence detection requires relatively slow phosphorylation of the transcription factor (*k_{ptf}* = 10^{-1.5}, *k_{dtt}* = 10^{-1.5}); this approximates a delay between TF phosphorylation and FRA production, an expected necessary condition.

Parameter regions where duration sensitivity is feasible are shown in Figure S6B (top), by plotting level sets (i.e. planes along which the value of the peak time metric is uniform). The region where *k_{oF}* is low (i.e. FRA is not rapidly degraded) corresponds to stabilization-independent super-linearity, due to the slow rise of the TF increasing the rate of FRA production; this is illustrated by producing the same plot with the phosphorylation rate for FRA (*k_{pF}*) set to zero (Figure S6B, bottom). The remaining region of super-linearity in Figure S6B (top) corresponds to the current conceptual model: FRA degradation (*k_{oF}*) is fast, while FRA^P degradation and dephosphorylation (*k_{dF}*, *k_{oFP}*) are slow. Selecting a point in this region (*k_{oF}* = 10¹, *k_{dF}* = 10⁻¹, *k_{oFP}* = 10⁻¹, red dot in Figure S6B, top), we explore the effects of varying remaining model parameters, especially as relates to the production of FRA (Figure S6C). Variation of FRA baseline and stimulated production clearly shows a tradeoff, that duration sensitivity can only exist when baseline production is small compared to stimulated production. From this model-based hypothesis, we designed experiments and analyses to evaluate FRA baseline levels and the contribution of ERK-dependent transcription, via phosphor-specific western blot time series and live cell imaging of the FIRE reporter that mimics the FRA degron on a naive promoter.

Simulations of ERK pulse experiments were performed using the DDE model. Starting from steady state conditions with no ERK, we simulated ERK pulses as square waves of various durations; simulations were continued for twice as long as the longest pulse (200 arbitrary time units). For each simulation, the total Fra-1 AUC was calculated by the trapezoidal method. We validated the AUC of Fra-1 abundance as a metric to assess persistence detection and confirmed that this metric is strictly linear in the long term with significant early curvature differences dependent on baseline and delay. From the biological perspective, the AUC is the metric most likely to reflect its net functional effect as a transcription factor, as the number of transcripts produced will scale with both the rate and duration of transcript production (Little et al., 2013), and we therefore focused our subsequent analysis on this output parameter.

DATA AND SOFTWARE AVAILABILITY

Datasets are available at <https://www.mcb.ucdavis.edu/faculty-labs/albeck/data.htm>.

Live-Cell Imaging and Analysis with Multiple Genetically Encoded Reporters

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Genetically encoded live-cell reporters measure signaling pathway activity at the cellular level with high temporal resolution, often revealing a high degree of cell-to-cell heterogeneity. By using multiple spectrally distinct reporters within the same cell, signal transmission from one node to another within a signaling pathway can be analyzed to quantify factors such as signaling efficiency and delay. With other reporter configurations, correlation between different signaling pathways can be quantified. Such analyses can be used to establish the mechanisms and consequences of cell-to-cell heterogeneity and can inform new models of the functional properties of signaling pathways. In this unit, we describe an approach for designing and executing live-cell multiplexed reporter experiments. We also describe approaches for analyzing the resulting time-course data to quantify correlations and trends between the measured parameters at the single-cell level. © 2018 by John Wiley & Sons, Inc.

Keywords: biosensor • fluorescent protein • live-cell microscopy • image informatics • reporter • signal transduction

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INTRODUCTION

Live-cell imaging has become a very useful tool for signal transduction research, and can be essential for making accurate measurements of dynamic signaling activity, particularly in heterogeneous cell populations (Locke & Elowitz, 2009; Purvis & Lahav, 2013). However, measuring any single signaling event in isolation leaves many questions unanswered, due to the highly interconnected nature of intracellular signaling networks. Each signaling event measured by a reporter may be influenced by many additional factors, including the activity of other pathways, metabolism, and the cell cycle, which may vary from cell to cell at any time. Recording multiple signaling components within the same cell allows the dynamics of related pathways to be directly correlated without the interference of cell-to-cell variance. The techniques in this unit focus on reliably measuring and determining the relationships among intracellular signaling components.

This unit describes techniques and design principles for the simultaneous dynamic measurement of multiple gene products or enzyme activities in single cells. Applied to multiple parts of a signaling pathway, this approach facilitates the identification and modeling of intracellular signaling and regulatory processes. An increasing array of signaling molecules can now be observed in live cells via fluorescence microscopy. Molecular processes including kinase activity (Komatsu et al., 2011; Regot, Hughey, Bajar, Carrasco, & Covert, 2014), transcription (Chubb, Trcek, Shenoy, & Singer, 2006), translation (Han et al., 2014), and intracellular translocation (Nelson et al., 2002) can be



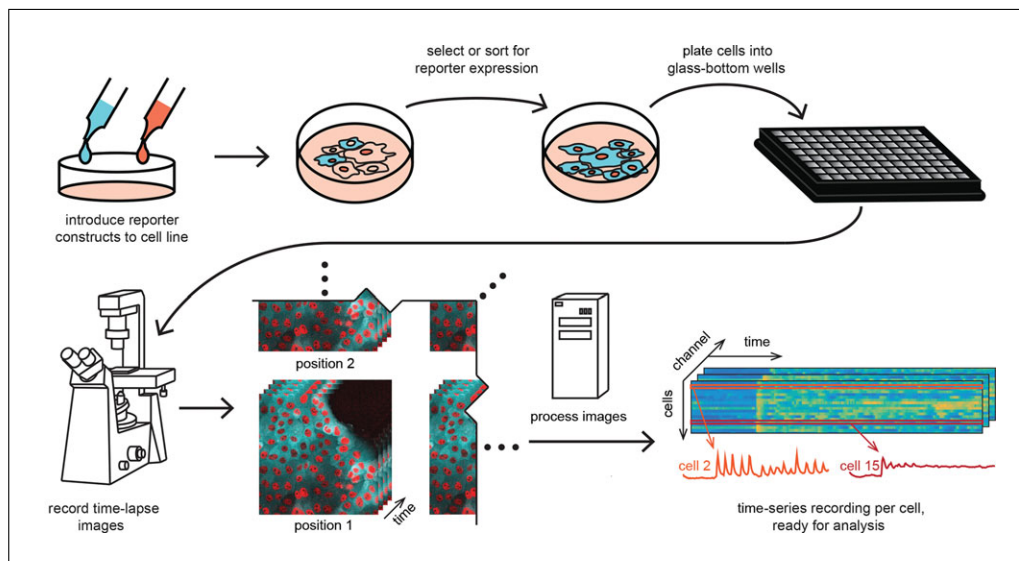


Figure 4.36.1 Schematic example figure showing the construction of a cell line with multiple reporters, live-cell imaging, and data processing.

reliably imaged using existing live-cell reporters. Moreover, CRISPR-based knock-in of fluorescent proteins to genomic loci is expected to add to the number and diversity of regulatory events that can be imaged in living cells (Stewart-Ornstein & Lahav, 2016). In each case, live-cell imaging allows the observation of signal dynamics in single cells (Albeck, Mills, & Brugge, 2013) and the evaluation of intra- vs. extra-cellular noise (Elowitz, Levine, Siggia, & Swain, 2002).

This unit is designed to provide a conceptual overview of experimental design and to deliver critical details for managing practical implementations. Four basic topics are covered: 1) Construction of suitable cell lines; 2) Long-term time-lapse imaging of live cells; 3) Image processing to automate cell segmentation and extract fluorescent reporter values; and 4) Signal analysis of relationships among the reporters.

STRATEGIC PLANNING

General

Efficiently executing a multiple reporter live-cell imaging experiment requires coordination of several key operations. Because the construction of cell lines bearing stable fluorescent reporter genes can be quite lengthy, careful planning is critical. In addition to the principles of the experiment, the targets to measure, fluorophores to use, and final analyses should be planned prior to embarking. Illustrated in Figure 4.36.1, the complete experiment consists of (1) acquiring or producing DNA constructs for reporters, (2) inserting all constructs into desired cell line(s); (3) imaging cells under desired treatments; and (4) processing image data and analyzing time-series data. Review the *Time Considerations* section for suggestions regarding time-consuming steps.

Selection of Reporters and Fluorophores

Reporter selection is a balance of choosing targets suitable for the study that are feasible to measure and minimally challenging to implement. Establishing fundamental reporter function can be very application-specific (Komatsu et al., 2011; Slattery & Hahn, 2014) and is beyond the scope of this unit. Instead, this protocol addresses the choice of fluorophores, especially fluorescent proteins, and methods to drive their expression. Five aspects of the fluorescent proteins themselves are emphasized: spectral overlap,

chromophore maturation rate, aggregation, photobleaching, and brightness. Each candidate fluorophore should be evaluated against alternatives based on these features.

PAIRED REPORTER CELL LINE CONSTRUCTION

This protocol addresses the preparation of cell lines expressing multiple fluorescent reporters, including the selection of reporters, transfection and transduction into the desired cell line, and selection and validation of the reporter cell lines. Specific DNA constructs are application-specific, and the methods of their construction are well established, so they are not addressed here.

Materials

- Cell line(s) to receive reporters
- Culture medium appropriate for selected cell line(s)
- DNA construct(s) for reporters
- Epifluorescence microscope

Additional reagents and equipment for mammalian cell culture (UNIT 1.1; Phelan & May, 2015), transfection of cultured cells (UNIT 20.7; Rose, 2003)

NOTE: Table 4.36.1 enumerates critical properties of fluorescent proteins and indicates the concerns when considering each. Comparative lists of properties for different fluorescent proteins are available from a variety of sources (e.g., <http://www.fpvis.org>), though none are exhaustive.

Select fluorophores for each reporter

1. Evaluate fluorophore options for spectral overlap. Review both the excitation and emission spectra for candidates and ensure that for each filter set, the final intensity passed from off-target fluorophores is less than 1% of on-target intensity. Minimizing spectral overlap becomes more important if reporters are not expressed at the same levels: higher concentration reporters will have proportionally greater effects on

Table 4.36.1 Criteria for Choosing FPs and Reporters

Property	Design preference	Complications
Spectra	Narrow, and distinct, for separability by filters (see Figure 4.36.2)	Spectra (excitation or emission) that are particularly broad, have multiple distant peaks, or overlap greatly (for two FPs) can lead to bleed-through noise
Quantum yield (QY)	High, for brightness	When low, FP is inefficient and dim, requiring more intense excitation
Extinction coefficient	High, for brightness	When low, FP absorbs light poorly, requiring more intense excitation
Maturation time	Low, for expression sensors	When high, protein is slow to develop its chromophore, and not suitable for reporting expression levels dynamically
Aggregation	Low, for image quality	When high, imagery is corrupted by puncta and activity may be altered by dimer/multimer formation
Photobleaching time	Long (slow bleaching), for dynamics	When short (rapid bleaching), dynamic measurements may be corrupted
pKa	Low, for selectivity	When near physiological pH (6 to 7.4), fluorescence can be sensitive to pH changes, esp. in lysosomes

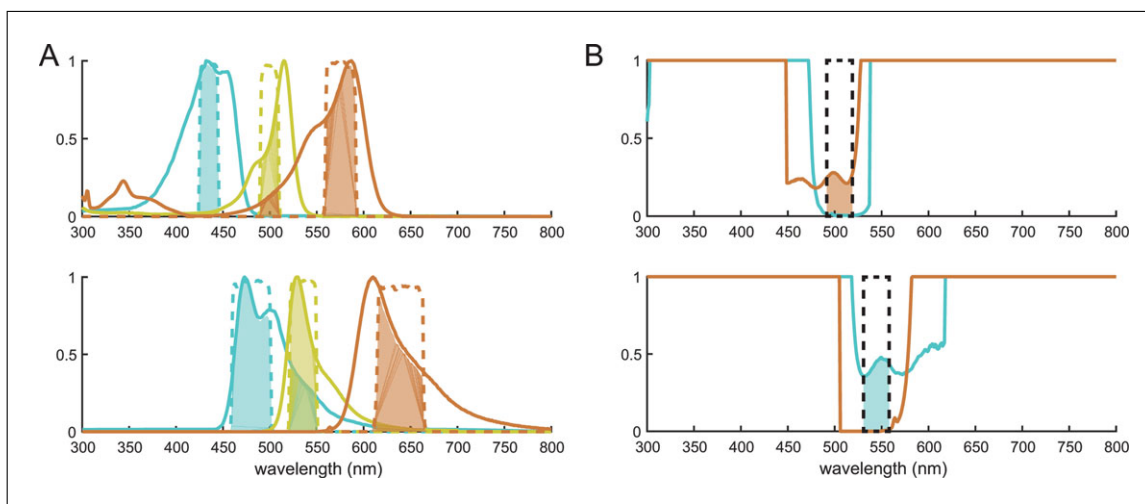


Figure 4.36.2 (A) Absorption (upper) and emission (lower) spectra of an example fluorescent protein combination suitable for multiplexed imaging, comprising a CFP (mTurquoise2, in cyan), a YFP (mVenus, in yellow), and a RFP (mCherry, in orange). Appropriate filters are indicated by dashed lines, color-coded according to the protein they are intended to measure. The relative contribution of each protein through each filter is shown as the shaded area beneath the filter band. The contributions of RFP to YFP excitation and CFP to YFP emission are allowable because neither contributes significantly to both filters. (B) Example filter selection for YFP, by plotting the interference ratio of the other fluorophores (RFP in orange, CFP in cyan). The ratio is defined as the interfering spectrum divided by the intended spectrum, bounded on 0 to 1 for convenient visualization. Suitable filter regions are shown by dashed black lines, with shaded regions indicating the interference contribution. Filters should be placed where the ratio is minimal, and contribution of any interfering fluorophore through both filters should be strictly avoided.

other filter channels. Contact filter manufacturers for details and recommendations. See Figure 4.36.2 for example spectral comparisons.

Ensure that a nuclear marker is available for image processing, either by a reporter confined to the nucleus or a dedicated nuclear marker. If a nuclear dye is to be added at the time of imaging, ensure that its spectra are evaluated alongside those of the other reporters. Typically, three colors can be imaged with little difficulty, e.g., cyan, yellow, and red fluorescent proteins (a UV dye, such as DAPI, may be used as well).

- For protein expression reporters, rule out fluorescent proteins that require long maturation times (>1 hr). Fast maturing fluorescent proteins include mNeonGreen, mVenus, mCherry, and mKate2.

Availability of maturation time measurements is not consistent. Refer to www.fpvis.org and primary literature regarding the development of particular fluorescent proteins.

- Evaluate the tendency of remaining fluorescent protein candidates to aggregate, a common issue for fluorescent proteins. While tolerable in some cases, aggregation is generally detrimental, leading to punctate imagery and an increased risk of perturbing the function of fusion proteins.

Direct data on aggregation are often difficult to find, and decisions rely on more qualitative commentary. It is often necessary to evaluate the trade-off between maturation rate, aggregation, and brightness (see step 4). Selections are generally qualitative based on which characteristics are most important for a particular application.

- Consider the quantum yield, extinction coefficient, and relative photo-bleaching time of remaining fluorescent protein candidates, and determine an acceptable compromise between brightness (estimated as quantum yield times extinction coefficient) and bleaching.

Compiled properties may be found at www.fpvis.org, and primary literature typically includes quantification of these properties.

Table 4.36.2 Comparison of Reporter Gene Delivery and Expression Strategies

Technique	Description	Additional requirements	Pros/cons
Transfection	Insertion of plasmid DNA only; genomic integration occurs at a low frequency	None	↓ Transient (X-Y days)
Transposon	Use a co-transfected transposase to insert the reporter into genome (Cadinanos & Bradley, 2007; Geurts et al., 2003)	Transposase plasmid	↑ Stable integration with high efficiency in transfected cells ↓ Limited by transfection frequency of target cell
Retrovirus	Use retroviral particles to insert the reporter into the genome (Cepko & Pear, 2001)	Packaging cell line Viral packaging plasmids Filtration devices Polybrene	↑ Stable integration ↑ High efficiency in a broad range of target cells ↓ Virus handling
CRISPR	Introduce a fluorescent protein under the control of a genomic promoter of interest (Dickinson, Ward, Reiner, & Goldstein, 2013)	Cas9 Homology plasmid	↑ Stable integration ↑ Targeted insertion ↓ Low success rate

Select reporter gene delivery and co-expression strategy

5. Compare co-expression options to determine a suitable compromise between the expected expression conditions and the complexity of the reporter insertion process. Multiple reporters may be included on one plasmid using an internal ribosome entry site (IRES), or a “self-cleaving” 2A peptide. Multiple plasmids or viruses may also be used to introduce reporters.

It is useful to consult experimental comparisons of bicistronic expression systems (Goedhart et al., 2011) and promoter expression output (Qin et al., 2010) when choosing a co-expression strategy. Due to possible transcriptional interference, multiple promoters on any one plasmid is not recommended.

6. Determine which reporter gene delivery technique to use. See Table 4.36.2 for a comparison of insertion techniques, including references for appropriate protocols.

Transient expression can be achieved by simple transfection of plasmid DNA (e.g., using polyethylenimine, FuGene, Lipofectimine, electroporation, etc.). However, expression levels will be variable over time and typically fade 5 to 8 days after transfection, as cell division dilutes the plasmid DNA. The useful length of the experiment is thus limited and frequently yields noisy data that are difficult to interpret. Transient transfection is therefore not recommended.

Insert reporter constructs into target cells

7. Prepare purified plasmid DNA for each reporter, and for each associated genomic insertion method as needed. Modify any reporters via molecular cloning techniques of choice. Similarly, prepare any constructs needed for transposon expression, genomic targeting, or retroviral packaging.

See step 9 for commentary on selection antibiotic resistance genes that may be included.

DNA for transfection into a cell line should be highly purified, for optimal performance. While DNA from a standard alkaline lysis “mini-prep” can be used, it is advantageous to purify by alcohol precipitation or, preferably, by paramagnetic beads.

8. Introduce reporters to the target cells by transfection, transduction, etc., depending on the technique chosen in steps 5 and 6.
9. Select or sort cells for adequate stable expression of reporter constructs. Selection can be carried out by inclusion of an appropriate antibiotic resistance gene in the same construct as the reporter. To select for multiple reporters, each construct must bear a different resistance gene, such as puromycin, neomycin, blasticidin, or hygromycin. Alternatively, cells can be sorted by flow cytometry, avoiding the need for selection antibiotics. As multiple antibiotic-resistance genes can become cumbersome, sorting is recommended for more than 2 insertions. If the insertion efficiency of each reporter is high enough, it is feasible to transfect or transduce in tandem and sort (or select) for multiple insertions directly rather than sorting after each.

Validate reporter insertion and function

10. Evaluate the fluorescent reporters initially by viewing cells on an epifluorescence microscope. In particular, look for side-by-side pairs of positive cells suggestive of a successful cell division.

If expression is weak and the insertion efficiency low, it may be quite difficult to visually identify the presence of positive cells even when there are enough to successfully sort. Analysis by flow cytometry provides the best assessment of brightness and frequency.

11. After selection or sorting, evaluate reporter function via a preliminary experiment (i.e., Basic Protocol 2). Identify two conditions that will induce minimal and maximal reporter response. Plate cells for a live-cell experiment (Basic Protocol 2 and the Support Protocol), and apply both conditions in the same experiment, preferably in the same well via spike-in of a drug or stimulant. Evaluate the resulting movie for appropriate responses.

For example, using an ERK reporter in a cell line responsive to EGF stimulation, withdraw growth factors for >2 hr before imaging. Begin imaging and treat cells with saturating levels of EGF. Image for enough time (1 to 2 hr) to observe the response. Then treat cells with saturating levels of ERK, MEK, or EGFR inhibitors and continue imaging (1 to 2 hr) to observe the inhibitory response.

12. If any insertions are targeted to specific sites in the genome, validate the insertion site by sequencing the local region of the genome or the appropriate transcript.
13. Store frozen aliquots of cells lines with successful reporter integration for future use.

It is often useful to freeze cells after each successful insertion to retain stocks that can be used for future combinations of reporters.

If a genetically uniform clonal line is desired, it can be produced at this point (e.g., by limiting dilution).

BASIC PROTOCOL 2

Multi-Reporter Live Cell Imaging

4.36.6

TIME-LAPSE IMAGING OF LIVING CELLS

This protocol describes the culture and imaging of cells for a long-term live-cell imaging experiment. The process with multiple reporters is nearly identical to that with a single reporter. See (Pargett, Gillies, Teragawa, Sparta, & Albeck, 2017) for a specific implementation focused on a single reporter in MCF-10A epithelial cells. The goal is to keep the cells viable in an environment accessible to the microscope with high throughput

and the ability to add treatments. To achieve this, cell monolayers are produced on a glass-bottom multi-well plate by coating with collagen, laminin, or another appropriate substrate. To provide adequate media for longer experiments (those longer than 24 hr), use larger wells (e.g., a 24-well plate) and spot only a small portion with the substrate. To facilitate robust image processing, it is advantageous to plan for one well to be left without cells; background images will be taken from this well.

Materials

Substrate coating solution, e.g., collagen or laminin (see recipes)

Imaging medium without phenol red, riboflavin, and folic acid, to reduce background fluorescence (e.g., Fluorobrite from Invitrogen, or custom media formulations that are available from a variety of vendors)

Glass-bottom culture plates for imaging: 24-well (Cellvis, cat. no. P24-1.5H-N); 96-well (Cellvis, cat. no. P96-1.5H-N); square well 96-well (Brooks Life Science Systems, cat. no. MGB096-1-2-LG-L)

Wide-field epifluorescence microscope equipped with a digital camera, automated stage and capture controls, and an environmental control chamber for samples (e.g., Nikon Eclipse Ti with NIS Elements control software, Andor Zyla 5.5 camera, Lumencor SOLA light source, and OKO Labs stage-top incubator)

Prepare imaging culture plate

1. Coat each well of the glass-bottom plate with the substrate solution, using enough solution to cover the desired region. For short-term experiments, simply coat the entire well. For long-term experiments (>24 hr), it is preferable to increase the ratio of media volume to cells by using a square well 96-well plate or a 24-well plate and applying only a 3- μ l “spot” of the substrate solution in the center of each well. This strategy creates a region of cells at a density suitable for imaging, while reducing the total number of cells and the depletion of medium factors need for growth. Cover and incubate at least 30 min.

The substrate is intended to promote cell adhesion, which is often poor on bare glass, and may also provide substrate-specific interactions that modify cell signaling and phenotype. Choose substrates as desired for the particular cell type and experiment.

When using a substrate spot to limit cell adhesion area, sparse adhesion outside of the spot is of no concern. However, if the chosen cell type grows robustly on bare glass, the area outside the substrate may be treated to repel them (e.g., with bovine serum albumin), or non-adhered cells may be carefully washed out after step 6. In larger wells (e.g., 24-well), a larger spot may be used, but 3 μ l typically provides more than enough area for multiple frames viewed at 20 $\times$ magnification.

2. Aspirate the substrate solution using a Pasteur pipette or vacuum aspirator. Wash gently with sterile PBS, and aspirate.

Avoid touching the plate with pipet tips, as it will mar the coated substrate.

3. Obtain a suspension of the desired cells by treating with trypsin and passaging as usual. Determine the cell concentration using a hemocytometer or automated cell counter. Cells will settle; remix suspension prior to use.
4. Add the desired number of cells to each well. Determining a suitable cell count may require experimentation. For a medium density of MCF-10A cells, 15,000 cells per well is sufficient for a 96-well plate. If only a “spot” was coated in step 1, add 5,000 cells in 10 μ l media pipetted directly onto the substrate spot to yield near confluence. It is recommended to leave a well empty of cells to provide a convenient background measurement during imaging.

When seeding cells onto a substrate spot, the goal is to cover the entire spot without allowing the cell suspension to contact the wall, as it will wick away from the center.

5. Add PBS or sterile water to spaces between wells to provide humidity and prevent the cell suspension from drying. Cover the plate and incubate for 2 to 2.5 hr.
6. Observe with an inverted microscope to determine if cells have adhered. Elongated cells and small processes indicate adhesion and that more media may be added. If cells still appear spherical, incubate for an additional hour.
7. When cells have adhered, gently add media (e.g., 100 μ l per well for 96-well plates, or 500 μ l per well for 24-well plates). Pipet slowly and avoid contacting the adhered cells to minimize disturbance. Incubate overnight to allow cells to fully attach and acclimate.

If using a substrate spot and the chosen cell type grows well enough on bare glass to compete for nutrients in the media, the cells growing on glass may be washed off before adding media. Some experimentation may be required to determine the best time to wash cells from bare glass without washing cells from the substrate.

8. Using an inverted microscope, verify the attachment and appearance of cells in each well. When cells appear fully attached and healthy, experimental treatments may be started. Switch to an imaging medium, if available, well before imaging (4 hr or more) to avoid observing transient perturbations caused by changing the medium.

For optimal fluorescent imaging, it is recommended to use imaging media that do not autofluoresce in the spectra of interest. Phenol red, riboflavin, folic acid, and serum are often the primary contributors to background fluorescence. Media without these components can significantly lower background intensity and improve the ability to resolve faint expression and to quantify spectral shifts in reporters (e.g., Förster Resonance Energy Transfer, or FRET).

Prepare microscope and imaging procedure

9. Power on the microscope and environmental controller. Set the environment temperature and gas mixture, and allow time for the system to stabilize (time required will vary depending on the hardware).
10. When the environmental controller is stable at the set point, securely mount the imaging plate to the microscope stage. Any flexibility in the mounting often results in frame shifts during imaging, especially when any treatments are added.
11. Start the microscope control software, focus on cells, and set desired acquisition parameters in the software (i.e., filters to use, plate positions to image, acquisition interval, total number of time points, etc.). See the Support Protocol for instructions and recommendations specific to NIS Elements control software.

All mechanical movements require time, so it is beneficial to minimize movement distances by choosing plate positions in an order that always moves to an adjacent well and minimizes the total travel. Similarly, selecting filters in the order they are physically positioned limits the movement from one to the next.

When choosing excitation intensity and exposure time, there are several factors to consider: 1) maximum image intensities should be well below the saturation point of the camera; 2) the intensities of different channels should be kept similar for later image processing steps; 3) cellular phototoxicity can often be reduced by using lower intensity excitation for longer exposure times.

Perform imaging experiment

12. When all imaging parameters are set, begin imaging. It is recommended to review a trial run of all filters at all plate positions to verify that the views are suitable.

13. If timed treatments are required, pause imaging after completion of all plate positions, prior to the start of the next time point. Apply treatments (e.g., drug addition, washing, or replacement of media) as quickly as possible while being careful to avoid disturbing or contaminating the plate.

Contacting the plate directly often results in minor image frame shifts even when it is firmly secured. Shifts can be troublesome for image processing, but software implementations of image registration can correct minor cases.

A spike solution containing a 20-fold concentration of drug in imaging medium allows for easy pipetting and minimal (5%) change in the culture volume. Direct addition of solutions with different viscosity than the imaging medium (such as DMSO or glycerol) should be avoided due to the potential for poor mixing. The disturbance of the imaging medium upon treatment can also stimulate responses in signaling pathways due to redistribution of localized signaling molecules or changes in fluorescence readings due to local photobleaching of imaging medium. Vehicle-only treatments are therefore important controls.

14. After all imaging is complete, copy the image file(s) to a suitable location for processing, and retain a backup copy in a separate location.

PROCESSING FOR MULTI-CHANNEL TIME-SERIES IMAGES

This protocol describes the procedure and algorithms for automated processing of time-lapse images to extract single cell data. As computation procedures often vary widely between implementations, we describe the concepts and provide notes regarding specific software implementations using MATLAB. Image processing procedures are complex with multiple layers of subroutines, so this protocol should be reviewed carefully to understand the full process prior to developing or implementing software. A detailed implementation for MATLAB is available in Pargett et al. (2017). Alternative software solutions that perform the same functions are equally appropriate. For example, CellProfiler provides a publicly available solution with many of the features described here in a graphical user interface (Carpenter et al., 2006).

Materials

Computer with at least 4 GB RAM
Scripting software (e.g., MATLAB)
(Optional) Bio-Formats software (<https://www.openmicroscopy.org/site/products/bio-formats>)
Particle tracking software, e.g., uTrack (Jaqaman et al., 2008)

Preparation of an image processing pipeline

1. Install all necessary software per distributor instructions (e.g., MATLAB, Python, etc.). The Bio-Formats library is useful for reading images from proprietary microscopy files. Particle tracking software (such as uTrack) is used to track cells over time.
2. Make images available to the processing computer by mounting a network drive or copying the image data file(s) locally.

As accessing network storage is often relatively slow, it is preferable to copy the data to the processing computer ahead of time.

3. Set up access to microscopy files, or extract data to image files (e.g., TIFF). If using Bio-Formats, review the documentation (<https://www.openmicroscopy.org/site/support/bio-formats5.4/>) for methods to access image data from different file types using the chosen scripting software. Microscopy data or image files will be read one frame at a time, each followed by the processing below.

BASIC PROTOCOL 3

Microscopy

4.36.9

It is convenient to load all color channels for each frame. If these are exported to different TIFF files, all channels will need to be loaded at once (e.g., into a Xpixels-by-Ypixels-by-Channels matrix).

Correct images for known biases and background intensity

4. (Optional) Correct non-uniform intensity via a recorded image of a uniform field. Scale each pixel intensity by dividing by the relative “intensity” it receives from a uniform field, with the highest intensity pixel valued at 1. With a recorded image of a uniform field, simply divide by the maximum value to give the pixel scalings. It is prudent to filter (i.e., smooth) noise or remove outlier pixels first.
5. Identify background intensity in each color channel and subtract from images. To get intensity values, define the well and region to sample for background, load the relevant image(s) if necessary, and take the mean value of the selected pixels for each color channel.

Ideally, a background value should be determined for each time point. Retaining and imaging a control well with medium and treatment compounds, but no cells, provides a convenient reference for background. In this case, it is more efficient to evaluate and store background values prior to processing other frames. In the absence of a designated control well, a cell-free region from the current or other wells may be used.

Segment images to estimate nuclear regions

6. Working with the channel containing the nuclear marker, reduce noise for segmentation by filtering (i.e., smoothing). A Gaussian filter sized to cover approximate 1/10th of a cell nucleus is typically sufficient.
7. (Optional) If the only reliable marker in the cells is a nuclear-excluded cytoplasmic marker, use a gradient magnitude filter to highlight “edges”, i.e., where the image intensity changes quickly, targeting the border between the bright cytoplasm and dark nucleus. Invert the resulting image intensities by subtracting it from its maximum value (this makes the cytoplasm dark and both the nuclei and the background bright, which aligns with the following procedures).

Gradient magnitude filtering may be carried out by applying a Sobel filter in both the X and Y directions and taking the L^2 norm of the two filtered images (square root of sum of squares).

8. Segment nuclei by selecting regions that exceed a threshold value and filtering for shape and size. To be robust to varied expression levels, consider multiple thresholds spanning the range of intensities in the image. Using 20 thresholds linearly spaced between the 5th and 95th percentile intensity values has been successful and robust over a variety of imaging conditions and cell types.

Binary morphological operations and analyses are commonly included in packages focused on image analysis. For example, MATLAB includes the function `regionprops` that calculates metrics for each region of a binary image (e.g., area, perimeter, etc.).

9. Remove “salt and pepper” noise in the binary image by morphological dilation and erosion. Both dark and light spots can be removed by first dilating by 1 pixel, eroding by 2 pixels, and re-dilating by 1 pixel. Dilation and erosion sizes may be scaled up to suit the application, e.g., when desired regions (nuclei) are larger.
10. Identify each distinct binary feature and select only those matching the feasible size and shape of a cell nucleus. For size, evaluate the total area, and for shape, use the squared ratio of the ideal perimeter of a circle (with the measured area) to the actual perimeter. Store passing features by setting corresponding pixels in a logical mask to TRUE.

A feature found again for the next threshold will be the same size or larger, and will simply overwrite the logical mask.

Create representative masks over the nucleus and cytoplasm of each segmented cell

11. Create nuclear masks. The logical mask from segmentation may be used directly for nuclear masks. If appropriate, regions may be eroded to confine masks further within nuclei to ensure robust sampling. Such approaches may be adjusted based on performance for a particular cell type.
12. Create cytoplasmic masks by dilating the nuclear mask to extend over a portion of the (presumed) cytoplasm region. From that expanded mask, remove a smaller dilation of the nuclear mask, making a hole over the nuclear region. The size of dilations may be scaled based on the expected nucleus size.
13. (Optional) Remove pixels in the cytoplasmic mask that could be associated with more than one nucleus. If using label matrices as masks (each nucleus or cytoplasm is represented by regions of a particular integer value), perform cytoplasm mask construction again with inverted (negative) label values. In grayscale morphological operations, this will give dilations that overlap in the opposite manner. Invert the resulting mask, then set to zero (i.e., background) all pixels with values that differ between the two masks.
14. Extract channel data by taking the average over each mask for each color channel. Optionally, to guard against outliers, exclude a maximum and minimum fraction of the intensity values within each mask. Robust performance can be achieved using only the values between the 20th and 80th percentiles, provided masks are large enough (hundreds of pixels). Store values in a matrix alongside X- and Y-coordinate values for the centroids of each nucleus, one row for each cell.

Track cells over time

After all images have been processed via steps 4 to 14, the cells may be tracked across frames using the X and Y coordinate data stored in step 14.

15. Track cells over time using the centroid coordinates and a particle tracking algorithm (or similar software). uTrack is a suitable software for the MATLAB environment (see Pargett et al., 2017 for an example).
16. Align track coordinates with segmentation centroid coordinates. The values extracted in step 14 need to be rearranged (within each time point) to provide coherent cell tracks. Find the nearest centroid coordinate to each tracked coordinate, and rearrange the order of cells (e.g., the row entries in a cells-by-time-by-channel data matrix) for each time point to create a dataset where each track is a single row.

PAIRED SIGNAL ANALYSIS

This protocol provides a framework and describes a variety of analyses that may be valuable for paired time-series data. Analyses comprise quality control, correlation metrics, and modeling (see Figure 4.36.3 for examples of selected analyses). These procedures are independent and may be included as desired, though initial quality control is highly recommended. All detailed computational procedures are presented for use with MATLAB, but any such scripting software is suitable with some variation in the availability and features of packages. See Anticipated Results for discussion of the outputs from these analyses.

Additional Materials (also see Basic Protocol 3)

Tracked image data (from Basic Protocol 3)

BASIC PROTOCOL 4

Microscopy

4.36.11

Quality control

Preliminary processing to ensure the quality of tracks is critical for the accuracy of all further analyses. Quality control corrects for the errors that arise in automated image processing, as well as the occasional outlier behavior from the biological system.

1. Correct for gaps in tracking. Tracking software will typically allow for some gaps in the data (e.g., linking points that appear in frames 3 and 5, but not 4), but many signal analyses require the track to be complete. Choose a maximum gap size that is acceptable, and fill any gaps up to that size by interpolating between the data on either side. If multiple track segments remain that are separated by oversized gaps, keep the longest of these tracks.

It is recommended to choose a maximum gap size according to the expected rate of change for the signals at hand, relative to the imaging interval. If the signal changes rapidly, and especially if it may be pulsatile or oscillatory, a shorter gap size will be necessary to ensure that relevant activity is not missed. Gaps are expected even with effective image processing, as cells may transiently change shape such that they no longer fit the filters, and passing debris in the media may occlude the view of some cells for a short time.

2. Filter tracks for desired length by completely removing any tracks that are shorter than a desired threshold.

Choose a minimum track length that is suitable for the desired analyses (e.g., based on the duration of the expected activity). When a timed stimulation or drug treatment has been applied early in the experiment, it may be convenient to remove tracks that do not start at the beginning of imaging (e.g., the first time point).

3. Calculate any derived signals, such as normalized values of translocation reporters (ratio of cytoplasmic to nuclear signal) or bi-color reporters, like FRET, where the values of two color channels are divided. This calculation may be performed at any point up to here, but it can be important to complete before the following filtering step.
4. Filter data for consistency by removing tracks that contain obvious outliers. Examples of outlier behavior include values outside of a reasonable range and changes that occur too quickly (i.e., sudden shifts in value, evaluated as derivative magnitudes beyond a bound). Bounds need to be determined with care based on the system being measured and the imaging interval. To avoid biasing the data, it is not recommended at this stage to reject outliers based solely on a statistical basis (e.g., lying far from a normal distribution).

The primary aim on this quality-filtering step is to limit errors from image processing (e.g., poor segmentation of the nucleus vs. cytoplasm or background, or misaligned tracking due excess motion of nearby cells). Reasonable bounds should be chosen based on the combination of the biochemical process and underlying signal being recorded, and should err on the side of keeping noisy data. For example, both FRET and translocation reporters are automatically normalized with a distinct range of signal values for any given reporter; if typical ratios fall in the range of 0.5 (when inhibited) and 2 (under saturating stimulation), tracks with many values over 4 could safely be excluded. Note that this, and all, filtering steps should be reported in publication.

Direct signal comparisons

Because each cell contains multiple reporters, direct comparisons may be made between the reporters in each cell. These comparisons are essentially forms of correlation, some of which accommodate non-linearity or dynamic lag.

5. Compute correlation coefficients for each track (Fig. 4.36.3A). The most common correlation coefficient, Pearson's rho, specifically evaluates linear relationships,

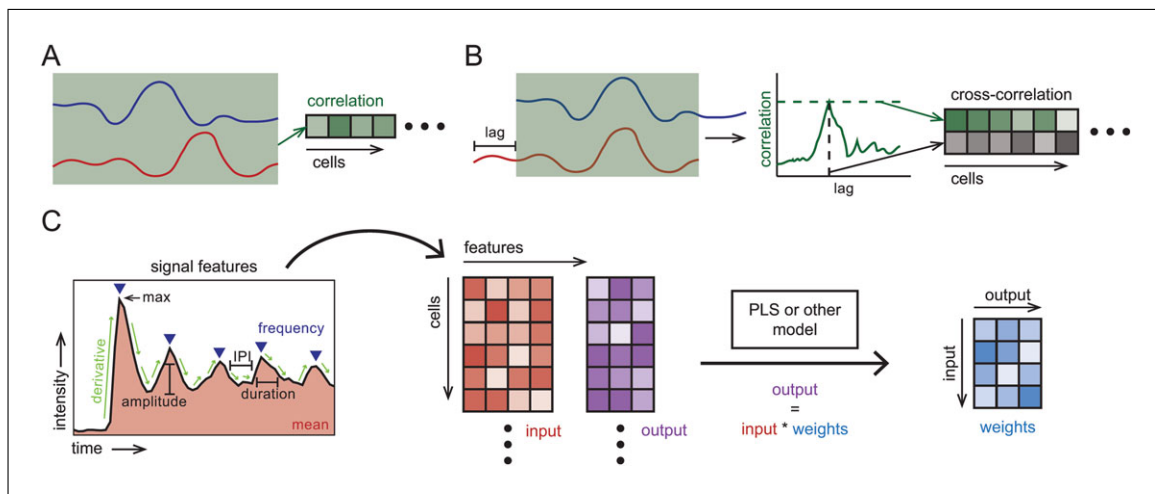


Figure 4.36.3 Examples of paired signal analyses. **(A)** Correlation, computed for each cell, yielding a vector of coefficients. **(B)** Cross-correlation, computed from the correlation of shifted (lagged) traces. For each cell, the peak correlation and corresponding lag are stored, yielding a vector for each. **(C)** Signal response modeling example, using a feature decomposition (left) extracting the: mean value, max value, frequency, amplitudes, derivatives, pulse durations, and inter-pulse intervals (IPI). Mean or maximum values of the latter four features would be stored (center). Stored features are used to fit model weights (right; e.g., via PLS), so that output values may be estimated by these weights and the input values.

while Spearman's tau does not diminish for non-linear relationships. Both are typically implemented in basic scripting software or statistics packages for scripting software.

6. Compute mutual information for each track. Mutual information is the most general form of "correlation," and evaluates how much knowing one signal's value restricts possible values of the other. It is implemented in some statistical packages for scripting software, though less widely than basic correlation coefficients.

Computation of mutual information relies on binning data to make a histogram, or on summing up nearest neighbors. As such, it is particularly important to have many data points for a robust estimate of mutual information.

7. Compute cross-correlation for each track (Fig. 4.36.3B). Cross-correlation is equivalent to correlation, but allows for a dynamic lag between signals. Properly normalized, it represents the linear correlation coefficient evaluated for each possible time lag (i.e., shifting one signal backward in time and calculating correlation, neglecting points that no longer overlap at the ends). Typically, the critical values to record are the peak (maximum) correlation value and the lag at which it occurs. Cross-correlation is typically included in signal processing packages.

Signal response modeling

Signal response models may be constructed to estimate the specific relationship between signals across a population of cells. Each pair of input and output signals from individual cells becomes one observation, and the mean relationship is estimated over all observations. The time-series signals are broken down into a smaller number of "characteristics" to comprise an observation. Partial least squares (PLS) modeling is particularly useful as it extracts the most informative features from the covariance structure, allowing many input and output characteristics to be used simultaneously. See Figure 4.36.3C.

8. Determine a suitable set of characteristics to describe the single-cell tracks. Basic statistics, such as mean value, variance, maximum and minimum values, etc. are likely to be informative. Signals that fluctuate significantly may be informed by mean (or max and min) rates of change. Signals with pulsatile or oscillatory behavior may

require evaluation of mean (or max and min) frequency and amplitude. It is often useful to apply some degree of smoothing when calculating maximum and minimum values, or rates of change, as these are highly sensitive to noise.

There are no hard-and-fast limitations on what decompositions are applicable. If convenient for the data and further desired analyses, rigorous mathematical expansions may be applied, such as the Fourier Transform or an orthogonal basis decomposition. Such expansions offer a relatively unbiased breakdown of the signal, but are often more difficult to interpret conceptually.

9. Calculate the values for each characteristic of each signal. Each track will result in an observation vector containing the values for each of the characteristics, for each of the signals recorded.
10. Infer models using one or more characteristics of two selected signals as input and output. Any model fitting routine is applicable, and the selection and application of such a scheme is beyond this protocol. However, it is highly recommended to use PLS regression as it can simultaneously consider all of the characteristics in the input or output.

PLS regression and similar procedures are fundamentally linear problems with each of the terms considered being one of the signal characteristics. However, any non-linear combinations of characteristics may be added as additional terms when desired, e.g., x^2 or $e^{(x*y)}$.

SUPPORT PROTOCOL

IMAGING WITH NIS ELEMENTS

This protocol describes the steps to set up and run an imaging experiment using the NIS Elements software. Alternative microscopy control software may have substantially different interfaces, but will accomplish approximately the same tasks.

Additional Materials (also see Basic Protocol 2)

1. In NIS Elements, open the ND Acquisition panel.
2. Set path and filename for saving data. Data are automatically saved when experiment is complete.
3. In the Time tab, select the imaging interval and total imaging duration.
4. In the Wavelength tab, select the desired fluorescent channels for the experiment.
5. Navigate to the first well in the imaging sequence and find cells. Turn on “Perfect Focus” and set the appropriate focal plane using the focus wheel.
6. Open the Zyla Settings panel and set the exposure for each channel to be used in imaging experiment.
7. Using the joystick, navigate to a suitable area for imaging; try to avoid areas where cells are overcrowded.
8. In the ND Acquisition panel select the XY tab. Navigate to the first desired ROI using the joystick and select the XY point by checking the box in the XY selection tab.
9. Continue selecting ROIs in each desired well by navigating to each ROI and selecting the coordinate using the checkbox. Adjust focal plane at each ROI as necessary using Perfect Focus.

10. To select a background ROI (optional), navigate to an XY position that contains no cells in the field of view. This will be used later to calculate background during image processing.
11. When all ROIs have been selected return to first ROI and check that focus and exposure are set correctly and that the plate has not shifted.
12. Start imaging experiment by hitting the “Run Now” button.
13. To add treatments during imaging, pause imaging using the “Pause” button on the Acquisition Window. Then, remove plate lid and environmental control box, add treatments to each well, minimize contact with plate. Return lid and environmental control box to plate, and resume imaging using the “Resume” button on the Acquisition Window.
14. Once the time course has completed, the file will be saved in the location specified during imaging set up. Alternatively, terminate the image acquisition using the “Finish” button.
15. When finished, close NIS Elements and shut down the microscope.

REAGENTS AND SOLUTIONS

Prepare all solutions with distilled, deionized water.

Collagen coating solution

Prepare 20 µg/ml rat tail type I collagen (Thermo Fisher Scientific, cat. no. A1048301) in 0.1 N acetic acid. Sterilize acetic acid and water by filtration through 0.2 µm filter before adding irradiated collagen; do not filter after adding collagen. Store at 4° C.

Laminin coating solution

Prepare 50 mg/ml laminin 111 (Thermo Fisher Scientific, cat. no. 23017015) in 20 mM sodium acetate (pH 4), containing 1 mM CaCl₂. Prepare fresh for each experiment.

COMMENTARY

Background Information

Live-cell studies of signal transduction have revealed kinetic aspects of signaling that are invisible to both population-based measurements (such as immunoblots) and static single-cell methods (such as immunofluorescence). For example, live-cell reporters of the p53 tumor suppressor protein have revealed that certain forms of DNA damage activate this key regulator of DNA repair in discrete hour-long bursts that vary in both spacing and number (Batchelor, Loewer, Mock, & Lahav, 2011; Lahav et al., 2004). Kinetics observed in single cells are often incompatible with existing models for signaling pathways, prompting significant revisions to long-standing models (Batchelor, Mock, Bhan, Loewer, & Lahav, 2008). However, to move beyond simple description, the single-cell kinetics of a given molecule must be related to its upstream and downstream processes, which requires that the

variables of interest be imaged simultaneously in the same cell. In the case of p53, simultaneous imaging of p53 activity and the resolution of DNA damage (upstream) or activation of the target gene p21 (downstream) was necessary to understand the importance of p53 kinetics in encoding responses to different types of DNA damage (Loewer, Karanam, Mock, & Lahav, 2013; Purvis et al., 2012). Similar examples can be found in multiplexed single-cell studies of death receptor-induced apoptosis (Albeck, Burke, Spencer, Lauffenburger, & Sorger, 2008), EGFR/Ras/ERK-induced proliferation (Albeck et al., 2013; Shankaran et al., 2009), and NF-κB-mediated gene induction (Nelson et al., 2004).

Single-cell experiments have revealed that “noise” plays a significant role in determining the signaling response of a given pathway to a stimulus, resulting in a distribution of responses to a uniform stimulus within a

population of cells (Cheong, Rhee, Wang, Nemenman, & Levchenko, 2011; Raser & O'Shea, 2005). While this variation may result from stochastic effects in the biochemical process of signaling, the observed variation in any one pathway can also result from deterministic but unmeasured cell-to-cell variation in other pathways and cellular processes (Selimkhanov et al., 2014). Multiparameter analysis, as presented here, can be useful in identifying the sources of such variation by making it possible to quantify the amount of variance in one pathway that can be explained by measured variance in a second (or additional) pathways. When choosing reporters for multiparameter analysis, it is important to perform a careful literature search to take into account known dependencies (or “crosstalk”) between the pathways of interest and consider how these dependencies will influence the quality of the data collected.

Critical Parameters

Throughout the processes of establishing, imaging, and analyzing a multiplexed reporter cell line, it is important to double-check quality at every opportunity. In cell line construction, cells should be evaluated after each reporter insertion, sorting (or selection), or cloning. Verify that cells retain the phenotype and any characteristic behavior of the parental line, and that reporter expression levels are suitable for measurement—but not so high that they perturb cell growth or viability or interfere with the measurement of other reporters. Additionally, be certain to perform the experiments described (Basic Protocol 1, step 11) to validate the function of the reporters.

When setting up the microscopy experiment, take care to choose frame positions with minimal obstructions (floating debris, distortions from the substrate, cells overlaying each other, etc.), and carefully double-check the focus. Ensure that a background region is available (see Basic Protocol 3, step 5). After starting imaging, it is recommended to observe at least one frame of each position to ensure that it meets expectations. When treating cells (Basic Protocol 2, step 13), be especially cautious not to disturb the plate, and observe the initial image frames after the treatment to verify that they align with frames prior to treatment. If the plate was disturbed and a large shift occurred, it is possible to realign it (to some degree) with minimal loss of data by carefully pressing the plate in the direction needed and checking the view (select a notable feature in the desired view as a point of reference).

Prior to image processing (Basic Protocol 3), ensure that the parameters are appropriate for the current experiment. Previously used parameters can be overlooked and give sub-optimal results in subsequent experiments. In particular, check that the limits on nucleus size (see step 8) are suitable for the cell type and the magnification that was used. Similarly, an inappropriate background can degrade processing. If the background is to be taken from frames containing cells, carefully ensure that neither cells nor fluorescent materials are present in the background regions. Accurate background removal is particularly important when using ratiometric measures, such as FRET or translocation reporters.

While a well-constructed image processing and quality control pipeline enables high-throughput analysis, it is critical to review the data at different points. Review the raw imagery for microscopy issues, such as out-of-focus frames, misaligned filters, or obstructions in some frames. After processing, visualize tracks in bulk to ensure both that a reasonable number were generated and that overall quality is acceptable. The presence of many jagged or extremely noisy tracks suggests a processing error. Review tracks again after quality control (Basic Protocol 4, steps 1 to 4) to ensure that final data are high quality. In such review, it is best to view all individual tracks, as well as averages, to ensure all outliers have been addressed. Individual tracks may be viewed simultaneously by plotting them overlaid, as grossly aberrant tracks will remain apparent. If the data are used for modeling (steps 8 to 10), carefully consider how the models are interpreted, as with any modeling endeavor. In particular, it is essential to evaluate for a parsimonious model, i.e., the simplest model that satisfies the available data (Burnham and Anderson, 2003).

Anticipated Results

Each imaging experiment in which multiple reporters are measured (Basic Protocol 2) will result in a series of digital images corresponding to each time point, fluorescence channel, and microscope stage position. Software such as ImageJ, MATLAB, or packages from the microscope manufacturer can be used to view each recorded image series. Processing of the raw images using Basic Protocol 3 will result in a dataset of tens to thousands of individual cell “tracks” per stage position, with each track consisting of time-course measurements of all measured reporters for an individual cell. Heatmap visualizations are often

helpful in quickly obtaining an overview of the full dataset and identifying major trends for each reporter (Fig. 4.36.1). It is also helpful to display a line plot of the mean or median for each of the measured reporters, along with lines corresponding to 25th and 75th percentile values for the population; overlaying line plots of individual cells on top of the mean can also be of use in determining the extent to which individual cells depart from the average population behavior. Visualizing the relationship between reporter measurements within each cell is also critical but can be more tedious, as it is difficult to display multiparameter data for more than one cell at a time on a single 2-dimensional chart. It is often necessary to adjust the limits of the axes or the normalization to obtain the best visualization for each cell. An appropriate strategy is to combine visual inspection of at least 20 individual cells with the multiparameter analyses described in Basic Protocol 4 for each condition.

The paired signal analyses described in Basic Protocol 4 will result in scalar values that allow for quantitation of the relationship between the reporters analyzed. Correlation analysis, for example, will produce a single value between 0 and 1 for each cell analyzed, with higher numbers indicating increasing similarity between the reporter signals. Cross-correlation analysis will produce two values for each cell, one indicating the lag time between signals and one indicating correlation value (between 0 and 1). Mutual information will also produce a single positive value for each cell, with higher values indicating an increased level of dependence between the reporter signals analyzed. In the case of signal response modeling, each dataset modeled will produce a series of coefficients that indicate the weight with which each considered parameter contributes to the overall response, with negative values indicating an opposing influence and positive values a supporting influence. Interpretation of the biological significance of these values will depend on the particular experimental context. It may be appropriate to compare, for example, the average cross correlation for all cells in one condition in which two reporters are expected to behave similarly (such as when both pathways are induced by the same stimulus) to the same measurement for all cells in a condition in which the reporters are expected to behave differently (e.g., when one pathway is stimulated and the other is blocked by an inhibitor). Alternatively, it may be useful to compare the calculated values for one

pair of reporters to similar measures in a different pair of reporters, such as control fluorescent proteins that are not expected to vary specifically in response to similar stimuli. In the absence of either different conditions or different reporters that can serve as points of reference, reporter signals from each cell in the dataset can be “scrambled” such that reporter signals are mismatched; this calculation can be used as a point of comparison to determine whether correlations in the original dataset are meaningful.

Independent replicates of experiments are also critical for establishing reproducibility and statistical significance for comparisons between conditions. It is not recommended to perform a standard t-test between conditions using each cell as an independent measurement, as the large number of cellular measurements collected will result in very low p values and statistically “significant” differences even between replicates of the same condition. It is more appropriate to perform a t-test on measurements of the average correlation (or other metrics) value for each conditions performed as experimental replicates on different days. As always, if more than two conditions are being compared, a t-test alone is inadequate and the family-wise error rate (or false discovery rate) must be considered, e.g., using the Bonferroni correction, Benjamini-Hochberg procedure, or a suitable statistical post-test following ANOVA.

Time Considerations

Several aspects of establishing successful live-cell experiments require long-term time investment. The most time-consuming operations are in producing the necessary DNA constructs and cell lines, which can require weeks to months. Naturally, establishing the computational pipeline for processing image data may also require significant time and troubleshooting, and should be started early. Once in place, image processing should consume only hours to days, depending on processor speeds and availability of parallel computing (via desktop machines or clusters).

Some of the experimental operations can be performed in parallel to avoid unnecessary delays, especially the preparation of different DNA constructs, and the (co-)transfection of cells for reporter insertion, when feasible. However, for cell lines or techniques with low insertion rates (such as targeted genomic insertions via CRISPR), transfection is more efficiently done in series, selecting for positive cells between insertions. Establishing a clonal

population of reporter-bearing cells can require an additional month; evaluate the trade-off between the extra time invested and having uniform genotype and reporter expression. In practice, it is productive to begin performing and refining imaging experiments as soon as cells bearing a reporter are available, rather than waiting for the perfect cell line to be complete.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Literature Cited

- Albeck, J. G., Burke, J. M., Spencer, S. L., Lauf-
fenburger, D. A., & Sorger, P. K. (2008). Model-
ing a snap-action, variable-delay switch control-
ling extrinsic cell death. *PLoS Biology*, 6, 2831–
2852. doi: 10.1371/journal.pbio.0060299.
- Albeck, J. G., Mills, G. B., & Brugge, J. S. (2013). Frequency-modulated pulses of
ERK activity transmit quantitative prolifera-
tion signals. *Molecular Cell*, 49, 249–261. doi:
10.1016/j.molcel.2012.11.002.
- Batchelor, E., Loewer, A., Mock, C., & Lahav, G. (2011). Stimulus-dependent dynamics of p53 in
single cells. *Molecular Systems Biology*, 7, 488.
doi: 10.1038/msb.2011.20.
- Batchelor, E., Mock, C. S., Bhan, I., Loewer, A., &
Lahav, G. (2008). Recurrent initiation: A mech-
anism for triggering p53 pulses in response to
DNA damage. *Molecular Cell*, 30, 277–289.
doi: 10.1016/j.molcel.2008.03.016.
- Burnham, K. P., & Anderson, D. R. (2003). Model
selection and multimodel inference: A practical
information-theoretic approach (Springer Sci-
ence & Business Media).
- Cadinanos, J., & Bradley, A. (2007). Genera-
tion of an inducible and optimized piggyBac
transposon system. *Nucleic Acids Research*, 35,
e87. doi: 10.1093/nar/gkm446.
- Carpenter, A. E., Jones, T. R., Lamprecht, M. R.,
Clarke, C., Kang, I. H., Friman, O., ... Saba-
tini, D. M. (2006). CellProfiler: Image anal-
ysis software for identifying and quantifying
cell phenotypes. *Genome Biology*, 7, R100. doi:
10.1186/gb-2006-7-10-r100.
- Cepko, C., & Pear, W. (2001). Overview of the
retrovirus transduction system. *Current Proto-
cols in Molecular Biology*, Chapter 9, Unit9 9.
doi: 10.1002/0471142727.mb0909s36.
- Cheong, R., Rhee, A., Wang, C. J., Nemenman, I.,
& Levchenko, A. (2011). Information transduc-
tion capacity of noisy biochemical signaling net-
works. *Science*, 334, 354–358. doi: 10.1126/sci-
ence.1204553.
- Chubb, J. R., Trcek, T., Shenoy, S. M., & Singer, R.
H. (2006). Transcriptional pulsing of a develop-
mental gene. *Current Biology*, 16, 1018–1025.
doi: 10.1016/j.cub.2006.03.092.
- Dickinson, D. J., Ward, J. D., Reiner, D. J., & Gold-
stein, B. (2013). Engineering the *Caenorhabditis*
elegans genome using Cas9-triggered homolo-
gous recombination. *Nature Methods*, 10, 1028–
1034. doi: 10.1038/nmeth.2641.
- Elowitz, M. B., Levine, A. J., Siggia, E. D., &
Swain, P. S. (2002). Stochastic gene expression
in a single cell. *Science*, 297, 1183–1186. doi:
10.1126/science.1070919.
- Geurts, A. M., Yang, Y., Clark, K. J., Liu, G.,
Cui, Z., Dupuy, A. J., ... Hackett, P. B. (2003). Gene transfer into genomes of hu-
man cells by the sleeping beauty transposon
system. *Molecular Therapy*, 8, 108–117. doi:
10.1016/S1525-0016(03)00099-6.
- Goedhart, J., van Weeren, L., Adjobo-Hermans,
M. J., Elzenaar, I., Hink, M. A., & Gadella,
T. W., Jr (2011). Quantitative co-expression of
proteins at the single cell level—application to a
multimeric FRET sensor. *PLoS One*, 6, e27321.
doi: 10.1371/journal.pone.0027321.
- Han, K., Jaimovich, A., Dey, G., Ruggero,
D., Meyuhas, O., Sonenberg, N., & Meyer,
T. (2014). Parallel measurement of dynamic
changes in translation rates in single cells.
Nature Methods, 11, 86–93. doi: 10.1038/
nmeth.2729.
- Jaqaman, K., Loerke, D., Mettlen, M., Kuwata, H.,
Grinstein, S., Schmid, S. L., & Danuser, G. (2008). Robust single-particle tracking in live-
cell time-lapse sequences. *Nature Methods*, 5,
695–702. doi: 10.1038/nmeth.1237.
- Komatsu, N., Aoki, K., Yamada, M., Yukinaga,
H., Fujita, Y., Kamioka, Y., & Matsuda, M. (2011). Development of an optimized backbone
of FRET biosensors for kinases and GTPases.
Molecular Biology of the Cell, 22, 4647–4656.
doi: 10.1091/mbc.E11-01-0072.
- Lahav, G., Rosenfeld, N., Sigal, A., Geva-Zatorsky,
N., Levine, A. J., Elowitz, M. B., & Alon, U. (2004). Dynamics of the p53-Mdm2 feedback
loop in individual cells. *Nature Genetics*, 36,
147–150. doi: 10.1038/ng1293.

- Locke, J. C., & Elowitz, M. B. (2009). Using movies to analyse gene circuit dynamics in single cells. *Nature Reviews. Microbiology*, 7, 383–392. doi: 10.1038/nrmicro2056.
- Loewer, A., Karanam, K., Mock, C., & Lahav, G. (2013). The p53 response in single cells is linearly correlated to the number of DNA breaks without a distinct threshold. *BMC Biology*, 11, 114. doi: 10.1186/1741-7007-11-114.
- Nelson, D. E., Ihekweba, A. E., Elliott, M., Johnson, J. R., Gibney, C. A., Foreman, B. E., ... White, M. R. (2004). Oscillations in NF-kappaB signaling control the dynamics of gene expression. *Science*, 306, 704–708. doi: 10.1126/science.1099962.
- Nelson, G., Paraoan, L., Spiller, D. G., Wilde, G. J., Browne, M. A., Djali, P. K., ... White, M. R. (2002). Multi-parameter analysis of the kinetics of NF-kappaB signalling and transcription in single living cells. *Journal of Cell Science*, 115, 1137–1148.
- Pargett, M., Gillies, T. E., Teragawa, C. K., Sparta, B., & Albeck, J. G. (2017). Single-Cell Imaging of ERK Signaling Using Fluorescent Biosensors. In *Kinase Signaling Networks* (Springer) (pp. 35–59).
- Phelan, K. and May, K. M. (2015). Basic techniques in mammalian cell tissue culture. *Current Protocols in Cell Biology*, 66, 1.1.1–1.1.22. doi: 10.1002/0471143030.cb0101s66.
- Purvis, J. E., Karhohs, K. W., Mock, C., Batchelor, E., Loewer, A., & Lahav, G. (2012). p53 dynamics control cell fate. *Science*, 336, 1440–1444. doi: 10.1126/science.1218351.
- Purvis, J. E., & Lahav, G. (2013). Encoding and decoding cellular information through signaling dynamics. *Cell*, 152, 945–956. doi: 10.1016/j.cell.2013.02.005.
- Qin, J. Y., Zhang, L., Clift, K. L., Hlur, I., Xi, A. P., Ren, B. Z., & Lahn, B. T. (2010). Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. *PLoS One*, 5, e10611. doi: 10.1371/journal.pone.0010611.
- Raser, J. M., & O'Shea, E. K. (2005). Noise in gene expression: Origins, consequences, and control. *Science*, 309, 2010–2013. doi: 10.1126/science.1105891.
- Regot, S., Hughey, J. J., Bajar, B. T., Carrasco, S., & Covert, M. W. (2014). High-sensitivity measurements of multiple kinase activities in live single cells. *Cell*, 157, 1724–1734. doi: 10.1016/j.cell.2014.04.039.
- Rose, J. K. (2003). Optimization of Transfection. *Current Protocols in Cell Biology*, 19, 20.7.1–20.7.4. doi: 10.1002/0471143030.cb2007s19.
- Selimkhanov, J., Taylor, B., Yao, J., Pilko, A., Albeck, J., Hoffmann, A., ... Wollman, R. (2014). Systems biology. Accurate information transmission through dynamic biochemical signaling networks. *Science*, 346, 1370–1373. doi: 10.1126/science.1254933.
- Shankaran, H., Ippolito, D. L., Chrisler, W. B., Resat, H., Bollinger, N., Opresko, L. K., & Wiley, H. S. (2009). Rapid and sustained nuclear-cytoplasmic ERK oscillations induced by epidermal growth factor. *Molecular Systems Biology*, 5, 332. doi: 10.1038/msb.2009.90.
- Slattery, S. D., & Hahn, K. M. (2014). A high-content assay for biosensor validation and for examining stimuli that affect biosensor activity. *Current Protocols in Cell Biology*, 65, 14.15.11–14.15.31. doi: 10.1002/0471143030.cb1415s65.
- Stewart-Ornstein, J., & Lahav, G. (2016). Dynamics of CDKN1A in single cells defined by an endogenous fluorescent tagging toolkit. *Cellular Reprogramming*, 14, 1800–1811. doi: 10.1016/j.celrep.2016.01.045.

Internet Resources

www.openmicroscopy.org/site/products/bio-formats

Website for Bio-Formats.

www.utsouthwestern.edu/labs/danuser/software

Website hosting u-Track software.

www.fpviz.org

Website hosting aggregated fluorescent protein properties.

Oncogenic mutant RAS signaling activity is rescaled by the ERK/MAPK pathway

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Abstract

Activating mutations in RAS are present in ~30% of human tumors, and the resulting aberrations in ERK/MAPK signaling play a central role in oncogenesis. However, the form of these signaling changes is uncertain, with activating RAS mutants linked to both increased and decreased ERK activation *in vivo*. Rationally targeting the kinase activity of this pathway requires clarification of the quantitative effects of RAS mutations. Here, we use live-cell imaging in cells expressing only one RAS isoform to quantify ERK activity with a new level of accuracy. We find that despite large differences in their biochemical activity, mutant KRAS isoforms within cells have similar ranges of ERK output. We identify roles for pathway-level effects, including variation in feedback strength and feedforward modulation of phosphatase activity, that act to rescale pathway sensitivity, ultimately resisting changes in the dynamic range of ERK activity while preserving responsiveness to growth factor stimuli. Our results reconcile seemingly inconsistent reports within the literature and imply that the signaling changes induced by RAS mutations early in oncogenesis are subtle.

Keywords computational modeling; epidermal growth factor; FRET biosensor; RAS disease; single-cell kinetics

Subject Categories Cancer; Signal Transduction

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Introduction

The RAS GTPases act as molecular switches, alternating between an inactive GDP-bound state and an active GTP-bound state. In the active state, RAS proteins have a greatly increased binding affinity for their effectors (Gremer *et al*, 2011), which in mammalian cells drive multiple cell growth signaling pathways. The net signaling activity of RAS in the cell represents a balance between two classes of proteins: GTPase-activating proteins (GAPs), which inactivate RAS by increasing its GTPase activity, and guanine nucleotide

exchange factors (GEFs), which catalyze the dissociation of GDP and return RAS to the active GTP-bound state. Though RAS proteins are considered binary switches on the molecular level, the collective behavior of the thousands of RAS proteins present inside each cell is analog in nature. The relative activity of GAPs vs. GEFs in the cell determines the fraction of RAS molecules in the active state, which in turn regulates the activity of downstream processes.

RAS mutations occur frequently in cancers, especially those of the pancreas, lung, or colon (Fernandez-Medarde & Santos, 2011), and typically have the effect of increasing the signaling output of one of the RAS isoforms. Most oncogenic RAS mutations (85%) occur in the KRAS isoform, with 11% in NRAS and 4% in HRAS (An & Harper, 2018). Across all isoforms, 98% of oncogenic mutations are located at G12, G13, and Q61 (Prior *et al*, 2012) and render the RAS proteins GAP-insensitive to varying degrees. The net effect of these mutations is to increase the fraction of RAS proteins in the GTP-bound active state, which enhances their binding affinity to effectors, including the RAF kinases (Gremer *et al*, 2011; Smith *et al*, 2013; Hunter *et al*, 2015). RAF initiates a kinase cascade involving MEK and ERK (Fig 1A), which plays a primary role in tumor development and is a pharmacological target for cancer therapy. ERK phosphorylates hundreds of downstream targets (Yoon & Seger, 2006), many of which are transcription factors controlling cell cycle progression and cell migration.

While RAS mutations are widely thought to initiate tumors by enhancing the activity of RAF/MEK/ERK signaling to drive tumorigenic cellular behaviors, this model is not consistent with all of the data available. A number of observations deviate from this simple linear view of RAS signaling. First, the observed frequency of RAS mutations in cancer does not correlate with the strength of their effect on RAS GTPase activity. Mutations of intermediate strength are most prevalent, and the strongest mutations are found infrequently (Li *et al*, 2018). Second, the mutational status of RAS is poorly correlated with average levels of active dually-phosphorylated ERK (ppERK) both in tumor cell lines (Omerovic *et al*, 2008; Yeh *et al*, 2009) and in genetically engineered mouse models. In fact, converting a wild-type *Kras* gene to an activating mutant can actually *reduce* average ppERK levels, despite inducing tumor formation (Tuveson *et al*, 2004). These data contrast with

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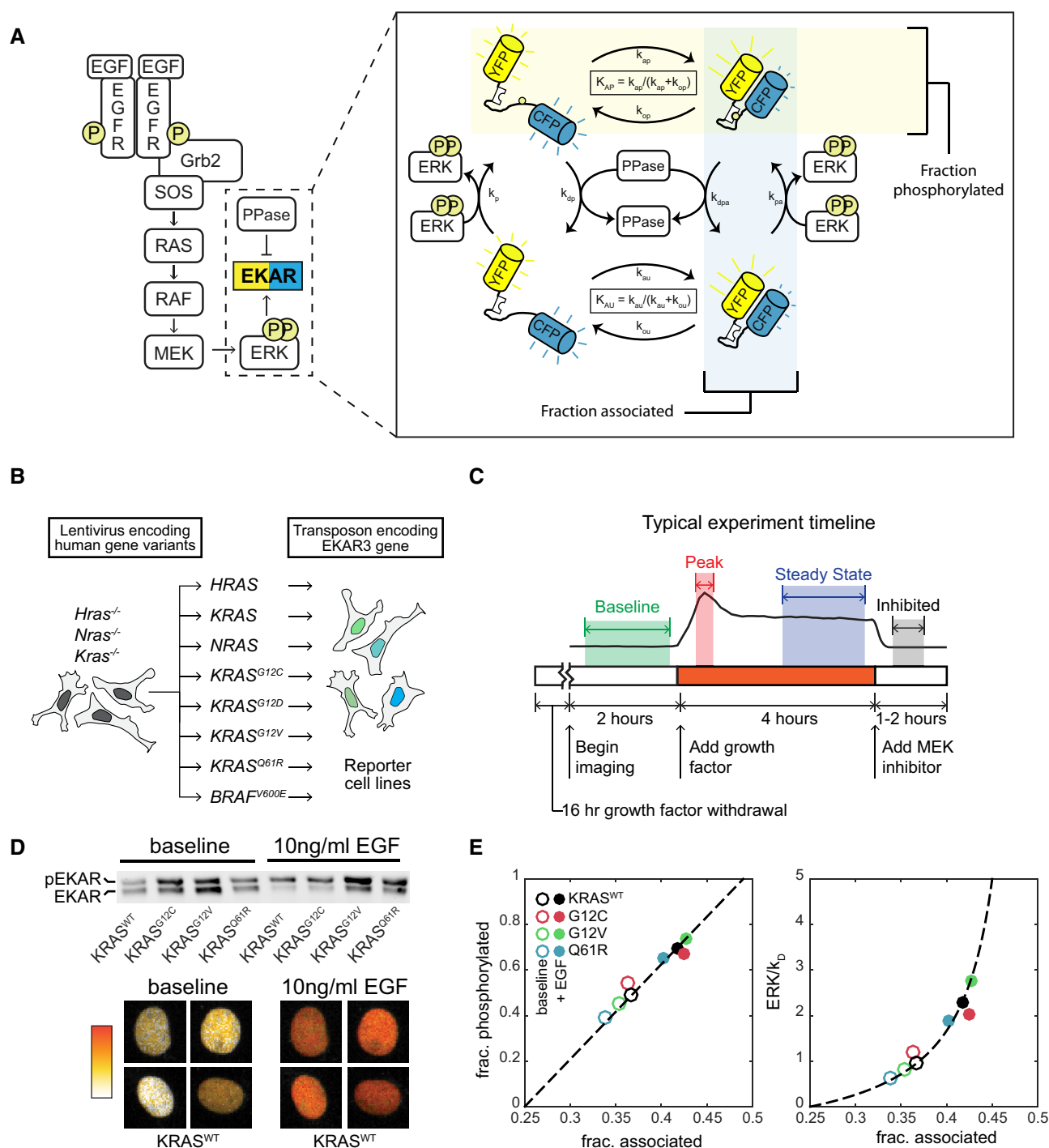


Figure 1. Platform for ERK activity measurement in MEF cell lines expressing a single RAS isoform.

- A Schematic of EGF signaling through RAS to ERK, including the EKAR3 sensor. The detail image at right depicts the cycle of EKAR3 phosphorylation by ERK, binding and unbinding of the internal WW domain to the phosphorylated threonine residue, and removal of the phosphate by phosphatases. Spontaneous association of the fluorophores in the absence of phosphorylation contributes to background signal and is included in activity calculations.
- B Construction scheme for cell lines bearing a single RAS isoform, using H/K/N-RAS knockouts.
- C Diagram of the typical experiment timeline. Shaded regions indicate time windows that are averaged for each measurement.
- D Sample calibration data for the EKAR3 reporter, consisting of Phos-Tag immunoblot for phospho-EKAR (upper) and live-cell imaging of reporter FRET activity (lower) under matched conditions for 4 cell lines that span the full range of ERK activity levels. Ratiometric images of four individual nuclei from the $KRAS^{WT}$ line, which show the largest change from baseline to peak, are shown before and after stimulus as a representative example of the image data.
- E Calibration curves for ERK activity. Fraction of EKAR3 phosphorylated is shown vs. the fraction in the associated conformation by FRET (left). The ERK to phosphatase activity ratio (right) is derived from a model of EKAR3 (see Appendix Supplementary Methods). Each marker represents the mean value from one cell line with (filled circle) or without (open circle) EGF treatment, from 3 replicate live-cell samples and 4 replicate immuno blot samples.

observations that ectopic expression of RAS mutants does drive strong over-activation of ERK, as would be expected from simple amplification by RAF/MEK/ERK (Park *et al*, 2006; Konishi *et al*, 2007).

These contradictory observations could arise for various reasons, including feedback in the RAS/ERK pathway (Courtois-Cox *et al*, 2006), oncogene-induced senescence (Sarkisian *et al*, 2007), additional mutations, or tissue- or cell type-specific effects (Brandt *et al*, 2019). However, these possibilities cannot be disentangled without first addressing a major technical limitation inherent in the immunoblots and kinase assays that have been used almost exclusively to date. Because immunoblot measurements are relative, not absolute, it is not typically possible to compare the magnitude of ERK activation across datasets or studies. Furthermore, ERK activation has been shown to be pulsatile and heterogeneous (Albeck *et al*, 2013; Regot *et al*, 2014), so the relative differences observed when blotting for active ERK could have multiple biochemical interpretations: More ERK proteins may be active per cell, or cells with active ERK may be more frequent in the population (Birtwistle *et al*, 2012; Purvis & Lahav, 2013). Increases in the magnitude, frequency, or duration of ERK activation pulses would all yield the same result via immunoblot, though each of these signaling changes would imply different effects on gene expression and warrant different approaches for pathway directed therapy.

To clearly distinguish the forms of ERK activity that result from RAS mutations, we combined live-cell and immunoblot techniques to study a panel of cell lines each expressing only one wild type or mutant isoform of human RAS in an isogenic background. To unequivocally measure ERK activity, we employed a genetically encoded Förster resonance energy transfer (FRET)-based sensor (EKAR3) and calibrated it to deliver a quantitative linear readout of ERK substrate phosphorylation. The live-cell sensor allows measurement of cell-to-cell heterogeneity and signaling dynamics for a more detailed view of ERK activity at the cellular level. Complementing live-cell data with immunoblot measurements of RAS/ERK pathway components and computational modeling, we found that ERK activity is strikingly constrained in cells expressing mutant KRAS. When unstimulated, KRAS mutant cells exhibit only moderately elevated ERK activity compared to the wild type, and when stimulated reach peak activity no greater than the wild type. These findings outline a new unified model for how elevated RAS activity is modulated by downstream effectors and for which signaling characteristics may be relevant in cancer.

Results

A platform to quantify ERK activity downstream of individual RAS isoforms

To evaluate the cellular signaling capacity of each RAS isoform individually, we utilized a panel of genetically engineered mouse embryonic fibroblasts (MEFs) in which the genes for the three major RAS isoforms (*Hras*, *Kras*, and *Nras*) have been functionally deleted and complemented with a single constitutively expressed human cDNA (Drosten *et al*, 2010; Fig 1B). Human proteins expressed are: HRAS, KRAS, NRAS, KRAS^{G12C}, KRAS^{G12D}, KRAS^{G12V}, KRAS^{Q61R}, or the oncogenic RAF gene BRAF^{V600E}, in which case no RAS isoform

is expressed. In these cell lines, the signaling behavior of each RAS protein isoform can be characterized in isolation both from other isoforms and from locus-specific variations in transcriptional regulation. To track the resulting signaling activity with high temporal resolution, we transfected each MEF cell line with EKAR3, a live-cell FRET-based ERK activity reporter (Harvey *et al*, 2008; Sparta *et al*, 2015). EKAR3 is directly phosphorylated by ERK, acting as a synthetic substrate. Intramolecular binding of the reporter's WW domain to the phosphorylated residue in the substrate domain induces a FRET interaction that can be visualized by observing changes in the CFP/YFP ratio using time-lapse fluorescence microscopy. This interaction is reversible by phosphatases, allowing the reporter to indicate transient changes in the ERK:phosphatase activity ratio (Fig 1A). The resulting imaging data were analyzed with a custom image analysis pipeline (see Materials and Methods), typically yielding 100–300 single-cell time series measurements of ERK activity from each replicate of an experimental condition.

To enable accurate comparisons between the single RAS cell lines, we developed a workflow to make quantitative live-cell measurements of ERK activity (Fig 1C). Signaling activity in the absence of external stimulation, a condition we term “baseline”, was quantified in cells cultured with neither serum nor growth factors for at least 16 h prior to imaging. Responses to receptor stimulation were quantified by introducing growth factor after several hours of baseline imaging. As a negative control for ERK reporter measurements, we treated cells with the highly specific MEK inhibitor PD0325901 (MEKi), which rapidly inhibited the EKAR3 signal in all cell lines. In all live-cell experiments, a 100 nM MEKi treatment was applied just prior to ending the experiment; this measures the cell-specific residual EKAR3 signal, accounting for non-specific fluorescence. The signal from the EKAR3 reporter was derived from the intensity ratio of the cyan and yellow fluorescent channels (CFP/YFP) and corrected for background as well as excitation and filter spectra. The corrected EKAR3 signal linearly reflects the fraction of reporter molecules in a FRET conformation, which is in turn linearly related to the fraction of molecules phosphorylated by ERK (Birtwistle *et al*, 2011). To calibrate, we used Phos-Tag immunoblotting to quantify the fraction of the EKAR3 reporter that is phosphorylated in various samples and conditions (Fig 1D) and fit these values against the average corrected EKAR3 signal for the same cell lines and conditions. For this calibration experiment, we selected conditions which capture a wide range of ERK activity, including the highest observed ERK activity measurement in KRAS^{WT} stimulated with 10 ng/ml EGF. In concert with a mass action model of substrate phosphorylation, this calibration yields a linear measure of the ERK:phosphatase ratio (Fig 1E), i.e., the concentration of active ERK divided by the concentration of any active phosphatases that dephosphorylate the reporter (see Appendix Supplementary Methods for details). As these competing phosphatases also presumably act on endogenous ERK targets, the ERK activity measurement reflects not just the levels of active ERK, but the *net* effect of ERK on its substrates. These data confirm that our ERK activity measurement remains linear across the ERK activity ranges investigated in this study.

To demonstrate the utility of this platform for assessing inhibitor activity, we treated the panel of reporter cells with ARS-853, an inhibitor specific to KRAS^{G12C}. Following treatment with ARS-853, ERK activity decreased over the course of 60 min in KRAS^{G12C} MEFs, but

not in any of the other KRAS cell lines (Fig 2A). Thus, allele-specific drug responses can be identified and quantified using the reporter cell panel. Furthermore, because ARS-853 inhibits the only KRAS isoform present in KRAS^{G12C} cells, we used this condition to estimate the RAS-independent background level of ERK activity. Following ARS-853 treatment, EKAR3 signal decreased to a level approximately equivalent to that of untreated KRAS^{WT}, followed by a small rebound. This similarity suggests that the ERK activity contributed by RAS-independent sources is near the minimal baseline value.

We next used our platform to perform a comprehensive survey of the effects of various growth factors on each cell line. We stimulated the MEF cell line panel with six growth factors known to activate RAS/ERK signaling: EGF, IGF, FGF, HGF, PDGF, and amphiregulin. Two to three concentrations of each growth factor were tested across three biological replicates, yielding activity “traces” from approximately 400 cells per condition (Fig 2B). ERK kinetics differed depending on the growth factor. For example, FGF induced sustained ERK activity without pulsatile behavior, while

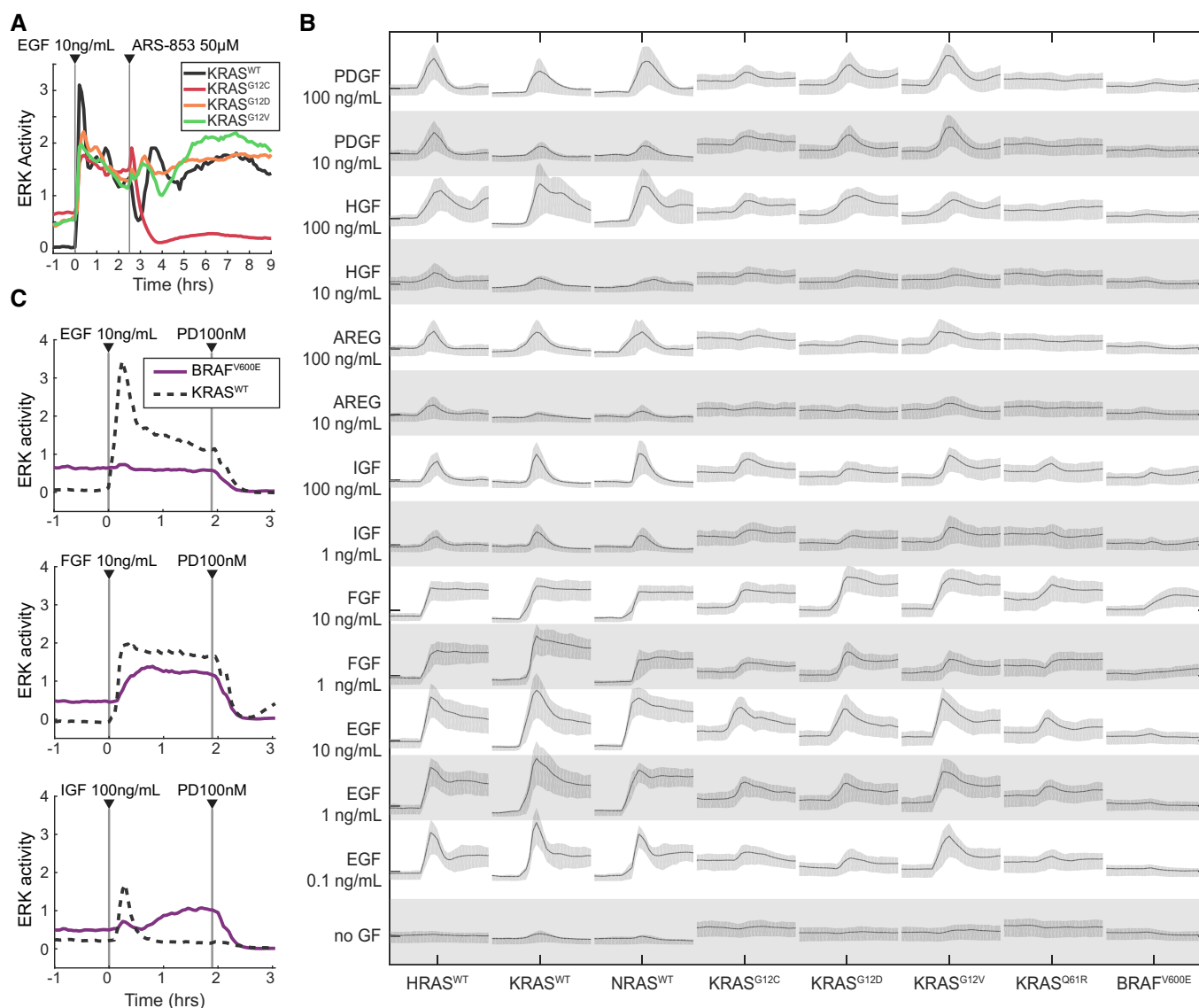


Figure 2. Activity profiles of MEF cell lines expressing a single RAS isoform.

- A Demonstration of the system measuring a cell line-specific response via ARS-853, a RAS activity inhibitor specific to the KRAS^{G12C} mutant. Traces are median values from a representative experiment. Experiment was replicated 3 times.
- B Graphical summary of single RAS isoform cell lines (labeled along bottom) stimulated by a panel of growth factors (labeled along left). Each panel of the matrix shows the time series of ERK activity with the indicated growth factor spiked in after beginning imaging. All scales are equal; x-axis: time; y-axis: ERK activity. Lines indicate median of single-cell measurements over time, and shaded regions denote the 25th–75th percentile region, across 3 replicate cultures (6 for no GF).
- C Demonstration of RAS-independent activity from ligands other than EGF, evidenced by response in the BRAF^{V600E} cell line lacking H/K/N-RAS. Traces are median values from a representative experiment. Experiment was replicated 5 times.

Source data are available online for this figure.

IGF induced a single ERK activity pulse, approximately 30–40 min in duration, immediately following stimulation. The BRAF^{V600E} cell line showed moderate baseline ERK activity, consistent with previous studies in which this allele was expressed in MEFs at endogenous levels (Mercer *et al*, 2005), but it was not expected to respond to any growth factor stimulus because it lacked all RAS genes. However, both FGF and IGF induced elevated ERK activity in BRAF^{V600E} cells (Fig 2C), indicating an ERK response that is not mediated via HRAS, KRAS, or NRAS, which could occur through other GTPases with the potential to activate RAF, such as RRAS or RAP1. By contrast, EGF induced high amplitude ERK activity in both mutant and wild-type RAS cells, without evidence of H/K/NRAS-independent activity in the BRAF^{V600E} cell line. The remaining growth factors, PDGF, HGF, and amphiregulin, did not induce activity in BRAF^{V600E} cells, but induced weaker or more transient ERK responses than did EGF in RAS-expressing cells. We therefore focus on EGF for the bulk of our subsequent analysis, because it induces a strong ERK response without H/K/NRAS-independent effects.

RAS mutants only moderately elevate ERK activity, and only without stimulation

With quantitative single-cell resolution available, we addressed the question of how the RAS protein isoforms differ in their ERK activity patterns. After growth factor withdrawal, cells were stimulated with either media alone, or media with EGF to a final concentration of 10 ng/ml (Fig 3A–C). Across all cell lines, EGF stimulation initiated a rapid ERK activity peak ~ 15 min after stimulation, followed by attenuation over 1.5–2 h to reach a steady-state level (Fig 3B and C), with HRAS^{WT} and NRAS^{WT} cells exhibiting slower attenuation than any of the KRAS isoforms. Responses in single cells were qualitatively similar to the average, though each cell showed variation over time (Fig 3C). To statistically compare responses across single cells, we decomposed each single-cell ERK trace into parameters: average baseline activity, peak stimulated activity, stimulated amplitude, and average steady-state activity 2 h after stimulation. To compare the tendency for sporadic and time-varying activity, we sought a metric similar to the coefficient of variation (CV). However, when used on time series data, the CV neglects time and only reflects how far samples deviate from the mean regardless of when they occurred. We instead compute a metric we term “volatility”. This is calculated by first differentiating the ERK activity per cell, then taking the absolute value and averaging over the time series. As with the CV, we scale volatility by the mean value for that cell. This metric is the time-dependent equivalent of the CV in that it is the mean-scaled average of deviations from the past time point, where the CV is the mean-scaled average of deviations from the mean. Low volatility indicates flatter more consistent activity, while higher volatility indicates more pulses, or changes in activity level over time.

Baseline ERK activity was detectable in all cells but varied in magnitude among the RAS isoforms (Fig 3D). All KRAS mutant cell lines, as well as HRAS^{WT}, exhibited significantly elevated baseline ERK activity compared to KRAS^{WT}, with the highest levels observed in KRAS^{Q61R} and KRAS^{G12C}. Mutant KRAS and BRAF^{V600E} cells were less volatile over time than KRAS^{WT} cells under baseline conditions (Fig 3E). Minimal differences in volatility were detected between HRAS^{WT}, KRAS^{WT}, and NRAS^{WT} cells (Fig 3E). The higher baseline

activity in mutant KRAS isoforms compared to wild type is qualitatively consistent with constitutively higher GTP loading of these GTPase-deficient RAS proteins, which would also reduce variability in RAS activation by obscuring minor spontaneous activation events, such as autocrine signals.

The quantitative parameters of the ERK response to growth factor stimulation, including the amplitude and duration of activity, play an important part in shaping downstream cellular responses (Ebisuya *et al*, 2005; Nakakuki *et al*, 2010). We therefore explored the differences between these parameters in mutant and wild-type KRAS cells. While the rise and fall of ERK activity occurred with similar kinetics across all KRAS variants, the average peak ERK activity in the KRAS mutant cell lines was unexpectedly equal to or lower than KRAS^{WT} (Fig 3B). However, differences in the average ERK activity could result from heterogeneity between cells, and upon examination, we found that the percentage of cells with a detectable ERK response to EGF was significantly reduced in KRAS mutant lines (Fig 3F and G). KRAS^{Q61R} cells in particular exhibited drastically reduced response rates. This reduced response could arise from a functionally resistant subpopulation of cells, but could also result from the difficulty of detecting smaller amplitude responses in cells with elevated baseline activity. Therefore, to validate the response measurement, we examined the correlation of response frequency with baseline activity. While the response rate does vary with average baseline activity for most mutants (Fig 3H), correlation at the single-cell level is quite poor for all cell lines (Fig 3I and J); many high baseline cells clearly respond and many low baseline cells do not. Thus, the population-averaged peak ERK activity is genuinely reduced in KRAS mutant cells by a lower probability of response for each cell.

To remove the bias introduced by non-responding cells and more accurately compare average ERK responses, we filtered the ERK activity dataset to include only cells with a distinguishable response (Fig 3K–M). In this filtered dataset, the peak ERK responses in KRAS mutant cells were still equivalent to or less than those of the KRAS^{WT} cells (Fig 3K). Kinetics of the growth factor response also remained similar after correction for non-responding cells (Fig 3L and M). The only distinction observed was that steady-state ERK activity 2 h after stimulation was higher in NRAS^{WT} and KRAS^{G12V} cells, compared with KRAS^{WT}, implying a slower attenuation (Fig 3L). Thus, even accounting for a reduced frequency of response, KRAS mutant cells exhibit peak ERK activity no higher than wild-type lines and show no other notable differences. The similarity of peak ERK responses across mutants is unexpected given the range of RAS GTPase activities represented in this panel of cell lines and implies that the upper limit of ERK activity is subject to tight regulation. Altogether, when individual cell variability is accounted for, the only broadly consistent distinction between ERK activity in KRAS^{WT} and mutant isoforms in this system is moderate elevation of unstimulated activity.

Immediate feedback from ERK is distributed and relatively weak in RAS mutants

The RAS-ERK pathway is subject to multiple feedback effects triggered by ERK activity that could account for the strict moderation of mutant RAS signaling. We therefore assessed the involvement of these feedback loops by comparing activity at several points in

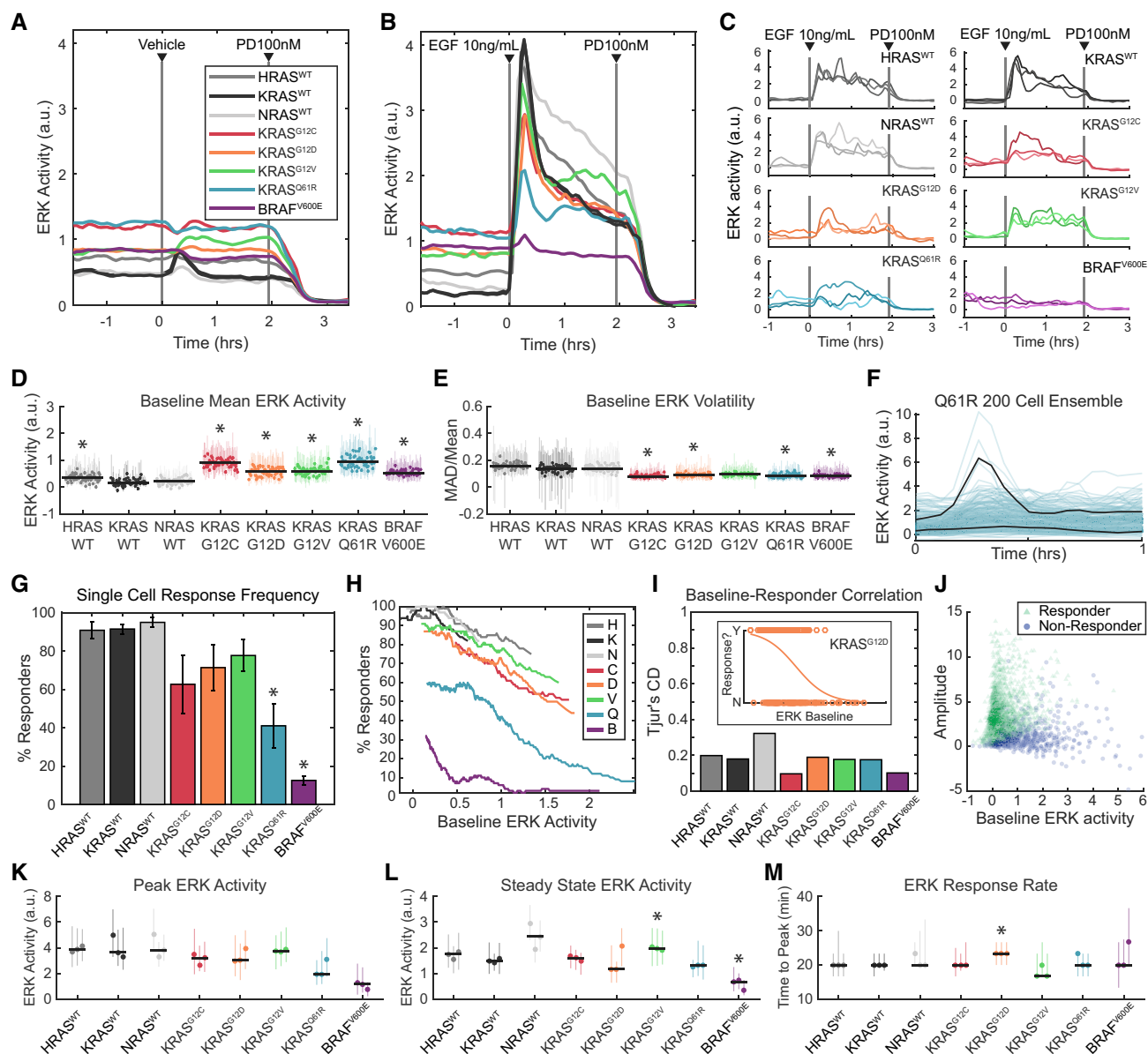


Figure 3. Kinetic analysis of ERK activity for each RAS isoform in response to EGF stimulation.

A–E ERK activity in each of the 8 MEF cell lines, after growth factor withdrawal for 16–24 h, followed by stimulus consisting of (A) media only, or (B) 10 ng/ml EGF. (A, B) Mean values over 3 replicate cultures. (C) Three example single-cell traces per cell line, randomly selected from 3 replicate experiments. (D) Average baseline (pre-stimulus) ERK activity over 58 replicate cultures per cell line. Each dot represents the median value across cells in an experiment and vertical lines represent the 25th–75th percentiles. Black horizontal bars denote the median across all replicates. Asterisks indicate significance by t-test (pFDR < 0.05). (E) Average volatility (pre-stimulus) over 58 replicate cultures per cell line, reflecting the scale of variation over time, displayed as in D. Dots show medians and error bars show 25th–75th percentiles. Asterisks indicate significance by t-test (pFDR < 0.05).

F–J Analysis of single-cell response likelihood after EGF stimulus. (F) Demonstration of many cells not responding to EGF stimulus in the Q61R cell line, in a representative experiment. Black lines highlight one responder and one non-responder cell, with 200 individual cell traces shown. (G) Likelihood of single cells responding to EGF stimulus, for each cell line, showing mean of 3 replicates with error bars showing one standard deviation. Asterisks indicate significance by t-test (pFDR < 0.05). (H) Relationship between response likelihood and average baseline ERK activity as a possible correlate, for each cell line. Means taken over 3 replicate cultures. (I) Weakness of correlation between baseline ERK activity and response likelihood, measured by Tjur's coefficient of discrimination (i.e., correlation coefficient for a binary response). Inset shows an example from the KRAS^{G12D} mutant, where dots are scattered per cell by baseline ERK activity (x-axis) and whether that cell responded to EGF (binary y-axis). Orange line indicates the logistic fit. Correlations calculated from single-cell data from 3 replicate experiments. (J) Scattered single-cell measurements of baseline ERK activity and amplitude of the change after EGF stimulus. Green triangles: cells that responded; blue circles: cells that did not respond.

K–M Analysis of the response to EGF, by filtering to remove cells that do not respond, presented as in (D), with dots showing medians and error bars showing 25th–75th percentiles. Asterisks indicate significance by t-test (pFDR < 0.05). Data from 3 independent culture replicates. (K) Peak ERK activity reached after EGF stimulus. (L) Average ERK activity after 2 h in the presence of EGF. (M) Delay between EGF stimulus and peak ERK activity.

Data information: All t-tests herein were performed as detailed in Methods and Protocols, “Statistical Analysis: t-tests for Single-Cell Data”.

the pathway in the absence or presence of the ERK inhibitor SCH7272984 (ERKi; Fig 4A; Morris *et al*, 2013). As an ATP competitive inhibitor with an allosteric mode, ERKi suppresses both the activity of ppERK and the phosphorylation of ERK by MEK (Chai-kwad *et al*, 2014). Consistent with this allosteric inhibition, this treatment had a partial effect on ERK phosphorylation (Fig 4A and B). To test for ERK-mediated feedback effects, we treated KRAS^{WT}, KRAS^{G12C}, and KRAS^{Q61R} cells with 100 nM ERKi 1 h prior to EGF stimulation and measured the phosphorylated or active forms of EGFR, RAS, AKT, and MEK by immunoblotting. In Fig 4B, the comparison between untreated and ERKi-treated cells is shown side by side (dark- and light-shaded bars, respectively) to emphasize the effect of ERK-mediated feedback on each species. Treatment with ERKi resulted in increased MEK dual phosphorylation at Ser217/Ser221 (ppMEK) to different extents under both resting and EGF-stimulated conditions in all three cell lines (Fig 4B), confirming the presence of feedback effects on pathway activity upstream of MEK.

To explore which steps in the pathway are subject to ERK-mediated feedback, we compared immunoblots of ppMEK, EGFR phosphorylation at Tyr1068 (pEGFR), AKT phosphorylation at Ser473 (pAKT), and RAS activation by pulldown of GTP-bound RAS (RAF-RBD PD; Fig 4A and B). In unstimulated mutant and wild-type KRAS cells, only ppMEK was elevated by ERKi treatment, indicating a significant negative feedback effect due to ERK-mediated inhibitory phosphorylation of RAF (Dougherty *et al*, 2005). Upon EGF stimulation of KRAS^{WT} cells, we observed the expected increases in pEGFR, pAKT, ppMEK, and RAF-RBD bound RAS. With the exception of pEGFR, all of these species were further increased significantly by ERKi treatment, indicating that under stimulated conditions, ERK-mediated negative feedback also acts at the level of RAS and/or recruitment of GEFs and GAPs, but not receptor activation. These data argue that ERK-mediated negative feedback is distributed throughout the pathway to constrain ERK activation.

However, a different pattern was observed in stimulated mutant KRAS^{G12C} and KRAS^{Q61R} cells. While pEGFR, pAKT, and ppMEK were all significantly increased by EGF stimulation, no significant increase was detected in RAF-RBD pulldown of RAS for either mutant (Fig 4A and B). The increases in pEGFR and ppMEK, though significant, were lower in magnitude in both KRAS^{G12C} and KRAS^{Q61R} compared with KRAS^{WT}, indicating a decreased pathway responsiveness relative to baseline, which is consistent with reduced ERK activity responses for these cell lines (Fig 3). The EGF-stimulated increase in ppERK in KRAS^{G12C} cells was similar to KRAS^{WT} cells, but reduced in KRAS^{Q61R} cells, also consistent with EGF-stimulated ERK activity measurements for these cell lines. As in the KRAS^{WT} cells, negative feedback was assessed by the increase in EGF-stimulated phosphorylation of each protein in the presence of ERKi relative to the vehicle treatment. Unlike KRAS^{WT} cells, ppMEK and pAKT were increased by ERKi treatment to a much lesser degree in KRAS^{G12C} and KRAS^{Q61R} cells than in KRAS^{WT}. These data suggest that ERK-mediated feedback is weaker in mutant cells relative to wild type, although they do not rule out the possibility that in the mutant cells, one or more steps in the pathway reaches saturation under these conditions, limiting the ERKi-driven increase.

ERK activity is rescaled bidirectionally, independent of pathway expression levels

The similarity in ERK activity between wild type and mutant KRAS cells contrasts starkly with the conceptual model of RAS mutations hyperactivating the pathway. As this difference is not clearly explained by direct feedback effects from ERK, we employed a mathematical modeling approach to more carefully consider other variables that could account for it (Fig 5A). In a simple linear view of RAS-to-ERK transduction, the mutant RAS GTPase activities being 50- to 800-fold lower than wild type would be expected to produce correspondingly large changes in both pre-stimulus and stimulated ERK activity. However, additional variables may modulate the mutant activity. First, variation in expression level of pathway components could compensate for the differences in RAS activity, especially if expression levels become limiting. Second, the affinities of GTP-bound RAS mutants for their effectors are not equivalent to wild type; mutation lowers the affinity for RAF up to 7-fold (Hunter *et al*, 2015). We therefore designed our modeling approach to predict the expected increase in ERK activity based on known properties of KRAS mutants.

Our modeling approach requires the relative protein concentration for components of the pathway, which we evaluated using immunoblots. Initial experiments indicated that the cell lines varied both in the total amount of protein extracted by our protocol, and in the abundance of typical loading control proteins such as actin. We therefore employed an alternative approach to normalize each sample by total protein (see Materials and Methods). Using this approach, each cell line was assessed at baseline, peak (~15 min), and steady state (~2 h) following a 10 ng/ml EGF stimulus (Fig 5B). In these samples, we also measured the fraction of ERK that is dually-phosphorylated via Phos-Tag immunoblot (Aoki *et al*, 2013). This measurement of ppERK confirmed the trends observed by FRET measurements: EGF induced a peak in ppERK which then diminished at steady state, and ppERK was elevated in mutant RAS cells only under baseline conditions (Fig 5C and D). As expected, measurements of ppERK were well correlated with the fraction of dually-phosphorylated ERK (Fig EV1). This quantitative dataset rules out two simple explanations for low ERK activity in KRAS mutants. First, compared with KRAS^{WT} cells, total RAS levels were higher in KRAS^{G12C} and KRAS^{G12V} lines, precluding the possibility that the expression level of RAS was compensating for the excess mutant activity (Fig 5B). Second, levels of BRAF, MEK, and ERK, but not CRAF, varied significantly among cell lines, but without a clear pattern or correlation structure. When total expression levels were compared with ERK activity (via EKAR), no significant correlations were found, suggesting that no individual component acts as a limiting factor. Moreover, dually-phosphorylated ERK ranged from only ~1 to ~30% of total ERK across all samples (Fig 5C), confirming that it does not reach saturation.

We parameterized our model using the measured protein levels, along with the biochemical activity of wild type and mutant KRAS proteins previously reported *in vitro* (Gremer *et al*, 2011; Smith *et al*, 2013; Hunter *et al*, 2015; Appendix Table S1). As the goal of our modeling approach was to identify potential explanations for restrained ERK signaling in KRAS mutant cells, we omitted any additional regulation, such as feedback. Effectively, the model separates operation of the core GTPases and kinases of the cascade,

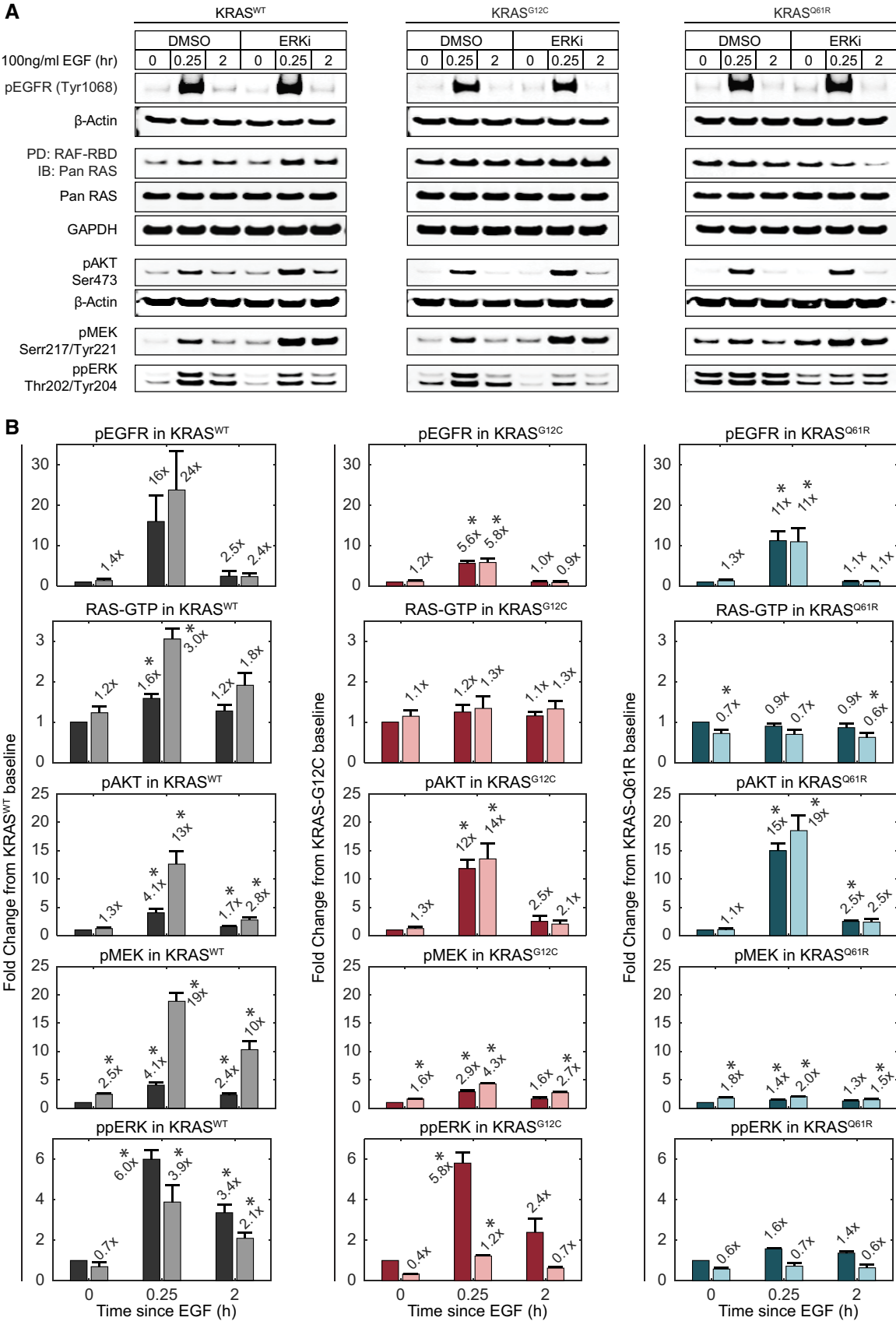


Figure 4.

Figure 4. Analysis of ERK-dependent feedback in RAS mutants.

A, B Immunoblot analysis of RAS-ERK pathway activity at multiple levels in the absence or presence of ERK inhibition by 100 nM SCH772984 (ERKi). Lysates for the indicated cell lines were collected at baseline, peak (15 min), and steady state (2 h) time points after treatment with 100 ng/ml EGF. (A) Sample blot imagery for each measurement. (B) Quantified measurements, shown as fold change relative to the DMSO-treated baseline sample. Values for pEGFR, pAKT, ppMEK, and ppERK were normalized to β -actin; data for RAF-RBD PD/Pan RAS were normalized to total Pan RAS. Bars represent the mean of triplicate measurements and error bars the standard error of the mean. Mean fold change values are printed above each bar. Dark bars: DMSO-treated; light bars: ERKi-treated. x-axis indicates the duration of EGF treatment. Asterisks indicate statistical significance by *t*-test ($P < 0.05$).

Source data are available online for this figure.

which we term “internal” factors, from “external” factors that include feedbacks, adapters, scaffolding proteins, and/or phosphatases. By comparing model predictions against experimental data, this approach identifies the differences attributable to external factors. Using a steady-state solution of this model, we predicted the baseline and EGF-stimulated steady-state levels of ppERK for the isoforms for which biochemical data is available: KRAS^{WT}, KRAS^{G12C}, KRAS^{G12D}, KRAS^{G12V}, and KRAS^{Q61R} (Fig 5E). These simulations confirmed that the experimentally measured ppERK is indeed much lower in KRAS mutants than expected, especially at baseline. Conversely, the amplitude (fold change) in ppERK upon stimulation is greater in the experimental system than in the model, except for KRAS^{Q61R} where differences are indistinguishable (Fig 5F). This analysis clarifies the role of the external factors in the ERK pathway, revealing that they have a bidirectional effect: They suppress ppERK under both baseline and stimulated conditions, but also amplify the difference between these conditions. The external factors therefore effectively increase the responsiveness of ERK to growth factor stimulation.

To gain further insight into the relative importance of internal and external factors modulating ERK activity, we extended our analysis to test whether ppERK correlates with expression level of pathway components, using partial least squares regression (PLSR). We fit both the simulated and measured ppERK against the measured protein expression and the presence of EGF stimulation. PLSR explained 75% of the variance in simulated ppERK, but only 55% of the variance in experimentally measured ppERK (Fig 5G). In the simulated ppERK data, we found significant correlations with the abundance of RAS, MEK, and ERK proteins. In contrast, ppERK in the experimental system was only significantly correlated with the presence of growth factor stimulation (Fig 5H). Thus, another key function of external factors is to confer robustness to expression level variation in cascade components, extending previous observations that the regulation of ERK phosphorylation is robust to changes in ERK expression level (Fritsche-Guenther *et al*, 2011). Altogether, our model analysis reveals that external factors increase the dynamic range of ERK response to EGF and that nearly all of this control lies in mechanisms outside of the linear RAS-to-ERK kinase cascade. This bidirectional effect on ERK activity and the moderate strength of feedback observed in mutant KRAS cells (Fig 4) imply that ERK activity in the cell is rescaled by mechanisms beyond simple negative feedback (Dougherty *et al*, 2005; Amit *et al*, 2007).

Phosphatases dynamically shape the functional ERK output

Multiple phosphatases are dynamically regulated during growth factor responses (Amit *et al*, 2007), some directly by ERK (Yoon & Seger, 2006), raising the question of whether such regulation could

contribute to the observed rescaling of ERK dynamic range. While phosphatase protein levels can be quantified, phosphatase activity is typically difficult to assay, especially in living cells. However, our experimental system provided a unique opportunity to estimate phosphatase activity acting on ERK substrates by comparing the datasets for ERK activity (measured by FRET, Figs 2 and 3) and the abundance of active ERK molecules (measured by immunoblot, Fig 4). While these measures are typically considered equivalent under the assumption that phosphatase activity should be stable, our measurements made under identical conditions reveal some differences. For example, we observe significant variation in ppERK across cell lines, while ERK activity is indistinguishable in the same lines, especially at the steady-state time point (comparing Figs 3K and L to 5D). As noted in our calibration of EKAR, the activity measurement is a ratio of the concentrations of active ERK and any phosphatases that act on ERK substrates. We therefore inferred how this phosphatase activity varied by examining the correspondence between ppERK and activity measurements (Fig 6A) and estimating the relative phosphatase activity as the ratio of these values (Fig 6B). All data used for these ratios fell within the linear range for the respective measurements (see Figs 1E and EV1).

At baseline, phosphatase activity appears nearly uniform across cell lines, except for the KRAS^{WT} measurement, which is likely an outlier (Fig 6B). This consistency in phosphatase activity between cell lines results in a significant linear correlation between ppERK and ERK activity, as expected (Fig 6A). However, after stimulation, both the slope and correlation are diminished, and variance in the estimated phosphatase activity increases. By the steady-state time point, estimated phosphatase activity rises significantly, as the different RAS cell lines settle to very similar levels of ERK activity (Fig 3L) despite varying levels of ppERK (Fig 5D). This correlational analysis implies that after stimulation, phosphatase activities and/or levels are regulated in such a manner that they act to normalize the levels of ERK activity, despite residual differences in concentration of ppERK. However, the observation that apparent phosphatase activity at steady state is uncorrelated to ppERK at baseline implies dynamic complexity beyond simple regulation by ERK.

To corroborate the implied regulation of phosphatase activity, we used an independent indicator of phosphatase activity, the decay of EKAR FRET signal following MEK inhibition. Because all of our experiments included MEK inhibitor as a final treatment, we were able to fit decay curves for individual cells with single exponential functions, whose time constant is in principle proportional to the phosphatase activity acting on the reporter (Fig 6C). Across all cell lines and treatments, a pattern emerges of increased phosphatase activity in KRAS mutants and increases, with some treatments, in cells expressing a wild-type RAS (Fig 6D), though the current dataset lacks sufficient replication to establish statistical significance

across the many conditions. We performed a more focused statistical analysis of the decay rates, comparing each cell line to $KRAS^{WT}$ at baseline (Fig 6E), and comparing each treatment to the baseline within $KRAS^{WT}$ (Fig 6F). This analysis demonstrates significantly elevated activity at baseline in several mutant cell lines, and with FGF treatment in $KRAS^{WT}$, supporting the indication that phosphatases are differentially regulated. While these analyses are

specific to phosphatases acting at the level of ERK substrates, it is likely that a similar mechanism is functionally relevant at the level of ERK or MEK, contributing to the bidirectional modulation of ERK activity in cell lines with severe $KRAS$ mutations. We validated this concept by immunoblot analysis examining DUSP6, a phosphatase that acts on ERK1/2 (Fig 6G). The level of DUSP6 is consistently elevated in the presence of ERK inhibitor, compared to baseline

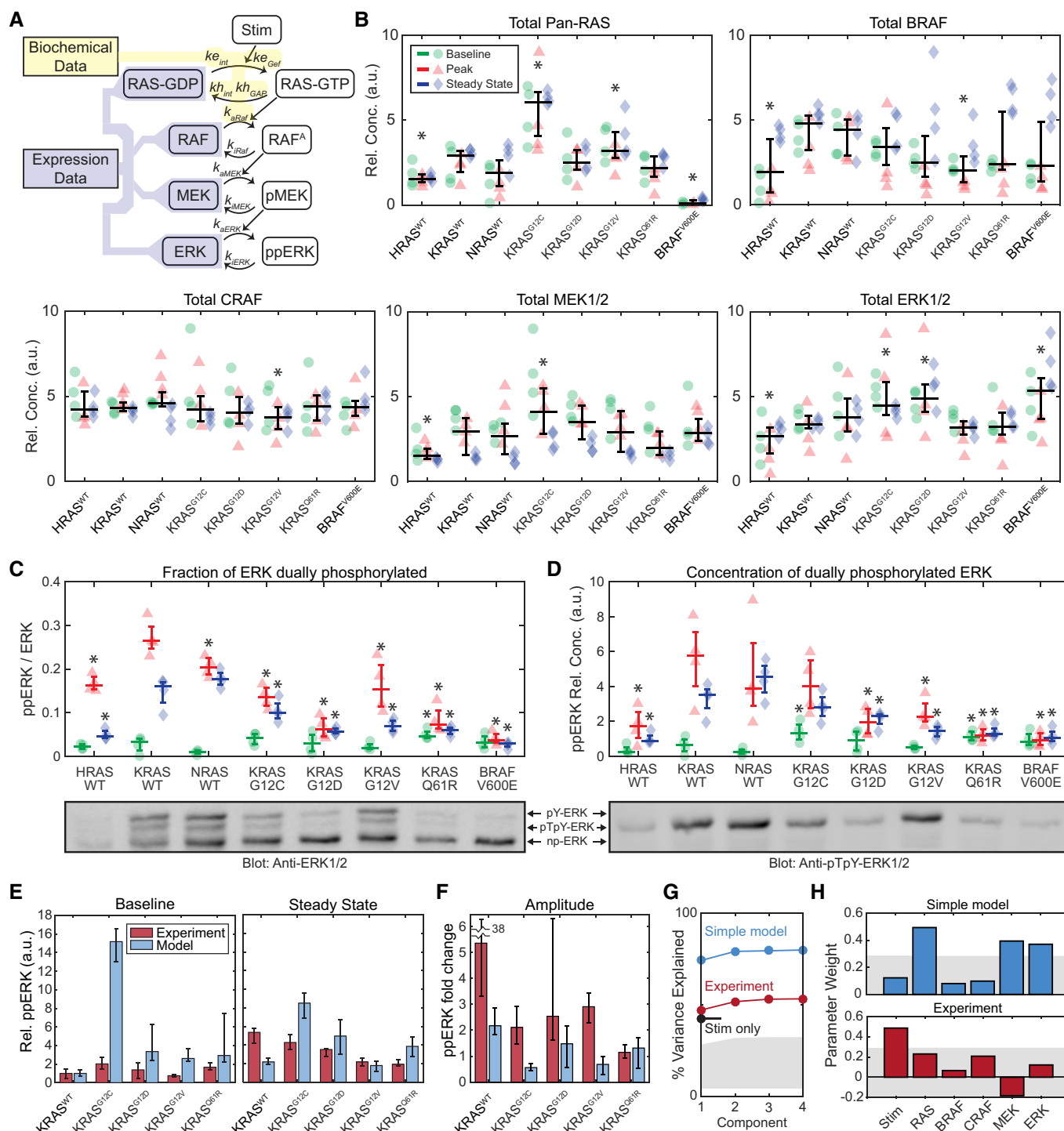


Figure 5.

Figure 5. Quantitative analysis of ERK phosphorylation in response to RAS mutation.

- A Schematic of a model of the internal factors of the RAS-ERK pathway, showing parameters associated with each reaction. Shaded regions indicate portions of the model for which parameter values are available from either (yellow) published biochemical assays or (blue) our immunoblot expression data (B–D).
- B Immunoblot measurement of RAS-ERK pathway components in each cell line, at baseline (green circles), peak activity (15 min, red triangles), and steady-state activity (2 h, blue diamonds), four replicates each. Overlaid plots indicate the median (horizontal bars) and 25th–75th percentiles (vertical whiskers) over all conditions. Asterisks indicate statistical significance from the KRAS^{WT} cell line, by t-test (pFDR < 0.05).
- C, D Phos-Tag immunoblot measurement of ERK fractional phosphorylation (C) and the relative concentration of dually-phosphorylated ERK (D), annotated as in (B), but with median and percentile ranges indicated per treatment condition, for 4 replicate cultures. Sample blot imagery shows anti-ERK1/2 (below C) and anti-ppERK1/2 (below D) for the same blot replicate. Asterisks indicate significance by t-test (pFDR < 0.05) from the KRAS^{WT} cell line measurement for each condition.
- E, F Comparison of the internal RAS-ERK model to experimental data. (E) Relative ppERK as predicted by the internal model and measured by immunoblot for the 4 replicates collected, showing the median of the baseline and steady state after EGF treatment (E), and the amplitude of stimulation (F). Error bars show 25th–75th percentiles.
- G, H Partial least squares regression of both experimental ppERK measurements and predictions via the simple RAS-ERK model. Regression was based on presence/absence of EGF, and expression levels of RAS, BRAF, CRAF, MEK, and ERK. (G) Percent of variance explained by each PLS model considered, based on how many component terms are allowed. Stim only refers to a PLS model using experimental ppERK data, but only predicting based on the presence/absence of EGF. (H) Weights assigned to each parameter in the PLS models. Gray shaded regions indicate the bounds of statistical significance, determined via bootstrapping with scrambled data. Only values that extend beyond the gray regions are statistically significant from zero ($P < 0.05$).

Source data are available online for this figure.

without growth factor. Furthermore, DUSP6 levels are rapidly and transiently suppressed on stimulation with EGF in the KRAS^{WT} and KRAS^{G12C} lines, an effect which appears at least partially mitigated by ERK inhibition. While DUSP6 is known to be primarily specific for ERK due to a direct binding interaction, its dynamic regulation demonstrates that phosphatase expression can shift on a time scale consistent with the inferred phosphatase activity. Additional work will be needed to more clearly identify the phosphatases that act on EKAR or on endogenous ERK substrates.

Discussion

Here, we used a single-cell approach to bring increased temporal resolution and quantitative rigor to the question of how oncogenic RAS mutants alter signaling behavior within the cell. Our analysis provides two major conclusions. First, our systematic dataset reconciles previously conflicting observations of ERK activity driven by RAS mutations. Second, we find that mutant-driven ERK activity is not simply suppressed by negative feedback as previously postulated, but instead that multiple mechanisms cooperate to constrain its dynamic range in response to stimuli.

A unified model of ERK activity as stimulated by growth factors and mutant RAS activity

The canonical view is that RAS mutations hyperactivate the ERK/MAPK cascade. Yet, several experimental models of the conversion of a single *Kras* allele from wild type to GTPase-defective mutant have found that this alteration results in no increase, or even a decrease, in activated ERK (Guerra *et al*, 2003; Tuveson *et al*, 2004; Konishi *et al*, 2007; Huang *et al*, 2014). While similar ERK signaling could result from negative feedback that restrains mutant-driven activity, it is less clear what mechanisms would result in lower ERK activity.

Based on the data presented here, these differences can now be attributed to the temporal and quantitative limitations of the methods previously used to measure ERK activation (primarily uncalibrated ppERK immunoblots). Our dataset recapitulates the reported attributes of mutant KRAS signaling that in isolation appear

contradictory: elevated baseline signaling, retained capacity for GF stimulation, and reduced absolute peak upon stimulus. Additionally, we find that RAS mutant cells have a reduced probability of response that is independent of their current ERK activity, especially in the case of the severe Q61R mutation. This tendency toward unresponsiveness may contribute to the reduced ERK activation observed in RAS mutant cells in the presence of serum or growth factors.

In addition to reconciling previous observations, our analysis also reveals a previously unquantified phenomenon, which is that ERK activity remains unexpectedly responsive to growth factor stimulation in cells carrying mutant KRAS (Fig 7). This responsiveness arises because, while both baseline and peak GF-stimulated ERK activity are limited in KRAS mutant cells, the relative strength of suppression is greater at baseline than at peak. This observation is consistent with the idea that dynamic range of a signaling pathway is a physiologically important parameter (Janes *et al*, 2008) and that mechanisms exist to buffer it from deleterious mutations. This effect is only reliably observable through comparisons between isogenic mutant and non-mutant cells using calibrated ERK activation measurements, underscoring the importance of a quantitative, systematic approach to complex signaling networks.

The MAPK pathway as a robust interpreter moderating mutant RAS signaling

The consistency of ERK signaling in the context of RAS mutations or changes in pathway expression has been attributed mainly to negative feedback from ERK (Courtois-Cox *et al*, 2006; Fritsche-Guenther *et al*, 2011). However, our systematic analysis reveals a more complex situation with differential suppression of ERK distributed across multiple factors. ERK-mediated negative feedback plays a significant role in restraining MEK activation, but that role appears lesser in mutant KRAS cells rather than greater (Fig 4). At least two models may explain the bidirectional rescaling of ERK signaling that we observe. One possibility is that the RAF/MEK/ERK cascade could act as fold change detector for variation in EGFR activity (Cohen-Saidon *et al*, 2009). Fold change detector models are expected to incorporate motifs such as an incoherent feedforward loop or a non-linear integral feedback loop (Adler *et al*, 2017). An alternative

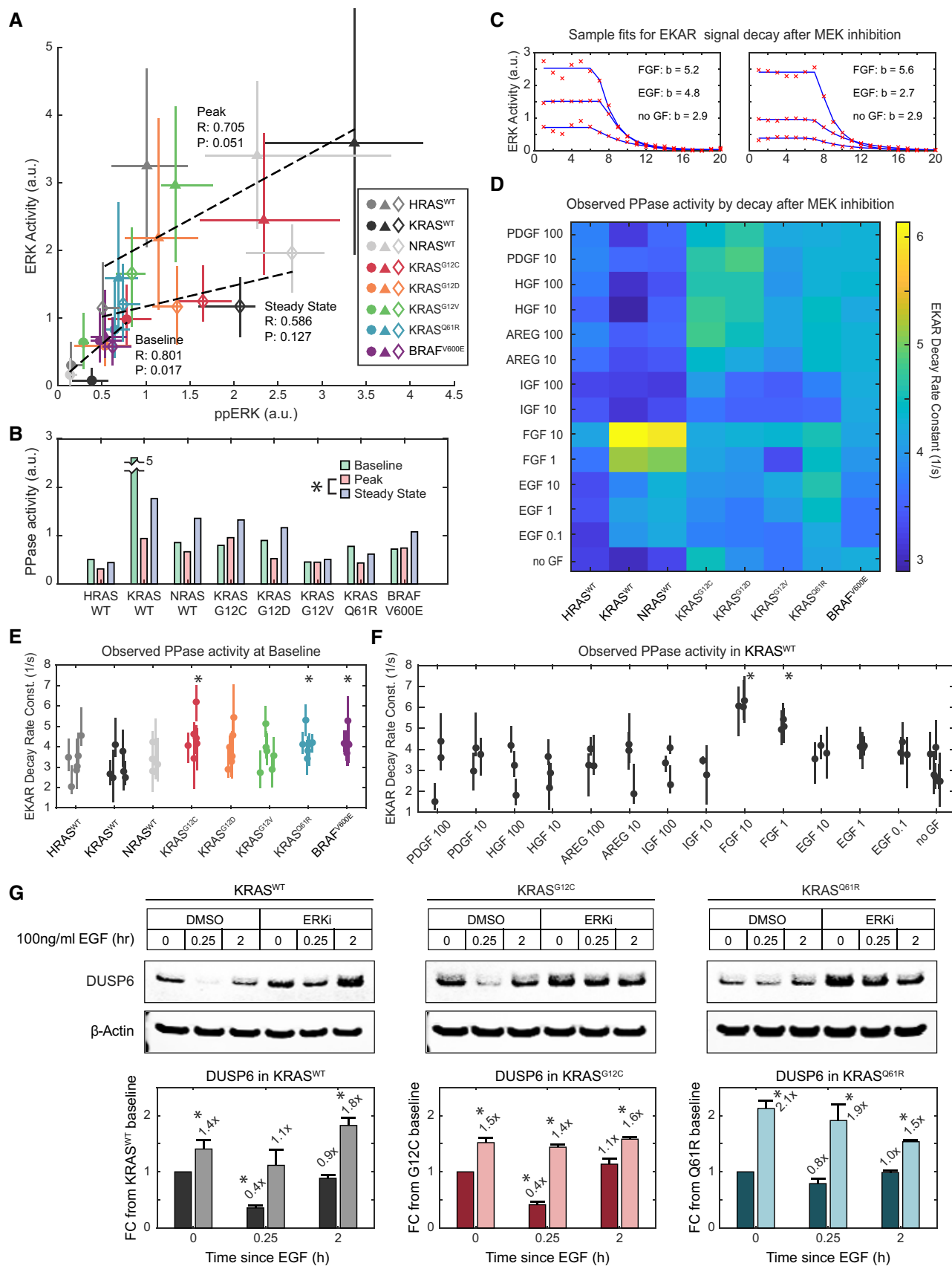


Figure 6. Inference of phosphatase activity on ERK substrates.

- A Correlation of ERK activity and ppERK concentration, median of 3 and 4 replicates, respectively, per condition. Error bars denote 25th–75th percentiles, including single-cell distributions for ERK activity. Markers are color-coded by cell line, and marker shape indicates treatment (circle: baseline, triangle: peak, diamond: steady state). Dotted lines show linear regression for each treatment; Pearson's correlation coefficients (R), and associated P -values (P) are printed alongside.
- B Estimate of substrate level phosphatase activity per cell line and treatment, calculated as ppERK/ERK activity. Asterisk indicates significance when comparing all cell lines, by t -test ($pFDR < 0.05$).
- C EKAR signal decay after MEK inhibition, example single-cell data (red x's) fit to a decaying exponential model (blue lines), with decay rate constants (b) printed.
- D Heatmap of median decay rate constants fit for each cell line and treatment.
- E, F Statistical analysis of phosphatase activities observed by EKAR signal decay at (E) baseline (i.e., no GF treatment prior to MEKi) for all cell lines compared with KRAS^{WT}, with 6 replicates, and (F) in KRAS^{WT} for all treatments, compared with no GF, with 3 replicates. Dots denote median values, and bars 25th–75th percentiles. Asterisks indicate significance by t -test ($pFDR < 0.05$); t -tests performed as detailed in Methods and Protocols "Statistical Analysis: t -tests for Single-Cell Data".
- G Immunoblot analysis of DUSP6 levels, subject to stimulation by EGF and inhibition of ERK by 100 nM SCH772984. Bars represent the mean of triplicate measurements and error bars the standard error of the mean. Asterisks indicate statistical significance by t -test ($P < 0.05$).

Source data are available online for this figure.

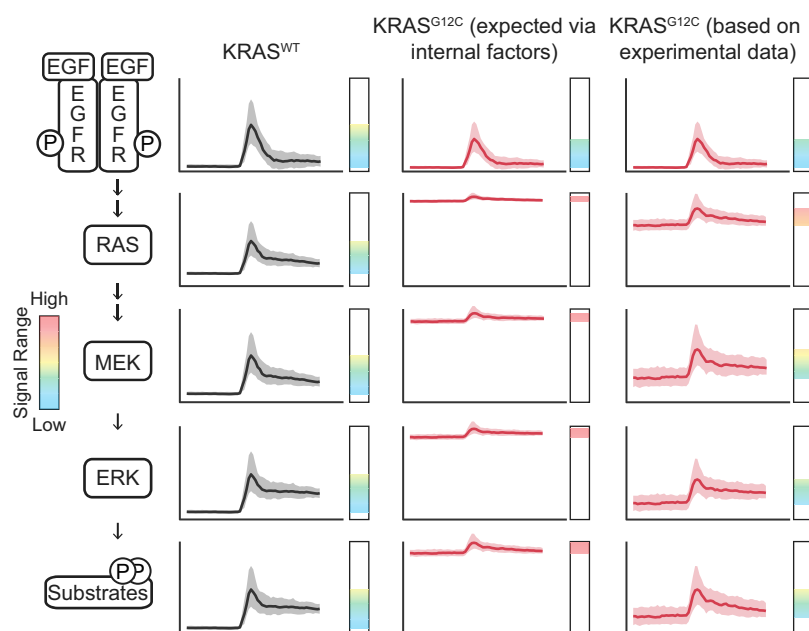
**Figure 7. Rescaling and expansion of dynamic range through the RAS/ERK pathway.**

Diagram depicts the activities we expect at various levels in the RAS/ERK pathway, as time series in our typical EGF stimulation experiment, extrapolated from our live-cell data and immunoblot measurements. From left to right, columns depict the response in KRAS^{WT}, the expected response in KRAS^{G12C} cells (as an example KRAS mutant) considering only the internal factors of the RAS-ERK pathway, and observed behavior of KRAS^{G12C} cells. Vertical bars indicate the dynamic range of the activity of that node in the network, with the colored spectrum indicating signal levels from low (blue) to high (yellow-orange) and excessive (red).

model is that the pathway is arranged for dose-response alignment (Brent, 2009), which ideally employs push–pull mechanisms or combines negative feedback with a comparator (Andrews *et al*, 2016). In both models, ERK-mediated negative feedback could play a critical part but would operate in concert with additional regulatory interactions. Such models could explain the complex behavior we observe of restraint of excessive activity from mutant KRAS that also preserves and enhances the ability of the pathway to respond to growth factor stimulus.

Another factor affecting the output of the pathway is the apparent fine-tuning of phosphatase activity acting on ERK substrates. As ERK phosphorylation is often used as a *de facto* measurement of its activity, quantitative effects at the level of substrates have received less attention. Nonetheless, the ability of ERK to maintain phosphorylation of its substrates is inherently limited by the opposing

process of dephosphorylation, making this a critical but understudied control point. Our data imply that regulation of this process is significant for an exogenous FRET-based substrate whose sequence is based on the endogenous substrate Cdc25A, warranting further study of this effect on endogenous substrates. This effect could be mediated by direct control of phosphatase activity, or through competition of substrates for the phosphatase (Rowland *et al*, 2015); future work will be needed to elucidate this mechanism.

Lastly, the potential for each RAS variant line to have been subject to selection during the process of cell line construction and propagation may play a significant role (Li *et al*, 2018). Cells receiving a RAS insertion that produces sufficiently high levels of expression to drive truly excessive ERK activity could be driven into senescence and thus prevented from establishing a cell line. Therefore, cells bearing epigenetic modifications or point mutations that

moderate the output of ERK could be overrepresented in the surviving population. While our analysis indicates that the expression level of pathway components is insignificant in determining ERK activity in the cell lines assayed, this rules out neither activity-modifying mutations that do not alter expression, nor the existence of an activity threshold above which cells are eliminated by selection. Furthermore, while our limited immunoblot-based analysis was unable to identify differences in expression that explain the divergence of ERK signaling from the expected, a more precise and comprehensive proteomic analysis could reveal overlooked correlations (Shi *et al*, 2016). Naturally, the same caveats apply to the vast majority of cell-based experiments on RAS signaling (including transient expression experiments that typically exceed at least one cell cycle). Thus, experimental strategies in which RAS isoforms are abruptly exchanged, and the resulting cellular changes monitored with high temporal resolution, would be informative in understanding the adaptation to a RAS mutation.

Constraints on RAS-driven signaling in oncogenesis

The ability of the ERK/MAPK pathway to constrain the quantitative effects of mutant KRAS raises important questions for how these mutations function in oncogenesis. In many cancers, RAS mutations are thought to occur very early in oncogenesis, and therefore, the homeostatic nature of the pathway likely plays a central role in determining whether a RAS mutant cell progresses toward malignancy (Li *et al*, 2018). Our data from cells with few other genetic abnormalities can be considered a model for signaling at this early stage, unique from studies that have investigated mutant RAS in fully developed cancers and focused on treatment of later-stage disease. However, if mutations in RAS lead to only modest changes in ERK signaling, how do they drive progression toward malignancy? One potential model was that excess ERK activity could engage lower-affinity substrates, expanding the effective ERK-driven phosphoproteome to non-traditional targets. However, given the constraints we observe on the magnitude of ERK signaling, it is impractical for these KRAS mutant cells to promote phosphorylation of non-typical ERK targets. Furthermore, KRAS mutant-bearing cells do not show longer duration of peak ERK activity following stimulus

than those with KRAS^{WT}, so excess activation after growth factor stimulus is also unlikely. Instead, our finding that the over-activating effect of KRAS mutants is limited to chronic baseline elevation implies (i) that chronic moderate signaling is sufficient to drive deleterious phenotypes and (ii) that mutant cells are unlikely to respond to normal low-level signaling.

A strong downstream effect from chronic moderate ERK activity is consistent with current models of some effectors. The ERK target gene Fra-1, a transcription factor whose expression is correlated with cancer invasiveness (Tam *et al*, 2013), integrates ERK activity over time (Gillies *et al* 2017). With its slow decay rate [half-life > 5 h (Basbous *et al*, 2007)], Fra-1 can accumulate to relatively high levels over a long period of moderately elevated ERK activity. Any ERK-induced gene products with similar degradation kinetics will also accumulate over time in cells with baseline ERK elevation. Conversely, gene products subject to rapid degradation kinetics such as c-Fos and Egr-1 would be only weakly elevated in RAS mutants, compared to the large changes in expression driven by sporadic wild-type activity. Products under negative regulation, such as those that degrade rapidly even with extended activity (Wilson *et al*, 2017), may actually be suppressed by chronic ERK activity. Thus, while enhanced ERK kinase activity as an indicator of early RAS mutant cells is difficult to detect without live-cell measurements, the resulting expression profile—particularly the ratio between long-term and short-term responsive genes—may be more informative.

While the damping of mutant RAS-driven signals at the level of ERK may appear to be a tumor suppressive mechanism; this is not necessarily the case. KRAS mutation frequencies in human cancer and data from mouse models suggest that a limited quantitative range of RAS signal (a “sweet spot”) is critical for the development of tumors (Sarkisian *et al*, 2007; Li *et al*, 2018). Pathway constraints could help RAS mutant cells to stay within this range and evade senescence or cell death due to excessive ERK activation. This paradox raises the question of whether RAS mutations are more common than downstream mutations (such as MEK or ERK) in cancer and related syndromes such as RASopathies because they are strong enough to induce increased ERK activity, or rather because they are more constrained and able to escape selection by senescence.

Materials and Methods

Reagents and Tools table

Reagent or resource	Source	Identifier
Antibodies		
Anti-ERK1/2, Rabbit	CST	9102
Anti-ERK1/2, Mouse	CST	4696
Anti-phospho-ERK1/2(Y202/Y204), Rabbit	CST	4370
Anti-MEK1/2, Mouse	CST	4694
Anti-phospho-MEK1/2(S217/S221), Rabbit	CST	9121
Anti-CRAF, Mouse	CST	12552
Anti-BRAF, Mouse	Invitrogen	MA5-15495
Anit-panRAS, Mouse	Cytoskeleton	AESA02
Anti-phospho-AKT(S473)	CST	4060

Reagents and Tools table (continued)

Reagent or resource	Source	Identifier
Anti-beta-Actin	Sigma-Aldrich	A2228
Anti-Tubulin	CST	3873
Anti-GAPDH	CST	2118
IRDye 800CW Donkey anti-Rabbit IgG	Licor	925-32213
IRDye 800CW Donkey anti-Mouse IgG	Licor	926-32212
IRDye 680RD Donkey anti-Rabbit IgG	Licor	926-68073
IRDye 680RD Donkey anti-Mouse IgG	Licor	925-68072
Chemicals, peptides, and recombinant proteins		
Amphiregulin	Peprotech	100-55B
EGF	Peprotech	AF-100-15
FGF	Peprotech	100-18B
HGF	Peprotech	100-39
IGF-I	Peprotech	100-11
PDGF-AB	Peprotech	100-00AB
ARS-853	MedChemExpress	HY-19706
PD0325901	Selleck Biochemicals	S1036
SCH772984	Selleck Biochemicals	S7101
Blasticidin	Corning	30-100-R1
Bovine serum albumin	Sigma-Aldrich	A7906
Collagen I, rat tail	Life Technologies	A10483-01
Dextrose	Fisher	C6H1206
L-Glutamine	Life Technologies	25030-081
Puromycin dihydrochloride	Sigma-Aldrich	P8833
DMEM	Life Technologies	11965-092
Fetal bovine serum	Gemini Bio-products	100-106
Penicillin streptomycin	Life Technologies	15070-063
0.25% Trypsin-EDTA	Life Technologies	25200-056
Tris Base	Fisher	BP152
Glycine (Crystalline Powder)	Fisher	BP381
Sodium dodecyl sulfate (SDS), Micropellets	Fisher	BP8200500
Ponceau S solution, suitable for electrophoresis, 0.1% (w/v) in 5% acetic acid, 1L	Sigma-Aldrich	P7170-1L
Bromophenol blue	Sigma-Aldrich	B5525
Dithiothreitol	Fisher	BP172
Critical commercial assays		
Amaya MEF 2 Nucleofector kit	Lonza	VPD-1005
Experimental models: cell lines		
RAS-less MEF (Mouse Embryonic Fibroblast) cell lines with transduced human RAS or BRAFV600E	Gift from Dom Esposito, Frederick National Laboratory	
HRAS ^{WT} EKAR3	This paper	Available on request
KRAS4B ^{WT} EKAR3	This paper	Available on request
NRAS ^{WT} EKAR3	This paper	Available on request
KRAS ^{G12C} EKAR3	This paper	Available on request
KRAS ^{G12D} EKAR3	This paper	Available on request

Reagents and Tools table (continued)

Reagent or resource	Source	Identifier
KRAS ^{G12V} EKAR3	This paper	Available on request
KRAS ^{Q61R} EKAR3	This paper	Available on request
BRAF ^{V600E} EKAR3	This paper	Available on request
Recombinant DNA		
Plasmid: pPBJ-EKAR3nls-puro	Sparta <i>et al</i> (2015)	Addgene # forthcoming
Software and Algorithms		
NIS Elements AR ver. 4.20	Nikon	RRID:SCR_014329
Bio-Formats ver. 5.1.1 (May 2015)	OME	RRID:SCR_000450
uTrack 2.0	Jaquaman <i>et al</i> (2008)	http://www.utsouthwestern.edu/labs/danuser/software/
MATLAB	Mathworks	SCR_001622
Other		
Glass-Bottom Plates, #1.5 cover glass	Cellvis	P24-1.5H-N, P96-1.5H-N
12% Mini-PROTEAN [®] TGX [™] Precast Protein Gels, 15-well, 15 μ l	Bio-Rad	4561046
SuperSep Phos-tag gels (50 μ mol/l), 12.5%, 17 wells	Wako-Chem	195-17991
GE Healthcare Amersham [™] Protran [™] NC Nitrocellulose Membranes: Rolls, 0.1 μ m pore	Fisher	45-004-000

Methods and Protocols

Cell culture

Mouse embryonic fibroblasts expressing a single RAS isoform were obtained from the Frederick National Laboratory of the National Cancer Institute, Frederick, MD. Cells were authenticated through Whole Exome Sequencing, PCR, and immuno blot methods at the Frederick National Laboratory. Mycoplasma testing was performed on a regular basis with negative results of no contamination. Cells were cultured in DMEM supplemented with 0.2% bovine serum albumin (BSA) and 2.5 μ g/ml puromycin or 4 μ g/ml blasticidin. For imaging experiments, cells were cultured in a custom imaging media composed of DMEM lacking phenol red, folate and riboflavin, glucose, glutamine, and pyruvate, supplemented with 0.1% BSA, 4 mM L-glutamine, and 25 mM glucose.

Reporter cell line construction

Cells were electroporated using a Lonza Nucleofector electroporator. EKAR3 was stably integrated into cells using the piggyBAC transposase system (Pargett *et al*, 2017). Positive integrants were selected by fluorescence-based cell sorting.

Live-cell microscopy

Multi-well plates with #1.5 glass bottoms were coated with collagen and seeded with reporter cell lines 1 day prior to imaging. Prepared culture plates were imaged on a Nikon Ti-E inverted microscope with a stage-top incubator to maintain the culture at 37°C and 5% CO₂ throughout the experiment. Microscopy and image processing performed as described in (Pargett *et al*, 2017). Imaging sites within each well were selected and imaged sequentially at each acquisition time, automated via the NIS Elements AR software. Images were captured using a 20 $\times$ /0.75 NA objective and an Andor Zyla 5.5 scMOS

camera. Filter sets used were Chroma #49001 (ET-CFP) and #49003 (ET-YFP).

Immunofluorescence microscopy

After growth and treatment as indicated on glass-bottom 96-well plates, cells were fixed for 30 min at room temperature with a freshly prepared solution of 12% paraformaldehyde in PBS and permeabilized with 1% Triton X-100. Samples were then stained with primary and secondary antibodies in PBS + 0.1% Triton X-100 + 2% bovine serum albumin, and images were captured on a Nikon Ti-E inverted microscope with a 20 $\times$ /0.75 NA objective with an Andor Zyla 5.5 scMOS camera.

Image processing

Imaging data were processed to segment and average pixels within each identified cell's nucleus and cytoplasm, using a custom procedure written for MATLAB (Pargett *et al*, 2017). The procedure accessed image data from ND2 files generated by NIS Elements, using the Bio-Formats MATLAB toolbox, and tracked single-cell positions over time using uTrack 2.0 (Jaquaman *et al*, 2008). The resulting single-cell time series traces were filtered for quality (minimum length of trace, maximum number of contiguous missing, or corrupt data points), and ratiometric reporter levels calculated. EKAR3 level was calculated as $1 - ((CFP/YFP)/R_p)$ as, where CFP and YFP are the pixel intensities of the cyan and yellow channels, respectively, and R_p is the ratio of total power collected in cyan over that of yellow (each computed as the spectral products of relative excitation intensity, exposure time, molar extinction coefficient, quantum yield, light source spectrum, filter transmissivities, and fluorophore absorption and emission spectra). See Appendix Supplementary Methods for detailed interpretation of the EKAR3 signal.

Immunoblotting

For immunoblot experiments, assaying pathway activity and feed-back sensitivity (all blots in Fig 4), cells were seeded at a density of 2.5×10^6 cells per 10 cm plate and starved of growth factor for 6 h in imaging media. Cells were pre-treated with DMSO or 100 nM SCH727283 (Selleckchem) (Morris *et al*, 2013) for the last hour of starvation. Cells were then stimulated with vehicle or 100 ng/ml EGF for 15 min or 2 h and lysed with Cell Lysis Buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, 2% Igepal) (Cytoskeleton Inc.) containing protease and phosphatase inhibitors (Pierce/Thermo Fisher Scientific). Lysates were clarified by centrifugation at $>5,000 g$ for 2 min at $4^\circ C$ and snap-frozen in liquid nitrogen with protein concentrations measured using the BCA protein assay (Pierce/Thermo Fisher Scientific). For RAS activation assays, 300 μg of total cell protein was used to pull-down GTP-bound RAS/RAF-RBD complexes according to the manufacturer's instructions (Cytoskeleton). Activated RAS or 20 μg of total cell protein were separated using NuPAGE Novex Bis-Tris gels (Invitrogen/Thermo Fisher Scientific) and transferred to PVDF membrane using an iBlotTM 2 dry blotting system (Invitrogen/Thermo Fisher Scientific). Immunoblot data for this assay were analyzed using the Odyssey[®] application software v3.030 as described previously (Silva & McMahon, 2014). Statistical significance was determined by *t*-test analyses of three independent experiments.

For immunoblot analysis of pathway expression levels (all blots in Fig 5), a different loading and normalization procedure was followed to allow cell lines to be compared despite reproducible differences in protein extraction efficiency and loading control protein expression. We prepared four replicate samples of each treatment (baseline, stimulation, or steady state) for each of the eight cell lines. Cells were plated in 10 cm plates at a density equivalent to that in our imaging experiments (1.7×10^6 cells, $\sim 30,000$ cells/cm²). Cultures to be stimulated were treated with EGF at 10 ng/ml EGF for 15 min or 2 h, matching the timing of peak activity in live-cell data. Cultures were lysed in 500 μl RIPA buffer with protease and phosphatase inhibitors and clarified as above. These lysates were assayed for protein content by DC protein assay (Bio-Rad) and frozen. Immunoblotting was performed using SDS-PAGE with 12.5% acrylamide or 4–15% gradient gels (Bio-Rad, Cat #4561046, Cat #4561086). To include the many samples in this dataset on the same scale via immunoblot, we loaded lysates based on volume and employed lane-to-lane normalization by total protein load and blot-to-blot normalization by including one sample, the “control”, from each blot together on a reference blot. In this way, variations in staining efficiency among membranes were accounted for by scaling all lanes (per target protein) such that the control sample matched its intensity on the reference blot. Normalization to protein load was performed by Ponceau S stain and is applied for all samples prior to normalization across blots, such that variation in the Ponceau stain is also included in the reference blot. Intensity measurements were performed using ImageJ, with background samples collected adjacent to each band/region of interest.

Phos-Tag immunoblotting

We employed the Phos-Tag method using precast gels (SuperSep Phos-Tag 12.5% Cat #195-17991, and 7.5% Cat #192-18001,

FUJIFILM Wako Chemicals). However, in accordance with previous observations with these gels (Kinoshita-Kikuta *et al*, 2012) and the observation that they likely have excess Phos-Tag reagent, we use samples collected in EDTA-containing RIPA buffer and we performed electrophoresis with Tris-Glycine running buffer (as with above). Phos-Tag fractional phosphorylation measurements are internally controlled and required neither cross-load or cross-blot normalization. See Appendix for detailed Appendix Supplementary Methods.

Statistical analysis

For all imaging experiments shown, a minimum of 100 cells were imaged and tracked for each condition. Single-cell data points were excluded as outliers if greater than six standard deviations from the dataset mean. For all analyses, at least three independent experimental replicates were performed. Where indicated, single-cell data were normalized to the median value of the PD0325901-treated period. All statistical and computational tasks were performed using MATLAB. Each single-cell trace was normalized to the minimum value in a 1 h window following treatment with 100 nM PD0325901. Baseline values were calculated by taking the mean of the 2 h window prior to stimulation for each cell. The mean was calculated from a 2 h window following treatment with the specified growth factor or vehicle control.

Statistical analysis: single-cell metrics

Volatility is calculated as the scaled mean absolute derivative, i.e., the sum of the absolute value of the derivative over a 2 h window following stimulation, divided by the mean of the same window. Responders were defined as cells with (i) post-stimulation ERK activity that significantly increased at least 5% compared to the average baseline value, and (ii) a higher maximum derivative at the time of stimulation compared to the baseline region. Response rate was defined as the time from stimulation to the peak ERK activity in each responding cell.

Statistical analysis: t-tests for single-cell data

Unless otherwise indicated, each statistical comparison was made by *t*-test with unequal variances, and false discovery rate was controlled within each dataset via the Benjamini and Hochberg Step-Up procedure ($\alpha = 0.05$). Where replicates were available at the single-cell level as well as across experiments, the variance of the mean for each experiment was determined from single-cell samples and added to variance across experiments. This corresponds to a linear error model: $\epsilon_i = \epsilon_{\text{cell}} + \epsilon_{\text{exp}}$, where the error (from the mean) of an individual cell ϵ_i equals the sum of the errors arising from cell-to-cell variation ϵ_{cell} and from experiment variation ϵ_{exp} .

Data availability

All per cell fluorescence data and immunoblot images are provided as source data files for each figure. Note that data presented graphically in Figs 1 and 3 are drawn from the large dataset associated with Fig 2. Raw image data are available via BioStudies: <https://www.ebi.ac.uk/biostudies/studies/S-BSST511>.

Expanded View for this article is available online.

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Author contributions

MP, JGA, FM: Conceptualized the study; TEG, MP, JMS, FM, JGA: Designed the experiments; TEG, CKT, JMS, FM: Prepared cell lines; TEG, CKT: Performed live-cell experiments; TEG, MP, JMS: Performed immuno blot experiments; MP: Prepared mathematical models; TEG, MP, JMS, JGA: Analyzed the data; TEG, MP, JGA: Wrote the manuscript; TEG, MP, JMS, CT, FM, JGA: Reviewed/edited the manuscript.

Conflict of interest

The authors acknowledge the following potential sources for conflicts of interest. Frank McCormick is a consultant for the following companies: Aduro Biotech, Amgen, Daiichi Ltd., Ideaya Biosciences, Kura Oncology, Leidos Biomedical Research, Inc., PellePharm, Pfizer Inc., PMV Pharma, Portola Pharmaceuticals, and Quanta Therapeutics. Dr. McCormick has received research grants from Daiichi Sankyo Ltd. and is a recipient of funded research from Gilead Sciences. Dr. McCormick is a consultant and co-founder for the following companies (with ownership interest including stock options): BridgeBio, DNATRIX Inc., Olema Pharmaceuticals, Inc., and Quartz. Dr. McCormick is Scientific Director of the NCI Ras Initiative at Frederick National Laboratory for Cancer Research/Leidos Biomedical Research Inc. John Albeck has received research grants from Kirin Corporation.

References

- Adler M, Szekely P, Mayo A, Alon U (2017) Optimal regulatory circuit topologies for fold-change detection. *Cell Syst* 4: 171–181 e178
- Albeck JG, Mills GB, Brugge JS (2013) Frequency-modulated pulses of ERK activity transmit quantitative proliferation signals. *Mol Cell* 49: 249–261
- Amit I, Citri A, Shay T, Lu Y, Katz M, Zhang F, Tarcic G, Siwak D, Lahad J, Jacob-Hirsch J et al (2007) A module of negative feedback regulators defines growth factor signaling. *Nat Genet* 39: 503–512
- An H, Harper JW (2018) Systematic analysis of ribophagy in human cells reveals bystander flux during selective autophagy. *Nat Cell Biol* 20: 135–143
- Andrews SS, Peria WJ, Yu RC, Colman-Lerner A, Brent R (2016) Push-Pull and feedback mechanisms can align signaling system outputs with inputs. *Cell Syst* 3: 444–455 e442
- Aoki K, Takahashi K, Kaizu K, Matsuda M (2013) A quantitative model of ERK MAP kinase phosphorylation in crowded media. *Sci Rep* 3: 1541
- Basbos J, Chalbos D, Hipskind R, Jariel-Encontre I, Piechaczyk M (2007) Ubiquitin-independent proteasomal degradation of Fra-1 is antagonized by Erk1/2 pathway-mediated phosphorylation of a unique C-terminal destabilizer. *Mol Cell Biol* 27: 3936–3950
- Birtwistle MR, von Kriegsheim A, Kida K, Schwarz JP, Anderson KI, Kolch W (2011) Linear approaches to intramolecular Forster resonance energy transfer probe measurements for quantitative modeling. *PLoS ONE* 6: e27823
- Birtwistle MR, Rauch J, Kiyatkin A, Aksamitiene E, Dobrzynski M, Hoek JB, Kolch W, Ogunnaike BA, Kholodenko BN (2012) Emergence of bimodal cell population responses from the interplay between analog single-cell signaling and protein expression noise. *BMC Syst Biol* 6: 109
- Brandt R, Sell T, Luthen M, Uhlitz F, Klinger B, Riemer P, Giesecke-Thiel C, Schulze S, El-Shimy IA, Kunkel D et al (2019) Cell type-dependent differential activation of ERK by oncogenic KRAS in colon cancer and intestinal epithelium. *Nat Commun* 10: 2919
- Brent R (2009) Cell signaling: what is the signal and what information does it carry? *FEBS Lett* 583: 4019–4024
- Chaikuad A, Tacconi EMC, Zimmer J, Liang Y, Gray NS, Tarsounas M, Knapp S (2014) A unique inhibitor binding site in ERK1/2 is associated with slow binding kinetics. *Nat Chem Biol* 10: 853–860
- Cohen-Saidon C, Cohen AA, Sigal A, Liron Y, Alon U (2009) Dynamics and variability of ERK2 response to EGF in individual living cells. *Mol Cell* 36: 885–893
- Courtois-Cox S, Genther Williams SM, Reczek EE, Johnson BW, McGillicuddy LT, Johannessen CM, Hollstein PE, MacCollin M, Cichowski K (2006) A negative feedback signaling network underlies oncogene-induced senescence. *Cancer Cell* 10: 459–472
- Dougherty MK, Müller J, Ritt DA, Zhou M, Zhou XZ, Copeland TD, Conrads TP, Veenstra TD, Lu KP, Morrison DK (2005) Regulation of Raf-1 by direct feedback phosphorylation. *Mol Cell* 17: 215–224
- Drosten M, Dhawahir A, Sum EY, Urosevic J, Lechuga CG, Esteban LM, Castellano E, Guerra C, Santos E, Barbacid M (2010) Genetic analysis of Ras signalling pathways in cell proliferation, migration and survival. *EMBO J* 29: 1091–1104
- Ebisuya M, Kondoh K, Nishida E (2005) The duration, magnitude and compartmentalization of ERK MAP kinase activity: mechanisms for providing signaling specificity. *J Cell Sci* 118: 2997–3002
- Fernandez-Medarde A, Santos E (2011) Ras in cancer and developmental diseases. *Genes Cancer* 2: 344–358
- Fritsche-Guenther R, Witzel F, Sieber A, Herr R, Schmidt N, Braun S, Brummer T, Sers C, Blüthgen N (2011) Strong negative feedback from Erk to Raf confers robustness to MAPK signalling. *Mol Syst Biol* 7: 489
- Gillies TE, Pargett M, Minguet M, Davies AE, Albeck JG (2017) Linear integration of ERK activity predominates over persistence detection in Fra-1 regulation. *Cell Syst* 5: 549–563
- Gremer L, Merbitz-Zahradnik T, Dvorsky R, Cirstea IC, Kratz CP, Zenker M, Wittinghofer A, Ahmadian MR (2011) Germline KRAS mutations cause aberrant biochemical and physical properties leading to developmental disorders. *Hum Mutat* 32: 33–43
- Guerra C, Mijimolle N, Dhawahir A, Dubus P, Barradas M, Serrano M, Campuzano V, Barbacid M (2003) Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context. *Cancer Cell* 4: 111–120
- Harvey CD, Ehrhardt AG, Cellurale C, Zhong H, Yasuda R, Davis RJ, Svoboda K (2008) A genetically encoded fluorescent sensor of ERK activity. *Proc Natl Acad Sci USA* 105: 19264–19269
- Huang H, Daniluk J, Liu Y, Chu J, Li Z, Ji B, Logsdon CD (2014) Oncogenic K-Ras requires activation for enhanced activity. *Oncogene* 33: 532–535
- Hunter JC, Manandhar A, Carrasco MA, Gurbani D, Gondi S, Westover KD (2015) Biochemical and structural analysis of common cancer-associated KRAS mutations. *Mol Cancer Res* 13: 1325–1335
- Janes KA, Reinhardt HC, Yaffe MB (2008) Cytokine-induced signaling networks prioritize dynamic range over signal strength. *Cell* 135: 343–354

- Jaqaman K, Loerke D, Mettlen M, Kuwata H, Grinstein S, Schmid SL, Danuser G (2008) Robust single-particle tracking in live-cell time-lapse sequences. *Nat Methods* 5: 695–702
- Kinoshita-Kikuta E, Kinoshita E, Koike T (2012) A laborsaving, timesaving, and more reliable strategy for separation of low-molecular-mass phosphoproteins in Phos-tag affinity electrophoresis. *Int J Chem* 4: 1–8
- Konishi H, Karakas B, Abukhdeir AM, Lauring J, Gustin JP, Garay JP, Konishi Y, Gallmeier E, Bachman KE, Park BH (2007) Knock-in of mutant K-ras in nontumorigenic human epithelial cells as a new model for studying K-ras mediated transformation. *Cancer Res* 67: 8460–8467
- Li S, Balmain A, Counter CM (2018) A model for RAS mutation patterns in cancers: finding the sweet spot. *Nat Rev Cancer* 18: 767–777
- Mercer K, Giblett S, Green S, Lloyd D, DaRocha Dias S, Plumb M, Marais R, Pritchard C (2005) Expression of endogenous oncogenic V600EB-raf induces proliferation and developmental defects in mice and transformation of primary fibroblasts. *Cancer Res* 65: 11493–11500
- Morris EJ, Jha S, Restaino CR, Dayananth P, Zhu H, Cooper A, Carr D, Deng Y, Jin W, Black S et al (2013) Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. *Cancer Discov* 3: 742–750
- Nakakuki T, Birtwistle MR, Saeki Y, Yumoto N, Ide K, Nagashima T, Brusch L, Ogunnaike BA, Okada-Hatakeyama M, Kholodenko BN (2010) Ligand-specific c-Fos expression emerges from the spatiotemporal control of ErbB network dynamics. *Cell* 141: 884–896
- Omerovic J, Hammond DE, Clague MJ, Prior IA (2008) Ras isoform abundance and signalling in human cancer cell lines. *Oncogene* 27: 2754–2762
- Pargett M, Gillies TE, Teragawa CK, Sparta B, Albeck JG (2017) Single-cell imaging of ERK signaling using fluorescent biosensors. *Methods Mol Biol* 1636: 35–59
- Park KS, Jeon SH, Kim SE, Bahk YY, Holmen SL, Williams BO, Chung KC, Surh YJ, Choi KY (2006) APC inhibits ERK pathway activation and cellular proliferation induced by RAS. *J Cell Sci* 119: 819–827
- Prior IA, Lewis PD, Mattos C (2012) A comprehensive survey of Ras mutations in cancer. *Cancer Res* 72: 2457–2467
- Purvis JE, Lahav G (2013) Encoding and decoding cellular information through signaling dynamics. *Cell* 152: 945–956
- Regot S, Hughey JJ, Bajar BT, Carrasco S, Covert MW (2014) High-sensitivity measurements of multiple kinase activities in live single cells. *Cell* 157: 1724–1734
- Rowland MA, Harrison B, Deeds EJ (2015) Phosphatase specificity and pathway insulation in signaling networks. *Biophys J* 108: 986–996
- Sarkisian CJ, Keister BA, Stairs DB, Boxer RB, Moody SE, Chodosh LA (2007) Dose-dependent oncogene-induced senescence *in vivo* and its evasion during mammary tumorigenesis. *Nat Cell Biol* 9: 493–505
- Shi T, Niepel M, McDermott JE, Gao Y, Nicora CD, Chrisler WB, Markillie LM, Petyuk VA, Smith RD, Rodland KD et al (2016) Conservation of protein abundance patterns reveals the regulatory architecture of the EGFR-MAPK pathway. *Sci Signal* 9: rs6
- Silva JM, McMahon M (2014) The fastest Western in town: a contemporary twist on the classic Western blot analysis. *J Vis Exp* 84: e51149
- Smith MJ, Neel BG, Ikura M (2013) NMR-based functional profiling of RASopathies and oncogenic RAS mutations. *Proc Natl Acad Sci USA* 110: 4574–4579
- Sparta B, Pargett M, Minguet M, Distor K, Bell G, Albeck JG (2015) Receptor level mechanisms are required for epidermal growth factor (EGF)-stimulated extracellular signal-regulated kinase (ERK) activity pulses. *J Biol Chem* 290: 24784–24792
- Tam WL, Lu H, Buikhuisen J, Soh BS, Lim E, Reinhardt F, Wu ZJ, Krall JA, Bieri B, Guo W et al (2013) Protein kinase C alpha is a central signaling node and therapeutic target for breast cancer stem cells. *Cancer Cell* 24: 347–364
- Tuveson DA, Shaw AT, Willis NA, Silver DP, Jackson EL, Chang S, Mercer KL, Grochow R, Hock H, Crowley D et al (2004) Endogenous oncogenic K-ras (G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell* 5: 375–387
- Wilson MZ, Ravindran PT, Lim WA, Toettcher JE (2017) Tracing information flow from Erk to target gene induction reveals mechanisms of dynamic and combinatorial control. *Mol Cell* 67: 757–769 e755
- Yeh JJ, Routh ED, Rubinas T, Peacock J, Martin TD, Shen XJ, Sandler RS, Kim HJ, Keku TO, Der CJ (2009) KRAS/BRAF mutation status and ERK1/2 activation as biomarkers for MEK1/2 inhibitor therapy in colorectal cancer. *Mol Cancer Ther* 8: 834–843
- Yoon S, Seger R (2006) The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. *Growth Factors* 24: 21–44



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