Frequency-Modulated Pulses of ERK Activity Transmit Quantitative Proliferation Signals

John G. Albeck,1 Gordon B. Mills,2 and Joan S. Brugge1,*
1Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
2Department of Systems Biology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA
*Correspondence: joan_brugge@hms.harvard.edu
http://dx.doi.org/10.1016/j.molcel.2012.11.002

SUMMARY
The EGF-stimulated ERK/MAPK pathway is a key conduit for cellular proliferation signals and a therapeutic target in many cancers. Here, we characterize two central quantitative aspects of this pathway: the mechanism by which signal strength is encoded and the response curve relating signal output to proliferation. Under steady-state conditions, we find that ERK is activated in discrete, asynchronous pulses with frequency and duration determined by extracellular concentrations of EGF spanning the physiological range. In genetically identical sister cells, cell-to-cell variability in pulse dynamics influences the decision to enter S phase. While targeted inhibition of EGFR reduces the frequency of ERK activity pulses, inhibition of MEK reduces their amplitude. Continuous response curves measured in multiple cell lines reveal that proliferation is effectively silenced only when ERK pathway output falls below a threshold of ~10%, indicating that high-dose targeting of the pathway is necessary to achieve therapeutic efficacy.

INTRODUCTION
Signal transduction networks transmit information about the external environment of the cell and integrate these inputs to trigger discrete cell fate decisions. The biochemical events involved in signal transduction have been studied in many systems, providing a detailed view of the molecular paths through which information flows from cell surface receptors to transcription factors and other effectors of cell state. However, much less is known about how quantitative information is transmitted by these networks. In the simplest cases, the number or fraction of responding signaling molecules activated inside the cell is proportional to the extracellular concentration of the stimulating ligand (Brent, 2009). In other cases, quantitative information about a constant extracellular stimulus is carried not by the number of molecules responding (or signal “amplitude”), but by the frequency with which the pool of responding signaling molecules shifts between “on” and “off” states (and thus termed “frequency modulation”) (Cai et al., 2008; Hao and O’Shea, 2012).

While many quantitative studies of signal transduction have focused on unicellular systems, much remains to be learned in metazoans, where quantitative signaling properties play a central role in development and disease. Appropriate responses to quantitative variations in morphogen gradients are essential in developmental processes, and detailed “response curves” have been mapped in which cellular response is plotted as a continuous function of the strength of an upstream signal (Gregor et al., 2007). In cancer, key oncogenes such as c-Myc and Ras elicit different cellular responses depending on the extent to which they are activated, but these determinations have been made for only three to five discrete signal levels (Murphy et al., 2008; Sarkisian et al., 2007). Continuous signal-response maps spanning the full dynamic range of output for pathways involved in tumor growth and survival would facilitate rational cancer therapy by indicating the level of pathway inhibition necessary to achieve a biologically significant change in proliferation rate (Figure 1A).

The EGFR-ERK/MAPK (epidermal growth factor receptor extracellular-regulated kinase/mitogen-activated protein kinase) signaling cascade is a central driver of cell proliferation in many cancers and the target of clinically relevant inhibitors. While quantitative and systems-level analyses of EGF-stimulated ERK activity have been performed (Amit et al., 2007; Chen et al., 2009; Nakakuki et al., 2010; Santos et al., 2007; Sturm et al., 2010; Zwang et al., 2011), these studies have focused on acute restimulation of cells with growth factors following a period of withdrawal, which induces ERK signaling within minutes, followed by proliferation many hours later. This temporal separation between signal and response obscures the signal-response relationship because multiple characteristics of the initial signal pulse—including delay, amplitude, frequency, or duration—may contribute to control of phenotype (Asthagiri et al., 2000; Traverse et al., 1994). Signaling and proliferation can be more easily related when both processes have reached steady state (at the population level), because the magnitude of each can be represented by a single time-independent average (Figure 1A). Steady-state conditions also more accurately model the cellular response to chronic EGF exposure, which occurs in many physiological and tumor environments.

Here, to understand how quantitative information is transmitted by the EGFR-ERK signaling pathway, we utilize live and fixed single-cell methods to measure signal strength and dynamics under conditions of steady-state EGF stimulation. We find that this pathway incorporates both frequency- and amplitude-modulated elements: ERK is activated in discrete pulses...
that are integrated to set graded levels of downstream effectors. We show that inhibitors acting at different levels of the ERK cascade can modify the amplitude or the frequency of ERK activity, and we use our quantitative measurements to map a continuous signal–response relationship between ERK pathway output and proliferation rate across the pathway’s full dynamic range.

RESULTS

ERK Pathway Dynamics at Steady State

Working with MCF-10A mammary epithelial cells, which require EGF-stimulated ERK signaling for proliferation (Et hier and Moorth y, 1991), we assembled a panel of live-cell and high-content immunofluorescence (HCIF) measurements for ERK pathway signaling and proliferation (Figure 1B). ERK activity was monitored by HCIF detection of phosphorylated ERK (pERK) and by live-cell measurement of a Förster resonance energy transfer (FRET)-based ERK activity reporter, EKAR-EV, with rapid response kinetics (Harvey et al., 2008; Komatsu et al., 2011). Downstream output of the ERK pathway was monitored in fixed cells by HCIF for ERK effectors, including c-Fos, c-Myc, and Fra-1, and in live cells by a newly developed live-cell reporter consisting of YFP fused to the PEST domain of Fra-1, termed FIRE (Fra-1-based integrative reporter of ERK; Figure 1C). Because phosphorylation by ERK (or the downstream kinase RSK) stabilizes the Fra-1 PEST domain (Casalino et al., 2003; Vial and Marshall, 2003), FIRE intensity increases by up to 7-fold upon ERK induction (Figure 1D), and decays upon inhibition of ERK or EGFR (Figure 1E). Cellular proliferation was monitored by HCIF detection of phosphorylated Rb protein (pRb) or a geminin-based live-cell reporter (Sakaue-Sawano et al., 2008), both of which initiate near the onset of S phase and remain “on” until the beginning of the next cell cycle (Figure S1 available online). Control experiments confirmed that the fraction of pRb-positive (fpRb+) cells within a population is closely correlated to the population doubling rate (Figure S1).

Using live-cell imaging, we monitored populations of 200–1,000 cells over the course of 4 days, allowing individual cells to be tracked for four to five successive cell cycles (Movie S1). Although individual cells vary in signaling and cell-cycle status over the course of the experiment (Figure 1F), it was possible to maintain the population-average ERK signaling and proliferation at nearly constant steady-state levels between 48 and 72 hr after stimulation (Figure 1G). Depletion of EGF from the medium (resulting in periodic signal decay, Figure 1H) was prevented by use of a high ratio of medium to cells (Kim et al., 2009; Knauer et al., 1984). Using a range of EGF concentrations, we established a series of steady-state conditions under which quantitative characteristics of ERK signaling could be linked to proliferation rates ranging from ~3% to ~80% pRb-positive (Figures 1I and 1J).

We next measured ERK network states associated with each EGF concentration under steady-state conditions (58–60 hr after stimulation). HCIF imaging of Fra-1 revealed a detectable range for pathway output of ~15-fold, with unimodally distributed populations of cells indicative of a graded change in signal strength in response to increasing EGF (Figure 2A). Similar behavior was observed with other ERK effectors including c-Fos, c-Myc, and Ets-1 (Figure S2). In contrast to its downstream targets, pERK exhibited a bimodal staining pattern, in which the fraction of cells in the positive peak varied as a function of EGF, implying all-or-none regulation. Paradoxically, at intermediate EGF concentrations in which only a subset of cells were positive for pERK or pRb, the pERK-positive (pERK+) population did not correlate with the pRb-positive population (pRb+; Figures 2B and 2C), despite the absolute requirement of ERK activity for cell-cycle progression.

This apparent discrepancy was resolved by dynamic analysis of EKAR-EV, the live-cell ERK activity reporter. EKAR-EV displayed intermittent bursts of activity alternating with periods of low activity, at a frequency dependent on the concentration of EGF (Figures 3A and 3B). EKAR-EV emission ratios in the

Figure 1. Steady-State Signaling and Proliferation in Mammary Epithelial Cells

(A) Determining signal–response relationships. Left, a signal–response curve (yellow) relates the strength of pharmacological signal inhibition (e.g., of ERK) to the resulting reduction in proliferation (arrows). Right, this curve can be evaluated using steady-state measurements of signal intensity and proliferation rate at various EGF concentrations.

(B) EGFR-ERK signaling pathway measurements used in this study.

(C) Design of FIRE. Amino acids 163–271 of Fra-1 were fused in-frame to the C terminus of mVenus, with a nuclear localization sequence (NLS) at the N terminus.

(D) Induction of FIRE fluorescence. Curves depict the median fluorescence intensity for populations of 100–1,000 cells treated with EGF at time 0 (green) or untreated (black) cells.

(E) Decay of FIRE fluorescence upon ERK pathway inhibition. MCF-10A cells expressing FIRE were grown in the presence of 20,000 pg/ml EGF and treated with 1 µM gefitinib (EGFR inhibitor), 1 µM PD325901 (MEK3), or 1 µM PD184161 (MEK4) at the indicated time. Curves depict the mean fluorescence intensity for a population of 100–1,000 cells for EGF-treated (green) or untreated (black) cells. Associated time-lapse images are shown in Movie S1.

(F) Live-cell measurements of ERK output (FIRE reporter) and cell cycle progression (RFP-geminin) in a single cell deprived of growth factors for 48 hr and re-stimulated at time 0 with 20,000 pg/ml EGF. Medium was replaced at ~24 hr intervals (dashed lines).

(G) Population-level measurements of signaling and proliferation in mammary epithelial cells. Curves represent the median intensity of FIRE signal (green; shaded region indicates the 25th–75th percentile) and the fraction positive for RFP-geminin (f-GMNN+; red) as determined by automated live-cell microscopy for cells treated as in (C). Yellow shaded region indicates a time period over which both FIRE and f-GMNN+ signals remain stable. Measurements were made under high-volume culture conditions (see the Experimental Procedures).

(H) Fluctuations in signaling and proliferation under standard culture conditions. Measurements were performed as in (G), with standard culture conditions (see the Experimental Procedures).

(I) Establishment of multiple proliferative steady states. Cells were treated and analyzed as in (G), with varying concentrations of EGF.

(J) Percentage of pRb-positive cells at steady state. Error bars indicate standard deviation of six replicates. See Figure S1 for characterization of pRb dynamics in proliferation.

Data shown are from one experiment representative of three or more independent replicates. See also Figure S1 and Movie S1.
nucleus and cytoplasm were closely correlated (Figure 3C), and pERK staining was distributed throughout the cell (Figure 3D), suggesting that activity pulses were not constrained to specific subcellular domains. EKAR-EV pulses were sporadic in the absence of EGF, while low concentrations of EGF induced isolated bursts lasting 20–30 min (mean 27 min) interspersed by dormant periods of 1–4 hr (Figure 3A). With increasing concentrations of EGF, pulses increased in duration and decreased in spacing, with individual bursts becoming indistinguishable at higher concentrations of EGF (>200 pg/ml). Thus, pRb+/pERK– cells can be observed at low EGF concentrations because the population of ERK molecules within the cell alternates between “on” (mostly active) and “off” (mostly inactive) states at least 10 to 20 times during a single cell cycle. Because essentially all cells within the population display pulsatory behavior (Figure 3B and Movie S2), the fraction of pERK+ cells cultured at steady state with 200 pg/ml EGF and analyzed as in (A).

Data shown are from one experiment representative of three or more independent replicates. See also Figure S2 and Movie S2.

Control of S Phase Entry by ERK Pulses
To understand how sporadic pulses of ERK activity at low EGF concentrations affect S phase entry, we analyzed sister cell pairs in which one sister cell entered S phase at least 5 hr prior to the other sister (Figures 4A and S3). Pulses of ERK activity were identified with a combination of automated and manual scoring (Figure 4B), and ERK activity profiles for 223 sister cell pairs were aligned by the time of RFP-geminin induction (Figure 4C). Analysis of the aligned profiles revealed a shift in ERK pulse dynamics specific to the time interval preceding S phase entry: median pulse length rose sharply at 12 hr prior to RFP-geminin induction among the cells entering S phase, but not in the corresponding sister cells. This increase in pulse length suggested that longer periods of ERK activity stimulate S phase entry.

To test this idea, we performed a statistical analysis of the mean pulse length per cell, the longest pulse in each cell, and the fraction of time spent in the ERK on state. All of these metrics were significantly greater in the earlier-committing cell (“cell 1”) for both the 12 hr immediately preceding S phase entry and for the entire interval between the previous mitosis and S phase entry, although the differences were largest within the 12 hr period (Table S1). When cell 1 and cell 2 populations were compared as a whole (Figure 4D), mean pulse length and longest pulse were ~50% larger in the cell 1 population (1.8 versus 1.2 hr for mean pulse; 3.8 versus 2.6 hr for longest pulse) and the
fraction of time spent in the ERK\textsubscript{on} state was greater (70\% versus 56\%). Comparison of the same pulse parameters within each pair also revealed a highly significant enhancement of ERK\textsubscript{on} time for cell 1 (Figure 4E). In contrast, the number of pulses in cell 1 was smaller than in cell 2 for the majority of pairs (Table S1), suggesting that the length of time spent in the ERK\textsubscript{on} state, and not the number of pulses, is a significant determinant of each cell’s decision to enter S phase.

**Modulation of EGFR-ERK Pathway Dynamics by Inhibitors**

To understand the role of ERK dynamics in response to pharmacological inhibition, we evaluated the effects of the EGFR inhibitor gefitinib or the highly selective MAPK/ERK kinase (MEK) inhibitor PD0325901 (PD) (Bain et al., 2007). Similar to titration with EGF, varying concentrations of gefitinib produced a bimodal shift in pERK intensity measured by HCIF (Figure 5A). In accord, live
Figure 4. Stimulation of Cell-Cycle Progression by Sustained ERK Activity Pulses

(A) Imaging of ERK activity pulses and cell-cycle progression. MCF-10A cells expressing EKAR-EV and RFP-geminin were imaged in the presence of 50 pg/ml EGF. Pairs of sister cells were analyzed in which one sister cell entered a subsequent round of DNA replication (as indicated by RFP-geminin induction) >5 hr prior to the other sister. One representative cell pair is shown; see Figure S3 and Movie S3 for additional examples and time-lapse images.

(B) Automatic detection of ERK activity pulses. Top, regions of increased EKAR-EV activity (red circles) identified by a peak detection algorithm for a representative cell pair. Bottom, heatmap of EKAR-EV signal in the same cell pair, with binary EKAR-EV values (solid blue and yellow) shown for each pair.

(legend continued on next page)
imaging of EKAR-EV upon gefitinib addition revealed complete inhibition of the EGF-induced signal at high gefitinib concentrations, but pulsatory behavior at intermediate concentrations (Figure 5B). These data, taken from cells stimulated with saturating levels of EGF, demonstrate that ERK activity pulses are not simply the result of fluctuations in the local concentration of EGF, but are intrinsic to the intracellular signaling machinery.

Integration of ERK Dynamics by Downstream Effectors

Because ERK activity can vary in both amplitude and frequency, measuring the integrated activity level of ERK directly poses technical challenges. We therefore turned to downstream effectors as potential indicators of integrated ERK pathway output, focusing on Fra-1 because it displayed the largest measurable dynamic range (Figure S2). To understand how unimodal distributions of Fra-1 arise downstream of FM ERK activity, we performed a kinetic analysis of changes in effector concentration. When cells growing in full growth medium were treated with MEK inhibitor, ERK phosphorylation was fully abrogated within 10 min, while Fra-1 levels decayed slowly ($t_{1/2} > 12$ hr; Figure 6A). A simplified kinetic model of Fra-1 stabilization based on reported half-lives for phosphorylated Fra-1 (Casalino et al., 2003) and pERK (Kleiman et al., 2011) was consistent with these decay times (Figure 6B; see the Supplemental Experimental Procedures and Tables S2 and S3 for details of model construction). Using the rate of phosphorylation of ERK as an input parameter to the model, we simulated the experimentally observed patterns of ERK pulses and found that the level of Fra-1 changed in a graded manner as a function of ERK frequency (Figures 6C and 6D). This simulated behavior is consistent with the observation that the median intensity of Fra-1 changes in a graded manner as the frequency of ERK pulses varies (Figure 2A). The model also predicts that variation over time in ERK pulse frequency, which is observed in individual cells (Figures 3A and 3B) would result in slow fluctuations in Fra-1 (Figure 6E). This prediction is confirmed in individual cells expressing the Fra-1-based FIRE reporter; at concentrations of EGF under which ERK activity is highly pulsatory (50 pg/ml; see Figure 3A), fluctuations in Fra-1-based FIRE intensity occurred on a much slower time scale (~12 hr; Figure 6F). The sporadic nature of ERK activity pulses at intermediate EGF concentrations should also result in increased variability in effector expression, which is indeed observed as a broader distribution of Fra-1 and c-Myc at 50 pg/ml EGF (Figure 2A).

Because the fraction of time spent in the ERK$_{on}$ state controls entry to S phase (see Figure 4), our model would further predict that individual cells with higher Fra-1 levels, which have experienced a greater integrated ERK$_{on}$ time, would be more likely to enter S phase. To test this idea, we analyzed immunofluorescence data from a population of cells treated with intermediate levels (50 pg/ml) of EGF. Fra-1 expression was divided into bins, and the cells at each Fra-1 level were assessed for the frequency of pERK staining (Figure 6G,H). Across the range of Fra-1 levels, the fraction of pRb+ cells increased from <0.05 at low Fra-1 to >0.6 at high Fra-1. These data confirm that variations in ERK pulse activity, which are integrated over a 12–24 hr window by Fra-1 expression, strongly influence the proliferative activity of individual cells.

The correlation of Fra-1 intensity with both the frequency of pERK+ cells under FM conditions and the intensity of pERK staining under AM conditions (Figure 7A) makes it a useful steady-state indicator of ERK pathway output independent of ERK dynamics. Across a wide range of EGF and PD concentrations, we compared ERK pathway output (measured by steady-state Fra-1 intensity) to proliferation rate (measured by f-pRb+; Figures 7B and 7C). Strikingly, these data fit a single curvilinear relationship, suggesting that ERK output is the main quantitative factor controlling steady-state proliferation within this system. The inverted “L” shape of this relationship indicates that at low levels of ERK pathway output (yellow region in Figure 7C), small changes in signal intensity correspond to large changes in proliferative rate, while large changes in signal intensity near the high end of the dynamic range have little impact on proliferation. This relationship predicts, and experiments confirm (Figures 7D and 7E), that under saturating concentrations of EGF, inhibition of up to 85% of ERK output will have less than a 2-fold effect on proliferation rate. However, once this threshold of inhibition is
passed, proliferation declines rapidly; 95% inhibition is sufficient to reduce proliferation by 10-fold. Similar curves were measured for 184A1 cells, an unrelated mammary epithelial cell line displaying similar ERK activity patterns to MCF-10A (Figures 7F and S4), and for SUM159, a triple-negative breast cancer line dependent on ERK for proliferation (Figure 7G). Although the slopes of these curves vary, in all cases the relationship between ERK output and proliferation is nonlinear and steepest at the lower end of the ERK dynamic range.

DISCUSSION

Information Transfer from EGFR to the Cell Cycle

The EGFR-ERK system functions to relay information about the extracellular concentration of growth factor ligands to the core cell cycle circuitry; here we trace the representation of quantitative information along this pathway with single-cell methods. At the level of ERK, we find that the steady-state concentration of EGF is represented by the frequency and duration of pulses of ERK activity. At the next stage of the pathway, expression levels of effectors such as Fra-1 and c-Myc are controlled by ERK activity via an integrative process, such that the total levels of these proteins are proportional to the frequency of ERK activity pulses. From a measurement perspective, exploiting this integrative control by fusing phosphorylation-regulated degradation domains to a fluorescent protein, such as in the FIRE reporter, represents an underutilized approach to generating high dynamic range biosensors for kinase activity. Finally, at the cell fate level, the average rate of entry into the S phase of the cell cycle, a commitment that is made discrete by positive feedback loops (Yao et al., 2008), displays a nonlinear dependence on the output of the ERK pathway.

We examined the quantitative relationship between ERK activity and proliferation rate at two different scales. At the level of individual cells (Figure 3), the pattern of ERK activity is unique for each cell, and the total duration of ERK pulses regulates the rate of entry into S phase (Figure 4). This trend may reflect the existence of downstream network motifs that respond preferentially to sustained ERK activity (Murphy et al., 2002; Yamamoto et al., 2006). Our analysis of cell-wide changes in ERK activity likely overlooks subtle effects of ERK activity localization (Harling et al., 2005). Moreover, competition of EKAR-EV with endogenous substrates for ERK and phosphatases, may perturb the output of the system, which is sensitive to substrate levels (Kim et al., 2011). Population-level studies have identified distinct periods of competency for cell-cycle induction by ERK (Jones and Kazlauskas, 2001; Zwang et al., 2011); an individual cell’s decision to enter into S phase may depend on the extent that its unique pattern of ERK pulses falls within these windows of competency, or on the timing of ERK pulses relative to other signaling events. At the population scale, however, data from analyzed by HCIF. Dashed lines indicate the approximate median of the pERK-positive population; a shift in this value indicates decreased amplitude of the ERK-on state.

Data shown are from one experiment representative of two or more independent replicates.
thousands of cells can be averaged to identify a continuous steady-state relationship between ERK and proliferation rate (Figure 7). The shape of this curve is conserved across multiple cell lines and indicates that, in general, proliferation can be stimulated by very low levels of ERK output.

**Figure 6. Integration of ERK Pulses by Downstream Effectors**

(A) Decay of endogenous pERK and Fra-1 levels upon MEK inhibition. Cells were grown in full growth medium and treated with MEK inhibitor for the indicated times prior to fixation and HCIF analysis.

(B) Simulation of pERK and Fra-1 decay upon MEK inhibition. See the Supplemental Experimental Procedures and Tables S2 and S3 for details of model construction and simulation.

(C) Simulation of Fra-1 concentration in response to varying frequencies of ERK activity pulses.

(D) Simulated steady-state levels of Fra-1 as a function of the fraction of time pERK spends in the “on” state.

(E) Simulation of slow fluctuations in Fra-1 levels driven by variation in ERK pulse frequency over time.

(F) Representative individual cell trace of FIRE expression at 50 pg/ml. Cells were cultured as in Figure 1F.

(G) Scatter plot of pRb and Fra-1 intensities detected for HCIF for MCF-10A cells growing at steady state (59 hr) with 50 pg/ml EGF. Pink line indicates the threshold for determining pRb+ or pRb– status; gray vertical lines indicate bins dividing Fra-1 into graded expression levels.

(H) Frequency of pRb+ cells as a function of Fra-1 expression. For each Fra-1 expression bin (gray lines), the fraction of pRb+ cells was determined (red circles); error bars indicate the standard deviation for triplicate samples. The overall Fra-1 distribution is shown in green.

Data shown are from one experiment representative of three or more independent replicates. See also Tables S2 and S3.

**Generation of ERK Activity Pulses**

The data obtained here at physiological EGF concentrations (Tanaka et al., 2008) in nontumor epithelial cells with no known mutations in the EGFR-ERK pathway, suggest that frequency-modulated ERK signaling is pertinent to cells exposed chronically to
EGF within normal epithelia. The bursts of ERK activity observed here are distinct from highly regular, frequency-invariant oscillations in ERK localization (Shankaran et al., 2009) and damped oscillations immediately after EGF stimulation (Cohen-Saidon et al., 2009; Nakayama et al., 2008). While the capacity of ERK to respond in an all-or-none manner has been noted
by ERK-mediated phosphorylation (Brondello et al., 1997; Bron-
dello et al., 1999), and inhibitory phosphorylation of SOS (Lan-
glois et al., 1995), Raf (Brunner et al., 2003; Dougherty et al., 2005), or MEK (Pagès et al., 1994) by ERK. Positive feedback may also control the rapid onset of ERK pulses and may arise from the allosteric activation of SOS by GTP-bound Ras (Boyke-
visch et al., 2006; Margarit et al., 2003), phosphorylation of RKIP by ERK (Shin et al., 2009), phosphorylation-induced degradation of the MKP-3 (Marchetti et al., 2005), or stimulatory phosphoryla-
tion of Raf by ERK (Balan et al., 2006). The presence of many redundant feedback pathways complicates analysis of the roles of individual processes. However, given the ~5–10 min time-
scale of ERK pulse induction and inactivation, feedbacks involv-
ing direct phosphorylation events are more likely to be involved in pulse generation than slower processes involving transcrip-
tion, degradation, or receptor internalization.

Interpretation of Quantitative Changes in ERK Signaling
ERK phosphorylation and activity are frequently measured as indicators of the activity of upstream oncoproteins such as re-
ceptor tyrosine kinases or Ras. Very often, however, the impact on cell phenotype of an observed change in ERK activity is not clear; for example, what does a 2-fold change in pERK signify for the cell? Our analysis of response curves provides a prototype for translating such measurements of signal strength into ex-
pected cellular behaviors. However, the nonlinear nature of the response curves measured here implies that the significance of a quantitative difference cannot be established without knowing in what region of the dynamic range ERK is operating. A 2-fold change in ERK activity near the top of the dynamic range may have little effect on proliferation, while a 2-fold effect on ERK activity near the bottom of the dynamic range may stimulate a 5-fold change in proliferation rate. This characteristic under-
scores the need for quantitative techniques for measuring sig-
naling events. Transfer curves, which describe the input-output behavior of system components, are ubiquitous and essential in electrical and systems engineering; measurement of analogo-
gous relationships for mammalian signaling systems will be a key step in developing clinically useful predictive models.

The quantitative characteristics of information transfer in the ERK network determined here suggest several avenues for clinical intervention in tumors dependent on this pathway for growth. Because inhibition of up to 85% of ERK output has little effect on proliferation (a finding consistent with clinical data [Bollag et al., 2010]), our findings provide rationale for combined inhibition of multiple nodes in the ERK pathway, to constrain ERK output below the threshold required for proliferation. Alternatively, it may be advantageous to identify agents that shift the quantita-
tive relationship between ERK output and proliferation, such that less stringent ERK inhibition is required; the quantitative methods developed here will be of use in identifying such compounds. Finally, as prolonged ERK activation is more effect-
ive in committing a single cell to proliferate (Figures 4D and 4E), intermittent high-dose inhibition may provide a usable thera-
petic index.

EXPERIMENTAL PROCEDURES

Experimental Culture Conditions
Signaling and proliferation experiments were performed in glass-bottom 12-, 24-, or 96-well plates (MatTek, Matrical). For "standard" culture conditions (Figure 1H only), the entire well bottom was pretreated with type I collagen (BD Biosciences) to promote cell adherence and seeded with 2,500–5,000 cells/well in 200 μl culture medium (in a 96-well plate). To reduce the effect of cell-mediated EGFR depletion, all other experiments were performed in "high-volume" culture conditions, in which wells were pretreated with a 3–10 μl droplet of collagen solution (depending on the size of the plate) to create a region of high adherence ~2–4 mm in diameter (area ~0.03–0.12 cm²). Cells (2 × 10^3–1 × 10⁴) were then seeded directly on the collagen-coated region; during the course of the experiments, cells remained confined to the coated region, within which they were 50%–100% confluent. Wells were then filled with the practical maximum volume of medium (0.4, 2.5, or 5 ml/well in 96-, 24-, or 12-well plates, respectively) and replenished daily by removing 80% of the culture volume and adding an equivalent volume of fresh medium.

For steady-state experiments, cells were initially plated in full growth medium to promote adherence, and then shifted to GM-GFS for 2 days. After this starvation period, medium was replaced with GM-GFS supplemented with varying concentrations of EGF; this time point is designated "time 0." For the duration of the experiment (up to 96 hr), medium was replaced daily as described above. Importantly, while serum and insulin are required for long-
term propagation of MCF-10A cells, we found that these cells will proliferate normally in response to EGF alone for at least 2 weeks (data not shown). All steady-state experiments were performed within this time window to enable EGF-stimulated signaling and proliferation to be analyzed in isolation from signals induced by insulin or growth factors present in the serum.

For sister-cell experiments, cells were plated as above in full growth medium and shifted to GM-GFS supplemented with 50 pg/ml EGF immediately prior to beginning live-cell microscopy. This protocol allowed a large fraction of the population to enter S/G2 phase and become poised for cell division prior to imaging; cells dividing during the imaging phase were identified manually immediately following division and then tracked using automated routines (see below) through their subsequent cell cycles.

Live-Cell Microscopy
Time-lapse images were captured with a 20 x 0.75 NA objective on Nikon Eclipse Ti or Applied Precision Instruments Deltavision inverted fluorescence microscopes equipped with environmental chambers; cells were maintained at 37°C and ~5% CO₂ for the duration of the experiments. Images were collected at intervals of 5–20 min with a Hamamatsu Orca-ER digital camera with 2 × 2 binning. For higher-frequency imaging (<10 min intervals), neutral density filters were used to reduce phototoxicity.

High-Content Immunofluorescence
After growth and treatment as indicated on glass-bottom 96-well plates, cells were fixed for 15 min at room temperature with a freshly prepared solution of 2% paraformaldehyde in PBS and permeabilized with 100% methanol; rapid
Image Processing

Immunofluorescence image analysis and was performed in MATLAB as previously described (Worster et al., 2012), with routines derived from the CellProfiler platform (Lamprecht et al., 2007). In brief, image segmentation was performed on the nuclear (DAPI-stained) image for each field; after background subtraction, the fluorescence intensity for each channel was calculated as the mean pixel value for either the nuclear or cytoplasmic region of each cell.

Population-level live-cell analysis of FIRE and RFP-geminin was performed as for immunofluorescence, with NLS-mCerulean used as the segmentation marker. FIRE and RFP-geminin signals were normalized to the NLS-mCerulean intensity to correct for changes in cell shape. Segmentation and quantitation were performed independently for each time point. Analysis of FIRE and RFP-geminin in individual live cells for time periods of >48 hr was performed manually, to avoid tracking errors resulting from failure of automated tracking to identify cell division events.

Analysis of EKAR-EV was performed with a custom MATLAB algorithm, and FRET intensity was calculated as the mean CFP/YFP ratio (calculated pixel-by-pixel on background-subtracted images) for a dilated nuclear region that included both cytoplasmic and nuclear regions. To avoid the introduction of nonlinearities into the FRET measurement, the CFP-excitation, YFP-emission channel was not used (Birtwistle et al., 2011). Analysis of EKAR-EV activity pulses was performed automatically with MATLAB; transitions between the ERK “on” and “off” state were identified as peaks in a vector calculated by subtracting the CFP/YFP ratio in frame n from that in frame n-3, followed by manual editing (blinded to cell fate) to correct errors.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, three tables, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2012.11.002.

ACKNOWLEDGMENTS

Imaging facilities were provided by the Institute of Chemistry and Cell Biology and the Nikon Imaging Center at Harvard Medical School. Plasmid DNA for reporter constructs was provided by K. Aoki (EKAR-EV), A. Miyawaki (geminin), A. Bradley (PiggyBAC), and the Harvard Institute of Proteomics (Fra-1). We thank S. Spencer and Y.P. Hung for advice on the manuscript. This work was supported by the National Institutes of Health (5-R01-CA105134-07 to J.S.B.) and by a Department of Defense Breast Cancer Research Program postdoctoral fellowship (W81XWH-08-1-0609 to J.G.A.).

Received: July 22, 2012
Revised: October 9, 2012
Accepted: November 2, 2012
Published: December 6, 2012

REFERENCES


Molecular Cell
Frequency-Modulated ERK Signaling in Proliferation


