JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

COMMUNICATION

pubs.acs.org/JACS

Light-Activated Kinases Enable Temporal Dissection of Signaling Networks in Living Cells

Arnaud Gautier,[†] Alexander Deiters,[‡] and Jason W. Chin*[,†]

[†]Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 0QH, United Kingdom
[‡]Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States

Supporting Information

ABSTRACT: We report a general strategy for creating protein kinases in mammalian cells that are poised for very rapid activation by light. By photoactivating a caged version of MEK1, we demonstrate the specific, rapid, and receptor independent activation of an artificial subnetwork within the Raf/MEK/ERK pathway. Time-lapse microscopy allowed us to precisely characterize the kinetics of elementary steps in the signaling cascade and provided insight into adaptive feedback and rate-determining processes in the pathway.

Signal transduction pathways use cascades of protein and lipid kinase mediated phosphorylation to elicit specific responses to distinct extracellular stimuli. Kinase pathways are complex, dynamic, multistep processes that cross-talk, feed-back and -forward, and contain elementary steps that may operate at very different rates. A complete, and ultimately quantitative, understanding of kinase pathways will require: (1) an understanding of the network connectivity and regulation in space and time of each kinase and (2) a description of how the individual steps within a pathway regulate the kinetics of signal transmission through the pathway.

The utility of genetic or si-RNA-based approaches for understanding the architecture of kinase networks using constitutively active, knocked-out or knock-down kinases may be compromised by the evolvability or adaptability of signaling networks in response to these slowly effected perturbations. In addition, these strategies do not, in general, allow the role of kinase catalysis to be separated from other roles a kinase may play, for example, in scaffolding signaling networks. These approaches also generally fail to provide information about contributions of elementary steps to the kinetics of signal transmission. Efforts to reconstitute kinase pathways in vitro may not capture the complexity of the in vivo situation and may be thwarted by the challenge of expressing every signaling protein from the membrane to the nucleus.

Shokat and co-workers have addressed the creation of mutant kinases that are selectively sensitized to inhibition by adenine derivatives, allowing the creation of kinase alleles in which the catalytic activity can be specifically inhibited within minutes by the addition of a small molecule.[1] This approach has allowed 'chemical-genetic' inhibition of kinases, addressed many limitations of classical genetics for loss of function studies, and provided new insights into kinase network connectivity.

Strategies for the selective and rapid activation of the catalytic activity of a single kinase in the cell would complement insights gained by classical genetic approaches and chemical genetic inhibition. In particular, rapid activation of a particular kinase might allow us to observe the kinetics of steps within a pathway that are unresolved within the entire network and provide insight into the temporal control of signal transmission in a pathway. Activatable kinases might also provide tools to uncover the architecture of pharmacologically important, time-dependent, adaptive control processes, such as feedback inhibition. Cole and co-workers have demonstrated chemical rescue of an inactivating mutation in a kinase,[2] Lawrence and co-workers demonstrated photochemical destruction of an inhibitor leading to kinase activation,[3] and Hahn and co-workers recently reported a promising strategy for allosteric activation of chimeric kinases containing a rapamycin binding domain.[4]

We recently demonstrated that the photocaged lysine **1** (Figure 1) can be site-specifically incorporated into proteins in

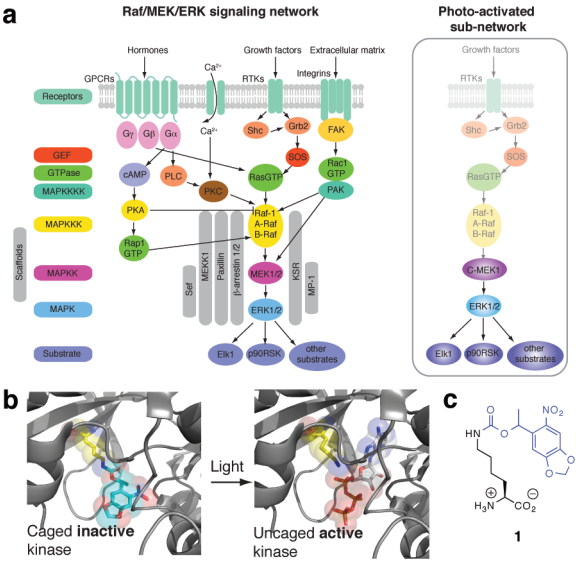

Figure 1. Isolating a subnetwork in MAP kinase signaling via genetically encoding a photocaged lysine in MEK1 active site. (a) Schematic of the MAP kinase signaling pathway and the synthetic, photoactivable subnetwork. (b) Caging a near universally conserved lysine (K97) in the MEK1 active site inactivates the enzyme by sterically blocking ATP binding. Decaging with light rapidly removes the caging group and activates the kinase (figures created using Pymol and MEK1 structure PDB: 1S9J). (c) Photocaged lysine **1**.

Received: December 7, 2010
Published: January 27, 2011

   dx.doi.org/10.1021/ja1109979 | J. Am. Chem. Soc. 2011, 133, 2124–2127

response to the amber codon in mammalian cells using the PCKRS/$tRNA_{CUA}$ pair.[5] This pair was synthetically evolved from the pyrrolysyl-tRNA synthetase/$tRNA_{CUA}$ pair that directs the incorporation of pyrrolysine in certain methanogens. Here we report a new, potentially general, strategy for very rapidly and selectively activating the catalytic activity of a user-defined kinase within the cell using light, taking advantage of the PCKRS/$tRNA_{CUA}$ pair.

Our strategy for creating a kinase poised for photoactivation involves: (1) replacing a near universally conserved lysine residue, in the ATP binding pocket of the conserved catalytic domain, that anchors and orientates ATP and is required for the phosphorylation activity of protein kinases,[6] with the photocaged lysine **1** (Figure 1) and (2) introducing activating mutations into the kinase.

To begin to demonstrate the feasibility of this approach, we created a photoactivatable version of the MAP kinase kinase MEK1. MEK1 is central to the Raf/MEK/ERK signaling pathway (Figure 1) and controls diverse responses, including cell proliferation, that are activated by growth factors or other extracellular stimuli. Upon stimulation of the pathway, MEK1 is activated by phosphorylation and phosphorylates the downstream extracellular signal-regulated protein kinases ERK1 and ERK2.[7] ERK1/2 are phosphorylated on a threonine and a tyrosine, within a conserved Thr-Glu-Tyr (TEY) motif.[8] To encode a caged version of MEK1 that would be fully active in resting conditions after light-induced uncaging, we first introduced an amber stop codon in place of the codon for lysine 97 in the gene of A-MEK1-ΔN (a constitutively active MEK1 mutant in which residues 30−49 are deleted;[9] A denotes active), creating *mek1-ΔN-97TAG*. When transiently expressed with the PCKRS/$tRNA_{CUA}$ pair in human embryonic kidney (HEK)293ET cells grown in presence of **1** (2 mM), *mek1-ΔN-97TAG* allows the expression of a full-length photocaged mutant C-MEK1-ΔN (C denotes that the catalytic residue K97 is caged) [Figure S1, Supporting Information (SI)]. Immunoblotting showed that the expression level of C-MEK1-ΔN was comparable with that obtained when tyrosine was incorporated instead of **1** in response to the amber codon using a human tyrosine amber suppressor $tRNA^{Tyr}_{CUA}$ and slightly lower than that of the mutants A-MEK1-ΔN and D-MEK1-ΔN (inactive MEK1 mutant with the inactivating mutation K97M; D denotes dead) (Figure S1, SI).

To confirm that the introduction of the photocaged lysine **1** prevents kinase activity, we coexpressed C-MEK1-ΔN with ERK2 fused to an enhanced green fluorescent protein (EGFP-ERK2) in resting HEK293ET cells. Immunoblotting revealed no phosphorylation of the TEY motif in both endogenous ERK1/2 and EGFP-ERK2 (Figures 2 and S2, SI), showing that the caged lysine **1** blocks the catalytic activity of C-MEK1-ΔN as efficiently as the K97 M mutation in D-MEK1-ΔN.

We next demonstrated that C-MEK1-ΔN could be activated by light-induced uncaging. We illuminated cells expressing C-MEK1-ΔN and EGFP-ERK2 for 1 min using a 365 nm light-emitting diode (LED) lamp placed underneath the culture plate. Phosphorylation of EGFP-ERK2 and endogenous ERK2 was detectable by immunoblotting 1 min after illumination, with a maximum phosphorylation after 10 min (Figure 2). Illumination of cells coexpressing C-MEK1-ΔN and EGFP-ERK2 for increasing times led to increased phosphorylation of EGFP-ERK2 (Figure S4, SI), demonstrating that it is possible to finely tune the cellular concentration of active kinase by simply modulating the illumination time. When C-MEK1-ΔN was replaced with the

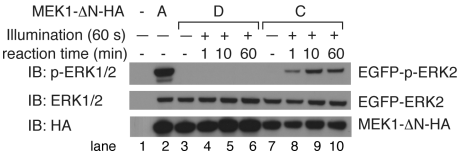

Figure 2. Specific phosphorylation of ERK2 upon photoactivation of the caged MEK1. HEK293ET cells cotransfected with plasmids encoding PCKRS, pyrrolysyl $tRNA_{CUA}$, EGFP-ERK2, and either A-MEK1-ΔN-HA (A, lane 2), D-MEK1-ΔN-HA (D, lanes 3−6), or C-MEK1-ΔN-HA (C, lanes 7−10) were grown in medium supplemented with 2 mM of **1** and 0.1% FBS for 24 h. Cells expressing D-MEK1-ΔN-HA and C-MEK1-ΔN-HA were illuminated with a 365 nm LED lamp for 60 s. Cells were lysed 1, 10, and 60 min after illumination. Cell lysates were resolved by SDS-PAGE, followed by immunoblotting (IB) with the indicated antibodies.

inactive kinase D-MEK1-ΔN (Figure 2) or with no protein (Figure S3, SI), there was no detectable phosphorylation of EGFP-ERK2 upon illumination. These data demonstrate that illumination alone is not sufficient to induce phosphorylation of ERK.

To demonstrate that photoactivation of C-MEK1-ΔN led to activation of ERK1/2, we probed the phosphorylation state of two ERK1/2 downstream substrates: p90 ribosomal S6 kinase p90RSK and the transcription factor Elk-1. Illumination of resting cells coexpressing C-MEK1-ΔN and EGFP-ERK2 led to an increase of endogenous phosphorylated p90RSK and Elk-1, which was not observed when D-MEK1-ΔN was used instead of C-MEK1-ΔN or when the MEK1 inhibitor U0126 was added (Figure S5, SI). These data demonstrate that photoactivation of C-MEK1-ΔN allows the specific activation of a subnetwork of the MAP kinase pathway in the cell, independent of extracellular stimuli. In this subnetwork ERK1/2 is phosphorylated and activated and then phosphorylates p90RSK and Elk-1.

We next investigated whether our ability to specifically and rapidly activate a subnetwork of a kinase pathway might enable us to provide insights into signaling that are challenging to obtain when the whole pathway is activated. Aside from its role as an activator, MEK1 also acts as a cytoplasmic anchor protein for ERK1/2.[10,11] Upon dual phosphorylation, ERK1/2 detaches from MEK1 and its other cytoplasmic anchors and translocates into the nucleus,[10,12] where it regulates gene expression by phosphorylating transcription factors.[13] Nuclear ERK1/2 returns to the cytoplasm upon deactivating dephosphorylation by nuclear MAPK phosphatases (MKPs).[14,15]

Stimulation of resting cells expressing wild-type MEK1 and EGFP-ERK2 with epidermal growth factor (EGF, 100 ng/mL) activates the entire MAP kinase pathway resulting in EGFP-ERK2 translocation from the cytoplasm to the nucleus. Quantification of fluorescence time-lapse microscopy images demonstrates that the nuclear accumulation of EGFP-ERK2 exhibits sigmoidal kinetics, with a lag phase of 3 min prior to rapid nuclear accumulation of EGFP-ERK2 (Figure S6a,b, SI and movie 1). The nuclear accumulation of EGFP-ERK2 peaks and then dissipates to prestimulus levels, despite continued stimulation (Figure S6a,b).

This type of desensitization to persistent stimuli is a well-known effect in cellular systems known as exact (or nearly complete) adaptation[16] and is likely to involve negative feedback. However the balance between activation and desensitization and the molecular targets of feedback are poorly

dx.doi.org/10.1021/ja1109979 |*J. Am. Chem. Soc.* 2011, 133, 2124–2127

understood. A very recent model for exact adaptation proposes that it involves multiple ERK-dependent negative feedback steps, including desensitization of upstream Ras-guanine nucleotide exchange factor (GEF) recruitment by hyperphosphorylation of Sos, desensitization of upstream Raf isoforms through phosphorylation, and transcriptional up-regulation of MAPK phosphatases (MKPs) that dephosphorylate ERK (Figure S6c, SI).[17] Among these, the desensitization of Raf, the kinase that directy activates MEK1, is proposed to have the strongest effect on adaptation.[17]

In order to obtain additional insights into the elementary steps of this process, we characterized the dynamics of ERK2 nuclear translocation using a photocaged MEK1 poised for photoactivation. For these experiments we created a new caged MEK1 (C-MEK1-DD) by photocaging the conserved lysine K97 of A-MEK1-DD, a constitutively active MEK1 in which Ser218 and Ser222, the residues normally phosphorylated by Raf to activate MEK1, are substituted with Asp residues to mimic the phosphorylated state[9] (see text S1 for detailed explanation of the requirement to create a new caged MEK1 and Figure S7, SI for full characterization of C-MEK1-DD).

First, we demonstrated that photoactivation of C-MEK1-DD specifically induces ERK2 nuclear translocation through phosphorylation. Confocal fluorescence imaging showed that C-MEK1-DD retains EGFP-ERK2 in the cytoplasm, demonstrating that its anchoring function is maintained, while its catalytic activity is eliminated (Figure S8a, SI). Upon illumination with a microscope metal halide lamp equipped with a UV filter (2 s, 365 nm, 1 mW/cm^2), cells under resting conditions that show cytoplasmic C-MEK1-DD and EGFP-ERK2, rapidly accumulated EGFP-ERK2 in the nucleus (Figure S8a,b, SI). A 4-fold increase in the ratio of the nuclear to cytoplasmic EGFP fluorescence F(n/c) within 10 min following illumination was observed (Figure S8b). These experiments in combination with numerous additional controls (see text S2 and Figure S8a,b) demonstrate that activation of ERK2 by MEK1-induced phosphorylation of the TEY motif is sufficient to trigger ERK2 nuclear translocation in resting cells.

Real-time measurements of EGFP-ERK2 nuclear translocation (Figure 3a,b and movie 3) show that the translocation process in the subnetwork is initiated much faster after photoactivation of C-MEK1-DD than for EGF-stimulated activation of the whole pathway ($t_{1/2}$ = 1.5 min vs 4.5 min, Figures 3b and S9, SI). Moreover, in contrast to EGF stimulation, photoactivation of C-MEK1-DD allows the nuclear accumulation of EGFP-ERK2 to be sustained for long periods (Figure 3a,b and movie 3). This demonstrates that the pool of photoactivated C-MEK1-DD acts as a stationary stimulus and that, unlike the whole EGF stimulated pathway, the activity of the subnetwork is not subjected to exact adaptation. Since our light-sensitive subnetwork is insensitive to upstream ERK-dependent negative feedback, because C-MEK1-DD can only be activated by light and is not regulated by Raf, this result is in accordance with a model in which ERK-dependent Raf desensitization is a regulatory process more important than the transcriptional up-regulation of MAPK phosphatases in ERK signaling adaption.[17]

Our results also demonstrate that the activity of MAPK phosphatases is sufficient to counteract nuclear import of ERK1/2 by promoting nuclear dephosphorylation of ERK1/2 and its export,[14,15] leading to an apparent steady-state of nuclear ERK2. However this process is not sufficient to produce an adaptive decrease in ERK2 in the nucleus in these cells. To unveil the compensating

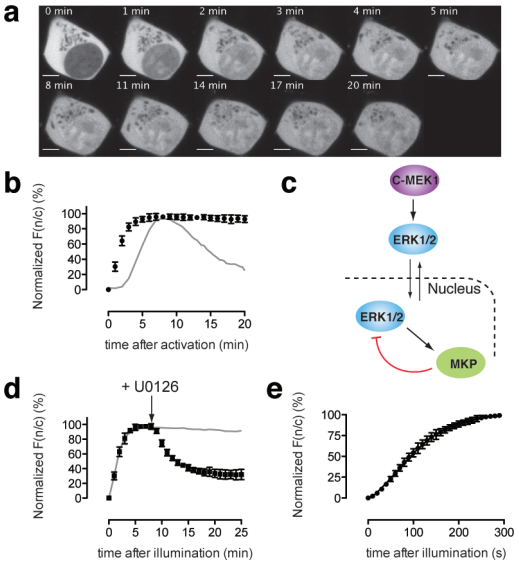

Figure 3. EGFP-ERK2 nuclear translocation upon photoactivation of the caged MEK1. (a) Representative EGFP-ERK2 subcellular fluorescence at different time points after photoactivation (2 s, 365 nm, 1 mW/cm^2) of coexpressed C-MEK1-DD. Scale bars represent 5 μm (see movie 2). (b) Normalized F(n/c) (ratio of nuclear to cytoplasmic EGFP fluorescence intensities) as a function of time after photoactivation (mean ± SD of 10 independent experiments). The gray line shows the normalized F(n/c) observed when cells are stimulated with EGF (see Figure S6 and movie 1, SI). (c) Scheme of the potential negative feedback within the photoactivatable subnetwork. (d) U0126 (10 μM) was added 8 min after photoactivation of C-MEK1-DD (black line), control experiment without inhibitor addition is gray line (Figure S10 and movie 3, SI). (e) High-resolution kinetics of translocation following C-MEK1-DD photoactivation (mean ± SD of 10 independent experiments) is shown. Data were fitted with a sigmoidal function (Figure S12 and movie 5, SI).

nuclear export of ERK2, we induced EGFP-ERK2 nuclear translocation by C-MEK1-DD photoactivation and then blocked MEK1-induced ERK2 nuclear translocation by addition of the MEK1 inhibitor U0126. This caused a rapid loss of EGFP nuclear fluorescence ($t_{1/2}$ < 3 min) (Figure 3d, Figure S10, SI, and movie 4), in accordance with a rapid dephosphorylation-induced nuclear export of the imported ERK2.[15]

The curves for EGF stimulated translocation and photoactivated translocation have comparable slopes once translocation is initiated (as judged by the slope when 50% of the net translocation has occurred), but the initial lag phase in the EGF stimulated experiment is much longer than in the photoactivated experiment (compare Figures S6b, SI and 3b,e). These observations suggest that steps upstream of MEK1 in the pathway function introduce a delay prior to activating the translocation process but do not significantly affect the translocation rate once translocation has begun. The rate of translocation is therefore set between MEK1 and ERK2 in the pathway. This is consistent with the rate of ERK2 phosphorylation being rate determining for nuclear import.

Recent work has characterized the kinetics of nuclear accumulation and phosphorylation of a previously reported ERK mutant (ERK1-Δ4) following EGF stimulation. Researchers have provided evidence that this mutant accumulated in the nucleus more slowly than wild-type ERK and was phosphorylated more slowly than wild-type ERK following EGF stimulation.[18] Consistent

dx.doi.org/10.1021/ja1109979 |J. Am. Chem. Soc. 2011, 133, 2124–2127

with these observations, we confirmed that ERK2-Δ4 was phosphorylated more slowly and accumulated in the nucleus more slowly than wild-type ERK following MEK1 photoactivation (see text S3, for detailed explanation, Figure S11, and movie 4, SI). While these experiments demonstrate that phosphorylation of ERK2 can become rate determining when using a mutant ERK2, this does not demonstrate conclusively that MEK1-mediated ERK2 phosphorylation is rate-determining in the native pathway with wild-type ERK2.

In vitro it is known that phosphorylation of ERK2 on each of its phosphorylation sites by MEK1 is nonprocessive (distributive)[19] and that distributive phosphorylation of ERK1/2 leads to ultrasensitive, switch-like, sigmoidal kinetics for formation of the diphosphorylated form.[19,20] However in vivo, where scaffolds and other proteins may organize and regulate kinases,[21] it is unknown if sigmoidal kinetics for this elementary step are conserved and whether these phosphorylations, or steps upstream of MEK, control ERK1/2 translocation.[18] We reasoned that if ERK2 phosphorylation by MEK1 is rate determining for nuclear import and the phosphorylation kinetics in vivo reflect those seen in vitro, then we should observe sigmoidal kinetics of ERK2 nuclear import following MEK1 photoactivation. Indeed the high temporal resolution of our method allowed us to directly observe sigmoidal kinetics for ERK2 import upon photoactivation of C-MEK1-DD (Figures 3e and S12 and movie 5, SI), consistent with the suggestion that ERK2 import rate is, at least in part, determined by distributive dual phosphorylation.

In conclusion, we have demonstrated a—potentially general—strategy for creating kinases that can be activated in living mammalian cells by a rapid light pulse. This approach allows the design of subnetworks controlled solely by light, which enables the study of the kinetics of single steps in signaling cascades and the dissection of temporal regulation. Due to the substantial conservation of the targeted lysine residue (present in 95% of human protein kinases),[6] the light-activation method reported here should be generally and readily applicable to creating photoactivated versions of other protein kinases. Moreover, by applying the lysine photocaging to each kinase in a pathway, further precise quantitative insights into the kinetics of kinase networks, and the substrates of individual kinases will be possible. Such quantitative insights in specifically and rapidly activated single-cell subnetworks will help to rapidly constrain the experimental parameters in quantitative models of signal transduction and may ultimately inform pharmacological strategies.[22]

■ ASSOCIATED CONTENT

Supporting Information. Movies 1−5, Figures S1−S12, additional text S1−S3, and methods and materials. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
chin@mrc-lmb.cam.ac.uk

■ ACKNOWLEDGMENT

A.G. thanks the Federation of European Biochemical Societies (FEBS) for a long-term fellowship.

■ REFERENCES

(1) Bishop, A. C.; Ubersax, J. A.; Petsch, D. T.; Matheos, D. P.; Gray, N. S.; Blethrow, J.; Shimizu, E.; Tsien, J. Z.; Schultz, P. G.; Rose, M. D.; Wood, J. L.; Morgan, D. O.; Shokat, K. M. *Nature* **2000**, *407*, 395. Kim, J. S.; Lilley, B. N.; Zhang, C.; Shokat, K. M.; Sanes, J. R.; Zhen, M. *Neural Dev.* **2008**, *3*, 23. Justman, Q. A.; Serber, Z.; Ferrell, J. E., Jr.; El-Samad, H.; Shokat, K. M. *Science* **2009**, *324*, 509. Li, S.; Makovets, S.; Matsuguchi, T.; Blethrow, J. D.; Shokat, K. M.; Blackburn, E. H. *Cell* **2009**, *136*, 50.

(2) Qiao, Y.; Molina, H.; Pandey, A.; Zhang, J.; Cole, P. A. *Science* **2006**, *311*, 1293.

(3) Li, H.; Hah, J. M.; Lawrence, D. S. *J. Am. Chem. Soc.* **2008**, *130*, 10474. Lee, H. M.; Larson, D. R.; Lawrence, D. S. *ACS Chem. Biol.* **2009**, *4*, 409.

(4) Karginov, A. V.; Ding, F.; Kota, P.; Dokholyan, N. V.; Hahn, K. M. *Nat. Biotechnol.* **2010**, *28*, 743. Karginov, A. V.; Zou, Y.; Shirvanyants, D.; Kota, P.; Dokholyan, N. V.; Young, D. D.; Hahn, K. M.; Deiters, A. *J. Am. Chem. Soc.* **2011**, *133*, 420.

(5) Gautier, A.; Nguyen, D. P.; Lusic, H.; An, W.; Deiters, A.; Chin, J. W. *J. Am. Chem. Soc.* **2010**, *132*, 4086.

(6) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. *Science* **2002**, *298*, 1912.

(7) Shaul, Y. D.; Seger, R. *Biochim. Biophys. Acta* **2007**, *1773*, 1213.

(8) Payne, D. M.; Rossomando, A. J.; Martino, P.; Erickson, A. K.; Her, J. H.; Shabanowitz, J.; Hunt, D. F.; Weber, M. J.; Sturgill, T. W. *EMBO J.* **1991**, *10*, 885.

(9) Mansour, S. J.; Matten, W. T.; Hermann, A. S.; Candia, J. M.; Rong, S.; Fukasawa, K.; Vande Woude, G. F.; Ahn, N. G. *Science* **1994**, *265*, 966.

(10) Rubinfeld, H.; Hanoch, T.; Seger, R. *J. Biol. Chem.* **1999**, *274*, 30349.

(11) Fukuda, M.; Gotoh, Y.; Nishida, E. *EMBO J.* **1997**, *16*, 1901.

(12) Khokhlatchev, A. V.; Canagarajah, B.; Wilsbacher, J.; Robinson, M.; Atkinson, M.; Goldsmith, E.; Cobb, M. H. *Cell* **1998**, *93*, 605.

(13) Lenormand, P.; Sardet, C.; Pages, G.; L'Allemain, G.; Brunet, A.; Pouyssegur, J. *J. Cell. Biol.* **1993**, *122*, 1079. Kim, K.; Nose, K.; Shibanuma, M. *J. Biol. Chem.* **2000**, *275*, 20685. Chen, R. H.; Sarnecki, C.; Blenis, J. *Mol. Cell. Biol.* **1992**, *12*, 915. Brunet, A.; Roux, D.; Lenormand, P.; Dowd, S.; Keyse, S.; Pouyssegur, J. *EMBO J.* **1999**, *18*, 664.

(14) Volmat, V.; Camps, M.; Arkinstall, S.; Pouyssegur, J.; Lenormand, P. *J. Cell. Sci.* **2001**, *114*, 3433.

(15) Costa, M.; Marchi, M.; Cardarelli, F.; Roy, A.; Beltram, F.; Maffei, L.; Ratto, G. M. *J. Cell. Sci.* **2006**, *119*, 4952. Ando, R.; Mizuno, H.; Miyawaki, A. *Science* **2004**, *306*, 1370.

(16) Horgan, A. M.; Stork, P. J. *Exp. Cell. Res.* **2003**, *285*, 208. Burack, W. R.; Shaw, A. S. *J. Biol. Chem.* **2005**, *280*, 3832. Adachi, M.; Fukuda, M.; Nishida, E. *EMBO J.* **1999**, *18*, 5347. Cohen-Saidon, C.; Cohen, A. A.; Sigal, A.; Liron, Y.; Alon, U. *Mol. Cell* **2009**, *36*, 885.

(17) Cirit, M.; Wang, C. C.; Haugh, J. M. *J. Biol. Chem.* **2010**, *285*, 36736.

(18) Lidke, D. S.; Huang, F.; Post, J. N.; Rieger, B.; Wilsbacher, J.; Thomas, J. L.; Pouyssegur, J.; Jovin, T. M.; Lenormand, P. *J. Biol. Chem.* **2010**, *285*, 3092.

(19) Salazar, C.; Hofer, T. *FEBS J.* **2009**, *276*, 3177. Ferrell, J. E., Jr.; Bhatt, R. R. *J. Biol. Chem.* **1997**, *272*, 19008.

(20) Burack, W. R.; Sturgill, T. W. *Biochemistry* **1997**, *36*, 5929. Markevich, N. I.; Hoek, J. B.; Kholodenko, B. N. *J. Cell. Biol.* **2004**, *164*, 353.

(21) Bashor, C. J.; Helman, N. C.; Yan, S.; Lim, W. A. *Science* **2008**, *319*, 1539. Malleshaiah, M. K.; Shahrezaei, V.; Swain, P. S.; Michnick, S. W. *Nature* **2010**, *465*, 101.

(22) Fujioka, A.; Terai, K.; Itoh, R. E.; Aoki, K.; Nakamura, T.; Kuroda, S.; Nishida, E.; Matsuda, M. *J. Biol. Chem.* **2006**, *281*, 8917. Barkai, N.; Leibler, S. *Nature* **1997**, *387*, 913. Aldridge, B. B.; Burke, J. M.; Lauffenburger, D. A.; Sorger, P. K. *Nat. Cell Biol.* **2006**, *8*, 1195.