A genetically encoded fluorescent sensor of ERK activity

Christopher D. Harvey
Watson School of Biomedical Sciences

Anka G. Ehrhardt
University of Massachusetts Medical School

Cristina Arrigo Cellurale
University of Massachusetts Medical School

See next page for additional authors
A genetically encoded fluorescent sensor of ERK activity

**Authors**
Christopher D. Harvey, Anka G. Ehrhardt, Cristina Arrigo Cellurale, Haining Zhong, Ryohei Yasuda, Roger J. Davis, and Karel Svoboda

**Keywords**
fluorescence lifetime imaging microscopy, FRET, MAPK

**Rights and Permissions**

Publisher PDF posted as allowed by the publisher’s author rights policy at [http://www.pnas.org/site/aboutpnas/authorfaq.xhtml](http://www.pnas.org/site/aboutpnas/authorfaq.xhtml).

This article is available at eScholarship@UMMS: [http://escholarship.umassmed.edu/davis/70](http://escholarship.umassmed.edu/davis/70)
A genetically encoded fluorescent sensor of ERK activity

Christopher D. Harveya,b, Anka G. Ehrhardtc, Cristina Cellurale, Haining Zhonga, Ryohei Yasudad, Roger J. Davisc, and Karel Svobodaa,b,1

*Janelia Farm Research Campus, Howard Hughes Medical Institute, Ashburn, VA 20147; †Watson School of Biological Sciences, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; ‡Howard Hughes Medical Institute and Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605; and §Neurobiology Department, Duke University, Durham, NC 27710

Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved October 16, 2008 (received for review May 13, 2008)

The activity of the ERK has complex spatial and temporal dynamics that are important for the specificity of downstream effects. However, current biochemical techniques do not allow for the measurement of ERK signaling with fine spatiotemporal resolution. We developed a genetically encoded, FRET-based sensor of ERK activity (the extracellular signal-regulated kinase activity reporter, EKAR), optimized for signal-to-noise ratio and fluorescence lifetime imaging. EKAR selectively and reversibly reported ERK activation in HEK293 cells after epidermal growth factor stimulation. EKAR signals were correlated with ERK phosphorylation, required ERK activity, and did not report the activities of JNK or p38. EKAR reported ERK activation in the dendrites and nucleus of hippocampal pyramidal neurons in brain slices after theta-burst stimuli or trains of back-propagating action potentials. EKAR therefore permits the measurement of spatiotemporal ERK signaling dynamics in living cells, including in neuronal compartments in intact tissues.

The MAPK family is a class of serine/threonine kinases that includes the ERK, p38, and JNK subfamilies. Members of the ERK subfamily are essential for numerous, diverse physiological functions, including cellular differentiation, proliferation, and neuronal plasticity, and their activities are up-regulated in many cancers (1). ERK signaling spans multiple subcellular compartments (1, 2). For example, in neurons ERK is activated at synapses and regulates gene transcription in the nucleus, hundreds of micrometers away (1). The spatial and temporal dynamics of ERK activity are likely critical in establishing the specificity of downstream signals. In PC12 cells, for example, epidermal growth factor (EGF) induces transient ERK activity only in the cytoplasm, leading to cellular proliferation; whereas, neural growth factor (NGF) triggers long-lasting ERK activity in both the cytoplasm and nucleus, resulting in cellular differentiation (2).

Traditional methods to measure ERK signaling, by Western blotting or immunostaining for phosphorylated, active ERK, have provided valuable insight into ERK function. However, these methods present a static snapshot of cellular events; they do not allow for the dynamic examination of ERK activity with fine spatial resolution. Recently developed imaging approaches that use fluorescent sensors of signaling activities can overcome these shortcomings (3). FRET-based reporters have been used in living cells to monitor the spatiotemporal patterns of Ca2+ signaling and enzymatic activities (4). We therefore created a genetically encoded FRET-based sensor of ERK activity that selectively reports ERK signaling in living cells.

Results

Design and Function of EKAR. To create a genetically encoded fluorescent sensor of ERK activity, we customized a generic design for FRET-based kinase activity reporters (5–8). Our EKAR activity sensor, named EKAR (extracellular signal-regulated kinase activity reporter), includes a fluorescent protein FRET pair well suited for 2-photon fluorescence lifetime imaging (2pFLIM; EGFP and mRFP1) (9, 10), a substrate phosphorylation peptide from Cdc25C containing the consensus MAPK target sequence (PRTP) (11), and the proline-directed WW phospho-binding domain (12) (Fig. 1). ERK activation leads to phosphorylation of the substrate sequence and subsequent binding by the phospho-binding domain. The resulting conformational rearrangement triggers a change in FRET between the donor (EGFP) and acceptor (mRFP1) fluorophores. Because specificity in MAPK signaling depends on docking domains (13), we added an ERK-specific, 4-aa (FOQFP) docking site adjacent to the phosphorylation sequence (14). Finally, we included a central linker consisting of 72 glycine residues (15). EKAR expression was restricted to the nucleus likely because of the nuclear localization of the WW domain (16). Addition of a C-terminal nuclear export sequence resulted in cytoplasmic expression, providing a cytoplasmic form of the sensor (EKARcyto).

To test the sensor’s function in living cells, HEK293 cells expressing EKAR were stimulated with EGF (100 ng/ml) to strongly activate ERK signaling. FRET was quantified and imaged by using 2pFLIM (9, 10, 17, 18). The fluorescence lifetime of the donor fluorophore (EGFP), defined as the average time between fluorophore excitation and photon emission, is related to FRET efficiency and the fraction of donors interacting with acceptors (binding fraction) (3). A shorter lifetime implies higher FRET. The fluorescence lifetime of EKARcyto decreased rapidly following stimulation with EGF ($\Delta$Lifetime = $-2.92 \pm 0.07\%$, $P < 0.001$; Fig. 2A, B, and F). In cells coexpressing EKARcyto and EKARNuclear, the time courses and magnitudes of EGF-induced responses in the nucleus and cytoplasm were similar (Fig. 2B). We also measured the responses of a CFP-YFP version of EKARcyto containing Cerulean (19) and Venus (20), by using intensity-based ratiometric methods. EGF stimulation triggered an increase in the ratio ($R$) of acceptor to donor fluorescence in HEK293 cells expressing this version of EKARcyto ($\Delta R/R_{YFP/CFP} = 20.9 \pm 1.0\%$, $P < 0.001$; Fig. 2C). EKARcyto therefore undergoes a stimulus-dependent FRET change.

The ERK-specific docking site (FOQFP) was necessary for this FRET change. The inclusion of docking sites with lower affinities for ERK (14) greatly reduced the signal of EKARcyto (Fig. 2C).


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1To whom correspondence should be addressed. E-mail: svobodak@janelia.hhmi.org.

This article contains supporting information online at www.pnas.org/cgi/content/full/0804598105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA
phosphodiesterase inhibition in HEK293 cells (PKA activity, in response to adenylate cyclase activation and EGFP-mRFP1 version of AKAR2 (21), a FRET-based sensor of (10 EKARcyto reversibly reports ERK activity with rapid kinetics. Application of U0126 following EGF-induced ERK activation slightly above baseline levels (Fig. 2).

We mutated the MAPK phosphorylation site in the Cdc25C P/H11022 peptide (Thr-to-Ala substitution). The mutant sensor was insensitive to stimulation with EGF (P < 0.01; Fig. 2E). Consistently, the 72-glycine linker also improved the signal of an EGFP-mRFP1 version of AKAR2 (21), a FRET-based sensor of PKA activity, in response to adenylate cyclase activation and phosphodiesterase inhibition in HEK293 cells (Δ lifetime = −2.41 ± 0.18%, Δ lifetime(short linker) = −1.45 ± 0.29%, P < 0.02; Fig. 2E). Other components of EKAR, including the fluorescent proteins, phospho-binding domain and substrate peptide, were also optimized [see supporting information (SI) Table S1].

Relationship Between EKAR Signals and ERK Activity. To examine the relationship between ERK activation and the EKARcyto signal, we mutated the MAPK phosphorylation site in the Cdc25C peptide (Thr-to-Ala substitution). The mutant sensor was insensitive to stimulation with EGF (Δ lifetime = −0.08 ± 0.11%, P > 0.6; Fig. 2F), indicating that the phosphorylation of the MAPK substrate peptide is necessary for the FRET change. In addition, preincubation with the ERK pathway inhibitor U0126 (10 µM) eliminated the EGF-induced decrease in fluorescence lifetime (Δ lifetime = −0.05 ± 0.09%, P > 0.8). Furthermore, application of U0126 following EGF-induced ERK activation caused a rapid (~5 min) increase of the fluorescence lifetime to slightly above baseline levels (Fig. 2F). These results suggest that EKARcyto reversibly reports ERK activity with rapid kinetics.

To examine further the relationship between EKARcyto signals and ERK activity, we simultaneously measured ERK phosphorylation and EKARcyto phosphorylation at the Cdc25C peptide by using a biochemical approach. Both ERK and EKARcyto had low levels of baseline phosphorylation in COS7 cells (Fig. 3). Expression of a constitutively active form of MEK1 (ΔMEK1), to activate the ERK subfamily of MAPKs (22), caused a robust phosphorylation of both ERK and EKARcyto (Fig. 3A). Overexpression of a MAPK phosphatase (MKP1), to inhibit ERK activation (23), eliminated phosphorylation of both EKARcyto and ERK (Fig. 3A). Consistently, in the presence of ΔMEK1 or MKP1, EKARcyto-expressing cells had, on average, lower or higher fluorescence lifetimes, respectively, in comparison to control cells. Furthermore, stimulation of COS7 cells with phorbol myristate acetate (PMA, 1 µM) triggered strong phosphorylation of ERK and EKARcyto that was prevented by the addition of the ERK pathway blocker U0126 (10 µM; Fig. 3B). The addition of PMA also triggered an ERK-dependent decrease in fluorescence lifetime (Fig. 3B). Therefore, EKARcyto phosphorylation and EKARcyto signals correlate with the activation of endogenous ERK.

Because the MAPK family members have similar target phosphorylation sequences, EKAR may also be sensitive to p38 and JNK activities, despite the inclusion of the ERK-specific docking domain. To address this possibility, we irradiated COS7 cells with UV light, which strongly induces p38 and JNK activity, while only modestly elevating ERK activity (Fig. S1) (24). UV irradiation triggered EKARcyto phosphorylation that was eliminated in the presence of the ERK pathway blocker U0126 (10 µM) but was insensitive to p38 (10 µM PD169316) and JNK (10 µM SP600126) inhibitors (Fig. 3C). Consistently, UV irradiation decreased the fluorescence lifetime, on average, in EKARcyto-expressing cells in an ERK-dependent manner (Fig. 3C). EKARcyto therefore selectively reports the activity of ERK and does not report the activities of closely related members of the MAPK family.
EKAR phosphorylation, and EKAR signals in COS7 cells. (Top and Middle) Cells were transfected with EKARcyto or EKARcyto (Thr-to-Ala). ERK activity was modulated by cotransfection with a constitutively active MEK (ΔMEK1) or a MAPK phosphatase (MKP1). An example Western blot is shown. Relative phosphorylation is the ratio of phosphorylated protein to total protein. Data are mean ± SEM from 3 experiments. (Bottom) Fluorescence lifetimes in cells expressing EKARcyto in control, ΔMEK1, and MKP1 conditions. Data for each condition are mean ± SEM for >20 cells from 2 dishes. (B) PMA-induced ERK phosphorylation, EKAR phosphorylation, and EKAR signals in COS7 cells transfected with EKARcyto. Cells were stimulated with PMA (1 μM; 10 min). MAPK pathways were blocked by preincubation (30 min) with ERK (10 μM U0126), JNK (10 μM SP600125), and p38 (10 μM PD169316) pathway inhibitors. (Top and Middle) An example Western blot is shown. Data are mean ± SEM from 3 experiments. (Bottom) Changes in lifetime after PMA application with and without U0126 (10 μM). Data for each condition are mean ± SEM for 10 cells from 2 dishes. (C) UV irradiation-induced ERK phosphorylation, EKAR phosphorylation, and EKAR signals in COS7 cells transfected with EKARcyto. Cells were irradiated with 60 J/m² UV-C and then incubated for 1 h. Drugs are the same as in B. (Top and Middle) An example Western blot is shown. Data are mean ± SEM from 3 experiments. (Bottom) Fluorescence lifetimes in cells expressing EKARcyto after UV-C treatment with and without U0126 (10 μM). Data for each condition are mean ± SEM for >20 cells from 2 dishes.

**EKAR in Pyramidal Neurons.** We also examined EKARcyto function in pyramidal neurons in organotypic hippocampal brain slices. We monitored EKARcyto signals in apical dendrites following high-frequency trains of back-propagating action potentials (40 action potentials at 83 Hz, repeated 8 times at 0.2 Hz), which cause relatively global Ca²⁺ influx through voltage-gated Ca²⁺ channels (VGCCs) to activate Ras (10, 25). VGCC-dependent Ca²⁺ influx triggered ERK activation in proximal dendrites, as reported by EKARcyto, that peaked within several minutes and decayed to baseline levels within 30 min (∆Lifetime = −0.97 ± 0.12%, P < 0.01; tpeak, ERK = 5.5 ± 0.7 min; τdecay, ERK = 10.7 ± 1.1 min, obtained by dividing the area by the peak amplitude of the transient; Fig. 4). The time course of ERK activation was longer than for Ras activation following the same stimulus (tpeak, Ras < 2 min; τdecay, Ras = 4.7 ± 0.6 min) (10), which is consistent with ERK’s role as a downstream effector of Ras. EKAR’s sensitivity is therefore sufficient to study ERK signaling in neurons in intact tissue.

We used EKAR to study the dynamics of ERK signaling in the cytoplasm and nucleus of hippocampal pyramidal neurons in cultured brain slices. In neurons, ERK signals trigger downstream effects in both the cytoplasm and the nucleus (1). However, it is not known whether the dynamics and regulation of ERK activation are similar in these compartments. We therefore cotransfected pyramidal neurons with EKARcyto and EKARNuclear and used synaptic stimulation (5 stimuli at 100 Hz repeated 10 times at 5 Hz, all repeated 3 times at 0.1 Hz) to activate neurons in a theta-burst pattern (2–3 action potential bursts repeated at a theta frequency of 5 Hz), which mimics hippocampal firing patterns in awake animals and induces long-term potentiation (26). Theta-burst stimuli triggered robust ERK activation in the somatic cytoplasm and the nucleus (∆Lifetimecytoplasm = −0.88 ± 0.04%, ∆Lifetimeseptoplasmand = −0.83 ± 0.05%; Fig. 5 A–C) with similar time courses; ERK activity peaked within 10 min (tpeak, cytoplasm = 7.8 ± 1.2 min, tpeak, nuclear = 8.4 ± 1.0 min, P > 0.5; Fig. 5 B and C) and decayed to baseline levels within 35 min (τcytoplasm = 10.9 ± 2.8 min, τnuclear = 14.7 ± 2.1 min, P > 0.2; Fig. 5 A–C). Previous studies suggest that ERK signaling may be preferentially activated by Ca²⁺ influx through L-type VGCCs (27–29). Application of the L-type VGCC blocker nimodipine (20 μM) reduced ERK activation to similar extents in the nucleus and cytoplasm (∆Lifetimeseptoplasm = −0.54 ± 0.08%, ∆Lifetimeseptoplasm = −0.63 ± 0.08%, P > 0.4; Fig. 5D), indicating that Ca²⁺ influx...
through L-type VGCCs is an important trigger for ERK signaling. The dynamics and regulation of ERK activity following theta-burst stimuli are therefore similar in the somatic cytoplasm and nucleus, possibly because of action potential-evoked ERK activation by relatively global Ca2+ influx (30) and rapid diffusional exchange between the 2 compartments (31).

**Comparison of ERK Activity Reporters.** Several fluorescent indicators of ERK activity have been described previously (32–34). We compared the response of EKARCyo with published reporters (Mu2 and Erkus) side-by-side in HEK293 cells stimulated with EGF. Mu2 consists of ERK tagged with CFP and YFP and undergoes a conformational change upon unbinding of MEK (33). Erkus is a sensor based on design principles similar to those used in EKAR, but with a different substrate peptide, phospho-binding domain, docking site, and linkers (34). EGF stimulation triggered small increases in the ratio of donor to acceptor fluorescence in cells expressing Mu2 or Erkus (ΔR/ΔCFP, Mu2 = 6.1 ± 2.5%, P < 0.05; ΔR/ΔCFP, Erkus = 7.3 ± 2.1%, P < 0.01; Fig. 6). The signal of EKARCyo under the same conditions was approximately 3 times as large as those of Mu2 and Erkus (ΔR/ΔCFP, Erkus = 20.9 ± 1.0%; versus Mu2: P < 0.01; versus Erkus: P < 0.01; Fig. 6). Consistently, the fluorescence lifetime change for a mRFP1-EGFP version of Mu2 was smaller than that of EKARCyo (ΔLifetimeMu2 = +0.56 ± 0.16%, ΔLifetimeEKAR = −2.92 ± 0.07%, P < 0.01; Table S1). EKAR is therefore likely preferable for monitoring ERK activity, especially in small compartments.

**Signal-to-Noise Ratios of FLIM and Ratiometric FRET Measurements.**

We measured EKAR FRET signals by using both 2pFLIM (e.g., Fig. 2A) and intensity-based ratiometric measurements (Fig. 2C). Theory predicts that ratiometric methods may have a higher signal-to-noise ratio (SNR) than FLIM measurements (3, 35). We therefore used EKAR to compare the SNRs directly. We
acquired interleaved intensity and FLIM images of HEK293 cells expressing the CFP-YFP version of EKARcyto before and after EGF stimulation (Fig. 7A). The excitation and fluorescence detection parameters were identical for both sets of images. No corrections were made for spectral bleed-through in the intensity images. The SNR was calculated as the EGF-induced change in YFP/CFP ratio (ΔR/R) or fluorescence lifetime divided by the standard deviation of baseline fluctuations. Intensity-based measurements provided a slightly higher SNR than FLIM across a wide range of photon counts (P < 0.05; Fig. 7C), although the differences were surprisingly small (<20%). Therefore, intensity-based FRET measurements may be preferable to FLIM in certain experimental situations (see Discussion). We also attempted to compare SNRs using the EGFP-mRFP1 version of EKARcyto; however, the acceptor fluorescence of this sensor was too dim to provide a useful signal, likely because of the poor quantum yield of mRFP1 (36).

Discussion

We have designed and tested EKAR, a genetically encoded, FRET-based sensor of ERK activity. EKAR selectively and reversibly reported ERK activity after EGF stimulation in HEK293 cells and following back-propagating action potentials or theta-burst stimuli in hippocampal pyramidal neurons in brain slices. EKAR therefore allows for the analysis of ERK signaling in living cells.

Is EKAR’s signal sufficient to address biological questions related to ERK signaling? We used EKAR to measure simultaneously the dynamics of ERK activity in the nucleus and cytoplasm of pyramidal neurons following physiological theta-burst stimuli (Fig. 5). Ca2+ influx through L-type VGCCs was an important trigger for ERK activity in both compartments (Fig. 5D). We also used EKAR in pyramidal neurons to monitor ERK activity in small dendrites following back-propagating action potentials (Fig. 4), and we measured EGF-induced ERK activation in 2 subcellular compartments simultaneously in HEK293 cells (Fig. 2B). EKAR responses in HEK293 cells following EGF stimulation had high SNRs even with modest numbers of photons (Fig. 7C), indicating that the SNR is sufficient to measure ERK activity following physiological stimuli and in small compartments with limited photon counts. Furthermore, the CFP-YFP version of EKARcyto provided an acceptor-to-donor fluorescence ratio change of ~20% (Fig. 2C), which is comparable to those of other sensors, such as AKAR2 (21) and the CaMKII sensor Cameleon (37), that have been used to study the spatiotemporal dynamics of signaling events in living cells. We therefore conclude that EKAR’s signal is sufficient to examine the spatial and temporal dynamics of ERK signaling under biologically relevant conditions in living cells. We note, however, that EKAR’s signal is smaller than those of the best reporters, such as Ras sensors (10, 38) and AKAR3 (39). Also, as with all sensors, EKAR expression may perturb endogenous signaling, requiring experiments to measure sensor-dependent effects in each experimental preparation (4, 25, 40).

The design principles demonstrated here should be applicable to the design of similar kinase activity reporters. In particular, the addition of EKAR’s central linker will likely improve the signals of other sensors of this type, including the PKC sensor CKAR (41), Src reporters (42), and reporters of receptor phosphorylation (6, 7), as we demonstrated for AKAR2 (Fig. 2E). Also, the inclusion of docking sites will aid the design of new, selective sensors for kinases with nonspecific substrate phosphorylation sequences, such as p38 and JNK (13).

We measured stimulus-induced EKAR FRET changes with both 2pFLIM and intensity-based ratiometric methods. Ratiometric FRET methods provided a slightly higher SNR than did FLIM measurements. It might therefore be advantageous to use ratiometric intensity methods in certain experimental situations, such as when using an intramolecular sensor to measure FRET signals before and after a stimulus in the same sample. However, under most experimental conditions, FLIM is likely preferable, especially for quantitative FRET measurements and comparisons of FRET across samples in light scattering tissue (3).

Materials and Methods

Sensor Construction. EKAR was assembled in the pCI vector (Promega) behind the CMV promoter. EKARnuclear contains the 5’ to 3’ direction: mRFP1 (36), the WW domain of Pin1 (amino acids 1–54 of human Pin1), a 72-glycine linker (3 24-Gly segments separated by Ala-Arg and Ala-Ser) (15), a Cdc25C peptide (PDVPRTPVGK, amino acids 43–52 of human Cdc25C) (43), the ERK docking site (PDVPR, respectively. The nuclear export sequence of MEK (LQKKLEELELDE) (44) was included at the 3’ end of EGFP for EKARcyto. The CFP-YFP versions of EKARnuclear and EKARcyto were made by changing the fluorescent proteins to monomeric Cerulean (A206K mutation) (19) and monomeric Venus (A206K mutation) (20) at the N and C terminus, respectively. The linkers adjacent to Cerulean and Venus are RHR and PRAREL, respectively.

Preparations. HEK293 cells were cultured in DMEM with 10% FBS and were transfected with EKAR by using Lipofectamine 2000 (Invitrogen) or FuGENE (Roche). One-to-2 days after transfection, the cells were serum starved in 0.2% FBS overnight (~12 h). Imaging was carried out in a solution containing: 25 mM Hepes (pH 7.4), 114 mM NaCl, 2.2 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 22 mM NaHCO3, 1.1 mM NaH2PO4 and 2 mM glucose at room temperature. EGF (100 ng/ml final concentration, Calbiochem) was applied to cells plated in a 35-mm culture dish by using a pipette. Cells expressing AKAR2 were stimulated with an adenylate cyclase activator (50 µM forskolin) and a phosphodiesterase inhibitor (100 µM IBMX).

Hippocampal slice cultures were prepared from postnatal day 6–8 rats, as described (45), in accordance with the animal care and use guidelines of Cold Spring Harbor Laboratory and Janelia Farm Research Campus. After 5–8 days in culture, cells were transfected by ballistic gene transfer. Neurons were imaged 2 days after transfaction in artificial cerebral spinal fluid (ACSF) containing: 127 mM NaCl, 2.5 mM KCl, 4 mM CaCl2, 4 mM MgCl2, 25 mM NaHCO3, 1.25 mM NaH2PO4 and 25 mM glucose aerated with 95% O2 and 5% CO2 at room temperature.

Imaging and Electrophysiology. 2pFLIM was performed as described previously (10, 25). In brief, we used a custom-built 2-photon microscope with custom software integrated into ScanImage (46). A Ti:Sapphire laser tuned to 910 nm was used to excite EGFP for fluorescence lifetime measurements. Fluorescence
decay curves were measured by comparing the times of laser pulses (80 MHz) detected by a photodiode (FD5010; Thorlabs) and photon pulses from a fast PMT (R3896; Hamamatsu). Only epifluorescence photons were collected. “Green” and “red” fluorescence photons were separated with a dichroic mirror (565 nm) and barrier filters (510/30, 635/90; Chroma). A similar setup was used for CFP-YFP EKAR imaging, except with 800-nm excitation light and different detection optics (dichroic mirror:565 nm; filters:480/40, 535/50).

Perforated patch-clamp recordings (Figs. 4 and 5), back-propagating action potential stimuli (Fig. 4), and synaptic stimulation (Fig. 5) were performed as previously described (10, 25, 48). See SI Text for details.

**Biochemistry.** EKARcyto-expressing COS7 cells were cotransfected with constitutively active MEK or MKP1, stimulated with 60 μM UVC, or activated with 1 μM PMA (10 min). Standard Western blot analysis was performed. See SI Text for details.

**Data Analysis.** We used fluorescence lifetime measurements to quantify the FRET signals reported by EKAR. Fluorescence lifetimes were measured by using time-correlated single-photon counting following pulsed excitation, as described previously (10) (see SI Text).

Fluorescence lifetime changes were measured for the entire field of view, typically containing 2 or 3 HEK293 cells or a ~20 μm stretch of pyramidal neuron dendrite. Because EGF was applied to HEK293 cells in a dish by using a pipette, the onset kinetics of the EKAR signal depended on both the diffusion of EGF to the cell of interest and the activation of ERK, resulting in large jitter of response onset times. We therefore calculated the lifetime change as the maximum lifetime change within 20 min after EGF application. All P values are from 2-tailed t tests. The null hypothesis for all tests stated that the mean was equal to zero, except for the comparisons between linkers (Fig. 2E), sensors (Fig. 6D), and SNRs (Fig. 7C) for which the null hypothesis stated that the means were the same.

**SNR Comparison of FLIM and Intensity-Based Measurements.** Standard ratio-metric and FLIM imaging methods were performed in HEK293 cells expressing the CFP-YFP version of EKARcyto. See SI Text and Fig. S2 for details.

**ACKNOWLEDGMENTS.** We thank B. Burbach, C. Zhang, H. White, B. Shields, S. Winfrey, and N. Ghitani for technical assistance; D. Yue (John Hopkins, Baltimore) for the glycine linker; D. Litchfield (University of Western Ontario, London, Ontario, Canada) for Pin1; D. Morrison (National Cancer Institute, Rockville, MD) for Cdc25C; J. Blenis (Harvard Medical School, Boston) for murine RSK2; A. Miyawaki (RIKEN, Saitama, Japan) for the circularly permuted Venus variants; R. Tsien (University of California at San Diego, La Jolla, CA) for AKAR2; M. Matsuda (Osaka University, Osaka, Japan) for MiU2; and Y. Umesaka (University of Tokyo, Tokyo, Japan) for Erk2. This work was supported by the Howard Hughes Medical Institute, the National Institutes of Health, and a David and Fanny Luke Fellowship (to C.D.H.).