Letters

Controlling Enzymatic Action in Living Cells with a Kinase-Inducible Bimolecular Switch

Vedangi Sample,[†] Qiang Ni,[†] Sohum Mehta,[†] Takanari Inoue,[‡] and Jin Zhang*[,†,§]

[†]Department of Pharmacology and Molecular Sciences, [‡]Department of Cell Biology, and [§]The Solomon H. Snyder Department of Neuroscience and Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205, United States

S *Supporting Information*
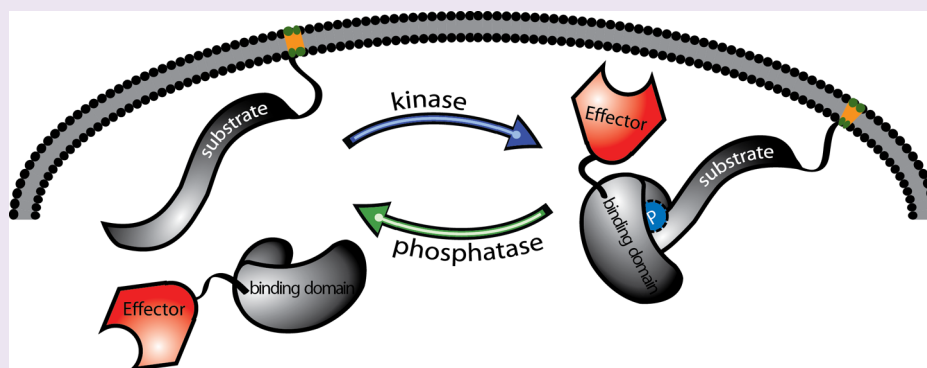
ABSTRACT: Molecular probes designed to monitor or perturb signaling events in living cells rely on engineered molecular switches. Here, we show that a kinase-inducible bimolecular switch comprising a kinase-specific substrate and a phosphoamino acid binding domain can be used for acute regulation of cellular events. As a proof of concept, we employed a Protein Kinase A (PKA)-dependent switch and coupled it to a lipid phosphatase to manipulate the level of phosphatidylinositol 4,5-bisphosphate $(PI(4,5)P_2)$ in living cells. PKA activation results in rapid degradation of $PI(4,5)P_2$. Conversely, when PKA is inhibited, dephosphorylation of the switch leads to the replenishment of $PI(4,5)P_2$. Thus, this strategy can be used for reversibly controlling enzymatic action in living cells. Furthermore, its genetic encodability and modular design should facilitate the adaptation of this approach to control different cellular activities as a function of phosphorylation-dependent input signals, thereby providing versatile tools for potentially perturbing or rewiring signaling pathways.

In order to cope with an ever-changing environment, all cells and organisms have evolved molecular strategies for signal transduction. One such strategy involves using molecular switches to convert an input signal, such as small molecule binding, post-translational modifications, light absorption, and changes in pH, to an output response. A classic example of an allosteric protein switch is protein kinase A (PKA), a cAMP dependent heterotetrameric enzyme consisting of two regulatory and two catalytic subunits. When cAMP concentration increases, cAMP binding to the regulatory subunits induces an allosteric conformational change within the regulatory subunit resulting in the release of the catalytic subunit.[1] In addition to naturally occurring switches, various molecular switches have been engineered and utilized in different molecular probes. Cameleon, for example, is a fluorescence resonance energy transfer (FRET)-based biosensor that utilizes calmodulin and M13 peptide as the molecular switch. The input of calcium binding generates a conformational change within the calmodulin-M13 switch, resulting in a change in the FRET efficiency between a fluorescent protein pair that flanks the switch.[2] A rather unconventional example of an engineered switch system is the rapamycin induced heterodimerization of FK506 binding protein (FKBP) and Rapamycin-binding domain (FRB), where rapamycin can be considered as an input signal that triggers heterodimerization between FKBP and FRB.[3] This molecular switch has been used in a variety of contexts to regulate cellular events.[4−7]

Phosphorylation-dependent molecular switches, consisting of a substrate designed for the kinase of interest and a phosphoamino acid binding domain that binds the phosphorylated substrate, have been successfully utilized in engineering a family of genetically encoded kinase activity reporters.[8,9] Salient features of this type of molecular switches include reversibility, modularity, and generalizability. However, their potential in cellular perturbation or rewiring has yet to be explored. Here, we demonstrate that an inducible bimolecular switch that responds to kinase activity changes can be used to alter levels of membrane lipid messengers in living cells in an inducible and reversible manner.
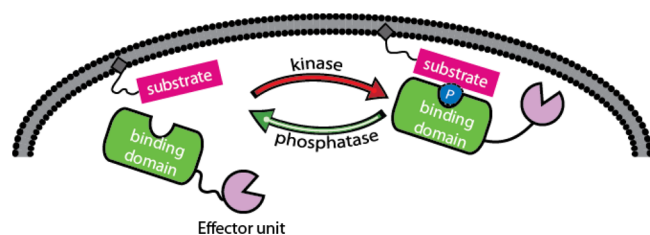
We previously utilized a kinase-inducible bimolecular switch in designing bioluminescence and FRET-based kinase activity reporters. In this bimolecular switch, the kinase-specific substrate, such as a substrate specific for PKA, and the corresponding phosphoamino acid binding domain are in two separate polypeptides.[10] We reasoned that one way to utilize this kinase-inducible bimolecular switch to regulate a specific cellular event is to couple it to signaling enzymes that serve as effector units. In one specific design (Scheme 1), half of the

switch, the phosphoamino acid binding domain, is coupled to an active enzyme, which is typically cytosolic and separated from its substrates at the plasma membrane. The other half of the switch, the kinase substrate, is tethered to the plasma membrane. Phosphorylation of the substrate should induce the intermolecular binding between the phosphorylated substrate and the phosphoamino acid binding domain, thereby localizing the active enzyme to the plasma membrane in close proximity to its substrate. In this way, manipulation of the location of the enzyme through the state of the bimolecular switch should allow us to control the action of the signaling enzyme and subsequent signaling events.

We first tested whether the activation of a bimolecular switch could induce protein translocation from the cytosol to the plasma-membrane. As a proof of principle, we chose to make the switch sensitive to PKA and targeted a PKA substrate sequence (LRRATLVD)[11,12] to the plasma membrane (PM) via a CAAX targeting motif derived from K-Ras.[12] A monomeric red fluorescent protein (RFP) mCherry[13] was fused to the C-terminus of the substrate (PKA substrate-RFP)

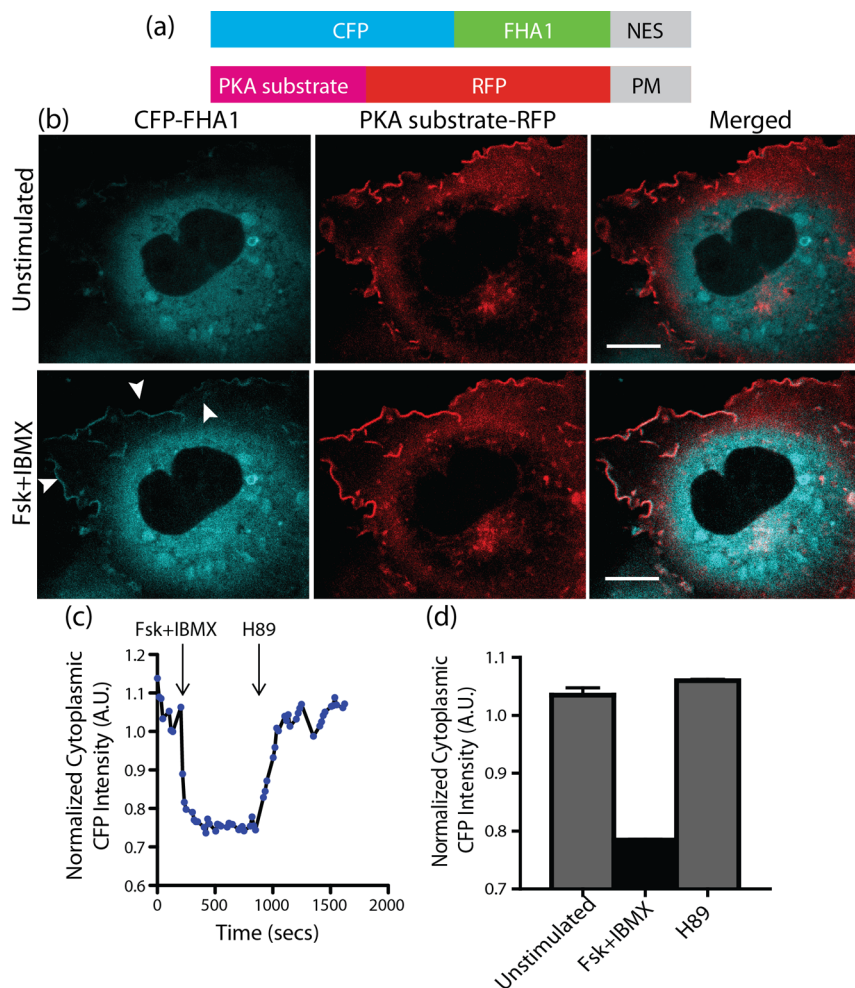**Scheme 1. Utilization of a Kinase-Inducible Bimolecular Switch to Regulate Cellular Events**

Figure 1. PKA inducible bimolecular switch recruits CFP to the plasma membrane. (a) Schematic representation of the fusion constructs including the plasma membrane (PM) targeted PKA substrate labeled with a RFP and phosphoamino acid binding domain FHA1 fused to CFP and excluded from nucleus via an NES. (b) Confocal images of Cos7 cells showing localization of CFP-FHA1. In unstimulated cells, CFP is mostly cytosolic. Upon elevation of cAMP levels with cotreatment of Fsk (50 μM) and IBMX (100 μM), CFP is recruited to the plasma membrane regions, marked by arrows. The addition of H89, a PKA inhibitor, results in its relocalization back to the cytosol. Scale bar, 10 μm. (c) Representative time course showing changes in cytoplasmic fluorescence. (d) Bar graph of normalized cytoplasmic fluorescence intensity of CFP upon Fsk/IBMX and H89 treatment ($n = 6$). Fluorescence intensities were normalized to the point before Fsk/IBMX addition.
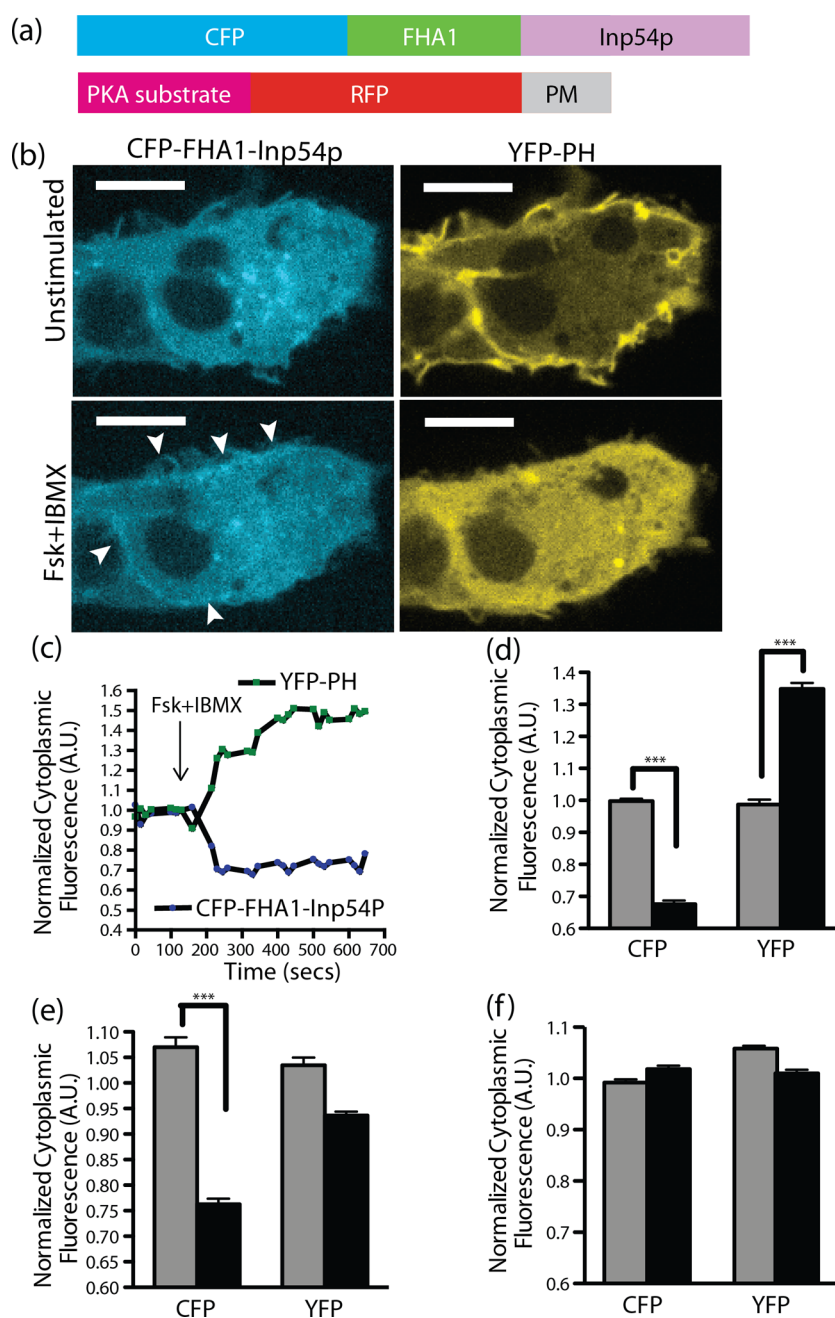
Figure 2. PKA-induced turning-on of the switch results in rapid PI(4,5)P$_2$ hydrolysis. (a) Schematic representation of a PKA-inducible bimolecular switch coupled with Inp54p, an inositol phosphatase. (b) Confocal images of Cos7 cells showing localization of CFP-FHA1-Inp54p (left) and YFP-PH (right). The localization of plasma membrane-targeted PKA substrate (PKA substrate-RFP) is not shown here. In unstimulated cells, CFP-FHA1-Inp54p is mostly cytosolic, while YFP-PH is localized to the plasma membrane. Upon cotreatment with Fsk and IBMX (at 130 s), Inp54p is recruited to the plasma membrane regions, marked by arrows, and YFP-PH is dissociated from the membrane and distributed into the cytosol. Scale bar, 10 μm. (c) Representative time course of Fsk (50 μM) and IBMX (100 μM) cotreated Cos7 cells expressing CFP-FHA1-Inp54p, PKA substrate-RFP and YFP-PH. (d) Bar graph of normalized fluorescence intensity of CFP and YFP upon Fsk/IBMX treatment ($n = 6$). (e) Bar graphs showing normalized cytosolic fluorescence intensities for CFP and YFP in Cos7 cells expressing PKA substrate-RFP, YFP-PH, and a phosphatase dead mutant of Inp54p fused to CFP-FHA1 (CFP-FHA1-Inp54p(D281A)), unstimulated (gray) or stimulated with Fsk/IBMX (black). (f) Bar graphs showing normalized cytosolic fluorescence intensities for CFP and YFP in Cos7 cells expressing CFP-FHA1-Inp54p, YFP-PH, and a T/A mutant of the PKA substrate (PKAsubstrate (T/A)-RFP), unstimulated (gray) or stimulated with Fsk/IBMX (black).

and used as a marker for expression as well as localization. Forkhead-associated (FHA1) domain[14] was used as the phosphoamino acid binding domain and fused C-terminally to Cerulean,[15] a variant of cyan fluorescent protein (CFP). This chimeric protein, CFP-FHA1, was excluded from the nucleus by appending a nuclear export signal (NES) (Figure 1a and Supplementary Figure 1). When Cos7 cells coexpressing these

two constructs were treated with a cocktail of cAMP elevating agents including forskolin (Fsk, a transmembrane adenylyl cyclase agonist) and 3-isobutyl-1-methylxanthine (IBMX, a general phosphodiesterase inhibitor) (Supplementary Figure 2), we observed a rapid and marked decrease in cyan fluorescence in the cytosol and concomitant increase in fluorescence at the plasma membrane (Figure 1b,c; $t_{1/2} = 83$

± 6 s (n = 6) [average ± SD (n value, number of cells)]). Treatment with H89, a PKA inhibitor, reversed these effects (Figure 1c,d). This data indicated that binding between FHA1 and the phosphorylated substrate was able to drive the translocation of some of the fusion protein CFP-FHA1 to the plasma membrane.

Next, we tested the ability of this bimolecular switch to control enzymatic action. We set out to perturb $PI(4,5)P_2$ levels at the plasma membrane by inducing membrane translocation of Inp54p, a yeast inositol polyphosphate 5-phosphatase (Supplementary Figures 2 and 3). This phosphatase specifically cleaves the phosphate at the 5 position of $PI(4,5)P_2$.[16] A truncated Inp54p was fused to CFP-FHA1 to generate CFP-FHA1-Inp54p (Figure 2a). To monitor $PI(4,5)P_2$ levels in living cells, we used yellow fluorescent protein (YFP) tagged pleckstrin homology (PH) domain from phospholipase C-δ1 (YFP-PH), a fluorescent biosensor for $PI(4,5)P_2$.[17] Cos7 cells were transfected with plasma membrane targeted PKA substrate-RFP, CFP-FHA1-Inp54p, and YFP-PH. In naïve cells, CFP-FHA1-Inp54p was distributed throughout the cytosol, while the $PI(4,5)P_2$ sensor, YFP-PH, was localized to the plasma membrane (Figure 2b) suggesting that the cytosolic, constitutively active phosphatase had minimal effect on $PI(4,5)P_2$ levels at the plasma membrane. The addition of Fsk/IBMX cocktail induced the translocation of CFP-FHA1-Inp54p to the plasma membrane (Figure 2b−d), thereby bringing the enzyme in close proximity to its substrate. Concurrently, YFP-PH was dissociated from the plasma membrane and translocated into the cytosol (Figure 2b−d), suggesting that the level of $PI(4,5)P_2$ was reduced as a result of the enzymatic action of Inp54p . The kinetics of translocation of CFP-FHA1-Inp54p ($t_{1/2}$ = 80 ± 3 s (n = 6)) were comparable to that of CFP-FHA1 alone ($t_{1/2}$ = 83 ± 6 s (n = 6)), indicating that fusion of Inp54p did not affect the rate of translocation to the membrane. Degradation of $PI(4,5)P_2$ also occurred in the same time frame ($t_{1/2}$ = 83 ± 6 s (n = 6)), suggesting that, under these conditions, the enzymatic reaction catalyzed by Inp54p is not kinetically limiting.

To test if this alteration of $PI(4,5)P_2$ level depends on the catalytic activity of Inp54p, we utilized a catalytically inactive mutant of Inp54p with Asp281 mutated to Ala (D281A).[18] The fusion chimera of this mutant, CFP-FHA1-Inp54p(D281A), translocated to the plasma membrane upon Fsk/IBMX treatment; however, no translocation of YFP-PH was observed in these cells indicating that the observed changes in $PI(4,5)P_2$ level require active Inp54p (Figure 2e). In addition, we mutated the phospho-acceptor threonine in the PKA substrate to alanine (PKA substrate (T/A)) and showed that this mutation abolished both the translocation of CFP-FHA1-Inp54p to the plasma membrane and the changes in $PI(4,5)P_2$ (Figure 2f), demonstrating that this acute regulation of $PI(4,5)P_2$ is dependent on a functional molecular switch.

Many signaling events in a cell are reversible; therefore, it is advantageous to have a molecular switch that allows us to control these events in either direction. To test the reversibility of this system, we treated the cells that were depleted of $PI(4,5)P_2$ with H89, a small molecule inhibitor of PKA. The addition of H89 resulted in relocalization of CFP-FHA1-Inp54P to the cytosol (Figure 3), consistent with our previous finding that this bimolecular switch was reversible. Importantly, removal of the phosphatase from its site of action allowed $PI(4,5)P_2$ to be replenished at the plasma membrane, indicated by relocalization of the YFP-PH biosensor to the plasma-
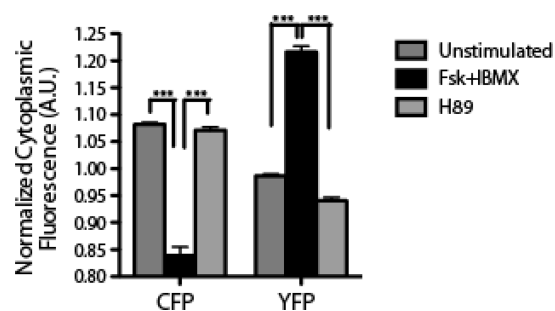

Figure 3. Treatment with PKA inhibitor H89 relocalizes CFP-FHA1-Inp54p to the cytosol resulting in replenishment of $PI(4,5)P_2$ levels at the plasma membrane. CFP-FHA1-Inp54p, PKA substrate-RFP, and YFP-PH are coexpressed. Bar graph of normalized fluorescence intensity of CFP and YFP upon Fsk/IBMX treatment and after H89 treatment (n = 6).

membrane (Figure 3). Furthermore, the fluorescence intensity of YFP-PH returned to baseline (Figure 3). These results suggest that control of enzymatic action via this bimolecular switch is fully reversible.

We have demonstrated that a kinase-inducible bimolecular switch can be utilized to control enzymatic action in living cells. Some of the features of this system can be beneficial for the acute regulation of cellular events. First, all the components are genetically encodable, providing ease of use as well as flexibility and specificity. With introduction of various targeting sequences, site-specific control of signaling events can be achieved, provided that kinase activity is present at the desirable locations. Second, because of its reversibility, a desirable feature not easily achieved with available methodologies such as FKBP-FRB[4,18] and degradation-based systems,[19,20] and this approach makes it possible to examine the functional effects of both turning on and off a signaling activity. Although the kinetics achieved here, with a $t_{1/2}$ of 80 s, are not as fast as the FKBP-FRB based system, they are within the same time scale of many signaling events such as enzyme activation or production and degradation of small molecule second messengers. Moreover, the duration of the on state could be precisely controlled to facilitate functional evaluation of temporal control of signaling events.[21] Lastly, the modular design of the system makes it amenable to being adapted for using switches that are sensitive to different kinases and for perturbing other signaling pathways of interest. The test case presented in the study utilizes endogenous PKA to demonstrate the feasibility of the approach. Although it is clear that changes in $PI(4,5)P_2$ levels shown here are dependent on the recruitment of Inp54p to the plasma membrane and that PKA activation alone does not change the level of $PI(4,5)P_2$, the activation of cAMP/PKA induces a wide range of responses, which can complicate the functional output in other situations. Therefore, future efforts will focus on generating kinase-inducible bimolecular switches that are orthogonal to target signaling networks by introducing non-native kinases from other organisms or kinases only present in specialized cell types and their corresponding substrates. These efforts will aim to make this kinase-inducible bimolecular switch strategy a more practical strategy that complements the existing biochemical perturbation methods.[4−7,18−20,22−24] However, by coupling two previously uncoupled signaling events, this system can be potentially used to rewire signaling circuitry. New connections between existing molecular components could allow us to probe the

properties of existing circuitry and examine the functional importance of specific feedback interactions.[25,26] In addition, synthetic circuitry may be established in model systems to help us better understand the fundamental principles and logics of signaling networks.[27] Given the generalizable switch design,[10] this system could be used to connect many different kinase pathways to novel signal outputs. This bimolecular switch based strategy is therefore a versatile and valuable addition to the chemical and synthetic biology toolkit.

■ METHODS

Gene Construction. CFP-FHA1 was PCR amplified and subcloned using *Bam*HI and *Eco*RI restriction sites into a modified pcDNA3 (Invitrogen), containing an NES sequence (5′-LPPLERLTL) at the C terminal end of the MCS between *Eco*RI and *Xba*I restriction sites. Inp54p was PCR amplified and subcloned in pcDNA3-NES containing CFP-FHA1 between *Sal*I and *Eco*RI. To generate PKA substrate-RFP, mCherry with no stop codon was cloned into pRSET B vector between SacI and *Eco*RI. In the same pRSET B vector, PKA substrate was inserted between *Bam*HI and SacI using drop-in primers. This entire fusion insert was then cut with *Bam*HI and *Eco*RI and subcloned into pcDNA3 vector containing a plasma membrane targeting motif from K-Ras ((KKKKKSKTKCVIM) between *Eco*RI and *Xba*I sites.

Cell Culture. Cos7 cells were maintained in high glucose DMEM (10% FBS and 1% pen/strep) at 37 °C with 5% CO_2. For imaging, cells were plated onto sterilized poly-D-lysine coated glass coverslips in 35 mm dishes and transfected with lipofectamine2000 (Invitrogen) at 60−80% confluency with 1 μg of total DNA at a ratio of 3:1:1 of PKA substrate-RFP/CFP-FHA1(or CFP-FHA1-Inp54p)/YFP-PH. Cells were then allowed to grow for 16−24 h before imaging.

Live-Cell Confocal Microscopy. For live-cell imaging, cells were washed twice with and maintained in Hanks' balanced salt solution buffer. Cos7 cells expressing appropriate levels of all the three fusion constructs were selected for time lapse imaging and were treated with forskolin (Fsk; Calbiochem), IBMX (3-isobuytl-1-methylxanthine; Sigma), and H89 (Sigma) as indicated. Time lapse imaging was performed on a spinning disk confocal microscope (CSU10; Yokogawa). A helium cadmium laser and argon laser (CVI-Melles Griot) were used for CFP and YFP excitations with a 420DF20 for CFP and a 514DF25 excitation filter for YFP (Chroma Technology), and dual CFP-YFP dichroic mirrors (Semrock). Fluorescence images were procured with a CCD camera (Orca ER, Hamamatsu Photonics), controlled by Metamorph 7.5 imaging software (Molecular Devices) using a 40× objective (Zeiss) fitted on an inverted Axiovert 200 microscope. RFP images were acquired with a DPSS 561 laser, a 560DF40 excitation filter, a RQFT 405/488/568/647 dichroic mirror and a 629DF62 emission filter. The fluorescence signal was calculated by choosing a region of interest that covered a major portion of the cytoplasmic region devoid of vacuoles. We obtained background subtracted averaged pixel intensities for this region and then normalized our data by dividing each value with the intensity value right before the point of first drug treatment.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Domain structures of key constructs used in this study; schematic representation of PKA-inducible bimolecular switch; Inp54p expression does not interfere with IP_3-mediated Ca^{2+} signaling. This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*Phone: (410) 502-0173. Fax: (410) 955-3023. E-mail: jzhang32@jhmi.edu.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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