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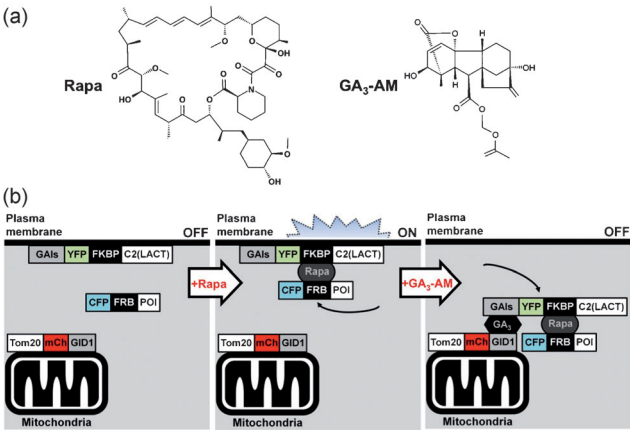
Rapidly Reversible Manipulation of Molecular Activity with Dual Chemical Dimerizers**

Yu-Chun Lin, Yuta Nihongaki, Tzu-Yu Liu, Shiva Razavi, Moritoshi Sato, and Takanari Inoue**

The four main characteristics of cellular signaling events are that they are rapid, local, specific, and reversible. With these features, cells spatiotemporally choreograph dynamic signaling. In particular, reversibility enables cells to adjust the duration of a signaling event and efficiently utilize their finite resources. This characteristic is exemplified by small GTPases (enzymes that hydrolyze guanosine triphosphate, GTP) and phosphatidylinositol lipids, which trigger diverse cellular processes, including proliferation, transformation, migration, and apoptosis.[1] To generate the precise command for each function, these signaling molecules are tightly regulated by a pair of enzymes that switch their activity on or off: guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins for small GTPases, and phosphatidylinositol kinases and phosphatases for phosphatidylinositol lipids.[1b,2]

The chemically inducible dimerization (CID) technique has been widely used to rapidly manipulate molecular activities.[3] In a CID system, a chemical dimerizer, such as rapamycin (Scheme 1a), induces the dimerization of two proteins: FK506 binding protein (FKBP) and the FKBP12–rapamycin binding protein (FRB). When FKBP is prelocalized to the plasma membrane and the FRB-fused protein of interest (FRB–POI) to the cytosol, rapamycin-induced dimerization results in the relocation of cytosolic FRB–POI to the plasma membrane (left and middle panels in Scheme 1b).[4] The accumulation of the POI at the plasma membrane subsequently triggers a biological effect that is pertinent to the specific POI molecule. The entire process can be induced on a timescale of seconds in intact living cells. Owing to the rapid, local, and specific induction of signaling, the CID technique has proven powerful and versatile as an experimental perturbation tool. To fulfill the fourth characteristic of signaling, namely, reversibility, one may consider washing rapamycin out to dissociate the dimerized complex. However, the clearance of rapamycin from cells is extremely slow.[5] Furthermore, the binding affinity between rapamycin and FKBP is extremely high (200 pM).[3b,c,6] Accordingly, once rapamycin-induced manipulation has been turned on, it is challenging to turn it off on a comparable timescale.[3b,c,7]



Scheme 1. a) Structure of the dimerizers rapamycin (Rapa) and GA_3-AM used in this study. b) Schematic representation of the rapid, local, specific, and reversible modulation of molecular activity by dual CID systems: Rapamycin binds to FKBP and traps FRB, and thus causes the relocation of CFP–FRB–POI from the cytosol to the GAIs–YFP–FKBP–C2(LACT)-labeled plasma membrane and the activation of the POI-dependent signaling event at the plasma membrane (as indicated by "ON"). The subsequent addition of GA_3-AM induces dimerization between the GAIs and GID1 and thus results in relocation of the GAIs–YFP–FKBP–C2(LACT)/rapamycin/CFP–FRB–POI complex as a whole from the plasma membrane to the Tom20–mCherry–GID1-labeled mitochondria and termination of the POI-dependent signaling event at the plasma membrane (as indicated by "OFF").

Nevertheless, the rapamycin-dissociation kinetics should be a function of experimental conditions, such as the washout protocol, cell type, dimerizer concentration, and the protein configuration and expression level of both the FKBP and FRB constructs. Therefore, we began to evaluate the reversibility of CID by using a series of CID probes previously developed in our laboratory. Specifically, we co-transfected COS-7 cells with fluorescently tagged FKBP and FRB proteins that each reside in a distinct compartment within the cell: CFP–FRB (CFP = cyan fluorescent protein) is cytosolic, whereas YFP–FKBP (YFP = yellow fluorescent

[*] Dr. Y.-C. Lin, T.-Y. Liu, S. Razavi, Prof. T. Inoue
Department of Cell Biology, Center for Cell Dynamics
Johns Hopkins University, Baltimore, MD 21205 (USA)
E-mail: ylin54@jhmi.edu
jctinoue@jhmi.edu
Homepage: http://www.jhu.edu/inouelab
Y. Nihongaki, Prof. M. Sato
Department of General Systems Studies
Graduate School of Arts and Sciences, University of Tokyo (Japan)
T.-Y. Liu
Emory College of Arts and Sciences, Atlanta, GA 30322 (USA)
S. Razavi
Department of Biomedical Engineering, Johns Hopkins University
Baltimore, MD 21205 (USA)
Prof. T. Inoue
JST, 4-1-8 Honcho Kawaguchi, Saitama 332-0012 (Japan)

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protein) is expressed at the plasma membrane once fused with the Lyn N-terminal signal sequence (Lyn–YFP–FKBP). We then added rapamycin to induce the relocation of CFP–FRB to the plasma membrane (see Figure S1a in the Supporting Information). To evaluate the dissociation kinetics of the induced dimerization complex, we then washed away the rapamycin-containing medium ten times. In each washing step, the washout efficiency was over 95%; thus, extracellular rapamycin was diluted theoretically by a factor of 10^{15}. Even 3 h after the extensive washout procedure, CFP–FRB remained primarily at the plasma membrane (see Figure S1b). We then took a more proactive approach and used chemical competitors of rapamycin.[8] In one study, FK506 reversed rapamycin-induced dimerization reasonably well on a timescale of 10 min in HEK-293T cells.[9] To test the efficiency of this competition strategy in our dimerization system, we first treated cells with rapamycin to induce the rapid relocation of CFP–FRB from the cytosol to the Lyn–YFP–FKBP-expressing plasma membrane. Subsequently, we added FK506 at increasing concentrations to compete with the interaction between FKBP and rapamycin. However, even treatment for 1 h with FK506 failed to dislodge CFP–FRB from the plasma membrane (see Figure S2). Other rapamycin competitors, such as FK506M and synthetic ligand of FKBP (SLF) also had little to no effect (data not shown). Thus, neither extensive washout nor an excess of a competitor could reverse the rapamycin-induced dimerization on a timescale of seconds. These results highlight the inherent limitations of these approaches.

We therefore explored a different strategy to induce the rapid reversal of dimerization-induced cellular effects. We reasoned that the sequestration of a whole dimerized complex containing a POI from the plasma membrane could deactivate the POI-triggered effect. We recently developed a novel CID system that is completely orthogonal to the existing rapamycin system.[4b] This new system employs GA_3-AM (Scheme 1a) as a chemical dimerizer that induces the rapid dimerization of GAIs (N-terminal 92 amino acids from gibberellin-insensitive) and GID1 (gibberellin-insensitive dwarf 1).[4b] We believed it might be possible to induce the relocation of the POI to the plasma membrane with rapamycin, and then to relocate the rapamycin-induced dimerization complex as a whole to other places in cells, such as mitochondria, by the use of GA_3-AM (Scheme 1b, middle and right panels). With this double-relocation strategy, it is crucial that FKBP is localized to the plasma membrane firmly enough that the FRB–POI fusion protein is accumulated for the induction of biological effects, but loosely enough to be dissociated from the plasma membrane: a delicate criterion that the Lyn sequence does not satisfy as a membrane anchor. We therefore chose to use a lipid-binding domain, such as the C2 domain from lactadherin (C2(LACT)), as the membrane anchor.[10] C2(LACT) binds to phosphatidylserine and primarily localizes to the inner leaflet of the plasma membrane, yet shuttles between membrane and cytosol with biased retention at the membrane.[11]

We first evaluated C2(LACT) as a targeting motif for the double-relocation strategy. To this end, we introduced three constructs into the COS-7 cells: CFP–FRB, GAIs–YFP–FKBP–C2(LACT), and Tom20–mCherry–GID1 (Tom20 is a mitochondrial transmembrane protein; mCherry is a red fluorescent protein). These fusion proteins localized to the cytosol, the plasma membrane, and the mitochondria, respectively (Figure 1a). The addition of rapamycin induced rapid accumulation of CFP–FRB at the plasma membrane (Figure 1a; see also Movie S1 in the Supporting Information). Upon the subsequent addition of a second dimerizer, GA_3-AM, both CFP and YFP fluorescence decreased at the plasma membrane, with a concomitant increase at the mitochondria (Figure 1a,b; see also Movie S1); these observations indicated that the rapamycin-induced dimerization complex (i.e., CFP–FRB and GAIs–YFP–FKBP–C2(LACT)) had been successfully sequestered to the mitochondria through GA_3-

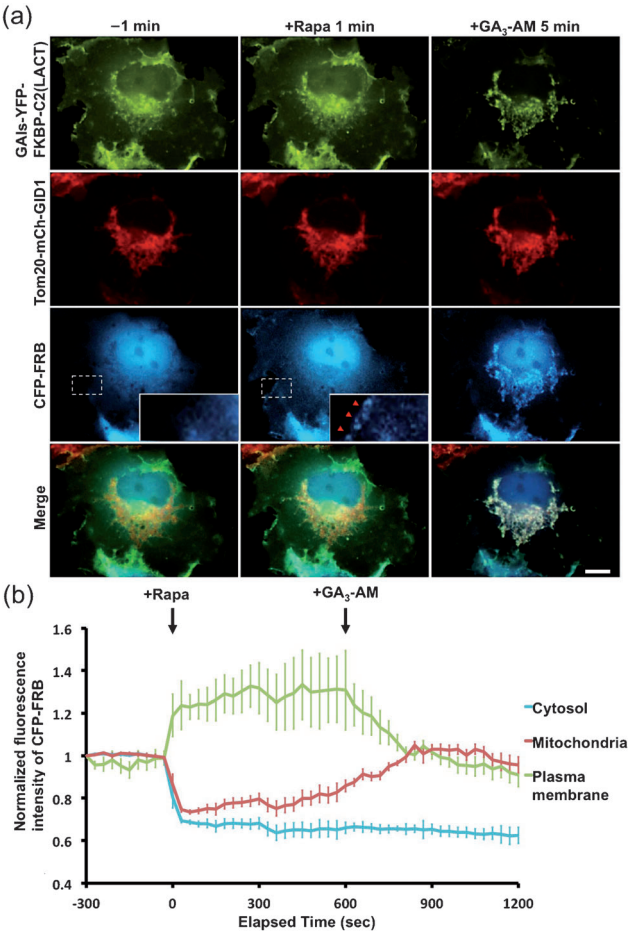

Figure 1. Double relocation of a cytosolic protein by dual CID systems. a) GAIs–YFP–FKBP–C2(LACT) (green), CFP–FRB (blue), and Tom20–mCherry–GID1 (red) were expressed in COS-7 cells. Treatment with rapamycin (100 nM) induced the relocation of CFP–FRB to the plasma membrane. Subsequent treatment with GA_3-AM (10 μM) induced relocation of the GAI–YFP–FKBP–C2(LACT)/rapamycin/CFP–FRB complex to the mitochondria. Insets show enlarged views of the regions in the dashed boxes with a higher contrast. The rapamycin-induced relocation of CFP–FRB to the plasma membrane is highlighted by red arrowheads. Scale bar: 10 μm. b) The fluorescence intensity of CFP–FRB in the regions of interest was measured at the indicated time points. The values indicate fluorescence intensity divided by the initial fluorescence. Error bars represent the standard error of the mean (SEM; $n = 10$ cells from three independent experiments).

AM-induced dimerization with Tom20–mCherry–GID1. The kinetics of the CFP–FRB relocations were quantified by calculating the time required for half-maximal accumulation ($t_{1/2}$) at the plasma membrane ((26.7 ± 4.6) s for the initial relocation) and the mitochondria ((97.8 ± 17.3) s for the second relocation; Figure 1b). Whereas the rate of the initial relocation to the plasma membrane reflects rapamycin-induced dimerization alone, that of the second relocation from the plasma membrane is a function of two factors: the dissociation of C2(LACT) from the plasma membrane and the dimerization of the GAIs and GID1. In control experiments, we replaced one of the chemical dimerizers with dimethyl sulfoxide and observed a single relocation event for CFP–FRB (see Figure S3a–c). We also confirmed that the FRB domain is indispensable for the dual relocation strategy (see Figure S3d,e). These results clearly demonstrate that dual CID systems can rapidly sequester the initial dimerized complex as a whole.

To test whether this double-relocation strategy can be used to rapidly turn on and then off molecular activity, we first chose the small GTPase Rac as a model. Previously, we showed that the recruitment of a Rac-specific GEF, Tiam1, to the plasma membrane activates endogenous Rac and induces membrane ruffling in a "sustained" manner.[4a,12] For "transient" Rac activation, we generated CFP–FRB–Tiam1, which was co-transfected into cells with GAIs–YFP–FKBP–C2-(LACT), Tom20–mCherry–GID1, and mCherry–CAAX (a plasma-membrane marker). Treatment with rapamycin triggered the accumulation of CFP–FRB–Tiam1 at the plasma membrane (Figure 2a; see also Movie S2). Upon the subsequent addition of GA_3-AM, the rapamycin-induced dimerization complex (i.e., CFP–FRB–Tiam1 and GAIs–YFP–FKBP–C2(LACT)) was rapidly recruited from the plasma membrane to the mitochondria (Figure 2a; see also Movie S2). Quantitative analysis of mCherry fluorescence in the plasma-membrane region indicated that the initial dimerization significantly increased membrane ruffling, and the subsequent dimerization decreased this ruffling to the basal level (Figure 2b–d). These results clearly demonstrate that the double-relocation strategy can be used to rapidly turn on and off the Rac signaling pathway.

We next determined whether this strategy could be applied to other signaling molecules. We showed previously that the recruitment of an inter-SH2 domain (iSH) from a regulatory PI3K subunit, p85, to the plasma membrane activates phosphatidylinositol 3-kinase (PI3K) and produces phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which results in membrane ruffling.[13] We therefore constructed CFP–FRB–iSH, which was co-transfected with Lyn–mCherry–FKBP and the YFP-labeled PH domain from AKT (YFP–PH(AKT)) as a PIP_3 biosensor. The accumulation of CFP–FRB–iSH at the plasma membrane upon the addition of rapamycin was followed by the accumulation of YFP–PH-(AKT) at the plasma membrane and the "sustained" induction of membrane ruffles (see Figure S4 and Movie S3). We then co-transfected CFP–FRB–iSH with GAIs–YFP–FKBP–C2(LACT), Tom20–mCherry–GID1, and mCherry–CAAX for reversibility experiments. After the rapamycin-induced dimerization of the FKBP and FRB fusion proteins, we added

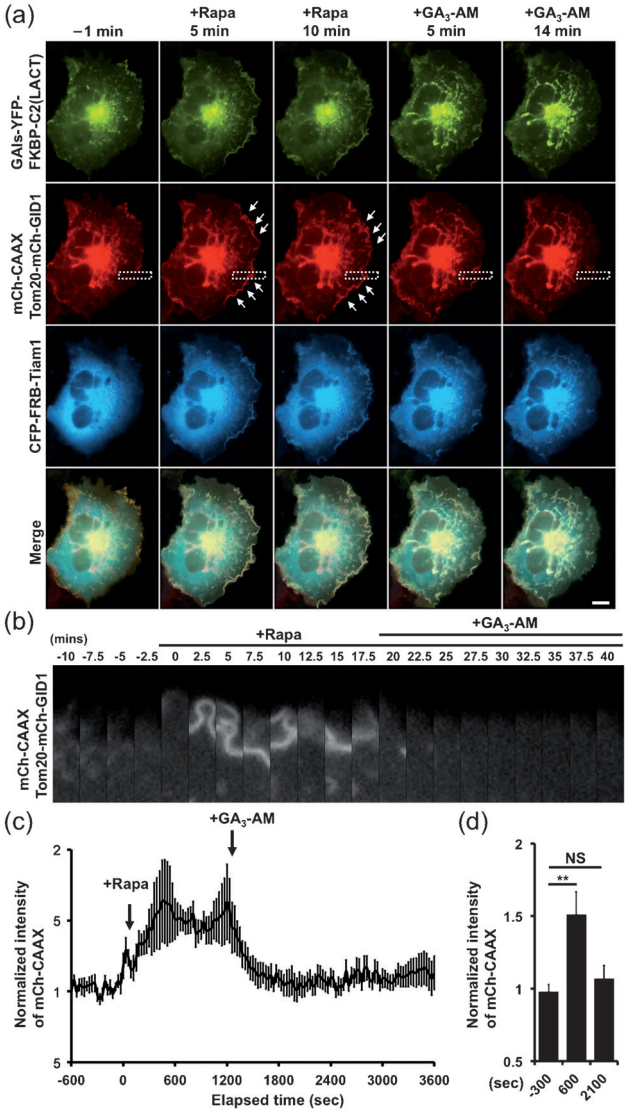

Figure 2. Rapidly reversible induction of Rac-dependent membrane ruffling. a) GAIs–YFP–FKBP–C2(LACT) (green), CFP–FRB–Tiam1 (blue), Tom20–mCherry–GID1 (red), and mCherry–CAAX (also red) were expressed in COS-7 cells. After serum starvation for 2 h, the addition of rapamycin (100 nM) induced the relocation of CFP–FRB–Tiam1 to the plasma membrane, and subsequent treatment with GA_3-AM (10 μM) induced the relocation of the chemically dimerized complex (i.e., GAIs–YFP–FKBP–C2(LACT)/rapamycin/CFP–FRB–Tiam1) as a whole from the plasma membrane to the mitochondria. Arrows indicate membrane ruffles. b) Video frames of enlarged views of the regions in the dotted boxes in (a) illustrating the reversible induction of Rac-dependent membrane ruffling. c) Ruffling activity was quantified by measuring the fluorescence intensity of mCherry–CAAX at the cell periphery. d) Fluorescence intensity of mCherry–CAAX at the indicated time points. $^{**}p < 0.01$. Error bars represent the SEM ($n = 8$ cells from three independent experiments). The values indicate the change in fluorescence (fluorescence at the time shown divided by the initial fluorescence). Scale bar: 10 μm.

GA_3-AM, which triggered the relocation of the dimerized complex of CFP–FRB–iSH and GAIs–YFP–FKBP–C2-(LACT) from the plasma membrane to the mitochondria (Figure 3a). Quantitative analysis of mCherry fluorescence at

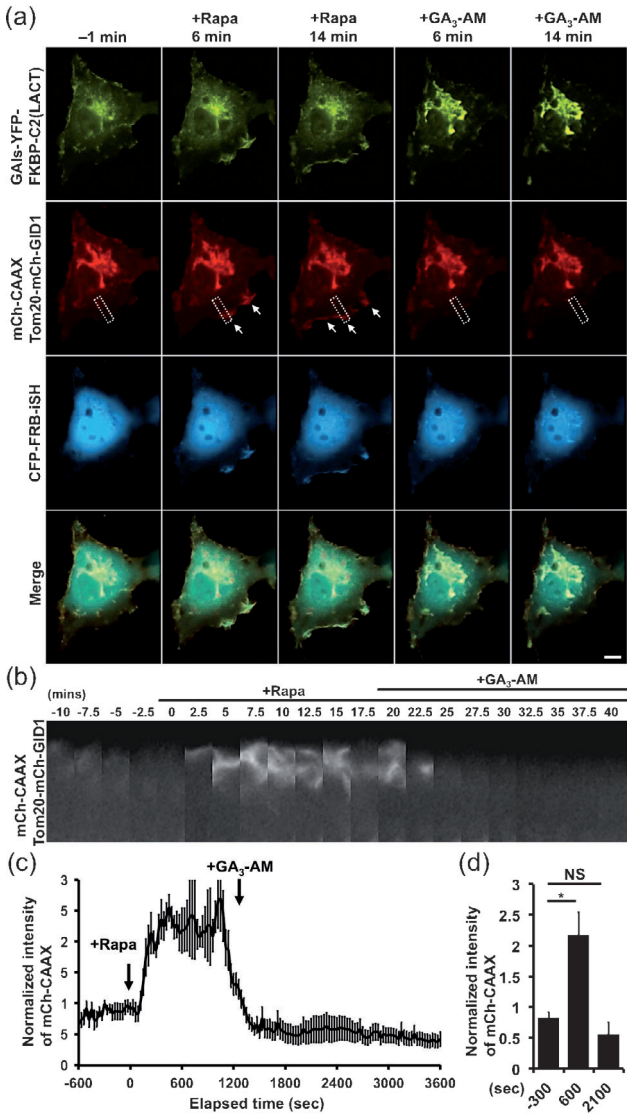

Figure 3. Rapidly reversible induction of PI3K-dependent membrane ruffling. a) GAIs–YFP–FKBP–C2(LACT) (green), CFP–FRB–iSH (blue), Tom20–mCherry–GID1 (red), and mCherry–CAAX (also red) were expressed in COS-7 cells. Treatment with rapamycin (100 nM) induced the relocation of CFP–FRB–iSH to the plasma membrane, and subsequent treatment with GA_3-AM (10 μM) induced relocation of the chemically dimerized complex (i.e., GAIs–YFP–FKBP–C2(LACT)/rapamycin/CFP–FRB–iSH) as a whole from the plasma membrane to the mitochondria. Arrows indicate membrane ruffles. b) Video frames of enlarged views of the regions in the dotted boxes in (a) illustrating the reversible induction of PI3K-dependent membrane ruffling. c) Ruffling activity was quantified by measuring the fluorescence intensity of mCherry–CAAX at the cell periphery. d) Fluorescence intensity of mCherry–CAAX at the indicated time points. $*p < 0.05$. Error bars represent the SEM ($n = 5$ cells from two independent experiments). The values indicate the change in fluorescence (fluorescence at the time shown divided by the initial fluorescence). Scale bar: 10 μm.

the plasma membrane indicated that the first dimerization induced membrane ruffling, which was then abolished by the second dimerization (Figure 3b–d; see also Movie S4). To rule out the possibility that treatment with GA_3-AM and/or a sudden decrease in the concentration of GAIs–YFP–FKBP–C2(LACT) at the plasma membrane affected membrane ruffling, we performed the following control experiment: Cells were transfected with GAIs–YFP–FKBP–C2(LACT), Tom20–mCherry–GID, and mCherry–CAAX, and exposed to epidermal growth factor to induce membrane ruffling. We then administered GA_3-AM to trigger the relocation of GAIs–YFP–FKBP–C2(LACT) from the plasma membrane to the mitochondria. However, this procedure did not affect the formed membrane ruffles (see Figure S5 and Movie S5).

There are many perturbation tools available with an emphasis on different aspects of signaling events. For example, Neddermann et al. established a eukaryotic gene-expression system based on engineered bacterial quorum sensing.[14] Kinzel et al. developed an orthogonal estrogen-receptor-based gene-switch system that was responsive to ligands.[15] Shimizu-Sato et al. developed a light-switchable gene promoter system.[16] In distinct contrast to these techniques, the double-relocation strategy turns on and off signaling events on a timescale of seconds in living intact cells. Furthermore, this strategy could be applied to different intracellular locations. We used C2(LACT) for plasma-membrane targeting in this study. Alternatively, a PH domain from FAPP (PH(FAPP)) can be used for targeting of the Golgi apparatus. PH(FAPP) binds to phosphatidylinositol 4-phosphate at the Golgi and still diffuses into the cytosol.[12] By using PH(FAPP) in place of C2(LACT), it should be possible to reversibly manipulate molecular activity at the Golgi. In summary, the technique described herein is a powerful tool for gaining deeper insight into the spatiotemporal dynamics of cell signaling.

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