Reversible Dimerization

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A Bioorthogonal Small-Molecule-Switch System for Controlling Protein Function in Live Cells**

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Abstract: *Chemically induced dimerization (CID) has proven to be a powerful tool for modulating protein interactions. However, the traditional dimerizer rapamycin has limitations in certain in vivo applications because of its slow reversibility and its affinity for endogenous proteins. Described herein is a bioorthogonal system for rapidly reversible CID. A novel dimerizer with synthetic ligand of FKBP′ (SLF′) linked to trimethoprim (TMP). The SLF′ moiety binds to the F36V mutant of FK506-binding protein (FKBP) and the TMP moiety binds to E. coli dihydrofolate reductase (eDHFR). SLF′-TMP-induced heterodimerization of FKBP(F36V) and eDHFR with a dissociation constant of 0.12 μM. Addition of TMP alone was sufficient to rapidly disrupt this heterodimerization. Two examples are presented to demonstrate that this system is an invaluable tool, which can be widely used to rapidly and reversibly control protein function in vivo.*

Methods to perturb and control the activity or localization of proteins in cells are enormously useful to probe a variety of biological processes.[1] Chemically induced dimerization (CID) systems have been used to bring two proteins of interest into close proximity, and can result in modulation of their function and perturbation of associated cellular processes.[2] Since the discovery that the natural product rapamycin induces heterodimerization of FK506-binding protein (FKBP) and the FKBP-rapamycin-binding (FRB) domain of the mammalian target of rapamycin (mTOR),[3] rapamycin-based CID has been widely used to control a variety of protein functions including gene transcription, signal transduction, post-translational protein modification, and protein degradation.[1a,4]

However, because rapamycin also binds to endogenous proteins, it can produce off-target effects. For example, heterodimerization of FKBP and mTOR by rapamycin inhibits the kinase activity of mTOR, thus leading to undesirable biological activities including immunosuppression and induction of autophagy.[5] And, FKBP-binding ligands such as rapamycin and FK506 have been shown to interfere with the cellular functions of FKBP proteins, such as regulation of intracellular calcium release.[6] Moreover, since FKBPs are ubiquitous and abundant in mammals, they could sequester rapamycin and attenuate its efficacy as a dimerizer. To eliminate the off-target effects of rapamycin on the native mTOR protein, extensive work has been done to design improved rapamycin analogues (rapalogues) which only bind to a mutant of the FRB domain.[7] However, these rapalogues are not truly bioorthogonal, as they still interact with endogenous FKBP. Moreover, chemical modifications based on the already complex rapamycin molecule are highly restricted. Therefore, it is not surprising that only limited progress has been achieved in the development of selective rapalogues. Similarly, the "bump and hole" strategy was used to engineer a synthetic homodimerizer (SLF) which is only recognized by the F36V mutant of FKBP.[8] More recently, bioorthogonal CID systems which use plant hormones, such as abscisic acid (ABA) and a gibberellin analogue (GA_3-AM), as chemical dimerizers have been introduced.[9] These developments have substantially expanded the toolkit of CID systems.[2a,10]

Another challenge in the development of CID systems is the reversible control of dimerization. This type of control is necessary to dissect the complexity of many biological processes, such as signal transduction, which are often regulated in a reversible manner (e.g., phosphorylation and dephosphorylation, activation and inactivation of molecular switches, and reversible protein–protein interactions). Yet most CID systems are essentially irreversible. For the rapamycin CID system, this irreversibility is due to the high affinity of rapamycin for its binding partners and the extremely slow dissociation of the dimerization complex.[11] Attempts to disrupt rapamycin-induced dimerization using medium exchange or competition with FK506 have not been very successful.[12] And, although the heterodimerization of the tobacco 14-3-3 protein and the C-terminal domain of

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tobacco H^+-ATPase (PMA), induced by the natural product fusicoccin, was reversed by medium exchange, both the dimerization and the dissociation proceeded at relatively slow rates.[13] Activation and deactivation of signal transmission in cells has been shown by the reversible homodimerization of FKBP induced by the addition of FK1012 (a homodimeric variant of FK506) with subsequent treatment with the competitor FK506.[2c] Similarly, FK506-induced heterodimerization of FKBP and calcineurin was blocked by rapamycin, which competes for binding to FKBP.[14] However, once again, the small molecules used in these studies bind to endogenous proteins, thus leading to undesirable biological activities.

A recent study that employed the sequential application of two orthogonal CID systems (the rapamycin and gibberellin systems) was used to turn Rac-/PI3K-dependent membrane ruffling on and off.[12b] However, because each of the CID systems used in this study is irreversible, multiple rounds of recruitment and dissociation are not possible with this approach. Moreover, in addition to the off-target effects of rapamycin, this approach is also subject to the side effects of gibberellin, side effects which have been shown to induce cell acidification.

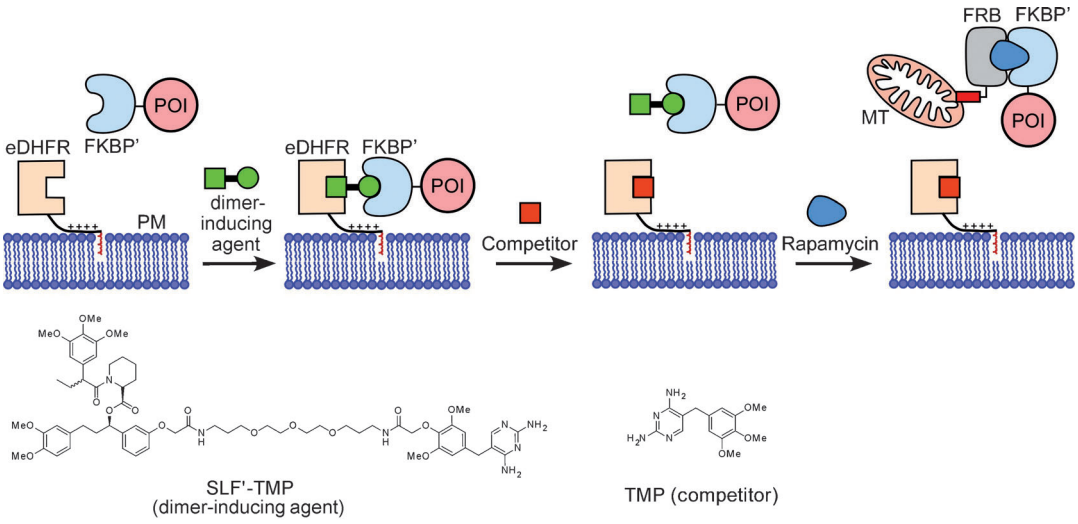
Therefore, despite significant advances in the development of CID systems, there remains a high demand for reversible CID systems with fast reaction rates, minimal disruption of endogenous functions, and low cytotoxicity.[2b,15] Herein, we report a novel bioorthogonal and reversible CID system for modulating protein function in living cells. To our knowledge, the present work is the first to establish a bioorthogonal small-molecule-switch system for controlling protein function in cells.

The dimerizer contains two moieties, each of which binds to a separate bioorthogonal protein module. The trimethoprim (TMP) moiety has a high affinity ($K_I = 1$ nM) for *E. coli* dihydrofolate reductase (eDHFR), an 18 kDa monomeric protein and a substantially lower affinity ($K_I = 4$–8 μM) for the mammalian form of DHFR (Scheme 1).[16a] The other moiety consists of synthetic ligand of FKBP′ (SLF′), which binds with high affinity (subnanomolar) to the F36V mutant of FKBP (hereafter referred to as FKBP′) and displays a 1000-fold selectivity for this mutant over the wild-type FKBP.[8] The TMP molecule alone is used as a competitor to dissociate the SLF′-TMP-induced dimerization. Importantly, the selectivity of TMP and SLF′ conjugates has been proven before in cellular labeling studies from other groups and our group, thus showing that those components also do not significantly interact with other cellular components.[16b–d] Therefore, we reasoned that SLF′-TMP should display minimal off-target effects and should not interfere with native biological systems.

The crystal structures of the eDHFR:TMP and FKBP′:SLF' complexes provide a good structural basis for the selection of crosslinking sites on each of the moieties, as well as the selection of an appropriate linker length.[8,16a,17] The 4′-O of the trimethoxybenzyl group of TMP and 3-O of the phenyl group of SLF' (Scheme 1) were chosen as crosslinking sites since previous studies have shown that modifications at these positions do not significantly disrupt the binding of the individual moieties to their cognate proteins.[8,18] SLF′ was connected to TMP with a triethyleneglycol (TEG) linker (Scheme 1). The synthesis of SLF′ and the SLF′-TMP conjugate is described in the Supporting Information.

To test the ability of SLF′-TMP to induce heterodimerization, recombinant eDHFR and FKBP′ proteins were mixed together in either the presence or the absence of an equimolar amount of the dimerizer, and subsequently subjected to size-exclusion chromatography (Figure 1 A). A higher molecular weight species was eluted in the presence of SLF′-TMP, thus indicating formation of the heterodimer.

Importantly, neither the SLF′-TMP dimerizer nor the TMP competitor showed signs of cytotoxicity toward COS-7 cells up to a concentration of 50 μM. This is in contrast to that of rapamycin, which displays significant cytotoxicity at 50 μM.



Scheme 1. Schematic presentation of reversible chemically induced dimerization and translocation between two subcellular sites using a combination of SLF′-TMP and rapamycin systems. MT = mitochondria, PM = plasma membrane, POI = protein of interest.

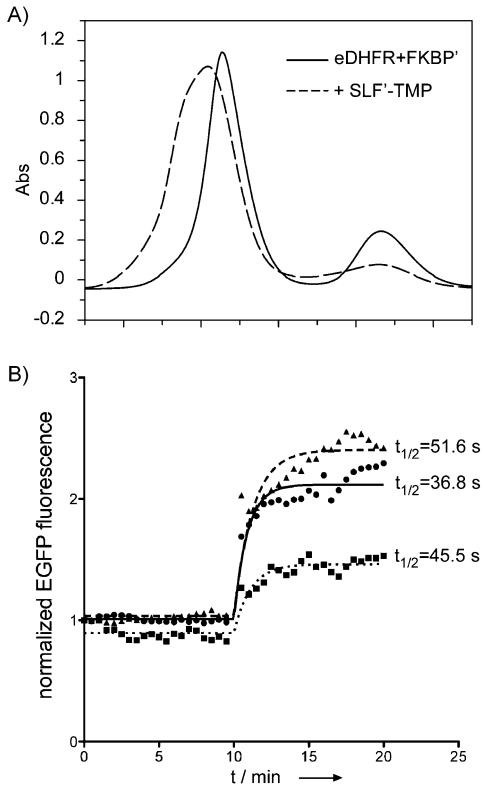


Figure 1. SLF′-TMP induces heterodimerization of eDHFR and FKBP' in vitro and in cells. A) Size-exclusion chromatograms of a mixture of 100 μM eDHFR and 100 μM FKBP′ in the absence (solid line) or the presence of 100 μM SLF′-TMP (dashed line). B) Measurement of EGFP fluorescence intensity by TIRF was used to monitor the plasma membrane recruitment dynamics of EGFP-2×FKBP′ constructs after addition of 10 μM SLF′-TMP. Neuro-2a cells were co-transfected with TagBFP-2×eDHFR-CAAX and EGFP-2×FKBP′.

This data demonstrates that the SLF′-TMP dimerizer and the TMP competitor are orthogonal to biological systems (see Figure S3 in the Supporting Information).

To further confirm the efficiency of SLF′-TMP-induced heterodimerization, we made ECFP-eDHFR and Citrine-FKBP′ fusion proteins. Fluorescence resonance energy transfer (FRET) between ECFP and Citrine was used to assess the extent of heterodimerization. Addition of the dimer inducer led to a dose-dependent increase of FRET as judged by a significant decrease of the ECFP fluorescence intensity at $\lambda =$ 475 nm and a concomitant increase of the fluorescence intensity at $\lambda =$ 527 nm upon excitation at $\lambda =$ 370 nm (see Figure S1 in the Supporting Information). A titration profile of ECFP-eDHFR and Citrine-FKBP′ with SLF′-TMP was fitted to a binding equation, thus giving a dissociation constant of (0.12 ± 0.04) μM.

To test the ability of SLF′-TMP to induce heterodimerization in cells, we expressed EGFP with two copies of FKBP′ (EGFP-2×FKBP′) and TagBFP with two copies of eDHFR and a plasma-membrane-targeting sequence from K-Ras (TagBFP-2×eDHFR-CAAX) in Neuro-2a cells. Total internal reflection fluorescence microscopy (TIRF-M) was used to observe the recruitment of EGFP-2×FKBP′ to the plasma membrane. About 10 μM SLF′-TMP rapidly induced dimerization in cells as observed by measuring the kinetics of EGFP-2×FKBP′ translocation. The half-life for maximal recruitment ($t_{1/2}$) was calculated to be (44 ± 4.3) s, which is similar to that of rapamycin-induced dimerization in cells.[12b] To confirm the chemically induced dimerization in cells using FRET, we expressed mCherry-2×FKBP′ and Citrine-2×eDHFR-CAAX in HeLa cells. If FRET occurs, energy transfer from the donor molecule to the acceptor molecule will cause a decrease in the lifetime of the donor, and can be measured by fluorescence lifetime imaging microscopy (FLIM). Since FLIM-based FRET measurements are insensitive to the concentrations of the fluorophores, artifacts caused by changes in concentration and emission intensity can be avoided. Therefore, FLIM is a very useful technique for monitoring protein interactions in cells.[19] Addition of 1 μM SLF′-TMP to cells expressing Citrine-2×eDHFR-CAAX and mCherry-2×FKBP′ led to a time-dependent decrease of the fluorescence lifetime of Citrine, a decrease which was not observed in control cells expressing Citrine-2×eDHFR-CAAX and mCherry (Figure 2). In those experiments, a lower concentration of SLF′-TMP was used (1 μM). Therefore, the association kintetics were slower compared to the experiments shown in Figure 1B. These results suggest that SLF′-TMP-induced heterodimerization of eDHFR and FKBP′ also occurs in living cells. Subsequent addition of

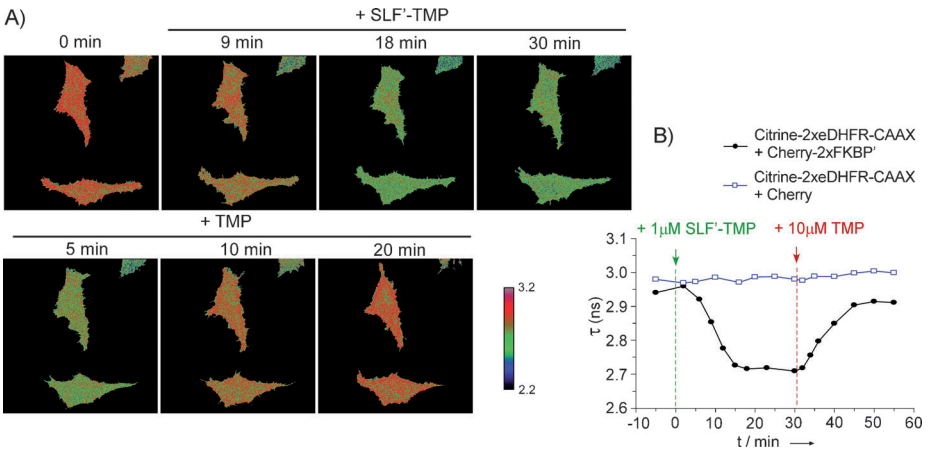


Figure 2. FLIM measurements of the reversible heterodimerization of eDHFR and FKBP′ in live cells. A) Lifetime images demonstrating the reversible heterodimerization of Citrine-2×eDHFR-CAAX and mCherry-2×FKBP′ in HeLa cells induced by the addition of 1 μM SLF′-TMP followed by 10 μM TMP. B) Measurement of the average lifetime of Citrine in HeLa cells coexpressing Citrine-2×eDHFR-CAAX and mCherry-2×FKBP′ (solid circles) and in HeLa cells coexpressing Citrine-2×eDHFR-CAAX and mCherry (open squares) upon addition of 1 μM SLF′-TMP followed by 10 μM TMP.

10 μM of the competitor (TMP) led to the recovery of the fluorescence lifetime (Figure 2), thus showing that the induced protein–protein interaction was fully reversed (Figure 3, and see Figure S2 in the Supporting Information). When the sequential application of the dimerizer and competitor is combined with medium exchange, multiple rounds of protein dimerization and dissociation can be induced. FLIM measurements were used to detect switches between the dimerization on and off states (Figure 3B; see Figure S2). The rapid translocation of mCherry-2×FKBP′ between the cytosol and the plasma membrane was also monitored by confocal microscopy (Figure 3A).

Because rapamycin can also bind to FKBP′, it is possible to combine the SLF′-TMP system (ST system) and the rapamycin system to induce double relocation of the FKBP′ protein in cells. In light of this idea, we made a construct containing mCherry with two copies of the FRB domain and a mitochondrial localization sequence from the *Listeria monocytogenes* protein ActA at its C terminus (mCherry-2×FRB-ActA). For COS-7 cells coexpressing TagBFP-2×eDHFR-CAAX, Citrine-2×FKBP′, and mCherry-2×FRB-ActA, confocal microscopy revealed that these three proteins can be found at the plasma membrane, in the cytosol and the nucleus, and at mitochondria, respectively (Figure 4). Addition of SLF′-TMP to the cells induced translocation of Citrine-2×FKBP′ to the plasma membrane. And, subsequent addition of TMP together with rapamycin led to translocation of Citrine-2×FKBP′ to the mitochondria, because of the disruption of the ST-dimerization and the concomitant formation of the rapamycin dimerization (Figure 4 and Scheme 1).

We next tested whether the ST system could be used to reversibly induce a biological process in cells. The signaling activity of Rac1, a small Rho GTPase, has been shown to lead to the formation of thin actin-based cell protrusions called lamellipodia.[19] The post-translational prenylation of wild-type Rac1 enables it to bind to the plasma membrane, where it is often activated by membrane-bound guanine nucleotide exchange factors (GEFs).[20] Once it is active, Rac1 recruits downstream effectors to the plasma membrane, which promote lamellipodial actin assembly.[21,22] Therefore, to control Rac1 signaling activity in cells, we generated a constitutively active Rac1 mutant which is largely cytosolic because of the removal of its membrane anchor (Rac1[Q61L; C189S; Δ190-192], hereafter referred to as Rac1Q61LΔCAAX) and we fused this mutant to the C terminus of EGFP-2×FKBP′. We expressed this cytosolic fusion protein together with the plasma membrane anchored TagBFP-2×eDHFR-CAAX in Neuro-2a cells, a cell line which forms extensive Rac1-dependent lamellipodia.[23] TIRF-M was used to observe plasma membrane translocation of the Rac1Q61LΔCAAX. These cells were also co-transfected with mCherry to facilitate detection of changes in the cell adhesion area independent of fluorescence intensity changes (Figure 5A,C). The reversible plasma membrane translocation of Rac1Q61LΔCAAX (Figure 5B) was closely correlated with a reversible increase in cell adhesion area (Figure 5C). This correlation shows that SLF′-TMP-mediated plasma membrane targeting of Rac1Q61LΔCAAX can be

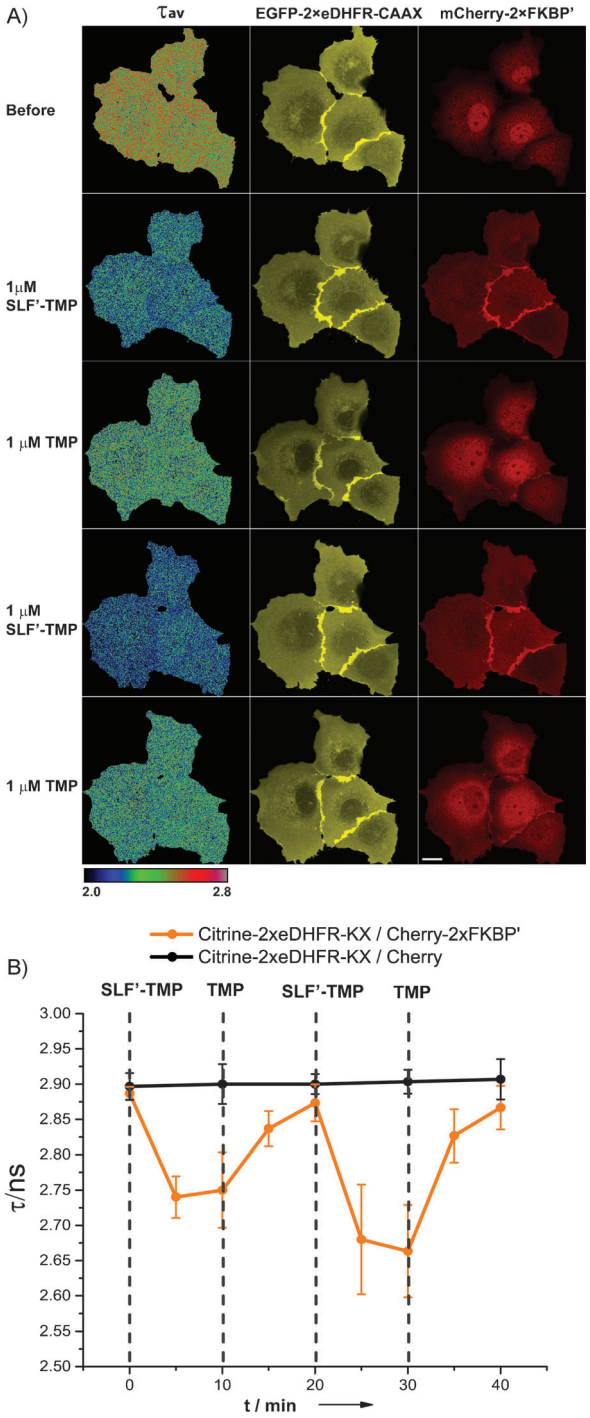


Figure 3. Confocal microscopy and FLIM measurements for multiple rounds of chemically induced protein dimerization and dissociation. A) Confocal and lifetime images demonstrating multiple rounds of dimerization and dissociation of EGFP-2×eDHFR-CAAX and mCherry-2×FKBP′ in COS-7 cells in response to sequential treatment with 1 μM SLF′-TMP and 1 μM TMP. Scale bar: 15 μm. B) Measurement of the oscillating average lifetime of Citrine in HeLa cells coexpressing Citrine-2×eDHFR-CAAX and mCherry-2×FKBP′ or control mCherry produced by switching dimerization on with 2 μM SLF′-TMP and off with 2 μM TMP ($n=3$ cells).

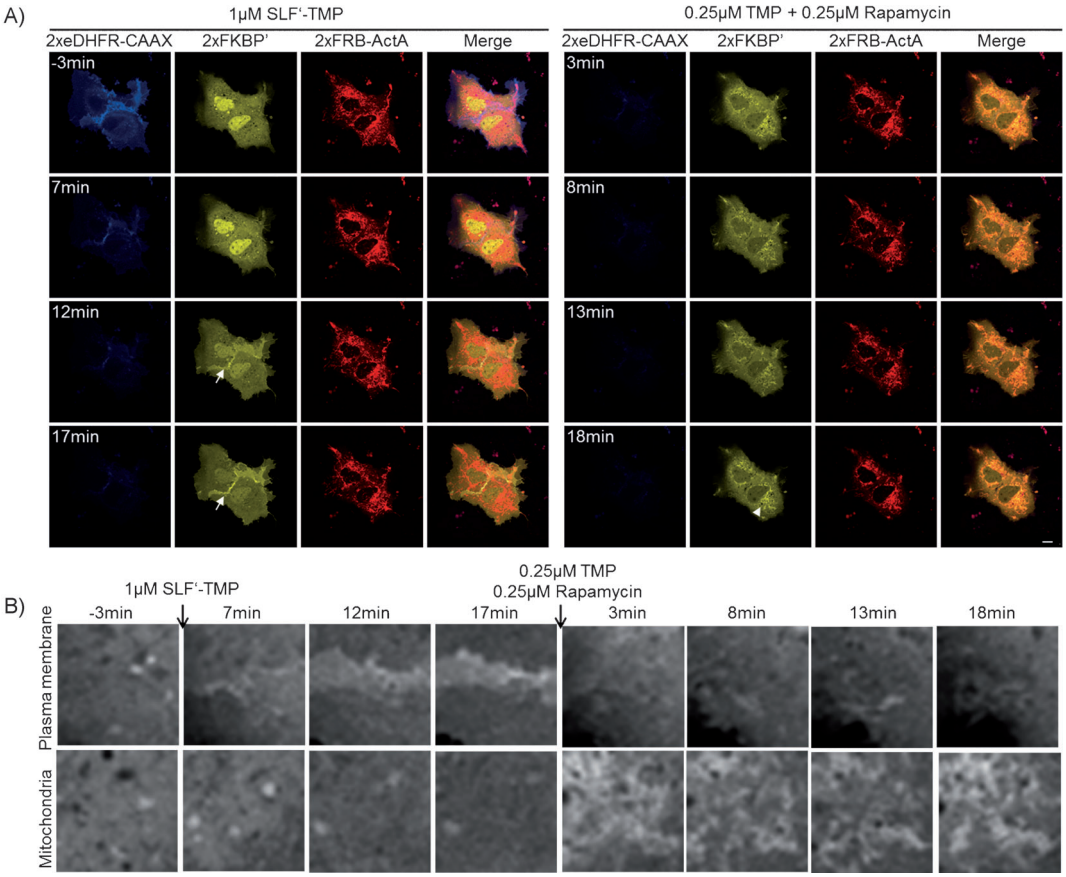


Figure 4. Double relocation of proteins in live cells. A) Confocal microscopy images showing the translocation of Citrine-2×FKBP' between two intracellular organelles. COS-7 cells coexpressing TagBFP-2×eDHFR-CAAX, Citrine-2×FKBP′ and mCherry-2×FRB-ActA were treated with 1 μM SLF′-TMP to induce plasma membrane targeting (arrow) and subsequently with 0.25 μM TMP together with 0.25 μM rapamycin to re-route Citrine-2×FKBP′ to the mitochondria (arrow head). Scale bar: 10 μm. B) Enlarged views of parts of the plasma membrane and mitrochondria.

used to acutely switch on Rac1 signaling activity and associated cellular processes, such as the induction of cell protrusions, in living cells. Furthermore, this signaling activity can also be acutely switched off by addition of the TMP competitor. Interestingly, the rapid reversal of Rac1 signaling activity caused the cell adhesion area to shrink to a value that was similar to that observed before addition of the dimerizer.

The ST system described here offers several advantages over existing CID systems for the control of protein function in cells. First, the dimerizer SLF′-TMP and the competitor TMP molecules selectively bind to exogenously expressed protein modules. Therefore, they are bioorthogonal, thus exhibiting minimal interference with endogenous cellular systems. Second, the dimerization induced by SLF′-TMP can be rapidly disrupted by the addition of the competitor TMP, thus making this a reversible CID system. Third, this approach can be used to induce multiple rounds of dimerization and dissociation. Finally, the features of the ST system make it possible to carry out the temporally controlled, double relocation of a protein of interest to two distinct subcellular locations when combined with the rapamycin system. Although this strategy is subject to the limitations of the rapamycin system (e.g., irreversibility and off-target effects), it nevertheless offers advantages over the recently reported approach.[12b] It only requires three protein modules, while the previous one requires four protein modules. Furthermore, with the approach presented here, the initial translocation step could be performed in a reversible way before the irreversible rapamycin-based translocation to a final distinct site.

In conclusion, we have developed a bioorthogonal and reversible system of chemically induced protein dimerization. We demonstrated that SLF′-TMP rapidly induces the heterodimerization of eDHFR and FKBP′ in live cells and that this dimerization is rapidly disrupted by addition of the competitor TMP. We also demonstrated that this CID system could be used to reversibly target a constitutively active Rac1 mutant to the plasma membrane in live cells, which led to the rapid and reversible formation of lamellipodia. We believe that this system possesses many useful features which will make it an invaluable tool for controlling protein function in vivo.

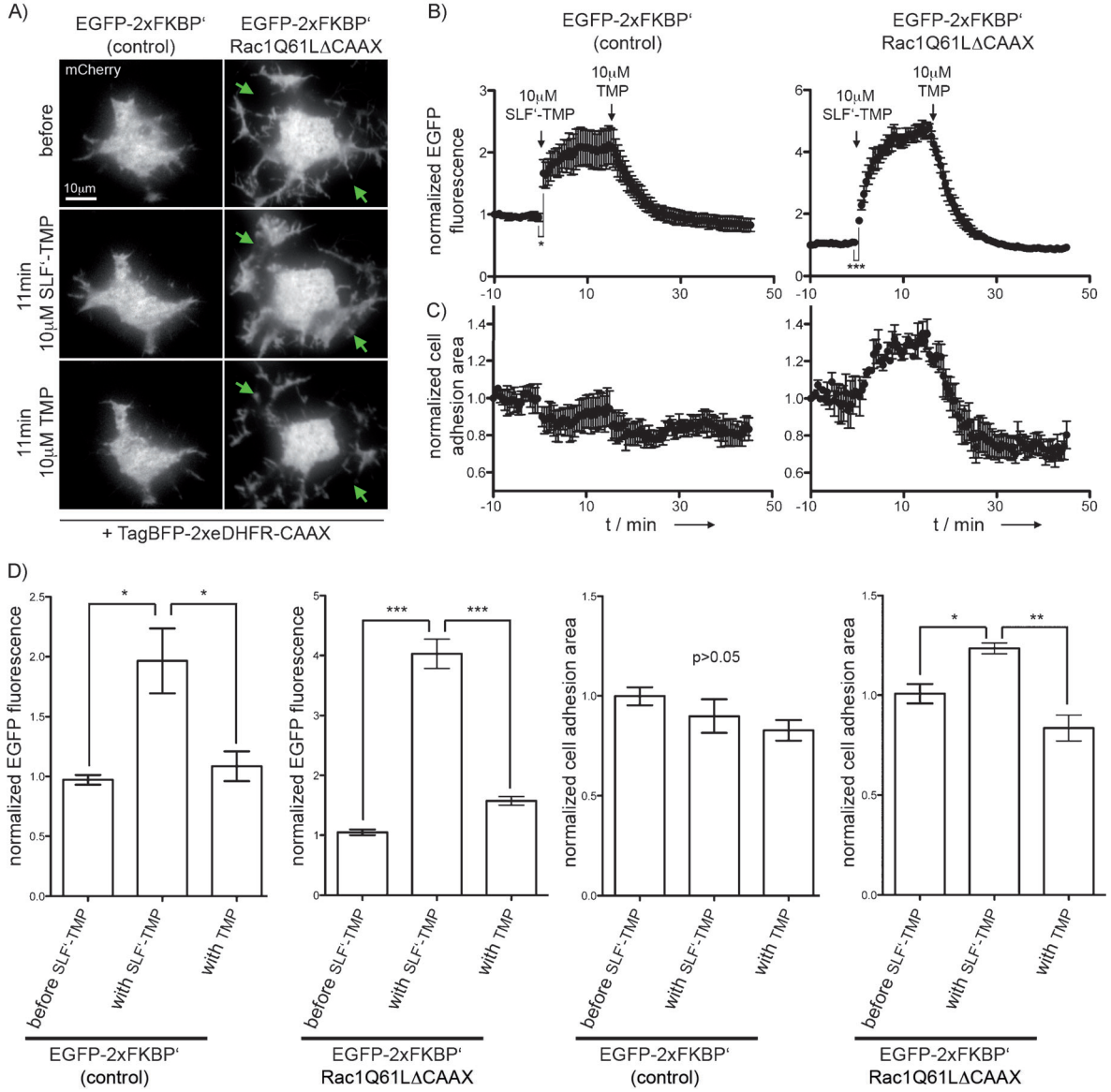


Figure 5. Reversible induction of lamellipodia formation in cells. Neuro-2a cells were co-transfected with mCherry, TagBFP-2×eDHFR-CAAX and either EGFP-2×FKBP′ (control) or EGFP-2×FKBP′-Rac1Q61LΔCAAX. A) TIRF-M images of mCherry before and after addition of either 10 μM SLF′-TMP or TMP. Green arrows point to regions of reversible lamellipodium formation. B) Measurement of EGFP fluorescence intensity in the TIRF field was used to monitor the reversible plasma membrane recruitment dynamics of EGFP-2×FKBP′ constructs after addition of 10 μM SLF′-TMP and TMP. Within 30 sec after SLF′-TMP addition, a significant increase in fluorescence intensity of EGFP-2×FKBP′ or EGFP-2×FKBP′-Rac1Q61LΔCAAX was observed ($n=3$ cells; *: $p<0.05$; ***: $p<0.001$; Student's t-test). C) Morphometric analysis of cell adhesion area corresponding to the recruitment kinetics shown in (B). D) Representation of average EGFP fluorescence intensity and average cell area measurements from panel (C) during the time periods before addition of SLF′-TMP, with addition of SLF′-TMP, and with addition of TMP ($n=3$ cells; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; One-way ANOVA with Tukey's multiple comparison test. All significant changes are marked with brackets).

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