Engineered Networks of Synthetic and Natural Proteins To Control Cell Migration

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Abstract: Mammalian cells reprogrammed with engineered transgenes have the potential to be useful therapeutic platforms because they can support large genetic networks, can be taken from a host or patient, and perform useful functions such as migration and secretion. Successful engineering of mammalian cells will require the development of modules that can perform well-defined, reliable functions, such as directed cell migration toward a chemical or physical signal. One inherently modular cellular pathway is the Ca^{2+} signaling pathway: protein modules that mobilize and respond to Ca^{2+} are combined across cell types to create complexity. We have designed a chimera of Rac1, a GTPase that controls cell morphology and migration, and calmodulin (CaM), a Ca^{2+}-responsive protein, to control cell migration. The Rac1-CaM chimera (named RACer) controlled lamellipodia growth in response to Ca^{2+}. RACer was combined with LOVS1K (a previously engineered light-sensitive Ca^{2+}-mobilizing module) and cytokine receptors to create protein networks where blue light and growth factors regulated cell morphology and, thereby, cell migration. To show the generalizability of our design, we created a Cdc42-CaM chimera that controls filopodia growth in response to Ca^{2+}. The insights that have been gained into Ca^{2+} signaling and cell migration will allow future work to combine engineered protein systems to enable reprogrammed cell sensing of relevant therapeutic targets in vivo.

Keywords: Rac1, calmodulin, LOV2, protein engineering, Ca^{2+}

Novel platforms for therapeutic interventions in humans at the cellular and molecular level include nanoparticles, viruses, and biomaterials. Each class of platform has its particular strengths and weaknesses for a given application; for example, nanoparticles can simultaneously combine multiple modalities such as imaging and payload delivery but are often difficult to target to sites of interest with fidelity, such as a particular cell population within a tumor. Viruses can ensure efficient and reliable transgene delivery to a population of host cells but can have unpredictable consequences on the host such as inflammation and production of viral-neutralizing antibodies. The use of metazoan cells as a platform for therapeutic application, that is, not simply correcting a genetic defect as is the goal in conventional gene therapy but rather introducing novel function to cells, is a relatively unexplored field. Metazoan cells have several desirable properties as a therapeutic platform including the ability to support large networks of transgenes, the potential to be drawn from the host or patient themselves, and the inherent ability to perform useful functions such as migration, secretion, membrane fusion, and target-cell lysis. Some general limitations of metazoan cells as a therapeutic platform are their complexity and heterogeneity, making their overall behaviors often difficult to predict, and stringent handling requirements compared to nanoparticles, viruses or small-molecule drugs. Notwithstanding these limitations, there are several recent reports in the literature of host or patient metazoan cells being reprogrammed by the delivery of transgenes to perform therapeutically valuable functions in vivo, including secreting inflammatory cytokines from tumor cells, targeting and destruction of chronic lymphocytic leukemia cells by host T-cells, and expression of RNA-based anti-HIV moieties from hematopoietic stem cells.

Successfully using metazoan cells as therapeutic platforms will require the modular combination of genetic and protein units that can carry out well-defined functions, such as migration, protein secretion, and cell death. Cell migration is a particularly important function because cells will need to migrate from their source (for example, an injection site, the circulation, or bone marrow) to the site of disease or injury. The ability of therapeutically engineered cells to migrate in a predictable way will determine the efficiency and efficacy of a particular strategy and the likelihood of off-target effects. Cell migration is often considered to be under control of the three principal Rho GTPases Rac1, Cdc42, and RhoA. The simplistic paradigm of the three Rho GTPases is that Cdc42...
initiates filopodia outgrowths, which give way to Rac1-dependent lamellipodia based on actin polymerization at the leading edge while RhoA contracts the rear of the cell enabling locomotion. While this model does not capture the complexity of Rho-dependent migration, for example, the contribution of the some 20 “other” Rho GTPases that are not as frequently studied or the different modes of migration (e.g., mesenchymal and amoeboid), it has been used to create light-sensitive protein switches that control cell migration. While the reported chimeric proteins have been able to reprogram cells such that they migrate in response to light, light-dependent cellular functions are not ideal for use in large organisms, although PARac has been used to study cell migration in Drosophila. Chemical, mechanical, or physical cues that can penetrate tissue are more appropriate for use in large organisms; however, the existing chimeras cannot be readily altered to respond to chemical or other stimuli because they were developed using specific allosteric or binding interactions between light-sensitive proteins and Rho GTPases.

One cellular messenger pathway that can be reconfigured to respond to different chemical and physical cues is the Ca²⁺ signaling pathway. Ca²⁺ is a versatile second messenger that regulates many physiological pathways through Ca²⁺-responsive proteins and can be regulated by a large “tool-box” of Ca²⁺-mobilizing proteins. The tool-box of Ca²⁺-mobilizing proteins can control the cytoplasmic Ca²⁺ concentration in time and space.
space in response to a variety of chemical cues including neurotransmitters, growth factors, purines, and physical signals such as light and voltage. Combinations of Ca²⁺-mobilizing proteins and Ca²⁺-responsive proteins, each of which have specific isoforms depending on cell type, allow cells to decode Ca²⁺ signals into physiological actions. We have shown previously that the Ca²⁺-responsive protein calmodulin (CaM) and its binding peptides can be used to rationally design protein switches that have specific responses to a variety of chemical and physical cues. In particular, we have engineered a Ca²⁺-responsive RhoA chimera, named CaRQ, that regulates blebbing morphology in a set of epithelial cell lines. While blebbing and amoeboid-like migration have been shown to govern cell locomotion in a number of contexts, we are motivated by developing a migration system for other physiological contexts such as in high-adhesion environments, to complement control over amoeboid-like migration. Our goal is to create a Ca²⁺-responsive Rac1 chimera that can be used in a variety of cell lines, exploits mesenchymal cell migration, and enables direction-oriented migration toward chemical and light cues.

## RESULTS

### Conceptual Design of a Rac1-CaM Chimera.

Rac1 was rendered sensitive to Ca²⁺ by a fusion of CaM and two of its binding peptides: those from myosin V (IQ2p) and myosin light chain kinase (MLCKp). The IQ2p peptide has moderate, Ca²⁺-independent affinity for CaM (low micromolar range), while the MLCKp peptide has low affinity for apoCaM and very high affinity for Ca²⁺-CaM (low nanomolar range). We have previously shown that embedding the IQ2p peptide in the surface-exposed loop between amino acids 47 and 48) sensitive to Ca²⁺-CaM. We included a tandem amino-terminal fusion of CaM-MLCKp to promote dissociation of CaM from IQ2p upon Ca²⁺ increase (Figure 1A, Supplementary Figure 1). YFP was added as an amino-terminal fusion for visualization. When the YFP-CaM-MLCKp-Rac1(Q61L E91H ΔCAAX)/IQ2p chimera was initially expressed in HEK293 cells, there was a strong fluorescent signal from the nucleus. There appeared to be more Rac1-like activity of RACer at basal Ca²⁺ than Rac1(Q61L) (Figure 1B, Supplementary Figures 3 and 4). Using both methods, RACer demonstrated significantly less Rac1 activity than Rac1(Q61L) (P < 0.001 by morphology method and P = 0.032 by PCC method, n = 10 in both cases). There appeared to be more Rac1-like activity of RACer at basal Ca²⁺ by the PCC method than by morphology; this was not surprising since our primary motivation is to regulate morphology and migration.

RACer activation by Ca²⁺ resulted in increased Rac1 activity and morphology changes in cells (Figure 1C–I). We continued to use morphology and PCC analyses to measure Rac1 activity after stimulation by ionomycin, a Ca²⁺-mobilizing drug. Strictly in vitro analyses were not performed because RACer was not well expressed in E. coli. Cells expressing RACer and stimulated by ionomycin developed new lamellipodia, and large increases in total area began 2–3 min after the addition of ionomycin and typically lasted for 10–15 min (Figure 1C and D and Supplementary Movies 1 and 2). Rather than reporting the number of lamellipodia per cell or the number of cells with increased lamellipodia formation, the number of cells with an increase in area and the average peak area change are reported. Identifying lamellipodia is subjective, whereas area can be computed with minimal human bias (see Methods for more details). Perfect focus during the imaging time-course was impossible to maintain due to the cell’s lamellipodia occupying more than one focal plane after stimulation. Changes in morphology and cell area were not observed when ionomycin was added to cells expressing RACer(T17N), pLyn-YFP alone, or YFP-Rac1(Q61L) alone (Figure 1E–H). Changes in morphology and area were generally insensitive to Y-27632 (Rho kinase inhibitor) and blebbistatin (myosin ATPase inhibitor) although calmidazolium (CDZ, CaM inhibitor) abrogated Ca²⁺-induced changes (Figure 1G and H). Co-expressing the PBD probe with RACer also prevented Ca²⁺-induced changes in cell morphology and area; the PCC values with the PBD probe increased by approximately 27.8% with RACer and 4.5% with RACer(T17N) (Figure 11).

The concentration of free Ca²⁺ needed for RACer to cause an increase in area in 50% of cells, or EC₅₀, was approximately 24 μM (Figure 2). The EC₅₀ value was determined by stimulating cells with ionomycin using a CaEGTA-K₄EGTA buffer to provide reliable concentrations of extracellular free Ca²⁺. The fit to a standard sigmoid expression was achieved by minimizing the sum of square differences between the data and the model using the three parameters indicated. The quality of the fit, determined by the square of the Pearson coefficient between data and model values, was 0.996, indicating a high-quality fit. For decreasing concentrations of extracellular Ca²⁺ the duration of area increase diminished as shown for representative examples (Figure 2B). The average peak fold area change also decreased as a function of Ca²⁺ concentration, although more as step-function than a sigmoid (Figure 2C). These experiments suggest that there is a strong Ca²⁺-dependence on the changes in cell morphology and area that have been observed.

RACer mediated Ca²⁺-dependent morphology changes in a variety of cell types (Figure 3). One motivation for developing a Ca²⁺-sensitive Rac1 chimera was that our Ca²⁺-sensitive RhoA chimera caused morphology changes (i.e., bleb formation) only in a set of epithelial-like cell lines (HEK293, HeLa and CHO) whereas we believed that a Rac1 chimera could...
control morphology in more cell lines since Rac1-dependent lamellipodia formation has been reported in a wide variety of cell types. RACer was overexpressed in COS7 (primate fibroblast), RAW264.7 (murine macrophage), and HeLa (human-origin epithelial-like) cells and stimulated with ionomycin as with the above experiments. The response was mostly consistent with our earlier observations: a small, but consistent, area increase was observed in all three cell types. The extent of the area increase was generally less than with the above experiments. The response was mostly consistent with our earlier observations: a small, but consistent, area increase was observed in all three cell types. The extent of the area increase was generally less than with the above experiments.

**Figure 2.** Effect of Ca\(^{2+}\) concentration on RACer activation. (A) The percent of cells with an area increase after ionomycin stimulation for the indicated Ca\(^{2+}\) concentration in the extracellular buffer (for example, −6 is 10\(^{-6}\) M or 1 μM). The dashed line is the fitted curve using the equation at the top left and the indicated EC\(_{50}\). The data are the mean ± SEM, n = 3 with at least 10 cells. See Methods for more details. (B) Representative fold area data for stimulated single cells corresponding to each Ca\(^{2+}\) concentration: black (1000 μM), dark gray (100 μM), light gray (10 μM), open (1 μM), dashes (0.1 μM). The 1000 μM is the average data reproduced from Figure 1D. (C) Peak fold area change for each Ca\(^{2+}\) concentration. The data are the mean ± SEM, n = 3 with at least 10 cells.

RACer(T17N) than RACer (Figure 3A) (P = 0.013, 0.044, and 0.019 for COS7, RAW264.7, and HeLa cells, respectively). While the RACer-mediated Ca\(^{2+}\)-induced changes in these other cell lines were not as extensive as they were in HEK293, taken together they show that RACer has a potentially broad applicability to several cell types, which may be optimized on an application-dependent basis.

**RACer Is Robustly Activated by Several Distinct Ca\(^{2+}\)-Mobilizing Signals.** Engineered systems that utilize Ca\(^{2+}\) signaling, such as RACer, can act as modules that can be controlled by distinct Ca\(^{2+}\)-mobilizing signals (Figure 4). A remarkable property of Ca\(^{2+}\) signaling pathways is that proteins that regulate different stages of Ca\(^{2+}\) signaling such as Ca\(^{2+}\)-mobilization, response, buffering, and removal are combined in different ways in different cell types to achieve complexity and endow Ca\(^{2+}\) signals with specificity. Therefore it should be possible for a Ca\(^{2+}\)-responsive protein, such as RACer, to be activated by some other signal if that signal enables a Ca\(^{2+}\)-mobilizing protein to mobilize Ca\(^{2+}\), subject to the spatial and temporal Ca\(^{2+}\) needs of the Ca\(^{2+}\)-responsive protein, as we have established in other cases previously. Such a scheme has obvious advantages in the design of therapeutically useful engineered cells; cues to direct the engineered cells may take on a variety of chemical or physical forms depending on the application.

RACer can be activated by at least three stimuli using three classes of Ca\(^{2+}\)-mobilizing modules: endogenous, exogenous, and engineered proteins. Adenosine S'-triphosphate (ATP) binds purinergic receptors on the plasma membrane and causes a brief Ca\(^{2+}\) transient via the IP\(_3\) pathway and IP\(_3\)-R on the endoplasmic reticulum (endogenous). Acetylcholine (ACh) binds to nicotinic ACh-receptor (nAChR-α4) and opens a Ca\(^{2+}\)-permeable pore for a short Ca\(^{2+}\) transient, but ACh does not cause a Ca\(^{2+}\)-response without nAChR-α4 in HEK293 cells; however, it can be introduced as a transgene (exogenous). LOVS1K, a recently reported synthetic protein, enables blue light to reversibly activate Orai channels, leading to a specific, tunable flux of Ca\(^{2+}\) ions when LOVS1K and Orai1 are delivered as transgenes (engineered) (Figure 4A). For each of these three stimuli, cells expressing RACer and the Ca\(^{2+}\)-mobilizing protein of interest showed morphological changes and area increases (P = 0.003, 0.020, and 0.032 for ATP, ACh, and blue light, respectively) consistent with the Rac1 activation observed with ionomycin (Figure 4B–E and Supplementary Movies 3 and 4). The peak fold area changes were relatively small for the transient-inducing stimuli (ATP and ACh) but were similar between blue light/LOVS1K and ionomycin (Figure 4C). In the case of blue light-activated RACer with LOVS1K, area increase occurred gradually over 20–30 min, which is similar to the reported duration for cytoplasmic Ca\(^{2+}\) accumulation due to LOVS1K (Figure 4F). Further, cell area increase stopped and partially reverted after blue light illumination ceased. Similarly, for ACh-activated RACer, the duration of the area increase corresponded to a typical Ca\(^{2+}\) transient seen from ACh binding to nAChR-α4. ATP stimulation also resulted in relatively small changes in cell area, and this was also likely because the ATP-induced Ca\(^{2+}\) transient in these cells is short, typically less than 30 s in duration. Taking the results of the three stimuli conditions together, this suggests there may be a proportional relationship between the cell area change and the magnitude and/or duration of the Ca\(^{2+}\) signal induced by a given stimulus. The ability to activate RACer using exogenous stimuli such as ACh...
and blue light show that RACer can be used as a Ca^{2+}-responsive module in combination with potentially many upstream signals.

**Directed Cell Migration Mediated by RACer.** Prolonged activation of RACer increased cell migration in wound closure and transwell assays (Figures 5 and 6). The role of Rho GTPases in cell migration has been well studied, and Rac1 is known to regulate lamellipodia formation at the leading edge of migrating cells. We hypothesized that long-term activation of RACer would enhance the migration of HEK293 cells leading to faster penetration and repopulation in a wound closure assay. Cells co-expressing RACer, LOVS1K and Orai1 were illuminated with pulsed blue light (cycling on for 1s, off for 14s) for 24 h resulting in S2.1 ± 4.6% wound closure (Figure 5). Without illumination, replacing RACer with RACerT17N or illuminating cells without LOVS1K resulted in significantly smaller wound closures of 25.2 ± 3.7%, 17.9 ± 3.1%, and 19.4 ± 2.0%, respectively (P = 0.011, 0.007, and 0.004, respectively). The myosin ATPase inhibitor blebbistatin also significantly reduced wound closure to 15.8 ± 6.0% (P = 0.009). The Rho kinase inhibitor Y-27632 also appeared to reduce wound closure to 35.8 ± 3.8%, but the difference was not significant (P = 0.052). The CaM inhibitor CDZ could not be applied here because we find that cells do not survive 24 h when this inhibitor is present at useful concentrations.

The protein networks used here to increase the migration of cells were delivered by transient transfection, resulting in less than 100% transfection efficiency and a population of cells in the wound assay not expressing RACer (Figure 5B). In wounds that were highly closed, the migrating cells were a mixture of the RACer-expressing and wild type populations, suggesting that the presence of RACer-expressing cells was able to alter the migratory phenotype of neighboring wild type cells. This observation is supported by other reports of migrating cells that can “pull” or “guide” neighboring cells along with them through cell–cell adhesions and is also consistent with our earlier work.

Engineered cells migrated in response to concentration gradients of VEGF-A, as well as light, in a transwell migration assay (Figure 6). The wound closure assay demonstrated that light could act as a “permissive” signal to induce migration but did not explicitly provide a directional cue. Further, chemical concentration gradients could not be presented easily using a wound assay. We turned to a transwell migration assay to provide a directional signal for cell migration and the ability to present chemicals such as VEGF-A to guide cell migration. Above we showed that ATP- and ACh-induced Ca^{2+} transients initiated lamellipodia formation, and the literature reports of VEGF-A induced VEGFR2 Ca^{2+} transients appear to be a similar shape, amplitude, and duration to ATP and ACh transients. This appeared to be the case in our hands as well (Figure 6A). Cells expressing RACer seeded onto transwell inserts robustly migrated through the porous filters in response to illumination (LOVS1K/Orai co-expression) and 10 ng/mL VEGF-A (VEGFR2 co-expression) (Figure 6B–D). For light/LOVS1K, illumination of RACer significantly increased migration over RACer(T17N), pLyn-YFP, no illumination, or ATP and ACh would not be suitable stimulants in this assay because their small molecular size would prevent the porous transwell filter from maintaining an effective chemical concentration gradient, thereby obscuring the cue for cell migration. In experiments where VEGF-A was added to both the apical and basal chambers, cell migration was still significantly different than when VEGF-A was present in only the basal chamber (P = 0.038). These experiments show that chemical signals and light can instruct engineered cells to migrate. Further, the directionality of the inducing signal is important to guide cell migration.

**A Ca^{2+}-Sensitive Cdc42 Using the RACer Design.** The modifications made to Rac1 to create RACer were applied to Cdc42 to create a Ca^{2+}-sensitive Cdc42 chimera (Figure 7). We wanted to test the generalizability of our design with another Rho protein using the same layout of RACer to create a Ca^{2+}-sensitive Cdc42 chimera: pLyn-YFP-CaM-MLCKp-Cdc42-ΔCAAX/IQ2p with IQ2p inserted between amino acids 47 and 48 (Supplementary Figure 5). Cells co-expressing the Cdc42 chimera and the PBD probe from above had a similar extent of co-localization by PCC as with RACer, and after ionomycin stimulation the co-localization was not signiﬁcantly different than when VEGF-A was present in only the basal chamber (P = 0.038). These experiments show that chemical signals and light can instruct engineered cells to migrate. Further, the directionality of the inducing signal is important to guide cell migration.
PAK in addition to regulators of filopodia growth such as WASP.

**DISCUSSION**

The long-term goal of this work is to develop modular systems to reprogram mammalian cells for useful purposes such as therapeutic platforms or other applications. Specifically, the system presented here enabled cell morphology changes and directed cell migration in response to exogenous stimuli by bringing Rac1 under control of the Ca²⁺ second messenger. In this design, a CaM-binding peptide, IQ2p, with Ca²⁺-independent CaM affinity, was embedded in a surface-exposed loop of Rac1. A second peptide, MLCKp, with strong, Ca²⁺-dependent CaM affinity was added to the fusion so that increases in local free Ca²⁺ result in CaM dissociating from IQ2p and associating with the nearby MLCKp. This shows that CaM’s ability to recognize target peptides is highly modular, since CaM was able to bind them in the context of a structurally modified chimera.

The inherent modularity of Ca²⁺ signaling was leveraged so that RACer was activated by exogenous stimuli such as ACh and blue light. RACer, a Ca²⁺-responsive protein, was combined with either nAChR-α4 or LOVS1K, which are Ca²⁺-mobilizing proteins, to create a multimodule protein network based on Ca²⁺ signaling. These two examples are hardly exhaustive; Ca²⁺-mobilizing proteins exist that can respond to yellow-red wavelengths of light, cytokines and growth factors, and membrane potential. Existing systems that regulate cell migration in response to exogenous stimuli such as PARAcα and the phytochrome-PIF system rely on direct interaction between Rac1 and the stimuli-sensing proteins. This means that these systems cannot be adapted to respond to other stimuli such as biochemicals or physical cues. In contrast, RACer is one module that can be combined with other protein modules to sense stimuli, using Ca²⁺ as the intermediary. Input flexibility is a powerful motivation for the further development of synthetic systems based on Ca²⁺ signaling or other modular signaling pathways. Additionally, new insights into Ca²⁺ signaling in nature have been gained: Ca²⁺ can act as a cellular messenger to relay messages between proteins that are foreign to the cellular milieu (here, our chimera and exogenous Ca²⁺-mobilizing domains). This suggests that Ca²⁺-based signaling may have been important in the evolution of new cellular pathways.

The Ca²⁺ EC₅₀ value of 24 μM for RACer was determined by stimulating cells expressing RACer with ionomycin in buffers of different Ca²⁺ concentrations. The output that we considered was the percentage of cells with an increase in cell area indicative of Rac1 activation. Ionomycin treatment of cells results in an equilibrium of extracellular and cytoplasmic Ca²⁺ concentrations. For Ca²⁺ concentrations below 100 μM, where buffering effects of cellular and extracellular proteins can be significant, a mixture of CaEGTA and K₂EGTA was used to establish buffers with known free Ca²⁺. The value reported here is similar to the EC₅₀ value of 27 μM we determined previously for CaRQ. Given that the same CaM protein and peptides were used in both chimeras, the similarity between EC₅₀ values is not surprising, provided that Rac1 and RhoA have similar binding affinities for their downstream targets and abilities to generate the morphology changes that were used as reporters of chimera activity.

Wound and transwell assays were used to demonstrate that prolonged activation of RACer for 24 h increased the migration of
of HEK293 cells. For the wound assays, blue light was used as the stimulus and LOVS1K/Orai1 as the Ca\textsuperscript{2+}-mobilizing module because light can be controlled more easily than chemicals in solution; a continuous stimulus could be provided rather than a bolus dose of ionomycin, ACh, or some other biochemical signal. However, in the transwell assays where a concentration gradient could be maintained for some time a VEGF-A stimulus was used with VEGFR2 as the Ca\textsuperscript{2+}-mobilizing domain to direct cell migration. Overexpression of dominant positive Rho proteins, including Rac1, has usually been noted to suppress cell migration,\textsuperscript{32} as we have also seen in our wound assay (Figure 5A and B). However, when dominant positive Rac1 is activated with directionality, for example, with PARac, then dominant positive Rac1 can promote cell migration.\textsuperscript{10a,c} A similar phenomenon likely accounts for increased cell migration in these wound assays: periodic activation of RACer via LOVS1K leads to lamellipodia growth in the direction of the wound because this is the only open space available. Growth of lamellipodia into the wound provides a default directionality cue to the cell, enabling migration in that direction and wound closure.

As an extension to the development of RACer and to show that our design strategy is applicable to other Rho family proteins, we created a chimera of Cdc42 analogous to RACer. The Cdc42 chimera displayed activation kinetics similar to that of RACer, that is, morphology changes could typically be seen in cells 2–3 min after adding ionomycin, and the duration of the morphology change was similar. The morphology change included an initial growth of filopodia followed by lamellipodia in most cells. This suggests that activated Cdc42 was able to interact with PAK, which is known to activate αPIX, a GEF for Rac1.\textsuperscript{27} Expanding the chimeric Ca\textsuperscript{2+}-control strategy presented here to other Rho family proteins may create insights into the functioning of highly homologous members such as RhoA, RhoB, and RhoC. If the approach is further generalizable, novel control strategies may be possible for other Ras superfamily proteins with diverse functions such as Rab (endosomal shuttling), Ran (nucleocytoplasmic transport), Rap (cell adhesion), and others.

Future work with the RACer construct will further the goal of using it as a functional module for reprogramming cells. The construct may need to be optimized for particular Ca\textsuperscript{2+}-mobilizing signals: for example, using RACer with signals that cause short Ca\textsuperscript{2+} transients such as ACh may require modifications to increase the duration of the Ca\textsuperscript{2+}-mediated cell response. This may be accomplished by changing or mutating the CaM-binding peptides used in RACer or by adding buffering proteins to the vicinity of RACer or the Ca\textsuperscript{2+}-

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**Figure 5.** Light-activated cell migration with RACer and LOVS1K. (A) Wound width at 0 h (dark bars) and 24 h (light bars) with percentage change indicated above the bars, for conditions indicated beneath the charts. Data are the mean ± SEM, n = 9 wounds in 3 independent experiments. (B) Representative images of RACer and RACer(T17N) in both YFP and brightfield channels at 0 and 24 h. Scale bar is 100 μm.
mobilizing module. RACer may also be tested with other protein modules that have been developed to induce cellular apoptosis or promote membrane fusion, as first steps toward creating a multifunctional reprogrammed cell. Combining RACer with other Ca\textsuperscript{2+}-mobilizing modules, for example, VEGFR2, may enable reprogrammed cells to directly migrate toward sources of VEGF \textit{in vitro} and \textit{in vivo} such as areas of active angiogenesis during tumor formation.

**Conclusion.** We have developed a chimera of Rac1, CaM, and two CaM binding peptides that enabled Rac1-like activity in cell lines when cytoplasmic Ca\textsuperscript{2+} was elevated. The Rac1-CaM chimera, RACer, was used as a Ca\textsuperscript{2+}-responsive module with a variety of Ca\textsuperscript{2+}-mobilizing protein modules including ATP/purinergic receptors, ACh/nAChR-\textalpha 4, and blue light/LOVS1K. The combination of RACer and LOVS1K enabled light-sensitive cell migration with repeated activation over 24 h.

The development of Ca\textsuperscript{2+}-based synthetic protein networks provides new insights into the role of Ca\textsuperscript{2+} in nature and will ultimately enable mammalian cells to perform complex novel functions that may be of practical therapeutic or industrial value.
Plasmid Construction. The RACer plasmid was created with methods described previously. Primers used to amplify the amino- and carboxy-terminal fragments of Rac1 and the PBD domain are given in the Supplementary Methods. Plasmids for Rac1, nAChR-α4, Orai1, and PBD were Addgene plasmids 13720, 15245, 19756 and 13723, respectively. Mutations to Rac1 (T17N and E91H) were performed by self-hybridizing PCR; primers are given in the Supplementary Methods.

Cell Culture and Transfection. COS7, HeLa, and HEK293 cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS, 25 mM d-glucose, 1 mM sodium pyruvate, and 4 mM L-glutamine (Invitrogen, Carlsbad, CA) in a T-25 flask. Cells were passaged at 95% confluence using 0.05% trypsin with EDTA (Sigma Aldrich, St. Louis, MO). RAW264.7 cells were maintained in RPMI-1640 supplemented with 10% FBS and 10 mM HEPES. RAW264.7 cells were passaged by cell scraping at 90% confluence. All cell media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. After passaging, cells were seeded onto glass-bottom dishes at 1:15 dilution. (Mattek, Ashland, MA). Cells were transiently transfected using Lipofectamine 2000 according to manufacturer’s directions (Invitrogen).

Reagents Used. Cells were treated with Y-27632 (10 μM), CDZ (50 μM), or (−)-blebbistatin (10 μM) by preincubating the inhibitor with cells for 1 h prior to imaging. ATP (10 μM), ACh (1 mM), and ionomycin (1.5 μM) were added as a 1:10 dilution directly into the imaging medium. These six chemicals were purchased from Sigma Aldrich. VEGF-A (10 ng/mL), dextran (10,000 MW), and Fura-2 AM (Invitrogen, Carlsbad, CA) in a T-25 flask. Cells were illuminated with CFP excitation light.

Cellular Imaging and Analysis. Imaging was performed using an inverted IX81 microscope with Lambda DG4 xenon lamp source and QuantEM 512SC CCD camera with 40x and 60x oil immersion or 10x objectives (Olympus, Markham, ON, Canada). For LOVS1K, all characterization on the microscope stage (Figure 4 and Movies 3 and 4) received 300 ms pulses of blue light every 10 s. Overnight illumination (Figures 5 and 6) was provided by an iPod programmed to deliver blue light on/14 s off; this was the fastest cycling that could be achieved using our method of cycling light on the iPod. The power output of the iPod display (blue screen) was approximately 1 mW/cm². The power output of the xenon lamp at the microscope stage is 25 mW/cm². CA²⁺ transients were recorded using the TN-XL biosensor; the data are the ratio of YFP channel intensity to CFP channel intensity when cells were illuminated with CFP excitation light.

Data Analysis. Significance, where discussed, was calculated using the unpaired Student’s t test without assuming equal variances. The alpha value for this study was set at 0.05, and therefore P < 0.05 was considered significant.

For fold area change versus time graphs, cell area was determined using the lowest intensity threshold that captured the whole cell area with ImageJ. For cells that were too close to be delineated automatically, area was calculated by visual inspection. The fold area change was normalized to the whole cell area with ImageJ. For cells that were too close to be delineated automatically, area was calculated by visual inspection. The fold area change versus time graph. For the Cdc42 chimera experiments, a cell was counted as having a morphology change if filopodia or lamellipodia began growing after the stimulus was added up to 20 min later. The peak fold area change where reported was the global maximum of the fold area change versus time graph.

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