ACS SyntheticBiology

Letter

pubs.acs.org/synthbio

Development of an Artificial Calcium-Dependent Transcription Factor To Detect Sustained Intracellular Calcium Elevation

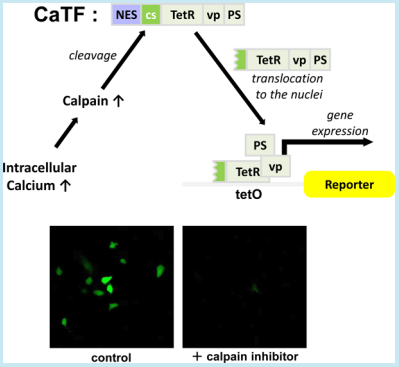
Shingo Suzuki,*[,†] Kazutoshi Murotomi,[‡] Yoshihiro Nakajima,[‡] Katsuhisa Kawai,[†] Ken-ichi Ohta,[†] Katsuhiko Warita,[†] Takanori Miki,[†] and Yoshiki Takeuchi[†]

[†]Department of Anatomy and Neurobiology, Faculty of Medicine, Kagawa University, Miki-cho, Kagawa 761-0793, Japan

[‡]Health Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Takamatsu, Kagawa 761-0395, Japan

Supporting Information

ABSTRACT: The development of a synthetic transcription factor that responds to intracellular calcium signals enables analyzing cellular events at the single-cell level or "rewiring" the intracellular information networks. In this study, we developed the calcium-dependent transcription factor (CaTF), which was cleaved by calpain and then translocated to the nuclei where it induced reporter expression. Our results demonstrated that CaTF-mediated reporter expression was stable and responded to the intracellular calcium level and calpain activity. In addition, CaTF detected the sustained calcium increase that was induced by physiological stimulation with epidermal growth factor (EGF). These results suggest that CaTF could be a useful tool to analyze intracellular calcium signals and be an interface between an endogenous signal network and synthetic gene network.



KEYWORDS: *mammalian synthetic biology, synthetic transcription factor, biosensor, intracellular calcium, interface, cell therapy*

Intracellular calcium is a secondary messenger that is involved in a broad range of biological processes, including transduction of survival signals, muscle contraction, and synaptic transmission.[1] Several types of protein sensors for imaging intracellular calcium signals have been developed to better understand the roles of calcium signaling, including cameleon,[2] pericam,[3] GCaMP6,[4] and GECO family proteins,[5] many of which are aimed at real-time imaging of intracellular calcium. In addition, indirect methods to detect intracellular calcium with activity-dependent promoters, such as c-fos or arc, have been exploited.[6−11] The transduction of intracellular calcium signals to reporter gene expression by synthetic protein sensors, however, has not been investigated. If the intracellular calcium activity is artificially converted to reporter gene expression, one could potentially use alternative methods to measure cellular activity. Further, a synthetic transcription factor that is activated by physiological stimuli would be a useful interface to connect endogenous cell systems to synthetic systems. For example, physiological calcium signaling could be used as the "Input" signal for synthetic networks.

Calpain is a calcium-dependent cysteine protease that is ubiquitously expressed in mammalian cells.[12] In response to the increase of intracellular calcium, calpain activates intracellular signaling by cleaving substrate proteins.[13] Calcium-mediated calpain activation is critical for cell mobility, cell cycle progression, the formation of long-term potentiation or dendritic morphology.[14,15] Therefore, several synthetic protein sensors that can visualize calcium transients by monitoring the calpain activity have been developed to investigate these effects. The sensor design is based on the structure containing the fluorescence resonance energy transfer (FRET) domain and the α-spectrin-derived sequence that is specifically cleaved by calpain.[16−19] Calpain activity is detected in these sensor proteins as a change in the FRET efficiency.

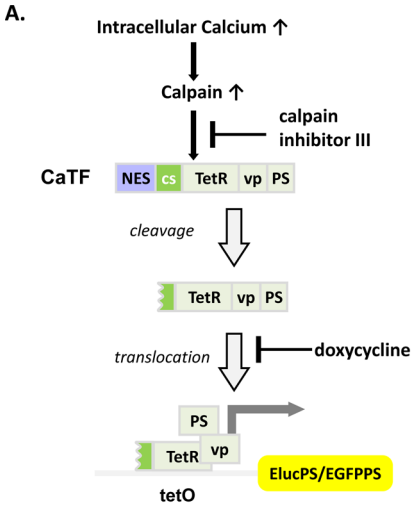
Here, we designed and created a novel synthetic protein sensor to transduce intracellular calcium signals to reporter expression. To accomplish this, calpain activity and an α-spectrin-derived sequence were used. Assessment of this synthetic sensor revealed that this probe accurately responded to the intracellular calcium signals under our culture conditions.

RESULTS AND DISCUSSION

CaTF-Mediated Intracellular Calcium Sensing. To transduce the intracellular calcium signal to reporter systems, an artificial calcium-dependent transcription factor (CaTF) was designed (Figure 1A and B). The CaTF contains 329 amino acids and encodes a fusion protein mainly consisting of five protein domains. To verify the detection of calcium-dependent transcription, the CaTF-expression plasmid was introduced into baby hamster kidney (BHK) cells with a luciferase reporter plasmid that encoded for enhanced green-emitting luciferase

Special Issue: Engineered DNA-Binding Proteins

Received: February 3, 2014
Published: September 4, 2014



B.

MNLVDLQKKL	EELELDEQQG	SGSGQQEVY*G	MMPRDGSGKD	PMSRLDKSKV
INSALELLNE	VGIEGLTTRK	LAQKLGVEQP	TLYWHVKNKR	ALLDALAIEM
LDRHHTHFCP	LEGESWQDFL	RNNAKSFRCA	LLSHRDGAKV	HLGTRPTEKQ
YETLENQLAF	LCQQGFSLEN	ALYALSAVGH	FTLGCVLEDQ	EHQVAKEERE
TPTTDSMPPL	LRQAIELFDH	QGAEPAFLFG	LELIICGLEK	QLKCESGRGP
ADALDDFDLD	MLPADALDDF	DLDMLPADAL	DDFDLDMLSR	HGFPPEVEEQ
AAGTLPMSCA	QESGMDRHPA	ACASARINV		

Figure 1. Design of calpain-dependent transcription factor (CaTF). (A) CaTF-mediated reporter expression system. An increase in the intracellular Ca^{2+} level induced activation of the calcium-dependent protease, calpain, which cleaved the cs site of CaTF in the cytosol. A cleaved CaTF was translocated to the nuclei, bound to the tetO element, and induced the expression of reporter genes. Calpain inhibitor and doxycycline inhibited the cleavage and nuclear translocation of CaTF, respectively. (B) Amino acid sequence of CaTF. CaTF mainly consists of NES (underlined), cs (in blue), TetR (in red), vp (in green), and PS (in gray). The linker and spacer sequences are in black.

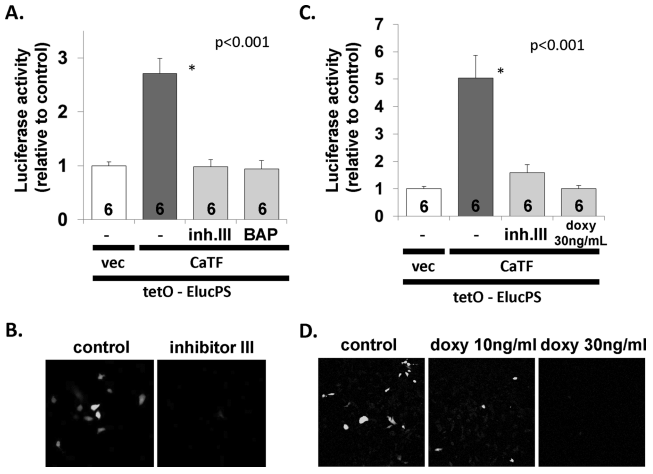


Figure 2. CaTF sensed the intracellular calcium level of BHK cells at steady-state. CaTF gene and reporter construct (tetO-Eluc or tetO-EGFP) were introduced into BHK cells. After 24 h incubation, luciferase activity (A and C) or fluorescence of EGFP (B and D) was analyzed. An empty expression vector (vec), without the CaTF gene, was introduced as a control. The number associated with each column represents the number of wells in the same experiment. (A and B) Cells were cultured in the presence or absence of 20 μM Calpain Inhibitor III (Inh.III; calpain inhibitor) or 10 μM BAPTA-AM (BAP; intracellular calcium chelator). Note that CaTF-dependent reporter activation was inhibited by treatment with Inh.III or BAPTA-AM. (C and D) Cells were cultured in the presence or absence of 20 μM Inh.III or 10 ng/mL or 30 ng/mL doxycycline (doxy). Note that fluorescent signals of EGFP were decreased depending on the dosage of doxycycline. $p < 0.001$. In this and all other figures, results are shown as the mean ± SEM. One-way ANOVA and Tukey's posthoc test were used for the statistical analyses of differences. The asterisk indicates a significant difference from all other groups ($p < 0.001$).

(Eluc) with a PEST sequence, an ornithine decarboxylase-derived degradation sequence, after the Tet operator (tetO) binding sites.[20] As shown in Figure 2A, when compared to an empty expression vector, introduction of the CaTF plasmid induced Eluc expression, indicating that endogenous intracellular calcium signals were possibly converted into reporter expression. We then investigated the dependency of calpain activity on CaTF-mediated Eluc expression. Calpain activity is inhibited by a selective calpain inhibitor, Calpain Inhibitor III (also known as MDL28170), which is a cell-permeable peptide with a sequence of Z-Val-Phe-CHO. In the presence of Calpain Inhibitor III, CaTF-dependent Eluc expression was clearly inhibited. We further examined the dependency of the CaTF-mediated Eluc expression on the intracellular calcium signals. As shown in Figure 2A, treatment with an intracellular calcium chelator, 10 μM BAPTA-AM, strongly inhibited the CaTF-dependent Eluc expression, suggesting that the CaTF-mediated reporter expression was dependent upon intracellular calcium levels. In addition to the Eluc-based reporter system, the dependency of CaTF-mediated reporter expression on calpain activity was also confirmed with an enhanced green fluorescent protein (EGFP) reporter system. A PEST sequence was also introduced at the C-terminal of EGFP. Our results showed that CaTF-induced EGFP expression was strongly inhibited by the presence of Calpain Inhibitor III (Figure 2B), indicating that CaTF responded to intracellular calcium levels and induced reporter expression that was dependent on calpain activity. It was previously reported that calpain was potentially activated in a calcium-independent manner,[21−24] possibly involving mitogen-activated protein kinase (MAPK), Rho GTPase, and phosphatidylinositol 4,5-bisphosphate (PIP_2). The Rho GTPases[25−27] activate PIP5K, which in turn phosphorylates PI4P to $PI(4,5)P_2$. Thus, the constitutively active RhoA (caRhoA) was cointroduced with CaTF and the reporter constructs to investigate whether Rho GTPase or PIP_2 induced the CaTF-mediated reporter expression.[28] As shown in Supporting Information (SI) Figure S1, caRhoA did not induce reporter expression, but rather suppressed it. The effect of MAPK phosphatase inhibitor, sanguinarine, was also investigated.[29] However, the effect of MAPK activation could not be evaluated because this inhibitor affected cell viability in our system (data not shown). Finally, we determined whether CaTF activity was controlled by the application of an exogenous compound. Doxycycline treatment inhibits the translocation of a tet-repressor-derived DNA binding domain (TetR) to nuclei. As expected, CaTF-dependent Eluc expression was blocked by treatment with doxycycline (Figure 2C). Correspondingly, this inhibitory effect of doxycycline was also observed when the EGFP reporter plasmid was used (Figure 2D). These results confirmed that reporter expression by CaTF was TetR-dependent and suggested that the strength of reporter expression by CaTF was controllable by treatment with doxycycline.

Stability of CaTF-Mediated Reporter Gene Expression. Reporter expression induced by CaTF should be stable and

dx.doi.org/10.1021/sb500070c | ACS Synth. Biol. 2014, 3, 717−722

detectable over at least several days. To confirm this, real-time monitoring for Eluc activity was carried out over 4 days following transient transfection.[20] Eluc activity was measured for 1 min at 10 min intervals with a luminescence detector embedded with a CO_2 incubation system. In response to the expression of CaTF with the reporter construct, Eluc activity robustly increased 12 h post-transfection and peaked at 41 h (Figure 3A). This activation slightly decreased but was sustained

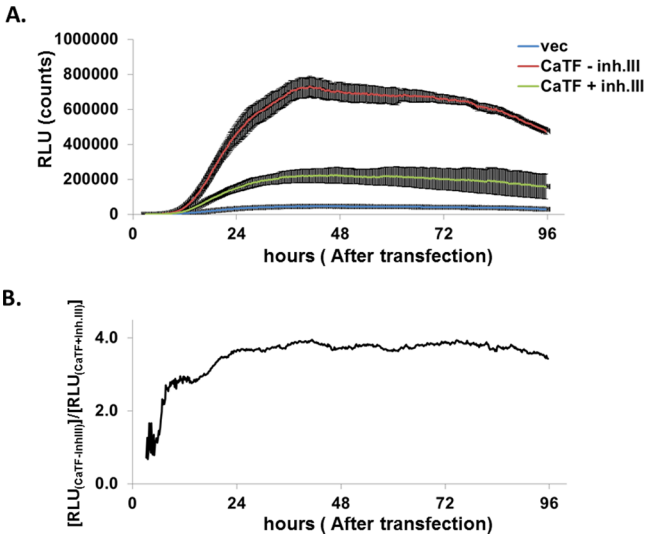


Figure 3. Stability of CaTF-derived signals in BHK cells. CaTF gene and reporter construct (tetO-Eluc) were introduced into BHK cells. Cells were cultured in the presence or absence of 20 μM Calpain Inhibitor III (Inh.III). An empty expression vector (vec), without the CaTF gene, was introduced as a control. (A) Time-course of luciferase activity. After transfection, Eluc activity was monitored by Kronos. (B) Ratio of Eluc activities in the presence or absence of Calpain Inhibitor III in A. Note that $[RLU_{(CaTF\text{-}inh.III)}]/[RLU_{(CaTF+inh.III)}]$ was virtually constant after a 24 h incubation.

for 96 h following transfection. In the presence of Calpain Inhibitor III, Eluc activity slightly increased after transfection, peaked at 47 h, and was sustained for 96 h. Without CaTF expression, Eluc activity was quite lower than that of the other two groups. As shown in Figure 2A and C, the Eluc activity for CaTF in the absence of Calpain inhibitor III was different in these images. This may be because the CaTF-mediated reporter expression had not peaked at 24 h post-transfection. To investigate the net increase of Eluc activity by calpain activation, the ratio of the Eluc activity in the presence of Calpain Inhibitor III to that in the absence of Calpain Inhibitor III was calculated (Figure 3B). A significant increase was observed up to 12 h, after which the ratio was sustained from 24 to 96 h. Surprisingly, the ratio during this period did not change greatly, suggesting that the CaTF response to calpain activity was highly stable.

Detection of EGF-Induced Intracellular Calcium Increase. Finally, we investigated whether CaTF could detect an increase of intracellular calcium resulting from EGF stimulation, which is known to induce intracellular calcium signals.[30] BHK cells were starved with serum-free DMEM and treated with EGF 4 h following transfection of the CaTF expression plasmid and the Eluc reporter plasmid. In the presence of EGF, Eluc activity was significantly increased when compared to Eluc activity in the absence of EGF (Figure 4A). A similar result was observed using the EGFP reporter plasmid (Figure 4B). EGF treatment resulted in increased EGFP expression levels in each of the

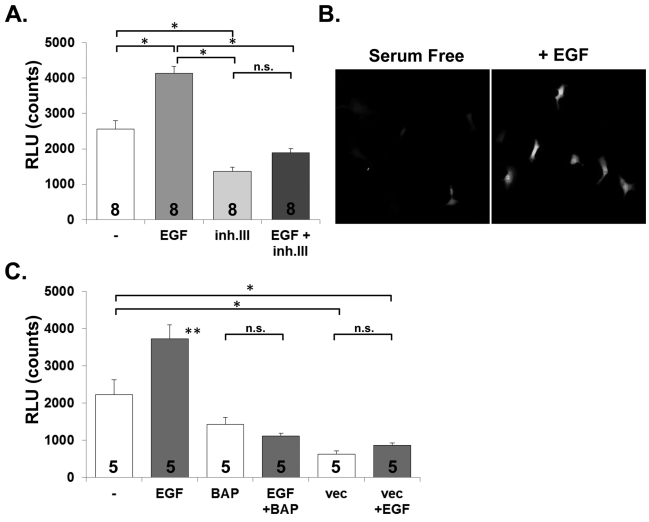


Figure 4. Detection of physiological calcium increase by CaTF. CaTF gene and reporter construct (tetO-Eluc or tetO-EGFP) were introduced into BHK cells. (A–C) Cells were cultured with serum-free medium in the presence or absence of 200 ng/mL EGF, 20 μM Calpain Inhibitor III (Inh.III), and 10 μM BAPTA-AM (BAP). Instead of CaTF, an empty expression vector (vec) was introduced as the control. After incubation for 24 h, luciferase activity (A and C) or fluorescence of EGFP (B) was analyzed. The number associated with each column represents the number of wells in the same experiment. One-way ANOVA and Tukey's posthoc test were used for the statistical analyses of differences. Single asterisks indicate a significant difference from the control ($p < 0.05$). The double asterisks indicate a significant difference from all other groups ($p < 0.03$). Other combinations of groups did not show any significant differences. "n.s." means "no significant difference".

cells compared to the control culture. Additionally, EGF-mediated Eluc expression was blocked by the treatment with Calpain inhibitor III (Figure 4A) or BAPTA-AM (Figure 4C), suggesting that CaTF could monitor the increased calpain activity induced by treatment with EGF to stimulate intracellular calcium signaling. In contrast, Eluc activity did not significantly change between EGF-treated and untreated cells without the CaTF construct (vec; Figure 4C). In conclusion, we confirmed that the synthetic transcription factor, CaTF, responds to intracellular calcium signals and induces reporter activation by detecting intracellular calpain activity.

Engineering of the Intracellular Calcium-Dependent Transcription Factor. In this study, we created a synthetic transcription factor, CaTF, which senses and converts intracellular calcium signals to reporter expression, and assessed its activity *in vitro*. To our knowledge, this is the first synthetic transcription factor that transduces cytosolic calcium signals to activate reporter genes in the nuclei. The present study indicates that CaTF can detect intracellular calcium increases resulting from EGF stimulation. As shown in SI Figure S2, the effect of basic fibroblast growth factor (bFGF) was also detected by CaTF. We anticipate that this sensor will be used to investigate intracellular calcium signaling, with high-throughput screening as just one potential application for the CaTF.

In addition to its potential as a calcium sensor, CaTF is a beneficial interface for synthetic biology. Though several types of synthetic transcription factors have been created,[31−34] a synthetic transcription factor that is activated by physiological stimuli has not been well studied. An interface between endogenous cell systems and synthetic systems is quite

dx.doi.org/10.1021/sb500070c | ACS Synth. Biol. 2014, 3, 717−722

important for the development of the synthetic tools that modify innate cell systems. CaTF was shown to connect a physiological stimulus to synthetic gene networks. Calpain activation is involved in many severe diseases.[35] As such, CaTF-mediated sensing of calpain activity and induction of synthetic gene expression may provide a platform for new viral-based therapeutics.

The expression levels and types of calpain are different for different cell types and their specific states.[36] Though these calcium-independent factors make CaTF-mediated calcium sensing qualitative, the CaTF response to calpain activity in the same cells was stable (Figure 3). Therefore, calcium sensing by CaTF may be suitable to detect alterations in calcium levels in the same cell populations for prolonged periods. It was suggested that calpain could be activated independently of calcium via MAPK-mediated phosphorylation.[37] However, under normal culture conditions, CaTF-mediated reporter expression was primarily dependent upon intracellular calcium signals because the effects of BAPTA-AM on CaTF-mediated reporter expression were comparable to those resulting from treatment with Calpain inhibitor III. Therefore, MAPK would not be the main activator of calpain under normal culture conditions. In contrast, reporter expression induced by EGF was, at least in part, mediated through the direct activation of calpain by MAPK, as previously reported.[21] In addition, our study using caRhoA indicated that activation of Rho GTPase did not induce reporter expression. Although the reason for the reporter suppression by caRhoA was not known, these results suggested that intracellular calcium would be a main factor that activates CaTF. However, we could not completely rule out the possibility that CaTF is activated by calcium-independent manner.

In this study, reporter expression without CaTF was observed (Figure 2). This leaky signal was mainly derived from a reporter plasmid that was cotransfected with the CaTF expression plasmid. In general, exogenous gene expression in a stable cell line or transgenic animal is more stable and has a lower background signal than that in cotransfected cells. Thus, to improve data quality, CaTF and the reporter genes should be expressed from genomic DNA. In the present study, doxycycline treatment was used to decrease the baseline reporter expression by calcium signals. Doxycycline has been used to control gene expression *in vitro* and *in vivo* in numerous reports. Therefore, despite the presence of a background signal, the gain of reporter expression by CaTF would be controllable for *in vitro* or *in vivo* studies.

CaTF-mediated reporter expression was strongly inhibited by 10 μM BAPTA-AM (Figure 2). However, the ratiometric calcium measurement study using Fura-2-AM showed that treatment with 10 μM BAPTA-AM did not result in complete chelation of intracellular calcium and that the 30 μM BAPTA-AM treatment more strongly suppressed the intracellular calcium level (SI Figure S3). This result indicated that CaTF-mediated reporter expression was not induced by low levels of calcium concentration. Although the molecular mechanisms are not known, it is expected that CaTF-mediated reporter expression would need a certain amount of calcium in the cell.

While FRET-based sensors can directly monitor intracellular calcium levels more than CaTF,[16−19] the accumulation of cleaved probes and saturation of endogenous calpain activation were shown to limit the time-resolution and the use of various cell types *in vivo*, respectively.[17] CaTF-mediated reporter systems indirectly monitor calpain activity unlike FRET-based sensors. However, the limitation of the CaTF duration with a PEST sequence provides time-resolution and the use of a TetR domain provides the potential for gain control by doxycycline, which are properties of the CaTFs that distinguish them from FRET-based calcium sensors. Although PEST sequence in CaTF does not provide the ability to respond to short time calcium oscillations or unsustained impulsive calcium signals, we consider that CaTF would respond to the accumulation of them within a few hours since the half-life of the fusion protein with PEST sequence is less than 3 h.[38] CaTF-mediated reporter expression is similar to reporter systems, such as c-fos promoter or arc elements.[6−11] However, the activity of these promoters and elements could also be affected by calcium-independent transcription factors, calcium-independent signals, and cell type. Although the CaTF activity is affected by some of the aforementioned factors, CaTF-mediated reporter expression is less complex than reporter systems using endogenous calcium responsive promoters. Therefore, CaTFs potentially provide alternative methods to monitor intracellular calcium signaling instead of reporter systems that use promoters or elements.

The TetR-tetO system was used in the CaTF to transduce the intracellular calcium signals to reporter expression. With the use of other appropriate combinations of its DNA-binding motif, the output signals could be easily changed. A PEST sequence from ornithine decarboxylase was used in the design of CaTF. In general, degradation tags can influence the expression levels and life span of proteins, and replacement of degradation tags could also provide a means to improve signal efficiency. The cleavage site from α-spectrin was used to detect the calpain activity in the design of the CaTF. Since calpain has several substrates,[39] alteration of the cleavage site may improve the efficiency of CaTF-mediated reporter expression. Instead of using calpain sensitive sites in the CaTF, interfaces for other physiological proteases could be developed. These derivatives would be widely applicable for manipulating intracellular signal networks in response to physiological stimulation.

METHODS

Cell Culture and Transfection. BHK cells were maintained in a 100 mm dish in Dulbecco's modified Eagle's medium (DMEM) containing 5% (v/v) heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS) in a humidified CO_2 incubator. For transfection, BHK cells were seeded at the following densities: 1 × 10^4 cells/well in 96-well plates, 5 × 10^5 cells/well in 35 mm dishes, and 2 × 10^4 cells/well in 8-well coverglass chamber plates. Cells were maintained in DMEM supplemented with 5% (v/v) FBS. For immunofluorescence evaluation, BHK cells were seeded in 8-well coverglass chamber and maintained in phenol red-free DMEM supplemented with 5% (v/v) FBS. After 1 day of culture, transfection was carried out using Lipofectamine 2000 (Invitrogen). Expression and reporter plasmids were then added to cells in the following quantities: 2.4 ng to wells of 96-well plates, 16 ng to the 35 mm dish, and 3.6 ng to wells of 8-well coverglass chamber plates.

Luciferase Assay. The luciferase assay was performed according to the method of Nakajima et al. (2010).[20] The CaTF expression vector and the reporter vector were cotransfected into BHK cells seeded in 96-well plates in medium supplemented with 100 μM D-luciferin (TOYOBO). Bioluminescence was measured using a fluorescence microplate reader (SH9000Lab, Corona) 1 day following transfection. For real-time monitoring of bioluminescence, cells were plated in a 35 mm dish in medium supplemented with 200 μM D-luciferin

dx.doi.org/10.1021/sb500070c | *ACS Synth. Biol.* 2014, 3, 717−722

and overlaid with mineral oil (Sigma-Aldrich) to prevent evaporation. Bioluminescence was recorded using an AB2500 Kronos luminometer (ATTO) starting at 4 h post-transfection. Signals were collected at 10 min intervals for a period of 1 min under 5% CO_2 and 37 °C atmospheric conditions. The luciferase activity was expressed as relative light units (RLU).

Fluorescence Microscopy. Cells plated in a chambered coverglass were fixed with 4% paraformaldehyde at room temperature for 30 min, and then rinsed three times with phosphate-buffered saline (PBS). Fluorescence images of EGFP-expressing cells were obtained using a laser confocal microscope (LSM700, Carl Zeiss, New York).

Ratiometric Intracellular Calcium Measurement. Intracellular calcium levels were estimated using the Fura-2-AM calcium indicator, according to the manufacturer's instructions (Calcium kit-Fura 2, Dojindo). BHK cells seeded in 96-well plates were preincubated with 5 μg/mL of Fura-2-AM reagent in 1× reaction buffer in the presence or absence of an intracellular calcium chelator, BAPTA-AM, for 1 h. After washing once with the 1× reaction buffer, the BHK cells were incubated with 1× reaction buffer in the presence of 1.25 mM probenecid with or without BAPTA-AM. Fluorescence intensity was measured at 510 nm using a fluorescence microplate reader (SH9000Lab, Corona Electric, Ibaraki, Japan) with two different excitation wavelengths of 340 and 380 nm.

Design of a Calcium-Dependent Transcription Factor. The CaTF mainly consisted of five protein domains (Figure 1A and B): a nuclear export signal (NES) from 1Met to 19Gln, a calpain-sensitive sequence (cs) from 25Gln to 35Asp, a TetR from 42Met to 247Gly, three repeats of a minimal VP16 activation domain (vp) from 250Pro to 288Leu, and an ornithine decarboxylase-derived PEST sequence (PS) from 291His to 329Val. Linker or spacer sequences were introduced between each domain, and the codons for these domains were optimized for expression in mammalian cells. The NES at the N-terminal region was derived from MAPK kinase (MAPKK).[40] The cs sequence was derived from α-spectrin with a cleavage site between 29Tyr and 30Gly, and the sequence was then used for monitoring or imaging calpain activity under physiological and pathological conditions.[18,41] The sequence was designed such that following an increase of intracellular calcium, the NES was cleaved from the CaTF in response to calpain activation, and then, the cleaved CaTF or TetR-vp-PS was translocated into the nuclei. After binding to tetO sites, the cleaved CaTF robustly induced transcription of a reporter gene through the vp domain.[42] The PEST sequence was added at the C-terminal region of CaTF to prevent prolonged reporter expression from transient calcium activation. The CaTF gene was subcloned into the pcDNA3 expression vector.

Gene Sequence. ATGAACCTGGTGGACCTCCAAAAGAAGCTGGAGGAGCTGGAGCTGGACGAGCAGCAGGGATCTGGAAGCGGCCAGCAGGAGGTGTATGGCATGATGCCTAGAGATGGAAGCGGAAAGGATCCAATGAGCCGGCTGGACAAGAGCAAAGTGATCAACAGCGCCCTGGAACTGCTGAACGAAGTGGGCATCGAGGGCCTGACCACCCGGAAGCTGGCCCAGAAACTGGGCGTGGAACAGCCCACCCTGTACTGGCATGTGAAGAACAAGCGGGCCCTGCTGGACGCTCTGGCCATCGAGATGCTGGACCGGCACCACACCCACTTTTGCCCCCTGGAAGGCGAGAGCTGGCAGGACTTCCTGCGGAACAACGCCAAGAGCTTCAGATGCGCCCTGCTGAGCCACCGGGACGGCGCCAAAGTGCACCTGGGCACCAGACCCACCGAGAAGCAGTACGAGACACTGGAAAACCAGCTGGCCTTCCTGTGCCAGCAGGGCTTCAGCCTGGAAAACGCCCTGTACGCCCTGAGCGCCGTGGGACACTTCACCCTGGGCTGCGTGCTGGAAGATCAGGAACACCAGGTCGCCAAAGAGGAAAGAGAGACACCCACCACCGACAGCATGCCCCCCCCTGCTGAGACAGGCCATCGAGCTGTTCGATCATCAGGGCGCCGAGCCCGCCTTCCTGTTCGGCCTGGAACTGATCATCTGCGGCCTGGAAAAGCAGCTCAAGTGCGAGAGCGGCCGCGGACCAGCCGACGCACTCGATGACTTCGACCTCGACATGCTCCCCGCCGATGCCCTGGATGATTTTGATCTCGATATGCTGCCTGCAGACGCTCTCGACGATTTCGATCTGGATATGCTGTCTAGACACGGCTTCCCTCCCGAGGTGGAGGAGCAGGCCGCCGGCACCCTGCCCATGAGCTGCGCCCAGGAGAGCGGCATGGATAGACACCCTGCTGCTTGCGCCAGCGCCAGGATCAACGTCTAG

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: 81-87-891-2087. Fax: 81-87-891-2088. Email: ssuzukineurosci@gmail.com.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge Dr. Makoto Fujii and Dr. Youhei Egami for their insightful suggestions. This work was supported by Grants-in-Aid for Scientific Research (No. 24791111 and No. 26461635) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Astellas Foundation for Research on Metabolic Disorders. The DNA sequencer, fluorescence microplate reader and confocal microscopy used here were maintained and supported by the Division of Instrument and Equipment, Kagawa University.

■ ABBREVIATIONS

CaTF, calcium-dependent transcription factor; FRET, fluorescence resonance energy transfer; NES, nuclear export signal; Eluc, enhanced green-emitting luciferase; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; cs, a calpain-sensitive sequence; vp, a minimal VP16 activation domain; RhoA, Ras homologue gene family, member A; caRhoA, constitutive active RhoA

■ REFERENCES

(1) Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol. 1*, 11−21.

(2) Miyawaki, A., Griesbeck, O., Heim, R., and Tsien, R. Y. (1999) Dynamic and quantitative Ca^{2+} measurements using improved cameleons. *Proc. Natl. Acad. Sci. U.S.A. 96*, 2135−2140.

(3) Nagai, T., Sawano, A., Park, E. S., and Miyawaki, A. (2001) Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. *Proc. Natl. Acad. Sci. U.S.A. 98*, 3197−3202.

(4) Chen, T. W., Wardill, T. J., Sun, Y., Pulver, S. R., Renninger, S. L., Baohan, A., Schreiter, E. R., Kerr, R. A., Orger, M. B., Jayaraman, V., Looger, L. L., Svoboda, K., and Kim, D. S. (2013) Ultrasensitive

fluorescent proteins for imaging neuronal activity. *Nature 499*, 295−300.
(5) Zhao, Y., Araki, S., Wu, J., Teramoto, T., Chang, Y. F., Nakano, M., Abdelfattah, A. S., Fujiwara, M., Ishihara, T., Nagai, T., and Campbell, R. E. (2011) An expanded palette of genetically encoded Ca^{2+} indicators. *Science 333*, 1888−1891.
(6) Kawashima, T., Okuno, H., Nonaka, M., Adachi-Morishima, A., Kyo, N., Okamura, M., Takemoto-Kimura, S., Worley, P. F., and Bito, H. (2009) Synaptic activity-responsive element in the Arc/Arg3.1 promoter essential for synapse-to-nucleus signaling in activated neurons. *Proc. Natl. Acad. Sci. U.S.A. 106*, 316−321.
(7) Guenthner, C. J., Miyamichi, K., Yang, H. H., Heller, H. C., and Luo, L. (2013) Permanent genetic access to transiently active neurons via TRAP: Targeted recombination in active populations. *Neuron 78*, 773−784.
(8) Guzowski, J. F., McNaughton, B. L., Barnes, C. A., and Worley, P. F. (1999) Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. *Nat. Neurosci. 2*, 1120−1124.
(9) Barth, A. L., Gerkin, R. C., and Dean, K. L. (2004) Alteration of neuronal firing properties after *in vivo* experience in a FosGFP transgenic mouse. *J. Neurosci. 24*, 6466−6675.
(10) Smeyne, R. J., Schilling, K., Robertson, L., Luk, D., Oberdick, J., Curran, T., and Morgan, J. I. (1992) fos-lacZ transgenic mice: Mapping sites of gene induction in the central nervous system. *Neuron 8*, 13−23.
(11) Wang, K. H., Majewska, A., Schummers, J., Farley, B., Hu, C., Sur, M., and Tonegawa, S. (2006) In vivo two-photon imaging reveals a role of arc in enhancing orientation specificity in visual cortex. *Cell 126*, 389−402.
(12) Ono, Y., and Sorimachi, H. (2012) Calpains: An elaborate proteolytic system. *Biochim. Biophys. Acta 1824*, 224−236.
(13) Goll, D. E., Thompson, V. F., Li, H., Wei, W., and Cong, J. (2003) The calpain system. *Physiol Rev. 83*, 731−801.
(14) Amini, M., Ma, C. L., Farazifard, R., Zhu, G., Zhang, Y., Vanderluit, J., Zoltewicz, J. S., Hage, F., Savitt, J. M., Lagace, D. C., Slack, R. S., Beique, J. C., Baudry, M., Greer, P. A., Bergeron, R., and Park, D. S. (2013) Conditional disruption of calpain in the CNS alters dendrite morphology, impairs LTP, and promotes neuronal survival following injury. *J. Neurosci. 33*, 5773−5784.
(15) Staubli, U., Larson, J., Thibault, O., Baudry, M., and Lynch, G. (1988) Chronic administration of a thiol-proteinase inhibitor blocks long-term potentiation of synaptic responses. *Brain Res. 444*, 153−158.
(16) Vanderklish, P. W., Krushel, L. A., Holst, B. H., Gally, J. A., Crossin, K. L., and Edelman, G. M. (2000) Marking synaptic activity in dendritic spines with a calpain substrate exhibiting fluorescence resonance energy transfer. *Proc. Natl. Acad. Sci. U.S.A. 97*, 2253−2258.
(17) Stockholm, D., Bartoli, M., Sillon, G., Bourg, N., Davoust, J., and Richard, I. (2005) Imaging calpain protease activity by multiphoton FRET in living mice. *J. Mol. Biol. 346*, 215−222.
(18) Bartoli, M., Bourg, N., Stockholm, D., Raynaud, F., Delevacque, A., Han, Y., Borel, P., Seddik, K., Armande, N., and Richard, I. (2006) A mouse model for monitoring calpain activity under physiological and pathological conditions. *J. Biol. Chem. 281*, 39672−39680.
(19) Takatsuka, K., Ishii, T. M., and Ohmori, H. (2005) A novel Ca^{2+} indicator protein using FRET and calpain-sensitive linker. *Biochem. Biophys. Res. Commun. 336*, 316−323.
(20) Nakajima, Y., Yamazaki, T., Nishii, S., Noguchi, T., Hoshino, H., Niwa, K., Viviani, V. R., and Ohmiya, Y. (2010) Enhanced beetle luciferase for high-resolution bioluminescence imaging. *PLoS One 5*, e10011.
(21) Glading, A., Bodnar, R. J., Reynolds, I. J., Shiraha, H., Satish, L., Potter, D. A., Blair, H. C., and Wells, A. (2004) Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. *Mol. Cell. Biol. 24*, 2499−2512.
(22) Leloup, L., Shao, H., Bae, Y. H., Deasy, B., Stolz, D., Roy, P., and Wells, A. (2010) m-Calpain activation is regulated by its membrane localization and by its binding to phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem. 285*, 33549−33566.
(23) Glading, A., Lauffenburger, D. A., and Wells, A. (2002) Cutting to the chase: Calpain proteases in cell motility. *Trends Cell Biol. 12*, 46−54.
(24) Kulkarni, S., Saido, T. C., Suzuki, K., and Fox, J. E. (1999) Calpain mediates integrin-induced signaling at a point upstream of Rho family members. *J. Biol. Chem. 274*, 21265−21275.
(25) Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994) The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell. 79*, 507−13.
(26) Ren, X. D., and Schwartz, M. A. (1998) Regulation of inositol lipid kinases by Rho and Rac. *Curr. Opin. Genet. Dev. 8*, 63−7.
(27) Weernink, P. A., Meletiadis, K., Hommeltenberg, S., Hinz, M., Ishihara, H., Schmidt, M., and Jakobs, K. H. (2004) Activation of type I phosphatidylinositol 4-phosphate 5-kinase isoforms by the Rho GTPases, RhoA, Rac1, and Cdc42. *J. Biol. Chem. 279*, 7840−9.
(28) Nobes, C. D., and Hall, A. (1999) Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J. Cell Biol. 144*, 1235−44.
(29) Vogt, A., Tamewitz, A., Skoko, J., Sikorski, R. P., Giuliano, K. A., and Lazo, J. S. (2005) The benzo[c]phenanthridine alkaloid, sanguinarine, is a selective, cell-active inhibitor of mitogen-activated protein kinase phosphatase-1. *J. Biol. Chem. 280*, 19078−19086.
(30) Wells, A. (1999) EGF receptor. *Int. J. Biochem. Cell Biol. 31*, 637−643.
(31) Weber, W., and Fussenegger, M. (2011) Molecular diversity—The toolbox for synthetic gene switches and networks. *Curr. Opin Chem. Biol. 15*, 414−420.
(32) Maeder, M. L., Linder, S. J., Reyon, D., Angstman, J. F., Fu, Y., Sander, J. D., and Joung, J. K. (2013) Robust, synergistic regulation of human gene expression using TALE activators. *Nat. Methods 10*, 243−245.
(33) Perez-Pinera, P., Kocak, D. D., Vockley, C. M., Adler, A. F., Kabadi, A. M., Polstein, L. R., Thakore, P. I., Glass, K. A., Ousterout, D. G., Leong, K. W., Guilak, F., Crawford, G. E., Reddy, T. E., and Gersbach, C. A. (2013) RNA-guided gene activation by CRISPR-Cas9-based transcription factors. *Nat. Methods 10*, 973−976.
(34) Lohmueller, J. J., Armel, T. Z., and Silver, P. A. (2012) A tunable zinc finger-based framework for Boolean logic computation in mammalian cells. *Nucleic Acids Res. 40*, 5180−5187.
(35) Huang, Y., and Wang, K. K. (2001) The calpain family and human disease. *Trends Mol. Med. 7*, 355−362.
(36) Sorimachi, H., Hata, S., and Ono, Y. (2011) Calpain chronicle—An enzyme family under multidisciplinary characterization. *Proc. Jpn. Acad. Ser. B, Phys. Biol. Sci. 87*, 287−327.
(37) Zadran, S., Jourdi, H., Rostamiani, K., Qin, Q., Bi, X., and Baudry, M. (2010) Brain-derived neurotrophic factor and epidermal growth factor activate neuronal m-calpain via mitogen-activated protein kinase-dependent phosphorylation. *J. Neurosci. 30*, 1086−1095.
(38) Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., Huang, C. C., and Kain, S. R. (1998) Generation of destabilized green fluorescent protein as a transcription reporter. *J. Biol. Chem. 273*, 34970−34975.
(39) Wu, H. Y., and Lynch, D. R. (2006) Calpain and synaptic function. *Mol. Neurobiol. 33*, 215−236.
(40) Fukuda, M., Gotoh, I., Gotoh, Y., and Nishida, E. (1996) Cytoplasmic localization of mitogen-activated protein kinase kinase directed by its NH_2-terminal, leucine-rich short amino acid sequence, which acts as a nuclear export signal. *J. Biol. Chem. 271*, 20024−20028.
(41) Stockholm, D., Bartoli, M., Sillon, G., Bourg, N., Davoust, J., and Richard, I. (2005) Imaging calpain protease activity by multiphoton FRET in living mice. *J. Mol. Biol. 346*, 215−222.
(42) Ogura, E., Okuda, Y., Kondoh, H., and Kamachi, Y. (2009) Adaptation of GAL4 activators for GAL4 enhancer trapping in zebrafish. *Dev. Dyn. 238*, 641−655.