

Letter

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Development of an artificial calcium-dependent transcription factor to detect sustained intracellular calcium elevation

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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

INTRODUCTION

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 transmission¹. Several types of protein sensors for imaging intracellular calcium signals have been developed to better understand the roles of calcium signaling, including cameleon², pericam³, GCaMP6⁴, and GECO family proteins⁵, 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 exploited⁶⁻¹¹. 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 cells¹². In response to the increase of intracellular calcium, calpain activates intracellular signaling by cleaving substrate proteins¹³. 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 alpha-spectrin-derived sequence that is specifically cleaved by calpain¹⁶⁻¹⁹. 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 alpha-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 (**Figs. 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 (Eluc) with a PEST sequence, an ornithine decarboxylase-derived degradation sequence, after the Tet operator (tetO) binding sites²⁰. 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 μ M 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 (Fig. 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 manner²¹⁻²⁴, possibly involving mitogen-activated protein kinase (MAPK), Rho GTPase, and phosphatidylinositol 4,5-bisphosphate (PIP₂). The Rho GTPases²⁵⁻²⁷ activate PIP5K, which in turn phosphorylates PI4P to PI(4,5)P₂. Thus, the constitutively active RhoA (caRhoA) was co-introduced with CaTF and the reporter constructs to investigate whether Rho GTPase or PIP₂ induced the CaTF-mediated reporter expression²⁸. As shown in Supplemental Figure 1, caRhoA did not induce reporter expression, but rather suppressed it. The effect of MAPK phosphatase inhibitor, sanguinarine, was also

investigated²⁹. 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 (Fig. 2C). Correspondingly, this inhibitory effect of doxycycline was also observed when the EGFP reporter plasmid was used (Fig. 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 detectable over at least several days. To confirm this, real-time monitoring for Eluc activity was carried out over 4 days following transient transfection²⁰. Eluc activity was measured for 1 min at 10-min intervals with a luminescence detector embedded with a CO₂ 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 (Fig. 3A). This activation slightly decreased, but was sustained 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 Figures 2A and 2C, the Eluc activity for CaTF in the absence of Calpain inhibitor III was different in these figures. 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 (Fig. 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 signals³⁰. 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 (Fig. 4A). A similar result was observed using the EGFP

reporter plasmid (Fig. 4B). EGF treatment resulted in increased EGFP expression levels in each of the cells compared to the control culture. Additionally, EGF-mediated Eluc expression was blocked by the treatment with Calpain inhibitor III (Fig. 4A) or BAPTA-AM (Fig. 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; Fig. 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 Figure S2 (Supporting Information), 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 created³¹⁻³⁴, 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 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 diseases³⁵. 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 states³⁶. Though these calcium-independent factors make CaTF-mediated calcium sensing qualitative, the CaTF response to calpain activity in the same cells was stable (Fig. 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 phosphorylation³⁷. 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 reported²¹. 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 (Fig. 2). This leaky signal was mainly derived from a reporter plasmid that was co-transfected 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 co-transfected 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 μ M BAPTA-AM (Fig. 2). However, the ratiometric calcium measurement study using Fura-2-AM showed that treatment with 10 μ M BAPTA-AM did not result in complete chelation of intracellular calcium and that the 30 μ M BAPTA-AM treatment more strongly suppressed the intracellular calcium level (Fig. 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 CaTF¹⁶⁻¹⁹, 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*, respectively¹⁷. 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

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1 CaTF does not provide the ability to respond to short time calcium oscillations or
2 unsustained impulsive calcium signals, we consider that CaTF would respond to the
3 accumulation of them within a few hours since the half-life of the fusion protein with
4 PEST sequence is less than 3 h³⁸. CaTF-mediated reporter expression is similar to
5 reporter systems, such as c-fos promoter or arc elements⁶⁻¹¹. However, the activity of
6 these promoters and elements could also be affected by calcium-independent
7 transcription factors, calcium-independent signals, and cell type. Although the CaTF
8 activity is affected by some of the aforementioned factors, CaTF-mediated reporter
9 expression is less complex than reporter systems using endogenous calcium responsive
10 promoters. Therefore, CaTFs potentially provide alternative methods to monitor
11 intracellular calcium signaling instead of reporter systems that use promoters or
12 elements.

13 The TetR-tetO system was used in the CaTF to transduce the intracellular calcium
14 signals to reporter expression. With the use of other appropriate combinations of its
15 DNA-binding motif, the output signals could be easily changed. A PEST sequence from
16 ornithine decarboxylase was used in the design of CaTF. In general, degradation tags
17 can influence the expression levels and life span of proteins, and replacement of
18 degradation tags could also provide a means to improve signal efficiency. The cleavage
19 site from alpha-spectrin was used to detect the calpain activity in the design of the
20 CaTF. Since calpain has several substrates³⁹, alteration of the cleavage site may
21 improve the efficiency of CaTF-mediated reporter expression. Instead of using calpain
22 sensitive sites in the CaTF, interfaces for other physiological proteases could be
23 developed. These derivatives would be widely applicable for manipulating intracellular
24 signal networks in response to physiological stimulation.

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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 CO₂ 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 co-transfected into BHK cells seeded in 96-well plates in medium supplemented with 100 μ M 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 μ M D-luciferin, 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% CO₂ 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, NY).

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

1 seeded in 96-well plates were pre-incubated with 5 μ g/mL of Fura-2-AM reagent in 1 $\times$
2 Reaction Buffer in the presence or absence of an intracellular calcium chelator,
3 BAPTA-AM, for 1 h. After washing once with the 1X Reaction Buffer, the BHK cells
4 were incubated with 1X Reaction Buffer in the presence of 1.25 mM probenecid with or
5 without BAPTA-AM. Fluorescence intensity was measured at 510 nm using a
6 fluorescence microplate reader (SH9000Lab, Corona Electric, Ibaraki, Japan) with two
7 different excitation wavelengths of 340 nm and 380 nm.

9 **Design of a Calcium-dependent Transcription Factor**

10 The CaTF mainly consisted of five protein domains (**Figs.1A and B**): a nuclear export
11 signal (NES) from 1Met to 19Gln, a calpain-sensitive sequence (cs) from 25Gln to 35Asp,
12 a TetR from 42Met to 247Gly, three repeats of a minimal VP16 activation domain (vp)
13 from 250Pro to 288Leu, and an ornithine decarboxylase-derived PEST sequence (PS)
14 from 291His to 329Val. Linker or spacer sequences were introduced between each
15 domain, and the codons for these domains were optimized for expression in mammalian
16 cells. The NES at the N-terminal region was derived from MAPK kinase (MAPKK)⁴⁰.
17 The cs sequence was derived from alpha-spectrin with a cleavage site between 29Tyr
18 and 30Gly, and the sequence was then used for monitoring or imaging calpain activity
19 under physiological and pathological conditions^{18,41}. The sequence was designed such
20 that following an increase of intracellular calcium, the NES was cleaved from the CaTF
21 in response to calpain activation, and then the cleaved CaTF or TetR-vp-PS was
22 translocated into the nuclei. After binding to tetO sites, the cleaved CaTF robustly
23 induced transcription of a reporter gene through the vp domain⁴². The PEST sequence
24 was added at the C-terminal region of CaTF to prevent prolonged reporter expression
25 from transient calcium activation. The CaTF gene was subcloned into the pcDNA3
26 expression vector.

28 **Gene sequence**

29 ATGAACCTGGTGGACCTCCAAAAGAAGCTGGAGGAGCTGGAGCTGGACGAGCAGC
30 AGGGATCTGGAAGCGGCCAGCAGGAGGTGTATGGCATGATGCCTAGAGATGGAAG
31 CGGAAAGGATCCAATGAGCCGGCTGGACAAGAGCAAAGTGATCAACAGCGCCCTG
32 GAACTGCTGAACGAAGTGGGCATCGAGGGCCTGACCACCCGGAAGCTGGCCCAGA
33 AACTGGGCGTGGAACAGCCCACCCTGTACTGGCATGTGAAGAACAAGCGGGCCCT
34 GCTGGACGCTCTGGCCATCGAGATGCTGGACCGGCACCAACCCACTTTTGCCCC
35 CTGGAAGGCGAGAGCTGGCAGGACTTCCTGCGGAACAACGCCAAGAGCTTCAGAT
36 GCGCCCTGCTGAGCCACCGGGACGGCGCCAAAGTGCACCTGGGCACCAGACCCAC

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1 CGAGAAGCAGTACGAGACACTGGAAAACCAGCTGGCCTTCCTGTGCCAGCAGGGC
2 TTCAGCCTGGAAAACGCCCTGTACGCCCTGAGCGCCGTGGGACACTTCACCCTGG
3 GCTGCGTGCTGGAAGATCAGGAACACCAGGTGCGCAAAGAGGAAAGAGAGACACC
4 CACCACCGACAGCATGCCCCCCTGCTGAGACAGGCCATCGAGCTGTTTCGATCATC
5 AGGGCGCCGAGCCCGCCTTCCTGTTCGGCCTGGAACTGATCATCTGCGGCCTGGA
6 AAAGCAGCTCAAGTGCAGAGAGCGGCCGCGGACCAGCCGACGCACTCGATGACTTC
7 GACCTCGACATGCTCCCCGCCGATGCCCTGGATGATTTTGATCTCGATATGCTGCC
8 TGCAGACGCTCTCGACGATTTTCGATCTGGATATGCTGTCTAGACACGGCTTCCCTC
9 CCGAGGTGGAGGAGCAGGCCGCCGGCACCCTGCCCATGAGCTGCGCCCAGGAGA
10 GCGGCATGGATAGACACCCTGCTGCTTGCGCCAGCGCCAGGATCAACGTCTAG

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1 **ASSOCIATED CONTENT**
2 **Supporting Information**
3 Supplemental materials are available free of charge via the internet at
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Notes

The authors declare no competing financial interest.

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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 homolog gene family, member A. caRhoA, constitutive active RhoA.

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Figure legends

Fig. 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*.

Fig. 2 CaTF sensed the intracellular calcium level of BHK cells at steady-state

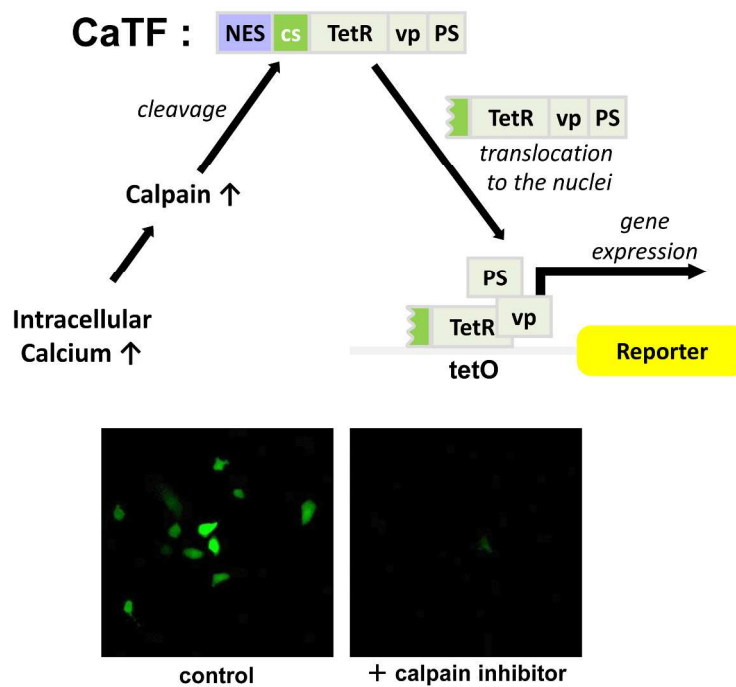
The CaTF gene and reporter construct (tetO-Eluc or tetO-EGFP) were introduced into BHK cells. After 24 h incubation, luciferase activity (*A&C*) or fluorescence of EGFP (*B&D*) was analyzed. An empty expression vector (vec), without the CaTF gene, was introduced as a control. The number associated with each column represents the number of wells in the same experiment. (*A&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&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 $\pm$ SEM. One-way ANOVA and Tukey's post-hoc test were used for the statistical analyses of differences. The asterisk indicates a significant difference from all other groups ($p < 0.001$).

Fig. 3 Stability of CaTF-derived signals in BHK cells.

The 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 $[\text{RLU}_{(\text{CaTF-inh.III})}]/[\text{RLU}_{(\text{CaTF+inh.III})}]$ was virtually constant after a 24 h incubation.

Fig. 4 Detection of physiological calcium increase by CaTF

The 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 μ M Calpain Inhibitor III (Inh.III), and 10 μ M BAPTA-AM (BAP). Instead of CaTF, an empty expression vector (vec) was introduced as the control. After incubation for 24 h, luciferase activity (A&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 post-hoc 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".



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Fig 1

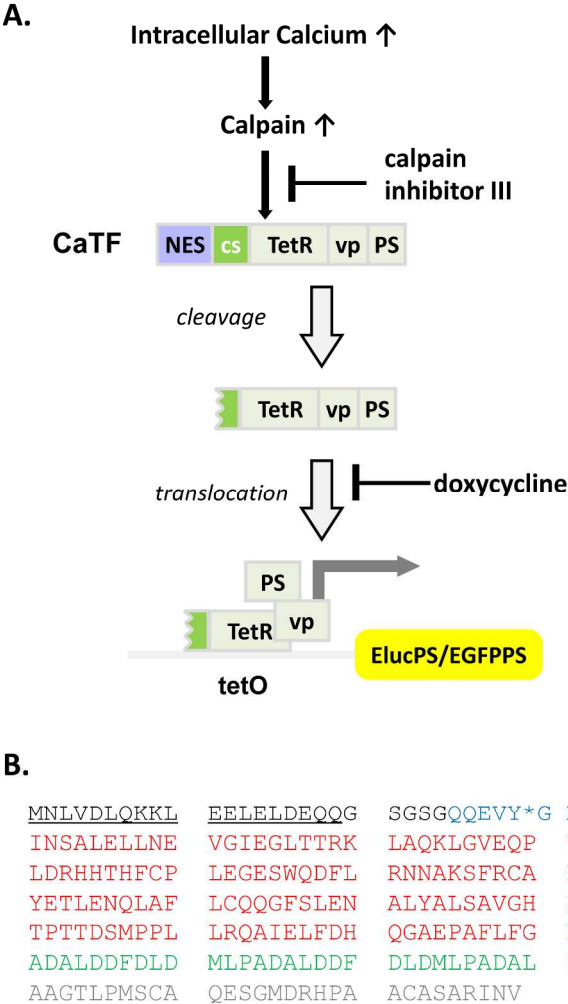


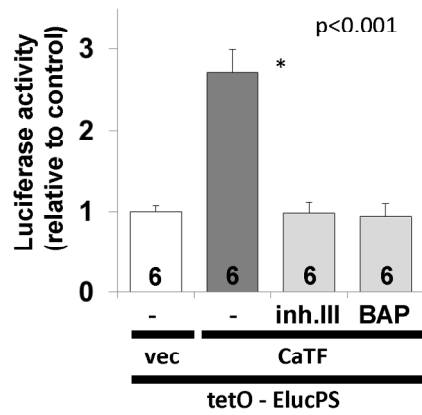
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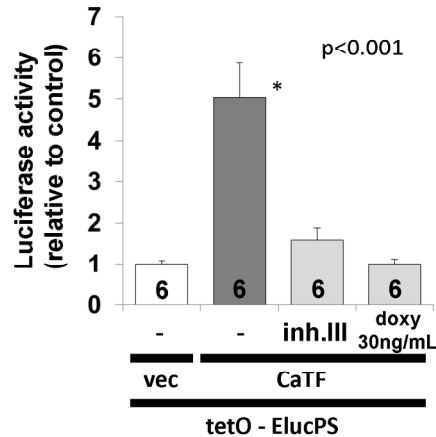
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Fig 2

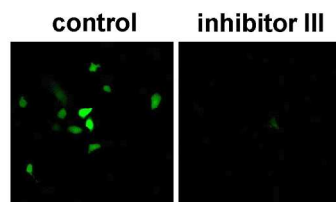
A.



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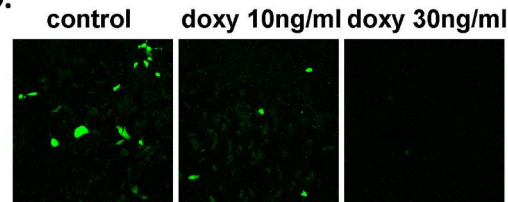


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Fig 3

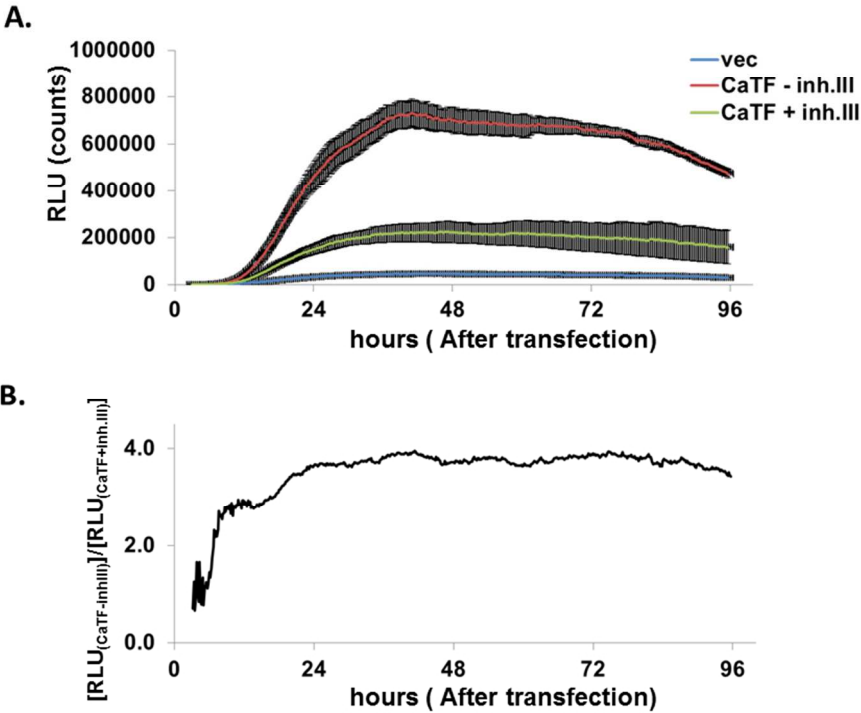


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254x190mm (96 x 96 DPI)

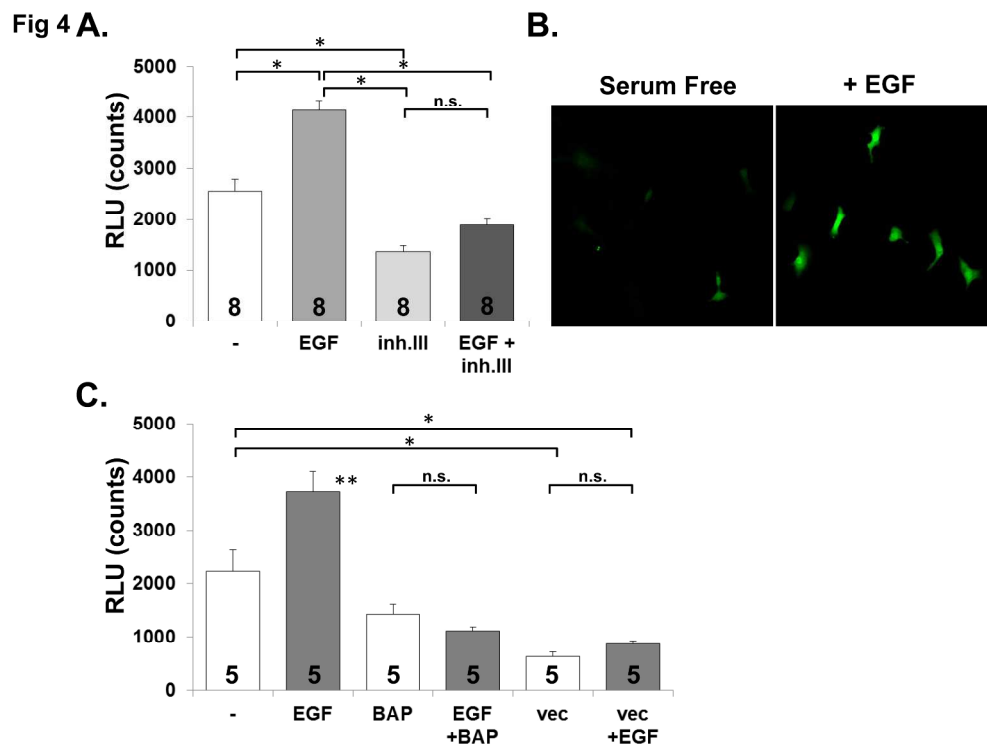


Fig. 4 Detection of physiological calcium increase by CaTF

The 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 μ M Calpain Inhibitor III (Inh.III), and 10 μ M BAPTA-AM (BAP). Instead of CaTF, an empty expression vector (vec) was introduced as the control. After incubation for 24 h, luciferase activity (A&C) or fluorescence of EGFP (B) was analyzed. The number associated with each column represents the number of wells in the same experiment.

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254x190mm (300 x 300 DPI)