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Enhanced Bioluminescent Sensor for Longitudinal Detecting of CREB Activation in Living Cells

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Abstract

Cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) is associated with memory formation and controls cell survival, and proliferation via regulation of downstream gene expression in tumorigenesis. As a transcription factor, CREB binds to cAMP response elements. Phosphorylation of CREB triggers transcriptional activation of CREB downstream genes following the interaction of the kinase-inducible domain (KID) of CREB with the KID interaction domain (KIX) of CREB-binding protein. Nevertheless, because of the lack of single-cell analytical techniques, little is known about spatiotemporal regulation of CREB phosphorylation. To analyze CREB activation in single living cells, we developed genetically encoded bioluminescent sensors using luciferase-fragment complementation: the sensors are designed based on KID–KIX interaction with a single-molecule format. The luminescence intensity of the sensor, designated as CREX (a sensor of CREB activation based on KID(CREB)–KIX interaction), increased by phosphorylation of CREB. Moreover, the luminescence intensity of CREX was sufficient to detect CREB activation in live-cell bioluminescence imaging for single-cell analysis because of the higher sensitivity. CREX sensor is expected to contribute to elucidation of the spatiotemporal regulation of CREB phosphorylation by applying single-cell analysis.

Introduction

Cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) has been characterized as a transcription factor that binds to cAMP response elements.[1] CREB is important for memory formation and synaptic plasticity.[2] Furthermore, CREB controls cell survival and cell proliferation via the regulation of the downstream gene expression in tumorigenesis, including Hodgkin lymphoma,[3] non-small lung carcinoma,[4] and colorectal cancer.[5] CREB is phosphorylated at the 133rd serine residue in the kinase-inducible domain (KID). The phosphorylation results from kinases including protein kinase A (PKA),[6] Ca^{2+}/calmodulin-dependent kinase IV,[7] and p90 ribosomal protein S6 kinase,[8, 9] resulting in recruitment of the transcriptional coactivators, CREB-binding protein (CBP) or its paralogue, p300. The phosphorylated KID interacts with the KID interaction domain (KIX) of CBP,[10, 11] which triggers the transcriptional activation of CREB downstream genes. Monitoring CREB activation is important for elucidating the pathophysiological mechanisms *in vitro* and *in vivo*.

To visualize CREB activation, fluorescent probes on CREB activation have been developed using fluorescence resonance energy transfer (FRET).[12, 13] Those probes were capable of detecting KID–KIX interaction based on phosphorylation of the 133rd serine residue in CREB. However, application of the FRET probes in deep tissues has not been

realized yet because of the necessity of excitation light. Instead, bioluminescence imaging is beneficial for quantitative analysis with the wider dynamic range of signals and high signal-to-background ratio.[14] Therefore, bioluminescence imaging is adapted to long-term monitoring using living cells and *in vivo*. We earlier developed bioluminescent probes to monitor CREB activation by luciferase-fragment complementation assays in living cells.[15] The probes comprise two fragments of firefly luciferase (FLuc), each of which is connected with KID and KIX. When the KID–KIX interaction occurs in the probes, FLuc fragments of the probes are complemented intermolecularly. Actually, the probes have been applied to drug screening for the identification of an adenylyl cyclase inhibitor and a PKA inhibitor,[16, 17] but their luminescence intensity was insufficient for their application to *in vitro* long-term monitoring of CREB activation or *in vivo* experiments for spatiotemporally elucidating pathophysiological mechanisms in deep tissues.

Therefore, we improved a pair of bioluminescent probes (C6 and D5 probes, Fig. S1 of the ESI) based on KID–KIX interaction[15] in terms of transfection efficiency for application to live-cell imaging for single-cell analysis. All DNA fragments including FLuc fragments, KID, and KIX were integrated into a vector as a single-molecule-format bioluminescent sensor. Results show that the absolute

bioluminescence intensity was markedly improved. The sensor allowed for detection of the CREB activation at a single-cell level. The sensor might further elucidate spatiotemporal regulation of CREB phosphorylation by performing single-cell analysis.

Experimental Section

Vector construction

DNA fragments encoding mouse KID of CREB and mouse KIX of CBP including a nuclear localization signal (NLS: DPKKKRKVDPKKKRKV) were amplified by PCR using commercially synthesized DNA fragment (Integrated DNA Technologies, Inc., Coralville, IA) as a template. The amino acid sequences of KID and KIX were selected from 101 to 160 amino acids (NCBI reference sequence: NP_034082.1) and from 586 to 666 amino acids (NCBI reference sequence: NP_001020603.1), respectively, as reported earlier.[15] DNA fragments encoding FLuc were amplified by PCR using the synthesized DNA fragment and D5 probe as templates.[15] The N-terminal and C-terminus of FLuc were selected, respectively, from 1-415 (FLucN) and 416-550 amino acids (FLucC). All these fragments were inserted into pcDNA4/V5-His (B) vector (Invitrogen-Thermo Fisher Scientific, Carlsbad, CA) using restriction enzyme sites. The region between FLucC and FLucN (or KIX and

KID) was replaced by a flexible linker, $(GGGGS)_4$ or alpha-helical linkers including di-glycine as a hinge, EAAAREAGGEAAAREA or $(EAAAR)_2EAAGG(EAAAR)_2EAA$,[18, 19] using In-Fusion HD cloning kit (Clontech-Takara, Mountain View, CA). The DNA fragments of the linkers were generated by oligonucleotide annealing. The constructed probes are designated as sensor_A to sensor_G. To ascertain whether inhibition of phosphorylation in CREB decreases bioluminescence intensity of sensor_G (hereinafter designated as CREX: a sensor of CREB activation based on KID(CREB)–KIX interaction), the 133rd serine residue in CREB was replaced by alanine using a kit (KOD Plus Mutagenesis Kit; Toyobo Co. Ltd., Osaka, Japan), which is designated as CREX (S133A).

Cell cultures, transfection, and stable cell line establishment

Human embryonic kidney (HEK) 293T cells (abcam, Inc., Cambridge, UK) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) under 5% CO_2 at 37°C. Each constructed probe was transfected into HEK293T cells using TransIT-LT1 reagent (Mirus-Takara, Madison, WI) in according to the manufacturer's instructions. To establish a stable cell line expressing CREX, the cells were selected using 800 μg/ml Zeocin (Invitrogen-Thermo

Fisher Scientific, Carlsbad, CA). The stable cell line was established from a single clone.

Luciferase assay

HEK293T cells were spread on 96-well solid white microplates and were cultured in DMEM supplemented with 10% FBS for 1 day. The cells were transfected transiently with each of the constructed sensors for 48 h. Then the cells were treated with phenol red-free DMEM containing 25 mM HEPES and 10 or 50 μM forskolin (an adenylyl cyclase activator) or vehicle (dimethyl sulfoxide) for 1 h. The vehicle means the solvent of forskolin. The medium was added with an equal amount of phenol red-free DMEM containing 0.4 mM (Fig. 1c) or 1 mM D-Luciferin (Figs. 2a, 2c). The luminescence intensity was then measured using a microplate reader (TriStar LB 941; Berthold Technologies GmbH and Co. KG, Bad Wildbad, Germany) at room temperature.

To confirm whether the KID–KIX interaction depends on phosphorylation of the 133rd serine residue in the CREB of CREX, cells transfected with CREX were treated with 20 μM H-89, a PKA inhibitor, for 30 min before forskolin treatment.

The stable cell line of CREX was spread on 96-well solid white microplates and was cultured in DMEM supplemented with 10% FBS for 2 days. Then the cells were treated with phenol red-free DMEM containing 25 mM HEPES and 10, 20, or 50 μM forskolin or vehicle for 1 h. The medium was added with an equal amount of phenol red-free DMEM containing 0.4 mM D-Luciferin. The luminescence intensity was then measured using a microplate reader at room temperature. The data show relative luminescence intensity when the vehicle control is set to 1.

Western blotting

HEK293T cells were lysed with RIPA buffer containing protease inhibitor cocktail, Complete EDTA-free (Roche, Mannheim, Germany), and were centrifuged at 15,000 rpm at 4°C for 10 min. The cell supernatants were boiled for 5 min, were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk or 2% bovine serum albumin for 1 h. Western blot analysis was performed using Anti-V5 antibody (Novex-Thermo Fisher Scientific, Carlsbad, CA), Anti-phospho-CREB (Ser133) antibody (Upstate-Merck Millipore, Burlington, MA), and Anti-β-Actin antibody (Sigma-Aldrich Corp., St. Louis, MO). beta-actin was used

as an internal standard. The amounts of proteins were measured by a lumino image analyzer (LAS 4000 mini; GE Healthcare, Ltd., UK).

Live-cell imaging for single-cell analysis

The stable cell line expressing CREX was plated on a poly-L-lysine-coated glass-base dish (AGC TECHNO GLASS CO., LTD, Shizuoka, Japan) and was cultured for 2 days. Cell nuclei were stained using NucRed Live 647 ReadyProbes Reagent (Molecular Probes – Thermo Fisher Scientific, Eugene, OR) according to the manufacturer's instructions. The medium of the cells was replaced by phenol red-free DMEM containing 25 mM HEPES, 0.5 mM D-Luciferin and 1% FBS. The luminescence intensity of the complemented FLuc was imaged directly at room temperature with an inverted fluorescence and luminescence microscope (IX81; Olympus Corp., Tokyo, Japan), which was used with a 20× oil-immersion objective (0.85 NA). The luminescence images were acquired with an electron-multiplying charge-coupled device camera (ImagEM; Hamamatsu Photonics K.K., Shizuoka, Japan) at 3-min exposures with 1-min intervals. Fluorescence images and bright field images were taken, respectively, for 600 ms and 150 ms at the interval. After images were acquired for 1 h, the cells were treated dropwise with 50 μM forskolin (final

concentration) or vehicle. Then the images were acquired for 3 h. The obtained data were analyzed using imaging software (Meta Morph; Molecular Devices Corp., LLC, San Jose, CA). The luminescence intensity of time-lapse imaging indicates the average intensity in arbitrary areas including the cell nuclei of individual cells. The average intensity in an area without cells was subtracted as a background signal.

Statistical analysis

Data of multiple groups and two series of data were analyzed, respectively, using one-way and two-way analysis of variance with a post hoc multiple comparison test (Bonferroni/Dunn procedure). Two series of data for live-cell imaging for single-cell analysis were analyzed using repeated measures two-way ANOVA with Games–Howell's multiple comparison test.

Results

Development of single-molecule-format bioluminescent sensors to monitor CREB activation based on luciferase-fragment complementation

Because live-cell imaging for single cell analysis requires higher luminescence intensities than those of whole cells in a dish or in individual wells of a microtiter plate,

we improved the KID-KIX probes to enhance the luminescence intensity: we designed single-molecule-format bioluminescent sensors (sensor_A to sensor_G), in which KID and KIX interact intramolecularly (Figs. 1a, 1b, Fig. S2 of the ESI). Each N-terminal end of sensor_A to sensor_G contains an NLS. When the 133rd serine residue of CREB in the KID is phosphorylated by kinases, KID–KIX interaction occurs, resulting in bioluminescence based on complementation of the FLucN (1–415 amino acids) and FLucC (416–550 amino acids) fragments (Fig. 1a). The complemented luciferase recovers its enzymatic activity, thereby producing bioluminescence in the presence of D-Luciferin. To compare the differences in luminescence intensity related to linker flexibility, we designed GS linkers and alpha-helical linkers including di-glycine as a hinge (Fig. 1b).

Luminescence intensity of the constructed sensors based on KID–KIX interaction under forskolin treatment in HEK293T cells

To confirm whether the constructed KID–KIX sensors detect CREB activation in living cells, HEK293T cells were transfected transiently with each sensor (sensor_A to sensor_G or the KID-KIX probes) for 48 h. Then the cells were treated with 10 or 50 μM forskolin or vehicle for 1 h. The luminescence intensity of the cells treated with 50

μM forskolin showed an approximately 1.8- to 2.3-fold increase over the respective vehicle control for all the sensors. Of all the sensors, sensor_G showed the highest intensity of luminescence. To apply *in vitro* live-cell imaging for single-cell analysis of CREB activation or *in vivo* experiments including deep tissues, we focused on the brighter sensor_G. When the vehicle control of the KID-KIX probes was set to 1, the luminescence intensity of sensor_G was each 2.8×10^2-fold (vehicle), 4.4×10^2-fold (10 μM forskolin), or 6.6×10^2-fold (50 μM forskolin) increase (Fig. 1c). The luminescence intensity of the cells treated with 10 or 50 μM forskolin showed an approximately 1.6- or 2.3-fold increase over the vehicle control, respectively. Therefore, we selected sensor_G (hereinafter designated as CREX) as a sensor to detect CREB activation. These results indicate that CREX allowed for sensitive detection of the phosphorylation of CREB in living cells.

KID–KIX interaction depending on phosphorylation at the amino acid position 133 in the CREB

To clarify whether the KID–KIX interaction of CREX depend on the phosphorylation of the 133rd serine residue in CREB in the KID, HEK293T cells were transfected transiently with CREX for 48 h and then were treated with 20 μM H-89, a

PKA inhibitor, for 30 min. The cells were also treated with 10 or 50 μM forskolin or vehicle for 1 h. Then the luminescence intensity of the cells was measured using a microplate reader (Fig. 2a). The luminescence intensity of cells treated with 20 μM H-89 and 50 μM forskolin was 0.6-fold lower than that of the cells without H-89 in the presence of 50 μM forskolin. This result suggests that the KID–KIX interaction of CREX depended on phosphorylation of the CREB.

To elucidate whether the bioluminescence intensity of CREX reflects the phosphorylation of the 133rd serine residue in CREB within the KID, the serine residue of CREX was replaced by alanine (named CREX (S133A)). HEK293T cells were transfected transiently with CREX or CREX (S133A). The amount of the phosphorylated CREB protein was analyzed 1 h after treatment of 10 or 50 μM forskolin or vehicle (Fig. 2b). The molecular weight of the open reading frame of CREX and CREX (S133A) sensors indicates approximately 88 kDa. The phosphorylation of CREX was confirmed by detection of the protein band using an anti-pCREB antibody. CREX allowed for the phosphorylation at the 133rd serine residue in CREB, whereas the phosphorylated band of CREX (S133A) was not detected. On the other hand, endogenous CREB in the cells expressing these probes was phosphorylated by forskolin treatment (Fig. 2b). To elucidate whether inhibition of the

phosphorylation within the KID decreased the luminescence intensity, we measured the luminescence intensity under the same conditions as those used for Western blot (Fig. 2c). No significant difference was found among the luminescence intensities of the cells expressing CREX (S133A), irrespective of whether the cells were treated with forskolin. The luminescence intensity of the cells under vehicle or forskolin treatment conditions expressing CREX (S133A) was significantly lower than that of the cells expressing CREX treated with vehicle. The luminescence intensity ratios (CREX/CREX (S133A)) were 1.5-fold (vehicle), 2.0-fold (10 μM forskolin), or 2.4-fold (50 μM forskolin), respectively. These results indicate that the value obtained by subtracting the luminescence intensity of CREX (S133A) from that of CREX reflects the phosphorylation state of CREB.

Next, to exclude the possibility that the decrease in luminescence intensity of CREX (S133A) originated from the decrease in the amount of protein, we analyzed the protein levels of cells expressing CREX or CREX (S133A). We found no significant difference in the protein levels between the cells expressing CREX and the cells expressing CREX (S133A) (Fig. 2d). Furthermore, the amounts of these proteins were unaffected by forskolin treatment. These results indicate that the CREX luminescence is engendered by the phosphorylation of the 133rd serine residue in the CREB within the

KID.

Time-lapse bioluminescence imaging of a CREX-expressing stable cell line under forskolin treatment in HEK293T cells

To monitor CREB activation using time-lapse imaging, we established a stable cell line expressing CREX in HEK293T cells. After the cells were treated with 10, 20, or 50 μM forskolin or vehicle for 1 h, the luminescence intensity of the cells was measured using a microplate reader (Fig. 3a). The luminescence intensity of the cells treated with 10, 20, or 50 μM forskolin increased significantly respective 1.7-, 2.0-, or 2.5-fold compared to that of the vehicle control. The luminescence intensity increased in proportion to forskolin concentrations: in comparison of 10 μM and 20 μM forskolin, the luminescence intensity increased approximately 1.2-fold. Also, the difference between 20 μM and 50 μM forskolin was an approximately 1.3-fold increase. Therefore, the luminescence intensity of CREX would be proportional to the CREB phosphorylation. Next, to monitor the alteration in KID–KIX interaction caused by forskolin treatment at the single-cell level, we performed time-lapse imaging of CREB activation using the stable cell line in the presence and absence of forskolin (Figs. 3b–3d, Supplementary Movie 1 of the ESI). After time-lapse images were acquired for 1 h,

the cells were treated with 50 μM forskolin or vehicle. Then the images were further acquired for 3 h. As portrayed in the integrated image of 4–7 min after the forskolin treatment (the image is shown as 'time 70' in Figs. 3b–3d), the luminescence intensities of these cells increased significantly compared to those of the vehicle control. The increase in luminescence intensity was 2.1-fold at time 70, then the luminescence peak was a 2.9-fold increase at time 78 (within 15 min after the forskolin treatment), as compared to each vehicle control (Fig. 3d). Up to 3 h (time 246) after forskolin treatment, the increase in the luminescence intensity was maintained for single-cell analysis (Figs. 3c, 3d). To ascertain whether CREX was localized in the cell nuclei by the NLS in the probe, the cell nuclei were stained with a fluorescent reagent (NucRed Live 647 ReadyProbes Reagent) and were merged with the luminescence image (Fig. 3b, FL–BL). The fluorescent reagent is excited at 638 nm when it binds to DNA. The luminescence signal colocalized with the cell nuclei that had been stained with the fluorescent reagent. These results indicate that CREX can be used for analysis of CREB activation at the single-cell level *in vitro*.

Discussion

HEK293T cells expressing CREX showed an increase in luminescence with increasing forskolin. In addition, the luminescence intensity of CREX was 2.8×10^2-fold

higher than that of the previously reported KID–KIX probes. Furthermore, no significant difference was found in the signal-to-background ratios between the two probes. Therefore, CREX might be more appropriate for long-term monitoring of CREB activation with single-cell analysis. In our earlier manuscript, the phosphorylation of endogenous CREB in HEK293T cells peaked at 5 min after forskolin treatment. Besides, the increase was continued for 6 h after the forskolin treatment.[15] Also, the phosphorylation of CREB in CREX significantly increased in the integrated image of 4–7 min after the forskolin treatment, and the phosphorylation was kept for approximately 3 h after the treatment. These results indicate that the luminescence intensity of CREX reflects the phosphorylation of endogenous CREB.

To clarify whether the KID–KIX interaction of CREX depends on the phosphorylation of the 133rd serine residue in the CREB within KID, we inhibited the phosphorylation of CREB using a PKA inhibitor H-89 because earlier reports described that H-89 does not inhibit the luminescence reaction of FLuc.[20] We demonstrated that the luminescence intensity of CREX was reduced by H-89 in the presence of forskolin. This result indicates that the CREB of CREX is phosphorylated by PKA following upregulation of cAMP production. Using CREX (S133A), we also demonstrated that the phosphorylation of the 133rd serine residue in CREB occurs specifically in CREX,

except for CREX (S133A). The luminescence intensity of the cells expressing CREX (S133A) showed a slight background luminescence, which might have originated from spontaneous complementation of luciferase fragments independent of KID–KIX interaction. Although such background luminescence occurred, the luminescence signals from KID–KIX interaction were sufficiently strong for single-cell analysis, indicative of the usefulness of the sensor sensitivity for CREB phosphorylation. However, CREB activation does not necessarily depend on the phosphorylation of the 133rd serine residue in CREB. Under the exposure to cAMP and calcium, CREB-regulated transcription co-activators can enhance CRE-dependent transcription independently of the phosphorylation of the 133rd serine residue.[21, 22] Thus, the CREB activation in CREX reflects only the phosphorylation-dependent CREB activation.

CREB is associated with tumorigenesis through enhanced cell proliferation, reduced sensitivity to undergo apoptosis, increased angiogenesis, and radiation-induced differentiation.[4] CREX might be applicable to high-throughput screening to explore anticancer reagents and evaluation of the drug treatment. Moreover, CREB participates in neuronal maturation and survival in hippocampal neurogenesis.[23] In the adult brain, neurogenesis occurs in the subgranular zone of the dentate gyrus of the hippocampus.[23] For *in vivo* imaging of CREB phosphorylation in mouse brain, a transgenic mouse line

harboring the KID–KIX (C6 and D5) probes was established.[24] The images were acquired from the cerebral cortex of an awake-mouse. However, it is not easy to monitor deep brain tissues in mouse, especially using luciferase-fragment complementation, because of the following reasons: in general, the luminescence intensity of split-luciferase is lower than that of the full-length luciferase. Besides, to deliver FLuc substrate of D-Luciferin to deep brain tissues, the substrate must pass through the blood–brain barrier (BBB). Although small lipophilic compounds traverse the BBB, D-Luciferin with relative polarity is predicted to pass through the BBB to a modest degree.[25, 26] The lower D-Luciferin concentration in brain tissues hampers sensitive detection of the complemented luciferase. In such a severe experimental condition, higher sensitivity of CREX will enable the detection of the complemented luciferase because of the markedly improved sensitivity.

Conclusion

We established CREX to detect CREB activation using luciferase fragment complementation assays and demonstrated that CREX was useful for time-lapse imaging of CREB activation at a single-cell level. The CREX might allow for the development of therapeutic agents and might elucidate pathophysiological mechanisms

including memory consolidation and tumorigenesis.

Conflicts of interests

There are no conflicts to declare.

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Figure legends

Figure 1. Bioluminescence engendered by KID–KIX interaction within one vector in the presence of forskolin in HEK293T cells

(a) When CREB is phosphorylated at the 133rd serine residue within the kinase-inducible domain (KID) by kinases, the KID interacts with the KID interaction domain (KIX) of CBP. The interaction causes complementation of FLucN and FLucC in a vector, resulting in the restoration of luminescence in the presence of D-Luciferin. 'P' on the KID in the figure represents phosphorylation of the 133rd serine residue in the CREB. NLS denotes a nuclear localization signal. V5/His respectively show an epitope tag (V5) and a tag composed of six histidine residues (His). (b) Schematic diagrams of constructed probes to detect KID–KIX interaction based on luciferase-fragment complementation. (c) HEK293T cells were transfected transiently with each sensor (sensor_A to sensor_G or the KID-KIX probes) for 48 h. The cells were treated with phenol red-free DMEM containing 10 or 50 μM forskolin or vehicle for 1 h; then the luminescence intensity of the cells was measured in the presence of 0.2 mM D-Luciferin. Data were expressed as mean ± SE (*n*=4). The data shows relative luminescence intensity when the vehicle control of the KID-KIX probes is set to 1.

Figure 2. KID–KIX interaction depending on phosphorylation at the amino acid

position 133 in the CREB

(a) HEK293T cells were transfected transiently with CREX for 48 h; then the cells were treated with phenol red-free DMEM containing 25 mM HEPES and 20 μM H-89 or vehicle for 30 min. The medium of the cells was replaced by phenol red-free DMEM containing 10 or 50 μM forskolin or vehicle for 1 h. The luminescence intensity of the cells was measured using a microplate reader in the presence of 0.5 mM D-Luciferin. Data were expressed as mean ± SD (n=4). **$p < 0.01$. '-' denotes untransfected cells. (b-d) HEK293T cells were transfected transiently with CREX or CREX (S133A) for 48 h; then the cells were treated with phenol red-free DMEM containing 10 or 50 μM forskolin or vehicle for 1 h. '-' denotes untransfected cells. (b) The amounts of proteins were analyzed using Western blotting. pCREB indicates CREB phosphorylated at the 133rd serine residue. (c) The luminescence intensity of the cells was measured using a microplate reader in the presence of 0.5 mM D-Luciferin. Data are expressed as mean ± SD (n=5). **$p < 0.01$ versus respective CREX. +$p < 0.05$ versus vehicle of CREX. ++$p < 0.01$ versus vehicle of CREX. (d) The amounts of proteins were analyzed using Western blotting. V5 epitope tag was used to detect CREX and CREX (S133A) proteins.

Figure 3. Time-lapse bioluminescence imaging of a CREX-expressing stable cell line under forskolin treatment in HEK293T cells

(a) A CREX-expressing stable cell line was treated with phenol red-free DMEM containing 10, 20, or 50 μM forskolin or vehicle for 1 h; then the luminescence intensity of the cells was measured in the presence of D-Luciferin. Data were expressed as mean ± SE (n=3). **$p < 0.01$ versus vehicle. +$p < 0.05$. ++$p < 0.01$. (b–d) The cell nuclei of the CREX-expressing stable cell line were stained with a fluorescent reagent, of which the medium was replaced by DMEM containing 1% FBS and D-Luciferin. After bioluminescence images were acquired at the single-cell level for 1 h, the cells were treated with 50 μM forskolin or vehicle. Then images were acquired for 3 h. (b) Red and green images respectively show cell nuclei stained with a fluorescent reagent and bioluminescence based on luciferase-fragment complementation depending on KID–KIX interaction. Arrowheads indicate cells measured the luminescence intensity: BF, bright field; BL, bioluminescence; FL, fluorescence. Time-lapse luminescence images were acquired over 4 h at 3-min exposures with 1-min intervals. Time 0 denotes the initiation of the time-lapse bioluminescence imaging. The image at time 66 was obtained after initiation of 50 μM forskolin treatment. Scale bar: 10 μm. (c) The time-lapse bioluminescence images were analyzed at the single-cell level. The

bioluminescence intensity represents the average intensity in arbitrary areas including the cell nuclei of individual cells. Arrow indicates the time of initiation of forskolin treatment. Vehicle control indicates a representative cell. 'For' and 'Veh' respectively denote forskolin and vehicle treatments. (d) Average intensity of the time-lapse bioluminescence images was, respectively, shown in two groups of forskolin or vehicle treatment. Arrow indicates the time of initiation of forskolin treatment. Data are expressed as mean ± SD (forskolin, n=10; vehicle, n=6). *$p < 0.05$ versus respective vehicle controls. **$p < 0.01$ versus respective vehicle controls.

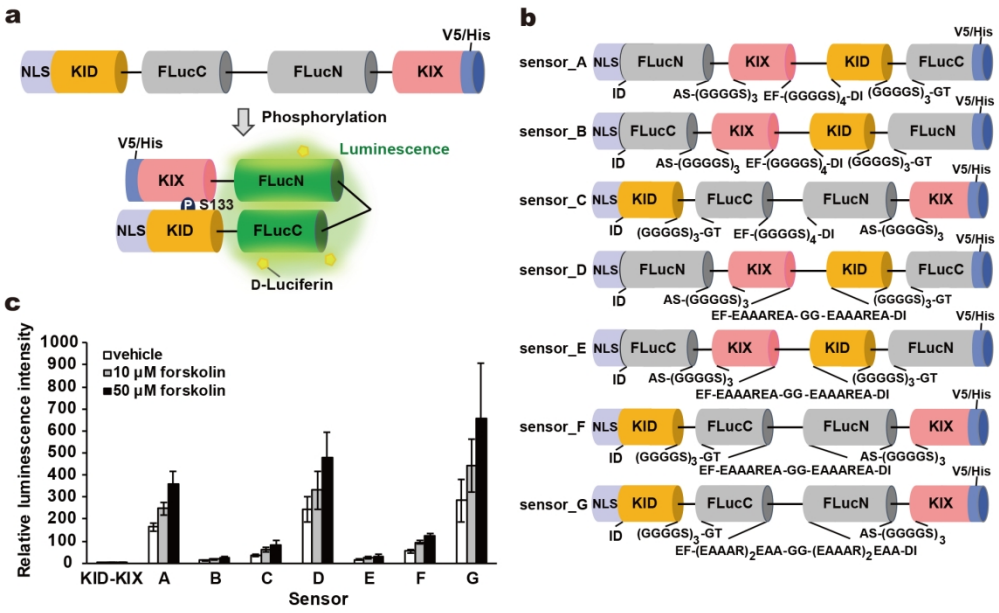

Fig. 1. Bioluminescence engendered by KID–KIX interaction within one vector in the presence of forskolin in HEK293T cells

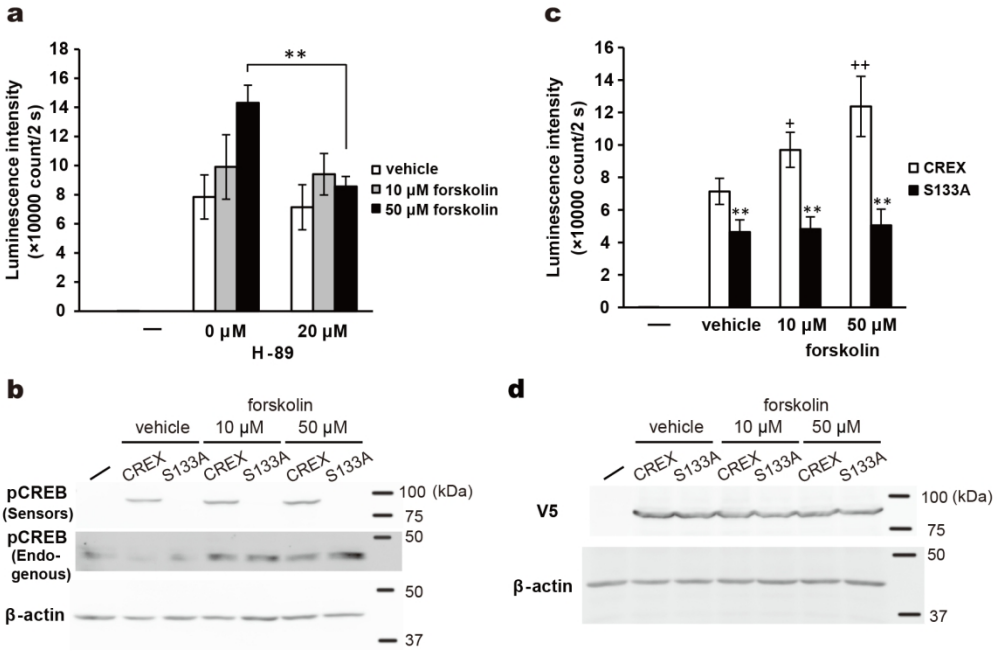

Fig. 2. KID–KIX interaction depending on phosphorylation at the amino acid position 133 in the CREB

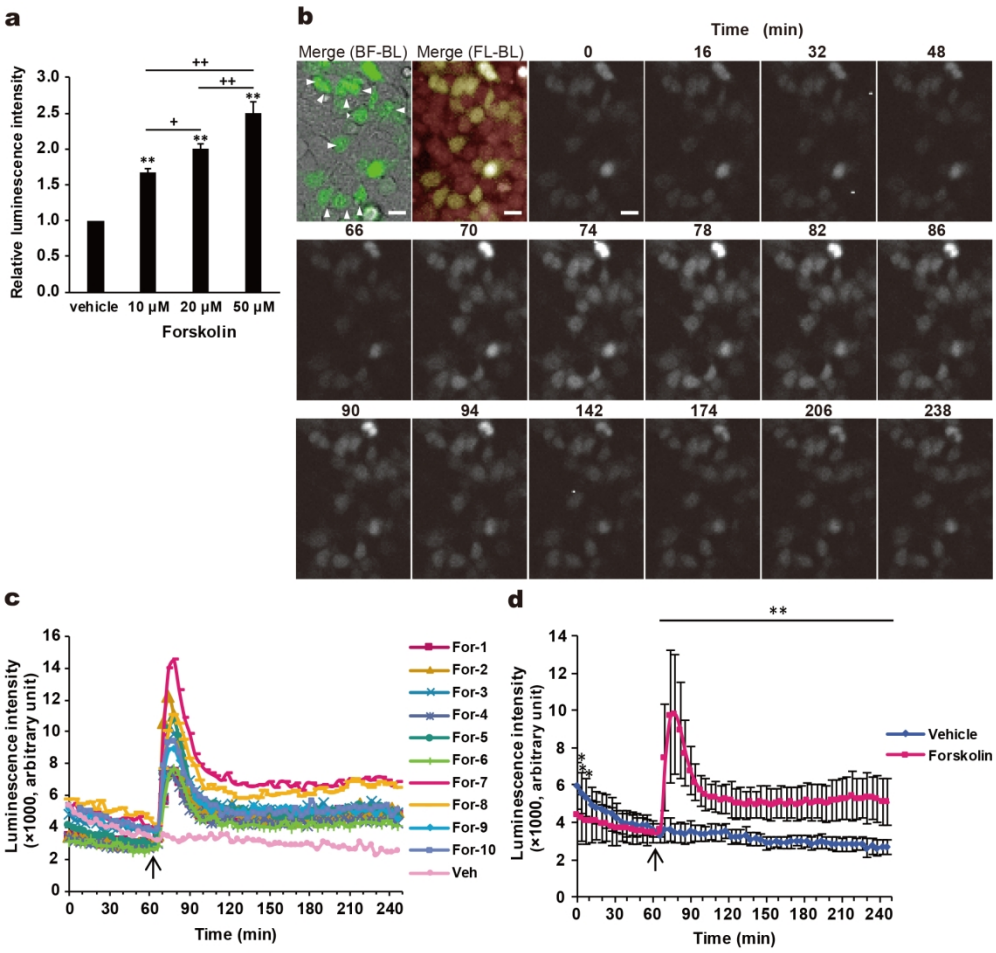

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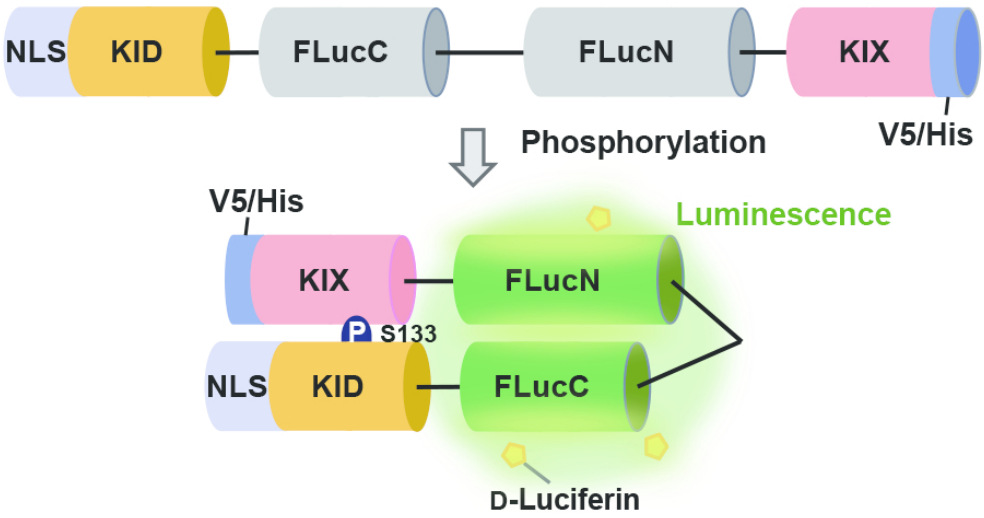
Table of contents:

Development of a single-molecule-format bioluminescent sensor to monitor CREB activation based on luciferase-fragment complementation.


NLS
KID
FLucC
FLucN
KIX
V5/His
Phosphorylation
V5/His
Luminescence
KIX
FLucN
P
S133
NLS
KID
FLucC
D-Luciferin