Light generation of intracellular Ca\(^{2+}\) signals by a genetically encoded protein BACCS

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Ca\(^{2+}\) signals are highly regulated in a spatiotemporal manner in numerous cellular physiological events. Here we report a genetically engineered blue light-activated Ca\(^{2+}\) channel switch (BACCS), as an optogenetic tool for generating Ca\(^{2+}\) signals. BACCS opens Ca\(^{2+}\)-selective ORAI ion channels in response to light. A BACCS variant, dmBACCS2, combined with Drosophila Orai, elevates the Ca\(^{2+}\) concentration more rapidly, such that Ca\(^{2+}\) elevation in mammalian cells is observed within 1 s on light exposure. Using BACCSs, we successfully control cellular events including NFAT-mediated gene expression. In the mouse olfactory system, BACCS mediates light-dependent electrophysiological responses. Furthermore, we generate BACCS mutants, which exhibit fast and slow recovery of intracellular Ca\(^{2+}\). Thus, BACCSs are a useful optogenetic tool for generating temporally various intracellular Ca\(^{2+}\) signals with a large dynamic range, and will be applicable to both in vitro and in vivo studies.
Precise spatial and temporal coordination of molecular events are the basis of many cellular functions. Recently, there has been growing interest in using optogenetic tools to investigate cellular functions, because light is non-invasive and can be easily controlled spatiotemporally. Optical control enables precise regulation of intracellular signals in the target cells or even in the local area of single cells. The most successful optogenetic tool, channelrhodopsin (ChR) from the green alga *Chlamydomonas reinhardtii*, modulates the membrane voltage in response to light and has been widely applied in the field of neurosciences. More recently, in addition to natural photosensory proteins, there have been advances in the development of genetically encoded photosensory modules for investigating cellular functions. One such photosensory module is the *Avena sativa* (oat) LOV2-J domain, which has been fused with various effector domains of proteins to generate novel engineered light-controlled molecules.

**Ca**$^{2+}$ is a ubiquitous second messenger in nearly all cells and regulates a wide variety of cell functions from cell division to cell death, including gene expression, cell migration, secretion, neural activities and muscle contraction. **Ca**$^{2+}$ signals function over a wide timescale, from milliseconds for synaptic vesicle release to hours for gene expression leading to cell development and differentiation. Moreover, **Ca**$^{2+}$ signals play important roles in subcellular level, such as in learning and memory at spiny dendrites and in neurotransmitter release at synaptic endings in a single neuron. A genetically encoded light-activated protein that regulates intracellular **Ca**$^{2+}$ signals could be useful and has been eagerly awaited.

Compared with caged compounds, genetically encoded light-sensitive proteins are more convenient to apply in vivo, because they usually do not require an external supply of chromophores to absorb photons. A light-activated cation channel, ChR2 (refs 16, 17), efficiently open ORAI1 channels and is designated human blue light-activated **Ca**$^{2+}$ channel. One of the BACCS variants induces an increase in intracellular global **Ca**$^{2+}$ signals within 1 s. BACCS is tuneable for its recovery kinetics, enabling fast and slow recovery of intracellular **Ca**$^{2+}$. We further demonstrate the application of BACCS *in vitro* and in *ex vivo* whole-mount preparations. Thus, BACCS is a useful optogenetic tool for regulating a wide variety of cellular events via intracellular **Ca**$^{2+}$ signals in various cell types both *in vitro* and *in vivo*.

### Results

**Engineering of a photoswitch for a **Ca**$^{2+}$-selective channel.** We generated a series of fusion genes of the LOV2-J domain from *A. sativa* phototropin 1 (refs 4, 5) as a photosensory module and the regulatory sequence for ORAI1 from human STIM1 (refs 23–26) as a signal effector (Fig. 1a; Supplementary Fig. 1). We assumed that the closer the photosensor and the signal effector were, the more efficient steric hindrance of the STIM1 interaction would be, thereby enabling inhibition of the signalling function in the dark. The basic core unit of BACCS was selected by combining the following three screening steps (see Supplementary Fig. 1 for details). First, the minimal signal effector domain of the STIM1 fragment for ORAI1 activation was defined. Second, the minimal STIM1 fragment was fused to the C terminus of a deletion series of LOV2-J to find the structure causing steric hindrance of the STIM1 interaction by LOV2-J. Third, the candidate fusion proteins were expressed with nuclear factor of activated T cells (NFAT):CFP ('::' represents a fusion) to examine light-induced NFAT translocation from the cytoplasm to the nucleus. It has been reported that elevation of intracellular **Ca**$^{2+}$ (>200–300 nM) induces dephosphorylation of NFAT, followed by its nuclear translocation. LOV2-J (amino acids 404–538)::STIM1 (amino acids 347–448) was the most efficient photoswitch, displaying high sensitivity and low basal activity, and is designated human blue light-activated **Ca**$^{2+}$ channel switch 1 (hBACCS1). hBACCS1 has the structural feature that a leucine residue (originally an isoleucine in LOV2-J) at the junction of the photoswitch protein, which is important for the function of both LOV2-J and STIM1, is shared between them (Fig. 1a). Therefore, the dark-state form of LOV2-J will block the function of STIM1 through steric hindrance over the leucine residue. Three variants of BACCS were designed (Fig. 1b): (1) hBACCS2, a dimer of hBACCS1; (2) ORAI1::hBACCS2, a fusion protein of human ORAI1 and hBACCS2; and (3) dmBACCS2, protein with the same structure as hBACCS2 except that *Drosophila* Stim was used instead of human STIM1. The designs of hBACCS2 and ORAI1::hBACCS2 were based on the observation that a tandem dimer of STIM1 (336–485) and its ORAI1 fusion protein can efficiently open ORAI1 channels.
Calcium response of ORAI1 activated by hBACCS. To evaluate the hBACCS function, we performed Ca\(^{2+}\) imaging in HEK293T cells expressing hBACCS2 (Fig. 2a). The widespread expression of ORAI proteins in most tissues\(^{29–31}\) will enable the omission of additional introduction of ORAI proteins in most cell types. We compared the following four constructs: hBACCS1, hBACCS2, hBACCS2-IRES-ORAI1 and ORAI1::hBACCS2 (Fig. 2b,c; Supplementary Fig. 2a,b). The IRES sequence allows co-translation of hBACCS2 with ORAI1 from a bicistronic messenger RNA. More than 90% of cells expressing hBACCS2 or ORAI1::hBACCS2 showed a Ca\(^{2+}\) increase on light exposure, while only 46% of cells expressing hBACCS1 showed an increase (Supplementary Fig. 2a; Supplementary Table 1), suggesting that hBACCS2 and ORAI1::hBACCS2 are more efficient photoswitches than hBACCS1. The time courses of the intracellular Ca\(^{2+}\) increase revealed that hBACCS2 induced a faster intracellular Ca\(^{2+}\) increase than ORAI1::hBACCS2 (Fig. 2b,c). Exogenous expression of ORAI1 in addition to hBACCS2 (hBACCS2-IRES-ORAI1) did not enhance the Ca\(^{2+}\) response in HEK293T cells compared with expression of hBACCS2 alone (Fig. 2b). Two ORAI1::hBACCS2 variants with shorter and longer linkers were also examined, resulting in no apparent improvement in Ca\(^{2+}\) response (Supplementary Fig. 2c). Removal of Ca\(^{2+}\) from the extracellular Ringer’s solution prevented the light-induced increase in intracellular Ca\(^{2+}\) in cells expressing hBACCS2 (Fig. 2b), suggesting that the Ca\(^{2+}\) increase occurs through influx of extracellular Ca\(^{2+}\), rather than release of Ca\(^{2+}\) from intracellular organelles such as the endoplasmatic reticulum or mitochondria. The Ca\(^{2+}\) indicator dye Fura-2/AM was used to measure the intracellular Ca\(^{2+}\) concentrations. The geometric means of the basal Ca\(^{2+}\) level in cells expressing ORAI1::hBACCS2 or hBACCS2 in the dark were both around 100 nM, slightly higher than that in control cells but within the range of physiological basal intracellular Ca\(^{2+}\) concentrations (Fig. 2d). In cells expressing hBACCS2, the intracellular Ca\(^{2+}\) concentration was elevated ~4.5-fold after 30 s and to beyond 1 \(\mu\)M (>10-fold) after 60 s in response to ultraviolet light (Fig. 2e). The efficiency of photoactivation of hBACCS2 was light intensity dependent. The magnitude of the Ca\(^{2+}\) response increased with increasing intensity in the range 0.6–180 \(\mu\)W mm\(^{-2}\) of continuous 470-nm light emitting diode (LED) illumination (Supplementary Fig. 2d). Activation of hBACCS2 was reversible (Supplementary Fig. 2e), and repeated photoactivation resulted in similar levels of Ca\(^{2+}\) elevation each time (Supplementary Fig. 2f).

Using whole-cell patch-clamp recording, inward currents were recorded at a holding potential of ~60 mV in HEK293T cells expressing ORAI1::hBACCS2 or hBACCS2 on blue light exposure (Supplementary Fig. 3a,c). Inward currents were also recorded in Jurkat cells (Supplementary Fig. 3e), which are known to have significant endogenous Ca\(^{2+}\) release-activated Ca\(^{2+}\) currents. The inward rectification of the light-induced current (Supplementary Fig. 3b,d,f) is consistent with the characteristic of ORAI1 channel\(^ {32–35}\). Furthermore, photostimulation did not generate outward currents representing eflux of Na\(^+\) or K\(^+\) in depolarized HEK293T cells expressing hBACCS2 (Supplementary Fig. 3d), suggesting that hBACCS2 does not activate non-selective cation channels, such as TRPC (transient receptor potential canonical) channels, which give large outward cationic currents at depolarized potential\(^ {36}\), and that Ca\(^{2+}\) is the only charge carrier of photoactivated currents under the whole-cell current recording condition. These results provide further indications that light-activated hBACCS2 can open ORAI channels in the plasma membrane and induce influx of extracellular Ca\(^{2+}\).

Characterization of dmBACCS2. To examine the applicability of hBACCS2 to other species, we performed Ca\(^{2+}\) imaging in Drosophila S2 cells expressing hBACCS2. However, we found no intracellular Ca\(^{2+}\) increase on photoactivation (Supplementary Fig. 4a), suggesting that interaction between hBACCS2 and Drosophila Orai (dOrai) was absent or insufficient. Since currents through a store-operated Ca\(^{2+}\) channel were recorded\(^ {37}\), and dOrai was identified as a gene involved in store-operated Ca\(^{2+}\) entry in S2 cells by genome-wide RNA interference screens\(^ {33,34,38}\), dOrai should be expressed in S2 cells. Therefore, we performed Ca\(^{2+}\) imaging in S2 cells expressing dmBACCS2, a Drosophila version of BACCS2, and found that intracellular Ca\(^{2+}\) signals were generated in a subset of cells after stimulation with a 488-nm laser (Supplementary Fig. 4b). The time course of the Ca\(^{2+}\) changes in S2 cells revealed that the concentration of intracellular Ca\(^{2+}\) reached its maximum ~50 s after illumination (Supplementary Fig. 4c). When Ca\(^{2+}\) imaging was performed in HEK293T cells expressing dmBACCS2 alone, intracellular Ca\(^{2+}\) was not elevated by photoactivation (Fig. 2b), suggesting that dmBACCS2 does not activate human ORAI1. As dmBACCS2 appeared to activate dOrai according to the Ca\(^{2+}\) imaging experiments in S2 cells, we examined HEK293T cells expressing dmBACCS2-IRES-dOrai. Surprisingly, activation of dmBACCS2 in dOrai expressing HEK293T cells caused fast and robust intracellular Ca\(^{2+}\) elevation (Fig. 2a–c). Removal of Ca\(^{2+}\) from the extracellular Ringer’s solution prevented the light-induced increase in intracellular Ca\(^{2+}\) in these cells (Fig. 2b), suggesting that the Ca\(^{2+}\) increase occurred through influx of extracellular Ca\(^{2+}\). Intracellular Ca\(^{2+}\) elevation was observed in 98.0% of cells expressing dmBACCS2-IRES-dOrai (Supplementary Table 1). To closely observe the Ca\(^{2+}\) changes, the cells were photoactivated with a 488-nm laser and observed every 0.1 s. Intracellular Ca\(^{2+}\) elevation was clearly observed within 1 s (Fig. 2f,g). Activation of dmBACCS2 was reversible, and repeated activation resulted in similar levels of intracellular Ca\(^{2+}\) elevation each time (Supplementary Fig. 2e,f). We performed whole-cell patch-clamp recording in HEK293T cells expressing dmBACCS2-IRES-dOrai, and confirmed the stimulus-dependent response amplitude and the inward rectification (Fig. 2h,i), consistent with the electrical characteristics of store-operated calcium channels in S2 cells\(^ {37}\). LOVS1K (ref. 20), which is composed of LOV2-Jx (404–546) and STIM1 (233–450) with mRFP, was also analysed for comparison. Photoactivation of LOVS1K in HEK293T cells resulted in slightly faster intracellular Ca\(^{2+}\) elevation compared with that due to hBACCS2 in our system (Fig. 2b). However, the speed of the Ca\(^{2+}\) elevation and amplitude of the Ca\(^{2+}\) increase by dmBACCS2 were significantly greater than those induced by LOVS1K or hBACCS2 (Fig. 2c).

The geometric mean of the basal Ca\(^{2+}\) level in cells expressing dmBACCS2-IRES-dOrai was 119.8 nM (Fig. 2d), higher than that in control cells but almost within the physiological range. It tended to be elevated with increases in the expression level (Supplementary Fig. 5a). We also examined NFAT::GFP subcellular localization in the dark, an alternative physiological assessment of the basal intracellular Ca\(^{2+}\) level (Supplementary Fig. 5b,c). We observed that NFAT::GFP was distributed in the cytoplasm, but not in the nucleus, in cells expressing hBACCS2 or dmBACCS2-IRES-dOrai in the dark, indicating a low basal intracellular Ca\(^{2+}\) level. In contrast, a large population (64.8%) of cells expressing LOVS1K showed nuclear localization of NFAT::GFP without light illumination, suggesting that the basal Ca\(^{2+}\) concentration in cells expressing LOVS1K was high.

Generation of Ca\(^{2+}\) signals in various cell types. To examine the applicability to various types of cells, hBACCS2 or dmBACCS2-IRES-dOrai was expressed in other cell types, namely
Figure 2 | Characterization of BACCSs expressed in HEK293T cells. (a) Representative Ca\textsuperscript{2+} responses in cells transiently expressing hBACCS2-ires-mCherry or dmBACCS2-ires-dOrai-ires-mCherry with Fluo-8/AM during the course of photoactivation (488-nm laser; 0.2 Hz). Pseudocoloured images are shown. A key for the heatmap is on the right. Scale bar, 40 μm. (b) Time course of Fluo-8/AM fluorescence changes in cells transiently expressing various constructs in the presence or absence of extracellular Ca\textsuperscript{2+} during the course of photoactivation (488-nm laser; 0.2 Hz; illumination indicated by blue vertical lines). (c) Mean Fluo-8/AM fluorescence changes at 5 and 20 s, and mean maximum fluorescence changes in each cell during the course of photoactivation in b. Significance was assessed by t-test. (d) Basal intracellular Ca\textsuperscript{2+} concentrations in cells expressing DsRed (n = 313), ORAI1::hBACCS2-ires-mCherry::tdTomato (n = 163), hBACCS2-ires-mCherry::tdTomato (n = 201) or dmBACCS2-ires-dOrai-ires-mCherry::tdTomato (n = 181). Purple bars indicate geometric means. Significance was assessed by analysis of variance and Fisher’s PLSD test. (e) Rates of [Ca\textsuperscript{2+}] increase. The times after ultraviolet illumination for BACCS activation are indicated. Because of high fluorescence signals beyond the Fura-2/AM detection limit (>1 μM) immediately after illumination, dmBACCS2 is not included. Data represent mean ± s.d. Significance was assessed by t-test. (f) Images of Fluo-8/AM responses in HEK293T cells transiently expressing dmBACCS2-ires-dOrai-ires-mCherry during the course of photoactivation (488 nm; 10 Hz). Pseudocoloured images are shown as in a. Scale bar, 20 μm. (g) Time course of Fluo-8/AM fluorescence changes in HEK293T cells transiently expressing dmBACCS2-ires-dOrai-ires-mCherry, hBACCS2-ires-mCherry or mCherry during the course of photoactivation (488 nm; 10 Hz; blue vertical lines). (h,i) Whole-cell inward current responses in HEK293T cells expressing dmBACCS2-ires-dOrai. (h) Current responses with LED blue light stimulation of increasing duration as indicated by different colours (1, 2, 3 and 5 s) at a holding potential of −60 mV. (i) Current responses of HEK293T cells at various holding potentials (left) and I-V relationships obtained by a ramp protocol. The blue bar indicates the timing of blue light (470 nm) stimulation. Data in b,c and g represent means ± s.e.m. The cell numbers analysed in b and g are indicated in parentheses. All data were obtained from more than three independent experiments. Significance: *P<0.05, **P<0.01, ***P<0.001.
COS-7 cells (derived from African green monkey kidney), mouse cultured hippocampal neurons and HIT-T15 hamster pancreatic β-cells, and Ca²⁺ imaging was performed (Fig. 3). Efficient elevation of intracellular Ca²⁺ was observed in most COS-7 cells (Fig. 3a,b) and mouse hippocampal neurons (Fig. 3c,d) on photoactivation. The extracellular solution for the hippocampal

**Figure 3 | Activation of hBACCS2 and dmBACCS2 in various cell types.** Cells were illuminated with a blue laser (488-nm; 0.2 Hz; illumination indicated by blue vertical lines). (a) Representative Ca²⁺ responses in Fluo-8/AM-loaded COS-7 cells transiently expressing hBACCS2-IRES-mCherry or dmBACCS2-IRES-dOrai-IRES-mCherry. Scale bar, 50 μm. (b) Time course of Fluo-8/AM fluorescence changes in a during the course of photoactivation. Ca²⁺ elevation was observed in 94.7 and 79.5% of cells expressing hBACCS2 and dmBACCS2-IRES-dOrai, respectively. (c) Representative Ca²⁺ responses in mouse hippocampal neurons transiently expressing hBACCS2-IRES-mCherry or dmBACCS2-IRES-dOrai-IRES-mCherry. Flu-0/AM fluorescence images corresponding to the boxed areas in the left panels are shown. Scale bar, 50 μm. (d) Time course of Fluo-8/AM fluorescence changes in the hippocampal neurons in c during the course of photoactivation. Cells transiently expressing mCherry were used as a negative control. Ca²⁺ elevation was observed in 86.7 and 95.5% of cells expressing hBACCS2 and dmBACCS2-IRES-dOrai, respectively. (e) Representative Ca²⁺ responses in Fluo-8/AM-loaded hamster pancreatic β-cell line HIT-T15 cells transiently expressing hBACCS2-IRES-mCherry, hBACCS2-IRES-ORAI1-IRES-mCherry or dmBACCS2-IRES-dOrai-IRES-mCherry. Images of the fluorescence changes (ΔF) between the images at 0 and 100 s or 0 and 15 s are shown. Scale bar, 30 μm. (f) Ca²⁺ elevation in individual HIT-T15 cells after 100 or 15 s of stimulation shown in e. The expression levels of mCherry (relative fluorescence unit, r.f.u.) and Fluo-8/AM fluorescence changes (ΔF/F) of individual HIT-T15 cells are plotted. The red lines represent the regression lines. Data are shown for n = 144 cells (hBACCS2), n = 133 cells (hBACCS2-i-ORAI1), and n = 141 cells (dmBACCS2-i-dOrai). r, Pearson correlation coefficient. Pseudocoloured images are shown in a, c, and e as in Fig. 2a. Data in b and d represent means ± s.e.m. The cell numbers analysed are indicated in parentheses in b and d. All data were obtained from more than three independent experiments.
neurons contained tetrodotoxin, a sodium channel blocker, thus excluding any effect of the action potential. In general, excitatory cells such as neuronal cells express Orai proteins, but they provide little contribution to any Ca^{2+} influx10. Our results suggest that hBACCS2 can be applied to both excitatory and non-excitable cells.  

In HIT-T15 cells, a comparison of hBACCS2 expression and intracellular Ca^{2+} elevation revealed that higher expression of hBACCS2 resulted in a smaller response, while lower expression resulted in a larger response (Fig. 3e,f). In contrast, a Ca^{2+} response was observed in a large population of HIT-T15 cells expressing hBACCS2-RES-ORAI1 or dmBACCS2-RES-dOrai regardless of the expression level (Fig. 3e,f). These results indicate that the level of endogenous Orai expression could vary substantially among HIT-T15 cells.  

These findings suggest that our strategy can generate Ca^{2+} signals in most mammalian cell types under blue light illumination, and that dmBACCS2 induces faster and larger intracellular Ca^{2+} elevation compared with hBACCS2 regardless of the cell types involved.

Spatiotemporal generation of Ca^{2+} signals. To examine whether Ca^{2+} signals could be generated with spatial specificity, Ca^{2+} imaging was performed in HEK293T cells expressing hBACCS2 with focused light illumination on a specific population of cells. In this experiment, near-infrared fluorescent protein (iRFP) was used as a marker for transfected cells, thereby making it possible to image hBACCS2-expressing cells with simultaneous blue light stimulation and Ca^{2+} imaging. Five cells loaded with the Ca^{2+} indicator dye Rhod-3/AM were sequentially illuminated with a 488-nm laser, and intracellular Ca^{2+} elevation was specifically observed in the targeted cells (Fig. 4a). Next, mouse cultured hippocampal neurons were examined for subcellular local photostimulation. We confirmed that hBACCS2 and ORAI1 were detected throughout the neuronal structure, including the dendrites and axon, by fluorescence imaging and immunostaining (Fig. 4b,c). We then carried out Fluo-4/AM Ca^{2+} imaging of neurons expressing hBACCS2. The photostimulation was initially focused on the tip of the axon and then focused on a dendrite. Ca^{2+} signals were clearly elevated in the axon terminal first and then in the dendrite (Fig. 4d,e). The Ca^{2+} elevation was restricted to a small area and the signal did not show long-distance transmission (<20 μm) (Fig. 4f,g). We also performed a subcellular light illumination in hippocampal neurons expressing dmBACCS2-RES-dOrai, and observed much faster local intracellular Ca^{2+} elevation than that for hBACCS2 (Fig. 4h).

Control of cellular events. To evaluate BACCS for cell biological applications, we first tested the light-activated switch in transcriptional regulation. NFAT is a transcription factor that regulates the expressions of cytokines, growth factors and their receptors, proteins involved in cell–cell interactions and many microRNAs40; elevation of intracellular Ca^{2+} induces dephosphorylation of NFAT, followed by its nuclear translocation22. HEK293T cells expressing hBACCS2-RES-NFAT::mCherry or dmBACCS2-RES-dOrai-RES-NFAT::mCherry were stimulated with a 488-nm laser every 2 min. Live-cell imaging of NFAT::mCherry revealed that NFAT molecules, which were initially distributed in the cytoplasm, translocated to the nucleus within 10 min (Fig. 5a). To examine whether BACCS photoactivation could induce gene expression, HEK293T cells were co-transfected with dmBACCS2-RES-dOrai and PHY41 (P_{NFAT}-YFP-pA)19 encoding yellow fluorescent protein (YFP) under the control of a promoter with an NFAT response element. After 12 h of blue light exposure, YFP expression was induced (Fig. 5b). To quantify the level of induction, we performed luciferase assays. hBACCS2, LOVS1K and dmBACCS2-RES-dOrai were compared (Fig. 5c,d). Significant luciferase induction was observed for hBACCS2 after 6 h of light exposure. Low, but slightly elevated, basal luciferase activity in the dark was observed in cells expressing hBACCS2. This could reflect a small population of cells with an elevated basal Ca^{2+} level. LOVS1K induced luciferase activity at a similar level to that of hBACCS2 on light exposure, but showed high basal activity in the dark, consistent with a higher basal Ca^{2+} level. dmBACCS2 induced robust luciferase activity on light exposure compared with hBACCS2 and LOVS1K. dmBACCS2 also showed basal activity in the dark, which decreased with lower amounts of transfected DNA. The dynamic ranges of the induced luciferase activity for hBACCS2 and dmBACCS2 in this assay were much higher than that for LOVS1K.  

We also used hBACCS2 to initiate the formation of blebs, spherical membrane protrusions, in HEK293 cells in combination with Lyn-CaRQ41, an engineered membrane-anchored Ca^{2+}-sensitive RhoA protein that becomes activated in response to Ca^{2+} signals. Plasma membrane blebs have been implicated in apoptosis, cytokinesis and cell migration42. Cells expressing both hBACCS2 and Lyn-CaRQ were photoactivated with a 488-nm laser. Blebs were induced in a stimulation-dependent manner (Fig. 5e), and the number of induced blebs reached the maximum level within 3.5 min (Fig. 5f).  

Thus, BACCS can be applied to the control of cellular events such as NFAT-dependent transcription and cell morphological changes.

Electrophysiological response in mouse olfactory epithelium. To examine whether BACCS is applicable to mouse tissues, we generated a recombinant adenovirus for expression of hBACCS2-RES-mem::tdTomato, and infected the adenovirus into the olfactory epithelium (Fig. 6a). We recorded local field potentials from the surface of the olfactory epithelium in an ex vivo whole-mount preparation, the electro-olfactogram (EOG)43. Figure 6c shows the waveforms of the average EOG responses to blue light and the odontor amyl acetate as a positive control. Significant responses to both blue light and amyl acetate were recorded at the infected surface of the olfactory epithelium on blue light exposure, while the olfactory epithelium of wild-type mice only showed responses to amyl acetate (Fig. 6c,d), suggesting that hBACCS2 was photoactivated in olfactory sensory neurons (OSNs).  

To further consolidate the EOG results, we generated a transgenic mouse strain possessing a subset of OSNs that expressed ORAI1::hBACCS2. A DNA fragment containing the odorant receptor gene M71 (also known as Olfr151) was used to generate the transgenic mouse strain44, in which neurons expressing the transgene M71 co-expressed ORAI1::hBACCS2 and mem::tdTomato (Supplementary Fig. 6a). The M71 transgene was expressed in a subset of OSNs (Fig. 6b), and their axonal projection to the olfactory bulb and neural survival appeared normal (see Supplementary Fig. 6b–f for details). We carried out EOG using the transgenic mice, and observed electrophysiological responses in these mice (Fig. 6c,d). The responses in the transgenic mice were smaller, but less variable, than those in the adenovirus-infected mice, suggesting that the specific and stable expression of BACCS in transgenic animals enabled the acquisition of more stable EOG responses.

Thus, BACCS can be used in the mouse olfactory epithelium and will be applicable to in vivo studies.

Fast and slow recovery kinetic mutants of dmBACCS2. dmBACCS2 is an efficient Ca^{2+} photoswitch in mammalian cells, but still suffers from slow recovery kinetics (time constant of
the whole-cell current decay phase) (Fig. 2h)\textsuperscript{45}, rendering it difficult to precisely control its temporal signalling activities. Recently, mutational analysis of the LOV2 domain was conducted to obtain fast- and slow-cycling LOV2 variants\textsuperscript{46,47}. We introduced such mutations into the LOV2 domain of dmBACCS2; N425S and V416L\textsuperscript{46} resulted in fast and slow

Figure 4 | Local generation of intracellular Ca\textsuperscript{2+} signals. (a) Time series of fluorescence images of Rhod-3/AM-loaded HEK293T cells transiently expressing hBACCS2-ires-iRFP during the course of photoactivation. Five target cells in circles were sequentially illuminated with a 488-nm laser (15 times; 0.2 Hz; illumination indicated by blue vertical lines). Scale bar, 50 \textmu m. (b) Fluorescence images of a hippocampal neuron expressing mCherry::hBACCS2 (magenta) and ORAI1::YFP (green). Scale bar, 20 \textmu m. (c) Fluorescence images of a hippocampal neuron expressing mCherry::hBACCS2::RES-ORAI1. The localization of mCherry::hBACCS2 is shown in magenta and a putative axon is stained with an anti-Tau1 antibody (Alexa 488; green). Scale bar, 40 \textmu m. (d) Fluorescence images of a Fluo-4/AM-loaded hippocampal neuron transiently expressing hBACCS2-ires-mem::iRFP. The left panel shows the iRFP image with the positions of the first and second local activations with a 458-nm laser (blue boxes). Scale bar, 50 \textmu m. (e) Higher-magnification views of the activated areas in d. The time points of photoactivation are indicated by blue boxes. (f) Higher-magnification views at the border of the first photoactivation in d. The time points and location of photoactivation are indicated by blue labels. Scale bar, 50 \textmu m. (g) Kymograph representing the spatiotemporal activation pattern in f. The intensity of Fluo-4/AM fluorescence along the axon is shown as a heatmap. The positions indicated by the arrowhead and arrow correspond to the locations of the axons indicated by the same symbols in d and f. The location of the photoactivated area is indicated by the blue bar. The border of the photoactivation is set to distance 0. (h) Fluorescence images of a Fluo-4/AM-loaded hippocampal neuron transiently expressing dmBACCS2-ires-dOrai::ires-iRFP. The left panel shows the iRFP image with the position of the local activation with a 458-nm laser (blue circle). Higher-magnification views of the activated area (box) are shown below. Scale bar, 50 \textmu m. Pseudocoloured images are shown in a, d, e, f, g and h as in Fig. 2a.
from the analysis. Data represent means and standard errors of means, and independent experiments. Significance was assessed by t-test: *P < 0.05, **P < 0.01. (a) Time-lapse imaging of NFAT::mCherry translocation in HEK293T cells. Cells transiently expressing hBACCS2-ires-NFAT::mCherry or dmBACCS2-ires-dOrai::NFAT::mCherry were illuminated with a 488-nm laser every 2 min. Scale bar, 20 μm. (b) Light-dependent YFP upregulation in HEK293T cells transfected with dmBACCS2-ires-dOrai and P_{NAFAT}-YFP. Cells were continuously illuminated with 470-nm LED blue light of 60 μW mm⁻² or left in the dark for 12 h. Scale bar, 200 μm. (c) and (d) Light-dependent induction of luciferase expression after 6 h in HEK293T cells co-transfected with pGL4.30 (P_{NAFAT}-luc2P), pRL-SV40 (P_{SV40}-hRluc, internal control,) and various Ca²⁺ photwitch constructs. Cells were left in the dark or continuously illuminated with 470-nm LED blue light of 60 μW mm⁻² for 6 h (n = 4–6 independent experiments). Significance was assessed by t-test: *P < 0.05, **P < 0.01. (e) Representative images of bleb induction in HEK293 cells. Cells transiently expressing hBACCS2-ires-m::iRFP and Lyn-CaRQ (YFP fusion) were stimulated (0.1 Hz) and observed with a 488-nm laser. Scale bar, 20 μm. (f) Time course of bleb induction by stimulation (488-nm laser, 0.1Hz; illumination indicated by blue vertical lines). Among cells transfected with both hBACCS2-ires-m::iRFP and Lyn-CaRQ, 35 of 78 cells initiated blebbing after photoactivation, compared with 0 of 83 cells transfected with Lyn-CaRQ alone. The numbers of blebs per cell were counted during the time course of photoactivation (n = 35), and cells in which blebs were not induced were excluded from the analysis. Data represent means ± s.e.m. in (c), (d), and (f). switch-off phenotypes, respectively. We performed Ca²⁺ imaging in HEK293T cells expressing dmBACCS2-ires-dOrai, dmBACCS2-N425S (dmBACCS2NS)-ires-dOrai or dmBACCS2-V416L (dmBACCS2VL)-ires-dOrai, and compared the Ca²⁺ responses. The amplitude of the Ca²⁺ increase in cells expressing the dmBACCS2NS construct was smaller than those in cells expressing the dmBACCS2 or dmBACCS2VL constructs (Fig. 7a–c). To analyse the recovery kinetics, the intracellular Ca²⁺ decrease was observed every 5 s after 170 s of photostimulation (Fig. 7d). Notably, dmBACCS2NS had faster recovery kinetics and dmBACCS2VL had slower recovery kinetics than dmBACCS2 (Fig. 7e,f). Whole-cell patch-clamp recording revealed the precise time courses of dOrai activation and inactivation for dmBACCS2 and the two mutants (Fig. 8a). The results for three kinetic parameters, amplitude, time to peak and recovery time constant (Fig. 8b,c) were consistent with the Ca²⁺ imaging observations. The recovery time constant for dmBACCS2NS is less than one-half of that for dmBACCS2, while the time constant for dmBACCS2VL was > 100 times larger than that for dmBACCS2.

Thus, the recovery kinetics of BACCS are tuneable, and both fast and slow recovery switches can be developed for specific experimental purposes.

Discussion

We have engineered BACCS, an optogenetic tool for generating intracellular Ca²⁺ signals. Compared with previously reported Ca²⁺ optogenetic tools, some variants of BACCS were endowed with features of fast and large response induction and better off-recovery efficiency, thereby making BACCS a more useful tool for precisely generating Ca²⁺ signals.

The four BACCS variants, hBACCS1, hBACCS2, ORAI1:: hBACCS2 and dmBACCS2, had different properties in their...
Ca\(^{2+}\) responses. Although the stoichiometry of ORAI1 and STIM1 remains controversial, Li et al.\(^{29}\) proposed a model in which one STIM1 dimer can interact with one ORAI1 subunit in the pore formed by tetrameric ORAI1 and octameric STIM1 to maximally open the ORAI1 channel. Consistent with this model, hBACCS2, a tandem dimer of hBACCS1, was more efficient photoswitch than hBACCS1. A fusion protein of hBACCS2 with the C terminus of ORAI1 was also functional as a switch in HEK293T cells, but the photoactivation time course of ORAI1:hBACCS2 was slower than that of hBACCS2. Although we observed no drastic differences in Ca\(^{2+}\) responses among the three ORAI1–hBACCS2 fusion constructs with different linker lengths, the location and length of the linker might still influence the function. Alternatively, endogenous ORAI1 and ORAI1:hBACCS2 might form a heterogeneous tetramer, resulting in insufficient maximal activation of the channel. dmBACCS2 was a much more efficient photoswitch but required coexpression of dOrai in mammalian cells. We observed that the intracellular Ca\(^{2+}\) increase occurred within 1 s in HEK293T cells expressing dmBACCS2-IRES-dOrai, although the Ca\(^{2+}\) response was not as fast as in Drosophila S2 cells expressing dmBACCS2. Mammalian cells may lack negative regulatory mechanisms for dOrai function. Although we cannot exclude the possibility that the fast Ca\(^{2+}\) response arose through functionally better stoichiometry of dmBACCS2 and dOrai in cells expressing dmBACCS2-IRES-dOrai, this seems unlikely because expression of hBACCS2-IRES-ORAI1 did not result in faster or larger intracellular Ca\(^{2+}\) elevation compared with expression of hBACCS2 alone. It has been reported that Orai1-interacting STIM1 domains, CAD\(^{24}\) and SOAR\(^{25}\), can interact with voltage-gated Ca\(^{2+}\) Ca\(^{1,2}\) channels\(^{8,10}\) and Trpc channels\(^{39}\), although the domains themselves do not activate these channels. Therefore, hBACCS2, which contains a similar sequence to CAD/SOAR, may interact with such molecules, but should generate no signals through them.

We examined the basal Ca\(^{2+}\) level by two methods, comprising direct measurement by Fura-2/AM and indirect measurement by the NFAT::GFP assay. Although the Fura-2 method measures exact intracellular Ca\(^{2+}\) concentrations, we cannot exclude the possibility that BACCS could be slightly activated in the darkroom because of the difficulty in achieving complete darkness during the experiments. However, the NFAT::GFP assay would not be affected by this problem because NFAT::GFP was fixed in the darkroom before possible NFAT::GFP translocation, if any. The results for both the NFAT::GFP assay and the measurement by Fura-2/AM indicated that the mean basal Ca\(^{2+}\) levels in hBACCS2-expressing cells and dmBACCS2-IRES-dOrai-expressing cells were within the physiological range. However, BACCS-expressing cells, especially dmBACCS2-dOrai-expressing cells, contained a population of cells with a high basal Ca\(^{2+}\) level, probably because of intrinsic activity of BACCS or the difficulty in achieving complete darkness for the experiments, and these cells are likely to have influenced the results of the luciferase assay. The basal Ca\(^{2+}\) concentration could be controlled and kept at a lower level by avoiding high-level expression of the BACCS gene, at least partially, as demonstrated in the luciferase assay findings.

In what kinds of cells can BACCS be used? dmBACCS2-dOrai was successfully used in Drosophila S2 cells, mammalian HEK293T cells, COS-7 cells, HIT-T15 cells and hippocampal neurons, suggesting that it can be used in a wide variety of species and cell types. hBACCS2 also functioned in all mammalian cell types examined, and can be used to photoactivate endogenous Orai. The efficiency of intracellular Ca\(^{2+}\) signal elevation by light relies on the level of endogenous Orai expression in each cell. The cytosolic STIM1 C terminus has been reported to stimulate not only human ORAI1, but also ORAI2 and ORAI3 (ref. 51), indicating that hBACCS2 can activate all ORAI proteins by light illumination. On the basis of previous observations that Orai proteins are widely expressed in most mouse and human tissues\(^{29–31}\), hBACCS2 expression alone is likely to be sufficient for the generation of Ca\(^{2+}\) signals through endogenous Orai in most mammalian cells on light illumination. Although Stim and Orai proteins are evolutionarily conserved across metazoans\(^{52,53}\), direct cross-species interactions between Stim and Orai molecules appear to be inefficient between humans and Drosophila.

Two Ca\(^{2+}\)-selective photoswitches have been reported, PACR (ref. 21) and LOVS1K (ref. 20). PACR is composed of calmodulin fused with the M13 peptide and LOV2-Jz, and releases Ca\(^{2+}\) upon light exposure. The expression can be restricted to a particular organelle, thereby allowing the control of Ca\(^{2+}\) signals at the organelle level. PACR induces Ca\(^{2+}\) concentration changes of 10–90 nM in HeLa cells, which are not sufficient to control many Ca\(^{2+}\)-regulated cellular events because micromolar changes in Ca\(^{2+}\) concentration are observed in many biological responses. LOVS1K and hBACCS are both composed of the same photosensory module, LOV2-Jz, and effector domain, STIM1, with different sizes of each fragment: LOV2-Jz (404–538) and STIM1 (347–448) for hBACCS and LOV2-Jz (404–546) and STIM1 (233–450) for LOVS1K. Pham et al.\(^{50}\) reported that repeated photoactivation of LOVS1K co-expressed with ORAI1 gradually caused intracellular Ca\(^{2+}\) accumulation to ~500 nM over 40 min. However, it can be estimated from our experiments that intracellular Ca\(^{2+}\) elevation to ~500 nM can be achieved in

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**Figure 6 | EOG analyses of odorant- and light-induced responses.** (a) Representative fluorescence image of part of the C57BL/6 wild-type (WT) mouse olfactory epithelium infected with the adenovirus (AV) hBACCS2-IRES-mem::tdTomato. Scale bar, 200 μm. (b) Representative fluorescence image of part of the olfactory epithelium from an M71-ORAI1:hBACCS2-IRES-mem::tdTomato transgenic (TG) mouse. The inset shows a higher-magnification view of the olfactory epithelium. Scale bar, 200 μm. The mice in a and b were 8 weeks of age. (c) Light-induced depolarizing potentials recorded from BACCS-expressing olfactory sensory neurons. (d) Average EOG responses of WT mice (left), AV-infected mice (middle) and TG mice (right) to blue light illumination (blue bars). The arrows indicate the timing of the 100-ms amyl acetate application to the olfactory epithelium. Shaded regions around the EOG response traces represent the s.e.m. (d) Box plot of the maximal EOG responses to 4 min of blue light illumination. The y axis represents the EOG responses induced by blue light illumination relative to those induced by amyl acetate exposures. The data shown are for n = 10 WT mice, n = 16 AV-infected mice and n = 12 TG mice. Significance was assessed by t-test: *P < 0.05, **P < 0.01.
30 s in cells expressing LOVS1K alone (Fig. 2b,e), probably reflecting differences in the experimental setup. One non-negligible defect of LOVS1K is its high basal activity, which was observed in our NFAT::GFP assay and luciferase assay in the dark. The basal Ca$^{2+}$ level, which affects NFAT-dependent transcription, could lead to considerable interference with cell function.

BACCS has the following features and advantages. hBACCS2 can be used to induce intracellular Ca$^{2+}$ elevation to $\approx 500$ nM in 30 s and $> 1$ μM in 60 s, and its basal activity in the dark is...
Figure 8 | Characterization of light-activated whole-cell inward currents recorded from HEK293T cells expressing mutant dmBACCS2-ires-dOrai. (a) Average current responses in HEK293T cells expressing mutant dmBACCS2-ires-dOrai to 30 s of LED light (470 nm) illumination (blue bars): n = 14 (dmBACCS2); n = 12 (dmBACCS2NS); n = 11 (dmBACCS2VL). The shaded region around the trace represents s.e.m. The dotted lines indicate the baseline. (b) Parameters for the current analysis. Response amplitude, time to peak (time from start of illumination to peak of response) and time constant of termination (sum of t₁ and t₂ determined by fitting the decay phase of the response to a double exponential equation; magenta) were calculated. (c) Box plots for a summary of the kinetic analyses of mutant dmBACCS2. The blue box indicates the timing of the illumination. Significance was assessed by t-test: *P<0.05, **P<0.01, ***P<0.001.

relatively low compared with LOVS1K. hBACCS2 functions in the mouse olfactory system. In M71 transgenic mice, OSNs expressing ORAI1::hBACCS2 appeared normal in terms of cell survival and axonal projection54, suggesting that the basal Ca²⁺ concentration induced by ORAI1::hBACCS2 was sufficiently low to have no effect on the development of olfactory neurons. The relatively low basal activity and high Ca²⁺ response (micromolar change) make hBACCS2 suitable for application to many Ca²⁺-dependent biological events both in vitro and in vivo. dmBACCS2-dOrai is by far the most efficient and robust Ca²⁺ photoswitch in mammalian cells. Although basal activity in the dark can be observed when dmBACCS2-dOrai is expressed at a high level, the dynamic range of the Ca²⁺ response is wide, and fast Ca²⁺ intracellular elevation can be induced. We observed that both its magnitude and speed of Ca²⁺ elevation were better in all types of tested cells expressing dmBACCS2-ires-dOrai than in cells expressing hBACCS2, suggesting that dmBACCS2-dOrai is a more useful tool to precisely induce Ca²⁺ responses. Furthermore, as dmBACCS2 does not appear to interact with other endogenous molecules in mammalian cells. Therefore, hBACCS2 and dmBACCS2 are alternative candidates for practical use in mammalian cells (Supplementary Fig. 7), hBACCS2 will be an appropriate tool to control the activity of endogenous Orai and Orai-mediated cellular events, such as induction of T-cell activation22, turning of growth cones55 and cell migration56. The dmBACCS2-dOrai system may be used with better temporal resolution for controlling general Ca²⁺-dependent cellular events without interfering with the functions of other endogenous molecules.

Although the activation time was improved by using dmBACCS2, the recovery kinetics would be a limiting factor for inter-trial frequency. In cases of synaptic transmission or cardiac contraction, the Ca²⁺ effector systems respond to pulses within the microsecond to millisecond range9. The photocycles of PACR, LOVS1K, hBACCS2 and dmBACCS2 all depend on LOV2-Jα and their recovery kinetics will be similar; LOV2-Jα recovers spontaneously with half-times of ~50 s (ref. 45). As an effort to tune the recovery kinetics, we introduced mutations implicated in the photocycle of LOV2-Jα (refs 46,47) into dmBACCS2 to obtain various recovery kinetic mutants (Supplementary Fig. 7). The fast recovery mutant showed significantly faster Ca²⁺ recovery in the dark than wild-type dmBACCS2. This improvement will help substantially towards the manipulation of cellular events controlled by oscillations of Ca²⁺ transients on the second to minute timescale, including growth-cone
migration/turning, axonal growth, cell migration, muscle development and release of cytokines, although this mutant still falls short for the control of synaptic transmission or cardiac contraction. Further mutagenesis in the LOV2-2α domain will produce improved dmBACCS2 with faster reversibility. The slow recovery kinetic mutant showed prolonged intracellular Ca\(^{2+}\) elevation after the lights were turned off. It will require less light illumination for sustained activation of the photoswitch, which could avoid undesirable phototoxicity to cells and unfocused activation through drifting of the target sample. The fact that the introduction of the mutations for the fast- and slow-cycling LOV2-2α into dmBACCS2 resulted in fast and slow recovery kinetics of dmBACCS2-mediated oDrai responses, respectively, is consistent with the idea that these mutations changed the LOV2-2α kinetics, resulting in the tuning of the binding of dmBACCS2 to oDrai.

One of the advantageous features of photoactivatable molecules is the ability to control their activity with high spatial resolution. By focused illumination of a blue light laser, we successfully activated local Ca\(^{2+}\) signals at axon terminals and dendrites of hippocampal neurons. Ca\(^{2+}\) microdomains are sites with localized high Ca\(^{2+}\) concentrations near Ca\(^{2+}\) channels at the cell surface or internal stores. Ca\(^{2+}\) microdomains enable different subcellular domains to execute many distinct cellular processes with specificity in single cells. For example, Ca\(^{2+}\) microdomains in spiny/aspy dendrites, soma, mossy fibres and synaptic endings are responsible for distinct neuronal functions of learning and memory, gene transcription and transmitter synaptic endings are responsible for distinct neuronal functions of learning and memory, gene transcription and transmitter.

The ability of BACCS to generate global and subcellular Ca\(^{2+}\) signals, and our application of BACCS to various cell types and transgenic animals suggest that BACCS will be a useful optogenetic tool for generating intracellular Ca\(^{2+}\) signals both in vitro and in vivo. Precise and non-invasive exploration of intracellular Ca\(^{2+}\) functions at the subcellular level by light illumination will contribute towards a deeper understanding of the numerous Ca\(^{2+}\)-dependent physiological events.

Methods

DNA constructs. HA-NFAT1 (1–460)-GFP, human STIM1-CFP and Orai1-YFP were kind gifts from Anjana Rao (Addgene plasmids #11107, #19755 and #19756), pLyn::tdTomato (tdTomato with a membrane localization signal) from Kazunari Rac1-I539E from Kraus Hahn (Addgene plasmids #22007, #22021 and #22026); pTriEx-mVenus-PA-Rac1, pTriEx-mVenus-PA-Rac1-C450A and pTriEx-PA-pLyn-CaRQ and LOVS1K from Kevin Truong (Addgene plasmids #34559 were kind gifts from Anjana Rao (Addgene plasmids #11107, #19755 and #19756); Drosophila Jurkat cells were seeded and transfected with plasmids using Lipofectamine 2000 and COS-7 cells were seeded on glass-bottom dishes (Greiner) coated with

Immunostaining. mCherry::hBACCS2 was transfected into hippocampal cells 6 days after dissection. The cells were fixed with 4% paraformaldehyde/PBS for 20 min, permeabilized with 0.1% Triton X-100/PBS for 15 min and incubated in 3% bovine serum albumin (BSA)/PBS for 15 min. The cells were then incubated with a mouse anti-Tau1 monoclonal antibody (PC16; Millipore; 1:4,000 dilution) in 2% BSA/PBS for 1 h. After blocking with 5% BSA/PBS for 15 min, the cells were incubated with an Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen; 1:500 dilution) in 2% BSA/PBS for 30 min. Images were obtained using an LSM510 META laser scanning microscope (Carl Zeiss).

Ca\(^{2+}\) imaging. Cells were loaded with Fluo-4/AM, Fluo-8/AM (2.5 μM for both; Invitrogen) or Rhod-3/AM (10 μM; Invitrogen) according to the manufacturer’s instructions. Fluo-4/Fluo-8 and Rhod-3 were used for different wavelengths, and there were no specific reasons underlying the use of Fluo-4 and Fluo-8 for the experiments. Laser light for photoactivation of hippocampal neurons, Fluo-4 and Fluo-8 for the experiments. Laser light for photoactivation of hippocampal neurons, Fluo-4 and Fluo-8 for the experiments.

Live-cell imaging and photoactivation. Time-lapse images of NFAT translocation were obtained using the FV1200 laser scanning microscope.

13-acetate (PMA; Wako Chemical Co.), a specific activator of protein kinase C, was added (final concentration: 50 ng ml\(^{-1}\)) to enhance the cytomegalovirus promoter

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The cytoplasm were defined as cells with nuclear NFAT. 

Transfection into HEK293T cells, and the cells were fixed with 4% paraformaldehyde. 

was transfected into HEK293T cells. The cells were photoactivated every 2 min at a laser dose of (488 nm: 20%) for NFAT translocation, and the NFAT:mCherry localization was imaged using the 568-nm laser every 2 min (n = 3 independent experiments for hBACCS2-RES::mCherry; n = 7 independent experiments for dmbBACCS2-RES::dOrai1-RES::NFAT::mCherry). Time-lapse images of bleb formation were obtained using the LSM510 META laser scanning microscope. Ly-CaRq (containing YFP) and hBACCS2-RES::IRFP were co-transfected into HEK293 cells. hBACCS2-transfected cells were verified by iRFP expression. The cells with YFP fluorescence were first imaged using the 514-nm laser (no photoactivation), and then photoactivated and imaged with the 488-nm laser. Cells that showed bleb formation before photoactivation were excluded from the analyses for both Ly-CaRq/hBACCS2-RES::IRFP double-transfected cells and Ly-CaRq single-transfected cells (control). These experiments were performed at room temperature.

NFAT translocation assay. For screening of BACCS constructs, NFAT::CFP and tdTomato::LOV::STIM1 were co-transfected into HEK293T cells. Cells were either illuminated with a 470-nm LED light (cat. no. NF-1050-B; NIHON EIDO) or not illuminated, for 20 min in a darkroom. The cells were then fixed with 4% paraformaldehyde.

For analyses of the basal NFAT location, NFAT::GFP, hBACCS2-RES::NFAT::GFP, dmbBACCS2-RES::NFAT::GFP, or LOVS1-K-RES::NFAT::GFP were transfected into HEK293T cells, and the cells were fixed with 4% paraformaldehyde in the darkroom 24 h after transfection. The cells were counterstained with 4,6-diamidino-2-phenylindole (Nacalai Tesque) to visualize the nuclei. Images were obtained using the LSM510 META laser scanning microscope. Cells with brighter NFAT::CFP (or GFP) fluorescence in the nucleus compared with that in the cytoplasm were defined as cells with nuclear NFAT.

Luciferase assay and YFP induction. For the luciferase assay, HEK293T cells in a 96-well format were transfected with 50 ng per well pRepG-tuc2P (pGL4.30[b]/Luc2P/ NFAT RE); Promega), 50 ng per well pRL (pRL-SV40; Promega) and 0–10 ng per well various Ca2 + photowitch constructs, and incubated for 24 h. hRluc (Renilla luciferase) activity was used as an internal control for cell viability and transfection efficiency. After the medium was replaced with MEM supplemented with 10% FBS containing 10 ng/ml PMA, the cells were either illuminated with a 470-nm LED light (60 μW mm⁻²) or not illuminated, for 6 h in CO2 incubators. The firefly and Renilla luciferase activities were quantified using a Dual-Glo Luciferase assay system (Promega). Luminescence was measured using an ARVO X5 (PerkinElmer). For YFP induction, HEK293T cells in a glass-bottomed 35-mm dish with four compartments (Greiner Bio-One) were transfected with 1 μg per well pHLY1 and 500 ng per well dmbBACCS2-RES::dOrai1, and incubated for 24 h. The medium was then replaced with MEM supplemented with 10% FBS containing 10 ng/ml PMA, and the cells were either illuminated with a 470-nm LED light (60 μW mm⁻²) for 12 h or not illuminated, in CO2 incubators. YFP expression was observed using the FV1200 laser scanning microscope.

Recombinant adenosine infection. The recombinant cosmid vector was packaged with Gigapack III Plus Packaging Extract (Agilent) according to the manufacturer’s instructions and amplified. The extracellular solutions contained 140 mM NaCl, 5 mM CaCl2, 10 mM HEPES, 10 mM CaCl2, 1.2 mM MgCl2 and 30 mM 3-glucose (pH 7.4). The electrode solution contained 140 mM CaCl2, 10 mM NaCl, 10 mM HEPES, 2.84 mM CaCl2, 2.47 mM MgCl2 and 5 mM EGTA-2Ca (pH 7.4). Nystatin was dissolved in methanol containing 100 mM N-methyl-d-glucamine, 100 mM methanesulfonic acid and 10 mM phenol red to a concentration of 5 mg ml⁻¹. Subsequently, 50 μl of this nystatin solution was evaporated, and the precipitate was dissolved in 1 ml of electrode solution. The solution was loaded into the electrode through a 0.22-μm syringe filter. After G2 seal formation by manual suction, 10–15 min was allowed for stable perfusion.

For EOG recording, mice at 5–7 weeks of age were anesthetized with diethyl ether and euthanized by decapitation. The heads of the mice were split along the midline with a scalpel, and the nasal septum was removed so that the olfactory turbinates could be exposed. The head was placed on a recording agar chamber. Single-labeled neuronal potential signals were amplified with a 30-Hz low-pass digital filter. Odorant solutions were prepared in 25 ml of water as 0.1% w/v solutions. Vapour-phase odorant stimulation was performed by collection of the head space above 25 ml of odorant solution in a sealed 100-ml glass bottle. Vapour stimuli were injected into a stream of air under the control of a custom-made transistor–transistor logic-controlled electric valves (Cole-Parmer). hBACCS2 was photoactivated with an LED and a 438 ± 24-nm band filter (SPECTRA light engine, Lumenmicon). The timing of the light stimulation was controlled by a custom-made transistor–transistor logic signal controller.

Measurement of intracellular Ca2 + concentration. Transfected HEK293T cells were loaded with 2.5 μM Fura-2/AM for 30 min. Fluorescence was measured with an Aquacosmos Ca2 + imaging system and a CCD camera (ORCA-ER: Hamamatsu Photonics). Fura-2/AM was excited by a Xenon lamp (Lambda DG-4 PLUS; Sutter Instruments). For the measurement of the intracellular Ca2 + concentration after completion of the experiments, ionomycin Ca2 + salt (5 μM) and the Ca2 + chelator EGTA (10 mM) were added to chelate the Ca2 + in the chamber and obtain the minimal Fura-2 fluorescence. CaCl2 (10 mM) was then added to the imaging chamber to obtain the maximal Fura-2 fluorescence.

The intracellular free Ca2 + concentration was calculated from the Fura-2 fluorescence using the following equation:

\[
\text{Ca}^{2+} = K_d \times R / (R - R_m)/(R_m - R)
\]

where \(K_d\) is the dissociation constant of the calcium complex, \(B\) is the minimum/ maximum fluorescence ratio of Fura-2 observed at the reference wavelength of 380 nm, and \(R\) is the fluorescence ratio of 340/380 nm.

Statistical analysis. Statistical analyses were performed using Excel software (Microsoft) for cell counts (t-test) and HIT-T15 cell analyses (Pearson correlation coefficient), and StatsView (Hulinks) for EOG and whole-cell current analyses (t-test) and measurements of intracellular Ca2 + concentrations (one-way analysis of variance and post hoc Fisher’s Protected Least Significant Difference (PLSD) test). All tests were two sided.

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Author contributions

T.I. and T.N. conceived and designed the entire project; K.S. designed and performed the experiments to establish the CRACM1 method in Drosophila S2 cells; J. Gen. Physiol. 123, 167–182 (2004).

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by K.S.; K.T. discussed the results; T.I. performed all other experiments, analysed the data
and wrote the manuscript; T.N. edited the manuscript.

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Competing financial interests: T.I. and T.N. are inventors of the patent-pending BACCS
constructs (polypeptide, isolated nucleic acid, recombinant vector, gene transfer kit, trans-
formant and method for regulating intracellular calcium signalling; laid-open disclosure
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