Supplementary Information

Generation of ER Ca\textsuperscript{2+} indicator (split-YC7.3er)

We modified the GFP-based Ca\textsuperscript{2+} indicator (yellow cameleon) to monitor Ca\textsuperscript{2+} concentration within the ER. The yellow cameleon consists of enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) linked by calmodulin and the M13 peptide (a calmodulin-binding peptide) (Miyawaki et al., 1997). The indicator detects Ca\textsuperscript{2+} concentration, because the Ca\textsuperscript{2+}-dependent intramolecular binding of calmodulin and M13 increases the fluorescence resonance energy transfer (FRET) efficiency between ECFP and EYFP. We introduced three modifications (Supplementary Fig. 1A). First, a Gln69Met (Q69M) mutation was introduced to reduce the environment sensitivity of EYFP, which is called “Citrine” after the modification (Griesbeck et al., 2001). Second, we split the indicator into two parts, namely, ECFP-calmodulin and M13-Citrine, to reduce the background intramolecular FRET, and attached ER targeting signals to each part. Most importantly, the Ca\textsuperscript{2+} sensitivity of the indicator was altered. One of the previous ER-targeted cameleons (YC3er) had an apparent dissociation constant ($K'_{d}$) of 4.4 µM, while another (YC4er), in which one of the glutamate residues in the Ca\textsuperscript{2+} binding site of calmodulin was substituted by glutamine (E31Q), showed a biphasic response with $K'_{d}$ values of 83 nM and 700 µM (Miyawaki et al., 1997). We altered the mutation in the Ca\textsuperscript{2+}-binding site to a more conservative one (E31D) expecting to obtain an intermediate $K'_{d}$, which is more suitable for measuring the full range of changes in [Ca\textsuperscript{2+}]\textsubscript{er}. 

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We examined the properties of the modified indicator split-YC7.3er using recombinant proteins. Upon the addition of Ca$^{2+}$, the fluorescence spectrum with excitation at 434 nm was altered due to FRET between ECFP-calmodulin and M13-Citrine: the fluorescence intensity at 535 nm (F535) increased at the expense of F480 (Supplementary Fig. 1B). Measurement of F535/F480 at various Ca$^{2+}$ concentrations (Supplementary Fig. 1C) showed that split-YC7.3er has $K'_d$ of 130 µM (Hill coefficient = 1.4).
**Supplementary Figure 1.** Structure and property of split-YC7.3er. (A) Domain structure of split-YC7.3er. CaM; calmodulin, CRsig; calreticulin signal sequence, KDEL; ER retention signal; E31D, glutamate at residue 31 of CaM was substituted by aspartate; Citrine, EYFP after Q69M mutation. (B) Emission spectra (excitation at 434 nm) at zero (blue) and saturated Ca\(^{2+}\) (orange). (C) Ca\(^{2+}\) calibration curve at pH 7.4. The changes in emission ratio (F535/F480) were normalized by the ratio in the absence of Ca\(^{2+}\). Data are representative of three experiments.
**Supplementary Figure 2.** Localization of the CFP-CaM7-er (left) and M13-Citrine-er (middle) in HeLa cells. CFP-CaM7-er and M13-Citrine-er were monitored at 458 nm excitation, and 463-510 nm and 525-560 nm emissions, respectively, under a confocal microscope. A merged image is also shown (right). In each panel, the area within the white box was expanded. Upper scale bar, 8 µm. Lower scale bar, 4 µm. The expression of indicator had no noticeable effect on cell morphology or growth.
**Supplementary Figure 3.** Mitochondrial Ca$^{2+}$ indicator. Ca$^{2+}$ calibration curve of inverse-pericam2-mt at pH 8.0. The fluorescence intensity at 535 nm was normalized to that in the absence of Ca$^{2+}$. The continuous line shows a Hill plot with a $K'_d$ of 80 nM and Hill coefficient = 1.4. Data are representative of three experiments.
**Supplementary Figure 4.** Mitochondrial Ca^{2+} indicator. Localization of inverse-pericam2-mt (left) and the Mito-tracker (middle) in cells transduced with retrovirus encoding inverse-pericam2-mt. The two images were merged (right). In each, panel the area within the white box was expanded. Upper scale bar, 16 µm. Lower scale bar, 4 µm. The expression of inverse-pericam2-mt did not have a noticeable effect on cell morphology and growth.
**Supplementary Figure 5.** Absence of effect of CGP-37157 on histamine-induced intracellular IP$_3$ response. Cells were transduced with retrovirus encoding an IP$_3$ indicator GFP-PHD (Hirose et al., 1999). The intensity of cytoplasmic fluorescence of GFP-PHD was monitored at 500-555 nm with 488-nm excitation using a confocal microscope (TCS SP2 AOBS; Leica) equipped with a 63× objective (NA 0.9) at a rate of one frame per 2 s in the absence of extracellular Ca$^{2+}$. The size of peak responses to 10 μM histamine was compared in the absence ($n = 9$) and the presence ($n = 7$) of 10 μM CGP-37157. Mean ± SEM. The application of CGP-37157 was initiated 10 min before the measurements.
Supplementary Methods

**Construction of indicators.** pcDNA3-CFP-CaM4-er and pcDNA3-M13-EYFP V68L/Q69K-er were constructed by a PCR technique using primers 1 and 2 for the former, and primers 3 and 4 for the latter with pcDNA3-YC4.1er (gift from Dr. A. Miyawaki (RIKEN, Wako, Japan)) as the template. PCR products were self-ligated after phosphorylation by T4 polynucleotide kinase (TAKARA, Japan). The fragment encoding CFP-CaM4-er digested from pcDNA3-CFP-CaM4-er was cloned into the *Hin* dIII / *Eco* RI site of pBluescript SK(-) (Stratagene), yielding the pBluescript SK(-)-CFP-CaM4-er. The fragment encoding M13-EYFP V68L/Q69K-er digested from pcDNA3-M13-EYFP V68L/Q69K-er was cloned into the *Hin* dIII / *Eco* RI site of pBluescript SK(-), yielding pBluescript SK(-)-M13-EYFP V68L/Q69K-er.

The cDNA fragments encoding CFP-CaM7-er and M13-Citrine-er were generated using a primer containing the corresponding mutation (primers 5 and 6 for the former, and primers 7 and 8 for the latter) with pBluescript SK(-)-CFP-CaM4-er and pBluescript SK(-)-M13-EYFP V68L/Q69K-er as templates, respectively. The PCR products were self-ligated after phosphorylation by T4 polynucleotide kinase, yielding pBluescript SK(-)-CFP-CaM7-er and pBluescript SK(-)-M13-Citrine-er. For the construction of the *E. coli* expression vector, the cDNA fragment encoding CFP-CaM7-er or M13-Citrine-er digested from pBluescript SK(-)-CFP-CaM7-er or pBluescript SK(-)-M13-Citrine-er, respectively, was cloned into pET23a at the Nde I / Xho I site. For the construction of the retroviral vector, the fragments encoding CFP-CaM7-er and M13-Citrine-er with blunted Not I and *Eco* RI sites digested
from pBluescript SK(-)-CFP-CaM7-er or pBluescript SK(-)-M13-Citrine-er were cloned into
the blunted Bam HI and Eco RI site of pMX (a generous gift from Dr. Kitamura, The
University of Tokyo) (Onishi et al., 1996).

The cDNA encoding inverse-pericam2 was generated using primers 7 and 8 with
pcDNA3-inverse-pericam (gift from Dr. A. Miyawaki) as the template. The PCR product was
self-ligated after phosphorylation by T4 polynucleotide kinase, yielding pcDNA3-inverse-
pericam2. The fragment encoding inverse-pericam2 digested from pcDNA3-inverse-pericam2
was cloned into the Xho I / Xba I site of pCMV/myc/Mito (Invitrogen), yielding pCMV/myc-
inverse-pericam2-mt. For the construction of the E. coli expression and retrovirus vectors, the
fragment encoding inverse-pericam2-mt was cloned into pET19b at the Nde I / Eco RI site
and into pMX at the Bam HI / Eco RI site, respectively. All PCR products were confirmed by
sequencing. The sequences of all primers used in PCR amplification are listed below.

Primer 1: 5’-GGGATCATCCCTCGGCAAGGACGAGCTG-3’
Primer 2: 5’-CCCCTTCTGCTCATCATTTGTACAAACTC-3’
Primer 3: 5’-GGGCGGAAGAGCGCTGGAAGAAAAAC-3’
Primer 4: 5’-TCGGCGGCACGCCCAGCAGGCAGCCAG-3’
Primer 5: 5’-GGAAGCTTTAGATCTTTGCAACCGTTATGAGGTCG-3’
Primer 6: 5’-CCGAATTTCAGATCTTTGTGTTGATGGTGGCCTGC-3’
Primer 7: 5’-CCCAGATATCCCGACCACATGAAGGACGC-3’
Primer 8: 5’-GGGATATCGGCGAAGCACATCAGGCGGAGCTGAGGGTG-3’
**Bacterial transformation and protein purification.** The *E. coli* BL21-Codon Plus (DE3)-RIL strain (Stratagene) was transformed with plasmids harboring the expression construct and selected on ampicillin (50 μg/ml)-containing LB plates. The selected clones were cultured in 100 mL of 2×YT medium containing ampicillin. Cultures were incubated at 37°C with shaking. After the optical density at 600 nm reached 0.6, at which point the bacterial cells were induced with 0.4 mM IPTG, the cell culture was further incubated for 12 h at 16°C. The cells were then harvested by centrifugation and the cell pellet was stored at -80°C.

The frozen cell pellet was thawed on ice and suspended in an extraction buffer (50 mM Na₂HPO₄ and 300 mM NaCl, pH 7.5). The cell suspension was lysed with a French press (Aminco). The supernatant collected after the centrifugation of the cell homogenate at 20,400 g for 30 min was suspended in the extraction buffer with TALON metal affinity resin (Clontech). After a 30-min incubation of the suspension at 4°C, the resin was washed with an extraction buffer, and proteins were then eluted with an imidazole-containing elution buffer (50 mM Na₂HPO₄, 300 mM NaCl and 300 mM imidazole; pH 7.5).

**Generation of retrovirus encoding the indicators.** Vesicular somatititis virus G glycoprotein (VSV-G) pseudotyped retroviruses encoding indicators (i.e., CFP-CaM7-er, M13-Citrine-er, and inverse-pericam2-mt) were generated as described previously (Miyakawa et al., 2001). A pantropic packaging cell line, GP293, (Clontech) was cotransfected with 12 μg of indicator plasmid (pMX-CFP-CaM7-er, pMX-M13-Citrine-er, or pMX-inverse-pericam2-mt) and 1 μg of pVSV-G (Clontech) using LipofectAmine 2000 (Invitrogen). Two days later, retroviral
particles were collected from the medium and used to infect HeLa cells after concentration by centrifugation (6,000 g for 16 h). For the production of HeLa cells expressing split-YC7.3er, cells were first infected with the retrovirus encoding CFP-CaM7-er. One week later, the infected cells were additionally infected with the retrovirus encoding M13-Citrine-er. Cell clones emitting both CFP and Citrine fluorescence were isolated.

**Ca^{2+} calibrations.** The fluorescence intensity of the recombinant split-YC7.3er (CFP-CaM7-er and M13-Citrine-er) was measured in a solution containing 130 mM KCl and 50 mM MOPS (pH 7.0), using a fluorescence spectrometer (FP-6500; JASCO). Small aliquots of CaCl\(_2\) were added to the solution, and a fluorescence emission spectrum was taken after each addition. Inverse-pericam2-nt was calibrated in a solution containing 130 mM KCl and 50 mM Hepes (pH 8.0). Ca\(^{2+}\) concentration was buffered by adding appropriate concentrations of Ca\(^{2+}\)-free and Ca\(^{2+}\)-saturated BAPTA assuming a \(K_d\) of 0.1 \(\mu\)M for Ca\(^{2+}\) (total BAPTA concentration, 10 mM).

**Cell culture and expression.** HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml). HeLa cells were infected with retroviruses encoding the indicator proteins.

**Assessment of spectral cross-talk.** The extent of spectral cross-talk (bleed-through) contamination resulting from the excitation spectral overlap between CFP-CaM7-er and indo-
5F was assessed using cells expressing CFP-CaM7-er and wild type cells loaded with indo-5F. The intensity of CFP-CaM7-er signal at 346-nm excitation was typically 8.5% that of indo-5F. On the other hand, the intensity of the indo-5F signal at 434-nm excitation was 3.1% that of the CFP-CaM7-er signal. Similarly, the extent of the spectral bleed-through between inverse-pericam-2-mt and fura-2 was assessed using cells expressing inverse-pericam-2-mt and wild type cells loaded with fura-2. The intensity of the inverse-pericam-2-mt signal at 380-nm excitation was typically 1.4% that of fura-2. On the other hand, the intensity of the fura-2 signal at 490-nm excitation was 2.4% that of the inverse-pericam-2-mt signal. These magnitudes of bleed-through did not have noticeable effects on the measurements.

Subcellular localization of the GFP-based indicators. Cells expressing split-YC7.3er were observed at 458 nm excitation under a confocal microscope (TCS SP2 AOBS; Leica). Localization of the CFP-CaM7-er and M13-Citrine-er were monitored at 463-510 nm and 525-560 nm emissions, respectively. Cells expressing the inverse-pericam2-mt were incubated for 30 min at 37°C in the dark in PSS containing 200 nM MitoRed (Dojindo, Japan) and 0.1% BSA. Inverse-pericam2-mt and MitoRed were monitored at 488 nm and 543 excitations and 500-580 nm and 600-700 nm emissions, respectively, under the confocal microscope.

Averaging of data from different experiments after normalization. The time courses of $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{er}$ (or $[Ca^{2+}]_{mt}$) during the first $Ca^{2+}$ oscillation in different experiments were averaged after normalization. The time course was first normalized by the interval
between the onset of the first Ca\textsuperscript{2+} oscillation and the bottom of [Ca\textsuperscript{2+}]\text{cyt} after the peak. The amplitude of signals was then normalized by the magnitude of the peak of the first Ca\textsuperscript{2+} oscillation. The time courses of [Ca\textsuperscript{2+}]\text{cyt} and [Ca\textsuperscript{2+}]\text{er} (or [Ca\textsuperscript{2+}]\text{mit}) during the second and third oscillations in different experiments were averaged after the following normalization. The time course was first normalized by the interval between the midpoints of the rising phase of the second and third Ca\textsuperscript{2+} oscillations. The amplitude of signals was then normalized by the magnitude of difference between the bottom and the peak of the third Ca\textsuperscript{2+} oscillation.
References


Miyakawa, T., Mizushima, A., Hirose, K., Yamazawa, T., Bezprozvanny, I., Kurosaki, T. and Iino, M. (2001) Ca\textsuperscript{2+}-sensor region of IP\textsubscript{3} receptor controls intracellular Ca\textsuperscript{2+} signaling. EMBO J, 20, 1674-1680.