

A FRET Biosensor for ROCK Based on a Consensus Substrate Sequence Identified by KISS Technology

Chunjie Li¹, Ayako Imanishi¹, Naoki Komatsu¹, Kenta Terai², Mutsuki Amano³, Kozo Kaibuchi³, and Michiyuki Matsuda^{1*}

¹Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan, ²Imaging Platform for Spatio-Temporal Information, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan, ³Department of Cell Pharmacology, Graduate School of Medicine, Nagoya University, Showa-ku, Nagoya, Aichi 466-8550, Japan

ABSTRACT. Genetically-encoded biosensors based on Förster/fluorescence resonance energy transfer (FRET) are versatile tools for studying the spatio-temporal regulation of signaling molecules within not only the cells but also tissues. Perhaps the hardest task in the development of a FRET biosensor for protein kinases is to identify the kinase-specific substrate peptide to be used in the FRET biosensor. To solve this problem, we took advantage of kinase-interacting substrate screening (KISS) technology, which deduces a consensus substrate sequence for the protein kinase of interest. Here, we show that a consensus substrate sequence for ROCK identified by KISS yielded a FRET biosensor for ROCK, named Eevee-ROCK, with high sensitivity and specificity. By treating HeLa cells with inhibitors or siRNAs against ROCK, we show that a substantial part of the basal FRET signal of Eevee-ROCK was derived from the activities of ROCK1 and ROCK2. Eevee-ROCK readily detected ROCK activation by epidermal growth factor, lysophosphatidic acid, and serum. When cells stably-expressing Eevee-ROCK were time-lapse imaged for three days, ROCK activity was found to increase after the completion of cytokinesis, concomitant with the spreading of cells. Eevee-ROCK also revealed a gradual increase in ROCK activity during apoptosis. Thus, Eevee-ROCK, which was developed from a substrate sequence predicted by the KISS technology, will pave the way to a better understanding of the function of ROCK in a physiological context.

Key words: ROCK, FRET, cytokinesis, apoptosis

Introduction

Genetically-encoded biosensors based on Förster/fluorescence resonance energy transfer (FRET) have been widely used to visualize spatio-temporal changes of signaling molecule activities, small molecule concentrations, and physical properties (Oldach and Zhang, 2014; Miyawaki and Niino, 2015). Recent advances in the techniques for the stable expression (Aoki *et al.*, 2012) and generation of transgenic mice (Kamioka *et al.*, 2012; Johnsson *et al.*, 2014)

are further widening the application of the FRET biosensors. However, even with an optimized backbone for the FRET biosensor (Komatsu *et al.*, 2011), the development of a FRET biosensor for protein kinases is still a time-consuming pursuit, because many of the peptide sequences known to be phosphorylated by a given kinase do not reliably work in the context of a FRET biosensor.

The small GTPase RhoA controls cell adhesion and motility via actin-cytoskeleton reorganization and actomyosin contraction (Etienne-Manneville and Hall, 2002; Fukata *et al.*, 2003). Rho-associated protein kinase (ROCK) was isolated as a target protein of RhoA (Leung *et al.*, 1995; Ishizaki *et al.*, 1996; Matsui *et al.*, 1996), and has been shown to play pivotal roles in actomyosin contraction and the induction of actomyosin bundles (Narumiya *et al.*, 2009). ROCK has been shown to induce actomyosin contraction via phosphorylation of the myosin-binding subunit of myosin phosphatase or myosin light chain (Amano *et al.*, 1996; Kimura *et al.*, 1996; Uehata *et al.*, 1997). ROCK has

*To whom correspondence should be addressed: Michiyuki Matsuda, Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan.

Tel: +81-75-753-4421, Fax: +81-75-753-4655

E-mail: matsuda.michiyuki.2c@kyoto-u.ac.jp

Abbreviations: FRET, Förster/fluorescence resonance energy transfer; ROCK, Rho-associated protein kinase; KISS, kinase-interacting substrate screening; FKBP, FK506-binding protein; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; siRNA, small interfering RNA; DH, Dbl homology; FRB, FK506-rapamycin-binding.

two isoforms, ROCK1, also called ROCK I, ROK β , rho-kinase β , or p160ROCK, and ROCK2, also called ROCK II, ROK α , or Rho kinase (Riento and Ridley, 2003). The two ROCK homologs share 64% identity in their primary amino acid sequences, with the highest homology (92%) being within the kinase domains. Tissue-specific expressions of ROCK1 and ROCK2 were reported previously (Schmandke *et al.*, 2007; Iizuka *et al.*, 2012), but later analysis of ROCK1 and ROCK2 expressed sequence tag distribution revealed that the distribution patterns of ROCK1 and ROCK2 are similar and that there are few specific organs and/or tissues with dramatically higher expression levels than the others (Julian and Olson, 2014).

The ROCK-dependent actomyosin contraction is typically manifested in two biological phenomena: cytokinesis and blebbing in the apoptotic cells (Coleman and Olson, 2002; Amano *et al.*, 2010; Ohgushi *et al.*, 2010; Thumkeo *et al.*, 2013). ROCK phosphorylates intermediate filaments beneath the cleavage furrow and thereby promotes cytokinesis (Kosako *et al.*, 1997; Goto *et al.*, 1998). During apoptosis, caspases cleave and activate ROCK1 and induce blebbing (Coleman *et al.*, 2001; Sebbagh *et al.*, 2001, 2005). However, the precise timing of ROCK activation has not been reported due to technical difficulty of its measurements.

Recently, Amano *et al.* reported a technique named kinase-interacting substrate screening (KISS) to identify the consensus sequence of protein kinases (Amano *et al.*, 2015). Because this method deduces the consensus sequence from a huge number of phospho-peptides identified by liquid chromatography tandem mass spectrometry, the peptide sequence may be used to develop FRET biosensors for protein kinases with high sensitivity. Here, we show that the consensus substrate sequence for ROCK identified by KISS can be used to develop a sensitive FRET biosensor for ROCK.

Experimental Procedures

Reagents

Blasticidin S was purchased from InvivoGen (San Diego, CA). EGF and cycloheximide were purchased from Sigma-

Aldrich (St. Louis, MO). Rapamycin was obtained from LC Laboratories (Woburn, MA). Y27632 dihydrochloride was purchased from Cosmo Bio (Tokyo, Japan). GSK429286 and HA1077 were purchased from Tocris Bioscience (Bristol, UK) and Tokyo Chemical Industry (Tokyo, Japan), respectively. TNF- α was purchased from Toyobo Co., Ltd. (Osaka, Japan). Phorbol 12-Myristate 13-Acetate (PMA), Forskolin, and 3-isobutyl-1-methylxanthine (IBMX) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

FRET biosensors and plasmids

FRET biosensors were constructed as described previously using the optimized Eevee backbone (Komatsu *et al.*, 2011). From the N-terminus, the Eevee backbone consists of YPet, a spacer (Leu-Glu), the FHA1 domain of yeast Rad53, a spacer (Gly-Thr), the EV Linker, a spacer (Ser-Gly), a substrate peptide sequence, a spacer (Gly-Gly-Arg), enhanced CFP, a spacer (Ser-Arg), and a subcellular localization signal. The substrate peptide sequences are summarized in Table I. Eevee-ROCK-HRasCT, Eevee-ROCK-KRasCT, Eevee-ROCK-Lyn, Eevee-ROCK-Mito, and Eevee-ROCK-NLS were developed based on Eevee-iAkt-pm (HRas), Eevee-iAkt-pm (KRas), Eevee-iAkt-pm (lyn), Eevee-iAkt-mito, and Eevee-iAkt-nls (Miura *et al.*, 2014). We used pCAGGS (Niwa *et al.*, 1991) and pPBbsr (Yusa *et al.*, 2009) for transient and stable expressions, respectively. Plasmids for RhoA and Raichu-RhoA, the FRET biosensor for RhoA, were reported previously (Yoshizaki *et al.*, 2003; Kurokawa and Matsuda, 2005).

Cell culture and transfection

HeLa cells were purchased from the Human Science Research Resources Bank (Sennanashi, Japan) and maintained in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS) at 37°C, 5% CO₂. HeLa cells were transfected with the plasmids by using 293fectin (Invitrogen, San Diego, CA) according to the manufacturers' instructions. For stable expression, the pPBbsr-based expression vector and pCMV-mPBase (neo-) encoding the piggyBac transposase (Yusa *et al.*, 2009) were cotransfec-

Table I. ROCK PHOSPHORYLATION SITES USED FOR FRET BIOSENSORS

Plasmids	Target proteins	Peptide sequences*	Ref
3654NES	Protein phosphatase 1 regulatory subunit 12A (T696)	ARQSRSSpTQG[V/D]TLTD	(Wooldridge <i>et al.</i> , 2004)
3655NES	ARHGAP35/p190A RhoGAP (S1150)	LERGRKV[pT]IV[S/D]KPVL	(Mori <i>et al.</i> , 2009)
3656NES	Ezrin (T567)	QGRDKYKpTLR[Q/D]IRQG	(Tran Quang <i>et al.</i> , 2000)
4146NES	CONSENSUS	KRRNRKpTLV[L/D]LPLD	(Amano <i>et al.</i> , 2015)
4149NES	TA mutant	KRRNRK[T/A]LV[L/D]LPLD	

*The letter p indicates a phospho-amino acid. Amino acid substitutions are shown in parenthesis. Aspartate (D) and threonine (T) are introduced to meet the consensus sequence of the FHA1 domain. The T/A substitution in 4149NES is for the negative control.

ted and selected with 20 $\mu\text{g}/\text{ml}$ blasticidin S for at least 10 days. The rapamycin-inducible Akt or RhoA activation system was constructed as described previously (Suh *et al.*, 2006; Miura *et al.*, 2014). Human ROCK1 cDNA was cloned from human cDNA library. Human ROCK2 cDNA (KIAA0619) was kindly provided from Kazusa DNA Research Institute (Chiba, Japan). ROCK1 cDNA and ROCK2 cDNA were subcloned into pEF-BOS-GST and pEGFP-c1 vectors, respectively. pEF-BOS-GST-bRho-kinase and pEGFP-c1-bRho-kinase are expression vectors for bovine ROCK2/Rho kinase tagged with GST and GFP, respectively (Amano *et al.*, 2015). pCX4bsr-3HA-FKBP-p63RhoGEF-DH encodes FKBP-p63RhoGEF-DH, which was comprised of the FK506-binding protein (FKBP) fused to the Dbl homology (DH) of the guanine nucleotide exchange factor p63RhoGEF as described previously (van Unen *et al.*, 2015). pCX4puro-LDR encodes LDR, which was comprised of the FK506-rapamycin-binding (FRB) domain of mTOR fused to the myristoylation signal of Lyn (Suh *et al.*, 2006). Lentiviruses were prepared from the pCX4-derived plasmids and used to infect HeLa cells as described previously (Akagi *et al.*, 2003), generating HeLa-LDR/3HA-FKBP-p63RhoGEF-DH cells. After transfection of an expression plasmid for a FRET biosensor, cells were stimulated with 50 nM rapamycin to translocate p63RhoGEF to the plasma membrane.

Time-lapse FRET imaging

FRET imaging was performed as previously reported (Aoki and Matsuda, 2009). Cells were plated on 35-mm glass base dishes, transfected with plasmids, incubated for 24 h, and serum-starved for 3–6 h in phenol red-free M199 medium (Invitrogen, Carlsbad, CA) containing 20 mM HEPES (Invitrogen, Carlsbad, CA) and 0.1% BSA before imaging. Cells were imaged with an IX83 inverted microscope (Olympus, Tokyo, Japan) equipped with a PlanApo 60 $\times$ /1.40 oil objective lens, a Cool SNAP K4 CCD camera (Roper Scientific, Tucson, AZ), a CoolLED precisExcite LED illumination system (Molecular Devices, Sunnyvale, CA), an IX2-ZDC laser-based autofocus system (Olympus), and an MD-XY30100T-Meta automatically programmable XY stage (SIGMA KOKI, Tokyo, Japan). The following filters used for the dual-emission imaging studies were obtained from Omega Optical (Brattleboro, VT): an XF1071 440AF21 excitation filter, an XF2034 455DRLP dichroic mirror, and an XF3075 480AF30 emission filter for CFP, and an XF3079 535AF26 emission filter for FRET. For the imaging of cytokinesis, an LCV110 inverted microscope (Olympus, Tokyo, Japan) was used. After background subtraction, FRET/CFP ratio images were created with Meta-Morph software (Universal Imaging, West Chester, PA), and represented in the intensity-modulated display mode. In the intensity-modulated display mode, eight colors from red to blue are used to represent the FRET/CFP

ratio, with the intensity of each color indicating the mean intensity of FRET and CFP. For the quantification, the FRET and CFP intensities were averaged over the whole cell area, and the results were exported to Excel software (Microsoft Corporation, Redmond, WA). The FRET/CFP values before stimulation were averaged and used as a reference. The ratio of the raw FRET/CFP value to the reference value was defined as the normalized FRET/CFP value.

Small interfering RNA transfection

HeLa cells were transfected with small interfering RNA (siRNA) by Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The control siRNA was purchased from Dharmacon (Lafayette, CO). siRNA sequences of ROCK1 and ROCK2 were obtained from previous reports (Vega *et al.*, 2011; Iizuka *et al.*, 2012).

Western blotting

Cells were lysed in SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 12% glycerol, 2% SDS, 0.004% bromophenol blue and 5% 2-mercaptoethanol). The samples were separated by SDS-PAGE on SuperSep Ace 5–20% or SuperSep Ace 5–20% pre-cast gels (Wako Pure Chemical), and transferred to PVDF membranes (Merck Millipore, Darmstadt, Germany). After 60-min incubation with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) at room temperature, the membranes were incubated for overnight at 4°C with rabbit monoclonal anti-ERK1/2 antibody (1:1,000) (No. 9101; Cell Signaling Technology, Danvers, MA), rabbit monoclonal anti-ROCK1 antibody (1:1,000) (ab45171; Abcam, Cambridge, MA), rabbit monoclonal anti-ROCK2 antibody (1:1,000) (ab125025; Abcam), mouse Anti-ERK antibody (1:1,000) (No. 610123; BD biosciences, San Jose, CA) or mouse monoclonal anti-GFP antibody (1:8,000) (No. 632381, Clontech Laboratories, Inc., Mountain View, CA). The immunoreactivities were visualized with IRDye 800CW-conjugated donkey anti-mouse IgG antibodies (1:15,000; LI-COR) and IRDye 680LT-conjugated goat anti-rabbit IgG antibodies (1:15,000; LI-COR). All antibodies were diluted in Odyssey blocking buffer. Proteins were detected by an Odyssey Infrared Imaging System (LI-COR) and analyzed by using the Odyssey imaging software.

Results

Eevee-ROCK based on the consensus sequence identified by KISS technology

The FRET biosensor was based on the optimized Eevee

backbone (Komatsu *et al.*, 2011), which was comprised of, from the amino-terminus, YPet yellow fluorescent protein, the phospho-serine/threonine-binding FHA1 domain, the long flexible EV-linker, a substrate peptide for ROCK, and enhanced cyan fluorescent protein (CFP) (Fig. 1A). We added a nuclear export signal at the C-terminus unless specified otherwise. The resulting FRET biosensor is expected to increase its FRET signal upon phosphorylation by ROCK (Fig. 1A). As the substrate for ROCK, we first used the previously reported ROCK substrate sequences derived from protein phosphatase 1 regulatory subunit 12A, ARHGAP35/p190A RhoGAP (S1150), and ezrin (T567) (Table I). In the original ROCK substrate consensus sequence, the threonine residue at the +3 position from the phosphothreonine was modified to aspartate to increase the affinity to the FHA1 domain. These FRET biosensors were expressed in HeLa cells and time-lapse imaged to examine the response to EGF and a ROCK inhibitor, Y27632. However, none of the developed FRET biosensors, 3654NES, 3655NES, or 3656NES, responded significantly to either EGF or ROCK inhibitor Y27632 (Fig. 1B). Next, we used the consensus substrate sequence of ROCK, which was identified by the KISS technology (Table I). In HeLa cells expressing the resulting FRET biosensor 4146NES, the FRET ratio was significantly increased upon EGF stimulation and decreased by Y27632 (Fig. 1B, Movie 1). The increase in the FRET ratio was caused by the increase in YFP intensity and decrease of CFP intensity as anticipated (Fig. 1C). Similar responses were observed by stimulators of RhoA, serum and lysophosphatidic acid (Fig. 1D). We further investigated the specificity of 4146NES by another ROCK inhibitor, GSK429286, and obtained essentially the same results as by Y27632 (Fig. 1E). Because all of the stimulators used here also activate ERK MAP kinase, we excluded the involvement of ERK by pretreatment with the MEK inhibitor U0126 (Fig. 1F). As expected, U0126 pretreatment did not affect EGF-induced activation or Y27962-induced suppression of the ROCK activity. Finally, the requirement of phosphorylation was confirmed by using 4149NES, in which the threonine at the phosphorylation site was substituted for alanine. The mutant did not respond to either EGF or Y27632 (Fig. 1B). Taken together, these results showed that 4146NES could be used as a ROCK biosensor and, therefore, we renamed 4146NES as Eevee-ROCK.

Specificity of Eevee-ROCK

To further confirm that Eevee-ROCK monitored ROCK activation, we applied the rapamycin-inducible RhoA activation system. This system employs two fusion proteins, FKBP-p63RhoGEF-DH and LDR. Stimulation with rapamycin leads to rapid translocation of FKBP-p63RhoGEF-DH to the membrane-anchored LDR via heterodimerization of FKBP and FRB (Fig. 2A). The

translocation of p63RhoGEF is expected to activate RhoA, and thereby ROCK. Indeed, the FRET/CFP ratio of Eevee-ROCK rapidly increased upon stimulation with rapamycin (Fig. 2B, C). The activation could be cancelled by Y27632 as expected. Thus, Eevee-ROCK could faithfully monitor the RhoA activation induced by a Rho GEF.

ROCK is a member of AGC-family kinases, which includes protein kinase A (PKA), protein kinase C (PKC), and Akt. Therefore, we examined the response of Eevee-ROCK against stimulators specific to these kinases in the presence of Y27632 (Fig. 2D). As expected from the results of Fig. 1, EGF did not increase the FRET/CFP ratio of Eevee-ROCK. Unexpectedly, we observed slight response to forskolin/IBMX, which increases cytoplasmic cAMP level, suggesting that Eevee-ROCK could be phosphorylated by PKA also. PMA, a potent stimulator of PKC did not increase in the FRET/CFP ratio of Eevee-ROCK. To stimulate Akt, we expressed FKBP-p85 subunit of PI-3K and LDR. Rapamycin-induced Akt activation failed to increase the FRET/CFP ratio of Eevee-ROCK. Thus, Eevee-ROCK can be phosphorylated by PKA to some extent, but not by PKC or Akt.

Effect of siRNA-mediated silencing of ROCK expression and RhoA mutants on the FRET ratio of Eevee-ROCK

To confirm the specificity of Eevee-Rock, we knocked down ROCK1 and ROCK2 by small interfering RNA (siRNA). The lysates were analyzed by immunoblotting with anti-ROCK1, anti-ROCK2, and anti-ERK antibodies. The specificity of the anti-ROCK antibodies was examined by the reactivity to recombinant ROCK1 and ROCK2 (Supplementary Fig. 1). The anti-ROCK1 antibody used in this study cross-reacted with ROCK2/Rho kinase; whereas the anti-ROCK2 antibody detected only ROCK2. In agreement with this specificity, the mixture of two siRNAs against ROCK1 partially decreased the band detected by anti-ROCK1 antibody (Fig. 3A). Meanwhile, the mixture of two siRNAs against ROCK2 decreased not only the band detected by anti-ROCK2 antibody but also the band detected by ROCK1. The mixtures of all four siRNAs almost completely diminished the bands detected by anti-ROCK1 antibody and anti-ROCK2 antibody. Therefore, we concluded that both siRNA mixtures against ROCK1 and ROCK2 worked efficiently. Under this condition, knockdown of ROCK1 did not affect the FRET ratio, whereas knockdown of ROCK2 decreased the FRET ratio modestly. Knockdown of both ROCK1 and ROCK2 markedly decreased the FRET ratio, indicating that both ROCK1 and ROCK2 contributed to the basal FRET signal in HeLa cells (Fig. 3B).

To further confirm the contribution of RhoA to the basal FRET signal of Eevee-ROCK, the dominant negative RhoA (RhoA-T19N) and the constitutively activated RhoA (RhoA-F30L) were expressed in the Eevee-ROCK-

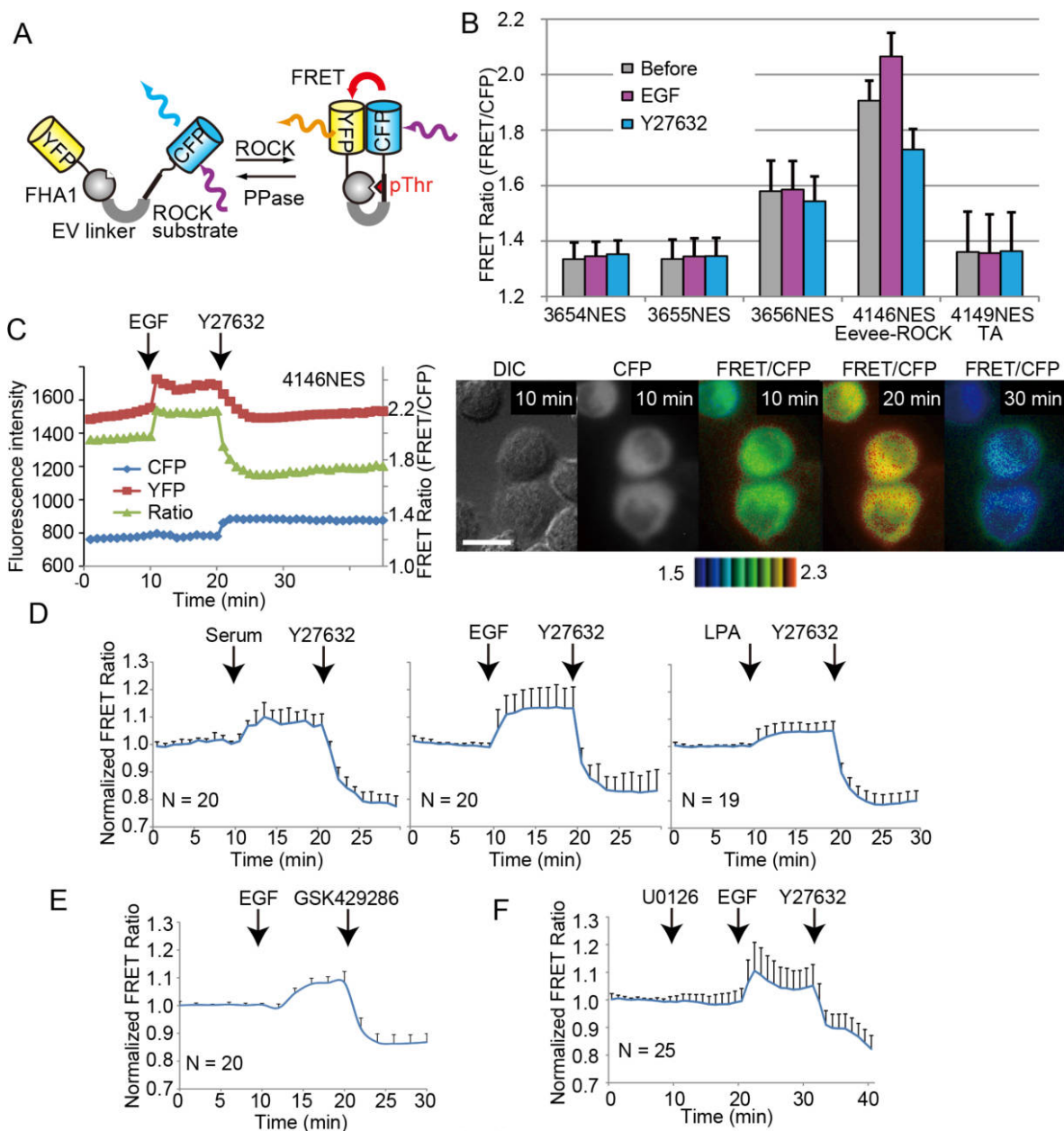


Fig. 1. Development of a FRET biosensor for ROCK, 4146NES/Evee-ROCK. (A) Mode of action of an intramolecular FRET biosensor for ROCK. Phosphorylation of the ROCK substrate peptide induces binding of the FHA1 domain to the substrate peptide, resulting in a conformational change and concomitant increase in FRET efficiency from the FRET donor CFP to the acceptor YFP. (B) HeLa cells expressing the FRET biosensor indicated at the bottom were serum-starved for more than 3 h and time-lapse imaged with an inverted epifluorescence microscope. Cells were stimulated with 10 ng/ml EGF at 10 min after the start of imaging, and then treated with 30 μM Y27632, a ROCK inhibitor, at 20 min after the start of imaging. Images of DIC, CFP, and FRET (YFP fluorescence excited at 440 nm) were acquired at 1-min intervals. In each cell (N=24), the average FRET ratios from 0 to 10 min, 12 to 20 min, and 22 to 30 min were used to represent values before stimulation and after EGF and Y27632 stimulation. (C) Shown are actual time courses of CFP, FRET, and FRET/CFP values and representative images for 4146NES/Evee-ROCK. FRET images are shown in the intensity-modulated display mode with the ratio range on the bottom. Scale bar, 20 μm. (D) Similar experiments were performed with 5% serum, 10 ng/ml EGF, or 10 μM LPA. The FRET/CFP ratio was normalized by the averaged FRET/CFP value before stimulation. (E) HeLa cells expressing 4146NES/Evee-ROCK were stimulated with 10 ng/ml EGF and treated with 10 μM GSK429286, another ROCK inhibitor. (F) HeLa cells expressing 4146NES/Evee-ROCK were treated with the MEK inhibitor U0126 (10 μM) at 10 min, EGF (10 ng/ml) at 20 min, and Y27632 (30 μM) at 30 min after the start of imaging. Bars are the S.D. The numbers in the panels indicate the numbers of analyzed cells.

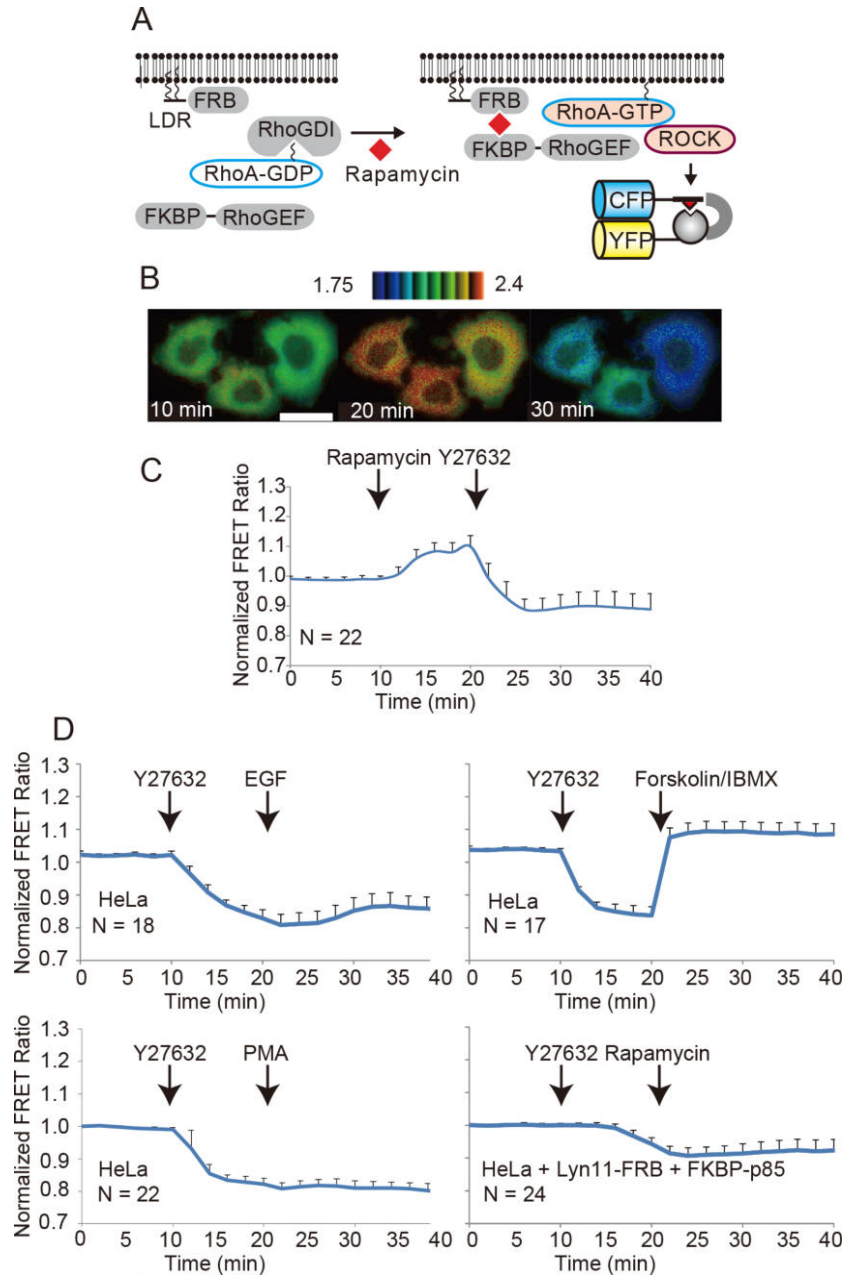
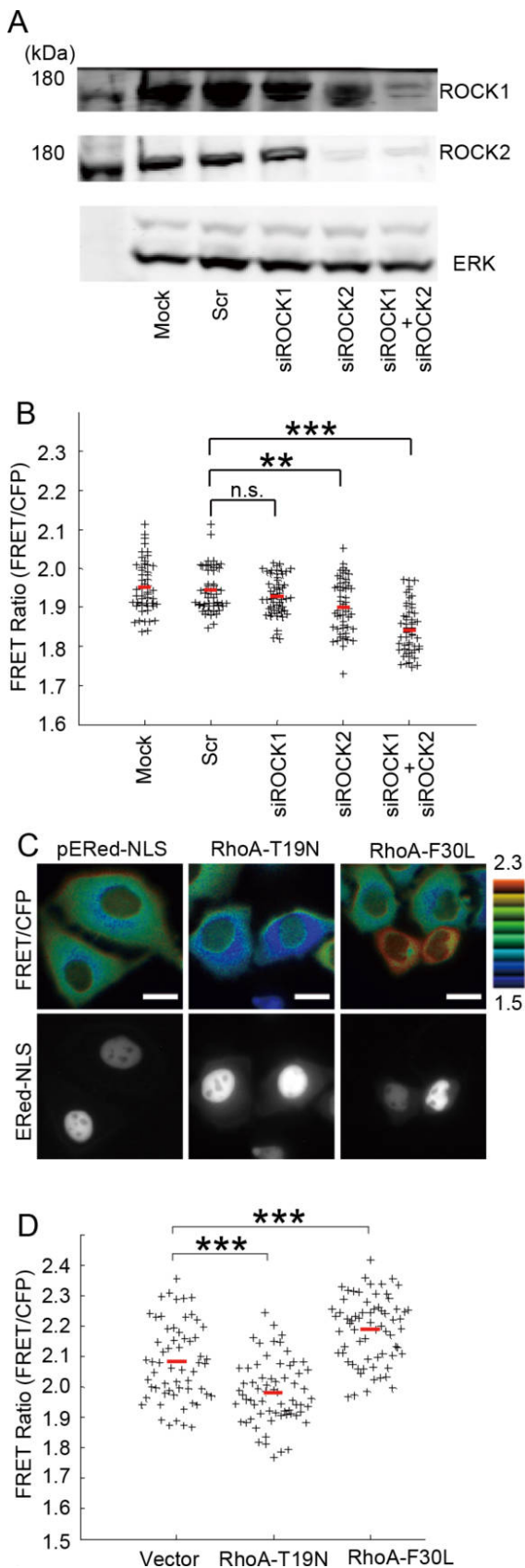


Fig. 2. Specificity of Eevee-ROCK. (A) Schematic representation of the rapamycin-inducible ROCK activation. (B, C) HeLa cells expressing 4146NES/Eevee-ROCK and the two fusion proteins LDR and FKBP-p63RhoGEF were serum-starved for at least 3 h, incubated with 50 nM rapamycin for 10 min, and then treated with 30 μ M Y27632. Representative images of FRET/CFP (B) and the mean normalized FRET/CFP ratios are shown with the S.D. Scale bar, 20 μ m. (D) HeLa cells expressing 4146NES/Eevee-ROCK with or without LDR and FKBP-p85 were serum-starved for more than 3 h and time-lapse imaged. Cells were pretreated with 30 μ M Y2763 at 10 min, and stimulated with 10 ng/ml EGF, 1 μ M Forskolin/50 μ M IBMX, 1 μ M PMA, or 50 nM rapamycin at 20 min after the start of imaging.

expressing HeLa cells. The expression vector carries an ERed fluorescent protein tagged to a nuclear localization signal as a marker (Fig. 3C). The FRET ratio was decreased by RhoA-T19N and increased by RhoA-F30L, indicating that the FRET ratio of Eevee-ROCK was correlated with RhoA activity, supporting the notion that Eevee-ROCK

served as the ROCK biosensor (Fig. 3D). Note that cells that did not express ERed-NLS in the RhoA-T19N-transfected or RhoA-F30L-transfected dish exhibited FRET/CFP ratios similar to cells transfected with empty vector.



Effect of subcellular localization on ROCK activity

For the analysis of subcellular ROCK activity, various localization sequences were added at the C-termini as reported for Akt biosensors (Miura *et al.*, 2014). Plasma membrane recruitment of the ROCK biosensor by the farnesylation signal of HRas or KRas attenuated the serum-induced increase in FRET ratio (Fig. 4). Moreover, we found that Y27632 did not decrease the FRET ratio any further than the basal level, indicating that the basal phosphorylation level was markedly suppressed when the biosensor was placed on the plasma membrane. Interestingly, plasma membrane targeting by the myristoylation signal of Lyn did not significantly perturb the serum-induced increase in FRET ratio. When Eevee-ROCK was localized to the mitochondria or nucleus, both serum-induced activation and Y27632-mediated inhibition were markedly attenuated. Taken together, these results showed that the localization of the FRET biosensor markedly affected its response, strongly suggesting that the regulation of ROCK may be subject to the subcellular localization.

ROCK activation at early G1 phase

Next, we tested whether the Eevee-ROCK biosensor could monitor the activity change of ROCK under the conditions where ROCK is known to be activated—i.e., cytokinesis (Fig. 5A, B, Movie 2). The FRET/CFP ratio was increased rapidly upon entering into M phase and decreased at the completion of cytokinesis (arrows in Fig. 5A, B). Interestingly, the FRET/CFP ratio rapidly increased again when the cells began to spread and adhere to the dish. After entering the G1 phase, the FRET/CFP ratio gradually decreased until M phase. To examine the contribution of ROCK to the increase in FRET/CFP ratio, we performed similar experiments in the presence of three ROCK inhibitors, Y27632, GSK429286, and HA1077 (Fig. 5B–D). All three inhibitors markedly decreased the FRET/CFP ratio during interphase.

Fig. 3. Effect of ROCK1 and/or ROCK2 depletion and expression of RhoA mutants on Eevee-ROCK. (A, B) HeLa cells expressing Eevee-ROCK were mock-transfected or transfected with scramble siRNA (scr) or siRNAs against ROCK1 and/or ROCK2. Cells were analyzed by immunoblotting with antibodies against ROCK1, ROCK2, and ERK (A). In parallel, fluorescence images were acquired as in Fig. 1. The FRET ratio (FRET/CFP) was calculated for 52 cells under each condition, and the resulting ratios are shown in a bee swarm plot (B). Red bars represent the averages. Results of unpaired Student's *t*-tests are shown. n.s., not significant ($p > 0.05$); ** $p < 0.01$; *** $p < 0.001$. (C, D) HeLa cells were transfected with the empty vector pERed-NLS, the expression plasmid for the dominant negative mutant pERed-NLS-RhoA-T19N, or the expression plasmid for the constitutively active mutant pERed-NLS-RhoA-F30L. Fluorescence images were acquired and FRET/CFP values were calculated. Shown in (C) are representative images of the FRET/CFP ratio and the nuclear ERRed fluorescent protein, which was used to identify transfected cells. The obtained bee swarm plot is shown in (D). Scale bars, 20 μ m.

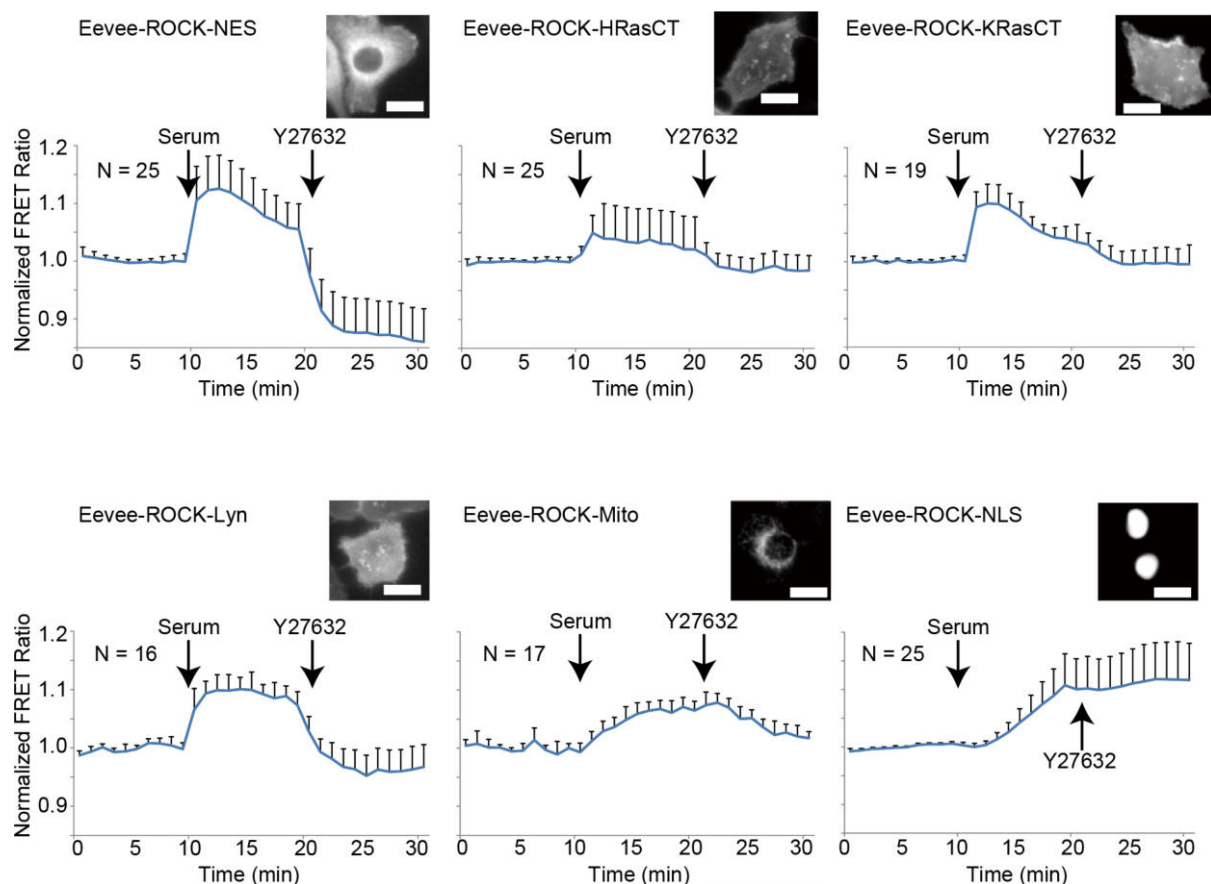


Fig. 4. Subcellular ROCK activity in HeLa cells. Eevee-ROCK biosensors with subcellular localization signals were transfected into HeLa cells, which were serum-starved for more than 3 h, stimulated with 5% serum at 10 min, and then treated with 30 μ M Y27632 at 20 min after the start of imaging. Images of CFP and FRET were acquired at 1-min intervals with an inverted fluorescence microscope equipped with a CCD camera. Shown in the insets are representative CFP images. The FRET/CFP ratio was normalized by the averaged FRET/CFP value before stimulation. The normalized FRET/CFP ratios and S.D. are shown. The numbers in the panels are the numbers of analyzed cells. Scale bars, 20 μ m.

However, the inhibitors did not suppress the rapid increase in FRET/CFP ratio in M phase, indicating that kinases other than ROCK contributed to the increased FRET/CFP in M phase. Significant number of cells treated with the ROCK inhibitors failed to complete cytokinesis, generating binuclear cells (Fig. 5C, D). Importantly, in the presence of ROCK inhibitors, the FRET/CFP ratio was not increased after the completion of cytokinesis, indicating that the increased FRET/CFP ratio after abscission (Fig. 5A) is due to ROCK activation. In agreement with this note, we observed that RhoA activity was initially decreased in M phase and increased during and after cytokinesis (Fig. 5E) (Yoshizaki *et al.*, 2003). Thus, ROCK appears to contribute to cell adhesion in the early G1 phase.

ROCK activation during apoptosis

Another phenomenon during which ROCK is known to be activated is apoptosis. We therefore observed the timing of

ROCK activation during apoptosis induced by TNF- α and cycloheximide (CHX). The FRET/CFP ratio of HeLa cells stably-expressing Eevee-ROCK was increased gradually for 3–4 h without remarkable morphological changes (Fig. 6A, movie 3). Then the cells suddenly started to shrink, concomitant with a rapid surge and then a drop in the FRET/CFP ratio. These marked morphological changes occurred 208 $\pm$ 27 min after the addition of TNF- α and CHX. After this period, the cells exhibited membrane blebbing without any increase in FRET/CFP ratio (Fig. 6C). When the cells were pre-treated with Y27632 and GSK429286 before TNF- α and cycloheximide (CHX), the FRET/CFP ratio remained low throughout the observation period (Fig. 6B). After several hours without remarkable morphological changes, the cells rapidly retracted as observed in the control cells. The striking difference was that the membrane blebbing in the ROCK inhibitor-treated cells was not as obvious as that in the control cells. The periods between the addition of reagents and apoptosis in

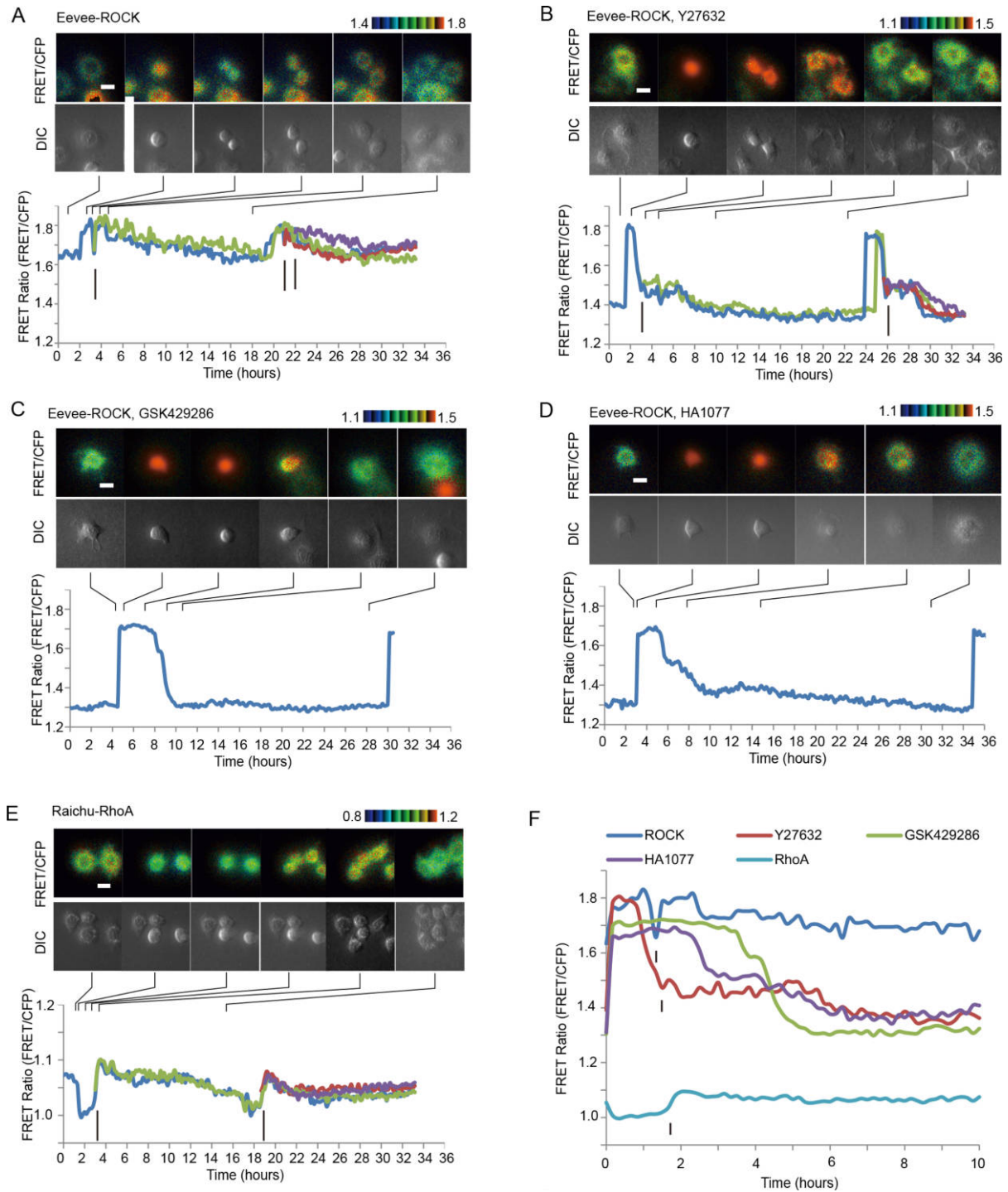


Fig. 5. ROCK activation after cytokinesis. (A-D) With an LCV110 incubator-type inverted fluorescence microscope, HeLa cells stably-expressing Eevee-ROCK were time-lapse imaged in the absence (A) or presence of ROCK inhibitors, 300 μ M Y27632 (B), 100 μ M GSK429286 (C), and 100 μ M HA1077 (D). Images were acquired every 10 min for two to three days. FRET/CFP ratio images and DIC images (upper panels) were acquired at the time points indicated on the lower panels. The FRET ratios (FRET/CFP) of a representative cell and its daughter cells were plotted against time (lower panels). The exposure times for CFP and FRET were 500 and 500 msec, respectively. Scale bar, 20 μ m. The arrows in the upper panels indicate the completion of cytokinesis (abscission). (E) HeLa cells stably-expressing Raichu-RhoA were time-lapse imaged as described. (F) Overlay of the time courses of FRET/CFP ratio changes. The origin was set to the nuclear breakdown at M phase.

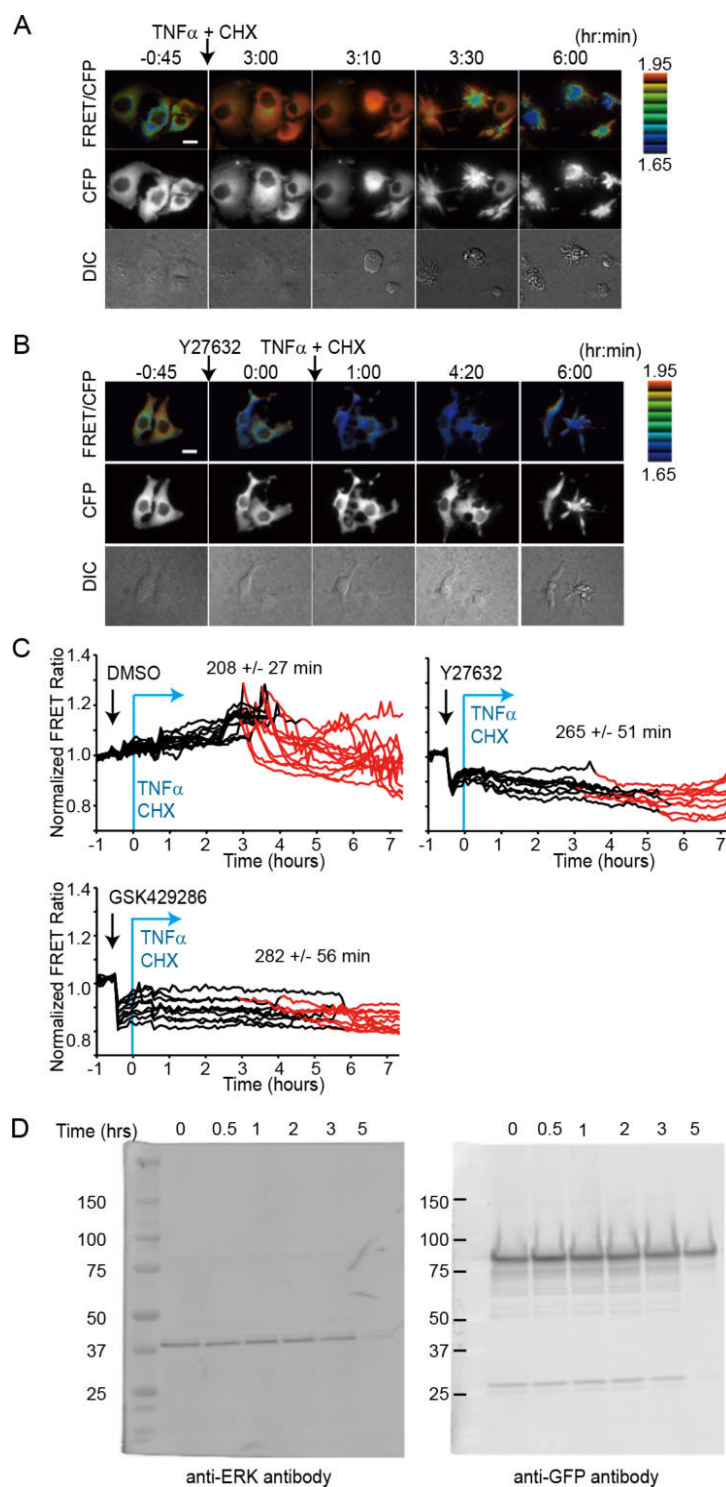


Fig. 6. ROCK activation during apoptosis. HeLa cells stably-expressing Eevee-ROCK were time-lapse imaged with an LCV110 incubator-type inverted fluorescence microscope. Images were acquired every 5 min for at least 8 h. DMSO as a solvent control, 30 μ M Y27632, or 10 μ M GSK429286 was added at 30 min after the start of imaging. For the induction of apoptosis, 10 μ g/mL TNF- α and 100 μ g/mL cycloheximide (CHX) were added at 60 min. Representative images of FRET/Ratio, CFP, and DIC images for the solvent control (A) and Y27632-treated cells are shown. Scale bars, 20 μ m. (C) Time courses of FRET/CFP ratios normalized to the first 30 min. The timing of apoptosis was determined by the nuclear membrane breakdown on the CFP images. Time points after apoptosis are shown by red lines. (D) In a parallel experiment, cells were lysed at the indicated time points and analyzed by immunoblotting with antibodies against ERK and GFP.

HeLa cells treated with Y27632 and GSK429286 were 265 $\pm$ 51 and 282 $\pm$ 56 min, respectively. Thus, inhibition of ROCK inhibited membrane blebbing and delayed the onset of, but did not prevent, apoptosis. Another interesting observation was the rapid decrease in FRET/CFP ratio after the initiation of apoptosis. We speculated that the cleavage of the linker region might have caused this rapid decrease and tested the integrity of the probe by immunoblotting (Fig. 6D). Against our expectation, we detected only the intact Eevee-ROCK of 81.9 kDa, but not cleaved forms. Therefore, the decrease in the FRET/CFP ratio might indeed indicate the decrease in the ROCK activity.

Discussion

The specificity of FRET biosensors for protein kinase activity depends on the kinase-specific substrate peptide (Sample *et al.*, 2014). Most, if not all, FRET biosensors adopt peptide sequences from the known substrate proteins, except for AKAR, a PKA biosensor, in which the PKA phosphorylation consensus sequence is used (Zhang *et al.*, 2001). In fact, the consensus phosphorylation sequence has not been known for many protein kinases, which forces developers to use the peptides known to be phosphorylated by the kinase of interest. In this study, we also attempted in vain to generate FRET biosensors for ROCK based on the phosphorylation sites derived from protein phosphatase 1, ARHGAP35, and ezrin (Tran Quang *et al.*, 2000; Wooldridge *et al.*, 2004; Mori *et al.*, 2009). In contrast, the consensus sequence for ROCK phosphorylation that was determined by the kinase-interacting substrate screening (KISS) served as a sensitive and specific substrate for ROCK in the context of the FRET biosensor based on the Eevee backbone (Komatsu *et al.*, 2011). In KISS technology, the consensus sequences for phosphorylation are determined experimentally from a huge number of peptides (Amano *et al.*, 2015), which may underlie the high sensitivity and selectivity in the context of the FRET biosensor. Meanwhile, the substrate sequence used in Eevee-ROCK was found to be phosphorylated also by PKA, a member of AGC-family kinases. Furthermore, ROCK inhibitors failed to suppress robust increase in FRET/CFP ratio in M phase (Fig. 5), indicating that kinases activated in M phase also phosphorylates Eevee-ROCK. Therefore, it should be kept in mind that the responses observed by using Eevee-ROCK should be validated in the presence of specific inhibitors of ROCK.

Cytokinesis generates two separate daughter cells through the actions of the actin-myosin-rich contractile ring. The role of the Rho/ROCK pathway in cytokinesis has been established by many research groups (Schwayer *et al.*, 2016). Importantly, ROCK accumulates at the cleavage furrow and phosphorylates several proteins, including the myosin regulatory light chain (MLC), during cytokinesis in

several cultured cell lines (Kosako *et al.*, 2000). Because ROCK inhibitors failed to suppress the increased FRET/CFP ratio during M phase, the activation of ROCK in M phase could not be evidenced with Eevee-ROCK. A substrate sequence specific to ROCK or a method to target the FRET biosensor to ROCK may be required for this purpose. Although we failed to show ROCK activation during M phase, we found that, after the completion of cytokinesis, ROCK activity rapidly increased when cells spread and adhered to the dishes. Because ROCK plays critical roles in the formation of stress fibers and focal contacts in interphase (Narumiya *et al.*, 2009), the reactivation of ROCK in early G1 phase is reasonable. In agreement with this observation, we reported previously that RhoA activity was decreased in M phase and increased during and after cytokinesis (Yoshizaki *et al.*, 2003).

The role of ROCK as a critical effector of the membrane blebbing, a hallmark of apoptotic cells, is well recognized (Coleman and Olson, 2002). During the execution phase, caspase-mediated cleavage and activation of ROCK-1 induce actomyosin contraction and dynamic membrane blebbing (Coleman *et al.*, 2001; Sebbagh *et al.*, 2001). As expected, we observed the rapid increase in the FRET ratio before the contraction of apoptotic cells (Fig. 5A). However, the rapid decrease of FRET ratio during the execution phase of apoptosis was unexpected. Cleavage of the linker region was the most likely explanation; however, we failed to detect the cleaved FRET biosensor (Fig. 6D). The question of whether the decrease in FRET ratio indeed reflects a decrease in ROCK activity awaits further analysis.

The role played by ROCK during apoptosis has been attracting more interest because the ROCK inhibitor Y27632 was discovered to prevent apoptosis of human embryonic stem cells (hESCs) during passage (Watanabe *et al.*, 2007). Further studies revealed that RhoA-dependent activation of ROCK causes apoptosis of hESCs during the passage (Chen *et al.*, 2010; Ohgushi *et al.*, 2010; Walker *et al.*, 2010). This apoptosis of hESCs critically differs from that triggered by death receptors such as the TNF receptor in the time lag between the membrane blebbing and nuclear breakdown of the apoptotic cells. In hESCs, membrane blebbing continues for several hours before cell death, whereas in the death receptor-mediated cell death, the membrane blebbing is immediately followed by the cell death (Ohgushi and Sasai, 2011). In agreement with these previous reports, we found that ROCK activation and membrane blebbing were observed immediately before the nuclear breakdown of the cells treated with TFN α and CHX (Fig. 6). We also noticed that the timing of cell contraction and nuclear breakdown was delayed approximately 1 h in the ROCK inhibitor-treated cells. Therefore, the ROCK-mediated activation of mitochondria may accelerate the apoptosis induced by TFN- α and CHX (Ohgushi *et al.*, 2010).

In summary, we developed a novel genetically-encoded

FRET biosensor specific to ROCK activity, Eevee-ROCK, and demonstrated its versatility as a tool for examining the temporal activity changes of ROCK during the cell cycle and apoptosis. The successful development and application of Eevee-ROCK has validated the usefulness of the substrate peptides identified by means of KISS technology, and will encourage researchers to develop FRET biosensors for the protein kinases of their interest.

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