

# Development of FRET-Based Indicators For Visualizing Homophilic *Trans* Interaction of A Clustered Protocadherin

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## Research Article

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# Abstract

Clustered protocadherins (Pcdhs), which are cell adhesion molecules, play a fundamental role in self-recognition and non-self-discrimination by conferring diversity on the cell surface. Although systematic cell-based aggregation assays provide information regarding the binding properties of Pcdhs, direct visualization of Pcdh *trans* interactions across cells remains challenging. Here, we present Förster resonance energy transfer (FRET)-based indicators for directly visualizing Pcdh *trans* interactions. We developed the indicators by individually inserting FRET donor and acceptor fluorescent proteins (FPs) into the ectodomain of Pcdh molecules. They enabled successful visualization of specific *trans* interactions of Pcdh and revealed that the Pcdh *trans* interaction is highly sensitive to changes in extracellular  $\text{Ca}^{2+}$  levels. We expect that FRET-based indicators for visualizing Pcdh *trans* interactions will provide a new approach for investigating the roles of Pcdh in self-recognition and non-self-discrimination processes.

## Introduction

Self-recognition and non-self-discrimination among neurons play a fundamental role in the assembly of neural circuits to form neural connections with other neurons but not with themselves. This process is called self-avoidance and is mediated in part by clustered protocadherins (Pcdhs). Pcdh is a cell adhesion molecule; in mice, its 58 isoforms are encoded by the *Pcdha*, *Pcdhb*, and *Pcdhy* gene clusters (14 $\alpha$ , 22 $\beta$ , and 22 $\gamma$ ), which are tandemly arranged on the same chromosome<sup>[1, 2]</sup>. Individual neurons have been shown to constitutively express five C-type *Pcdh* isoforms (*aC1*, *aC2*,  *$\gamma$ C3*,  *$\gamma$ C4*, and  *$\gamma$ C5*) and stochastically express the remaining 53 isoforms with variable combinations<sup>[3–5]</sup>. Pcdh shows adhesive activity solely between cells with the same combinatorial expression patterns of isoforms<sup>[6, 7]</sup>. Therefore, it is proposed that Pcdh functions as a diversified molecule that confers individualities on cells and that the self is recognized by the homophilic interaction of Pcdh across neurites derived from identical neurons; thereafter, these neurites repel each other by unidentified mechanisms<sup>[8–10]</sup>. On the basis of experimental evidence, *Pcdhy* has been proven to be necessary for the self-avoidance of dendrites in Purkinje cells and retinal starburst amacrine (SAC) cells. Genetic ablation of *Pcdhy* caused dendrites derived from identical Purkinje cells and retinal SAC cells to be crossed, and functional neural connections were constructed between dendrites of identical retinal SAC cells<sup>[11–13]</sup>. Notably, a single Pcdh isoform was able to rescue these abnormalities in retinal SAC cells<sup>[11, 12]</sup>. In addition, Pcdh has been observed to be involved in a wide range of biological phenomena, such as axonal coalescence of olfactory sensory neurons<sup>[14, 15]</sup>, axonal projection of serotonergic neurons<sup>[16–18]</sup>, formation of dendrite branching complexity<sup>[19–21]</sup>, and synapse maturation<sup>[22, 23]</sup>.

Till date, Pcdh interaction analysis has relied primarily on cell aggregation assays using K562 cells<sup>[6, 7, 24–28]</sup>. Because K562 cells lack endogenous cell adhesion molecules and they are observed as separate cells under a microscope, the binding property of the cell adhesion molecules introduced into K562 cells can be evaluated as cell aggregates<sup>[29]</sup>. This elegant and systematic experimental system has revealed that Pcdh interacts homophilically, and a single mismatched Pcdh is sufficient to interfere with this

homophilic interaction<sup>[6, 7]</sup>. However, direct visualization of Pcdh interactions across living cells remains challenging.

Förster resonance energy transfer (FRET) between two fluorescent proteins (FPs) has been employed to directly visualize protein-protein interactions in living cells. Of particular note is the indicator for detecting the N-cadherin *trans* interaction across living cells. FRET donor and acceptor FPs are separately inserted into the ectodomain of N-cadherin molecules, which are close to the *trans*-interacting interface<sup>[30]</sup>. Using this FRET-based indicator for N-cadherin *trans* interaction, Kim et al. investigated the Ca<sup>2+</sup>-responsive dynamics of N-cadherin *trans* interaction across cells<sup>[30]</sup>.

In this study, we developed indicators for directly visualizing the Pcdh *trans* interaction across cells by separately inserting FRET donor and acceptor FPs into Pcdh molecules, similar to the FRET-based indicator for N-cadherin *trans* interaction. The indicators showed a FRET efficiency of over 25 % at the cell adhesion sites. We successfully visualized the Pcdh *trans* interactions across cells using simple ratio imaging. By creating indicators with chimeric Pcdh that do not interact with the original indicators, we confirmed that these indicators specifically visualize the homophilic Pcdh *trans* interaction. We additionally confirmed a Ca<sup>2+</sup> dependency of Pcdh *trans* interaction, through the FRET ratio change by chelation of extracellular Ca<sup>2+</sup>. These results suggest that the FRET-based Pcdh indicators could serve as a new means for analyzing Pcdh *trans* interactions.

## Results

### Insertion of FPs into Pcdh for development of the FRET-based Pcdh indicators

To develop FRET-based Pcdh indicators, we used the protocadherin- $\gamma$ B2 ( $\gamma$ B2) isoform because structural information was available regarding a *trans*-dimer formed by longer ectodomain fragments (EC1-EC5) of  $\gamma$ B2 (PDB: 5T9T) <sup>[27]</sup>, which was helpful in determining the insertion sites of FPs for the FRET donor and acceptor. We expected that the loop between the  $\beta$ B and  $\beta$ C strands in EC1 of one  $\gamma$ B2 molecule and the loop between the  $\beta$ C and  $\beta$ D strands in EC5 of another  $\gamma$ B2 molecule would be close to each other with the formation of a *trans*-dimer. On the basis of this expectation, we individually inserted yellow FP Venus into these regions (amino acid position 29th and 472nd residues in the mature form, respectively; Fig. 1a) and examined the effect of FP insertion on the localization of  $\gamma$ B2. Venus-inserted  $\gamma$ B2s,  $\gamma$ B2-EC1-Venus, and  $\gamma$ B2-EC5-Venus were mainly localized at the perinuclear region in HEK293T cells, similar to C-terminal Venus-fused  $\gamma$ B2 ( $\gamma$ B2-Venus) (Fig. 1b). This result indicates that the insertion of Venus into  $\gamma$ B2 hardly compromised the localization of  $\gamma$ B2. Since the low localization of  $\gamma$ B2 at the plasma membrane hampers the development of FRET-based indicators for monitoring  $\gamma$ B2 *trans* interactions across cells, we deleted the intracellular domain (ICD) to efficiently localize FP-inserted  $\gamma$ B2s at the plasma membrane<sup>[31]</sup>. We prepared  $\gamma$ B2 $\Delta$ ICDs in which cyan FP mTurquoise2 (mTQ2) and Venus were inserted into the EC1 and EC5 domains as the FRET donor and acceptor, respectively ( $\gamma$ B2 $\Delta$ ICD-EC1-mTQ2 and  $\gamma$ B2 $\Delta$ ICD-EC5-Venus) (Fig. 1c). They were efficiently localized at the plasma membrane similar to  $\gamma$ B2 $\Delta$ ICD-Venus (Fig. 1d).

## FRET-based $\gamma$ B2 $\Delta$ ICD indicators can monitor homophilic trans interaction of $\gamma$ B2

To examine whether FRET occurs by  $\gamma$ B2 *trans* interaction across cells (Fig. 2a), we co-cultured HEK293T cells individually expressing  $\gamma$ B2 $\Delta$ ICD-EC1-mTQ2 and  $\gamma$ B2 $\Delta$ ICD-EC5-Venus and assessed FRET by acceptor photobleaching at the cell adhesion sites (Fig. 2c-d). The average FRET efficiency was  $14.1 \pm 2.42$  % (Fig. 2e, mean  $\pm$  SD, pre-optimization). To increase FRET efficiency, we optimized the linkers between the FPs and  $\gamma$ B2. We eventually obtained  $\gamma$ B2 $\Delta$ ICD-EC1-G4S-mTQ2 $\Delta$ C6 and  $\gamma$ B2 $\Delta$ ICD-EC5-Venus $\Delta$ N3C9-P3 as post-optimized FRET-based  $\gamma$ B2 $\Delta$ ICD indicators (Fig. 2b). FRET at cell adhesion sites was assessed (Fig. 2c-d), and the average FRET efficiency was  $27.4 \pm 5.67$  % (Fig. 2e, mean  $\pm$  SD, post-optimization), which was approximately twice as high as the pre-optimized FRET efficiency.

A cell aggregation assay using K562 cells in previous studies showed that Pcdh isoforms mediate specific interactions only between identical isoforms<sup>[6, 7]</sup>. To examine the effect of FP-insertion into  $\gamma$ B2 on the binding specificity, we performed a cell aggregation assay using K562 cells individually transfected with expression plasmids encoding FRET-based  $\gamma$ B2 $\Delta$ ICD indicators and C-terminal tdTomato-fused  $\gamma$ B2 ( $\gamma$ B2-tdTomato) and  $\gamma$ A3 ( $\gamma$ A3-tdTomato) isoforms. Both types of cells expressing  $\gamma$ B2 $\Delta$ ICD-G4S-mTQ2 $\Delta$ C6 and  $\gamma$ B2 $\Delta$ ICD-EC5-Venus $\Delta$ N3C9-P3 aggregated with cells expressing  $\gamma$ B2-tdTomato; however, they did not aggregate with cells expressing  $\gamma$ A3-tdTomato and segregated into homophilic aggregates (Fig. 2f), indicating that FRET-based  $\gamma$ B2 $\Delta$ ICD indicators retain homophilic interactions. We observed that  $\gamma$ B2 $\Delta$ ICD-EC1-G4S-mTQ2 $\Delta$ C6 and  $\gamma$ B2 $\Delta$ ICD-EC5-Venus $\Delta$ N3C9-P3 mediate homophilic interactions with each other (Fig. 2g). Finally, we assessed FRET by acquiring a Venus/mTQ2 ratio image of cell aggregates formed by cells individually expressing  $\gamma$ B2 $\Delta$ ICD-EC1-G4S-mTQ2 $\Delta$ C6 and  $\gamma$ B2 $\Delta$ ICD-EC5-Venus $\Delta$ N3C9-P3. Consequently, a higher ratio was confirmed at the cell adhesion sites between cells expressing  $\gamma$ B2 $\Delta$ ICD-EC1-G4S-mTQ2 $\Delta$ C6 and  $\gamma$ B2 $\Delta$ ICD-EC5-Venus $\Delta$ N3C9-P3 (Fig. 2h), indicating that the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators enable monitoring of Pcdh-mediated cell-cell adhesions as a Venus/mTQ2 ratio readout.

## FRET-based $\gamma$ B2 $\Delta$ ICD indicators detect specific $\gamma$ B2 trans interaction

To confirm the specificity of the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators, we prepared FRET-based indicators to monitor the interaction of other Pcdh isoforms and examined whether FRET occurs between the indicators with  $\gamma$ B2 $\Delta$ ICD and other Pcdh. We initially prepared mTQ2- and Venus-inserted  $\gamma$ A3 $\Delta$ ICDs with insertion sites corresponding to those of the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators. However, almost no FRET was observed between them (data not shown). A previous cell aggregation assay using K562 cells indicated that chimeric Pcdhs whose EC2-EC3 domains were swapped by those of other Pcdh isoforms interact homophilically; however, they did not interact with parental Pcdhs<sup>[25]</sup>. On the basis of this knowledge, we prepared FRET-based chimeric  $\gamma$ B2 $\gamma$ A3 $\Delta$ ICD indicators (Fig. 3a). Both  $\gamma$ B2 $\gamma$ A3 $\Delta$ ICD-EC1-G4S-mTQ2 $\Delta$ C6 and  $\gamma$ B2 $\gamma$ A3 $\Delta$ ICD-EC5-Venus $\Delta$ N3C9-P3 were localized at the cell adhesion sites in HEK293T cells (Fig. 3b). Next, we examined the binding specificity of FRET-based chimeric  $\gamma$ B2 $\gamma$ A3 $\Delta$ ICD indicators using a cell aggregation assay in K562 cells. While cells individually expressing FRET-based chimeric  $\gamma$ B2 $\gamma$ A3 $\Delta$ ICD indicators aggregated with each other, they segregated into discrete aggregates

with cells expressing  $\gamma$ B2-tdTomato or  $\gamma$ A3-tdTomato (Fig. 3c). This result indicates that the FRET-based chimeric  $\gamma$ B2 $\gamma$ A3 $\Delta$ ICD indicators mediate homophilic interactions. To examine the specificity of the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators, we individually expressed the FRET-based  $\gamma$ B2 $\Delta$ ICD and chimeric  $\gamma$ B2 $\gamma$ A3 $\Delta$ ICD indicators in HEK293T cells and assessed FRET by acceptor photobleaching at the cell adhesion sites. Although FRET between the same pairs of indicators was confirmed, different pairs did not exhibit FRET (Fig. 3d). We further analyzed the specificity of the indicators in K562 cells as a FRET readout. Since the FRET-based  $\gamma$ B2 $\Delta$ ICD and chimeric  $\gamma$ B2 $\gamma$ A3 $\Delta$ ICD indicators mediate homophilic interactions (Fig. 2f, 3c), the cells expressing them should be segregated. N-cadherin has been reported to bridge K562 cells expressing different Pcdh isoforms<sup>[7]</sup>. We co-expressed N-cadherin with the FRET-based  $\gamma$ B2 $\Delta$ ICD or chimeric  $\gamma$ B2 $\gamma$ A3 $\Delta$ ICD indicators in K562 cells and co-cultured them. As expected, cell aggregation was confirmed even between the cells expressing a different pair of Pcdh (Fig. 3e), and FRET was confirmed only in the same pairs of indicators at the mTQ2- and Venus-positive cell adhesion sites (Fig. 3f, white arrowheads). These results indicate that the FRET-based  $\gamma$ B2 $\Delta$ ICD and chimeric  $\gamma$ B2 $\gamma$ A3 $\Delta$ ICD indicators specifically monitor  $\gamma$ B2 and  $\gamma$ B2 $\gamma$ A3 *trans* interactions across cells, respectively.

### **Ca<sup>2+</sup> dependency of $\gamma$ B2 *trans* interaction revealed by the FRET-based $\gamma$ B2 $\Delta$ ICD indicators**

Previous reports have examined the Ca<sup>2+</sup>-dependency of Pcdh *trans* interactions in K562 cells, using different approaches, and their conclusions were not consistent<sup>[6, 25]</sup>. To clarify the Ca<sup>2+</sup>-dependency of the Pcdh *trans* interaction, we examined the effect of Ca<sup>2+</sup> chelation by EGTA on the pre-formed cell adhesions mediated by the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators in K562 cells. After treatment with EGTA, the Venus/mTQ2 ratio at the cell adhesion sites was reduced (Fig. 4a). Time-lapse imaging showed that a decrease in the Venus/mTQ2 ratio was confirmed within a second (Fig. 4b). Furthermore, emission spectra before and after EGTA treatment indicated that a decrease in the Venus/mTQ2 ratio by Ca<sup>2+</sup> chelation resulted from an increase in the emission of mTQ2, corresponding to a decrease in the emission of Venus (Fig. 4c). Quantitative analysis strongly supported that the Pcdh *trans* interaction was dependent on Ca<sup>2+</sup> (Fig. 4d).

## **Discussion**

We have developed FRET-based  $\gamma$ B2 indicators,  $\gamma$ B2 $\Delta$ ICD-EC1-G4S-mTQ2 $\Delta$ C6/ $\gamma$ B2 $\Delta$ ICD-EC5-Venus $\Delta$ N3C9-P3, that enabled direct visualization of the Pcdh *trans* interaction and revealed that Pcdh senses changes in extracellular Ca<sup>2+</sup> levels within a second. The Ca<sup>2+</sup>-dependency of the Pcdh *trans* interaction is controversial. Previously, a cell-based pull-down assay using K562 cells showed that Pcdh *trans* interaction is less sensitive to Ca<sup>2+</sup><sup>[6]</sup>. In contrast, Rubinstein et al. concluded that Pcdhs mediate cell-cell interactions in a Ca<sup>2+</sup>-dependent manner on the basis of a cell aggregation assay using K562 cells;<sup>[25]</sup> consistent with these observations, our result for the FRET-based  $\gamma$ B2 $\Delta$ ICD in K562 cells clearly demonstrated that the Pcdh *trans* interaction is dependent on Ca<sup>2+</sup> (Figs. 4).

To show the specificity of the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators, we first prepared  $\gamma$ A3 $\Delta$ ICD in which FRET donor and acceptor FPs were individually inserted at the corresponding sites to  $\gamma$ B2. However, we could not confirm FRET at the cell adhesion sites formed by the cells individually expressing them (data not shown). A previous report showed that dimer structures formed by the EC1-EC5 domains of  $\gamma$ B2 and EC1-EC4 domains of  $\gamma$ B7 are closely related<sup>[27]</sup>. In contrast, root mean square deviations (RMSDs) between a dimer structure formed by EC1-EC4 of  $\gamma$ A1 and dimer structures formed by EC1-EC5 domains of  $\gamma$ B2 and EC1-EC4 domains of  $\gamma$ B7 were relatively large<sup>[27]</sup>. These notions suggest that the overall structures of *trans* dimers between the  $\gamma$ A and  $\gamma$ B subfamilies are varied. In addition, considering that the FRET phenomenon is sensitive to the distance and orientation of the donor and acceptor FPs, the FRET of FP-inserted  $\gamma$ A3 was not detected because of the improper relative orientation. Crystal structures of *trans* dimers formed by longer ectodomains will enable further development of individual indicators for other Pcdh isoforms.

The Pcdh *trans* interaction was analyzed using a cell aggregation assay in K562 cells lacking endogenous cell adhesion molecules. Since this method relies on the observation of cell aggregates, we cannot strictly distinguish the Pcdh *trans* interaction from the other factors that promote cell aggregation. A previous report<sup>[7]</sup> and our results (Fig. 3e) showed that cells individually expressing different Pcdh isoforms co-aggregated in the presence of N-cadherin, indicating that homophilic Pcdh *trans* interactions cannot be confirmed as cell aggregates in the presence of other cell adhesion molecules. In contrast, the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators visualized homophilic Pcdh *trans* interactions, even in the presence of N-cadherin (Fig. 3f). Furthermore, merged images of fluorescence and DIC in Fig. 4A showed that cell-cell contacts were sustained even after EGTA treatment. However, the ratio images indicated that the Pcdh *trans* interaction was diminished by extracellular  $\text{Ca}^{2+}$  chelation, suggesting that the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators can detect Pcdh *trans* interaction independent of visual cell aggregation.

Cell-cell interactions have been visualized using a bimolecular fluorescence complementation (BiFC) technique<sup>[32–37]</sup>. Although BiFC-based indicators are potent tools for visualizing cell-cell interactions, they have limitations. The complementation reaction is irreversible, which hampers the monitoring of the dissociation of cell-cell interactions. In contrast, since FRET is reversible, we successfully monitored the dissociation of Pcdh *trans* interactions across cells using the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators (Fig. 4a).

We have accomplished only the development of FRET-based  $\gamma$ B2 indicators and observation of the Pcdh *trans* interaction across cells by introducing indicators in heterologous cells such as K562 and HEK293T cells. One of the most important issues to be addressed is determination of the spatio-temporal roles of the Pcdh *trans* interaction in biological phenomena such as dendritic self-avoidance. For examination of self-avoidance in neurons, interactions between dendrites derived from identical neurons should be analyzed. In such a case, the FRET-based  $\gamma$ B2 indicators must be expressed in the same cells. On the basis of this theory, to examine whether co-expressed FRET-based  $\gamma$ B2 $\Delta$ ICD indicators monitor the Pcdh *trans* interaction across cells, we co-cultured K562 cells co-expressing the indicators. However, although FRET was hardly detected, cell aggregates were formed (data not shown). It is possible that the same

pairs of dimers (donor-donor and acceptor-acceptor) were formed between the cells, which reduced the net FRET. One method to avoid this problem is analysis of cell-cell contact sites between two different neurons lacking endogenous Pcdhs. We previously showed that  $\gamma$ A3-Venus and  $\gamma$ A3-tdTomato co-localized at the process-process contact sites formed by *Pcdha $\beta$  $\gamma$* -deficient neurons individually expressing them while they localized at the processes as discrete puncta in wild-type neurons<sup>[38]</sup>. Furthermore, Kostadinov and Sanes revealed that retinal SAC cells expressing a single  $\gamma$ C3 isoform showed a reduction in intercellular functional connections, which was considered as self-avoidance between cells<sup>[12]</sup>. These previous findings suggest that combinatorially expressed endogenous Pcdhs prevent an exogenous Pcdh isoform from forming a *trans* interaction; therefore, an exogenous Pcdh isoform can interact homophilically across processes of two different neurons lacking endogenous Pcdhs. Pcdh *trans* interactions could be visualized in the self-avoidance process using *Pcdha $\beta$  $\gamma$* -deficient neurons individually expressing the FRET-based  $\gamma$ B2 indicators. We believe that FRET-based Pcdh indicators are helpful in unveiling the spatio-temporal roles of Pcdh *trans* interactions in self-recognition and non-self-discrimination in neurons.

## Methods

### Plasmid construction

All expression plasmids were subcloned into the pCX vector. Full-length mouse Pcdhs C-terminally fused with fluorescent proteins were generated as described previously<sup>[38]</sup>. We used monomeric Venus (A206K) for the following constructions and described them as Venus. The amino acid positions of Pcdh are described in the immature form in this section. To create Venus-inserted  $\gamma$ B2s,  $\gamma$ B2-EC1-Venus, and  $\gamma$ B2-EC5-Venus, we inserted Venus flanked by NheI sites at amino acid position 58 in the EC1 domain and 501 in the EC5 of  $\gamma$ B2 C-terminally fused with the PA tag<sup>[39]</sup> by overlapping PCR.  $\gamma$ B2 $\Delta$ ICD constructs were created by the deletion of ICD (739-940 amino acids). To generate  $\gamma$ B2 $\Delta$ ICD constructs inserted with linker-modified FPs for FRET imaging, we inserted PCR products encoding linker-modified FPs flanked by NheI sites into  $\gamma$ B2 $\Delta$ ICD constructs C-terminally fused with the PA tag. For the construction of  $\gamma$ B2 $\gamma$ A3 $\Delta$ ICD chimeric constructs, an EC1 domain containing a propeptide (1-58 amino acids), EC4-EC6 domains containing a transmembrane region, and a part of the cytoplasmic region (343-940 amino acids) from  $\gamma$ B2, and EC2-EC3 domains (127-340 amino acids) from  $\gamma$ A3 were connected using an overlapping PCR.

### Cell culture and plasmid transfection

HEK293T cells (RIKEN BRC) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10 % (v/v) fetal bovine serum (FBS, Biowest) at 37 °C in humidified air containing 5 % CO<sub>2</sub>. K562 cells (RIKEN BRC) were maintained in Iscove's modified Dulbecco's medium (IMDM, FUJIFILM) supplemented with 10 % (v/v) FBS (Thermo Fisher Scientific) and 50  $\mu$ g/mL kanamycin at 37 °C in humidified air containing 5 % CO<sub>2</sub>. HEK293T cells were transfected using

polyethylenimine “MAX” (Cosmo Bio). K562 cells were electroporated using an Amaxa 4D-Nucleofector (Lonza).

## Cell imaging

HEK293T cells grown on glass-bottomed dishes coated with Cellmatrix Type I-C (Nitta gelatin) were transiently transfected with expression plasmids and incubated overnight. Before imaging, the medium was replaced with phenol red-free DMEM/F12 (Thermo Fisher Scientific). Fluorescence and DIC images were acquired using an Olympus FV-1000 laser scanning confocal microscope with an IX81 microscope equipped with a x60, 1.35 numerical aperture (NA) oil-immersion objective lens (UPLSAP060XO) (Olympus). For co-culture experiments, HEK293T cells were individually transfected with expression plasmids and incubated overnight. The cells were detached from dishes using trypsin-EDTA (0.25 %) and phenol red (Thermo Fisher Scientific) and mixed in 10 % FBS/DMEM. Imaging was performed after 24 h.

Transfected K562 cells were rotated at 30 rpm overnight at 37 °C in humidified air containing 5 % CO<sub>2</sub>. Before imaging, the cells were decanted into glass-bottomed dishes coated with 0.1 % (w/v) polyethylenimine (Sigma-Aldrich) for 1 h at 37 °C under humidified air containing 5 % CO<sub>2</sub>. The images were acquired using an LSM780 confocal microscope equipped with a Plan-Apochromat ×10, 0.45 NA objective lens (for observation of cell aggregates), and a Plan-Apochromat ×63, 1.40 NA oil immersion objective lens (for ratio imaging and spectrum imaging) (Carl Zeiss).

For ratio imaging, mTQ2 was excited with a 405-nm laser. The donor and acceptor emissions were collected at 463-500 nm and 520-620 nm, respectively. IMD images were created using the MetaMorph software (Molecular Devices).

For spectrum imaging, mTQ2 was excited with a 405-nm laser. Emission was collected over a spectrum with a width of 8.9 nm.

## Acceptor photobleaching

Photobleaching was performed as previously described<sup>[40,41]</sup>. mTQ2 and Venus were excited using a 405 nm laser diode and the 515 nm line of a multi-argon laser, and their emissions were collected using bandpass filters set at 460-500 nm and 530-630 nm, respectively. The image size was set to 512×512 pixels. The pinhole diameter was set to 300 μm. For each time-lapse series, an optical zoom of 3.0 was used, and images were acquired without intervals at 200×50 pixels. Venus signals at the cell adhesion sites were photobleached using the main scanner in tornado scan mode with the 515 nm line of a multi-argon laser set at 100 % transmission on one frame. The bleached region was set as a circle with a diameter of 20 pixels. The fluorescence intensities within the bleached region and cell-free area (background) were measured using the Olympus FV10-ASW software. After background subtraction, the fluorescence intensities of mTQ2 and Venus in the bleached region were normalized to the mean fluorescence intensity of 10 frames before photobleaching and plotted over time. The mean fluorescence

intensities of mTQ2 from 10 frames before and after photobleaching were calculated, and the FRET efficiency was calculated using Eq. (1).

$$FRET \text{ efficiency (\%)} = 1 - \frac{F_{BB}}{F_{AB}} \times 100 \text{ (\%)}, \quad (1)$$

where  $F_{BB}$  and  $F_{AB}$  are the fluorescence intensities of mTQ2 before and after photobleaching, respectively.

## Declarations

### Data availability

The data and materials supporting this research are available from the authors upon request.

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### Author contributions

T.K. and N.H. contributed equally to this work. T.K. planned and performed the experiments, analyzed the data, and wrote the manuscript. N.H. performed the experiments and analyzed the data. T.N. provided advice regarding the experiments and data analyses. T.M. and T.Y. designed the study and wrote the manuscript.

### Additional information

**Competing interests:** The authors declare no competing interests.

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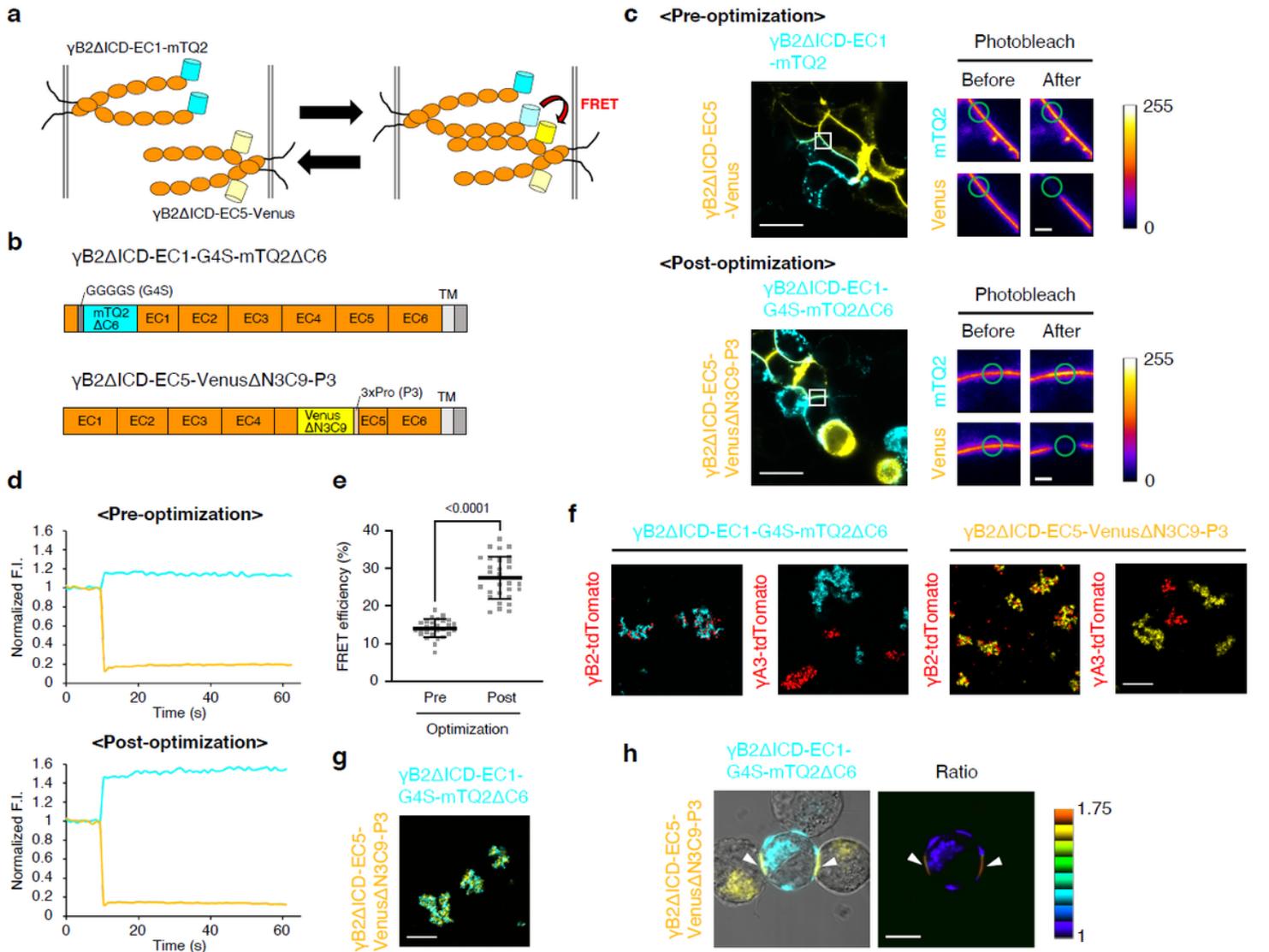
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## Figures

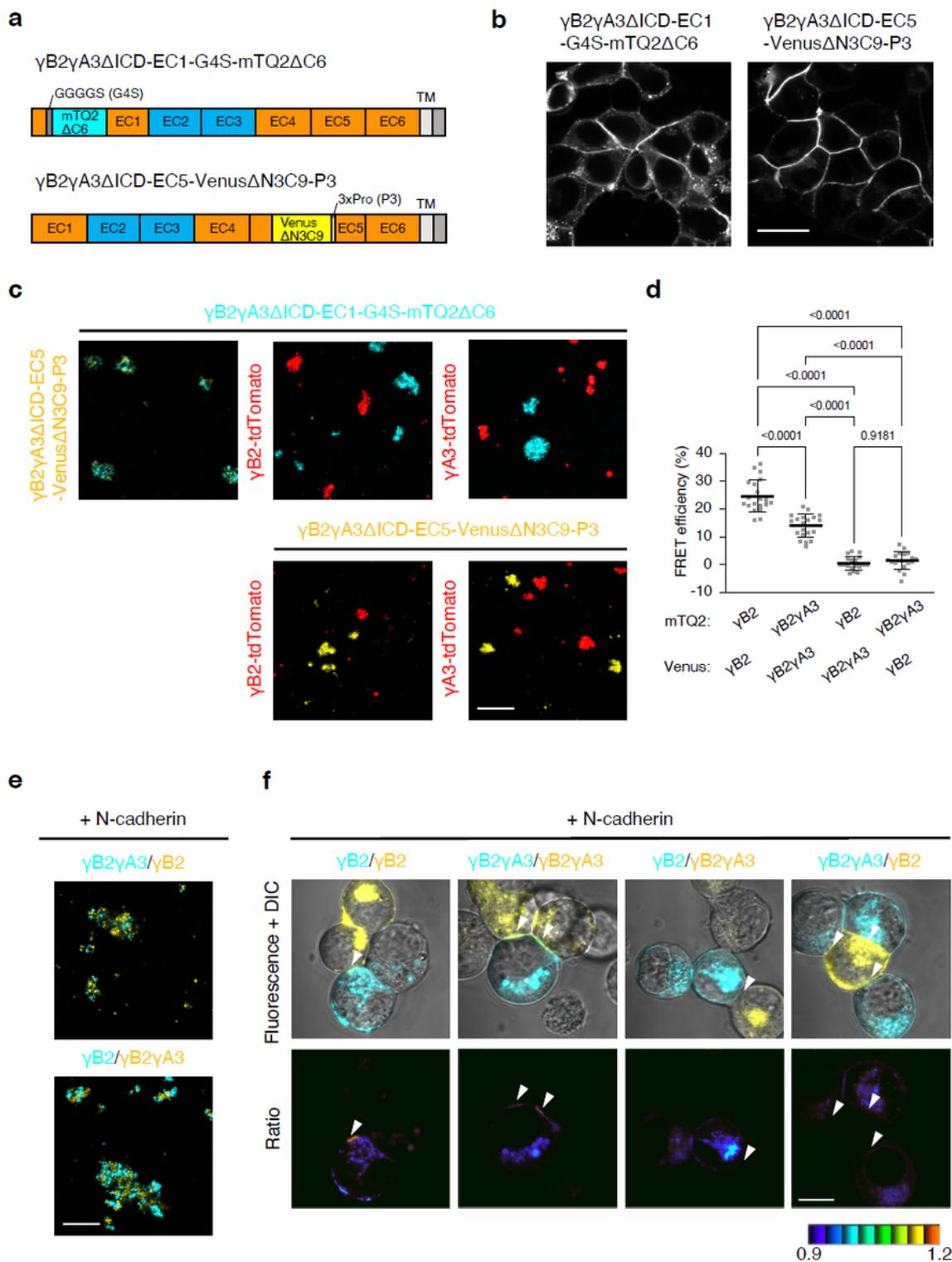




**Figure 2**

The  $\gamma$ B2 trans interaction monitored by FRET-based  $\gamma$ B2 indicators. (a) Schematics of the  $\gamma$ B2 trans interaction, as visualized using the FRET-based  $\gamma$ B2 indicators. The orange ovals represent the EC domains of  $\gamma$ B2. mTQ2 and Venus are represented as cyan and yellow cylinders, respectively. (b) Schematics of linker-optimized FRET-based  $\gamma$ B2 indicators. The C-terminal 6 amino acids of mTQ2 are deleted, and a GGGGS (G4S) linker is inserted at the N-terminus of mTQ2 in  $\gamma$ B2 $\Delta$ ICD-EC1-G4S-mTQ2 $\Delta$ C6. The 3 N-terminal amino acids and the 9 C-terminal amino acids of Venus are deleted, and a 3xPro (P3) linker is inserted at the C-terminus of Venus in  $\gamma$ B2 $\Delta$ ICD-EC5-Venus $\Delta$ N3C9-P3. (c) Representative acceptor photobleaching experiments of the pre- and post-optimized FRET-based  $\gamma$ B2 $\Delta$ ICD indicators in HEK293T cells. HEK293T cells individually expressing the indicated constructs were co-cultured (left panels). The panels on the right represent magnified fluorescence images of white squared regions in left panels before and after acceptor photobleaching at green circles. The color bars indicate the range of the fluorescence intensities of mTQ2 and Venus (arbitrary units). Scale bars, 20  $\mu$ m (left panel) and 2  $\mu$ m (right panel). (d) Alterations in fluorescence intensities of mTQ2 and Venus by acceptor

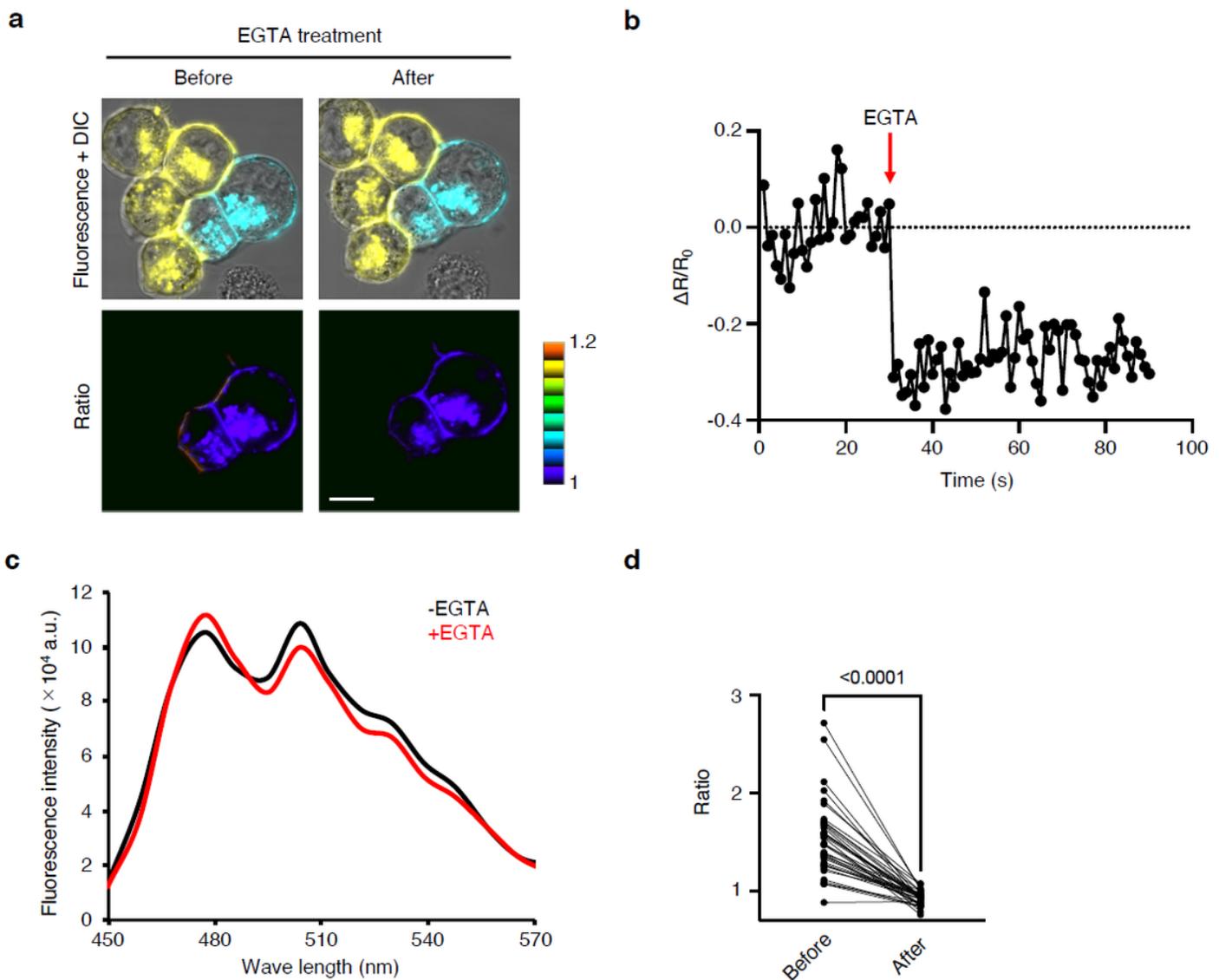
photobleaching in (c) were followed and plotted over time. (e) Mean values of FRET efficiency for the pre- and post-optimized FRET-based  $\gamma$ B2 $\Delta$ ICD indicators (n = 28 in both pre- and post-optimization). Data are shown as the means  $\pm$  SD. Significant difference was analyzed by Welch's t test. p value is described in the graph. (f) Binding specificity of the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators by cell aggregation assay. K562 cells individually expressing the indicated constructs were co-cultured. Scale bar, 200  $\mu$ m. (g) The interaction between  $\gamma$ B2 $\Delta$ ICD-EC1-mTQ2 $\Delta$ C6 and  $\gamma$ B2 $\Delta$ ICD-EC5-Venus $\Delta$ N3C9-P3, as revealed by cell aggregation assay. K562 cells individually expressing the indicated constructs were co-cultured. Scale bar, 200  $\mu$ m. (h) Direct visualization of the  $\gamma$ B2 trans interaction using the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators. K562 cells individually expressing the indicated constructs were co-cultured. The merged fluorescence and DIC image is shown in the left panel. The ratio image of Venus/mTQ2 is shown as intensity-modulated display (IMD) in the right panel. Scale bar, 10  $\mu$ m.



**Figure 3**

Specificity of FRET-based  $\gamma B2\Delta ICD$  indicators. (a) Schematics of FRET-based chimeric  $\gamma B2\gamma A3\Delta ICD$  indicators. The EC2-EC3 domains of the FRET-based  $\gamma B2\Delta ICD$  indicators were swapped by those of  $\gamma A3$ . The orange and light blue boxes are the EC domains from  $\gamma B2$  and  $\gamma A3$ , respectively. (b) The localization of the FRET-based chimeric  $\gamma B2\gamma A3\Delta ICD$  indicators in HEK293T cells. Scale bar, 20  $\mu m$ . (c) Binding specificity of the FRET-based chimeric  $\gamma B2\gamma A3\Delta ICD$  indicators, as revealed by cell aggregation assay.

K562 cells individually expressing the indicated constructs were co-cultured. Scale bar, 200  $\mu\text{m}$ . (D) Evaluation of the FRET-based chimeric  $\gamma\text{B2}\gamma\text{A3}\Delta\text{ICD}$  indicators and the FRET-based  $\gamma\text{B2}\Delta\text{ICD}$  indicators by acceptor photobleaching in HEK293T cells. Acceptor photobleaching at the cell adhesion sites was performed using co-cultured HEK293T cells in the indicated combination, and FRET efficiency was calculated [ $n = 20$  ( $\gamma\text{B2}-\gamma\text{B2}$ ),  $n = 20$  ( $\gamma\text{B2}\gamma\text{A3}-\gamma\text{B2}\gamma\text{A3}$ ),  $n = 18$  ( $\gamma\text{B2}-\gamma\text{B2}\gamma\text{A3}$ ),  $n = 19$  ( $\gamma\text{B2}\gamma\text{A3}-\gamma\text{B2}$ ). The combination is described as mTQ2-Venus pair]. Data are shown as the means  $\pm$  SD. Significant differences were analyzed by Welch's ANOVA test, followed by Dunnett's T3 multiple comparison test.  $p$  values are indicated in the graph. (e) Cell aggregation between the FRET-based  $\gamma\text{B2}\Delta\text{ICD}$ - and chimeric  $\gamma\text{B2}\gamma\text{A3}\Delta\text{ICD}$  indicator-expressing cells, mediated by co-expressing N-cadherin. K562 cells individually expressing the indicated FRET constructs and N-cadherin were co-cultured. Scale bar, 200  $\mu\text{m}$ . (f) FRET between the FRET-based  $\gamma\text{B2}\Delta\text{ICD}$ - and chimeric  $\gamma\text{B2}\gamma\text{A3}\Delta\text{ICD}$  indicators. K562 cells expressing N-cadherin and the FRET-based  $\gamma\text{B2}\Delta\text{ICD}$ - or the chimeric  $\gamma\text{B2}\gamma\text{A3}\Delta\text{ICD}$  indicators were co-cultured in the indicated combination. The merged fluorescence and DIC images are shown in the upper panels. The ratio images of Venus/mTQ2 are shown as IMD in the lower panels. mTQ2- and Venus-positive cell adhesion sites are indicated by white arrowheads. Scale bar, 10  $\mu\text{m}$ .



## Figure 4

Ca<sup>2+</sup>-dependency of  $\gamma$ B2 trans interaction. (a) The effect of EGTA treatment on the FRET at the cell adhesion sites. K562 cells individually expressing the FRET-based  $\gamma$ B2 $\Delta$ ICD indicators were co-cultured. The merged fluorescence and DIC images (upper panels) and the ratio images of Venus/mTQ2 (lower panels) were acquired before and after 5 mM EGTA treatment. Scale bar, 10  $\mu$ m. (b) Change in Venus/mTQ2 ratio ( $\Delta R/R_0$ ) at a cell adhesion site with EGTA treatment over time. EGTA (5 mM) was added to the medium at 30 s.  $R_0$  was calculated as the average Venus/mTQ2 ratio value of 30 frames before EGTA treatment. (c) Emission spectra before and after EGTA treatment. The emission spectra excited by 405 nm light at the cell adhesion site before and after 5 mM EGTA treatment were acquired. (d) Quantitative analysis of ratio changes by EGTA treatment. The FRET ratio values at the cell adhesion sites before and after 5 mM EGTA treatment were measured (n = 38). Significant difference was analyzed by Wilcoxon matched-pairs signed rank test. p value is indicated in the graph.