Supporting Information

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SI Materials and Methods

Equations. Bioluminescence resonance energy transfer (BRET) ratios were calculated using the following equation (bioluminescence signal given as average radiance [BL]) (Eq. S1):

$$\text{BRET RATIO} = \frac{\text{BL emission(long-wavelength)}}{\text{BL emission(short-wavelength)}} - \frac{\text{BL emission(long-wavelength) donor only}}{\text{BL emission(short-wavelength) donor only}}. \quad [S1]$$

The double ratio (DR) is defined as (Eq. S2)

$$\text{DR} = \frac{\text{BL (long-wavelength)} \times \mu_t\,\text{(long-wavelength)}}{\text{BL (short-wavelength)} \times \mu_t\,\text{(short-wavelength)}} \Big/ \frac{\text{BL (long-wavelength) donor only} \times \mu_t\,\text{(long-wavelength)}}{\text{BL (short-wavelength) donor only} \times \mu_t\,\text{(short-wavelength)}}, \quad [S2]$$

which is independent of μ_t (total attenuation coefficient).

Plasmid Construction. Previously developed pCMV-GFP^2-MCS-*Renilla reniformis* luciferase (RLuc)8 vector [derived from the commercially available plasmid pGFP^2-MSC-RLuc(h); BioSignal Packard] was used as a backbone for creating all BRET constructs. To introduce the red fluorescent protein (RFP) acceptors, fusion constructs were made by replacing GFP^2 sequence with either PCR-amplified TagRFP or TurboFP635 sequence between the 5′ NheI and 3′ BspEI site. These RFPs were PCR-amplified using the pTagRFP and pTurboFP635 (Evrogen) as templates. To replace the RLuc8 donor sequence with RLuc8.6 sequence, restriction-digested RLuc8.6 sequence from pcDNA3.1-RLuc8.6 was introduced in the RFP-RLuc8 vector developed. The two mammalian target of rapamycin (mTOR) pathway proteins, FKBP12 and FRB, were PCR-amplified from a previously developed plasmid using a BglII restriction enzyme and inserted into the pTurboFP635-RLuc8.6 vector to generate pTurboFP635-FRB-FKBP12-RLuc8.6. Similarly, pRLuc8.6-FRB-FKBP12-TurboFP635, pTurboFP635-FKBP12-FRB-RLuc8.6, and pRLuc8.6-FKBP12-FRB-TurboFP635 were generated to serve as PCR templates for creating constructs containing all orientations for bimolecular BRET6 FRB/FKBP12 sensors. The PCR product of TurboFP635-FRB, FKBP12-RLuc8.6, and all other orientation products was inserted in a pCMV vector using NheI and XhoI restriction sites. The clones were sequence-analyzed against the template DNAs. Low-salt bacto-agar plates containing 25 μg/mL zeocin (Invitrogen) were used for all clonal selections of the BRET plasmids. For generating a double stable cell line expressing both FKBP12-TurboFP635 and FRB-RLuc8.6, a pcDNA3.1-PURO-FRB-RLuc8.6 was generated by subcloning FRB-RLuc8.6 from the pCMV plasmid using NheI and XhoI restriction enzymes. The clones were in *E. coli* selected using ampicillin (100 μg/mL).

Cell Culture, Transfection, and Clonal Isolation. The HT1080 cells were grown in DMEM (high glucose) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were plated in 12-well plates in complete growth media. Transient transfection of all plasmids was done 24 h later using SuperFect (Qiagen) according to the manufacturer's protocol. Stable selection of HT1080 cells expressing pcDNA-RLuc8 and pcDNA-RLuc8.6 was performed with 350 μg/mL geneticin (Invitrogen). pCMV-TagRFP(TurboFP635)-RLuc8 (RLuc8.6), pCMV-FKBP12-TurboFP635, and pCMV-FRB-RLuc8.6 plasmids were selected with 350 μg/mL zeocin. Single-cell colonies with the highest expression of reporter protein were selected by measuring RLuc8/RLuc8.6 activity using the coelenterazine substrate and cell sorting for stable cells expressing pCMV-FKBP12-TurboFP635. These cells were then used in a second step for transfection with pcDNA3.1-PURO-FRB-RLuc8.6. Single-cell colonies were selected by bioluminescence imaging and maintained using puromycin (8 μg/mL) and zeocin (350 μg/mL).

Western Blot Analysis. Stable HT1080 cells expressing the BRET vectors, RLuc8 or RLuc8.6 were harvested by trypsinization and lysed with passive lysis buffer (Promega) for 30 min on ice. Total protein concentration of the lysates was determined by Bradford assay. Equal amounts of total protein were loaded on 4–12% NuPAGE gels (Invitrogen) and subsequently transferred to a nitrocellulose membrane (Amersham) using a wet transfer system. The membrane was incubated for 1 h at room temperature with 5% nonfat milk powder in Tris-buffered saline containing 0.01% Tween 20, pH 7.6 (TBS-T). Subsequent to blocking, the membrane was incubated with anti-RLuc mouse monoclonal antibody (1:5,000; Millipore) or anti-tRFP rabbit polyclonal antibody (1:10,000; Evrogen) diluted in 5% milk solution in TBS-T at 4 °C overnight. The membrane was washed (three times for 5 min each) with TBS-T and subsequently incubated with HRP-conjugated anti-mouse secondary antibody or HRP-conjugated anti-rabbit secondary antibody (both 1:5,000 dilution). An anti–α-tubulin antibody (Aldrich) was used as loading control. Note that the second band noticed for some RFP fusion proteins results from the partial hydrolysis of the acylimine C = N bond, which is contained in the chromophores of RFPs, during sample heating under denaturing conditions (1).

Imaging the Rapamycin-Induced FRB/FKBP12 Interaction in Mice. Mice were first injected through the tail vein with 40 μg (in 20 μL DMSO) rapamycin diluted in 130 μL PBS. Control mice were injected only with DMSO (20 μL in 130 μL PBS). At 2 h post-rapamycin injection, mice were injected through the tail vein with HT1080 cells (3×10^6 in 150 μL PBS) stably expressing the FRB/FKBP12 BRET6 bimolecular sensor (FRB-RLuc8.6 and FKBP12-TurboFP635). Two hours later, mice were injected i.v. with CLZ (35 μg/mouse) and imaged in sequence using an IVIS-200 imaging system with open/donor/acceptor filters. In a different experiment, mice were injected with cells expressing only the donor unit of the sensor (FRB-RLuc8.6) and imaged similarly.

1. Martynov VI, et al. (2003) A purple-blue chromoprotein from Goniopora tenuidens belongs to the DsRed subfamily of GFP-like proteins. *J Biol Chem* 278:46288–46292.

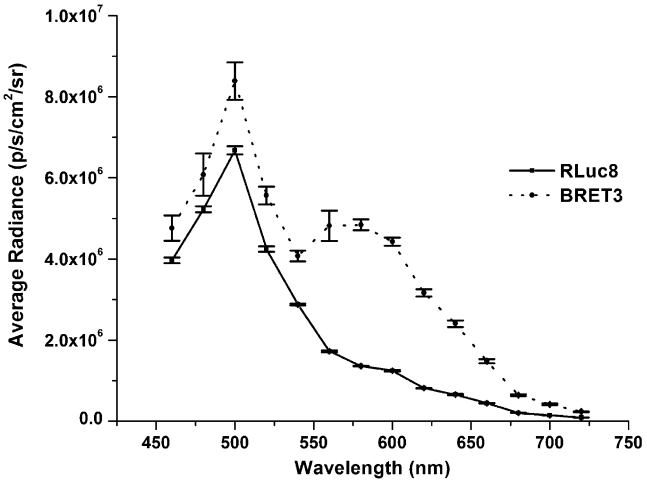

Fig. S1. Spectral imaging for the BRET3 systems and RLuc8 donor-only control. Spectral imaging of HT1080 cells expressing either RLuc8.6 or BRET6 using 20-nm filers in the 460–720 nm range. EnduRen luciferase substrate was used for this experiment. Error bars represent SD (n = 4).

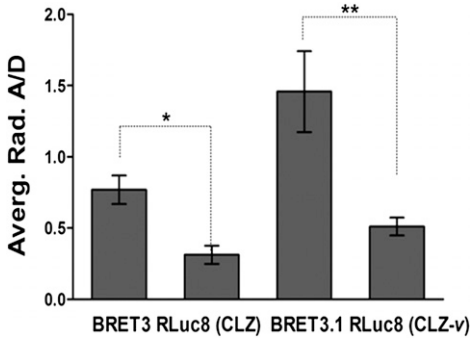

Fig. S2. The ratios of acceptor and donor bioluminescence emission (A/Ds) for BRET systems obtained from mice imaging experiments. A/Ds for BRET3, BRET3.1, and donor-only RLuc8 and RLuc8.6. Average A/Ds for BRET3, BRET3.1, RLuc8 [coelenterazine (CLZ)], and RLuc8.6 (CLZ-v) calculated from mice bioluminescence imaging experiments; for BRET3 and RLuc8 (CLZ), 480- and 560-nm filters were used, respectively. For BRET3.1 and RLuc8 (CLZ-v), 520- and 560-nm filters were used, respectively. *$P = 3.8 \times 10^{-12}$; **$P = 2.1 \times 10^{-5}$ (n = 10). Error bars = SD.

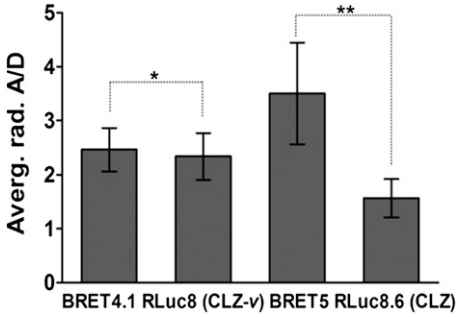

Fig. S3. A/Ds for BRET4.1, BRET5, and donor-only RLuc8 and RLuc8.6. Average A/Ds for BRET4.1, BRET5, RLuc8 (CLZ-v), and RLuc8.6 (CLZ) calculated from mice bioluminescence imaging experiments. For BRET4.1 and RLuc8 (CLZ-v), 520- and 580-nm filters were used, respectively. For BRET5 and RLuc8.6 (CLZ), 540- and 580-nm filters were used, respectively. *P = 0.6; **$P = 7.3 \times 10^{-7}$ (n = 10). Error bars = SD.

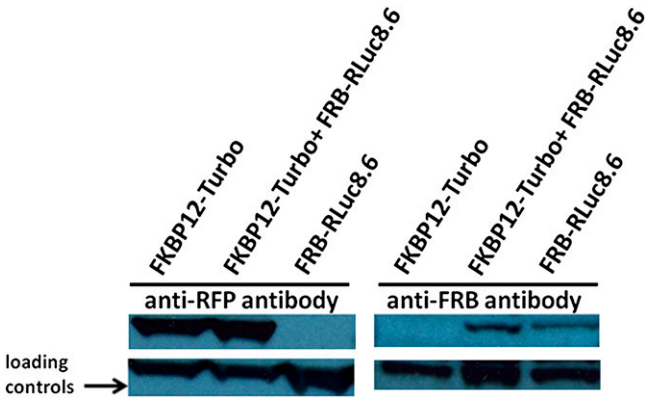

Fig. S4. Western blot analysis of BRET6 FRB/FKBP12 sensor proteins expression in stable HT1080 cells. HT1080 cells constitutively expressing the two fusion proteins of FRB/FKBP12 sensor, donor and acceptor fusion proteins, were lysed with passive lysis buffer. Equal amounts of protein were loaded on 4–12% NuPage gels and then, transferred to a nitrocellulose membrane. The membranes were probed with either anti-RFP (1:10,000 dilution) or anti-FRB (1:100 dilution) primary antibodies. Anti–α-tubulin primary antibody was used as a loading control for the anti-RFP blot. For the anti-FRB blot, a nonspecific band was used as a loading control.