

Supporting Information

Single-molecule dynamics of site-specific labeled transforming growth factor type II receptors on living cells

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Material and Methods

Materials and Plasmid Construction. ACPK were synthesized as previously reported.¹ The plasmid pCMV-Mb-ACPK-RS encoding Pyl tRNA synthetase (ACPK-RS) and its cognitive tRNA_{cuA}^{Pyl} were prepared as reported.² DBCO-PEG-TAMRA was purchased from Click Chemistry Tools (Scottsdale, AZ). The DNA fragments encoding full-length TβRII were point mutation in Asn40, Lys82, and Glu91 on the TβRII extracellular domain. All plasmids of three HA-TβRII-APCK variants and TβRII-APCK-GFP variants were prepared by QuikChange (Stratagene, La Jolla, CA) mutagenesis. The plasmids were confirmed by DNA sequencing.

Cell Culture, Transfection and Ligand Stimulation. HeLa and MCF-7 cells (Cell Resource Center, IBMS, CAMS/PUMC) were cultured in a 5% CO₂, water-saturated atmosphere and grown in DMEM (Gibco) medium supplemented with 10% fetal bovine serum (Hyclone), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Transfection was performed using Lipofectamine2000 (Invitrogen). Before transfection, cells were seeded in a 35-mm glass-bottom dish and incubated with 1 mM ACPK for twenty-four hours. The 0.5 ug plasmid pCMV-Mb-ACPK-RS was cotransfected with 0.5 ug plasmid carrying the target genes with an amber mutation (TβRII-ACPCK-GFP or HA-TβRII-ACPCK) into HeLa or MCF-7 cell lines for eight hours. For the ligand stimulation experiments, the transfected cells were added with 200 pM TGF-β1 (R&D, USA) in DMEM medium for 15 min at 37 °C .

Luciferase Assay. HeLa cells were transfected with 0.3 μg TβRII-GFP variants or HA-TβRII variants 0.3 μg ACPK-RS, 0.5 μg CAGA12-luciferase and the internal control vector pRenilla-TK (10 ng) using Lipofectamine2000 (Invitrogen). The cells were then serum-starved for 8 h before treating with TGF-β1 (200 pM) and luciferase activity was measured 16 h later using the Dual Luciferase Assay (Promega).

Fluorescent Labeling of TβRII-ACPCK on HeLa Cells. For TβRII-ACPCK labeling on living cells, cells transfected with TβRII-ACPCK were incubated with 10 nM DBCO545 at room temperature for thirty minutes. After incubation, cells were washed three times with D-PBS buffer (pH=7.4).

Single-Molecule TIRF Imaging. For single-molecule imaging, we used a home-built TIRFM system described previously.³ Briefly, the experiment was performed with objective-type total internal reflection fluorescence (TIRF) microscopy using an inverted Olympus IX71 microscope equipped with a total internal reflective fluorescence illuminator, 100×/1.45NA Plan Apochromatic TIR objective and an a 14-bit back-illuminated electron-multiplying charge-coupled device (EMCCD) camera (Andor iXon DU-897 BV). The microscope was equipped with a CO₂ incubation system (TOKAI HIT) and all living cell imaging was performed at 37 °C. Sample was excited by an argon laser (Melles Griot) with the power of 1 mW measured after the laser passing through the objective. The collected fluorescent signals were passed through two filters, BA510IF and HQ 525/50 (Chroma Technology) or HQ 605/50(Chroma Technology), before directed to the CCD camera. Movies of 200-300 frames were acquired for each sample at a frame rate of 20 Hz. Sequences of images were stored directly to a computer hard drive for subsequent analysis by IQ live cell imaging software (Andor, BT).

Image Analysis. The single-molecule images and movie were treated with Image J software (National Institutes of Health). The images were background subtracted using rolling ball method, and were threshold (six times of the mean intensity of an area with no fluorescent spots) to show single-molecule image of the receptor, such as Figure 2A.

A user-defined Matlab (Math Works Corp.) program was used to identify the single molecule spots in the images.³ The spots lasted less than 2 frames and covered less than 3 pixels were discarded to exclude the random noise. Moreover, only the spots which were diffraction limited (<3*3 pixels) and their PSF could be fitted well with a Gaussian function (the convergence of the fitting was reached, the roundness of the fitting ranged between 0.7 and 1.4, and the sharpness (FWHM of Gaussian fitting) ranged between 2/3 and 3 pixels) will be regarded as single molecule spots and chosen for the next analysis. With the pixel corresponding to the Gaussian fitting peak value as the central pixel, intensity values of the central pixel and surrounding 8 pixels were recorded. The averaged count of this 3x3 pixels area was used as the intensity (A.U.) of one single molecule to plot the photobleaching traces for each molecules and intensity distributions of different molecules. In photobleaching steps

analysis, the intensities were averaged with the two adjacent frames to minimize noise and show the steps clearly.

Single-Molecule Tracking. For dimer lifetime analysis, we selected from the movies by eye the two T β RII-40APCK-DBCO545 single-molecule spots which merged together, co-diffused for a period of time (> 100 ms, longer than 2 frames to exclude the accidental merge), and then separated. We calculated the T β RII-40APCK-DBCO545 co-diffusion time which accompanied with intensity change as the dimer lifetime. The dimer lifetime histogram is fitted with monoexponential decay function in Origin software to obtain the mean dimer lifetime.

To characterize the diffusion rate of single T β RII-40APCK-DBCO545 receptors, the positions of the single molecules in each frame were firstly determined by detecting significant local intensity maxima and fitting with two-dimensional Gaussian function. Then the positions of each molecule were linked between frames using a single-particle tracking algorithm based on u-track methods.^{4,6} The distance between two adjacent positions of one molecule was set to be less than 3 pixels.⁶ The traces longer than three frames were selected to exclude noise, and the linking gap was set to two frames to avoid photoblinking effect.

Colocalization Imaging. 0.5 μ g HA-T β RII-40ACPK or HA-T β RII were transfected in HeLa cells for 12 h and then fixed in cold 4% paraformaldehyde / PBS solution for 15 min. After that, the cells were incubated with 1:500 mouse anti-HA conjugated Alexa Fluor 488 (Invitrogen) and 10^{-8} M DBCO545 for one hour at 37 $^{\circ}$ C. The samples were rinsed several times with D-PBS (pH = 2) medium and then imaged by dual-color TIRF microscopy.

Supporting Figures

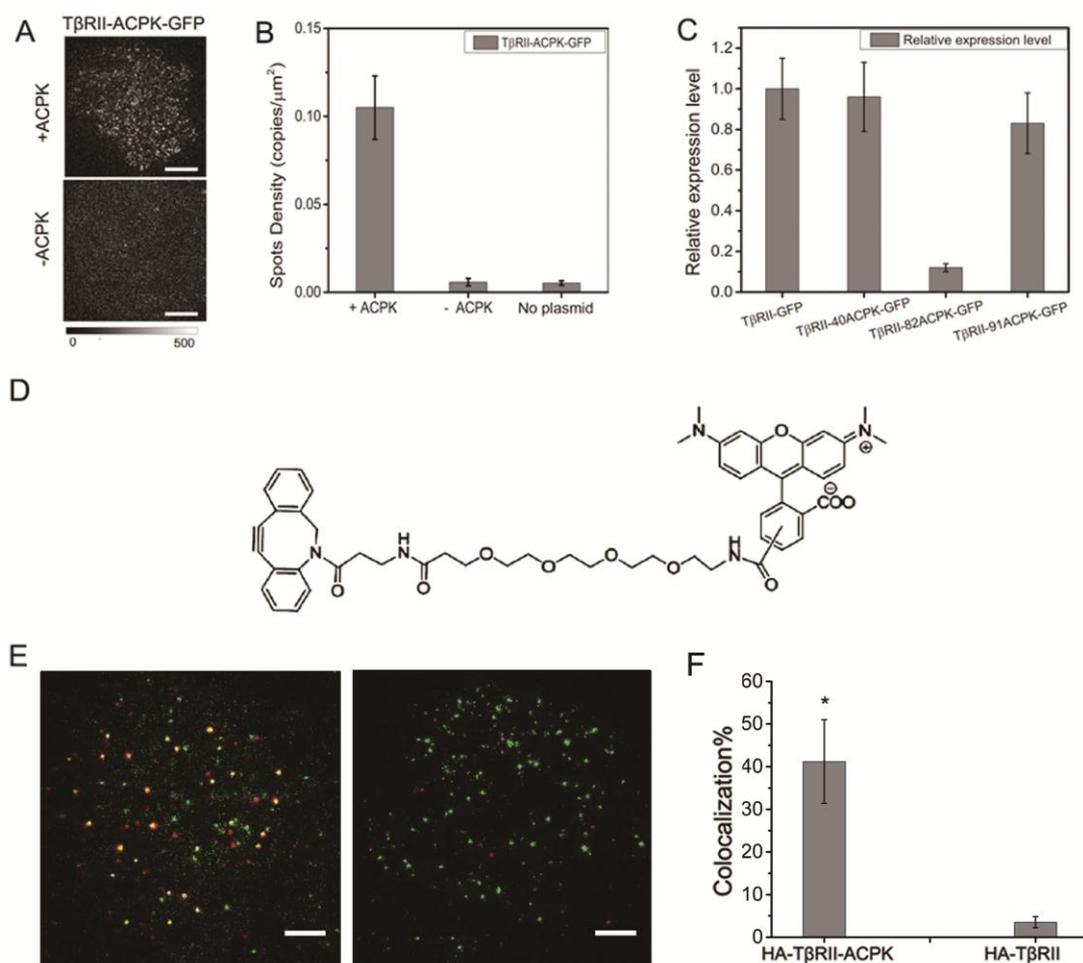


Figure S1. (A) Representative images showed the diffraction-limited fluorescent spots of TβRII-ACPK-GFP on resting HeLa cell membrane with (upper) and without (lower) ACPK incubation. Scale bar equals 10 μm . (B) The densities of GFP spots on the membranes of TβRII-ACPK-GFP-transfected cells with or without ACPK incubation and the untransfected cells. (C) Relative expression level of TβRII and TβRII-ACPK variants, measured by mean GFP fluorescence intensity. (D) Structure of the DBCO545. (E) Colocalization of HA488 with DBCO545 in fixed HeLa cells transfected with HA-TβRII-ACPK (left) or HA-TβRII (right). Cells were stained with HA488 (green) and DBCO545 (red) and imaged by multicolor TIRF microscopy. The yellow spots indicate colocalization. The scale bar was 5 μm . (F) Percentage of colocalization of HA488 with DBCO545 in cells expressing HA-TβRII-ACPK and HA-TβRII, respectively. Five cells were measured under each condition. Error bars represent SEM, *statistically difference from control group values by t-test, $p < 0.05$.

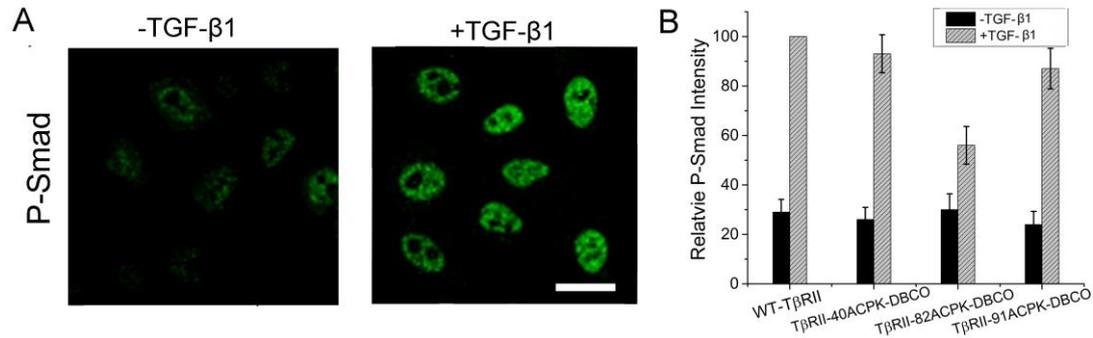


Figure S2. The signaling activity of TβRII-ACPK variants labeled with DBCO545 was examined by fluorescent intensity of P-Smad in the nuclear region. A) Typical confocal images of P-Smad in MCF-7 cells transfected with TβRII-40ACPK labeled by DBCO545 before (left) and after (right) 200 pM TGF-β1 treatment. The scale bar is 20 μm. B) The intensities of P-Smad in nuclear region were measured from confocal images and normalized by that with WT-TβRII and TGF-β1 treatment. Each data were obtained from 10 cells.

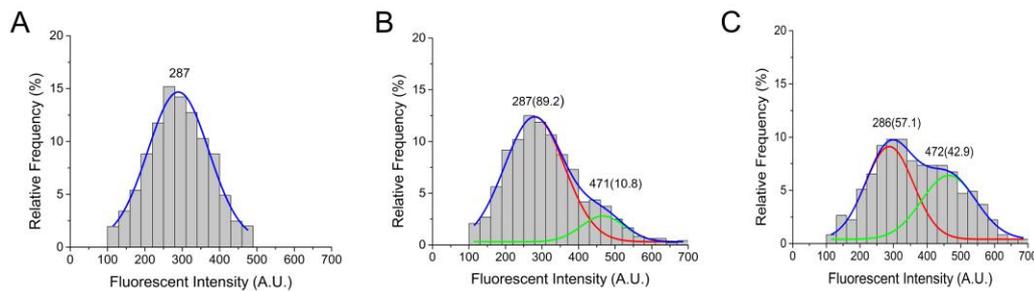


Figure S3. (A) The distributions of the fluorescent intensities of single DBCO545 molecules (n=200) on the coverslip. (B) The distributions of the fluorescent intensities of the TβRII-40APCK-DBCO545 spots (n=500) on living cells before and (C) after TGF-β1 stimulation. The solid curves indicate the fitting of Gaussian function and correlation coefficient is 0.97. The two peaks represented TβRII-40APCK monomers and dimers, respectively. The peak values of the fitting curves are shown above the curves, numbers in the parentheses are the percentage of the fractions. All the experiments have been repeated for three times.

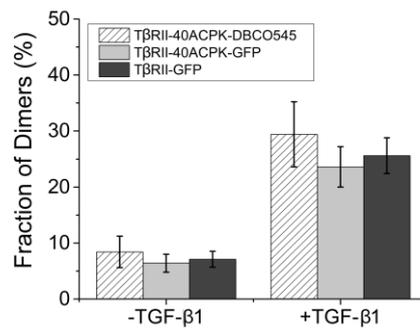


Figure S4. Dimer populations for TβRII-40ACPK-DBCO545 (white dashed bar), TβRII-40ACPK-GFP (gray bar) and TβRII-GFP (black bar) on cell membrane of the fixed MCF-7 cells with or without TGF-β1 treatment.

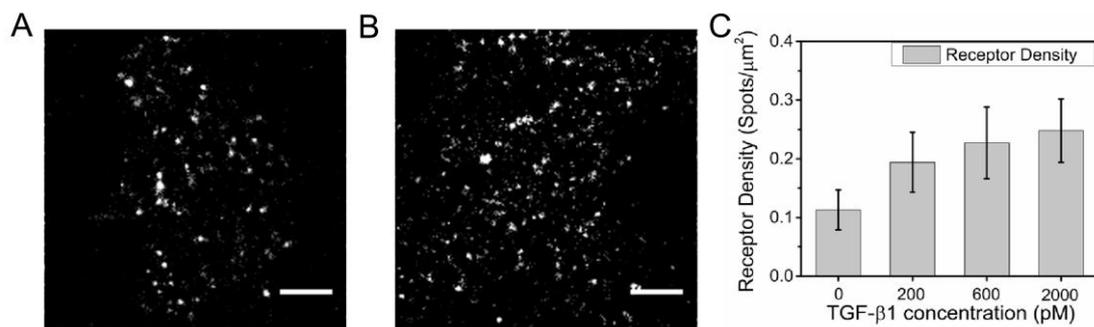


Figure S5. Density of TβRII receptors on cell membrane increased after ligand stimulation. (A) Typical TIRF image of TβRII-40APCK-DBCO545 in living cells before and (B) after TGF-β1 stimulation after background subtracted. The scale bar is 5μm. (C) Quantification of TβRII receptor density on cell membrane before and after 200 pM, 600 pM or 2000 pM ligand treatment. The data were obtained from five cells.

Supporting Movie 1. Imaging of single TβRII-40APCK-DBCO545 molecules on one living cell from which Figure 2A was derived. Image size: 41.6 um × 44.9 um.

Supporting Movie 2. Imaging of single TβRII-40APCK-DBCO545 molecules on another living cell. Image size: 21.1 um × 28.3 um.

Supporting Movie 3. Imaging of single TβRII-40APCK-DBCO545 molecules showing the monomer-dimer transition among two molecules. Image size: 4.8 um × 4.8 um.

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