Supporting Information

Bioorthogonal approach for imaging the binding between Dasatinib and its target proteins inside living cells

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Experimental sections

Construction of kinase-mCherry-Rab5b and LifeAct-TagRFP-kinase expression vectors

The wild type genes, ABL1 (NM_007313), SRC (BC011566), and CSK (BC106073) were obtained from Korean UniGene Information (KUGI, Korea) and Open Biosystems (Thermo Fisher Scientific Inc.). FRB-mCherry-Rab5b^{Q79L} vector was kindly provided by Professor W. D. Heo (KAIST, Korea). LifeAct-TagRFP vector was purchased from ibidi (Martinsried, Germany).

To make the kinase (ABL1, SRC and CSK)-mCherry-Rab5b expression vector, the FRB gene was removed from the pFRB-mCherry-Rab5b^{Q79L} using the restriction enzymes NheI and AgeI. The PCR-amplified ABL1, SRC, and CSK genes were cloned into the FRB gene-removed FRB-mCherry-Rab5b^{Q79L} vector. To construct the LifeAct-TagRFP-kinase (ABL1, SRC and CSK) expression vector, the EGFP gene was removed from the pEGFP-C3 (Clontech). The PCR-amplified LifeAct-TagRFP gene was cloned into the EGFP gene-removed EGFP-C3 vector. After construction of the pLifeAct-TagRFP-C3, the PCR-amplified ABL1, SRC, and CSK gene were cloned into the LifeAct-TagRFP-C3 vector. Primers and vectors used in this study are listed in Table S1 and Figure S2.

Cell culture and western blotting

HeLa cells were purchased from American Tissue Type Collection (ATTC) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂.

For the Western blotting experiments, antibodies against SRC (# 2123 for SRC-mCherry-Rab5b and # 2018 for LifeAct-TagRFP-SRC) and CSK (# 4980) were purchased from Cell Signaling Technology. HeLa cells were grown on culture plate to 50-70% confluence. Cells were transfected using a TurboFect (Thermo Fisher Scientific Inc.) according to the manufacturer's standard protocol. To generate protein lysates, transiently transfected cells were washed twice with cold phosphate-buffered saline (PBS), harvested by use of a cell scraper, and collected by centrifugation. Cell pellets were washed with PBS, lysed with pro-prep protein extraction buffer (Intron biotech. Korea), and then incubated on ice for 1h. The lysates were centrifuged and the supernatants were collected. Total protein samples were boiled for 10 min, separated by electrophoresis on a 12% polyacrylamide gel and electro-transferred to a nitrocellulose membrane. Nonspecific binding sites that were blocked in Tris-buffered saline (TBS) containing 5% BSA and 0.1% Tween-20 at room temperature for 1h. Membranes were rinsed and then incubated with anti-SRC (1:1,000) or anti-CSK (1:1,000) at 4 °C overnight followed by horseradish peroxidase conjugate goat anti-rabbit IgG (1:2,000, sc-2004, Santa Cruz Biotechnology) at

room temperature for 2 h. After rinsing with buffer, the immunocomplexes were visualized with chemiluminescence using the ImageQuant LAS 4000 (GE Healthcare) according to the manufacturer's instructions.

SRC inhibition assays

HeLa cells were transiently transfected with SRC-mCherry-Rab5b or LifeAct-TagRFP-SRC. Transfected cells were washed with serum free DMEM. Cells were treated with either Dasatinib or Dasatinib-TCO (1 and 3 \Box M, 0.1% DMSO in serum free DMEM) for 1 h. Cells were then washed with cold PBS, harvested by use of a cell scraper, and collected by centrifugation. Immunoblotting analysis was performed with pY416-SRC (1:1,000, # 2101, Cell Signaling Technology) and SRC antibodies.

Cell imaging

HeLa cells were grown on 25 mm round coverslips (Knittel glaser Inc.) in a 6-well culture plate to 50–70% confluence. Cells were transfected using a TurboFect according to the manufacturer's standard protocol. Transfected cells were rinsed once with serum free DMEM and treated with 1µM Dasatinib-TCO (0.1% DMSO in serum free DMEM) for 30 min. Cells were rinsed once and left in culture medium (DMEM with 10% v/v FBS) for a further 1 h at 37 °C to allow excess unreacted Dasatinib-TCO to wash out of the cells. Cells were treated with 200 nM CFDA-Tz (0.1% DMSO in serum free DMEM) for 10 min at 37 °C, then rinsed twice with culture medium, and kept at 37 °C in order for excess dye to wash out. The culture medium was replaced twice more, 30 min later, to improve the washout. The cells on round coverslips were mounted on a home-made perfusion chamber, which was connected to a temperature controller set at 37 °C, and 1 mL of culture medium was added. Live cells were imaged using a laser-scanning confocal microscope (Zeiss LSM 710 and Nanoscope systems K1-Fluo) with a 40X water immersion lens.

In vitro kinase assays

The IC50 of Dasatinib-TCO for ABL1, SRC, and CSK were determined using the Kinase-Glo® Plus luminescent assay kit (Promega). Recombinant active kinases against ABL1 and SRC were purchased from SignalChem, and the corresponding peptide substrates (ABL1: EAIYAAPFAKKK and SRC: KVEKIGEGTYGVVYK) were used. The ATP and substrate peptide concentrations used in the assay were 100 μ M and 100 μ M, respectively. Dose-dependent inhibition assays were performed by varying the concentration of the probes under kinase concentration of ~ 25 nM. The IC50 values of the probes were calculated from the percentage activity vs. log [concentration of probe] curves generated using GraphPad Prism software.

Cytotoxicity assays

HeLa cells (non-transfected) and each of the vector-expressing HeLa cells were cultured in a 96well plate for 12–18 h and then incubated with either none, 0.1% DMSO (vehicle), or different concentrations of Dasatinib-TCO (300 nM–10 μ M, 0.1% DMSO in serum free DMEM) at 37 °C for 1 h. Cell viability was evaluated using a Cell Counting Kit-8 (Dojindo Molecular Technologies Inc.) according to the manufacture's protocol. Absorbance at 450 nm was measured using a SpectraMax M4 plate reader (Molecular Devices).

Synthesis and Characterization Data



Preparation of Dasatinib-TCO

Dasatinib-TCO was synthesized from 4Chem Laboratory (Suwon, Korea). All reagents were obtained from commercial sources and used without further purifications. Dasatinib was obtained from LC Laboratories (Boston, MA). (*E*)-Cyclooct-4-enyl-2,5-dioxo-1-pyrrolidinyl carbonate (TCO-NHS ester) was purchased from Jena Bioscience (Jena, Germany). Triethylamine (TEA) and *N*,*N*-diisopropylethylamine (DIPEA) were distilled from CaH₂ and stored over anhydrous KOH. Flash chromatography was carried out using silica gel (F60, 230-400 mesh) and thin layer chromatography (TLC) was conducted on silica gel 60F-254, 0.25 mm pre-coated TLC plates that were purchased from Merck. TLC plates were visualized using UV254 and acidic *p*-anisaldehyde solution or 7% ethanolic phosphomolybdic acid with charring. 1H and 13C nuclear magnetic resonance spectra were recorded on a Bruker AVANCE 400, 500 and 900 MHz spectrometer. Mass spectrum was measured on a Waters Synapt G2 quadrupole time-of-flight (TOF) mass spectrometer equipped with an electrospray ion source and Agilenet 1200 HPLC system with UV-VIS Diode array detector (J'sphere ODS-H80 column, particle size 4 μ m, pore size 8 nm, 150 x 4.6 mm I.D.).

Dasatinib-amine was prepared starting from commercially available Dasatinib according to the literature method. ¹H NMR (500 MHz, DMSO- d_6): δ 9.85 (br s, 1H), 8.21 (s, 1H), 7.39 (dd, J = 7.5,

1.5 Hz, 1H), 7.27 (m, 2H), 6.05 (s, 1H), 3.50 (t, J = 5.0 Hz, 4H), 2.63 (t, J = 6.5 Hz, 2H), 2.43 (t, J = 5.0 Hz, 4H), 2.40 (s, 3H), 2.33 (t, J = 6.5 Hz, 2H), 2.28 (s, 3H). See reference: Fischer, J. J.; Dalhoff, C.; Schrey, A. K.; Baessler, O. Y. G.; Michaelis, S.; Andrich, K.; Glinski, M.; Kroll, F.; Sefkow, M.; Dreger, M.; Koester, H. J. Proteomics 2011, 75, 160–168.

To a solution of amine-modified Dasatinib (30.1 mg, 0.0618 mmol) in DMF (1 mL) was added TCO-NHS ester (19.8 mg, 0.0741 mmol) and DIPEA (32.1 μ L, 0.184 mmol). The mixture was stirred for 12 h and the solvent was removed under reduced pressure. The crude reside was purified by flash column chromatography (CH₂Cl₂:MeOH = 10:1 to 7:1) to give the desired product Dasatinib-TCO (25 mg, 63%) as a white solid. ¹H-NMR (900 MHz, DMSO-*d*₆): δ 11.42 (br s, 1H), 9.86 (s, 1H), 8.21 (s, 1H), 7.40 (d, *J* = 6.5 Hz, 1H), 7.29-7.24 (m, 2H), 6.85 (t, *J* = 6.0 Hz, 1H), 6.04 (s, 1H), 5.58-5.55 (ddd, *J* = 16.0, 10.5, 4.0 Hz, 1H), 5.45-5.41 (ddd, *J* = 16.0, 11.0, 3.0 Hz, 1H), 4.20 (br s, 1H), 3.49 (br s, 4H), 3.09 (m, *J* = 7.0 Hz, 2H), 2.44 (m, 4H), 2.40 (s, 3H), 2.35 (t, *J* = 7.0 Hz, 2H), 2.28-2.26 (m, 2H), 2.23 (s, 4H), 1.90-1.82 (m, 4H), 1.64-1.52 (m, 3H). ¹³C-NMR (225 MHz, DMSO-*d*₆): 165.14, 162.53, 162.35, 159.89, 156.91, 155.77, 140.82, 138.79, 134.89, 133.49, 132.49, 128.99, 128.14, 126.98, 125.67, 82.60, 79.02, 59.72, 57.23, 52.21, 43.56, 40.66, 38.19, 37.64, 33.72, 32.13, 30.58, 25.55, 20.76, 18.27, 14.06 ppm. ESI-HRMS m/z [M+H]⁺: calculated 639.2633 for C₃₁H₄₀ClN₈O₃S, found 639.2632. Preparation of **CFDA-Tz**



Carboxyfluorescein diacetate-tetrazine (CFDA-Tz) was prepared starting from commercial available 3-(*p*-benzylamino)-1,2,4,5-tetrazine-5-fluorescein (Tz-F) (Jena Bioscience, Germany) according to the literature method. ¹H NMR (400 MHz, DMSO-*d*₆): δ 10.57 (s, 1H), 9.38 (t, *J* = 5.86 Hz, 1H), 8.45 (d, *J* = 8.58 Hz, 2H), 8.25 (m, 1H), 8.12 (dd, *J* = 0.70, 8.05 Hz, 1H), 7.76 (dd, *J* = 0.74, 1.38 Hz, 1H), 7.57 (d, *J* = 8.63 Hz, 2H), 6.70 (m, 2H), 6.59 (m, 4H), 4.56 (d, *J* = 5.82 Hz, 2H), 2.37 (s, 6H). ESI-MS m/z [M+Na]⁺: calculated 652.15 for C₃₄H₂₃N₅O₈Na, found 652.14. See references: (a) Liu, D. S.; Tangpeerachaikul, A.; Selvaraj, R; Taylor, M. T.; Fox, J. M.; Ting, A. Y. *J. Am. Chem. Soc.* **2012**, *134*, 792–795. (b) Yang, K. S.; Budin, G.; Reiner, T.; Vinegoni, C.; Weissleder, R. *Angwe. Chem. Int. Ed.* **2012**, *51*, 6958–6603.



¹H NMR spectrum of Dasatinib-amine (500 MHz, DMSO-*d*₆)

¹H NMR spectrum of Dasatinib-TOC (900 MHz, DMSO-*d*₆)

1H of Dasatinib-TCO in DMSO



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¹³C NMR spectrum of Dasatinib-TOC (225 MHz, DMSO-*d*₆)

13C of Dasatinib-TCO in DMSO

65.14 62.53 62.35 62.35 59.89 55.91 .55.77	40.82 34.89 34.89 333.49 333.49 28.94 28.94 28.94 25.67 25.67	9.02	0.7.23 2.21 2.21 2.20 0.00 0.44 0.50 0.50 0.41 0.31	4.06 4.06 4.06 5.55 4.06 4.06 4.06	NAME EXPNO PROCNO	0729_kbsi_1kb_900 2 1
		8			F2 - Acq Date Time INSTRUM PROBHD PULPROG TD SOLVENT NS DS SWH FIDRES	uisition Parameters 20160729 9.44 5 mm CPTCI 1H- zgpg30 65536 DMSO 720 8 56818.184 Hz 0.866977 Hz
		N O			AQ RG DW DE TE D1 D11 TD0 ======== SF01	0.5767168 sec 2050 8.800 used 298.1 K 3.0000000 sec 0.03000000 sec 1 = CHANNEL f1 ======= 226.3901776 MHz
		н		1	NUC1 P1 PLW1 SF02 NUC2 CPDPRG[2 PCPD2 PLW2 PLW12 PLW13	13C 11.80 usec 117.48999786 W CHANNEL f2 ======= 900.2343211 MHz 1H Waltz16 55.00 usec 12.58899975 W 0.32817000 W 0.16528000 W
					F2 - Pro SI SF WDW SSB LB GB PC	ocessing parameters 65536 226.3631271 MHz EM 0 2.00 Hz 0 1.40
160 150	140 130 120 110 100	90 80 7	0 60 50 40	30 20 ppm		

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Current Data Parameters

ESI-HRMS spectrum of Dasatinib-TCO



HPLC trace of Dasatinib-TCO



ESI-MS spectrum of CFDA-Tz





¹H NMR spectrum of CFDA-Tz (400 MHz, DMSO-*d*₆)

Table S1: List of primers in this study.

5'-CTA <u>GCTAGC</u> CCACCATGGTGTCCCCCAACTACGACAAGTG-3' (NheI) 5'-GACT <u>ACCGGT</u> AGGGCGCCGGCGCCGGCGCCC CTGGAACATTGTTTCAAAGGCTT-3' (AgeI)	pFRB-mCherry- Rab5b ^{Q79L}	pABL1-mCherry-Rab5b
5'-CTA <u>GCTAGC</u> CCACC ATGGGTAGCAACAAGAGCAAGCC-3' (NheI) 5'-GACT <u>ACCGGT</u> AGGGCGCCGGCGCCGGCGCC GAGGTTCTCCCCGGGCTGGT-3' (AgeI)	pFRB-mCherry- Rab5b ^{Q79L} □	pSRC-mCherry-Rab5b
5'-CTA <u>GCTAGC</u> CCACCATGTCAGCAATACAGGCCGC-3' (NheI) 5'-GACT <u>ACCGGT</u> AGGGCGCCGGCGCGGCGCC CAGGTGCAGCTCGTGGGTTTT-3' (AgeI)	pFRB-mCherry- Rab5b ^{Q79L}	pCSK-mCherry-Rab5b
5'-CTA <u>GCTAGC</u> CGCCACCATGGGTGTCGCAGATTTGATCAAG-3' (NheI) 5'-GA <u>AGATCT</u> GAATTAAGTTTGTGCCCCAGTTTGC-3' (BgIII)	pEGFP-C3	pLifeAct-TagRFP-C3
5'-CTA <u>CTCGAG</u> CTCATGGTGTCCCCCAACTACGACAAGTG-3' (XhoI) 5'-GCC <u>GAATTC</u> CTACTGGAACATTGTTTCAAAGGCTT-3' (EcoRI)	pLifeAct-TagRFP-C3	pLifeAct-TagRFP- ABL1
''-CTA <u>CTCGAG</u> CTCATGGGTAGCAACAAGAGCAAGCC-3' (XhoI) ''-GCC <u>GAATTC</u> CTAGAGGTTCTCCCCGGGCTG-3' (EcoRI)	pLifeAct-TagRFP-C3	pLifeAct-TagRFP-SRC
I'-CTA <u>CTCGAG</u> CTCATGTCAGCAATACAGGCCGCC-3' (XhoI) I'-CGC <u>GGATCC</u> TCACAGGTGCAGCTCGTGGGGTT-3' (BamHI)	pLifeAct-TagRFP-C3	pLifeAct-TagRFP-CSK
5'-5'- 5'- 5'- 5'- 5'- 5'- 5'- 5'	-CTA <u>GCTAGC</u> CCACCATGGTGTCCCCCAACTACGACAAGTG-3' (NheI) -GACT <u>ACCGGT</u> AGGGCGCCGGCGCGCGCGCGCCCCTGGAACATTGTTTCAAAGGCTT-3' (AgeI) -CTA <u>GCTAGC</u> CCACC ATGGGTAGCAACAAGAGCAAGCC-3' (NheI) -GACT <u>ACCGGT</u> AGGGCGCCGGCGCGGCGCCGCGCGCGCGCGCGCGCGCGC	CTAGCTAGCCACCATGGTGTCCCCCAACTACGACAAGTG-3' (NheI) pFRB-mCherry- Rab5b ^{079L} CTAGCTAGCCCACC ATGGGTAGCAACAAGAGCAAGCC-3' (NheI) pFRB-mCherry- Rab5b ^{079L} CTAGCTAGCCCACC ATGGGTAGCAACAAGAGCAAGCC-3' (NheI) pFRB-mCherry- Rab5b ^{079L} CTAGCTAGCCCACCATGTCAGCAATACAGGCCGCC GAGGTTCTCCCCGGGCTGGT-3' (AgeI) pFRB-mCherry- Rab5b ^{079L} CTAGCTAGCCCACCATGTCAGCAATACAGGCCGCC-3' (NheI) pFRB-mCherry- Rab5b ^{079L} CTAGCTAGCCGCCACCATGGGTGTCGCAGATTTGATCAAG-3' (NheI) pEGFP-C3 -CTAGCTAGCCGCCACCATGGGTGTCCCCAACTACGACAAGTG-3' (XhoI) pLifeAct-TagRFP-C3 -CTACTCGAGCTCATGGTAGCAACAAGAGCAAGCC-3' (XhoI) pLifeAct-TagRFP-C3 CTACTCGAGCTCATGGTAGCAACAAGAGCAAGCC-3' (XhoI) pLifeAct-TagRFP-C3 CTACTCGAGCTCATGGTAGCAACAAGGCCAGCCC-3' (XhoI) pLifeAct-TagRFP-C3 CTACTCGAGCTCATGGTAGCAACAAGAGCCAGCC-3' (XhoI) pLifeAct-TagRFP-C3 CTACTCGAGCTCATGGTAGCAACAAGGCCAGCC-3' (XhoI) pLifeAct-TagRFP-C3 CTACTCGAGCTCATGTCAGCAATACAGGCCGCC-3' (XhoI) pLifeAct-TagRFP-C3 CTACTCGAGCTCATGTCAGCAATACAGGCCGCC-3' (XhoI) pLifeAct-TagRFP-C3

^aABL1 kinase catalytic domain (amino acids 247 ~ 517) was used in this study.



Figure S1. Spatially-localized expression of Dasatinib target kinases (ABL1, SRC, and CSK). mRFP vector (expressed in the cytoplasm), Rab5b vector (expressed on endosome), and LifeAct vector (expressed on F-actin). All scale bars are $10 \mu m$.



Figure S2. Ribbon representation of the structure of the c-ABL (A) and c-SRC (B) kinase domain in complex with Dasatinib (yellow, left), Dasatinib-TCO (blue, middle), and overlay (right).



Figure S3. Schematic representation of the constructed expression vectors. (A) Kinase-mCherry-Rab5b expression vectors. (B) LifeAct-TagRFP-kinase expression vectors.



Figure S4. Western blot analysis of the HeLa cells transfected with different constructed expression vectors. ABL1catalytic domain was not detected by commercially available antibody.



Figure S5. Cell viability results of HeLa (non-transfected) and each of vector-expressing HeLa cells treated with different concentration of Dasatinib-TCO for 1 h.



Figure S6. Confocal images of endosome-localized, (A) kinase-mCherry-Rab5b and F-actin-localized, (B) LifeAct-TagRFP-kinase. Nuclei were stained with DAPI. All scale bars are 10 µM.

(A) Kinase-mCherry-Rab5b





Figure S7. Colocalization between Dasatinib-TCO and expressed its target kinases ((A) kinasemCherry-Rab5b and (B) LifeAct-TagRFP-kinase) in fixed HeLa cells. Live cells were first incubated with Dasatinib-TCO and fixed. The fixed cells were permeabilized, followed by staining with fluorescein-Tz (F-Tz). Nuclei were stained with DAPI. All scale bars are 10 μ M.



Figure S8. Control experiment without treatment of Dasatinib-TCO. Dasatinib target kinases ((A) kinase-mCherry-Rab5b and (B) LifeAct-TagRFP-kinase)-expressed HeLa cells were directly treated with CFDA-Tz. All scale bars are 10μ M.