Bioorthogonal Chemical Activation of Kinases in Living Systems

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1. Supplementary Experimental Procedures

1.1 Plasmid Construction

The plasmids pCMV-*Mm*PylRS-306A/384F and pSupAR-*Mm*PylRS-306A/384F encoding the *Mm*PylRS-306A/384F mutant synthetase-$tRNA^{Pyl}_{CUA}$ pair for site-specific incorporation of unnatural amino acids (UAAs, including TCOK/BCNK/CbzK) in eukaryotic and prokaryotic cells, respectively, as well as the plasmid pBAD-GFP-N149TAG for GFP expression have been all reported in our previous work.[1] The plasmids for expression of MEK1 variants from E. *coli* (pBAD-MEK1-ΔN, pBAD-MEK1-ΔN-K97R, pBAD-MEK1-ΔN-K97TAG) were constructed on the pBAD-mycHisA vector. The plasmids for eukaryotic protein expression including MEK1 variant (pcDNA4-MEK1-ΔN-K97TAG), FAK variants (pcDNA4-FAK, pcDNA4-FAK-YM, pcDNA4-FAK-YM-KD, pcDNA4-FAK-YM-Y397F, pcDNA4-FAK-YM-K454TAG, pcDNA4-FAK-YM-KD-K454TAG, pcDNA4-FAK-Y397F-K454TAG) and Src variants (pcDNA4-Src-Y527F, pcDNA4-Src-K295R-Y527F, pcDNA4-Src-K295TAG-Y527F, pcDNA4-Src-Y527F-EGFP, pcDNA4-Src-K295R-Y527F-EGFP, pcDNA4-Src-K295TAG-Y527F-EGFP) were all constructed on the pcDNA4-mycHisB vector. Plasmids for GST-ERK expression and Elk luciferase reporter (Elk-Gal4, Gal4-fLuc, pRL-TK) were kind gifts from Prof. Feng Shao (National Institute of Biological Science, China).[2] LifeAct-mRFP plasmid was a generous gift from Prof. Li Yu (Tsinghua University, China) to visualize filament actin.[3,4] The plasmid for firefly luciferase expression (pcDNA3.1-fLuc-K529TAG) was reported in our previous work.[5]

1.2 Reagents and Instruments

TCOK(**2**), Me_2Tz(**3**) and ONPK(**1**) were synthesized according to the previously reported procedure.[1,6] CbzK was purchased from J&K Scientific, while BCNK was purchased from Synaffix (a mixture of exo/endo =5:1). Primary antibodies were purchased from Cell Signaling Technology (CST), ThermoFisher or ZSGB-BIO as listed below: Myc-tag (#2276, CST), His-tag (#2366, CST), ERK (#9102, CST),

p-ERK (#9101, CST), pY397-FAK (PA5-17084, ThermoFisher), Src (#2110, CST), pY416-Src (#6943, CST), p-Tyr-100 (#9411, CST) and actin (TA-09, ZSGB-BIO). HRP-linked secondary antibody were purchased from CST. Fluorescence secondary antibodies and Phalloidin-Alexa Fluor 568 were purchased from Invitrogen. Protein immunoprecipitation was performed using protein A/G plus agarose beads (sc-2003, Santa Cruz). Protein purifications were performed on an AKTA START protein purification system (GE Healthcare). Protein LC-MS was performed using an ACQUITY UPLC I-Class SQD-2 (Waters) system with electrospray ionization (ESI). Western blot images were captured on a ChemiDoc XRS+ molecular imager (Bio-Rad) or Tanon 5200 Multi image system (Tanon). Luminescence of cell samples were acquired on a Synergy H4 microplate reader (Bio-Tek), while the luminescence in living mice was measured on IVIS Lumina II imaging system (PerkinElmer). Confocal microscopy images were obtained on LSM 700 laser scanning confocal microscope (Zeiss).

1.3 Cell Culture

HEK293T, NIH3T3, HeLa and CHO cell lines were purchased from American Type Culture Collection (ATCC). The 293A cell line was purchased from Invitrogen, ThermoFisher. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C in 5% CO_2.

2. Supplementary Figures

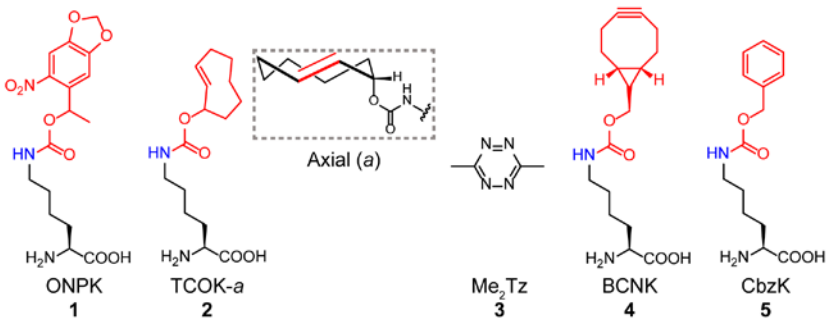

Figure S1. Chemical structures of unnatural amino acids and chemical reagents used in this study. Caging group (red) blocks the primary amine group (blue) of native lysine.

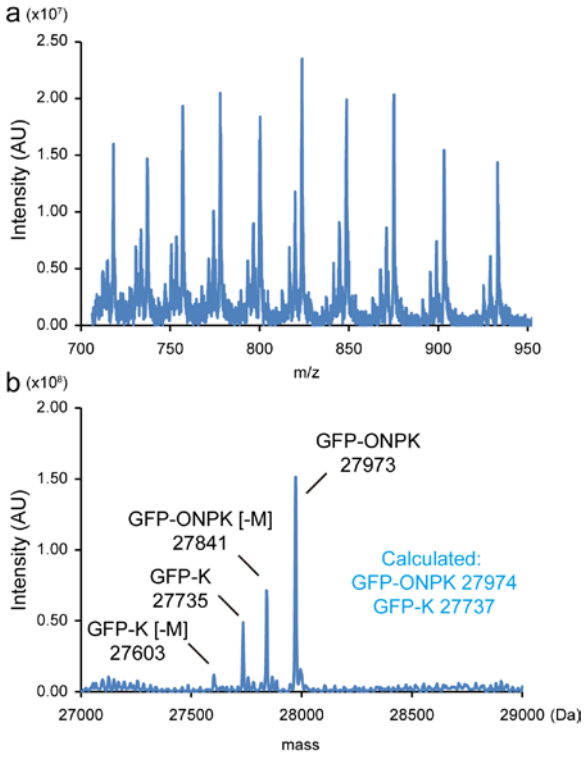

Figure S2. LC-MS analysis of GFP-N149ONPK. (a) The original ESI-MS results of GFP-N149ONPK expressed and purified from E. *coli* (DH10B). (b) The deconvoluted spectrum of GFP-N149ONPK purified from E. *coli*. The MS spectra showed peaks of intact GFP-N149ONPK protein (MW: 27973 Da) and the corresponding -Met form (MW: 27841 Da), as well as naturally reduced and decaged GFP-N149K (MW: 27735 Da) and the corresponding -Met form (MW: 27603 Da), indicating partial spontaneous reduction of ONPK. Theoretical MW: GFP-N149ONPK, 27974 Da; GFP-N149K, 27737 Da. About 25% of total proteins were decaged under the condition we tested measured by relative intensity ratio.

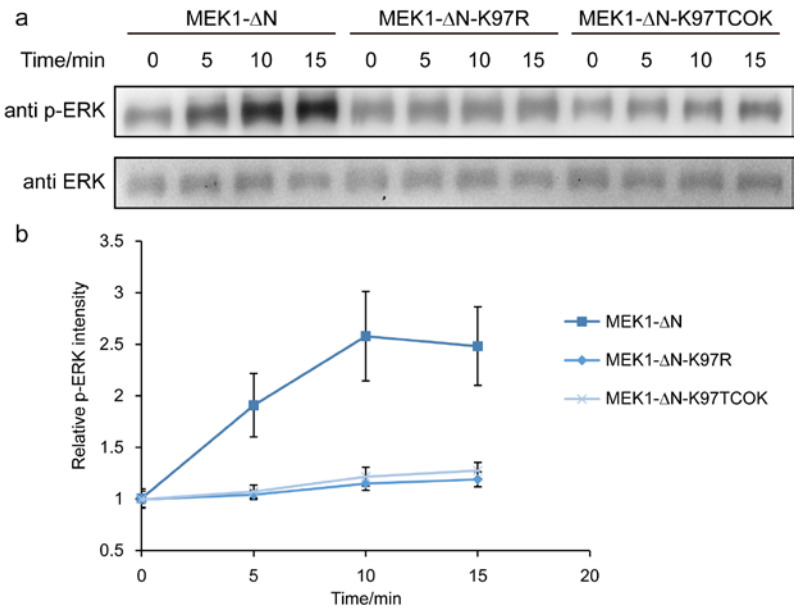

Figure S3. *In vitro* kinase assay on MEK1. (a)The phosphorylation level of ERK indicated the blocked activity of both MEK1-ΔN-K97R mutant and MEK1-ΔN-K97TCOK caged MEK1 mutant. (b) Quantification of relative p-ERK level from three independent experiments. The relative p-ERK phosphorylation level of MEK1-ΔN-K97TCOK was similar to MEK1-ΔN-K97R dead mutant and much lower than active MEK1-ΔN protein.

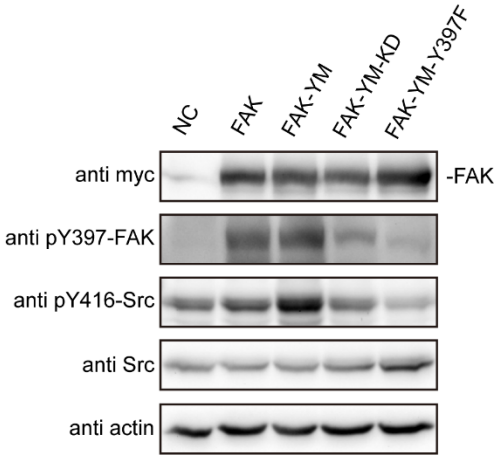

Figure S4. Phosphorylation analysis of several FAK mutants. FAK-YM mutant showed higher activity than wild-type FAK on autophosphorylation and phosphorylation of downstream Src, while KD or Y397F mutation strongly eliminated the phosphorylation activity. YM: Y180A/M183A hyperactive mutation, KD: D546R kinase dead mutation, Y397F: Y397F phosphorylation site mutation.

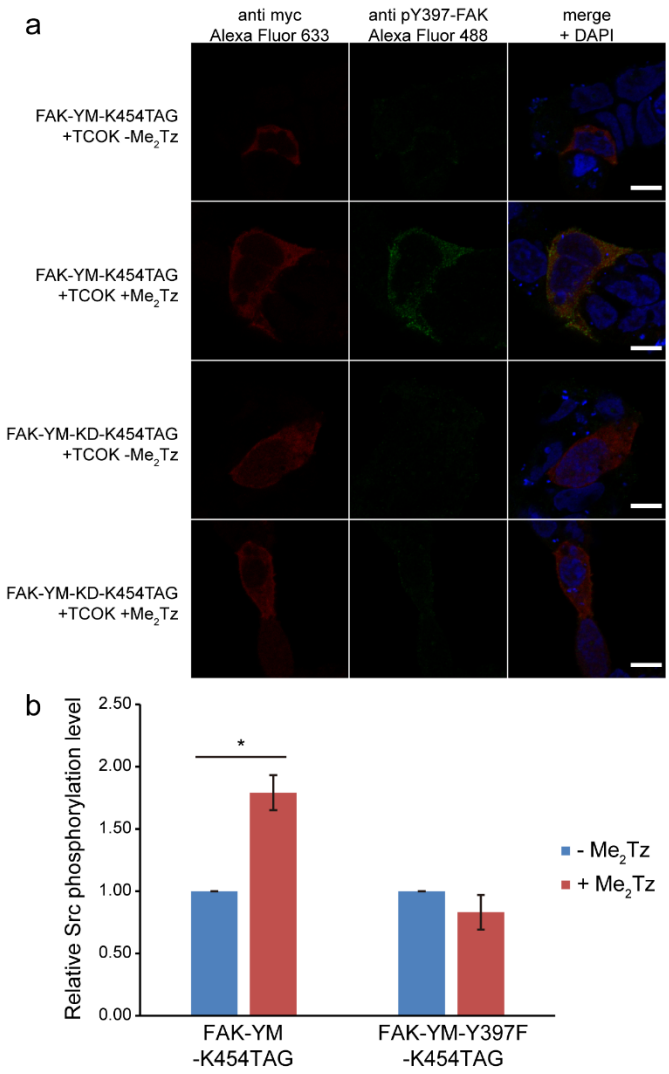

Figure S5. Bioorthogonal cleavage mediated FAK activation. (a) Representative images of immunofluorescence staining of FAK and pY397-FAK. FAK phosphorylation level was increased in FAK-YM-K454TCOK expressing cells upon Me_2Tz treatment. Scale bars, 5 μm. (b) Statistical analysis (mean ± s.d., n = 3) of Figure 2d. Src phosphorylation level was increased in Me_2Tz treated hyperactive FAK-YM-K454TCOK group but not in the Y397F mutant group. *P = 0.0142 by ANOVA. Cells were treated with 100 μM Me_2Tz for 1 h.

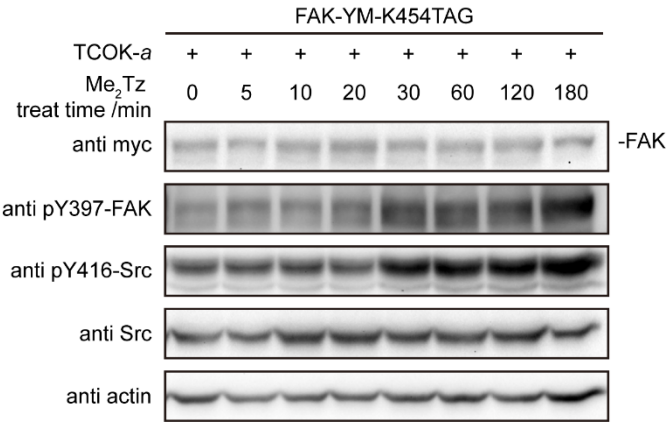

Figure S6. Activation of FAK at different time points after Me_2Tz treatment. FAK autophosphorylation level and Src phosphorylation level both reached saturation 30 min after Me_2Tz addition. Me_2Tz, 100 μM.

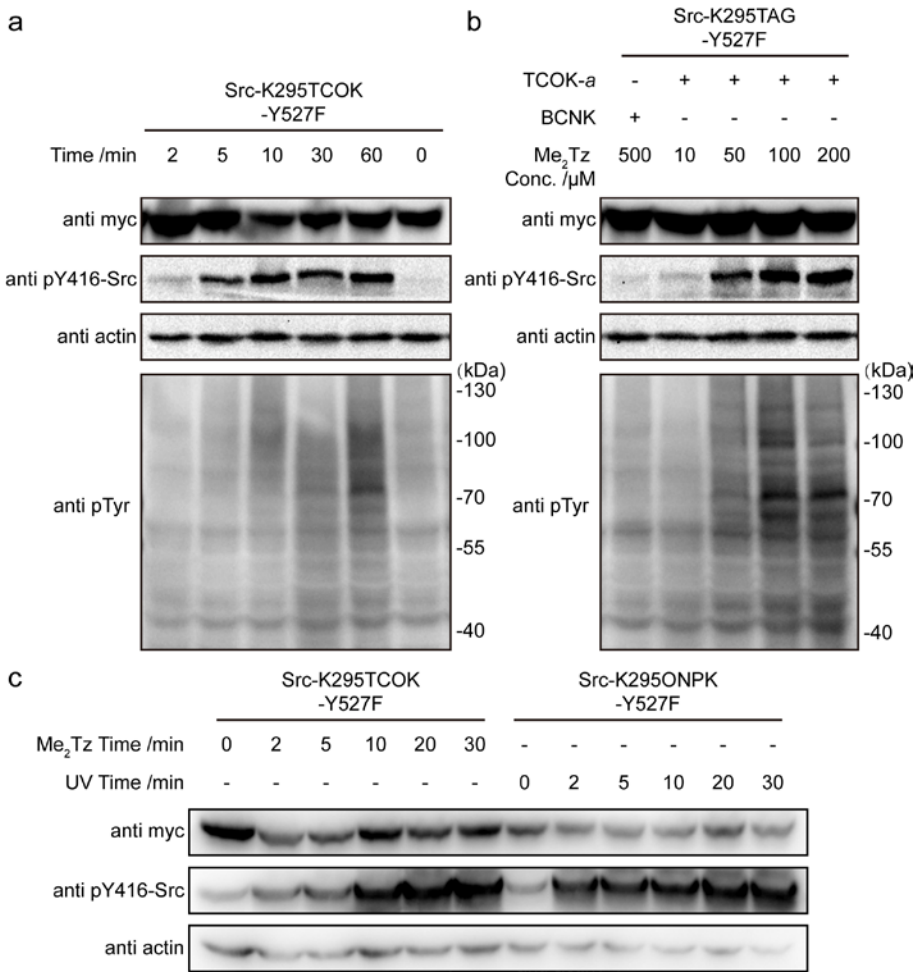

Figure S7. Time and concentration dependence of chemical decaging mediated oncogenic Src activation and the comparison with UV-mediated decaging. (a) Src autophosphorylation and total Tyr phosphorylation in Src-K295TCOK-Y527F expressing cells at different time points after Me_2Tz treatment (100 μM). (b) The restored Src autophosphorylation and total Tyr phosphorylation by different concentrations of Me_2Tz in the Src-K295TCOK-Y527F expressing cells within 1 h. (c) Side by side comparison of Me_2Tz-mediated chemical activation (100 μM Me_2Tz) and UV-mediated activation (365 nm). Me_2Tz-mediated chemical activation reached saturation of p-Src level in about 10 min, while UV-mediated activation reached saturation in 2-5 min.

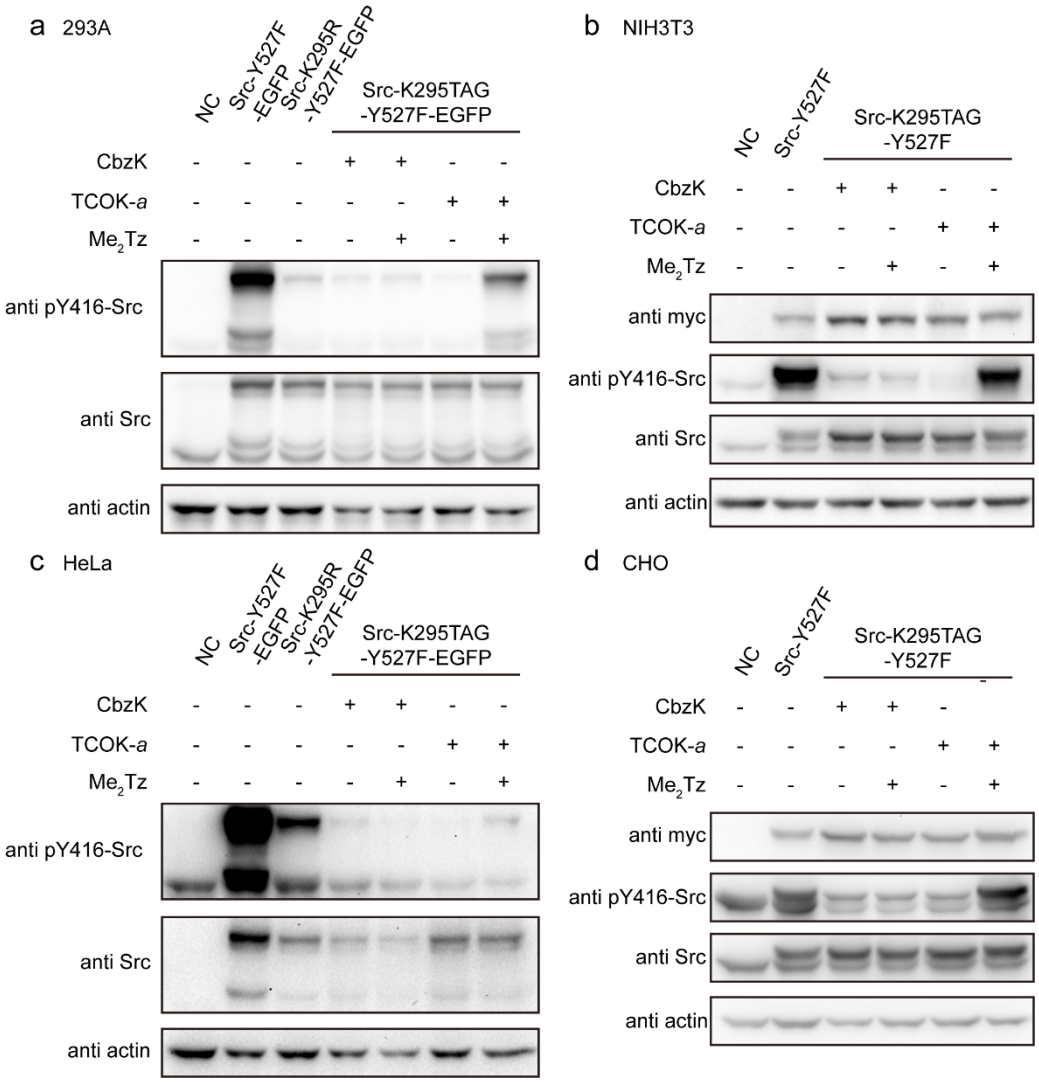

Figure S8. Activation of oncogenic Src variant in different cell lines. Cells expressing Src-K295TCOK-Y527F was treated with Me_2Tz to allow bioorthogonal cleavage mediated kinase activation in 293A cells (a), NIH3T3 cells (b), HeLa cells (c) and CHO cells (d). The endogenous Src was detected using Src or pY416-Src antibody at a smaller molecular weight, while the overexpressed Src with myc-tag was at a higher molecular weight. Cells were treated with 100 μM Me_2Tz for 1 h.

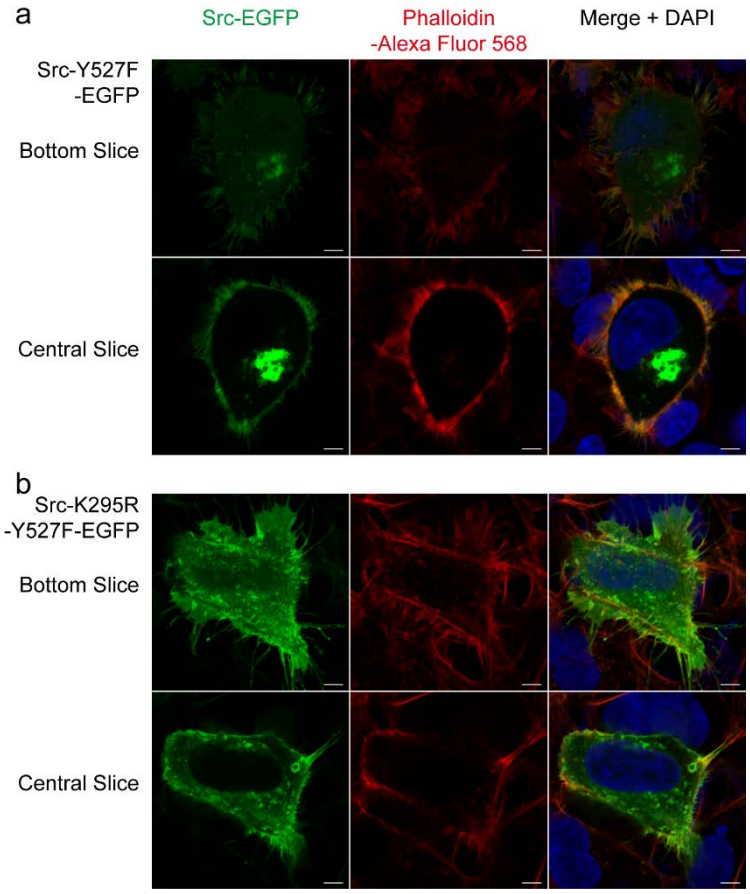

Figure S9. Phenotypes of 293A cells expressing Src variants. (a) Representative images of cells expressing the oncogenic Src variant (Src-Y527F). (b) Representative images of cells expressing the kinase dead Src mutant (Src-K295R-Y527F). Bottom slices and central slices were captured with different focal plane. Src localization was indicated by GFP and filament scaffold was stained with Phalloidin-Alexa Fluor 546. Cells expressing Src-Y527F showed rounded, detached and disassembled filaments. Scale bars, 5 μm.

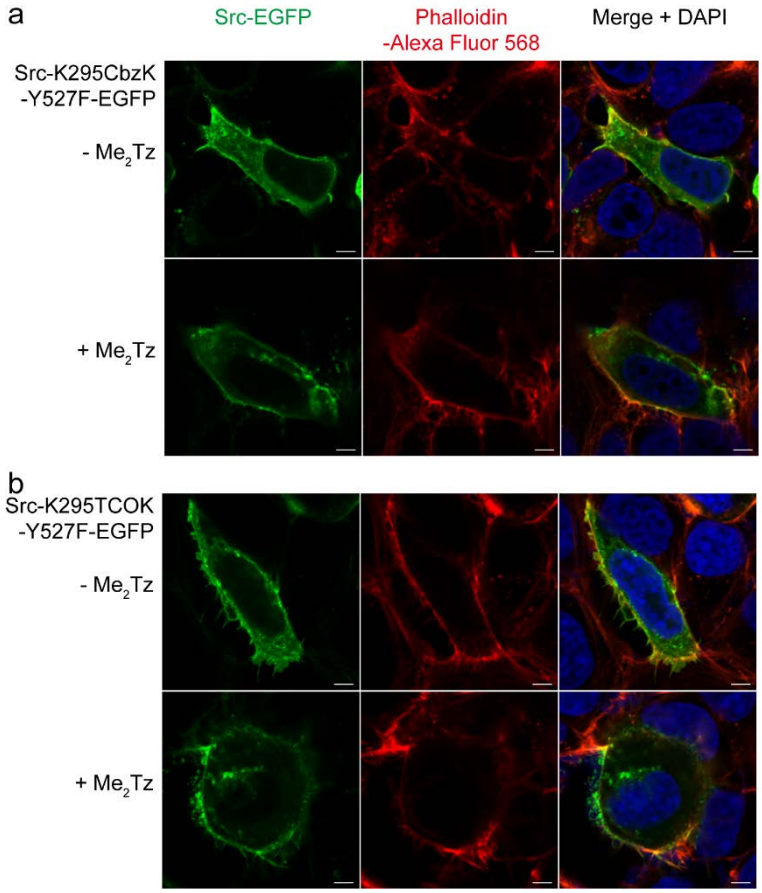

Figure S10. Phenotype change of 293A cells expressing caged oncogenic Src mutant with or without Me_2Tz-mediated activation. (a) Representative images of cells expressing the CbzK caged Src variant (Src-K295CbzK-Y527F). (b) Representative images of cells expressing the TCOK-*a* caged Src variant (Src-K295TCOK-Y527F). Src localization was indicated by GFP while filament scaffold was stained with Phalloidin-Alexa Fluor 546. Cells expressing the Src-K295TCOK-Y527F variant showed cell rounding and detaching after Me_2Tz treatment (100 μM). Scale bars, 5 μm.

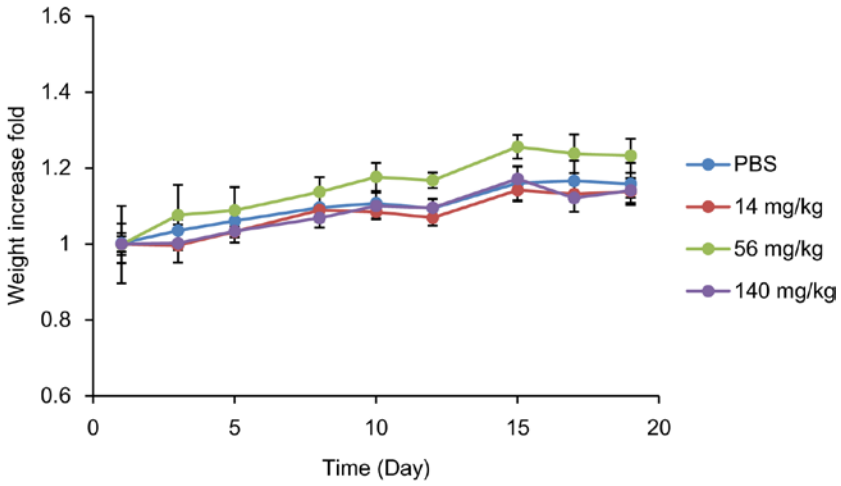

Figure S11. Toxicity study on Me_2Tz. The weights of mice after intravenous Me_2Tz or PBS injection were monitored over 20 days (mean ± s.d., n = 3) after initial injection. 50 μL Me_2Tz of 50/200/500 mM concentration (equal to 14/56/140 mg/kg body weight for an ~20 g mouse) or PBS was injected through tail vein three times a week. The growth of mice indicated by weight increase was not affected.

3. References

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(5) Zhao, J.; Lin, S.; Huang, Y.; Zhao, J.; Chen, P. R. Mechanism-based design of a photoactivatable firefly luciferase. *J. Am. Chem. Soc.* **2013,** *135*, 7410-7413.

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