Supplementary Information (SI)

Genetically Encoded Green-light-responsive Photocaged Lysine for Sequential Control of Protein Function

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Supplementary figures

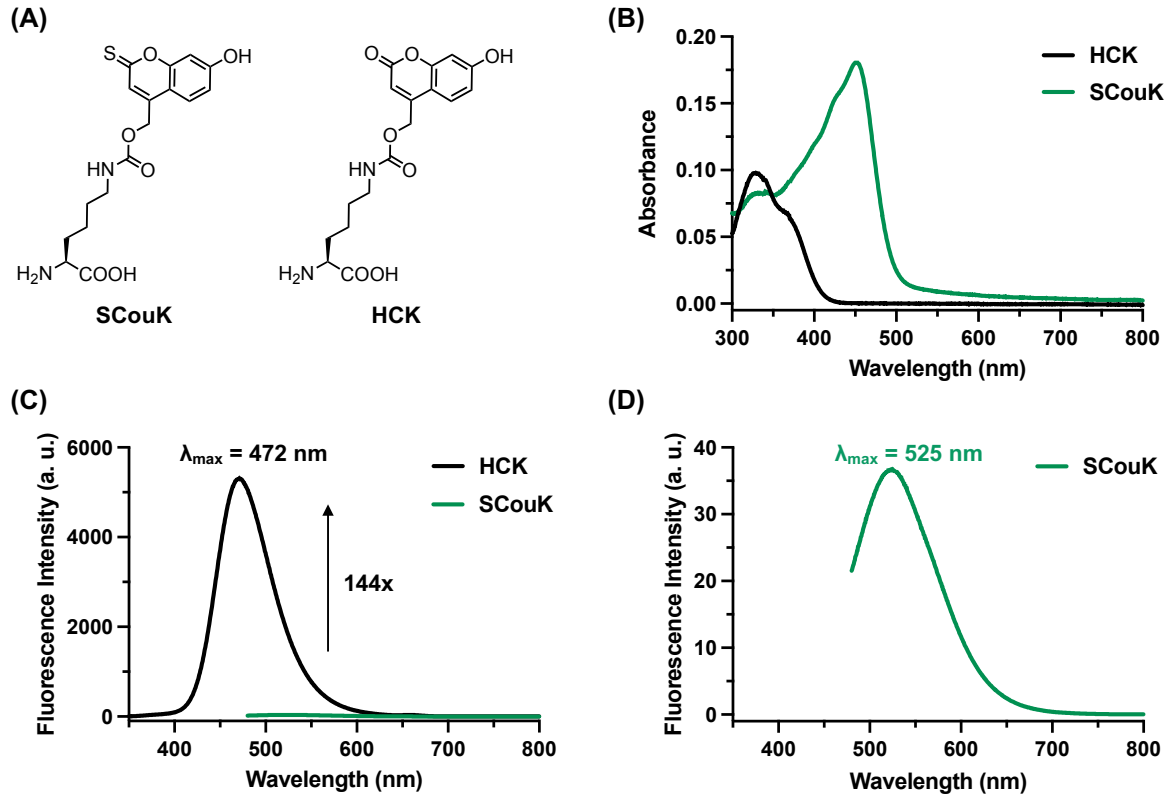


Fig. S1 Photophysical properties of SCouK. (A) Structures of SCouK and HCK. (B) Absorption spectra of SCouK ($\lambda_{ab,\ max}$ = 453 nm) and HCK ($\lambda_{ab,\ max}$ = 329 nm). (C) Fluorescence emission spectra of SCouK and HCK. (D) Enlarged fluorescence emission spectrum of SCouK. SCouK and HCK were dissolved in phosphate-buffered saline (PBS) (20 mM, pH 7.4; containing 0.01% DMSO) at 10 μM concentrations. Fluorescence emission spectra of SCouK and HCK were recorded with excitation at 453 nm and 329 nm, respectively. Note: SCouK exhibits stronger absorption but approximately 144-fold weaker fluorescence compared to HCK. Data in (B), (C), and (D) are representative of three independent experiments.

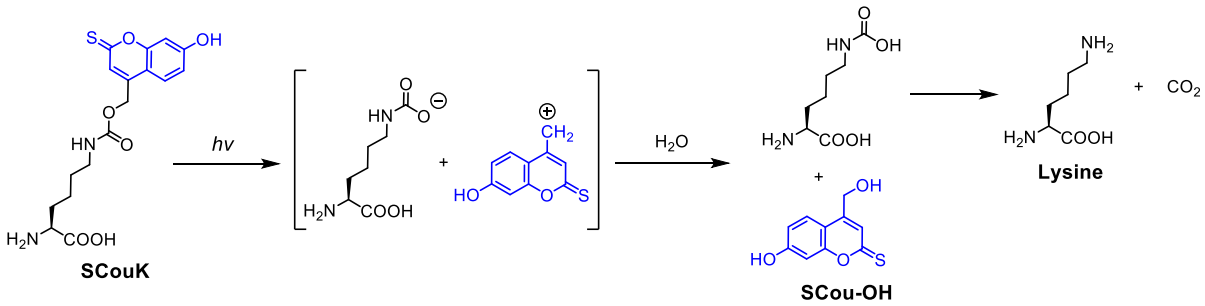


Fig. S2 Proposed mechanism of photolysis of the thiocoumarin lysine SCouK to generate a native lysine and thiocoumarin SCou-OH.

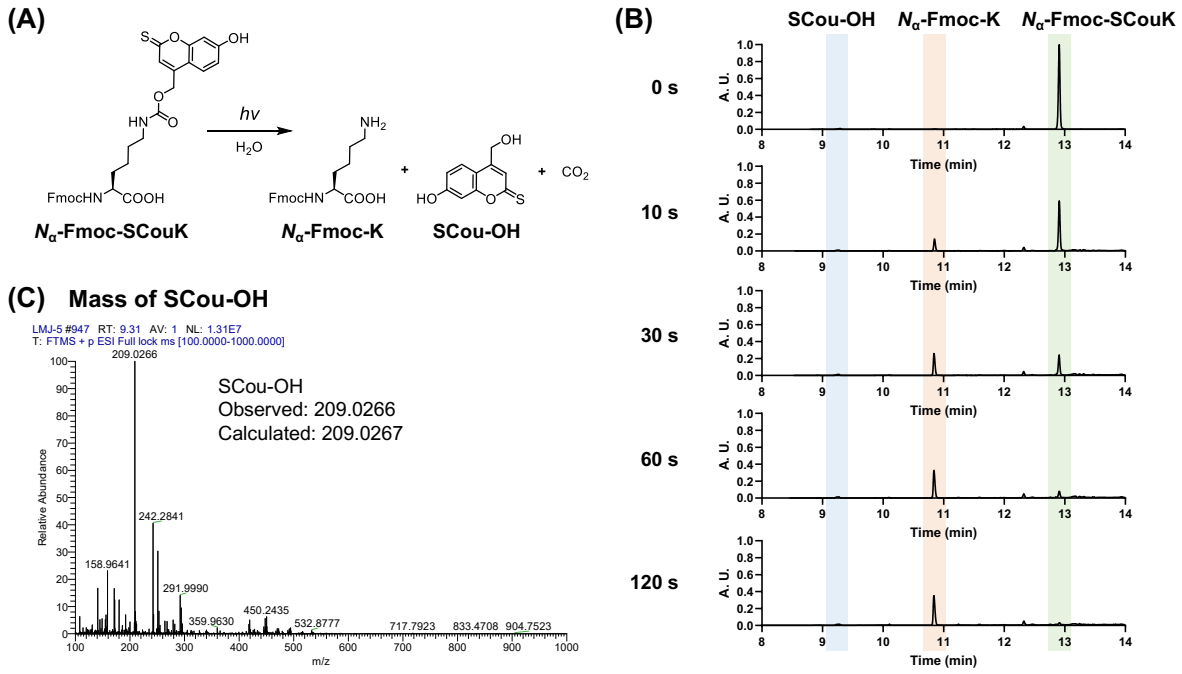


Fig. S3 Characterization of photolysis of the thiocoumarin lysine derivative N_α-Fmoc-SCouK *in vitro*. (A) The photolysis reaction of N_α-Fmoc-SCouK to generate decaged N_α-Fmoc-K and SCou-OH. (B) Representative LC-MS analysis of N_α-Fmoc-SCouK photolysis showing the efficient generation of N_α-Fmoc-K. N_α-Fmoc-SCouK (50 μM) was dissolved in CH_3CN and PBS (CH_3CN/PBS = 2:8). The solution was irradiated with a 520 nm LED (22 mW/cm^2) for varying periods (10, 30, 60, and 120 s). The resulting mixture was analyzed by HPLC-MS, with a UV detector set at the wavelength of 254 nm. The experiments were performed in four replicates with reproducible results. The peak areas of N_α-Fmoc-SCouK in the HPLC traces were used to calculate its conversions, using the non-irradiated N_α-Fmoc-SCouK sample as the reference. Each replicate dataset of N_α-Fmoc-SCouK conversion was individually fitted to a nonlinear one-phase exponential decay model to calculate the half-life ($t_{1/2}$) of SCouK photodecaging. The resulting half-life values were averaged. (C) Mass spectrum of the generated SCou-OH with a retention time at 9.31 min. Data in (B) and (C) are representative of three independent experiments.

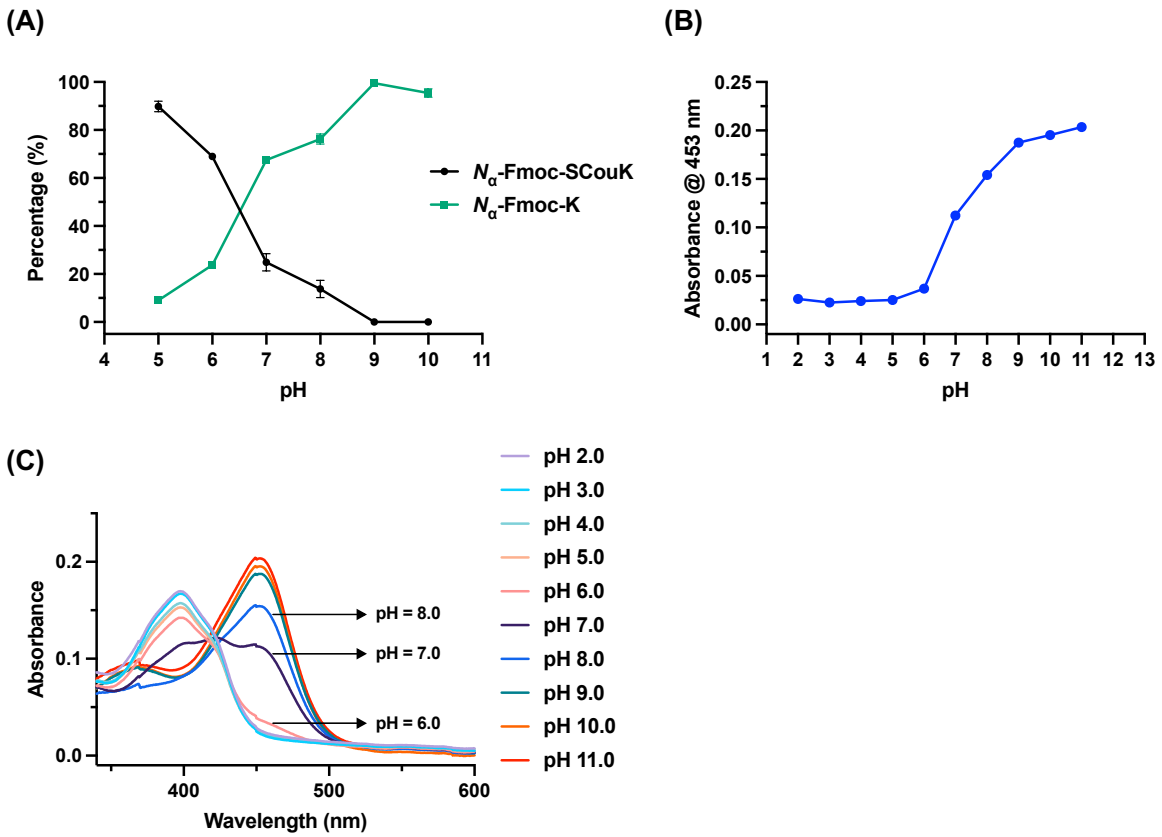


Fig. S4 (A) Photolysis of the thiocoumarin lysine derivative N_α-Fmoc-SCouK under different pH conditions. N_α-Fmoc-SCouK (50 μM) was dissolved in CH_3CN and PBS at different pH values (CH_3CN/PBS = 2:8). The solution was irradiated with a 520 nm LED (22 mW/cm^2) for 30 s with a distance of 20 cm. The resulting mixture was analyzed by HPLC-MS, with a UV detector set at the wavelength of 254 nm. The peak areas of N_α-Fmoc-SCouK and N_α-Fmoc-K in the HPLC traces were used to calculate the conversions and yields, using known concentrations of N_α-Fmoc-SCouK and N_α-Fmoc-K as references. The experiments were performed in three replicates with reproducible results. Data are shown as mean ± SD (n = 3). (B) The maximal absorbance of SCouK at 453 nm under different pH conditions. (C) Absorption spectra of SCouK under different pH conditions. For the measurement of absorption, SCouK (10 μM) was dissolved in PBS (20 mM, containing 0.01% DMSO) at different pH values. Data in (B) and (C) are representative of three independent experiments.

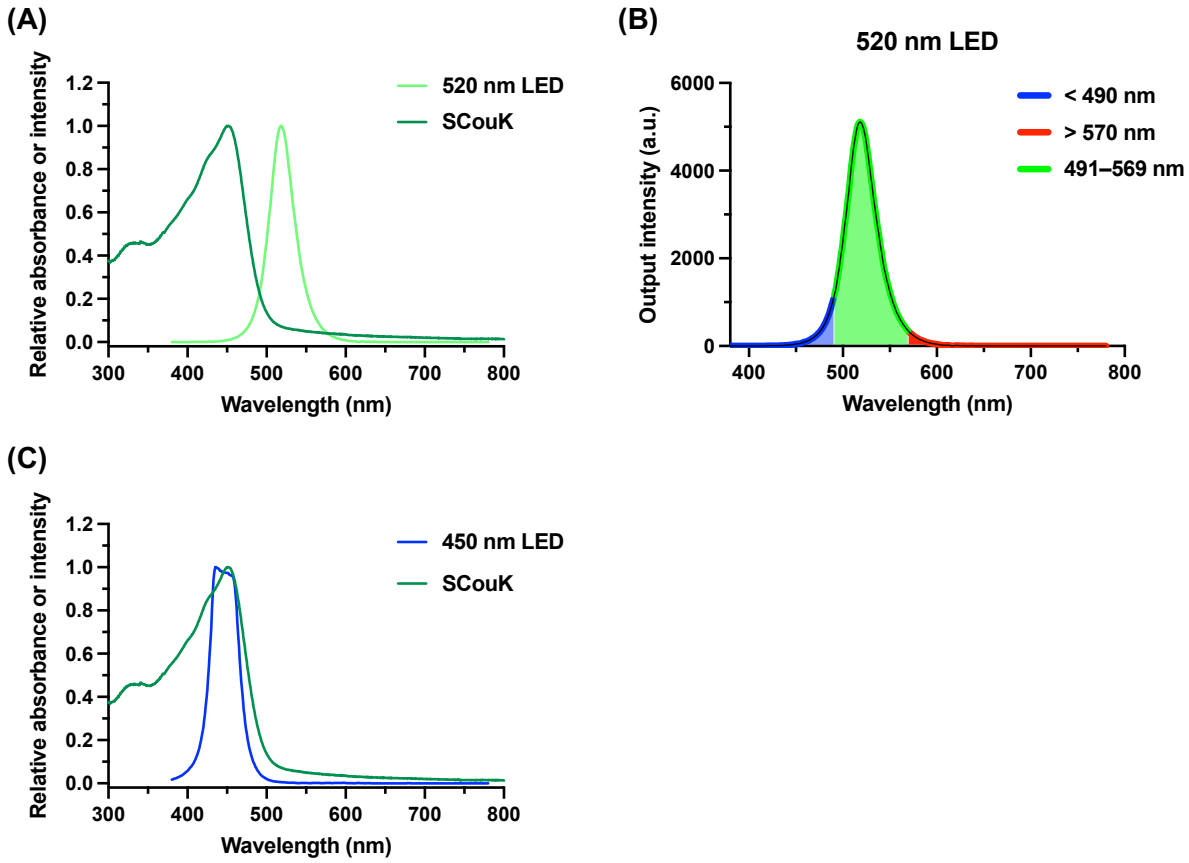


Fig. S5 (A) Overlay of the absorption spectrum of SCouK (10 μM in PBS at pH 7.4 containing 0.01% DMSO) with the emission spectrum of the 520 nm LED used in this study. (B) Emission spectrum of the 520 nm LED used in this study. The emission below 490 nm and above 570 nm contributed approximately 6% and 2%, respectively, to the total output, confirming that the LED predominantly emits green light centered around 520 nm. (C) Overlay of the absorption spectrum of SCouK (10 μM in PBS at pH 7.4 containing 0.01% DMSO) with the emission spectrum of the 450 nm LED used in this study. Data are representative of three independent experiments.

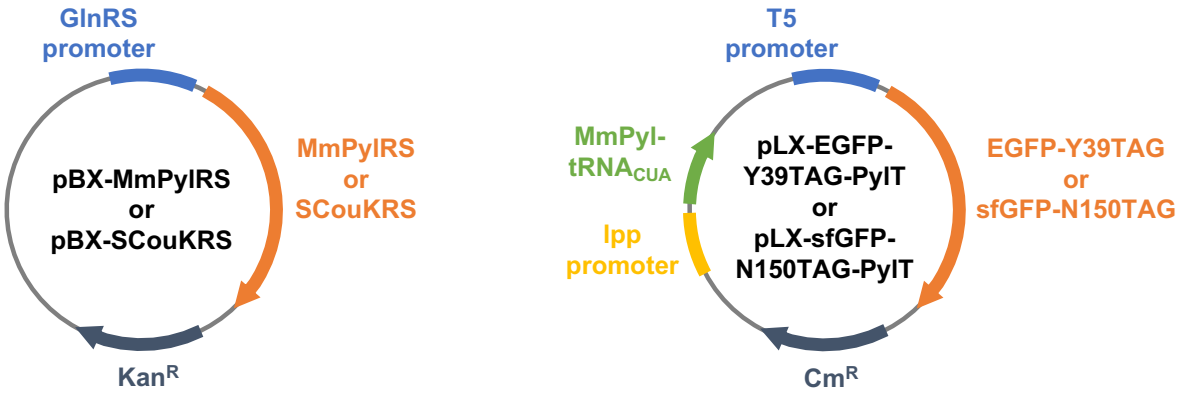


Fig. S6 Maps of plasmids used in this study for protein expression in *E. coli*.

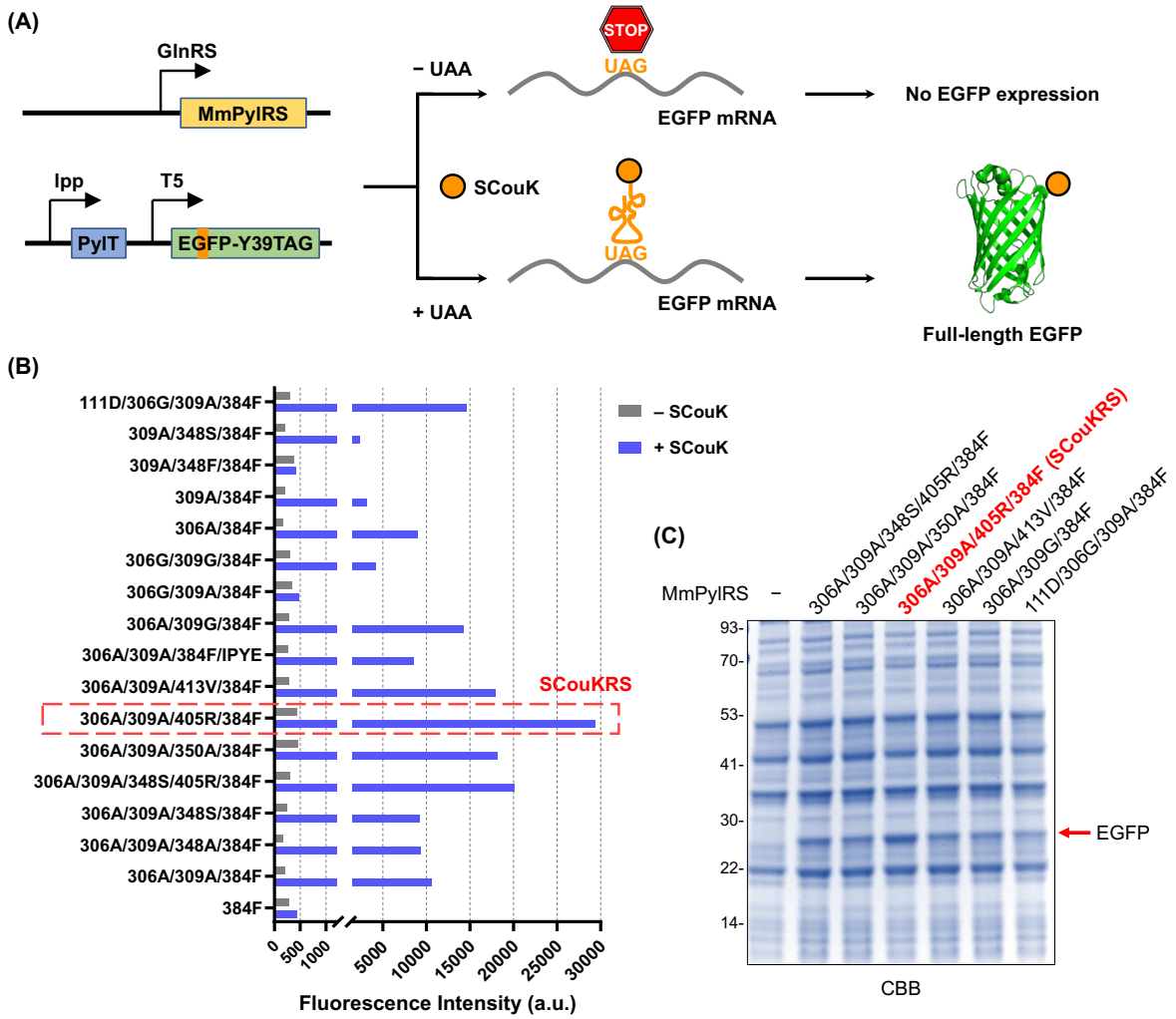


Fig. S7 (A) Schematic of the principle for site-specifically encoding SCouK into EGFP-Y39TAG *via* the genetic code expansion technology. (B) Screening of MmPylRS mutants for site-specific incorporation of SCouK into EGFP-Y39TAG in *E. coli*. The pLX-EGFP-Y39TAG-PylT plasmid was co-transformed with individual pBX-MmPylRS variants into *E. coli* strain BL21(DE3). The transformed bacteria cells were grown in LB medium overnight at 37 °C and then inoculated by 1:100 dilution into LB medium. SCouK (1 mM) was added into the bacterial culture when OD_{600} reached 0.6. After 0.5 h incubation, protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 10 h at 37 °C. The EGFP fluorescence intensity of individual bacterial culture was measured on a microplate reader and compared with the control culture in the absence of SCouK. The fluorescence was normalized to OD_{600}. (C) Validation of MmPylRS mutants for site-specific incorporation of SCouK into EGFP-Y39TAG in *E. coli*. The indicated bacterial cultures were lysed and analyzed by Coomassie Brilliant Blue (CBB) staining. The screening experiment shown in (B) was performed once for initial assessment, and selected variants were subsequently validated in (C), which represents reproducible results from two biological replicates.

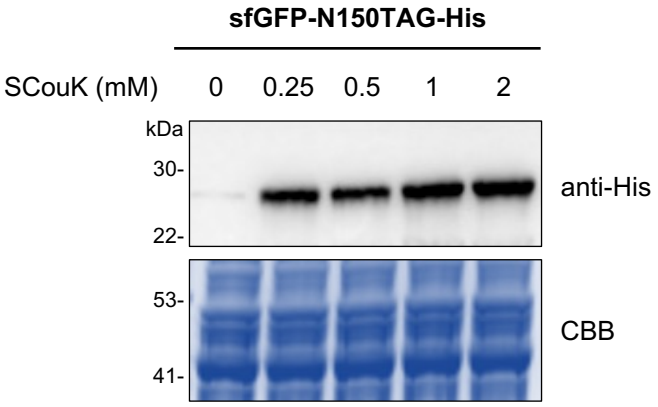


Fig. S8 Concentration-dependent incorporation of SCouK into 6*His-tagged sfGFP-N150TAG in *E. coli*. The pLX-sfGFP-N150TAG-PylT plasmid was co-transformed with the pBX-SCouKRS plasmid into *E. coli* strain BL21(DE3). After overnight culture at 37 °C, the transformed bacteria cells were inoculated by 1:100 dilution into LB medium. SCouK at different concentrations (from 0 to 2 mM) was added into the bacterial culture when OD_{600} reached 0.6. After 0.5 h, protein expression was induced with 1 mM IPTG for 10 h at 37 °C. Cells were harvested and lysed with 4% SDS lysis buffer. Cell lysates were analyzed by western blotting and Coomassie Brilliant Blue (CBB) staining. Data are representative of three independent experiments.

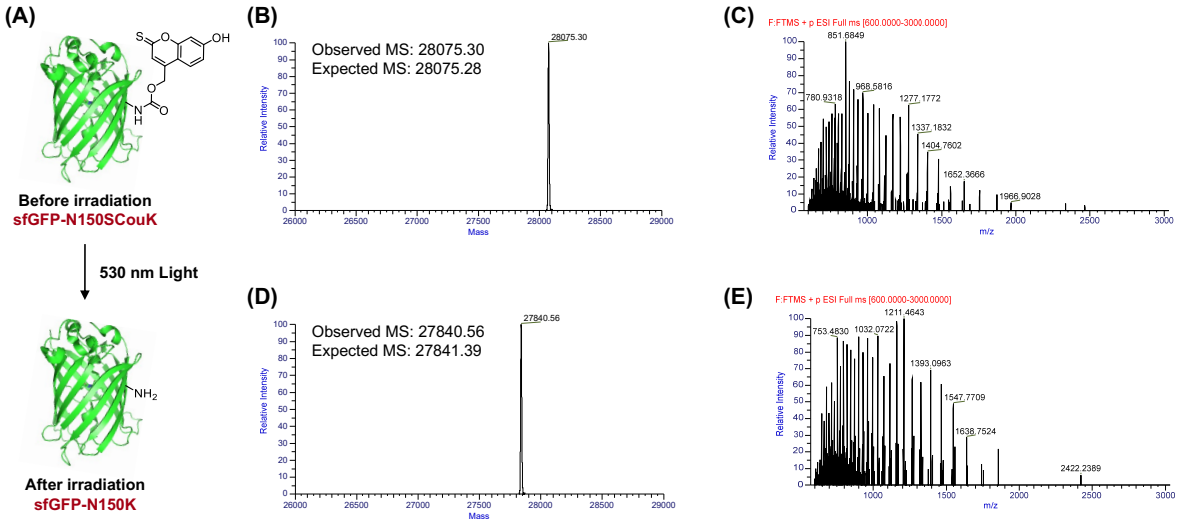


Fig. S9 Electrospray ionization mass spectrometry (ESI-MS) analyses of photolysis of SCouK at the protein level. (A) Schematic for photolysis of 6*His-tagged sfGFP-N150SCouK to generate 6*His-tagged sfGFP-N150K. (B) Deconvoluted and (C) original ESI-MS spectra of the sfGFP-N150SCouK protein. Calculated mass of sfGFP-N150SCouK: 28075.28 Da; observed mass: 28075.30 Da. (D) Deconvoluted and (E) original ESI-MS spectra of the sfGFP-N150SCouK protein after photoirradiation. Calculated mass of sfGFP-N150K: 27841.39 Da; observed mass: 27840.56 Da. The pLX-sfGFP-N150TAG-PylT plasmid was co-transformed with the pBX-SCouKRS plasmid into *E. coli* strain BL21(DE3). After overnight culture at 37 °C, the transformed bacteria cells were inoculated by 1:100 dilution into LB medium. SCouK (1 mM) was added into the bacterial culture when OD_{600} reached 0.6. After 0.5 h, protein expression was induced with 1 mM IPTG for 10 h at 37 °C. Cells were lysed and the proteins were purified by Ni-NTA Sefinose Resin for ESI-MS analysis. For photolysis of the recombinant protein *in vitro*, sfGFP-N150SCouK (1 μM) in PBS was irradiated with a 520 nm LED (22 mW/cm^2) for 30 s and analyzed by ESI-MS. Data are representative of two independent experiments.

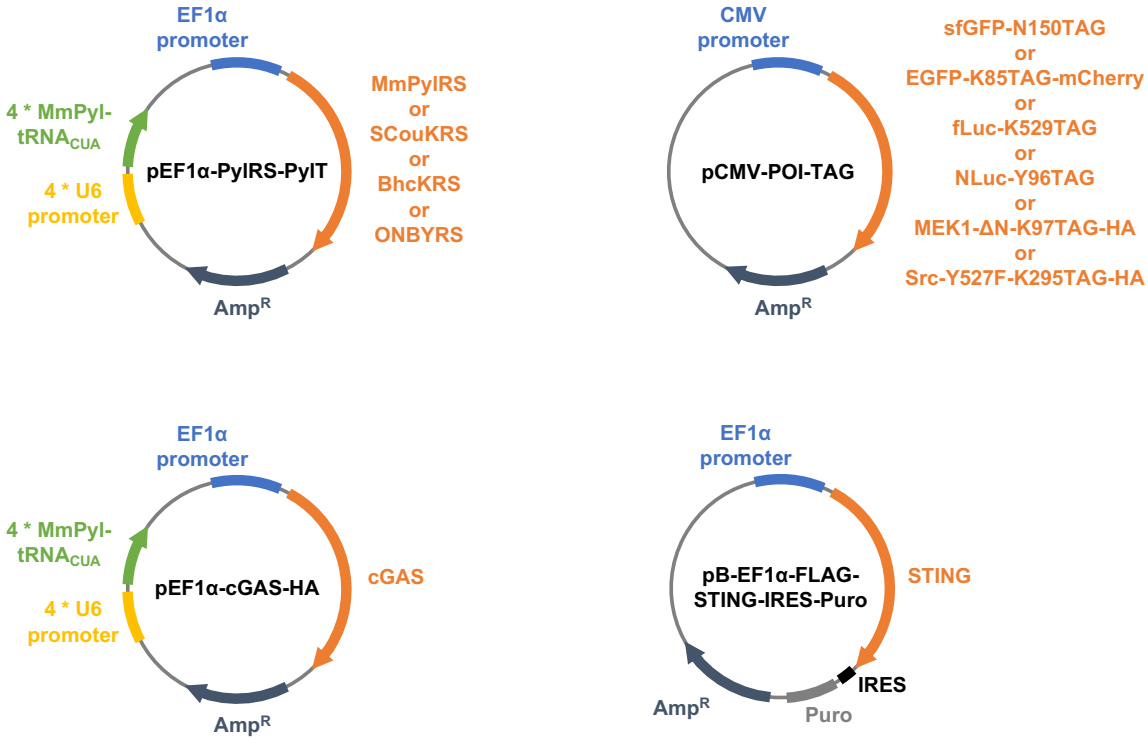


Fig. S10 Maps of plasmids used in this study for protein expression in mammalian cells.

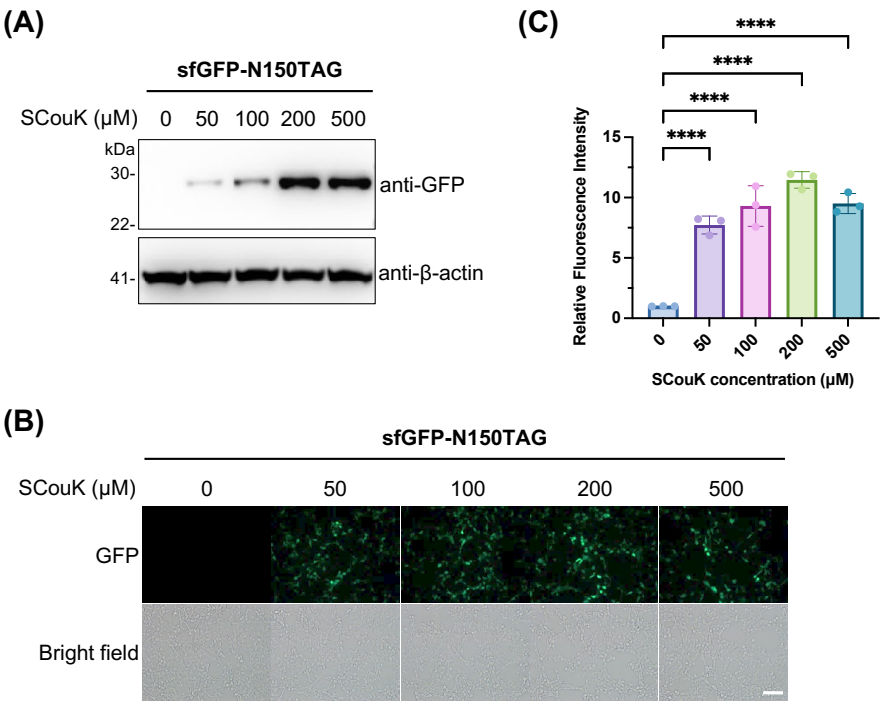


Fig. S11 Site-specific incorporation of SCouK into sfGFP-N150TAG in mammalian cells. (A) Concentration-dependent incorporation of SCouK into sfGFP-N150TAG in HEK293T cells analyzed by western blotting. (B) Representative widefield fluorescence imaging of sfGFP-N150SCouK expression in the presence of varying concentrations of SCouK in live HEK293T cells. Scale bar represents 100 μm. (C) Quantification of fluorescence images shown in (B). Data are shown as mean ± SD (*n* = 3). Statistical analysis was performed with one-way ANOVA, ****p < 0.0001. HEK293T cells were transfected with plasmids of pCMV-sfGFP-N150TAG and pEF1α-SCouKRS-PylT in the presence of SCouK at indicated concentrations for 24 h and analyzed by western blotting or widefield fluorescence microscopy. Data in (A) are representative of three independent experiments.

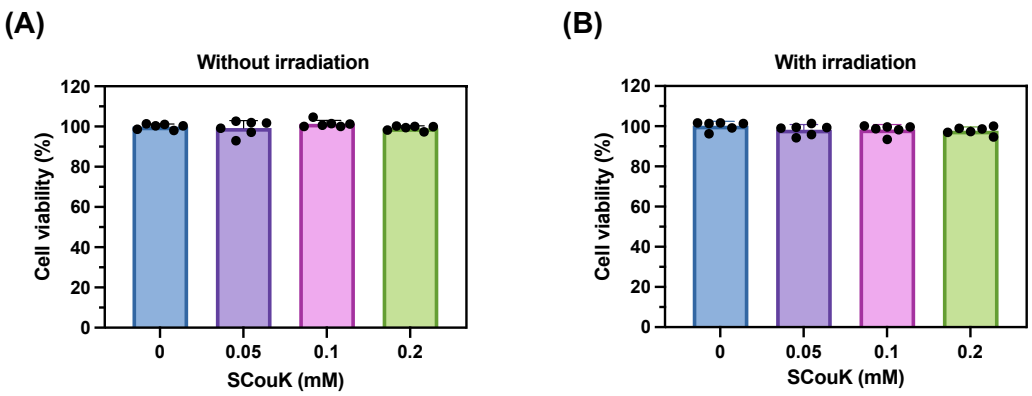


Fig. S12 Cytotoxicity of SCouK and its decaging product. (A) HEK293T cells were treated with SCouK at different concentrations for 24 h. (B) HEK293T cells were treated with SCouK at different concentrations for 0.5 h, irradiated with a 520 nm LED (22 mW/cm^2) for 1 min, and further cultured for 24 h. Cell viability was measured by the CCK-8 assay. Data are shown as mean ± SD (*n* = 6).

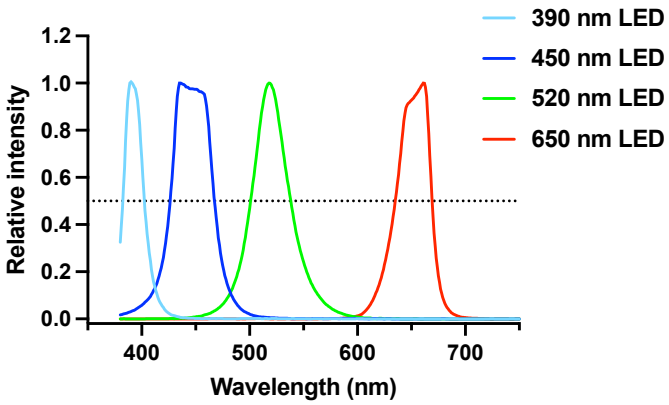


Fig. S13 Emission spectra of the LEDs used in this study. Detailed parameters of the LEDs are shown in Table S2.

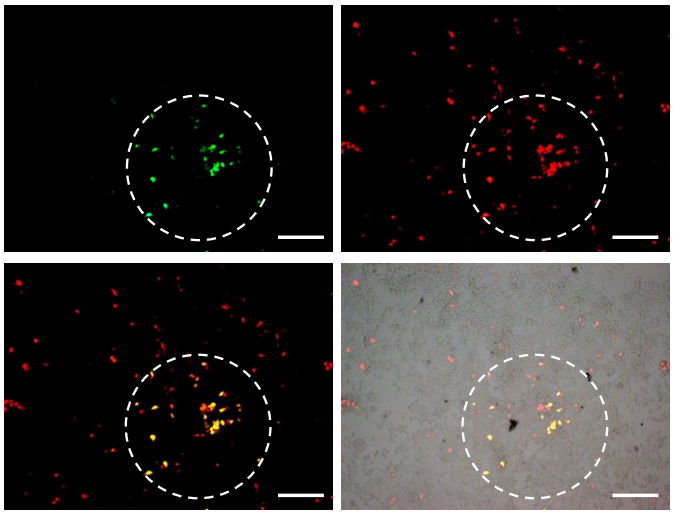


Fig. S14 Spatial activation of EGFP fluorescence using SCouK through localized photoirradiation. HEK293T cells expressing EGFP-K85SCouK-mCherry were transferred to an Olympus IX73 inverted fluorescence microscope, focused using the bright-field channel, and locally irradiated for 10 s using a mercury lamp equipped with a green excitation filter set (bandpass 530–550 nm), followed by widefield fluorescence imaging of the live cells. Scale bars represent 100 μm. The white dashed circle indicates the area subjected to photoirradiation. Data are representative of two independent experiments.

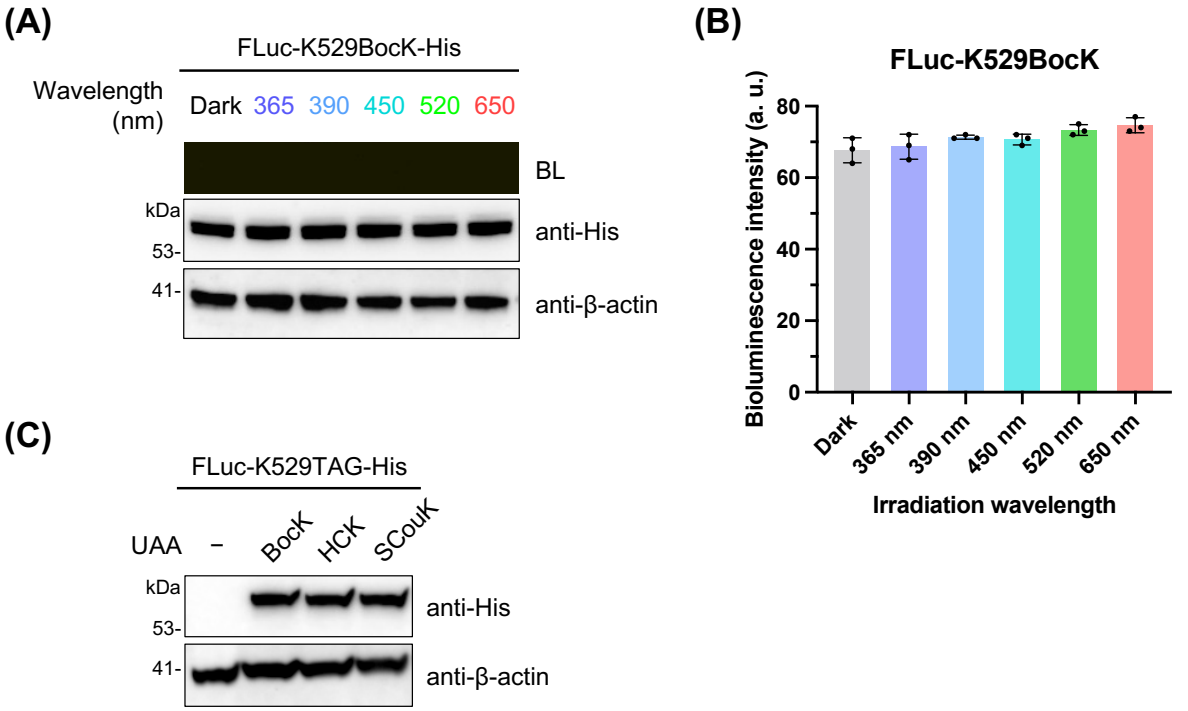


Fig. S15 Photoactivation of FLuc-K529BocK using light at different wavelengths in HEK293T cells. (A) Cells were transfected with pCMV-FLuc-K529TAG and pEF1α-MmPylRS-PylT in the presence of BocK (0.2 mM) and illuminated with light at indicated wavelengths for 60 s, followed by western blot analysis. (B) Cells expressing FLuc-K529BocK were illuminated with light at indicated wavelengths for 60 s and examined for bioluminescence activities. Data in (B) are shown as mean ± SD (n = 3). (C) Western blot analysis of FLuc-K529TAG expression in the presence of UAAs (0.2 mM). HEK293T cells were transfected with pCMV-FLuc-K529TAG and the corresponding synthetase/tRNA plasmids in the presence of BocK, HCK, or SCouK (0.2 mM) for 24 h, followed by western blot analysis. Data in (A) and (C) are representative of three independent experiments.

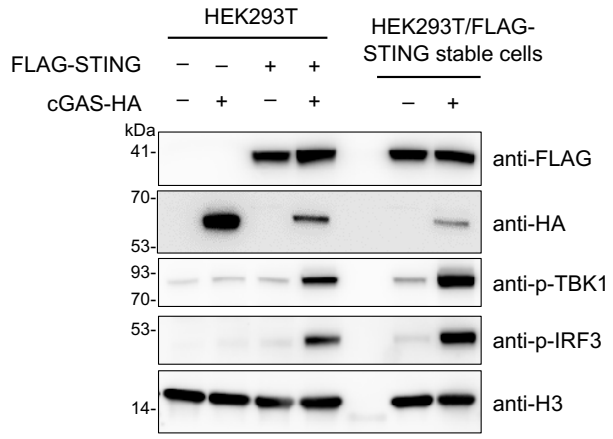


Fig. S16 Activation of TBK1 and IRF3 phosphorylation in HEK293T cells by co-expression of cGAS and STING. Wild-type HEK293T cells were transfected with plasmids of pEF1α-cGAS-HA and pB-EF1α-FLAG-STING-IRES-Puro as indicated for 24 h, whereas HEK293T cells stably expressing FLAG-tagged STING were transfected with the plasmid of pEF1α-cGAS-HA as indicated for 24 h. After that, the cells were lysed and analyzed by western blotting. Data are representative of two independent experiments.

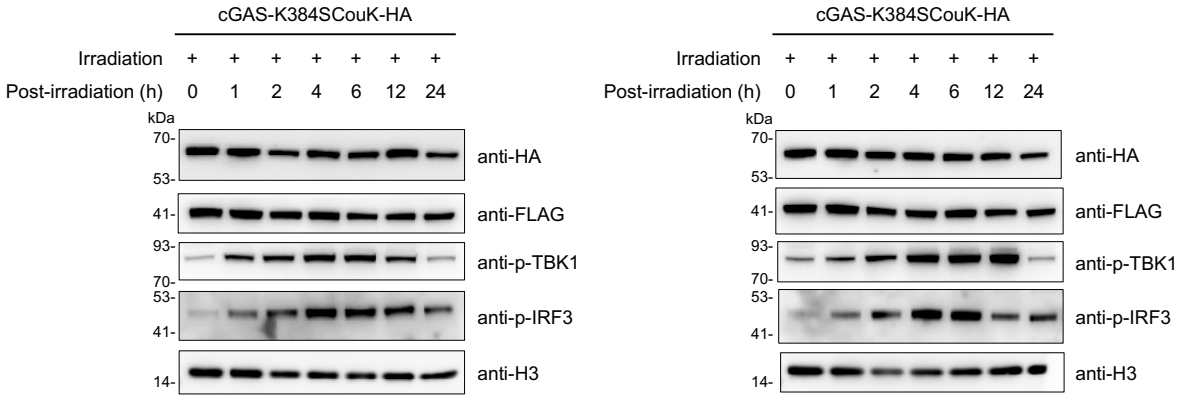


Fig. S17 Two replicates of photoactivation of cGAS-K384SCouK in HEK293T cells stably expressing FLAG-STING. Cells were transfected with plasmids of pEF1α-cGAS-K384TAG-HA and pEF1α-SCouKRS-PylT in the presence of SCouK (0.2 mM) for 24 h and irradiated with a 520 nm LED (22 mW/cm^2) for 1 min. After that, the cells were cultured for indicated periods and lysed for western blotting analysis. Data are representative of three independent experiments.

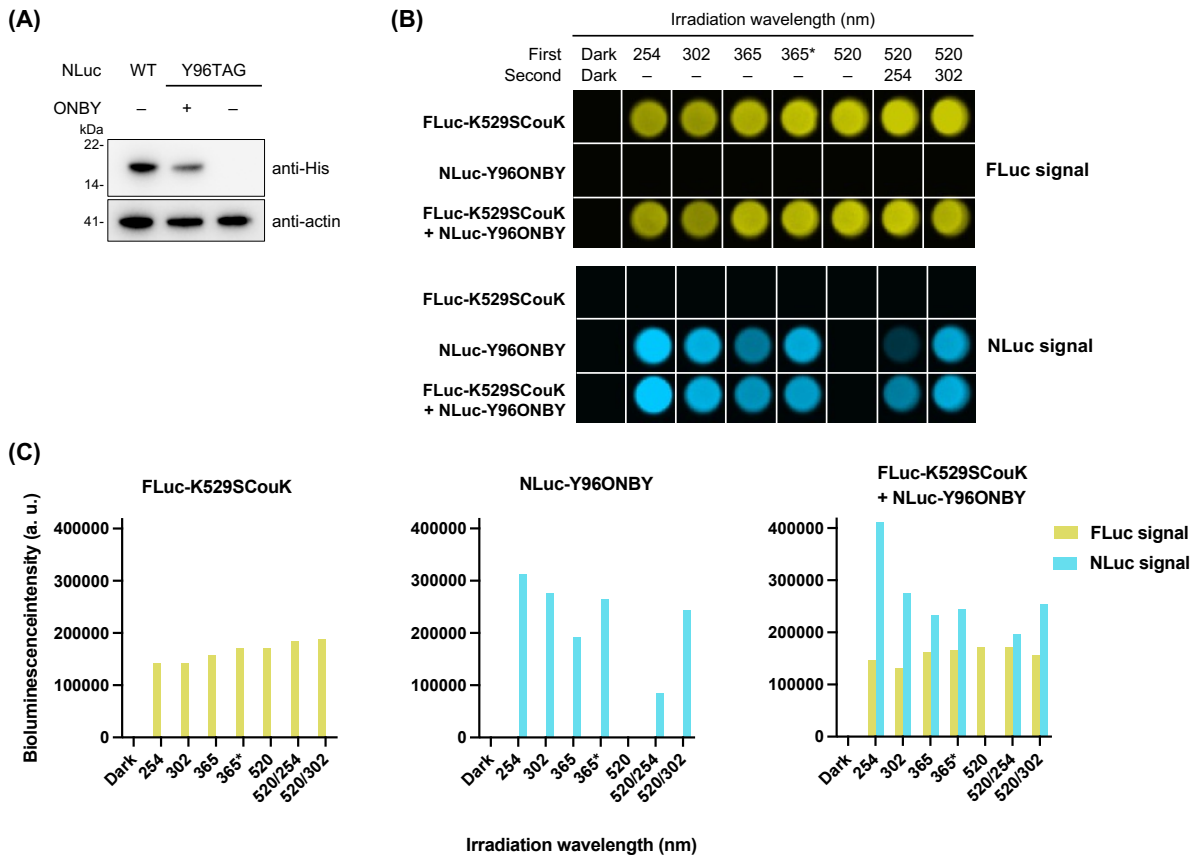
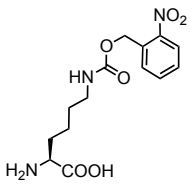
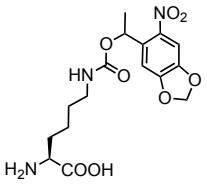
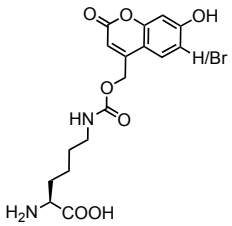
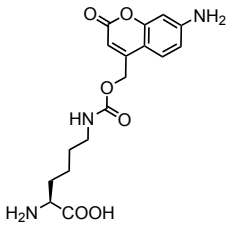
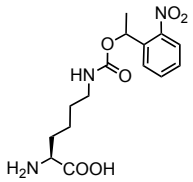
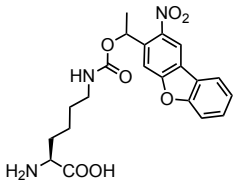
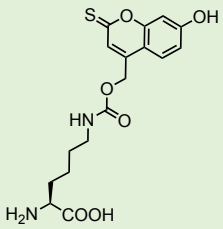


Fig. S18 Sequential photoactivation of two proteins using SCouK and ONBY. (A) Expression of NLuc-Y96ONBY in HEK293T cells. (B) Sequential photoactivation of FLuc-K529SCouK and NLuc-Y96ONBY in HEK293T cells. Cells were co-transfected with a plasmid encoding FLuc-K529TAG and a plasmid encoding SCouKRS and MmPyl-tRNA$_{CUA}$ in the presence of SCouK (0.2 mM) to express FLuc-K529SCouK. Cells were co-transfected with a plasmid encoding NLuc-Y96TAG and a plasmid encoding ONBYRS and MmPyl-tRNA$_{CUA}$ in the presence of ONBY (0.2 mM) to express NLuc-Y96ONBY. The cells expressing FLuc-K529SCouK, NLuc-Y96ONBY, or FLuc-K529SCouK and NLuc-Y96ONBY were sequentially irradiated with light at 365 nm or 520 nm (22 mW/cm^2) for 30 s as indicated. “First” and “Second” denotes the first and second irradiation events, respectively. After irradiation, FLuc and NLuc activities were examined and quantified. The asterisk (“365*”) indicates irradiation with 365 nm light for 1 min. (C) Quantification of chemiluminescence data shown in (B). Data in (A) are representative of three independent experiments. Data in (B) and (C) were obtained in one biological replicate, but key findings here were repeated with three independent experiments shown in Fig. 7 of the main text.

Supplementary table

Table S1. Summary of photoactivatable lysine-derived UAAs. [a]

UAA	Decaging wavelength (nm)	Decaging time	Orthogonal RS/tRNA pair used for incorporation	Organism	Target protein	Year	Ref
 **ONBK**	365	20 min	*Mm*PylRS/ *Mm*Pyl-tRNA$_{CUA}$	*E. coli*, mammalian cells	EGFP	2009	[1]
 **PCK**	365	1–5 s	*Mb*PylRS/ *Mb*Pyl-tRNA$_{CUA}$	*E. coli*, mammalian cells	Nuclear localization signal (NLS), p53	2010	[2]
 **HCK, BHCK**	365, 405, [b] 760 [c]	4 min (365 nm)	*Mb*PylRS/ *Mb*Pyl-tRNA$_{CUA}$	*E. coli*, mammalian cells	EGFP, luciferase	2014	[3]
 **ACK**	405	2 min	*Mb*PylRS/ *Mb*Pyl-tRNA$_{CUA}$	*E. coli*, mammalian cells	Luciferase, Cre recombinase, Protein Kinase A, NRAS	2023	[4]

UAA	Decaging wavelength (nm)	Decaging time	Orthogonal RS/tRNA pair used for incorporation	Organism	Target protein	Year	Ref
 **NPEK**	365	30 s	*Mb*PylRS/ *Mb*Pyl-tRNA$_{CUA}$	Mammalian cells	NLS, luciferase	2024	[5]
 **NDBFK**	405	15 s	*Mb*PylRS/ *Mb*Pyl-tRNA$_{CUA}$	Mammalian cells	NLS, luciferase	2024	[5]
 **SCouK**	254, 302, 365, 390, 450, 520 [d]	30–60 s (520 nm) [e]	*Mm*PylRS/ *Mm*Pyl-tRNA$_{CUA}$	*E. coli*, mammalian cells	EGFP, luciferase, MEK1, Src, cGAS	2025	This work

[a] Only photocaged lysine derivatives are listed here.

[b] 405 nm: 30 mW diode laser, 20% laser power, 12.6 μs dwell time, 8 cycles.[3]

[c] 760 nm (two-photon): 130 mW laser, 30 cycles of scanning.[3]

[d] 390 nm, 450 nm, and 520 nm LEDs were used (Table S2).

[e] 520 nm LED: Peak wavelength, 519 nm; Central wavelength, 520 nm; Dominant wavelength, 521 nm; Centroid wavelength, 521 nm; Half width, 38 nm; Irradiance, 22 mW/cm^2.

Table S2. Parameters of the LEDs used in this study. [a]

LED name	390 nm LED	450 nm LED	520 nm LED	650 nm LED
Peak wavelength (nm) [b]	389	436	519	662
Central wavelength (nm) [c]	392	447	520	652
Dominant wavelength (nm) [d]	447	454	521	634
Centroid wavelength (nm) [e]	402	447	521	650
Half width (nm) [f]	20	41	38	34
Irradiance (mW/cm^2) [g]	18	34	22	58

[a] The spectra parameters of the LEDs used in this study were measured using a spectroradiometer. Each LED is referred to by its central wavelength.

[b] Peak wavelength: the wavelength at which the emission intensity of the light source is maximum.

[c] Central wavelength: the arithmetic midpoint between the wavelengths at which the emission intensity falls to half of the maximum value on either side of the peak.

[d] Dominant wavelength: the wavelength of monochromatic light that would appear visually the same color to the human eye as the light source.

[e] Centroid wavelength: the weighted average wavelength of a spectrum, calculated by taking the ratio of the quantum flux to the radiant flux or using a weighted average of intensity and wavelength.

[f] Half width: also called full width at half maximum (FWHM), the spectral width of the emission curve measured between the two points where the intensity falls to half of the peak intensity.

[g] Measured at the sample plane with a distance of 20 cm.

Table S3. Plasmids used in this study.

Plasmid	Promoter	Gene	Organism
pBX-MmPylRS	GlnRS	Wild-type MmPylRS	*E. coli*
pBX-SCouKRS	GlnRS	SCouKRS (MmPylRS mutant)	*E. coli*
pLX-EGFP-Y39TAG-PylT	lpp	MmPyl-tRNA$_{CUA}$	*E. coli*
	T5	EGFP-Y39TAG	
pLX-sfGFP-N150TAG-PylT	lpp	MmPyl-tRNA$_{CUA}$	*E. coli*
	T5	sfGFP-N150TAG	
pEF1α-MmPylRS-PylT	EF1α	Wild-type MmPylRS	Mammalian cells
	4 × U6	4 × MmPyl-tRNA$_{CUA}$	
pEF1α-SCouKRS-PylT	EF1α	SCouKRS (MmPylRS mutant)	Mammalian cells
	4 × U6	4 × MmPyl-tRNA$_{CUA}$	
pEF1α-BhcKRS-PylT	EF1α	BhcKRS (MbPylRS mutant)	Mammalian cells
	4 × U6	4 × MmPyl-tRNA$_{CUA}$	
pEF1α-ONBYRS-PylT	EF1α	ONBYRS (MbPylRS mutant)	Mammalian cells
	4 × U6	4 × MmPyl-tRNA$_{CUA}$	
pCMV-sfGFP-N150TAG	CMV	sfGFP-N150TAG	Mammalian cells
pCMV-EGFP-K85TAG-mCherry	CMV	EGFP-K85TAG-mCherry	Mammalian cells
pCMV-FLuc-K529TAG	CMV	FLuc-K529TAG	Mammalian cells
pCMV-NLuc-Y96TAG	CMV	NLuc-Y96TAG	Mammalian cells
pCMV-MEK1-ΔN-K97TAG-HA	CMV	MEK1-ΔN-K97TAG	Mammalian cells
pCMV-Src-Y527F-K295TAG-HA	CMV	Src-Y527F-K295TAG	Mammalian cells
pEF1α-cGAS-HA	EF1α	cGAS	Mammalian cells
	4 × U6	4 × MmPyl-tRNA$_{CUA}$	
pB-EF1α-FLAG-STING-IRES-Puro	EF1α	STING	Mammalian cells

Chemical synthesis

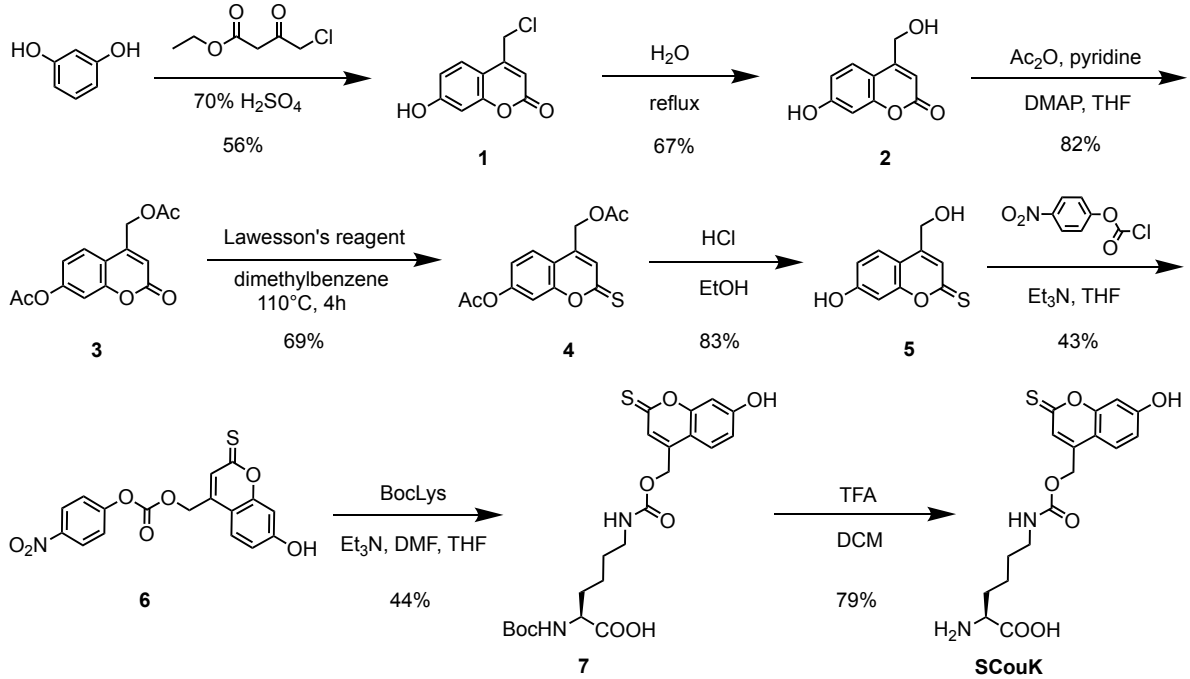
General information

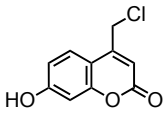
Chemicals and solvents were obtained from commercial sources and used directly as received. Chemical reactions were performed in oven-dried round-bottom flasks and monitored by TLC on silica gel 60 GF254 glass plates (Qingdao Haiyang Chemical). Spots on TLC were visualized by UV irradiation (254 nm) and staining with potassium permanganate ($KMnO_4$). Flash column chromatography was performed with silica gel (200–300 mesh, reagent grade) from Accela. Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$, CD_3OD, or $(CD_3)_2SO$ at room temperature on Bruker Avance NMR Spectrometers. Chemical shifts (δ) are reported in ppm and coupling constants (*J* values) are reported in Hz. ^1H NMR chemical shifts are calibrated using tetramethylsilane (TMS, δ = 0.00 ppm) in $CDCl_3$ as the internal standard or with the residual solvent peaks of CD_3OD (δ = 3.31 ppm) or $(CD_3)_2SO$ (δ = 2.50 ppm). ^{13}C NMR chemical shifts are calibrated with the residual solvent peaks of $CDCl_3$ (δ = 77.16 ppm), CD_3OD (δ = 49.00 ppm), or $(CD_3)_2SO$ (δ = 39.52 ppm). High resolution mass spectra (HRMS) were recorded on a Q Exactive Focus mass spectrometer (ThermoFisher).

Synthesis of the unnatural amino acid SCouK

SCouK was synthesized as shown in Scheme S1.

Scheme S1. Synthetic scheme of SCouK.

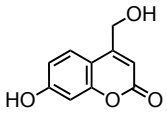




1

4-(Chloromethyl)-7-hydroxy-2*H*-chromen-2-one (1)

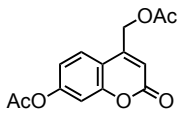
Compound **1** was prepared following literature procedures.[6] Briefly, to a solution of resorcinol (10 g, 91.0 mmol) in 70% sulfuric acid (100 mL) at 0 °C was added ethyl 4-chloro-3-oxobutanoate (12.5 mL, 91.0 mmol) dropwise. The reaction mixture was stirred at room temperature overnight. After that, the solution was poured into 500 mL of ice water. The precipitate was filtered, washed with water, and dried to give the desired product **1** as a white solid (10.7 g, 56% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 (d, *J* = 8.6 Hz, 1H), 6.84 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.82 (d, *J* = 2.4 Hz, 1H), 6.37 (s, 1H), 4.63 (s, 2H). The ^{1}H NMR data are consistent with previously reported.[6]



2

7-Hydroxy-4-(hydroxymethyl)-2*H*-chromen-2-one (2)

A solution of compound **1** (10.7 g, 51.0 mmol) in 400 mL of H_2O was heated to reflux for 48 h. After that, the solution was cooled to room temperature and placed at 0 °C for 24 h. The precipitate was filtered, washed with water, and dried to give the desired product **2** as a white solid (6.6 g, 67% yield). ^{1}H NMR (500 MHz, CD_3OD) δ 7.50 (d, *J* = 8.7 Hz, 1H), 6.79 (dd, *J* = 8.7, 2.4 Hz, 1H), 6.72 (d, *J* = 2.4 Hz, 1H), 6.36 (s, 1H), 4.81 (d, *J* = 1.6 Hz, 2H). The ^{1}H NMR data are consistent with previously reported.[6]

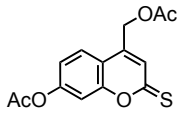


3

(7-Acetoxy-2-oxo-2*H*-chromen-4-yl)methyl acetate (3)

To a solution of compound **2** (6.6 g, 34.1 mmol) in anhydrous tetrahydrofuran (THF; 100 mL) was added acetic anhydride (19.0 mL, 204.8 mmol), pyridine (2.76 mL, 34.1 mmol), and 4-dimethylaminopyridine (DMAP; 417 mg, 3.41 mmol) successively. The reaction mixture was stirred at room temperature for 2 h and quenched with water. The mixture was extracted with ethyl acetate (EA), washed with water and brine, and dried with anhydrous Na_2SO_4. After filtration, the organic phase was concentrated under vacuum. The crude product was purified by flash column chromatography (petroleum ether (PE)/EA = 5:1) to give product **3** as a white

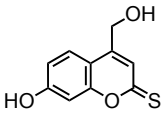
solid (7.7 g, 82% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (d, *J* = 8.7 Hz, 1H), 7.17 (d, *J* = 2.3 Hz, 1H), 7.10 (dd, *J* = 8.6, 2.3 Hz, 1H), 6.48 (s, 1H), 5.28 (d, *J* = 1.5 Hz, 2H), 2.35 (s, 3H), 2.22 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.2, 168.8, 160.2, 154.5, 153.4, 148.7, 124.5, 118.6, 115.1, 112.9, 111.0, 61.2, 21.2, 20.8. HRMS calcd for $C_{14}H_{12}O_6$ $[M+H]^+$: 277.0707; found: 277.0708.



4

(7-Acetoxy-2-thioxo-2*H*-chromen-4-yl)methyl acetate (4)

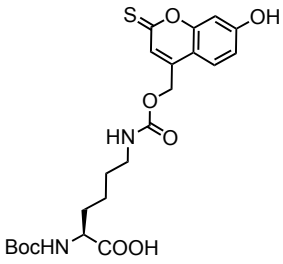
To a solution of compound **3** (7.7 g, 28.0 mmol) in anhydrous m-xylene (150 mL) was added Lawesson's reagent (9.1 g, 22.4 mmol). The mixture was heated to reflux in the dark for 6 h. After that, the mixture was concentrated under vacuum. The crude product was purified by flash column chromatography (PE/EA = 5:1) to give product **4** as an orange solid (5.6 g, 69% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 (d, *J* = 8.7 Hz, 1H), 7.29 (d, *J* = 2.2 Hz, 1H), 7.26 (s, 1H), 7.13 (dd, *J* = 8.7, 2.2 Hz, 1H), 5.21 (d, *J* = 1.3 Hz, 2H), 2.35 (s, 3H), 2.20 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 196.9, 170.2, 168.6, 156.8, 153.6, 139.6, 126.4, 124.4, 119.6, 116.6, 110.7, 60.9, 21.3, 20.9. HRMS calcd for $C_{14}H_{12}O_5S$ $[M+H]^+$: 293.0478; found: 293.0478.



5

7-Hydroxy-4-(hydroxymethyl)-2*H*-chromene-2-thione (5)

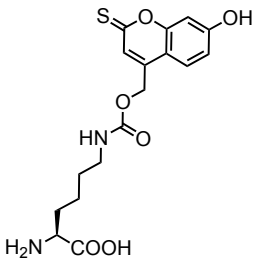
To a solution of compound **4** (5.6 g, 19.3 mmol) in ethanol (80 mL) was added concentrated HCl (8 mL). The mixture was heated to reflux in the dark for 1 h. After that, the reaction mixture was concentrated under vacuum and diluted with EA. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. The resulting crude product was purified by flash column chromatography (CH_2Cl_2/MeOH = 60:1) to give product **5** as an orange solid (3.3 g, 83% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 7.57 (d, *J* = 9.0 Hz, 1H), 7.23 (s, 1H), 6.89 – 6.85 (m, 1H), 6.85 (d, *J* = 1.3 Hz, 1H), 4.77 (d, *J* = 1.4 Hz, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 199.7, 163.3, 159.3, 149.4, 126.3, 122.7, 115.7, 113.1, 103.3, 60.5. HRMS calcd for $C_{10}H_8O_3S$ $[M+H]^+$: 209.0267; found: 209.0267.



7

***N*^{2}-(*tert*-Butoxycarbonyl)-*N*^{6}-(((7-hydroxy-2-thioxo-2*H*-chromen-4-yl)methoxy)carbonyl)-*L*-lysine (7)**

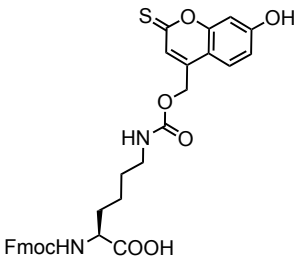
To a solution of compound **5** (1.8 g, 8.6 mmol) in anhydrous THF (30 mL) at 0 °C was added a solution of triethylamine (TEA; 1.8 mL, 13.0 mmol) and nitrophenyl chloroformate (2.6 g, 13.0 mmol) in THF (20 mL) under a N_2 atmosphere. The reaction was then allowed to warm to room temperature and stirred in the dark for 4 h. The reaction mixture was diluted with EA and washed with a saturated aqueous solution of $NaHCO_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum to give the crude product **6** as a yellow solid (1.4 g, 43% yield) without further purification. To a solution of N^{α}-Boc-*L*-Lys (0.99 g, 4.0 mmol) in dimethylformamide (DMF; 10 mL) was added TEA (0.19 mL, 1.3 mmol) and a solution of compound **6** (1.0 g, 2.7 mmol) in DMF (10 mL) dropwise. The reaction mixture was stirred at room temperature in the dark overnight and concentrated under vacuum. The residue was diluted with EA, washed with 0.1 M aqueous HCl solution, and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH = 20:1) to provide compound **7** as a viscous orange oil (0.57 g, 44% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 7.55 (d, *J* = 8.7 Hz, 1H), 7.30 (br, 1H), 7.06 (s, 1H), 6.86 (dd, *J* = 8.7, 2.4 Hz, 1H), 6.82 (d, *J* = 2.4 Hz, 1H), 5.24 (s, 2H), 4.09 – 4.06 (m, 1H), 3.15 (t, *J* = 6.6 Hz, 2H), 1.84 – 1.81 (m, 1H), 1.69 – 1.65 (m, 1H),1.58 – 1.52 (m, 2H), 1.42 (s, 11H). ^{13}C NMR (101 MHz, CD_3OD) δ 199.0, 176.3, 163.5, 159.4, 158.2, 157.9, 144.7, 126.5, 123.2, 115.8, 112.7, 103.4, 80.5, 62.4, 54.8, 41.6, 32.5, 30.3, 28.7, 24.1. HRMS calcd for $C_{22}H_{28}N_2O_8S$ $[M+H]^+$: 479.1494; found: 479.1495.



SCouK

***N*^{6}-(((7-Hydroxy-2-thioxo-2*H*-chromen-4-yl)methoxy)carbonyl)-*L*-lysine (SCouK)**

To a solution of compound **7** (0.57 g, 1.2 mmol) in anhydrous DCM (5 mL) was added trifluoroacetic acid (TFA; 5 mL) under a N_2 atmosphere. After stirring for 1 h at room temperature in the dark, the reaction mixture was concentrated under vacuum to remove the solvent and TFA. The crude product was dissolved in MeOH and precipitated with Et_2O. The precipitate was collected, redissolved in MeOH, and precipitated again with Et_2O. The precipitate was collected and dried to give compound **SCouK** as an orange solid (0.35 g, 79% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 7.62 (d, *J* = 8.7 Hz, 1H), 7.39 (br, 1H), 7.11 (s, 1H), 6.92 – 6.88 (m, 2H), 5.29 (s, 2H), 3.74 – 3.71 (m, 1H), 3.21 – 3.19 (m, 2H), 1.96 – 1.94 (m, 1H), 1.90 – 1.86 (m, 1H), 1.62 – 1.60 (m, 2H), 1.51 – 1.47 (m, 2H). ^{13}C NMR (101 MHz, *d*^{6}-DMSO) δ 196.4, 170.3, 162.8, 157.7, 155.3, 144.8, 125.9, 121.0, 115.2, 110.6, 102.2, 60.5, 53.9, 40.3, 30.6, 29.0, 22.3. HRMS calcd for $C_{17}H_{20}N_2O_6S$ [M-H]$^-$: 379.0969; found: 379.0972.



Fmoc-SCouK

***N*^{2}-(((9*H*-Fluoren-9-yl)methoxy)carbonyl)-*N*^{6}-(((7-hydroxy-2-thioxo-2*H*-chromen-4-yl)methoxy)carbonyl)-*L*-lysine (Fmoc-SCouK)**

To a solution of *N*^{α}-Fmoc-*L*-Lys (0.59 g, 1.6 mmol) in DMF (5 mL) was added TEA (0.08 mL, 0.5 mmol) and a solution of compound **6** (0.4 g, 1.1 mmol) in DMF (5 mL) dropwise. The reaction mixture was stirred at room temperature in the dark overnight and concentrated under vacuum. The residue was diluted with EA, washed with 0.1 M aqueous HCl solution, and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH = 20:1) to provide compound **Fmoc-SCouK** as a viscous orange oil (0.27 g, 41% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 7.75 (d, *J* = 7.4 Hz, 2H), 7.64 (dd, *J* = 7.2, 4.1 Hz, 2H), 7.46 (d, *J* = 8.4 Hz, 1H), 7.35 (t, *J* = 7.4 Hz, 2H), 7.30 – 7.26 (m, 3H), 7.05 (s, 1H), 6.83 (dd, *J* = 6.9, 1.8 Hz, 1H), 6.80 (s, 1H), 5.17 (s, 2H), 4.36 – 4.29 (m, 2H), 4.20 – 4.15 (m, 2H), 3.17 (t, *J* = 5.8 Hz, 2H), 1.96 – 1.88 (m, 1H), 1.75 – 1.71 (m, 1H), 1.63 – 1.44 (m, 4H). ^{13}C NMR (101 MHz, CD_3OD) δ 199.0, 175.9, 163.4, 159.3, 158.7, 157.9, 145.3, 145.2, 144.7, 142.5, 128.7, 128.2, 126.3, 126.2, 123.0, 120.9, 115.7, 112.6, 103.4, 68.0, 62.3, 55.2, 41.5, 32.3, 30.3, 24.0. HRMS calcd for $C_{32}H_{30}N_2O_8S$ [M+H]$^+$: 603.1796; found: 603.1798.

Biological methods and materials

Reagents and antibodies

High-fidelity DNA polymerase, restriction enzymes, and dNTPs were obtained from New England Biolabs. Oligonucleotide primers and gene fragments were synthesized by Genewiz or Tsingke. Site-directed mutagenesis was performed with QuikChange II Site-Directed Mutagenesis Kit (Agilent) with primers designed by Agilent Primer Design Program. Gibson assembly was carried out with Seamless Cloning Kit (Beyotime). Plasmid DNA isolation was carried out with Plasmid Mini/Midi Kit (Omega). Polyethylenimine (PEI) was purchased from Polysciences. Protease inhibitor cocktail (cOmplete ULTRA mini-Tablets) was purchased from Roche. Isopropyl-β-D-thiogalactoside (IPTG), kanamycin, and chloramphenicol were purchased from Sangon Biotech.

N$^{\varepsilon}$-Boc-L-Lysine (BocK) was purchased from Adamas and dissolved in 0.1 M NaOH as the stock solution (2 M). *O*-(2-nitrobenzyl)-tyrosine (ONBY) was purchased from Sigma-Aldrich. HCK was synthesized in house according to literature.[3] SCouK was synthesized as described in this manuscript and is available from the corresponding author upon request. Unless otherwise noted, unnatural amino acids (UAAs) were dissolved in DMSO as the stock solution (500 mM) and stored at −20 °C. All photocaged amino acids (ONBY, HCK, and SCouK) were protected from light during storage and handling. All UAAs were freshly diluted to the indicated working concentrations with culture medium immediately before use.

Anti-6*His-HRP (HRP-66005, 1:5000 dilution), anti-GFP-HRP (66002-1-Ig, 1:10000 dilution), anti-α-tubulin-HRP (HRP-66031, 1:5000 dilution), anti-β-actin-HRP (HRP-60008, 1:5000 dilution), and anti-histone H3 (17168-1-AP, 1:10000 dilution) antibodies for western blotting were purchased from Proteintech. Anti-p-Src (6943T, 1:1000 dilution), anti-p-ERK-1/2 (4370S, 1:2000 dilution), anti-ERK-1/2 (4695T, 1:1000 dilution), anti-p-TBK1 (5483S, 1:1000 dilution), anti-p-IRF3 (phospho S386, 37829S, 1:1000 dilution) were purchased from Cell Signaling Technology. Anti-HA-Peroxidase (clone 3F10, 12013819001, 1:10000 dilution) was purchased from Roche. Anti-FLAG M2 antibody (F1804, 1:10000 dilution) was purchased from Sigma. Goat anti-rabbit and anti-mouse HRP secondary antibodies (1:10000 dilution) were purchased from Jackson ImmunoResearch Laboratories. α-Tubulin, β-actin, or histone H3 was used as the loading control for western blotting analysis, depending on reagent availability in the lab.

Plasmids and cloning

For site-specific incorporation of UAAs in *E. coli*, the pBX-MmPylRS plasmid expressing wild-type PylRS from *Methanosarcina mazei* (Mm) was constructed from a

pBR322-derived plasmid pBK-JYRS (developed by Prof. Peter Schultz group),[7], in our laboratory previously[8] and used in this study. The wild-type MmPylRS in the pBX-MmPylRS plasmid was under the control of constitutive *E. coli* GlnRS promoter and terminator. Wild-type MmPylRS in pBX-MmPylRS was mutated at indicated sites with site-directed mutagenesis to generate PylRS mutation plasmids, such as pBX-SCouKRS. The pLX-EGFP-Y39TAG-PylT plasmid containing the MmPyl-tRNA$_{CUA}$ gene and the *C*-terminally 6*His-tagged EGFP-Y39TAG gene was constructed from the pACYC184-derived pYC-J17 backbone (developed by Prof. Peter Schultz group)[7] in our laboratory previously[8] and used in this study. The pLX-sfGFP-N150TAG-PylT plasmid was generated by replacing the EGFP-Y39TAG gene in the pLX-EGFP-Y39TAG-PylT plasmid with the sfGFP-N150TAG gene. The pLX-EGFP-Y39TAG-PylT and pLX-sfGFP-N150TAG-PylT plasmids contain the MmPyl-tRNA$_{CUA}$ gene under the *lpp* promoter and *rrnC* terminator, as well as the EGFP-Y39TAG or sfGFP-N150TAG gene under the control of a bacteriophage *T5* promoter and t_0 terminator. The plasmid maps for pBX-MmPylRS, pBX-SCouKRS, pLX-EGFP-Y39TAG-PylT, and pLX-sfGFP-N150TAG-PylT are shown in Fig. S6 in Supplementary Information.

For site-specific incorporation of UAAs in mammalian cells, the pEF1α-MmPylRS-PylT plasmid was previously reported by Prof. Jason Chin group[9] and constructed in our laboratory.[8] The pEF1α-MmPylRS-PylT plasmid contains the FLAG-tagged MmPylRS gene under the control of an EF1α core promoter and a SV40 poly(A) terminator, as well as four copies of MmPyl-tRNA$_{CUA}$ under U6 promoters. A similar plasmid can be obtained from Addgene (a kind gift from Prof. Simon Elsaesser, Addgene #140009). To generate the pEF1α-SCouKRS-PylT plasmid, the desired mutations (Y306A/L309A/Y384F/I405R) were introduced into the pEF1α-MmPylRS-PylT plasmid via site-directed mutagenesis. To generate the pEF1α-BhcKRS-PylT plasmid, the BhcKRS gene[3] was synthesized by Genewiz and cloned into the pEF1α-MmPylRS-PylT plasmid to replace the original MmPylRS gene via Gibson assembly. To generate the pEF1α-ONBYRS-PylT plasmid, the ONBYRS gene[10] was synthesized by Genewiz and cloned into the pEF1α-MmPylRS-PylT plasmid to replace the original MmPylRS gene via Gibson assembly. The plasmid maps for pEF1α-MmPylRS-PylT, pEF1α-SCouKRS-PylT, pEF1α-BhcKRS-PylT, and pEF1α-ONBYRS-PylT are shown in Fig. S10 in Supplementary Information.

To generate the plasmids of pCMV-sfGFP-N150TAG, pCMV-EGFP-K85TAG-mCherry, pCMV-FLuc-K529TAG, and pCMV-NLuc-Y96TAG, the sfGFP-N150TAG, EGFP-K85TAG-mCherry, fLuc-K529TAG, and NLuc-Y96TAG genes (synthesized by Genewiz) were individually cloned into the pCMV-HA vector (Clontech) via Gibson assembly. Similarly, the *C*-terminally HA-tagged MEK1-ΔN-K97TAG gene was synthesized by Genewiz and cloned into the pCMV-HA vector (Clontech) via Gibson assembly to generate the pCMV-MEK1-ΔN-K97TAG-HA plasmid. The *C*-terminally HA-tagged Src-Y527F-K295TAG gene was

synthesized by Genewiz and cloned into the pCMV-HA vector (Clontech) via Gibson assembly to generate the pCMV-Src-Y527F-K295TAG-HA plasmid. The *C*-terminally HA-tagged cGAS gene was synthesized by Genewiz and cloned into the pEF1α-MmPylRS-PylT plasmid to replace the original MmPylRS gene via Gibson assembly, generating the pEF1α-cGAS-HA plasmid. An amber TAG codon was introduced into the pEF1α-cGAS-HA plasmid at the K384, K394, or K414 site by site-directed mutagenesis to generate the pEF1α-cGAS-K384TAG-HA, pEF1α-cGAS-K394TAG-HA, and pEF1α-cGAS-K414TAG-HA plasmids. The maps for these plasmids are shown in Figure S10 in Supplementary Information.

For the generation of STING-expressing stable cells, the *N*-terminally FLAG-tagged STING gene was synthesized by Genewiz and cloned into the pB-EF1a-NLuc-IRES-Puro plasmid (a gift from James Thomson, Addgene #130936) to replace the original NLuc gene via Gibson assembly, generating the pB-EF1a-FLAG-STING-IRES-Puro plasmid. The hyPBase plasmid used for stable integration was obtained from System Biosciences (#PB200A-1). The IFN-Beta_pGL3 plasmid was purchased from Miaoling Biology (#P5314).

All plasmids used in this study are available from the corresponding author upon request. The key plasmids are also in the process of being deposited to Addgene.

General information for photoirradiation

For photoirradiation at 254 nm, 302 nm, and 365 nm, UV lamps (16 W; Shanghai GuCun, China) at the indicated wavelengths were used. For photoirradiation at 390 nm, 450 nm, 520 nm, and 650 nm, flat-type light-emitting diode (LED) bulbs (45 W; Xuzhou AiJia, China) labeled at the indicated wavelengths (390–400 nm, 450–460 nm, 520–530 nm, and 640–660 nm, respectively) were used. Samples were generally placed on ice and irradiated with the bulb or lamp positioned above the samples at a distance of 20 cm to ensure uniform light illumination. The spectral characteristics of the LEDs (named 390 nm LED, 450 nm LED, 520 nm LED, and 650 nm LED) were determined at the sample plane using an HP350 portable spectroradiometer (380–780 nm; Zhejiang ShuangSe, China). The peak wavelength, central wavelength, dominant wavelength, centroid wavelength, half width, irradiance, and emission spectrum were characterized for each LED.

Photolysis of N_α-Fmoc-SCouK *in vitro*

N_α-Fmoc-SCouK was dissolved in CH_3CN and diluted with PBS (20 mM, pH 7.4; CH_3CN/PBS = 2:8, v/v) to a final concentration of 50 μM. For pH-dependent photolysis, PBS solutions (20 mM) of the indicated pH values were prepared and used to dilute the N_α-Fmoc-SCouK stock solution to a final concentration of 50 μM (in CH_3CN/PBS = 2:8, v/v). The resulting solutions were irradiated with a 520 nm LED (22 mW/cm^2) for varying periods (10,

30, 60, and 120 s). The resulting mixture was analyzed by LC-MS on a Q Exactive Focus LC-MS/MS System (ThermoFisher). The UV detector was set at 254 nm. Samples were eluted from a Hypersil C18 UHPLC column (100 × 2.1 mm, 1.9 μm) with a linear gradient from 10% acetonitrile/90% water containing 0.1 % FA to 95% acetonitrile/5% water containing 0.1 % FA at a flow rate of 0.28 mL/min. LC-MS spectra were analyzed with the Xcalibur software (ThermoFisher). The peak areas of N_α-Fmoc-SCouK and N_α-Fmoc-K in the HPLC traces were used to calculate the conversions and yields at different irradiation times, using known concentrations of N_α-Fmoc-SCouK and N_α-Fmoc-K as references. The experiments were performed in four replicates with reproducible results. Each replicate dataset of N_α-Fmoc-SCouK conversion was individually fitted to a nonlinear one-phase exponential decay model in the Prism software (GraphPad Software) to calculate the half-life ($t_{1/2}$) of SCouK photodecaging. The resulting half-life values were averaged, and the standard deviation (SD) was calculated.

Screening of MmPylRS variants for SCouK incorporation

The *E. coli* strain BL21(DE3) was co-transformed with the pLX-EGFP-Y39TAG-PylT plasmid and individual pBX-MmPylRS mutant plasmids. The transformed bacterial cells were grown in Luria Broth (LB) medium containing kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) overnight at 37 °C. The cultures were then inoculated by 1:100 dilution into fresh LB medium supplemented with kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C. When the OD_{600} reached 0.6, SCouK (1 mM) was added into the bacterial cultures. After 0.5 h, IPTG (1 mM) was added into the culture to induce the protein expression at 37 °C for 10 h. EGFP fluorescence intensities of individual bacterial cultures were measured on a Biotek Synergy H1 microplate reader and compared with the corresponding control cultures without the addition of SCouK.

Incorporation of SCouK into proteins in *E. coli*

The *E. coli* strain BL21(DE3) was co-transformed with the pLX-sfGFP-N150TAG-PylT plasmid and the pBX-SCouKRS plasmid. The transformed bacterial cells were grown in LB medium containing kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) overnight at 37 °C. The culture was then inoculated by 1:100 dilution into fresh LB medium supplemented with kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C. SCouK (1 mM) was added into the bacterial culture when the OD_{600} reached 0.6. For concentration-dependent incorporation of SCouK into sfGFP-N150TAG, SCouK at different concentrations (from 0 mM to 2 mM) was added into the bacterial culture. After 0.5 h, IPTG (1 mM) was added into the culture to induce the protein expression at 37 °C for 10 h. Cells were harvested and lysed with

4% SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM triethanolamine, pH 7.4) at 95 °C for 5 min. The resulting cell lysates were centrifuged at 16,000g for 5 min at room temperature. Protein concentrations were determined by the BCA assay (Pierce). Finally, the proteins were separated on 4–20% SDS-PAGE gels (Genscript) and analyzed by Western blotting and Coomassie Brilliant Blue staining.

Recombinant purification of SCouK-incorporated sfGFP from *E. coli*

For purification of 6*His-tagged sfGFP-N150SCouK from the *E. coli* culture, the BL21(DE3) strain was co-transformed with the pLX-sfGFP-N150TAG-PylT plasmid and the pBX-SCouKRS plasmid. The transformed bacterial cells were grown in LB medium containing kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) overnight at 37 °C. The culture was then inoculated by 1:100 dilution into fresh LB medium supplemented with kanamycin (40 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C. SCouK (1 mM) was added into the bacterial culture when the OD_{600} reached 0.6. After 0.5 h, IPTG (1 mM) was added into the culture to induce the protein expression at 37 °C for 10 h. Cells were harvested and lysed with a sonic disruptor (Scientz, JY92-IIN) in the binding buffer (20 mM Tris-HCl, 500 mM NaCl, 10% glycerol, 10 mM imidazole, pH 8.0) containing the protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF), deoxyribonuclease I, and lysozyme. After centrifugation, the supernatant was purified by Ni-NTA Sefinose Resin 6FF (Sangon, cat#C600033-0025) according to the manufacturer's protocol and eluted with the elution buffer (binding buffer supplemented with 250 mM imidazole). The purified protein was stored at −80 °C. For photoactivation of the recombinant protein *in vitro*, sfGFP-N150SCouK (1 μM) in PBS was irradiated with a 520 nm LED (22 mW/cm^2) for 30 s.

Mass spectrometry analysis of recombinant proteins

The recombinant proteins were desalted with 10 kDa Amicon centrifugal filters (Millipore, cat#MRCPRT010), washed three times with water, and reconstituted into 0.01% ammonia solution. The resulting samples were analyzed on a Q Exactive Focus LC-MS/MS system (ThermoFisher) equipped with a nano-ESI ionization source. The mass spectra were deconvoluted with the BioPharma Finder software (ThermoFisher).

Mammalian cell culture and transfection

HEK293T cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Cytiva, cat#SH30243.01) supplemented with 10% fetal bovine serum (FBS; Biological Industries, cat#04-001-1ACS) in a humidified incubator at 37 °C with 5% CO_2. For transfection, cells were grown to 70%

confluence and transfected with indicated plasmids using PEI (Polysciences) for 18–24 h in cell growth media.

To generate HEK293T-STING stable cell line, the PiggyBac transposon system was used. Briefly, HEK293T cells seeded in 6-well plate were transfected with 1 μg of the pB-EF1a-FLAG-STING-IRES-Puro plasmid (constructed in this study) together with 1 μg of the hyPBase plasmid (System Biosciences) for 24 h. Transfected cells were further grown in fresh growth media for 24 h and then selected with 3-6 μg/mL puromycin for 7 days. The stable cell line was maintained in growth media containing 1 μg/mL puromycin after selection.

Incorporation of SCouK into proteins in mammalian cells

HEK293T cells were seeded in 6-well plates and cultured overnight in cell culture media. On the next day cells were co-transfected with the pCMV-sfGFP-N150TAG plasmid (0.65 μg per well) and the pEF1α-MmPylRS-PylT or pEF1α-SCouKRS-PylT plasmid (0.35 μg per well) using PEI (2.5 μg per well) in cell culture media in the absence or presence of UAA (0.5 mM unless otherwise stated). For the expression of SCouK-modified proteins, pEF1α-SCouKRS-PylT was used, while pEF1α-MmPylRS-PylT was used for the incorporation of BocK. After transfection for 24 h, cells were lysed with 4% SDS lysis buffer containing benzonase by sonication and vortexing. The resulting cell lysates were centrifuged at 16,000g for 5 min at room temperature. Protein concentrations were determined by the BCA assay (Pierce). Proteins were separated on 4–20% SDS-PAGE gels (Genscript) and analyzed by western blotting. Alternatively, cells were imaged live in FluoroBrite DMEM (ThermoFisher) using an Olympus IX73 inverted fluorescence microscope equipped with a 10× objective lens. The blue filter set (bandpass 460–495 nm) was used for recording EGFP signals.

Widefield fluorescence images were captured under identical acquisition settings and exposure times for all samples and processed in the cellSens Dimension software (Olympus). Images were saved in the native VSI format for Olympus cellSens software and exported as uncompressed TIFF files for quantification. Image analysis and quantification were performed with ImageJ software (NIH) as described previously.[11] Briefly, images were opened in ImageJ and converted to 8-bit grayscale versions (*Image → Color → Split channels*). The green sfGFP channel was used and thresholded using the *Image → Adjust → Threshold* functions to isolate fluorescent signals from background and select all fluorescent cells for quantification. The default threshold was generally used across different samples. The measurement parameters were selected using the *Analyze → Set Measurements* functions to make sure that gray value parameters and the *Limit to Threshold* option was enabled. Finally, the mean fluorescence intensity of the areas above the threshold was measured and exported for analysis. Each experimental condition was performed in at least three biological replicates. The number of

biological replicates was specified in the corresponding figure legend. For each biological replicate, at least three representative images from different fields of view per well were quantified and averaged to obtain the mean fluorescence intensity of the replicate. All mean fluorescence intensity data were pooled and normalized for statistical analysis. The relative mean fluorescence intensity was reported as mean ± SD.

Photophysical characterization of SCouK

For the photophysical characterization, SCouK and HCK were dissolved in DMSO to make the stock solutions. UV-Vis absorption spectra of SCouK and HCK were recorded on a UV-Vis spectrophotometer (Techcomp UV-1000) at 10 μM concentrations in PBS (20 mM, pH = 7.4) containing 0.01% DMSO. For pH-dependent absorption measurement, PBS solutions (20 mM) at indicated pH values were prepared and used to dilute the SCouK stock solution to a final concentration of 10 μM. Fluorescence spectra of SCouK and HCK were recorded on a Hitachi F-4600 fluorescence spectrophotometer at 10 μM concentrations in PBS (20 mM, pH = 7.4) containing 0.01% DMSO.

Cytotoxicity of SCouK and its decaging product

Cell Counting Kit-8 (CCK-8) was used to measure the cytotoxicity of SCouK and its decaging product. Briefly, HEK293T cells were seeded in 96-well plates (~5,000 cells per well) and cultured overnight. Cells were treated with SCouK at different concentrations for 24 h. For assessing the decaging product of SCouK, cells were treated with SCouK at different concentrations for 0.5 h, irradiated with a 520 nm LED (22 mW/cm^2) for 1 min, and further cultured for 24 h. After that, CCK-8 solution (HY-K0301, MedChemExpress) was added to the cells (20 μL per well) and incubated at 37 °C for 1–2 h. The absorbance of each well was measured at the wavelength of 450 nm with a Cytation 5 Multi-Mode Reader (BioTek).

Photoactivation of EGFP in live mammalian cells

HEK293T cells were seeded in poly-D-lysine-coated 35-mm dishes and cultured overnight in growth media. On the next day cells were co-transfected with the plasmids of pCMV-EGFP-K85TAG-mCherry (0.65 μg per dish) and pEF1α-SCouKRS-PylT (0.35 μg per dish) using PEI (2.5 μg per dish) in cell culture media in the presence of SCouK (0.2 mM). After incubation for 24 h, cells were washed with DMEM twice over 2 h and irradiated under different wavelengths (365, 390, 450, 520, and 650 nm; Table S2) for 1 min, or with a 520 LED (22 mW/cm^2) for varying periods (10, 30, 60, 120, and 300 s) in FluoroBrite DMEM (ThermoFisher). After that, cells were cultured in a humidified incubator at 37 °C with 5% CO_2 for 2 h. Cells were imaged live in FluoroBrite DMEM using a Nikon A1R confocal fluorescence

microscope equipped with a 20× objective lens. For the EGFP channel, the 488 nm laser was used as the excitation, and emission was collected between 500 nm to 550 nm. For the mCherry channel, the 561 nm laser was used as the excitation, and emission was collected between 570 nm to 620 nm.

For localized irradiation, HEK293T cells were transfected and prepared as described above. The culture dish was transferred to an Olympus IX73 inverted fluorescence microscope, focused using the bright-field channel under a 60× objective lens, and locally irradiated for 10 s using a mercury lamp equipped with a green excitation filter set (bandpass 530–550 nm). After irradiation, cells were cultured for 2 h and then imaged live in FluoroBrite DMEM using an Olympus IX73 inverted fluorescence microscope equipped with a 10× objective lens. The blue filter set (bandpass 460–495 nm) and yellow filter set (bandpass 560–580 nm) were used for recording EGFP and mCherry signals, respectively.

Confocal fluorescence images were captured under identical acquisition settings and exposure times for all samples and processed in the NIS Elements software (Nikon). Images were saved in the native ND2 format for Nikon NIS Elements software and exported as uncompressed TIFF files for quantification. Image analysis and quantification were performed with ImageJ software (NIH) as described previously.[11] Briefly, images were opened in ImageJ and converted to 8-bit grayscale versions (*Image → Color → Split channels*). The green EGFP and red mCherry channels were used and thresholded using the *Image → Adjust → Threshold* functions to isolate fluorescent signals from background and select all fluorescent cells for quantification. The default threshold was generally used across different samples. The measurement parameters were selected using the *Analyze → Set Measurements* functions to make sure that gray value parameters and the *Limit to Threshold* option was enabled. Finally, the mean fluorescence intensity of the areas above the threshold was measured and exported for analysis. Each experimental condition was performed in at least three biological replicates. The number of biological replicates was specified in the corresponding figure legend. For each biological replicate, at least three representative images from different fields of view per well were quantified and averaged to obtain the mean fluorescence intensity of the replicate. The ratio of mean EGFP intensity to mean mCherry intensity was calculated. All EGFP/mCherry ratio data were pooled and normalized for statistical analysis. The relative EGFP/mCherry ratio was reported as mean ± SD.

Photoactivation of luciferase in live mammalian cells

HEK293T cells were seeded in 60-mm dishes and cultured overnight in growth media. On the next day cells were co-transfected with the plasmids of pCMV-FLuc-K529TAG (1.3 μg per dish) and pEF1α-SCouKRS-PylT (0.7 μg per dish) using PEI (5 μg per dish) in cell culture

media in the presence of SCouK (0.2 mM). After incubation for 24 h, cells were washed with DMEM twice over 2 h and collected. Cells were resuspended in PBS and divided into a 96-well black plate, which allowed consistent expression across wells, minimized reagent consumption, and enabled quantitative bioluminescence detection using a plate reader. Cells were then irradiated in PBS under different wavelengths (365, 390, 450, 520, and 650 nm; Table S2) for 1 min. Finally, luciferase activities were measured using a Bright-Lumi luciferase assay kit (Beyotime, RG051M) on a Biotek Synergy H1 microplate reader. Bioluminescence images were recorded on a Chemidoc MP imaging system (Biorad) using the chemiluminescence detection mode. For the analysis of cells expressing FLuc-K529HCK, HEK293T cells were co-transfected with the plasmids of pCMV-FLuc-K529TAG (1.3 μg per dish) and pEF1α-BhcKRS-PylT (0.7 μg per dish) using PEI (5 μg per dish) in cell culture media in the presence of HCK (0.2 mM). For the analysis of cells expressing FLuc-K529BocK, HEK293T cells were co-transfected with the plasmids of pCMV-FLuc-K529TAG (1.3 μg per dish) and pEF1α-MmPylRS-PylT (0.7 μg per dish) using PEI (5 μg per dish) in cell culture media in the presence of BocK (0.2 mM).

Photoactivation of kinase in live mammalian cells

HEK293T cells were seeded in 12-well plates and cultured overnight in growth media. On the next day cells were co-transfected with the plasmids of pEF1α-SCouKRS-PylT (0.35 μg per well) and pCMV-MEK1-ΔN-K97TAG-HA or pCMV-Src-Y527F-K295TAG-HA (0.65 μg per well) using PEI (2.5 μg per well) in cell culture media in the presence of SCouK (0.2 mM). For the negative control experiments, HEK293T cells were co-transfected with the plasmids of pEF1α-MmPylRS-PylT (0.35 μg per well) and pCMV-MEK1-ΔN-K97TAG-HA or pCMV-Src-Y527F-K295TAG-HA (0.65 μg per well) using PEI (2.5 μg per well) in cell culture media in the presence of BocK (0.2 mM). After incubation for 24 h, cells were washed with DMEM twice over 2 h and irradiated with a 520 nm LED (22 mW/cm^2) for 1 min in FluoroBrite DMEM (ThermoFisher). After that, cells were cultured in growth media in a humidified incubator at 37 °C with 5% CO_2 for 2 h. Cells were harvested and lysed with 4% SDS lysis buffer containing benzonase for 5 min at 95 °C. The resulting cell lysates were centrifuged at 16,000g for 5 min at room temperature. Protein concentrations were determined by the BCA assay (Pierce). Proteins were separated on 4–20% SDS-PAGE gels (Genscript) and analyzed by western blotting.

Photoactivation of cGAS in live mammalian cells

HEK293T-STING cells were seeded in 12-well plates and cultured overnight in growth media. On the next day cells were co-transfected with the plasmids of pEF1α-SCouKRS-PylT

(0.28 μg per well) and pEF1α-cGAS-HA with K384, K394, or K414 mutated to TAG (0.52 μg per well) using PEI (2 μg per well) in cell culture media in the presence of SCouK (0.2 mM). After incubation for 24 h, cells were washed with DMEM twice over 2 h and irradiated with a 520 nm LED (22 mW/cm^2) for 1 min in FluoroBrite DMEM (ThermoFisher). After that, cells were cultured in culture media in a humidified incubator at 37 °C with 5% CO_2. For SCouK incorporation sites analysis, cells were harvested at 4 h after irradiation, and lysed with 4% SDS lysis buffer containing benzonase for 5 min at 95 °C. For kinetics analysis, cells were harvested at indicated time points after irradiation. The resulting cell lysates were centrifuged at 16,000g for 5 min at room temperature. Protein concentrations were determined by the BCA assay (Pierce). Proteins were separated on 4–20% SDS-PAGE gels (Genscript) and analyzed by western blotting.

For luciferase-based IFNβ expression assay, HEK293T-STING cells were co-transfected with pEF1α-SCouKRS-PylT (0.28 μg per well), pEF1α-cGAS-K384TAG-HA (0.4 μg per well), and IFN-Beta_pGL3 (0.14 μg per well) using PEI (2 μg per well) in cell culture media in the presence of SCouK (0.2 mM). The culture and irradiation conditions were the same as above. Cells were harvested at 6 h after irradiation and divided into a 96-well black plate for luciferase assay or lysed for SDS-PAGE. Luciferase activities were measured using a Bright-Lumi luciferase assay kit (Beyotime, RG051M) on a Biotek Synergy H1 microplate reader. Bioluminescence images were recorded on a Chemidoc MP imaging system (Biorad) using the chemiluminescence detection mode.

Sequential photoactivation

For the sequential photoactivation experiment, FLuc-K529SCouK and NLuc-Y96ONBY were separately expressed in HEK293T cells. FLuc-K529SCouK was expressed as above. NLuc-Y96ONBY was expressed by co-transfecting cells with plasmids of pCMV-NLuc-Y96TAG and pEF1α-ONBYRS-PylT in the presence of 0.2 mM ONBY. After 24 h transfection, cells were washed with DMEM twice over 2 h and then resuspended in PBS. Fluc-K529SCouK- and NLuc-Y96ONBY-expressing cells were mixed at 1:1 ratio or mixed with non-transfected HEK293T cells at 1:1 ratio. The cells were divided into a 96-well black plate and irradiated in PBS under different wavelengths for 1 min or sequentially with two wavelengths for 30 s each. Luciferase activities were measured using the Nano&Firefly-Glo Luciferase Reporter Assay Kit (Meilunbio, MA0522) on a Biotek Synergy H1 microplate reader. Bioluminescence images were recorded on a Chemidoc MP imaging system (Biorad) using the chemiluminescence detection mode.

Western blotting

SDS-PAGE gels were transferred to nitrocellulose membranes using Genscript eBlot™ L1 wet protein transfer system. The membranes were blocked with 5% nonfat milk in PBST (0.05% Tween-20 in PBS) for 30 min at room temperature and incubated with the primary antibody at 4 °C overnight. Membranes were washed with PBST three times and incubated with the second antibody at room temperature for 2 h. Membranes were finally washed with PBST three times, developed using ECL substrates, and imaged with a Chemidoc MP imaging system (Biorad).

Western blots grouped together were performed using cell lysates prepared from the same cell batches. For probing multiple proteins, membranes were sectioned into strips by molecular weight using a razor blade, and the strips were incubated with the respective antibodies. When proteins of similar apparent molecular weights (e.g., actin and FLAG-STING) were detected, membranes were first incubated with the antibody for the first protein of interest, stripped with Restore Western Blot Stripping Buffer (ThermoFisher), and then reprobed with the antibody for the second protein of interest. If a single membrane after sectioning was insufficient to detect all targets, identical lysate aliquots were electrophoresed in parallel with equal loading amounts. Consistent, specific, and non-overlapping detection across all experiments was ensured throughout the study. Unprocessed original images of all blots, with the membrane edges and molecular weight marker bands visible, are provided in this document.

Quantification and statistical analysis

Data were generally shown as mean ± SD calculated from biological replicates (shown as *n*). The method (one-way ANOVA) for determining significance is indicated in the corresponding figure legend, with the replication number of independent biological experiments specified. Significance was denoted as follows: *ns* = non-significant ($p > 0.05$), ∗ $p < 0.05$, ∗∗ $p < 0.01$, ∗∗∗ $p < 0.001$, and ∗∗∗∗ $p < 0.0001$. Statistical analyses were performed with the Prism software (GraphPad Software).

Protein sequences

Wild-type MmPylRS:

MDYKDDDDKMDKKPLNTLISATGLWMSRTGTIHKIKHHEVSRSKIYIEMACGDHLVV
NNSRSSRTARALRHHKYRKTCKRCRVSDEDLNKFLTKANEDQTSVKVKVVSAPTRTKKAM
PKSVARAPKPLENTEAAQAQPSGSKFSPAIPVSTQESVSVPASVSTSISSISTGATASALVK
GNTNPITSMSAPVQASAPALTKSQTDRLEVLLNPKDEISLNSGKPFRELESELLSRRKKDLQ
QIYAEERENYLGKLEREITRFFVDRGFLEIKSPILIPLEYIERMGIDNDTELSKQIFRVDKNFCL

RPMLAPNLYNYLRKLDRALPDPIKIFEIGPCYRKESDGKEHLEEFTMLNFCQMGSGCTRENL
ESIITDFLNHLGIDFKIVGDSCMVYGDTLDVMHGDLELSSAVVGPIPLDREWGIDKPWIGAGF
GLERLLKVKHDFKNIKRAARSESYYNGISTNL

SCouKRS (mutations compared to wild-type MmPylRS are marked in red):

MDYKDDDDKMDKKPLNTLISATGLWMSRTGTIHKIKHHEVSRSKIYIEMACGDHLVV
NNSRSSRTARALRHHKYRKTCKRCRVSDEDLNKFLTKANEDQTSVKVKVVSAPTRTKKAM
PKSVARAPKPLENTEAAQAQPSGSKFSPAIPVSTQESVSVPASVSTSISSISTGATASALVK
GNTNPITSMSAPVQASAPALTKSQTDRLEVLLNPKDEISLNSGKPFRELESELLSRRKKDLQ
QIYAEERENYLGKLEREITRFFVDRGFLEIKSPILIPLEYIERMGIDNDTELSKQIFRVDKNFCL
RPMLAPNLANYARKLDRALPDPIKIFEIGPCYRKESDGKEHLEEFTMLNFCQMGSGCTREN
LESIITDFLNHLGIDFKIVGDSCMVFGDTLDVMHGDLELSSAVVGPRPLDREWGIDKPWIGA
GFGLERLLKVKHDFKNIKRAARSESYYNGISTNL

BhcKRS (mutations compared to wild-type MbPylRS are marked in red):

MDKKPLDVLISATGLWMSRTGTLHKIKHHEVSRSKIYIEMACGDHLVVNNSRSCRTA
RAFRHHKYRKTCKRCRVSDEDINNFLTRSTESKNSVKVRVVSAPKVKKAMPKSVSRAPKPL
ENSVSAKASTNTSRSVPSPAKSTPNSSVPASAPAPSLTRSQLDRVEALLSPEDKISLNMAKP
FRELEPELVTRRKNDFQRLYTNDREDYLGKLERDITKFFVDRGFLEIKSPILIPAEYVERMGI
NNDTELSKQIFRVDKNLCLRPMLAPTLANYMRKLDRILPGPIKIFEVGPCYRKESDGKEHLE
EFTMVNFCQMGSGCTRENLEALIKEFLDYLEIDFEIVGDSCMVYGDTLDIMHGDLELSSAVV
GPVSLDREWGIDKPWIGAGFGLERLLKVMHGFKNIKRASRSESYYNGISTNL

ONBYRS (mutations compared to wild-type MbPylRS are marked in red):

MDKKPLDVLISATGLWMSRTGTLHKIKHHEVSRSKIYIEMACGDHLVVNNSRSCRTA
RAFRHHKYRKTCKRCRVSDEDINNFLTRSTESKNSVKVRVVSAPKVKKAMPKSVSRAPKPL
ENSVSAKASTNTSRSVPSPAKSTPNSSVPASAPAPSLTRSQLDRVEALLSPEDKISLNMAKP
FRELEPELVTRRKNDFQRLYTNDREDYLGKLERDITKFFVDRGFLEIKSPILIPAEYVERMGI
NNDTELSKQIFRVDKNLCLRPMLAPTFYNYMRKLDRILPGPIKIFEVGPCYRKESDGKEHLE
EFTMVGFGQMGSGCTRENLEALIKEFLDYLEIDFEIVGDSCMVFGDTLDIMHGDLELSSAVV
GPVSLDREWGIDKPWIGAGFGLERLLKVMHGFKNIKRASRSESYYNGISTNL

EGFP-Y39TAG (* denotes the UAA site):

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDAT*GKLTLKFICTTGKLPVP
WPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG
DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLA

DHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYKRS
HHHHHH

sfGFP-N150TAG (* denotes the UAA site):

MVSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPV
PWPTLVTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFE
GDTLVNRIELKGIDFKEDGNILGHKLEYNFNSH*VYITADKQKNGIKANFKIRHNVEDGSVQL
ADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMDELYKG
SHHHHHH

EGFP-K85TAG-mCherry (* denotes the UAA site):

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVP
WPTLVTTLTYGVQCFSRYPDHMKQHDFF*SAMPEGYVQERTIFFKDDGNYKTRAEVKFEG
DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLA
DHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYKSG
LRSRVATMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVT
KGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFNWERVMNFEDGGVVTVTQ
DSSLQDGEFIYKVKLRGTNFPSDGPVMQCRTMGWEASTERMYPEDGALKGEIKQRLKLKD
GGHYDAEVKTTYKAKKPVQLPGAYNVDIKLDILSHNEDYTIVEQYERAEGRHSTGGMDELY
K

FLuc-K529TAG (* denotes the UAA site):

MEDAKNIKKGPAPFYPLEDGTAGEQLHKAMKRYALVPGTIAFTDAHIEVDITYAEYF
EMSVRLAEAMKRYGLNTNHRIVVCSENSLQFFMPVLGALFIGVAVAPANDIYNERELLNSM
GISQPTVVFVSKKGLQKILNVQKKLPIIQKIIIMDSKTDYQGFQSMYTFVTSHLPPGFNEYDFV
PESFDRDKTIALIMNSSGSTGLPKGVALPHRTACVRFSHARDPIFGNQIIPDTAILSVVPFHH
GFGMFTTLGYLICGFRVVLMYRFEEELFLRSLQDYKIQSALLVPTLFSFFAKSTLIDKYDLSNL
HEIASGGAPLSKEVGEAVAKRFHLPGIRQGYGLTETTSAILITPEGDDKPGAVGKVVPFFEA
KVVDLDTGKTLGVNQRGELCVRGPMIMSGYVNNPEATNALIDKDGWLHSGDIAYWDEDEH
FFIVDRLKSLIKYKGYQVAPAELESILLQHPNIFDAGVAGLPDDDAGELPAAVVVLEHGKTMT
EKEIVDYVASQVTTAKKLRGGVVFVDEVPKGLTG*LDARKIREILIKAKKGGKIAVHHHHHH

MEK1-ΔN-K97TAG (* denotes the UAA site):

MPKKKPTPIQLNPAPDGSAVNGTSSAETNLEALQKKLEELELDEQQRKRLEAFLTQ
KQKVGELKDDDFEKISELGAGNGGVVFKVSHKPSGLVMAR*LIHLEIKPAIRNQIIRELQVLHE
CNSPYIVGFYGAFYSDGEISICMEHMDGGSLDQVLKKAGRIPEQILGKVSIAVIKGLTYLREK
HKIMHRDVKPSNILVNSRGEIKLCDFGVSGQLIDSMANSFVGTRSYMSPERLQGTHYSVQS

DIWSMGLSLVEMAVGRYPIPPPDAKELELMFGCQVEGDAAETPPRPRTPGRPLSSYGMDS
RPPMAIFELLDYIVNEPPPKLPSGVFSLEFQDFVNKCLIKNPAERADLKQLMVHAFIKRSDAE
EVDFAGWLCSTIGLNQPSTPTHAAGVLMAMSYPYDVPDYA

Src-Y527F-K295TAG (* denotes the UAA site):
MGSNKSKPKDASQRRRSLEPAENVHGAGGGAFPASQTPSKPASADGHRGPSAAFAPAAA
EPKLFGGFNSSDTVTSPQRAGPLAGGVTTFVALYDYESRTETDLSFKKGERLQIVNNTEGD
WWLAHSLSTGQTGYIPSNYVAPSDSIQAEEWYFGKITRRESERLLLNAENPRGTFLVRESE
TTKGAYCLSVSDFDNAKGLNVKHYKIRKLDSGGFYITSRTQFNSLQQLVAYYSKHADGLCH
RLTTVCPTSKPQTQGLAKDAWEIPRESLRLEVKLGQGCFGEVWMGTWNGTTRVAI*TLKP
GTMSPEAFLQEAQVMKKLRHEKLVQLYAVVSEEPIYIVTEYMSKGSLLDFLKGETGKYLRLP
QLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENLVCKVADFGLARLIEDNEYTARQGA
KFPIKWTAPEAALYGRFTIKSDVWSFGILLTELTTKGRVPYPGMVNREVLDQVERGYRMPC
PPECPESLHDLMCQCWRKEPEERPTFEYLQAFLEDYFTSTEPQYQPGENLLMAMSYPYDV
PDYA

Wild-type STING:
MDYKDHDGDYKDHDIDYKDDDDKMPHSSLHPSIPCPRGHGAQKAALVLLSACLVTL
WGLGEPPEHTLRYLVLHLASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLR
RGALLLLSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEKGNFNVAH
GLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQRLYILLPLDCGVPDNLSMADPNIR
FLDKLPQQTGDRAGIKDRVYSNSIYELLENGQRAGTCVLEYATPLQTLFAMSQYSQAGFSR
EDRLEQAKLFCRTLEDILADAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTV
GSLKTSAVPSTSTMSQEPELLISGMEKPLPLRTDFS

cGAS-K384TAG (* denotes the UAA site):
MQPWHGKAMQRASEAGATAPKASARNARGAPMDPTESPAAPEAALPKAGKFGPA
RKSGSRQKKSAPDTQERPPVRATGARAKKAPQRAQDTQPSDATSAPGAEGLEPPAAREP
ALSRAGSCRQRGARCSTKPRPPPGPWDVPSPGLPVSAPILVRRDAAPGASKLRAVLEKLK
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PRIQLEEYSNTRAYYFVKFKRNPKENPLSQFLEGEILSASKMLSKFRKIIKEEINDIKDTDVIM
KRKRGGSPAVTLLISEKISVDITLALESKSSWPASTQEGLRIQNWLSAKVRKQLRLKPFYLVP
KHAKEGNGFQEETWRLSFSHIE*EILNNHGKSKTCCENKEEKCCRKDCLKLMKYLLEQLKE
RFKDKKHLDKFSSYHVKTAFFHVCTQNPQDSQWDRKDLGLCFDNCVTYFLQCLRTEKLEN
YFIPEFNLFSSNLIDKRSKEFLTKQIEYERNNEFPVFDEFLMAMSYPYDVPDYA

NLuc-Y96TAG (* denotes the UAA site):

MVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLKI
DIHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILH*GTLVIDGVTPNMIDYFGRPYEGIAV
FDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLCERILAHHHHHH

Reference

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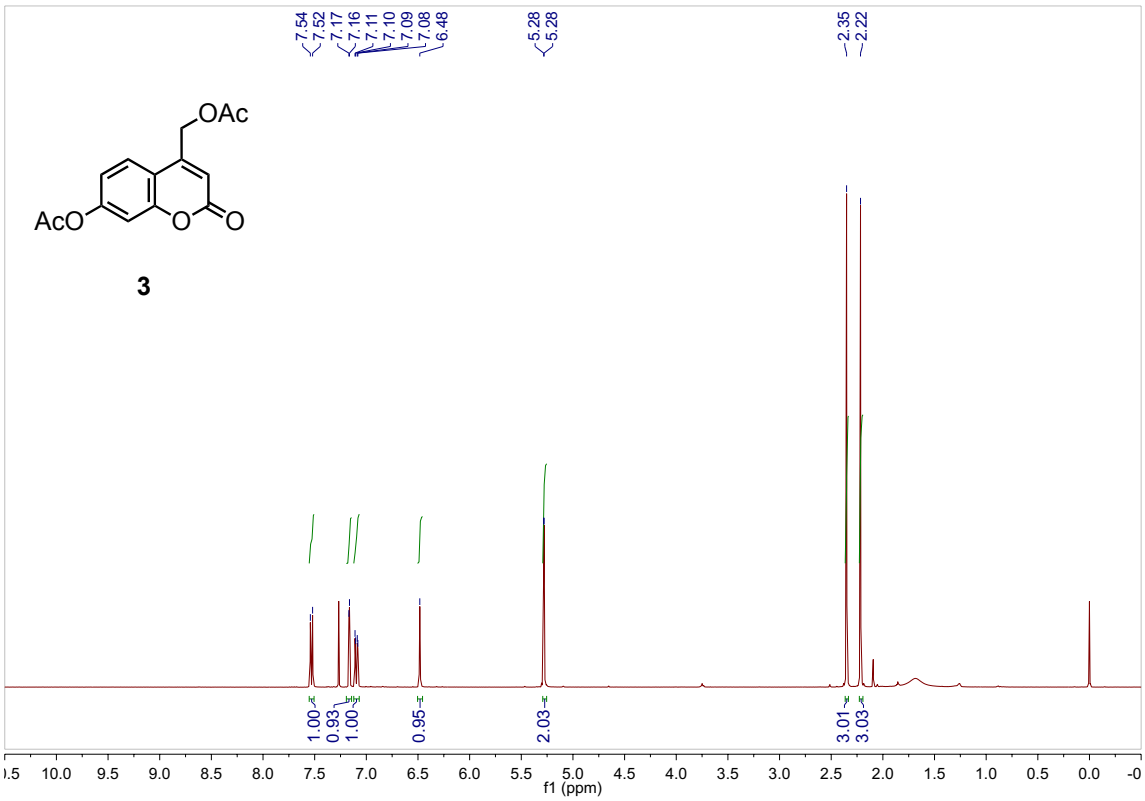
NMR spectra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5.28
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2.22
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AcO
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O
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0.93
1.00
0.95
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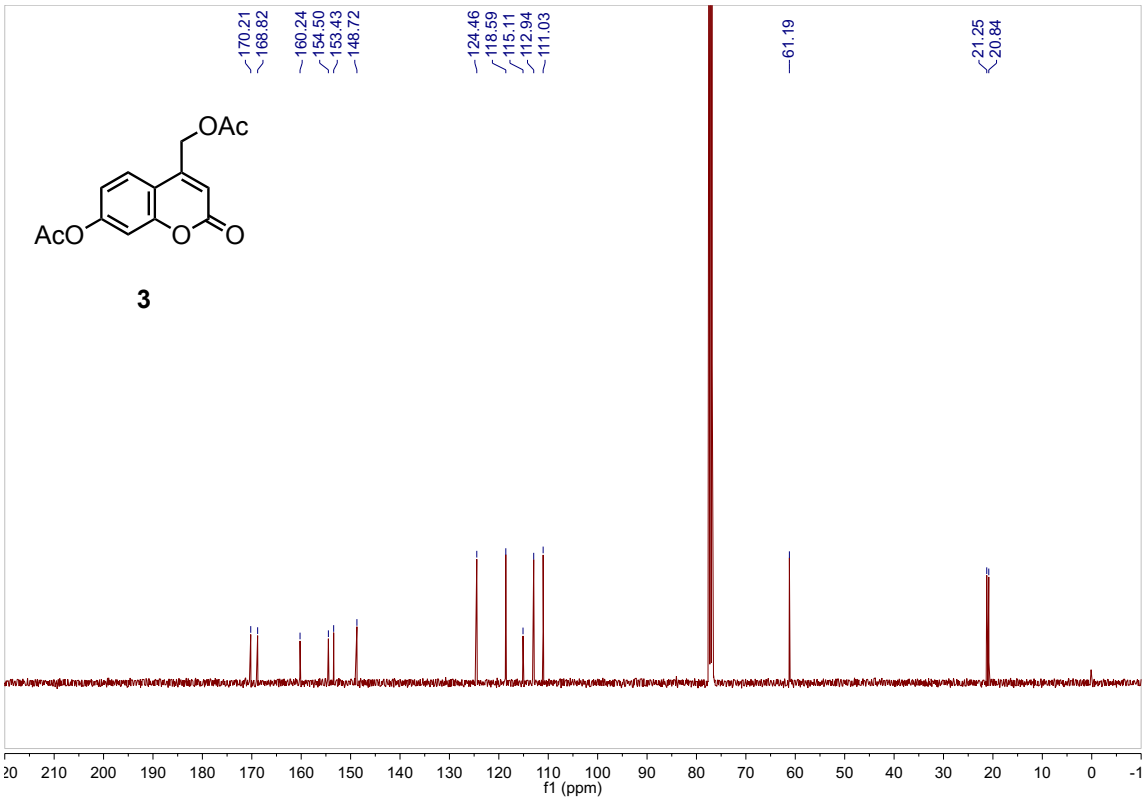
3.03
f1 (ppm)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OAc
AcO
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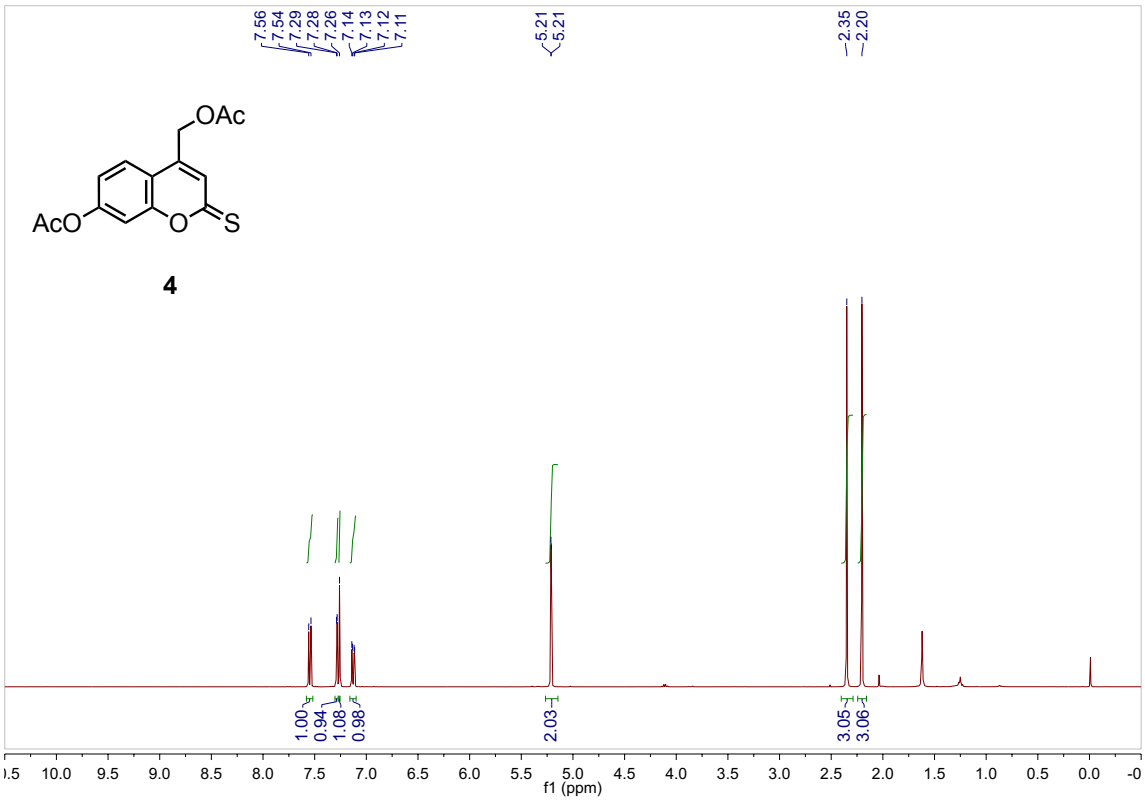
O
3
f1 (ppm)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2.20
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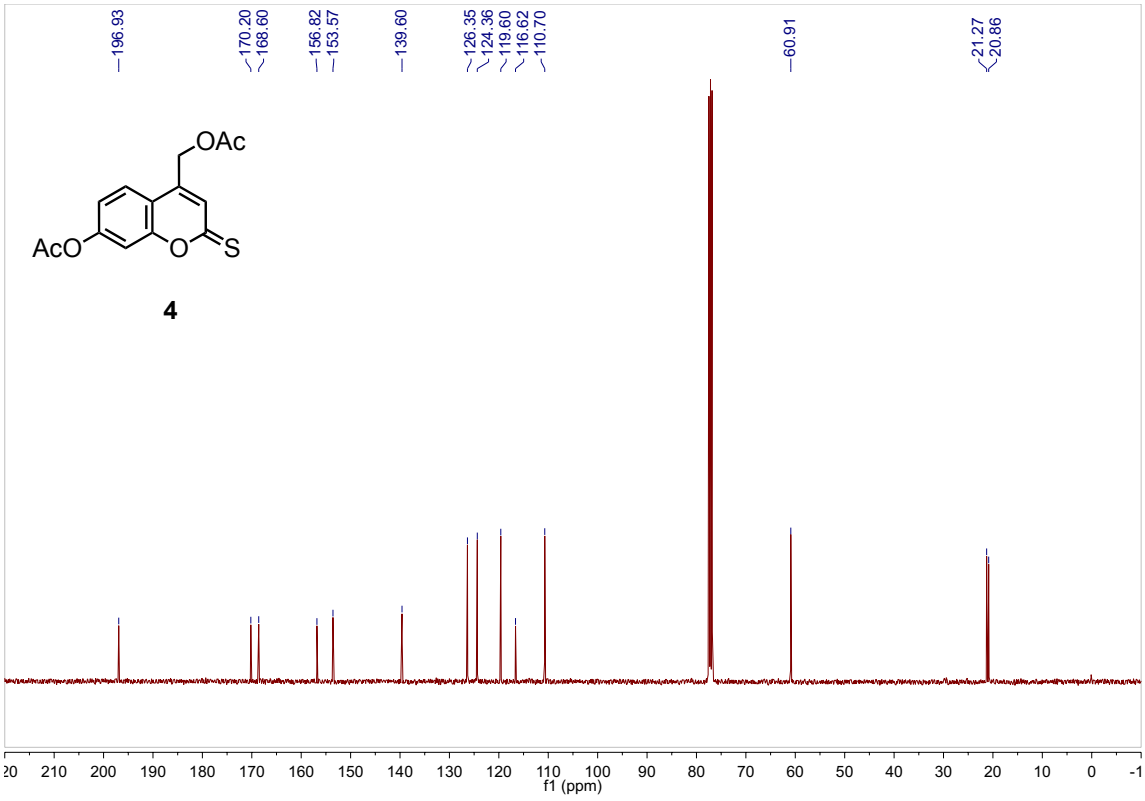
3.06
f1 (ppm)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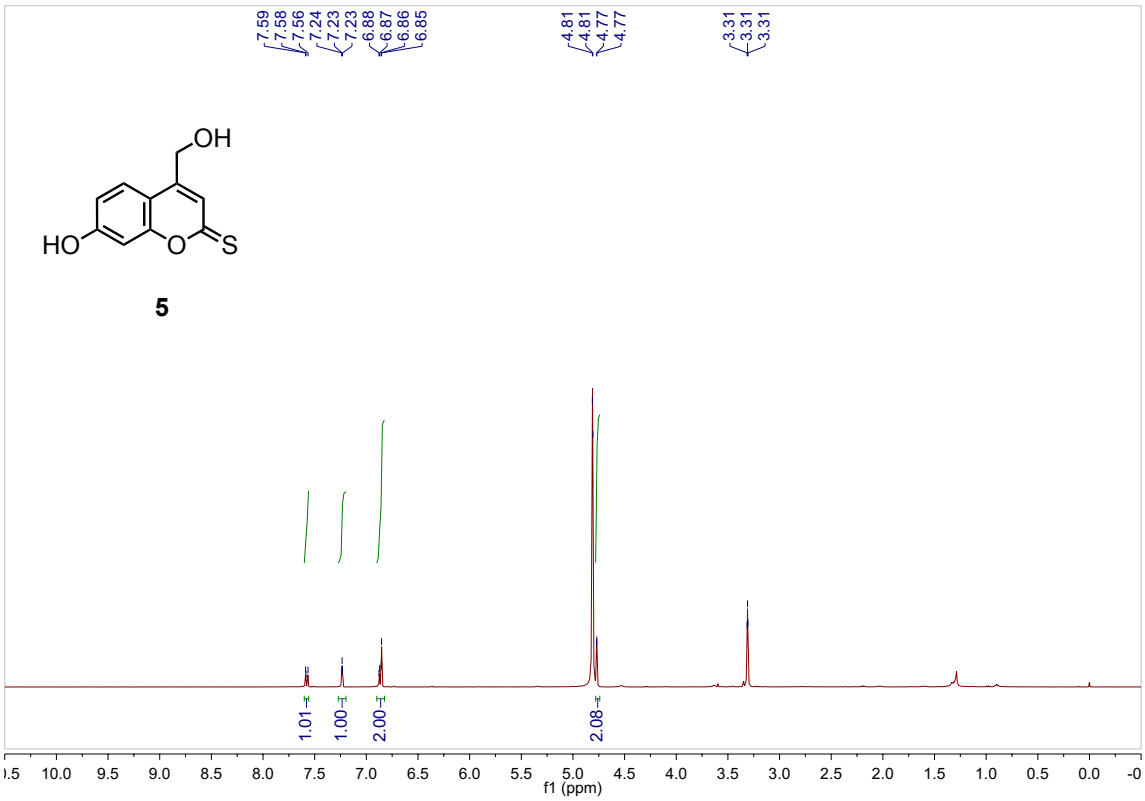
AcO
O
S
4
f1 (ppm)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4.77
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3.31
OH
HO
O
S
5
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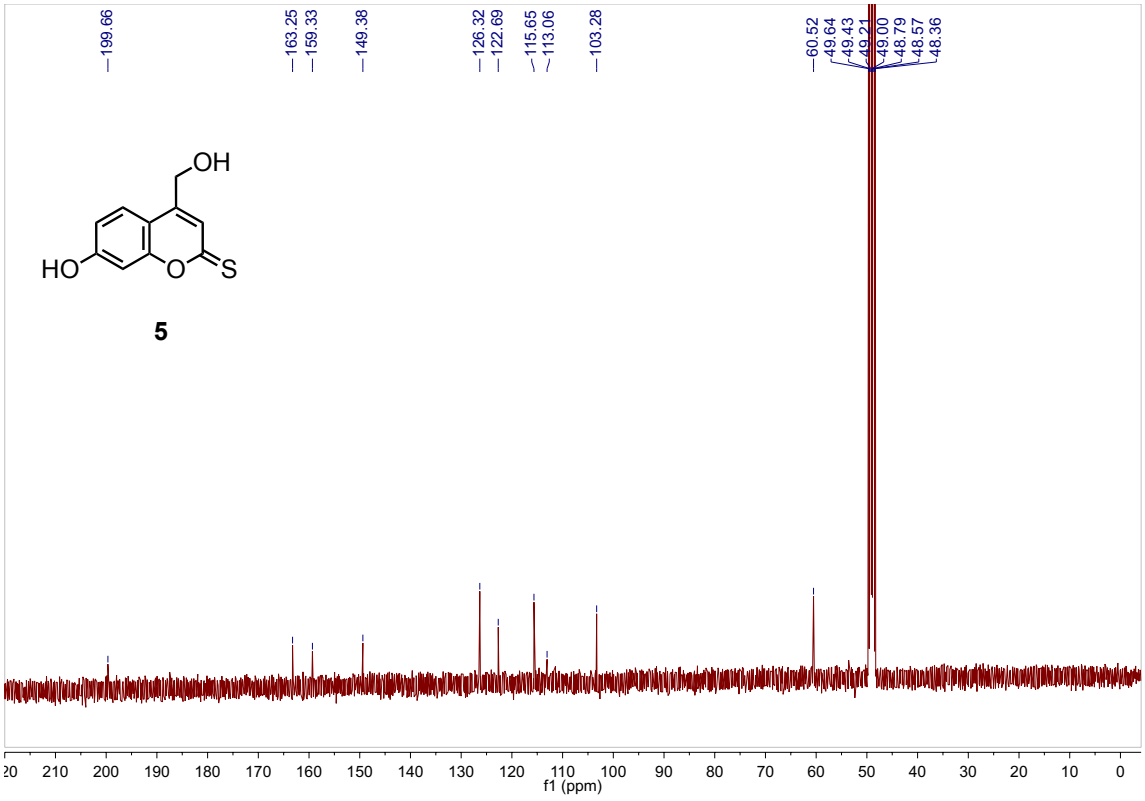
f1 (ppm)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S
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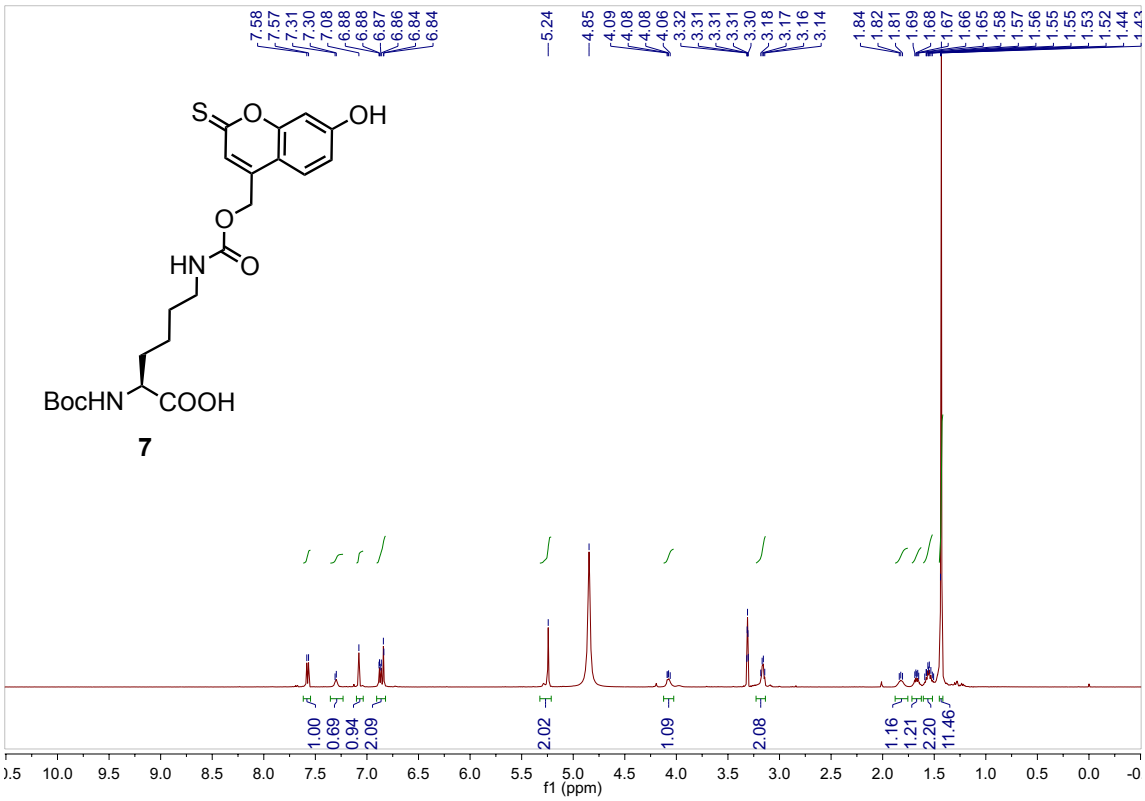
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4.09
4.08
4.08
4.06
3.32
3.31
3.31
3.31
3.30
3.18
3.17
3.16
3.14
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1.67
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1.57
1.56
1.55
1.55
1.53
1.52
1.44
S
O
OH
O
HN
O
BocHN
COOH
7
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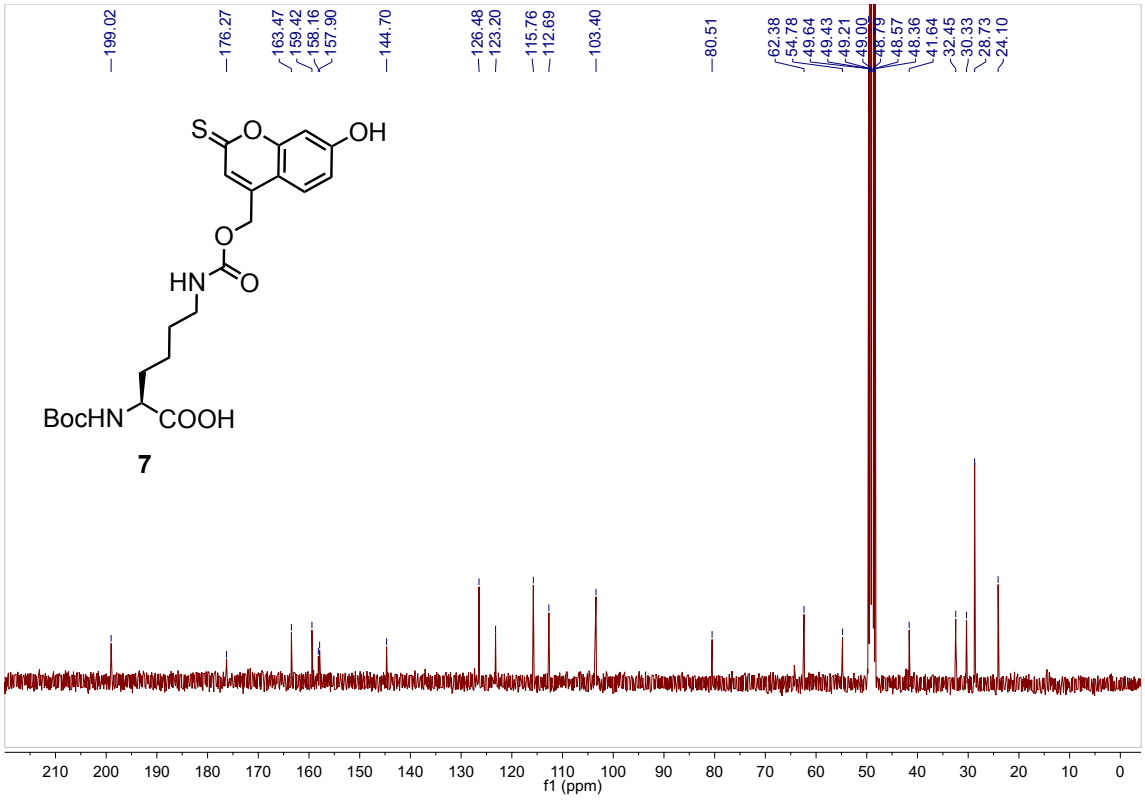
f1 (ppm)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49.21
49.00
48.79
48.57
48.36
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S
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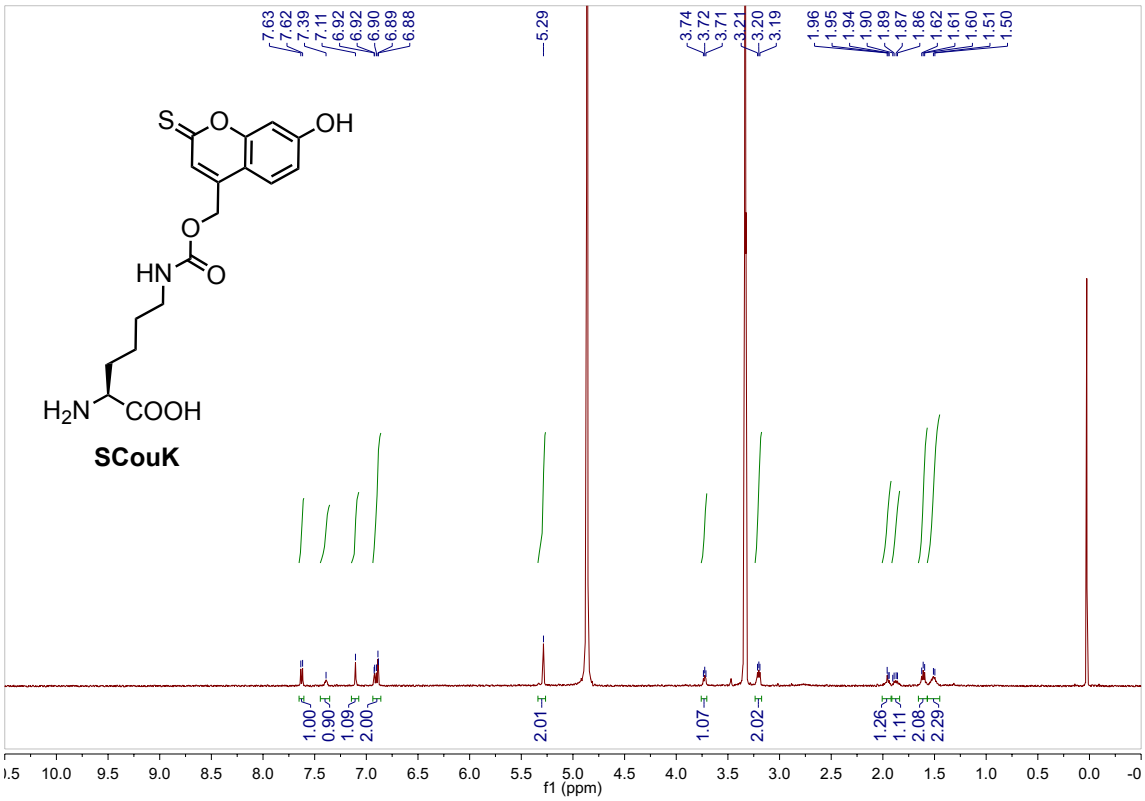
OH
O
HN
O
BocHN
COOH
7
f1 (ppm)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6.92
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3.19
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1.89
1.87
1.86
1.62
1.61
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1.50
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2.00
2.01
1.07
2.02
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S
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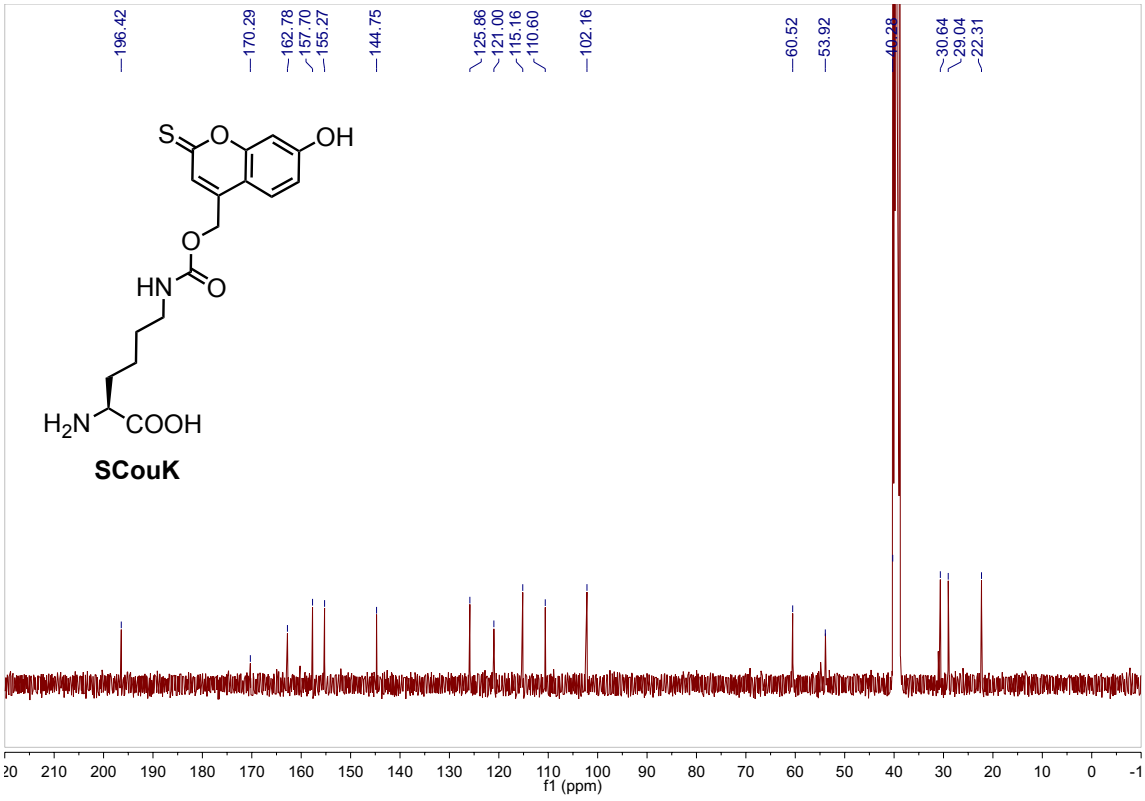
OH
O
HN
O
H2N
COOH
SCouK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O
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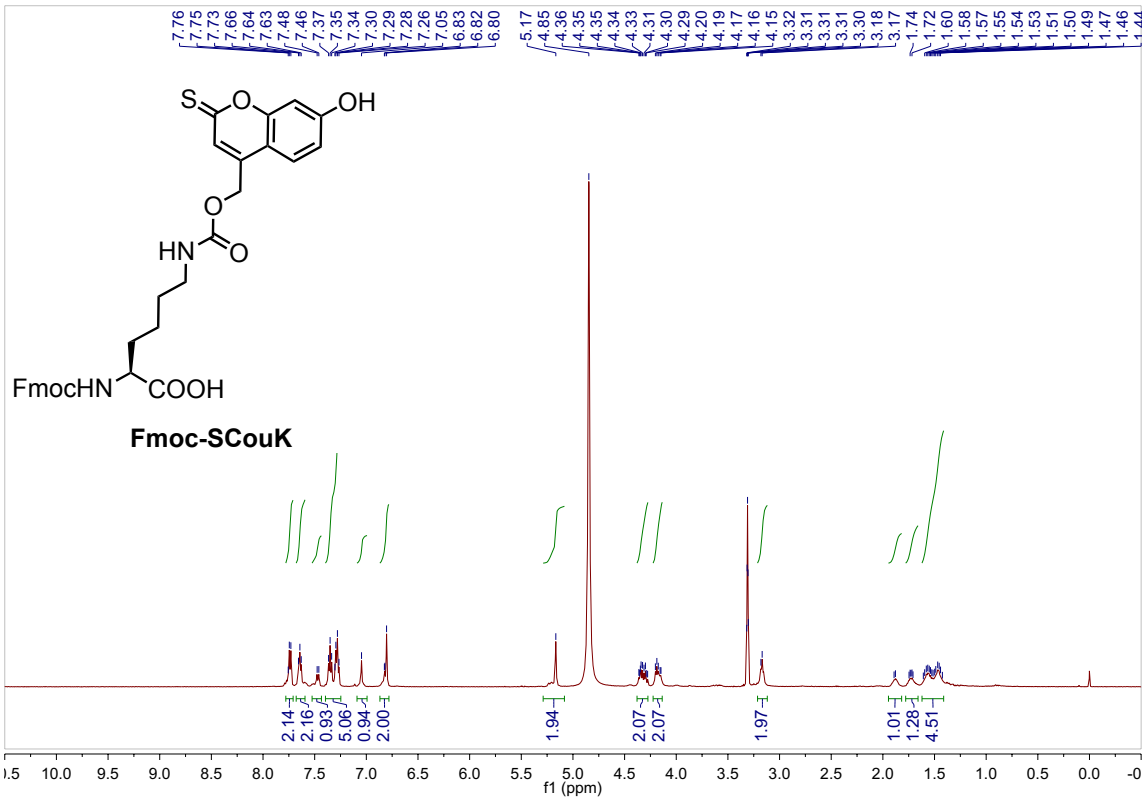
SCouK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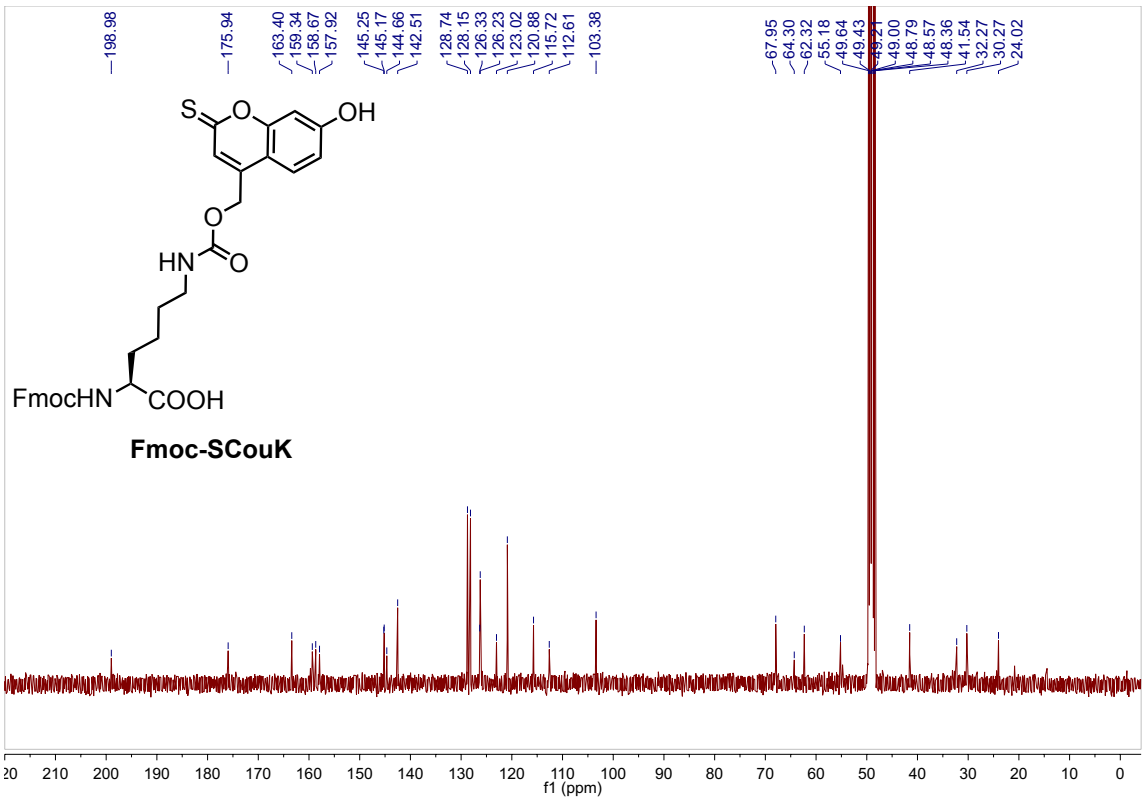
S
O
OH
O
HN
O
FmocHN
COOH
Fmoc-SCouK
2.14 2.16 0.93 5.06 0.94 2.00 1.94 2.07 2.07 1.97 1.01 1.28 4.51
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)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67.95 64.30 62.32 55.18 49.64 49.43 49.21 49.00 48.79 48.57 48.36 41.54 32.27 30.27 24.02
S
O
OH
O
HN
O
FmocHN
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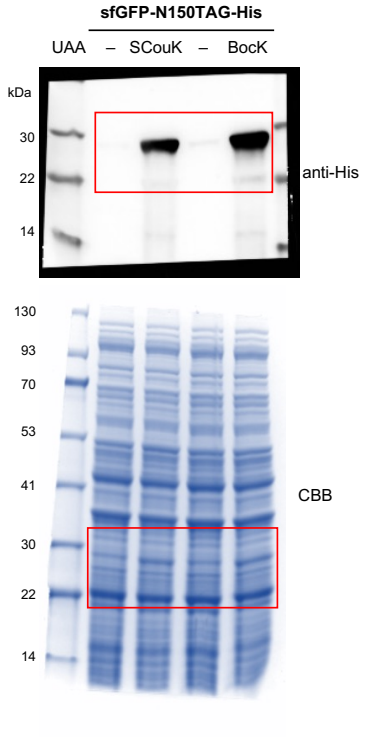
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

Un-edited originally captured gels and blots

Fig. 2C



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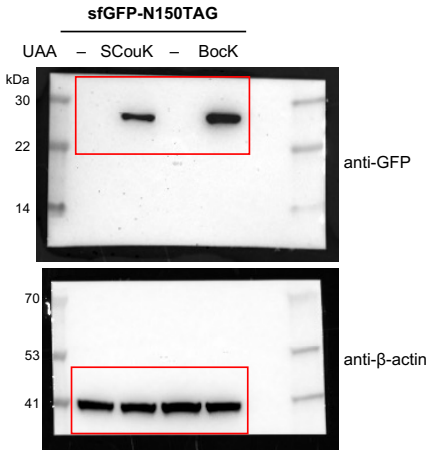

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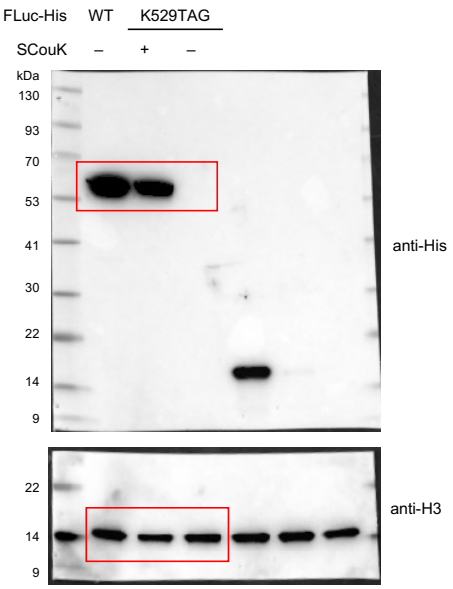

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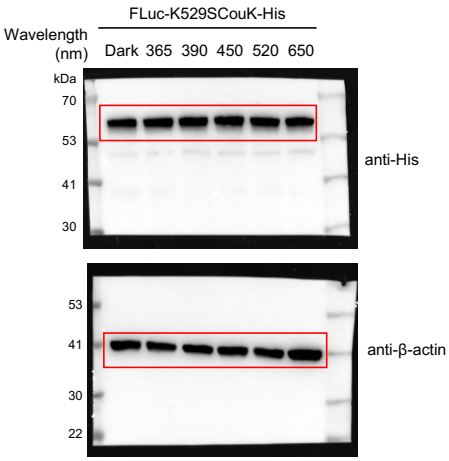

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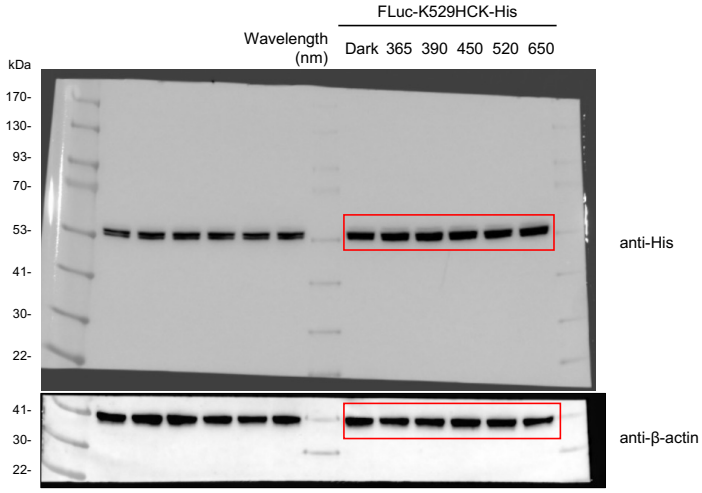

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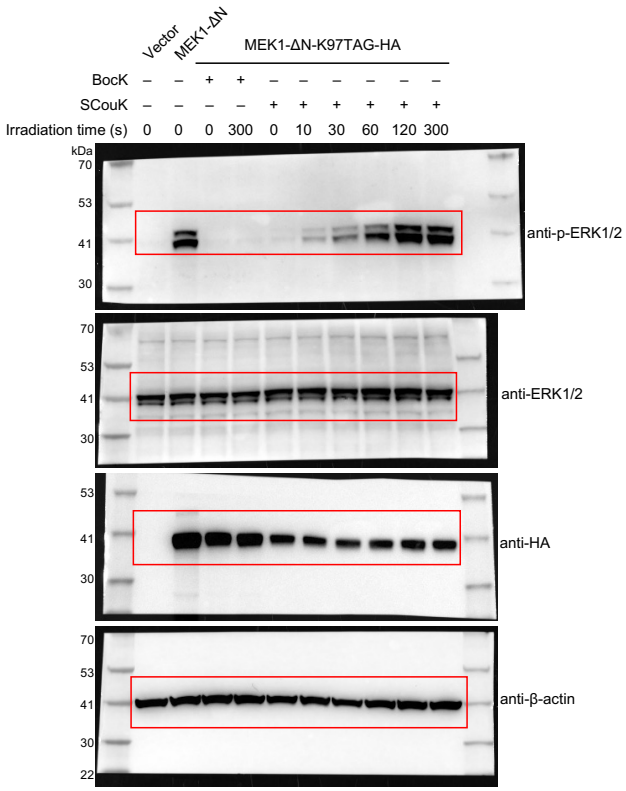
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MEK1-ΔN
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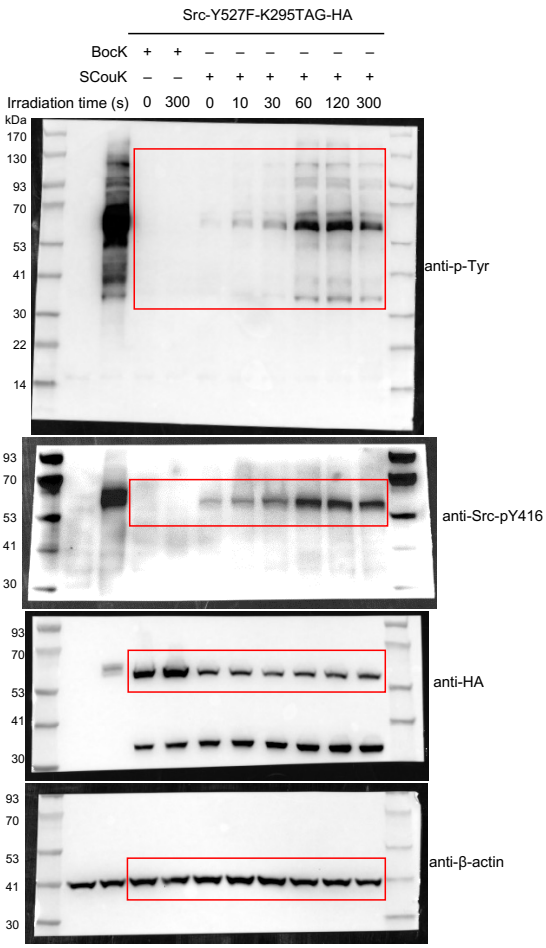
BocK – – + + – – – – – –
SCouK – – – – + + + + + +
Irradiation time (s) 0 0 0 300 0 10 30 60 120 300
kDa
70
53
41
30
anti-p-ERK1/2
70
53
41
30
anti-ERK1/2
53
41
30
anti-HA
70
53
41
30
22
anti-β-actin

Fig. 5D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BocK + + – – – – – –
SCouK – – + + + + + +
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kDa
170
130
93
70
53
41
30
22
14
anti-p-Tyr
93
70
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70
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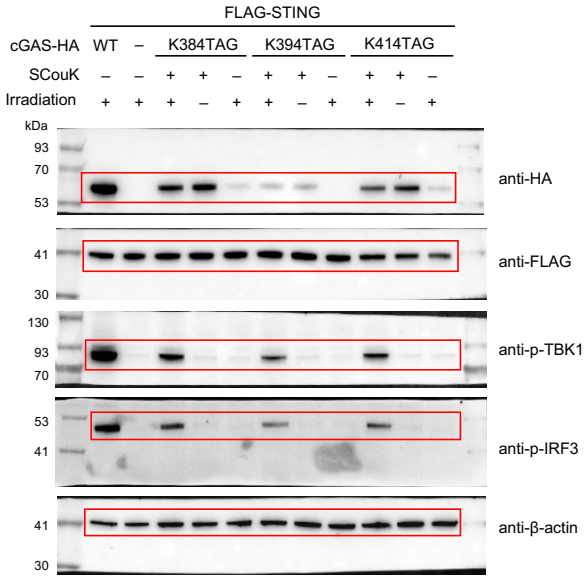
anti-β-actin

Fig. 6E



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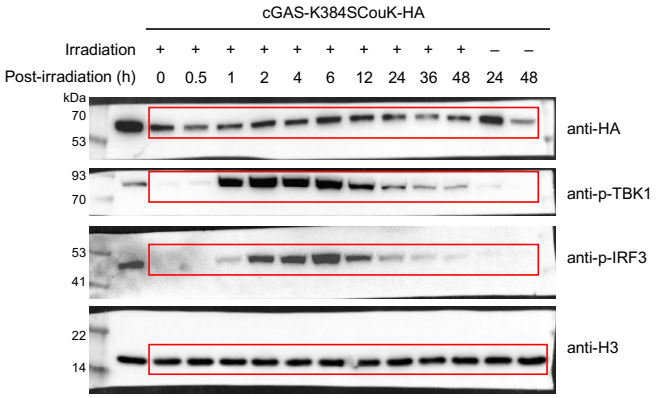

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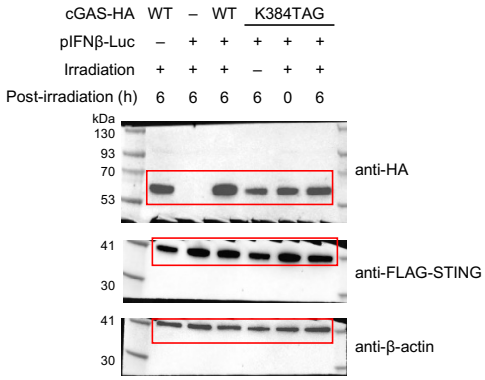

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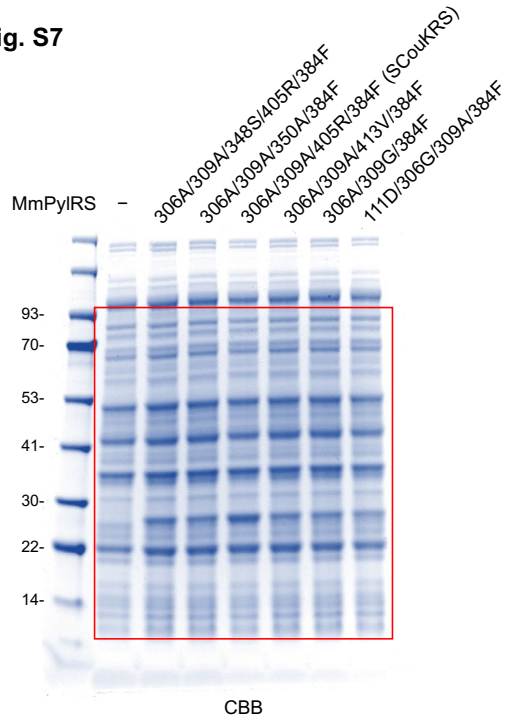
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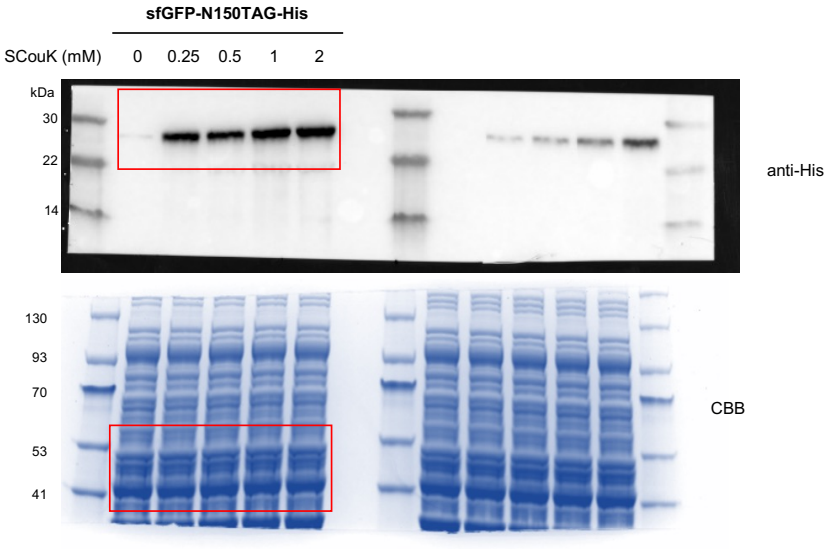
306A/309A/413V/384F
306A/309G/384F
111D/306G/309A/384F
93-
70-
53-
41-
30-
22-
14-
CBB

Fig. S8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1
2
kDa
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93
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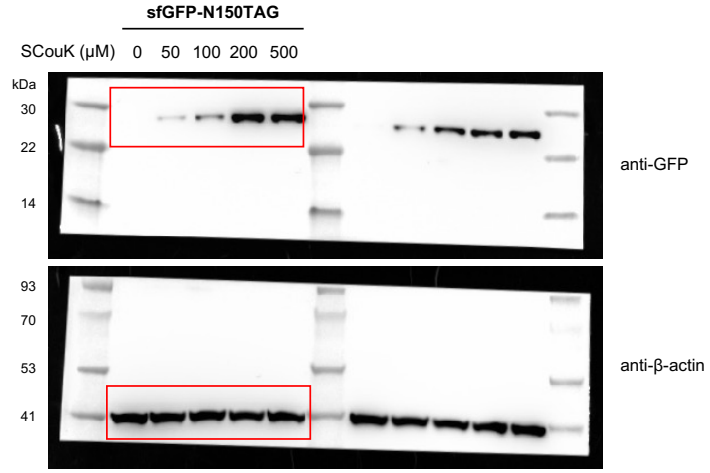
53
41
CBB

Fig. S11



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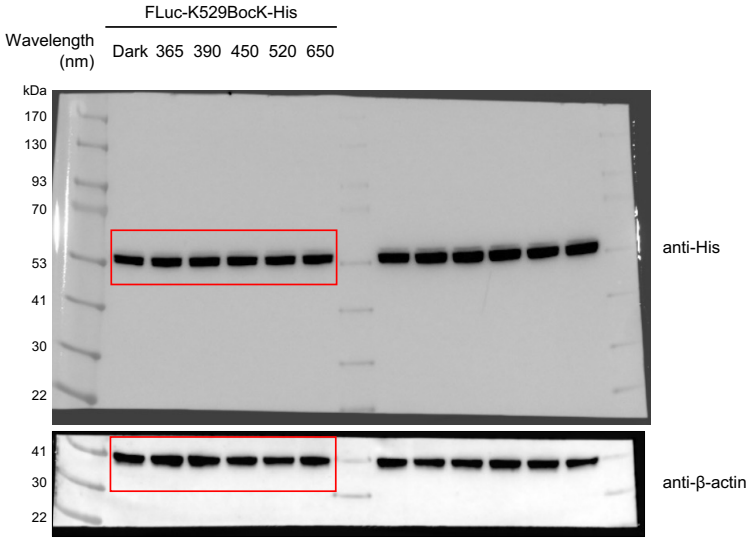

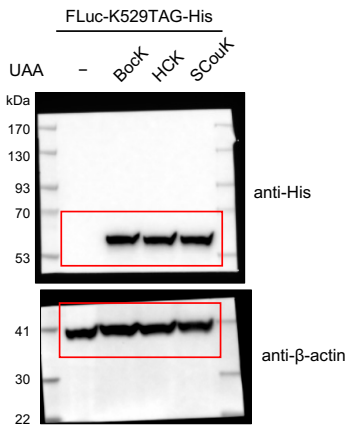

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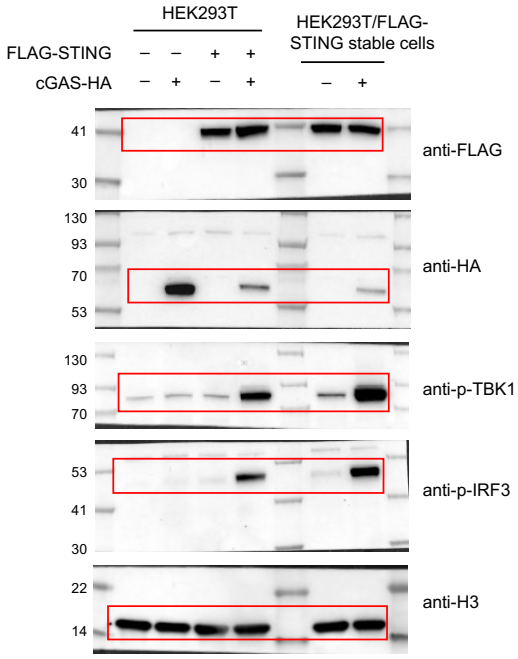

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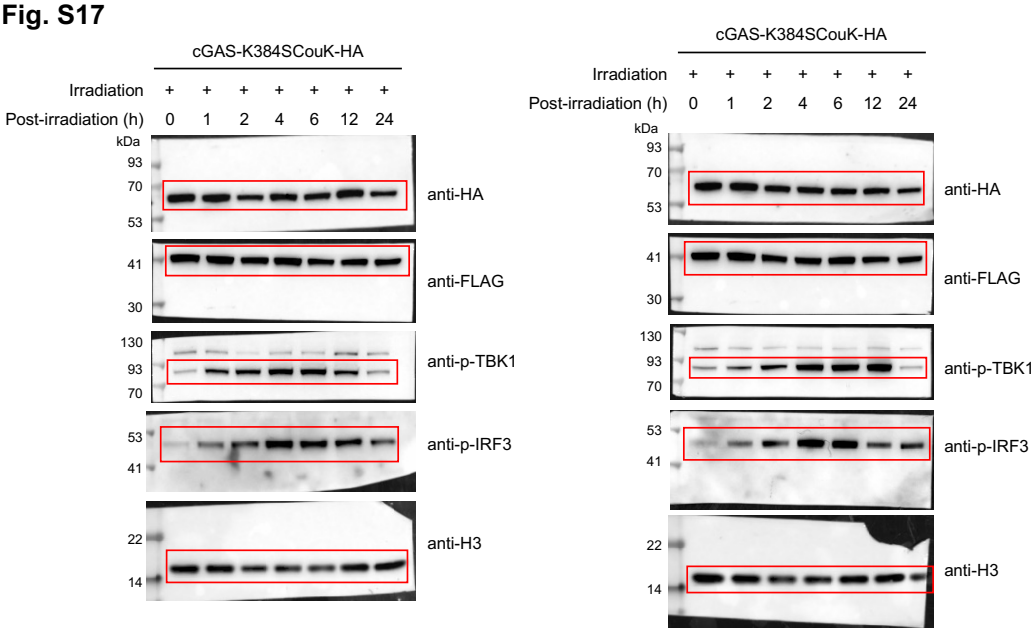

Fig. S18