Supporting Information

Genetic encoding of photocaged cysteine allows photoactivation of TEV protease in live mammalian cells

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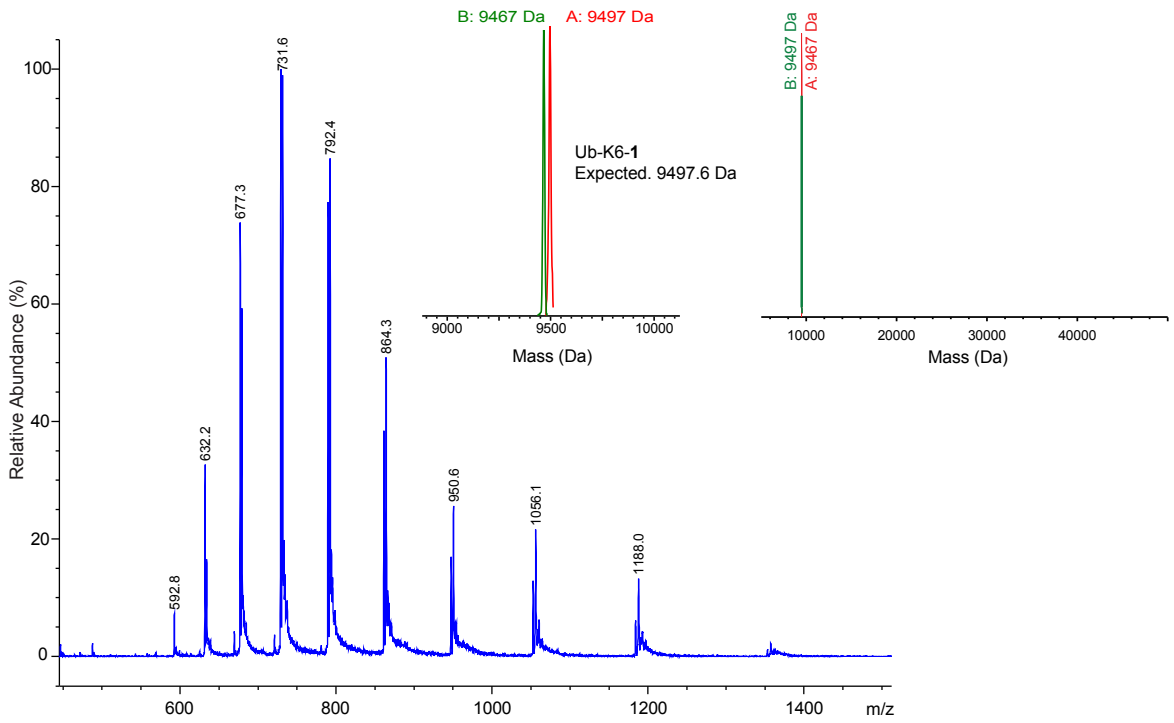
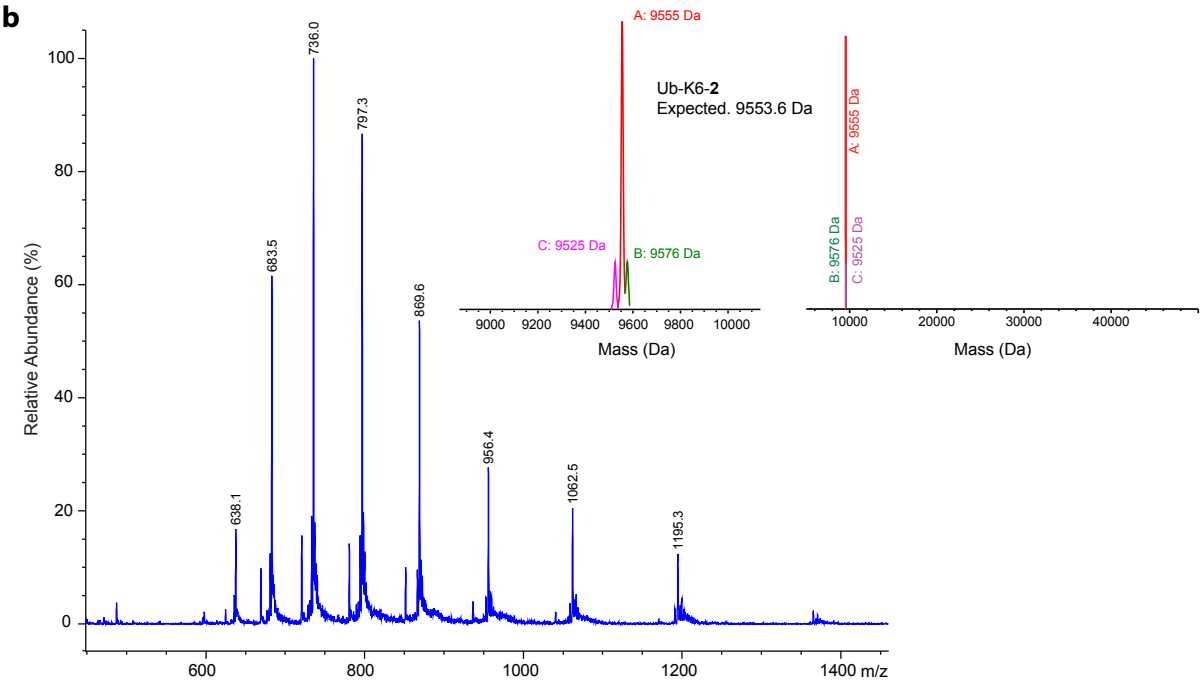
Table S1. Sequences of PylRS variants selected for the specific incorporation of the photocaged cysteine **1**. Clone 1 was used for further protein expression and purification.

Clones	Mutations						Frequency
	311	**313**	**366**	**382**	**386**	**Non-programmed**	
PylRS	N	C	V	W	G		
1	M	Q	G	N	G	R85H	68/80
2	Q	S	G	N	G	S131N	9/80
3	Q	A	G	N	G	E170G	2/80
4	Q	S	G	N	G		1/80

S1a


B: 9467 Da
A: 9497 Da
Ub-K6-1
Expected. 9497.6 Da
B: 9497 Da
A: 9467 Da
Mass (Da)
Mass (Da)
Relative Abundance (%)
m/z
731.6
792.4
677.3
864.3
632.2
950.6
1056.1
1188.0
592.8

S1b


A: 9555 Da
Ub-K6-2
Expected. 9553.6 Da
C: 9525 Da
B: 9576 Da
A: 9555 Da
B: 9576 Da
C: 9525 Da
Mass (Da)
Mass (Da)
Relative Abundance (%)
m/z
736.0
797.3
683.5
869.6
956.4
1062.5
1195.3
638.1

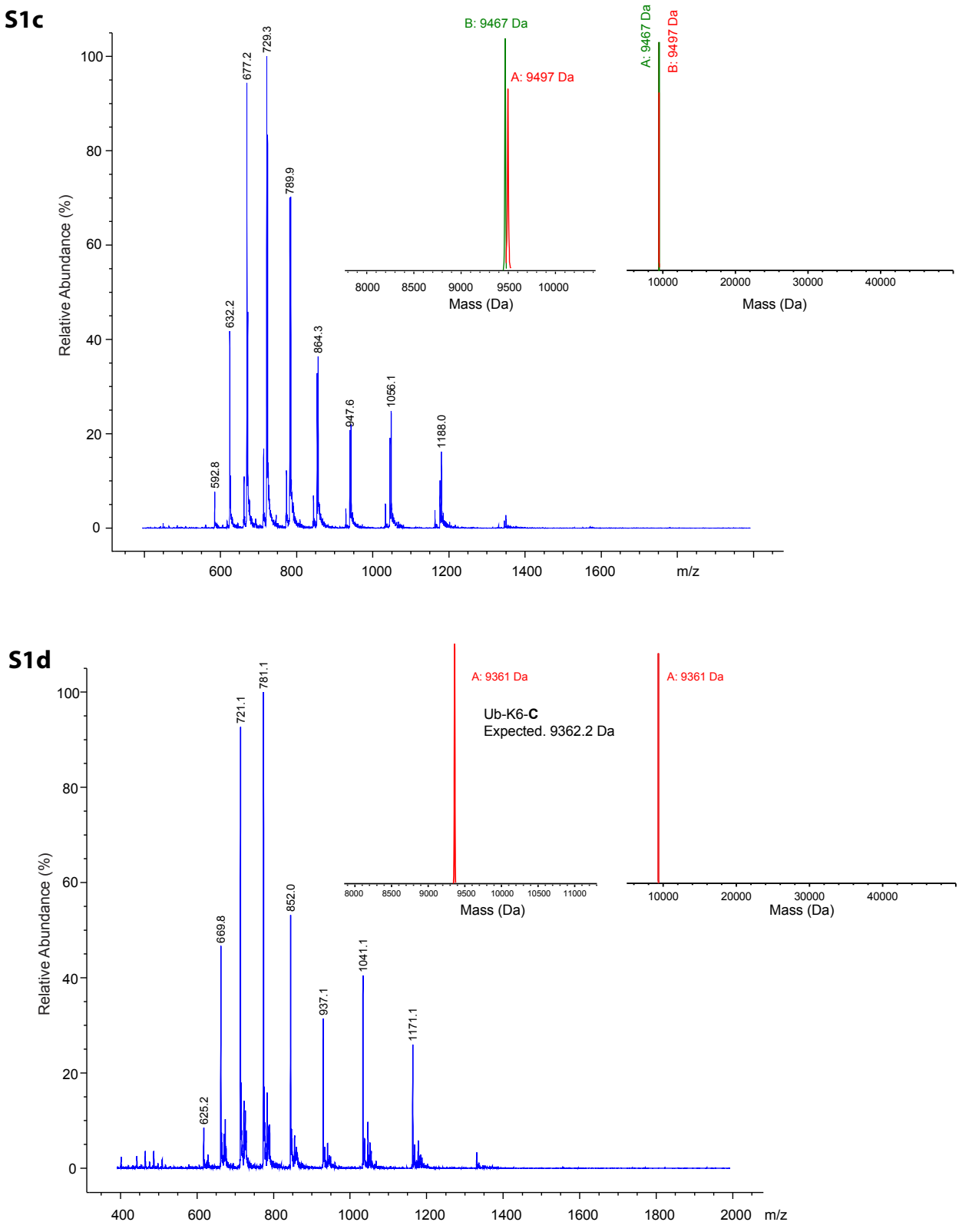
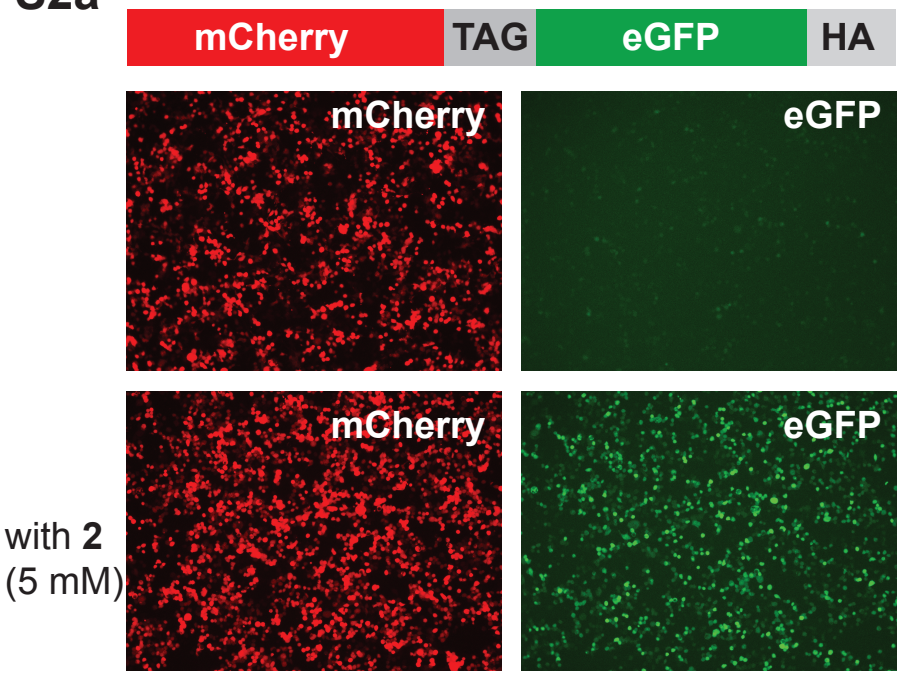


Figure S1. (a). ESI-MS analysis of C-terminally hexahistidine-tagged ubiquitin (Ub-His6) produced by PCC1RS/tRNA$_{CUA}$ pair revealed a mass of 9497 Da (peak A; expected mass 9497.6 Da).

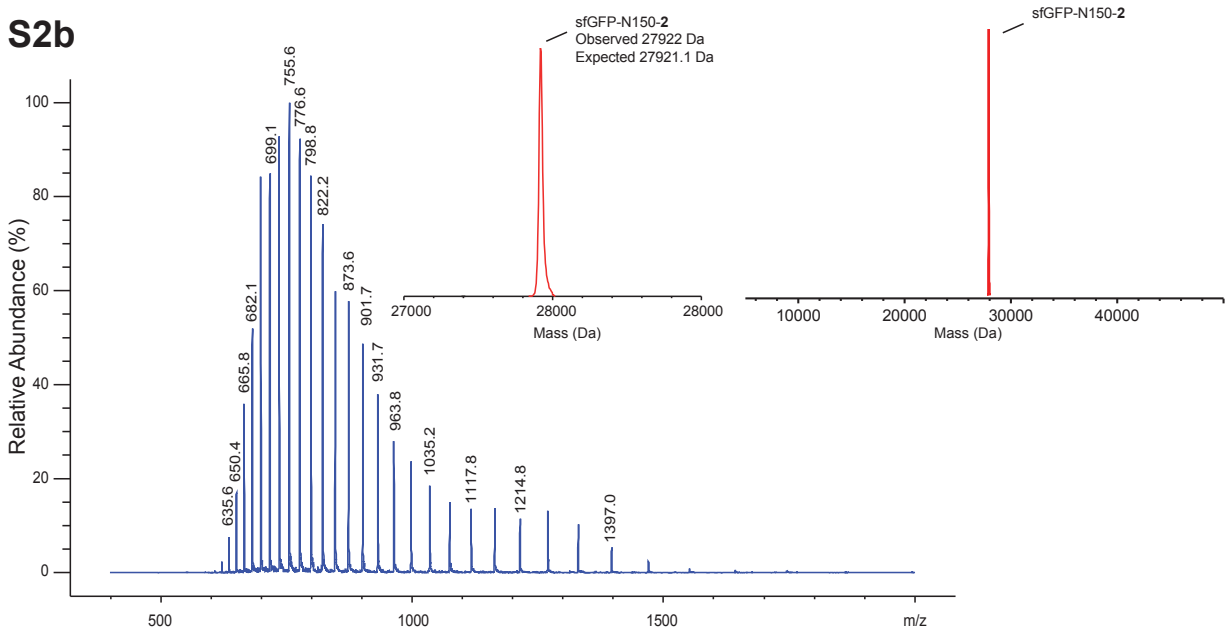
The second peak corresponds to Ub-His6 containing the reduced nitro group (peak B; expected mass 9467.6 Da). (b). ESI-MS analysis of Ub-His6 produced by PCC2RS/$tRNA_{CUA}$ pair revealed a mass of 9555 Da (peak A; expected mass 9553.6 Da). Two minor peaks B (9576 Da) and C (9525 Da) correspond to species containing sodium adduct and reduced nitro group respectively. (c). ESI-MS analysis of Ub-His6 incorporating **1** after photolysis for 10 min with 365 nm showed no change in the masses of the protein. The absence of a peak corresponding to the uncaged protein indicates the inefficient deprotection of the cage group at the noncytotoxic wavelength, 365 nm. (d). ESI-MS analysis of Ub-His6 incorporating **2** after photolysis for 1 min with 365 nm revealed a single peak of 9361 Da corresponding to the uncaged protein (expected mass, 9362.2 Da).

S2a



with **2**
(5 mM)

S2b



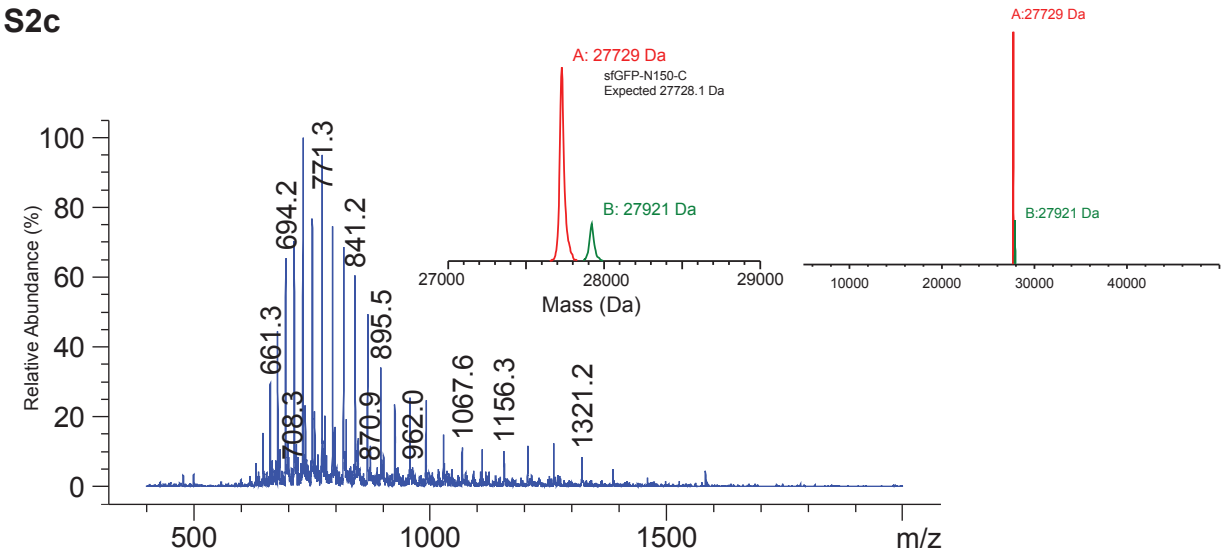


Figure S2. Genetically directed incorporation and photo-decaging of 2 in mammalian cells. (a) Fluorescence micrographs of HEK293T cells expressing *mCherry-TAG-egfp-ha* and PCC2RS/tRNA$_{CUA}$ pair with or without **2** (5 mM). (b) ESI-MS analysis of sfGFP-N150-**2** purified from anti-GFP immunoprecipitation confirmed the genetically directed incorporation of **2** in sfGFP. Expected mass: 27921.1 Da; Observed mass: 27922 Da. (c) ESI-MS analysis of sfGFP-**2** after photolysis in living cells. HEK 293T cells transfected with the PCC**2**RS/tRNA$_{CUA}$ pair in the presence of **2** (5 mM) were illuminated at 365 nm for 1 min. Expected mass of sfGFP150Cys: 27728.1 Da; Observed mass: 27729 Da (peak A). Peak B corresponds to sfGFP-N150-**2**.

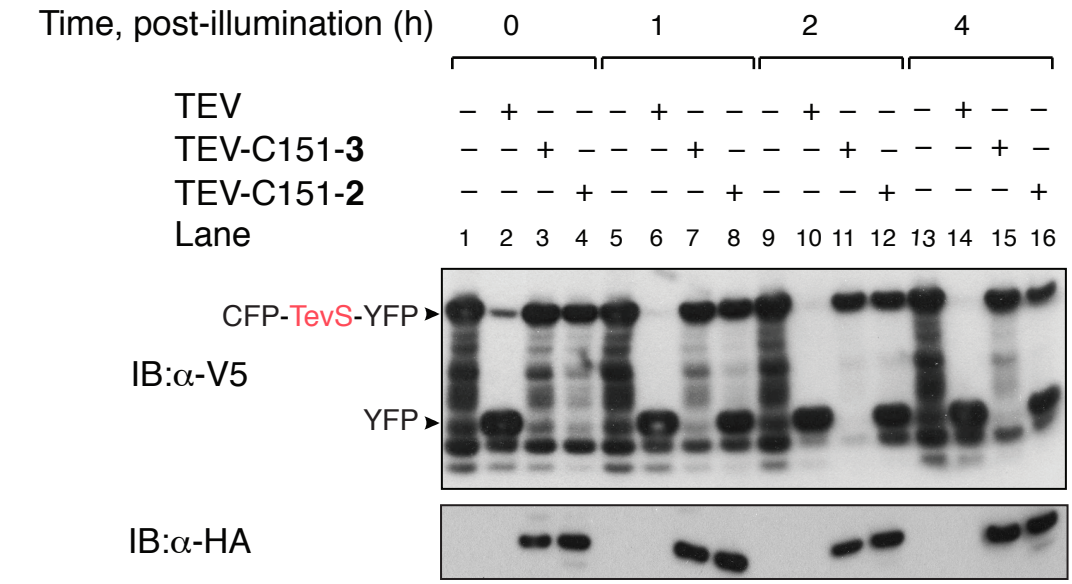
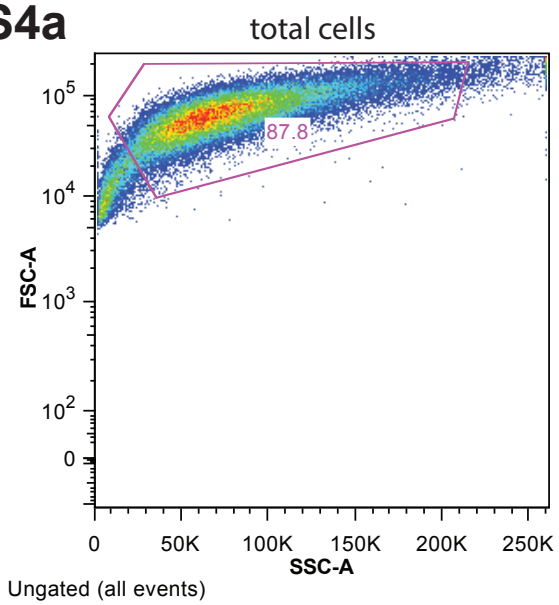
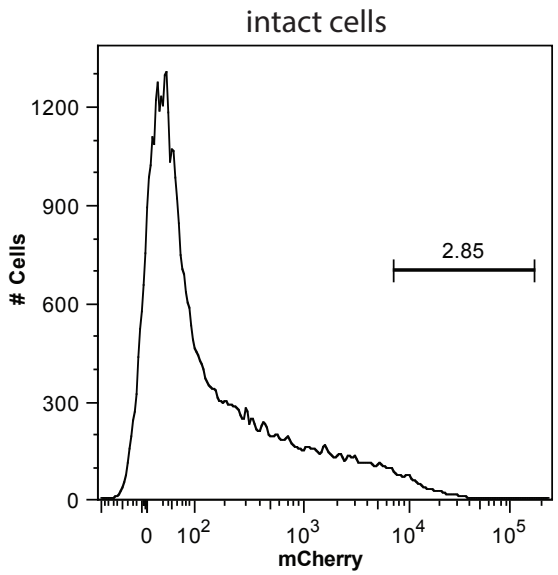


Figure S3. Photocontrol of TEV activity. TEV protease can be efficiently activated with light in HEK 293T cells. Controls: cells expressing only FRET sensor (lanes 1, 5, 9 and 13); cells coexpressing FRET sensor and wild-type TEV protease (lanes 2, 6, 10 and 14); cells coexpressing FRET sensor and TEV-C151-**3** (lanes 3, 7, 11 and 15). Cells expressing FRET sensor and TEV-C151-**2** (lane 4, 8, 12, and 16) were irradiated for 1 min at 365 nm and further incubated for 1 h, 2 h or 4 h. Immunoblotting of cells expressing only the FRET sensor, TEV, TEV-C151-**2** or TEV-C151-**3** was performed with anti-V5 antibody to probe for full length CFP-TevS-YFP or truncated YFP and anti-HA for expressed synthetases. The sensor appears to be slightly less well expressed upon co-expression of TEV and TEV mutants. This may be because the sensor, TEV constructs and tRNA constructs are all CMV enhancer or promoter driven and components required for CMV directed transcription may become limiting in these experiments.

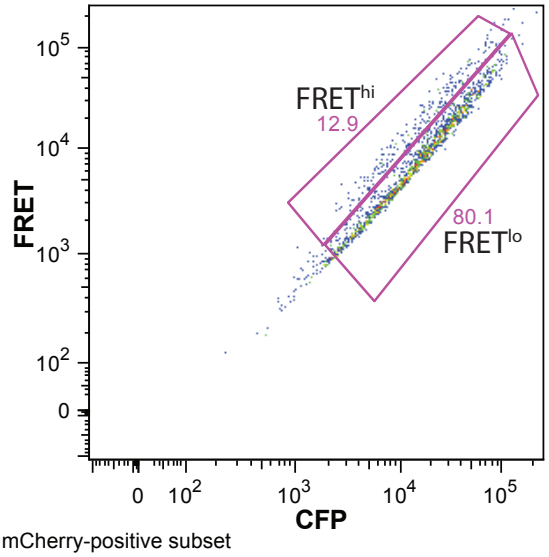
S4a



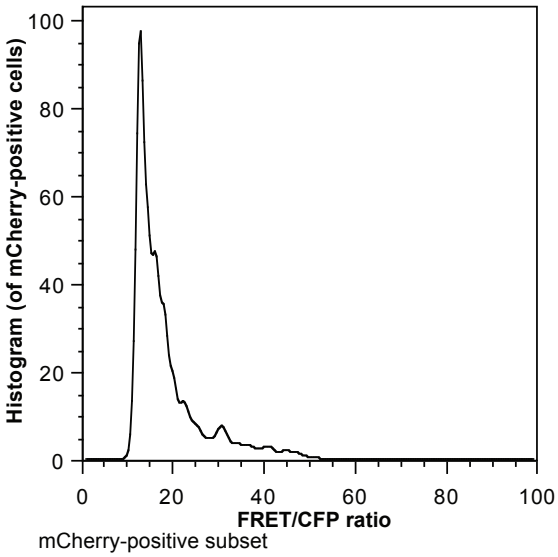
Ungated (all events)
TEV-C151-**2** 4h
Event Count: 62716



Intact cells subset
TEV-C151-**2** 4h
Event Count: 55075

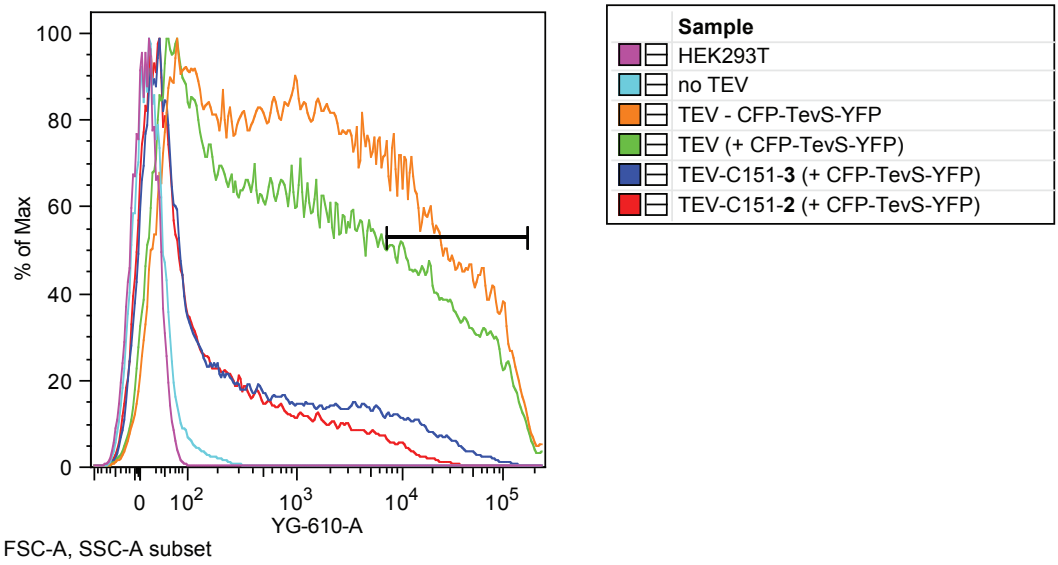


mCherry-positive subset
TEV-C151-**2** 4h
Event Count: 1575

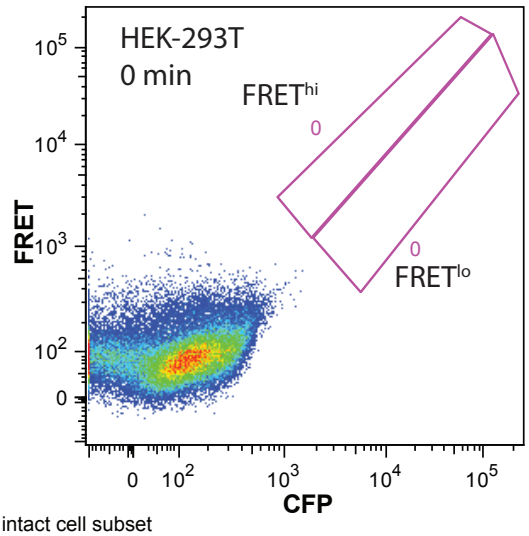


mCherry-positive subset
TEV-C151-**2** 4h
Event Count: 1575

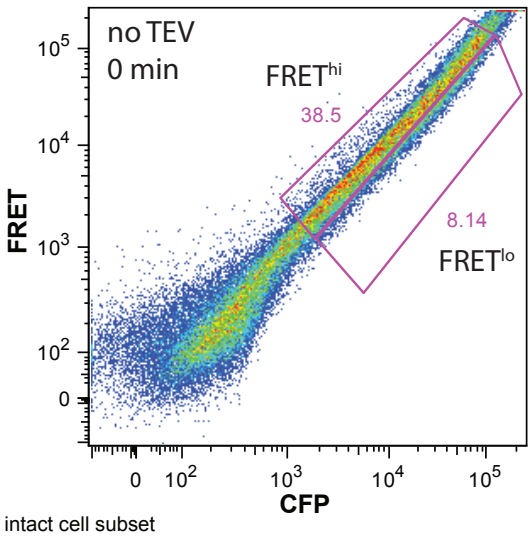
S4b


100
80
60
40
20
0
% of Max
0
10^2
10^3
10^4
10^5
YG-610-A
FSC-A, SSC-A subset
Sample
HEK293T
no TEV
TEV - CFP-TevS-YFP
TEV (+ CFP-TevS-YFP)
TEV-C151-**3** (+ CFP-TevS-YFP)
TEV-C151-**2** (+ CFP-TevS-YFP)

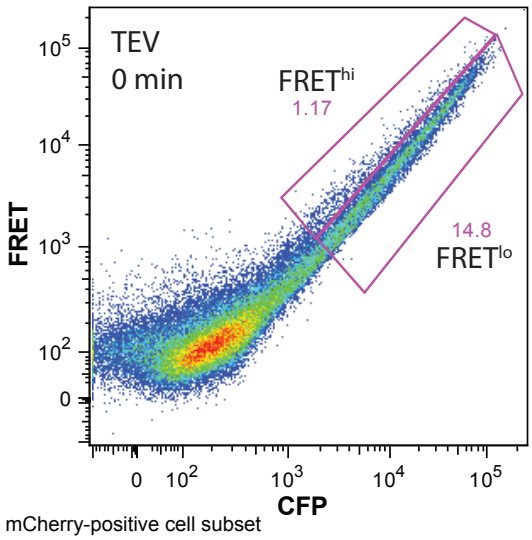
S4c



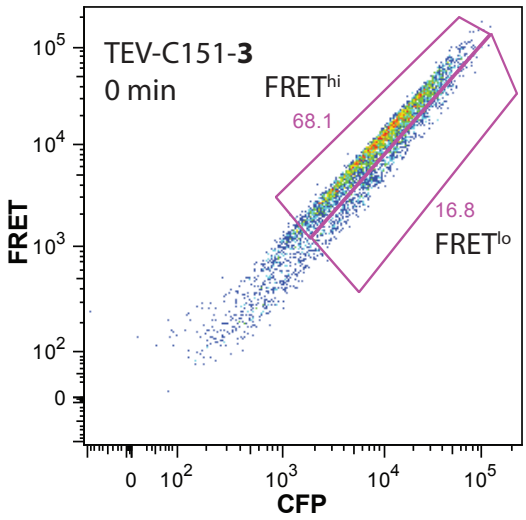
intact cell subset
HEK-293T
Event Count: 52801



intact cell subset
CFP-TevS-YFP
Event Count: 53313

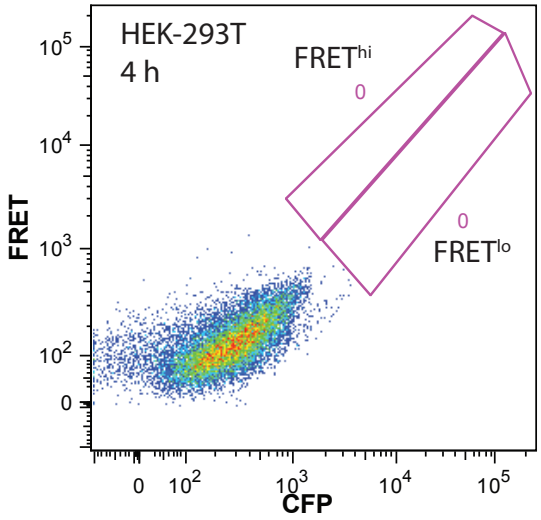


mCherry-positive cell subset
TEV
Event Count: 53490

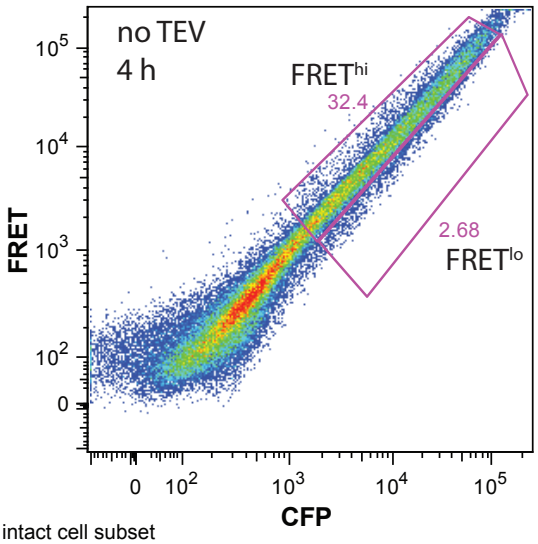


mCherry-positive cell subset
TEV-C151-**3**
Event Count: 5052

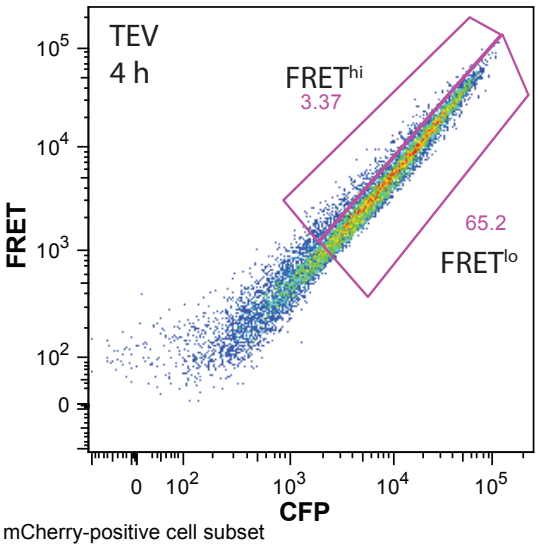
S4d



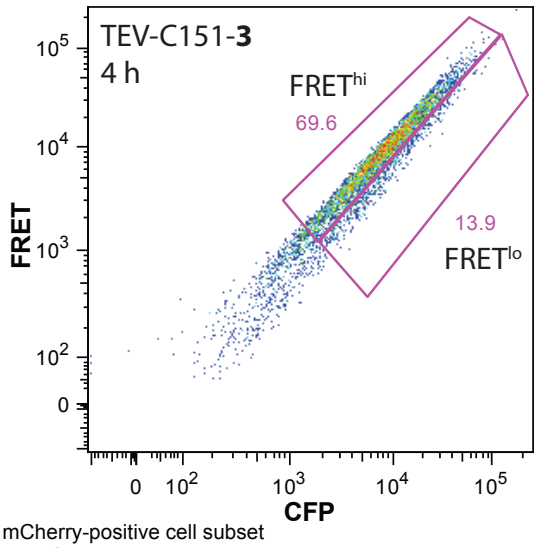
intact cell subset
HEK293T
Event Count: 13938



intact cell subset
CFP-TevS-YFP
Event Count: 56767

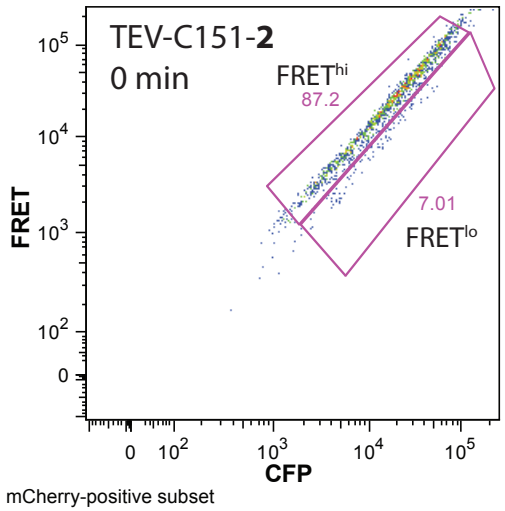


mCherry-positive cell subset
TEV
Event Count: 12267

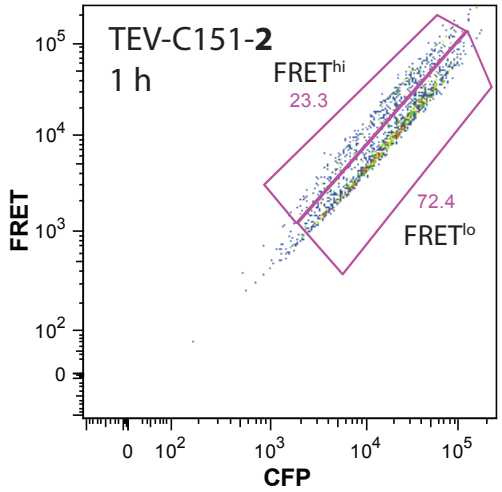


mCherry-positive cell subset
TEV-C151-**3**
Event Count: 5046

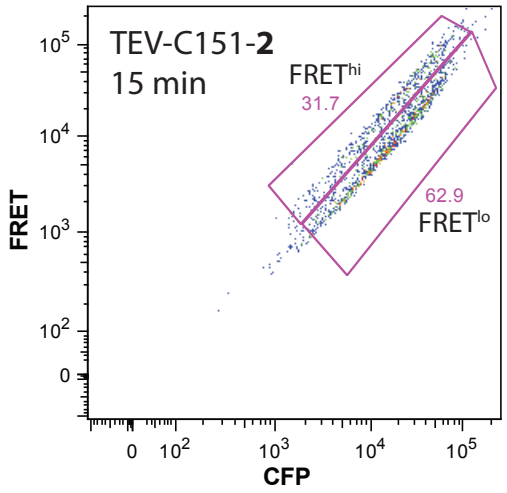
S4e



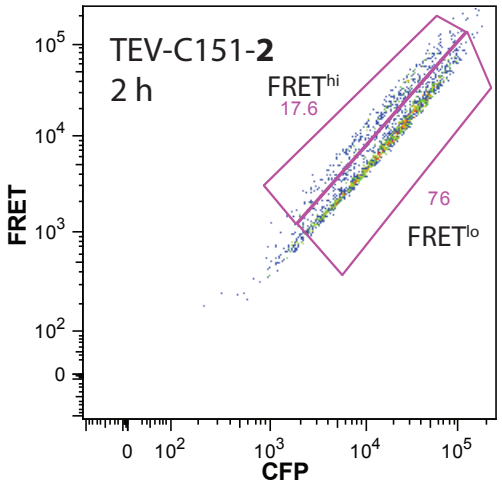
mCherry-positive subset
Event Count: 1498



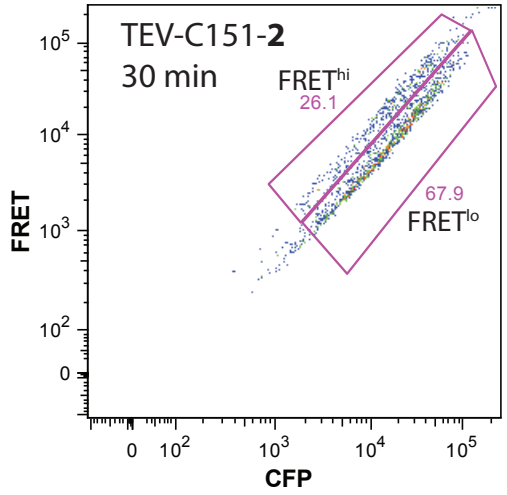
mCherry-positive subset
Event Count: 1724



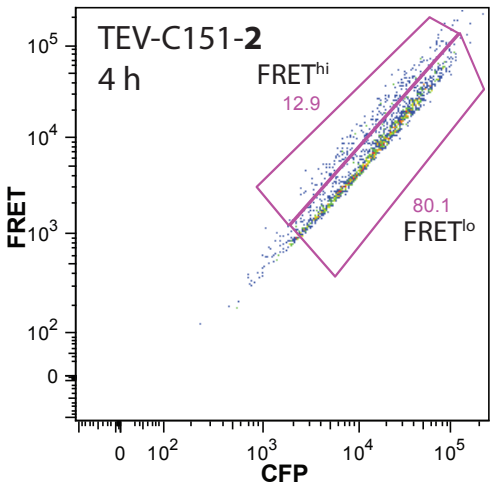
mCherry-positive subset
Event Count: 1519



mCherry-positive subset
Event Count: 1931



mCherry-positive subset
Event Count: 1487



mCherry-positive subset
Event Count: 1575

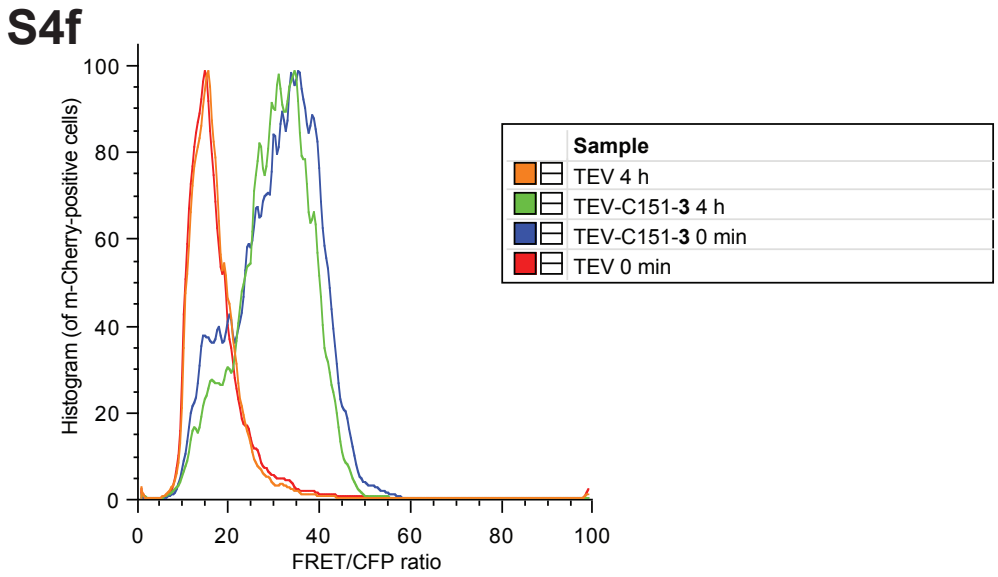
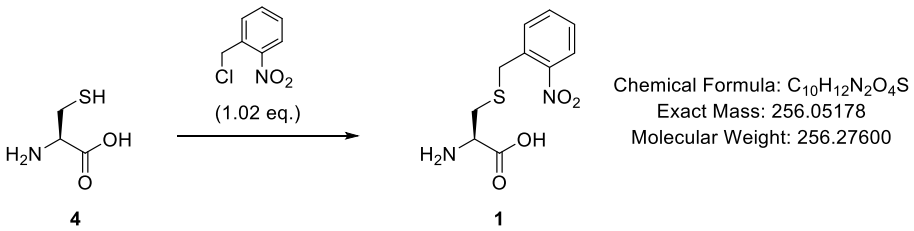


Figure S4. FRET-FACS analysis. (**A**) FRET FACS analysis on cells expressing TEV-**2** at 4 h after illumination. Top left: all events; intact cells were gated by FSC/SSC (forward/side scattering). Top right: intact cells were further gated based on red fluorescence to include only mCherry-positive cells for analysis. Bottom left: a plot of FRET (V-540) over CFP (V-450) revealed two populations, low FRET ($FRET^{lo}$) and high FRET ($FRET^{hi}$) in the absence and presence of active TEV respectively. Bottom right: a plot of FRET/CFP ratio over a histogram of mCherry-positive cells showed a bimodal distribution of high-FRET/low-FRET. FACS analysis for mCherry-positive cells indicated that 2.85% of transfected cells contain all genes for expressing TEV-C151-**2** and protease sensor. (**B**) Gating of cells to select mCherry-positive subpopulations. Controls: TEV incorporating **3** in place of Cys151 (TEV-C151-**3**) (with TevS-FRET reporter); wildtype TEV with TevS-FRET reporter (TEV); wildtype TEV without TevS-FRET reporter (TEV – CFP-TevS-YFP); only TevS-FRET reporter (no TEV); non-transfected cells (HEK-293T). (**C**) FRET/CFP plots of cells without illumination. (**D**) FRET/CFP plots of cells at 4 h after illumination for 1 min. (**E**) FRET/CFP plots of cells expressing TEV-C151-**2** at various time points after illumination. (**F**) Plots of FRET/CFP ratio over a histogram of mCherry-positive cells in samples expressing wildtype TEV or TEV-C151-**3** before and after illumination revealed distinct FRET populations in controls. The observed timescale of TEV cleavage may be limited by TEV levels in our experiment.

Materials and methods

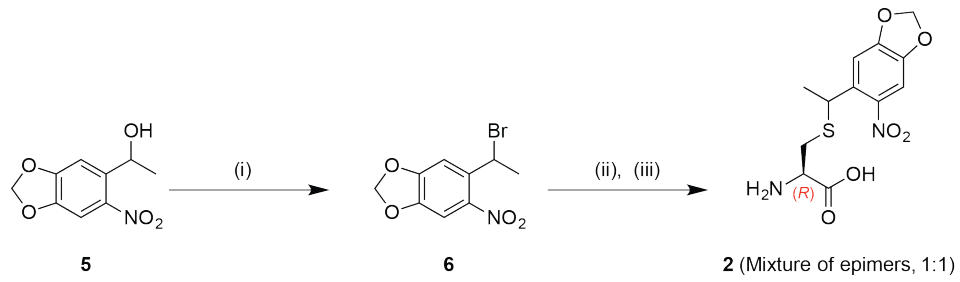
Synthesis of Photocaged Cysteines

Scheme S1[a]. Synthesis of *S*-(2-nitrobenzyl)-L-cysteine, 1



[a] *Reagents and conditions:* (i) L-Cysteine **4** (0.1 mol), aq. NaOH (1.0 eq., 0.5 M), r.t., 0.5 h, argon atmosphere, then 2-nitrobenzyl chloride (1.02 eq.) in degassed 1,4-dioxane (1 M, dropwise 1.5 h addition), then additional 1.5 h, r.t., argon atmosphere, 76% (**1** as HCl salt); (ii) Desalted on Amberlite® XAD-4.

SCHEME S2[a]. Preparation of *S*-[(*R*,*S*)-1-{4',5'-(methylenedioxy)-2'-nitrophenyl}ethyl]-L-cysteine, **2**



[a] *Reagents and conditions:* (i) **5** (75 mmol), PBr_3 (0.4 eq., dropwise addition), dry CH_2Cl_2, 0 °C, then dry pyridine (cat.), 0 °C, 15 min, then r.t., 1.5 h, 89% (**6**); (ii) L-Cysteine **4** (50 mmol), aq. NaOH (1.0 eq., 0.5 M), r.t., 0.5 h, argon atmosphere, then **6** (1.1 eq.) in degassed 1,4-dioxane (1 M, dropwise 1.5 h addition), then additional 3 h, r.t., argon atmosphere, 76% (**2** as HBr salt); (iii) Desalted on Amberlite® XAD-4.

***S*-(2-nitrobenzyl)-L-cysteine (1).** 2-Nitrobenzyl chloride (17.5 g, 0.102 mol, 1.02 eq.) was dissolved in degassed 1,4-dioxane (100 mL) in a 500 mL single-neck round-bottomed flask and the solution was purged with a stream of argon gas for a period of 30 min and kept aside. L-cysteine **4**

(12.1 g, 0.1 mol, 1.0 eq.) was dissolved in freshly prepared aq. NaOH solution (200 mL, 0.1 mol, 1.0 eq., 0.5 M in degassed and de-ionised H_2O) in a 1 L single-neck round-bottomed flask fitted with a magnetic stirring bar. The clear cysteine salt solution was purged with a stream of argon gas and stirred at r.t. After 0.5 h, the solution of 2-nitrobenzyl chloride in 1,4-dioxane was slowly added to the cysteine solution. A pale-yellow precipitate appeared during the course of the addition and the contents were stirred vigorously for a further period of 1.5 h at r.t. under an argon atmosphere and the progress of the reaction was monitored by LC-MS analysis. Upon completion of the reaction, the mixture was neutralized by addition of HCl (1 M) and the pH of the reaction mixture was adjusted to 6 – 6.5. The heavy yellow precipitate was then filtered through Whatman® filter paper and the off-white cake was washed sequentially and rapidly with de-ionised H_2O (2 × 200 mL), 2-propanol (10 mL), Et_2O (3 × 200 mL) and finally with *n*-hexane (300 mL). The product was dried under vacuum overnight to obtain *S*-(2-nitrobenzyl)-L-cysteine hydrochloride **1** as a fine off-white powder (22.1 g, 76%). m.p. 183.9-185.0 ^{o}C. ^{1}H NMR (400.13 MHz, CF_3COOD) (referenced to residual protonated solvent peak = 11.50 (s) ppm) δ 3.19 (dd, J = 15.3, 8.5 Hz, 1H), 3.39 (dd, J = 15.3, 3.0 Hz, 1H), 4.22 (app. d, J = 13.3 Hz, 1H), 4.27 (app. d, J = 13.3 Hz, 1H), 4.59 (dd, J = 8.5, 3.0, 1H), 7.45-7.57 (m, 2H), 7.63-7.72 (m, 1H), 8.13 (app. d, J = 8.1, 1H); ^{13}C NMR (100.61 MHz, CF_3COOD) [referenced to solvent peak = 116.6 (q), 164.2 (q) ppm] δ 33.2 (CH_2), 35.8 (CH_2), 55.4 (CH), 128.2 (CH), 131.6 (CH), 134.38 (C), 134.43 (CH), 136.8 (CH), 149.8 (C), 173.6 (C); MS (ESI +) *m/z* (rel intensity) 257 [$(M+H)^+$, 55%], 240 (26), 196 (24), 136 (100), 130 (2); HRMS (ESI +) *m/z* calc'd for $C_{10}H_{12}N_2O_4S$ $[M+H]^+$: 257.0591, found 257.0585 (Δ = -2.12 ppm).

A sample of *S*-(2-nitrobenzyl)-L-cysteine hydrochloride **1** (1.85 g) was desalted on a column packed with Amberlite® XAD-4 resin (20-60 mesh) using de-ionized H_2O. This was first washed with deionized H_2O to remove salt residues and then eluted with EtOH/H_2O (1:1). Pure fractions containing the amino acid were concentrated to obtain neutral *S*-(2-nitrobenzyl)-L-cysteine as an off-white solid. This was purified by trituration by dissolving in a minimum volume of dry CH_3OH and addition of copious amount of dry Et_2O, the off-white precipitate was filtered through a sintered funnel, dried *in vacuo* to obtain neutral *S*-(2-nitrobenzyl)-L-cysteine **1** as a fine off-white powder (1.51 g).

(*R*,*S*)-1-Bromo-1-[4',5'-(methylenedioxy)-2'-nitrophenyl]ethane (6). (*R*,*S*)-1-[4',5'-(methylenedioxy)-2'-nitrophenyl]ethanol **5** was prepared as previously reported.[1] A dry sample of

5 (15.8 g, 75 mmol, 1.0 eq.) was loaded onto an oven-dried 1 litre, 3-necked round-bottomed flask. The flask was dried *in vacuo* for 15 min and purged with dry argon gas and the procedure was repeated 3 times. The compound was dissolved in dry CH_2Cl_2 (375 mL, sonication required for complete solubility), cooled to 0 ^oC under an argon atmosphere. After 20 min, PBr_3 (2.82 mL, 30 mmol, 0.4 eq.) was added dropwise for a period of 10 min using a syringe pump followed by addition of dry pyridine (0.5 mL) at 0 ^oC. The yellow reaction mixture was stirred at 0 ^oC for 15 min, then brought to r.t. and stirred at r.t. for a further 1.5 h (total time = 15 + 90 = 105 min) under an argon atmosphere. After this time, the reaction was judged to be complete by TLC analysis (SiO_2, TLC eluent: 100% CH_2Cl_2), the reaction mixture was re-cooled to 0 ^oC, quenched by addition of dry CH_3OH (15 mL), warmed to r.t. and stirred for 30 min under an argon atmosphere. After the quenching was complete, the reaction mixture was evaporated to dryness under reduced pressure using rotary evaporator and the resulting yellow gum was re-dissolved in CH_2Cl_2 (300 mL) and saturated aq. $NaHCO_3$ solution (300 mL). The organic phase was washed sequentially with further saturated aq. $NaHCO_3$ solution (1 × 300 mL) and saturated aq. NaCl solution (3 × 300 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to obtain a yellow solid. The crude product was purified by flash chromatography on SiO_2 [eluent: CH_2Cl_2/*n*-hexane (1:1), then 100%CH_2Cl_2] to obtain a pure sample of (*R*,*S*)-1-bromo-1-[4',5'-(methylenedioxy)-2'-nitrophenyl]ethane **6** (18.330 g, 89%) as yellow crystals. The compound was stored in a freezer at -20 ^oC in a dark and dry atmosphere. R_f = 0.17 (CH_2Cl_2/*n*-hexane, 1:4); m.p. 76.1-77.8 ^oC; ^1H NMR (400.13 MHz, $CDCl_3$) δ 2.04 (d, J = 6.8 Hz, 3H), 5.89 (q, J = 6.8 Hz, 1H), 6.13 (s, 2H), 7.27 (s, 1H), 7.35 (s, 1H); ^{13}C NMR (100.61 MHz, $CDCl_3$) δ 27.6 (CH_3), 42.9 (CH), 103.3 (CH_2), 105.1 (CH), 108.8 (CH), 134.8 (C), 141.6 (C), 147.7 (C), 152.1 (C); IR (CH_2Cl_2) ν_{max} 2981, 2970, 2930, 1615, 1504, 1481, 1420, 1395, 1385, 1328, 1305, 1257, 1156, 1141, 1057, 1028, 1014, 957, 925, 872, 815, 752, 730, 719, 698; HRMS (ESI+) *m/z* calc'd for $C_9H_8{}^{79}BrNO_4$ $[M+Na]^+$: 295.9529, found 295.9519 (Δ = -3.45 ppm).

***S*-[(*R*,*S*)-1-{4',5'-(Methylenedioxy)-2'-nitrophenyl}ethyl]-L-cysteine (2).** A freshly prepared sample of (*R*,*S*)-1-bromo-1-[4',5'-(methylenedioxy)-2'-nitrophenyl]ethane **6** (15.074 g, 55 mmol, 1.1 eq.) was dissolved in degassed 1,4-dioxane (50 mL) in a 100 mL single-necked round-bottomed flask and a stream of argon gas was purged through the solution for a period of 30 min and the flask was kept aside. Simultaneously, L-cysteine **4** (6.058 g, 50 mmol, 1.0 eq.) was dissolved in freshly prepared aq. NaOH solution (100 mL, 50 mmol, 0.5 M in degassed and de-ionized H_2O) in a 500 mL single-neck round-bottomed flask fitted with a magnetic stirring bar. A

stream of argon gas was purged through the clear cysteine salt solution for a period of 30 min at r.t. After this period, the solution of **6** in 1,4-dioxane was slowly added to the flask containing cysteine salt solution, dropwise *via* a cannula, for a period of 1.5 h, whilst the reaction mixture was left stirring. A pale-yellow precipitate appeared during the course of the addition and the contents were vigorously stirred for a further period of 3 h at r.t. under an argon atmosphere. During this period, the progress of the reaction was periodically monitored by LC-MS analysis. After the reaction was judged to be complete (total time = 1.5 h addition + 3 h), the reaction mixture was neutralized by addition of minimum aq. HBr (1 M) and the pH of the reaction mixture was adjusted to 6-6.5. The heavy yellow precipitate was then filtered through Whatman™ filter paper using a Buchner funnel. The pale-yellow cake was washed rapidly and sequentially with de-ionised H_2O (3 × 200 mL), 2-propanol (5 mL), Et_2O (3 × 200 mL) and finally with *n*-hexane (2 × 150 mL). Finally, the product was dried in high vacuum overnight to obtain *S*-[(*R*,*S*)-1-{4',5'-(methylenedioxy)-2'-nitrophenyl}ethyl]-L-cysteine hydrobromide **2** as a pale yellow powder (15.0 g, 76%). The amino acid **7** was obtained as a mixture of 1:1 epimer as determined by ^{1}H NMR spectroscopic analysis.

A sample of *S*-[(*R*,*S*)-1-{4',5'-(methylenedioxy)-2'-nitrophenyl}ethyl]-L-cysteine hydrobromide **2** (15.0 g) was desalted on a column packed with Amberlite® XAD-4 resin (20-60 mesh) using de-ionized H_2O in a dark atmosphere. The salt impurities were removed by initially washing with deionized H_2O and then with EtOH/deionized H_2O (1:9) and the washings were discarded. The column was later eluted with EtOH/deionized H_2O (1:3 to 1:1). Pure fractions containing the amino acid **2** were concentrated to obtain a pale yellow solid. This was purified further by trituration by dissolving in minimum dry CH_3OH and addition of dry Et_2O, the pale yellow precipitate was filtered through a sintered funnel, dried *in vacuo* to obtain neutral *S*-[(*R*,*S*)-1-{4',5'-(methylenedioxy)-2'-nitrophenyl}ethyl]-L-cysteine **2** as a fine pale yellow powder (10.3 g). The amino acid was stored at 4 ^{o}C in a dark and dry atmosphere. m.p. 165.0-166.9 ^{o}C; ^{1}H NMR (400.13 MHz, DMSO-d_6) δ (Mixture of epimers) 1.50 (2 × t, J = 7.0 and 7.0 Hz, 3H), 2.47-2.52 and 2.53-2.59 (2 × m, 1H), 2.72 and 2.85 (2 × dd, J = 13.7, 3.8 and 13.5, 3.8 Hz, 1H), 3.11 and 3.16 (2 × dd, J = 8.6, 3.8 and 9.1, 3.8 Hz, 1H), 3.17-3.80 (broad s, 2H), 4.61 and 4.64 (2 × t, J = 7.0 and 7.0, 1H), 6.21 and 6.23 (2 × s, 2H), 7.34 and 7.35 (2 × s, 1H), 7.49 and 7.50 (2 × s, 1H); ^{13}C NMR (100.61 MHz, DMSO-d_6) δ (Mixture of epimers) 22.38 (CH_3), 22.44 (CH_3), 32.7 (CH_2), 33.1 (CH_2), 38.0 (CH), 38.9 (CH), 53.0 (CH), 53.6 (CH), 103.3 (2 × CH_2), 104.4 (CH), 104.5 (CH), 107.6 (2 × CH), 134.7 (C), 135.0 (C), 142.7 (C), 142.9 (C), 146.5 (C), 146.6 (C), 151.58

(C), 151.65 (C), 168.0 (C), 168.2 (C); MS (ESI+) *m/z* (rel intensity) 337 [$(M+Na)^+$, 11%], 315 [$(M+H)^+$, 100], 194 (12); HRMS (ESI+) *m/z* calc'd for $C_{12}H_{14}N_2O_6S$ $[M+H]^+$: 315.0645, found 315.0633 (Δ = -3.79 ppm).

Library construction and selection of aminoacyl-tRNA synthetases specific for unnatural amino acids 1 and 2

Using plasmid pBK-pylS as a template[2], the library was generated by two consecutive rounds of inverse PCR reactions: (round 1) P179 (5'- GCGCAGGAAAGGTCTCACGTGAATGGGGCATTGATAAACCGNNKATTGGCGCGNNKTTTGGCCTGGAACGTCTGCTGAAAGTGATGC-3') and P180 (5'- GCGCAGAGTAGGTCTCACACGATCCAGGCTAACCGGACCCACMNNCGCGCTGCTCAGTTCCAGATCGCCATGCATAATATCC-3'); (round 2) P181 (5'-GCGCAGGAAAGGTCTCAATGGTTNNKTTTNNKCAAATGGGCAGCGGCTGCACCCGTGAAAACC-3') and P182 (5'-GCGCAGAGTAGGTCTCACCATGGTGAATTCTTCCAGGTGTTCTTTGCCATCG-3'). These primers randomized the codons for positions N311, C313, V366, W382, and G386 of the PylS gene to the codons for all 20 natural amino acids. The resulting PCR products were digested with BsaI and DpnI, and circularised with T4 ligase. This DNA was amplified using ϕ29 polymerase (New England Biolabs) and N6 random hexamer primer at 30°C for 16 h, digested with NcoI and re-circularised with T4 ligase. Ethanol precipitated ligation product was transformed into electrocompetent Mega-X DH10B (Invitrogen) following the manufacturers instructions and inoculated into overnight culture with appropriate antibiotic to prepare plasmid DNA. Diversity was estimated by plating serial dilutions of the transformation rescue culture on agar plates with appropriate antibiotic. A library of 10^8 transformants was isolated that covered the theoretical diversity of the library with 97% confidence. Selection of synthetase mutants specific for **1** and **2** was carried out as previously reported.[2]

Protein expression and purification

Recombinant sfGFP and ubiquitin were expressed and purified using procedures similar to those previously reported.[3,4] For expressing sfGFP containing the genetically encoded unnatural amino acid **1** or **2**, *E. coli* DH10B cells were transformed with pGFP150TAG-pylT and pBK-PCC**1**RS variants or pBK-PCC**2**RS and grown overnight (16h, 37°C, 250 r.p.m.) in TB supplemented with 50 μg/mL kanamycin and 25 μg/mL tetracycline. Overnight culture (10 mL) was inoculated into 500 mL TB supplemented with 25 μg/mL kanamycin and 12 μg/mL tetracycline. At O.D. ~1.0, the cells were pellted and resuspended in 100 mL TB supplemented with 25 μg/mL

kanamycin, 12 μg/mL tetracycline, 0.2% L-arabinose and **1** (10 mM) or **2** (5 mM). 3 h after induction (37°C, 250 r.p.m.), the cells were harvested. A 1-mL aliquot of cells was also collected by centrifugation (5 min, 16000 g), resuspended in 200 μL of LDS Sample buffer (Life Technologies) supplemented with 5% β-mercaptoethanol. The cells were heated at 95°C for 10 min and centrifuged at 16000 g for 5 min. The whole cell lysate was analyzed by immunoblotting using anti-His_6 antibody (Cell Signaling, His-tag 27E8 mouse mAb #2366).

For purification of sfGFP, the bacterial pellets were resuspended in lysis buffer (20 mM Tris-HCl buffer pH 8.0, 200 mM NaCl, 20 mM imidazole, 1 mg/mL lysozyme, 100 μg/mL DNaseI, 1 mM PMSF, Roche protease inhibitor, 5 mM β-mercaptoethanol) and lysed by sonication at 4 ºC. The extract was clarified by centrifugation (30 min, 21000 g, 4 ºC). After Qiagen Ni-NTA beads (300 μL) were added to the extract, the mixture was incubated with agitation for 1 h at 4 ºC. Beads were collected by centrifugation (10 min, 1000 g). The beads were twice resuspended in 50 mL wash buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 40 mM imidazole, 5 mM β-mercaptoethanol) and pelleted at 1000 g. Subsequently, the beads were resuspended in 20 ml of wash buffer and transferred to a column. Protein was eluted in 1 ml of wash buffer supplemented with 300 mM imidazole and analysed by SDS-PAGE.

For the expression of ubiquitin-His_6 incorporating **1** and **2**, *E. coli.* BL21(DE3) were transformed with pCDF-Ub6TAG-pylT and pBK-PCC**1**RS or pBK-PCC**2**RS. The expression and purification were performed using the same protocol as described above.

Mammalian cell culture

Adherent HEK-293T cells were cultured at 37°C in DMEM-Glutamax medium (Gibco) supplemented with 10% FBS in a 5% CO_2 atmosphere before transfection. The cells were transiently transfected with Lipofectamine 2000 (Life Technologies) according to manufacturer's protocol. After transfection for 24 h, growth medium was replaced with fresh DMEM medium supplemented with 10% FBS and the photocaged cysteine **2** (5 mM), and cells were further grown for 48 h before analysis. The growth medium was then replaced with fresh DMEM medium with 10% FBS and further incubated for 1 h before photo-activation experiments. For western blot analysis, cells were washed with PBS and lysed with RIPA buffer at 4°C for 10 min. Immunoblotting was performed using antibodies against HA-tag (Roche 3F10 rat mAb), Flag-tag (Cell Signaling rabbit mAb #2368) and V5-tag (Life technologies mouse mAb R960).

Photolysis and mass spectrometry

For the analysis of the uncaging process *in vitro*, purified ubiquitin was photolysed at 365 nm with a high power LED source module at 365 nm (Black-led-365, Prizmatix, ~35 mW/cm^2).[5] For analyzing the uncaging in cultured cells, cells were seeded in a 10-cm plate, transfected with *sfGFP150TAG* and the PCC**2**RS/tRNA$_{CUA}$ pair for 24 h. The cells were illuminated at 365 nm for 1 min using UVP Benchtop 2UV Transilluminator (~4 mW/cm^2). Cells were then washed with cold PBS and lysed in 500 µL of RIPA buffer supplemented with Roche protease inhibitor for 10 min. The cell lysate was collected by centrifugation (10 min, 16000 g, 4 ºC) and incubated with 50 µL of GFP-Trap_M (Chromotek) at 4ºC for 4 h. The beads were magnetically separated, and washed three times with cold PBS and once with PBS supplemented with 0.5 M NaCl. The protein was eluted in 40 µL of 0.1% acetic acid for ESI-MS analysis.

Electrospray mass spectrometry was carried out using an Agilent 1200 LC-MS system equipped with a 6130 Quadrupole spectrometer. The solvent system consisted of 0.1 % formic acid in H2O as buffer A, and 0.1 % formic acid in acetonitrile (MeCN) as buffer B. Protein UV absorbance was monitored at 214 and 280 nm. Protein MS acquisition was carried out in positive ion mode and total protein masses were calculated by deconvolution within the MS Chemstation software (Agilent Technologies).

Microscopy

To image cells expressing *mcherry-tag-egfp-ha*, cells were seeded in 6-well plates, transfected with *mCherry-TAG-egfp-ha* and the PCC**2**RS/tRNA$_{CUA}$ pair for 24 h, and grown in the presence of **2** as described above. Fluorescence microscopy was performed using a Leica DM IL LED inverted microscope equipped with a HI PLAN 10x/0.22 objective and a GFP/RFP filter set.

For monitoring TEV activity in individual cells, cells were seeded in µ-Dish (Ibidi) and transfected with *cfp-tevs-yfp*,[6] *tevp151tag-t2a-mcherry* and the PCC**2**RS/tRNA$_{CUA}$ pair for 24 h. The cells were then grown in the presence of **2** as described above. Live cells were imaged at room temperature with a Zeiss LSM 780 inverted confocal microscope equipped with a Plan Apochromat 63x/1.4 oil immersion objective. Cells were illuminated for 5 s (power: ~570 mW/cm^2) with an EXFO X-Cite 120 XL System as previously described.[5] The cells containing the mCherry signal were imaged for 30 minutes at 30 s intervals using lambda mode with excitation wavelength 412 nm at room temperature. Microscope settings: scan resolution 512x512, averaging 16, scanning speed 10. Fluorescent images were background corrected, and the CFP and YFP fluores-

cent intensities were quantified using ImageJ at wavelength range 468-495 nm and 521-547 nm, respectively, to yield ratiometric CFP/YFP values.

Fluorescence Resonance Energy Transfer Analysis by FACS

For monitoring TEV activity in individual cells, cells expressing CFP were seeded and transfected in 6-well plates in a similar protocol as described above. The cells were illuminated at 365 nm for 1 min using UVP Benchtop 2UV Transilluminator and further incubated at 37^{o}C. At various time points, cells were trypsinized and fixed with 1% Formaldehyde in PBS, washed twice with PBS and analyzed on a BD LSR II Flow Cytometer equipped with 405 nm (V), Green 488 nm (G) and Yellow/Green 633 nm (Y/G) lasers. CFP fluorescence and YFP FRET was measured in the V-450 (450BP50), V-540 (550BP40) channels, respectively. YFP fluorescence was measured separately in the G-525 (525BP50) and mCherry in Y/G-610 (610/20). Single live cells were gated using SSC/FSC and included only transfected (mCherry positive) cells in the FRET analysis. Plotting V-540 (FRET) over V-450 (CFP) revealed two populations, FRET-high and FRET-low in the absence and presence of active TEV, respectively. The proportion of FRET-low cells was calculated as compared to total transfected cells as a measure of TEV activity. The V-540/V-450 ratio was further analyzed on a single-cell level in a histogram, showing a bimodal FRET-high/FRET-low distribution at all time points.

Plasmids

The plasmids pCDNA3.1-TEV-FRET and pHR-TEV-FLAG for expressing CFP-Tevs-YFP and TEV in mammalian cells were kind gifts from James A. Wells (UCSF). The genes *Mm*PCC2RS, codon-optimized for expression in mammalian cells, was purchased from GeneArt (Life Technologies). Plasmids pU6-pylT, pPB-CMV-sfGFP150TAG-4xU6-pylT-U25C, pPB-CMV-mCherry-TAG-eGFP-HA-4xU6-pylT-U25C, pPB-EF1a-MCS-4xU6-pylT-U25C and pCDH-T2A-mCherry were constructed by Simon J. Elsässer.[7]

1. Construction of pHA-MmPylRS-CMVE-U6-pylT-U25C, pFLAG-MmPylRS-CMVE-U6-pylT-U25C, pHA-MmPCC2RS-CMVE-U6-pylT-U25C and pFLAG-MmPCC2RS-CMVE-U6-pylT-U25C.

The genes HA-*Mm*PylRS and HA-*Mm*PCC**2**RS (from codon-optimized GeneArt synthetic gene) were amplified using primers DN379/DN380, and the genes FLAG-*Mm*PylRS and FLAG-*Mm*PCC**2**RS were amplified using primers DN393/DN380. The PCR fragments were digested and ligated into NotI and XhoI sites of pBUDCE4.1 (Life Technologies), giving pHA-*Mm*PylRS, pHA-*Mm*PCC**2**RS, pFLAG-*Mm*PylRS and pFLAG-*Mm*PCC**2**RS plasmids.

Quikchange mutagenesis was performed on the plasmid pU6-pylT using primers DN351/DN352 to introduce mutation U25C on the gene *pylT*,[8] producing pU6-pylT-U25C. The CMV enhancer sequence from the CMV promoter of pmCherry-C1 (Clontech) was amplified with primers DN349/DN350. The PCR product was digested with XbaI and SpeI, and ligated into pU6-pylT-U25C using the same sites, giving pCMVE-U6-pylT-U25C. The $tRNA_{CUA}$ operon was then amplified with primers DN387/DN388. The plasmids were obtained by ligating the PCR fragment CMVE-U6-pylT-U25C into the PvuI and BamHI of pHA-*Mm*PylRS, pHA-*Mm*PCC**2**RS, pFLAG-*Mm*PylRS and pFLAG-*Mm*PCC**2**RS.

2. Construction of pPB-EF1a-FLAG-MmPCC2RS-4xU6-pylT-U25C

The gene FLAG-MmPCCRS was amplified with primers DN583/DN584. The PCR fragment was inserted into the BamHI site of pPB-EF1a-MCS-4xU6-pylT-U25C using Infusion cloning kit (Clontech).

3. Construction of pTEV-T2A-mCherry and pTEV151TAG-T2A-mCherry

Two PCR fragments were generated by using primers DN377/DN368 and primers DN367/DN378 on pHR-TEV-FLAG as the template. The PCR fragment of TEV151TAG was obtained by using primers DN377/DN378 to assemble these two fragments by overlapping PCR. The PCR was digested with HindIII and SalI, and ligated into HindIII and XhoI of pCDNA4/TO (Life Technologies), producing the plasmid pCDNA4-TEV151TAG. The PCR products TEV-T2A-mCherry and TEV151TAG-T2A-mCherry were assembled by using primers DN605/DN606 from two fragments using overlapping PCR: a TEV fragment (generated with primers DN605/DN608 and pCDNA4-TEV151TAG or pHR-TEV-FLAG as template) and a T2A-mCherry fragment (generated with primers DN606/607 and pCDH-T2A-mCherry as template). The PCR products were digested and ligated into AflII and XbaI sites of pCDNA4/TO to yield the plasmids pTEV-T2A-mCherry and pTEV151TAG-T2A-mCherry.

Primer list

Name	Sequence
DN349	TATTCTCTAGAATGCATTAGTTATTAATAGTAATC
DN350	CAGTCACTAGTGTCCCGTTGATTTTGGTGCCAAAAC
DN351	GATCATGTAGATCGAACGGACTCTAAATCCGTTC
DN352	GAACGGATTTAGAGTCCGTTCGATCTACATGATC
DN367	CAAACCAAGGATGGGCAGTAGGGCAGTCCATTAGTATCAAC
DN368	GTTGATACTAATGGACTGCCCTACTGCCCATCCTTGGTTTG
DN377	CATGATAAGCTTGCCACCATGGGCGAGAGCCTTTTCAAGG
DN378	CAGATGTCGACTTATCACTTATCGTCGTCATC
DN379	ATTATGCGGCCGCCACCATGTACCCATACGATGTTCCAGATTACGCTGACAAGAAGCCCCTGAA CACCCTG
DN380	CACTACTCGAGTTATCACAGGTTGGTGCTGATGCCG
DN387	TATTCCGATCGATGCATTAGTTATTAATAGTAATC
DN388	CATGATGGATCCAAAAACCGCACTTGTCCGGAAACCC
DN393	ATTATGCGGCCGCCACCATGGACTACAAGGACGACG
DN583	AATTTAAATCGGATCCGCCACCATGGACTACAAGGACGACG
DN584	GCGCGGCCGCGGATCTTATCACAGGTTGGTGCTGATGCCG
DN605	CTAGCGTTTAAACTTAAGGCCACCATGGGCGAGAGCCTTTTCAAGGGCC
DN606	GTTTAAACGGGCCCTCTAGATTATTTATACAGCTCGTCCATTCCGCC
DN607	GACGACGATAAGGAGGGCAGAGGCTCTCTGCTGACATG
DN608	GCCTCTGCCCTCCTTATCGTCGTCATCCTTGTAATC

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