Supporting Information

Inducible Regulation of CRISPR-Cas9 Gene Editing Enable by Oxidative Signaling Molecule

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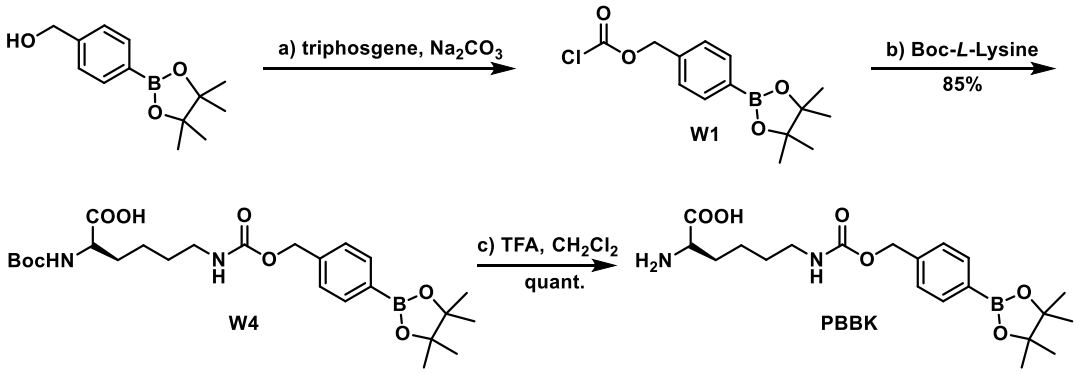
MATERIALS.

Cas9 Nuclease, *Streptococcus pyogenes* (product # M0646), T7 Endonuclease I (product # M0302) Ribonucleotide solution mix (NTPs) and deoxy-ribonucleoside triphosphates (dNTPs) were purchased from *New England Biolabs* (USA). Transcript Aid T7 High Yield Transcription kit (product # K0441) and Glycogen (product # R0561) were purchased from *Thermo Fisher Scientific*. Pyrobest™ DNA Polymerase and PrimeSTAR HS DNA Polymerase were purchased from *Takara Shuzo Co. Ltd*. (Tokyo, Japan). DNA Clean & Concentrator™-5 kit (product # D4014) was purchased from *Zymo Research Corp*. The DNeasy Blood & Tissue Kit was purchased from *QIAGEN*. The oligonucleotides at HPLC purity were obtained from *Takara company* (Dalian, China). The nucleic acid stains Super GelRed (NO. S-2001) was bought from *US Everbright Inc*. (Suzhou, China). DPBA (Cas # 17261-28-8), TCEP (Cas # 51805-45-9), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES, Cas # 7365-45-9) and Thiazolyl Blue Tetrazolium Bromide (MTT, Cas # 298-93-1) were purchased from *Sigma-Aldrich Inc.* (Shanghai, China). DPBS (Cas # 63995-75-5) was purchased from *TCI* (Shanghai) *Development Co., Ltd*. The concentration of DNA or RNA was quantified by NanoDrop 2000c (*Thermo Scientific*, USA). Gel Imaging was performed using Pharos FX Molecular imager (*Bio-Rad, USA*). MST experiment was carried out and the data was analyzed on an MST instrument NT115.

Anhydrous solvents were used and redistilled using standard procedures. All solid reagents were dried under a high vacuum line prior to use. Air sensitive reactions were carried out under nitrogen. RNase-free water, tips and tubes were used for RNA purification, crystallization, and thermodynamic studies. Analytical TLC plates pre-coated with silica gel F254 (Dynamic Adsorbents) were used for monitoring reactions and visualized by UV light and spots were visualized under UV light and/ or phosphomolybdic acid-ethanol. Flash column chromatography was performed using silica gel (32-63 μm). All the chemicals as well as solvents were purchased from *Sigma-Aldrich*, Taufkirchen, Germany, and were used without further purification. All ^{1}H-, and ^{13}C-NMR spectra were recorded on a Brucker 400 spectrometer. Chemical shift values are in ppm. ^{13}C-NMR signals were determined by using APT technique. High-resolution MS were achieved by ESI at Huazhong University of Science and Technology, Wuhan.

SYNTHETIC PROCEDURES FOR PBBK (2).



Scheme S1. Synthetic route for PBBK.

Synthetic procedure

1. Synthesis of 2-*tert*-butoxycarbonylamino-6-[4-(4, 4, 5, 5-tetramethyl-[1, 3, 2]dioxaborolane-2-yl)-benzyloxycarbonylamino]-hexanoic acid (**W4**).

Phenylboronic acid pinacol ester (500.2 mg, 2.1 mmol) was dissolved in THF (5 mL), containing Na_2CO_3 (226.8 mg, 2.1 mmol), and cooled to 0°C. To the solution was added triphosgene (635.4 mg, 2.1 mmol) and the reaction was kept stirring for 12 hours at r.t. The reaction was filtered and the volatiles were subsequently evaporated without heating and the residue dried under vacuum **to give crude W1 used directly without further purification.**

To a solution of Boc-lysine (550.2 mg, 2.4 mmol) in THF/1.0 M NaOH (aq.) (1: 4 v/v, 9.0 mL) at 0°C was added **W1** (634.6 mg, 2.1 mmol). After the reaction was stirred for 12 hours at r.t., aqueous layer was washed with Et_2O (5 mL) and subsequently acidified with ice-cold 1.0 M HCl (20 mL) to pH = 1 and extracted with EtOAc (30 mL). The organic layer was dried over Na_2SO_4, filtered, and the volatiles were evaporated, compound **W4** (548.1 mg) was obtained in 51% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25-7.16 (m, 1H), 6.86-6.69 (m, 1H), 4.25 (t, *J* = 8.6 Hz, 2H), 3.50 (t, *J* = 7.2 Hz, 5H), 1.97-1.77 (m, 2H), 1.69 (td, *J* = 14.6, 9.5 Hz, 2H), 1.65-1.52 (m, 3H), 1.24 (s, 12H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 175.71, 156.74, 135.04, 127.08, 83.85, 77.57, 77.25, 76.93, 40.59, 31.96, 29.23, 28.32, 24.71 (d, *J* = 16.7 Hz), 22.40. **MS (ESI)**: $[M-H]^-$ = 505.3; Found: 505.7. **HRMS**: Calcd. for $C_{25}H_{39}BN_2NaO_8$ $[M+Na]^+$: 529.2697; Found: 529.2692.

2. 2-Amino-6-[4-(4, 4, 5, 5-tetramethyl-[1, 3, 2]-dioxa-borolan-2-yl)-benzyloxy-carbonylamino]-hexanoic acid (**PBBK**).

Compound **W4** (50.0 mg, 0.1 mmol) was dissolved in CH_2Cl_2 (1.0 mL), followed by TFA (1.0 mL), at 0°C under N_2 atmosphere. Then, the reaction mixture was stirred 0.5 h at r.t. Petro ether (10 mL) was added to the reaction, and the **PBBK** (47.4 mg) was separated out at 0°C in 91% yield.

^{1}H NMR (400 MHz, D_2O) δ 7.70 (d, *J* = 7.9 Hz, 2H, Ph), 7.34 (d, *J* = 7.8 Hz, 2H, Ph), 5.04 (s, 2H, CH_2Ph), 3.81 (t, *J* = 4.0 Hz, 1H, $CH(NH_2)(COOH)CH_2$), 3.05 (t, *J* = 4.0 Hz, 2H, CH_2NH), 1.88-1.78 (m, 2H, CH_2), 1.49-1.42 (m, 2H, CH_2), 1.37-1.29 (m, 2H, CH_2), 1.13 (**s, 12H, CH_3** × 4).

^{13}C NMR (101 MHz, D_2O) δ 172.00 (COOH), 158.02(CO, isomer 157.57), 139.90 (Ph), 139.09 (Ph), 133.94 (Ph, isomer 134.62), 126.71, 84.17 ($CH(Me)_2$), 75.53 (CH_2Ph), 66.45 ($CHNH_2$(COOH)), 52.66 ($CH_2(CH_3)_2$), 39.76 (CH_2), 29.31 (CH_2, isomer 29.39), 28.38 (CH_2, isomer 28.59), 23.71 (CH_2, isomer 24.05), 21.43 (CH_2, isomer 21.58).

MS (ESI): $[M+H]^+$ = 407.6.

HRMS: Calcd for $C_{20}H_{32}BN_2O_6$ $[M+H]^+$: 407.2353; Found: 407.2356.

NMR and MS spectra

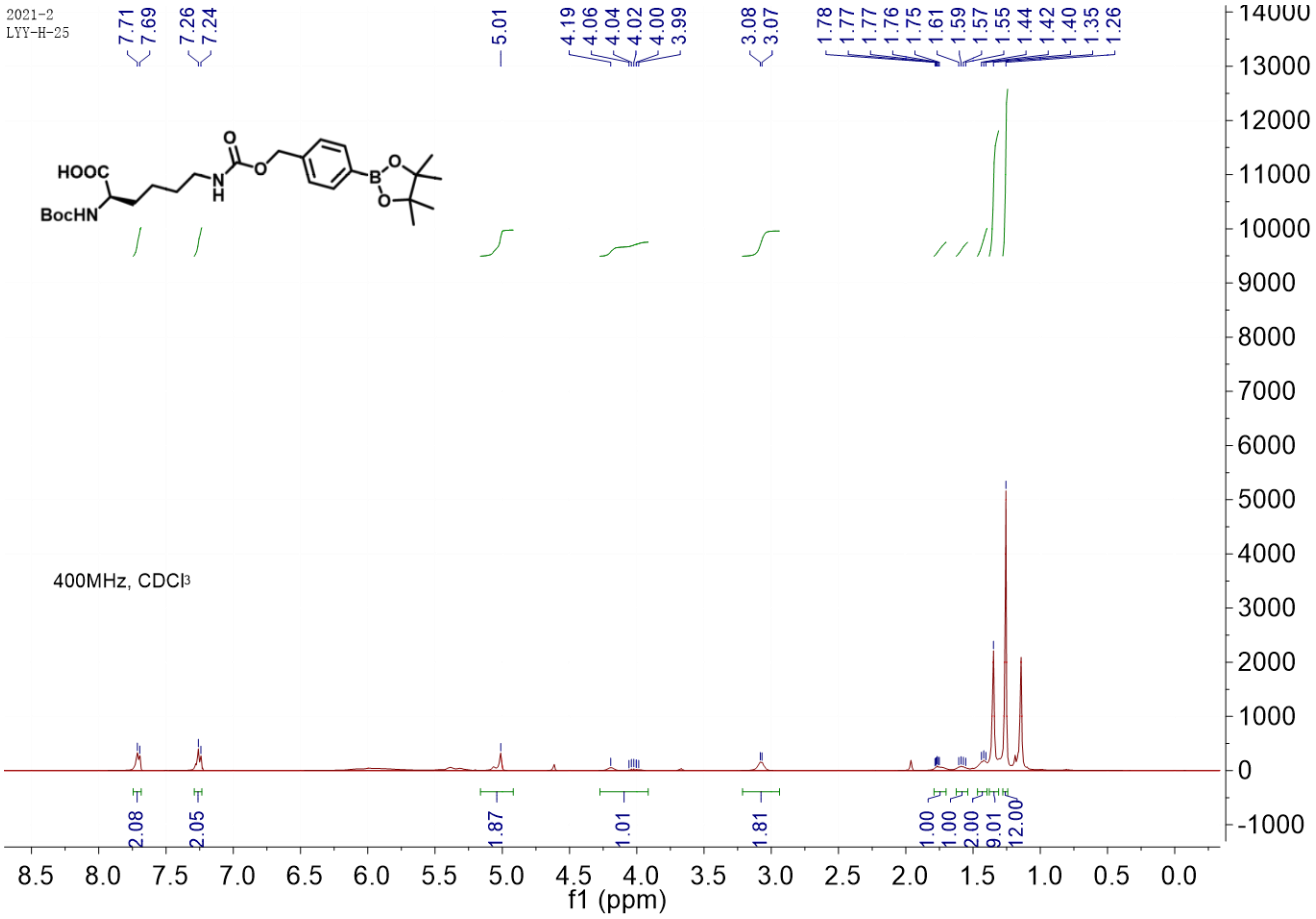


Figure S1. ^{1}H NMR spectrum for compound **W4**.

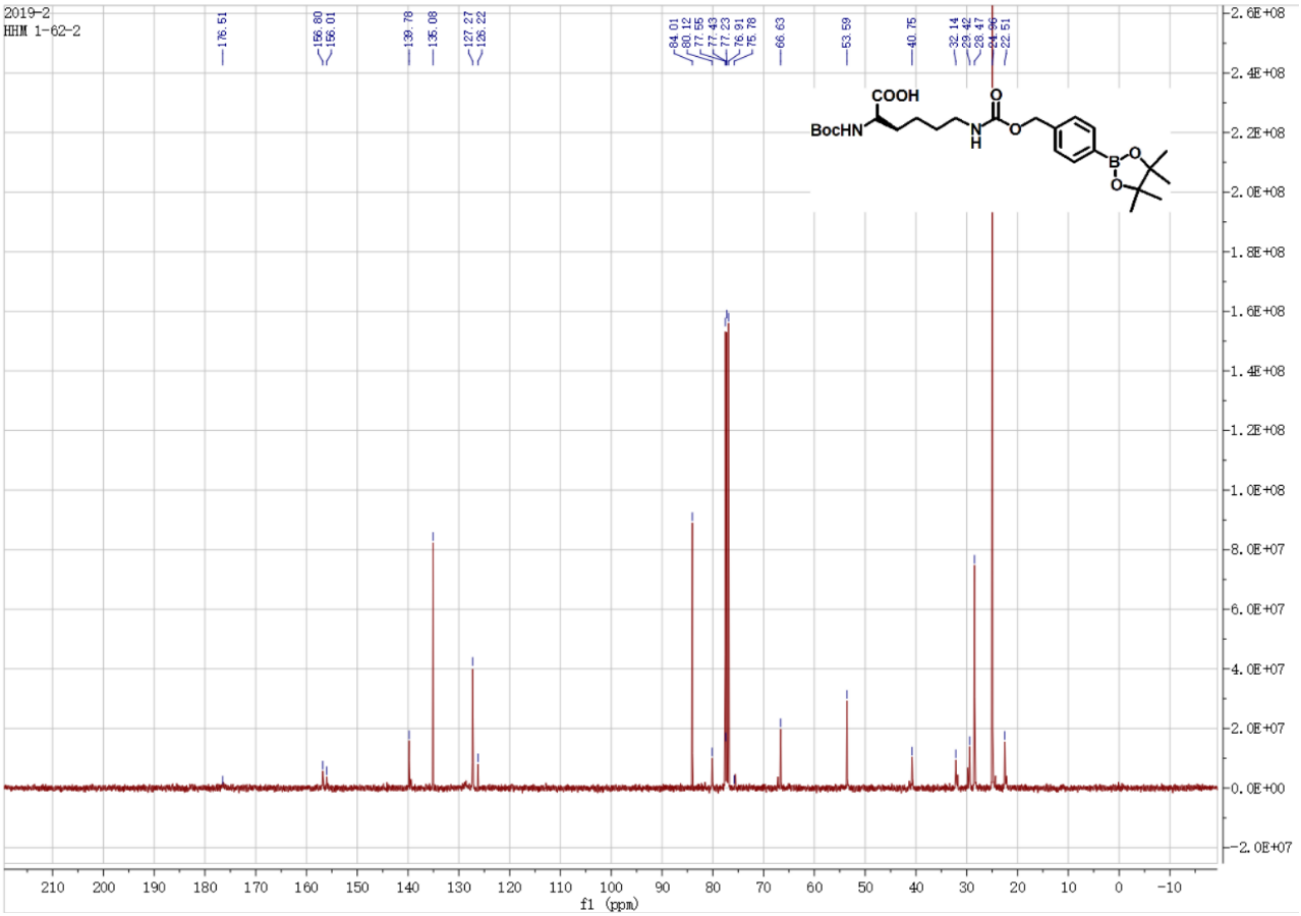


Figure S2. ^{13}C NMR spectrum for compound **W4**.

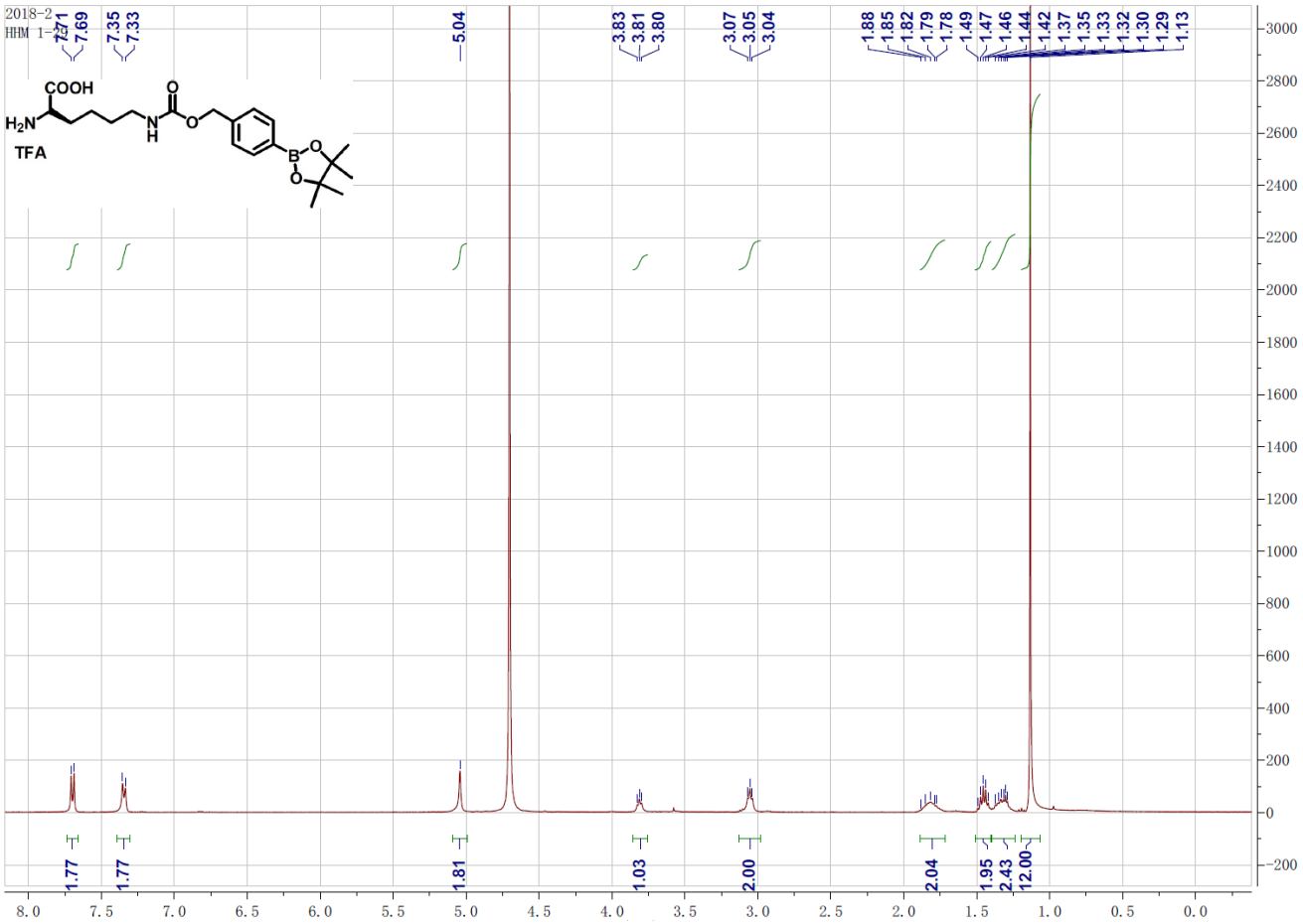


Figure S3. ^{1}H NMR spectrum for **PBBK**.

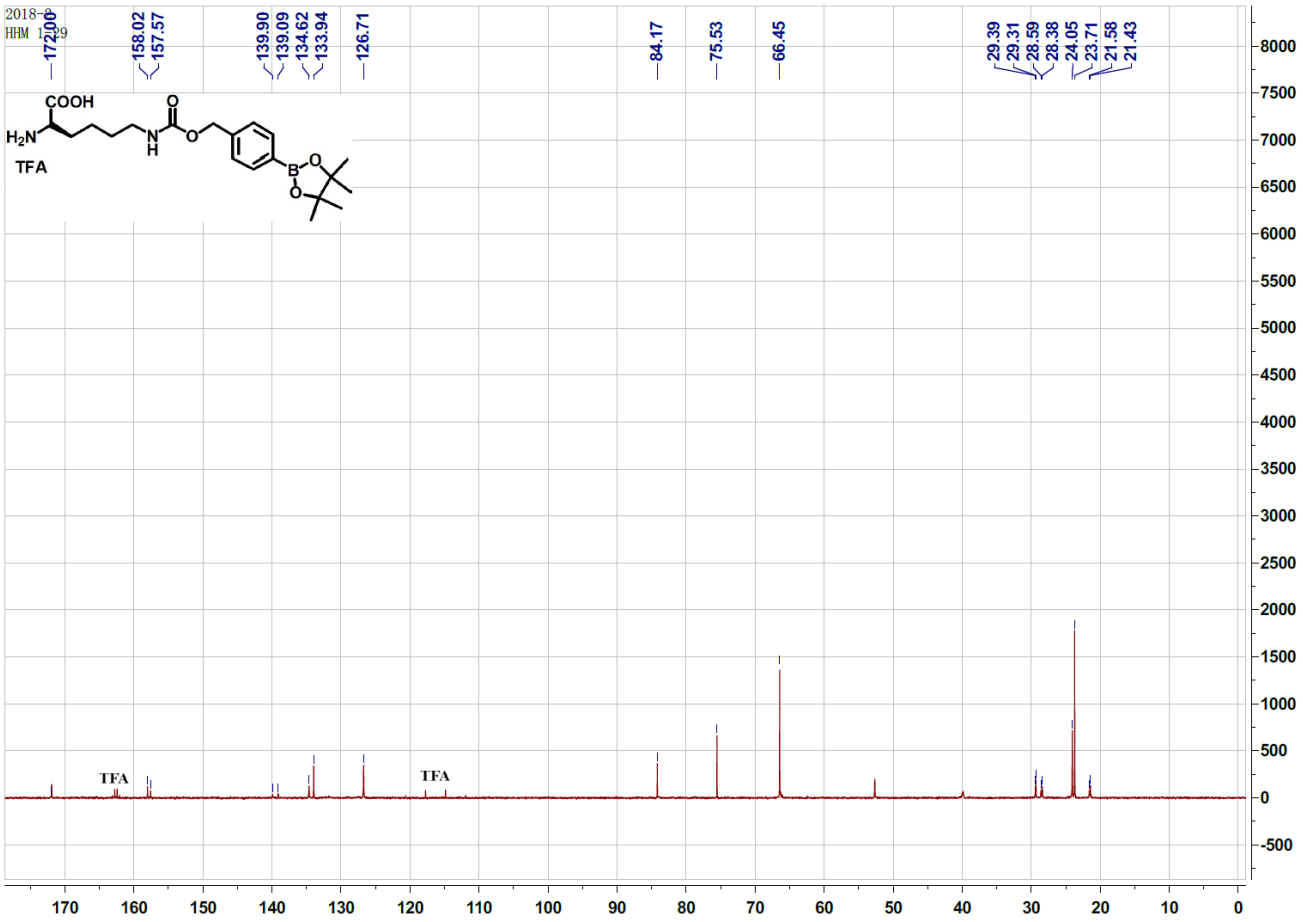
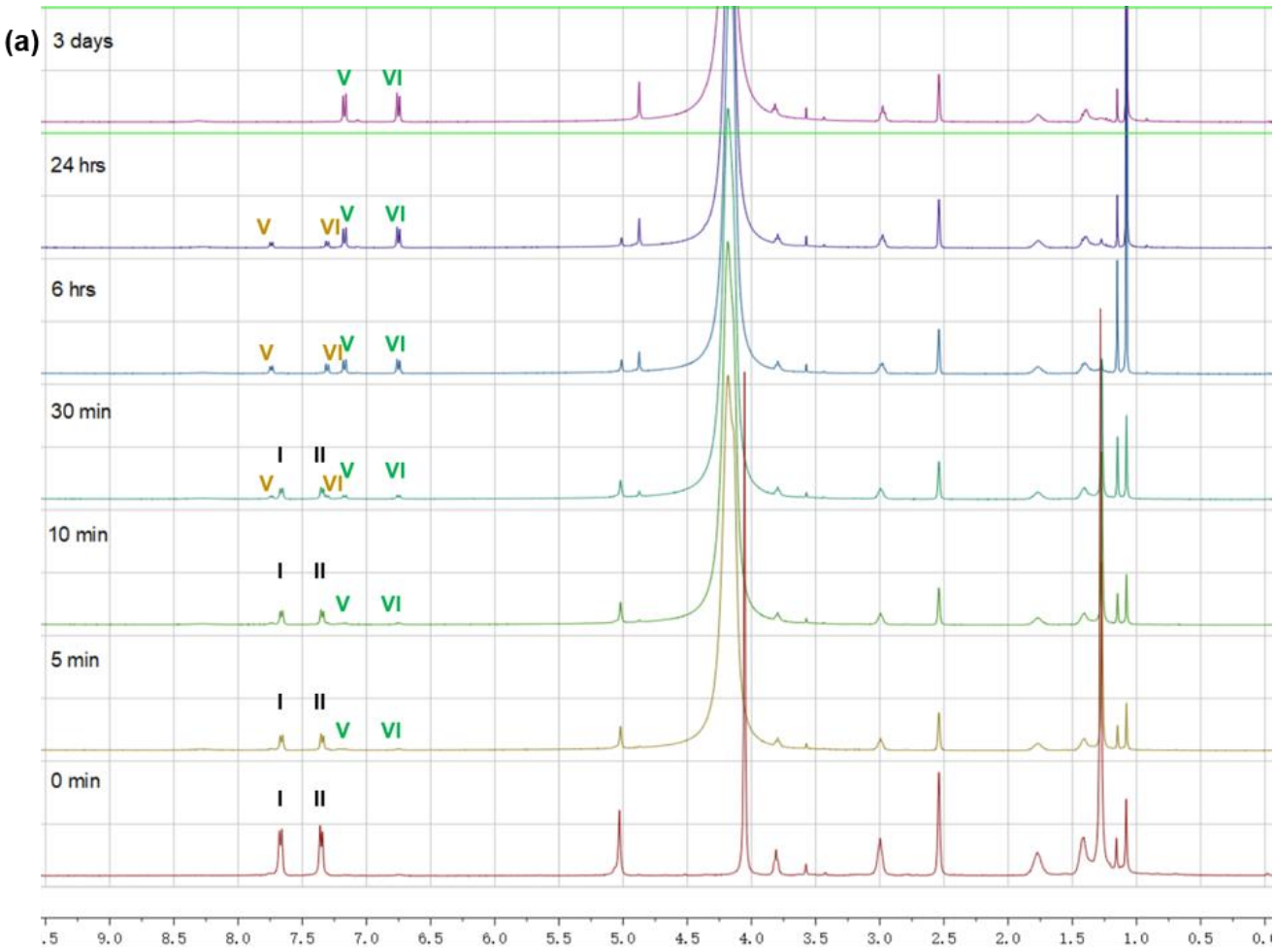


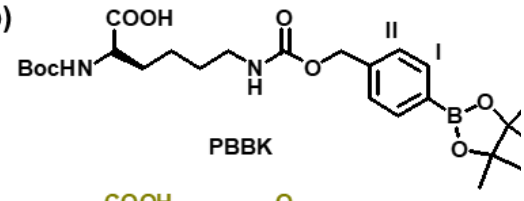
Figure S4. ^{13}C NMR spectrum for **PBBK**.

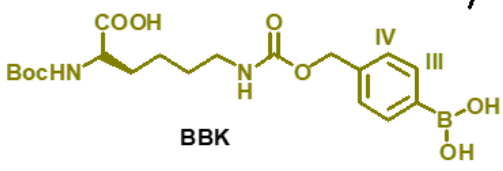
DYNAMIC EXPERIMENT OF PBBK WITH ROS SPECIES.

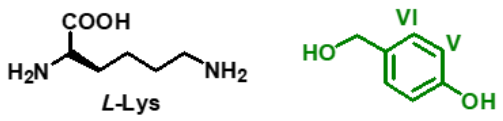
In an NMR tube, PBBK (2.0 μM, final concentration) was added into D_2O (0.7 mL), then this mixture was treated with 30% H_2O_2 (200 μM, final concentration) at varied times monitored by NMR spectroscopy.



(b)







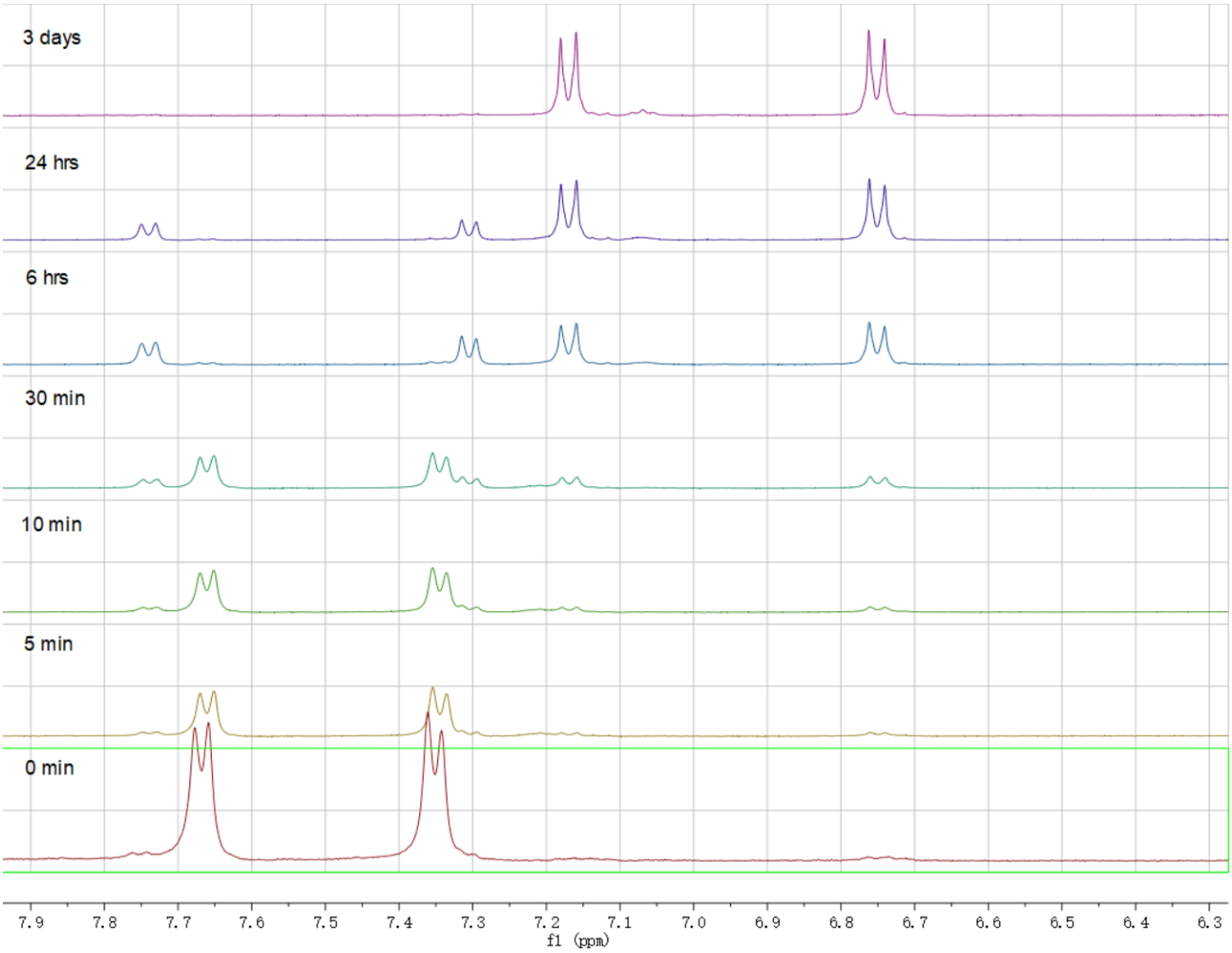
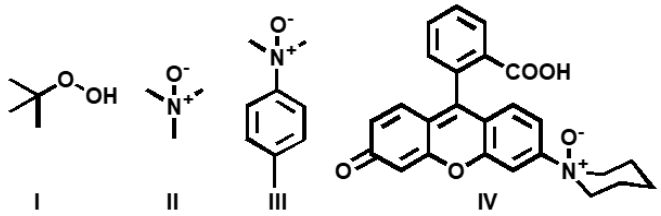
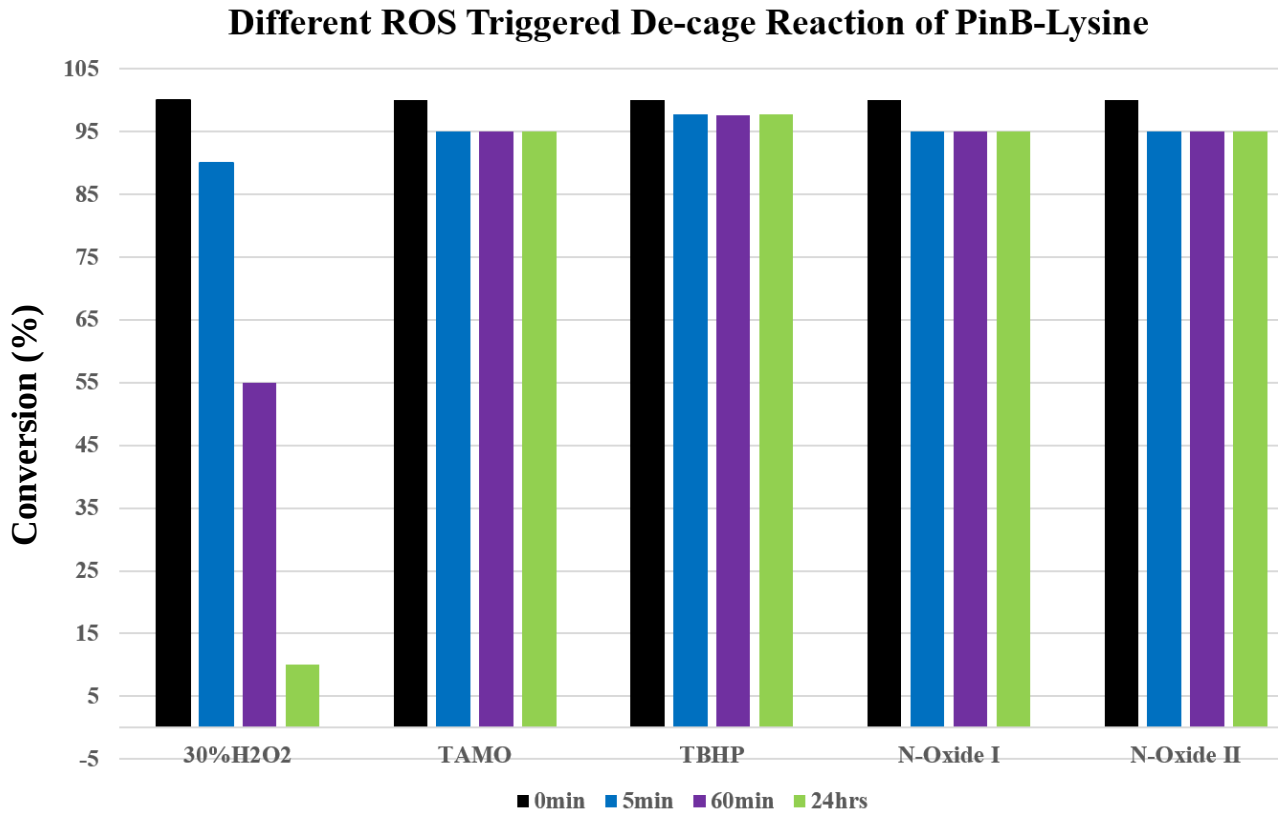


Figure S5. Time-lapse proton NMR of uncaged-reaction of PBBK with ROS (H_2O_2). *Notes (a) Proton NMR in varied times. Time-lapse scope: 0 min to 3 days. (b) Starting material, possible intermediate and products involved in this transformation. PBBK (2.0 μM) treated with 30% H_2O_2 (200 μM) in varied times.

REACTIVITY SCREENING OF OTHER ROS SPECIES.





Note: (1) 150 uM PinB-Lys reacted with 200 mM ROS at r.t., the reaction was monitored by RP-HPLC; (2) *N*-Oxide 1: Dimethyl-p-tolyl-amine *N*-Oxide; *N*-Oxide 2: Fluorescent *N*-Oxide;

Chart S1. Reaction-lapse of ROS species-triggered bond cleavage of PBBK. PBBK (150 μM) reacted with ROS (200 mM) at r.t. in varied times (5, 40, 60 and 120 min were marked as black, blue, purple and green, respectively), and the reaction was monitored by RP-HPLC. *N*-Oxide I: dimethyl-p-tolyl-amine *N*-Oxide; *N*-Oxide II: fluorescent *N*-Oxide. All the synthetic *N*-oxide were consistent with the reported data. Zhu, C.; Wang, R.; Falck, J. R. *Mild and rapid hydroxylation of aryl/heteroaryl boronic acids and boronate esters with N-oxides*. *Org. Lett., 14*, 3494-3497 (**2012**).

CYTOTOXIC EXPERIMENT OF NON-CANONCIAL AMINO ACIDS PBBK IN VARIED TIMES.

The CCK8 kit (*Yeasen Biotechnology*) was used to detected the HeLa cell viability when treated with varied concentration of PBBK. The operation is strictly accordance with the vector's instructions. Two thousand HeLa cells was firstly planted in 96-well plate. While the cells were adhered to the plate, the HeLa cells were treated with increasing concentration of PBBK (0, 0.1, 1.0, 5.0 mM) for 12 h and 24 h. Then cell viability was detected by CCK8, each well added CCK8 (10 μl) and was treated for 2 h at 37℃. Finally, a multifunctional microplate reader was used to detect the absorbance.

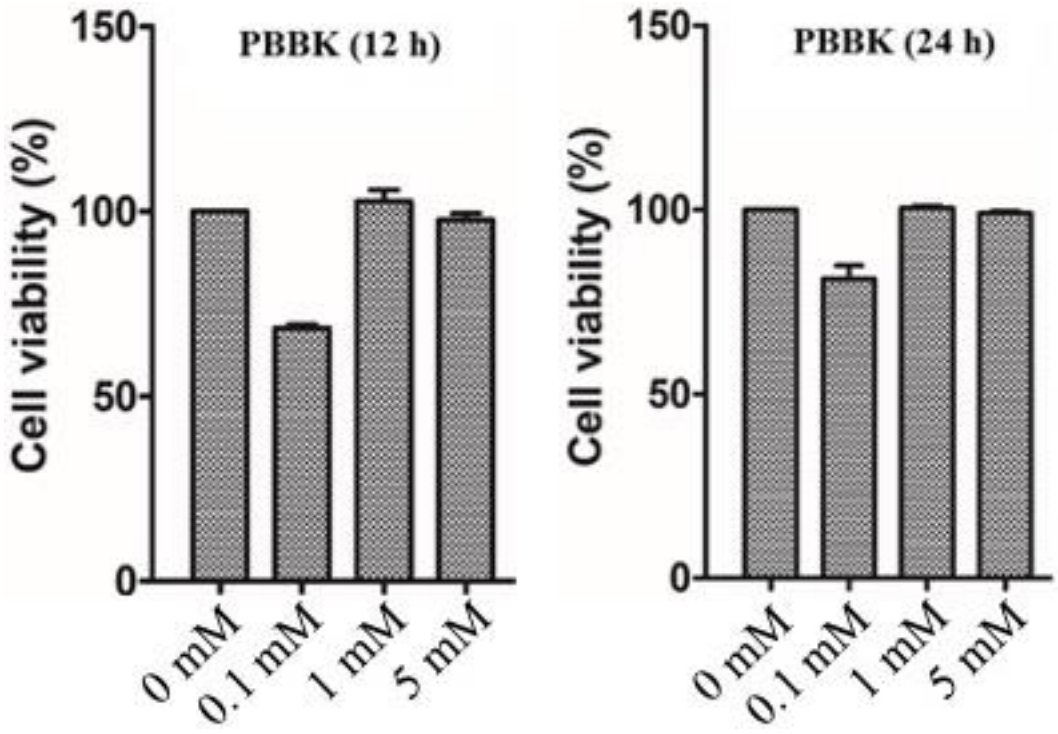


Chart S2. mM-level of PBBK treatment observed non-visible cell death towards HeLa cells in either 12 h (left) or 24 h (right).

WESTERN BLOT RESULTS OF CAS9 EXPRESSION WITH VARIED SITE-SPECIFIC MUTATIONS.

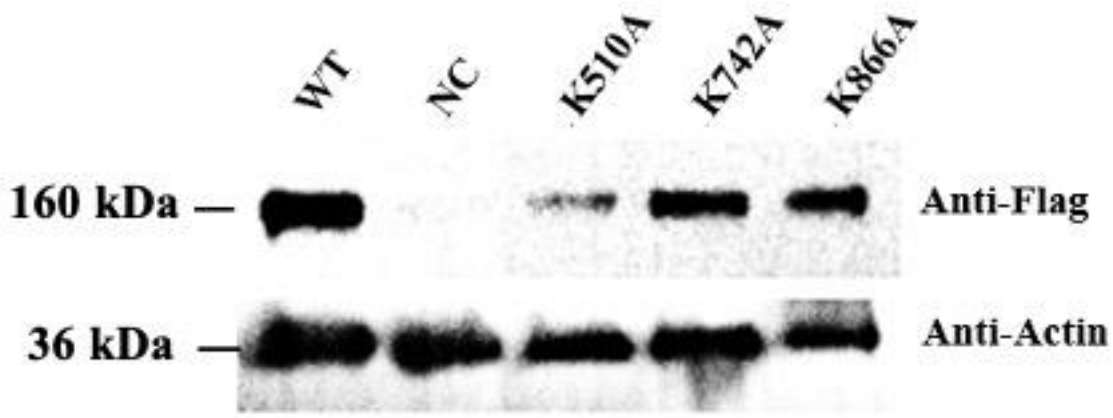


Figure S6. Western blot assays of Cas9 expression with varied mutations.

CONSTRUCTION OF CAS9 ACTIVATION DUAL-FLUORESCENCE DETECTION SYSTEM.

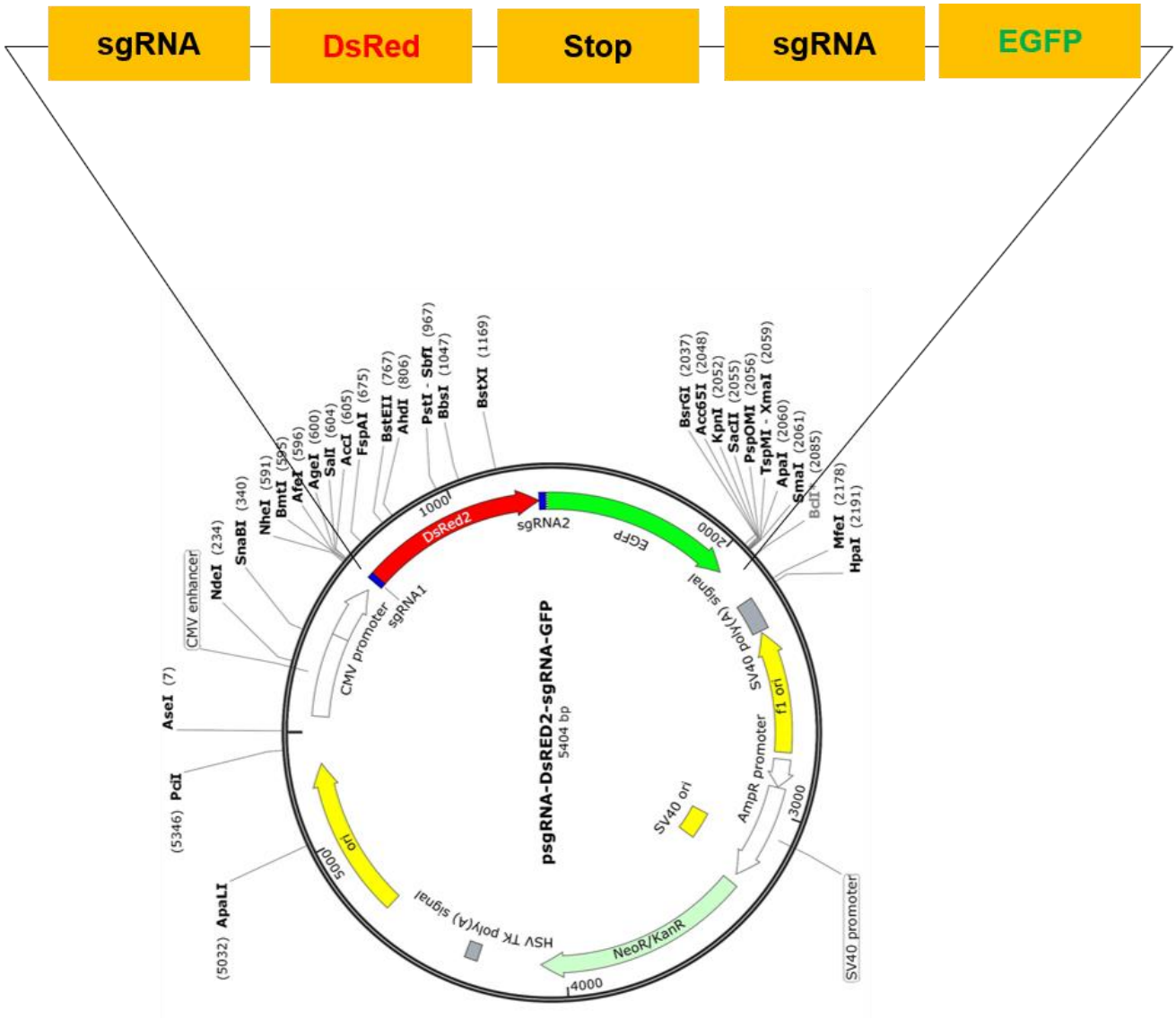


Figure S7. Map of construction of non-natural amino acids replacement system for Cas9 endonuclease.

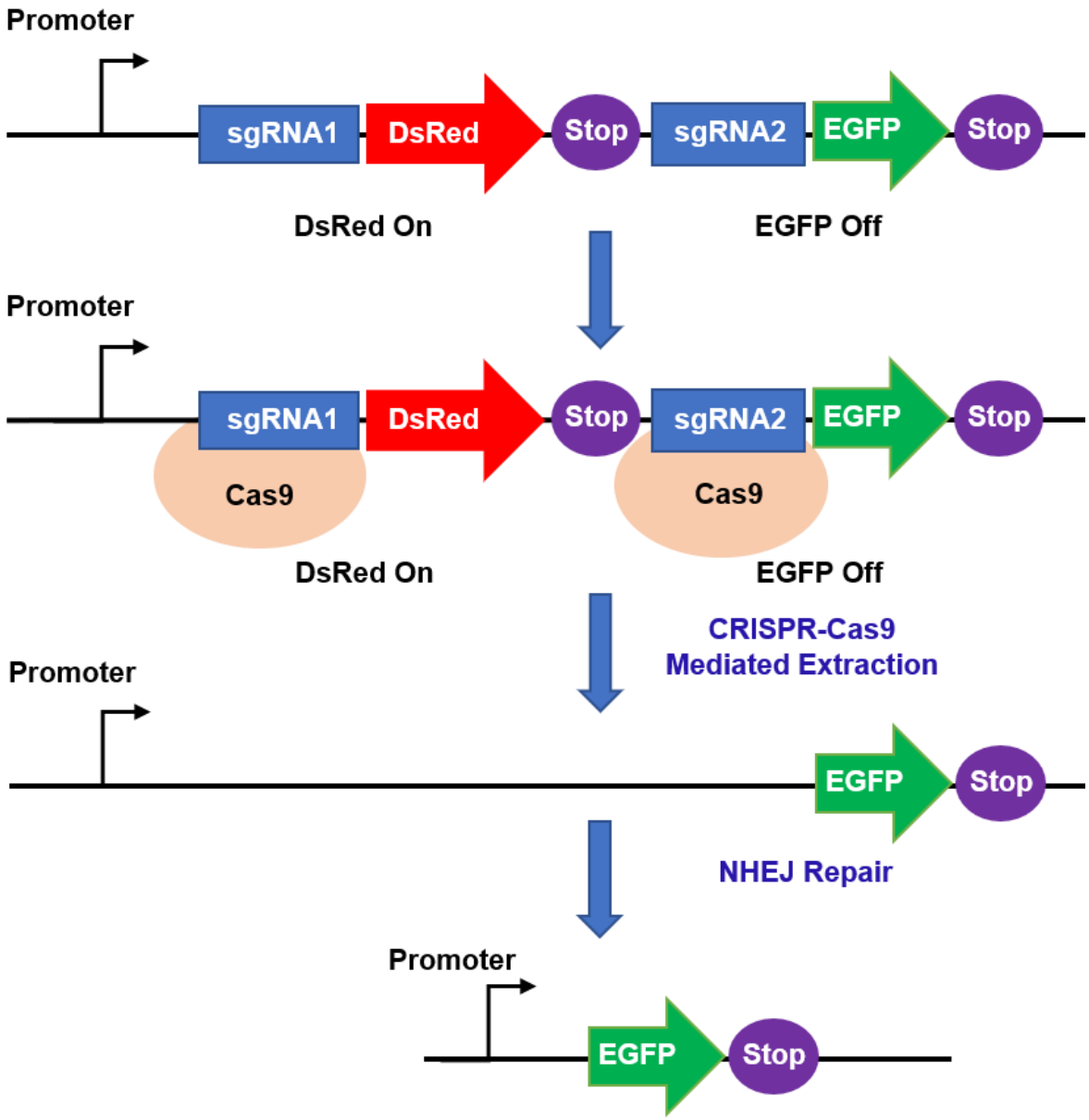


Figure S8. Schematic illustration of non-natural amino acids replacement system for Cas9 endonuclease.

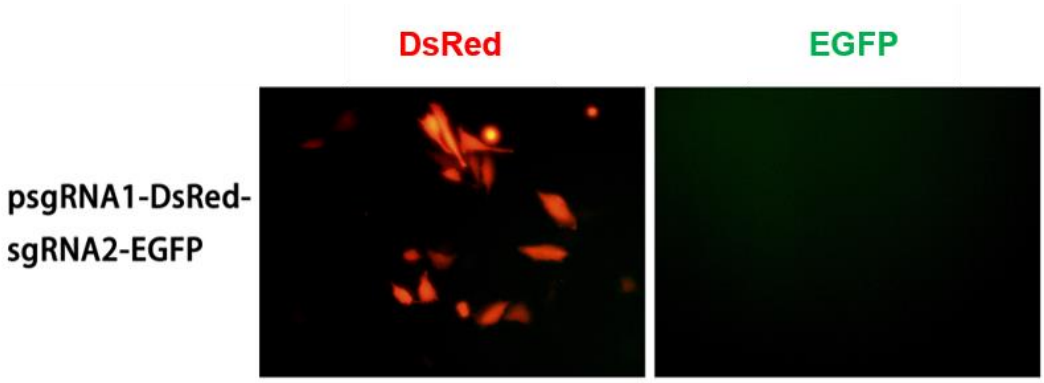
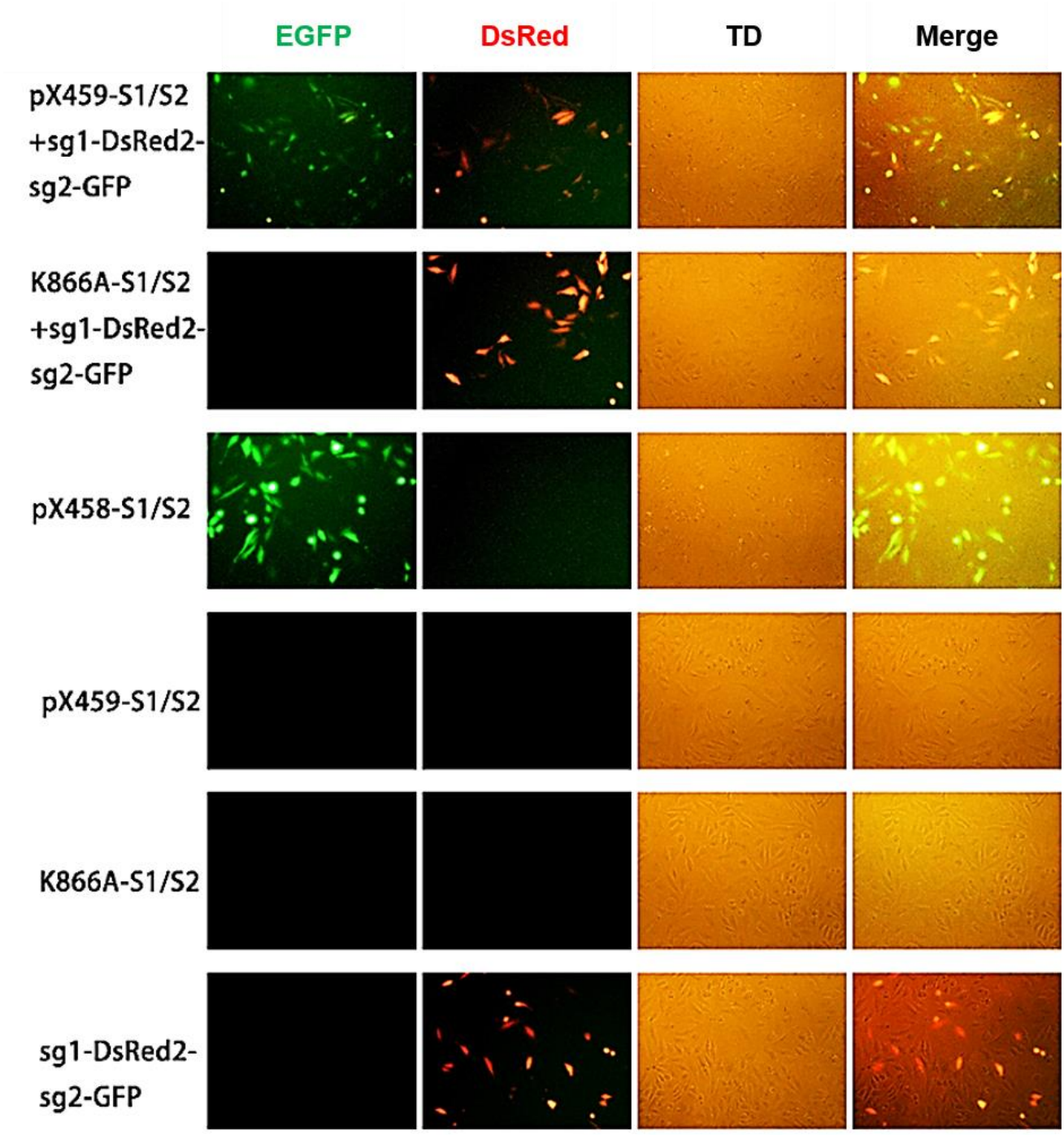


Figure S9. Fluorescent imaging results of the dual reporter system for Cas9 endonuclease.

DETECTION OF THE ACTIVITY OF CAS9 MUTANT.



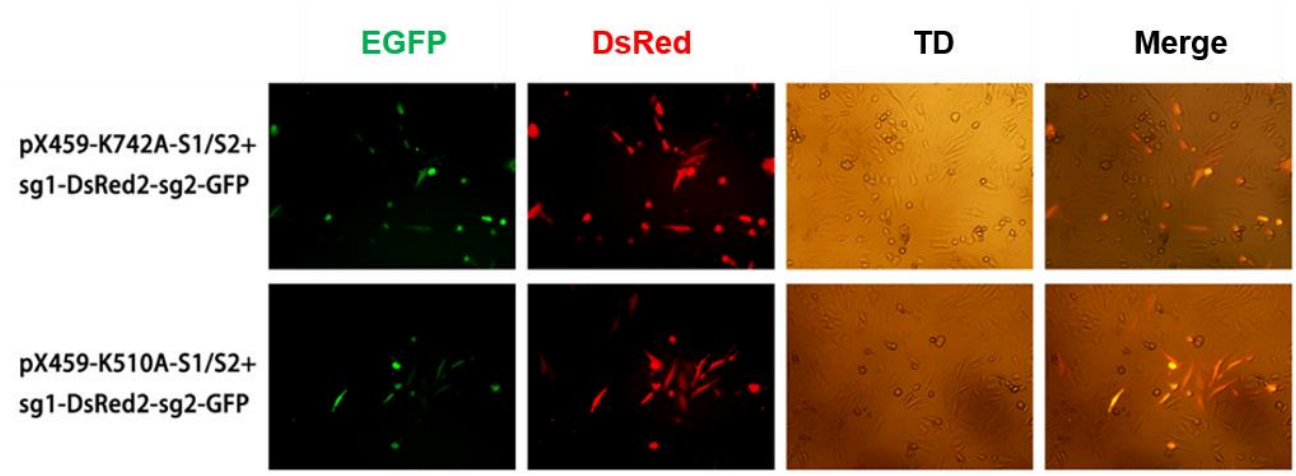


Figure S10. Fluorescent imaging of the dual reporter system for Cas9 endonuclease. Both pX458 and pX459 are the names of plasmids. S1 and S2 are the sgRNA expression sequences containing sg1 and sg2, respectively.

REPLACEMENT OF LYSINE ON KEY POSITION OF CAS9 ENDONUCLEASE WITH PBBK.

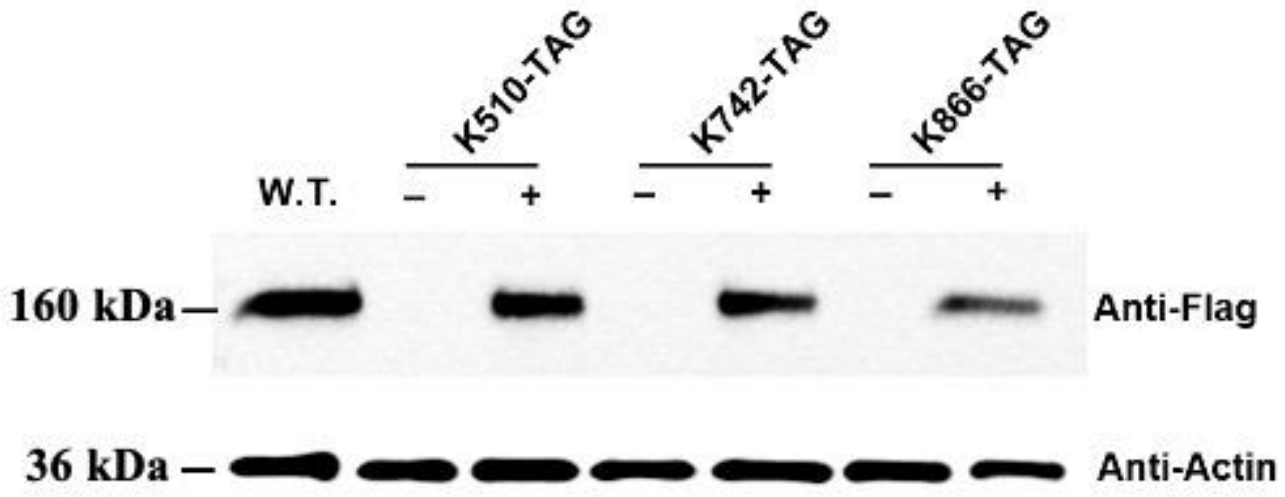


Figure S11. Western blot detection of the Cas9 variants expression with lysines on varied positions. PBBK concentration is 1.0 mM.

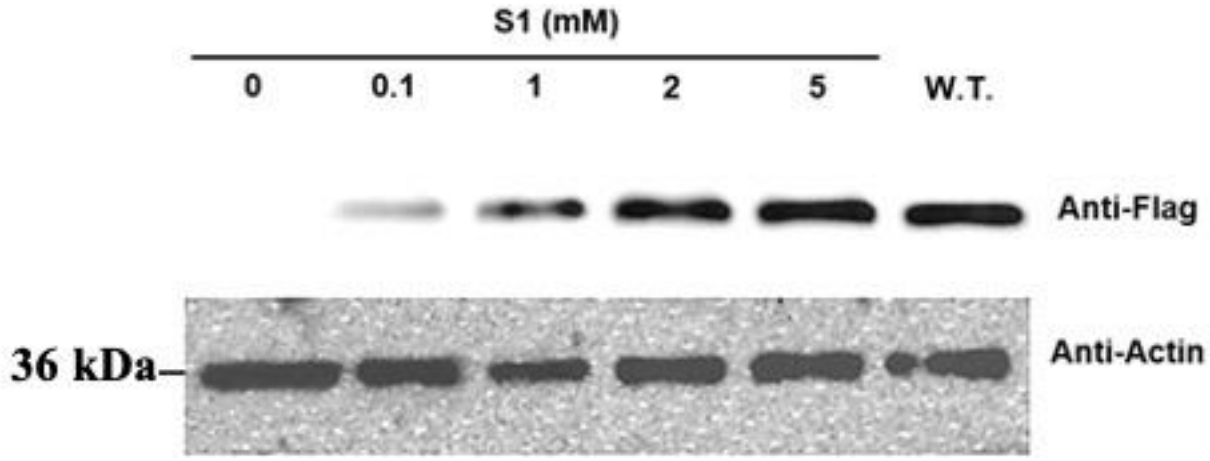


Figure S12. Screenings of PBBK concentration. It was observed that there of no obvious difference between PBBK (2.0 mM) and PBBK (5.0 mM).

DETECTION OF H_2O_2-TRIGGERED RELEASE PROCESS OF PBBK-MODIFIED CAS9.

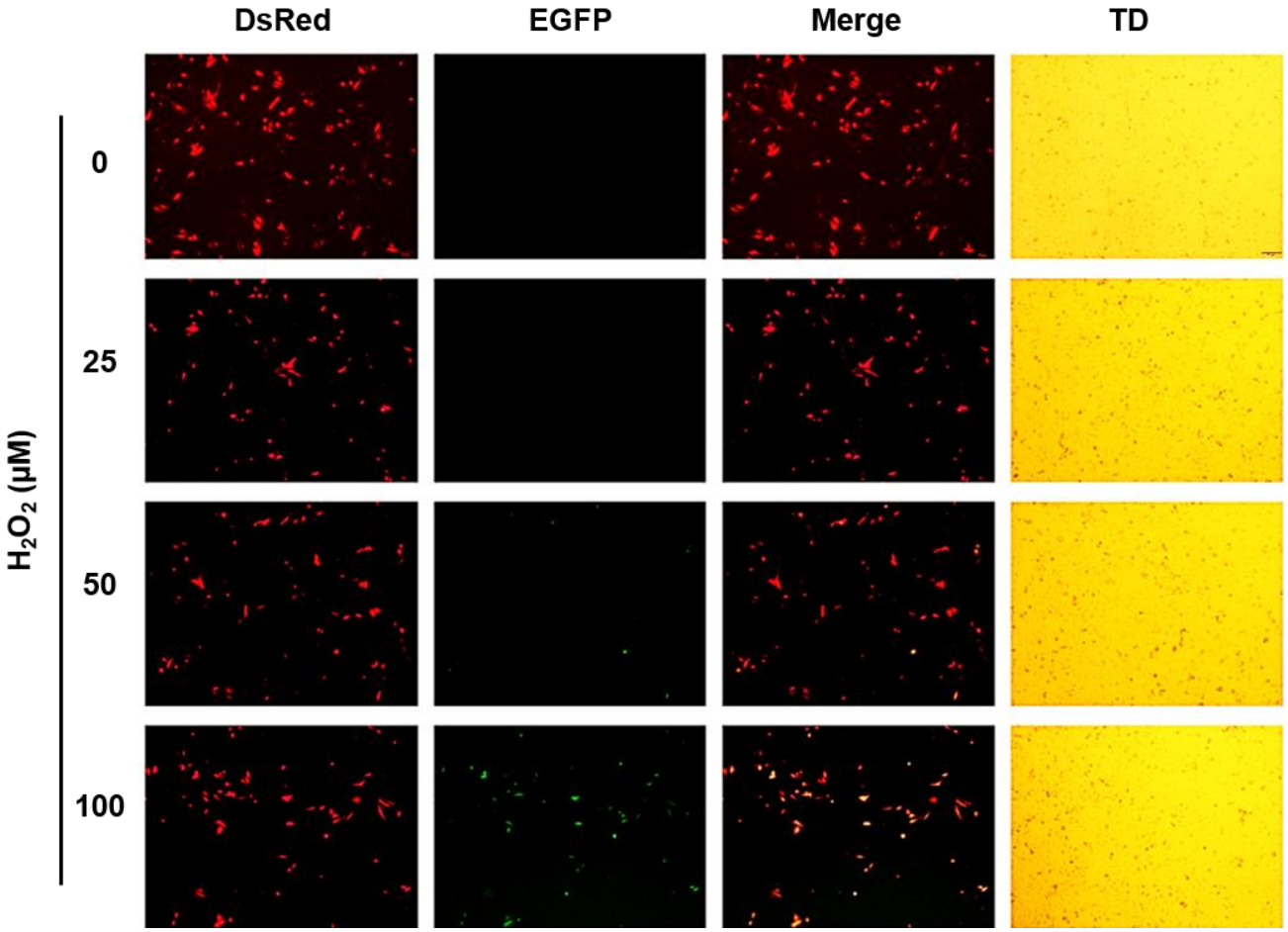


Figure S13. Fluorescent imaging assays of H_2O_2-triggered rescuing of Cas9 variant with PBBK (1.0 mM) mutant in K866 position in various concentration.

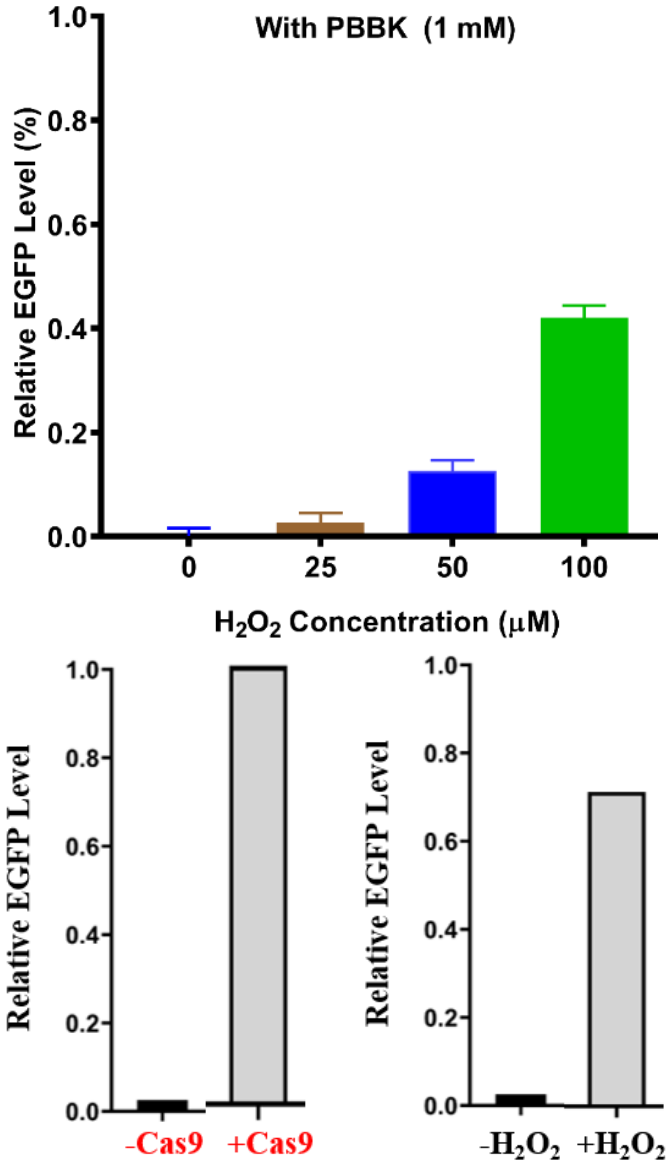


Chart S4. EGFP analysis of H_2O_2-triggered rescuing of Cas9 variant with PBBK (1.0 mM) mutant in K866 position in various concentration.

The procedure is the same as the description stated above. But the cells were cultured in DMEM medium with PBBK-caged lysine (2.0 mM).

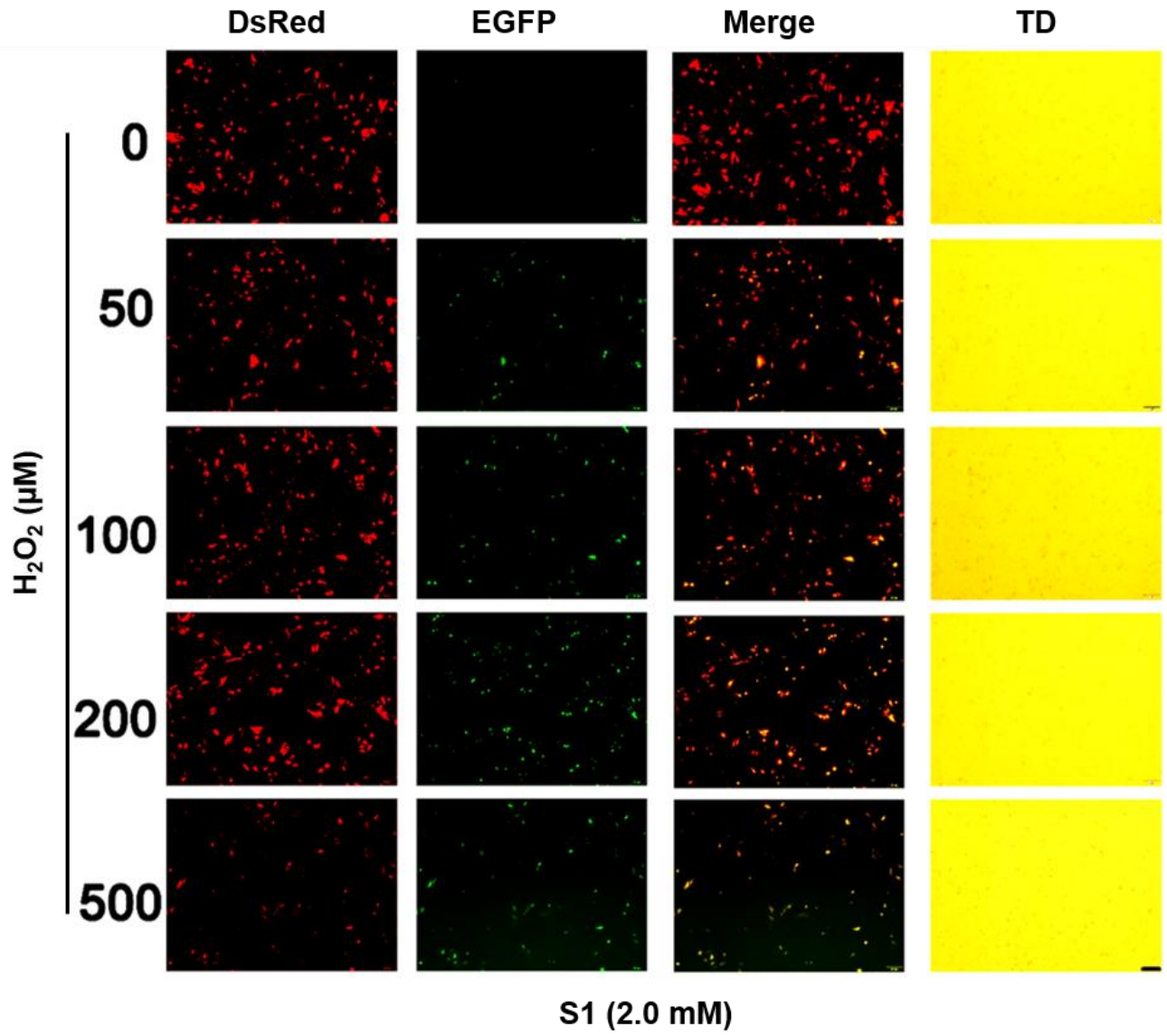


Figure S14. Fluorescent imaging assays of H_2O_2-triggered rescuing of Cas9 variant with PBBK (2.0 mM) mutant in K866 position in various concentration.

NOTCH1 KNOCKDOWN SYSTEM CONSTRUCTION.

Six pairs of sgRNA were designed target to the first six exons of *Notch1* gene.

(1) sgRNA design.

To target the temple DNA, search for 5'-N_{20}-NGG-3' and the sequence of N_{20} will be used directly as base-pairing region of the sgRNA. NGG is the PAM sequence that is recognized by the Cas9 protein. U6 promoter requires a G at the 5' end for efficient transcription. To maximize U6 promoter activity for sgRNA expression, add an extra G at the 5' end of the sense guide oligo if the first nucleotide of the transcribed sgRNA is not a G, E.G., 5'-G-H-N_{19}-3', and in this case also put an extra C at the 3' end of the antisence guide oligo.

Add CACC at the 5' end of the sense guide oligo, and put AAAC at the 5' end of the antisense guide oligo to 5' protruding ends for cloning as shown below:

Sense guide oligo	5'-CACC-G-N_{20}-3'
Antisence guide oligo	5'-AAAC-N_{20}-3'
Notch1-sgRNA1-F	CACCGAGCAGCGCCAGGCAGAGCAG
Notch1-sgRNA1-R	AAACCTGCTCTGCCTGGCGCTGCTC
Notch1-sgRNA2-F	CACCGTGAAGCGGCCAATGGCACGG
Notch1-sgRNA2-R	AAACCCGTGCCATTGGCCGCTTCAC
Notch1-sgRNA3-F	CACCGAACGCCGGGACATGCCACG
Notch1-sgRNA3-R	AAACCGTGGCATGTCCCGGCGTTC
Notch1-sgRNA4-F	CACCGCGGAGGCACCTGCCACAACG
Notch1-sgRNA4-R	AAACCGTTGTGGCAGGTGCCTCCGC
Notch1-sgRNA5-F	CACCGGAAACAACTGCAAGAACGG
Notch1-sgRNA5-R	AAACCCGTTCTTGCAGTTGTTTCC
Notch1-sgRNA6-F	CACCGACCTGCCACAACACCCACGG
Notch1-sgRNA6-R	AAACCCGTGGGTGTTGTGGCAGGTC

Table S1. Primers design for the synthesis of sgRNA (1-6) targeted Cas9.

(2) sgRNA anneal.

First, dissolve the oligonucleotides into 100 μM with autoclaved distillated water.

Second, prepare 10× annealing buffer:

Entry 1	K-acetate (1.0 M)
Entry 2	HEPES-KOH, pH =7.4 (0.3 M)
Entry 3	Mg-acetate (20 mM)

Third, set up annealing mixture:

Entry 1	Sense guide oligo	9 μl
Entry 2	Antisense guide oligo	9 μl
Entry 3	10× annealing buffer	2 μl

Fourth, anneal in a thermocycler using the following parameters: 95 ℃, 5 min, then ramp down to 25 ℃ at 1 ℃/min.

(3) Plasmid enzyme digestion.

pX459、pX459-Cas9-K866-TAG、pX459-Cas9-K742-TAG、pX459-Cas9-K510-TAG was digested by ***BbsI***.

Entries	**Volume (μl)**
BbsI	1.0
G buffer	2.0
Plasmid	3.0
ddH_2O	14.0
Total 20 μl	

The mixture was cut overnight in 37 ℃. Then, the enzyme-excised plasmid was extracted by Gel DNA Mini Purification Kit for the next step.

(4) sgRNA was linked to the enzyme-excised plasmid

The annealing-sgRNA was linked to the enzyme-excised plasmid by T4 DNA Ligase (*Solusion I*, Takara).

Entries	**Volume (μl)**
Solusion I	5.0
sgRNA (sg1/sg2)	3.0
Linear plasmids	2.0
Total 10 μl	

The system was put in 16 ℃ for at least 6 h.

(5) Preservation and detection of the new plasmid.

The new plasmid was transfected into DH5α and the single clone was picked for expand cultivation. pX459-sgRNA1, pX459-sgRNA2, pX459-sgRNA3, pX459-sgRNA4, pX459-sgRNA5, pX459-sgRNA6 were firstly constructed. The others will be constructed after the pX459-sgRNA were detected in section III.

THE EFFECT OF SIX sgRNA SEQUENCE INHIBITING *NOTCH1* EXPRESSION WAS DETECTED.

HeLa cells were seeded into 6-well plates and incubated overnight in DMEM growth media supplemented with 10% FBS at 37°C in 5% CO_2. Transfections were performed with 5 μg of each plasmid using 2 μL Vigofect transfection reagent in 2 ml DMEM media at 37°C for 4 h. After 4 h, the DMEM media transfection mixtures were removed from the cells and replaced with fresh DMEM growth media for a 48-h incubation. Then the proteins were extracted and *Notch1* was detected by western-blot.

PBBK-MODIFIED CAS9 REGULATE ENDOGENOUS *NOTCH1* EXPRESSION.

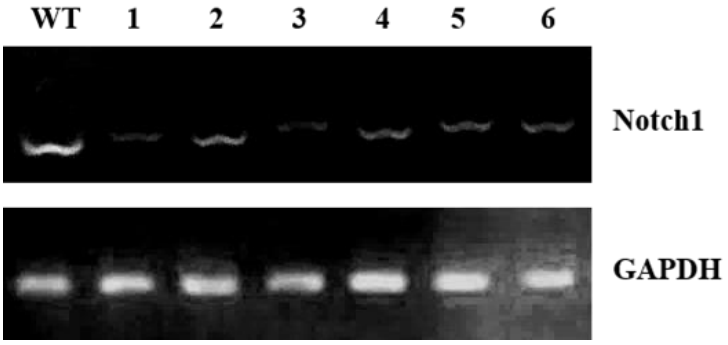


Figure S15. RT-PCR assays of gRNAs targeting different sites of *Notch1* exon. 1-6 represent sgRNA (1 to 6).

As stated before, regulation effect of the sgRNA (1 to 6) on *Notch1* expression was detected by Western blots (instead of reverse transcription PCR).

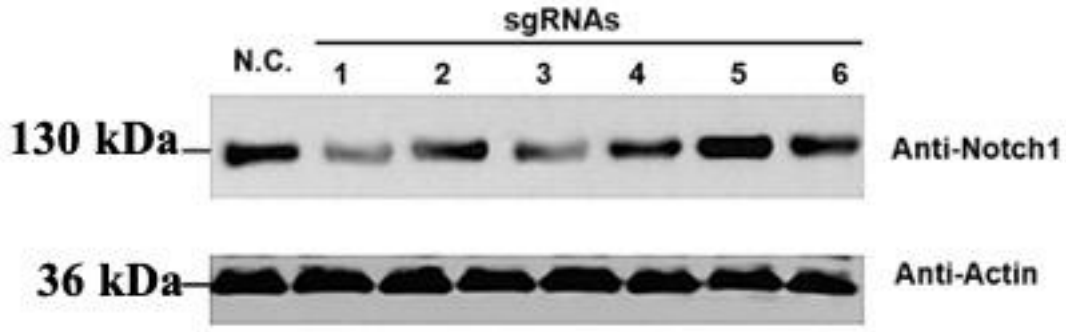


Figure S16. Western blot assays of gRNAs targeting different sites of *Notch1* extron.

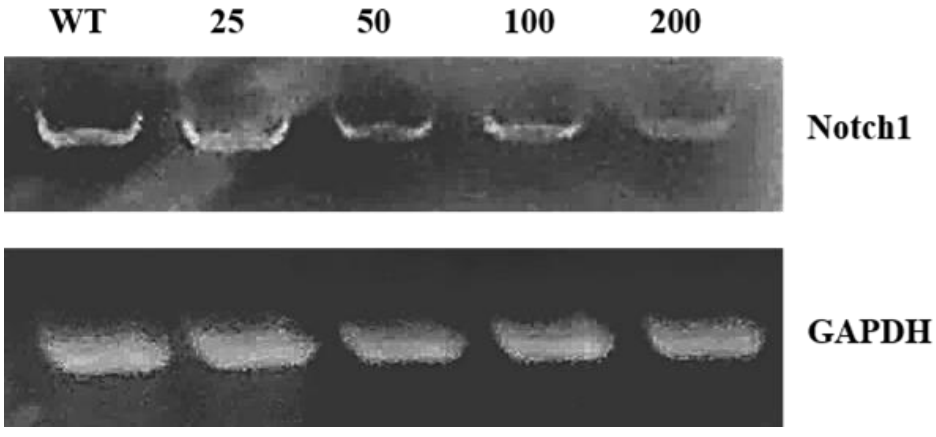


Figure S17. RT-PCR assays of H_2O_2-triggered rescuing of Cas9 variant with PBBK mutants in varied positions targeting *Notch1* gene *in vivo*.

As stated above, this section was designed to regulate *Notch1* expression by controlling Cas9 activation with H_2O_2. The expression level of *Notch1* was detected by Western blots.

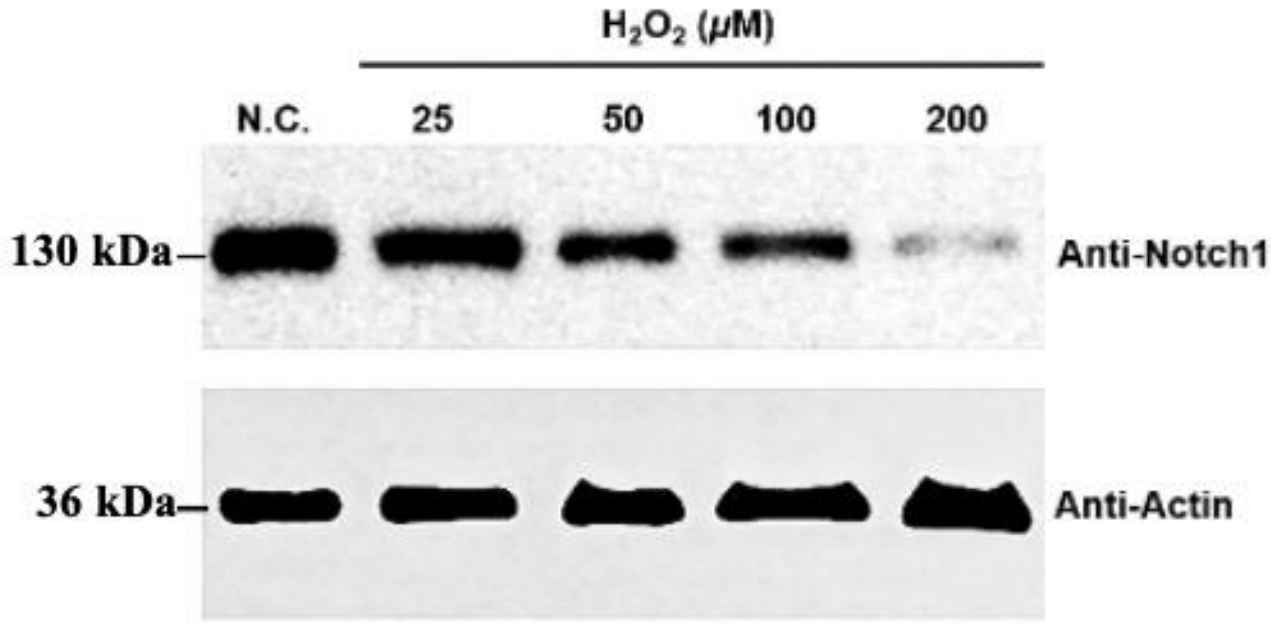


Figure S18. Western blot assays of H_2O_2-triggered rescuing of Cas9 variant with PBBK mutants in varied positions targeting *Notch1* gene in *vivo*.

DETECTION OF ROS LEVEL IN HELA CELLS USING VARIED CONCENTRATION OF H_2O_2.

(1) HeLa cell resuscitation.

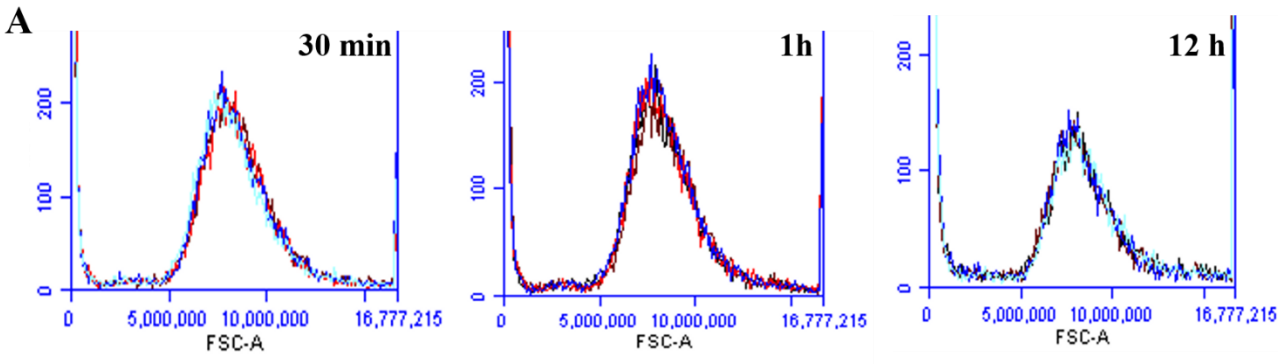
HeLa cells were taken out from the -80℃ refrigerator and thawed rapidly in a water bath at 37℃. The cell suspension was quickly added into a centrifuge tube containing 5 ml DMEM medium with a pipette and centrifuged at 1000 rpm for 5 min. the supernatant was discarded in a super clean table, and the cell precipitate was resuspended with fresh medium and transferred to a 100 mm culture dish. The cells were gently blown and evenly mixed. The cells were cultured in a constant temperature incubator for 24 h. The HeLa cells were cultured in 37℃ and 5% CO_2 incubator.

(2) Cell passage.

The cells were passaged when the cell density was 80%. Firstly, the cells were washed with PBS buffer, digested with appropriate amount of T, and then the pancreatin was discarded. Then, DMEM medium was added to gently blow until the cells were dispersed. Then, 10 μL cell suspension was added to the cell counting plate for counting. The cells were seeded into a 100 mm culture dish according to the cell density of 2×10^6 cells / ml. the cells were gently blown with a pipette to make them evenly distributed and cultured in the incubator.

(3) Detection of ROS Kit.

HeLa cells in logarithmic growth phase were seeded in 6-well plates at a density of 1×10^5 cells / well, and 2 ml DMEM medium was added to each well. Blank group and H_2O_2 group (50 μM, 100 μM, 200 μM) were set. After 24 h, discard the old medium, add different concentrations of H_2O_2 (50 μM, 100 μM, 200 μM) to stimulate for 30 min, remove the medium, replace the fresh medium, and continue to culture for varied time at 30 min, 1 h, and 12 h. According to the instructions of ROS kit, DCFH-DA was diluted in 1:1000 serum-free medium, and the final concentration was 10 μM. Remove the cell culture medium, add 1.5 ml diluted DCFH-DA, and incubate in 37℃ incubator for 20 min. Remove the medium from the 6-well plate, wash it with PBS buffer twice, add trypsin to digest the cells, and then stop the digestion with M. Then transfer the cells to 2 ml EP tube, filter with 300 mesh filter, and detect the fluorescence intensity by flow cytometry. The results are as follows:



Group 1: 0 μM; Group 2: 50 μM; Group 3: 100 μM; Group 4: 200 μM

Figure S19. Evaluation of the ROS Effect of on HeLa cells viability using varied concentration.

MIGRATION OF HELA CELLS.

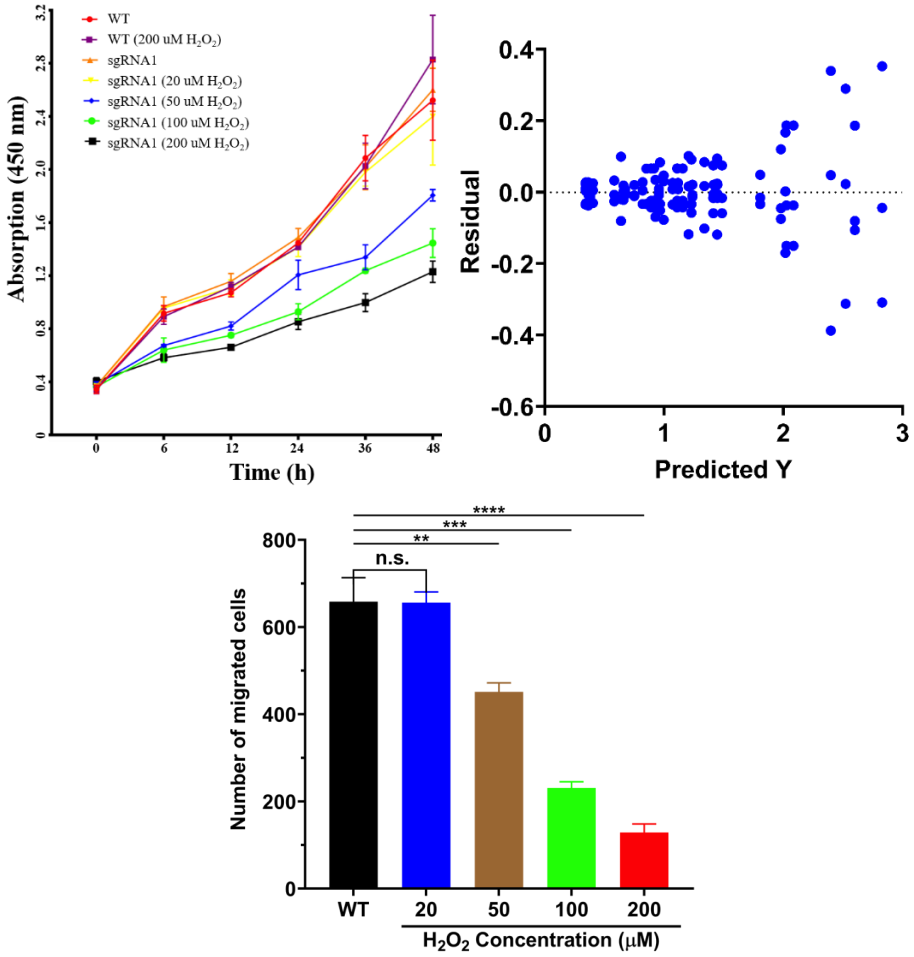


Figure S20. HeLa cells migration assays of H_2O_2-triggered rescuing of Cas9 variant with PBBK mutants targeting *Notch1* gene *in vivo.* Cell migration investigation under our PBBK-caged and H_2O_2-rescuing approaches. Statistics results of cell migration by our approaches using chemical chaperone PBBK-H_2O_2.

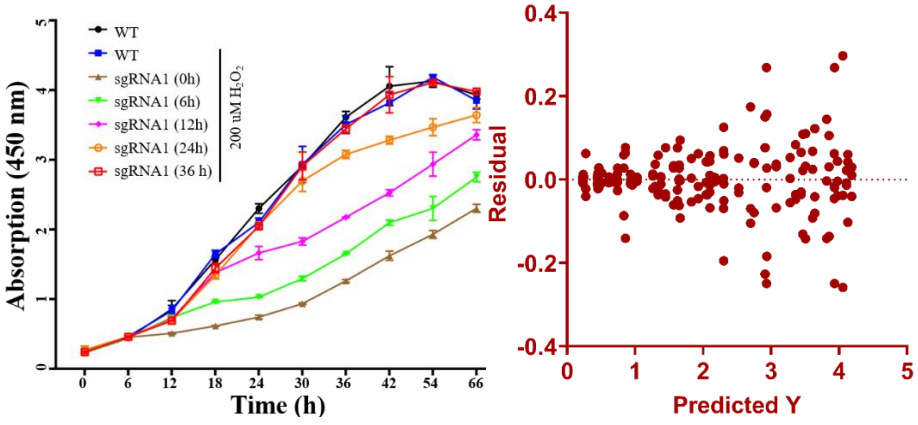


Figure S21. HeLa cells proliferation assays of H_2O_2-triggered rescuing of Cas9 variant with PBBK mutants targeting *Notch1* gene in *vivo.*

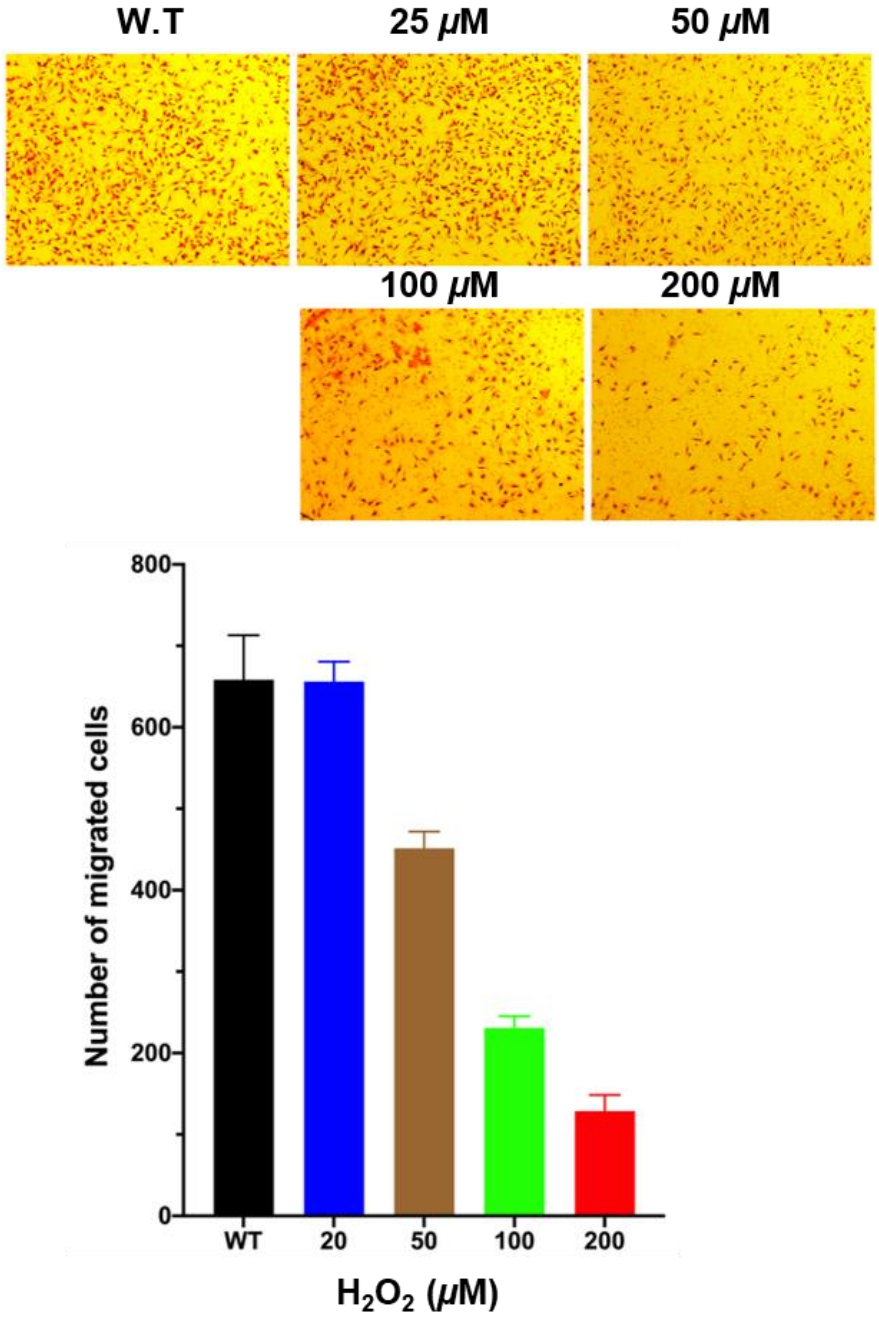


Figure S22. HeLa cells proliferation assays of H_2O_2-triggered rescuing of Cas9 variant with PBBK mutants targeting *Notch1* gene *in vivo* at different concentration.

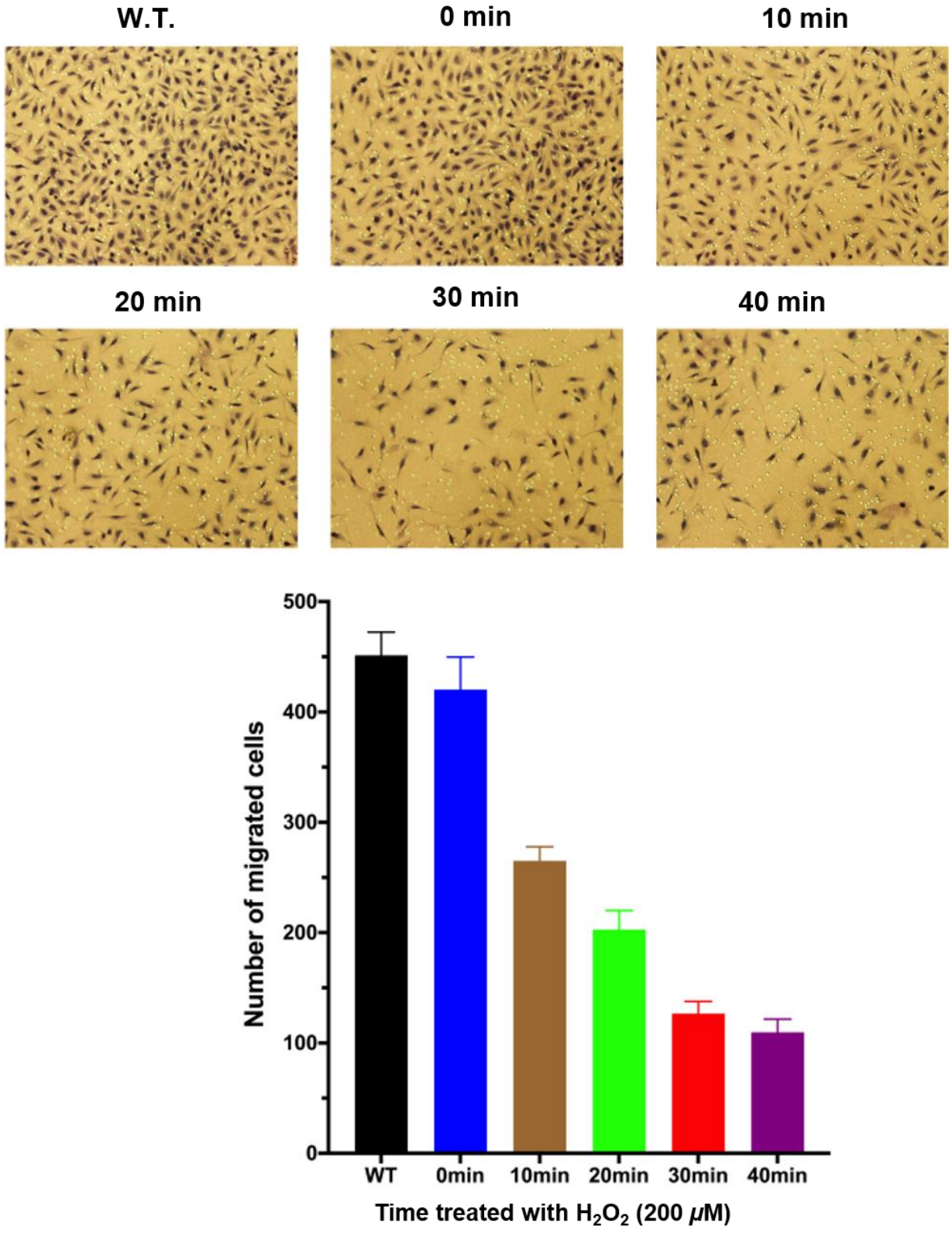


Figure S23. HeLa cells proliferation assays of H_2O_2-triggered rescuing of Cas9 variant with PBBK mutants targeting *Notch1* gene *in vivo* in time intervals.

DYNAMIC INVESTIGATION OF PBBK WITH VRIED *N*-OXIDE.

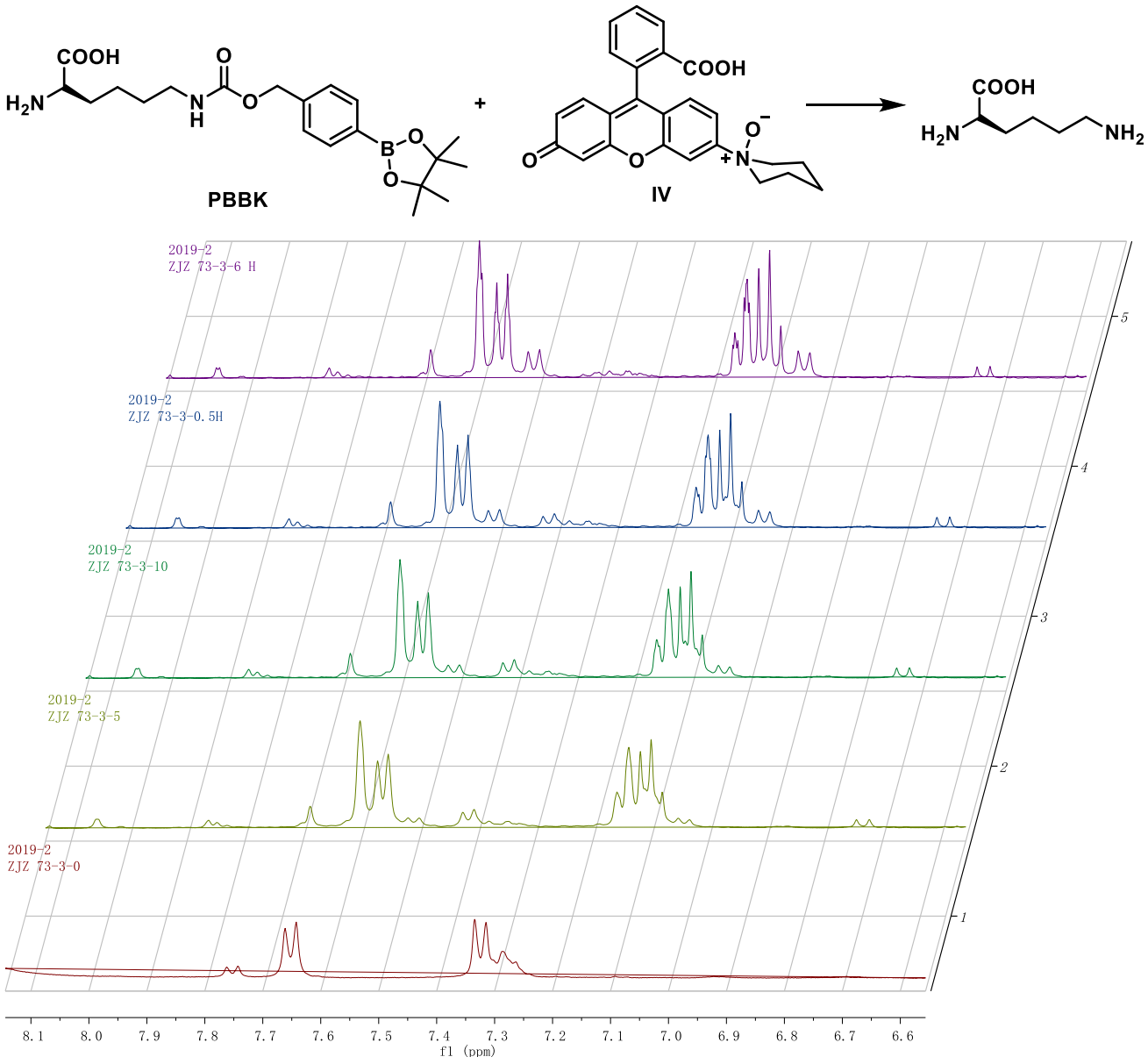


Figure S24. PBBK (2.0 μM) in DMSO-d^6 reacted with fluorescent *N-oxide* (**IV**, 10 μM) at r.t. at indicated time.

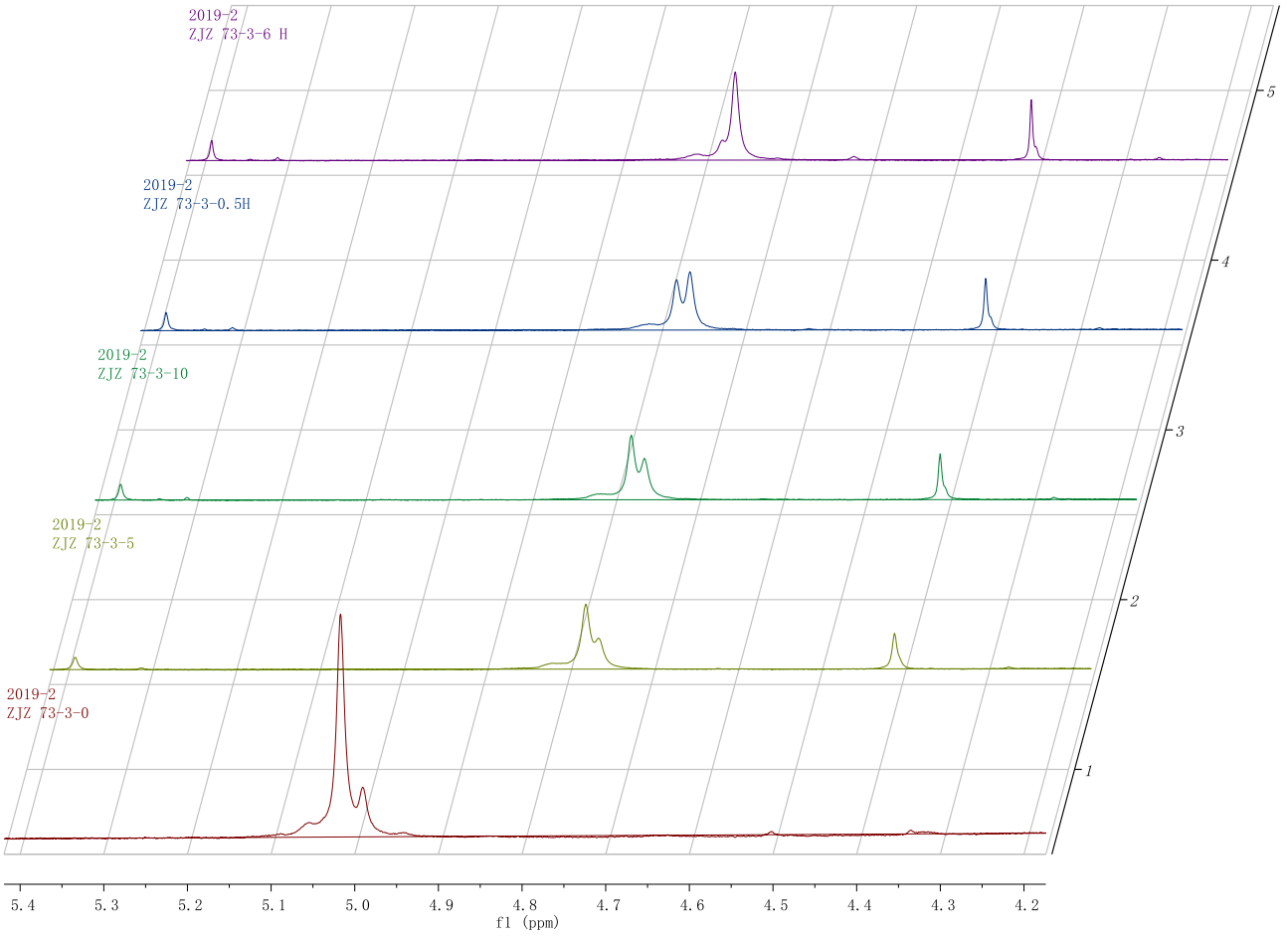


Figure S25. PBBK (2.0 μM) in DMSO-d^6 reacted with fluorescent *N*-oxide (**IV**, 10 μM) at r.t. at indicated time.

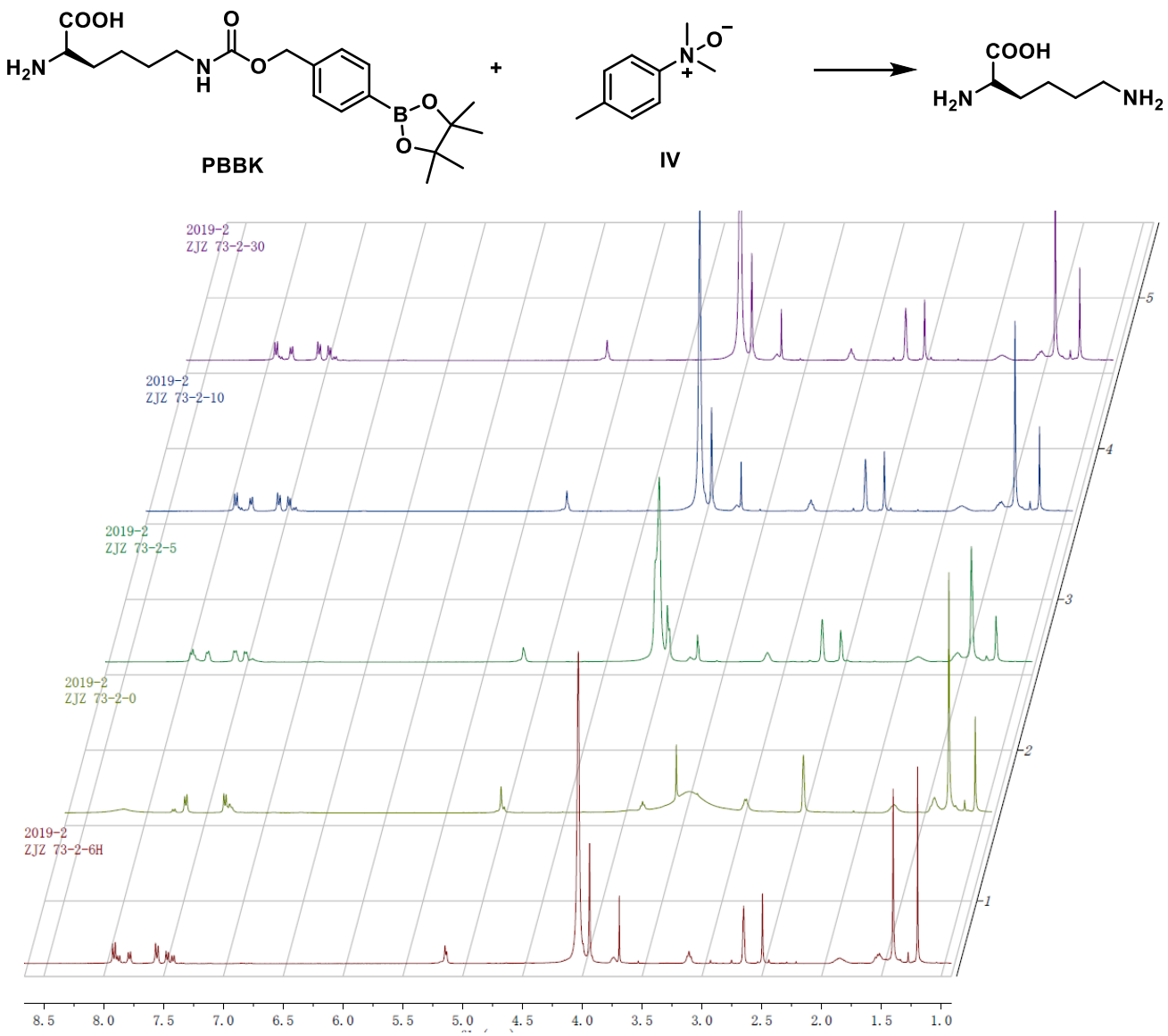


Figure S26. PBBK (2.0 μM) in DMSO-d^6 reacted with *N*, *N*-dimethyl-4-methylbenzene amino *N*-oxide (**III**, 10 μM) at r.t. at indicated time.

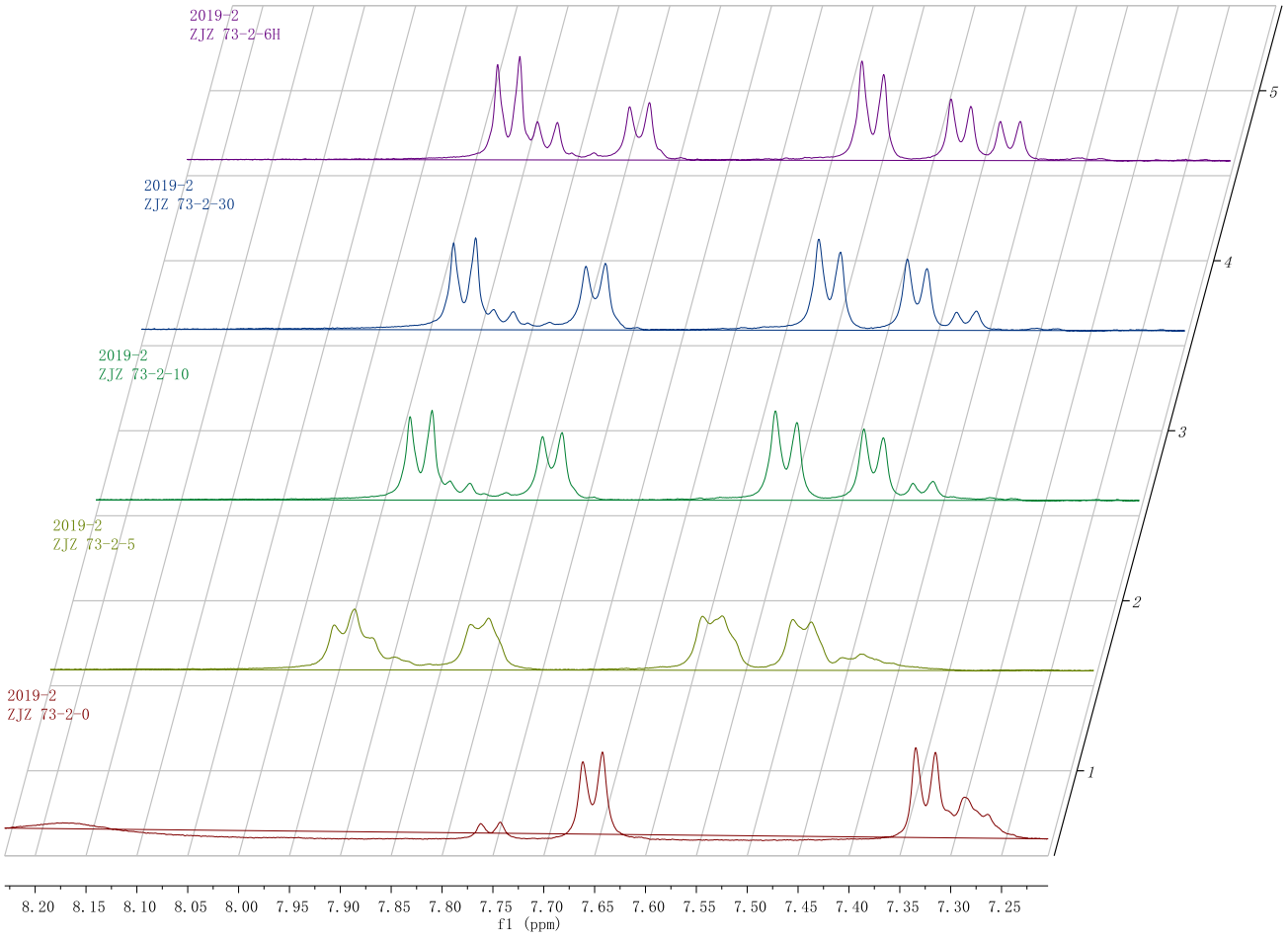


Figure S27. PBBK (2.0 μM) in DMSO-d^6 reacted with *N*, *N*-dimethyl-4-methylbenzene amino *N*-oxide (**III**, 10 μM) at r.t. at indicated time.

REFERENCES

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2. S.-R. Wang, L.-Y. Wu, H.-Y. Huang, W. Xiong, J. Liu, L. Wei, P. Yin, T. Tian, X. Zhou, *Nat. Commun.*, **2020**, *11*, 91.