Supplementary Information for

A Split CRISPR/Cas13b for Conditional RNA Regulation and Editing

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Materials and Methods

Plasmids Construction

Split pairs of wild type Cas13b used for screening were constructed by fusing the N-terminal fragments and C-terminal fragments with PYL-HA and Flag-ABI-P300 with the XTEN (SGSETPGTSESATPES) or (GGGGS)3 linkers. The N-dCas13b (H133A) fragment and Flag-ABI-C-dCas13b (H1058A) were amplified with dPspCas13b-longkerlinker-ADAR2DD (wt), a gift from Feng Zhang (Addgene, #103866) and fused with METTL3 domain with zinc finger RNA-binding motifs removed, which was amplified with dCas13-M3nls, a gift from David Liu (Addgene, #155366) via XTEN linker to get Flag-ABI-C-dCas13b-METTL3. All sgRNAs used were constructed via Golden gate assembly by ligation of the targeting sequence and the linearized backbone of PspCas13b sgRNA, a gift from Feng Zhang (Addgene, #103854) using T4 ligase according to the manufacturer's protocol (New England Biolabs, M0202S). The sequences of all plasmids were validated by sanger sequencing.

Mammalian cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle medium (Thermo Fisher Scientific, 11965118) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, A4766801) and 1% penicillin/streptomycin (Sigma-Aldrich, P4333). Cells were incubated at 37 ℃ with 5% CO2. HEK293T cells were seeded into the cell culture dishes or plates and incubated for about 16 h to reach a confluency of 80% for transfection. All the transfection experiments were performed with the Lipofectamine 2000 (Thermo Fisher Scientific, 11668019) according to the manufacturer's protocol at the ratio of 100 ng plasmid /0.2 μL lipofectamine 2000 reagents.

Expression of proteins

HEK293T cells were seeded into 12-well plate and transfected with corresponding split pairs of N-Cas13b-PYL-HA and Flag-ABI-C-Cas13b. After 24 h post-transfection, cells were harvested for whole protein extraction. Briefly, the cell culture media was removed and PBS was used to wash the cells twice. Then cells were lysed on ice with 100 μL RIPA buffer (Thermo Fisher Scientific, 89901) supplemented with protease inhibitor cocktail (Thermo Fisher Scientific, 78430).

Cell lysates were gently vortexed and sonicated 3 times within 30 min at 4 ℃, followed by centrifugation at 4 ℃ at 12,000 g for 20 min. The supernatants were reserved and protein concentrations were measured with Pierce 660 assay (Thermo Fisher Scientific, 22660) following the manufacturer's protocol. Then 20 μg proteins were loaded for gel electrophoresis with the current of 100 mA for 90 min and transferred onto the PVDF membrane with the current of 10 mA at 4 ℃ for 16 h. The membrane was blocked at room temperature (RT) for 1 h with 5% milk in TBST and washed 3 times with TBST, followed by incubation with anti-HA (Invitrogen, 26183), anti-Flag antibodies (Sigma, F1804) or anti-GAPDH (Cell Signaling Technology, 5174) at RT for 2 h and wash for 3 times with TBST followed by incubation with corresponding secondary antibody for 1 h and wash for 3 times. The membrane was imaged with ChemiDocTM MP Image System (Bio-Rad Laboratories). GAPDH was set as the reference control.

The re-assembly of split Cas13b pairs (Co-Immunoprecipitation)

HEK293T cells were seeded into 10 cm dish and transfected with 5 μg N-Cas13b-PYL-HA (761) and 5 μg Flag-ABI-C-Cas13b (761). After 24 h post-transfection, cells were treated with 100 μM ABA or DMSO, followed by another 24 h-incubation before 90 μL formaldehyde (Sigma, F8775) was added into the dish for crosslink for 10 min. Then 1 mL (0.2 g/mL) glycine (Fisher Bioreagents, BP381-1) was added to quench the crosslink. Then cells were collected by scrape and centrifugation for 5 min at 1000 g. Whole Proteins were then extracted and used for immunoprecipitation and a small portion was saved as input for western blotting. Briefly, 500 μg proteins were incubated with Anti-Flag@M2 Magnetic Beads (Sigma, M8823) or protein G magnetic beads (Thermo Fisher Scientific, 10004D) on rotation overnight at 4 ℃. 3X Flag peptide were used to elute the proteins according to the manufacturer's protocol (Sigma, F4799). Finally, 20 μg proteins before or after IP were used for western blotting assay to analyze the interactions of Flag and HA tagged proteins.

RNA cleavage induced by ABA treatment

HEK293T cells were seeded into 96-well plate and transfected with 100 ng Cas13b/dCas13b or 50 ng of N-Cas13b-PYL-HA, 50 ng Flag-ABI-C-Cas13b and 100 ng sgRNA targeting KRAS or non-targeting sgRNA (NT-sgRNA) when cells were at 80% confluency. After 24 h of transfection, cells were treated with 100 μM ABA or DMSO and incubated for another 24 h before harvest.

RNA was extracted using TRIzolTM reagent (Thermo Fisher Scientific, 15596026) according to the manufacturer's protocol. Then cDNA was synthesized with the iScriptTM cDNA Synthesis Kit (Biorad, 1708891) and used as template for the quantitative PCR reactions (qPCR). The mRNA level was analyzed via qPCR with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, A25778) according to the manufacturer's protocol. GAPDH was used as the internal control to normalize for sample quantity variations.

Reversibility of RNA cleavage with ABA removal

HEK293T cells were transfected with the corresponding plasmids and after 24 h incubation, cells were treated with 100 μM ABA and incubated for another 24 h followed by replacement with fresh DMEM media to remove ABA. After incubated with the ABA-free media for specific time, cells were harvested for RNA extraction and RT-PCR, qPCR were performed to analyze the mRNA level similarly to the procedures described above.

RNA cleavage induced by light using photo-caged ABA

HEK293T cells were transfected with split Cas13b-761 pairs and KRAS sgRNA for 24 h, followed by treatment with ABA, pre-cleaved ABA-DMNB and ABA-DMNB with or without light exposure (365 nm light for 2 min) and incubated for another 24 h. Then cells were harvested for RNA extraction and qPCR assays to quantify the KRAS mRNA level with similar procedures described above.

m^{6}A enrichment on ACTB mRNA by split dCas13b-M3 with ABA addition/removal.

HEK293T cells were seeded into 10 cm dishes and transfected with 6 μg N-dCas13b-PYL-HA, 6 μg Flag-ABI-C-dCas13b-METTL3 and 4 μg sgRNA targeting ACTB or SOX2 (dCas13b-PYL + sgRNA was used negative control for normalization). 100 μM ABA or DMSO was added into the cells at 24 h post-transfection. After another 24 h incubation, cells were harvested for RNA extraction or replaced with ABA-free DMEM media and incubated for specific time period before RNA extraction.

RNA was fragmented with RNA fragmentation buffer (10 mM Tris-HCl pH = 7.0, 10 mM ZnCl2) at 94 ℃ for 1 min and precipitated by incubation at -80 ℃ with one-tenth volumes of 3 M sodium acetate and 2.5 volumes of 100% ethanol. Fragmented RNA was collected by centrifugation at 4 ℃

for 25 min at 21,000 g and washed with 75% ethanol followed by air-dry and resuspended in 200 μL nuclease-free water. 120 μg RNA were incubated with 5 μg m6A antibody (Cell Signal and Technology, 56593) in 80 μL of 5x IP buffer (0.5 mL 1 M Tris-HCl (pH 7.4), 1.5 mL 5 M NaCl, 0.5 mL 10% of Igeal CA-630, and 7.5 mL of Nuclease-free water) supplemented with 4 μL of RNasin Plus RNase Inhibitor (Promega, N2611), 4 μL of RVC (Sigma, R3380) and 312 μL nuclease free water with protocols reported previously. After 2 h incubation on rotation at 4 ℃, 40 μL of protein G magnetic beads (Thermo Fisher Scientific, 10004D) washed with 1x IP buffer twice were added into the mixture and incubated overnight at 4 ℃ with head-tail rotation. The beads were then washed 3 times with 1XIP buffer. Next, RNA was eluted twice with 100 μL elution buffer (20 μL 5x IP buffer, 33.33 μL 20 mM m6A salt, 1.56 μL RNasin Plus, and 45.11 μL nuclease-free water) with rotation at 4 ℃ for 1 h and purified with RNA Clean & ConcentratorTM-5 kits (Zymo Research, R1014). The purified RNA before or after m6A IP was reverse transcribed into cDNA and qPCR assay was performed to analyze the m6A enrichment.

Assembly/disassembly of split dCas13b-M3 on ACTB mRNA with ABA addition/removal

HEK293T cells were seeded into 10 cm dishes and transfected with 6 μg N-dCas13b-PYL-HA, 6 μg Flag-ABI-C-dCas13b-METTL3 and 4 μg sgRNA targeting ACTB (dCas13b-PYL + sgRNA was used negative control for normalization). 100 μM ABA was added into the cells at 24 h post-transfection. After another 24 h incubation, 280 μL 37% formaldehyde was added into the media and incubated for 10 min. Then the fixed cells were added with 1 ml (1 g/5 mL) 125 mM glycine and incubated for 5 min to quench the crosslink, followed by scrape and centrifugation at 4 ℃ for 5 min at 1000 g to collect the cell pellets. 600 μL RIPA buffer supplemented with PIC and RNase inhibitor were used to lyse the cells. Then the cells were incubated on ice for 10 min and the sonicated for 2 min with a 30 s on/off cycle on a Bioruptor sonicator (Diagenode), followed by centrifugation at 16,000 g for 10 min at 4 ℃. The clear supernatant containing lysate was then used for RNA-protein immunoprecipitation. 20 uL lysate was saved as input sample.

For RNA-protein immunoprecipitation, 35 μL of Dynabeads Protein A were washed with 200 μL of wash buffer (PBS with 0.02% Tween 20 (Sigma)). Then, 5 μg of anti-Flag antibody (Sigma, F1804) or anti-HA antibody (Invitrogen, 26183) and 200 μL wash buffer was added into the beads and incubated on a rotator at RT for 2 h. After that, beads were carefully washed twice with wash buffer and resuspended in 250 μL of RIPA buffer supplemented with PIC and RNase inhibitor.

Then, 250 μL lysate was added into the beads mixture and incubated at 4 ℃ overnight with rotation. After incubation, beads were washed with wash buffer twice and proteins were digested by Proteinase K (New England BioLabs) and incubated at 55 ℃ for 3 h. Urea was then added for purpose of denaturation. RNA was purified with RNA Clean & Concentrator-5 kits (Zymo Research). Purified RNA was reverse transcribed and quantified with qPCR described above.

Destabilization of ACTB mRNA mediated by the m^{6}A writing

HEK293T cells were seeded in 24-well plate and transfected with 150 ng N-dCas13b-PYL-HA, 150 ng Flag-ABI-C-dCas13b-METTL3 and 100 ng sgRNA targeting ACTB (dCas13b-PYL+sgRNA was used as negative control for normalization), followed by adding 100 μM ABA for 24 h and then incubated with/without removal of ABA for another 24 h before cells were treated with 5 μg/mL actinomycin D for 6 h. After wash with PBS, total RNA was isolated by TRIzol^{TM} reagent (Thermo Fisher Scientific, #15596026) and purified according to the manufacturer's protocol. After reverse transcription, mRNA levels of target transcripts were analyzed by qPCR. GAPDH was used as the internal control to normalize for sample quantity variations.

Light-inducible m^{6}A writing

Cells were seeded in 10 cm dishes and incubated for about 16 h to reach 80% confluency before transfection with different components of ABA-induced m6A modification system for 24 h (dCas13b-PYL+gRNA, split dCas13b-M3+gRNA) using the lipofectamine 2000 according to the manufactures' protocol. After 24h, cells were treated with 20 μM ABA-DMNB with or without exposure to the 405 nm light for 2 min and incubated for another 24 h before RNA extraction, then m6A-IP and the RT-qPCR were performed to quantify the m6A level on A1216 of ACTB mRNA as described above.

Statistical analyses

Information regarding error bars, numbers of replicates or samples and statistical analyses are described in the corresponding figure legends. Representative results of at least three independent experiments are shown unless otherwise indicated.

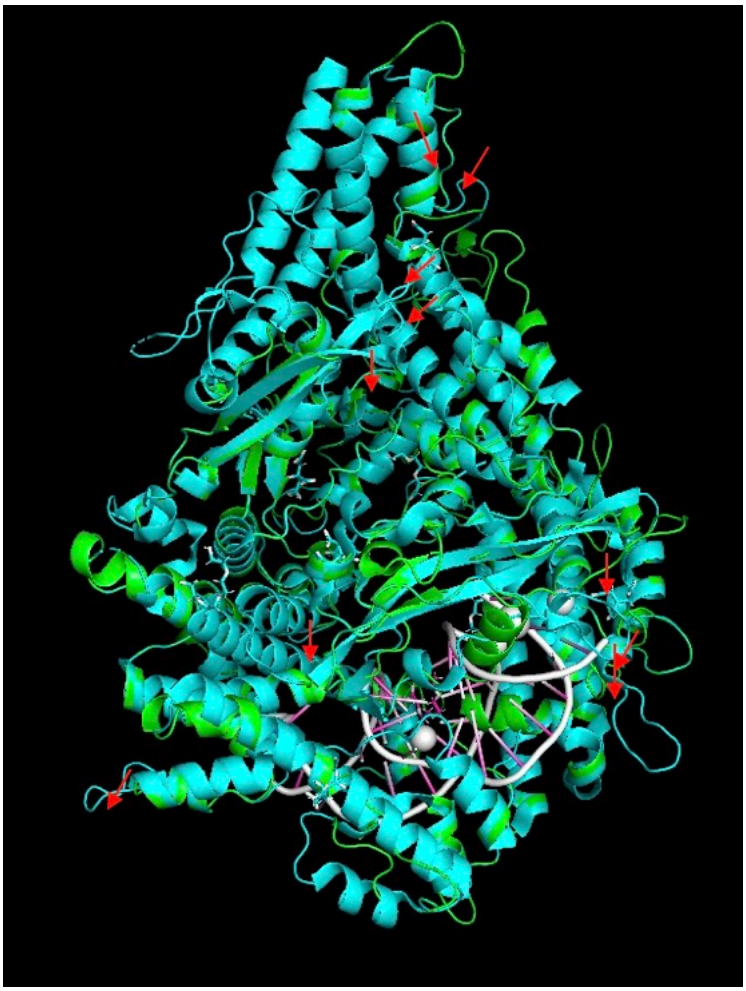

Fig. S1.

Predicted PspCas13b (cyan) structure and its alignment to the reported structure of PbuCas13b (green). The prediction and alignment of structures of these two Cas13b were obtained by the Phyre2 web portal based on the comparison of their amino acid sequences.

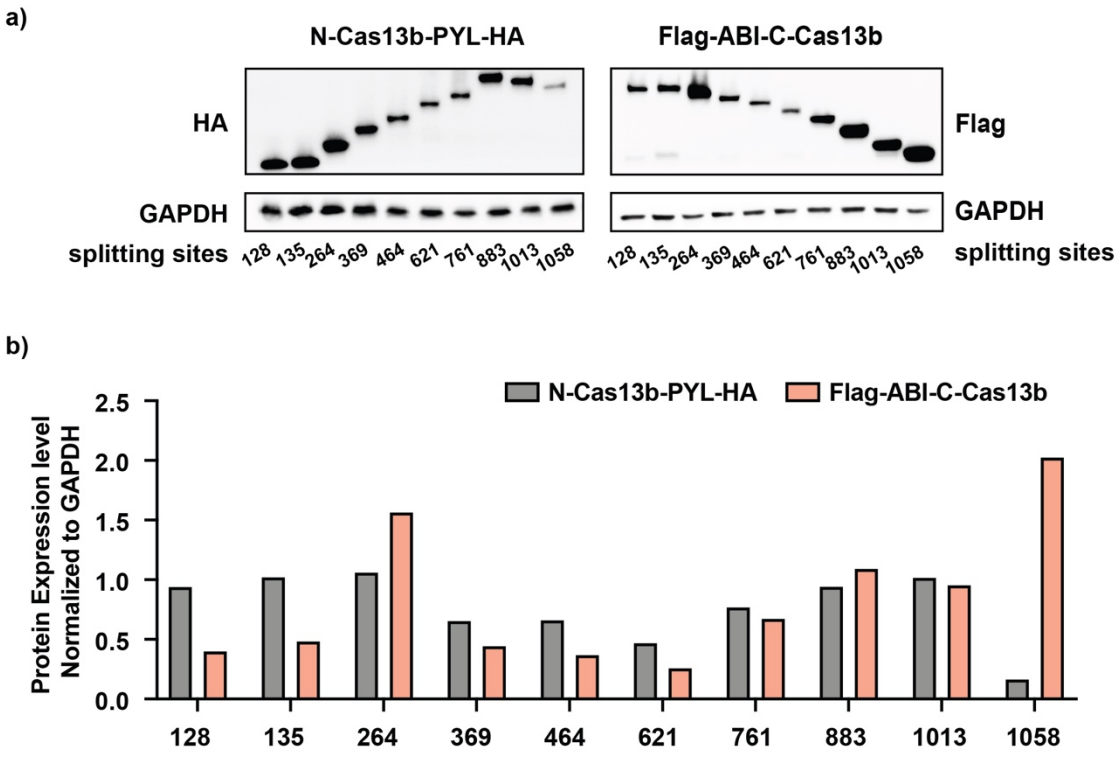

Fig. S2.

Fusion protein expressions of the 10 split Cas13b pairs. (a) The cellular expression of the N-Cas13b-HA and Flag-ABI-C-Cas13b proteins were analyzed by western blotting. The N-terminal and C-terminal fragments of Cas13b were probed with the corresponding anti-HA and anti-Flag antibodies. GAPDH was used as the loading control. (b) Quantification of western blotting data from (a) and the relative levels were obtained by normalizing the expression of split pairs to that of GAPDH. Shown in (a) and (b) were the representative results from 3 independent experiments.

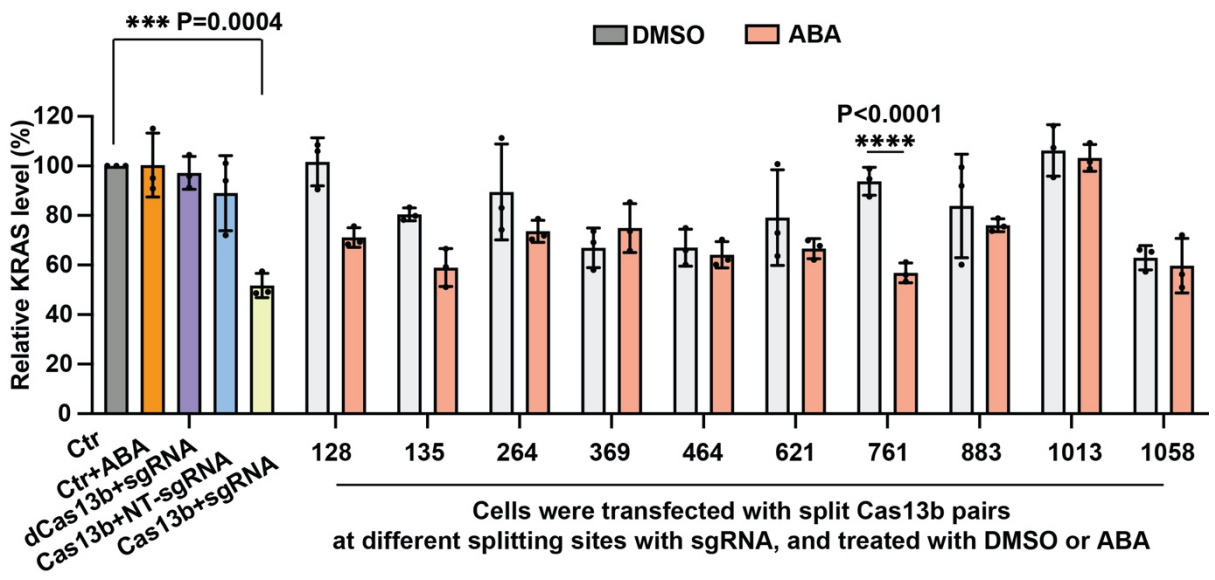

Fig. S3.

The screening of split Cas13b pairs via KRAS cleavage. HEK293T cells were transfected with different pairs of split Cas13b and the sgRNA targeting KRAS followed by incubation with 100 μM of ABA or DMSO for another 24 h. The KRAS mRNA levels were measured via RT-qPCR. GAPDH was used as the internal control to normalize for sample quantity variations. The KRAS level percentage changes were calculated by comparing to the non-transfected and non-treated cells (Ctr). Values and error bars represent the mean and s.e.m. of 3 independent biological experiments. P values were determined by one-way ANOVA.

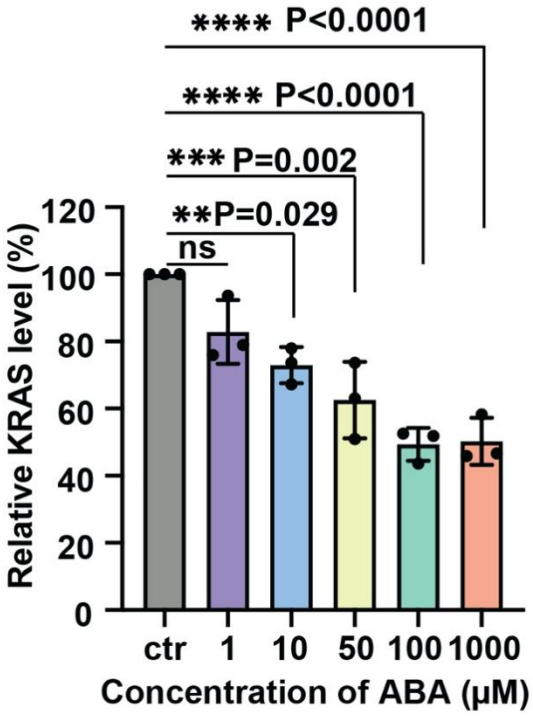

Fig. S4.

The ABA dosage dependent KRAS cleavage by split Cas13b-761. Cells were transfected with split Cas13b-761 and the KRAS sgRNA followed by ABA treatment at different concentrations. The KRAS mRNA levels were quantified via qPCR. GAPDH was used as the reference control to normalize for sample quantity variations. The KRAS level percentage changes were calculated by comparing to the non-transfected and non-treated cells (Ctr). Values and error bars represent the mean and s.e.m. of 3 independent biological experiments. P values were determined by one-way ANOVA.

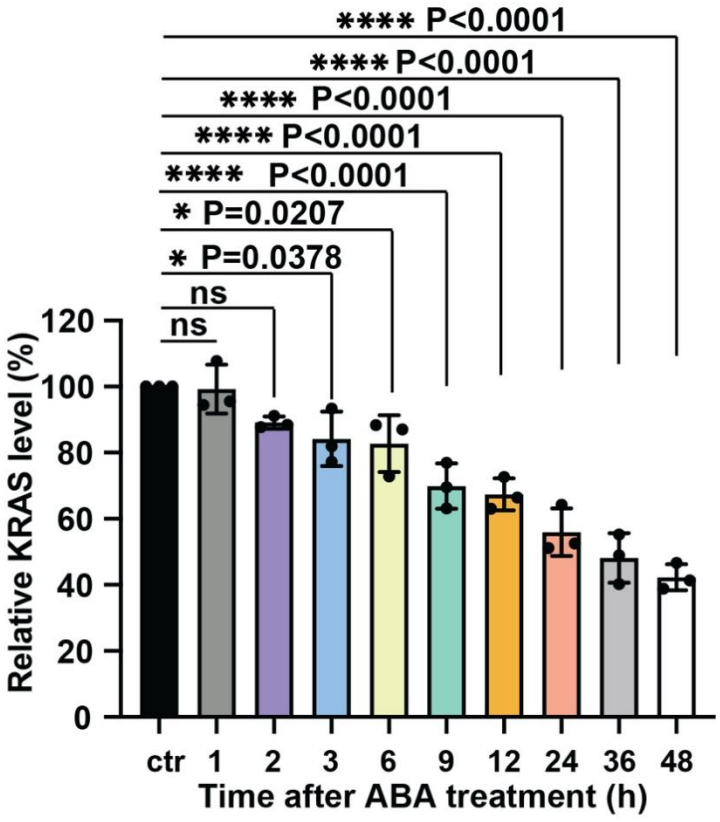

Fig. S5.

The time-course of ABA-induced KRAS cleavage by split Cas13b-761. Cells were transfected with split Cas13b-761 and the KRAS sgRNA followed by ABA incubation for specific time periods. The KRAS mRNA levels were quantified via RT-qPCR. GAPDH was used as the reference control to normalize for sample quantity variations. The KRAS level percentage changes were calculated by comparing to the non-transfected and non-treated cells (Ctr). Values and error bars represent the mean and s.e.m. of 3 independent biological experiments. P values were determined by one-way ANOVA.

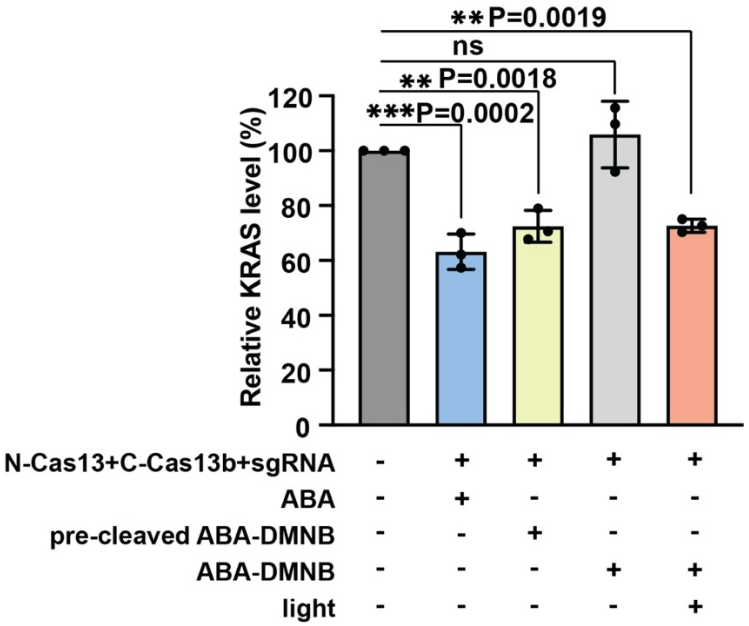

Fig. S6.

Light controlled activation of the split Cas13b system. Cells were transfected with split Cas13b-761 and the KRAS sgRNA followed by treatment of ABA, pre-cleaved ABA-DMNB or ABA-DMNB with or without light exposure. After 24 h, the KRAS mRNA levels were quantified via RT-qPCR. GAPDH was used as the reference control to normalize for sample quantity variations. The KRAS level percentage changes were calculated by comparing to the non-transfected and non-treated cells. Values and error bars represent the mean and s.e.m. of 3 independent biological experiments. P values were determined by one-way ANOVA.

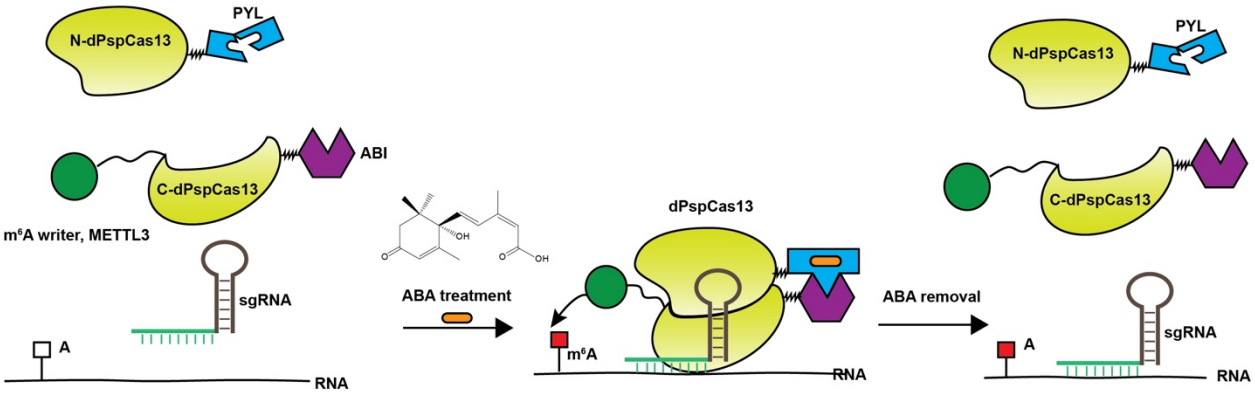

Fig. S7.

Scheme of the chemically inducible split PspCas13b-based m^{6}A writing platform. The N- and C- terminal of dCas13b were fused to PYL and ABI separately, and the METTL3 was fused with C-terminal of dCas13b. The addition of ABA initiates the assembly of functional dCas13b fusion protein and the subsequent m^{6}A writing at the sgRNA-targeted RNA locus, which can both be reversed after ABA removal.

Fig. S8.

Expression of the split dCas13b-M3 fusion proteins. The N-terminal and C-terminal fragments of Cas13b were probed with the corresponding anti-HA or anti-Flag antibody. GAPDH was used as the loading control. Shown were the representative images from 3 independent experiments.

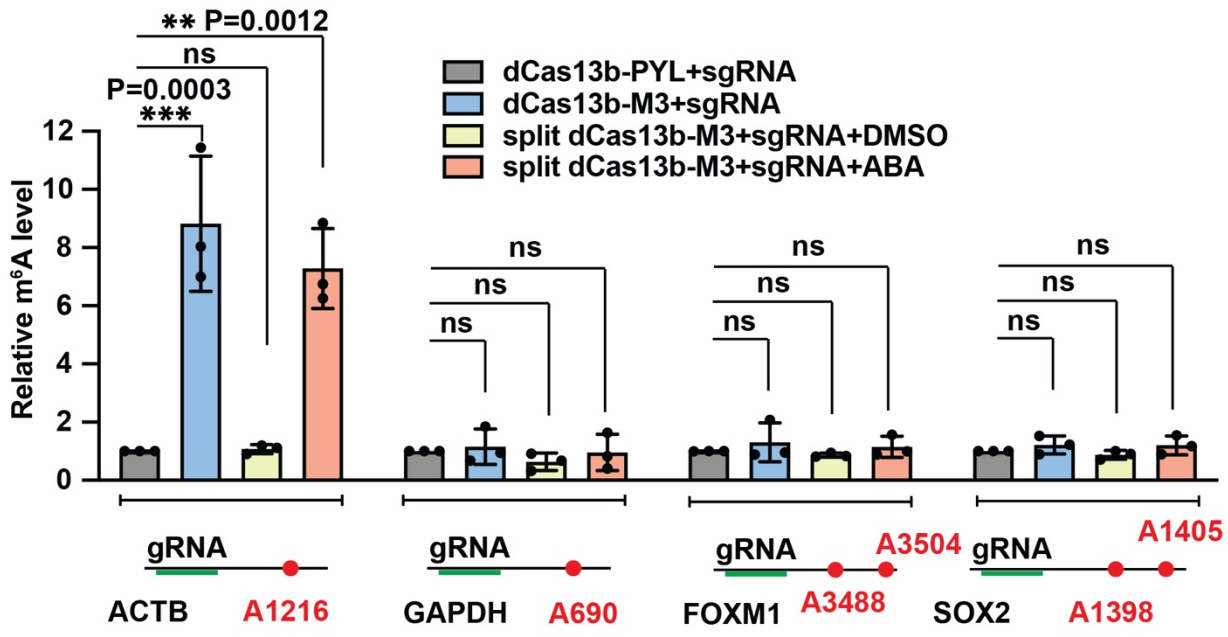

Fig. S9.

Relative m^{6}A levels at different loci on ACTB and other non-targeted RNAs. The relative m^{6}A levels were determined by m^{6}AIP-qPCR assays and by normalizing to the result from the condition of dCas13b-PYL plus sgRNA. Values and error bars represent the mean and s.e.m. of 3 independent biological experiments. P values were determined by one-way ANOVA.

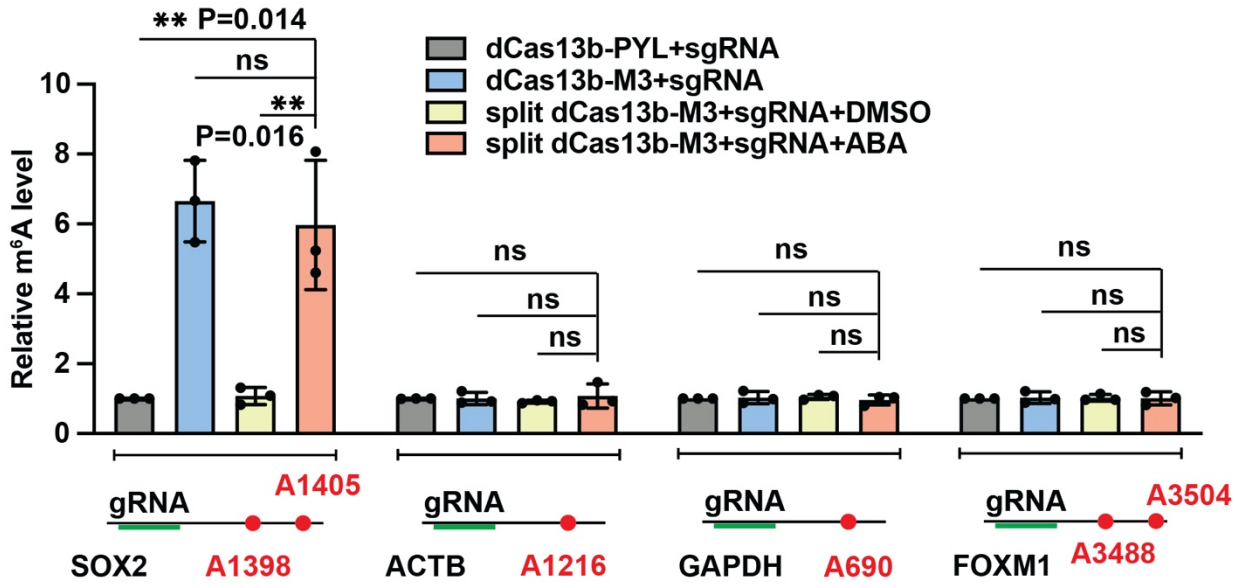

Fig. S10.

Relative m^{6}A levels at different loci on SOX2 and other non-targeted RNAs. The relative m^{6}A levels were determined by m^{6}AIP-qPCR assays and by normalizing to the result from the condition of dCas13b-PYL plus sgRNA. Values and error bars represent the mean and s.e.m. of 3 independent biological experiments. P values were determined by one-way ANOVA.

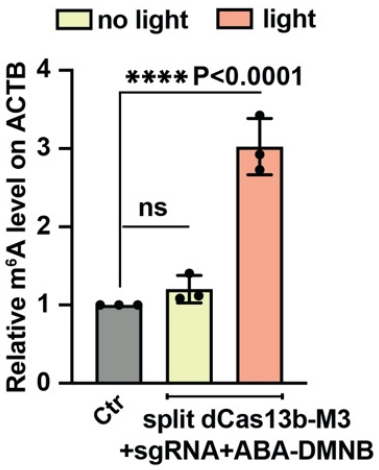

Fig. S11.

Light induced m^{6}A writing on ACTB mRNA A1216 site by the split dCas13b-M3 platform. The m^{6}A levels on ACTB written by the split dCas13b-M3 with the addition of 20 μM photo-caged ABA (ABA-DMNB) with or without light exposure. The relative m^{6}A levels were calculated by normalizing to the result from the condition of dCas13b-PYL plus sgRNA (Ctr). Values and error bars represent the mean and s.e.m. of 3 independent biological experiments. P values were determined by one-way ANOVA.

sgRNA	Spacer sequences (5'-3')
KRAS	AATAATACTAAATCATTTGAAGATATTCAC
ACTB sgRNA for A1216 targeting	TCCATCGTCCACCGCAAATGCTTCTAGGCG
SOX2 sgRNA	AACGGCACACTGCCCCTCTCACACATGTGA

Table S1. Spacer sequences of sgRNAs used in this work. All oligo sequences were obtained from Thermo Fisher Scientific.

Primers	Oligo sequences (5'-3')
Gapdh forward	GGTGTGAACCATGAGAAGTATGA
Gapdh Reverse	GAGTCCTTCCACGATACCAAAG
KRAS forward	TCTTGGATATTCTCGACACAGC
KRAS reverse	CCATAGGTACATCTTCAGAGTCC
ACTB forward	TCCCAAGTCCACACAGG
ACTB reverse	CACGAAGGCTCATCATTCAAA
Actb-m^{6}A-forward	AGATGTGGATCAGCAAGC
Actb-m^{6}A-reverse	TCATCTTGTTTTCTGCGC
Gapdh-m^{6}A-forward	CATCACTGCCACCCAGAAGA
Gapdh-m^{6}A-reverse	CAGTAGAGGCAGGGATGATGTT
Foxm1-m^{6}A-forward	TGCCCAGATGTGCGCTATTA
Foxm1-m^{6}A-reverse	CTTCTCAAGCCTCCACCTGA
Sox2-m^{6}A-forward	GGCCATTAACGGCACACTG
Sox2-m^{6}A-reverse	TCTTTTGCACCCCTCCCATT

Table S2. Primers for RT-qPCR used in this work. All oligos were obtained from the Thermo Fisher Scientific.

Antibody	Vendor	Cat. No	Dilution for WB
anti-m6A (D9D9W)	Cell Signal and Technology	56593	N/A
HA Tag Monoclonal Antibody (2-2.2.14)	Invitrogen	26183	1:1000
Flag Tag Monoclonal Antibody (M2)	Sigma	F1804	1:1000
Anti-Gapdh Antibody GAPDH (D16H11) XP® Rabbit mAb	Cell Signal and Technology	5174	1:2000
anti-rabbit IgG HRP-linked antibody	Cell Signaling Technology	7074	1:2500
anti-mouse IgG HRP-linked antibody	Cell Signaling Technology	7076	1:2500

Table S3. Information regarding the antibodies used in this work