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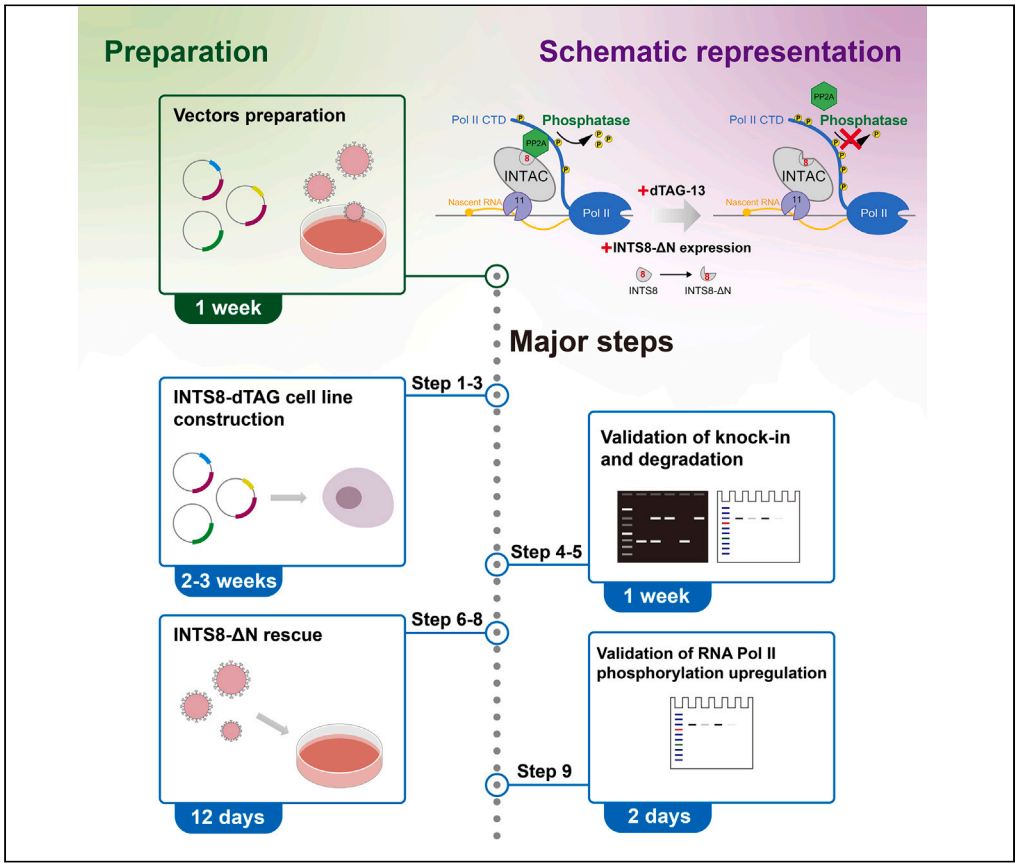
Protocol for rapidly inducing genome-wide RNA Pol II hyperphosphorylation by selectively disrupting INTAC phosphatase activity



While several inhibitors targeting RNA polymerase II (Pol II) kinases have been applied for inhibiting RNA Pol II phosphorylation, there are few approaches for inducing RNA Pol II hyperphosphorylation. Here, we present a protocol for constructing the INTS8 degradation tag (dTAG) system combined with ectopic expression of N-terminally truncated INTS8 (INTS8-ΔN) in DLD-1 cells. We describe steps for INTS8-dTAG cell line construction, validation of knockin and degradation, and INTS8-ΔN rescue. We then detail validation of RNA Pol II phosphorylation upregulation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A platform for rapidly and specifically inducing RNA Pol II hyperphosphorylation

Create INTS8-dTAG+ΔN cell line to target INTAC phosphatase without affecting PP2A

Applicable to studying the direct functions of Pol II phosphorylation in editable cell lines

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Protocol for rapidly inducing genome-wide RNA Pol II hyperphosphorylation by selectively disrupting INTAC phosphatase activity

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SUMMARY

While several inhibitors targeting RNA polymerase II (Pol II) kinases have been applied for inhibiting RNA Pol II phosphorylation, there are few approaches for inducing RNA Pol II hyperphosphorylation. Here, we present a protocol for constructing the INTS8 degradation tag (dTAG) system combined with ectopic expression of N-terminally truncated INTS8 (INTS8-ΔN) in DLD-1 cells. We describe steps for INTS8-dTAG cell line construction, validation of knockin and degradation, and INTS8-ΔN rescue. We then detail validation of RNA Pol II phosphorylation upregulation.

For complete details on the use and execution of this protocol, please refer to Hu et al. (2023).[1]

BEFORE YOU BEGIN

The prospect for understanding the direct functions of Pol II phosphorylation in transcriptional regulation necessitates methodologies that rapidly, specifically, and efficiently up- and downregulate genome-wide Pol II phosphorylation levels. Rapid and specific decreases in Pol II phosphorylation levels can be achieved by selective inhibitors targeting the catalytic activities of kinases including CDK7,[2] CDK9,[3,4] and CDK12/13.[5] However, different from Pol II kinases that are often assembled into a small number of complexes primarily functioning in transcription, Pol II phosphatases, such as PP2A and PP1, bind with varying adapters to assemble dozens of holoenzymes that have functions far beyond direct transcriptional control.[6,7] Accordingly, inhibitors targeting the catalytic activities of PP2A and PP1 will introduce a broad spectrum of pleiotropic effects, which would inevitably squelch the transcriptional effects resulting from Pol II hyperphosphorylation. We and others have identified Integrator-PP2A (INTAC) complex as a major RNA Pol II phosphatase and that PP2A is recruited by Integrator subunit 8 (INTS8) to direct its dephosphorylation of RNA Pol II.[8–10] Therefore, to induce RNA Pol II hyperphosphorylation rapidly and specifically, we provide a platform through inducing rapid degradation of INTS8 using the degradation TAG (dTAG) system[11] combined with the expression of an N-terminally truncated mutant INTS8-ΔN lacking PP2A interaction. This strategy results in the dislodgment of PP2A from INTAC without impairing the integrity of Integrator and thus its RNA endonuclease activity. This protocol describes details of INTS8-dTAG+ΔN cell line construction in colorectal adenocarcinoma cell line DLD-1, which can also be applied in other cell lines.

Preparation: Plasmid preparation for generating INTS8-dTAG cell line

Timing: 5 days

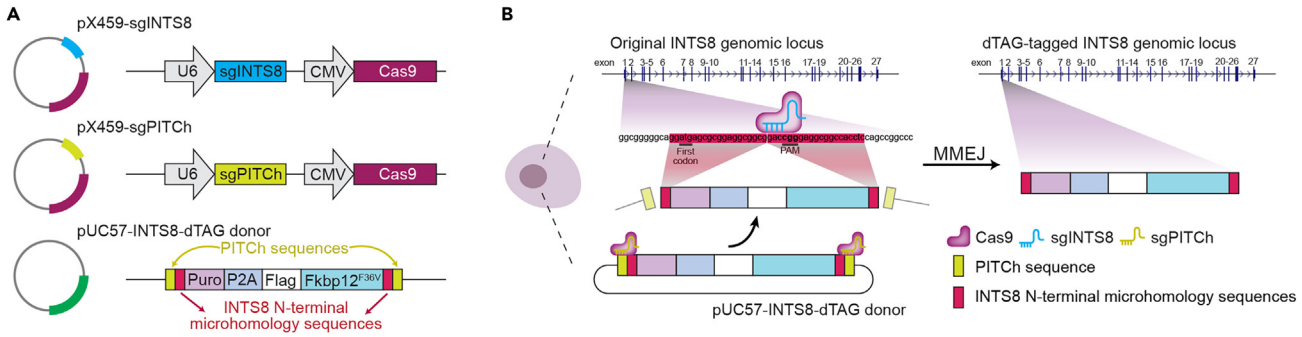

Figure 1. Schematic representation of the gene editing strategy to tag endogenous INTS8 at the N-terminal locus with a puromycin resistance gene and FKBP12^{F36V}

(A) The construction of pX459-sgINTS8, pX459-sgPITCh, and pUC57-INTS8-dTAG donor for generating INTS8-dTAG cell line.

(B) Schematic of MMEJ-mediated knockin using the three-plasmid PITCh system for generating INTS8-dTAG cell lines.

The dTAG system offers an efficient and cost-effective approach for endogenous knockin of the dTAG cassette using the microhomology-mediated end joining (MMEJ)-based Precise Integration into Target Chromosome (PITCh) strategy.[12] This step describes the preparation of plasmids used in this three-plasmid PITCh system (Figure 1A) for the knockin of puromycin resistance gene (Puro), cleaving peptide (P2A), 3× Flag tag and Fkbp12^{F36V} tag [Puro-P2A-Flag-Fkbp12^{F36V}] encoding DNA sequences into the INTS8 N-terminal genomic locus (Figure 1B). The three-plasmid system comprises (1) a donor vector (pUC57-INTS8-dTAG donor) containing the knockin sequence, (2) the pX459-sgINTS8 plasmid with a gene-specific sgRNA targeting the INTS8 N-terminal genomic locus, and (3) the pX459-sgPITCh plasmid with the PITCh-specific sgRNA targeting the pPUC57-INTS8-dTAG construct. By transfecting this set of plasmids into a specific cell line, such as DLD-1, endogenous INTS8 protein will be tagged with FKBP12^{F36V}, enabling rapid and selective depletion of the endogenous INTS8 protein upon the application of dTAG-13 ligand.

1. Preparation of pX459-sgPITCh and pX459-sgINTS8 plasmids through restriction enzyme cloning.
   a. Linearize the pX459 vector.
      i. Set up digestion reaction (Table 1).
      ii. Incubate the reaction at 37°C for 3 h.

   ⏸ **Pause point:** The vector can be digested at 37°C overnight (12–16 h).

      iii. Run the digestion product on a 1% (w/v) agarose gel and cut out the band at the size of 9153 bp.
      iv. Purify the digested plasmid using AxyPrep DNA Gel Extraction Kit (following the manufacturer's instructions) and elute in 20 μL ddH_2O.
      v. Determinate the concentration of linearized pX459 vector with Nanodrop 2000c.

   ⏸ **Pause point:** The purified linearized pX459 vector can be stored at −20°C for up to several months.

   b. Anneal oligonucleotides of PITCh-sgRNA and INTS8-sgRNA.

Table 1. Digestion reaction

Reagent	Amount
pX459	~3 μg
10X rCutsmart buffer	2 μL
BbsI-HF	1 μL
ddH_2O	to 20 μL

Table 2. Oligo annealing

Reagent	Amount
PITCh (INTS8)-sgRNA-F (10 μM)	10 μL
PITCh (INTS8)-sgRNA-R (10 μM)	10 μL

i. Set up oligo annealing (Table 2).

Note: All oligonucleotide sequences in this protocol are provided in the key resources table.

⚠ CRITICAL: The sgINTS8 and INTS8 N-terminal microhomology sequences were designed for the human cell line DLD-1. For application in cell lines from other species, these sequences should be redesigned.

ii. Put the two mixtures into a thermocycler and anneal according to the following conditions (Table 3).

Pause point: The annealed heteroduplexes can be stored at −20°C for up to several months.

iii. Dilute the annealed heteroduplex 1:100 in ddH_2O.

c. Ligation.
   i. Set up ligation reaction (Table 4).
   ii. Incubate the three reactions in a thermocycler at 25°C for 5 min.
d. Transform 4 μL of the ligation product into 50 μL DH5α competent cells.
e. Gently spread transformed cells on pre-warmed LB-Amp-plates (LB-agar plates containing 100 μg/mL ampicillin) and incubate at 37°C overnight (12–16 h).
f. Pick colonies from plates and grow each colony in 300 μL LB-Amp-Broth (LB broth containing 100 μg/mL ampicillin). (troubleshooting 1).

Note: We usually pick 3 colonies from each of the sgPITCh and sgINTS8 plates for amplification and Sanger sequencing.

g. In our case, 200 μL liquid cultures of each clone were shipped to Boshang Biotechnology (Shanghai) Co., Ltd. to perform Sanger DNA sequencing with a U6 primer.

Alternatives: If the Sanger sequencing results take a long time to be returned, the plasmid purification step can be done prior to the Sanger sequencing of plasmids.

h. Amplify the 10 μL from the remaining liquid culture of each clone in 10 mL LB-Amp-Broth and purify the successfully constructed plasmids with EndoFree Mini Plasmid Kit II according to the sequencing result.
i. Determinate the concentration of pX459-sgPITCh or pX459-sgINTS8 with Nanodrop 2000c.
j. Store the plasmids at –20°C until use.

Pause point: The plasmid DNA can be stored at −20°C for long-term storage.

2. pUC57-INTS8-dTAG donor plasmid preparation

Table 3. Annealing program

Steps	Temperature	Time	Cycles
Denaturation	95°C	5 min	1
Annealing	–0.1°C/cycles	6 s	700
Hold	25°C	Forever	-

Table 4. Ligation reaction

Reagent	sgPITCh or sgINTS8	Control
Linearized pX459 from preparation step 1.a.iv	~20 ng	~20 ng
Annealed heteroduplex from preparation step 1.b.iii	0.5 μL	-
TaKaRa Ligation Mix	2 μL	2 μL
ddH_2O	to 4 μL	to 4 μL

a. Synthesize the sequence of INTS8-dTAG donor with flanking PITCh sequence (provided in the key resources table) and clone into a vector (e.g., pUC57) by ordering from Boshang Biotechnology (Shanghai) Co., Ltd.
b. Amplify the plasmid in 10 mL LB-Amp-Broth.
c. Purify the plasmid with EndoFree Mini Plasmid Kit II (following the manufacturer's instructions).
d. Determinate the concentration of pUC57-INTS8-dTAG donor plasmid with Nanodrop 2000c.
e. Store the plasmid at –20°C until use.

Pause point: The plasmid DNA can be stored at −20°C for long-term storage.

Preparation: Lentivirus preparation for generating INTS8-dTAG+ΔN cell line

Timing: 1 week

This step describes the lentivirus packaging for INTS8-ΔN overexpression in INTS8-dTAG cells.

Alternatives: Other laboratory-available vectors can also serve as an alternative solution for INTS8-ΔN overexpression.

3. Plasmids preparation for Lentivirus packaging
   a. Linearize the pLVX vector.
      i. Set up digestion reaction (Table 5).
      ii. Incubate the reaction at 37°C for 3 h.

Pause point: The vector can be digested at 37°C overnight (12–16 h).

      iii. Run the reaction product on a 1% (w/v) agarose gel and cut out the band at the size of 7,953 bp.
      iv. Purify the digested plasmid using AxyPrep DNA Gel Extraction Kit (following the manufacturer's instructions) and elute in 20 μL ddH_2O.
      v. Determinate the concentration of linearized pLVX vector with Nanodrop 2000c.

Pause point: The purified linearized pLVX vector can be stored at −20°C for up to several months.

   b. Prepare insert of INTS8-ΔN211 (2,436bp).
      i. Isolate the total RNA of the DLD-1 cell using TRIzol.
      ii. Add reaction components into a 0.2 mL PCR tube:

Reagent	Amount
Total RNA from preparation step 3.b.i	1 μg
$Oligo(dT)_{24}$ (100 μM)	1 μL
dNTP Mix, 10 mM each	1 μL
DEPC-treated water	To 14.5 μL

iii. Incubate the tube at 65°C for 5 min and chill on ice immediately to denature RNA.
iv. Add the remaining reaction component in the following order:

Reagent	Amount
5× RT Buffer	4 μL
RNase inhibitor	0.5 μL
Maxima H Minus Reverse Transcriptase	1 μL

v. Perform reverse transcription reaction with the following parameters (Table 6):
vi. Prepare the Polymerase Chain Reaction (PCR) master mix for the first round amplification of the protein-coding sequence of INTS8-ΔN211 (Table 7):
vii. Perform PCR reaction with the following parameters (Table 8):
viii. Purify the PCR product using AxyPrep PCR Clean-up Kit and elute the DNA in 20 μL ddH_2O (following the manufacturer's instructions).
ix. Prepare the master mix for the second round amplification of the protein-coding sequence of INTS8-ΔN211 (Table 9):
x. Perform PCR reaction with the following parameters (Table 10):
xi. Purify the PCR product using AxyPrep PCR Clean-up Kit and elute the DNA in 20 μL ddH_2O (following the manufacturer's instructions).

Note: After two rounds of PCR amplification, the Kozak sequence, and FLAG coding sequence will be added to the 5′ of the INTS8-ΔN211 coding sequence with flanking homology sequence used for recombination with linearized vector.

xii. Determinate the concentration of the insert of pLVX-Flag-hINTS8-N211 plasmid with Nanodrop 2000c

c. Recombination

Note: We used ClonExpress II One Step Cloning Kit to perform recombination reaction. Referring to the Kit manual is recommended for successfully conducting the experiment.

i. Set up recombination reaction on ice (Table 11):
ii. Put the mixture into a thermocycler and incubate at 37°C for 30 min, then hold on ice.

d. Transformation of 1 μL of the recombination product into 9 μL DH5α competent cells.
e. Gently spread transformed cells on pre-warmed LB-Amp-plates and incubate at 37°C overnight (12–16 h).
f. Pick colonies from plates and amplify each in 300 μL LB-Amp-Broth.

Note: Typically, no or fewer than 5 clones will grow on the control plates. We usually pick 5 clones from recombination plate for Sanger sequencing.

g. In our case, a 200 μL liquid culture of each clone was shipped to Boshang Biotechnology (Shanghai) Co., Ltd. to perform Sanger DNA sequencing with CMV primer. (troubleshooting 2).

Alternatives: If the local Sanger sequencing results take a long time to be returned, the plasmid purification step can be done prior to Sanger sequencing of plasmids.

Table 5. Digestion reaction mix

Reagent	Amount
pLVX	~3 μg
10X rCutsmart buffer	2 μL
EcoRI-HF	1 μL
ddH_2O	to 20 μL

Table 6. Reverse transcription conditions

Steps	Temperature	Time	Cycles
Reverse transcription	50°C	30 min	1
	65°C	15 min	1
Enzyme inactivation	85°C	5 min	1
Hold	4°C	forever	-

h. Amplify 10 μL from the remaining liquid culture of each clone in 10 mL LB-broth and purify the successfully constructed plasmids with EndoFree Mini Plasmid Kit II according to the sequencing result.
i. Determinate the concentration of pLVX-Flag-hINTS8-N211 plasmid with Nanodrop 2000c.
j. Store the plasmids at –20°C until use.

Pause point: The plasmid DNA can be stored at −20°C for long-term storage.

4. Lentivirus preparation for generation of INTS8-ΔN211 overexpression cell line.
   a. One day before transfection, harvest 293T cells in exponential phase in DMEM medium with 10% FBS.
   b. Seed 2 × 10^6 cells in 2 mL medium in each well of a 6-well plate.
   c. Culture at 37°C in a 5% CO_2 incubator for 24 h.
   d. When cell confluency reaches 70%–80%, prepare transfection mixture:
      i. Add 0.68 μg psPAX2 plasmid, 0.22 μg pMD2.G plasmid, and 1.2 μg pLVX-Flag-hINTS8-N211 plasmid into 200 μL serum-free medium in a sterilized 1.5 mL EP tube. Brief vortex to mix well.
      ii. Add 6 μL PEI transfection reagent and vortex to mix well.
      iii. Incubate for 15–20 min at room temperature (25°C).
   e. Gently add the transfection mixture to 293T cells, and gently shake the 6-well plate to mix well.
   f. Incubate at 37°C in a 5% CO_2 incubator for 6–8 h.
   g. Replace the transfection medium with fresh complete medium.
   h. Continue to incubate at 37°C in a 5% CO_2 incubator for 48 h.
   i. Collect the culture medium and centrifuge at 6000 × *g* for 10 min at 4°C to remove cell debris.
   j. Filter the supernatant containing the virus with 0.45 μm filters.
   k. Store the virus at 4°C until use.

Pause point: Store the virus at 4°C for several days or –80°C for long-term storage.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rpb1 NTD (D8L4Y) (1:4000)	CST	Cat.#14958; RRID:AB_2687876
Phospho-Rpb1 CTD (Ser5) (D9N5I) (1:4000)	CST	Cat.#13523S; RRID:AB_2798246
Phospho-Rpb1 CTD (Ser2) (E1Z3G) (1:4000)	CST	Cat.#13499S; RRID:AB_2798238
INTS8 (1:100)	Atlas Antibodies	Cat.# HPA057299; RRID: AB_2683403
Vinculin (1:10000)	Abclonal	Cat.#A2752; RRID:AB_2863020
HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H + L) (1:10000)	Proteintech	Cat.#SA00001-2; RRID:AB_2722564
Biological samples		
Trelief 5α Chemically Competent Cells	TSINGKE	Cat.#TSC-C01

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Commercially available plasmids		
pX459	Addgene	Cat.#62988
psPAX2	Addgene	Cat.#12260
pMD2.G	Addgene	Cat.#12259
Chemicals, peptides, and recombinant proteins		
dTAG-13 ligand	Tocris	Cat.#6605
Puromycin	Meilunbio	Cat.#MB2005
Blasticidin	Meilunbio	Cat.#3513-03-9
Cryopreservation medium	Applied cell	Cat.#AC-1001041
Proteinase K	Sangon Biotech	Cat.#A610451
Tween 20	Sangon Biotech	Cat.#A100777
Tris-Base	Sangon Biotech	Cat.#A501492
HCl	Sigma-Aldrich	Cat.#H1758
EDTA	Sigma-Aldrich	Cat.#E5134-500g
NaOH	Sigma-Aldrich	Cat.#S8045-1KG
DEPC	Sigma-Aldrich	Cat.#D5758-100ML
Critical commercial assays		
ClonExpress II One Step Cloning Kit	Vazyme	Cat.#C112-01
AxyPrep PCR Cleanup Kit	Axygen	Cat.#AP-PCR-250
EndoFree Mini Plasmid Kit II	TIANGEN	Cat.#DP118-03
AxyPrep DNA Gel Extraction Kit	Axygen	Cat.#AP-GX-50
Neon Transfection system 100 μL Kit	Invitrogen	Cat.#MPK10025
Experimental models: Cell lines		
DLD-1	ATCC	Cat.#CCL-221
293T	ATCC	Cat.#CRL-3216
Oligonucleotides		
pLVX-Flag-INTS8-N211_F1: GACTACAAAG ACGATGACGACAAGGGCAGCATGGATC TTTATGTCCATACGATGAG	This paper	N/A
pLVX-Flag-INTS8-N211_F2: CTATTTCCGGT GAATTCACCATGGACTACAAAGACGATGA CGACAAGGGCAGC	This paper	N/A
pLVX-Flag-INTS8-N211_R: CAAGCGGCTTCGGCCAGTAACGTTAAA AGTAAAGTTTTGCCATTGC	This paper	N/A
pX459-sgPITCH_F: CACCGGCATCGTACGCGTACGTGTT	This paper	N/A
pX459-sgPITCH_R: AAACAACACGTACGCGTACGATGCC	This paper	N/A
pX459-sgINTS8_F: CACCGTGAGCGCGGAGGCGGCGGAC	This paper	N/A
pX459-sgINTS8_R: AAACGTCCGCCGCCTCCGCGCTCAC	This paper	N/A
donor INTS8-N_F: TACGCGTACGTGTTTGGgatgagcgcg gaggcggcgATGACCGAGTACAAGC	This paper	N/A
donor INTS8-N_R: TACGCGTACGTGTTTGGgaggtggccgcc tcccggtcCGATCCAGAACCTCCGCCAC	This paper	N/A
hINTS8_genomic_F: GGATTGTGTGAGTTTCCGGG	This paper	N/A
hINTS8_genomic_R: CCGGGCATGTTTAAGGGAAG	This paper	N/A
U6 primer: GAGGGCCTATTTCCCATGATT	This paper	N/A
CMV primer: GTGATGCGGTTTTGGCAGTA	This paper	N/A
Oligo(dT)$_{24}$: TTT TTT TTT TTT TTT TTT TTT TTT	This paper	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
INTS8-dTAG donor with flanking PITCh: GCATCGTACGCGTACGTGTTTGGgatg agcgcggaggcggcgATGACCGAGTACAA GCCCACGGTGCGCCTCGCCACCCGCG ACGACGTCCCCAGGGCCGTACGCACC CTCGCCGCCGCGTTCGCCGACTACCC CGCCACGCGCCACACCGTCGATCCGG ACCGCCACATCGAGCGGGTCACCGAG CTGCAAGAACTCTTCCTCACGCGCGTC GGGCTCGACATCGGCAAGGTGTGGGT CGCGGACGACGGCGCCGCGGTGGCG GTCTGGACCACGCCGGAGAGCGTCGA AGCGGGGGCGGTGTTCGCCGAGATCG GCCCGCGCATGGCCGAGTTGAGCGGTT CCCGGCTGGCCGCGCAGCAACAGATG GAAGGCCTCCTGGCGCCGCACCGGCC CAAGGAGCCCGCGTGGTTCCTGGCCA CCGTCGGCGTCTCGCCCGACCACCAGG GCAAGGGTCTGGGCAGCGCCGTCGTG CTCCCCGGAGTGGAGGCGGCCGAGCG CGCCGGGGTGCCCGCCTTCCTGGAGAC CTCCGCGCCCCGCAACCTCCCCTTCTAC GAGCGGCTCGGCTTCACCGTCACCGCC GACGTCGAGGTGCCCGAAGGACCGCGC ACCTGGTGCATGACCCGCAAGCCCGGTG CCGGAAGCGGAGCTACTAACTTCAGCCT GCTGAAGCAGGCTGGAGACGTGGAGGA GAACCCTGGACCTGACTACAAAGACCAT GACGGTGATTATAAAGATCATGACATCGA TTACAAGGATGACGATGACAAGAGCGGA ATGGGAGTGCAGGTGGAAACCATCTCCC CAGGAGACGGGCGCACCTTCCCCAAGCG CGGCCAGACCTGCGTGGTGCACTACACC GGGATGCTTGAAGATGGAAAGAAAGTTG ATTCCTCCCGGGACAGAAACAAGCCCTTT AAGTTTATGCTAGGCAAGCAGGAGGTGAT CCGAGGCTGGGAAGAAGGGGTTGCCCAG ATGAGTGTGGGTCAGAGAGCCAAACTGAC TATATCTCCAGATTATGCCTATGGTGCCACT GGGCACCCAGGCATCATCCCACCACATGCC ACTCTCGTCTTCGATGTGGAGCTTCTAAAAC TGGAAGGCATGGACGAGCTGTACAAGGGC GGTTCGGGTGGCGGTTCGGGTTCGGGCGG AGGTCGTATGGAAAGGCGGATGCAGCCAT CGCCCGGTGGAGGTTCAGGTGGCGGAGGT TCTGGATCGgaccgggaggcggccacctcCCAAAC ACGTACGCGTACGATGC	This paper	N/A
Recombinant DNA		
pLVX vector with blasticidin resistance gene	This paper	N/A
pLVX-Flag-hINTS8-N211	This paper	N/A
pX459-sgPITCH	This paper	N/A
pX459-sgINTS8	This paper	N/A
pUC57-INTS8-dTAG donor	This paper	N/A
Other		
10× rCutsmart Buffer	New England Biolabs	Cat.#B6004S
BbsI-HF	New England Biolabs	Cat.#R3539S
EcoRI-HF	New England Biolabs	Cat.#R3101S
Ligation Mix	TaKaRa	Cat.#6023
PEI	Yeasen	Cat.#40816ES
Polybrene	Yeasen	Cat.#40804ES86
2× Phanta Max Master Mix	Vazyme	Cat.#P515-03
Maxima H minus RT enzyme	Invitrogen	Cat.#EP0752
dNTP Mix	New England Biolabs	Cat.#N0447L
RNase inhibitor	Invitrogen	Cat.#AM2696

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
TRIzol	Invitrogen	Cat.#15596018
Stripping buffer	EpiZyme	Cat.#PS107
Blocking buffer	EpiZyme	Cat.#PS108
Primary antibody dilution buffer	EpiZyme	Cat.#PS114
Nitrocellulose membrane	Millipore	Cat.#HATF00010
High-sig ECL Western Blotting Substrate	Tanon	Cat.#180-506
5× Protein Loading Buffer	Meilunbio	Cat.#MA0003-D
PBS	BasalMedia	Cat.#B320KJ
DMEM	BasalMedia	Cat.#L110KJ
Fetal bovine serum	Yeasen	Cat.#40130ES
Ampicillin sodium	Meilunbio	Cat.#MB1378
Penicillin-streptomycin	Meilunbio	Cat.#MA0110
10× TBST	EpiZyme	Cat.#PS103

MATERIALS AND EQUIPMENT

- 1× TBST: Dilute 50 mL 10× TBST in 450 mL ddH_2O.

Store at room temperature (25°C) for up to 12 months.

- Proteinase K (20 mg/mL): Dissolve 200 mg proteinase K powder in 10 mL ddH_2O.

Store at –20°C for up to 6 months.

- Ampicillin (100 mg/mL): Dissolve 1 *g* Ampicillin Sodium powder in 10 mL ddH2O.

Store at –20°C for up to 12 months.

- 10% (vol/vol) Tween-20: Dilute 1 mL of Tween 20 with 10 mL of ddH_2O.

Sterilize the solution through a 0.22-μm filter. Store at room temperature (25°C) for up to 12 months.

- DEPC-treated water: Add 1 mL of DEPC to 1 L of ddH_2O in a bottle.

Shake the bottle to mix and incubate at room temperature (25°C) overnight (12–16 h). Autoclave the solution at 121°C and 15 psi pressure for 15 min. Store at 4°C for up to 12 months.

LB-Amp-Broth: add 10g Tryptone, 5g Yeast extract, and 10g NaCl in 1L ddH_2O in a glass container		
Reagent	Final concentration	Amount
Tryptone	N/A	10 g
Yeast extract	N/A	5 g
NaCl	10 mg/mL	10 g
Ampicillin (100 mg/mL)	100 μg/mL	1 mL
ddH_2O	N/A	Up to 1 L

Prepare LB Broth without ampicillin in a glass container, and secure it with an autoclave-safe cap to prevent contamination during the sterilization process. Autoclave the LB broth at 121°C and 15 psi pressure for 15 min to sterilize the medium. Store at 4°C for up to 1 month.

⚠ CRITICAL: After sterilization, allow the medium to cool down to room temperature (25°C) before adding ampicillin for use.

LB-Amp-plates		
Reagent	Final concentration	Amount
Tryptone	10 mg/mL	10 g
Yeast extract	5 mg/mL	5 g
NaCl	10 mg/mL	10 g
Agar	15 mg/mL	15 g
Ampicillin (100 mg/mL)	100 μg/mL	1 mL
ddH_2O	N/A	Up to 1 L
Prepare LB-Agar Broth in a glass container without adding ampicillin. Secure the container with an autoclave-safe cap to minimize the risk of contamination during sterilization. Autoclave the LB-Agar Broth at 121°C and 15 psi pressure for 15 min to ensure complete sterilization.		

⚠ CRITICAL: After sterilization, allow the LB-Agar Broth to cool to 50°C before adding ampicillin. Stir well to ensure even distribution.

Immediately pour the LB-Agar mixture into sterile Petri dishes. Allow the agar to solidify at room temperature (25°C). Once solidified, the LB agar plates are ready for use or store the plates inverted at 4°C for up to 1 month.

Complete DMEM medium		
Reagent	Final concentration	Amount
DMEM medium	N/A	445 mL
Fetal bovine serum (FBS)	10%	50 mL
Penicillin-streptomycin	1%	5 mL
Store at 4°C for up to 4 weeks.		

0.5 M EDTA pH 8.0		
Reagent	Final concentration	Amount
$Na_2EDTA{\cdot}H_2O$	0.5 M	1.86 g
ddH_2O	N/A	8 mL
Adjust to pH 8.0 with NaOH	N/A	
ddH_2O	N/A	up to 10 mL
Sterilize the solution through a 0.22-μm filter. Store at 4°C for up to 12 months.		

1 M Tris-HCl pH 8.0		
Reagent	Final concentration	Amount
Tris-Base	1 M	6.06 g
ddH_2O	N/A	30 mL
Adjust to pH 8.0 with HCl	N/A	
ddH_2O	N/A	up to 50 mL
Sterilize the solution through a 0.22-μm filter. Store at 4°C for up to 12 months.		

Lysis buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl pH 8.0	50 mM	500 μL
0.5M EDTA	1 mM	20 μL
10% Tween-20	0.5%	500 μL
20 mg/mL Proteinase K	200 μg/mL	100 μL
ddH_2O	N/A	up to 10 mL
Sterilize the solution through a 0.22-μm filter. Store at 4°C for up to 12 months.		

⚠ CRITICAL: Added proteinase K freshly prior to use.

Table 7. PCR reaction master mix

Reagent	Amount
2× Phanta Max Master Mix	10 μL
ddH_2O	8 μL
pLVX-Flag-INTS8-N211 F1	0.5 μL
pLVX-Flag-INTS8-N211 R	0.5 μL
RT product from preparation step 3.b.v	1 μL

STEP-BY-STEP METHOD DETAILS

Generation of INTS8-dTAG cell line

Timing: 3–4 weeks

The successful construction of the INTS8-dTAG cell line is a prerequisite for generating the platform of rapidly upregulating global Pol II phosphorylation levels. This step details the procedures of the generation of the INTS8-dTAG cell line, including plasmid transduction of DLD-1 cells, single colony selection, PCR validation, and western blotting (WB) validation.

1. Plasmid transfection of colorectal adenocarcinoma cell line DLD-1.

Note: We used Neon transfection system to perform plasmid transfection of DLD-1. Referring to the Neon manual is recommended for successfully conducting the experiment.

a. Grow the DLD-1 cells in DMEM (Gibco) supplemented with 10% FBS and 1× Penicillin-Streptomycin in a 10-cm cell culture dish at 37°C, 5% CO_2.
b. When cell confluence reaches 80%, prepare the plasmid pools and control in 1.5 mL EP tubes as suggested in Table 12:
c. Plate 2 mL antibiotic-free DMEM medium supplemented with 10% FBS into 6-well plate per well and pre-warm in a 37°C/5% CO_2 incubator.
d. Set up a Neon Tube with 3 mL Buffer E2 into the Neon Pipette Station.
e. Set the pulse conditions (1300 V, 20 ms, 2 pulses) on the Neon device.
f. Trypsinize the cells with TrypLE Express.
g. After neutralization, transfer the cell suspension to a 15 mL conical tube and centrifuge the cells at 200 × *g* for 5 min.
h. Aspirate off the supernatant, resuspend cells in PBS, and count the cell density.
i. Transfer 4×10^6 of the cell suspension to a 1.5 mL microcentrifuge tube and centrifuge at 200 × *g* for 5 min and resuspend in 240 μL Resuspension Buffer R.
j. Add 110 μL cells into each 1.5 mL EP tube containing plasmid pools from major step 1.b and gently mix by pipetting.
k. Use Neon pipette and 100 μL Neon Tips to pipette up the cell-plasmid mixture.

⚠ CRITICAL: Avoid air bubbles during pipetting as air bubbles can lead to lowered or failed transfection

Table 8. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	15 s	20
Annealing	51°C	15 s	
Extension	72°C	1.5 min	
Final extension	72°C	5 min	1
Hold	4°C		1

Table 9. PCR reaction master mix

Reagent	Amount
2× Phanta Max Master Mix	10 μL
ddH_2O	8 μL
pLVX-Flag-INTS8-N211_F2	0.5 μL
pLVX-Flag-INTS8-N211_R	0.5 μL
PCR product from preparation step 3.b.viii	1 μL

l. Insert the Neon Pipette with the sample into the Neon Tube placed in Neon Pipette Station and press "Start" on the screen.

m. After "Complete" displayed on the screen, slowly remove the Neon Pipette from the Neon Pipette Station, and pipette the cells into the prepared 6-well plate containing prewarmed medium.

n. Gently rock the plate to ensure the cells are evenly distributed. Incubate the plate at 37°C in a 5% CO_2 incubator for 24 h.

2. Puromycin selection

a. Discard the medium and trypsinize the cells with 0.5 mL TrypLE Express per well at 37°C for 5 min.

b. After neutralization with 1 mL complete medium, transfer the cells suspension into 10 mL complete medium in a 10 cm culture dish.

c. Add puromycin to a final concentration of 1 μg/mL.

d. Gently rock the plate to ensure the cells and puromycin are evenly distributed.

e. Culture the cells at 37°C in a 5% CO_2 incubator.

f. Change fresh medium supplemented with 1 μg/mL puromycin every 2 days.

Note: During puromycin selection, observe the cells under microscope every day. Typically, after 10 days of puromycin selection, control cells die completely, and sample cells in the sample dish form tens to hundreds of single-cell colonies that are large enough to pick up (Figure 2).

3. Single-cell colony selection (troubleshooting 3).

a. After 10 days of puromycin selection, remove the medium of the sample dish, and wash the cells once with PBS.

b. Add 15 mL PBS to the sample dish.

c. Prepare a 96-well plate with 20 μL TrypLE Express in each well.

d. Put the sample dish on the microscope stage, pick a colony into a well of prepared 96-well plate under the 4× objective with 10 μL tip, and mix by pipetting more than 10 times.

e. Repeat major step 1.3.d for 16 times.

f. Incubate the plate at 37°C for 5 min in a 5% CO_2 incubator to trypsinize the cells.

g. Add 150 μL complete medium to neutralize, and gently mix by pipetting.

h. Pick 48 to 96 colonies in total by repeating major step 1.3.d to major step 1.3.g.

i. Culture the cells in the 96-well plate at 37°C in a 5% CO_2 incubator.

Note: After this step, the cells from a single colony are evenly divided into the corresponding wells of two 96-well plates, one of which is used to continue culturing the cells, and the other is used for PCR validation.

Table 10. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	15 s	15
Annealing	65°C	15 s	
Extension	72°C	1.5 min	
Final extension	72°C	5 min	1
Hold	4°C		1

Table 11. Recombination reaction mix

Reagent	Recombination	Control-1	Control-2
Linearized Vector from preparation step 3.a.iv	40 ng	40 ng	0 ng
Insert from preparation step 3.b.xi	25 ng	0 ng	25 ng
5 × CE II Buffer	1 μL	0 μL	0 μL
Exnase II	0.5 μL	0 μL	0 μL
ddH_2O	to 5 μL	to 5 μL	to 5 μL

4. PCR validation of INTS8-dTAG homozygous knockin cells
   a. 2–4 days after single-cell colony picking, remove the medium, trypsinize the cells in each well with 30 μL TrypLE.
   b. Add 200 μL complete medium to neutralize, gently mix by pipetting, and transfer 115 μL cells to another 96-well plate for PCR validation.

   Note: After this step, the cells from a single colony are evenly divided into the corresponding wells of two 96-well plates, one of which is used to continue culturing the cells, and the other is used for PCR validation.

   c. One day after subculturing, remove the medium of the PCR validation plate and add 50 μL lysis buffer to each well.
   d. After sealing the 96-well plate with a sealing film, digest the cells for 1 h in a 55°C oven.
   e. After the cell lysates cool down to room temperature (25°C), gently mix the cell lysate in each well by pipetting and transfer to 8-strip tubes.
   f. Inactivate the proteinase K at 95°C for 12 min.
   g. During this time, prepare genotyping PCR master mix following Table below (Table 13) and dispense 9.5 μL aliquot to 96 PCR tubes.
   h. After the cell lysates cool down to room temperature (25°C), transfer 0.5 μL of each lysate to the master mix in corresponding PCR tubes.
   i. Perform PCR reaction with the following parameters (Table 14).
   j. Run 5 μL PCR product of each colony on a 1% (w/v) agarose gel.
   k. Take an image of the gel, the PCR product of the knockin allele will display at the size of 1609 bp while the PCR product of the WT allele will display at the size of 430 bp (Figure 3). Record the numbers of homozygous knockin colonies.
   l. Ship the remaining PCR product of PCR-validated homozygous knockin colonies to Boshang Biotechnology (Shanghai) Co., Ltd. And perform Sanger DNA sequencing with hINTS8_genomic_F and hINTS8_genomic_R.
5. Further validation by WB (troubleshooting 4).
   a. Subculture PCR validated clones in the 96-well plate to 24-well plates.
   b. After reaching 80% confluency, for each colony, subculture to 3 wells of a 24-well plate. Cells in one of the wells continue culturing and cells in the other two wells are used for WB validation after dTAG-13 treatment.
   c. One day after subculturing, for each clone, treat cells with DMSO or 100 nM dTAG-13 for 3 h.
   d. Remove the medium, wash cells once with PBS, then add 50 μL 1× Protein Loading Buffer per well.
   e. Shake to thoroughly lyse the cells and transfer to 1.5 mL microcentrifuge tubes.

Table 12. Transfection

Plasmid	Sample	Control
pX459-sgPITCH	2 μg	-
pX458	-	4 μg
pX459-sgINTS8	2 μg	-
pUC57-INTS8-dTAG donor	4 μg	4 μg

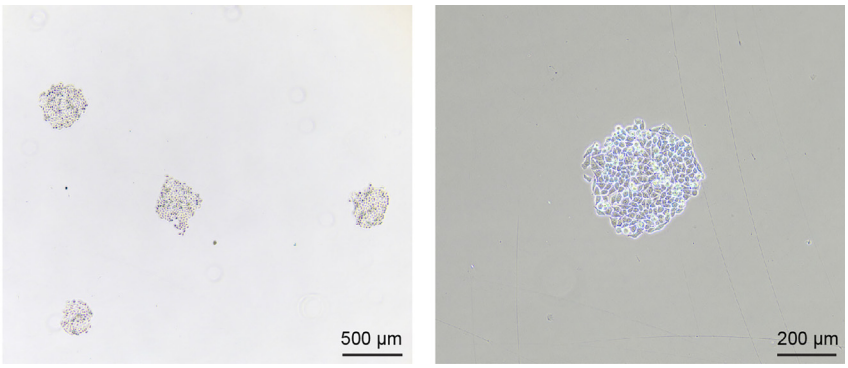

Figure 2. Representative images of selected DLD-1 single-cell colonies after 10 days of puromycin treatment
Scale bar: 500 μm (left) and 200 μm (right).

f. Boil the protein lysates for 9 min.
g. Run protein on a 10% SDS-PAGE gel.
h. Transfer proteins to nitrocellulose membrane.
i. Block the membrane with 1× Blocking Buffer for 10 min.
j. Incubate the membrane with primary antibodies diluted in Primary Antibody Dilution Buffer overnight (12–16 h) at 4°C.
k. Wash the membrane with 1× TBST for 15 min. Repeat this washing step twice more.
l. Incubate the membrane with secondary antibodies diluted in TBST for 1 h at room temperature (25°C).
m. Wash the membrane with 1× TBST for 15 min. Repeat this washing step twice more.
n. Detect the signal of INTS8 and vinculin after treatment with High-sig ECL Western Blotting Substrate (Figure 4).
o. Expand the WB-validated and Sanger DNA sequencing-validated colonies.
p. Take one validated colony to continue to generate the INTS8-dTAG+ΔN cell line.

Note: Validated colonies should satisfy nearly complete deletion of INTS8 and corrected knockin on INTS8 N-terminal genomic locus.

Generation of INTS8-dTAG+ΔN cell line

Timing: 10–14 days

In this section, we describe the procedures of expressing N-terminus deletion INTS8 in the generated INTS8-dTAG cells.

6. Seed 5 × 105 INTS8-dTAG cells per well into 2 wells of a 6-well plate.
7. After reaching 70–80% confluency, transduce the cells with lentivirus.
   a. Remove the medium.
   b. Add 1 mL fresh medium, 1 mL lentivirus, and 1.5 μL polybrene to the sample well. Add 2 mL fresh medium to the control well.

Table 13. PCR reaction master mix

Reagent	Amount per reaction	Amount for 96 reactions
2× Phanta Max Master Mix	5 μL	550 μL
ddH2O	3.5 μL	385 μL
hINTS8_genomic_F	0.5 μL	55 μL
hINTS8_genomic_R	0.5 μL	55 μL
Cell lysate	0.5 μL	-

Table 14. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	15 s	30
Annealing	58°C	15 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	4°C		1

c. Incubate at 37°C in a 5% CO_2 incubator for 24 h.
d. Replace medium containing lentivirus with 2 mL fresh medium.
e. Continue to incubate the cells at 37°C in a 5% CO_2 incubator for 48 h.

8. Blasticidin selection.
   a. Discard the medium and trypsinize the cells with 0.5 mL TrypLE Express per well at 37°C for 5 min.
   b. After neutralization with 1 mL complete medium, transfer the cells suspension into 10 mL complete medium in a 10 cm culture dish.
   c. Add blasticidin to a final concentration of 10 μg/mL.
   d. Gently rock the plate to ensure the cells and puromycin are evenly distributed.
   e. Culture the cells at 37°C in a 5% CO_2 incubator.
   f. Change fresh medium supplemented with 10 μg/mL blasticidin every 2 days until the cells in the control dish are all killed.
   g. Frozen the antibiotic-selected cells for long-term storage.

   Note: Typically, 10 days of antibiotic selection is enough to kill all untransduced cells.

9. Validate INTS8-ΔN expression and upregulation of global Pol II phosphorylation level by WB (troubleshooting 5).
   a. Subculture the antibiotic-selected cells in two wells of a 6-well plate.
   b. Treat cells with DMSO or 100 nM dTAG-13 for 3 h.
   c. Remove the medium, wash cells once with PBS, then add 150 μL 1× protein loading buffer per well.
   d. Perform the WB processes according to major step 1.5.e to 1.5.n with primary antibodies of INTS8, Rpb1, pSer2, pSer5, and Vinculin (Figure 5).

EXPECTED OUTCOMES

This protocol describes a method allowing us to rapidly and specifically induce global RNA Pol II hyperphosphorylation, which can be used as a valuable tool to study the direct functions of RNA Pol II

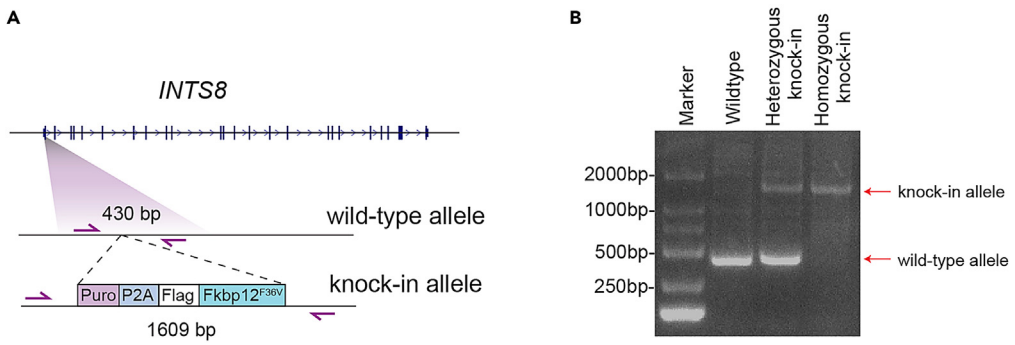

Figure 3. PCR validation of the integration of the dTAG into N-terminal INTS8 encoding genomic locus
(A) schematic representation of genotyping primers targeting locus.
(B) Representative agarose gel separating colony PCR amplicons.

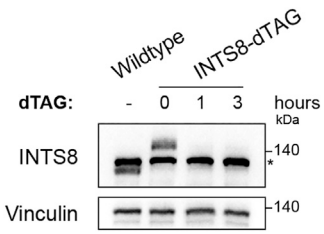

Figure 4. Validation of INTS8 deletion efficiency by western blotting

Western blotting of whole-cell lysates of Wildtype cells and INTS8-dTAG cells treated with dTAG-13 for 0, 1, or 3 h. Vinculin is a loading control. Stars represent non-specific bands.

phosphorylation in the processes of transcription, mRNA splicing, etc. An example of the outcomes after 1-h and 3-h dTAG-13 treatment is shown in Figure 5: the expression of INTS8-ΔN and the substantial elevation of the levels of phosphorylated RNA Pol II, including pSer5 and pSer2, upon INTS8 loss.

LIMITATIONS

We provide a protocol for inducing RNA Pol II hyperphosphorylation by replacing INTS8 with INTS8-△N in DLD-1 cells. While this protocol can be modified and applied to other cell lines, the rapidity and efficiency of INTS8 degradation may vary among cell lines. Researchers should validate the INTS8 degradation and Pol II hyperphosphorylation using western blotting in a specific cell line. Additionally, since the dTAG system relies on genetic editing, this method is limited to cell types amenable to genome editing.

Although unprecedented rapid upregulation of Pol II phosphorylation levels allows us to minimize the interference of indirect effects, the secondary responses of Pol II hyperphosphorylation cannot be ruled out due to the highly dynamic nature of the transcription processes. To minimize indirect effects, combining this protocol with time-resolved techniques for detecting nascent RNA and exploring faster protein deletion methods will enhance the applicability of this approach.

TROUBLESHOOTING

Problem 1

Too many colonies grow on the control plate (related to preparation step 1.f).

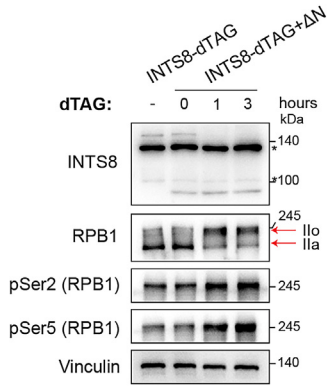

Figure 5. Rapid upregulation of global RNA Pol II phosphorylation levels by acute INTS8 deletion in INTS8-dTAG+ΔN cells

Western blotting of whole-cell lysates of INTS8-dTAG cells and INTS8-dTAG+ΔN cells treated with dTAG-13 for 0, 1 or 3 h. IIo and IIa indicate hyper- and hypophosphorylated RNA Pol II, respectively. Vinculin is a loading control. Stars represent non-specific bands.

Potential solution

Typically, no or fewer than 5 colonies will grow on the control plate, and tens to hundreds of colonies will grow on each of the sgPITCh and sgINTS8 plates. We usually pick 3 colonies on each of the sgPITCh and sgINTS8 plates for Sanger sequencing. Colonies growing on the control plate may suggest incomplete linearization of the pX459 vector. If the number of colonies on the sample plates is much more than on the control plate, pick more colonies for Sanger sequencing. Otherwise, extend the digestion time of the pX459 vector (16h, overnight) and run an agarose gel to confirm complete digestion.

Problem 2

The coding sequence for INTS8-ΔN211 was not successfully inserted (related to preparation step 3.*g*).

Potential solution

If the wrong sequence has been inserted according to the results of Sanger sequencing, please perform gel extraction and cut the gel at the size of 2436bp to recover the insert obtained in preparation step 3.b.xi, and check the integrity of the total RNA obtained in preparation step 3.b.i.

Problem 3

Puromycin-selected cells are too many or colonies are too dense to pick up. (related to major step 3).

Potential solution

Trypsinize the puromycin-selected cells and dilute to 1 cell per 100 μL culturing medium and add 100 μL to each well of a 96-well plate.

Problem 4

The extent of INTS8 degradation is insufficient (related to major step 5).

Potential solution

Protein degron systems, including the dTAG system, have a common limitation that the degron system may have variable efficiency under specific experimental conditions in a certain cell line. Based on our experience with DLD-1 cells, ensuring that cells are in the logarithmic growth phase during dTAG-13 treatment can enhance the effectiveness of INTS8 degradation.

Problem 5

Pol II phosphorylation levels do not increase after INTS8 depletion (related to major step 9).

Potential solution

Proteins and their phosphorylation modifications are unstable. Therefore, boiling the proteins as soon as possible after cell lysis is recommended. Alternatively, protease inhibitors and phosphatase inhibitors can be added to the lysis buffer in advance.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fei Xavier Chen (feixchen@fudan.edu.cn).

Materials availability

All materials used in this experiment are available through commercial resources and Addgene, as indicated in the key resources table.

Data and code availability

No new datasets or code were generated in this protocol.

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AUTHOR CONTRIBUTIONS

F.X.C. and S.H. conceived and designed the project. Y.-X.J. and S.H. conducted the experiments. Y.-X.J. and F.X.C. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Hu, S., Peng, L., Song, A., Ji, Y.X., Cheng, J., Wang, M., and Chen, F.X. (2023). INTAC endonuclease and phosphatase modules differentially regulate transcription by RNA polymerase II. Mol. Cell *83*, 1588–1604.e5. https://doi.org/10.1016/j.molcel.2023.03.022.

2. Kwiatkowski, N., Zhang, T., Rahl, P.B., Abraham, B.J., Reddy, J., Ficarro, S.B., Dastur, A., Amzallag, A., Ramaswamy, S., Tesar, B., et al. (2014). Targeting transcription regulation in cancer with a covalent CDK7 inhibitor. Nature *511*, 616–620. https://doi.org/10.1038/nature13393.

3. Wang, Z., Song, A., Xu, H., Hu, S., Tao, B., Peng, L., Wang, J., Li, J., Yu, J., Wang, L., et al. (2022). Coordinated regulation of RNA polymerase II pausing and elongation progression by PAF1. Sci. Adv. *8*, eabm5504. https://doi.org/10.1126/sciadv.abm5504.

4. Chao, S.H., Fujinaga, K., Marion, J.E., Taube, R., Sausville, E.A., Senderowicz, A.M., Peterlin, B.M., and Price, D.H. (2000). Flavopiridol inhibits P-TEFb and blocks HIV-1 replication. J. Biol. Chem. *275*, 28345–28348. https://doi.org/10.1074/jbc.C000446200.

5. Zeng, M., Kwiatkowski, N.P., Zhang, T., Nabet, B., Xu, M., Liang, Y., Quan, C., Wang, J., Hao, M., Palakurthi, S., et al. (2018). Targeting MYC dependency in ovarian cancer through inhibition of CDK7 and CDK12/13. Elife *7*, e39030. https://doi.org/10.7554/eLife.39030.

6. Sents, W., Ivanova, E., Lambrecht, C., Haesen, D., and Janssens, V. (2013). The biogenesis of active protein phosphatase 2A holoenzymes: a tightly regulated process creating phosphatase specificity. FEBS J. *280*, 644–661. https://doi.org/10.1111/j.1742-4658.2012.08579.x.

7. Peti, W., Nairn, A.C., and Page, R. (2013). Structural basis for protein phosphatase 1 regulation and specificity. FEBS J. *280*, 596–611. https://doi.org/10.1111/j.1742-4658.2012.08509.x.

8. Zheng, H., Qi, Y., Hu, S., Cao, X., Xu, C., Yin, Z., Chen, X., Li, Y., Liu, W., Li, J., et al. (2020). Identification of Integrator-PP2A complex (INTAC), an RNA polymerase II phosphatase. Science *370*, eabb5872. https://doi.org/10.1126/science.abb5872.

9. Huang, K.L., Jee, D., Stein, C.B., Elrod, N.D., Henriques, T., Mascibroda, L.G., Baillat, D., Russell, W.K., Adelman, K., and Wagner, E.J. (2020). Integrator Recruits Protein Phosphatase 2A to Prevent Pause Release and Facilitate Transcription Termination. Mol. Cell *80*, 345–358.e9. https://doi.org/10.1016/j.molcel.2020.08.016.

10. Vervoort, S.J., Welsh, S.A., Devlin, J.R., Barbieri, E., Knight, D.A., Offley, S., Bjelosevic, S., Costacurta, M., Todorovski, I., Kearney, C.J., et al. (2021). The PP2A-Integrator-CDK9 axis fine-tunes transcription and can be targeted therapeutically in cancer. Cell *184*, 3143–3162.e32. https://doi.org/10.1016/j.cell.2021.04.022.

11. Nabet, B., Roberts, J.M., Buckley, D.L., Paulk, J., Dastjerdi, S., Yang, A., Leggett, A.L., Erb, M.A., Lawlor, M.A., Souza, A., et al. (2018). The dTAG system for immediate and target-specific protein degradation. Nat. Chem. Biol. *14*, 431–441. https://doi.org/10.1038/s41589-018-0021-8.

12. Sakuma, T., Nakade, S., Sakane, Y., Suzuki, K.I.T., and Yamamoto, T. (2016). MMEJ-assisted gene knock-in using TALENs and CRISPR-Cas9 with the PITCh systems. Nat. Protoc. *11*, 118–133. https://doi.org/10.1038/nprot.2015.140.