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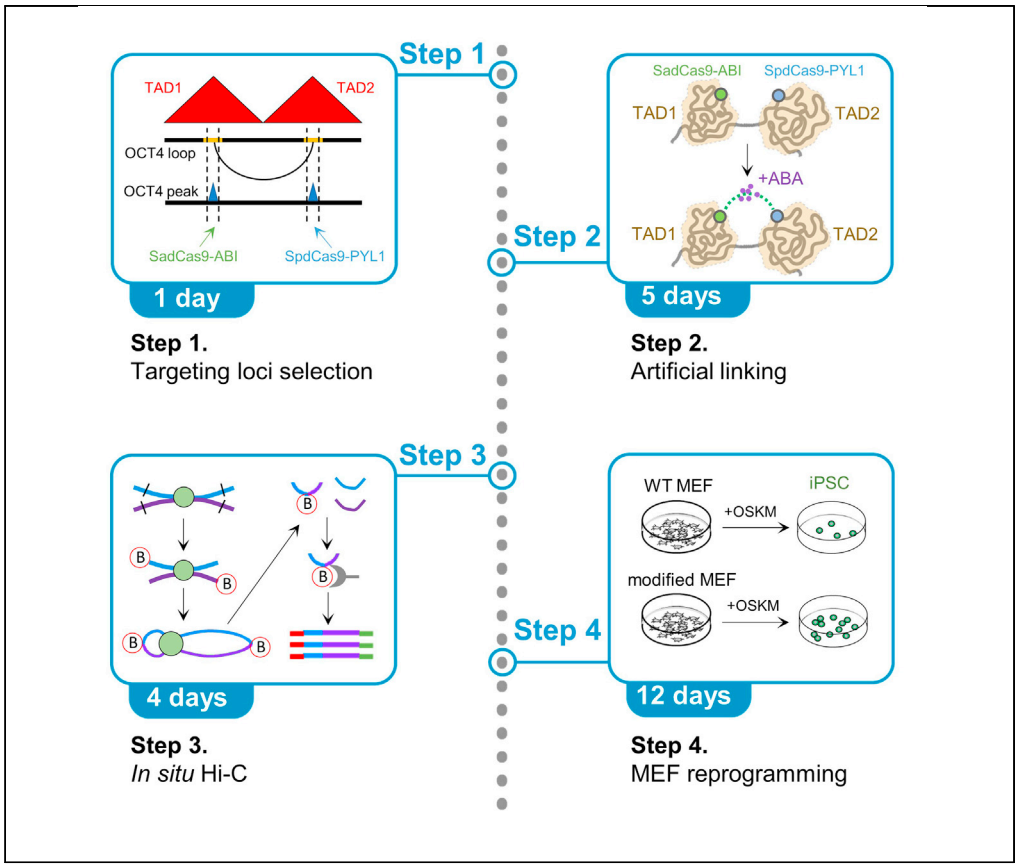
Manipulation of TAD reorganization by chemical-dependent genome linking



Reorganization of topologically associated domain (TAD) is considered to be a novel mechanism for cell fate transitions. Here, we present a protocol to manipulate TAD via abscisic acid (ABA)-dependent genome linking. We use this protocol to merge two adjacent TADs and evaluate the influence on cell fate transitions. The advantages are that the manipulation does not change the genome and is reversible by withdrawing ABA. The major challenge is how to select linking loci for efficient TAD reorganization.

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Highlights

Artificial linking is established at dCas9-targeting loci by small molecule chemical

Chemical-dependent genome linking induces TAD merging by Hi-C

Quality control after each key step can improve Hi-C outcome

Wang et al., STAR Protocols 2, 100799
September 17, 2021
https://doi.org/10.1016/j.xpro.2021.100799

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Manipulation of TAD reorganization by chemical-dependent genome linking

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SUMMARY

Reorganization of topologically associated domain (TAD) is considered to be a novel mechanism for cell fate transitions. Here, we present a protocol to manipulate TAD via abscisic acid (ABA)-dependent genome linking. We use this protocol to merge two adjacent TADs and evaluate the influence on cell fate transitions. The advantages are that the manipulation does not change the genome and is reversible by withdrawing ABA. The major challenge is how to select linking loci for efficient TAD reorganization.

For complete details on the use and execution of this protocol, please refer to Wang et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific strategy and workflow for inducing TAD merging by a dCas9-based chemical-dependent artificial genome linking. This manipulation can also contribute to cell fate transitions.

Loci selection

Timing: 1 day

1. Analyze the HiC data during somatic cell reprogramming and summarize all reorganized TAD sites with HiC-Pro.
2. Select TADs with the following characteristics.
   a. Two TADs merge into one large TAD from mouse embryonic fibroblast (MEF) to induced pluripotent stem cell (iPSC) transitions.
   b. The TADs contain key pluripotency genes which may be activated upon TAD merging.
   c. The TADs contain key cis-regulatory elements such as enhancers or super-enhancers (SEs).
3. Select potential dCas targeting loci within the selected TADs. The loci should be close to the center of TAD to enhance TAD merging efficiency.

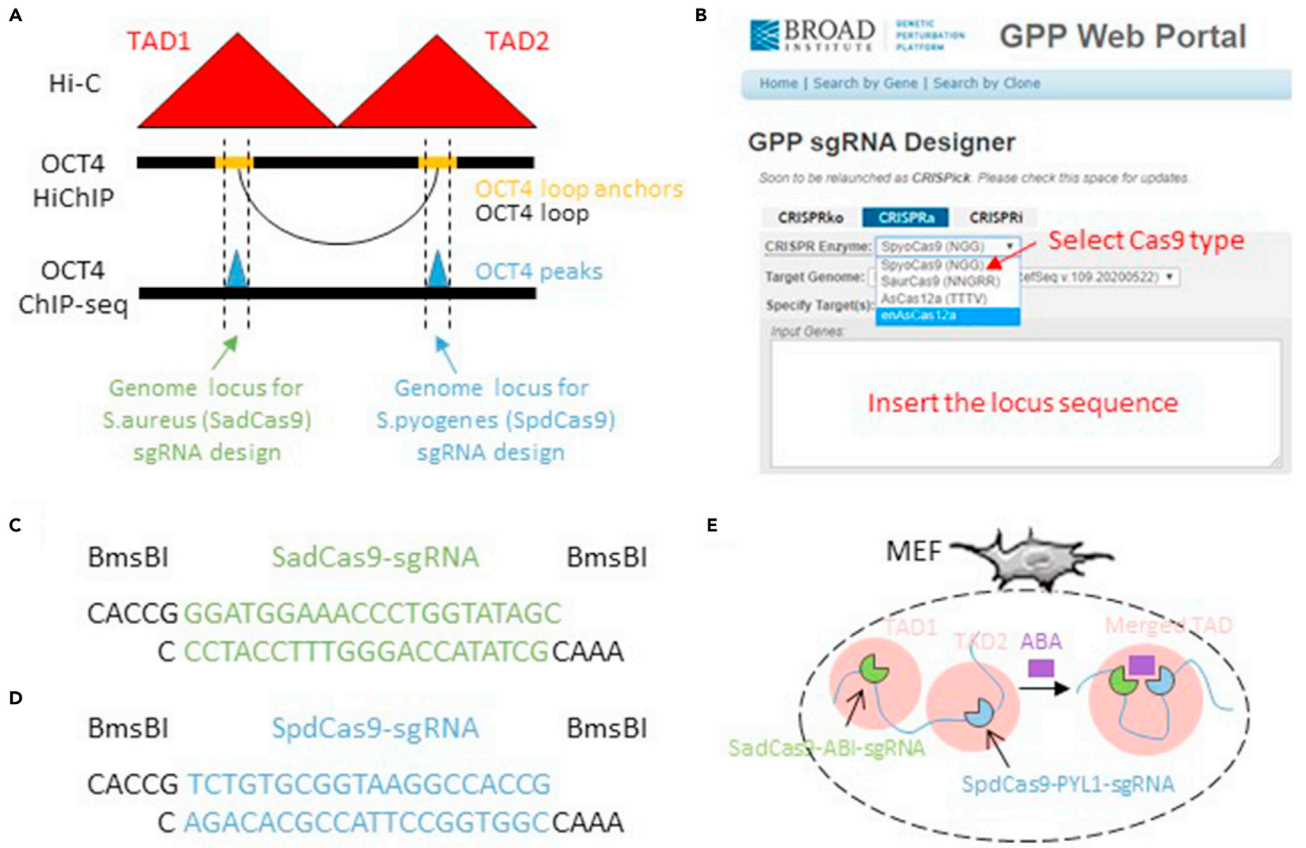

Figure 1. Selection of genome loci and sgRNA design for artificial linking

(A) To enhance the TAD merging rate, the loci selected for artificial linking should be close to the center of each TAD. Moreover, the loci should be the overlapping genome regions between the OCT4 loop anchors identified by OCT4 HiChIP and OCT4 peaks identified by OCT4 ChIP-seq in PSC. The loci are about 500–1000 bp length. We design sgRNA within the loci for dCas9 targeting.

(B) The website for sgRNA design.

(C and D) The BmsBI restriction site sequence is added into the end of sgRNA and its complementary sequence.

(E) Artificial linking in MEF can reorganize TAD structure in a chemical-dependent manner.

Note: To enhance the TAD merging rate, the loci selected for artificial linking should be close to the center of each TAD. Moreover, the loci should be the overlapping genome regions between the OCT4 loop anchors identified by OCT4 HiChIP and OCT4 peaks identified by OCT4 ChIP-seq in PSC (Figure 1A). The loci are about 500–1000 bp length. We design sgRNA within the loci for dCas9 targeting.

sgRNA design

Timing: 1 day

4. Design sgRNAs of SadCas9 and SpdCas9.
   a. The potential dCas9 targeting loci obtained in step 3 were input into the website (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design) for sgRNA designing. Select SaurCas9(NNGRR) or SpyoCas9(NGG) in the *CRISPR Enzyme* button for S.aureus (SadCas9) or S.pyogenes (SpdCas9) sgRNA design, respectively (Figure 1B).
   b. Select 3–5 sgRNAs with high score of on-target efficiency for each interested locus.

Note: the website will automatically score the on-target efficiency of the candidate sgRNAs, and usually we select the top 3–5 highly scored sgRNAs.

c. The BmsBI restriction sites were selected for inserting the sgRNA into SadCas9-ABI or SpdCas9-PYL1 plasmids. Therefore, add the sequences of restriction site at the end of sgRNA sequence and their complementary sequence for future manipulation (Figures 1C and 1D).
d. For the strategy to induce TAD merging by the artificial linking, we will target two genome loci belonging to two neighboring TADs by SadCas9-ABI and SpdCas9-PYL1 respectively. The artificial linking is established by adding abscisic acid (ABA) which can co-bind with ABI and PYL1. The linking may shorten the distance of the two TADs and even induce their merging (Figure 1E).

5. sgRNA Synthesis.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CTCF (dilution 1:1000)	Santa Cruz	Cat# sc-398149
CTCF (dilution 1:1000)	Millipore	Cat# 07–729; RRID: AB_441965
Normal Mouse IgG (dilution 1:2000)	Millipore	Cat# 12–371; RRID: AB_145840
Normal Rabbit IgG (dilution 1:2000)	Millipore	Cat# 12–370; RRID: AB_145841
Chemicals, peptides, and recombinant proteins		
DMEM Medium	HyClone	Cat# SH30022.01
DMEM/F12 1:1 Medium	Gibco	Cat# C11330500BT
Fetal Bovine Serum	VISTECH	Cat# SE100-B
Trypsin/EDTA	Coring	Cat# 25–051
KnockOut™ Serum Replacement	Gibco	Cat# 10828028
PD0325901	Selleck	Cat# S1036
CHIR99021	Selleck	Cat# S1263
N2 Supplement	Thermo Fisher	Cat# 17502-048
Puromycin	Sigma-Aldrich	Cat# 540222
B27 Supplement	Gibco	Cat# 17504044
β504044ptoethanol	Sigma	Cat# M6250
NEAA	Thermo Fisher	Cat# 11140050
GlutaMax	Thermo Fisher	Cat# 35050061
MboI	New England Biolabs	Cat# R0147
Biotin-14-dATP	Thermo Fisher	Cat# 19524016
dCTP	Invitrogen	Cat# 18253013
dGTP	Invitrogen	Cat# 18254011
dTTP	Invitrogen	Cat# 18255018
DNA Polymerase I, Large (Klenow) Fragment	New England Biolabs	Cat# M0210
T4 DNA Ligase	New England Biolabs	Cat# M0202
Proteinase K	New England Biolabs	Cat# P8102
UltraPure™ 10% SDS	Thermo Fisher	Cat# 15553-035
Triton X-100	Sigma	Cat# 93443
T4 PNK	New England Biolabs	Cat# M0201
T4 DNA Polymerase I	New England Biolabs	Cat# M0203
Klenow (3′-5′exo-)	New England Biolabs	Cat# M0212
Quick Ligase	New England Biolabs	Cat# M2200
User Enzyme	New England Biolabs	Cat# E7338A
Proteinase Inhibitor	Roche	Cat# 1169749800
RNaseA	Thermo Fisher	Cat# EN0531
Lenti-X Concentrator	Clontech	Cat# 631231
HEPES-KOH	Sigma	Cat# 7365-45-9
LiCl solution	Sigma	Cat# 7447-41-8

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
UltraPure™ 0.5 M EDTA	Thermo Fisher	Cat# 15575-038
EGTA	Sigma	Cat# 67-42-5
Glycerol	Sigma	Cat# 56-81-5
NP-40	Sangon Biotech	Cat# 9016-45-9
Tween-20	Amresco	Cat# 0777-1L
50× TAE buffer	Sangon Biotech	Cat# B548101-0500
DMSO	Sigma	Cat# D2650-100ML
IGEPAL CA-630	Sigma	Cat# 19996-50ML
Na-Deoxycholate	Sigma	Cat# 302-95-4
N-Lauroylsarcosin	Sigma	Cat# 97-78-9
Agarose	TSINGKE	Cat# TSJ001
LB Broth Agar	Sangon Biotech	Cat# A507003-0250
1 M Tris-HCl buffer	Solarbio	Cat# T1140
5 M NaCl solution	Sigma	Cat# S5150
Abscisic acid	Sigma	Cat# A1049
Critical commercial assays		
RNAzol	MRC	Cat# RN190-500
EndoFree Plasmid Midi Kit	Cwbio	Cat# CW2105S
FuGENE	Promega	Cat# E2311
TruePrep DNA Library Prep Kit V2 for Illumina	Vazyme	Cat# TD501
TruePrep Index Kit V2 for Illumina	Vazyme	Cat# TD202
CLOuD9	System Biosciences	Cat# CASCL9-100A
E.Z.N.A. Gel Extraction Kit	Omega	Cat# D2500-02
TIANquick Midi Purification Kit	TIANGEN	Cat# DP204-03
TIANamp Genomic DNA Kit	TIANGEN	Cat# DP304-02
SYBR qPCR Master Mix	Vazyme	Cat# Q711-00
Leukocyte Alkaline Phosphatase Kit	Sigma	Cat# 86R
Alkaline Phosphatase Stain Kit	Yeasen	Cat# 40749ES60
Phenol:chloroform pH 6.7/8.0	Amresco	Cat# 0883-400ML
AMPure XP Beads	Beckman Coulter	Cat# A63881
Dynabeads MyOne Streptavidin T1 Beads	Life Technologies	Cat# 65602
Protein G Agarose Beads	Thermo Scientific	Cat# 10004D
NEBNext Multiplex Oligos for Illumina	New England Biolabs	Cat# E7335
Qubit™ 1× dsDNA HS Assay Kit	Invitrogen	Cat# Q33230
Primescript RT Master Mix	Takara	Cat# RR036A
Cell lines		
Mouse embryonic stem cell line V6.5	Laboratory of R. Jaenisch	RRID: CVCL_C865
Mouse OG2 MEFs	Laboratory of Jiekai Chen	N/A
Mouse OD14 MEFs	Laboratory of Jiekai Chen	N/A
Human 293T cells	ATCC	Cat# CRL-3216; RRID: CVCL_0063
Software and algorithms		
mm10	ENSEMBL release 90	https://asia.ensembl.org/Mus_musculus/
Trim Galore 0.4.4_dev	https://www.bioinformatics.babraham.ac.uk/projects/trim_galore	https://github.com/FelixKrueger/TrimGalore
bowtie2 v2.3.0	(Langmead and Salzberg, 2012)	https://github.com/BenLangmead/bowtie2
samtools v1.3.1	(Li et al., 2009)	https://github.com/samtools/samtools
Bedtools v2.26.0	(Quinlan and Hall, 2010)	https://github.com/arq5x/bedtools
macs2 v2.1.2	(Zhang et al., 2008)	https://github.com/taoliu/MACS
HTSeq-count v0.11.2	(Anders et al., 2015)	https://github.com/simon-anders/htseq
edgeR v3.26.5	(Robinson et al., 2010)	https://bioconductor.org/packages/release/bioc/html/edgeR.html
HiC-Pro v2.10.0	(Servant et al., 2015)	https://github.com/nservant/HiC-Pro
HiCRep v1.8.0	(Yang et al., 2017)	https://bioconductor.org/packages/release/bioc/html/hicrep.html
TopDom v0.0.2	(Shin et al., 2016)	https://github.com/HenrikBengtsson/TopDom

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
diffHic v1.16.0	(Lun and Smyth, 2015)	https://bioconductor.org/packages/release/bioc/html/diffHic.html
Hichipper v0.7.5	(Lareau and Aryee, 2018)	https://github.com/aryeelab/hichipper
Other		
Covaris sonicator E220	Covaris	N/A
Qubit™ 4 Fluorometer	Invitrogen	Cat# Q33226

STEP-BY-STEP METHOD DETAILS

Vector construction

Timing: 1 day

This step describes how to create plasmid and insert the sgRNA into the plasmid.

1. BsmBI enzyme was used to digest SadCas9-ABI or SpdCas9-PYL1 plasmids (CLOuD9, System Biosciences, Cat# CASCL9-100A) at 37°C for 12 h. On the next day, the mixture was incubated at 65°C for 20 min to inactivate the enzyme. Run the mixture on 1.5% agarose DNA gel, cut the correct fragments and purify the DNA by Omega DNA purification Kit. The size of SpdCas9-PYL1 and SadCas9-ABI vectors are 14049 bp and 12984 bp respectively (Morgan et al., 2017).

Material	Volume
dcas9-ABI/PYL vector	5ug
BsmBI (10000 units/mL)	3ul
10× Buffer Tango (thermo)	5ul
DTT (20 mM)	1ul
H_2O	Add to 50ul

2. Annealing and phosphorylation of sgRNA oligoes

Material	Volume
Oligo 1 (100uM)	1 ul
Oligo 2 (100uM)	1 ul
10× Ligation Buffer (NEB)	1 ul
T4 PNK (10000 units/mL)	0.5 ul
H2O	6.5 ul

Steps	Temperature	Time	Cycles
	37°C	30 min	1
	95°C	5 min	1
	5 °C/min decrease to 25°C	14 min	1
	12°C	Forever	1

3. Ligation at 20°C for 10 min, and at 37°C for 10 min

Material	Volume
Digested vectors	50 ng
Annealed Oligoes	1 ul
2× Quick ligase buffer (NEB)	5 ul
Quick ligase (NEB M2200)	1 ul
H2O	Add to 11 ul

Virus packaging

Timing: 3 days

Packaging virus for infection and artificial linking.

4. Package virus using VSV-G and psPAX2 system
   a. 18–24 h before transfection seed 12.5× 10^6 293T cells in a 10 cm plate by using DMEM/ 10% FBS/without P/S, and ensures cells reach near 80%–90% confluence when transfection.
   b. Add 16.7ug of SadCas9-ABI-sgRNA or SpdCas9-PYL1-sgRNA plasmids respectively with 11.17ug psPAX2, 7.4ug VSV-G and 450uL water to an Eppendorf tube, then mix.

Plasmid	Amount
VSV-G	7.4 ug
psPAX2	11.17 ug
SadCas9-ABI-sgRNA1.1	16.7 ug
or SpdCas9-PYL1-sgRNA1.2	16.7 ug
or SadCas9-ABI-sgRNA2.1	16.7 ug
or SpdCas9-PYL1-sgRNA2.2	16.7 ug
H2O	450 uL

   c. Add 62.5uL 2 M $CaCl_2$ to water-DNA mixture.
   d. Add 500uL 2× BES in a 15 mL conical tube.
   e. Using a Pasteur pipet and a pipet aid, release bubbles into the 2× BES-buffered saline, followed by adding the DNA mixture dropwise to the 2× BES buffered saline.
   f. Incubate at 20°C for 15 min. Solution will be slightly cloudy after 15 min.
   g. In the meantime, aspirate media off the 293T cells seeded the day before and add 10 mL fresh media (without P/S) with 25nM chloroquine.
   h. Add virus droplets to 293T cells, distributing evenly.
   i. Incubate plate at 37°C for 12 h.
   j. 18–24 h post incubation, change media and check for reporter gene expression (if applicable).
   k. Collect supernatant at 48–72 h post transfection. Virus is good for about 2 weeks at 4°C, Store concentrated virus at −80°C.

Note: The virus was concentrated by Lenti-X Concentrator (Clontech, Cat# 631231) which provides a fast and simple method for concentrating lentiviral stocks. Concentration is achieved by mixing a lentiviral supernatant with this concentration reagent, followed by a short incubation step and centrifugation in a standard centrifuge. The process is easily scaled up to accommodate larger supernatant volumes.

Virus infection

Timing: 6 days

This step describes how to infect cells with the virus.

5. Thaw MEFs from liquid nitrogen storage and plant into 24-well plate. For each well, there are 500,000 cells with 0.5 mL medium.
6. Infect MEFs with lentivirus containing SpdCas9-PLY1-purocymin and SadCas9-ABI-hygromycin.

   Note: As the proportion of the virus and medium is up to 1:3, the volume of the virus is about 166ul. Add polybrene (Stock concentration: 8mg/mL; final concentration: 10ug/mL) to the system to enhance infection efficacy.

7. Starting from the next day, the infected MEFs were selected by 2ug/mL puromycin and 300ug/mL hygromycin in DMEM medium for 5 days.
8. After selection, the second passage MEFs were used in the next step.

Chemical-dependent artificial linking

Timing: 2 days

This step describes how to induce the linking by chemical.

9. The infected MEFs containing the chemical inducible SadCas9-ABI and SpdCas9-PYL1 system were treated with 3 μM abscisic acid (ABA) for 48 h before harvest. Equal amount of DMSO (3 μM) was added into the MEF medium without ABA as control.

Chromosome conformation capture (3C)

Timing: 3 days

3C is performed to 1) identify the PSC-specific loops which are not existent in MEF, and 2) validate whether artificial linking is successfully established.

10. Crosslink
    a. 10^7 cells were harvested and digested by trypsin.
    b. The cells were washed by PBS for three times, and resuspended in 50 mL crosslink solution containing 43 mL PBS and 1.3 mL formaldehyde (stock solution 37.5%; final concentration: 1%), rotated at 20°C for 15 min.
    c. 4 mL of 2 M glycine was added to the solution and rotated 5 min at 20°C to stop crosslink.
    d. Centrifuge at 2500×*g* to remove the supernatant, and the pellet was washed with PBS for three times.

11. Cell lysis and enzyme digestion
    a. Resuspended 10^7 cells with 250 μL Hi-C lysis buffer containing proteinase inhibitor Cocktail, incubate on ice for 15 min, centrifuge on 2500×*g* at 4°C for 5 min to pellet nucleus.

Hi-C lysis buffer	Volume
1 M Tris-HCl (pH=8.0)	500 μL
5 M NaCl	100 μL
Igepal CA630	100 μL
H_2O	49.3 mL
Total volume	50 mL

Note: Hi-C lysis buffer can be stored at 4°C for three months.

b. Resuspended the nucleus pellet with 50 μL of 0.5% SDS, incubated at 62°C for 8 min.

c. 145 μL ddH_2O and 25 μL 10% Triton X-100 were added to neutralize SDS, incubated at 37°C for 15 min.

d. 25μL 10×NEBuffer2 and 20 μL 100 U MboI enzyme were added to digest DNA, incubated at 37°C for 12 h.

12. DNA end filling, ligation and reverse crosslink

a. Inactivate MboI by incubating at 62°C for 20 min, then cooled the mixture to 20°C.

b. Using dNTP to end fill the DNA fragments. Incubating the mixture at 37°C for 1 h.

Material	Volume
Reaction from the previous step	250 μL
H_2O	40.5μL
10 mM dNTP	1.5 μL
5 U/μL DNA Polymerase I, Large (Klenow) Fragment	8 μL
Total volume	300 μL

c. Then ligate the product by adding 900 μL ligation solution by incubating at 20°C for 4 h.

Material	Volume
Reaction from the previous step	300 μL
ddH2O	663 μL
10× NEB T4 DNA ligase buffer	120 μL
10% Triton X-100	100 μL
10 mg/mL BSA	12 μL
400 U/ μL T4 DNA Ligase	5 μL
Total volume	1200 μL

d. 50 μL of 20 mg/mL proteinase K and 120 μL of 10% SDS were added into the mixture and incubated at 55°C for 30 min to reverse crosslink.

e. 130μL of 5 M NaCl was added into the mixture and incubated at 68°C for 12 h.

13. DNA purification

a. Cooled the mixture to 20°C, equally separated the mixture into two 2 mL fresh tube (750 μL each). 1.6×volume absolute EtOH and 0.1×volume sodium acetate (3 M, pH 5.2) were added into each tube, mixed, and incubated at −80°C for 4 h.

b. Centrifuge on 20000×*g* at 4°C for 15 min, and remove supernatant.

c. Wash the DNA pellet twice with 800 μL of 75% EtOH, dry the pellet and dissolved by H_2O. Concentration of the DNA solution by nanodrop.

14. Check the PSC-specific DNA bands (PSC-specific looping) which are not existent in MEF by PCR

a. Collect supernatant, and using the following system for PCR amplification.

Material	Volume
DreamTaq Green PCR Master Mix 2×	25 ul
Forward primer (10uM)	1 ul
Reverse primer (10uM)	1 ul
DNA template (1 ng/ul)	6 ul
H_2O	17 ul
Total volume	50 ul

b. PCR condition

Steps	Temperature	Time	Cycles
	95°C	1min	1
	95°C	30s	35
	60°C	30s	
	72°C	1min	
	72°C	5min	1
	12°C	Forever	

c. PSC-specific loops in the two TADs selected for merging were identified (Figure 2A). The candidate loop anchors for SadCas9-ABI (green bars) or SpdCas9-PYL1 (blue bars) were selected according to H3K27ac and OCT4 peaks (Figure 2B). We compared the DNA bands between MEF and PSC, and identified three PSC-specific loops in this region based on the difference of band strength (Figure 2C, yellow arrows).

Pause point: the assay would be paused at step 13a and the sample should be stored at −80°C no more than one week.

⚠ CRITICAL: to ensure the ligation efficiency at Step 12c, the ligation time can be prolonged.

ChIP-qPCR

Timing: 4 days

CTCF ChIP-qPCR is performed to evaluate CTCF binding change in response to artificial linking.

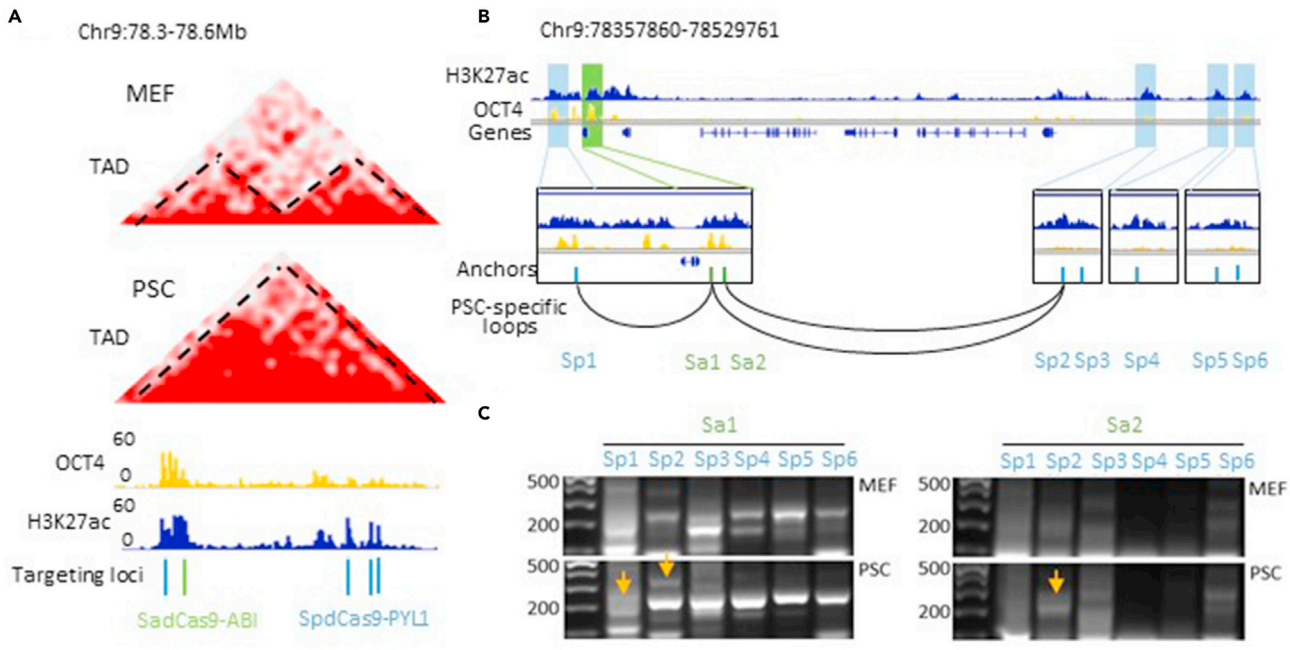

Figure 2. Identification of PSC-specific Loops by 3C

(A) The potential looping anchors by SadCas9-ABI (green bars) and SpdCas9-PYL1 (blue bars) are provide.

(B) The looping anchors for designing 3C primers are provided.

(C) DNA agarose gel showed the different bands between MEF and PSC. The yellow arrows stand for PSC-specific loops.

15. Crosslink
    a. 5×10^7 MEFs treated with or without ABA were harvested. 1% formaldehyde in PBS was used to crosslink the cells for 10 min, followed by quenching with 4 mL glycine (stock solution 2 M; final concentration: 125 mM) on ice.
    b. Cells were collected and flash frozen in liquid nitrogen, then stored at −80°C for use.

16. Cell lysis
    a. Frozen crosslinked cells were thawed on ice and then resuspended in lysis buffer I (final concentration: 50mMHEPES-KOH, pH 7.5, 140mMNaCl, 1 mMEDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, protease inhibitors).

Lysis buffer I	Volume
1M HEPES-KOH	2.5 mL
5 M NaCl	1.4 mL
0.5 M EDTA	100 μL
Glycerol	5 mL
NP-40	250 μL
Triton X-100	125 μL
1000× protease inhibitors	50 μL
H_2O	40.575 mL
Total volume	50 mL

Note: Lysis buffer I can be stored at 4°C for six months.

    b. After rotated for 10 min at 4°C, the cells were collected, and resuspended in lysis buffer II (final concentration: 10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, protease inhibitors).

Lysis buffer II	Volume
1 M Tris-HCl	500 μL
5 M NaCl	2 mL
0.5 M EDTA	100 μL
EGTA (380 g/mol)	9.51 mg
1000× protease inhibitors	50 μL
H_2O	47.35 mL
Total volume	50 mL

Note: Lysis buffer II can be stored at 4°C for six months.

    c. After rotated for 10 min at 4°C, the cells were collected, and resuspended in lysis buffer III (final concentration: 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, 0.5% N-lauroylsarcosine, protease inhibitors).

Lysis buffer III	Volume
1 M Tris-HCl	500 μL
5 M NaCl	1 mL
0.5 M EDTA	50 μL
EGTA (380 g/mol)	9.51 mg
Na-Deoxycholate (414 g/mol)	50 mg
N-lauroylsarcosine (293 g/mol)	250 mg
1000× protease inhibitors	50 μL
H_2O	48.4 mL
Total volume	50 mL

Note: Lysis buffer III can be stored at 4°C for six months.

17. Sonication
    a. Transfer cells to tube for sonication. Sonication condition: work 1 s pause 0.5 s total 30 s cycle 11.
    b. Sonicated lysates were transferred into 1.5 mL tube and centrifuged at 16000×*g* for 10 min at 4°C. The supernatant was collected for immunoprecipitation.
    c. Save 50ul as input DNA and store at −20°C.

18. Antibody precipitation
    a. Add 100ul of beads to 1.5 mL tube; add 1 mL block solution, 4°C. Collect Dynal beads using Dynal MPC, remove supernatant. Add 1.5 mL block solution and gently resuspend beads. Collect beads using Dynal MPC, remove supernatant. Wash again by 1.5 mL block solution.
    b. Resuspend beads in 250ul block solution and add 10ug antibody. Incubate at 4°C for 12 h on a rotator. Wash beads three times in 1 mL block solution, resuspend beads in 100ul block solution.
    c. The sonicated DNA was incubated with magnetic beads bound with antibody to enrich for DNA fragments at 4°C for 12 h.
    d. Beads were washed with wash buffer (final concentration: 50 mM HEPES-KOH pH 7.5, 500 mM LiCl, 1 mM EDTA pH 8.0, 0.7% Na-Deoxycholate, 1% NP-40) and TE buffer (final concentration: 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl) in order.

Wash buffer	Volume
1 M HEPES-KOH	2.5 mL
8 M LiCl	3.125 mL
0.5 M EDTA	100 μL
NP-40	500 μL
Na-Deoxycholate (414 g/mol)	350 mg
H_2O	43.775 mL
Total volume	50 mL

Note: Wash buffer can be stored at 20°C for six months.

TE buffer	Volume
1 M Tris-HCl	500 μL
0.5 M EDTA	100 μL
H_2O	49.4 mL
Total volume	50 mL

Note: TE buffer can be stored at 20°C for six months.

    e. Beads were removed by incubation at 65°C for 30 min in elution buffer (final concentration: 50mMTris-HCl pH 8.0, 10mMEDTA,1%SDS).

Elution buffer	Volume
1 M Tris-HCl	2.5 mL
0.5 M EDTA	1 mL
10% SDS	5 mL
H_2O	41.5 mL
Total volume	50 mL

Note: Elution buffer can be stored at 20°C for six months.

19. Reverse crosslink
    a. Cross-links were reversed at 65°C for 12 h.
20. DNA purification
    a. To purify eluted DNA, 200 mL TE was added and then RNA was degraded by incubation after addition of 8ul 10 mg/mL RNase A at 37°C for 2 h.
    b. Protein was degraded by addition of 4ul 20 mg/mL proteinase K and incubation at 55°C for 2 h.
    c. Phenol: chloroform: isoamyl alcohol extraction was performed followed by an ethanol precipitation.
    d. The DNA was then resuspended in 50 mL TE and used for qPCR.
21. qPCR
    a. qPCR was performed using SYBR qPCR Master mix. All qPCR experiments were triplicated.

In situ HiC

Timing: 4 days

In situ HiC is performed to validate whether TADs are reorganized in response to artificial linking.

22. Crosslink
    a. The MEFs treated with or without ABA were harvest and digested by trypsin.
    b. The MEFs were washed by PBS for three times, and resuspended in 50 mL crosslink solution containing 43 mL PBS and 1.3 mL formaldehyde (stock solution 37.5%; final concentration: 1%), rotated at 20°C for 15 min.
    c. 4 mL of 2 M glycine was added to the solution and rotated 5 min at 20°C to stop crosslink. Centrifuge at 2500×*g* to remove the supernatant, and the pellet was washed with PBS for three times.
23. Cell lysis and enzyme digestion
    a. Resuspended 10^7 cells with 250 μL Hi-C lysis buffer containing proteinase inhibitory Cocktail, incubate on ice for 15 min and centrifuge at 2500×*g* to collect nucleus pellet. Resuspended the nucleus pellet with 50 μL of 0.5% SDS, incubated at 62°C for 8 min. 145 μL ddH2O and 25 μL 10% Triton X-100 were added to neutralize SDS, incubated at 37°C for 15 min. 25μL 10×NEBuffer2 and 20 μL 100 U MboI enzyme were added to digest DNA, incubated at 37°C for 12 h (see step 11).

24. DNA end filling, ligation and reverse crosslink
    a. Inactivate MboI by incubating at 62°C for 20 min, then cooled the mixture to 20°C.
    b. Using dNTP to end fill the DNA fragments, in which dATP was biotin labeled. Incubating the mixture at 37°C for 1 h.

Material	Volume
0.4 mM Biotin-14-dATP	37.5μL
10 mM dCTP	1.5 μL
10 mM dGTP	1.5 μL
10 mM dTTP	1.5 μL
5 U/μL DNA Polymerase I, Large (Klenow) Fragment (NEB M0210)	10μL

    c. Then ligate the product by adding 948 μL ligation solution and incubating at 20°C for 4 h.

Material	Volume
ddH2O	660 μL
10× NEB T4 DNA ligase buffer	150 μL
10% Triton X-100	125 μL
50 mg/mL BSA	3 μL
400 U/ μL T4 DNA Ligase	10 μL

d. 50 μL of 20 mg/mL proteinase K and 120 μL of 10% SDS were added into the mixture and incubated at 55°C for 30 min to reverse crosslink.
e. 130μL of 5 M NaCl was added into the mixture and incubated at 68°C for 12 h.

25. Sonication and fragment collection
    a. Cooled the mixture to 20°C, equally separated the mixture into two 2 mL fresh tube (0.75 mL each). 1.6 × volume absolute alcohol and 0.1 × volume sodium acetate (3 M, pH 5.2) were added into each tube, incubated at −80°C for 4 h.
    b. Centrifuge on 20000×*g* at 4°C for 15 min, and remove supernatant. Wash the DNA pellet twice with 800 μL of 75% alcohol, dry the pellet and dissolved by 130 μL of H_2O.
    c. Transfer the solution to Covaris millitube, sonicate to make the DNA fragment enriching at 300–500 bp (Figure 3A).

Equipment	Covaris LE220 (Covaris, Woburn, MA)
Volume of Library	130μL in a Covaris microTUBE
Fill Level	10
Duty Cycle	15
PIP	50
Cycles/Burst	200
Time	200 s

    d. Transfer the DNA solution from Covaris millitube to a fresh tube; add 70ul H2O to 200 μL total volume. Add 140 μL (0.7× volume) AMPure XP beads, incubate at 20°C for 5 min.
    e. Magnetically separate AMPure XP beads from solution, and transfer the supernatant to a fresh tube. The size of the fragments in the supernatant is less than 500 bp (Figure 3B).
    f. Add another 30 μL (0.15× volume; to a final 0.85× volume) AMPure XP beads to the solution, incubate at 20°C for 5 min. Magnetically separate AMPure XP beads from solution and remove the supernatant. The size of the fragments on AMPure XP beads is between 300–500 bp while the residues in the supernatant is less than 300 bp (Figure 3C).
    g. Wash the beads twice with 700 μL of 75% alcohol, air dry the beads absolutely. 100 μL of H_2O was added to dissolve DNA, incubated at 37°C for 15 min, and concentrated by nanodrop.

26. Biotin pull down
    a. For each sample, prepare 75 μL of 10 mg/mL Dynabeads MyOne Streptavidin T1 beads, wash the beads with 200 μL of 1× tween washing buffer (TWB). The beads were resuspended in 100 μL of 2× binding buffer (BB), 100 μL DNA sample was added and incubated at 20°C for 45 min.

Tween washing buffer	Volume
1 M Tris-HCl	250 μL
0.5 M EDTA	50 μL
5 M NaCl	10 mL
Tween 20	25 μL
H_2O	39.675 mL
Total volume	50 mL

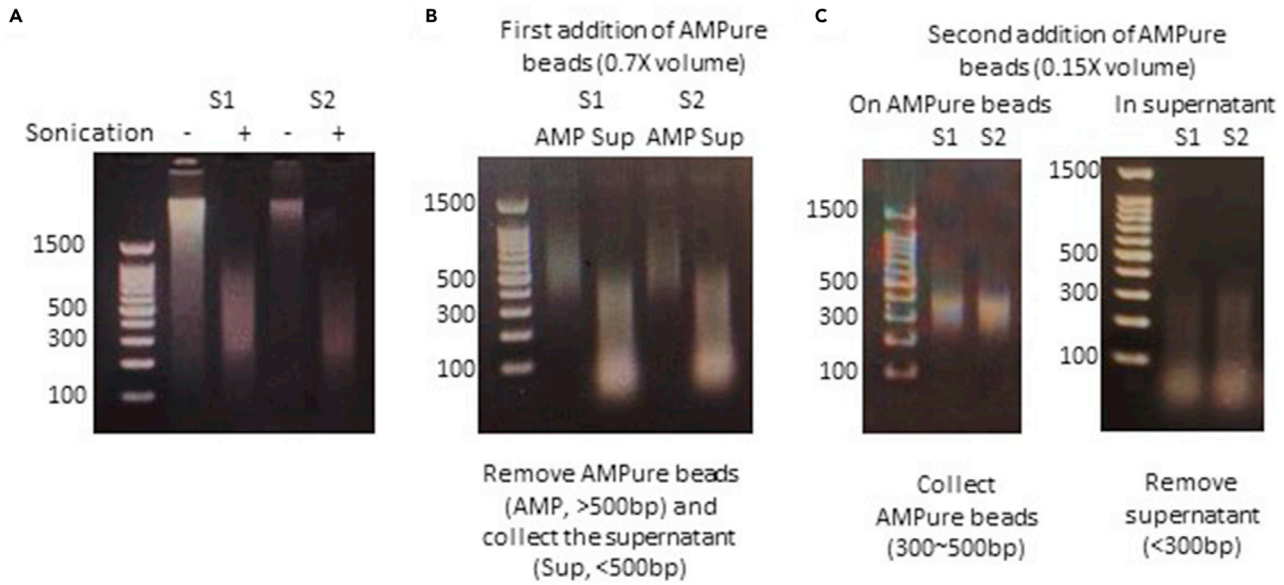

Figure 3. The length of DNA fragments from sonication to selection by AMPure beads

(A) DNA was sheared by sonication. Sonication condition should be optimized to ensure that DNA fragments are enriched around 300–500 bp. S1 and S2 stand for sample 1 and 2.

(B) First, 0.7× volume (as compared to DNA solution) of AMPure beads were added to separate DNA fragments more than 500 bp length. The larger DNA fragments (>500 bp) were maintained on the beads while the smaller fragments (<500 bp) were in the supernatant.

(C) Second, another 0.15× volume of AMPure beads were added to the supernatant of last step to separate DNA fragments less than 300 bp length. The larger DNA fragments (300–500 bp) were maintained on the beads (left panel) while the smaller fragments (<300 bp) were in the supernatant (right panel). The beads were collected and the DNA fragments were dilute by H_2O for next step use.

Note: TWB buffer can be stored at 20°C for six months.

2× Binding buffer	Volume
1 M Tris-HCl	500 μL
0.5 M EDTA	100 μL
5 M NaCl	20 mL
H_2O	29.4 mL
Total volume	50 mL

Note: 2× Binding buffer can be stored at 20°C for six months.

b. Magnetically separate T1 beads, and washed with 500 μL of 1× TWB at 55°C for 2 min, remove the supernatant. Repeat washing.

27. End filling and remove dangling ends

a. Wash the beads with 100 μL of 1× T4 buffer, and transfer to a fresh tube. 100 μL of the following solution was added to the beads, and incubated at 20°C for 30 min to fill the end and remove dangling ends.

88 μL	1× NEB T4 DNA ligase buffer with 10 mM ATP
2 μL	25 mM dNTP mix
5 μL	10 U/μL NEB T4 PNK
4 μL	3 U/μL NEB T4 DNA polymerase I
1 μL	NEB DNA polymerase I, Large (Klenow) Fragment

b. Magnetically separate T1 beads, and washed with 500 μL of 1× TWB at 55°C for 2 min, remove the supernatant. Repeat washing.

28. A-tailing
    a. Wash the beads with 100 μL of 1× NEB buffer 2, and transfer to a fresh tube. 100 μL of the following solution was added to the beads, and incubated at 37°C for 30 min to A-tailing.

90 μL	1× NEB buffer 2
5 μL	10 mM dATP
5 μL	5 U/μL NEB Klenow 3′→5′ exo minus

    b. Magnetically separate T1 beads, and washed with 500 μL of 1× TWB at 55°C for 2 min, remove the supernatant. Repeat washing.

29. Adapter adding
    a. Wash the beads with 100 μL of 1× Quick ligation reaction buffer, and transfer to a fresh tube. 50 μL of the following solution was added to the beads, and incubated at 20°C for 15 min to add adapter.

25 μL	2× Quick ligation reaction buffer
2 μL	NEB quick ligase
3 μL	Illumine indexed adapter
20μL	H_2O

    b. Add 2.5 μL of USER enzyme to the solution and incubate at 37°C for 15 min. Magnetically separate T1 beads, and washed with 500 μL of 1× TWB at 55°C for 2 min, remove the supernatant. Repeat washing.
    c. Add 50 μL of 1× Tris buffer (10 mM) to wash, and transfer to a fresh tube. Add 20 μL of H2O and incubate at 98°C for 10 min to dissolve DNA. Measure concentration by Qubit.

1× Tris buffer	Volume
1 M Tris-HCl buffer	0.5 mL
H_2O	49.5 mL
Total volume	50 mL

Note: 1× Tris buffer can be stored at 20°C for six months.

30. PCR and fragment selection
    a. Collect supernatant, and using the following system for PCR amplification. These materials come from the TruePrep DNA Library Prep V2 for illumina Kit.

Material	Volume
5 × TAB	10 ul
PPM	5 ul
N5XX	5 ul
N7XX	5 ul
TAE	1 ul
H2O	24 ul

b. PCR condition

Steps	Temperature	Time	Cycles
	72°C	3min	1
	98°C	30s	1
	98°C	15s	5–9
	60°C	30s	
	72°C	3min	
	72°C	5min	1
	12°C	Forever	

c. After PCR reaction, 32.5 μL (0.65× volume) of AMPure XP beads were added and incubate at 20°C for 5 min to remove fragments longer than 600 bp. The supernatant was transferred to a fresh tube.
d. Another 7.5 μL (0.15× volume; to a final 0.8× volume) of AMPure XP beads were added and incubate at 20°C for 5 min to collect fragments between 300–500 bp.
e. Wash beads with 700 μL of 75% alcohol twice, air dry absolutely.
f. 20 μL 1×Tris buffer was added to the beads, incubate at 37°C for 15 min to dissolve DNA. Concentrate DNA by Qubit.
g. The DNA sample is ready for sequencing.

Pause point: the assay would be paused at step 25a and the sample should be stored at −80°C no more than one week.

⚠ CRITICAL: to ensure the ligation efficiency at step 24c, the ligation time can be prolonged. Select exact sonication time and cycle to ensure the fragments enriched at 300–500bp at step 25c (Figure 3A).

MEF reprogramming

Timing: 12 days

MEF reprogramming is performed to investigate the influence of TADs reorganization on cell fate transitions.

31. Experimental design
    a. MEFs are recovered into 6 cm plate and expanded.
    b. The MEFs are sub-cultured and planted into 12-well plates containing 1.5×10^4 cells/well as follows:
    c. Plate A contains 12 wells (half treated with ABA and half without ABA) which are used to Alkaline Phosphatase (AP) staining and count OCT4-GFP positive clones after reprogramming.
    d. Plate B contains 14 wells (half treated with ABA and half without ABA) for RNA extraction and RT-qPCR. The time points for cell collection is day0, 2, 4, 6, 8, 10 and 12.
    e. Plate C contains 4 wells (half treated with ABA and half without ABA) for collection of iPSC clones and further culture.
32. Reprogramming
    a. MEFs are cultured in iPSC medium (DMEM supplemented with 10% FBS, 10% Knockout serum replacement, 0.1 mM b-mercaptoethanol, NEAA, 2 mM Glutamax, Nucleoside MIX, leukemia inhibitory factor (LIF)) with or without 3uM ABA. Medium is changed every day for 12 days to get the iPSC clones.

iPSC medium	Volume
DMEM	375 mL
FBS	50 mL
Knockout serum replacement	50 mL
Nucleoside MIX	5 mL
Glutamax	5 mL
NEAA	5 mL
b-mercaptoethanol	0.5 mL
LIF	9.5 mL
Total volume	500 mL

Note: iPSC medium can be stored at 4°C for three months.

RT-qPCR

Timing: 1 day

RT-qPCR is performed to check the expression change of related genes in response to TAD reorganization

33. RNA extraction and reverse transcription
    a. Total RNA was extracted from cell pellets using RNAzol reagent (MRC) and cDNA was synthesized using Primesoript RT Master Mix (Takara).
34. Real time qPCR
    a. qPCR was performed using SYBR qPCR Master Mix on LightCycler 480 II system.
35. Data analysis
    a. The fold change (FC) of experimental group versus control group was calculated. △Ct was calculated as △Ct = Ct (test gene) – Ct (Ref. gene). △△Ct was calculated as △△Ct = △Ct (experimental group) – △Ct (control group). The FC of a test gene in experimental group versus control group was calculated as FC = 2^(-△△Ct). Each gene tested in triplicates in every independent experiment, and all experiments were triplicated.

Colony formation assay (CFA)

Timing: 1 day

CFA is performed to evaluate reprogramming efficiency in response to manipulation of TAD reorganization.

36. After reprogramming, the iPSC colonies are stained with alkaline phosphatase (AP) for 1 h, followed by washing with PBS for three times. The positively stained clones are counted and compared between the experimental and control groups.

Flow cytometry

Timing: 1 day

Flow cytometry is performed to evaluate reprogramming efficiency by counting OCT4-positive cells in response to manipulation of TAD reorganization.

37. The cells were harvested at 12th day during reprogramming, wash twice with PBS, and the OCT4-GFP positive cell number was also considered for reprogramming efficiency by flow

cytometry. All colony formation assays and OCT4-GFP flow cytometry assays were performed for three biological replicates.

EXPECTED OUTCOMES

The hypothesis is that we can promote cell fate transitions through manipulating of TAD structures. For the strategy to induce TAD reorganization, two genome loci belonging to two neighboring TADs were targeted by SadCas9-ABI and SpdCas9-PYL1 respectively, and a linking would be established by addition of ABA. The linking would provide a driving force to shorten the distance of the two TADs, which results in TAD merging. TAD reorganization in response to the artificial linking is validated by HiC, as evidenced by 1) the HiC heatmap that showed the merging of the two TADs in MEFs with artificial linking compared to the control MEFs, and 2) an increased insulation score. The insulation score was obtained by aggregating the amount of interactions that occur across chromosome bins which were divided by mean interaction amount of the whole chromosome and then logarithmized (Crane et al., 2015). In general, bins with a high insulation effect have a low insulation score whereas bins with low insulation effect have a high insulation score. Therefore, a more positive score (+ABA) means less insulation between the two TADs. The MEFs with the modified TAD structure were reprogrammed by adding the four Yamanaka factors OCT4, SOX2, KLF4 and MYC. The reprogramming efficiency was increased in TAD modified group, as evidenced by increased iPSC colonies and OCT4-GFP positive cell numbers compared to those of the control MEFs. All these data can be seen in the Figure 2 of (Wang et al., 2021).

LIMITATIONS

dCas9-based genome targeting provides a powerful tool to precisely target any genome loci interested. Previous strategy using this tool to intervene 3D chromatin structure is to construct lower-ordered structure such as chromatin loops (Morgan et al., 2017). This paper is the first paper using this powerful tool to reorganize TAD structure (Wang et al., 2021). However, this protocol also has limitations for manipulating TAD structures. For instance, it is only suitable to merge two TADs into one large TAD, which cannot be used to separate one TAD into two small TADs. Moreover, whether any loci within TAD for genome linking can efficiently induce TAD merging is needed to validate. Furthermore, how to regulate other higher-ordered chromatin 3D structure such as A/B compartment is still unknown. The issue that whether the tool is suitable for manipulating A/B compartment switch is valuable to discuss. Together, the potential wide application of this tool for intervening 3D genome is expected and promising.

TROUBLESHOOTING

Problem 1

Enhanced reprogramming efficiency may be caused by the artificial linking rather than TAD reorganization (step 33–37).

Potential solution

To exclude the possibility that only artificial linking works, the expressions of other genes involved in the *Dppa5a* TAD were tested. The hypothesis is that if reprogramming is regulated by TAD reorganization, all the genes in the reorganized TAD will be influenced rather than *Dppa5a* alone. Instead, if reprogramming is regulated by the artificial linking, only *Dppa5a* expression will be influenced without affecting other genes. The qPCR results showed that the overall genes in the TADs were activated after the artificial manipulation. However, as a negative control, overexpression of *Dppa5a* only did not significantly induce the expression of other genes in the same TAD. These results demonstrate that TAD reorganization rather than artificial linking takes effect. All these data can be seen in the Figure S2 of (Wang et al., 2021).

Problem 2

Which genome region within TAD selected for artificial linking are most potentially effective to induce TAD reorganization (step 3 in before you begin)?

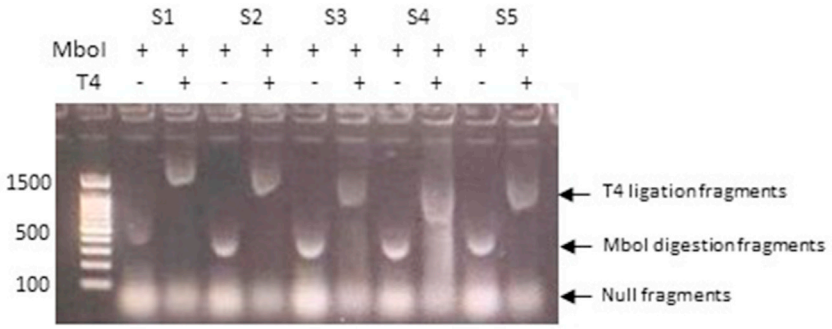

Figure 4. The DNA bands distribution upon MboI digestion followed by T4 ligation

DNA was digested to less than 500 bp length by MboI, and was ligated to more than 1000 bp length by T4 ligase. Null fragments mean useless small fragments. S1-S5 stands for sample 1–5.

Potential solution

New loci locating at different places within TAD should be selected for dCas9-based genome linking, followed by testing the efficiency of TAD merging.

Problem 3

Low efficiency for artificial linking (step 9).

Potential solution

Low efficiency for artificial linking may occur for several reasons. We need to (1) increase the viral amount of sgRNA, if the problem is due to a low lentiviral transduction, (2) measure dCas9 expression levels in MEFs, (3) increase the amount of ABA, and (4) if the problem continues, design and test more sgRNAs.

Problem 4

Low pulldown efficacy of biotin-labeled DNA fragments by Dynabeads MyOne Streptavidin T1 beads in Hi-C (step 26).

Potential solution

To keep a higher pulldown efficacy, the experimenter should make sure high efficacy of MboI digestion and T4 ligation (Figure 4). The technician should perform PCR for quality control in each step. The time of ligation can be prolonged if the ligation efficacy is lower.

Problem 5

Low effective reads of Hi-C data (step 30).

Potential solution

Low effective reads maybe mainly result from high unmapped pairs, high rate of dangling ends and high duplicate rate by PCR. To reduce the adverse influence by these factors, the experimenter should make sure the DNA fragments are between 300–500 bp length (Figure 3C), prolong the time and increase the amount of Large (Klenow) Fragment to effectively remove dangling ends, and increase the biotin pulldown efficacy to reduce duplicates.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Junjun Ding (dingjunj@mail.sysu.edu.cn).

Materials availability

This study did not generate any unique reagents.

Data and code availability

The accession number for HiC data reported in this paper is Sequence Read Archive (SRA): PRJNA650173.

ACKNOWLEDGMENTS

This research was funded by grants from the National Key Research and Development Program (2017YFA0102800), the National Natural Science Foundation of China (grant nos. 31970811, 31771639, and 32170798), the Frontier Research Program of Bioland Laboratory (Guangzhou Regenerative Medicine and Health Guangdong Laboratory) (2018GZR110105007), the Guangdong Innovative and Entrepreneurial Research Team Program (2016ZT06S029) to J.D., and the National Natural Science Foundation of China (grant no. 81703086) to J.W.

AUTHOR CONTRIBUTIONS

J.D. conceptualized and supervised the project. J.W. developed, wrote, and edited the protocol. Q.M., P.F., Q.T., and J.S. performed the experiments and wrote and edited the protocol. H.Y. performed bioinformatic analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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