Protocol

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.
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.
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. 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 |
| j504044ptoethanol | Sigma | Cat# M6250 |
| NEAA | Thermo Fisher | Cat# 11400050 |
| GlutaMax | Thermo Fisher | Cat# 35050061 |
| Mbol | 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 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| UltraPure™ 0.5 M EDTA | Thermo Fisher | Cat# 15575-038 |
| EGTA | Sigma | Cat# E7-42-5 |
| Glycerol | Sigma | Cat# 56-81-5 |
| NP-40 | Sangon Biotech | Cat# 9016-45-9 |
| Tween-20 | Amresco | Cat# 0777-1L |
| 50X TAE buffer | Sangon Biotech | Cat# B548101-0500 |
| DMSO | Sigma | Cat# D2650-100ML |
| EGTA-Ca | 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# TD140 |
| 5 M NaCl solution | Sigma | Cat# S5150 |
| Abscisic acid | Sigma | Cat# A1049 |

Critical commercial assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| RNAzol | MRC | Cat# RN190-500 |
| EndoFree Plasmid Midi Kit | Cwbio | Cat# CW21055 |
| 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# D5200-02 |
| TIANquick Midi Purification Kit | TIANGEN | Cat# DP204-03 |
| TIANamp Genomic DNA Kit | TIANGEN | Cat# DP304-02 |
| SYBR qPCR Master Mix | Vazyme | Cat# G711-00 |
| Leukocyte Alkaline Phosphatase Kit | Sigma | Cat# 86R |
| Alkaline Phosphatase Stain Kit | Yeasen | Cat# 40749E560 |
| 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™ 1x dsDNA HS Assay Kit | Invitrogen | Cat# Q33230 |
| Primerscript RT Master Mix | Takara | Cat# RR036A |

Cell lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse embryonic stem cell line V6.5 | Laboratory of R. Jaenisch | RRID: CVCL_C86S |
| 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

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| mm10 | ENSEMBL release 90 | [https://asia.ensembl.org/Mus_musculus/](https://asia.ensembl.org/Mus_musculus/) |
| TrimmGalore 0.4.4_dev | [https://www.bioinformatics.babraham.ac.uk/projects/trim_galore](https://www.bioinformatics.babraham.ac.uk/projects/trim_galore) | [https://github.com/FelixKrueger/TrimmGalore](https://github.com/FelixKrueger/TrimmGalore) |
| bowtie2 v2.3.0 | [https://bioinformatics.babraham.ac.uk/projects/trim_galore](https://bioinformatics.babraham.ac.uk/projects/trim_galore) | [https://github.com/BenLangmead/bowtie2](https://github.com/BenLangmead/bowtie2) |
| samtools v1.3.1 | [https://github.com/samtools/samtools](https://github.com/samtools/samtools) | [https://github.com/FelixKrueger/TrimmGalore](https://github.com/FelixKrueger/TrimmGalore) |
| Bedtools v2.26.0 | [https://github.com/arq5x/bedtools](https://github.com/arq5x/bedtools) | [https://github.com/FelixKrueger/TrimmGalore](https://github.com/FelixKrueger/TrimmGalore) |
| macs2 v2.1.2 | [https://github.com/taoiu/MACS](https://github.com/taoiu/MACS) | | |
| HTSeq-count v0.11.2 | [https://github.com/simon-anders/htseq](https://github.com/simon-anders/htseq) | | |
| edgeR v3.26.5 | [https://bioconductor.org/packages/release/bioc/html/edgeR.html](https://bioconductor.org/packages/release/bioc/html/edgeR.html) | | |
| HiC-Pro v2.10.0 | [https://github.com/servent/HiC-Pro](https://github.com/servent/HiC-Pro) | | |
| HiCRep v1.8.0 | [https://bioconductor.org/packages/release/bioc/html/hicrep.html](https://bioconductor.org/packages/release/bioc/html/hicrep.html) | | |
| TopDom v0.0.2 | [https://github.com/HenrikBengtsson/TopDom](https://github.com/HenrikBengtsson/TopDom) | | |

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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).

2. Annealing and phosphorylation of sgRNA oligoes

3. Ligation at 20°C for 10 min, and at 37°C for 10 min
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 \times 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.
   c. Add 62.5uL 2 M CaCl\textsubscript{2} to water-DNA mixture.
   d. Add 500uL 2× BES-buffered saline, followed by adding the DNA mixture dropwise to the 2× BES buffered saline.
   e. Incubate at 20°C for 15 min. Solution will be slightly cloudy after 15 min.
   f. 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.
   g. Add virus droplets to 293T cells, distributing evenly.
   h. Incubate plate at 37°C for 12 h.
   i. 18–24 h post incubation, change media and check for reporter gene expression (if applicable).
   j. 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

| Material                        | Amount   |
|---------------------------------|----------|
| Digested vectors                | 50 ng    |
| Annealed Oligos                 | 1 ul     |
| 2× Quick ligase buffer (NEB)    | 5 ul     |
| Quick ligase (NEB M2200)        | 1 ul     |
| H\textsubscript{2}O             | Add to 11 ul |

| 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  |
| H\textsubscript{2}O             | 450 ul   |
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 166μL. Add polybrene (Stock concentration: 8mg/mL; final concentration: 10μg/mL) to the system to enhance infection efficacy.

7. Starting from the next day, the infected MEFs were selected by 2μg/mL puromycin and 300μg/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. 10⁷ 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 10⁷ 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 |
| H₂O | 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 ddH2O 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.

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

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 at 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 H2O.
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  |
|-----------------------------------------------|---------|
| 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 |

| Material                                      | Volume  |
|-----------------------------------------------|---------|
| DreamTaq Green PCR Master Mix 2x             | 25 ul   |
| Forward primer (10uM)                        | 1 ul    |
| Reverse primer (10uM)                        | 1 ul    |
| DNA template (1 ng/ul)                       | 6 ul    |
| H2O                                           | 17 ul   |
| Total volume                                  | 50 ul   |
b. PCR condition

| Steps   | Temperature | Time  | Cycles |
|---------|-------------|-------|--------|
| 1       | 95°C        | 1min  | 1      |
| 2       | 95°C        | 30s   | 35     |
| 3       | 60°C        | 30s   |        |
| 4       | 72°C        | 1min  |        |
| 5       | 72°C        | 5min  | 1      |
| 6       | 12°C        | Forever|       |

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) or SpdCas9-PYL1 (blue bars) are provided.

(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 × 10^4 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: 50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, protease inhibitors).

   Lysis buffer I
   | Component                        | 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 |
   | 1000X protease inhibitors        | 50 μL  |
   | H2O                              | 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
   | Component                        | 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|
   | 1000X protease inhibitors        | 50 μL  |
   | H2O                              | 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
   | Component                        | 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 |
   | 1000X protease inhibitors        | 50 μL  |
   | H2O                              | 48.4 mL|
   | Total volume                     | 50 mL  |
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  | 350 mg |
| (414 g/mol)      |        |
| H₂O              | 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 |
| H₂O              | 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   |
| H₂O              | 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 8 µl 10 mg/mL RNase A at 37°C for 2 h.
   b. Protein was degraded by addition of 4 µl 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 2500xg to remove the supernatant, and the pellet was washed with PBS for three times.

23. Cell lysis and enzyme digestion
   a. Resuspended 10⁷ cells with 250 µL Hi-C lysis buffer containing proteinase inhibitory Cocktail, incubate on ice for 15 min and centrifuge at 2500xg 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. 25 µL 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.
   c. Then ligate the product by adding 948 µL ligation solution and incubating at 20°C for 4 h.
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. 130 μL 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 x volume absolute alcohol and 0.1 x volume sodium acetate (3 M, pH 5.2) were added into each tube, incubated at −80°C for 4 h.
b. Centrifuge on 20000 x 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 H2O.
c. Transfer the solution to Covaris millitube, sonicate to make the DNA fragment enriching at 300–500 bp (Figure 3A).

d. Transfer the DNA solution from Covaris millitube to a fresh tube; add 70μl H2O to 200 μL total volume. Add 140 μL (0.7 x 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 x volume; to a final 0.85 x 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 H2O 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 x tween washing buffer (TWB). The beads were resuspended in 100 μL of 2 x binding buffer (BB), 100 μL DNA sample was added and incubated at 20°C for 45 min.

| 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 |

| 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 |

| 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  |
| H2O                  | 39.675 mL |
| Total volume         | 50 mL  |
Note: TWB buffer can be stored at 20°C for six months.

| 2x Binding buffer | Volume   |
|-------------------|----------|
| 1 M Tris-HCl      | 500 µL   |
| 0.5 M EDTA        | 100 µL   |
| 5 M NaCl          | 20 mL    |
| H₂O               | 29.4 mL  |
| Total volume      | 50 mL    |

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

b. Magnetically separate T1 beads, and washed with 500 µL of 1x 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 1x 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   | H2O                        |

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.

**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 x 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 x volume; to a final 0.8 x 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 x 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 $1.5 \times 10^4$ 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 3μM 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 Primescript 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. \( \Delta \text{Ct} \) was calculated as \( \Delta \text{Ct} = \text{Ct} \text{ (test gene)} - \text{Ct} \text{ (Ref. gene)} \). \( \Delta \Delta \text{Ct} \) was calculated as \( \Delta \Delta \text{Ct} = \Delta \text{Ct} \text{ (experimental group)} - \Delta \text{Ct} \text{ (control group)} \). The FC of a test gene in experimental group versus control group was calculated as \( \text{FC} = 2^{\Delta \Delta \text{Ct}} \). 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 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)?
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.

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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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