Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a
- Confirmed

☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ The statistical test(s) used AND whether they are one- or two-sided

☐ Only common tests should be described solely by name; describe more complex techniques in the Methods section.

☐ A description of all covariates tested

☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

- RT-qPCR: CFX 384 detection system (Bio-Rad)
- Next-generation sequencing: NovaSeq 6000 System (Illumina)

Data analysis

- fastqc 0.11.8
- MultiQC 1.0.dev0
- trimmomatic 0.38
- Bowtie2 2.2.5
- SAMtools 1.9
- deepTools 3.1.3
- capC-MAP 1.1.3
- bedGraphToBigWig 2.10
- bigWigAverageOverBed 2
- bigWigMerge 2
- bedtools 2.27.1
- Housekeeping and Reference Transcript Atlas database 1.0
- MACS2 2.1.1.20160309
- cooTools 0.4.0-dev
- hic2cool 0.8.3
- R 4.3.2
- WebGestalt R package 0.4.6
- Inflection R package 1.3.6
CTCF R package 0.99.11
UCSC genome browser (https://genome.ucsc.edu/)
AMIGO browser (https://amigo.geneontology.org/amigo/)
3D Genome browser (http://3dgenome.fsm.northwestern.edu/)
4DN Data portal (https://data.4dnucleome.org/)
rcompanion R package 2.4.36

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy.

All the generated ChIP-seq data are publically available through GEO (GSE252218).
All the generated Capture-C data are publically available through GEO (GSE252080).

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

- Reporting on sex and gender: NA
- Reporting on race, ethnicity, or other socially relevant groupings: NA
- Population characteristics: NA
- Recruitment: NA
- Ethics oversight: NA

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

- Sample size: For all the transgenic ESC lines we generated at least two clonal lines with the same genotype. Taking into account the amount of different cell lines generated and that the clones from the same cell line were generally responding in a similar manner, we decided to keep a minimum of two clones per investigated genotype.
- Data exclusions: No data was excluded.
- Replication: All experiments were performed independently at least twice for each clonal line (i.e. at least four biological replicates per genotype) and all the attempts at data replication were successful. The exact number of biological replicates is described in the corresponding figure legends.
- Randomization: Randomization was not relevant for our study as the sample sizes of the different experiments were too small for randomization.
- Blinding: The investigators were not blinded to allocation during experiments and outcome assessment. There was one person in charge of generating and characterizing all the transgenic cell lines and we did not have additional personal that could be solely in charge of analyzing the data. Blinding is typically used with randomization and large sample sizes, which, as stated before, does not apply to our experiments.
### Behavioural & social sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Study description | NA |
|-------------------|----|
| Research sample   | NA |
| Sampling strategy | NA |
| Data collection   | NA |
| Timing            | NA |
| Data exclusions   | NA |
| Non-participation | NA |
| Randomization     | NA |

### Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Study description | NA |
|-------------------|----|
| Research sample   | NA |
| Sampling strategy | NA |
| Data collection   | NA |
| Timing and spatial scale | NA |
| Data exclusions   | NA |
| Reproducibility   | NA |
| Randomization     | NA |
| Blinding          | NA |

**Did the study involve field work?**

- [ ] Yes
  - [x] No

### Field work, collection and transport

| Field conditions | NA |
|------------------|----|
| Location         | NA |
| Access & import/export | NA |
| Disturbance      | NA |

### Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.
### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ❑ | Antibodies |
| ❑ | Eukaryotic cell lines |
| ❑ | Palaeontology and archaeology |
| ❑ | Animals and other organisms |
| ❑ | Clinical data |
| ❑ | Dual use research of concern |
| ❑ | Plants |

### Antibodies

**Antibodies used**

| RAD21 (abcam, ab154769, lot#GR32241) | 10 μg /ChIP-seq sample |
| H3K27ac (Active Motif, 39133, lot#31521015) | 3 μg /ChIP-seq sample |

**Validation**

RA021 antibody was validated by the manufacturer: Rhodes JDP et al., Cohesin Disrupts Polycomb-Dependent Chromosome Interactions in Embryonic Stem Cells. Cell Rep. 2020 Jan 21;30(3):820-835.e10. doi: 10.1016/j.celrep.2019.12.057. PMID: 31968256; PMCID: PMC6988126.

H3K27ac antibody was validated in: Cruz-Molina, S. et al. PRC2 Facilitates the Regulatory Topology Required for Poised Enhancer Function during Pluripotent Stem Cell Differentiation. Cell Stem Cell 20, 1–17 (2017).

### Eukaryotic cell lines

**Policy information about** [cell lines and Sex and Gender in Research](#)

**Cell line source(s)**

Male mouse ESC line E14Tg2a was used for all experiments. This cell line was a kind gift from Joanna Wysocka’s lab (Stanford University). All the rearrangements were done using CRISPR/Cas9 and the gRNAs are described in Supplementary Data 3. Using the E14Tg2a mESC, the following cell lines were generated in this work:

- Gbx2/Akb18 locus: Δ3xCTCF, 71 Kb INV, Δ3xCTCF:71 Kb INV, ∆Prom Gbx2, Δ3xCTCF:ΔProm Gbx2, ΔCTCF SE Gbx2, Gbx2 INV, Gbx2 INV:Δ3xCTCF
- Six3/Six2 locus: Δ6xCTCF, 156 Kb INV, Δ6xCTCF:156 Kb INV, Six3-/-, Δ6xCTCF:Six3-/-, Δ4xCTCF, Δ4xCTCF:Six3-/-, Δ6xCTCF:Six2-/-, 226 Kb INV

**Authentication**

All the rearrangements were authenticated by PCR genotyping (see supplementary figures).

**Mycoplasma contamination**

The WT E14 mESC are regularly tested and no contamination has been detected.

**Commonly misidentified lines**

None

### Palaeontology and Archaeology

**Specimen provenance**

NA

**Specimen deposition**

NA

**Dating methods**

NA

Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

**Ethics oversight**

NA

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Animals and other research organisms

**Policy information about** [studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research](#)

**Laboratory animals**

NA

**Wild animals**

NA
Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Clinical data

Policy information about [clinical studies](#)

All manuscripts should comply with the ICMJE [guidelines for publication of clinical research](#) and a completed [CONSORT checklist](#) must be included with all submissions.

| Clinical trial registration | NA |
|-----------------------------|----|
| Study protocol              | NA |
| Data collection             | NA |
| Outcomes                    | NA |

### Dual use research of concern

Policy information about [dual use research of concern](#)

#### Hazards

Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

| No | Yes |
|----|-----|
| ☒  |     |
| ☐  | Public health |
| ☒  | National security |
| ☐  | Crops and/or livestock |
| ☒  | Ecosystems |
| ☐  | Any other significant area |

#### Experiments of concern

Does the work involve any of these experiments of concern:

| No | Yes |
|----|-----|
| ☒  | Demonstrate how to render a vaccine ineffective |
| ☒  | Confer resistance to therapeutically useful antibiotics or antiviral agents |
| ☒  | Enhance the virulence of a pathogen or render a nonpathogen virulent |
| ☒  | Increase transmissibility of a pathogen |
| ☒  | Alter the host range of a pathogen |
| ☒  | Enable evasion of diagnostic/detection modalities |
| ☒  | Enable the weaponization of a biological agent or toxin |
| ☒  | Any other potentially harmful combination of experiments and agents |
# Plants

**Seed stocks**

NA

**Novel plant genotypes**

NA

**Authentication**

NA

# ChIP-seq

## Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

### Data access links

May remain private before publication.

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE252218

## Files in database submission

| Accession | Sample Description |
|-----------|--------------------|
| GSM7996810 | WT_ESC_RAD21       |
| GSM7996811 | WT_ESC_H3K27ac     |
| GSM7996812 | Δ3XCTCF_ESC_RAD21  |
| GSM7996813 | Δ3XCTCF_ESC_H3K27ac|
| GSM7996814 | ΔPromGbx2_ESC_RAD21|
| GSM7996815 | ΔPromGbx2_ESC_H3K27ac|
| GSM7996816 | Δ3XCTCF_ΔPromGbx2_ESC_RAD21 |
| GSM7996817 | Δ3XCTCF_ΔPromGbx2_ESC_H3K27ac |
| GSM7996818 | WT_NPC_RAD21       |
| GSM7996819 | WT_NPC_H3K27ac     |
| GSM7996820 | Δ6XCTCF_NPC_RAD21  |
| GSM7996821 | Δ6XCTCF_NPC_H3K27ac|
| GSM7996822 | Six3KO_NPC_RAD21   |
| GSM7996823 | Six3KO_NPC_H3K27ac |
| GSM7996824 | Δ6XCTCF_Six3KO_NPC_RAD21 |
| GSM7996825 | Δ6XCTCF_Six3KO_NPC_H3K27ac |

## Genome browser session

(e.g. UCSC)

BigWig files are provided through GEO (GSE252218) for all generated ChIP-seq samples. BigWig files can be easily uploaded in the UCSC browser for visualization purposes.

## Methodology

### Replicates

Chip-seq experiments were performed as single replicates for each condition.

### Sequencing depth

Truseq ChIP-seq + Novaseq6000_150PE (2x150bp) _40Mrd/spl

### Antibodies

RAD21 (abcam, ab154769, lot#GR32241); 10 μg /ChIP-seq sample

H3K27ac (Active Motif, 39133, lot#31521015); 3 μg /ChIP-seq sample

### Peak calling parameters

Peak calling was not performed

### Data quality

Basic read quality check was performed using FastQC (Babraham Bioinformatics) and MultiQC. The removal of read adapters and low-quality filtering was done with trimmomatic.

### Software

Reads were mapped to the mm10 reference genome with Bowtie2 and duplicated reads were discarded with SAMtools. Bigwig files were generated with bamCoverage from deepTools applying the reads per genome coverage normalization.
Flow Cytometry

Plots

Confirm that:

☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☐ All plots are contour plots with outliers or pseudocolor plots.
☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Instrument

Software

Cell population abundance

Gating strategy

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type

Design specifications

Behavioral performance measures

Acquisition

Imaging type(s)

Field strength

Sequence & imaging parameters

Area of acquisition

Diffusion MRI

☐ Used ☒ Not used

Preprocessing

Preprocessing software

Normalization

Normalization template

Noise and artifact removal

Volume censoring

Statistical modeling & inference

Model type and settings

Effect(s) tested

Specify type of analysis: ☐ Whole brain ☐ ROI-based ☐ Both
| Statistic type for inference | NA |
|-----------------------------|----|
| Correction                  | NA |

### Models & analysis

| n/a | Involved in the study |
|-----|-----------------------|
|     | Functional and/or effective connectivity |
|     | Graph analysis |
|     | Multivariate modeling or predictive analysis |

| Functional and/or effective connectivity | NA |
|------------------------------------------|----|
| Graph analysis                           | NA |
| Multivariate modeling and predictive analysis | NA |