**Supplementary Note**

**ATAC-seq libraries preparation and genomic analysis**

ATAC-seq libraries were made according to the published protocols [1,2] using the Nextera DNA Library Preparation Kit (illumine, cat# FC-121-1030) or home-made Tn5 transposon as described in [3]. We also supplemented 0.05ng drosophila genomic DNA purified from S2 cells as spike-in control right before adding the tagmentation mixture to improve comparison between samples. Multiplexed ATAC-seq libraries (barcodes were adapted from [1] ) were sequenced on Illumina HiSeq 2500 or NextSeq 500/550 high and/or mid throughput 150 cycles at Janelia quantitative Genomics Core, with a run configuration of 75 bp paired-end sequencing, either as single indexed or dual indexed runs. All samples were quantitated on Roche 480 lightcycler using FAST qPCR program and normalized and pooled at 2nM. Libraries were loaded at varied final concentrations across HiSeq (10pM) and NextSeq (1.5pM to 1.9pM). Illumina's Bcl2tofastq2 v2.17 was used to convert BcL files to fastq files and to demultiplex the samples.

To analyze ATAC-seq libraries, pair-end reads were first adapter removed by Cutadapt and mapped to mm10 genome build using Bowtie2 with the following parameters: --no-discordant --no-mixed --phred33 -X2000 –threads32. Reads mapped to mitochondria/uncharacterized chromosomes (chrM/chrUn/random) and PCR duplicates were removed by samtools. The pair-end reads from the drosophila genomic DNA was mapped to dm6 genome build. To compare the coverage among ATAC-seq libraries, sequencing reads were normalized to 1×sequence depth defined by total number of mapped reads × fragment length / effective genome size (2,150,570,000) or by the spike-in drosophila genomic DNA reads. ATAC-seq peaks were called using the MACS2 callpeak function using the –f BAMPE parameter. Fragment length distribution, transcription start site (TSS) enrichment, scatter plots were analyzed as previously described [4]. ATAC-seq experiments in Extended Figure 1d-g were performed once and other ATAC-seq

experiments had at least one replicate. Raw sequencing data were deposited to NCBI GEO with the accession number of GSE126112.

CTCF and RAD21 ChIP-seq data sets from wild type ESCs were obtained from [5] under GSE number GSE90994. The TSS annotation was downloaded from the UCSC Table Browser mouse mm10 build (GRCm38/mm10, Dec.2011) NCBI RefSeq genes and the promoters were defined as -400bp to +100bp relative to the TSS. ESC enhancer coordinates were retrieved from the H3K27ac ChIP-seq peak regions as described previously [6].


**Cell culture and generation of auxin-inducible ESC lines**

JM8.N4 mouse ESCs from the C57BL/6N strain and their genome edited derivatives were cultured on 0.1% gelatin coated plates without feeders at 37°C and 5% $CO_2$. The ESC medium was composed of the knockout DMEM(1X) optimized for ESCs (Thermo Fisher Scientific 10829-018), 15% ESC qualified Fetal Bovine Serum (ATCC SCRR-30-2020), 1 mM GlutaMAX (Thermo Fisher Scientific 35050-061), 0.1 mM MEM nonessential amino acids (Thermo Fisher Scientific 11140-50), 0.1 mM 2-mercaptoethanol (Thermo Fisher Scientific 21985-023),1000 units of Leukemia inhibitory factor (LIF) (Millipore) and Antibiotic-Antimycotic (Thermo Fisher Scientific 15240-062). The JM8.N4 cells were authenticated and approved by the NIH 4D Nucleome project as a Tier2 cell line. Mouse embryonic fibroblast (MEF) cells were derived from 14 d.p.c pregnant C57BL female mice and cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum and 1× Antibiotic-Antimycotic. The HEK293T cells used for producing lentiviral particles (Lenti-X 293T cell line) were purchased from TaKaRa ( Catlog number 632180) and cultured according to manufacture's protocol.

To implement the auxin-inducible degron system (AID), the pLenti-EF1-osTir1-9myc-P2A-Bsd lentivirus was used to infect the low passage JM8.N4 wild type ESCs and selected with

10µg/mL Blasticidin (Thermo Fisher Scientific R21001). Blasticidin resistant clones were manually picked and tested for osTir1 expression by Western Blot using an anti-Myc antibody (Santa Cruze, 9E10/sc-40). Cell cycle kinetics and ATAC-Seq analysis of Tir1 ESCs showed no discernable difference to parental JM8.N4 ESCs.

To generate auxin inducible degradation for CTCF, 1.5 µg/µl wild type SpCas9_sgRNA_PGK-Venus construct and 3 µg/µl of CTCF donor constructs were nucleofected into ~3X10^6 Tir1 ESCs using the Amaxa™ 4D-Nucleofector and the P3 Primary Cell 4D-Nucleofector™ X Kit following the manufacture's protocol. ~24 hours following nucleofection, Venus positive cells were FACS sorted as a pool and grown for another ~3-5 days. Cells were then stained with 50nM Janelia Fluor® 549 HaloTag ligand ($JF_{549}$) for 30min, washed 3X with ESC medium for 15min, and subjected to another round of FACS sorting. $JF_{549}$ positive cells were plated sparsely at 10cm tissue culture plates and grown for another 5-7 days. Single colonies were picked for genotyping by designing PCR primers outside of the homology arms. Bi-allelic knock-in clones were verified by Sanger Sequencing and expanded for downstream analysis. We also generated the HaloTag labeled H2A.Z and MED1 line in the similar fashion for two color PALM imaging. The GFP version of CTCF-AID ESCs were similarly generated except that ESCs were sorted for GFP fluorescence ~5 days after initial sorting for Venus signal. Genotyping primers for *Ctcf* knock-in are: 5'-CCGCCCAGTCATTTCACCTACA-3', 5'-GGCCGTTCTGGAGTGGTTTACG-3', for H2A.Z are 5'-CGCAGAGGTAAGCGGGAGTACG-3', 5'-TGGAATCACCAACACTGGACAGC-3', for Med1 are 5'-CGGGGAGGACGAAATCAGCCAACC-3', 5'-CGTCCCTCGTCCCGCAGAAAACCT-3'. The sgRNA sequence are 5'-GAGCCTTCATCCTGACAGCG-3' (Med1), 5'-GAAGACTGTTTAAGGATGCC-3' (H2A.Z). The sgRNA for CTCF knock-in was described previously [5].

We tested a wide range of auxin concentration for target degradation and found that 50-500μM IAA induced robust CTCF (data not shown). Unless indicated, we used 100μM final concentration of IAA throughout our experiments.

**Chemicals and plasmids**

The plant auxin analog indole-3-acetic acid (IAA) (Sigma, Cat# I3750-5G-A) was dissolved in Ethanol at a stock concentration 500mM and aliquoted to store at −20°C.

The pLenti-EF1-osTir1-9Myc-P2A-Bsd was constructed by PCR amplifying the Oryza Sativa Tir1 (osTir1) cDNA from the pBabe Puro osTIR1-9Myc (Addgene #80074) and inserted into the AgeI/BamHI site of the lentiCas9-Blast construct (Addgene #52962).

The donor plasmids for CTCF were modified from previous HaloTag-CTCF donor constructs as described from [5] by in-frame insertion of the 71-104aa of full length auxin-inducible degron (miniAID) [78]. The CRISPR/Cas9 and sgRNA constructs used for targeted CTCF knock-in were used as previously described [5]. The corresponding GFP version of CTCF donor constructs were generated by replacing the HaloTag with mNeonGreen by Gibson assembly. The donor plasmids for H2.AZ-Halo and MED1 were constructed by amplifying homology arms from mouse genomic DNA followed by Gibson assembly.

The PiggyBac_EF1_mNeonGreen_HP1α construct was made by inserting the mNeonGreen and HP1α gBlock into the PiggyBac_EF1_HaloTag_SOX2 backbone by Gibson Assembly (NEB, E5520S). Relevant plasmids have been deposited to Addgene. Maps of plasmids used in this study are also available upon request.

**RNAi**

Lenti-viral vectors expressing shRNA against mouse CTCF and preparation of lentiviral particles were described in our previous report [9]. Two different lentiviral vectors expressing CTCF shRNAs and an empty vector control were used to infect CTCF-HaloTag ESCs [5] in the presence of 5ng/mL Polybrene (Millipore, cat# TR-1003-G). One day after infection, cells were selected with 1 µg/mL Puromycin (Thermo Fisher Scientific, cat# A1113803) for additional 48 hours before analysis with immunofluorescence, ATAC-seq and 3D ATAC-PALM microscopy. The endogenously HaloTag labeled CTCF was labeled with 100nM $JF_{646}$ HaloTag ligand [10] for 30min to monitor the degree of CTCF depletion by shRNA.

**Western Blots**

ESCs were lysed in 1XSDS sampling buffer (200mM Tris HCl pH7, 10% glycerol, 2% SDS, 4% beta-mercaptoethanol, 400mM DTT, 0.4% bromophenol blue) preheated at 95°C. Lysates were further sonicated and denatured at 95°C for 5min. Proteins from each sample was resolved by SDS-PAGE using Mini-PROTEAN® TGX™ Precast Gels (Biorad). Primary antibodies used include: CTCF (Millipore, cat#07729), OCT4 (Santa Cruz, sc-5279). We used HRP conjugated secondary antibodies (Pierce) at a dilution of 1:3000. Western blot was exposed to Western Lightning Plus-ECL (PerkinElmer) and imaged in a ChemiDoc MP (Bio-Rad) detection system.

**Confocal Imaging and analysis**

Immunofluorescence and HaloTag $JF_{549}$ imaging for CTCF or RAD21 depletion was performed under the Nikon A1-R confocal system using the Galvo scanning mechanism. The antibodies used for immunofluorescence are anti-OCT4 (Santa Cruz, Cat# C-10). We used the default excitation lines: 405/488/561/640 nm lasers and the 4 channel detector--Ch1 450/50 DAPI (multi-alkali), Ch2 525/50 GFP (GaAsP), Ch3 600/50 RFP (GaAsP), Ch4 685/70 Cy5 (multi-alkali).

Multiple z-stack images (300nm) were acquired and the fluorescence intensity was obtained from the maximum intensity projection under ImageJ/Fiji.

**Colony Formation Assay and Cell proliferation analysis**

Colony forming assays were performed by plating 600 cells per well on 12-well plates (150/cm$^2$) coated with gelatin. Plates were fixed after up to 7 days of IAA treatment upon initial plating and stained for alkaline phosphatase (AP) activity (Sigma, cat. 86R-1KT) according to the manufacturer's protocol.

CTCF depleted ESCs were assayed for DNA replication by the Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Thermo Fisher Scientific, cat#C-10420) according to the manufacture's protocols. Prior to Flow Cytometry analysis, DNA was stained with 1µg/mL FxCycle™ Violet stain (Thermo Fisher Scientific, cat#F10347) at room temperature for 30min. DNA synthesis was measured by incorporating 5-ethynyl-2'-deoxyuridine (EdU) coupled with an alkyne group, followed by the click reaction with Alexa Fluor 488 dye coupled with the picolyl azide group. Flow Cytometry was performed on the CytoFLEX S system from Beckman with 50,000 cells recorded and analyzed by FlowJo.

**Oligopaint FISH experiment and analysis**

The Oligopaint FISH probe libraries were constructed as described previously [11]. A Tier 15 ssDNA oligo pool was ordered and synthesized from Twist Bioscience (San Francisco, CA). Each oligo consists of a 32 nucleotide (nt) homology to the mm9 genome assemble discovered by the OligoArray2.0 with the following parameters -n 22 -D 1000 -l 32 -L 32 -g 52 -t 75 -T 85 -s 60 -x 60 -p 35 -P 80-m "GGGGG;CCCCC; TTTTT;AAAAA" run from the algorithm developed from the laboratory of Dr.Ting Wu (https://oligopaints.hms.harvard.edu/). Each library subpool consists of

a unique sets of primer pairs for orthogonal PCR amplification and a 20 nt T7 promoter sequence for *in vitro* transcription and a 20 nt region for reverse transcription. Individual Oligopaint probes were generated by PCR amplification, *in vitro* transcription, and reverse transcription, in which ssDNA oligos conjugated with ATTO565 and ATTO647 fluorophores were introduced during the reverse transcription step as described previously [12,13]. The Oligopaint covered genomic region (mm9) used in this study are listed below:

| Domain Name | Genome coordinates (mm9) | Size (kb) | Probe number |
|---|---|---|---|
| Domain R1 | Chr4: 53067000-53700000 | 633 | 8948 |
| Domain P1 | Chr4: 53767000-54900000 | 1133 | 14618 |
| Domain R2 | Chr4: 54925000-55568000 | 643 | 9324 |
| Domain P2 | Chr4: 55650000-56750000 | 1100 | 15725 |
| Domain R3 | Chr4: 56810000-57930000 | 1120 | 16553 |
| Domain R4(or A3) | Chr6: 120150000-121400000 | 1250 | 17717 |
| Domain P3(or I3) | Chr6: 121410000-122150000 | 740 | 4725 |
| Domain R5(or A2) | Chr6: 122160000-122800000 | 640 | 8007 |
| Domain P4(or I2) | Chr6: 122810000-123500000 | 690 | 4116 |
| Domain P5(or I1) | Chr6: 123510000-124200000 | 690 | 4323 |
| Domain R6(or A1) | Chr6: 124210000-125200000 | 990 | 11763 |

| Domain Name | Forward primer (5'-3') | Reveres primer (5'-3') | RT oligo (5'-3') |
|---|---|---|---|
| Domain R1 | GCGGGACGTAAGGGCAACCG | GCGTTGCGGTGCGATCTTCT | CCTATCCCCTGTGTGCCTTG |
| Domain P1 | GATTTCGGTAAGGCGGGCCG | GCGCCGAGCCTCATCTACCG | CCTATCCCCTGTGTGCCTTG |
| Domain R2 | TTGGGTCCGGTTGTGATCCG | GCGATGCCCGGGTAACACAA | AATTCGGCAGACCCGAATGC |

| | | | |
|---|---|---|---|
| Domain P2 | ATTCATATGCGCTCCGGCGG | GAGCCCGCTGATACACGCGC | AATTCGGCAGACCCGAATGC |
| Domain R3 | TGATAACCCACGCACGGCTG | GACCCGGGCCACTAACACGA | AATTCGGCAGACCCGAATGC |
| Domain R4 | CACGGCAACCCTCAGAACGG | CAGTTCGGTGGGACCGGGTT | CCTATCCCCTGTGTGCCTTG |
| Domain P3 | TAATGACGAGCGCGTGCCTG | GTCGCACATCGGACCTTCGG | AATTCGGCAGACCCGAATGC |
| Domain R5 | TTGGACGGCGCGCGTAAGAC | GGATTGCGCTCATGCCGTCT | CCTATCCCCTGTGTGCCTTG |
| Domain P4 | ACGTCCGCCGCATCTACGAG | GCCGTGCATTCAGGACCGTT | CCTATCCCCTGTGTGCCTTG |
| Domain P5 | GGGAGTAGGGTCCTTTGTGTG | TTCTCTAGAACGATCCAGCGA | CCTATCCCCTGTGTGCCTTG |
| Domain R6 | ACGTCCATGCAAGGAACGGG | CACGTGACGTCGGTTGGACG | AATTCGGCAGACCCGAATGC |

The oligo sequences for conjugating fluorophores during reverse transcription are:

/5ATTO565N/ or /5ATTO647N/AATTCGGCAGACCCGAATGC

/5ATTO565N/ or /5ATTO647N/CCTATCCCCTGTGTGCCTTG

For 3D DNA FISH on ESCs, #1.5 round glass coverslips (Electron Microscopy Sciences) were pre-rinsed with anhydrous ethanol for 5min, air dried, and coated with 0.1% gelatin or equivalent for at least 2 hours. Fully dissociated ESCs were seeded onto the coverslips and recovered for at least 6 hours before experiments. Cells were fixed with 4% (v/v) methanol free paraformaldehyde (Electron Microscopy Sciences, Cat# 15710) diluted in 1X PBS at room temperature for 10min. Then cells were washed 2X with 1XPBS and permeabilized in 0.5% Triton-X100 in 1XPBS for 30min. After 2X wash in 1XPBS, cells were treated with 0.1M HCl for 5min, followed by 3X washes with 2XSSC and 30 min incubation in 2X SSC + 0.1% Tween20 (2XSSCT) + 50% (v/v) formamide (EMD Millipore, cat#S4117). For each sample, we prepare 25ul hybridization mixture containing 2XSSCT+ 50% formamide +10% Dextran sulfate (EMD Millipore,

cat#S4030) supplemented with 0.5µl 10mg/mL RNaseA (Thermo Fisher Scientific, cat# 12091-021) +0.5µl 10mg/mL salmon sperm DNA (Thermo Fisher Scientific, cat# 15632011) and 20pmol probes with distinct fluorophores. The probe mixture was thoroughly mixed by vortexing, and briefly microcentrifuged. The hybridization mix was transferred directly onto the coverslip which was inverted facing a clean slide. The coverslip was sealed onto the slide by adding a layer of rubber cement (Fixo gum, Marabu) around the edges. Each slide was denatured at 78°C for 3 min followed by transferring to a humidified hybridization chamber and incubated at 42°C for 16 hours in a heated incubator. After hybridization, samples were washed 2X for 15 minutes in pre-warmed 2XSSCT at 60 °C and then were further incubated at 2XSSCT for 10min at RT, at 0.2XSSC for 10min at RT, at 1XPBS for 2X5min with DNA counterstaining with DAPI. Then coverslips were mounted on slides with Prolong Diamond Antifade Mountant (Thermo Fisher Scientific Cat#P36961) for imaging acquisition.

3D DNA FISH images were acquired on the ZEISS LSM 880 Inverted Confocal microscope attached with a Airyscan 32 GaAsP (gallium arsenide phosphide)-PMT area detector [14]. Before imaging, the beam position was calibrated centering on the 32 detector array. Images were taken under the Airyscan Super-resolution mode with a Plan Apochromat 63X/NA1.40 oil objective in a lens immersion medium having a refractive index 1.515. We used 405nm (Excitation wavelength) and 460nm (Emission wavelength) for the DAPI channel, 561nm (Excitation wavelength) and 579nm (Emission wavelength) for the ATTO565 channel and 633nm (Excitation wavelength) and 654nm (Emission wavelength) for the ATTO647 channel. Z-stacks were acquired under Super-resolution mode for the optimal z sectioning thickness around 190nm. The Airyscan super-resolution technology used a very small pinhole (0.2AU) at each of its 32 detector elements to increase SNR ~4-8 fold and enables ~1.7-fold improvement of resolution upon linear deconvolution in both lateral (xy) and axial (z) directions. After image acquisition, Airyscan image

was post-processed and reconstructed using the provided algorithm from ZEISS LSM880 platform.

3D DNA FISH analysis was performed in Imaris 9.1 installed in Windows 10 X64 OS with the GeForce GTX 760/PCIe/SSE2 (version 4.5.0 NVIDIA 369.09). We applied a background subtraction filter to the 3D Airyscan processed images before the downstream analysis. To characterize the 3D DNA FISH domain, we employed the synthetic model—Surfaces object from Imaris and applied a Gaussian filter (σ = 1 voxel in xy) before the downstream 3D segmentation and quantification. The 3D volume of the DNA FISH defined domain is estimated by the number of voxels within the detected objects with the voxel size (48.9nmX48.9nmX199nm).The sphericity score is estimated by the ratio of the surface area of a sphere to that of the object (with equal volume) defined by equation $\frac{\pi^{\frac{1}{3}}(6V)^{2/3}}{S}$ (V and S are volume and surface area of the object, respectively).

For two-color 3D Oligopaint FISH and ATAC-PALM imaging experiments, cells were first mounted onto the 5mm coverslip embedded with nano-gold fiducial and treated with Tn5-PA-JF$_{549}$ transposome. With minimal exposure to light, samples were further processed for 3D DNA FISH experiments, except that HCl treatment is omitted and replaced with serial dehydration (immersed in 70%, 80%, 95% and 100% ethanol for progressive dehydration) step to maximally preserve the ATAC-PALM signal during imaging. The image stack of Oligopaint FISH channel was taken before the ATAC-PALM experiment under the Lattice light-sheet microscopy. The Oligopaint FISH image stacks were de-skewed and de-convoluted. The centroid of the diffraction limit spot of DNA FISH signal was identified by 3D Gaussian fitting and a cluster of 200 points were generated centering on the centroid to visualize the Oligopaint FISH signal represented in ViSP through Gaussian random sampling with a standard deviation σ=200 nm.

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