Molecular Cell

Supplemental Information

Cause and Consequence of Tethering a SubTAD to Different Nuclear Compartments

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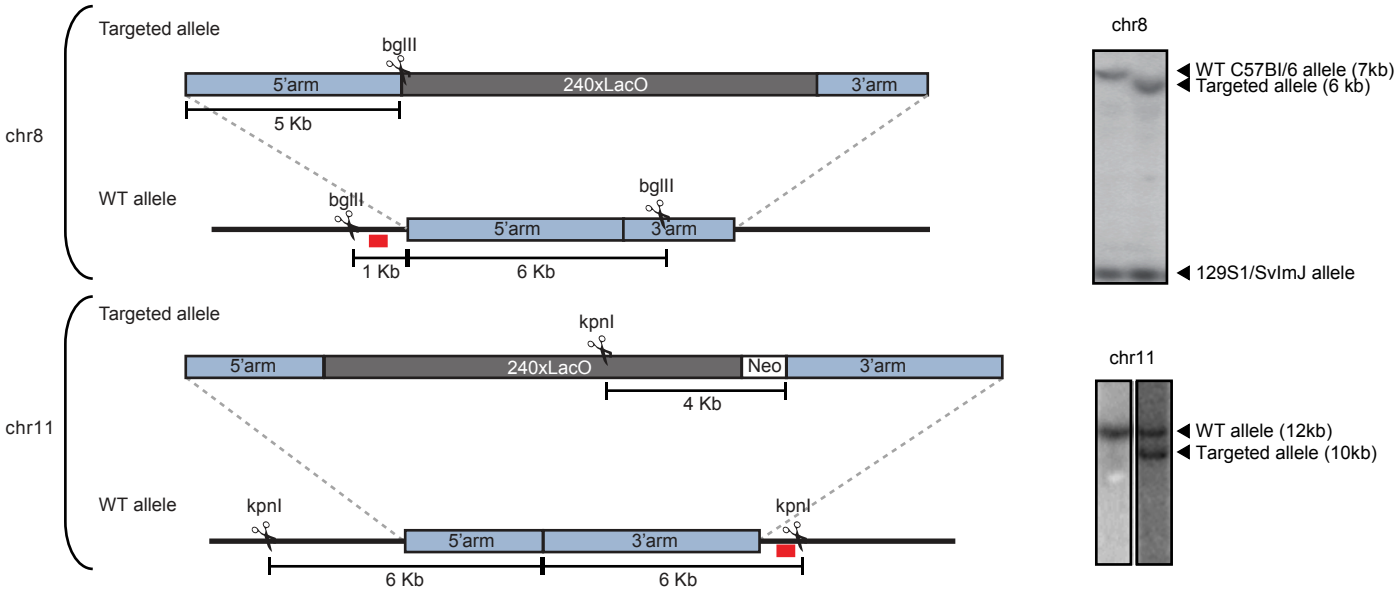
Figure S1

A


Targeted allele
bglII
5'arm
240xLacO
3'arm
5 Kb
chr8
WT allele
bglII
bglII
5'arm
3'arm
1 Kb
6 Kb
chr8
WT C57Bl/6 allele (7kb)
Targeted allele (6 kb)
129S1/SvImJ allele
Targeted allele
kpnI
5'arm
240xLacO
Neo
3'arm
4 Kb
chr11
WT allele
kpnI
kpnI
5'arm
3'arm
6 Kb
6 Kb
chr11
WT allele (12kb)
Targeted allele (10kb)

B

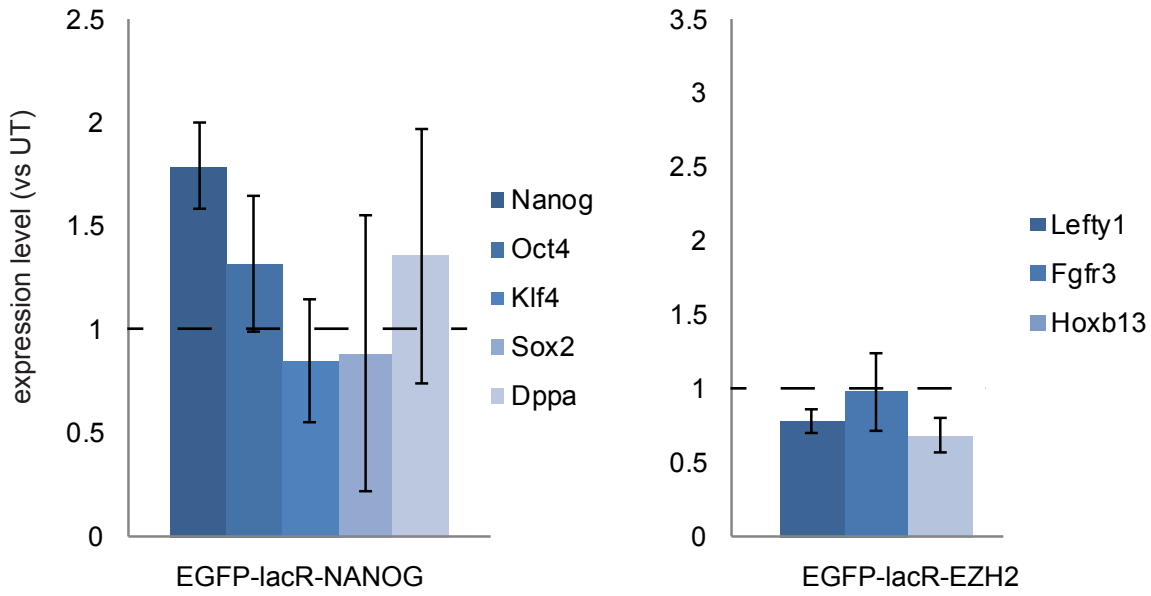

expression level (vs UT)
2.5
2
1.5
1
0.5
0
Nanog
Oct4
Klf4
Sox2
Dppa
EGFP-lacR-NANOG
3.5
3
2.5
2
1.5
1
0.5
0
Lefty1
Fgfr3
Hoxb13
EGFP-lacR-EZH2

Figure S1, related to Figure 1. A lacO/lacR recruitment platform to induce nuclear repositioning.

A) Strategy and data from Southern blot analysis confirming correct integration of the *lacO* cassette on chr8 (top) and chr11 (bottom). Red blocks indicate probes used for hybridization.

B) Quantitative expression analysis (qPCR on cDNA) for a number of endogenous target genes of Nanog and of Ezh2. The analysis reveals that the ectopic expression of the corresponding LacR fusion genes has little impact on the expression of the presumed target genes. Note that the primers used for Nanog also analyze transgene expression, showing that there is less than 2-fold overexpression. Error bars indicate standard deviation (SD).

Figure S2

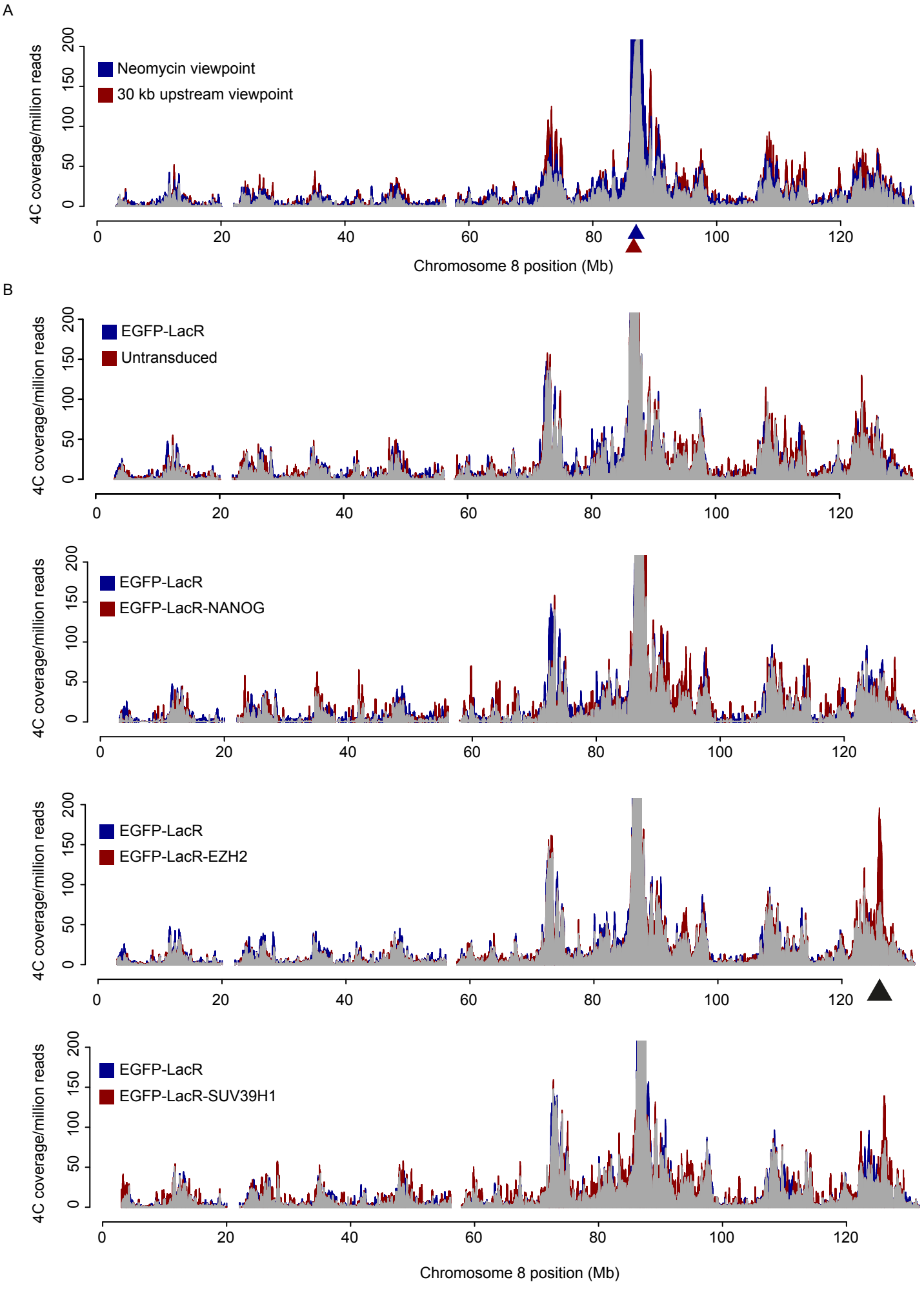

A
4C coverage/million reads
Neomycin viewpoint
30 kb upstream viewpoint
Chromosome 8 position (Mb)
B
EGFP-LacR
Untransduced
EGFP-LacR-NANOG
EGFP-LacR-EZH2
EGFP-LacR-SUV39H1

Figure S2, related to Figure 2. Susceptibility to spatial repositioning depends on genomic location and trans-acting factors.

A) Overlays of 4C profiles on chromosome 8, comparing contacts made by the '*Neomycin*' gene in untransduced ES cells carrying only a *lacO-Neomycin* transgene on chr8, *versus* contacts made by a viewpoint '30 kb upstream' of the integration site. Viewpoints are indicated by arrowheads. Note that the profiles show some quantitative differences in contact frequencies, but only at the chromosomal sites that are contacted by both viewpoints (no 'new' interactions).

B) As in A) but for the double-transgenic *lacO* cells transduced with the different EGFP-lacR fusions. Note here that all contact profiles are highly similar and that only upon EZH2 recruitment a single 'new' contact is seen, near the telomere of chromosome 8 (indicated with an arrowhead).

Figure S3

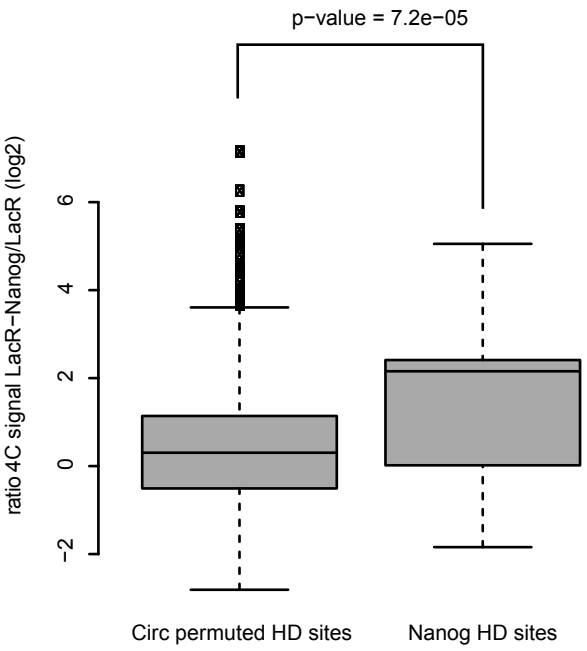
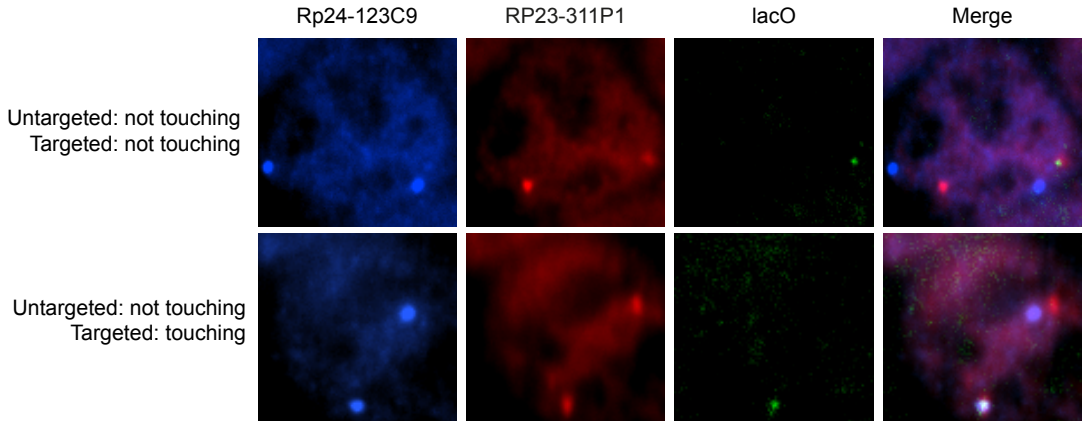

p−value = 7.2e−05
ratio 4C signal LacR−Nanog/LacR (log2)
−2
0
2
4
6
Circ permuted HD sites
Nanog HD sites

Figure S3, related to Figure 3. Local chromatin signature influences direction of nuclear repositioning.

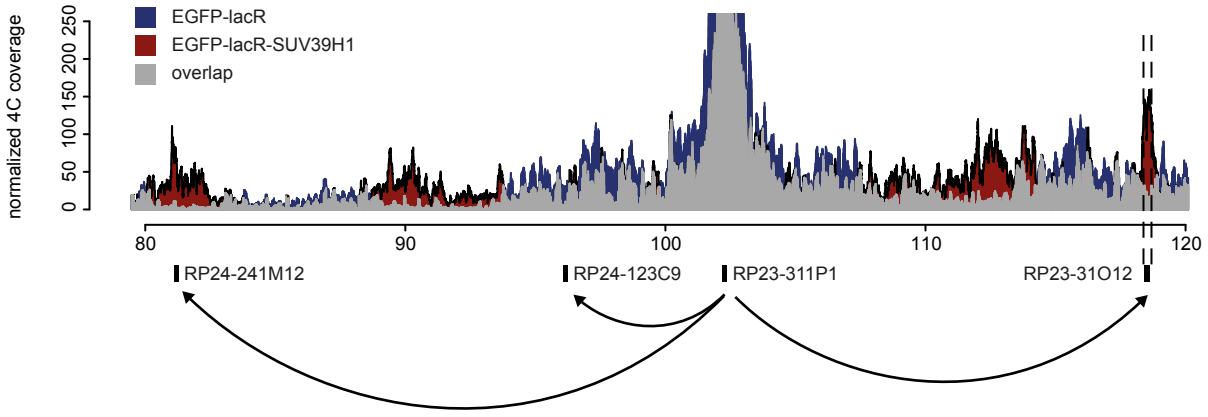
Boxplot showing the ratio in 4C signals of EGFP-lacR-NANOG and EGFP-lacR cells across high-density NANOG binding sites and upon circular permutation of these positions. Note that NANOG-binding significantly increased contact frequency on high-density NANOG binding sites.

Figure S4

A



B



	RP24-241M12	RP24-123C9	RP23-31O12
SUV39H1	44.8% (47/105)	29.3% (22/75)	46.7% (43/92)
Untargeted	25.6% (66/258)	32.4% (155/478)	32.2% (86/267)
Untransduced	21.2% (11/52)	34.1% (29/85)	27.8% (25/90)
EZH2	n.d.	72.5% (95/131)	n.d.

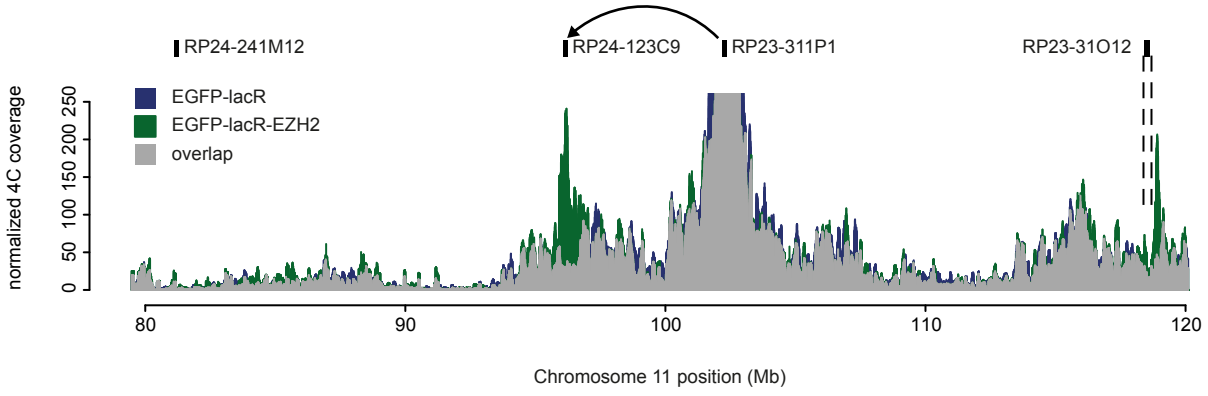


Figure S4. FISH validation of 4C contacts. Related to Figure 3 and Figure 4.

A) Examples of FISH results. Shown are (top) a cell in which, upon LacR-EZH2 expression, neither the untargeted nor the targeted site on chromosome 11 visually touches the *HoxB* locus 6 Mb upstream of *lacO* on chromosome 11, and (bottom) one in which only the *LacO* targeted site touches *HoxB* (bottom). Shown are z-stacks recording the signals of the different probes (three leftmost panels), and their merge (right panels).

B) Summary of the quantification of the FISH results. ImageJ (Image5D plug-in) software was used to count touching (no unstained pixel in between FISH signals) and non-touching (with unstained pixel(s) in between) alleles. Depending on the BAC probe used, the average diameter of the fluorescent signal varied between 0.8-1µm, implying that the centers of touching signals can be up to 1µm apart. Location and name of BAC is indicated, and the percentage of touching FISH signals is indicated for the 'untargeted' chromosome (i.e. the homologous chromosome that does not carry the *lacO* array), the *lacO* untransduced allele (which carries *lacO* without any associated LacR (fusion) protein), the LacR-EZH2 associated *lacO* allele and the LacR-SUV39H1 associated *LacO* allele. The plotted differential 4C contact profiles comparing LacR (blue) versus LacR-SUV39H1 (red) (top) and LacR (blue) versus LacR-EZH2 (green) (bottom) enable a direct comparison between differential contact frequencies measured by 4C and by FISH. Note that the most telomeric BAC probe hybridizes to the SUV39H1 contacted region, not to the *Cbx* locus contacted by EZH2. N.d.: not done.

Figure S5

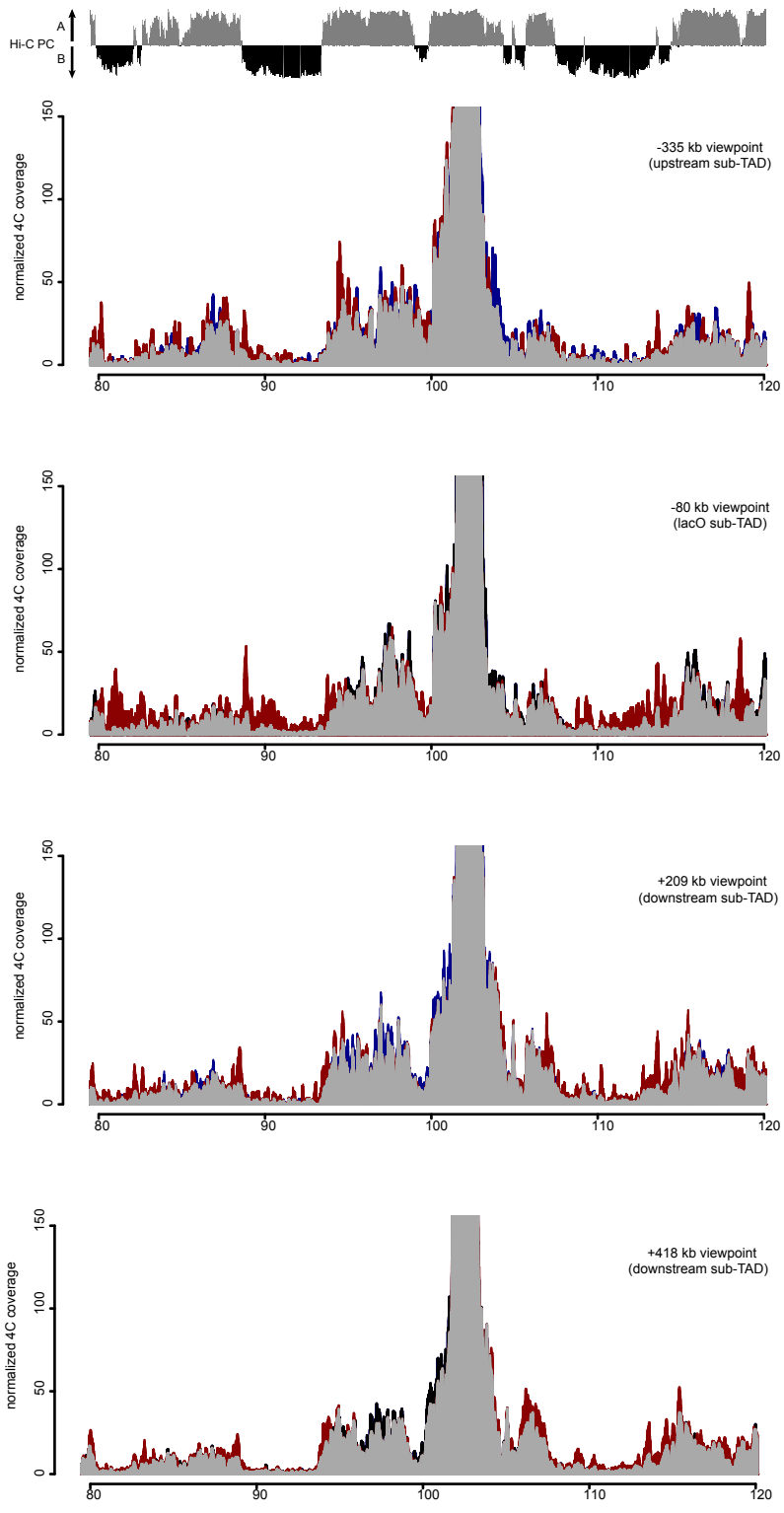
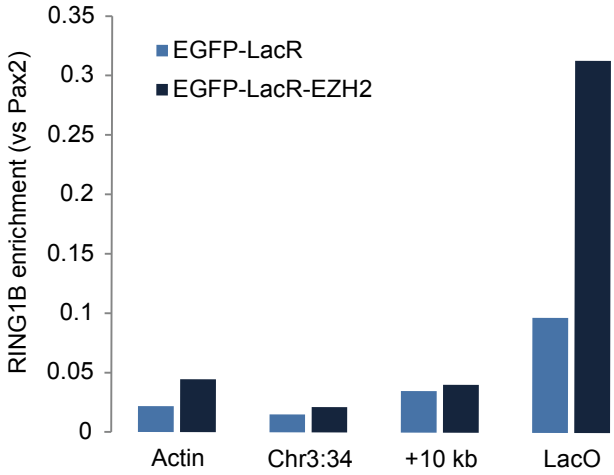

Hi-C PC
A
B
normalized 4C coverage
-335 kb viewpoint
(upstream sub-TAD)
-80 kb viewpoint
(lacO sub-TAD)
+209 kb viewpoint
(downstream sub-TAD)
+418 kb viewpoint
(downstream sub-TAD)
0
50
100
150
80
90
100
110
120

Figure S5, related to Figure 4. A repositioned locus drags along its associated sub-TAD.

Comparative allele-specific 4C profiles comparing the lacO and wild type alleles upon binding of SUV39H1, for a viewpoint 335 kb upstream of the lacO in the neighbouring sub-TAD, 80 kb upstream within the same sub-TAD and 209 and 418 kb downstream in immediately neighbouring sub-TADs. Note the increased contact frequency with regions in the B compartment (displayed on top) only for the viewpoint within the *lacO* sub-TAD.

Figure S6

A


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
RING1B enrichment (vs Pax2)
EGFP-LacR
EGFP-LacR-EZH2
Actin
Chr3:34
+10 kb
LacO

B

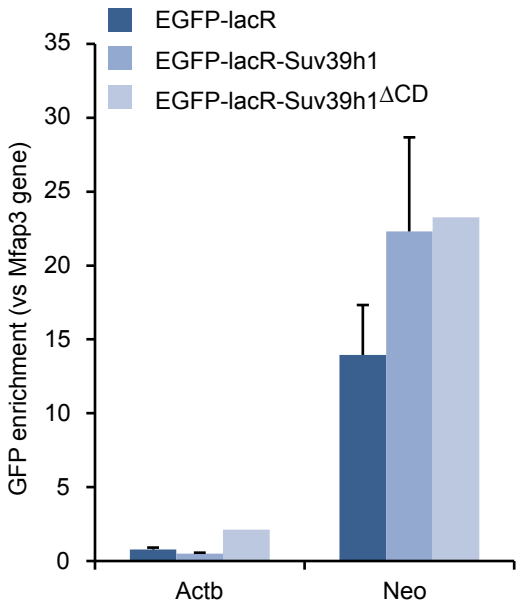

35
30
25
20
15
10
5
0
GFP enrichment (vs Mfap3 gene)
EGFP-lacR
EGFP-lacR-Suv39h1
EGFP-lacR-Suv39h1ΔCD
Actb
Neo

Figure S6, related to Figure 6. The role of chromatin in spatial repositioning.

A) ChIP data showing that EZH2 recruitment to LacO facilitates Ring1B association.

B) ChIP data confirming the binding of EGFP-lacR-SUV39H1 and EGFP-lacR- SUV39H1$^{\Delta CD}$ to the *Neo* gene on the recruitment platform. Error bars indicate standard deviation (SD).

Supplemental Experimental Procedures

Cell culture

Mouse embryonic stem cells C57Bl/6-129/Sv were cultured as previously described (de Wit et al., 2013). In brief, they were cultured on gelatin-coated plates in BRL (buffalo–rat liver cells)-conditioned DMEM (Hooper et al., 1987)(high glucose, Gibco) with 15% FBS, 1x non-essential amino acids (NEAA; Gibco), 1x penicillin–streptomycin (Gibco), 1:1000 b-mercaptoethanol (Invitrogen), 1x L-glutamine (Gibco) and 1000 U ml-1 leukaemia inhibitory factor (LIF; Gibco).

Generation of *lacO* targeted cell line

First, the *lacO* array (Lau et al., 2003) was inserted in chromosome 8 (chr8)(AatII site, mm9 chr8:87321244), as described (de Wit et al., 2013). Cre transfection (Splinter et al., 2006) was used to delete *Neomycin* at Chr8. Subcolonies were picked, and deletion was confirmed by PCR on genomic DNA from clonal cell lines. Then, the same *lacO* array was introduced into chromosome 11 (AatII site, mm9 chr11:102209489) using the same gene targeting protocol. Homology arms were excised with KpnI from bacterial artificial chromosome (BAC) RP23-311P1, and the *lacO* array inserted at the unique AatII site. The linearized targeting construct was introduced by electroporation. After 14 days of neomycin selection, positive colonies were screened by Southern blot (Figure S1). Probes for Southern blot were PCR-amplified from genomic DNA using the following primers:
chr8_forward: TGTGTGGTGATCATGTGTGC
chr8_reverse: TGCCACTCCTGTGTCTCAAG
chr11_forward: CCTCCTTTGGATACCTTCC
chr11_reverse: CTTTAAATCGGTGGCTGAGG

LacR-fusion constructs and transduction

Proteins of interest were cloned in frame downstream of EGFP-LacR . To transduce EGFP-LacR fusions under control of the EF1α promoter, they were introduced in place of the DsRed gene of the phage2-EF1α-DsRed-IRES-PURO vector (Wilson et al., 2008) by blunt cloning. The chromodomain of the EGFP-lacR-SUV39H1 fusion protein was removed using BstZ17I (NEB) and SphI (Roche), and re-ligated after Klenow fill in. *LacO*-transgenic cells were transduced and selected with Puromycin (P8833, SIGMA at 1µg/µl) for approximately 10 days when cells had reached sufficient numbers for collection and tested for purity by flow cytometry (minimum 70% GFP-positive).

Western blot

Nuclear extracts were made as described in (Andrews and Faller, 1991), except for EGFP-lacR-SUV39H1, where nuclei were boiled for 5 minutes in Laemmli buffer. Immunoblot analyses were performed with antibodies against GFP (ab290, Abcam) or NANOG (A300-397A, Bethyl Laboratories).

GFP distribution analysis

Stably transduced *lacO* cell lines were grown overnight on gelatin-coated coverslips. They were crosslinked with 4% paraformaldehyde (rT) (10 minutes), washed once with 0.125M glycine in PBS and PBS+0.1% Tween 20, and permeabilized with 0.2% Triton X-100 in PBS (5 minutes). After a final wash with PBS+0.1% Tween 20, VectaShield containing DAPI (Vector Labs) was applied and coverslips were sealed with nail polish. Images were taken with a Leica SPE confocal microscope and analyzed using ImageJ software. Maximum projections were made to allow simultaneous visualization of both *lacO* alleles.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was done using Millipore's protocol with minor modifications. In brief, 5-10 million cells were fixed with 1% formaldehyde, then lysed in cell lysis buffer (10 mM Tris, pH 8.0; 10 mM NaCl; 0.2% NP-40; 10 mM Na-Butyrate; Proteinase inhibitor 1x) and nuclei lysis buffer (50 mM Tris, pH 8.0; 10 mM EDTA; 1% SDS; 10 mM Na-Butyrate; Proteinase inhibitor 1x). Isolated chromatin was sonicated to 500-1000 bp, processed on Covaris S2 as 30 seconds on and 30 seconds off, and then precipitated with GFP antibody overnight. After washing, chromatin was eluted, purified, and used for quantification analysis by qPCR, and data normalized to input and enrichment plotted over the untransduced cells. ChIP for histone modifications were performed based on the protocol in (Schmidt et al., 2009) with slight modifications. Briefly, cross-linked cells were lysed (50 mM Tris, pH 7.5; 150 mM NaCl; 5mM EDTA; 0.5% NP-40; 1% Triton X-100) and sonicated to 500-1000bp using a Bioruptor (Diagenode, 15 seconds on/15 seconds off) or a Covaris (20 cycles 30 seconds on/30 seconds off).

Chromatin equivalents of five million cells were used per IP, with antibodies against GFP (ab290, Abcam), H3K27me3 (ab6002, Abcam), H3K9me3 (ab8898, Abcam) and RING1B (#5694, Cell Signaling Tech). After immunoprecipitation, isolated DNA samples were used for quantification analysis by qPCR, and data normalized to input and enrichment calculated over plotted over the (transcriptionally active) actB promoter. Primers used:

genomic site	forward	reverse
Actb	GCAGGCCTAGTAACCGAGACA	AGTTTTGGCGATGGGTGCT
Pax2	CGGAGGGAATGAAGCAGGTT	GTTTCGAAGAGGTTCCCCGT
Mfap3	TCTGCAAGGAAGGCATCAGG	ACTCTTTCCCCCTCCCCTTT
lacO	TTCGATACCTTTATCCGCTCA	GCGGATAACAATTGCTGAAG
kan^{R}	TGATAATCCTGATATGAATAAATTGC	TTGGCACCTTTGCTAGATTAGAA
-90 kb	ATTTTGCCTGCTGTGTGCAG	TCCCTTCTCCACAGGGACAT
-60 kb	ACTATGTGGTCTTGGCTGGC	GGAGTGGCAGGAGAGAGGTA
-10 kb	GAGTAAGCCTGACGCCTGTT	AGACACACACTGTCCTGGTG
+10 kb	GACTTGCTCTCACCCCAACA	ATCTGGGTGAAAGGTGCCTG
+60 kb	GCCAGCACTGACTACACCTT	GCTGGGCTGAGCAGTGAATA
+90 kb	GGCTGATGGGGTGTGTTACC	ACCCACTTTCAGACGCAAGA
Chr3:34	GAACTGATCACAGGGACGTG	CAGGCCTGGTGAAGTATCTG

Gene expression analysis

RNA was isolated using TRIzol (Life technology) from cells trypsinized and then converted into cDNA using random primers (Promega) using standard manufacturer providing protocol (Promega). Quantitative PCR was performed and data normalized to the *Actb* gene. Allelic specific gene expression using high throughput sequencing was applied on cDNA synthesized in the same way. Primers used to detect gene transcripts flanked a SNP to discriminate C57Bl/6 allele from the 129S1/SvImJ allele. PCR product amplified from cDNA was sequenced on Illumina GAII. Sequencing reads were mapped to each allele to determine the fraction of reads coming from the *lacO*-transgenic BL/6 allele. Fold-changes were expressed relative to the BL/6 fraction in untransduced cells.

Primer used:

genomic site	forward	reverse
hprt	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC
neo (set-1)	ATGCCTGCTTGCCGAATA	CCACAGTCGATGAATCCAGA
neo (set-2)	GCAGGATCTCCTGTCATCTCA	TAGCCGGATCAAGCGTATG
Grn	GCCCGTTCTCTAAGGGTGTG	ACAGCACCCAAGGGGTTATC
Slc25a39	AGGCAGTATCTTGGCCCCAT	GCACACGTACCCCAAGACA
mRundc3a	AAGGGCGAAGTTCTGGATGG	GGTAGTCGTAGCTTTGGGTGA
Atxn7l3	GGTTTGGGGCTCTGAGGAAA	TGGGAGGTGGGATACAGGTC
Ubtf	GCTCCTCTAACTGCTTGCCA	GGAGAGCCTACTTCCCACCT
Oct4	CTCCCTACAGCAGATCACTC	GAACCATACTCGAACCACAT
Nanog	CCATTCTGAACCTGAGCTAT	ACCATTGCTAGTCTTCAACC
Klf4	CAGGTACCCCTCTCTCTTCT	TGACAGCCATGTCAGACTC
Sox2	GGAGCAACGGCAGCTA	GTAGCGGTGCATCGGT
Dppa	ACGCCAGGACAGACTCGTAG	TGCTGCTCACTCGTTTCTTCT
Lefty	CAGCTCGATCAACCGCCAGT	GGCTGGCATGGCTGTGTT
Fgfr3	CTTAAGCGACAGGTGTCCT	CTGGATAGCTCCCACTTGG
HoxB13	CGTTTGCAGAGCCCAGTGTC	CTGCATACTCCCGCTCCAAC

4C-seq analysis

Standard 4C experiments were done as previously described (Splinter et al., 2012). For allele specific 4C we have used a paired-end 4C strategy (Holwerda et al., 2013), where the forward primer analyses the ligation product and the reverse primer flanks a SNP. After sequencing, this SNP is used to demultiplex the two alleles, to create two separate 4C profiles. Allelic specific 4C from the chr8 *lacO* viewpoint and reciprocal single-end 4C used a combination of HindIII and DpnII, single-end 4C from chr11 *lacO* viewpoint used a combination of HindIII and NlaIII. 4C data mapping and analysis was as described (de Wit et al., 2013). To allow direct comparisons of 4C profiles of different samples, data was normalized to equalize the number of reads along the chromosomes, and plotted as the 4C coverage per million mapped reads. Primers used:

	primer 1	primer 2
chr8	AATGATACGGCGACCACCGAG ATCTACACTCTTTCCCTACACG ACGCTCTTCCGATCTCTGGAAC TAAATGGAGGATC	CAAGCAGAAGACGGCATACGA GATCGGTCTCGGCATTCCTGCT GAACCGCTCTTCCGATCTCAA GCAGAAGACGGCATACGAGAT CGGTCTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTTACCAGG ACCCCTGGGACCC
Neo	AATGATACGGCGACCACCGAA CACTCTTTCCCTACACGACGCT CTTCCGATCTCGAAGTTATCGA TCGAAGCTT	CAAGCAGAAGACGGCATACGA AGAAAAGCGGCCATTTTCCA
HoxB	AATGATACGGCGACCACCGAA CACTCTTTCCCTACACGACGCT CTTCCGATCTTCCCCTGGATGA GGAAGCTT	CAAGCAGAAGACGGCATACGA GAGCGGTTGACGCTGAGATC
Asci2	AATGATACGGCGACCACCGAA CACTCTTTCCCTACACGACGCT CTTCCGATCTTGTTAGGTGGCA CCAAGCTT	CAAGCAGAAGACGGCATACGA ACTAATGATGGGCACAGTTT
-335 kb viewpoint	AATGATACGGCGACCACCGAA CACTCTTTCCCTACACGACGCT CTTCCGATCTTGCACTGTCAGC CCAAGCTT	CAAGCAGAAGACGGCATACGA GATCGGTCTCGGCATTCCTGCT GAACCGCTCTTCCGATCTCAGT GCCACAGACTGCCC
-80 kb viewpoint	AATGATACGGCGACCACCGAA CACTCTTTCCCTACACGACGCT CTTCCGATCTAACTTGAGTGGA GAAAGCTT	CAAGCAGAAGACGGCATACGA GATCGGTCTCGGCATTCCTGCT GAACCGCTCTTCCGATCTCCTG GGTCTCTTGTCTACTCA
+209 kb viewpoint	AATGATACGGCGACCACCGAG ATCTACACTCTTTCCCTACACG ACGCTCTTCCGATCTGCTCCGC CTCCTAAAAGCTT	CAAGCAGAAGACGGCATACGA GATCGGTCTCGGCATTCCTGCT GAACCGCTCTTCCGATCTACGT CTTTGCAAGCTATTCGC
+418 kb viewpoint	AATGATACGGCGACCACCGAA CACTCTTTCCCTACACGACGCT CTTCCGATCTGTCTAAAACAGC CTAAGCTT	CAAGCAGAAGACGGCATACGA AAAGCATAGTCATTCAGGGA

Mapping and processing of 4C data

4C sequencing reads were aligned to the mouse reference genome (UCSC, mm9) using a mapping pipeline written in Perl as described in (van de Werken et al., 2012). In short, we first identify 4C captures by trimming primer

sequences for each bait specific 4C sequencing primer. Capture sequences are then aligned to a reduced genome consisting of sequences that flank restriction sites of the primary restriction enzyme (4C fragends). Non-unique 4C fragends were removed in subsequent analysis. Mapped reads are normalized for sequencing depth by multiplying by a factor such that the total sum of mapped reads in cis (i.e. on the bait chromosome), after discarding the 4C fragend with the highest multiplicity, equals one million. For visualization, we compute the average 4C signal across windows of 51 consecutive fragends (centered on the 25th) per experiment. We plot the average of this signal as taken from two independent biological replicates.

Statistical analysis of 4C data

For comparison of two 4C profiles we first binarize the data, such that at each 4C fragend we only record whether it was captured (at least 1 read mapping to the fragend) or not. We then create bins of 50 consecutive fragends and for each profile compute the number of captured fragends in each bin. We select bins in which at least 5 fragends are covered in at least 1 experiment for further analysis. For each bin we test the null hypothesis that the proportion of covered fragends in each experiment is equal with Fisher's exact test. We reject this null hypothesis for bins with a p-value < 0.001.

All statistical analyses were performed under R/Bioconductor (Gentleman et al., 2004). Manipulation with and computation of statistics on genomic intervals and domains was done using the GenomicRanges (Lawrence et al., 2013) package.

For comparative analysis, Hi-C and principle component analysis data from embryonic stem cells was taken from Geeven, Zhu et al., in prep., density maps of Nanog from (Marson et al., 2008), Smc1 ChIP-seq data from (Kagey et al., 2010), RNA-seq and CTCF, H3K4me3, H3K27me3 and H3K9me3 ChIP seq data from the ENCODE project (The ENCODE Project Consortium, 2011).

FISH

The FISH procedure was adapted from (Bienko et al., 2013; Solovei and Cremer, 2010). In short, cells were grown overnight on gelatin coated coverslips, fixed in 4% paraformaldehyde (441244, Sigma) for 10 minutes, permeabilised with 0.5% Triton X-100 for 10 minutes, and then subjected them to four freeze-thaw cycles in 20% glycerol/PBS. We incubated the cells in 0.1N HCl for 10 minutes, and subsequently kept them in 50% formamide 2xSSC until hybridization. Locus-specific FISH probes were prepared from BACs as previously described (Splinter et al., 2011). The following BACs were used: RP24-241M12 (chr11:81096552-81279234) (Asci2 locus), RP23-31O12 (chr11:118411048-118618579), RP24-123C9 (chr11:96069113-96245395) (HoxB locus) and RP23-311P1 (chr11:102184420-102354519) (integration site). We designed a custom LacO probe and ordered it as an HPLC purified 3' Quasar-670 linked oligomer from Stellaris (LacO-1: 5'- acctttatccgctcacaattcggttacccctgctagaggt-Quasar670 -3'). Per slide, 1 pM LacO probe was mixed with the appropriate BAC probes before dehydration. Probes were denatured with the cellular DNA for 5 minutes at 80°C and subsequently hybridized for 16 hours at 37 °C in a hybridization buffer consisting of 50% formamide, 2xSSC, 2.5x Denhardt's solution, 50mM EDTA and 10% Dextran Sulphate. After hybridization, cells were washed twice for 30 minutes in 25% formamide 2xSSC at 30°C, counter stained with DAPI and embedded on glass slides with Prolong Gold (P36934, Thermo Scientific) and imaged on a Leica MM-AF microscope. ImageJ (Image5D plug-in) software was used to score touching (no unstained pixel in between FISH signals) and non-touching (with unstained pixel(s) in between) alleles. Depending on the BAC probe used, the average diameter of the fluorescent signal varied between 0.8-1µm, implying that the centers of touching signals can be up to 1µm apart.

Supplemental References

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