Spatiotemporal allele organization by allele-specific CRISPR live-cell imaging (SNP-CLING)

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Imaging and chromatin capture techniques have provided important insights into our understanding of nuclear organization. A limitation of these techniques is the inability to resolve allele-specific spatiotemporal properties of genomic loci in living cells. Here, we describe an allele-specific CRISPR live-cell DNA imaging technique (SNP-CLING) to provide the first comprehensive insights into allelic positioning across space and time in mouse embryonic stem cells and fibroblasts. With 3D imaging, we studied alleles on different chromosomes in relation to one another and relative to nuclear substructures such as the nucleolus. We find that alleles maintain similar positions relative to each other and the nucleolus; however, loci occupy unique positions. To monitor spatiotemporal dynamics by SNP-CLING, we performed 4D imaging and determined that alleles are either stably positioned or fluctuating during cell state transitions, such as apoptosis. SNP-CLING is a universally applicable technique that enables the dissection of allele-specific spatiotemporal genome organization in live cells.

The 3D organization of DNA is critical in the establishment of cellular states and is frequently dysregulated in disease1–4. This organization relies on regulatory aspects, such as nonrandom chromosomal positioning, chromosomal substructures and properties, and ultimately the position of a given locus in the nucleus5–7. Understanding the importance of these processes, several recent studies have revealed mechanistic links between chromosomal topology and disease. For example, structural chromosomal aberrations can result in pathogenic rewiring of enhancer–promoter interactions8,9. This raises the question of how heterozygous structural aberrations affect nuclear organization properties. Addressing this has been difficult for several reasons. (1) An allele-specific imaging approach is required, but so far, there are only two allele-specific techniques for fixed cells: using copy number variations and oligopainting10,11. (2) The Hi-C method has been adopted to study molecular interactions of haplotype genomes but lacks the ability to assess spatiotemporal dimensions12,13. (3) These techniques all encompass a multitude of previously intractable questions on chromosomal positioning, chromosomal substructures and properties, and ultimately the position of a given locus in the nucleus5–7. This finding suggests that not only are chromosome territories stably positioned, but also, specific spatial distances are maintained between alleles or loci. Moreover, through time, these distances are preserved, suggesting that there is no random movement of alleles relative to each other or relative to nuclear substructures such as the nucleolus. Altogether, SNP-CLING is broadly applicable in deciphering a multitude of previously intractable questions on chromatin biology and nuclear architecture in living cells.

Results

Implementing allele-specific SNP-CLING. To visualize each allele of a locus simultaneously in a living cell, we leveraged a nuclease-null mutant of the Streptococcus pyogenes Cas9 protein (dCas9) with pools of two to three single-guide RNAs (sgRNAs) targeting each gous chromosomes. Importantly, all of these factors can be investigated in living cells.

We applied SNP-CLING to two long noncoding RNA (lncRNA) loci, Firre and CISTR-ACT, which are involved in heterozygous structural aberrations that cause Mendelian disease16–18. Amplifications encompassing FIRRE have been found in patients with periventricular nodular heterotopia with polymicrogyria19, and translocations of CISTR-ACT are causally associated with brachydactyly20. Current methods cannot resolve the resulting implications of these heterozygous aberrations on higher-order nuclear architecture. Here, we first validate the specificity and accuracy of SNP-CLING and explore allelic positioning across space and time. Using 3D imaging, we determined that alleles stably maintain similar positions close to the nucleolus, although each studied locus occupied a unique localization within the nucleus. Next, we extended our analysis and performed allele-specific imaging across time (4D) to elucidate spatiotemporal allele positioning in relation to the major subnuclear compartment of the nucleolus. We found that alleles are stably positioned through time in human and mouse cells. This finding suggests that not only are chromosome territories stably positioned, but also, specific spatial distances are maintained between alleles or loci. Moreover, through time, these distances are preserved, suggesting that there is no random movement of alleles relative to each other or relative to nuclear substructures such as the nucleolus.
allele. Each sgRNA was internally appended with RNA-apta-mer motifs (MS2, PP7, or PufI) and cotransfected with the correspond-
 ing RNA-binding proteins (MS2, PP7, or PUM1) fused to a fluores-
 cent protein 19–23 (mVenus, mCherry, or IRFP670; Fig. 1a).

To reach our goal of allele-specific labeling of loci in living cells, we
exploited the need of S. pyogenes dCas9 to have a protospe-cimen-
directed motif (PAM, 5′-NRG-3′) located next to its target 14 and
 asked whether dCas9 could distinguish SNPs within the PAM motif,
thereby resolving specific alleles. To test whether SNP-CLING can
specifically label different alleles, we used mouse embryonic stem
 cells (mESCs) and mouse embryonic fibroblasts (MEFs), derived
from a hybrid 129S1× castaneous (129S1/CAST) mouse cross. First,
we identified suitable SNPs genome-wide in the PAM motif ‘NGR’
by filtering for either C or T substitutions at the second position
or any nucleotide other than G at the third position of the motif
by filtering for either C or T substitutions at the second position

Next, we measured distances of alleles on gene-dense and gene-
poor chromosomes. The alleles of the locus on a gene-rich chro-
some (3,223 ± 2,582 nm) were further apart from each other than the
Sox9 (2,288 ± 2,080 nm) or Cistr-act alleles on gene-poor chro-
mosomes (2,539 ± 1,776 nm; Fig. 2b and Supplementary Fig. 4b,
chr. 7 versus Sox9, P = 0.02). These observations demonstrate the
feasibility of SNP-CLING to detect spatial allelic distances and sug-
gest that relative allelic positioning can be slightly different for each
chromosome, supporting the notion that the positions of chro-
mosomal territories are stable.

Multiple genomic loci of local chromatin neighborhoods can be
clustered in spatial proximity to orchestrate tissue-specific gene regu-
lation 12–23. Because SNP-CLING is multiplexable, we were able to
address whether interallelic distances change relative to intergenic
distances to a third locus. Specifically, we labeled the alleles on chro-
some 7 by SNP-CLING (PP7-mCherry for maternal 129S allele,
MS2-mVenus for paternal CAST allele) and included a non-allele-
specific label (CLING) on a third locus on chromosome 18 (PufI-
PUM1-iRFP670). Notably, measuring interallelic distances between
the two alleles of chromosome 7 (129S1/CAST) relative to intergenic
distances of the locus on chromosome 18 showed highly similar and
stable distances independently of the genetic backgrounds 129S1 or
CAST (P < 0.0001, Pearson's r² = 0.71; Fig. 2c). This finding suggests
that both interallelic and intergenic distances relative to another
locus are similar. Among the loci we tested, one allele seemed to
maintain the same spatial distance relative to the other, underscor-
ing stable allelic positioning and the maintenance of these distances.

Collectively, SNP-CLING offers the advantage of measuring allelic
properties in living cells and directly assaying genomic positioning
in relation to other loci. In contrast, other imaging techniques that
are capable of labeling individual genomic loci, such as DNA-FISH,
require cross-linked cells and do not allow distinguishing between
parental alleles in live cells.

Allele positioning is preserved and unique for each locus. Despite
advances in characterizing chromosomal interactions and nuclear
architecture, we still do not have an understanding of how specific
alleles are positioned relative to subnuclear compartments in live
cells. Gene positioning relative to nuclear bodies (such as Cajal
bodies, sc35 speckles, nucleolus, etc.) gives insight into functional
nuclear organization 19; hence, we selected the nucleolus, as the larg-
est subnuclear compartment, to address the relative positioning of
individual alleles and whether any parental bias occurs. We labeled
the alleles of the loci studied above by SNP-CLING and used the
rRNA-GFP system for live-cell staining of nucleoli in female 129S1/CAS-
T MEFs (Fig. 3a and Methods).

By measuring distances between the alleles and the nucleoli, we
determined that between each locus, the distributions and distances
between 129S1 and CAST alleles to the nucleoli were highly similar
(Fig. 3b). We also measured distances of the individual alleles to
the nuclear periphery (Fig. 3c) and found that the distributions of
allelic distances to the nuclear periphery were also highly similar
between 129S1 and CAST backgrounds (Fig. 3d). This observation
is consistent with the finding that the tested loci are quite stable in
their relative nuclear positioning and neither randomly organized
nor undergoing random motion in the nucleus.

In order to assess whether every locus exhibits unique nuclear
positioning, we compared the allele–nucleoli distances with the
allele–nucleolar peripheries distances. Notably, both the maternal
129S1 and paternal CAST alleles were significantly closer to the

Interallelic differences are modest. Chromosomal territories are
nonrandomly organized 29,30, and the features of genomic loci,
such as chromosomal size, DNA (GC) content, gene density, and
transcriptional activity, differ. These properties are interde-pendent
and determine individual locus positioning 18. The 3D dynamics of
alleles have not yet been able to be addressed, and we still do not
know whether alleles occupy random or stable positions. However,
SNP-CLING can discriminate between alleles and determine allelic
positioning in living cells. To this end, we chose genes that are
located on chromosomes with different properties (size and gene
density) and are involved in heterozygous structural aberrations
causing brachydactyly (Hdac4, Sox9, and Cistr-act) 31,33. Specifi-
cally, we targeted alleles on a large chromosome (chr. 1: Hdac4), a small
chromosome (chr. 18: 41.21 Mb), a gene-dense chromosome (chr.
7: 99.55 Mb, chr. 11: Sox9), and a gene-poor chromosome (chr. 15:
Cistr-act; chr. 18: 41.21 Mb) in female 129S1/CAST MEFs (PP7-
mCherry for the maternal 129S allele and MS2-mVenus for the
paternal CAST allele; Supplementary Fig. 4a,b).

In order to address differences of spatial allelic distances that
may be influenced by chromosome size, we first measured distances
between alleles on the largest chromosome (chr. 1: Hdac4) and
between alleles on a small chromosome (chr. 18: random locus;
Fig. 2a). Despite differences in the chromosomal size, interallelic
distances on the large and the small chromosomes were highly simi-
lar to one another in a majority of cells (Figs. 2a, 2.948 ± 2.399 nm at
Hdac4, 2.664 ± 1.938 nm locus at 41.21 Mb on chr. 18).

To further test the ability of SNP-CLING to label alleles
related with the cells’ karyotypes, and these foci were the brightest
among the foci exhibiting two-color colocalization, thus indicating locus specificity (Fig. 1d and Supplementary Fig. 3). Moreover, we successfully
resolved maternal alleles separately from paternal alleles in 83% of
cells (Supplementary Fig. 3).

Predicted off-target sgRNA sites tend to occur at sites distant
from the primary target 19. We thus tested foci specificity without dis-
tinguishing alleles in human retinal epithelial RPE-1 cells 19 and C28/
12 chondrocytes 18. We targeted two different sgRNA pools (MS2-
mVenus and PP7-mCherry) or paternal CAST (two sgRNAs,
MS2-mVenus) Ypel4 (Fig. 1b). The expected number of foci cor-
related with the cells’ karyotypes, and these foci were the brightest
and largest nuclear signals compared to background signals in liv-
ing cells (Supplementary Figs. 2 and 3). Moreover, we successfully
resolved maternal alleles separately from paternal alleles in 83% of
cells (Supplementary Fig. 3).

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SNP-CLING or CLING labeling in live cells. a, sgRNAs harboring internal protein-binding RNA motifs (MS2, PP7, or Puf1) direct noncatalytic dCas9 to each targeted locus. The corresponding RNA-binding proteins MS2, PP7, and PUM1 are fused to mVenus, mCherry, and iRFP670, respectively, and fluorescently label up to three different loci. For allele-specific labeling, either the second or third nucleotide in the dCas9 PAM motif 5'-NGG-3' was substituted by a heterozygous SNP to a nonspecific dCas9 motif, thereby preventing dCas9 binding to either the 129S1 or CAST alleles in mouse hybrid cells. Sanger sequencing of selected SNPs confirmed heterozygosity. Y indicates C or T; H indicates A, C, or T.

b, Left, genotypes of 129S1–CAST cells used in this study: male mESCs and female MEFs. Right, allele-specific visualization of 129S1-Ypel4 (yellow) and CAST-Ypel4 (red) in 129S1–CAST mESCs. In total, 35 nuclei from four independent transfections were visualized; representative image is shown. Dashed line, nucleus. Arrowheads, SNP-CLING foci of 129S1 and CAST Ypel4 alleles. Scale bars, 5 µm.

c, Scheme of the experimental set up to assess specificity of SNP-CLING. Three sgRNAs harboring MS2 motifs (sgRNA pool 1, red) and three sgRNAs harboring PP7 motifs (sgRNA pool 2, yellow) used target sequences localized 1 kb apart from each other within the CISTR-ACT locus. d, All measurable foci exhibited two-color colocalization, indicating locus specificity in female RPE-1 cells. Edges of foci were closer than one voxel to each other, corresponding to 50 nm or less. In total, 35 nuclei from four independent transfections were visualized; representative image is shown. Arrowheads, CLING foci of CISTR-ACT. Scale bars, 500 nm.

e, Scheme of the experimental set up to assess resolution of SNP-CLING. Two sgRNA pools used, each consisting of three sgRNAs, target XIST (yellow) or TSIX (red) loci, separated by a topologically associating domain (TAD) boundary corresponding to a genomic linear distance of ~69 kb. f, Distinct, non-colocalized signals between XIST and TSIX, with spatial displacements of ~163–638 nm in all three dimensions in RPE-1 cells. An image representative of six out of ten cells is shown, and the distance of 163 nm between the signals along the x–y axis is indicated. Arrowheads, CLING foci of XIST and TSIX. Owing to the resolution limit and the magnification of the representative image, the scale bar (white spot, 100 nm) is blurry.
Each other. Internuclei distances assess whether subnuclear positioning is altered when multiple nucleoli occur. We found a range of one to six nucleoli, but more nucleoli in MEFs (average = 3) than in RPE-1 cells (average = 2.2, P < 0.0001; Fig. 3f). The internuclei distances were highly similar independent of the number of nucleoli (Fig. 3g), suggesting that nucleoli positioning may also be a stable feature in the nucleus and conserved between human and mouse.

Taken together, our results demonstrate that SNP-CLING can be used to label alleles in conjunction with subnuclear organelles. Our findings support the notion that irrespective of genetic backgrounds (129S1/CAST), the spatial positioning of the studied alleles was similar and preserved in interphase architecture and often close to the nucleoli.

**Firre's positioning and its interallelic interactions.** The human IncRNA *FIRRE* locus has previously been shown to associate with the nucleolar periphery using FISH in fixed cells48. Therefore, we questioned whether there were differences in nucleolar positioning to the loci described above. To this end, we measured spatial distances of *FIRRE* (MS2-mCherry), with GAPDH (PP7-mCherry) as a control locus, to their closest nucleoli (Fig. 4a and Supplementary Fig. 5). Consistent with the results in fixed cells, *FIRRE* was closer to the nucleolar periphery with an average locus–nucleus distance of 1,189 ± 1,291 nm, whereas GAPDH was 1,744 ± 1,508 nm distal from the nucleolus (P < 0.0001, Fig. 4a).

Because *FIRRE* is perinucleolar in many nuclei, we next asked whether there is an allele-specific bias in *Firre's* positioning that would be suggestive of a 3D imprinting mechanism. To this end, we labeled maternal (129S1, MS2-mVenues) or paternal *Firre* (CAST, PP7-mCherry) and quantified *Firre*-nucleoli distances in female 129S1/CAST MEFs (Fig. 4b). We found that either one of the alleles (maternal in 60% of nuclei, paternal in 63% of nuclei) or both of the alleles (47% of nuclei) were closer than 1 µm to the nucleoli (average 1,049 ± 1,096 nm (129S1), 1,094 ± 1,096 nm (CAST); Fig. 4b). Consistent with our previous findings, both *Firre* alleles were closer to the nucleoli than to the nuclear periphery (P < 0.0001; Fig. 4b). The mean distance of *Firre* and the other tested loci to the nucleoli was 1,019 ± 1,036 nm. Both human and mouse *FIRRE* loci were frequently perinucleolar without any allele bias (Fig. 4b). These findings indicate the preserved genomic positioning of *Firre* with low cell-to-cell variability, independent of the inherited genetic background.

Having resolved that SNP-CLING can identify allele positioning, we next investigated its feasibility to measure cross-chromosomal interactions of two genomic loci in live cells, which could also be indicative of a 3D imprinting mechanism, for example, maternal–maternal cross-chromosomal interactions. To this end, we focused on the previously described interaction of *Firre* (chr. X) with *Ypel4*, a locus on the nonhomologous chromosome 2 (ref. 14) (Fig. 4c). We targeted maternal *Firre* (129S1, MS2-mVenues) and either maternal or paternal *Ypel4* (PP7-mCherry) and quantified *Firre–Ypel4* colocalizations in male 129S1/CAST mESCs (Fig. 4d and Supplementary Fig. 5a, each combination >130 nuclei). Signals were deemed to be colocalized as monoallelic or biallelic interactions if observed at <50 nm spatial distance (Methods). We identified colocalizations between *Firre* and the 129S1 *Ypel4* allele in 52% of the nuclei and CAST *Ypel4–Firre* interactions in 67% of nuclei (P = 0.0012), confirming previous FISH results14. Thus, there is a slight but significant skewing of the parental origin for cross-chromosomal interactions that can be identified using SNP-CLING (Fig. 4d).

**Firre alleles are stably positioned over time.** Thus far, the temporal properties of alleles could not have been assessed by traditional imaging (such as DNA-FISH) or molecular methods (3C–Hi-C). Because SNP-CLING can detect allele-specific locus properties over time (4D), we selected to study *Firre's* temporal positioning relative...
Fig. 3 | Allelic distances to the nucleoli or to the nuclear periphery in 129S1/CAStr MEFs. In total, 80 nuclei from five independent transfections were visualized for each locus assessed. Arrowheads: red, 129S1 allele; yellow, CAST allele. **a**, Representative images demonstrate positioning of the studied alleles and the nucleoli (rRNA-GFP). Scale bars, 5 μm. **b**, Allelic distances and distributions of alleles to the nucleoli between 129S1 and CAST alleles. Allelic distances of the studied loci to the nucleoli are shown as means ± s.d. Measurements were normalized to nuclei sizes. **c**, Representative images demonstrate positioning of the studied alleles versus the nuclear periphery. Scale bars, 5 μm. **d**, Allelic distances and distributions of alleles to the nuclear periphery between 129S1 or CAST alleles. Allelic distances of the studied loci to the nucleoli (data shown as means ± s.d. in **b**) were significantly smaller than their distances to the nuclear periphery (data shown here as means ± s.d.). Two-tailed Mann–Whitney rank-sum testing, *P* values are indicated in the distribution graphs. **e**, Spearman’s rank correlation coefficient of all allelic distances to the nucleoli (left) or to the nuclear periphery (right). 61% of 129S1- or 64% CAST-inherited loci were closer than 1 μm to the nucleolus. 35% of 129S1 or 30% of CAST loci were closer than 1 μm to the periphery. In total, distances of 620 alleles were measured. **f**, Number of nucleoli in human RPE-1 and MEFs used in this study. Nucleoli were counted in 120 RPE-1 and 250 MEF cells. Two-tailed Mann–Whitney rank-sum testing, ***P* < 0.0001. **g**, Internucleoli distances in human RPE-1 or MEFs. Kruskal–Wallis multiple comparisons testing (not adjusted). Box plot midlines show medians, box edges show 25th and 75th percentiles, and whiskers show minimum and maximum values. Graphs indicate sample sizes. n.s., not significant; RPE-1, CI_{lower} = 3.02–3.31 μm and CI_{upper} = 3.67–3.99 μm; MEFs, CI_{lower} = 3.06–3.35 μm and CI_{upper} = 3.63–4.7 μm.
Fig. 4 | Firre’s positioning and its interallelic interactions. a, Representative images of CLING-labeled Firre and Gapdh loci with nucleoli (rRNA-GFP) in female RPE-1 cells. Loci–nuclei distances revealed that Firre was closer to the perinucleolar space than Gapdh. Two-tailed Mann–Whitney rank-sum test, ***P < 0.0001. In total, 108 nuclei were visualized for Firre and 107 nuclei for Gapdh measurements. Arrowheads, CLING-labeled loci. Scale bars, 5 μm. b, Left, representative image showing Firre alleles in 129S1/CAST MEFs. Arrowheads: yellow, 129S1 allele; red, CAST allele. Scale bars, 5 μm. Middle, Firre’s allelic distances to the nucleoli (129S1 allele CI = 0.77–1.32 μm; CAST allele CI = 0.81–1.38 μm). Right, Firre alleles’ distances to the nuclear periphery (129S1 allele CI = 1.38–1.84 μm; CAST allele CI = 1.48–1.89 μm). Firre was closer to the nucleolus than to the nuclear periphery. Two-tailed Mann–Whitney rank-sum test, ***P < 0.0001. In total, 80 nuclei were visualized. Means ± s.d. are shown. c, Scheme of SNP-CLING or CLING targeting two interacting loci (red and yellow) between two nonhomologous chromosomal territories. d, Left, scheme of SNP-CLING targeting 129S1-derived Firre with either Ypel4-129S1 or Ypel4-CAST in 129S1/CAST mESCs. Right, representative image of a colocalization event of Firre and maternal Ypel4-129S1 Arrowheads: yellow, 129S1 allele; red, CAST allele. Scale bar, 5 μm. e, Quantification of colocalization events of Firre with Ypel4 alleles determined the allele-biased interaction of Firre with the paternal Ypel4-CAST allele in 129S1/CAST mESCs. Each quantification was done in at least 130 nuclei from five independent transfections. χ² test, **P = 0.0012.

To address how alleles behave over time in the 3D space of the nucleus, we further performed allele-specific 4D-SNP-CLING of the maternal and paternal Firre alleles for up to 4.5 h in 129S1/CAST hybrid MEFs (Supplementary Table 2). First, we measured interallelic distances between the maternal 129S1-Firre and paternal CAST-Firre alleles. We detected that the distance between the alleles was stable on average over time (Fig. 5c). This finding recapitulates our results that alleles are stably positioned in the 3D space of the nucleus across time.

Next, by measuring spatiotemporal distances as described above, we addressed how the Firre alleles are positioned in relation to the nucleoli over time. Both of the maternal and paternal Firre alleles were equally distant to its closest nucleolus (Fig. 5d). We also determined average speed and tortuosity (3D directional changes in space) and detected no differences between the alleles and in comparison to the nucleoli (Fig. 5e,f), thus indicating preserved and stable spatiotemporal allele positioning.

Allele-specific spatiotemporal dynamics in cell-cycle stages and state transitions. The question of how cell-cycle and apoptotic actions reorganize the genome and influence allele positioning has not been investigated in live cells thus far. To address this, we used serum starvation of 129S1/CAST hybrid MEFs and then induced the cell cycle or apoptosis by serum exposure before imaging to provoke changes of the mitotic stage or to induce apoptosis38. In extended time-lapse experiments (up to 4 h per nucleus, Supplementary Table 2), we measured Firre’s allelic distances to the nucleoli across space and time, as described above. We separated the acquired datasets of 4D-SNP-CLING in living cells into two groups. First, we analyzed time lapses in which we observed allele-specific signals from the sister chromatids, indicating S-G2 phases (Fig. 5g). We determined that Firre’s interchromatid distances and the distances of each chromatid to the closest nucleolus were on average stable over time (Fig. 5h,i), suggesting that replication does not have a major impact on spatial repositioning of loci.

In our second group of time-lapse experiments, we analyzed cells undergoing apoptosis, implicating major genomic reorganization by chromatin condensation (Fig. 5). As far as we were able to track stable signals successfully, we determined that the interallelic distances increased over time toward the point at which apoptosis was visually detectable by condensed, nonpatterned chromatin (Fig. 5j,k). Moreover, the allelic–nucleoli distances fluctuated much more, especially at later time points (Fig. 5l). These findings suggest that apoptosis-mediated changes of the genome organization cause locus repositioning by dissolving and separating the nucleoli from alleles. Altogether, these results indicate that genomic reorganization affects allelic repositioning and that SNP-CLING can be used to study conformational changes during mitotic and apoptotic stages of the nucleus. Importantly, 4D-SNP-CLING is a versatile tool that can specifically discern spatiotemporal allele positioning in relation to subnuclear compartments in various fields of cell biology.
Fig. 5 | Firre’s allelic positioning over time. a, Scheme of 4D time-lapse imaging: the individual distances, paths, and intervals of signals (loci or alleles) were tracked over time to address spatiotemporal dynamics. b, Ratios of loci-nucleolus distances of FIRRE and GAPDH in RPE-1 cells over time. Means ± s.e.m. are shown. FIRRE was closer to the perinucleolar region than GAPDH. Two-tailed Mann–Whitney rank-sum test, **P < 0.001. In total, nine nuclei were visualized for FIRRE and seven nuclei for GAPDH measurements. Individual samples shown in Supplementary Fig. 6a. c, The interallelic distances of Firre alleles were measured with 4D-SNP-CLING over time in 129S1/CAST MEFs. Means ± s.d. of measurements for eight nuclei are shown; individual samples shown in Supplementary Fig. 6b. d, The allelic distances of Firre alleles to the closest nucleolus were measured with 4D-SNP-CLING over time in 129S1/CAST MEFs. Means ± s.d. of measurements for eight nuclei are shown; individual samples shown in Supplementary Fig. 6c. e, Average speed of Firre alleles and nucleoli measured by 4D-SNP-CLING in MEFs over time. Means ± s.d. and individual data points from measurements for eight nuclei are shown. f, Tortuosity (3D changes in direction) of Firre alleles and nucleoli in MEFs. Means ± s.d. and 64 individual data points from measurements for eight nuclei are shown. g, Left, representative images of nucleoli and 4D-SNP-CLING detecting alleles of Firre on sister chromatids during S-G2 phase. Arrowheads: yellow, 129S1 allele; red, CAST allele. Scale bars, 5µm. Right, scheme of measured interchromatid distances and distances to the closest nucleolus. In total, three nuclei of 129S1/CAST MEFs were tracked over time. h, Distances between sister chromatids in 129S1/CAST MEFs over time. Means ± s.d. of measurements for three nuclei are shown. Individual samples shown in Supplementary Fig. 6d. i, Distances of sister chromatids to the nucleolus in 129S1/CAST MEFs over time. Means ± s.d. of measurements for three nuclei are shown. Individual samples shown in Supplementary Fig. 6e. j, Left, representative images of nucleoli and 4D-SNP-CLING detecting alleles in a cell undergoing apoptosis. Arrowheads: yellow, MS2-mVenus, 129S1 allele; red, PP7-mCherry, CAST allele. Scale bar, 5µm. Right, scheme of measured interallelic distances and distances to the closest nucleolus. In total, five nuclei of 129S1/CAST MEFs were tracked over time. k, Fluctuating interallelic distances in apoptotic 129S1/CAST MEFs. Means ± s.d. of measurements for five nuclei are shown. Individual samples shown in Supplementary Fig. 6f. l, Fluctuating distances between the Firre alleles and the nucleolus in apoptotic 129S1/CAST MEFs. Means ± s.d. measurements for five nuclei are shown. Individual samples shown in Supplementary Fig. 6g.
Discussion
Several studies have shown that distinct gene positioning is crucial for gene regulation and molecular pathogenesis\(^{5,14,39}\). Yet, most of our current understanding derives from the average of both homologous chromosomes and does not have the resolution for spatiotemporal assessment of specific allele positioning. This important aspect allows us to move forward in understanding how chromosomal aberrations on one allele affect nuclear organization. SNP-CLING provides a solution to this knowledge gap by detecting allelic characteristics in live cells that yield insight into gene regulation and nuclear architecture.

The applications of SNP-CLING cover a wide range of biological questions, including allele-specific studies to evaluate distances of genomic interactions in fixed and in living cells, spatiotemporal gene positioning in relation to cellular or nuclear subcompartments\(^{40}\), epigenetic phenomena like imprinting\(^1\), karyotyping, or allele-specific transcriptional control (SNP-CLING with smRNA-FISH)\(^5\). SNP-CLING\(^5\) facilitates testing these genomic identities by using heterozygous SNPs and the multiplexable CRISPR–Cas9 sgRNA-aptamer system to label individual DNA loci with unique aptamer-binding fluorescent proteins.

We applied SNP-CLING to address the question of how alleles on different chromosomes are positioned relative to one another and to nuclear subcompartments such as the nucleolus, which has not yet been tractable in a facile manner, especially in live cells. We tested two possible outcomes: (i) alleles move in a random motion in the nucleus and exhibit significantly different distances from each other, the nucleolus and/or other chromosomes (three-color SNP-CLING); (ii) alleles are stably positioned in space and time relative to each other, other chromosomes and/or the nucleolus.

To distinguish between these two possibilities, we selected alleles on chromosomes with different features of size and gene density and observed that their interallelic distances were mostly similar, either in relation to one another or to other loci. Although individual alleles are stably positioned in space and time, different chromosomes have unique positions. These findings are consistent with results of fixed cells and the chromosome territory models\(^5,7,43\).

We also found that allele positions are preserved and similar in live cells, irrespective of the genetic backgrounds (129S1/CAST) in 3D space (Fig. 3e). Particularly, we detected that the studied alleles are equally positioned proximal to the nucleoli with unique distances from each other, the nucleolus and/or other chromosomes (Figs. 2, 3 and 5). Similar results were obtained by studying chromosomal territories, where each territory showed a unique pattern with minor differences between homologous chromosomes in fixed cells\(^5\). A broader analysis is required to dissect the characteristic chromosomal features of size, gene density\(^5\), transcriptional activity\(^5\), local chromatin states and protein recruitment\(^5\) and whether they directly influence allele positioning proportional to the spatial order of whole chromosomes.

Remarkably, the majority (>60%) of the studied alleles were positioned at discrete subnuclear positions around the perinuclear space (Fig. 3e). Particularly, we detected that the studied alleles are equally positioned proximal to the nucleoli with unique distances for each locus, and in the case of Firre, both alleles stayed stably associated with the nucleoli across time. Therefore, it is plausible that structural chromosomal rearrangements encompassing these loci can alter their constrained and preserved allele positions and contribute to disease\(^5,14,46\).

We also demonstrated that SNP-CLING can assess interchromosomal contacts in live cells. Firre displayed a slight allelic skewing for its cross-chromosomal interaction with Ypeh (Fig. 4e). However, further studies are required to assess whether allele-biased interactions between nonhomologous chromosomes are common and stable, as in the case of the Firre–Ypeh cross-chromosomal contact. Our finding may support the idea that a deterministic parental-origin grouping of haploid chromosomes and allele-specific interactions may exist across chromosomes, but not across alleles of the same chromosome.

In summary, allele-specific SNP-CLING is designed for dissecting nuclear architecture phenomena at allele-specific resolution across space, through time, and in relation to other loci or subnuclear compartments. SNP-CLING and CLING are easily applicable and versatile tools that will shed new light on the contribution of genome architecture to congenital disease and cancer that are often linked to allele-specific aneuploidy and heterozygous chromosomal aberrations\(^44,46\). Further characterization of spatial and temporal properties of allele-specific alterations occurring in disease will provide significant insight into the genetic, genomic, and structural features underlying nuclear architecture.

Methods
Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41594-017-0015-3.

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References
1. Dekker, J. Two ways to fold the genome during the cell cycle: insights obtained with chromosome conformation capture. *Epigenetics Chromatin* 7, 25 (2014).
2. Luján, D. G. et al. Disruptions of topological chromatin domains cause pathogenic rewiring of gene-enhancer interactions. *Cell* 161, 1012–1025 (2015).
3. Dixon, J. R. et al. Chromatin architecture reorganization during stem cell differentiation. *Nature* 518, 331–336 (2015).
4. Gorkin, D. U., Leung, D. & Ren, B. The 3D genome in transcriptional regulation and pluripotency. *Cell Stem Cell* 14, 762–775 (2014).
5. Jost, K. L. et al. Gene repositioning within the cell nucleus is not random and is determined by its genomic neighborhood. *Epigenetics Chromatin* 8, 36 (2015).
6. Chubb, J. R., Boyle, S., Perry, P. & Bickmore, W. A. Chromatin motion is constrained by association with nuclear compartments in human cells. *Curr. Biol.* 12, 439–445 (2002).
7. Kumaran, R. I. & Spector, D. L. A genetic locus targeted to the nuclear periphery in living cells maintains its transcriptional competence. *J. Cell Biol.* 180, 51–65 (2008).
8. Meaburn, K. J., Godla, P. R., Khan, S., Lockett, S. J. & Misteli, T. Disease-specific gene repositioning in breast cancer. *J. Cell Biol.* 187, 801–812 (2009).
9. Ferrai, C., de Castro, I. J., Lavitas, L., Chotalia, M. & Pombo, A. Gene positioning. *Cold Spring Harb. Perspect. Biol.* 2, a000588 (2010).
10. Franke, M. et al. Formation of new chromatin domains determines pathogenicity of genomic duplications. *Nature* 538, 265–269 (2016).
11. Weise, A. et al. POD-FISH: a new technique for parental origin determination based on copy number variation polymorphism. *Methods Mol. Biol.* 659, 291–298 (2010).
12. Beliveau, B. J. et al. Single-molecule super-resolution imaging of chromosomes and in situ haplotype visualization using Oligopaint FISH probes. *Nat. Commun.* 6, 7147 (2015).
13. Giorgetti, L. et al. Predictive polymer modeling reveals coupled fluctuations in chromosome conformation and transcription. *Cell 157*, 950–963 (2014).
14. Darrow, E. M. et al. Deletion of DXZ4 on the human inactive X chromosome alters higher-order genome architecture. *Proc. Natl. Acad. Sci. USA* 113, E4504–E4512 (2016).
15. Hoffman, E. A., Frey, B. L., Smith, L. M. & Aubele, D. T. Formaldehyde crosslinking: a tool for the study of chromatin complexes. *J. Biol. Chem.* 290, 26404–26411 (2015).
16. Hacisuleyman, E. et al. Topological organization of multichromosomal regions by the long intergenic noncoding RNA Firre. *Nat. Struct. Mol. Biol.* 21, 198–206 (2014).
17. Abe, Y. et al. Xq26.1-26.2 gain identified on array comparative genomic hybridization in bilateral periventricular nodular heterotopia with overlying polymicrogyria. *Dev. Med. Child Neurol.* 56, 1221–1224 (2014).
18. Maas, P. G. et al. A misplaced IncRNA causes brachydactyly in humans. *J. Clin. Invest.* 122, 3990–4002 (2012).
19. Stiehn, D. M., Hacisuleyman, E., Younger, S. T. & Rinn, J. L. Multiplexable, locus-specific targeting of long RNAs with CRISPR-Display. *Nat. Methods* 12, 664–670 (2015).
20. Chao, J. A., Patksovsky, Y., Almo, S. C. & Singer, R. H. Structural basis for the coevolution of a viral RNA-protein complex. Nat. Struct. Mol. Biol. 15, 103–105 (2008).

21. Shcherbakova, D. M. & Verkhusha, V. V. Near-infrared fluorescent proteins for multicolor in vivo imaging. Nat. Methods 10, 751–754 (2013).

22. Filippovska, A., Razif, M. F., Nygaard, K. K. & Rackham, O. A universal code for RNA recognition by PUF proteins. Nat. Chem. Biol. 7, 425–472 (2011).

23. Cheong, C. G. & Hall, T. M. Engineering RNA sequence specificity of Pumilio repeats. Proc. Natl. Acad. Sci. USA 103, 13635–13639 (2006).

24. Anders, C., Niewoehner, O., Duerst, A. & Jinek, M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature 513, 569–573 (2014).

25. Doench, J. G. et al. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat. Biotechnol. 34, 184–191 (2016).

26. Bodnar, A. G. et al. Extension of life-span by introduction of telomerase into normal human cells. Science 279, 349–352 (1998).

27. Goldring, M. B. et al. Interleukin-1 beta-modulated gene expression in immortalized human chondrocytes. J. Clin. Invest. 94, 2307–2316 (1994).

28. Nora, E. P. et al. Spatial partitioning of the regulatory landscape of the X-inactivation centre. Nature 485, 381–385 (2012).

29. Sehgal, N. et al. Large-scale probabilistic 3D organization of human chromosome territories. Hum. Mol. Genet. 25, 419–436 (2016).

30. Cremer, T. & Cremer, M. Chromosome territories. Cold Spring Harb. Perspect. Biol. 2, a003889 (2010).

31. Williams, S. R. et al. Haploinsufficiency of HDAC4 causes brachydactyly, delays, and behavioral problems. Am. J. Hum. Genet. 87, 219–228 (2010).

32. Beagrie, R. A. et al. Complex multi-enhancer contacts captured by genome architecture mapping. Nature 543, 519–524 (2017).

33. Shopland, L. S., Johnson, C. V., Byron, M., McNeil, J. & Lawrence, J. B. Clustering of multiple specific genes and gene-rich R-bands around SC-35 domains: evidence for local euchromatic neighborhoods. J. Cell Biol. 162, 981–990 (2003).

34. Edelman, L. B. & Fraser, P. Transcription factories: genetic programming in three dimensions. Curr. Opin. Genet. Dev. 22, 110–114 (2012).

35. Lamond, A. I. & Spector, D. L. Nuclear speckles: a model for nuclear organelles. Nat. Rev. Mol. Cell Biol. 4, 605–612 (2003).

36. Shea, J. R. Jr & Leblond, C. P. Number of nucleoli in various cell types of the mouse. J. Morphol. 119, 425–433 (1966).

37. Yang, P. et al. The lncRNA Firre anchors the inactive X chromosome to the nucleolus by binding CTCF and maintains H3K27me3 methylation. Genome Biol. 16, 52 (2015).

38. Kulkarni, G. V. & McCulloch, C. A. Serum deprivation induces apoptotic cell death in a subset of Balb/c 3T3 fibroblasts. J. Cell Sci. 107, 1169–1179 (1994).

39. Morimoto, M. & Boerkoel, C. F. The role of nuclear bodies in gene expression and disease. Biology (Basel) 2, 976–1033 (2013).

40. Thul, P. J. et al. A subcellular map of the human proteome. Science 356, eaal3321 (2017).

41. Chess, A. Monoallelic gene expression in mammals. Annu. Rev. Genet. 50, 317–327 (2016).

42. Cui, C., Shu, W. & P. Fluorescence in situ hybridization: cell-based genetic diagnostic and research applications. Front. Cell Dev. Biol. 4, 89 (2016).

43. Wiichers, P. J. et al. Cause and consequence of tethering a SubTAD to different nuclear compartments. Mol. Cell 61, 461–473 (2016).

44. Peric-Hupkes, D. et al. Molecular maps of the reorganization of genome-nuclear lamina interactions during differentiation. Mol. Cell 38, 603–613 (2010).

45. Lupiñez, D. G., Spielmann, M. & Mundlos, S. Breaking TADs: how alterations of chromatin domains result in disease. Trends Genet. 32, 225–237 (2016).

46. Wijchers, P. J. & de Laat, W. Genome organization influences partner selection for chromosomal rearrangements. Trends Genet. 27, 63–71 (2011).

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Author contributions

P.G.M and J.L.R conceived the study. P.G.M. performed the experiments and wrote the manuscript with J.L.R and A.R.B. C.L.W. performed the mESC experiments, M.M. analyzed the availability of suitable SNP-CLING SNPs, and D.M.S. provided intellectual input and plasmids.

Competing interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41594-017-0015-3.

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Methods

**Cell lines.** hTERT RPE-1 cells (ATCC), C28/I2 chondrocytes, 129S1-SvImJ × CAST-EiJ hybrid 2–3 × 10^6 cells were seeded per well of a LabTek chamber slide. Animals were handled according to the Harvard University IACUC guidelines (protocol AEP 11–13).

**SNP selections in human or mouse PAM motifs.** We downloaded hg19 and mm10 genome assemblies and masked regions with repetitive elements using RepeatMasker (http://www.repeatmasker.org). We looked for all instances in which a SNP annotated in the dbSNP database (version 147 for human and version 142 for mouse) disrupted the PAM sequence 5′-NRG-3′ in either the sense or the antisense orientation in the corresponding assembly.

**sgRNA design for CLING and SNP-CLING.** Nonrepetitive genomic regions several kilobases upstream or downstream of the target genes, avoiding interference with transcription, were selected in the hg19 or mm10 UCSC Genome Browser. These regions served as the input to design sgRNAs (http://www.broadinstitute.org/rnaai/public-analysis-tools/sgrna-design-v1). Four highly specific sgRNAs with sgRNA scores around 1 and low off-target scores were chosen and aligned back to the reference genome using BLAT (UCSC) to determine specificity. Flanking BbsI restriction sites were used to ligate sgRNAs with a backbone expressing three MS2, three Pp7, or six PuF1 motifs (Addgene #68442, #68424). mCherry and mVenus fluorescent proteins were expressed as fusion proteins with Pp7- and MS2-binding proteins (Addgene #68420), respectively, independent of dCas9 (Addgene #68416). All combinations of three of the four sgRNAs were pooled after endonuclease-free MAXI preparations to test off-target binding in confocal microscopy (Supplementary Fig. 5). The sgRNA pools with the most specific signals and reduced off-target-binding effects were used for super-resolution microscopy. Three sgRNAs, each with three RNA stem–loops for the RNA-binding proteins bound to fluorescent proteins, resulting in a specific CLING-signal derived from 18 molecules (Fig. 4c and Supplementary Table 1).

**For SNP-CLING, genomic regions were selected in the MGI browser (http://www.informatics.jax.org), and heterozygous SNPs were extracted for the 129S1-SvImJ × CAST-EiJ hybrid cells.** The SNP-CLING selection was made based on heterozygous SNPs at the second or third position of the PAM sequence (5′-NRG-3′) that were selected from either the 129S1-SvImJ or the CAST-EiJ allele. The heterozygous state of the SNPs was validated by Sanger sequencing. A list of sgRNA sequences can be found in Supplementary Table 1.

**mESC culture.** Male 129S1-SvImJ × CAST-EiJ hybrid 2–3 × 10^6 cells were grown on a 0.2% gelatinized petri dish in 2i media (125 ml DMEM-F12 (Thermo Fisher Scientific, #11330-032), 125 ml Neurobasal Medium (Thermo Fisher Scientific, #21030-049), 1.25 ml B-27 Supplement 50 (Millipore, #21103-049), 1.25 ml NDiff Neuro-2 Medium Supplement 200 (Millipore, #SCM012), 83.5 µl β-mercaptoethanol, 25 µl LIF ESGRO (107 units LIF/ml stock) (Millipore, #21528-010), 1 µl NucBlue Live ReadyProbes (Thermo Fisher Scientific, #A1896701), and one drop of NucBlue Live ReadyProbes (Thermo Fisher Scientific, #A1896701), and one drop of NucBlue Live ReadyProbes (Thermo Fisher Scientific, #A1896701), and one drop of NucBlue Live ReadyProbes (Thermo Fisher Scientific, #A1896701). Media was changed 24 h post transfection and cells were imaged 48 h post transfection.

Prior to imaging, the medium was changed to FluoroBrite DMEM Media (Thermo Fisher Scientific, #A1896701), and one drop of NucBlue Live ReadyProbes Reagent (Thermo Fisher Scientific #R37605) was added to stain the nuclei. A 1:2,500 dilution of the CytoPainter Nucleolar Staining Kit (Abcam, #ab139475), according to the manufacturer instructions, was used to stain the nucleoli. After a 10-min incubation at 37°C, 5% CO2, the cells were washed twice with PBS and prepared for imaging as described above. None of the studied loci were observed inside the nucleoli.

**Confocal super-resolution microscopy (Airyscan).** The LSM880 with Airyscan (Zeiss) and the incubation module at 37°C and 5% CO2, were used to visualize all experiments in this project. A 32-channel gallium arsenide phosphide photodiode multiplier tube (GaP-APT) was used as a detector (Airyscan) to acquire whole-cell plane image at every scan position. The Airyscan detector system enhances sensitivity 4–8-fold and resolution beyond the diffraction limit of light of up to 1.7-fold (~130 nm) compared to standard confocal microscopy (as described in a recent Application Note advertising feature in Nature Methods, https://www.nature.com/articles/nmeth.f.388). For all acquisitions, −5 to −10× les laser power was used compared to standard maximum intensity projection (MIP) imaging software version 2 (Zeiss) was used for acquisition. All shown images were acquired in super-resolution (SR) mode with 16 bit, 0.17 µm slices, and fitted zoom level, whereas quantification experiments were done in resolution-versus-sensitivity (R-S) mode with 8 bit and 0.25–0.3 µm slices to increase imaging speed. The oil immersion objective Plan-Apochromat 63×/1.4 OIL DIC M27 was used for all acquisitions. If the edges of foc were closer than one voxel distance to each other which corresponds to <0.1 nm, this distance represented the smallest measurable volume (distance) in the 3D space by combination of CLING and Airyscan microscopy. For the quantifications of colocalization events, CLING signals were either merged or overlapping (<50 nm distance, see below) in contrast to distinctly separated signals (>50 nm distance) indicating no colocalization.

**4D time-lapse imaging.** The conditions for time lapses, acquired in R-S mode with 8 bit and 0.25–0.45 µm slices to minimize the acquisition time, were set up in the experiment designer (ZEN black edition version 2) with delay blocks (breaks) of either 10 or 20 s between every z stack, favoring reduced fading or phototoxic effects. The time intervals and features of the 4D experiments are given in the Supplementary Information.

**Post-processing and analyzing imaging data.** The Airyscan super-resolution raw data were deconvoluted and processed in ZEN black edition version 2.1 (release version 11.0, Zeiss). Maximum intensity projection (MIP) was used to merge the z stacks and to intensify the signals. Pixel intensity display settings were automatically and manually optimized with a ‘linear best-fit analysis’ applied to the entire field of view of each channel separately in ZEN blue edition (Zeiss). Using the voxel centers, distance measurements were done in ZEN editions and ImageJ (https://imagej.nih.gov/ij/). Maximum intensity projection images were measured between each nuclear center, and loci–nucleoli distances were measured from each SNP-CLING signal (voxel center) to its closest nucleolus. All measurements were normalized to the nuclei sizes (x–y–dimensions). To measure the minimal distance between two non-colocalizing signals, the distance between the signals was measured in ZEN software. The smallest unit was one pixel, corresponding to approximately ~50 nm. If this one-pixel distance was not clearly determined, the signals were considered as colocalization. All images were exported as non-compressed TIFF images.

The raw data of 4D time-lapses were processed and inspected for signal integrity over time in ZEN black edition. Datasets with insufficient and dim signals due to photobleaching effects at later time points were cropped. The adjusted post-processed time-lapse datasets were then imported in arvis Vision4D versions 2.12 and 2.11 software. CLING-signals were selected according to their appearance in every first and last plane of every time frame and exchanged by artificial centroids representing the raw signals to simplify visualization. 3D coordinates were calculated depending on the position of the centroids’ center representing the raw CLING voxel’s center. Hoechst nuclei staining was visualized with the ‘skin’ default setting in Vision4D v2.11. Ratios of distances were calculated based on the distance measurements between signals. For normalization, all signals were divided by the mean distance of signals at time point 1 of every time-lapse experiment. The plotted values shown are means (±s.e.m. or ±s.d.) of all signals.

**Statistical analysis.** Signals for every experiment were quantified in ≥2 stacks of 80–100 nuclei. Additional numbers (n) of experiments or analyzed nuclei, or alleles, are mentioned in the manuscript or in the figure legends. Significance was determined by nonparametrical two-tailed Wilcoxon Mann–Whitney rank-sum test or Student’s t-test (***P < 0.0001, **P < 0.001, *P < 0.05). To show how median, box edges show 25th to 75th percentiles, and whiskers show minimum and maximum values. Plots and statistics were generated in either GraphPad Prism version 6.00 or MATLAB R2015b (MathWorks).
Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. The datasets of spatial distances analyzed during the current study (Figs. 2–3) are available from the corresponding authors on reasonable request.

References
47. Doench, J. G. et al. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. Nat. Biotechnol. 32, 1262–1267 (2014).
48. Engreitz, J. M. et al. The Xist lncRNA exploits three-dimensional genome architecture to spread across the X chromosome. Science 341, 1237973 (2013).
Experimental design

1. Sample size

Describe how sample size was determined.

We haven't done a sample-size calculation. Instead, we imaged 80 - 130 nuclei for each experiment for any statistical calculations. 80-100 nuclei is the gold-standard in cytogenetics.

2. Data exclusions

Describe any data exclusions.

No data were excluded.

3. Replication

Describe whether the experimental findings were reliably reproduced.

The imaged nuclei "represent" replicates in this study. The variations (distance measurements) within these 100 nuclei are depicted in CDF plots.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

We generated descriptive data or generated two datasets (i.e. measurements of maternal vs. paternal allele) that we compared directly. Randomizing these data would not provide novel insights, not validate the experimental data.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Blinding was not relevant for most experiments of this study, since conditions / allocations were not compared with one another. We analyzed all occurring spatial distances within a population of 80-100 nuclei. With regards to co-localizations in mESCs, two investigators analyzed the images independently.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- [ ] The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- [ ] A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- [ ] A statement indicating how many times each experiment was replicated
- [ ] The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- [ ] A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- [ ] The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted
- [ ] A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- [ ] Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Zen black 2.0, 2.1 & blue editions (Zeiss) were used to process Airyscan raw images, including deconvolution, signal detection, maximum intensity projection, and image export. Arivis Vision4D v2.11, 2.12 was used to track time-lapse signals and to extract three-dimensional coordinates to calculate spatial distances, speeds, tortuosity. GraphPad Prism 6.00, MATLAB R2015b and Excel were used for statistical calculations. Repeatmasker (http://www.repeatmasker.org) was used to identify repetitive elements. sgRNA design (http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design-v1). Mouse genomic regions were selected by using http://www.informatics.jax.org.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

Materials are available from the authors or from Addgene, as described in the methods.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

No antibodies were used.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

RPE-1 cells from ATCC, C28/I2 cells from Mary B. Goldring, mESCs from Jesse Engreitz, MEFs were generated by Jordan Lewandowski

b. Describe the method of cell line authentication used.

ATCC validated the karyotype of RPE-1 cells. C28/I2, mESCs or MEFs were not validated

c. Report whether the cell lines were tested for mycoplasma contamination.

Mycoplasma tests were performed on all used cell lines.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Mouse embryonic fibroblasts (MEFs) were isolated with standard procedures from 129S1-SvImJ x CAST-EiJ mouse strains. Animals were handled according to the Harvard University IACUC guidelines (protocol AEP 11-13).

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

Human participants were not involved in this study.