eLife

Reviewed Preprint
v2 • February 24, 2025
Revised by authors

Reviewed Preprint
v1 • July 16, 2024

Genetics and Genomics

Beyond A and B Compartments: how major nuclear locales define nuclear genome organization and function

Omid Gholamalamdari, Tom van Schaik, Yuchuan Wang, Pradeep Kumar, Liguo Zhang, Yang Zhang, Gabriela A Hernandez Gonzalez, Athanasios E Vouzas, Peiyao A Zhao, David M Gilbert, Jian Ma, Bas van Steensel, Andrew S Belmont

Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, United States • Division of Gene Regulation and Oncode Institute, Netherlands Cancer Institute, Amsterdam, Netherlands • Ray and Stephanie Lane Computational Biology Department, School of Computer Science, Carnegie Mellon University, Pittsburgh, United States • San Diego Biomedical Research Institute, San Diego, United States • Center for Biophysics and Quantitative Biology, University of Illinois at Urbana-Champaign, Urbana, United States • Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, United States

eLife Assessment

In this **valuable** study, the authors integrate several datasets to describe how the genome interacts with nuclear bodies across distinct cell types and in Lamin A and LBR knockout cells. They provide **convincing** evidence to support their claims and particularly find that specific genomic regions segregate relative to the equatorial plane of the cell when considering their interaction with various nuclear bodies. The authors are encouraged to consider citing the relevant work of other labs who have shown the presence of different types of Lamin Associated Domains (LADs).

https://doi.org/10.7554/eLife.99116.2.sa2

Abstract

Models of nuclear genome organization often propose a binary division into active versus inactive compartments yet typically overlook nuclear bodies. Here we integrated analysis of sequencing and image-based data to compare genome organization in four human cell types relative to three different nuclear locales: the nuclear lamina, nuclear speckles, and nucleoli. Whereas gene expression correlates mostly with nuclear speckle proximity, DNA replication timing correlates with proximity to multiple nuclear locales. Speckle attachment regions emerge as DNA replication initiation zones whose replication timing and gene composition vary with their attachment frequency. Most facultative LADs retain a partially repressed state as iLADs, despite their positioning in the nuclear interior. Knock out of two lamina proteins, Lamin A and LBR, causes a shift of H3K9me3-enriched LADs from lamina to nucleolus, and a reciprocal relocation of H3K27me3-enriched partially repressed iLADs from nucleolus to lamina. Thus, these partially repressed iLADs appear to compete with LADs for nuclear lamina attachment with consequences for replication timing. The nuclear organization in adherent cells is polarized with nuclear bodies and genomic regions segregating both radially

and relative to the equatorial plane. Together, our results underscore the importance of considering genome organization relative to nuclear locales for a more complete understanding of the spatial and functional organization of the human genome.

Introduction

Over 100 years of cytology has recognized characteristic features of nuclear genome organization, including blocks of condensed chromatin enriched at the nuclear and nucleolar peripheries and varying degrees of condensation/decondensation throughout the nuclear interior. Foci of active transcription are concentrated at the edges of chromosome territories and sub-territory condensed chromatin masses and depleted at the nuclear periphery (Belmont, 2022; Bernhard and Granboulan, 1963; Cremer et al., 2015; Cremer and Cremer, 2006; Misteli, 2020; Politz et al., 2013, 2016; Takizawa et al., 2008; van Steensel and Belmont, 2017). Two current models for nuclear genome organization prevail (Belmont, 2022).

The radial genome organization model describes a radial gradient of higher gene expression activity towards the nuclear center (Bickmore, 2013; Croft et al., 1999; Girelli et al., 2020; Kolbl et al., 2012; Kupper et al., 2007; Takizawa et al., 2008). The binary model of genome organization describes the differential intranuclear positioning of two discrete chromatin states: heterochromatin primarily to the nuclear lamina and to a lesser extent the nucleolar periphery and clusters of PCH and euchromatin to the remaining nuclear space or “interior”. Originally supported by the differential intranuclear segregation of Alu repeat-enriched, early replicating versus LINE1 repeat-enriched, late replicating chromosome bands [18-20], three orthogonal genomic methods-DamID, Repli-seq, and Hi-C-have segmented the genome into similar Lamin Associated Domains (LADs)/ late replicating/ B compartment versus inter-LAD (iLADs), early replicating/ A compartment binary divisions (Guelen et al., 2008; Kind et al., 2013; Lieberman-Aiden et al., 2009; Peric-Hupkes et al., 2010; Ryba et al., 2010; White et al., 2004).

However, neither model acknowledges the considerable cell-type specific variability in nuclear shape and size, as well as the size, number, and relative positioning of nuclear bodies. This variability could dramatically affect chromosome trajectories and the distribution of active and inactive chromatin regions among nuclear locales. Additionally, neither model acknowledges the variation between cell types in how different heterochromatin regions distribute between the nuclear periphery, the nucleolar periphery, and the nuclear interior as well as how active genes might segregate to different regions of the nuclear interior. In fact, these differences comprise a major element of histology and pathology textbooks and are used routinely for clinical diagnosis (Fischer, 2020; Skinner and Johnson, 2017).

Indeed, high-resolution Hi-C has identified two active A subcompartments and three major repressive B subcompartments (Rao et al., 2014). Similarly, TSA-seq, NAD-seq, and SPRITE have distinguished between types of heterochromatin localizing preferentially at nucleoli versus the nuclear lamina and have revealed a type of active chromatin specifically localizing near nuclear speckles (Chen et al., 2018; Nemeth et al., 2010; Quinodoz et al., 2021, 2018; van Koningsbruggen et al., 2010; Vertii et al., 2019; Zhang et al., 2020). However, a major limitation with these previous studies is that they typically focused on one type of measurement (i.e. Hi-C, DamID, or NAD-seq), one or two nuclear compartments, and/or one or a limited number of cell types.

To address these problems, we integrated multiple spatial and functional genomic measurements and light microscopy across multiple cell lines. We focused on the specific goal of relating nuclear genome features to three nuclear locales-the nuclear periphery, nucleoli, and nuclear speckles-while also developing improvements to DamID (van Schaik et al., 2020), TSA-seq (Zhang et al., 2020), and Repli-seq (Zhao et al., 2020). In a companion paper, we extended and validated our

improved TSA-seq 2.0 protocol to map the nuclear genome relative to the nuclear lamina and nucleoli (Kumar et al., 2024), adding to our previous TSA-seq 2.0 mapping relative to nuclear speckles (Zhang et al., 2020) and our improved-resolution 16-fraction Repli-seq (Zhao et al., 2020).

Here we integrate this nuclear locale genomic mapping with light microscopy imaging of the same three nuclear locales to investigate how the genome is both spatially and functionally organized relative to these nuclear locales across four human cell types-hTERT-immortalized human foreskin fibroblasts (HFFc6, abbreviated hereafter as HFF), H1 human embryonic stem cells (hESCs), HCT116 (HCT) colon carcinoma epithelial cells, and K562 erythroleukemia cells.

Our results show that both radial and/or binary divisions of the genome are oversimplified. Multiple types of active versus inactive chromatin states can be recognized by their varying association with nuclear locales. Moreover, nuclear genome organization varies among cell types both because of changes in genome positioning relative to individual nuclear locales as well as changes in the intranuclear positioning of these locales relative to each other. Gene expression shows much stronger correlation with distance to nuclear speckles versus either the nuclear lamina or nucleoli. Finally, we demonstrate a nuclear polarity to the genome organization beyond a simple radial axis which further spatially divides active versus repressive genomic states as well as DNA replication timing.

Results

Cell types differ widely in morphology and arrangement of nuclear locales

Prior genomic analyses of nuclear locales have largely ignored variation in nuclear morphology. However, differences in position relative to nuclear locales can result from either differences in chromatin location or to differences in the relative positioning or even morphologies of nuclear locales. Therefore, we began by comparing nuclear, nucleolar, and nuclear speckle sizes and shapes, as well as nucleolar and nuclear speckle positioning relative to each other and the nuclear periphery, across the four human cell lines-H1, HCT116, HFF and K562.

To measure sizes and shapes of nuclei and nuclear bodies, cell lines were stained for nuclear speckles (hereafter referred to simply as speckles), nucleoli, and the nuclear periphery (NP) with anti-SON, anti-MKI67IP, and anti-glycosylated nucleoporin (anti-RL1) antibodies, respectively, and for DNA using DAPI. Deconvolved wide-field microscopy images (**Fig. 1A**) were then segmented to generate nuclear 3D models (**Fig. 1B**, **SFig. 1A**).

K562 and H1 nuclei have larger volume (V), lower surface area (SA), and are rounder (lower SA/V) as compared to HCT and HFF nuclei (**Fig. 1C**). Whereas K562 and H1 speckles distribute radially in 3D, surrounding nucleoli and concentrating within the nuclear interior, HCT and HFF speckles preferentially distribute in the equatorial (center) z-planes while being depleted radially near the nuclear periphery within these equatorial planes (**Fig. 1B**).

However, other nuclear morphology features did not segregate with flat versus round nuclei. K562 nuclei are lobulated and have deep invaginations of the nuclear lamina (**Fig. 1A**). Individual nuclear speckles are significantly larger in HCT116 cells, even though total nuclear speckle volumes are similar across all four cell lines (**Fig. 1D**). H1 and HCT116 have large, often single, nuclear-centrally located nucleoli, while HFF and K562 have smaller, multiple nucleoli distributed between speckles (**Fig. 1A, B, D**). Total nucleolar volumes are largest in HCT116 and lowest in HFF (**Fig. 1D**).

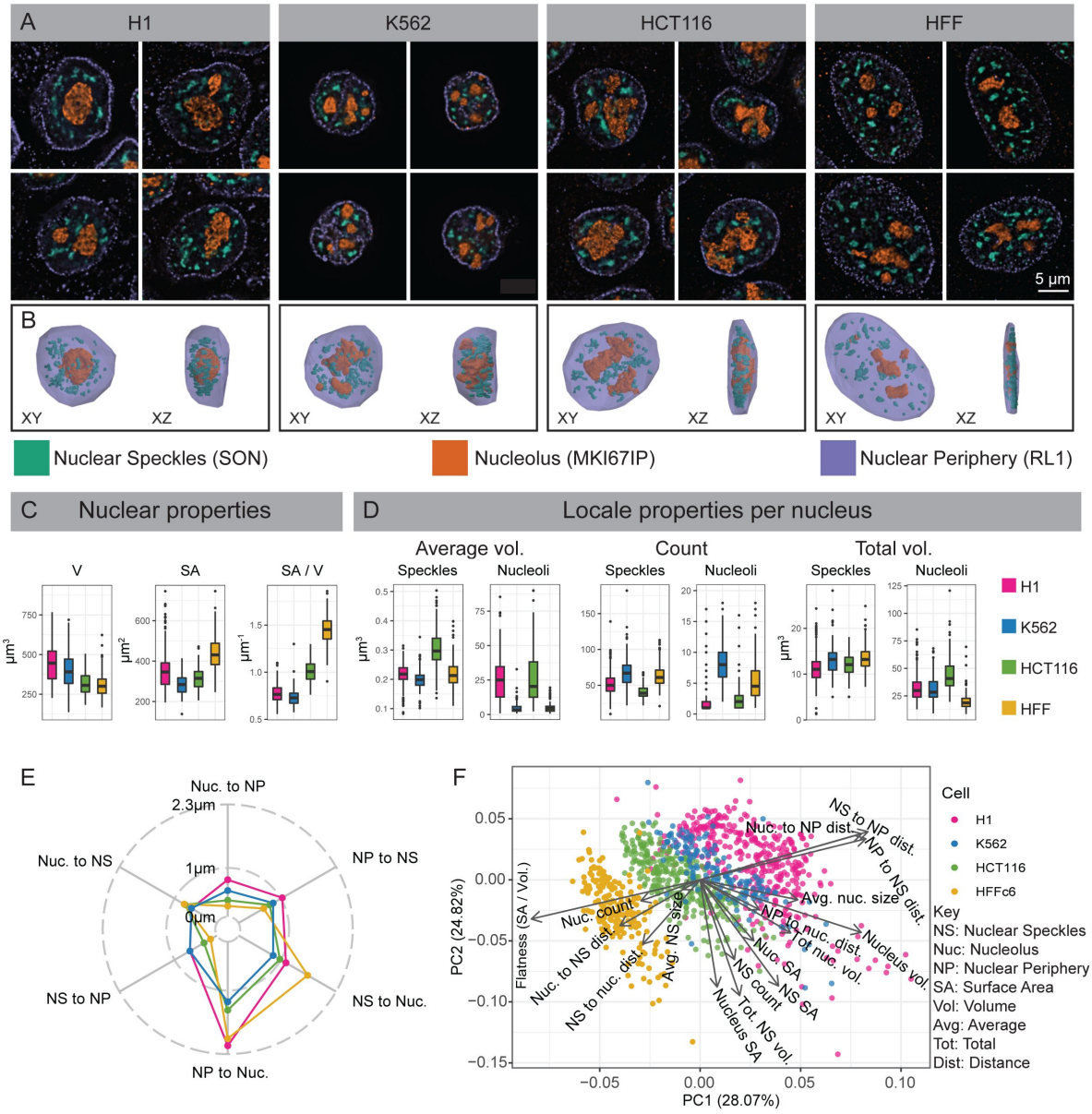


Figure 1

Differences in nuclear and nuclear body morphology and relative positioning among four cell types

(**A-B**) Wide-field deconvolution light microscopy (**A**) and 3D solid models in XY and XZ orientations (**B**). Immunostaining of nuclear periphery (NP), nucleoli, and nuclear speckles using antibodies against RL1 (nuclear pore, purple), MKI67IP (nucleolar GC, orange), and SON (nuclear speckle, green) in H1, K562, HCT116, and HFF cells. (**C**) Comparing Volume (V), surface area (SA), and roundness (SA/V) of nuclei measured from NP 3D solid models in H1 (red), K562 (blue), HCT116 (green), and HFF (yellow) cells. (**D**) Comparison across cell lines of nucleolar and nuclear speckle (NS) numbers, average volumes, and summed volumes per nucleus. Individual NS are significantly larger in HCT116 cells, even though total NS volumes are similar across all four cell lines. (**E**) Pairwise average distances between locales (asymmetric, as defined in **SFig. 1B**). (**F**) Principal Component (PC) 1 (x-axis) versus PC2 (y-axis) scatterplot using PCA of 14 morphological features reveals adjacent clustering of H1 and K562 cells with HCT116 and HFF clusters closer to each than to H1 and K562 cells. Each scatterplot point represents an individual cell. Arrow lengths show the quality of representation and arrow direction show the loadings on PC1 and PC2.

We compared relative arrangements of these nuclear locales across cell lines by measuring the nearest distances between these locales (**SFig. 1B**). Rounder nuclei (K562, H1) have significantly larger speckle-to-NP and nucleolus-to-NP distances compared to flatter nuclei (HFF, HCT116) as expected (**SFig. 1C**, **Fig. 1E**). But speckle-to-nucleoli distances in K562 are lower than other cell lines, with HFF showing noticeably larger speckle-to-nucleoli distances (**SFig. 1C**, **Fig. 1E**). While total speckle and nucleolar volumes scale linearly with nuclear volume in all cell lines, curiously total speckle volume correlates more strongly with nuclear surface area versus volume (**SFig. 1D**).

Overall, we found a large variation in the sizes and shapes of nucleoli and nuclear speckles as well as their spatial arrangement relative to each other and to the nuclear periphery. However, despite various cell line specific differences in nuclear locale morphologies, the four cell lines divide approximately into round (K562, H1) nuclei with radial organization of nuclear bodies versus flat nuclei (HCT116, HFF) with nuclear bodies distributed differentially relative to distance from both the x-y equatorial plane as well as distance from the nuclear center. This division is further supported by Principal Component Analysis (PCA) using 14 morphological measures (**Fig. 1F**, **SFig. 1F**).

Global differences in nuclear genome organization in different cell types revealed by DamID and TSA-seq

We next surveyed genome organization relative to these nuclear locales using a combination of DamID and TSA-seq. DamID is a well-established molecular proximity assay; DamID applied to the nuclear lamina divides the genome into lamina-associated domains (LADs) versus non-associated "inter-LADs" or "iLADs" (Guelen et al., 2008; van Steensel and Belmont, 2017). In contrast, TSA-seq measures relative distances to targets on a microscopic scale corresponding to 100s of nm to ~ 1 micron based on the measured diffusion radius of tyramide-biotin free-radicals (Chen et al., 2018). Exploiting the measured exponential decay of the tyramide-biotin free-radical concentration, we showed how the mean distance of chromosomes to nuclear speckles could be estimated from the TSA-seq data to an accuracy of ~50 nm, as validated by FISH (Chen et al., 2018). While lamin DamID segments LADs most accurately, lamin TSA-seq provides distance information not provided by DamID-for example, variations in relative distances to the nuclear lamina of different iLADs and iLAD regions. These differences between the lamin DamID and TSA-seq signals are also crucial to a computational approach, SPIN, that segments the genome into multiple states based on their varying nuclear localization, including biochemically and functionally distinct lamina-associated versus near-lamina states (Consortium et al., 2024; Wang et al., 2021).

Thus, lamin DamID and TSA-seq complement each other, providing more information together than either one separately. For these reasons, we set out to develop both SON and nucleolar DamID and nucleolar TSA-seq with the goal of mapping the genome relative to the nuclear lamina, nuclear speckles, and nucleoli in each case using both DamID and TSA-seq. We successfully developed nucleolar TSA-seq, which we extensively validated using comparisons with two different orthogonal genome-wide approaches (Kumar et al., 2024)-NAD-seq, based on the biochemical isolation of nucleoli, and previously published direct microscopic measurements using highly multiplexed immuno-FISH (Su et al., 2020). As previously demonstrated for both SON and lamin TSA-seq (Chen et al., 2018), nucleolar TSA-seq was also robust in the sense that multiple target proteins showing similar nucleolar staining showed similar TSA-seq results (Kumar et al., 2024); this robustness is intrinsic to TSA-seq being a microscopic rather than molecular proximity assay, and therefore not sensitive to the exact molecular binding partners and molecular distance of the target proteins to the DNA.

In contrast, we were unsuccessful with both the SON and nucleolar DamID. SON DamID showed narrow, several hundred bp peaks near the promoters of most active genes, similar to previous SON ChIP-seq profiles (Kim et al., 2016), consistent with measurement of the local accumulation of SON molecules at these sites rather than chromosome contact with nuclear speckles. Nucleolar DamID instead showed broad positive peaks over large chromatin domains, largely overlapping with LADs mapped by LMNB1 DamID (Wang et al., 2021). However, this nucleolar DamID signal, while strongly correlated with lamin DamID, showed poor correlation with either NAD-seq or nucleolar distances mapped by multsiiplexed immuno-FISH (Kumar et al., 2024). We suspect the problem is that DamID is a molecular rather than microscopic proximity assay; therefore, both the SON and nucleolar DamID output signals should be disproportionally dominated by the small fraction of target proteins juxtaposed in sufficient proximity to the DNA to produce a signal, rather than by the amount of protein concentrated in the microscopically adjacent target nuclear body.

For these reasons, our analyses focused on those measurements that proved robust in measuring relative proximity to the nuclear lamima, nucleoli, and nuclear speckles locales: lamina (LMNB1) DamID and, as described in previous papers, lamina (LMNB1) and nucleolar (MKI67IP) (Kumar et al., 2024), and speckle (SON) (Zhang et al., 2020) TSA-seq 2.0.

Speckle TSA-seq is highly conserved across all four cell types (**Fig. 2A**), as described previously (Zhang et al., 2020) and supported here by the high correlations for speckle TSA-seq comparisons between pairs of cell lines, despite a lower dynamic range in HCT116 and especially HFF (**Fig. 2B**).

In contrast, genome-wide correlations in pair-wise cell line comparisons were substantially lower for lamina DamID and for both lamina and nucleolus TSA-seq (**Fig. 2A-B**, **SFig. 2A-C**). For example, LADs near the ends of long chromosome arms switch from a strong lamina association in HFF and HCT116 to a more interior and nucleolar association in H1 and K562 (**Fig. S2A**). While in HCT116 cells local maxima in nucleolar TSA-seq align with LADs, in HFF, H1, and K562 cells small peak and “peak-within-valley” nucleolar TSA-seq local maxima instead align with speckle TSA-seq peaks (**Fig. S2B**).

Thus, whereas genome positioning relative to speckles is highly conserved across cell lines, despite variations in speckle intranuclear positioning (**Fig. 1**), genome positioning relative to the lamina and nucleoli shows major variations across different cell types.

Differences in gene expression primarily correlate with differences in genome positioning relative to speckles whereas differences in DNA replication timing correlate with differences in relative distances to all three nuclear locales

Given both the binary and radial models of nuclear genome organization, there has been a long-standing expectation that changes in gene expression would correlate with changes in distance relative to the nuclear periphery. More recently, the strong correlation of gene expression levels with position along a nuclear lamina – nuclear speckle axis in K562 cells, as measured by TSA-seq, led to the suggestion that distance along this axis was a more directly functionally relevant metric with respect to gene expression (Chen et al., 2018).

The large variations in genome positioning relative to the lamina and nucleoli among the four cell types, described in the previous section, now allowed us to ask how exactly differences in gene expression and/or DNA replication timing correlate with differences in distances to each of the different nuclear locales.

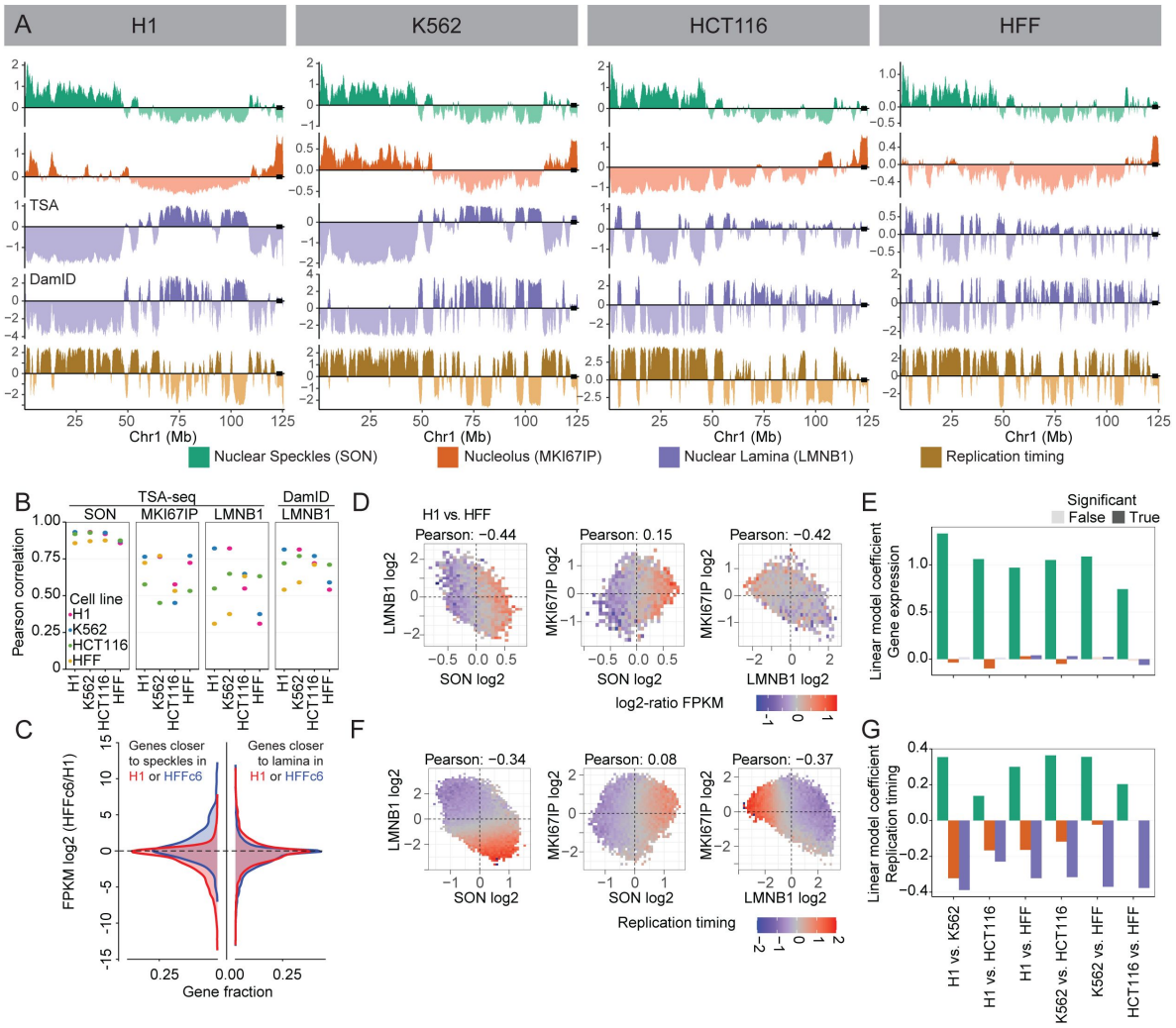


Figure 2

Global changes in nuclear genome intranuclear positioning and their correlations with changes in gene expression and DNA replication timing

(**A**) Chr1 left arm browser view suggests chromosome trajectory alternating between positioning of early replicating regions near nuclear speckles and late-replicating regions at either the nucleolar (H1, K562) or the nuclear periphery (HCT116, HFF); Top to bottom-nuclear speckle (SON, green), Nucleolus (MKI67IP, orange), and nuclear lamina (LMNB1, purple) TSA-seq, LMNB1 (purple) DamID, and 2-fraction Repli-seq (Replication timing, brown). Left to right-H1, K562, HCT116, and HFF cells. (**B**) Comparison of Pearson correlations of TSA-seq and DamID datasets between different cell lines (H1,K562,HCT116, HFF) reveals much higher conservation of genome positioning relative to nuclear speckles, which is largely conserved, versus nucleoli or nuclear lamina; (**C**) Left-Genes positioned closer (blue)/ further (red) to nuclear speckles show a bias towards increased / decreased gene expression in HFF versus H1 cells. Fraction of genes showing significant difference in relative positioning to nuclear speckles (gene fraction, x-axis) versus log2 (HFF FKPM / H1 FKPM) (y-axis); Right-Similar comparison for genes positioned closer (blue) / further (red) to nuclear lamina does not show such bias; (**D-E**) Changes in gene expression between H1 and HFF cells vary largely as function of changes in nuclear speckle rather than nuclear periphery or nucleolar relative positioning; (**D**) 2D histograms showing mean ratio changes in gene expression between H1 versus HFF cells (log2 ratios of FKPM, color-coded) for binned genes as function of their changes in z-normalized TSA-seq scores (left to right: LMNB1 (y) vs SON (x), MKI67IP (y) vs SON (x), MKI67IP (y) vs LMNB1 (x)); (**E**) Linear modeling of changes in gene expression versus z-normalized TSA-seq score changes reveals significantly larger coefficients (dependence) for SON (green) versus MKI67IP (orange) or LMNB1 (blue). (**F-G**) Similar comparison as in (D-E), but for replication timing (2-fraction Repli-seq), shows changes in DNA replication timing are function of both changes in SON and LMNB1 TSA-seq.

We had previously identified genomic regions 100 kb or larger, comprising ~10% of the genome, that showed statistically significant differences in speckle TSA-seq smoothed scores in pairwise comparisons between the same four cell lines (Zhang et al., 2020). Genes within these regions showed a strong positive correlation between differences in their gene expression and speckle positioning. Chromosome regions with different position relative to speckles typically show inverse differences in their position relative to the lamina (**SFig. 2D**, top panels), though individual genome regions have variable magnitude and even direction of these differences (**SFig. 2E**). However, the reverse is not true. Pairwise cell-line comparisons reveal ~40-70% of the genome shows statistically significant differences in lamina TSA-seq over regions 100 kb or larger, with most of these regions showing little or no differences in speckle TSA-seq scores (**SFig. 2D-E**, bottom panels).

Genes within genomic regions displaying significant differences in speckle TSA-seq in cell line comparisons tend to show increased expression when they are closer to speckles and decreased expression when they are further from speckles (**Fig. 2C**), as described previously (Zhang et al., 2020). In contrast, genes within genomic regions which in pair-wise comparisons of cell lines show a statistically significant difference in lamina TSA-seq show no obvious trend in their expression differences (**Fig. 2C**).

Binning all genes as a function of differences in their normalized speckle, nucleolar, and lamina TSA-seq scores show a positive correlation of differences in gene expression with changes in speckle TSA-seq scores but no apparent correlation with differences in lamina or nucleolar TSA-seq scores (**Fig. 2D**). Linear modeling for correlation between changes in gene expression and changes in TSA-seq reveals a substantially stronger correlation for positioning relative to speckles, as compared to positioning relative to the lamina or nucleoli (**Fig. 2E**, **SFig. 2F**); this pattern holds genome-wide (**Fig.2E**) as well as for correlations over specific genome region types (LAD, iLAD, speckle TSA-seq maxima) (**SFig. 2F**). Instead, pair-wise cell type comparisons revealed significant correlations between changes in DNA replication timing and changes in TSA-seq with respect to all three locales-speckle, lamina, and nucleoli (**Fig. 2F-G**).

In summary, whereas gene expression primarily correlates with relative distance to nuclear speckles, DNA replication timing correlates with relative distance to all three nuclear locales, despite the variable positioning of nuclear speckles and nucleoli across cell types.

Both Type I and Type II speckle attachment regions are gene expression hot-zones and DNA replication IZs but show varying gene composition and DNA replication timing

Previously, we showed that SON TSA-seq local maxima align with gene expression “hot-zones” embedded within larger iLAD/A compartment regions (Chen et al., 2018). Larger amplitude, “Type I” SON TSA-seq peaks were largely centered within A1 Hi-C subcompartments, and flanked by A2 Hi-C subcompartments, while smaller amplitude, “Type II” SON TSA-seq peaks were contained within A2 Hi-C subcompartments (Chen et al., 2018).

Given the predominant correlation of differences in gene expression with differences in relative position specifically to speckles among the three nuclear locales, as described in the previous section, here we further characterize these singular iLAD/A compartment subregions. As before (Chen et al., 2018), operationally we identified Type I SON TSA-seq local maxima as those embedded within A1 Hi-C subcompartments versus Type II local maxima as those embedded within A2 Hi-C subcompartments (red versus blue tick marks, **Fig. 3A**).

Type I peaks showed a near unimodal distribution of distance to the nearest speckle with a peak at ~100 nm distance (**Fig. 3B**), based on IMR90 fibroblast multiplexed FISH data (Su et al., 2020). In this case we used HFF fibroblast SON TSA-seq measurements as a proxy for IMR90 fibroblast

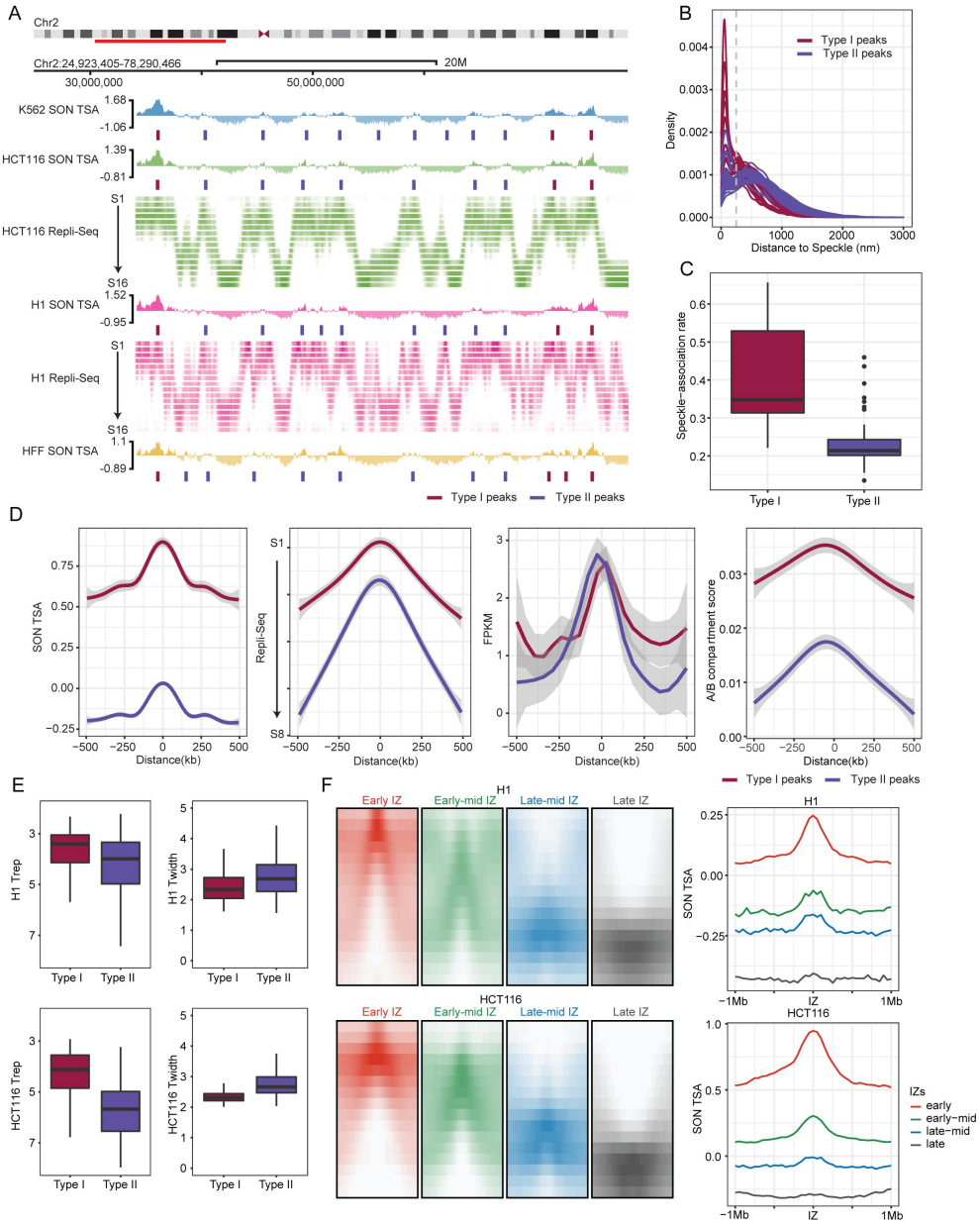


Figure 3

Varying gene composition, DNA replication timing, and speckle proximity of Type I versus Type II SON TSA-seq peaks which align with gene expression “hot-zones” and DNA replication initiation zones (IZs)

(**A**) SON TSA-seq Type I (red ticks) and Type II (blue ticks) local maxima (“peaks”) align with DNA early replication IZs identified in 16-fraction (S1 (early) – S16 (late)) Repli-seq. Top to bottom: K562 SON TSA-seq, HCT116 SON TSA-seq and Repli-seq, H1 SON TSA-seq and Repli-seq, HFF SON TSA-seq; (**B-C**) Histograms of distances (x-axis, nm) (**B**) and boxplots showing speckle association fractions (<250 nm) (**C**) of HFF Type I and II peaks located in IMR90 fibroblasts [43] from nuclear speckles show higher and unimodal (Type I) versus lower and bimodal (Type II) nuclear speckle attachment frequencies; (**D**) Pileup plots showing SON TSA-seq (left), Repli-seq (2nd to left), FKPM RNA-seq (2nd from right), and Hi-C compartment scores (right) flanking (+/-500 kb) Type I (red) versus Type II (blue) SON TSA-seq peaks; (**E**) Boxplots of Trep (timing of replication, left) and Twidth (variation in replication timing, right) in H1 (top) and HCT116 (bottom) show earlier and less variable DNA replication timing for Type I versus II peaks; (**F**) Pileup Repli-seq profiles for early, early-mid, late-mid, and late IZs (left, +/-1 Mbp) show progressively later timing of replication correlating with the lower amplitude of SON TSA-seq peaks (right) centered at the IZ center (right).

SON TSA-seq (see Section "Polarity of Nuclear Genome Organization" for comparison of IMR90 versus HFF SON TSA-seq). In contrast, Type-II peaks showed a bimodal speckle distance distribution (Hartigan's dip test (Hartigan and Hartigan, 1985), p-value < 0.05, (**SFig. 3A**)), with one narrow peak similarly located at ~100 nm distance and a broader peak centered at ~500 nm from speckles (**Fig. 3B**). Notably, Type II peaks show a lower speckle association frequency (<250 nm threshold, **Fig. 3B**, dashed line) than Type I peaks (**Fig. 3C**), suggesting that while Type II peaks still associate specifically with speckles their interaction with speckles is weaker and/or less frequent as compared to Type 1 peaks.

Both Type I (red) and Type II (blue) average peak widths are ~400kb (**Fig. 3D**); however, Type I peaks have higher speckle TSA-seq and Hi-C compartment scores (**Fig. 3D**) and contain shorter genes, genes with smaller exon and intron length, and genes with lower fractional intron composition (**SFig. 3B**). Across the four cell types, Type I peaks also are more conserved than Type II peak (Jaccard indexes of 0.85 versus 0.67 for Type 1 versus Type 2) (**SFig. 3C**).

Although gene expression levels are similar over Type I and II peaks (**Fig. 3D**, 2nd from right), the IMR90 multiplexed image data reveals a trend of gene loci within Type I peaks showing a higher fraction of "ON" states among the cell population as compared with gene loci within Type II peaks (**SFig. 3D**).

Both Type I and Type II peaks align with DNA replication initiation zones (IZs), identified as peaks in 16-fraction Repli-seq (**Fig. 3A**). Type I peaks replicate slightly earlier than Type II peaks (**Fig. 3E-F**, **SFig. 3E**), although this difference is noticeably larger in HCT116 versus H1 cells (**Fig. 3A,E,F**). Speckle TSA-seq scores progressively decrease with later replication timing of IZs (**Fig.3F**). However, DNA replication timing variability (Twidth) is notably larger for Type II peaks, particularly in HCT116 (**Fig. 3E**, **SFig. 3E**) suggesting a possible link between variability in speckle association (**Fig. 3B**) and variability in DNA replication timing (**Fig. 3E**).

We next used live-cell imaging to show that chromosome regions close to nuclear speckles show the earliest DNA replication timing; this is consistent with the earliest firing DNA replication IZs, as determined by Repli-seq, aligning with Type I peaks that are closely associated with nuclear speckles. Specifically, in HCT116 cells expressing EGFP-SON and mCherry-PCNA, the first PCNA foci appearing within the first 30 mins of S-phase were nearly 100% localized within 500 nm of speckles (**SFig. 3F**). From 30 to 135 mins after initiation of S-phase, more PCNA foci appeared at increasing distances from speckles (**SFig. 3F**).

In summary, these results show that iLADs are punctuated with two types of gene expression "hot-zones" of ~400 kb width that also act as DNA replication initiation zones. Although average levels of elevated gene expression are similar among these two types of gene expression hot-zones, they contain different types of genes, different speckle association rates, and different DNA initiation zone replication timing.

LAD to iLAD conversions mostly correspond to transitions from a repressed/ late-replicating to an intermediate repressed/ late-to-middle DNA replicating chromatin state

One important class of nuclear genome transitions are chromosome regions which undergo transitions from LADs to iLADs, as determined by their LMNB1 DamID signals. LADs and iLADs show a close correspondence to B and A Hi-C compartments, respectively, with LADs and B compartments generally associated with repressive, late replicating chromatin states versus iLADs and A compartments which have generally been associated with active, early replicating chromatin states (Lieberman-Aiden et al., 2009; van Steensel and Belmont, 2017)(Consortium et al., 2024). For this reason, previously it has been generally assumed that LAD to iLAD transitions signify transitions from a repressed to active chromatin state. However, the low

correlation in comparisons between cell types between differences in gene expression with differences in lamina and/or nucleolar TSA-seq and lamina DamID scores (**Fig. 2D-E**, **SFig. 2D-E**) suggested that transitions from facultative LAD (fLAD) to facultative iLAD (fiLAD) might not always represent a binary transition from repressed to active chromatin.

Therefore, we next asked whether regions within iLADs might show differential localization relative to the lamina and/or nucleoli and, if so, whether these regions would show different levels of gene expression. More specifically, analogously to how gene expression hot-zones appeared as local maxima in speckle TSA-seq with early DNA replication timing, we asked whether iLAD regions that appeared as local maxima in lamina or nucleolar proximity mapping signals would correspond to iLAD regions with locally reduced gene expression levels and later DNA replication timing.

Indeed, analysis across cell lines reveals that lamina DamID scores of fLAD genomic regions can continuously transition from positive peaks in lamina DamID (red rectangles), to peak-within-valley (p-w-v) local maxima (yellow rectangles), or to valleys (v) (blue rectangles) (**Fig. 4A**, **SFig. 4A**). A subset of p-w-v fiLADs also showed p-w-v local maxima in their lamina TSA-seq signals together with p-w-v local maxima or small positive peaks in their nucleolus TSA-seq signals (**SFig. 4A**).

We implemented an algorithm to define these three domain classes genome-wide based on their local relative increase ("enrichment") in DamID score (**SFig. 4B**) (See Methods), revealing that p-w-v fiLADs are more common than v-fiLADs in all cell lines except HFF, which has few fiLADs of either type (**Fig. 4B-C**). The transitions between LADs, p-w-v fiLADs and v-fiLADs are highly dynamic across the four cell lines (**Fig. 4A**, **SFig. 4C**), with some regions transitioning between all three domain types among the four cell lines (**Fig. 4A**, **SFig. 4A**&**C**). We observe an increasing number (**Fig. 4B**) and total genome coverage (**Fig. 4C**) of LADs versus p-w-v fiLADs and v-fiLADs comparing H1, K562, HCT116, and HFF cell lines.

p-w-v fiLADs show late to middle DNA replication timing and reduced gene expression levels, rather than the early DNA replication timing and higher gene expression typical of constitutive iLADs (ciLADs) and a subset of v-fiLADs (**Fig. 4A-B**, **SFig. 4D**). Moreover, transitions between cell lines from p-w-v-to v-fiLADs show an increase in gene expression of comparable magnitude to that observed in LAD to p-w-v fiLAD transitions (**Fig. 4D**, **SFig. 4E**). While p-w-v fiLADs are classified as A compartment, with positive Hi-C compartment scores, in pile-up plots they show lower compartment scores than iLAD or v-fiLAD regions (**Fig. 4E**). Both p-w-v-fiLADs and v-fiLADs have earlier replication timing than LADs, but p-w-v fiLADs replicate later than v-fiLADs (**Fig. 4E-F**). Additionally, p-w-v fiLADs typically have an intermediate chromatin state between LADs and v-fiLADs, as defined by their relatively increased levels of "repressive" and decreased levels of "active" histone marks (**SFig. 4F**).

In summary, our analysis revealed that most LAD to iLAD transitions between different cell types correspond to a transition to a partially repressive heterochromatic state which still shows some residual association with the lamina and, in some cases, with nucleoli. These partially repressive iLAD regions can exist as more fully repressed LADs or more fully active iLAD chromatin domains in other cell types.

p-w-v fiLADs compete with LADs for nuclear lamina association; linking nuclear lamina association with later DNA replication timing but not lower gene expression

In a previous Section we described how differences in gene expression showed little dependence on differences in relative distances to the nuclear lamina (**Fig. 2**, **SFig. 2**), challenging key predictions of previous models of genome organization. One possibility is that while many

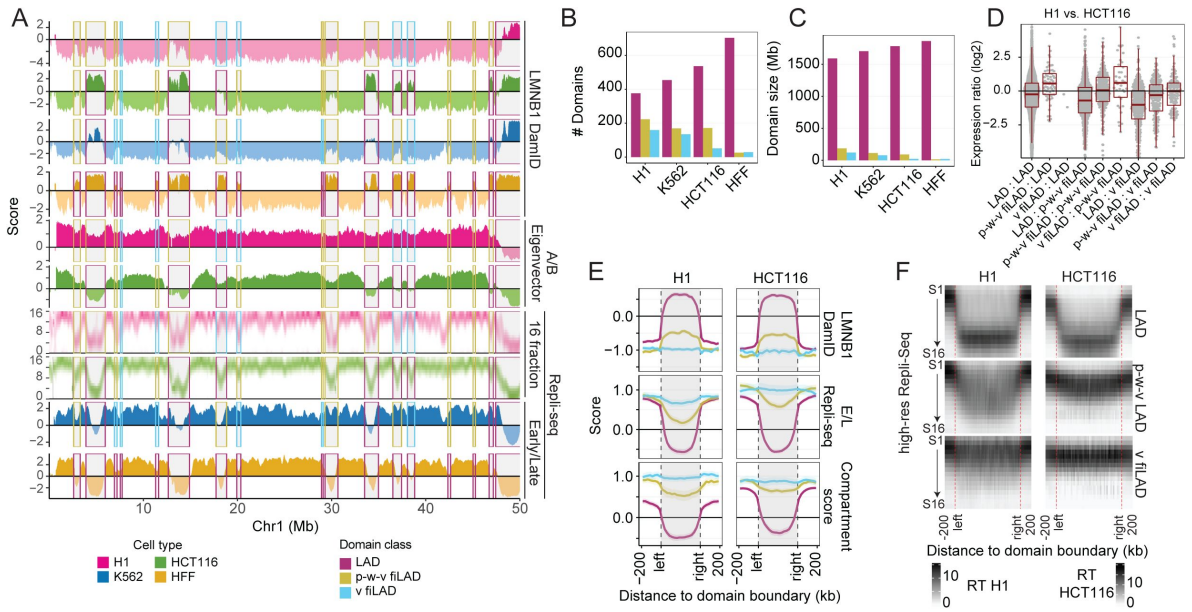


Figure 4

Facultative LADs transition most frequently to a partially repressed, middle-to-late replicating iLAD and less frequently to an active, early replicating facultative iLAD in different cell types

(**A**) Chr1 region with examples of LADs (red rectangles) showing peaks in LMNB1 DamID in one cell type changing in other cell types to facultative iLADs (fiLADs) showing either valleys ("v") (blue rectangles) or peak-within-valleys ("p-w-v") (yellow-orange rectangles) in their LMNB1 DamID signals. Replication timing is late for fLADs, changes to early for v-fiLADs but remains late-to-middle for p-w-v fiLADs, despite Hi-C A compartment scores for both p-w-v and v-fiLADs. Top to bottom - LMNB1 DamID for H1, HCT116, K562, and HFF, Hi-C A/B Eigenvector score for H1 and HCT116, 16-fraction Repli-seq for H1 and HCT116, and 2-fraction Repli-Seq for K562 and HFF (H1-red; HCT116-green; K562-blue; HFF-brown) (some v-fiLADs and iLADs are p-w-v fiLADs classified as v-fiLADs or missed by our classification scheme); (**B-C**) Numbers (**B**) and mean sizes (**C**) of LADs (red), p-w-v fiLADs (yellow/orange), and v-fiLADs (blue) in the four cell types. HFF cells have smallest number of p-w-v and v fiLADs; (**D**) Genes in same domain type show similar expression mean levels but show increased expression in p-w-v fiLADs versus LADs or v fiLADs versus p-w-v fiLADs in H1 versus HCT116. Log2 (FKPM(H1)+1 / FKPM(HCT116)+1) for genes in one type of domain in H1 and the same or another type of domain in HCT116 (H1 domain type : HCT116 domain type); (**E**) Pileup plots for LMNB1 DamID (top), E/L 2-fraction Repli-seq (middle), and Hi-C compartment score (bottom) for LADs (red), p-w-v fiLADs (yellow/orange), v fiLADs (blue) in H1 (left) versus HCT116 (right) reveals overall trend towards higher LMNB1 DamID signal, less early replication timing, and lower Hi-C compartment score of p-w-v fiLADs versus v fiLADs which resemble flanking iLAD regions; (F) 16-fraction Repli-seq pileup plots of LADs (top), p-w-v fiLADs (middle), and v fiLADs (bottom) confirms late, middle, versus early DNA replication patterns, respectively, for these domains in both H1 (left) and HCT116 (right).

heterochromatic regions might exchange a repressive environment near the lamina for another repressive environment within the nuclear interior, a smaller number of chromosome regions might instead show a dependence of gene expression on differences in distance relative to the lamina. Indeed, we did observe that most of the ~10% of chromosome regions between cell types which showed differences in location relative to nuclear speckles also showed inverse differences in location relative to the lamina (**SFig. 2D**).

Therefore, we set out to experimentally manipulate genome association with the lamina by making LMNA, LBR, and double LMNA/LBR K562 knockout (KO) lines to probe the functional consequences of changes in genome distance to the lamina. In at least some post-mitotic mouse cell types, LMNA/LBR double KO creates an 'inverted" nuclear morphology, with loss of peripheral heterochromatin, accumulation of most heterochromatin in the nuclear interior, and positioning of euchromatin towards the nuclear periphery (Solovei et al., 2013). This inverted nuclear morphology is seen normally in retinal rod cells of nocturnal mammals, which lack both LBR and LMNA expression (Solovei et al., 2013), but Hi-C reveals largely unchanged A and B compartments, despite the inversion of euchromatin and heterochromatin nuclear positioning in these cells (Falk et al., 2019).

Whereas the LMNA KO shows little change in lamina DamID from K562 wild-type (wt) or the parental clone, the LBR KO and the LMNA/LBR double knockout (DKO) lines show similar but partial reduction in the lamina DamID for most LADs and increases for some iLADs (**SFig. 5A**). Therefore, we focused our attention on comparisons between wt K562 and the DKO line.

DKO cells showed loss of the nuclear lobulation characteristic of wtK562 nuclei, the appearance of a DAPI-dense perinucleolar rim not apparent in the wtK562 nuclei, and an increased number of DAPI-dense foci in the nuclear interior (**Fig. 5A**). Immunostaining against LMNB1 revealed the normal ring of staining around the nuclear periphery seen in wt cells (images deposited as metadata in the deposited sequencing data sets). Based on DamID and TSA-seq, most LADs in DKO cells decrease their interaction and relative proximity with the lamina and increase their proximity to nucleoli but maintain their positioning to speckles (**Fig. 5B**, **SFig. 5B**); nuclear and nuclear locale morphologies change as well in the DKO cells compared to wt K562 (**SFig. 5C**). Unexpectedly, most p-w-v-fiLADs instead increase their lamina association while decreasing their proximity to nucleoli (**Fig. 5B**, **SFig. 5B**&**D**). Indeed, most of these p-w-v fiLADs become actual LADs in the DKO (**SFig. 5E**); some v-fiLADs also become LADs in the DKO (which may be due to a misclassification of v-versus p-w-v fiLADs) (**SFig. 5D-E**).

Scatterplots comparing changes in lamina and nucleolar TSA-seq scores between wt and DKO showed an inverse relationship (**Fig. 5C**). This shift correlates with ChIP-seq data, showing that regions enriched in H3K27me3 generally shift away from nucleoli towards the lamina in the DKO, while the opposite trend is observed for regions enriched in H3K9me3 (**Fig. 5D**) (consistent with the enrichment of H3K27me3 in p-w-v fiLADs versus H3K9me3 in LADs (**SFig. 4F**)).

Despite the wide-spread genomic shifts relative to the lamina and nucleolus, only a small number of genes significantly change their gene expression in the KO and DKO lines (**Fig. 5F**). Moreover, there is no obvious bias in the direction of gene expression changes as a function of changes in the lamina DamID between the DKO and wt (**Fig. 5G**). Similarly, there is no obvious bias towards increased gene expression among those genes contained specifically within wt LADs that change their expression and also show reduced lamina interaction (decreased lamina DamID) in the DKO (**SFig. 5F**). Curiously, there is an increased fraction of differentially expressed genes in LADs versus iLADs in all KO cell lines (**SFig 5G-I**), but the significance of this bias, in the absence of bias in the direction of the changes in gene expression, remains unclear.

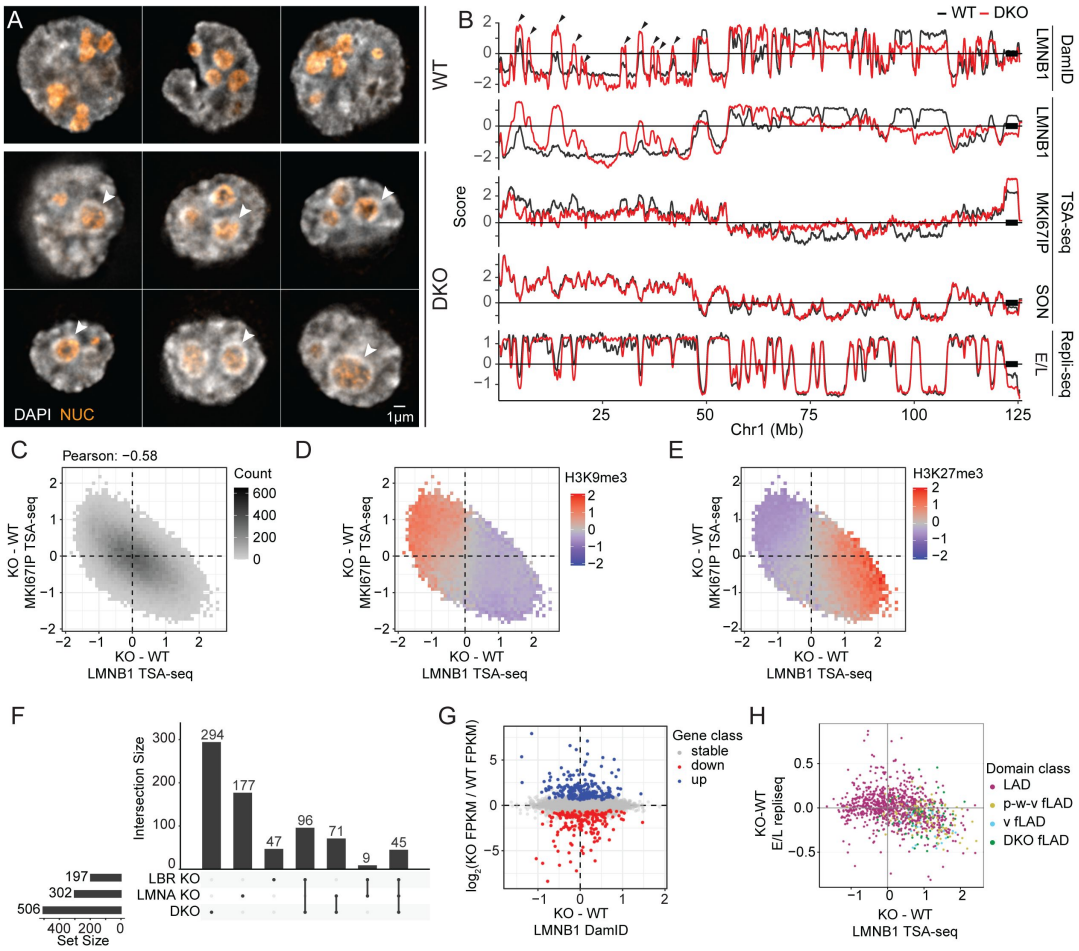


Figure 5

LADs shift towards nuclear interior but peak-within-valley (p-w-v) fiLADs shift towards nuclear lamina and replicate later in LMNA/LBR double knockout (DKO) K562 cells

(A) Representative deconvolved widefield images showing wildtype (WT) and DKO K562 cells. Scale bar = 1 μm. DNA staining (DAPI, grey) shows increased numbers of condensed chromatin foci (arrowheads) at nucleolar (orange) periphery as well as within the nuclear interior in DKO (middle and bottom rows) versus WT (top row) nuclei. DKO nuclei also are rounder and less lobular than wt nuclei; (**B**) Browser views over Chr1 left arm showing differences in (top to bottom) LMNB1 DamID, LMNB1, MKI67IP (nucleolar), and SON (nuclear speckle) TSA-seq, and 2-fraction Repli-seq (Early (E) / Late (L)) for WT (black) versus DKO (red) cells. Whereas LADs shift towards the nuclear interior, p-w-v fiLADs (arrowheads) shift towards the nuclear lamina, or become actual LADs, with slightly later replication timing; in contrast, there is no significant change in nuclear speckle positioning. (**C**) Chromosome shifts away from (towards) nuclear lamina are inversely correlated with shifts towards (away from) nucleoli: 2D-histogram showing changes (KO – wt) in LMNB1 (x-axis) versus MKI67IP (y-axis) TSA-seq. For each histogram bin (pixel), the number of overlapping 25 kb genomic bins is grey-scale coded. Only pixels with at least 10 overlapping genomic bins are plotted. Pearson correlation = −0.58; (**D-E**) H3K9me3-enriched regions shift away from nuclear periphery and towards nucleoli whereas H3K27me3-enriched regions shift towards nuclear periphery and away from nucleoli in DKO versus wt cells; 2D histograms from (C) overlaid with mean wt H3K9me3 (**D**) or H3K27me3 (**E**) ChIP-seq color-coded values. (**F**) Upset plot showing numbers of differentially expressed genes in each of the 3 KO cell lines and overlap of these differentially expressed genes between KO lines; (**G**) No trend in gene expression changes as a function of increased or decreased nuclear lamin association after DKO: Scatterplot of gene expression differences (log2-ratio FKPM, y-axis) versus differences in LMNB1 DamID (x-axis) (DKO-wt). Only active genes are shown (blue, upregulated; red, downregulated; grey, no change); (**H**) Domains that shift closer to nuclear lamina in DKO cells also shift to later DNA replication: Scatterplot comparing mean changes over chromosome domains in E/L 2-fraction Repli-seq (DKO – wt) (y-axis) versus changes in LMNB1 TSA-seq (DKO – wt) (x-axis). Domain data points are colored based on their transition class (see text) (wt LADs, p-w-v fiLADs, and v-fiLADs, and DKO fLADs).

In contrast, plotting 2-fraction Repli-seq versus changes in DamID shows a consistent trend of slightly later DNA replication timing for regions (primarily p-w-v fiLADs) moving closer to the lamina (**Fig. 5B** &**G**). However, LADs that shift further away from the lamina, do not show an obvious progressive shift towards earlier DNA replication timing (**Fig. 5B** &**G, I-J**).

In summary, upon LMNA and LBR DKO in K562 cells the lamina associated heterochromatin (LADs) enriched in H3K9me3 move away nuclear periphery. In contrast, heterochromatin regions localized to nuclear interior and enriched in H3K27me3 (p-w-v fiLADs) move toward the nuclear periphery in DKO K562 cells. These movements show no specific dependence of gene expression on relative distances to the lamina, although a subset of chromosome regions corresponding to p-w-v fiLADs might show altered DNA replication timing as a function of increased lamina association.

Differential positioning relative to speckles and lamina identifies different types of LAD regions

In a previous Section, we showed a large fraction of the genome showed variations in localization relative to the lamina and/or nucleoli without showing a correlation with differences in gene expression (**Fig. 2**, **SFig. 2**). However, these chromosome regions showed a correlation between these differences in locale localization and DNA replication timing (**Fig. 2F-G**), suggesting that we might use these differences in nuclear positioning to identify different types of heterochromatic regions with differential biochemical and functional properties.

More specifically, we next asked whether we could use differences in lamina versus speckle TSA-seq scores to identify heterochromatin regions with differential biochemical and functional properties (**Fig. 6**, **SFig. 6**).

First, whereas an overall inverse relationship between lamina and speckle TSA-seq observed in K562 cells is largely preserved in H1 cells, scatterplots reveal off-diagonal genomic bins ("H1 ROI" orange bins in **Fig. 6A**, "H1 ROI C1&C2" in **SFig. 6A**) with disproportionally low lamina TSA-seq scores (**Fig. 6A-B**, **SFig A-B**). These genomic bins are largely within several Mbp from centromeres (**Fig. 6C**, **SFig. 6E**), are H3K9me3-enriched (**Fig. 6D**), and are more associated with the nucleolus specifically in H1 cells in which they are less associated with the lamina (**SFig. 6B-C**). Many genomic regions contained within the H1 ROI-C1 display a similar position in the K562 DKO scatterplot (**SFig. 6D**), suggesting a LMNA and/or LBR dependence for nuclear lamina association for this subgroup of LADs.

Second, deviations in the inverse relationship between SON and lamin TSA-seq in HCT116 and HFF cells with flat nuclei reveals four types of LAD regions, defined by DamID (**SFig. 6F**), varying in their histone modifications (**Fig. 6E-F**): 1) H3K9me3-enriched LAD Cluster C1: low SON, high LMNB1; 2) H3K27me3-enriched LAD Cluster C2: moderate SON, high LMNB12; 3) H3K9me2- and H2A.Z-enriched LAD Cluster C3: low SON, low LMNB1; 4) low in multiple histone marks LAD Cluster C4: moderate SON, low LMNB1. Superimposing these histone mark enrichments over the HCT116 scatterplot more precisely revealed the differential spatial distribution of LAD regions with different types of histone mark enrichments (**Fig. 6G**).

These four types of LAD regions show different correlations with both gene expression levels and DNA replication timing (**Fig. 6H**). H3K9me3-enriched C1 LAD regions show the lowest levels of gene expression. H3K27me3-enriched C2, H3K9m2/H2A.Z-enriched C3, and C4 LAD regions show gene expression levels significantly lower than iLADs but slightly higher than C1 LAD regions. DNA replication timing is latest and most uniform for the C1 LAD regions and shows progressively earlier DNA replication timing for the C3, C2, and C4 LAD regions which all still replicate later than iLADs. We defined a "constitutive LAD (cLAD) score" as the number of cell lines out of 7 overall in

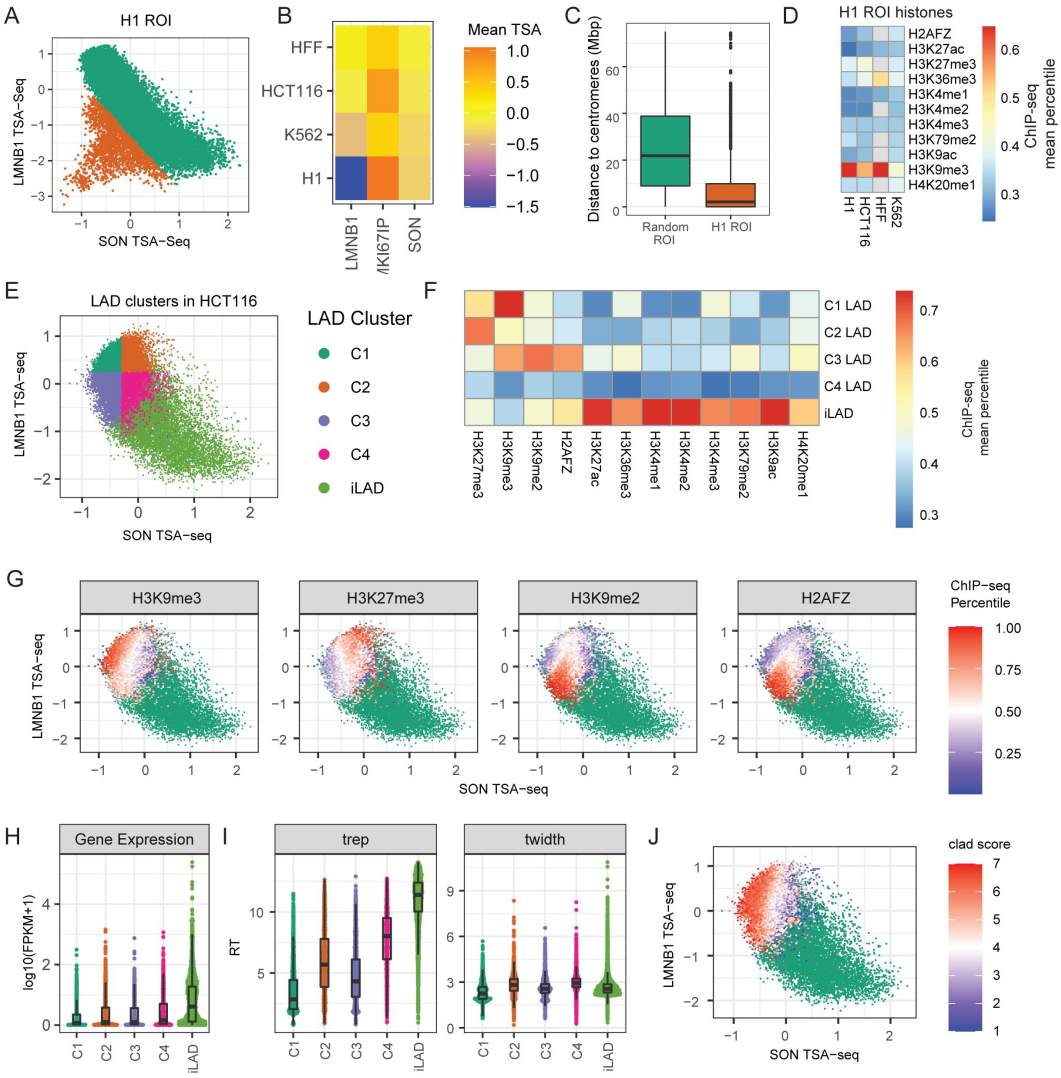


Figure 6.

Spatial segregation within nuclei of different heterochromatin types revealed by LMNB1 and SON TSA-seq

(**A**) Genomic ROI (ROIs C1 and C2 in **SFig. 6**) in H1 cells with disproportionately reduced nuclear lamina interactions (orange) which deviate from otherwise inverse correlation (green) between SON and LMNB1 TSA-seq. H1 SON(x-axis) versus LMNB1 (y-axis) TSA-seq scatterplot; (**B**) Mean TSA-seq LMNB1, MKI67IP, SON values for H1 ROI in HFF, HCT116, K562, and H1 cells (top to bottom) reveals decreased nuclear lamina but increased nucleolar proximity in H1 cells; (**C**) Most H1 ROIs are located within several Mbp of centromeres; distance (Mbp) boxplots of H1 ROIs versus other regions; (**D**) Higher enrichment of H3K9me3 versus other histone marks (mean ChIP-seq percentile values) in H1 ROIs in H1, HCT116, HFF, K562 cell lines; (**E**) Subdivision of HCT116 LAD genomic bins (100 kb) into four, color-coded clusters (C1-4) based on their SON and LMNB1 TSA-seq scores; light green points are iLAD bins; (**F**) LAD bins in C1-4 clusters show different histone marks; mean percentile ChIP-seq values of indicated histone marks for the C1-4 LAD clusters and iLADs; (**G**) H3K9me3, H3K27me3, H3K9me2, and H2AFZ (H2A.Z) (left to right) enriched regions show differential nuclear localization relative to nuclear speckles and lamina roughly paralleling C1-C4 clusters; SON versus LMNB1 TSA-seq 2D histograms of LADs. The color-code represents the average histone modification levels in each bin (iLADs, green points); (**H-I**) C1-4 LAD clusters vary functionally. C1 especially but also C3 LAD bins have lower gene expression, later DNA replication timing (Trep), and more uniform replication timing (Twidth) than C2 and C4 LAD bins. iLAD bins have highest gene expression, earliest replication timing, but are less variable in replication timing than C2 and C4 LAD bins. Boxplot distributions for log2(FKPM) (**H**), Trep (**I**, left), and Twidth (**I**, right); (**J**) cLAD segregate spatially differentially from fLADs largely based on their greater distance to nuclear speckles (iLADs, green). Color-coded cLAD score (# cell lines out of 7 in which a HCT116 LAD bin maps within a LAD) superimposed on SON versus LMNB1 TSA-seq scatterplot.

which a chromosome region is a LAD. cLADs are enriched over LAD regions with the lowest SON TSA-seq scores (C1 and C3) while fLADs are enriched over LAD regions with moderate SON TSA-seq scores (C2 and C4) (**Fig 6J**).

In summary, LAD regions that segregate differentially in certain cell types relative to speckles and the lamina show different histone mark-enrichments and functional properties. Why these different types of heterochromatin segregate differentially in certain cell types remains unclear, but these results imply that such heterochromatin regions can assume very different nuclear locale positioning in different cell types.

Polarity of Nuclear Genome Organization

The loss of the strong inverse relationship between speckle and lamina TSA-seq in the HCT116 and HFF cell lines was unexpected and puzzling. Whereas in K562 cells the latest replicating, lowest expressing LADs enriched in H3K9me3 and cLADs map to the highest LMNB1 and lowest SON TSA-seq scores (Chen et al., 2018), those same cLAD regions in HCT116 cells show lower lamin B1 TSA-seq scores than fLADs enriched in H3K27me3 (**Fig. 6**). What is the actual explanation for these fLADs showing even higher lamin B TSA-seq scores than cLADs in HCT116 cells but not in K562 cells? The noticeably weaker inverse relationship between speckle and lamina TSA-seq in cells with flat (HCT116, HFF) versus round nuclei (K562, H1) was due specifically to changes in lamina TSA-seq as lamina DamID scores showed less variation across cell lines (**SFig. 2C**).

The inactive X chromosome, a classic example of facultative heterochromatin, was previously inferred to be localizing preferentially to the nuclear periphery contained within the equatorial plane of fibroblast nuclei (Belmont et al., 1986). We hypothesized that LADs with the highest lamin B1 TSA-seq signal in flat HFF and HCT116 nuclei, enriched in the H3K27me3 mark (**Fig. 6G**), might also correspond to genomic regions enriched specifically at the nuclear lamina contained within the nuclear equatorial plane. We hypothesized that these LADs would have the highest lamina TSA-seq scores: after lamina TSA-staining they would be exposed to a higher concentration of tryamide free-radicals generated and diffusing from the nearby side, top, and bottom of the nuclear lamina as compared to LADs at either the top or bottom of the nuclear lamina.

To test this we turned to a highly multiplexed, immuno-FISH dataset from IMR90 human fibroblasts (Su et al., 2020), published after our TSA-seq profiling of HFF human fibroblasts. We reasoned that the nuclear genome organization in the two human fibroblast cell lines would be sufficiently similar to justify using the IMR90 FISH dataset as a proxy for our analysis of HFF TSA-seq data. Indeed, the high inverse correlation (R= −0.86) of distances to speckles measured by MERFISH in IMR90 cells with HFF SON TSA-seq scores is nearly identical to the inverse correlation (R= −0.89) measured instead using IMR90 SON TSA-seq scores, published elsewhere (Alexander et al., 2021) (**Fig. 7A**). Similarly, distances to the nuclear lamina and nucleoli show high inverse correlations with lamina and nucleolar TSA-seq, respectively (**Fig. 7A-B**). These correlations were cell type specific, particularly for the lamina and nucleolar distance correlations, as these correlations were reduced if we used TSA-seq data from other cell types (**SFig. 7**). Therefore, the high correlation between IMR90 microscopic distances and HFF TSA-seq scores can be considered a lower bound on the likely true correlation, justifying the use of IMR90 as a proxy for HFF for testing our predictions.

We first simulated this effect of LADs at the equatorial plane being exposed to a higher tyramide free radical concentration during TSA staining. Convolving the HFF anti-RL1 immunostaining image with a kernel corresponding to the exponential diffusion gradient of tyramide-biotin TSA labeling (Chen et al., 2018), did indeed produce a notably elevated TSA signal in the equatorial plane of the nuclear periphery (**Fig. 7C**). These simulation results predict that the LADs with the

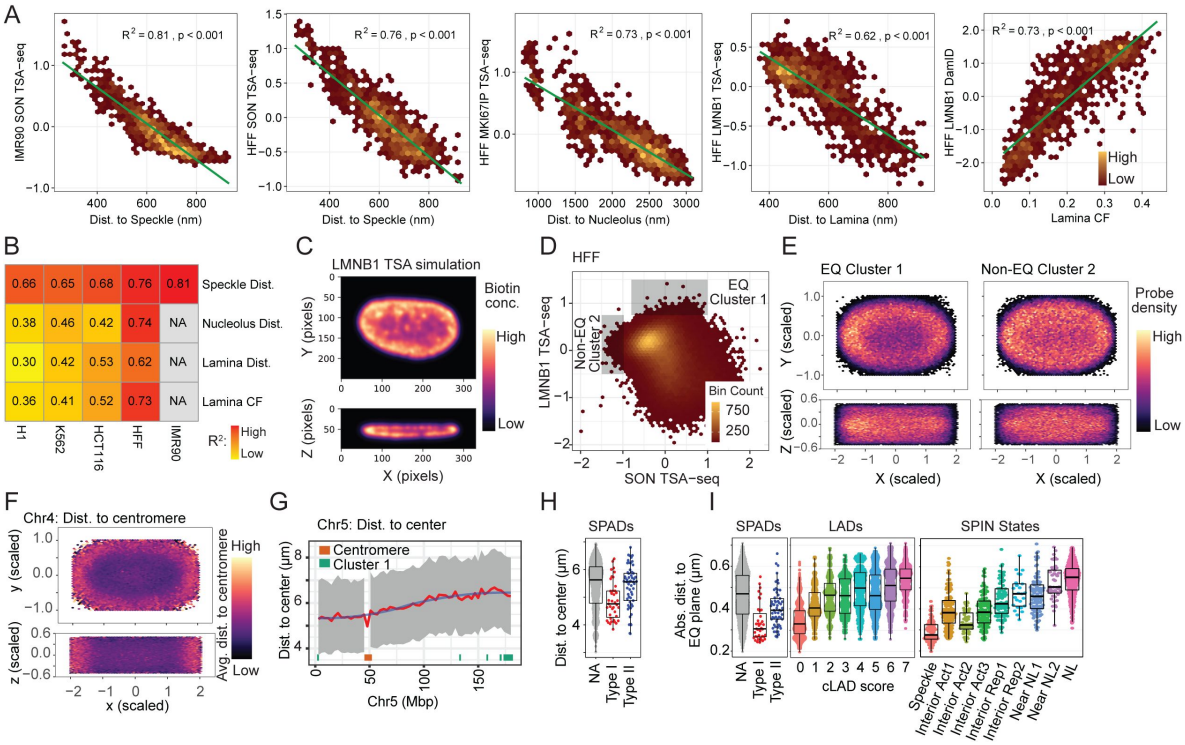


Figure 7

Polarity of nuclear genome organization

(**A-B**) Microscopic distances measured by MERFISH (Su et al., 2020) inversely correlate with TSA-seq scores while lamina contact frequencies measured by MERFISH correlate with lamina DamID. (A) Left to second to right: Scatterplots showing distances of FISH probes to nuclear locales (x-axis) in IMR90 fibroblasts versus TSA-seq scores (y-axis) for speckles in IMR90, for speckles, nucleoli (MKI67IP), and lamina (LMNB1) in HFF fibroblasts; right panel: lamina contact frequencies measured by microscopy in IMR90 (x-axis) versus lamina (LMNB1) DamID score (y-axis); (B) Best correlations are seen for comparisons of IMR90 data with the similar fibroblast line HFF as compared to other cell lines: correlation coefficients (R^2) for comparison of MERFISH distances and contact frequencies (lamina) with TSA-seq or DamID measured in different cell lines; (**C-E**) LMNB1 TSA-seq provides a readout of nuclear genome polarity in flat nuclei due to the diffusion radius of tyramide free-radicals, identifying a LAD subset preferentially localizing at the nuclear equatorial plane periphery. (C) nuclear pore immunostaining from HFF nucleus convolved with the 3D exponential decay function of TSA staining (pseudo-colored intensity) predicts higher biotin-labeling of lamina-associated chromatin lying in nuclear equatorial plane versus top or bottom of nucleus. Top (x-y cross section); bottom (x-z cross section). Pixel size = 80 nm; (D) 2D color-coded histogram showing number of LAD genomic bins with given SON (x-axis) and LMNB1 (y-axis) TSA-seq mean values in HFF. Rectangular boxes show selection of EQ Cluster 1, with the highest LMNB1 TSA-seq values, and the Non-EQ Cluster 2, with low to moderate LMNB1 and low SON TSA-seq; (E) Cluster 1 LADs preferentially localize to the equatorial plane of the nuclear periphery. Heat map showing FISH probe location density over many IMR90 fibroblast nuclei from Cluster 1 (left) versus Cluster 2 (right) LADs superimposed on normalized nuclear shape. Top: x-y projection; bottom: x-z projection; (**F**) Distal chromosome arms also preferentially localize to equatorial plane. Same as (E) but using all the FISH probes mapped to Chr4 and color-coded by distance (averaged over probes) to centromere in Mbp; (**G**) Distance to IMR90 nucleus x-y center also varies with chromosome distance from centromeres: IMR90 FISH probe distances (y-axis) from nuclear center (projected x-y plane distances) as a function of Chr5 position (x-axis). Grey-interquartile range of probe distances; blue-mean probe distance; red-smoothed mean probe distance. Bottom track-red rectangle marks centromere position, green rectangles mark Cluster 1 LADs.; (**H and I**) Overall nuclear genome polarization in fibroblasts (IMR90); (H) Boxplots of mean distance to nuclear center (projected x-y plane distance) for all probes (NA), or Type I (red) or Type II (blue) SON TSA-seq peaks; (I) Boxplots of mean distance to equatorial plane for SON TSA-seq peaks, facultative versus constitutive LADs, and SPIN states. Strong versus moderate bias of Type I (red) versus Type II (blue) HFF SON TSA-seq peaks, respectively, to locate close to x-y nuclear center (H) and near to the equatorial plane of nuclear interior (I, left panel). Facultative LADs (low cLAD scores) localize closer to equatorial plane than constitutive LADs (high cLAD scores) (I, middle panel). SPIN states show progressive trend of increased mean distances to equatorial plane from “speckle” to” interior active” to “interior repressed” and “near lamina 1”, to “near lamina 2” and “lamina” SPIN states (Wang, 2021) (I, right panel).

highest lamina TSA-seq scores lie within the equatorial plane, while LADs that show lower lamina TSA-seq scores, but similar lamina DamID scores, either distribute over the nuclear periphery randomly or are biased towards the top or bottom of the nucleus.

To test these predictions, we identified LADs with very different lamina TSA-seq scores, measured in human HFF fibroblasts, and compared their microscopic localization within IMR90 human fibroblast nuclei, measured by immuno-FISH.

Specifically, we compared the localization in HFF cells of 26 LADs mapping to regions of highest lamina TSA-seq (EQ Cluster 1, **Fig. 7D**) versus 27 LADs mapping to regions of lowest speckle TSA-seq but with low to moderate lamina TSA-seq (Non-EQ Cluster 2, **Fig. 7D**). Cluster 1 LADs showed a biased localization in IMR90 fibroblasts towards the equatorial plane as compared to Cluster 2 LADs that distributed more uniformly over the x-y nuclear projection (**Fig. 7E**). More generally, Cluster 1 and 2 LADs show a similar differential localization in lamina versus SON TSA-seq scatterplots in both HFF and HCT116 cells with flat nuclei which is notably different than in K562 and H1 cells with round nuclei (**SFig. 8A**). Cluster 1 LADs correspond to LADs located towards the ends of long chromosome arms (**SFig. 8B**). Overall, in fibroblasts there is a trend of distal chromosome arms localizing preferentially towards the nuclear equator versus centromeric regions localizing preferentially towards the nuclear center (**Fig. 7F-G and SFig. 8C-D**).

We next examined the nuclear polarity of genomic regions associated with different nuclear locales in fibroblasts. Single cell data (**SFig. 8E**) and average trends over all nuclei (**SFig. 8F**), show speckle-associated regions concentrated towards the equatorial plane, consistent with the direct localization of speckles within the equatorial plane (**Fig. 1B**). Nucleolus-associated regions were distributed towards the nuclear center but throughout the z-plane (**SFig. 8E-F**). Type I speckle attachment regions localize more centrally in the x-y plane and closer to the equatorial plane than Type II speckle attachment regions (**Fig. 7H-I**, **SFig. 8G**). The more peripheral, less central positioning of Type II peaks is consistent with the shorter genomic distances of Type II versus I peaks to the nearest LADs (Chen et al., 2018). fLADs localize closer to the equatorial plane (**Fig. 7I**) consistent with the enrichment of H3K27me3 modification in these regions, while cLADs localize furthest away from this plane (**Fig. 7I**). Additionally, v-fiLADs cluster closer to the equatorial plane and further from the nuclear center as compared to p-w-v fiLADs (**SFig. 8I**).

To more comprehensively survey how the genome distributes relative to nuclear polarity, we turned to the use of SPIN states, which combine TSA-seq and DamID with Hi-C data to segment the genome into 9-10 states based on their intranuclear localization; SPIN states show differential epigenetic marks, gene expression, and DNA replication timing (Wang, 2021; Wang et al., 2021). SPIN states ordered according to highest to lowest gene expression, and earliest to latest DNA replication timing, showed a pronounced, nearly monotonic increasing distance from the equatorial plane (**Fig. 7I**) as well as differential localization relative to the x-y nuclear center (**SFig. 8H**).

Thus, in adherent cells with flat nuclei the distance from the equatorial plane emerges as an additional key axis in nuclear genome organization in addition to the x-y plane radial position.

Discussion

Here we integrated imaging with both spatial (DamID, TSA-seq) and functional (Repli-seq, RNA-seq) genomic readouts across four human cell lines. Overall, our results significantly extend previous nuclear genome organization models, while also demonstrating a cell-type dependent complexity of nuclear genome organization. Briefly, in contrast to the previous radial model of genome organization, we reveal a primary correlation of gene expression with relative distances

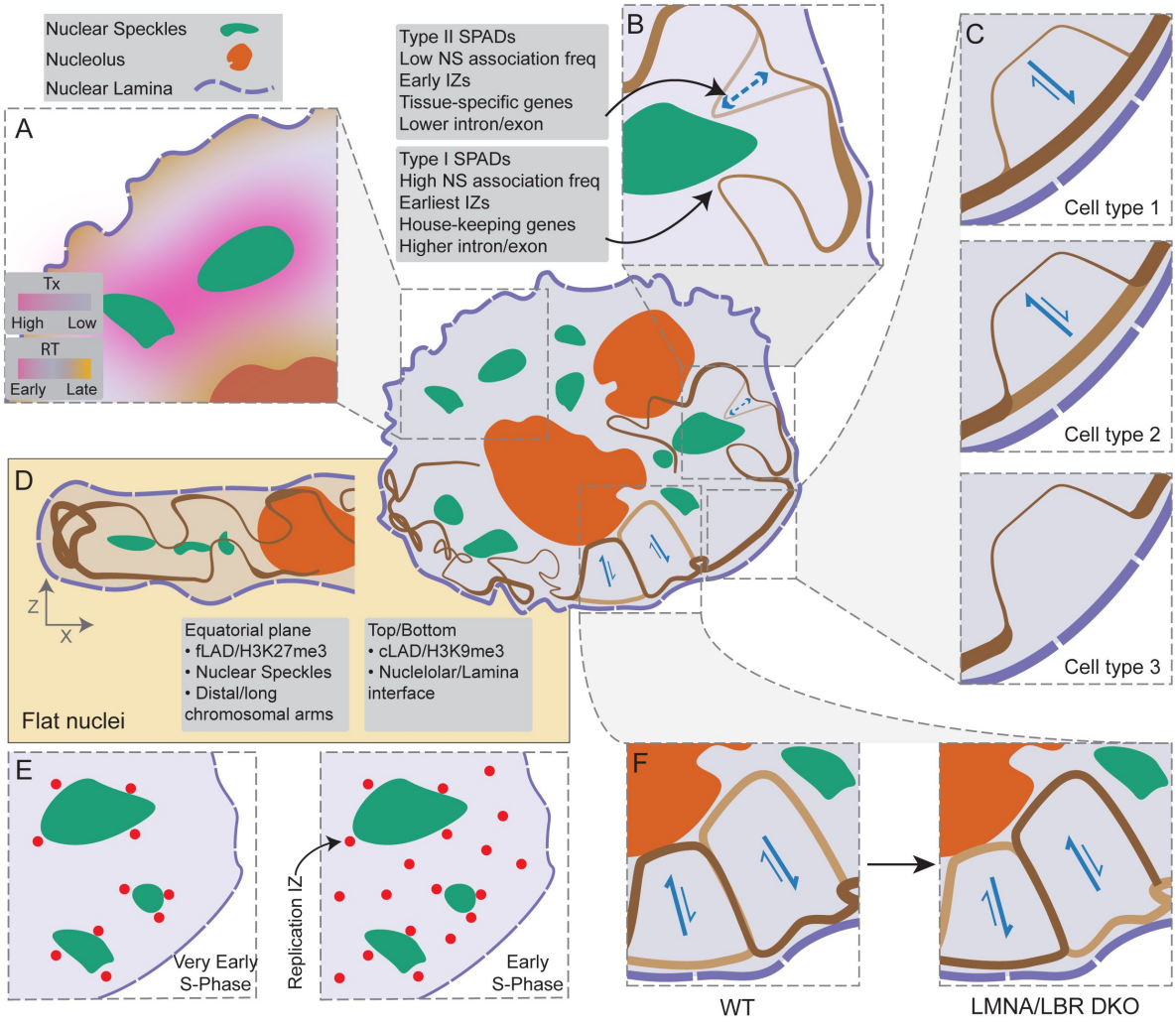


Figure 8.

New insights into nuclear genome organization revealed by nuclear locale genome mapping

Human nucleus schematic (middle) shows nuclear speckles (NS, green), nucleolus (orange), and nuclear lamina (NL, purple). (A) Changes in the transcriptional activity of genes (pink to purple gradient) primarily correlates with changes in their distance to nuclear speckles (green), rather than nuclear lamina or nucleolus, whereas changes in DNA replication timing (pink-purple-yellow gradient) correlates with distance to all three nuclear locales. Regions close to nuclear speckles replicate early, while regions close to nucleolus and/or nuclear lamina replicate late during S-phase. (B) Two types of speckle association domains (SPADs), with similarly elevated levels of gene expression but genes of different lengths and relative intron/exon fraction, associate with NS. Type-I SPADs, with shorter, more exon-rich genes, have higher NS association frequencies compared to Type-II SPADs, with longer, more intron-rich genes. (C) fLADs can assume at least three different chromatin states across different cell types: i) a B-compartment LAD (positive lamina DamID signal) with high NL association, low gene expression levels and late DNA replication (cell type 1); ii) an A-compartment p-w-v-fiLAD (peak-within-valley lamina DamID signal) with low NL association, intermediate gene expression and middle-to late DNA replication (cell type 2); iii) an A-compartment v-fiLAD (valley lamina DamID signal) with no NL association, high gene expression and early DNA replication (cell type 3). (D) In cells with flat nuclei, the genome is differentially organized relative to the nuclear equatorial plane: facultative heterochromatin regions are enriched near the NL within the equatorial plane, while constitutive heterochromatin regions are enriched near the NL towards the top or bottom of the nucleus. Nuclear speckles localize towards the nuclear equatorial plane and towards the nuclear center, with Type I SPADs closer than Type II SPADs to the equatorial plane and nuclear center. (E) Type I and II SPADs align with DNA replication initiation zones with the earliest-firing DNA replication initiation zones mapping to Type I SPADs and the earliest DNA replication foci appearing adjacent to NS. (F) LADs and p-w-v fiLADs compete for NL versus nucleoli association: in a LMNA/LBR double knockout cell line, LADs shift from the NL towards nucleoli and the nuclear interior while p-w-v fiLADs shift towards the NL.

to nuclear speckles rather than radial position. Additionally, beyond a correlation of nuclear genome organization with radial position, in cells with flat nuclei we show a pronounced correlation of nuclear genome organization with distance from the equatorial plane. In contrast to previous binary models of genome organization, we describe how both iLAD / A compartment and LAD / B compartment contain within them smaller chromosome regions with distinct biochemical and/or functional properties that segregate differentially with respect to relative distances to nuclear locales and geometry.

More specifically, we describe five significant new insights into genome organization (**Fig. 8**). First, across the four cell lines examined, differences in gene expression correlate primarily with differences in the genome positioning of these genes relative to nuclear speckles (**Fig. 8A**) rather than distance from the nuclear lamina and despite variations in speckle positioning relative to the nuclear lamina and nucleoli. Consistent with previous observations showing a more interior nuclear localization of genes after their activation (Bickmore, 2013; Takizawa et al., 2008), we demonstrate an inverse trend between changes in speckle and lamina distances for the small fraction of genomic regions that show significant changes in their relative speckle distances. For these regions, there is a strong bias towards increased (decreased) gene expression with decreased (increased) speckle distance. However, several-fold more genomic regions significantly change their relative lamina distances without significantly changing their speckle distances or gene expression. Similarly, LBR/LMNA DKO in K562 cells results in many genomic regions increasing or decreasing their lamina association without changes in their speckle distance or gene expression. In contrast, changes in DNA replication timing correlate with changes in positioning relative to all three nuclear locales-speckles, lamina, and nucleoli (**Fig. 8A**).

Second, Hi-C A compartment/iLADs are punctuated by strong (Type I) and weaker (Type II) speckle attachment regions which align with DNA replication initiation zones and show elevated gene expression levels above flanking iLAD regions (**Fig. 8B**). Type I regions contain shorter, more exon-rich genes as compared to Type II regions and are more highly conserved across cell lines than Type II regions. IZs within strong speckle attachment sites replicate earlier than IZs within weak speckle attachment zones with the very earliest DNA replication foci appearing adjacent to nuclear speckles (**Fig. 8E**).

Third, Hi-C A compartment/iLADs are also punctuated by partially repressed fiLADs which retain weak lamina interaction and, in some cases, elevated nucleolar interactions. Identified as local maxima in lamina TSA-seq and/or DamID, these “p-w-v-fiLADs” comprise ~2/3 of all fiLADs across the four cell lines examined. P-w-v-fiLADs show gene expression levels intermediate between LADs and typical iLADs while retaining late or shifting to middle DNA replication timing. Only approximately 1/3 of fiLADs, identified as valleys in lamina DamID (v-fiLADs), convert to an active, early-replicating, interior chromatin state. The same fLADs across different cell lines can associate strongly with the lamina as a LAD, retain weak lamina association as a partially repressive p-w-v-fiLAD, or show a true transition to an interior, active, early DNA replicating state (**Fig. 8C**). Upon LMNA/LBR double KO, p-w-v-fiLADs shift closer to the lamina, suggesting they compete with LADs for lamina association in wt cells (**Fig. 8F**).

Fourth, we show how LAD regions showing different histone marks-either enriched in H3K9me3, H3K9me2 plus H2A.Z, H3K27me3, or none of these marks-can differentially segregate within nuclei. These results support the previous suggestion of different “flavors” of LAD regions, based on the autonomous targeting of BAC transgenes to the lamina (Bian et al., 2013). Differential nuclear localization also was recently inferred by the appearance of different Hi-C B-subcompartments, which similarly were differentially enriched in either H3K9m3, H3K27me3, or the combination of H3K9me2 and H2A.Z (Spracklin et al., 2023). Here, our use of TSA-seq directly measures and assigns the intranuclear localization of different LAD regions to different nuclear locales.

Fifth, beyond the previously described dependence of genome organization relative to the radial axis in the x-y plane, there exists an unexpectedly high degree of nuclear polarity with respect to an orthogonal z-axis in HCT116 and HFF cells with flat nuclei (**Fig. 8D**). Strong speckle attachment regions localize closer to the equatorial plane than weak speckle attachment regions. A subset of LADs, primarily at the ends of long chromosome arms, preferentially localize at the lamina within this equatorial plane. Using SPIN states, ordered by DNA replication timing and levels of gene expression, we show that this overall nuclear polarity applies to nearly the entire genome, suggesting a corresponding overall gradient in both later DNA replication timing and lower gene expression with increased distance from the equatorial plane.

In conclusion, by examining genome organization relative to multiple nuclear locales, we were able to demonstrate a higher correlation than previously apparent between nuclear location and functions such as DNA replication timing and gene expression. We anticipate future mapping of the genome relative to additional nuclear locales will reveal a still more deterministic relationship between nuclear positioning and genome function.

Materials and methods

Cell culture

H1, HCT116, and HFF cells were cultured according to standard operation protocols established by the NIH 4D Nucleome Consortium (*https://www.4dnucleome.org/cell-lines.html*). K562 cells were cultured according to the ENCODE Consortium protocol (*http://genome.ucsc.edu/ENCODE/protocols/cell/human/K562_protocol.pdf*).

Immunostaining

Adherent cells (HCT116, HFF, and H1) were seeded on 12mm round coverslips and cultured for two days prior to immunostaining. For K562 cells, 150µl of cell suspension was added to coverslips treated with poly-L-lysine as described previously (Chen et al., 2018). All washing steps were 3 ξ 10 mins in Ca, Mg-free PBS at room-temperature (RT), unless specified elsewise. Cells were fixed with freshly made 2% paraformaldehyde (PFA) in PBS for 10 mins at RT and washed. Cells were permeabilized for 5 mins on ice with 1X PBS containing 0.2% Triton X-100 (Sigma-Aldrich T8787*)* and 1% natural goat serum (NGS) and washed with 1XPBS/1% NGS (PBS/NGS) 3 x 10 mins at RT. Each coverslip was then incubated with 100µl of the primary antibody cocktail in a dark humid chamber at 4°C overnight. The cocktail contained moue-anti-RL1 (Santa Cruz cat# sc-58815, 1:100 dilution), rabbit-MKI67IP (Atlas antibodies cat# HPA035735, 4ng/ml dilution) and rabbit-anti-SON (Pacific Immunology Corp, custom-raised, 4ng/ml dilution) (Chen et al., 2018) diluted in PBS/NGS. The anti-MKI67IP and anti-SON antibodies were directly labelled with CF-594 and CF-640R fluorophores using Mix-n-Stain™ CF® Dye Antibody Labeling Kits (Biotium cat #92256 and #92258) according to manufacturer's instructions. After immunostaining, coverslips were washed and RL1 antibody was immunostained with goat-anti-mouse-Alexa-Fluor-488 secondary antibody (Jackson ImmunoResearch Laboratories, cat#115-545-003) diluted 1:500 in PBS/NGS) for 1 hr in a dark humid chamber at RT and washed. The DNA was counterstained with 200 ng/ml DAPI in PBS for 10 mins at RT and washed. Finally, the coverslips were mounted on glass slides using an antifade mounting media (10% w/v Mowiol 4-88(EMD Millipore)/1% w/v DABCO (Sigma-Aldrich)/25% glycerol/0.1 M Tris, pH 8.5) and cured overnight before proceeding to imaging.

Microscopy and image segmentation

Locales were imaged using the OMX-V4 microscope (GE Healthcare) equipped with a U Plan S-Apo 100×/1.40-NA oil-immersion objective (Olympus), two Evolve EMCCD cameras (Photometrics). Z-sections were 125 nm apart. Images were segmented in 3D using a combination of in-house softwares and the Allen Cell and Structure Segmenter (Segmenter) toolkit in Python (Chen et al.,

2020). Briefly, nuclei were first identified by segmenting DAPI using Otsu thresholding method. RL1 signal was first segmented using a modified nup153 workflow from Segmenter. In H1 cells, there are some cytoplasmic signals for RL1 which were removed using the DAPI nucleus mask. RL1 signals are puncta and the alpha-shapes algorithm (Edelsbrunner et al., 1983) was used to calculate a 3D mesh-surface enclosing all the puncta (**SFig 1A**). This surface was used to measure the nuclei's volume (V), surface area (SA), and SA/V. We segmented SON using the SON workflow, and MKI67IP using "masked object thresholding" workflow from Segmenter. The binary segmented images were used to measure the total number of locales in each nucleus and average and total V, SA. Nuclei images and segmentations were manually curated for quality control and are available online. To measure locale distances in each cell line, the SON and MKI67IP binary images were converted to mesh surfaces using a marching cube algorithm. An asymmetric locale to locale distance was then calculated by measuring the closest distance between a pair of vertices from locales mesh-surfaces (**SFig. 1B**). The PCA analysis was performed in R using 15 numerical metrics for each nucleus (**Fig. 1F** and **SFig. 1F**). A similar approach was used to compare K562 wt and LMNA/LBR double knockout cells.

HCT116 EGFP-SON KI

A Cas9/sgRNA RNP complex and linear dsDNA donor were transfected into HCT116 cells using the Amaxa Nucleofector II device (Lonza) set for program D-032 with the Cell Line Nucleofector Kit V. We obtained the guide and CRISPR RNA using the GeneArt Precision gRNA Synthesis Kit (Thermo Fisher Scientific) with our designed SON guide sequence 5'-gagagaacggagcggacgcca −3'. The Cas9 protein Alt-R S.p. Cas9 Nuclease V3 was purchased from Integrated DNA Technologies (IDT). Donor DNA was amplified by PCR from a previously modified version of the SON-containing BAC RP11-165J2 (Invitrogen) with EGFP sequence at the NH2 terminus of SON (Khanna et al., 2014). We used modified primers containing a 5' amine group with a C6 linker (Yu et al., 2020) 5'-GTGCTCACTGATTGGTCCCTC −3' and 5' -GAACGACTGCGTCTCCGAAG −3' (amC6, IDT) to obtain a 1044bp fragment that includes the EGFP sequence flanked by a 160bp upstream and a 165bp downstream homologous region of SON. After 5 days of recovery, EGFP-positive cells were sorted using the FACS ARIA II sorter at the UIUC Roy J. Carver Biotechnology Center. Individual HCT116 clones expressing SON-GFP were genotyped by PCR with primers 5'-CAAGAGAGACGGCTCCTGTAATG −3' and 5'-TCGGAAAAGGCGAAGTTCCTCG −3' which amplify the complete EGFP integration to identify the double allele knock-in clone "C4". HCT116 EGFP-SON clone C4 was transduced with a lentivirus carrying the Lenti-mCherry-PCNA construct (Xiong et al., 2023) by supplementing media with 8 µg/ml polybrene (Santa Cruz Biotechnology).

Live-cell Imaging and analysis

HCT116 EGFP-SON clone C4 cells expressing mCherry-PCNA were seeded in 35-mm dishes with a #1.5-thickness glass coverslip bottom (MatTek, P35G-1.5-14-C) and grown to ~90% confluency. Live cell imaging was performed using a OMX V4 (Applied Precision) microscope with a 100×/1.4 NA oil immersion objective (Olympus), two Evolve EMCCDs (Photometrics), and a live-cell incubation chamber. The chamber contains a temperature control (37°C) for the incubator and the objective lens, and a humidified CO2 supply. 3D images were acquired using a z-spacing of 300nm once every 5 mins for 12 hrs. Each z-slice was imaged at 2.0% transmittance and 10ms exposure for 488nm excitation (GFP, Speckles), and 5.0% T with 10ms exposure for 568nm (mCherry, PCNA). We used FIJI to smooth images and remove noise. A median filter of 0.24µm and "rolling ball" background subtraction of ~0.5µm was used. All measurements were done from corresponding individual Z-sections per timepoint. To identify PCNA foci we used the FIJI maximum entropy threshold plugin followed by watershed segmentation of the grayscale image. FIJI's particle analysis was used to identify the center of each outlined PCNA foci, and the distance to nuclear speckle was measured from the PCNA foci center to the visible edge of the nearest nuclear speckle.

K562 LMNA and LBR single and double knock-out generation

To create LMNA and LBR knockout (KO) lines and the LMNA/LBR double knockout (DKO) line, we started with a parental "wt" K562 cell line, clone #17, expressing an inducible form of Cas9 (Brinkman et al., 2018). The single KO and DKO were generated by CRISPR-mediated frameshift mutation according to the procedure described previously (Schep et al., 2021). The "wt" K562 clone #17 was used for comparison with the KO clones. The LBR KO clone, K562 LBR-KO #19, was generated, using the LBR2 oligonucleotide GCCGATGGTGAAGTGGTAAG to produce the gRNA, and validated previously, using TIDE (Brinkman et al., 2014) to check for frameshifts in all alleles as described elsewhere (Schep et al., 2021). The LMNA/LBR DKO, K562 LBR-LMNA DKO #14, was made similarly, starting with the LBR KO line and using the combination of two oligonucleotides to produce gRNAs: LMNA-KO1: ACTGAGAGCAGTGCTCAGTG, LMNA-KO2: TCTCAGTGAGAAGCGCACGC. Additionally, the LMNA KO line, K562 LMNA-KO #14, was made the same way but starting with the "wt" K562 cell line. Validation was as described above; additionally, for the new LMNA KO and LMNA/LBR DKO lines, immunostaining showed the absence of anti-LMNA antibody signal under confocal imaging conditions used to visualize the wt LMNA staining while the RNA-seq from these clones revealed an ~20-fold reduction in LMNA RNA reads relative to the wt K562 clone.

TSA-seq

SON and MKI67IP TSA-Seq was performed using Condition E (labeling with 1:300 tyramide biotin, 50% sucrose and 0.0015% hydrogen peroxide) (Zhang et al., 2020) with the following minor modification: 150ul of Dynabeads M-270 streptavidin (Invitrogen, catalog no. 65306) was used to purify the biotinylated DNA. For LMNB1 TSA-Seq, Condition A2 (Zhang et al., 2020) (labeling with 1:10000 tyramide biotin, 50% sucrose, 0.0015% hydrogen peroxide and reaction time 20 min at RT) was used.

DamID-seq and data processing

The DamID-seq and subsequent processing was performed according to a previously published protocol (Leemans et al., 2019).

RNA-seq (for K562 DKO)

RNA was isolated using the Qiagen RNeasy column purification kit, after which sequencing libraries were prepared using the Illumina TruSeq polyA stranded RNA kit.

Repli-seq and data processing (for K562 DKO)

The 2-fraction Repli-seq used in analysis of the K562 DKO cell line was done according to a previously published protocol (Marchal et al., 2018). Briefly, BrdU was added to a final concentration of 100 μM and cells were incubated at 37°C for another 2 h. Cells were fixed in ice-cold ethanol and processed for Repli-seq as described before.

Genomic segmentations

For detecting SON TSA-seq Type I and Type II peaks, the SON TSA-seq score (25 kb bins) first was smoothed using locally weighted regression (LOESS). Next, a local maximum filter with window size (w) was applied, which recorded the maximum SON TSA-seq score within this window for each genomic locus. Then the data after the maximum filter were subtracted from the original smoothed data. Peaks were retained only if they showed a reduction in value, which indicates true local peaks. A parameter optimization procedure was conducted in order to maximize the peak agreement between two SON TSA-seq replicates. As a result, a smoothing factor (span=0.005) and a window size (w=50) were selected. To distinguish local peaks into Type I and Type II, we

overlapped them with Hi-C subcompartments (Rao et. al, 2014). Genomic loci overlapping A1 subcompartments were classified as Type I peaks, while those overlapping A2/B1 were designated as Type II peaks.

ROIs in H1 cells corresponding to off-diagonal 100 kb genomic bins in SON versus LMNB1 TSA-seq scatterplots were defined as those bins below the line LMNB1 = −1.5ξSON – 1.3, in these scatterplots. H1 ROI genomic regions were further subdivided into H1 ROI-C1 and C2 sub-regions (**Supp. Fig. 6A**), in which the H1 ROI-C2 regions were defined as the genomic bins below the LMNB1= −1.5ξSON – 2.2 line, while ROI-C1 regions were all remaining ROI bins.

To define LADs, p-w-v-fiLADs, and v-fiLADs, first we identified a consensus set of all chromosome regions which are LADs in at least one of the four examined cell types. Segmentation into these three domain classes was implemented heuristically using the local relative increase ("enrichment") of the domain DamID score relative to its flanking regions (**SFig. 4B**): LAD domains, defined by their positive DamID scores using a Hidden Markov model, showed the largest positive local enrichment. We defined v-fiLADs as those domains which changed from LADs in a different cell line to iLADs and which showed lower local enrichment than 95% of the LADs. P-w-v-fiLADs were then defined as all other fiLAD domains and showed intermediate local enrichment levels.

To define LAD clusters in HCT116 cells based on LMNB1 vesus SON TSA-seq scatterplots, all 100kb genomic bins corresponding to LADs based on the HCT116 DamID (4DN file) were divided into 4 quadrants (**Fig. 6E**) centered at SON = −3.0 and LMNB1= 0.25. The C1, C2, C3, and C4 clusters were defined as the top-left, top-right, bottom-left, and bottom right quadrants, respectively.

Equatorial (Cluster 1) and non-equatorial (Cluster 2) LADs were defined in HFF cells (**Fig. 7B**) as those 100 kb bins which were LADs as defined by the LMNB1 DamID which had LMNB1 TSA-seq (4DN file number) scores higher than 0.75 for the equatorial LADs or SON TSA-seq (4DN file number) scores less than 0.98 for the non-equatorial LADs.

Gene expression analysis

Regarding **Fig. 6H**, the genes overlapping with C1-C4 LAD clusters were identified and their expression was evaluated in HCT116 RNA-seq.

Correlations with other genomic features

Data sets used for this study (Supplementary Table 1) include TSA-seq, DamID-seq, Repli-seq, RNA-seq, ChIP-seq and HiC compartment scores. TSA-seq, DamID, Repli-seq, and ChIP-seq data were mapped to hg38 and binned in 25 kb genomic windows, except for analysis included in **Fig. 6** and **SFig. 6**.

For the ChIP-seq analysis in **Fig. 6**, fold-change over input was calculated for 25kb bins and was converted to percentile values across the genome. The percentile values were averaged over all the bins corresponding to the C1-C4 LAD clusters in the **Fig. 6F** heatmap or were overlayed on the LMNB1 versus SON TSA-seq binned scatterplots in **Fig. 6G**.

A cLAD score was calculated using the DamID LAD calls in 7 available human cell lines (STable 1). First the union of all LAD calls among the 7 cell lines was computed. For each 100kb genomic bin a number between 1 to 7 was assigned depending on the number of cell lines in which the bin was called as a LAD. The average cLAD score was overlayed on the LMNB1 versus SON TSA-seq binned scatterplot in **Fig. 6J**.

Correlating omics data with the IMR90 multiplex FISH data

For the LMNB1 TSA simulation (**Fig. 7A**), a 3D image of the segmented HFF nuclear periphery (anti-RL1 staining) was used. The binary image was convolved with a symmetrical 3D kernel based on the exponential decay function, Be^{-R*d}, of the TSA-biotin signal in which R is the decay constant and d is the distance to the central element of the kernel (B= 5.86, R= 3).

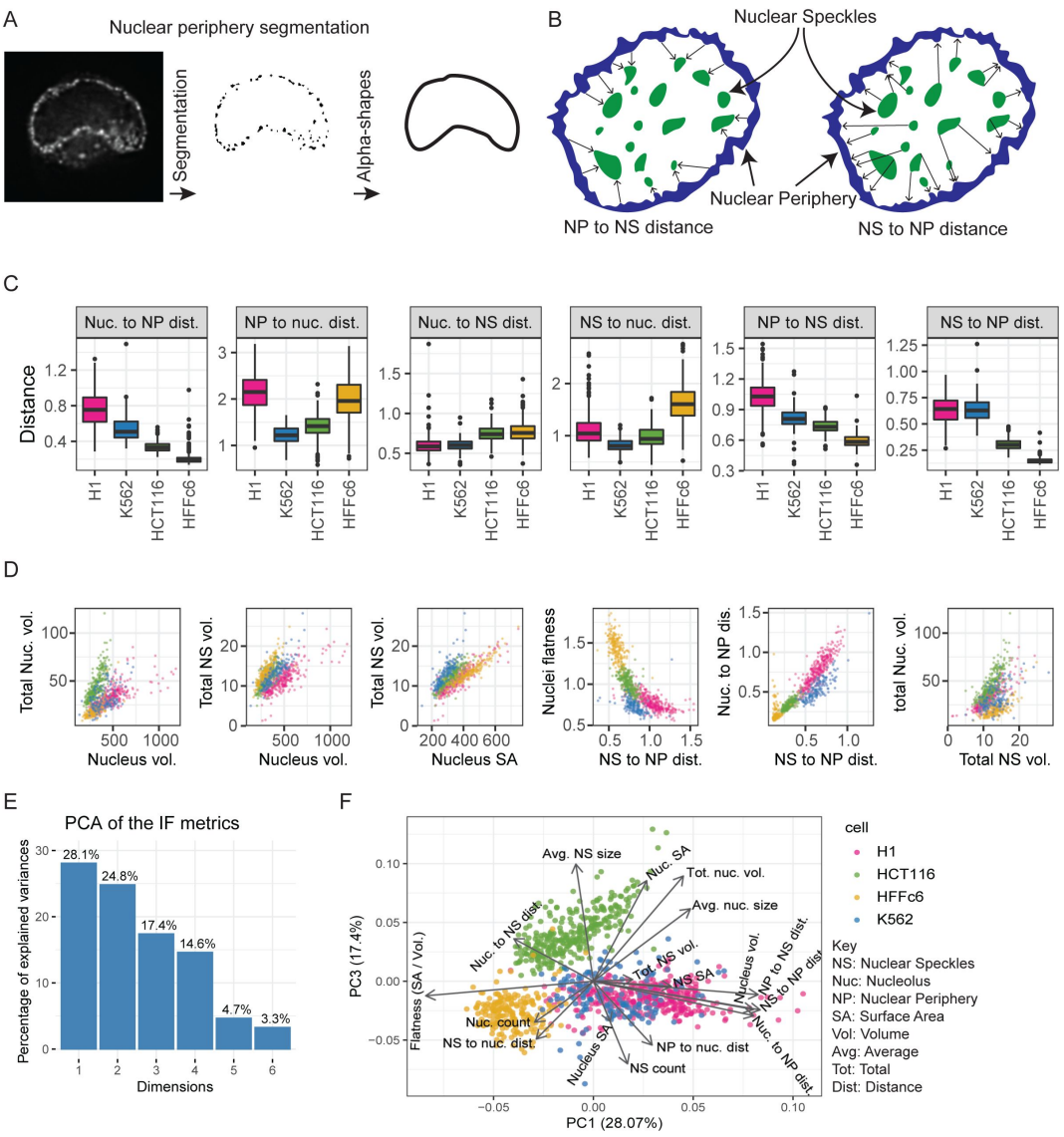
To correlate HFF LAD clusters with published FISH data (Su et al., 2020) (**Fig. 7C-D**) the x-y coordinates of FISH probes were first rotated using Principal Component Analysis (PCA) such that the length (PC1) and width (PC2) of each nucleus aligns with its PC1 (X) and PC2 (Y) axes. The size of each nucleus was then normalized to fit in a 4×2×1 (X*Y*Z, arbitrary units) cuboid using a min-max normalization. Probes mapping within equatorial (EQ, Cluster 1) and non-equatorial (non-EQ, Cluster 2) LAD clusters were selected and overlayed to generate **Fig. 7C**. Similarly, to correlate high-throughput FISH data with distance to centromeres, probes mapping to chromosome 4 (**Fig. 7D**) were selected and their distances to centromeres were calculated using UCSC chromosome banding data (**STable 1**).

Data access and code availability

Bed files corresponding to the genomic domains and datasets that we are uploading or previously have uploaded to 4DN can be found on the manuscript webpage on the 4DN website (*https://data.4dnucleome.org/belmont_lab_nuclear_locale*).

The K562 LMNA, LBR, and LMNA/LBR DamID-seq, Repli-seq, and RNA-seq raw and processed data has been deposited to GEO (GSE263012). The locale imaging data and the corresponding segmentations can be found on Illinois Data Bank (*https://doi.org/10.13012/B2IDB-9792611_V1*). The code used to generate figures in this manuscript, along with processed input and output files can be found have been reposited to the Illinois Data Bank (*https://doi.org/10.13012/B2IDB-4383352_V1*).

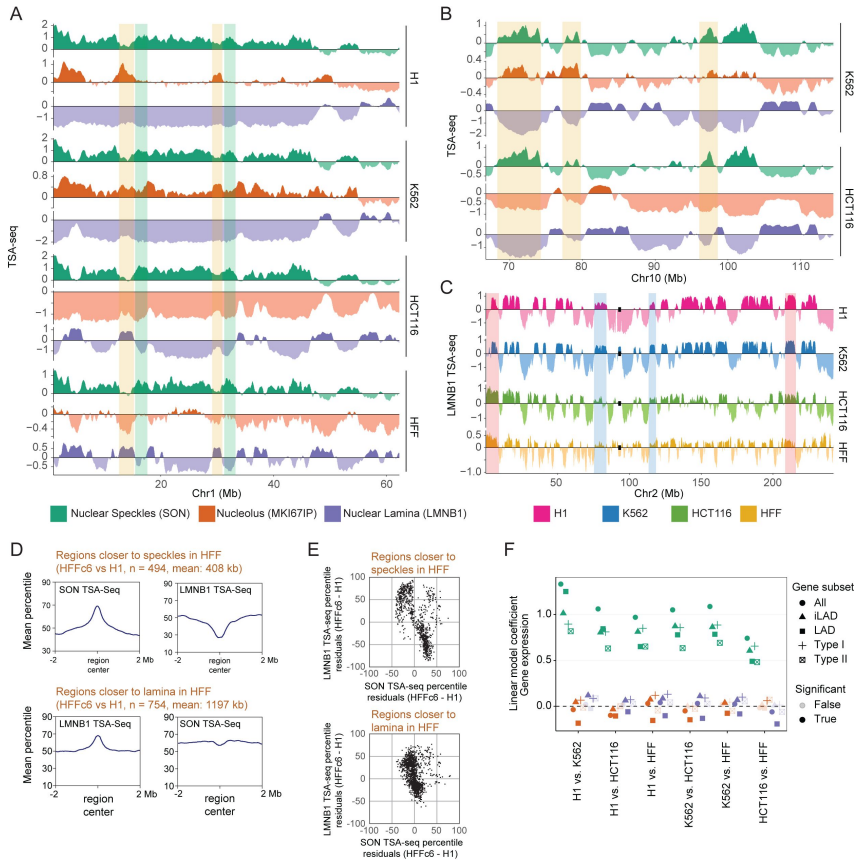
Supplementary Figures



Supplementary Figure 1

Comparisons of nuclear and nuclear body morphologies and the intranuclear relative positioning of nuclear bodies across four cell types

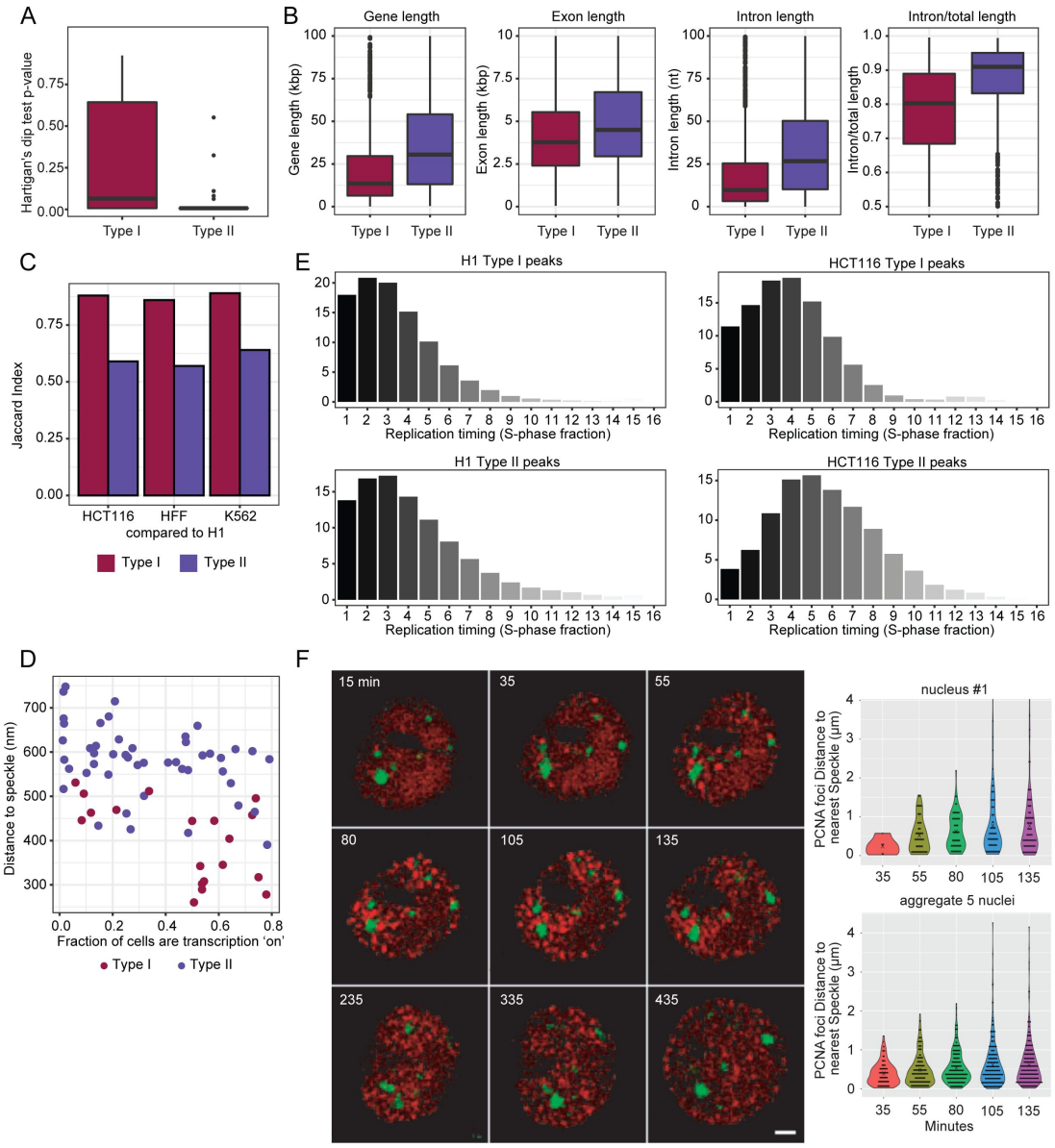
(A) Nuclear periphery segmentation was achieved by first segmenting the anti-RL1 nuclear pore signal, which approximated point sources, followed by fitting alpha-shapes to define the convex hull fitting this staining. We chose nuclear pore rather than nuclear lamina staining to mitigate the “missing cone” of spatial frequency along the optical axis which has the effect of attenuating signals from flat surfaces such as the nuclear lamina top and bottom surfaces [51]; (**B**) Schematic illustrating asymmetric distance metric: for example, NP (nuclear periphery) to NS (nuclear speckle) distance measurements refer to the nearest distance to a nuclear speckle from each NP surface point, whereas NS to NP distance measurements refer to the nearest distance to the NP from NS; (**C**) Inter-locale distance distributions-pairwise distances between NP, NS, and NUC (nucleolus)-compared across cell types; (**D**) Scatterplot comparisons between cell types (H1, red; K562, blue; HCT116, green, HFF, yellow) of different morphological metrics (Volume, vol; Surface Area, SA; Distance, dist; Flatness; Nucleolus, Nuc.; Nuclear speckle, NS; Nuclear periphery, NP); each scatterplot dot represents measurements from a single nucleus. (**E**) Percentage of explained variances (y-axis) for PC1-6 (x-axis) using Principal Component Analysis (PCA) of 14 morphological features from the immunofluorescence (IF) staining; (**F**) Principal Component (PC) 1 (x-axis) versus PC3 (y-axis) scatterplot reveals overall morphological similarity of H1 (red) and K562 (blue) nuclei with extensive overlap in both PC1 and PC3, with HCT116 and HFF nuclei varying from H1 and K562 in their lower PC1 values and HCT116 nuclei varying in their higher PC3 values from the nuclei in the other three cell lines. Each point in the scatterplot represents a cell in principal component space. Arrow length shows the quality of representation and arrow direction show the loadings on PC1 and PC2.



Supplementary Figure 2

Global changes in nuclear genome intranuclear positioning and their correlations with changes in gene expression and DNA replication timing

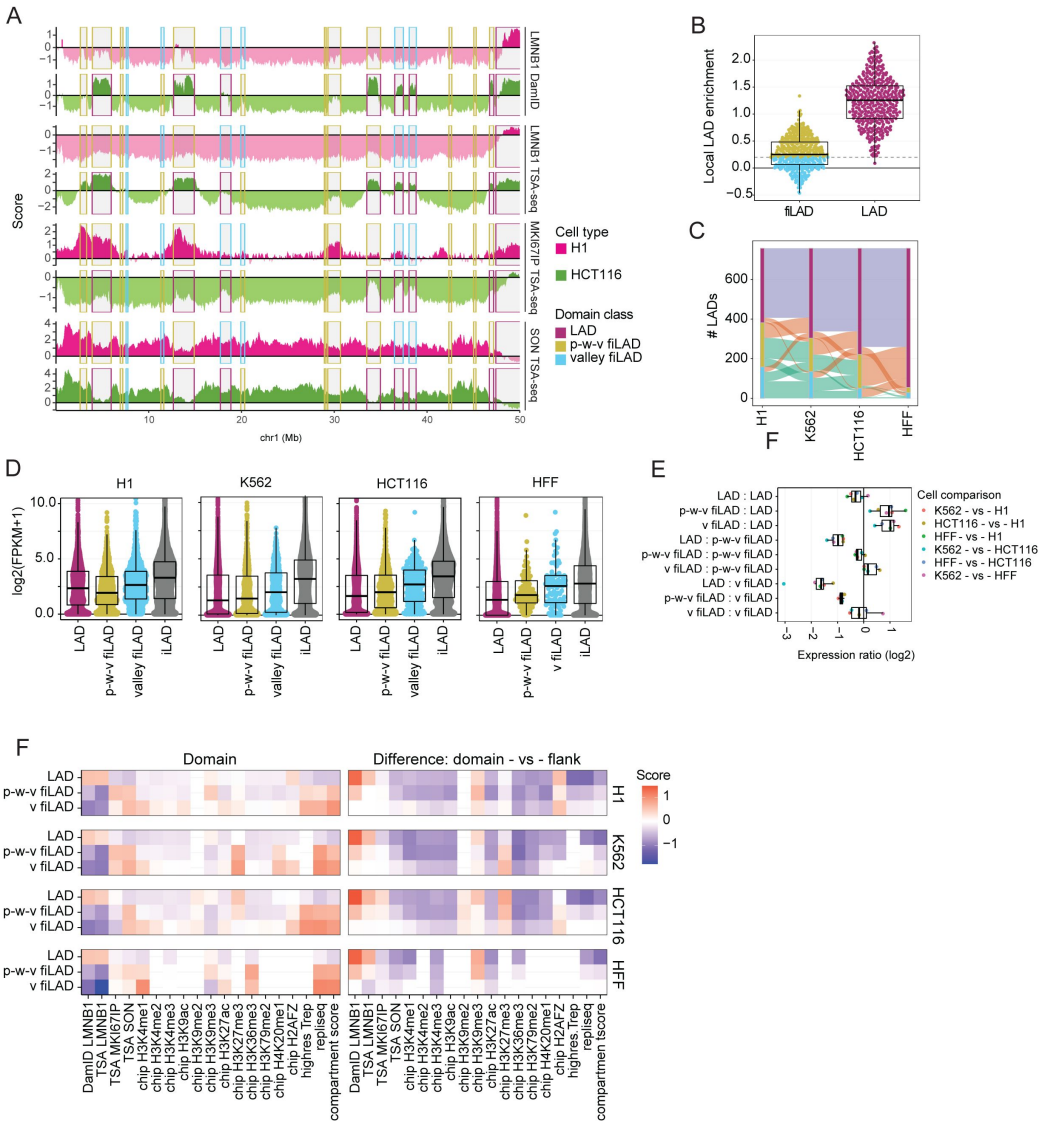
(**A**) Chr1 left arm (0-60 Mbp) showing heterochromatin chromatin domains (orange highlights) which associate near nucleoli and away from the nuclear lamina in H1 and K562 cells but near the nuclear lamina in HCT116 and HFF cells. These heterochromatin regions are flanked by speckle-associated regions (green highlights) in all cell lines, suggesting a Chr1 left arm trajectory alternating between speckles and nucleoli in H1 and K562 cells versus between speckles and the lamina in HCT116 and HFF cells. Top to bottom: SON (green), MKI67IP (brown), and LMNB1 (blue) TSA-seq repeated for H1, K562, HCT116, and HFF cells; (**B**) Nuclear speckle-associated regions align with nucleolar TSA-seq peaks in K562 cells but nucleolar TSA-seq valleys in HCT116 cells. Top to bottom: SON (green), MKI67IP (brown), and LMNB1 (blue) TSA-seq repeated for K562 versus HCT116 cells; (**C**) Chromosome-wide pattern of increased LMNB1 TSA-seq peak amplitudes of LADs towards the ends of long chromosome arms (orange highlights) versus decreased peak amplitudes for centromere-proximal LADs (blue highlights); these differences are enhanced in HCT116 and HFF versus H1 and K562 cells. Top to bottom: Chr2 LMNB1 TSA-seq for H1 (red), K562 (blue), HCT116 (green), HFF (orange) over Chr2; (**D**) Regions shifted closer to nuclear speckles in HFF versus H1 cells on average are shifted away from the nuclear lamina, but not vice versa. Top: Peak in the summed SON TSA-seq signals over chromosome regions with significantly higher SON TSA-seq values in HFF versus H1 cells correlates with a valley in the summed LMNB1 TSA-seq signals for these same regions. Bottom: peak in summed LMNB1 TSA-seq for regions significantly closer to the nuclear lamina in HFF versus H1 cells but little change in summed SON TSA-seq for these same regions; (**E**) Scatterplots showing overall inverse relationship between changes in SON versus LMNB1 TSA-seq in H1 versus HFF cells for chromosome regions with different positions relative to nuclear speckles (top) but small changes in SON TSA-seq for chromosome regions with different positions relative to nuclear lamina (bottom). Top-percentile-normalized SON versus LMNB1 TSA-seq for genomic bins from chromosome regions shifting closer to nuclear speckles in HFF cells. Despite an overall inverse correlation trend, there is a widespread in changed LMNB1 scores. Additionally, some bins show positive correlation between SON and LMNB1 TSA-seq. Bottom-Corresponding scatterplot for regions that shift significantly closer to nuclear lamina in HFF cells shows small changes in SON TSA-seq; (**F**) Linear modeling of changes in gene expression versus z-normalized TSA-seq score changes reveals significantly larger coefficients (dependence) for changes in SON (green) versus MKI67IP (orange) or LMNB1 (blue) TSA-seq, as modeled for genes in all genomic regions, in LADs only, in iLADs only, or in Type I versus II SON TSA-seq local maxima. “True” versus “False” tests for statistical significance are at a p=0.05 threshold.



Supplementary Figure 3

Varying gene composition, DNA replication timing, and speckle proximity of Type I and II SON TSA-seq peaks which align both with gene expression "hot-zones" and DNA replication initiation zones (IZs)

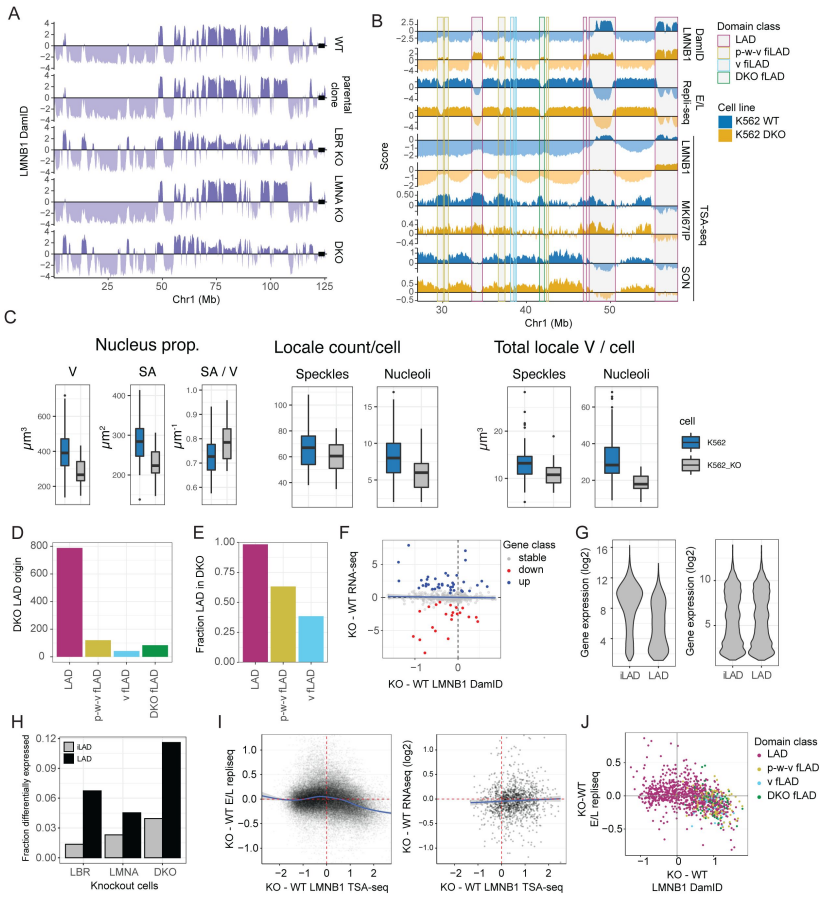
(**A**) Boxplots of p-value of multimodality for Type I and II peaks (The Hartigan's dip test) supports the unimodal versus bimodal distance distributions (**Fig. 3B**) of Type I versus II SON TSA-seq peaks, respectively, from nuclear speckles; (**B**) Boxplots showing different gene composition for Type I versus II SON TSA-seq peaks. Left to right: Gene length, exon length, intron length, and intro length/ exon length ratio; (**C**) Jaccard index shows greater conservation for Type I (red) versus II (blue) SON TSA-Seq peaks across the four cell lines; (**D**) Fraction of gene alleles transcriptionally active (x-axis) versus mean distance to nuclear speckles (y-axis) for Type I (red) versus II (blue) peaks, identified in HFF cells, from IMR90 cell FISH data [43] shows higher "ON" fraction for Type I genes closer to nuclear speckles; (**E**) Repli-seq fraction distribution of Type I (top) versus II (bottom) SON TSA-seq peaks in H1 (left) and HCT116 (right); (**F**) Live-cell imaging reveals earliest PCNA replication foci cluster near nuclear speckles in HCT116 cells, consistent with Type I SON TSA-seq peaks corresponding to IZs with the earliest replication timing. Left: Mid-nuclear optical z-sections at varying times (mins) after S-phase initiation defined by first appearance of PCNA foci showing PCNA foci (red, mCherry-PCNA) versus nuclear speckles (green, EGFP-SON); Right: Boxplots showing PCNA foci distance (y-axis) distributions from nuclear speckles at different times (mins) after S-phase began (x-axis). Top: statistics for nucleus 1; Bottom: statistics for foci from 5 nuclei. "X"= median. Scale bar = 2 μm.



Supplementary Figure 4

Facultative LADs transition most frequently to a partially repressed, middle-to-late replicating iLAD and less frequently to an active, early replicating iLAD in different cell types

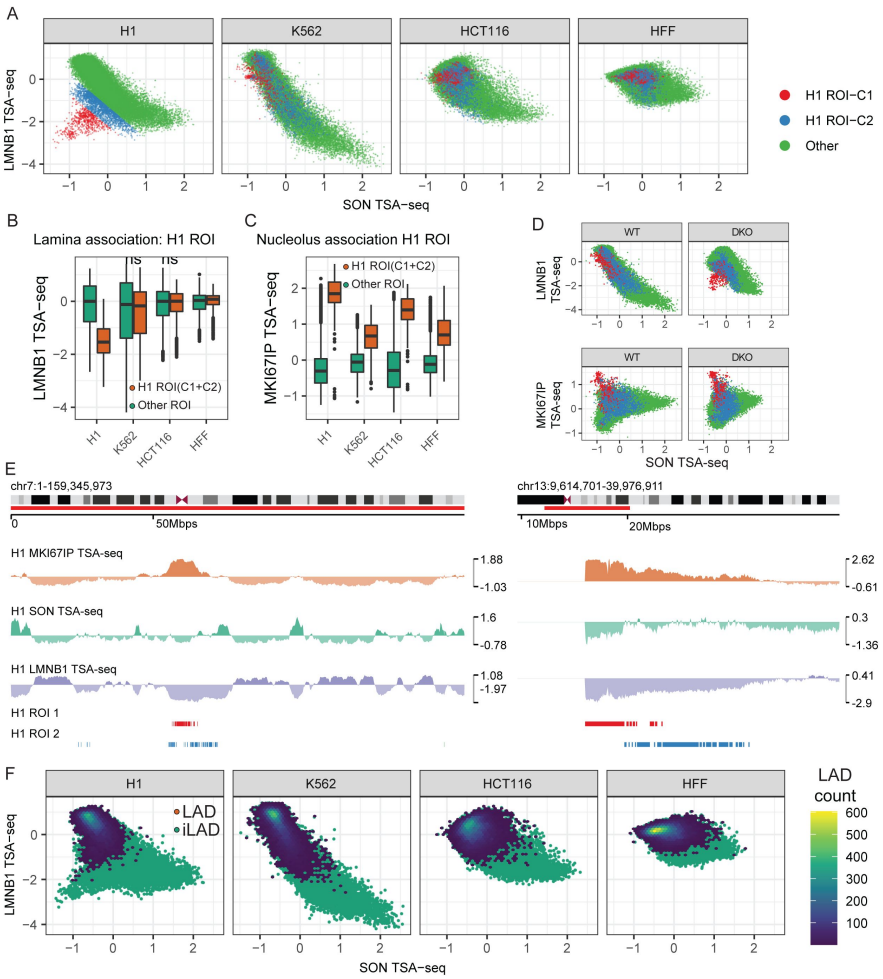
(**A**) Chr1 distal left arm in H1 (red tracks) or HCT116 (green tracks) with examples of LADs (red rectangles) with peaks in LMNB1 DamID signals (top) in one cell type changing in other cell type to facultative iLADs (fiLADs) with either valley (“v”) (blue rectangles) or peak-within-valley (“p-w-v”) (yellow/orange rectangles) LMNB1 DamID signal (top). p-w-v fiLADs appear either as peak-within-valleys or valleys in the LMNB1 TSA-seq (2nd from top). LADs appear as peak-within-valleys whereas p-w-v fiLADs appear frequently as peaks in MKI67IP (nucleolar) TSA-seq (3rd from top). SON TSA-seq (bottom) over these regions show local minimums which often are deeper for LADs versus p-w-v fiLADs; (**B**) Local differences in LMNB1 DamID (y-axis) over chromosome domain versus flanking regions are shown for fiLADs (left) versus LADs (right, red); a cutoff corresponding to the lowest 5% of LADs was used to segment v-fiLADs (blue) (below this cutoff) from p-w-v fiLADs (olive); (**C**) LAD numbers (y-axis) and their transitions to p-w-v fiLADs (olive) or v-fiLADs (blue) between H1, K562, HCT116, and HFF cell types; (**D**) Comparisons of gene expression levels in different domain types in the same and different cell lines. LADs and p-w-v fiLADs show similar ranges of expression levels, with median expression actually slightly lower in p-w-v fiLADs versus LADs in H1 cells. Compared to p-w-v fiLAD genes, genes in v-fiLADs show a higher range of gene expression levels which in turn is lower than the range of gene expression levels in iLADs. (**E**) Boxplots for ratios of gene expression levels (log2((FKPM(cell type 1) +1)/FKPM(cell type 2)+1)) for genes in one type of chromosome domain in one cell type versus another type of chromosome domain in a second cell type; (**F**) Heat map summary of DamID, TSA-seq, ChIP-seq, and Repli-seq signals over different chromatin domain types (left) and the relative signal differences comparing domains with their flanking regions.



Supplementary Figure 5

LADs shift towards nuclear interior but peak-within-valley (p-w-v) fiLADs shift towards the nuclear lamina and replicate later after LMNA/LBR double knockout (DKO)

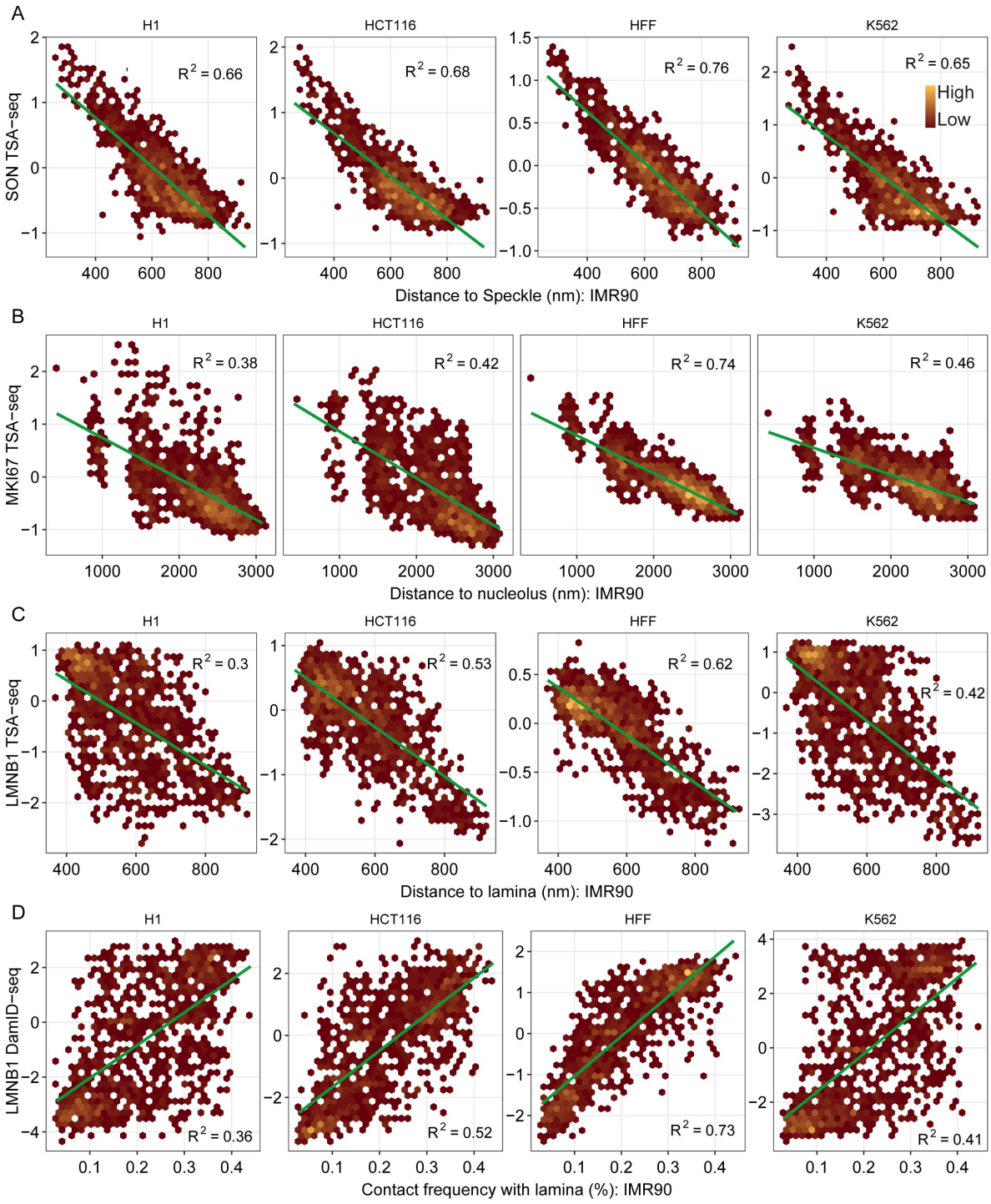
(A) Comparisons over Chr1 left arm between: (Top to bottom) LMNB1 DamID signals in wildtype (wt) K562 cells, parental clone expressing CRISPR Cas9 fused to a destabilization domain, LBR knockout (KO) clone, LMNA KO clone, and LBR/LMNA DKO clone. Black box at right end of plots shows centromere region. (**B**) Comparisons between wt (blue tracks) and DKO (orange tracks) over left arm Chr1 for: (Top to bottom) LMNB1 DamID, 2-fraction Repli-seq (Early / Late (E/L) ratio, and LMNB1, MKI67IP, and SON TSA-seq. Rectangles highlight specific domain classes in wt K562 defined by comparison of cell types (see text and **Fig. 4**) (LADs, red; p-w-v fiLADs, orange; v-fiLADs, blue). Also shown are "DKO fLADs" (green rectangles), a domain class defined as wt iLADs, not previously defined by cell type comparisons, which become LADs in the DKO; (**C**) Comparison of nuclear and nuclear locale morphologies measured by microscopy for K562 versus K562 DKO. (**D**) Numbers of LADs in DKO that arise from wt LADs, p-w-v fiLADs, and v-fiLADs, and "DKO fLADs" which arise from wt iLADs; (**E**) Fraction of wt LADs, p-w-v fiLADs, and v-fiLADs that are LADs in DKO; (**F**) No trend in LAD gene expression changes as a function of increased or decreased nuclear lamin association: scatterplot of gene expression differences (log2-ratio FKPM, y-axis) versus differences in LMNB1 DamID (x-axis). Only active LAD genes are shown (blue, upregulated; red, downregulated; grey, no change); (**G**) Generation of a matched set of iLAD genes. LAD genes are expressed at lower expression levels than iLAD genes (left panel). To compare LAD and iLAD genes, we selected a matching set of iLAD genes with similar gene expression to the set of LAD genes (right panel). This was repeated 100 times for a robust result; (**H**) Fractions (y-axis) of differentially expressed genes in iLADs (grey) versus LADs (black) (defined in wt cells) in LBR KO (left), LMNA KO (middle), or DKO (right); (**I**) (Left) Differences in E/L Repli-seq ratios (y-axis) versus changes in LMNB1 TSA-seq for individual genomic bins corresponding to LADs in wt and/or DKO cells. Curve fitting through data points reveals a trend for later DNA replication timing for LAD genomic bins which shift closer to nuclear lamina in DKO; (Right) Scatterplot showing log2-ratios of gene expression levels (y-axis) versus changes in LMNB1 TSA-seq for individual genes in LADs within wt and/or DKO cells. No consistent trend for changes in gene expression in DKO versus wt cells is observed; **(J)** Scatterplot comparing mean changes over chromosome domains in E/L 2-fraction Repli-seq (DKO – wt) (y-axis) versus changes in LMNB1 Dam-ID (DKO – wt) (x-axis) further supports trend towards later DNA replication timing for LADs, p-w-v fiLADs, and v-fiLADs which show increased association with nuclear lamina in DKO (similar to **Fig. 5H** comparing E/L Repli-seq to LMNB1 TSA-seq).



Supplementary Figure 6

LMNB1 and SON TSA-seq reveal differential intranuclear spatial segregation of centromeric and pericentromeric regions and LADs in different cell types

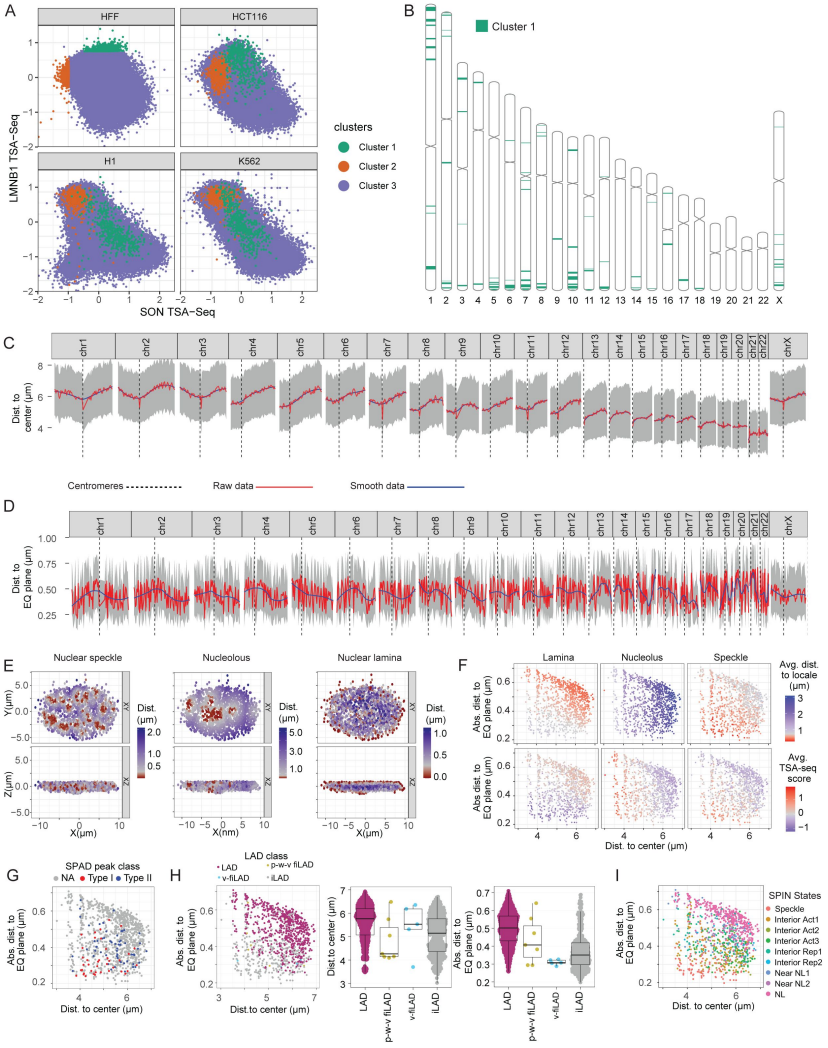
Further dividing LMNB1 versus SON TSA-seq scatterplot off-diagonal genomic bins into two Region-of-Interests (ROIs) reveals two subsets of genomic regions with low SON TSA-seq which lose nuclear lamina interactions specifically in H1 cells- the furthest off-diagonal ROI-C1 bins correspond to pericentric chromosome regions, particularly enriched on NOR-containing chromosomes, while ROI-C2 bins correspond to more distal chromosome regions flanking centromere regions. (**A**) An overall inverse relationship between LMNB1 and SON TSA-seq exists in both H1 and K562 cells creating a diagonal pattern in the LMNB (y-axis) versus SON (x-axis) TSA-seq scatterplot. Specifically in H1 cells, off-diagonal bins with low SON TSA-seq but also low LMNB1 TSA-seq appear. These were further divided into two H1 ROIs (C1, orange; C2, blue) relative to other genomic regions (green). These H1 ROI-C1 and ROI-C2 regions map to local clusters that lie within the overall LMNB1 versus SON scatterplot patterns in K562, HCT116, and HFF cells; (**B-C**) H1 C1 and C2 ROIs associate with both nucleoli and nuclear lamina in other cell lines but lose nuclear lamina association while gaining stronger nucleolar association in H1 cells: LMNB1 (**B**) and MKI67IP (nucleolus) (**C**) TSA-seq boxplots for H1 ROI-C1 and C2 (orange) bins versus other genomic regions (green); (**D**) (Top) The LMNB1 (y-axis) versus SON (x-axis) TSA-seq scatterplot on-diagonal positions of H1 C1 and C2 ROIs in wt K562 cells (left) shift closer to the off-diagonal distribution visualized in H1 cells after LBR, LMNA double-knockout (KO) (right); (Bottom) MKI67IP (y-axis) versus SON (x-axis) TSA-seq scatterplots reveal these H1 C1 ROIs show high nucleolar associations in both wt and double knockout K562 cells; (**E**) Centromeric and pericentromeric locations of H1 ROI C1 (red), C2 (blue) segmented regions (bottom 2 tracks) shown in H1 cells for non-NOR containing Chr7 (left) and NOR-containing Chr13 (right) regions with MKI67IP (brown), SON (green), LMNB1 (blue) TSA-seq (top 3 tracks) showing high nucleolar and low nuclear speckle and lamina interactions; (**F**) Majority of LAD bins have high LMNB1 TSA-seq scores in H1 and K562 (round cells), but medium scores in HCT116 and HFF (flat cells). Distribution of LAD (blue) versus iLAD (green) genomic bins with LAD count superimposed over corresponding bins (color-coded) in LMNB1 (y-axis) and SON (x-axis) TSA-seq scatterplots for H1, K562, HCT116, and HFF cells (left to right).



Supplementary Figure 7

IMR90 fibroblast MERFISH data serves as a good proxy for HFF fibroblast genomic data. (A-C)

Scatterplots showing comparison of IMR90 FISH probe microscopic distances (x-axes) to speckles (SON) (A), lamina (LMNB1) (B), or nucleoli (MKI67IP) (C) TSA-seq scores (y-axes) from H1, HCT116, HFF, or K562 cells. The highest correlations are seen comparing the IMR90 distance measurements with HFF TSA-seq data. (**D**) Similar scatterplot comparisons of IMR90 contact frequencies (0-1) (x-axis) with H1, HCT116, HFF, or K562 DamID scores (y-axis). The highest correlation with the IMR90 FISH data (Su et al., 2020) is for the human fibroblast TSA-seq and DamID data.



Supplementary Figure 8

Polarity of nuclear genome organization

(A) HFF Cluster 1 LAD bins (green), defined by their lowest SON TSA-seq scores, show low to moderate LMNB1 TSA-seq scores in both HFF and HCT116. In contrast, HFF Cluster 2 LAD bins, defined as LAD regions with the highest LMNB1 TSA-seq scores in HFF, while also showing high LMNB1 TSA-scores in HCT116 instead show lower LMNB1 TSA-seq scores relative to HFF Cluster 1 LAD bins in both K562 and H1 cells: SON (x-axis) versus LMNB1 (y-axis) TSA-seq scatterplots color-coded by HFF Cluster 1 and 2 LADs shown for HFF, HCT116, H1, and K562 cells; (**B**) Chromosome ideograms showing location of HFF1 Cluster 1 LAD regions, which show a bias towards the equatorial plane in IMR90 cells, towards the ends of long chromosome arms; (**C-D**) Distances to IMR90 nucleus center (**C**) and equatorial plane (**D**) vary with chromosome position, particularly distance from centromeres (dashed vertical lines): IMR90 multiplex FISH probe distances [43] from center and equatorial plane along each chromosome. Grey-interquartile range of probe positions; blue-mean probe position distance; red-smoothed mean probe distance. Ends of long chromosome arms, and chromosome arm regions containing Cluster 1 LADs, show trend of localizing away from nuclear center but close to equatorial plane; **(E)** Projection of IMR90 FISH probe distances (color-coded) from nuclear speckles (left), nucleoli (middle), and nuclear periphery (right) from a single nucleus onto the x-y plane (top) or x-z plane (bottom); (**F**) Scatterplots of mean distance from equatorial plane (y-axis) and from nuclear center (x-axis) of FISH probes in IMR90 nuclei with the mean distance in IMR90 nuclei (top) or mean TSA-seq scores in HFF nuclei of same FISH probe regions relative to the nuclear lamina (left), nucleolus (middle), or nuclear speckle (right) (FISH probe distances from [43]); (**G-I**) Scatterplots similar to (F) but color-coded with the genomic classification of each FISH probe: type I/II SPADs (**G**), LADs, p-w-v fILADs, v-fiLADs, iLADs (**H**, left panel), SPIN states (**I**). (**H, middle and right panels**) Boxplots for IMR90 mean FISH probe distances to nuclear center (middle panel) or nuclear equatorial plane (right panel) for HFF LADs, p-w-v fiLADs, v-fiLADs, and iLAD regions.

Acknowledgements

This work was supported by the National Institutes of Health Common Fund 4D Nucleome Program grants U54DK107965 (A.S.B., B.v.S., D.M.G., and J.M.) and UM1HG011593 (J.M., A.S.B., and D.M.G.). Research at the Netherlands Cancer Institute is supported by an institutional grant of the Dutch Cancer Society and of the Dutch Ministry of Health, Welfare and Sports. The Oncode Institute is partially funded by the Dutch Cancer Society.

Additional information

Author contributions

OG, TvS, YW, PK, LZ, YZ are co-first authors based on their contributions described below. This study was a highly collaborative group effort between the laboratories of ASB, BvS, DMG, and JM as a research center during the of NIH's 4D-Nucleome's consortium phase I. The analysis performed here were led by two PhD students, OG and TvS from the laboratories of ASB and BsV, respectively. They organized weekly online meetings among all the trainees with monthly guidance from the corresponding authors.

Data gathering: OG (HCT116 and K562) and PK (H1, HFF and K562-LMNA/LBR DKO) performed the immunofluorescence staining of locales and collected images. LZ (HCT116, H1 and HFF) and PK (K562, H1 and HFF) performed LMNB1 TSA-seq experiments. PK performed and processed MKI67IP TSA-seq experiments. PK performed MKI67IP, LMNB1 and SON TSA-seq in K562 WT and K562-LMNA/LBR DKO cell lines. TvS performed LMNB1 DamID experiments. AEV performed the early/late repli-seq in K562-LMNA/LBR DKO cell lines. YZ and YW curated and performed quality control on RNA- and ChIP-seq datasets from external sources. They also created the infrastructure needed for organization and hosting of data generated for this study and external data for trainees to use.

Hypothesis generation and analysis: All authors contributed to hypothesis generation with the following specifics. OG created software for the segmentation and the analysis of nuclear locales microscopy data (**Fig. 1** and **SFig. 1**). TvS performed most of the analysis for **Fig. 2** and **SFig. 2**. LZ performed analysis for **Fig. 2C** and **SFig. 2D-E**. YW performed most of the analysis for **Fig. 3** and **SFig. 3**. PAZ performed the initial analysis for **Fig. 3F**, which was then reproduced by YW. GAHG performed live-cell imaging and the analysis of related to **SFig. 3F**. Initial idea for p-w-v fiLADS (**Fig. 4**) was developed by PK and ASB which was then realized in **Fig. 4** and **SFig. 4** by analysis done by TvS. Analysis in **Fig. 5** and **SFig. 5** were done by TvS. Analysis in **Fig. 6** and **SFig. 6** were done by OG. Analysis in **Fig. 7** and **SFig. 8** were done by OG and TvS. Comparison of TSA-seq and IMR90 multiplexed FISH data in **Fig. 7** and **SFig. 7** was done by PK, YZ, and OG. Ideas behind **Fig. 8** were developed by OG and ASB and was realized by OG.

Writing: Original draft was written by OG, TvS, YW, and ASB. Later drafts and final manuscript were written by OG and ASB with inputs from others.

Additional files

Supplemental Table 1

Note

This reviewed preprint has been updated to amend the co-authorship.

References

Alexander KA, Cote A, Nguyen SC, Zhang L, Gholamalamdari O, Agudelo-Garcia P, Lin-Shiao E, Tanim KMA, Lim J, Biddle N, Dunagin MC, Good CR, Mendoza MR, Little SC, Belmont A, Joyce EF, Raj A, Berger SL 2021) **p53 mediates target gene association with nuclear speckles for amplified RNA expression** *Mol Cell* **81**:1666–1681 https://doi.org/10.1016/j.molcel.2021.03.006

Belmont AS 2022) **Nuclear Compartments: An Incomplete Primer to Nuclear Compartments, Bodies, and Genome Organization Relative to Nuclear Architecture** *Cold Spring Harb Perspect Biol* **14** https://doi.org/10.1101/cshperspect.a041268

Belmont AS, Bignone F, Ts'o PO 1986) **The relative intranuclear positions of Barr bodies in XXX non-transformed human fibroblasts** *Exp Cell Res* **165**:165–79

Bernhard W, Granboulan N 1963) **The Fine Structure of the Cancer Cell Nucleus** *Exp Cell Res* **24**:19–53 https://doi.org/10.1016/0014-4827(63)90243-x

Bian Q, Khanna N, Alvikas J, Belmont AS 2013) **. beta-Globin cis-elements determine differential nuclear targeting through epigenetic modifications** *J Cell Biol* **203**:767–83 https://doi.org/10.1083/jcb.201305027

Bickmore WA 2013) **The spatial organization of the human genome** *Annu Rev Genomics Hum Genet* **14**:67–84 https://doi.org/10.1146/annurev-genom-091212-153515

Brinkman EK, Chen T, Amendola M, van Steensel B. 2014) **Easy quantitative assessment of genome editing by sequence trace decomposition** *Nucleic Acids Research* **42**:e168 https://doi.org/10.1093/nar/gku936

Brinkman EK, Chen T, de Haas M, Holland HA, Akhtar W, van Steensel B. 2018) **Kinetics and Fidelity of the Repair of Cas9-Induced Double-Strand DNA Breaks** *Mol Cell* **70**:801–813 https://doi.org/10.1016/j.molcel.2018.04.016

Chen J, Ding L, Viana MP, Lee H, Sluezwski MF, Morris B, Hendershott MC, Yang R, Mueller IA, Rafelski SM 2020) **The Allen Cell and Structure Segmenter: a new open source toolkit for segmenting 3D intracellular structures in fluorescence microscopy images** *bioRxiv* https://doi.org/10.1101/491035

Chen Y, Zhang Y, Wang Y, Zhang L, Brinkman EK, Adam SA, Goldman R, van Steensel B, Ma J, Belmont AS. 2018) **Mapping 3D genome organization relative to nuclear compartments using TSA-Seq as a cytological ruler** *J Cell Biol* **217**:4025–4048 https://doi.org/10.1083/jcb.201807108

Consortium 4D Nucleome, Dekker J, Oksuz BA, Zhang Y, Ye Wang, Minsk MK, Kuang S, Yang L, Gibcus JH, Krietenstein N, Rando OJ, Xu J, Janssens DH, Henikoff S, Kukalev A, Willemin A, Winick-Ng W, Kempfer R, Pombo A, Yu M, Kumar P, Zhang L, Belmont AS, Sasaki T, Schaik T van, Brueckner L, Peric-Hupkes D, Steensel B van, Wang P, Chai H, Kim M, Ruan Y, Ran Zhang, Quinodoz SA, Bhat P, Guttman M, Zhao W, Chien S, Liu Y, Venev SV, Plewczynski D, Azcarate II, Szabó D, Thieme CJ, Szczepińska T, Chiliński M, Sengupta K, Conte M, Esposito A, Abraham A, Ruochi Zhang, Yuchuan Wang, Wen X, Wu Q, Yang Y, Liu J, Boninsegna L, Yildirim A, Zhan Y, Chiariello AM, Bianco S, Lee L, Hu M, Li Y, Barnett RJ, Cook AL, Emerson DJ, Marchal C, Zhao P,

Park P, Alver BH, Schroeder A, Navelkar R, Bakker C, Ronchetti W, Ehmsen S, Veit A, Gehlenborg N, Wang T, Li D, Wang X, Nicodemi M, Ren B, Zhong S, Phillips-Cremins JE, Gilbert DM, Pollard KS, Alber F, Ma J, Noble WS, Yue F. 2024) **An integrated view of the structure and function of the human 4D nucleome** *bioRxiv* https://doi.org/10.1101/2024.09.17.613111

Cremer T, Cremer C 2006) **Rise, fall and resurrection of chromosome territories: a historical perspective** *Part I. The rise of chromosome territories. Eur J Histochem* **50**:161–76

Cremer T, Cremer M, Hubner B, Strickfaden H, Smeets D, Popken J, Sterr M, Markaki Y, Rippe K, Cremer C 2015) **The 4D nucleome: Evidence for a dynamic nuclear landscape based on co-aligned active and inactive nuclear compartments** *FEBS Lett* **589**:2931–43 https://doi.org/10.1016/j.febslet.2015.05.037

Croft JA, Bridger JM, Boyle S, Perry P, Teague P, Bickmore WA 1999) **Differences in the localization and morphology of chromosomes in the human nucleus** *J Cell Biol* **145**:1119–31

Edelsbrunner H, Kirkpatrick D, Seidel R 1983) **On the shape of a set of points in the plane** *IEEE Transactions on Information Theory* **29**:551–559 https://doi.org/10.1109/TIT.1983.1056714

Falk M, Feodorova Y, Naumova N, Imakaev M, Lajoie BR, Leonhardt H, Joffe B, Dekker J, Fudenberg G, Solovei I, Mirny LA 2019) **Heterochromatin drives compartmentalization of inverted and conventional nuclei** *Nature* **570**:395–399 https://doi.org/10.1038/s41586-019-1275-3

Fischer EG 2020) **Nuclear Morphology and the Biology of Cancer Cells** *Acta Cytol* **64**:511–519 https://doi.org/10.1159/000508780

Girelli G, Custodio J, Kallas T, Agostini F, Wernersson E, Spanjaard B, Mota A, Kolbeinsdottir S, Gelali E, Crosetto N, Bienko M 2020) **GPSeq reveals the radial organization of chromatin in the cell nucleus** *Nat Biotechnol* **38**:1184–1193 https://doi.org/10.1038/s41587-020-0519-y

Guelen L, Pagie L, Brasset E, Meuleman W, Faza MB, Talhout W, Eussen BH, de Klein A, Wessels L, de Laat W, van Steensel B. 2008) **Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions** *Nature* **453**:948–51

Hartigan JA, Hartigan PM 1985) **The Dip Test of Unimodality** *The Annals of Statistics* **13**:70–84 https://doi.org/10.1214/aos/1176346577

Kim JH, Baddoo MC, Park EY, Stone JK, Park H, Butler TW, Huang G, Yan X, Pauli-Behn F, Myers RM, Tan M, Flemington EK, Lim ST, Ahn EY 2016) **SON and Its Alternatively Spliced Isoforms Control MLL Complex-Mediated H3K4me3 and Transcription of Leukemia-Associated Genes** *Mol Cell* **61**:859–73 https://doi.org/10.1016/j.molcel.2016.02.024

Kind J, Pagie L, Ortabozkoyun H, Boyle S, de Vries SS, Janssen H, Amendola M, Nolen LD, Bickmore WA, van Steensel B. 2013) **Single-cell dynamics of genome-nuclear lamina interactions** *Cell* **153**:178–92 https://doi.org/10.1016/j.cell.2013.02.028

Kolbl AC, Weigl D, Mulaw M, Thormeyer T, Bohlander SK, Cremer T, Dietzel S 2012) **The radial nuclear positioning of genes correlates with features of megabase-sized chromatin domains** *Chromosome Res* **20**:735–52 https://doi.org/10.1007/s10577-012-9309-9

Kumar P, Gholamalamdari O, Zhang Y, Zhang L, Vertii A, van Schaik T, Peric-Hupkes D, Sasaki T, Gilbert DM, van Steensel B, Ma J, Kaufman PD, Belmont AS. 2024) **Nucleolus and centromere**

Tyramide Signal Amplification-Seq reveals variable localization of heterochromatin in different cell types *Commun Biol* **7**:1–18 https://doi.org/10.1038/s42003-024-06838-7

Kupper K, Kolbl A, Biener D, Dittrich S, von Hase J, Thormeyer T, Fiegler H, Carter NP, Speicher MR, Cremer T, Cremer M. 2007) **Radial chromatin positioning is shaped by local gene density, not by gene expression** *Chromosoma* **116**:285–306

Leemans C, van der Zwalm MCH, Brueckner L, Comoglio F, van Schaik T, Pagie L, van Arensbergen J, van Steensel B. 2019) **Promoter-Intrinsic and Local Chromatin Features Determine Gene Repression in LADs** *Cell* **177**:852–864 https://doi.org/10.1016/j.cell.2019.03.009

Lieberman-Aiden E, van Berkum NL, Williams L, Imakaev M, Ragoczy T, Telling A, Amit I, Lajoie BR, Sabo PJ, Dorschner MO, Sandstrom R, Bernstein B, Bender MA, Groudine M, Gnirke A, Stamatoyannopoulos J, Mirny LA, Lander ES, Dekker J. 2009) **Comprehensive mapping of long-range interactions reveals folding principles of the human genome** *Science* **326**:289–93

Marchal C, Sasaki T, Vera D, Wilson K, Sima J, Rivera-Mulia JC, Trevilla-García C, Nogues C, Nafie E, Gilbert DM 2018) **Genome-wide analysis of replication timing by next-generation sequencing with E/L Repli-seq** *Nat Protoc* **13**:819–839 https://doi.org/10.1038/nprot.2017.148

Misteli T 2020) **The Self-Organizing Genome: Principles of Genome Architecture and Function** *Cell* **183**:28–45 https://doi.org/10.1016/j.cell.2020.09.014

Nemeth A, Conesa A, Santoyo-Lopez J, Medina I, Montaner D, Peterfia B, Solovei I, Cremer T, Dopazo J, Langst G 2010) **Initial genomics of the human nucleolus** *PLoS Genet* **6**:e1000889 https://doi.org/10.1371/journal.pgen.1000889

Peric-Hupkes D, Meuleman W, Pagie L, Bruggeman SW, Solovei I, Brugman W, Graf S, Flicek P, Kerkhoven RM, van Lohuizen M, Reinders M, Wessels L, van Steensel B. 2010) **Molecular maps of the reorganization of genome-nuclear lamina interactions during differentiation** *Molecular cell* **38**:603–13 https://doi.org/10.1016/j.molcel.2010.03.016

Politz JC, Scalzo D, Groudine M 2013) **Something silent this way forms: the functional organization of the repressive nuclear compartment** *Annu Rev Cell Dev Biol* **29**:241–70 https://doi.org/10.1146/annurev-cellbio-101512-122317

Politz JCR, Scalzo D, Groudine M 2016) **The redundancy of the mammalian heterochromatic compartment** *Curr Opin Genet Dev* **37**:1–8 https://doi.org/10.1016/j.gde.2015.10.007

Quinodoz SA, Jachowicz JW, Bhat P, Ollikainen N, Banerjee AK, Goronzy IN, Blanco MR, Chovanec P, Chow A, Markaki Y, Thai J, Plath K, Guttman M 2021) **RNA promotes the formation of spatial compartments in the nucleus** *Cell* **184**:5775–5790 https://doi.org/10.1016/j.cell.2021.10.014

Quinodoz SA, Ollikainen N, Tabak B, Palla A, Schmidt JM, Detmar E, Lai MM, Shishkin AA, Bhat P, Takei Y, Trinh V, Aznauryan E, Russell P, Cheng C, Jovanovic M, Chow A, Cai L, McDonel P, Garber M, Guttman M 2018) **Higher-Order Inter-chromosomal Hubs Shape 3D Genome Organization in the Nucleus** *Cell* **174**:744–757 https://doi.org/10.1016/j.cell.2018.05.024

Rao SS, Huntley MH, Durand NC, Stamenova EK, Bochkov ID, Robinson JT, Sanborn AL, Machol I, Omer AD, Lander ES, Aiden EL 2014) **A 3D Map of the Human Genome at Kilobase Resolution Reveals Principles of Chromatin Looping** *Cell* **159**:1665–80 https://doi.org/10.1016/j.cell.2014.11.021

Ryba T, Hiratani I, Lu J, Itoh M, Kulik M, Zhang J, Schulz TC, Robins AJ, Dalton S, Gilbert DM 2010) **Evolutionarily conserved replication timing profiles predict long-range chromatin interactions and distinguish closely related cell types** *Genome Res* **20**:761–70 https://doi.org/10.1101/gr.099655.109

Schep R, Brinkman EK, Leemans C, Vergara X, van der Weide RH, Morris B, van Schaik T, Manzo SG, Peric-Hupkes D, van den Berg J, Beijersbergen RL, Medema RH, van Steensel B. 2021) **Impact of chromatin context on Cas9-induced DNA double-strand break repair pathway balance** *Molecular Cell* **81**:2216–2230 https://doi.org/10.1016/j.molcel.2021.03.032

Skinner BM, Johnson EE 2017) **Nuclear morphologies: their diversity and functional relevance** *Chromosoma* **126**:195–212 https://doi.org/10.1007/s00412-016-0614-5

Solovei I, Wang AS, Thanisch K, Schmidt CS, Krebs S, Zwerger M, Cohen TV, Devys D, Foisner R, Peichl L, Herrmann H, Blum H, Engelkamp D, Stewart CL, Leonhardt H, Joffe B 2013) **LBR and lamin A/C sequentially tether peripheral heterochromatin and inversely regulate differentiation** *Cell* **152**:584–98 https://doi.org/10.1016/j.cell.2013.01.009

Spracklin G, Abdennur N, Imakaev M, Chowdhury N, Pradhan S, Mirny LA, Dekker J 2023) **Diverse silent chromatin states modulate genome compartmentalization and loop extrusion barriers** *Nat Struct Mol Biol* **30**:38–51 https://doi.org/10.1038/s41594-022-00892-7

Su JH, Zheng P, Kinrot SS, Bintu B, Zhuang X 2020) **Genome-Scale Imaging of the 3D Organization and Transcriptional Activity of Chromatin** *Cell* **182**:1641–1659 https://doi.org/10.1016/j.cell.2020.07.032

Takizawa T, Meaburn KJ, Misteli T 2008) **The meaning of gene positioning** *Cell* **135**:9–13 https://doi.org/10.1016/j.cell.2008.09.026

van Koningsbruggen S, Gierlinski M, Schofield P, Martin D, Barton GJ, Ariyurek Y, den Dunnen JT, Lamond AI. 2010) **High-resolution whole-genome sequencing reveals that specific chromatin domains from most human chromosomes associate with nucleoli** *Mol Biol Cell* **21**:3735–48 https://doi.org/10.1091/mbc.E10-06-0508

van Schaik T, Vos M, Peric-Hupkes D, Hn Celie P, van Steensel B. 2020) **Cell cycle dynamics of lamina-associated DNA** *EMBO Rep* **21**:e50636 https://doi.org/10.15252/embr.202050636

van Steensel B, Belmont AS 2017) **Lamina-Associated Domains: Links with Chromosome Architecture, Heterochromatin, and Gene Repression** *Cell* **169**:780–791 https://doi.org/10.1016/j.cell.2017.04.022

Vertii A, Ou J, Yu J, Yan A, Pages H, Liu H, Zhu LJ, Kaufman PD 2019) **Two contrasting classes of nucleolus-associated domains in mouse fibroblast heterochromatin** *Genome Res* **29**:1235–1249 https://doi.org/10.1101/gr.247072.118

Wang Y. 2021) **Computational Methods for Integrative Analysis of Nuclear Genome Compartmentalization** Cernegie Mellon University

Wang Y, Zhang Y, Zhang R, van Schaik T, Zhang L, Sasaki T, Peric-Hupkes D, Chen Y, Gilbert DM, van Steensel B, Belmont AS, Ma J. 2021) **SPIN reveals genome-wide landscape of nuclear compartmentalization** *Genome Biol* **22**:36 https://doi.org/10.1186/s13059-020-02253-3

White EJ, Emanuelsson O, Scalzo D, Royce T, Kosak S, Oakeley EJ, Weissman S, Gerstein M, Groudine M, Snyder M, Schubeler D 2004) **DNA replication-timing analysis of human**

chromosome 22 at high resolution and different developmental states *Proc Natl Acad Sci U S A* **101**:17771–6 https://doi.org/10.1073/pnas.0408170101

Xiong X, Tasan I, Yang C, Zhang M, Hernandez Gonzalez GA, Liu S, Chaturvedi P, Belmont AS, Zhao H 2023) **Imaging Method Using CRISPR/dCas9 and Engineered gRNA Scaffolds Can Perturb Replication Timing at the HSPA1 Locus** *ACS Synth Biol* **12**:1424–1436 https://doi.org/10.1021/acssynbio.2c00433

Zhang L, Zhang Y, Chen Y, Gholamalamdari O, Wang Y, Ma J, Belmont AS 2020) **TSA-seq reveals a largely conserved genome organization relative to nuclear speckles with small position changes tightly correlated with gene expression changes** *Genome Res* https://doi.org/10.1101/gr.266239.120

Zhao PA, Sasaki T, Gilbert DM 2020) **High-resolution Repli-Seq defines the temporal choreography of initiation, elongation and termination of replication in mammalian cells** *Genome Biol* **21**:76 https://doi.org/10.1186/s13059-020-01983-8

Author information

Omid Gholamalamdari[#]
Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, United States
ORCID iD: 0000-0002-5773-1205

[#]These authors contributed equally to this work

Tom van Schaik[#]
Division of Gene Regulation and Oncode Institute, Netherlands Cancer Institute, Amsterdam, Netherlands

[#]These authors contributed equally to this work

Yuchuan Wang[#]
Ray and Stephanie Lane Computational Biology Department, School of Computer Science, Carnegie Mellon University, Pittsburgh, United States

[#]These authors contributed equally to this work

Pradeep Kumar[#]
Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, United States

[#]These authors contributed equally to this work

Liguo Zhang[#]
Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, United States

[#]These authors contributed equally to this work

Yang Zhang[#]
Ray and Stephanie Lane Computational Biology Department, School of Computer Science, Carnegie Mellon University, Pittsburgh, United States

[#]These authors contributed equally to this work

Gabriela A Hernandez Gonzalez
Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, United States

Athanasios E Vouzas
San Diego Biomedical Research Institute, San Diego, United States

Peiyao A Zhao
San Diego Biomedical Research Institute, San Diego, United States

David M Gilbert
San Diego Biomedical Research Institute, San Diego, United States
ORCID iD: 0000-0001-8087-9737

For correspondence: gilbert@sdbri.org

Jian Ma
Ray and Stephanie Lane Computational Biology Department, School of Computer Science, Carnegie Mellon University, Pittsburgh, United States
ORCID iD: 0000-0002-4202-5834

For correspondence: jianma@cs.cmu.edu

Bas van Steensel
Division of Gene Regulation and Oncode Institute, Netherlands Cancer Institute, Amsterdam, Netherlands
ORCID iD: 0000-0002-0284-0404

For correspondence: b.v.steensel@nki.nl

Andrew S Belmont
Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, United States, Center for Biophysics and Quantitative Biology, University of Illinois at Urbana-Champaign, Urbana, United States, Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, United States
ORCID iD: 0000-0002-6540-0801

For correspondence: asbel@illinois.edu

Editors

Reviewer #2 (Public review):

Summary:

Golamalamdari, van Schaik, Wang, Kumar Zhang, Zhang and colleagues study interactions between the speckle, nucleolus and lamina in multiple cell types (K562, H1, HCT116 and HFF). Their datasets define how interactions between the genome and the different nuclear landmarks relate to each other and change across cell types. They also identify how these relationships change in K562 cells in which LBR and LMNA are knocked out.

Strengths:

Overall, there are a number of datasets that are provided, and several "integrative" analyses performed. This is a major strength of the paper, and I imagine the datasets will be of use to the community to further probed and the relationships elucidated here further studied. An especially interesting result was that specific genomic regions (relative to their association with the speckle, lamina, and other molecular characteristics) segregate relative to the equatorial plane of the cell.

Weaknesses:

The experiments are primarily descriptive, and the cause-and-effect relationships are limited (though the authors do study the role of LMNA/LBR knockdown with their technologies).

https://doi.org/10.7554/eLife.99116.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***eLife Assessment***
>
> *(1) This is a valuable manuscript that successfully integrates several data sets to determine genomic interactions with nuclear bodies.*

In this paper we both challenge and/or revise multiple long-standing "textbook" models of nuclear genome organization while also revealing new features of nuclear genome organization. Therefore, we argue that the contributions of this paper extend well beyond "valuable". Specifically, these contributions include:

a. We challenge a several decades focus on the correlation of gene positioning relative to the nuclear lamina. Instead, through comparison of cell lines, we show a strong correlation of di4erences in gene activity with di4erences in relative distance to nuclear speckles in contrast

to a very weak correlation with di4erences in relative distance to the nuclear lamina. This inference of little correlation of gene expression with nuclear lamina association was supported by direct experimental manipulation of genome positioning relative to the nuclear lamina. Despite pronounced changes in relative distances to the nuclear lamina there was little change relative to nuclear speckles and little change in gene expression.

b. We similarly challenge the long-standing proposed functional correlation between the radial positioning of genes and gene expression. Here, and in a now published companion paper (doi.org/10.1038/s42003-024-06838-7), we demonstrate how nuclear speckle positioning relative to nucleoli and the nuclear lamina varies among cell types, as does the inverse relationship between genome positioning relative to nuclear speckles and the nuclear lamina. Again, this is consistent with the primary correlation of gene activity being the positioning of genes relative to nuclear speckles and also explains previous observations showing a strong relationship between radial position and gene expression only in some cell types.

c. We identified a new partially repressed, middle to late DNA replicating type of chromosome domain- “p-w-v fILADs”- by their weak interaction with the nuclear lamina, which, based on our LMNA/LBR KO experimental results, compete with LADs for nuclear lamina association. Moreover, we show that when fLADs convert to iLADs, most conversions are to this p-w-v fiLAD state, although ~ one third are to a normal, active, early replicating iLAD state. Thus, fLADs can convert between repressed, partially repressed, and active states, challenging the prevailing assumption of the division of the genome into two states – active, early replicating A compartment/iLAD regions versus inactive, late replicating, B compartment/LAD regions.

d. We identified nuclear speckle associated domains as DNA replication initiation zones, with the domains showing strongest nuclear speckle attachment initiating DNA replication earliest in S-phase.

e. We describe for the first time an overall polarization of nuclear genome organization in adherent cells with the most active, earliest replicating genomic regions located towards the equatorial plane and less expressed genomic regions towards the nuclear top or bottom surfaces. This includes polarization of some LAD regions to the nuclear lamina at the equatorial plane and other LAD regions to the top or bottom nuclear surfaces.

We have now rewritten the text to make the significance of these new findings clearer.

> *(2) Strength of evidence: The evidence supporting the central claims is varied in its strength ranging from solid to incomplete. Orthogonal evidence validating the novel methodologies with alternative approaches would better support the central claims.*

We argue that our work exploited methods, data, and analyses equal to or more rigorous than the current state-of-the-art. This indeed includes orthogonal evidence using alternative methods which both supported our novel methodologies as well as demonstrating their robustness relative to more conventional approaches. This explains how we were able to challenge/revise long-standing models and discover new features of nuclear genome organization. More specifically:

a. Unlike most previous analyses, we have integrated both genomic and imaging approaches to examine the nuclear genome organization relative to not one, but several di4erent nuclear locales and we have done this across several cell types. To our knowledge, this is the first such integrated approach and has been key to our success in appreciating new features of nuclear genome organization.

b. The 16-fraction DNA replication Repli-seq data we developed and applied to this project represents the highest temporal mapping of DNA replication timing to date.

c. The TSA-seq approach that we used remains the most accurate sequence-based method for estimating microscopic distance of chromosome regions to di4erent nuclear locales. As implemented, this method is unusually robust and direct as it exploits the exponential micron-scale gradient established by the di4usion of the free-radicals generated by peroxidase labeling to measure relative distances of chromosome regions to labeled nuclear locales. We had previously demonstrated that TSA-seq was able to estimate the average distances of genomic regions to nuclear speckles with an accuracy of ~50 nm, as validated by light microscopy. The TSA-seq 2.0 protocol we developed and applied to this project maintained the original resolution of TSA-seq to estimate to an accuracy of ~50 nm the average distances of genomic regions from nuclear speckles, as validated by light microscopy, while achieving more than a 10-fold reduction in the required number of cells.

We have rewritten the text to address the reviewer concerns that led them to their initial characterization of the TSA-seq as novel and not yet validated.

First, we have added a discussion of how the use of nuclear speckle TSA-seq as a “cytological ruler” was based on an extensive initial characterization of TSA-seq as described in previous published literature. In that previous literature we showed how the conventional molecular proximity method, ChIP-seq, instead showed local accumulation of the same marker proteins over short DNA regions unrelated to speckle distances. Second, we reference our companion paper, now published, and describe how the extension of TSA-seq to measure relative distances to nucleoli was further validated and shown to be robust by comparison to NAD-seq and extensive multiplexed immuno-FISH data. We further discuss how in the same companion paper we show how nucleolar DamID instead was inconsistent with both the NAD-seq and multiplexed immuno-FISH data as well as the nucleolar TSA-seq.

Third, we have added scatterplots showing exactly how highly the estimated microscopic distances to all three nuclear locales, measured in IMR90 fibroblasts, correlate with the TSA-seq measurements in HFF fibroblasts. This addresses the concern that we were not using the exact same fibroblast cell line for the TSA-seq versus microscopic measurements. The strong correlation already observed would only be expected to become even stronger with use of the exact same fibroblast cell lines for both measurements.

Fourth, we have addressed the reviewer concern that the nuclear lamin TSA-seq was not properly validated because it did not match nuclear lamin Dam-ID. We have now added to the text a more complete explanation of how microscopic proximity assays such as TSA-seq measure something di4erent from molecular proximity assays such as DamID or NAD-seq. We have added further explanation of how TSA-seq complements molecular proximity assays such as DamID and NAD-seq, allowing us to extract further information than either measurement alone. We also briefly discuss why TSA-seq succeeds for certain nuclear locales using multiple independent markers whereas molecular proximity assays may fail against the same nuclear locales using the same markers. This includes brief discussion from our own experience attempting unsuccessfully to use DamID against nucleoli and nuclear speckles.

> ***Reviewer #1 (Public Review):***
>
> *(1) The weakness of this study lies in the fact that many of the genomic datasets originated from novel methods that were not validated with orthogonal approaches, such as DNAFISH. Therefore, the detailed correlations described in this work are based on methodologies whose efficacy is not clearly established. Specifically, the authors*

> *utilized two modified protocols of TSA-seq for the detection of NADs (MKI67IP TSA-seq) and LADs (LMNB1-TSA-seq).*

We disagree with the statement that the TSA-seq approach and data has not been validated by orthogonal approaches. We have now addressed this point in the revised manuscript text:

a) We added text to describe how previously FISH was used to validate speckle TSA-seq by demonstrating a residual of ~50 nm between the TSA-seq predicted distance to speckles and the distance measured by light microscopy using FISH:

"In contrast, TSA-seq measures relative distances to targets on a microscopic scale corresponding to 100s of nm to ~ 1 micron based on the measured diffusion radius of tyramide-biotin free-radicals (Chen et al., 2018). Exploiting the measured exponential decay of the tyramide-biotin free-radical concentration, we showed how the mean distance of chromosomes to nuclear speckles could be estimated from the TSA-seq data to an accuracy of ~50 nm, as validated by FISH (Chen et al., 2018)."

b) We note that we also previously have validated lamina (Chen et al, JCB 2018) and nucleolar (Kumar et al, 2024) TSA-seq and further validated speckle TSA-seq (Zhang et al, Genome Research 2021) by traditional immuno-FISH and/or immunostaining. The overall high correlation between lamina TSA-seq and the orthogonal lamina DamID method was also extensively discussed in the first TSA-seq paper (Chen et al, JCB 2018). Included in this discussion was description of how the di4erences between lamina TSA-seq and DamID were expected, given that DamID produces a signal more proportional to contact frequency, and independent of distance from the nuclear lamina, whereas TSA-seq produces a signal that is a function of microscopic distance from the lamina, as validated by traditional FISH.

c) We added text to describe how the nucleolar TSA-seq previously was validated by two orthogonal methods- NAD-seq and multiplexed DNA immuno-FISH:

"We successfully developed nucleolar TSA-seq, which we extensively validated using comparisons with two different orthogonal genome-wide approaches (Kumar et al., 2024)-NAD-seq, based on the biochemical isolation of nucleoli, and previously published direct microscopic measurements using highly multiplexed immuno-FISH (Su et al., 2020)."

d) We have now added panels A&B to Fig. 7 and a new Supplementary Fig. 7 demonstrating further validation of TSA-seq based on showing the high correlation between the microscopically measured distances of many hundreds of genomic sites across the genome from di4erent nuclear locales and TSA-seq scores. As discussed in response #2 below, we have used comparison of distances measured in IMR90 fibroblasts with TSA-seq scores measured in HFF fibroblasts. We would argue therefore that these correlations are a lower estimate and therefore the correlation between microscopic distances and TSAseq scores would likely have been still higher if we had performed both assays in the exact same cell line.

> *(2) Although these methods have been described in a bioRxiv manuscript by Kumar et al., they have not yet been published. Moreover, and surprisingly, Kumar et al., work is not cited in the current manuscript, despite its use of all TSA-seq data for NADs and LADs across the four cell lines.*

The Kumar et al, Communications Biology, 2024 paper is now published and is cited properly in our revision. We apologize for this oversight and confusion our initial omission of this citation may have created. We had been writing this manuscript and the Kumar et al manuscript in parallel and had intended to co-submit. We planned to cross-reference the two at the time we co-submitted, adding the Kumar et al reference to the first version of this manuscript once we obtained a doi from bioRxiv. But we then submitted the Kumar et al

manuscript several months earlier, but meanwhile forgot that we had not added the reference to our first manuscript version.

> *(3) Moreover, Kumar et al. did not provide any DNA-FISH validation for their methods.*

As we described in our response to Reviewer 1's comment #1, we had previously provided traditional FISH validation of lamina TSA-seq in our first TSA- seq paper as well as validation by comparison with lamina DamID (Chen et al, 2018).

We also described how the nucleolar TSA-seq was extensively cross-validated in the Kumar et al, 2024 paper by both NAD-seq and the highly multiplexed immuno-FISH data from Su et al, 2020).

We note additionally that in the Kumar et al, 2024 paper the nucleolar TSA-seq was additionally validated by correlating the predicted variations in centromeric association with nucleoli across the four cell lines predicted by nucleolar TSA-seq with the variations observed by traditional immunofluorescence microscopy.

> *(4) Therefore, the interesting correlations described in this work are not based on robust technologies.*

This comment was made in reference to the Kumar et al paper not having been published, and, as noted in responses to points #2 and #3, the paper is now published.

But we wanted to specifically note, however, that our experience is that TSA-seq has proven remarkably robust in comparison to molecular proximity assays. We've described in our responses to the previous points how TSA-seq has been cross-validated by both microscopy and by comparison with lamina DamID and nucleolar NAD-seq. We note also that in every application of TSA-seq to date, all antibodies that produced good immunostaining showed good TSA-seq results. Moreover, we obtained nearly identical results in every case in which we performed TSA-seq with different antibodies against the same target. Thus anti-SON and antiSC35 staining produced very similar speckle TSA-seq data (Chen et al, 2018), anti-lamin A and anti-lamin B staining produced very similar lamina TSA-seq data (Chen et al, 2018), antinucleolin and anti-POL1RE staining produced very similar DFC/FC nucleolar TSA-seq data (Kumar et al, 2024), and anti-MKI67IP and anti-DDX18 staining produced very similar GC nucleolar TSA-seq data (Kumar et al, 2024).

This independence of results with TSA-seq to the particular antibody chosen to label a target differs from experience with methods such as ChIP, DamID, and Cut and Run/Tag in which results can differ or be skewed based on variable distance and therefore reactivity of target proteins from the DNA or due to other factors such as non-specific binding during pulldown (ChIP) or differential extraction by salt washes (Cut and Tag).

Our experience in every case to date is that antibodies that produce similar immunofluorescence staining produce similar TSA-seq results. We attribute this robustness to the fact that TSA-seq is based only on the original immunostaining specificity provided by the primary and secondary antibodies plus the diffusion properties of the tyramide-free radical.

We've now added the following text to our revised manuscript:

"As previously demonstrated for both SON and lamin TSA-seq (Chen et al., 2018), nucleolar TSA-seq was also robust in the sense that multiple target proteins showing similar nucleolar staining showed similar TSA-seq results (Kumar et al., 2024); this robustness is intrinsic to TSA-seq being a microscopic rather than molecular proximity assay, and therefore not sensitive to the exact molecular binding partners and molecular distance of the target proteins to the DNA."

> *(5) An attempt to validate the data was made for SON-TSA-seq of human foreskin fibroblasts (HFF) using multiplexed FISH data from IMR90 fibroblasts (from the lung) by the Zhuang lab (Su et al., 2020). However, the comparability of these datasets is questionable. It might have been more reasonable for the authors to conduct their analyses in IMR90 cells, thereby allowing them to utilize MERFISH data for validating the TSA-seq method and also for mapping NADs and LADs.*

We disagree with the reviewer's overall assessment that that the use of the IMR90 data to further validate the TSA-seq is questionable because the TSA-seq data from HFF fibroblasts is not necessarily comparable with multiplexed immuno-FISH microscopic distances measured in IMR90 fibroblasts.

In response we have now added panels to Fig. 7 and Supplementary Fig. 7, showing:

a) There is very little di4erence in correlation between speckle TSA-seq and measured distances from speckles in IMR90 cells whether we use IMR90 or HFF cells SON TSA-seq data (R^2 = 0.81 versus 0.76) (new Fig. 7A).

b) There is also a high correlation between lamina (R^2 = 0.62) and nucleolar (R^2 = 0.73) HFF TSA-seq and measured distances in IMR90 cells. Thus, we conclude that this high correlation shows that the multiplexed data from ~1000 genomic locations does validate the TSA-seq. These correlations should be considered lower bounds on what we would have measured using IMR90 TSA-seq data. Thus, the true correlation between distances of loci from nuclear locales and TSA-seq would be expected to be either comparable or even stronger than what we are seeing with the IMR90 versus HFF fibroblast comparisons.

c) This correlation is cell-type specific (Fig. 7B, new SFig. 7). Thus, even for speckle TSAseq, highly conserved between cell types, the highest correlation of IMR90 distances with speckle TSA-seq is with IMR90 and HFF fibroblast data. For lamina and nucleolar TSA-seq, which show much lower conservation between cell types, the correlation of IMR90 distances is high for HFF data but much lower for data from the other cell types. This further justifies the use of IMR90 fibroblast distance measurements as a proxy for HFF fibroblast measurements.

Thus, we have added the following text to the revised manuscript:

"We reasoned that the nuclear genome organization in the two human fibroblast cell lines would be sufficiently similar to justify using IMR90 multiplexed FISH data [43] as a proxy for our analysis of HFF TSA-seq data. Indeed, the high inverse correlation (R= -0.86) of distances to speckles measured by MERFISH in IMR90 cells with HFF SON TSA-seq scores is nearly identical to the inverse correlation (R= -0.89) measured instead using IMR90 SON TSA-seq scores (Fig. 7A). Similarly, distances to the nuclear lamina and nucleoli show high inverse correlations with lamina and nucleolar TSA-seq, respectively (Fig. 7A). These correlations were cell type specific, particularly for the lamina and nucleolar distance correlations, as these correlations were reduced if we used TSA-seq data from other cell types (SFig. 7A). Therefore, the high correlation between IMR90 microscopic distances and HFF TSA-seq scores can be considered a lower bound on the likely true correlation, justifying the use of IMR90 as a proxy for HFF for testing our predictions."

> ***Reviewer #2 (Public Review):***
>
> *Weaknesses:*
>
> *(1) The experiments are largely descriptive, and it is difficult to draw many cause-andeffect relationships…The study would benefit from a clear and specific hypothesis.*

This study was hypothesis-generating rather than hypothesis-testing in its goal. Our research was funded through the NIH 4D-Nucleome Consortium, which had as its initial goal the development, benchmarking, and validation of new genomic technologies. Our Center focused on the mapping of the genome relative to different nuclear locales and the correlation of this intranuclear positioning of the genome with functions- specifically gene expression and DNA replication timing. By its very nature, this project took a discovery-driven versus hypothesis-driven scientific approach. Our question fundamentally was whether we could gain new insights into nuclear genome organization through the integration of genomic and microscopic measurements of chromosome positioning relative to multiple different nuclear compartments/bodies and their correlation with functional assays such as RNA-seq and Repliseq.

Indeed, this study resulted in multiple new insights into nuclear genome organization as summarized in our last main figure. We believe our work and conclusions will be of general interest to scientists working in the fields of 3D genome organization and nuclear cell biology. We anticipate that each of these new insights will prompt future hypothesis-driven science focused on specific questions and the testing of cause-and-effect relationships.

However, we do want to point out that our comparison of wild-type K562 cells with the LMNA/LBR double knockout was designed to test the long-standing model that nuclear lamina association of genomic loci contributes to gene silencing. This experiment was motivated by our surprising result that gene expression differences between cell lines correlated strongly with differences in positioning relative to nuclear speckles rather than the nuclear lamina. Despite documenting in these double knockout cells a decreased nuclear lamina association of most LADs, and an increased nuclear lamina association of the “p-w-v” fiLADs identified in this manuscript, we saw no significant change in gene expression in any of these regions as compared to wild-type K562 cells. Meanwhile, distances to nuclear speckles as measured by TSA-seq remained nearly constant.

We would argue that this represents a specific example in which new insights generated by our genomics comparison of cell lines led to a clear and specific hypothesis and the experimental testing of this hypothesis.

> *(2) Similarly, the paper would be very much strengthened if the authors provided additional summary statements and interpretation of their results (especially for those not as familiar with 3D genome organization).*

We appreciate this feedback and agree with the reviewer that this would be useful, especially for those not familiar with previous work in the field of 3D genome organization. In an earlier draft, we had included additional summary and interpretation statements in both the Introduction and Results sections. At the start of each Results section, we had also previously included brief discussion of what was known before and the context for the subsequent analysis contained in that section. However, we had thought we might be submitting to a journal with specific word limits and had significantly cut out that text.

We have now restored this text and, in certain cases, added additional explanations and context.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Figures 1C and D. Please add the units at the values of each y-axis.*

We have done that.

> *The representation of Figure 2C lacks clarity and is diJicult to understand. The x-axis labeling regarding the gene fraction number needs clarification.*

We've modified the text to the Fig. 2C legend: "Fraction of genes showing significant di=erence in relative positioning to nuclear speckles (gene fraction, x-axis) versus log2 (HFF FKPM / H1 FKPM) (y-axis);"

> *"We next used live-cell imaging to corroborate that chromosome regions close to nuclear speckles, primarily Type I peaks, would show the earliest DNA replication timing." This sentence requires modification as Supplementary Figure 3F does not demonstrate that Type I peaks exhibit the earliest DNA replication timing; it only indicates that the first PCNA foci in S-phase are in proximity to nuclear speckles.*

We've modified the text to: "We next used live-cell imaging to show that chromosome regions close to nuclear speckles show the earliest DNA replication timing; this is consistent with the earliest firing DNA replication IZs, as determined by Repli-seq, aligning with Type 1 peaks that are closely associated with nuclear speckles."

> *In Figure 5, the authors employed LaminB1-DamID to quantify LADs in LBR-KO and LMNA/LBR-DKO K562 cells. These are interesting results. However, for these experiments, it is crucial to assess LMNB1 signal at the nuclear periphery via immunofluorescence (IF) to confirm the absence of changes, ensuring that the DamID signal solely reflects contacts with the nuclear lamina. Furthermore, in this instance, their findings should be validated through DNA-FISH.*

Immunostaining of LMNB1 was performed and showed a normal staining pattern as a ring adjacent to the nuclear periphery. Images of this staining were included in the metadata tied to the sequencing data sets deposited on the 4D Nucleome Data portal. We thank the reviewer for bringing up this point, and have added a sentence mentioning this result in the Results Section:

"Immunostaining against LMNB1 revealed the normal ring of staining around the nuclear periphery seen in wt cells (images deposited as metadata in the deposited sequencing data sets)."

Because both TSA-seq and DamID have been extensively validated by FISH, as detailed in our previous responses to the public reviewer comments, we feel it is unnecessary to validate these findings by FISH.

> *p-w-v-fiLADs should be labelled in Figure 5B.*

We've added labeling as suggested.

> *"The consistent trend of slightly later DNA replication timing for regions (primarily p-w-v fiLADs) moving closer to the lamina" is not visible in the representation of the data of Figure 5G.*

We did not make a change as we believed this trend was apparent in the Figure.

> *To reduce the descriptive nature of the data, it would be pertinent to conduct H3K9me3 and H3K27me3 ChIP-seq analyses in both the parental and DKO mutant cells. This would elucidate whether p-w-v-fiLADs and NADs anchoring to the nuclear lamina undergo changes in their histone modification profile.*

We believe further analysis of the reasons underlying these shifts in positioning, including such ChIP-seq or equivalent analysis, is of interest but beyond the scope of this publication. We see such measurements as the beginning of a new story but insuJicient alone to determine mechanism. Therefore we believe such experiments should be part of that future study.

> *The description of Figure 7 lacks clarity. Additionally, it appears that TSA-seq for NADs and LADs may not be universally applicable across all cell types, particularly in flat cells, whereas DamID scores demonstrate less variation across cell lines, as also stated by the authors.*

TSA-seq is a complement to rather than a replacement for either DamID or NAD-seq. TSAseq reports on microscopic distances whereas both DamID and NAD-seq instead are more proportional to contact frequency with the nuclear lamina or nucleoli, respectively, and insensitive to distances of loci away from the lamina or nucleoli. Thus, TSA-seq provides additional information based on the intrinsic diJerences in what TSA-seq measures relative to molecular proximity methods such as DamID or NAD-seq. The entire point is that the convolution of the exponential point-spread-function of the TSA-seq with the shape of the nuclear periphery allows us to distinguish genomic regions in the equatorial plane versus the top and bottom of the nuclei. The TSA-seq is therefore highly "applicable" when properly interpreted in discerning new features of genome organization. As we stated in the revised manuscript, the lamina DamID and TSA-seq are complementary and provide more information together then either method along. The same is true for the NAD-seq and nucleolar TSA-seq comparison, as described in more detail in the Kumar, et al, 2024 paper.

> *Introduction:*
>
> *The list of methodologies for mapping genomic contacts with nucleoli (NADs) should also include recent technologies, such as Nucleolar-DamID (Bersaglieri et al., PMID: 35304483), which has been validated through DNA-FISH.*

We did not include nucleolar DamID in the mention in the Introduction of methods for identifying diJerential lamina versus nucleolar interactions of heterochromatin- either from our own collaborative group or from the cited reference- because we did not have confidence in the accuracy of this method in identifying NADs. In the case of the published nucleolar DamID from our collaborative group, published in Wang et al, 2021, we later discovered that despite apparent agreement of the nucleolar DamID with a small number of published FISH localization the overall correlation of the nucleolar DamID with nucleolar localization was poor. As described in detail in the Kumar et al, 2024 publication, this poor correlation of the nucleolar DamID was established using three orthogonal methods- nucleolar TSA-seq, NAD-seq, and multiplexed immuno-FISH measurements from ~1000 genomic locations. Instead, we found that this nucleolar DamID showed high correlation with lamina DamID. We note that many strong NADs are also LADs, which we think is why validation with only several FISH probes is inadequate to demonstrate overall validation of the approach.

We could not compare our nucleolar-DamID data in human cells with the alternative nucleolar-DamID results cited by the reviewer which were performed in mouse cells. We note that in this paper the nucleolar DamID FISH validation only included several putative NAD chromosome regions and, I believe, one LAD region. However, our initial comparison of the nucleolar DamID cited by the reviewer with unpublished TSA-seq data from mouse ESCs produced by the Belmont laboratory and with NAD-seq data from the Kaufman laboratory shows a similar lack of correlation of the nucleolar DamID signal with nucleolar TSA-seq and NAD-seq, as well as multiplexed immuno-FISH data from the Long Cai laboratory, as we saw in our analysis of own nucleolar DamID data in human cells.

We have added explanation concerning the lack of correlation of our nucleolar DamID with orthogonal measurements of nucleolar proximity in the added text (below) to our revised manuscript:

"Nucleolar DamID instead showed broad positive peaks over large chromatin domains, largely overlapping with LADs mapped by LMNB1 DamID (Wang et al., 2021). However, this nucleolar DamID signal, while strongly correlated with lamin DamID, showed poor correlation with either NAD-seq or nucleolar distances mapped by multiplexed immunoFISH (Kumar et al., 2024). We suspect the problem is that with molecular proximity assays the output signals are disproportionally dominated by the small fraction of target proteins juxtaposed in su=icient proximity to the DNA to produce a signal rather than the amount of protein concentrated in the target nuclear body. "

Our mention of nucleolar TSA-seq was in the context of why we focused on nucleolar TSAseq and excluded our own nucleolar DamID. We chose not to discuss the second nucleolar DamID method cited above 1) because it was not appropriate to our discussion of our own experimental approach and 2) also because we cannot yet make a definitive statement of its accuracy for nucleolar mapping.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) The authors start the manuscript by describing the 'radial genome organization' model and contrast it with the 'binary model' of genome organization. It would be helpful for the authors to contextualize their results a bit more with regard to these two diJerent models in the discussion.*

We have added several sentences in the first paragraph of the Discussion to accomplish this contextualization. The new paragraph reads:

"Here we integrated imaging with both spatial (DamID, TSA-seq) and functional (Repli-seq, RNA-seq) genomic readouts across four human cell lines. Overall, our results significantly extend previous nuclear genome organization models, while also demonstrating a cell-type dependent complexity of nuclear genome organization. Briefly, in contrast to the previous radial model of genome organization, we reveal a primary correlation of gene expression with relative distances to nuclear speckles rather than radial position. Additionally, beyond a correlation of nuclear genome organization with radial position, in cells with flat nuclei we show a pronounced correlation of nuclear genome organization with distance from the equatorial plane. In contrast to previous binary models of genome organization, we describe how both iLAD / A compartment and LAD / B compartment contain within them smaller chromosome regions with distinct biochemical and/or functional properties that segregate di=erentially with respect to relative distances to nuclear locales and geometry."

> *(2) Data should be provided demonstrating KO of LBR and LMNA - immunoblotting for both proteins would be one approach. In addition, it would be helpful to provide additional nuclear morphology measurements of the DKO cells (volume, surface area, volume of speckles/nucleoli, number of speckles/nucleoli).*

We've added additional description describing the generation and validation of the KO lines:

"To create LMNA and LBR knockout (KO) lines and the LMNA/LBR double knockout (DKO) line, we started with a parental "wt" K562 cell line, clone #17, expressing an inducible form of Cas9 (Brinkman et al., 2018). The single KO and DKO were generated by CRISPR-mediated frameshift mutation according to the procedure described previously (Schep et al., 2021). The "wt" K562 clone #17 was used for comparison with the KO clones.

The LBR KO clone, K562 LBR-KO #19, was generated, using the LBR2 oligonucleotide GCCGATGGTGAAGTGGTAAG to produce the gRNA, and validated previously, using TIDE (Brinkman et al., 2014) to check for frameshifts in all alleles as described elsewhere (Schep et al., 2021). The LMNA/LBR DKO, K562 LBR-LMNA DKO #14, was made similarly, starting with the LBR KO line and using the combination of two oligonucleotides to produce gRNAs:

LMNA-KO1: ACTGAGAGCAGTGCTCAGTG, LMNA-KO2: TCTCAGTGAGAAGCGCACGC.

Additionally, the LMNA KO line, K562 LMNA-KO #14, was made the same way but starting with the "wt" K562 cell line. Validation was as described above; additionally, for the new LMNA KO and LMNA/LBR DKO lines, immunostaining showed the absence of anti-LMNA antibody signal under confocal imaging conditions used to visualize the wt LMNA staining while the RNA-seq from these clones revealed an ~20-fold reduction in LMNA RNA reads relative to the wt K562 clone."

As suggested, we also added morphological data for the DKO line in a modified SFig.5.

> *(3) The rationale for using LMNB1 TSA-seq and LMNB1 DAMID is not immediately clear. The LMNB1 TSA-seq is more variable across cell types and replicates than the DAMID. Could the authors please compare the datasets a bit more to understand the diJerences? For example, the authors demonstrate that "40-70% of the genome shows statistically significant diJerences in Lamina TSA-seq over regions 100 kb or larger, with most of these regions showing little or no diJerences in speckle TSA-seq scores." If the LMNB1 DAMID data is used for this analysis or Figure 2D, is the same conclusion reached? Also, in Figure 6, the authors conclude that C1 and C3 LAD regions are enriched for constitutive LADs, while C2 and C4 LAD regions are fLADs. This is a bit surprising because the authors and others have previously shown that constitutive LADs have higher LMNB1 contact frequency than facultative LADs (Kind, et al Cell 2015, Figure 3C).*

Indeed, in the first TSA-seq paper (Chen et al, 2018) we did observe that cLADs had the highest LMNB TSA-seq scores; this was for K562 cells with round nuclei in which there is therefore no diJerence in lamina TSA-seq scores produced by nuclear shape over the entire nucleus.

However, there are diJerences between TSA-seq and DamID in terms of what they measure and we refer the reviewer to the first TSA-seq paper (Chen et al, 2018) that explains in greater depth these diJerences. This first paper explains how DamID is indeed related to contact frequency but how the TSA-seq instead estimates mean distances from the target, in this case the nuclear lamina. This is because the diJusion of tyramide free radicals from the site of their constant HRP production produces an exponential decay gradient of tyramide free radical concentration at steady state.

We have summarized these diJerences in in text we have added to introduce both DamID and TSA-seq in the second Results section:

"DamID is a well-established molecular proximity assay; DamID applied to the nuclear lamina divides the genome into lamina-associated domains (LADs) versus nonassociated “inter-LADs” or “iLADs” (Guelen et al., 2008; van Steensel and Belmont, 2017). In contrast, TSA-seq measures relative distances to targets on a microscopic scale corresponding to 100s of nm to ~ 1 micron based on the measured diJusion radius of tyramide-biotin free-radicals (Chen et al., 2018)... While LMNB1 DamID segments LADs most accurately, lamin TSA-seq provides distance information not provided by DamID- for example, variations in relative distances to the nuclear lamina of diJerent iLADs and iLAD regions. These diJerences between the LMNB1 DamID and LMNB TSA-seq signals are also crucial to a computational approach, SPIN, that segments the genome into multiple states based on their varying nuclear

localization, including biochemically and functionally distinct lamina-associated versus near-lamina states (Consortium et al., 2024; Wang et al., 2021).

Thus, lamin DamID and TSA-seq complement each other, providing more information together than either one separately."

We note that these diJerences in lamina DamID and TSA-seq are crucial to being able to gain additional information by comparing variations in the lamina TSA-seq for LADs in Figs. 6&7. See our response to point (4) below, for further explanation.

> *(4) In 7B/C, the authors show that the highest LMNB1 regions in HFF are equator of IMR90s. However, in Figure 7G, their cLAD score indicates that constitutive LADs are not at the equator. This is a bit surprising given the point above and raises the possibility that SON signals (as opposed to LMNB1 signals) might be more responsible for correlation to localization relative to the equator. Hence, it might be helpful if the authors repeat the analyses in Figures 7B/C in regions with diJering LMNB1 signals but similar SON signals (and vice versa).*

Again, this is based on the apparent assumption by the reviewer that DamID and TSA-seq work the same way and measure the same thing. But as explained above in the previous point, this is not true.

In our first TSA-seq paper (Chen et al, 2018) we showed how we could use the exponential decay point-spread-function produced by TSA, measured directly by light microscopy, to convert sequencing reads from the TSA-seq into a predicted mean distance from nuclear speckles, approximated as point sources. These mean distances predicted from the SON TSA-seq data agreed with measured FISH distances to nuclear speckles to within ~50 nm for a set of DNA probes from diJerent chromosome regions. Moreover, varying TSA staining conditions changed the decay constants of this exponential decay, thus producing diJerences in the SON TSA-seq signals. By using these diJerent exponential decay functions to convert the TSA-seq scores from these independent data sets to estimated distances from nuclear speckles, we again observed a distance residual of ~50 nm; in this case though this distance residual of ~50 nm represented the mean residual observed genome-wide. This gives us great confidence that the TSA-seq is working as we have modeled it.

As we mentioned in our response to point 3 above, we did see the highest LMNB TSA-seq signal for cLADs in K562 cells with round nuclei (Chen et al, 2018).

But as we now show in our simulation performed in this paper for Fig. 7, the observed tyramide free radical exponential decay gradient convolved with the flat nuclear lamina shape produces a higher equatorial LMNB1 TSA-seq signal for LADs at the equatorial plane. We confirmed that LADs with this higher TSA-seq signal were enriched at the equatorial plane by mining the multiplexed IMR90 imaging data. Similar mining of the multiplexed FISH IMR90 data showed localization of cLADs away from the equatorial plane.

We are not clear about the rationale for what the reviewer is suggesting about SON signals "being more responsible for correlation to localization to the equator". We have provided an explanation for the higher lamina TSA-seq scores for LADs near the equator based on the measured spreading of the tyramide free radicals convolved with the eJect of the nuclear shape. This makes a prediction that the observed variation in lamina TSA-seq scores for LADs with similar DamID scores is related to their positioning relative to the equatorial plane as we then validated through our mining of the IMR90 multiplexed FISH data.

> *(5) FISH of individual LADs, v-fiLADs, and p-w-v-fiLADs relative to the lamina and speckle would be helpful to understand their relative positioning in control and LBR/LMNA double KO cells. This would significantly bolster the claim that "histone mark*

> *enrichments..more precisely revealed the diJerential spatial distribution of LAD regions...".*

Adequately testing these predictions made from the lamina/SON TSA-seq scatterplots by direct FISH measurements would require measurements from large numbers of diJerent chromosome regions through a highly multiplexed immuno-FISH approach. We are not equipped currently in any of our laboratories to do such measurements and we leave this therefore for future studies.

Rather our statement is based on our use of TSA-seq analyzed through these 2D scatterplots and should be valid to the degree that our TSA-seq measurements do indeed correlate with microscopy derived distances.

However, we do now include demonstration of a high correlation of speckle, lamina, and nucleolar TSA-seq with highly multiplexed immuno-FISH measurement of distances to these locales in a revised Fig. 7. The high correlation shown between the TSA-seq scores and measured distances does therefore add additional support to our claim that the reviewer is discussing, even without our own multiplexed FISH validation.

> *(6) "In contrast, genes within genomic regions which in pair-wise comparisons of cell lines show a statistically significant diJerence in lamina TSA-seq show no obvious trend in their expression diJerences (Figure 2C).". This appears to be an overstatement based on the left panel of 2D.*

We do not follow the reviewer's point. In Fig. 2C we show little bias in the diJerences in gene expression between the two cell types for regions that showed diJerences in lamina TSA-seq. The reviewer is suggesting something otherwise based on their impression, not explicitly stated, of the left panel of Fig. 2D. But we see similar shades of blue extending vertically at low SON values and similar shades of red extending vertically at high SON values, suggesting a correlation of gene expression only with the SON TSA-seq score but not with the LMNB1 TSA-seq score displayed on the y-axis. This is also consistent with the very small and/or insignificant correlation coeJicients measured in our linear model relating diJerences in LMNB1 TSA-seq to diJerences in expression but the large correlation coeJicient observed for SON TSA-seq (Fig. 2E). Thus, we see Fig. 2C-E as self-consistent.

> *(7) In the section on "Polarity of Nuclear Genome Organization" - "....Using the IMR90 multiplexed FISH data set [43]...." - The references are not numbered.*

We thank the reviewer for this correction.

> *(8) I believe there is an error in the Figure 7B legend. The descriptions of Cluster 1 and 2 do not match those indicated in the figure.*

We again thank the reviewer for this correction.

https://doi.org/10.7554/eLife.99116.2.sa0