

## H3K9me2 orchestrates inheritance of spatial positioning of peripheral heterochromatin through mitosis

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## Abstract

**Cell-type-specific 3D organization of the genome is unrecognizable during mitosis. It remains unclear how essential positional information is transmitted through cell division such that a daughter cell recapitulates the spatial genome organization of the parent. Lamina-associated domains (LADs) are regions of repressive heterochromatin positioned at the nuclear periphery that vary by cell type and contribute to cell-specific gene expression and identity. Here we show that histone 3 lysine 9 dimethylation (H3K9me2) is an evolutionarily conserved, specific mark of nuclear peripheral heterochromatin and that it is retained through mitosis. During mitosis, phosphorylation of histone 3 serine 10 temporarily shields the H3K9me2 mark allowing for dissociation of chromatin from the nuclear lamina. Using high-resolution 3D immuno-oligoFISH, we demonstrate that H3K9me2-enriched genomic regions, which are positioned at the nuclear lamina in interphase cells prior to mitosis, re-associate with the forming nuclear lamina before mitotic exit. The H3K9me2 modification of peripheral heterochromatin ensures that positional information is safeguarded through cell division such that individual LADs are re-established at the nuclear periphery in daughter nuclei. Thus, H3K9me2 acts as a 3D architectural mitotic guidepost. Our data establish a mechanism for epigenetic memory and inheritance of spatial organization of the genome.**

## 1    **Introduction**

2    In order for a dividing cell of a given lineage to maintain its identity, it must pass along to its  
3    progeny not only a complete copy of its genome, but also the memory of its specific cellular  
4    identity (Buchwalter et al., 2019, Towbin et al., 2013, Amendola and van Steensel, 2014). It is  
5    well appreciated that the spatial arrangement of the genome inside the nucleus contributes to  
6    regulation of cell-fate choices and differentiation (Peric-Hupkes et al., 2010, Phillips-Cremins et  
7    al., 2013). However, the mechanistic underpinnings of how the blueprint for cell-type-specific  
8    nuclear architecture is transmitted from mother to daughter cells in order to maintain cell identity  
9    remain poorly understood (Dekker et al., 2017).

10   The chromatin in eukaryotic cells is organized both structurally and functionally into subnuclear  
11   compartments (Towbin et al., 2013, Kohwi et al., 2013, Stadhouders et al., 2019) and recent  
12   developments in super-resolution microscopy (Cremer et al., 2017, Ricci et al., 2017),  
13   chromosome capture methods (Dekker et al., 2002, Dekker et al., 2013), and chromatin  
14   immunoprecipitation (ChIP) (Collas, 2010, Kubben et al., 2010) have greatly increased our  
15   understanding of 3D nuclear architecture (Naumova et al., 2013). Separation of transcriptionally  
16   active and inactive chromatin in three-dimensional space reinforces efficient regulation of gene  
17   expression and maintains silencing of heterochromatic loci (reviewed in (Andrey and Mundlos,  
18   2017, Buchwalter et al., 2019, Amendola and van Steensel, 2014, Bickmore, 2013)). This is  
19   illustrated by examples of aberrant gene expression patterns that occur upon disruption of  
20   topological domains and, in extreme cases, are associated with oncogenic transformation (Andrey  
21   and Mundlos, 2017, Flavahan et al., 2016). Heterochromatin is segregated into spatially distinct  
22   subnuclear compartments including peripherally located lamina-associated domains (LADs)  
23   (Guelen et al., 2008), which encompass approximately 30-40% of the genome (Peric-Hupkes et  
24   al., 2010, Poleshko et al., 2017). Multiple examples in mammalian cell types indicate that proper  
25   positioning of LADs contributes to cell-type-specific gene expression (Peric-Hupkes et al., 2010,  
26   Poleshko et al., 2017, Robson et al., 2016). Likewise, in *Drosophila*, competence of neuroblasts to  
27   respond to inductive signals depends upon stage-specific reorganization of peripheral  
28   heterochromatin (Kohwi et al., 2013), and muscle differentiation in *Caenorhabditis elegans*  
29   requires anchoring of heterochromatin to the nuclear periphery (Gonzalez-Sandoval et al., 2015).  
30   These findings, combined with the observation that many developmental and lineage-specific  
31   genes reside in LADs, suggests a key role for peripheral heterochromatin in establishment and

32 maintenance of cellular identity (Zullo et al., 2012, Poleshko et al., 2017, Peric-Hupkes et al.,  
33 2010). LADs are defined by their interaction with the nuclear lamina which is disassembled during  
34 cell division, posing a conundrum as to how cell-type specific LADs are remembered through  
35 mitosis.

36 The molecular mechanisms by which LADs are established and maintained at the nuclear  
37 periphery remain poorly understood. For example, there does not appear to be a clear targeting  
38 sequence that localizes areas of the genome to the nuclear periphery (Zullo et al., 2012, Meuleman  
39 et al., 2013). However, histone post-translational modifications have been implicated in LAD  
40 regulation. Proline Rich Protein 14 (PRR14) has been shown to recognize H3K9me3, found on  
41 both peripheral and nucleoplasmic heterochromatin, through an interaction with HP1 (Poleshko et  
42 al., 2013). In addition, work from our group and others has demonstrated a specific enrichment for  
43 H3K9me2 at the nuclear periphery, raising the possibility of a regulatory role in LAD positioning  
44 (Poleshko et al., 2017, Kind et al., 2013). CEC-4, a *C. elegans* chromodomain-containing protein,  
45 localizes to the nuclear periphery and has been shown to be a reader of H3K9 methylated chromatin  
46 (Gonzalez-Sandoval et al., 2015). Depletion studies using RNAi and loss-of-function mutants  
47 demonstrated that CEC-4 is required for peripheral heterochromatin anchoring but not  
48 transcriptional repression. While not all of the tethering complexes and molecular determinants  
49 responsible for the interaction of heterochromatin with the nuclear lamina have been determined,  
50 it is clear that these associations must be disrupted upon mitotic entry when the nuclear envelope  
51 breaks down and the chromosomes condense. Furthermore, these interactions must be precisely  
52 re-established upon mitotic exit when the cell reforms an interphase nucleus.

53 Entry into mitosis involves eviction of proteins, including RNA polymerase and many  
54 transcription factors, and reorganization of chromosomes into their characteristic metaphase form  
55 (Naumova et al., 2013). Remarkably, at mitotic exit, cell-type-specific chromatin architecture,  
56 transcription factor binding, and gene expression are re-established (reviewed in (Oomen and  
57 Dekker, 2017, Palozola et al., 2019, Hsiung and Blobel, 2016, Probst et al., 2009, Festuccia et al.,  
58 2017)). While both interphase nuclear architecture and post-mitotic restoration of transcription  
59 factor association with the genome have been extensively studied (Palozola et al., 2019, Kadauke  
60 and Blobel, 2013), our understanding of how cell-type-specific genome organization including  
61 LADs is restored in daughter cells after mitosis is less well developed.

62

63 Pioneering studies in the 1980s revealed the necessity for DNA in the process of nuclear lamina  
64 reassembly after mitosis, and the activity of kinases and phosphatases were implicated in  
65 mediating interactions between lamin and chromosomes (Foisner and Gerace, 1993, Newport,  
66 1987, Burke and Gerace, 1986, Gerace and Blobel, 1980), although the mechanistic explanation  
67 for the dependence of reassembly on chromatin has been unclear. Here, we utilize high resolution,  
68 single-cell imaging and oligopaints to simultaneously track 82 LAD and non-LAD genomic loci  
69 through mitosis. We show that the H3K9me2 modification of nuclear lamina-associated  
70 heterochromatin, revealed upon dephosphorylation of H3S10 at mitotic exit, provides a 3D spatial  
71 guidepost for genomic regions that are to be re-localized to the nuclear periphery following mitosis  
72 and that the nuclear lamina of daughter cells reassembles around the exposed H3K9me2 mark.

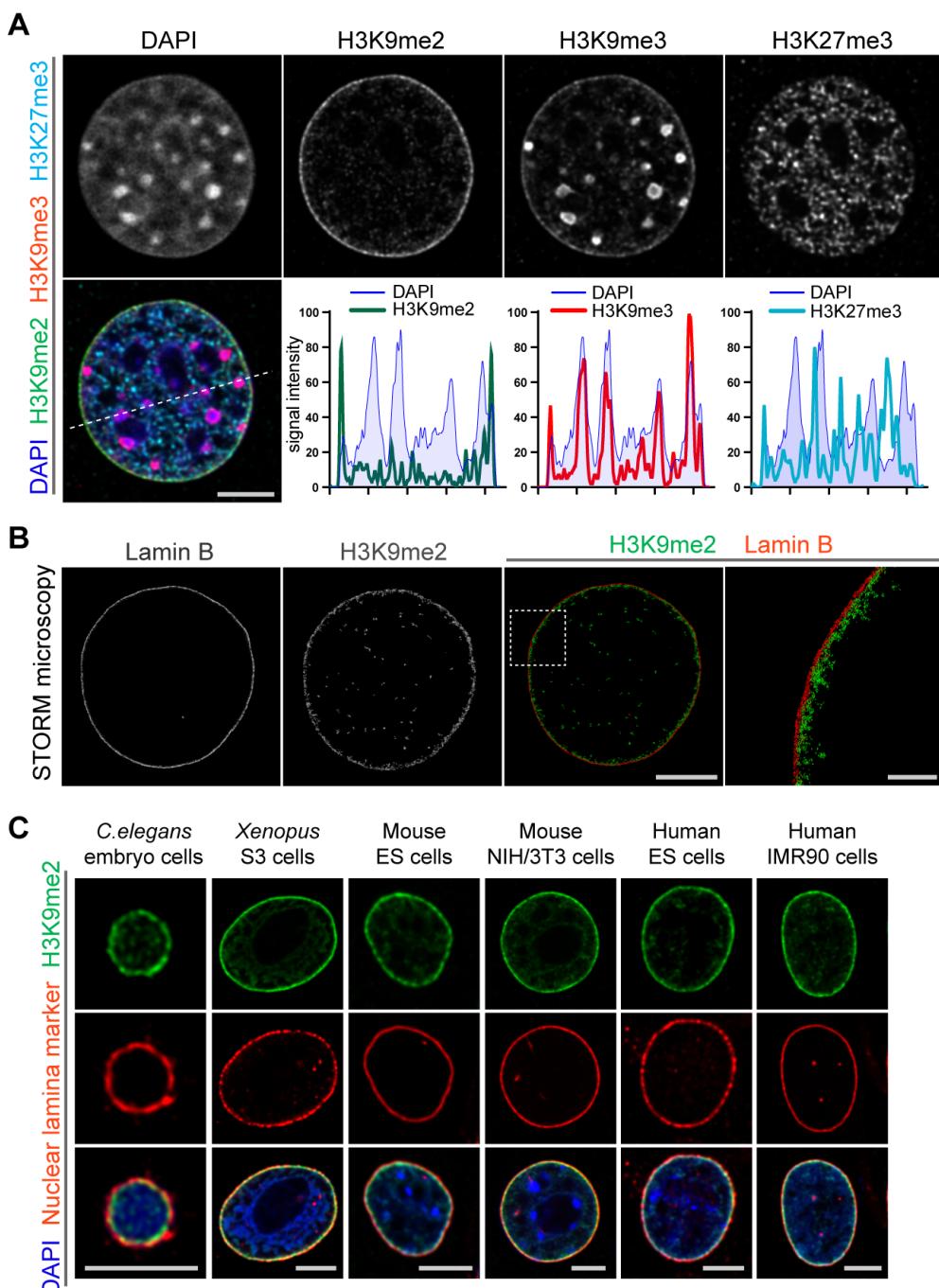
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## 74 **Results**

### 75 **H3K9me2 is an evolutionarily conserved mark of peripheral heterochromatin**

76 Heterochromatin is organized in multiple compartments throughout the nucleus (Pueschel et al.,  
77 2016), and H3K9me2 is a posttranslational histone modification that specifically marks  
78 heterochromatin at the nuclear periphery (Poleshko et al., 2017). Immunostaining of murine  
79 NIH/3T3 fibroblasts for repressive histone modifications demonstrates the distribution of the  
80 major types of heterochromatin in the nucleus of a single cell (Figure 1a). H3K9me2 marks only  
81 peripheral heterochromatin, whereas H3K9me3 and H3K27me3 co-localize with heterochromatin  
82 in the nuclear interior, or at both the interior and the periphery (Figure 1a, Figure S1). The close  
83 association between H3K9me2 and Lamin B in single cell immunostaining is consistent with the  
84 correlation between H3K9me2 and Lamin B ChIP-seq data (Figure S1). The adjacency of  
85 H3K9me2 chromatin to the nuclear lamina was verified by super-resolution microscopy (Figure  
86 1b). Stochastic Optical Reconstruction Microscopy (STORM) using a Voronoi tessellation  
87 confirms a non-random distribution of the H3K9me2 signal at the periphery of the nucleus (Figure  
88 S2). We further examined H3K9me2-marked heterochromatin across species and observe that  
89 restriction to the nuclear periphery is evolutionarily conserved from *C. elegans* to humans (Figure  
90 1c) suggesting functional significance of the localization of this histone post-translational  
91 modification.

Figure 1



92

93 **Figure 1. Localization of H3K9me2-marked chromatin at the nuclear periphery is evolutionarily**  
94 **conserved.** (A) Immunofluorescent confocal images illustrating localization of the indicated repressive

95 chromatin marks in the nucleus of a NIH/3T3 cell, counterstained with DAPI; dashed line indicates position

96 of the line signal intensity profiles. Scale bar: 5 $\mu$ m (B) Representative super-resolution images of a

97 NIH/3T3 cell stained for H3K9me2 and Lamin B obtained using Stochastic Optical Reconstruction

98 Microscopy (STORM). Scale bar: 5 $\mu$ m (C) Localization of H3K9me2-marked chromatin in distinct

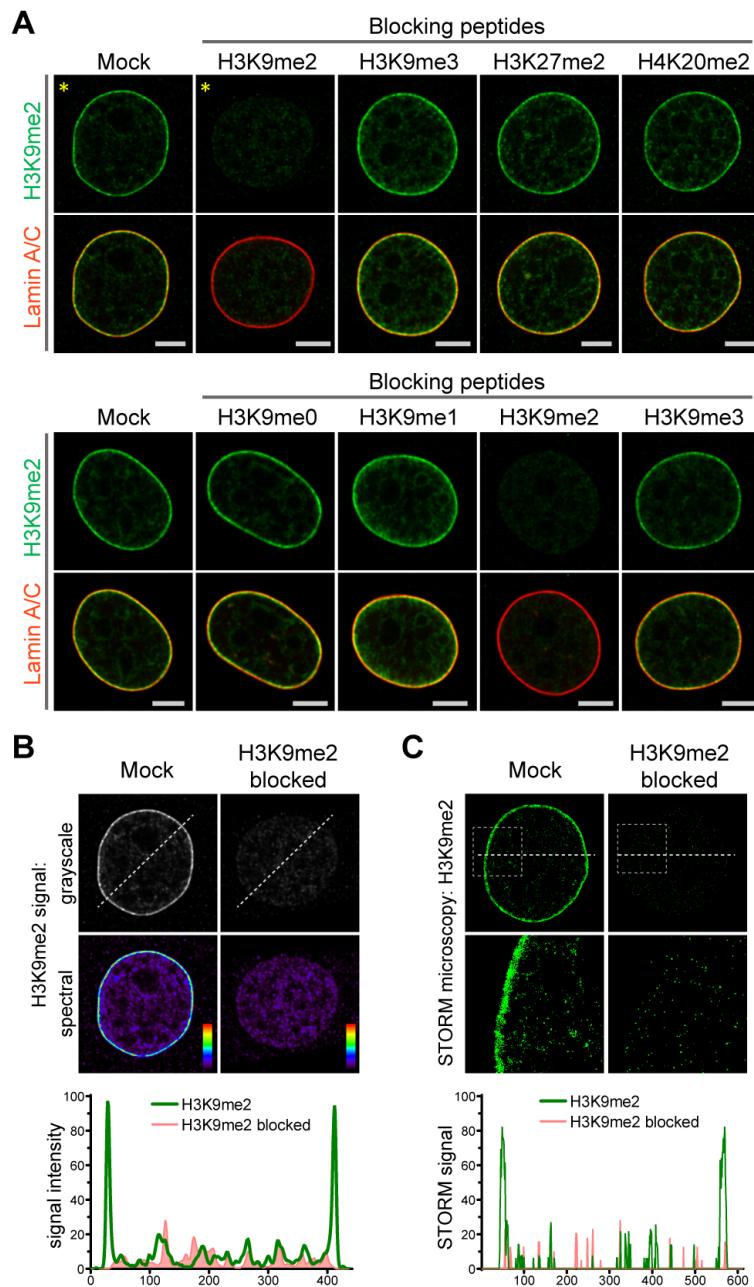
99 species, co-stained with nuclear lamina markers (Lamin 1 for *C. elegans*; Lamin B all others),

100 counterstained with DAPI. Scale bars: 5 $\mu$ m.

101 Previously, distinctions between genomic regions marked by H3K9me2 versus H3K9me3 were  
102 unclear, perhaps because of lack of specificity of relevant antibodies. Therefore, we extensively  
103 characterized the specificity of the H3K9me2 antibody employed in these studies (Figure 2). By  
104 preincubating the anti-H3K9me2 antibody with peptides representing each of the possible histone  
105 tail modifications before use in immunostaining, we were able to determine that the H3K9me2  
106 antibody detects only the dimethyl modification and only on lysine 9 of histone H3 (Figure 2a).  
107 Additionally, by blocking the H3K9me2 antibody with an H3K9me2 peptide, the specific signal  
108 observed at the nuclear periphery can be distinguished from non-specific background signal  
109 observed in the nuclear interior and detected with signal intensity analysis (Figure 2b). This  
110 observation was further confirmed by STORM imaging (Figure 2c).

111

Figure 2



112

113 **Figure 2. Anti-H3K9me2 antibody used in immunofluorescence assays is specific.** (A) Mouse C2C12  
114 cells stained with nuclear marker Lamin A/C and H3K9me2 antibodies preincubated with indicated  
115 blocking peptides. (B) Highlighted images (\*) from panel A, displayed in grayscale and signal intensity  
116 spectral view; line signal intensity profile illustrates H3K9me2-specific signal (green) and non-specific  
117 antibody background (red). (C) STORM images of NIH/3T3 cell stained for H3K9me2 and blocked with  
118 mock or H3K9me2 peptide; line signal intensity profile below as in panel B.

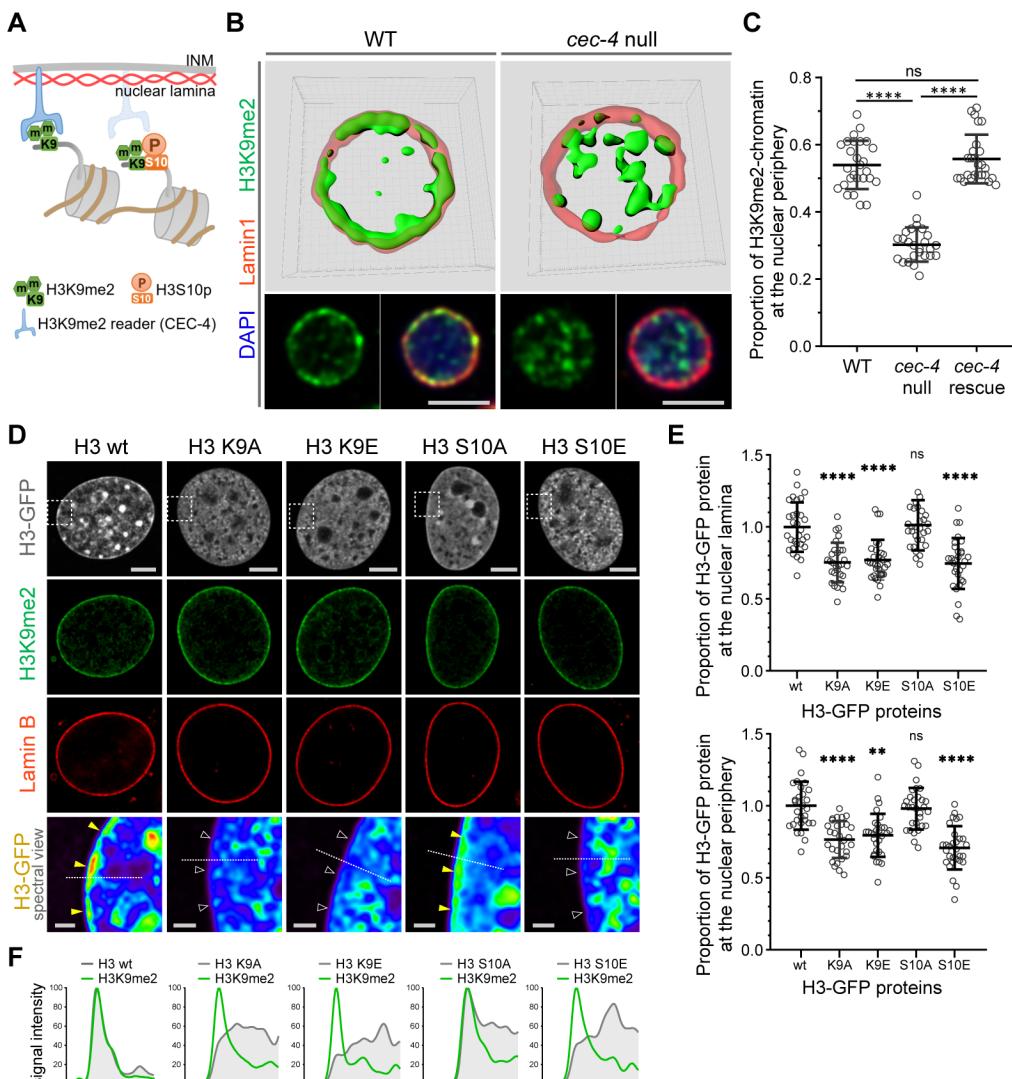
119 **H3K9me2 is required for nuclear peripheral localization of chromatin**

120 Given the specificity of H3K9me2 for peripheral heterochromatin, we hypothesized that this  
121 epigenetic histone modification is necessary for peripheral localization of chromatin and might be  
122 recognized by a nuclear peripheral protein “reader” to tether chromatin to the nuclear lamina  
123 (Figure 3a). In *C. elegans*, CEC-4 functions as a reader of methylated H3K9 and is localized to the  
124 nuclear periphery where it is thought to function as part of a tethering complex for peripheral  
125 heterochromatin (Gonzalez-Sandoval et al., 2015). Mammalian functional orthologues of CEC-4  
126 have not yet been identified. Since CEC-4 is required for peripheral heterochromatin anchoring  
127 (Gonzalez-Sandoval et al., 2015), we compared the localization of H3K9me2 in wild-type and *cec-*  
128 *4*-null embryo cells. Immunostaining revealed a dramatic alteration in spatial patterning in which  
129 H3K9me2 is no longer restricted to the periphery in *cec-4*-null cells (Figures 3b and 3c).  
130 Localization of the H3K9me2-marked chromatin at the nuclear lamina was restored by expression  
131 of the CEC-4-mCherry transgene (Figure 3c, Figure S3). Loss of CEC-4 does not have the same  
132 effect on H3K9me3. H3K9me3 is found both at the nuclear periphery and in the nucleoplasm, but  
133 its localization does not vary between wild-type and *cec-4*-null embryo cells (Figure S3). These  
134 data suggest loss of a peripheral heterochromatin tether, CEC-4, results in a specific effect on  
135 H3K9me2-marked chromatin and not H3K9me3-marked chromatin.

136 To extend our results and probe the role of H3K9 in chromatin positioning in mammalian cells,  
137 we expressed GFP-tagged histone H3 (hereafter H3) or GFP-tagged mutant forms of H3 in which  
138 Lys9 was substituted with alanine (H3K9A) or glutamic acid (H3K9E); both substitutions preclude  
139 methylation at this position in H3. GFP-tagged proteins were expressed in NIH/3T3 cells at  
140 relatively low levels compared to endogenous H3 (Figure S4) and attempts to drive higher levels  
141 of expression resulted in cell death. Wild-type GFP-H3 was observed throughout the nucleus  
142 including at the nuclear periphery, where it overlapped with endogenous H3K9me2 staining,  
143 immediately adjacent to Lamin B (Figure 3d). In contrast, GFP-H3K9A and GFP-H3K9E failed  
144 to partition to the nuclear periphery (Figures 3d-f). This indicates that H3K9, or a residue at this  
145 position that can be methylated, is necessary for peripheral localization of H3. Combined with the  
146 CEC-4 results, this suggests that dimethylation of H3K9 orchestrates positioning of chromatin to  
147 the nuclear periphery.

148

Figure 3



149  
150 **Figure 3. H3K9me2 is essential for histone H3 positioning at the nuclear periphery.** (A) Schematic  
151 illustrating *C. elegans* protein CEC-4 tethering H3K9me2-marked chromatin to the nuclear periphery; INM:  
152 inner nuclear membrane. (B) Localization of H3K9me2-marked chromatin (green) in wild-type (WT) and  
153 *cec-4*-null *C. elegans* embryo cells, counterstained with Lamin 1 (red) and DAPI (blue); 3D reconstruction  
154 (top); immunofluorescent confocal images of *C. elegans* embryo cells (bottom). Scale bars: 3 $\mu$ m (C) Dot  
155 plot of the proportion of total H3K9me2-marked chromatin at the nuclear lamina in WT, *cec-4*-null, and  
156 *cec-4*-rescued embryo cells (mean $\pm$ SD); n=25 cells per condition. (D) Localization of indicated histone H3-  
157 GFP fusion proteins in NIH/3T3 cells; counterstained with H3K9me2 (green) and Lamin B (red); spectral  
158 views (magnifications of top panels as indicated by dashed squares) illustrate H3-GFP signal intensity.  
159 Localization of the H3-GFP at the nuclear periphery (yellow arrowheads) or loss of peripheral localization  
160 (white arrowheads). Scale bars: 5 $\mu$ m (top panels) and 1 $\mu$ m (bottom panels). (E) Dot plot of the proportion  
161 of indicated H3-GFP fusion protein at the nuclear lamina (marked by Lamin B, top) or within the layer of  
162 peripheral heterochromatin (marked by H3K9me2, bottom), normalized to wt H3-GFP, calculated using  
163 Lamin B or H3K9me2 signal as a mask (mean $\pm$ SD); n=30 cells per condition. (F) Line signal intensity  
164 profiles of corresponding images in panel D indicated by dashed lines. Statistical analyses performed using  
165 one-way ANOVA non-parametric Kruskal-Wallis test; \*\*\*\* p<0.0001, \*\*p=0.0024, ns: not significant.  
166

167

## 168 **A phospho-methyl switch controls peripheral heterochromatin localization**

169 H3S10 phosphorylation is associated with mitotic chromosome condensation (Wei et al., 1999,  
170 Prigent and Dimitrov, 2003) and, together with the neighboring Lys9 residue, has been proposed  
171 to function as a ‘phospho-methyl switch’ to modulate binding of H3 to effector proteins (Varier et  
172 al., 2010, Fischle et al., 2003, Wang and Higgins, 2013). Expression of a GFP-tagged H3 mutant  
173 in which Ser10 is replaced with the phospho-mimic glutamic acid (H3S10E) resulted in  
174 distribution of the GFP-H3S10E throughout the nucleus, but notably not at the nuclear periphery  
175 (Figures 3d-f). This is consistent with the ability of phosphorylated Ser10 to inhibit interaction of  
176 the reader with H3K9me2 and suggests that phosphorylation of Ser10 can prevent H3 peripheral  
177 localization. Replacement of H3 Ser10 with an alanine (H3S10A) precludes phosphorylation at  
178 this site and did not disrupt peripheral localization. Instead, H3S10A produced a pattern similar to  
179 wild-type GFP-H3 in interphase cells (Figures 3d-f). These results suggest that H3K9me2 is  
180 required for localization of heterochromatin to the nuclear periphery. Further, they indicate that  
181 phosphorylation of Ser10 can prevent or disrupt this association as part of a phospho-methyl  
182 switch.

183

## 184 **H3K9me2 persists through mitosis and associates with reassembling nuclear lamina in 185 daughter cells at mitotic exit**

186 Given the requirement for H3K9me2 to position heterochromatin at the nuclear lamina in  
187 interphase, we asked whether the H3K9me2 mark is maintained through cell division or if the  
188 histone modification is lost and re-acquired *de novo* in daughter cells. Examination of cells  
189 progressing through the consecutive phases of mitosis revealed persistence of H3K9me2 on  
190 mitotic chromatin (Figure 4a). Prior to disassembly of the nuclear lamina in prophase, H3K9me2-  
191 marked chromatin begins to detach from the nuclear periphery. Concordant with this detachment,  
192 we observe phosphorylation of Ser10 on the H3 tail adjacent to dimethylated Lys9  
193 (H3K9me2S10P) beginning in prophase and persisting until late telophase (Figures 4a and 4b). As  
194 for the anti-H3K9me2 antibody, we carefully tested the specificity of the anti-H3K9me2S10P  
195 antibody used in these experiments and verified that it does not recognize the H3K9me2 epitope  
196 without an adjacent phosphate group on S10 (Figure S5). H3S10 phosphorylation in prophase may

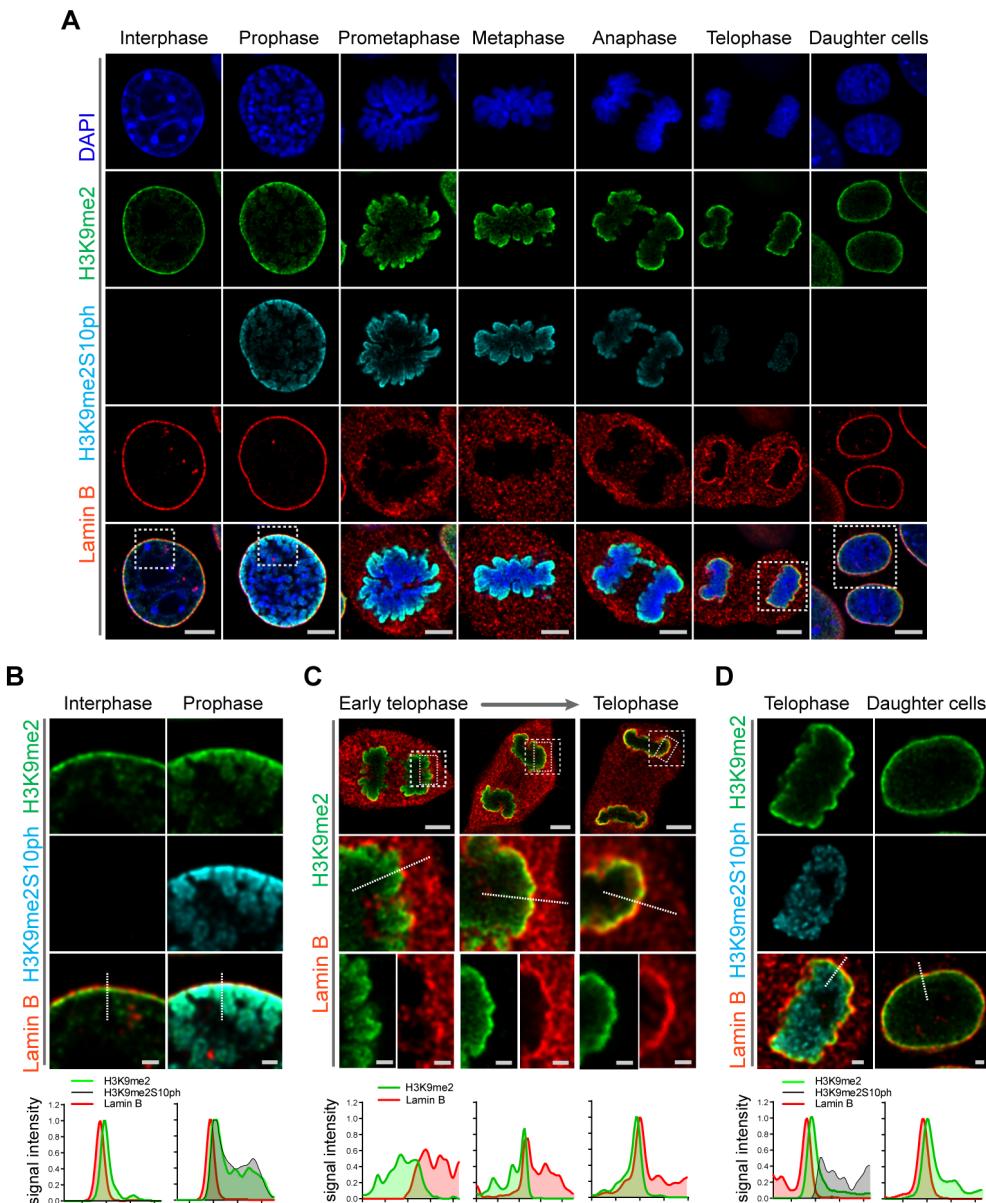
197 contribute to release of H3K9me2 readers/tethers (Eberlin et al., 2008, Hirota et al., 2005) and  
198 detachment from the nuclear periphery.

199 We also examined cells at successive points in telophase. As telophase progresses, re-  
200 establishment of the H3K9me2 layer occurs in parallel with reassembly of the nuclear lamina. We  
201 observed aggregation of H3K9me2-marked chromatin and the reformation of this heterochromatin  
202 layer at the interface with the newly forming nuclear lamina structure (Figure 4c). However,  
203 chromatin marked with H3K9me2S10P was not enriched at the interface of the forming nuclear  
204 lamina but remained in the nucleoplasm (Figure 4d), suggesting that loss of S10 phosphorylation  
205 occurs prior to association of chromatin with the nuclear lamina. We detected little or no  
206 H3K9me2S10P in daughter cells after mitosis was complete (Figure 4d).

207 A subset of H3K9me3-marked chromatin is at the nuclear periphery, though it is not restricted to  
208 the periphery as is H3K9me2. H3K9me3 is enriched in microsatellite heterochromatin and persists  
209 through mitosis (Figure 5a). In addition, in telophase we noted strong differences in localization  
210 of other repressive (H3K9me3, H3K27me3) and active (H3K4me3) histone marks in contrast to  
211 H3K9me2 (Figure 5b). Trimethylated H3K9 is also distinct from H3K9me2 in that H3K9me3  
212 chromatin is not enriched at the interface with the forming nuclear lamina during telophase and  
213 mitotic exit. In the newly formed daughter cells, we observed H3K9me2- but not H3K9me3-  
214 marked chromatin preferentially associated with the nuclear lamina.

215

Figure 4



216  
217

218 **Figure 4. H3K9me2-marked chromatin is maintained throughout mitosis to be re-established at the**  
219 **nuclear lamina during nuclear lamina reassembly.** (A) Immunofluorescent confocal images of mouse

220 C2C12 cells illustrating localization of H3K9me2- and H3K9me2S10ph-marked chromatin and Lamin B

221 during different stages of mitosis; DNA visualized with DAPI. Scale bars: 5 $\mu$ m. (B) Magnified images of

222 Interphase and Prophase from panel (A) demonstrating detachment of the H3K9me2-chromatin from the

223 nuclear lamina concomitant with H3K9me2S10ph phosphorylation; scale bar: 1 $\mu$ m. (C) Representative

224 images of cells progressing through telophase as the layer of peripheral H3K9me2-marked heterochromatin

225 (green) is re-established and nuclear lamina (Lamin B, red) is reassembled; dashed boxes in top panels

226 indicate higher resolution images. Scale bars: 5 $\mu$ m (top) and 1 $\mu$ m (bottom panels). (D) Magnified images

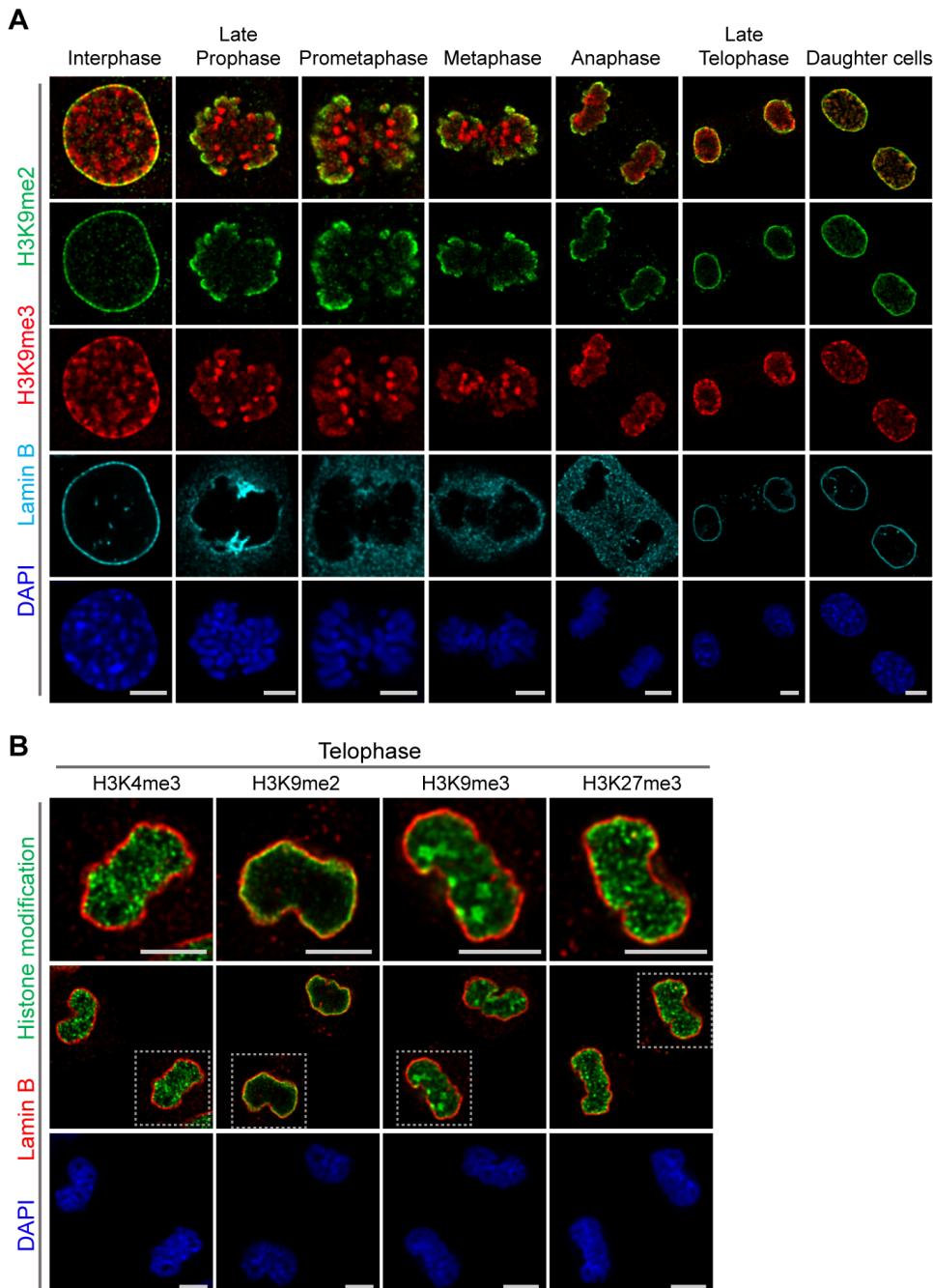
227 of telophase and daughter cells from panel A demonstrating de-phosphorylated H3K9me2-chromatin

228 (green) assembled at the nuclear lamina (Lamin B, red), while the phosphorylated form (H3K9me2S10ph,

229 cyan) remains localized in the nuclear interior; scale bar: 1 $\mu$ m. Dashed lines indicate location of

230 corresponding line signal intensity profiles (bottom row).

Figure 5



231

232 **Figure 5. Localization of H3K9me2- and H3K9me3-marked chromatin differs during mitosis. (A)**  
233 Representative immunofluorescent confocal images illustrating a difference in localization of H3K9me2  
234 (green) and H3K9me3 (red) chromatin marks in interphase, during mitosis, and upon mitotic exit; co-  
235 stained with Lamin B (cyan) and DAPI (blue). **(B)** Representative immunofluorescent confocal images of  
236 telophase cells illustrating difference in localization of different histone modifications (green) in relation to  
237 Lamin B (red); co-stained with DAPI (blue). Dashed boxes in middle panels indicate higher resolution  
238 images. Scale bars: 5 $\mu$ m.

239 **Specific LADs positioned at the nuclear periphery prior to mitosis re-associate with forming**  
240 **nuclear lamina in telophase**

241 Restoration of H3K9me2-marked chromatin at the nuclear lamina prior to mitotic exit suggests a  
242 mechanism for inheritance of spatial localization of specific genomic loci within the peripheral  
243 heterochromatin layer. Our experiments thus far demonstrate that H3K9me2-marked chromatin,  
244 in general, is re-established at the nuclear lamina. Conflicting reports have emerged regarding  
245 whether LADs are reshuffled at every cell division to stochastically localize in other, non-lamina-  
246 associated heterochromatic subcompartments (Kind et al., 2013, Zullo et al., 2012, Kind et al.,  
247 2015). To determine whether specific genomic regions are re-established at the nuclear periphery  
248 at mitotic exit, we used fluorescence *in situ* hybridization (FISH)-based imaging to monitor the  
249 localization of individual genomic regions in single cells. We designed libraries of fluorescent  
250 DNA oligo probes (oligopaints) targeting domains of the genome that were identified through  
251 population-based studies (Meuleman et al., 2013, Peric-Hupkes et al., 2010, Poleshko et al., 2017)  
252 to be either cell-type invariant regions of nuclear peripheral, H3K9me2-marked heterochromatin  
253 (LADs) or cell-type invariant regions of euchromatin (non-LADs). The pool of probes (41 LAD  
254 and 41 non-LAD regions) includes regions from every mouse autosome (Figure S6, Table S1). We  
255 performed immunofluorescent *in situ* hybridization with the probes in individual cells in interphase  
256 and mitosis; reconstruction of stacks of confocal images allowed us to visualize the 3D position of  
257 specific genomic loci (Movies S1-S3).

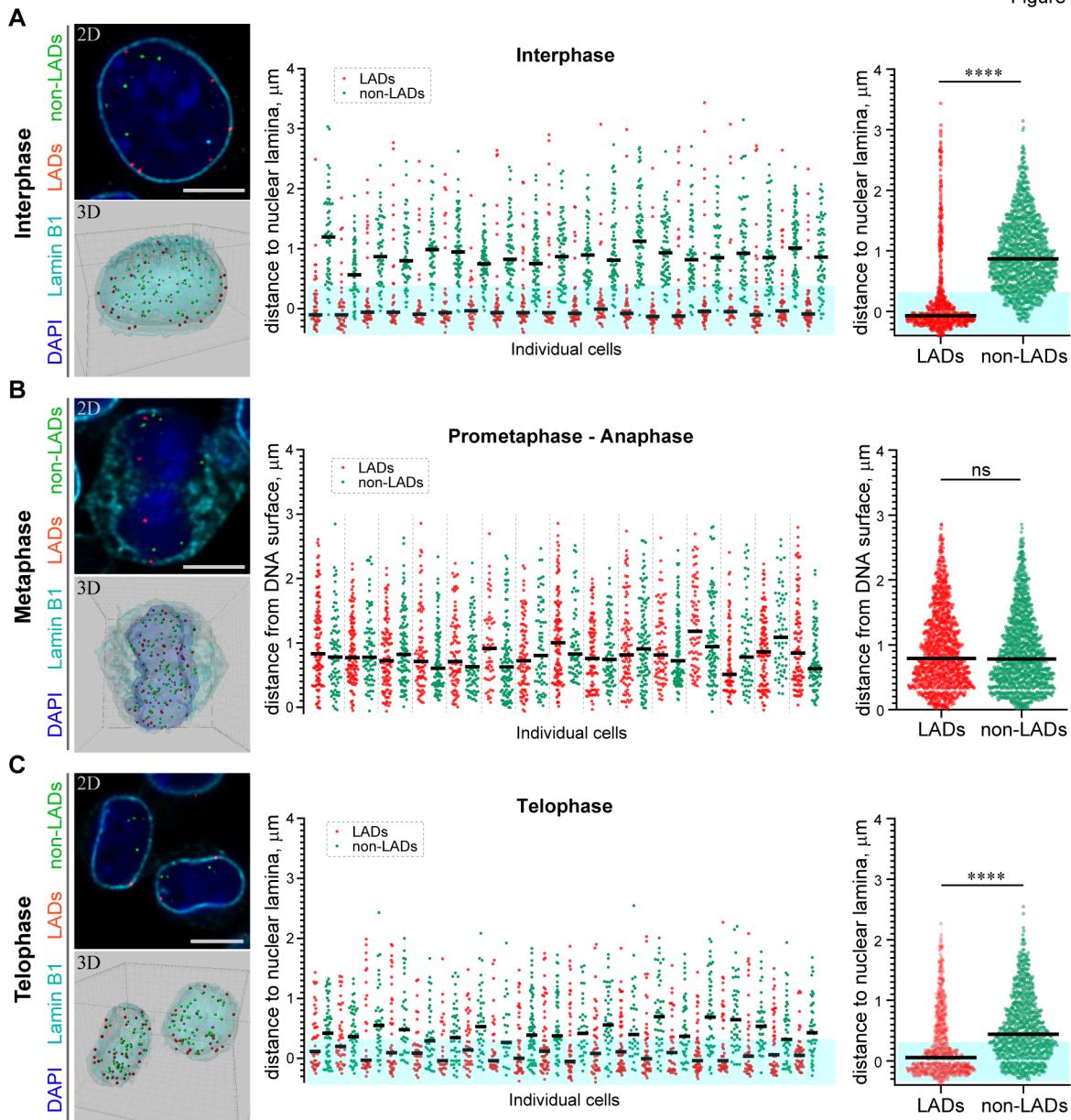
258 In a population of interphase cells, we found the LAD probes to be at the periphery of individual  
259 nuclei at a frequency consistent with previous observations of haploid cells in studies using single-  
260 cell DamID (Kind et al., 2015). An average of 82% of LAD probes (74-90% in individual cells)  
261 were positioned at the nuclear periphery within the measured thickness of the H3K9me2 chromatin  
262 layer in interphase cells (Figure 6a, Movie S1). Non-LAD probes, assessed in each of the same  
263 interphase cells, were more frequently found in the nucleoplasm, as expected: an average of 89%  
264 of non-LAD probes (79-95% in individual cells) segregated outside of the peripheral chromatin  
265 layer (Figure 6a).

266 Next, we examined the location of these representative LAD and non-LAD genomic loci in cells  
267 undergoing mitosis. Both LAD and non-LAD probes are present at similar distances from the DNA  
268 surface in cells in metaphase, a point in mitosis at which the nuclear lamina has disassembled (Figure

269 6b, Movie S2). However, by telophase, LAD probes have repositioned to the nuclear periphery  
270 (Figure 6c, Movie S3), indicating that H3K9me2-marked domains that were at the periphery in  
271 parent cells are specifically repositioned at the periphery in daughter nuclei before mitotic exit. In  
272 these same cells in telophase, non-LAD probes remained largely in the nucleoplasm, away from  
273 the nuclear lamina (Figure 6c, Movie S3). Thus, specific LADs found at the nuclear periphery in  
274 parental cells are repositioned at the periphery at mitotic exit.

275

Figure 6



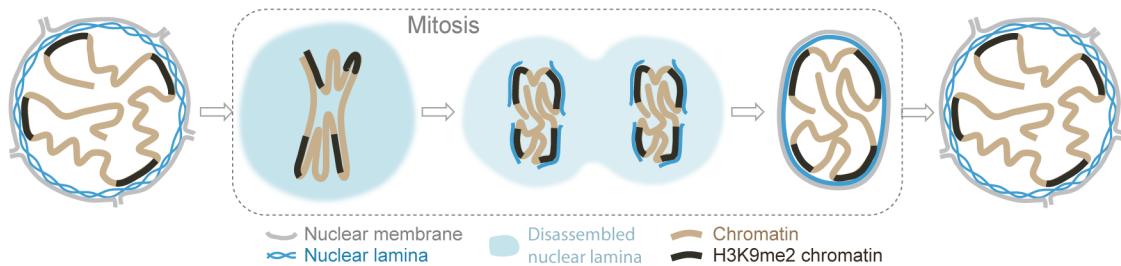
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277 **Figure 6. H3K9me2-enriched LADs are positioned at the nuclear lamina in interphase cells and the**  
278 **position is inherited through mitosis. (A) Localization of LADs and non-LADs in interphase mESCs.**  
279 Left panels show representative immuno-FISH image (top) and 3D image reconstruction (bottom) of cells  
280 hybridized with fluorescent DNA oligopaint probes targeting individual LADs (red) and non-LADs (green),  
281 and immunostained for Lamin B1 (cyan) and DAPI (blue). Scale bar: 5 μm. Dot plots show distribution and  
282 median of distances to the nuclear periphery of individual LAD and non-LAD probes for individual cells  
283 (middle) and cumulative over all cells (right) in interphase. (B) As in panel A for prometaphase-metaphase-  
284 anaphase cells. (C) As in panel A for telophase cells. For dot plots, nuclear periphery defined by Lamin B1  
285 or DNA edge; black line: median value; cyan boxes indicate average thickness of H3K9me2 peripheral  
286 heterochromatin layer. n≥20 individual nuclei; N=870-1399 individual LADs or non-LADs per condition.  
287 Statistical analysis performed using two-tailed t-test; \*\*\*\* p<0.0001; ns: not significant.

288 **Discussion**

289 Our results provide experimental support of a model for nuclear peripheral localization and mitotic  
290 inheritance of lamina-associated heterochromatin (Figure 7). We show that H3K9me2 marks  
291 chromatin domains that are specifically positioned at the nuclear lamina during interphase. In  
292 mitosis, these domains retain and are bookmarked by H3K9me2. H3S10 phosphorylation  
293 promotes release from the nuclear periphery, likely by masking the Lys9 dimethyl modification  
294 from recognition by its reader/tether (Fischle et al., 2003, Wang and Higgins, 2013, Eberlin et al.,  
295 2008). In late stages of mitosis, dephosphorylation of H3S10 unmasks bookmarked LADs which  
296 are then reassembled at the nuclear periphery during nuclear lamina reformation in the nuclei of  
297 daughter cells.

Figure 7



298  
299  
300  
301  
302

**Figure 7. Model illustrating the role of the H3K9me2 chromatin modification in inheritance of peripheral heterochromatin localization through cell division.**

303 How cells convey information related to cellular identity to daughter cells has been a long-standing  
304 focus of investigation. Although mitotic chromosomes are condensed and transcriptionally silent,  
305 it is now appreciated that many nuclear factors remain associated with specific regions of mitotic  
306 chromatin, and some histone post-translational modifications are also retained. The concept of  
307 “mitotic bookmarking” has been put forth to describe mechanisms by which transcriptionally  
308 active regions of euchromatin may be “remembered” and rapidly re-activated upon mitotic exit  
309 (Kadauke and Blobel, 2013, Palozola et al., 2019, Sureka et al., 2018). Here, we extend this  
310 concept by elucidating a mechanism for transmitting a blueprint of the 3D organization of the  
311 genome from mother to daughter cell with a specific focus on peripheral heterochromatin  
312 associated with the inner nuclear lamina. Our data indicate that H3K9me2 acts as a 3D architectural  
313 mitotic guidepost.

314 Our data highlight the role of H3S10 phosphorylation adjacent to dimethylated Lys9 in 3D mitotic  
315 bookmarking, allowing for dissociation of peripheral heterochromatin from the nuclear lamina  
316 while retaining memory of genomic regions that will be reattached to the newly formed nuclear  
317 lamina upon dephosphorylation and mitotic exit. This example of a phospho-methyl switch  
318 extends previous studies that implicated related phospho-methyl switch mechanisms in  
319 transcriptional bookmarking without invoking regulation of 3D genome organization or nuclear  
320 reassembly. For example, H3S10 phosphorylation can displace HP1 binding to trimethylated Lys9  
321 during mitosis (Hirota et al., 2005, Fischle et al., 2005). In another example, the active histone  
322 mark H3K4me3 is bound by TFIID and the basal transcriptional machinery during interphase.  
323 While H3K4me3 is maintained through mitosis, phosphorylation of Thr3 results in dissociation of  
324 TFIID and transcriptional silencing. The retention of H3K4me3 is thought to allow for rapid re-  
325 initiation of transcription after mitosis when Thr3 is dephosphorylated (Varier et al., 2010,  
326 Sawicka and Seiser, 2014). Our results supporting an H3K9me2S10 phospho-methyl switch  
327 suggest that this conserved mechanism also is employed for mitotic memory of nuclear  
328 architecture. During cell division, this mechanism is utilized to release all peripheral  
329 heterochromatin from the nuclear lamina, but it will be of interest to determine if a similar process  
330 occurs during interphase to release specific LADs from the periphery, perhaps endowing these  
331 domains with competence to be accessed by nuclear regulators of transcription. Histone  
332 phosphorylation, including H3S10, has been well documented to occur in response to classic signal  
333 transduction pathways such as Mapk signaling (Winter et al., 2008) suggesting a potential  
334 mechanism for the regulation of LAD release as a component of signal transduction.

335 The importance of the spatial organization of the genome has attracted increasing attention in  
336 recent years with a growing appreciation for unique, lineage-specific LADs and other architectural  
337 features. Large-scale efforts have focused on characterizing genome organization in interphase,  
338 with less attention to how 3D architecture is transmitted through mitosis. Indeed, an early study  
339 suggested that LADs might be stochastically formed *de novo* following each cell division rather  
340 than inherited from the mother cell following mitosis (Kind et al., 2013). Unless all  
341 heterochromatic subcompartments are functionally equivalent, this would be somewhat  
342 inconsistent with the role that LADs are thought to play in cell identity (Robson et al., 2016, Peric-  
343 Hupkes et al., 2010, Kohwi et al., 2013, Gonzalez-Sandoval et al., 2015, Poleshko et al., 2017).  
344 Many reports have documented consistent, cell-type-specific LAD architecture as well as

345 restoration of particular heterochromatin domains at the lamina after cell division (Zullo et al.,  
346 2012, Kind et al., 2015). It is conceivable that cell-type-specific LAD organization is  
347 “rediscovered” after mitosis rather than “remembered.” But our results, including those produced  
348 with LAD-specific oligopaints, indicate that at least a subset of LADs is re-established at the  
349 nuclear periphery at the end of mitosis, concomitant with nuclear lamina re-assembly. Thus, a  
350 daughter cell inherits the key aspects of 3D chromatin organization required to retain cellular  
351 identity.

352 Mitosis and the period shortly following in G1 may provide a vulnerable period to regulate or  
353 modify genome organization. Consistent with this, pioneering experiments artificially tethering  
354 areas of the genome to the nuclear lamina noted the requirement for a mitotic event to precede  
355 efficient tethering of the genome to the nuclear lamina (Finlan et al., 2008, Reddy et al., 2008,  
356 Kumaran and Spector, 2008). Moreover, nuclear transfer experiments demonstrated that mitotic  
357 chromatin can be reprogrammed to activate the core pluripotency network 100 times more  
358 efficiently than interphase chromatin (Halley-Stott et al., 2014). This may be, in part, because  
359 three-dimensional reorganization of the genome after mitosis helps to regulate accessibility. In  
360 particular, it is possible that the period during which H3S10 phosphorylation is lost in late mitosis,  
361 but before H3K9me2-marked chromatin is fully re-established as lamina-associated  
362 heterochromatin at the nuclear periphery, is a particularly vulnerable time to change LAD  
363 positioning in daughter cells. Hence, this may also coincide with a window in which cell fate  
364 changes associated with modifications in nuclear architecture occur (Gilbert, 2010). This would  
365 be in accord with the “quantal theory of differentiation” put forth by Howard Holtzer over 50 years  
366 ago which proposed that major steps in lineage determination and cell fate restriction required  
367 mitotic events (Holtzer et al., 1972).

368 Classic cell biology experiments have demonstrated the necessity of kinase-phosphatase activity  
369 for mitotic progression and the requirement for chromatin to allow nuclear membranes to reform  
370 in daughter cells after mitosis (Gerace and Blobel, 1980, Newport, 1987, Foisner and Gerace,  
371 1993, Burke and Gerace, 1986, Wei et al., 1999, Prigent and Dimitrov, 2003, Wandke and Kutay,  
372 2013, Haraguchi et al., 2008). Our model provides a mechanistic explanation for these  
373 requirements and advances current models of mitotic bookmarking by introducing the concept of  
374 3D architectural mitotic bookmarking. This model for epigenetic inheritance may have  
375 implications for understanding how cells adopt new fates in the setting of asymmetric cell

376 divisions, and how cellular identity may be lost or altered in the context of cancer or trans-  
377 differentiation. For example, it will be of great interest to determine if the re-establishment of  
378 spatial chromatin organization is disrupted in cells as they undergo oncogenic transformation  
379 and/or cellular reprogramming.

380

381 **Author Contributions**

382 A.P., C.L.S., R.J., and J.A.E. conceived of the project and designed experiments. A.P. performed  
383 experiments. P.S. and J.I.M. contributed reagents. C.L.S., S.C.N. and E.F.J. performed oligo probe  
384 design and production. M.L. provided algorithms for image analysis. A.P., C.L.S., R.J., and J.A.E.  
385 analyzed data and wrote the manuscript. R.J. and J.A.E. supervised the project.

386

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389 Matt Good and Nicolas Plachta for discussions and comments on the manuscript. This work was  
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394

395 **Declaration of Interests:**

396 Authors declare no competing interests.

397

398

399 **Methods**

400

401 **Cell lines**

402 Murine NIH/3T3 fibroblast and C2C12 skeletal myoblast, and human IMR90 fibroblast cells were  
403 maintained at 37°C in DMEM supplemented with 10% FetalPlex serum complex (Gemini,  
404 cat#100-602), penicillin, and streptomycin. Mouse ES cells were maintained at 37°C on a feeder  
405 layer of mitotically inactivated MEFs in DMEM with 15% FBS (Fisher Scientific #SH3007003)  
406 and ESGRO LIF (EMD Millipore, cat#ESG1106). Human ES cells were maintained at 37°C in  
407 StemMAC™ iPS-Brew XF media (Miltenyi Biotec GmbH, cat#130-104-368), supplemented  
408 with penicillin, and streptomycin. Xenopus S3 cells were maintained at 25°C in 66% L-15 media  
409 (Gibco, cat#11415-064) with 10% fetal bovine serum (Atlanta Biologicals, cat#S11550), sodium  
410 pyruvate, penicillin, and streptomycin.

411

412 **Plasmids, mutagenesis and transfection**

413 Expression plasmids for Histone H3-mEmerald was received from Addgene (cat#54115, deposited  
414 by Michael Davidson). This plasmid was used to create Histone H3 tail mutant constructs: H3  
415 K9A, H3 K9E, H3 S10A and H3 S10E using QuikChange II XL Site-Directed Mutagenesis Kit  
416 (Agilent technologies, cat#200521) according to manufacturer's instruction. Plasmid transfections  
417 were performed with FuGENE 6 (Promega, cat#E2691) according to manufacturer instructions.  
418 For confocal imaging cells were plated on coverslips (EMS, cat#72204-01), then transfected at  
419 50% confluency and fixed 48 hours post-transfection. Primers used for mutagenesis:  
420 H3 K9A (5'-ACTAACACAGACAGCTCGGGCATCCACCGGGCGGTAAAGCG, 5'-  
421 CGCTTACCGCCGGTGGATGCCGAGCTGTCTGTTAGT); H3 K9E (5'-  
422 ACTAACACAGACAGCTCGGAATCCACCGGGCGGTAAAGCG, 5'-  
423 CGCTTACCGCCGGTGGATTCCCGAGCTGTCTGTTAGT); H3 S10A (5'-  
424 ACTAACACAGACAGCTCGAAAGCCACCGGGCGGTAAAGCG, 5'-  
425 CGCTTACCGCCGGTGGCTTCCGAGCTGTCTGTTAGT); H3 S10E (5'-  
426 ACTAACACAGACAGCTCGAAAGAAACCGGGCGGTAAAGCG, 5'-  
427 CGCTTACCGCCGGTTCTTCCGAGCTGTCTGTTAGT).

428

429 ***C. elegans* strains, embryo cell isolation for immunofluorescence**

430 The wild-type strain is N2; the *cec-4* null is deletion strain RB2301 from the *Caenorhabditis*  
431 Genetics Center (CGC); CEC4-mCherry transgene is the GW849 strain (gwSi17 [*cec-4p::cec-*  
432 4::WmCherry::cec-4 3'UTR] II) obtained from Susan Gasser (Gonzalez-Sandoval et al., 2015).  
433 The rescue strain was created by crossing *cec-4* mutant [*cec-4* (ok3124) deletion] males to GW849  
434 hermaphrodites. Animals were grown as previously described (Stiernagle, 2006). For  
435 immunostaining, worms were bleached, then washed off the plate with M9 solution (86mM NaCl,  
436 42mM Na<sub>2</sub>HPO<sub>4</sub>, 22mM KH<sub>2</sub>PO<sub>4</sub>, and 1mM Mg<sub>2</sub>SO<sub>4</sub>, pH 6.5). They were washed with a bleach  
437 solution (15ml MilliQ water, 4ml Clorox, and 2ml 5 M KOH) with shaking until adult bodies were  
438 dissolved. Then, embryos were washed twice with M9 solution, fixed with 4% formaldehyde  
439 solution (incubated at room temperature (RT) for 15 min). Embryos were then flash freeze by  
440 immersing tube in an ethanol/dry ice bath for 2 minutes, thawed to RT, and then incubated on ice  
441 for 20 min and wash twice with PBS. Fixed embryos were spun on the coverslips at 1000g for 10  
442 min in cushion buffer (100mM KCl, 1mM MgCl<sub>2</sub>, 0.1mM CaCl<sub>2</sub>, 10mM HEPES pH7.7, 250mM  
443 sucrose, 25% glycerol), then post-fixed with 2% PFA for 10 min at RT. A single-cell suspension  
444 of embryonic cells was prepared in a similar manner, but after the beach solution washing step  
445 embryos were washed three times in L15 media (Corning Cellgro, cat#10-045-CV), and then  
446 incubated in the 0.5mg/ml Chitinase (Sigma, cat#C6137) in Boyd Buffer (25 mM HEPES pH 7.3,  
447 118 mM NaCl, 48 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>) at RT with rotation/aspiration to  
448 dissociate cells. Cells were pelleted at 1000g for 5 min at 4oC and dissolved in PBS. Cells were  
449 kept at 4°C before immunostaining.

450

## 451 **Immunofluorescence**

452 NIH/3T3 cells, C2C12 cells, undifferentiated mouse and human ES cells, *Xenopus laevis* S3 cells  
453 utilized for immunofluorescence experiments were grown on glass coverslips, fixed with 2%  
454 paraformaldehyde (PFA) (EMS, cat#15710) for 10 minutes at RT, washed 3 times with DPBS  
455 (Gibco, cat#14190-136), then permeabilized with 0.25% Triton X-100 (Thermo Scientific,  
456 cat#28314) for 10 minutes. After permeabilization, cells were washed 3 times with DPBS for 5  
457 min, then blocked in 1% BSA (Sigma, cat#A4503) in PBST (DPBS with 0.05% Tween 20, pH 7.4  
458 (Thermo Scientific, cat#28320)) for 30-60 min at RT. Incubated with primary antibodies for 1 hour  
459 at RT, then washed three times with PBST for 5 min. Incubated with secondary antibodies for 30-  
460 60min at RT, when washed two times with PBST for 5min. Samples were counterstained with

461 DAPI solution (Sigma, cat#D9542) for 10 min at RT, then rinsed with PBS. Coverslips were  
462 mounted on slides using 80% glycerol mounting media: 80% glycerol (Invitrogen, cat#15514-  
463 011), 0.1% sodium azide (Sigma, cat#S2002), 0.5% propyl gallate (Sigma, cat#02370), 20mM  
464 Tris-HCl, pH 8.0 (Invitrogen, cat#15568-025).

465

#### 466 **Immunofluorescence and DNA oligo FISH**

467 Mouse ESCs were grown on 0.1% porcine gelatin (Sigma, cat#G2500) coated glass coverslips  
468 (EMS, cat#3406), fixed with 2% PFA for 10 minutes at RT. Then cells were immunostained as  
469 described above. DNA oligo hybridization protocol was adopted from Rosin et al., 2018 (Rosin et  
470 al., 2018). In brief, after incubation with secondary antibodies, samples were washed with DPBS  
471 and post-fixed with 2% PFA for 10 minutes at RT, washed 3 times with DPBS and permeabilized  
472 with 0.7% Triton X-100 for 10 minutes at RT, then rinsed with DPBS. Incubate coverslips in 70%  
473 ethanol, 90% ethanol, and 100% ethanol for 2 minutes each, then incubate in 2X SSC (Corning,  
474 cat#46-020-CM) for 5 min. Incubate coverslips in 2X SSCT (2X SSC with 0.1% Tween) for 5 min  
475 at RT, then incubate in 2X SSCT with 50% Formamide for 5 min at RT. DNA denaturation was  
476 performed in 2X SSCT with 50% Formamide for 2.5 minutes at 92°C, then additional 20 min at  
477 60°C. After DNA denaturation, samples were cooled to RT in humid conditions for 2-3 min, then  
478 hybridized with DNA oligo probes in ~50-100 pmol primary DNA probe coverslips were heated  
479 at 92°C for 2.5 min on a heat block. Samples were hybridized with DNA oligo probes overnight  
480 at 37°C in a humid chamber. After hybridization with primary DNA oligo probes samples were  
481 washed in 2X SSCT for 15 min at 60°C, then for 10 min in 2X SSCT for 10 min at RT, then  
482 transferred in 2X SSC for 5 min. Next samples were hybridized with a secondary fluorescent DNA  
483 oligo probes in dark humidified chamber for 3 hours at RT. Hybridization mix: 10% Formamide,  
484 10% dextran sulfate, 10 pmol secondary DNA probe. After secondary hybridization samples were  
485 washed for 5 min in 2X SSCT at 60°C, then 2X SSCT at RT, and 2X SSC buffer with DAPI.  
486 Samples were rinsed with DPBS and mounted on a slide.

487

#### 488 **Image acquisition**

489 All confocal immunofluorescent images were taken using a Leica TCS SP8 3X STED confocal  
490 microscope. 3D images were taken as Z-stacks with 0.05µm intervals with a range of 80-250 Z-  
491 planes per nucleus. Confocal 3D images were deconvoluted using Huygens Professional software.

492 Stochastic Optical Reconstruction Microscopy (STORM) images were obtain using Vutara SRX  
493 STORM system. Cells for STORM imaging were plated on confocal plates (MatTek, cat#P35GC-  
494 1.5-14-C). After immunostaining cells were kept in DPBS until image acquisition. STORM  
495 imaging was performed in fresh imaging buffer (50mM Tris-HCl, pH 8.0, 10mM NaCl, 10% (w/v)  
496 glucose (Sigma, cat#G8270), 1.5mg MEA (Sigma, cat#30070), 170 AU Glucose oxidase (Sigma,  
497 cat#G2133), 1400 AU Catalase (Sigma, cat#C40)). Confocal channel shift alignment and STORM  
498 point spread function (PSF) calibration and channel shift alignment were performed using 0.1 $\mu$ m  
499 TetraSpeck fluorescent beads (Invitrogen, cat#T7279).

500

### 501 **Image analysis**

502 Image analysis were performed using Image J, Imaris 9.0.1, and Vutara SRX software.  
503 Representative confocal images show a single focal plane. 2D image analysis was performed using  
504 Image J software (National Institute of Health, USA). Line signal intensity profile plots were  
505 created using Plot Profile tool. Measurement of localization of the IF signal at the nuclear periphery  
506 was performed as a proportion of the signal at the nuclear periphery measured using a mask of the  
507 nuclear lamina or H3K9me2 signals, to total signal in the nucleus. 3D image reconstructions were  
508 performed using Imaris 9.0.1 software (Bitplane AG, Switzerland) as described (Poleshko et al.,  
509 2017). In brief, nuclear lamina, nuclear DNA volume, and H3K9me2-marked chromatin structure  
510 were created using Surfaces tool with automatic settings based on the fluorescent signals from the  
511 anti-Lamin B, DAPI staining, and anti-H3K9me2 antibodies. DNA oligo FISH probe spots were  
512 generated using the Spots tool with a 250nm diameter, created at the intensity mass center of the  
513 fluorescent probe signal. Distance from the center of the DNA oligo FISH spot to the edge of the  
514 nuclear lamina surface was quantified using the Distance Transformation tool. Thickness of the  
515 peripheral heterochromatin layer was calculated as the distance from the H3K9me2 surface inner  
516 edge to nuclear lamina inner edge again using the Measurement Points tool. If the distance from  
517 the DNA oligo FISH spot to the nuclear lamina was smaller than (or equal to) the average thickness  
518 of peripheral chromatin, then the spot was counted as localized to nuclear periphery. In cases when  
519 the DNA oligo FISH signal was imbedded into the nuclear lamina layer, the measurement returned  
520 negative distances. STORM image and cluster analysis were performed using Vutara SRX  
521 software (Bruker, USA) and Voronoi Tessellation Analysis of H3K9me2 STORM images was  
522 performed in MATLAB 2016a in a fashion similar to Andronov et al., 2016 (Andronov et al.,

523 2016). First, the lateral x,y localizations were input into the ‘delaunayTriangulation’ function, and  
524 then used to construct Voronoi polygons using the ‘Voronoidiagram’ function. Areas of the  
525 Voronoi polygons were determined from the vertices with the function ‘polyarea’. Multiscale  
526 segmentation of the STORM images was carried out using an automatic thresholding scheme in  
527 which the thresholds were defined by comparing the Voronoi area distribution of the localizations  
528 to a reference distribution of the expected Voronoi areas of random coordinates drawn from a  
529 spatial uniform distribution (Levet et al., 2015). The reference distribution was estimated with a  
530 Monte-Carlo simulation. The first threshold was selected as  $\rho=\delta$ , where  $\rho$  is the threshold and  $\delta$  is  
531 the average Voronoi area for a uniform distribution of localizations. After applying this first  
532 threshold, the intersection between the Voronoi polygon area distribution and the distribution of  
533 Voronoi polygon areas corresponding to the Monte Carlo simulation was identified and applied as  
534 the second threshold. This procedure was iterated multiple times to define several thresholds at  
535 increasing density.

536

### 537 **Antibodies**

538 The following antibodies were used in this study:

Antibodies	Company	cat#
Rabbit anti-H3K9me2	Active Motif	39239
Mouse anti-H3K9me2S10ph	Active Motif	61429
Rabbit anti-H3K9me3	Abcam	ab8898
Rabbit anti-H3K27me3	EMD Millipore	07-499
Rabbit anti-Lamin B1	Abcam	ab16048
Goat anti-Lamin B	Santa Cruz	sc-6216
Goat anti-Lamin B	Santa Cruz	sc-6217
Mouse anti-Lamin A/C	Santa Cruz	sc-376248
Mouse NPCs/mAb414	Abcam	Ab24609
Mouse anti-LMN1	HBUI	LMN1
Rabbit anti-histone H3	Abcam	ab1791
Rabbit anti-GFP	Abcam	ab290
Blocking peptides		
H3K9me2	Abcam	ab1772

H3K9me3	Abcam	ab1773
H3K27me2	Abcam	ab1781
H4K20me2	Abcam	ab14964
H3K9me0	EpiCypher	12-0001
H3K9me1	EpiCypher	12-0010
H3K9me2	EpiCypher	12-0011
H3K9me3	EpiCypher	12-0012
H3K9me2S10ph	EpiCypher	12-0093
<b>Secondary Antibodies</b>		
Donkey anti-rabbit AlexaFluor 555	Invitrogen	A11010
Donkey anti-Rabbit AlexaFluor 488	Invitrogen	A21206
Donkey anti-Rabbit AlexaFluor 568	Invitrogen	A10042
Donkey anti-Rabbit AlexaFluor 647	Invitrogen	A31573
Donkey anti-Mouse AlexaFluor 488	Invitrogen	A21202
Donkey anti-Mouse AlexaFluor 568	Invitrogen	A10037
Donkey anti-Goat AlexaFluor 488	Invitrogen	A11055
Donkey anti-Goat AlexaFluor 568	Invitrogen	A11057
Donkey anti-Goat AlexaFluor 647	Invitrogen	A21447
anti-rabbit IgG, HRP-linked	Cell Signaling	7074

539

540 **Antibody validation**

541 To test anti-H3K9me2 (Active Motif, cat#39239, lot#28214002) antibody specificity for  
542 immunofluorescence assay, a set of short peptides mimicking histone tail lysine methylation was  
543 used. H3K9me2 antibodies were preincubated with blocking peptides according to manufacturer's  
544 recommendations (1µg of the antibody with 1µg of a peptide) in 1 ml of antibody blocking buffer  
545 (1% BSA in PSBT), then used for immunostaining.

546

547 **DNA oligo FISH probe design and generation**

548 Target regions were based on constitutive LADs (LADs) or constitutive inter-LADs (non-LADs)  
549 as previously defined (Meuleman et al., 2013). For LADs, regions were selected only if they were  
550 also defined as LADs according to both LaminB and H3K9me2 ChIPseq data from (Poleshko et  
551 al., 2017); for non-LADs, regions were selected only if they were also defined as non-LADs

552 according to both LaminB and H3K9me2 ChIPseq data from Poleshko et al., 2017 (Poleshko et  
553 al., 2017). Two to three of each, LAD and non-LAD, regions per mouse autosome were chosen  
554 for generation of DNA oligo libraries (Table S1). Oligopaint libraries were designed using the  
555 OligoMiner pipeline (Beliveau et al., 2018). Sequences of 42 nucleotides of homology to the  
556 regions of interest were mined from the mouse mm9 genome build using the default parameters of  
557 OligoMiner. Each probe was designed to target a 250 kb region of sequence at a density of 4  
558 probes/kb when possible. Single stranded probes were produced using PCR, T7 RNA synthesis,  
559 and reverse transcription as described previously (Rosin et al., 2018).

560

### 561 **Western blot**

562 Lysates were run on 4-12% Bis-Tris protein gels (Invitrogen #NP0335) and blots were probed  
563 with anti-GFP (Abcam #ab290, 1:5000) or anti-H3 (Abcam #ab1791, 1:7500) primary antibodies  
564 according to the instructions of the manufacturer. Anti-rabbit HRP-conjugated secondary antibody  
565 (Cell Signaling #7074) was used at 1:7500. Visualization was achieved using ECLPrime (GE Life  
566 Sciences #RPN2232).

567

### 568 **ChIP-seq tracks**

569 The accession number for the ChIP-seq data referenced (Poleshko et al., 2017) is NCBI GEO:  
570 GSE97878.

571

### 572 **Statistical analysis**

573 Statistical analyses were performed with Graphpad PRISM 8.0.1 software (Graphpad Software,  
574 Inc.) using ANOVA one-way non-parametric Kruskal-Wallis test with multiple comparison  
575 correction using Dunn's test, or unpaired non-parametric Student's t-test (Mann-Whitney).

576

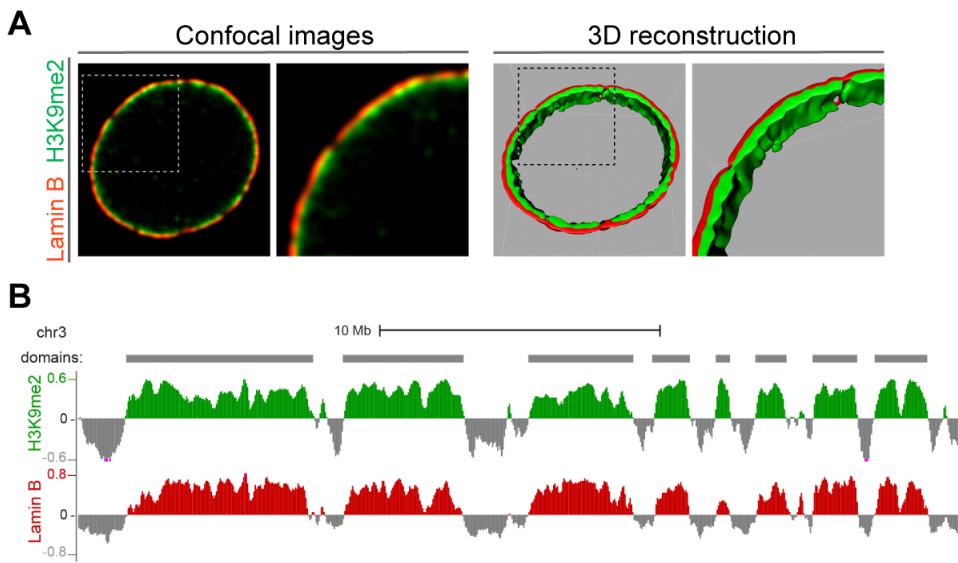
577 **Supplemental Information**

578 Supplemental Information includes six supplemental figures, one supplemental table, and three  
579 movies.

580

581

Supplemental Figure 1



582

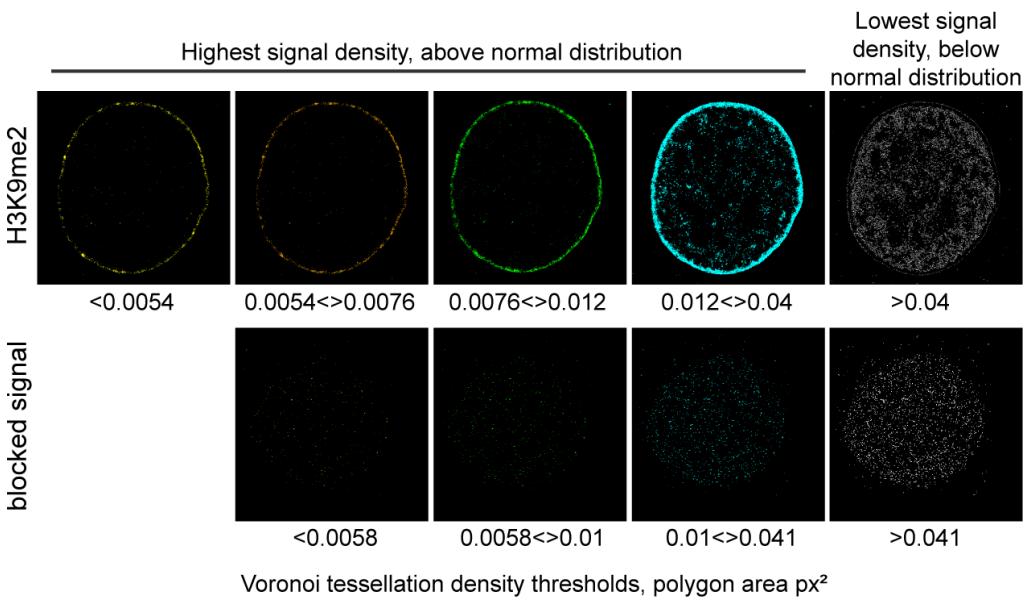
583

584

585 **Supplemental Figure S1. H3K9me2-marked chromatin localizes specifically at the nuclear periphery**  
586 **and forms large heterochromatin domains.** (A) Representative confocal images of the H3K9me2-marked  
587 chromatin (green) localizes at the nuclear lamina (Lamin B, red) of mouse ESCs. 3D-image reconstruction  
588 (right panels) demonstrates H3K9me2 heterochromatin layer at the nuclear lamina. (B) Representative  
589 H3K9me2 and Lamin B ChIP-seq tracks from mESCs illustrating lamina-associated domains specifically  
590 enriched for H3K9me2 and Lamin B.

591

Supplemental Figure 2



592

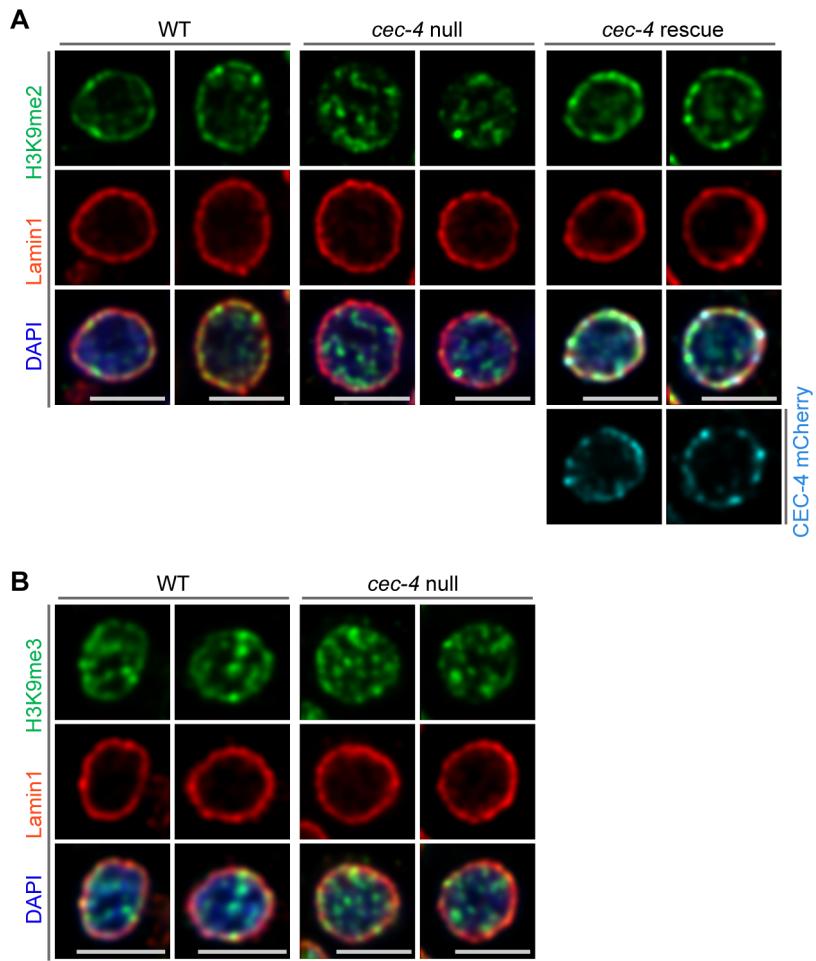
593

594 **Supplemental Figure S2. H3K9me2 signal distribution is specific at the nuclear periphery.**

595 Representative STORM images of the H3K9me2 signal with or without blocking peptides after applying  
596 the automatic thresholding based on Voronoi tessellation (see methods) and shown from highest density  
597 (yellow) to lowest density (gray), illustrating separation of H3K9me2 and blocked signal. Specific  
598 H3K9me2 signal is localized at the nuclear periphery forming a layer of peripheral heterochromatin.

599

Supplemental Figure 3



600

601

602 **Supplemental Figure S3. Localization of H3K9me3-marked chromatin in *C. elegans* wild-type (WT),**

603 *ce $c$ -4-null*, and *ce $c$ -4-rescue* embryo cells. (A) Additional representative immunofluorescent confocal

604 images of *C. elegans* embryo cells illustrate H3K9me2 (green) localized to the nuclear periphery as stained

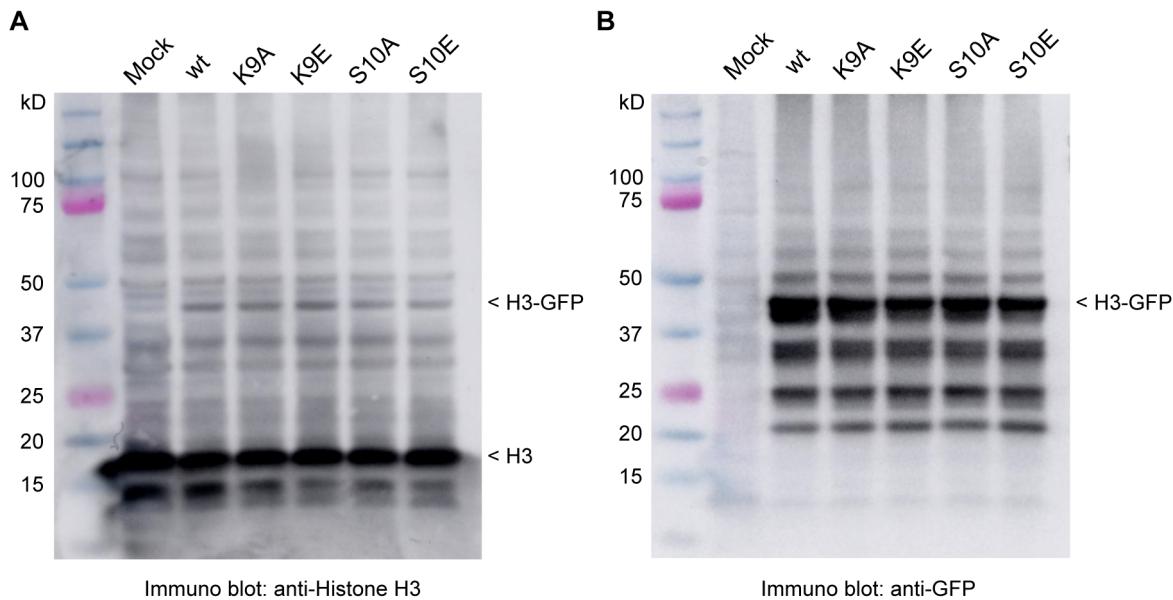
605 with Lamin1 (red) in WT and *ce $c$ -4-null* cells rescued with *ce $c$ -4* transgene (CEC-4 mCherry), but not in

606 *ce $c$ -4-null* cells; counterstained with DAPI (blue). (B) Representative immunofluorescent confocal images

607 illustrate H3K9me3 (green) distribution, counterstained with Lamin 1 (red) and DAPI (blue); Scale bars:

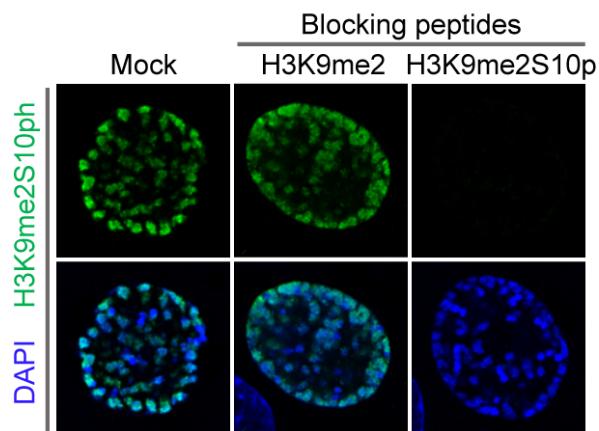
608 3 $\mu$ m.

## Supplemental Figure 4



609 Immuno blot: anti-Histone H3 Immuno blot: anti-GFP  
610  
611  
612 **Supplemental Figure S4. Expression of histone H3-GFP fusion proteins.** Histone H3 immunoblot  
613 demonstrating expression of exogenous H3-GFP fusion proteins. **(A)** anti-histone H3 immunoblot; and **(B)**  
614 anti-GFP immunoblot.

Supplemental Figure 5



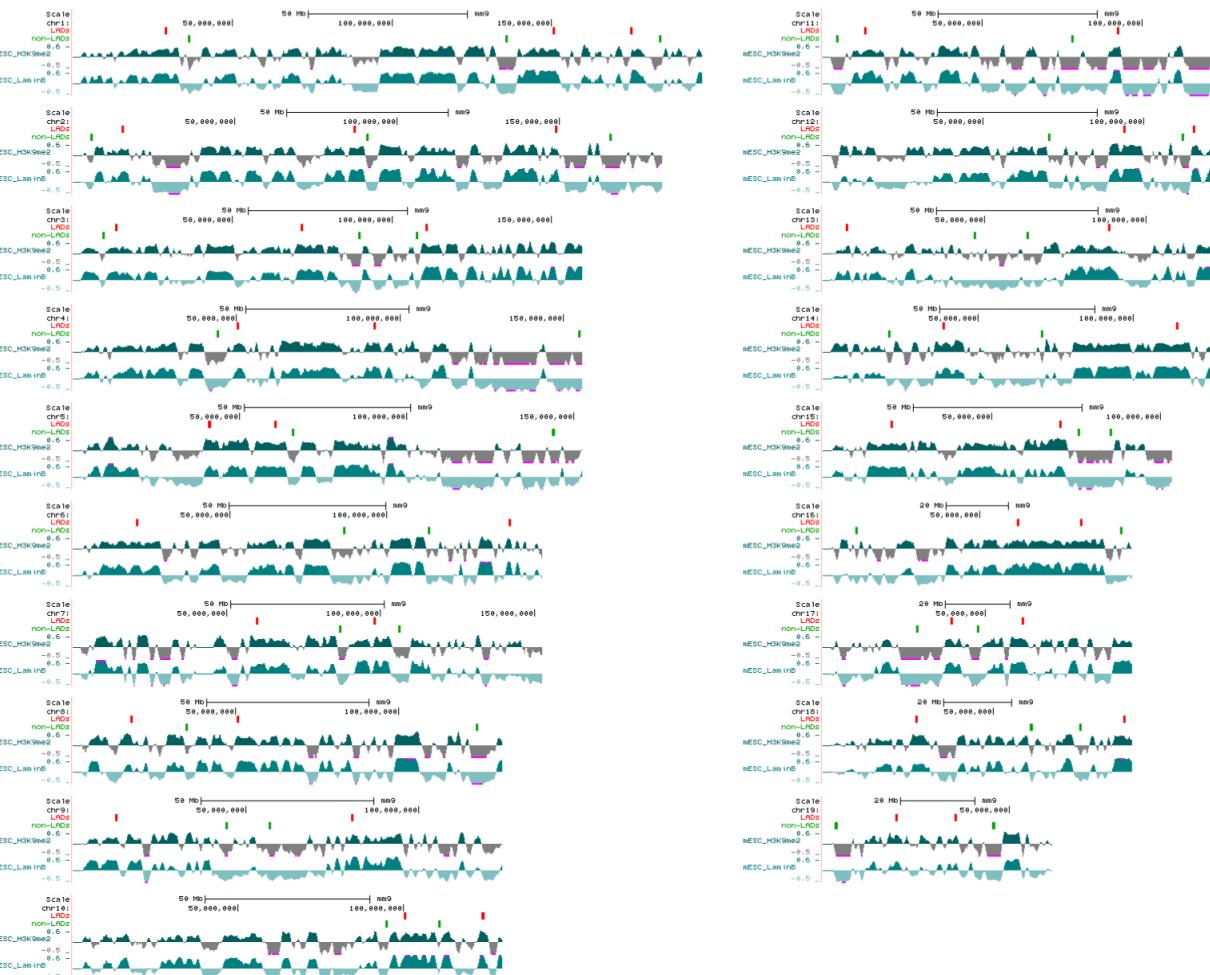
616

617

618 **Supplemental Figure S5. Anti-H3K9me2S10p antibody specificity validation.** C2C12 cells in prophase  
619 stained with H3K9me2S10p antibody preincubated with indicated blocking peptides, counterstained with  
620 DAPI.

621

Supplemental Figure 6



622

623

624

625 **Supplemental Figure S6. Location of the oligopaint DNA probes targeting LADs and non-LADs on**  
626 **mouse chromosomes.** Displayed are H3K9me2 and LaminB ChIP-seq tracks from mESCs with each  
627 region of 41 'LADs' (enriched for H3K9me2 and Lamin B signal) shown as a red bar above tracks and  
628 each region of 41 'non-LADs' (depleted for H3K9me2 and Lamin B) shown as a green bar; probes for each  
629 region span 250kb of the mouse genome (mm9).

630

631 **Supplemental Table S1.** Genomic coordinates (mm9) of regions targeted with oligopaint DNA probes.

632

	start	end	type	size		start	end	type	size	
<b>chr1</b>	29104230	29354230	LAD	250000		chr1	36434433	36684433	non-LAD	250000
<b>chr1</b>	150673087	150923087	LAD	250000		chr1	135865407	136115407	non-LAD	250000
<b>chr1</b>	174920593	175170593	LAD	250000		chr1	183973123	184223123	non-LAD	250000
<b>chr10</b>	100527334	100777334	LAD	250000		chr10	94872520	95122520	non-LAD	250000
<b>chr10</b>	124051755	124301755	LAD	250000		chr10	110869061	111119061	non-LAD	250000
<b>chr11</b>	13179019	13429019	LAD	250000		chr11	4582747	4832747	non-LAD	250000
<b>chr11</b>	92370539	92620539	LAD	250000		chr11	77940228	78190228	non-LAD	250000
<b>chr12</b>	93813781	94063781	LAD	250000		chr12	70473766	70723766	non-LAD	250000
<b>chr12</b>	115634346	115884346	LAD	250000		chr12	111962534	112212534	non-LAD	250000
<b>chr13</b>	7489538	7739538	LAD	250000		chr13	47028288	47278288	non-LAD	250000
<b>chr13</b>	88441235	88691235	LAD	250000		chr13	63199709	63449709	non-LAD	250000
<b>chr14</b>	38703956	38953956	LAD	250000		chr14	21358915	21608915	non-LAD	250000
<b>chr14</b>	113906951	114156951	LAD	250000		chr14	70419884	70669884	non-LAD	250000
<b>chr15</b>	20289182	20539182	LAD	250000		chr15	75828606	76078606	non-LAD	250000
<b>chr15</b>	70422540	70672540	LAD	250000		chr15	85276609	85526609	non-LAD	250000
<b>chr16</b>	61890914	62140914	LAD	250000		chr16	10659379	10909379	non-LAD	250000
<b>chr16</b>	82120430	82370430	LAD	250000		chr16	94760705	95010705	non-LAD	250000
<b>chr17</b>	39538078	39783931	LAD	245853		chr17	28970900	29220900	non-LAD	250000
<b>chr17</b>	61496613	61746613	LAD	250000		chr17	47803613	48053613	non-LAD	250000
<b>chr18</b>	27523365	27738657	LAD	215292		chr18	61099822	61349822	non-LAD	250000
<b>chr18</b>	88450557	88700557	LAD	250000		chr18	75521659	75771659	non-LAD	250000
<b>chr19</b>	19633267	19883267	LAD	250000		chr19	3551051	3801051	non-LAD	250000
<b>chr19</b>	35509829	35759829	LAD	250000		chr19	45622537	45872537	non-LAD	250000
<b>chr2</b>	15379049	15629049	LAD	250000		chr2	5573731	5823731	non-LAD	250000
<b>chr2</b>	86720000	86970000	LAD	250000		chr2	90721129	90971129	non-LAD	250000
<b>chr2</b>	148980799	149230799	LAD	250000		chr2	165772679	166022679	non-LAD	250000
<b>chr3</b>	71596078	71846078	LAD	250000		chr3	9641987	9891987	non-LAD	250000
<b>chr3</b>	110735097	110985097	LAD	250000		chr3	89658552	89908552	non-LAD	250000
<b>chr3</b>	13525990	13775990	LAD	250000		chr3	107840306	108090306	non-LAD	250000
<b>chr4</b>	50271294	50521294	LAD	250000		chr4	44223436	44473436	non-LAD	250000
<b>chr4</b>	92067850	92317850	LAD	250000		chr4	154687066	154937066	non-LAD	250000
<b>chr5</b>	40813624	41063624	LAD	250000		chr5	65928768	66178768	non-LAD	250000
<b>chr5</b>	60638162	60884800	LAD	246638		chr5	143797763	144047763	non-LAD	250000
<b>chr6</b>	20389410	20639410	LAD	250000		chr6	86325063	86575063	non-LAD	250000
<b>chr6</b>	139025301	139275301	LAD	250000		chr6	113420393	113670393	non-LAD	250000
<b>chr7</b>	59721791	59971791	LAD	250000		chr7	86892565	87142565	non-LAD	250000
<b>chr7</b>	97983011	98233011	LAD	250000		chr7	105952531	106202531	non-LAD	250000
<b>chr8</b>	17936222	18186222	LAD	250000		chr8	34947799	35197799	non-LAD	250000
<b>chr8</b>	50541295	50791295	LAD	250000		chr8	123933822	124183822	non-LAD	250000
<b>chr9</b>	12396200	12646200	LAD	250000		chr9	44431734	44681734	non-LAD	250000
<b>chr9</b>	80651724	80901724	LAD	250000		chr9	56821780	57071780	non-LAD	250000

633

634 **Movie S1. 3D reconstruction of mouse ES cells in interphase.** Immunostained for Lamin B1  
635 (cyan) and hybridized with fluorescent oligo probes for LADs (red) and non-LADs (green), and  
636 counterstained with DAPI (blue).

637

638 **Movie S2. 3D reconstruction of mouse ES cells in metaphase.** Immunostained for Lamin B1  
639 (cyan) and hybridized with fluorescent oligo probes for LADs (red) and non-LADs (green), and  
640 counterstained with DAPI (blue).

641

642 **Movie S3. 3D reconstruction of mouse ES cells in telophase.** Immunostained for Lamin B1  
643 (cyan) and hybridized with fluorescent oligo probes for LADs (red) and non-LADs (green), and  
644 counterstained with DAPI (blue).

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