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The impact of DNA methylation on CTCF-mediated 3D genome organization

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24 **Abstract**

25 Cytosine DNA methylation is a highly conserved epigenetic mark in eukaryotes. While the role
26 of DNA methylation at gene promoters and repetitive elements has been extensively studied,
27 the function of DNA methylation in other genomic contexts remains less clear. In the nucleus
28 of mammalian cells, the genome is spatially organized at different levels, and strongly
29 influences myriad genomic processes. There are a number of factors that regulate the 3D
30 organization of the genome, with the CTCF insulator protein being among the most well-
31 characterized. Pertinently, CTCF binding has been reported as DNA methylation sensitive in
32 certain contexts, perhaps most notably in the process of genomic imprinting. Therefore, it
33 stands to reason that DNA methylation may play a broader role in regulation of chromatin
34 architecture. Here we summarize the current understanding that is relevant to both the
35 mammalian DNA methylation and chromatin architecture fields, and attempt to assess the
36 extent to which DNA methylation impacts the folding of the genome. The focus is in early
37 embryonic development and cellular transitions, when the epigenome is in flux, but we also
38 describe insights from pathological contexts such as cancer, when the epigenome and 3D
39 genome organization are misregulated.

40 Introduction

41 Proper chromosome organization within the nucleus of eukaryotic cells is critical for genome-
42 associated processes, including transcription, gene regulation, DNA replication,
43 recombination and repair and cell division. Ever-improving genomics and imaging assays
44 have revealed that chromosomal DNA in the mammalian cell nucleus adopts an intricate
45 hierarchical organization (Fig. 1). Most coarsely, chromatin is organized into chromosome-
46 scale territories, resulting in a strong preference for intra- over inter-chromosomal contacts<sup>1-
47 3</sup>. These territories are further subdivided into multi-megabase (Mb) compartments that are
48 defined by their transcriptional activity³: the **more active** “A compartments” that are typically
49 gene rich, **transcriptionally active** and located in the interior of the nuclear space, and the **less**
50 **active** “B compartments” that are gene poor and **frequently associated** with the nuclear lamina
51 and the nucleolus⁴⁻⁸. Using Hi-C, a sequencing-based assay to quantify contacts between
52 genomic domains, these compartments were found to preferentially engage in homotypic
53 contacts. Compartments further divide into Topologically Associating Domains (TADs), which
54 are sub-Mb scale insulated domains that are relatively invariant between cell-types and whose
55 position is highly conserved in mammals⁹. Within TADs, DNA loops can be formed, which
56 either facilitate or insulate interactions between promoters and *cis*-regulatory elements, such
57 as enhancers. Unlike TADs, these loops can vary tremendously between cell types, where
58 they can be crucial for determining proper cell identity¹⁰. This three-dimensional (3D)
59 conformation of the genome is not static though, with a particularly dramatic reorganization of
60 the genome occurring during mitosis¹¹. The **plastic** 3D chromosome organization is important
61 during other time-frames as well, such as cell-state transitions, aging and in disease^{12,13}.

62 Understanding the processes that underpin chromosome conformation is an area of
63 intense research interest. Perhaps the most studied factor that regulates 3D genome
64 organization is the CCCTC-binding factor (CTCF). CTCF is conserved among bilaterians and
65 in mammalian cells it binds tens of thousands of sites in the genome¹⁴⁻¹⁶. CTCF plays a key
66 role at the boundaries between TADs where it stabilizes the residence of the Cohesin protein
67 complex. In turn, the ring-shaped Cohesin complex regulates genome folding by creating DNA
68 loops through a process of “loop extrusion” until CTCF boundaries are encountered¹⁷⁻¹⁹. In
69 parallel, CTCF also plays a direct role in transcriptional control by regulating DNA loops
70 between enhancers and promoters^{20,21}. CTCF is essential in mammals, likely owing to its role
71 in coordinating gene expression programs. Indeed, the complete knockout (KO) of CTCF in
72 mice is lethal during early embryogenesis, and heterozygous knockouts show a predisposition
73 to cancer^{22,23}.

74 What then drives the dynamic landscape of CTCF binding? While multiple mechanisms
75 are likely at play, including co-factors, other transcription factors, chromatin remodeling
76 complexes and **RNA binding**²⁴⁻²⁶, one compelling candidate is 5-methylcytosine DNA
77 methylation (5meC). Indeed, CTCF-binding is 5meC sensitive in certain contexts²⁷⁻²⁹. In
78 mammals, 5meC is typically found in the CpG dinucleotide context, where it is usually
79 associated with transcriptional repression when present at regulatory elements. Outside of
80 promoter regions, the vast majority of CpGs (approximately 80%) are methylated in somatic
81 tissues. While 5meC is typically considered a stable epigenetic mark, key exceptions are intra-
82 and intergenic transcription factor binding sites, where turnover of the methyl-mark is high³⁰.
83 Moreover, 5meC is dramatically remodeled during embryonic development and subsequent
84 cell lineage specification. After fertilization, most gametic 5meC marks are erased, followed
85 by a major wave of *de novo* methylation during implantation of the embryo via the action of
86 the DNA METHYLTRANSFERASE 3A and 3B (DNMT3A and 3B) enzymes, after which
87 embryonic DNA methylation is globally maintained by DNMT1³¹. **Dynamic changes** in DNA
88 methylation at certain promoters and enhancers **are** essential for the exit of pluripotency and
89 lineage specification³²⁻³⁴. Mouse embryos deficient for DNA methylation machinery exhibit
90 **significant** developmental abnormalities, which leads to embryonic lethality in the most
91 extreme cases^{35,36}. Notably, substantial changes in chromatin conformation are also observed
92 during this period³⁷⁻³⁹.

93 How chromatin architecture and DNA methylation help to determine cellular identity has
94 been the topic of heightened research interest⁴⁰. Despite large amounts of high-resolution data
95 across many tissue types in healthy, mutant, and pathological conditions, the broad
96 implications of the interplay between 5meC and CTCF still remain nebulous. In this review,
97 we will summarize direct and indirect evidence to provide the most up-to-date model of the
98 relationship between DNA methylation and CTCF-mediated chromosome organization and its
99 biological importance for cellular identity and function. Moreover, we will discuss how 5meC
100 may influence genome architecture outside of the CTCF context.

101 **The molecular basis for 5meC-mediated CTCF regulation**

102 CTCF contains 11 zinc fingers (ZFs), of which ZF3-ZF7 bind to an essential but variable core
103 motif of 15 nucleotides²⁸. This degenerate core motif contains potential cytosine methylation
104 sites, most prominently at positions 2 and 12 (C2 and C12) within the 15-base pair binding
105 motif, which can be CpG dinucleotides (Fig. 2a). *In vitro* fluorescence-based DNA binding
106 assays have shown that methylation of the C2 reduces the affinity in the binding of CTCF
107 around 23 fold, whereas the methylation at C12 did not noticeably impair binding²⁸. By
108 concurrently analyzing bisulfite sequencing data and chromatin immunoprecipitation followed
109 by sequencing (ChIP-Seq) data from diverse human cell types, it was observed that
110 approximately 40% of the variability in CTCF binding is associated with differential DNA
111 methylation. This variation is particularly concentrated at the C2 and C12 positions, which
112 could indicate an *in cellula* role for C12 that was not observed *in vitro*, or simply could be a
113 correlation⁴¹. In a systematic inventory of bound CTCF motifs in mouse embryonic stem cells
114 (mESCs), roughly 10% of motifs contained a CpG at one or both positions, suggesting the
115 possibility for direct impact of 5meC at these sites⁴². While this is a minority of sites, it still
116 represents a sizeable number—upwards of 17,000—of genomic loci where 5meC may
117 regulate the binding of CTCF, which may in turn impact gene regulation (Fig. 2b,c). Recently,
118 single molecule footprint analysis combined with ChIP-Seq data in mESC showed that the
119 presence of a CpG at C4 in the CTCF binding site was found to affect CTCF binding affinity
120 as well, thereby further expanding the potential for 5meC-mediated regulation⁴³. At a subset
121 of sites, additional upstream and downstream motifs have also been identified, which may
122 contain CpG dinucleotides, and thus may affect the affinity of CTCF for DNA binding or
123 otherwise affect its binding kinetics^{28,29,44}. Finally, CTCF binding can also be hindered by
124 presence of 5meC outside of its binding motifs, for example through combinatorial action with
125 other 5meC sensitive transcription factors, or through more local 5meC-dependent changes
126 to chromatin accessibility that may impair the access of CTCF to the DNA^{45,46}. Combined,
127 these mechanisms can explain sequence-independent 5meC-mediated reorganization of
128 CTCF binding during normal cellular differentiation and lineage specification and perturbed
129 organization in developmental defects and disease.

130 **CTCF regulation of genomic imprints**

131 The antagonistic relationship between CTCF binding and 5meC was first described at gene
132 clusters harboring genomic imprints^{47,48}. Genomic imprinting refers to mono-allelic gene
133 expression in a parent-of-origin-specific manner, which affects between 100 to 200 genes in
134 human and mouse cells⁴⁹. Imprinted gene clusters are subject to *cis* regulation by Imprinting
135 Control Regions (ICRs), which exhibit differential 5meC marks on the parental alleles. The
136 *Igf2-H19* imprinted domain, which is conserved from mice to humans, is a prime example of
137 how 5meC can regulate CTCF binding, genome conformation and gene expression. At this
138 domain, the ICR known as the *H19* differentially methylated region (DMR) is methylated on
139 the paternal allele. CTCF binds to the unmethylated maternal *H19* DMR, thereby blocking the
140 activation of the *Igf2* gene by upstream enhancers, which in turn allows the expression of the
141 *H19* long non-coding RNA (lncRNA). Conversely, on the paternal allele, 5meC impairs CTCF
142 binding between the enhancer and the *Igf2* promoter, thus allowing the activation of this gene
143 (Fig. 3a)^{47,48}. Chromosome conformation capture (3C) techniques performed in mESCs that
144 maintain genomic imprinting have provided much greater depth of insight into how imprinted
145 CTCF binding can regulate mono-allelic gene activity: the presence of CTCF on the maternal

146 chromosome creates a sub-TAD that increases the insulation between the regulatory
147 elements near the *H19* DMR and the more telomeric *Igf2* gene (Fig. 3b)^{50–52}.

148 Although imprinted chromatin structure at the *Igf2-H19* is the most well-characterized,
149 there are other imprinted genes that are regulated by allele-specific CTCF binding, such as at
150 the *Peg13-Kcnk9*⁵³ and *Kcnq1*⁵⁴ domains, where CTCF binds to the unmethylated paternal
151 allele. It has also been shown in mESCs and mouse neural progenitor cells (NPCs) that
152 binding of CTCF on the maternal unmethylated allele of the imprinted *Meg3-Dlk1* locus is
153 necessary for the formation of an allelic sub-TAD, which is required for the proper activation
154 of the imprinted *Dlk1* gene during neural differentiation⁵⁰. Given the preponderance of 5meC-
155 mediated CTCF binding regulation at imprinted gene clusters, it would not be unreasonable to
156 think this is a universal regulatory mechanism throughout the genome. However, the study of
157 imprinted gene regulation is greatly facilitated by the coexistence of both imprinted alleles
158 within the same nucleus, allowing for direct comparison. In contrast, characterizing the impact
159 of 5meC on genome-wide CTCF binding dynamics during differentiation, development and
160 disease remains a much more complex undertaking.

161 **5meC DNA methylation impacts CTCF occupancy at a substantial number of binding** 162 **sites**

163 At least 30% of CTCF binding sites in human cells, obtained from different tissues, are cell-
164 type specific. However, the mechanisms that govern the full repertoire of differential CTCF
165 binding at these sites remain to be identified. Nonetheless, around 40% of variability could be
166 linked to 5meC status in the binding site²⁷. It should be noted that this data was generated
167 over a decade ago as of this writing, and meta-analysis of more recent data sets may arrive
168 at a different value. Functional studies have further been carried out to demonstrate that the
169 methyl-mark, *per se*, antagonizes CTCF binding *in vivo*. For example, deposition of 5meC in
170 key CpG(s) of a CTCF motif by epigenome editing at specific loci caused reduced CTCF
171 binding, with a loss of insulation mediated by enhancer-promoter interactions in human
172 HEK293 cells⁵⁵. Moreover, CTCF binding in DNMT3B/DNMT1 double knockout (DKO)
173 HCT116 human colon cancer cells were moderately reorganized as well. In these cells, the
174 decrease in 5meC levels had a minor effect on global CTCF occupancy, but specific binding
175 sites were detected that either presented increased CTCF occupancy or *de novo* binding sites
176 when compared to the control cells⁵⁶. And thirdly, CTCF binding in mESCs with mutations in
177 the *Ten-eleven translocation 1* and 2 (*Tet1* and *Tet2*) methyl-cytosine dioxygenases, which
178 induce 5meC demethylation, confirmed that gain in methylation can create loss of CTCF
179 binding⁴⁶. This study showed that CTCF binding was lost at sites with low CpG density and
180 was retained at sites with high CpG density. These findings support the idea that CTCF binding
181 within CpG islands (regions of exceptional CpG density) remain mostly constant, while outside
182 of CpG islands, CTCF binding is influenced by competition with nucleosomes. These
183 experiments further demonstrated that DNA methylation can also affect CTCF binding by re-
184 positioning of nucleosomes in the vicinity of the binding site⁴⁶, thereby confirming that the
185 epigenomic context is important as well for CTCF binding. Recently, a single molecule
186 footprinting study of *cis* regulatory elements (CREs) in mESCs showed that DNA methylation
187 can either be antagonistic or neutral to CTCF depending on the locus, with a subset of binding
188 sites bearing a nucleosomal, inaccessible signature when methylated⁴³.

189 These aforementioned studies come with caveats. Firstly, it is important to highlight that
190 the 5meC-CTCF relationship is not unidirectional. The strength of CTCF binding is associated
191 with the likelihood of a cytosine within the binding site being in a demethylated state,
192 suggesting that CTCF occupancy can play a role in actively influencing the turnover of DNA
193 methylation⁵⁷. This observation is corroborated with the fact that mutations in the ZF8 of CTCF,
194 affecting its chromatin residence time, have the potential to trigger extensive genome-wide
195 hypermethylation⁵⁸.

196 Secondly, although the relationship between 5meC and CTCF has been extensively
197 investigated, there is also interest in understanding how TET-mediated oxidation of 5meC
198 could affect CTCF binding to DNA. TET enzymes progressively catalyze 5-
199 hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), which

200 lead to demethylation via active and passive mechanisms^{46,59}. However, data suggests that
201 5hmC and 5fC, *per se*, could alter nucleosome stability that would facilitate CTCF binding^{46,60}.
202 Reinforcing this idea, enzymatic mapping of 5hmC in mESCs also showed the presence of
203 this modification in and around CTCF binding sites⁶¹. Moreover there is evidence that 5caC
204 leads to increased CTCF binding at normally weakly bound sites⁶². Collectively, these findings
205 illustrate that oxidated derivatives of cytosines are not only precursors to demethylation, but
206 also may themselves impact the CTCF binding landscape.

207 In sum, genomic analyses in both mouse and human cells indicate that DNA methylation
208 can indeed act as antagonist to CTCF binding at a substantial number of binding sites. TET
209 activity may promote CTCF binding either through DNA demethylation or oxidizing 5meC.
210 However, the vast majority of CTCF binding sites are insensitive to 5meC, either because they
211 are constitutively unmethylated or because CpGs are present at positions where CTCF is
212 impervious to the mark. These differences can be due to a combined involvement of both the
213 CTCF core motif as well as the surrounding chromatin context. Given its well-known role as a
214 transcriptional regulator, it is reasonable to presume that at least a proportion of the subset of
215 5meC-sensitive CTCF binding sites in the genome can impact gene expression⁶³. Yet, given
216 the highly confounding effects in both *Dnmt* and *Ctcf* mutants, definitively assigning changes
217 in gene expression to 5meC dynamics and differential CTCF binding remains challenging.
218 Continued research, possibly involving precise manipulations of selected gene loci—for
219 instance certain imprinted loci—will be necessary to ascertain the full scope of this form of
220 gene control.

221 **DNA methylation plays a minor role in TAD (re)organization**

222 During early mammalian embryogenesis, both 3D genome organization and DNA methylation
223 are in flux, thereby providing an interesting system for insights into how 5meC shapes
224 chromatin architecture. Hi-C experiments in early mouse development showed that TAD
225 structure in zygotes is weak and diffuse. In mice throughout embryogenesis, TAD formation
226 gradually increases, with highly resolvable organization detected in the epiblast stage after
227 implantation (Embryonic Day 7.5 [E7.5])^{39,64}. A similar pattern is observed in humans, where
228 data indicate that TADs are mostly absent in 2-cell embryos, exhibit weak presence in 8-cell
229 embryos, and become progressively more apparent in embryos during the implantation
230 stage⁶⁵. In mammals, 5meC is mostly erased after fertilization, but by E7.5, DNA methylation
231 patterns have been reestablished, and remain high in all somatic tissues (Fig. 4). Particularly
232 notable is the fact that at E3.5-E4.5 in mouse cells, when DNA methylation levels are at their
233 lowest (~20% CpG methylation), the structure of TADs has essentially been established and
234 the subsequent wave of *de novo* DNA methylation does not appear to grossly alter the TAD
235 organization landscape. The occurrence of comparable chromatin architecture embryonic
236 transitions in zebrafish, a non-mammalian vertebrate lacking 5meC reprogramming during
237 embryo development, further strengthens the idea that *de novo* methylation does not
238 significantly influence large-scale 3D chromatin structure in vertebrates^{66,67}.

239 Experiments in relevant cell culture systems undergird these *in vivo* observations.
240 mESCs are derived from the inner cell mass of the blastocyst—the stage when the genome
241 is DNA hypomethylated. In addition to their ability to differentiate to all embryonic tissue types,
242 they can also withstand a complete absence of the 5meC machinery⁶⁸, which is not the
243 case for human ESCs⁶⁹. Combined, this makes these cells a convenient and widely used
244 model to study the involvement of 5meC in developmental processes. Electron microscopy
245 revealed that the absence of 5meC in *Dnmt3A/B* DKO and *Dnmt1/3A/B* triple KO (*Dnmt* TKO)
246 cells did not affect the ultra-structure of chromatin⁷⁰. Hi-C in DKO and TKO mESCs indicates
247 a minimal, if not entirely absent, impact on global TAD organization as well⁷¹. This result is not
248 unexpected, as the CTCF binding landscape in TKO ESCs is globally unperturbed as well³⁰.
249 Similarly, during differentiation of DKO/TKO mESCs towards cardiac myocytes there is
250 virtually no effect in A/B compartment establishment or TAD formation⁷¹.

251 Altogether, these data suggest that DNA methylation does not have a major impact on
252 genome wide higher-order 3D genome organization and particularly TAD structure. At many
253 TAD boundaries, multiple CTCF binding sites are grouped together⁷² and CTCF presence is

254 needed for the correct formation of TADs during embryonic development⁶⁵; the combination
255 and strength of the individual binding sites, and the potential for non-CpG containing sites to
256 compensate, may provide most TADs with resilience to buffer 5meC changes. Instead, 5meC
257 may influence boundaries at specific loci, like at imprinted domains, thereby having a more
258 local impact on chromatin architecture (Fig. 2c). However, much of the data described in this
259 section does not have the resolution to detect finer scale interactions.

260 **CTCF & DNA methylation in cancer**

261 While studies in mESCs have provided key insights into mammalian developmental dynamics,
262 models of human disease can also reveal and illuminate aspects of 5meC- / CTCF-mediated
263 regulation. DNA methylation is misregulated in many diseases, with cancer as a notable
264 example. In many cancers, the promoters of cancer suppressor genes are hypermethylated,
265 whereas hypomethylation of repeats, transposable elements and oncogenes can influence
266 the initiation or progression of tumors, as well⁷³. Furthermore, CTCF itself has been reported
267 to act as a tumor suppressor⁷⁴, with its haploinsufficiency in mouse cells both destabilizing
268 DNA methylation and orienting cells towards tumour progression²³. The genome-wide analysis
269 of six types of human cancers revealed that a substantial number of changes in CTCF binding
270 could be explained by changes in DNA methylation in the region where the factor binds⁷⁵. In
271 human gastrointestinal stromal tumors, the hypermethylation of over 600 CTCF binding sites
272 caused a loss of binding. CRISPR-mediated excision of one of these CTCF motifs in a
273 gastrointestinal cancer cell line (GIST-T) generated disrupted chromatin contacts around a
274 super-enhancer, resulting in a strong transcriptional upregulation of the *FGF4* oncogene⁷⁶.
275 Similarly, multiple studies have identified examples of impaired CTCF binding that are directly
276 associated with changes in 5meC and oncogene activation (Fig. 5a). For example, in human
277 *IDH* mutant gliomas, the hypermethylation of a CTCF binding site causes a reduction in CTCF
278 binding, a loss of insulation between two TADs, and ultimately the activation of a glioma
279 oncogene^{77,78}. Furthermore, at the *INK4B-ARF-INK4A* (*INK/ARF*) locus—composed of three
280 tumor suppressor genes—the gene activity is regulated by a 5meC sensitive CTCF binding
281 site that is highly methylated in some cancer types. Treatment of such cells with the
282 demethylating agent 5-aza-2'-deoxycytidine leads to CTCF binding and de-repression of the
283 tumor suppressor locus⁷⁹ (Fig. 5b). Based on these data, it could be argued that key genes
284 are regulated by methyl-sensitive CTCF binding sites.

285 Hypomethylation and loss of imprinting of the *IGF2-H19* imprinted domain have been
286 described as an important factor of tumor progression. Although there is no formal evidence
287 yet that epimutations at this locus affect chromatin structure^{80,81}. Nonetheless, such
288 methylation changes have been shown to affect *H19* and *IGF2* expression, and the
289 methylation status of the CTCF binding site^{82,83}. This may indicate that 3D organization is
290 probably also affected in these tumors. Global and local aberrations in 5meC patterns in cells
291 can thus have an influence on chromatin contacts and gene expression, and directly affect
292 cancer emergence and progression.

293 **CTCF-independent impact of 5meC changes on 3D genome organization**

294 Although we have mostly focused on the impact of 5meC through the key genome organizer
295 CTCF, other DNA methylation-sensitive chromatin-associated machineries may regulate 3D
296 chromosome organization as well. Notably, Polycomb Repressive Complex (PRC) 1 and 2
297 modulate chromatin topology both at the local scale and over long distances^{84–86}. Indeed,
298 within extensive genomic regions characterized by minimal 5meC methylation, known as
299 "DNA methylation canyons," the presence of elevated H3K27me3 levels becomes essential
300 for preserving large-scale 3D genomic interactions⁸⁷. Analysis of mESCs that lack an intact
301 Cohesin complex reveals that DNA contacts between specific sets of genomic regions remain
302 despite a nearly complete loss of TADs and strongly perturbed A/B compartment organization.
303 Most of these maintained contacts correspond to loci that are marked by Polycomb group
304 (PcG) proteins, and these contacts disappear in the absence of PRC1⁸⁸. In mESCs, many of
305 the chromatin contacts that are controlled by PRC1 have a role during the naïve-to-primed
306 pluripotency transition, where they regulate developmental genes such as the *Hox* cluster^{84,89}.

307 Developmental genes often present unusually large hypomethylated regions—termed “DNA
308 methylation valleys”—that are marked by the PRC2-associated histone 3 lysine 27
309 trimethylation (H3K27me3) and that serve as a platform for PRC1 binding⁹⁰. In the absence of
310 *Eed*, a core PRC2 subunit, these DNA methylation valleys gain methylation and the chromatin
311 organization of some developmental genes, such as *Hox* clusters, is lost⁹⁰. During embryonic
312 development, broad Polycomb-marked domains recede as 5meC is deposited, likely linked
313 with the antagonism between DNA methylation and Polycomb group (PcG) proteins^{91–93}. This
314 may indicate that 5meC is impacting Polycomb-mediated genomic contacts during this
315 window. Moreover, in situations where DNA methylation is aberrant, such as in cancer, 5meC
316 may be affecting the presence of PcG proteins in some regions and thereby affecting
317 chromatin contacts and gene expression. Future work will be needed to formally demonstrate
318 the extent to which DNA methylation is antagonizing PcG, and furthermore if impaired
319 Polycomb-mediated 3D genome organization has a biologically relevant impact on underlying
320 gene expression.

321 Beyond the CTCF and PcG proteins, we can envision that other components of
322 chromatin regulation can also interact with 5meC to organize the genome in 3D space. [The
323 introduction of murine DNA methyltransferases into *Saccharomyces cerevisiae* results in
324 heightened chromatin condensation in peri-centromeric regions. This process reduces the
325 overall flexibility of DNA and promotes a heterochromatin state, suggesting an involvement in
326 chromatin condensation, even in the absence of specific protein machinery capable of
327 recognizing methylation signals⁹⁴. Bolstering this concept is nuclear lamina organization. The
328 nuclear lamina line the nuclear envelope and are generally associated with H3K9me2-marked
329 heterochromatic regions in the genome^{7,95}. Although, DNA methylation is weakly associated
330 with Lamina Associated Domains \(LADs\)⁹⁶, there is evidence that DNA methylation
331 misregulation is correlated with reorganization of LADs in aging and cancer⁹⁷. However, it
332 remains to be determined if the 5meC signature is a cause or consequence of this
333 phenomenon, and the extent to which the shifting LAD landscape affects cellular processes.
334 Moreover, other 5meC-sensitive DNA binding factors, like certain transcription factors, could
335 also influence 3D organization, for instance through DNA looping^{98,99}. To our knowledge,
336 examples of such 5meC sensitive loops have not been reported \[but it should be stressed that
337 this may potentially be a technical limitation, as a significant number of Hi-C datasets
338 generated in these studies lack the resolution required for identifying loops\]\(#\). Suffice it to say,
339 our knowledge of the full extent to which DNA methylation regulates the 3D genome and the
340 underlying genes remains from complete.](#)

341 **Conclusions and perspectives**

342 Genome-wide profiling of CTCF has identified thousands of binding sites in the mouse and
343 human genomes. Whereas most of these binding sites are conserved among cell types,
344 roughly 30% are cell type specific²⁷. While DNA methylation only appears to impact a minority
345 of CTCF binding sites, this still represents a vast number—upwards of 17,000 by some
346 estimates⁷². Given the outsized role DNA methylation plays on CTCF-mediated gene control
347 at imprinted loci, it is attractive to expand such regulation to the genome-wide scale. This
348 sentiment is bolstered by cancer studies where methyl-sensitive binding sites can regulate the
349 activity oncogenes and tumor suppressors^{76,77,79}.

350 However, in cells with induced DNA hypomethylation, no major structural changes are
351 observed at the level of TADs or compartments⁷⁰. Recently published single cell data in mouse
352 and human neuronal tissues indicate that DNA hypomethylation is associated with stronger
353 loop strength and boundary probabilities⁴⁰. Therefore, it is likely that the effect of DNA
354 methylation should be investigated at a finer scale, at specific sites where methyl-sensitive
355 CTCF binding sites may be affecting shorter-range chromatin contacts. Recent refinements of
356 chromosome conformation capture techniques, such as Hi-ChIP¹⁰⁰, Capture-C [and Capture
357 Hi-C^{101,102}](#) and (Capture) Micro-C^{103,104}, have vastly increased both resolute powers and
358 signal-to-noise ratios. These assays may therefore allow for more sensitive interrogation of
359 *cis* regulatory contacts, including those affected by differences in 5meC levels.

360 Another challenge for the study of 5meC-associated changes in 3D genome
361 organization has been that loss-of-function studies are hampered by lethal phenotypes in most
362 cell types. We envision two strategies that can circumvent these difficulties. Firstly, the use of
363 genetic tools such as inducible degrons that can induce rapid degradation of DNA methylation
364 machinery (e.g., DNMT1)¹⁰⁵. In the window before cell death, it will be possible to understand
365 how 5meC affects genome conformation in a cell-type specific manner. These tools have been
366 already used with great success by researchers to understand the role of chromatin
367 architecture proteins such cohesin¹⁰⁶ and CTCF¹⁰⁷. Secondly, epigenome editing tools can
368 allow for local modulation of the 5meC state without global changes to the DNA methylation
369 landscape and associated confounding effects¹⁰⁸. Such tools have already been utilized at
370 CTCF binding sites^{109,110}, and provide an avenue to dissect novel loci uncovered from
371 aforementioned genome-wide approaches.

372 We believe that in the coming years, investigation using these emerging techniques
373 will greatly bolster our understanding of [how DNA methylation and the 3D genome interact](#)
374 and its consequences for gene regulation in development and disease.

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383 **Competing Interests**

384 [The authors declare no competing interests.](#)

386 **References**

- 387 1. Cremer, T. & Cremer, M. Chromosome territories. *Cold Spring Harb. Perspect. Biol.* **2**,
388 1–22 (2010).
- 389 2. Branco, M. R. & Pombo, A. Intermingling of chromosome territories in interphase
390 suggests role in translocations and transcription-dependent associations. *PLoS Biol.* **4**,
391 780–788 (2006).
- 392 3. Lieberman-Aide, E. *et al.* Comprehensive mapping of long range interactions reveals
393 folding principles of the human genome. *Science* **236**, 289–293 (2009).
- 394 4. Chen, Y. *et al.* Mapping 3D genome organization relative to nuclear compartments
395 using TSA-Seq as a cytological ruler. *J. Cell Biol.* **217**, 4025–4048 (2018).
- 396 5. Briand, N. & Collas, P. Lamina-associated domains: Peripheral matters and internal
397 affairs. *Genome Biol.* **21**, 1–25 (2020).
- 398 6. Van Koningsbruggen, S. *et al.* High-resolution whole-genome sequencing reveals that
399 specific chromatin domains from most human chromosomes associate with nucleoli.
400 *Mol. Biol. Cell* **21**, 3735–3748 (2010).
- 401 7. Guelen, L. *et al.* Domain organization of human chromosomes revealed by mapping of
402 nuclear lamina interactions. *Nature* **453**, 948–951 (2008).
- 403 8. Vieux-Rochas, M., Fabre, P. J., Leleu, M., Duboule, D. & Noordermeer, D. Clustering
404 of mammalian Hox genes with other H3K27me3 targets within an active nuclear
405 domain. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 4672–4677 (2015).
- 406 9. Dixon, J. R. *et al.* Topological domains in mammalian genomes identified by analysis
407 of chromatin interactions. *Nature* **485**, 376–380 (2012).
- 408 10. Schoenfelder, S. & Fraser, P. Long-range enhancer–promoter contacts in gene
409 expression control. *Nat. Rev. Genet.* **20**, 437–455 (2019).
- 410 11. Gibcus, J. H. *et al.* A pathway for mitotic chromosome formation. *Science.* **359**, (2018).
- 411 12. Zheng, H. & Xie, W. The role of 3D genome organization in development and cell
412 differentiation. *Nat. Rev. Mol. Cell Biol.* **20**, 535–550 (2019).
- 413 13. Bonev, B. & Cavalli, G. Organization and function of the 3D genome. *Nat. Rev. Genet.*
414 **17**, 661–678 (2016).
- 415 14. Heger, P., Marin, B., Bartkuhn, M., Schierenberg, E. & Wiehe, T. The chromatin
416 insulator CTCF and the emergence of metazoan diversity. *Proc. Natl. Acad. Sci. U. S.*
417 *A.* **109**, 17507–17512 (2012).
- 418 15. Fang, C. *et al.* CTCF binding facilitates oncogenic transcriptional dysregulation.
419 *Genome Biol.* **21**, 1–30 (2020).
- 420 16. Kim, T. H. *et al.* Analysis of the Vertebrate Insulator Protein CTCF-Binding Sites in the
421 Human Genome. *Cell* **128**, 1231–1245 (2007).
- 422 17. de Wit, E. *et al.* CTCF Binding Polarity Determines Chromatin Looping. *Mol. Cell* **60**,
423 676–684 (2015).
- 424 18. Kim, Y. *et al.* Human cohesin compacts DNA by loop extrusion. *Science.* **366**, 1345–
425 1349 (2020).
- 426 19. Li, Y. *et al.* The structural basis for cohesin–CTCF-anchored loops. *Nature* **578**, 472–
427 476 (2020).
- 428 20. Arzate-Mejía, R. G., Recillas-Targa, F. & Corces, V. G. Developing in 3D: the role of
429 CTCF in cell differentiation. *Development* **145**, (2018).
- 430 21. Kubo, N. *et al.* Promoter-proximal CTCF binding promotes distal enhancer-dependent
431 gene activation. *Nat. Struct. Mol. Biol.* **28**, 152–161 (2021).
- 432 22. Moore, J. M. *et al.* Loss of maternal CTCF is associated with peri-implantation lethality
433 of CtCf null embryos. *PLoS One* **7**, (2012).
- 434 23. Kemp, C. J. *et al.* CTCF haploinsufficiency destabilizes DNA methylation and
435 predisposes to cancer. *Cell Rep.* **7**, 1020–1029 (2014).
- 436 24. Kaaij, L. J. T., Mohn, F., van der Weide, R. H., de Wit, E. & Bühler, M. The ChAHP
437 Complex Counteracts Chromatin Looping at CTCF Sites that Emerged from SINE
438 Expansions in Mouse. *Cell* **178**, 1437–1451.e14 (2019).

- 439 25. Barisic, D., Stadler, M. B., Iurlaro, M. & Schübeler, D. Mammalian ISWI and SWI/SNF
440 selectively mediate binding of distinct transcription factors. *Nature* **569**, 136–140
441 (2019).
- 442 26. Saldaña-Meyer, R. *et al.* RNA Interactions Are Essential for CTCF-Mediated Genome
443 Organization. *Mol. Cell* **76**, 412–422.e5 (2019).
- 444 27. Wang, H. *et al.* Widespread plasticity in CTCF occupancy linked to DNA methylation.
445 *Genome Res.* **22**, 1680–1688 (2012).
- 446 28. Hashimoto, H. *et al.* Structural Basis for the Versatile and Methylation-Dependent
447 Binding of CTCF to DNA. *Mol. Cell* **66**, 711–720.e3 (2017).
- 448 29. Nakahashi, H. *et al.* A Genome-wide Map of CTCF Multivalency Redefines the CTCF
449 Code. *Cell Rep.* **3**, 1678–1689 (2013).
- 450 30. Stadler, M. B. *et al.* DNA-binding factors shape the mouse methylome at distal
451 regulatory regions. *Nature* **480**, 490–495 (2011).
- 452 31. Wang, L. *et al.* Programming and inheritance of parental DNA methylomes in mammals.
453 *Cell* **157**, 979–991 (2014).
- 454 32. Ng, R. K. *et al.* Epigenetic restriction of embryonic cell lineage fate by methylation of
455 *Elf5*. *Nat. Cell Biol.* **10**, 1280–1290 (2008).
- 456 33. Auclair, G., Guibert, S., Bender, A. & Weber, M. Ontogeny of CpG island methylation
457 and specificity of DNMT3 methyltransferases during embryonic development in the
458 mouse. *Genome Biol.* **15**, 545 (2014).
- 459 34. Oda, M. *et al.* DNA Methylation Restricts Lineage-specific Functions of Transcription
460 Factor *Gata4* during Embryonic Stem Cell Differentiation. *PLoS Genet.* **9**, 1–17 (2013).
- 461 35. Li, E., Bestor, T. H. & Jaenisch, R. Targeted Mutation of the DNA Methyltransferase
462 Gene Results in Embryonic Lethality. **69**, 915–926 (1992).
- 463 36. Okano, M., Bell, D. W., Haber, D. A. & Li, E. DNA Methyltransferases *Dnmt3a* and
464 *Dnmt3b* Are Essential for De Novo Methylation and Mammalian Development. *Cell* **99**,
465 1–11 (1999).
- 466 37. Argelaguet, R. *et al.* Multi-omics profiling of mouse gastrulation at single-cell resolution.
467 *Nature* **576**, 487–491 (2019).
- 468 38. Ke, Y. *et al.* 3D Chromatin Structures of Mature Gametes and Structural
469 Reprogramming during Mammalian Embryogenesis. *Cell* **170**, 367–381.e20 (2017).
- 470 39. Du, Z. *et al.* Allelic reprogramming of 3D chromatin architecture during early mammalian
471 development. *Nature* **547**, 232–235 (2017).
- 472 40. Tian, W. *et al.* Single-cell DNA methylation and 3D genome architecture in the human
473 brain. *Science* . **174**, 1–20 (2023).
- 474 41. Wang, H. *et al.* Widespread plasticity in CTCF occupancy linked to DNA methylation.
475 *Genome Res.* **22**, 1680–1688 (2012).
- 476 42. Chang, L. H. *et al.* Multi-feature clustering of CTCF binding creates robustness for loop
477 extrusion blocking and Topologically Associating Domain boundaries. *Nat. Commun.*
478 **14**, (2023).
- 479 43. Kreibich, E., Kleinendorst, R., Barzaghi, G., Kaspar, S. & Krebs, A. R. Single-molecule
480 footprinting identifies context-dependent regulation of enhancers by DNA methylation.
481 *Mol. Cell* **83**, 787–802.e9 (2023).
- 482 44. Huang, H. *et al.* CTCF mediates dosage- and sequence-context-dependent
483 transcriptional insulation by forming local chromatin domains. *Nat. Genet.* **53**, 1064–
484 1074 (2021).
- 485 45. Sahu, B. *et al.* Sequence determinants of human gene regulatory elements. *Nat. Genet.*
486 **54**, 283–294 (2022).
- 487 46. Wiehle, L. *et al.* DNA (de)methylation in embryonic stem cells controls CTCF-
488 dependent chromatin boundaries. *Genome Res.* **29**, 750–761 (2019).
- 489 47. Hark, A. T. *et al.* CTCF mediates methylation-sensitive enhancer-blocking activity at the
490 *H19/Igf2* locus. *Nature* **405**, 486–489 (2000).
- 491 48. Bell, A. C. & Felsenfeld, G. Methylation of a CTCF-dependent boundary controls
492 imprinted expression of the *Igf2* gene. *Nature* **405**, 2–5 (2000).
- 493 49. Babak, T. *et al.* Genetic conflict reflected in tissue-specific maps of genomic imprinting

- 494 in human and mouse. *Nat. Genet.* **47**, 544–549 (2015).
- 495 50. Llères, D. *et al.* CTCF modulates allele-specific sub-TAD organization and imprinted
496 gene activity at the mouse Dlk1-Dio3 and Igf2-H19 domains. *Genome Biol.* **20**, 1–17
497 (2019).
- 498 51. Yoon, Y. S. *et al.* Analysis of the H19ICR Insulator . *Mol. Cell. Biol.* **27**, 3499–3510
499 (2007).
- 500 52. Murrell, A., Heeson, S. & Reik, W. Interaction between differentially methylated regions
501 partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. *Nat.*
502 *Genet.* **36**, 889–893 (2004).
- 503 53. Court, F. *et al.* The PEG13-DMR and brain-specific enhancers dictate imprinted
504 expression within the 8q24 intellectual disability risk locus. *Epigenetics and Chromatin*
505 **7**, 1–13 (2014).
- 506 54. Battistelli, C., Busanello, A. & Maione, R. Functional interplay between MyoD and CTCF
507 in regulating long-range chromatin interactions during differentiation. *J. Cell Sci.* **127**,
508 3757–3767 (2014).
- 509 55. Tarjan, D. R., Flavahan, W. A. & Bernstein, B. E. Epigenome editing strategies for the
510 functional annotation of CTCF insulators. *Nat. Commun.* **10**, 1–8 (2019).
- 511 56. Maurano, M. T. *et al.* Role of DNA Methylation in Modulating Transcription Factor
512 Occupancy. *Cell Rep.* **12**, 1184–1195 (2015).
- 513 57. Feldmann, A. *et al.* Transcription Factor Occupancy Can Mediate Active Turnover of
514 DNA Methylation at Regulatory Regions. *PLoS Genet.* **9**, (2013).
- 515 58. Soochit, W. *et al.* CTCF chromatin residence time controls three-dimensional genome
516 organization, gene expression and DNA methylation in pluripotent cells. *Nat. Cell Biol.*
517 **23**, 881–893 (2021).
- 518 59. Wu, X. & Zhang, Y. TET-mediated active DNA demethylation: Mechanism, function and
519 beyond. *Nat. Rev. Genet.* **18**, 517–534 (2017).
- 520 60. Teif, V. B. *et al.* Nucleosome repositioning links DNA (de)methylation and differential
521 CTCF binding during stem cell development. *Genome Res.* **24**, 1285–1295 (2014).
- 522 61. Sun, Z. *et al.* High-Resolution Enzymatic Mapping of Genomic 5-
523 Hydroxymethylcytosine in Mouse Embryonic Stem Cells. *Cell Rep.* **3**, 567–576 (2013).
- 524 62. Nanan, K. K. *et al.* TET-Catalyzed 5-Carboxylcytosine Promotes CTCF Binding to
525 Suboptimal Sequences Genome-wide. *iScience* **19**, 326–339 (2019).
- 526 63. Dehingia, B., Milewska, M., Janowski, M. & Pękowska, A. CTCF shapes chromatin
527 structure and gene expression in health and disease . *EMBO Rep.* **23**, 1–22 (2022).
- 528 64. Zhang, Y. *et al.* Dynamic epigenomic landscapes during early lineage specification in
529 mouse embryos. *Nat. Genet.* **50**, 96–105 (2018).
- 530 65. Chen, X. *et al.* Key role for CTCF in establishing chromatin structure in human embryos.
531 *Nature* **576**, 306–310 (2019).
- 532 66. Wike, C. L. *et al.* Chromatin architecture transitions from zebrafish sperm through early
533 embryogenesis. *Genome Res.* **31**, 981–994 (2021).
- 534 67. Skvortsova, K. *et al.* Retention of paternal DNA methylome in the developing zebrafish
535 germline. *Nat. Commun.* **10**, 1–13 (2019).
- 536 68. Tsumura, A. *et al.* Maintenance of self-renewal ability of mouse embryonic stem cells
537 in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b. *Genes to Cells*
538 **11**, 805–814 (2006).
- 539 69. Liao, J. *et al.* Targeted disruption of DNMT1, DNMT3A and DNMT3B in human
540 embryonic stem cells Jing. *Nat. Genet.* **47**, 469–478 (2015).
- 541 70. Hassan-Zadeh, V., Rugg-Gunn, P. & Bazett-Jones, D. P. DNA methylation is
542 dispensable for changes in global chromatin architecture but required for chromocentre
543 formation in early stem cell differentiation. *Chromosoma* **126**, 605–614 (2017).
- 544 71. Nothjunge, S. *et al.* DNA methylation signatures follow preformed chromatin
545 compartments in cardiac myocytes. *Nat. Commun.* **8**, (2017).
- 546 72. Chang, L.-H. *et al.* A complex CTCF binding code defines TAD boundary structure and
547 function. *bioRxiv* 2021.04.15.440007 (2021).
- 548 73. Baylin, S. B. & Jones, P. A. Epigenetic determinants of cancer. *Cold Spring Harb.*

- 549 *Perspect. Biol.* **8**, 1–35 (2016).
- 550 74. Fiorentino, F. P. & Giordano, A. The tumor suppressor role of CTCF. *J. Cell. Physiol.*
551 **227**, 479–492 (2012).
- 552 75. Fang, C. *et al.* Cancer-specific CTCF binding facilitates oncogenic transcriptional
553 dysregulation. *Genome Biol.* **21**, 1–30 (2020).
- 554 76. Flavahan, W. A. *et al.* Altered chromosomal topology drives oncogenic programs in
555 SDH-deficient GISTs. *Nature* **575**, 229–233 (2019).
- 556 77. Flavahan, W. A. *et al.* Insulator dysfunction and oncogene activation in IDH mutant
557 gliomas. *Nature* **529**, 110–114 (2016).
- 558 78. Steinhäuser, S. *et al.* Isocitrate dehydrogenase 1 mutation drives leukemogenesis by
559 PDGFRA activation due to insulator disruption in acute myeloid leukemia (AML).
560 *Leukemia* **37**, 134–142 (2023).
- 561 79. Rodriguez, C. *et al.* CTCF is a DNA methylation-sensitive positive regulator of the
562 INK/ARF locus. *Biochem. Biophys. Res. Commun.* **392**, 129–134 (2010).
- 563 80. Cui, H. *et al.* Loss of imprinting in colorectal cancer linked to hypomethylation of H19
564 and IGF2. *Cancer Res.* **62**, 6442–6446 (2002).
- 565 81. Tian, F. *et al.* Loss of imprinting of IGF2 correlates with hypomethylation of the H19
566 differentially methylated region in the tumor tissue of colorectal cancer patients. *Mol.*
567 *Med. Rep.* **5**, 1536–1540 (2012).
- 568 82. Takai, D., Gonzales, F. A., Tsai, Y. C., Thayer, M. J. & Jones, P. A. Large scale mapping
569 of methylcytosines in CTCF-binding sites in the human H19 promoter and aberrant
570 hypomethylation in human bladder cancer. *Hum. Mol. Genet.* **10**, 2619–2626 (2001).
- 571 83. Ulaner, G. A. *et al.* Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied
572 by reciprocal methylation changes of a CTCF-binding site. *Hum. Mol. Genet.* **12**, 535–
573 549 (2003).
- 574 84. Schoenfelder, S. *et al.* Polycomb repressive complex PRC1 spatially constrains the
575 mouse embryonic stem cell genome. *Nat. Genet.* **47**, 1179–1186 (2016).
- 576 85. Denholtz, M. *et al.* Long-range chromatin contacts in embryonic stem cells reveal a role
577 for pluripotency factors and Polycomb proteins in genome organization. *Cell Stem Cell*
578 **13**, (2013).
- 579 86. Blackledge, N. P. *et al.* PRC1 Catalytic Activity Is Central to Polycomb System Function.
580 *Mol. Cell* **77**, 857-874.e9 (2020).
- 581 87. Zhang, X. *et al.* Large DNA Methylation Nadirs Anchor Chromatin Loops Maintaining
582 Hematopoietic Stem Cell Identity. *Mol. Cell* **78**, 506-521.e6 (2020).
- 583 88. Rhodes, J. D. P. *et al.* Cohesin Disrupts Polycomb-Dependent Chromosome
584 Interactions in Embryonic Stem Cells. *Cell Rep.* **30**, 820-835.e10 (2020).
- 585 89. Kraft, K. *et al.* Polycomb-mediated genome architecture enables long-range spreading
586 of H3K27 methylation. *Proc. Natl. Acad. Sci. U. S. A.* **119**, 1–10 (2022).
- 587 90. Li, Y. *et al.* Genome-wide analyses reveal a role of Polycomb in promoting
588 hypomethylation of DNA methylation valleys. *Genome Biol.* **19**, 1–16 (2018).
- 589 91. Zheng, H. *et al.* Resetting Epigenetic Memory by Reprogramming of Histone
590 Modifications in Mammals. *Mol. Cell* **63**, 1066–1079 (2016).
- 591 92. Statham, A. L. *et al.* Bisulfite sequencing of chromatin immunoprecipitated DNA
592 (BisChIP-seq) directly informs methylation status of histone-modified DNA. *Genome*
593 *Res.* **22**, 1120–1127 (2012).
- 594 93. Brinkman, A. B. *et al.* Sequential ChIP-bisulfite sequencing enables direct genome-
595 scale investigation of chromatin and DNA methylation cross-talk. *Genome Res.* **22**,
596 1128–1138 (2012).
- 597 94. Buitrago, D. *et al.* Impact of DNA methylation on 3D genome structure. *Nat. Commun.*
598 **12**, (2021).
- 599 95. Kind, J. *et al.* Single-cell dynamics of genome-nuclear lamina interactions. *Cell* **153**,
600 178–192 (2013).
- 601 96. Berman, B. P. *et al.* Regions of focal DNA hypermethylation and long-range
602 hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains.
603 *Nat. Genet.* **44**, 40–46 (2015).

- 604 97. Zhou, W. *et al.* DNA methylation loss in late-replicating domains is linked to mitotic cell
605 division. *Nat. Genet.* **50**, 591–602 (2018).
- 606 98. Y, Y. *et al.* Impact of cytosine methylation on DNA binding specificities of human
607 transcription factors. *Science.* **356**, (2017).
- 608 99. Kaluscha, S., Domcke, S., Wirbelauer, C., Burger, L. & Schübeler, D. *Direct inhibition*
609 *of transcription factor binding is the dominant mode of gene and repeat repression by*
610 *DNA methylation. Nature Genetics* **54**, (2022).
- 611 100. Mumbach, M. R. *et al.* HiChIP: Efficient and sensitive analysis of protein-directed
612 genome architecture. *Nat. Methods* **13**, 919–922 (2016).
- 613 101. Schoenfelder, S., Javierre, B. M., Furlan-Magaril, M., Wingett, S. W. & Fraser, P.
614 Promoter capture Hi-C: High-resolution, genome-wide profiling of promoter
615 interactions. *J. Vis. Exp.* **2018**, 1–17 (2018).
- 616 102. Davies, J. O. J. *et al.* Multiplexed analysis of chromosome conformation at vastly
617 improved sensitivity. *Nat. Methods* **13**, 74–80 (2015).
- 618 103. Hsieh, T. H. S. *et al.* Mapping Nucleosome Resolution Chromosome Folding in Yeast
619 by Micro-C. *Cell* **162**, 108–119 (2015).
- 620 104. Goel, V. Y., Huseyin, M. K. & Hansen, A. S. Region Capture Micro-C reveals
621 coalescence of enhancers and promoters into nested microcompartments. *Nat. Genet.*
622 **55**, 1048–1056 (2023).
- 623 105. Yesbolatova, A. *et al.* The auxin-inducible degron 2 technology provides sharp
624 degradation control in yeast, mammalian cells, and mice. *Nat. Commun.* **11**, (2020).
- 625 106. Rao, S. S. P. *et al.* Cohesin Loss Eliminates All Loop Domains. *Cell* **171**, 305-320.e24
626 (2017).
- 627 107. Luan, J. *et al.* Distinct properties and functions of CTCF revealed by a rapidly inducible
628 degron system. *Cell Rep.* **34**, (2021).
- 629 108. Policarpi, C., Dabin, J. & Hackett, J. A. Epigenetic editing: Dissecting chromatin function
630 in context. *BioEssays* **43**, 1–16 (2021).
- 631 109. Liu, X. S. *et al.* Editing DNA Methylation in the Mammalian Genome. *Cell* **167**, 233-
632 247.e17 (2016).
- 633 110. Policarpi, C., Munafò, M., Tsagkris, S., Carlini, V. & Hackett, J. A. Systematic
634 Epigenome Editing Captures the Context-dependent Instructive Function of Chromatin
635 Modifications. *bioRxiv* 2022.09.04.506519 (2022).
- 636
- 637

638

639 **Figure Legends**

640

641 **Figure 1 – Hierarchical layers of 3D genome organization.** DNA exists in chromosome
642 territories, which are further divided into chromatin compartments A and B. Finer scale
643 structures, such as TADs and the DNA loops within them, are notably regulated by cohesin
644 and CTCF. Chromatin architecture influences many genomic processes such as transcription,
645 DNA replication and repair.

646

647 **Figure 2 – CTCF, its DNA binding motif and the impact of 5meC.** **a.** Schematic structure
648 of the CTCF zinc fingers. The DNA binding is recognized by the zinc fingers (ZFs) 3–7. Black
649 lollipops represent dinucleotides that may contain a CpG and thus have the potential to carry
650 5meC that can antagonize CTCF binding. **b.** Representation of a potential CpG methylation-
651 sensitive CTCF binding site in a genome browser screenshot. Looping can be determined by
652 chromosome conformation assays, such as Hi-C. High methylation conditions tracks are
653 represented in green and low methylation in orange. **c.** Potential impact of the gain of
654 methylation on a CpG methylation-sensitive CTCF binding site. Loss of CTCF binding results
655 in the erosion of the chromatin loop and decreased contacts between the promoter and the
656 enhancer leads to changes in gene expression (meCpG = methylated CpG site).

657

658 **Figure 3 – CTCF-mediated regulation of genomic imprints.** **a.** Classical model of *Igf2-H19*
659 imprinted domain regulation, based on allele-specific promoter-enhancer loops. Figure
660 adapted from Murrell, Heeson, and Reik 2004. Panel not drawn to scale. **b.** A more global
661 view of the same locus obtained from sequencing-based chromosome conformation capture
662 studies reveal specific TAD organization. Here, the *Igf2* gene is located within a different sub-
663 TAD as the enhancers on the maternal chromosome, whereas on the paternal chromosome
664 they colocalize within the same 3D domain. Figure adapted from Llères et al. 2019.

665

666 **Figure 4 – Global DNA methylation and 3D genome dynamics in early mammalian**
667 **development.** Over large genomic distances, chromatin architecture appears to be only
668 minimally impacted by embryonic *de novo* DNA methylation.

669

670 **Figure 5 – Methyl-sensitive CTCF site misregulation in cancer.** **a.** Hypermethylation of a
671 CTCF binding site leading to oncogene overexpression. Figure adapted from Flavahan et al.,
672 2016. **b.** Similar CTCF regulation as in a., but leading to tumor suppressor repression. Figure
673 adapted from Rodriguez et al., 2010. It should be noted that hypomethylated CTCF binding
674 sites may lead to aberrant CTCF binding, and cancer-associated gene misregulation as well,
675 although putative examples are less well-described in literature.

Figure 1

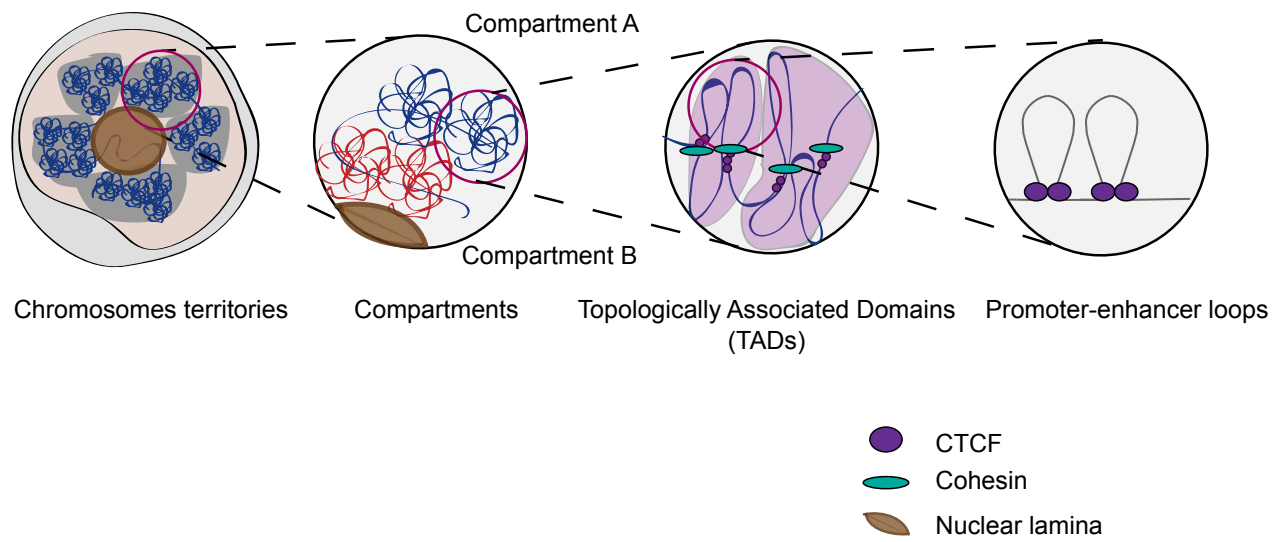
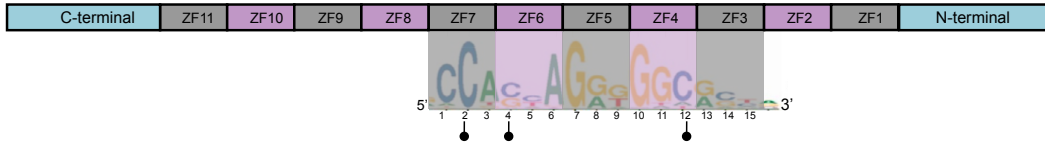
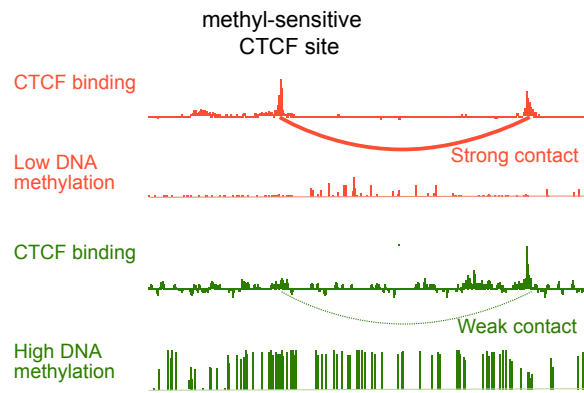


Figure 2

a.



b.



c.

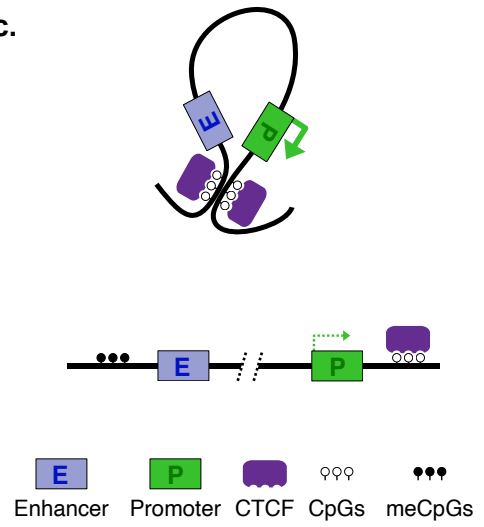
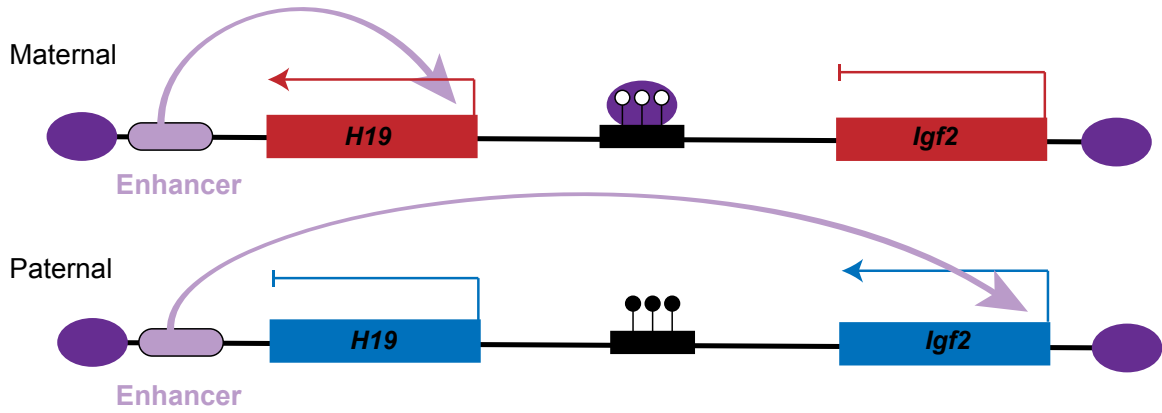


Figure 3

a.



b.

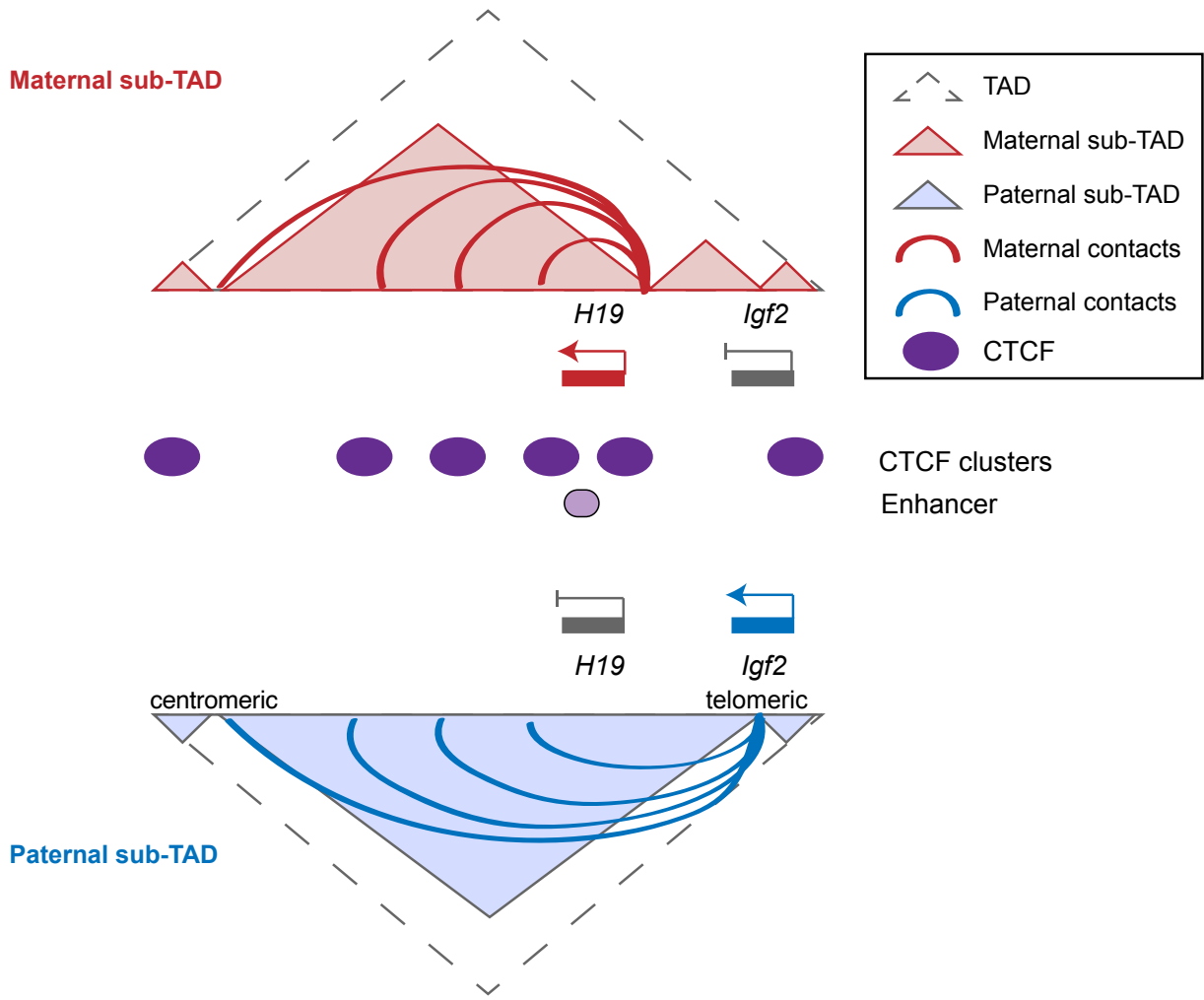


Figure 4

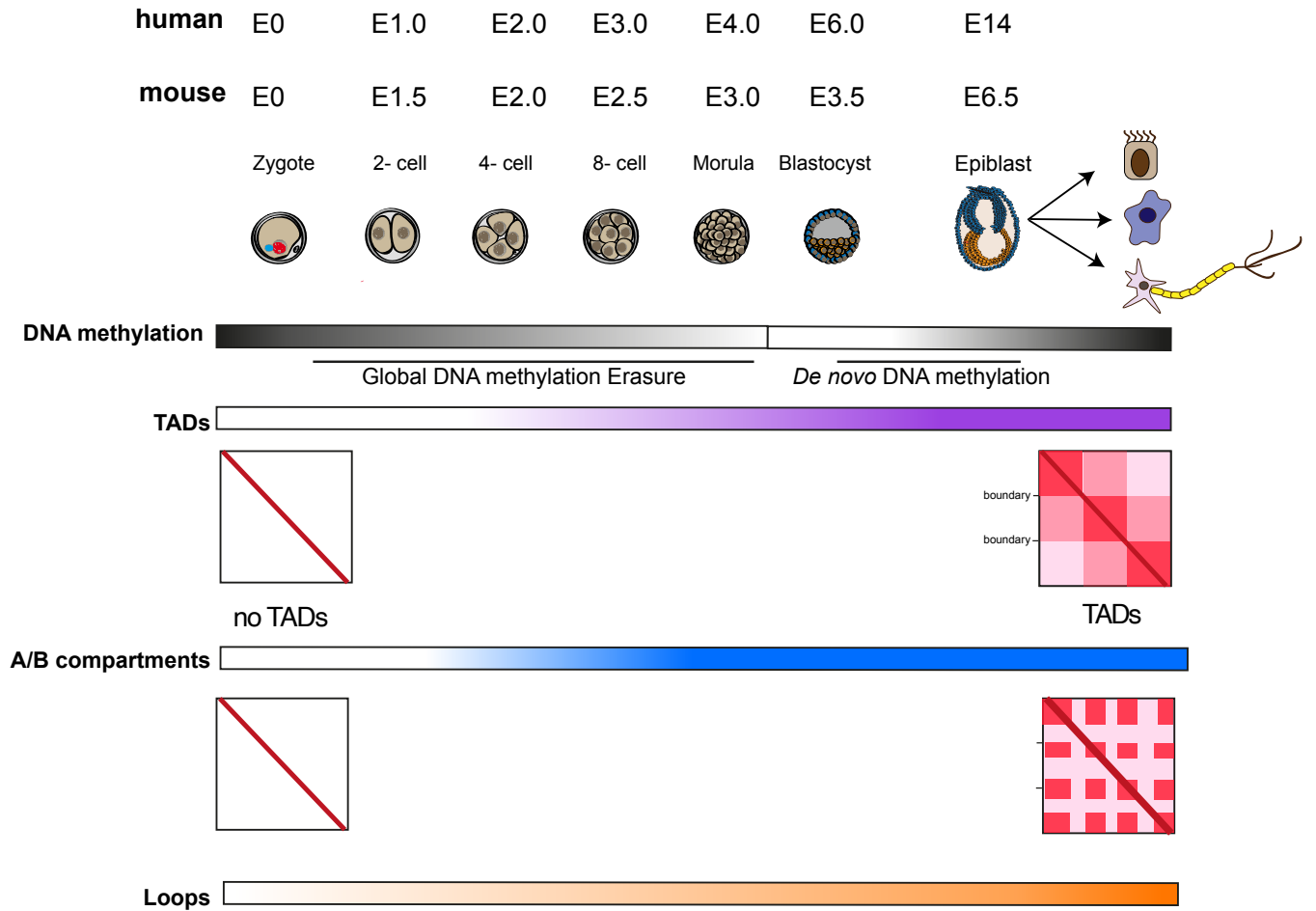
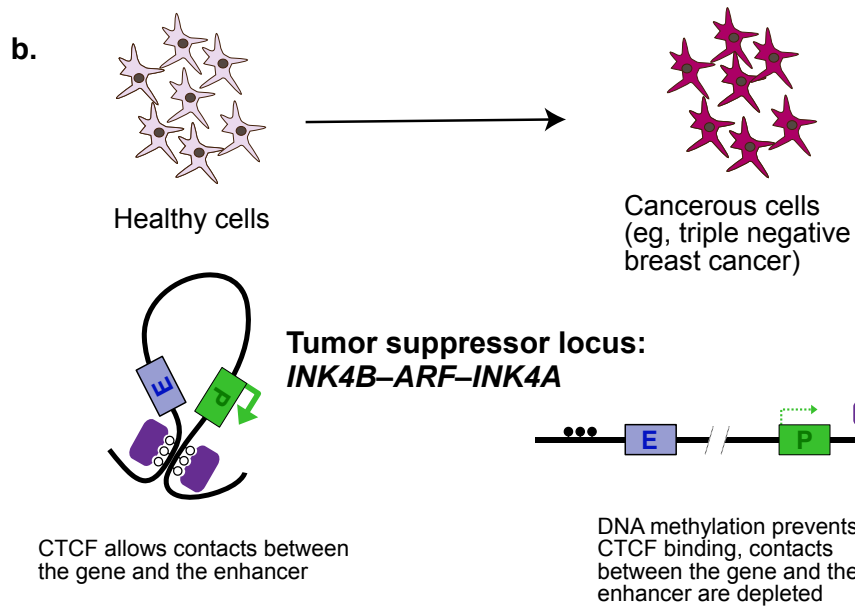
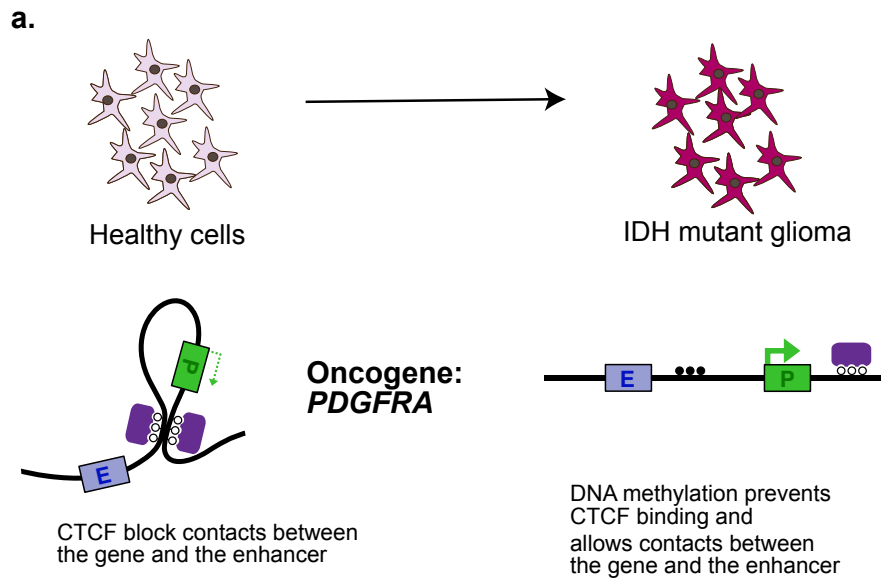


Figure 5



E **P**
Enhancer Promoter CTCF CpGs meCpGs