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1 **Differential 3D chromatin organization and gene activity in genomic imprinting**

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16 **Abstract**

17 Genomic imprinting gives rise to parent-of-origin dependent allelic gene expression. Most
18 imprinted genes cluster in domains where differentially methylated regions (DMRs)—carrying
19 CpG methylation on one parental allele—regulate their activity. Several imprinted DMRs bind
20 CTCF on the non-methylated allele. CTCF structures TADs (“Topologically Associating
21 Domains”), which are structural units of transcriptional regulation. Recent investigations show
22 that imprinted domains are embedded within TADs that are similar on both parental
23 chromosomes. Within these TADs, however, allelic sub-domains are structured by
24 combinations of mono- and bi-allelic CTCF binding that guide imprinted expression. This
25 emerging view indicates that imprinted chromosomal domains should be considered at the
26 overarching-TAD level, and questions how CTCF integrates with other regulatory proteins and
27 lncRNAs to achieve imprinted transcriptional programs.

28 **Introduction**

29 Genomic imprinting, the process whereby parental origin dictates mono-allelic gene expression
30 in the offspring, is amongst the best-studied epigenetic phenomena in mammals [1]. It is
31 controlled by CpG methylation marks that are inherited from the mother, via the oocyte, or
32 from the father, via the sperm. These epigenetic “imprints”, and the associated balancing of
33 transcriptional output, is essential for development, growth, metabolism and behavior [2].
34 Several hundred imprinted genes have been identified in humans and mice, which include
35 many non-coding RNAs (ncRNAs) including several well-characterized regulatory long
36 ncRNAs (lncRNAs) [3-5].

37 A majority of imprinted genes clusters in chromosomal domains of up to several mega-bases in
38 size. Each domain contains a CpG island that is marked by a parent-of-origin DNA methylation
39 imprint in the germline, a so-called germline “Differentially Methylated Region” (gDMR) or
40 “Imprinting Control Region” (ICR). These gDMRs are maintained in the developing embryo
41 where they are essential for mono-allelic gene expression in the entire domain [6]. A plethora
42 of nuclear proteins contribute to the somatic stability of gDMRs, which shows considerable
43 overlap with chromatin repression at endogenous retroviruses [7].

44 How imprinted DMRs instruct mono-allelic gene expression is less well understood. At some
45 domains, the gDMR encompasses a gene promoter, which may directly induce allelic
46 repression of the linked gene. At most imprinted domains however, more complex mechanisms
47 are involved that can affect genes at considerable distances from the gDMR [3,4,6]. These
48 observations have raised the question if methylation imprints regulate their target genes through
49 long-range chromatin interactions. In this review, we focus on this aspect of imprinted domain
50 organization.

51

52 **Binding dynamics of the CTCF insulator protein at imprinted domains**

53 A key finding after the discovery of gDMRs was the allelic recruitment of the “CCCTC-
54 binding factor” (CTCF) to a subset of gDMRs [8-10]. This zinc-finger protein, whose DNA
55 binding is inhibited by CpG methylation, was initially recognized for its chromatin boundary
56 function [11-13]. At the imprinted *Igf2-H19* (Insulin-like growth factor-II) domain, the gDMR
57 (“*H19* ICR”) is intergenic and methylated on the paternal allele (Figure 1a). Within this 2-kb
58 ICR, multiple CTCF sites are bound on the (non-methylated) maternal allele. Various studies
59 have revealed that this maternal CTCF binding insulates the *Igf2* gene from enhancers located

60 on the other side of the domain. Consequently, *Igf2* is expressed from the paternal chromosome
61 predominantly [14-17]. In mouse cells, perturbation of CTCF binding at the *H19* ICR causes
62 bi-allelic (and increased) *Igf2* expression [18]. In human patients, similarly, maternally
63 transmitted micro-deletions that affect CTCF binding increase *IGF2* activity, which leads to the
64 congenital overgrowth syndrome Beckwith-Wiedemann Syndrome (BWS) [19].

65 Allelic binding of CTCF has also been identified at other imprinted domains, including the
66 growth-related *Dlk1-Dio3*, *Kcnq1* and *PEG13* domains (Figure 1b-d) [20-23]. Similar to the
67 *Igf2-H19* domain, CTCF is recruited to imprinted DMRs at these domains, where it controls the
68 activity of distant promoters. At the *Dlk1-Dio3* domain, recruitment occurs not at the gDMR
69 though, but within a secondary DMR that is established in the pre-implantation embryo (Figure
70 1b) [20,23]. At the *Kcnq1* and *PEG13* domains, CTCF recruitment occurs at the non-
71 methylated (paternal) copy of the gDMR (Figure 1c, d). Mutations in the gDMR of the human
72 *KCNQ1* domain can give rise to BWS upon paternal transmission, by affecting the distant,
73 growth-related, *CDKN1C* gene [24].

74 A different CTCF binding dynamic has been described in a non-allelic study of the imprinted
75 *Zdbf2* domain (Figure 1e) [25]. In a model for early embryonic development, loss of CTCF
76 binding at a single site in the domain perturbs the essential switch between two alternative
77 promoters of the *Zdbf2/Liz* transcription unit.

78 These examples highlight the importance of allelic and dynamic CTCF recruitment on
79 imprinted gene regulation, and prompted the interest in their underlying structural mechanisms.

80

81 **First snapshots of CTCF-structured chromatin architecture at imprinted domains**

82 After the development of Chromosome Conformation Capture (3C) in 2002 [26], the imprinted
83 *Igf2-H19* domain proved an attractive model to explore whether allelic CTCF recruitment
84 mediates differential chromatin organization. The paternally-expressed *Igf2* is positioned about
85 100-kb from the maternally-expressed *H19* lncRNA gene. Although limited in the number of
86 interactions that were probed, these 3C studies yielded the valuable conclusion that *H19* and
87 the nearby enhancers had a reduced propensity for contacts with *Igf2* on the maternal
88 chromosome, as compared to the paternal chromosome (Figure 1a and 2, and e.g. [16,17]).
89 Combined, these studies suggested that the *H19* ICR -bound by CTCF on the maternal
90 chromosome- acts as a chromatin boundary that blocks interactions between regions located on
91 opposite sides of this gDMR (further discussed in [23]). In a similar fashion, 3C detected DNA

92 interactions, including promoter-enhancer loops, linked to differential CTCF binding at the
93 paternal copy of the *Kcnq1* and *PEG13* gDMRs (Figure 1c, d) [21,22,27,28].

94 Although these 3C-based studies were instrumental in instigating the first structural models of
95 imprinted domains, their non-comprehensive nature prevented a comprehensive view of
96 differential chromatin organization on the parental chromosomes (Figure 2).

97

98 **The genomics era: global views of CTCF binding, 3D chromatin architecture and** 99 **imprinted gene expression**

100 The ever-expanding toolbox of genomics assays increasingly allows the study of chromatin
101 structure and function without prior selection of genomic regions of interest. A first finding
102 with major implications for imprinted domains was the genome-wide co-localization of the
103 ring-shaped Cohesin complex at CTCF-bound sites during interphase [29,30]. Based on the
104 observation that Cohesin rings keep the sister chromatids together during mitosis, the co-
105 localization with CTCF was hypothesized to anchor DNA loops. Indeed, depletion of Cohesin
106 ablated CTCF-structured loops at the human *IGF2-H19* domain [31].

107 The subsequent discovery of “Topologically Associating Domains” (TADs), which appear as
108 discrete triangles in Hi-C matrixes, drastically changed models for genome structure and
109 transcriptional regulation [32,33]. TADs are chromatin domains, generally of several hundred
110 kb in length, with about two-fold increased interactions over surrounding regions [34]. Within
111 TADs, genes and their associated regulatory elements cluster, whereas CTCF binding is
112 strongly enriched at their boundaries [32,35]. Within TADs, additional DNA loops and
113 domains may be observed, resulting in nested sub-TAD structures (e.g. [36,37]). Depletion of
114 CTCF or Cohesin components results in the loss of domain organization, confirming their
115 essential roles in structuring (sub-)TADs [38,39].

116 Genes within imprinted domains all depend on the gDMR *in cis* for their regulation, and thus it
117 may be expected that these domains reside within overarching TADs. Moreover, the
118 identification of differential CTCF binding at gDMRs may suggest the presence of allele-
119 specific (sub-)TAD structures. Intersection of the *Igf2-H19*, *Dlk1-Dio3* and *Zdbf2* domains with
120 Hi-C data revealed that they are indeed embedded within much larger TADs. 4C-seq
121 experiments confirm that the imprinted domains focus their contacts within the TADs, with no
122 difference in the position of the boundaries between the parental chromosomes or during
123 cellular development (Figure 3) [23,25,33].

124 In contrast, major allelic differences in 3D contacts can be observed within the *Igf2-H19* TAD
125 (Figure 2) [23]. The CTCF-bound maternal *H19* ICR interacts with four bi-allelic CTCF sites
126 elsewhere in the locus. As a result, the domain is split into two sub-TADs that physically
127 insulate *Igf2* from the enhancers that located near the *H19* gene. In contrast, the absence of
128 CTCF binding at the paternal *H19* ICR allows *Igf2* to outcompete *H19* for regulatory
129 interactions (Figure 1 and 2) [23]. Interestingly, the bi-allelic CTCF sites that interact with the
130 *H19* ICR on the maternal allele extend their loops on the paternal allele towards another bi-
131 allelic site near *Igf2*. A first level of sub-TAD organization is therefore present on the paternal
132 chromosome, which is further subdivided by absence of DNA methylation and CTCF binding
133 to the *H19* ICR on the maternal allele (Figure 2) [23].

134 A similar allele-specific sub-TAD organization was detected at the *Dlk1-Dio3* domain (Figure
135 1) [23], which precedes the activation of the protein-coding *Dlk1* gene at this imprinted locus.
136 Compartmentalization of imprinted domains by allelic binding of CTCF at DMRs may thus be
137 a commonly employed strategy to facilitate the setting and maintenance of imprinted
138 transcriptional programs. How other imprinted loci are embedded within TADs, and if allelic
139 CTCF binding implements further structural differences, remains largely to be determined.

140 In parallel, powerful transcriptome studies allowed the systematic assessment of allele-specific
141 gene expression in embryonic and extra-embryonic tissues, and at different developmental
142 stages [3,4]. These comprehensive data-sets identified many new imprinted genes located near
143 known ICRs, thus providing more accurate estimates of the size of these domains. Some
144 imprinted domains—including the *Kcnq1* and *Igf2r* (*Igf2*-receptor) loci—are likely much larger
145 than previously thought [3,4]. Intersection with TADs may be instrumental to further delineate
146 the maximum span of imprinted domains.

147

148 **A mechanistic outlook: integrating the multiple levels of imprinted gene regulation**

149 Multi-omics approaches have started to reveal how allele-specific and bi-allelic chromatin
150 features, including 3D genome organization, CTCF binding, DNA methylation and histone
151 modifications, establish imprinted transcriptional programs (e.g. [3,6,23,25,40]). How all these
152 mechanisms intersect with CTCF remains interesting to further explore. Moreover, little is
153 known with which proteins CTCF interacts at imprinted domains and how CTCF itself is
154 modified. At the *H19* ICR, CTCF can acquire high levels of poly(ADP-ribosylation) through

155 interaction with PARP1. Like perturbation of CTCF binding at the *H19* ICR, prevention of this
156 modification of CTCF directly influences *Igf2* expression [18,41].

157 At all DMRs where CTCF is allele-specifically recruited, a lncRNA is transcribed from the
158 same chromosome (Figure 1). For instance at the *Dlk1-Dio3* domain, the maternal CTCF
159 binding occurs in the first intron of the lncRNA gene *Meg3*, whose maternal expression is
160 essential for the imprinting of nearby protein-coding genes [20,23,40]. For now, it remains
161 unexplored whether these lncRNAs are mechanistically linked to CTCF, or whether they
162 contribute independently to imprinted regulation. A recently characterized RNA binding
163 domain (RBD) within the CTCF protein controls its recruitment to many binding sites in the
164 genome [42,43]. Moreover, at the *Igf2-H19* domain, CTCF interacts with the RNA binding
165 protein p68, which regulates boundary function [44]. The domain-structuring function of CTCF
166 may thus directly or indirectly be guided by RNA-binding.

167 Another major challenge will be to unravel how these mechanisms act at the individual alleles
168 in single cells, either in isolation or within the integrated framework of regulatory mechanisms.
169 It remains for instance to be determined if regulatory DNA interactions occur in “hubs” or
170 rather in a pair-wise fashion. At the cell population level, the maternal *H19* ICR is observed to
171 interact with four CTCF-bound regions (Figure 2), but if these regions concurrently interact on
172 individual chromosomes has not been established. To tackle this issue, “multi-contact” 3C
173 approaches like Multi-contact 4C and Tri-C may be promising, as they can pinpoint
174 simultaneous interactions between gene promoters and pairs of enhancers [45,46].

175 Finally, several genomics studies have shown that TADs overlap with domains of DNA
176 replication timing, with replication early in S phase being associated with increased gene
177 activity [47-49]. Such a link is particularly intriguing for imprinted domains, where genes are
178 differentially expressed between the parental chromosomes. Indeed, at the *Igf2-H19*, *Dlk1-Dio3*
179 and other imprinted domains, imaging detected differential timing of replication between the
180 parental chromosomes [50-53]. The extent of these replication domains, and how they
181 originate, remains undetermined. Their integration in future multi-omics studies will add an
182 important functional component to the sub-TAD structure of imprinted domains.

183

184 **Conclusions**

185 At in-depth characterized imprinted domains, CTCF creates sub-domains that act as a structural
186 framework for enhancer-promoter contacts. Allelic CTCF binding to DMRs structures sub-

187 TADs at the *Igf2-H19* and *Dlk1-Dio3* domains, thus restricting enhancer-promoter contacts and
188 modulating imprinted gene activation (Figure 1a, b and 2). Despite similar patterns of CTCF
189 binding at other imprinted loci, additional characterization will be required to determine if
190 CTCF structures sub-TADs here as well (Figure 1c, d). At the *Zdbf2* domain, CTCF-mediated
191 restructuration contributes to the developmental dynamics of imprinted gene expression [25]
192 (Figure 1e). In contrast, at the *Dlk1-Dio3* locus the structure precedes imprinted gene activation
193 [23], raising the question if imprinted domains are commonly reorganized during
194 differentiation.

195 The underlying mechanisms that guide the CTCF-structured reorganization between alleles and
196 cell types remain mostly to be identified. Locus-specific integration of CTCF binding with
197 lineage-specific (transcription) factors, histone modifying complexes, lncRNAs and larger
198 structures like replication domains may be essential. Such integration at imprinted domains
199 may also provide new avenues for exploring gene expression defects in BWS and other human
200 imprinting disorders.

201

202 **Conflict of Interest statement**

203 Nothing declared.

204

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211

212 **Authorship statements**

213 D. N.: drafted and edited the manuscript.

214 R. F.: drafted and edited the manuscript.

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408 **Figure legends**

409 ***Figure 1: Examples of dynamic chromatin structures and domains at imprinted domains.***

410 Schematic depiction of differential chromatin organization at imprinted domains. Domain sizes
411 and the positions of elements are not to scale.

412 **A.** At the *Igf2-H19* domain, CTCF binds the intergenic gDMR (“*H19* ICR”) on the maternal
413 allele, which prevents distal enhancers from activating the proximal *Igf2* gene [10,16-18,23].

414 **B.** At the *Dlk1-Dio3* domain, the gDMR (ICR) functions as an enhancer on the maternal allele,
415 to activate the nearby *Meg3* lncRNA polycistron (encompassing the *Rian* and *Mirg* ncRNAs as
416 well). CTCF binds the DMR comprising the promoter of *Meg3*, which contributes to the
417 imprinted expression of the *Dlk1* gene [20,23,51]. The activity and DNA contacts of the *Dlk1*
418 enhancer have not been determined in an allele-specific manner.

419 **C.** At the *Kcnq1* domain, the intragenic gDMR (ICR, acting as the promoter for the *Kcnq1-ot1*
420 lncRNA) binds CTCF on its unmethylated paternal allele, which prevents activation of the
421 *Cdkn1c* gene [28]. On the opposite end of the domain, a DNA loop is formed between the
422 *Kcnq1* promoter and its enhancers [22,27]. The activity and the DNA contact of the *Kcnq1*
423 enhancer have not been determined in an allelic manner.

424 **D.** At the *PEG13-KCNK9* domain, CTCF binds to the unmethylated allele of the gDMR (ICR,
425 acting as the promoter for the *PEG13* lncRNA), which prevents activation of *KCNK9* [21]. For
426 the chromatin loop interactions, parental alleles were not told apart.

427 **E.** At the developmentally regulated mouse *Zdbf2* locus, CTCF binding in embryonic stem
428 cells (mESCs) activates *Liz*, an extended *Zdbf2*-isoform that shares characteristics with
429 lncRNAs. Upon differentiation into epiblast-like cells (mEpiLCs), loss of CTCF binding allows
430 *Zdbf2* to interact with its enhancers [25]. CTCF binding and chromatin loops were not
431 distinguished in an allele-specific manner.

432

433 ***Figure 2: Allelic CTCF binding to the H19 ICR structures allelic sub-TADs at the imprinted***
434 ***Igf2-H19 domain.***

435 **A.** Non-allelic Hi-C experiments revealed that the *H19* and *Igf2* genes are located in a TAD
436 that spans 450 kb [33].

437 **B.** 4C-seq interactions (*H19* ICR viewpoint) and CTCF ChIP-seq signal on the maternal (red)
438 and paternal (blue) chromosomes within the *Igf2-H19* TAD. Genes and enhancers are indicated
439 as in Figure 1. Maternal-allele CTCF binding to the *H19* ICR (red arrow) coincides with the
440 formation of four DNA loops towards four bi-allelic CTCF binding clusters at the left part of
441 the TAD. Without CTCF binding to the ICR, on the paternal chromosome, these loops are
442 absent. Relative to the overarching TAD structure, a single sub-TAD is present on the paternal
443 chromosome that contains the active *Igf2* gene, the inactive *H19* gene and the nearby enhancers
444 (blue ovals). CTCF binding at the maternal *H19* ICR splits this organization into two sub-
445 TADs (red ovals) [23]. In-between the maternal and paternal 4C data, results from two 3C
446 studies are plotted [16,17]. Blue arches indicate paternal-specific DNA loops and red arches
447 maternal-specific loops. The 3C data support the notion that DNA contacts are contained within
448 sub-TADs, but the investigated region probed in all 3C studies (white domain) was too
449 restricted to detect this overarching organization.

450

451 **Figure 3: The TAD overarching the *Igf2-H19* domain is similarly positioned on the maternal**
452 **and paternal chromosomes.**

453 **A.** TAD structure surrounding the *Igf2-H19* domain as identified by non-allelic Hi-C. The
454 positions of the TAD boundaries are indicated with black dashed lines [33].

455 **B.** 4C-seq interactions of 3 viewpoints located within the *Igf2-H19* TAD, with signal on the
456 maternal chromosome in red and signal on the paternal chromosome in blue (viewpoints:
457 arrowheads) [23]. The three viewpoints (black triangles) have different positions within the
458 TAD and different allele-specific patterns of intra-TAD contacts. Yet, on both chromosomes
459 they restrict their contacts within the same overarching TAD, as reflected by the strong drop of
460 interactions outside the boundaries.