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Differential 3D chromatin organization and gene activity in genomic imprinting

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

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Introduction

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

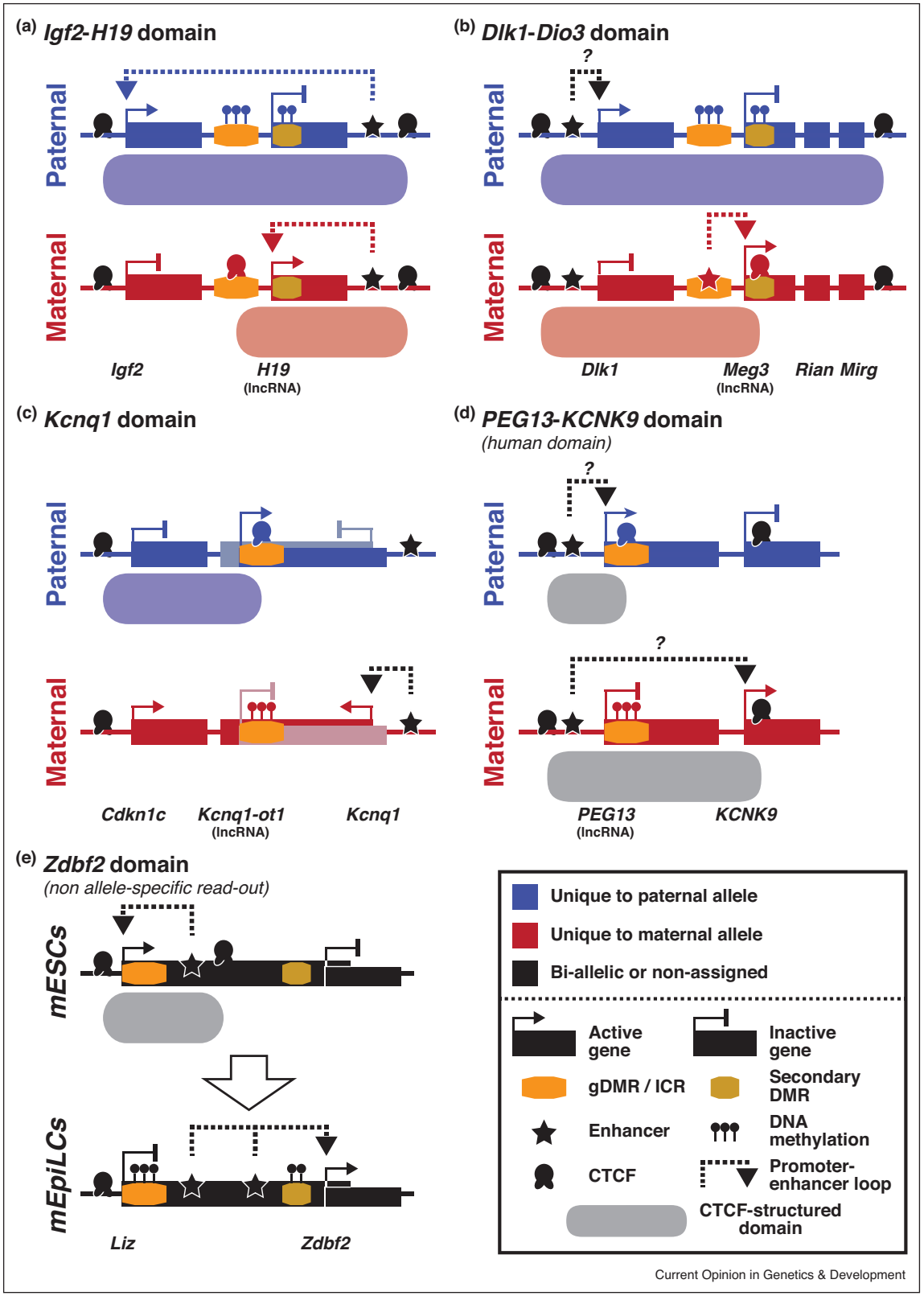
A majority of imprinted genes clusters in chromosomal domains of up to several megabases in size. Each domain contains a CpG island that is marked by a parent-of-origin DNA methylation imprint in the germline, a so-called germline 'Differentially Methylated Region' (gDMR) or 'Imprinting Control Region'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

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

Binding dynamics of the CTCF insulator protein at imprinted domains

A key finding after the discovery of gDMRs was the allelic recruitment of the 'CCCTC-binding factor' (CTCF) to a subset of gDMRs [8–10]. This zinc-finger protein, whose DNA binding is inhibited by CpG methylation, was initially recognized for its chromatin boundary function [11–13]. At the imprinted *Igf2-H19* (Insulin-like growth factor-II) domain, the gDMR ('*H19* ICR') is intergenic and methylated on the paternal allele (Figure 1a). Within this 2-kb ICR, multiple CTCF sites are bound on the (non-methylated) maternal allele. Various studies have revealed that this maternal CTCF binding insulates the *Igf2* gene from enhancers located on the other side of the domain. Consequently, *Igf2* is expressed from the paternal chromosome predominantly [14–17]. In mouse cells, perturbation of CTCF binding at the *H19* ICR causes bi-allelic (and increased) *Igf2* expression [18]. In human patients, similarly, maternally transmitted micro-deletions that affect CTCF binding increase

Figure 1



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

IGF2 expression, which leads to the congenital overgrowth syndrome Beckwith-Wiedemann Syndrome (BWS) [19].

Allelic binding of CTCF has also been identified at other imprinted domains, including the growth-related *Dlk1-Dio3*, *Kcnq1* and *PEG13* domains (Figure 1b–d) [20–22,23••]. Similar to the *Igf2-H19* domain, CTCF is recruited to imprinted DMRs at these domains, where it controls the activity of distant promoters. At the *Dlk1-Dio3* domain, recruitment occurs not at the gDMR though, but within a secondary DMR that is established in the pre-implantation embryo (Figure 1b) [20,23••]. At the *Kcnq1* and *PEG13* domains, CTCF recruitment occurs at the non-methylated (paternal) copy of the gDMR (Figure 1c,d). Mutations in the gDMR of the human *KCNQ1* domain can give rise to BWS upon paternal transmission, by affecting the distant, growth-related, *CDKN1C* gene [24].

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

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

First snapshots of CTCF-structured chromatin architecture at imprinted domains

After the development of Chromosome Conformation Capture (3C) in 2002 [26], the imprinted *Igf2-H19* domain proved an attractive model to explore whether allelic CTCF recruitment mediates differential chromatin organization. The paternally expressed *Igf2* is positioned about 100-kb from the maternally expressed *H19* lncRNA gene. Although limited in the number of interactions that were probed, the 3C studies yielded the valuable conclusion that *H19* and the nearby enhancers had a reduced propensity for contacts with *Igf2* on the maternal chromosome, as compared to the paternal chromosome (Figures 1a and 2, and e.g. [16,17]). Combined, these studies suggested that the *H19* ICR—bound by CTCF on the maternal chromosome—acts as a chromatin boundary that blocks interactions between regions located on opposite sides of this gDMR (further discussed in Ref. [23••]). In a similar fashion, 3C detected DNA interactions, including promoter-enhancer loops, linked to differential CTCF binding at the paternal copy of the *Kcnq1* and *PEG13* gDMRs (Figure 1c,d) [21,22,27,28].

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

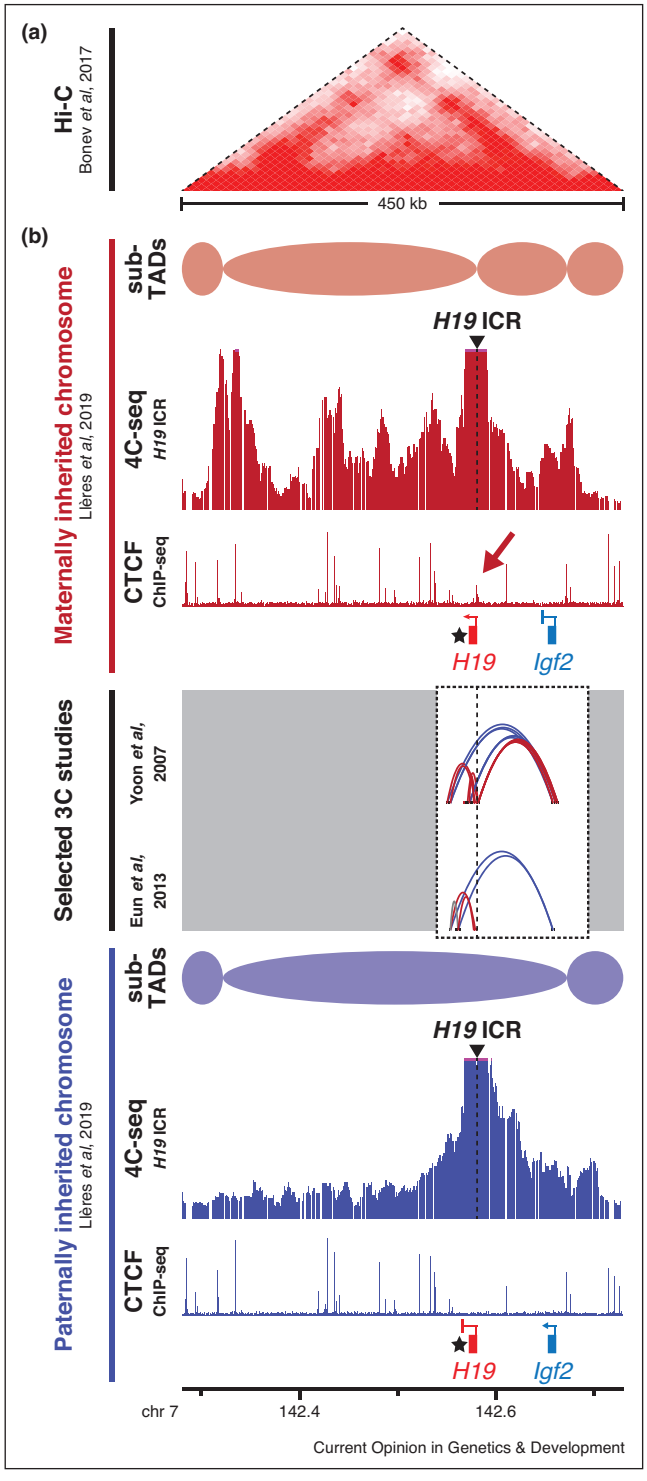
The genomics era: global views of CTCF binding, 3D chromatin architecture and imprinted gene expression

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

The subsequent discovery of 'Topologically Associating Domains' (TADs), which appear as discrete triangles in Hi-C matrixes, drastically changed models for genome structure and transcriptional regulation [32,33]. TADs are chromatin domains, generally of several hundred kilobases in length, with about twofold increased interactions over surrounding regions [34]. Within TADs, genes and their associated regulatory elements cluster, whereas

(a) At the *Igf2-H19* domain, CTCF binds the intergenic gDMR ('*H19* ICR') on the maternal allele, which prevents distal enhancers from activating the proximal *Igf2* gene [10,16–18,23••].
(b) At the *Dlk1-Dio3* domain, the gDMR (ICR) functions as an enhancer on the maternal allele, to activate the nearby *Meg3* lncRNA polycistron (encompassing the *Rian* and *Mirg* ncRNAs as well). CTCF binds the DMR comprising the promoter of *Meg3*, which contributes to the imprinted expression of the *Dlk1* gene [20,23••,51]. The activity and DNA contacts of the *Dlk1* enhancer have not been determined in an allele-specific manner.
(c) At the *Kcnq1* domain, the intragenic gDMR (ICR, acting as the promoter for the *Kcnq1-ot1* lncRNA) binds CTCF on its unmethylated paternal allele, which prevents activation of the *Cdkn1c* gene [28]. On the opposite end of the domain, a DNA loop is formed between the *Kcnq1* promoter and its enhancers [22,27]. The activity and the DNA contact of the *Kcnq1* enhancer have not been determined in an allelic manner.
(d) At the *PEG13-KCNK9* domain, CTCF binds to the unmethylated allele of the gDMR (ICR, acting as the promoter for the *PEG13* lncRNA), which prevents activation of *KCNK9* [21]. For the chromatin loop interactions, parental alleles were not told apart.
(e) At the developmentally regulated mouse *Zdbf2* locus, CTCF binding in embryonic stem cells (mESCs) activates *Liz*, an extended *Zdbf2*-isoform that shares characteristics with lncRNAs. Upon differentiation into epiblast-like cells (mEpiLCs), loss of CTCF binding allows *Zdbf2* to interact with its enhancers [25•]. CTCF binding and chromatin loops were not distinguished in an allele-specific manner.

Figure 2



Allelic CTCF binding to the *H19* ICR structures allelic sub-TADs at the imprinted *Igf2-H19* domain.
(a) Non-allelic Hi-C experiments revealed that the *H19* and *Igf2* genes are located in a TAD that spans 450 kb [33].
(b) 4C-seq interactions (*H19* ICR viewpoint) and CTCF ChIP-seq signal on the maternal (red) and paternal (blue) chromosomes within the *Igf2-H19* TAD. Genes and enhancers are indicated as in Figure 1. Maternal-allele CTCF binding to the *H19* ICR (red arrow) coincides with the formation of four DNA loops towards four bi-allelic CTCF binding clusters at the left part of the TAD. Without CTCF binding to the ICR, on the paternal chromosome, these loops are absent. Relative to the overarching TAD structure, a single sub-TAD is present on the paternal chromosome that contains the active *Igf2* gene, the inactive *H19* gene and the nearby enhancers (blue ovals). CTCF binding at the maternal *H19* ICR splits this organization into two sub-TADs (red ovals) [23••]. In-between the maternal and paternal 4C data, results from two 3C studies are plotted [16,17]. Blue arches indicate paternal-specific DNA loops and red arches maternal-specific loops. The 3C data support the notion that DNA contacts are contained within sub-TADs, but the investigated region probed in all 3C studies (white domain) was too restricted to detect this overarching organization.

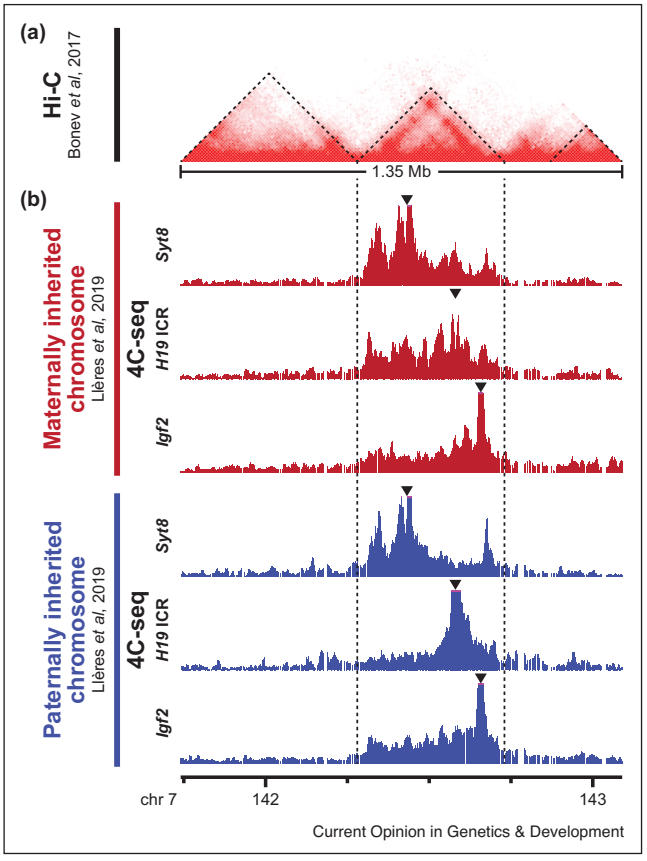
CTCF binding is strongly enriched at their boundaries [32,35]. Within TADs, additional DNA loops and domains may be observed, resulting in nested sub-TAD structures (e.g. [36,37]). Depletion of CTCF or Cohesin components results in the loss of domain organization, confirming their essential roles in structuring (sub)TADs [38,39].

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

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

A similar allele-specific sub-TAD organization was detected at the *Dlk1-Dio3* domain (Figure 1) [23••], which precedes the allele-specific developmental activation of the protein-coding *Dlk1* gene at this imprinted locus. Compartmentalization of imprinted domains by allelic

Figure 3



The TAD overarching the Igf2-H19 domain is similarly positioned on the maternal and paternal chromosomes.
(a) TAD structure surrounding the *Igf2-H19* domain as identified by non-allelic Hi-C. The positions of the TAD boundaries are indicated with black dashed lines [33].
(b) 4C-seq interactions of three viewpoints located within the *Igf2-H19* TAD, with signal on the maternal chromosome in red and signal on the paternal chromosome in blue (viewpoints: arrowheads) [23••]. The three viewpoints (black triangles) have different positions within the TAD and different allele-specific patterns of intra-TAD contacts. Yet, on both chromosomes they restrict their contacts within the same overarching TAD, as reflected by the strong drop of interactions outside the boundaries.

binding of CTCF at DMRs may thus be a commonly employed strategy to facilitate the setting and maintenance of imprinted transcriptional programs. How other imprinted loci are embedded within TADs and whether allelic CTCF binding implements further structural differences, remains largely to be determined.

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

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

Multi-omics approaches have started to reveal how allele-specific and bi-allelic chromatin features, including 3D genome organization, CTCF binding, DNA methylation and histone modifications, establish imprinted transcriptional programs (e.g. [3,6,23••,25•,40•]). How all these mechanisms intersect with CTCF remains interesting to further explore. Moreover, little is known about the proteins that CTCF interacts with at imprinted domains and how CTCF itself is modified. At the *H19* ICR, CTCF can acquire high levels of poly(ADP-ribosylation) through interaction with PARP1. Like perturbation of CTCF binding at the *H19* ICR, prevention of this modification of CTCF directly influences *Igf2* expression [18,41].

At all DMRs where CTCF is allele-specifically recruited, an lncRNA is transcribed from the same chromosome (Figure 1). For instance at the *Dlk1-Dio3* domain, the maternal CTCF binding occurs in the first intron of the lncRNA gene *Meg3*, whose maternal expression is essential for the imprinting of a nearby protein-coding gene [20,23••,40•]. It it still unclear whether Meg3 lncRNA itself confers this role [40•]. It remains unexplored also whether the lncRNAs are mechanistically linked to CTCF, or whether their expression contributes independently to imprinted regulation. A recently characterized RNA binding domain (RBD) within the CTCF protein controls its recruitment to many binding sites in the genome [42••,43••]. Moreover, at the *Igf2-H19* domain, CTCF interacts with the RNA binding protein p68, which regulates boundary function [44]. The domain-structuring function of CTCF may thus directly or indirectly be guided by RNA-binding.

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

Finally, several genome-wide studies have shown that TADs overlap with domains of DNA replication timing, with replication early in S phase being associated with increased gene activity [47•,48,49]. Such a link is particularly intriguing for imprinted domains, where genes are differentially expressed between the parental chromosomes. Indeed, at the *Igf2-H19*, *Dlk1-Dio3* and other imprinted domains, microscopic imaging detected differential timing of replication between the parental chromosomes [50–53]. The extent of these replication domains, and how they originate, remains undetermined. Their integration in future multi-omics studies will add an important functional component to the sub-TAD structure of imprinted domains.

Conclusions

At in-depth characterized imprinted domains, CTCF creates subdomains that act as a structural framework for enhancer-promoter contacts. Allelic CTCF binding to DMRs structures sub-TADs at the *Igf2-H19* and *Dlk1-Dio3* domains, thus restricting enhancer-promoter contacts and modulating imprinted gene activation (Figures 1a, b and 2). Despite similar patterns of CTCF binding at other imprinted loci, additional characterization will be required to determine if CTCF structures sub-TADs here as well (Figure 1c,d). At the *Zdbf2* domain, CTCF-mediated restructuration contributes to the developmental dynamics of imprinted gene expression [25•] (Figure 1e). In contrast, at the *Dlk1-Dio3* locus the structure precedes imprinted gene activation [23••], raising the question if imprinted domains are commonly reorganized during differentiation.

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

Authorship statements

D. N.: drafted and edited the manuscript.

R. F.: drafted and edited the manuscript.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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