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Causality in transcription and genome folding: Insights from X inactivation

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Abstract

The spatial organization of genomes is becoming increasingly understood. In mammals, where it is most investigated, this organization ties in with transcription, so an important research objective is to understand whether gene activity is a cause or a consequence of genome folding in space. In this regard, the phenomena of X-chromosome inactivation and reactivation open a unique window of investigation because of the singularities of the inactive X chromosome. Here we focus on the cause–consequence nexus between genome conformation and transcription and explain how recent results about the structural changes associated with inactivation and reactivation of the X chromosome shed light on this problem.

KEYWORDS
chromatin, genome organization, transcription, X-inactivation, X-reactivation

INTRODUCTION

The last decade has seen unprecedented progress in our understanding of genome organization in space and time, thanks to technological and conceptual advances. Most notably, the Hi-C technology allowed us to assay chromosomal contacts at a genome-wide scale,[1] super-resolution microscopy pushed the boundaries of the observable,[2] and polymer physics revealed some of the mechanisms that structure the nucleus and the genome.[3]

With so many connections between transcription and organization of the genome in space,[4–6] the question naturally arises as to which is a cause and which is a consequence. This problem has become a central

Abbreviations: CTCF, CCCTC-binding factor; H2AK119u1, mono-ubiquitylation of histone H2A lysine 119; H3K27me3, tri-methylation of histone H3 lysine 27; PRC1, polycomb repressive complex 1; PRC2, polycomb repressive complex 2; TADs, Topologically Associating Domains; Xic, X-inactivation center; Xist, X-inactive specific transcript.

research theme in the field of gene regulation, and if many questions were recently answered, many others still remain.

Two difficulties make it challenging to disentangle causes from consequences in this area: the multiple layers of feedback in gene regulation and the difficulty of modifying genome conformation at scale, as there is presently no technology to force genomes to adopt a given 3D configuration. For now, the question is best addressed within the realm of naturally occurring phenomena where transcription is either initiated or shut down on a large scale. Such experimental systems include the reinitiation of transcription after mitosis,[7,8] the zygotic genome activation in early development,[9–12] and the inactivation of the X chromosome in female mammals.[13] All three systems have defined initial states and clear kinetics, but X chromosome inactivation stands out because the presence of an active X chromosome in the same nucleus gives the unique opportunity to disentangle *cis*-acting from *trans*-acting signals. Recent advances in the field of X-chromosome inactivation and reactivation shed light on some of the key mechanisms and help tease apart causes from consequences.

WHAT MAKES X INACTIVATION SPECIAL?

X-chromosome inactivation happens twice in the early development of female mice. The first time, inactivation is imprinted, that is, the X chromosome inherited from the father's sperm is specifically inactivated. Imprinted X-inactivation occurs from the 4-cell stage onward in all the cells of the embryo and is later on maintained in the extraembryonic lineages, like the placenta.[15,16] At the blastocyst stage, imprinted X-inactivation is erased in the pluripotent epiblast by the reverse process of X-reactivation.[17,18] The second time, inactivation is random.[19] This occurs in the postimplantation epiblast (the embryo proper) after the paternal X has been reactivated. In both cases, a cascade of events initiated by the transcription of the long non-coding RNA Xist causes a shutdown of transcription on the whole chromosome,[20–22] except for the so-called escapee genes, which remain active on both X chromosomes.[23] However, the nature of the inactive X manifests itself not only by transcriptional inactivation but also by distinctive heterochromatic landscape and three-dimensional organization.[24]

The inactive X is silenced by a succession of chromatin alterations that synergize with each other.[25–28] Active marks, such as histone acetylation, are depleted by the transcriptional repressor SPEN, itself recruited by the Xist RNA.[29–32] Silencing marks are deposited by the Polycomb repressive complexes 1 and 2 (PRC1 and PRC2),[33–36] and finally the silent state is locked by DNA methylation of CpG islands at promoters.[37] Moreover, the inactive X adopts a characteristic structure where topologically associating domains (TADs, see **Box 1**, Figures 1 and 2) are strongly attenuated[38,39] except at escapee genes,[38] and with an organization into two mega-domains [27,38,40,41] separated by a boundary element called *Dxz4*.

Escapee regions extend outside of the silencing territory of the inactive X and come in contact with each other, which was proposed to enable their expression.[42,43] To fully appreciate the scope of X inactivation within the larger picture, it is important to highlight its most unique constraint: a second X chromosome with a near-identical sequence has to remain fully active in the same nucleus. This implies that all the transcription factors and diffusible signals that promote the transcription of X-linked genes must be fully active, while the inactive X must remain completely impervious to them.

Box 1: Hi-C reveals the main features of genome conformation

The Hi-C technology[1] revealed several key features that are used to describe the conformation of eukaryotic genomes (Figure 1). Hi-C is based on the 3C technology,[14] whereby a genome is fixed in its native form in cells, cut and ligated, resulting in the formation of chimeras between loci in close proximity to each other. In Hi-C, the chimeras are purified and sequenced, producing genome-wide contact maps, that is, heat maps showing the contact frequencies between all the pairs of loci in the genome. Most of the signal is along the diagonal because the majority of the contacts are between loci that are on the same chromosome and within a few kb of each other.

Chromosome territories appear on Hi-C contact maps as strong enrichments of intra-chromosomal contacts (they were originally revealed by microscopy using whole-chromosome staining). Chromosomes occupy autonomous territories that mostly exclude each other in the nucleus. Chromosome territories have a characteristic scale of ~100 Mb.

A/B compartments are defined by a succession of computational steps including a principal component analysis. Genomic loci form two distinct clusters, where the coordinates of loci from the A and B compartments on the first principal component have opposite signs. Those clusters correspond to regions of the genome that come in contact more often than expected by chance, and correlate with transcriptionally active and inactive domains of the genome. They have a scale of ~1–10 Mb and appear as a characteristic "checkerboard" or "plaid" pattern on Hi-C maps.

Topologically Associating Domains (TADs) typically appear as squares along the diagonal of Hi-C contact maps (triangles when half of the matrix is visible). They correspond to chromosomal domains isolated from each other by borders (TAD boundaries) with defined positions. Their sizes range from ~0.1 to 1 Mb.

Loops or loop domains appear as prominent dots away from the diagonal of Hi-C contact maps. They correspond to recurrent or stable contacts between loci that are sufficiently separated to be considered distinct. They have a characteristic scale of 5–500 kb.

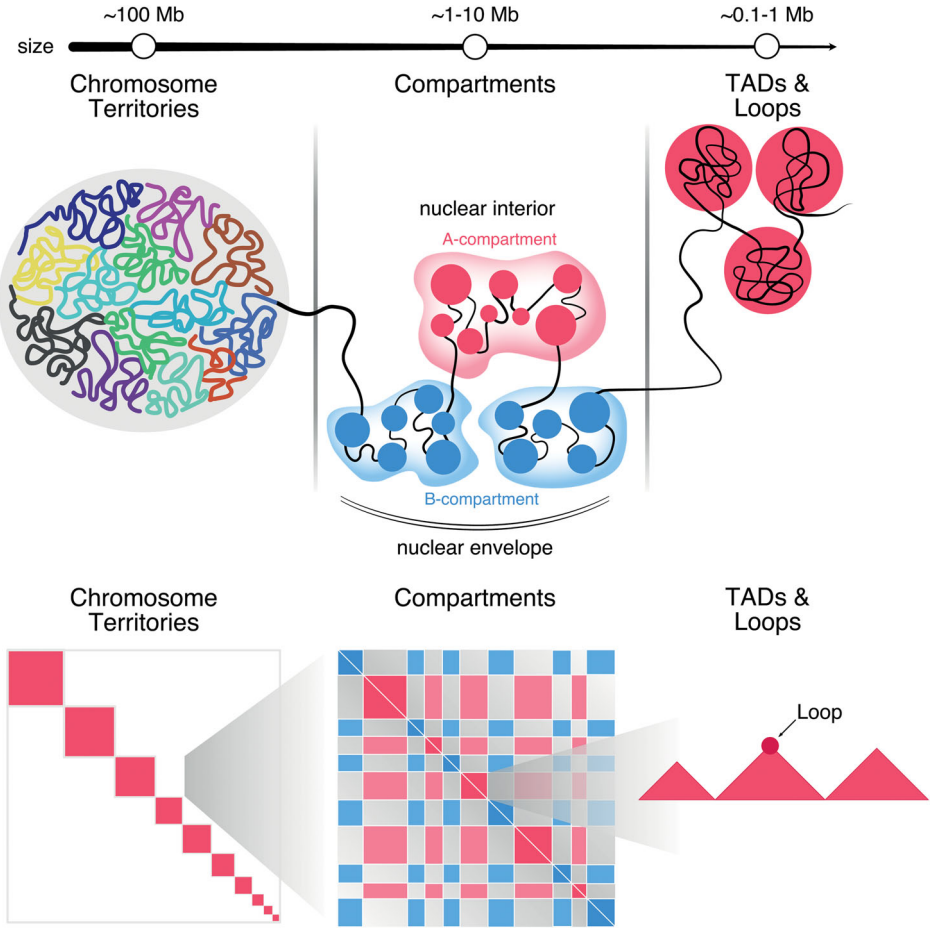

FIGURE 1 **Illustration of the features described in box 1**. From larger to smaller scale, the picture shows chromosome territories, A/B compartments, TADs and loop domains. The top panel represents the chromatin fiber at different scales, the bottom panel represents the Hi-C matrix at different zoom levels (in the rightmost panel, the matrix is rotated by 45° and only the top half is shown). Mb, mega base pairs

This contrasts with the control of developmentally-regulated genes, which are typically activated when lineage-specific transcription factors are present in the nucleus.[44] There are cases where developmentally-regulated genes are unresponsive to transcription factors, such as the targets of the PRC2 complex for instance,[45] but in the case of the inactive X, the phenomenon takes place at a larger scale and shows the effects of chromosome-wide removal of transcription factors.[42] Incidentally, transcription factors typically bind only a select subset of their sequence motifs in the genome,[46] the rest being inaccessible. The mechanisms that regulate accessibility are essential for gene regulation, and yet they are still poorly understood. In this regard, the inactive X chromosome may also be viewed as a model to study how transcription factors gain or lose access to their potential binding sites.

In sum, X-chromosome inactivation should not be viewed as a mere silencing process, but also as a process, which makes promoters and enhancers inaccessible to the transcription machinery. In other words, to achieve dosage compensation through inactivation, it is essential that transcription factors are locked out of the inactive X, which can be seen as the ultimate outcome of the process. This property is unique to the inactive X chromosome at the chromosome-wide scale, making it an ideal model to study the interactions between transcription factors, chromosome conformation and transcription.

PROMOTER–ENHANCER CONTACTS

The conformation of a genome is dynamic and ever-changing, but typical structures emerge from recurrent contacts between loci. Among those, promoter–enhancer contacts are particularly important as they drive complex gene regulatory networks. Promoter–enhancer contacts are an indispensable aspect to review before addressing more specific aspects of genome organization.

The textbook view of enhancer–promoter interactions is often represented in picture by a model whereby a chromosome loop brings an enhancer close to a promoter.[47] In other words, enhancers would act as assembly hubs for macromolecular complexes and bring them in contact with promoters via looping of the chromatin fiber. The essential aspects of these "contact-via-looping" models are twofold: First, enhancers must pre-assemble transcriptional complexes, so they must bring the subunits close to each other in an orderly fashion. Second, in order to deliver the assembled complexes, enhancers

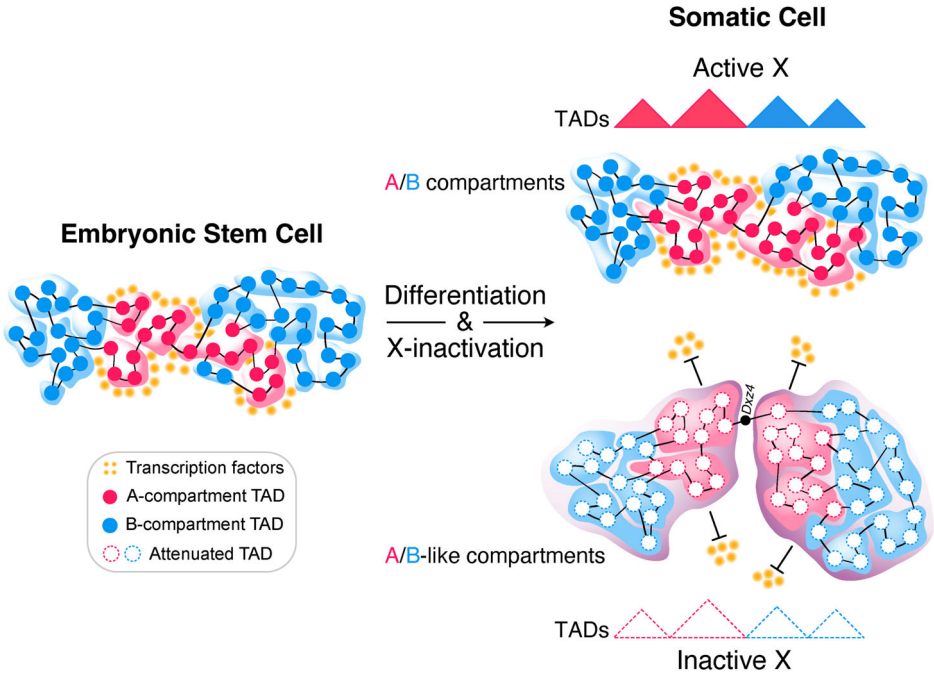

FIGURE 2 **Distinct 3D conformation of the inactive X chromosome**. Both X chromosomes are active in embryonic stem cells, and one becomes inactivated upon differentiation into somatic cells. The active X chromosome shows A/B compartmentalization (A is shown in red and B in blue) and TADs (circles; triangles on Hi-C maps) and allows transcription factor binding (yellow dots). The inactive X chromosome shows A/B-like compartments and attenuated TADs. The inactive X is moreover organized in two mega-domains that are separated by the *Dxz4* boundary region. The repressive epigenetic environment (purple shade) set up by the long non-coding RNA Xist, repels transcription factors

must come in extreme proximity to promoters, either transiently or stably.[47]

However, neither the stable nor the transient version of the looping model can explain the following observations: (1) Transcription factors remain bound to their motif for a few seconds at most,[47] while transcription bursts can last minutes.[48,49] This is incompatible with the view that transcription factors directly initiate transcription. Instead, transcription factors must take part in a process that facilitates transcription even after they detach; (2) Promoters regulated by the same enhancer often fire synchronously, at least in *Drosophila*,[50] requiring that enhancers exclusively engage in multi-way loops with their target promoters; (3) Almost any combinations of a dozen distinct binding sites were shown to be sufficient to create synthetic enhancers in transient transfection experiments,[51] raising questions as to how preassembled macromolecular complexes could emerge from almost any combination of motifs; and (4) Some enhancers are more potent as they lie further away from their target promoter.[52]

But abandoning the looping model raises an important question: how does transcription factor binding at enhancers translate to transcription initiation at promoters, that is, at another locus?

There is presently no firm answer, but several models suggest how this difficulty could be addressed. In the transcription factor activity gradient (TAG) model,[47] enhancers are proposed to act as "fountains" of modified transcription factors. Based on the observation that histone-modifying enzymes also target transcription factors,[53] the authors propose that the enzymatic activities that deposit specific histone marks at enhancers also modify transcription factors. After binding enhancers, transcription factors would be modified and thereby become activated, they would then detach and diffuse randomly, occasionally hitting promoters. A key assumption of the model is that transcription factors would promptly return to their ground state so that enhancers could only activate promoters in the close vicinity of the enhancer that modified them.

A second model draws from the observation that proteins with intrinsically disordered domains can form short-lived condensates in the nucleus, a phenomenon referred to as "condensation", "phase transition" or "liquid demixion".[54] Transcription factors are enriched in intrinsically disordered domains compared to the rest of the proteome,[55,56] making them prone to form condensates. In the model that we will refer to as the "condensates model", enhancers and promoters nucleate the formation of aggregates with high concentration of transcription factors.[57–61] This aggregate facilitates the assembly of transcriptional complexes in a local area and can produce a transcription burst at promoters within the condensate.[62] At some point, the condensate disperses and the transcription burst stops.[63]

Both models are compatible with observations (1)–(4) above. To give one example, in the TAG model, an active enhancer keeps releasing modified transcription factors, so all nearby promoters will fire synchronously; whereas in the condensates model, promoters in the vicinity are typically part of the same aggregate, so they all fire when the aggregates are formed. Overall, those models make similar predictions, except for the residence time of transcription factors. In the TAG model, very long residence times on the enhancer should be associated with low activity because transcription factors are supposed

to leave the enhancer to activate promoters. On the contrary, in the condensates model, very long residence times should be associated with higher activity because transcription factors must reach a critical concentration to initiate the formation of an aggregate. Quantitative experiments will help us choose the most plausible model, or come to the realization that both are possible.

The mechanistic models of activation by enhancers have changed over the years, but the views on their activity have remained remarkably unaltered. Simply put, enhancers are active when they are bound by multiple activating transcription factors. The Achilles heel of this model is that transcription factors usually bind a small fraction of their sequence motifs, following rules that are only partially understood, so the code of enhancer regulation remains unbroken. It is even debated whether there is a code at all, that is, whether only some combination of transcription factors in some specific order can create an enhancer.[44] The answer seems to be that multiple enhancer architectures are possible. For instance, the enhanceosome of the beta-interferon gene follows the rules of a formal grammar where any change to the layout of the binding sites ruins the enhancer activity.[64] But in most cases, the architecture of the enhancer is such that transcription factors contribute to the overall activity either individually or in small groups that bind synergistically.[44] In summary, enhancers are diverse enough that they may correspond to different biological entities that abide by different rules.

How much transcription factors contribute to genome conformation is an open question. The transcription factor with the largest impact is CTCF, which mediates the formation of chromatin loops.[40,65] Indeed, loop domains disappear from Hi-C maps when CTCF is removed using a degron system,[66] to the extent that it is considered as a structural factor by many authors. The collective contribution of other transcription factors is harder to evaluate. During the reprogramming of mouse iPS cells, the loci bound by OCT4 and KLF4 were also the ones with the largest conformational changes at the end of the process,[67] suggesting that transcription factors may contribute to genome conformation through mechanisms that are yet to be discovered. The formation of enhancers and their potential contacts with promoters is another important aspect to consider. In experiments where artificial enhancers were mobilized to different locations of a TAD in mouse embryonic stem cells, the Hi-C maps showed only mild differences.[68] This suggests that enhancer–promoter pairs have a modest impact on the global conformation of the genome, and therefore that transcription factors have little room to play an influential role, but additional experiments will be required to determine if these conclusions can be generalized.

In summary, present research is at a critical point where our knowledge of enhancers is advancing at a fast pace and future developments will shed light on the mechanisms that link enhancer–promoter contacts with the initiation of transcription.

ACCESSIBILITY OF THE X CHROMOSOME

These considerations are key to understanding the conformation of the X chromosome. Upon reactivation, transcription factors regain access to the chromosome, or at least to some binding sites, and the question is whether this contributes to the acquisition of the active conformation, or whether this is a neutral event. It is still unclear what event licenses the return of transcription factors; the most prominent candidates being the loss of PRC2 and H3K27me3 (tri-methylation of histone H3 lysine 27), the loss of DNA methylation at CpG islands, or the loss of Xist coating. It is possible that all events contribute to some extent, but it is presently difficult to pinpoint the exact mechanism by which they cause the disappearance of the mega-domains and the reappearance of Topologically Associating Domains during X-reactivation (see **Box 1**). The upregulation of Xist RNA leads to the eviction of cohesin from specific sites on the inactive X,[27,69] and its downregulation during X-reactivation are potentially key steps. Indeed, deleting *Xist* in differentiated cells allows the return of cohesin on the inactive X and leads to the reappearance of TADs.[27,43]

It is not yet clear how transcription factors return to the X chromosome upon reactivation. A possibility is that pioneer transcription factors[70,71] break into the inactive X and pave the way for other transcription factors to bind their target sites. In particular pluripotency transcription factors, such as c-MYC, KLF4, ESRRB, PRDM14 and ZFP42 have been implicated in X-reactivation.[18,72–74] Yet, a challenge for this model is to explain the reactivation kinetics. In a model of induced pluripotency,[75] the ATAC-seq signal on the active X is already in its final state while transcription factors have not yet returned to the reactivating X. This suggests that all the transcription factors that are required to generate accessibility of the active X chromosome are already present, including the pioneer factors, but they seem to not be sufficient to open the inactive X in the early phase of the process.

Nevertheless, a recent study has shown that enhancer regions are opened before promoters during reprogramming.[76] Intersecting gene regulatory network analysis with enriched binding motifs on the X chromosome, the study identified the naive pluripotency factor ZFP42 as the most prominent candidate, which becomes upregulated during the time point of X-reactivation with several other naive pluripotency factor such as NANOG and PRDM14.[75] As these factors have been also implicated indirectly or indirectly promoting the downregulation of *Xist*,[72,77–79] this further demonstrates the intricate intertwining between the X-reactivation process and the pluripotency network.[74,80] The MYC family is also potentially involved, as suggested by the enrichment of binding sites near early reactivated genes in mouse blastocysts.[18] Future work will need to test if pluripotency factors not only indirectly lift the accessibility barrier on the inactive X by downregulating Xist RNA, but rather have an active role in remodeling the chromatin to turn on X-linked gene transcription.

Related to this issue, it is also unclear whether pioneer transcription factors can bind their target on the inactive X. ATAC-seq data shows no evidence that they do,[75] although they might not immediately leave a print in ATAC-seq signal.[70,71] Progress on this question will require allele-resolution ChIP-seq data for pluripotency factors with a pioneer activity, such as OCT4, SOX2 and KLF4.

The most straightforward scenario is that transcription factors return as soon as the signal that excluded them is removed from the inactive X chromosome. Known exclusion mechanisms include the repressive marks H3K27me3,[33,81] CpG methylation of

promoters,[25,37,82] and the Xist RNA itself.[27] It is unclear which would be the ultimate barrier and how it would be removed. The Xist RNA is depleted as a consequence of its down-regulation at the outset of the reactivation process. Without Xist RNA, H3K27me3 is no longer maintained and therefore evicted from the inactive X.[83] The loss of H3K27me3 could be at least in part due to active demethylation mechanisms, as mouse blastocysts deficient for the H3K27-demethylase *Utx/Kdm6a* showed a less efficient removal of the mark and reactivation of some X-linked genes.[18] Removing CpG methylation is more complex because the mark has its own independent maintenance mechanism via the DNA-methyltransferase DNMT1.[84] How the mark is removed has not yet been investigated in detail, but this may be a critical step for transcription factors to return and for the chromosome to adopt the conformation of an active X chromosome.

In summary, the contributions of transcription factors remain elusive. To this day, CTCF remains the most important player in mammals and additional experiments are required to know if regular transcription factors can collectively have a significant impact on the shape of the genome.

A/B COMPARTMENTS

One of the first insights brought by the Hi-C technology was to establish that the genome consists of two compartments called A and B (see **Box 1**) that have some self-affinity.[85] Therefore, a site in the A compartment contacts other sites in the A compartment more frequently than sites in the B compartment. The same holds for the B compartment; the main difference being that transcriptionally active gene-rich regions reside in the A compartment, while silent heterochromatic regions reside in the B compartment.

Interesting insight into the nature of A/B compartments came from studies of X inactivation (Figure 2). It was initially observed that the mouse inactive X has no compartments, that is, that it contains neither A nor B regions and consists of its own separate compartment; the only structure being a bipartite organization in two mega-domains that are separated by the *Dxz4* macrosatellite region.[38,41]

Moreover, claims of a compartmentless inactive X were partially challenged in a later study showing that compartments were present on the inactive X of human cell lines while maintaining the partitioning into two mega-domains,[86] leading to the interim conclusion that mice differed from humans in this regard. It was proposed that in mice, compartments disappeared because they were merged during the X-inactivation process by the architectural protein SMCHD1[39,87] (but similar studies were not performed in humans). However, a recent study showed that a compartment structure appears on the mouse inactive X if the mega-domains are examined separately.[75] It is not yet clear whether those A/B-like compartments are a static feature of the inactive X or if they vary between cell types. Since some genes are escapees in some cell types but not in others,[88,89] it is probable that the A/B-like compartments might change accordingly, but new data will be required to answer this question.

Here it is important to emphasize the key role of computations and formal definitions in the seemingly discordant results described above. A/B compartments are defined from a computational intermediate involving a technique called Principal Component Analysis (see **Box 1**). The two mega-domains can "capture" the first principal component, obscuring any underlying structure at a smaller scale. Analyzing the mega-domains separately is a way to release the first principal component so that it can reveal other existing structures. In that sense, the discovery of A/B-like compartments on the inactive X of mice[75] is not in conflict with previous studies, it is merely the realization that mega-domains can overshadow chromosome-wide structures when applying the original definition of A/B compartments.[85]

The A/B-like compartments of the inactive X chromosome may be the computational equivalent of the A/B compartments of autosomes, but they are different biological entities. The A-like and B-like compartments of the mouse inactive X correspond to H3K27me3-rich and H3K9me3-rich regions,[26,75,86] respectively (instead of transcriptionally active and transcriptionally silent regions), as previously described also for the human inactive X chromosome.[26,86] Of note, A/B compartments have been further subdivided into sub-compartments[40,90] but it is presently unknown whether the A/B-like compartments of the inactive X correspond to some of those subtypes.

In mice, the emergence of H3K27me3 domains during X inactivation gives some functional insight into the possible structure of A/B-like compartments. After the induction of *Xist*, Xist RNA initiates gene silencing by recruiting the protein SPEN.[29–31,91] The histone marks H2AK119ub1 (mono-ubiquitylation of histone H2A lysine 119) and H3K27me3 are later deposited by PRC1 and PRC2, respectively.[35,92] There is agreement that PRC1 is recruited first, by Xist RNA through hnRNPK,[93] but it is debated whether PRC2 is indirectly recruited by PRC1[94,95] or directly by Xist RNA and JARID2,[96–98] — as the PRC2 complex was not pulled down by the full length Xist RNA in three independent studies.[27,31,99] More important for this discussion, the activity of the PRC1 complex induces the coalescence of its target sites,[100,101] which may be one of the driving mechanisms for the formation of A/B-like compartments on the inactive X,[75,87] further highlighting their ontological differences with the A/B compartments of autosomes.

The novel insight from these results is that different mechanisms may lead to relatively similar compartment structures. As a result, it is doubtful that there exists a simple and unique relationship between A/B compartments and transcription. More likely, A/B compartments may emerge from different or even concurrent processes. Along those lines, H3K27me3 delineates chromosomal compartments in mouse oocytes and two-cell embryos,[102,103] which are both transcriptionally inactive. In such conditions, it becomes relevant to find out whether A/B compartments all emerge from the same mechanisms before asking if they are the cause or the consequence of transcription.

TOPOLOGICALLY ASSOCIATING DOMAINS

A puzzling feature of genome organization is the segmentation in Topologically Associating Domains or TADs (see **Box 1**). TADs are chromosomal domains enriched in self contacts; they typically appear as a succession of squares on the diagonal of Hi-C matrices (or triangles in

half-matrix representations, see Figure 2). TADs were first discovered on the X chromosome[104] and it was immediately established that they are a common feature of all chromosomes.[105]

A difficulty with TADs is that they appear as individual domains only when stacking the Hi-C contacts from several cells. At the single-cell level, the enrichment of contacts within TADs exists,[106] but it is usually insufficient to identify boundaries, raising some questions regarding the physiological relevance of TADs. The current consensus is that TADs are formed by loop extrusion, a mechanism whereby chromatin loops are formed through the combined action of cohesins and CTCF.[107] Under that model, TADs are dynamic structures maintained by an active process.

Presently, the causal relationships between TADs and transcription are unclear, and conflicting results were obtained in different systems. For instance, in the early development of *Drosophila* and mice, TADs are formed before the zygotic genome activation,[9,108] whereas in humans, the embryonic genome activation is necessary for the formation of TADs.[109] While TADs might be of a different nature in vertebrates and invertebrates, the apparent contradiction still has to be resolved.

Significant insight came from degron systems, showing that TADs disappear if either CTCF or cohesins are removed.[66,110–112] CTCF is maternally expressed in mouse embryos[113] but not in human embryos.[109] The strict requirement for CTCF thus explains that TADs cannot form before the embryonic genome activation in humans. These results suggest that transcriptional activity does not in itself structure the genome into TADs. However, inhibiting transcription can attenuate the formation of TADs, as shown in cultured *Drosophila* cells for instance.[110] The current consensus is that cohesin and CTCF are necessary to form TADs, but their distribution itself depends on the global transcriptional activity and vice versa.[110,111]

Since CTCF and cohesins are major factors for the structuration of chromosomes, it is plausible that the return of transcription factors per se (as opposed to the events that permit this to happen) is one of the most important events for the acquisition of a new conformation on the reactivating X chromosome. Symmetrically, the loss of transcription factors during the inactivation process probably has significant consequences in terms of conformation. Here it is important to emphasize that CTCF is not completely removed from the inactive X. First, it remains at the loci containing escapees, suggesting that it may be important to maintain a transcriptionally active chromatin.[38,114] And second, it is present at the *Dxz4* locus that separates the mega-domains.[115,116]. In experiments where the cohesin subunit RAD21 was removed from mouse cells,[117] the active X chromosome acquired some features of the inactive X, such as the loss of TADs and the formation of a superloop between *Dxz4* and *Firre*. However, the active X did not form mega-domains, showing that the characteristic structure of the inactive X is not simply due to the eviction of cohesin.

Another key factor for the inactive X chromosome is the non-canonical SMC protein SMCHD1 (structural maintenance of chromosomes hinge domain containing 1),[118] which plays important roles in gene silencing[91,119] and CpG island methylation.[37] Importantly, SMCHD1 also contributes to the 3D conformation of the inactive X: it attenuates TADs by affecting the binding of CTCF[120] and the underlying compartment structure is more visible upon SMCHD1 knockout in mice.[39,75,87,120] SMCHD1 is recruited during X-inactivation in an Xist- and PRC1-dependent manner,[121] facilitating further chromosome-wide Xist spreading[87] and the formation of H3K9me3 blocks,[122] highlighting the complex interplay of factors in shaping the chromatin structure of the inactive X.

Reciprocally, the effect of TADs on transcription is somewhat intricate. The transcriptome is only mildly affected when CTCF or cohesins are removed with a degron system.[66,110–112] Likewise, ablating TADs by removing CTCF in leukemic B cells does not prevent trans-differentiation into macrophages,[123] arguing that TADs are required neither for maintaining nor for changing cell identity. However, removing CTCF in macrophages affects their ability to trigger inflammation,[123] showing that TADs may play a role in stimulus response.

TAD borders intervening between a promoter and an enhancer can act as insulators, preventing illegitimate interactions, also known as enhancer hijacking, which could otherwise lead to accidental spikes of transcription. In this regard, TADs and especially TAD borders represent an additional layer of transcriptional regulation, even though this mechanism may not apply to every gene.[124] Recent enhancer-mobilization experiments in mouse ES cells showed that strong enhancers can bypass a TAD border that weaker enhancers cannot,[68] demonstrating the potentially complex effect of TAD borders on transcription.

Other mobilization experiments revealed that some TAD borders play a key role in gene regulation. For instance, at the X-inactivation center (Xic), *Xist* and its postitive *cis*-regulators such as the non-coding RNA *Jpx* lie in one TAD, whereas *Tsix*, the non-coding antisense regulator of *Xist*, and its regulatory elements occupy a separate neighboring TAD.[104,125,126] When switching the orientation and swapping *Xist* and *Tsix* in opposite TADs, *Xist* became regulated like *Tsix* normally would and vice versa, indicating that the TAD boundary has an important function in shielding developmentally regulated genes such as *Xist* and *Tsix* from inappropriate regulatory inputs from enhancers in the neighboring TADs.[127,128] In sum, TADs might increase the precision of gene regulation, in particular when it comes to developmental transition contexts[124,127] or during specific cellular responses as in the case of inflammation in macrophages.[123]

Once again, the study of X inactivation led to key insight into the relationships between TADs and transcription. Xist RNA upregulation leads to the eviction of CTCF and cohesins from the inactive X through direct[27] or indirect mechanisms.[31,69,99] At the single-cell level though, inactive X chromosomes still harbor TAD-like domains that fluctuate from cell to cell.[129] Thus, the lack of TADs is not due to the absence of contact domains, but to the volatility of the borders that separate them. This is in line with the view that residual levels of cohesin are necessary for the inactive X to maintain the two mega-domains, as cohesin depletion hinders their formation.[117] It is therefore likely that loop extrusion is at work on the inactive X, but the depletion of CTCF makes it impossible to form static borders, so TADs fail to emerge at the population level. This example shows a concrete

link between the exclusion of transcription factors to the folding of the inactive X chromosome (Figure 2).

It is worth mentioning that escapees retain some TAD-like structures while TADs disappear from the rest of the inactive X.[38,102] After removing the *Dxz4* mega-domain boundary, one study found escapees to lose both expression and TAD-like structure,[38] while other studies observed no major effect on TAD-like structure or expression of escapees.[114,130] During imprinted inactivation of the paternal X chromosome in mouse preimplantation development, TADs loss is a late event not preceding but rather following or occurring concomitantly with gene silencing while escapee regions retain TADs.[102] This suggests that loss of TAD structures might be facilitated by the loss of transcription or the specific chromatin state established on the inactive X during silencing.[102] Moreover, the gene bodies of escapees were found to display lower Xist enrichment compared to silenced genes of the inactive X chromosome.[131] A simple explanation could be that the local concentration of Xist is insufficient to evict CTCF and cohesins, but the *Xist* locus itself suggests that there may be more to it: While showing high enrichment for Xist RNA,[131] the *Xist* locus, which escapes its own inactivation, features a prominent TAD present also on the inactive X.[75] Therefore, Xist does not prevent the formation of TADs in escapee regions. It is possible that escapees self-associate in space via active chromatin, which would explain their clustering into distinct subcompartments.[75] It is presently unknown if TAD-like structures around escapees are functionally important or if clustering in 3D facilitates escapee from silencing.

Symmetrically, during the reactivation process, TADs reappear on the X chromosome before transcription restarts.[75] This is consistent with the view that transcription factors must bind their targets before transcription can resume; but if transcription factors are present, then so are CTCF and cohesin, allowing TADs to form again. The precise timing of transcription relative to the formation of TADs on the reactivating X chromosome is probably accidental. Indeed, both events are downstream of transcription factors binding, so they are initiated at the same time, but it may take them different times to complete.

In summary, TADs and transcription share a common cause, namely, the binding of transcription factors and architectural factors. This may explain why they seem to coincide at so many levels, while the issue of their causal relationships remains generally confusing. Overall, it seems that TADs require mostly CTCF and cohesins to start forming, and that TAD borders can tweak promoter–enhancer interactions in ways that still need to be understood.

CONCLUDING REMARKS

X-chromosome inactivation sheds light on the general mechanisms of chromosome folding. In particular, the signature depletion of transcription factors from the inactive X allows us to investigate their role by comparison with the active X. However, it is important to highlight that the depletion of transcription factors is by no means the only characteristic feature of the inactive X, so one has to interpret the experimental results with caution. For instance, the Xist RNA and Polycomb repressive complexes are key factors that also shape the inactive X chromosome.[27,87]

Future research should investigate in greater details the relationships between pioneer transcription factors and the special heterochromatin of the inactive X chromosome. Likewise, an important stake will be to understand the mechanism that maintains transcription factors locked away from the inactive X. It will also be important to determine whether this mechanism is the same as that of gene occlusion,[132] whereby genes can become impervious to transcription factors. Addressing these fundamental issues will allow us to better understand the relationships between transcription factors and genome conformation.

In conclusion, transcription and genome folding intersect at multiple levels and it becomes increasingly clear that multiple concurrent mechanisms contribute to shaping the genome. Parallel to the overarching principles that govern the formation of TADs or loop domains, other mechanisms operating at a local scale can also influence the conformation. As a result, disentangling causes and consequences will require a better knowledge of the mechanisms at work in the nucleus in order to understand how genome conformation and transcriptome emerge in a given cell type.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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