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Three-dimensional chromatin organization in cardiac development and disease

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ABSTRACT

Recent technological advancements in the field of chromatin biology have rewritten the textbook on nuclear organization. We now appreciate that the folding of chromatin in the three-dimensional space (i.e. its 3D "architecture") is non-random, hierarchical, and highly complex. While 3D chromatin structure is partially encoded in the primary sequence and thereby broadly conserved across cell types and states, a substantial portion of the genome seems to be dynamic during development or in disease. Moreover, there is growing evidence that at least some of the 3D structure of chromatin is functionally linked to gene regulation, both being modulated by and impacting on multiple nuclear processes (including DNA replication, transcription, and RNA splicing). In recent years, these new concepts have nourished several investigations about the functional role of 3D chromatin topology dynamics in the heart during development and disease. This review aims to provide a comprehensive overview of our current understanding in this field, and to discuss how this knowledge can inform further research as well as clinical practice.

1. Introduction

Chromatin dynamics in the heart have fascinated cardiac biologists and pathologists for decades. More than 50 years ago it became apparent that not only gene expression but also nuclear shape, size, and chromatin density experience remarkable changes during postnatal cardiomyocyte development [1,2]. Cardiomyocyte hypertrophy was also shown to modulate nuclear structure by inducing fetal-like nuclear features [1], mirroring the well-known reactivation of the fetal gene program. Moreover, hypoxia was reported to reversibly increase chromatin condensation [3,4], suggesting that nuclear organization can be highly dynamic. However, for many years such early morphological observations could not be translated into molecular-level details due to the lack of high-throughput methods to measure the dynamics of individual loci. Thus, the potential link between changes in nuclear organization and heart development and disease remained largely speculative.

Meanwhile, classical gene mapping and subsequent large-scale exome and genome sequencing studies provided ample evidence that alterations of nuclear function can cause both developmental defects and heart disease in adults. On the one hand, rare mutations in core cardiac transcription factors (TFs), such as *NKX2-5* [5], *TBX5* [6,7], and *GATA4* [8,9], cause Mendelian congenital heart disease (CHD). Moreover, mutations in chromatin modifiers contribute to ~30% of all CHD cases associated to single nucleotide variants appearing for the first time in a family member (*de novo*), and to ~3% of all CHD cases [10–13]. On

Abbreviations: 3C, chromatin conformation capture; CHD, congenital heart disease; ChIP, chromatin immunoprecipitation; CM, cardiomyocyte; cPIR, cardiac promoter-interacting region; CVD, cardiovascular disease; DCM, dilated cardiomyopathy; eQTL, expression quantitative trait locus; FIRE, frequently-interacting region; FISH, fluorescence *in situ* hybridization; GWAS, genome-wide association study; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; hPSC-CM, human pluripotent stem cell-derived cardiomyocyte; LAD, lamina-associated domain; PCHi-C, promoter capture after high-throughput chromosome conformation capture; SNP, single nucleotide polymorphism; TAC, transverse aortic constriction; TAD, topologically associating domain; TF, transcription factor.

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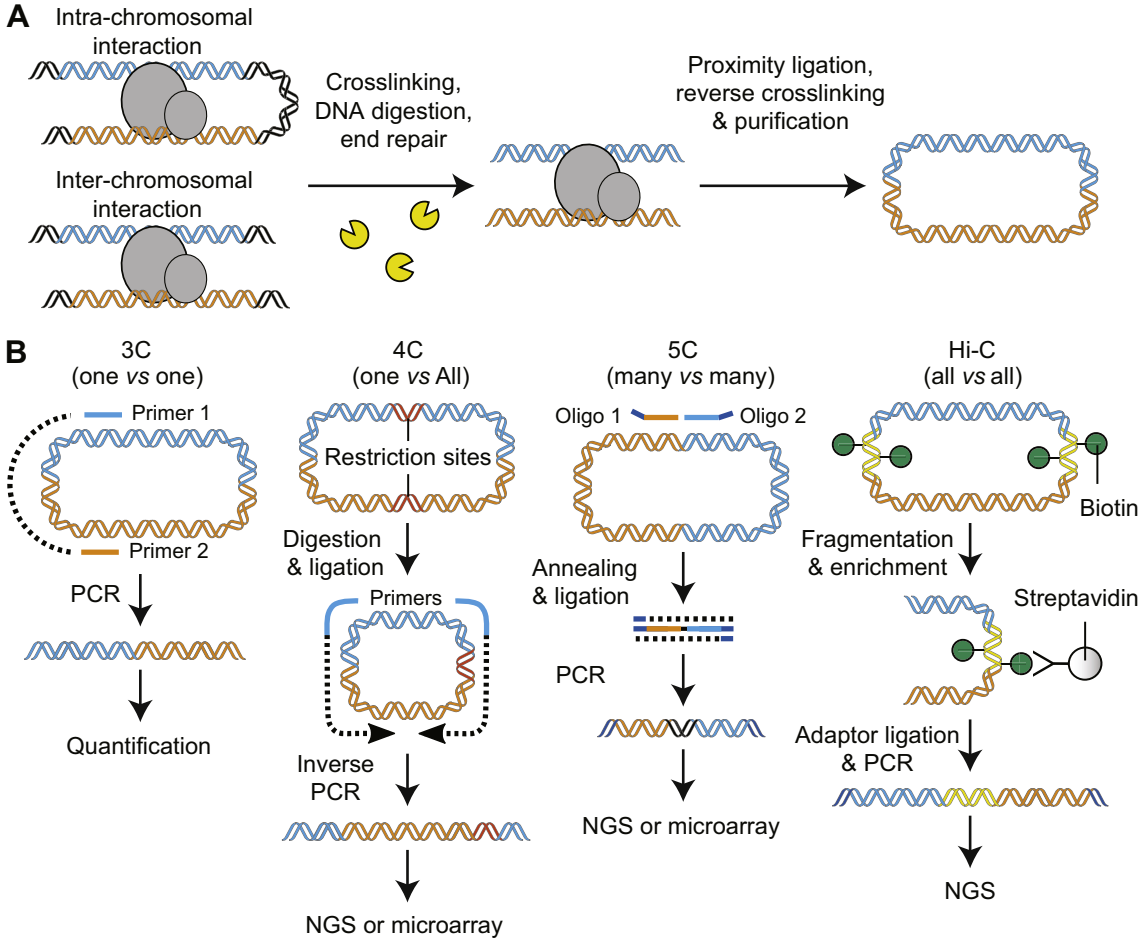

Fig. 1. Chromatin conformation capture technology.
(A) Basic pipeline for most chromatin conformation capture (3C)-based experiments. Naïve 3D chromatin structure is preserved by crosslinking to stabilize interactions between proteins (grey ovals) and nucleic acids. Chromatin is then fragmented using endonucleases (i.e. restriction enzymes, DNase, or MNase) to generate free DNA ends. After end repair, proximity ligation generates hybrid molecules containing DNA regions that may have been far away on the primary sequence (or even located on different chromosomes), but were nearby in the 3D space. (B) Specific steps for 3C, 3C on chip (4C), 3C carbon copy (5C), and high-throughput 3C (Hi-C). 3C quantifies the interaction between two loci by amplifying a specific proximity ligation product. 4C can capture the interactions between a locus of interest and the rest of the genome: proximity ligation products are "trimmed", re-circularized, and amplified by inverse PCR. 5C can reveal all the contacts in a large genomic region: proximity ligation products from restriction enzyme-based 3C are annealed with a complex mix of oligos designed to recognize the regions next to predicted cuts sites; oligos facing each other are ligated and amplified. Hi-C can identify any interactions across the genome: proximity ligation products are generated so as to incorporate biotin molecules that allow streptavidin-based enrichment of ligation products. 4C, 5C, and Hi-C are nowadays generally analyzed by paired-end next generation sequencing (NGS), which allows to map the location of the two DNA fragments contributing to each hybrid molecule obtained after proximity ligation.

the other hand, ~10% of dilated cardiomyopathy (DCM) cases are directly attributable to *de novo* or inherited mutations of just two genes involved in nuclear regulation of gene expression, *LMNA* (encoding nuclear intermediate filaments) and *RBM20* (encoding a muscle-specific splicing factor), both of which lead to particularly malignant and aggressive disease [14–17]. These and other observations prompted a plethora of studies on the mechanisms involved in chromatin regulation in cardiac development and disease. However, until recently most such studies had focused on the so-called "nucleosomal scale", which spans from a single DNA base pair (bp; which can be modified by dynamic 5'-cytosine-phosphate-guanine-3' [CpG] methylation), to ~150 bp (the size of an individual nucleosome that can be modulated by post-transcriptional modifications of histone tails), to a few kilobases (kb; the range of action of nucleosome remodelers and other local-acting chromatin regulators). In this context, the role of "supra-nucleosomal" (i.e. up to megabase-sized, or chromosome-level) and "nuclear" scale (i.e. up to gigabase-sized, or genome-level) chromatin organization had remained largely unknown.

Over the last decade, the development of powerful technologies to map three dimensional (3D) chromatin folding (i.e. its "architecture"), particularly proximity ligation-based chromosome conformation capture (3C) technologies (Fig. 1 and Glossary; reviewed in [18]), has finally enabled cardiac biologists to address the long-standing question about the functional implications of chromatin topology in cardiac pathobiology. The central goal of this review is to provide a critical summary of the first series of reports on this topic, most of which have emerged during the last five years. We begin by providing a brief overview of the state of the art in the chromatin organization field, and follow with two main sections describing notable findings and open questions in the context of cardiac development and disease, respectively. We conclude by suggesting what we see as promising future directions for the field, including possible paths for the translation of this knowledge into clinical practice.

Throughout this review we focus on studies that specifically provided insights into the supra-nucleosomal and/or nuclear scale 3D architecture of chromatin in cardiomyocytes. Indeed, the topic of cardiac chromatin regulation at the nucleosomal scale has been extensively reviewed elsewhere [19–21]. While we attempt to introduce new

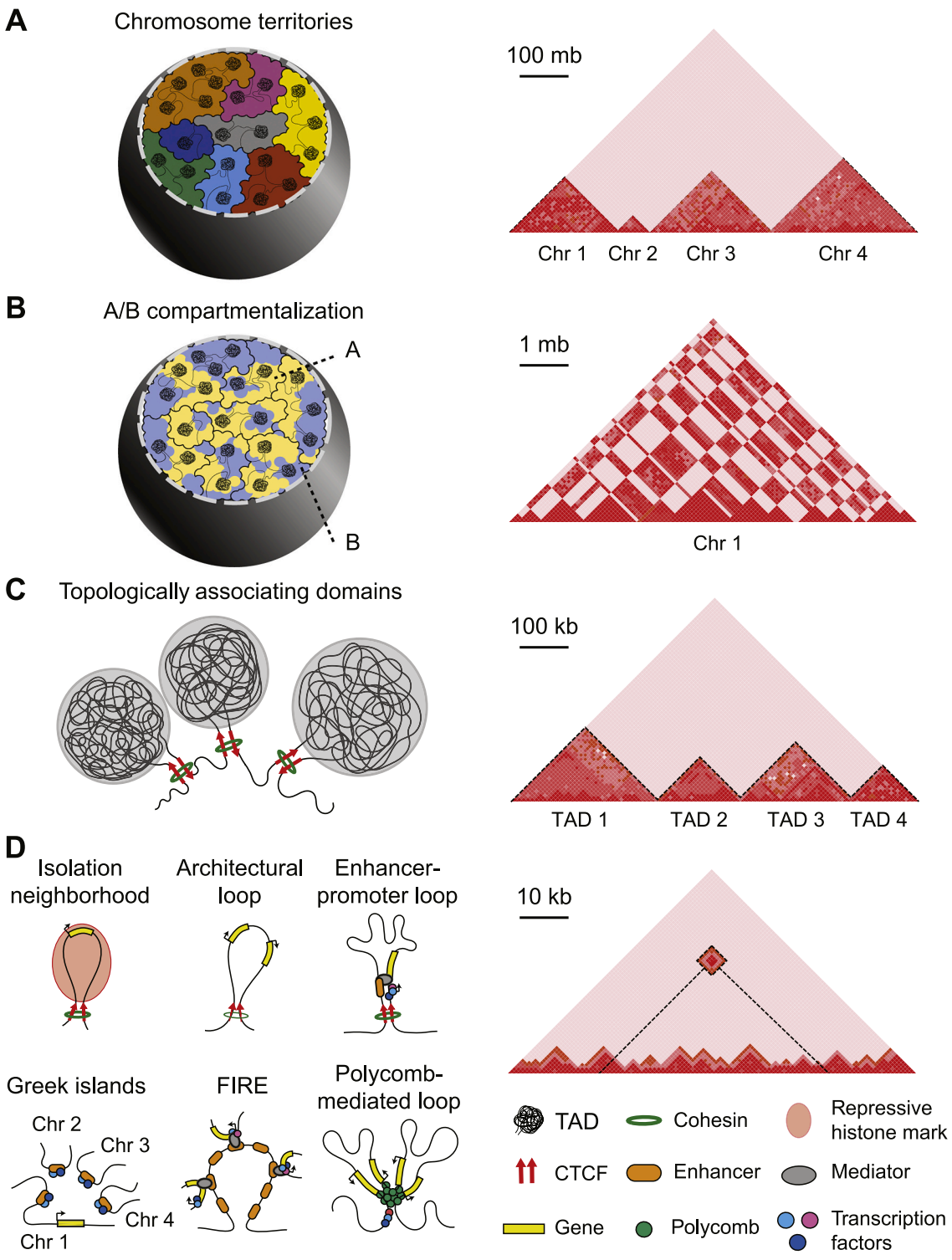

Fig. 2. Higher order chromatin organization.
Schematics of the various hierarchical layers of 3D chromatin architecture (left), and exemplative Hi-C chromatin contact heatmaps showcasing the underlying DNA interactions (right; each square indicates the interaction frequency between the matching chromosomal coordinates based on a color coding where darker shades of red represent stronger interaction). (A) At the nuclear scale, chromosomes mostly occupy specific territories: this is visually indicated by the low degree of inter-chromosomal interactions on genome-wide Hi-C maps. (B) At the chromosomal scale, chromatin segregates into megabase-sized active, "A", and inactive, "B", compartments: this is captured on Hi-C maps by the "checkerboard" interaction pattern within individual chromosomes. (C) At the sub-megabase scale, chromatin is subdivided into regions of preferential self-interaction called topologically associating domains (TADs): on finely binned Hi-C maps TADs are identified as "triangles" separated by boundary regions. (D) The supra-nucleosomal chromatin architecture within TADs consists of various loop-shaped structures: these are identified as "spots" on high-resolution Hi-C maps.

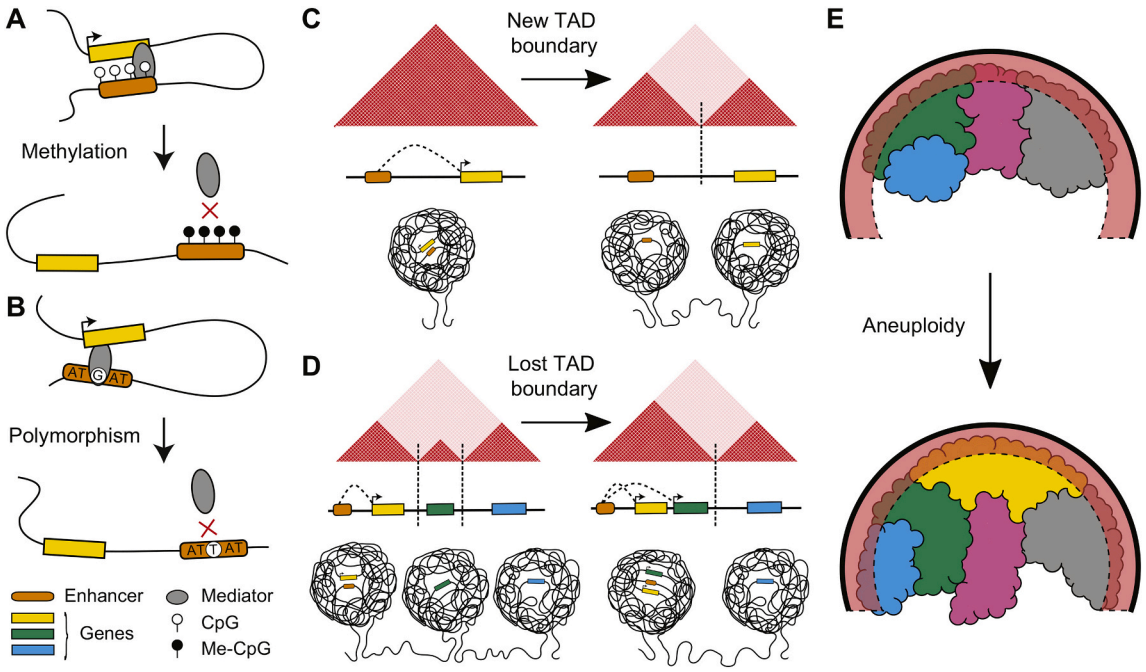

Fig. 3. Dysregulation of 3D chromatin organization.
Examples of potential mechanisms by which genetic and environmental factors may functionally affect chromatin topology. (A-B) Post-transcriptional modifications (A) or mutations (B) in enhancers may disrupt long-range gene regulation, for instance by preventing binding of a loop mediator. (C-D) Cytogenetic abnormalities such as translocations, inversions, or copy number variations may change TAD architecture by generating new boundaries (C) or by removing existing boundaries (D). The resulting changes in the topological constraints of regulatory regions (i.e. enhancers) may lead to inappropriate gene regulation. (E) Aneuploidies may affect the relative positioning of chromosome territories with respects to active and inactive nuclear neighborhoods.

concepts and present key findings as accessibly as possible, we encourage non-specialist readers to take advantage of the glossary and the list of abbreviations provided.

2. Three-dimensional chromatin architecture

Studies over the last decade have shown that interphase (non-mitotic) chromosomes organize into hierarchical domains at different genomic scales (Fig. 2; reviewed more extensively in [22–24]). At the highest order of nuclear architecture, individual chromosomes preferentially occupy specific regions called "chromosome territories" (Fig. 2A). The relative positioning of such territories is quite conserved in different cell types, with small, gene-rich chromosomes and large, gene-poor chromosomes forming two separate clusters [25]. At this scale, gene expression correlates with the positioning of loci with respect to specialized nuclear "neighborhoods". Thus, while vicinity to heterochromatin environments like nuclear lamina-associated domains (LADs) or nucleolus-associated domains is linked with low gene expression, proximity to active euchromatin domains such as nuclear speckles fosters gene transcription [26,27]. These domain types also tend to differ both in their genomic composition and histone marking. For instance, LADs are generally gene poor and enriched for repressive histone marks [28]. Nevertheless, LADs are quite heterogeneous, including both regions of constitutive (i.e. stable, generally housekeeping) and facultative (i.e. reversible, generally cell type-specific) heterochromatin. While the majority of DNA interactions generally occur within individual chromosomes (intra-chromosomal, or in *cis*), interactions across chromosomes (inter-chromosomal, or in *trans*) can account for up to half of all contacts in certain cell types [29]. Recent work has shown that in olfactory neurons multiple active enhancers located on different chromosomes (imaginatively named "Greek islands") form a multi-enhancer hub in *trans* that transcriptionally activates one specific olfactory receptor gene [30–32]. This example highlights the potential functional relevance of *bona fide trans* chromatin interactions, which have so far been difficult to distinguish from background noise arising from the random intermixing of nuclear material during proximity ligation assays.

At the sub-chromosomal level, megabase-sized chromatin domains broadly segregate into two mutually exclusive "compartments", customarily referred to as "A" (active) and "B" (inactive) [25,33] (Fig. 2B). The A compartment generally contains highly-accessible, gene-rich regions characterized by active histone marks (i.e. H3K36me3, H3K79me2, H3K27ac, and H3K4me1). In contrast, the B compartment tends to be compacted, gene-poor, and decorated with repressive histone marks (i.e. H3K27me3 and H3K9me3) [25,34]. Chromatin domains from different chromosomes characterized by A compartmentalization tend to also preferentially interact in *trans*, and to be localized within the nuclear interior. On the contrary, the B compartment is not enriched for *trans* interactions, and these domains are generally associated with the nuclear lamina. Indeed, the B compartment is thought to contain the majority of LADs [35,36]. Thus, compartmentalization is directly linked to the organization of nuclear-scale chromatin topology [29,35–38]. The mechanisms that control chromatin compartmentalization are only partially understood, but it seems that they converge to dictate liquid-liquid phase separation of heterochromatin from the rest of nuclear plasma containing the euchromatin [39–41]. Combined with the anchoring of heterochromatin at the nuclear periphery by nuclear lamina proteins, these biophysical forces determine the "classic" radial separation of A and B compartments at the nuclear interior and periphery, respectively, observed in most cell types [42]. Notably, at least 20% of the genome shows cell-specific compartmentalization [43–47], indicating a substantial degree of dynamicity during differentiation and, possibly, disease.

At the sub-megabase scale, chromatin is organized into regions of preferential intra-domain interaction called topologically-associating domains (TADs; Fig. 2C) [43,48]. TADs are largely stable between different cell types, highly conserved across species, and often regarded as basic units of chromosome folding [49]. Indeed, TADs are the

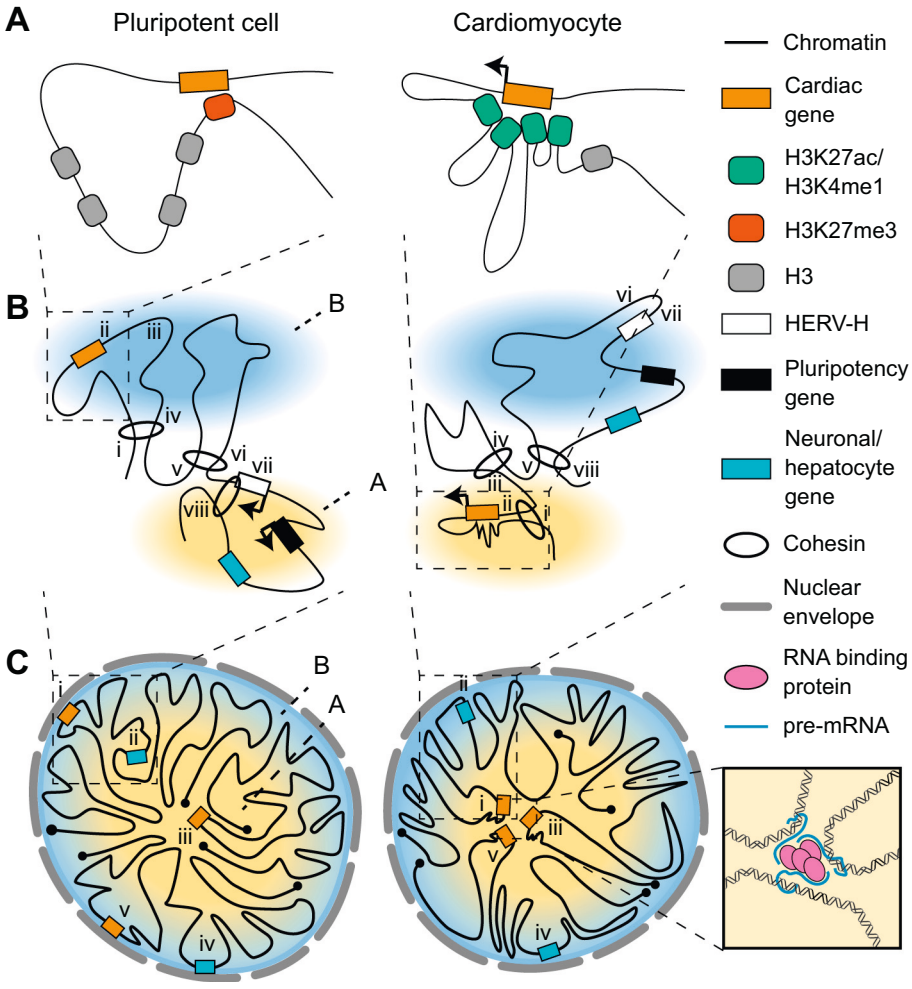

Fig. 4. Chromatin topology dynamics during cardiac development.
Developmental changes in gene expression during cardiogenesis can be associated with multi-scale changes in 3D chromatin organization. (A) Cardiac gene promoters are often involved in dynamic long-range chromatin interactions with repressive domains in the pluripotent state, and with enhancer clusters after differentiation. (B) Selected TADs also restructure, with novel cohesin-enriched boundaries appearing nearby upregulated cardiac genes (arrow). On the other hand, pluripotency-specific boundaries induced by the transcription of endogenous retroelements of the HERV-H family are lost upon silencing of such elements. As a result, the enhancer activity of HERV-H on nearby pluripotency genes is weakened. (C) At the nuclear scale, transition of certain cardiac genes to from the inactive, B, to the active, A, compartment is reflected by gene repositioning from the nuclear lamina to the nuclear interior. Alternative lineage genes (i.e. neuronal or hepatocyte-specific) can follow an opposite transition. In specific cases, cardiac genes found on distinct chromosomes may occupy the same chromatin neighborhood (magnified inset), a process that relies on nucleic acid-binding proteins targeting such loci and/or their transcriptional output. In B and C, Roman numerals point at specific genomic regions to facilitate the comparison of chromatin folding patterns in pluripotent cells and cardiomyocytes.

building blocks of the A and B compartments, which are the result of inter-TAD interactions, as well as of other chromosomal neighborhoods (i.e. peripherally-localized TADs that contribute to LADs; reviewed in [50]). The formation of TADs generally relies on the interplay of two highly conserved factors: cohesin and CTCF. Cohesin is a ring-shaped complex that was initially identified as a mediator of sister chromatid cohesion [51]. CTCF uses distinct combinations of its 11 zinc-finger domains to interact with other proteins and to bind across the genome in a sequence-specific manner [52]. TADs are formed by "extrusion" of chromatin by the cohesin complex, which pulls a fragment of chromatin within the ring to form a loop [53,54]. DNA extrusion progresses until cohesin encounters a physical barrier (i.e. a "TAD boundary"), often a CTCF bound site [55,56]. CTCF binding motifs are not palindromic, and their orientation plays an important role in controlling the length of an extruded loop. While convergent CTCF motifs (i.e. forward- *versus* reverse-oriented) are typically found at the anchors of small loops, a divergent direction (i.e. forward-forward or reverse-reverse) is frequently observed at TAD boundaries [57]. Importantly, not all TAD boundaries are CTCF-dependent, as in other instances cohesin-dependent loop extrusion can be halted by the RNA polymerase machinery in the context of highly transcribed genes [58]. At least in some cases the organization of genes and regulatory regions within TADs ensures the appropriate cross-talk between enhancers and promoters, participates in the preservation of gene expression patterns, and facilitates gene co-regulation [59–61]. Notably, alteration of TAD boundaries due to copy number variations or expansion of short tandem repeats can lead to congenital developmental disorders [62–64] (Fig. 3C-D). Moreover, CTCF/cohesin binding sites are often mutated in cancers [65], possibly leading to TAD alterations. On the other hand, certain loci are robust to perturbations of TAD structure [66], and acute disruption of cohesin-dependent TADs has surprisingly limited effects on global gene expression [67]. Thus, the exact function of TADs is still being debated.

Within TADs chromatin is organized into loops involving genomic regions that can be hundreds of kb away (Fig. 2D). Loops are formed by the 3D folding of the chromatin fiber, the most basic unit of genome organization consisting of DNA wrapped around histone octamers to form nucleosomes interconnected by linker DNA. Some loops can be formed through a CTCF/cohesin-dependent extrusion mechanism. Such CTCF-mediated loops are very conserved and are involved in the formation of chromatin environments [68]. Other loops are CTCF-independent: these are generally more dynamic and mainly involved in direct control of transcription, for instance by mediating enhancer-promoter interactions or by promoting the formation of polycomb complexes [69]. Loop formation within TADs creates an organization that can be markedly cell type-specific. TAD-nested structures include sub-TADs, insulation neighborhoods, and frequently interacting regions (FIREs). Sub-TADs and insulation neighborhoods have a strong influence on gene expression by participating in the formation of local chromatin microenvironments. While sub-TADs aggregate several loops, insulation neighborhoods are typically formed by a CTCF-mediated loop to ensure isolation of the contained genes [70]. FIREs are regions with significant *cis*-connectivity located toward the middle of TADs, and are strongly enriched with active enhancers and super-enhancers (i.e. clustered enhancer arrays strongly bound the Mediator complex). FIREs are strongly tissue-specific, and their activity has been related to the regulation of gene expression programs associated with cell identity and

tissue function [46]. Organizational variability of these local structures contrasts with TAD-level consistency across cell types, and suggests that lineage-specific genome regulation is controlled primarily by unique topological signatures established at this fine level of chromatin organization [71].

3. Chromatin architecture dynamics during cardiac development

Developmental changes in chromatin organization have now been reported in multiple lineages [43–47]. Here we examine how cardiogenesis impacts the various levels of chromatin organization described in the previous section, zooming back out from the fine supranucleosomal scale all the way to the nuclear scale (Fig. 4).

3.1. Long range chromatin interactions

Functional interactions between cardiac promoters and distal regulatory regions during heart development have been the focus of many classical genetics studies. However, the hypothesis that these result from physical chromatin interactions lacked experimental validation until recently. Application of 3C technology revealed that this is indeed often the case. For instance, 3C experiments demonstrated that in cardiac progenitor cells the Isl1/Ldb1 complex promotes long-range enhancer-promoter interactions, including those involving the core cardiac transcription factors Hand2 and Mef2c [72].

More recently, two groups independently reported the first genome-wide maps of promoter interactions in cardiomyocytes differentiated from human pluripotent stem cells (hPSC-CMs; from either embryonic stem cells [hESCs] or induced pluripotent stem cells [hiPSCs], both of which are valid *in vitro* models to study human development [73,74]). In both cases the authors performed promoter capture after high-throughput chromosome conformation capture (PCHi-C), a method in which genome-wide proximity ligation products are enriched for promoter-interacting regions through capture by a large number of probes [75]. Choy et al. performed PCHi-C on hESC-derived cardiomyocytes (hESC-CMs) and identified ~180,000 cardiac promoter interacting regions (cPIRs) for coding or non-coding genes [76]. They found that cPIRs interfacing with transcriptionally active promoters were significantly enriched in active histone marks (H3K4me3 and H3K36me3), while those engaging with inactive promoters were enriched for the repressive mark H3K27me3. Moreover, cPIRs significantly overlapped with validated cardiac enhancers and with left ventricular expression quantitative trait loci (eQTLs, pairs of genes and single nucleotide polymorphisms [SNP] in which the expression of a gene is associated with the allelic configuration of the matching SNP). However, such overlaps were relatively modest (~11% and ~7%, respectively). Interestingly, validation experiments for a putative enhancer overlapping with a cPIR for the gene *EDNRA* revealed an unexpected repressive role, showcasing the importance of orthogonal assays and functional validation in the interpretation of this data type.

Montefiori et al. performed PCHi-C from hiPSCs and hiPSC-derived cardiomyocytes (hiPSC-CMs), identifying ~400,000 significant promoter interactions in each condition [77]. Key findings regarding long-range chromatin interactions included: (1) both cPIRs and hiPSC PIRs involved distal regions (median distance of 170 kb) and were strongly enriched for the CTCF binding motif, confirming its central role in long-range chromatin organization; (2) nearly half of cPIRs were not observed in hiPSCs, indicating substantial dynamicity of long-range regulatory interactions during cardiogenesis; (3) cPIRs and hiPSC PIRs were modestly enriched for motifs of lineage-specific transcription factors (such as MEIS1 and OCT4, respectively), indicating that these may contribute to developmental chromatin dynamics; (4) cardiac-specific PIRs were enriched in cardiac-specific H3K27ac domains, compared to hiPSC-specific ones, and depleted for cardiac-specific H3K27me3, suggesting that cardiac differentiation drives both engagement of novel enhancers and decommissioning of regulatory domains that are inactive or poised in hiPSCs. Correlation of cPIRs with left ventricle eQTLs confirmed a significant association, though only ~12% of eQTL-gene interactions could be validated from promoter interaction data, similarly to what was observed by Choy et al.

Long-range chromatin interactions can also be determined in an unbiased fashion from standard Hi-C (i.e. without promoter capture) if the sequencing depth is very high. In their original preprint [78], Zhang et al. analyzed chromatin looping from their deeply sequenced Hi-C data at multiple stages of hESC differentiation into ventricular cardiomyocytes (~1 billion unique long-range interactions per point). Overall, they found ~14,000 significant chromatin loops at 10 kb resolution, 70% of which involved promoter-promoter or promoter-enhancer interactions, and 23% of which showed stage-specific strengthening and enrichment for motifs of relevant TFs. Interestingly, the GATA4 motif was enriched already at two days of differentiation, suggesting that it may be important in the early reorganization of chromatin during cardiogenesis (possibly because of its pioneering factor activity), while MEF2C was enriched only in day 80 ventricular hESC-CMs. The authors also identified that exit from the pluripotent state involved abrupt loss of loops enriched for H3K27me3 and appearance of H3K27ac-marked loops, suggesting an interaction switch from inactive or poised regulatory regions to active enhancers. Moreover, enhancers were characterized by strong interconnectivity specifically in the context of TFs (e.g. the *HAND2* locus) and not of housekeeping genes. These findings reinforce the conclusions of Montefiori et al. regarding the decommissioning of poised or inactive enhancers and the engagement of novel activating enhancers during cardiogenesis, and indicate that this mechanism leads to a multi-enhancer architecture that may ensure consistent expression of certain key cardiac TFs.

Direct experimental support to the model of enhancer cooperativity in the regulation of cardiac TFs expression comes from the aforementioned study of the role of Isl1/Ldb1 in promoter enhancer interactions in cardiac progenitors [72]. Indeed, the *Mef2c* anterior heart field enhancer was shown to interact with and promote the expression of multiple other key cardiac development genes in a Ldb1-dependent fashion. Further evidence of enhancer cooperativity also in the context of cardiac ion channels is provided by a recent report which demonstrated that *SCN5A* expression is exquisitely sensitive to multiple downstream regulatory regions which act cooperatively as a super-enhancer [79]. Such super-enhancer controls the topology of the neighboring region, and is essential for normal cardiac conduction and embryonic development. Enhancer- and super-enhancer-enriched cardiac-specific FIREs have been identified in Hi-C data from human left and right ventricle [46], but their dynamicity and functional role during cardiogenesis has not yet been explored. Overall, it appears that dynamic changes in cooperative long-range chromatin interactions may be key to the regulation of stage-specific gene expression during cardiogenesis. CTCF may have a critical role in the formation of such regulatory chromatin loops, but there may be other players. For instance, the transcriptional and architectural modulator Yy1 mediates enhancer-promoter looping during mouse cardiac development [80], possibly through Ctcf-independent stalling of cohesin extrusion and/or through Yy1 dimerization [81,82]. Importantly, most Hi-C-predicted promoter interactions await functional validation. Therefore, the pervasiveness and mechanisms of developmental gene regulation by *cis* chromatin interactions remain areas of active investigation.

3.2. Topologically associating domains

Moving beyond the 10-200 kb scale, we and others examined TAD dynamics during human cardiogenesis [29,58]. In Bertero et al. we generated genome-wide chromatin contact maps during cardiac differentiation of hESCs and hiPSCs using Hi-C. We observed that ~70% of CTCF-enriched TAD boundaries are stable throughout cardiac

specification[29], confirming earlier reports that TADs are generally quite conserved across cell types [46]. Moreover, correlating Hi-C with RNA-seq revealed that TAD dynamicity had only a minimal effect on global gene expression. Differences were observed only in the context of regions that acquired a new TAD boundary in hESC-CMs, which correlated with significant upregulation of the closest gene. However, we did not test whether such correlation implied causality. A recent report suggested that during mouse cardiac development the expression of *Hand2* is negatively regulated by a nearby long non-coding RNA, *Handsdown,* through a mechanism that may involve reorganization of the local TAD structure [83]. However, dynamic changes in the chromatin interactions involving the *Handsdown* locus were independent from changes in Ctcf binding, arguing against a change in TAD boundaries. Moreover, the authors did not examine the overall chromatin architecture of the locus using Hi-C. Thus, whether developmental TAD dynamics can be functionally linked to activation of cardiac genes remains currently unclear.

Zhang et al. also examined TAD dynamics during *in vitro* cardiogenesis using the aforementioned deeply-sequenced Hi-C maps [58]. Their dataset included mature ventricular hESC-CMs, purified after 80 days of differentiation using a genetic fluorescent reporter. They observed that TAD numbers decreased during differentiation by 20%-40% (depending on the TAD identification algorithm used). This happened in two waves of approximately equal magnitude: first after exit from pluripotency, and secondly during cardiac maturation and ventricular specification. The authors determined that TAD boundaries lost during early differentiation were adjacent to the H family of human endogenous retroviruses (HERV-H), which were strongly transcribed in hESCs and promoted the expression of upstream genes. Specific HERV-H sequences were also shown to be necessary and sufficient to create TAD boundaries, possibly by interfering with DNA extrusion. On the other hand, the mechanism that dictates the loss of additional TADs during maturation of ventricular cardiomyocytes and its functional implications remain an open question.

A crucial role for the chromatin organizers CTCF and cohesin during cardiogenesis was established by multiple loss of function studies. Ctcf knockout in murine Nkx2.5-positive cardiac progenitor cells was shown to lead to myocardial thinning and enlargement, pericardial edema, and embryonic lethality at approximately embryonic day 12.5 [84]. This did not result from overt changes in cardiomyocyte proliferation or apoptosis, but rather from dysregulation of the balance between cardiac development and maturation. On the one hand, Ctcf knockout upregulated cardiac maturation genes involved in protein translation and mitochondrial oxidative phosphorylation, and led to premature sarcomere assembly. On the other hand, developmental regulators were downregulated. Intriguingly, Ctcf binding proved different for the two classes of genes: upregulated genes showed Ctcf binding close to the transcription start sites, suggesting that in this context Ctcf could act as a repressor by inducing intragenic looping [85] or *via* other mechanisms, while downregulated genes interacted with distal Ctcf sites located on cardiac enhancers, indicating that in this case Ctcf may act as an activator by promoting enhancer-promoter interactions. A recent study found that Trim33, another gene essential for early mouse heart development, often interacts with Ctcf on cardiac enhancers, further supporting the importance of Ctcf loops in the positive regulation of cardiac developmental genes [86]. A specific subset of the cohesin complex was also recently reported to be essential for heart morphogenesis. Indeed, global knockout of Stag2, but not its homologous Stag1 [87], impaired atrial and ventricular septation, right ventricle development, and outflow tract morphogenesis [88]. Distinctly from the Ctcf knockout model, this phenotype appeared to result from the combination of reduced cellular proliferation and defective migration of second heart field progenitors. Heterozygous loss of the cohesin loading factor Nipbl was also shown to induce atrial septal defects in mice and zebrafish [89,90], which was proposed to result from the defective expansion of second heart field cardiac progenitors [91]. In summary, CTCF and cohesin are both key players during cardiogenesis, but they seem to act through partially non-overlapping mechanisms. It remains unknown whether control of higher order chromatin architecture by CTCF/cohesin beyond facilitation of long-range interactions has important functional effects during cardiac development.

3.3. Chromatin compartmentalization

Beyond the megabase scale, multiple groups provided important insights into changes in active *versus* inactive (A/B) chromatin compartmentalization during cardiogenesis. We found that during cardiac differentiation of hESCs and hiPSCs ~20% of the genome shows dynamic compartmentalization [29]. For ~80% of such regions, changes in compartmentalization occurred in a single direction (i.e. B to A or A to B; ~50% and ~30%, respectively), with only few domains showing a transient switch (i.e. B-A-B or A-B-A). This was in striking contrast with the highly stage-specific gene expression signatures during differentiation. Accordingly, the correlation between gene expression changes and compartmentalization dynamics was generally limited. A notable exception to this were regions transitioning from B to A, which were strongly enriched for heart development genes upregulated during cardiogenesis. Similar observations were made in other studies examining cardiac differentiation of hPSCs [58,77], as well as in the context of mouse heart development [92], confirming the *in vivo* relevance of these mechanisms.

Intriguingly, we found that compared to other upregulated genes loci transitioning from B to A during human cardiogenesis were larger (median size > 100 kb), more isolated from nearby genes, and more cardiac specific, all characteristics that may facilitate control of gene expression *via* large-scale chromatin topology rearrangements with high specificity and minimal requirement for additional regulatory layers [29]. A notable example for this regulation was *TTN*, encoding the largest human protein, titin. By performing 3D DNA fluorescence *in situ* hybridization (FISH) we further demonstrated that the B to A transition of *TTN* corresponded to repositioning of the locus from the nuclear periphery to the nuclear interior. An analogous topological rearrangement was independently reported during mouse embryonic stem cell differentiation by the Jain and Epstein labs, which described the release of key cardiac loci from *bona fide* LADs enriched with the repressive histone mark H3K9me2 [26,93]. They also found that in this context anchoring of cardiac loci to the nuclear lamina relied on the histone deacetylase Hdac3, though intriguingly it did not require its enzymatic activity [26]. Impairment of this mechanism due to Hdac3 inducible knockout led to premature cardiac differentiation, while upregulation of Hdac3 impaired cardiogenesis, indicating a correlation between release of cardiac loci from the nuclear lamina and the cardiogenic potential of mouse embryonic stem cells. Notably, Hdac3 was previously shown to promote mouse secondary heart field development by epigenetically repressing Tgfβ1 expression in a deacetylase-independent manner [94], though no connections with chromatin organization were made at the time.

While examining regions of increased chromatin accessibility within B to A domains in hESC-CMs, we observed a strong enrichment for binding motifs of the core cardiac TFs GATA4, NKX2-5, and TBX5 [29]. Moreover, GATA4 already bound such regions in cardiac progenitors, suggesting that it may represent a pioneer factor that contributes to the release of these genomic loci from the repressive environment of the nuclear lamina (an hypothesis that still requires experimental validation). By contrast, we observed loss of CTCF binding in at least some B to A genes (i.e. *TTN*), which corresponded to decreased intragenic chromatin interactions. Overall, activation of topologically-regulated cardiac genes may result from the interplay of epigenetic remodelers (such as HDAC3), transcriptional regulators (possibly GATA4), and chromatin organizers (possibly CTCF), whose dynamic binding or release leads to chromatin repositioning from the lamina to the nucleoplasm, decreased gene compaction, and transcriptional activation. However, the order

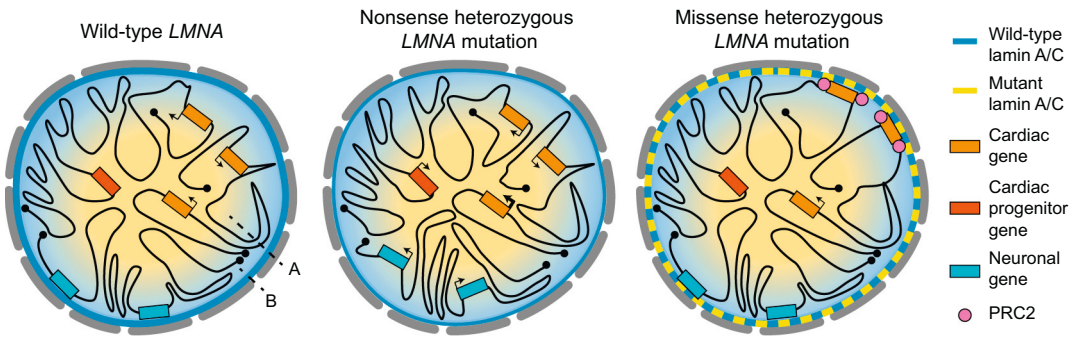

Fig. 5. Chromatin topology rearrangements in cardiac laminopathy.
Nonsense and missense heterozygous mutations in *LMNA* have distinct effects on the 3D chromatin architecture of cardiac myocytes. Nonsense mutations that lead to Lamin A/C haploinsufficiency (center) disrupt the sequestration into the B compartment of specific non-cardiac loci. In some cases, this results in ectopic expression (i.e. the neuronal P/Q-type calcium channel *CACNA1A*). Regulatory mechanisms other than compartmentalization changes lead to the inefficient silencing of some cardiac progenitor genes (i.e. the tyrosine kinase receptor *PDGFRB*) and to the upregulation of certain cardiac genes (i.e. the L-type calcium channel *CACNA1C*). Missense mutations in lamin A/C (right) that strengthen the association of chromatin with the Polycomb Repressive Complex 2 (PRC2) lead to increased peripheral localization and downregulation of specific cardiac genes (i.e. the fast sodium channel *SCN5A*). In both contexts, aberrant gene expression contributes to disease-associated electrophysiological abnormalities.

and causal relationship between these events remains to be clarified, as does the precise identity of the *trans*-acting factors involved.

Chromatin switching from A to B during hPSC-CM differentiation proved to be largely independent of gene expression changes [29]. Nevertheless, as described in more detail in Section 4.1 below, we subsequently observed that in hiPSC-CMs haploinsufficient for lamin A/C impairment of A to B transitions corresponded to incomplete segregation of non-cardiac loci from the nucleoplasm to the nuclear lamina, and in some cases led to their ectopic expression in hiPSC-CMs [95]. Along the same lines, in Chapski et al. we found that in mouse cardiomyocytes the A compartment was comparatively depleted of non-cardiac (liver) genes compared to hepatocytes, which could "tolerate" the presence of cardiac genes in the active compartment while maintaining tissue-specific regulation [96]. Moreover, 3D models of chromatin compartmentalization indicated that only cardiomyocytes radially segregated the A and B compartments towards the nuclear interior and periphery, respectively [96]. This suggests that cardiomyocytes rely more strongly on topological silencing of alternative lineage loci at the nuclear lamina than other differentiated cell types. In other words, as recently proposed by Jain and Epstein, chromatin compartmentalization may define cardiac cell fate competence [97]. However, to date this model has not been formally tested by varying A/B compartmentalization independently of gene expression (for instance by forcing nuclear lamina or nucleoplasm localization of a given locus without altering the activity of the *trans*-acting regulators involved in nuclear lamina localization or release).

DNA methylation has been unveiled as a reliable predictor of A/B compartmentalization [98]. Strikingly, Nothjunge et al. [92] showed that the organization of A/B compartments precedes the establishment of DNA methylation patterns during cardiomyocyte differentiation. Moreover, A/B compartmentalization was not altered after ablation of the two enzymes responsible for *de novo* methylation (Dnmt3A and Dnmt3B). On the other hand, DNA methylation is an established modulator of CTCF binding [99–101]. Moreover, a recent report demonstrated that impairment of DNA demethylation in the heart (double knockout of Tet2 and Tet3) disrupts binding of Yy1, impairing long-range chromatin interactions [80]. Therefore, while DNA methylation seems dispensable for nuclear-scale organization of cardiac chromatin, it plays an important and only partially understood regulatory role at the level of TADs and chromatin loops (Fig. 3A).

3.4. Chromosomal organization

Nuclear dynamics up to the gigabase scale are only beginning to emerge. We found that during cardiac differentiation of hESCs the relative proportion of interactions between chromosomes (inter-chromosomal, or in *trans*) increased from ~30% to ~50% [29]. Despite this, the relative topology of chromosome territories was overall maintained. Throughout differentiation, *trans* chromatin interactions were enriched in the A compartment and depleted in the B. Interestingly, Hi-C from mouse cardiomyocytes revealed that significant *trans* interactions were markedly enriched for cardiac-specific genes (and not liver-specific genes), suggesting that at least some *trans* interactions may be associated with the formation of functional chromatin neighborhoods [96].

Supporting this notion, we found that during hESC-CM differentiation several cardiac loci from at least 10 different chromosomes formed a *trans*-interacting chromatin domain centered around the gene *TTN* [29]. We further identified that these *trans* interactions were dependent on a common splicing factor, RBM20, which formed foci proximal to the *TTN* gene and nucleated by its many binding sites onto the *TTN* pre-mRNA. Preventing *TTN* transcription not only disrupted this chromatin feature, but also had a *trans*-acting effect on the alternative splicing of other RBM20 target genes. This suggests that RBM20 foci at the *TTN* loci represent cardiac-specific splicing factories that arise from specific inter-chromosomal chromatin associations. It has been proposed that cooperative binding of transcriptional and epigenetic regulators may lead to spatial clustering of their shared targets into specialized transcription factories [102]. However, the existence of multiple non-random transcription factories with some degree of structural stability has not been yet established. Indeed, the lack of robust methods to identify and validate significant *trans* chromatin interactions remains an important bottleneck in the field.

4. Chromatin architecture dynamics in cardiac disease

Emerging evidence implicates disruption of chromatin architecture in the pathogenesis of multiple diseases (recently reviewed in [103]). Here we examine our current knowledge about the impact of disease-causing gene mutations, genetic variants, and environmental factors on chromatin organization in cardiomyocytes.

4.1. Monogenic disorders

Studies of 3D chromatin organization changes in cardiac monogenic diseases have so far largely focused on DCM due to heterozygous *LMNA* mutations (Fig. 5). *LMNA* encodes lamin A and lamin C (henceforth lamin A/C), two key components of the nuclear lamina that can interact directly with chromatin within LADs [104,105]. Thus, after the

discovery that mutations in *LMNA* can cause striated muscle pathology [106], it was proposed that this may be at least partially explained by pathological alterations in chromatin architecture. Early work in patient-derived fibroblasts reported repositioning of chromosomes 13 and 18 from the nuclear periphery to the interior [107]. A subsequent report observed a similar mislocalization of two proximal gene clusters on chromosome 13, which was associated with decondensation of the local genomic region and upregulation of gene expression both in fibroblasts and in the heart [108]. More direct evidence for lamin A/C in the regulation of cardiac chromatin architecture came from studies in a mouse cardiomyocyte cell line, which showed that knockdown of lamin A/C reduced the peripheral localization of three randomly-selected lamin A/C-bound loci [109]. Interestingly, however, none of such genes were upregulated, indicating that topological rearrangements of chromatin were insufficient for transcriptional activation.

More recently, three studies examined changes in association of chromatin with the nuclear lamina in *LMNA* DCM using patient-derived hiPSC-CMs [110,111] and primary cardiomyocytes [112]. Lee et al. studied a haploinsufficient hiPSC model of arrhythmogenic *LMNA* DCM (K117fs) [110]. Lamin A/C association was altered for ~25% of all LADs and involved both decreased and increased occupancy (accounting for ~5% and ~2% of the genome, respectively). These dynamic LADs were also relatively short, with a mean size of ~250 kb (compared to ~1.5 Mb for unaffected LADs). Importantly, ~80% of the 250 differentially expressed genes were located outside of LADs, while LADs lost or acquired in mutant hiPSC-CMs contained only ~2% and ~4% of all differentially expressed genes, respectively, which were mostly upregulated in both cases. Mechanistically, the authors found that abnormal activation of PDGF signaling due to upregulation of the receptor gene *PDGFRB* led to arrhythmic manifestations. *PDGFRB* is normally expressed in cardiac progenitors, and its incomplete silencing in mutant hiPSC-CMs was mirrored by increased active histone marks and chromatin accessibility at its promoter. However, this was not associated to changes in lamin A/C association. Accordingly, *PDGFRB* is constitutively located in the A compartment throughout cardiac differentiation of hPSCs [29], implying that its transitory upregulation in cardiac progenitors and subsequent downregulation in hPSC-CMs must rely on mechanisms other than broad rearrangements of chromatin compartmentalization. Overall, this work showed that while lamin A/C haploinsufficiency leads to selective changes in LAD topology and gene upregulation, the majority of gene expression changes occurs in other genomic regions.

In Salvarani et al. [111], we studied a distinct hiPSC model of *LMNA* DCM caused by a heterozygous missense mutation in the rod domain of lamin A/C (K219T) and characterized by impairment in cardiac conduction. This was mechanistically associated with epigenetic silencing of the fast sodium channel gene *SCN5A*, which proved to be more closely associated to the nuclear lamina and more strongly enriched with Polycomb Repressive Complex 2 (PRC2) and H3K27me3. Interestingly, similar results were obtained in the context of another heterozygous missense mutation located in the same domain of lamin A/C (R190W). These results suggested that certain missense mutations in *LMNA* may have dominant negative effects that can lead to gene silencing. However, this study did not address global changes in LADs, and therefore the pervasiveness of this mechanism at the genome wide level mechanism remains unclear.

Cheedipudi et al. examined lamin A/C association of chromatin and gene expression changes in cardiomyocyte nuclei isolated from the hearts of *LMNA* DCM patients undergoing cardiac transplantation [112]. Five patients were analyzed, four with missense mutations in the rod domain (two with R166P, and one each with G312H or R335W) and one with a nonsense mutation truncating the rod domain (G353X). When comparing the aggregated list of LADs from patients with LADs from five control healthy individuals, the authors found differences on regions collectively accounting for ~10% of all coding genes. Interestingly, control-specific LADs accounted for a total of 95 Mb, while *LMNA* DCM-specific LADs accounted for 539 Mb. Whether missense and nonsense mutations led to similar or distinct LAD dynamics was not reported. In general, gain and loss of LADs was associated with lower and higher gene expression, respectively. Nevertheless, only ~15% and ~5% of differentially expressed genes were found in gained and lost LADs, respectively, once again indicating that a majority of gene expression changes in *LMNA* DCM does not involve LAD dynamics. Differentially expressed genes found in dynamic LADs were largely specific for *LMNA* DCM compared to non-*LMNA* DCM, indicating that they could include genes associated to the particularly severe manifestations of *LMNA* DCM [14–17]. It is worth pointing out that Lee et al., Salvarani et al., and Cheedipudi et al. all relied on chromatin immunoprecipitation (ChIP) for lamin A/C to define LADs. However, considering that in all models lamin A/C was downregulated and/or mutated, it is not clear whether such ChIP-defined domains correspond to *bona fide* LADs (i.e. peripherally located regions also interacting with other nuclear lamina proteins such as B-type lamins and the lamin B receptor, LBR [113]). While Salvarani et al. provided validation of *SCN5A* mislocalization by DNA FISH, this or other orthogonal assays were not implemented by Lee et al. and by Cheedipudi et al.

In Bertero et al. we used Hi-C to examine genome-wide changes in chromatin organization in a hiPSC model of *LMNA* DCM due to lamin A/C haploinsufficiency (R225X) and characterized by irregular and slower beat rate and prolonged calcium transients [95]. Surprisingly, A/B compartment changes involved only ~1% of the genome. Chromatin domains normally in B but found in A in mutant hiPSC-CMs were enriched for regions that during cardiogenesis transition from A to B, and DNA FISH confirmed that such domains were generally localized further away from the nuclear lamina in mutant cells. Notably, however, genes in most of these domains were not differentially expressed. We found that in mutant hiPSC-CMs only three genomic hotspots were characterized both by ectopic retention in the A compartment and modest upregulation of some genes contained within. Such genes were strongly enriched for neuronal factors, such as the P/Q-type calcium channel *CACNA1A*. We found that this gene aggravated the electrophysiological abnormalities in mutant hiPSC-CMs, suggesting that inappropriate silencing of specific neuronal genes due to lamin A/C haploinsufficiency may contribute to the pathogenesis of *LMNA* DCM. These findings provide further support for the conclusion that lamin A/C haploinsufficiency can lead to selective disruptions of developmental LAD dynamics, but that only some of these result in gene expression alterations. On the other hand, we also observed that electrophysiological abnormalities in mutant hiPSC-CMs were primarily caused by upregulation of the cardiac L-type calcium channel *CACNA1C*, which was not associated with changes in chromatin compartmentalization (similarly to *PDGFRB*, this gene is constitutively found in the A compartment during cardiogenesis).

All in all these and other studies established that nuclear lamina association is not the sole determinant of gene repression at the nuclear periphery. Whether lamin A/C impairment leads to selective dysregulation of cardiac progenitor and cardiac genes through more localized changes in chromatin organization (i.e. at the level of TADs or looping) or *via trans*-acting effects involving other chromatin regions remains unclear. Interestingly, B-type lamins have been shown to indirectly affect the expression of lamina-distal genes by modulating inter-TADs interactions as a result of altered LADs compaction [36]. If A-type lamins played a similar role, their disruption may lead to ripple effects throughout multiple layers of 3D chromatin organization. Alternatively, A/B compartment-independent gene expression changes may be driven by alterations in intracellular signalling pathways with established links to the nuclear lamina, such as MAPK and mTOR, which are upregulated in animal models of cardiac laminopathy (reviewed in [114]). Overall, to what extent changes in chromatin organization represent a key primary driver of pathology in cardiac laminopathy remains an open question.

Besides *LMNA* DCM, the potential involvement of 3D chromatin

organization in the many other known monogenic cardiac diseases (for instance CHD due to mutations in TFs or chromatin modifiers, and *RBM20* DCM) remains virtually unexplored, and is thus an attractive subject for future investigations.

4.2. Genetic variants

Over the last decade, large-scale genotyping with classical methods or *via* exome and whole genome sequencing have provided an ever growing list of genetic associations to multiple cardiovascular diseases (reviewed in [115,116]). Genome-wide association studies (GWAS) have revealed that a vast majority of such disease-associated genetic variants are non-coding and often located in intergenic regions, and are thus not easily interpreted. Since most such variants are believed to regulate expression of genes within neighboring regions, integration of GWAS with 3C-type data has the potential to allow the identification of how disease variants directly interact with their target loci.

Choy et al., Montefiori et al., and Zhang et al. all explored this approach by integrating their long range chromatin interaction maps from cardiomyocytes with cardiac GWAS data [76–78]. Choy et al. found a modestly significant association ($p < 0.045$) between significant cPIRs and GWAS hits for cardiac conduction and rhythm disorders [76]. These included regions that were previously predicted to affect expression of *TFPI*, *SC35F1*, and *HCN4*, based on their proximity to such genes, but which were found to actually interact with *ZSWIM2*, *PLN*, and *NPTN*, respectively. Another aspect of note was that the combined use of promoter interactome data and eQTLs allowed the authors to confirm a functional interaction between *ACTN4* and *CAPN12* in correspondence to a GWAS peak that did not reach significance in previous studies, possibly due to an insufficient study sample size. While none of such potential interactions were functionally validated in the study, these examples showcase the potential for chromosomal maps to refine GWAS predictions beyond what is possible by simply looking at the closest gene and/or at known eQTLs. Notably Choy et al. found no significant association between cPIRs and GWAS hits for coronary artery disease, and virtually no association with hits for CHD. Both of these phenotypes also involve non-cardiomyocyte lineages, which may explain this somewhat unexpected finding. However, it is also possible that correlation between cPIRs and CHD GWAS hits may become more apparent in analogous analyses of cardiac progenitors, which may possess unique and transitory PIRs.

Montefiori et al. examined the correlation of cPIRs with GWAS hits for cardiac arrythmias, heart failure, and myocardial infarction [77]. They found that just ~20% of the lead SNPs or the other variants in high linkage disequilibrium with the lead SNPs were located in a significant cPIR. This indicates that most cardiac GWAS hits may involve rare or transient chromatin interactions that are hard to identify with confidence from bulk PCHi-C. Alternatively, the underlying genetic variants may affect other cell types or states, and/or act independently of chromatin looping. Interestingly, this study revealed that over 90% of chromatin interactions between GWAS SNPs and promoters do not involve the closest gene, with over 50% of them skipping five or more genes. Indeed, the median distance between such interactions was 185 kb. Moreover, ~40% of SNPs interacted with more than one gene. Interestingly, cPIRs overlapping with GWAS SNPs were enriched for genes that lead to cardiovascular phenotypes when knocked out in mice, suggesting an important and evolutionarily conserved role in cardiac gene regulation.

Zhang et al. found a strong enrichment of GWAS SNPs located in chromatin loop anchors (i.e. one of the two interacting regions in a given loop) when examining heart rhythm phenotypes, and a moderate enrichment in the context of congenital heart disease and coronary heart disease [78]. Accordingly, the matching genes were enriched for cardiac ion channels and other factors involved in cardiac function. Notably, the authors also provided experimental evidence that two of the GWAS target predictions based on chromatin looping data proved more accurate than the one originally made based on gene proximity alone. Indeed, deletion of a SNP-containing enhancer led to reduced expression of its interacting locus *WNT3* during cardiac differentiation, while it did not affect the nearest gene *GOSR2*. A similar experiment established the long-range regulatory function of a SNP-associated enhancer located ~200 kb away from *MSX1* (while this region did not influence expression of the closest gene *STX18*). These results provide a strong proof of principle and outline a promising pipeline for the validation of cardiac GWAS hits through the integration of genome-wide chromatin organization data.

Chromatin conformation capture data can also inform GWAS studies beyond the interrogation of direct long-range chromatin interactions. For instance, a GWAS study for gene modifiers increasing the risk of developing Tetralogy of Fallot in patients with DiGeorge syndrome relied on TAD maps to predict an interaction between an intronic SNP in the gene *ADGRV1/GPR98* and the cardiac transcription factor *MEF2C* locus [117]. Since TADs are quite conserved across cell types, the authors leveraged on Hi-C data from non-cardiac cells to identify the TAD containing *ADGRV1*. Then, under the assumption that *cis* effects of the GWAS hit would be restricted within such TAD, they identified *MEF2C* as the most likely transcriptional target within the TAD (also considering its established role in heart development and its specific expression in the secondary heart field). This approach may prove particularly valuable in the many cases where GWAS hits cannot be mapped to long-range interactions, and ought to be even more predictive when applied to the recently reported cardiomyocyte Hi-C maps.

Direct evidence of the impact of GWAS-associated long-range chromatin interactions and cardiac disease comes from the study of *PITX2* regulation in atrial fibrillation [118,119]. 3C analyses reveled that a genomic region containing risk alleles for atrial fibrillation and located ~170 kb upstream of mouse *Pitx2* includes multiple regulatory elements that physically interact with the *Pitx2* promoter [118]. A subsequent study confirmed these observations with circularized chromosome conformation capture (4C) analyses, and also demonstrated that deletion of a ~20 kb enhancer within this region leads to reduced *Pitx2* expression and increased atrial fibrillation propensity [119]. Deletion of an intronic Ctcf binding site in *Pitx2* also had a similar effect, suggesting that Ctcf-dependent chromatin looping was required for the enhancer to interact with *Pitx2* (though this was not formally tested). Another example of integrating GWAS and chromatin conformation capture data comes from the aforementioned study of the *SCN5A* super-enhancer [79]. The authors identified such enhancer structure by integrating hits from a GWAS study of heart rhythm phenotypes, TAD maps, and H3K27ac enrichment. They then demonstrated with 4C that the super-enhancer interacts with the *Scn5a* promoter in the mouse. Finally, they proved that the super-enhancer is essential for *Scn5a* transcription during development. Notably, they also identified a heart rhythm-associated genetic variant within the enhancer whose minor allele reduced enhancer activity in response to Gata4 and Nkx2.5 (though such evidence was limited to a luciferase reporter assay and not tested at the endogenous locus). Finally, a recent study relied on the aforementioned high-resolution Hi-C data for hiPSC-CMs of Zhang et al. [58] to functionally annotate a heart failure GWAS hit to an enhancer of the *ACTN2* gene [120]. Notably, such annotation could not be made based on eQTL data alone, demonstrating the added value of the Hi-C data. Deletion of a ~2 kb fragment containing the enhancer reduced *ACTN2* expression in hiPSC-CMs, confirming its regulatory function. However, it is important to note that neither this study nor any of those previously described provided evidence that patient-associated disease variants were sufficient to affect the expression of their putative target genes, nor did they test whether this was associated with changes in chromatin interactions (Fig. 3A). Therefore, while it appears clear that chromatin conformation capture data can be effectively leveraged to identify key regulatory elements associated to GWAS hits, the causative role of these genetic variants in functional chromatin interactions remains to be established.

The potential impact of genetic variation on cardiac chromatin

structure may go well beyond what can be detected by GWAS. Aneuploidies and copy number variations account for ~25% of all CHD cases [121]. Such large scale chromatin rearrangements may have profound effects on cardiac nuclear organization, as already shown in other contexts such as trisomy 21, cancer, and limb development [62,63,122,123]. Thus, future studies may explore whether genetic variation impacts not only the formation and activity of long-range chromatin interactions, but also higher order chromatin architecture at the level of TADs, A/B compartments, and even chromosome territories (Fig. 3).

4.3. Environmental stressors

Cardiovascular disease (CVD) remains the leading cause of death globally [124]. CVD risk factors such as high blood pressure, smoking, and diabetes, are well known to induce profound transcriptional changes in the heart (reviewed in [125]). The pathogenic role of epigenetic changes at the level of DNA methylation and histone modifications is also well established (reviewed in [20]). In recent years, alterations of 3D chromatin architecture at multiple levels have been proposed to also play a role in the adaptation and maladaptation of the heart to humoral and mechanical stress.

At the level of chromatin looping, He et al. demonstrated that pressure overload by transverse aortic constriction (TAC) activates Gata4 binding at enhancers, subsequently activating gene expression by recruitment of p300 and H3K27ac deposition [126]. This process restores Gata4 binding at a subset of fetal enhancers that reactivate the fetal gene program, a hallmark of cardiomyopathy. Papait et al. mapped cardiac enhancers associated with genes involved in the progression of cardiac hypertrophy, and demonstrated that their activity is mediated by Mef2 [127]. Although these studies did not directly examine the structural dynamics of chromatin, their results suggest that enhancer-promoter interactions that control the pathological gene program rely on the formation of chromatin loops that are physically mediated by cardiac disease-associated transcription factors.

In the context of genome organizers, in Monte et al. we showed a reciprocal regulation of Ctcf and Hmgb2 [128], a non-histone chromatin structural protein upregulated after TAC [129]. We found that at the same locus Ctcf serves as a boundary to prevent heterochromatin spreading (thereby promoting gene accessibility), while Hmgb2 increases DNA compaction and gene repression. Notably, we subsequently found that CTCF is downregulated in primary cardiac samples from patients with heart failure [130]. These findings suggest that changes in the CTCF to HMGB2 ratio allow for varied genomic plasticity.

At the nuclear scale, in Karbassi et al. [131] we demonstrated that cardiac stress due to phenylephrine treatment or TAC increases the number or size of RNA Pol II clusters in neonatal or adult cardiomyocytes, respectively, and also affects their positioning, with the most active clusters being found in the nuclear interior. Moreover, we found that gene movement towards the nuclear periphery and/or other heterochromatic regions correlated with gene repression after TAC (i.e. the sarcoplasmic reticulum calcium pump *Atp2a2*), and *vice versa* (i.e. the stress response gene *Nppa*). Finally, we showed that chromatin structure reorganization after TAC promoted the physical interaction between genes that change expression in the same direction, supporting a model where the congregation of co-regulated genes coordinates transcription during stress.

We and others recently applied Hi-C to examine the global changes in chromatin 3D structure during mouse models of heart failure [130,132]. In Rosa-Garrido et al. we examined the effects of Ctcf depletion (as a model of chromatin disruption) and of TAC on 3D chromatin architecture in the heart [130]. We generated a cardiac-specific inducible Ctcf knockout mouse to selectively deplete Ctcf in the adult heart, and observed that this is sufficient to induce heart failure through mechanisms that involve reorganization of genome architecture. Remarkably, such changes in chromatin architecture measured were similar to those observed after TAC. In summary, we showed that induction of heart failure promotes: (1) profound loss of intra-chromosomal interactions, reducing the number of chromatin loops and enhancer-promoter contacts by ~20%–25%; and (2) switching of A/B compartmentalization for ~4% of the genome, which correlated with gene expression changes towards the anticipated direction. Interestingly, although widespread changes in the strength of TAD boundaries were detected, TAD integrity was overall conserved.

Following a similar approach, Lee et al. independently confirmed that cardiac-specific depletion of Ctcf in the mouse leads to heart failure and alterations of 3D chromatin architecture [132]. In contrast to our earlier observations the authors detected a strong disruption of TADs after Ctcf depletion. This discrepancy is likely explained by the different methodology used to ablate Ctcf: in Rosa-Garrido et al. we used a tamoxifen-inducible Cre-Lox system which downregulated Ctcf by ~80% at the protein level, while Lee al. delivered Cre via an adeno-associated virus and reached nearly complete depletion of Ctcf protein. These results suggest that some highly-conserved TAD boundaries are bound by Ctcf so strongly and/or stably that some degree of chromatin structure is retained when Ctcf is not completely depleted (as also proposed by others in non-cardiac systems [133]). This notion aligns with the results of Nora et al., who used an auxin-inducible degron system to demonstrate that while Ctcf is essential for TAD integrity in mouse embryonic stem cells, TAD integrity is maintained with as low as 4% of normal Ctcf expression [134]. Importantly, while CTCF levels decrease after induction of cardiac disease in some mouse models and in patients with heart failure, CTCF never disappears completely [130]. Thus, the approach of Lee et al. is valuable for studying chromatin structure dynamics after complete loss of CTCF, while the method used in Rosa-Garrido et al. offers a clinically relevant approach to investigate changes in high order chromatin with cardiac disease.

Overall, there is increasing evidence that 3D chromatin organization in the adult heart is modulated by and may contribute to heart failure due to pressure overload and chronic humoral stimuli. However, the causal relationships and underlying mechanisms are yet to be explored in detail. Moreover, the effect of other CVD-causing environmental stressors on 3D chromatin topology remains an open area for future investigation.

5. Future outlook

Three-dimensional chromatin organization in cardiac development and disease is a young field with many open questions, some of which are mentioned throughout the previous sections. Here we summarize what we view as other “big picture” areas for future investigation.

While most studies have so far focused on cardiac myocytes, little is known about genome topology dynamics in other cardiovascular cell types such as endothelial cells, smooth muscle cells, fibroblasts, and immune cells. All of these play key roles in CHD and CVD, undergo dramatic phenotypic changes after stress, and represent promising targets for therapeutic intervention. For instance, it was very recently reported that cardiac fibroblasts undergo a major pathological rearrangement of chromatin accessibility in heart failure, which can be mitigated by inhibition of bromodomain-containing (i.e. histone lysine acetylation-reading) coactivators [135]. Endothelial cells were shown to undergo marked changes in 3D chromatin architecture during differentiation [136], further showcasing the potential for phenotypic regulation *via* genome topology dynamics in non-myocyte cells.

The genome organization field is rapidly advancing both conceptually and methodologically. Important areas that deserve particular attention are: (1) the integration of biophysics and molecular biology, including the recent development of tools to perturb the biophysical properties of chromatin [137]; (2) the orthogonal application of imaging- and sequencing-based assays to elucidate 3D chromatin organization, particularly the application of single-molecule, super-resolution, and highly multiplexed imaging [138,139]; (3) the advances in

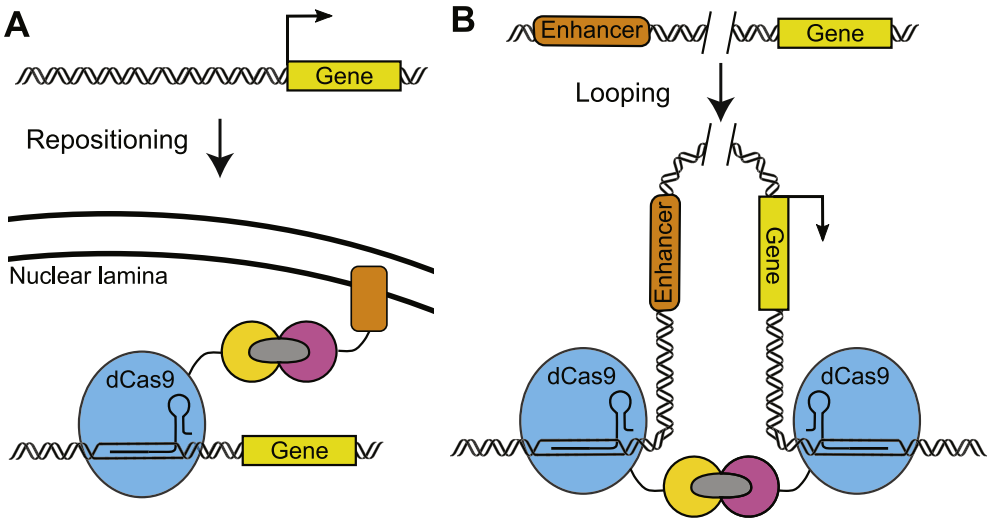

Fig. 6. Modulation of 3D chromatin architecture.
Synthetic biology approaches such as those based on CRISPR/Cas9 technology can be employed to alter chromatin structure-function. For instance, the interaction of a catalytically inactive Cas9 (dCas9) and single guide RNA complex with a nuclear lamina protein (i.e. Emerin) can be leveraged to reposition gene loci to the repressive gene environment of the nuclear periphery (A). Similarly, promoter-enhancer loops can be engineered by dCas9 multimeric complexes (B). Both approaches have been successfully implemented by creating fusion proteins with the ABI and PYL1 heterodimerization domains, which interact in the presence of abscisic acid (ABA) [140,141].

synthetic biology tools to elucidate the structure-function relationship of chromatin, for example by engineering chromatin loops [140], or by repositioning loci to specific nuclear sub-compartments [141]; (4) the development of single cell assays for chromatin architecture [142], including multi-omics approaches to examine multiple layers of nuclear function in the same cell [143–145]; and (5) the growing appreciation of the key role of non-coding RNAs [146–148] and DNA methylation [100,101] in genomic structuring. The timely incorporation of these and other innovations in cardiac biology would offer remarkable opportunities to study the functional role of 3D chromatin organization in key physiological contexts, such as the most common birth defect (CHD) and the leading cause of death in adults (CVD). Going forward, it will be particularly important to move from describing and correlating changes in chromatin organization due to broad-acting perturbations (an approach that by necessity has characterized most studies so far), to mechanistically probe the role of individual chromatin structure regulations.

Importantly, we believe that such basic knowledge has a strong potential to inform clinical practice through identification of novel CHD and/or CVD biomarkers, clarification of genetic associations for non-coding variants, and identification of druggable mechanisms. This last goal could be pursued in at least two ways: (1) the use of small molecules that modulate the activity of epigenetic modifiers (i.e. histone writers, readers, and remodelers) to indirectly affect chromatin looping and compartmentalization; (2) the application of CRISPR/Cas9-based gene therapy strategies aimed at modulating specific chromatin interactions (i.e. deletion of loop anchors or TAD boundaries, or re-wiring of chromatin looping or locus compartmentalization; Fig. 6).

Overall, we think that future work in the field of 3D nuclear biology will reveal a novel dimension to our understanding of cardiac pathology and, hopefully, guide treatment of both developmental and acquired cardiac disease.

Disclosures

None.

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Glossary

Chromosome conformation capture: general methodological framework to study 3D chromatin architecture based on the quantification of contact frequencies between regions that interact in the 3D space, as revealed by proximity ligation assays (Fig. 1A).
3C: basic chromosome conformation capture technique to detect physical interaction between two specific loci (Fig. 1B).
4C: 3C-derivative technique to detect all interactions between one specific locus and the rest of the genome (Fig 1B).
Hi-C: genome-wide extension of the 3C technique to determine all pairwise 3D genomic interactions (Fig 1B).
Chromatin contact heatmap: graphic representation of 3D interaction frequencies between genomic loci. Often plotted as a triangle where the base (x axis) covers a genomic

interval and each point on the y axis indicates the interaction frequency of the matching genomic coordinates (Fig. 2).

Chromosome territory: nuclear region preferentially occupied by specific chromosomes. On a genome-wide, triangular chromatin contact heatmap, it is indicated by a distinct triangular interaction pattern delimiting a given chromosome (Fig. 2A).

A/B compartmentalization: separation of megabase-sized chromosomal domains within active (A) or inactive (B) chromatin environments. On a chromosome-wide, triangular chromatin contact heatmap, it is indicated by the "checkerboard" structure building upward from the base (Fig. 2B).

TAD: sub-megabase chromosomal domain characterized by preferential self-interaction. On a sub-chromosomal, triangular chromatin contact heatmap it appears a triangle onto its base (Fig. 2C).

TAD boundary: genomic interval that delimitates two neighboring TADs.

Chromatin loop: 3D interaction between two genomic regions that are located several kilobases away from each other on the same chromosome. On a high-resolution, triangular chromatin contact heatmap it appears as an isolated dot separated from the base (Fig. 2D).

CTCF/cohesin complex: protein complex involved in the formation of some chromatin loops and TAD boundaries.

LAD: megabase-sized chromosomal domain in close proximity to the nuclear lamina and possibly physically associated to its proteinaceous components; generally associated with poor transcriptional activity.

Transcription factories: nuclear foci of RNA Pol II associated to high transcriptional activity.

trans chromatin interaction: physical proximity between loci located on different chromosomes.

eQTL: genomic locus that explains a portion of the genetic variance of a gene expression phenotype.

GWAS: study that aims to identify genetic variants associated with a phenotype, often a disease, by mapping its segregation with many genetic markers in a population.