

3D genome organization in health and disease: emerging opportunities in cancer translational medicine

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Organizing the DNA to fit inside a spatially constrained nucleus is a challenging problem that has attracted the attention of scientists across all disciplines of science. Increasing evidence has demonstrated the importance of genome geometry in several cellular contexts that affect human health. Among several approaches, the application of sequencing technologies has substantially increased our understanding of this intricate organization, also known as **chromatin interactions**. These structures are involved in transcriptional control of gene expression by connecting distal regulatory elements with their target genes and regulating co-transcriptional splicing. In addition, chromatin interactions play pivotal roles in the organization of the genome, the formation of structural variants, recombination, DNA replication and cell division. Mutations in factors that regulate chromatin interactions lead to the development of pathological conditions, for example, cancer. In this review, we discuss key findings that have shed light on the importance of these structures in the context of cancers, and highlight the applicability of chromatin interactions as potential biomarkers in molecular medicine as well as therapeutic implications of chromatin interactions.

Chromatin Interactions in Health and Disease: An Introduction

The orchestrated expression of genes is a key underlying feature of a living cell and distinct gene expression signatures can be found depending on different cellular contexts such as cell growth, division, survival and response to stimuli. Therefore, it is not surprising that aberrant gene expression patterns often correlate with development and progression to pathological situations. Mechanistically, different elements such as enhancers, promoters, and their target genes have to be brought together in close spatial

proximity to permit the desired transcriptional outcome. In order for this to occur, there are 2 problems that need to be surmounted. First regulatory elements are interspersed along considerable physical distance through the long linear DNA. Second, the complex packing of DNA inside the nucleus introduces issues of accessibility to factors. DNA associates with histone proteins and is organized into structures called nucleosomes. A nucleosome in its simplest form contains 145bp of DNA wound around a histone octamer. The nucleosome in turn associates with other nucleosomes to form higher order structures known as chromatin, which further is organized into distinct entities known as chromosomes. Further, the chromosomes occupy distinct territories in the nucleus, referred to as “chromosome territories” (Fig. 1).¹

This hierarchical organization of the chromatin poses a challenge regarding the accessibility of cis-regulatory elements such as promoters, enhancers and insulators to regulate transcription. How do these genomic elements, which are situated far from a target gene, influence its transcription? The solution to this problem arose when it was discovered that distant loci can be brought into spatial proximity through the formation of “loops.” These looped structures, also known as chromatin interactions) refer to 2 or more distal regions of the genome that are brought together in close spatial proximity by factors such as protein and RNA. Interest in these structures deepened with findings that chromatin interactions regulate many cellular processes such as transcription, cell growth and cell cycle, recombination, repair and homeostasis in the cellular environment. These structures assume translational and clinical importance in light of the fact that dysregulation of chromatin interactions could potentially lead to altered cellular health and therefore, to diseases such as cancer²⁻⁵ as has been elaborated in subsequent sections.

In this review, we discuss the mechanisms by which chromatin interactions can play roles in disease, the factors associated with chromatin interactions and their relationship with disease, the prospects for translation of this basic understanding to cancer therapies and biomarkers, as well as future directions for the field.

Chromatin Conformation Analysis: The Molecular Toolkit to Probe Chromatin Interactions

Over the past 2 decades, several techniques have made great headway in understanding the formation of these loops.

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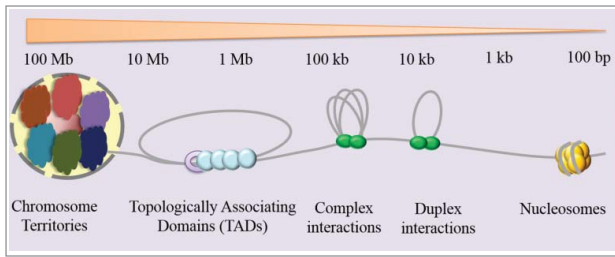


Figure 1. The 3-Dimensional chromatin architecture of the cell. The fundamental organizational unit of chromatin are the nucleosomes, which are approximately 147 bp, which makes up the 10 nm “beads on a string” structure seen by Electron Microscopy (EM).¹¹⁶ chromatin interactions, in the form of duplex interactions in which 2 loci come together, or complex interactions in which more than 2 loci interact with each other, further organize chromatin at the kilobase level. Megabase-sized Topologically Associating Domains (TADs) further organize chromatin, and finally, chromosomes occupy distinct “chromosome territories” in cell nuclei.

An important precursor to these techniques was the discovery of the nuclear ligation assay which has been adapted to yield a range of techniques.⁶ Subsequent methodological developments have led to the advent of techniques such as Genome conformation capture^{7,8} as well as 3C¹ and higher-throughput variants of 3C, such as 4C,^{9,10} 5C,¹¹ as well as ChIA-PET,¹² and Hi-C¹³ which allows for high-throughput genome-wide analyses (Fig. 2). Analysis of the 3D-genome organization using these techniques has led to breakthroughs in characterizing chromatin interactions.

Chromosome Conformation Capture (3C) involves first cross-linking chromatin with an appropriate agent such as formaldehyde. Next, the chromatin is fragmented, typically with a restriction enzyme. The chromatin is then diluted in a large volume for “proximity” ligation wherein intra-complex ligations are formed as a result of ligation. Finally, the chromatin is reverse cross-linked, purified and subjected to PCR amplification¹⁴ or quantitative PCR amplification (Fig. 2) to assess the frequency of the amplified ligated fragments.¹⁵

To analyze chromatin interactions in a more high-throughput manner, Circular Chromosome Conformation Capture (4C) identifies chromatin interactions genomic fragments that

interact with a known bait, through inverse PCR of a particular bait region to obtain all interacting fragments, which are then deconvoluted by microarrays or sequencing.^{9,10} Chromosome Conformation Capture Carbon Copy (5C) uses multiplexed oligonucleotide annealing and ligation followed by microarrays or sequencing to interrogate many specific sites in the genome in a high-throughput manner.

Chromatin Interaction Analysis with Paired-End Tag (ChIA-PET) sequencing allows for the analysis of chromatin interactions between genomic regions bound to specific factors under study.¹² The inclusion of the ChIP step in ChIA-PET allows for greater specificity as well as a reduction in the complexity of the library that is sequenced.^{12,16}

Hi-C examines all interacting chromatin regions without ChIP enrichment and, therefore, describes chromatin interactions on a global level. Previously, due to cost limitations in terms of sequencing, complexity reduction to allow successful Hi-C analysis of the human genome meant that a low-resolution picture was built through the use of 1 Mb bins,¹³ although application in organisms with smaller genomes allowed for a kilobase-resolution picture.¹⁷ However, recently the Lieberman-Aiden and Lander labs took advantage of further decreases in next-generation sequencing costs to profile chromatin interactions with Hi-C at kilobase resolution.¹⁸

Fluorescence *in-situ* hybridization (FISH) using fluorescently labeled DNA or RNA probes against chromatin are capable of low-resolution, but single-cell, measurements of chromatin proximity using microscopy.¹⁹⁻²¹ C techniques have also been successfully combined with microscopy to yield powerful tools to investigate chromatin interactions. For example, Fraser and colleagues described single-cell Hi-C (so-called “1C”), which combines the single-cell nature of microscopy with Hi-C.²² In this method, reactions including proximity ligation are performed within individual nuclei, which are then selected and sequenced.

Genome-wide analyses of chromatin interactions by Hi-C and ChIA-PET have demonstrated a plethora of intermediate chromatin interactions between 1Kb and 100 Mb (Fig. 1) with the vast majority of these chromatin interactions being intrachromosomal as compared to interchromosomal interactions.^{12,13,23} A major hurdle in the tools is the aspect of combining single cell

resolution with genome-wide analysis as the current tools seem to be mutually exclusive with very little overlap. In other words, “C” techniques that enable the visualization of the genome organization are constrained in the context of single-cell analysis. Likewise, a technique like FISH, which affords single-cell resolution, is not amenable to whole genome analysis. Hence, an active area of research is the development of new techniques, improving the current techniques to permit single-cell genome-wide analysis, as well as computational tools to aid in uncovering chromatin interactions at the genome-wide level.

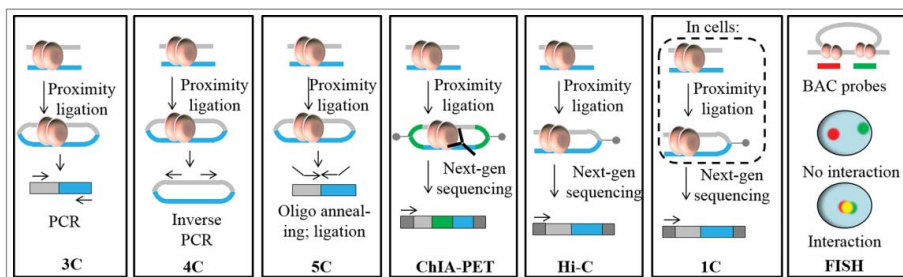


Figure 2. Chromatin conformation structure analysis methods. The “C” methods take advantage of proximity ligation, whereby interacting chromatin regions stabilized by cross-linking cell fixation are ligated, and the ligation products are deconvoluted by PCR, next-generation sequencing or other methods. FISH is a microscopy-based method that uses differently-labeled Bacterial Artificial Chromosomes (BACs) to detect genomic regions in close proximity.

Constructing Chromatin Interactions: Key Players

Formation and maintenance of chromatin interactions is an intricate process mediated by an array of RNA and protein factors including CTCF, Cohesin, and the Mediator complex. CTCF is a zinc finger DNA-binding tumor suppressor that is mutated or deleted in many cancers and is required for the formation of certain chromatin interactions.^{24,25} Depletion of CTCF led to fewer intradomain interactions, but more interdomain interactions.²⁶ Examination of CTCF by ChIA-PET in mouse cells showed categories of CTCF-bound chromatin interactions, including chromatin interactions associated with active gene transcription, chromatin interactions associated with gene repression, insulator properties with active gene transcription separated from gene repression by the CTCF loops and no specific pattern of regulation.²⁷ CTCF-bound chromatin interactions tended to be intrachromosomal loops over a large range of sizes, but a number of interchromosomal interactions were also detected.²⁷ While chromatin interactions were found to be enriched for CTCF, certain long-range chromatin interactions were found to loop over many CTCF-bound sites in the genome, suggesting that these sites might not act as a barrier to looping events in the classical idea of an insulator.²⁸

A possible mechanism by which CTCF might function is to compartmentalize the genome, as opposed to classical insulator properties. CTCF could have insulator functions by forming other loops, thereby blocking active enhancer-promoter contacts,²⁹ but in addition, CTCF could also promote gene repression and gene activation through forming activatory or inhibitory chromatin loops. The different functions of CTCF in turn could be mediated through associations with other proteins such as P300, ZNF143 and Cohesin. P300 is a tissue-specific enhancer binding protein, associated with long-range chromatin interactions.³⁰ P300 associates with CTCF-bound loops containing active genes and enhancers.²⁷ Similarly, ZNF143 a transcriptional activator that colocalizes with CTCF and Cohesin, has been associated in chromatin interactions through the analysis of ChIA-PET libraries ChIPed with histone modification marks (H3K4me1, H3K4me2, H3K4me3 and H3K27ac).³¹ We speculate that an interaction between these proteins could help in the maintaining the compartmentalization of the genome, and a possible consequence of mutations in these pathways could be the development of altered loops leading to tumorigenesis.

Cohesin is a component of the structural maintenance of chromosome protein complexes (SMCs).³² Cohesin “pairs” sister chromatids, possibly by encircling them in the ring structure of cohesin.^{33,34} This pairing, termed “cohesion,” is essential to ensure each daughter cell receives the correct number of chromosomes during cell division.^{35,36} Loss of cohesin subunits (e.g. RAD21) causes aneuploidy,³⁷ a common feature of tumor cells, and mutations in cohesin subunits have been linked to colorectal cancer.³⁸ The connection between cohesins and chromatin interactions came through the analysis of the long-range chromatin interactions at the cytokine locus *IFNG*.³⁹ Cohesin depletion led to reduced local chromatin interactions, but had no effect on TADs.²⁶ Analysis of the cohesin subunit SMC1A in mouse limbs

through ChIA-PET showed abundant SMC1A-bound chromatin interactions during development, suggesting that cohesin-associated chromatin interactions may be involved in developmental processes.⁴⁰ ChIA-PET on several histone modifications and RAD21 showed that chromatin interactions with RAD21 bound at both ends had higher interaction scores than chromatin interactions with RAD21 bound at only one end or neither end,³¹ suggesting an association between cohesin and chromatin interactions.

The mediator complex is a conserved transcriptional coactivator that helps to recruit RNA polymerase II to promoters of the target genes.⁴¹ Mediator is important in development and has been implicated in many diverse diseases, including cancer.⁴² Interestingly, Mediator and cohesin can physically interact, and these 2 proteins are associated with DNA looping events.⁴³ By contrast, mediator rarely interacts with CTCF alone, but mediator, cohesin, and CTCF may cooperate to form small scale interactions (<1 Mb).⁴⁴

Chromatin Interactions in Health and Disease: Understanding Functionality of the Genome

Formation and function of chromatin interactions is crucial for cellular health.²⁻⁵ On this basis, we speculate that errors in these processes could drive the formation of disease states through various mechanisms. Some of the pathogenic mechanisms include dysregulation of transcriptional and epigenetic changes by chromatin interactions,⁴⁵ including dysregulation of transcription, dysregulation of DNA replication, and structural variants and recombination, whereby DNA breaks and repair at chromatin interactions can facilitate the formation of translocations and fusion genes.^{46,47} We will illustrate these processes in this section and summarize the hypothesized mechanisms in **Figure 3**.

An important mechanism by which chromatin interactions influences the functionality of the genome is through transcriptional control of gene expression. A classic situation in this context is the looping of enhancers to their respective target gene promoters to control transcription.³⁰ This was first demonstrated in the case of the locus control region of the β -globin gene cluster where it was established that this region is heavily regulated by chromatin interactions.⁴⁸ Similarly, the Sonic hedgehog (*Shh*) enhancer, which is 1 Mb away from the *Shh* gene, interacts with the *Shh* target gene promoter through looping.⁴⁹⁻⁵¹

There is a growing body of evidence suggesting that enhancers located at distal positions in the linear genome loop over through chromatin interactions to influence several transcriptional processes. Further, recent advances in chromatin conformation structure profiling by ChIA-PET, Hi-C and other methods have demonstrated that these enhancers are not isolated examples, but rather that chromatin interactions constitute major mechanisms by which enhancers and other genomic elements connect with target genes to regulate gene transcription. To add further complexity, studies have revealed that chromatin interactions do not always link enhancers with the nearest gene. For example,

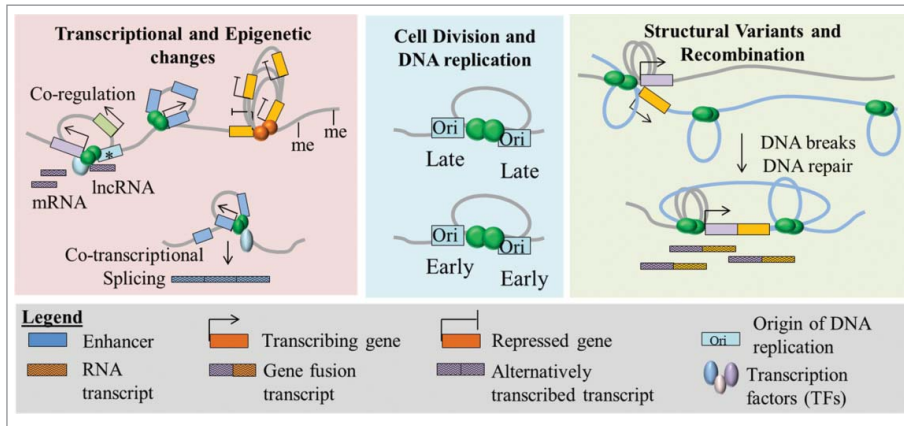


Figure 3. Mechanisms by which aberrant chromatin conformation structures may result in diseases such as cancer. These mechanisms include: (1) Regulation of transcriptional and epigenetic changes by chromatin interactions, including aberrant methylation and dysregulation of transcription. Aberrant chromatin interactions could lead to altered long non-coding RNA (lncRNA) and mRNA levels. In addition, aberrant chromatin interactions could lead to dysregulated co-transcriptional splicing, and loss of gene co-regulation, thus resulting in cancer. Epigenetic factors and long non-coding RNA may control chromatin interactions. (2) DNA replication. Early and late replicating origins of replication have been found to cluster together through chromatin interactions. Dysregulation of chromatin interactions could interfere with the organization of DNA replication, leading to aberrant DNA replication and consequent cancer formation. (3) Structural variants and recombination, whereby DNA breaks and repair at chromatin interactions can facilitate the formation of translocations and fusion genes, thus resulting in cancer. The formation of translocations, deletions or insertions and other structural variants could also lead to the formation or disruption of chromatin interactions. Genome organization may also facilitate virus and transposon integration at particular locations in the genome.

ChIA-PET on RNA polymerase II in human cancer cells showed that about 40% of enhancers bypass nearby genes to link to more distant genomic loci.²³ Evidence from 5C experiments further corroborated the observation by demonstrating that only 7% of looping interactions are with the nearest gene.²⁸ Additionally, these studies also showed the existence of loops spanning across several genes and that multiple chromatin interactions may occur at a specific genomic locus. As these findings are the result of sequencing many cells, one point to note is that these loops may not occur in the same cells at the same time, but reflect an aggregate picture of looping conditions.

Similarly, ChIA-PET on RNA Polymerase II in human cancer cell lines and mouse cells demonstrated abundant complex chromatin interactions which involved multiple genes, and may be co-regulated, suggesting that chromatin interactions may organize the genome into coordinated gene expression foci.^{23,52} A high degree of coordinated transcription is especially seen at genomic loci that contain members of the same gene family, such as the keratin locus, the *HOX* locus and the histone locus.²³ Indeed, many RNA polymerase II-bound chromatin interactions could be connected into a large hierarchical network of chromatin communities with specific functions.⁵³ Besides RNA polymerase II, the phenomenon of coordinated gene expression can be seen with H3K4me2 ChIA-PET.⁵⁴ Disruption of chromatin interactions in a multigene complex using TALENs to create double-strand breaks at one site directly led to transcriptional alterations at the interacting genes.⁵⁵

It should be noted we cannot conclude that the looping structure is critical to producing transcription from this evidence alone. It is unknown whether transcription leads to the production of the looping structure or vice versa, and under what circumstances. In an interesting study, disruption of gene loops using TALENs to create double-strand breaks and visualization of its effects using microscopy at a NF- κ B-regulated multi-gene complex which forms upon tumor necrosis factor α (TNF α) stimulation, showed that perturbing one end of the chromatin conformation structure directly altered transcription of the genes on the other end of the loop.⁵⁵ Therefore, at least at certain loci, the formation of specific chromatin structures leads to transcription.

Taken together, these results demonstrate that chromatin interactions are a mechanism used by the cell to coordinate gene transcription. Dysregulation of coordinated gene expression through aberrant chromatin interactions, as well as disruption of “transcription factories” may uncouple transcript levels, thereby leading to disease. Complexes of chromatin loops may form a “cage” maintaining high local concentrations of factors in certain locations of the genome, and possibly sequestering the factors from other locations in the nucleus. Specifically, complex transcription-associated chromatin interactions might be a manifestation of multiple genes coming together at the same “transcription factory”.^{23,56} Transcription factories are foci of concentrated RNA polymerase II engaged in active transcription that remain even in the absence of transcription.⁵⁷ In support of the “transcription factory” hypothesis, inhibition of transcription by drugs did not affect tested chromatin interactions in one experiment, indicating that at least some chromatin interactions are not dependent on active RNA polymerase II transcription.⁵⁸

The prevalence of chromatin interactions that connect multiple loci together could be one mechanism leading to pleiotropic effects.⁵³ Multiple enhancers may allow for fine-tuning of gene expression levels under different conditions and confer robustness in non-optimal conditions. Both ChIA-PET on RNA Polymerase II and 5C on targeted regions of the genome were performed in K562 chronic myelogenous leukemia as well as 3–4 additional cell lines as part of the ENCODE Consortium project. Both methods demonstrated highly interconnected chromatin, with abundant chromatin interactions. Many chromatin interactions could only be seen in one tissue type, demonstrating tissue specificity. For example, RNA polymerase II-bound chromatin interactions identified by ChIA-PET showed differences between embryonic stem cells, neural stem cells and neurosphere

stem/progenitor cells⁵² highlighting the developmental specificity of chromatin interactions. Given the complex nature of transcriptional regulation of target genes through looping, perturbation of a single enhancer, for example by mutation or epigenetic alterations, could lead to multiple and unexpected effects on different genes.

Intriguingly, chromatin interactions have been found to connect promoters and terminators at specific genes.⁵⁹ An estrogen-dependent promoter-terminator loop occurs at the *BRCA1* gene.⁶⁰ Chromatin loops between the promoter and terminator of genes may be a method for rapid cycling of the transcriptional machinery,⁶¹⁻⁶³ thus resulting in high levels of expression. Newly-formed aberrant loops between promoters and terminators could lead to extremely high levels of particular genes while disrupted loops would lead to lower levels of genes, both of which could result in disease.

Chromatin interactions have been connected with cotranscriptional splicing.⁶⁴ The human genome shows high levels of cotranscriptional splicing. A combination of ChIA-PET, DNase hypersensitivity data, and exon data has shown that chromatin interactions are associated with exons, particularly alternatively spliced exons.⁶⁵ As an example, cancer is associated with aberrant alternative splicing – over 15,000 splice variants have been discovered in different cancers through genome-wide studies.⁶⁶ Therefore, it may be possible that aberrant chromatin interactions lead to aberrant cotranscriptional splicing which results in disease-associated spliced variants. These pathological variants might be another potential mechanism by which chromatin interactions could trigger a disease condition.

Besides transcription activation, chromatin interactions can also regulate repression. Repressive states may be marked by H3K27me₃, under the control of the EZH2 component of the polycomb repressor complex (PRC), which is associated with cancer.^{67,68} PRC2 mutations have been found in several cancers such as B-cell lymphoma and myeloid disorders.^{69,70} Polycomb group proteins are known to mediate certain chromatin interactions, for example, at the *Drosophila* bithorax complex and the homologous human Hox clusters.⁷¹ The intersection between chromatin interactions and repression arises from the findings that certain repressive CTCF interactions may cooperate with Polycomb group proteins.⁷² The chromatin interactions may create a complex structure that excludes transcriptional machinery.⁷² Long-Range Epigenetic Silencing (LRES) refers to large tracts of DNA on the order of megabases that are silenced and occurs frequently in cancers.⁷³ Examination of estrogen signaling in normal cells showed that multiple DNA loops could form in an LRES region for coordinated repression.⁷⁴

In addition to the formation of repressive chromatin interactions, another possibility is that complexes of chromosome loops might sequester transcription factors and cofactors away from other locations in the nucleus due to limited amounts of such factors in the cell, thus leading to repression or gene activation in these regions of the cell. The sequestration of the transcription factor Estrogen receptor α and subsequent repression of its target genes is an example of by which chromatin interactions mediates repression through sequestration.²³ Given these different

methods by which chromatin interactions could mediate gene repression, dysregulation in chromatin interactions could lead to dysregulation of gene silencing, thus resulting in aberrant activation of oncogenes or silencing of tumor suppressor genes, leading to cancer.

Chromatin interactions may facilitate the formation of specific structural variants of the DNA.^{47,75} Further, deletions and insertions of genomic regions may remove or introduce new enhancers or other DNA elements. Chromosomal rearrangements may place DNA elements near other DNA elements. These processes could lead to the disruption of existing chromatin interactions and the formation of new chromatin interactions, which could have pathological consequences through the dysregulation of transcription or other factors.

This concept has been demonstrated in the context of a distal GATA2 enhancer in the case of EVI1-driven Acute Myeloid Leukemia (AML). Certain high-risk AML involve a chromosomal inversion between 3q21 and 3q26. Analysis of breakpoints in AML samples as well as ChIP-Seq studies demonstrated the presence of a superenhancer 77 kb upstream of GATA2, which activates GATA2 in normal haematopoietic cells.⁷⁶ A translocation can bring the enhancer near *Evi1*, which it upregulates through chromatin interactions as identified by 4C.⁷⁷ A linked Bacterial Artificial Chromosome (BAC) recapitulating the inversion of the enhancer found in AML led to leukemia in mice.⁷⁶ At the same time, the loss of enhancer-GATA2 chromatin interactions caused GATA2 haploinsufficiency.⁷⁷ Excision of the enhancer by genome editing techniques and JQ1 inhibition of BET bromodomains associated with superenhancers led to differentiation of AML cell lines, indicating how structural variants influence pathological outcomes through dysregulation of chromatin interactions.⁷⁷ It is likely that similar mutations in other loci will be seen in different cancers.

Similarly, chromatin interactions may facilitate the formation of translocations. During translocation formation, a Double-Strand Break (DSB) forms in DNA. Upon a double strand break, imaging studies have shown that the majority of DNA ends undergo locally restricted movement to find nearby partners, after which the DNA ends join together.⁷⁸ Given this phenomenon, DNA ends involved in pre-existing chromatin interactions would preferentially join to each other. Alternatively, during the DNA repair process, new chromatin interactions may arise as multiple chromatin fragments share repair foci.⁷⁹ Thus, chromatin interactions would facilitate the formation of specific translocations, leading to cancer. Hi-C combined with translocation sequencing studies of pro-B cell genomes showed that translocation frequency correlated with the level of chromatin interactions.⁸⁰ This result suggests that genomic regions rich in chromatin interactions have a higher propensity to form translocations which would result in cancer, and thus provides an explanation for the formation of several clinically observed translocations in cancer.

The relationship between translocations and chromatin interactions has been investigated in the context of the *TMPRSS2-ERG* fusion in prostate cancer. In the presence of androgen, binding of Androgen Receptor to DNA induces chromatin

interactions between the androgen-regulated gene *TMPRSS2* and the transcription factor *ERG*.^{47,81} DNA double strand breaks followed by DNA repair then facilitate the formation of the *TMPRSS2-ERG* fusion gene that is observed in about 50% of human prostate cancers.^{47,81} This has also been shown for the *TMPRSS2-ETV1* translocation in human prostate cancers.⁴⁷ The DNA double strand breaks could arise from external factors such as gamma radiation,⁸¹ but Androgen Receptor also promotes site-specific DNA double-stranded breaks at the points of translocation through altering chromatin structure and recruiting stress-induced enzymes such as Activation-Induced cytidine Deaminase (AID) and LINE-1 repeat-encoded ORF2 endonuclease.⁴⁷ The double-stranded breaks are then repaired by the nonhomologous end joining pathway.⁴⁷ Moreover, overexpression of *ERG* leads to alterations in chromatin interactions throughout the cell, as detected by Hi-C,⁸² highlighting the relevance of chromatin interactions in the process of carcinogenesis through functional alteration of the genome.

Transposons and viruses insert new genetic material into the genome, and one of the downstream effect, of this insertion is the development of new translocations and dysregulated gene expression, leading to cancer. For example, human papilloma virus (HPV) integrates into the human genome as a part of the infection cycle and a downstream consequence of this integration is the formation of cancer. Besides this mechanism, transposons and virus integrations could provide new material for the possible formation of new chromatin interactions, as well as the disruption of existing chromatin interactions, thus leading to cancer. A study that investigated several hundreds of thousands of unselected integrations in the mouse genome for Sleeping Beauty and Piggybac transposons and the Mouse Mammary Tumor Virus (MMTV) showed differences in integration potential arising from chromatin features. In particular, retroviral and transposon integration hotspots are associated with chromatin interactions defined by Hi-C. All systems showed a preference for DNA insertions in the boundaries of TADs, suggesting that chromatin interactions might facilitate DNA integration at particular regions while preventing DNA integration at other regions that may have very tight chromatin interactions.⁸³

Errors in cell division are key features of cancer development. The cell cycle, therefore, is a tightly regulated process. Chromatin interactions are associated with the cell cycle and were characterized further by Hi-C in cancer cell lines.⁸⁴ It was found that distinct chromatin interactions signatures could be observed at various stages of the cell cycle. For example, interphase was marked by cell-type-specific compartments, while metaphase showed a locus-independent folding structure marked by loops around 100 kb across all chromosomes in all cell types examined,⁸⁴ indicating that chromosome structures are lost during the cell cycle and must be re-patterned following completion of cell division. In particular, chromatin organization is associated with the replication stage of the cell cycle, which is dysregulated in diseases such as cancer⁸⁵ - accurate duplication of the genome is critical for the maintenance of genomic integrity and tightly synchronized with the cell cycle. Mammalian cells demonstrate large (up to megabases) tracts of "replication domains," which

replicate at particular times. Interestingly, Hi-C analysis indicated that regions that replicate early tend to cluster together, while late-replicating regions clustered separately, suggesting that these "replication domains" are structural units in the genome.⁸⁶

To understand the relationship between replication timing, long-range chromatin conformation structure information and somatic copy number alterations found in cancer, De and Michor integrated a large dataset of somatic copy number alterations found in 26 cancers⁸⁷ with replication timing identified through sequencing of newly replicated DNA⁸⁸ and Hi-C data.¹³ De and Michor found that genomic regions not connected by Hi-C-identified chromatin interactions and which had different replication timing were highly unlikely to form somatic copy number alterations, suggesting that chromatin conformation structure and replicating timing information could help to predict the appearance of cancer-associated mutations.⁸⁹ The need to unfold and re-fold chromatin accurately into specific chromatin structures upon the completion of cell division could pose a novel form of cell stress, related to replication stress. Hence, it may be possible that dysregulated formation of chromatin interactions form could eventually lead to the development of genomic instability, and thus promote cancer events.

Chromatin Interactions in Translational Cancer Medicine: Genomic Annotations for Personalized Medicine

As mentioned earlier, emerging evidence suggests a link between chromatin interactions and disease. 3-dimensional chromatin analysis will be critical for basic understanding of normal cellular functioning and the dysregulation of cellular functioning in diseases such as cancer. In addition, the insights that we will gain from understanding distal genomic elements and the role of chromatin interactions could have potential translational benefits. Inclusion of chromatin interactions in efforts to identify target genes for non-coding mutations or variants associated with disease could further identify actionable genes in diseases.

The incorporation of 3D genome organization has become a critical piece of many Genome-Wide Association Studies (GWAS). GWAS make use of the phenomenon of linkage disequilibrium to identify markers, usually Single Nucleotide Polymorphisms (SNPs) associated with a particular trait of interest. The challenge in GWAS studies is identifying the disease-associated locus and the target gene from the "tagging" SNP on the genotyping chip.⁹⁰ Half or more of disease-associated SNPs from published GWAS are in non-coding regions.³⁰ For example, a cluster of SNPs associated with insulin resistance, type II diabetes and coronary heart disease⁹¹ could be associated with *IRS1* using RNA Polymerase II-associated chromatin interactions.²³ In another example, the SNP rs385893 associated with platelet count could be associated with *JAK2* using RNA Polymerase II-associated chromatin interactions.⁹²

Several GWAS studies have begun to incorporate 3C, 4C or other chromatin interaction assays to test chromatin interactions, for traits associated with cancer as well as diseases and traits

besides cancer.⁹³⁻⁹⁵ For example, breast cancer-associated SNPs at the 10q21.2 risk locus have been found to loop to *ZNF365* and *NRBF2*, which code for DNA-binding proteins.⁹⁶ The 10q26 locus in the second intron of *FGFR2*, which is associated with estrogen receptor-positive breast cancer could interact with the promoter of *FGFR2*.⁹⁷ In another example, a papillary thyroid cancer-associated risk locus could be associated with *FOXE1*, which codes for a DNA-binding protein, and *PTCSC2*, a long non-coding RNA, via chromatin interactions.⁹⁸

In an effort to make this process more unbiased and higher-throughput, a capture Hi-C method which allows the analysis of specific chromatin interactions associated with various loci was applied to 14 colorectal cancer risk loci.⁹⁹ All 14 loci showed significant long range (> 10 kb) interactions, including known interactions between the rs6983267 SNP and *MYC* as well as *CCAT1*. Interestingly, several interactions connected the loci to known cancer-associated genes, such as the *ETSI* oncogene. Taken together, gene-distal risk loci may be connected with target genes through chromatin interactions. In the future, more studies looking at cancer risk loci in connection with chromatin interactions will help to assign function to these important non-coding genomic regions.

In support of clinical sequencing and annotation efforts, a number of bioinformatics databases have been developed. Genome-Wide Annotation of Variants (GWAVA)¹⁰⁰ integrate genomic and epigenomic annotations to assign functions to non-coding mutations and prioritize them for further experiments. Moreover, an annotator has been developed which incorporates 3D Genome Organization data: GWAS3D, which integrates chromatin states, sequence motifs, cross-species conservation information, and chromatin interaction information from the ENCODE project.¹⁰¹

Chromatin Interactions in Translational Cancer Medicine: Biomarkers

Chromatin interactions are good candidates for biomarkers¹⁰² - besides 3C and 1C, FISH probes against chromatin interactions could be used as the basis for FISH-based biomarkers. One of the main advantages of using chromatin interactions as a biomarker would be the ability to visualize the functional anatomy of the whole genome as against selected few genes or regions. Additionally, chromatin interactions based biomarkers can be personalized, thereby providing an unprecedented resolution in the analysis of personalized genomes. Further, analysis of chromatin interactions can be coupled with other molecular techniques to enhance/validate the specificity of the results obtained from other investigations.

There are several challenges in using chromatin interactions as biomarkers. Foremost among them is the necessity for abundant starting material which poses a huge challenge in the applicability of chromatin interactions in molecular medicine. Heterogeneity of the cell population in a given clinical sample is another challenge when it comes to delineating chromatin signatures in a particular clinical specimen. Similarly, the turnaround time for these assays is long and hence lowers its utility in clinical applications.

FISH is one method of interest in terms of biomarkers.¹⁰³ The finding that certain chromatin interactions can precede translocations also raises the possibility that FISH-based biomarkers that investigate translocations may provide earlier detection.¹⁰⁴ This can be investigated through the use of both FISH and PCR to interrogate translocations. During the early stages of disease, PCR would not show any positive results as the translocation would not have occurred yet, while FISH would show a positive result because the chromatin interaction that preceded the translocation might have occurred. To apply this concept, scientists designing FISH probes at a particular translocation would first need to determine whether early stages of disease are associated with the occurrence of particular chromatin structures that facilitate the formation of specific disease-causing translocations. Next, the scientists would need to determine whether the occurrence of the chromatin structures is disease-specific and predictive of disease - if not, such biomarkers would lead to high false positive rates. Finally, the FISH probe would need to be designed in a specific manner such that the chromatin conformation can be captured and imaged easily.

One challenge associated with FISH-based biomarkers is that it has a poor resolution in the case of intrachromosomal interactions and can only be applied to chromatin interactions at least 1 Mb, ideally longer in distance. Moreover, FISH probe-based assays are tedious to perform and require much manual labor for counting. In the future, super-resolution microscopy may assist with the challenges of visualizing intrachromosomal interactions.¹⁰⁵ Moreover, automated imaging methods and computer-assisted interaction scoring may reduce the burden of this method.

The development of *Episwitch*, a proprietary 3C-based platform developed by Oxford biodynamics is a promising entry into this field.^{106,107} The platform utilizes blood as the specimen to identify chromosomal conformations in the target genes of interest and reports the results in few hours. This method could open up new possibilities for less invasive blood-based biomarkers for monitoring. Another advantage of using 3C-based chromatin interactions as biomarkers is that cross-linking is relatively stable, and following proximity ligation, give rise to a stable DNA product. In the coming days, it is likely that more players with different detection systems will enter this nascent arena of chromatin interactions-based biomarkers.

Chromatin Interactions in Translational Cancer Medicine: Therapies

Targeting chromatin interactions offers several advantages as therapies. First, enhancer-promoter interactions offer a method of fine-tuning expression levels. Enhancer-promoter interactions tend to be cell-specific^{23,28} and slightly raise gene expression levels.^{108,109} As compared with directly inhibiting *MYC* and other important oncogenes, which could cause many off-target effects because *MYC* is expressed in many tissues and its expression is essential for many cellular functions,¹¹⁰ targeting chromatin interactions that regulate *MYC* could allow for slightly reduced expression levels in specific cells of interest.

At the same time, beyond targeting gene-specific enhancer-promoters interactions, the widespread nature of chromatin interactions means that targeting chromatin interactions associated with particular factors of interest could allow for fine-tuning of the expression levels of these gene products associated with a factor of interest. One such example is SATB1, a genome organizer. SATB1 is associated with bad prognosis in breast cancer.¹¹¹ Reduction of SATB1 levels by RNAi changed expression levels for over a thousand genes and reversed tumorigenesis.¹¹¹ While specific chromatin interactions are known to be controlled by SATB1, it is unknown whether the reduction of SATB1 levels altered chromatin interactions, and whether these chromatin interaction alterations led to the reversal of tumorigenesis or if this was from other pathways.

Second, many disease-associated distal non-coding regions have been found to regulate target genes through chromatin interactions.¹¹² In contrast with genome editing which could result in undesirable permanent alterations to the genome in this location and in other off-target locations, perturbing enhancer-promoter chromatin interactions could allow us to target these distal non-coding regions and their mechanism of action in a reversible, specific manner.

Likewise, therapies based on chromatin interactions face several challenges that need to be overcome. First, chromatin interactions and modulators of chromatin interactions lie in the nucleus. While certain nuclear factors, such as estrogen receptor, may be perturbed by small molecules such as Selective Estrogen Receptor Modulators (SERMs), many nuclear targets are difficult to perturb using small molecule inhibitors or antibodies. This may be addressed through novel drug delivery methods such as viral-like particles, liposomes, and nanoparticles.¹¹³ Second, CTCF, cohesin and mediators, the factors that are known to regulate chromatin interactions, regulate many chromatin interactions and are also involved in many pathways. Moreover the mechanisms by which these factors operate are not clear. Inhibiting these factors may cause many off-target and general effects on the rest of the body. The factors that confer specificity in terms of chromatin interactions will be a critical piece of the puzzle for further investigations.

Another important question is that many epigenetic regulators have been found to be perturbed in cancer cells through large-scale cancer sequencing experiments.¹¹⁴ However, their relationship, if any, to chromatin interactions is unclear. It will be important to determine whether these epigenetic regulators operate via chromatin interactions, and if so, whether perturbation of the epigenetic regulators in cancer leads to dysfunctional chromatin interactions. Finally, several epigenetic drugs are being developed that target specific epigenetic regulator. It would be interesting to note if epigenetic drugs affect chromatin interactions in any manner, and to characterize this as part of better understanding the drug mechanism of action or off-target effects. In this context, recent work from the laboratory of Tom Misteli has thrown up some exciting possibilities.¹¹⁵ The group utilized a combination of high-throughput FISH as well as high-throughput imaging pipeline to develop a versatile tool- HIPMap, or high-throughput imaging position mapping to map the endogenous organization

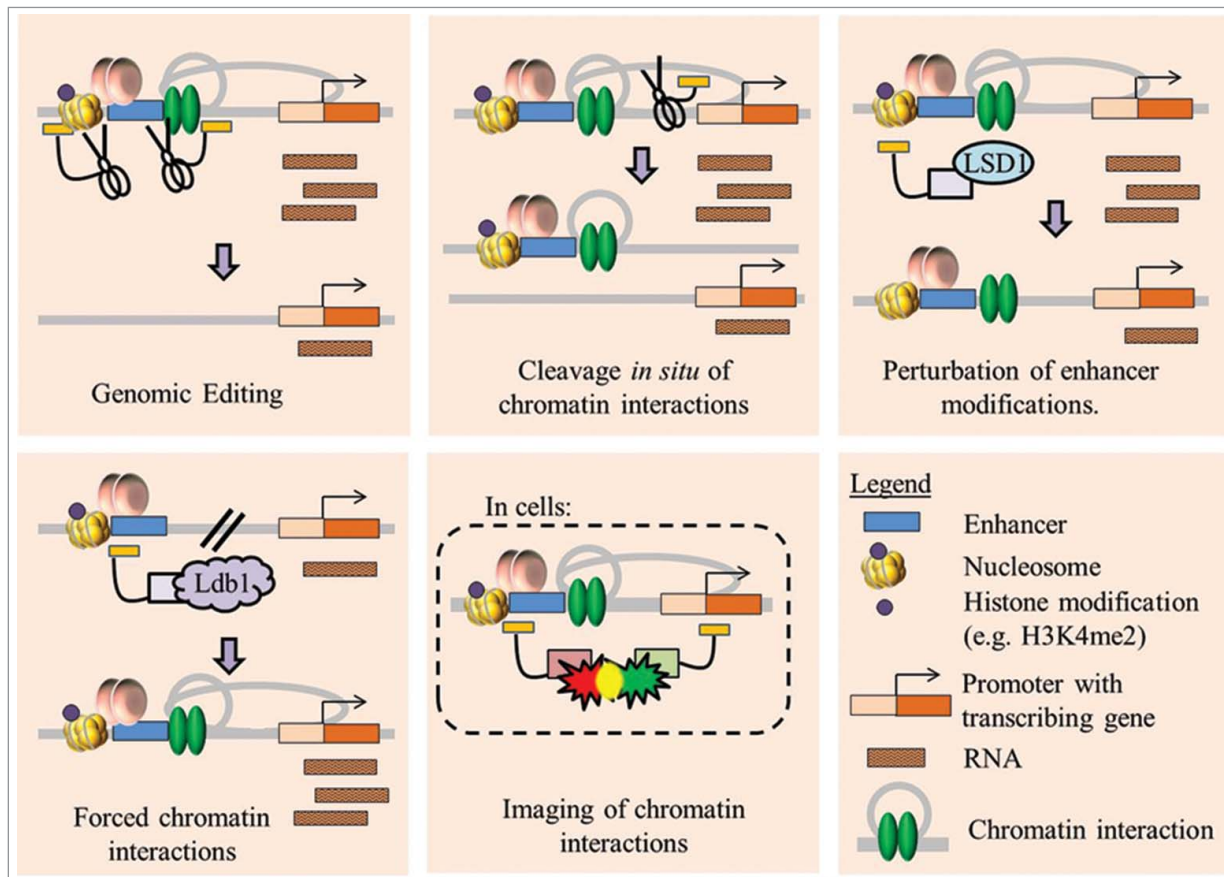
of genomic loci. They further carried out an siRNA-mediated knock-down screen along with HIPMap which identified 50 factors that are important for genome organization.¹¹⁵ Some of these hits include centromeric proteins (for example, centromere protein E *CENPE*), factors involved in chromatin remodeling (for example, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 2-*SMARCD2*), components of the nuclear envelope (for example, nuclear pore complex protein *NUP85*) and DNA replication (for example, Proliferating cell nuclear antigen *PCNA*) and repair factors (for example MutS homology6 repair protein *MSH6*). In future, results from such screens would help uncover novel candidates for targeted drug design.

Future Prospects

Further research will be required to better understand the role of chromatin interactions in disease, as well as how this knowledge can be harnessed for translational benefits. One important question in the nascent field of chromatin interactions in disease is regarding the series of events that takes place upon the formation of a new chromatin conformation structure, for example, following cell division or upon hormone stimulation. How are these biochemical events orchestrated? Answering this question will allow us to understand how to target chromatin interactions, for example by small molecule inhibitors. While CTCF, cohesin, mediator and several other factors have been shown to lead to the loss of chromatin interactions upon knockdown, and several more have been hypothesized to be linked, such as chromatin remodelling proteins seen in cancer, the sequence as well as the biochemical basis for their involvement in chromatin interactions is unclear. The creation of deletion mutants for these factors could assist in identifying domains in these proteins that are needed for looping formation. In addition, drug, shRNA, or CRISPR based screens targeting chromatin conformation structure alterations could facilitate the discovery of novel factors that may play roles in chromatin conformation structure formation, allowing for possible therapeutic modulation of chromatin interactions.¹¹⁵

Another important question is what are the chromatin conformation structure changes that occur in cells upon transformation from normal cells into cancerous states? How common are these changes? Answering these questions will facilitate the development of chromatin interactions to assess pre-cancerous states. While much of our understanding comes from mouse models and human cancer cell lines, chromatin conformation structure analysis with patient tumor samples will be vital for further understanding of chromatin interactions in cancer. New breakthroughs in technologies for identifying chromatin interactions such as the recently-developed single-cell Hi-C as well as advancements in genomic editing (Box 1) could allow for detailed functional characterization of chromatin interactions from clinical samples. Further, in terms of translational medicine, the technologies that are closest to becoming clinical reality include

Box 1. Genomic Engineering Methods for investigating chromatin conformation structures.



New genomic engineering technologies such as TALENs,¹¹⁷ zinc fingers,¹¹⁸ and CRISPR¹¹⁹ have been shown to be fast, specific and reliable for genetic engineering, as well as a host of other applications. They may be applied to the study of chromatin interactions as well.

Genomic Editing of chromatin conformation structures. One application to understanding chromatin interactions is using genomic engineering to excise non-coding regions involved in chromatin interactions to observe the effects on cells and whole organisms.^{77,120}

Cleavage *in situ* of chromatin conformation structures. Besides genomic engineering, these new tools can be used in other ways: TALENs have been used to cut chromatin interactions in cells without excising any regions.⁵⁵

Perturbation of enhancer modifications. A TALE-LSD1 histone demethylase fusion was used to perturb enhancer modifications at specific loci.¹²¹

Forced chromatin conformation structures. Ldb1, which is associated with chromatin interactions at the β -globin locus, fused with zinc fingers, forced the formation of a chromatin conformation structure in cells, establishing Ldb1 as a mediator of particular chromatin interactions.¹²²

Imaging of chromatin conformation structures. In addition, CRISPR can be modified to allow imaging of genetic loci in live mammalian cells.¹²³ In this approach, cas9 is modified with a Green Fluorescent Protein (GFP) tag and has its nuclease activity removed. Guide RNAs against the target locus then bind the CAS9, thus allowing imaging of the location of the genetic locus in the cell. By using different CAS systems tagged with different fluorescent proteins and different guide RNAs, it is theoretically possible to adapt the method to a multicolor system, allowing the visualization of chromatin interactions in living cells.

chromatin-interaction-based biomarkers and personalized medicine advances from using chromatin interactions to understand the target genes to which non-coding mutations and polymorphisms are associated.

While the study of chromatin interactions is still in its infancy, one thing that is clear is that the roles of chromatin interactions in cancer deserves further study, and is likely to bear fruit in

terms of understanding the basic biology of cancer as well as provide platforms for new therapeutic approaches to the management of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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