

# An Overview of Genome Organization and How We Got There: from FISH to Hi-C

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## SUMMARY

In humans, nearly two meters of genomic material must be folded to fit inside each micrometer-scale cell nucleus while remaining accessible for gene transcription, DNA replication, and DNA repair. This fact highlights the need for mechanisms governing genome organization during any activity and to maintain the physical organization of chromosomes at all times. Insight into the functions and three-dimensional structures of genomes comes mostly from the application of visual techniques such as fluorescence *in situ* hybridization (FISH) and molecular approaches including chromosome conformation capture (3C) technologies. Recent developments in both types of approaches now offer the possibility of exploring the folded state of an entire genome and maybe even the identification of how complex molecular ma-

chines govern its shape. In this review, we present key methodologies used to study genome organization and discuss what they reveal about chromosome conformation as it relates to transcription regulation across genomic scales in mammals.

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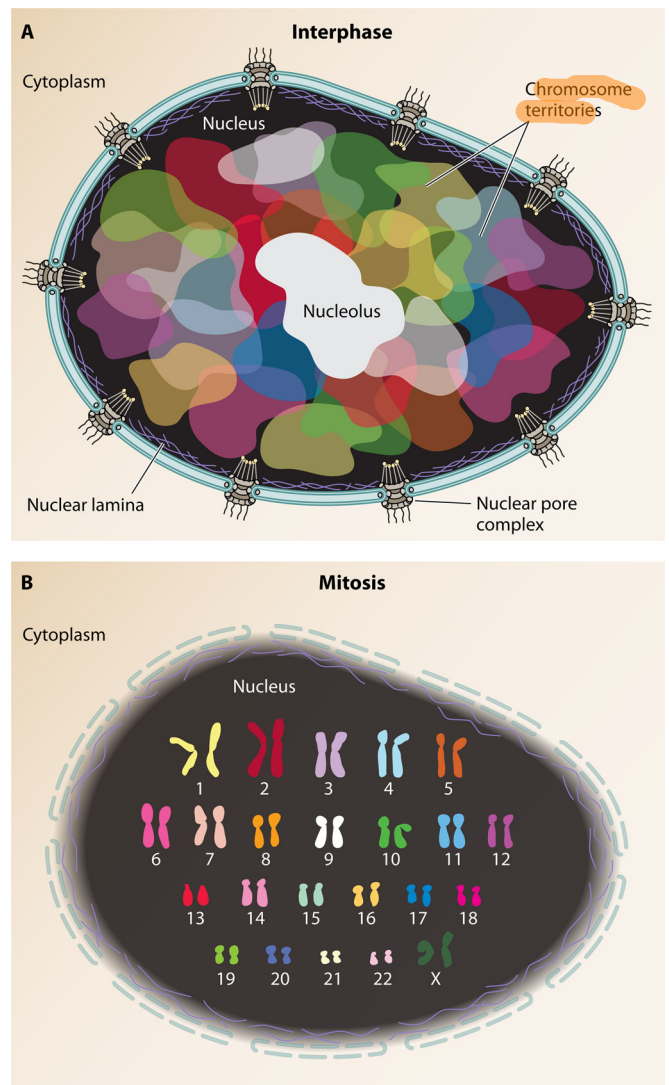
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## INTRODUCTION

Human genomic DNA was serendipitously identified in 1869 by Friedrich Miescher while searching for new proteins in the pus of wounded soldiers (reviewed in reference 1). We have since come to recognize that DNA is the genetic material containing all the information essential for life and the basis for heredity (2). The human genome is divided into 46 DNA molecules, or chromosomes, consisting of pairs of chromosomes 1 to 22 (autosomes), numbered sequentially according to their size, and of two sex chromosomes that determine whether an individual is male or female (Fig. 1). Together, these molecules contain over 6 billion letters that when joined would measure ~2 m in length. It stands to reason that the human genome must be extensively packaged in order to fit inside the nucleus, the size of which is in the micrometer range.

The physiological state of genomic DNA is in the form of chromatin, where it is bound to histone and nonhistone proteins. Histones are by far the most abundant proteins in chromatin and bind DNA mainly as nucleosomes composed of two copies each of H2A, H2B, H3, and H4. Wrapping of DNA around nucleosomes represents the first level in packaging, which effectively shortens the length of chromosomes by 7-fold. Histones, particularly their amino- and carboxy-terminal tails, are subject to posttranslational modifications (PTMs) on multiple residues, including methylation, acetylation, phosphorylation, sumoylation, ADP-ribosylation, or ubiquitinylation (3–5). PTMs regulate the activity of underlying genomic regions by altering how nucleosomes interact with each other and the DNA, thereby controlling access to given sequences, and/or by recruiting effector proteins that bind PTMs directly and interpret whether a region should be active or not. As such, chromatin could be considered the basic regulatory unit of genomes, and further packaging within the confines of the three-dimensional (3D) nuclear space can have a direct impact on its activity. The importance of three-dimensional chromatin organization both for reducing chromosome size and for other genome functions such as transcription is indeed recognized.

In healthy cells, higher-order chromatin organization is necessarily consistent with genome function and regulation. This level of organization is poorly described, with even the fundamental principles guiding interphase chromatin folding and unfolding still being unknown. How the genome folds is particularly important for transcription because control DNA elements and their target genes are not always next to each other along the linear genome sequence, a fact which has been apparent since Barbara McClintock's early studies on transposition (6). More recently, it was found that gene regulation by distal control elements such as enhancers is often associated with physical contacts between them. Given that tissue specificity is achieved through the combined action of regulatory sequences on target genes, this observation raises a compelling conundrum about specificity: if elements like enhancers can act long distance on genes located anywhere in the genome, how is specificity achieved? Three-dimensional genome organization appears to play an important role in this process by both promoting and restricting the access of control DNA elements to genes. This review provides an overview of genome architecture in mammals and the methods used to study its function and regulation. While some of the principles derived from these studies might be applicable to different species,



**FIG 1** Human genome organization in a three-dimensional nucleus. (A) Chromosome territories observed during interphase. Nuclear pore complexes are shown perforating the nuclear envelope. The nucleolus is shown in white. The nuclear lamina is represented as a filamentous mesh inside the double nuclear membrane. (B) Example of a normal female karyotype as would be observed by SKY (322) of mitotic cells.

they might not be applicable to others, including the budding yeast *Saccharomyces cerevisiae*.

## THE GENOME IN A THREE-DIMENSIONAL NUCLEUS

### The Nuclear Lamina

In mammals, the genome is contained within the cell nucleus, a double-membrane organelle that effectively segregates the transcription machinery from the cytoplasm, where protein production occurs (Fig. 1). At its lowest resolution, genome organization is guided by contacts with several nuclear substructures. The nuclear envelope (NE) and its lamina are such structures. The outer nuclear membrane (ONM) and inner nuclear membrane (INM) of the NE are populated by nuclear envelope transmembrane proteins (NETs), which associate with the lamin proteins on the INM face to form the nuclear lamina (reviewed in references 7 and 8).

The lamins are intermediate filament proteins, which, together with NETs, can bind many different proteins, including chromatin components such as heterochromatin protein 1 (HP1) and histones. Interactions between the lamina and the chromatin can regulate the position of chromosomes in the nucleus (9) and various other activities (10–12).

Chromatin interacts with the nuclear lamina through lamin-associated domains (LADs) that vary in size from 0.1 to 10 Mb and frequently contain transcriptionally inactive heterochromatin (13, 14). LADs are generally conserved but can also be cell type specific (15). While conserved LADs span regions with very low GC content and are gene poor, cell type-specific LADs usually have a higher GC content and correlate with tissue-specific gene expression (16). These observations suggest that inactive chromatin regions, even those that are gene rich, tend to localize at the nuclear periphery or the nucleolus (see below). In fact, simply relocating a given region to the nuclear lamina is often sufficient to reduce gene expression (17, 18), but this is not always the case (19).

### The Nuclear Pore Complex

The nuclear pore complex (NPC) is another nuclear substructure involved in regulating gene expression that might play a role in chromatin organization (20). NPCs are evolutionarily conserved structures that mediate all transport between the nucleus and the cytoplasm (21). They are very large, ranging in mass from ~60 million to 100 million Da, depending on the organism, and exhibit an 8-fold symmetrical/cylindrical geometry around a central transport channel. NPCs “perforate” the two lipid bilayers of the nuclear envelope and are composed of >30 different nucleoporin proteins. Nucleoporins either are part of the core (integral proteins), form filaments extending from the NPC core toward the cytoplasm or nucleoplasm (those containing FG repeats, or FG-Nups), or are part of the nuclear fibers (nuclear basket) (Fig. 1).

The chromatin environment around the nucleoplasmic face of NPCs differs from that of the rest of the inner nuclear membrane despite their physical proximity to the lamina. Early electron microscopy studies of nuclei in higher eukaryotes revealed the presence of heterochromatin exclusion zones (HEZs) around NPCs (22, 23). These gaps in the heterochromatin landscape of the inner membrane vary in size, tend to be cone-like, and are populated by euchromatin that extends from the NPC fibers to the nucleoplasm. Interestingly, contacts between nucleoporins and chromatin could be captured by double cross-linking (24, 25). Although direct physical NPC-chromatin interactions cannot be concluded from these experiments, they nonetheless suggest an intimate link between chromatin and the nuclear transport machinery. Accordingly, several nucleoporins have been involved in the activation of transcription (26, 27), and although a large fraction of nucleoporins is found free in the nucleoplasm, NUP98 has been shown to bind genes on its own or as part of the NPC (28). Nucleoporins might also contribute to the compartmentalization of chromatin marks along human chromosomes given that they have been linked to insulation in yeast (29) and are required for HEZ establishment in human (30).

### The Nucleolus

A third type of nuclear landmark involved in genome organization is the nucleolus. Nucleoli are dense structures, visible by light microscopy, where rRNA synthesis and preribosome assembly oc-

cur. They form around grouped rRNA genes from different chromosomes that are transcribed by RNA polymerase I (Pol I) and are located where nascent rRNA transcripts are processed and packaged into preribosomes. Several RNA polymerase II genes were found to copurify with the nucleolus when transcriptionally inactive (31, 32). These nucleolus-associated domains (NADs) significantly overlap LADs (33) and have similar GC-poor and gene-poor contents. Loci were actually found to colocalize with either NADs or LADs, suggesting that a certain amount of redistribution occurs between the two regions after mitosis and possibly that similar factors target the inactive chromatin to either the lamina or nucleolus (33, 34). Other substructures have also been linked to chromosome organization and include Cajal and promyelocytic leukemia (PML) bodies (35, 36).

## VISUAL AND MOLECULAR ANALYSIS OF GENOME ORGANIZATION

In addition to anchors with nuclear landmarks, human genome organization is guided by chromatin interactions within (*cis*) and possibly between (*trans*) chromosomes. These interactions are driven by the chromatin landscape and are thus often tissue specific and regulated. Our current view of genome organization is based largely on, and perhaps is limited by, data derived mainly from only two types of approaches. A variety of microscopy techniques, including several fluorescence *in situ* hybridization (FISH) procedures, visualized by conventional or superresolution light microscopy, is currently used to directly measure the proximity between DNA segments. These methods yield information-rich data about genome topography in individual cells that are well complemented by insights obtained from cell populations using molecular techniques such as chromosome conformation capture (3C) and its derivatives. This second type of technique infers DNA proximity by quantifying the frequencies of contacts between DNA segments and considering them to be inversely proportional to their original distance *in vivo*. Representative methods from each category are presented below.

### Visualizing Genome Organization

Until the advent of molecular techniques such as 3C and its high-throughput derivatives, the predominant method for determining nuclear organization and chromatin conformation was FISH. This cytogenetic approach has been used for a variety of applications, from clinical diagnostics to the study of genome architecture. Sensitivity and resolution are limiting factors to consider when designing a FISH experiment. Sensitivity depends on the light-capturing capability of a particular microscope, therefore determining the size of the probe (larger probes will generally produce stronger signals). Probe size then brings in one aspect of resolution: being able to distinguish between two points along the length of a chromosome. For example, fosmid probes are frequently used to measure chromatin compaction or to identify the colocalization of genes with remote regulatory elements. However, due to their size (~40 kb), genomic distances of <100 kb cannot be resolved. However, oligonucleotide-based probes of ~10 kb in size with high hybridization efficiencies that produce strong signals allow the chromatin conformation of sub-100-kb genomic regions to be determined (37–40). There is also the resolution of the light microscope to consider: conventional light microscopy cannot resolve structures with sizes of <200 nm in the *x* and *y* planes and <500 nm in the *z* plane. However, superreso-



lution microscopy is bringing the light diffraction limit down to the tens-of-nanometers scale (see “New Insight from Superresolution Microscopy,” below).

**2D-, 3D-, and cryo-FISH.** The observation that complementary nucleotide sequences could hybridize to each other and form more stable complexes than noncomplementary sequences was the basis for the first *in situ* hybridization analysis that identified the position of ribosomal DNA within the nucleus of a frog egg (41). Molecular cytogenetics is based on this procedure, with the replacement of radioactive labels with more stable fluorochromes (42) providing improved safety and ease of detection. The FISH technique, then, relies upon probe sequences that target genomic DNA, which either are directly labeled with a fluorochrome or have been modified to contain a hapten (such as biotin) and are then rendered fluorescent indirectly by enzymatic or immunological detection. Target and probe DNAs must go through a denaturation step to allow target-probe hybridization. The fluorescently labeled genomic loci can then be visualized by using a fluorescence microscope.

There are now a diverse number of FISH assays that can be applied to megabase (metaphase chromosomes), submegabase (interphase chromosomes), and even nucleotide (oligonucleotide arrays) resolutions (43, 44). The two-dimensional FISH (2D-FISH), 3D-FISH, and cryo-FISH variations of the FISH process have been used to directly visualize and measure the nuclear distance between DNA segments, the nuclear location of DNA segments or indeed whole chromosomes, and the location of a DNA segment in relation to the rest of the chromosome (i.e., within or “looped out” of the chromosome territory [CT]). In the 2D-FISH procedure, cells are fixed in methanol acetic acid (MAA), which generates looser chromatin packaging due to the flattening out of the cells on the slide; however, results are comparable to those for paraformaldehyde (pFA) fixation of the same cells (45–49). The advantages conferred by 2D- over 3D-FISH are clearer visualization and rapid image analysis. This technique has been predominantly used for determining the nuclear location of genes or translocation from the nuclear periphery to the center and vice versa (17, 50) and for determining the location of a DNA segment in relation to the rest of the chromosome (45, 48, 51). We have used 2D-FISH to determine changes in chromatin condensation at the submegabase level, during differentiation and across a polarizing axis during development (45, 49) or between wild-type and mutant cells (46, 52).

Not all direct analyses of chromatin structure can be adequately visualized by 2D-FISH. Colocalization of discrete genomic loci, for example, such as promoter-enhancer interactions, requires 3D reconstruction of nuclei. In 3D-FISH, cells or tissue sections are fixed in 2 to 4% pFA, and image capture requires confocal microscopy or deconvolution software if images are taken with a wide-field fluorescence microscope that has the capacity to generate image stacks through the *z* dimension. Applying this method to tissue sections and cell lines derived from embryonic day 10.5 (E10.5) limb buds of mouse embryos, we found that the colocalization frequency of *Hoxd13*, crucial for distal limb development, with a limb-specific long-range enhancer is increased in expressing cells (49, 53). This type of analysis, in combination with 3C, has also been done on the *Shh* locus at the same stage of limb development (see below) (54) and has been used for visualizing the colocalization of a single olfactory receptor allele and an enhancer element in individual sensory neurons (55). More recently,

the Lomvardas laboratory identified the colocalization of multiple putative enhancers with individual olfactory receptor (OR) alleles by chromosome conformation capture-on-chip (4C) and 3D-FISH and determined by Hi-C that many of these colocalizations occurred in *trans*, which was also confirmed by FISH (56). Each olfactory sensory neuron expresses only one of ~2,800 olfactory receptor alleles, and by generating a DNA FISH probe that simultaneously detected most OR loci, this group showed that the silent OR alleles converge to form exclusive heterochromatic foci in a cell type-specific and differentiation-dependent manner (57).

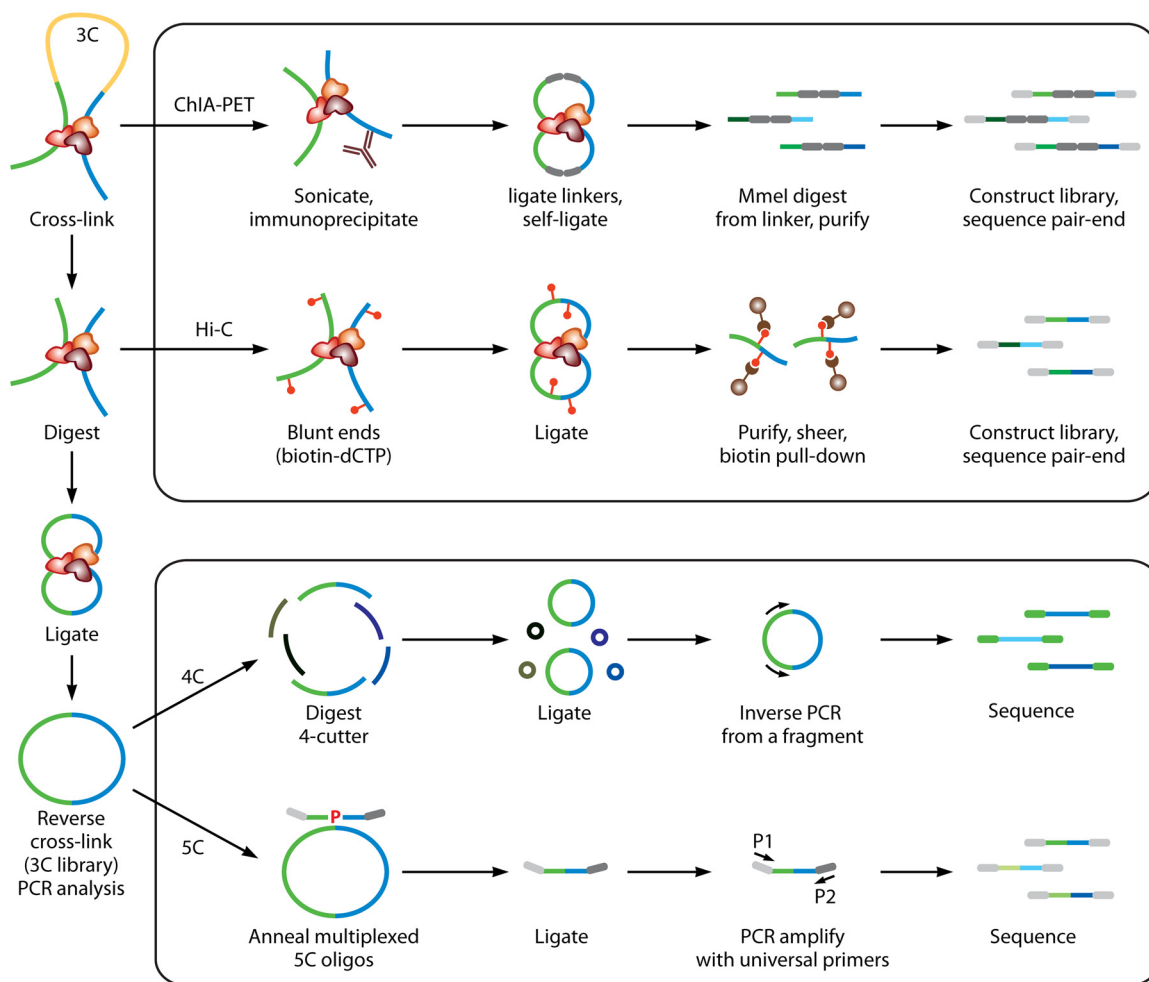
FISH has been combined with live-cell imaging to show that targeted transcriptional activation of a chromosome locus can induce movement from predominantly peripheral to more interior nuclear locations (58, 59) and that chromatin movement is restrained by the nuclear architecture (60). Live-cell imaging involves the incorporation of LacO or TetO arrays into genomic regions of interest and subsequent illumination through the binding of a vector cassette containing LacR/TetR with a fused fluorescent protein. Tagged loci can then be visualized, and their movement can be monitored while cells are maintained under suitable conditions. Live-cell imaging of a whole chromosome has also been achieved by combining LacO/LacI tagging with photoactivatable histones (61). Three-dimensional FISH has been used in conjunction with live-cell imaging and mathematical models to probe chromatin topography at the immunoglobulin heavy-chain locus. This combined approach has elucidated the compartmentalization and large chromatin changes that occur during B lymphocyte development and has generated a strong model for how the widely spread *Igh* coding elements (within three domains over >2 Mb) can frequently interact (62, 63). Spatial confinement of the interacting domain was posited to be the main driver in genomic interactions between the *Igh* coding elements, which is a scenario that we identified by 3D-FISH combined with chromosome conformation capture carbon copy (5C) for increased gene enhancer colocalization (53).

The cryo-FISH technique has been applied for determining the spatial intermingling of interphase chromosome territories (64) and for validating results from 4C technology that identified functionally significant long-range chromosomal interactions (65). Cryo-FISH involves pFA fixation and then embedding of cell pellets in sucrose before cells are frozen in liquid nitrogen. Ultrathin cryosections (150 to 200 nm) can be generated to allow 2D, wide-field microscopy analysis of sequential sections through nuclei with no reduction in *z* resolution while improving the hybridization efficiency and preserving the chromatin ultrastructure.

### Inferring Genome Organization

Whereas distances between genetic loci can be measured directly in single cells by microscopy, the physical proximity of chromatin can also be deduced based on the frequency at which DNA segments interact with each other in cell populations *in vivo*.

This approach is based on the premise that interactions between close regions are more likely to be captured by cross-linking than are those between regions located far away and that the contact frequency over the cell population at a given time essentially reflects how chromatin is organized in the nucleus of individual cells. Genome architecture can be modeled with this type of data by considering the frequency to be inversely proportional to the physical distance. Several molecular techniques are available to quantify chromatin contacts, including 3C and 3C-related meth-



**FIG 2** Inferring chromatin organization. The original 3C method is outlined from top to bottom on the left. Formaldehyde cross-linking captures interactions between DNA segments (blue and green lines) mediated by protein complexes (colored shapes). The chromatin is next digested with a restriction enzyme, and the free DNA ends are joined by proximity ligation before reverse cross-linking and purification. The genome-wide ChIA-PET and Hi-C techniques are related to 3C, and key steps are shown from left to right. The Y-shaped molecule represents antibodies. Biotinylated nucleotides are shown as red dots. Streptavidin beads are shown in brown. The genome-scale 4C and 5C methods indicated at the bottom require the production of 3C libraries, and specific key steps are outlined from left to right. Green arrows represent PCR primers specific to the bait region. 5C primers used during the ligation-mediated amplification step are illustrated with green and blue lines, where the light and dark gray moieties represent universal primer sequences.

ods (3C technologies) and chromatin interaction analysis by paired-end tag sequencing (ChIA-PET). In contrast to FISH, where analysis is performed at the single-cell level, these techniques always capture contacts in cell populations and yield average structure models, with the exception of one study where single cells were analyzed (66).

**3C.** The “chromosome conformation capture” (3C) technique was developed by Dekker et al. >10 years ago (67) and is routinely used to study the organization of short genomic regions at high resolution compared to the resolution of most visual techniques (Fig. 2) (68). During 3C, a population of cells is first chemically fixed with formaldehyde to create covalent bonds between chromatin segments (67, 69, 70). The cross-linked chromatin is then digested with a restriction enzyme, which cuts at specific sites across the genome. The type of enzyme selected defines the resolution of the 3C experiment, with those recognizing palindromes of 4 bp yielding higher-resolution libraries (256 bp) than 6-cutters (i.e., enzymes that recognize 6-bp sequences; 4,096 bp). The di-

gested DNA is next diluted, and ligase is added to join cross-linked fragments pairwise. This step generates unique DNA junctions measurable by various PCR methods (67, 71, 72).

As interactions are measured individually, 3C is generally used for small-scale analysis and was first applied to confirm the Rabl-like organization of chromosomes in *Saccharomyces cerevisiae* (67, 73, 74). It was then used to explain long-range regulation at the  $\beta$ -globin cluster during erythroid differentiation (75, 76) in studies that followed the very first demonstration of enhancer-promoter looping at the rat prolactin gene (77). Long-distance *cis* and *trans* physical contacts have since been found genome-wide and regulate the activity of enhancers at promoters, insulator function, transcriptional silencing, imprinting, and X inactivation (68, 78, 79).

Although the data generated with 3C and its related technologies have largely been corroborated by other methods like FISH, it appears that we still have much to learn about how these methods work (80). For instance, it was originally assumed that dilution of

the 3C reaction mixture prior to ligation was required to favor the ligation of cross-linked restriction fragments. Recent work shows that this is likely not the case, since a substantial portion of the digested DNA remains trapped within the cross-linked nuclei (81). The nucleus-bound DNA was found to actually contribute most of the measured 3C signal, indicating that ligation occurs mainly while the DNA is still bound to the nuclei rather than in solution (81). Dilution of 3C reaction mixtures might therefore simply be required to decrease SDS concentrations prior to ligation. In another study, it was found that enhancer-promoter ligation products actually represent <1% of all the restriction fragments subjected to ligation (82). This result likely reflects the frequency of interactions between regulatory DNA elements *in vivo*; the many different types of products generated at the ligation step; as well as the efficiency, specificity, and stability of the formaldehyde cross-links formed during a 3C experiment (82, 83). Also, we recently demonstrated that data from FISH and 5C are sometimes discordant at high resolution, suggesting that parameters other than distance might influence the interaction frequency (53).

**4C.** The chromosome conformation capture-on-chip (4C) techniques were the first set of methods designed to improve the throughput and resolution of 3C (55, 65, 84, 85). Each technique was developed independently to identify all contacts between a given region and the rest of the genome without any prior knowledge of the contacting domains (68). Generally, these methods work through the generation of very short ligation products between a specific restriction fragment (the “bait” or “anchor”) and the rest of the genome, which are quantified by using either microarrays or sequencing.

The “open-ended” 3C methodology (84) was used to identify HoxB1-associated loci throughout the genome in mouse embryonic stem (ES) cells. This approach identified interacting DNA segments by sequencing of short ligation products amplified from the 3C library by inverse PCR with nested HoxB1 primers. The Hoffman group developed the “associated chromosome trap” (ACT) technique to find genomic domains that interact with the mouse insulin-like growth factor (Igf2)/H19 imprinting control region (86). This method differed from the open-ended 3C technique in that short 3C ligation products were first ligated to linkers before amplification with linker primers and nested Igf2/H19-specific primers. The “circular chromosome conformation capture” approach (85) used the mouse H19 imprinting control region (ICR) as a general model to introduce the technique. It was also similar to the open-ended 3C technique but used custom arrays specific to the 4C libraries to quantify novel chromatin interactions. In contrast, the 4C method (65) used microarrays containing genome-wide probes, which were later phased out in favor of the more sensitive high-throughput sequencing (Fig. 2) (87).

The chromosome conformation capture-on-chip method (65) was the only easily scalable 4C approach. Here, ligation products from a 3C library are further digested with a restriction enzyme that cuts more frequently and are religated into circular DNA. PCR primers designed to face outwards on either side of the bait are then used to simultaneously amplify all interacting fragments, and the amplicons are quantified by deep sequencing. The application of sequencing greatly increased the scale and sensitivity of the 4C assay, allowing genome-wide profiling of interactions with the bait region. No other 3C-related technique can yet generate

comparable high-resolution interaction profiles. 4C was also modified to include an immunoprecipitation step before the first ligation reaction, which enables the capture of chromatin interactions mediated by specific proteins (88, 89).

**5C.** Since 4C elucidates only interactions between a single restriction fragment and the rest of the genome, it cannot be used to predict the conformation of entire domains or chromosomes (90, 91). The chromosome conformation capture carbon copy (5C) method, on the other hand, is suitable for this type of analysis, as it can detect up to millions of 3C ligation junctions between many restriction fragment pairs simultaneously (92–97).

5C was designed to increase the throughput and accuracy of 3C by combining 3C with a modified version of the ligation-mediated amplification (LMA) technique (Fig. 2). For 5C, a series of primers is computationally designed (98) at the restriction site of each fragment in the region of interest. These 5C primers are then pooled with a 3C library where they anneal to targeted 3C fragment ends. Primers located next to each other across the 3C junction are next ligated together by *Taq* ligase, generating new synthetic DNA molecules. This 5C library is amplified by using the common tails present in the 5C primers and is quantified by using high-throughput sequencing. This process results in the interrogation of all chromatin interactions between fragments represented by 5C primers. As the LMA step quantitatively ligates primers onto 3C junctions, the 5C library is essentially a “carbon copy” of existing 3C products.

5C can be used at different scales to probe various biological questions. That it uses predefined primer sets to measure chromatin contacts, however, implies that 5C can measure contacts only for regions covered by the primer library. 5C has been used to study chromosome-sized regions at high resolution (99) by multiplexing large numbers of primers. We determined the physical organization of the human *Hox* clusters in various cell systems with 5C (90, 100–102) and used the data to model spatial chromatin organization computationally (103, 104). Other groups have used 5C to study the alpha-globin cluster (105), the three-dimensional organization of the bacterial *Caulobacter crescentus* genome (106), the regulatory landscape of mouse X inactivation (91), and changes in developmentally regulated chromatin domains (107).

**Hi-C, GCC, and TCC.** The Hi-C technology, sometimes called genome-wide chromosome conformation capture, uses high-throughput sequencing to directly quantify proximity ligation products in contact libraries and therefore can be used to probe the spatial organization of an entire genome (108–110) (Fig. 2). The type of data produced with Hi-C is therefore more comprehensive than what other 3C-type methods usually yield, as was previously described in detail (111). The production of Hi-C libraries is similar to the production of 3C libraries: cell populations are first chemically fixed with formaldehyde, and the chromatin is digested with a restriction enzyme or DNase I, which was recently reported to achieve higher resolutions (112). The restriction fragment overhangs are then filled with Klenow enzyme and a mixture of deoxynucleoside triphosphates (dNTPs) that includes biotin-14-dCTP. This is followed by blunt-end ligation with T4 DNA ligase to join cross-linked DNA fragments, reverse cross-linking, and purification. These unprocessed Hi-C libraries are then sheared by sonication and size selected prior to pulldown on streptavidin-coated beads, which enriches the samples for DNA sequences containing the informative ligation junctions.

Illumina’s paired-end sequencing has so far been the method



of choice to identify the sequences on either side of Hi-C junctions, but longer read lengths will increasingly make single-read-through approaches viable alternatives for Hi-C analysis. Current methods map each side of the sequence reads separately to a reference genome, where the quantities of all valid Hi-C pairs are catalogued in a matrix format spanning the entire genome. Since the complexity of Hi-C libraries is very high, a sizeable amount of sequencing is required. The data are typically binned at different sizes based on sequence depth and library quality. The organizations of large genomes such as those of human and mouse have consequently been described mainly at resolutions ranging from 40 kb to 1 Mb (109, 113), except for a recent study where the Hi-C protocol was modified to achieve kilobase resolutions (114). Hi-C data have been used to correlate genome architecture with several genomic features such as replication timing. It was found that differential firing at origins could be explained by the spatial compartmentalization of origins into different units of three-dimensional chromatin architectures (115, 116). Hi-C was also used to show that the proximity of chromatin correlates well with the incidence of intra- and interchromosomal translocations from double-stranded DNA breaks (117). The generation of very-high-resolution Hi-C data sets is prohibitively expensive at present, and until sequencing costs decrease or alternative Hi-C protocols are developed, approaches such as 4C and 5C remain ideally suited for high-resolution analyses.

Two techniques similar to Hi-C were reported at around the same time as the original Hi-C report and were used to explore the genome organization of the yeast *Saccharomyces cerevisiae*. The “genome conformation capture” (GCC) technique reported a global map of chromosomal interactions in yeast by shearing and directly sequencing conventional 3C libraries (118). Since the majority of the DNA in 3C libraries does not contain any 3C ligation junctions, this approach yielded a small number of usable reads that was nonetheless sufficient to identify contact networks in the small *S. cerevisiae* genome. GCC has since been used to map the spatial organization of the *Escherichia coli* nucleoid (119). Another group developed a 4C-inspired approach, which involved the ligation of biotinylated adaptors to mark ligation junctions (120). This study generated kilobase-resolution maps that were used to construct a three-dimensional model of the yeast genome.

Hi-C itself has been modified into the “tethered conformation capture” (TCC) method, which generates the same chromatin interaction data but boasts higher signal-to-noise ratios (121). The premise of TCC is that random intermolecular ligation products between non-cross-linked DNA fragments are the largest source of noise and appear most frequently as interchromosomal contacts in the data (120, 122). In TCC, the frequency of this event was decreased by biotinylating the cross-linked protein-DNA complexes and tethering them to streptavidin-coated magnetic beads prior to the Hi-C ligation step. This modification was suggested to improve the efficiency of ligation between cross-linked DNA strands compared to that of traditional Hi-C ligation under diluted conditions. Given that most contacts were recently shown to form in the nucleus-bound fraction instead of in solution (81), it will be interesting to see how immobilization of complexes on beads decreases the incidence of nonspecific ligation products. Comparable signal-to-noise ratios have since been obtained with the original Hi-C procedure (113), but the fact that the levels of random ligation products tend to vary significantly between ex-

periments nonetheless highlights the importance of optimizing this aspect of library production to improve read depth (123).

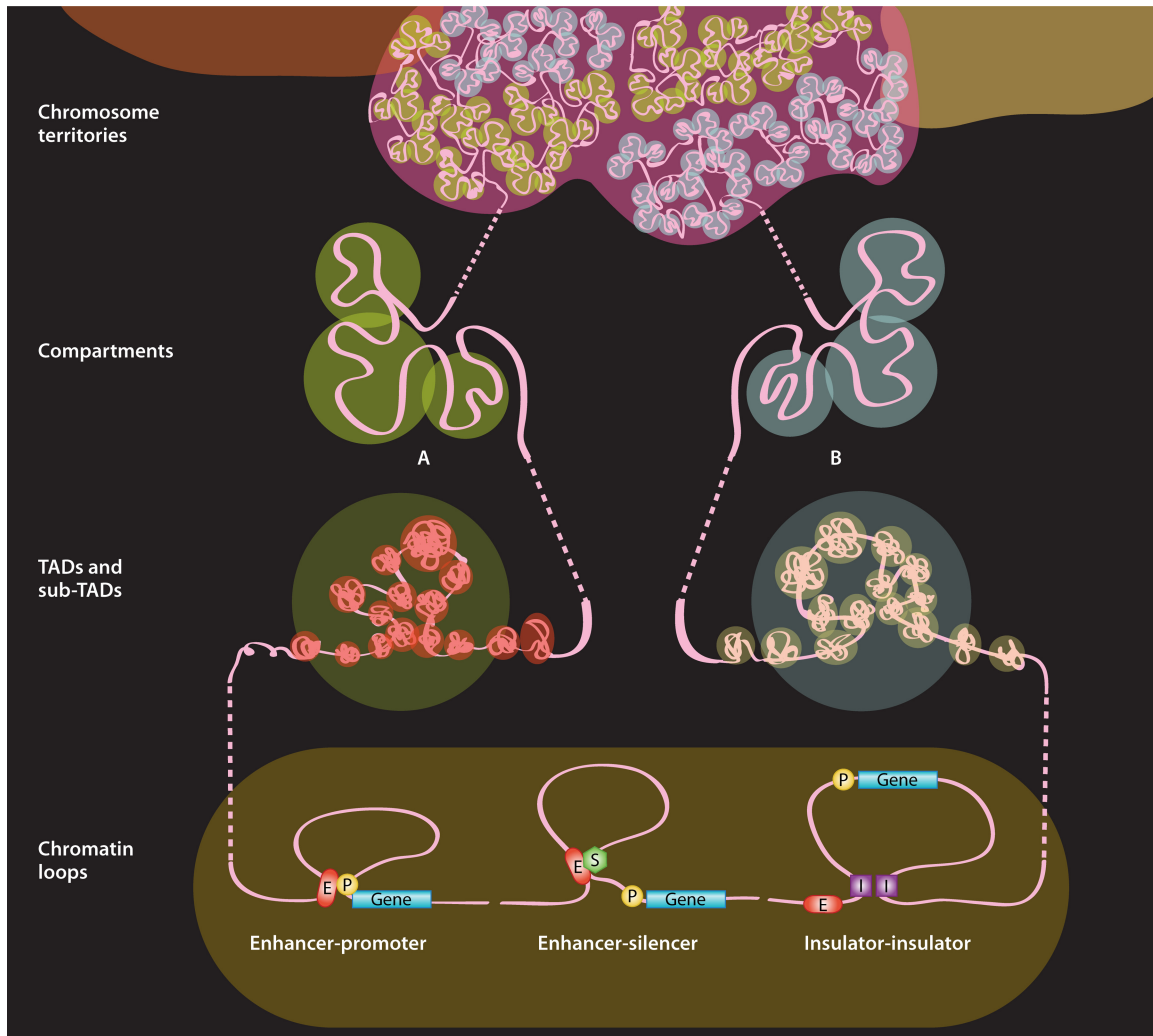
**ChIA-PET.** While Hi-C can be used to identify contacts genome-wide, it does not provide information about the nature or the function of these interactions. The “chromatin interaction analysis by paired-end tag sequencing” (ChIA-PET) method was developed to probe this type of question by mapping chromatin networks associated with specific proteins (124, 125). ChIA-PET is a genome-wide technique that uses a chromatin immunoprecipitation (ChIP) step to isolate interactions between all regions bound by a particular protein. Like Hi-C, ChIA-PET identifies contacts in cell populations fixed with formaldehyde, but the fixed cells are sonicated and used first for ChIP of the protein of interest. Biotinylated DNA linkers are next added at the ends of the coimmunoprecipitated DNA segments, and the resulting cross-linked DNA fragments are ligated together intramolecularly. The ChIA-PET junctions generated by this process are then excised with restriction sites featured in the linkers prior to purification on streptavidin beads and paired-end sequencing. Each half of the ChIA-PET products is finally mapped to a reference genome and joined to reveal the location of protein-mediated chromatin contacts. This method has been used to map networks associated with RNA polymerase II, CCCTC-binding factor (CTCF), and the estrogen receptor genome-wide (124, 126, 127).

#### CHROMOSOME ORGANIZATION IN THE NUCLEAR SPACE

A general model of genome architecture wherein chromosomes are organized in hierarchical length scales has recently emerged (128). From low to high resolution, chromosomes first fold to occupy distinct territories and positions in the nuclear space defined in part by interactions with nuclear subdomains, including heterochromatic regions. Individual chromosomes are then folded into compartments A (open/active) and B (closed/silent) that preferentially interact together, respectively (109) (Fig. 3). Within compartments, the chromatin is packaged in the form of topologically associated domains (TADs), largely conserved between cell types and across species. The chromatin is further folded into sub-TADs, the topologies of which can vary in a tissue-specific manner. Ultimately, genomic DNA is wrapped around nucleosomes, which represents the first level of genome folding; however, how DNA is packaged between the resolution of the 10-nm fiber and sub-TAD scales is still largely unknown. Below, we explore the organization of chromatin across genomic scales, from chromosome territories to individual genes.

#### Chromosome Territories

The work of Carl Rabl and Theodor Boveri suggested long ago that animal interphase chromosomes adopt a form of territorial organization where interchromosomal contacts are minimized. Chromosome territories (CTs) were visualized under a light microscope in these early studies and identified while the movement of DNA during the cell’s life cycle was tracked (73, 129, 130). More specialized approaches using UV irradiation and pulse labeling later supported the existence of CTs by demonstrating the preferential distribution of DNA aberrations within chromosomes (131). FISH has since been used to visualize the location of chromosomes and clearly demonstrates their propensity to form individual domains (132–134). 3C-based data corroborate the existence of CTs. For instance, 4C analyses and genome-wide Hi-C studies capture more intrachromosomal contacts than interac-



**FIG 3** Chromatin organization across genomic scales. The chromatin fiber from one chromosome is unraveled to illustrate four different organization levels described previously in the text. Chromatin conformations are presented from low (top) to high (bottom) resolutions. The chromatin fiber and corresponding chromosome territory are shown in pink. A and B compartments (multimegabase scale) are shown separately to highlight their inherently distinct nature, although there is no evidence that their conformations differ at the level of TADs (megabase scale). Three examples of chromatin looping (submegabase scale) are shown: (i) enhancer-promoter, (ii) enhancer-silencer, and (iii) insulator-insulator. E, enhancer; P, promoter; S, silencer; I, insulator.

tions between chromosomes, even for loci hundreds of megabases apart on a given chromosome (65, 87, 109). Accordingly, Heride et al. demonstrated by 3D-FISH that homologous chromosomes in a diploid cell are far apart from each other (135). Recent work on haplotype reconstruction using Hi-C data supports these findings by demonstrating that chromosome haplotypes in diploid cells do not interact frequently with each other (136).

Although chromosomes mostly keep to themselves, they can considerably interact with other CTs. For instance, contacts between small, gene-rich chromosomes in Hi-C libraries of human lymphocytes were shown to occur more frequently than would be expected based on their size (109). Several loci were shown to loop out of their chromosome territory, coinciding with both an open conformation and active expression and suggesting that the space between chromosome territories might be important (48, 85, 86, 137–141). It is important to note, however, that our understanding of the structure and biology of CTs is derived largely from FISH experiments using probe sets that do not cover entire chro-

somes, and thus, such looping out might sometimes reflect only extrusion from the visualized regions rather than the actual CT (142). Nevertheless, comparison of the FISH signals from conventional whole-chromosome “painting” (i.e., hybridization with fluorescently labeled chromosome-specific probes) to those from exome painting of the entire chromosome revealed that chromatin segments at the surface of CTs are enriched for exons, residing largely away from the more compact CT core, which is consistent with looping out (39). Several groups demonstrated a significant amount of intermingling between different chromosome territories on a cell-specific basis (64, 143, 144), although the extent of these contacts remains an open question. Similarly, interchromosomal contacts between a select set of highly transcribed regions were captured by TCC, and it was suggested that access to the transcription machinery, possibly within transcription factories, can drive the formation of contacts (121). Other studies using genome-wide Hi-C data from mouse and human show that physical proximity prior to chromosomal rearrangement correlates



well with the incidence of translocations genome-wide (117, 145–147).

Chromosomes have preferred radial positions in the nucleus of mammalian cells (132, 148). 3D-FISH and chromosome painting analyses in various cell types showed that chromosomes tend to localize at either the nuclear center or the periphery according to gene density. Human chromosome 19, for example, is a small gene-rich chromosome more frequently found at the center of the nucleus than chromosome 18, which is similar in size but gene poor (149–152). This behavior has been observed for multiple species, including other primates, rodents, cattle, and birds, and thus appears to reflect a general feature of eukaryotic nuclear organization (153–157). Accordingly, Hi-C analysis showed that all small gene-rich human chromosomes interact more frequently with each other than with the similarly sized chromosome 18 (109). Computational modeling of TCC data further indicated that gene-dense chromosomes tend to localize to the center of the nucleus, while a group of gene-poor chromosomes localized to the periphery (121). Other studies argue that transcription activity, chromatin remodeling, replication timing, chromosome size, GC content, and gene density within megabase-sized genomic windows contribute to the preferential position of chromosomes relative to the nucleus center (50, 132, 150, 155, 158–164).

Whether the position of a specific chromosome and its interacting partners is functionally important is unknown. The position of CTs was shown to be cell type specific, suggesting that boundaries shared between a given chromosome and its neighbors, along with their relative nuclear position, might be functionally relevant (161, 165, 166). However, while the CT position tends to be stable within a given cell (167), it can vary significantly from cell to cell, indicating that not all *trans* contacts are required and that an exact nuclear position may not be essential (128). This is supported by the observation that tethering to the nuclear periphery mostly alters the expression of genes in *cis*, with little effect on transcription from other chromosomes, even though *trans* contacts must be altered in these experiments (17). The positions of CTs might be influenced by their internal chromatin organization. Chromosome folding into a CT was suggested to act as a barrier to internal nuclear movement (128, 167, 168), and accordingly, genes embedded deep within a chromosome territory are more difficult to activate (39, 169).

While the function of CTs as a first level of compartmentalization is recognized, there is at least one example where they can serve a very different role. It was found that the CT position in the rod photoreceptor cells of nocturnal mammals is inverted relative to the conventional architecture seen in diurnal animals and most eukaryotic cells (170). In nocturnal retina rod cells, the heterochromatin localizes at the center of the nucleus, and the euchromatin lines the nuclear periphery. Computational modeling of this nuclear organization in the eye indicates that rod nuclei can act as collecting lenses that efficiently channel light in this configuration, thus contributing to an adaptation to the nocturnal lifestyle.

### Chromosome Compartments

The original Hi-C study reported the genome-wide chromatin organizations of two human cell lines at a resolution of 1 Mb. The resulting Hi-C contact matrices displayed a type of checkerboard-like contact pattern where multimegabase regions interact even across large distances along chromosomes (109, 171). Principal

component analysis (PCA) of the Hi-C data was used to segregate contact frequencies into pairwise states, which uncovered the existence of two types of chromosome compartments (109). The open “A” compartments include regions with high GC content that are enriched in genes, transcription activity, DNase I hypersensitivity, and histone modifications associated with active chromatin (H3K36me3) and poised chromatin (H3K27me3). In contrast, B compartments show higher interaction frequencies, a stronger tendency toward self-association, and high levels of the silencing H3K9me3 mark. The position of B compartments was also found to be highly correlated with late replication timing and LADs, suggesting a proximity to the nuclear periphery not observed for A compartments (116).

The segregation of CTs into A and B compartments has thus far been observed for all autosomes and for all mammalian cell types examined. Their distribution along chromosomes is also highly stable across cell types but can vary, pointing to a regulatory role (172). However, contacts within compartments tend to be weak and spread over large groups of restriction fragments throughout the domains, suggesting that compartments may exist only transiently or may even form simply as a consequence of shared features.

### TADs and Sub-TADs

While exploring chromosome organization at smaller scales using 5C and Hi-C, blocks of dense chromatin were identified in human, mouse, and *Drosophila melanogaster*, which interact more frequently within themselves than with neighboring regions (91, 113, 173, 174). The existence of topologically associating domains (TADs) is supported by the finding that FISH probes intermingle more frequently within TADs than between them (91). TADs are clearly visible in 5C and Hi-C data and are defined by sharp changes in the contact frequency from one region to the next. TADs are thought to partition genomes into distinct globular units that can remain spatially distant even if they are adjacent along chromosomes. Perhaps because of their small genome sizes, TADs have not been identified in bacteria or yeast (106, 120). They are also not found in the larger plant genomes, where other types of chromatin domains exist (106, 120, 175).

The first identified TADs had an average size of between 0.5 and 1 Mb and were argued to have distributions that are highly conserved between cell types and species (113, 174). Similar-sized domains had previously been observed by microscopy (176). The domains were visible at the edges between pairs of chromosome territories and were suggested to represent basic CT building blocks (177, 178). The fact that both TADs and CT domains exist at approximately the same scale suggests that they may be the same chromatin structures (128).

The actual molecular makeup of TADs and the mechanisms by which they are formed are the subjects of much investigation. Genes within TADs tend to be coexpressed during differentiation, and the position of TADs correlates well with the distribution of activating and repressing histone marks (91). The boundaries delineating the original TADs were found to be enriched in transcription start sites (TSSs), active transcription and the corresponding histone marks, housekeeping genes, tRNA genes, and short interspersed nuclear elements (SINEs) (91, 107, 113, 174). TAD boundaries are also enriched in binding sites for the architectural proteins CTCF and cohesin (see below). The fact that deletion of the sequence at a TAD boundary results in a partial

fusion of the domains and affects nearby gene expression highlights the importance of this level of compartmentalization in the regulation of genes (91). Also, the observation that the positions of TADs viewed at the megabase scale are largely conserved suggests that sequence-related factors either within TADs or at their boundaries are involved in their formation (179). This is supported by the observation that most long-range gene regulation by enhancers is constrained within TADs (180–182).

High-resolution 5C analysis later demonstrated that TADs are further divided into submegabase-sized structures that are loosely referred to as “sub-TADs” (107). Finer substructures were actually already visible within the original lower-resolution TAD data (113) but were subsumed owing to lower confidence. The average size and position of TADs appear to depend on both data resolution and the parameters used to identify them. TADs are usually defined computationally by the position of their boundaries, identified with a directionality index (DI) (113). The DI is a measure of the difference between upstream and downstream interactions along a chromosome, and boundaries are defined as locations where high contact frequencies shift from downstream to upstream regions. As such, DI data can vary significantly depending on the sliding window size selected. Whereas small window sizes return smaller TADs, larger ones yield larger TADs that often contain groups of smaller domains (183). In fact, when the original Hi-C data from which megabase-sized TADs were first identified (113) were reanalyzed with a different algorithm that uses smaller window sizes, it was found that the larger conserved TADs tend to consist entirely of smaller domains with an average size of 0.2 Mb (183). These domains were stable between cell lines and persisted across resolutions, and their boundaries were also enriched in CTCF binding and activating histone marks.

The finding that TADs can be substratified into smaller domains displaying a pronounced hierarchical organization (183) suggests that topologies from different length scales interact with each other to yield functional genome architectures. However, how domains at the sub-TAD scale relate to each other and the more conserved TADs is unknown. The notion that domains might sequentially interact with each other in progressively larger structures challenges the functional significance of domain classification based on size. In contrast to TADs, chromatin organization at the submegabase scale was found to be more tissue specific and mediated by various protein complexes (107). Whereas invariant subdomains relied on CTCF and the cohesin complex forming long-range interactions, the more tissue-specific enhancer/promoter contacts within and across subdomains required Mediator and cohesin. The unique topological signatures of sub-TADs therefore appear to reflect the levels and types of genomic activities. Whether boundaries at the invariant subdomains are functionally distinct from the ones characterized at larger TADs is unclear, and it will be interesting to see if the structures defined by them are one and the same.

### Chromatin Looping and Looping Out

The finer structures observed at the submegabase scale in high-resolution conformation data highlight the existence of long-range contacts that either form the base of stable domains or are directly involved in regulating processes such as transcription. These long-distance interactions reflect the ability of chromatin fibers to fold into “loops.” Chromatin looping was evident as early as 1878, when Walther Flemming first reported the existence of

“strange and delicate structures” in the nucleus of amphibian oocytes (184). It was J. Ruckert, however, who concluded that they were looped chromosomes, calling them “lampbrush chromosomes” for their resemblance to the bristled brushes then used to clean the soot off oil-burning lamps. A detailed visual and biochemical account of chromatin loops came only some 50 years later through the work of Joseph Gall (185) and facilitated the discovery of DNA loops in human cells, most of which are several orders of magnitude smaller than the very large loops of the lampbrush chromosomes containing upwards of several hundred kilobases of DNA (186, 187).

As it relates to transcription in mammals, the function of chromatin looping appears chiefly to either promote or prevent contacts between gene promoters and regulatory elements, particularly enhancers. Since their initial discovery in viral genomes (188, 189), cellular enhancers have been found genome-wide and are largely responsible for the tissue-specific expression of genes (190, 191). Despite the fact that enhancers and promoters can each initiate bidirectional transcription (192), enhancers can activate transcription in either orientation, whereas promoters cannot. Enhancers can also drive transcription from a position upstream of, downstream of, or within target genes (193). Enhancers are found at various distances from the promoters that they regulate and can work over very long distances in *cis* or even from different chromosomes (56, 86, 141, 194–196). The observation that chromosome territories intermingle significantly in the nuclei of human cells (64) supports the existence of *trans*-regulatory mechanisms, although the functional relevance of most contacts with respect to transcription remains largely undefined.

A striking example of long-range transcription regulation was described for the mouse *Sonic hedgehog* (*Shh*) gene, which is activated during limb development by an enhancer known as the zone of polarizing regulatory sequence (ZRS; also known as MFCS1), imbedded within the intron of another gene located >1 Mb away (197, 198). 3C analysis revealed that *Shh* activation by the ZRS correlates with physical contacts between them (54), suggesting a looping of the chromatin path between the enhancer and *Shh*. A study that combined genetic manipulation of the enhancer locus, transgenics, and 3D-FISH showed that while the 5' end of the ~800-bp ZRS is sufficient to drive an adjacent reporter gene, the 3' end of the enhancer is required for long-range regulation and full expression of *Shh* in the developing forelimbs and hind limbs and, consequently, complete digit sets (199). The *Shh* locus was further observed by 3D-FISH to loop out of its chromosome territory when the gene is active (54). Such looping out suggests extensive unfolding of the locus and should not be confused with chromatin looping *per se*, which in essence refers to proximity between distal regions.

Whether or not looping out of a CT accompanies the formation of chromatin loops during long-range regulation might actually depend on the type of enhancer mechanism used to activate transcription. Indeed, while chromatin looping has been the preferred model to explain how enhancers activate promoters, there are several variations differing in the way in which the enhancer-promoter interaction is established (200). Enhancers have been suggested to use either free or facilitated diffusion to reach their promoters or to use an active mechanism such as “tracking” (201) or “oozing” (202–204). The process used by enhancers to find their targets might significantly impact whether activation is accompanied by looping out of CTs, since active mechanisms like tracking

will invariably transform the chromatin composition, while others involving diffusion might not.

Given that control elements are not necessarily next to each other in the linear genome, mapping of physical contacts is particularly important to define functional connectivity. It was previously thought that enhancers mostly regulate and interact with their nearest gene(s), provided that this interaction does not cross sites bound by CTCF and cohesin (205). A survey of enhancer-promoter contacts assessed by 5C in 1% of the human genome sequence, which identified >1,000 long-range contacts in different cell lines, indicated that this is not likely the case (206). It was found that long-range promoter interactions were often not blocked by CTCF and cohesin binding sites and that enhancers physically interact with the nearest gene in only 7% of cases. Interactions between promoters and sequences were most frequent 120 kb upstream of transcription start sites and were asymmetric, suggesting a directionality of chromatin looping (111, 206). It is important to note that in this study, as in all similar studies, chromatin interaction peaks were depicted as loop configurations, i.e., two contacting regions with the intervening sequence excluded, but that this is not the only possible interpretation, since physical contacts are captured in cell populations by the 3C technologies (207, 208). Peak 3C signals may reflect only the frequent occurrence of interactions in the sample, particularly in the presence of high cell-to-cell variation. Looping conformations within TADs actually have not yet been shown by complementary methods such as FISH.

The facts that one enhancer can have more than one target gene and, conversely, that multiple enhancers can regulate a single gene (190, 209) further highlight the need for mapping of chromatin contacts to understand the functional connectivity of regulatory elements. The  $\beta$ -globin locus provides a good example of this type of complex regulatory network. The  $\beta$ -globin locus harbors multiple  $\beta$ -globin genes that are expressed sequentially and in a tissue-specific manner during development. The  $\beta$ -globin protein produced from this region along with  $\alpha$ -globin together form the two subunits of hemoglobin, which transports oxygen in the blood of mammals. Not surprisingly, disruptions of the  $\beta$ -globin gene or its regulation are known to cause diseases such as sickle cell anemia and  $\beta$ -thalassemia (210, 211).

The  $\beta$ -globin locus and its regulation have been extensively characterized and are highly conserved in human and mouse. The locus spans a region of ~60 kb on human chromosome 11 and mouse chromosome 7 and features an ~15-kb domain upstream of the genes called the “locus control region” (LCR) (212, 213). The LCR consists of numerous enhancers required for proper  $\beta$ -globin expression during development. Specific looping of the LCR and expressed  $\beta$ -globin genes was first demonstrated with 3C in mouse erythroid cells, where chromatin around the LCR preferentially interacted with the active genes compared to brain tissue (76). A closer proximity between the LCR and expressed  $\beta$ -globin genes was also inferred in a separate study of the locus using RNA tagging and recovery of associated proteins (RNA-TRAP) in embryonic liver cells (214).

These data strongly suggested that looping between the LCR and genes was important for transcription. Further analyses showed that insertion of the LCR into a gene-dense region affected transcription at distances of up to 150 kb and was frequently associated with positioning of the LCR outside its chromosome territory (215). Contacts between the LCR and the active  $\beta$ -globin

genes persisted after transcription inhibition, demonstrating that they form independently of RNA Pol II binding (216). Looping between the LCR and its targets at the  $\beta$ -globin locus was later shown to require several protein complexes. Erythroid-specific transcription factors, which include GATA-1, EKLF1, and TAL1, were independently found to be required for looping at the locus (217–220). In one study, the Ldb1 protein complex composed of GATA-1, TAL1, LMO2, and Ldb1 was shown to mediate loop formation (221). The Ldb1 complex is known to promote the transcription of numerous erythroid genes, including Myb, where long-range interactions similar to the ones found at the  $\beta$ -globin locus have been identified (222–224), and might therefore promote loop formation genome-wide in erythrocytes.

Transcription factor complexes do not appear entirely responsible for the chromatin conformation at the  $\beta$ -globin cluster. First, the observation that the locus itself loops out of its chromosome territory prior to gene activation, possibly toward more active regions between chromosomes, suggests considerable changes in chromatin composition during activation (225). Also, in addition to contacts between the LCR and the active  $\beta$ -globin genes, a network of interactions was found to link DNase I-hypersensitive sites from both sides of the locus, the LCR, and the active gene(s) (76, 226). A similar 3D clustering pattern, termed an “active chromatin hub” (ACH), was observed at the active  $\alpha$ -globin locus, suggesting that both hemoglobin components are regulated by a conserved spatial mechanism (105). The ACH is thought to reflect the types of *cis*-acting regulatory elements that come together in the three-dimensional space to coordinate gene expression, and the nuclear compartmentalization provided by the formation of an ACH was suggested to promote transcription irrespective of the surrounding chromatin activity (75, 78, 227). CTCF binds at hypersensitive sites on either side of the  $\beta$ -globin locus and is required for ACH formation. Interestingly, depletion of CTCF destabilized chromatin looping at the  $\beta$ -globin locus and altered its histone acetylation and methylation profiles but did not significantly affect gene expression, pointing to a predominant insulator role in ACH function (228–231).

CTCF looping is also important to control the physical access of enhancers to promoters. Studies have thus far identified many more active enhancers than promoters in the human genome, and contacts between them therefore must be tightly regulated (190, 191). Such regulation might be achieved by compartmentalizing inactive genes away from enhancers by differential CTCF looping, as was described for the apolipoprotein locus (232), or by domain formation at the level of sub-TADs and TADs to insulate and/or alter the three-dimensional path of chromatin.

### Transcription Factories and *trans* Contacts

The physical clustering of actively transcribed genes into “transcription factories” was first observed when nascent transcripts were monitored by pulse labeling in HeLa cells (233, 234). These transcripts were shown to colocalize with RNA polymerase II in foci that also contained splicing and transcription factors as well as chromatin-remodeling enzymes (235–237). The number of foci observed in interphase nuclei ranges from hundreds (238) to a few thousand (239) and appears to depend on both the cell type and the imaging technique used to detect them. In any case, multiple genes can be predicted to share the same transcription factory given that active genes far outnumber the total foci detected at any given time in the nuclei of cells (240). Accordingly, active genes



were shown to colocalize into factories (238), and the transient crowding of enzymes at these sites is thought to enhance transcription and splicing efficiency (241–243).

In some cases, gene activation has actually been found to correlate well with relocalization into transcription factories containing other active genes (225, 238, 244). Although some colocalized genes may be coregulated by the same set of transcription factors (88), there more often appears to be little in common between the genes other than their transcription state. For instance, work on the globin genes shows that they can localize in factories with other active but unrelated genes (65, 105, 238, 245, 246), but they have also been found around splicing speckles (239). Although focal concentrations of RNA polymerase II have been shown to occur transiently and thus may not always represent real factories (241), genome-wide chromatin conformation analysis with TCC supports their existence genome-wide as a cluster of active genes without a shared purpose or function (121).

Long-range contacts are likely to constrain how genes are organized in chromosomes and how chromosomes are positioned in the nucleus. The overall tendency of active gene-rich regions to cluster into transcription factories may thus play an important role in genome organization as a source of chromatin loops and by compartmentalizing coregulated genes (246–248). The extent to which transcription factories may function in genome architecture is unknown, but their limited number and spatial positions were previously suggested to promote self-organization into tissue-specific conformations (249). Regardless of the mechanisms at play, three-dimensional modeling of chromatin interaction data supports a major role for looping in genome organization because of its considerable impact on the entropy of chromatin fibers (250).

### New Insight from Superresolution Microscopy

Several fluorescence imaging methods have overcome the diffraction resolution limit of light (251). Of these, there are three main techniques (required for imaging of internal cell structures): structured illumination microscopy (SIM), stimulated emission depletion (STED), and photoactivation localization microscopy/stochastic optical reconstruction microscopy (PALM/STORM) (252).

While STED and PALM/STORM have been used predominantly to image large protein clusters and organelles located in/on the cell membrane or in the cytoplasm, SIM has been employed to gain increased insight into nuclear ultrastructures and interchromosomal topography. SIM illuminates a sample with a series of high-spatial-frequency stripes, recording a series of frames at different stripe orientations with different shift positions, which results in 9 to 15 frames per final superresolution image (253). It is a hybrid technique: the whole field of view is imaged as in standard wide-field microscopy while the sample is being scanned with the stripe pattern in the manner of confocal microscopy. By illuminating the sample using a striped pattern while rotating the orientation, fluorescing signals can be captured at different times, thus resolving structures that are closer to each other than would otherwise be permitted by the actual light diffraction limit. The lateral resolution limit is halved to ~100 nm, and indeed, the resolution is doubled in all dimensions (252).

At the subnuclear level, SIM was first used to resolve peripheral nuclear ultrastructures such as the nuclear pore complex and the nuclear lamina (254). The Cremer group combined

3D-SIM with 3D-FISH in a proof-of-principle study that showed that key chromatin features were largely well preserved (but with some perturbations) after 3D-FISH down to the resolution limit imposed by 3D-SIM (255). This combined technique, in conjunction with 5C, revealed that adjacent DNA sequences within the same TAD colocalized to a greater extent than did adjacent sequences in different TADs (91). Also, the combination of 3D-SIM and 3D-FISH showed that chromatin decompaction occurs at key differentiation genes and that these genes migrate to the center of the nucleus as neural differentiation progresses during embryonic development (256). 3D-SIM was also used to show a striking difference in the functional organizations of transcriptionally active CTs and the Barr body (257). In a novel approach based on SIM but replacing the single light beam with a lattice light sheet that enabled single-molecule live imaging, Sox2 binding sites were mapped and shown to form discrete clusters in live ES cells (258).

The three techniques described here all have strengths and weaknesses. The decision of which one to use depends on several parameters such as resolution and whether the specimen is fixed or live imaging is to be done. So far, only SIM has been combined with FISH to investigate chromatin ultrastructure in reported studies; however, the other techniques should also be able to provide further insight into chromatin topography, particularly STED microscopy when looking at interprobe distances within compact domains.

### KEY REGULATORS OF GENOME ARCHITECTURE

Despite the fact that radial chromosome positions can vary significantly between generations (34, 167, 259), whether chromatin organization is itself epigenetic at high resolution is unknown, although it was recently suggested that by mutually affecting each other, the chromatin state and architecture take part in a self-enforcing feedback process to propagate cell fate memory (260). Proteins that can both physically shape and regulate the composition of chromatin are thus likely to play important roles in spatial inheritance. The CTCF protein and the cohesin complex are two chromatin components thought to shape the human genome in hierarchical length scales, which have been linked to transcription regulation, imprinting, and X chromosome inactivation.

#### CTCF as a Master Genome Organizer

The CCCTC-binding factor (CTCF) is an essential protein that is highly conserved from fly to human (231, 261, 262). It is known to exert vastly diverse nuclear functions (263), and its functional diversity is thought to originate from the way in which it binds DNA. CTCF binds genomic DNA through a central 11-zinc-finger DNA binding domain with close to 100% homology between chicken, mouse, and human. It can bind to a wide range of sequences by the combinatorial use of its zinc fingers, but most binding sites (75 to 90%) contain a core consensus of 11 to 15 bp. It was postulated that both CTCF and DNA adopt different conformations upon binding to accommodate different zinc finger combinations based on the underlying sequence and that these allosteric shifts determine the kinds of proteins that can bind CTCF (262). CTCF is thus viewed as a “multivalent factor” because it binds to different proteins depending on the sequence with which it interacts, leading to different posttranslational mod-



ifications of itself and surrounding proteins and different functional outcomes (262–264).

CTCF is a vertebrate protein shown to bind insulator sequences directly and to help establish their activity (265). Insulators are DNA elements that control transcription either by stopping the spread of histone marks (266) or by preventing contacts that activate transcription (267). CTCF is known to regulate gene expression by both mechanisms (196, 215). For instance, it can act as an insulator/barrier at heterochromatin boundaries and divide chromatin into silent and active domains (268–270). It is also known for its enhancer-blocking function to repress transcription (271, 272), an activity that likely plays a pivotal role in promoting or preventing long-distance contacts between regulatory elements and target genes.

Highlighting its importance in the regulation of gene expression, CTCF was found to bind >30,000 sites across the human genome, and many of these sites are conserved across cell types and species (269, 273–276). Genome-wide analyses showed that groups of genes within regions flanked by CTCF are likely to be coregulated, in contrast to gene pairs divided by CTCF binding, a characteristic that may be linked to CTCF's ability to demarcate chromatin domains (276, 277). Regions that frequently bind CTCF often contain genes featuring different tissue-specific promoters, suggesting that it might be involved in the complex regulation of such genes (276). CTCF also appears to be involved in the formation of lamina-associated domains, as suggested by its enrichment at LAD boundaries (13) and by ChIA-PET interaction data (126). Together, these results point to a general role for CTCF in genome function, partly by segregating transcriptional activity to specific nuclear areas.

The exact mechanism(s) by which CTCF contributes to insulator function is unknown, but evidence strongly suggests that it involves the manipulation of chromatin architecture (263, 278). Indeed, CTCF was shown to mediate long-range chromatin interactions such as those observed during enhancer-promoter looping (231, 232, 263, 279–282). In fact, CTCF's roles in transcription, imprinting, and X chromosome inactivation could likely be explained mainly by its ability to form long-range DNA contacts and spatially organize the chromatin. In addition, CTCF was found to mediate contacts both within and between chromosomes (85, 86, 283). One of the mechanisms by which CTCF might physically recruit remote sites along and between chromosomes is through its ability to oligomerize (284, 285). Exactly how the protein achieves this is unknown, but CTCF was found to bind asymmetrically across strong topological borders in a manner that predicts the directionality of CTCF-CTCF interactions, suggesting that the types of long-range contacts made by the protein are defined by the position and orientation of binding sites along the linear sequence (114, 286).

One of the most well-characterized CTCF chromatin architectures was identified at the imprinted *Igf2/H19* locus and regulates imprinting. It was found that *Igf2* repression on the maternal allele is achieved by preventing the interaction between the gene and a distal enhancer through the formation of chromatin loops mediated by CTCF (282, 287). In contrast, CTCF binding at the ICR and insulator looping are prevented by DNA methylation on paternal alleles, allowing the *Igf2* gene to contact the distal enhancer by transcription factor-mediated looping. These studies were the first ones to demonstrate cross talk between “classical” epigenetics and spatial chromatin organization (282, 287, 288).

CTCF looping is essential for gene regulation and relevant to human health. For example, mutations that affect CTCF binding at the *H19/Igf2* locus were shown to result in serious human syndromes (289–291). Improper CTCF binding has also been linked to other diseases, such as Huntington's disease, where mutations that destabilize CTCF binding sites appear to cause trinucleotide repeat expansion (292–294).

### Cohesin as a Cell Type-Specific Regulator of Chromatin Organization

Cohesin is another important genome organizer involved in transcription regulation. Cohesin is a multisubunit protein complex composed of the *Smc1A*, *Smc3*, *Rad21*, and *Stag1/2* (*SA1/2*) proteins. It was initially recognized for its role in sister chromatid cohesion, mitotic and meiotic chromosome segregation, and DNA repair (295–297). The first indication that cohesin regulates transcription was the finding that mutations in *Nipped-B* facilitate the activation of the *Drosophila cut* and *Utrabithorax* homeobox genes by distal transcriptional enhancers (298). *Nipped-B* and its human orthologue *Nipped-B-like* (*NIPBL*) are factors required for the loading of cohesin onto the DNA that colocalize with CTCF/cohesin but also bind at independent sites like promoters (299). Mutations in the cohesin *Smc1A* or *Smc3* subunit and in the *NIPBL* gene are responsible for many cases of Cornelia de Lange syndrome (*CdLS*) (300–303). Like CTCF, cohesin was also found to bind thousands of sites in interphase nuclei, but its binding is much more tissue specific (304–308). Cohesin was shown to colocalize with Mediator genome-wide and facilitated enhancer-promoter looping at the *Nanog* gene (304). Although it was originally thought to be important for mouse stem cell maintenance by directly regulating the *Oct4* and *Nanog* pluripotent genes (304), a more recent study indicates that this is not likely the case (309).

Cohesin colocalizes extensively with CTCF throughout the genome. In fact, many of the original CTCF-mediated looping contacts were later found to require cohesin. However, CTCF does not exclusively colocalize with cohesin and vice versa. Also, sites bound by CTCF and cohesin, colocalized or not, can each colocalize with Mediator. Together, these findings point to the existence of functionally distinct CTCF and cohesin complexes that are DNA bound and involved in defining the chromatin architecture. How distinct CTCF/cohesin complexes relate to each other and genes to coordinate architecture and transcription is unknown. However, the fact that CTCF and cohesin were found to be enriched at TAD boundaries suggests that they at least play a role in partitioning the transcriptional landscape of the genome (113).

### Roles of CTCF and Cohesin in TAD Formation

The enrichment of CTCF and cohesin at TAD boundaries is one of the most interesting TAD features and the subject of much scrutiny. While clearly enriched at boundaries, the absolute number of CTCF and cohesin binding sites within the TADs themselves is much greater, suggesting multiple functions for the proteins and/or that TAD structures may be more complex than currently thought (113). CTCF is nonetheless required for proper TAD formation since its depletion results in fewer intra-TAD contacts and in more inter-TAD interactions (299).

If CTCF truly contributes to delineating TADs, it might do so by mechanisms similar to the ones used at heterochromatin boundary sites. Supporting this possibility is one study where deletion of

a TAD boundary led to the formation of contacts across the deleted region and transcription misregulation (91). The interaction profiles of TADs point to higher enhancer-promoter interaction frequencies within domains than between them. Thus, by physically segregating chromatin regions into topological domains, CTCF and cohesin might define functional microenvironments for regulatory elements and target genes where contacts are more easily nucleated while preventing chromatin states from spreading and limiting contacts with the rest of the genome (128, 310). This model is supported by the fact that partitioning of the genome into TADs correlates with enhancer-promoter units, clusters of co-regulated promoters and enhancers (179, 311). Physical modeling of 3C-type data that explored all possible TAD conformations within population-averaged data sets further suggested that contacts between control elements dynamically fluctuate rather than exist as stable structures (208).

The role of cohesin at TAD boundaries and its relationship with CTCF are unclear. In contrast to CTCF, cohesin depletion only reduces the intensity of intra-TAD interactions without affecting the actual TAD location or organization (299, 312, 313), which is consistent with a role for cohesin in mediating tissue-specific enhancer-promoter contacts at the submegabase scale (107). Cohesin is known to be required for CTCF-based insulation (113, 308, 314, 315), and depletion affects insulation patterns genome-wide, correlates with global gene expression changes, and negatively affects hierarchical long-range interactions between TAD boundaries separated by multiple domains (312, 313). These findings collectively suggest that cohesin may organize chromatin in such a way as to prevent interactions between particular TADs and isolate gene expression states from one another (312). The mechanism(s) by which cohesin exerts this regulation on chromatin organization remains unclear but might depend on CTCF.

## FUTURE OUTLOOK

For over a decade, 3C-based approaches have frequently been revised and improved upon to fill certain roles and explore new areas of the genome. 3C paved the way for the second-generation technologies 4C and 5C, which in turn enabled the development of Hi-C and related third-generation technologies. The introduction of Hi-C presents a potentially scalable approach for enabling genome-wide chromatin interaction analyses that can supersede the benefits of first- and second-generation 3C technologies. Even still, is it possible to improve upon Hi-C in much the same way that it improved upon second-generation technologies? What form might the fourth generation of 3C-based technologies take? What else could they be used for?

## Limitations of 3C-Type Analyses

**The challenge of cell populations.** The ultimate goal of studying chromatin conformation is to understand how it behaves at the single-cell level. As discussed above, FISH is perfectly suited for this type of analysis, and the information that it provides is well complemented by 3C-type analyses over large domains and even genome-wide. Both FISH and 3C approaches have previously pointed to significant differences in chromatin conformations between individual cells (14, 148, 167, 316, 317). Variations may stem from multiple sources, including the cellular state and the cell cycle stage.

Perhaps the most overlooked aspect of 3C-type data is the fact that they are derived from populations of cells, and thus, these

data inherently reflect averaged chromatin interaction patterns. This type of data does not provide information, for instance, about the stability or the strength of interactions, whether these interactions occur in all cells, or whether additional parameters such as chromatin composition and flexibility affect the contact frequency. Defining these aspects of chromatin interactions will be essential to distinguish between what actually represents *in vivo* chromatin architecture and the contacts captured by way of the accessibility of chromatin fragments to each other, both of which are likely important in the overall scheme of transcription regulation. Also, during 3C-based analyses, only one ligation event is ever possible for each restriction fragment end such that at most, two different contacts are detectable for each fragment and from each chromosome copy of a karyotypically normal cell. Thus, the observation that contacts can be observed simultaneously for most genomic regions in Hi-C data sets (109, 113) implies the existence of many potential interaction partners and certain variability in chromatin organization. A single study demonstrated this variability by applying Hi-C at the single-cell level (66, 318). Although little information about chromatin architecture is individually contained in these data sets due to the pairwise nature of Hi-C contacts, data pooling recapitulated population-based Hi-C data remarkably well, including the existence of TAD-like structures.

A potentially major contributor to chromatin conformation variability is the cell cycle stage. Cycling populations exist as a mixture of G<sub>1</sub>-, S-, G<sub>2</sub>-, and M-phase (mitotic) cells, where the entire genome is duplicated, folded into metaphasic chromosomes, and unfolded upon cell cycle reentry. Few conformation studies thus far have applied any form of synchronization or sorting to obtain homogeneous cell populations. At least at the megabase scale, interphase chromatin organization appears remarkably stable, since the Hi-C contact profiles of G<sub>1</sub>-, S-, and G<sub>2</sub>-phase and nonsynchronized HeLa S3 cells were shown to be highly correlated (99). In contrast, the chromatin of mitotic cells, which represent ~3% of asynchronous cell populations, was strikingly different, having lost the hallmark plaid interaction patterns of higher-order chromosome compartments and the smaller topologically associating domains. A different study comparing the Hi-C contact profiles of G<sub>1</sub>-sorted and unsynchronized neural stem cells also found a high level of correlation (313). As both studies focused on chromatin organization at larger scales, it will be interesting to see how much change occurs at higher resolutions where more differences would be expected.

**Ploidy.** For genomes such as those of human and mouse, all autosomes exist as two copies, each inherited from separate parents and bearing slightly different DNA sequences. Distinguishing between haplotypes is generally difficult without deep sequencing, since sequence variations are rather small. For this reason, 3C-type analyses usually ignore the diploid nature of the samples, and sequencing reads are averaged for each chromosome. In much the same way, any differences arising from chromosome numbers and structural abnormalities are simply combined and averaged based on a reference genome, although such aberrations are easily identified in the data (see below).

There have been surprisingly few Hi-C studies using haploid cells, unlike 5C, which has been used to model the X chromosome in male and female cells (91, 208). With the exception of one 4C study using single nucleotide polymorphisms (SNPs) to tease sequence reads apart to examine the inactive X chromosome (87),

the chromosome organization in haploid cells has not yet been explored. Such data would be highly valuable for generating accurate chromatin models of individual chromosomes, which could then be used as a reference to help elucidate their structure within diploid cells.

### Improving Genome-Wide 3D Mapping

While Hi-C provides genome-wide coverage, it does so at a cost, and any advance improving sequencing depth will benefit this approach. The sequence complexity of Hi-C libraries is orders of magnitude higher than that of the original genome sequence because of the combinatorial nature of 3C products. This high complexity currently demands very deep sequencing to obtain high-resolution data, and for this reason, techniques such as 4C and 5C remain better suited for analysis at the submegabase scale. If progress in high-throughput sequencing maintains its current pace, it will soon be feasible to generate high-confidence restriction fragment contact maps of Hi-C libraries. Such advances in sequencing depth would also make it possible to further increase resolution by analyzing ultracomplex Hi-C libraries generated with combinations of restriction enzymes or with enzymes that cut more frequently. This type of assay might be necessary to understand the true nature of TADs. Analysis of Hi-C libraries at this resolution scale would require specialized bioinformatics tools, to deal with both the sheer quantity of data and the potential additional biases appearing at ultrahigh resolution (123).

Better genome-wide 3D maps might additionally be improved by amending the Hi-C protocol. First, simply sequencing longer paired-end reads (~100 bp) that map more frequently than shorter reads will produce more Hi-C read pairs. Increasing the quality of Hi-C libraries will also directly influence data resolution. Decreasing the incidence of random ligations, which materializes as higher interchromosomal interaction frequencies and a shift of the *cis/trans* contact ratio, would increase the amount of usable Hi-C data (121). Although the presence of random ligation products can be corrected during analysis (113), eliminating them at the source would improve data quality.

Another way to increase the number of usable Hi-C reads is to introduce a genome capture step in the Hi-C protocol. Most genomes contain regions that cannot be mapped with high confidence because they either are highly repetitive or have low complexity. A large portion of human Hi-C libraries is composed of these regions, and removing them prior to sequencing will yield a larger number of informative reads. Whole-genome capture could be applied by hybridizing all nonrepetitive sequences onto beads or by removing repetitive sequences from libraries. Alternatively, selective genome capture may be performed with specific oligonucleotide to bind Hi-C libraries. The latter approach, considered a fourth-generation 3C technology, has already been successfully used by several groups to study *cis*-regulatory landscapes (capture-C [319]), to identify targets of breast cancer risk variants (capture-Hi-C [320]), and to obtain more detailed maps of the H19/Igf2 and  $\beta$ -globin networks (targeted chromatin capture [321]).

### Alternative Uses of 3C Technologies

**Karyotyping with Hi-C.** Karyotype abnormalities featuring one or more chromosome aberrations are often found in human disease, particularly in cancers. Chromosome anomalies can be numerical or structural and lead to the misexpression of genes. The

large-scale translocations identified in leukemia, for example, can be recapitulated in the production of oncogenic fusion proteins driving uncontrolled cell proliferation. Generally, these types of aberrations can be visualized with a form of chromosome painting called spectral karyotyping (SKY), where each chromosome is represented by a different color (322). Translocations identified with SKY are easily detected by microscopy as molecules containing fluorescence markers from different chromosomes.

While SKY is one of the more accurate techniques used to identify structural abnormalities, its 1- to 2-Mb resolution limit implies that structural defects smaller than 1 Giemsa band will not be detected (322). However, these aberrations are well within the resolution range of 3C-based data and could easily be identified by using this type of technology. The value of 3C-based methods in identifying chromosomal rearrangements was first shown with 4C by examining baits at frequently rearranged sites (320, 323). High interaction frequencies are usually biased toward sites close to each other along the linear genome and are not found between chromosomes in cells with normal karyotypes. Thus, regions displaying very high interaction frequencies must be next to each other on the same DNA molecule. It was shown that 4C contact profiles reflect the position of breakpoints as very high contact frequencies between the bait region and the translocated domain on the other side of the fusion site. Chromosomal rearrangements can therefore be mapped genome-wide at high resolution with Hi-C simply by identifying highly interacting regions that map to different chromosomes on a reference genome (99, 324).

**Genome and haplotype assembly with Hi-C.** *De novo* sequencing and assembly of genomes remain challenging, mainly because the grouping of short reads into “contiguous sequences” (contigs) is difficult. Even though high sequence coverage is now possible with deep sequencing technologies, the small size and generally low quality of the reads render assembly difficult and result most often in fragmented genome maps, particularly for large and repeat-rich genomes (325, 326). Centromeres, telomeres, and other regions rich in repetitive sequences all contribute to this challenge. The approaches currently used to build large-scale genome models from short sequence reads actually rely on preexisting detailed genetic and physical maps like the ones generated by the Human Genome Project. Still, there remain unplaced contigs even in the well-studied human reference genome (327). Analyses of less-well-studied organisms for which no such reference genome has yet been established must therefore rely entirely on the billions of short reads obtained with current deep sequencing approaches.

In 2013, two groups used slightly different approaches to explore the value of three-dimensional chromatin data in defining the positions of contigs along chromosome sequences. In one study, a program named “LACHESIS” (ligating adjacent chromatin enables scaffolding *in situ*) was developed to assemble genomes *de novo* from Hi-C and shotgun sequencing data (324). LACHESIS first assembles contigs by using shotgun sequencing data and aligns Hi-C read pairs onto them. Hierarchical clustering is next applied to classify contigs into chromosome groups, the number of which is initially specified. The location and orientation of each contig within a chromosome are then identified, guided again by the Hi-C read pair information. LACHESIS has 99% accuracy with regard to the order and orientation of contigs, and errors tend to involve regions enriched in duplications and repeats.

This approach works primarily because intrachromosomal Hi-C interactions are more frequent than those between chromo-



somes. Any interchromosomal interactions in the Hi-C data would incorrectly be considered part of the same chromosome and might lead to fusions. It is therefore not surprising that LACHESIS generates such artifacts in the reconstruction of small gene-rich human chromosomes, which are known to interact more frequently with each other than their gene-poor counterparts.

A second group used Hi-C data in a similar way to map the locations of 65 previously unplaced contigs in the human genome (328). This group found that long-range Hi-C interactions between regions located >1 Mb apart were sufficient to place the contigs. A comparison of this approach to another method that uses admixture mapping and SNP data showed a high level of similarity, validating the use of Hi-C (327). Although Hi-C appears well suited to the assembly of genomes *de novo*, the  $1 \times 10^7$  cells required for a Hi-C experiment may not always be feasible and limit its use (324, 329). More recently, however, Marbouty et al. developed a metagenomic chromosome conformation capture approach called “meta3C,” which can be used to define the average chromosome organization of known and new microorganisms in complex populations (330).

Because chromosomes fold into distinct territories, all of them, including homologues, should remain physically separated from each other, and 3C-type chromatin contacts should primarily derive from within individual chromosomes. If this were the case, separation of homologous pairs would be possible by using Hi-C and whole-genome sequencing data (136). By using Hi-C data of hybrid mouse embryonic stem cells with a known haplotype, it was shown that only 2% of the intrachromosomal interactions actually derive from between chromosome copies. A total of 99.5% of the known cell haplotypes could be reconstructed from only the Hi-C data by using a modified version of HapCUT tuned to deal with Hi-C data (136, 331). This included correctly linking haplotypes across metacentric centromeres, rendered feasible by the larger insert sizes of the Hi-C read pairs. These studies demonstrate previously unrealized uses for Hi-C beyond 3D chromatin organization.

## CONCLUSION

Work from around the world altogether paints the human genome as a complex molecular machine performing a myriad of functions that is built on a four-letter language. Everything happening within cells is ultimately derived from this simple language. Regardless of what came first, a change in what binds to the DNA or in how the chromatin folds, it is now clear that chromosome organization both reflects and guides transcription regulation. Understanding the chromatin structure–function relationship might therefore require a change in the types of questions that we ask, from what the roles of contacts are to how they are formed. A closer look at what leads to given structures might indeed point to which control mechanisms could explain the RNA quantities measured in cells. The only nontrivial issue remaining is that we do not yet know how to read these mechanisms from 3C-type data. Combining 3C-based approaches, paired or not with genome capture, with other types of epigenomics data might help bridge this knowledge gap and reveal fundamental principles underlying genome structure and function.

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