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Evolution of 3D Chromatin Folding

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Abstract

Studies examining the evolution of genomes have focused mainly on sequence conservation. However, the inner working of a cell implies tightly regulated crosstalk between complex gene networks controlled by small dispersed regulatory elements of physically contacting DNA regions. How these different levels of chromatin organization crosstalk in different species underpins the potential for genome evolutionary plasticity. We review the evolution of chromatin organization across the Animal Tree of Life. We introduce general aspects of the mode and tempo of genome evolution to later explore the multiple layers of genome organization. We argue that both genome and chromosome size modulate patterns of chromatin folding and that chromatin interactions facilitate the formation of lineage-specific chromosomal reorganizations, especially in germ cells. Overall, analyzing the mechanistic forces involved in the maintenance of chromatin structure and function of the germ line is critical for understanding genome evolution, maintenance, and inheritance.

1. INTRODUCTION

3D genome: higher-order genome organization in the 3D space of the nucleus

Comparative genomics: systematic comparison of the similarities and differences of biological information derived from whole-genome sequences within and across species

Homologous syntenic blocks (HSBs): genomic regions where the gene order has been conserved among species

Evolutionary breakpoint regions (EBRs): genomic regions where chromosomal reorganization has disrupted synteny

Genome organization across deep divisions in the Tree of Life is highly diverse. Understanding of why some species have stable genomes, whereas others have undergone extensive rearrangement across evolutionary timescales, has puzzled biologists since the first microscopic description of chromosomes (1). Compelling evidence suggests the role of different factors associated with genome plasticity, including intrinsic genomic features such as repetitive elements (making DNA more susceptible to genomic reshuffling) together with functional constraints (i.e., gene expression related to species-specific phenotypes) (2–7). This coupled with the highly dynamic state of chromatin is key for determining mammal genome plasticity (8–10).

Exploring divergent patterns of chromatin conformation interactions (**Figure 1**) across taxa is fundamental for understanding the mechanism(s) responsible for the evolution and plasticity of genome architecture. Distant loci within the genome interact during the cell cycle in 3D space (10–12). This spatial compartmentalization into regulatory neighborhoods permits a fine-tuned genome regulation while maintaining genome stability. Yet, little is known about the evolutionary dynamics and mechanisms of 3D genome inheritance during species diversification.

Here we provide an overview of chromatin organization across the Animal Tree of Life. In the context of the mode and tempo of genome evolution, we introduce general aspects of the hierarchical 3D genome organization to later explore the role of chromosome folding in large-scale genome reorganizations (genome reshuffling). We highlight how diversification within the Animal Tree of Life was accompanied by evolutionary innovations that were permitted to accommodate the regulation and stability of large genomes. Moreover, we propose that chromatin remodeling in germ cells played a critical role in shaping lineage-specific trends of genome evolution.

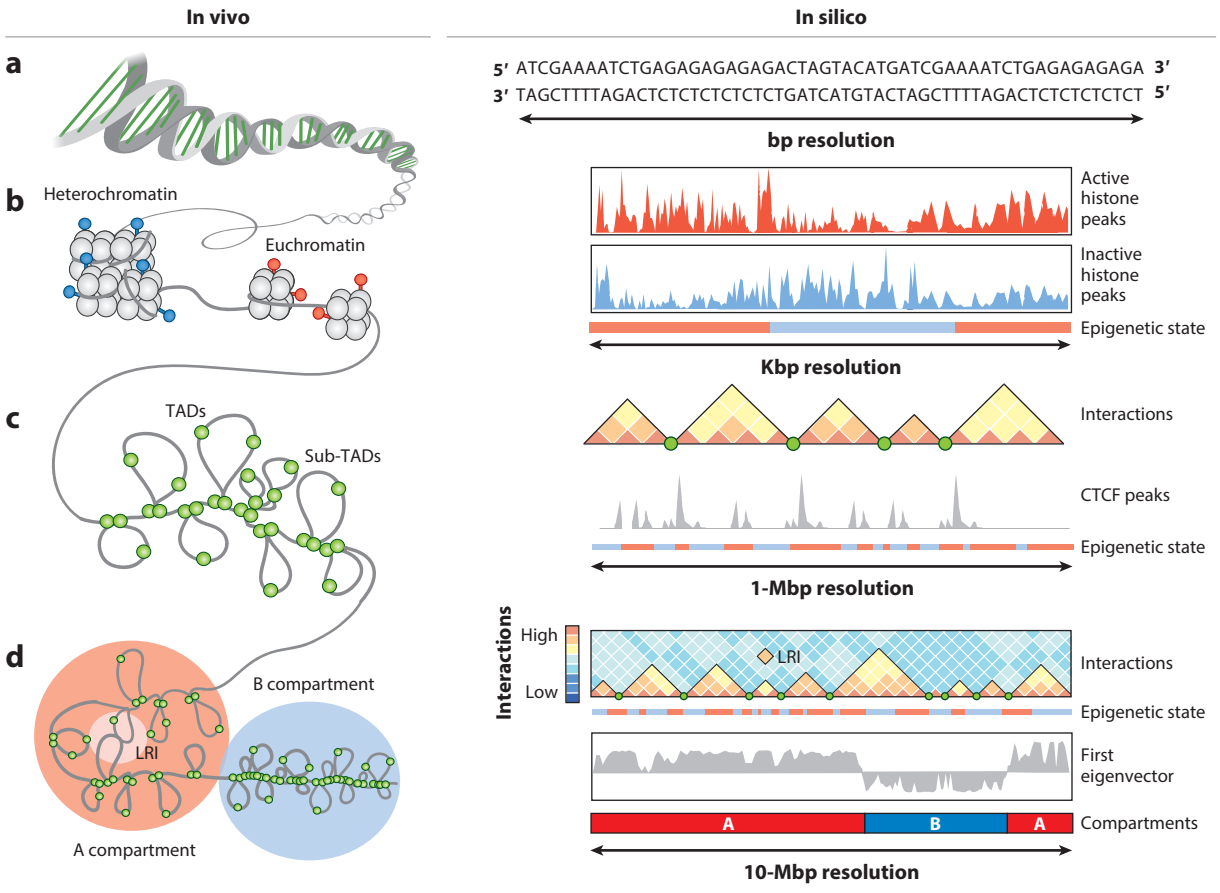
2. MODE AND TEMPO OF GENOME EVOLUTION

Organisms exhibit a wide range of genome sizes, from the small genomes found in some bacteria (i.e., few hundreds of kbp) to larger sizes in animals (i.e., 40 Gbp in the African lungfish) and plants (i.e., 149 Gbp in the Japanese canopy) (13, 14). This diversity is the result of a complex interplay between genetic, ecological, and evolutionary factors (14). Whereas in some vertebrate species the accumulation of repetitive elements and noncoding DNA sequences played a role in genome expansion over evolutionary time, other lineages experienced reductions in genome size since recent common ancestors as genes became redundant or nonfunctional (14, 15). In all cases, chromosomal reorganizations, or CRs (fusions and fissions, reciprocal and nonreciprocal translocations, and inversions), have shaped genome architecture through evolutionary time. Thus, fluctuations in structure hold critical insights into adaptation, speciation, and evolutionary strategies, making the study of genome architecture one of the most fascinating aspects of evolutionary biology.

2.1. Leveraging Whole-Genome Sequences to Reconstruct Ancestral Genomes

Comparative genomics studies of both closely and distantly related species allow the identification of genomic regions implicated in CRs across evolutionary timescales. In this context, genomic reconstructions of regions of homology (homologous syntenic blocks, HSBs) across lineages permit an accurate estimation of rates of genome reshuffling together with the identification of genomic features associated with these evolutionary changes (evolutionary breakpoint regions, EBRs) (16–18). Therefore, defining rates of CRs can be instrumental for studying the phylogenetic relationships between different taxonomic groups (phylogenomics), as it permits distinguishing between derived (synapomorphy) and ancestral (symplesiomorphy) chromosomal forms (3).

Initial comparative cytogenetics within mammalian species permitted the reconstruction of ancestral karyotypes for different phylogroups, including Marsupials (19), Carnivora (20),



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

The hierarchical 3D genome organization. Representation of the different layers of 3D genome organization described in animal species. Diagrams on the left represent in vivo structural features, whereas diagrams on the right represent the in silico patterns that can be defined using Hi-C and ChIP-seq data. The combined use of high-throughput sequencing technologies such as Hi-C, RNA-Seq, and ChIP-seq has revealed different hierarchical levels of genome organization. (*a*) The DNA double helix is wrapped around histones forming the chromatin fiber, which (*b*) can be found in two different states (heterochromatin or euchromatin) depending on its accessibility and histone modifications. (*c*,*d*) The chromatin fiber is folded into TADs and sub-TADs that can be contained in either A or B compartments. (*d*) Within compartments, LRIs between distant TADs can also be stablished. (*e*, *i*) Commonly, chromosomes are positioned within the nucleus in the so-called chromosome territories. However, recent studies in nonmodel species (9, 73, 109) have revealed different configurations characterized by the presence of interchromosomal interactions: (*ii*) centromere clustering, (*iii*) centromere–telomere clustering, and (*iv*) telomere clustering. Abbreviations: CTCF, CCCTC-binding factor; LRI, long-range interaction; TAD, topologically associated domain.

Perissodactyla (21), Cetartiodactyla (22), and Primates (23, 24), among others. All these studies led to a robust hypothesis (based on data from more than 100 mammalian species) that the ancestral Boreoeutherian karyotype consisted of 23 pairs of chromosomes (25–27). But the advent of next-generation sequencing technologies expedited comparative genomic studies, making it possible to define patterns of genome evolution at an unprecedented level of resolution. The combination of long read– and short read–based sequencing methods and liked reads has facilitated the generation of de novo genomes in relatively short periods of time (28), boosted by the assemblage of large international consortia (29–32). In conjunction with population genomic data, this exceptional number of genomic resources is currently allowing researchers to tackle unanswered questions related to biodiversity monitoring, conservation, and restoration efforts (33, 34).

The availability of whole genomes of representative species of the Animal Tree of Life has permitted modeling of ancestral genomes spanning at least 690 million years of evolution since the multicellular progenitor of animals (35–37). The animal phyla (Metazoa) encompass five lineages, the evolutionary relationships of which are far from resolved (38, 39). These include bilaterally symmetrical animals (i.e., vertebrates and invertebrates), cnidarians (i.e., jellyfish and anemones), placozoans (flat marine and free-living animals), and sponges and ctenophores (a group of gelatinous zooplankton). Despite the uncertainty in reconstructing early branching of the group, emerging comparative genomics are providing new hypotheses on the minimal set of ancestral chromosomal linkage groups (ALGs): (*a*) at least 16 ALGs in the last metazoan common ancestor, (*b*) 19 ALGs in the cnidarian ancestor, and (*c*) a slightly rearranged 16 ALGs for the bilaterian ancestor (36) (**Figure 2**; see **Supplemental Table 1**).

Extant cnidarian species have maintained small ancestral genomes with tiny and compacted chromosomes, with essential gene regulatory regions and proximal *cis*-regulatory regions (i.e., short intergenic distances) (36). In the case of Bilateria, however, diversification of the branching leading to vertebrates ~490 Mya was accompanied by at least two rounds of whole-genome duplications and allotetraploidization (37, 40, 41). This resulted in a highly reorganized ancestral vertebrate genome consisting of 54 chromosomes (41). Further genome reshuffling led to an estimated ancestral genome of 49 chromosomes in the Amniota (sauropsids and mammals) ancestor approximately 310 Mya (41) (**Figure 2**).

As for amniote lineages, examination of extant species suggests lineage-specific trends of genome evolution, from generally conserved genomes in sauropsids (including birds and non-avian reptiles) to a high diversity of diploid numbers in mammals (26). Sauropsids are further characterized by having their genomes organized into two types of chromosomes with distinct features: large macrochromosomes and tiny GC- and gene-rich microchromosomes (42, 43). Recent sequence comparisons between birds and reptiles suggest that bird microchromosomes best represent the ancestral amniote (44). In fact, syntenic homologies between microchromosomes

Supplemental Material >

Ancestral genomes: reconstruction of ancestral order of genes in a common ancestor of a given set of species

Whole-genome duplications: duplications of the entire set of chromosomes within a cell

Allotetraploidization: generation of four sets of chromosomes derived from the hybridization of different species

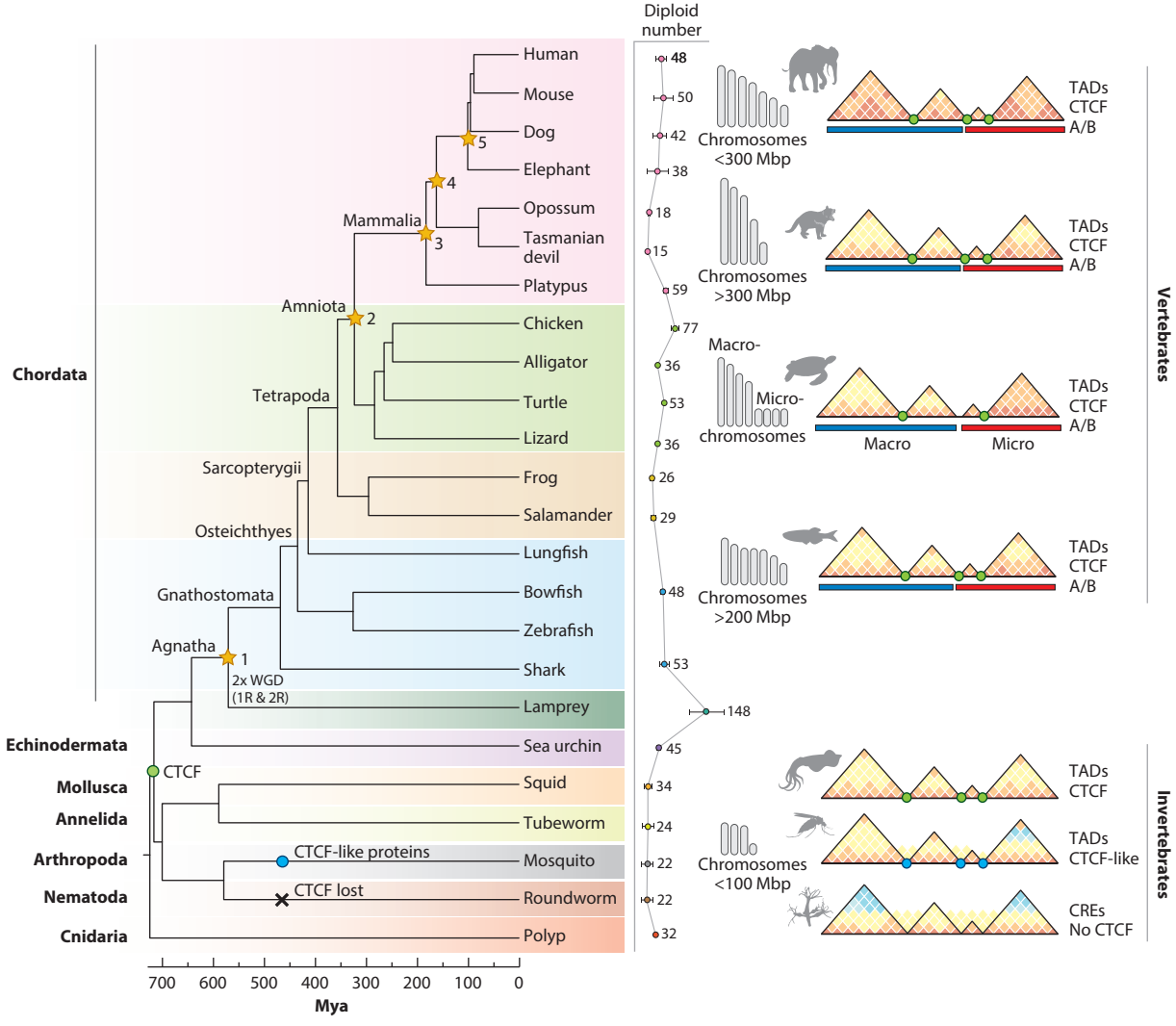


Figure 2

Diversity of genome architecture across the Animal Tree of Life. Schematic representation of the diversity of key genomic structural features across the Animal Tree of Life. Phylogenetic groups are color coded, including Cnidaria, Nematoda, Arthropoda, Annelida, Mollusca, Echinodermata, and Chordata [fishes (*blue*), amphibians (*yellow*), sauropsids (*green*), mammals (*pink*)]. Species representative for each group with reported Hi-C data are shown (see **Supplemental Table 1**). The phylogenetic tree was generated using data from Kumar et al. (40). Divergent nodes from which ancestral genomes are known are depicted with a yellow star (5, 41). Median diploid numbers (along with the standard deviation) are shown for each phylogroup (data extracted from 148). The increase in diploid number is accompanied by the appearance of the characteristically organized 3D structure of vertebrates. Invertebrates have low diploid numbers and a less defined 3D structure caused by the absence of CTCF in some groups. Conversely, vertebrates' 3D organization is represented by well-defined TADs, A/B compartments, and higher diploid numbers. Abbreviations: CRE, *cis*-regulatory element; CTCF, CCCTC-binding factor; TAD, topologically associated domain; WGD, whole-genome duplication.

and the small chromosomes of amphioxus (the lancelet, an early-branching chordate) suggest an ancient origin of these chromosomes of at least 690 Mya (37).

Supplemental Material >

In the case of mammals, the diversification within the group ~180 Mya was accompanied by a high degree of genome reshuffling (an average estimated rate of 3.9 EBRs per millions of years) (5).

This resulted in a wide range of diploid numbers and chromosome morphologies, ranging from $2n = 6/7$ in the Indian muntjac to $2n = 102$ in the red viscacha rat (26). This trend suggests that from an ancestral vertebrate genome with a high number of chromosomes organized into macro- and microchromosomes (41, 44), mammalian evolution resulted in the formation of highly reorganized macrochromosomes, with an ancestral diploid number of 40 chromosomes (5) (**Figure 2**).

2.2. Drivers of Evolutionary Reshuffling

Given the diversity of evolutionary trends across the Tree of Life, what could have driven extensive genome rearrangement across evolutionary timescales, especially in the case of mammals? Initial ancestral mammalian genome reconstruction permitted modeling of the mode and tempo of genome evolution (5, 6, 16–18, 26, 27, 45, 46). These studies revealed that evolutionary CRs that disrupt regions of synteny are clustered in regions that are more prone to break and reorganize across evolutionary time, although rates of reshuffling can vary among phylogroups (16, 17). Importantly, the occurrence of CRs can be accompanied by changes in gene expression of key genes that might provide selective advantage through the development of new adaptive characters relevant to specific mammalian lineages (6, 16, 45, 46). As a result, genomes can be considered as a mosaic of highly conserved functional regions and fragile unstable regions prone to reorganization (6, 18). In this context, HSBs can act as functional and regulatory units that have remained unchanged through evolution because they contain genes that are essential for development and cellular function (16, 45). Conversely, EBRs demarcate fragile and unstable regions that can provide novel grounds for genetic variation in a lineage-specific manner (4, 5, 18).

Overall, evidence supports that the genomic distribution of mammalian EBRs has a multifactorial basis, which can involve repetitive elements (i.e., turning DNA more susceptible to chromosomal change) (7, 18, 27, 47); functional constraints (i.e., genes related to species-specific phenotypes) (8, 16, 45, 46); and, more importantly, the way in which genomes are folded inside cells and its effect on gene function and regulation (8, 48). This view was encapsulated by the Integrative Breakage Model (IBM) (6), which postulates that chromatin conformation could influence the permissiveness of some genomic regions to undergo chromosomal breakage and genomic rearrangements. The IBM (6) presents a balanced scenario where the presence of not only repetitive sequences but open chromatin states together with chromatin accessibility can predispose some genomic regions to undergo double-strand breaks (DSBs) and therefore be resolved as CRs. These genomic features are extremely dynamic and vary depending on cell type and cell cycle. Therefore, understanding the cellular and chromatin context in which DSBs occur and are repaired is key to better understanding the processes that contribute to CR formation and subsequent fixation within populations. Recent comprehensive comparative studies combining 3D genome structure with the reconstructions of ancestral karyotypes for marsupials, afrotherians, and rodents have permitted the identification of different patterns of genome reshuffling and lineage-specific CRs across mammalian phylogroups that could be related to different trends in chromosome folding configurations (8, 9).

3. MULTIPLE LAYERS OF 3D GENOME ORGANIZATION

3.1. The 3D Genome: An Overview

But how does two meters of DNA (in the case of human cells) fit into a nucleus of a few micrometers? The answer lies in a complex chromatin structure, the regulation of which depends on several superimposed layers of organization: (*a*) chemical modifications of the DNA,

(*b*) nucleosomes with DNA wrapped around core histones (which can be also modified), and (*c*) the high-order chromatin organization inside the nucleus (**Figure 1**).

Histone modifications (i.e., deacetylation, acetylation, and methylation) constitute the very first layer of genome remodeling and regulation, defining highly dynamic chromatin contexts and epigenetic states (active, inactive, and poised chromatin) (49, 50). Advances in high-throughput chromosome conformation capture–based methods (i.e., Hi-C) have revealed that the 3D chromatin structure is complex and highly dynamic, including different levels of organization: (*a*) topologically associated domains (TADs) and DNA looping interactions established and maintained by structural proteins including cohesins and the CCCTC-binding factor (CTCF) at the kilobase pair level (11, 51); (*b*) open/active (A) and closed/inactive (B) compartments at the megabase pair scale; and (*c*) chromosomal territories (CTs) distributed within the interphase nucleus (**Figure 1**).

Chromatin fibers can be topologically organized as DNA loops and TADs, which can range from 40 kbp to 3 Mbp in size in the human genome (11). TADs can be defined as self-interacting genomic regions that act as functional and structural units. In fact, TADs facilitate enhancer–promoter interactions in the 3D space and prevent crosstalk with neighboring regions (52–55) (**Figure 1**). TADs can be quantitatively defined by two features: (*a*) an increase of chromatin interactions within TADs and (*b*) insulation of their boundaries (insulation score) (51). Other structural features, such as sub-TADs and DNA loops, are normally detected at high resolutions. TADs are considered an inherent property of mammalian genomes, as they are maintained across different cell types and species (12, 52, 56). TAD boundaries tend to be enriched for insulator proteins such as CTCF (a zinc finger protein that binds to a conserved 20-bp consensus motif) (57, 58), active transcription marks such as H3K4me3 and H3K36me3, nascent transcripts, housekeeping genes, and repetitive elements (52, 59). Although CTCF can be found at most TAD boundaries, it can also mediate long-range genomic interactions (LRIs), connecting promoters with distant regulatory elements of the genome (11, 60). In fact, the depletion of CTCF and cohesins is translated into a general attenuation of TADs, although some degree of 3D chromatin organization is still detected (61, 62), suggesting the existence of additional mechanisms in the formation of the 3D genome structure. In line with this view, it has been suggested that both active transcription and RNA polymerase deposition can also remodel chromatin (63–65).

At the megabase pair scale, chromatin is organized into genomic compartments that can contain several TADs (52, 55, 66). Computationally, compartments are provided by the first principal component of Hi-C interaction matrices and can be captured by the correspondent eigenvector, which discriminates between interaction frequencies (51, 67) (**Figure 1**). Overall, chromosomes are organized into two types of compartments [A (open) and B (closed)] that can vary from 1–10 Mbp in mammals (68) down to 15 kbp in *Drosophila* (69). Compartments correlate with the transcriptional state of the chromatin following the classical description of euchromatin (A compartments) and heterochromatin (B compartments) (70). A compartments are defined as genomic regions of high genomic interaction and often coincide with gene-rich, transcriptionally active regions and active histone marks such as H3K9ac, H3K27ac, H3K36me3, H3K79me2, and H3K4me1/3 (11, 71). Conversely, B compartments correspond to genomic regions with low interaction frequencies and are positively correlated with low gene expression levels and inactive histone modifications such as H3K27me3 and H3K9me2/3 (52, 72).

Beyond compartments, chromatin is ultimately organized into chromosomes, organization and positioning of which within the nucleus in CTs can vary depending on the cell type and the evolutionary origin of the studied species (9, 44, 73). Seminal studies by Carl Rabl and Theodor Boveri initially proposed the territorial organization of chromosomes (74), but it was not until later

Topologically associated domains (TADs): self-interacting genomic regions obtained from Hi-C data

Cohesins: ring-shaped protein complex that mediates sister chromatid cohesion, homologous recombination, and DNA looping

CTCF: zinc finger protein implicated in myriad regulatory functions, including transcriptional activation/repression and DNA looping

Chromosomal territories (CTs): specific regions of the nucleus preferentially occupied by particular chromosomes

Long-range genomic interactions (LRIs): regions of high nuclear interactions; can occur intra-chromosomally between regions of the same chromosome or inter-chromosomally between different chromosomes

that FISH (fluorescence in situ hybridization) techniques allowed the visualization of CTs using fluorescence microscopy (75). Later on, the combined use of chromosomal probes and oligopaints in 3D-FISH approaches together with high-resolution microscopy permitted delineation of patterns of nonrandom distribution of CTs and chromosomal subregions within nuclei (76). Current Hi-C data allow the analysis of chromosomal territoriality using two types of interactions: (*a*) intrachromosomal (*cis*) interactions within the same chromosome and (*b*) interchromosomal (*trans*) interactions occurring between different chromosomes (68). In mammalian cells, intrachromosomal interactions are normally higher than interchromosomal interactions due to the territorial nature of their chromosomes (11, 12, 73). In fact, it has been extensively reported that CTs are nonrandomly distributed within the nucleus because their organization plays a key role in maintaining genome stability and gene expression, facilitating specific interactions between genomic regions (73, 77). Thus, one of the main factors influencing territoriality is chromosome size, as large chromosomes tend to occupy peripheral positions, whereas small chromosomes are orientated toward the center of the nucleus (78). This radial disposition is further influenced by gene density and replication dynamics, as peripheral chromatin replicates later in S-phase than internal chromatin (79).

Given the relevance of the 3D chromatin structure in demarcating the limits of gene-regulatory domains, architectural disturbances would represent a means for rapid (or nongradual) change in gene expression over evolutionary timescales. It is critical then to understand the evolutionary plasticity and function of higher-order vertebrate genome organization, and how this is transmitted to the offspring to ultimately define patterns of evolution. In this context, is genome plasticity a conserved feature in different vertebrate linages? To answer this question, it is important to consider the different levels of resolution in the hierarchical 3D genome organization, from chromosomal occupancy to TAD conservation at the kilobase pair level of resolution.

3.2. Chromosomal Size Modulates Patterns of Chromatin Folding Across Species

The initial view that the hierarchical 3D genome organization is conserved across mammals was based on the study of few boreoeutherian species (human, mouse, dog, macaque, and gibbon) (11, 12, 52, 56). But, because mammalian phylogeny is highly diverse [including more than 6,000 estimated species encompassing ~180 million years of evolution (40, 80)], the analysis of ancestral and highly divergent mammalian orders is key to gain deeper insights into patterns of chromosome folding, especially at the root of the three major lineages of mammals, including Prototheria (monotremes), Metatheria (marsupials), and Eutheria (Afrotheria, Xenarthra, Laurasiatheria, and Euarchontoglires) (40, 80). Recent reports on representative species of chordates, plants, and fungi have revealed at least four patterns of nuclear architecture at the scale of whole chromosomes (9, 73): (*a*) CTs in most vertebrate species; (*b*) telomere-to-centromere axes in frogs and some fishes; (*c*) centromere clustering in marsupials, mosquitos, and sea urchins; and (*d*) telomere clustering in some mollusks (**Figure 1**). In the case of mammals, comparative 3D genomics studies (9) have reported different features of genome organization, including the existence of divergent patterns of chromosome occupancy across taxa and the presence of different degrees of structural conservation among species.

Although mammalian species have equivalent genome sizes [~3.5 Gb (15)], average chromosome size and diploid numbers are diverse across phylogroups (26) (**Figure 2**). This diversity is especially relevant between eutherians and marsupials, which shared a common ancestor circa 190 Mya (81), following distinct genomic evolutionary trajectories thereafter. Marsupials are characterized by low numbers [$2n = 14–22$ (82)] of large chromosomes (>500 Mbp in

size), larger than any other mammalian phylogroup. Importantly, chromosomal size might impose structural constraints, as marsupials' chromosomes exhibit low intrachromosomal and high interchromosomal interactions, accentuated by centromere clustering associated with a Rabl-like chromosomal configuration (9) (**Figure 2**). These observations, together with the detection of low CTCF genomic density (83), suggest that long marsupial chromosomes form a floppy distribution that extends across the nucleus, with presumably lower numbers of longer DNA loops anchored by their centromeres that are orientated toward the center of the cell. Eutherians, on the other hand, have relatively highly compacted chromatin and medium to short chromosomes [average size of ~200 Mbp (15)] organized in CTs, characterized by high intrachromosomal and low interchromosomal interactions, which correlate with high CTCF densities (9) (**Figure 2**).

Rabl-like configuration: clustering of centromeres in nuclei, observed mainly in species with low chromosome number

Initial structural models showed that chromosome folding in somatic cells follows a globular fractal model, where chromatin is organized in a sphere-like structure, with genomic regions folding and interacting in a pattern that repeats itself at different genomic scales (51, 84). Under this model, chromatin reaches an equilibrium state when chromatin entanglements are reduced to a minimum. In this context, the contrasting patterns of chromosome folding detected across mammalian species suggest that, in the case of species with long chromosomes (i.e., marsupials), the state of equilibrium can be reached with lower intrachromosomal interactions. This reduction of interactions can be translated in longer chromatin loops and therefore in less subloops within them.

Nonmammalian vertebrates, however, exhibit variations of this configuration, showing clustering of small chromosomes (microchromosomes) toward the center of the nucleus. Amniote vertebrates shared a last common ancestor approximately 325 Mya (85) and are characterized by distinctive chromosome morphology and evolutionary adaptations. This is the case of sauropsids (lizards, snakes, turtles, crocodiles, and birds), whose genomes are organized into two types of chromosomes: macrochromosomes and microchromosomes (44, 86, 87). This clustering of microchromosomes initially detected by cytogenetic studies (88) is reflected in increased interchromosomal interactions extracted from Hi-C maps (44). Moreover, other nonanimal species, such as flies, mosquitos, and yeast, present different nuclear occupancy patterns in which the interchromosomal interactions are higher due to centromere or telomere clustering within the nucleus according to the so-called Rabl-like configuration (69, 89, 90) (**Figure 1**).

3.3. Conservation of Genomic Compartments Correlates with Sequence Divergence

The generation of Hi-C somatic contact maps of mammalian species representative of major phylogroups has permitted identification of different levels of conservation of the 3D genome. Whereas genome compartmentalization decreased as sequence divergence increased, TADs maintain high conservation levels across closely and distantly related species. For example, 80% of the HSBs detected between human and mouse genomes [~80 million years of divergence (40)] showed similar compartment patterns (9). Conversely, the level of compartment conservation decreased to 50% in the HSBs detected between Tasmanian devil and human genomes [~160 million years of divergence (40)] (**Figure 3*a***). Evolutionary stability of compartments between closely related species has been also reported in carnivores (91) and between elephants and mammoth (92). In the case of TADs, these structural features are highly conserved among the studied species, even between distantly related species (i.e., marsupials versus human) (**Figure 3*a***). This could be expected, as TADs represent functional genomic units of regulatory gene networks (93, 94). In fact, TAD formation is linked to sequence conservation, and their insulator capacity is defined by the

presence of CTCF (along with cohesins), which has a specific sequence-conserved binding motif (95–97).

Meiosis: conserved process in sexually reproducing organisms that results in production of haploid gametes, sperm, and oocytes

Altogether, these observations can be interpreted in the context of the dynamic nature of genomic compartmentalization. Compartments combine properties of chromatin folding (i.e., interaction frequency) with epigenetics (i.e., histone modifications and DNA accessibility). This is highlighted by the high dynamism of genomic compartments during the cell cycle [compartments are attenuated during cell division in both meiosis and mitosis (10, 98, 99)] and even during the life of an individual (i.e., aging) (100). Therefore, the organization of the genome into compartments would permit rapid activation or inactivation of genomic blocks without altering TADs. Within this framework, genomic compartments represent a highly dynamic epigenetic-regulated layer of organization present in vertebrates (11, 12, 101–103). Further studies are needed to test whether

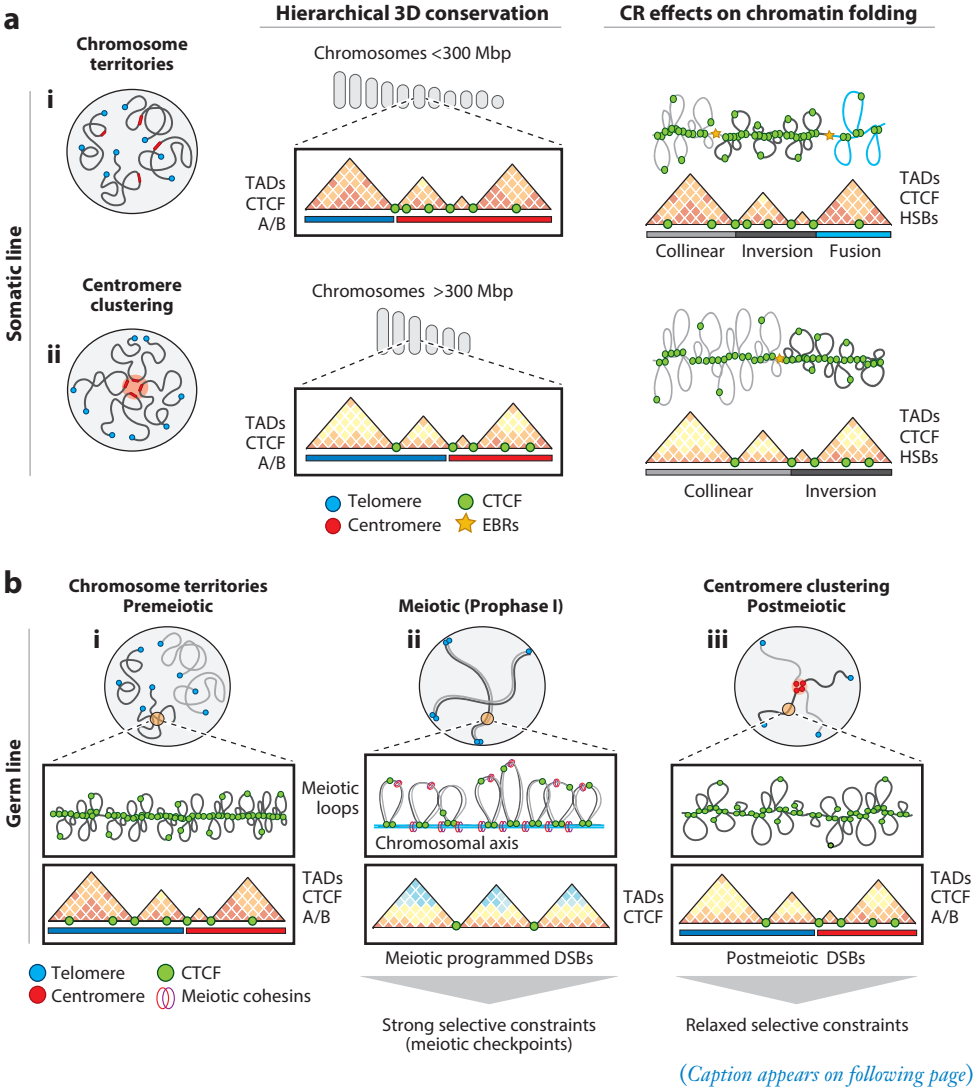


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Figure 3 (*Figure appears on preceding page*)

Evolutionary divergence of 3D genome folding in somatic and germ cells. (*a*) Patterns of chromosome occupancy and chromatin folding in somatic cells of (*i*) eutherians and (*ii*) marsupials according to recent data (9). Eutherian mammals display chromosomal territories, whereas marsupials exhibit centromere clustering. This pattern has been associated with chromosome number and size. Medium-sized eutherian chromosomes tend to have high intrachromosomal interactions and a high density of CTCF and short loops, whereas (*ii*) marsupials present opposite patterns: low intrachromosomal interactions and a low density of CTCF and long loops. Moreover, A/B compartments represent a dynamic layer more conserved between eutherians than between marsupials and eutherians. Importantly, genome reshuffling (chromosomal fusions, CRs) impacts chromosome folding and occupancy, exemplified by differences in DNA loop number and size depending on the type of evolutionary CR (fusions and inversions). In both (*i*) eutherians and (*ii*) marsupials, a higher compaction of inverted regions was detected, associated with a high number of short DNA loops. Conversely, collinearly maintained or fused genomic regions present low numbers of long DNA loops. Within this context, EBRs are located on somatic-specific TAD boundaries characterized by being low in gene density. (*b*) Representation of chromatin and epigenetic remodeling during mouse spermatogenesis and their relation to the formation of chromosomal reorganizations according to recent reports (8). (*i*) Premeiotic cells (i.e., spermatogonia) present similar chromatin folding and accessibility as somatic cells. They are characterized by strong TAD boundaries and well-defined A/B compartments. (*ii*) In meiotic cells (i.e., primary spermatocytes), chromatin is highly remodeled with an attenuation of compartments and TADs. Moreover, the chromatin is organized in DNA loops anchored to the chromosomal axis through cohesins. This chromatin remodeling is characterized by increased chromatin accessibility and the presence of meiotic checkpoints that ensure correct meiosis progressions. During this stage, highly controlled meiotic DSBs take place. (*iii*) Postmeiotic cells (i.e., round spermatids) recover a floopy somatic-like folding pattern with compartments and TADs. In the house mouse, centromeres are clustered at the chromocenter. Chromatin presents a high accessibility pattern in which postmeiotic DSBs can occur. Abbreviations: CR, chromosomal rearrangement; CTCF, CCCTC-binding factor; DSB, double-strand break; EBRs, evolutionary breakpoint regions; HSB, homologous syntenic block; TAD, topologically associated domain.

this compartmentalization is related to gene expression or to epigenetically associated regulatory networks.

3.4. Diversification Within the Animal Tree of Life Was Accompanied by an Increase in Genome Complexity and Structure

Given the divergent patterns of chromatin folding in mammalian species, how was this genome organization acquired during evolution? Is chromatin folding conserved across deeper, ancient animal lineages? As we have already discussed (Section 2.1), species diversification within the Animal Tree of Life was accompanied by an increase in genome complexity [from 16 ALGs in the metazoan ancestor to 49 chromosomes in the Amniota ancestor (41)].

Emerging applications of Hi-C methods in nonmodel species are revealing that early-branching animals (i.e., cnidarians, annelids, and nematodes) lack TAD-like structures (104–106). Basal metazoan phylogroups are characterized by small, gene-dense genomes, with little intergenic distances. Interestingly, Zimmermann et al. (36) proposed that gene proximity would be sufficient for maintaining *cis*-regulation without invoking the formation and maintenance of compartments and TADs. This would also apply to the case of nematodes and annelids, species in which the chromatin is organized into *cis*-regulatory elements instead of TADs (37). Therefore, the lack of genomic complexity and compaction present in deep metazoan lineages would have provided sufficient genomic plasticity for the extensive rearrangements that led to the subsequent burst of species (37, 107).

Importantly, the absence of topological structures in these basal groups has been related to the absence of CTCF-like proteins, linking the appearance of TAD-like structures with the evolutionary emergence of CTCF. In fact, invertebrate species expressing CTCF (i.e., flies and mosquitoes)

have their genomes organized into genomic compartment-like structures (69, 73, 108, 109) (**Figure 2**). These compartments are characterized by different degrees of gene density and expression levels, combining TAD and compartments at the same layer of organization (69, 109). Thus, the emergence of CTCF in the Animal Tree of Life might represent an evolutionary innovation that permitted the fine-tuned regulation of high-complexity genomes in bilaterian species, invertebrates, and vertebrate species.

Vertebrate species (all characterized by expressing CTCF and cohesins), on the other hand, increased genome complexity via whole-genome duplications and allotetraploidization (see Section 2.1). The appearance of larger genomes was accompanied by highly insulated chromatin structures as a result of a lineage-specific evolutionary acquisition of more stable genome configurations. This would have been accomplished by introducing additional layers of organization in the 3D chromatin hierarchical organization (i.e., TADs, compartments, and CTs) to bring into close proximity distantly located regulatory regions. As a result, TADs appear to be under strong selection pressure to maintain regulatory neighborhoods.

4. THE ROLE OF 3D CHROMOSOME FOLDING IN GENOME RESHUFFLING

Another evolutionary advantage of highly organized vertebrate genomes is the maintenance of genome stability. Highly regulated folding maintains genome integrity by, for example, suppressing the illegitimate repair of DSBs between repetitive sequences (110). On the other hand, DSB formation can modify chromosome architecture and organization in the nuclear space. As a result, chromatin compartmentalization can regulate the response to DNA damage (111), determining the outcomes of CRs.

4.1. Chromatin Interactions Facilitate Lineage-Specific Reorganizations

In an evolutionary scenario, reconstructions of ancestral karyotypes for marsupials, afrotherians, and rodents have allowed the identification of lineage-specific CRs across mammalian phylogroups and emphasized the presence of different chromosome folding configurations (8, 9) (**Figure 3*a***). In the case of marsupials, diversification within the group from a proposed marsupial ancestor of $2n = 14$ chromosomes was accompanied mainly by intrachromosomal reorganizations (i.e., pericentric inversions) without substantially reducing diploid numbers (9, 82). The extensive centromeric heterologous interactions detected in marsupials, together with low levels of chromosome compaction, suggest that centromeres might have imposed structural constraints during the group's evolution, facilitating the formation of pericentric inversions and selecting against the occurrence of extensive CRs that might alter TADs (9) (**Figure 3*a***). In fact, it has been suggested that centromeres can act as strong topological barriers that prevent contact between the two chromosome arms (112, 113). These observations align with prior cytogenetic studies, which already speculated on the important role of centromeres in marsupial genome evolution (114–117). In this regard, it can be hypothesized that centromeric heterologous interactions can facilitate the occurrence of pericentric inversions as evolutionary mechanisms to stabilize genomic regions.

Afrotherians and rodents, on the other hand, have been subjected to extensive genome reorganization (fusions, fissions, translocation, and inversions) since their divergence from their respective common ancestors (8, 9) (**Figure 3*a***). High rates of CRs in afrotherians and rodents (and probably other eutherians too) indicate a distinctive evolutionary trend when compared to marsupials, suggesting that the way genomes are organized at the 3D level can influence the occurrence of CRs. Having chromosomes organized into CTs in eutherians might facilitate chromatin contacts between heterologous chromosomes. In this way, when DSBs occur and are repaired

using error-prone DNA repair mechanisms, interactions between nonhomologous chromosomes (6, 8) can facilitate the rejoining of DSBs of heterologous chromosomes and can explain the excess of interchromosomal rearrangements in some eutherian phylogroups. In fact, CTs can promote heterologous chromatin intermingling of heterochromatin and repetitive regions on peripheral nuclear domains while preserving essential interactions and TADs by insolating those regions from other chromosomes (52, 118, 119). In this context, the occurrence of interchromosomal CRs would be not deleterious but advantageous and positively selected.

Recombination: process by which homologous chromosomes exchange genetic material during the first meiotic division

Genomic islands of divergence: highly differentiated regions of the genome with reduced gene flow between diverging lineages

4.2. Inversions as Structural Genomic Islands of Divergence

Remarkably, lineage-specific CRs can result in distinct configurational genomic interactions that can be conserved over millions of years of evolution. This is the case for inversions, which (irrespective of the clade studied) could result in different DNA loop sizes and distance-dependent interaction contact frequencies when compared with collinear genomic regions (9). This has been observed for lineage-specific inversions in the Tasmanian devil and the African elephant, where chromatin was packaged differently (high levels of intrachromosomal interactions, high density of CTCF, and short DNA loops) from neighboring, non-reorganized regions (i.e., collinear) on the same chromosome (9) (**Figure 3*a***).

Chromosomal inversions reportedly play an important role in speciation, evidenced by the initial cytological descriptions (120) and the most recent genomic approaches (121, 122). Inversions are commonly associated with phenotypic changes and adaptation in multiple species, from mammals and birds to butterflies, mosquitos, pea aphids, fishes, and plants (123–126). The mechanisms behind this pattern are still under discussion but have been related to reductions in recombination, facilitating the fixation of new alleles involved in local adaptation (4, 45). In fact, highly species-divergent genomic regions associated with inversions, commonly referred to as genomic islands of divergence, have been reported in the literature (126–128).

In this context, comparative analysis of 3D genome folding across species can improve our understanding of the presence of genomic islands of divergence, adding another layer of complexity: structural islands of divergence (9) (**Figure 3*a***). That is, the existence of physical and functional constraints in inverted genomic regions when compared to surrounding, collinear genomic regions might confer specific 3D genome folding properties that facilitate gene regulation within inverted regions. Because genomic islands of divergence have been reported to contain genes essential for reproduction and survival (126, 128), condensed chromatin folding within these regions can influence their stabilization by favoring loop interactions, facilitating microenvironments of lineage-specific gene regulation. This might lead to distinctive, functional, species-specific gene contacts within highly compacted inverted regions that would enable the appearance of new species-specific traits (**Figure 3*a***). This view aligns with novel studies that show the existence of novel CTCF binding sites associated with gene transcription, different from the conserved CTCF binding sites associated with essential TAD maintenance (95). Conversely, interchromosomal reorganizations such as fusions/fissions are associated with high interchromosomal interaction values and long DNA loops (**Figure 3*a***). Thus, collinear genomic regions can be considered less compacted and more dynamic regions than inversions (9). In this regard, it can be speculated that whereas inversions isolate and compact speciation-relevant genomic regions, fusions can rewire new chromatin contacts, enabling the evolution of new traits.

5. SEX MATTERS: REVISITING THE CHROMOSOMAL SPECIATION THEORY IN LIGHT OF CHROMATIN REMODELING OF GERM CELLS

In searching for the origin (and consequences) of genome evolutionary plasticity, several models have been proposed over the years to explain how CRs can contribute to speciation by

reproductive isolation (129). The initial chromosomal speciation theory (also known as the hybrid dysfunction model) (120) relied on the development of chromosomal incompatibility between divergent lineages by invoking post-zygotic isolating mechanisms that lead to a point when a species eventually becomes two under a model of bifurcating evolutionary history. This view was later complemented by the suppressed recombination model of speciation, which suggested that CRs facilitate lineage divergence in the face of continuing gene flow and reduced recombination between chromosomes carrying different rearrangements (129). Because chromatin is highly remodeled during spermatogenesis (10, 98, 130, 131) and evolutionary CRs must occur during germ cell formation to be transmitted to the offspring, analyzing the mechanistic forces involved in the maintenance of chromatin structure and germ line function is relevant for understanding genome evolution. This is where meiotic chromatin remodeling plays a critical role.

Meiotic checkpoints: molecular mechanisms that control chromosomal pairing, synapsis, and double-strand break formation and repair during meiosis

5.1. The 3D Organization of Mammalian Meiotic Chromosomes

Haploid germ cells (sperm and oocytes) are generated through a complex and highly regulated process known as gametogenesis involving two consecutive meiotic divisions accompanied by extensive chromatin remodeling (131, 132). At premeiotic stages, spermatogonia presents a somatic-like chromatin organization with clear TADs and compartments, which dramatically change as spermatogenesis progresses (10, 98, 130). Chromosome condensation that accompanies prophase I is translated into extensive chromatin remodeling. Throughout prophase I, chromatin is organized into large DNA anchored to the chromosomal axes formed by the axial element of the synaptonemal complex and meiotic cohesins (i.e., REC8 or RAD21L) (**Figure 3*b***). This chromosomal assembly permits the accommodation of the major events that take place during prophase I, including the repair of meiotic DSBs, and allows recombination to occur in the physical contact of the chromosomal axis (10, 131, 133–136). As such, chromosome condensation that accompanies prophase I is translated into extensive chromatin remodeling, which is highly regulated by meiotic checkpoints (137, 138).

After meiosis, postmeiotic cells (i.e., round spermatids) undergo further remodeling necessary for the replacement of histones by protamines for formation of a highly condensed sperm head. This is accompanied by the reappearance of A/B compartments and TADs. However, TAD borders in this stage present less defined borders, suggesting a different pattern of genomic folding (10, 131) (**Figure 3*b***). Importantly, the transition from histone to protamines that takes place during spermiogenesis requires the generation of postmeiotic DSBs needed for the elimination of free DNA supercoils formed during histone replacement to ensure protamine deposition (139, 140). And because postmeiotic cells lack cellular checkpoints to safeguard DSB repair, chromatin structural constraints might be more relaxed during spermiogenesis (8). Therefore, if DSBs are not ligated correctly after formation, the repair of DNA lesions via either nonhomologous or microhomology-mediated end joining (141, 142) can generate potential sources of genome instability that can be transmitted to subsequent generations.

5.2. Evolutionary Chromosomal Rearrangements Alter 3D Chromatin Conformation in Male Germ Cells

Among CRs, chromosomal fusions such as Robertsonian (Rb) fusions represent the most common large-scale chromosomal structural change in nature, from animals to plants (143, 144). Importantly, Rb fusions can alter chromatin folding in germ cells, including increased heterologous interactions in primary spermatocytes and alterations in both chromosome synapsis and axis length (48). Moreover, disturbances in the 3D nuclear topology of spermatocytes have been

associated with changes in genomic landscapes of recombination (48, 145), resulting in detectable genomic footprints of divergence (48).

The redistribution of chromosomal nuclear occupancy in spermatocytes that results from Rb fusions can bring new genomic regions into proximity, predisposing them to the occurrence of additional rearrangements (48, 146). In this context, remodeled chromosomal domains can expose novel regulatory environments absent prior to CR formation, potentially affecting gene expression and/or regulation, as the IBM initially proposed (6). In this way, new chromosomal interactions resulting from chromosomal fusions would rewire or attenuate gene networks, providing new grounds for evolution (48). This has been the case for olfactory receptor (OR) family clusters detected in meiotic-specific interchromosomal interactions in Rb mice (48).

The study of chromatin remodeling of germ cells has also revealed the existence of cell-specific LRIs enriched in relevant gene functions, forming 3D contact hubs. This has been the case of the house mouse, where LRIs enriched in differentially expressed OR genes in postmeiotic cells can recapitulate ancestral chromosomal states (8). This suggests that genomic regions involved in LRIs in mouse spermatids were once present as single chromosomes in the ancestral Muridae karyotype (8). Thus, the landscape of CRs (fusions and fissions) that took place during genome evolution in rodents can be linked to the chromatin context now present in mouse round spermatids (8). Under this scenario, cell-specific LRIs can represent 3D contact hubs where DSBs are repaired. The resulting outcome (CRs) may either separate interacting regions or conversely bring them together. Alternatively, when chromosomal fissions occur, new interchromosomal LRIs may be created to maintain critical associations between formerly contiguous regions (8).

5.3. Chromatin Folding of Postmeiotic Cells as a Key Player for Genome Evolution

We can then consider that chromatin remodeling in the germ line represents an emerging framework for understanding how evolutionary genomic variation can be generated and transmitted to offspring, as previously suggested (8, 48, 131). Recent studies have revealed that permissiveness of some genomic regions to undergo chromosomal breakage can also be associated with changes in chromatin accessibility in the germ line (8). This is sustained by two main observations: (*a*) EBRs are significantly associated with regions that are inactive and highly compacted in somatic and premeiotic cells but are activated and accessible during spermatogenesis, and (*b*) EBRs are preferentially located in epigenetically active and accessible chromatin regions in postmeiotic cells (i.e., round spermatids), when relaxed functional and structural constraints and the absence of cellular checkpoints facilitate CR occurrence and transmission (8) (**Figure 3*b***).

Importantly, the study of chromatin remodeling in early stages of meiosis in the house mouse suggests that CRs do not disturb the essential architecture of meiotic chromosomes, because EBRs are devoid of meiotic cohesins and DSBs (8). Because cohesins are necessary structural parts of the DNA loops attached to the chromosomal axes and DSB formation and repair are tightly regulated during meiosis (i.e., meiotic checkpoints), the genomic distribution of EBRs suggests the presence of purifying selection for CR occurrence in the germ line, at least in the mouse (8, 45, 147). Overall, genomic regions prone to reorganization during rodent evolution exhibit specific features: (*a*) depletion of essential genes, (*b*) an epigenetically accessible and dynamic chromatin context, and (*c*) a relaxed chromatin folding needed for efficiently rewiring relevant chromatin regions. Whether these observations account for other species remains to be tested.

6. FUTURE PROSPECTS

We have summarized key aspects of 3D genome evolution, integrating different levels of resolution and cellular contexts across evolutionary timescales. This included describing general

aspects of the mode and tempo of genome evolution across species, highlighting the importance of chromatin folding in regulating the formation of lineage-specific CRs. Within this framework, deciphering the complex interplay between chromatin folding and remodeling and genome reshuffling in the germ line context is key to understanding genome evolution. More specifically, it can provide new clues for the mechanisms driving CR formation and fixation in offspring. As more genomic resources become available, it will soon be possible to develop integrated models of genome function and the high structural organization of genomes across the Tree of Life. Exploring how 3D genome remodeling affects gene regulation in the germ line may reveal the origin, function, and plasticity of genome architecture at the chromosomal, cellular, and species levels.

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