Chapter 23

Chromatin, Nuclear Organization, and Genome Stability in Mammals

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Chapter Outline

1. INTRODUCTION

In mammalian cells, the long DNA molecules comprising the genome are wrapped with proteins to form a complex called chromatin. Chromatin fibers are then folded multiple times within the nucleus of the cell, allowing lengthy genomes to fit inside much smaller cells. Apart from overcoming space constraints, the folding of the genome also has a regulatory function, influencing fundamental processes such as gene expression, genome replication, and DNA-damage repair (DDR).

Chromatin is a complex structure with different levels of organization. At the most basic level, chromatin is made up of nucleosomes—147 base pairs (bp) of the DNA sequence wrapped around a protein octamer composed of eight histone proteins. Arrays of nucleosomes are then further folded to form a fiber measuring 30-nm in diameter; 30-nm fibers are then arranged into larger-scale structures forming domains with differing structural and functional properties and which ultimately form chromosomes—the largest units of chromatin organization.

A cell's genome is frequently exposed to factors that have the potential to introduce changes in the DNA sequence ranging from point mutations to chromosome structural aberrations and even chromosome gain or loss. Classically, threats to genome integrity were perceived to mainly come from external factors, such as drugs, chemical compounds, or UV radiation. A more current view is that internal factors and fundamental cellular processes such as transcription and replication also pose a risk to genome stability.

Whatever the source of the threat, chromatin is the context in which the genome is assaulted and then repaired. However, chromatin is more than just a passive bystander in the DNA-damage response. It forms a dynamic structure which plays an active role in a cell's response to genome damage and reacts to DNA damage with extensive changes to its structure and composition. The best accepted model describing chromatin dynamics upon induction of damage is the so-called "access, repair, restore" model [1]. It postulates that to fully repair a damaged locus, chromatin first has to be disrupted to allow access to the damaged template, followed by recruitment of factors that facilitate the repair process and finally, a reestablishment of the initial chromatin structure and eviction of the DNA-damage marks from the region. Failure in these processes can result in a serious predisposition to genomic damage and catastrophic consequences for the cell and the

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organism; therefore, our knowledge of mammalian DNA-damage response is incomplete without considering the contribution of chromatin context and the 3D organization of the genome.

2. HISTONES

Apart from DNA, chromatin contains numerous proteins with structural and regulatory functions. Among them, histone proteins are the most prominent. Core histones form nucleosomes—the basic repeating unit of chromatin, while linker histones provide the connections between nucleosomes. Histone proteins can be posttranslationally modified on their N-terminal tails, with different modifications exerting different effects on the chromatin fiber structure, adding a regulatory as well as a structural role to the range of histone functions. These posttranslational modifications (PTMs) include acetylation, methylation, and phosphorylation, as well as other, less well-characterized marks. In addition to the canonical histone proteins, the histone family also includes many histone variants, which can replace their classical counterparts in chromatin in a carefully regulated manner and in specific circumstances.

The histone proteins that form the nucleosome particle are called "core histones" and include H2A, H2B, H3, and H4, as well as their variants. Each nucleosome is an octamer consisting of two copies of each core histone, arranged as an H3/H4 tetramer and two H2A/H2B dimers. The core histones are positively charged proteins, rich in lysine and arginine residues. They bind to DNA noncovalently, through electrostatic interactions between positive charges on histones and the negatively charged DNA molecule. Nucleosomes are separated by linker DNA, whose length is not constant, but can vary from 10 to 100 bp between species and cell types. Histone proteins binding to this linker DNA are called linker histones and include H1 and its variant H5. Like core histones, H1 is also positively charged and is associated with both the linker DNA and the nucleosome particle. The H1 molecule consists of a globular domain and two tails, with the globular domain binding at the nucleosome dyad, while the tails contact the linker DNA and can drape along the chromatin fiber to stabilize the folding of nucleosomes into a 30-nm fiber structure.

2.1 Histone Variants

The classical histone molecules may be replaced in the nucleosomes and linker regions by histone variants—proteins with a high degree of sequence similarity to their common counterparts. H2A, H2B, H3, and H1, all have noncanonical variants which can replace canonical histones in the fiber in different circumstances. This replacement can have many effects, including a change in the fiber conformation (causing chromatin to become more or less tightly folded) or recruitment of regulatory proteins. Histone variant incorporation into chromatin can be independent of replication [2] and is carefully regulated by a class of proteins called histone chaperones.

An interesting example of a histone variant with an important role is CENP-A, an H3 variant present specifically at centromeres, deposited there by a histone chaperone called HJURP. Multiple studies have found evidence for a distinct chromatin structure at mammalian centromeres [3], which may be affected by the presence of CENP-A-containing nucleosomes. The presence of CENP-A is also important for recruitment of kinetochore components. Although the precise effects of CENP-A incorporation into nucleosomes in vivo are unclear, a 2011 study found that nucleosomal arrays containing CENP-A are more condensed compared to arrays containing canonical H3, suggesting that the presence of CENP-A helps to establish an unusual chromatin structure at centromeres [4].

As an important function of chromatin, locating and signaling DNA damage is also associated with a separate histone variant—H2AX, which comprises up to 32% of H2A throughout the genome. H2AX is phosphorylated at serine 139 as one of the primary events at sites of DNA damage and plays an essential role for DNA-damage signaling, detection, and repair, as discussed later in this chapter.

2.2 Histone Modifications

In addition to histone variants, chromatin fiber structure and composition can also be affected by PTMs, which are frequently present on the N-terminal tails of the histone proteins. These modifications include acetylation, methylation, phosphorylation, and ubiquitination and similarly to the presence of histone variants, can act by directly modifying chromatin structure or by recruiting regulatory factors recognizing specific posttranslational marks. Numerous posttranslational marks exist and their effects, both individual and combinatorial, are still under active investigation.

Acetylation of lysine residues in the N-terminal tails of H3 and H4 are marks often associated with active transcriptional states. Acetylation neutralizes the charge of the lysine residue, which is expected to weaken histone–histone and histone–DNA interactions, resulting in the opening of the chromatin fiber. However, a careful in vitro study performed on

short arrays of nucleosomes reconstituted on a repetitive DNA sequence failed to demonstrate significant unfolding of the fiber, suggesting the effects of acetylation may depend on the wider chromatin context [5]. H3 and H4 can be acetylated at numerous positions, including H3K9, H3K14, H3K18, H4K5, H4K8, H4K12, and H4K16 providing binding sites for bromodomains that are present within some transcriptional activators and chromatin remodelers. The acetylation mark is put on histone molecules by a class of enzymes called histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs). Interestingly, loss of HDAC function is associated with genome instability, including aneuploidy and lagging chromosomes [6].

Methylation occurs on lysine and arginine residues. Up to three methyl groups can be added on lysines, while arginines can only be mono- or dimethylated; unlike acetylation, methylation does not alter the charge of the residue affected. Lysines are methylated by lysine methyltransferases (HKMTs), which are very specific and only methylate-specific residues. Multiple HKMTs have been identified, all of which share a SET protein domain. Arginine residues are modified by arginine methyltransferases, also known as PRMTs, while removal of the methyl residues is done by demethylases. A few classes of lysine demethylases exist, including lysine-specific demethylase 1 (LSD1), which can demethylate different lysine residues depending on different accessory proteins and the jumonji domain demethylases, which act on trimethylated lysine residues. Methyl marks on H3 and H4 residues can be associated with active and inactive chromatin states. Examples include H3K9me3–a repressive mark which recruits the heterochromatin protein HP1 and H3K4me3–a mark present in actively transcribed regions. A direct relationship between appropriate methylation patterns and genome instability has been demonstrated via depletion of Suv39h, an H3K9 methyltransferase involved in establishing H3K9me3 at pericentromeric chromatin. Mice lacking Suv39h are prone to tumor formation, while embryonic fibroblasts derived from the animals have extremely unstable karyotypes [7]. While the H3K9 methylation mark probably exerts its effects on genomic stability through maintaining the structural state of certain genomic regions, another methylation mark, H3K79me, has been implicated in the DNA-damage response in a signaling manner. This mark is established by the DOT1 lysine methylase and is important for recruitment of 53BP1, a protein integral to the DDR, to a break site. 53BP1 recruitment by H379Kme is through a Tudor domain in the 53BP1 protein, a domain recognizing methylated residues; however, it is unclear whether the mark is established in response to a DNA break or whether chromatin changes in the vicinity of a break cause the mark to be exposed and recognized by 53BP1 [8].

Another important PTM, phosphorylation, can be added on serine, threonine, and tyrosine residues. This modification is placed by kinases and removed by phosphatases. Unlike acetylation and methylation, which are related to establishing chromatin domains with different properties, phosphorylation also has a major role in cell-cycle progression. The serine 10 position of H3 is phosphorylated genome-wide by the Aurora B kinase as cells progress through late G2 and into mitosis [9] in a manner that is interdependent on other histone modifications, such as H3K9me [10]. This modification is required for the mitotic condensation of chromosomes—a process in which chromosomes are compacted to facilitate chromosome separation and minimize entanglements during cell division.

Phosphorylation of the H2AX histone variant at serine 139 (phosho-H2AX or γ-H2AX) is the most widely studied DNA damage–associated histone modification. H2AX Serine 139 is rapidly phosphorylated in response to DNA damage and phosphorylation is dependent on the ATM, ATR, and DNA-PK kinases. The γ-H2AX mark spreads in large, megabase-sized domains surrounding the break region [11] and is essential for DNA-damage signaling and response. γ-H2AX–containing chromatin then serves as a platform for recruiting additional repair components, including 53BP1 and BRCA1 [12]. Interestingly, studies in which the H2AX phosphorylation site is disrupted indicate that lack of H2AX phosphorylation does not preclude initial recruitment of repair factors to the site (NBS1, BRCA1, and 53BP1); however, it affects their retention, suggesting that γ-H2AX provides a platform for maintaining factors necessary for repair. Following repair, H2AX phosphorylation is reversed by phosphatase complexes including PP2A and PP4 [13] and through histone exchange mediated by the FACT complex [14]. Mammalian cells lacking H2AX exhibit enhanced susceptibility to genomic instability and cancer [15]. Given the coordinated structural and signaling functions of other histone modifications, it is tempting to speculate that phosphorylation of H2AX may impact on chromatin fiber structure as well as via signaling in the DDR cascades. However, no such structural effects have been convincingly demonstrated and while changes in chromatin compaction are known to occur as a consequence of damage, they have been shown to be independent of the presence of γ-H2AX [16]. Other histone marks which may have a small role in the DNA-damage response include H2A ubiquitination [17], H2B phosphorylation at serine 14 [18], and H3 threonine 45 phosphorylation [19].

While the establishment of histone marks in response to DNA damage is well characterized, a 2015 publication by the Misteli Lab [20] explored the opposite idea—can certain histone PTMs predispose genomic regions to instability? Surprisingly, the study found enrichment of H3K4me1 and H3K27ac and depletion of the repressive H3K9me3 mark in genes frequently involved in translocations when compared to genes with similar expression patterns and levels. To demonstrate the correlation is causal, the authors tethered an H3K4 methyltransferase and an H3/H4 lysine acetyltransferase to a *LacO*

array incorporating an artificially introduced unique restriction enzyme site. When the frequency of breaks was assessed, the authors found elevated rates in the presence of both the H3K4 methyltransferase and the H3/H4 lysine acetyltransferase, leading them to speculate that this created a more open chromatin environment making the genome more prone to instability. Interestingly, the H3K4me3 mark is also associated with the introduction of double-strand DNA breaks during V(D)J recombination in lymphocytes [21].

3. NUCLEOSOMES AND THE 30-NM FIBER

Independent of any variants or PTMs that may be present, core histones are invariably arranged in nucleosome structures, containing two H2A:H2B dimers and two H3:H4 dimers. One hundred and forty-seven base pairs of DNA are wrapped around each nucleosome with 10–100 bp "linker" DNA bound to histone H1, linking up different nucleosomes. With the help of linker histones, the arrays of nucleosomes fold into a fiber measuring 30 nm in diameter, the exact structure of which is still under intense debate (Fig. 23.1).

A number of models have been proposed for the arrangement of nucleosomes in the 30-nm fiber structure, including a solenoid model, where nucleosomes are organized in a helical array, a "zigzag" model with a zigzag arrangement of nucleosomes and an "irregular fiber" model with a disorganized arrangement and variable spacing of nucleosomes. Various techniques have been used since 1963 to try and resolve the structure of the 30-nm fiber, including variations of electron microscopy, X-ray diffraction, and in early 2010s, superresolution microscopy [22,23]. While successful observation of the 30-nm fiber structure is possible in chromatin reconstituted in situ and in some rare types of nuclei, it has proven impossible to resolve the fibers in intact nuclei, with chromatin appearing as a densely staining mass.

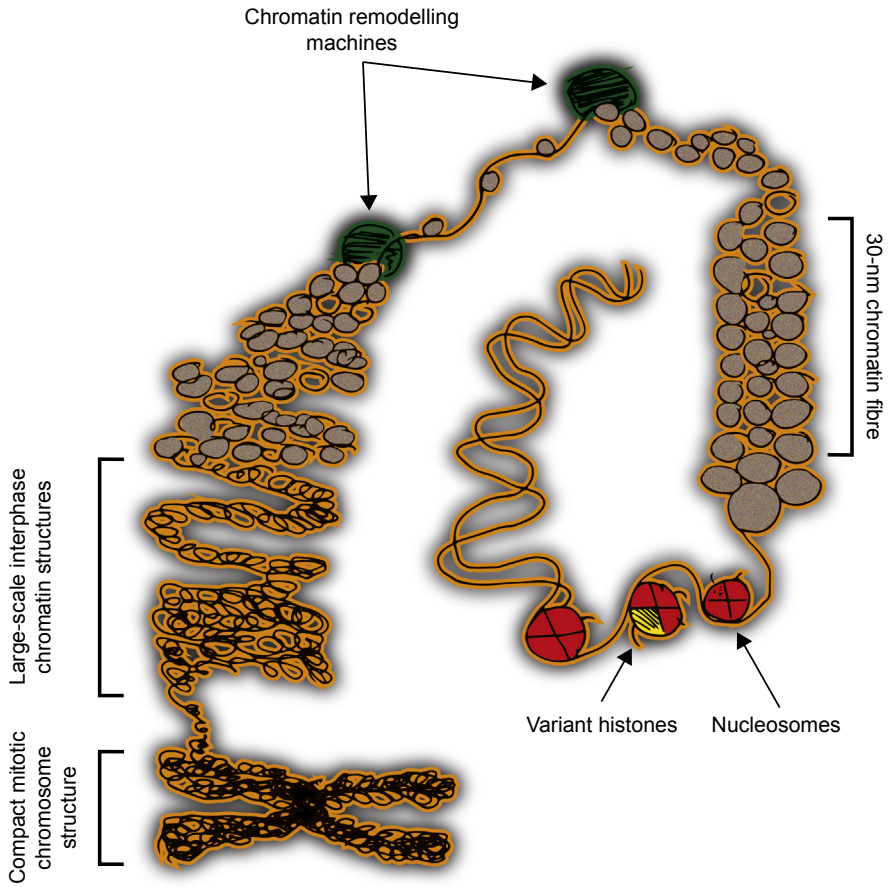

FIGURE 23.1 Levels of chromatin organization. At the primary level of chromatin folding, the DNA molecule is wrapped around histone octamers to form nucleosomes. Nucleosomes may contain core histones or histone variants and the N-terminal tails of the histones can carry various posttranslational marks. Interactions between nucleosomes cause further folding into a 30-nm fiber. The exact arrangement of nucleosomes within the 30-nm fiber is unknown, but it is likely not homogeneous and local disruptions caused by chromatin remodeling events are present. Larger-scale structures are formed by further folding of the 30-nm fibers. Interphase chromatin is additionally compacted for mitosis, giving rise to mitotic chromosomes.

In reality, as chromatin structures are very dynamic, it is likely the structure of the 30-nm fiber in living nuclei is not homogeneous, but instead is made up of a mixture of the models proposed with some regions being more compact and others more disrupted. In another illustration of the structure–function relationship, it has been shown that constitutively transcriptionally inactive parts of the genome (eg, centromeric heterochromatin) show a regular folding at the 30-nm level, while the bulk genome has a less regular conformation, interspersed with irregularities [3]. Nucleosomes can be moved and shuffled by proteins called chromatin remodelers to enable proteins such as transcription factors, replication-related proteins, and DNA-repair proteins to bind to the naked DNA template. It is easy to envisage how these movements of nucleosomes can introduce transient local disruptions in the chromatin fiber. A frequently used method to investigate nucleosome disruptions and 30-nm fiber structure is performed by testing the accessibility of the naked DNA by DNase I digestion, or a 2013 approach taking advantage of next-generation sequencing called ATAC-seq [24].

4. HIGHER-ORDER STRUCTURES

At a further level of chromatin organization, interactions between the 30-nm fibers give rise to so-called "large-scale" structures, an example of which are chromonema fibers measuring 100-nm in diameter, as observed by electron microscopy. The fine details of this level of organization are unknown (although looping of fibers is likely to be involved) and currently not many methods are available to investigate the mechanical composition of higher-order chromatin structures. Generally, these structures are organized into segments with differing functional properties, determined by a combination of sequence composition (AT:GC content), transcriptional state and the presence of different histone modifications and chromatin-bound proteins. A simplistic classical view is to split the genome into gene-rich segments with more open structures and gene-poor regions enriched in repeats and satellites where the folding of higher-order structures are more compact. However, a 2011 classification of the differing properties of chromatin types splits them into five categories based on the prevalence of histone modifications: yellow (constitutively transcriptionally active regions), red (tissue-specific active regions), blue (repressed development and differentiation-related regions), black (silenced regions containing genes), and green (constitutively inactive repeats and satellites) chromatin [25]. The first two categories contain the transcriptionally active portion of the genome, which is enriched in acetylated H3 and H4. The chromatin structure in such regions is likely to be enriched in disruptions at the 30-nm level, particularly at regulatory elements, for example, promoters and enhancers, while large-scale domains will be more unfolded, facilitating easy access of transcription, replication and DNA repair factors to the DNA template. In contrast, the chromatin structure within green regions is likely to be more compacted and less dynamic. Processes that require access to the DNA template such as replication and DNA repair may necessitate chromatin remodeling to open up chromatin in these regions of the genome. In fact, some 2008 data suggest that permanently silenced regions may act as a barrier to the DNA-damage response and that breaks within them may take longer to detect and repair [26].

The segmentation of the genome into higher-order domains with differing structures is essential for its correct function. A small number of human diseases related to perturbations of chromatin structure have been described, including ICF syndrome (immunodeficiency, centromeric instability, and facial anomalies syndrome) and Rett syndrome. ICF syndrome is caused by mutations in the *DNMT3B* gene, coding for a DNA methyltransferase, and patients show instability and breakage of the silenced, repeat-rich regions at the centromeres of chromosomes 1, 9, and 16 in lymphoblastoid cells [27].

5. CHROMATIN REMODELERS

Apart from histones, chromatin contains a range of other proteins with diverse roles, some of which function to prevent genomic instability and respond to DNA damage. One of the most important classes of nonhistone chromatin–associated proteins is the remodelers: proteins which can reposition and remove nucleosomes or change their composition in an ATP-dependent manner. Consequently, they introduce small-scale alterations in the state of the chromatin fiber and alter the accessibility of the DNA template. Chromatin remodelers are required for many nuclear processes, including transcription, replication, cell-cycle progression, and of course, DNA repair [28,29]. Numerous mammalian chromatin remodelers exist and they can be broadly divided into four families: SWI/SNF, ISWI, INO80, and CHD and their roles in DNA repair are summarized in Table 23.1.

Genes encoding chromatin remodelers of the SWI/SNF family are frequently mutated in cancer and components of the SWI/SNF members BAF and PBAF have been shown to localize to sites of DNA damage. PBAF subunit BAF180 has a role in silencing transcription at sites of DNA breaks [30], while Brg1, a subunit common to BAF and PBAF, is involved in sister chromatid decatenation at the G2/M boundary and its inhibition results in anaphase bridges and lagging chromosomes [31]. Hinting at the wide range of roles these remodelers have, the PBAF complex was also found to promote sister chromatid cohesion, especially at centromeres, with chromosomal breaks and abnormalities following its inhibition [32].

TABLE 23.1 Roles of Chromatin Remodelers in DNA-Damage Repair

Family	Features	Complexes	Role in DNA Repair	Subunit Implicated
SWI/SNF	Bromodomains	BAF	Decatenation of sister chromatids	Brg1
		PBAF	Silencing transcription at breaks Sister chromatid cohesion at centromeres	BAF180, Brg1
ISWI		ACF	Facilitates NHEJ	ACF1, SNF2H
INO80	Histone exchange	TIP60	Restores chromatin environment by removing gamma H2AX	p400
		INO80	Promotes HR repair	
CHD	Chromodomains	NuRD	Released from heterochromatin to promote relaxation	CHD3
			Recruited to heterochromatin to promote relaxation	CHD4

ACF-1, a component of two ISWI-type complexes, ACF and CHRAC, was also found to bind at laser-induced DNA breaks, colocalizing with γ-H2AX [33]. Cells depleted of ACF-1 are very sensitive to DNA damage, and the authors showed that ACF-1 facilitates the binding of NHEJ protein Ku at double-strand DNA breaks.

The CHD class of remodelers are characterized by the presence of chromodomains, which can read methyl marks on histones. An example is the nucleosome remodeling and deacetylase complex (NuRD), which promotes nucleosome compaction in heterochromatin. The CHD4 subunit of the NuRD complex is phosphorylated by ATM in response to genome damage [34] and is rapidly recruited to sites of damage [35]. In the same studies the authors observed increased rates of genomic breaks in CHD4-depleted cells, suggesting not only that CHD4 is essential for repair, but that its depletion might also make chromatin more susceptible to breaks. In contrast, NuRD complexes containing an alternative CHD3 isoform were released from heterochromatin upon treatment with ionizing radiation, promoting chromatin relaxation [36].

Mammalian cells depleted of the INO80 remodeler also exhibit DNA-repair problems, with homologous recombination (HR) specifically affected as INO80 seems to be involved with 5′- to 3′-DNA resection at break sites [37]. Depletion of p400, an INO80 component, primarily involved in the incorporation of the H2AZ variant at transcriptionally active regions, makes cells sensitive to DNA damage [38]. p400 also incorporates H2AZ at double-strand breaks, contributing to chromatin opening in the break region to facilitate access for repair proteins [39]. A further INO80 subunit, TIP60, which acetylates H2A and H4, has been implicated in restoring the chromatin environment following DNA-damage response by removing the phosphorylated H2AX from the affected regions [40]. An additional role for TIP60 also involves histone acetylation in heterochromatic breaks to potentiate chromatin relaxation before repair [41].

Overall, the study of chromatin remodelers in DNA repair is a very active field of research but complicated by the many functions of these enzymes. In addition, as chromatin remodelers tend to have a serious impact on gene expression, studies have to exclude indirect effects on genome instability due to altered transcription. This underlies the need for better and more representative in vitro chromatin models, which could be used to study the direct structural effects of the remodelers, separately from their other roles.

Apart from chromatin remodelers and the specialized repair factors described elsewhere in the book, a number of other nonhistone chromatin–associated proteins also assist with DNA repair, often as a secondary function; examples include topoisomerases, helicases, and structural scaffolding proteins. A particularly interesting example of this is the cohesin complex, a large molecular complex essential for sister chromatid cohesion and chromosome segregation. A ring-shaped structure, cohesin associates chromatin fibers not through direct binding, but rather topologically and contributes to the 3D organization of the genome [42]. It brings together the two sister chromatids following replication and functions in the HR pathway, ensuring proximity between the damaged chromatid and the repair template. Cohesin is recruited to DNA-damage sites following laser irradiation only in the S and G2 phases of the cell cycle through an interaction with Rad50 [43]. Other nonhistone proteins are implicated in maintenance of genomic stability indirectly by working to avoid the formation of DNA:RNA hybrids, conflicts between the transcription and replication machinery, and by rescuing stalled replication forks.

6. ACCESS, REPAIR, RESTORE

As illustrated by the extensive role of chromatin remodelers in the DNA-damage response, changes in chromatin conformation are essential for the repair process (Fig. 23.2).

There is some controversy about whether these changes are limited to the chromatin environment local to the break or whether they spread globally. Local changes have been demonstrated convincingly, using a variety of methods: HATs and HDACs are recruited to laser-induced tracks [44], while high-resolution imaging of chromatin in DNA repair foci shows chromatin in a state resembling a 10 nm fiber [45]. Consistent with this, a live cell imaging study utilizing the *SceI*/*LacO* system mentioned earlier demonstrated local chromatin remodeling in the proximity of a break [46]. In this study, authors used a photo-activated GFP fused to H2A, allowing them to induce damage and photoactivate chromatin within the damaged region simultaneously. They then measured changes in the H2A-GFP spot size and were able to show rapid expansion of the spot area lasting 1.5 min, followed by a recompaction phase lasting 15 min and then hyper-condensation beyond baseline level (20–30 min). A brief local decompaction, as demonstrated in this study, would enable access of the DDR

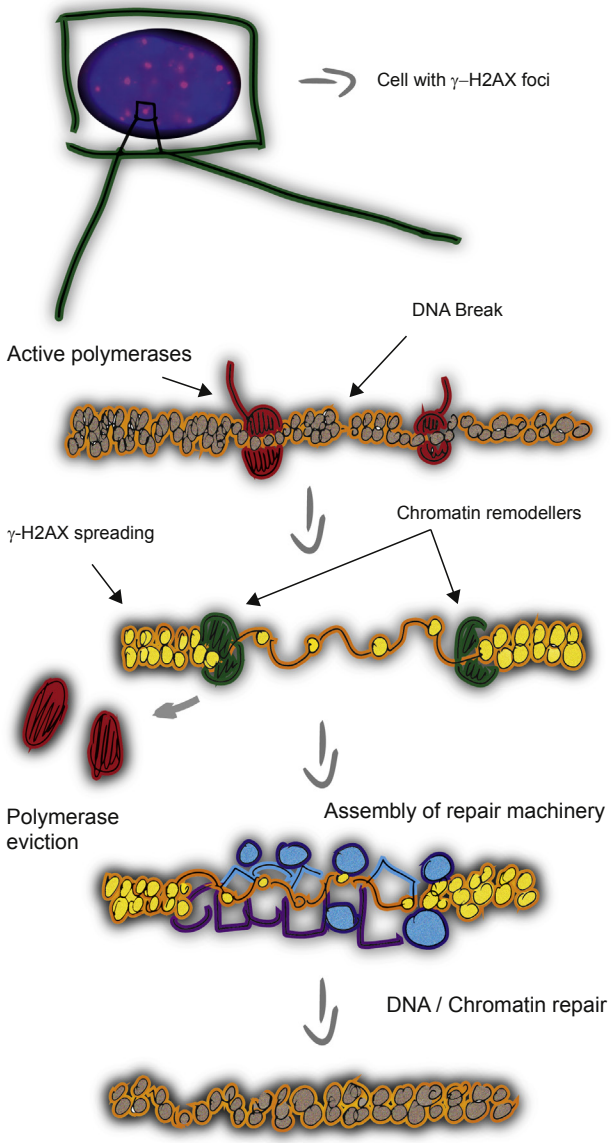

FIGURE 23.2 **Changes in local chromatin structure upon dsDNA breaks.** DNA breaks are accompanied by local changes in chromatin compaction and transcription. Upon breakage, chromatin remodelers alter the chromatin surrounding the break to be more accessible. Transcription stops and H2AX is phosphorylated (*yellow*) in a megabase-sized domain surrounding the break. The DDR components are recruited onto chromatin and retained there through γ-H2AX. Following repair, the normal chromatin environment is restored and the γ-H2AX mark is removed.

proteins to breaks. Alterations in the transcriptional activity of a locus in the vicinity of a DNA break also accompany local compaction changes. Ubiquitination of H2A at break sites was shown to correlate with transcriptional silencing near break regions [47] and recruitment of the SWI/SNF remodeler PBAF is found to contribute to this silencing [30]. A somewhat opposing finding was published in 2012, when Francia et al. [48] found evidence that transcription of small noncoding RNAs within a damaged region is required for the DNA-damage response. Whether the local changes in compaction and transcription spread globally is debatable. A 2006 study, using MNase digestion to assess genome-wide chromatin states, found evidence of global decondensation following DNA-damage induction [49]. However, a 2011 study from our lab found no evidence for global decompaction using the same approach or by sucrose gradient sedimentation to analyze the structure of soluble chromatin fibers [50].

Once the appropriate chromatin environment has been established, repair of the damage can proceed. The earliest step in the DDR involves rapid targeting of repair factors to the lesion and formation of DNA repair foci. The primary sensor is the MRN complex, composed of three different factors: MRE11, RAD50, and NBS1. The MRN complex activates ATM, which in turn phosphorylates H2AX at the damage site and the flanking chromatin up to a megabase away [11], amplifying the damage signal. An interesting question in the field is whether a full DDR is initiated only in response to DNA breaks. Surprisingly, not. Tethering of early repair components to genomic regions resulted in a full DNA-damage response and cell-cycle arrest, indicating that breaks are not needed beyond the initial recruitment of factors [51]. Consistently, treatment of cells with the HDAC-inhibitor TSA resulted in the activation of ATM raising the possibility that DDR can also be triggered by stimuli other than breaks, such as unusual chromatin structures [52].

Once the necessary factors have been recruited, repair can proceed. There are two main pathways for repair of double-strand DNA breaks—nonhomologous end joining (NHEJ) and HR, which are described in detail elsewhere in the book. Briefly, NHEJ works by joining the ends of the break together and is active throughout the cell cycle, while in HR, which is only possible in S and G2, the nondamaged homologous locus on the sister chromatid is used as a repair template. Interestingly, the 2014 evidence showed that breaks located in transcriptionally active segments of the genome are preferentially repaired with HR, while breaks in less-active regions are more frequently repaired vie NHEJ even as the cells transition into S and G2 [53]. The preferential recruitment of the HR machinery to breaks in transcribed regions is found to be dependent on an interaction between the H3K36me3 mark and LEDGF, a protein component of HR.

7. NUCLEAR ORGANIZATION OF CHROMATIN

Within cells, chromatin is contained within the nucleus—a complex organelle shaping the 3D organization of the genome. Positioning of the genome in the nucleus has important functional consequences; nuclear position is a significant characteristic of a locus, impacting on its transcriptional activity, replication timing, and proximity to other loci. Changes in the nuclear positioning of loci accompany development and differentiation, demonstrating the biological importance of nuclear organization.

The exact positioning of loci within the nucleus is probabilistic—it is not the same in every cell but is guided by a set of rules. With few exceptions, in mammalian cells, gene-rich, transcriptionally active regions of the genome are located toward the nuclear interior, while the gene-poor and heterochromatic regions are located toward the periphery. As a result, rather than having precisely defined locations, chromosomes have preferred radial positions in the nucleus. Centromeres also tend to be located toward the periphery [54], while telomeres are distributed through the nuclear volume.

The nuclear periphery is defined by its interaction with the nuclear lamina—a part of the inner nucleoplasmic membrane. The genomic regions that interact with the lamina are known as lamina-associated domains (LADs); they measure 0.1–10 Mb in size and overlap with chromatin features such as low gene density and repressive histone marks. LADs can be divided into a *facultative* and a *constitutive* class. Facultative LADs are cell type specific, while constitutive LADs are shared between cell types. Interestingly, disruptions in the lamina structure have been associated with genome instability, as illustrated by a class of diseases known as laminopathies, caused by mutations in the genes coding for the proteins that make up the nuclear lamina. The best studied among them is the Hutchinson–Gilford progeria syndrome (HGPS), a rare premature aging syndrome caused by mutations in the *LMNA* gene. Cells from patients with HGPS show microscopically visible disruptions to the shape of the nuclear envelope, loss of the heterochromatic protein HP1 at the nuclear periphery, and altered histone modifications pattern. Although not deficient in any of the components of the DDR response, HGPS cells are sensitive to ionizing radiation and accumulate DNA damage when grown in culture [55]. They also display increased levels of γ-H2AX and ATR/ATM activation. In addition, Werner's syndrome, a disease that is phenotypically related to HGPS, is caused by mutations in the WRN protein—a DNA helicase that prevents DNA damage by resolving stalled replication forks. However, despite all the evidence that the lamina disruption observed in HGPS cells ultimately lead to increased DNA damage, the underlying molecular mechanisms are yet unknown.

8. CHROMOSOME TERRITORIES

Rather than being dispersed throughout the nucleus, each chromosome occupies a distinct volume, called a *chromosome territory*. This has been demonstrated by *chromosome painting*—a FISH-based technique where the genome is hybridized to a large number of chromosome-specific probes to allow visualization of individual chromosomes within the nucleus. The radial positioning of a chromosome is strongly influenced by its composition—gene-poor chromosomes tend to occupy positions closer to the nuclear periphery, while gene-rich chromosomes are more frequently located toward the interior [56]. This trend is illustrated by human chromosomes 18 and 19, which are very similar in size but have very different sequence composition: chromosome 18 is gene poor, while 19 is gene rich. The Bickmore lab used chromosome territory FISH to investigate the positions of the two chromosomes in the nucleus and found that chromosome 18 was consistently located closer to the nuclear periphery than chromosome 19 in both lymphoblastoid and fibroblast cell lines [57]. The radial positioning of chromosomes in the nucleus was also found to be tissue specific, with more closely related cell types exhibiting more similar chromosome positioning [58]. The human genome also contains five acrocentric chromosomes, containing rDNA sequences—chromosomes 13, 14, 15, 21, and 22 which are usually clustered around the nucleolus—the site of transcription and processing of ribosomal RNA.

The radial rule of chromosome positioning also influences the positioning of alternating gene-rich and gene-poor segments within chromosomes—in this case, gene-rich segments are located more centrally, while gene-poor regions occupy regions closer to the periphery. In addition, within chromosome territories, transcriptionally inactive segments are located internally and transcriptionally active segments are at the surface of the territory [59]. This arrangement allows transcriptionally active regions ready access to the transcription machinery and domains rich in mRNA metabolic factors such as SC-35 foci [60]. However, the fine-detail structure of chromosome territories is yet unclear, reflecting our lack of knowledge of the chromatin structures that shape them.

From a genome stability perspective, an important consequence of chromosome positioning patterns relates to translocations, the most frequent chromosomal abnormality seen within the human population. It is well established that the physical proximity of two chromosomes in the nucleus affects the probability of a translocation occurring between them (Fig. 23.3).

An analysis between the frequencies of different nonpathogenic translocations in the human population and the preferred radial positions of chromosomes in the nucleus found that chromosomes with similar nuclear positions form translocations

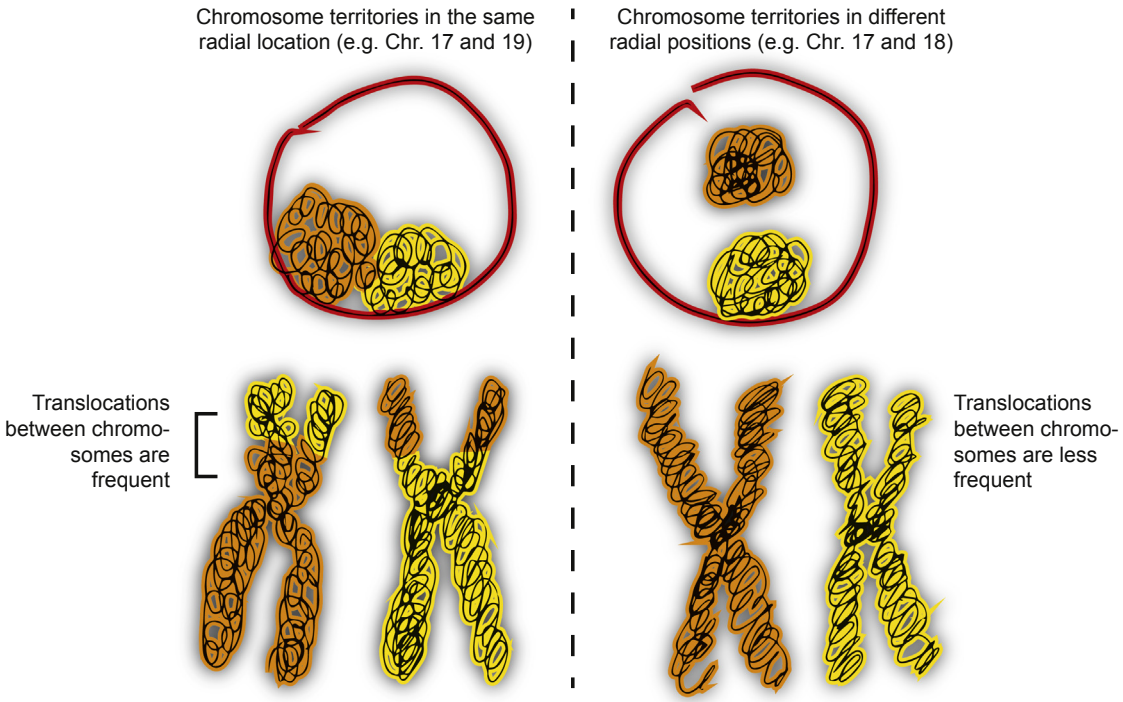

FIGURE 23.3 **Preferred positions of chromosomes in the nucleus influences translocation frequency.** Chromosomes with the same preferred radial position in the nucleus (eg, chromosomes 17 and 19) are more likely to be involved in translocations than chromosomes with different radial positions (eg, chromosomes 17 and 18).

more frequently than expected by chance [61]. Another study was able to demonstrate close proximity between the BCR and ABL loci, involved in the well-characterized t(9; 22) translocation forming a "Philadelphia" chromosome in chronic myeloid leukemia. The authors showed that the BCR and ABL loci were closer in B-lymphocytes than in hematopoietic progenitor cells, suggesting that cell type–specific aspects of nuclear organization may contribute to the association of certain translocations with particular cancer types. In 2013, the Misteli lab published a study [62] exploring the dynamics of double-strand breaks and subsequent translocation formation in an elegant system: NIH3T3duo cells encode a small number of *Sce*I restriction enzyme sites integrated on different chromosomes, with some sites adjacent to a *LacO* array and other sites neighboring a *TetO* array. Upon break induction by the *Sce*I enzyme, it was possible to track the breaks which were marked by fluorescently tagged Lac (LacR) and Tet (TetR)-repressor proteins; translocation formation was indicated by long-lasting, stable co-localization of the LacR and TetR signals. The authors were able to demonstrate that most translocations are formed by loci that are closely located prior to break induction (*contact-first model*), rather than as a result of a movement of double-strand breaks to proximal locations (*breakage-first model*).

Beyond methods for analysis of chromosome territories, two main complementary methods are used to study the 3D organization of the genome at the level of higher-order domain structure: FISH-based methods and chromosome confirmation capture methods [63]. FISH relies on hybridization of fluorescently labeled probes to visualize individual loci, defined portions of the genome or whole chromosomes. It provides a snapshot of nuclear structure at the single cell level, but disadvantages are that it is time-consuming and provides a limited amount of information at a low resolution. Chromatin conformation capture (3C) techniques rely on "freezing" the nuclear structure by cross-linking interactions within the nucleus, ligating DNA fragments held in proximity by the cross-links, followed by PCR or next-generation sequencing to identify hybrid DNA fragments, indicative of contacts. At the most sophisticated end, these techniques can theoretically identify all possible interactions throughout the genome, but there are also disadvantages. Unlike FISH, 3C techniques work on populations of cells rather than at a single cell level, producing a population average which may reflect a number of different contact configurations at the single cell level. Despite the caveats, 3C methodologies have been very influential in the field of 3D genome organization, contributing the concept of topologically associating domains (TADs). TADs are defined as regions measuring ~900 kb, where contact maps show increased interactions; FISH-based studies have shown that probes located within a TAD are physically closer than probes not located within the same TAD but separated by a similar "linear" genomic distance [63]. The full human genome is divided into approximately 2000 TADs which also overlap with the distribution of histone marks and other genomic features such as replication timing (described later). However, they are not cell-type specific and the question of what level of structural organization they reflect and their functional importance is still open to debate. Interestingly, the translocation frequency pattern seen with chromosome territories can be also traced to the TAD level of organization—a study conducted in B-cells found that the likelihood of translocation between two loci is strongly related to the contact frequency between them, as defined by chromosome confirmation capture-generated contact maps [64].

9. TRANSCRIPTION AND REPLICATION IN THE NUCLEUS

As we have seen earlier, the nucleus is a site of many correlations: radial position, gene density, histone mark enrichments, and transcriptional activity. Another correlation comes from the process of replication: the exact timing of replication of a locus also correlates with its nuclear position, as well as with its transcriptional activity. Replication proceeds in a well-controlled timely manner across the genome-alternating segments of chromosomes replicate at different times throughout S-phase, with gene-rich, transcriptionally active segments replicating early in S-phase and heterochromatic regions replicating last. These replication domains measure from 400 to 800 kb and control of replication timing is achieved by simultaneous firing of clusters of origins within the replication domains at defined times during S-phase. The correlation between replication timing and nuclear position is so strong that it gives rise to striking S-phase patterns visible in nuclei stained with markers of active replication (Fig. 23.4): early replicating cells show diffuse staining with markers excluded from the nuclear periphery; cells in mid-S have speckled patterns; and in nuclei in the latest stages of replication the staining overlaps with the nuclear periphery and heterochromatic regions. Replication timing domains partially overlap with TADs, however some replication domains are cell-type specific and change during development and differentiation, along with changes in transcription. About 80% of the genome has constant replication timing between cell types, with 50% showing development and differentiation-related changes.

A few studies to date have tried to separate out the effects of chromatin state, transcription and replication timing to investigate the real determinants of nuclear positioning. A 2014 study by the Bickmore Lab indicated that the chromatin compaction state may be the primary factor [65], with transcriptional activation influencing nuclear position, while replication timing was shown to be a consequence of transcriptional state. However, other studies have argued that replication plays a role in the

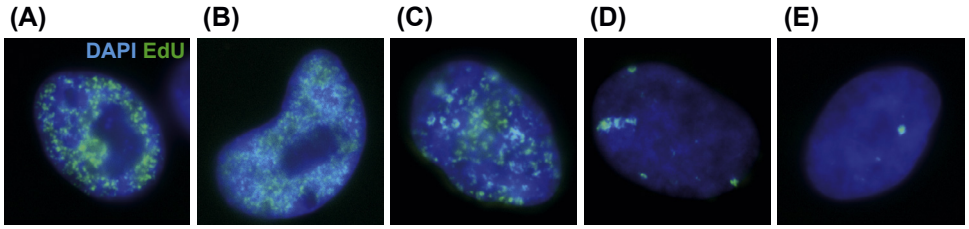

FIGURE 23.4 **Replication timing in the nucleus.** Correlation between replication timing and nuclear position gives rise to striking patterns in replicating cells, which discriminate between early (A,B), mid (C), and late (D,E) replicating cells. In this experiment, cells were pulsed with the thymidine analogue EdU which is incorporated into newly replicated DNA and can then be readily visualized (*green*). The nuclei are stained with the DNA dye 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI, *blue*). EdU staining, indicating sites of active replication is diffuse in early S, speckly and close to the periphery in mid-S, and coinciding with heterochromatin in late-S.

establishment of nuclear organization. A 2015 chromatin confirmation capture study revealed that TAD structure is established during early G1, at the same time as the replication timing program [66]. Another 2015 study used high-throughput FISH to screen for factors affecting nuclear positioning of a small number of loci; it found that a number of replication-related proteins significantly affected positioning and also that replication was needed to maintain correct nuclear positioning [67].

The processes of replication and transcription have been at the heart of a conceptual shift in the field of genome stability since 2014. While historically research on the DNA-damage response was focused on external and severe mutagens such as UV light and carcinogenic drugs, recently it has become clear that DNA damage resulting from internal factors and fundamental cellular processes may be more physiologically relevant. A succession of recent studies has implicated replication and transcription as contributors to genome instability. For example, a study in 2015 determined that regions of very high mutation rates within the genome overlap with Okazaki fragment junctions; the underlying mechanism was found to be retention of short segments spanning the junctions synthesized by the error-prone DNA polymerase Pol-α [68]. An earlier study identified replication stress, physiologically present in cancer cells, as the root cause of structural and numerical chromosome instability in colorectal cancers with unstable karyotypes [69]. Transcription was implicated as a contributor to genomic instability in a publication by the Svejstrup Lab—the authors found that inhibition of a transcription-associated helicase caused transcription speed to increase, resulting in recurrent chromosomal rearrangements at particular genomic regions [70]. Another example is provided by the *RNU1*, *RNU2*, *RN5S*, and *PSU1* loci, all coding for tandemly repeated, highly transcribed small RNA sequences. These four loci exhibit fragility and appear as breaks on metaphase chromosomes upon either adenovirus infection or in the absence of the Cockayne syndrome group B (CSB) protein, which is mutated in Cockayne syndrome, a rare disorder characterized by neurological and developmental defects. As CSB functions as a transcription elongation factor, it has been speculated that its loss causes RNA polymerase stalling and blockage at the *RNU1*, *RNU2*, *RN5S*, and *PSU1* loci, which then interferes with chromosome condensation and consequently, the stability of the four regions [71].

Unlike external factor-mediated instability, which usually arises from stoichiometric interactions of the damage-inducing agents with DNA and results in predictable outcomes, internally mediated instability is stochastic: it is likely to result from a combination of factors, including the exact chromatin context at the location where problems arise. While in the past most common strategies for studying the role of chromatin in genome stability involve triggering DNA damage through methods such as irradiation, laser marks, or harsh damage-inducing agents such as hydroxyurea, it is clear that this new view of the field will require novel models and methods. A good model for how complex relationships between transcription, replication, and chromatin influence genome stability is presented by common fragile sites (CFS).

CFS are regions of the genome prone to instability in response to replication stress, manifesting as breaks, gaps, and constrictions on metaphase chromosomes. While it is known that CFS fragility is triggered by replication stress, the exact events leading up to genomic instability are unknown. As CFS fragility is cell-type specific—different genomic locations are fragile in different cell types—it is clear that factors beyond their sequence composition contribute to fragility; in particular, replication timing and transcription are considered important, while chromatin context is a promising but understudied potential contributor.

Three models have been proposed to explain how the induction of replication stress results in genomic instability in a locus-specific manner (Fig. 23.5).

The *replication fork collapse* model suggests that the AT-rich sequence of CFS makes them prone to forming secondary structures which contribute to replication fork stalling and collapse [72]. The *transcription–replication collisions* model is based on the observation that fragile sites frequently span long genes, raising the possibility that CFS instability can be the result of concomitant transcription and replication [73]. The *replication–initiation paucity* model explains CFS fragility as a consequence of cell type–specific features of replication timing [74].

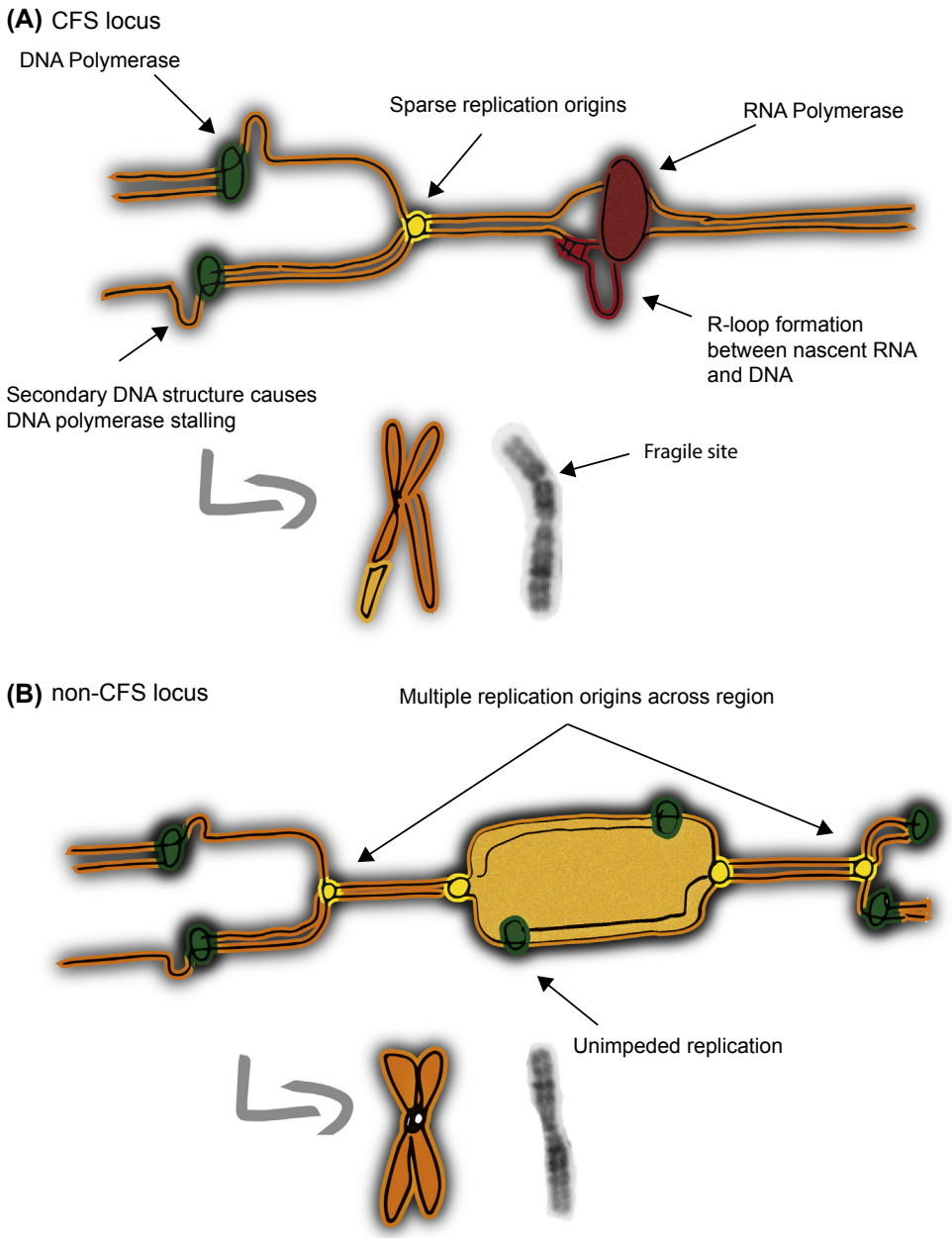

FIGURE 23.5 **Models of CFS formation.** Multiple models have been proposed to explain the cell type–specific fragility of CFS. (A) CFS region in a cell type–specific fragility inducing chromatin environment. (B) CFS region in a noninducing chromatin environment. In (A), the AT-rich sequence of fragile regions causes DNA polymerases to stall; transcription/replication encounters in the region result in the formation of R-loops; paucity of replication origins (*yellow*) means that the region is replicated very late in the cell cycle or remains unreplicated. In (B), the AT-rich sequence also causes stalling, but the lack of transcription in the vicinity of the CFS and frequently spaced replication origins across the region allow replication to proceed in time.

In support of the fork collapse model, two genetic disorders characterized by increased fragile site formation, Bloom syndrome, and Werner syndrome are caused by deficiencies of RecQ helicases specialized in resolving stalled replication intermediate structures [75]. Werner syndrome is caused by a deficiency of the Werner syndrome protein (WRN), an ATP-dependent helicase which efficiently unwinds structures resembling stalled replication bubbles such as Holliday Junctions (HJ). Cells derived from WRN-deficient patients form breaks at CFS spontaneously in the absence of aphidicolin treatment, while in wild-type cells, an increased frequency of CFS formation is observed following WRN depletion [76]. BLM syndrome is caused by a deficiency of the Bloom Syndrome protein (BLM) and is characterized by increased susceptibility to early onset cancers. BLM resolves structures that mimic replication and recombination intermediates, such as HJs, via homologous repair in a manner which does not result in a crossover and BLM has been shown to localize to stalled replication forks in vivo [77]. Cells from Bloom syndrome patients show an increased sensitivity to aphidicolin and an increased

frequency of sister chromatid exchanges which could result from crossover-mediated repair of HJs by alternate nuclease complexes. Interestingly, in the absence of BLM and other Holliday junction dissolution mechanisms, extreme chromosome abnormalities resembling multiple fragile site breaks are observed [78]. Further evidence supporting the fork collapse model comes from observations that in the presence of aphidicolin, the replicative helicase complex becomes uncoupled from the replication machinery, giving rise to long stretches of single-stranded DNA [79]. Additional supporting evidence comes from a 2011 study demonstrating replication fork stalling at AT-rich sequences at the FRA16D fragile site [80]. A major disadvantage of this model however is that it fails to explain the cell-type specificity of CFS expression.

The tendency of fragile regions to encompass large genes has inspired a model suggesting that CFS instability results from collisions between the transcription and replication machinery. Large genes require longer times for transcription, sometimes exceeding the length of a full cell cycle, indicating that transcription might be ongoing during S-phase. Normally, S-phase transcription and replication are spatially separated in eukaryotic cells; most actively transcribed genes are early replicating and changes in transcription during development are accompanied by changes in replication timing [81]. In this model, aphidicolin treatment interferes with the temporal and spatial separation of replication and transcription at large genes, causing the occurrence of transcription and replication at fragile sites. The model speculates that concurrent transcription and replication can cause instability through the formation of *RNA–DNA (R-loop) hybrids* or through head-on collisions of the transcription machinery and the replication bubble, causing replication fork collapse. Efforts to correlate CFS fragility with gene expression in a cell type–specific manner have given conflicting results. A 2011 study showed a correlation between expression of the FHIT gene at the FRA3B fragile site and FRA3B fragility, accompanied by an increase in R-loop formation in the presence of aphidicolin [73]. However, a study from 2013 failed to find a correlation between expression and fragility on a more genome-wide scale [82]. Furthermore, breaks at CFS are not restricted to transcribed regions and can also occur at intergenic sequences. Therefore, unlike the RNU loci, active transcription is not required for induction of fragility at CFS, suggesting that the transcription–replication collision model does not fully explain CFS lesion formation.

In the *replication initiation paucity* model of CFS formation, instability is caused by a cell type–specific lack of initiation events across fragile regions, forcing the forks to travel long distances to replicate CFS loci and causing the regions to remain unreplicated at the end of S-phase in the presence of replication stress. Evidence supporting the model comes from a study demonstrating that a lack of initiation events across the well-studied FRA3B site correlates with its fragility in lymphoblastoid cells [74]; in contrast, initiation events across the site were observed in fibroblasts, where FRA3B is stable. In addition, the authors demonstrated increased use of origins in response to aphidicolin treatment at the flanking regions, but not the core of FRA3B, showing that a failure to utilize additional origins during replication stress may also contribute to fragility.

To date, no model has been found to exclusively explain the cell type–specific fragility or CFS loci and it is likely that aspects of all three models contribute to CFS instability. This complexity makes CFS a good model for studying how genomic instability develops in the complex landscape of the cell.

10. CONCLUSIONS

In summary, the study of the roles of chromatin and nuclear organization in maintaining genome stability is an active and developing field and the advance of novel technologies promises exciting new discoveries. CRISPR, the new genome-editing technology, will allow researchers to easily engineer specific mutations within chromatin-associated proteins and study their effects on genome stability; this technology can also be used to recruit proteins such as DDR components, repressors and activators, or fluorescent tags to endogenous genomic loci, in contrast with the *LacO*/*TetO* systems described earlier which are based on repeat arrays artificially integrated within the genome. Development of biologically faithful in vitro chromatin models is also a major aim of the chromatin field; such a system would be important both as a model to study chromatin structure and as a template which can be used to study how various components of the DDR interact with chromatin. The reduced cost of high-throughput sequencing has transformed the field, while high-throughput imaging approaches are also becoming more accessible. Still, numerous questions remain to be answered before our understanding of chromatin response to genomic instability is complete.

GLOSSARY

Breakage-first model Another model for the generation of translocations, in which the frequency of translocations between two chromosomes is independent of their preferred nuclear positions. This model hypothesizes that double-stranded breaks are brought together in the nucleus for repair, sometimes resulting in translocations. Recent data contradicts the breakage-first model.

Chromatin remodelers Proteins which can reposition and remove nucleosomes or change their composition in an ATP-dependent manner.

Chromonema A level of large-scale organization of the chromatin fiber measuring 100–130 nm in diameter. Although chromonema fibers have been observed by electron microscopy, their fine-scale organization is unknown.

Chromosome painting A FISH-based technique based on hybridization of chromosome-specific probes which allows visualization of whole chromosome territories.

Chromosome territory The defined nuclear volume occupied by a chromosome.

Contact-first model A model for the generation of translocations which suggests that the likelihood of a translocation occurring between two chromosomes depends on their proximity in the nucleus prior to generation of double-stranded breaks.

Laminopathy A class of diseases resulting from defects in the structure of the lamina component of the nuclear envelope. Laminopathies are caused by mutations in genes encoding the components of the nuclear lamina, including *LMNA* and *LMNB2*, and have diverse phenotypic characteristics such as muscular dystrophy and premature aging.

RNA–DNA (R-loop) hybrids RNA:DNA hybrid structure which can occur during transcription if the nascent RNA hybridizes to the complement DNA strand and displaces the nontemplate DNA strand. When such structures are resolved, ds DNA breaks are generated, implicating R-loops in genomic instability.

Sister chromatid decatenation The process of separating entanglements and catenanes between sister chromatids following replication and prior to cell division.

LIST OF ABBREVIATIONS

ATP Adenosine triphosphate
CFS Common fragile sites
DDR DNA-damage repair
DNA Deoxyribonucleic acid
FISH Fluorescence in situ hybridization
GFP Green fluorescent protein
HAT Histone acetyltransferase
HDAC Histone deacetylase
HGPS Hutchinson–Gilford progeria syndrome
HJ Holliday junction
HKMT Lysine methyltransferase
HR Homologous recombination
ICF Immunodeficiency, centromeric instability, facial anomalies syndrome
LAD Lamina-associated domain
MNase Micrococcal nuclease
mRNA Messenger RNA
NHEJ Nonhomologous end joining
PRMT Arginine methyltransferases
PTM Posttranslational modification
rDNA Ribosomal DNA
RNA Ribonucleic acid
TAD Topologically associated domains
UV Ultraviolet

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