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Mechanisms and Consequences of Double-strand DNA Break Formation in Chromatin

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Abstract

All organisms suffer double-strand breaks (DSBs) in their DNA as a result of exposure to ionizing radiation. DSBs can also form when replication forks encounter DNA lesions or repair intermediates. The processing and repair of DSBs can lead to mutations, loss of heterozygosity, and chromosome rearrangements that result in cell death or cancer. The most common pathway used to repair DSBs in metazoans (non-homologous DNA end joining) is more commonly mutagenic than the alternative pathway (homologous recombination mediated repair). Thus, factors that influence the choice of pathways used DSB repair can affect an individual's mutation burden and risk of cancer. This review describes radiological, chemical and biological mechanisms that generate DSBs, and discusses the impact of such variables as DSB etiology, cell type, cell cycle, and chromatin structure on the yield, distribution, and processing of DSBs. The final section focuses on nucleosome-specific mechanisms that influence DSB production, and the possible relationship between higher order chromosome coiling and chromosome shattering (chromothripsis).

Keywords

Base excision repair; chromatin; chromothripsis; DNA double-strand break formation; double-strand break repair; homologous recombination; non-homologous end-joining; microhomology-mediated end joining; ionizing radiation; nucleosomes; replication forks; reactive oxygen species

1. MECHANISMS THAT GENERATE DOUBLE-STRAND BREAKS IN DNA

Double-strand breaks (DSBs) in DNA form as a result of exposure to exogenous agents such as radiation and certain chemicals, as well as through endogenous processes, including DNA replication and repair. In addition to these inadvertent occurrences, meiosis I entails the deliberate induction of DSBs, which triggers homologous recombination, thus helping to ensure normal chromosome segregation (reviewed in (de Massy, 2013)). Programmed formation of DSBs also occurs during the development of somatic nuclei in protozoans (reviewed in (Duharcourt et al., 2009)), mating-type switching in yeast (reviewed in (Haber,

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2012)), T-cell receptor formation in T-lymphocytes, and immunoglobulin class switching in B-lymphocytes (reviewed in (Soulas-Sprauel et al., 2007)). In this review, we will focus solely on inadvertently produced DSBs. This first section describes the nature and major sources of ionizing radiation (IR), the concept of radiation quality, and mechanisms that link radiation-exposure to the formation of DSBs. We also describe the production of DSBs by radiomimetic chemicals, and the endogenous production of DSBs during DNA replication.

What is ionizing radiation and where does it come from?

IR can be defined as any subatomic particle or electromagnetic wave that possesses enough energy to liberate electrons from atoms. A loss of electrons can disrupt covalent bonds and produce reactive oxygen species (ROS) that can react chemically with, and thereby damage, biological molecules. High-energy protons, electrons, and other charged particles, can dislodge electrons through direct electrostatic (Coulombic) interactions. High-energy but electrically neutral particles, such as neutrons, can interact with atomic nuclei to produce new isotopes. These are commonly unstable (i.e. radioactive), and emit charged α-particles (consisting of two protons and two neutrons) or β-particles (electrons produced by the decay of neutrons into protons, electrons, and neutrinos) as they decay. Photons are electrically neutral ‘wave packets’ that carry the electromagnetic force. Given sufficient energy, they can dislodge electrons via the photoelectric effect (Einstein, 1905).

The particles with the highest energies are found in cosmic rays, which originate mainly from the supernovae of massive stars and from supermassive black holes that reside at the center of the Milky Way and many other galaxies. Virtually all (~99%) of these particles are atomic nuclei stripped of their electron shells and traveling at relativistic speeds. Of these, ~90% are simple protons, derived from hydrogen, while another ~9% are α particles, derived from helium. The remaining ~1% are high-Z, high-energy (HZE) atomic nuclei, derived from heavier elements (Mewaldt, 1994; NASA, 2006). Some of these particles, electrons especially (Baker et al., 2014), are deflected or trapped by the Earth’s magnetic field, forming the Van Allen radiation belts. Particles that penetrate the radiation belts and enter the earth’s atmosphere collide with atomic nuclei in the air. These collisions produce highly energetic protons, antiprotons, and other, much less stable free hadrons, which decay to form electrons, neutrons, protons, α particles, and photons. The resulting cascade of ionized particles and photons, known as an air shower, can be many km wide (Hinton and Hofmann, 2009). Air shower-associated particles with energies that vastly exceed those needed for ionization (in the range of 1 GeV) arrive on earth at a rate of ~10,000 per square meter per second (Pierre Auger Observatory). For comparison, the electrons and protons that make up the solar wind have energies that range between 1.5 and 10 keV. Thus, the sun is responsible for only a small fraction of our cosmic radiation-derived exposure (Kim et al., 1999). Indeed, particles associated with coronal mass ejections (solar flares) may sometimes act as a barrier to lower energy cosmic rays (NASA and Phillips, 2005).

When IR meets biological tissues

As will be detailed in later sections, the type and energy of IR critically affects the kinds of DNA damage created, which in turn may influence the cell’s survival and the means by which the damaged DNA is repaired. IR-associated particles and photons penetrate

biological tissues in straight tracks, producing secondary tracks as they interact with atoms in the tissues. Linear energy transfer (LET) is defined as the energy transferred from an IR particle or photon per unit of distance traveled, and is reported as kiloelectron volts per micrometer. As summarized in Table 1, protons, α particles, and high atomic number HZE ions may ionize virtually every atom in their path, giving them high LET coefficients. In contrast, photons and electrons may travel several hundred nanometers between ionization events, giving them much lower LET coefficients. Thus, while protons and α particles will not penetrate as deeply as photons or electrons of equivalent energies, they are far more damaging per unit volume of tissue penetrated.

High LET radiation is a particular risk to astronauts as well as cancer patients treated with heavy ion radiation. Astronauts orbiting the earth are protected from cosmic rays, to a degree, by the Van Allen Radiation belts, whereas those traveling to the moon or beyond are at greater risk. There is considerable uncertainty about the actual magnitude of the increased radiation risk (NASA, 1998), due mainly to sparse information on the biological responses to high LET radiation. The cosmic ray flux, measured on Earth, varies considerably with latitude and longitude, mainly because the Earth's magnetic field diminishes near the north and south poles. As a results, cosmic ray-derived IR may thus account for a large fraction of the total radiation exposure for airline flight crews that routinely fly polar routes. However, radon accounts for a much larger fraction (~42%) of the general population's annual exposure to high LET radiation. Radon forms as an intermediate in the decay of thorium and uranium into lead, and is the only gas that consists entirely of radioactive isotopes. Other radioactive isotopes present in food, soil and building materials, account for another ~26% of one's average annual IR exposure.

The largest source of low LET IR comes in the form of high-energy photons, although β particles produced by radioactive decay also contribute to low LET IR in the environment. A photon's energy is inversely proportional to its wavelength, and photons that carry visible light (wavelengths of ~380 to ~750 nm) are not energetic enough to ionize atoms. Longer wavelength photons, such as those used in microwaves and transmission of radio and television signals, carry even less energy. The shorter wavelength photons associated with the other side of the visible light spectrum include ultraviolet rays, X-rays, and gamma rays, and carry much higher energies. Photons with wavelengths shorter than ~100 nm are energetic enough to ionize hydrogen atoms and break chemical bonds. The energies associated with airport security, medical X-rays and CT scans can be 1,000 to 10,000 times higher than this. Collectively, these devices are estimated to account for ~20% of the average person's annual IR exposure.

Direct induction of double-strand breaks by IR

By the late 1930s, experimental geneticists had established that IR can break chromosomes (Sax, 1938). With the advent of methods to isolate very long DNA, it became possible to demonstrate, using velocity sedimentation of DNA through neutral and alkaline sucrose gradients, that IR breaks the DNA within chromosomes. These breaks can occur either directly or indirectly. The most direct path entails a collision between a high-energy particle or photon and a strand of DNA, breaking the phosphodiester backbone. More commonly, IR

splits water molecules near DNA, creating hydrogen and hydroxyl free radicals ((Yamaguchi et al., 2005) and reviewed in (Barnard et al., 2013; Ward, 1988)). The short-lived, but highly reactive hydroxyl radicals may react with nearby DNA, producing single-strand DNA breaks (SSBs). Closely opposed SSBs, created by either route, may spontaneously convert into a double-strand break (DSB). In the simplest version of the SSB to DSB conversion hypothesis, each SSB forms independently. The number of SSBs that form each day in mammalian cells has been estimated at ~55,000 (Tice and Setlow, 1985), roughly one SSB per ~10^5 bp, or one SSB per ~10^4 helical turns. The probability of two opposing SSBs forming within one or two DNA helical turns of one another would thus be on the order of $(10^{-4} \times 10^{-4}) = 10^{-8}$. This frequency could account for the estimated 10–50 DSBs that form every day in every nucleated human cell ((Vilenchik and Knudson, 2003); and reviewed in (Mehta and Haber, 2014)), but only if SSBs persist for a large fraction of the cell cycle. As described below, some do but many do not. The studies that called into question the simple SSB to DSB conversion hypothesis tested the prediction that a 10-fold increase in rates of SSB formation, (e.g. from 10^{-4} to 10^{-3} per one or two helical turns) would be expected to increase the rate of DSB formation by ~100-fold (from 10^{-8} to $(10^{-3} \times 10^{-3}) = 10^{-6}$). The predicted quadratic relationship proved true for peroxide-generated DSBs but not for radiation-induced DSBs, which increased linearly with radiation doses up to several hundred Gray (Dahm-Daphi et al., 2000). The simplest interpretation of these results was that each peroxide-generated SSB reflects a single independent event, but that a collision with a single IR particle generates multiple damages, including closely opposed SSBs. These closely opposed lesions, initially dubbed “locally multiply damaged sites”, or LMDSs (Ward, 1988), are now commonly referred to as “clustered damages.” They are defined as two or more DNA lesions, created by a single track of radiation, that reside within one or two helical turns of DNA. Low or high LET radiation doses as low as 1 Gray (100 rad) can produce clustered lesions (Goodhead et al., 1993; Rydberg, 1996; Sutherland et al., 2000a), and Monte Carlo-based modeling of radiation tracks suggests that low and high LET radiation can generate, respectively, up to 10 and 25 lesions per damage cluster (Semenenko and Stewart, 2004). These may hinder the DNA processing events associated with DSB repair (see “A short primer on double-strand break repair,” below). As well, DNA ligase cannot reseal a SSB unless the break is “clean,” that is consisting of one 3’ hydroxyl end and one 5’ phosphate end. “Dirty” single and double strand DNA breaks created by high or low LET IR may contain virtually unprocessable, unligatable ends (Weinfeld and Soderlind, 1991). Tyrosyl-DNA phosphodiesterase, for example, can require several hours to remove phosphoglycolate moieties from 3’ DNA ends (Zhou et al., 2009).

Indirect generation of DSBs during attempted base excision repair (BER) of clustered lesions

Although the above-described direct mechanisms account for many of the DSBs that form in cells, a significant fraction is generated by indirect mechanisms. The reactive oxygen species (ROS) produced by IR-mediated radiolysis of water generate not only SSBs, but also a wide array of other DNA damages, including oxidized bases and sites of base loss (De Bont and van Larebeke, 2004). Most such lesions are subject to base excision repair (BER), which entails production of gapped repair intermediates (for reviews, see (David et al., 2007; Duclos et al., 2012; Hegde et al., 2008; Krokan and Bjoras, 2013; Robertson et al., 2009;

Wallace, 2014)). Normally, these 1–2 nucleotide DNA gaps are filled by DNA polymerase β and sealed by DNA ligase IIIα. However, the near-simultaneous attempted BER of clustered oxidative lesions can produce SSBs in both DNA strands, which may convert to a DSB, as illustrated in Figure 1. BER-dependent formation of DSBs has been observed in multiple *in vitro* studies (for reviews, see (Eccles et al., 2011; Sage and Harrison, 2011)) and now forms the basis for assays to measure the abundance of *non-strand break* clustered damages (Sutherland et al., 2000b; Sutherland et al., 2003). Specifically, one treats oxidatively damaged DNA with a mixture of recombinant, prokaryote-derived DNA glycosylases and endonucleases, fractionates the resulting DNA fragments by gel electrophoresis, and then calculates the break frequency from the number average size of the DNA fragments. There is compelling evidence that BER-driven DSB formation also occurs *in vivo*, in both prokaryotes and eukaryotes (Blaisdell et al., 2001; Blaisdell and Wallace, 2001; Yang et al., 2006; Yang et al., 2004). Specifically, mutation or knockdown of DNA glycosylases reduced the frequency of DSB formation in cells that had been exposed to IR. Conversely, overexpression of those same glycosylases sensitized cells to radiation and increased DSBs. The section below, “Impact of chromatin on the formation and persistence of DSBs”, describes mechanisms by which nucleosomes suppress (but do not fully eliminate) BER-dependent production of DSBs in eukaryotes.

DNA damaging ROS are produced not only by IR-mediated radiolysis of water but also during normal oxidative metabolism. Most of these endogenously-generated ROS are neutralized, but enough escape to generate roughly 10,000 oxidative base lesions and another 10,000 base loss events per cell per day ((Fraga et al., 1990; Lindahl and Nyberg, 1972) and reviewed in (Friedberg, 2006)). Given this, one might ask why the frequency of DSB formation is no higher than observed. The answer seems to be that, unlike the multiple, highly localized ROSs produced by a single IR particle or photon, each endogenously-generated ROS is produced independently and, therefore, rarely form clustered lesions.

DNA replication-associated DSBs

Most of the DSBs that can be attributed to endogenous processes are produced during DNA replication (Syeda et al., 2014). As depicted on the left in Figure 2, a replicative polymerase that encounters a SSB in the template strand may stall, leading to a collapse of the replicative fork and subsequent DSB formation (Pfeiffer et al., 2000). The SSB itself may be present as a DNA repair intermediate. DSBs can also occur as a result of replication fork stalling due unusual DNA secondary structures, bulky lesions, polymerase blocking oxidative lesions, abasic sites, chemical or IR-generated inter-strand crosslinks, or as a result of collisions with transcription complexes and certain DNA binding proteins (Dextraze et al., 2010; Mirkin and Mirkin, 2007; Prado and Aguilera, 2005). As depicted on the right in Figure 2, stalled replication forks may regress and partially displace newly synthesized DNAs from their template strands, enabling the 3’ end of the leading strand to anneal to the 5’ end of the lagging strand. The resulting “chicken foot” is structurally identical to a Holliday junction, which can be cleaved by Holliday junction resolvases or structure-directed nucleases, forming a DSB in the process (reviewed in (Mehta and Haber, 2014)). Importantly, DSBs generated at collapsed replication forks may sometimes be repaired by the highly mutagenic microhomology-mediated end-joining pathway (see section “A short

primer on double-strand break repair") rather than by error-free recombination mediated repair (Truong et al., 2013).

Transcription and R-loop-related formation of DSBs

Numerous studies have reported higher than average mutation rates in heavily transcribed regions of the genome. Some of these mutations may be due to elevated rates of DSB formation in these same regions. Variable rates of DSB formation across the genome are influenced by chromatin structure, as discussed in Section 3, below. However, some DSBs in heavily transcribed regions appear to result from transcription *per se*. DNA unwinding during the formation of replication and transcription bubbles generates superhelical stress in flanking DNA, which is relieved by topoisomerases. Once formed, transcription bubbles, and the ~8 bp RNA-DNA hybrids that form within them, remain relatively constant in size during transcription, because DNA unwinding ahead of the elongating RNA polymerase is matched by the progressive displacement of nascent RNA and DNA rewinding behind the RNA polymerase. However, transcription elongation may occasionally outpace the action of topoisomerases, resulting in a transient accumulation of positive superhelical density ahead of the RNA polymerase, and a corresponding accumulation of negative superhelical density in the trailing region (Liu and Wang, 1987). Negative superhelical density may delay reannealing of DNA, and enable a portion of the nascent RNA to reanneal to its DNA complement, after it exits the RNA polymerase. The result is an R-loop. At least in yeast, R-loop formation also requires Rad51p and Rad52p, which normally act in homologous recombination, and is suppressed by the DNA helicase and Rad51p antagonist, Srs2p (Wahba et al., 2013). One might expect the packaging and processing of nascent RNAs to reduce the probability of R-loop formation, and RNA-DNA helicases and RNase H enzymes to restrict the lifetime of R-loops that do form. Consistent with this prediction, defects in certain RNA processing enzymes produce hyper-recombination phenotypes can be suppressed by overexpression of RNase H (reviewed in (Hamperl and Cimprich, 2014)). Exactly how R-loops are transformed into DSBs is unclear, but candidates include the DNA and RNA editing enzymes in the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) family (Hamperl and Cimprich, 2014). The prototype enzyme in this family is activation-induced cytidine deaminase (AID), which converts cytidine to uracil during class switching in B cells (Stavnezer, 2011). The resulting dU-dG mismatches are substrates for uracil-DNA glycosylase (UDG), which excises uracil moieties, leaving an abasic site. The abasic site could subsequently halt progression of a replication fork, leading in some cases to fork collapse and rescue via homologous recombination. Alternatively, the abasic site might be processed further via BER, with formation of a single-strand gapped intermediate that might then be converted to a DSB during replication. The postulated involvement of DNA replication in R-loop mediated DSB formation is consistent with the observation that the hyper-recombination phenotype seen in certain RNA processing mutants is confined to genes transcribed during S phase, and is not evident among genes transcribed in G2 phase (Wellinger et al., 2006). Various genome wide genetic and proteomic screens have further cemented the links between defective RNA processing, R-loop formation, and genome instability. For example, certain RNA processing factors are among those proteins targeted by the DNA damage response kinases, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) (Matsuoka et al., 2007) (Smolka et al., 2007).

Induction of DSBs by radiomimetic compounds

Exogenous chemicals that damage DNA are referred to as *clastogens*. These include anti-cancer drugs, such as the DNA-alkylating agents methyl methanosulfonate and temozolomide, and the DNA cross-linking reagents cisplatin and mitomycin C. Clastogens can induce DSBs indirectly, via the above-described cellular processes. Chemical agents that induce strand breaks directly are commonly referred to as "radiomimetic" compounds because of their ability to induces DSBs. Bleomycin, for example, is a radiomimetic compound that the World Health Organization has included in its 2013 list of essential medicines for treatment of high priority diseases. "Activated bleomycin" contains a reduced transition metal (Fe(II) or Cu(I)), oxygen and a one-electron reductant (often hydrogen peroxide). Decomposition of activated bleomycin in the presence of DNA can generate strand breaks and create additional hydroxyl radicals. Although bleomycin carries just one reactive moiety, it somehow produces about one DSB for every 10 single-strand breaks it generates (Chen and Stubbe, 2005; Povirk, 1996).

Even more efficient than bleomycin at generating DSBs are the bicyclic enediynes, molecules that contain a C-C double bond flanked by C-C triple bonds. These molecules can cyclize to form a 1,4-dehydrobenzene intermediate. Neocarzinostatin, which is produced by *Streptomyces carzinostaticus*, is a small protein with a tightly bound chromophore. Cyclization of the chromophore forms a highly reactive para-benzyne diradical that can abstract hydrogens from the C-1', C-4' and C-5' positions of deoxyribose moieties in both DNA strands. Subsequent reactions between these carbon atoms and molecular oxygen can produce DSBs. Several enediyne compounds are in clinical trials for possible use in cancer therapies. However, until it becomes possible to deliver these compounds to specific cellular targets, it is unlikely that their use will supplant radiation therapy.

Topoisomerases play critical roles in DNA metabolism, relieving local super-helical stress associated with formation of transcription and replication bubbles, and with the assembly and disassembly of nucleosomes. Class I topoisomerases generate transient SSBs in DNA, while Class II topoisomerases generate transient DSBs, which enables them to carry out decatenation reactions. Camptothecins are topoisomerase I inhibitors, while etoposides are topoisomerase II inhibitors (Hande, 1998; Koster et al., 2007). Both act by stabilizing topoisomerase-DNA intermediates, preventing re-ligation. The topoisomerases can thus be trapped through covalent linkages with DNA ends, creating protein-associated SSBs or DSBs. The protein-DNA moieties must be removed by specific endonucleases before repair can ensue. Thus, repair of such damages necessarily entails deletion of short segments of DNA (Takahashi et al., 2011).

Can our knowledge of mechanisms of DSB formation help guide public policy on radiation exposure?

There is widespread agreement among scientists and policy experts about the importance of limiting exposure to high doses of IR. There is much less agreement on the risks associated with very low dose IR (e.g. (Doss et al., 2014)). The adoption of assays based on counting radiation-induced DSB repair foci, visualized by fluorescent tagging of antibodies to phosphorylated histone H2AX (γ-H2AX), tumor suppressor P53 binding protein 1 (53BP1),

or auto-phosphorylated ATM, has made it possible to investigate the occurrence and rates of repair of DNA damage resulting from radiation doses far lower than were commonly used in early studies. With the development of biomarkers for radiation exposure it should be possible to gather more precise epidemiological information (Pernot et al., 2012). However, the number of potentially confounding variables has increased along with the precision and quality of exposure and repair assays. For example, cardiovascular disease may increase mortality following radiation exposure (Little et al., 2012). It is also likely that cancer risks associated with lose dose IR will vary with genetically-determined differences in DNA repair pathways and immune surveillance. Such variables should be considered in the design and evaluation of studies of low dose IR risks. Unfortunately, in doing so, one may lose the very statistical power needed to assess risk. Because of this conundrum, it is unlikely that debates on the risks of low dose IR will be settled anytime soon.

2. A SHORT PRIMER ON DOUBLE-STRAND BREAK REPAIR

Although this review focuses on mechanisms of DSB formation, the impact of DSBs, and the manner in which they are repaired, varies with their etiology, for example whether DSBs result from high or low LET radiation. To facilitate this discussion, this section briefly summarizes the damage response pathways that process DSBs. There are several excellent, recent reviews of DSB repair for readers looking for more detailed expositions (e.g. (Asaithamby and Chen, 2011; Lieber, 2010; Mehta and Haber, 2014; Polo and Jackson, 2011; San Filippo et al., 2008; Thompson, 2012)).

Non-homologous end-joining (NHEJ)

NHEJ is the only DSB pathway that normally operates in G0 and G1 phases of the cell cycle. NHEJ is initiated by the binding of Ku70/80 heterodimers to DNA ends created by a DSB event. Ku heterodimers exist as preformed rings that encircle 3–4 bp of DNA, fitting along the contours of the major and minor grooves (Walker et al., 2001). Once bound to DNA, Ku recruits the X-ray repair cross-complementing protein 4 (XRCC4) and the Cernunnos-X4-like factor (Cer-XLF), which help bridge the two DNA ends. If the DNA ends are complementary and undamaged, they can be joined directly by the XRCC4-associated DNA ligase IV (Reynolds et al., 2012). If the DNA ends are damaged or not fully complementary, ligation cannot occur without further end processing. In this case, Ku recruits DNA protein kinase (DNA-PK). The binding of DNA-PK activates its catalytic subunit (DNA-PKcs), which leads to the recruitment and phosphorylation of the endonuclease Artemis. Provided that one or two complementarity nucleotides in the two DNAs are able to anneal with one another, Artemis will remove excess single-strand DNA. This generates a substrate that can be ligated by DNA ligase IV, acting in concert with XRCC4 and Cer-XLF.

Homology-directed recombination-mediated repair (HRR)

A large fraction of the DSBs that form during the S or G2 phase of the cell cycle are repaired by HRR, which begins with resection of 5’ DNA ends that flank a DSB, as illustrated in Figure 3. This resection is catalyzed primarily by MRN, a trimeric complex containing MRE11, RAD50, and Xrs2/NBS1. MRE11 possesses both single-strand

endonuclease and 3’ to 5’ exonuclease activities that can process ‘clean’ DNA ends, such as those created by endogenous expression of restriction endonucleases. However, the processing of DNA ends that form hairpins or are attached to proteins requires additional help from the endonuclease Sae2/Ctp1/CtIP. Acting together, MRE11 and CtIP remove 5’ strands in 50 to 100 NT increments (reviewed in (Mimitou and Symington, 2009)). The single-strand endonuclease DNA2 or the FLAP endonuclease EXO1, acting in concert with the RecQ family helicase Sgs1/BLM, catalyze further resection. The 3’ ssDNA tails generated by these resection events are bound first by the ssDNA binding protein RPA. RPA is subsequently replaced by the RAD51 recombinase, which catalyzes the search for complementary DNA, usually in the form of a sister chromatid. As the RAD51–3’ single-strand DNA filament from one side of the DNA break invades and anneals to complementary sequences in its sister chromatid, it displaces the opposing strand in the sister chromatin, forming a D-loop. The invading 3’ single-strand DNA then primes new DNA synthesis. This extends the D-loop, exposing bases that can anneal to the 3’ single-strand DNA from other side of the DNA break. This second annealing event, when it occurs, primes leading strand synthesis of DNA on the opposing strand. Ultimately, these cross-over structures (“double Holliday junctions”) are resolved, leaving widely separated, single-strand DNA nicks that are sealed by DNA ligase. In some instances, the first invading strand primes new DNA synthesis, but the second annealing event fails to occur. Instead, the original invading strand is displaced and, having been lengthened by new DNA synthesis, can now anneal to 3’ single-strand DNA from other side of the DNA break (Figure 3E). This synthesis-dependent strand annealing (SDSA) provides a template for repair synthesis. HRR is commonly viewed as an error-free repair pathway, and indeed one is less likely to observe the highly localized short deletions and base changes that often accompany NHEJ. On the other hand, the fidelity of DNA repair synthesis is not as high as one sees in normal genomic replication; thus one may find more broadly distributed base substitutions in the wake of HRR (Malkova and Haber, 2012).

Alternative (microhomology-mediated) end-joining (alt-EJ/MMEJ)

Microhomology-mediated end-joining is linguistically related to NHEJ but shares key mechanistic features with HRR ((Truong et al., 2013) and references therein). MMEJ appears to operate mainly during S-phase of the cell cycle, in a Ku- and DNA-PK-independent fashion. As with HRR, MMEJ begins with the resection of 5’ DNA ends at the DSB, leaving behind 3’ single-strand DNA. Although MMJE and HRR employ the same end processing enzymes, subsequent steps diverge. MMEJ occurs when end-resection exposes micro-homologies of 5–25 bp that enable DNA single strands to anneal. This creates a substrate that, following removal of non-annealed DNA ends, is competent for gap filling and ligation (by DNA ligase IV in yeast and DNA ligase III in mammalian cells), as also occurs during single strand annealing repair (see (Frankenberg-Schwager et al., 2009)).

Regulation of pathway choice during DSB repair

The pathway that cells use to process DSBs depends on multiple variables, including the cell cycle stage during which the DSB is generated, and whether the DSB itself is ‘clean’ or ‘dirty.’ DSB formation during late S-phase or G2 phase triggers a DNA damage checkpoint that prevents cells from progressing through mitosis until DNA is repaired. Likewise, DSB

formation during G1 phase triggers a checkpoint that prevents cells from entering into S phase. Interestingly, the G1/S phase checkpoint is absent in mouse embryonic stem (ES) cells and, as a result, ES cells that suffer DSBs during G1 phase commonly undergo apoptosis; DSBs that form in ES cells during S or G2 phases are repaired by HRR. This may explain why ES cells are hypersensitive to IR, and also why surviving cells accumulate far fewer mutations than do isogenic embryonic fibroblasts (Tichy et al., 2010). In addition to checkpoint control-related mechanisms, the choice of HRR over NHEJ in S and G2 phases is influenced by the cell cycle-dependent, activating phosphorylation of the resection factor Sae2 (Huertas et al., 2008). Presumably, the absence of active Sae2 in G0 or G1 phase suppresses attempted HRR at a stage where cells lack the homologous DNA that HRR requires. It is not clear if there are analogous mechanisms that suppress NHEJ during S and G2 phase, beyond the fact that end-resection at DSBs inhibits the binding of Ku proteins that initiate NHEJ. Interestingly, the checkpoint kinase ATR, when activated, modulates the relative activity of EXO1 and CtIP: ATR-mediated phosphorylation of EXO1 renders it vulnerable to proteolysis, whereas ATR-mediated phosphorylation of CtIP is required for its chromatin binding and resection activity (Peterson et al., 2013). These phosphorylation thus events may influence the choice between MMEJ and HRR. Replicative age may also influence pathway choice. In aging mice, the frequency of MMEJ appears to increase while that of NHEJ declines (Vaidya et al., 2014).

Impact of DSB etiology on DSB repair

Whether DSBs are ‘clean’ or ‘dirty’ can influence both the pathway used to repair a DSB, and the overall rate of repair. As noted earlier, DNA that flanks DSBs produced by high-energy protons and α particles may contain numerous additional damages that slow or inhibit the processing of DNA ends for NHEJ. (Pinto et al., 2005), for example, reported that some DSBs generated by exposure to relatively massive α particles had half-lives of one hour or less, whereas others had half-lives of 12–16 hours; a small fraction of the DSBs persisted so long as to appear unrepairable. (Staaf et al., 2012) reported that gamma (photon) radiation generates as many or more γ-H2AX-marked DSB repair foci than do α particles. However, the rates of repair were faster for gamma radiation-induced DSBs than for α particle-induced DSBs. Taken together, these studies suggest that lesions induced by high LET radiation, even if no greater in total extent, are generally more difficult to repair, making them all the more damaging. For DSBs that form during late S or G2 phase, this may shift the balance toward homologous recombination-mediated repair (or to MMEJ), owing to the capacity of the CtIP endonuclease to bypass complex DNA structures. Because HR requires cells to enter into S or G2 phase, one might predict that complex DSBs that form in G0 or G1 can be repaired only if cells are able to bypass the G1/S phase checkpoint. It appears, however, that CtIP and other end resection factors can be recruited to complex DSBs in G1 phase (Averbeck et al., 2014; Yajima et al., 2013) where, presumably, they initiate MMEJ-mediated repair of complex damages. Interestingly, CtIP must be phosphorylated to act in HRR, but apparently not when acting in MMEJ (Yun and Hiom, 2009).

DSB repair mechanisms and the optimization of radiotherapy for cancer patients

As described above, DSBs induced by high LET radiation are difficult to repair, and may thus be more frequently lethal than DSBs generated by equivalent doses of low LET radiation. However, these predicted differences in cell lethality do not seem to have translated to significantly better outcomes for radiotherapy patients (Terasawa et al., 2009). There may be several reasons for this, including differences in IR dose fractionation and delivery. For example, the high LET coefficients of protons has meant using proton beam energies of 70–250 MeV to ensure sufficient penetration, as compared to photon beam energies of 6–15 MeV for equivalent tumors (e.g. (Sung et al., 2012)). On a more optimistic note, advances in our understanding of DSB repair mechanisms are beginning to pay dividends in the form of adjuvant therapies for treatment of certain cancers. For example, cancer cells containing BRCA 1 or BRCA 2 mutations are generally unable to repair DSBs by HRR, making them heavily reliant on NHEJ and MMEJ. Such cells exhibit elevated vulnerability to IR when treated with inhibitors of poly-ADP ribose polymerase 1 (PARP1), which is required for MMEJ. Accordingly, some PARP1 inhibitors have already been approved for treatment of certain cancers, and others are in phase III clinical trials (Sonnenblick et al., 2015).

3. IMPACT OF CHROMATIN STRUCTURE ON DSB FORMATION

Nucleosome structure and changes in the chromatin landscape as a function of transcription and cell cycle progression

Most of the DNA in nuclei is packaged in chromatin, which consists of regularly spaced nucleosomes, punctuated by nucleosome-free DNA segments that associate with factors engaged in transcription, DNA repair, DNA replication, and telomere-related activities. Each nucleosome consists of ~147 bp DNA, wrapped in a left-handed toroidal helix around a protein core, containing two copies each of histones H2A, H2B, H3 and H4 (Luger et al., 1997). Individual nucleosomes are separated from one another by segments of linker DNA that, in humans, average ~50 bp in length, but can vary from ~20 bp (e.g. in *S. cerevisiae*) to ~110 bp (e.g. in sea urchin sperm). Most nucleosomes assemble during replication, in a stepwise fashion that is coordinated with movement of the DNA replication fork and facilitated by specific histone chaperones. In the first step, DNA associates with a histone H3–H4 tetramer. This is followed by the addition of histone H2A–H2B dimers to either side of the central H3–H4 tetramer. Prior to and following assembly of nucleosomes, histones are subject to numerous (and in most cases reversible) secondary modifications that influence interactions between nucleosomes and various regulatory and structural factors. In addition to functional heterogeneity imparted through histone modifications, cells contain chaperones that catalyze the exchange of histone primary sequence variants in a replication-independent fashion. Chromatin associated with transcriptionally active regions is generally in a relatively “open” or extended configuration, commonly referred to as a 10 nm filament (or fiber) (Finch and Klug, 1976; Olins and Olins, 1974; Oudet et al., 1975; Woodcock et al., 1976). 10 nm chromatin fibers can coil to produce more compact, 30 nm filaments that, generally, are transcriptionally inactive (Finch and Klug, 1976; Olins and Olins, 1979; Pooley et al., 1974; Ris and Kubai, 1970; Worcel and Benyajati, 1977). In advance of mitosis or meiosis, the 30 nm fibers assemble into radially arrayed 50–100 kbp loops, each

anchored to a central axis that forms the long axis of meiotic and mitotic chromosomes. Clearly, chromatin is a dynamic entity, and one might expect its impact on DSB formation to vary across the genome (as a function of transcription, for example) and at different points in the cell cycle. We discuss these variables below.

Nuclear factors and conformational variables in DNA that influence the susceptibility of DNA to free radical-mediated damage

The direct production of DNA strand-breaks is commonly driven by reactions between hydroxyl radicals (OH·) and hydrogen atoms in deoxyribose (Balasubramanian et al., 1998). As summarized in Table 2, those atoms are less accessible in DNA with unusually narrow minor grooves (Isabelle et al., 1995). Variations in groove width are sometimes sequence-directed, which may, for example, explain why 5'-AATT-3' sequences are less sensitive than average to radiation-induced strand-breaks (Franchet-Beuzit et al., 1993; Sy et al., 1997). In other instances, variations in groove geometry are induced by DNA circularization, supercoiling, or bending, as when DNA winds about the histone octamer. This might explain why plasmid DNA is more radiosensitive in a relaxed configuration than when supercoiled (Swenberg and Speicher, 1995). As well, small molecules, such as the polyamine spermine, can moderate reactions between ROS and DNA (Chiu and Oleinick, 1998; Spotheim-Maurizot et al., 1995; Warters et al., 1999). Spermine may exert this protective effect by displacing water molecules from DNA, thereby reducing the local yield of hydroxyl radicals formed by collisions between IR and water (van Dam et al., 2002); spermine may also react with, and thereby neutralize, local ROS. In a similar fashion, histones and other chromatin associated proteins protect DNA by physically masking reactive moieties, and by scavenging or reacting with ROS. Evidence of this includes studies in which the frequency of DSB formation increased after chromatin-associated proteins were stripped from DNA, using high salt (reviewed in (Lavelle and Foray, 2014)). Although results from such studies vary with methodology, cell type, and radiation dose, they suggest that non-histone proteins and the linker histone H1 provide a two to five-fold protection from DSBs, while the core histones provide the most protection: the frequency of DSBs increased on the order of ~20–40-fold with the removal of histone H2A–H2B dimers, and up to 70-fold with the removal of all four core histones.

Nucleosomes suppress formation of DSBs during attempted BER of clustered lesions

The close association between DNA and the histone octamer inhibits access by the large radiomimetic compounds described earlier (e.g. (Wu et al., 1999)). As a result, damage from such compounds is confined largely to linker DNA and regions such as promoters, where nucleosome occupancy is lower than in transcriptionally quiescent regions. ROS, on the other hand, are much smaller, and can react with most of the DNA in nucleosomes. Thus, the overall extent to which nucleosomes mask ROS-reactive moieties in DNA is modest, although one commonly sees a ~10 bp periodicity in ROS reactivity, which matches the helical repeat of DNA in nucleosomes (Franchet-Beuzit et al., 1993; Hayes et al., 1990). This periodicity, which is also evident when nucleosomes are probed with DNA endonucleases whose activities are similarly sensitive to groove width (e.g. (Prunell, 1983)), can be attributed to three structural features, acting in concert. First, most DNA segments adopt a discrete helical orientation when bent, either free in space or when wrapped about a

histone octamer (Drew and Travers, 1985; Satchwell et al., 1986). Second, moieties on the outside of a bend are typically more reactive than those on the inside because of differences in the width of the minor groove. Finally, reactions between ROS and nucleosomal DNA are impeded by the discrete interactions between histones and the DNA backbone at every helical turn (Luger et al., 1997).

In vitro studies indicate that BER enzymes can repair oxidative lesions at many sites in nucleosomes, without irreversibly disrupting the host nucleosome (reviewed in (Odell et al., 2013)). Thus, BER may differ from other DNA repair pathways, where nucleosome disruption is thought to precede or accompany repair. The efficiency with which single oxidative lesions are repaired varies both with the distance between the lesion and the edge of the nucleosome, and the lesion's helical orientation relative to the underlying histone octamer ((Odell et al., 2013); also, (Maher et al., 2013; Menoni et al., 2012; Rodriguez and Smerdon, 2013; Ye et al., 2012)). Given that nucleosomes are semi-permissive for BER of single oxidative lesions, one might expect that nucleosomes would offer only limited protection from the BER-dependent conversion of clustered lesions to DSBs. When tested however, nucleosomes suppressed DSB formation at clustered lesions far more than predicted (Cannan et al., 2014). The underlying reasons for this are, first, that the processing of one oxidative lesion will interfere with processing of an opposing strand lesion unless the two lesions are offset from one another by at least three bp (Figure 4). Second, BER of clustered, opposing strand lesions will generate DSBs only if the repair at each lesion is initiated more or less simultaneously and proceeds with similar kinetics. This can only occur in nucleosomes if both opposing strand lesions are sterically accessible. BER factors are able to bind and process sterically occluded lesions in nucleosomes, but only during the relatively infrequent episodes of spontaneous, transient partial unwrapping of DNA from the histone octamer (Maher et al., 2013; Prasad et al., 2007). The upshot is that only a small fraction of the clustered lesions that form in nucleosomes exposed to IR will exhibit a configuration that satisfies both of these geometric constraints.

The extent to which nucleosomes suppress DSB formation *in vitro* is substantial, but it is difficult to estimate the magnitude of protection that nucleosomes offer in cells. Dynamic behaviors intrinsic to nucleosomes, such as transient, partial unwrapping of DNA from the histone octamer, will likely reduce their protective effect, as may extrinsic factors such as histone chaperones and chromatin remodeling agents. (The possibility that chromatin-remodeling agents facilitate BER in cells has yet to be convincingly demonstrated.) On the other hand, linker associated factors such as histones H1 and H5, which limit nucleosome mobility (Pennings et al., 1994) and stabilize higher order coiling of the chromatin fiber, probably enhance the nucleosome-mediated protection from DSB formation. This prediction is consistent with studies summarized below, which indicate, collectively, that transcriptionally competent or active euchromatin is more vulnerable to IR than is heterochromatin. It is likely that the elevated vulnerability of euchromatin to DSBs is due in part to more efficient attempted BER of clustered lesions.

The impact of higher order chromatin structure on rates of DSB formation

The distinction between hetero- and euchromatic regions in chromosomes dates to the early 20th century (Heitz, 1928; Heitz, 1929). “Regions of increased gene expression,” or RIDGEs (Caron et al., 2001; Versteeg et al., 2003), reside within euchromatic regions. The chromatin within these regions is in a relatively open configuration (Goetze et al., 2007). Controversy over whether euchromatin is more susceptible to IR-induced DSB formation than heterochromatin persisted for many years, due mainly to differing experimental conditions and methods. Early studies that relied on counting chromosome aberrations in mitotic spreads suggested that heterochromatin is more vulnerable to chromosome breaks than euchromatin (e.g. (Natarajan and Ahnstrom, 1969). However, later studies indicated that translocations occur more frequently in euchromatin (reviewed in (Folle et al., 1998)). By combining pulsed field gel electrophoresis with Southern blotting, (Chiu et al., 1982) were able to show that housekeeping genes are more vulnerable to IR-induced DSBs than α-satellite or bulk DNA. As well, active genes were often found to occur in clusters, surrounded by more radiosensitive chromatin (Bunch et al., 1995). Later studies that used immunostaining of γ-H2AX to monitor DSB formation generally confirmed results from the pulsed field gel electrophoresis studies. Despite the near universal adoption of the immunostaining assays, it is still difficult at times to compare results from different labs. A particularly critical variable in accurately assessing the relative abundance of DSBs in hetero- and euchromatin is the time interval between irradiation and visualization of γ-H2AX foci. If the interval is too brief, foci formation will not be maximal; if too long, a significant portion of the damage may have already been repaired. A related challenge is the apparent suppression of γ-H2AX foci formation in heterochromatin (Kim et al., 2007) and the correspondingly slow repair of DSBs in heterochromatin ((Slijepcevic and Natarajan, 1994); reviewed in (Falk et al., 2010)). Indeed, DSB repair may not occur at all until heterochromatin decondenses (Kruhlak et al., 2006; Lorat et al., 2012). Thus, slow or delayed repair could explain reports of elevated radiation sensitivity in condensed chromatin (Chapman et al., 2001; Stobbe et al., 2002). One of the most compelling studies on the vulnerability of transcriptionally active chromatin to DSB formation combined use of γ-H2AX immunofluorescence with Immuno-FISH, to quantify IR-induced DSBs in individual chromosomes (Falk et al., 2008). The authors compared human chromosome 18, which is largely transcriptionally silent, to chromosome 19, which is similar in size but highly transcribed. Chromosome 19 proved to be ~four-fold more sensitive to IR-induced DSBs than chromosome 18. The authors also compared two 11 Mbp regions (one RIDGE and one ‘anti-RIDGE’ region), both on Chromosome 11. Here too, the RIDGE region was ~four-fold more vulnerable to DSBs than was the anti-RIDGE region. This study strongly suggests that gene density alone can account for a higher than normal sensitivity to IR.

X-ray scattering studies of isolated nuclei have revealed diffraction peaks at 30–40 nm (reflecting side-by-side packing of ~30 nm chromatin filaments), 11 and 6 nm (reflecting the packing constraints of individual nucleosomes), and 2.1 and 2.7 nm (reflecting the diameter of DNA and its pitch within nucleosomes, respectively) ((Langmore and Paulson, 1983), and references therein). This is evidence of relatively uniform DNA packaging at multiple levels. Given this, one might expect chromatin to influence DSB patterns on a scale ranging from ~150 bp (the amount of DNA in one nucleosome) to ~100,000 bp (corresponding to the

distance between adjacent chromatin loops in mitotic chromosomes). In asking if IR-induced DSB patterns reflect the packaging of DNA in chromatin, or if higher order chromatin coiling protects DNA from DSB formation, one should also consider whether the damaging agent is low or high LET IR. As noted earlier, low LET IR consists largely of electrons and photons that liberate secondary electrons (β particles) and produce ROS. Although low LET IR can create closely-spaced, clustered lesions (Chatterjee and Holley, 1991), collisions between low LET particles and atoms in tissues are infrequent, and thus low to moderate levels of low-LET may produce DSBs that are more or less randomly distributed. High LET α particles and heavy ions travel along straight tracks and exhibit far higher collision rates, producing DSB patterns that appear to be non-random (Lobrich et al., 1996; Prise et al., 2001; Rydberg, 2001). The distribution of high LET IR-induced damage became more random when chromatin proteins were removed prior to IR (Radulescu et al., 2004). This is direct evidence that non-random DSB patterns from high LET IR reflect the packaging of DNA in chromatin. It also supports the use of IR-induced DSB patterns to determine nucleosome positions and investigate the structure of the 30-nm chromatin fiber (Bernhardt et al., 2003; Rydberg, 2001; Rydberg et al., 1998). A discussion of how these DSB patterns relate to the competing zig-zag and “solenoid” models of higher order chromatin would take us beyond the scope of this review, but interested readers may wish to consult one or more of the recent reviews of the subject (Grigoryev and Woodcock, 2012; Joti et al., 2012; Mozziconacci and Lavelle, 2009; Tremethick, 2007; van Holde and Zlatanova, 2007).

Chromothripsis

In cancers of all kinds, 2–3% of cells contain chromosomes that show signs of having suffered numerous DSBs, inversions, and deletions. When such scars are evident in multiple chromosomes, this phenomenon is referred to as chromoplexy. In chromothripsis (“chromosome shattering”), the chromosome rearrangement and deletion events can number in the hundreds, but are usually confined to a single chromosome (reviewed in (Forment et al., 2012)). Other distinguishing features of chromothripsis include a low gene copy number within shattered regions, and alternations between retained heterozygosity and loss of heterozygosity (Maher and Wilson, 2012)). The chromosome rearrangement and deletion events point to the involvement of NHEJ during the repair of shattered chromosomes, while DNA regions that exhibit loss of heterozygosity may result from break induced DNA replication.

But what generates so many DSBs in the first place? There are several hypotheses, none mutually exclusive. One hypothesis is that chromothripsis reflects an aborted apoptosis (Tubio and Estivill, 2011), where DNA was partially fragmented by endonucleases that are activated during apoptosis. Although reversal of apoptosis has been observed (Tang et al., 2012), this model does not explain why the endonucleolytic activity would have been confined to a single chromosome. A second model posits that NHEJ of eroded telomeres generates sister chromatid fusions. This produces a dicentric chromosome that may be pulled to opposite poles during mitosis. Such events often end when the chromosome breaks, producing new DSBs. This sets the stage for another round of NHEJ that restores the dicentric chromosome, allowing the breakage-fusion cycle to repeat (Colnaghi et al., 2011;

Meyerson and Pellman, 2011). In this model, the chromothriptic phenotype develops over the course of many cell cycles. One problem with this theory is that chromosomal termini are *not* usually lost during chromothripsis. Thus, the postulated initial sister chromosome fusion event would more likely be triggered by a localized set of DSBs than by telomere attrition. A third, and perhaps the most widely accepted, model begins with the mis-segregation of a chromosome during mitosis, which would occur if sister chromatids were slow to decatenate following DNA replication or sister chromatid recombination. As a result, the lagging chromosome may be excluded from the daughter nucleus that reassembles after telophase. Instead, the lagging chromosome is packaged into a separate, small micronucleus that may be starved for DNA replication and repair factors, owing to an insufficient number of nuclear pores. The resulting lag in DNA replication of the chromosome within the micronucleus may not become apparent until nuclei break down in advance of mitosis. At that point, the incompletely replicated chromosome will trigger a DNA damage response, but one that may decay before replication is complete (Giunta et al., 2010; van Vugt et al., 2010). As cells enter metaphase, the incompletely replicated chromosome is "pulverized," perhaps as a result of premature chromosome condensation. Micronuclei may repeatedly reassemble around chromosomes or chromosome fragments that are no longer capable of normal disjunction. Thus pulverization and rearrangement may occur over multiple cell divisions, until the damaged, rearranged chromosome, or fragments thereof, are taken up by the nucleus (Crasta et al., 2012).

The findings and concepts outlined in this review suggest one additional model that might also contribute to chromothripsis, namely that the numerous, localized DSBs could be generated by collisions between a few high LET particles and a single mitotic chromosome. As noted earlier, a single high LET particle can generate multiple DSBs in relatively small region of the nucleus. Chromatin packaging can bring widely spaced DNA segments into close proximity with one another, increasing the likelihood that both might be damaged by the same high LET particle. Collisions between a high LET particle and a mitotic chromosome may thus produce DSBs separated by hundreds of kilobases. NHEJ of these multiple DSBs during the subsequent G1 phase might then produce the multiple deletions and rearrangements characteristic of chromothripsis. We are attracted to this hypothesis because exposure to a single dose of high energy IR readily fits the descriptions of both non-random damage and a "single catastrophic cellular event."

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The abbreviations used are

γ-H2AX	phosphorylated histone H2AX
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related

BER	base excision repair
bp	base pair
Cer-XLF	Cernunnos-X4-like factor
DSB	double-strand break
HRR	homology-directed, recombination-mediated repair
HZE	high-Z, high energy
IR	ionizing radiation
LET	linear energy transfer
MMEJ	microhomology-mediated end-joining
NHEJ	non-homologous end joining
PARP1	poly-ADP ribose polymerase 1
RIDGE	regions of highly expressed genes
ROS	reactive oxygen species
SDSA	synthesis-dependent strand annealing
SSB	single-strand break
XRCC1	X-ray repair cross-complementing protein 1
XRCC4	X-ray repair cross-complementing protein 4

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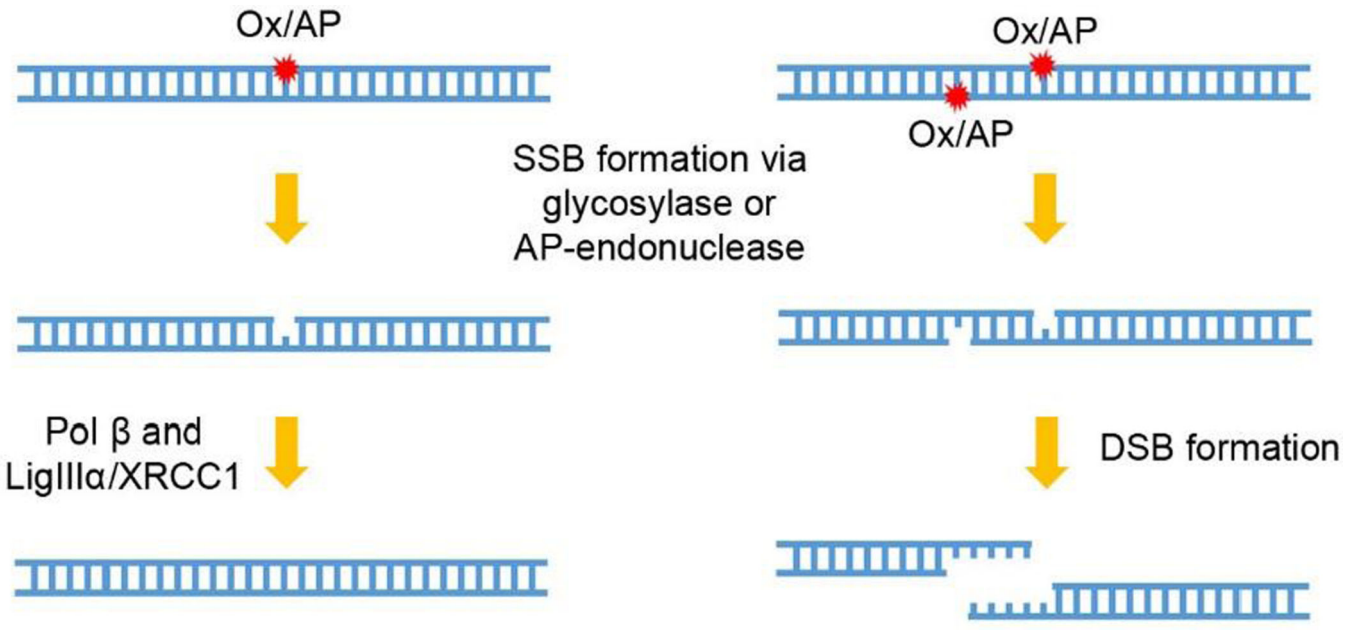

FIGURE 1. DSB formation via attempted base excision repair of closely opposed oxidative lesions

Reactive oxygen species (ROS) can produce oxidized DNA bases, sites of base loss and DNA nicks. The base excision repair (BER) of single lesions (**left**) begins with the excision of oxidized bases (Ox) by DNA glycosylases, or with the excision of apurinic/apyrimidinic (AP) sites by apurinic endonuclease. In either case, apurinic endonuclease goes on to generate a single base gap that is filled by DNA polymerase β and sealed by DNA ligase IIIα, in complex with XRCC1. Because a single IR particle can produce multiple clustered ROS, IR often generates a cluster of oxidative lesions in DNA. Near-simultaneous BER of closely opposed lesions in such a cluster can generate closely spaced nicked or gapped repair intermediates in opposing DNA strands. These may spontaneously convert to DSBs before BER is complete (**right**).

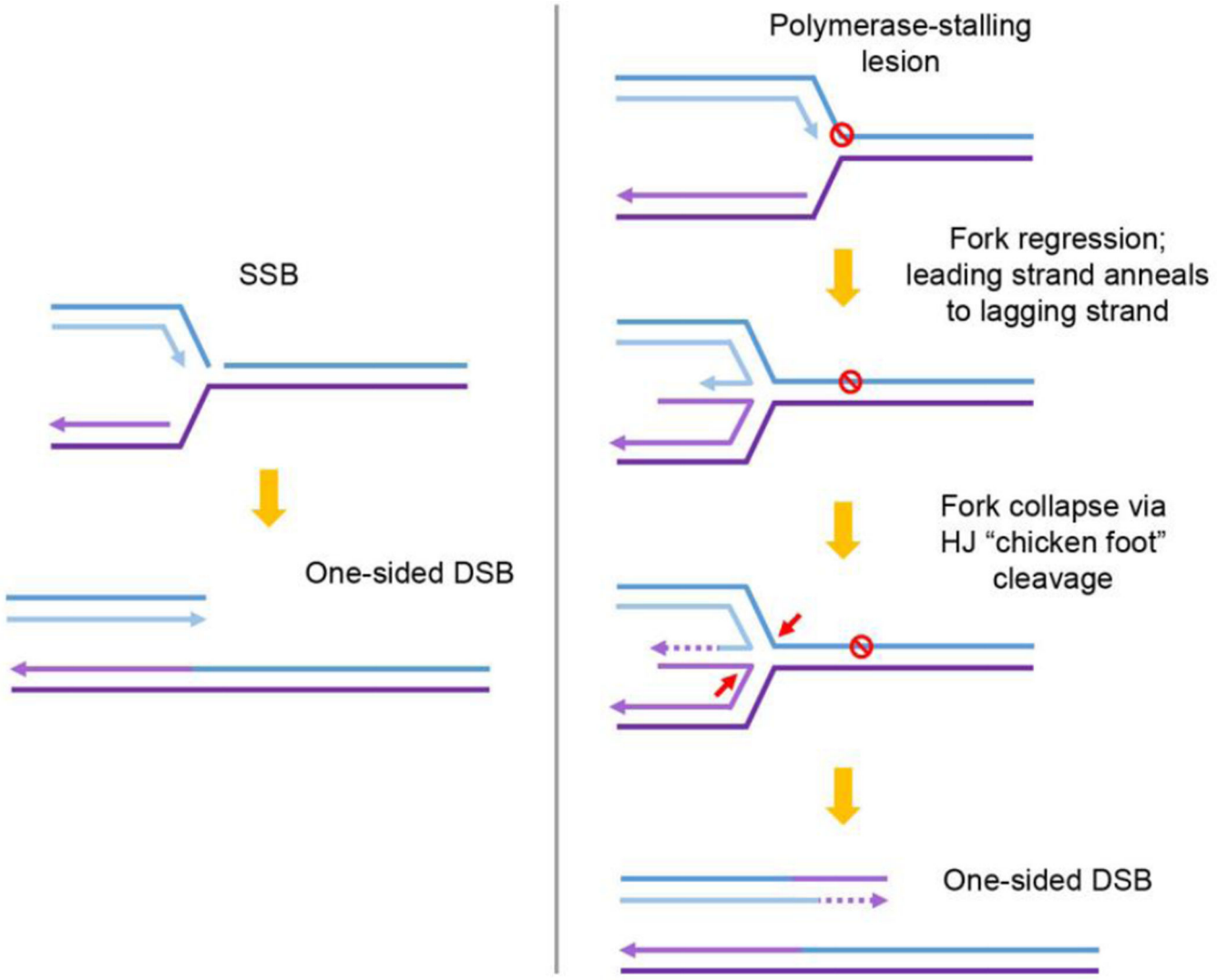

FIGURE 2. DSB formation via replication fork collapse

(**Left**) In the simplest scenario, leading strand synthesis is halted by a single-strand break (SSB), created via exogenous damaging agents or as a repair intermediate (see Figure 1). Collapse of the replication fork converts the SSB into a one-sided DSB. The attempted repair of one-sided DSBs by NHEJ would potentially lead to chromosomal rearrangements or translocations. More commonly, one-sided DSBs may initiate break-induced DNA replication (reviewed in (Malkova and Ira, 2013)). (**Right**) Several kinds of DNA lesions, including thymine dimers, certain oxidized bases (e.g. thymine glycol), abasic sites, and inter-strand crosslinks (such as those caused by cisplatin) can cause replication forks to stall. If the stalled replication fork regresses, it will partially displace newly synthesized leading and lagging strands, allowing them to anneal, as depicted. The newly synthesized lagging strand may then serve as a template to further extend the leading strand, producing the "chicken foot" intermediate shown. This may resolve if the replication block is removed, allowing replication to restart. Alternatively, because the chicken foot is structurally

analogous to a Holliday Junction, it may be cleaved by resolvases, producing, once again, a one-sided DSB.

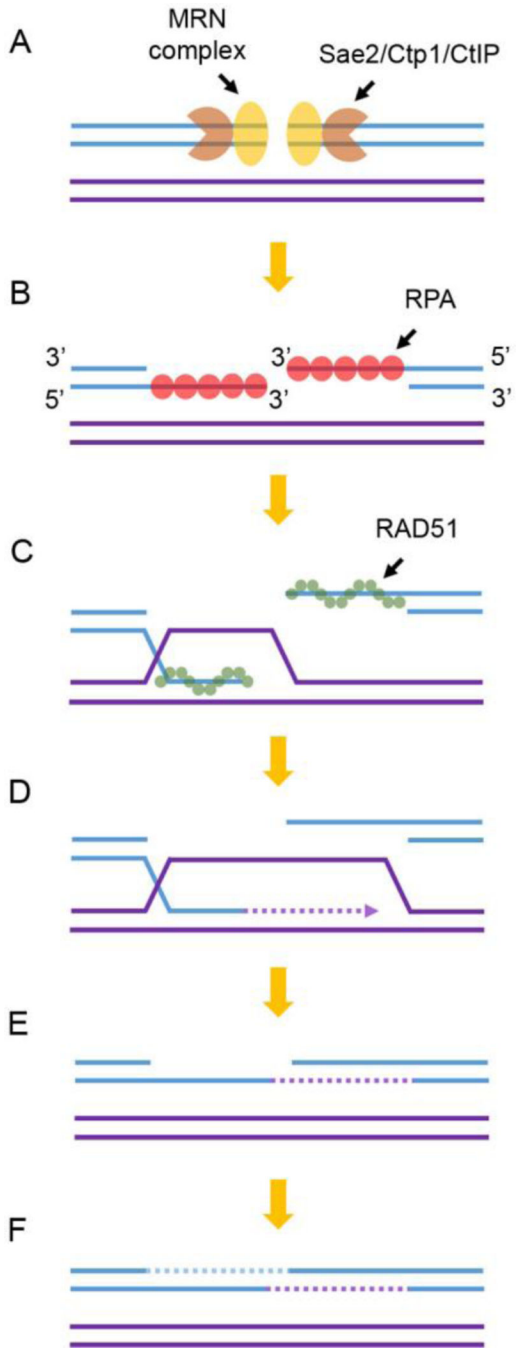

FIGURE 3. Homology-directed recombination-mediated repair (HRR) via synthesis-dependent strand annealing (SDSA)

The Figure depicts key factors and the sequence of steps in SDSA, a common homology-mediated DSB repair sub-pathway. For clarity and simplicity, other HR-initiated pathways, accessory proteins and replication machinery are not shown, but are described in (Mehta and Haber, 2014; San Filippo et al., 2008) and other HRR-related reviews. Step (**A**) depicts the initiation of HRR by the MRN complex which, together with the endonuclease Sae2/Ctp1/CtIP resects 5' DNA ends. This creates 3' single strand DNA (ssDNA) tails that are bound

by the ssDNA binding protein RPA (step (**B**)). RPA is then replaced by RAD51, which catalyzes a homology search; annealing of the Rad51-ssDNA filament to its homolog displaces the homolog's normal complement, creating a "D-loop" (step (**C**)). Using the sister chromatid as a template, the invading strand is able to prime DNA synthesis, thereby extending the D-loop (step (**D**)). Further extension of the D-loop may enable it to anneal with the second 3'-ssDNA tail, which could then prime DNA synthesis in the opposite direction (not shown). Alternatively, the original, newly-extended invading strand may be displaced, allowing it to anneal with the second 3'-ssDNA tail (step (**E**)). DNA synthesis primed by the second 3'-ssDNA tail, followed by ligation, would then complete the repair (step (**F**)).

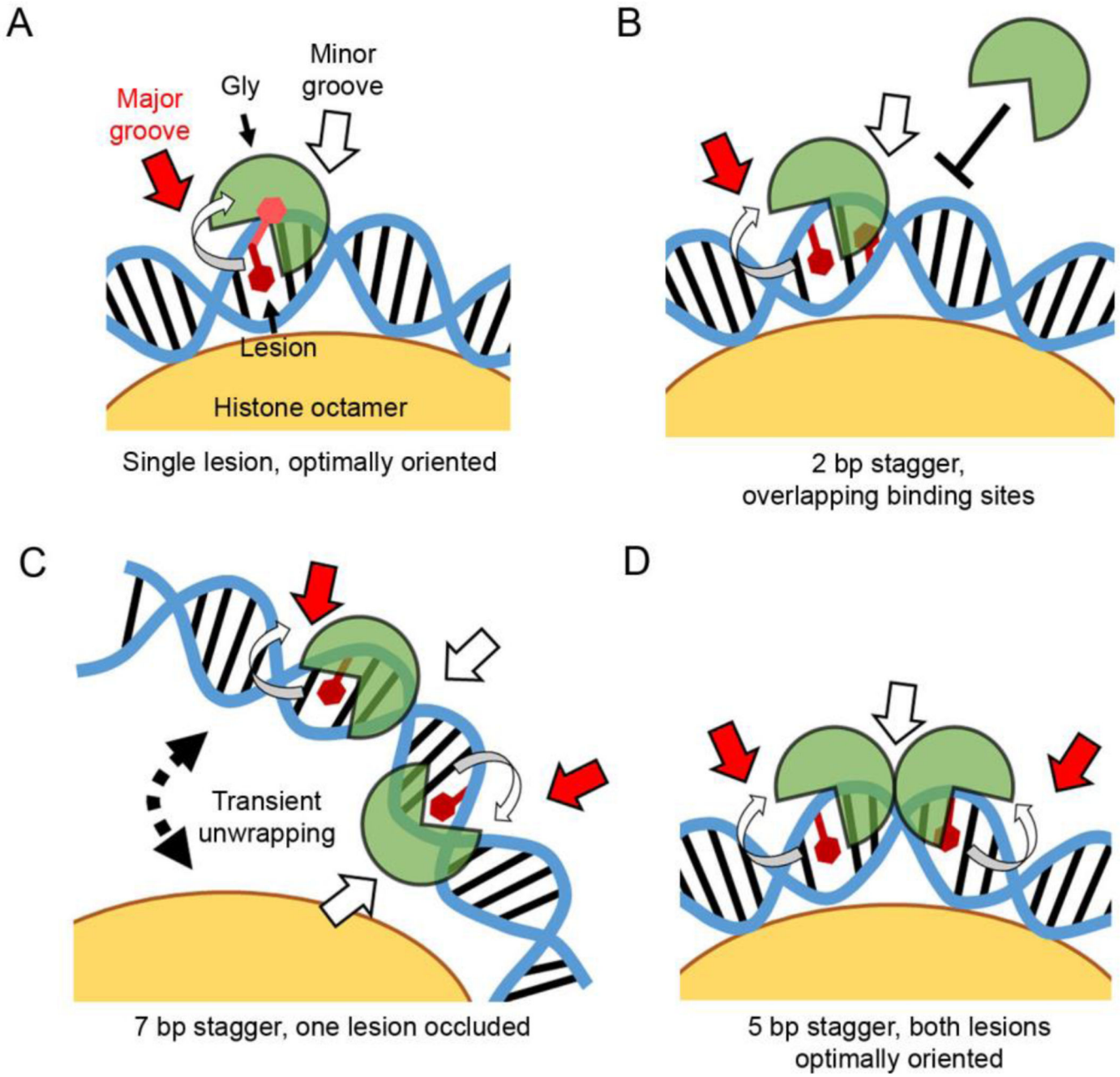

FIGURE 4. Nucleosomes suppress BER-mediated double strand break formation

(**A**) depicts the excision of an oxidized base (red hexagon) from a nucleosome by a DNA glycosylase ("Gly"). This excision reaction is relatively high efficient when the lesion is oriented so that it can flip through the major groove (red arrow) without steric hindrance from the histone octamer or nearby DNA, into the active site of the glycosylase, which must be able to bind via the minor groove (white arrow). Provided both these constraints are satisfied, base excision repair can proceed to completion, as depicted in Figure 1 (left). If DNA glycosylases initiate repair of two, closely-spaced lesions on opposing strands at about the same time, subsequent steps in BER will generate single strand break or gapped repair intermediates. If these intermediate are present at the same time they may spontaneously

convert into a DSB, as described in Figure 1 (right). However, if the opposing strand lesions are separated by fewer than 3 bps, as depicted in (**B**), near-simultaneous repair cannot occur, probably because processing of one lesion degrades the binding site needed to initiate repair of the second lesion. This restriction is evident in repair reactions with both DNA and nucleosomal substrates. If the opposing strand lesions are more optimally spaced with respect to one another (e.g. 3 or 7 bp), access to one or both lesions may be hindered by the histone octamer, as depicted in (**C**). In this case, the more accessible lesion will likely be repaired more rapidly than the opposing strand lesion, where repair can begin only when the lesion is exposed by spontaneous, transient partial unwrapping of DNA from the histone octamer (Maher et al., 2013; Prasad et al., 2007). If the opposing strand lesions are optimally spaced (~4–6 bp), and optimally oriented with respect to the underlying histone octamer, as depicted in (**D**), near-simultaneous BER may ensue, resulting in a DSB (for additional details, see (Cannan et al., 2014)).

TABLE 1

Summary of ionizing radiation types, “quality” (i.e. low or high LET), and damage hallmarks.

Ionizing radiation type	Common sources	Linear Energy Transfer (LET)*	% Annual Exposure **	Particle track	Damage clustering †	Damage Hallmarks
Photons	Gamma rays & X-Rays	Low	~35%	High penetration, crooked track	≤10 lesions per cluster	Highly penetrating but sparse damage; Random distribution of DSBs
β particles						
Protons	Cosmic rays & radon emissions	High	~65%	Low penetration, straight track	≤25 lesions per cluster	Complex clustered lesions surrounding DSB; “Dirty breaks”; Non-random DSBs (average break size 2kbp); Lagging repair
α particles						
HZE ions						

*While photons cannot directly be described by LET, the secondary electrons (β particles) created by them will have low LET.

**(World Health Organization, 2009)

†Theoretically determined by Monte Carlo algorithm (Semenenko and Stewart, 2004)

TABLE 2

Multiple levels of protection against DSBs.

Compaction level	Structural factors that suppress reactions between DNA and reactive oxygen species (ROS).	Structural factors that suppress formation of DSB during replication, transcription, or attempted base excision repair (BER).
DNA alone (and DNA-associated small molecules)	Narrow minor grooves (created by either sequence context or DNA bending) limit access by ROS. Spermine and similar small molecules may partially displace water from DNA, reducing the number of molecules that can be transformed by IR into ROS. They also provide reactive moieties that may help quench local ROS.	Local, sequence-directed bending of DNA can influence the relative rates of lesion discovery and repair at sites containing multiple clustered damages. In principle, this might reduce the probability of DSB formation from near-simultaneous generation of nicked or gapped repair intermediates in opposing DNA strands.
Nucleosome	Minor groove narrowing imposed by wrapping of DNA about the histone octamer, and histones themselves partially limit access to ROS reactive moieties in DNA; histones also provide reactive moieties that may help quench local ROS.	As depicted in Figure 4, nucleosomes substantially restrict the fraction of clustered oxidative lesions that can be transformed into DSBs during attempted BER.
Hetero-chromatic regions & higher order chromatin structures *	Linker histones and other chromatin-associated proteins limit nucleosome mobility, increasing the total fraction of DNA protected by histones. Higher order coiling (particularly the 'zig-zag' or crossed-linker configurations) may reduce access of ROS to linker DNA.	Linker histones and other chromatin-associated proteins that limit nucleosome mobility may partially suppress BER-dependent formation of DSBs while higher order coiling may abolish BER altogether. Transcription-associated formation of DSBs would be similarly affected.

* While higher order chromatin compaction generally confers greater protection from DSBs, high LET radiation creates non-random damage within these structures, potentially creating more deleterious damage than their decondensed counterparts.