STRUCTURAL ASPECTS OF DOUBLE STRAND BREAK REPAIR

Peter Botev, George Russev, Boyka Anachkova and Anastas Gospodinov
Bulgarian Academy of Sciences, Institute of Molecular Biology, Sofia, Bulgaria
Correspondence to: Anastas Gospodinov
E-mail: agg@bio21.bas.bg

ABSTRACT
Double strand breaks are extremely deleterious to the stability of the genome as they lead to loss of whole chromosome fragments or chromosomal rearrangements with the consequent cell death or tumor development. The principal players in the cellular response to DNA damage have been rigorously studied. At the same time, knowledge about the organization of the repair process in the context of local chromatin and the nucleus as a whole has started to emerge only recently. Here, we summarize the most pertinent results regarding the spatial organization of the double strand break repair. On the basis of the data obtained by others and us, we propose a possible chain of events that may take place in chromatin in the vicinity of the breaks to balance the needs for spatial stability and accessibility of the damaged DNA.

Introduction
Double strand breaks (DSBs) are the most dangerous type of damage for the cell. Breaks result from various factors, both endogenous and exogenous. Normal processes such as V(D)J recombination, yeast mating type switching and meiosis as well as replication fork collapse lead to DSBs. They are also the result of exogenous causes: ionizing radiation, crosslinking agents that may stall replicative polymerases, topoisomerase poisons, etc. In higher eukaryotes, failure to repair the break, may lead to loss of genetic material or to chromosomal rearrangements that may result in tumor development. Cells have evolved mechanisms collectively termed DNA damage response that sense DSBs, arrest the cell cycle and activate DNA repair pathways to repair the lesions.

DSBs are a unique type of lesion in the sense that the phosphodiester backbones of both DNA strands get severed. This imposes specific spatial requirements for repair – the most obvious one being to keep the broken ends together. During homologous recombination repair, the homologous duplexes should find one another and align for proper transfer of the genetic information. The complexities of DSB repair require that multiple factors interact and this in turn necessitates mechanisms to recruit and retain repair and signaling molecules.

In this text we examine the pathways that deal with DSBs and the mechanisms which ensure that the spatial requirements of the processes are met, namely keeping partner DNAs aligned and changing the chromatin structure in a way most conductive to repair.

Repair of double strand breaks
Profound advances in the study of the DNA damage responses, during the last 15 years, have clarified in detail the enzymology of the DSB repair. DSBs are subjected to two major repair pathways – nonhomologous end joining (NHEJ) and homologous recombination (HR) repair, carried out by distinct sets of factors. In mammalian cells NHEJ is the predominant form of DSB repair, active in all phases of the cell cycle, whereas HR functions in late S and G2 phases (86) when a homologous sister chromatid becomes available (Fig. 1).

The MRN complex is believed to be the primary sensor of DSBs. After limited end processing, NHEJ is initiated by the Ku70 and Ku80 proteins forming a heterodimer with affinity for DSB ends. Ku proteins recruit the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which autophosphorylates itself. It has been suggested that DNA-PKcs can bridge DNA ends (11). The coordinated assembly of Ku proteins and DNA-PKcs on DNA ends is followed by recruitment of the DNA ligase IV complex, which is responsible for the rejoining step. The ligase is associated with a dimer of XRCC4 (49) and XLF (Cernunnos) (1). The nuclease Artemis helps the repair of a subset of IR-induced DSBs and opens hairpins formed during V(D)J recombination (28, 67). NHEJ is an error-prone way to repair a break since it is not guided by a DNA template (Fig. 1).

In contrast, homologous recombination repair is considered an error-free mechanism as it uses homologous sequences somewhere else in the genome to repair the break. It functions in late S and G2 phases, normally using the sister chromatid as a repair template. To carry out HR repair, broken ends are first processed to long 3′-ssDNA tails covered by RPA. Early studies implicated the MRN (Mre11-Rad50–Nbs1) complex in the generation of a ssDNA stretch (26, 52) with a 3′-overhang
able to invade the homologous duplex. However, the Mre11 nuclease lacks the necessary 5' to 3' exonuclease activity (27, 89). The Ctp1 nuclease that functions in 5' to 3' resection of DSB ends was first found in S. pombe (39). Data suggest a two-step mechanism for DSB processing: first Mre11 and Sae2 (the Ctp1 homolog in S. cerevisiae) remove a small oligonucleotide from DNA ends, and then Exo1 and Sgs1 process this intermediate to generate extensive tracts of ssDNA (46) in a mechanism conserved from yeast to mammals (21). Single strand DNA is covered by RPA, which is later replaced by Rad51. The Rad51-covered nucleoprotein filament is capable of searching for, invading, and transferring strands with a homologous duplex. Since Rad51 has little preference for ssDNA over dsDNA (73), its specificity to ssDNA is enhanced by interactions with the Rad51 paralogs (58, 68, 88) and Rad52 (6, 55, 74). Rad51 filament invasion into the duplex donor DNA is ATP dependent (4, 80, 85) and is stimulated by Rad54 – a SWI2/SNF2 ATPase (63, 76). After joint molecule formation, the invading strand is extended by DNA polymerases and branch migration leads to restoration of the genetic information spanning the break (58, 98) (Fig. 1).

Large-scale organization of the repair process

Major nuclear processes such as replication and transcription take place in spatially limited compartments that are thought to increase the efficiency by concentrating factors and templates together (47). Similarly, the repair of DSB breaks is organized in repair foci that can be easily visualized by immunofluorescence with antibodies against repair proteins. Existing research results indicate that in yeast multiple DSBs get recruited to a single repair centre. This is supported by the observation that, if 2–4 repair foci appear at a low dose of irradiation, higher doses do not lead to a proportional increase of foci relative to the number of DNA breaks (40, 41).

Real-time imaging and immunoprecipitation studies in yeast showed migration of DSBs to the nuclear periphery, where they associated with the nuclear pore complex components (51, 56). However, it seems that after 1–2 hours only persistent DSBs are re-localized to the nuclear periphery. The process requires DSB end processing and the Rad51 recombinase (56). DSBs that are rapidly repaired (<2 hours) do not move to the nuclear periphery as they fail to induce robust cell cycle checkpoint activation (31, 51). The movement of breaks to the nuclear periphery requires that the chromatin is relatively mobile and indeed it has been shown that a tagged domain in the yeast nucleus is capable of traveling <0.5 mm in about 10 seconds (of a total nuclear diameter of about 2 mm) (18). Direct evidence for long-range motion of broken chromosomes has been found by the visualization of two independent DSBs that coalesced into a common repair focus (40). The situation, however, seems to be rather different in mammalian cells. Indeed, an elegant study using fluorescently tagged markers to follow the ends of a site-specific DSB, found that the broken chromosome ends are essentially immobile in nuclear space (79). Still, this could not rule out the possibility that breaks are organized in shared repair centers, since repair foci grow in size but diminish in number with time, suggesting that closely positioned ones may coalesce. In proof of increased break mobility, at least under certain circumstances, are the reports that 53BP1 participates in a mechanism to augment the dynamic behavior of local chromatin that facilitates joining of distant sites by NHEJ during V(DJ) recombination (12, 13).

Keeping DNA molecules together

In both yeast and mammals cohesin is required for DSB repair (33, 77, 78). Cohesin is a protein complex, which consists of Smc1, Smc3, Scc1 and Scc3. Its primary function is to link sister chromatids. SMC (structural maintenance of chromosomes) proteins are the molecular clips in a variety of processes that require the physical proximity of DNA molecules (23). They share a common architecture with an ATPase domain separated into N- and C-terminal halves by a long linker. The two terminally positioned halves of the ATPase are brought together by folding the linker at the hinge domain (where the amino acid sequence turns back on itself) (54, 99). Cohesin is loaded on chromatin in G1 but cohesion is established in S phase and maintained until anaphase (53, 84). It is believed that cohesin topologically embraces the two sister chromatids inside its ring. Physical binding of cohesin to chromatin in the vicinity of DNA lesions was reported in chromatin immunoprecipitation studies at specific endonuclease-induced break sites (83, 91).

Data in yeast indicate that DSB induction leads to binding of cohesin not only in the vicinity of DSBs, but throughout the genome in G2 (82, 92). Although it is not firmly established, available data suggest the conservation of the genome-wide damage-induced cohesion in mammalian cells (14, 32). It has also been suggested that cohesin may influence the DSB repair pathway choice (70).

Another protein considered a member of the Smc family is Rad50. It is structurally and functionally related to the SMC proteins, however, there are functionally related differences in the way the Rad50-containing MRN complex and the cohesin complexes are assembled (9, 38, 60). Available data indicate that the MRN complex keeps broken ends together. The core of the complex is a heterotrimer that consists of two Rad50 and two Mre11 subunits. While Smc proteins are bound at their hinge domains, dimerization of Rad50 is mediated by Mre11 dimer binding near the ATPase domain of RAD50 at the opposite side of the latter molecule (10, 24). MRN binding to DNA induces a conformational change that favors the interaction between Rad50 belonging to different complexes. Multiple MRN complexes present a dense group of protruding hooks that thus function as molecular Velcro for broken DNA (35).

Chromatin changes at the break site

The processes that maintain spatial large-scale chromatin dynamics and spatial proximity of DNA ends are only part of the changes induced in response to DSBs. The chromatin neighboring a DSB break is subject to post-translational modifications of histones and ATP-dependent chromatin
Fig. 1. Pathways to repair a DSB. (A) MRN complex is considered to be the sensor of DSBs. It is essential for HR repair, and though debated, for NHEJ in mammals as well. (B) NHEJ is initiated by the binding of Ku proteins, which in turn, bind DNA-PKcs and activate DNA-PK kinase activity (C). DNA-PK regulates limited processing of DNA ends (D) and stimulates the recruitment of the factors that carry out rejoining of the DNA ends: DNA ligase IV complexed with XRCC4 and XLF (E). Extensive processing of DSB ends into long 3'-ssDNA overhangs in a two-step mechanism: first by Mre11 and Sae2 to remove a small oligonucleotide from DNA ends (F), and then by Exo1 and Sgs1 to process this intermediate and generate extensive tracts of ssDNA covered with single strand binding protein RPA (G). RPA is replaced by RAD51 with the help of RAD52 and RAD51 paralogues (H) and then RAD51-covered nucleoprotein filament carries out the search, invasion and strand exchange with the homologous duplex (I).
remodeling which 1) control chromatin accessibility around the lesion, and 2) provide binding motifs for DSB repair and signaling proteins. Phosphorylation of the histone variant H2AX is the classic example of a modification that regulates the binding of the proteins involved in DSB repair.

**Phosphorylation of H2AX**
Phosphorylation of the histone variant H2AX at serine 139 is one of the earliest events following break formation (69). In yeast there is no H2AX and canonical H2A is phosphorylated on S129 (for convenience, it is referred to as H2AX throughout this text). Accumulation of phosphorylated H2AX could be visualized inside the nucleus as prominent microscopically detectable foci. The principal kinase phosphorylating H2AX after DSB induction is ATM (8). During S phase, in response to replication stress, H2AX is phosphorylated by ATR (97). Phosphorylated H2AX covers a large chromatin domain surrounding the break: several tens of kilobases in yeast (75) and megabase-sized regions in mammalian cells (69). The larger size of the mammalian γ-H2AX domain is attributed to an amplification mechanism that involves the MDC1 protein. MDC1 binds phosphorylated H2AX (81) and stabilizes chromatin bound NBS1 at the DSB site (42). This, as NBS1 is part of the MRN complex, recruits more ATM (17, 101) and ATM further phosphorylates H2AX, thus establishing a positive feedback loop (34). The principle “reader” of the phosphorylated H2AX mark is again the MDC1 protein. MDC1 binds phosphorylated H2AX, but also a number of other repair-related factors in addition to NBS1 and ATM (30): CHK2, Rad51, RNF8. Some of these binding partners cause different downstream modifications that create binding surfaces for protein–protein interactions during repair. RNF8 induces ubiquitylation of H2A/H2AX and facilitates the binding of RNF168 (15), BRCA1 and 53BP1 (5, 43). MMSET methyltransferase increases locally after the appearance of DSBs and mediates H4K20 methylation (62). Thus, directly or indirectly, phosphorylated H2AX serves as the principal spatial organizer of the DSB repair process. This is manifested by the formation of H2AX foci and the reliance of other repair factors on γ-H2AX for their retention on chromatin surrounding a DSB. While retention is linked to the presence of docking sites afforded by γ-H2AX, and downstream ubiquitylation and methylation; the entry of these factors, however, depends on chromatin compaction and local nucleosome structure, controlled by histone acetyltransferases and chromatin remodelers.

**Histone acetylation and accessibility of DNA lesions**
Histone acetylation is a key modulator of chromatin accessibility. It changes the charge of positively charged lysine and arginine residues and weakens their interaction with the negatively charged DNA backbone. The importance of histone acetylation for repair was first recognized in respect to nucleotide excision repair. Early studies that dealt with repair of UV-induced cyclobutanepyrimidine dimers found that total protein acetylation increases in response to UV irradiation (65) and that repair DNA synthesis is enhanced in hyperacetylated nucleosomes (66). Though DSB repair and nucleotide excision repair are mechanistically different, they both share the need for histone acetylation change to properly restore the damaged DNA.

Initially it was found that the yeast NuA4 complex, which acetylates all histone H4 tail lysines, was required for HR and NHEJ repair, and localized to a break site (7). Tamburini and Tyler (87) demonstrated that acetylation and deacetylation of histone H3 and H4 N-terminal tails were important for viability following DSB repair and that histone acetyltransferases Gen5 and Esa1 as well as deacetylases Sir2, Rpd3 and Hst1 were dynamically recruited to a site-specific DSB. The mammalian NuA4 homologue – the TIP60 complex – is essential for efficient DSB repair and facilitates access and loading of DNA repair proteins onto chromatin as demonstrated by others and us. Deletion of the TRRAP subunit of the complex compromised homologous recombination repair and recruitment of a subset of repair proteins (50). Work in our laboratory demonstrated that the knock-down of RUVBL proteins, stoichiometrically the most abundant subunits of TIP60 (as well as in INO80 and SRCAP complexes), impaired Rad51 redistribution to chromatin and nuclear foci formation in response to damage (19). As a proof that TIP60-mediated histone acetylation is needed to relax chromatin structure and improve accessibility of repair proteins, both studies found that forced relaxation of chromatin using histone deacetylase inhibitors, chloroquine or hypotonic conditions compensated the repair defects in TIP60 deficient cells. The acetylation status of chromatin around DSBs in mammalian cells, however, changes dynamically during the course of DSB repair. In response to damage, mammalian HDAC1 and HDAC2 are quickly localized to chromatin at DSBs, where they deacetylate H3K56 and H4K16. Depletion of HDAC1 and HDAC2 inhibited NHEJ repair (45). Intriguingly, deacetylation was transient and was rapidly followed by hyperacetylation. Work done in our laboratory indicated that the HDAC inhibitor sodium butyrate suppressed both NHEJ and HR as judged by repair foci dynamics and repair of plasmid reporter constructs. These results are consistent with a mechanism in which deacetylation of core histones in the vicinity of the break is required to compact chromatin. Chromatin compaction may be needed to prevent the ends from moving apart immediately following the break (36). If early break stability depends on compaction of local chromatin, it is likely that initial deacetylation lasts until other factors take over, such as sufficient accumulation of MRN complex. It should be noted, though, that while both in yeast and mammals histone deacetylation is involved in DSB repair, it appears that in yeast it follows hyperacetylation at the break site, whereas in mammalian cells it precedes the acetylation. This distinction may reflect differences in the chromatin organization and consequently dissimilar requirements of its modification in the course of repair.

Another way to control chromatin accessibility relies on ATP-dependent chromatin remodelers, which function by
weakening the histone-DNA interactions at the expense of ATP hydrolysis and can slide or evict individual nucleosomes. At that level of chromatin structure, chromatin remodelers present finer-grained means to alter it and accommodate repair factors.

**ATP-dependent chromatin remodeling**

Chromatin remodelers are large multi-subunit complexes that contain an ATPase of the SWI2/SNF2 class and a number of regulatory and targeting subunits (96). There are four related families of chromatin remodelers, based on the structure of their ATPase catalytic core: SWI/SNF (switching defective/sucrose nonfermenting), INO80 (inositol requiring 80), CHD (chromodomain, helicase, DNA binding) and ISWI (imitation switch).

A very good example of the importance of structural modifications induced by ATP dependent remodelers in the DDR, as well as their interplay with histone-modifying activities, is the recently described role of BRG1 in H2AX phosphorylation. BRG1 is a subunit of the mammalian SWI/SNF complex that binds to acetylated H3 but only in γ-H2AX nucleosomes, not in bulk chromatin. BRG1 stimulates the recruitment of Gcn5 acetyltransferase and causes further acetylation of γ-H2AX domains. Increased chromatin accessibility augments the recruitment of ATM kinase, which, in turn, promotes additional H2AX phosphorylation, and the three activities form an auto-amplification loop. Cells deficient for BRG1 and therefore lacking this mechanism have impaired H2AX phosphorylation (37).

Another example of interaction between chromatin remodeling and histone acetylation has been described in regard to mammalian NuRD chromatin remodeler, the loss of which leads to spontaneous accumulation of damage (61) and multiple other DDR-related effects. One of the NuRD complex subunits – CHD4, has been shown to be required for recruitment of HDAC1 and HDAC2 to sites of damage. In agreement with data regarding the role of HDACs in NHEJ, CHD4 deficiency leads to NHEJ defects (64).

The INO80 chromatin remodeler has been intensely studied with regard to DSB repair. The human INO80 complex is capable of sliding a mononucleosome along a piece of DNA under *in vitro* conditions (29), but the complex has also been implicated in nucleosome eviction (3). The exact role of INO80 in DSB repair has long been elusive. In yeast, deletion of its ARP5 or ARP8 subunits, specific to the complex, results in hypersensitivity to DSB-inducing agents (71, 72). Yeast INO80 shares a common mode of recruitment with NuA4 and Swr1 complexes: via ARP4-dependent binding to phosphorylated H2AX (16). In a study done in the laboratory of S. Gasser, INO80-deficient yeast strains were found less effective in the initial 5’–3’ resection at DSB ends prior to strand invasion in the homologous duplex template (94). An independent study did not replicate the impaired DSB end processing, but observed defective loading of the repair proteins Rad51 and Rad52 in strains lacking INO80 subunits (90).

Using mammalian cells, we observed that the knock-down of the INO80 expression caused DSB repair deficiency. The

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**Fig. 2.** A model of chromatin dynamics at a DSB. (A) Following DSB induction, the surrounding chromatin is rapidly deacetylated by HDACs recruited to the site of damage, leading to chromatin compaction. Compacted chromatin facilitates DSB immobility and prevents the ends from floating apart. (B) To allow entry of factors involved in initial DSB processing, chromatin remodeling activities such as INO80 induce smaller-scale changes in chromatin structure, while the chromatin domain remains compacted due to histone deacetylation. (C) After initial DSB ends processing and after accumulation of other factors that tether the ends, massive acetylation promotes decompaction of chromatin surrounding the break and permits the massive accumulation of repair factors needed to complete the repair.
mammalian INO80 protein associated with chromatin within 10 kb from a defined break site. INO80 depletion impaired the focal recruitment of 53BP1 but did not impede RAD51 foci formation, suggesting that INO80 was required for the early steps of repair. Further analysis indicated that INO80 deficient cells were compromised for 5’–3’ resection of DSBs (20). Using immunofluorescence of BrdU-labeled ssDNA to directly probe for the product of resection, as well as RPA foci, which require ssDNA to form, we found that Ino80 and Arp8 were necessary for normal resection of DSB ends.

The yeast SWR1 also belongs to the INO80 class of remodelers (2). Available data suggest that it is involved in transcriptional regulation and DNA repair. SWR1 participates in the exchange of H2A with H2AZ variant (48) at promoters of inactive genes and heterochromatin boundaries (44). It has been reported that SWR1 also catalyzes the exchange of H2AX (H2AS129Ph in yeast) with H2AZ and antagonizes INO80 complex in the maintenance of γ-H2AX levels in yeast (57). SWR1 has been found to facilitate the binding of yeast Ku80 protein and mediate NHEJ repair (93). The function of its mammalian analog has not been studied yet.

The precise mechanisms by which chromatin remodelers facilitate repair and the steps they are involved in are still under intense investigation. It is likely that chromatin remodelers function non-redundantly in successive steps of the process, with each providing gradual structural changes at a precise moment during the repair of a DSB. In spite of the fact that the field is fairly young, the current body of evidence makes it clear that chromatin remodeling complexes and post-translational modifiers function in concert to promote the changes in chromatin structure that regulate repair (3, 95). Based on our own data and that of others, we propose a possible chain of events that takes place on chromatin surrounding a DSB (Fig. 2). Upon induction of a DSB, the surrounding chromatin gets quickly deacetylated by the rapidly recruited histone deacetylases (Fig. 2A). Chromatin compaction limits the local movement of DSB ends, which restricts possible erratic recombinogenic events. Thus insulated, the broken DNA ends remain in mutual proximity, which is important for the subsequent repair, regardless of the particular repair pathway to be used. However, while in a deacetylated state, DSB ends need to be processed in order to initiate repair. At that point, chromatin remodeling complexes such as INO80 induce spatially limited changes within chromatin (we have found that the remodeler is mostly recruited within 10 kb from the break) that are required for DSB end-processing and recruitment of early repair factors (Fig. 2B). While this is taking place, break immobility starts to be provided by the accumulation of additional MRN complexes that mechanically tether broken ends and compacted chromatin structure is no longer needed. At that stage, further steps of repair require increased accessibility to the break site in order to accumulate the massive amounts of repair factors that form repair foci. To provide for that, chromatin modifiers such as Trrap-TIP60 begin to acetylate chromatin in the vicinity of the break (Fig. 2C), allowing recruitment of later repair factors such as Rad51, Brca1, etc. While at present we still have a fragmentary picture of chromatin changes at the break site, the proposed model fits well our current knowledge and provides a logical explanation of the dynamic nature of the histone acetylation at DSBs and its interplay with other activities that remodel chromatin.

Conclusions

The efficient repair of DSBs requires the provision of proper structure at multiple levels. Many studies indicate that break sites are organized in the context of the nucleus, but this field is just emerging and we have significantly better understanding of the local changes that occur at the site of damage. Phosphorylated H2AX marks the sites undergoing repair and provides the interfaces for the accumulation of repair proteins. It is the signal for on-going repair and blockage of the cell cycle until the repair process is complete. Acetylation of histones at DSBs is dynamic, as the requirements for accessibility should be balanced with that of spatial stability of chromatin surrounding the break. Histone deacetylases likely ensure that DSB ends are held together, while simultaneously, chromatin remodeling provides a finer and more controllable way to locally amend the chromatin structure for the initial processing of the DNA ends.

Deregulation of histone acetyltransferases has been directly linked to oncogenesis (100). Some of the genes coding for subunits of chromatin remodelers such as RUVBL proteins (TIP48 and TIP49) (25) or INO80 (59) have also been shown to carry mutations in cancer cells and other pathologies. Still, our knowledge of how chromatin modifiers/remodelers are altered in cancer is largely limited (22). As DSBs are among the primary means to induce apoptosis in cancer cells, it is within the set of factors of DSB repair where potential targets for therapy are sought. Interference with histone deacetylases is one of the mainstays of epigenetic therapy, but multi-subunit chromatin remodelers may also present opportunities for therapeutic targeting.

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