# REGULATION OF DNA DOUBLE STRAND BREAK REPAIR BY LOCAL CHROMATIN ARCHITECTURE

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# REGULATION OF DNA DOUBLE STRAND BREAK REPAIR BY LOCAL CHROMATIN ARCHITECTURE

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REVIEW
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# Processing of DNA Double-Strand Breaks by the MRX Complex in a Chromatin Context

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DNA double-strand breaks (DSBs) are highly cytotoxic lesions that must be repaired to ensure genomic stability and avoid cell death. The cellular response to DSBs is initiated by the evolutionarily conserved Mre11-Rad50-Xrs2/NBS1 (MRX/MRN) complex that has structural and catalytic functions. Furthermore, it is responsible for DSB signaling through the activation of the checkpoint kinase Tel1/ATM. Here, we review functions and regulation of the MRX/MRN complex in DSB processing in a chromatin context, as well as its interplay with Tel1/ATM.

**Keywords: Mre11, Rad50, Xrs2/NBS1, Sae2/CtIP, Tel1/ATM, MRX complex, double-strand break, resection**

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## INTRODUCTION

Chromosomal DNA double-strand breaks (DSBs) are potentially lethal DNA lesions that can form accidentally during DNA replication and transcription, or upon exposure to genotoxic agents, such as ionizing radiation or chemicals. Failure to repair them can result in loss of genetic information or cell death, whereas inaccurate repair can lead to chromosome rearrangements (Jackson and Bartek, 2009; Liu et al., 2012). Even though DSBs pose a significant threat to genome stability, DSBs are programmed recombination intermediates during gametogenesis or antigen-receptor diversity in lymphocyte development (Lam and Keeney, 2014; Arya and Bassing, 2017). In all cases, DSBs need to be repaired to preserve genomic integrity.

Eukaryotic cells possess two main mechanisms for repairing DSBs: non-homologous end-joining (NHEJ) and homologous recombination (HR). Repair by NHEJ requires the Ku70–80 heterodimer (hereafter referred to as Ku) that recruits the DNA ligase IV complex (Lig4/Dnl4 in *Saccharomyces cerevisiae*), which directly religates the two broken ends (Chang et al., 2017). By contrast, HR is a more complex process that uses DNA information stored in a homologous double-stranded DNA (dsDNA) as template to reconstitute any missing genetic information at the break site (Mehta and Haber, 2014; Kowalczykowski, 2015).

The key process in determining which pathway is used to repair DSBs is the initial processing of the DSB ends. While NHEJ requires little or no DNA end processing, HR is initiated by nucleolytic degradation of the 5′ terminated strands at both DNA ends by a concerted action of nucleases in a process termed DNA end resection (Bonetti et al., 2018). The preferential degradation of the 5′-terminated strands results in formation of 3′-ended single-stranded DNA (ssDNA) ends that are first coated by the Replication Protein A (RPA) complex. RPA is subsequently replaced by Rad51 to form a nucleoprotein filament that is used to search for a homologous dsDNA sequence (Kowalczykowski, 2015). Repair can then proceed via synthesis-dependent strand annealing or the canonical recombination pathway that involves formation of a double Holliday junction (Mehta and Haber, 2014).

Extended resection of the DSB ends not only commits DSB repair to HR, but it makes the DNA ends non-ligatable by NHEJ. In vegetatively growing cells, HR uses the sister chromatid as repair template and this restricts recombination to the S and G2 phases of the cell cycle when the sister chromatid is available. This cell-cycle control of recombination is based on activation of key resection proteins by cyclin-dependent kinase (CDK)-catalyzed phosphorylation events (Aylon et al., 2004; Ira et al., 2004; Huertas et al., 2008; Chen et al., 2011).

The evolutionarily conserved Mre11-Rad50-Xrs2/NBS1 complex (MRX in *S. cerevisiae*, MRN in humans) recognizes, signals and initiates repair of DSBs. MRX is rapidly recruited to DSBs, where it has structural and enzymatic activities to initiate DSB resection and to maintain the DSB ends tethered to each other for their repair (Syed and Tainer, 2018). MRX also recruits and activates the checkpoint protein Tel1 (ATM in mammals) to coordinate DSB repair with cell cycle progression (Villa et al., 2016). Germline hypomorphic mutations of human MRN complex components are associated with Ataxia Telangiectasia-like disorder (ATLD), Nijmegen Breakage Syndrome (NBS) and NBS-like disorder, which are characterized by cellular radiosensitivity, immune deficiency and cancer predisposition (O'Driscoll, 2012). Here we review structure, functions and regulation of the MRX complex in sensing, signaling and processing DSBs within a chromatin context, focusing mainly on the work done in the budding yeast *S. cerevisiae*.

## STRUCTURAL AND BIOCHEMICAL PROPERTIES OF MRX

In both yeast and mammals, the MRX complex exists as a hetero-hexameric assembly, in which the Mre11 subunit interacts independently with both Rad50 and Xrs2 (NBS1 in mammals), and dimerizes with itself. Mre11 has five phosphodiesterase motifs in the N-terminal region and exhibits 3′-5′ dsDNA exonuclease and ssDNA endonuclease activities *in vitro* (Bressan et al., 1998; Paull and Gellert, 1998; Trujillo et al., 1998; Usui et al., 1998). The Sae2 protein (CtIP in mammals) stimulates Mre11 endonuclease activity to cleave the 5′-terminated DNA strands at both DSB ends (Cannavo and Cejka, 2014;Reginato et al., 2017; Wang et al., 2017).

Rad50 is characterized by ATPase motifs at the N− and C− terminal regions of the protein, with the sequence in between forming two long coiled-coil domains that are separated by a zinc binding CXXC motif referred to as zinc hook (Syed and Tainer, 2018; **Figure 1**). The two ATPase motifs associate together to generate an ATP nucleotide binding domain and the coiled-coil domains fold back on themselves to form antiparallel intramolecular coiled coils (Hopfner et al., 2001; Moncalian et al., 2004; Williams et al., 2008; **Figure 1**). The zinc hook at the apex of the coiled-coil domains can form intralinked or interlinked complexes via tetrahedral coordination of a $zinc^{2+}$ atom and the interlinked assembly can account for the MRX ability to maintain the DSB ends in close proximity (de Jager et al., 2001; Hopfner et al., 2002; Kaye et al., 2004; Lobachev et al., 2004; Wiltzius et al., 2005; Hohl et al., 2011; Nakai et al., 2011; He et al., 2012). Recently, crystal structure and X-ray scattering analyses of human RAD50 Zn-hook with a portion of the coiled-coil domain indicate the existence of a novel eukaryotic-specific interface that stabilizes Rad50 coiled coils in an intramolecular dimer assembly (Park et al., 2017), suggesting that the intralinked arrangement is the predominant form of the complex.

Several studies have shown that ATP binding and hydrolysis activities of Rad50 are crucial to regulate DNA binding, tethering and nuclease functions of the MRX complex. Structural studies of Mre11 in complex with Rad50 core domains from bacteria and archaea indicate that, upon ATP binding, Rad50 closes into a rigid conformation, in which the N- and C-terminal domains interact with each other and form a central groove that can accommodate dsDNA. This closed ATP-bound state of Rad50 renders dsDNA inaccessible to the Mre11 nuclease active site (Lammens et al., 2011; Lim et al., 2011; Williams et al., 2011; Möckel et al., 2012; Liu et al., 2016; Seifert et al., 2016). Point mutations that stabilize the ATP-bound conformation of Rad50 increase DNA binding, NHEJ and end-tethering (Deshpande et al., 2014), suggesting that MRX exerts these functions when it is present in the ATP-bound state. By contrast, in the ATP-free or hydrolyzed state, the Rad50 ATPase subunits are flexible and relatively open, suggesting that ATP hydrolysis drives the rotation of the two nucleotide binding domains of Rad50 and the disengagement of the Rad50 dimer that makes DNA accessible to the Mre11 nuclease active sites (Lammens et al., 2011; Lim et al., 2011; Williams et al., 2011; Möckel et al., 2012; Deshpande et al., 2014). Consistent with this hypothesis, biochemical analyses demonstrate that ATP hydrolysis by Rad50 is a prerequisite for Mre11/Rad50-mediated nuclease activity on dsDNA molecules (Paull and Gellert, 1999; Hopfner et al., 2000; Trujillo and Sung, 2001; Herdendorf et al., 2011). Altogether, these findings lead to a model whereby these ATP-driven transitions regulate the balance between MRX functions in NHEJ and end-tethering, which require ATP binding, and those in resection and HR, which require ATP hydrolysis (**Figure 1**).

Rad50 has a slow ATP hydrolysis rate (Herdendorf et al., 2011; Majka et al., 2012; Deshpande et al., 2017; Saathoff et al., 2018), suggesting that other proteins can promote its ATP hydrolysis activity within a cell. In *S. cerevisiae*, MRX is known to interact with Rif2, which is recruited to telomeric DNA ends and negatively regulates telomerase-mediated telomere elongation (Wotton and Shore, 1997; Levy and Blackburn, 2004; Hirano et al., 2009; Martina et al., 2012). Interestingly, Rif2, which is recruited to DSBs in a manner partially dependent on MRX, enhances ATP hydrolysis by Rad50 (Cassani et al., 2016). This observation, together with the finding that the lack of Rif2 increases the efficiency of both end-tethering and NHEJ (Cassani et al., 2016), suggests that Rif2 can regulate MRX ATP-driven transitions.

While Mre11 and Rad50 are conserved in bacteria and archaea, only eukaryotes possess Xrs2, which is the only MRX component that harbors a nuclear localization signal and is necessary for translocation of the Mre11-Rad50 subcomplex into the nucleus (Desai-Mehta et al., 2001; Tsukamoto et al., 2005). Localization of Mre11 into the nucleus in the absence of Xrs2 restores Mre11-Rad50 functions in DSB resection,

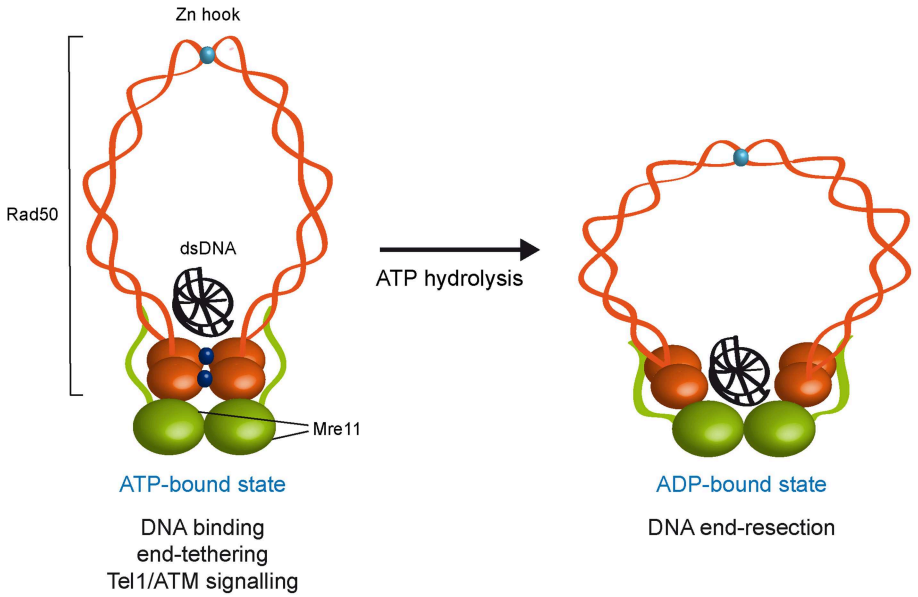


**FIGURE 1 |** ATP- and ADP-bound state of the MRX complex. The Mre11 dimer (green) is bound to Rad50 dimer (orange) with a double-stranded DNA molecule located on the top surface of Rad50. The ATP-bound state of Rad50 supports DNA binding, end-tethering, and Tel1/ATM signaling, whereas it renders the dsDNA inaccessible to the Mre11 nuclease active sites and therefore negatively regulates Mre11 nuclease activity. ATP hydrolysis by Rad50 opens the complex to allow the Mre11 active sites to access DNA. Whether the ADP-bound state maintains an interlinked assembly is unknown. ATP molecules are indicated as blue dots. $Zn^{2+}$ atoms are indicated as light blue dots. Xrs2 is not represented.

hairpin resolution and meiotic recombination, but not in NHEJ and Tel1 activation (Oh et al., 2016), indicating an essential role for Xrs2 in these two latter processes. This finding is consistent with the observation that stimulation of the Mre11 endonucleolytic clipping activity by Sae2 requires Rad50 but not Xrs2 (Cannavo and Cejka, 2014).

By contrast, human NBS1 is required to promote MRE11 endonuclease activity on blocked DNA ends and hairpin substrates (Paull and Gellert, 1999; Deshpande et al., 2016). Using a reconstituted system, it has been recently shown that human NBS1 stimulates the MRE11-RAD50 nuclease by directly interacting with the MRE11 subunit and this stimulation requires CtIP phosphorylation (Anand et al., 2019). By contrast, in the absence of NBS1, MRE11-RAD50 subcomplex exhibits a weak nuclease activity that requires CtIP but not its phosphorylation (Anand et al., 2019). These findings lead to a model in which CtIP promotes MRE11 nuclease activity in a phosphorylation-dependent mode in the presence of NBS1 and in a phosphorylation-independent mode in the absence of NBS1, suggesting a role for NBS1 in restricting the MRE11-RAD50 nuclease to S and G2 phases of the cell cycle when CtIP is phosphorylated by CDKs.

## ROLE OF MRX IN DSB RESECTION

The obligate step that initiates all recombination pathways is the degradation of the 5′-terminated DNA strands at both DSB ends to generate 3′-ended ssDNA overhangs that catalyze homologous pairing and strand exchange (Bonetti et al., 2018). In both yeast and mammals, DNA end resection occurs in two main steps (Garcia et al., 2011; Shibata et al., 2014; **Figure 2**). In the first step, Sae2 activates the endonuclease activity of Mre11 within the context of the MRX complex to cleave the 5′-terminated DNA strands at both DSB DNA ends (Cannavo and Cejka, 2014). This step is followed by 3′-5′ nucleolytic degradation by Mre11 that proceeds back toward the DNA ends (Reginato et al., 2017; Wang et al., 2017). The MRX-Sae2 ensemble can degrade the 5′-terminated strands up to ∼300 nucleotides away from the end and this processing is thus referred to as short-range resection. The resulting nick/gap provides an internal entry site for either Exo1 or the combined activities of the Sgs1 helicase and the Dna2 nuclease (Mimitou and Symington, 2008; Zhu et al., 2008; Cejka et al., 2010; Nicolette et al., 2010; Niu et al., 2010; Cannavo et al., 2013; Reginato et al., 2017; Wang et al., 2017). Exo1 and Dna2 are capable of resecting thousands of nucleotides in length in the 5′-3′ direction and this nucleolytic degradation is thus referred to as long-range resection.

### Short-Range Resection

Sae2 is known to be phosphorylated by multiple kinases, including CDKs and Mec1/Tel1 in a cell cycle- and DNA damage-dependent manner, respectively (Baroni et al., 2004; Cartagena-Lirola et al., 2006; Huertas et al., 2008; Manfrini et al., 2010). Using a reconstituted system, it has been shown that the ability of Sae2 to promote Mre11 endonuclease activity requires CDK-mediated Sae2 phosphorylation, and this control represents one of the key mechanisms that allow DSB resection to take place only during the S and G2 phases of the cell

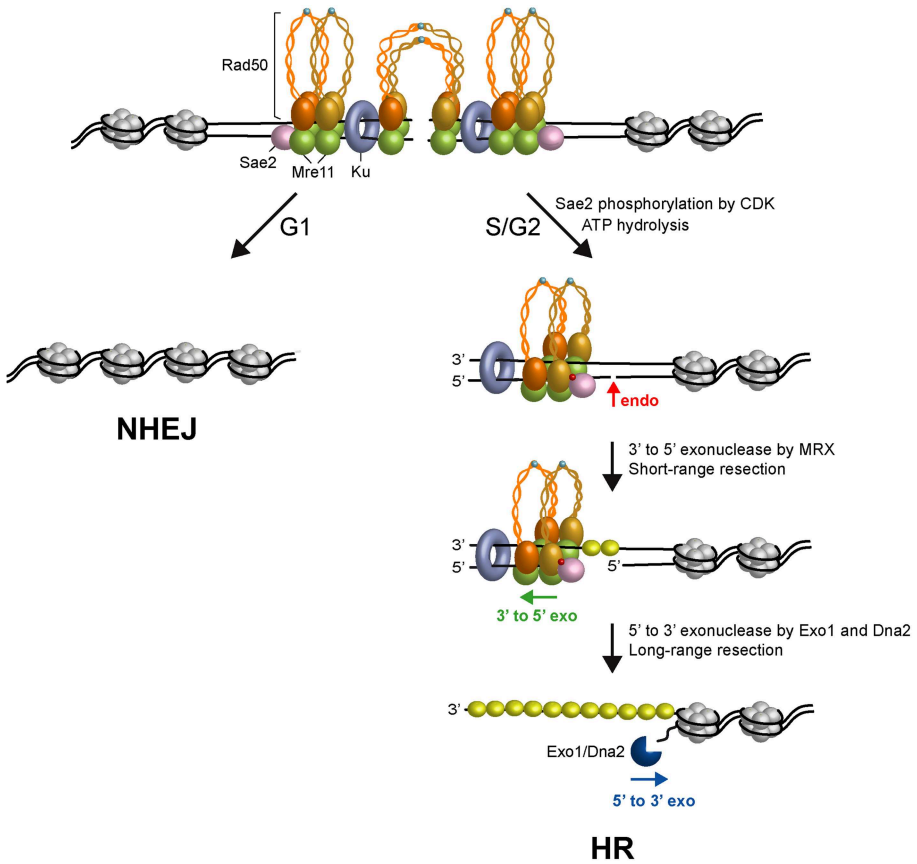


**FIGURE 2 |** Model for initiation of DSB repair. Two ATP-bound MRX complexes are loaded onto both sides of the DSB, together with Ku and Sae2 proteins. The Rad50 subunits interact through the Zn-hook to form intralinked complexes. Owing to their proximity, the Zn-hook and coiled-coil domain may switch to form interlinked complexes that maintain the DSB ends tethered to each other. In the interlinked assembly, the Mre11 and Rad50 molecules are pictured separated from each other to visualize the DNA interruption. In G1, the DSB is repaired mainly by NHEJ because Sae2 is not phosphorylated, Rad50 is an ATP-bound state that blocks the Mre11 nuclease and Ku inhibits Exo1. In the S and G2 phases of the cell cycle, upon Sae2 phosphorylation by CDK and ATP hydrolysis by Rad50, Rad50 dimerization interface opens and dsDNA becomes accessible to the Mre11 nuclease active sites. Phosphorylated Sae2 then stimulates the Mre11 endonuclease to incise the 5′-terminated strands (red arrows) at Ku-bound DNA ends or adjacent to nucleosomes. MRX proceeds back toward the DSB end using the Mre11 3′-5′ exonuclease activity. Exo1 or Sgs1-Dna2 nuclease then can degrade DNA in the 5′-3′ direction. ssDNA generated by resection is coated by RPA to initiate HR. Phosphorylation is indicated as red dots. $Zn^{2+}$ atoms are indicated as light blue dots. The Rad50 subunits belonging to a dimeric assembly are indicated with the same color (orange or gold). Xrs2 is not represented.

cycle when sister chromatids are available as repair templates (Huertas et al., 2008; Huertas and Jackson, 2009; Cannavo and Cejka, 2014; Anand et al., 2016). The phosphorylation state of Sae2 was shown to affect its oligomeric state that is critical for its activity (Kim et al., 2008; Fu et al., 2014; Andres et al., 2015; Davies et al., 2015). In particular, during the G1 phase of the cell cycle, Sae2 exists as unphosphorylated inactive soluble multimeric complexes (Cannavo et al., 2018). During S and G2 cell cycle phases or after DNA damage, phosphorylation at multiple Sae2 sites promotes formation of active Sae2 tetramers, which promote the Mre11 nuclease within the MRX complex (Cannavo et al., 2018). Furthermore, phosphorylation of the Sae2 C-terminus is necessary for a direct physical interaction between Sae2 and Rad50 (Cannavo et al., 2018). Since stimulation of Mre11 nuclease activity by Sae2 is dependent on ATP hydrolysis by Rad50 (Cannavo and Cejka, 2014; Wang et al., 2017), phosphorylated Sae2 might control the Mre11 nuclease by coupling ATP hydrolysis by Rad50 with Mre11 processing activity.

Genetic experiments have shown that MRX-Sae2-catalyzed cleavage is dispensable for resection of endonuclease-induced "clean" DSBs (Llorente and Symington, 2004), as Exo1 and Sgs1-Dna2 can directly access and resect the 5′-terminated strands of these DNA ends, although less efficiently. By contrast, MRX-Sae2-mediated cleavage is essential for removing hairpin-capped DSBs or protein blocks that render DNA ends refractory to Exo1- and Sgs1-Dna2-mediated resection (Lobachev et al., 2002; Neale et al., 2005). These end-binding factors can include trapped topoisomerases (Hoa et al., 2016) or Spo11, a meiosis-specific type II topoisomerase-like that generates programmed DSBs in meiosis by forming a covalent linkage between a conserved tyrosine residue and the 5′ end of the cleaved

strand (Bergerat et al., 1997; Keeney et al., 1997). Spo11 is then removed endonucleolytically by Mre11, which introduces internal incisions at short distance from Spo11-bound DNA ends and releases short Spo11-attached oligonucleotides (Neale et al., 2005; Garcia et al., 2011).

Interestingly, using a reconstituted system, it has been shown that phosphorylated Sae2, or CtIP in humans, promotes the Mre11 nuclease within the MRX/MRN complex to cleave endonucleolytically the 5′-terminated DNA strand ∼15–20 nucleotides away from a streptavidin block located at the end of a linear duplex DNA molecule (Cannavo and Cejka, 2014; Anand et al., 2016; Deshpande et al., 2016). Phosphorylated Sae2 was shown also to stimulate the MRX endonuclease activity on linear dsDNA substrates harboring either a streptavidin block or a catalytic inactive EcoRI restriction enzyme located at sites internal to the DSB end (Reginato et al., 2017; Wang et al., 2017). These findings suggest that any stable protein obstacle bound either internally or at the end of a DNA molecule can activate the 5′ DNA strand cleavage activity of MRX-Sae2.

The above observations raised the question of whether physiological protein blocks would also stimulate MRX-Sae2-catalyzed endonucleolytic cleavage. The Ku complex is rapidly recruited to DNA ends and protects them from degradation, particularly in the G1 phase of the cell cycle (Lisby et al., 2004; Clerici et al., 2008; Zierhut and Diffley, 2008). The lack of Ku partially restores DNA damage resistance in *sae2*Δ and *mre11* nuclease-deficient alleles (Clerici et al., 2008; Bonetti et al., 2010; Mimitou and Symington, 2010; Shim et al., 2010; Foster et al., 2011; Langerak et al., 2011), indicating that Ku bound to the DSB ends acts as a block to resection. Remarkably, Ku is as effective as a streptavidin block in stimulating the endonucleolytic cleavage by MRX in a manner that depends on phosphorylated Sae2 and ATP hydrolysis by Rad50 (Reginato et al., 2017; Wang et al., 2017). Furthermore, Ku shields DNA ends from the Mre11-catalyzed 3′-5′ degradation (Reginato et al., 2017; Wang et al., 2017). As MRX and Ku also promote NHEJ, these results support a model in which the presence of both MRX and Ku at the DSB ends in the G1 phase of the cell cycle first channels DSB repair into NHEJ (**Figure 2**). In S and G2 phases of the cell cycle, when Sae2 is phosphorylated by CDK and ATP hydrolysis by Rad50 is allowed, the presence of Ku at the DSB ends renders the 5′ DNA strand susceptible to endonucleolytic cleavage by MRX-Sae2 that directs the repair toward HR (**Figure 2**).

In any case, as Ku preferentially binds dsDNA ends over ssDNA (Griffith et al., 1992), the 3′-5′ MRX-Sae2 processing activity should cause the removal of Ku from DNA ends (Mimitou and Symington, 2010; Langerak et al., 2011; Chanut et al., 2016), raising the possibility that other proteins could stimulate 5′ strand scission by MRX-Sae2 to overcome any obstacles present not only at DNA ends but also at sites internal to the DSB. Interestingly, similar to Ku, binding of the RPA complex to either partially resected DNA ends or terminal hairpin structures also stimulates MRX-Sae2 cleavage of the 5′ strand (Wang et al., 2017), suggesting that RPA can allow MRX-Sae2 to generate an entry site in case the long-range resection machinery is disassembled from partially resected DNA ends. Furthermore, a recent reconstitution of

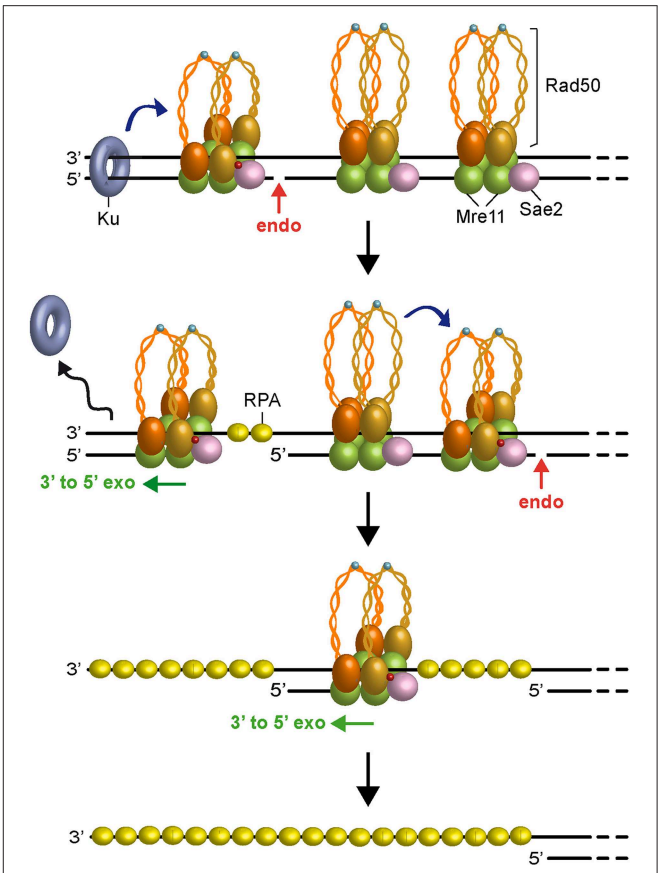


**FIGURE 3 |** Model for short-range resection. Upon Sae2 phosphorylation and ATP hydrolysis by Rad50, MRX-Sae2 introduces an endonucleolytic cleavage (red arrow) on DNA ends that are bound by Ku, which acts as protein block to stimulate MRX-Sae2 cleavage (blue arrow). Mre11 3′-5′ exonuclease proceeds back toward the DSB end to generate ssDNA that removes Ku from DNA ends. Degradation proceeds by stepwise endonucleolytic incisions, in which one MRX complex can promote (blue arrow) cleavage by another MRX complex that is bound at an adjacent site. The endonucleolytic cleavage is followed by Mre11 3′-5′ exonucleolytic degradation of the DNA fragments between the incision sites. $Zn^{2+}$ atoms are indicated as light blue dots. Phosphorylation is indicated as red dots.

the *S. cerevisiae* short-range resection machinery has shown that the Mre11-Rad50 subcomplex and phosphorylated Sae2 can cleave a 5′-terminated DNA strand by stepwise incision without the requirement for a separate protein block (Cannavo et al., 2019). Altogether, these data lead to a model (**Figure 3**), in which Ku bound to DNA ends acts as a protein block to stimulate MRX-Sae2 cleavage. 3′-5′ Mre11 exonuclease proceeds back toward the DSB end and removes Ku from the DSB. Then, MRX-mediated degradation can proceed by stepwise endonucleolytic incisions, in which one MRX-Sae2 ensemble can act by its own as protein block to stimulate DNA cleavage by another MRX-Sae2 ensemble that is bound at adjacent sites internal to the DSB. The endonucleolytic cuts are followed by 3′-5′ exonucleolytic degradation by Mre11 exonuclease of the short DNA fragments between the incision sites.

Rad50 prevents degradation of the 3′-terminated DNA strand by limiting Mre11 exonuclease activity in an ATP-binding-dependent manner, thus explaining why the 3′-5′ exonuclease activity of Mre11 does not resect 3′-terminated strands at DSB sites (Cannavo et al., 2019). By contrast, phosphorylated Sae2 can partially overcome this inhibition by stimulating Mre11 exonuclease when ATP hydrolysis is allowed (Cannavo et al., 2019). However, because phosphorylated Sae2 also promotes the endonuclease of MRX, the exonuclease and endonuclease activities of MRX-Sae2 likely compete with each other.

### Long-Range Resection

Long-range resection can be carried out by either of two partially overlapping pathways, dependent on the enzymatic activities of Dna2 and Exo1 nucleases (Mimitou and Symington, 2008; Zhu et al., 2008; Cejka et al., 2010; Nicolette et al., 2010; Niu et al., 2010; Cannavo et al., 2013; Reginato et al., 2017; Wang et al., 2017). Inactivation of a single pathway results in only a minor resection defect, whereas major resection defects are only observed when both pathways are inactivated simultaneously (Mimitou and Symington, 2008; Zhu et al., 2008). While Exo1 is a dsDNA-specific exonuclease capable to degrade 5′-terminated DNA strands within a duplex DNA molecule (Tran et al., 2002), Dna2 is loaded on ssDNA ends and degrades them endonucleolytically, resulting in products of ∼5–10 nucleotides in length (Kao et al., 2004). Dna2 resection activity requires an helicase activity that is provided by Sgs1 in yeast and by either BLM or WRN in human cells (Zhu et al., 2008; Sturzenegger et al., 2014; Pinto et al., 2016). In both yeast and mammals, Dna2 was shown to stimulate degradation of long ssDNA molecules by acting as a ssDNA translocase with 5′-3′ polarity (Levikova et al., 2017; Miller et al., 2017). This finding suggests that Sgs1 unwinds DNA in a 3′-5′ direction to provide Dna2 with ssDNA, and Dna2 translocates in a 5′-3′ direction to degrade the unwound 5′-terminated ssDNA strand.

In addition to provide an entry site for Dna2 and Exo1, MRX has also a structural role in promoting their resection activity, thus explaining why the resection defect of *mre11*Δ cells is more severe than that of *sae2*Δ or *mre11* nuclease defective mutants. Biochemical reconstitution experiments in both yeast and mammals have shown that MRX enhances the ability of Sgs1 to unwind dsDNA independently of Mre11 nuclease, possibly by increasing Sgs1 association to DNA ends (Cejka et al., 2010; Nicolette et al., 2010; Niu et al., 2010; Nimonkar et al., 2011; Cannavo et al., 2013). Furthermore, MRX/MRN enhances both the affinity to DNA ends and the processivity of Exo1 (Cejka et al., 2010; Nicolette et al., 2010; Niu et al., 2010; Nimonkar et al., 2011; Cannavo et al., 2013). Although Exo1 is a processive nuclease *in vitro*, single-molecule fluorescence imaging has shown that RPA strips Exo1 from DNA (Myler et al., 2016), implying that efficient resection requires multiple cycles of Exo1 rebinding at the same DNA end. Interestingly, MRX was shown to possess a weak ATP-dependent unwinding activity on dsDNA (Paull and Gellert, 1999; Cannon et al., 2013), which was proposed to be dependent on a rotation of the Rad50 nucleotide-binding domains (Liu et al., 2016). The recent identification of the hypermorphic *mre11-R10T* mutation, which increases Exo1 resection activity, has allowed us to demonstrate that this strand-separation function of MRX is important to stimulate Exo1 resection activity (Gobbini et al., 2018). In fact, molecular dynamic simulations have shown that the capping domains of wild type Mre11 dimer rapidly interact with the DNA ends and cause a partial unwinding of the dsDNA molecule, whereas the mutant Mre11-R10T dimer undergoes an abnormal rotation that leads one of the capping domain to wedge in between the two DNA strands and to persistently melt the dsDNA ends (Gobbini et al., 2018).

## ROLE OF MRX IN Tel1/ATM ACTIVATION

In both yeast and mammals, MRX is necessary for activation of the protein kinase Tel1/ATM (Carson et al., 2003; Uziel et al., 2003; Lee and Paull, 2004), which is a member of a serine/threonine protein kinase family with an N-terminal HEAT repeat domain and C-terminal kinase domain (Ciccia and Elledge, 2010; Gobbini et al., 2013). Mutations in the ATM gene are associated with the human syndrome Ataxia Telangiectasia (AT), whose clinical phenotypes are similar to those of ATLD and include neurodegeneration, sensitivity to IR, immunodeficiency, premature aging, radiosensitivity and predisposition to cancer (Shiloh and Ziv, 2013; Rothblum-Oviatt et al., 2016).

The exact mechanism of Tel1/ATM activation by MRX/MRN is mechanistically poorly understood. Indeed, in both yeast and mammals, MRX is required to recruit Tel1/ATM to DSBs through direct interaction between the N-terminal HEAT domain of Tel1/ATM and the C-terminal domain of the Xrs2/NBS1 subunit (Nakada et al., 2003; Falck et al., 2005; Lee and Paull, 2005; You et al., 2005). In *S. cerevisiae*, MRX and Tel1 association to DSBs is counteracted by Rif2, whose lack increases the association of MRX to DSBs in a Tel1-dependent manner (Hirano et al., 2009; Cassani et al., 2016). Co-immunoprecipitation experiments have shown that the C terminus of Xrs2 interacts with Rif2. As Tel1 also binds this Xrs2 region, Rif2 can limit Tel1 association to DSBs by interfering with MRX-Tel1 interaction (Hirano et al., 2009). Once Tel1 is recruited to DSBs by MRX, it plays a structural role in stabilizing the association of MRX to the DSB ends in a manner independently of its kinase activity (Cassani et al., 2016). This Tel1-mediated regulation of MRX retention on DNA ends is important to allow proper MRX-DNA binding that is needed for end-tethering and DSB repair (Cassani et al., 2016).

In any case, *in vitro* activation of human ATM by MRN requires ATP binding but not ATP hydrolysis (Lee et al., 2013), raising the possibility that MRX activates Tel1/ATM when it is present in the ATP-bound state. This hypothesis is supported by the identification of the separation-of-function *S. cerevisiae rad50-A78T* mutant allele, which specifically abolishes Tel1 activation without impairing MRX functions in DSB repair (Cassani et al., 2019). Molecular dynamics simulations have revealed that the mutant Mre11-Rad50$^{A78T}$ subcomplex bound to ATP undergoes conformational rearrangements similar to those observed when wild type Mre11-Rad50 subcomplex is bound to ADP (Cassani et al., 2019), suggesting that failure of

Mre11-Rad50$^{A78T}$ to activate Tel1 is due to the inability of the mutant complex to maintain the closed conformation.

In *S. cerevisiae*, the lack of Sae2 increases MRX and therefore Tel1 persistence at DSBs (Lisby et al., 2004; Clerici et al., 2006, 2014). *mre11-nd* cells also exhibit persistent MRX and Tel1 association at DSB ends (Lisby et al., 2004; Yu et al., 2018; Colombo et al., 2019). These findings suggest that MRX-Sae2 processing activity contributes to eliminate MRX bound to DNA ends and this MRX displacement limits Tel1 signaling activity. However, *sae2*Δ cells, but not *mre11-nd* cells, exhibit increased accumulation of the Rad9 protein at DSBs and enhanced activity of the Rad53 checkpoint kinase, both of which inhibit the resection activity of Dna2-Sgs1 and Exo1 (Usui et al., 2001; Bonetti et al., 2015; Ferrari et al., 2015; Yu et al., 2018; Colombo et al., 2019). Mutations that decrease either MRX/Rad9 association to DSBs or Rad53/Tel1 signaling restores DNA damage resistance in Sae2-deficient cells (Bonetti et al., 2015; Chen et al., 2015; Ferrari et al., 2015; Gobbini et al., 2015; Puddu et al., 2015; Yu et al., 2018). These findings indicate that Sae2 has an Mre11 nuclease-independent function in resection that counteracts the inhibition that Rad9 and Rad53 exert on Exo1 and Dna2-Sgs1. The identification of the *sae2-ms* allele, which upregulates MRX and Tel1 signaling activities at DSBs but does not cause increased Rad9 association at DSBs and persistent Rad53 activation, suggests that Sae2 functions in dampening MRX-Tel1 and Rad53 signaling activities can be uncoupled (Colombo et al., 2019). These findings lead to a model whereby Sae2 removes MRX and Tel1 from DNA ends by promoting Mre11 nuclease activity, whereas it limits Rad9 accumulation to DSBs independently of Mre11 nuclease activity. Both these Sae2 functions contribute to downregulate Rad53 activation, with the control of Rad9 association playing the major role in supporting DNA damage resistance and checkpoint activation (Colombo et al., 2019).

## DSB RESECTION IN A CHROMATIN CONTEXT

DNA is packaged through histone and non-histone proteins into a higher order structure called chromatin, which raises the question as to how DNA end resection occurs in the context of chromatin. Chromatin surrounding DSBs undergoes extensive modification and several highly conserved nucleosome remodelers are recruited to DNA DSBs. While some of them deposit covalent modifications on histone tails to facilitate DNA damage signaling and recruitment of repair factor, others alter chromatin structure either by replacing canonical histones with histone variants or by moving or evicting nucleosomes (Hauer and Gasser, 2017). These latter functions are carried out by proteins that use the energy of ATP hydrolysis to translocate on dsDNA and to disrupt histone-DNA contacts by nucleosome sliding, eviction or histone exchange (Osley et al., 2007).

Chromatin immunoprecipitation experiments support nucleosome disassembly near DSBs in both yeast and human cells (Li and Tyler, 2016; Tsabar et al., 2016), suggesting that nucleosome eviction occurs during resection. A key question is whether nucleosomes are evicted prior to the onset of resection or whether chromatin remodelers help the resection machinery to navigate through chromatin, with nucleosome loss occurring as a consequence of nucleolytic degradation. Genome-wide studies in meiotic cells suggest that MRX-Sae2 catalyzes the endonucleolytic cleavage preferentially on an internucleosomal DNA region at +1 and +2 nucleosomes proximal to meiotic DSB ends (Mimitou et al., 2017). Furthermore, MRX-Sae2 endonucleolytically cleaves the 5′ DNA strand bordering a nucleosome (Wang et al., 2017), thus explaining the ∼100-nucleotide incremental cleavages detected at endonuclease-induced DSBs in *sgs1*Δ *exo1*Δ cells (Zhu et al., 2008). Thus, if nucleosomes are evicted near a DSB, their removal might occur after Mre11-dependent incision of the 5′-terminated strands. Consistent with a coexistence of both nucleosomes and MRX bound at DSB ends, single-molecule imaging studies have shown that MRX can diffuse along dsDNA even in the presence of nucleosomes (Myler et al., 2017).

Interestingly, by using an *in vitro*-reconstituted chromatin assay, it has been shown that the presence of nucleosomes impedes resection by both Exo1 and Sgs1-Dna2, with Exo1-dependent resection much more strongly affected (Adkins et al., 2013). This finding suggests that nucleosome destabilization or removal occurs before nucleolytic processing by Exo1, with a constraint on resection length being how many nucleosomes are removed (Mimitou et al., 2017). In any case, removal of H2A-H2B dimers from nucleosomes was shown to enhance Exo1 activity (Adkins et al., 2013). Furthermore, biochemical and genetic evidence reveals that nucleosomes harboring H2AZ, an H2A variant that has been linked to DSB repair, are more accessible to Exo1 (Adkins et al., 2013). These findings suggest that ATP-dependent chromatin-remodeling enzymes promote Exo1-mediated resection *in vivo*.

Several chromatin remodelers are recruited to chromatin regions adjacent to DSBs and are candidates for nucleosome destabilization during DSB resection (Hauer and Gasser, 2017). Both the RSC and the SWI/SNF complexes appear to promote MRX association to DSBs and subsequent DSB processing by catalyzing eviction or mobilization of nucleosomes adjacent to a DSB (Chai et al., 2005; Shim et al., 2007; Wiest et al., 2017). Also the INO80 complex is recruited to DSBs and participates in eviction of nucleosomes to facilitate Rad51 nucleoprotein filament formation (Morrison et al., 2004; van Attikum et al., 2004, 2007; Tsukuda et al., 2009). Furthermore, two other remodelers have been shown to facilitate long-range resection. Both the SWR-C complex, which replaces the H2A/H2B dimers with H2A.Z in an ATP-dependent manner (Mizuguchi et al., 2004), and the Fun30/SMARCAD1 nucleosome remodeler promote Exo1-mediated degradation (Morillo-Huesca et al., 2010; Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012; Adkins et al., 2013). Interestingly, the resection defect of *fun30*Δ cells is suppressed by elimination of Rad9, suggesting that Fun30 stimulates Exo1 resection activity by alleviating a Rad9-dependent chromatin barrier (Chen et al., 2012; Eapen et al., 2012). Finally, mammalian CHD1, which belongs to the

chromodomain helicase DNA-binding CHD family of chromatin remodelers, is recruited to chromatin in response to DSBs in an MRE11-dependent manner and promotes the loading of CtIP onto damaged DNA (Kari et al., 2016).

## CONCLUSIONS

Work in the last years has advanced our understanding of the structure, biochemical activities, and regulation of the MRX complex. However, we still do not know at the mechanistic level how the functions of Sae2 and Rad50 ATPase integrate to regulate Mre11 nuclease activity, how the endonuclease activity of MRX is targeted locally, or how chromatin structure influence the MRX/Sae2-mediated DNA incision. Given the importance of this protein complex in ensuring genome stability and therefore in preventing carcinogenesis, answering these questions will be strongly relevant to human diseases.

## AUTHOR CONTRIBUTIONS

MPL conceptualized the work. EC, CR, and MPL wrote the manuscript. AM, MG, CVC, and DB revised and edited the manuscript.

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# The Cajal Body Protein WRAP53β Prepares the Scene for Repair of DNA Double-Strand Breaks by Regulating Local Ubiquitination

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Proper repair of DNA double-strand breaks is critical for maintaining genome integrity and avoiding disease. Modification of damaged chromatin has profound consequences for the initial signaling and regulation of repair. One such modification involves ubiquitination by E3 ligases RNF8 and RNF168 within minutes after DNA double-strand break formation, altering chromatin structure and recruiting factors such as 53BP1 and BRCA1 for repair via non-homologous end-joining (NHEJ) and homologous recombination (HR), respectively. The WD40 protein WRAP53β plays an essential role in localizing RNF8 to DNA breaks by scaffolding its interaction with the upstream factor MDC1. Loss of WRAP53β impairs ubiquitination at DNA lesions and reduces downstream repair by both NHEJ and HR. Intriguingly, WRAP53β depletion attenuates repair of DNA double-strand breaks more than depletion of RNF8, indicating functions other than RNF8-mediated ubiquitination. WRAP53β plays key roles with respect to the nuclear organelles Cajal bodies, including organizing the genome to promote associated transcription and collecting factors involved in maturation of the spliceosome and telomere elongation within these organelles. It is possible that similar functions may aid also in DNA repair. Here we describe the involvement of WRAP53β in Cajal bodies and DNA double-strand break repair in detail and explore whether and how these processes may be linked. We also discuss the possibility that the overexpression of WRAP53β detected in several cancer types may reflect its normal participation in the DNA damage response rather than oncogenic properties.

**Keywords: DNA repair, ubiquitin, WRAP53β, Cajal body, WD40, cancer, chromatin modification, RNF8**

## THE LEAD ROLE FOR WRAP53β: SCAFFOLDING RNA-PROTEIN COMPLEXES

### Function Through Organization

Structural organization within the nuclear space contributes significantly to functional regulation (Misteli, 2005; Nunez et al., 2009; Van Bortle and Corces, 2012). For example, organizing appropriate ribonucleoprotein complexes into the nuclear organelles known as Cajal bodies controls and accelerates reactions involved in pre-mRNA splicing and telomere elongation

(Carmo-Fonseca et al., 1992; Jády et al., 2004; Kiss et al., 2006; Matera and Shpargel, 2006; Stanek and Neugebauer, 2006). Similarly, upon DNA damage, repair factors are concentrated into foci, providing an environment beneficial for repair (Bekker-Jensen et al., 2006; Altmeyer et al., 2015).

Cajal bodies contain transcription factors and polymerases (Polak et al., 2003; Machyna et al., 2013; Hutten et al., 2014) and like other nuclear bodies, can be formed in association with transcription (Shevtsov and Dundr, 2011). For Cajal bodies, this nucleation occurs at specific genomic loci, including genes encoding small nuclear (sn)RNAs, small nucleolar (sno)RNAs, small Cajal body-specific (sca)RNAs and histones (Frey and Matera, 1995, 2001; Smith et al., 1995; Machyna et al., 2013). When transcribed, these loci are brought together in a transcriptional center within the Cajal body that accelerates RNA production (Sawyer et al., 2016a,b; Wang et al., 2016). The RNAs transcribed are subsequently processed, modified (methylated, pseudouridinylated) and/or function within the Cajal bodies themselves (Darzacq et al., 2002; Jády et al., 2003; Dominski and Marzluff, 2007; Enwerem et al., 2015). Similarly, RNAs are transcribed from sites of DNA damage (Francia et al., 2012; Wei et al., 2012; Michelini et al., 2017; Bonath et al., 2018), which can hybridize with the damaged DNA (Ohle et al., 2016; Lu et al., 2018), be processed by DICER and DROSHA (Francia et al., 2012; Michelini et al., 2017; Lu et al., 2018) or become methylated by METTL3 (Xiang et al., 2017), thereafter, regulating damage repair.

## The Genome and Cajal Bodies Come Together With WRAP53β

The scaffold protein WRAP53β (WD40-encoding RNA antisense to p53) (alias WRAP53, WDR79, and TCAB1), initially discovered in our laboratory as an antisense gene to p53 (Mahmoudi et al., 2009), plays several key roles in Cajal bodies. First, this protein is vital for their formation (Mahmoudi et al., 2010), bringing the necessary proteins and gene loci into close proximity (Mahmoudi et al., 2010; Wang et al., 2016). Loss of WRAP53β disrupts Cajal bodies, suppresses clustering of sn/sno/scaRNA/histone loci and downregulates transcription from these sites. Second, WRAP53β plays essential roles in maintaining Cajal bodies and targeting factors to these organelles (**Figure 1A**; Mahmoudi et al., 2010; Henriksson and Farnebo, 2015), probably by stabilizing interactions between Cajal body components. The direct interaction between the Cajal body marker Coilin and the splicing-related survival of motor neuron (SMN) protein is stabilized by WRAP53β (Mahmoudi et al., 2010), which can bind several proteins and RNAs simultaneously through its seven WD40 repeats (**Figures 1A,B**). WRAP53β also binds the telomerase RNA (TERC) and locates the telomerase complex to Cajal bodies and further on to telomeres (Venteicher et al., 2009).

Extensively interacting chromosomal loci are often fragile and enriched in DNA repair factors, indicating that they are primed for rapid DNA repair (Sobhy et al., 2019). WRAP53β is involved in DNA repair and its presence at Cajal body-associated gene loci may thus allow rapid repair. In addition, assembly of WRAP53β at DNA lesions may facilitate damage-induced genome reorganization and/or clustering of DNA breaks, which promotes efficient recognition and repair of lesions (Aymard et al., 2017; Stadler and Richly, 2017). WRAP53β may also stimulate transcription of RNA from the break site and/or its processing or concentrate repair factors into specialized foci to accelerate necessary reactions.

# SPOTLIGHT ON DNA REPAIR: WRAP53β CONTROLS LOCAL UBIQUITINATION

## Regulation of Protein Recruitment and Repair Pathway Choice by Ubiquitination

Among the most toxic DNA lesions are double-strand breaks, repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR) (reviewed e.g., by Ciccia and Elledge, 2010; Ceccaldi et al., 2016). Following such breakage, the damaged chromatin is modified chemically, including by ubiquitination, which facilitates recruitment of repair factors. Ubiquitination involves stepwise enzymatic activation, conjugation, and ligation of the small ubiquitin protein to lysine residues by E1, E2, and E3 enzymes, respectively (Pickart and Eddins, 2004). The presence of lysine residues in ubiquitin itself allows formation of various types of polyubiquitin chains, with ubiquitin chains linked at K48 typically targeting proteins for degradation, whereas K63-linked chains often signal protein recruitment (Panier and Durocher, 2009; Smeenk and van Attikum, 2013).

RNF8, the first ubiquitin ligase to arrive at DNA breaks (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007), initially catalyzes K63-linked ubiquitin chains on histone H1 (Thorslund et al., 2015), which recruits RNF168 to ubiquitinate histone H2A at K13/K15 (Mattiroli et al., 2012; Uckelmann and Sixma, 2017), thereby potentiating the local ubiquitin signal (Uckelmann and Sixma, 2017). This triggers recruitment of 53BP1 (via K15 ubiquitination of H2A), which then restricts DNA end resection and promotes NHEJ repair (**Figure 1C**; Nakamura et al., 2006;

**Abbreviations:** 53BP1, p53-binding protein 1; ATM, Ataxia-telangiectasia mutated; ATR, Ataxia telangiectasia and Rad3 related; BARD1, BRCA1-associated RING domain protein 1; BRCA1, Breast cancer type 1 susceptibility protein; BRCC36, BRCA1/BRCA2-containing complex subunit 36; CHD4, Chromodomain-helicase-DNA-binding protein 4; CHK1, Checkpoint kinase 1; CHK2, Checkpoint kinase 2; DUB, Deubiquitinating enzyme; FHA domain, Forkhead-associated domain; H1, Histone 1; H2A, Histone 2A; H2B, Histone 2B; H2AX, Histone variant 2AX; γH2AX, Phosphorylated histone H2AX; HR, Homologous recombination; MDC1, mediator of DNA damage checkpoint 1; METTL3, N6-adenosine-methyltransferase 70 kDa subunit; MRN, Complex with MRE11 homolog double strand break repair nuclease, RAD50 double strand break repair protein and Nibrin; NBS1, Nibrin; NHEJ, Non-homologous end joining; PARP, Poly (ADP-ribose) polymerase; POH1, proteasome 19S subunit; RAD17, DNA repair protein RAD17 homolog; RAD51, DNA repair protein RAD51 homolog; RAP80, Receptor-associated protein 80; RNF8, Ring finger protein 8; RNF20, Ring finger protein 20; RNF40, Ring finger protein 40; RNF168, Ring finger protein 168; scaRNA, Small Cajal body-specific RNA; SMN, Survival of motor neuron protein; snRNA, Small nuclear RNA; snoRNA, Small nucleolar RNA; UBE4A, Ubiquitin conjugation factor E4 A; WRAP53β, WD40-encoding RNA antisense to p53; XRCC4, X-ray repair cross-complementing protein 4.

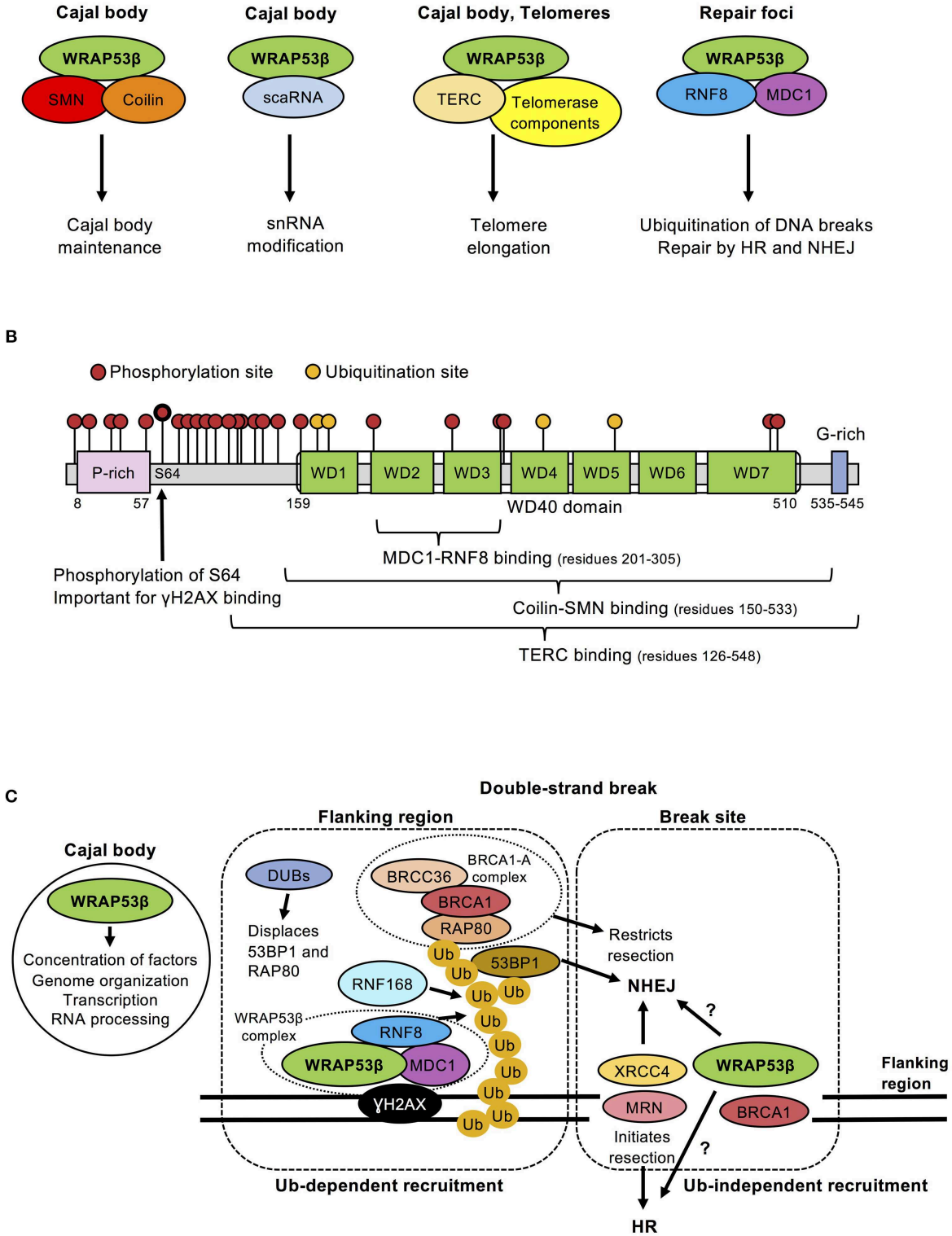


**FIGURE 1 | (A)** Schematic illustration of the different WRAP53β complexes, their localization and function. Note: scaRNA and TERC are RNA molecules. **(B)** Schematic illustration of the domains, binding, phosphorylation and ubiquitination sites in the WRAP53β protein. The sites for post-translational modifications were

*(Continued)*

**FIGURE 1 |** obtained from PhosphoSitePlus on April 17, 2019. The location of WD40 repeats were predicted using the WD40-repeat protein Structures Predictor (Wu et al., 2012; Wang et al., 2013, 2015; Ma et al., 2019); (WDSP, May 2nd 2019): WD40 1 (amino acid residues 159–197), WD40 2 (residues 207–259), WD40 3 (residues 266–305), WD40 4 (residues 313–354), WD40 5 (residues 358–397), WD40 6 (residues 402–442), WD40 7 (residues 450–510). **(C)** Schematic view of the functions of WRAP53β in Cajal bodies, at the break site and in surrounding chromatin. Ubiquitin-dependent recruitment of DNA repair factors occurs at regions flanking the break site. WRAP53β binds γH2AX and also scaffolds the interaction between MDC1 and RNF8, which is important for the recruitment of RNF8 to DNA breaks. Once there, RNF8 and RNF168 ubiquitinate proteins at damaged chromatin, which stimulates recruitment of downstream factors 53BP1, RAD51, and BRCA1. BRCA1 forms several sub-complexes with different functions, of which the BRCA1-A complex (containing BRCA1, RAP80, BRCC36, and additional proteins not discussed here) restrict resection. Recruitment to the break site appears to be ubiquitin-independent and the factors recruited here include XRCC4, which promotes NHEJ, or DNA break sensor proteins, such as the MRN complex that promote HR. Pools of WRAP53β and BRCA1 also locate at this site for reasons unknown. Functions performed by WRAP53β in Cajal bodies could potentially be performed at break sites. The recruitment of RAD51, a downstream protein of WRAP53β, to DNA lesions appears to occur via both ubiquitin-dependent and independent mechanisms.

Kolas et al., 2007; Fradet-Turcotte et al., 2013). Intriguingly, ubiquitin chains also recruit RAP80 along with the key HR factor BRCA1 (Sobhian et al., 2007; Wang and Elledge, 2007).

The choice of repair pathway beyond this point remains unknown. In addition to the known determinants [i.e., cell cycle phase, site of damage (e.g., gene-rich/poor regions) and local concentration of factors required], the BRCA1 recruited to ubiquitin as part of the BRCA1-A complex appears to be involved in fine-tuning the choice of repair pathway, since this complex attenuates end resection (**Figure 1C**; Sobhian et al., 2007; Wang and Elledge, 2007; Hu et al., 2011). In contrast, BRCA1 recruited via resected DNA belonging to other complexes (i.e., BRCA1-B, BRCA1-C, BRCA1-D) promotes end resection, strand invasion and RAD51 loading, crucial steps in the HR pathway (Greenberg et al., 2006; Sy et al., 2009; Zhang et al., 2009; Xie et al., 2012; Cruz-García et al., 2014; Savage and Harkin, 2015; Zhao et al., 2017). Moreover, BRCA1 has E3 ligase activity and, with the E3 ligase BARD1, can ubiquitinate H2A to remove 53BP1, thereby promoting HR (Densham et al., 2016).

Other ubiquitin ligases also promote HR and influence NHEJ by fine-tuning ubiquitination of damaged chromatin. For example RNF20 and RNF40 ubiquitinate histone H2B (Moyal et al., 2011; Nakamura et al., 2011; So et al., 2019), while UBE4A edits ubiquitin chains at breaks (Baranes-Bachar et al., 2018).

Deubiquitinating enzymes (DUBs) are also involved in the choice of repair pathway. Thus, removal of ubiquitin chains by POH1 promotes HR by displacing 53BP1 and RAP80 to the periphery of the repair foci (Butler et al., 2012; Kakarougkas et al., 2013; Nakada, 2016). Some BRCA1 complexes contain DUBs, including BRCC36, which functions together with the BRCA1-A complex; removal of this DUB results in unrestrained end resection and hyperactive HR (**Figure 1C**; Shao et al., 2009; Ng et al., 2016).

These observations emphasize the central role of ubiquitination in the DNA damage response, in which the RNF8/RNF168-pathway is a key upstream actor, regulating several steps of both NHEJ and HR repair. Notably, the RNF8 protein is unstable, so continuous splicing is required for its presence at DNA lesions (Pederiva et al., 2016). Consequently, even short term inhibition of splicing impairs repair (Pederiva et al., 2016), which can explain the defective repair associated with knockdown of various splicing factors detected in several genome-wide siRNA screens (Paulsen et al., 2009; Adamson et al., 2012).

## Alteration of Chromatin Structure by Ubiquitination

The structure of chromatin around DNA lesions influences the DNA damage response. Initial compaction stimulates early steps in this process, such as recruitment of the MRN complex, while persistent compaction is unfavorable to downstream repair and recovery, and attenuates phosphorylation of CHK2 (Burgess et al., 2014). Moreover, a collar of compact chromatin is formed around the DNA lesions, potentially to restrict repair to this site, since repair factors, including 53BP1, only localize within its decompacted interior (Lou et al., 2019).

Interestingly, this compaction around the break site is dependent on RNF8 (Lou et al., 2019), indicating a role for ubiquitination in regulating the higher-order structure of damaged chromatin. Since RNF8 can promote relaxation of chromatin by recruiting the remodeling factor CHD4 (Luijsterburg et al., 2012), it is possible that interior decondensation by RNF8 triggers the formation of a heterochromatic border around DNA breaks. Altogether, the ubiquitin response not only stimulates recruitment of repair factors and influences the choice of DNA double-strand break repair pathway but also appears to shape the local chromatin for proper progression of the DNA damage response.

## WRAP53β Orchestrates Ubiquitination of Damaged Chromatin via RNF8

WRAP53β was first implicated in DNA repair by several screens for novel repair proteins (Matsuoka et al., 2007; Paulsen et al., 2009; Adamson et al., 2012). Its direct involvement in the repair of DNA double-strand breaks by both HR and NHEJ was later confirmed and shown to involve scaffolding interactions between RNF8 and MDC1 (Henriksson et al., 2014) by simultaneously and independently binding the FHA domains of both proteins through its own WD40 domain (**Figure 1B**; Henriksson et al., 2014). In this manner, WRAP53β promotes assembly of RNF8 at DNA lesions, ubiquitination of damaged chromatin and downstream recruitment of 53BP1, BRCA1, and RAD51 (**Figure 1A**; Henriksson et al., 2014; Hedström et al., 2015). RNF8 and MDC1 can interact directly, but do not in the absence of WRAP53β, which appears to stabilize their interaction in a manner similar to the SMN-coilin interaction (**Figure 1A**). WRAP53β does not influences RNF8 levels, excluding indirect effects on splicing.

## BEHIND THE SCENES: WRAP53β PLAYS MULTIPLE ROLES AT DNA BREAKS

### WRAP53β Influences DNA Repair Beyond RNF8

Notably, depletion of WRAP53β reduces HR and NHEJ efficiency more than knockdown of RNF8 (Henriksson et al., 2014), indicating that WRAP53β plays additional roles, probably ubiquitin-independent, in DNA repair. Indeed, two distinct WRAP53β fractions are present at DNA double-strand breaks; one in regions surrounding the break (also positive for γH2AX/MDC1/RNF8) and another at the break site itself [normally devoid of/low in γH2AX/MDC1/RNF8 (Henriksson et al., 2014; Goldstein and Kastan, 2015), but instead enriched in NHEJ factors (e.g., XRCC4) and DNA break sensors (e.g., NBS1, part of the MRN complex) (Goldstein et al., 2013)]. WRAP53β is recruited to the break site itself more rapidly and remains there longer than in the surrounding regions (**Figure 1C**; Henriksson et al., 2014).

Interestingly, like WRAP53β, BRCA1 is recruited to both regions and to a higher extent to the break site. Its recruitment to the surrounding regions depends on the RNF8/RNF168/RAP80-pathway (and on ATM and PARP), while its accumulation at the break site is mediated by the MRN complex. BRCA1 appears to stimulate cell cycle checkpoints at the flanking regions and re-ligation of the breaks at the break site (Xu et al., 2001; Goldstein and Kastan, 2015). Thus, WRAP53β and BRCA1 both participate in the RNF8-mediated ubiquitination pathway, while promoting other aspects of repair at the break site itself (**Figure 1C**).

### What Regulates WRAP53β?

Recruitment of WRAP53β to repair foci, probably the regions surrounding DNA double-strand breaks, requires ATM, H2AX and MDC1 (Henriksson et al., 2014). Importantly, upon DNA damage, WRAP53β is phosphorylated by ATM at serine 64 (Matsuoka et al., 2007; Coucoravas et al., 2017) and a phosphomutant of WRAP53β (S64A) cannot rescue defects in DNA repair when the wild-type protein is knocked down (Rassoolzadeh et al., 2015; Coucoravas et al., 2017). ATM-mediated phosphorylation of WRAP53β does not influence its interaction with RNF8 and MDC1. However, WRAP53β also binds γH2AX and this interaction is enhanced by phosphorylation (**Figure 1B**; Rassoolzadeh et al., 2015; Coucoravas et al., 2017). Since WRAP53β binds RNF8 and MDC1 even before damage, these three proteins might pre-form a complex that can be activated and recruited to DNA breaks by ATM in a multistep manner, e.g., phosphorylation of MDC1 allows direct RNF8-MDC1 interaction, phosphorylation of WRAP53β stimulates WRAP53β-γH2AX interaction and phosphorylation of γH2AX allows MDC1-γH2AX interaction (Rassoolzadeh et al., 2015; Coucoravas et al., 2017).

Phosphorylated WRAP53β$^{S64}$ locates to both DNA breaks and Cajal bodies. However, the unphosphorylated form accumulates in Cajal bodies to a greater extent (Coucoravas et al., 2017), indicating that phosphorylation of WRAP53β by ATM relocates this protein from Cajal bodies to DNA breaks. WRAP53β targets several factors to Cajal bodies and maintains the structure of this organelle and these functions may be affected by WRAP53β relocation. For example, ionizing radiation moves WRAP53β to DNA breaks, while telomerase (Wong et al., 2002) and several other Cajal body components (including coilin, SMN, fibrillarin, and snRNAs) move to and around nucleoli (in nucleolar caps). This indicates that exit of WRAP53β from Cajal bodies displaces associated proteins to other sites. Moreover, Cajal bodies become disrupted several hours after DNA damage (Gilder et al., 2011).

In addition to S64, 23 other residues of WRAP53β are phosphorylated and four ubiquitinated by unknown enzymes for unclear reason (**Figure 1B**) (data from UniProt and PhosphoSitePlus websites) (Hebert and Poole, 2017). WRAP53β appears to be rate-limiting for both HR and NHEJ and, accordingly, its overexpression enhances the efficiency of both pathways by stimulating RNF8-mediated ubiquitination at damaged chromatin (Rassoolzadeh et al., 2016). Further studies on the complex interplay between the functions of WRAP53β in DNA repair, the Cajal body and telomere maintenance are required and post-translational modifications may be important in this context.

## WRAP53β ACTING OFF-SCRIPT: LOSS OF TUMOR SUPPRESSION AND ACTIVATION OF THE DNA DAMAGE RESPONSE IN CANCER

### Loss of Tumor Suppression by WRAP53β

Inactivating germline mutations in WRAP53β cause dyskeratosis congenita, characterized by bone marrow failure, premature aging and predisposition for cancer (Zhong et al., 2011). Moreover, downregulation of WRAP53β RNA or its loss from the nucleus in patients with head and neck, breast, and ovarian cancer is correlated with shorter survival (Garvin et al., 2015; Hedström et al., 2015; Silwal-Pandit et al., 2015). Furthermore, numerous genetic alterations in *WRAP53*, mainly deletions or mutations, are present in multiple cancers (**Figure 2A**) (cBioPortal, https://www.cbioportal.org/) (Cerami et al., 2012; Gao et al., 2013), further evidence that loss-of-WRAP53β-function promotes cancer development/progression. In addition, attenuated expression of WRAP53β correlates with resistance of patients with head and neck cancer and metastasized rectal cancer to radiotherapy (Zhang et al., 2012; Garvin et al., 2015).

At the same time, WRAP53β is overexpressed in other cancer types (see further below), but the clinical relevance remains unclear. Although we have suggested that WRAP53β has oncogenic properties (Mahmoudi et al., 2011), we now believe that this is a misinterpretation of the data, which instead reflects participation of WRAP53β in the DNA damage response (**Figure 2B**).

### Does WRAP53β Appear to be Oncogenic by Activating the DNA Damage Response?

Precancerous lesions are characterized by activation of DNA damage signaling and repair, often due to replication stress, which is believed to constrain tumor progression. This phenomenon includes formation of 53BP1 foci and activation

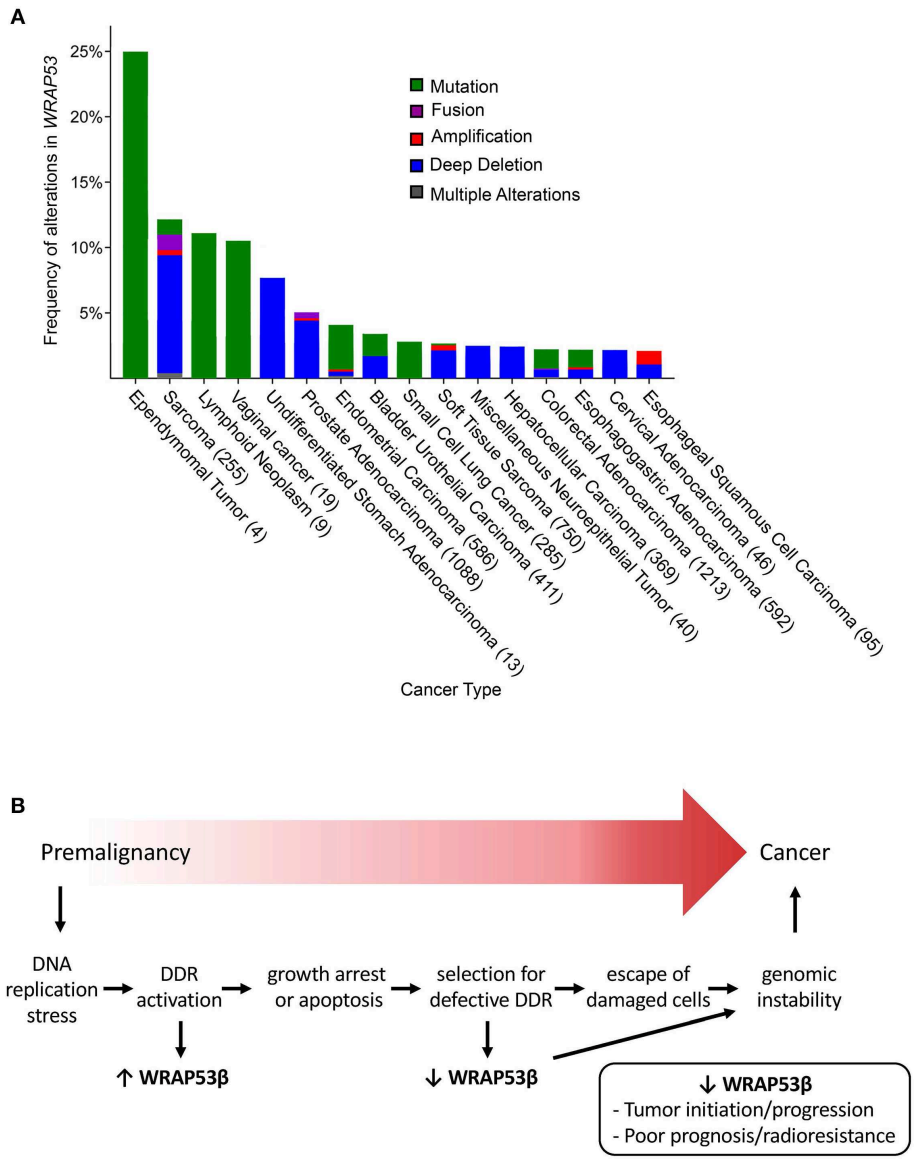


**FIGURE 2 | (A)** The frequency of genetic alterations in *WRAP53* associated with various cancer types (minimum threshold of 2%) (From cBioPortal, accessed April 17, 2019). The numbers in parenthesis represent the number of patients analyzed. **(B)** Proposed model for the involvement of WRAP53β and the DNA damage response in the development of cancer. Aberrant cell proliferation may cause replication stress, formation of DNA double-strand breaks and activation of the DNA damage response. Overexpression of WRAP53β as part of the response stimulates repair, growth arrest and/or apoptosis, but some damaged cells may escape (e.g., due to downregulation of WRAP53β), leading to genomic instability and potential progression into cancer.

of the ATM/ATR checkpoint; premalignant tumor samples stain positively for the phosphorylated forms of ATM, CHK1, CHK2, RAD17, p53, and H2AX (Bartkova et al., 2005; Gorgoulis et al., 2005). Potential inactivating mutations in key DNA damage response proteins, such as p53 and ATM (Olivier et al., 2010; Choi et al., 2016) allow survival of damaged and genetically unstable cells that upon clonal expansion progress into carcinoma (Bartkova et al., 2005; Gorgoulis et al., 2005).

WRAP53β is overexpressed in a variety of cancer cell lines and primary head and neck, lung and colorectal cancers (Zhang et al., 2012; Rao et al., 2014; Sun et al., 2014, 2016; Zhu et al., 2018). In addition, knockdown of this protein promotes apoptosis and reduces proliferation of cancer cell lines and xenografts (Mahmoudi et al., 2011; Sun et al., 2014, 2016; Wang et al., 2017; Yuan et al., 2017; Chen et al., 2018; Zhu et al., 2018). Such observations appeared to indicate that WRAP53β may act as an oncogene. However, in the vast majority of studies to date overexpression of WRAP53β was not significantly associated with worse patient survival.

Instead, overexpression of WRAP53β may reflect its involvement in DNA repair and thus be a response to the stress of rapid proliferation. Further support for this proposal

includes the following: (1) upregulation of WRAP53β promotes activation of the ATR-CHK1 pathway in nasopharyngeal carcinoma induced by Epstein Barr Virus (Wang et al., 2017); (2) upregulation of WRAP53β correlates with activation of the DNA damage response pathway in ovarian cancer (Hedström et al., 2015); (3) subsequent downregulation of WRAP53β in patients with ovarian cancer is significantly associated with higher mortality, while intitial upregulation was not, indicating that downregulation drives tumor progression (Hedström et al., 2015); and (4) similarly, downregulation of WRAP53β in patients with metastasized rectal cancer promotes resistance to radiotherapy and is associated with higher mortality, while initial upregulation did not influence patient survival (Zhang et al., 2012). Moreover, enhanced expression of WRAP53β in cancer cells may be linked to their greater number of Cajal bodies, reflecting a higher demand for associated functions (Spector et al., 1992).

Potential re-activation of telomerase is, as far as we can see, the only reasonable mechanism by which upregulation of WRAP53β could actually promote tumorigenesis. However, the telomerase gene is not an oncogene, since its product does not by itself cause uncontrolled growth and is active in normal embryonic stem and germline cells (Harley, 2002). Several studies have reported a correlation between overexpression of WRAP53β and increased telomere length or telomerase activity, but it remains to be determined whether telomerase activity is correlated with prognosis (Qiu et al., 2015; Wang et al., 2017; Sun et al., 2018).

## CONCLUDING REMARKS—WRAPPING IT ALL UP

The scaffolding protein WRAP53β organizes the genome so that formation of Cajal bodies is stimulated, the expression of associated genes enhanced and their products concentrated in these organelles. Similarly, WRAP53β concentrates factors important for repair of DNA double-strand breaks via ubiquitination of damaged chromatin. Upon DNA damage, a fraction of WRAP53β is phosphorylated, promoting its role in DNA repair, with several other pools of WRAP53β having different localizations and functions, orchestrated by various post-translational modifications. This complexity explains how this protein performs so many tasks within the cell in a coordinated fashion, as well as why disease may occur when it is lost or dysfunctional.

In addition to dyskeratosis congenita and sporadic cancer, loss of WRAP53β has been linked to the pathogenesis of spinal muscular atrophy (Mahmoudi et al., 2010; Di Giorgio et al., 2017). Furthermore, this protein is part of a repair machinery that organizes and resolves persistent DNA damage in neurons (Mata-Garrido et al., 2016) and accumulation of such damage is believed to contribute to neurodegenerative disorders such as spinal muscular atrophy and amyotrophic lateral sclerosis (Fayzullina and Martin, 2014; Mitra et al., 2019).

Thus, more in-depth understanding of the role of WRAP53β in DNA repair and other processes may help decipher the complicated mechanisms underlying tumorigenesis, premature aging and neurodegeneration and thereby lead to novel treatment strategies.

## AUTHOR CONTRIBUTIONS

SB, EO, and MF wrote the manuscript, were involved in the generation of the figures and critically revised the manuscript. All authors contributed to the conception of this review article and MF co-ordinated the work.

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# Regulation of DNA Double Strand Breaks Processing: Focus on Barriers

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In all the eukaryotic cells, nucleolytic processing (resection) of a double strand DNA break (DSB) is a key step to channel the repair of the lesion toward the homologous recombination, at the expenses of the non-homologous end joining (NHEJ). The coordinated action of several nucleases and helicases generates 3′ single strand (ss) DNA, which is covered by RPA and recombination factors. Molecular details of the process have been first dissected in the model organism *Saccharomyces cerevisiae*. When DSB ends are occupied by KU, a central component of the NHEJ, the Mre11-Rad50-Xrs2 (MRX) nuclease complex (MRN in human), aided by the associated factors Sae2 (CTIP in human), initiates the resection process, inducing a nick close to the DSB ends. Then, starting from the nick, the nucleases Mre11, Exo1, Dna2, in cooperation with Sgs1 helicase (BLM in human), degrade DNA strand in both the directions, creating the 3′ ssDNA filament. Multiple levels of regulation of the break processing ensure faithful DSB repair, preventing chromosome rearrangements, and genome instability. Here we review the DSB resection process and its regulation in the context of chromatin. Particularly, we focus on proteins that limit DSB resection, acting as physical barriers toward nucleases and helicases. Moreover, we also take into consideration recent evidence regarding functional interplay between DSB repair and RNA molecules nearby the break site.

**Keywords: resection barriers, DSB processing, NHEJ, HDR, DNA:RNA hybrid**

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## DSB END PROCESSING

DSBs are classically defined as broken chromosomes, however uncapped telomere ends and reversed forks are bound and processed by the same factors. In this review we generally focus on broken chromosomes, although proteins, and mechanisms that we mention are active on whole types of DSBs.

In all the eukaryotes, a DSB can be repaired through non-homologous end joining (NHEJ) or homology directed recombination (HDR). Both pathways are organized in distinct steps and sub-pathways, which involve the coordination of several factors and enzymes (Heyer et al., 2010; Symington, 2016). Of note, specific mechanisms are required to process DSB ends containing covalently-bound proteins (such as Topoisomerase), DNA alterations (oxidation, methylation, hairpin formation, and others) and associated RNA molecules (e.g., DNA:RNA hybrids), which interfere with their repair through NHEJ and HDR (**Figures 1A–C**). Moreover, an irreparable DSB can be eventually processed by telomerase and DNA polymerase alpha-primase (Pol α-Prim), together with other factors, leading to *de novo* telomere addition (Putnam and Kolodner, 2017). Given the heterogeneity and complexity of the mechanisms involved, multiple levels of

regulation have been identified, determining the repair of a DSB in the different cell cycle phases and chromatin context. Indeed, the uncontrolled DSB processing and repair greatly contribute to chromosome rearrangements (deletions, insertion, translocations), hallmarks of cancer and other pathological conditions associated to genome instability.

The nucleolytic processing (also called resection) of the DSB ends is a critical and finely regulated step to promote HDR over NHEJ. Indeed, the resection process generates an extended 3′-end ssDNA filament, which is then covered by RPA and the recombinase Rad51, depending on the sub-pathway (Symington, 2016).

The DSB resection is carried out by the coordinated actions of several nucleases, among which Mre11, Exo1, and Dna2 are the most involved from yeast to human. According to current models, Dna2 in cooperation with the helicase Sgs1 (BLM in human), and Exo1 process a DSB whose 5′ ends are accessible. Alternatively, a DSB with blocked or chemically modified ends needs the activity of the MRX (MRN in human) complex to initiate the process (**Figure 1D**). Indeed, *in vivo* and *in vitro* data (Neale et al., 2005; Shibata et al., 2014; Reginato et al., 2017; Wang et al., 2017, 2018) have shown that Mre11 is recruited nearby the DSB ends and induces a nick on the 5′-end filament, creating the entry point for both Exo1 and Dna2-Sgs1/BLM (**Figure 1D**). Then, starting from the nick, MRX/MRN processes the DNA in the 3′-to-5′ direction till the break site (short-range resection), while Exo1 and Dna2-Sgs1/BLM extensively process the DNA in the 5′-to-3′ direction (long-range resection) (**Figure 1D**). Interestingly, recent *in vitro* data indicate that BLM promotes the EXO1 resection processivity, too (Soniat et al., 2019). This nick-dependent mechanism for resection is activated in S and G2/M phases through the CDK1-dependent phosphorylation of Sae2 (CTIP in human) (Huertas et al., 2008; Huertas and Jackson, 2009), which associates with the Mre11 complex.

The importance of regulating the DNA ends resection in DSB repair is underlined by the increasing list of factors participating in the reaction in human cells, including the oncosuppressor BRCA1 (Zhao et al., 2019).

Below we review how specific factors and DNA/RNA transactions limit DSB resection, acting as physical barriers toward the nucleases. However, their antagonistic roles in the process appear very dynamic, likely exerting both negative and positive regulations on DSB repair.

## NUCLEOSOME-DEPENDENT BARRIER

There is a general agreement that DNA, wrapped around the histone octamer into the nucleosome, is refractory to be resected due to steric hindrance. Indeed, in yeast the resection of DSBs frequently terminates at nucleosome (Mimitou et al., 2017); moreover, *in vitro* assays showed that DNA with reconstituted nucleosomes is resected by both Exo1 and Dna2-Sgs1 slower than naked DNA (Adkins et al., 2013). Remarkably, other *in vitro* results showed that BLM is able to slide nucleosomes, if RPA is added in the assay, promoting DNA resection by EXO1 and DNA2 (Xue et al., 2019). Of importance, the phosphorylation of RPA is critical to limit resection at nucleosomes, interfering with the strand-switching of BLM helicase (Soniat et al., 2019). However, Exo1 can actively resect DNA packed into nucleosomes containing the H2A.Z histone variant, which promotes higher mobility and instability of the octamer (Adkins et al., 2013). As such, the dynamic deposition of H2A.Z, together with other histone modifications, might facilitate the long-range resection by Exo1, with processing rate similar to naked DNA. On the other hand, it has been also shown that H2A.Z and H3.3 variants facilitate the loading of the NHEJ factors KU and XRCC4 onto DSB, thus limiting resection initiation (Xu et al., 2012; Luijsterburg et al., 2016). Nevertheless, other modifications of the histone core have been recently shown to facilitate the recruitment at DSB of both NHEJ and pro-resection factors, leading to a more complex scenario. According to several *in vivo* results, current models support a fundamental role of chromatin remodelers to mobilize and/or dissociate nucleosomes 1-2 kb nearby a DSB, creating the entry-space for repair factors (Shim et al., 2007; Price and D'Andrea, 2013; Clouaire and Legube, 2019; **Figure 1D**).

## KU-DEPENDENT BARRIER

Soon after a DSB formation, the heterodimer Ku70-80 complex (KU) binds DNA ends in all the cell cycle phases, acting as a platform for the association of several factors involved in NHEJ (Frit et al., 2019). Along with its role in promoting NHEJ, KU plays also fundamental role in limiting chromosome translocations mediated by the annealing of ssDNA repeats in human cells (Weinstock et al., 2006). Indeed, KU-bound DSB ends are resistant to Exo1 and Dna2 processing (Shim et al., 2010; Symington, 2016; Wang et al., 2018), reducing recombination DNA repair by micro-homology mediated end joining (MMEJ, also called alternative end-joining or alt-EJ in higher eukaryotes) and single strand annealing (SSA) mechanisms (Symington, 2016). In yeast, KU-dependent resection barrier is predominant in G1 phase (Clerici et al., 2008), when MRX-Sae2 is not activated by CDK1, or in the absence of functional MRX complex or Sae2 (Mimitou and Symington, 2010). Accordingly, deletion of *KU70* partially suppressed the resection defect and sensitivity of *sae2* or *mre11* mutants to ionizing radiations (Bonetti et al., 2010; Mimitou and Symington, 2010; Foster et al., 2011).

These and other experimental evidence support the involvement of the Mre11 complex and Sae2/CTIP to overcome the KU barrier, through the nick-dependent resection initiation (**Figure 1D**). By this model, the short-range resection through the Mre11 complex, together with Sae2/CTIP, is responsible for KU removal from the ends (Chanut et al., 2016; Symington, 2016), leading to a more complex and functional interplay between NHEJ and HDR. This mechanism is also functional at one-ended DSB created at broken DNA replication forks in human cells (Chanut et al., 2016). Moreover, depending upon the organisms, it is known that KU binding to DSB is finely regulated through neddylation (Brown et al., 2015), ubiquitylation (Postow et al., 2008; Feng and Chen, 2012), sumoylation (Hang et al., 2014), acetylation (Kim et al., 2014), and phosphorylation by

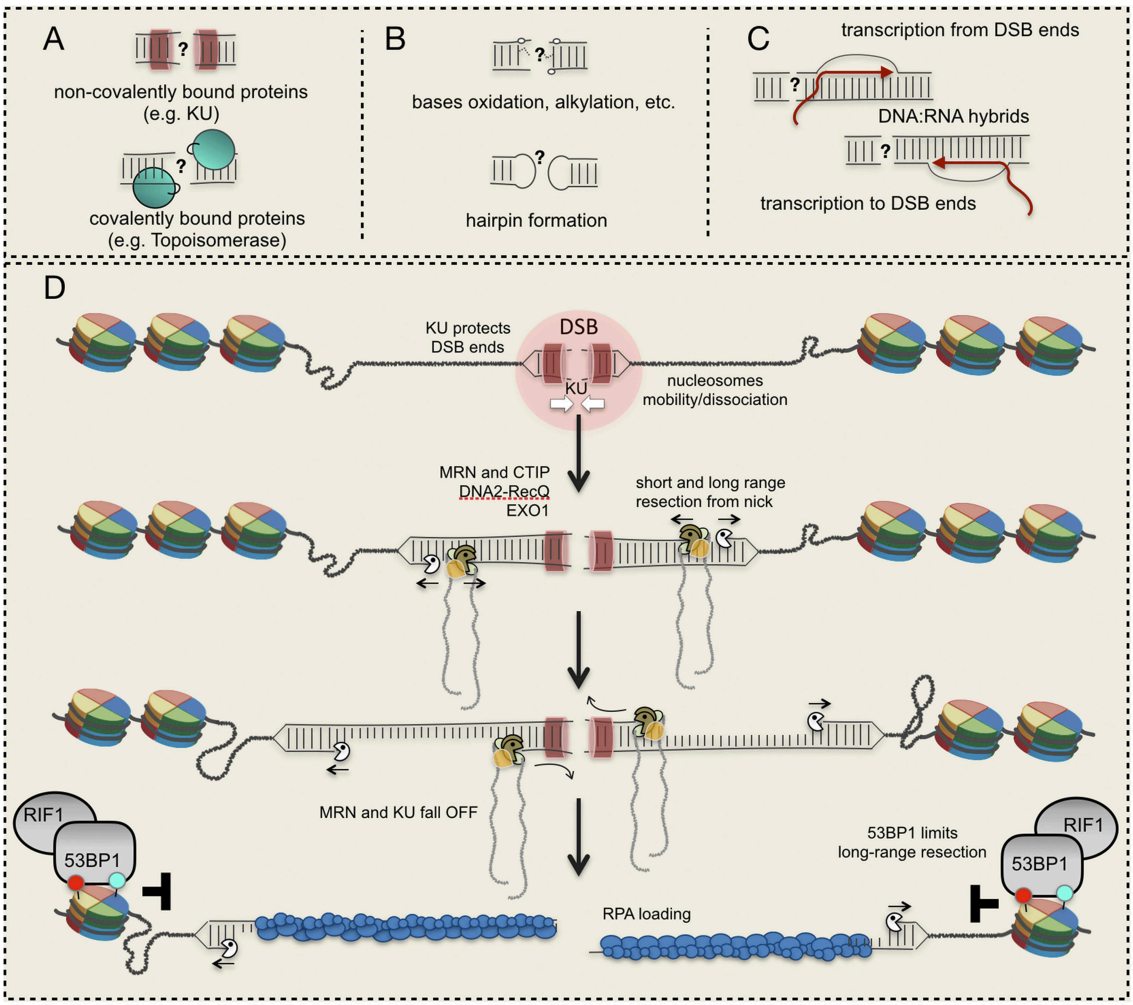


**FIGURE 1 |** DSB ends and their processing. **(A)** Proteins bound to DSB ends interfere with resection initiation; **(B)** structural/chemical modifications of DNA ends require specific processing; **(C)** DNA:RNA hybrid formation at a DSB; **(D)** a model to resect a DSB starting from a nick induced by Mre11 nearby the DSB site. Red and light green circles indicate histone modifications. See details in the text.

DNA-PKs (Chan et al., 1999). In particular, neddylation primes ubiquitylation of KU in human cells, facilitating the release of the complex and associated factors from repaired DNA (Brown et al., 2015). Moreover, it has been shown that the AAA-ATPase p97 also cooperates for the removal of ubiquitinated KU from DSBs, after completion of end joining in human cells (van den Boom et al., 2016). However, it is unknown whether these and/or other post-translational modifications of KU might also control DSB resection initiation through KU stability at the DNA ends.

Further studies will be required to define how these multiple post-translational modifications of KU are conserved throughout evolution, co-exist during the cell cycle, regulate resection, and modulate DSB repair pathways.

## 53BP1-DEPENDENT BARRIER

The mammalian p53-binding protein 1 (53BP1) and its yeast ortholog Rad9 are important regulators of the DSB repair pathway choice (Panier and Boulton, 2014). Remarkably, 53BP1 and Rad9 act on all types of DSBs, including reversed forks and uncapped telomeres. They are recruited to chromatin through direct recognition of a DSB-specific histone code and their function as an anti-resection factor is conserved throughout evolution. Both 53BP1 and Rad9 act as mediators, linking the upstream kinase ATR/Mec1 to the downstream effector kinases CHK2/Rad53 and CHK1. In yeast Rad9 oligomers are recruited to chromatin through three different pathways: (1) the constitutive interaction with the histone H3 methylated at the K79 residue by Dot1/DOT1L; (2) the binding to the histone H2A phosphorylated at the S129 residue by Mec1; (3) the interaction with Dpb11/TOPBP1. All of these three pathways cooperate for efficient checkpoint signaling and cell survival after genotoxic treatments throughout the cell cycle.

In higher eukaryotes 53BP1 protects DNA ends from inappropriate 5′ hyper-resection, facilitating NHEJ, and error-free gene conversion at the expense of mutagenic SSA and alt-EJ

(Ochs et al., 2016). Of note, extended ssDNA can lead to increased recombination events between repeats that are frequently present in eukaryotic genomes, leading to increased hypermutagenesis at breakpoint junctions (Sinha et al., 2017). Similarly to Rad9, 53BP1 recruitment requires the direct recognition of a DSB-specific histone code: it displays a strong binding affinity for the histone H4 constitutively mono- or di-methylated at the K20 (Botuyan et al., 2006) and for the histone H2A DSB-induced ubiquitination at K15 (Fradet-Turcotte et al., 2013). Moreover, 53BP1 oligomerization, mediated by DYNLL1, is essential for its recruitment to DSBs (Becker et al., 2018; West et al., 2019). Specifically, 53BP1 barrier is known to antagonize nucleases involved in the long-range resection, although its role to block resection initiation is supported by data in yeast, particularly in mutants affecting short-range resection. Interestingly, it has been shown that Rad9 accumulates at DSB ends in yeast cells lacking *SAE2*, blocking resection initiation by Dna2-Sgs1 (Bonetti et al., 2015; Ferrari et al., 2015; Yu et al., 2018). Moreover, resection initiation and capture of distant double-strand ends by CTIP is counteracted by 53BP1 in human cells (Guirouilh-Barbat et al., 2016).

These and other evidence indicate that 53BP1 exerts its action as a resection barrier in an extremely dynamic way, by mutual antagonism with BRCA1 and recruiting several downstream effectors (Panier and Boulton, 2014; Zimmermann and de Lange, 2014). Notably, following DSB-induced phosphorylation by ATM, 53BP1 recruits RIF1, the Shielding complex and the CST/ Pol α-Prim complex that fills in the resected DNA end, restoring dsDNA and allowing NHEJ [see a recent review here (Setiaputra and Durocher, 2019)]. It is an open debate whether 53BP1 and its partners exert their function to limit resection directly as a physical barrier to nucleases or indirectly reconstituting processed DNA ends (Setiaputra and Durocher, 2019). Most likely, both hypotheses are true (**Figure 2A**).

Of note, in the S/G2 phases of the cell cycle BRCA1 promotes phosphatase PP4C-dependent 53BP1 dephosphorylation and RIF1 release (Isono et al., 2017), promoting end resection and directing repair toward HDR. Inactivation not only of 53BP1, but also of its downstream effectors was shown to increase DNA damage tolerance of cancer-prone BRCA1$^{-/-}$ cells, most likely potentiating error prone HR pathways and increasing genome instability (Setiaputra and Durocher, 2019).

In addition to BRCA1, other factors and mechanisms modulate the mobility of the 53BP1-dependent barrier. For instance, the H2A ubiquitylation by BRCA1-BARD1 recruits the chromatin remodeler SMARCAD1, which then controls 53BP1 repositioning nearby a DSB and promotes long-range resection (Costelloe et al., 2012; Densham et al., 2016). In yeast, the SMARCAD1-ortholog Fun30 also acts on the Rad9-barrier, promoting long-range resection (Chen et al., 2012; Eapen et al., 2012; Bantele et al., 2017). Moreover, the Slx4-Rtt107 complex counteracts Rad9 binding to Dpb11/TOPBP1 and histones at the break, favoring DSB resection and HDR in yeast (Dibitetto et al., 2016).

It is important to keep in mind that the extensive resection is controlled by other factors and mechanisms than the 53BP1 barrier. For example, in human cells the $5'-3'$ translocase HELB limits EXO1 and DNA2/BLM nuclease activity (Tkac et al., 2016).

## IS THE DNA:RNA HYBRID A BARRIER TO DSB RESECTION?

It is an open debate how local transcription might interfere with DSB processing and repair. Indeed, DNA transcription might act as a physical barrier to DSB repair, especially during HDR, which requires long-range DSB resection. Accordingly, a reduction of DNA transcription nearby a DSB has been detected in both yeast (Lee et al., 2000; Manfrini et al., 2015) and mammals (Kruhlak et al., 2007; Chou et al., 2010; Shanbhag et al., 2010; Pankotai et al., 2012; Kakarougkas et al., 2014; Ui et al., 2015; Awwad et al., 2017; Iannelli et al., 2017; Vitor et al., 2019). While canonical ongoing transcription is switched off in response to DSB formation, mounting evidence suggests that DSB ends may act as transcriptional promoter-like elements, priming the formation of long non-coding RNA specie. In this context, transcription requires MRN-dependent recruitment of RNAPII at DNA lesions (Michelini et al., 2017) or, in the case of DSBs generated at promoter-proximal regions, cAbl-dependent tyrosine phosphorylation of RNAPII (Burger et al., 2019). The newly-synthetized non-coding RNAs at DSBs contribute to signal locally DNA damage and facilitate DNA repair (Francia et al., 2012; Wei et al., 2012) and, by changing chromatin structure, also possibly contribute to repress canonical transcription (Burger et al., 2019).

Since RNA synthesis nearby a DSB is both repressed and activated, it is unclear whether the transcription process *per se* and/or the formation of transcripts might antagonize locally the DSB resection and repair. Indeed, nascent RNA can be utilized as template to repair DSBs in transcribed genes via either error-free cNHEJ in human cells (Chakraborty et al., 2016), or HDR upon its assimilation into broken DNA by Rad52 protein, via an inverse strand exchange mechanism conserved from yeast to human (Keskin et al., 2014; Mazina et al., 2017). There are also evidence that DNA:RNA hybrids, generated at resected or minimally resected DNA ends, regulate the recruitment of RPA, BRCA1, BRCA2, RAD51, and RAD52, promoting HDR (Ohle et al., 2016; Cohen et al., 2018; D'Alessandro et al., 2018; Lu et al., 2018; Burger et al., 2019; Domingo-Prim et al., 2019). Another recent study in human cells showed that DSBs within transcriptionally active genes lead to the formation of R-loops, whose cleavage by the endonuclease XPG promotes an alternative way to initiate DSB resection and HDR (Yasuhara et al., 2018). Of interest, after their recruitment at XPG-processed DSBs, RAD52, and BRCA1 limit the 53BP1-RIF1 barrier. Remarkably, dysfunctions in the XPG-dependent mode to initiate resection lead to elevated NHEJ at transcribed loci and genome instability. Although DNA:RNA hybrids might not antagonize DSB resection initiation, they need to be dismantled by specific helicases or processed by RNases, allowing the HDR repair to proceed (Li et al., 2016; Ohle et al., 2016; Cohen et al., 2018). Interestingly, the RNase EXOSC10, a catalytic subunit of the RNA exosome complex, has been recently

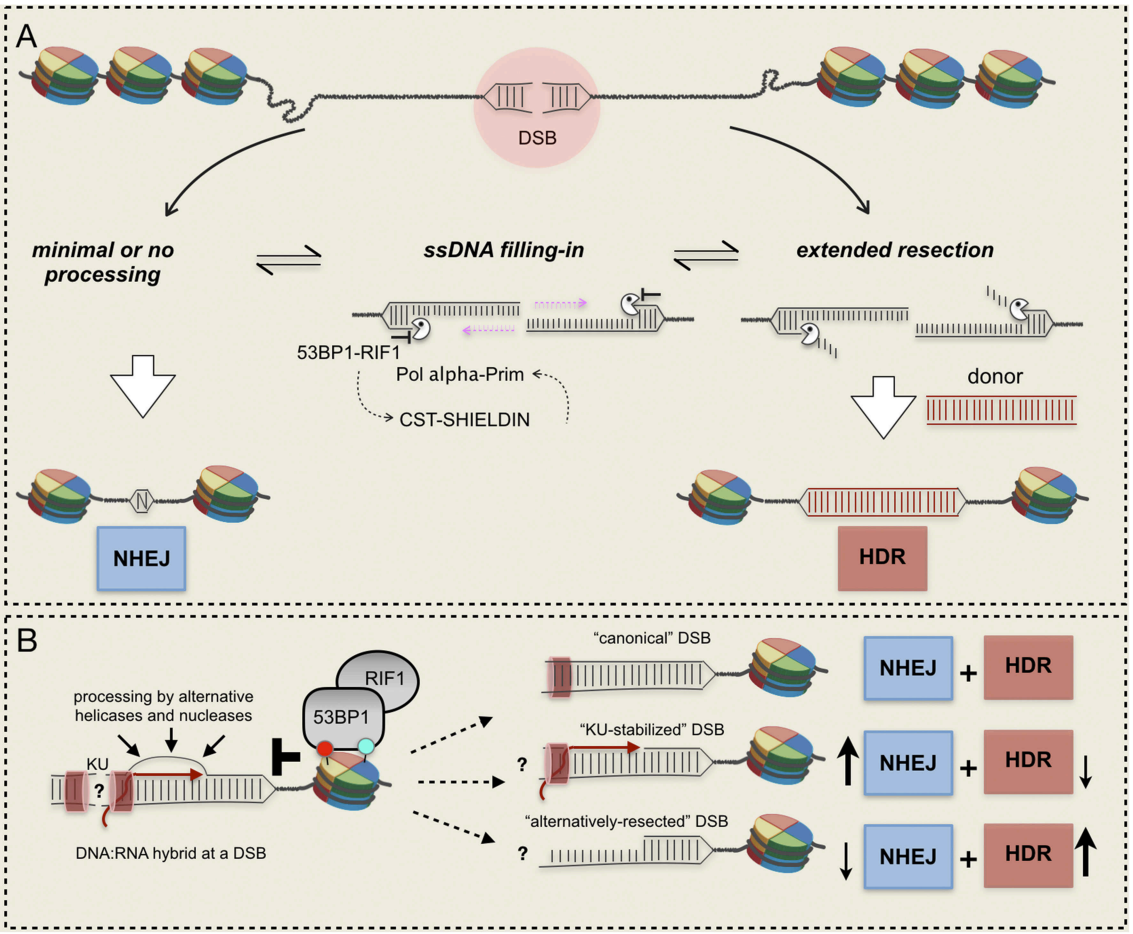


**FIGURE 2 |** Mechanisms and resection barriers influencing DSB repair pathways choice. **(A)** 53BP1-dependent axis antagonizes resection and promotes ssDNA re-filling, leading to NHEJ; **(B)** DNA:RNA hybrids in the context of other barriers can be processed by alternative mechanisms or can persist at the break, unbalancing the DSB repair pathway choice. RNA molecules can be present at the break in active transcribed gene or can be newly-synthetized after a DSB. Red and light green circles indicate histone modifications. See text for details.

involved to clear DNA:RNA hybrids at DSBs, preventing hyper-resection and coupling the nucleolytic processing with deposition of RPA and HDR repair in human cells (Domingo-Prim et al., 2019). Similarly, the accumulation of hybrids in cells depleted of Senataxin, a DNA/RNA helicase with R-loop-resolving activity, counteracts the binding of RAD51 and stimulates that of 53BP1 (Cohen et al., 2018), leading to illegitimate repair of broken ends and chromosome translocations (Brustel et al., 2018; Cohen et al., 2018). In this scenario, it is also important to mention that a recent work has reported that high levels of DNA:RNA hybrids at DSBs, due to the inactivation of human RNA binding protein HNRNPD, limit DSB resection, and HDR (Alfano et al., 2019).

Overall, current findings indicate that DNA:RNA hybrids at a DSB both promote and impair resection and HDR (**Figure 2B**), which might depend on local chromatin context. However, timely formation and dissociation of DNA:RNA hybrids impact on the DSB repair pathway choice and genome stability. Further investigations will be required to understand how the recruitment of the RNAPII complex and RNA synthesis impact locally on DSB resection and repair, favoring NHEJ or HDR.

## CONCLUSIVE REMARKS

Studies from yeast to human have shown that a wide variety of proteins and DNA/RNA transactions modulate resection, altering DSB repair pathway choice. Further investigations will be required to define their functional interplay. Moreover, an open debate regards the DSB resection initiation within active transcribed chromatin. It is unclear how the transcription machinery and the DNA:RNA hybrids influence the DSB repair pathway choice. Do the DNA:RNA hybrids at DSBs interfere with the resection antagonists, rather than with the resection machinery? Do the loading of the resection antagonists (such as KU and/or 53BP1) at the DSB is influenced by DNA:RNA hybrids?

Other relevant questions regard the role and mechanisms of resection barriers at stall or collapsed replication forks. Indeed, transcription, and the DNA damage response are highly influenced by the chromatin architecture changes occurring during DNA replication.

Remarkably, factors involved in DSB resection are deregulated in different cancers and genome instability syndromes, being also considered promising therapy targets. Indeed, the importance of all the factors involved in establishing and/or dampening resection barriers clearly emerged by treating tumor cells, which carry mutations in the BRCA1-axis, with the PARP1 inhibitor Olaparib. Notably, inactivation of the 53BP1-dependent resection barrier dramatically reduces the effectiveness of the treatment on BRCA1 defective cells, possibly leading to genome instability, poor prognosis, and cancer relapse (Lord and Ashworth, 2017; Setiaputra and Durocher, 2019).

## AUTHOR CONTRIBUTIONS

AP conceived the idea. All authors wrote the manuscript.

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REVIEW
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# Beyond the Trinity of ATM, ATR, and DNA-PK: Multiple Kinases Shape the DNA Damage Response in Concert With RNA Metabolism

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Our genome is constantly exposed to endogenous and exogenous sources of DNA damage resulting in various alterations of the genetic code. DNA double-strand breaks (DSBs) are considered one of the most cytotoxic lesions. Several types of repair pathways act to repair DNA damage and maintain genome stability. In the canonical DNA damage response (DDR) DSBs are recognized by the sensing kinases Ataxia-telangiectasia mutated (ATM), Ataxia-telangiectasia and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK), which initiate a cascade of kinase-dependent amplification steps known as DSB signaling. Recent evidence suggests that efficient recognition and repair of DSBs relies on the transcription and processing of non-coding (nc)RNA molecules by RNA polymerase II (RNAPII) and the RNA interference (RNAi) factors Drosha and Dicer. Multiple kinases influence the phosphorylation status of both the RNAPII carboxy-terminal domain (CTD) and Dicer in order to regulate RNA-dependent DSBs repair. The importance of kinase signaling and RNA processing in the DDR is highlighted by the regulation of p53-binding protein (53BP1), a key regulator of DSB repair pathway choice between homologous recombination (HR) and non-homologous end joining (NHEJ). Additionally, emerging evidence suggests that RNA metabolic enzymes also play a role in the repair of other types of DNA damage, including the DDR to ultraviolet radiation (UVR). RNAi factors are also substrates for mitogen-activated protein kinase (MAPK) signaling and mediate the turnover of ncRNA during nucleotide excision repair (NER) in response to UVR. Here, we review kinase-dependent phosphorylation events on RNAPII, Drosha and Dicer, and 53BP1 that modulate the key steps of the DDR to DSBs and UVR, suggesting an intimate link between the DDR and RNA metabolism.

**Keywords: DNA damage response, RNA metabolism, phosphorylation, RNA polymerase II, dicer, kinase, 53BP1**

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## INTRODUCTION

Chromosomes encode essential genetic information that needs to be faithfully inherited by daughter cells to maintain genome stability and prevent tumourigenesis. However, numerous exogenous and endogenous factors such as ionizing radiation (IR), ultraviolet radiation (UVR), pathogens, reactive oxygen species, or chemotherapeutic drugs can frequently induce DNA damage. If such lesions are not repaired correctly they have the potential to drive mutations of genes, leading to detrimental effects on genomic integrity. Various lesion-specific pathways repair

damaged DNA by a signaling network collectively termed the DNA damage response (DDR) to maintain genome stability and prevent alterations of genomic information (Jackson and Bartek, 2009).

DNA double-strand breaks (DSBs) are highly cytotoxic forms of DNA damage, which impair essential cellular processes including DNA replication and RNA synthesis, which if left unrepaired, can lead to cell death. However, various physiological processes such as meiosis (de Massy, 2013), V(D)J recombination, and immunoglobulin class-switch recombination (Soulas-Sprauel et al., 2007), also inherently involve formation, recognition and repair of DSBs. Mammalian cells employ two major types of DSB repair in the context of chromatin and the cell cycle: homologous recombination (HR) and non-homologous end joining (NHEJ) (Jackson and Bartek, 2009; Ciccia and Elledge, 2010; Polo and Jackson, 2011; Chapman et al., 2012; Her and Bunting, 2018; Hnízda and Blundell, 2019).

HR can be utilized in the S-/$G_2$-phase of the cell cycle, after a sister chromatid template with sufficient homology of >100 base pairs has been produced in replication. HR also requires extensive resection of DNA ends, which generates 3' single-stranded DNA overhangs and engages factors such as exonuclease Exo1, the single-strand DNA-binding protein RPA, and the Rad51 recombinase. Unlike HR, NHEJ requires no nucleotide homology and is active throughout the cell cycle. However, the precision of NHEJ repair is lower than in HR and can lead to mutagenesis. Important NHEJ factors include DNA end-binding heterodimer Ku70/80, the DNA ligase 4, DNA endonuclease Artemis/SNM1C and XRCC4. Interestingly, HR and NHEJ pathways compete for the DSB substrate in the S-/$G_2$-phase. Around 80% of DSBs are repaired by NHEJ, despite the second chromatid being available (Chapman et al., 2012; Pannunzio et al., 2018). Additional types of DSB repair include microhomology-mediated end joining (MMEJ), and single-strand annealing (SSA) (Verma and Greenberg, 2016; Chang et al., 2017).

Three key phosphatidylinositol 3-kinase-related kinase (PIKK) family members, Ataxia Telangiectasia mutated (ATM), Ataxia Telangiectasia and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK) orchestrate the DDR by phosphorylating hundreds of substrates (Kastan and Lim, 2000; Matsuoka et al., 2007; Blackford and Jackson, 2017). ATR and DNA-PK sense DSBs in cooperation with the Mre11-Rad50-Nbs1 (MRN) complex leading to the activation of downstream kinases such as checkpoint kinase 1/2 (Chk1/2) (Blackford and Jackson, 2017).

Downstream of the sensing kinases, 53BP1 is a key factor in DNA double strand break (DSB) repair, regulating repair pathway choice between HR and NHEJ. 53BP1 promotes NHEJ and represses HR by preventing DNA end resection at DSBs through antagonism with BRCA1 (Shibata, 2017). 53BP1 recruitment to DSBs begins following MRN recruitment to DSBs, whereupon ATM is recruited and phosphorylates histone H2AX on Ser139 (γH2AX) (Panier and Boulton, 2014; Mirza-Aghazadeh-Attari et al., 2019).

Additionally, several kinases beyond PIKKs act in the DDR. Intriguingly, about 40% of DSB-induced phosphorylation events occur independent of ATM and may regulate processes related to nucleic acid metabolism, including RNA processing and chromatin organization (Bennetzen et al., 2010; Bensimon et al., 2010). In fact, the majority of DSB-induced phospho-proteins indeed lack a PIKK consensus motif (Beli et al., 2012). These findings not only suggest an important role for downstream kinases as amplifiers of DSB signaling, but also establish regulatory links between the DDR and RNA metabolic enzymes. Moreover, the interplay between the DDR and RNA metabolic enzymes is not limited to the recognition and repair of DSBs. Some of the regulatory principles for DSB-induced regulation of the RNA metabolism are mirrored in response to UV damage. Here, we review recent advances in our understanding of the regulatory phosphorylation events that control the RNA-dependent DDR. We illustrate their relevance for genome stability by describing the various types of 53BP1 engagement in DSB repair. We will further compare similarities and differences of RNAi factors involved in DSB repair with their contribution to the recognition and repair of UV lesions.

## GLOBAL REPRESSION AND LOCAL INDUCTION OF TRANSCRIPTION IN RESPONSE TO DSBs

Unscheduled or excessive transcription is generally regarded as a threat to genome stability. Therefore, RNA synthesis is tightly controlled and coordinated with DNA replication timing to avoid collisions of the transcription and replication machineries, otherwise leading to replication fork collapse and accumulation of DNA-RNA hybrids (R-loops). Whilst R-loops can form as intermediates in certain cellular processes such as IgG class switch recombination and transcription (Skourti-Stathaki and Proudfoot, 2014; Skourti-Stathaki et al., 2014), formation of R-loops exposes single-stranded, non-template DNA strand, which can lead to an increase in mutagenesis, DNA breaks, and subsequent formation of DSBs (Huertas and Aguilera, 2003; Skourti-Stathaki and Proudfoot, 2014; Hamperl and Cimprich, 2016; Hamperl et al., 2017). RNAPII transcription of protein-coding genes is globally impaired in response to DSBs. Onset of ATM signaling triggers the damage-induced ubiquitination of RNAPII by Nedd4 ubiquitin ligase and its subsequent proteasomal degradation (Anindya et al., 2007; Shanbhag et al., 2010). Combination of site-specific induction of DSBs by the AsiSI endonuclease and sequencing of both steady-state and nascent RNA revealed that ATM-dependent downregulation of RNAPII at protein-coding genes occurs at the level of RNAPII initiation and elongation and also depends on the distance from the DSBs (Iannelli et al., 2017). Similarly, DNA-PK arrests elongating RNAPII at DSBs within protein-coding genes (Pankotai et al., 2012).

The recognition and repair of DSBs is accompanied by substantial changes in the chromatin landscape to allow DNA repair by HR and/or NHEJ pathways. Various chromatin-modifying enzymes and remodeling machineries such as PBAF facilitate silencing of actively transcribed loci by formation of non-permissive heterochromatin (Kakarougkas et al.,

2014). Furthermore, ATM-dependent phosphorylation of the transcription elongation factor ENL facilitates recruitment of the polycomb repressor complex to silence transcription (Ui et al., 2015). Finally, the Cohesin complex, well-known for sister chromatid cohesion and DSBs repair through HR, is required for repression of transcription in damaged interphase nuclei and the organization of DSBs into higher-order chromatin structures (Meisenberg et al., 2019). Thus, an active, damage-induced transcriptional response associated with DSBs might seem surprising.

Early *in vitro* studies suggest that RNAPII can transcribe linearised plasmids by recognizing DNA ends with 10–100 nts 3′overhangs (Kadesch and Chamberlin, 1982). The ability of RNAPII to transcribe RNA from linearised plasmids can also be observed in cells, where RNAPII components are part of the DNA end-binding proteome (Michalik et al., 2012; Berthelot et al., 2016). Surprisingly, physiological DSBs promote gene expression *in vivo*. A subset of early synaptic response genes is induced upon DNA damage by inhibition of topoisomerase II in neurons (Madabhushi et al., 2015). Additionally, stimulation of RNAPII activity by androgens or estrogens involves formation of DSBs, also mediated by topoisomerase II (Haffner et al., 2011). The stimulation of RNAPII elongation involves components of DSB signaling such as DNA-PK and topoisomerase II (Bunch et al., 2015). Intriguingly, DSBs are repaired faster if they occur at actively transcribed loci with transcriptionally active chromatin directing DSB repair toward the HR pathway (Chaurasia et al., 2013; Aymard et al., 2014). Data utilizing the sequence-specific AsiSI cleavage demonstrate that histone marks associated with active transcription, such as histone H4 acetylation, accumulate at a subset of AsiSI induced DSBs. Furthermore, RNAPII occupancy correlates with nucleosome-free regions rather than being disengaged from AsiSI-restricted chromatin (Iacovoni et al., 2010). More recently, systematic profiling of epigenetic marks in response to AsiSI cleavage has defined the histone H3 lysine120 (H3K120) ubiquitination mark as DSB-responsive molecular identifier of damaged DNA. H3K120 deubiquitination and acetylation depends on the SAGA multi-enzyme complex and may promote local permissive chromatin (Clouaire et al., 2018). These findings suggest that DSBs trigger chromatin breathing, which may result in a local, transiently open chromatin state to create a "window of opportunity" for transcription factors and nascent RNA synthesis (Price and D'Andrea, 2013). Indeed, the 55 kD large isoform of the major RNAPII transcription-regulating cyclin-dependent kinase 9 (Cdk9 55k), associates with the DNA end-binding Ku70 protein and depletion of Cdk9 55k induces accumulation of DSBs (Liu et al., 2010), further implying a close link between RNAPII transcription and genome stability. Given that the chromatin state impacts on genome stability—with poorly transcribed, heterochromatic regions driving mutation rates (Schuster-Böckler and Lehner, 2012)—it has been tempting to postulate that localized induction of RNA synthesis may have benefits for DSB repair. Indeed, increasing evidence suggests that an RNA-dependent response to DSB may involve the *de novo* production of strand-specific, long non-coding (lnc)RNA precursors. Such transcripts may originate from RNAPII activity

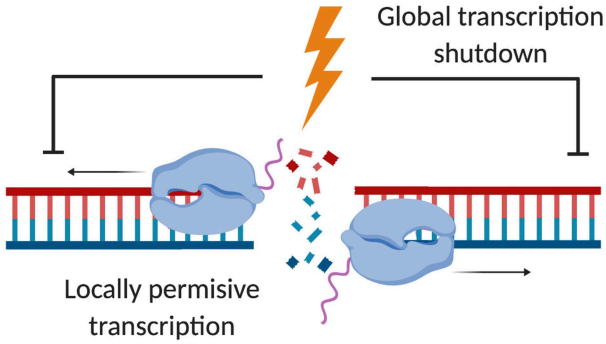


**FIGURE 1 |** Locally permissive transcription in response to DSBs despite global transcription shutdown. Upon DSB induction, RNAPII transcription of protein-coding genes is globally impaired via ATM signaling. Non-permissive heterochromatin forms to facilitate the silencing of actively transcribed loci. However, transcriptionally permissive open chromatin has been suggested to form locally in response to DSBs, allowing nascent RNA synthesis to occur at the site of the DSB (created by Biorender).

at both genic and intergenic DSBs, as well as at damaged ribosomal DNA (*rDNA*) genes (Michelini et al., 2017; Bonath et al., 2018; Burger et al., 2019; Vítor et al., 2019). Damage-induced lncRNA are prone to form hybrids, such as R-loops and/or double-stranded (ds)RNA, and undergo subsequent processing by RNAi factors Drosha and/or Dicer, but may also utilize alternative enzymes for trimming and clearance (Ohle et al., 2016; Burger et al., 2017; Burger and Gullerova, 2018; D'Alessandro et al., 2018; Lu et al., 2018; Yasuhara et al., 2018). Findings in *S. cerevisiae* suggest a model of RNA-templated DSB repair, which employs both exogenous RNA oligonucleotides and endogenous lncRNA as complimentary templates for DSB repair by HR (Storici et al., 2007; Keskin et al., 2014). Similarly, nascent RNA forms a complex with actively transcribing RNAPII and a subset of NHEJ factors to mediate error-free repair of DSBs (Chakraborty et al., 2016). DSB can further utilize pre-existing or damage-induced lncRNA to scaffold recruitment of DDR factors or modulate the activity of the p53 tumor suppressor (Huarte et al., 2010; Sharma and Misteli, 2013; Schmidts et al., 2016). In summary, the relevance of RNA in DSB repair is an emerging concept, where various modes of DDR signaling may coexist to modulate transcription at DSBs, depending on the chromatin landscape and the cell cycle stage (Chowdhury et al., 2013; Michelini et al., 2018; **Figure 1**).

## DAMAGE-INDUCED KINASES REGULATE RNA METABOLISM IN RESPONSE TO DSBs

The formation and processing of dsRNA is a consequence of damage-induced ncRNA synthesis and essential for efficient, RNA-dependent repair of DSBs. But how does DDR signaling fine-tune transcription and RNA processing at DNA lesions? Mounting evidence suggests that the DDR engages phospho-specific isoforms of RNAPII and Dicer for DSB repair by activation of damage-induced kinases, which often are activated

downstream of canonical PIKK signaling (Blackford and Jackson, 2017). We and others have recently shown that the RNA-dependent response to DSBs is initiated by active RNAPII transcription. The recruitment and activity of RNAPII at broken DNA ends not only involves PIKK signaling and the MRN complex, but also hyperphosphorylation of the RNAPII carboxy-terminal domain (CTD) (Napolitano et al., 2013; Michelini et al., 2017; Burger et al., 2019). The CTD is a low complexity domain of the largest RNAPII subunit, which comprises 52 repeats of the consensus heptad Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 and undergoes dynamic, regulatory post-translational modifications, commonly referred to as "CTD code" (Zaborowska et al., 2016; Harlen and Churchman, 2017). We have shown that the Abelson kinase c-Abl is required for the accumulation of a specific, catalytically active CTD Tyr1-phosphorylated RNAPII isoform at DSBs. Both chemical and genetic inhibition of c-Abl activity impairs the formation of CTD Tyr1-phosphorylated RNAPII foci at DSBs and attenuates its activity to produce damage responsive transcripts *de novo* (Burger et al., 2019). c-Abl is a promiscuous, nuclear tyrosine kinase with multi-faceted functions in the DDR (Colicelli, 2010; Meltser et al., 2011). c-Abl is activated by IR in a DNA-PK- and ATM-dependent manner (Baskaran et al., 1997; Kharbanda et al., 1997; Shafman et al., 1997), and phosphorylates various HR factors like Rad51 (Colicelli, 2010). Interestingly, c-Abl directly phosphorylates CTD Tyr1 residues *in vitro* (Baskaran et al., 1997; Mayer et al., 2012) and interacts with RNAPII CTD *in vivo* (Burger et al., 2019), suggesting that c-Abl, at least in part, directly regulates the accumulation and activity of CTD Tyr1-phosphorylated RNAPII at DSBs. This leads to the stepwise formation of damage-responsive transcripts and dsRNA, which are processed by the RNAi machinery and stimulate RNA-dependent recruitment of a subset of DDR factors. Intriguingly, the levels of RNAPII CTD Tyr1-phosphorylation are elevated in response to various stresses, including DNA damage, by the atypical tyrosine kinase Mpk1/Slt2 in budding yeast, whereas Mpk1/Slt2 deletion reduces, but not completely diminishes RNAPII CTD Tyr1-phosphorylation (Yurko et al., 2017). This suggests that RNAPII CTD Tyr1 phospho-marks associated with ncRNA synthesis are somewhat conserved under stress and that additional stress-responsive tyrosine kinases regulate RNAPII CTD Tyr1-phosphorylation levels. Indeed, activation of tyrosine kinase signaling is widespread during the DDR (Mahajan and Mahajan, 2015). It will be interesting to investigate additional roles for tyrosine kinases in the RNA-dependent DDR.

The requirement of Drosha and Dicer for an RNA-dependent DSB response involves formation of DSB-derived dsRNA and seems to occur independent from their canonical roles in RNAi pathway (d'Adda di Fagagna, 2014; Burger and Gullerova, 2015; Hawley et al., 2017; Pong and Gullerova, 2018). However, the molecular principles that control the formation of dsRNA or its recognition and turnover remain unclear. The endoribonuclease Dicer is a largely cytoplasmic enzyme and well-known for its canonical function in micro (mi)RNA biogenesis (Ha and Kim, 2014). During development or stimulation of growth factor signaling, however, a subset of cytoplasmic Dicer is phosphorylated by the mitogen-activated protein kinase (MAPK) signaling effector Erk1/2 in *C. elegans* and mammalian cells (Drake et al., 2014). In particular, MAPK signaling phosphorylates the two human Dicer residues Ser1728 and Ser1853–Ser1712 and Ser1836 in mouse—in the carboxy-terminal, catalytically active RNaseIII and dsRNA-binding domains of Dicer. Recent studies have confirmed the importance of these carboxy-terminal phospho-residues for Dicer localization and function in phospho-mimetic Dicer mouse models (Aryal et al., 2019). The constitutive carboxy-terminal phosphorylation of murine Dicer Ser1712 and Ser1836 residues is pathogenic and causes a hypermetabolic phenotype, which is accompanied by prominent nuclear Dicer localization, defective miRNA biogenesis, and sterility. Interestingly, we recently showed that a subset of the cytoplasmic Dicer pool translocates to the nucleus in response to DSBs to process damage-induced dsRNA on chromatin. Moreover, the localization and activity of nuclear phosphorylated Dicer requires an additional phospho-mark in the Dicer platform-PAZ connector helix residue Ser1016 (Burger et al., 2017). Dicer Ser1016 phosphorylation is induced by DSB signaling, depends on PIKK activity, and is necessary and sufficient for nuclear Dicer localization. The accumulation of nuclear, phosphorylated Dicer in response to DSB induction seems to be conserved in mammals and was confirmed in primary mouse embryonic fibroblasts that express an endogenously-tagged, full-length Dicer enzyme at physiological conditions (Burger and Gullerova, 2018).

Drosha and its cofactor DiGeorge syndrome critical region 8 (DGCR8) are also subject to stress-induced post-translational modifications. The MAPK effector p38 phosphorylates the amino-terminal Arg-Ser-rich region of Drosha upon oxidative stress (Yang et al., 2015). Drosha phosphorylation promotes its dissociation from DGCR8, causes nuclear export of phosphorylated Drosha and subsequent proteasomal degradation. Stress-induced Drosha phosphorylation alters miRNA biogenesis and causes hypersensitivity to hydrogen peroxide treatment. Alternative splicing variants of Drosha localize to the cytoplasm and do not seem to alter miRNA biogenesis severely (Dai et al., 2016; Link et al., 2016). In unperturbed cells, the glycogen synthase kinase 3 beta (GSK3β) phosphorylates the two Drosha residues Ser300 and Ser302 to facilitate nuclear accumulation of Drosha and promote primary miRNA processing. Upon infection with RNA viruses, which causes pleiotropic DNA damage (Weitzman and Weitzman, 2014), a substantial amount of nuclear Drosha functions as antiviral factor. Drosha translocates to the cytoplasm to interfere with the viral RNA metabolism by sponging viral RNA. The nuclear export of Drosha is dependent on the dephosphorylation of Ser300 and Ser302 residues and is accompanied by alterations in the host transcriptome. Remarkably, Drosha interferes with viral replication independent of its catalytic activity or DGCR8 (Shapiro et al., 2014; Aguado et al., 2017). DGCR8 itself is phosphorylated by c-Abl in response to treatment with the DNA-damaging agents doxorubicin or cisplatin (Tu et al., 2015). c-Abl targets the DGCR8 residue Tyr267, which stimulates processing of a specific miRNA precursor to promote the DDR at the post-transcriptional level. Thus, various forms of cellular stress, including DNA damage, control the localization and activity of RNAi factors. It will be important to further assess the

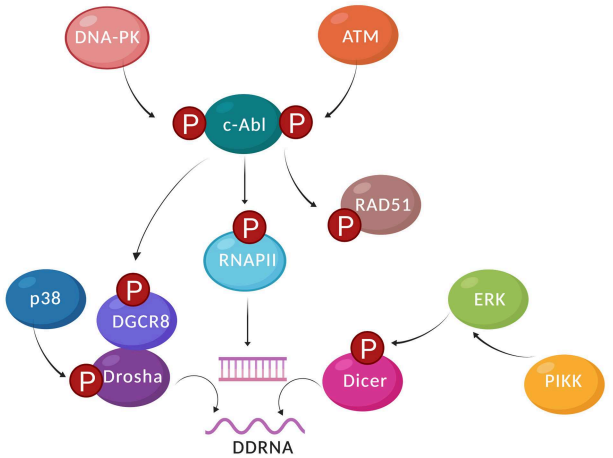


**FIGURE 2 |** Damage-induced kinases regulate the RNA metabolism in response to DSBs. In response to DSBs, DNA-PK, and ATM phosphorylate and activate c-Abl kinase, which can phosphorylate HR factors such as Rad51. c-Abl can also phosphorylate the CTD of RNAPII at Tyr1, which is required for the recruitment and activity of RNAPII at DSBs. Damage responsive transcripts and dsRNA can then be produced at the DSB, recruiting a subset of DDR factors. Alongside RNAPII, Drosha and Dicer are also required for the formation of DSB-derived dsRNA. Cytoplasmic Dicer is phosphorylated by Erk1/2 at Ser1728 and Ser1853. Dicer phosphorylation at Ser1016 is necessary and sufficient for nuclear Dicer localization. p38 phosphorylates Drosha, promoting its dissociation from DGCR8, nuclear export of phosphorylated Drosha, and subsequent proteasomal degradation. DGCR8 can be phosphorylated by c-Abl at Tyr267, which stimulates processing of a specific miRNA precursor to promote the DDR at the post-transcriptional level (created by Biorender).

impact of DDR signaling on the post-translational modifications of RNAi factors beyond phosphorylation (**Figure 2**).

## RNA-DEPENDENT AND INDEPENDENT MODES OF 53BP1 ENGAGEMENT AT DSBs

53BP1 is an important regulator of DSB signaling and pathway selection between HR and NHEJ at DSBs. Canonical recruitment of 53BP1 to DSBs involves recognition of broken DNA by the MRN complex, ATM activation, and γH2A.X accumulation. The γH2A.X mark mediates the recruitment of 53BP1, Mediator of DNA damage checkpoint 1 (MDC1), and the E3 ubiquitin ligases RNF8 and RNF168 to ubiquitinated H2A marks on damaged chromatin. The recruitment of 53BP1 to DSBs further involves recognition of the H4K20me2 mark and requires ATM-dependent phosphorylation of 53BP1 itself during the S-/$G_2$-phase of the cell cycle. In quiescent cells, 53BP1 is also phosphorylated by the vaccinia-related kinase 1 (VRK1) in response to IR in an ATM- and p53- independent manner. Loss of VRK1 impairs 53BP1 foci formation (Sanz-García et al., 2012). Upon recruitment to damaged chromatin, 53BP1 and its effector RIF1 promote NHEJ and repress HR by preventing BRCA1 access to DSBs. To license the HR pathway, BRCA1 together with the DNA endonuclease CtIP trigger dephosphorylation of 53BP1, which repositions 53BP1 to the periphery and allows recruitment of HR factors such as BRCA1, Exo1 and RPA to the center of the DDR focus (Daley and Sung, 2014; Lee et al., 2014; Panier and Boulton, 2014; Zimmermann and de Lange, 2014). Interestingly, the occupancy of 53BP1 on damaged chromatin may also be influenced by the Dicer-dependent regulation of the histone deacetylase sirtuin 7 (SIRT7). SIRT7 controls chromatin density and thus accessibility of 53BP1 to DSBs (Vazquez et al., 2016). In unperturbed cells, Dicer tethers a fraction of SIRT7 to the cytoplasm, thereby controlling nuclear SIRT7 levels. Upon DNA damage, however, Dicer expression may be upregulated, which further retains SIRT7 in the cytoplasm and restricts its access to chromatin. Tethering of SIRT7 to the cytoplasm decreases the levels of acetylated H3 lys18, which limits chromatin decondenzation and may eventually impair the efficient recruitment of NHEJ factors like 53BP1 (Zhang et al., 2016). However, whether or not SIRT7 deacetylation and subsequent increased H3K18 acetylation enhances or impairs NHEJ is not clear. It also remains to be clarified to what extent perturbations in miRNA biogenesis influence Dicer's contribution to the chromatin status.

More recent evidence suggests that additional, non-canonical modes of 53BP1 recruitment to DSBs exist, involving regulatory functions of additional kinases. The dual-specificity tyrosine-regulated kinase 1a (DYRK1A) is a pleiotropic kinase, present in both the nucleus and cytoplasm, and its deregulation has been linked to neurological diseases (Altafaj et al., 2001; Aranda et al., 2011). DYRK1A modulates the recruitment of 53BP1 to DSBs through interaction with RNF169, a paralogue of RNF168. RNF169 competes with RNF168 for binding of 53BP1 and has a function in repair pathway choice by limiting 53BP1 at DSBs (Poulsen et al., 2012; An et al., 2018). Thus, DYRK1A enhances NHEJ by regulating the recruitment of RNF169 and 53BP1 to DSB sites (**Figure 3**). The dual specificity tyrosine phosphorylation-regulated kinase 2 (DYRK2) is also involved in DSB repair. In non-damage conditions, DYRK2 is mostly cytoplasmic and nuclear DYRK2 is constitutively ubiquitinated and degraded. In response to DNA damage, however, DYRK2 is phosphorylated by ATM, which prevents degradation and causes nuclear accumulation. Stabilized DYRK2 phosphorylates p53 at residue Ser46, suggesting that DYRK2 plays a role in p53 dependent apoptosis. The knockdown of DRYK2 impairs the formation of 53BP1 foci and HR efficacy (Yamamoto et al., 2017).

Interestingly, 53BP1 is an RNA-binding protein and may recognize DSBs via interaction of its tudor domain with damage-induced lncRNA (dilncRNA) in a Dicer-dependent manner. Indeed, transfection of antisense oligonucleotides specific for dilncRNA, mutation of the 53BP1 tudor domain, or depletion of Dicer, attenuates the formation of 53BP1 foci. Strikingly, 53BP1 foci formation in response to IR is sensitive to treatment with structure-specific RNases and the addition of RNA purified from damaged, but not non-damaged cells, rescues 53BP1 foci formation following RNase treatment (Pryde et al., 2005; Francia et al., 2012; Burger et al., 2017, 2019; Michelini et al., 2017; Botuyan et al., 2018). Thus, the efficient recruitment of 53BP1 to DSBs may involve the specific interaction with damage-induced RNA and its dependence on RNAi factors like Dicer suggests the involvement of dsRNA. Indeed, site-specific, DNA damage

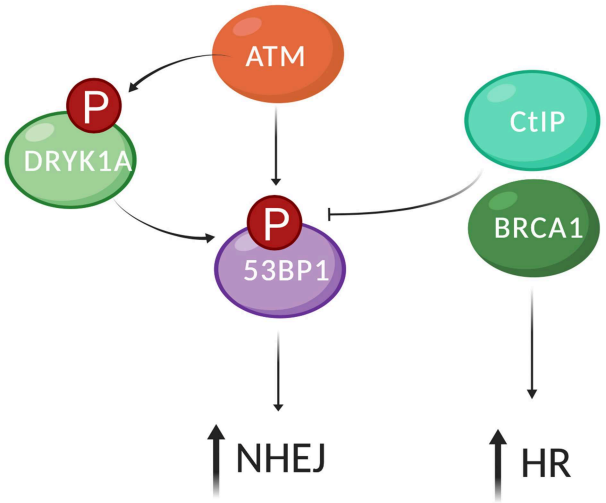


**FIGURE 3 |** 53BP1 recruitment DSBs and its regulation by phosphorylation. Recruitment of 53BP1 to DSBs involves recognition of the DSB by the MRN complex, ATM activation, and phosphorylation of histone H2A.X on residue Ser139 (γH2A.X). 53BP1 is phosphorylated on its 28 S/TQ sites by ATM. The ubiquitin ligases RNF8 and RNF168 are recruited downstream of ATM phosphorylation of γH2AX and are required for 53BP1 recruitment to chromatin. DYRK1A modulates the recruitment of 53BP1 to DSBs through interaction with RNF169. RNF169 competes with RNF168 for binding of 53BP1 and has a function in repair pathway choice by limiting 53BP1 at DSBs. Thus, DYRK1A enhances NHEJ by regulating the recruitment of RNF169 and 53BP1 to DSB sites. BRCA1 together with the DNA endonuclease CtIP trigger dephosphorylation of 53BP1, which repositions 53BP1 to the periphery and allows recruitment of HR factors such as BRCA1, Exo,1 and RPA to the center of the DDR focus, promoting HR (created by Biorender).

response RNA (DDRNA)/damage-induced (di)RNA accumulate in an RNAi factor-dependent manner in various organisms (Lee et al., 2009; Francia et al., 2012; Michalik et al., 2012; Wei et al., 2012). DDRNA/diRNA facilitate recruitment of a subset of secondary DDR factors, including 53BP1 and MDC1, to establish DSB foci and repair of DSBs, but are dispensable for the recruitment of primary DDR factors like the MRN complex (Francia et al., 2016).

Interestingly, additional DDR factors such as Rad51 and BRCA1 may also engage small ncRNA in an RNAi-like mechanism, where diRNA is complexed with the Argonaute family member Ago2 to guide the recruitment to DSBs. Using a DR-GFP/U2OS HR reporter system, the authors determine that Ago2 impairs HR comparably to RAD51 knockdown in mammals (Gao et al., 2014). Efficient recruitment of the acetyltransferase Tip60/KAT5 to DSBs also depends on small ncRNA (Wang and Goldstein, 2016). However, details on the structure of such transcripts are sparse and their physiological relevance remains controversial.

Furthermore, the tudor-interacting repair regulator (TIRR) was identified as binding partner of 53BP1 and regulator of DSB repair (Drané et al., 2017; Zhang et al., 2017). TIRR binds the tandem tudor domains of 53BP1, occupying the same binding site as H4K20me2. As binding of 53BP1 to TIRR occurs with ∼25-fold higher affinity than binding to H4K20me2, TIRR outcompetes H4K20me2 for 53BP1 binding, thereby preventing 53BP1 recruitment to chromatin (Dai et al., 2018; Wang et al., 2018). 53BP1 is phosphorylated and released from the 53BP1-TIRR heterodimer in a RIF1- and ATM-dependent manner upon IR. Intriguingly, an additional, RNA-dependent mechanism of 53BP1-TIRR dissociation has been proposed. TIRR is an RNA-binding protein that interacts with a variety of transcripts (Avolio et al., 2018), and RNA molecules can displace the 53BP1-TIRR interaction *in vitro* (Botuyan et al., 2018). However, the molecular mechanism of RNA-mediated dissociation of the 53BP1-TIRR complex remains enigmatic.

The above examples illustrate the relevance of RNA for DSB recognition and suggest a complex regulatory network to engage DDR factors with the RNA. Future studies likely will extend a growing list of examples for the RNA-dependent DDR.

## DAMAGE-INDUCED RNA METABOLIC ENZYMES IN RESPONSE TO UV IRRADIATION

Additionally to DSB repair, emerging evidence suggests that RNA metabolism plays a regulatory role in the repair of UV damage. Here, we review RNA-dependent DDR with focus on canonical and non-canonical responses to UV-induced DNA damage, highlighting novel, unexpected roles of RNAPII and phospho-isoforms of the RNAi machinery during nucleotide excision repair (NER).

Exposure to UVR triggers formation of DNA photo-adducts such as cyclobutane-pyrimidine dimers and is a natural driver of mutations. Two general pathways exist to recognize and repair UV-induced DNA damage in mammals: global genome (GG)- and transcription-coupled (TC)-NER (Marteijn et al., 2014). NER involves the formation of single-stranded DNA, potentially stalling of replication forks, and activation of ATM/ATR and downstream effector kinases, including Chk1 (Ciccia and Elledge, 2010). Critical factors for NER include Cockayne syndrome A/B (CSA/CSB), the UV-stimulated scaffold protein A (UVSSA), the ubiquitin-specific protease 7 (USP7), and Xeroderma pigmentosum factors A-F (XPA-XPF). During GG-NER, UV-induced signaling causes removal of RNAP II CTD phospho-marks and dynamic ubiquitination steps, which globally impairs both initiation and elongation of RNAPII transcription (Rockx et al., 2000; Sugasawa et al., 2005; Andrade-Lima et al., 2015). In TC-NER, the actively transcribing RNAPII machinery senses UV lesions and either stalls or performs trans-lesion RNA synthesis upon encountering DNA damage (Gregersen and Svejstrup, 2018). Thus, DDR signaling globally impairs RNAPII activity in response to UVR and triggers widespread ubiquitin-dependent proteasomal degradation, if TC-NER fails (Elia et al., 2015).

Interestingly, the comprehensive analysis of nascent RNA levels following UVR identified a subset of damage-induced transcripts such as ASCC3, which precede RNAPII inhibition and promote transcriptional recovery at DNA lesions (Williamson et al., 2017). In analogy to DSB signaling, the UV-induced DDR seems to be locally permissive to synthesize a subset of ncRNA transcripts with their production and/or processing

being relevant for recognition and repair of UV lesions (Izhar et al., 2015) and transcription-initiation associated NER (Frit et al., 2002). Thus, a growing body of evidence suggests that the UV-induced DNA damage response modulates various different RNA metabolic processes, including transcription, splicing and translation (Munoz et al., 2009; Paronetto et al., 2011; Tresini et al., 2015; Wickramasinghe and Venkitaraman, 2016).

The MAPK effector p38 is an integral transducer of cellular stress and activated by numerous stress-inducing agents, including UVR (Brancho et al., 2003). Recent studies investigating the UV-induced phospho-proteome define p38 signaling as a critical regulator of the RNA metabolism after UV damage, in addition to ATM/ATR signaling (Borisova et al., 2018). In particular, p38 signaling preferentially targets RNA-binding proteins such as splicing factors, proteins involved in the turnover of AU-rich elements-containing mRNA, mRNA polyadenylation, and translation (Dean et al., 2004). The spectrum of targets is somewhat different from the UV-induced ATM/ATR phospho-proteome, which primarily identifies DNA-binding DDR factors. For example, p38-dependent phosphorylation of the negative elongation factor (NELF) complex promotes RNAPII elongation in a subset of genes upon UV damage. Phosphorylation of the NELF complex subunit NELF-E at residue Ser115 causes binding of the 14-3-3 proteins to NELF and its rapid release from chromatin (Borisova et al., 2018). The stimulation of RNAPII elongation occurs independently of the positive transcription elongation factor b (pTEFb), but is dependent on both CSB and XPC (Donnio et al., 2019). To reinitiate stalled RNAPII after completion of TC-NER the CTD phospho-mark Ser2 is reintroduced in a CSB-dependent manner. Reinitiation further involves the general RNAPII transcription factor H (TFIIH) and Cdk9 (Lainé and Egly, 2006; Anindya et al., 2010; Donnio et al., 2019). The serine-threonine kinase STK19 also promotes reinitiation of stalled RNAPII. Interestingly, STK19 mutations are critical drivers of melanoma (Yin et al., 2019). During TC-NER, STK19 interacts with CSB and accumulates at UV lesions and the depletion of STK19 causes hypersensitivity to UV damage. However, the precise molecular role of STK19 in TC-NER remains elusive. In addition, UV-induced DDR signaling can also target the RNAPII holoenzyme itself (Boeing et al., 2016). It will be important to elucidate further regulatory principles that control RNAPII activity in response to UV irradiation.

UV-induced DDR signaling is not limited to the control of RNAPII activity. In reminiscence to DSB signaling, the response to UV damage involves the RNAi factors Ago2, Dicer, Drosha and DGCR8 to control the DDR in both a miRNA-dependent and -independent manner. At the post-transcriptional level, UV damage causes an immediate-early relocalization phenotype of Ago2 into stress granules, which is accompanied by changes in the miRNA signature and altered expression levels of critical cell cycle regulators such as the Cdc25a phosphatase (Garinis et al., 2005; Pothof et al., 2009). The rapid, ATR-dependent degradation of Cdc25a upon UV damage (Mailand et al., 2000) is accompanied by induction of miRNA miR-16 to further destabilize Cdc25a transcripts by post-transcriptional gene silencing. The depletion of Ago2 or Dicer, in turn, impairs DDR signaling and cellular survival in response to UVR. Interestingly, Ago2 relocalization requires Cdk activity, but appears to be independent of ATM/ATR. However, the precise mechanism of UV-induced Ago2 relocalization remains elusive.

More recent evidence suggests an involvement of Dicer, Drosha, and DGCR8 in the UV-induced DDR besides post-transcriptional gene silencing. A subset of the cellular Dicer molecules accumulate in the nucleus to promote chromatin decondenzation in UV-irradiated cells (Chitale and Richly, 2017). Dicer chromatin occupancy depends on interaction with the transcriptional repressor ZRF1. The Dicer-dependent accumulation of the methyltransferase MMSET and dimethylation of histone H4 lysine 20 residues at UV lesions further stimulates NER and involves the scaffolding factor XPA. The individual depletion of Drosha or DGCR8 also results in hypersensitivity to UV irradiation (Calses et al., 2017). The importance of DGCR8 for the NER pathway is underscored by epistatic effects, which are caused by combining DGCR8 depletion with defects in XPA, CSA or CSB functions. The DDR signaling involves DGCR8 in NER by specific placement of the UV-induced DGCR8 phospho-residue Ser153. DGCR8 phosphorylation involves the MAPK effector JNK1a and confers resistance to UVR. With >20 mapped phospho-sites, DGCR8 phosphorylation is common and canonically involved in DGCR8 stabilization and enhancement of miRNA biogenesis (Herbert et al., 2013). Surprisingly, the function of Ser153 phosphorylated DGCR8 in NER is independent of its RNA-binding capability or interaction with its binding partner Drosha, and therefore likely miRNA-independent. Instead, phosphorylated DGCR8 physically interacts with RNAPII and CSB and does not alter miRNA biogenesis, indicating that phosphorylated DGCR8 promotes NER on chromatin in an RNA-independent manner. Concomitant with DGCR8 phosphorylation upon stress, the damage-induced phosphorylation of DGCR8, and its function independent of Drosha, represent some analogy to the involvement of phosphorylated Dicer in DSB repair (Yang et al., 2015). Such findings further underscore the crosstalk between the DDR and RNA metabolic factors.

Taken together, we described various regulatory principles that control the localization and activity of RNAPII and various RNAi factors in response to various DSB-induced phosphorylation events, underscoring the contribution of damage-induced transcripts for the recognition and repair of DSBs and the relevance of RNA-dependent DSB recognition.

## CONCLUDING REMARKS

Collectively, we have discussed the various interconnections of RNA metabolic enzymes with DNA damage-induced signaling, pointing toward an intimate crosstalk of a subset of DDR factors, including key regulatory proteins such as 53BP1, with RNA metabolism in response to both DSBs and UV lesions. Growing evidence indicates that some of the observed RNA-dependent DDR phenotypes may be generally employed by the

DDR, while others seem to be locus-specific. With this in mind, a deeper understanding of Dicer functions in the DDR and its relevance for genome maintenance is of vital interest for cancer research.

Nevertheless, many questions remain in the emerging field of RNA-dependent DDR. How do RNAi factors discriminate transcripts at DNA lesions from canonical pri-/pre-miRNA substrates? Number of studies demonstrate the relevance of post-translational modifications for RNAi factors and their involvement in the DDR. It is tempting to speculate that complementary mechanisms exits that selectively direct phospho-isoforms to damage-induced transcripts rather than miRNA biogenesis. Interestingly, the epitranscriptomic mark $N^6$-methlyadenosine (m6A) transiently and very rapidly accumulates at UV lesions to promote efficient DNA repair (Xiang et al., 2017). m6A is not required for the recruitment of canonical DDR factors such as XPA or TFIIH, but involves DNA polymerase kappa, indicating that m6A promotes translesion synthesis. Placement of m6A, potentially in combination with other marks, may also alter the conformation of transcripts and thereby create a DNA damage-specific eptitranscriptomic signature complementary to the damage-induced changes in posttranslational modification of both canonical DDR factors and non-canonical RNA metabolic enzymes involved in genome maintenance.

The engagement of RNA metabolic enzymes at DNA lesions creates a steric conflict between canonical DNA-binding DDR factors, which tend to protect DNA lesions from unscheduled activity of large multi-enzymatic complexes like the replisome or the RNAPII machinery and RNA metabolic factors which may even produce transcripts *de novo*. The understanding of spatio-temporal integration and regulatory principles of such seemingly counterintuitive processes will be a major advancement in the field.

## AUTHOR CONTRIBUTIONS

KB, RK, and MG discussed the structure and content of the review. KB and RK wrote the first draft. MG and RK edited the draft.

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REVIEW
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# Moving Mountains—The BRCA1 Promotion of DNA Resection

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DNA double-strand breaks (DSBs) occur in our cells in the context of chromatin. This type of lesion is toxic, entirely preventing genome continuity and causing cell death or terminal arrest. Several repair mechanisms can act on DNA surrounding a DSB, only some of which carry a low risk of mutation, so that which repair process is utilized is critical to the stability of genetic material of cells. A key component of repair outcome is the degree of DNA resection directed to either side of the break site. This in turn determines the subsequent forms of repair in which DNA homology plays a part. Here we will focus on chromatin and chromatin-bound complexes which constitute the "mountains" that block resection, with a particular focus on how the breast and ovarian cancer predisposition protein-1 (BRCA1) contributes to repair outcomes through overcoming these blocks.

**Keywords: BRCA1, SMARCAD1, USP48, 53BP1, resection, homologous recombination**

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## INTRODUCTION

DNA DSBs occur as a result of exogenous agents such as irradiation and chemotherapy, but also as a result of cellular processes, such as replication and transcription. A single-ended DSB may be formed when replication forks encounter single-stranded DNA (ssDNA) breaks. In contrast, some cell types generate DSBs as part of specialist processes such as immunoglobulin gene rearrangements and recombination in meiosis. Experimentally, DSBs are also induced by rare-cutting restriction enzymes, such as the *I-Sce1* endonuclease, and sequence-guided nucleases, such as clusters of regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated protein 9 (CRISPR-Cas9).

DSBs in mammalian cells can be repaired by several means (**Figure 1**). Non-homologous end joining (NHEJ) involves the re-ligation of both ends of the break, and may or may not involve nucleic processing of the ends and polymerases to fill gaps prior to ligation to restore the backbone. NHEJ is rapid, predominates throughout the cell cycle, and undertakes the majority (∼80%) of DSBs repair (Mao et al., 2008; Beucher et al., 2009). When no end-processing occurs and the two correct ends are ligated this is error-free, but if the ends are processed or incorrect ends are ligated, material is lost or translocations occur. Alternative non-homologous end joining (aNHEJ), or microhomology-mediated end joining (MMEJ) is used when one of the core-components of NHEJ are absent (such as Ku70/80 or Ligase IV), aNHEJ uses short patches of microhomology (<25 nucleotides) so that minimal resection of either end is required (Seol et al., 2018). Homology-directed repair (HDR) requires a template to copy from and all HDR pathways share the same initial step of resection around the DNA DSB to create long 3′ ssDNA overhangs coated by the ssDNA binding protein replication protein A (RPA). When resection exposes direct repeats either side of the break, i.e., homologous sequences, repair can occur following direct annealing of the repeat sequences, in a process catalyzed by the DNA Repair Protein RAD52 Homolog (RAD52). This

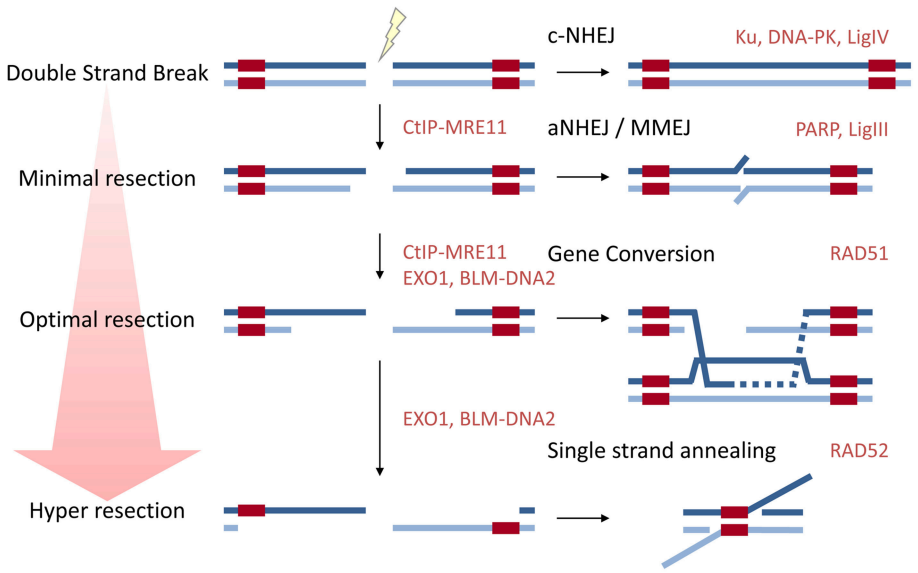


**FIGURE 1 |** A diagram to show how resection influences repair pathway choice. Approximately 80% of DSBs are repaired by classical NHEJ which does not require resection. aNHEJ or MMEJ requires minimal resection to expose regions of microhomology. Long range resection is required for the major HDR pathways gene conversion (GC) and single-strand annealing (SSA). Key proteins in each pathway are given in red.

process of single-strand annealing (SSA) is error prone due to deletion of the intervening sequence between the direct repeats (reviewed in Bhargava et al., 2016). In the form of HDR often referred to as "homologous recombination," herein called gene conversion (GC), the ssDNA participates in a homology search followed by DNA strand invasion. The critical step is the formation of the ssDNA-RAD51 [DNA Repair Protein RAD51 Homolog (RecA Homolog, *E. Coli*)] nucleofilament in which RPA loaded onto ssDNA exposed by resection either side of the DSB, is exchanged for RAD51. This ssDNA-RAD51 nucleofilament invades the homologous sister chromatid, displacing one strand of DNA and forming a synapse with the homologous sequence on the other strand in a DNA-loop (D-loop). The invading strand then acts as a primer for polymerases to extend along the template. Depending on how the structure is resolved determines whether the chromatids gain material from the partner or not: if the D-loop is dissolved they do not; but if the two crossed-over structures (Holliday junctions) are cleaved, cross-over products are formed in half of cases. GC is often referred to as "error free" as it uses the sister chromatid as the template and no genetic material is lost. Heterologous recombination, i.e., the use of near-homologous sequences is suppressed by Regulator of telomere elongation helicase 1 (RTEL1) and Bloom Syndrome RecQ-like helicase (BLM) (Leon-Ortiz et al., 2018).

The process of digesting one strand on the duplex on either side of the DSB, resection, is initiated by the endonuclease activity of Meiotic recombination 11 homolog A (MRE11)—a DSB repair nuclease, found in a complex with Nijmegen breakage syndrome 1/Nibrin (NBS1) and the DSB repair protein RAD50 Homolog (RAD50) [termed MRN (MRE11-RAD50-NBS1) complex] together with the carboxy-terminal binding protein interacting protein (CtIP) (Sartori et al., 2007; Stracker and Petrini, 2011; Anand et al., 2016; Daley et al., 2017). CtIP forms a tetramer and appears to have a preference for binding blocked DNA ends (Wilkinson et al., 2019), which may provide some explanation for the preferential use of HDR in tackling lesions resulting from Poly-ADP-Ribose-Polymerase (PARP) inhibition or topoisomerase-poisons, which produce DNA-protein adducts. The endonuclease cut of MRE11 occurs around 20–40 nucleotides from the blocked end (Anand et al., 2016, 2019). Intriguingly MRN-CtIP is found constitutively in an inactive form in complex with the resection repressor Cell cycle and apoptosis regulator 2 (CCAR2). The CtIP-CCAR2 interaction is disrupted locally on damaged chromatin and also by phosphorylation of CtIP (Lopez-Saavedra et al., 2016). Indeed the CtIP-MRN complex is subject to several post-translational modifications. Phosphorylation by cyclin dependent kinases in S/G2 promotes CtIP-MRN association and MRE11 activity (Huertas and Jackson, 2009; Buis et al., 2012; Orthwein et al., 2015; Anand et al., 2016), thereby limiting resection to when the sister chromatid template is present. After MRE11 endonuclease activity forms a DNA nick, the 3′-5′ exonuclease activity of MRE11 degrades back to the break site (Shibata et al., 2014; Cejka, 2015). This short-range resection is also promoted by another recently described nuclease, Exonuclease 3′-5′ domain containing 2 (EXD2) (Broderick et al., 2016; Nieminuszczy et al., 2016). CtIP is critical for resection and its depletion is sufficient to switch some HDR-committed breaks to repair by NHEJ (Shibata et al., 2011, 2014) indicating that, at least in some cases, lesions that might have undergone HDR can be re-directed to NHEJ. Long-range resection is performed 5′-3′ by two redundant pathways: the dominant pathway through Exonuclease-1 (Exo1); and a backup pathway of BLM and DNA

replication helicase/nuclease 2 (DNA2) (Gravel et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008; Nimonkar et al., 2011; Tomimatsu et al., 2012; Myler et al., 2016). Exo1 generates extensive 3′ ssDNA (Cejka, 2015; Myler et al., 2016), while the RecQ-helicase BLM (and to a lesser degree the Werner Syndrome RecQ Like Helicase) unwinds dsDNA, and the nuclease DNA2 is a ssDNA flap endonuclease without specificity to one end or the other (Kim et al., 2006). The ssDNA produced is bound by RPA which protects the 3′ end from DNA2 attack, since DNA2 can only displace RPA from the 5′-end to enable degradation (Niu et al., 2010; Nimonkar et al., 2011; Zhou et al., 2015).

In cells DNA breaks rarely occur on naked DNA, but on nucleic acid wrapped in chromatin and chromatin and chromatin signaling has emerged as a key aspect controlling the vital step of DNA resection that in turn determines the downstream repair strategy employed.

## CHROMATIN BARRIERS TO RESECTION

DNA is wound round an octamer of histones that make up the nucleosome. The basic nucleosome consists of two copies of each of the core histones Histone 2A (H2A), Histone 2B (H2B), Histone 3 (H3), and Histone 4 (H4). The linker histone, Histone 1 (H1), binds at the DNA entry and exit point, stabilizes nucleosomes, and can thereby promote higher-order chromatin architecture. There are multiple variants for each histone providing a complex array of variations in nucleosome structure that occurs at the level of histone composition. In addition, all histones have long flexible N-terminal tails that extend away from the nucleosome body and which are highly modified by post-translational modifications (reviewed in Armeev et al., 2018). Chromatin context is crucial for DNA repair outcome and the first challenge the cellular machinery meets is to deal with the underlying chromatin structure. Nucleosomes can block the progression of Exo1 *in vitro* (Adkins et al., 2013) and in yeast the heterogeneity of resection lengths has been at least partly attributed to the disruption of Exo1-resection by the position of nucleosomes (Mimitou et al., 2017).

It is perhaps surprising then that in mammalian cells immediately following DNA damage chromatin undergoes a rapid, but transient compaction in the environment local to the DSB. This has been visualized recently using Fluorescence lifetime imaging microscopy (FLIM)- Förster resonance energy transfer (FRET) techniques with fluorescently labeled H2B to assess nuclear-wide chromatin compaction and compaction around laser-induced DSBs. Local chromatin compaction around the break site is observed within 10 min of damage (Lou et al., 2019). This initial repressive state may prevent unwanted movement of the DSB keeping a relationship between DNA ends, act to strip the chromatin of irrelevant factors and prime the modification landscape ready for new alterations. It is clear that it is required for local transcriptional silencing (reviewed in Gursoy-Yuzugullu et al., 2016). A number of mechanisms drive this transient compaction including PARP-dependent recruitment of heterochromatin protein 1 (HP1), KRAB-associated protein-1 (KAP1), macroH2A variants, and methyltransferases (reviewed in Price and D'Andrea, 2013; Oberdoerffer, 2015). This initial repressive state must necessarily be overcome in order to permit repair. In FLIM-FRET analysis the kinase ataxia-telangiectasia mutated (ATM) and E3 ligase RING finger-8 (RNF8) regulate chromatin de-compaction and compact chromatin at later time points is found beyond the boundary of the repair locus (Lou et al., 2019).

Tri-methylated-lysine 9 Modified H3 domains (H3K9me3) generated adjacent to the break, bind and activate the lysine(K) acetyltransferase 5 (KAT5 also known as TIP60) (Sun et al., 2009; Ayrapetov et al., 2014). In turn, KAT5 acetylates and activates the master regulator of the DNA damage response, ATM (Sun et al., 2009) and also modifies the H4 tail. Acetylation of the H4 tail blocks the interaction of the tail with the acidic groove on adjacent nucleosome patches, facilitating a more open chromatin structure (reviewed in Price and D'Andrea, 2013). Once activated ATM disrupts the small ubiquitin like modifier (SUMO)-mediated interaction of KAP1 with Chromodomain helicase DNA binding protein 3 (CHD3), a member of the histone deacetylase complex referred to as the Nucleosome Remodeling Deacetylase (NuRD) complex (Goodarzi et al., 2011). KAP1 depletion can relieve chromatin compaction and allow subsequent repair (Ziv et al., 2006), and similarly the ATM-mediated CHD3 dissociation from chromatin, promotes chromatin relaxation and allows DNA repair. In addition, both the SUMO targeting E3 ubiquitin (ub) ligase RING Finger 4 (RNF4) and Valosin-containing protein/AAA+-type ATPase p97 (VCP/p97) interact with pS824-KAP1-SUMO. VCP/p97 can extract ubiquitinated proteins from membranes or cellular structures, or segregate them from binding proteins and RNF4-VCP/p97 promotes removal and degradation of SUMOylated KAP1 (Kuo et al., 2014), providing a further mechanism for chromatin de-compaction.

The canonical modification catalyzed by the RING Finger 20/RING Finger 40 dimer (RNF20-RNF40), H2B-K120ub, is associated with transcription in open chromatin (Nickel and Davie, 1989; Zhu et al., 2005). This modification is also induced by RNF20-RNF40 following DNA damage (Moyal et al., 2011; Nakamura et al., 2011), where the H2B-K120ub mark is required for recruitment of subsequent DNA repair factors, such as BRCA1 and RAD51 (Moyal et al., 2011; Nakamura et al., 2011). The requirement is likely to be an indirect effect of H2B-K120ub in promoting chromatin relaxation. Indeed, relaxation relieves the requirement for RNF20 in HDR (Nakamura et al., 2011). H2B-K120ub supports increased access to DNA by promoting both local and higher order chromatin de-compaction (Fierz et al., 2011; Debelouchina et al., 2017).

A further means to relieve histone-repression of resection is in the recruitment of chromatin remodelers to break sites. The INO80 chromatin remodeler complex promotes incorporation of the histone variant H2AZ, which in turn promotes an open chromatin structure, in part through facilitating H4 acetylation (Xu et al., 2012). Similarly the yeast "remodels the structure of chromatin," RSC, complex contributes to MRX and Ku recruitment to damage sites (reviewed in Chambers and Downs, 2012). In humans the SWI/SNF-related, matrix associated, actin-dependent regulator of chromatin, subfamily A, member 4

(SMARCA4 also known as BRG1), which is the ATPase subunit of the SWI/SNF-B polybromo-associated BRG1-associated factor (PBAF) chromatin remodeling complex, is required for RPA-RAD51 exchange (Qi et al., 2015), while the component, AT-Rich Interaction Domain 2 (ARID2), promotes RAD51 recruitment through direct protein interaction (de Castro et al., 2017). The SWI/SNF-related, matrix associated, actin-dependent regulator of chromatin, subfamily A, member 5 (SMARCA5 also called SNF2H) which is the catalytic subunit of ISWI chromatin remodeling complexes recruits to DNA damage sites through PARP1 and Sirtuin 6 (SIRT6) activity and through the structural Nuclear mitotic apparatus protein (NuMA) (Smeenk et al., 2013; Toiber et al., 2013; Vidi et al., 2014). The co-factor of SMARCA5, Remodeling and spacing factor-1 (RSF-1), similarly recruits to sites of damage, and does so in an ATM-dependent fashion (Min et al., 2014). In turn SMARCA5-dependent remodeling, for example of heterochromatin, requires H2B-ubiquitination by RNF20/RNF40 (Klement et al., 2014). Intriguingly, at least some of these remodelers share an interaction domain for binding nucleosomes in order to induce nucleosome sliding. For example INO80 and SMARCA5 require the acidic patch of H2A/B (Gamarra et al., 2018) that is also an interaction face for the unacetylated H4 tail, and for other signaling and repair factors, suggesting a mutually exclusive and perhaps sequential hierarchy of remodeling events directing repair responses.

The differences between repair in open, active euchromatin compared to closed, repressive heterochromatin have been reviewed elsewhere (Murray et al., 2012; Watts, 2016). For many years the view that a more open chromatin environment of euchromatin is conducive to HDR and that breaks within transcribed genes are repaired more frequently by HDR has persisted (Aymard et al., 2014; Lemaitre et al., 2014). Indeed a recent study using CRISPR-Cas9 to target specific loci found that open-chromatin may recruit insufficient p53 binding protein 1 (53BP1) (van den Berg et al., 2019), required to promote NHEJ and restrict HDR. Additionally, a more nuanced view has recently arisen in which repair choice is actively directed in different chromatin environments. For example, the chromatin-binding protein Lens epithelium-derived growth factor (LEDGF) binds preferentially to epigenetic methyl-lysine histone markers characteristic of active transcription and also interacts with CtIP in a damage dependent way, thereby improving resection within active genomic regions (Daugaard et al., 2012). In addition, specialist remodelers, such as the Snf2-like remodeler Helicase, lymphoid specific (HELLS), appear to enable HDR at some heterochromatic regions (Kollarovic et al., 2019) and heterochromatin-resident proteins such as HP1 and Sentrin/SUMO-Specific Protease SENP7 (SENP7) nevertheless facilitate HDR (Garvin et al., 2013; Lee et al., 2013). A recent study using CRISPR-Cas9 to quantify HDR- and NHEJ-derived gene editing events at single-target sequences subjected to distinct chromatin conformations found that NHEJ and not HDR was more sensitive to chromatin state. Reduction of Cas9 activity in G1 was a far greater determinant of the relationship between NHEJ and HDR than chromatin conformation (Janssen et al., 2019).

## Chromatin Signaling as a Barrier to Resection

When both strands of DNA are broken nearby, sheering the chromosome, a dramatic signaling cascade occurs to initiate repair. This cascade, often referred to as the DNA damage response (DDR), is reviewed extensively elsewhere (Jackson and Bartek, 2009; Altmeyer and Lukas, 2013; Setiaputra and Durocher, 2019) while signaling leading to 53BP1-Shieldin recruitment to damage sites is described briefly here. DSBs are detected by two protein complexes, the Ku70/80 dimer and the MRN-complex. MRN (MRE11-RAD50-NBS1) tethers to the two ends and recruits the serine/threonine kinase ATM through interaction with NBS1. Phosphorylation of the histone variant H2AX at serine-139, recruits the Mediator of DNA damage checkpoint 1 (MDC1), which in turn recruits more ATM, amplifying the signal either side of the dsDNA break. The E3 ub ligase RNF8 is recruited to damage sites by interaction with ATM-phosphorylated-MDC1. Once at DSBs, RNF8 modifies the linker histone H1 with K63-ub chains (Thorslund et al., 2015). Additionally L3MBTL Histone methyl-lysine binding protein 2 (L3MBTL2) may be recruited by MDC1 and also modified by RNF8 (Nowsheen et al., 2018). H1 modification is assisted by the HECT, UBA, and WWE domain containing E3 ub ligase 1 (HUWE1) (Mandemaker et al., 2017), and the polycomb repressor complex 1 (PRC1) (Ismail et al., 2013). RNF8 signaling promotes the recruitment of another E3 ub ligase, RING finger 168 (RNF168), which binds K63-ub chains (Doil et al., 2009; Stewart et al., 2009; Panier et al., 2012), and also interacts with the nucleosome acidic patch where it catalyzes mono-ubiquitination of H2A at K13/K15 to promote recruitment of 53BP1 (Doil et al., 2009; Mattiroli et al., 2012; Fradet-Turcotte et al., 2013). H2A N-terminal ubiquitination is critical for 53BP1 accumulation, where the H2AK15-conjugated ub acts to trap a portion of 53BP1 against the nucleosome surface (Wilson et al., 2016). KAT5 acetylation of H4K16 reduces the second mode of 53BP1 interaction with nucleosomes, interaction of its Tudor domain with H4K20me2 (Tang et al., 2013), while acetylation at H2AK15 blocks 53BP1 binding as this modification is mutually exclusive with H2AK15ub (Jacquet et al., 2016). Binding of Bromo-domain containing 2 (BRD2) to acetylated H4 protects the chromatin from histone deacetylases (HDACs) 2 Kb both sides of the break, and limits the 53BP1 competitive inhibitor L3MBTL Histone methyl-lysine binding protein 1 (L3MBTL1) from binding (Dhar et al., 2017; Gursoy-Yuzugullu et al., 2017). RNF168 recognizes its own H2AK13/K15ub mark and thereby auto-propagates this signal along chromatin (Chen J. et al., 2012; Panier et al., 2012; Poulsen et al., 2012).

53BP1 is heavily phosphorylated by ATM following damage and the phosphorylated protein interacts with two apparently independent effectors. PAX transcription activation domain interacting protein-1-like (PTIP) which in turn interacts with Artemis (Munoz et al., 2007; Wang et al., 2014) and RIF1 Replication timing regulatory factor 1 (RIF1) which interacts with the Shieldin complex (Manke et al., 2003; Silverman et al., 2004; Munoz et al., 2007; Wu et al., 2009; Chapman et al.,

2013; Daley and Sung, 2013; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013; Feng et al., 2013; Zimmermann et al., 2013; Wang et al., 2014; Boersma et al., 2015; Tomida et al., 2015, 2018; Xu et al., 2015; Bakr et al., 2016; Bluteau et al., 2016; Dev et al., 2018; Ghezraoui et al., 2018; Gupta et al., 2018; Mirman et al., 2018; Noordermeer et al., 2018; Zlotorynski, 2018). The identification of Shieldin has been an exciting advance in understanding how chromatin signaling acts to inhibit resection (reviewed in Setiaputra and Durocher, 2019). RIF1 bound to 53BP1 interacts with Shld3/RINN1-Rev7 and in turn Shld2/RINN2-Shld1/RINN3 (Names: Shieldin Complex Subunit 3/RINN1-REV7-Interacting Novel NHEJ Regulator 1, REV7 Homolog/ Mitotic Arrest Deficient 2 Like 2; Shieldin Complex Subunit 1/RINN3-REV7-Interacting Novel NHEJ Regulator 3; Shieldin Complex Subunit 2/RINN2-REV7-Interacting Novel NHEJ Regulator 2; and Shieldin Complex Subunit 1/RINN3-REV7-Interacting Novel NHEJ Regulator 3, respectively). Shld2 carries 3 OB folds which interact with ssDNA and are required for the promotion of 53BP1 mediated NHEJ and HDR inhibition. Surprisingly Shld2 can precipitate ssDNA of >50 nucleotides, but not smaller than 30 nucleotides (Dev et al., 2018; Findlay et al., 2018; Gao et al., 2018; Noordermeer et al., 2018), which is slightly greater than the length of minimally resected DNA. Shieldin in turn contacts a complex made up of CST telomere replication complex component 1 (CTC1)-Subunit of CST Complex (STN1)-Telomere Length Regulation Protein TEN1 Homolog (TEN1), known as the CST complex, together with DNA polymerase-alpha (Pol-α). CST-Pol-α is critical to the integrity of telomeres, where it performs C-strand fill in (reviewed in Stewart J. A. et al., 2018) and CST-pol-α appears to perform a similar role at resected DNA ends, filling in the short regions of resected DNA (Barazas et al., 2018; Mirman et al., 2018) so that 53BP1 and its effectors may not only block resection but also reverse it. This mechanism provides further options for repair; since filling in the region that has been processed by MRE11 potentially generates a "clean" end for NHEJ (Setiaputra and Durocher, 2019; illustrated in **Figure 2**). Indeed 53BP1 is required for the promotion of several forms of NHEJ, including class-type switching, a subset of VDJ recombination and the fusion of unprotected telomere ends (Manis et al., 2004; Ward et al., 2004; Nakamura et al., 2006; Difilippantonio et al., 2008; Dimitrova et al., 2008; Kibe et al., 2016). Dramatically, the impact of the 53BP1-complex on suppressing resection is clearest in cells lacking BRCA1 (Bothmer et al., 2010, 2011; Bouwman et al., 2010; Bunting et al., 2010).

The embryonic lethality of *Brca1* deficient mice is rescued by concurrent loss of *53bp1* and coincides with improved DNA resection, and improved measures of HDR-proficiency, such as RAD51 foci in irradiated cells, PARP inhibitor resistance and improved repair of integrated HDR-substrates together with a reduction of radial chromosomes, often referred to as a hallmark of toxic end joining (Cao et al., 2009; Bouwman et al., 2010; Bunting et al., 2010, 2012; Li et al., 2016; Nacson et al., 2018). These data demonstrate that BRCA1, of itself, is not needed for the promotion of resection, since the need for the protein is largely overcome by loss of 53BP1, but show BRCA1 is critical to overcoming the resection block mediated by 53BP1. A similar, though less potent, relationship is also clear between BRCA1 and members of the 53BP1-Shieldin complex (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Diaz et al., 2013; Feng et al., 2013; Zimmermann et al., 2013; Boersma et al., 2015; Tomida et al., 2015, 2018; Xu et al., 2015; Dev et al., 2018; Findlay et al., 2018; Ghezraoui et al., 2018; Gupta et al., 2018; Mirman et al., 2018; Noordermeer et al., 2018; Zlotorynski, 2018).

## Repair Structures

In G1-phase cells the 53BP1 protein is found as a dense focus around the DSB, whereas in S-phase cells 53BP1 accumulations are less dense and more dispersed from the focus center. At the central focus core of damage sites in S-phase cells BRCA1, CtIP and the ssDNA binding protein RPA are found (Chapman et al., 2012; Kakarougkas et al., 2013). These S-phase structures are large, with 53BP1 peak density mapping in an axis through a foci center as far as 1 μm across, presumably ∼0.5 μm from the DSB (Chapman et al., 2012; Kakarougkas et al., 2013). Loss of BRCA1 reduces the circumference of the 53BP1 localization, placing it in the center of the foci, resembling a G1-phase focus (Chapman et al., 2012; Kakarougkas et al., 2013). Thus BRCA1 plays a role in the physical localization of 53BP1, contributing to its placement away from the break core in S-phase cells.

Mapping of chromatin compaction reveals that substantial local chromatin changes accompany the repair response. The chromatin density is re-arranged so that the initial compaction seen proximal to the break at 10 min post damage is lost and at 30–60 min a ring of condensed chromatin forms further away from the break site, with the greatest density occurring beyond the regions bound by 53BP1. Chromatin peak density mapping through focus centers reveal that compaction occurs as much as 5 μm apart, presumably ∼2.5 μm from the DSB (Lou et al., 2019; illustrated in **Figure 3**). These large chromatin rearrangements are dependent on ATM and RNF8 (Lou et al., 2019). One speculative explanation for the chromatin "wave" beyond 53BP1 is that chromatin remodeling required to promote long-range resection, forces chromatin bunching outside the resected region (see below). Another possibility is that the liquid-like properties of 53BP1 assemblies, which show fusion and sensitivity to disruption of hydrophobic interactions by detergents (Kilic et al., 2019), displace chromatin. These later observations are particularly fascinating in view of reports that liquid phase-separation mechanically excludes chromatin as it grows (Shin et al., 2018). Understanding the role of remodeling factors and the three dimensional chromatin structures of both the damaged and template strands within the repair structures is needed to address what these structures represent and the reason for their large scale.

## BRCA1 Regulation of Resection

BRCA1 exists as an obligate heterodimer with its N-terminal binding partner BRCA1-associated RING domain protein 1 (BARD1) and in the absence of BARD1, BRCA1 is degraded (Joukov et al., 2006). The BRCA1-BARD1 heterodimer has the ability to act as an E3 ub ligase by improving the transfer of ub from an interacting and loaded E2 ub conjugating enzyme to target lysines (Brzovic et al., 2003). Several E2 conjugating

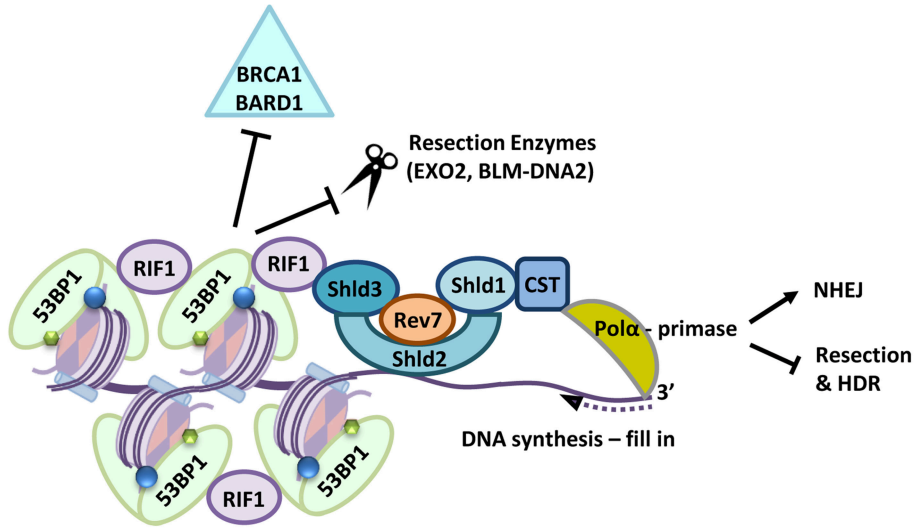


**FIGURE 2 |** Following a DSB 53BP1 interacts with modified histones, (H2A-K15-ubiquitin blue circles, H4K20-dimethylation, green hexagons), the 53BP1-RIF1-Shieldin (Shld1-Shld2-Shld3-Rev7-CST) complex is recruited to sites of DNA damage where it also prevents retention of BRCA1-BARD1. Shld2 binds directly to ssDNA stretches >50 nucleotides long via three OB-folds. Together the Shieldin complex recruits DNA Polα which in turn primes DNA synthesis to fill in resected DNA ends. This prevents long range resection and repair by HDR pathways and supports repair by NHEJ.

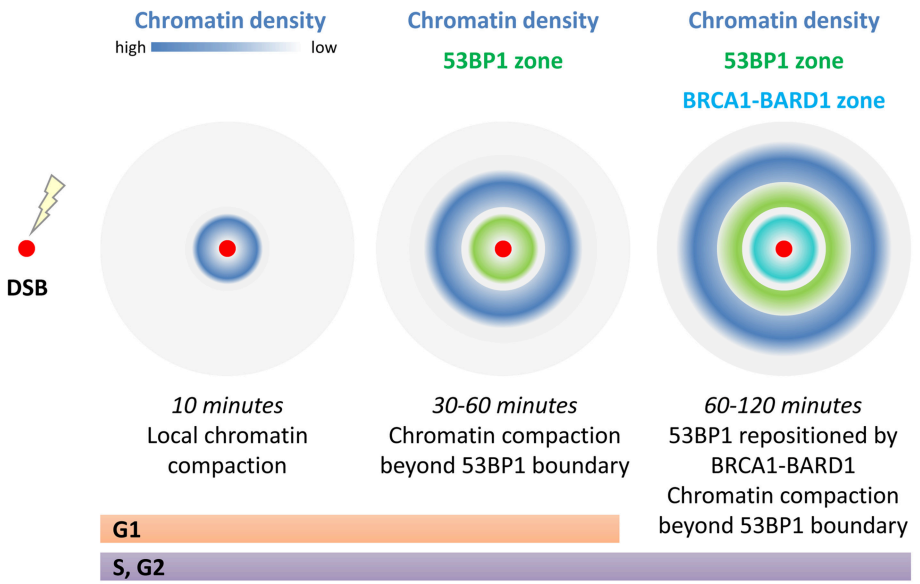


**FIGURE 3 |** Chromatin compaction around a single DSB changes with time. Immediately following damage (within 10 min) local compaction occurs which has been linked to transcriptional repression, limiting movement of the break ends, and to strip and prime chromatin modifications for repair. At 30–60 min post repair chromatin density is at its greatest beyond the 53BP1 boundary that marks the break site. In S/G2, the 53BP1 boundary is repositioned by BRCA1-BARD1 to open up the damage site for long range resection. In addition the 53BP1 damage complexes are thought to have liquid like phase properties which may be key to these large scale (5 μm diameter) effects on chromatin densities.

enzymes interact with the BRCA1-RING domain, and not the BARD1-RING domain, to catalyze the generation of ub conjugates (Christensen et al., 2007). Nevertheless, BARD1 brings more than protein stability to BRCA1, contributing a charged residue that interacts with ub to facilitate its transfer from the loaded E2 (Densham et al., 2016). Several targets of the BRCA1-BARD1 E3 ligase activity have been identified (reviewed in Wu et al., 2008) including several independent reports of H2A modification (Mallery et al., 2002; Zhu et al., 2011; Kalb et al., 2014a). Residues at the extreme C-terminus of H2A at K125/K127/K129 have been mapped as those modified (Kalb et al., 2014a). Modeling and mutagenesis approaches have

suggested that BRCA1-BARD1 contacts the H2A/B nucleosome acidic patch via an arginine anchor to promote ubiquitination of H2A (Buchwald et al., 2006; McGinty et al., 2014).

Ubiquitin modification of H2A at K118/K119 is associated with transcriptional repression (Blackledge et al., 2014; Kalb et al., 2014b), and de-repression of satellite DNA has been reported in human and mouse BRCA1-deficient cancers (Zhu et al., 2011). Recently cancer-associated germline patient variants in the BARD1-RING have been described which do not reduce BRCA1-BARD1 ligase activity, but do specifically prevent ubiquitination of H2A (Stewart M. D. et al., 2018). These mutations also suppress transcriptional repression, resulting in activation of estrogen metabolism genes in MCF10A breast cells (Stewart M. D. et al., 2018). Whether the de-repression of transcription can impact HDR directly is not clear, but there is potential for re-expressed genes, such as those involved in estrogen metabolism or satellite RNA to increase the demand for HDR (Santen et al., 2015; Kishikawa et al., 2018) the latter through the generation of RNA: DNA hybrids at repeat sequences and at replication forks (Zhu et al., 2018; Padeken et al., 2019).

Cells lacking BRCA1 are sensitive to a broad range of DNA damaging agents (reviewed in Costes and Lambert, 2012; Zimmermann and de Lange, 2013; Ceccaldi et al., 2016). However in a human cell system complemented with ligase defective BRCA1-BARD1, cells were sensitive to the PARP inhibitor, Olaparib, and the Topoisomerase inhibitor, camptothecin, but not sensitive to replication stressing agents, hydroxyurea, or aphidicolin (Densham et al., 2016). BRCA1 E3 ligase defective chicken DT40 cells also are sensitive to Topoisomerase inhibitors (Sato et al., 2012) and neither these cells, nor similarly altered mouse cells, nor human cells complemented with ligase defective BRCA1-BARD1, show sensitivity to DNA interstrand cross-linking agents (Reid et al., 2008; Sato et al., 2012; Densham et al., 2016) (although the engineered mouse cells do exhibit increased chromosome aberrations after cross-linking agent treatment Reid et al., 2008). BRCA1 loss, or loss of the ligase function, is associated with reduced long-range resection (Shibata et al., 2011, 2014; Alagoz et al., 2015; Densham et al., 2016; Drost et al., 2016). Intriguingly E3 ligase proficiency also correlates with the ability to position 53BP1 away from the break site in S-phase cells (Densham et al., 2016). Thus a subset of BRCA1-mediated responses relate to resection and to 53BP1 positioning.

Amongst the remodelers critical to DNA repair in yeast is the SNF2 family ATPase SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily A, containing DEAD/H Box-1 (SMARCAD1) homolog, Fun30. Fun30 promotes long range resection at camptothecin-induced lesions by facilitating the activity of Exonuclease 1 (Exo1) (Chen X. et al., 2012; Costelloe et al., 2012; Eapen et al., 2012). Significantly this remodeler is less important for resection in the absence of histone-bound Rad9, the 53BP1 ortholog, which like 53BP1 acts to block 5′ strand processing (Chen X. et al., 2012; Adkins et al., 2013). SMARCAD1 has two N-terminal ub-binding CUE domains (coupling of ub to ER degradation) (Kang et al., 2003; Shih et al., 2003) and these link BRCA1-BARD1 ligase function and H2A modification to 53BP1 positioning and resection (Densham et al., 2016). Moreover SMARCAD1 ATPase activity and the integrity of its ub binding domains are required for HDR repair and for the positioning of 53BP1 away from the BRCA1 core (Densham et al., 2016). These observations point to ub driven SMARCAD1 remodeling, rather than 53BP1:BRCA1 competition at chromatin, as critical to 53BP1 positioning. CUE domain interactions with ub are typically weak, with reported dissociation constants ranging from 20 to 160 μM (Kang et al., 2003; Prag et al., 2003; Shih et al., 2003) and while SMARCAD1 CUE domains and BRCA1-BARD1 are required for full SMARCAD1 recruitment to damage sites (Densham et al., 2016) an ATM consensus site at SMARCAD1-T906 is also required (Chakraborty et al., 2018). In addition, a recent peptide array screen has shown that SMARCAD1 binds to histone 3 modifications, including citrullinated Histone 3. Citrullination occurs when an arginine is deaminated and converted to the amino acid citrulline. SMARCAD1 binds modified Histone 3: H3R26Cit> H3K27ac>H3R17Cit>H3R26me2 (Xiao et al., 2017). Intriguingly H2A-K127/K129ub and H3R26Cit/K27ac are proximal on the nucleosome surface presenting the possibility that SMARCAD1 interaction with histone is through combined post-translational modification interactions. In yeast the CDK-mediated phosphorylation of Fun30 promotes interaction with Dpb11 [homolog of DNA Topoisomerase II Binding Protein 1 (TOPBP1)] and Mec1-Ddc2 [orthologs of ataxia telangiectasia and Rad3-related protein (ATR) and ATR-interacting protein (ATRIP)] resulting in improved Fun30 recruitment to damaged chromatin in S-phase. In human cells TOPBP1 similarly interacts through phosphorylated SMARCAD1 (Bantele et al., 2017). Further, purified Fun30 binds nucleosomes wrapped in ssDNA preferentially over dsDNA-wrapped nucleosomes and ssDNA-nucleosomes are effective at activating Fun30 (Adkins et al., 2017), providing a potential means for short-range resection to activate the remodeler. Taken as a whole, recent evidence suggest a model in which several components of SMARCAD1 recruitment prime it to locating and activating, not only at DNA break sites, but at minimally resected DNA (illustrated in **Figure 4**).

Intriguingly, independently of ubiquitination, SMARCAD1 constitutively interacts with KAP1 directly through its first CUE domain (Rowbotham et al., 2011; Ding et al., 2018; Lim et al., 2019). SMARCAD1 co-purifies with several other remodeling factors (Rowbotham et al., 2011) associated with gene silencing and heterochromatin formation, some of which have also been implicated in 53BP1 repositioning (Alagoz et al., 2015).

How SMARCAD1/Fun30 in turn promotes remodeling of 53BP1/Rad9 is less clear. Fun30 can promote nucleosome sliding or eviction of H2A-H2B from nucleosomes (Awad et al., 2010). Sliding might be expected to contribute to the compaction wave of condensed chromatin observed outside of 53BP1 domains (Lou et al., 2019) but this is not mutually exclusive with the model of 53BP1 phase separation and chromatin exclusion (Shin et al., 2018; Kilic et al., 2019). The related remodeler SMARCA5/SNF2h shifts DNA discontinuously with movement on the entry side preceding its exit (Sabantsev et al., 2019). A recent Cryo-electron microscopy model of a SMARCA5 dimer on nucleosomes suggests the disordered H2A-H2B

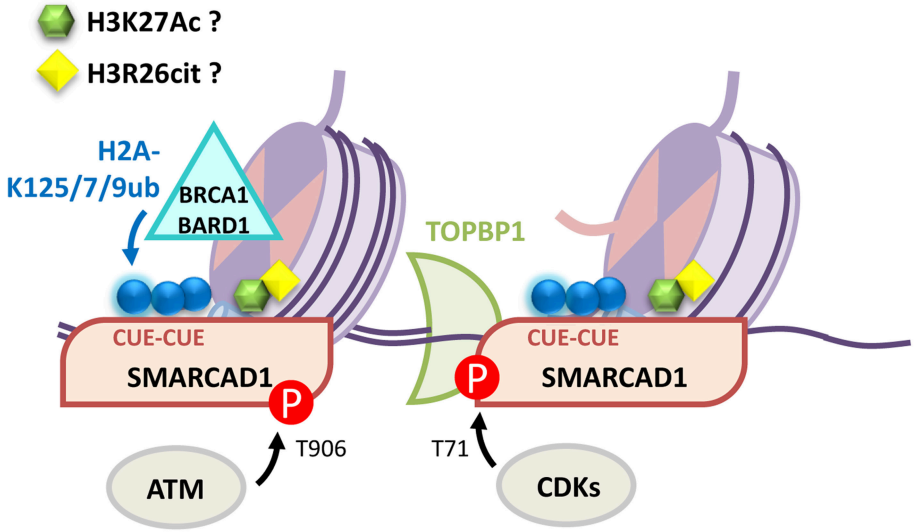


**FIGURE 4 |** Multiple mechanisms contribute to SMARCAD1 recruitment to DNA damage sites. BRCA1-BARD1 modification of H2A-K125/127/129ub is recognized by SMARCAD1 CUE domains. Phosphorylation events by ATM (SMARCAD1-T906) facilitate recruitment and CDKs (SMARCAD1-T71) promote TOPBP1 interaction. SMARCAD1 preferentially binds and is activated by ssDNA-nucleosomes. Finally, SMARCAD1 has been proposed to directly bind H3K27Ac and H3R26cit although the role of these interactions in the DDR has yet to be characterized.

acidic patch inhibits the second SMARCA5 protomer, while disorder near the bound SMARCA5 dyad stimulates directional DNA translocation (Armache et al., 2019). Thus, we might speculate that order induced by protein-protein interaction at the nucleosome acidic patch, for example by the bound 53BP1-ubiquitylation-dependent recruitment motif, could influence remodeling directionality or proficiency.

In the context of the DNA damage response the BRCA1-BARD1 E3 ub ligase contributes the third ub modification of H2A. Indeed H2A modification in a nucleosomal context is remarkably site specific both for the E3 ligases responsible and for the readers of these marks (reviewed in Uckelmann and Sixma, 2017). H2A has long tails at both N- and C-termini which can be modified by conjugation of ub at three major sites: K13/K15 by RNF168 (Mattiroli et al., 2012), K118/K119 by the PRC1 (Nickel and Davie, 1989), and K125/K127/K129 by BRCA1-BARD1 (Kalb et al., 2014a). The majority of H2A ubiquitination in the cell is at the K119 site (Nickel and Davie, 1989) which is ubiquitinated by proteins that form part of the PRC1 and the mark is associated with transcriptional gene repression and heterochromatin (Wang et al., 2004). Many de-ubiquitinating enzymes (DUBs) have been implicated in the removal of ub from H2A (reviewed in Vissers et al., 2008; Uckelmann and Sixma, 2017) but none had previously been reported to be specific for the BRCA1-H2Aub mark.

*In vitro* work from the group of Prof. Titia Sixma identified the highly conserved Ubiquitin Specific Peptidase 48 (USP48) (human has 77% identify with Xenopus, 95% with mouse Usp48) as a DUB specific for nucleosomal-H2A substrates and, more specifically, for nucleosomal-H2A modified at the BRCA1 K125/K127/K129 sites (Uckelmann et al., 2018). Additionally, like Ubiquitin Specific Peptidase 14 (USP14), the proteasome-associated DUB, USP48 requires a second "auxiliary" ub (i.e., not the substrate ubiquitin) to achieve full catalytic potential. This "auxiliary" ub can be at either the H2A-K125/K127/K129ub or H2A-K118/K119ub sites but it only increases activity toward ub removal from the three BRCA1 targeted sites, i.e., USP48 does not cleave H2A-K118/K119ub when H2A-K125/K127/K129ub is present (Uckelmann et al., 2018).

Modulating the levels of USP48 dramatically influences DNA resection lengths. Over-expression of USP48 results in restricted resection whereas low USP48 levels result in placement of 53BP1 further from the damage site and result in the extension of BRCA1 and SMARCAD1 dependent resection. The removal or depletion of 53BP1 results in extended resection lengths to the degree that SSA is favored over GC, leading to the suggestion that 53BP1 acts as to limit the extent of resection (Ochs et al., 2016). Intriguingly cells depleted of USP48 develop a dependence on SSA DNA repair even though they have normal levels of 53BP1 (Uckelmann et al., 2018). These findings suggest cells may fine-tune 53BP1 placement and HDR mechanisms through the opposing activities of the BRCA1-BARD1 ligase and USP48 DUB (illustrated in **Figure 5**). Additionally cells lacking the Fanconi anemia compatibility component A (FANCA), show improved survival to interstrand cross linking agents when they lack USP48 (Velimezi et al., 2018). These cells have enhanced BRCA1 dependent clearance of DNA damage that appears unrelated to resection proficiency (Velimezi et al., 2018). We speculate that BRCA1-BARD1 ligase function, amplified by loss of USP48, may provide a back-up role for the Fanconi anemia core complex.

The degree to which the BRCA1-BARD1-USP48 relationship is significant in regulating resection-driven repair pathway choice will be dependent on how the pathway is modulated in different environments. The identity of the "auxiliary" ub site on H2A is not clear and the ligase responsible for the modification

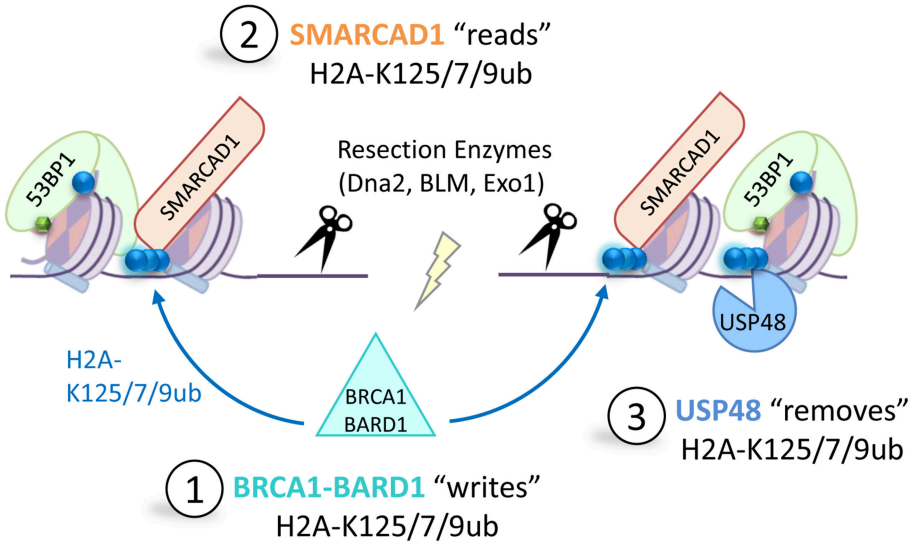


**FIGURE 5 |** A new BRCA1-circuit that controls DNA repair pathway choice. In S-phase BRCA1-BARD1 is retained at DNA double strand break sites where it mono-ubiquitinates the extreme C-terminus of H2A at K125/127/129 (1). This ubiquitination modification is recognized by the chromatin remodeler SMARCAD1 (2) which remodels nucleosomes to promote 53BP1 repositioning at the break site (53BP1 binds modified H2A-K15-ubiquitin blue circles, H4K20-dimethylation, green hexagons). This allows recruitment of long-range resection enzymes, such as DNA2, BLM or EXO1, required for homology-directed repair. The deubiquitinating enzyme USP48 specifically removes the BRCA1-mediated H2A-Ub modification (3) to prevent over-resection and limit use of the mutagenic single-strand annealing repair pathway.

not known. Similarly it is unclear if SMARCAD1: chromatin interaction favors ub-modification at any of one of the three lysines of H2A-K125/K127/K129 over another. It is possible that the dependency of USP48 for an auxiliary ub has the potential to regulate the degree of resection in particular chromatin environments; for example, in regions of heterochromatin marked by PRC1 mediated modification at H2A-K118/K119.

Given the potential for mutagenic DNA repair conferred by hyper-resection many regulatory mechanisms are to be expected. For example, incorporation of H2AZ at sites of damage has been proposed to limit resection and define chromatin boundaries (Xu et al., 2012). Interestingly, H2AZ, like H2AX, has shorter C-terminal tails than H2A and lacks the C-terminal lysines K125/K127/K129 present on H2A. H2AZ may thus be refractory to BRCA1 modification and SMARCAD1 remodeling.

In addition, positioning of 53BP1 by BRCA1 ligase activity is not the only means by which the block on resection is resisted. BRCA1 can counteract RIF1 recruitment in S-phase under conditions where no impact on 53BP1 is obvious (Chapman et al., 2013; Escribano-Diaz et al., 2013; Feng et al., 2013; Zimmermann et al., 2013). BRCA1 contributes to the recruitment of the protein phosphatase 4C (PP4C) to dephosphorylate 53BP1 and release RIF1 (Feng et al., 2015; Isono et al., 2017). BRCA1 is also reported to contribute to the recruitment of a further E3 ub ligase Ub-like with PHD and RING finger domains 1 (UHRF1), which mediates K63-linked polyubiquitination of RIF1, and results in its dissociation from 53BP1, thereby facilitating resection (Zhang et al., 2016). Further in S-phase cells RIF1 is gradually competed out from 53BP1 by the protein Suppressor of Cancer cell Invasion (SCAI), which binds 53BP1 to allow BRCA1-mediated repair (Isobe et al., 2017).

In addition ATM and CDK2 control the chromatin remodeling activity of the SWI2-SNF2 remodeler, Cockayne syndrome group B (CSB), which interacts with RIF1 and remodels chromatin by evicting histones, which limits RIF1-REV7 but promotes BRCA1 accumulation (Batenburg et al., 2017).

## CONCLUDING REMARKS

The response to DNA breaks drives both dramatic and subtle local chromatin changes. That resection is sensitive to chromatin state has been utilized by cells to regulate resection lengths in and of itself and chromatin has been used as a substrate to build inhibitory blocks, or mountains, upon. BRCA1-BARD1 and TOPBP1 are part of a signaling milieu that places and initiates the chromatin remodeling activity of SMARCAD1 at the right place to reposition 53BP1, while several mechanisms counter the interaction of 53BP1 with RIF1. The degree of resection is the net result of nuclease-digestion vs. Shieldin-CST-Pol-α fill in, where the positioning of the fill-in machinery further from the break site appears to give nucleases the upper-hand. Unrestrained BRCA1-mediated remodeling can lead to hyper-resection and bias HDR mechanisms from accurate GC to mutagenic SSA.

We understand comparatively little about the relative physical positioning of many of the factors critical to the regulation of resection including the 53BP1-binding proteins responsible for the block on resection, those that promote resection, and only recently has relationship of chromatin with these factors begun to emerge. The explosion in the number of components capable of promoting and, in particular, restricting resection, illustrates the premium that the cell places on tuning appropriate resection lengths. Given the critical role it plays in repair pathway choice,

how these factors are regulated will be key to understanding how chromatin context and HDR repair are interwoven.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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# Nucleosome Remodeling by Fun30$^{SMARCAD1}$ in the DNA Damage Response

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Many cellular pathways are dedicated to maintain the integrity of the genome. In eukaryotes, the underlying DNA transactions occur in the context of chromatin. Cells utilize chromatin and its dynamic nature to regulate those genome integrity pathways. Accordingly, chromatin becomes restructured and modified around DNA damage sites. Here, we review the current knowledge of a chromatin remodeler Fun30$^{SMARCAD1}$, which plays a key role in genome maintenance. Fun30$^{SMARCAD1}$ promotes DNA end resection and the repair of DNA double-stranded breaks (DSBs). Notably, however, Fun30$^{SMARCAD1}$ plays additional roles in maintaining heterochromatin and promoting transcription. Overall, Fun30$^{SMARCAD1}$ is involved in distinct processes and the specific roles of Fun30$^{SMARCAD1}$ at DSBs, replication forks and sites of transcription appear discordant at first view. Nonetheless, a picture emerges in which commonalities within these context-dependent roles of Fun30$^{SMARCAD1}$ exist, which may help to gain a more global understanding of chromatin alterations induced by Fun30$^{SMARCAD1}$.

**Keywords: Fun30/SMARCAD1, nucleosome remodeling, DNA double-stranded break, DNA end resection, cell cycle, post-translational modification, genome stability**

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Fun30 (function unknown now, budding yeast) and its homologs Fft3 (fission yeast) and SMARCAD1 (human; Etl1 in mouse) are non-essential Snf2-like Etl1-subfamily nucleosome remodelers which function in DNA replication, heterochromatin stability, transcription, meiotic hotspot activity, and regulation of DNA repair (Flaus, 2006; Okazaki et al., 2008; Neves-Costa et al., 2009; Rowbotham et al., 2011; Strålfors et al., 2011; Yu et al., 2011; Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012; Byeon et al., 2013; Steglich et al., 2015; Densham et al., 2016; Doiguchi et al., 2016; Bantele et al., 2017; Lee et al., 2017; Xiao et al., 2017; Chakraborty et al., 2018; Ding et al., 2018; Jahn et al., 2018; Storey et al., 2018; Terui et al., 2018; Sachs et al., 2019). Notably, during DNA double-strand break (DSB) repair a major function of Fun30 orthologs appears to be in DNA end resection, a process that requires the mobilization and likely eviction of nucleosomes (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012; Bantele et al., 2017). In apparent contrast, during DNA replication of heterochromatin, Fun30 orthologs in fission yeast and human cells seem to rather provide stability of nucleosomes and to prevent loss of heterochromatic histone marks (Rowbotham et al., 2011; Taneja et al., 2017).

With this review, we aim to summarize current data in order to show commonalities and highlight regulatory mechanisms controlling Fun30$^{SMARCAD1}$ remodelers with a special focus on the DNA damage response. The different Fun30$^{SMARCAD1}$ functions appear discrepant at first view, but in this review we will also attempt to point toward commonalities.

## DOMAIN STRUCTURE OF FUN30 AND ITS ORTHOLOGS

Fun30 and its orthologs are ∼1,000 amino acids large, single-subunit nucleosome remodelers, which appear to act in homodimeric form (Awad et al., 2010). A bioinformatic analysis showed that Fun30 shares the highest degree of homology with Swr1 and Ino80 of the Snf2 remodeler family (Flaus, 2006). It comprises the catalytic Snf2 nucleosome remodeling domain, but with a Fun30-specific yet uncharacterized insert at the C-terminus (Liu and Jiang, 2017). The N-terminal half of the protein appears to be regulatory and harbors specific regions with the ability to engage in protein-protein interactions (Flaus, 2006; Neves-Costa et al., 2009; Bantele et al., 2017). At the N-terminus, conserved Cyclin-dependent kinase (CDK) phosphorylation sites in yeast Fun30 and human SMARCAD1 (Chen X. et al., 2016; Bantele et al., 2017) are followed by ubiquitin-binding CUE (Coupling of Ubiquitin conjugation to ER degradation) domains, which exist in one or more copies in almost all Fun30 orthologs. In human SMARCAD1, further regulatory ATM phosphorylation sites and phosphorylation-dependent RING1 ubiquitylation sites are targeted after DNA damage and located at the C-terminus (Matsuoka et al., 2007; Densham et al., 2016; Chakraborty et al., 2018). In the following, we will view Fun30$^{SMARCAD1}$ from N to C and summarize the molecular role of the additional regulatory elements.

### CDK Phosphorylation at the N-Terminus of Fun30 and SMARCAD1

Several studies have established Fun30 as CDK substrate *in vitro* and *in vivo* (Ubersax et al., 2003; Chen et al., 2012; Chen X. et al., 2016; Bantele et al., 2017). Specifically, Fun30 is targeted by CDK on S20, S28, and S34 (Chen X. et al., 2016; Bantele et al., 2017). Similarly, SMARCAD1 can be phosphorylated by CDK on T71 (Bantele et al., 2017). Once phosphorylated, S20 and S28 in Fun30 and T71 in SMARCAD1 mediate a direct protein-protein interaction with the N-terminal BRCT repeats of the scaffold protein Dpb11 (in yeast) and TOPBP1 (in human) (Bantele et al., 2017). In yeast, this interaction leads to formation of a ternary complex with the 9-1-1 checkpoint clamp and contributes to targeting Fun30 to sites of DNA damage (Bantele et al., 2017, **Figure 1**). These data suggest that phosphorylation is a means to localize Fun30, but additionally it is possible that phosphorylation and the associated protein-protein interactions are involved in activating the remodeller toward its substrate.

### CUE Domains

CUE domains are known for their ability to bind ubiquitin (Donaldson et al., 2003; Kang et al., 2003; Shih et al., 2003), and the N-terminal CUE domain in SMARCAD1 was shown to mediate interactions with the chromatin regulator KAP1 (Rowbotham et al., 2011; Ding et al., 2018; Lim et al., 2019). Furthermore, a CUE domain-dependent interaction of SMARCAD1 with ubiquitylated histone H2A has been identified recently (Densham et al., 2016). Whether binding of ubiquitylated histones is a conserved property of Fun30 orthologs remains to be shown. While a CUE domain has been bioinformatically predicted for Fun30 as well (Neves-Costa et al., 2009), so far no binding partner of the Fun30 CUE domain could be identified. *In vitro* experiments also failed to provide evidence for Fun30 binding to ubiquitylated histones (Awad et al., 2010). Nonetheless, the CUE-dependent protein-protein interactions seem to contribute to context-dependent chromatin localization in the human protein (Densham et al., 2016; Ding et al., 2018). Interestingly, the SMARCAD1-KAP1 interaction has very recently been shown to occur between the SMARCAD1 N-terminal CUE domain and a specific patch in KAP1 that does structurally not resemble ubiquitin (Lim et al., 2019). This finding suggests alternative and still-to-be explored interaction modes of Fun30$^{SMARCAD1}$.

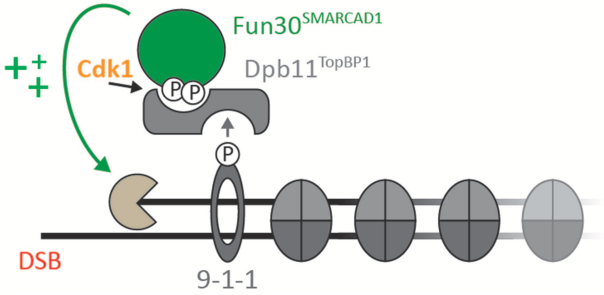


**FIGURE 1 |** Cell cycle- and DNA damage-activated kinases lead to formation of a ternary complex formed by Fun30$^{SMARCAD1}$, Dpb11$^{TOPBP1}$, and the 9-1-1 complex (adapted from Bantele et al., 2017; Bantele, 2018). Upon CDK-dependent phosphorylation of Fun30 S20/S28 or SMARCAD1 T71, respectively, Fun30 and SMARCAD1 associate with BRCT1+2 of Dpb11 or BRCT0/1/2 of TOPBP1. In yeast, binding to the 9-1-1 complex (in a DNA damage-induced manner) contributes to localization of Fun30-Dpb11 to sites of DNA end resection, where it stimulates long-range resection (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012; Bantele et al., 2017).

### DNA Damage-Dependent Phosphorylation at the C-Terminus of SMARCAD1

SMARCAD1 is a substrate of the ATM kinase and gets phosphorylated on T906 upon DNA damage (Matsuoka et al., 2007; Densham et al., 2016; Chakraborty et al., 2018). This modification is a prerequisite for the subsequent ubiquitylation on K905 in a RING1-dependent manner (Chakraborty et al., 2018). Both, DNA damage-dependent phosphorylation and ubiquitylation of SMARCAD1 were connected to functions in the DNA damage response, but do not seem to be conserved in the yeast protein. Interestingly, Fun30 was suggested to interact with other proteins of the DNA damage response, such as DNA end resection enzymes Exo1 and Dna2, as well as RPA (Chen et al., 2012). Where the specific interaction sites are located, whether all interactions are direct and in how far they are regulated by post-translational modification remains to be determined.

## BIOCHEMICAL ACTIVITIES OF FUN30$^{SMARCAD1}$

Nucleosome remodelers use ATP to remodel histone-DNA contacts in order to move or position nucleosomes, evict them or

change their composition (Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011). These molecular activities can be studied well *in vitro* and analogous experiments have been performed for Fun30 (Awad et al., 2010; Adkins et al., 2013, 2017).

Fun30 has the general ability to directly bind DNA *in vitro*. Interestingly, both single-stranded (ss) and double-stranded (ds) DNA as well as nucleosome-associated DNA was bound (Awad et al., 2010; Adkins et al., 2017). In line with these findings, presence of ssDNA, dsDNA, or chromatin stimulated the ATPase activity of Fun30 as was observed for other remodelers (Awad et al., 2010; reviewed in Zhou et al., 2016; Clapier et al., 2017). *In vitro*, nucleosomes were seen to be repositioned in the presence of Fun30 and H2A-H2B dimers were found to be liberated from chromatin templates, suggesting that Fun30 has nucleosome sliding and histone dimer exchange activity (Awad et al., 2010; Byeon et al., 2013). *In vivo*, evidence for a dimer exchange activity of Fun30 is currently lacking, but *fun30Δ* cells showed alterations in the nucleosome-free region at the 5′ end of gene bodies, as well as altered occupancy of −1, +2, and +3 nucleosomes (Byeon et al., 2013). Consistently, also fission yeast Fft3 was found to be required for chromatin architecture (Durand-Dubief et al., 2012). Overall, these data are in good agreement with a role of Fun30 and its orthologs in nucleosome sliding and perhaps positioning, but at this point indirect effects on cellular chromatin architecture cannot be ruled out.

In the context of DNA damage, it is unknown whether Fun30 is involved in nucleosome sliding and/or positioning or whether it plays other roles. While a previous study did not find evidence for Fun30 mediating changes in nucleosome positioning in the proximity of a DSB (Costelloe et al., 2012), it might be technically challenging to visualize such changes during dynamic repair.

A possible role in H2A-H2B dimer exchange may manifest in changes in occupancy of the H2A variant H2A.Z or perhaps also of post-translationally modified forms of H2A (such as γH2A). Distribution of H2A.Z was indeed influenced by Fft3 and Fun30, both genome-wide and particularly in centromeric, pericentromeric, and subtelomeric chromatin (Strålfors et al., 2011; Durand-Dubief et al., 2012). Given that H2A.Z is a well-known regulator of DSB repair (van Attikum et al., 2007; Kalocsay et al., 2009; Xu et al., 2012; Adkins et al., 2013; Lademann et al., 2017), it is tempting to hypothesize that Fun30 regulates H2A.Z at DSBs as well. So far, it remains to be determined whether the changes in H2A.Z distribution induced by Fun30 occur at DSBs and might potentially contribute to resection regulation.

Notably, Fun30 has particular binding preferences when it comes to nucleosome structure and modification, for example it seems to be repelled by S129-phosphorylated H2A (γH2A, induced by DNA damage) (Eapen et al., 2012; Adkins et al., 2017). One could therefore speculate that Fun30 might antagonize γH2A via an H2A/H2B dimer exchange activity. Experimental data however argue against such a model, as no changes in γH2A phosphorylation after DNA damage could be observed in mutants lacking Fun30 (Eapen et al., 2012).

Lastly, during maintenance of heterochromatin/transcriptionally silent chromatin, Fun30/Fft3/SMARCAD1 appear to function as stabilizers of chromatin marks (Durand-Dubief et al., 2012; Byeon et al., 2013; Taneja et al., 2017), but whether this can be explained by sliding/dimer exchange activities or whether this function involves an additional activity is not known.

Overall impressive progress has been made toward understanding the catalytic activities of Fun30$^{SMARCAD1}$, but nonetheless we currently do not understand the specific nature of the substrate toward which the remodeling activity is directed to, nucleosomes or modified nucleosomes are a possibility, but the function in DSB repair (see below) suggests that it might also be nucleosomes in complex with an additional protein(s) or maybe even a nucleosome-bound protein.

## BIOLOGICAL FUNCTIONS OF FUN30$^{SMARCAD1}$

At first glance, the biological functions of Fun30 and its orthologs appear at least as diverse as its biochemical activities (sliding, positioning, dimer exchange). In the following, we therefore aim to not only summarize the known functions, but also to highlight commonalities, since a common model describing Fun30 function would help discriminate direct from indirect consequences of a loss of Fun30 function and facilitate future research.

### Gene Expression Control

Orthologs of Fun30 promote gene expression. Fission yeast Fft3 facilitates the progression of RNA Polymerase II through actively transcribed genes by mediating nucleosome dissociation (Lee et al., 2017). Also, SMARCAD1 was found to act as transcriptional activator and enhances CBP-mediated histone acetylation (Doiguchi et al., 2016). The overall importance of the contribution of Fun30 orthologs to transcription regulation remains however to be elucidated. At least in budding yeast, absence of Fun30 caused only minor changes in the expression of few proteins (Chen et al., 2012), possibly reflecting redundancy with other nucleosome remodelers (Barbaric et al., 2007; Smolle et al., 2012).

### Maintenance of Silent Chromatin

All Fun30 orthologs were shown to localize to heterochromatic or repressed genomic loci and contribute to their establishment and preservation. Fission yeast Fft3 and budding yeast Fun30 localize to insulator elements and are involved in silencing at subtelomeres, centromeres, rDNA repeats, and mating type loci (Neves-Costa et al., 2009; Strålfors et al., 2011; Durand-Dubief et al., 2012; Steglich et al., 2015; Taneja et al., 2017; Jahn et al., 2018). In absence of Fft3, the composition and nuclear localization of heterochromatin is altered and accumulates euchromatic histone modifications such as H4K12Ac and H3K9Ac, as well as histone variants like H2A.Z (Strålfors et al., 2011; Steglich et al., 2015). Fun30 contributes to transcriptional repression of genes and across centromeres in order to ensure unhampered chromosome segregation (Strålfors et al., 2011; Durand-Dubief et al., 2012; Byeon et al., 2013). *In vivo*, Fft3, Fun30, and SMARCAD1 thus seem to ensure maintenance and

inheritance of boundaries between chromatin states by stabilizing nucleosomes and preserving heterochromatic histone marks (Durand-Dubief et al., 2012; Byeon et al., 2013; Taneja et al., 2017; Xiao et al., 2017; Ding et al., 2018; Sachs et al., 2019).

Notably, Fun30 and SMARCAD1 are not only involved in maintenance of heterochromatin or silent chromatin, but are also involved in generating repressed chromatin *de novo*, where an interaction with HDAC1/2 mediating H3/H4 deacetylation might be involved (Okazaki et al., 2008; Rowbotham et al., 2011; Yu et al., 2011).

Inheritance of nucleosomes is crucial for heterochromatin maintenance and therefore is tightly linked to DNA replication (Saredi et al., 2016; Yadav and Whitehouse, 2016; Yang et al., 2016; reviewed in Serra-Cardona and Zhang, 2018). In line with this, it was not only shown that SMARCAD1 is required for heterochromatin maintenance in proliferating cells, but SMARCAD1 was also shown to bind to the replication factor PCNA (Rowbotham et al., 2011) suggesting a possible mechanism for how it could be targeted to sites of DNA replication. Also, the CUE domains of SMARCAD1 are specifically required and could play a role in targeting (Rowbotham et al., 2011; Ding et al., 2018). The first CUE domain of SMARCAD1 binds to KAP1 (Ding et al., 2018), but a universal function of the CUE domains as well as the link between CUE-dependent interactors and PCNA has not been established. A putative role of Fun30$^{SMARCAD1}$ in chromatin inheritance during DNA replication is also interesting, since DNA replication involves formation of ssDNA and nucleosome eviction and therefore features mechanistic similarities to DNA resection, another process where Fun30 is crucially involved in (see below).

## DNA Damage Response and DSB Repair

First connections of Fun30 to the DNA damage response were made by several genetic screens in budding yeast—a screen identifying factors involved in chromosome stability and segregation (Ouspenski et al., 1999), several genetic interaction screens with DNA repair mutants (Krogan et al., 2003, 2006; Collins et al., 2007; Beltrao et al., 2009; Costanzo et al., 2010), a screen for mutants affecting gene targeting (Chen et al., 2012), and a screen for mutants affecting break-induced replication (Costelloe et al., 2012). Fun30 and SMARCAD1 were furthermore connected to the DNA mismatch repair pathway (MMR; Chen Z. et al., 2016; Goellner et al., 2018; Terui et al., 2018) and shown to be required for the resistance to irradiation and camptothecin (CPT) (Neves-Costa et al., 2009; Costelloe et al., 2012; Chakraborty et al., 2018). In 2012, a series of pioneering publications established a key role of Fun30 and SMARCAD1 during the repair of DNA DSBs by homologous recombination (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012). Together with more recent work (Chen X. et al., 2016; Densham et al., 2016; Bantele et al., 2017) these publications convincingly demonstrate a molecular function in promoting DNA end resection, the nucleolytic digestion of dsDNA at DSBs that leads to the formation of 3′ssDNA overhangs.

### Enhancement of DSB Resection

DNA DSBs can be repaired by non-homologous end-joining (NHEJ) or homologous recombination (HR) pathways (Symington and Gautier, 2011). The choice between these two repair regimes depends strongly on the cell cycle state and is determined at the step of DNA end resection, where DSB ends are nucleolytically digested so that 3′ overhangs are formed. These overhangs constitute crucial intermediates of repair by homologous recombination and moreover have a central signaling function at DSBs.

It is reasonable to assume that nucleosomes constitute a barrier to DNA end resection into undamaged chromatin, and indeed chromatinized DNA is resected less efficiently with increasing nucleosome density *in vitro* (Adkins et al., 2013). Notably, two nuclease complexes are mainly responsible for spreading of resection (long range resection) (Zhu et al., 2008; Mimitou and Symington, 2009). These nucleases—Exo1 alone and Dna2 in conjunction with the Sgs1-Top3-Rmi1 complex—bypass nucleosomes with distinct mechanisms, suggesting that they might require different forms of nucleosome remodeling (Adkins et al., 2013). Furthermore, different chromatin states such as heterochromatin might require additional means to promote resection (Baldeyron et al., 2011; Eapen et al., 2012; Soria and Almouzni, 2013; Batté et al., 2017).

Notably, while Fun30 and SMARCAD1 are required for efficient long-range resection through chromatin *in vivo* (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012; Bantele et al., 2017), initial studies could not demonstrate an effect of Fun30 on resection through chromatinized DNA *in vitro*, at least in case of Exo1 (Adkins et al., 2013). Most likely, the *in vitro* system therefore fails to recapitulate the *in vivo* situation. This allows to speculate that the specific substrate of Fun30 remodeling during DNA end resection might have been missing from the *in vitro* reaction.

In this regard, it is interesting to note that genetics have revealed a major function of Fun30 and SMARCAD1 in counteracting a resection inhibitor—Rad9 in yeast, 53BP1 in humans (Chen et al., 2012; Densham et al., 2016; Bantele et al., 2017). The role of Rad9 and 53BP1 as inhibitors of DNA end resection is clearly established. However, it is not clear whether the specific mechanism of resection inhibition is conserved through evolution. Notably, both Rad9 and 53BP1, as well as fission yeast Crb2 are nucleosome binders and appear to recognize several (modified) histones, suggesting multivalency (Huyen et al., 2004; Nakamura et al., 2004; Sanders et al., 2004; Wysocki et al., 2005; Botuyan et al., 2006; Toh et al., 2006; Grenon et al., 2007; Hammet et al., 2007; Fradet-Turcotte et al., 2013; Wilson et al., 2016). Again, the specific nature of binding sites does not appear to be conserved, but the multivalent interaction with nucleosomes is shared by Rad9 orthologs. In the absence of Rad9 or 53BP1, the remodeling activity of Fun30 or SMARCAD1 seems to be at least partly dispensable and phenotypes such as CPT sensitivity are suppressed (Chen et al., 2012; Densham et al., 2016; Bantele et al., 2017). Collectively, these data establish Rad9$^{53BP1}$-bound nucleosomes as excellent candidate substrate for Fun30 activity. Consistently, Rad9$^{53BP1}$ was shown to accumulate around DSBs when Fun30$^{SMARCAD1}$

was absent (Chen et al., 2012; Costelloe et al., 2012; Densham et al., 2016). In both yeast and human cells, the ATPase activity of the remodeler is required to facilitate resection, implying active remodeling as part of the resection-promoting mechanism (Bantele et al., 2017; Chakraborty et al., 2018). One can therefore conclude that the opposition to Rad9$^{53BP1}$ is a central task of Fun30$^{SMARCAD1}$-dependent remodeling.

The molecular nature of the Rad9$^{53BP1}$-Fun30$^{SMARCAD1}$ antagonism is currently elusive. **Figure 2** highlights plausible models for the yeast proteins, where Rad9 could act as specific inhibitor of resection, directly or indirectly inhibiting the resection nucleases via chromatin, for example by inhibiting Fun30 (**Figures 2A,B**). Conversely, Fun30 might overcome resection-inhibition by Rad9 and in this instance either directly remove Rad9 from chromatin, block its association in an indirect manner or counteract its downstream effects (**Figures 2C,D**). Since Rad9 is a nucleosome-binder, an indirect effect on Rad9 association could depend on the histone marks recognized by Rad9. For example, removal of the γH2A phosphorylation mark would lead to a defect in Rad9 chromatin association (Javaheri et al., 2006; Hammet et al., 2007; Eapen et al., 2012; Clerici et al., 2014). Such a model would be consistent with the described H2A-H2B dimer exchange ability of Fun30 (Awad et al., 2010), but would be inconsistent with experimental data, where cells lacking the γH2A modification still partially require Fun30 for efficient resection (Eapen et al., 2012). We anticipate that biochemical reconstitution will identify the remodeling substrate of Fun30$^{SMARCAD1}$ and allow to reveal the mechanism by which Rad9$^{53BP1}$ and Fun30$^{SMARCAD1}$ antagonize each other.

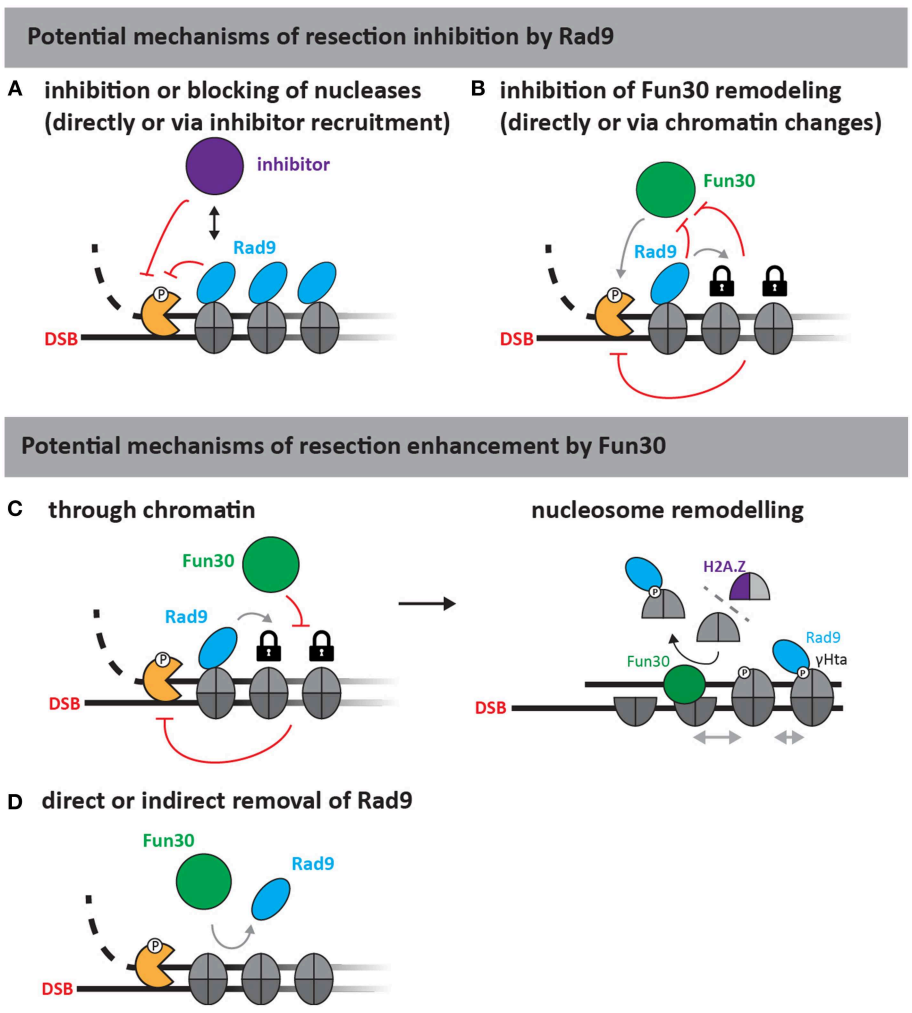


**FIGURE 2 |** Putative mechanisms of resection regulation by Fun30 and Rad9 (adapted from Bantele, 2018). As Rad9 is a chromatin-binding protein without apparent catalytic activity, at least two mechanisms of resection inhibition can be envisioned (upper part). First, Rad9 could directly block or slow down the progression of nucleases either by inhibiting the nucleases **(A)** or by stabilizing chromatin in a configuration that is non-permissive to resection **(B)** for example by inhibiting Fun30, if the latter was required to help overcome resection-inhibition by nucleosomes. Fun30 could also promote resection by several different mechanisms (lower part). As a nucleosome remodeler, Fun30 could either act through chromatin **(C)**, or by removing Rad9 from chromatin **(D)**. The action through chromatin could involve its putative remodeling activities and potentially H2A/H2B dimer exchange, which might affect γH2A and H2A.Z dynamics or repositioning of nucleosomes by nucleosome sliding (**C**, right side).

It is also interesting to note that several of the binding partners of Fun30 are shared by its antagonist Rad9. These include histones, but also the BRCT repeat protein Dpb11 (Javaheri et al., 2006; Hammet et al., 2007; Pfander and Diffley, 2011; Bantele et al., 2017). Notably, in case of Dpb11, Fun30 and Rad9 even share the binding site, suggesting direct competition (Bantele et al., 2017). However, while competition might contribute to the antagonistic relationship, it is certainly not the exclusive source of this antagonism, as resection depends on the catalytic activity of Fun30 even in a context of a Fun30-Dpb11 fusion protein (Bantele et al., 2017).

Also in human cells, SMARCAD1 antagonizes 53BP1, as depletion of SMARCAD1 leads to a stabilization of 53BP1 at DSBs (Densham et al., 2016). Compared to the budding yeast system, the situation in human cells appears to be more complex. First, human cells have a second, well-established pro-resection factor and 53BP1 antagonist—BRCA1 (Cao et al., 2009; Bothmer et al., 2010; Bouwman et al., 2010; Bunting et al., 2010, 2012; Escribano-Díaz et al., 2013). BRCA1 forms a ubiquitin ligase complex together with BARD1 and BRCA1-BARD1 were shown to mediate ubiquitylation of histone H2A (Kalb et al., 2014; Densham et al., 2016). Notably, this might be a point where the two pro-resection pathways converge, since ubiquitylated H2A appears to stabilize SMARCAD1 at the DSB site, likely via direct CUE domain-dependent binding of SMARCAD1 to ubiquitylated H2A (Densham et al., 2016). A second layer of complexity comes in the form of 53BP1 effectors, such as RIF1, REV3, and the Shieldin complex (Xu et al., 2015; Dev et al., 2018; Findlay et al., 2018; Gupta et al., 2018; Mirman et al., 2018; Noordermeer et al., 2018). These effectors may inhibit resection by changing PTMs on damaged chromatin (RIF1 is a PP1 phosphatase-associated factor) or even promoting fill-in DNA synthesis (Hiraga et al., 2014; Mirman et al., 2018; Bhowmick et al., 2019; Garzón et al., 2019). Similar mechanisms have not yet been described in yeast and might represent metazoan-specific additions to an evolutionary conserved chromatin-dependent control of DNA end resection.

### Cell Cycle Control and DSB Repair Pathway Choice

DSB repair by homologous recombination is coupled to the presence of a sister chromatid and therefore DSB repair pathway choice is cell cycle-regulated. This cell cycle-regulation impinges on the control of resection by Fun30, as Fun30 is phosphorylated by CDK (Chen X. et al., 2016; Bantele et al., 2017). Mechanistically, CDK-phosphorylation generates a binding site for the scaffold protein Dpb11, which in turn binds to the 9-1-1 checkpoint clamp thus leading to a ternary complex between Fun30, Dpb11, and 9-1-1 (**Figure 1**; Bantele et al., 2017). Formation of this complex mediates targeting to and likely activation of Fun30 at sites of DNA end resection (Bantele et al., 2017). A similar mechanism is likely occurring also in mammalian cells as SMARCAD1 can be phosphorylated by CDK as well, leading to interaction with the Dpb11 ortholog TOPBP1 (Bantele et al., 2017).

Budding yeast cells arrested in M phase show DNA end resection of DSBs that strongly depends on Fun30 and the Fun30 targeting complex (Bantele et al., 2017). Nonetheless, additional factors are clearly involved in the cell cycle control of DNA end resection. Overall, these findings raise the question of which specific DNA end resection pathway and pathway decision Fun30 is actually involved in. In this regard it has been shown that repair by homologous recombination requires resection of only a few 100 base pairs (Jinks-Robertson et al., 1993; Ira and Haber, 2002; Zhu et al., 2008), while for alternative recombination pathways and repair by single-strand annealing (SSA) in particular longer stretches of resected DNA are required (Zhu et al., 2008). One can therefore reason that the switch between NHEJ and HR is already done once resection initiates and that activation of long-range resection by Fun30 would rather further shift repair to an SSA-type mechanism. Indeed, a mild decrease of DNA end resection efficiency for example in an *exo1*Δ or *fun30*Δ mutant strain seems beneficial for HR efficiency (Lee et al., 2016), while it impedes SSA repair (Chen et al., 2012; Eapen et al., 2012; Bantele et al., 2017). Already now, one can however conclude that the Fun30-Rad9 switch and its effect on the DSB surrounding chromatin adds a further layer to DSB repair pathway choice and its changing nature during the cell cycle. It will be exciting to explore, in how far genetic tools such as a fusion of Fun30 to the 9-1-1 complex (Bantele et al., 2017) can be used to bypass these controls and whether they can be utilized for HR-dependent genome editing reactions.

## Role During Chromatin Disruption and Regulation by DNA Clamps

Past research has given us very valuable insights into the individual functions of Fun30$^{SMARCAD1}$, but is there commonality or can we link them to a specific enzymatic activity? The most obvious commonality at least between the function during DNA end resection and the function in the maintenance of silent chromatin regions during DNA replication is that both DNA end resection and DNA replication involve the formation of ssDNA and eviction of nucleosomes. While neither Fun30 nor any of its orthologs have been directly tested for an "evictase" function, the presence of Fun30 did not overcome the barrier function of nucleosomes toward the Exo1 exonuclease, arguing that Fun30 is not an "evictase" (Adkins et al., 2013). Moreover, the function of Fun30$^{SMARCAD1}$ at DSBs and replication forks seems generally discordant, since during DNA end resection and most likely also transcription Fun30$^{SMARCAD1}$ seems to open-up chromatin, while during replication it rather seems to be involved in stabilizing nucleosomes (Rowbotham et al., 2011; Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012; Lee et al., 2018).

Nonetheless, there is more commonality—in particular seen in the regulation of Fun30$^{SMARCAD1}$ by DNA clamps. During resection, Fun30 acts in complex with the 9-1-1 clamp (connected by the Dpb11 bridge) and this complex is likely conserved in humans as well (Takeishi et al., 2010; Ohashi et al., 2014; Bantele et al., 2017). Strikingly, SMARCAD1 was also found to bind to PCNA (Rowbotham et al., 2011), a processivity factor during DNA replication and key platform for protein recruitment at replication forks (Moldovan et al., 2007). While this similarity is striking, the connection to PCNA and 9-1-1 can in fact rather

offer an explanation for the discrepant roles of Fun30$^{SMARCAD1}$ during replication and DSB resection. PCNA and 9-1-1 were shown to be loaded onto double-stranded DNA in *in vitro* experiments and our unpublished data suggest that the same is true *in vivo* (Gomes and Burgers, 2001; Gomes et al., 2001; Majka et al., 2006; reviewed in Majka and Burgers, 2004; Peritore and Pfander, unpublished). Interestingly, the DNA clamps are located at very different positions, if one compares a replication fork to sites of DSB resection. At sites of resection, dsDNA is present upstream of the ss-dsDNA junction. The 9-1-1 complex and associated factors are therefore loaded "in front" of the resecting nucleases (see **Figure 1**). By associating with the 9-1-1 complex, Fun30$^{SMARCAD1}$ is therefore in an ideal position to remove potential obstacles ahead of the resecting nucleases. PCNA in turn is loaded at the primer-template junction and as such will travel behind the replicative polymerase and helicase (Moldovan et al., 2007). As such, binding to PCNA will allow SMARCAD1 not only to associate with the replisome, but exactly to the place where chromatin is restored (Rowbotham et al., 2011). Thus, while similar mechanisms are used to control Fun30$^{SMARCAD1}$ in different processes, the combination of localization and activity leads to different or discordant outcomes.

## CONCLUDING REMARKS AND FUTURE RESEARCH

Fun30 has a key role in promoting DNA end resection and is differentially regulated at different cell cycle stages. Such regulation appears to be of high importance for the maintenance of genomic integrity, and accordingly deregulation of the human ortholog of Fun30, SMARCAD1, was found to play a crucial role during the progression of triple-negative breast cancer, which is specifically characterized by an HR-defect (Kubaisy et al., 2016; Arafat et al., 2018).

It will therefore be highly exciting for future research to further unravel the different functions and molecular mechanisms that Fun30$^{SMARCAD1}$ employ to promote genome integrity. Notably, Fun30 is involved in several aspects of DNA metabolism. The major task here will be to elucidate commonalities and differences between the underlying mechanisms in order to achieve an overarching understanding of Fun30 remodeling activity.

## AUTHOR CONTRIBUTIONS

BP and SB wrote the review.

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# ALTernative Functions for Human FANCM at Telomeres

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The human FANCM ATPase/translocase is involved in various cellular pathways including DNA damage repair, replication fork remodeling and R-loop resolution. Recently, reports from three independent laboratories have disclosed a previously unappreciated role for FANCM in telomerase-negative human cancer cells that maintain their telomeres through the Alternative Lengthening of Telomeres (ALT) pathway. In ALT cells, FANCM limits telomeric replication stress and damage, and, in turn, ALT activity by suppressing accumulation of telomeric R-loops and by regulating the action of the BLM helicase. As a consequence, FANCM inactivation leads to exaggerated ALT activity and ultimately cell death. The studies reviewed here not only unveil a novel function for human FANCM, but also point to this enzyme as a promising target for anti-ALT cancer therapy.

**Keywords: FANCM, telomeres, ALT, R-loops, TERRA, BLM helicase**

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## FANCM

Human FANConi anemia, complementation group M (FANCM) is a highly conserved protein with ATPase and DNA translocase activity, belonging to the Fanconi anemia (FA) core complex (Meetei et al., 2005). FA is a hereditary disorder characterized by bone marrow failure, hypersensitivity to agents inducing DNA interstrand crosslinks (ICLs), chromosomal abnormalities and, later in life, cancer. Although FANCM is part of the FA complex, FANCM mutations are not causative of FA (Singh et al., 2009; Bogliolo et al., 2018; Catucci et al., 2018). Nonetheless, some *FANCM* mutations are associated with higher risk for breast and ovarian carcinomas; hence, this enzyme can be considered a tumor-suppressor (Catucci et al., 2018; Nurmi et al., 2019; Schubert et al., 2019).

Seven independent domains with separable functions have been identified in FANCM so far (**Figure 1**): (i) the N-terminal PIP-box (aa 5-12), which interacts with proliferating cell nuclear antigen (PCNA) (Rohleder et al., 2016); (ii) the DEAD/DEAH-motif (aa 77-590), with ATPase activity (Meetei et al., 2005) (iii) the MID-motif (aa 661-800), which interacts with the Major Histone Fold 1 and 2 (MHF1/2) heterotetramer (Yan et al., 2010); (iv) the MM1-motif (aa 826-967), which interacts with FANCF within the FA core complex (Deans and West, 2009); (v) the MM2-motif (aa 1218-1251), which interacts with RecQ-Mediated genome Instability protein 1 (RMI1), a component of the so-called BTR complex together with Bloom (BLM) and Topoisomerase IIIA (TOP3A) (Deans and West, 2009; Hoadley et al., 2012); (vi) the ERCC4-motif (aa 1818-1956), which is required for FANCM heterodimerization with its obligatory partner Fanconi Anemia core complex-Associated Protein 24 (FAAP24) (Ciccia et al., 2007); and (vii) the C-terminal HhH domain (aa 1971-2030), which equips FANCM with DNA binding activity (Coulthard et al., 2013; Yang et al., 2013). FANCM also comprises the MM3 domain (aa 1502-1708; **Figure 1**) of still unknown function (Deans and West, 2009).

FANCM association with the FA complex promptly suggested a role in the repair of ICL lesions (Meetei et al., 2005). Indeed, when a replication fork encounters an ICL, the FANCM-FAAP24-MHF1-MHF2 complex enhances the recruitment of the FA complex through interaction between FANCM MM1 and FANCF (Deans and West, 2009).

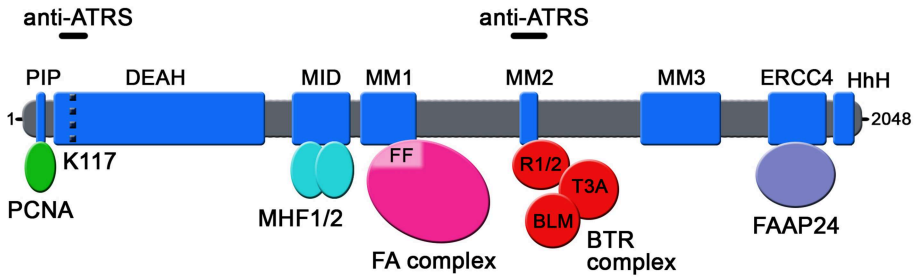


**FIGURE 1 |** Schematic representation of the domains so far identified in human FANCM protein. The position of lysine 117 (K117) within the ATPase pocket is indicated by a dotted black line. FF: FANCF; R1/2: RMI1 and RMI2; T3A: TOP3A; anti-ATRS: regions identified as necessary for FANCM function in suppressing ALT-associated telomeric replication stress.

This stimulates the monoubiquitination of FANCD2, another FA component, an essential event for ICL disengagement and DNA damage repair (Meetei et al., 2005; Mosedale et al., 2005; Yamamoto et al., 2011; Klein Douwel et al., 2014). However, in absence of FANCM, the FA complex still monoubiquitinates FANCD2, albeit less efficiently, and triggers repair (Bakker et al., 2009; Singh et al., 2009). This might explain why mutations in the *FANCM* gene are not causative of FA. FANCM also allows remodeling of arrested replication forks and traversing of ICL lesions independently of the FA complex (Huang et al., 2013). This requires FANCM ATPase activity and the interaction of the PIP-motif with PCNA (Huang et al., 2013; Rohleder et al., 2016).

FANCM promotes resolution of genome-wide spread R-loops (Schwab et al., 2015). The replication machinery might stall upon encountering R-loops and a lack of timely resolution of these structures can lead to genome instability (Crossley et al., 2019). In FANCM-deficient cells, R-loops accumulate across the genome and recombinant FANCM unwinds RNA:DNA hybrids in the presence of FAAP24 and ATP (Schwab et al., 2015). An ATPase-inactive mutant of FANCM (FANCM K117R) fails to suppress RNA:DNA hybrids both *in vitro* and *in vivo*, highlighting the importance of FANCM enzymatic activity in resolving R-loops (Schwab et al., 2015; Silva et al., 2019). FANCM involvement in R-loop metabolism appears to be evolutionarily conserved since budding yeasts deficient for the FANCM ortholog Mph1 accumulate genomic R-loops (Lafuente-Barquero et al., 2017). Notably, the FA components FANCD2, FANCA and FANCL also suppress R-loops in human and murine cells (Garcia-Rubio et al., 2015; Schwab et al., 2015). However, since FANCM ATPase activity is dispensable for FA complex function (Xue et al., 2008), FANCM and the other FA factors are likely to avert R-loops through separate mechanisms. FANCM ATPase activity also supports full activation of the ATR checkpoint cascade and common fragile site stability (Collis et al., 2008; Schwab et al., 2010; Wang et al., 2018).

FANCM interaction with RMI1 through its MM2-motif facilitates recruitment of the BTR complex to DNA lesions (Deans and West, 2009). Consistently, FANCM is required for the formation of BLM foci upon treatment with mitomycin C and camptopthecin (Deans and West, 2009). The BTR complex, also named "dissolvasome," promotes Holliday Junction branch migration and the dissolution of recombination intermediates that could lead to harmful sister chromatid exchange (SCE) events (Karow et al., 2000; Wu and Hickson, 2003). As a consequence, FANCM depletion in human cells causes SCE accumulation, a feature shared with BLM-deficient cells (Neff et al., 1999; Deans and West, 2009).

## ALTERNATIVE LENGTHENING OF TELOMERES

The ends of linear eukaryotic chromosomes, the telomeres, are actively transcribed heterochromatic nucleoprotein structures comprising repetitive DNA sequences (5′-TTAGGG-3′/5′-CCCTAA-3′ in vertebrates), shelterin proteins and the long noncoding RNA TERRA (Azzalin and Lingner, 2015; Shay and Wright, 2019). The inability of canonical DNA polymerases to fully replicate linear DNA molecules at each round of cell division causes progressive telomere shortening, which cannot be buffered in cells lacking mechanisms of *de novo* synthesis of telomeric DNA (Shay and Wright, 2019). Upon extensive shortening, critically short telomeres accumulate in cells and emanate an irreversible DNA damage signal causing permanent growth arrest and eventually cell death (Harley et al., 1990; Nassour et al., 2019). To gain unlimited replicative potential, 85–90% of human cancer cells reactivate the reverse transcriptase telomerase, which utilizes an associated RNA moiety to produce telomeric DNA (Kim et al., 1994; Shay and Bacchetti, 1997). The remaining 10–15% of human cancers elongate telomeres trough homology-directed repair (HDR) pathways collectively known as Alternative Lengthening of Telomeres or ALT (Apte and Cooper, 2017). ALT can thus be considered a specialized DNA repair mechanism assuring cell immortality. ALT was first described in budding yeast survivors arising after crisis induced by telomerase inactivation (Lundblad and Blackburn, 1993). Few years later, ALT was reported in human cells (Bryan et al., 1995, 1997; Dunham et al., 2000). Human ALT cancers are generally of mesenchymal or epithelial origin, and comprise among others some osteosarcomas, liposarcomas, glioblastomas and astrocytomas.

Besides being immortal and telomerase-negative, a number of features characterize ALT cells, including abundant extrachromosomal double-stranded (ds) or single-stranded (ss) telomeric DNA in circular or linear form (Ogino et al., 1998; Tokutake et al., 1998; Cesare and Griffith, 2004; Wang et al., 2004), and specialized nuclear bodies referred to as ALT-associated PML bodies (APBs). APBs contain ProMyelocytic Leukemia (PML), telomeric DNA, TERRA, shelterin components including TRF1 and TRF2, and DNA damage signaling and repair factors including RPA, RAD51, RAD52, BRCA1, and BLM and WRN helicases (Yeager et al., 1999; Johnson et al., 2001; Stavropoulos et al., 2002; Acharya et al., 2014; Arora et al., 2014; Pan et al., 2017). ALT cells are also characterized by elevated rates of exchange of DNA between sister telomeres (T-SCE) and increased transcription of TERRA, likely due to TERRA promoter hypomethylation (Bailey et al., 2004; Lovejoy et al., 2012; Arora et al., 2014). Finally, inactivation of one or both of the ATP-dependent chromatin remodelers Alpha-Thalassemia/mental Retardation X-linked (ATRX) and Death domain-Associated protein-6 (DAXX) are often found in ALT tumors (Heaphy et al., 2011; Lovejoy et al., 2012; Schwartzentruber et al., 2012). ATRX and DAXX form a complex that deposits the histone variant H3.3 at heterochromatic loci, including telomeres. Lack of ATRX and/or DAXX activity may explain the altered chromatin state of ALT telomeres, and possibly the deregulation in TERRA transcription and T-SCEs (Episkopou et al., 2014; Dyer et al., 2017). A recent report revealed that ALT telomeres are enriched in the heterochromatin mark H3 trimethylated at lysine 9 (H3K9me3), deposited by the histone methyltransferase SET Domain Bifurcated 1 (SETDB1). The same report proposed that H3K9me3 stimulates APB formation, telomeric recombination and TERRA transcription (Gauchier et al., 2019). Further studies are thus necessary to fully understand the intricate interplay between heterochromatin deposition and ALT establishment and/or maintenance.

ALT HDR occurs through Break-Induced Replication (BIR) in the G2/M phase of the cell cycle. BIR is a conservative DNA synthesis-based repair pathway engaging at one-ended DNA double-strand breaks (DSBs) and arrested replication forks (Kramara et al., 2018). Two types of BIR, either RAD51- or RAD52-dependent, were firstly identified in ALT yeasts (Le et al., 1999; Chen et al., 2001). In human ALT cells, BIR does not require RAD51 while it depends on RAD52 and on the DNA polymerase δ accessory subunits POLD3 and POLD4, and on PCNA (Roumelioti et al., 2016; Zhang et al., 2019). ALT dependence on telomeric HDR implies that at least a subset of telomeres is maintained physiologically damaged. This sustained damage is replication-dependent, explaining the constitutive association of replication stress-associated factors, such as RPA and its phosphorylation-modified versions, with ALT telomeres (Arora et al., 2014; Pan et al., 2017). Although the triggers of this ALT-specific Telomeric Replication Stress (herein referred to as ATRS) have not been fully elucidated, a variety of hypotheses can be envisaged. Telomeres are difficult to replicate regions because of the repetitive nature of their sequence, the tight association of telomeric DNA with heterochromatin marks and telomeric proteins, and their richness in higher order structures including T-loops, generated upon intramolecular invasion of the 3′ end ss tail of a telomere into its ds part, and telomeric R-loops (telR-loops), formed by annealing of TERRA with the C-rich strand of the telomere (Sfeir et al., 2009; Balk et al., 2013; Pfeiffer et al., 2013; Arora et al., 2014). Additionally, G-quadruplexes may form when the G-rich telomeric strand exists in ss state, for example at the displacement loop of a T-loop or a telR-loop (Tarsounas and Tijsterman, 2013). Improper handling of any of those features could contribute to ATRS.

Because replication stress impairs cell proliferation through activation of DNA damage checkpoints, alleviators of ATRS are constantly active in ALT cells. The endoribonuclease RNaseH1 associates with ALT telomeres, where it degrades the RNA moiety of telR-loops. Short interference RNA (siRNA)-mediated depletion of RNaseH1 in ALT cells increases telR-loops, ATRS and circular telomeric molecules comprising ss C-rich DNA (C-circles), and ultimately causes rapid loss of entire telomeric tracts (Arora et al., 2014). The ATP-dependent DNA annealing helicase SWI/SNF-related, Matrix-associated, Actin-dependent Regulator of Chromatin, subfamily A-Like 1 (SMARCAL1), which restarts arrested replication forks through fork regression, is enriched at ALT telomeres, and its depletion using siRNAs augments ATRS, telomeric DNA damage and ALT features including C-circles (Cox et al., 2016). The checkpoint kinase Ataxia Telangiectasia and Rad3-Related (ATR) is also found at ALT telomeres and its inactivation using siRNAs or small molecule inhibitors increases ATRS and leads to cell death specifically in ALT cells, although this last notion has been questioned (Flynn et al., 2015; Deeg et al., 2016).

## FANCM AND ALT

FANCM involvement in ALT was first reported by the Zhang laboratory in 2017 (Pan et al., 2017). The authors showed that FANCM and FAAP24 localize to telomeres in a variety of ALT cell lines. SiRNA-mediated depletion of FANCM, FAAP24, MHF1, or MHF2 induced ATRS in ALT cells, as demonstrated by the telomeric localization of phosphorylated RPA and the checkpoint kinase CHK1 (Pan et al., 2017). Single Molecule Analysis of Replicated DNA (SMARD) using telomeric DNA from FANCM-depleted ALT cells revealed diminished replication efficiencies, while replication genome-wide was only minimally affected (Pan et al., 2017). Overall those data indicate that in absence of FANCM the replication machinery fails to fully replicate telomeric DNA, thus leading to ATRS. FANCM depletion was also shown to cause accumulation of BLM and BRCA1 at ALT telomeres, and simultaneous depletion of either of those factors together with FANCM partly averted ATRS (Pan et al., 2017). The authors proposed that ATRS induced by FANCM depletion promotes recruitment of BLM and BRCA1 to telomeres, where the two factors enhance end resection in order to restart arrested forks and repair telomeric DNA through HDR. Apparently consistent with this model, co-depletions of FANCM with BLM or BRCA1 were shown to be synthetically lethal, specifically in ALT cells (Pan et al., 2017).

Two recent reports, from the Pickett laboratory and ours, have deepened our understanding of how FANCM functions at ALT telomeres. Both reports confirmed that FANCM depletion in ALT cells causes ATRS and telomeric DNA damage. Accumulation of phosphorylated RPA, ssDNA, and the DNA damage marks γH2AX and 53BP1 was observed at telomeres in cells depleted of FANCM using independent siRNAs (Lu et al., 2019; Silva et al., 2019). The two reports also established that FANCM suppresses ALT activity, likely as a consequence of ATRS alleviation. Augmented ALT features, including telomere clustering in large APBs, C-circle production and POLD3-mediated telomeric BIR in G2, were observed in FANCM-depleted ALT cells. Conversely, ALT features were not detected in telomerase-positive cells depleted of FANCM, indicating that FANCM inhibition does not cause ALT but rather FANCM has acquired specific telomeric functions in cells with already established ALT activity (Lu et al., 2019; Silva et al., 2019). Moreover, both studies revealed that FANCM inhibition alone is extremely toxic for ALT cells, as it leads to rapid arrest of cell cycle progression in G2/M phase followed by cell death (Lu et al., 2019; Silva et al., 2019). FANCM essentiality for ALT cell viability was further confirmed by interrogating publicly available catalogs of CRISPR/Cas9 gene knock-outs across cancer cell lines (Lu et al., 2019). These observations are in contrast with previous work from Pan and colleagues (Pan et al., 2017), who showed that FANCM depletion alone is not detrimental in ALT cells. It is likely that different siRNA efficiencies and experimental set ups for cell proliferation analysis account for those discrepancies.

Mechanistic insights from those two recent reports highlight the complexity of the mechanisms orchestrated by FANCM in ALT cells. We showed that telR-loops accumulate when FANCM is depleted, and orthogonal resolution of telR-loops through over-expression of RNaseH1 alleviates FANCM depletion-induced ATRS. FANCM likely restricts telR-loops directly, because FANCM can unwind telR-loops *in vitro* in an ATP-dependent manner and the K117R mutant fails to avert ATRS in FANCM-depleted cells (Silva et al., 2019). Moreover, we confirmed that BLM depletion alleviates ATRS in FANCM-depleted cells, and showed that combined RNaseH1 over-expression and BLM depletion fully eliminates ATRS induced by FANCM depletion (Silva et al., 2019). We thus proposed that deregulated telR-loops and BLM are the main triggers of ATRS, consequent ALT exacerbation and cell death when FANCM activity is inhibited. It remains possible that, besides R-loops resolution, other functions associated with the ATPase activity of FANCM (Collis et al., 2008; Schwab et al., 2010; Huang et al., 2013; Wang et al., 2018) could help support telomere stability and viability in ALT cells.

On the other side, the report by Lu and colleagues focused on the importance of the interaction between FANCM and the BTR complex. They showed that over-expression of FANCM suppresses ALT features including damaged telomeres and C-circles. Conversely, over-expression of two mutant versions of FANCM unable to bind the BTR complex did not suppress those features (Lu et al., 2019). Consistent with our results, also the K117R mutant failed to suppress ALT, while a mutant unable to interact with the FA core complex behaved as wild-type FANCM when over-expressed (Lu et al., 2019). Altogether, this set of data confirms the importance of the enzymatic activity of FANCM in suppressing ALT, reveals the relevance of the interaction between FANCM and the BTR complex, and excludes that FANCM suppresses ALT as a member of

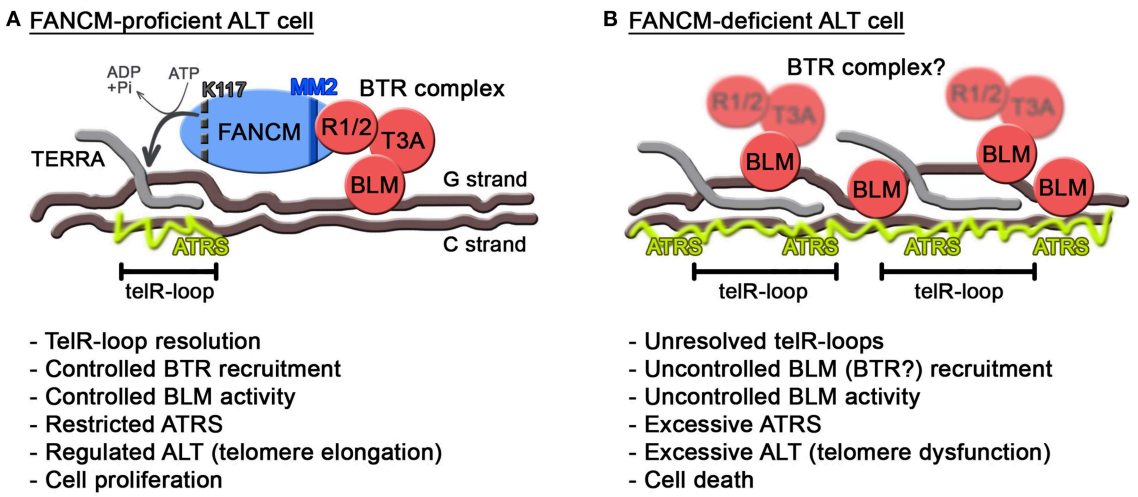


**FIGURE 2 |** Model for FANCM function at ALT telomeres. **(A)** In FANCM-proficient ALT cells, FANCM association with telomeric chromatin assures unwinding of harmful telR-loops through its ATPase activity. Additionally, FANCM interaction with RMI1/2 assures regulated recruitment and activity of BLM. In this situation, ATRS is maintained below toxic levels allowing telomere elongation and infinite cell proliferation. In FANCM, lysine K117 and the MM2 motif are indicated by a dotted black line and a blue line, respectively. **(B)** In FANCM-deficient ALT cells, telR-loops accumulate and BLM is aberrantly recruited and activated, leading to excessive ATRS and eventually cell death. RMI1/2 and TOP3A are blurred to indicate that their recruitment to telomeres has not been tested yet in FANCM-deficient cells.

the FA core complex. Moreover, the Pickett group utilized two independent approaches to prevent the interaction between FANCM and the BTR complex in cells: ectopic expression of a 28 aa long peptide from the MM2 sequence of FANCM or treatment with the small molecule inhibitor PIP-199. In both cases, FANCM-BTR interaction interference caused telomeric DNA damage and loss of cell viability specifically in ALT cells (Lu et al., 2019).

Collectively, the recent reports on FANCM in ALT established that FANCM is an alleviator of ATRS and unveiled two main co-players, telR-loops and BLM. TelR-loops are abundant at ALT telomeres and are kept in check by dedicated machineries including RNaseH1 and FANCM. Inactivation of such machineries induces telR-loop stabilization and ATRS (Arora et al., 2014; Silva et al., 2019). Although strongly suggesting that telR-loops are main triggers of ATRS, this evidence remains correlative, as systems to modulate TERRA transcription in cells are not available. It is now necessary to develop such systems and test the involvement of TERRA in telR-loop formation and ATRS. Moreover, while it is clear that RNaseH1 activity negatively regulates ATRS (Arora et al., 2014), the impact of its depletion or over-expression on the ALT mechanisms has not been fully tested. Analysis of ALT features including APBs and BIR in cells with altered RNaseH1 activity will help address this point.

As for BLM, this helicase seems to have intimate yet intricate connections with ATRS, in particular in the context of FANCM deficiency. While on one side decreasing BLM levels alleviates ATRS when FANCM is depleted (Pan et al., 2017; Silva et al., 2019), replacement of endogenous FANCM with a mutant unable to associate with the BTR complex exacerbates ATRS and ALT (Lu et al., 2019). Moreover, depletion of FANCM provokes BLM accumulation at ALT telomeres (Pan et al., 2017; Silva et al., 2019). Those apparently contradictory data can be reconciled by postulating independent activities for BLM. We propose that BLM supports regulated and productive ALT activity as long as it is properly controlled, possibly as a member of the BTR complex (**Figure 2A**). Consistently, depletion of any of the BTR members suppresses ALT features (Sobinoff and Pickett, 2017). Proper regulation of BLM at ALT telomeres would therefore depend on the BTR interaction with FANCM MM2 domain (**Figure 2A**). In the absence of this regulation, for example when FANCM is depleted or is replaced by a BTR interaction mutant, BLM could be recruited to telomeres through FANCM-independent routes and become hyperactive and therefore toxic (**Figure 2B**). It will be interesting to test whether RMI1 and TOP3A are also recruited to telomeres in FANCM-depleted cells and whether BLM localization at FANCM-depleted telomeres depends on the BTR complex.

Lastly, FANCM seems to be an optimal target for anti-ALT cancer therapies because it is a non-essential factor in normal and telomerase-positive cells. SiRNA-mediated depletion of FANCM in a large panel of non-ALT cells does not lead to cell cycle arrest or death (Lu et al., 2019; Silva et al., 2019). Telomerase-positive human colorectal carcinoma HCT116 cells, mouse embryonic fibroblasts and chicken DT40 cells knocked-out for FANCM proliferate normally unless challenged with DNA damaging agents (Mosedale et al., 2005; Bakker et al., 2009; Huang et al., 2013). Individuals with FANCM mutations reach adulthood without major complications (Meetei et al., 2005; Catucci et al., 2018). Targeting FANCM, more specifically its enzymatic activity or its interaction with the BTR complex, holds the potential for a successful treatment of ALT cancers. One possible caveat comes from the observations that human FANCM mutants might develop cancer, likely telomerase-positive, late in life (Catucci et al., 2018; Nurmi et al., 2019; Schubert et al., 2019). Nevertheless, considering the fast and dramatic effects of FANCM depletion on ALT cells, we anticipate that transient FANCM inhibition should be sufficient to extirpate ALT in absence of secondary effects on patients.

## AUTHOR CONTRIBUTIONS

BD-S, BS, and CA wrote, revised, and edited the manuscript.

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# DNA Double Strand Breaks and Chromosomal Translocations Induced by DNA Topoisomerase II

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DNA double strand breaks (DSBs) are the most cytotoxic lesions of those occurring in the DNA and can lead to cell death or result in genome mutagenesis and chromosomal translocations. Although most of these rearrangements have detrimental effects for cellular survival, single events can provide clonal advantage and result in abnormal cellular proliferation and cancer. The origin and the environment of the DNA break or the repair pathway are key factors that influence the frequency at which these events appear. However, the molecular mechanisms that underlie the formation of chromosomal translocations remain unclear. DNA topoisomerases are essential enzymes present in all cellular organisms with critical roles in DNA metabolism and that have been linked to the formation of deleterious DSBs for a long time. DSBs induced by the abortive activity of DNA topoisomerase II (TOP2) are "trending topic" because of their possible role in genome instability and oncogenesis. Furthermore, transcription associated TOP2 activity appears to be one of the most determining causes behind the formation of chromosomal translocations. In this review, the origin of recombinogenic TOP2 breaks and the determinants behind their tendency to translocate will be summarized.

**Keywords: DSB repair, DNA topoisomerase II, chromosomal translocations, genome instability, transcription**

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## INTRODUCTION

Chromosomal translocations are rearrangements of large fragments of DNA. When transcribed regions are affected, genome translocations usually result in the inactivation of one or a group of genes with the consequent deleterious effects for cellular survival. However, on occasion, translocations can generate chimeric proteins or deregulate transcription programmes creating abnormal growth capacities and contributing to malignancy and tumor development. Over 10,000 gene fusions have been found in cancer, most of which are considered passenger mutations, consequence of the intrinsic instability of tumor progression. Among them, more than 300 are recurrent and contribute to initial stages of cellular disarray (Mitelman et al., 2007; Mertens et al., 2015).

Recurrence of chromosomal translocations is determined by a large number of factors, starting from the nature of the DNA break and including the pathway involved in its repair, the cell cycle stage, the chromatin status of the locus, and the genomic location of the lesion. Since most of these factors are dynamic and interconnected, their relative relevance is difficult to establish, and many aspects of the origin of genomic translocations remain unclear. Recent studies have unveiled that transcription and 3D organization of the genome are two major determinants in the appearance

of DNA double-stranded breaks (DSBs) and they promote chromosomal translocations. In this brief review how DNA topoisomerase II (TOP2) appears at the crossroad of these factors will be discussed.

## DNA TRANSACTIONS AND TOP2 ACTIVITY

DNA topoisomerases are essential enzymes present in all cellular organisms with critical roles in DNA metabolism. DNA topoisomerases release the torsional stress generated in the DNA by a wide variety of processes including replication, transcription, 3D genome organization, and chromosome segregation (Pommier et al., 2016). According to their mechanism of action, DNA topoisomerases are classified in two types depending on whether they cut one (type I) or two strands (type II) of the DNA double helix. TOP2 is a type II enzyme that can pass an intact DNA duplex through a broken one while covalently bound to the DNA. Once strand passage is completed, the enzyme reseals the break (Nitiss, 2009a). Vertebrates express two TOP2 isoforms, TOP2α and TOP2ß. While TOP2ß is expressed throughout the cell cycle, TOP2α levels correlate with cellular proliferation and peak at S and G2/M phases. TOP2α has a major role in replication and chromosome segregation. TOP2ß activity has been mainly associated to transcription. It participates in: transcription elongation, conserving the structure of either active or inactive promoters, promoting the activation of hormone-driven, and early response genes and in the release of paused RNA polymerases (Ju, 2006; Haffner et al., 2010; Madabhushi et al., 2015; Dellino et al., 2019).

A key intermediate of topoisomerase activity is the cleavage complex (TOP2cc), formed when the topoisomerase cleaves the DNA and each subunit of the TOP2 dimer becomes covalently linked to the 5′-terminus of the break via a phosphotyrosyl bond (Vos et al., 2011) (**Figure 1**). Although the cleavage complex is normally transient, naturally due to unclear circumstances or induced by the presence of anti-tumor agents that act as topoisomerase "poisons" the cleaved intermediate can result in the formation of abortive (irreversible) TOP2cc, a singular DSB (Deweese and Osheroff, 2009; Nitiss, 2009b).

## TOP2 AND ONCOGENIC TRANSLOCATIONS

TOP2-associated translocations are main drivers of some common hematological and solid tumors (Felix et al., 2006; Haffner et al., 2010). Oncogenic translocations related to TOP2 have been mainly associated to TOP2ß activity (Nitiss, 2009a; Pommier et al., 2016; Madabhushi, 2018). However, after many years of study, we only start to understand the molecular mechanisms that direct TOP2-induced rearrangements.

In prostate cancer, androgen-regulated genes are frequently fused to transcription factors of the ETS family. For instance, the fusion of *TMPRSS2* and *ERG* occurs in more than 50% of prostate malignancies resulting in a hormone-dependent expression of *ERG* in prostate tissue (Kumar-Sinha et al., 2008). *TMPRSS2* and *ERG* expression has been linked to TOP2 activity since TOP2ß participates in the androgen-dependent activation of these genes. Androgen signaling promotes co-recruitment of androgen receptor and TOP2ß to *TMPRSS2* and *ERG* breakpoints, which can trigger recombinogenic DSBs (Haffner et al., 2010).

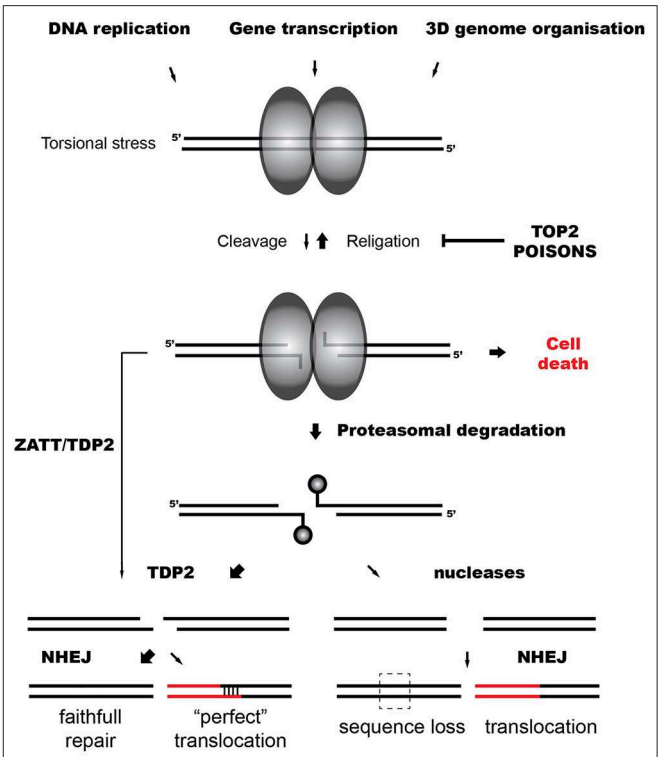


**FIGURE 1 |** TOP2-induced chromosomal translocations. Model representing the repair of TOP2 abortive breaks and the influence of TDP2-dependent and independent NHEJ on TOP2-induced translocations.

Recurrent fusions involving *MLL* and members of the super-elongation complex, such as *AF4* and *AF9,* account for 10–30% of secondary and infant acute myeloid leukemia (AML) (Mitelman et al., 2007; Mertens et al., 2015). Numerous potential mechanisms for *MLL* breakage have been proposed, from Alu-mediated recombination to TOP2ß-induced breaks (Cowell and Austin, 2012; Wright and Vaughan, 2014). Notably, secondary leukemias are those resulting from the use of genotoxic chemotherapeutical drugs, mainly alkylating agents or TOP2 inhibitors, uplifting the direct connection between TOP2 and translocations in *MLL* (Wright and Vaughan, 2014). The link of infant leukemia with TOP2 abortive activity is less clear but a correlation with dietary flavonoids, natural TOP2 poisons, has been proposed (Ross, 2000).

## THE CONTRIBUTION OF TOP2 IN THE CELLULAR POOL OF DSBs

The first factor influencing the propensity of a region to translocate is the frequency of DNA breakage. DSBs can arise directly from exogenous threats (clastogens), such as radiation and chemotherapeutic or industrial chemicals. Endogenous

threats are stochastic activity of apoptotic caspases, nucleases such as RAG1 and RAG2, and TOP2 (Ashour et al., 2015; Lieber, 2016). DSBs can also form indirectly from coincident single strand breaks (SSBs), induced exogenously by alkylating chemotherapeutical agents, or naturally by type I DNA topoisomerases, reactive oxygen species (ROS), or activation-induced cytidine deaminase (AID) (Xu et al., 2012; Rulten and Caldecott, 2013). DNA replication across SSBs also generates single ended DSBs (Kuzminov, 2001). This is a prominent source of DSBs, since SSBs are known to be as frequent as 50,000 per day per cell (Lindahl, 1993). Finally, replication stress, due to replication fork encountering with inter-strand crosslinks or non-B forms of DNA such as RNA-DNA hybrids (R-loops), is also known to promote DSB formation (Gómez-González and Aguilera, 2019).

The most precise information about endogenous DSBs comes from non-biased DSB mapping methods, developed to evaluate illegitimate cleavage by RAG nucleases, and AID in B cells or by CRISPR-Cas (Chiarle et al., 2011; Crosetto et al., 2013; Tsai et al., 2014; Frock et al., 2015; Canela et al., 2016; Lensing et al., 2016; Yan et al., 2017). These studies draw two major conclusions. The first one is that recurrent translocations (typically those that drive specific cancers) are mostly tissue-specific and triggered by recurrent DSBs. For instance, RAG off-target sites have been efficiently detected in activated mouse B-cells in which Rag1 and Rag2 are induced, supporting the role of stochastic activity of these nucleases in the formation of B-cell specific DSBs (Kuo and Schlissel, 2009; Chiarle et al., 2011; Canela et al., 2016).

The second conclusion of these studies, and probably the most ground-breaking, is that there are more stochastic sources of DSBs that are not cell-cycle nor tissue-specific but can be consistently detected in mice and human cells. Some of these are related to replication stress and frequently appear in long gene bodies, which are prone to undergo late replication and are predisposed to replication-transcription conflicts (Canela et al., 2016; Wei et al., 2016). Notably, others, a bulky group of them (over 60%), increase in frequency in the presence of the TOP2 poison etoposide (Canela et al., 2017). These breakpoints, concentrate in chromatin loop boundaries, gene bodies and promoter-proximal locations, frequently transcription start sites (TSS) (Chiarle et al., 2011; Schwer et al., 2016; Yan et al., 2017).

But, what is the origin of these DSBs? TOP2ß is positioned at loop anchors, this is, CTCF/cohesin (RAD21) binding sites that flank topologically associating domains, suggesting that it might be required to solve topological problems during loop extrusion dynamics (Uusküla-Reimand et al., 2016). Etoposide-induced TOP2cc can be detected in these loci, independently of transcription and replication activities (Canela et al., 2017). But these are reversible TOP2cc. Contrary, detection of abortive TOP2cc (irreversible) by DSB or protein-linked mapping has demonstrated that the induction of DSBs at loop anchors by TOP2 activity is largely depend on active transcription (Canela et al., 2019; Gittens et al., 2019; Gothe et al., 2019). In fact, a large number of TOP2ß-associated breaks also concentrate in gene bodies and around TSS, independently of RAD21 (Chiarle et al., 2011; Schwer et al., 2016; Canela et al., 2017; Yan et al., 2017; Gittens et al., 2019; Gothe et al., 2019). Importantly, distribution of TOP2 breaks around TSS positively correlates with transcription levels at these loci (Gittens et al., 2019). Indeed, the inhibition of transcription elongation prevents TOP2 breakage at these loci suggesting that transcription is a major driving force in TOP2 abortive cycles (Gómez-Herreros et al., 2017; Gothe et al., 2019). For instance, breaks at TSS associate with promoter fragility suggesting that events such as RNA polymerase II pause release requires TOP2 activity and is a source of DSBs (Dellino et al., 2019).

Regarding TOP2 isoforms, both TOP2α and TOP2ß influence DNA breakage at these hotspots (Yu et al., 2017; Gothe et al., 2019). Intriguingly, and despite a similar localization of both isoforms, TOP2ß-lacking cells reduce breakage at these loci, suggesting a dominant role of TOP2ß over TOP2α (Cowell et al., 2012; Canela et al., 2017).

In theory, any DSB can be a potential origin of a rearrangement. Interestingly, oncogenic breakpoints such as those found in *TMPRSS2, ERG, MLL, AF4,* and *AF9,* among many others, are localized to TOP2ß/CTCF/RAD21 breakpoints (Canela et al., 2017, 2019; Gothe et al., 2019). Moreover, TOP2-induced breaks have been detected by high-throughput, genome-wide translocation sequencing (HTGTS). HTGTS "fish" breaks genome wide using a bait DSB in a controlled locus (Chiarle et al., 2011; Frock et al., 2015). HTGTS has revealed the tendency of TOP2-induced breaks to translocate, with highly transcribed genes translocating more than with non-transcribed ones (Chiarle et al., 2011; Wei et al., 2016; Canela et al., 2019). Some of these hotspots are localized in TSS (Schwer et al., 2016).

## ILLEGITIMATE TOP2 DSB REPAIR

The illegitimate repair of DNA ends *in trans* is mediated by DNA repair pathways, but how often and why are breaks incorrectly joined is not clear. The two major pathways involved in the repair of DSBs in eukaryotic cells are non-homologous end joining (NHEJ) and homologous recombination (HR). HR occurs specifically in late S and G2 phases of the cell cycle, as it requires the presence of a sister chromatid for the repair process (Mehta and Haber, 2014; Wright et al., 2018). HR is considered an error-free pathway due to the fact that a very large homology, up to megabases, is used, ensuring the accuracy of the repair (Symington, 2016). The occurrence of recombination between homologous chromosomes or tandem repeats has been shown to be substantially low and HR-deficient cells exhibit higher rates of genome rearrangements, historically absolving HR for almost any responsibility in chromosomal translocations (Moynahan and Jasin, 1997, 2010; Lambert et al., 1999; Stark and Jasin, 2003). Exceptions to the HR paradigm are Rad51-independent but homology-directed pathways, Break-Induced Replication (BIR) and single strand annealing (SSA), which can promote exchanges *in trans* (Elliott et al., 2005; Malkova and Ira, 2013; Bhargava et al., 2016; Sakofsky and Malkova, 2017; Kramara et al., 2018).

In contrast to HR, NHEJ is active throughout the cell cycle and involves the efficient ligation of DNA ends with minimal processing at the site of joining. NHEJ is considered

an error-prone pathway since cellular nucleases trim DNA ends to make them compatible before ligation (Lieber, 2010). The homology required in this route is reduced to 1–2 nucleotides, in case there is any, making NHEJ the ideal scapegoat to provoke illegitimate joinings (Chang et al., 2017). However, in the absence of KU70/80 or XRCC4-LIG4, core factors of canonical NHEJ (cNHEJ), a genetically-different, alternative NHEJ (altNHEJ) pathway takes over (Yan et al., 2007). altNHEJ is characterized by a longer homology requirement at the site of break that goes up to 10–20 bp (McVey and Lee, 2008). Resection is mediated by CtIP and the MRN complex, similarly to initial stages of HR (Zhang and Jasin, 2010; Ghezraoui et al., 2014). Base pairing *in trans* of these ends results in translocations characterized by short microhomologies (Guirouilh-Barbat et al., 2004; Kent et al., 2015; Mateos-Gomez et al., 2015; Sfeir and Symington, 2015; Zahn et al., 2015).

In the case of TOP2-induced DSBs, trapped TOP2 represents a particular barrier for ligation, and DNA ends need to be processed. Abortive TOP2cc are denatured and degraded by the proteasome, leaving a TOP2-derived peptide of unknown length covalently bound to the 5′ phosphate of the DNA through a tyrosine residue (Zhang et al., 2006; Lin et al., 2008) (**Figure 1**). This protein adduct is a hallmark of TOP2 breaks and, like other blocking lesions, can condition DSB repair (Álvarez-Quilón et al., 2014). Resection can generate proficient substrates for HR independently of the nature of the DNA end and potentially remove these adducts. In accordance, HR-deficient cells are hypersensitive to TOP2 poisons, suggesting that TOP2 breaks can be repaired by HR when available (Gómez-Herreros et al., 2013). However, remaining topoisomerase can be precisely removed by Tyrosyl DNA phosphodiesterase 2 (TDP2), which cleaves the phosphotyrosyl bond between the tyrosine and the 5′ phosphate of the DNA (Cortés-Ledesma et al., 2009; Zeng et al., 2010). TDP2 can also remove non-degraded TOP2 in a proteasome-parallel route stimulated by the SUMO-ligase ZATT (Schellenberg et al., 2017). Once TOP2 is removed by TDP2, remaining four base-pair cohesive overhang is ready to be ligated by cNHEJ (Gómez-Herreros et al., 2013) (**Figure 1**).

TDP2-mediated pathways protect cells from TOP2 abortive activity, accelerating TOP2 DSB repair and preventing cell death and genome instability induced by TOP2 poisons (Gómez-Herreros et al., 2014, 2017; Zagnoli-Vieira et al., 2018). In accordance, breaks in *MLL* induced by the abortive activity of TOP2 during transcription accumulate in cells lacking TDP2 (Gómez-Herreros et al., 2017). Notably, TDP2 facilitates a faithful repair of TOP2 breaks suppressing chromosomal translocations generated by TOP2 during transcription (Gómez-Herreros et al., 2017) (**Figure 1**). Intriguingly, TDP2-processed ends can also originate chromosomal translocations. A four base pair homology at break joining can be generated in cells treated with etoposide and is dependent on TDP2 (Gómez-Herreros et al., 2017) (**Figure 1**). About 20% of secondary AML is characterized by this type of junction that is referred to as "perfect" (Whitmarsh et al., 2003; Meyer et al., 2005, 2017). A very high number of DSBs might challenge physiological repair capacities and promote this illegitimate pairing.

Since TDP2 prevents genome instability and chromosomal translocations, it has been hypothesized that marginal routes would promote them (Caldecott, 2012; Gómez-Herreros et al., 2013, 2017). If HR is not available, in contrast to the "clean" end processing mediated by TDP2, endonucleases would potentially generate the loss of information at DNA ends (**Figure 1**). However, it has been shown that MRE11, the nuclease activity of the MRN complex, can process abortive TOP2 DSBs regulated by a HR-independent role of BRCA1 (Hoa et al., 2016; Sasanuma et al., 2018). Notably, MRE11 H129N (nuclease deficient) mutants exhibit increased instability and translocations when treated with TOP2 poisons (Sasanuma et al., 2018; Gothe et al., 2019). The contribution of MRE11 and other nucleases such as ARTEMIS in the repair of physiological levels of TOP2 breaks, their relevance in TOP2 poison-based chemotherapy and their implication in TOP2-induced genome instability is under discussion.

Contrary to mouse cells, in which translocations depend mostly on altNHEJ, cNHEJ mediates translocations induced by nucleases and ionizing irradiation in humans (Ghezraoui et al., 2014; Biehs et al., 2017; So and Martin, 2019). However, it has also been shown that the DSB structure can predispose repair toward cNHEJ and altNHEJ suggesting that the nature of the DNA end can condition its repair (So and Martin, 2019). The role of NHEJ in the formation of TOP2-induced translocations is controversial. An epistatic effect of Ku70 over TDP2 in etoposide sensitivity in avian cells suggests that cNHEJ mediates TOP2-induced DSB repair (Gómez-Herreros et al., 2013). However LIG4 deficiency increases *MLL* translocations suggesting that different pathways such as altNHEJ might mediate TOP2 induced rearrangements in the absence of cNHEJ (Gothe et al., 2019). Further research is required to clarify this point.

Noteworthily, despite in the presence of a sister chromatid NHEJ still has a dominant role (Beucher et al., 2009; Karanam et al., 2012), it has been shown that transcriptionally-active regions are preferentially repaired by HR, promoted by open chromatin marks (Aymard et al., 2014; Pfister et al., 2014). This mechanism may moderate mutagenic pathways during the repair of critical sequences. Why TOP2-induced DSBs during transcription are so dependent on TDP2 and NHEJ remains unknown.

## THE SYNAPSIS OF TOP2 BREAKS

A major determinant for translocation propensity is the proximity between donor and acceptor DSBs (Roukos et al., 2013; Hu et al., 2016). Translocations occur preferentially in *cis* and are enhanced within the same topological domain due to pre-existing spatial proximity (Zhang et al., 2012). Notably, transcription may not only mediate TOP2 breakage but break proximity as well. Oncogenic translocation partners are known to share transcription factories (discrete concentrations of actively transcribed genes) (Ghamari et al., 2013). That is the case for *IgH* and *MYC* in Burkitt's lymphoma but also for TOP2 hotspots such as *TMPRSS2* and *ERG* in prostate and *MLL*, *AF4,* and *AF9* in bone marrow (Osborne et al., 2007; Lin et al., 2009; Cowell et al., 2012). Nevertheless, for other pairs such as *MLL* and *ENL,*

an inherent proximity exists, favoring synapsis independently of transcription (Gothe et al., 2019). The association of TOP2 breaks to loop anchors might also contribute to spatial proximity. However, a rational comparison of the 3D architecture of the genome with the genome-wide data of TOP2 abortive breaks is still missing.

Additionally, in G1, repair in highly transcribed loci is delayed and breaks dynamically cluster (Aten et al., 2004). The functional reason for this grouping remains unclear but the absence of a sister chromatid and the preferential use of HR over NHEJ would be an explanation (Aymard et al., 2017). However, grouping unrepaired, and may be partially resected, DSBs seems counter-productive for genome stability. Favoring TDP2-mediated repair in TOP2 breaks associated to transcription may be a mechanism to prevent this situation.

## CONCLUSION

High transcription and recurrent DSBs are hallmarks of oncogenic hotspots. These two factors get together with spatial proximity and NHEJ-mediated repair in transcription-associated TOP2 breaks generating the perfect breeding ground for chromosomal translocations.

## AUTHOR CONTRIBUTIONS

FG-H conceived this review and wrote the manuscript.

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# "An End to a Means": How DNA-End Structure Shapes the Double-Strand Break Repair Process

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Endogenously-arising DNA double-strand breaks (DSBs) rarely harbor canonical 5′-phosphate, 3′-hydroxyl moieties at the ends, which are, regardless of the pathway used, ultimately required for their repair. Cells are therefore endowed with a wide variety of enzymes that can deal with these chemical and structural variations and guarantee the formation of ligatable termini. An important distinction is whether the ends are directly "unblocked" by specific enzymatic activities without affecting the integrity of the DNA molecule and its sequence, or whether they are "processed" by unspecific nucleases that remove nucleotides from the termini. DNA end structure and configuration, therefore, shape the repair process, its requirements, and, importantly, its final outcome. Thus, the molecular mechanisms that coordinate and integrate the cellular response to blocked DSBs, although still largely unexplored, can be particularly relevant for maintaining genome integrity and avoiding malignant transformation and cancer.

**Keywords: DNA double strand break (DSB), Non-homologous DNA end joining, ATM, DNA-PK catalytic subunit, genome instability**

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Double-strand breaks (DSBs) are the most devastating lesion that DNA molecule can suffer. Indeed, they can cause dangerous chromosomal rearrangements or even cell death if they are not properly repaired. In general terms, there are two conceptually different pathways to repair DSBs that can be divided into those that use homologous sequences—either a sister chromatid or another sequence elsewhere in the genome—as a template in the repair (homologous recombination, HR), and those that directly rejoin the ends, without any template requirement (Lieber, 2008; San Filippo et al., 2008; Pannunzio et al., 2018), regardless of whether using minimal (non-homologous end-joining, NHEJ) or more extensive (microhomology-mediated end-joining, MMEJ) microhomologies to stabilize the junctions. Despite the general intrinsic diploidy of somatic mammalian cells, HR rarely uses the homologous chromosome as a template for DSB repair (Johnson, 2000). Consequently, HR is mostly restricted to late S/G2 phase, when a sister chromatid is available, whereas NHEJ can operate in any phase of the cell cycle. Besides this global distinction, there are additional peculiarities of DSB repair mechanisms based on the specific nature of each DNA lesion, specifically when it comes to the chemical configuration of the broken DNA ends. In this regard, since the HR will use the information of an intact template for repair (San Filippo et al., 2008), the ends of the break, both 5' and 3′, can be extensively degraded without compromising an efficient reconstitution of the initially lost DNA sequences. In contrast, the chemical modifications of DSB ends, and how these are solved, are pivotal in the NHEJ process and final repair outcome. It is therefore of great interest to understand how DSBs harboring complex DNA ends are repaired in the G1 phase of the cell cycle, during which HR is strongly limited.

## THE NHEJ PROCESS

The starting point of the NHEJ process takes place with the recognition and binding of double stranded DNA ends by the KU70/80 heterodimer, which occurs in an extraordinarily efficient manner due to its abundance and its strong avidity for this type of DNA substrate. DNA-bound KU heterodimer, in turn, recruits DNA-PKcs to form the DNA-PK holoenzyme, so that the two DNA-PKcs molecules bound to opposing sides of the DSB can interact one each other, contributing to synapsis of broken DNA ends (Meek et al., 2008; Neal and Meek, 2011). The DNA-PK complex is the main regulator of the NHEJ process, coordinating the recruitment of downstream NHEJ accessory factors, such as X-ray cross complementing group 4 (XRCC4), XRCC4-like factor/Cernunnos (XLF), or Paralog of XRCC4 and XLF (PAXX), and DNA ligase IV (LIG4), which contribute to the proper pairing of DSB ends and perform the final ligation of the break (Kakarougkas and Jeggo, 2014; Ochi et al., 2015; Conlin et al., 2017). In vertebrates, NHEJ further evolved an end processing capacity that allows for the repair of complex ends (e.g., hairpins), and which is also, in part, regulated by DNA-PK, as will be discussed below.

## RELEVANCE OF END STRUCTURE AND CONFIGURATION DURING NHEJ

It can be claimed that the only essential step of NHEJ process is the ligation of one of the DNA strands of the DSB (Waters et al., 2014). During this process, LIG4 activity requires compatible ends harboring canonical 5′-phosphate and 3′-hydroxyl termini. However, DSBs often have complex ends with chemical modifications or structures that do not allow straightforward joining of the termini, so they can be considered as blocked ends (**Figure 1**). These chemical variations can be sensed by LIG4 through the disruption of its catalytic cycle (Reid et al., 2017). Therefore, when DSBs harbor non-canonical chemical structures at the ends, they must be restored to conventional 5′-phosphate and 3′-hydroxyl termini so that DNA ligation can take place. There are two conceptually different ways by which these non-canonical DNA ends can be converted into ligatable substrates (**Figure 1**). On the one hand, cells have a variety of enzymes to directly restore the canonical chemical structure. Given that this event does not involve any sequence modification, it can be simply considered as an "unblocking" process. On the other hand, under certain circumstances, such as the presence of complex lesions, unblocking activities may be compromised or overwhelmed, resulting in DSBs that require additional "end processing" by the action of nucleases that cleave DNA sequence from the ends to remove the chemical modifications (**Figure 1**). Regarding unblocking, there is a large number of factors with different enzymatic activities that are available for this process during NHEJ (**Figure 2**), such as tyrosyl-DNA phosphodiesterases 1 and 2 (TDP1 and TDP2, respectively), polynucleotide kinase (PNKP), Aprataxin, and even KU. This, in turn, reflects the wide variety of damaged termini that can arise, as each of these factors removes specific chemical modifications at DNA ends (Povirk, 2012; Andres et al., 2015). These unblocking activities are essential in NHEJ, since they are responsible for facilitating accurate religation of the breaks, as opposed to the processing of DNA ends that may involve nucleotide loss or gain and, therefore, sequence modification. Interestingly, ionizing radiation, which is a common and well-established source of DSBs, mostly induces blocked termini with heterogeneous end structures. Damage occurs either directly, by high-energy particle collision with DNA, or indirectly, when these particles split water molecules leading to dangerous free radicals; in both cases this mainly results in breakage of the sugar backbone, and therefore needs to be processed, necessarily leading to loss of one nucleotide from the termini (Reisz et al., 2014).

Another aspect to highlight in NHEJ is the possible incompatibility among DSBs to be repaired due to the absence of sequence complementarity of DNA ends. This situation may occur when DSB ends have small protuberances, either with 5′ or 3′ polarity. The short stretches of single stranded DNA of these overhangs may be compatible (either fully or partially complementary sequences) or not. It has been shown that LIG4 can ligate across short gaps or rejoin several incompatible DNA end configurations that do not share even 1-bp of terminal microhomology (Gu et al., 2007). For this scenarios, NHEJ also takes advantage of several processing enzymes that can modify DNA ends until they become ligatable substrates (Strande et al., 2012). In this way, single-stranded DNA overhangs (as also may happen with blunt ends) can be trimmed by nucleases such as ARTEMIS giving rise to small gaps than can be efficiently filled-in by specialized X family DNA polymerases (see below) (Mahajan et al., 2002; Lee J. W. et al., 2004; Ma et al., 2004; McElhinny et al., 2005; Capp et al., 2007; Lieber, 2010). It is worth noting that non-complementary DNA ends are indeed the most likely result of end processing at initially chemically modified structures.

## NHEJ: AN ITERATIVE VS A HIERARCHICAL PROCESS

Although NHEJ is generally considered a single DNA repair pathway, a wide variety of factors are needed and different sub-routes can be distinguished depending on the different DSB end configurations (Pannunzio et al., 2018). In addition, there is still important debate about how these NHEJ accessory factors actually operate, and, in this sense, two apparently antagonistic positions can now be distinguished. On the one hand, some authors propose that NHEJ factors operate in an iterative way without an established order (Gu and Lieber, 2008; Lieber, 2008; Gu et al., 2010). This model highlights the flexibility of the NHEJ process and explains the diversity of repair products generated from the same type of DSB. The iterative nature of this process implies that multiple NHEJ components can act on the same DSB during multiple consecutive rounds of processing and that the involvement of factors is not mutually exclusive to the usage of other ones, all of them remaining active as long as the DSB continues unrepaired. On the other hand, other authors propose that there is a hierarchy in NHEJ, by which cells give precedence

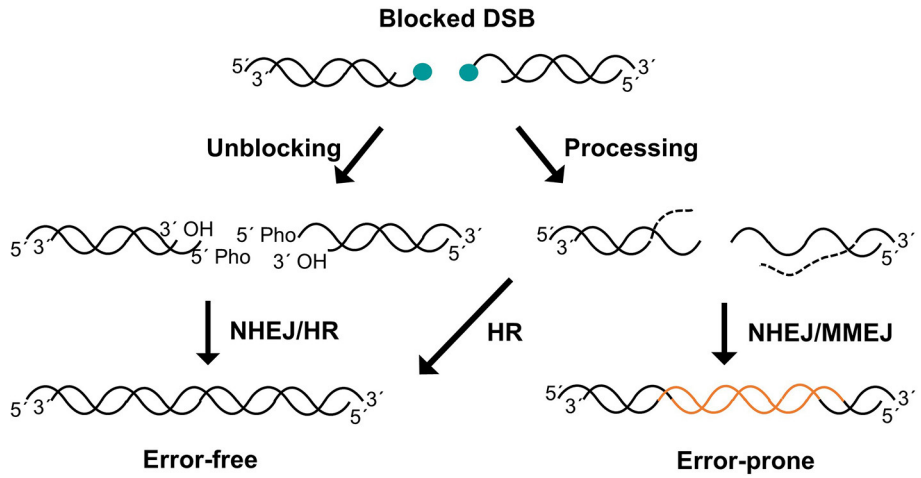


**FIGURE 1 |** Unblocking and processing of DSBs. Unblocking pathways directly convert ends into 5′-phospahte and 3′-hydroxyl but the nucleotide sequence remains intact, promoting error-free repair (**left**). Processing can also facilitate blocked DSBs repair removing aberrant structures from DNA ends by nucleotide trimming (**right**). This pathway can lead to error-prone repair when non-templated repair pathways such as NHEJ or MMEJ are used. 5′ blocks are depicted but similar situations could be generated on 3′ ends.

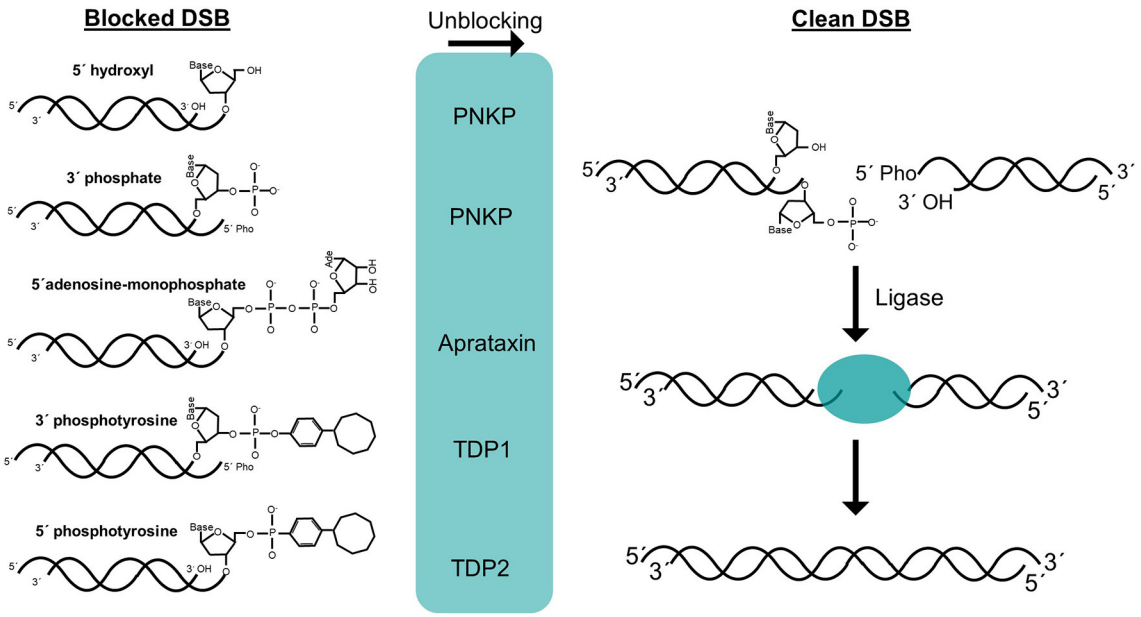


**FIGURE 2 |** Structure of DNA ends and unblocking enzymes. DSB termini can be blocked by numerous chemical structures *in vivo*. Several unblocking enzymes are present in mammalian cells and efficiently convert these structures to clean 5′-phosphate and 3′-hydroxyl DSB termini (**left**). These clean DSBs can be in theory, directly repaired with the only enzymatic activity of a ligase (**right**).

to resolution paths with the fewest number of enzymatic steps. This way, direct ligation is favored over more complex pathways that include end-processing and/or DNA synthesis (Waters et al., 2014). Consistent with this, LIG4 is the most flexible ligase known so far (Ma et al., 2004; Gu et al., 2007), and the differences in how their catalytic domains interact with different end structures trigger dramatic changes in the dynamics of the entire NHEJ complex, determining the steps taken to complete repair and the factors required (Conlin et al., 2017). A hierarchical order in the action of NHEJ components is also supported by the formation of a synapsis with two different stages (Graham et al., 2016). First, DNA ends are tethered sufficiently far apart, and are then closely aligned by DNA-PK, XLF, and the LIG4-XRCC4 complex. It has been suggested that this structural conversion can be coordinated with end-processing by changes in the phosphorylation profile of DNA-PKcs (Graham et al., 2016), which would provide a

mechanism for the regulation of end processing and ligation. Although both models could seem contradictory, they may not be mutually exclusive. While, NHEJ could behave as an iterative process in which various components can be loaded and act in various combinations without an established order, providing flexibility and efficiency to the repair process, the decision of how complex ends are repaired should not be stochastically determined, as specific unblocking activities must be preferred over end-processing in order to avoid sequence modification.

## NUCLEASES IN NHEJ

As mentioned above, under certain circumstances, DSBs require end processing by the action of nucleases. Usually, these nucleases remove chemical modifications and blockages or cleave mismatched ends by trimming 5′ or 3′ termini through exo- and/or endonucleolytic processing to expose short regions of microhomology between strands and promote end joining (Pannunzio et al., 2018). ARTEMIS is the major nuclease implicated in end-processing during NHEJ (Ma et al., 2002; Goodarzi et al., 2006; Yannone et al., 2008). Its main role takes place during V(D)J recombination, where it is responsible for the opening of DNA hairpins formed at coding joints, an endonucleolytic activity that is promoted by phosphorylation in the ABCDE cluster of DNA-PKcs. However, it has been also shown to have DNA-PKcs-independent 5′ exonuclease activity on ssDNA (Pawelczak and Turchi, 2010; Li et al., 2014). Beyond its role in V(D)J recombination, ARTEMIS contribution in NHEJ is still under study, and recent analysis demonstrated that the ARTEMIS-DNA-PKcs complex also promotes the ligation of incompatible overhangs *in vitro* (Chang et al., 2016; Pannunzio et al., 2018). Besides its versatility to act at many different types of DNA ends, there is a common feature in all ARTEMIS substrates: a ss-dsDNA boundary, which is present in a wide variety of different DNA end configurations (Chang et al., 2015; Chang and Lieber, 2016). Interestingly, a novel 3′ endonuclease activity of ARTEMIS has been recently described, that is promoted by XRCC4-LIG4 complex and also independent of DNA-PKcs (Gerodimos et al., 2017). The stimulation of this activity could be as a result of a conformational change due to the interaction with LIG4 (Pannunzio et al., 2018).

Another factor involved in the repair of complex ends requiring end processing is the MRE11 protein from the MRN complex (consisting of MRE11, RAD50, and NBS1). The MRN complex acts as a sensor of DSBs and promotes repair by NHEJ or HR. Specifically, MRE11 exhibits 3′-5′exonuclease and single-stranded and DNA hairpin endonuclease activities (Paull and Gellert, 1998; Trujillo et al., 2003; Lisby et al., 2004; Stracker and Petrini, 2011; Williams et al., 2011). Endonucleolytic cleavage may be of particular importance for DNA ends covalently-bound to Spo11 (Neale et al., 2005), terminated by hairpins (Lobachev et al., 2002) or generated by TOP1 and 2 poisons (Hartsuiker et al., 2009; Quennet et al., 2011; Hoa et al., 2016). Furthermore, recent *in vitro* studies described that NBS1 is essential to promote MRE11 nuclease activities on DNA ends containing protein adducts, while it inhibits MRE11 3′ to 5′ exonuclease degradation of clean ends (Deshpande et al., 2016). Additionally, the function of the MRN complex during resection is stimulated by the phosphorylated form of CTIP (Anand et al., 2016). Remarkably, the nuclease activity of CTIP has been reported to be specifically required for processing complex DSBs, such as those harboring topoisomerase adducts or generated by irradiation. This suggests that the endonuclease activity of CTIP is only necessary for the removal of DNA adducts and not for the resection of unmodified DNA breaks (Makharashvili et al., 2014). This differentiates catalytic and non-catalytic functions of CTIP during end resection, which requirement would be end-structure dependent.

## POLYMERASES IN NHEJ

As mentioned above, as a consequence of the processing of complex DSBs, the participation of other accessory factors such as DNA polymerases of the PolX family is often required. These polymerases are especially suited for filling in the small gaps that are generated when two ssDNA protruding ends with the same polarity and have either none or partial complementarity. The action of the different PolX polymerases during NHEJ seems to be determined by a gradient of template strand dependence after DSB ends are synapsed, with Polλ being completely template-dependent, Polμ having some template requirements and Terminal Deoxynucleotidyl Transferase (TdT) being fully template-independent (McElhinny et al., 2005). Therefore, when 3′-protruding ends at DSBs do not have any complementarity with each other, Polμ and TdT polymerases can add nucleotides for generating *de novo* terminal microhomology at DNA ends (Gu et al., 2007; Davis et al., 2008; Chang et al., 2016). PolX polymerases are recruited to DSBs through the specific interaction between their BRCT domains with NHEJ core factors (Mueller et al., 2008; Boubakour-Azzouz et al., 2012; Malu et al., 2012; Craxton et al., 2018). These interactions favor DSB repair efficiency (Tseng and Tomkinson, 2002; Craxton et al., 2018), and can be facilitated to some extent by DNA-PKcs-mediated phosphorylation (Sastre-Moreno et al., 2017). In fact, systematic analyses to determine how overhang sequence affects the activity of NHEJ polymerases has shown some DNA synthesis patterns that may be coordinated with ligation complex capabilities (Craxton et al., 2018).

## END-PROTECTING FACTORS

In addition to all these unblocking and processing factors, other accessory NHEJ components are required to inhibit or restrict degradation of DSB ends, and therefore avoid excessive DNA sequence loss. In this regard, modifications at the chromatin flanking the DSB, such as histone H2AX phosphorylation (Helmink et al., 2011), and the subsequent recruitment of downstream factors of the DNA damage response (DDR), such as MDC1, 53BP1, and BRCA1 (Bekker-Jensen and Mailand, 2010) represent crucial events for the choice of proper repair pathways, regulating to which extent DSB ends are processed. Accordingly, H2AX deficient mice show an increase in genome instability and,

in the absence of P53, are prone to tumor development (Celeste et al., 2002, 2003; Bassing et al., 2003). Moreover, in ARTEMIS deficient cells, H2AX was reported to limit the processing of DNA ends by CTIP endonuclease upon induction of blocked DSBs during V(D)J recombination, this function of H2AX being mediated by MDC1 (Helmink et al., 2011). In the same way, 53BP1 has been also shown to regulate end-processing during V(D)J and CSR recombination (Difilippantonio et al., 2008; Bothmer et al., 2010) and to inhibit CTIP-dependent resection in BRCA1 deficient cells at post-replicative stages of cell cycle, suggesting that H2AX phosphorylation may restrict resection by the recruitment of 53BP1 (Bunting et al., 2010). The protective role of DNA ends by 53BP1 requires the participation of some downstream factors, such as PTIP (Kurimasa et al., 2015) and RIF1 (Kienker, 2000; Lee K. J. et al., 2004; Douglas et al., 2005), and maybe other factors yet to be discovered. In this regard, the recently discovered ssDNA-binding complex shieldin has been proposed to act as ultimate effector of the 53BP-RIF1 pathway for end protection (Chan et al., 2002; Ding et al., 2003; Meek et al., 2007). Of note, ARTEMIS was previously identified as a PTIP-binding protein, and, strikingly, as one of main downstream effectors of 53BP1-PTIP pathway (Wang et al., 2014). This suggests that 53BP1 could be promoting limited end-trimming and the repair of DSBs through NHEJ, and therefore directly competing with the HR repair pathway that would entail long resection.

## DNA-PKcs, A MASTER REGULATOR OF ACCESS TO DSB ENDS

Despite not being conserved in lower eukaryotes, the activity of this phosphatidylinosytol 3-kinase-related kinase (PI3KK) is a clear requisite for its functioning during NHEJ in mammalian cells (Kienker, 2000; Kurimasa et al., 2015). Although there is a long list of DNA-PKcs substrates, mutational analysis (Lee K. J. et al., 2004; Douglas et al., 2005; Goodarzi et al., 2006; Meek et al., 2008) concludes that DNA-PKcs itself is the only NHEJ factor that has been shown to be a functionally relevant target of its own kinase activity (Chan et al., 2002; Ding et al., 2003; Soubeyrand et al., 2003; Cui et al., 2005; Douglas et al., 2007; Meek et al., 2007, 2008). The most well-accepted consequence of such DNA-PKcs autophosphorylation is its inactivation and dissociation from DNA ends, allowing subsequent joining by LIG4 (Chan and Lees-Miller, 1996; Douglas et al., 2001). Despite the fact that DNA end binding by DNA-PKcs is indifferent to distinct DNA end structures, some studies indicate that cisplatin-DNA adducts near the ends reduce kinase activation, suggesting that free termini could be involved in the activation of DNA-PKcs (Turchi, 2000; Pawelczak et al., 2005). It has been suggested that kinase activation occurs in trans, linking autophosphorylation of DNA-PKcs to synapsis. Although this point is still a matter of debate, this may provide an important mechanism by which DNA-PKcs protects DNA-ends to maintain genomic integrity. However, extensive studies have shown that in response to DSBs, DNA-PKcs autophosphorylation can occur in different residues, with each event having specific functional consequences (Meek et al., 2008; Davis et al., 2014). In human DNA-PKcs, amino acid clusters known as ABCDE, flanking Thr2609 residue, and PQR, around the Ser2056 residue, are the two major phosphorylation sites (Ding et al., 2003; Block et al., 2004; Reddy et al., 2004; Cui et al., 2005; Meek et al., 2007). Although both clusters can be autophosphorylated by DNA-PKcs itself, the ABCDE cluster can be also phosphorylated by ATM or ATR under different cellular stresses (Chen et al., 2007; Meek et al., 2008; Davis et al., 2010). Site-directed mutagenesis analyses and characterization of animal models of DNA-PKcs deficiency (Blunt et al., 1996; Araki et al., 1997; Taccioli et al., 1998; Beamish et al., 2000; Zhang et al., 2011; Danska et al., 2015; Jiang et al., 2015) have revealed that the specific defect resulting from blocking either ABCDE or PQR phosphorylation is DNA end processing deregulation. Both clusters show antagonistic functions, and whereas phosphorylation in the ABCDE cluster promotes DNA end processing, phosphorylation of sites within the PQR cluster inhibits DNA end resection. Specifically, the ABCDE cluster is reported to promote end processing by regulating the access of ARTEMIS to the ends (Ma et al., 2002; Cui et al., 2005; Goodarzi et al., 2006; Yannone et al., 2008). On the other hand, end-ligation requires a strict DNA-PKcs autophosphorylation, possibly in the PQR cluster, which is promoted by ligatable ends and synapsis. This way, possible unsuccessful ligation attempts are avoided. Thus, DNA-PKcs can be considered a molecular shift that coordinates end processing and ligation through its phosphorylation to maximize the efficiency of the NHEJ pathway.

## ATM, A KEY FACTOR TO ORCHESTRATE END PROCESSING

Ataxia Telangiectasia Mutated (ATM) kinase is another member of the PI3KK family, recognized by its function as an apical activator of the DDR in response to DSBs (McKinnon, 2004). Interestingly, the structure of ends is a crucial factor which determines the requirement of ATM for the repair of a DSB (Álvarez-Quilón et al., 2014). Specifically, ATM exclusively facilitates the repair of irreversibly blocked TOP2-mediated DSBs, arising by etoposide treatment in TDP2-deficient background (Álvarez-Quilón et al., 2014). Consistent with this, ATM-mediated repair promotes cell survival and the maintenance of genome integrity, avoiding micronuclei and chromosomal aberration formation after the induction of DSBs harboring termini that require end processing (Álvarez-Quilón et al., 2014). Although the underlying molecular mechanisms by which ATM deals with blocked DNA ends are still unclear, two complementary explanations have been proposed (Álvarez-Quilón et al., 2014). On the one hand, ATM can promote limited resection to eliminate the complex structures at DSB ends through the action of nucleases. In this regard, ATM phosphorylates ARTEMIS and DNA-PKcs at the ABCDE cluster (see above) (Chen et al., 2007; Meek et al., 2008; Davis et al., 2010). In addition, a functional interplay between ATM and the MRN complex has been widely reported. Indeed, the three components of the complex are all phosphorylated by ATM, which has been proposed as a modulator of its processing activity

(Kijas et al., 2015). Then, the MRN complex interacts with CtIP, which is also positively regulated by ATM to promote end-resection (You and Bailis, 2010; Wang et al., 2013). Finally, ATM regulates other nucleases that could be involved in resolving incompatible ends. This includes APLF (Aprataxin and PNKP-like factor) (Macrae et al., 2008; Fenton et al., 2013); DNA replication helicase/nuclease 2 (DNA2) (Paudyal et al., 2017) or EXO1 (Bolderson et al., 2010; Tomimatsu et al., 2017). On the other hand, ATM could restrict excessive nucleolytic degradation of DNA ends (Rahal et al., 2008). This can actually operate by a direct inhibitory action on aforementioned nucleases such as MRE11 (Rahal et al., 2010) or EXO1 (Bolderson et al., 2010), and/or by promoting modifications at the chromatin flanking the DSB and the recruitment of protecting factors. In this regard, the protective function of H2AX depends on its phosphorylation at Ser139 to form γ-H2AX in chromatin flanking DNA DSBs (Helmink et al., 2011), which is preferentially carried out by ATM (Takahashi et al., 2010). The γ-H2AX downstream factor MDC1 is also phosphorylated by ATM, promoting its oligomerization and spreading on chromatin (Maréchal and Zou, 2013). In addition, ATM phosphorylates 53BP1 (Anderson et al., 2002; Jowsey et al., 2007) and these phosphorylations are required for 53BP1 interaction with PTIP (Munoz et al., 2007) and RIF1 (Chapman et al., 2013). Finally, in addition to these dual end processing/-protective roles, ATM could operate at a later stage in the repair process. For example, after ionizing radiation-induced DSBs, ATM phosphorylates Polλ, which would promote conformational changes in Polλ that facilitate its interaction with NHEJ core factors at DSBs and, hence, stimulates gap-filling DNA synthesis during NHEJ (Sastre-Moreno et al., 2017).

The structure and conformation of DNA ends are therefore determinant to the repair process and outcome, especially in situations in which end-joining mechanisms are prevalent. Although many of the enzymatic activities required have been identified and characterized in detail, the mechanisms by which cells regulate and integrate these activities to keep sequence variation under control are still poorly understood. In this sense, it is tempting to think on blocked DSBs and a deregulated cellular response to these lesions as important threats to genome integrity, and, potentially, drivers of malignant transformation and cancer.

## AUTHOR CONTRIBUTIONS

AS-B, FC-L, and JR conceived and wrote the manuscript.

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REVIEW
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# Studying DNA Double-Strand Break Repair: An Ever-Growing Toolbox

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To ward off against the catastrophic consequences of persistent DNA double-strand breaks (DSBs), eukaryotic cells have developed a set of complex signaling networks that detect these DNA lesions, orchestrate cell cycle checkpoints and ultimately lead to their repair. Collectively, these signaling networks comprise the DNA damage response (DDR). The current knowledge of the molecular determinants and mechanistic details of the DDR owes greatly to the continuous development of ground-breaking experimental tools that couple the controlled induction of DSBs at distinct genomic positions with assays and reporters to investigate DNA repair pathways, their impact on other DNA-templated processes and the specific contribution of the chromatin environment. In this review, we present these tools, discuss their pros and cons and illustrate their contribution to our current understanding of the DDR.

**Keywords: DNA repair, homologous recombination (HR), non-homologous DNA end joining, chromatin, DNA damage**

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## DNA DOUBLE-STRAND BREAK DETECTION, SIGNALING AND REPAIR

DNA double-strand breaks (DSBs) are the most cytotoxic DNA lesions. Their detection, signaling, and repair require a comprehensive cellular response collectively known as the DNA damage response (DDR). The DDR requires the activation of the ATM kinase, a member of the phosphoinositide 3-kinase (PI3K)-related protein kinase family (Blackford and Jackson, 2017), which is rapidly recruited to chromatin in response to DSBs through the interaction with the MRE11-RAD50-NBS1 (MRN) complex (van den Bosch et al., 2003). This recruitment triggers the phosphorylation of a large number of substrates to initiate a signaling cascade that activates cell cycle checkpoints and promotes the recruitment of repair factors to the damage site. One of the substrates of ATM kinase activity is the serine 139 of the carboxyl terminus of the histone variant H2AX, which in its phosphorylated version is referred to as γH2AX (Burma et al., 2001). Once established, γH2AX promotes the recruitment of additional ATM molecules and the sequential accumulation of other DDR proteins, creating a positive feedback loop that fuels further spreading of γH2AX (van Attikum and Gasser, 2009; Polo and Jackson, 2011; Shi and Oberdoerffer, 2012).

DNA double-strand breaks repair can be achieved by different means that are commonly grouped in two broad categories depending on the use or not of a homologous DNA sequence as a template. Repair by non-homologous end joining (NHEJ) involves direct resealing of the two broken ends independently of sequence homology. Although being active throughout the cell cycle, NHEJ is relatively more important during G1 (Chang et al., 2017). A scheme showing the most

important steps of NHEJ is shown in **Figure 1**; Chang et al., 2017). NHEJ represents the simplest and fastest mechanism to heal a DSB, thus it is the most predominant DSB repair pathway within the majority of mammalian cells, even though it may occasionally lead to loss of genetic information (Chang et al., 2017).

In contrast to NHEJ, homologous recombination (HR) requires a homologous DNA sequence to serve as a template for DNA-synthesis-dependent repair and involves extensive DNA-end processing (Huertas, 2010). As expected, HR is extremely accurate, as it leads to precise repair of the damaged locus using DNA sequences homologous to the broken ends. HR predominantly uses the sister chromatid as a template for DSB repair, rather than the homologous chromosome (Johnson, 2000). This tight regulation is ensured thanks to both a strong inhibition of HR during G1 when a sister chromatid is absent (Hustedt and Durocher, 2016), but also thanks to the nature of the newly replicated chromatin, which favors HR (Saredi et al., 2016; Pellegrino et al., 2017; Nakamura et al., 2019). The key first step in HR, determinant for DSB pathway choice, is 5′ to 3′ resection: the processing of the 5′ DNA strand at the DSB by multiple nucleases and accessory proteins, resulting in 3′ single-stranded DNA (ssDNA) (Huertas, 2010; Symington, 2014). The 3′ ssDNA stretches created during resection are used for template search and recombination (**Figure 1**).

As described above, both HR and NHEJ safeguard genome integrity and proceed through a cascade of events whereby DNA damage sensors, transducers, and effectors detect and rejoin the broken DNA ends (Harper and Elledge, 2007). All these events take place within the chromatin environment, which is the actual substrate for the repair machinery. While the past 50 years have seen a mounting understanding of the DDR pathways, the contribution of the chromatin environment and nuclear organization to genome stability, particularly how it is organized upon the interplay between the DDR and the other cellular processes, has only begun to emerge over the past decade. Chromatin is modified *in cis* to the DSB and this break-induced chromatin landscape contributes to recruiting DNA repair factors, thanks to interactions between histone modifications and their readers (e.g., 53BP1 interacts with nucleosomes bearing H2AK15ub and H4K20me2). In addition, during DSB repair, the destabilization of nucleosomes further enhances accessibility and regulate the mobility of the broken DNA ends (Clouaire and Legube, 2019). Moreover, the original chromatin landscape of the damaged locus also contributes to the decision between DSB repair pathways (Clouaire and Legube, 2015; Fortuny and Polo, 2018; Bartke and Groth, 2019).

Most of our ever-growing knowledge of the DDR and, in particular, the DSB repair mechanisms has been possible due to a set of techniques that have allowed us to create DSBs in a programed manner. In this review we are coming back on those methodologies that have recently fostered our capacity to accurately study the full complexity of repair mechanisms, allowing us to consider the genomic position of the DSB and the contribution of chromatin, as well as their crosstalk with other DNA-templated processes.

# INDUCING DSBS AT RANDOM LOCATIONS

Historically, the study of the DDR relied mostly on the artificial induction of DSBs by either chemical or physical agents stochastically throughout the genome. The genomic location of these DSBs is not homogenous in the cell population and is poorly controlled. Importantly, the number of breaks can be modulated by adjusting either the dose or the duration of the treatments. Moreover, the stochastic induction of DSBs is usually very fast, requiring seconds or a few minutes, facilitating downstream kinetic studies.

## Ionizing Radiation-Induced Breaks

The exposure of cells to a source of ionizing radiation (IR) causes the appearance of a plethora of different genomic lesions (Kavanagh et al., 2013). They can arise from the radiation directly hitting the DNA, or indirectly by the effect of radiation-induced reactive species resulting from the ionization of several molecules, including water (**Figure 2**). The source of the DNA lesions depends on the type of radiation. For example, X-rays induce DNA damage mainly through indirect effects, whereas heavy particles, such as protons, interact more directly with the DNA backbone. Importantly, radiation creates many types of damage on the DNA, including all kinds of base modifications, loss of bases, single-strand breaks (SSBs) or DSBs. Indeed, it has been estimated that IR produces ten times more SSBs than DSBs (Ma et al., 2012). The degree of heterogeneity of the lesions created by IR also depends on the nature of the radiation, mostly on its LET (linear energy transfer: the amount of energy that the particle transfers to the medium along its trajectory per distance unit) (Zirkle and Tobias, 1953). In any case, all different types of DNA damage are quickly repaired, except for DNA breaks. DSBs formed upon ionizing radiation exposure are normally clustered SSBs, i.e., usually formed when two DNA lesions appear in opposite strands in close proximity ($<$10 bp) (Milligan et al., 1995). The broken DNA ends produced by radiation usually show chemical alterations, being considered "dirty" ends (Weinfeld and Soderlind, 1991). While IR induces breaks stochastically all over the genome, the randomness also depends on the LET of the radiation. Indeed, high LET particles tend to produce clusters of DSBs in close proximity (Löbrich et al., 1996; Newman et al., 1997). Additionally, high LET radiation seems to induce DSBs less randomly than photons in high-order chromatin structures (Radulescu et al., 2006).

Of interest, upon DSB induction following exposure to radiation, many DDR factors tend to accumulate temporarily at sites of DNA damage, forming the so-called Ionizing Radiation-Induced Foci (IRIF) (Ciccia and Elledge, 2010; Polo and Jackson, 2011). Importantly, some of these, including γH2AX, can spread over megabases along the DNA flanking the break (Iacovoni et al., 2010). If combined with the use of specific antibodies or fluorescent tagged-versions, this strong regional concentration allows for the visualization of IRIF under a fluorescent microscope (**Figure 2**). Hence IRIF formation has

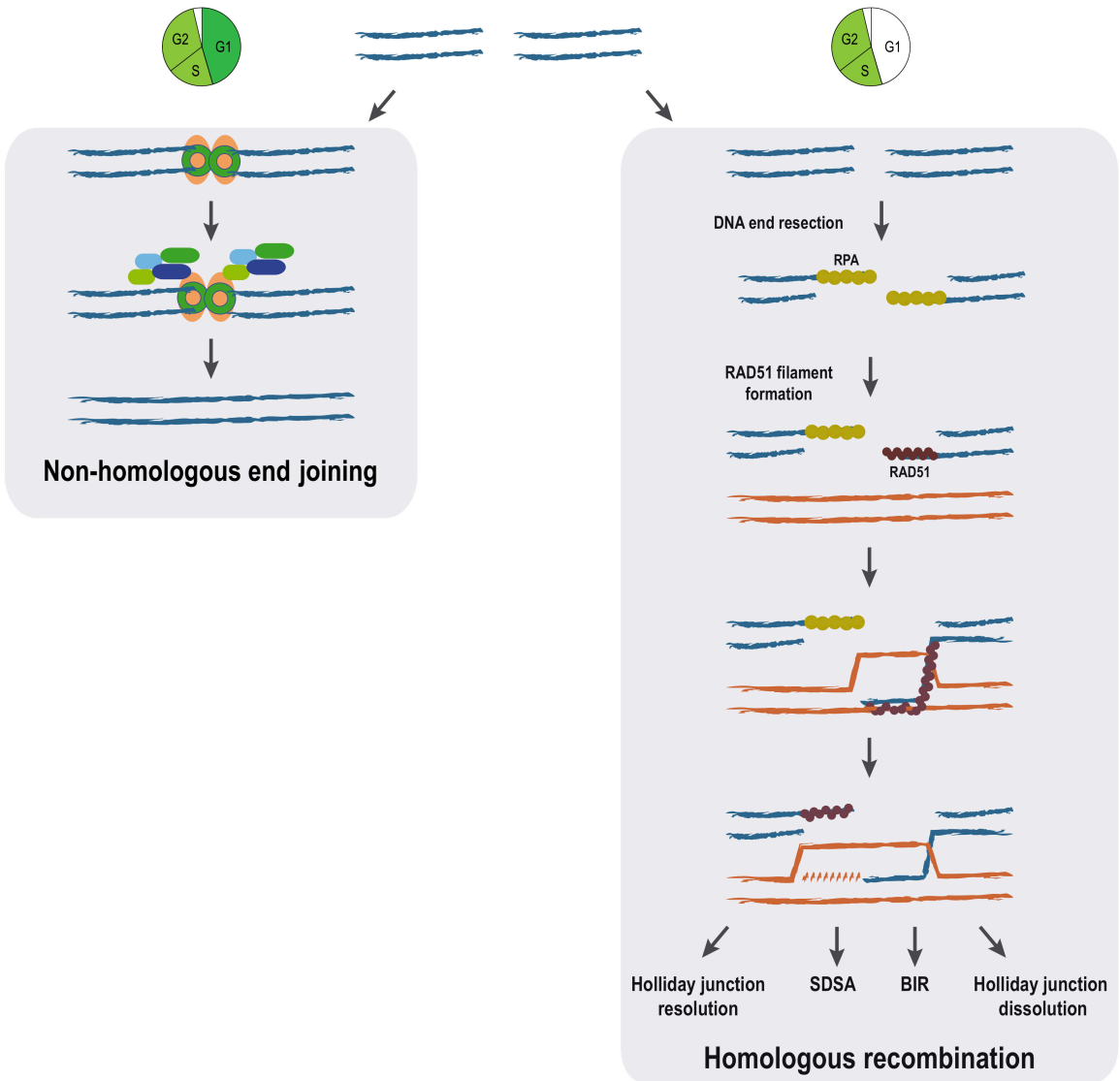


**FIGURE 1 |** Pathways for DSB repair. The main steps of non-homologous end joining and homologous recombination repair mechanisms are represented. Homologous recombination can proceed through distinct pathways (holiday junction resolution, synthesis-dependent strand annealing (SDSA), break-induced replication (BIR) and holiday junction dissolution) all sharing identical initial steps. The cell cycle is a major determinant of the choice between the DSB repair mechanisms. While NHEJ is available throughout interphase, homologous recombination pathways are restricted to S/G2 phases of the cell cycle.

been and still is, one of the easiest and most used tools to study the recruitment of DNA repair factors during the DDR. Additionally, since some of them, such as γH2AX foci, appear specifically in response to a DNA lesion and disappear when the repair process has been completed, clearance of IRIF provides a simple way to analyze the kinetics of DNA repair (Bouquet et al., 2006).

Analysis of IRIF can also inform on the DNA repair pathway choice. For instance, early steps of HR can be observed by the accumulation of the MRN complex or CtIP that will in turn be responsible of the formation of ssDNA (Mirzoeva and Petrini, 2001; Sartori et al., 2007). Resection products can also be observed by the accumulation of RPA (Sartori et al., 2007; Cruz-García et al., 2014; López-Saavedra et al., 2016; **Figure 2**). An alternative is the observation of BrdU-labeled ssDNA using non-denaturing conditions in cells treated with this thymidine analog for one cell cycle to ensure that one DNA strand is completely labeled in all chromosomes (Sartori et al., 2007). For later DNA repair steps, RAD51 accumulation is the preferred marker of recombination (Mirzoeva and Petrini, 2001; **Figure 2**). NHEJ proteins, however, are difficult to see at DNA damage foci due to the low number of units bound to each DSB and the high background levels. Thus, specific protocols have been developed for their observation (Britton et al., 2013). Alternatively, other accessory factors of NHEJ and HR, such as 53BP1 or BRCA1, respectively, can be used as a proxy for these DNA repair pathways

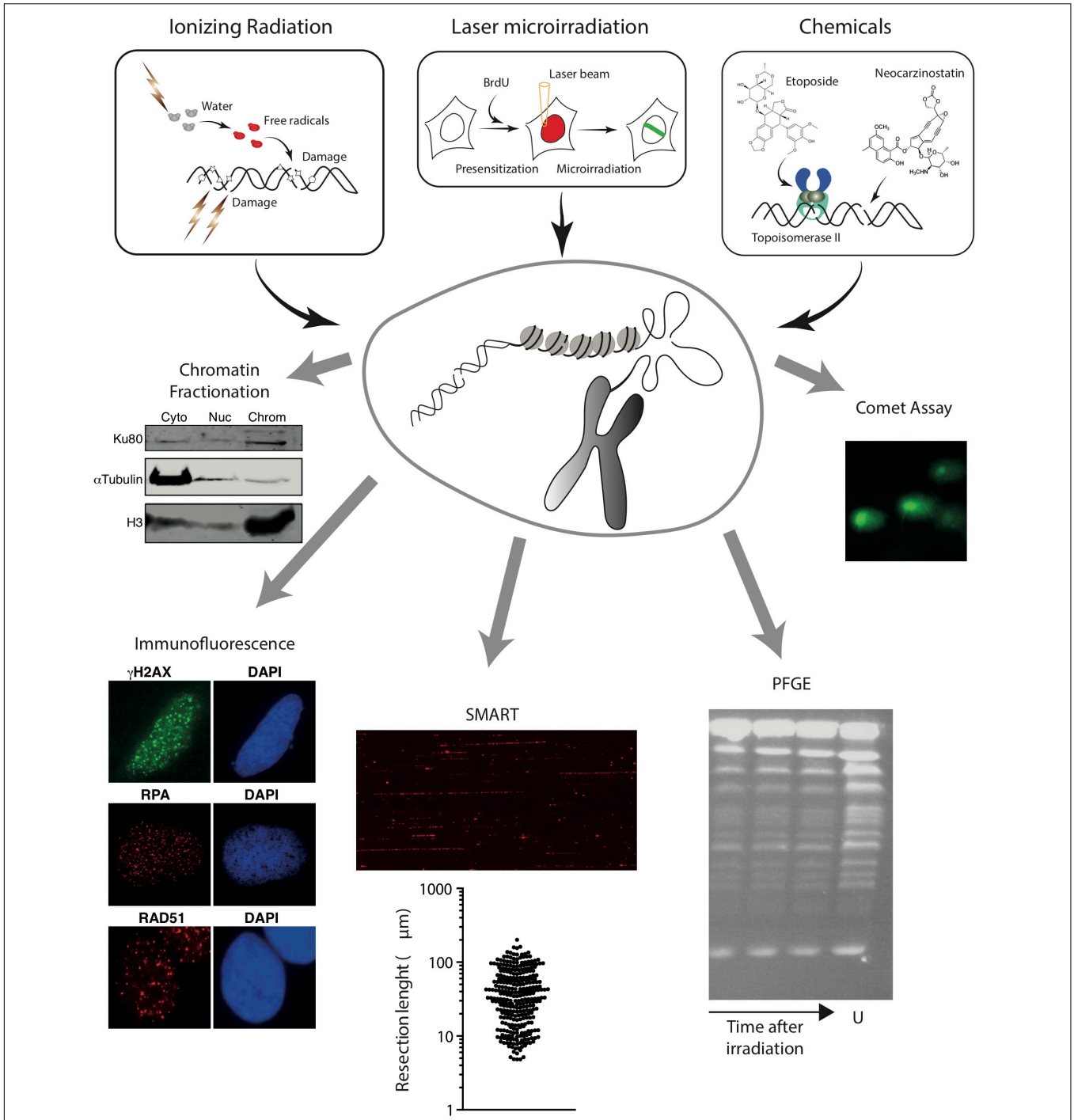


**FIGURE 2 |** Schematic overview of methods to induce random DNA breaks in the genome using radiation (top left) or chemical agents (top right). The energy of radiation can be transferred directly to the DNA molecule or can ionize other molecules like water that will then attack the DNA. In addition to DNA breaks, radiation damage induces additional modifications on the DNA, represented as stars, pentagons or triangles. Examples of chemical induction of DSBs by the direct attack of DNA (using drugs such as neocarzinostatin) or indirectly by affecting specific proteins (Etoposide inhibits the topoisomerase cycle) are shown. Experimental approaches that can be coupled with these methods to induce DSBs are also represented in the bottom. From left to right, chromatin fractionation, to observe the accumulation of a protein on the cytoplasmic (Cyto), nucleoplasmic (Nuc), or chromatin (Chrom) fractions; Immunofluorescence, to visualize the formation of nuclear foci using specific antibodies; SMART, to measure the length of resected DNA; PFGE, to visualize the presence of pieces of broken chromatin (in the figure, S. cerevisiae chromosomes untreatated (U) or at different times upon irradiation); and comet assay, to study the appearance of breaks at the single-cell level. For details, see the main text.

(Chapman et al., 2013; Escribano-Díaz et al., 2013; Zimmermann et al., 2013). Additionally, for low abundant factors, the signal can be boosted by using a Proximity Ligation Assay (PLA) to visualize if our protein or specific post-translational modification of interest is in close proximity to factors/modifications known to enrich at DSBs, such as γH2AX (Gullberg et al., 2003). A recent variation of the PLA, the DNA damage *in situ* ligation followed by proximity ligation assay (DI-PLA), allows detection and imaging of individual DSBs in cells (Galbiati et al., 2017).

If immunofluorescence analysis of IRIF is not appropriate (for instance, due to lack of antibodies or low amount of protein at DNA breaks precluding the observation of a positive signal under the microscope), the binding, recruitment, retention or release of specific proteins can be studied using a chromatin fractionation approach (**Figure 2**). First used to analyze the recruitment of NHEJ factors (Drouet et al., 2005), it can be adapted for any factor if there are specific antibodies that work in western blot. Briefly, chromatin fractionation consists in the separation of cytosolic, nucleoplasmic, and chromatin fractions from undamaged and radiation-exposed cells. The resolution of the proteins in SDS-PAGE followed by western blotting using appropriate antibodies from samples collected at different time-points upon DNA damage uncovers the dynamics of recruitment/retention/release of the studied factors. This method can be combined with depletion or inhibition of specific proteins, therefore uncovering the hierarchy of recruitment of different DNA repair factors to DSBs.

DNA resection can be specifically investigated with high resolution using single-molecule analysis of resection tracks (SMARTs). This is a modified DNA combing approach, in which resection of broken DNA ends leads to the exposure of otherwise inaccessible BrdU-epitopes previously incorporated in the DNA. When combined with an immunodetection protocol using fluorescence microscopy, SMARTs allows the direct visualization and quantification of individual tracks of resected DNA after IR (Cruz-García et al., 2014; Huertas and Cruz-Garcia, 2018; **Figure 2**).

Finally, approaches such as pulsed-field gel electrophoresis (PGFE) or single-cell gel electrophoresis (also known as comet assay) can also be combined with IR exposure to directly investigate DSB repair (**Figure 2**). IR-induced DSBs fragment the genome in smaller portions, which can be measured using PFGE to estimate the number and repair of DNA breaks. This technique allows the separation of rather large DNA pieces by forcing them to pass through an agarose matrix in response to changing electric fields (Schwartz and Cantor, 1984; Carle and Olson, 1985). Yeast chromosomes are small enough to be resolved in PFGE (Schwartz and Cantor, 1984; Carle and Olson, 1985; **Figure 2**), thus fragmentation due to DNA damage can be observed by the appearance of a smear of smaller bands (Contopoulou et al., 1987). The much larger mammalian chromosomes, on the contrary, remain on the wells during PFGE, and only smaller fragments caused by random DSBs will enter the gel (Ager et al., 1990). The size distribution of the DNA portions is dependent on the number of breaks. Thus, PFGE reveals the appearance of DSBs and estimates their number. Moreover, by taking samples at fixed times after exposure to a DNA damaging source, PFGE can be used to quantify the kinetics of DNA repair. A variation of this technique was developed in the Resnick laboratory using circular chromosomes in yeast or Epstein-Barr virus episomes in human cells (Ma et al., 2008, 2012). Another variation of this technique, the single-cell gel electrophoresis or comet assay, is a convenient way to estimate the number of DSBs created upon a given treatment with DNA damaging agents, such as IR, and to follow the kinetics of DNA repair in individual cells. Comet assays can be performed using either neutral or alkaline buffers to focus on DSBs or SSBs, respectively. Briefly, cells are treated with the DNA damage source, embedded in agarose to retain the nuclear structure, lysed and subjected to electrophoresis (Olive et al., 1991). DNA is attracted to the anode, but only broken fragments are small enough to abandon the nucleus (**Figure 2**). After staining with a DNA dye, nuclei are observed with a fluorescent microscope and the displacement of DNA from the nucleus depends on the number of breaks per genome (Olive et al., 1991). By analyzing samples at different time points after DSB induction, the kinetics of repair can be estimated.

### Key Points

(+) Radiation exposure provides an easy and robust way to analyze the recruitment of proteins to sites of DNA damage (provided that their level of binding is high enough) and to study the DNA repair kinetics using different approaches (e.g., γH2AX foci disappearance, comet assays, or PGFE).

(−) Radiation not only induces DSBs but also a plethora of other damages in the cell, and creates "dirty" ends, mostly in a random manner on the genome, hence likely biased toward the untranscribed genome in higher eukaryotes (given that genes represent a minority of the mammalian genome). Moreover, since radiation induces DSBs at unknown locations, and in a non-homogenous manner in the cell population, locus-specific analyses of DDR factor recruitment or chromatin modifications using chromatin immunoprecipitation (ChIP) studies, for instance, is not possible.

* Additionally, each readout of these stochastic DSBs has its own pros and cons. For example, ssDNA observed by SMART, RPA or BrdU foci might reflect unwound DNA; the COMET assay also detects apoptotic cells, albeit the tail shape is different; chromatin accumulation of some factors might occur independently of DNA damage and in response to other signals. Thus, in all cases, appropriate controls must be used.

## Non-ionizing Radiation: Laser Beams

In addition to IR-induced DNA damage, in which cells are exposed to an X-ray lamp or a Cesium irradiator, non-ionizing radiation can also be used to study DSBs. For instance, ultraviolet A (UVA) radiation can be used to create hundreds of DSBs along the path of a laser beam (line or spot) through laser scanning microscopy (Lukas et al., 2003). UVA does not directly generate DSBs. However, pre-treatment of cells with the thymidine analog BrdU for one cell cycle to allow its incorporation in one DNA strand, sensitizes DNA to UVA, causing the appearance of clustered SSBs and DSBs along the laser beam track. Laser irradiation provides two main advantages. First, one can decide

where to direct the laser beam in the nucleus, allowing to target specific subnuclear compartments, such as the nucleolus (Kruhlak et al., 2007). Second, the concentration of hundreds of breaks along a laser track facilitates the observation of the recruitment of factors that either do not spread at all, or gradually increase over time, and for which foci are therefore difficult to see, especially at early time points. As such, laser irradiation represents the most powerful tool to accurately determine the kinetics of DDR factors, providing a temporal resolution below 10 s.

Combined with the expression of fluorescently labeled proteins, laser microirradiation has provided unprecedented temporal resolution of the sequence of events following DNA damage (Kochan et al., 2017; Aleksandrov et al., 2018). This approach can also be complemented with FRAP and FLIP studies (see Mortusewicz and Leonhardt, 2007). The use of fluorescently-tagged histone proteins allowed the study of chromatin dynamics following damage with great resolution (Burgess et al., 2014; Luijsterburg et al., 2016; Sellou et al., 2016; Smith et al., 2018). Finally, this method has been useful to investigate the release of factors from DSBs and the post-translational modifications (PTMs) that drive such dynamics. In this case, the signal void created by the absence of the protein or by the removal of a specific PTM can be seen as a negative stripe (or anti-stripe) (Chou et al., 2010; López-Saavedra et al., 2016).

### Key Points

(+) Laser microirradiation represents the best technique today to temporally resolve the sequence of events at DSBs, allowing to observe very early (<10 s) and/or transient repair proteins recruitment and chromatin modifications.

(−) Microirradiation induces a large number of localized, clustered DNA lesions (not only DSBs), that may also initiate specific responses. Moreover, it is neither amenable for molecular characterization of the repair outcome at the sequence level, nor for ChIP, which limits the spatial resolution that can be achieved.

## Chemically Induced Breaks

In contrast to radiation treatments, that require specialized and expensive equipment, chemical-induction of DSBs is cheap and easy to implement in any laboratory and can be coupled with almost any experimental protocol. Usually, cells are treated with a defined concentration of a chemical agent for a fixed amount of time. It is important to distinguish between acute (from minutes to a few hours) versus chronic (for days) treatments, as the responses will vary enormously. Many types of chemical agents can indirectly cause DNA breaks. For example, chemical inhibition of topoisomerases I and II causes SSB and DSB respectively (Huang et al., 2003; **Figure 2**). SSBs caused by camptothecin, a common inhibitor of topoisomerase I, can, in turn, be converted to DSBs during replication. Replication inhibitors, such as HU or aphidicolin, and crosslinker agents, like cisplatin or mitomycin C, can also cause one ended DSBs due to fork collapse (Saintigny et al., 2001; Noll et al., 2006). Additionally, several chemical agents imitate the effect of ionizing radiation and break the DNA directly (**Figure 2**). These radiomimetic drugs include bleomycin, phleomycin or neocarzinostatin (Sleigh, 1976; Edo and Koide, 1997; Chen and Stubbe, 2005).

Of importance, given their different modes of action, all the above-mentioned drugs will produce different types of DSBs: either located at different genomic regions and/or introduced during different cell-cycle stages. For instance, DSBs created by radiomimetic drugs show a bias toward specific sequences (Murray and Martin, 1985; Burden et al., 1996). Moreover, topoisomerase II poisons such as etoposide preferentially induce lesions at CTCF binding loci located close or within transcriptionally active units (Canela et al., 2017, 2019; Gothe et al., 2019). Topoisomerase I and replication inhibitors induce DSBs specifically during S phase or the following mitosis (Saintigny et al., 2001; Huang et al., 2003).

The analysis of DSBs induced by chemical agents can be performed by the same approaches described for irradiation-induced breaks (**Figure 2**). In addition, a number of genome-wide methodologies have been recently developed to directly map DSB distribution at a nucleotide resolution across the genomes in a cell population (Bouwman and Crosetto, 2018) including for instance Break-seq, BLESS, iBLESS, BLISS, DSB-capture, End-seq and BrlTL (Hoffman et al., 2015; Canela et al., 2016; Lensing et al., 2016; Biernacka et al., 2018; Mirzazadeh et al., 2018; Shastri et al., 2018). These techniques are well suited to investigate DSBs that occur non-randomly across the genome such as those induced by topoisomerase II poisons for instance. Of importance they not only provide an information about DSBs positions on the genome, but they are also quantitative, hence providing an estimate of break frequency in the cell population (Aymard et al., 2017; Canela et al., 2019).

### Key Points

(+) Treatment with genotoxic compounds represents an easy to implement and robust way to analyze the recruitment of DSB repair factors and to study the repair kinetics using different approaches (kinetics of γH2AX foci, comet assays, and PGFE).

(−) Drugs produce different types of DNA damage, at different genomic loci, and most show a preference for specific cell cycle stages, which should be carefully considered during data interpretation.

# METHODS TO INDUCE ANNOTATED DNA BREAKS AT TRANSGENIC LOCI INSERTED IN THE GENOME

Different labs have sought to develop tools for the site-specific induction of DNA breaks making use of restriction enzymes targeting integrated exogenous cleavage sites, otherwise absent from the genome. Such tools overcome the ambiguity of DNA lesions introduced by previous methods and allow the inspection of protein recruitment during the DDR to a site-specific DSB and the assessment of chromatin remodeling events with nucleosome resolution. Moreover, they can be combined with strategies to control the timing of DSB induction, for instance by controlling the nuclear translocation of the restriction enzyme, affording a

valuable strategy to measure kinetic parameters of the DDR in live cells (Berkovich et al., 2007; Soutoglou et al., 2007).

The first reporter system, employing a site-specific DSB at a reporter transgene integrated into the genome of mammalian cells was developed in the mid-1990s. This genetic assay was devised by the Jasin lab to detect and quantify HR repair of DSBs induced by the rare-cutting endonuclease, I-*Sce*I (Rouet et al., 1994; **Figure 3**). Following this seminal work, a large number of labs further developed similar strategies based on I-*Sce*I cut of a transgenic locus to investigate various aspect of the DDR, including repair pathway preferences and efficiency (Gunn and Stark, 2012; Gelot et al., 2016), DNA-ends mobility and translocation (Soutoglou et al., 2007; Roukos et al., 2013), and the crosstalk with transcription (Shanbhag et al., 2010; Ui et al., 2015; Vítor et al., 2019). For example, Soutoglou et al. (2007) developed a cell system to visualize the dynamics of a single DSB induced at a defined genomic site in mammalian cells and demonstrated that broken ends are immobile in the nuclear space. For that, stable cell lines derived from mouse embryonic fibroblasts (NIH3T3) were generated containing a single I-*Sce*I restriction site flanked by arrays of lac-repressor binding sites and tetracycline-response elements (L-I-*Sce*I-T array) (**Figure 3**). Expression and binding of fluorescently-tagged lac and tetracycline-repressors to these arrays enabled

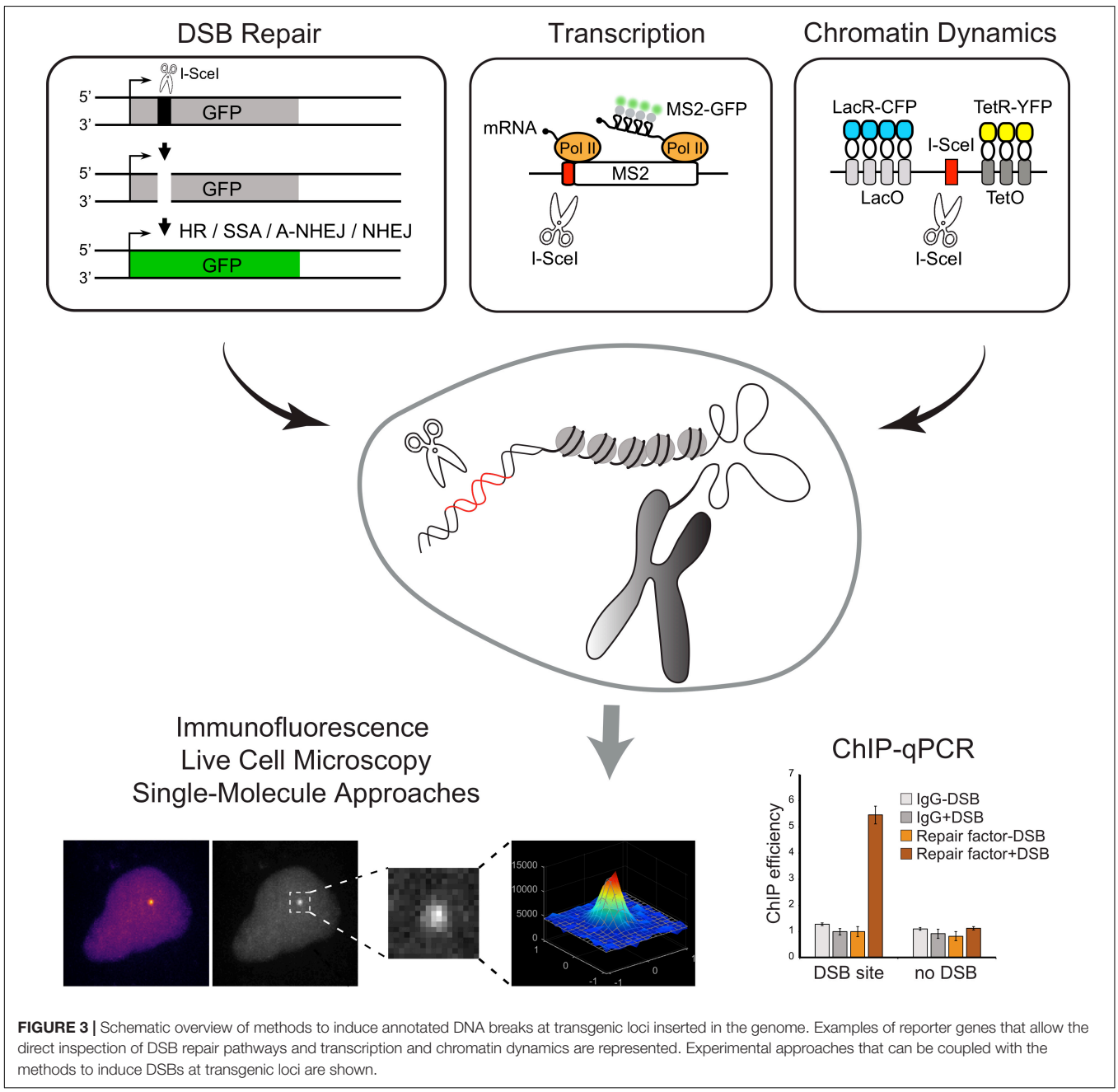


**FIGURE 3 |** Schematic overview of methods to induce annotated DNA breaks at transgenic loci inserted in the genome. Examples of reporter genes that allow the direct inspection of DSB repair pathways and transcription and chromatin dynamics are represented. Experimental approaches that can be coupled with the methods to induce DSBs at transgenic loci are shown.

the simultaneous detection of both DNA ends. The use of an I-*SceI* enzyme fused to a glucocorticoid receptor (I-*SceI*-GR) that translocated to the nucleus upon triamcinolone acetonide (TA) addition, allowed the controlled induction of a DSB at the L-I-*Sce*I-T array and the live-cell tracking of the broken DNA ends in real-time (Soutoglou et al., 2007).

Additional systems were further developed to generate multiple DSBs on a specific transgene, thus rendering the DNA repair easier to visualize. The Greenberg lab developed a noteworthy single-cell assay specifically designed to simultaneously analyze both the DSB repair and its effects on local transcription. The experimental procedure was based on the introduction of multiple nuclease-induced DSBs upstream the promoter of an inducible transgene, modified to enable the visualization of transcriptional and translational events (Shanbhag et al., 2010). The reporter system, integrated in the genome of a human osteosarcoma (U2OS) cell line, is visualized upon binding of the mCherry-fluorescently-tagged lac-repressor protein (mCherry-LacI) to a lac-operator array. Nascent transcription is visualized by the accumulation of fluorescent MS2-binding proteins at the transcription site, upon binding to nascent MS2 stem-loop structures present at the reporter gene RNA (Shanbhag et al., 2010). Expression of the *Fok*I nuclease domain fused to the mCherry-LacI creates DSBs at the lac operator array (Shanbhag and Greenberg, 2013). Of note, this approach leads to persistent and extensive DSB induction and the time of damage induction is dependent on the expression of mCherry-LacI-*Fok*I. A similar system to study transcription in proximity to DSBs was engineered by Ui et al. (2015). The authors established a U2OS cell line harboring multiple copies of an array of transcription units including tetracycline response elements (TRE) sites, MS2 sequences and I-*Sce*I restriction sites (Ui et al., 2015). Upon tamoxifen treatment, the mCherry-tTA-ER fusion proteins translocate into the nucleus and localize at transcription sites (TRE sites), to induce transcription activation, detected by the accumulation of fluorescently tagged-MS2 protein (Rafalska-Metcalf et al., 2010). Expression of a plasmid encoding I-*Sce*I generates DSBs at target restriction sites, thus enabling the study of the effect of DSBs on transcription. Using this experimental system the authors reported a DSB-induced transcriptional repression mechanism involving the transcription elongation factor ENL (Ui et al., 2015). More recently, the de Almeida lab developed a set of reporter genes that allow the direct visualization of transcription with single-molecule resolution upon the controlled induction of a unique DSB (Vítor et al., 2019; **Figure 3**). A single I-*Sce*I restriction site was inserted in either the promoter-proximal region or within an internal exon of a reporter gene. The binding of fluorescent proteins to MS2 and/or PP7 stem loops at the nascent transcripts allows measurements of transcription dynamics upon induction of the DSB. The exact timing of DSB induction is controlled using an I-*Sce*I-GR fusion protein. Using these reporters, the authors found that whereas induction of a DSB at the promoter region suppresses transcription, a DSB generated within an internal exon drives bidirectional break-induced transcription initiation (Vítor et al., 2019). In addition to live-cell microscopy imaging, these reporters may be combined with ChIP-qPCR, providing a valuable tool to directly inspect the recruitment of DNA repair factors to a DSB, to assess histone modifications or measure nucleosome occupancy at broken ends.

The direct visualization of DNA break-induced transcription activation using reporter genes, support a model whereby the DDR signaling involves the action of non-coding RNAs (ncRNAs) generated at sites of DNA damage (Michelini et al., 2018). To investigate the role of such DSB-induced ncRNAs in the DDR, the d'adda di Fagagna lab developed the RNase A treatment and reconstitution (RATaR) method, in which different RNA species of interest are used to reconstitute cells previously treated with recombinant RNase A (Michelini et al., 2019). RATaR may be employed to address the role of ncRNAs in the recruitment of repair proteins during the DDR using imaging approaches.

### Key Points

(+) I-*Sce*I or *Fok*I mediated DSB induction on transgenic loci are powerful systems to investigate the response to clean DSBs. These systems allow analyzing the repair event at a molecular resolution, the repair frequency (thanks to designed reporters cassettes) and the DNA damage repair/signaling in single cells (using imaging approaches). They can be combined with additional reporters to investigate with great detail the functional links between the DDR and transcriptional activity, chromatin modification and spatial organization, or DNA replication, for instance.

(−) These systems rely on transfection, transcriptional regulation, or nuclear localization of the endonuclease. Consequently, they cannot provide the same temporal resolution achieved using microirradiation, where DSB induction is immediate and highly synchronized. Moreover, the transgenic nature of the analyzed loci calls for caution, especially when repeat-rich transgenes are used (creating either multiple clustered DSBs or a single DSB but in a highly repeated transgenic locus, which may display a peculiar chromatin structure). Finally, the accurate repair of endonuclease-created breaks reconstitutes the target site, therefore being re-cleavable until the target site has been mutated. Hence, most of the outputs measured in these experimental contexts address mutagenic repair, leaving faithful repair out of reach.

## METHODS TO INDUCE DNA BREAKS AT SPECIFIC ENDOGENOUS LOCI IN THE GENOME

In order to bypass the need for introducing a transgene and to avoid potential, non-generalizable, side effects of transgenic loci on the repair process (e.g., in the case of LacI repeats, a high copy number triggering a peculiar chromatin state), efforts have been made recently to develop alternative systems where DSBs can be induced at endogenous, annotated loci on the genome (**Figure 4**). On one hand, homing endonucleases and type II restriction enzymes have been used, allowing to induce breaks at annotated but not controllable positions, and on the other hand, the development of transcription activator-like effector nucleases

(TALEN) and more recently of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system has opened the possibility to introduce breaks at chosen loci.

## Type II Restriction Enzymes and Homing Endonucleases: Induction of Multiple DSBs but at Constrained Locations

### I-*Ppo*I

The Kastan lab developed a system that uses the eukaryotic homing endonuclease I-*Ppo*I, which has a recognition sequence of 15bp, to form site-specific DSBs within endogenous target sites of the human genome (Berkovich et al., 2007). Expression of I-*Ppo*I in human cells results in the production of DSB at one site within the 28S ribosomal RNA gene, present in ∼300 copies, and fifteen additional unique loci. To tightly control DSB induction, a ligand-binding domain of the estrogen receptor (ER) was fused to I-*Ppo*I. The addition of 4-hydroxytamoxifen (4-OHT) promotes rapid nuclear localization of ER-I-*Ppo*I and the subsequent time-dependent cleavage of the endogenous sites. Using this system, the Kastan lab disclosed the distribution of Nbs1 and ATM, and histones (Berkovich et al., 2007; Goldstein et al., 2013) at DSBs by ChIP-qPCR. This system was used by others to investigate the dynamics of the transcription machinery following I-*Ppo*I DSB induction in RNA Polymerase II-transcribed genes, revealing a DNAPK-dependent break-induced transcriptional arrest (Pankotai et al., 2012; Caron et al., 2019), or to investigate the DDR induced in the nucleolus (Harding et al., 2015; Warmerdam et al., 2016; Pefani et al., 2018). I-*Ppo*I has been further applied to interrogate DSB repair mechanisms in other organisms, such as fission yeast (Sunder et al., 2012; Kuntz and O'Connell, 2013; Ohle et al., 2016) and mice (Kim et al., 2016). Of interest, in the latter, both temporal and spatial regulation of I-*Ppo*I activity was achieved by using a GFP-I-*Ppo*I endonuclease fused to an ER domain for tamoxifen-dependent temporal induction and whose tissue-specific expression was dependent on Cre recombinase. The results obtained using this *in vivo* model system showed transient, and DDR-dependent, decrease in gene expression of break-bearing - but not more distant - genes, further reversed upon DSB repair (Kim et al., 2016).

### I-*Cre*I

In Drosophila, the I-*Cre*I homing endonuclease has also been used to create annotated DSBs in the ribosomal DNA (Royou et al., 2010). This allowed the authors to uncover a new Bub1R/Bub3/Polo kinase-dependent pathway that contributes to handle unrepaired rDNA DSBs during mitosis and to ensure correct segregation of broken chromosomes (Royou et al., 2010; Derive et al., 2015).

### *Asi*SI

Another DSB-inducible tool developed to create multiple endogenous, sequence-specific breaks, makes use of the *Asi*SI - 8bp cutter - restriction enzyme. The Legube lab, fused *Asi*SI to a modified ER ligand-binding domain, which controls nuclear localization of *Asi*SI–ER fusion protein, and to an auxin-inducible degron enabling controlled ubiquitination and degradation of the enzyme (Iacovoni et al., 2010; Massip et al., 2010; Aymard et al., 2014). Stable integration of this construct in the genome of U2OS cells generated a DSB inducible via *Asi*SI (DIvA) system, where multiple annotated DSBs can be induced after 4-OHT treatment and DNA repair accurately monitored following auxin treatment. *Asi*SI induces 100-200 DSBs across the human genome [as determined by BLESS (Clouaire et al., 2018) and BLISS (Iannelli et al., 2017)], as well as one break in the ribosomal DNA repeat (Marnef et al., 2017). This system is then amenable to compare DNA repair at various genomic positions. Importantly, while *Asi*SI is not able to damage heterochromatin, likely due to both the DNA methylation status and decreased accessibility of compacted chromatin (Iacovoni et al., 2010; Clouaire et al., 2018), it induces DSBs at both transcribed and untranscribed loci (Aymard et al., 2014; Clouaire et al., 2018). The DIvA system has been used to inspect DNA repair pathway preferences at different chromatin regions (Aymard et al., 2014), to measure site-specific resection (Zhou et al., 2014) and repair kinetics and translocation frequency (Aymard et al., 2014; Cohen et al., 2018), and is instrumental to investigate the role of repair factors in HR or NHEJ [see, for example (Jacquet et al., 2016; Schrank et al., 2018)] Furthermore, combined with ChIP-seq, or any other high throughput genomic methods, it allows investigating DNA repair simultaneously at multiple DSBs and at high resolution. Consequently it has been extensively used to provide high-resolution maps of repair proteins and chromatin changes (Iacovoni et al., 2010; Caron et al., 2012, 2015; Aymard et al., 2014; Clouaire et al., 2018), of R-loops (Cohen et al., 2018; Lu et al., 2018), or long-range contacts (Aymard et al., 2017) around several breaks in the human genome. When combined to transcription mapping (RNA-seq, BrU-seq, Pol II ChIP-seq, or NET-seq) it disclosed insights into the interplay between γH2AX profile and transcription (Iacovoni et al., 2010), on the behavior of transcription at DSBs (Iacovoni et al., 2010; Iannelli et al., 2017; Cohen et al., 2018; Burger et al., 2019), as well as a novel DSB repair pathway coupled to transcription (Marnef et al., 2017).

### Key Points

(+) These systems represent powerful tools to compare DNA repair events that occur at different genomic loci and, because they induce DSBs at annotated positions in a homogenous manner in the cell population, are compatible with all high resolution, high throughput sequencing-based techniques such as ChIP-seq, Hi-C, etc.

(−) For all these systems, as for the above-mentioned I-*Sce*I based systems: (i) DSB production is not immediate nor synchronized in the cell population and (ii) accurately repaired DSB can be re-cleaved. Hence while being powerful to analyze the spatial distribution of repair protein and chromatin changes around DSBs, they preclude a fine temporal resolution of these events. Moreover, the position of the DSBs is dictated by the target site of the chosen

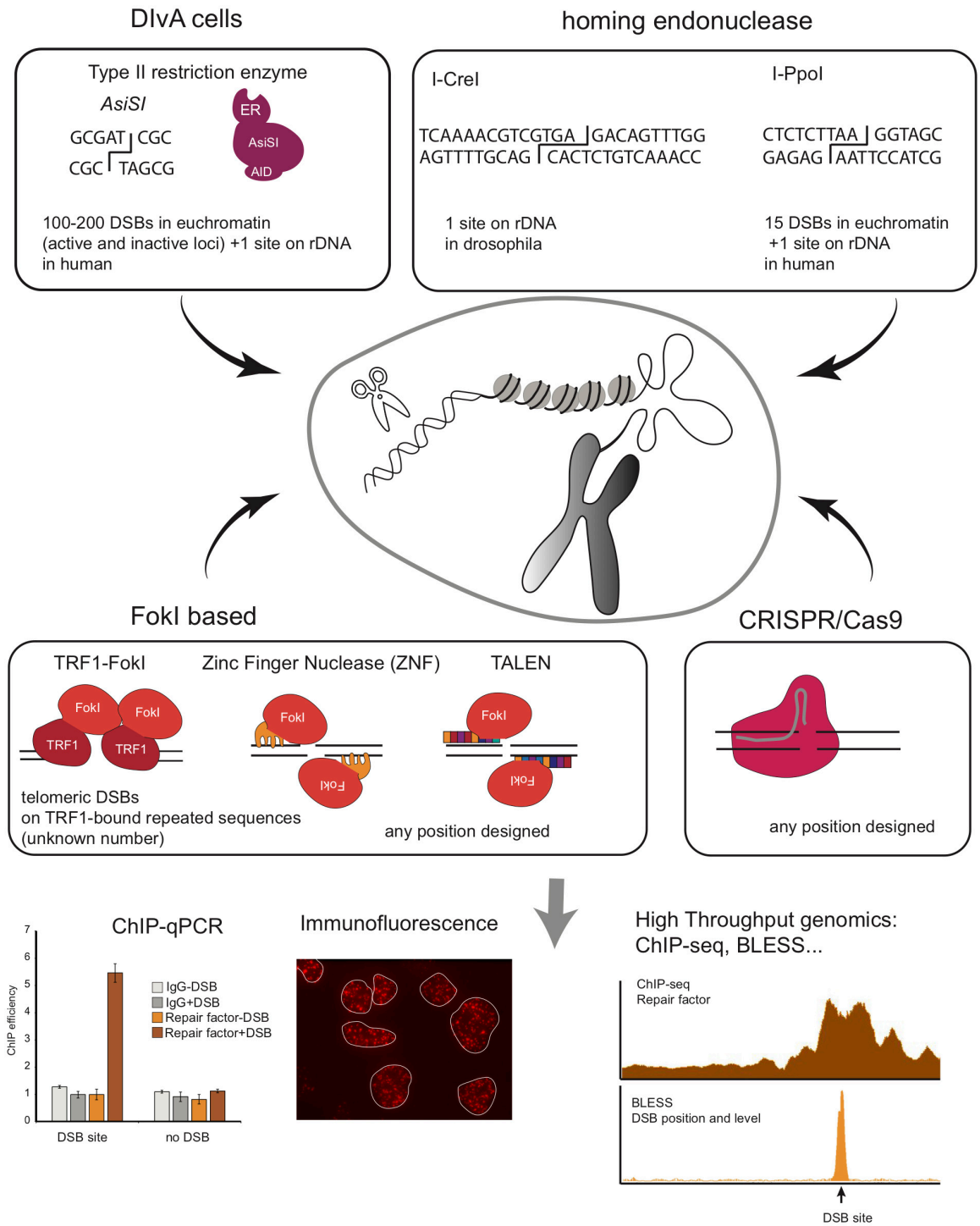


**FIGURE 4 |** Schematic overview of methods to induce annotated DNA breaks at endogenous loci, including Type II endonuclease, Homing endonucleases, *FokI* based system, and CRISPR/Cas9 system. Given that these DSBs are induced at annotated positions and in a homogeneous manner in the cell population, one can use ChIP to investigate protein recruitment at the site of damage. This can also be coupled to high throughput sequencing analyses to investigate simultaneously repair events at multiples breaks (ChIP-seq). Finally, BLESS, BLISS, Break-Seq and any other related genome wide methods to map DSB distribution across genomes can be used to analyze repair kinetics of these annotated DSBs.

enzyme, which can represent a limitation to the number of different loci analyzed.

## Zinc Finger Nucleases, TALEN and CRISPR/Cas9: Induction of a Single DSB but at a Chosen Locus

A number of specific tools have more recently allowed to induce DSBs at chosen endogenous genomic loci.

### Fusing *Fok*I to a Protein of Interest

To introduce DSBs at specific loci of interest, it is possible to fuse the *Fok*I endonuclease to a protein able to specifically target a particular locus. This approach was implemented for example to induce DSBs at telomeres by fusing *Fok*I to the shelterin protein TRF1 (Tang et al., 2013; Cho et al., 2014; Doksani and de Lange, 2016).

### Zinc Finger Nucleases and TALEN

Zinc finger nucleases (ZNF) are chimeric proteins comprised of both a zinc finger domain designed to recognize a specific locus and the *Fok*I nuclease. Using a pair of ZNF binding opposite strands allows the introduction of a DSB at a locus of interest. For instance, ZNF able to target the intron 1 of the PPP1R12C gene (p84-ZNF) (Urnov et al., 2005) were used in order to investigate chromatin changes by ChIP-qPCR (Xu et al., 2012; Ayrapetov et al., 2014; Gursoy-Yuzugullu et al., 2015) and translocation biogenesis (Ghezraoui et al., 2014).

TALE proteins were discovered as composed of a succession of 34aa monomers, each displaying the ability to recognize one nucleotide. Fused to *Fok*I, this system provides a rapid and easy way to design sequence-specific nucleases called TALEN. TALEN have been used to investigate DNA repair in a large number of organisms and genomic contexts, such as in CTG trinucleotide repeats in budding yeast (Mosbach et al., 2018), or to understand the influence of the transcription status of a locus on the repair pathway choice (Aymard et al., 2014).

### CRISPR/Cas9

The discovery of the CRISPR/Cas9 system in 2013 strongly revolutionized the DDR field by providing the ability to introduce DSBs at annotated loci, in a particularly simple and efficient manner, by the mean of a small guide RNA embedded in the Cas9 nuclease. For instance, this approach has been used successfully to induce DSBs and study DNA repair in rDNA (van Sluis and McStay, 2015; Korsholm et al., 2019). It allowed demonstrating RNA Pol I transcription inhibition *in cis* to rDNA DSBs and nucleolar reorganization upon rDNA breakage. CRISPR/Cas9 has also been instrumental to study the repair of heterochromatin. The Soutoglou lab used it to induce DSBs in a-satellites in mouse cells, demonstrating that, as for rDNA, heterochromatin foci are reorganized in G2 upon DSB induction (Tsouroula et al., 2016). CRISPR/Cas9 was also used to induce DNA breaks at multiple unique loci in order to study translocation biogenesis and repair mechanisms, such as on c-Myc, MLL, TMPRSS2, as well as G4 enriched or non-enriched genes (Ghezraoui et al., 2014; Day et al., 2017; Iannelli et al., 2017; Panchakshari et al., 2018; Wei et al., 2018; Meisenberg et al., 2019). However, of importance, it is yet unclear whether CRISPR/Cas9-induced breaks behave similarly to other types of DSBs. Indeed, recent studies indicated that Cas9-induced DSBs display highly mutagenic repair with nearly no accurate repair events (Brinkman et al., 2018; Richardson et al., 2018) and evidence suggests that they may be handled by the Fanconi Anemia repair pathway rather than canonical DSB repair machinery (Richardson et al., 2018).

### Key Points

(+) Methods to induce DNA breaks at specific endogenous loci in the genome are particularly powerful in that they provide the liberty to choose the locus to be analyzed. As for the other endonuclease-mediated DSB induction systems, they are amenable to both imaging and molecular high throughput sequencing-based technologies such as ChIP-seq.

(−) Yet, similarly to the other endonuclease-mediated DSB induction, they are less suited for thorough, careful kinetics analyses since they rely on the controlled expression of the Cas9, or transient transfection of the sgRNA and accurately repaired DSBs may be re-cleaved by Cas9. Moreover, the fact that Cas9-induced DSBs may be particularly refractory to repair, and hence biased in terms of repair pathway choice, call for caution when using these systems.

## Telomere Deprotection as a Tool to Generate DSBs at Chromosome Ends

Coating of telomeres with shelterin factors including telomeric repeat-binding factor 2 (TRF2), prevents fusions of linear chromosome ends and suppresses local DNA damage responses (de Lange, 2018). Dysfunctional telomeres induce cellular responses that are highly similar to the ones elicited by DSBs, such as DDR activation and cellular senescence (Fumagalli et al., 2012; Hewitt et al., 2012). Indeed, replicative telomere shortening, which eventually culminates in telomere deprotection, induces molecular markers characteristic of DSBs and may serve as models to investigate DNA damage signaling in the context of senescence and aging (D'Adda Di Fagagna et al., 2003).

Dysfunctional telomeres can be generated through telomere uncapping and other forms of telomere damage, which may be specifically induced to activate the DDR in cycling cells. In addition to *Fok*I fusion with TRF1 described above, DSB-signaling at telomeres can be activated upon *TRF2* deletion (Celli and de Lange, 2005). Deletion of *TRF2* provokes sustained DNA damage at mammalian chromosome ends, and the resulting uncapped telomeres are processed by the NHEJ pathway (Celli and de Lange, 2005). A plethora of methods - ranging from the visualization of DNA repair factors foci using immunofluorescence to the biochemical characterization of DDR complexes assembled at dysfunctional telomeres using ChIP - can be coupled to the *TRF2* inactivation to investigate the molecular details of different aspects of the DDR. Importantly, dysfunctional telomeres have been instrumental to discover the function of various proteins in DSB repair [e.g., Rif1 (Zimmermann et al., 2013); Pol θ (Mateos-Gomez et al., 2015); the LINC complex (Lottersberger et al., 2015); or CST and shieldin (Mirman et al., 2018)].

### Key Points

(+) Using dysfunctional telomeres as surrogates for DSBs is easy to implement and can be coupled with different imaging and biochemical approaches to directly inspect the molecular details of the DDR signaling.

(−) Telomeres possess several specific features that render them particularly refractory to repair, and, when uncapped through *TFR2* deletion, show a strong bias in terms of repair pathway choice toward NHEJ. The number of dysfunctional telomeres may vary considerably between cells and this heterogeneity may raise issues related with cells viability.

## CONCLUSION

Our capacity to create DSBs in a programed manner and in such a way that is compatible with a set of diverse methodologies to investigate the events that follow DNA damage, has led to our current deep understanding of the DDR. The induction of DSBs at random locations using different sources of radiation or genotoxic compounds, provides the easiest approach to analyze the recruitment kinetics of proteins to sites of DNA damage and is a powerful strategy to temporally resolve the sequence of DNA repair events. The development of methods to induce annotated DNA breaks at transgenic loci inserted in the genome, or at endogenous loci (restriction enzymes, CRISPR/Cas9) allowed the analysis of the DDR at molecular resolution and were instrumental in disclosing functional links between the DDR and processes such as transcriptional, chromatin dynamics, and DNA replication. Yet all the tools described here display significant drawbacks. For instance, nucleases-induced DSBs undergo consecutive cycles of repair/cleavage until these have been mutated, calling for caution when investigating DNA repair using these tools. A major challenge is now to refine these DSB-inducible systems and the subsequent methodologies to analyze repair in order to overcome these limitations.

## AUTHOR CONTRIBUTIONS

All authors contributed to discussing the review contents and to writing the manuscript.

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REVIEW
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# Genome Editing Fidelity in the Context of DNA Sequence and Chromatin Structure

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Genome editing by Clustered Regularly Inter Spaced Palindromic Repeat (CRISPR) associated (Cas) systems has revolutionized medical research and holds enormous promise for correcting genetic diseases. Understanding how these Cas nucleases work and induce mutations, as well as identifying factors that affect their efficiency and fidelity is key to developing this technology for therapeutic uses. Here, we discuss recent studies that reveal how DNA sequence and chromatin structure influences the different steps of genome editing. These studies also demonstrate that a deep understanding of the balance between error prone and error free DNA repair pathways is crucial for making genome editing a safe clinical tool, which does not induce further mutations to the genome.

**Keywords: chromatin, dna editing crispr, knock in, DNA repair, nucleus**

## INTRODUCTION

Genome editing is very valuable for both medical and research purposes. Future medical applications include the correction of disease-related mutations, disruption of disease-promoting genes or even introducing novel genes (e.g., for sensitising immune system to tumour cells). Research applications range from creating knock-out/knock in cell line or organisms, and/or introducing mutations, to study the role of a particular protein, pathway or processes to creating humanized disease models. Given the tempting scope of practical use, it is of no surprise that there has been considerable effort in developing genome editing methods. The traditional way for introducing changes to the genome was by the use of spontaneous recombination, either to introduce DNA mutations or to insert sequences that would allow further use of recombinases (such as Cre) to excise genes [reviewed in Sauer (2002)]. Subsequent discoveries of zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) allowed a considerable advance in the field by allowing the introduction of DNA breaks at desired, rather than random, genomic locations [reviewed in Gaj et al. (2013)]. Nevertheless, the biggest advance in genome editing has been the more recent discovery of clustered regularly interspaced palindromic repeat (CRISPR) associated (Cas) systems (Ishino et al., 1987; Jansen et al., 2002; Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013).

Shortly after its discovery, the CRISPR-Cas9 system, a bacterial defense mechanism, was repurposed as a powerful tool for genome editing in plant, animal and human cells due to its specificity and its easier implementation. Current and future potential uses cover a wide range of application in research and clinical areas, by allowing substitution, insertion or deletion to the DNA sequence in a targeted genomic location [reviewed in Hsu et al. (2014) and Wang and Qi (2016)]. The CRISPR-Cas9 system operates through the recruitment of the RNA-guided Cas9 nuclease at a specific genomic position. The targeting relies on the complementarity between the guide RNA and the targeted sequences and the presence of an adjacent DNA protospacer motif (PAM). The Cas9 nuclease generates a DNA double strand break (DSB) at the targeted sequence adjacent to the PAM sequence (Jiang and Doudna, 2017), which then leads to recruitment of DNA repair machinery to fix the break.

Typically, DNA DSBs are repaired by (i) the error free homologous recombination (HR) pathway, which occurs in S/G2 phases of the cell cycle as it uses the homologous sequences of the sister chromatids as a repair template, and (ii) the error prone non-homologous end joining (NHEJ) pathway, which occurs throughout the cell cycle and religates DNA ends without the presence of an undamaged template (Ciccia and Elledge, 2010). In addition, other alternative end joining pathways, which rely on the presence of microhomologies (MH mediated end joining, MMEJ), have been described, these DSB repair pathways are error prone and are often associated with long deletions (Decottignies, 2013; Chang et al., 2017).

DNA end resection is a major determinant influencing DNA repair pathway choice. Unresected DNA ends, processed by the NHEJ pathway, are bound to the Ku complex (Ku70-Ku80 heterodimer) which recruits NHEJ factors including DNA-PKcs (DNA dependent protein kinase catalytic subunit), XRCC4 (X ray repair cross complementing 4) and LIG IV (DNA ligase IV) to catalyze DNA ends ligation. In contrast, the MMEJ pathway requires minimal DNA ends resection (through the CtIP-MRN complex) that reveals homologies on opposite strands that will be further involved in annealing. DNA portion between homologies is removed, leading to deletion scars. Other MMEJ factors are further recruited to resolve the break, including DNA polymerase θ (POL Q), and the DNA ligases I and III (Decottignies, 2013; Chang et al., 2017).

DNA repair pathway choice is regulated at different levels: cell cycle stage, availability and post translational modifications of DNA repair factors, chromatin status and the position within the nucleus of the break [reviewed in Kalousi and Soutoglou (2016)]. The choice of pathway can have critical consequences for the cell, since the use of error prone pathways can lead to unwanted deleterious mutations. Despite the many efforts put into characterizing repair pathways, Cas9-induced DSB repair outcomes have not been yet extensively investigated. It is crucial, for both research and clinical purposes, to precisely understand how mutation profiles observed following Cas9-induced DSB are generated, to be able to predict repair outcomes. In this review, we will focus on recent work highlighting the outcome of CRISPR-Cas9-induced DSBs in mammalian cells. Interestingly, the CRISPR-Cas9 mutational pattern appears to be non-random, highly reproducible and mainly dependent on the targeted DNA sequence.

## CAS 9-MUTATIONAL PROFILES ARE LARGELY DEPENDENT ON THE TARGET DNA SEQUENCE

Several studies have revealed the prominent role of the target DNA sequence in Cas9-dependent DNA repair outcomes. In these studies, repair outcomes were profiled by classifying the mutations generated at Cas9 target sites by the type of insertion or deletion (indel) that occurred (e.g., size, position, microhomology), and monitoring the frequency of each class of indel. van Overbeek et al. (2016) were the first to conduct a systematic study of DNA repair profiles following Cas9 cleavage in human cell lines. They followed the repair outcomes after guide RNAs delivery targeting 69 different genomic sites and demonstrated that indel patterns differed from one targeted site to another and were very reproducible among replicates and between cell types. Nevertheless, the mutation frequencies of a given indel class varied with cell type. Taken together, this suggests that the characteristic DNA repair profile associated with a genomic location is influenced by the DNA sequence around the targeted area (van Overbeek et al., 2016). To further confirm this conclusion, guide RNAs matching multiple locations in human genome ("multiple target single spacers," MTSS) were designed and the associated indel profiles were assessed. In line with their previous observations, similarities between repair profiles for each site targeted by the same guide RNA are observed across replicates and cell type (van Overbeek et al., 2016).

Allen et al. (2018) confirmed such observations by specifically interrogating the influence of the DSB-flanking DNA sequence on repair outcomes. The authors designed and delivered synthetic constructs containing both a guide RNA and its target sequence flanked by variable DNA sequences, in human K562 cells. Indel profile analysis revealed that indels were highly reproducible and sequence-specific. Moreover, shorter deletions were more prominent compared to longer deletions, with nucleotide insertions (+1) and deletions (-1) being the most common. 58% of all Cas9-generated deletions, however, were at least 3 bp long and about a half of them occurred between at least two nucleotide repeats, referred to as microhomology (MH). The deletion frequency resulting from MH presence was inversely correlated with the distance between MH sequences. Introducing point mutation(s) in MH regions led to a remarkable drop in the associated repair outcome frequency (Allen et al., 2018). Intriguingly, although the indel patterns were similar across most cell types, stem cells had more large deletions and MH mediated products, whereas single nucleotide insertions (+1 insertions) were more frequent in differentiated cells. It was proposed that such observations correlate with different activities for the DNA repair pathways in different cell types.

Furthermore, indel profiling revealed that for almost half (49%) of the guide RNAs with a T (thymidine) before the cut site, a + 1 insertion involving another T dominates the repair outcome. A bias was also observed regarding small deletions: 77% of -1 deletions are associated with the removal of a repeated nucleotide at the break site. For half of the dinucleotide deletions, the removal of a two- base repeat was also quite common (Allen et al., 2018). These results are in agreement with Lemos et al. (2018), who demonstrated that single base insertions were shown to preferably repeat a PAM-distal nucleotide at the break site in yeast.

A recent large-scale study shed further light on the influence of genetic and epigenetic factors in CRISPR-Cas9 repair outcomes (Chakrabarti et al., 2019). Analysis of indel patterns at approximately 1,500 targeted locations in human cells (HepG2), revealed again that DNA editing precision differs across sites in a non-random and reproducible manner. The majority of examined targeted sites showed a preference for small indels (44% for 1 bp insertion and 26% for 1 bp deletion). However, a preference for large deletions (up to 41 nucleotides) was also observed for some sites. As a consequence of single nucleotide modifications, a considerable bias toward frameshifting mutations was observed (average of 80.1% compared to 66% of a random outcome).

Editing precision (recurrence of a specific indels) varied considerably between different targets with some targets associated with a large number (up to 79) of distinct, infrequent, deletions. In contrast, other targets showed one dominant mutation (representing up to 94% of all repair events). Overall, one fifth of all analyzed targets had at least a 50% chance of leading to a specific indel. Based on the distribution of commonest indel frequencies, the targeted sites were categorized into three groups: imprecise (commonest indel frequency below 25%), middle (commonest indel frequency below 50%), and precise (commonest indel frequency above 50%) sites. The vast majority of recurrent indels in precise targets (68.4%) are associated with a strong preference for insertions with a bias toward single nucleotide indels. In agreement with Allen et al. insertion, of a single nucleotide homologous to a PAM distal nucleotide (at position -4) at the break site was very common, especially when this nucleotide is T. These observations are consistent with Taheri-Ghahfarokhi et al. (2018), who also highlighted the importance of the 4th nucleotide before the PAM in the single nucleotide indel frequencies.

Strikingly, not only the indel pattern but also the editing precision could be predicted from the target site DNA sequence. Using a neural network Chakrabarti et al. found a significant correlation between the computational (estimated) and the observed indel frequencies. Despite a moderate predictive power of the model, it allowed the identification of key sequencing features. This computational quest also led to the conclusion that the nucleotide at position -4 from the PAM strongly influences the repair outcome in accordance with all previous experimental observations.

All in all, both by experimental studies and computer simulations, the Cas9-associated indel pattern and a presence of a dominant pattern appear to be mostly dependent on the DNA sequence around a break site, with the presence of MH in the target DNA sequence one of the main cues for predictability.

## CAS 9-MUTATIONAL PROFILES RELY ON MMEJ

The types of indel observed upon CRISPR-Cas9 cleavage suggest that Cas9-induced breaks are mainly repaired by NHEJ and MMEJ. It is generally assumed that small indels (<3 bp) occur via NHEJ and longer deletions occur via MMEJ. When analyzing the indel distribution following CRISPR-Cas9 activity over for a 48 h period, van Overbeek et al. showed that larger deletions are more prevalent at later points. They also observed that upon inhibition of NHEJ, +1 insertions and small indel (<3 bp) frequencies were decreased and, in contrast, large deletions (>3 bp) frequencies were increased (van Overbeek et al., 2016). The fact that alteration of NHEJ leads to increased MMEJ usage points to a tight balance between NHEJ and MMEJ pathways in repairing these breaks. Similar studies were performed later by Brinkman et al. for a single locus in human K562 cells. Targeting the *LBR* locus, the indel pattern analysis revealed a +1 insertion in balance with a -7 bp deletion. Addition of the NHEJ inhibitor NU7441 led to an increase of -7 deletions concomitant to a decrease in +1 insertions. Addressing the kinetics of the two processes revealed that MMEJ is delayed and initiated after NHEJ, and the delay is not observed when NHEJ is inhibited arguing for MMEJ predominantly being used as a back-up to repair breaks that, for unknown reason, failed to engage NHEJ (Brinkman et al., 2018).

Aiming to characterize in detail the contribution of the MMEJ pathway in the repair outcomes following Cas9 activity, Taheri et al. developed a computational platform called RIMA (Rational Indel Meta Analysis). Two datasets from the literature were reanalyzed using RIMA to validate their approach. They confirmed MMEJ pathway involvement in DNA repair after Cas9 cleavage and MMEJ-associated indels enrichment upon NU7441 (Bae et al., 2014; van Overbeek et al., 2016; Taheri-Ghahfarokhi et al., 2018). They also confirmed that larger indels and other MMEJ events relied on the activity of the known MMEJ factor POLQ (Taheri-Ghahfarokhi et al., 2018).

Experiments to determine the contribution of MH to the CRISPR-cas9 dependent DNA repair outcome by Chakrabarti et al. revealed that microhomologies of different sizes were responsible for a majority of deletions (73.3%). Strikingly, deletions associated with short microhomologies (1–4 bp), typically not considered as a substrate for MMEJ, were also enriched indicating a role for homology regions of any length MH, not restricted only to long regions of MH as had previously been believed (Chakrabarti et al., 2019). In line with these observations, Bae et al. found that a large subset of all observed deletions upon Cas9 activity were associated with 2–8 bp MH sequences. Based on this observation, the authors developed a computer program to predict MH-dependent deletions at a given site in order to increase the frequency of gene disruption (Bae et al., 2014).

Despite how incomplete our understanding of the exact role of MH involvement in the repair process is, it has already been flagged for its potential practical applications. In their recent work, Kim et al. demonstrated the possibility of using this genomic feature for obtaining a desired genome editing effect. They suggested an elegant two-step scheme for introducing point mutations in human iPS cells, associated with scar-less selection marker excision. Initially the desired mutation is introduced into the locus of interest as engineered MH sequences flanking a selection marker used as a donor. Although positive selection based on the presence of the selective marker represents an easy way to obtain clonal population, some applications require the removal of the selective marker. Therefore after positive selection, the selection marker can be excised using CRISPR-Cas9 induced DSBs targeting the region adjacent to the MH sequences, promoting the use of MMEJ for the selection marker excision while preserving the point mutation (Kim et al., 2018).

Overall, based on both computational and experimental studies, MH arises as a major factor influencing the DNA repair outcome at CRISP-Cas9 lesions. However, whether it is indeed an underestimated role of the MMEJ pathway or a lack of a deep understanding of NHEJ pathway functioning remains to be seen.

## CAS 9-MEDIATED LARGE DELETIONS AND COMPLEX REPAIR OUTCOMES

Most of the studies addressing repair of Cas9-induced breaks were focused on deletions of a relatively small size, based on the belief that NHEJ and MMEJ are the main pathways involved. However, large-scale indel pattern analysis highlights the complexity of Cas9-dependent repair outcomes. Such complexity is well depicted in the Shin et al. study where they analyzed the consequence of CRISPR-Cas9-mediated genome editing in founder mice (Shin et al., 2017). They showed that the majority of detected deletions were asymmetric (1.5-fold or more difference between deletion up-and downstream of the cutting site). Prevalence of asymmetric indels was observed for almost all targeted sites. Symmetric deletions were infrequent and tended to be small (less than 10 bp). Moreover, the deletions mostly occurred at repetitive regions, which is consistent with the conclusions of the above-mentioned studies relating to the role of MH in DSB repair.

Induction of DSBs with single guide RNAs in murine zygotes also revealed a 9 bp median deletion size, but larger deletions (up to 600 bp) were also present (Kim et al., 2018).

Testing whether sequential or simultaneous guide RNAs delivery would have any effect on an indel pattern and on a balance between small and large deletions, revealed that sequential guide RNAs delivery is more reliable than simultaneous in precisely deleting juxtaposed sites. Moreover, while no difference was observed for smaller deletions (less than 400 bp) between the two delivery strategies, deletions larger than 400 bp (up to 24 kb) were only present after simultaneous delivery. These large deletions didn't appear to rely on the presence of MH (Kim et al., 2018).

In light of the potential therapeutic use of Cas9, the findings of Kosicki et al., 2018 are especially striking. The authors explored large genetic alterations observed after CRISPR-Cas9 activity, focusing primarily on large deletions, which often are missing from repair outcome analysis due to a strong focus on a region proximal to the break (Kosicki et al., 2018). They performed knock-out experiments in mESC with single guide RNAs and observed that more than 20% of resulting alleles carried large (>250 bp and up to 6 kb) deletion. Even more surprisingly, in more than 15% of cases they observed additional DNA alterations (point mutations, large or small indels), distal to the cut site. Large inversions and duplications were also observed. Using mESCs obtained from a cross between two murine strains, Kosicki et al. also observed cases of loss of heterozygosity, presumably caused by using a homologous chromosome as a template. Despite differences in indel profile frequencies observed between stem cells and differentiated cells (Allen et al., 2018), larger deletions are not a unique feature associated with stem cells since they were observed in mouse hematopoietic progenitors cells and human RPE-1 cells (Kosicki et al., 2018).

Together, these data suggest, Cas9-mediated genome editing appears to be more complex and involves larger genome regions than was thought before. Thus, it is extremely important to understand the reasons for such an effect, and to take this into account while assessing using Cas9 for any medical purpose.

## CHROMATIN STRUCTURE INFLUENCES CAS 9 BINDING

The chromatin structure around DNA breaks influences DNA repair pathway choice (Kalousi and Soutoglou, 2016). However, regarding the repair of Cas9-mediated breaks, the question arises; which step of Cas9 editing (binding, cutting and/or repair) is most influenced by chromatin state? To dissect this, some *in vitro* and *in vivo* studies have been performed. First, Isaac et al. developed a biochemical assay to determine how nucleosomes and chromatin remodellers influence Cas9 activity. Using nucleosome assembly associated with poor breathing (a term that defines the dynamic binding of histones to DNA), they observed that Cas9 binding activity and cutting is inhibited. In contrast, Cas9-induced cleavage is achieved near to the entry/exit of a nucleosome assembly associated with higher breathing. Furthermore, the authors demonstrated that different classes of chromatin remodellers enhanced Cas9 activity, with an increase of Cas9-mediated cleavage in the presence of remodellers from the ISWI family promoting nucleosome sliding (SNF2h) or histone octamer eviction (RSC) (Isaac et al., 2016).

At the same time, a study conducted by Horlbeck et al. led to the same observations *in vivo* and *in vitro*. The authors first overlaid data obtained from a CRISPR screen (Gilbert et al., 2015) with MNase-seq experiments publicly available at ENCODE (performed in K562 human cells) and observed that high nucleosome occupancy is associated with low CRISPR interference activity (for CRISPR interference, catalytically inactive Cas9 is fused to a transcriptional repressor and guided to the targeted site in order to interfere with

gene transcription) (Horlbeck et al., 2016). Along similar lines, *in vitro* experiments argued for a block of Cas9 activity in the presence of DNA assembled into nucleosomes (Hinz et al., 2015; Horlbeck et al., 2016). Using an inducible system to control chromatin state (open or close) in human cells at a specific locus, Daer et al. observed reduced editing efficiency associated with heterochromatin (closed state) due to a reduction in Cas9 binding, for six over a total of nine guide RNAs used. This observation suggests that the effect of closed chromatin on Cas9 editing is guide RNA dependent or that in such inducible system the closed chromatin spreading is not covering equally all targeted sequences. Nevertheless, the mutation signature was not affected by the chromatin state. Interestingly, editing efficiency could be restored by artificial transcription activation (Daer et al., 2017).

Cas9 binding has also been studied in ChIP experiments in mouse ESC in which catalytically inactive Cas9 (dead Cas9) has been expressed. These studies also revealed that chromatin accessibility (assessed by DNAse I hypersensitivity experiments) is an important determinant of Cas9 binding *in vivo* and the vast majority of Cas9 off target sites are associated with active genes (Wu et al., 2014). Such findings were later confirmed by Kuscu et al. (2014) and O'Geen et al. (2015) that demonstrated a correlation between open chromatin and Cas9 off target binding in human and mouse cell lines, respectively.

Thus there is a general agreement that Cas9 activity is influenced by chromatin structure both *in vivo* and *in vitro*, with closed chromatin associated with less Cas9 binding and editing.

## THE ROLE OF CHROMATIN IN CAS 9-MEDIATED GENOME EDITING

The degree of influence of chromatin state over Cas9-induced mutagenesis has been the subject of studies by several research teams over the last few years. Chen et al. interrogated how chromatin status influences TALEN and CRISPR-Cas9 genome editing activity. For this purpose, a cellular system carrying a reporter in which chromatin status can be switched from compacted (H3K9me3 marked) to relaxed was used. Lower editing efficiency was observed when targeted sites were associated with heterochromatin for both TALENs and Cas9 nucleases, but the impact of chromatin state on editing was higher for TALENs. Interestingly, the efficiency of DSB formation was quite comparable (Chen et al., 2016). Subsequently, Chen et al. assessed the influence of chromatin structure on Cas9 editing in whole organisms. Zebrafish embryos were co-injected with guide RNAs and Cas9 mRNA. Editing efficiency positively correlated with chromatin accessibility (determined by ATAC-seq), and mutation rates were higher in an open chromatin. However, there was no correlation between nucleosome-occupancy and editing efficiency (Chen et al., 2017). The latter can be explained by high nucleosome dynamics in early zebrafish embryos, which is in line with the observations of Isaac et al., 2016 that pointed out that Cas9 activity is influenced by nucleosome breathing (Isaac et al., 2016). A study conducted by Kallimasioti-Pazi et al. induced Cas9 breaks at three different imprinted genes in mESC and demonstrated a delayed accumulation of mutations in heterochromatin compared to euchromatin. The allele-specific editing bias toward the active allele was particularly apparent in the case of low Cas9 expression or short Cas9 expression periods. In cells in which imprinting at the targeted locus had been lost, due to prolonged culture, there was a restoration of Cas9 editing efficiency, which again implies an heterochromatic environment impairs editing (Kallimasioti-Pazi et al., 2018). It does not appear to be the DNA methylation status of heterochromatin that is responsible for affecting cas9-mediated break editing, since Hsu et al. demonstrated that Cas9 mediated cleavage is not affected by CpG DNA methylation as supported by indel detection (around 8%) at the silent highly methylated SERPINB5 targeted locus (Hsu et al., 2013).

Kallimasioti-Pazi et al. (2018) could detect by allele-specific ChIP, that Cas9 binding was lower in heterochromatin, which correlated with the slowed rate of mutagenesis, thus confirming conclusions of Isaac et al. (2016) and Daer et al. (2017). Interestingly, despite distinct epigenetic statuses, the same mutation pattern was observed on maternal or paternal alleles arguing for an influence of heterochromatin on the kinetics but not on the outcome of Cas9 editing (Kallimasioti-Pazi et al., 2018). In line with such observations, using live cell single-molecule tracking in mouse cells, Knight et al. (2015) have demonstrated that even if Cas9 search efficiency is reduced in heterochromatic regions, Cas9 is still able to access successfully such regions (Knight et al., 2015).

Chakrabarti et al. have also come to similar conclusions. They observed that upon treatment with the histone deacetylase inhibitor TSA, indel formation is increased suggesting that chromatin decompaction augments Cas9 binding and editing efficiency (Chakrabarti et al., 2019). These results are in line with previous observations arguing for a lower editing efficiency associated with heterochromatin status (Chen et al., 2016; Daer et al., 2017; Kallimasioti-Pazi et al., 2018). In contrast, inhibition of the H3K27me3 methyltransferase EZH2, reduced indel formation, but with a less pronounced impact than TSA treatment. The fact that HDAC inhibition leads to the loss of constitutive heterochromatin and EZH2 inhibition, of facultative heterochromatin, suggests that different types of heterochromatin affect Cas9 editing in distinct ways (Chakrabarti et al., 2019). Nevertheless, these differences might not reflect only direct chromatin changes but indirect alterations on gene expression of DNA repair or other relevant genes. In agreement with this notion, even though both TSA and Ezh2i had an effect on indel formation, the authors were able to observe changes only in chromatin acetylation and not in H3K27me3 methylation. The same study demonstrated differences in ratios of different indels depending on a chromatin context. However, this did not affect dominant indels, suggesting that these changes are minor (Chakrabarti et al., 2019). Such results support the notion that in addition to the sequence around the break, certain chromatin context can modulate editing effectiveness.

Therefore, based on multiple studies with different experimental approaches and systems, we can conclude

that chromatin state influences Cas9-mediated genome editing efficiency with heterochromatin being an obstacle for this process. However, indel patterns are mostly unaffected.

## CAS 9 FOR KNOCK INS (KIS)

Utilization of the CRISPR-cas9 system for genetic replacement is particularly exciting as it can be implemented in the clinical setting for the cure of genetic diseases. Genetic replacement or KI is mediated by homology-directed repair (HDR).

Several recent studies have investigated the best ways to increase KI potential using Cas9. The most efficient way described so far is incorporation of a single stranded oligonucleotide DNA (ssODN), via single-strand template repair (SSTR). Farboud et al. performed a study in *C. elegans* to determine an efficient strategy to increase knock in efficiency. Their initial goal was to introduce point mutations as it is often required for therapeutic reasons. They used short single-stranded oligonucleotides as a template for recombination matching with the protospacer or with the spacer strand. Interestingly, they found that single nucleotide polymorphism (SNP) insertion was strongly biased toward 5′ or 3′ of the PAM according to the use of the protospacer or the spacer strand (respectively) as a repair template (Farboud et al., 2019). Such polarity can be mainly explained by synthesis-dependent strand annealing (SDSA) mechanism, an HDR pathway in which resected end is annealed to the repair template and extended. After template dissociation the extended end anneals to the other DSB end followed by DNA synthesis to fill the gap (Farboud et al., 2019).

Richardson et al. (2016) also discovered that the binding kinetics of Cas9 with the target DNA is asymmetric. Although Cas9 has a slow release from the template, it releases first the 3' end of the cleaved DNA strand that is not complementary to the sgRNA (or non-target strand). They observed that the use of an asymmetric donor DNA, complementary to the non-target strand, with 90 nt and 30 nt overlapping the PAM proximal and distal sites respectively, is associated with a higher HDR rate (Richardson et al., 2016). Such findings highlighted the importance for an optimal donor DNA design to ensure high HDR. The same strategy was used to increase HDR efficiency when using ssODN as a donor to correct the β-globin gene (HBB) carrying a mutation responsible for the sickle cell disease (SCD) in human hematopoietic stem/progenitor cells (DeWitt et al., 2016). Another recent study by Okamoto et al. demonstrated the influence of the Cas9 re-cutting capacity of the template DNA on the knock in efficiency using ssODNs. The authors found that either by introducing mutations at the donor sequences that resulted in blocking the re-cutting or either by expressing Cas9/sgRNA transiently using Cas9 protein/sgRNA ribonucleoprotein complexes had a substantial increase on the knock in efficiency (Okamoto et al., 2019).

The use of short single-strand templates was more efficient than a double-strand templates for knock in Farboud et al. (2019). It has recently been demonstrated that in human cells, repair based on a short single-stranded template is Rad51-independent and managed by the Fanconi anemia pathway (Richardson et al., 2018). Thus, differences in efficiencies could be explained by the use of different pathways, and potentially by differential requirements for the length of a template. In the case of a large DNA fragment insertion, the use of a double-stranded template becomes a requirement. For large fragments insertions, Farboud et al. were able to introduce a 9.3 kb fragment by adding a second DSB 340 bp from the initial DSB site. Interestingly, HR efficiency is influenced by the orientation of PAMs. Efficiency was much higher when recognition sites were selected on different strands rather than a single strand. These results suggest that the sequence around the break is important for Cas9-mediated knock in efficiency using larger DNA sequences as donors (Farboud et al., 2019). Insertion efficiency mediated by HR, for DNA fragment as long as 800 bp is also increased after NHEJ inhibition (using Scr7 ligase IV inhibitor treatment) in a bone marrow derived dendritic cell line (DC2.4) (Maruyama et al., 2015). Similarly, SSTR was increased in several genes and cell types when cells were baring a mutation into the human PRKDC gene (encoding for the DNA-PKcs protein) that suppress DNA-PKCs kinase activity (Riesenberg et al., 2019). Promoting homology directed repair (HDR) was also achieved through 53BP1 (a pro-NHEJ factor) inhibition in both human and mouse cells (Canny et al., 2018). This observation might be useful for knock in experimental design.

Since HR takes place during replicative and post replicative stages of the cell cycle, Gutschner et al. developed a system to restrict Cas9 expression to S/G2/M cell cycle phases. By fusing the Cas9 nuclease to geminin they were able to convert Cas9 into a substrate for the APC/Cdh1 complex, which promotes proteins ubiquitination and therefore degradation during late M and G1 phases. In a reporter assay, they monitored HDR-mediated EGFP expression restoration and showed an increase in HDR rate (up to 1.87-fold compare to wt Cas9). They also observed an increase of HDR at a target endogenous locus in HEK293T cells (Gutschner et al., 2016). Along the same lines, delivery of the Cas9/sgRNA ribonucleoprotein complex in cells arrested with nocodazole and aphidicolin and then released, increased SSTR (Lin et al., 2014).

Other groups developed strategies to increase HDR efficiency, allowing spatial proximity between the DSB site and the repair template. By fusing Cas9 to the PCV protein (porcine circovirus 2 rep), forming robust covalent link to a donor DNA, Aird et al. were able to increase HDR efficiency in human cell lines. Using different assays, they showed that covalent tethering of donor DNA template enhances (i) HDR mediated peptide-tag insertion (up to 30-fold) and (ii) HDR mediated mCherry fluorescence restoration (in reporter cells expressing a mutant mCherry) (Aird et al., 2018). Savic et al. came to the same conclusion using snap-tag technology to link donor DNA template to Cas9 and showed that repair template linkage enhances HDR efficiency in a fluorescent reporter cell line and, importantly, also at targeted endogenous loci in K562 and mES cells (Savic et al., 2018).

Another approach to increase HDR efficiency using the Cas9 nuclease fused to CtIP protein (an essential factor promoting

DNA end resection) has been described by Charpentier et al. They revealed that tethering CtIP next to the DSB site enhances GFP transgene integration in human fibroblasts. HDR stimulation was also observed in human iPS cells and rat oocytes but depends on the guide RNA (Charpentier et al., 2018).

Chromatin structure has a big influence on homologous recombination (Clouaire et al., 2018; Mitrentsi et al., 2020) but weather it has any influence on Cas9-mediated KI still remains elusive. The expectation is that it will be largely affected by the pre-existing structure of the chromatin surrounding the break. Kallimasioti-Pazi et al. however, found no consistent influence of pre-existing chromatin state on HDR efficiency across several imprinted genes. Systematic analysis on different genomic sites corresponding to different chromatin states will shed more light into the issue.

## CONCLUSION

In conclusion, genome editing using targeted nucleases, including Cas9, is a complex process, and its success depends on our understanding of specific mechanisms of DSB repair. It has become clear that repair outcome is predominantly sequence-specific and can minimally be altered by other factors. On the other hand, editing efficiency can be influenced by local chromatin structure and therefore can be improved by a change in the chromatin environment.

## AUTHOR CONTRIBUTIONS

LC and OM collected the literature and wrote the manuscript. ES wrote the manuscript.

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