Mechanisms and Regulation of Resection in DNA Damage Response

Sharad C. Paudyal
Washington University in St. Louis
Mechanisms and Regulation of Resection in DNA Damage Response
by
Sharad C. Paudyal

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Sharad C. Paudyal
Washington University in St. Louis
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Deoxyribonucleic acid (DNA) encodes genetic information essential for cell survival and function. However, it is constantly under assault from endogenous and exogenous damaging agents that not only threaten our own survival but also affect the faithful transmission of genetic information to our offspring. Double-strand breaks (DSBs) are one of the most hazardous forms of DNA damage, which if unrepaired or improperly repaired could lead to plethora of systemic human diseases including cancer. To deal with this problem, cells have evolved with a mechanism called DNA damage response (DDR) to detect, signal, and repair the breaks by inducing multiple cellular events. Resection is one of the key processes of cellular response to DSBs damage and is essential for genome maintenance, cell survival, and tumor suppression. Resection involves selective nucleolytic processing of the 5’ strand DNA at DSB ends to generate 3’ ssDNA overhangs, which in turn control both DNA repair and checkpoint response to the damage. Checkpoints coordinate the damage repair to other cellular processes including cell cycle regulation and gene expression. Despite its critical importance, the biochemical mechanisms and regulation of DSB resection is still not completely understood. Genetic studies in yeasts have suggested two steps mechanisms of resection: initiation by CtIP and MRN
(Mre11-Rad50-NBS1) complex and extension by Dna2 and Exo1. We took a multipronged approach to study the resection process and have determined new mechanisms and regulation of both initiation and extension pathways. Here, we report a novel mechanism for the initiation of resection at clean DSBs mediated by Dna2 endonuclease activity. Our results strongly suggest that resection of blocked and free DSB ends is initiated via distinct mechanisms. In addition, we have demonstrated that the extension of resection by Exo1 is regulated both positively and negatively by Poly(ADP-ribosyl)ation, a prominent posttranslational modification at the sites of DNA damage. Our results suggest that Poly(ADP-ribose) not only promote initial damage recruitment of Exo1 but also prevent unscheduled and improper extension of resection. These two separate studies demonstrating new mechanisms for both initiation and extension steps of resection provide some critical new insights into the cellular response to DSBs damage.
Chapter 1:

Sharpening the ends for repair: mechanisms and regulation of DNA resection

Sharad C. Paudyal and Zhongsheng You

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1.1 Introduction

DNA double-strand breaks (DSBs) are arguably the most toxic form of DNA damage, which, if unrepaired or improperly repaired, could cause genomic instability and a wide range of human diseases such as cancer, premature aging, immunodeficiency, neurodegeneration, and developmental disorders (1–4). Eukaryotic cells are equipped with a conserved mechanism called DNA damage response (DDR) to detect, signal, and repair the damage by activating multiple repair and checkpoint pathways (5–7). DSBs are repaired mainly by non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ repairs the break through direct re-ligation of the broken DNA ends with no or limited end processing and thus is error-prone. By comparison, HR repairs the break in an error-free manner, and is initiated by nucleolytic processing of the 5′ ends of a DSB through a process called DNA end resection (8–16).

Resection occurs in 5′→3′ direction to generate 3′ ssDNA overhangs, which are initially bound by ssDNA-binding protein replication protein A (RPA) and then replaced by Rad51 during HR. The Rad51-ssDNA filament mediates homology search and strand invasion, followed by DNA synthesis, Holliday junction resolution, and DNA ligation to restore the integrity of the DNA structure (9,10,15,16). The RPA-bound ssDNA structure also serves as the signal to activate the ATR checkpoint pathway that coordinates DNA repair with other cellular processes (17–19).

The generation of ssDNA by resection also indirectly inhibits NHEJ and attenuates the activation of the ataxia telangiectasia mutated (ATM) checkpoint pathway (8,9,11,20,21). Thus, resection is considered to be the major event in the DDR that dictates the pathway choice of both DNA repair and checkpoint signaling (Fig. 1.1). While DSB repair by NHEJ can occur at any time during the cell cycle, HR occurs primarily in S and G2 phases when sister chromatids are available (9–11,22–25). This cell cycle control of HR is in part mediated by the regulation of
DNA end resection by cyclin dependent kinase (CDK) activity (26–29). Resection is apparently also regulated by the checkpoint response to prevent deleterious consequences resulted from excessive resection (30–35). Besides its role in HR, resection also plays a role in the maintenance of 3’ overhangs at telomeres and repair of uncapped telomeres at the end of chromosomes (36–39). Likewise, resection also occurs at ssDNA-dsDNA junctions of stalled replication forks and at dsDNA ends of reversed forks, and is important for fork repair and restart (40–45). However, the detailed mechanisms of end resection in these contexts are much less understood. Genetic mutations in resection factors are associated with multiple genetic disorders and predisposition to cancer and premature aging (1,2,4). On the other hand, DNA end resection could also be a suitable target for cancer therapy because rapidly dividing cancer cells rely heavily on HR and the ATR checkpoint for growth and survival. In this review, we discuss our current understanding of the mechanisms and regulation of the DNA end resection process and their potential implications for cancer formation and treatment, focusing mainly on vertebrate systems.

1.2 Key steps and core factors of DNA end resection

1.2.1 Initiation of resection by MRN and CtIP

Studies in yeast, human cells, and in vitro reconstituted systems with purified proteins suggest that DSB end resection is initiated by a concerted action of MRN (Mre11-Rad50-NBS1) (MRX (Mre11-Rad50-Xrs2) in budding yeast) together with CtIP (Sae2 in budding yeast and Ctp1 in fission yeast) (46–51) (Fig. 1.2). MRN complex, which is among the first set of proteins to localize to sites of DNA damage, has a high affinity for DSB ends and plays a central role in sensing breaks in chromatin (52–54). MRN promotes the damage recruitment of the ATM
checkpoint kinase and its subsequent activation (55). They also promote the recruitment of CtIP to sites of damage (51). The NBS1 subunit of the MRN complex plays a key role in coupling these events through its direct interactions with Mre11, CtIP, and ATM (51,55–59). Rad50 is an ATPase that maintains the conformation of MRN complex and promotes DNA binding of the complex, as well as DNA resection and ATM activation by the complex (60–63). The Mre11 subunit possesses the catalytic function of MRN complex in resection and has both 5′ flab endonuclease activity and 3′→5′ exonuclease activity. Its endonuclease function is believed to initiate resection by internal cleavage of the 5′ strand to generate oligonucleotides that will be released, while the exonuclease activity processes the resulting 3′ ends on the DNA (64–71).

While MRN is necessary, the complex by itself is not sufficient to initiate resection. CtIP is also required for the initiation of DNA end resection by MRN complex (50,51,72–74). In vitro studies with purified yeast MRX and Sae2 proteins suggest that MRN-CtIP is a minimal system for resection initiation (46). CtIP interacts directly with NBS1 and promotes the endonuclease activity of Mre11 at the DSB ends (50,51). Both CtIP and Sae2 have also been shown to contain an endonuclease activity (75,76). While there is no direct evidence for the nuclease activity of CtIP or Sae2 in the resection initiation at ‘clean’ DSBs, they have been suggested to function to remove secondary DNA structures on the 5′ strand DNA at DSB ends (75–77). Resection initiation by MRN-CtIP is especially important when the ends are bound by chemical or protein adducts that prevent exonucleases from binding and processing them (69,70,78,79). A prominent example of such breaks is the DSBs generated during meiotic recombination, which are covalently linked to the Spo11 protein. Resection of these Spo11-blocked ends in yeast is initiated by the endonucleolytic activity of MRX-Sae2 to initiate the resection before further processing (69,70). For DSBs that are free of chemical or protein adducts, MRX-Sae2 function is
dispensable for end resection (47,71,80). In addition to end cleavage, studies in yeast suggest that MRX-Sae2 or MRN-Ctp1 plays a role in removing the NHEJ factor Ku from the DNA ends to promote the binding of nucleases Exo1 and Dna2 that mediate resection extension (81–83). Moreover, MRN-CtIP in human cells also provide structural and catalytic support to recruit Exo1 and Dna2 to the damage site to extend the resection (82–87).

### 1.2.2 Extension of resection by Exo1 and Dna2

Limited resection by MRN-CtIP alone could lead to DNA repair by a less common and highly error-prone mechanism called microhomology-mediated end joining (MMEJ), which involves the alignment of short ssDNA overhangs before ligation (9,88–90). While limited resection by MRX-Sae2 in yeast has been shown to be sufficient for HR repair, extended resection appears to be required to avoid MMEJ and promote HR (9,71,90–92). Resection extension is carried out by Exo1 and Dna2 in two parallel pathways, which can produce ssDNA of several kilobases long (47,71,93,94) (Fig. 1.2). Exo1 is a member of the RAD2 family of nucleases that possesses 5′→3′ dsDNA exonuclease and 5′-flap endonuclease activities, and plays a role in a plethora of biological processes including DNA replication, recombination, repair, checkpoint activation, and telomere maintenance (95–103). The resection function of Exo1 is positively regulated by MRN, Bloom syndrome RecQ-like helicase (BLM), RPA, proliferating cell nuclear antigen (PCNA), and 9-1-1 (87,103–106). MRN, PCNA, and likely 9-1-1 complex act to promote the processivity of Exo1 (87,104–106). It has been reported that CtIP promotes the loading of Exo1 to the damage site but negatively regulates Exo1 nuclease activity (107). However, CtIP has also been shown to be required for extensive resection and checkpoint maintenance, although the detailed mechanism remains to be determined (108).
Another major resection extension factor is the helicase/endonuclease Dna2, which is well known for its role in Okazaki fragment maturation and G-quadruplex DNA processing during DNA replication (109–113). During DNA end resection, Dna2 works together with Sgs1-Top3-Rmi1 complex in yeast and Sgs1 ortholog BLM in cultured human cells (71,85–87,114). Studies in *Xenopus* egg extracts as well as human cells show that another RecQ family of helicase Werner syndrome RecQ-like helicase (WRN) also promotes resection by unwinding the DNA ends and making it accessible for Dna2 (115–119). Although Dna2 functions as both a helicase and a nuclease, only the nuclease activity is essential for the extension of DNA end resection (87,120,121). The long stretch of ssDNA generated by Exo1 and Dna2 serves as the substrate for HR, and in the meantime prevents repair by NHEJ or MMEJ (9,92). The ssDNA-binding protein RPA promotes resection extension by enhancing the nuclease activity of Dna2 on the 5′ strand and by suppressing the inhibitory effects of the 3′ ssDNA resection product on Exo1 (85–87,92,117,118,122–124).

1.2.3 Termination of resection

Although ssDNA generated by resection is essential for ATR checkpoint and HR, uncontrolled excessive resection could be deleterious to genome integrity, as ssDNA is more prone to degradation that causes loss of genetic information. Excessive ssDNA generated by resection may also exhaust the RPA pool in cells, leading to unprotected ssDNA and genomic instability (123,125). Therefore, it is expected that when the length of ssDNA reaches a certain threshold, resection activities would stop processing the DNA ends. However, the control of the timing and the mechanism of resection termination are still unclear. Studies in yeast and human cells both suggest that the nuclease activity of major resection factor Exo1 is inhibited in a checkpoint-
dependent manner. In yeast, Exo1 nuclease activity is inhibited by both Mec1 and Rad53 at uncapped telomeres (30). Phosphorylation of Exo1 by Rad53 in yeast appears to inhibit its activity in processing DSB ends, unprotected telomeres and stalled replication forks. In human cells, direct phosphorylation of Exo1 by ATR leads to Exo1 degradation during replication stress (31–33,126). It is possible that Exo1 is also negatively regulated by the ATR checkpoint response during DNA end resection. Another study shows that ATM phosphorylates Exo1 and limits its activity after RPA is bound to ssDNA (34). Together, these observations suggest that checkpoint-mediated phosphorylation of Exo1 inhibits its activity to terminate the resection. Interestingly, Exo1 interacts with phospho-peptide binding proteins 14-3-3s, and this interaction inhibits its damage recruitment and subsequent DNA resection (127–129). Thus, it is plausible that phosphorylation of Exo1 by ATM, ATR, or their downstream kinases promotes the interaction of Exo1 with 14-3-3s, preventing its association with DNA damage, thereby promoting resection termination (Fig. 1.2). Interestingly, Durocher and colleagues have recently proposed another negative feedback mechanism for resection termination in which the recruitment of DNA helicase HELB by RPA to ssDNA inhibits the nuclease activities of Exo1 and BLM-Dna2, although the detailed biochemical mechanism of this inhibition remains to be defined (130). Another possible mechanism for resection termination is the second end capture during HR, which may prevent further resection by annealing the complimentary strands and formation of double Holliday junction (131–134). Recent studies have also shown that Dna2 is inhibited by fanconi anemia complementation group 2 (FANCD2) in human cells and Pxd1 in fission yeast (135,136), which could be the mechanisms to terminate Dna2-mediated DNA end resection.
1.2.4 RPA selects 5’ strand for resection and protects 3’ strand resection product

DSB resection occurs in the 5′→3′ direction, but what determines this directionality for the cleavage of the 5′ ends during resection initiation is still a mystery. An in vitro study by Petr Cejka and colleagues shows that MRX together with Sae2 selectively cleave the 5′ strand of a linear dsDNA substrate to initiate the resection, although the detailed mechanism for this strand selectivity in this ‘minimal’ resection initiation system remains to be determined (46).

Nevertheless, the mechanism for the strand selectivity during resection extension is better understood. Exo1 acts as a 5′→3′ exonuclease and thus has intrinsic polarity (95–98). RPA plays a key role in selecting the 5′ strand for processing by Dna2, which functions as a flap endonuclease in resection (85–87,120). Initial unwinding of the broken DNA ends by helicase BLM or WRN generates both 5′ and 3′ ssDNA strands. In vitro studies using purified proteins show that RPA binds to both strands but allows resection to occur only on the 5′ strand of the DNA (85–87,120). A recent structural study of Dna2-ssDNA-RPA complex and in vitro nuclease assays using mouse Dna2 shows that Dna2 physically interacts with RPA bound to both strands but can only displace RPA from the 5′ strand and hence the resection occurs only on the 5′ strand (120). Studies in Xenopus egg extracts also show that RPA interacts with both WRN and Dna2 to promote 3′→5′ helicase activity of WRN and 5′→3′ nuclease activity of Dna2 (117,118). In addition to its role in directing 5′→3′ resection, RPA binds promptly to the newly generated 3′ ssDNA and protects the resection product (85–87,92,118,120,123,124). Functional disruption of RPA in yeast not only abrogates resection extension, but also causes formation of hairpin structures on the short 3′ ssDNA generated by MRX-Sae2, which can be further processed, resulting in genomic instability (123). Binding RPA to 3′ ssDNA overhangs also suppresses DNA repair by MMEJ (92).
1.3 Regulation of DNA end resection

Either insufficient or excessive resection could compromise genome stability and cellular viability. While insufficient resection impairs the process of HR and ATR activation, over-resection could cause persistent checkpoint activation, loss of genetic information, and even cell death (123,137). In fact, accumulation of ssDNA is a major source of mutational load and genomic rearrangements in different forms of cancer (138–140). Hence, resection must be properly controlled to prevent under- or over-resection. To avoid HR in G1 phase of the cell cycle, DNA resection is also regulated by the cell cycle (9,11,13,23,25,74). While the key steps of resection and core factors have been widely studied, many questions remain open as to precisely how the overall extent of resection is controlled. Below we will discuss the regulation of the resection process by the cell cycle, checkpoint response, and other factors.

1.3.1 Cell cycle regulation of resection

In G1 phase of the cell cycle, DSBs are repaired mainly by NHEJ or MMEJ, two pathways that require no or little end resection (8,9,74,89,90,141). DNA end resection in G1 phase in general is suppressed by low activity of CDKs and higher activity of NHEJ factors (22,28). Ku70-Ku80 protein heterodimer, a major NHEJ factor, loads onto DSB ends during G1 to promote repair by NHEJ while indirectly inhibiting DNA end resection (142–146). Nevertheless, limited end processing is still possible during G1 due to the activities of MRN and CtIP (147,148). However, this limited resection by MRN-CtIP during G1 could be mechanistically different from their resection function during S and G2 phases. Suppression of DNA end resection in G1 phase is important as it prevents HR between homologous chromosomes that can lead to loss of heterozygosity.
During S and G2 phases of the cell cycle, DSBs can be repaired by HR that requires more extensive resection to generate a significant length of ssDNA for Rad51 binding and homology search on a sister chromatid (9,14–16). This increased resection results from the high level of CDK activity, which promotes the functions of the core resection factors including MRN, CtIP, Exo1, and Dna2 (26–29,149–156). The NBS1 subunit of the MRN complex is phosphorylated by CDKs at S432 in S, G2, and M phases (but not in G1), which is important for DNA end resection (149,150). Mre11 interacts directly with CDK2 and promotes phosphorylation of CtIP/Sae2 by CDK2, which is also crucial for resection in S and G2 phases (147,151–153). In budding yeast, Sae2 is phosphorylated at S267 by Cdc28 (CDK1) and mutation of this residue to alanine inhibits DNA resection in vivo (152). In human CtIP, the CDK phosphorylation sites S327 and T847 have been reported to be important for resection and subsequent HR in S and G2 phases of the cell cycle (153,154). Phosphorylation of CtIP by CDK2 promotes resection in part through increased damage recruitment of CtIP and association with MRN complex (72,153). CDKs also regulate resection extension by direct phosphorylation of Exo1 and Dna2, which also promotes their damage recruitment (155,156).

Compared to S and G2 phases, less is understood about DSB resection during M phase. The highly condensed nature of chromosomes in M phase may preclude the accessibility of repair factors to DSBs in M phase, thus cells could just exit mitosis with the DSBs that will be repaired by NHEJ in the next G1 phase (157,158). A recent study in Xenopus egg extracts and cultured human cells shows that limited resection still occurs at DSBs during M phase by the activity of MRN and CtIP (159). However, the high CDK1 activity also prevents the loading of ATR and Rad51 to the RPA-coated ssDNA. As a result, ATR checkpoint and HR are not activated in M phase (157,159).
1.3.2 Checkpoint regulation of resection

DNA end resection is also regulated by checkpoint kinases. Mass spectrometric studies have shown that hundreds of DDR proteins including major resection factors are phosphorylated by ATM/ATR after DNA damage (160,161). ATM promotes the damage recruitment of CtIP in human cells and *Xenopus* egg extracts (51). CtIP phosphorylation by ATR on T818 in *Xenopus* egg extracts also promotes its damage recruitment and resection activity (162). In yeast, Sae2 is phosphorylated by both Mec1 (ATR) and Tel1 (ATM), which promotes its function in DNA end processing (163,164).

Interestingly, checkpoint kinases not only promote resection but also prevent unscheduled and over-resection by nucleases. Consistently, Mec1 deletion in yeast causes accelerated rate of DSB resection (21). A study in *Xenopus* egg extracts showed that phosphorylation of Mre11 at SQ/TQ sites facilitates MRN complex dissociation from the damage site (165), which could be dependent on ATM/ATR to down-regulate Mre11 activity after initiation of resection. In human cells, ATM phosphorylates Mre11 on S676 and S678, which promotes Exo1 phosphorylation by ATM that attenuates its activity (34,35). Mec1 and its downstream kinase Rad53 in budding yeast inhibit Exo1 activity at unprotected telomeres and prevent the accumulation of ssDNA (30). Rad53-mediated phosphorylation of Exo1 attenuates its nuclease function and prevents uncontrolled resection at DSBs, telomeres, and stalled replication forks (32,33). In human cells, ATR phosphorylates Exo1 in response to replication stress, which promotes Exo1 degradation to prevent the aberrant processing of replication forks (31). Yeast Dna2 has also been suggested to be phosphorylated by checkpoint kinase Mec1, although whether this phosphorylation suppresses Dna2 resection activity remains to be determined (155). Overall, it appears that the
checkpoint kinases play a positive role in an early stage of resection and a negative role in a late stage of resection.

1.3.3 Regulation of resection by 53BP1 and BRCA1

The tumor suppressor BRCA1 promotes DNA end resection and is important for HR (72,166–169). The HR defects of BRCA1-deficient cells are synthetic lethal with inhibitors of PARP1 that is involved in base excision repair (170,171). Interestingly, the HR defects and cellular hyper-sensitivity to PARP1 inhibitors of BRCA1-deficient cells can be rescued by inactivation of 53BP1, which is important for NHEJ (168,172–177). While the detailed mechanisms of their respective functions in HR and NHEJ are still incompletely understood, BRCA1 and 53BP1 act antagonistically to regulate DNA end resection. 53BP1 inhibits DNA end resection through its associated factors Rap1 interacting factor 1 homolog (RIF1) and pax transactivation domain interacting protein (PTIP) (178–183). RIF1 inhibits BRCA1 damage recruitment during G1, inhibits resection, and hence promotes repair by NHEJ (179,180,183). During S and G2 phases of the cell cycle, BRCA1 together with CtIP inhibits the damage recruitment of RIF1, allowing for resection and repair by HR (147,167,174,184). BRCA1 also recruits the E3 ubiquitin ligase UHRF1 to the damage site where it ubiquitinates and removes RIF1 from the damage site, thereby promoting resection and HR (185). While the mechanism of how BRCA1 inhibits PTIP is unclear, it may involve the disruption of its interaction with 53BP1 and damage association (174,178,182). The striking functional relationship between BRCA1 and 53BP1 underscores the delicate balance between the HR and NHEJ pathways and the importance of proper regulation of the DNA resection process.
1.3.4 Mechanisms that prevent over-resection

DNA end resection must be properly controlled to prevent over-resection, as excessive ssDNA could cause cell death or genomic instability. Over-resection may result from unscheduled initiation, uncontrolled extension, or untimely termination. The function of Exo1 in resection is restrained by 14-3-3 proteins, which limit the damage recruitment of Exo1 by suppressing the binding of Exo1 to poly(ADP-ribose) (PAR) and PCNA, both of which promote Exo1’s damage association (104,127,186) (Fig. 1.3). Disruption of the Exo1-14-3-3 interaction causes over-resection and increased sensitivity to DNA damage (127). Exo1 activity in DSB resection may also be regulated by post-translational modifications such as phosphorylation, SUMOylation, and ubiquitination. In budding yeast, Exo1 is phosphorylated by Rad53 in response to DSBs, telomere uncapping, and replication stress, which inhibits its nuclease activity (32,33). In human cells, Exo1 is phosphorylated by ATR and SUMOylated by UBC9-PIAS1/PIAS4 in response to stalled replication, which induces its ubiquitination and degradation in a proteasome-dependent manner (31,126,187). It is possible that similar mechanisms exist to limit Exo1 activity during resection of DSBs. Recent studies have shown that Dna2 resection activity is restrained by FANCD2 in human cells and Pxd1 in fission yeast, although the detailed mechanisms remain to be defined (135,136). Studies in Xenopus egg extracts and human cells suggest that ATM-mediated phosphorylation of Mre11 inhibits its damage association as well as Exo1 nuclease activity preventing over-resection (35,165). The function of CtIP in resection is negatively regulated by phosphorylation-specific prolyl-isomerase PIN1, which binds to CtIP, and promotes its isomerization and subsequent ubiquitination and degradation (188). It is expected that these regulatory mechanisms function collectively to prevent uncontrolled excessive resection.
1.3.5 Other regulatory factors

In addition to the core factors described above, recent studies in multiple organisms have identified many other factors such as EXD2, PCNA, 9-1-1, PAR, lysine deacetylase SIRT6, chromatin-binding protein LEDGF/p75, chromatin remodelers SMARCAD1/Fun30 and SRCAP, ssDNA-binding protein SOSS1, and RNA-binding hnRNPU-like proteins in DNA end resection (104–106,186,189–197). These factors promote resection by promoting the damage recruitment of core resection factors, remodeling the chromatin structure at damage sites or enhancing activities of resection nucleases. The existence of these many regulatory factors further demonstrates that DNA resection is a highly orchestrated process that involves sophisticated coordination of nuclease and helicase activities.

1.4 DNA resection at telomeres, stalled replication forks, and heterochromatin

The 3′ ssDNA overhangs at telomeres are essential for telomerase binding and telomere maintenance (198–200). Replication of lagging strand DNA naturally generates ssDNA overhangs at telomeres in a sister chromatid. However, the leading strand is replicated completely, generating a blunt end that requires resection to produce a 3′ ssDNA overhang (198–200). This resection is carried out by an exonuclease called Apollo, which acts immediately after the completion of replication (201,202). Extensive resection by Apollo is inhibited by the binding of Pot1b to the ssDNA (201). In yeast, Dna2 has also been suggested to be involved in limited processing of the 5′ strand to maintain the telomere length and telomerase binding (203,204). While 5′ strand resection is important for telomere maintenance, over-resection could lead to telomere shortening, senescence, and other deleterious consequences (198). Indeed,
studies in yeast have shown that when the telomeric ends are not protected by capping proteins, Exo1 together with the Pif1 helicase could resect the ends of replicated DNA of both leading and lagging strands and initiates a protracted checkpoint response (205,206). To avoid this, it has been shown in human cells that resection of telomere ends by Exo1 is inhibited by Pot1b and RIF1 (176,181,201,202).

DNA resection is also highly regulated in DNA replication. Studies in yeast and human cells suggest that Exo1 degrades stalled forks and that checkpoint-dependent phosphorylation of Exo1 inhibits this activity (31–33). Mre11 has been suggested to play a major role in the processing and restart of stalled replication forks (43,207). However, uncontrolled resection by Mre11 could also lead to degradation of stalled forks, leading to fork collapse. Indeed, it has been shown that BRCA2 and PARP1 inhibit Mre11 nuclease activity to prevent fork degradation (43,208). WRN helicase also plays a major role in coordinating fork processing and restart by preventing unscheduled nascent DNA degradation by Mre11 and Exo1 (209,210). In Xenopus egg extracts, Rad51 binds to the newly synthesized DNA during replication and protects it from degradation by Mre11 (211). In yeast and human cells, Dna2-mediated end processing is important for the restart of stalled or reversed replication forks (44,45,212). However, upon replication fork stalling caused by interstrand crosslink, Dna2 activity is restrained by FANCD2 to prevent uncontrolled resection (135).

To date, little is known about the mechanisms and regulation of DNA end resection in heterochromatin. Recent studies in Drosophila melanogaster suggest that resection occurs efficiently in heterochromatin; however, DSBs are not repaired by HR until the DNA ends relocate outside of the heterochromatin domain (213,214). The relocalization of DSB is facilitated by resection and ATR (213). Counter-intuitively, resection of DSBs in
heterochromatin and subsequent loading of ATR interacting protein (ATRIP) and TopBP1 required for ATR activation appear to occur in a faster kinetics than in euchromatin, suggesting that resection is regulated differently in these two types of chromatin domains (213–215).

1.5 Relevance of DNA end resection for cancer formation and therapy

DNA end resection is essential for ATR checkpoint activation and HR, both of which play a critical role in genome maintenance and tumor suppression (1–4,7,10,18). Genetic knockout of the major resection factors Mre11, Rad50, NBS1, CtIP, and Dna2 in mice is embryonically lethal, and their deficiencies cause hypersensitive to DNA damaging agents (64,112,216–218). Exo1 knockout in mice leads to meiotic defects and cancer susceptibility (219). Mutations in Mre11, NBS1, Rad50, BLM, and WRN are causes of genetic diseases AT-like Disorder, Nijmegen Breakage Syndrome, NBS-like Disorder, Bloom Syndrome, and Werner Syndrome, respectively, all of which are associated with cancer predispositions (3,220–224). These findings further highlight the importance of DNA end resection in genome protection and tumor suppression. Paradoxically, DNA end resection may also be targeted for cancer therapy. The synthetic lethal relationship between PARP inhibition and HR deficiency suggests that combining inhibitors of PARPs with that of resection activities may be effective in cancer treatment (170,171). Moreover, over-resection (e.g. by disrupting Exo1-14-3-3 interaction) increases cellular sensitivity to DNA damage, and thus may also be exploited for cancer treatment (127).
1.6 Conclusions and perspective

DNA end resection is a key process in the DDR that controls both DNA repair and checkpoint response. Resection is initiated by an endocleavage step that is carried out by the MRN complex in collaboration with CtIP. Extended resection is mediated by two parallel pathways involving the Exo1 and Dna2 nucleases, respectively. The resection process is tightly regulated by multiple mechanisms and accessory factors to ensure proper repair at DSBs, telomere, replication forks, and heterochromatin. The DNA resection process is highly relevant to tumorigenesis and may be targeted for cancer therapy. Understanding the detailed mechanisms and regulation of DNA resection is the key to designing more efficient cancer therapeutics. Future work is needed to address many outstanding questions in the field, e.g. what determines the strand specificity during resection initiation? How do MRN and CtIP cooperate to initiate DNA resection? Are there any mechanistic differences in the initiation of resection of DNA ends with different structures? Do Exo1 and Dna2 pathways function redundantly at telomeres and stalled/collapsed replication forks? How is resection terminated? How is over-resection avoided? What does control the extent of DNA resection? How is resection regulated in heterochromatin? Can DNA end resection be exploited for cancer therapy, and if so, which resection activities can be targeted? The next few years will see major advances in addressing these important questions.

1.7 Acknowledgments

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Figure 1.1. DNA end resection dictates the pathway choice for both DNA repair and checkpoint response. DSBs can be repaired by NHEJ or by HR that requires 3’ ssDNA generated by end resection. Checkpoint kinase ATM is activated on the dsDNA flanking the break, whereas ATR is activated on the ssDNA structure generated by resection. Thus, DNA end resection promotes HR and ATR activation and attenuates NHEJ and ATM activation.
Figure 1.2. Key steps and core factors of DNA end resection. Resection is initiated on the 5′ strand of the DNA by the endocleavage activity of MRN-CtIP and extended by Exo1 and Dna2 in two parallel pathways. The underlying mechanism for the 5′ strand selectivity of MRN-CtIP in resection initiation is still not completely understood. The ssDNA generated from resection is bound and protected by RPA which promotes ATR activation and HR when replaced by Rad51. Precisely how resection is terminated remains unclear, but the ATR checkpoint pathway may help terminate resection via a feedback loop mechanism.
Figure 1.3. Human Exo1 is regulated by PCNA, poly(ADP-ribosyl)ation and 14-3-3s. The damage recruitment and resection activity of Exo1 are controlled by three sets of factors that directly interact with different domains in Exo1. PARylated proteins bind to the N-terminus of Exo1 and promote the initial damage recruitment of Exo1. PCNA binds to the PCNA-Interacting Protein box in the C-terminus of Exo1 and promotes the damage retention and processivity of Exo1. 14-3-3 Proteins interact with the central domain of Exo1 and inhibit its damage recruitment by suppressing the interactions of Exo1 with PARylated proteins and PCNA. These coordinated regulations of Exo1 by multiple factors with opposing activities ensure a highly orchestrated resection process and a proper level of ssDNA at DSBs.
1.8 References


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Chapter 2:

Dna2 initiates resection at clean DNA double-strand breaks

Sharad C. Paudyal, Shan Li, Hong Yan, Tony Hunter, and Zhongsheng You

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2.1 Introduction

DNA double-strand breaks (DSBs) are arguably the most hazardous forms of DNA damage in cells, which can be caused by ionizing radiation, reactive oxygen species, chemotherapeutic drugs and collapse of replication forks, or induced during genome engineering with CRISPR, ZFN and TALEN technologies (1–3). DSBs also occur as programmed recombination events during meiosis and V(D)J recombination in lymphocyte development (4,5). Regardless of their origin, DSBs pose a serious threat and can lead to genomic instability and even cell death if not properly repaired. To cope with this problem, cells have evolved with a highly sophisticated mechanism called DNA damage response (DDR) to detect, signal and repair these breaks (6–8). DSBs are repaired mainly by two largely competing pathways: non-homologous end joining (NHEJ) and homologous recombination (HR) (9–11). While NHEJ can occur throughout the cell cycle, HR is mainly limited to S and G2 phases (11–14). The choice between these repair pathways is dictated by end resection, a DNA processing mechanism that degrades specifically the 5’ strand DNA from the ends to generate long 3’ ssDNA overhangs required for HR in S and G2 phases of the cell cycle. By converting dsDNA ends into ssDNA structure, resection promotes HR and inhibits NHEJ (11,15–18). DSB resection also controls the checkpoint responses that coordinate DNA repair with other cellular processes such as cell cycle progression and gene expression (19–22). Checkpoint responses are controlled by ATM and ATR protein kinases, both of which are activated by DSBs (23–25). Whereas ATM activation occurs on double-strand DNA structure adjacent to the DNA break ends, ATR is activated on the ssDNA structure generated by resection (26–28). Consequently, DSB resection promotes the ATR checkpoint pathway and attenuates the ATM checkpoint pathway (29–31). Thus, resection governs both DNA repair and checkpoint signaling in the DSB damage response.
DSB resection involves multiple enzymatic activities including nucleases and helicases and is tightly regulated to ensure genomic stability (17,32). Studies in multiple organisms such as yeasts, *C. elegans, Xenopus laevis*, mice and humans have led to the proposal of a two-step, bi-directional model in which resection is initiated by cleavage of the 5’ strand DNA away from the DSB ends by MRN/MRX (Mre11, Rad50 and NBS1/XRS2) together with CtIP/Sae2 (functional ortholog of CtIP in budding yeast), generating clean 5’ and 3’ ends at the incision site (31,33–40). In this step, the endonuclease activity of the Mre11 subunit in MRN/MRX is believed to be responsible for this incision, although CtIP/Sae2 has also been suggested to contain endonuclease activity (33,34,41–45). In the subsequent extension stage of resection, the 3’ end generated by initiation is then processed by MRN and Exd2 in the 3’-5’ direction (35,46,47), whereas the 5’ end is further resected by Exo1 (together with PCNA or 9-1-1 complex) and Dna2 (together with BLM/WRN, RPA and Cdc24) in the 5’-3’ direction (33,34,48–64). The resulting long ssDNA overhangs then promote the activation of HR and the ATR checkpoint (10,11,28,29). The initial endocleavage step mediated by CtIP-MRN is essential for the resection of DSBs with protein or chemical adducts at the 5’ ends because exonuclease or flap endonuclease activities in Exo1, Dna2, Exd2 and MRN cannot directly process these ends (65–72). Consistent with this notion, it has been shown that MRN/MRX and CtIP/Sae2 are absolutely required for the resection of Spo11-linked DSBs in meiosis and Topoisomerase II-linked DSBs in somatic cells and *Xenopus* extracts (43,66–74).

It is generally believed that resection of clean DSBs without adducts—which can be generated at collapsed replication forks or by endonucleases or cancer drugs—is also initiated by CtIP-MRN (Sae2-MRX). In line with this idea, it has been shown that Sae2-MRX can initiate limited resection of clean DSBs generated by HO endonuclease in yeast in the absence of the Exo1 and
Dna2 pathways (33,34). However, unlike blocked DSBs, Sae2 and MRX are not essential for end processing and HR at clean DSBs (75–78). Moreover, in the *Xenopus* cytosolic extract the nuclease activity of MRN is dispensable for the overall resection of DNA substrate with free 5’ ends, which is in sharp contrast to the resection of the 5’ blocked ends where the nuclease activity of MRN is essential (79). Likewise, it has been shown that the catalytic function of CtIP is also dispensable for resection at “clean” DSBs in human cells (43). Furthermore, in the *in vitro* reconstituted reactions with purified proteins, Sae2-MRX or CtIP-MRN, prefer blocked ends over free ends for 5’ strand cleavage (41,42). In the absence of blocks, these proteins degrade 3’ free ends (41,42). Collectively, these observations raise the possibility that a different mechanism exists for the initiation of resection at clean DSBs.

To determine how resection is normally initiated at clean DSBs in the presence of all resection activities, we used the *Xenopus* nucleoplasmic extract (NPE) isolated from synthetic nuclei—a cell-free system that faithfully recapitulates the proper DNA damage response in S and G2 phases of the cell cycle (52,59,63,80–85). We found that resection initiation of clean DSBs also occurs via endocleavage of the 5’ strand DNA and that Dna2 is the primary nuclease for this process. Only in the absence of Dna2 function, CtIP initiates resection of clean DSBs. The CtIP pathway initiates resection also via endocleavage, but at different sites on the 5’ strand DNA. MRN promotes resection initiation by both Dna2 and CtIP, in part by facilitating their recruitment to the DNA substrate. The ssDNA-binding protein RPA also promotes both resection initiation pathways, but plays a minor role in the loading of Dna2, CtIP and MRN to DSBs.
2.2 Materials and methods

2.2.1 Xenopus nuclear extract, antibodies, immunodepletion, immunoblotting and co-immunoprecipitation

*Xenopus* nucleoplasmic extract (NPE) was prepared from synthetic nuclei assembled in the crude egg extract as previously described (80). Dna2 antibody was raised in rabbits against a bacterially expressed His-tagged fusion protein containing the N-terminal 712 amino acids of *Xenopus* Dna2 protein. Antibodies against *Xenopus* Exo1, PCNA, RPA, CtIP, NBS1 and Chk2, have been described before (26,31,52,94). For immunodepletion, 10 ml protein A agarose beads coupled with 50 ml of the protein antiserum or 50 ml each of two antisera for double-depletion were incubated with 50 ml NPE for 45 min at 4 °C. Beads were then removed from the extract by low-speed centrifugation (5,000 rpm) in a desktop microcentrifuge. The extract supernatant was then subjected to two additional rounds of depletion under the same conditions. Immunoblotting was performed using DyLight 800- and DyLight 680-conjugated secondary antibodies (Pierce) and an Odyssey Infrared Imaging System (LI-COR Biosciences), as described previously (52,84,85). To examine the interaction between RPA and Flag-Dna2 proteins shown in Fig. 2.4F, Protein A agarose beads bound by RPA antibodies were incubated with recombinant Flag-Dna2(WT) and Flag-Dna2(27-1053) proteins at 4 °C for 1 hour. The beads were then washed with 250 ml ELB buffer containing 0.5% NP-40 for 5 times followed by elution of proteins with sample buffer and analyzed by Western blotting.

2.2.2 Expression and purification of recombinant proteins

Baculoviruses expressing wild type, Flag-tagged *Xenopus* Dna2 were described previously (60). Flag-Dna2(D278A), Flag-Dna2(K655E) and Flag-Dna2(27-1053) expression constructs were
generated by PCR and cloned into the pFastBac1 vector by a Gibson Assembly method. Flag-tagged *Xenopus* CtIP in the FastBac1 vector was kindly provided by Dr. Jean Gautier (Columbia University) (69,100,108). All clones were verified by sequencing. Bacmids expressing CtIP and Dna2 proteins were generated in DH10Bac bacterial cells, and proteins were expressed in Sf9 cells using a Bac-to-Bac baculovirus expression system (Invitrogen), according to the manufacturer’s protocol. The Dna2 and CtIP recombinant proteins were affinity-purified using anti-Flag M2 beads (Sigma) following the standard protocol (109). Purified proteins were aliquoted to 3µl, frozen in liquid nitrogen and stored at – 80 °C. Expression and purification of wild-type RPA complex and the RPA (1NΔ) mutant complex were described previously (61). The RPA1 subunit in RPA (1NΔ) lacks 121 amino acids in the N-terminus (61).

2.2.3 DNA substrates and resection assays

To generate a model DNA substrate for assessing resection initiation at a clean DSB, PCR was performed to generate a one-end biotinylated 2 kb DNA fragment with a 5’ biotinylated primer and pBluescript SK(-) as the template. The free 5’ end was $^{32}$P-labeled with T4 polynucleotide kinase (PNK) in the presence of $^{32}$P-g-ATP. Another DNA substrate (2.1 kb) used in Fig. S2.1B and S2.1D was generated in the same way, but contains the DNA sequence that encodes the first 700 amino acids of human Exo1. DNA substrates with blunt and recessed ends used in 2. S2.2 were prepared by PCR using pBluescript SK(-) as the template to generate a 2 kb product, which were then digested with either KpnI or XhoI to generate 3’ or 5’ overhangs, respectively, on both ends. The substrates were then treated with Alkaline Phosphatase (New England BioLabs) to dephosphorylate the ends followed by 5’ $^{32}$P-labeling with PNK in the presence of $^{32}$P-g-ATP. Oligonucleotide marker was prepared by mixing 10-24, 30, 35, 40, 45 mer oligonucleotides
derived from the sequence at the radiolabeled 5’ end of the 2 kb DNA substrate used throughout the study. The marker mix was also 5’ 32P-labeled using PNK.

A typical resection assay involved 10 ml undeleted or depleted NPE supplemented with an ATP regenerating system (2 mM ATP, 20 mM Phosphocreatine and 5 ng/ml Creatine Phosphokinase) and 1 ml (5 ng/ml) of a radiolabeled DNA substrate. After incubation at room temperature, 2 ml reactions were withdrawn and mixed with 10 ml stop buffer (8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 0.2% bromophenol blue, 0.5% SDS, 80 mM Tris–HCl, pH 8.0) supplemented with 2 mg/ml Proteinase K. The samples were incubated at 37 °C for 2 hr, and then mixed with an equal volume of formamide, heated for 3 min at 95 °C, chilled on ice and run on a pre-run 16% polyacrylamide–urea (8 M) gel. After running, the gel was incubated with a protein destaining buffer (40% methanol and 10% acetic acid) overnight with gentle shaking for fixation and removal of urea. The gel was then placed on top of DE-81 filter paper (to retain small DNA resection products) and Whatman filter paper and dried before autoradiography.

To assay the flap endonuclease activity of recombinant Dna2 proteins, a flap DNA substrate was generated by annealing 3 ssDNA oligos (5’-CCA GTG AAT TCG AGC TCG GTA CCC GCT AGC GGG GAT CCT CTA-3’, 5’-32P-ATT GGT TAT TTA CCG AGC TCG AAT TCA CTG G-3’, 5’-TAG AGG ATC CCC GCT AGC GGG-3’) as described previously (110). The 5’ end of the flap ssDNA was labeled with 32P using PNK. 5’ 32P-labeled oligos of 10–13 nucleotides in length derived from the same sequence of the ssDNA flap were used as markers. The flap substrate was incubated in reaction buffer (40 mM Tris, pH 7.4, 50 mM NaCl, 5 mM MgCl2, 4 mM ATP, 2.5 mM DTT and 5% Glycerol) along with recombinant Flag-Dna2 proteins at room temperature. The reaction samples were then treated the same way as resections with 5’ 32P-labeled dsDNA substrate but resolved in a 20% polyacrylamide gel containing 8 M urea. The gel
was then incubated with the destaining buffer overnight for fixation and removal of urea before drying and autoradiography.

The 3’ 32P-labeled, 6 kb dsDNA substrate used in Fig. S2.5 resection assay was prepared by digestion of a ~ 6 kb plasmid pRS315 with XhoI and labeled by end filling using exonuclease-deficient Klenow Fragment in the presence of 32P-α-dCTP, dGTP, dTTP and ddATP as described previously (52,84,85). The resection samples were run on a 0.8% agarose gel followed by drying on top of Whatman filter paper and autoradiography.

### 2.2.4 DNA binding assay

For DNA binding assay in the extract, the one-end biotinylated 2 kb DNA fragment derived from pBluescript SK(-) was immobilized on streptavidin magnetic beads (New England BioLabs). 2.5 µl of beads coupled with 50 ng of biotinylated DNA fragment were incubated with 10 µl of NPE at room temperature for the indicated times. After incubation, beads were isolated and washed twice with 250 µl of egg lysis buffer. The beads were then treated with Lambda protein phosphatase (New England BioLabs) for 30 mins at 30 ºC to dephosphorylate DNA-bound proteins (to avoid gel mobility changes that could affect accurate detection of the proteins on western blots). The DNA-associated proteins were then detected by Western blotting.

### 2.3 Results

#### 2.3.1 Resection of clean DSBs is initiated through endocleavage, generating 5’ oligonucleotides

To determine how the resection is initiated at a clean DSB with a free 5’ end, we generated a one-end blocked 2 kb dsDNA fragment by PCR using a 5’ biotinylated primer. The other free 5’
end of the DNA fragment was labeled with $^{32}$P using PNK followed by gel purification (Fig. S2.1A). Using this model substrate, we performed resection assays in *Xenopus* NPE, which has been used extensively for the study of DNA end resection process (52,59,63,84,85). After incubating the DNA substrate in NPE, reactions were terminated at various time points, and resection initiation at the radiolabeled 5’ end was then monitored by resolving the resection products on 16% denaturing polyacrylamide gels followed by autoradiography. The result shown in Fig. 2.1A indicates that initiation of resection of clean DSB with blunt ends occurs through clipping of the free 5’ end, generating ssDNA oligos of ~ 10-20 nucleotides (nts) in length (due to its relative large size, a portion of the original substrate was “trapped” in the loading wells of the polyacrylamide gel). The kinetics of the generation of these resection products suggests that multiple cleavage events occurred on 5’ strand DNA at different locations. Incubation of another DNA substrate of similar length but with a different sequence also generated ssDNA oligos in a similar size range from the free 5’ end (Fig. S2.1B). Furthermore, we observed the release of oligonucleotides of ~ 10-20 nts in length from the 5’ ends of DNA substrates with 5’ or 3’ ssDNA overhangs (generated by restriction digestion) in NPE (Fig. S2.2A). However, the cleavage patterns differ significantly for the DNA substrates tested (Fig. 2.1A, S2.1B, S2.2A). Together, these results suggest that resection of clean DSBs is mediated by internal cleavage and that the cleavage sites are influenced by the end sequence and structure. To further dissect the mechanism of resection initiation at clean DSBs, we used primarily the one-end, 5’ $^{32}$P-labeled 2 kb DNA fragment with blunt ends for subsequent studies.

### 2.3.2 Cleavage of free 5’ DNA ends is mediated by Dna2
To define the mechanism for the initiation of resection of clean DSBs, we next determined which nuclease(s) are responsible for the generation of the oligonucleotides from 5’ free ends. Although Sae2-MRX can initiate resection from clean DSBs in the absence of Dna2 and Exo1 both in vivo and in vitro, it is not clear whether they also initiate resection at these breaks in the presence of all the other resection activities in cells (33,34,41,42,75–77). It is possible that other resection nucleases such as Dna2 and Exo1 can directly initiate resection of these free DNA ends. To test this idea, we immunodepleted Dna2 or Exo1 from the NPE and examined the effects of depletion on the initiation of resection of the 2 kb DNA substrate with a blunt end described above.

Depletion of Dna2 completely abrogated the generation of the 10-20 nts oligonucleotides from the radiolabeled 5’ end (Fig. 2.1B, S2.6A). Importantly, addition of purified recombinant Flag-Dna2 to the Dna2-depleted extract rescued resection initiation (Fig. 2.1C, 2.1E, S2.6B). Interestingly, we observed longer oligonucleotides (≥ 45 nts) released from the 5’ end of the DNA substrate in the Dna2-depleted extract (Fig. 2.1B), suggesting that there exists an alternative mechanism for resection initiation in the absence of Dna2 (see below). Similar results were also observed for the other DNA substrate with blunt or recessed ends (Fig. S2.1C, S2.1D, S2.2B, S2.2C). In contrast to Dna2, depletion of Exo1 did not affect the production of the 5’ endocleavage products at a clean DSB, suggesting that Exo1 does not play a major role in resection initiation at clean DSBs (Fig. S2.3A, S2.3B).

Dna2 has both nuclease activity and helicase activity (86–93). To determine whether Dna2 directly initiates resection of clean DSBs, we tested the requirement of the nuclease activity of Dna2 for the endocleavage of 5’ ends. In contrast to WT Flag-Dna2, a nuclease-inactive mutant Flag-Dna2(D278A) added to the Dna2-depleted extract failed to restore resection initiation (Fig. 2.1C, 2.1F, S2.6C). This result strongly suggests that Dna2 directly initiates resection of clean
DSBs through its 5’ flap endonuclease activity. In contrast to the nuclease-inactive mutant, the helicase-inactive mutant Flag-Dna2(K655E) was able to generate oligonucleotides from the free 5’ ends in the Dna2-depleted extract (Fig. 2.1C, 2.1F, S2.6C). However, at an equal amount, Flag-Dna2(K655E) was much less efficient in initiating resection in the Dna2-depleted extract, compared to WT Flag-Dna2, although these proteins exhibited a similar level of 5’ flap endonuclease activity in reconstituted reactions (Fig. 2.1D, 2.1F) (89,91,92). In addition, the 5’ oligonucleotides generated by Flag-Dna2(K655E) were significantly smaller in size, compared to that generated by WT Flag-Dna2 (Fig. 2.1F). We conclude that although the helicase activity of Dna2 is not essential for resection initiation at clean DSBs, it promotes Dna2 nuclease function and influences the cleavage sites on the 5’ strand DNA.

2.3.3 In the absence of Dna2, CtIP initiates resection via endocleavage at more distal sites

In the Dna2-depleted extract we observed the release of resection products of 45 nts or longer from the radiolabeled free 5’ end of the DNA substrate (Fig. 2.1B, S2.1D, S2.2C). The appearance of these oligonucleotides was much delayed compared with the short oligonucleotides generated by Dna2 (Fig. 2.1B, S2.1D). The long resection initiation products were also observed in the extract containing Flag-Dna2(D278A) (Fig. 2.1F). These results suggest that there exists a backup mechanism to initiate the resection of clean DSBs ends in the absence of Dna2 or its nuclease activity. Depletion of Exo1 from Dna2-depleted extract did not affect the production of these long 5’ oligonucleotides, indicating that Exo1 is not the nuclease that generates these products (Fig. S2.3C, S2.3D). Given the ability of Sae2 and MRX to initiate resection from HO-induced clean DSBs in yeast in the absence of Dna2 and Exo1 pathways (33,34), we hypothesized that CtIP-MRN is the alternative pathway that initiates resection of
clean DNA ends in the Dna2-depleted extract. In support of this idea, we found that co-depletion of CtIP from Dna2-depleted extract completely abrogated the generation of the long oligonucleotides from the free 5’ end (Fig. 2.2B, S2.6E). Addition of recombinant Flag-CtIP to the double-depleted extract restored the generation of the 5’ long oligonucleotide products (Fig. 2.2C, 2.2D, S2.6F). Depletion of CtIP alone had a very mild effect on the generation of the short oligonucleotide products by Dna2 (Fig. 2.2A, 2.2B). We conclude that while Dna2 normally initiates resection from the free 5’ ends of DSBs, CtIP can also initiate resection from these ends when Dna2 is absent. This CtIP-mediated “backup” mechanism also occurs through endocleavage, but at more distal sites from the DNA ends relative to the cleavage sites of the Dna2 pathway.

2.3.4 MRN promotes both Dna2- and CtIP-mediated resection initiation pathways

The exact role of MRN in the resection initiation at clean DSBs remains incompletely understood. While the overall resection of clean DSBs is less efficient in the absence of MRX in yeast, the complex is dispensable for resection of these ends and the downstream HR repair (75–77). This is in sharp contrast with 5’ blocked DSBs with chemical or protein adducts where resection cannot occur without MRX/MRN (35,65–72). To determine the role of MRN in resection initiation at clean DSBs, we disrupted the function of the NBS1 subunit of the complex in the extract by immunodepletion or by addition of previously characterized inhibitory antibodies (which inhibit the damage association of NBS1 as well as its phosphorylation by ATM in response to DSBs (Fig. S2.6G, S2.4A) (26,31,94). We then examined the effects on resection initiation at the free 5’ end of the radiolabeled DNA substrate. Both the depletion and inhibition of NBS1 dramatically decreased the levels of the 10-20 nts oligonucleotides generated.
by Dna2 from the free 5’ end, suggesting that MRN promotes the Dna2-mediated resection initiation at clean DSBs (Fig. 2.3A, 2.3B, S2.6G). In contrast to NBS1, depletion of CtIP from the extract had only a mild effect on Dna2-mediated cleavage of the free 5’ end, suggesting that MRN and CtIP play distinct roles in resection initiation by Dna2 at clean DSBs (Fig. 2.2A, 2.2B, 2.3A, 2.3B). Inhibition of NBS1 completely abrogated the generation of long oligonucleotide products in the Dna2-depleted extract, indicating that MRN also plays a crucial role in the CtIP-dependent resection initiation pathway in the absence of Dna2 (Fig. 2.3C, S2.6H).

2.3.5 MRN promotes the recruitment of Dna2 and CtIP to free DNA ends to initiate resection

To determine the mechanism by which MRN promotes Dna2-mediated resection initiation at clean DSBs, we performed DNA binding assay in the extract using the same one-end 5’ biotinylated DNA fragment described above that was immobilized on streptavidin beads. After incubation with NPE, the DNA-bound beads were pulled down and the proteins bound to the DNA substrate were detected by western blotting. As expected, in untreated NPE, Dna2 and other resection factors CtIP, NBS1 and Exo1 rapidly bound to the DNA substrate (Fig. S2.4B). Inhibition of NBS1 dramatically reduced the binding of Dna2 to the DNA substrate, suggesting that MRN promotes resection initiation by Dna2, at least in part, by facilitating its damage recruitment (Fig. 2.3D). This is consistent with previous studies which have shown that MRX/MRN promotes the association of Dna2 with DSB ends (51,57,95). Inhibition of NBS1 in the extract also inhibited the binding of CtIP in the Dna2-depleted extract (Fig. 2.3E, S2.6I). NBS1 inhibition did not affect the initial binding of Exo1 or PCNA to the DNA substrate, although a mild decrease in damage association was observed at a later time point in the presence
of the NBS1 antibodies (Fig. 2.3D, 2.3E). Consistent with the multiple roles of MRN in DNA resection, disruption of NBS1 function inhibited the DNA-binding of RPA (Fig. 2.3D, 2.3E). Depletion of Dna2 had no significant effects on the damage-association of NBS1 and CtIP (Fig. S2.4E). Dna2 depletion also did not affect the damage induced phosphorylation of NBS1, further supporting that Dna2 functions downstream of MRN (Fig. S2.4C, S2.4D). Together, these results indicate that MRN facilitates the loading of both Dna2 and CtIP onto free DSB ends to initiate resection.

2.3.6 Interaction with RPA is important for Dna2-mediated resection initiation at clean DSBs

It has been shown that the ssDNA-binding protein RPA is important for DSB resection (51,57,58,60,61,96–98). In addition, RPA has been shown to promote Dna2-mediated resection specifically on the 5’ strand DNA at DSBs in reconstituted reactions (51,57,58,99). To determine whether RPA is required for Dna2-mediated initiation of resection at a clean DSB, we immunodepleted RPA from NPE and then examined the effects on resection initiation at free 5’ ends. Depletion of RPA completely abrogated the radiolabeled endocleavage products generated by Dna2 (Fig. 2.4A, S2.6J). This effect could be rescued by the addition of purified WT RPA protein complex (Fig. 2.4I, S2.6N), indicating that RPA is required for Dna2-mediated resection initiation at free 5’ ends.

RPA has been shown to directly interact with Dna2 via the N-terminus of Dna2 and the N-terminus of RPA1 (the largest subunit of RPA) (60,61,99). To further investigate the role of RPA in resection initiation, we generated a Flag-Dna2(27-1053) mutant lacking the N-terminal 26 amino acids (Fig. 2.4E). The corresponding region in mouse Dna2 has been shown to interact
with RPA \textit{in vitro} (99). Consistently, we observed that the association of RPA with Flag-Dna2(27-1053) in the extract was significantly (but not completely) reduced compared to WT Flag-Dna2 in a co-immunoprecipitation experiment (Fig. 2.4F). Compared to WT Flag-Dna2, Flag-Dna2(27-1053)—which exhibited the same level of flap endonuclease activity \textit{in vitro}—was significantly less efficient in cleaving free 5’ ends in the extract, suggesting that the interaction with RPA promotes Dna2-mediated resection initiation (Fig. 2.4G, 2.4H, S2.6M). In further support of this idea, a Dna2 binding-deficient RPA(1NΔ) mutant complex containing a N-terminally truncated RPA1 subunit could not rescue Dna2-mediated resection initiation in the RPA-depleted extract (Fig. 2.4I, S2.6N), although this mutant retains DNA–binding activity (61). These data strongly suggest that the interaction between Dna2 and RPA is important for the Dna2-mediated resection initiation at clean DSBs.

In sharp contrast with MRN, which is critical for the damage recruitment of Dna2 (Fig. 2.3D), depletion of RPA had only a very modest effect on the association of Dna2 with the DNA substrate (Fig. 2.4C). This is consistent with the notion that RPA stimulates the nuclease activity of Dna2 towards RPA-bound 5’ ssDNA flaps after DNA unwinding at DSBs (51,57,58,99). Together, these data suggest that RPA promotes Dna2-mediated resection initiation at clean DSBs by stimulating the nuclease activity towards 5’ ends through their direct interaction.

\subsection*{2.3.7 RPA also promotes CtIP-mediated resection initiation in the absence of Dna2}

Unlike Dna2-depletion, no long endocleavage products were observed in the extract after RPA-depletion (Fig. 2.4A), raising the possibility that RPA is also required for the “backup” resection initiation pathway mediated by CtIP. Because at clean DSBs the CtIP resection initiation pathway operates only in the absence of Dna2 activity, we further examined the role of RPA in
resorption initiation in the Dna2-depleted extract. In contrast to the extract depleted of Dna2 alone, no long endocleavage products were generated from the free 5’ end of the DNA substrate in the extract depleted of both Dna2 and RPA (Fig. 2.4B, S.2.6K). This effect could be rescued by the addition of purified WT RPA complex (Fig. 2.4J, S.2.6O), demonstrating that RPA also plays a critical role in the CtIP-mediated resection initiation pathway. Interestingly, addition of the RPA(1NΔ) mutant complex to the double-depleted extract could also rescue CtIP-mediated resection initiation (Fig. 2.4J). This is in sharp contrast with its inability to support Dna2-mediated resection initiation (Fig. 2.4I), suggesting that RPA(1NΔ) is a separation-of-function mutant for these two pathways. Depletion of RPA only modestly inhibited the binding of CtIP and NBS1 to the DNA substrate in the presence or in the absence of Dna2 (Fig. 2.4C, 2.4D), indicating that RPA plays a minor role in the damage recruitment of these proteins.

2.4 Discussion

Using a Xenopus nuclear extract system, we have investigated specifically the mechanism of resection initiation at clean DSBs and identified Dna2 as the primary nuclease for this step (Fig. 2.1, S.2.1, S.2.2). CtIP can also initiate resection at clean DSBs, but this happens only in the absence of Dna2 function (Fig. 2.2). Both Dna2- and CtIP-dependent resection initiation is mediated by endonucleolytic cleavage of 5’ strand DNA; however, the incision sites apparently differ, with the CtIP pathway cleaving at more distal positions from the end (Fig. 2.1, 2.2). The MRN complex promotes both the Dna2 and CtIP resection initiation pathways, at least in part by facilitating the recruitment of Dna2 and CtIP to the break sites (Fig. 2.3, S.2.4). The ssDNA-binding protein RPA also promotes both Dna2- and CtIP-mediated resection initiation at clean DSBs, but with a minor role in facilitating the damage association of Dna2 and CtIP (Fig. 2.4).
Our findings suggest a new model for resection initiation at clean DSB ends without protein or chemical adducts (Fig. 2.5). In budding yeast, Sae2-MRX can initiate resection of HO-induced DSBs in the genetic background deficient of the Dna2 and Exo1 resection pathways (33,34), indicating that Sae2-MRX (CtIP-MRN) has the ability to initiate resection from free 5’ ends under this condition. However, this observation does not address whether or not Sae2-MRX (CtIP-MRN) normally initiates resection of clean DSBs when the Dna2 and Exo1 resection activities are present. Because free 5’ ends are accessible to both exonucleases and 5’ flap endonucleases, it is possible that nucleases such as Dna2 and Exo1 can directly initiate resection at these ends. Indeed, our results demonstrate that for clean DSBs the nuclease activity of Dna2 directly initiates resection. Dna2 does so by clipping the 5’ strand DNA internally via its flap endonuclease activity (which requires free 5’ ends), generating short oligonucleotides (Fig. 2.1, 2.5). This is in sharp contrast to blocked DSBs with chemical or protein adducts at 5’ (or 3’) ends whereby Sae2-MRX or CtIP-MRN is the primary mechanism for resection initiation (35,65–72).

Our results are in agreement with the observation that purified Sae2-MRX or CtIP-MRN prefers blocked 5’ DNA ends over free ends to initiate the resection in reconstituted reactions (41,42). Importantly, our model can also explain the observed limited resection by Sae2-MRX in the absence of the Dna2 and Exo1 pathways in yeast (33,34). It is also important to point out that although CtIP normally does not mediate resection initiation at clean DSBs, it contributes to overall resection of these breaks (Fig. S2.5) (31,33,34,48,100,101). In addition to nuclease activity, Dna2 exhibits helicase activity, whose function was less understood (55,88,89,91,92,102,103). Interestingly, we found that a mutation that inactivates the helicase activity of Dna2 partially reduced the rate of resection and affected the pattern of the endocleavage products (Fig. 2.1F). While this manuscript was in preparation, two recent studies
by the Sung group and Cejka group reported similar observations for yeast and human Dna2 whereby the helicase-deficient mutants also produce smaller resection products with a lower efficiency, compared to WT Dna2 (104,105). These findings support the idea that although the helicase function of Dna2 is not essential for DSB resection it promotes proper resection. The short oligonucleotide products generated from free 5’ ends of dsDNA substrates during resection initiation are stable in the extract for a prolonged period of time; the significance of which is currently unclear. A previous study has shown that ssDNA bound to MRN can promote ATM activation in the Xenopus extracts (106). Because no appreciable effects on NBS1 or Chk2 phosphorylation by ATM were detected in the Dna2-depleted extract, we suggest that the oligonucleotide products generated by Dna2 during resection initiation are not required for ATM activation (Fig. S2.4C, S2.4D).

MRN is also important for efficient resection initiation by Dna2 at free 5’ ends, but its role is to recruit Dna2 to the DNA substrate, although we cannot rule out the possibility that it also stimulates the nuclease activity of Dna2 during initiation (Fig. 2.3A, 2.3B, 2.3D). MRN also plays a key role in the CtIP-mediated resection initiation at clean DSBs in the absence of Dna2 (Fig. 2.3C). The nuclease activity responsible for this alternative initiation pathway at clean DSBs remains to be determined. Both CtIP and MRN have been shown to contain endonuclease activity (35,43–45,66,106), although recent studies by the Cejka group suggest that the Mre11 subunit of MRN, but not CtIP, confers the nuclease activity for 5’ end cleavage, at least at the blocked ends (41,42). The cleavage sites of the CtIP-MRN pathway on the 5’ strand DNA (≥ 45 nts) are more distal than that of the Dna2-MRN pathway (~10-20 nts), indicating that distinct mechanisms are employed by these pathways during resection initiation. Although the presence of Dna2 prevents CtIP-mediated end cleavage, Dna2 apparently does not delay the loading of
CtIP onto the DNA substrate (Fig. S2.4E). Because the CtIP pathway is operational in the presence of a nuclease-inactive Dna2 mutant (Fig. 2.1F) and because the CtIP-mediated resection initiation has a slower kinetics compared to the Dna2-pathway (Fig. 2.1B), we suggest that the fast engagement of Dna2 with the DNA substrate followed by immediate resection initiation precludes the action of CtIP. The physiological significance for the existence of this CtIP-MRN-mediated back-up pathway for initiating resection remains to be determined.

Our study has also provided new insights into the role of the ssDNA-binding protein RPA in DSB resection. Although it has been shown that RPA generally promotes DNA resection by both Dna2 and Exo1 (51,57,58,60,61,96–98), its function in resection initiation remained unclear. Our data indicate that RPA is required for the resection initiation at clean DSBs, with a role in both the Dna2 and CtIP-mediated pathways (Fig. 2.4). While previous studies have shown that RPA can stimulate the nuclease activity of Dna2 specifically towards the 5’ ends of a DSB in reconstituted reactions (51,57,58,99), a function of RPA in the CtIP-MRN mediated resection initiation is surprising, as Sae2-MRX or CtIP-MRN can directly clip 5’ strand DNA at blocked DSB ends in the absence of RPA in reconstituted reactions (41,42). The requirement of RPA for the CtIP pathway suggests that in the absence of Dna2, DNA needs to be unwound before CtIP-MRN can initiate resection at clean DSBs. RPA may stimulate the activity of CtIP-MRN or helps stabilize ssDNA substrate for CtIP-MRN-mediated cleavage. In agreement with the RPA requirement, a recent study has shown that the helicase activity of RECQL4 is required for CtIP-MRN-dependent resection in human cells (107). It will be interesting to determine whether RECQL4 is required for CtIP-MRN-mediated resection initiation at clean DSBs in the absence of Dna2. In a previous study using a heat-inducible degron system to deplete RPA from budding yeast, Symington and colleagues observed limited resection by Sae2-MRX at a clean DSB.
generated by the HO endonuclease in the td-RFA1 strain (96). This residual resection by Sae2-MRX could be caused by the incomplete depletion of RPA before break generation by HO, as pointed out by the authors (96). Alternatively, this discrepancy in RPA requirement could reflect the mechanistic differences in resection initiation between yeast and metazoans. While the direct interaction between the N-terminus of RPA1 and the N-terminus of Dna2 is required for Dna2-mediated resection initiation, the N-terminus of RPA1 is dispensable for the CtIP initiation pathway. The identification of RPA(1NΔ) as a potential separation-of-function mutant provides a unique opportunity for further elucidation of the two resection initiation pathways in the future.

2.5 Acknowledgments

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Figure 2.1. Dna2 initiates the resection of clean DSB ends via end cleavage of 5’ strand DNA.

A). Resection of a 5’ free DNA end in NPE is initiated via endocleavage at multiple sites, generating short oligonucleotides of various sizes (~10-20 nts). A gel-purified, one-end 5’ $^{32}$P-labeled 2 kb DNA fragment was incubated in NPE at room temperature. Reactions were stopped at the indicated times and the reaction products were resolved in a 16% polyacrylamide-urea gel. The top band represents the original DNA substrate that was “trapped” in the loading wells of the gel.

B). Immunodepletion of Dna2 completely abrogated the generation of short resection initiation products (~10-20 nts) from the 5’ end of the DNA substrate, but resulted in the generation of long oligonucleotides (≥ 45 nts).

C). Purified recombinant Flag-Dna2(WT), Flag-Dna2(D278A) and Flag-Dna2(K655E) expressed in insect cells.

D). Flag-Dna2(WT) and the helicase-inactive mutant Flag-Dna2(K655E) exhibited a similar level of nuclease activity on a dsDNA substrate with 5’ ssDNA flap in vitro. The nuclease-inactive mutant Flag-Dna2(D278A) had no detectable nuclease activity toward the same substrate.

E). Addition of recombinant wild type Flag-Dna2 to the Dna2-depleted extract rescued the generation of short resection initiation products and prevented the generation of long initiation products.

F). Comparison of the ability of recombinant Flag-Dna2(WT), Flag-Dna2(K655E) (helicaseinactive mutant) and Flag-Dna2(D278A) (nuclease-inactive mutant) in restoring resection initiation in the Dna2 depleted extract.
Figure 2.2. In the absence of Dna2, CtIP initiates resection at clean DSBs via cleavage of 5′ strand DNA at more distal sites.
A). Depletion of CtIP modestly affected the short resection initiation products generated by Dna2.

B). Co-depletion of CtIP with Dna2 completely abrogated the generation of long resection initiation products.

C). Purified recombinant Flag-CtIP expressed in insect cells.

D). Addition of recombinant Flag-CtIP rescued the long resection initiation products in the extract depleted of both Dna2 and CtIP.
**Figure 2.3. MRN promotes both Dna2- and CtIP-mediated resection initiation.**

A). Depletion of NBS1 dramatically inhibited the generation of short resection initiation products by Dna2 in the extract.

B). Addition of inhibitory NBS1 antibodies dramatically reduced the generation of short resection initiation products by Dna2 in the extract.

C). Addition of inhibitory NBS1 antibodies to the Dna2-depleted extract completely abrogated the generation of long resection initiation products by CtIP.
**D).** Effects of NBS1 inhibitory antibodies on the binding of NBS1, CtIP, Dna2, Exo1, RPA and PCNA to the DNA substrate immobilized on beads in the extract.

**E).** Effects of NBS1 inhibitory antibodies on the binding of NBS1, CtIP, Exo1, RPA and PCNA to the DNA substrate immobilized on beads in the Dna2-depleted extract.
A). Depletion of RPA completely abrogated the short resection initiation products generated by Dna2.

B). Removal of RPA abrogated the CtIP dependent long resection initiation products in the Dna2-depleted extract.

C). RPA depletion modestly inhibited the binding of Dna2 to the DNA substrate immobilized on beads in the extract.

D). Removal of RPA from the Dna2-depleted extract modestly inhibited the binding of CtIP to the beads immobilized DNA substrate.

E). Purified recombinant Flag-Dna2(WT) and Flag-Dna2(27-1053) expressed in insect cells.

F). Flag-Dna2(27-1053) exhibited reduced interaction with RPA in the extract, compared to Flag-Dna2(WT).

G). Flag-Dna2(WT) and Flag-Dna2(27-1053) exhibited a similar level of flap endonuclease activity in vitro.
**H)**. Flag-Dna2(27-1053) added to the Dna2-depleted extract generated smaller resection initiation products at a clean DSB with a slower kinetics, compared to Flag-Dna2(WT).

**I)**. Addition of WT RPA but not the RPA(1NΔ) mutant rescued the Dna2-dependent short resection initiation products in the RPA-depleted extract.

**J)**. Both WT RPA and RPA RPA(1NΔ) could rescue the CtIP-dependent long resection initiation products in the extract depleted of both Dna2 and RPA.
Figure 2.5. A model for resection initiation at clean or blocked DSBs.

DNA end resection at a blocked DSB with protein or chemical adducts is initiated through endocleavage by CtIP-MRN. In contrast, resection initiation at a clean DSB with free DNA ends is initiated by Dna2, which cleaves the 5’ strand DNA ~10-20 nts away from the end. The MRN complex promotes this step by facilitating the recruitment of Dna2 to the DNA end. In the absence of Dna2, CtIP together with MRN mediate an alternative pathway to initiate resection, which occurs also through 5’ endocleavage but at more distal sites (≥ 45 nts) from the end. RPA promotes resection by both Dna2 and CtIP, but plays a minor role in facilitating their damage recruitment. The Dna2 pathway requires the direct interaction of Dna2 with the N-terminus of RPA1 in the RPA complex, but this domain of RPA1 is dispensable for the CtIP pathway.
Figure S2.1 (related to Figure 2.1). Analysis of resection initiation on another DNA substrate with a blunt end.
A). A radiolabeled dsDNA substrate used for resection initiation analysis throughout the study. The one-end 5’ $^{32}$P-labeled dsDNA substrate of 2 kb was generated by PCR using a 5’ biotinylated primer and pBluescript SK(-) as a template, followed by $^{32}$P labeling using PNK and gel purification.

B). Resection of another one-end 5’ $^{32}$P-labeled, 2.1 kb dsDNA substrate with free 5’ end in the extract also generated endocleavage products of ~10-20 nts in length.

C). Immunodepletion of Dna2 from *Xenopus* NPE.

D). The depletion of Dna2 from the extract prevented the generation of the short resection initiation products of ~10-20 nts, but led to generation of long oligonucleotides ($\geq 45$ nts) from the radiolabeled 5’ end.
Figure S2.2 (related to Figure 2.1). Analysis of resection initiation at clean DSBs with 5’ or 3’ ssDNA overhangs.

A) Resection on a 2 kb dsDNA substrate with a blunt end, a 3’ ssDNA overhang or a 5’ ssDNA overhang in the extract generated endocleavage products of ~10-20 nts from free 5’ ends.

B) Immunodepletion of Dna2 from *Xenopus* NPE.

C) Depletion of Dna2 from the extract prevented the generation of short resection initiation products (~10-20 nts), but led to generation of long oligonucleotides (≥ 45 nts) from the substrates with a blunt end or with a 3’ or 5’ ssDNA overhang depicted in A).
Figure S2.3 (related to Figure 2.2). Exo1 apparently does not play a role in resection initiation at a clean DSB.

A). Immunodepletion of Exo1 from *Xenopus* NPE.
B). Depletion of Exo1 did not affect the generation of short resection initiation products by Dna2.

C). Immunodepletion of Dna2, Exo1 or both from *Xenopus* NPE.

D). Depletion of Exo1 did not affect the generation of long resection initiation products in the absence of Dna2.
Figure S2.4 (related to Figure 2.3). Effects of NBS1 inhibition on damage-induced NBS1 phosphorylation, and effects of Dna2 depletion on the damage-induced phosphorylation of NBS1 and Chk2 and on the damage association of NBS1, CtIP, Dna2, Exo1, RPA and PCNA.

A). Addition of inhibitory antibodies against NBS1 inhibited phosphorylation of NBS1 in the extract induced by DSB damage. Phosphorylation of NBS1 resulted in reduced mobility on polyacrylamide gels.
B). Time course of the binding of NBS1, Dna2, CtIP, Exo1, RPA and PCNA to the beads-immobilized DNA substrate in NPE.

C). Immunodepletion of Dna2 from *Xenopus* NPE.

D). Depletion of Dna2 did not affect the damage-induced phosphorylation of NBS1 and Chk2 in the extract. Phosphorylation of Chk2 also resulted in reduced mobility on polyacrylamide gels.

E). Dna2 depletion did not affect the binding of NBS1, CtIP, Exo1, RPA and PCNA to a dsDNA substrate immobilized on beads in the extract.
Figure S2.5 (related to Discussion). Effects of Dna2 or CtIP depletion on the overall resection of a 3’ end-labeled DNA substrate.

A). Immunodepletion of Dna2 or CtIP from Xenopus NPE.

B). Depletion of Dna2 or CtIP inhibited the resection of a 3’ $^{32}$P-labeled 6 kb dsDNA substrate.

Reactions were terminated at the indicated times and resection samples were resolved on a 0.8 % agarose gel followed by drying and autoradiography.
Figure S2.6 (related to Figures 2.1-2.4). Results of immunodepletion in *Xenopus* NPE.
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Chapter 3:

Poly(ADP-ribose)-binding promotes Exo1 damage recruitment and suppresses its nuclease activities

Abigail Cheruiyot*, Sharad C. Paudyal*, In-Kwon Kim*, Melanie Sparks, Tom Ellenberger, Helen Piwnica-Worms, and Zhongsheng You

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3.1 Introduction

First identified in *Schizosaccharomyces pombe*, Exo1 is an evolutionarily conserved nuclease that participates in DNA replication and repair pathways, including DNA double-strand break repair (DSBR), mismatch repair (MMR) and error-free post-replicative repair by template switching (1–3). Exo1 exhibits both 5’ to 3’ exonuclease activity and 5’ flap endonuclease activity (4). The exonuclease activity of Exo1 is crucial for the resection of DNA double-strand break (DSB) ends and the removal of mispaired nucleotides to enable the rejoining of DNA ends (1, 5, 6). During MMR, Exo1 excises DNA from the nick generated by Mlh1-Pms1 in the 5’ to 3’ direction to create a gap for subsequent repair steps (3, 5). The resection of DSBs generates long ssDNA tails that initiate DNA repair by homologous recombination (HR) and activate the ATR-dependent cell cycle checkpoint (7, 8). The process of DNA resection is thought to be initiated by endonucleolytic cleavage near the breakpoint that is mediated by the Mre11-Rad50-NBS1 (MRN) complex (Mre11-Rad5-Xrs2 in budding yeast) together with the CtIP (Sae2 in budding yeast) protein. Following this initial endocleavage, the resulting “clean” 5’ ends are further resected by Exo1 and Dna2, which act redundantly to generate long 3’ ssDNA overhangs (e.g. 2–4 kb in budding yeast) required for HR and the ATR checkpoint (9, 10). The resection of dsDNA ends to create ssDNA overhangs also inhibits DSB repair by nonhomologous end-joining (NHEJ) and attenuates the ATM-dependent checkpoint pathway (11, 12). Exo1’s 5’ flap endonuclease activity is thought to resolve DNA intermediates formed during replication and recombination. Genetic studies in yeast suggest that the flap endonuclease activity of Exo1 plays a redundant role with Fen1 (Rad27 in budding yeast) in Okazaki fragment maturation. Consistent with this notion, overexpression of yeast EXO1 or human Exo1 proteins functionally complements the replication defects of a *rad27* mutant (13).
The regulation of Exo1 activity ensures efficient break processing while avoiding unscheduled or uncontrolled DNA digestion that could lead to cell death or genomic instability. Indeed, Exo1 function is regulated in some manner by a number of proteins that function in DSB resection, including MRN, CtIP, Ku, RPA, SSO1, BLM, SWR1, ATM, ATR, Rad53 and CDK (14–32). We have previously shown that Exo1 activity during DSB resection is promoted by the sliding DNA clamp PCNA and inhibited by the 14-3-3 adaptor proteins through direct protein-protein interactions. PCNA binds to the PCNA-interacting protein box (PIP box) located in the C-terminus of Exo1 and supports the processive nuclease activity of Exo1 during DNA resection (33). 14-3-3 proteins contact the central domain of Exo1 and restrain its damage association and DNA resection activities (34). However, an Exo1 mutant lacking PCNA-binding activity is transiently recruited to sites of DNA damage by an unknown mechanism (33, 34). Here we have investigated the role of poly(ADP-ribose) produced in response to DNA damage as a regulator of Exo1.

A prominent early response to DNA breaks is protein PARylation by the enzyme poly(ADP-ribose) polymerase 1 (PARP1) (35, 36). PARP1’s enzymatic activity is activated by binding to DNA breaks, causing a localized burst of PARylation on many proteins at sites of DNA damage such as histone H1, XRCC1 as well as PARP1 itself. This posttranslational modification is transient, and is rapidly removed by the activity of poly(ADP-ribose) glycohydrolase (PARG), resulting in a robust, but transient pulse of protein PARylation at sites of DNA damage. The synthesis of PAR chains is an early response to DNA damage that creates docking sites for many checkpoint and repair proteins and chromatin remodeling factors (e.g. XRCC1, Ligase 4, NBS1, SSB1, BARD1, CHD4, ALC1, CHRF and APLF) with PAR-binding activity (36–44). Although the precise roles of the transient PARylation in the DNA damage response remains to be defined,
deficiencies in the PAR-binding activities of these factors affect chromatin structural remodeling and the kinetics of DNA repair (35, 45).

In this study, we show that Exo1 is a PAR-binding protein and that this PAR-binding activity contributes to the timely recruitment of Exo1 to DNA damage sites. Contrarily, PAR binding inhibits both the exonuclease and 5’ flap endonuclease activities of Exo1, suggesting that Exo1 activity may be held in check at damage sites until PAR is removed by the action of PARG. This delay could provide an opportunity for the cell to integrate various physiological signals before activating the long-range resection of DNA during DSBR or nucleotide excision during MMR.

3.2 Materials and methods

3.2.1 Plasmids, antibodies and chemicals

GFP-Exo1(WT), GFP-Exo1(1– 507), GFP-Exo1(508– 846) and GFP-Exo1(ΔPIP) in the pEGFP-C1 vector, mCherry-Difopein(WT) and mCherry-Difopein(MUT) in the pmCherry-C1 vector, and baculovirus expression constructs encoding C-terminally His-tagged Exo1(WT) and Exo1(ΔPIP) were described previously (33, 34). C-terminally His-tagged Exo1(1– 507) was cloned into the Gateway donor vector pDONR221 through PCR and BP recombination, and then transferred into pDEST8 through LR recombination, according to the manufacturer’s protocols (Life Technologies). GST-AF1521 in pGEX4T-1 was obtained from Dr. Michael Nielsen (University of Copenhagen). GST-PARP1C in pGEX-6P1 and His-PARP1(DBD) in pET28a(+) were described before (33, 46). Rabbit antibodies against *Xenopus* Exo1, Dna2 and PCNA were described previously (33). Anti-GST antibodies were home raised in rabbits using a GST-EGFP fusion protein as antigen. Anti-GFP (Clontech, 632569), anti-mCherry (BioVison, 5993– 100), anti-PAR polymer antibodies (Trevigen, 4335-MC– 100), Olaparib (Selleckchem, S1060), ADP-
3.2.2 **Cell culture, transfection, laser microirradiation, live-cell imaging and immunofluorescence staining**

Human U2OS and HEK293T cells were cultured in DMEM with 10% fetal bovine serum (FBS) at 37 °C with 5% CO₂. Plasmid DNA was transfected into U2OS and HEK293T cells using TransIT-LT1 (Mirus) transfection reagent, according to the manufacturer’s protocols. A customized laser microirradiation and live-cell imaging system was described before (33, 34, 47). Immunofluorescence staining to detect PAR signal at the DNA damage sites after laser irradiation was performed as previously described (33, 34). Anti-PAR primary antibodies were used at 1:100. Primary antibodies were detected with goat anti-mouse Alexa Fluor 488-conjugated secondary antibodies (Life Technologies, A11001, used at 1:250). DNA was visualized with Hoechst 33342 staining (1 µg/ml).

3.2.3 **Xenopus nuclear extract, immunodepletion, immunoblotting, chromatin binding and PARylation assays in the Xenopus extract**

*Xenopus* nucleoplasmic extract derived from unfertilized eggs was prepared from synthetic nuclei assembled in a crude egg extract, as previously described (48). To deplete xExo1 from the *Xenopus* nuclear extract, 10 µl protein A agarose beads coupled with 40 µl xDna2 antiserum or both xExo1 and xDna2 anti-serum were incubated with 50 µl extract for 45 min at 4 °C. Beads were then removed from the extract by centrifugation (Beckman Microfuge E with a single fixed speed, 1 min). The extract supernatant was then subjected to two additional rounds of depletion.
under the same conditions. Immunoblotting was performed using DyLight 800-or DyLight 680-conjugated secondary antibodies (Pierce) and an Odyssey Infrared Imaging System (LI-COR Biosciences), as described previously (33, 34).

To assay protein PARylation in the *Xenopus* extract, NPE supplemented with an ATP regenerating system (2 mM ATP, 20 mM Phosphocreatine and 5 µg/ml Creatine Phosphokinase) was incubated with a 400 bp dsDNA fragment (generated by PCR) (25 ng/µl). Samples were withdrawn at the indicated times in Fig. 3.4A and analyzed by SDS-PAGE followed by far-western blotting using GST-AF1521. Briefly, after protein transfer, the PVDF membrane was incubated with 3.75 µg/ml purified GST-AF1521 recombinant protein overnight at 4 °C. The membrane was then blotted for GST using anti-GST antibodies and DyLight 680-conjugated secondary antibodies.

### 3.2.4 Recombinant protein expression and purification

N-terminally GST-tagged AF1521 was expressed in the *E. coli* strain BL21(DE3) and was affinity-purified using FPLC with a GSTrap FF column (GE healthcare), according to the manufacturer’s protocol. N-terminally GST-tagged PARP1C containing the catalytic domain of human PARP1 (aa. 375–1014) was expressed in *E. coli* Rosetta cells, and was first affinity-purified on a glutathione Sepharose column, and then further purified on a Superdex 200 size-exclusion column (GE healthcare) in buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM DTT and 5% glycerol. N-terminally His-tagged DNA binding domain of human PARP1 (DBD; aa 1–374), and N-terminally His-tagged full length human PARP1 and PARP1C protein fragment in pET28a(+) were expressed in *E. coli* Rosetta cells and purified as described previously (46, 49). Purified proteins were dialyzed in PBS containing 10% glycerol, frozen in
liquid nitrogen and stored at −80 °C. C-terminally His-tagged Exo1(WT), Exo1(ΔPIP) or Exo1(1–507) was expressed in sf9 cells using the baculovirus expression system and purified using HisPur cobalt resin (Pierce) as described previously (33).

3.2.5 In vitro PARylation, PAR chain synthesis and purification, PAR binding and competition

For in vitro PARylation, GST-PARP1C (2 µM) was enzymatically auto-modified in a reaction containing the PARP1 DBD (2 µM), a 24 mer nicked DNA oligo (2 µM), and NAD⁺ (500 µM) for 1 hr at 37 °C, as described previously (46, 50). PAR chains were synthesized and purified as described previously (50, 51). Briefly, PAR polymers were synthesized in a reaction containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 12.5 µg/mL calf thymus DNA, 2 µM His-PARP1 protein, 4 µM His-PARP1C protein fragment, and 2 mM NAD⁺. PARylated PARP1 was precipitated in 10% ice-cold TCA, and the pellet was resuspended in 1 M KOH and 50 mM EDTA, and then incubated for 1 hour at 60 °C to detach PAR polymers from the denatured PARP1. The detached PAR chains were recovered from a dihydroxyboryl Bio-Rex 70 (DHBB) column and were desalted using a PD-10 desalting column (GE Healthcare). Purified PAR chains were air-dried and dissolved in water at a final concentration of 10 mM. The molar concentration of PAR (expressed as the concentration of ADP-ribose units) was estimated as follows: [PAR] = [(A₂₆₀ cm⁻¹)/[13,500 cm⁻¹M⁻¹]] (51). For the PAR-binding experiments, unmodified and PARylated GST-PARP1C (2 µM) were immobilized by incubating with GSH-sepharose beads for 30 min at room temperature in a binding buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM DTT and 1% NP-40. The beads were washed extensively with the binding buffer to remove DBD and the DNA oligo. For GST pull-down in cell lysate, HEK293T
cells expressing GFP, GFP-Exo1, GFP-Exo1(1–507) or GFP-Exo1(508–846) were lysed in the lysis/binding buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris, 1% NP-40, 5 mM NaF, 0.1 mM Na3VO4, 1 mM PMSF, 10 µM Olaparib, 3 µM ADP-HPD, pH8.0) for 1 hr at 4 °C. Bead-immobilized, modified or unmodified GST-PARP1C was then incubated with the cell lysate for 2 hr at 4 °C. Beads were then washed 3 times with the lysis/binding buffer. For GST pull-down with purified proteins, His-tagged Exo1(WT) (2.5 µg) or Exo1(1–507) (0.6 µg) was incubated with PAR-modified or unmodified GST-PARP1C for 20 min at 4 °C. Beads were then washed four times with the lysis/binding buffer above. For the PAR binding competition assay, Exo1(WT)-His recombinant protein was bound to PARylated GST-PARP1C immobilized on GSH-sepharose beads in a column. Beads were then washed extensively with the lysis/binding buffer described above. PAR polymer, ADP-ribose monomer, or polyadenylic acid was then loaded onto the columns three times for binding competition at room temperature. Bead-bound Exo1(WT)-His was then analyzed by SDS-PAGE and immunoblotting.

3.2.6 DNA substrate labeling, DNA end resection in Xenopus extracts and with purified proteins, 5’ flap cleavage assay

The 3’ 32P-labeled, 6 kb DNA substrate for resection was prepared as previously described (33). A typical resection reaction in the Xenopus extract contained 6 µl untreated or treated NPE (immunodepletion and/or addition of recombinant proteins) supplemented with an ATP regenerating system (2 mM ATP, 20 mM Phosphocreatine and 5 µg/ml Creatine Phosphokinase) and 1.5 µl radiolabeled DNA substrate (2 ng/µl). In a typical in vitro resection reaction, 30 nM Exo1-His and 2 ng/µl radiolabeled DNA substrate were incubated in the reaction buffer (20 mM Hepes pH 7.5, 50 mM KCl, 0.5 mM DTT, 5 mM MgCl2, 5% glycerol) at room temperature.
Following incubation, 1.5 µl reactions were stopped at indicated times by incubating with 10 µl stop buffer (8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 0.2% bromophenol blue, 0.5% SDS, 80 mM Tris-HCl, pH 8.0) supplemented with Proteinase K (2 mg/ml) for 2 hr at 37 °C. Samples were then resolved on 0.8% TAE-agarose gels overnight, followed by gel drying and autoradiography, as previously described (33). To assay the flap endonuclease activity of Exo1, a 5’ 32P-labeled flap DNA substrate was generated by annealing 3 ssDNA oligos (5’- CCA GTG AAT TCG AGC GTA CCC GCT AGC GGG GAT CCT CTA-3’, 5’-32P-ATT GGT TAT TTA CCG AGC TCG AGC GGG-3’) as described in Lee et al (4). 5’ 32P-labeled oligos of 10, 11, 12 and 13 nucleotides in length derived from the 5’ sequence of the flap oligo were used as markers. The reaction samples were treated the same way as resections with 3’ 32P-labeled dsDNA substrate but resolved in a 20% polyacrylamide gel containing 8 M urea. The gel was then incubated with a destaining buffer overnight for fixation and removal of urea before drying and autoradiography.

3.3 Results

3.3.1 The N-terminal domain of Exo1 mediates its initial recruitment to sites of DNA damage

The recruitment of Exo1 to sites of DNA damage is actively regulated. Using a laser-microirradiation method to create DNA breaks in human cells, we have shown that the interaction of PCNA with the C-terminal domain of Exo1 (751–846) promotes Exo1 damage association, whereas the interaction of 14-3-3 proteins with the central region (508–750) suppresses Exo1 damage recruitment (33, 34). Notably, deletion of the N-terminal domain (Exo1 residues 1–507) slowed the recruitment of Exo1 to damage sites, revealing its contribution to
Exo1 damage association (Fig. 3.1A). The N-terminal domain of Exo1 alone is robustly recruited to DNA damage (Fig. 3.1A) albeit in a transient manner lasting for only 5–10 mins compared to the full length Exo1 protein, which stably associates with the damage during the first 20 min after laser irradiation (Fig. 3.1A). These results suggest that the N-terminal domain of Exo1 increases its rate of association with DNA damage.

3.3.2 Protein PARylation promotes the early damage recruitment of Exo1

The transient association of the Exo1 N-terminal domain with DNA damage is reminiscent of the kinetics of protein PARylation at DNA damage sites. Using PAR-specific antibodies, we detected transient PARylation specifically at the sites of DNA damage for a period of 5–10 mins after laser microirradiation (Fig. 3.1B). These observations raised the possibility that protein PARylation may contribute to the damage recruitment activity of the Exo1 N-terminal domain. In support of this idea, blocking PAR synthesis with the PARP inhibitor Olaparib almost completely abrogated the damage recruitment activity of Exo1(1–507) (Fig. 3.1C). Consistent with the role of the N-terminal domain in Exo1 damage recruitment, Olaparib treatment also partially inhibited the damage association of the full length Exo1 protein, with more effects observed within the first 5 mins after laser irradiation (Fig. 3.1C). Furthermore, Olaparib completely inhibited the damage recruitment of a PIP-box mutant of Exo1 that lacks PCNA-binding activity (Fig. 3.1C). Taken together, these results strongly suggest that protein PARylation at DNA breaks promotes the initial recruitment of Exo1 through its N-terminal domain.
3.3.3 Exo1 directly binds to PAR through the N-terminal domain

To determine how PARylation activity promotes Exo1 damage recruitment, we tested the possibility that Exo1 is a PAR binding protein. Since the N-terminal domain of Exo1 is recruited to damage sites in a PAR-dependent manner (Fig. 3.1C) we asked whether this domain binds to PAR in vitro. To this end, we used an in vitro PARylation system to generate PAR chains on a GST-fused recombinant protein containing the C-terminal domain of PARP1 (aa. 375–1014; PARP1C) encompassing the automodification domain, WGR domain and the catalytic core of PARP1 (Fig. 3.2A) (46). PARylated GST-PARP1C protein (GST-PARP1C-PAR) was then immobilized on glutathione agarose beads and incubated with cell lysate containing expressed GFP, GFP-Exo1(WT), GFP-Exo1(1–507) or GFP-Exo1(508–846). As a control, bead-immobilized unPARylated GST-PARP1C was incubated with the cell lysates under the same condition. As shown in Fig. 3.2B, GFP-Exo1(WT) and GFP-Exo1(1–507) were associated with GST-PARP1C-PAR, whereas GFP-Exo1(508–846) or GFP alone was not. None of these proteins associated with GST-PARP1C lacking the PAR modification under the same condition (Fig. 3.2B). These results indicate that Exo1 associates with PARylated protein(s) and that the N-terminal domain of Exo1 is both necessary and sufficient for its PAR-binding activity. To test whether the N-terminal domain of Exo1 directly and specifically binds to PAR, we incubated purified recombinant, C-terminally His-tagged Exo1(WT) and Exo1(1–507) proteins with bead-immobilized GST-PARP1C-PAR or GST-PARP1C (Fig. 3.2C). Both Exo1(WT) and Exo1(1–507) associated with PARylated GST-PARP1C, but not unmodified GST-PARP1C (Fig. 3.2D). Importantly, the binding of Exo1 to PARylated PARP1C was effectively competed by protein-free PAR polymers, whereas monomeric ADP-ribose or poly(A) RNA had little or no effect on Exo1’s PAR-binding activity. These results demonstrate the specificity of Exo1’s PAR-binding
activity (Fig. 3.2E). We conclude that the N-terminal domain of Exo1 mediates the initial damage recruitment of Exo1 by directly binding to PAR generated at sites of DNA damage.

3.3.4 14-3-3 proteins suppress the PAR-binding activity and damage recruitment of Exo1

We previously showed that 14-3-3 proteins bind to the central domain of Exo1, suppressing its interaction with PCNA (34). To determine whether 14-3-3s also regulate Exo1’s PAR binding activity, we first examined the effects of deletion of the central domain of Exo1 on its PAR-binding activity. As shown in Fig. 3.3A, GFP-Exo1(ΔCR) exhibited a much higher level of PAR-binding than GFP-Exo1(WT), raising the possibility that interaction with 14-3-3s also restrains the PAR-binding activity of Exo1. To further test this idea, we overexpressed a peptide antagonist Difopein (Difopein(WT)) to specifically disrupt the interaction between Exo1 and all 14-3-3 isoforms in cells. A mutant form of Difopein (Difopein(MUT)) that lacks 14-3-3-binding activity was used as a control. We previously showed that overexpression of mCherry-Difopein (WT), but not mCherry-Difopein(MUT), abolishes the interaction of Exo1 with 14-3-3s (see Fig. 3.2B in (34)). Overexpression of mCherry-Difopein(WT) dramatically increased the PAR binding of GFP-Exo1(WT) expressed in mammalian cells in comparison to overexpression of the mCherry-Difopein(MUT) protein (Fig. 3.3B). In contrast, mCherry-Difopein(WT) overexpression had no or little effect on the PAR binding of GFP-Exo1(ΔCR) that lacks the 14-3-3-binding activity (Fig. 3.3B). These results, together with our recently published findings (34), reveal that 14-3-3 proteins negatively regulate the damage association of Exo1 by suppressing its binding to PAR and PCNA.
3.3.5 Inhibition of PARylation did not affect Exo1-mediated DNA end resection in the *Xenopus* egg extract

Although the PAR binding activity of Exo1 facilitates recruitment to sites of damage (Fig. 3.1), it is unknown how this interaction affects DSB resection. To examine DNA resection activity of Exo1, we used a cell-free *Xenopus* nucleoplasmic extract (NPE) system that faithfully recapitulates the DNA end resection activities observed in cells (33, 34, 52). We first determined whether protein PARylation occurs in NPE in response to DNA DSBs. PARylation was detected using the recombinant PAR binding protein AF1521 (53, 54) fused to GST (GST-AF1521). A far-western blot using GST-AF1521 to detect PARylated proteins demonstrated a robust, but transient, accumulation of PARylated proteins in the NPE in response to DSBs (Fig. 3.4A and Fig. S3.1A). This DSB-induced PARylation activity was significantly inhibited by the PARP inhibitor Olaparib, and the signal was enhanced by pretreatment of the NPE with the PARG inhibitor ADP-HPD (Fig. 3.4B). Together, these results indicate that DNA damage-dependent PARylation of proteins occurs efficiently and transiently in *Xenopus* NPE in response to DNA DSBs.

To determine whether protein PARylation is important for DSB resection by Exo1, we incubated NPE with Olaparib to block PAR synthesis and then added a $^{32}$P-labeled, 6 kb dsDNA fragment as a substrate for DNA resection. The rate of DNA resection in the NPE was unaffected by addition of Olaparib (Fig. 3.4C). Because Dna2 functions redundantly with Exo1 in this DNA resection assay, we also immunodepleted Dna2 from NPE then examined the effects of Olaparib on DNA resection (Fig. 3.4D). After depleting Dna2, Olaparib still had no effect on DNA resection activity (Fig. 3.4E). The interaction of PCNA with the C-terminus of Exo1 increases the processivity of Exo1 during resection, and this interaction could mask the effect of PAR.
binding activity on the DNA resection reaction. We therefore immunodepleted both Exo1 and Dna2 from the NPE then added recombinant Exo1(ΔPIP)-His protein that lacks PCNA-binding activity to the depleted extract (Fig. 3.4F and Fig. S3.1B). DNA resection activity was subsequently measured in the presence or in the absence of Olaparib. Again we did not see significant effects of PARP inhibition on DNA resection by Exo1 lacking PCNA binding activity (Fig. 3.4G) (33). Taken together, these results indicate that protein PARylation is dispensable for resection of DSBs with “clean” DNA ends.

3.3.6 PAR inhibits the exonuclease activity and 5’ flap endonuclease activity of Exo1

The N-terminal PAR-binding domain of Exo1 also contains the nuclease domain (aa 1–325), raising the possibility that PAR-binding affects the nuclease activities of Exo1. To test this, we first determined whether PAR binding affects the exonuclease activity of Exo1, which is required for DSB resection as well as MMR. To this end, PAR chains were enzymatically synthesized with recombinant PARP1 and purified by column chromatography (50) for use in in vitro DNA resection reactions. The purified PAR polymers, monomeric ADP-ribose, poly(A) RNA, or water was added to an in vitro DNA resection reaction containing Exo1-His and a 3’ 32P-labeled, 6 kb dsDNA fragment as substrate. As shown in Fig. 3.5A, PAR polymers dramatically inhibited the exonuclease activity of Exo1. In contrast, addition of monomeric ADP-ribose in an amount equal to the amount of ADP-ribose in the PAR chains had no effect on Exo1 exonuclease activity. Although poly(A) RNA partially inhibited Exo1’s exonuclease activity, RNA was a less potent inhibitor compared to PAR polymers (Fig. 3.5A). The inhibition of Exo1’s exonuclease activity by PAR chains may provide a mechanistic explanation for why blocking PAR synthesis with olaparib does not affect DSB resection in the NPE, despite its role in promoting the initial
damage recruitment of Exo1 (Figs. 3.1 and 3.2). In the presence of olaparib, the recruitment of Exo1 may be slowed, but conversely, exonuclease activity is uninhibited by PAR to generate a normal level of resection activity.

To determine whether PAR-binding also affects the 5’ flap endonuclease activity of Exo1, we generated a 42 bp dsDNA substrate with a \(^{32}\)P labeled 5’-flap located in the center (Fig. 3.5B). The 5’-flap endonuclease activity of Exo1 was similarly inhibited by PAR polymers, but not by monomeric ADP-ribose. As for exonuclease activity (Fig. 3.5A), poly(A) RNA also partially inhibited the flap endonuclease activity of Exo1 (Fig. 3.5B). Cleavage of the 5’ flap occurred at one nucleotide inwards in the double-strand region, consistent with published results (55). Together, these results indicate that PAR polymers specifically inhibit both the exonuclease and flap endonuclease activities of Exo1.

### 3.4 Discussion

In this study, we have identified Exo1 as a novel PAR-binding protein and that PAR-binding activity resides in the N-terminus of Exo1 containing the nuclease domain (Fig. 3.2). The DNA damage dependent activation of PAR synthesis in cells facilitates the initial damage recruitment of Exo1 (Fig. 3.1). In vitro, PAR polymers suppress the exonuclease and flap endonuclease activities of Exo1 (Fig. 3.5). This counterbalancing effect of PAR binding may explain why no overt effects on DSB end resection were observed in the Xenopus egg extract when protein PARylation was inhibited (Fig. 3.4). These data, together with our previously reported findings, suggest a model for the control of Exo1 function in DNA end resection through a multiple protein-protein interactions. In response to DNA DSBs, PARP1 quickly relocates to DNA breaks, leading to its activation and a transient wave of PARylation on multiple proteins. The
resulting PAR chains provide a docking platform for the rapid damage recruitment of Exo1 through the PAR-binding activity of its N-terminus. During this initial stage of damage association, Exo1 resection activity is held inactive by PAR until its clearance by PARG. The second stage, more sustained damage association of Exo1 is mediated by the sliding DNA clamp PCNA that also loads onto DSBs. The direct interaction between PCNA and the PIP-box in the C-terminus of Exo1 tethers Exo1 to the DNA substrate and promotes its processivity in resection. PAR and PCNA increase Exo1 damage association in an apparently additive manner, whereas both pathways are antagonized by the 14-3-3 adaptor proteins, which interact with the central domain of Exo1 to suppress its interactions with PAR and PCNA. This coordinated regulation of Exo1 activity by multiple interacting partners ensures an orchestrated resection process and a proper level of ssDNA for the activation of HR and the ATR checkpoint (Fig. 3.6). PARylation plays an important role in both DSBR and single-strand break repair (SSBR) and is a prominent target of cancer therapy (56–58). PAR synthesis at sites of DNA damage has been shown to recruit a number of protein factors through their PAR-binding activities, which promotes chromatin modification and remodeling, DNA repair and checkpoint activation (36, 59). Our results indicate that the initial damage recruitment of Exo1 is mediated by its direct PAR-binding activity resided in the N-terminal domain. However, PAR-binding also inhibits the DNA resection activity of Exo1, which could serve to slow or delay DNA end resection activity and provide time for cells to integrate cues from both inside and outside of the cell before committing to long-range resection. For example, PARylation promotes microhomology-mediated end joining (MMEJ) that requires only limited end resection by MRN and CtIP, but not by Exo1 (60, 61). By temporarily suspending Exo1 nuclease activity during its initial damage recruitment, PAR may prevent premature resection at a DSB before a commitment is made.
between HR, MMEJ and classic NHEJ repair pathways. Due to the highly negatively charged nature of PAR chains, it is likely that PAR inhibits Exo1 nuclease activity by directly competing with the binding of the DNA substrate to the nuclease domain. Alternatively, PAR-binding may function as an allosteric negative regulator of Exo1’s nuclease activities. Previously, Poirier and colleagues predicted a PAR-binding motif in Exo1 (residues 125–135; ITHAMAHKVIK) (62). However, mutations of the key residues predicted to be important for the PAR-binding activity (ITHAMAAAVIA) did not affect PAR-binding of Exo1 (data not shown), suggesting that the PAR-binding motif is located elsewhere in the N-terminal domain.

Inhibition of Exo1 nuclease activities by PAR may also play a role in the progression of DNA replication forks. In budding yeast lacking either the checkpoint kinase Rad53 or 14-3-3 genes, DNA resection by EXO1 leads to fork instability and DNA damage in response to replication stress (25, 26, 28). These observations imply that unscheduled or uncontrolled Exo1 activity is normally prevented at the stalled or collapsed replication forks. PAR-mediated inhibition of Exo1 nuclease activities may contribute to this regulation. Consistent with this idea, PARP1 is activated in response to replication stress and thought to be important for the restart of stalled forks (63–65). Previous studies suggest that PARP1 protects fork structure by promoting fork reversal or by preventing Mre11-dependent degradation of stalled forks (63–65). It will be interesting to determine in the future whether inhibition of Exo1 resection function by PAR represents another mechanism for fork protection and restart in the presence of replication stress.
3.5 Acknowledgments

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Figure 3.1. The N-terminal domain of Exo1 promotes its initial damage recruitment in a PARylation-dependent manner.

A). Left panel: Diagram of wild-type Exo1 and truncation mutants. Middle panel: Representative images for the damage association of GFP-Exo1, GFP-Exo1(508-846) and GFP-Exo1(1-507) shown in the left panel. Red lines indicate the sites of laser irradiation in cells. Right panel: Quantified results for the damage association of GFP-Exo1 and its mutants during the first 20 min after laser irradiation. Each data point is the average of independent measurements of 5 cells. Error bars represent standard deviation.

B). Immunofluorescence staining of PAR at sites of DNA damage at various times after laser irradiation.

C). Left panels: Representative images for the damage association of GFP-Exo1(1-507), GFP-Exo1(WT) and GFP-Exo1(ΔPIP) in cells treated with DMSO or Olaparib. Red lines indicate the sites of laser irradiation in cells. Right Panel: Quantified results for the damage association GFP-Exo1(1-507), GFP-Exo1(WT) and GFP-Exo1(ΔPIP) in cells treated with DMSO or Olaparib during the first 20 min after laser irradiation. Each data point is the average of independent measurements of 5 cells. Error bars represent standard deviation.
Figure 3.2. Exo1 directly interacts with PAR via its N-terminal domain.
A). Generation of PAR chains on PARP1C, through *in vitro* auto-PARylation in the presence of DBD, NAD+ and DNA fragments. PARylated PARP1C resulted in lower mobility on a polyacrylamide gel.

B). Pull-down of GFP, GFP-Exo1(WT), GFP-Exo1(1-507) or GFP-Exo1(508-846) expressed in 293T cells with bead-immobilized GST-PARP1C-PAR or GST-PARP1C. Note that GFP-Exo1(508-846) exhibited a slower gel mobility compared to GFP-Exo(1-507), although the latter has a larger molecular weight.

C). Purified recombinant Exo1(WT)-His and Exo1(1-507)-His expressed in Sf9 cells.

D). Pull-down of purified recombinant Exo1(WT)-His or Exo1(1-507)-His with bead-immobilized GST-PARP1C-PAR or GST-PARP1C.

E). Competition of PAR polymer, monomeric ADP-ribose or poly(A) RNA for binding to purified Exo1(WT)-His with bead-immobilized GST-PARP1C-PAR.
Figure 3.3. The PAR-binding activity of Exo1 is antagonized by 14-3-3 proteins that interact with the central domain of Exo1.

**A**). Pull-down of GFP-Exo1(WT) or GFP-Exo1(DCR) expressed in 293T cells with bead-immobilized GST-PARP1C-PAR.

**B**). Pull-down of GFP-Exo1(WT) or GFP-Exo1(DCR) in 293T cells overexpressing mCherry, mCherry-Difopein(WT) or mCherry-Difopein(MUT) with bead-immobilized GST-PARP1C-PAR.
Figure 3.4. PARylation Is dispensable for DNA end resection in the *Xenopus* egg extract.

A). Far-western using GST-AF1521 detected robust, but transient protein PARylation in *Xenopus* NPE induced by a dsDNA fragment.
B). Effects of the PARP inhibitor Olaparib and the PARG inhibitor ADP-HPD on protein PARylation in NPE incubated with a dsDNA fragment for 5 min.

C). Effects of Olaparib on end resection of a 6 kb dsDNA fragment in NPE. PARPi, Olaparib.

D). Depletion of Dna2 from the *Xenopus* NPE.

E). Effects of Olaparib on DNA end resection in NPE depleted of xDna2.

F). Depletion of both Exo1 and Dna2 from the *Xenopus* NPE.

G). Effects of Olaparib on DNA end resection in NPE depleted of both xDna2 and xExo1 and supplemented with Exo1(ΔPIP)-His.
Figure 3.5. PAR inhibits the nuclease activities of Exo1 in vitro.


B). Effects of PAR, ADP-ribose and polyA RNA on the 5’ flap endonuclease activity of purified Exo1.
Figure 3.6. A model for the regulation of Exo1 in DNA end resection by PAR, 14-3-3s and PCNA.

The damage association of Exo1 is regulated by three distinct domains in Exo1, which bind to PAR, 14-3-3s and PCNA, respectively. Immediately after DNA damage, protein PARylation at DNA damage sites promotes the initial damage recruitment of Exo1 through the direct interaction between PAR and the N-terminus of Exo1. At this point, the Exo1 nuclease activity is held until the clearance of PAR from the DNA damage sites by PARG. Through binding to the PIP-Box in the C-terminus of Exo1, PCNA facilitates the damage retention of Exo1 and increases its processivity in DNA resection. 14-3-3 proteins interact with the central domain of Exo1, suppress Exo1’s binding to PAR and PCNA and restrain DNA end resection. The coordinated regulation of Exo1 by multiple interacting factors ensures an orchestrated and controlled DSB resection process.
Figure S3.1. Recombinant proteins.

A). Recombinant GST-AF1521 expressed in bacteria.

B). Recombinant Exo1(ΔPIP)-His expressed in Sf9 cells.
3.6 References


Chapter 4:

Conclusions and future directions

Sharad C. Paudyal
4.1 Conclusions

DNA end resection is the central process of DNA damage response (DDR) that regulates both checkpoint signaling and DNA repair after double-strand breaks (DSBs) damage and therefore plays a significant role in genome maintenance, cell survival, and tumor suppression. Genetic studies in yeasts suggest two steps mechanisms for resection. First, resection is initiated by MRN (Mre11-Rad50-NBS1) complex in collaboration with CtIP through an internal cleavage of 5’ strand DNA. Following the initiation, the clean 5’end at the cleavage site is further resected by Dna2 and Exo1 to extend the resection in 5’-3’ direction. While this model fits the mechanisms of resection of blocked DNA ends with chemical or protein adducts, it does not address whether resection is initiated or extended the same way at clean DSBs with free 5’ ends, because these ends are directly accessible to both exonucleases and flap endonucleases like Exo1 and Dna2. In addition, while it is suggested that Dna2 and Exo1 extend the resection in two parallel pathways, it is still unclear how redundant their functions are and how the extension of resection is regulated so the DNA ends are resected sufficiently for repair but prevented from over-resection. We took multipronged approach to study DSB damage response using cultured human cells, *Xenopus* nuclear extract, and purified recombinant proteins and have determined novel mechanisms and regulation of both initiation and extension steps of DSB end resection.

4.1.1 Initiation of resection at clean DSB is dependent on Dna2 endonuclease activity

Using *Xenopus* nuclear extract and purified recombinant proteins, we investigated the mechanisms of resection initiation specifically at clean DSBs and identified Dna2 as the primary nuclease for the step. The flap endonuclease activity of Dna2 initiates the resection via internal cleavage of 5’ strand DNA generating oligonucleotide products. Interestingly, in the absence of
Dna2, CtIP initiates the resection at clean DSB also by endocleavage but at more distal sites from the end. While Exo1 plays no role in the initiation by endocleavage, MRN promotes both resection initiation pathways in part by promoting the recruitment of Dna2 and CtIP to the DNA ends. The ssDNA binding protein RPA also promotes both of these initiation pathways but plays a minor role in the damage recruitment of Dna2, CtIP, and MRN.

Our findings basically suggest a new model for resection initiation at clean DSB without protein or chemical adducts. Our model is in agreement with previous observations that purified Sae2-MRX and CtIP-MRN prefers blocked 5’ DNA ends over free ends to initiate the resection by endocleavage in reconstituted reactions (1,2). Our results also support the findings from genetic studies where Sae2-MRX has limited resection activity in the absence of Dna2 and Exo1 pathways (3,4). In addition to the nuclease activity, we also determined a role of Dna2 helicase activity in the initiation step of resection. The helicase function of Dna2 is dispensable for endocleavage per se but it is required to determine proper site and location of Dna2 nuclease activity on the 5’ strand DNA. Dna2 recruits relatively early to the clean 5’ DNA ends but plays no role in the recruitment of MRN and CtIP. In fact, MRN actually promotes the recruitment of both Dna2 and CtIP to the DNA ends. Interestingly, we have also identified a novel role of RPA in the initiation of resection. While it was known that RPA promotes Dna2 nuclease activity on the 5’ strand DNA, it remained unclear if it plays a role in the initiation of resection. Our results show that RPA is required for both Dna2 and CtIP mediated resection initiation at clean DSB. The requirement of RPA in CtIP mediated pathway also suggests that the DNA needs to be unwound before the endocleavage of 5’ strand by CtIP-MRN. The RPA mutant lacking interaction with Dna2 (N-terminus deletion of RPA1) is still able to support CtIP but not Dna2 mediated initiation of resection, suggesting distinct mechanisms of two initiation pathways.
4.1.2 Extension of resection by Exo1 is regulated by Poly(ADP-ribose)

While Exo1 does not play a role in the initiation of resection by endocleavage at clean DSB, it is involved in the extension of resection and is highly regulated by multiple proteins for its optimal resection activity. Using cultured human cells, *Xenopus* nuclear extract, and purified recombinant proteins, we have determined a new regulation of Exo1 by poly(ADP-ribose) (PAR) in response to DNA damage. PARylation by poly(ADP-ribose) polymerase 1 (PARP1) is the prominent post-translational modification on several proteins at the site of DNA damage. However, the modification is transient as it is rapidly removed by Poly (ADP-ribose) glycohydrolase (PARG). Our results show that Exo1 is a PAR-binding protein and PARylation promotes the timely recruitment of Exo1 to the DNA damage site. Exo1 binds to PAR directly via its N-terminal domain, which mediates its early damage recruitment. But interestingly PAR binding inhibits the exonuclease activity and 5’ flap endonuclease activity of Exo1 in reconstituted reactions *in vitro*. However, PARP inhibitor Olaparib has no significant effect on the Exo1 mediated resection in *Xenopus* nuclear extract. These results together suggest that PARylation, while promoting the damage recruitment of Exo1, also keeps Exo1 nuclease activity in check until dePARylation by PARG. We have also shown that the interaction of 14-3-3s to Exo1, which is known to inhibit the damage recruitment of Exo1, also inhibits Exo1 interaction with PAR.

4.2 Future directions

While great deal of progress has been made in determining the molecular mechanisms of DNA resection, some fundamental questions are still not completely understood. Our results strongly suggest that resection of blocked and free DSB ends is initiated via distinct mechanisms.
Previous studies using reconstituted systems in vitro have suggested that CtIP-MRN prefers blocked DNA ends instead of free ends to initiate the resection by endocleavage. Interestingly, same studies also show that 3’-5’ exonuclease function of Mre11 is preferred over 5’ endonuclease function on the free DNA ends (1,2). Therefore, more work is needed to figure out how the DNA end structures regulate the directionality and nuclease activity of MRN. It will also be interesting to find out if CtIP-MRN but not Dna2 initiates the resection of blocked 5’ DNA ends in the Xenopus nuclear extract. Moreover, future work is needed to address if the resection is initiated the same way in chromosomal breaks in cells because chromatin remodeling factors have also been identified to play a role in DNA resection in vivo (5,6).

DSB resection occurs in 5’-3’ direction but what determines this directionality is still a mystery. Our results suggest that RPA binding to the unwound DNA promotes the initiation of resection on the 5’ strand of clean DSB by both Dna2 and CtIP mediated pathways. In fact, a structural study of Dna2-ssDNA-RPA complex and in vitro nuclease assay using mouse Dna2 and RPA show that Dna2 physically interacts with RPA on both strands but can only displace RPA from the 5’ strand to resect the DNA in 5’-3’ direction (7). However, both Sae2-MRX and CtIP-MRN selectively cleave 5’ strand of the blocked DNA ends in reconstituted reactions in vitro without RPA (1,2). Therefore, the molecular mechanisms of 5’ strand selectivity in DSB end resection still remains to be determined.

We observed that CtIP initiates the resection in the absence of Dna2. This CtIP dependent ‘backup” mechanism also occurs through endocleavage but at more distal sites from the end compared to the sites of Dna2 dependent cleavage. However, Dna2 does not delay the loading of CtIP and NBS1 to the DNA substrate. Therefore, the physiological significance of the existence of this backup pathway of resection initiation remains to be determined. It is possible that CtIP-
MRN initiates the resection in cells that have low Dna2 expression or have defects in Dna2 nuclease activity. In addition, the nuclease responsible for this backup pathway is also still unclear because both CtIP and Mre11 have been shown to contain endonuclease activities (8–13). Studies have shown that Mre11 but not CtIP confers its endonuclease function to initiate the resection of blocked DNA ends in reconstituted reactions *in vitro*, (1,2). But since Mre11 endonuclease also prefers blocked ends over free ends *in vitro*, it will be interesting to figure out the nuclease involved in this backup resection initiation pathway of clean DSB in *Xenopus* nuclear extract.

We have identified a new role of RPA in the initiation of resection suggesting that DNA ends need to be unwound to generate ssDNA before the endocleavage of 5’ strand by Dna2 or CtIP-MRN. Future work is necessary to determine the helicases that are involved in this unwinding step. We also determined a separation-of-function mutant of RPA that distinguishes its role in Dna2 and CtIP mediated pathways. While it is known that RPA interacts directly with Dna2, it remains to be determined how it promotes the CtIP mediated initiation of resection. It is possible that RPA promotes CtIP-MRN function by either stabilizing the unwound DNA strands or by directly promoting their nuclease activity.

Among the core resection factors, Exo1 is the only protein that does not play a role in the initiation of resection by endocleavage at clean DSB. However, our results show that Exo1 recruits to the DNA ends as early as Dna2, CtIP, and NBS1. Therefore, it is possible that PAR binding promotes the early recruitment but inhibits the initiation activity of Exo1. It will be interesting to determine if PARylation also affects the initiation of resection by Dna2 or CtIP. Another important question to address in the future is the similarities and differences in roles of Dna2 and Exo1 in the extension of resection. It will be interesting to determine if Dna2 continues
to resect the DNA after initiation or is it possible that Exo1 alone extend the resection of clean DSB. It is expected that resection activities would eventually stop when the length of ssDNA reaches to a certain threshold for ATR checkpoint activation and homologous recombination. However, the mechanism of termination of resection remains unclear. Our study suggests that 14-3-3s interaction terminates Exo1 resection activity. However, the signal that promotes Exo1 interaction with 14-3-3s during DSB resection remains to be determined. It is possible that the phosphorylation of Exo1 by ATM, ATR or other downstream kinases result into its interaction with 14-3-3s leading to the termination of its resection activity. Studies have shown that Dna2 is inhibited by FANCD2 in human cells and Pxd1 in fission yeasts (14,15). Therefore, it will be interesting to determine if these factors inhibit the initiation and/or extension activities of Dna2 during DSB to help terminate the overall resection process. Addressing these questions in the future will help us understand more about the mechanisms and regulation of DNA resection and its significant role in the DNA damage response.
4.3 References


13. Jazayeri A, Balestrini A, Garner E, Haber JE, Costanzo V. Mre11–Rad50–Nbs1-dependent processing of DNA breaks generates oligonucleotides that stimulate ATM
