Summer 8-15-2017

Mechanisms and Regulation of Resection in DNA Damage Response

Sharad C. Paudyal
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
Molecular Cell Biology

Dissertation Examination Committee:
Zhongsheng You, Chair
Peter M. Burgers
Milan G. Chheda
Roberto Galletto
Nima Mosammaparast
Sheila A. Stewart
Jason D. Weber

Mechanisms and Regulation of Resection in DNA Damage Response

by

Sharad C. Paudyal

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2017
St. Louis, Missouri

Table of Contents

List of Figures

Chapter 1: Sharpening the ends for repair: mechanisms and regulation of DNA resection

Chapter 2: Dna2 initiates resection at clean DNA double-strand breaks

Chapter 3: Poly(ADP-ribose)-binding promotes Exo1 damage recruitment and suppresses its nuclease activities

Acknowledgments

First and foremost, I thank my thesis advisor and research mentor, Dr. Zhongsheng You. Zhongsheng supported me immensely for the successful completion of my thesis research in his laboratory. My dissertation would not have been completed without his generous funding, his availability to answer all my questions, and his technical support every steps of the way. He always encouraged my independency and offered me freedom to explore new ideas. From the day I joined his lab in May 2012 till today, Zhongsheng has always been an outstanding mentor, a good friend, and an inspiring colleague to me. I am truly grateful for his intellectual as well as financial support to my graduate training.

Next, I thank the members of my thesis committee: Dr. Peter M. Burgers, Dr. Milan G. Chheda, Dr. Roberto Galletto, Dr. Nima Mosammaparast, Dr. Sheila A. Stewart, and Dr. Jason D. Weber for guiding me through the entire process of my thesis research leading up to the dissertation defense. I am thankful for their tough questions, constructive comments, suggestions, and unique perspectives to refine my projects. I humbly appreciate their time for all the formal meetings as well as many of my informal walk-in into their offices. I also thank my program directors, Dr. Jason D. Weber and Dr. Heather L. True, for their continued advice and guidance on becoming a successful graduate student. I am extremely thankful to my program coordinator, Stacy Kiel, for her tireless effort to keep me on my toes and for helping me navigate through all the administrative requirements of graduate school. I also would like to thank Dr. Andrey S. Shaw and members of the Shaw lab at Washington University School of Medicine for all their help and support during my period as a technician in their lab as well as after I left to pursue graduate study. Andrey taught me the very basics of scientific research and truly inspired me to apply to the Washington University's graduate program.

My experience of graduate research would not have been so much fun without an amazing group of people in the You lab. I want to thank all the past and present members of the lab that I had a chance to meet and work with. I am grateful for their help and suggestions on my projects and for creating a uniquely fun working environment in the lab. I also thank our departmental chair Dr. David W. Piston, all the faculty, staff members, friends, and colleagues in the Department of Cell Biology and Physiology for an incredibly great workplace environment. I want to thank my next-door laboratories of Dr. Sergej Djuranovic and Dr. Kendall J. Blumer for their regular technical support to my projects. Most importantly, I am very thankful to all my friends at the Washington University as well as those in and outside of St. Louis for numerous fun and recreational activities outside the lab. They were always there for me, whether to celebrate my achievements and share my happiness or to console me through difficult times.

Lastly, I am incredibly grateful to my parents, my sister, and my brother for their unconditional love and support throughout my academic career. I went to boarding school for my fourth grade and have mostly been away from home for schooling ever since, including coming over to the United States for both undergraduate and graduate studies. However, my entire family and relatives in Nepal have always been my constant source of love, happiness, and inspiration. While my sister is no longer with us in this world to witness this, one of the biggest achievements of my academic career, her love and care for me were always my motivations to work hard through high school and college. I know from my heart that she would have been extremely proud of my accomplishments thus far.

Sharad C. Paudyal
Washington University in St. Louis
August 2017

ABSTRACT OF THE DISSERTATION

Mechanisms and Regulation of Resection in DNA Damage Response

by

Sharad C. Paudyal

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Cell Biology

Washington University in St. Louis, August 2017

Professor Zhongsheng You, Chair

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

This chapter was originally published in *Acta Biochimica et Biophysica Sinica (ABBS)*.
2016 July; 48(7): 647-57. Epub 2016 May 12.

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′ flap 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

The work was supported by the grants from the National Institutes of Health (No. R01GM098535) and the American Cancer Society Research Scholar Grant (No. RSG-13-212-01-DMC).

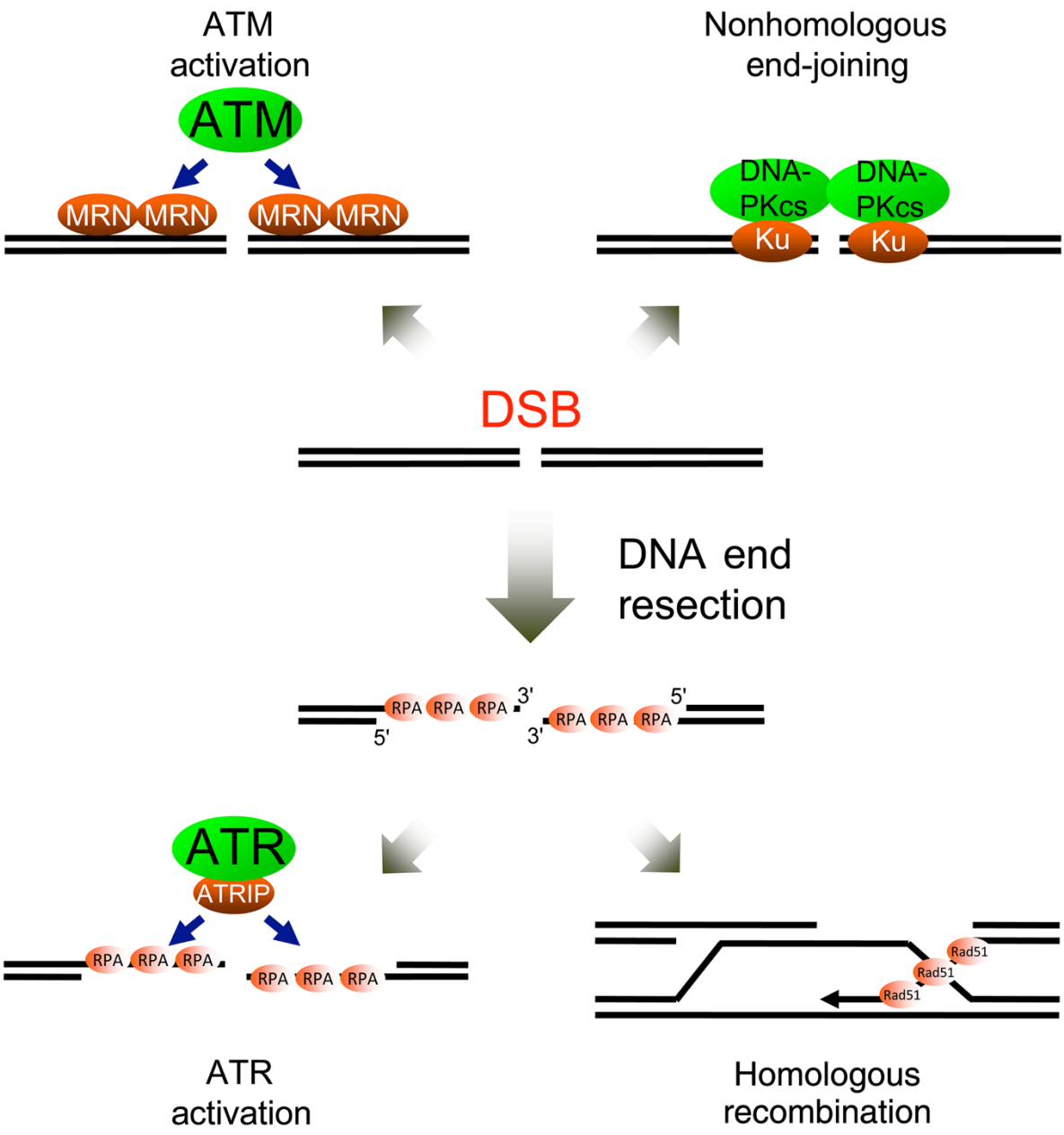


Figure 1.1. DNA end resection dictates the pathway choice for both DNA repair and checkpoint response. DSBs can be repaired by NHEJ or by HR that requires 3′ ssDNA generated by end resection. Checkpoint kinase ATM is activated on the dsDNA flanking the break, whereas ATR is activated on the ssDNA structure generated by resection. Thus, DNA end resection promotes HR and ATR activation and attenuates NHEJ and ATM activation.

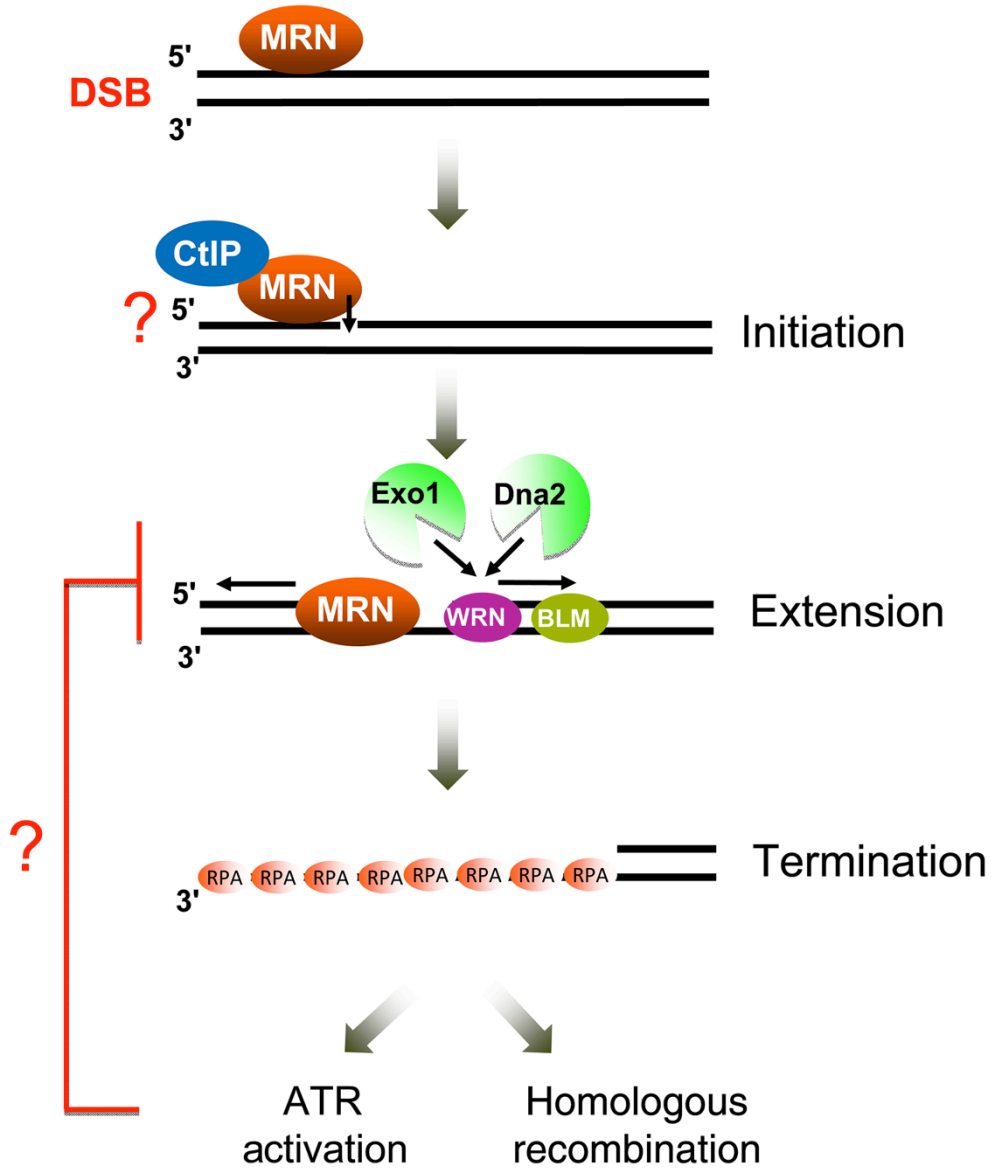


Figure 1.2. Key steps and core factors of DNA end resection. Resection is initiated on the 5′ strand of the DNA by the endocleavage activity of MRN-CtIP and extended by Exo1 and Dna2 in two parallel pathways. The underlying mechanism for the 5′ strand selectivity of MRN-CtIP in resection initiation is 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.

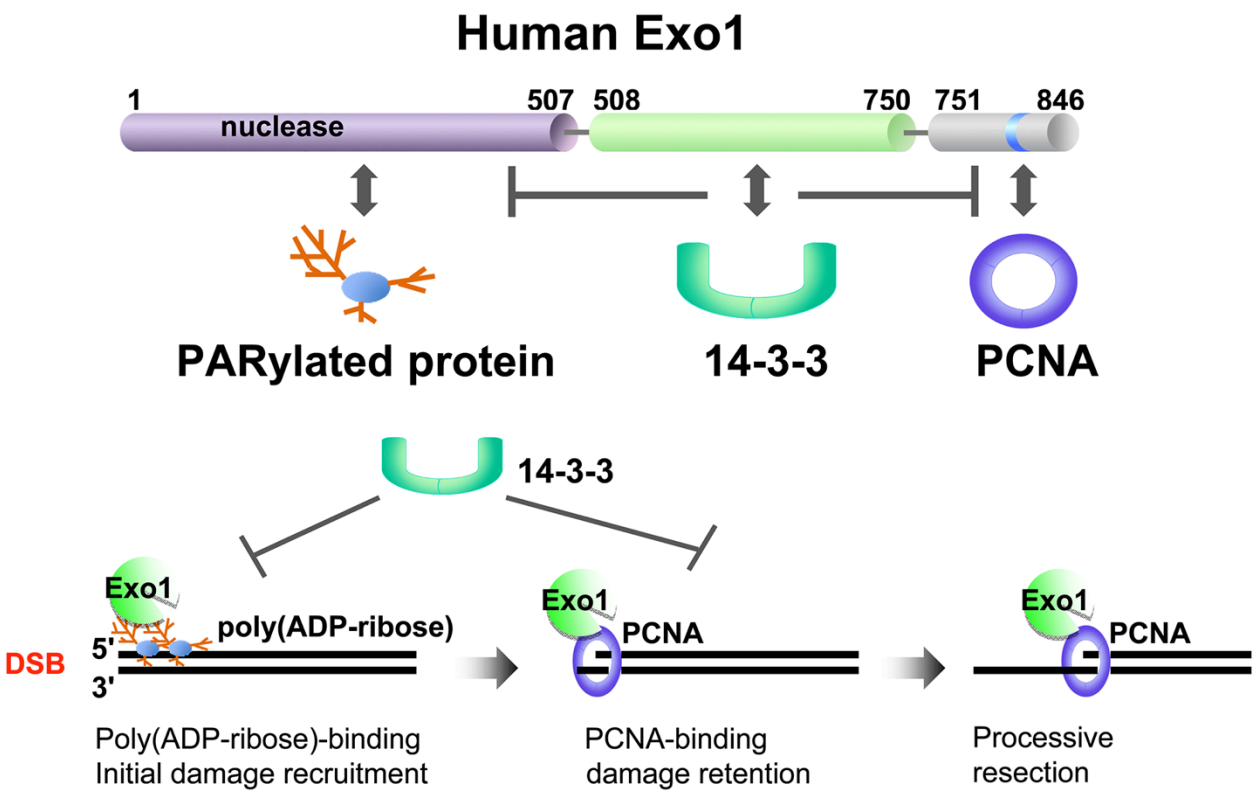


Figure 1.3. Human Exo1 is regulated by PCNA, poly(ADP-ribosyl)ation and 14-3-3s. The damage recruitment and resection activity of Exo1 are controlled by three sets of factors that directly interact with different domains in Exo1. PARylated proteins bind to the N-terminus of Exo1 and promote the initial 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

1. Aguilera A, Gómez-González B. Genome instability: a mechanistic view of its causes and consequences. *Nat Rev Genet* 2008, 9: 204–17.

2. McKinnon PJ, Caldecott KW. DNA Strand Break Repair and Human Genetic Disease. *Annu Rev Genomics Hum Genet* 2007, 8: 37–55.

3. Thompson LH, Schild D. Recombinational DNA repair and human diseases. *Mutat Res* 2002, 509: 49–78.

4. Jackson S, Bartek J. The DNA-damage response in human biology and disease. *Nature* 2009, 461: 1071–8.

5. Ciccia A, Elledge SJ. The DNA Damage Response: Making It Safe to Play with Knives. *Mol Cell* 2010, 40: 179–204.

6. Bakkenist CJ, Kastan MB. Initiating cellular stress responses. *Cell* 2004, 118: 9–17.

7. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature* 2004, 432: 316–23.

8. Lieber MR. The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End-Joining Pathway. *Annu Rev Biochem* 2010, 79: 181–211.

9. Symington LS, Gautier J. Double-Strand Break End Resection and Repair Pathway Choice. *Annu Rev Genet* 2011, 45: 247–71.

10. Aparicio T, Baer R, Gautier J. DNA double-strand break repair pathway choice and cancer. *DNA Repair (Amst)* 2014, 19: 169–75.

11. Kass EM, Jasin M. Collaboration and competition between DNA double-strand break repair pathways. *FEBS Lett* 2010, 584: 3703–8.

12. Bernstein KA, Rothstein R. At Loose Ends: Resecting a Double-Strand Break. *Cell* 2009, 137: 807–10.

13. Huertas P. DNA resection in eukaryotes: deciding how to fix the break. *Nat Struct Mol Biol* 2010, 17: 11–6.

14. Cejka P. DNA End Resection: Nucleases Team Up with the Right Partners to Initiate Homologous Recombination. *J Biol Chem* 2015, 290: 22931–8.

15. Moynahan ME, Jasin M. Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. *Nat Rev Mol Cell Biol* 2010, 11: 196–207.

16. Sung P, Klein H. Mechanism of homologous recombination: mediators and helicases take

on regulatory functions. *Nat Rev Mol Cell Biol* 2006, 7: 739–50.

17. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* 2003, 300: 1542–8.

18. Cimprich KA, Cortez D. ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol* 2008, 9: 616–27.

19. Jazayeri A, Falck J, Lukas C, Bartek J, Smith GCM, Lukas J et al. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat Cell Biol* 2006, 8: 37–45.

20. Shiotani B, Zou L. Single-Stranded DNA Orchestrates an ATM-to-ATR Switch at DNA Breaks. *Mol Cell* 2009, 33: 547–58.

21. Clerici M, Trovesi C, Galbiati A, Lucchini G, Longhese MP. Mec1/ATR regulates the generation of single-stranded DNA that attenuates Tel1/ATM signaling at DNA ends. *EMBO J* 2014, 33: 198–216.

22. Branzei D, Foiani M. Regulation of DNA repair throughout the cell cycle. *Nat Rev Mol Cell Biol* 2008, 9: 297–308.

23. Shrivastav M, De Haro LP, Nickoloff JA. Regulation of DNA double-strand break repair pathway choice. *Cell Res* 2008, 18: 134–47.

24. Kadyk LC, Hartwell LH. Sister chromatids are preferred over homologs as substrates for recombinational repair in Saccharomyces cerevisiae. *Genetics* 1992, 132: 387–402.

25. Orthwein A, Noordermeer SM, Wilson MD, Landry S, Enchev RI, Sherker A et al. A mechanism for the suppression of homologous recombination in G1 cells. *Nature* 2015, 528: 422–6.

26. Ferretti LP, Lafranchi L, Sartori AA. Controlling DNA-end resection: A new task for CDKs. *Front Genet* 2013, 4: 1–7.

27. Scully R, Xie A. In my end is my beginning: control of end resection and DSBR pathway "choice" by cyclin-dependent kinases. *Oncogene* 2005, 24: 2871–6.

28. Aylon Y, Liefshitz B, Kupiec M. The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle. *EMBO J* 2004, 23: 4868–75.

29. Ira G, Pellicioli A, Balijja A, Wang X, Fiorani S, Carotenuto W et al. DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. *Nature* 2004, 431: 1011–7.

30. Jia X, Weinert T, Lydall D. Mec1 and Rad53 Inhibit Formation of Single-Stranded DNA

at Telomeres of Saccharomyces cerevisiae cdc13-1 Mutants. *Genetics* 2004, 166: 753–64.

31. El-Shemerly M, Hess D, Pyakurel AK, Moselhy S, Ferrari S. ATR-dependent pathways control hEXO1 stability in response to stalled forks. *Nucleic Acids Res* 2008, 36: 511–9.

32. Morin I, Ngo H-P, Greenall A, Zubko MK, Morrice N, Lydall D. Checkpoint-dependent phosphorylation of Exo1 modulates the DNA damage response. *EMBO J* 2008, 27: 2400–10.

33. Cotta-Ramusino C, Fachinetti D, Lucca C, Doksani Y, Lopes M, Sogo J et al. Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells. *Mol Cell* 2005, 17: 153–9.

34. Bolderson E, Tomimatsu N, Richard DJ, Boucher D, Kumar R, Pandita TK et al. Phosphorylation of Exo1 modulates homologous recombination repair of DNA double-strand breaks. *Nucleic Acids Res* 2010, 38: 1821–31.

35. Kijas AW, Lim YC, Bolderson E, Cerosaletti K, Gatei M, Jakob B et al. ATM-dependent phosphorylation of MRE11 controls extent of resection during homology directed repair by signalling through Exonuclease 1. *Nucleic Acids Res* 2015, 43: 8352–67.

36. Longhese MP, Bonetti D, Manfrini N, Clerici M. Mechanisms and regulation of DNA end resection. *EMBO J* 2010, 29: 2864–74.

37. Longhese MP. DNA damage response at functional and dysfunctional telomeres. *Genes Dev* 2008, 22: 125–40.

38. Dewar JM, Lydall D. Similarities and differences between “uncapped” telomeres and DNA double-strand breaks. *Chromosoma* 2012, 121: 117–30.

39. Wellinger RJ. When the caps fall off: Responses to telomere uncapping in yeast. *FEBS Lett* 2010, 584: 3734–40.

40. Petermann E, Helleday T. Pathways of mammalian replication fork restart. *Nat Rev Mol Cell Biol* 2010, 11: 683–7.

41. Carr AM, Lambert S. Replication Stress-Induced Genome Instability: The Dark Side of Replication Maintenance by Homologous Recombination. *J Mol Biol* 2013, 425: 4733–44.

42. Atkinson J, McGlynn P. Replication fork reversal and the maintenance of genome stability. *Nucleic Acids Res* 2009, 37: 3475–92.

43. Bryant HE, Petermann E, Schultz N, Jemth A, Loseva O, Issaeva N et al. PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. *EMBO J* 2009, 28: 2601–15.

44. Thangavel S, Berti M, Levikova M, Pinto C, Gomathinayagam S, Vujanovic M et al. DNA2 drives processing and restart of reversed replication forks in human cells. *J Cell Biol* 2015, 208: 545–62.

45. Peng G, Dai H, Zhang W, Hsieh H-J, Pan M-R, Park Y-Y et al. Human Nuclease/Helicase DNA2 Alleviates Replication Stress by Promoting DNA End Resection. *Cancer Res* 2012, 72: 2802–13.

46. Cannavo E, Cejka P. Sae2 promotes dsDNA endonuclease activity within Mre11-Rad50-Xrs2 to resect DNA breaks. *Nature* 2014, 514: 122–5.

47. Mimitou EP, Symington LS. Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. *Nature* 2008, 455: 770–4.

48. Limbo O, Chahwan C, Yamada Y, de Bruin RAM, Wittenberg C, Russell P. Ctp1 Is a Cell-Cycle-Regulated Protein that Functions with Mre11 Complex to Control Double-Strand Break Repair by Homologous Recombination. *Mol Cell* 2007, 28: 134–46.

49. Takeda S, Nakamura K, Taniguchi Y, Paull TT. Ctp1/CtIP and the MRN Complex Collaborate in the Initial Steps of Homologous Recombination. *Mol Cell* 2007, 28: 351–2.

50. Sartori AA, Lukas C, Coates J, Mistrik M, Fu S, Bartek J et al. Human CtIP promotes DNA end resection. *Nature* 2007, 450: 509–14.

51. You Z, Shi LZ, Zhu Q, Wu P, Zhang YW, Basilio A et al. CtIP Links DNA Double-Strand Break Sensing to Resection. *Mol Cell* 2009, 36: 954–69.

52. Petrini J. The cellular response to DNA double-strand breaks: defining the sensors and mediators. *Trends Cell Biol* 2003, 13: 458–62.

53. Rupnik A, Lowndes NF, Grenon M. MRN and the race to the break. *Chromosoma* 2010, 119: 115–35.

54. Kanaar R, Wyman C. DNA Repair by the MRN Complex: Break It to Make It. *Cell* 2008, 135: 14–6.

55. You Z, Chahwan C, Bailis J, Hunter T, Russell P. ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. *Mol Cell Biol* 2005, 25: 5363–79.

56. Williams RS, Dodson GE, Limbo O, Yamada Y, Williams JS, Guenther G et al. Nbs1 Flexibly Tethers Ctp1 and Mre11-Rad50 to Coordinate DNA Double-Strand Break Processing and Repair. *Cell* 2009, 139: 87–99.

57. Lloyd J, Chapman JR, Clapperton JA, Haire LF, Hartsuiker E, Li J et al. A Supramodular FHA/BRCT-Repeat Architecture Mediates Nbs1 Adaptor Function in Response to DNA

Damage. *Cell* 2009, 139: 100–11.

58. Paull TT, Deshpande RA. The Mre11/Rad50/Nbs1 complex: Recent insights into catalytic activities and ATP-driven conformational changes. *Exp Cell Res* 2014, 329: 139–47.

59. Wang H, Shi LZ, Wong CCL, Han X, Hwang PYH, Truong LN et al. The Interaction of CtIP and Nbs1 Connects CDK and ATM to Regulate HR-Mediated Double-Strand Break Repair. *PLoS Genet* 2013, 9: e1003277.

60. Hopfner KP, Karcher A, Shin DS, Craig L, Arthur LM, Carney JP et al. Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell* 2000, 101: 789–800.

61. Deshpande RA, Williams GJ, Limbo O, Williams RS, Kuhnlein J, Lee JH et al. ATP-driven Rad50 conformations regulate DNA tethering, end resection, and ATM checkpoint signaling. *EMBO J* 2014, 33: 482–500.

62. Bhaskara V, Dupré A, Lengsfeld B, Hopkins BB, Chan A, Lee J-H et al. Rad50 adenylate kinase activity regulates DNA tethering by Mre11/Rad50 complexes. *Mol Cell* 2007, 25: 647–61.

63. Raymond WE, Kleckner N. RAD50 protein of S.cerevisiae exhibits ATP-dependent DNA binding. *Nucleic Acids Res* 1993, 21: 3851–6.

64. Buis J, Wu Y, Deng Y, Leddon J, Westfield G, Eckersdorff M et al. Mre11 Nuclease Activity Has Essential Roles in DNA Repair and Genomic Stability Distinct from ATM Activation. *Cell* 2008, 135: 85–96.

65. Shibata A, Moiani D, Arvai AS, Perry J, Harding SM, Genois M-M et al. DNA Double-Strand Break Repair Pathway Choice Is Directed by Distinct MRE11 Nuclease Activities. *Mol Cell* 2014, 53: 7–18.

66. Jazayeri A, Balestrini A, Garner E, Haber JE, Costanzo V. Mre11–Rad50–Nbs1-dependent processing of DNA breaks generates oligonucleotides that stimulate ATM activity. *EMBO J* 2008, 27: 1953–62.

67. Paull TT, Gellert M. The 3′ to 5′ Exonuclease Activity of Mre11 Facilitates Repair of DNA Double-Strand Breaks. *Mol Cell* 1998, 1: 969–79.

68. Williams RS, Moncalian G, Williams JS, Yamada Y, Limbo O, Shin DS et al. Mre11 Dimers Coordinate DNA End Bridging and Nuclease Processing in Double-Strand-Break Repair. *Cell* 2008, 135: 97–109.

69. Garcia V, Phelps SEL, Gray S, Neale MJ. Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1. *Nature* 2011, 479: 241–4.

70. Neale MJ, Pan J, Keeney S. Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. *Nature* 2005, 436: 1053–7.

71. Zhu Z, Chung WH, Shim EY, Lee SE, Ira G. Sgs1 Helicase and Two Nucleases Dna2 and Exo1 Resect DNA Double-Strand Break Ends. *Cell* 2008, 134: 981–94.

72. Chen L, Nievera CJ, Lee AY-L, Wu X. Cell Cycle-dependent Complex Formation of BRCA1.CtIP.MRN Is Important for DNA Double-strand Break Repair. *J Biol Chem* 2008, 283: 7713–20.

73. Yuan J, Chen J. N terminus of CtIP is critical for homologous recombination-mediated double-strand break repair. *J Biol Chem* 2009, 284: 31746–52.

74. You Z, Bailis JM. DNA damage and decisions: CtIP coordinates DNA repair and cell cycle checkpoints. *Trends Cell Biol* 2010, 20: 402–9.

75. Lengsfeld BM, Rattray AJ, Bhaskara V, Ghirlando R, Paull TT. Sae2 Is an Endonuclease that Processes Hairpin DNA Cooperatively with the Mre11/Rad50/Xrs2 Complex. *Mol Cell* 2007, 28: 638–51.

76. Makharashvili N, Tubbs AT, Yang SH, Wang H, Barton O, Zhou Y et al. Catalytic and Noncatalytic Roles of the CtIP Endonuclease in Double-Strand Break End Resection. *Mol Cell* 2014, 54: 1022–33.

77. Wang H, Li Y, Truong LN, Shi LZ, Hwang PY-H, He J et al. CtIP Maintains Stability at Common Fragile Sites and Inverted Repeats by End Resection-Independent Endonuclease Activity. *Mol Cell* 2014, 54: 1012–21.

78. Hartsuiker E, Mizuno K, Molnar M, Kohli J, Ohta K, Carr AM. Ctp1CtIP and Rad32Mre11 nuclease activity are required for Rec12Spo11 removal, but Rec12Spo11 removal is dispensable for other MRN-dependent meiotic functions. *Mol Cell Biol* 2009, 29: 1671–81.

79. Hartsuiker E, Neale MJ, Carr AM. Distinct Requirements for the Rad32Mre11 Nuclease and Ctp1CtIP in the Removal of Covalently Bound Topoisomerase I and II from DNA. *Mol Cell* 2009, 33: 117–23.

80. Llorente B, Symington LS. The Mre11 nuclease is not required for 5’ to 3' resection at multiple HO-induced double-strand breaks. *Mol Cell Biol* 2004, 24: 9682–94.

81. Langerak P, Mejia-Ramirez E, Limbo O, Russell P. Release of Ku and MRN from DNA ends by Mre11 nuclease activity and Ctp1 is required for homologous recombination repair of double-strand breaks. *PLoS Genet* 2011, 7: e1002271.

82. Shim EY, Chung W-H, Nicolette ML, Zhang Y, Davis M, Zhu Z et al. Saccharomyces cerevisiae Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2

with DNA breaks. *EMBO J* 2010, 29: 3370–80.

83. Mimitou EP, Symington LS. Ku prevents Exo1 and Sgs1-dependent resection of DNA ends in the absence of a functional MRX complex or Sae2. *EMBO J* 2010, 29: 3358–69.

84. Nicolette ML, Lee K, Guo Z, Rani M, Chow JM, Lee SE et al. Mre11–Rad50–Xrs2 and Sae2 promote 5′ strand resection of DNA double-strand breaks. *Nat Struct Mol Biol* 2010, 17: 1478–85.

85. Cejka P, Cannavo E, Polaczek P, Masuda-Sasa T, Pokharel S, Campbell JL et al. DNA end resection by Dna2–Sgs1–RPA and its stimulation by Top3–Rmi1 and Mre11–Rad50–Xrs2. *Nature* 2010, 467: 112–6.

86. Niu H, Chung W-H, Zhu Z, Kwon Y, Zhao W, Chi P et al. Mechanism of the ATP-dependent DNA end-resection machinery from Saccharomyces cerevisiae. *Nature* 2010, 467: 108–11.

87. Nimonkar A V, Genschel J, Kinoshita E, Polaczek P, Campbell JL, Wyman C et al. BLM – DNA2 – RPA – MRN and EXO1 – BLM – RPA – MRN constitute two DNA end resection machineries for human DNA break repair. *Genes Dev* 2011, 2: 350–62.

88. Ma J-L, Kim EM, Haber JE, Lee SE. Yeast Mre11 and Rad1 proteins define a Ku-independent mechanism to repair double-strand breaks lacking overlapping end sequences. *Mol Cell Biol* 2003, 23: 8820–8.

89. McVey M, Lee SE. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends Genet* 2008, 24: 529–38.

90. Truong LN, Li Y, Shi LZ, Hwang PY-H, He J, Wang H et al. Microhomology-mediated End Joining and Homologous Recombination share the initial end resection step to repair DNA double-strand breaks in mammalian cells. *Proc Natl Acad Sci U S A* 2013, 110: 7720–5.

91. Ira G, Haber JE. Characterization of RAD51 -Independent Break-Induced Replication That Acts Preferentially with Short Homologous Sequences. *Mol Cell Biol* 2002, 22: 6384–92.

92. Deng SK, Gibb B, de Almeida MJ, Greene EC, Symington LS. RPA antagonizes microhomology-mediated repair of DNA double-strand breaks. *Nat Struct Mol Biol* 2014, 21: 405–12.

93. Gravel S, Chapman JR, Magill C, Jackson SP. DNA helicases Sgs1 and BLM promote DNA double-strand break resection. *Genes Dev* 2008, 22: 2767–72.

94. Mimitou EP, Symington LS. DNA end resection : Many nucleases make light work. *DNA Repair (Amst)* 2009, 8: 983–95.

95. Tran PT, Erdeniz N, Symington LS, Liskay RM. EXO1- A multi-tasking eukaryotic nuclease. *DNA Repair (Amst)* 2004, 3: 1549–59.

96. Lee BI, Wilson DM. The RAD2 domain of human exonuclease 1 exhibits 5' to 3' exonuclease and flap structure-specific endonuclease activities. *J Biol Chem* 1999, 274: 37763–9.

97. Keijzers G, Bohr V, Juel Rasmussen L. Human exonuclease 1 (EXO1) activity characterization and its function on FLAP structures. *Biosci Rep* 2015, 35: e00206.

98. Liao S, Toczylowski T, Yan H. Mechanistic analysis of Xenopus EXO1's function in 5'-strand resection at DNA double-strand breaks. *Nucleic Acids Res* 2011, 39: 5967–77.

99. Tomimatsu N, Mukherjee B, Deland K, Kurimasa A, Bolderson E, Khanna KK et al. Exo1 plays a major role in DNA end resection in humans and influences double-strand break repair and damage signaling decisions. *DNA Repair (Amst)* 2012, 11: 441–8.

100. Tsubouchi H, Ogawa H. Exo1 roles for repair of DNA double-strand breaks and meiotic crossing over in Saccharomyces cerevisiae. *Mol Biol Cell* 2000, 11: 2221–33.

101. Schaetzlein S, Chahwan R, Avdievich E, Roa S, Wei K, Eoff RL et al. Mammalian Exo1 encodes both structural and catalytic functions that play distinct roles in essential biological processes. *Proc Natl Acad Sci U S A* 2013, 110: E2470–9.

102. Bertuch AA, Lundblad V. EXO1 contributes to telomere maintenance in both telomerase-proficient and telomerase-deficient Saccharomyces cerevisiae. *Genetics* 2004, 166: 1651–9.

103. Nimonkar A V., Ozsoy AZ, Genschel J, Modrich P, Kowalczykowski SC. Human exonuclease 1 and BLM helicase interact to resect DNA and initiate DNA repair. *Proc Natl Acad Sci U S A* 2008, 105: 16906–11.

104. Chen X, Paudyal SC, Chin R-I, You Z. PCNA promotes processive DNA end resection by Exo1. *Nucleic Acids Res* 2013, 41: 9325–38.

105. Ngo GHP, Balakrishnan L, Dubarry M, Campbell JL, Lydall D. The 9-1-1 checkpoint clamp stimulates DNA resection by Dna2-Sgs1 and Exo1. *Nucleic Acids Res* 2014, 42: 10516–28.

106. Ngo GHP, Lydall D. The 9-1-1 checkpoint clamp coordinates resection at DNA double strand breaks. *Nucleic Acids Res* 2015, 43: 5017–32.

107. Eid W, Steger M, El-Shemerly M, Ferretti LP, Peña-Diaz J, König C et al. DNA end resection by CtIP and exonuclease 1 prevents genomic instability. *EMBO Rep* 2010, 11: 962–8.

108. Kousholt AN, Fugger K, Hoffmann S, Larsen BD, Menzel T, Sartori AA et al. CtIP-dependent DNA resection is required for DNA damage checkpoint maintenance but not initiation. *J Cell Biol* 2012, 197: 869–76.

109. Bae S-H, Kim DW, Kim J, Kim J-H, Kim D-H, Kim H-D et al. Coupling of DNA helicase and endonuclease activities of yeast Dna2 facilitates Okazaki fragment processing. *J Biol Chem* 2002, 277: 26632–41.

110. Kumar S, Burgers PM. Lagging strand maturation factor Dna2 is a component of the replication checkpoint initiation machinery. *Genes Dev* 2013, 27: 313–21.

111. Levikova M, Cejka P. The Saccharomyces cerevisiae Dna2 can function as a sole nuclease in the processing of Okazaki fragments in DNA replication. *Nucleic Acids Res* 2015, 43: 7888–97.

112. Lin W, Sampathi S, Dai H, Liu C, Zhou M, Hu J et al. Mammalian DNA2 helicase/nuclease cleaves G-quadruplex DNA and is required for telomere integrity. *EMBO J* 2013, 32: 1425–39.

113. Masuda-Sasa T, Polaczek P, Peng XP, Chen L, Campbell JL. Processing of G4 DNA by Dna2 helicase/nuclease and replication protein A (RPA) provides insights into the mechanism of Dna2/RPA substrate recognition. *J Biol Chem* 2008, 283: 24359–73.

114. Daley JM, Chiba T, Xue X, Niu H, Sung P. Multifaceted role of the Topo IIIα-RMI1-RMI2 complex and DNA2 in the BLM-dependent pathway of DNA break end resection. *Nucleic Acids Res* 2014, 42: 11083–91.

115. Toczylowski T, Yan H. Mechanistic analysis of a DNA end processing pathway mediated by the Xenopus Werner syndrome protein. *J Biol Chem* 2006, 281: 33198–205.

116. Liao S, Toczylowski T, Yan H. Identification of the Xenopus DNA2 protein as a major nuclease for the 5'->3' strand-specific processing of DNA ends. *Nucleic Acids Res* 2008, 36: 6091–100.

117. Yan H, Toczylowski T, McCane J, Chen C, Liao S. Replication protein A promotes 5'-3' end processing during homology-dependent DNA double-strand break repair. *J Cell Biol* 2011, 192: 251–61.

118. Tammaro M, Liao S, McCane J, Yan H. The N-terminus of RPA large subunit and its spatial position are important for the 5′->3′ resection of DNA double-strand breaks. *Nucleic Acids Res* 2015, 43: 8790–800.

119. Sturzenegger A, Burdova K, Kanagaraj R, Levikova M, Pinto C, Cejka P et al. DNA2 cooperates with the WRN and BLM RecQ helicases to mediate long-range DNA-end resection in human cells. *J Biol Chem* 2014, 289: 27314–26.

120. Zhou C, Pourmal S, Pavletich NP. Dna2 nuclease-helicase structure, mechanism and regulation by Rpa. *Elife* 2015, 4: 1–19.

121. Levikova M, Klaue D, Seidel R, Cejka P. Nuclease activity of Saccharomyces cerevisiae Dna2 inhibits its potent DNA helicase activity. *Proc Natl Acad Sci U S A* 2013, 110: E1992–2001.

122. Krasner DS, Daley JM, Sung P, Niu H. Interplay between Ku and Replication Protein A in the Restriction of Exo1-mediated DNA Break End Resection. *J Biol Chem* 2015, 290: 18806–16.

123. Chen H, Lisby M, Symington L. RPA Coordinates DNA End Resection and Prevents Formation of DNA Hairpins. *Mol Cell* 2013, 50: 589–600.

124. Cannavo E, Cejka P, Kowalczykowski SC. Relationship of DNA degradation by Saccharomyces cerevisiae Exonuclease 1 and its stimulation by RPA and Mre11-Rad50-Xrs2 to DNA end resection. *Proc Natl Acad Sci* 2013, 110: E1661–8.

125. Toledo LI, Altmeyer M, Rask M-B, Lukas C, Larsen DH, Povlsen LK et al. ATR prohibits replication catastrophe by preventing global exhaustion of RPA. *Cell* 2013, 155: 1088–103.

126. El-shemerly M, Janscak P, Hess D, Jiricny J, Ferrari S. Degradation of Human Exonuclease 1b upon DNA Synthesis Inhibition. *Cancer Res* 2005, : 3604–9.

127. Chen X, Kim I, Honaker Y, Paudyal SC, Koh WK, Sparks M et al. 14-3-3 Proteins Restrain the Exo1 Nuclease to Prevent Overresection. *J Biol Chem* 2015, 290: 12300–12.

128. Engels K, Giannattasio M, Muzi-falconi M, Lopes M, Ferrari S. 14-3-3 Proteins Regulate Exonuclease 1 – Dependent Processing of Stalled Replication Forks. *PLoS Genet* 2011, 7: e1001367.

129. Andersen SD, Keijzers G, Rampakakis E, Engels K, Luhn P, El-Shemerly M et al. 14-3-3 checkpoint regulatory proteins interact specifically with DNA repair protein human exonuclease 1 (hEXO1) via a semi-conserved motif. *DNA Repair (Amst)* 2012, 11: 267–77.

130. Tkáč J, Xu G, Adhikary H, Young JTF, Gallo D, Escribano-Díaz C et al. HELB Is a Feedback Inhibitor of DNA End Resection. *Mol Cell* 2016, 61: 1–14.

131. Nimonkar A V., Kowalczykowski SC. Second-end DNA capture in double-strand break repair: How to catch a DNA by its tail. *Cell Cycle* 2009, 8: 1816–7.

132. Nimonkar A V, Sica RA, Kowalczykowski SC. Rad52 promotes second-end DNA capture in double-stranded break repair to form complement-stabilized joint molecules. *Proc Natl Acad Sci U S A* 2009, 106: 3077–82.

133. McIlwraith MJ, West SC. DNA Repair Synthesis Facilitates RAD52-Mediated Second-End Capture during DSB Repair. *Mol Cell* 2008, 29: 510–6.

134. Lao JP, Oh SD, Shinohara M, Shinohara A, Hunter N. Rad52 Promotes Postinvasion Steps of Meiotic Double-Strand-Break Repair. *Mol Cell* 2008, 29: 517–24.

135. Karanja K, Lee EH, Hendrickson E, Campbell JL. Preventing over-resection by DNA2 helicase/nuclease suppresses repair defects in Fanconi anemia cells. *Cell Cycle* 2014, 13: 1540–50.

136. Zhang J-M, Liu X-M, Ding Y-H, Xiong L-Y, Ren J-Y, Zhou Z-X et al. Fission yeast Pxd1 promotes proper DNA repair by activating Rad16XPF and inhibiting Dna2. *PLoS Biol* 2014, 12: e1001946.

137. Rossiello F, Herbig U, Longhese MP, Fumagalli M, d'Adda di Fagagna F. Irreparable telomeric DNA damage and persistent DDR signalling as a shared causative mechanism of cellular senescence and ageing. *Curr Opin Genet Dev* 2014, 26: 89–95.

138. Roberts SA, Sterling J, Thompson C, Harris S, Mav D, Shah R et al. Clustered mutations in yeast and in human cancers can arise from damaged long single-strand DNA regions. *Mol Cell* 2012, 46: 424–35.

139. Stephens PJ, Greenman CD, Fu B, Yang F, Bignell GR, Mudie LJ et al. Massive Genomic Rearrangement Acquired in a Single Catastrophic Event during Cancer Development. *Cell* 2011, 144: 27–40.

140. VanHulle K, Lemoine FJ, Narayanan V, Downing B, Hull K, McCullough C et al. Inverted DNA Repeats Channel Repair of Distant Double-Strand Breaks into Chromatid Fusions and Chromosomal Rearrangements. *Mol Cell Biol* 2007, 27: 2601–14.

141. Rothkamm K, Krüger I, Thompson LH, Kru I, Lo M. Pathways of DNA Double-Strand Break Repair during the Mammalian Cell Cycle Pathways of DNA Double-Strand Break Repair during the Mammalian Cell Cycle. *Mol Cell Biol* 2003, 23: 5706–15.

142. Barlow JH, Lisby M, Rothstein R. Differential Regulation of the Cellular Response to DNA Double-Strand Breaks in G1. *Mol Cell* 2008, 30: 73–85.

143. Zierhut C, Diffley JFX. Break dosage, cell cycle stage and DNA replication influence DNA double strand break response. *EMBO J* 2008, 27: 1875–85.

144. Shao Z, Davis AJ, Fattah KR, So S, Sun J, Lee KJ et al. Persistently bound Ku at DNA ends attenuates DNA end resection and homologous recombination. *DNA Repair (Amst)* 2012, 11: 310–6.

145. Sun J, Lee K-J, Davis AJ, Chen DJ. Human Ku70/80 Protein Blocks Exonuclease 1-mediated DNA Resection in the Presence of Human Mre11 or Mre11/Rad50 Protein

Complex. *J Biol Chem* 2012, 287: 4936–45.

146. Downs J a, Jackson SP. A means to a DNA end: the many roles of Ku. *Nat Rev Mol Cell Biol* 2004, 5: 367–78.

147. Yun MH, Hiom K. CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. *Nature* 2009, 459: 460–3.

148. Quennet V, Beucher A, Barton O, Takeda S, Löbrich M. CtIP and MRN promote non-homologous end-joining of etoposide-induced DNA double-strand breaks in G1. *Nucleic Acids Res* 2011, 39: 2144–52.

149. Wohlbold L, Merrick KA, De S, Amat R, Kim JH, Larochelle S et al. Chemical genetics reveals a specific requirement for Cdk2 activity in the DNA damage response and identifies Nbs1 as a Cdk2 substrate in human cells. *PLoS Genet* 2012, 8: e1002935.

150. Falck J, Forment J V, Coates J, Mistrik M, Lukas J, Bartek J et al. CDK targeting of NBS1 promotes DNA-end resection, replication restart and homologous recombination. *EMBO Rep* 2012, 13: 561–8.

151. Buis J, Stoneham T, Spehalski E, Ferguson DO. Mre11 regulates CtIP-dependent double-strand break repair by interaction with CDK2. *Nat Struct Mol Biol* 2012, 19: 246–52.

152. Huertas P, Cortés-Ledesma F, Sartori AA, Aguilera A, Jackson SP. CDK targets Sae2 to control DNA-end resection and homologous recombination. *Nature* 2008, 455: 689–92.

153. Huertas P, Jackson SP. Human CtIP Mediates Cell Cycle Control of DNA End Resection and Double Strand Break Repair. *J Biol Chem* 2009, 284: 9558–65.

154. Yu X, Chen J. DNA Damage-Induced Cell Cycle Checkpoint Control Requires CtIP , a Phosphorylation-Dependent Binding Partner of BRCA1 C-Terminal Domains. *Mol Cell Biol* 2004, 24: 9478–86.

155. Chen X, Niu H, Chung W-H, Zhu Z, Papusha A, Shim EY et al. Cell cycle regulation of DNA double-strand break end resection by Cdk1-dependent Dna2 phosphorylation. *Nat Struct Mol Biol* 2011, 18: 1015–9.

156. Tomimatsu N, Mukherjee B, Catherine Hardebeck M, Ilcheva M, Vanessa Camacho C, Louise Harris J et al. Phosphorylation of EXO1 by CDKs 1 and 2 regulates DNA end resection and repair pathway choice. *Nat Commun* 2014, 5: 3561.

157. Morrison C, Rieder CL. Chromosome damage and progression into and through mitosis in vertebrates. *DNA Repair (Amst)* 2004, 3: 1133–9.

158. Giunta S, Belotserkovskaya R, Jackson SP. DNA damage signaling in response to double-strand breaks during mitosis. *J Cell Biol* 2010, 190: 197–207.

159. Peterson SE, Li Y, Chait BT, Gottesman ME, Baer R, Gautier J. Cdk1 uncouples CtIP-dependent resection and Rad51 filament formation during M-phase double-strand break repair. *J Cell Biol* 2011, 194: 705–20.

160. Matsuoka S, Ballif B a, Smogorzewska A, McDonald ER, Hurov KE, Luo J et al. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science (80-)* 2007, 316: 1160–6.

161. Stokes MP, Rush J, Macneill J, Ren JM, Sprott K, Nardone J et al. Profiling of UV-induced ATM/ATR signaling pathways. *Proc Natl Acad Sci U S A* 2007, 104: 19855–60.

162. Peterson SE, Li Y, Wu-Baer F, Chait BT, Baer R, Yan H et al. Activation of DSB Processing Requires Phosphorylation of CtIP by ATR. *Mol Cell* 2013, 49: 657–67.

163. Baroni E, Viscardi V, Cartagena-Lirola H, Lucchini G, Longhese MP. The functions of budding yeast Sae2 in the DNA damage response require Mec1- and Tel1-dependent phosphorylation. *Mol Cell Biol* 2004, 24: 4151–65.

164. Cartagena-Lirola H, Guerini I, Viscardi V, Lucchini G, Longhese MP. Budding yeast Sae2 is an in vivo target of the Mec1 and Tel1 checkpoint kinases during meiosis. *Cell Cycle* 2006, 5: 1549–59.

165. Di Virgilio M, Ying CY, Gautier J. PIKK-dependent phosphorylation of Mre11 induces MRN complex inactivation by disassembly from chromatin. *DNA Repair (Amst)* 2009, 8: 1311–20.

166. Schlegel BP, Jodelka FM, Nunez R. BRCA1 promotes induction of ssDNA by ionizing radiation. *Cancer Res* 2006, 66: 5181–9.

167. Cruz-García A, López-Saavedra A, Huertas P. BRCA1 Accelerates CtIP-Mediated DNA-End Resection. *Cell Rep* 2014, 9: 451–9.

168. Bunting SF, Callén E, Wong N, Chen H-T, Polato F, Gunn A et al. 53BP1 Inhibits Homologous Recombination in Brca1-Deficient Cells by Blocking Resection of DNA Breaks. *Cell* 2010, 141: 243–54.

169. Aparicio T, Baer R, Gottesman M, Gautier J. MRN , CtIP , and BRCA1 mediate repair of topoisomerase II–DNA adducts. *J Cell Biol* 2016, 212: 399–408.

170. Farmer H, McCabe N, Lord CJ, Tutt ANJ, Johnson D a, Richardson TB et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 2005, 434: 917–21.

171. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 2005, 434: 913–7.

172. Bouwman P, Aly A, Escandell JM, Pieterse M, Bartkova J, van der Gulden H et al. 53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers. *Nat Struct Mol Biol* 2010, 17: 688–95.

173. Cao L, Xu X, Bunting SF, Liu J, Wang R-H, Cao LL et al. A Selective Requirement for 53BP1 in the Biological Response to Genomic Instability Induced by Brca1 Deficiency. *Mol Cell* 2009, 35: 534–41.

174. Panier S, Boulton SJ. Double-strand break repair: 53BP1 comes into focus. *Nat Rev Mol Cell Biol* 2013, 15: 7–18.

175. Bothmer A, Robbiani DF, Feldhahn N, Gazumyan A, Nussenzweig A, Nussenzweig MC. 53BP1 regulates DNA resection and the choice between classical and alternative end joining during class switch recombination. *J Exp Med* 2010, 207: 855–65.

176. Bothmer A, Robbiani DF, Di Virgilio M, Bunting SF, Klein IA, Feldhahn N et al. Regulation of DNA End Joining, Resection, and Immunoglobulin Class Switch Recombination by 53BP1. *Mol Cell* 2011, 42: 319–29.

177. Jaspers JE, Kersbergen A, Boon U, Sol W, Van Deemter L, Zander S a. et al. Loss of 53BP1 causes PARP inhibitor resistance in BRCA1-mutated mouse mammary tumors. *Cancer Discov* 2013, 3: 68–81.

178. Callen E, Di Virgilio M, Kruhlak MJ, Nieto-Soler M, Wong N, Chen HT et al. 53BP1 mediates productive and mutagenic DNA repair through distinct phosphoprotein interactions. *Cell* 2013, 153: 1266–80.

179. Chapman JR, Barral P, Vannier J-B, Borel V, Steger M, Tomas-Loba A et al. RIF1 Is Essential for 53BP1-Dependent Nonhomologous End Joining and Suppression of DNA Double-Strand Break Resection. *Mol Cell* 2013, 49: 858–71.

180. Di Virgilio M, Callen E, Yamane A, Zhang W, Jankovic M, Gitlin AD et al. Rif1 prevents resection of DNA breaks and promotes immunoglobulin class switching. *Science* 2013, 339: 711–5.

181. Zimmermann M, Lottersberger F, Buonomo SB. 53BP1 Regulates DSB Repair Using Rif1 to Control 5' End Resection. *Science (80-)* 2013, 339: 700–4.

182. Feng L, Li N, Li Y, Wang J, Gao M, Wang W et al. Cell cycle-dependent inhibition of 53BP1 signaling by BRCA1. *Cell Discov* 2015, 1: 15019.

183. Feng L, Fong KW, Wang J, Wang W, Chen J. RIF1 counteracts BRCA1-mediated end resection during DNA repair. *J Biol Chem* 2013, 288: 11135–43.

184. Escribano-Díaz C, Orthwein A, Fradet-Turcotte A, Xing M, Young JTF, Tkáč J et al. A Cell Cycle-Dependent Regulatory Circuit Composed of 53BP1-RIF1 and BRCA1-CtIP

Controls DNA Repair Pathway Choice. *Mol Cell* 2013, 49: 872–83.

185. Zhang H, Liu H, Chen Y, Yang X, Wang P, Liu T et al. A cell cycle-dependent BRCA1–UHRF1 cascade regulates DNA double-strand break repair pathway choice. *Nat Commun* 2016, 7: 10201.

186. Cheruiyot A, Paudyal SC, Kim I, Sparks M, Ellenberger T, Piwnica-worms H et al. Poly (ADP-ribose) -binding promotes Exo1 damage recruitment and suppresses its nuclease activities. *DNA Repair (Amst)* 2015, 35: 106–15.

187. Bologna S, Altmannova V, Valtorta E, Koenig C, Liberali P, Gentili C et al. Sumoylation regulates EXO1 stability and processing of DNA damage. *Cell Cycle* 2015, 4101: 2439–50.

188. Steger M, Murina O, Hühn D, Ferretti LP, Walser R, Hänggi K et al. Prolyl Isomerase PIN1 Regulates DNA Double-Strand Break Repair by Counteracting DNA End Resection. *Mol Cell* 2013, 50: 333–43.

189. Broderick R, Nieminuszczy J, Baddock HT, Deshpande RA, Gileadi O, Paull TT et al. EXD2 promotes homologous recombination by facilitating DNA end resection. *Nat Cell Biol* 2016, 18: 271–80.

190. Liberti SE, Andersen SD, Wang J, May A, Miron S, Perderiset M et al. Bi-directional routing of DNA mismatch repair protein human exonuclease 1 to replication foci and DNA double strand breaks. *DNA Repair (Amst)* 2011, 10: 73–86.

191. Zhang F, Shi J, Chen S-H, Bian C, Yu X. The PIN domain of EXO1 recognizes poly(ADP-ribose) in DNA damage response. *Nucleic Acids Res* 2015, 43: 10782–94.

192. Kaidi A, Weinert BT, Choudhary C, Stephen P. Human SIRT6 promotes DNA-end resection through CtIP deacetylation. *Science (80-)* 2010, 329: 1348–53.

193. Daugaard M, Baude A, Fugger K, Povlsen LK, Beck H, Sørensen CS et al. LEDGF (p75) promotes DNA-end resection and homologous recombination. *Nat Struct Mol Biol* 2012, 19: 803–10.

194. Costelloe T, Louge R, Tomimatsu N, Mukherjee B, Martini E, Khadaroo B et al. The yeast Fun30 and human SMARCAD1 chromatin remodellers promote DNA end resection. *Nature* 2012, 489: 581–4.

195. Dong S, Han J, Chen H, Liu T, Huen MSYSY, Yang Y et al. The Human SRCAP Chromatin Remodeling Complex Promotes DNA-End Resection. *Curr Biol* 2014, 24: 2097–110.

196. Yang S-H, Zhou R, Campbell J, Chen J, Ha T, Paull TT. The SOSS1 single-stranded DNA binding complex promotes DNA end resection in concert with Exo1. *EMBO J* 2013,

32: 126–39.

197. Polo SE, Blackford AN, Chapman JR, Baskcomb L, Gravel S, Rusch A et al. Regulation of DNA-End Resection by hnRNPU-like Proteins Promotes DNA Double-Strand Break Signaling and Repair. *Mol Cell* 2012, 45: 505–16.

198. O'Sullivan RJ, Karlseder J. Telomeres: protecting chromosomes against genome instability. *Nat Rev Mol Cell Biol* 2010, 11: 171–81.

199. Chai W, Du Q, Shay JW, Wright WE. Human telomeres have different overhang sizes at leading versus lagging strands. *Mol Cell* 2006, 21: 427–35.

200. Chow TT, Zhao Y, Mak SS, Shay JW, Wright WE. Early and late steps in telomere overhang processing in normal human cells: the position of the final RNA primer drives telomere shortening. *Genes Dev* 2012, 26: 1167–78.

201. Wu P, Takai H, de Lange T. Telomeric 3' overhangs derive from resection by Exo1 and Apollo and fill-in by POT1b-associated CST. *Cell* 2012, 150: 39–52.

202. Wu P, van Overbeek M, Rooney S, de Lange T. Apollo Contributes to G Overhang Maintenance and Protects Leading-End Telomeres. *Mol Cell* 2010, 39: 606–17.

203. Budd ME, Campbell JL. Dna2 is involved in CA strand resection and nascent lagging strand completion at native yeast telomeres. *J Biol Chem* 2013, 288: 29414–29.

204. Tomita K, Kibe T, Kang H-Y, Seo Y-S, Uritani M, Ushimaru T et al. Fission yeast Dna2 is required for generation of the telomeric single-strand overhang. *Mol Cell Biol* 2004, 24: 9557–67.

205. Dewar JM, Lydall D. Pif1- and Exo1-dependent nucleases coordinate checkpoint activation following telomere uncapping. *EMBO J* 2010, 29: 4020–34.

206. Maringele L, Lydall D. EXO1-dependent single-stranded DNA at telomeres activates subsets of DNA damage and spindle checkpoint pathways in budding yeast yku70Δ mutants. *Genes Dev* 2002, 16: 1919–33.

207. Trenz K, Smith E, Smith S, Costanzo V. ATM and ATR promote Mre11 dependent restart of collapsed replication forks and prevent accumulation of DNA breaks. *EMBO J* 2006, 25: 1764–74.

208. Ying S, Hamdy FC, Helleday T. Mre11-Dependent Degradation of Stalled DNA Replication Forks Is Prevented by BRCA2 and PARP1. *Cancer Res* 2012, 72: 2814–21.

209. Iannascoli C, Palermo V, Murfuni I, Franchitto A, Pichierri P. The WRN exonuclease domain protects nascent strands from pathological MRE11/EXO1-dependent degradation. *Nucleic Acids Res* 2015, 43: 9788–803.

210. Machwe A, Xiao L, Groden J, Orren DK. The Werner and Bloom syndrome proteins catalyze regression of a model replication fork. *Biochemistry* 2006, 45: 13939–46.

211. Hashimoto Y, Ray Chaudhuri A, Lopes M, Costanzo V. Rad51 protects nascent DNA from Mre11-dependent degradation and promotes continuous DNA synthesis. *Nat Struct Mol Biol* 2010, 17: 1305–11.

212. Hu J, Sun L, Shen F, Chen Y, Hua Y, Liu Y et al. The intra-S phase checkpoint targets Dna2 to prevent stalled replication forks from reversing. *Cell* 2012, 149: 1221–32.

213. Chiolo I, Minoda A, Colmenares SU, Polyzos A, Costes S V., Karpen GH. Double-Strand Breaks in Heterochromatin Move Outside of a Dynamic HP1a Domain to Complete Recombinational Repair. *Cell* 2011, 144: 732–44.

214. Ryu T, Spatola B, Delabaere L, Bowlin K, Hopp H, Kunitake R et al. Heterochromatic breaks move to the nuclear periphery to continue recombinational repair. *Nat Cell Biol* 2015, 17: 1401–11.

215. Peterson CL. The Ins and Outs of Heterochromatic DNA Repair. *Dev Cell* 2011, 20: 285–7.

216. Luo G, Yao MS, Bender CF, Mills M, Bladl AR, Bradley A et al. Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation. *Proc Natl Acad Sci U S A* 1999, 96: 7376–81.

217. Zhu J, Petersen S, Tessarollo L, Nussenzweig A. Targeted disruption of the Nijmegen breakage syndrome gene NBS1 leads to early embryonic lethality in mice. *Curr Biol* 2001, 11: 105–9.

218. Chen P, Liu F, Cai S, Lin X, Chen Y, Gu B et al. Inactivation of CtIP Leads to Early Embryonic Lethality Mediated by G1 Restraint and to Tumorigenesis by Haploid Insufficiency. *Mol Cell Biol* 2005, 25: 3535–42.

219. Wei K, Clark AB, Wong E, Kane MF, Mazur D, Parris T et al. Mutation of Exonuclease 1 results in DNA mismatch repair defects, male and female sterility and increased cancer susceptibility in older mice. *Genes Dev* 2003, 17: 603–14.

220. Stewart G. The DNA Double-Strand Break Repair Gene hMRE11 Is Mutated in Individuals with an Ataxia-Telangiectasia-like Disorder. *Cell* 1999, 99: 577–87.

221. Waltes R, Kalb R, Gatei M, Kijas AW, Stumm M, Sobeck A et al. Human RAD50 deficiency in a Nijmegen breakage syndrome-like disorder. *Am J Hum Genet* 2009, 84: 605–16.

222. Bogdanova N, Feshchenko S, Schürmann P, Waltes R, Wieland B, Hillemanns P et al. Nijmegen Breakage Syndrome mutations and risk of breast cancer. *Int J cancer* 2008,

122: 802–6.

223. Cheok CF, Bachrati CZ, Chan KL, Ralf C, Wu L, Hickson ID. Roles of the Bloom's syndrome helicase in the maintenance of genome stability. *Biochem Soc Trans* 2005, 33: 1456–9.

224. Rossi ML, Ghosh AK, Bohr VA. Roles of Werner syndrome protein in protection of genome integrity. *DNA Repair (Amst)* 2010, 9: 331–44.

Chapter 2:

Dna2 initiates resection at clean DNA double-strand breaks

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

This chapter is currently under revision for publication in *Nucleic Acids Research.*

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 nucleoplamic 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' ^{32}P-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 ^{0}C for 2 hr, and then mixed with an equal volume of formamide, heated for 3 min at 95 ^{0}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'-^{32}P-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 ^{32}P using PNK. 5' ^{32}P-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' ^{32}P-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' ^{32}P-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 ^{0}C 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 *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 *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.

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

resection 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, S2.6K). This effect could be rescued by the addition of purified WT RPA complex (Fig. 2.4J, S2.6O), 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, S2.1, S2.2). 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, S2.4). 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 ($\geq$ 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

We are grateful to Dr. Jean Gautier (Columbia University) for the gift of FastBac1-Flag-CtIP expression construct. We thank Wenjuan Wei and Won Kyun Koh for their assistance in generating the *Xenopus* Dna2 antibodies and cloning recombinant Dna2 mutant constructs, respectively. This work was supported by NIH grants (R01GM098535 to Z.Y.; R01CA80100 to T. H.) and an American Cancer Society Research Scholar Grant (RSG-13-212-01-DMC to Z. Y.).

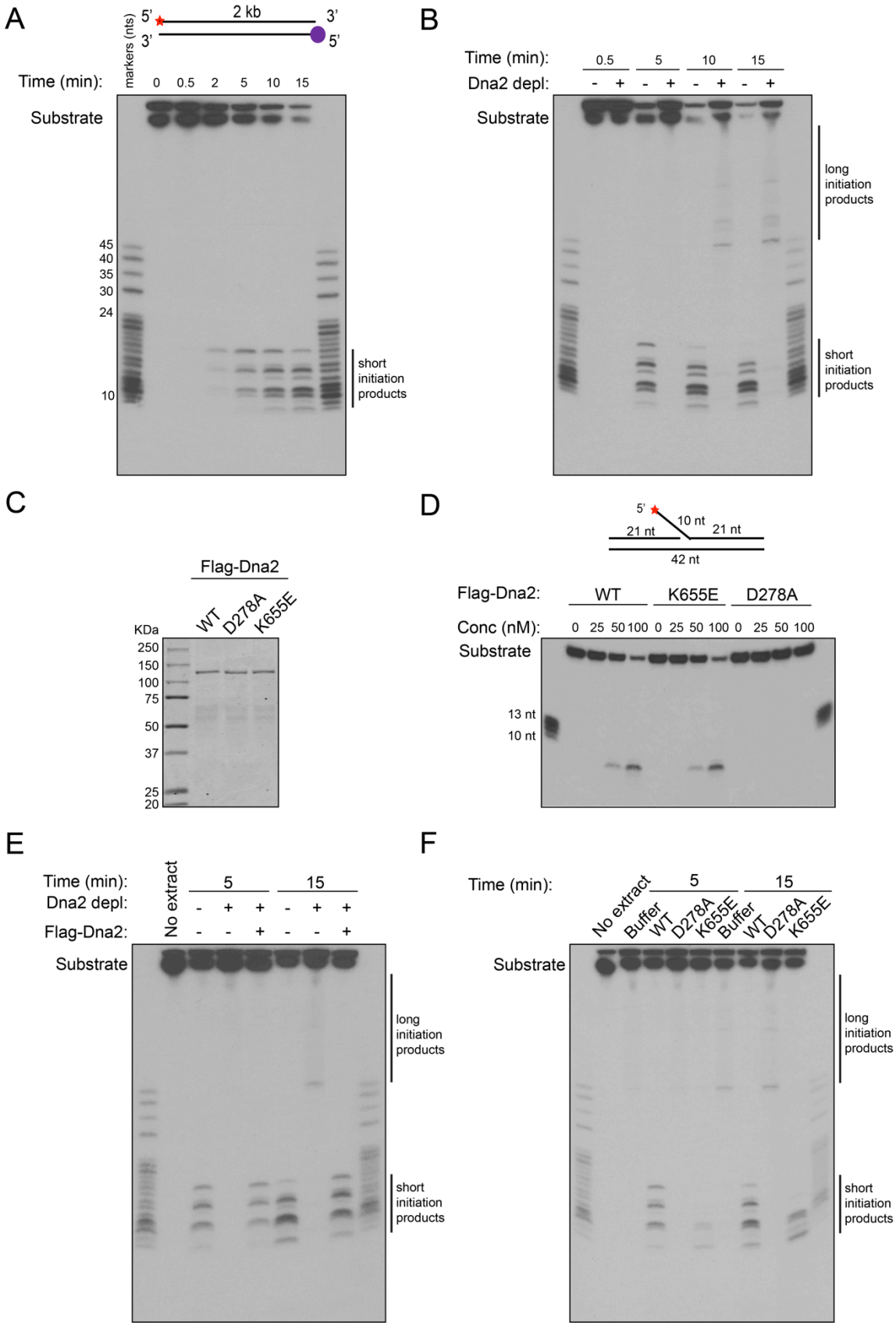

A
5'
2 kb
3'
3'
5'
markers (nts)
Time (min):
0 0.5 2 5 10 15
Substrate
45
40
35
30
24
10
short initiation products
B
Time (min):
0.5 5 10 15
Dna2 depl:
- + - + - + - +
Substrate
long initiation products
short initiation products
C
Flag-Dna2
KDa
WT D278A K655E
250
150
100
75
50
37
25
20
D
5'
10 nt
21 nt
21 nt
42 nt
Flag-Dna2:
WT K655E D278A
Conc (nM):
0 25 50 100 0 25 50 100 0 25 50 100
Substrate
13 nt
10 nt
E
Time (min):
5 15
Dna2 depl:
- + + - + +
Flag-Dna2:
- - + - - +
No extract
Substrate
long initiation products
short initiation products
F
Time (min):
5 15
No extract
Buffer
WT
D278A
K655E
Buffer
WT
D278A
K655E
Substrate
long initiation products
short initiation products

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) (helicase-inactive mutant) and Flag-Dna2(D278A) (nuclease-inactive mutant) in restoring resection initiation in the Dna2 depleted extract.

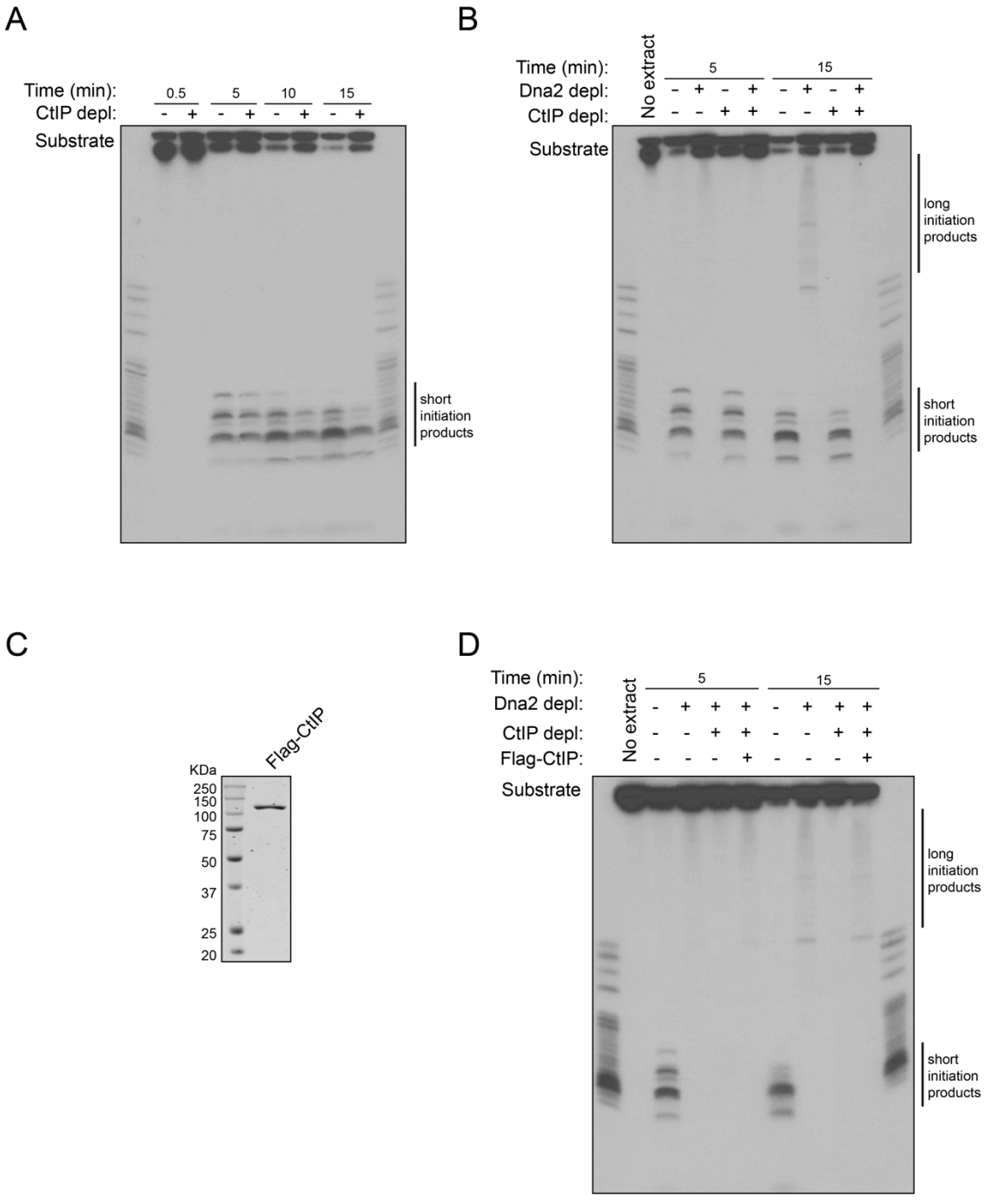


Figure 2.2. In the absence of Dna2, CtIP initiates resection at clean DSBs via cleavage of 5' strand DNA at more distal sites.

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.

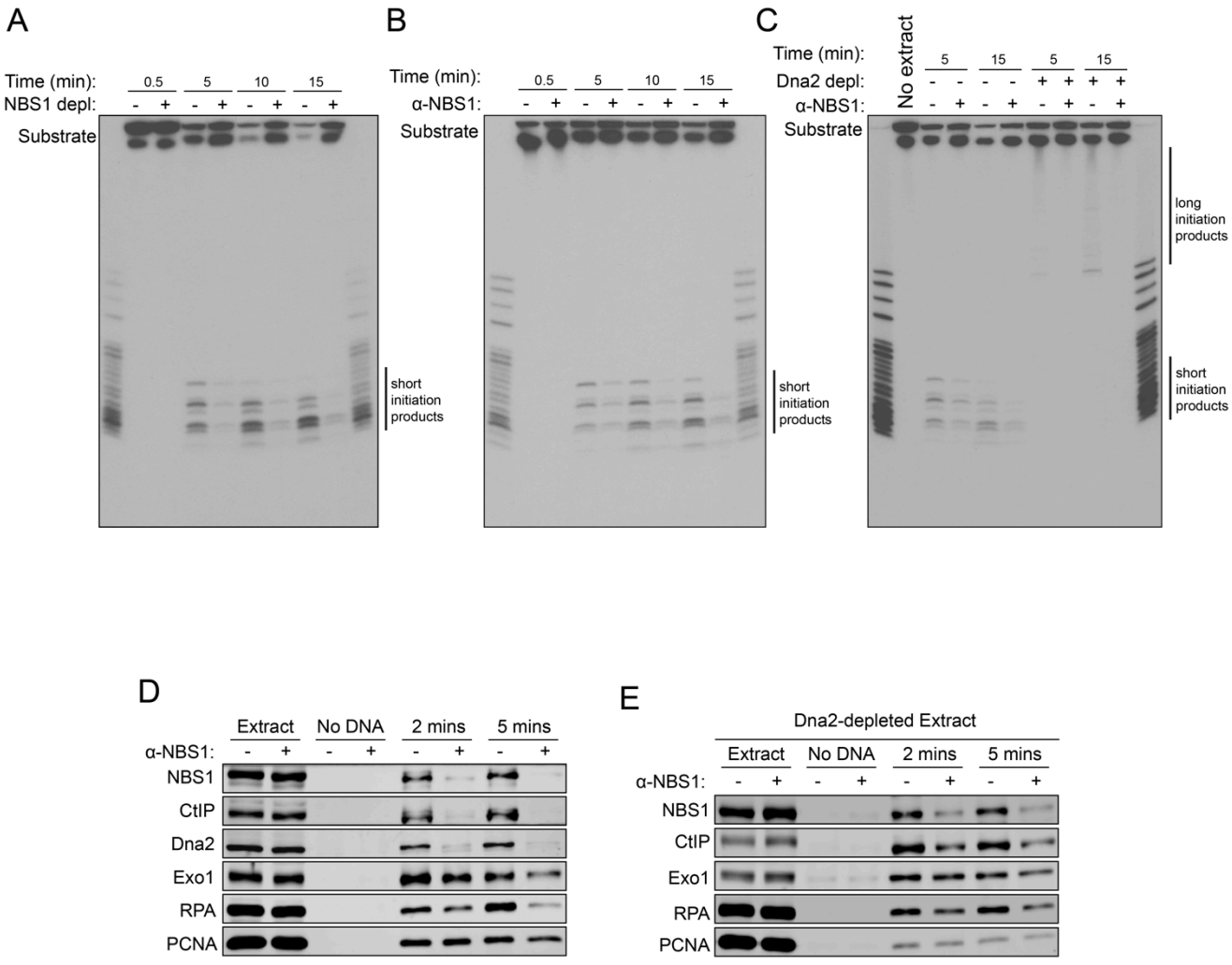


Figure 2.3. MRN promotes both Dna2- and CtIP-mediated resection initiation.

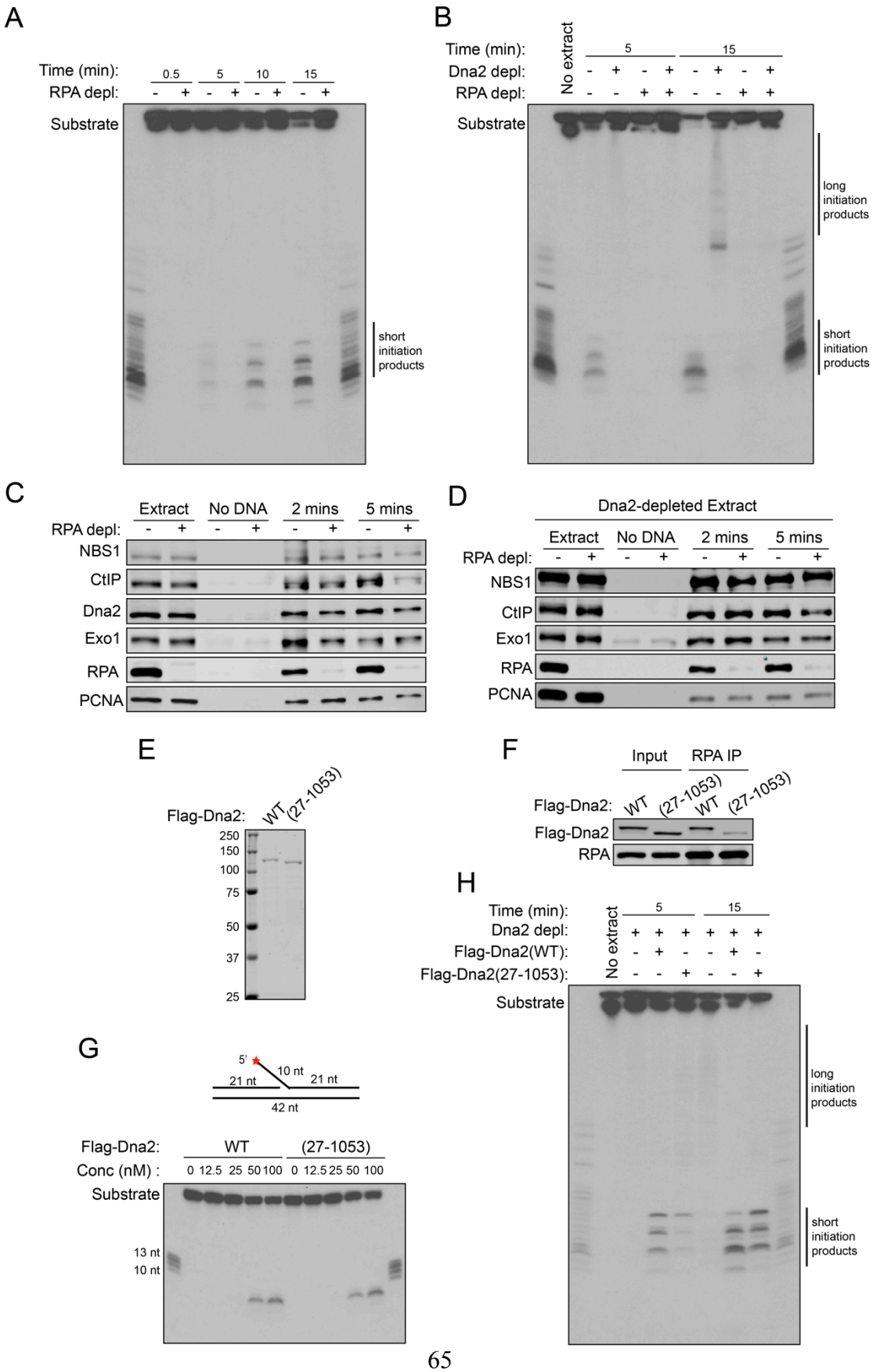
A). Depletion of NBS1 dramatically inhibited the generation of short resection initiation products by Dna2 in the extract.

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
Time (min):
0.5
5
10
15
RPA depl:
- + - + - + - +
Substrate
short initiation products
B
Time (min):
No extract
5
15
Dna2 depl:
- + - + - + - +
RPA depl:
- - + + - - + +
Substrate
long initiation products
short initiation products
C
Extract
No DNA
2 mins
5 mins
RPA depl:
- + - + - + - +
NBS1
CtIP
Dna2
Exo1
RPA
PCNA
D
Dna2-depleted Extract
Extract
No DNA
2 mins
5 mins
RPA depl:
- + - + - + - +
NBS1
CtIP
Exo1
RPA
PCNA
E
Flag-Dna2:
WT
(27-1053)
250
150
100
75
50
37
25
F
Input
RPA IP
Flag-Dna2:
WT
(27-1053)
WT
(27-1053)
Flag-Dna2
RPA
G
5'
10 nt
21 nt
21 nt
42 nt
Flag-Dna2:
WT
(27-1053)
Conc (nM) :
0 12.5 25 50 100 0 12.5 25 50 100
Substrate
13 nt
10 nt
H
Time (min):
No extract
5
15
Dna2 depl:
+ + + + + +
Flag-Dna2(WT):
- + - - + -
Flag-Dna2(27-1053):
- - + - - +
Substrate
long initiation products
short initiation products

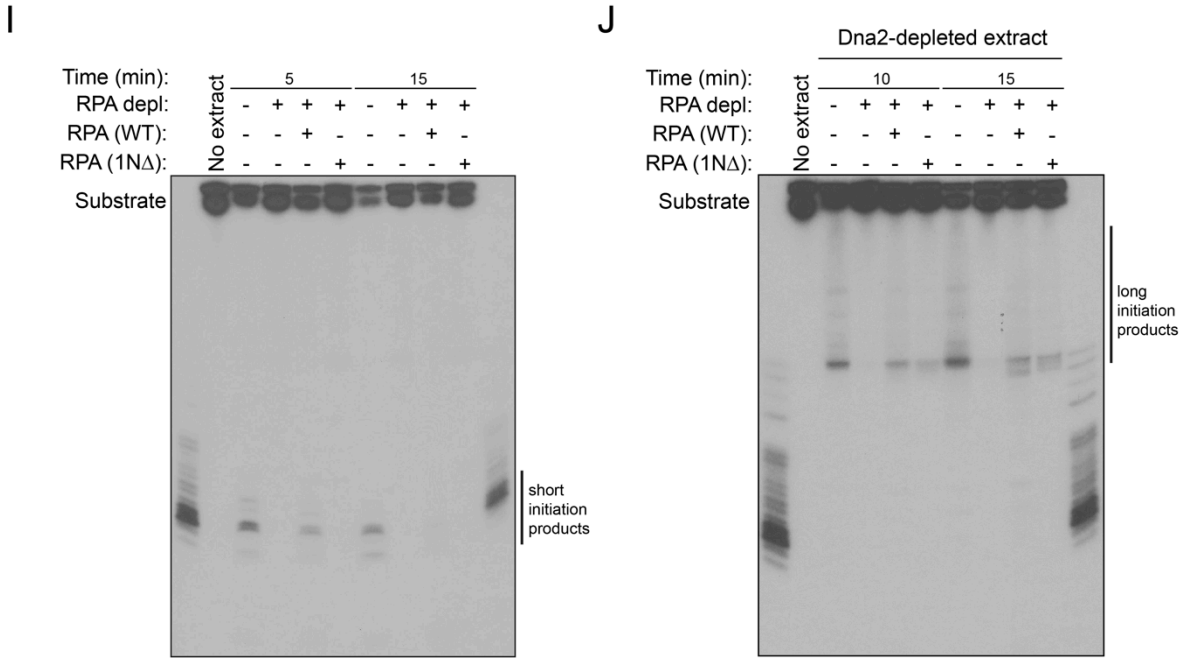


Figure 2.4. RPA plays a key role in resection initiation by both Dna2 and CtIP pathways.

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.

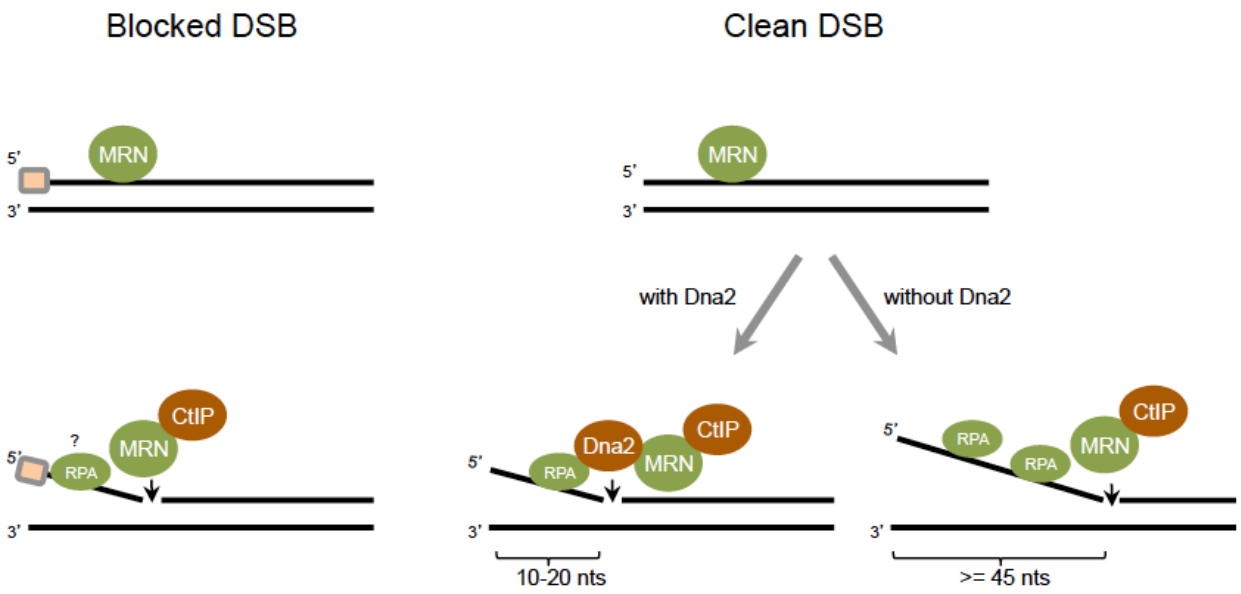


Figure 2.5. A model for resection initiation at clean or blocked DSBs.

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.

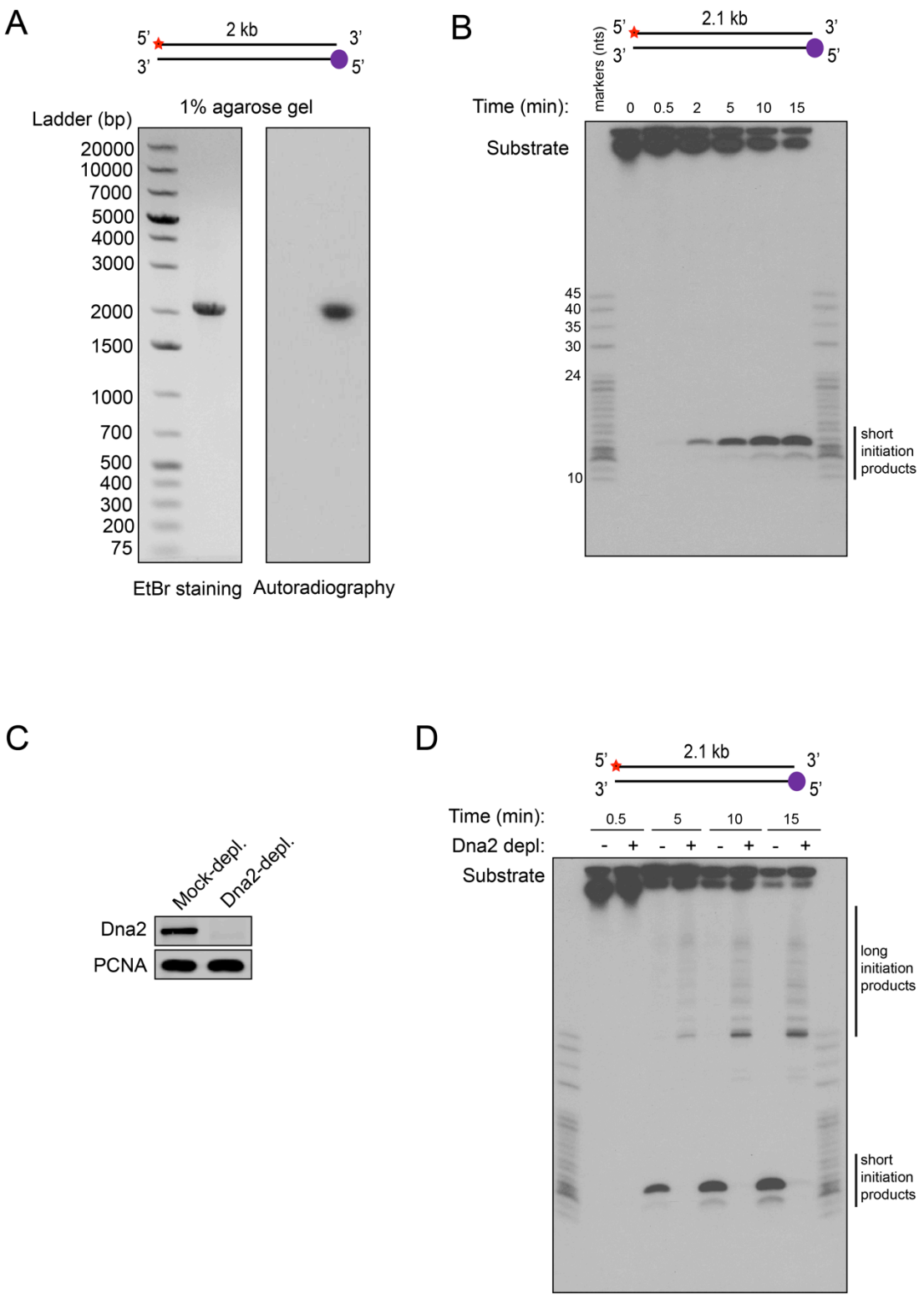


Figure S2.1 (related to Figure 2.1). Analysis of resection initiation on another DNA substrate with a blunt end.

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 (≥ 45 nts) from the radiolabeled 5' end.

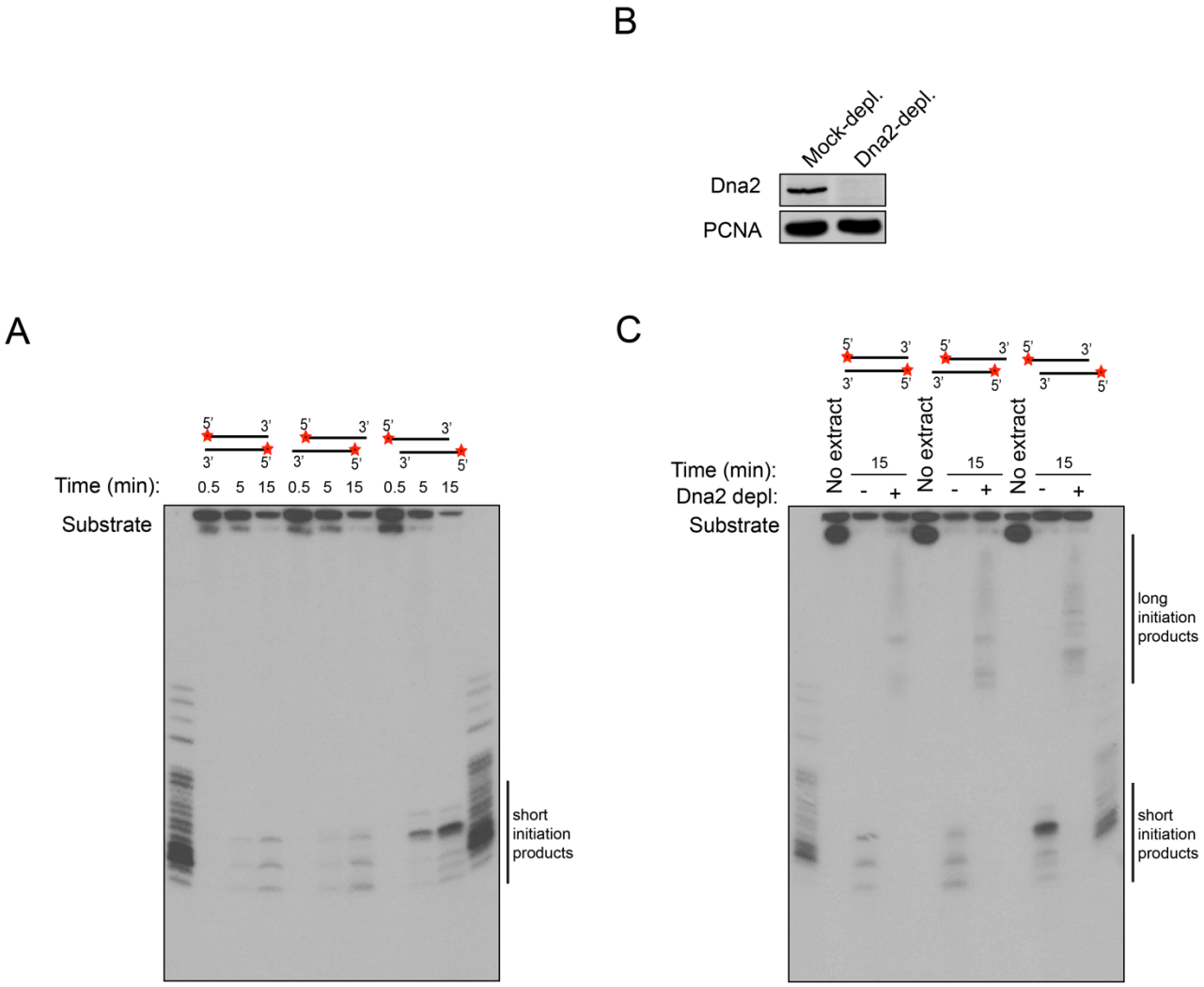


Figure S2.2 (related to Figure 2.1). Analysis of resection initiation at clean DSBs with 5' or 3' ssDNA overhangs.

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).

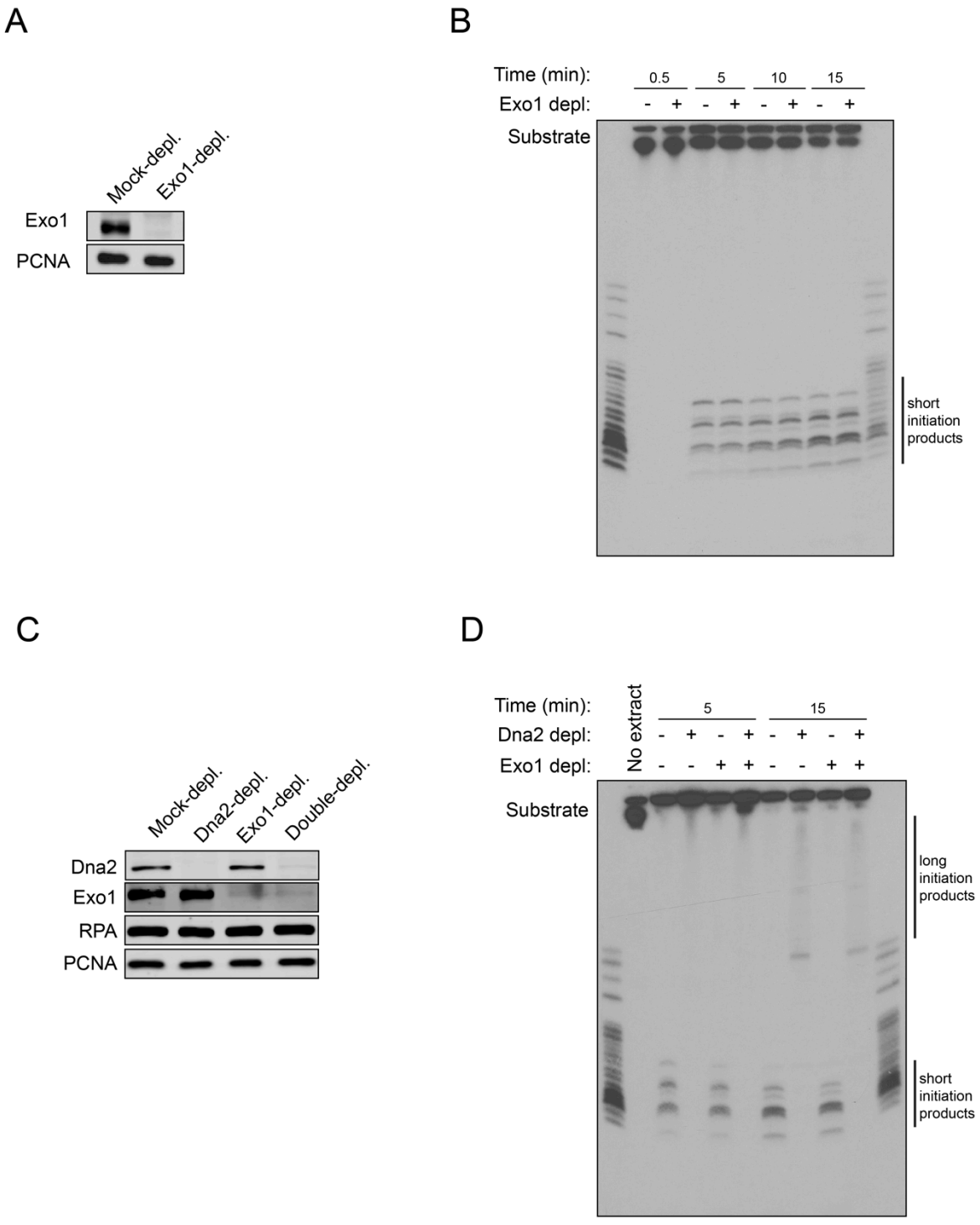


Figure S2.3 (related to Figure 2.2). Exo1 apparently does not play a role in resection initiation at a clean DSB.

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.

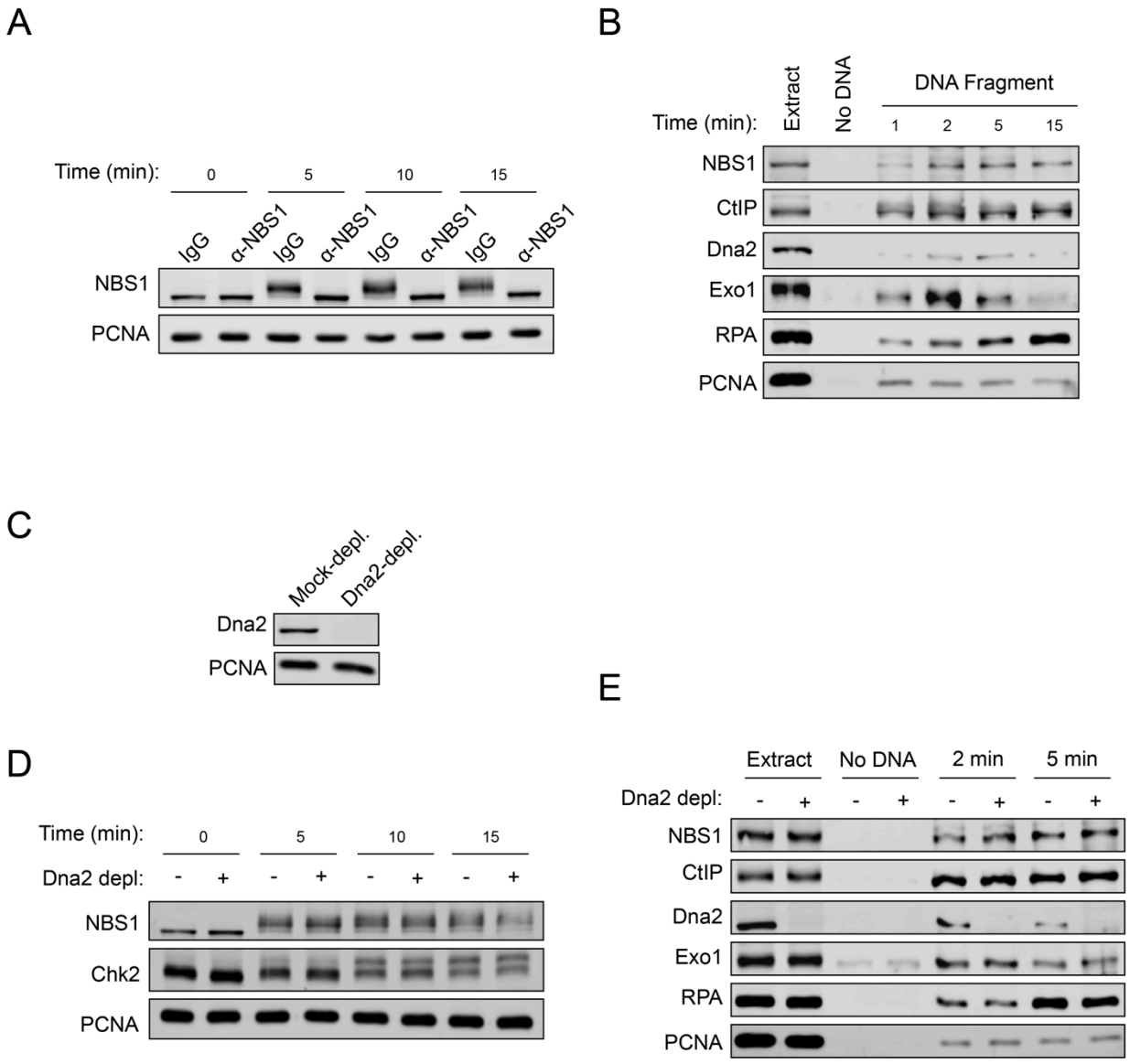


Figure S2.4 (related to Figure 2.3). Effects of NBS1 inhibition on damage-induced NBS1 phosphorylation, and effects of Dna2 depletion on the damage-induced phosphorylation of NBS1 and Chk2 and on the damage association of NBS1, CtIP, Dna2, Exo1, RPA and PCNA.

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.

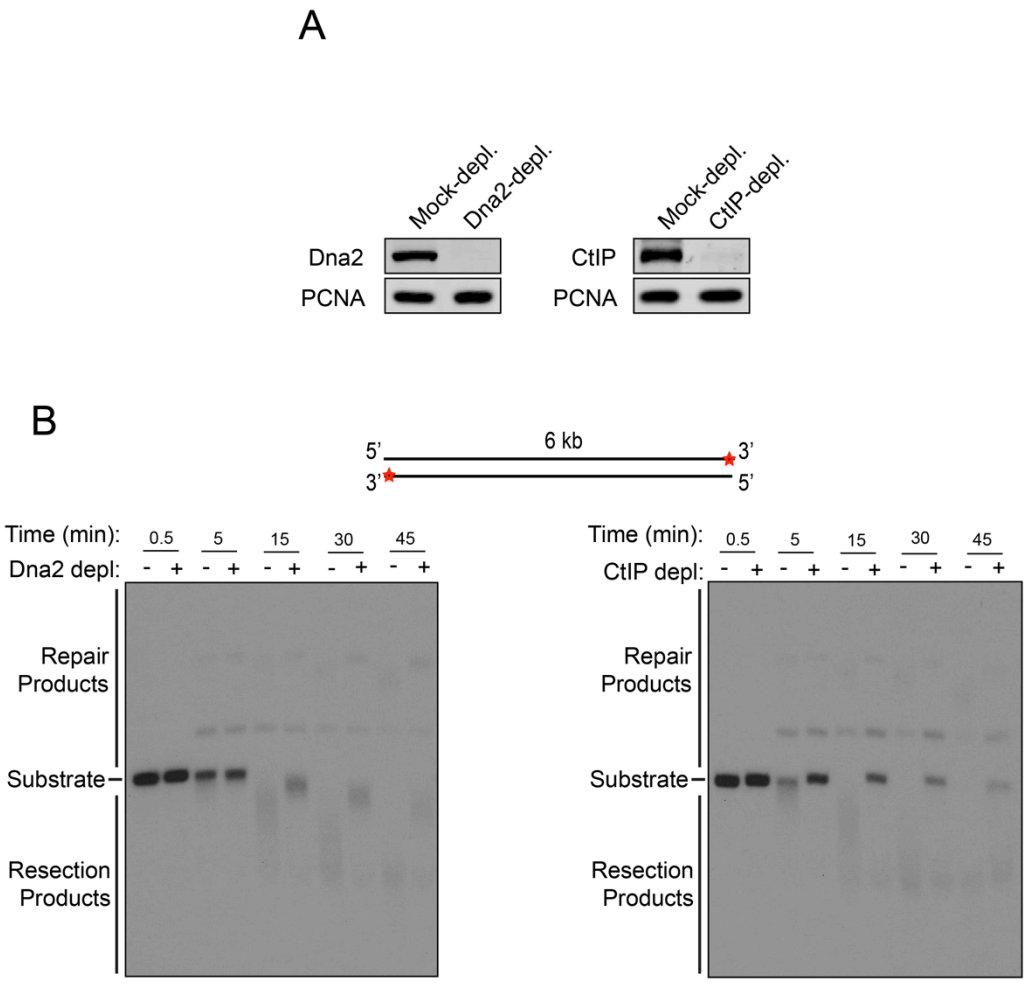


Figure S2.5 (related to Discussion). Effects of Dna2 or CtIP depletion on the overall resection of a 3' end-labeled DNA substrate.

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.

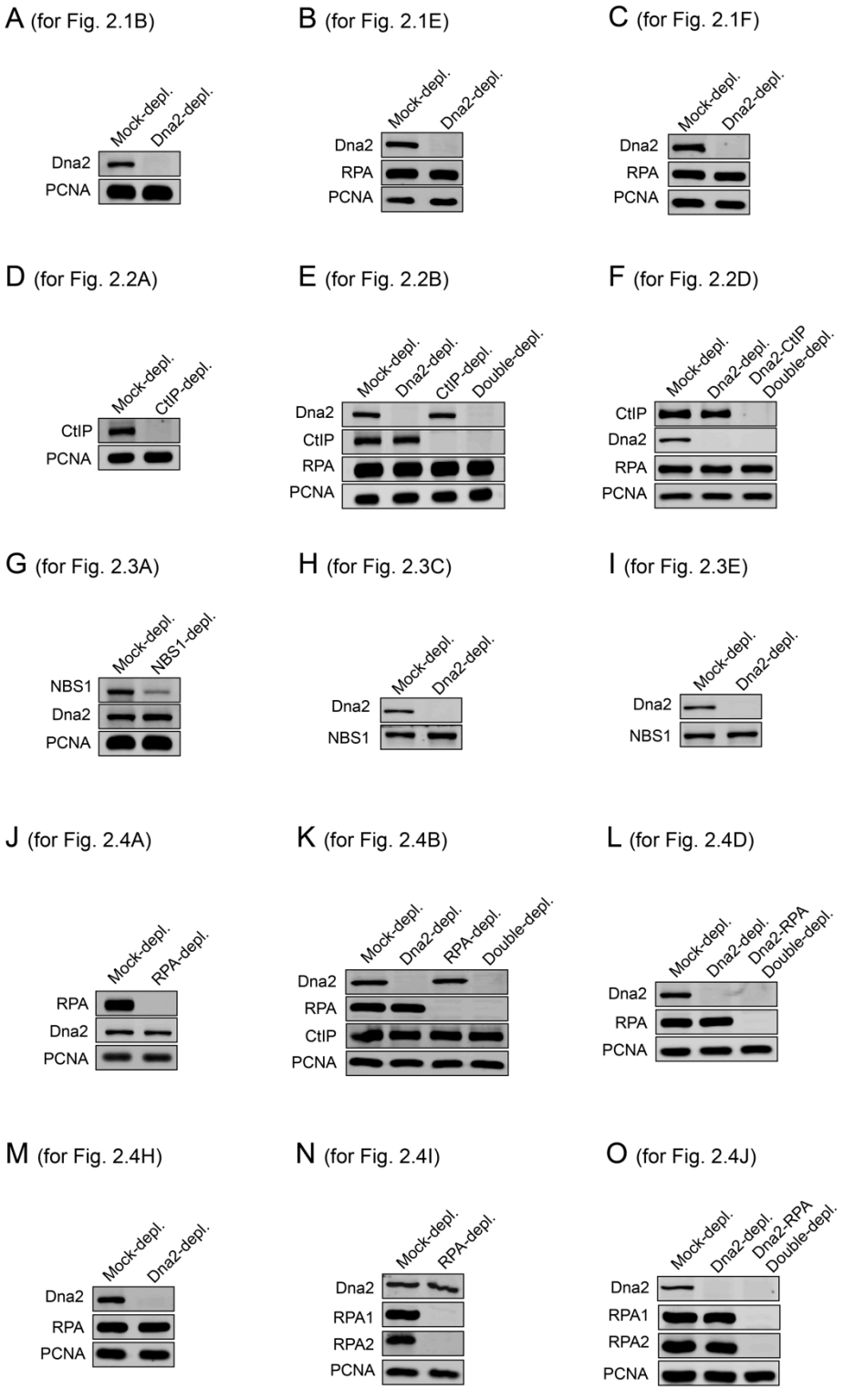


Figure S2.6 (related to Figures 2.1-2.4). Results of immunodepletion in *Xenopus* NPE.

2.6 References

1. Mehta A, Haber JE. Sources of DNA Double-Strand Breaks and Models of Recombinational DNA Repair. Cold Spring Harb Perspet Biol. 2014;6:a016428.

2. Zeman MK, Cimprich KA. Causes and consequences of replication stress. Nat Cell Biol. 2014;16(1):2–9.

3. Kim H, Kim JS. A guide to genome engineering with programmable nucleases. Nat Rev Genet. 2014;15(5):321–34.

4. Lam I, Keeney S. Mechanism and control of meiotic recombination initiation. Cold Spring Harb Perspet Biol. 2015;52:1–53.

5. Schatz DG, Swanson PC. V(D)J Recombination: Mechanisms of Initiation. Annu Rev Genet. 2011;45(D):167–202.

6. Ciccia A, Elledge SJ. The DNA Damage Response: Making It Safe to Play with Knives. Mol Cell. 2010;40(2):179–204.

7. Jackson S, Bartek J. The DNA-damage response in human biology and disease. Nature. 2009;461(7267):1071–8.

8. Polo S, Jackson S. Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. Genes Dev. 2011;25(5):409–33.

9. Kass EM, Jasin M. Collaboration and competition between DNA double-strand break repair pathways. FEBS Lett. 2010;584(17):3703–8.

10. Sung P, Klein H. Mechanism of homologous recombination: mediators and helicases take on regulatory functions. Nat Rev Mol Cell Biol. 2006;7(10):739–50.

11. Symington LS, Gautier J. Double-Strand Break End Resection and Repair Pathway Choice. Annu Rev Genet. 2011;45(1):247–71.

12. Branzei D, Foiani M. Regulation of DNA repair throughout the cell cycle. Nat Rev Mol Cell Biol. 2008;9(4):297–308.

13. Orthwein A, Noordermeer SM, Wilson MD, Landry S, Enchev RI, Sherker A, et al. A mechanism for the suppression of homologous recombination in G1 cells. Nature. 2015;528(7582):422–6.

14. Rothkamm K, Krüger I, Thompson LH, Lobrich M. Pathways of DNA Double-Strand Break Repair during the Mammalian Cell Cycle Pathways of DNA Double-Strand Break Repair during the Mammalian Cell Cycle. Mol Cell Biol. 2003;23(16):5706–15.

15. Paudyal SC, You Z. Sharpening the ends for repair : mechanisms and regulation of DNA resection. Acta Biochim Biophys Sin (Shanghai). 2016;48(May):647–57.

16. Daley JM, Niu H, Miller AS, Sung P. Biochemical mechanism of DSB end resection and its regulation. DNA Repair (Amst). 2015;(32):66–74.

17. Cejka P. DNA End Resection: Nucleases Team Up with the Right Partners to Initiate Homologous Recombination. J Biol Chem. 2015;290(38):22931–8.

18. Paull TT. Making the best of the loose ends: Mre11/Rad50 complexes and Sae2 promote DNA double-strand break resection. DNA Repair (Amst). 2010;9(12):1283–91.

19. Symington LS. Mechanism and regulation of DNA end resection in eukaryotes. Crit Rev Biochem Mol Biol. 2016;9238(212):1–18.

20. You Z, Bailis JM. DNA damage and decisions: CtIP coordinates DNA repair and cell cycle checkpoints. Trends Cell Biol. 2010;20(7):402–9.

21. Huertas P. DNA resection in eukaryotes: deciding how to fix the break. Nat Struct Mol Biol. 2010;17(1):11–6.

22. Bernstein KA, Rothstein R. At Loose Ends: Resecting a Double-Strand Break. Cell. 2009;137(5):807–10.

23. Abraham RT. Cell cycle checkpoint signaling through the ATM an ATR kinases. Genes Dev. 2001;15:2177–96.

24. Shiloh Y, Ziv Y. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. Nat Rev Mol Cell Biol. 2013;14(4):197–210.

25. Cimprich KA, Cortez D. ATR: an essential regulator of genome integrity. Nat Rev Mol Cell Biol. 2008;9(8):616–27.

26. You Z, Bailis JM, Johnson SA, Dilworth SM, Hunter T. Rapid activation of ATM on DNA flanking double-strand breaks. Nat Cell Biol. 2007;9(11):1311–8.

27. Paull TT. Mechanisms of ATM Activation. Annu Rev Biochem. 2015;(December):1–28.

28. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science. 2003;300(June):1542–8.

29. Shiotani B, Zou L. Single-Stranded DNA Orchestrates an ATM-to-ATR Switch at DNA Breaks. Mol Cell. 2009;33(5):547–58.

30. Clerici M, Trovesi C, Galbiati A, Lucchini G, Longhese MP. Mec1/ATR regulates the generation of single-stranded DNA that attenuates Tel1/ATM signaling at DNA ends.

EMBO J. 2014;33(3):198–216.

31. You Z, Shi LZ, Zhu Q, Wu P, Zhang YW, Basilio A, et al. CtIP Links DNA Double-Strand Break Sensing to Resection. Mol Cell. 2009;36(6):954–69.

32. Mimitou EP, Symington LS. DNA end resection : Many nucleases make light work. DNA Repair (Amst). 2009;8:983–95.

33. Mimitou EP, Symington LS. Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. Nature. 2008;455(7214):770–4.

34. Zhu Z, Chung WH, Shim EY, Lee SE, Ira G. Sgs1 Helicase and Two Nucleases Dna2 and Exo1 Resect DNA Double-Strand Break Ends. Cell. 2008;134(6):981–94.

35. Garcia V, Phelps SEL, Gray S, Neale MJ. Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1. Nature. 2011;479(7372):241–4.

36. Limbo O, Chahwan C, Yamada Y, de Bruin RAM, Wittenberg C, Russell P. Ctp1 Is a Cell-Cycle-Regulated Protein that Functions with Mre11 Complex to Control Double-Strand Break Repair by Homologous Recombination. Mol Cell. 2007;28(1):134–46.

37. Takeda S, Nakamura K, Taniguchi Y, Paull TT. Ctp1/CtIP and the MRN Complex Collaborate in the Initial Steps of Homologous Recombination. Mol Cell. 2007;28(3):351–2.

38. Sartori AA, Lukas C, Coates J, Mistrik M, Fu S, Bartek J, et al. Human CtIP promotes DNA end resection. Nature. 2007;450(7169):509–14.

39. Chin GM, Villeneuve AM. C.elegans mre-11 is required for meiotic recombination and DNA repair but is dispensable for the meiotic G 2 DNA damage checkpoint. Genes Dev. 2001;15:522–34.

40. Penkner A, Portik-Dobos Z, Tang L, Schnabel R, Novatchkova M, Jantsch V, et al. A conserved function for a Caenorhabditis elegans Com1/Sae2/CtIP protein homolog in meiotic recombination. EMBO J. 2007;26(24):5071–82.

41. Cannavo E, Cejka P. Sae2 promotes dsDNA endonuclease activity within Mre11-Rad50-Xrs2 to resect DNA breaks. Nature. 2014;514(7520):122–5.

42. Anand R, Ranjha L, Cannavo E, Cejka P. Phosphorylated CtIP Functions as a Co-factor of the MRE11-RAD50-NBS1 Endonuclease in DNA End Resection. Mol Cell. 2016;64(5):940–50.

43. Makharashvili N, Tubbs AT, Yang SH, Wang H, Barton O, Zhou Y, et al. Catalytic and Noncatalytic Roles of the CtIP Endonuclease in Double-Strand Break End Resection. Mol Cell. 2014;54(6):1022–33.

44. Wang H, Li Y, Truong LN, Shi LZ, Hwang PY-H, He J, et al. CtIP Maintains Stability at Common Fragile Sites and Inverted Repeats by End Resection-Independent Endonuclease Activity. Mol Cell. 2014;54(6):1012–21.

45. Lengsfeld BM, Rattray AJ, Bhaskara V, Ghirlando R, Paull TT. Sae2 Is an Endonuclease that Processes Hairpin DNA Cooperatively with the Mre11/Rad50/Xrs2 Complex. Mol Cell. 2007;28:638–51.

46. Shibata A, Moiani D, Arvai AS, Perry J, Harding SM, Genois M-M, et al. DNA Double-Strand Break Repair Pathway Choice Is Directed by Distinct MRE11 Nuclease Activities. Mol Cell. 2014;53(1):7–18.

47. Broderick R, Nieminuszczy J, Baddock HT, Deshpande RA, Gileadi O, Paull TT, et al. EXD2 promotes homologous recombination by facilitating DNA end resection. Nat Cell Biol. 2016;18(3):271–80.

48. Nicolette ML, Lee K, Guo Z, Rani M, Chow JM, Lee SE, et al. Mre11–Rad50–Xrs2 and Sae2 promote 5′ strand resection of DNA double-strand breaks. Nat Struct Mol Biol. 2010;17(12):1478–85.

49. Gravel S, Chapman JR, Magill C, Jackson SP. DNA helicases Sgs1 and BLM promote DNA double-strand break resection. Genes Dev. 2008;22(20):2767–72.

50. Nimonkar A V., Ozsoy AZ, Genschel J, Modrich P, Kowalczykowski SC. Human exonuclease 1 and BLM helicase interact to resect DNA and initiate DNA repair. Proc Natl Acad Sci U S A. 2008;105(44):16906–11.

51. Nimonkar A V, Genschel J, Kinoshita E, Polaczek P, Campbell JL, Wyman C, et al. BLM – DNA2 – RPA – MRN and EXO1 – BLM – RPA – MRN constitute two DNA end resection machineries for human DNA break repair. Genes Dev. 2011;2:350–62.

52. Chen X, Paudyal SC, Chin R-I, You Z. PCNA promotes processive DNA end resection by Exo1. Nucleic Acids Res. 2013;41(20):9325–38.

53. Ngo GHP, Balakrishnan L, Dubarry M, Campbell JL, Lydall D. The 9-1-1 checkpoint clamp stimulates DNA resection by Dna2-Sgs1 and Exo1. Nucleic Acids Res. 2014;42(16):10516–28.

54. Ngo GHP, Lydall D. The 9-1-1 checkpoint clamp coordinates resection at DNA double strand breaks. Nucleic Acids Res. 2015;43(10):5017–32.

55. Sturzenegger A, Burdova K, Kanagaraj R, Levikova M, Pinto C, Cejka P, et al. DNA2 cooperates with the WRN and BLM RecQ helicases to mediate long-range DNA-end resection in human cells. J Biol Chem. 2014;289(39):27314–26.

56. Daley JM, Chiba T, Xue X, Niu H, Sung P. Multifaceted role of the Topo IIIα-RMI1-

RMI2 complex and DNA2 in the BLM-dependent pathway of DNA break end resection. Nucleic Acids Res. 2014;42(17):11083–91.

57. Cejka P, Cannavo E, Polaczek P, Masuda-Sasa T, Pokharel S, Campbell JL, et al. DNA end resection by Dna2–Sgs1–RPA and its stimulation by Top3–Rmi1 and Mre11–Rad50–Xrs2. Nature. 2010;467(7311):112–6.

58. Niu H, Chung W-H, Zhu Z, Kwon Y, Zhao W, Chi P, et al. Mechanism of the ATP-dependent DNA end-resection machinery from Saccharomyces cerevisiae. Nature. 2010;467(7311):108–11.

59. Liao S, Toczylowski T, Yan H. Identification of the Xenopus DNA2 protein as a major nuclease for the 5'->3' strand-specific processing of DNA ends. Nucleic Acids Res. 2008;36(19):6091–100.

60. Yan H, Toczylowski T, McCane J, Chen C, Liao S. Replication protein A promotes 5'-3' end processing during homology-dependent DNA double-strand break repair. J Cell Biol. 2011;192(2):251–61.

61. Tammaro M, Liao S, McCane J, Yan H. The N-terminus of RPA large subunit and its spatial position are important for the 5′->3′ resection of DNA double-strand breaks. Nucleic Acids Res. 2015;43:8790–800.

62. Tammaro M, Liao S, Beeharry N, Yan H. DNA double-strand breaks with 5 adducts are efficiently channeled to the DNA2-mediated resection pathway. Nucleic Acids Res. 2016;44(1):221–31.

63. Toczylowski T, Yan H. Mechanistic analysis of a DNA end processing pathway mediated by the Xenopus Werner syndrome protein. J Biol Chem. 2006;281(44):33198–205.

64. Zhang H, Hua Y, Li R, Kong D. Cdc24 is essential for long-range end resection in the repair of double-stranded DNA breaks. J Biol Chem. 2016;291(48):24961–73.

65. Langerak P, Mejia-Ramirez E, Limbo O, Russell P. Release of Ku and MRN from DNA ends by Mre11 nuclease activity and Ctp1 is required for homologous recombination repair of double-strand breaks. PLoS Genet. 2011;7(9):e1002271.

66. Neale MJ, Pan J, Keeney S. Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. Nature. 2005;436(7053):1053–7.

67. Hartsuiker E, Mizuno K, Molnar M, Kohli J, Ohta K, Carr AM. Ctp1CtIP and Rad32Mre11 nuclease activity are required for Rec12Spo11 removal, but Rec12Spo11 removal is dispensable for other MRN-dependent meiotic functions. Mol Cell Biol. 2009;29(7):1671–81.

68. Hartsuiker E, Neale MJ, Carr AM. Distinct Requirements for the Rad32Mre11 Nuclease

and Ctp1CtIP in the Removal of Covalently Bound Topoisomerase I and II from DNA. Mol Cell. 2009;33(1):117–23.

69. Aparicio T, Baer R, Gottesman M, Gautier J. MRN , CtIP , and BRCA1 mediate repair of topoisomerase II–DNA adducts. J Cell Biol. 2016;212(4):399–408.

70. Deshpande RA, Lee JH, Arora S, Paull TT. Nbs1 Converts the Human Mre11/Rad50 Nuclease Complex into an Endo/Exonuclease Machine Specific for Protein-DNA Adducts. Mol Cell. 2016;64(3):593–606.

71. Hoa NN, Shimizu T, Zhou ZW, Wang ZQ, Deshpande RA, Paull TT, et al. Mre11 Is Essential for the Removal of Lethal Topoisomerase 2 Covalent Cleavage Complexes. Mol Cell. 2016;64(3):580–92.

72. Rothenberg M, Kohli J, Ludin K. Ctp1 and the MRN-complex are required for endonucleolytic Rec12 removal with release of a single class of oligonucleotides in fission yeast. PLoS Genet. 2009;5(11):e1000722.

73. Nakamura K, Kogame T, Oshiumi H, Shinohara A, Sumitomo Y, Agama K, et al. Collaborative action of Brca1 and CtIP in elimination of covalent modifications from double-strand breaks to facilitate subsequent break repair. PLoS Genet. 2010;6(1):E1000828.

74. Deng C, Brown JA, You D, Brown JM. Multiple endonucleases function to repair covalent topoisomerase I complexes in Saccharomyces cerevisiae. Genetics. 2005;170(2):591–600.

75. Moreau S, Ferguson JR, Symington LS. The nuclease activity of Mre11 is required for meiosis but not for mating type switching, end joining, or telomere maintenance. Mol Cell Biol. 1999;19(1):556–66.

76. Llorente B, Symington LS. The Mre11 nuclease is not required for 5’ to 3’ resection at multiple HO-induced double-strand breaks. Mol Cell Biol. 2004;24(21):9682–94.

77. Ivanov EL, Sugawara N, White CI, Fabre F, Haber JE. Mutations in XRS2 and RAD50 delay but do not prevent mating-type switching in Saccharomyces cerevisiae. Mol Cell Biol. 1994;14(5):3414–25.

78. Clerici M, Mantiero D, Lucchini G, Longhese MP. The Saccharomyces cerevisiae Sae2 protein promotes resection and bridging of double strand break ends. J Biol Chem. 2005;280(46):38631–8.

79. Liao S, Tammaro M, Yan H. The structure of ends determines the pathway choice and Mre11 nuclease dependency of DNA double-strand break repair. Nucleic Acids Res. 2016;44(12):5689–701.

80. Walter J, Sun L, Newport J. Regulated Chromosomal DNA Replication in the Absence of a Nucleus. Mol Cell. 1998;1:519–29.

81. Lupardus PJ, Van C, Cimprich KA. Analyzing the ATR-mediated checkpoint using Xenopus egg extracts. Methods. 2007;41(2):222–31.

82. Srinivasan S V, Gautier J. Study of Cell Cycle Checkpoints Using Xenopus Cell-Free Extracts. Methods in molecular biology. 2011. 119-158 p.

83. Garner E, Costanzo V. Studying the DNA damage response using in vitro model systems. DNA Repair (Amst). 2009;8(9):1025–37.

84. Cheruiyot A, Paudyal SC, Kim I, Sparks M, Ellenberger T, Piwnica-worms H, et al. Poly (ADP-ribose) -binding promotes Exo1 damage recruitment and suppresses its nuclease activities. DNA Repair (Amst). 2015;35:106–15.

85. Chen X, Kim I, Honaker Y, Paudyal SC, Koh WK, Sparks M, et al. 14-3-3 Proteins Restrain the Exo1 Nuclease to Prevent Overresection. J Biol Chem. 2015;290(19):12300–12.

86. Budd ME, Campbell JL. A yeast gene required for DNA replication encodes a protein with homology to DNA helicases. Proc Natl Acad Sci U S A. 1995;92(17):7642–6.

87. Imamura O, Campbell JL. The human Bloom syndrome gene suppresses the DNA replication and repair defects of yeast dna2 mutants. Proc Natl Acad Sci U S A. 2003;100(14):8193–8.

88. Liu Q, Choe W, Campbell JL. Identification of the Xenopus laevis Homolog of Saccharomyces cerevisiae DNA2 and Its Role in DNA Replication. J Biol Chem. 2000;275(3):1615–24.

89. Masuda-sasa T, Imamura O, Campbell JL. Biochemical analysis of human Dna2. Nucleic Acids Res. 2006;34(6):1865–75.

90. Balakrishnan L, Polaczek P, Pokharel S, Campbell JL, Bambara RA. Dna2 Exhibits a Unique Strand End-dependent Helicase. J Biol Chem. 2010;285(50):38861–8.

91. Pinto C, Kasaciunaite K, Seidel R, Cejka P. Human DNA2 possesses a cryptic DNA unwinding activity that functionally integrates with BLM or WRN helicases. Elife. 2016;1–24.

92. Levikova M, Klaue D, Seidel R, Cejka P. Nuclease activity of Saccharomyces cerevisiae Dna2 inhibits its potent DNA helicase activity. Proc Natl Acad Sci U S A. 2013;110(22):E1992-2001.

93. Lin W, Sampathi S, Dai H, Liu C, Zhou M, Hu J, et al. Mammalian DNA2

helicase/nuclease cleaves G-quadruplex DNA and is required for telomere integrity. EMBO J. 2013;32(10):1425–39.

94. You Z, Chahwan C, Bailis J, Hunter T, Russell P. ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. Mol Cell Biol. 2005;25(13):5363–79.

95. Shim EY, Chung W-H, Nicolette ML, Zhang Y, Davis M, Zhu Z, et al. Saccharomyces cerevisiae Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks. EMBO J. 2010;29(19):3370–80.

96. Chen H, Lisby M, Symington L. RPA Coordinates DNA End Resection and Prevents Formation of DNA Hairpins. Mol Cell. 2013;50(4):589–600.

97. Cannavo E, Cejka P, Kowalczykowski SC. Relationship of DNA degradation by Saccharomyces cerevisiae Exonuclease 1 and its stimulation by RPA and Mre11-Rad50-Xrs2 to DNA end resection. Proc Natl Acad Sci. 2013;110(18):E1661–8.

98. Deng SK, Gibb B, de Almeida MJ, Greene EC, Symington LS. RPA antagonizes microhomology-mediated repair of DNA double-strand breaks. Nat Struct Mol Biol. 2014;21(4):405–12.

99. Zhou C, Pourmal S, Pavletich NP. Dna2 nuclease-helicase structure, mechanism and regulation by Rpa. Elife. 2015;4:1–19.

100. Peterson SE, Li Y, Wu-Baer F, Chait BT, Baer R, Yan H, et al. Activation of DSB Processing Requires Phosphorylation of CtIP by ATR. Mol Cell. 2013;49(4):657–67.

101. Liao S, Guay C, Toczylowski T, Yan H. Analysis of MRE11's function in the 5'->3' processing of DNA double-strand breaks. Nucleic Acids Res. 2012;40(10):4496–506.

102. Kim J, Kim H, Ryu G, Kim D, Hurwitz J, Seo Y. Isolation of human Dna2 endonuclease and characterization of its enzymatic properties. Nucleic Acids Res. 2006;34(6):1854–64.

103. Duxin JP, Moore HR, Sidorova J, Karanja K, Honaker Y, Dao B, et al. Okazaki fragment processing-independent role for human Dna2 enzyme during DNA replication. J Biol Chem. 2012;287(26):21980–91.

104. Miller AS, Daley JM, Pham NT, Niu H, Xue X, Ira G, et al. A novel role of the Dna2 translocase function in DNA break resection. Genes Dev. 2017;1–8.

105. Levikova M, Pinto C, Cejka P. The motor activity of DNA2 functions as an ssDNA translocase to promote DNA end resection. Genes Dev. 2017;1–10.

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

activity. EMBO J. 2008;27(14):1953–62.

107. Lu H, Shamanna RA, Keijzers G, Anand R, Rasmussen LJ, Cejka P, et al. RECQL4 Promotes DNA End Resection in Repair of DNA Double-Strand Breaks. Cell Rep. 2016;16(1):1–13.

108. Peterson SE, Li Y, Chait BT, Gottesman ME, Baer R, Gautier J. Cdk1 uncouples CtIP-dependent resection and Rad51 filament formation during M-phase double-strand break repair. J Cell Biol. 2011;194(5):705–20.

109. Isaac C, Piwnica-worms H, Grünwald S, William G, Francis N, Fan H. Expression and Purification of Recombinant Proteins Using the Baculovirus System. Curr Protoc Mol Biol. 2004;16.11.1-16.11.14.

110. Lee BI, Wilson DM. The RAD2 domain of human exonuclease 1 exhibits 5' to 3' exonuclease and flap structure-specific endonuclease activities. J Biol Chem. 1999;274(53):37763–9.

Chapter 3 :

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

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

*Sharad C. Paudyal is a co-first author to this work.

This chapter was originally published in *DNA Repair*.
2015 Nov; 35:106-15. Epub 2015 Sep 30.

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 nonohomologous 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, SOSS1, 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-

HPD (EMD Biosciences, 118415), ADP-ribose (Sigma, A0752) and Poly(A) RNA (Roche, 10108626001) were purchased from the respective vendors.

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% CO_2. 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 $MgCl_2$, 1 mM DTT, 12.5 ug/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: $[PAR] = [(A_{260})\ cm^{-1}]/[13{,}500\ cm^{-1}M^{-1}]$ (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' ^{32}P-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 $MgCl_2$, 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' ^{32}P-labeled 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'-^{32}P-ATT GGT TAT TTA CCG AGC TCG AAT TCA CTG G-3', 5'-TAG AGG ATC CCC GCT AGC GGG-3') as described in Lee et al (4). 5' ^{32}P-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' ^{32}P-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-microirradidation 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' ^{32}P-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

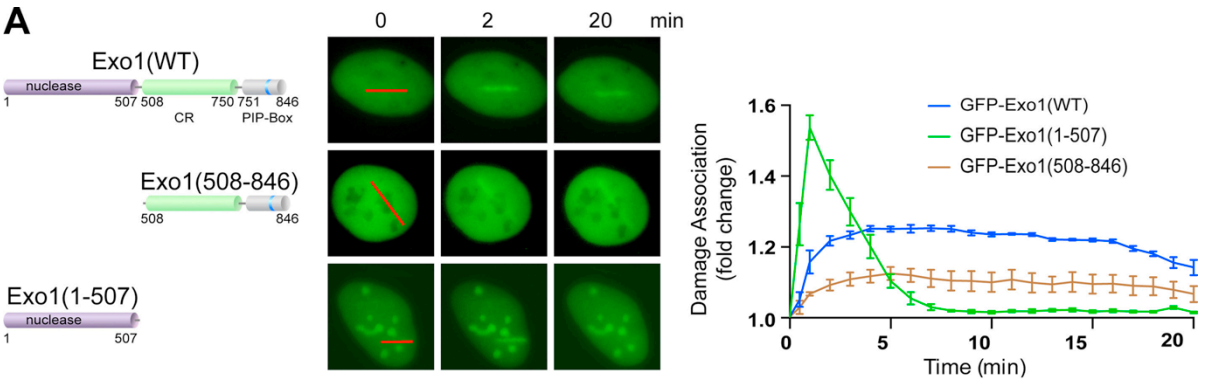
resulting PAR chains provide a docking platform for the rapid damage recruitment of Exo1 through the PAR-binding activity of its N-terminus. During this initial stage of damage association, Exo1 resection activity is held inactive by PAR until its clearance by PARG. The second stage, more sustained damage 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

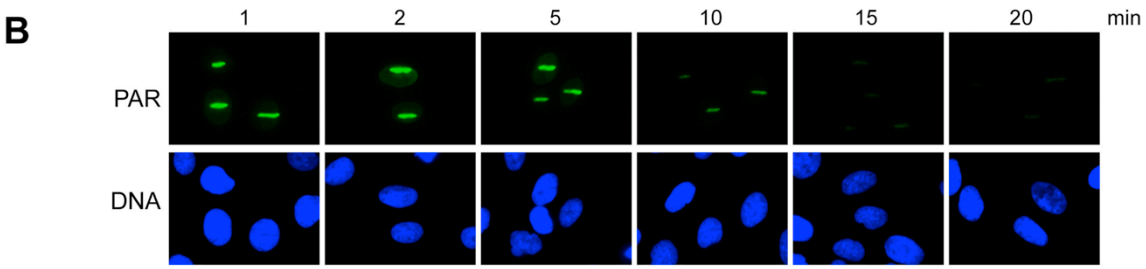
between HR, MMEJ and classic NHEJ repair pathways. Due to the highly negatively charged nature of PAR chains, it is likely that PAR inhibits Exo1 nuclease activity by directly competing with the binding of the DNA substrate to the nuclease domain. Alternatively, PAR-binding may function as an allosteric 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

We thank Dr. Michael Nielsen for the gift of the GST-AF1521 construct. This work was supported by NIH grants (R01GM098535 to Z.Y.; P01CA92584 to T.E.) and an American Cancer Society Research Scholar Grant (RSG-13-212-01-DMC to Z.Y.).


A
Exo1(WT)
nuclease
1
507 508
750 751
846
CR
PIP-Box
Exo1(508-846)
508
846
Exo1(1-507)
nuclease
1
507
0
2
20
min
Damage Association (fold change)
1.6
1.4
1.2
1.0
0
5
10
15
20
Time (min)
GFP-Exo1(WT)
GFP-Exo1(1-507)
GFP-Exo1(508-846)


B
1
2
5
10
15
20
min
PAR
DNA

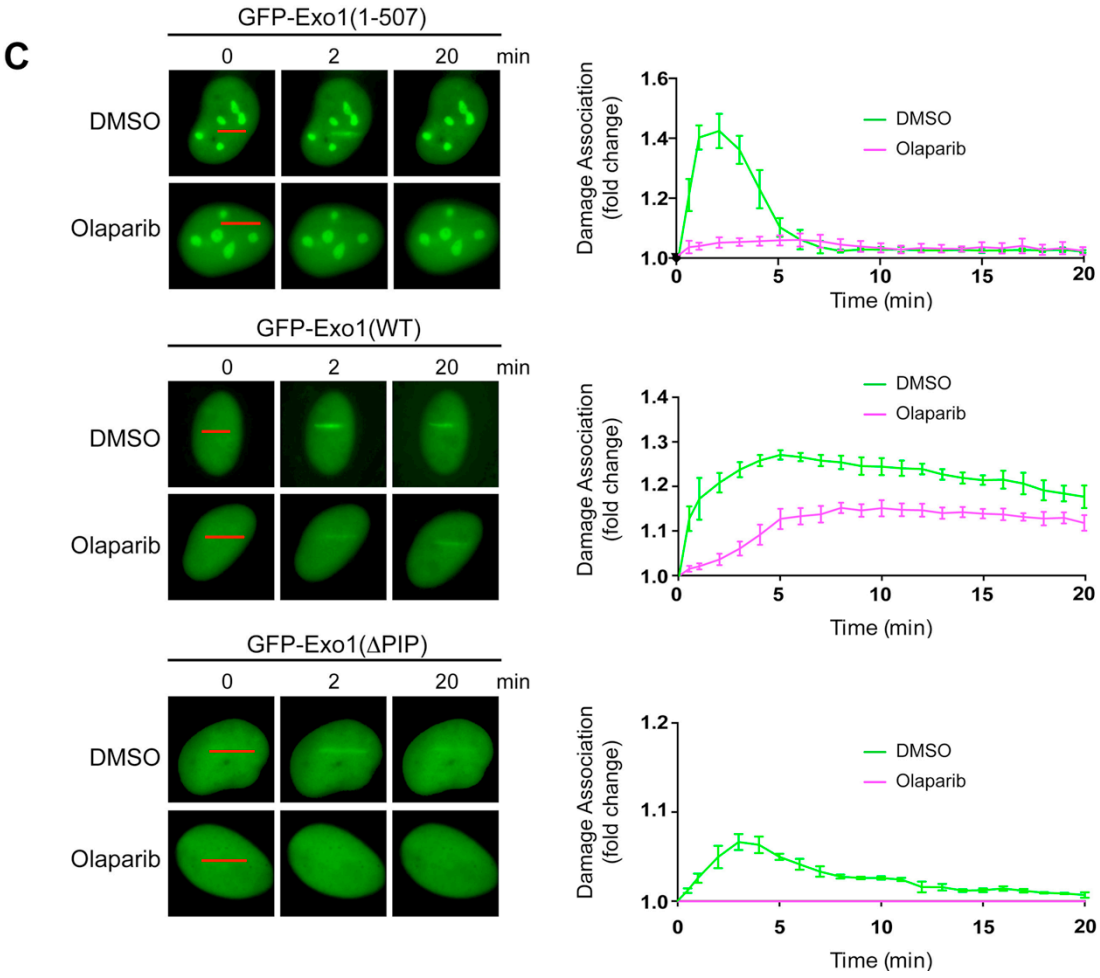

C
GFP-Exo1(1-507)
0
2
20
min
DMSO
Olaparib
Damage Association (fold change)
1.6
1.4
1.2
1.0
0
5
10
15
20
Time (min)
DMSO
Olaparib
GFP-Exo1(WT)
0
2
20
min
DMSO
Olaparib
Damage Association (fold change)
1.4
1.3
1.2
1.1
1.0
0
5
10
15
20
Time (min)
DMSO
Olaparib
GFP-Exo1(ΔPIP)
0
2
20
min
DMSO
Olaparib
Damage Association (fold change)
1.2
1.1
1.0
0
5
10
15
20
Time (min)
DMSO
Olaparib

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.

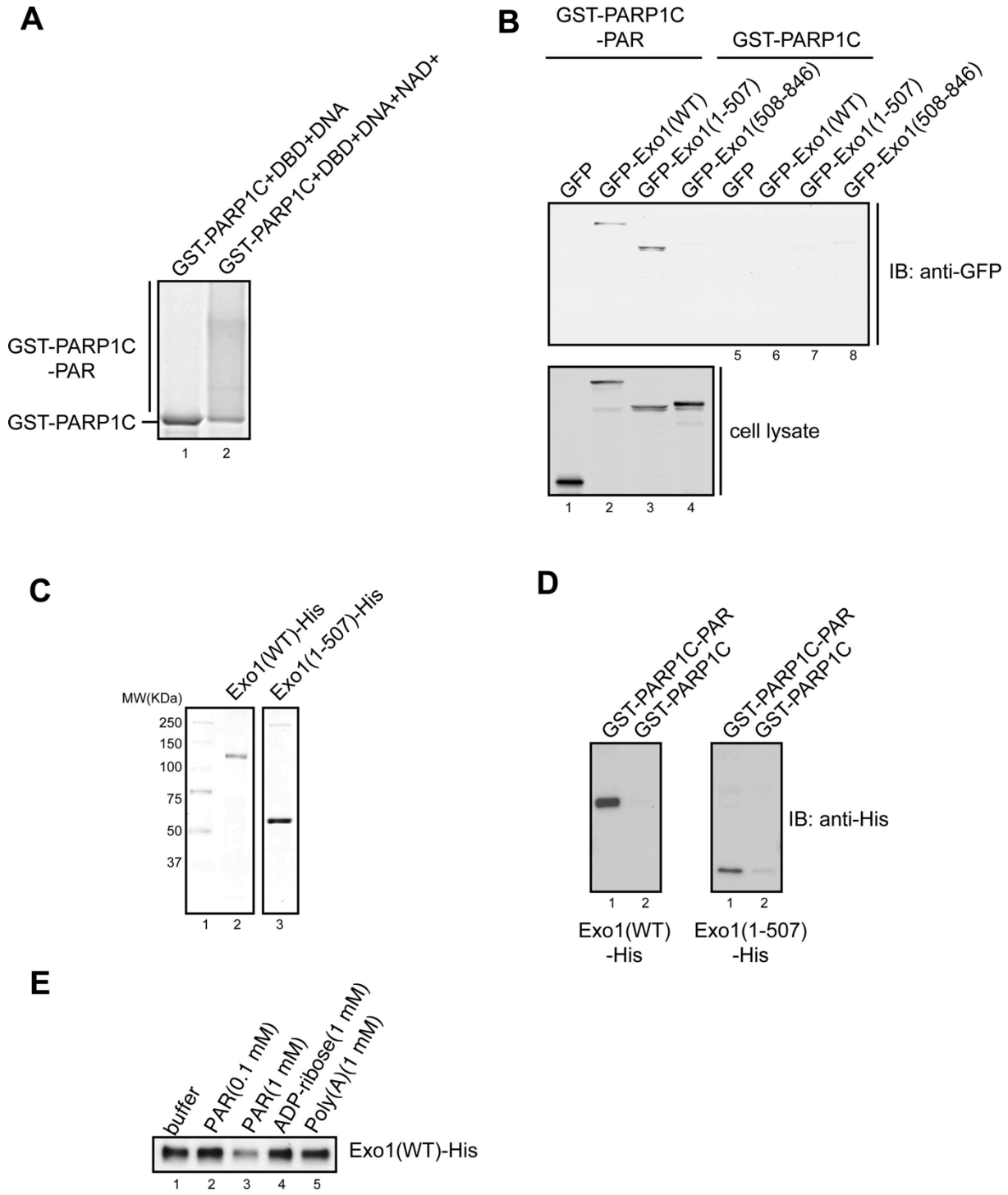


Figure 3.2. Exo1 directly interacts with PAR via its N-terminal domain.

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-immobilizied 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.

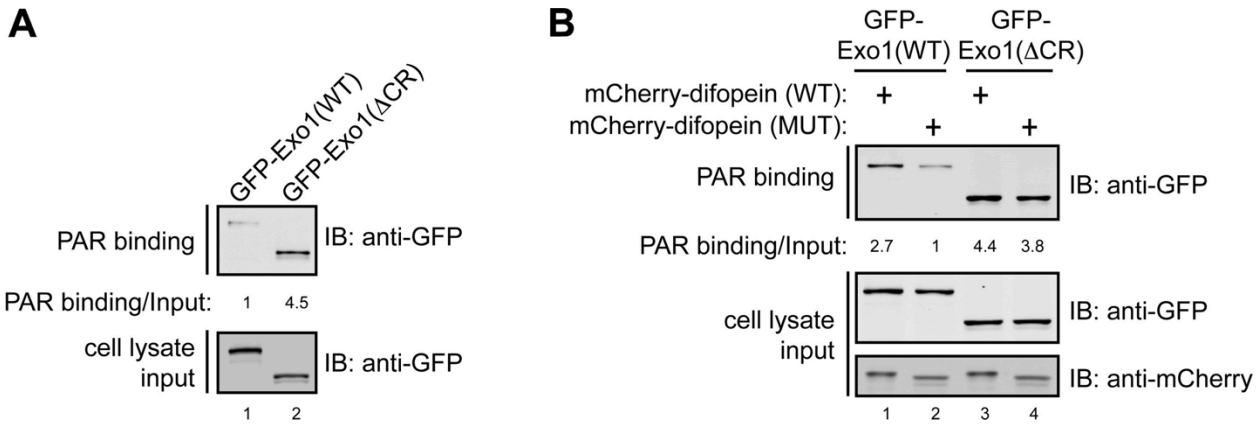


Figure 3.3. The PAR-binding activity of Exo1 is antagonized by 14-3-3 proteins that interact with the central domain of Exo1.

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

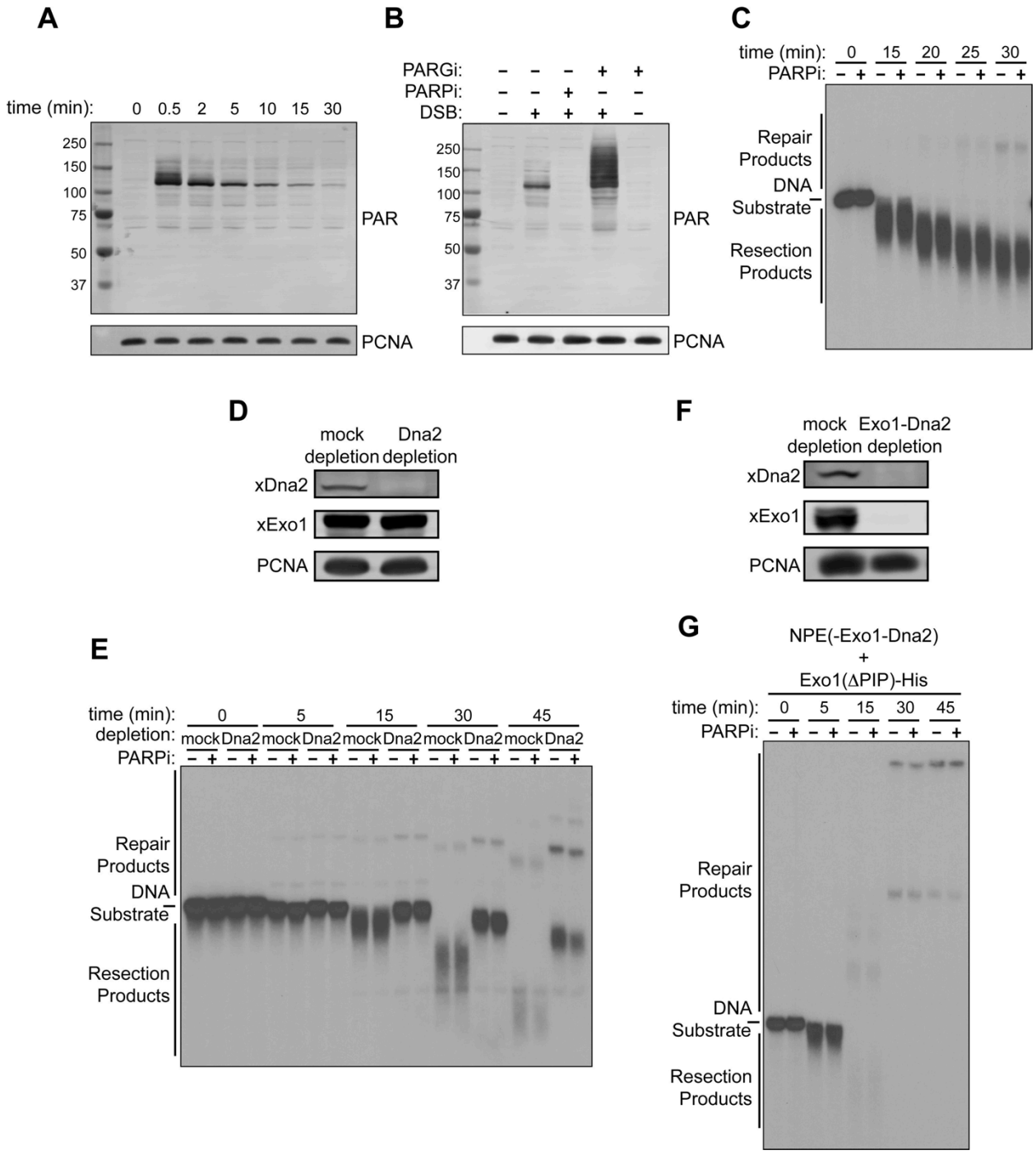


Figure 3.4. PARylation Is dispensable for DNA end resection in the *Xenopus* egg extract.

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.

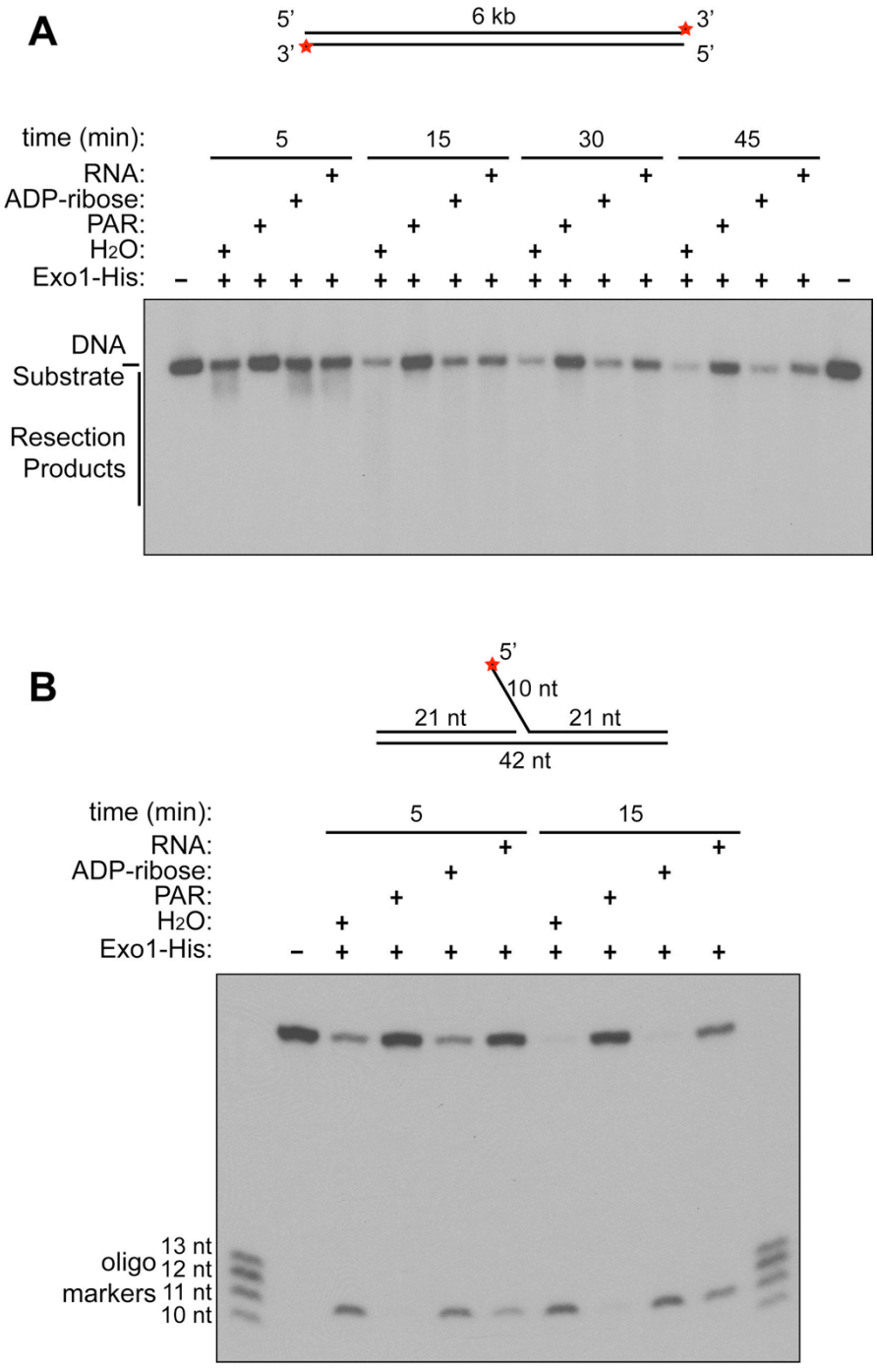


Figure 3.5. PAR inhibits the nuclease activities of Exo1 *in vitro.*

A). Effects of PAR, ADP-ribose and polyA RNA on the exonuclease activity of purified Exo1.

B). Effects of PAR, ADP-ribose and polyA RNA on the 5' flap endonuclease activity of purified Exo1.

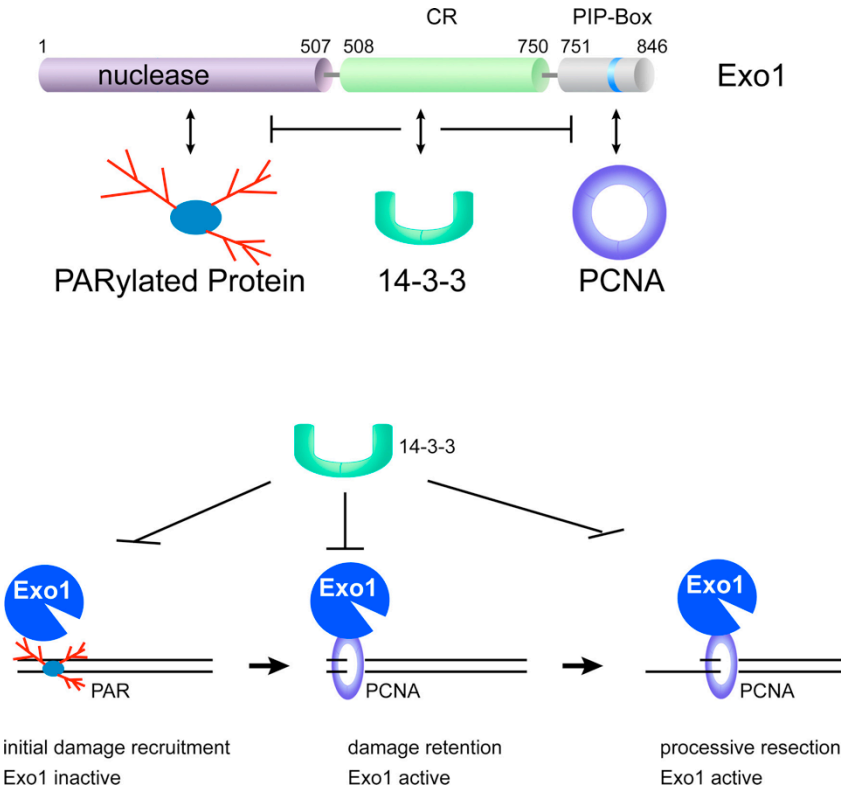


Figure 3.6. A model for the regulation of Exo1 in DNA end resection by PAR, 14-3-3s and PCNA.

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.

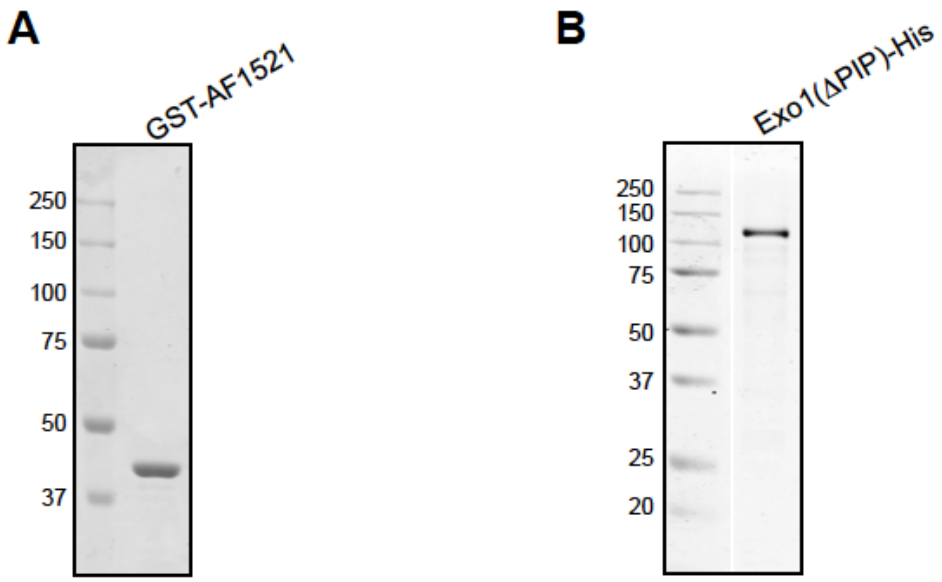


Figure S3.1. Recombinant proteins.

A). Recombinant GST-AF1521 expressed in bacteria.

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

3.6 References

1. Tran, P.T., N. Erdeniz, L.S. Symington, and R.M. Liskay, EXO1-A multi-tasking eukaryotic nuclease. DNA Repair 2004. 3(12): 1549-1559.

2. Karras, G.I., M. Fumasoni, G. Sienski, F. Vanoli, D. Branzei, and S. Jentsch, Noncanonical role of the 9-1-1 clamp in the error-free DNA damage tolerance pathway. Mol Cell 2013. 49(3): 536-546.

3. Goellner, E.M., C.D. Putnam, and R.D. Kolodner, Exonuclease 1-dependent and independent mismatch repair. DNA Repair 2015.

4. Lee, B.I. and D.M. Wilson, 3rd, The RAD2 domain of human exonuclease 1 exhibits 5' to 3' exonuclease and flap structure-specific endonuclease activities. J Biol Chem 1999. 274(53): 37763-37769.

5. Modrich, P., Mechanisms in eukaryotic mismatch repair. J Biol Chem 2006. 281(41): 30305-30309.

6. Shao, H., C. Baitinger, E.J. Soderblom, V. Burdett, and P. Modrich, Hydrolytic function of Exo1 in mammalian mismatch repair. Nucleic Acids Res 2014. 42(11): 7104-7112.

7. Symington, L.S. and J. Gautier, Double-strand break end resection and repair pathway choice. Annu Rev Genet 2011. 45: 247-271.

8. You, Z. and J.M. Bailis, DNA damage and decisions: CtIP coordinates DNA repair and cell cycle checkpoints. Trends Cell Biol 2010. 20(7): 402-409.

9. Chung, W.H., Z. Zhu, A. Papusha, A. Malkova, and G. Ira, Defective resection at DNA double-strand breaks leads to de novo telomere formation and enhances gene targeting. PLoS Genet 2010. 6(5): e1000948.

10. Mimitou, E.P. and L.S. Symington, DNA end resection: many nucleases make light work. DNA Repair 2009. 8(9): 983-995.

11. Daley, J.M., H. Niu, A.S. Miller, and P. Sung, Biochemical mechanism of DSB end resection and its regulation. DNA Repair 2015. 32: 66-74.

12. Chapman, J.R., M.R. Taylor, and S.J. Boulton, Playing the end game: DNA double-strand break repair pathway choice. Mol Cell 2012. 47(4): 497-510.

13. Qiu, J., Y. Qian, V. Chen, M.X. Guan, and B. Shen, Human exonuclease 1 functionally complements its yeast homologues in DNA recombination, RNA primer removal, and mutation avoidance. J Biol Chem 1999. 274(25): 17893-17900.

14. Nicolette, M.L., K. Lee, Z. Guo, M. Rani, J.M. Chow, S.E. Lee, and T.T. Paull, Mre11-Rad50-Xrs2 and Sae2 promote 5' strand resection of DNA double-strand breaks. Nat Struct Mol Biol 2010. 17(12): 1478-1485.

15. Shim, E.Y., W.H. Chung, M.L. Nicolette, Y. Zhang, M. Davis, Z. Zhu, T.T. Paull, G. Ira, and S.E. Lee, Saccharomyces cerevisiae Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks. EMBO J 2010. 29(19): 3370-3380.

16. Langerak, P., E. Mejia-Ramirez, O. Limbo, and P. Russell, Release of Ku and MRN from DNA ends by Mre11 nuclease activity and Ctp1 is required for homologous recombination repair of double-strand breaks. PLoS Genet 2011. 7(9): e1002271.

17. Nimonkar, A.V., J. Genschel, E. Kinoshita, P. Polaczek, J.L. Campbell, C. Wyman, P. Modrich, and S.C. Kowalczykowski, BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA break repair. Genes Dev 2011. 25(4): 350-362.

18. Sun, J., K.J. Lee, A.J. Davis, and D.J. Chen, Human Ku70/80 protein blocks exonuclease 1-mediated DNA resection in the presence of human Mre11 or Mre11/Rad50 protein complex. J Biol Chem 2012. 287(7): 4936-4945.

19. Nimonkar, A.V., A.Z. Ozsoy, J. Genschel, P. Modrich, and S.C. Kowalczykowski, Human exonuclease 1 and BLM helicase interact to resect DNA and initiate DNA repair. Proc Natl Acad Sci U S A, 2008. 105(44): 16906-16911.

20. Mimitou, E.P. and L.S. Symington, Ku prevents Exo1 and Sgs1-dependent resection of DNA ends in the absence of a functional MRX complex or Sae2. EMBO J, 2010. 29(19): 3358-3369.

21. Eid, W., M. Steger, M. El-Shemerly, L.P. Ferretti, J. Pena-Diaz, C. Konig, E. Valtorta, A.A. Sartori, and S. Ferrari, DNA end resection by CtIP and exonuclease 1 prevents genomic instability. EMBO Rep 2010. 11(12): 962-968.

22. El-Shemerly, M., D. Hess, A.K. Pyakurel, S. Moselhy, and S. Ferrari, ATR-dependent pathways control hEXO1 stability in response to stalled forks. Nucleic Acids Res 2008. 36(2): 511-519.

23. Bolderson, E., N. Tomimatsu, D.J. Richard, D. Boucher, R. Kumar, T.K. Pandita, S. Burma, and K.K. Khanna, Phosphorylation of Exo1 modulates homologous recombination repair of DNA double-strand breaks. Nucleic Acids Res, 2010. 38(6): 1821-1831.

24. Morin, I., H.P. Ngo, A. Greenall, M.K. Zubko, N. Morrice, and D. Lydall, Checkpoint-dependent phosphorylation of Exo1 modulates the DNA damage response. EMBO J, 2008. 27(18): 2400-2410.

25. Segurado, M. and J.F. Diffley, Separate roles for the DNA damage checkpoint protein kinases in stabilizing DNA replication forks. Genes Dev 2008. 22(13): 1816-1827.

26. Cotta-Ramusino, C., D. Fachinetti, C. Lucca, Y. Doksani, M. Lopes, J. Sogo, and M. Foiani, Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells. Mol Cell 2005. 17(1): 153-159.

27. Andersen, S.D., G. Keijzers, E. Rampakakis, K. Engels, P. Luhn, M. El-Shemerly, F.C. Nielsen, Y. Du, A. May, V.A. Bohr, S. Ferrari, M. Zannis-Hadjopoulos, H. Fu, and L.J. Rasmussen, 14-3-3 checkpoint regulatory proteins interact specifically with DNA repair protein human exonuclease 1 (hEXO1) via a semi-conserved motif. DNA Repair 2012. 11(3): 267-277.

28. Engels, K., M. Giannattasio, M. Muzi-Falconi, M. Lopes, and S. Ferrari, 14-3-3 Proteins regulate exonuclease 1-dependent processing of stalled replication forks. PLoS Genet 2011. 7(4): e1001367.

29. Yang, S.H., R. Zhou, J. Campbell, J. Chen, T. Ha, and T.T. Paull, The SOSS1 single-stranded DNA binding complex promotes DNA end resection in concert with Exo1. The EMBO J 2013. 32(1): 126-139.

30. Cannavo, E., P. Cejka, and S.C. Kowalczykowski, Relationship of DNA degradation by Saccharomyces cerevisiae Exonuclease 1 and its stimulation by RPA and Mre11-Rad50-Xrs2 to DNA end resection. Proc Natl Acad Sci U S A 2013. 110(18): E1661-1668.

31. Adkins, N.L., H. Niu, P. Sung, and C.L. Peterson, Nucleosome dynamics regulates DNA processing. Nat Struct Mol Biol 2013. 20(7): 836-842.

32. Tomimatsu, N., B. Mukherjee, M. Catherine Hardebeck, M. Ilcheva, C. Vanessa Camacho, J. Louise Harris, M. Porteus, B. Llorente, K.K. Khanna, and S. Burma, Phosphorylation of EXO1 by CDKs 1 and 2 regulates DNA end resection and repair pathway choice. Nat Commu, 2014. 5: 3561.

33. Chen, X., S.C. Paudyal, R.I. Chin, and Z. You, PCNA promotes processive DNA end resection by Exo1. Nucleic Acids Res 2013. 41(20): 9325-9338.

34. Chen, X., I.K. Kim, Y. Honaker, S.C. Paudyal, W.K. Koh, M. Sparks, S. Li, H. Piwnica-Worms, T. Ellenberger, and Z. You, 14-3-3 proteins restrain the exo1 nuclease to prevent overresection. J Biol Chem 2015. 290(19): 12300-12312.

35. Rouleau, M., A. Patel, M.J. Hendzel, S.H. Kaufmann, and G.G. Poirier, PARP inhibition: PARP1 and beyond. Nat Rev Cancer, 2010. 10(4): 293-301.

36. Gibson, B.A. and W.L. Kraus, New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs. Nature reviews. Mol Cell Biol 2012. 13(7): 411-424.

37. Li, M., L.Y. Lu, C.Y. Yang, S. Wang, and X. Yu, The FHA and BRCT domains recognize ADP-ribosylation during DNA damage response. Genes Dev 2013. 27(16): 1752-1768.

38. Ahel, D., Z. Horejsi, N. Wiechens, S.E. Polo, E. Garcia-Wilson, I. Ahel, H. Flynn, M. Skehel, S.C. West, S.P. Jackson, T. Owen-Hughes, and S.J. Boulton, Poly(ADP-ribose)-dependent regulation of DNA repair by the chromatin remodeling enzyme ALC1. Science, 2009. 325(5945): 1240-1243.

39. Li, G.Y., R.D. McCulloch, A.L. Fenton, M. Cheung, L. Meng, M. Ikura, and C.A. Koch, Structure and identification of ADP-ribose recognition motifs of APLF and role in the DNA damage response. Proc Natl Acad Sci U S A 2010. 107(20): 9129-9134.

40. Chou, D.M., B. Adamson, N.E. Dephoure, X. Tan, A.C. Nottke, K.E. Hurov, S.P. Gygi, M.P. Colaiacovo, and S.J. Elledge, A chromatin localization screen reveals poly (ADP ribose)-regulated recruitment of the repressive polycomb and NuRD complexes to sites of DNA damage. Proc Natl Acad Sci U S A 2010. 107(43): 18475-18480.

41. Ahel, I., D. Ahel, T. Matsusaka, A.J. Clark, J. Pines, S.J. Boulton, and S.C. West, Poly(ADP-ribose)-binding zinc finger motifs in DNA repair/checkpoint proteins. Nature, 2008. 451(7174): 81-85.

42. Masson, M., C. Niedergang, V. Schreiber, S. Muller, J. Menissier-de Murcia, and G. de Murcia, XRCC1 is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage. Mol Cell Biol 1998. 18(6): 3563-3571.

43. Li, M. and X. Yu, Function of BRCA1 in the DNA damage response is mediated by ADP-ribosylation. Cancer Cell, 2013. 23(5): 693-704.

44. Zhang, F., Y. Chen, M. Li, and X. Yu, The oligonucleotide/oligosaccharide-binding fold motif is a poly(ADP-ribose)-binding domain that mediates DNA damage response. Proc Natl Acad Sci U S A 2014. 111(20): 7278-7283.

45. Ciccia, A. and S.J. Elledge, The DNA damage response: making it safe to play with knives. Mol Cell, 2010. 40(2): 179-204.

46. Kim, I.K., J.R. Kiefer, C.M. Ho, R.A. Stegeman, S. Classen, J.A. Tainer, and T. Ellenberger, Structure of mammalian poly(ADP-ribose) glycohydrolase reveals a flexible tyrosine clasp as a substrate-binding element. Nat Struct Mol Biol 2012. 19(6): 653-656.

47. Pei, H., L. Zhang, K. Luo, Y. Qin, M. Chesi, F. Fei, P.L. Bergsagel, L. Wang, Z. You, and Z. Lou, MMSET regulates histone H4K20 methylation and 53BP1 accumulation at DNA damage sites. Nature, 2011. 470(7332): 124-128.

48. Walter, J., L. Sun, and J. Newport, Regulated chromosomal DNA replication in the absence of a nucleus. Mol Cell, 1998. 1(4): 519-529.

49. Langelier, M.F., K.M. Servent, E.E. Rogers, and J.M. Pascal, A third zinc-binding domain of human poly(ADP-ribose) polymerase-1 coordinates DNA-dependent enzyme activation. J Biol Chem 2008. 283(7): 4105-4114.

50. Kim, I.K., R.A. Stegeman, C.A. Brosey, and T. Ellenberger, A quantitative assay reveals ligand specificity of the DNA scaffold repair protein XRCC1 and efficient disassembly of complexes of XRCC1 and the poly(ADP-ribose) polymerase 1 by poly(ADP-ribose) glycohydrolase. J Biol Chem 2015. 290(6): 3775-3783.

51. Tan, E.S., K.A. Krukenberg, and T.J. Mitchison, Large-scale preparation and characterization of poly(ADP-ribose) and defined length polymers. Analyt Biochem 2012. 428(2): 126-136.

52. Liao, S., T. Toczylowski, and H. Yan, Identification of the Xenopus DNA2 protein as a major nuclease for the 5'->3' strand-specific processing of DNA ends. Nucleic Acids Res, 2008. 36(19): 6091-6100.

53. Karras, G.I., G. Kustatscher, H.R. Buhecha, M.D. Allen, C. Pugieux, F. Sait, M. Bycroft, and A.G. Ladurner, The macro domain is an ADP-ribose binding module. EMBO J 2005. 24(11): 1911-1920.

54. Jungmichel, S., F. Rosenthal, M. Altmeyer, J. Lukas, M.O. Hottiger, and M.L. Nielsen, Proteome-wide identification of poly(ADP-Ribosyl)ation targets in different genotoxic stress responses. Mol Cell, 2013. 52(2): 272-285.

55. Keijzers, G., V.A. Bohr, and L.J. Rasmussen, Human exonuclease 1 (EXO1) activity characterization and its function on flap structures. Biosci Rep 2015. 35(3).

56. D'Amours, D., S. Desnoyers, I. D'Silva, and G.G. Poirier, Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem J 1999. 342 (Pt 2): 249-268.

57. Beck, C., I. Robert, B. Reina-San-Martin, V. Schreiber, and F. Dantzer, Poly(ADP-ribose) polymerases in double-strand break repair: focus on PARP1, PARP2 and PARP3. Exp Cell Res 2014. 329(1): 18-25.

58. Lord, C.J., A.N. Tutt, and A. Ashworth, Synthetic lethality and cancer therapy: lessons learned from the development of PARP inhibitors. Annu Rev Med 2015. 66: 455-470.

59. Rouleau, M., R.A. Aubin, and G.G. Poirier, Poly(ADP-ribosyl)ated chromatin domains: access granted. J Cell Sci 2004. 117(Pt 6): 815-825.

60. Cheng, Q., N. Barboule, P. Frit, D. Gomez, O. Bombarde, B. Couderc, G.S. Ren, B. Salles, and P. Calsou, Ku counteracts mobilization of PARP1 and MRN in chromatin damaged with DNA double-strand breaks. Nucleic Acids Res 2011. 39(22): 9605-9619.

61. Wang, M., W. Wu, B. Rosidi, L. Zhang, H. Wang, and G. Iliakis, PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. Nucleic Acids Res 2006. 34(21): 6170-6182.

62. Gagne, J.P., M. Isabelle, K.S. Lo, S. Bourassa, M.J. Hendzel, V.L. Dawson, T.M. Dawson, and G.G. Poirier, Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes. Nucleic Acids Res 2008. 36(22): 6959-6976.

63. Bryant, H.E., E. Petermann, N. Schultz, A.S. Jemth, O. Loseva, N. Issaeva, F. Johansson, S. Fernandez, P. McGlynn, and T. Helleday, PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. EMBO J 2009. 28(17): 2601-2615.

64. Ying, S., F.C. Hamdy, and T. Helleday, Mre11-dependent degradation of stalled DNA replication forks is prevented by BRCA2 and PARP1. Cancer Res 2012. 72(11): 2814-2821.

65. Yang, Y.G., U. Cortes, S. Patnaik, M. Jasin, and Z.Q. Wang, Ablation of PARP-1 does not interfere with the repair of DNA double-strand breaks, but compromises the reactivation of stalled replication forks. Oncogene, 2004. 23(21): 3872-3882.

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 endocleavge 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

1. Cannavo E, Cejka P. Sae2 promotes dsDNA endonuclease activity within Mre11-Rad50-Xrs2 to resect DNA breaks. Nature. 2014;514(7520):122–5.

2. Anand R, Ranjha L, Cannavo E, Cejka P. Phosphorylated CtIP Functions as a Co-factor of the MRE11-RAD50-NBS1 Endonuclease in DNA End Resection. Mol Cell. 2016;64(5):940–50.

3. Mimitou EP, Symington LS. Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. Nature. 2008;455(7214):770–4.

4. Zhu Z, Chung WH, Shim EY, Lee SE, Ira G. Sgs1 Helicase and Two Nucleases Dna2 and Exo1 Resect DNA Double-Strand Break Ends. Cell. 2008;134(6):981–94.

5. Costelloe T, Louge R, Tomimatsu N, Mukherjee B, Martini E, Khadaroo B, et al. The yeast Fun30 and human SMARCAD1 chromatin remodellers promote DNA end resection. Nature. 2012;489(7417):581–4.

6. Dong S, Han J, Chen H, Liu T, Huen MSYSY, Yang Y, et al. The Human SRCAP Chromatin Remodeling Complex Promotes DNA-End Resection. Curr Biol. 2014;24(18):2097–110.

7. Zhou C, Pourmal S, Pavletich NP. Dna2 nuclease-helicase structure, mechanism and regulation by Rpa. Elife. 2015;4:1–19.

8. Garcia V, Phelps SEL, Gray S, Neale MJ. Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1. Nature. 2011;479(7372):241–4.

9. Makharashvili N, Tubbs AT, Yang SH, Wang H, Barton O, Zhou Y, et al. Catalytic and Noncatalytic Roles of the CtIP Endonuclease in Double-Strand Break End Resection. Mol Cell. 2014;54(6):1022–33.

10. Wang H, Li Y, Truong LN, Shi LZ, Hwang PY-H, He J, et al. CtIP Maintains Stability at Common Fragile Sites and Inverted Repeats by End Resection-Independent Endonuclease Activity. Mol Cell. 2014;54(6):1012–21.

11. Lengsfeld BM, Rattray AJ, Bhaskara V, Ghirlando R, Paull TT. Sae2 Is an Endonuclease that Processes Hairpin DNA Cooperatively with the Mre11/Rad50/Xrs2 Complex. Mol Cell. 2007;28:638–51.

12. Neale MJ, Pan J, Keeney S. Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. Nature. 2005;436(7053):1053–7.

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

activity. EMBO J. 2008;27(14):1953–62.

14. Karanja K, Lee EH, Hendrickson E, Campbell JL. Preventing over-resection by DNA2 helicase/nuclease suppresses repair defects in Fanconi anemia cells. Cell Cycle. 2014;13(10):1540–50.

15. Zhang J-M, Liu X-M, Ding Y-H, Xiong L-Y, Ren J-Y, Zhou Z-X, et al. Fission yeast Pxd1 promotes proper DNA repair by activating Rad16XPF and inhibiting Dna2. PLoS Biol. 2014;12(9):e1001946.