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The Mechanism of DNA Double-Strand Break (DSB) Resection in Human Cells

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The Mechanism of DNA Double-Strand Break (DSB) Resection in Human Cells

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2013

Dedication

I dedicate this dissertation to my wonderful family.

Acknowledgements

First, I would like to express my sincere gratitude to Dr. Tanya T. Paull for giving me the opportunity to work in her lab. Her guidance and persistant help has made my graduate career a thoughtful and rewarding journey. She has been a true mentor and has inspired me as a scientist throughout my whole graduate school years.

I would also like to thank my dissertation committee members: Dr. Alan M. Lambowitz, Dr. Rasika M. Harshey, Dr. Jon M. Huibregtse, and Dr. Lauren J. Webb for providing me with their valuable comments and time over the years.

I also thank the past and present members of the Paull lab for their insightful comments about my work and for providing a joyful atmosphere to work in the lab.

Finally, I would like to thank my father, Chunwoo Yang for his prayers and encouragement, my mother, Younghyo Yun for her endless prayers and love for me, and my brother, David S. Yang who has been the best brother in the world. Also I would like to thank my father in-law, Kyeongjin Min, my mother in-law, Jung Ae Jung, and my sister in-law Song Ran Min for their encouragement and support. And the person that I would like to thank most is my wonderful husband Jae Hong Min who has been providing me with endless love, support, and happiness. Most of all, I would like to thank God for his immense love and guidance.

The Mechanism of DNA Double-Strand Break (DSB) Resection in Human Cells

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Homologous recombination (HR) repair is critical for the maintenance of genomic stability, as it is involved in the precise repair of DNA double-strand breaks (DSBs) using an intact homologous template for repair. The initiation of 5' strand resection of DNA ends is a critical determinant in this process, which commits cells to HR repair and prevents repair by non-homologous end joining (NHEJ). The human single-stranded DNA (ssDNA) binding complexes, RPA and SOSS1, are involved in regulating DSB signaling and HR repair. In this study, I demonstrate a novel function of SOSS1 in HR repair, in which SOSS1 stimulates hExo1-dependent resection. Despite its poor activity in binding duplex DNA, SOSS1 facilitates hExo1 recruitment to duplex DNA ends and promotes its activity in resection independently of MRN in vitro. MRN(X) is a highly conserved complex that is involved in the early steps of HR repair by regulating DSB resection. MRN interacts with CtIP and constitutes resection machinery that can perform limiting processing on DNA ends. In this study, I also examine the biochemical activities of MRN and CtIP in DSB resection through reconstituted in vitro assays. I show that the

ATP-dependent DNA unwinding activity of MRN is responsible for overcoming Ku inhibition of hExo1- and Dna2/BLM-dependent resection activity in vitro. I propose that this unwinding step displaces Ku away from the DNA ends and facilitates the recruitment of hExo1 to the DNA ends for efficient resection. In addition, I show that CtIP can promote overcoming the inhibitory effect of Ku in resection together with MRN. Further, I demonstrate that MRN nuclease activity is required for efficient processing of covalent adducts from DNA ends in vitro, suggesting that the nucleolytic removal of covalent adducts by MRN generates free ends for hExo1- and Dna2/BLM binding. Overall, this study provides mechanistic insight into the regulation of DSB resection in human cells.

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CHAPTER 1: INTRODUCTION

DNA DOUBLE-STRAND BREAKS AND REPAIR

Genomes are under constant attack from both exogenous and endogenous sources. Exogenous factors such as ultraviolet (UV) light can damage DNA by generating thymine dimers, which are cross-links between pyrimidine bases (Douki et al, 2003). Endogenous factors including nucleases and metabolic products such as reactive oxygen species can also induce chemical modification of DNA, generating modified bases and sugars, DNA-protein adducts, base-free sites, and tandem lesions. DNA double-strand breaks (DSBs) are one of the most deleterious forms of damage in cells. DSBs can arise from direct exposure to ionizing radiation (IR) or indirectly through chemical modifications of DNA that cause replication fork stalling and collapse in dividing cells. DSBs can also arise during programmed cellular events such as mitotic and meiotic recombination, DNA replication, and V(D)J recombination (Haber, 1999; Jeggo, 1998; Olive, 1998; Pastink & Lohman, 1999; Wallace, 1998). These DSBs require precise repair for genome stability since alteration or loss of genetic sequences from DNA DSBs can not only induce apoptosis but also promote carcinogenesis with the activation of oncogenes or inactivation of tumor suppressor genes (Barteck & Lukas, 2007; Bartkova et al, 2005; Friedberg, 2003; Hoeijmakers, 2001; Rich et al, 2000).

Eukaryotic cells possess two primary mechanisms to repair DNA DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR) (Figure 1.1) (Symington & Gautier, 2011). In vertebrates, NHEJ is the major pathway for DSB repair which occurs in all phase of the cell cycle and repairs DSBs by direct joining of the broken DNA ends, often accompanied by deletions or insertions at the break site. The NHEJ pathway is considered to be error prone due to the fact that it stimulates DSB repair without using specific sequence homology. In contrast, HR repair occurs primarily in the S and G_2 phase of the cell cycle and is involved in the precise repair of DSBs by using the intact sister chromatid as a template for repair (Bartek et al, 2004; Daley et al, 2005; Lieber et al, 2003; Sonoda et al, 2006; Sung & Klein, 2006; West, 2003).

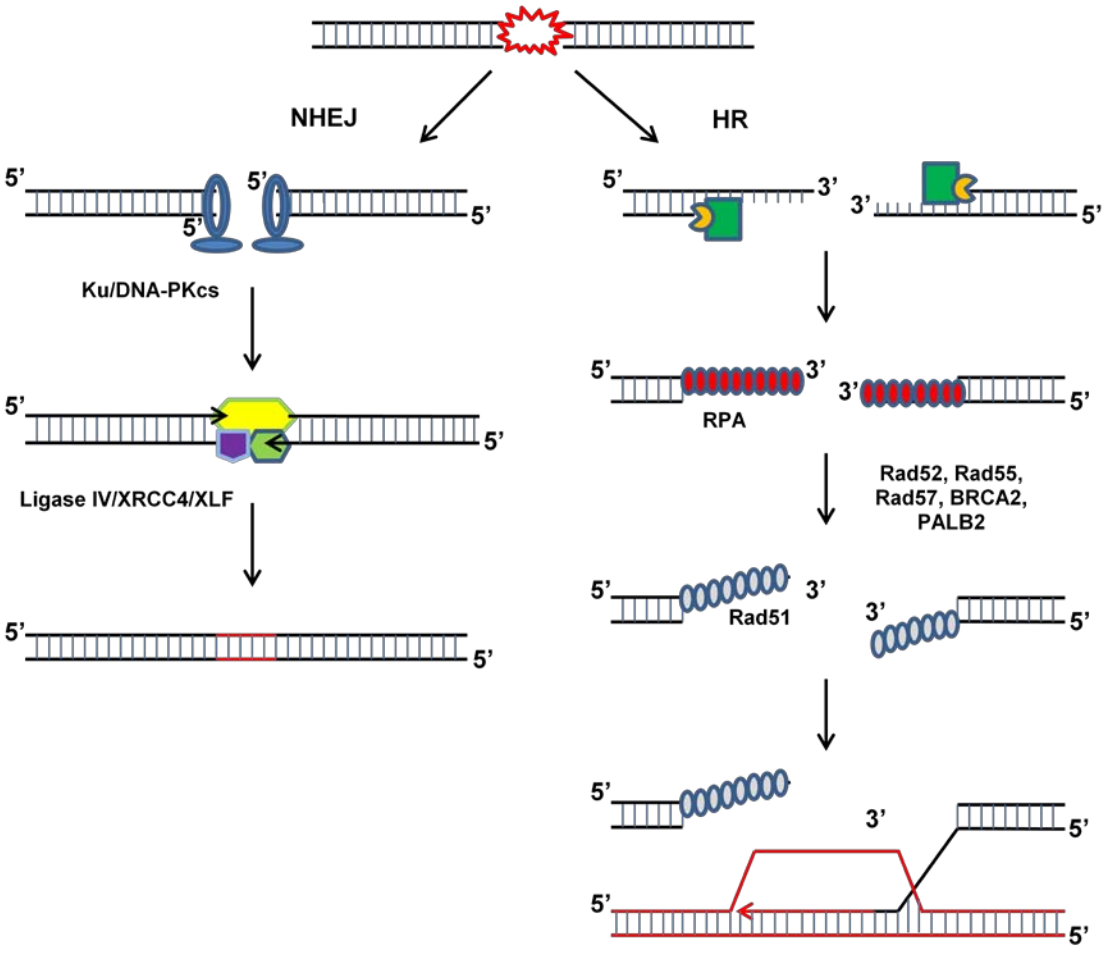

Figure 1.1: Schematic Model of DNA DSB repair by NHEJ and HR in human cells.

HOMOLOGOUS RECOMBINATION (HR) REPAIR

Homologous recombination (HR) repair is critical to maintain genome stability due to its error free repair mechanism that utilizes an intact homologous template. Homologous recombination is initiated by processing of the 5' strand at DSB ends to produce a 3' single-stranded DNA (ssDNA) (Figure 1.1). The resulting 3' ssDNA tail is rapidly bound by replication protein A (RPA), which promotes the activation of DNA damage signaling kinase ATR. The RPA-ssDNA complex is subsequently displaced by Rad51, a key recombinase enzyme, with the help of mediator proteins such as BRCA2, Rad52, Rad54, Rad55, and Rad57 to form a nucleoprotein filament which allows strand invasion and search for homologous intact sequences. Subsequently, DNA strand exchange occurs and generates a joint molecule between damaged and undamaged duplex DNAs. Sequence information that is lost at the damaged DSB site is recovered by DNA synthesis using the intact homologous strand as a template. The stand invasion intermediates are then processed by branch migration, Holliday junction resolution, and DNA ligation (van den Bosch et al, 2002).

Among the several steps that are present during HR repair, the initial processing of DSB ends to produce the 3' ssDNA tail is essential for cells to commit to HR as the processed ends can no longer be utilized by NHEJ (Symington & Gautier, 2011). Despite the importance of the generation of the 3' ssDNA tail in HR, however, the biochemical mechanism and regulation of this process remains poorly understood in human cells.

THE MECHANISM OF DNA DSB RESECTION IN PROKARYOTES

The molecular mechanisms of 5’ strand processing are well understood in bacteria where the RecBCD pathway and the RecQ/RecJ pathway exist to recognize DSBs and process the 5’ strand of DSBs to generate 3’ ssDNA for subsequent HR repair (Figure 1.2) (Amundsen & Smith, 2003; Dillingham & Kowalczykowski, 2008). The RecBCD pathway exists as the main pathway to recognize DSB ends and process the ends through both its exonuclease and helicase activities. As the RecBCD complex binds to DNA ends, it preferentially unwinds and resects the 3’ end of the DNA until it encounters a specific DNA sequence *Chi* (*X*) that it binds to with higher affinity than random sequence. Once the RecBCD complex encounters the *Chi* (*X*) site, the vigorous 3’ to 5’ exonuclease activity is attenuated whereas the 5’ to 3’ exonuclease is upregulated, resulting in the production of a 3’ ssDNA tail terminating at the *Chi* (*X*) sequence (Amundsen et al, 2000; Bianco et al, 1998). The resulting 3’ ssDNA is coated by the ssDNA binding protein SSB, which is subsequently replaced by the recombinase protein RecA for strand exchange to complete HR.

The RecQ/RecJ pathway exists redundantly with the RecBCD pathway to perform DSB resection when the RecBCD pathway is nonfunctional (Spies & Kowalczykowski, 2005). The RecQ bipolar helicase unwinds the duplex DNA at DSB ends to generate a splayed structure with both 5’ and 3’ ssDNA regions. The RecJ exonuclease digests the 5’ end of the ssDNA region to produce a 3’ ssDNA tail that recruits SSB. Unlike the

RecBCD pathway, the mediator RecFOR complex acts in the RecQ/RecJ pathway to stimulate nucleation and extension of RecA onto the SSB-coated ssDNA to initiate strand invasion and continue HR repair (Handa et al, 2009).

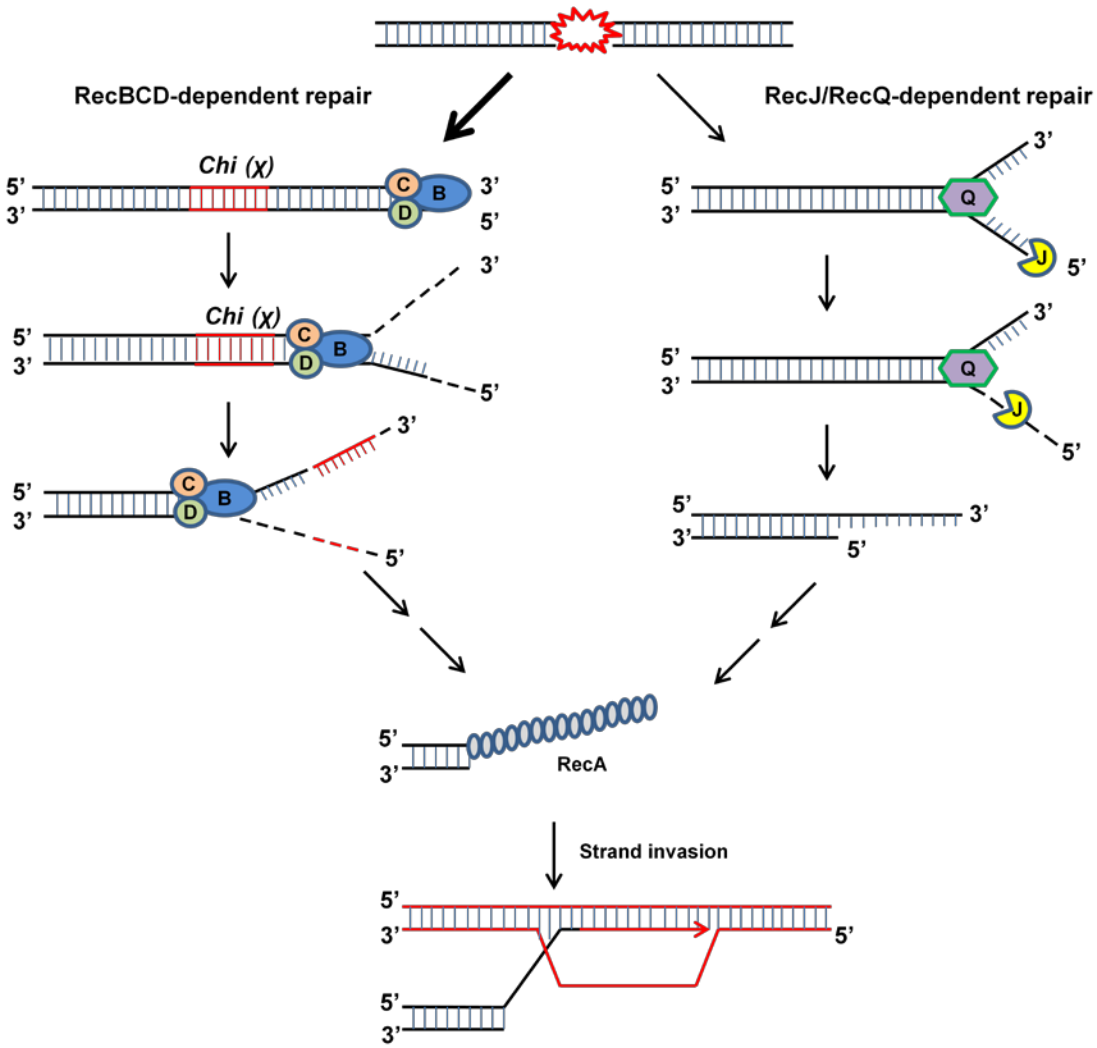

Figure 1.2: Model of the RecBCD pathway and RecQ/RecJ pathway in regulating DSB 5' strand resection in bacteria.

THE MRE11/RAD50/NBS1 COMPLEX

Despite the dominant function of the RecBCD complex in processing the 5' strand of DNA at DSBs in prokaryotes, no functional homolog of this complex has been found in any other kingdoms. Genetic studies from eukaryotic cells have shown the Mre11/Rad50/Nbs1 (Xrs2 in *S. cerevisiae*) complex to be involved in DNA DSB sensing, processing, and repair. Mre11 is a member of the lambda phosphatase family of phosphoesterases which exhibits manganese-dependent 3' to 5' exonuclease activity on double-stranded DNA (dsDNA) and endonuclease activity on single-strand and double-strand junctions and on hairpin loops in vitro (Paull & Gellert, 1998; Trujillo & Sung, 2001; Trujillo et al, 1998). Recombinant Mre11 also exhibits weak endonuclease activity on the 5' strand of linear DNA ends in the presence of magnesium (Hopkins & Paull, 2008; Nicolette et al, 2010). Mre11 binds to Rad50, which is a member of the structural maintenance of chromosome (SMC) proteins containing ATPase and adenylate kinase activities and together with Mre11, it is responsible for holding DNA ends in close proximity (Chen et al, 2001a; de Jager et al, 2001; Hirano & Hirano, 2002). In addition, Rad50 has been shown to be involved in ATP-dependent DNA unwinding activity of the complex (Cannon et al, 2013; Lee & Paull, 2005; Paull & Gellert, 1999). In eukaryotic cells, Nbs1/Nibrin (or Xrs2 in *S. cerevisiae*) associates with Mre11 and Rad50 and is responsible for the localization of the MRN(X) complex to DNA DSB sites, signal transduction of DNA damage through ATM (Tel1/Mec1 in *S. cerevisiae*), and regulation of the DNA unwinding and nuclease activities of the MRN(X) complex (Dong et al,

1999; Kobayashi et al, 2002; Lee & Paull, 2005; Mirzoeva & Petrini, 2001; Tauchi et al, 2002).

THE ROLE OF MRN(X) IN DNA DSB RESECTION

In *S. cerevisiae*, cells with mutations in *MRE11*, *RAD50*, and *XRS2* exhibit poor vegetative growth, increased sensitivity to ionizing radiation (IR), deficiency in meiotic recombination, and defects in telomere maintenance (D'Amours & Jackson, 2002; Symington, 2002). In addition, the *mrx* null mutants exhibit a delay in DSB resection in vegetative cells (Ivanov et al, 1994), suggesting a role of MRX in DSB repair by regulating resection. Indeed, in vivo studies in *S. cerevisiae* show that MRX functions in the early steps of resection with the Sae2 endonuclease to initiate short-range processing of 5' strands at DSBs (Gravel et al, 2008; Mimitou & Symington, 2008; Zhu et al, 2008). Previous studies have reported the initiation of 5' resection to occur at a slower rate compared to the rate of resection measured for processive resection occurring far from the initial break site in yeast cells (Frank-Vaillant & Marcand, 2002; Zierhut & Diffley, 2008). Overall, these reports propose the action of MRX and Sae2 to be the rate-limiting step in DSB resection by regulating the initiation of resection in cells.

Genetic studies from higher eukaryotes have shown the MRN(X) complex to be essential in HR-dependent repair, where deletion of Mre11, Rad50, and Nbs1 genes in

mouse and DT40 chicken cell lines lead to cell death by defective HR (Nakahara et al, 2009; Tauchi et al, 2002; Xiao & Weaver, 1997; Yamaguchi-Iwai et al, 1999). In addition, the role of the MRN complex in DNA repair in human cells was emphasized with the finding that mutations in these genes cause sensitivity to IR, DSB repair defects, and impaired cell cycle. Also these mutations cause cancer-prone syndromes in humans. For example, hypomorphic mutations in *MRE11* and *NBS1* were shown to be associated with cancer-prone diseases Ataxia-Telangiectasia-Like-Disorder (ATLD) and Nijmegen Breakage Syndrome (NBS), respectively (Carney et al, 1998; Matsuura et al, 1998; Stewart et al, 1999; Tauchi et al, 2002; Taylor et al, 2004; Varon et al, 1998).

In vitro studies with the archaeal MR complex and the eukaryotic MRN(X) complex have demonstrated the direct involvement of the Mre11/Rad50 proteins in resection, where the Mre11/Rad50 proteins promote short-range resection of 5' strands at DNA ends through endonucleolytic activity and also stimulate extensive resection through the recruitment of 5' to 3' exonucleases and helicases (Cejka et al, 2010a; Hopkins & Paull, 2008; Nimonkar et al, 2011; Niu et al, 2010). This evidence not only highlights the significance of the Mre11/Rad50 complex in DSB resection but also the involvement of redundant 5' to 3' exonucleases that act to generate 3' ssDNA tails.

CTIP AND ITS ROLE IN DNA REPAIR

CtIP was initially identified as an interactor for CtBP (C-terminal region of adenovirus E1A) and the tumor suppressor proteins RB and BRCA1(Fusco et al, 1998; Schaeper et al, 1998; Wong et al, 1998; Yu et al, 1998). CtIP is considered to be the functional ortholog of Sae2 in DNA repair, possessing a short sequence homology in the C-terminus of the protein (Sartori et al, 2007). CtIP has been shown to recruit to DSB sites after DNA damage and regulate DSB resection activity in human cells (Sartori et al, 2007). As a result, CtIP regulates Chk1 phosphorylation by the ATR kinase in response to DSB-inducing agents during the S and G_2 phases of the cell cycle (Chen et al, 2008; Huertas & Jackson, 2009; Sartori et al, 2007; You et al, 2009; Yu & Chen, 2004; Yuan & Chen, 2009). Studies of CtIP othologs in *S. pombe* (Ctp1), *C. elegans* (COM-1), *A. thaliana* (AtGR1), Xenopus, and chicken have substantiated the role of these proteins in DSB resection, as depletion of these CtIP orthologs result in a defect in DSB resection in cells (Akamatsu et al, 2008; Limbo et al, 2007; Penkner et al, 2007; Uanschou et al, 2007; You et al, 2009; Yun & Hiom, 2009).

CtIP has been shown to interact both physically and functionally with MRN to regulate DSB resection and promote HR-dependent repair (Chen et al, 2008; Lloyd et al, 2009; Sartori et al, 2007; Williams et al, 2009). In addition, the function of CtIP in DSB resection has been shown to be regulated by CDK phosphorylation in human cells and chicken DT40 cells, restricting its repair activities to the S/G_2 phases of the cell cycle

(Huertas & Jackson, 2009; Yun & Hiom, 2009). In yeast, MRX and Sae2 have been shown to stimulate the initiation of short-range resection and the recruitment of exonucleases to further promote the extent of resection at DSBs (Gravel et al, 2008; Mimitou & Symington, 2008; Zhu et al, 2008). However, the precise mechanism of how CtIP and MRN cooperate in DSB resection is unclear.

Sae2(Ctp1) and MRX(N) play a critical role in processing DSBs during meiosis, where the type II topoisomerase-like protein Spo11 forms a covalent 5'-phosphodiester bond with the nascent 5' DNA end with its catalytic tyrosine residue (Alani et al, 1990; Keeney et al, 1997; Keeney & Kleckner, 1995; Moreau et al, 1999; Nairz & Klein, 1997; Prinz et al, 1997; Usui et al, 1998). Notably, CtIP is also essential for repairing DSBs generated by IR and chemotherapeutic drugs such as topoisomerase inhibitors (Sartori et al, 2007), which generate DSBs with modified bases and covalent protein adducts (Barker et al, 2005; Henner et al, 1983; Lawley & Phillips, 1996). This suggests that CtIP may function in the removal of protein adducts from DSB ends together with MRN, generating free DNA ends for extensive resection by nucleases.

EXONUCLEASE 1 (EXO1) AND DNA REPAIR

Exonuclease 1 (Exo1) is a member of the Rad2 family of nucleases and possesses 5' to 3' exonuclease activity and 5' overhang flap endonuclease activity (Lee et al, 2002;

Qiu et al, 1999). Exo1 is essential for genomic stability maintenance by processing DNA intermediates involved in repair and recombination through its nucleolytic activity. Exo1 is implicated in several DNA repair pathways including mismatch repair (MMR), post-replication repair, meiotic and mitotic recombination (Fiorentini et al, 1997; Kirkpatrick et al, 2000; Tsubouchi & Ogawa, 2000). In addition, Exo1 has been shown to be involved in telomere maintenance by promoting the formation of ssDNA and activation of ATR in response to telomere dysfunction as well as recombination at transcription-induced telomeric structures (Schaetzlein, 2007; Vallur & Maizels, 2010b). Consistent with the role of Exo1 in DNA repair, mutation of Exo1 generates genomic instability in cells, which can lead to cancer development in mammalian organisms. For example, Exo1-deficient mice show an increase in lymphoma development (Wei et al, 2003) and germ-line mutations in humans have been linked to non-polypopsis colon cancer and other forms of colorectal cancer in humans related to mismatch repair (Sun et al, 2002; Wu et al, 2001).

Recent studies have highlighted the function of Exo1 in regulating HR repair. Depletion of Exo1 in human cells generates increased sensitivity to IR and defects in HR-dependent DSB repair, which lead to genome instability (Bolderson et al, 2009; Hartlerode & Scully, 2009). In addition, human cells lacking Exo1 display a defect in the recruitment of RPA and Rad51 to DSB sites (Bolderson et al, 2009), confirming that Exo1 acts in HR repair by generating ssDNA at DSB ends.

In *S. cerevisiae*, Exo1 functions in a redundant manner with the nuclease/helicase complex Dna2/Sgs1 and its co-factors Top3 and Rmi1 to promote long range resection (>1kb) at DSBs (Gravel et al, 2008; Mimitou & Symington, 2009; Mimitou & Symington, 2008), while in *S. pombe*, Exo1 functions as the major nuclease to promote long-range resection at DSBs (Langerak et al, 2011). Human Exo1 has been shown to function in parallel with the BLM helicase in vivo, while in vitro results with the purified recombinant Exo1 and BLM demonstrate a direct interaction between the proteins, which leads to increased activation on 5' strand processing of DNA ends (Gravel et al, 2008; Nimonkar et al, 2011). Overall, these results suggest that Exo1 functions in resection in both a BLM-dependent and –independent manner.

DNA2 AND DNA REPAIR

Dna2 is an enzyme that possesses weak helicase activity and bipolar endonuclease activity that can degrade ssDNA (Kim et al, 2006; Masuda-Sasa et al, 2006). Dna2 has been shown to play an important role in DNA replication and DNA repair by regulating Okazaki fragment processing and DSB resection, respectively (Bae & Seo, 2000; Budd & Campbell, 1997; Budd et al, 2005). In addition, Dna2 is responsible for genomic stability, as depletion of Dna2 has been shown to result in chromosomal aberrations resulting in aneuploidy and generation of internuclear chromatin bridges (Duxin et al, 2009). In vivo and in vitro studies from several organisms have shown that the Dna2 nuclease interacts

with the Sgs1/BLM helicase to mediate DNA end resection in an ATP-dependent manner (Cejka et al, 2010a; Nimonkar et al, 2011; Niu et al, 2010; Zhu et al, 2008). In addition, in vitro results with recombinant proteins from *S. cerevisiae* have shown Top3 and Rmi1, which are associated with Sgs1, further stimulate resection by increasing the recruitment of Sgs1 to DNA (Cejka et al, 2010a).

IN VITRO RECONSTITUTION OF RESECTION BY EXO1 AND DNA2

Recently, an in vitro study with recombinant yeast Exo1 has demonstrated that MRX and Sae2 stimulate Exo1-mediated 5' strand resection on linear double-stranded DNA (dsDNA) (Nicolette et al, 2010). At low concentrations of Exo1 (Exo1 to DNA ratios of 3-5:1), where Exo1 displays limited resection activity, MRX and Sae2 significantly increase Exo1 enzymatic activity on DNA ends. On the other hand, when Exo1 is present at higher concentrations, Exo1 is able to process the 5' strand of DNA independently from MRX and Sae2 (Nicolette et al, 2010). The stimulatory effect of MRX and Sae2 on Exo1 activity was attributed to an increased recruitment of Exo1 to DNA ends in vitro (Nicolette et al, 2010), consistent with in vivo results where loading of Exo1 at HO-induced breaks is significantly decreased in *mre11*Δor *rad50*Δ strains (Shim et al, 2010). As no protein-protein interaction between Exo1 and either MRX or Sae2 was observed, it was postulated that MRX and Sae2 create a specific DNA structure that increases Exo1 affinity to DNA ends.

Other in vitro studies have addressed the role of MRX in stimulating Dna2/Sgs1-Top3-Rmi1 (STR) dependent resection activity on DNA ends (Cejka et al, 2010a; Niu et al, 2010). While Sae2 has no effect on Dna2/STR activity (Niu et al, 2010), addition of MRX to reactions containing Dna2/STR stimulates the resection activity of Dna2 and Sgs1 on DNA (Cejka et al, 2010a; Niu et al, 2010). Unlike with Exo1, a physical interaction of MRX was observed with Dna2 and Sgs1 (Cejka et al, 2010a; Niu et al, 2010). As it was reported previously that Dna2 recruitment to HO-induced DNA breaks depends on MRX in vivo (Shim et al, 2010), these results suggests MRX is likely to promote Dna2 and Sgs1 activity by enhancing their recruitment to DNA ends through direct protein-protein interactions. On the other hand, efficient resection activity of Dna2/STR on DNA substrates with 3' ssDNA tails is observed even in the absence of MRX (Niu et al, 2010), suggesting MRX could also be creating specific DNA substrates for efficient recruitment of Dna2/STR.

INTERPLAY BETWEEN NHEJ FACTORS AND HR FACTORS IN REGULATING DSB RESECTION

While NHEJ and HR exist as the major pathways for DSB repair in eukaryotic cells, the mechanisms that determine which pathway is used are not well understood. The cell cycle is believed to be one of the major factors that affects this decision. Since a homologous template is required in HR repair, HR occurs primarily during the S and G_2

phase of the cell cycle when sister chromatids are present to serve as a replication template (Aylon et al, 2004; Ira et al, 2004; Jazayeri et al, 2006; Zhang et al, 2009). On the other hand, NHEJ can occur throughout the cell cycle as it does not require a homologous template for repair and it has been shown to be the dominant repair pathway during G_0 and G_1 phases of the cell cycle (Moore & Haber, 1996; Takata et al, 1998). Another key factor for distinguishing repair pathway choice is DSB resection of the 5' strand as processed DNAs are incompatible for repair by NHEJ (Sonoda et al, 2006; Symington & Gautier, 2011; You et al, 2009).

Studies in yeast have emphasized the competition between HR factors, Mre11/Rad50/Xrs2(Nbs1) and the NHEJ factor Ku in determining the choice between the two repair pathways (Sonoda et al, 2006; Wu et al, 2008), as the assembly of the proteins at DSB sites affects how DNA ends are processed before the ends are ligated. The Ku heterodimer (Ku70/80) and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) contribute to genomic integrity through their ability to bind DNA DSBs and facilitate repair by NHEJ (Boulton & Jackson, 1996b; Chen et al, 2001b; Feldmann et al, 2000; Gottlieb & Jackson, 1993; Liang et al, 1996). Ku is an abundant nuclear protein that binds to duplex DNA ends with high affinity by forming a ring-like structure that clamps on DSB ends (Walker et al, 2001). Ku binding to DSBs promotes recruitment of DNA-PKcs and other NHEJ factors including Artemis, XLF, and DNA ligase IV/XRCC4 to the ends to facilitate NHEJ repair (Dynan & Yoo, 1998).

Recent studies have also suggested a role for Ku in the inhibition of HR repair by inhibiting DSB resection activity of the resection enzymes. In *S. cerevisiae*, Ku inhibits DSB resection in the absence of the MRX complex by blocking the binding of enzymes involved in resection such as Exo1 (Shim et al, 2010). This anti-resection effect of Ku, however, is suppressed in the presence of the MRX complex in vivo. The MRX complex suppresses Ku accumulation at DSB ends and stimulates Exo1 recruitment to DSB ends, leading to a stimulation of DSB resection in vivo (Shim et al, 2010). The recruitment of Exo1 to DSBs and its activation is less dependent on the MRX complex in the absence of Ku (Shim et al, 2010), suggesting that the primary function of MRX in stimulating resection is to remove Ku from DSB ends. In *S.pombe*, Ku also exhibits an inhibitory effect on Exo1-dependent resection on DSBs and Mre11 functions to remove Ku from DNA ends (Langerak et al, 2011). Overall, these yeast results suggest that the MRX(N) complex stimulates DSB resection by recruiting nucleases and by promoting HR-dependent repair by removing Ku from DSB ends, although it remains to be examined whether this applies to the human proteins as well.

In summary, two major pathways exists to promote long-range resection in eukaryotic cells: Exo1-dependent and Dna2/STR(BLM)-dependent resection pathways. The MRX(N) complex is proposed to be involved in various activities to stimulate long-range resection activities of Exo1 and Dna2/STR(BLM) on DSB ends (Figure 1.3). In budding yeast, MRX acts with Sae2 to increase the recruitment of downstream enzymes by creating DNA ends that are preferable for binding. In addition, MRX and Sae2 are

involved in processing covalent modifications from DNA ends with Sae2. Moreover, MRX(N) competes with Ku for DNA ends and can block Ku from binding DNA ends, which allows loading of Exo1 and Dna2 to DSB ends for resection. However, the mechanistic function of MRX(N) in repressing Ku binding on DNA ends is unclear.

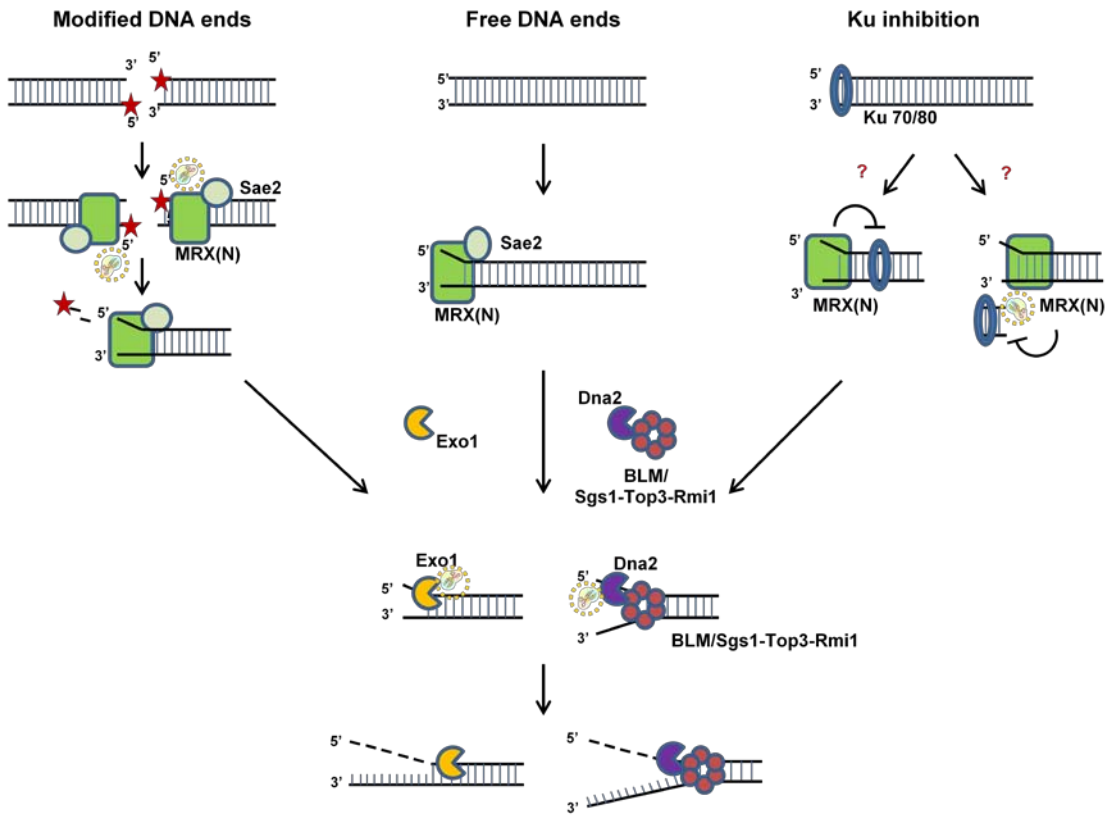

Figure 1.3: Model of MRX(N) stimulation of Exo1- and Dna2/Sgs1-Top3-Rmi1(BLM)-dependent pathways in DSB resection.

THE BACTERIAL SSB PROTEIN AND ITS ROLE IN HR REPAIR

Single-stranded DNA (ssDNA) binding proteins are essential in DNA replication, recombination, DNA damage signaling, and repair in all organisms. ssDNA binding proteins associate with ssDNA with high affinity in a sequence-independent manner, protecting ssDNA from chemical and nucleolytic attacks (Shereda et al, 2008). In bacteria, the SSB protein contains a single oligonucleotide/oligosaccharide-binding (OB) domain, which consists of beta sheets that form beta-barrel structures that can wrap around ssDNA to bind ssDNA through a combination of electrostatic and base-stacking interactions with the phosphodiester backbone and nucleotide bases, respectively (Murzin, 1993; Shereda et al, 2008).

In prokaryotic cells, the RecBCD pathway and RecJ/RecQ pathway share some common components such as the RecA recombinase and the SSB protein, which promote homologous DNA pairing. SSB in HR repair is well known for its ability to bind and protect the 3’ ssDNA tail generated by 5’ strand resection. The SSB-ssDNA complex serves as a template for the loading of the RecA recombinanse with the assistance of mediator proteins such as RecO and RecR to facilitate subsequent homologous pairing events (Umezu et al, 1993). However, bacterial SSB has also been shown to function during the initial steps in HR-dependent repair by regulating 5’ strand resection. For instance, SSB physically interacts with the RecQ helicase and stimulates the DNA unwinding activity of RecQ on dsDNA (Shereda et al, 2007; Umezu & Nakayama, 1993).

In addition, SSB promotes the recruitment of the RecJ 5’ to 3’ exonuclease on DNA and stimulates its enzymatic activity to process DNA to generate the 3’ ssDNA to proceed in HR repair (Figure 1.4) (Butland et al, 2005; Han et al, 2006b; Handa et al, 2009; Sharma & Rao, 2009).

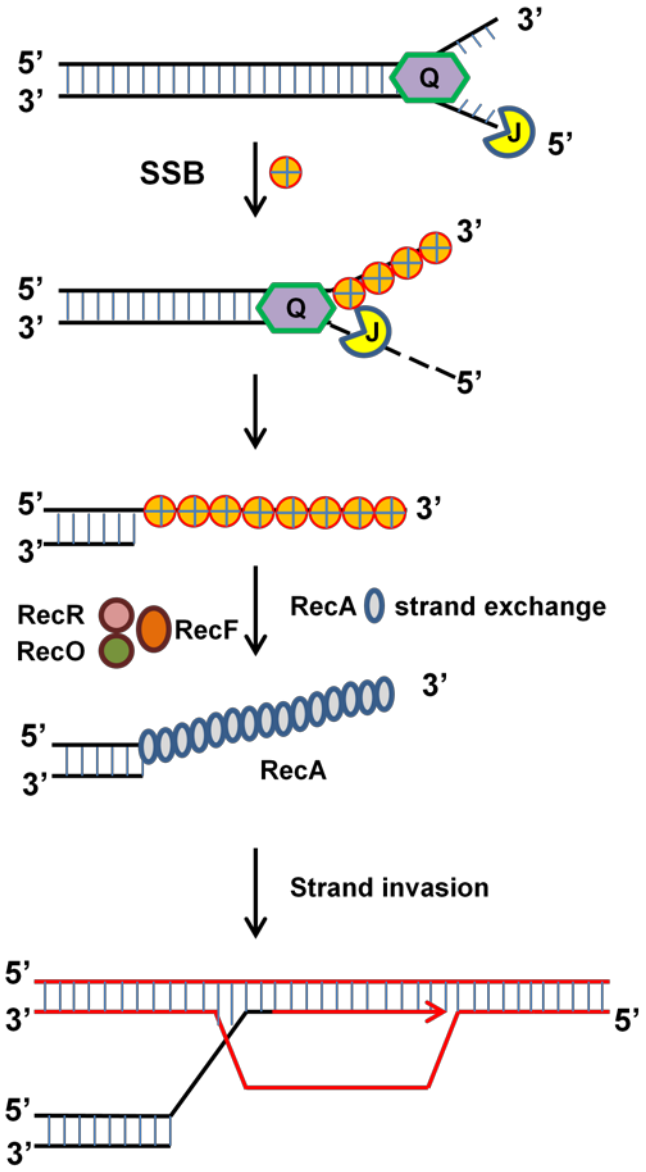

Figure 1.4: The role of SSB in DNA DSB resection in E. coli.
SSB functions in the initial resection steps in HR repair by stimulating RecJ resection activity and RecQ helicase activity on DNA ends. SSB further binds to the 3' ssDNA strand for exchange by RecA to proceed in strand invasion.

REPLICATION PROTEIN A (RPA)

Replication Protein A (RPA) is one of the primary single-stranded DNA binding complexes (SSBs) in eukaryotic cells that consists of 70 kDa, 32 kDa, and 14 kDa subunits, which are termed RPA1, RPA2, and RPA3, respectively. RPA contains a total of six oligonucleotide/oligosaccharide binding (OB) domains, which is a common structural feature in ssDNA binding proteins (Figure 1.5) (Bochkarev et al, 1999; Bochkarev et al, 1997; Murzin, 1993).

RPA is well known to function in DNA replication by stimulating both initiation and elongation of DNA through its interaction with DNA polymerases (Tanaka & Nasmyth, 1998; Waga et al, 1994; Waga & Stillman, 1994; Zou & Stillman, 2000) as well as by promoting Okazaki fragment processing through its interaction with Dna2 (Bae et al, 2003; Bae et al, 2001). RPA is also involved in several DNA repair pathways including nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) (Oakley & Patrick, 2010). Moreover, RPA is known to function in DNA DSB repair by regulating homologous recombination (HR) (Heyer et al, 1990). Following DNA 5' strand resection and the generation of 3' ssDNA at DSBs, RPA binds to the 3' ssDNA, protects the DNA, and prevents secondary structure formation. RPA subsequently promotes the recruitment of checkpoint proteins to activate cell cycle arrest after DNA damage (Zou & Elledge, 2003). RPA coated DNA is then displaced by the recombinase protein Rad51 with the aid of recombination mediator proteins, such as

yeast Rad52 or human BRCA2, to initiate strand invasion and subsequent steps to complete HR repair (Krogh & Symington, 2004; Sung & Klein, 2006).

Recently, biochemical studies with the recombinant RPA from yeast and human have demonstrated a novel function of RPA in the early steps in HR repair by regulating 5' strand resection (Cejka et al, 2010a; Nimonkar et al, 2011; Niu et al, 2010). Both yeast and human RPA stimulate Dna2/Sgs1 or Dna2/BLM dependent resection on dsDNA by promoting the helicase activity of Sgs1 or BLM, respectively (Cejka et al, 2010a; Nimonkar et al, 2011; Niu et al, 2010). While Dna2 is known to possess bipolar nuclease activity and degrade both 5' and 3' strands in vitro, the mechanism that restricts the directionality of Dna2 to the 5' strand was unknown. Notably, RPA was found to be the key component to serve this function by enhancing 5' strand resection performed by Dna2 while repressing 3' strand resection (Cejka et al, 2010a; Nimonkar et al, 2011; Niu et al, 2010). At the same time, RPA binds to the 3' ssDNA and protects the 3' strand from being degraded by Dna2 (Cejka et al, 2010a; Nimonkar et al, 2011; Niu et al, 2010). While Dna2/Sgs1 or Dna2/BLM functions with RPA to promote DSB resection in HR repair, it remains to be determined if RPA interacts with other HR-promoting factors to promote DSB resection as well. MRN and CtIP are required for RPA localization to DSBs upon DNA damage (Richard et al, 2008). Therefore, it is possible that RPA acts together with MRN and CtIP in stimulating DSB resection.

Figure 1.5: Organization of RPA.
RPA is a heterotrimeric complex that consists of 70kDa, 32kDa, 14kDa subunits, termed RPA1, RPA2, and RPA3, respectively. RPA1 contains 4 oligonucleotide/oligosaccharide binding (OB) domains two within the central region and one each in the N-terminus and C-terminus of the protein. RPA2 contains a single OB domain between amino acids 43-171. RPA3 contains a single OB domain that is formed from the entire amino acids 1-121 of the protein.

SENSOR OF SINGLE-STRANDED DNA COMPLEX 1 (SOSS1)

In eukaryotic cells, the heterotrimeric complex RPA is considered to be the primary ssDNA binding complex (Richard et al, 2009; Wold, 1997). However, two novel ssDNA binding proteins hSSB1 and hSSB2 were recently identified in the human genome (Richard et al, 2008). hSSB1 (SOSSB1) contains a single OB domain and exists as a member of a heterotrimeric complex called Sensor of Single-Stranded DNA complex 1 (SOSS1), together with SOSSA (INTS3) and SOSSC (C9orf80) (Figure 1.6) (Huang et al, 2009; Skaar et al, 2009). SOSSA functions as a scaffold protein to assemble and stabilize the SOSS1 complex as well as to localize the protein to DSB sites through its interaction with the MRN complex during the S phase, while the function of SOSSC is uncharacterized (Huang et al, 2009).

Depletion of SOSS1 in human cells results in loss of checkpoint activation, increased sensitivity to ionizing radiation (IR), defects in DSB resection, and reduced HR (Huang et al, 2009; Richard et al, 2008), suggesting a role for SOSS1 in HR repair. Both RPA and SOSS1 are recruited to DNA DSB sites but, paradoxically, they exhibit low co-localization (Richard et al, 2008). Unlike RPA, SOSS1 does not localize to replication foci, suggesting that the novel SOSS1 complex functions in DNA damage repair through HR but not in DNA replication (Richard et al, 2008).

Recent in vitro studies with RPA have suggested an important role for RPA in promoting DSB resection by stimulating the nuclease/helicase complex Dna2/Sgs1 in yeast and Dna2/BLM in humans (Cejka et al, 2010a; Nimonkar et al, 2011; Niu et al, 2010). However, the role of the SOSS1 complex in DSB resection in HR repair is not understood. Both RPA and SOSS1 are ssDNA binding protein complexes involved in HR repair. However, not only do they display low co-localization at DSB sites but also they are regulated in a distinct manner to recruit to DSB sites. For example, while RPA depends on MRN and CtIP to be recruited to DSB sites throughout the whole cell cycle, SOSS1 is independent of CtIP and depends on only MRN during S phase of the cell cycle to be recruited to DSB sites (Huang et al, 2009). Overall this suggests that RPA and SOSS1 may have different functions at DSBs. Therefore, the functional relationship between SOSS1 and RPA in regulating DSB repair requires further study.

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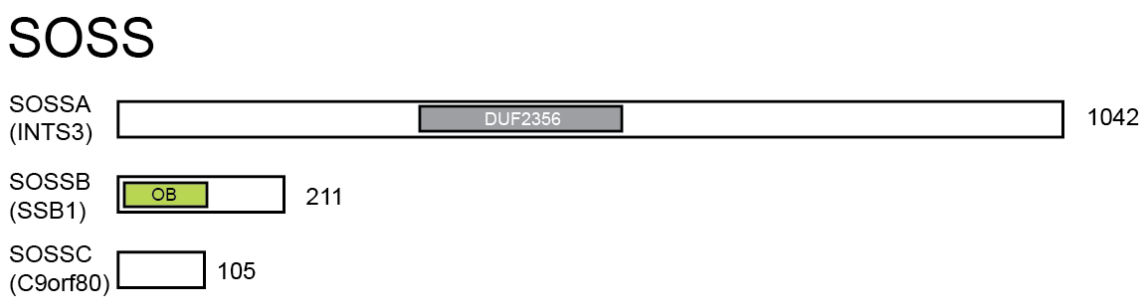

Figure 1.6: Organization of the SOSS1 complex.
SOSS1 is a heterotrimeric complex that consists of SOSSA (INTS3), SOSSB1 (SSB1), and SOSSC (C9orf80). SOSSA contains DUF2356 domain that is responsible for complex formation with SOSSB1 and SOSSC. SOSSB1 contains a single OB domain in the N-terminus and is responsible for DNA binding of the complex. The function of SOSSC is unknown.

HYPOTHESIS AND GOALS

The Function of Single-stranded DNA binding complexes in DSB resection

Single-stranded DNA binding proteins are critical for DNA replication, recombination, DNA damage signaling, and repair in all organisms. Recently, SOSS1 complex has been identified as a novel single-stranded DNA binding complex in addition to RPA in human cells (Huang et al, 2009; Richard et al, 2008). While both of these complexes are responsible for ssDNA binding activity in human cells they contain a different number of OB domains, suggesting SOSS1 and RPA may exhibit differences in their ability to bind ssDNA. This idea is supported by the localization pattern of SOSS1 and RPA to DNA in cells: Unlike RPA, SOSS1 does not form replication foci. In addition, both SOSS1 and RPA localize to DSBs after DNA damage but they exhibit low co-localization at DSBs in cells (Richard et al, 2008). hSSB1 in the SOSS1 complex was also shown to localize at DSBs rapidly after DNA damage to regulate Rad51 foci formation and to physically interact with MRN in cells, suggesting a role for the SOSS1 complex in the initiating steps in DSB resection (Richard et al, 2008; Richard et al, 2010). In this study I hypothesize that SOSS1 regulates DSB resection by stimulating the activity of DNA processing enzymes. To examine this question, I characterized the DNA binding specificities, affinities, and kinetics of SOSS1 in comparison to RPA in vitro. I also investigated the effects of the SOSS1 complex on hExo1 and Dna2/BLM and found

that SOSS1 has a unique ability to stimulate DSB resection by hExo1. In this study I demonstrate SOSS1 stimulates the enzymatic activity of hExo1 on DNA by increasing hExo1 recruitment to DNA ends in vitro, whereas RPA specifically activates Dna2, as reported previously (Cejka et al, 2010a; Nimonkar et al, 2011; Niu et al, 2010).

Role of MRN and CtIP in DNA DSB resection

Previous studies from yeast have shown that MRN(X) regulates the nuclease activity of Exo1 by antagonizing the effect of DNA end binding proteins such as Ku70/80 (Langerak et al, 2011; Shim et al, 2010). This led me to hypothesize that the human MRN complex stimulates hExo1 or Dna2 activity in processing DSBs in a context where DNA ends are blocked by Ku. In this study, I demonstrate that MRN in concert with either SOSS1 or RPA can overcome Ku inhibition of hExo1 and Dna2. Moreover, I examined how the nuclease activity and the ATP-driven DNA unwinding activity of the MRN complex affect DSB resection in the presence of Ku in vitro. I show that the nuclease activity of Mre11 is not required whereas the ATP-driven DNA unwinding activity of Rad50 is required for overcoming Ku inhibition of hExo1 resection activity in vitro. This supports the idea that MRN may be pushing Ku away from the DNA ends through its Rad50-dependent DNA unwinding activity rather than removing Ku from DNA ends through endonucleolytic cleavage by Mre11.

MRN has been shown to interact with CtIP to promote HR repair in human cells by stimulating ssDNA generation (Bolderson et al, 2009; D'Amours & Jackson, 2002; Lavin, 2004; Sartori et al, 2007). However, the biochemical mechanism of how these proteins function in regulating DSB resection remains further study. In this study, I hypothesize that MRN and CtIP cooperate to promote resection activity by hExo1 or Dna2/BLM at DSBs. I demonstrate in vitro that MRN and CtIP can stimulate the resection activity of hExo1 and Dna2/BLM on DSBs when the exonucleases are present in limiting concentrations, consistent with in vitro results observed with yeast Exo1 with MRX and Sae2 (Nicolette et al, 2010). However, I found that MRN and CtIP do not cooperate with SOSS1 in stimulating hExo1 resection activity, suggesting MRN and CtIP function independently from SOSS1 in regulating DSB resection by hExo1. Further, I show that CtIP can increase MRN activity in overcoming Ku inhibition of Dna2/BLM activity on DSB ends. Overall this study characterizes the biochemical mechanisms of MRN and CtIP in regulating resection in vitro.

CHAPTER 2: METHODS AND MATERIALS

EXPRESSION CONSTRUCTS

Baculovirus transfer vectors used for expression of his-tagged SSB1 (SOSS1), GST-tagged SOSSA (INTS3), and SOSSC (C9orf80) were provided by Junjie Chen (Huang et al, 2009). The plasmid encoding SSB1 in pDEST10 was modified to include the T117E mutation to yield transfer vector pTP1725. Human Exo1 wild-type and D173A expression plasmids were gifts from Paul Modrich. Transfer vectors and viruses expression His-Ku70 and Ku-80 were gifts from Dale Ramsden. Expression of human wild-type BLM was from transfer vector pTP1487, made by transfer of the gene from pYES2 (gift from Ian Hickson) into pFastBac1 (Invitrogen) with an N-terminal Flag tag. Point mutations were generated using QuikChange site-directed mutagenesis (Stratagene) and the mutations were confirmed by DNA sequencing All transfer vectors were used to generate bacmids and baculovirus according to the manufacture's instructions (Invitrogen).

PROTEIN EXPRESSION AND PURIFICATION

The SOSS1(T117E) complex was expressed in Sf21 insect cells using the baculovirus expression system. Lysis of the cell pellet was performed as described (Lee & Paull, 2006), with the following modifications. The complex was eluted from Nickel-NTA resin (Qiagen) with low-salt NiB buffer (50mM KCl, 50mM potassium phosphate pH 7.0, 10% glycerol, 20mM β-mercaptoethanol, and 125mM imidazole). Fractions containing the SOSS1 complex were loaded onto a 1mL HiTrap GST column (G.E.) and washed with buffer A (25mM Tris pH 8.0, 100mM NaCl, 10% glycerol, and 1mM DTT). The complex was eluted with buffer A containing 10mM glutathione. The fractions containing SOSS1 were loaded onto a 1mL HiTrap SP column (G.E.) and washed with buffer A then eluted with buffer A containing 500mM NaCl. The fractions containing SOSS1 were loaded onto a Superdex 200 gel filtration column (G.E.) equilibrated in buffer A and fractions containing SOSS1 were aliquoted and stored at -80°C (Figure 2A).

The human MRN complex was purified as described previously (Bhaskara et al, 2007). Human Exo1 was purified as described previously (Genschel et al, 2002) with the following modification: After purification through Q Sepharose (G.E.), Heparin (G.E.), and SP resin (G.E.), hExo1 was loaded onto a Superdex 200 gel filtration column (G.E.) equilibrated with buffer A and the fractions containing hExo1 were aliquoted and stored at -80°C.

Purification of human Ku70/80 complex was performed as with MRN (Bhaskara et al, 2007) but the eluate from the nickel resin was loaded onto a 1mL HiTrap Q column (G.E.) prewashed with buffer A. The Ku70/80 complex was eluted with buffer A containing 500mM NaCl. Concentrated fractions of Ku70/80 were loaded onto a Superdex 200 equilibrated with buffer A and fractions containing Ku were aliquoted and stored at -80°C.

Cell pellets with BLM were lysed as described for MRN and the protein was purified by Nickel-NTA and 1mL HiTrap SP (G.E.) as described above. The protein was then loaded onto a column containing ~2mL M2 anti-Flag antibody-conjugated agarose resin (Sigma) that was prewashed with buffer A. The protein was eluted from the Flag column with 5mL buffer A containing 0.1mg/mL Flag peptide (Sigma).

Dna2 expressing cells were lysed by homogenization and sonicated three times for 20 seconds in A buffer containg 0.5% tween-20 and 1mM PMSF (phenylmethylsulfonyl fluoride). The lysate was centrifuged for 1 hour at 35,000 rpm at 4°C. The supernatant was incubated with ~1mL M2 anti-Flag antibody-conjugated agarose resin (Sigma) with rotation at 4°C for 1 hour. After incubation the lysate with resin was centrifuged for 3 min at 1,000g. After removing the supernatant, the remaining resin was washed with 20mL buffer A twice and was eluted with 5mL of buffer A containing 0.8mg/mL 3X Flag peptide (Sigma). The peptide was incubated with resin for 20 min before elution. The Flag eluent was loaded onto a 1mL HiTrap SP column (G.E.)

prewashed with buffer A. The protein was eluted with buffer A containing 500mM NaCl and the eluted fractions were dialyzed in A buffer. The dialyzed fractions were aliquoted, and stored at -80°C.

BRCA1 expressing cells were purified as Dna2 and the protein was purified by ~2mL M2 anti-Flag antibody-conjugated agarose resin (Sigma) and 1mL HiTrap Q as described above.

OLIGONUCLEOTIDE DNA SUBSTRATES

The substrate in Figure 3.2A consisted of 5'[^{32}P]-labeled TP46 (5'-CTGCAGGGTTTTTGTTCCAGTCTGTAGCACTGTGTAAGACAGGCCAGATCCCATG CTGTCCTACGTGCCAGGTCACTGA-3') annealed to TP494 (5'-ACACAGTGCTACAGACTG GAACAAAAACCCTGCAG-3'). Substrates used in the binding experiments quantified in Figure 3.2B include the 3' overhang substrate used in Figure 3.2A, a 5' overhang substrate consisting of TP46 annealed to TP2756 (5'-TCAGTGACCTGGCACGTAGGACAGCATGGGATCTG-3'), and duplex DNA consisting of 5' [^{32}P]-labeled TP74 (5'-CTGCAGGGTTTTTGTTCCAGTCTGTAGCACTGTGTAAGACAGGCCA-3') annealed to TP504 (5'-TGGCCTGTCTTACACAGTGCTACAGACTGGAACAAAAACCCTGCAG-3').

Substrates used for binding assays quantified in Figure 3.2C included 5’ [^{32}P]-labeled TP70 (5'- CGGAATTCGATGTAATCCCTCGATGAGG -3'), TP144 (5’-CCTTGTTGGCCTGAAGTAGATGCTTACTAGG -3’), TP236 (5'-GACGGATCCGATCCAGACATGATAAGATACATTG-3'), and 5’ [^{32}P]-labeled TP8 (5’-GACCTGGCACGTAGGACAGCATGGGATCTGGCCTGTCTTACACAGTGCTACAGACTGGAACAAAAACCCTGCAG-3’) annealed to TP74, TP2999 (5'-CTGCAGGGTTTTTGTTCCAGTCTGTAGCACTGTGTAAGACAGG-3'), or TP507 (5'- CTGCAGGGTTTTTGTTCCAGTCTGTAGCACTGTGTAAGAC-3').

The substrate in Figure 3.2D and E consisted of 5’ [^{32}P]-labeled TP158 (5’-CATGTAATCCCTCGATGAGGTCTAGAACTG CAGTGGC TGCACATCTGGCCTGTCTTACACAGTGCTACAGACTGGAACAAAAACCCTGCAG-3’) annealed to TP2791 (5’- AGACCTCATCGAGGGATTACATG-3’) and TP2572 (5’- CTGCA GGG TTTTTGTTCCAGTCTGTAGCACT-3’).

Oligonucleotides used in single-molecule FRET experiments included:

1. 5’- /Cy3/ GCC TCG CTG CCG TCG CCA - /biotin/ - 3’
2. 5’- TGG CGA CGG CAG CGA GGC $(T)_{16}$ /Cy5/ $(T)_{24}$ - 3’
3. 5’- /Cy5/ GCC TCG CTG CCG TCG CCA -/biotin/- 3’
4. 5’- TGG CGA CGG CAG CGA GGC $(T)_{15}$ /iAmMC6T/ $(T)_{42}$ - 3’
5. 5’- TGG CGA CGG CAG CGA GGC $(T)_{31}$ /iAmMC6T/ $(T)_{39}$ - 3’
6. 5’- TGG CGA CGG CAG CGA GGC $(T)_{68}$ /iAmMC6T/ $(T)_{8}$ - 3’

The amine-modified thymine (/iAmMC6T/) shown in the sequence enables the oligonucleotides to be labeled with the monofunctional NHS ester form of Cy3 dyes (G.E.). /Cy5/ represents the Cy5 dye that was inserted directly to the DNA backbone using phosphoramidite chemistry.The partial duplex DNA substrates (18 bp dsDNA) with poly(T) single-stranded tails carrying fluorescence dyes were annealed by mixing ~5μM of biotinylated strand and ~7μM of poly(T) strand in 10mM Tris:HCl (pH 8.0) and 50mM NaCl followed by slow cooling from 90°C to room temperature for ~ 2 hours.

GEL MOBILITY SHIFT ASSAYS

Gel shift assays were performed with purified SOSS1(T117E) complex and RPA on DNA substrates labeled with [^{32}P] at the 5' end of the DNA strand. The radiolabeled substrates were incubated with SOSS1(T117E) or RPA at the concentrations indicated in the figure legends in reactions containing 2nM DNA substrate, 25mM MOPS pH 7.0, 1mM DTT, 5mM EDTA, 1mM ATP, 0.1% Tween 20, and 60mM NaCl for 20 minutes on ice. The reaction was loaded and separated in an 8% native acrylamide gel with 0.5X TBE buffer at 100 V/cm for 2 hrs. The gel was dried and analyzed by phosphorimager (G.E.).

IN VITRO RESECTION ASSAYS

The resection assays with plasmid DNA substrates were performed with pNO1 (4.5kb derivative of pBR322) linearized with SphI-HF, which generates 4nt 3' overhangs. The linearized DNA substrate was incubated in reactions containing 25mM MOPS pH 7.0, 1mM DTT, 5mM $MgCl_2$, and 60mM NaCl for 1 hour at 37°C. Each reaction contained 0.135nM linearized DNA in a total volume of 10ul. Each reaction was terminated by adding 0.1% SDS and 10mM EDTA. The reactions were loaded and separated on a 1% non-denaturing agarose gel and visualized by SYBR green (Invitrogen) staining. Each reaction were further analyzed by non-denaturing southern hybridization, in which the agarose gel was washed with 20X SSC (3M NaCl and 0.3M sodium citrate) and DNA was transferred onto nylon membranes (NEN) by capillary actions overnight in 20X SSC. The membranes were probed with RNA complementary to the 5' strand of the DNA substrate in a 1kb region adjacent to the SphI-HF site. The RNA probes were internally labeled with [α-^{32}P] CTP (NEN) and were made using Riboprobe System T7 (Promega) and purified with RNAeasy extraction kit (Qiagen) according to the manufacturer's instructions.

In vitro exonuclease assays were performed using a 1.7kb DNA substrate internally labeled with [^{32}P] by PCR, with conditions as described above except with 3nM DNA substrate in each reaction. Reactions were terminated by the addition of 0.1% SDS and 10mM EDTA and analyzed by Thin Layer Chromatography (TLC). The amount

of labeled NMP released by the exonuclease activity of hExo1 was quatified using ImageQuant software (G.E.).

The in vitro endonuclease assay was performed with 717bp Cy3/cy5 labeled DNA constructed by PCR amplification of pTP466 with TP2758 (5'-CCTCT[Cy5]ACAAATGTGGTATGGCTGATTATG-3') and TP2759 (5'-CTTGC[Cy3]ATGCCTCAGCTATTCCGGATTATTCATACCGTCCCA-3'). Each reaction was performed as described above but in a reaction buffer containing 25mM MOPS pH 7.0, 1mM DTT, 5mM $MgCl_2$, 1mM ATP, and 54nM oligonucleotide duplex, in a total volume of 40ul. Each reaction was ethanol precipitated and resuspended in formamide containing 1mM EDTA, bromophenol blue, and xylene cyanol. The reactions were further separated in a 20% denaturing acrylamide sequencing gel and analyzed by phosphorimage (G.E.). In vitro endonuclease assay with oligonucleotide substrate was performed as above but with [^{32}P]-labeled substrate.

QUANTITATIVE PCR

The amount of single-stranded DNA produced by resection was quantified (Nicolette et al, 2010) with the following modification. Resection reactions were performed as described above but terminated with a final concentration of 0.01% SDS. The reactions were diluted 10-fold or 20-fold and half of the mixture was digested

overnight at 37°C with 10 units or 20 units NciI (NEB) and the remaining half was incubated in the same buffer at 37°C overnight but without the enzyme. 1ul of the digested or undigested DNA sample was used as a template in a 25ul reaction with 0.5uM of each primer, 0.2uM probe, and 1X Taqman universal master mix (ABI). Quantitative PCR assay was performed on 7900HT Fast Real-Time PCR System (ABI) or ViiA 7 Real-Time PCR System (ABI) under standard thermal cycling conditions. Results were analyzed with ViiA 7 software (ABI).

PROTEIN AND DNA STRAND SPECIFIC CROSS-LINKING

hExo1 D78A/D173A binding to DNA 5' ends in the presence of SOSS1 or RPA was demonstrated by UV cross-linking of the protein to the DNA followed by a gel mobility shift assay. The substrate was a 717bp PCR product internally labeled with [^{32}P] containing azidophenacryl bromide (APB) at the 5' end. The substrate was generated by PCR amplification of pTP466 with TP20 (5'-T*A*T*TCCGGATTATTCATACCGTCCC-3') and TP1030 (5'-G*A*T*CCTCTAGTACTTCTCGACAAGC-3') in the presence of [α-^{32}P]dATP (NEN) (askterisks indicate positions of phosphorothioate bonds). After nucleotide removal (Qiagen), covalent incorporation of APB at phosphorothioate positions was performed as previously described (Yang & Nash, 1994).

The binding reactions contained 2nM substrate, 25mM MOPS pH 7.0, 1mM DTT, 5mM EDTA, 1mM ATP and were incubated on ice for 20 minutes with protein concentrations as indicated in the figure legends. After incubation, each reaction was UV crosslinked (254nm) on ice with a handheld UV source at 3cm for 5 minutes, 5% glycerol was added, and samples were separated in a 0.7% non-denaturing agarose gel in 1X TAE buffer. The gel was dried and analyzed by phosphorimager (G.E.).

PROTEIN-DNA STRAND-SPECIFIC CROSS-LINKING AND PULL-DOWN ASSAY

DNA binding assays with hExo1 D78A/D173A were performed using a 717 bp PCR product containing both biotin and three azide groups on each 5' ends of the DNA strand as shown in Figure 3.11B. The substrate was constructed by PCR amplification of pTP466 with TP1037 (5'-Biotin-TATTCCGGATTATTCATACCGTCCC-3') and TP1030. After gel purification APB was covalently coupled to the phosphorothioate containing DNA as previously described (Yang & Nash, 1994). Approximately 250ng Biotin-APB-DNA was pre-incubated with Dynal streptavidin-coated magnetic beads (Invitrogen) for immobilization according to the manufacturer's instructions in a total volume of 100ul. Cross-linking experiments were performed in 25ul reactions containing 25mM MOPS pH 7.0, 1mM DTT, 5mM EDTA, 1mM ATP, and 2ul of Dynabead/DNA with hExo1 and SOSS1 as indicated in the figure legends. The reactions were incubated

on ice for 15 minutes and UV cross-linked (254nm) on ice for 5 minutes. The Dynabead/DNA was separated using a magnetic stand, washed 3 times with wash buffer (25mM MOPS pH 7.0, 50mM NaCl, 0.2% CHAPS, 2mM DTT) and eluted with 1X SDS-PAGE loading buffer. The reaction was further separated on a 6% SDS-PAGE and transferred to a PVDF membrane, which was probed with antibody against hExo1 (Genetex: GTX92126) and further visualized with horseradish peroxidase-conjugated anti-mouse secondary antibody by chemiluminescence (Pierce).

GENERATING PROTEIN CONJUGATED DNA SUBSTRATES

Protein conjugated DNA substrates were made by conjugating Sulfo-S-Hynic ($C_{14}H_{14}N_3NaO_7S$) modified protein or DNA to S-4FB (succinimidyl-4-formylbenzamide) modified DNA or protein using quick chemistry according to the manufacturer's protocol (Solulink). To generate 5' Ku-conjugated DNA, 1.5 mmole of Ku was dialyzed into modification buffer (100mM Sodium phosphate, 150mM NaCl, pH 7.4) for 2 hours at 4°C. After dialysis, Ku was incubated with 25 mmole Sulfo-S-Hynic at room temperature for 2 hours. Ku was then dialyzed into conjugation buffer (100mM Sodium phosphate, 150mM NaCl, pH 6.0) for 2 hours at 4°C. The Sulfo-S-Hynic modified Ku was incubated with DNA substrate containing 5' S-4FB overnight at 4°C with rotation. The 5' S-4FB DNA substrate was generated by PCR amplification of pTP466 with TP2814 (4FB-TATTCCGGATTATTCATACCGTCCC) and TP2815 (4FB-

CCTCTACAAATGTGGTATGGCTG), which was subsequently purified by PCR cleanup (Qiagen) followed by Micro Bio-spin 6 column (Biorad) pre-washed with the conjugation buffer. The Sulfo-S-Hynic Ku and 5' S-4FB DNA mix was separated in a 1.5% agarose gel (1X TAE), running 85V/cm for 3 hours. The Ku conjugated DNA was gel purified followed by electroelution in dialysis tubing and was further concentrated with a centricon unit (Millipore) prewashed with 150mM NaCl.

The 3' Ku-conjugated DNA was generated as described above with the following modifications. The 3'end of the PCR substrate was labeled with ddUTP-NH_2 using Tdt modification. The modified PCR substrate was purified through Micro Bio-spin 6 column equilibrated with 1X modification buffer then dried down to 1/10 of volume. The substrate was then modified with S-4FB according to the manufacturer's instruction (Solulink). 3' S-4FB modified PCR substrate was then incubated with Sulfo-S-Hynic modified Ku as described above.

CHAPTER 3: THE SOSS1 SINGLE-STRANDED DNA BINDING COMPLEX PROMOTES DNA END RESECTION IN CONCERT WITH EXO1

Portions of this chapter have been published in EMBO J, 32(1), 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."

ABSTRACT

The human SSB homolog 1 (SSB1) has been shown to facilitate homologous recombination and double-strand break signaling in human cells. Here we compare the DNA binding properties of the SOSS1 complex, containing SSB1, with RPA, the primary single-strand DNA binding complex in eukaryotes. Ensemble and single-molecule approaches show that SOSS1 binds ssDNA with lower affinity compared to RPA, and exhibits less stable interactions with DNA substrates. Nevertheless, the SOSS1 complex is uniquely capable of promoting interaction of human Exo1 with double-strand DNA ends and stimulates its activity independently of the MRN complex in vitro. Both MRN and SOSS1 also act to mitigate the inhibitory action of the Ku70/80 heterodimer on Exo1 activity in vitro. These results may explain why SOSS complexes do not localize with

RPA to replication sites in human cells, yet have a strong effect on double-strand break resection and homologous recombination.

INTRODUCTION

DNA double-strand breaks (DSBs) are induced by exogenous factors such as ionizing radiation (IR), and genotoxic agents as well as endogenous factors including replication fork collapse and oxidative stress. Eukaryotic cells possess two primary mechanisms to repair DNA DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR) (Symington & Gautier, 2011). In vertebrates, NHEJ is the major pathway for DSB repair which occurs in all phases of the cell cycle and repairs DSBs by direct joining of the broken DNA ends. In contrast, HR occurs primarily in the S and G_2 phases of the cell cycle and is initiated by processing of the 5’ strand at DSB ends to produce a 3’ single-stranded DNA (ssDNA) that is essential for Rad51 filament formation and strand invasion.

The molecular mechanisms of 5’ strand processing are well understood in bacteria where the RecBCD pathway and the RecQ/RecJ pathway exist to recognize DSBs and process the 5’ strand of DSB ends to generate 3’ ssDNA for subsequent HR repair (Dillingham & Kowalczykowski, 2008). RecBCD does not exist in eukaryotes, but several partially redundant pathways function to perform resection of 5' strands (Mimitou

& Symington, 2009). In budding yeast, the Mre11/Rad50/Nbs1(Xrs2) (MRN(X)) complex cooperates with the Sae2 endonuclease to initiate short-range processing of 5' strands, then the Exo1 and Dna2 exonuclease function redundantly to continue the resection of DNA ends from a few hundred to several thousand nucleotides from the original break site (Gravel et al, 2008; Mimitou & Symington, 2008; Zhu et al, 2008). In vitro studies with the archaeal MR complex and the eukaryotic MRN/X complex have demonstrated that Mre11/Rad50 proteins perform at least two important roles in resection: short-range endonucleolytic resection of 5' strands at DSB ends, and also stimulation of extensive resection through recruitment of 5’ to 3’ exonucleases and helicases (Hopkins & Paull, 2008; Nicolette et al, 2010; Nimonkar et al, 2008; Niu et al, 2010).

Single-stranded DNA binding proteins are critical for DNA replication, recombination, DNA damage signaling, and repair in all organisms. In bacteria, Single-Stranded DNA binding proteins (SSBs) are essential because of their roles in replication, but also interact with and stimulate many other factors involved in recombination and repair (Shereda et al, 2008). For instance, SSB was shown to stimulate the 5' to 3' exonucleolytic activity of RecJ on both ssDNA and double-stranded DNA (dsDNA) substrates in vitro, using proteins from *E. coli* and *H. influenza* (Butland et al, 2005; Han et al, 2006a; Handa et al, 2009; Sharma & Rao, 2009).

In eukaryotes, the heterotrimeric complex Replication Protein A (RPA) is considered to be the primary single-stranded DNA-binding activity in eukaryotic cells (Richard et al, 2009; Wold, 1997). However, two novel SSB proteins hSSB1 and hSSB2 were recently identified in the human genome (Richard et al, 2008). hSSB1 exists as a member of a heterotrimeric complex called Sensor of Single-Stranded DNA complex 1 (SOSS1), together with SOSSA(INTS3) and SOSSC(C9orf80) (Huang et al, 2009; Skaar et al, 2009). Unlike RPA, SOSS1 does not localize to replication foci, suggesting that the novel SOSS1 complex functions in DNA damage repair through HR but not in DNA replication (Richard et al, 2008). Both RPA and SOSS1 are recruited to DNA DSB sites but, paradoxically, they exhibit low co-localization (Richard et al, 2008). Depletion of SOSS1 results in loss of checkpoint activation, increased sensitivity to ionizing radiation (IR), defects in DSB resection, and reduced HR (Huang et al, 2009; Richard et al, 2008), suggesting a role for SOSS1 in HR-mediated repair. Recent in vitro studies have suggested an important role for RPA in promoting DSB resection through stimulation of the nuclease-helicase complex Dna2/Sgs1 in yeast and Dna2/BLM in human cells (Cejka et al, 2010a; Nimonkar et al, 2008; Niu et al, 2010). However, the role of the SOSS1 complex in resection and HR-mediated repair has not been characterized and the functional relationship between SOSS complexes and RPA in regulating DSB repair is unclear. Recent reports have shown that the hSBB1 protein localizes at DSB sites very early after DNA damage and binds directly to the MRN complex, suggesting a role in the initiating stages of DSB processing (Richard et al, 2011; Richard et al, 2010).

In this study, we examine the DNA-binding properties of the SOSS1 complex in comparison to RPA, using both ensemble and single-molecule techniques. While RPA exhibits significantly higher affinity for single-stranded DNA and a smaller minimum binding site, SOSS1 has a unique ability to stimulate DSB resection by human Exonuclease 1 (hExo1), a member of the Rad2 family of nucleases which is implicated in numerous DNA repair pathways including mismatch repair (MMR), post-replication repair, meiotic and mitotic recombination, and HR repair (Tran et al, 2004). SOSS1 stimulates the enzymatic activity of hExo1 on DNA by increasing hExo1 recruitment to DNA ends in vitro, whereas RPA specifically activates Dna2, as reported previously (Cejka et al, 2010a; Nimonkar et al, 2008; Niu et al, 2010).

In addition, we demonstrate that MRN in concert with either SOSS1 or RPA can overcome Ku inhibition of hExo1 and Dna2. Overall, we find that SOSS1 binds DNA and affects the activity of DNA processing enzymes in a distinct manner compared to RPA, and propose that the divergent functions of these complexes can be understood in light of these differences.

RESULTS

RPA binds ssDNA with higher affinity compared to SOSS1 T117E

Both RPA and SOSS1 are recruited to DNA double-strand breaks (DSBs) although they exhibit low co-localization (Richard et al, 2008). Unlike RPA, SOSS1 does not localize to replication sites (Huang et al, 2009), indicating that RPA and SOSS1 may have different affinity and specificity for DNA substrates. To address this question, we expressed and purified human RPA and SOSS1 complexes from *E. coli* and insect cell expression systems, respectively. High level expression of the SOSS1 complex required a phosphomimic mutation at threonine 117 of SSB1 (T117E), consistent with previous observations that ATM phosphorylation at this residue stabilizes the SSB1 protein (Richard et al, 2008). All the experiments shown here utilize the T117E form of the SOSS1 complex. The requirement for complex formation between the SOSSA(IntS3) component and the SOSSB (hSSB1) component of SOSS1 has been disputed (Skaar et al, 2009), but here we found that SOSSB and SOSSC (C9orf80) both co-purify with GST-tagged SOSSA with approximately 1:1:1 stoichiometry, consistent with complex formation between these factors as previously reported (Huang et al, 2009).

With the purified recombinant complexes (Figure 3.1), gel mobility shift assays were performed on several [^{32}P]-labeled DNA substrates containing different lengths and

configurations of single-stranded DNA (ssDNA) (Fig. 3.2). Both RPA and SOSS1 complexes bound specifically to ssDNA and showed little binding to double-stranded duplex DNA (Figure 3.2A, B). However, RPA showed significantly higher affinity for the ssDNA overhangs compared to SOSS1, with a K_d of ~15 nM on the substrate containing a 44 nt ssDNA overhang, compared with ~45nM for SOSS1 (Fig. 3.2A). DNA substrates containing either 3' or 5' ssDNA overhangs were also tested, showing that neither complex exhibits a polarity preference (Fig. 3.2B). At least two distinct RPA-ssDNA complexes were apparent in these gels, whereas the complexes of SOSS1 were not resolved under these conditions. The minimum length of ssDNA required for SOSS1 binding was ~35 nt, consistent with previous results with SSB1 protein alone (Richard et al, 2008), which is much larger than the 10 nt minimal binding site reported for RPA (Fanning et al, 2006). SOSS1 binding was also observed with shorter regions of ssDNA (28 nt) when present adjacent to duplex DNA, and RPA binding also increased in efficiency with overhangs present (Figure 3.2C).

Since RPA binding in cells is specific for replication foci (Huang et al, 2009), we also analyzed a DNA substrate with an internal ssDNA gap flanked by double-stranded junctions, which recapitulates the form of DNA present at replication sites (Figure 3.2D, E). Both RPA and SOSS1 exhibited robust binding to this substrate, and again showed that the affinity of RPA is higher than that of SOSS1 (~7 nM for RPA compared to ~35 nM for SOSS1). Taken together, these results suggest that RPA binds to ssDNA with higher affinity than SOSS1, and also can bind more efficiently to a smaller length of

ssDNA. With all the single-stranded DNA substrates tested, it was apparent that SOSS1 exhibits marked cooperativity in binding (Hill coefficients calculated from binding data ranged from 3.0 to 6.8 depending on the length of the single-stranded DNA and the presence of adjacent duplex), whereas RPA exhibited less cooperativity in binding (H=1.6 to 3.1)(Table 3.1).

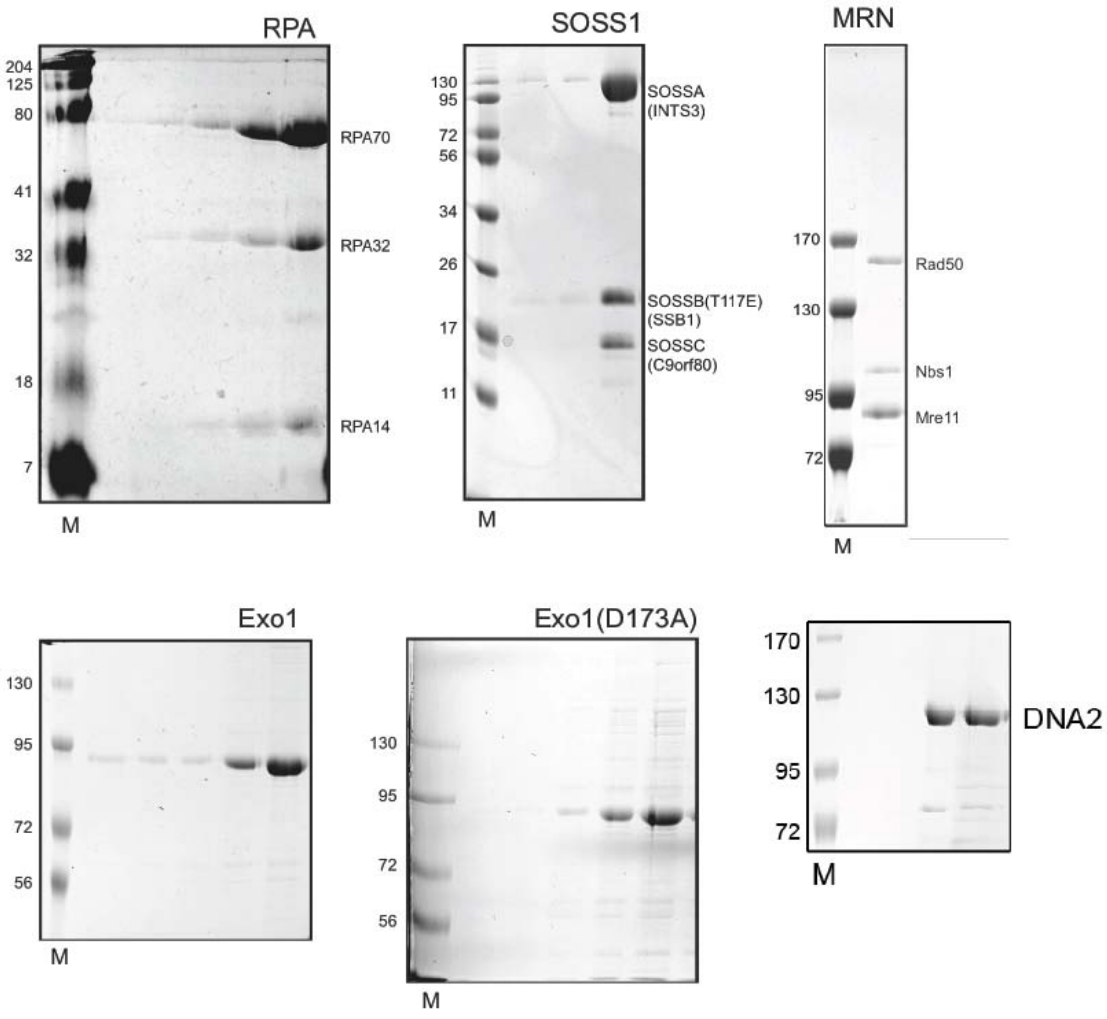

Figure 3.1 Recombinant proteins used in this study.
RPA, SOSS1, MRN, Exo1, nuclease-deficient Exo1 (D173A), and Dna2 were separated by SDS-PAGE and stained with Coomassie Blue.

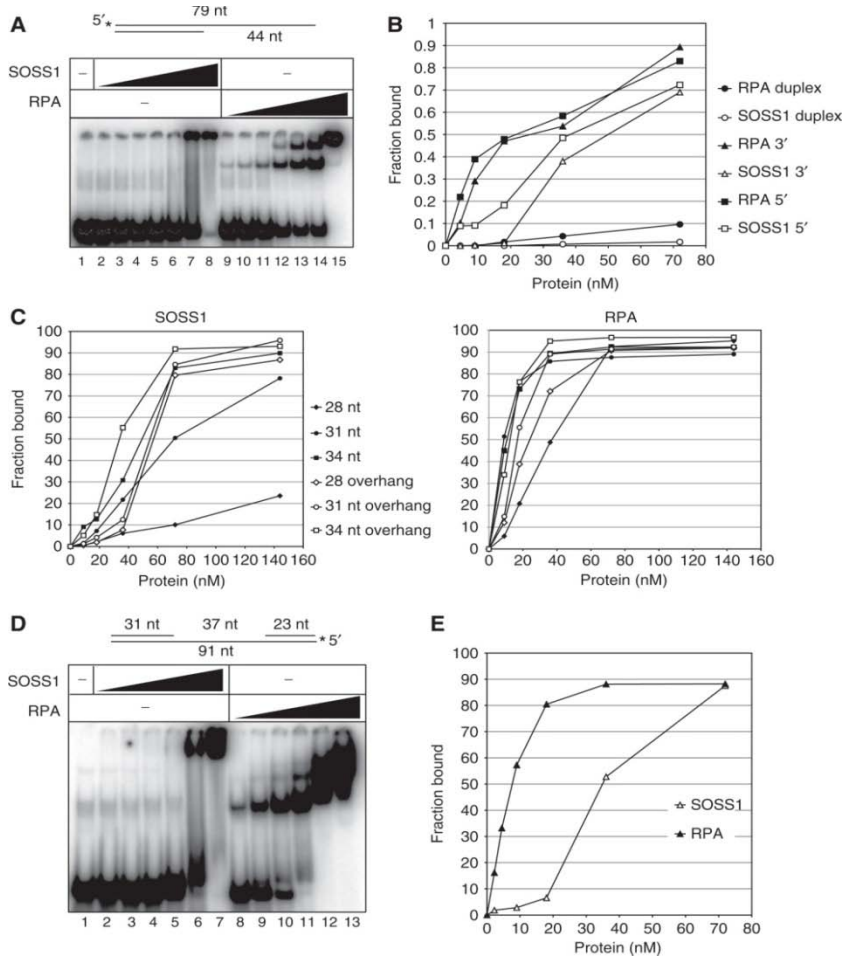

Figure 3.2 Gel shift assays with recombinant SOSS1 T117E and RPA.
(A) Gel shift assays were performed with SOSS1 complex and RPA on a ^{32}[P]-labeled subtrate containing a 44 nt ssDNA 3' overhang and a 35 bp duplex region as shown. Each reaction contained 1.1, 2.2, 4.5, 9, 18, 36, or 72 nM SOSS1 complex or RPA. The reactions were separated in a native 8% acrylamide gel which was analyzed by phosphorimager. (B) DNA binding assays were performed as in (A) with substrates containing a 44 nt 5' overhang, a 44 nt 3' overhang, or a 50 bp duplex DNA, and quantification of the results from a representative experiment are shown. Reactions contained 4.5, 9, 18, 36, or 72 nM SOSS1 or RPA. (C) Binding assays were performed with SOSS1 as in (A) with various lengths of radiolabeled ssDNA (28, 31, or 34 nt) or substrates containing 28, 31, or 34 nt 5' overhangs adjacent to 31nt duplex DNA and the quantified results from a representative set of experiments is shown. (D) Binding assays were performed with SOSS1 and RPA as in (A) with a substrate containing an internal ssDNA gap as shown. Each reaction contained 2.25, 4.5, 9, 18, 36, or 72 nM of SOSS1 or RPA. Quantification of these results is shown in (E).

Summary of SOSS1 and RPA binding coefficients for single-stranded DNA substrates

	SOSS				RPA			
	28 nt overhang	31 nt overhang	34 nt overhang	37 nt internal	28nt overhang	31 nt overhang	34 nt overhang	37 nt internal
h	6.808	5.564	3.025	4.061	2.282	3.151	2.813	1.636
Kd	50.66	50.5	32.05	33.37	21.08	15.65	11.28	6.283
Std. Error:								
h	*0.4025*	*0.5272*	*0.3627*	*0.4373*	*0.1199*	*0.217*	*0.09751*	*0.131*
Kd	*1.089*	*1.775*	*1.471*	*0.8346*	*0.5627*	*0.3747*	*0.139*	*0.3463*

Table 3.1 Equilbrium binding constants (Kd, nM) and Hill coefficients (h) calculated from binding data in Figure 3.2.

The data was fit by nonlinear regression and standard error is shown.

Less stable, more dynamic binding of SOSS1 to ssDNA compared to RPA

To measure the binding of RPA and SOSS1 to ssDNA with greater accuracy, we employed single-molecule Fluorescence Resonance Energy Transfer (smFRET), a single molecule method to sensitively monitor the distribution and changes of distance between a donor and an acceptor fluorophore in the range of 3-8 nm (Roy et al, 2008). We used partial duplex DNA molecules containing a 3' Poly (T) tail ($(dT)_{m+n}$; Figure 3.3A). The donor (Cy3) and the acceptor (Cy5) were attached to the ss-dsDNA junction and the middle of the ssDNA tail, respectively, separated by $(dT)_m$. A small distance between the two fluorophores would result in high FRET efficiencies and vice versa.

We first tested RPA and SOSS1 binding to a partial duplex containing a 3' 40 nt tail ($(dT)_{16+24}$; Cy3 and Cy5 were separated by 16 nt). A relatively high FRET peak centered at 0.53 was initially observed in the single molecule histogram of FRET efficiency for $(dT)_{16+24}$ only (i.e. without any protein; Figure 3.3B), due to the fact that ssDNA is very flexible (Murphy et al, 2004). Protein binding is expected to decrease FRET via ssDNA stretching unless ssDNA wraps around the protein. After adding 200 pM to 2 nM RPA, two new peaks at low FRET efficiencies (~ 0.42 and ~ 0.29) appeared in the FRET histogram as we increased the RPA concentration, suggesting that one and two RPA bind to each $(dT)_{16+24}$ DNA molecule respectively. We recorded the time evolution of the FRET histogram after the removal of the excess unbound RPA from our sample chamber (Figure 3.3C). The 0.29 FRET population shifted to the 0.42 FRET

population over time, indicating one of the two bound RPA dissociated from $(dT)_{16+24}$. The occluded site size for RPA is 30 nt with all of the four major ssDNA-binding domains involved, but RPA can bind to 10 nt ssDNA using only two ssDNA-binding domains with a lower affinity (Fanning et al, 2006). $(dT)_{16+24}$ that contains a 40 nt tail is hence sufficient for two RPA binding : one RPA tightly binds in the 30 nt binding mode and the other RPA weakly binds in the 10 nt binding mode. Indeed, 95 min after flushing out unbound RPA, the 0.29 FRET peak almost disappeared. Dissociation events of the second, weakly bound RPA could also be found in some single molecule FRET-time traces (Figure 3.3D). In contrast, the remaining RPA likely in the 30 nt binding mode, yielding the 0.42 FRET peak, was very stable and we could not detect its dissociation even 1-2 hours after flushing out unbound RPA (Figure 3.3C, Figure 3.4B and G).

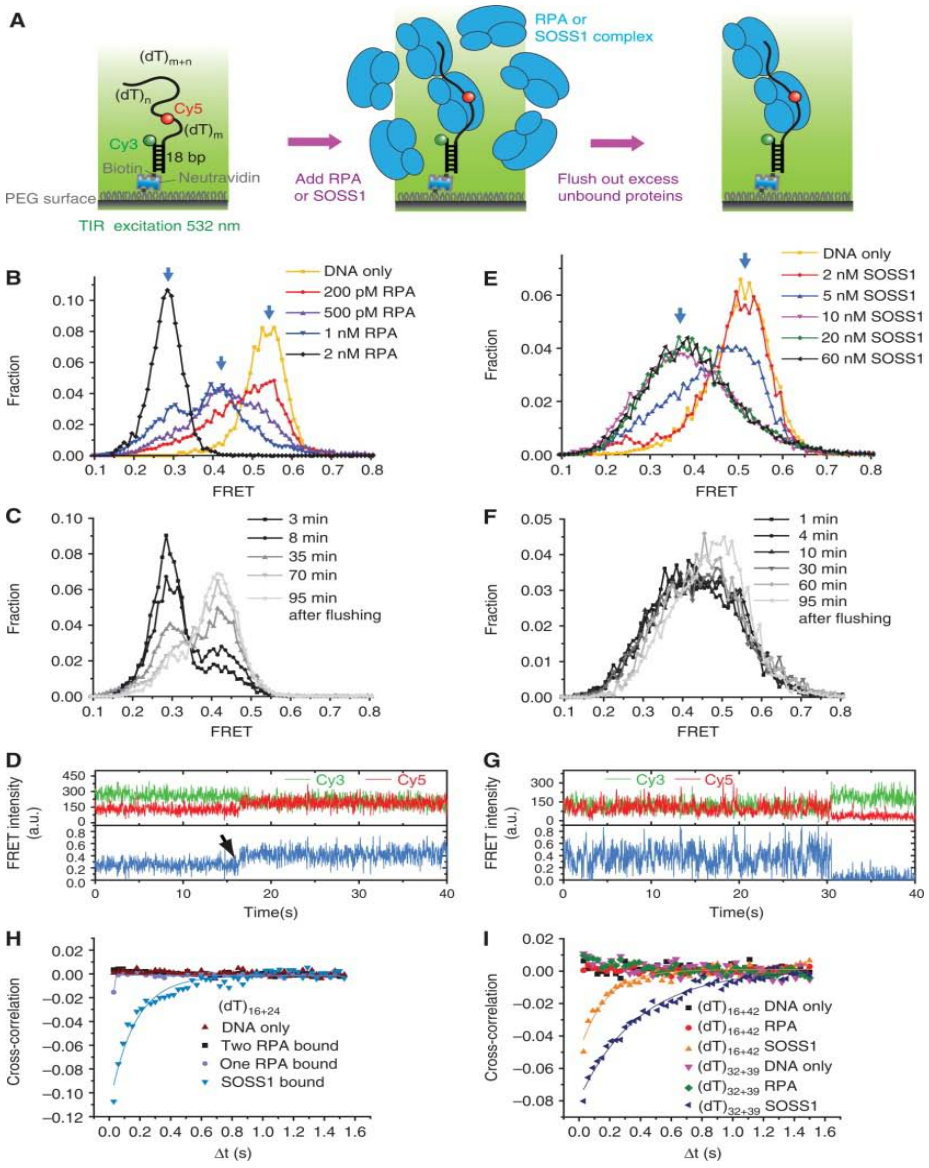

Figure 3.3 Single-molecule studies of RPA and SOSS1 binding to ssDNA.
(A) A schematic representation of reaction steps for single molecule FRET measurements. *m* and *n* are in the unit of nucleotides (B) FRET efficiency histograms for $(dT)_{16+24}$ DNA only, and RPA binding to $(dT)_{16+24}$ at different protein concentrations. Three distinct peaks centered at FRET = 0.53, 0.42 and 0.29 were observed at varying RPA concentrations (blue arrows), representing 0, 1 and 2 RPA binding to $(dT)_{16+24}$ respectively. (C) Time evolution of the FRET histogram for $(dT)_{16+24}$ after incubating with 2 nM RPA and flushing out the excess unbound RPA. (D) A single-molecule FRET-time trace for $(dT)_{16+24}$ obtained after incubating with 2 nM RPA and flushing out the excess unbound RPA, showing a RPA dissociation event (the black

arrow). After the event, there was still one RPA remaining bound, giving a FRET of 0.42. (E) FRET efficiency histograms for $(dT)_{16+24}$ DNA only, and SSOS binding to $(dT)_{16+24}$ at different protein concentration. Two distinct peaks centered at FRET = 0.53 and 0.37 were observed at varying SOSS concentrations (blue arrows), representing 0 and 1 SOSS binding to $(dT)_{16+24}$ respectively. (F) Time evolution of the FRET histogram for $(dT)_{16+24}$ after incubating with 60 nM SOSS-1 and flushing out the excess unbound SOSS. (G) A representative single-molecule FRET-time trace for $(dT)_{16+24}$ obtained after incubating with 60 nM SSOS and flushing out the excess unbound SSOS. At t = 30 s, Cy5 was photobleached. (H) Average cross-correlations of Cy3 and Cy5 intensity time traces for $(dT)_{16+24}$ DNA only, one or two RPA bound, and one SSOS bound. Each cross-correlation was averaged over more than 100 molecules. (I) Average cross-correlations of Cy3 and Cy5 intensity time traces for other two DNA substrates, $(dT)_{16+46}$ and $(dT)_{32+39.}$ The FRET-time traces for cross-correlation analysis were obtained after flushing out unbound RPA or SSOS, in order to assure the time-resolved anti-correlation comes only from the bound proteins. Single exponential fits are also shown (solid lines).

Previously, we showed that *E coli* SSB diffuses along ssDNA based on smFRET signal fluctuations and that the diffusion time scale can be estimated by performing a cross-correlation analysis of donor and acceptor intensity-time traces (Roy et al, 2009; Zhou et al, 2011). Here, we calculated the average cross-correlation functions between the Cy3 and Cy5 intensity-time traces separately for 0.53, 0.42 and 0.29 FRET states from many $(dT)_{16+24}$ molecules (Figure 3.3H). Other than a very short time scale anti-correlation for the 0.42 FRET state, yielding a characteristic time (τ) of 5 ± 2 ms, faster than our time resolution of 30 ms, we could not observe any evidence of anti-correlated fluctuations of the donor and acceptor intensities, indicating that movement of RPA on ssDNA is either minimal or too fast to be detected within the 30 ms time resolution.

Next, we repeated the smFRET experiment for SOSS1 binding to $(dT)_{16+24}$. Besides the DNA only peak (~0.53 FRET), only one additional peak (~0.37 FRET) was observed at all SOSS1 concentrations (2 to 60 nM), suggesting that the 40 nt tail is sufficient for only one binding unit of SOSS1 (Fig. 3.3E). After flushing out the excess unbound SOSS1, we found that SOSS1 dissociates slowly (Figure 3.3F and Figure 3.4). Clear and large amplitude FRET fluctuations were observed in FRET-time trace for the SOSS1 bound state (i.e., 0.37 FRET state; Figure 3.3G). The average cross-correlation between the Cy3 and Cy5 intensity-time traces shows a single exponential function with τ = 163 ± 12 ms, suggesting that SOSS1 on ssDNA is very dynamic in the milliseconds time scale (Figure 3.3H).

We further tested two other partial duplex DNA molecules, $(dT)_{16+42}$ and $(dT)_{32+39}$. The cross-correlation analysis suggests that SOSS1 is also more dynamic when bound to these two DNA molecules ($\tau = 170 \pm 15$ ms for $(dT)_{16+42}$ and 388 ± 22 ms for $(dT)_{32+39}$) compared to RPA. τ, which is the measure of the time scale of the dynamics, increases as the tail length of the partial duplex increases. We could rule out transient destabilization of the duplex region by SOSS1 as a source of FRET fluctuations (Figure S2). Therefore, it is possible that SOSS1 diffusion on the ssDNA, as was demonstrated for *E coli* SSB (Roy et al, 2009; Zhou et al, 2011), is responsible for the observed FRET fluctuations although a definitive demonstration requires further experiments.

Neither RPA nor SOSS1 binding to ssDNA resembles the 'closed wrapping' structure observed in the *E coli* SSB-DNA complexes (Lohman & Ferrari, 1994) (Figure 3.4). Rather, both RPA and SOSS1 binding increase the end-to-end distance of the ssDNA region to which they bind, consistent with the extended structure of RPA-ssDNA complexes in microscopy experiments (Blackwell et al, 1996). Additionally, SOSS1 binds to ssDNA less tightly than RPA (30 nt binding mode) (See also Figure 3.4) or *E coli* SSB (*E coli* SSB stays bound to ssDNA in its 65 nt binding mode for 5 hours after flushing out the unbound proteins) (Roy et al, 2009; Zhou et al, 2011).

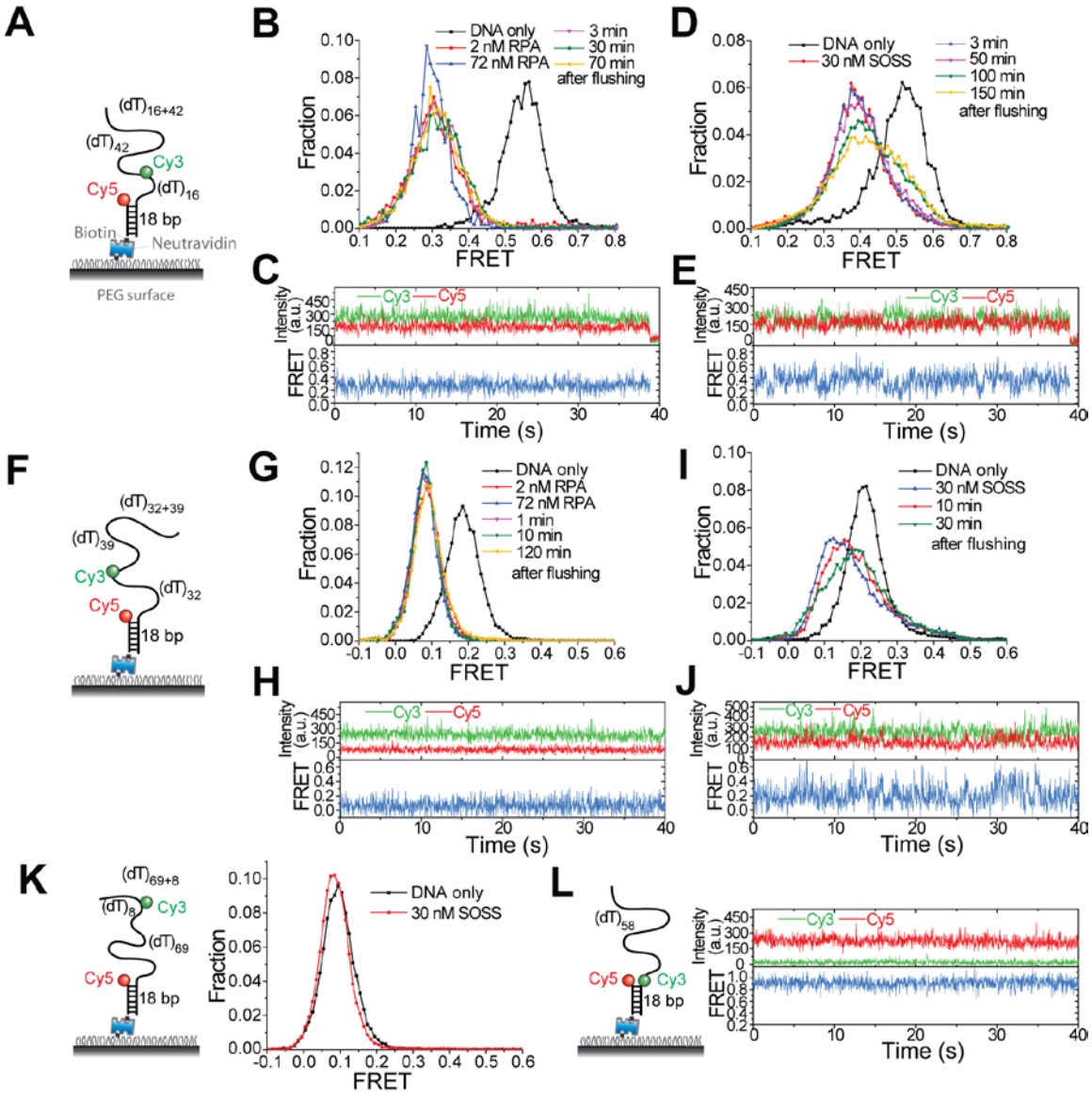

Figure 3.4 RPA and SOSS1 binding to ssDNA substrates is more stable with increasing length of ssDNA.

(A) A schematic representation of $(dT)_{16+42}$. The ssDNA tail contains 58 nt and may allow two RPA to bind with the 30 nt binding mode. Cy3 and Cy5 was separated by 16 nt as in $(dT)_{16+24}$. (B) FRET efficiency histograms for $(dT)_{16+24}$ DNA only and RPA binding to $(dT)_{16+24}$ in 2 and 72nM RPA, and the time evolution of the FRET histogram after incubating with 72nM RPA and flushing out the excess unbound RPA. Similar to the data for $(dT)_{16+24}$ (Figure 3.3B), the peaks centered at 0.52 and 0.29 FRET represent 0 and 2 RPA binding to $(dT)_{16+24}$ respectively. (C) A representative single-molecule FRET-time

tract for $(dT)_{16+42}$ obtained after incubating with 72nM RPA and flushing out the excess unbound RPA. (D) FRET efficiency histograms for $(dT)_{16+46}$ DNA only and SOSS1 binding to $(dT)_{16+42}$ in 30nM SOSS1, and the time evolution of the FRET histogram after incubating with 30nM SOSS1 and flushing out the excess unbound SOSS1. The peaks centered at 0.52 and 0.38 FRET represents 0 and 1 SOSS1 binding to $(dT)_{16+42}$ respectively. (E) A representative single-molecule FRET-time trace for $(dT)_{16+42}$ obtained after incubating with 30nM SOSS1 and flushing out the excess unbound SOSS1. (F) A schematic representation of $(dT)_{32+39}$. The ssDNA tail contains 71nt and may allow two RPA to bind with the 30nt binding mode and one RPA bind with the 10nt binding mode. (G) FRET efficiency histograms for $(dT)_{32+39}$ DNA only and RPA binding to $(dT)_{32+39}$ in 2 and 72nM RPA, and the time evolution of the FRET histogram after incubating with 72nM RPA and flushing out the excess unbound RPA. The peak centered at 0.08 FRET may represent 2 or 3 RPA binding. (H) A representative single-molecule FRET-time trace for $(dT)_{32+39}$ obtained after incubating with 72nM RPA and flushing out the excess unbound RPA. (I) FRET efficiency histograms for $(dT)_{32+39}$ DNA only and SOSS1 binding to $(dT)_{32+39}$ in 30nM SOSS1, and the time evolution of the FRET histogram after incubating with 30nM SOSS1 and flushing out the excess unbound SOSS1. (J) A representative single-molecule FRET-time trace for $(dT)_{16+42}$ obtained after incubating with 30nM SOSS1 and flushing out the excess unbound SOSS1. (K) FRET histograms for $(dT)_{69+8}$ in the absence and presence of 30nM SOSS1. (L) Test for duplex destabilization by SOSS1. A partial duplex with a 59nt tail was used where Cy3 and Cy5 were placed next to each other at the ss-dsDNA junction such that high FRET (~0.9) is observed for DNA only. This DNA construct and $(dT)_{16+42}$ only differ in fluorophore labeling locations. Any significant local breathing (or melting) of the duplex DNA at the junction that lasts longer than our experimental time resolution (30ms) would be reported as a drop in FRET. A representative single-molecule FRET-time trace is shown, which was obtained after incubating with 30nM SOSS1 and flushing out the excess unbound SOSS1.

SOSS1 T117E and MRN stimulate hExo1 resection of DSBs in vitro

SOSS1 and RPA complexes have both been shown to influence DSB resection in human cells (Richard et al, 2008; Richard et al, 2010). In vitro, RPA stimulates the activity of Dna2/Sgs1 complexes (Cejka et al, 2010a; Niu et al, 2010) and also coats ssDNA once resection has occurred, prior to loading of Rad51 (Flynn & Zou, 2010). However, the role of SOSS1 in resection and how it relates to the role of RPA is unknown.

To examine if SOSS1 and RPA function directly in 5' strand processing, in vitro resection assays were performed with a 4.5 kb linear DNA and purified recombinant human Exonuclease 1 (Exo1) in the presence of SOSS1 or RPA (Figure 3.5A). The products of the reactions were visualized by DNA stain (SYBR Green) (top panel) and by nondenaturing southern hybridization using an RNA probe complementary to a 1 kb region of the 3' strand at one end of the DNA substrate (middle panel). To further characterize the extent of resection, quantitative PCR (qPCR) was also used to determine the level of ssDNA generated by resection at sites located 29 nt and 1025 nt from the DNA end (Figure 3.5B) as previously described (Nicolette et al, 2010). With limiting concentrations of Exo1 (0.375 nM under these conditions), SOSS1 complex strongly stimulated hExo1-mediated resection of the 5' end of DNA (Figure 3.5A), generating 11-fold and 55-fold higher levels of ssDNA at sites located 29 nt and 1025 nt from the DNA end, respectively (Figure 3.5B). This stimulation depends on the nuclease activity of

hExo1, as SOSS1 was unable to stimulate 5’ strand resection in the presence of a nuclease-deficient mutant of hExo1 (Exo1 D78A/D173A) (Lee et al, 2002; Orans et al, 2011) (Figure 3.5A, lane 11). In contrast, RPA was not stimulatory and in fact actually limited the resection activity of Exo1 at DNA ends (Figure 3.5A, lane 4) similar to previous results shown with yeast Exo1 in vitro (Nicolette et al, 2010). This was also the case when higher levels of Exo1, RPA, and substrate were used in order to compare directly to previously published data (Nimonkar et al, 2011) (Figure 3.6).

Previous studies have shown the direct involvement of Mre11/Rad50 and Mre11/Rad50/ Xrs2 complexes in DSB resection (Hopkins & Paull, 2008; Langerak et al, 2011; Nicolette et al, 2010; Nimonkar et al, 2011; Sartori et al, 2007). In addition, MRN has been reported to affect RPA foci formation at DNA damage sites in vivo and to interact with hSSB1 to stimulate the endonuclease activity of MRN in vitro (Richard et al, 2008; Richard et al, 2010). This led us to further test the effect of human MRN in DNA end processing in vitro with Exo1 in the presence of SOSS1 and RPA complexes. In reactions performed with MRN and Exo1, MRN stimulated Exo1 activity in 5’ strand processing 6 to 18-fold, similar to our previous results with yeast MRX and Exo1 (Nicolette et al, 2010). SOSS1 and MRN each stimulated Exo1-mediated 5' strand resection independently, and did not exhibit cooperative functional interactions. In contrast to a previous report with hSSB1 and MRN (Richard et al, 2011), we also did not observe any stimulation of MRN nuclease activity by SOSS1 (Figure 3.7). These data demonstrate that SOSS1 and RPA have very different effects on Exo1 activity. SOSS1

stimulates Exo1 activity in 5’ strand resection similar to the effect of MRN, whereas RPA limits the extent of Exo1 activity in resection.

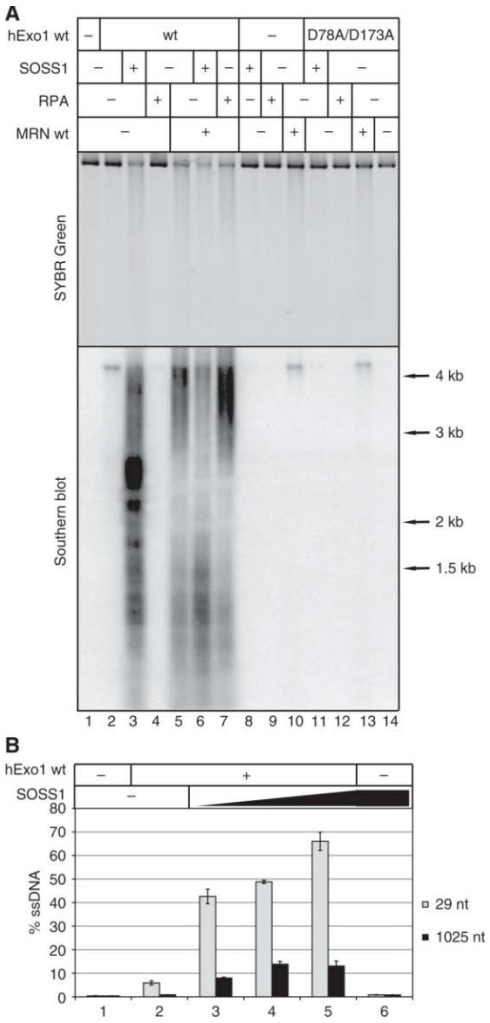

Figure 3.5 SOSS1 promotes resection by hExo1.
(A) Resection reactions were performed with 0.375 nM Exo1 wild-type or nuclease-deficient (D78A/D173A), 40 nM SOSS1(T117E) complex or 40 nM RPA, and 31.25 nM wild-type MRN. Reactions were separated in a 1% non-denaturing agarose gel and further analyzed by SYBR Green staining (top panel) for total DNA and by non-denaturing Southern hybridization (middle panel) using an RNA probe complementary to the 3' end of DNA adjacent to one of the break sites. The small products observed in the southern are likely generated by simultaneous digestion of the DNA from both ends of the molecule (Hopkins & Paull, 2008). (B) Resection reactions were performed with 0.375 nM Exo1 and 14, 42, or 126 nM SOSS1 and the amount of single-stranded DNA produced was quantified through quantitative PCR (qPCR) using two sets of primers to measure ssDNA levels at sites located 29 nt or 1025 nt from the DNA end as described previously (Nicolette et al, 2010). Shown is the average percent of ssDNA from 3 experiments; error bars indicate standard deviation.

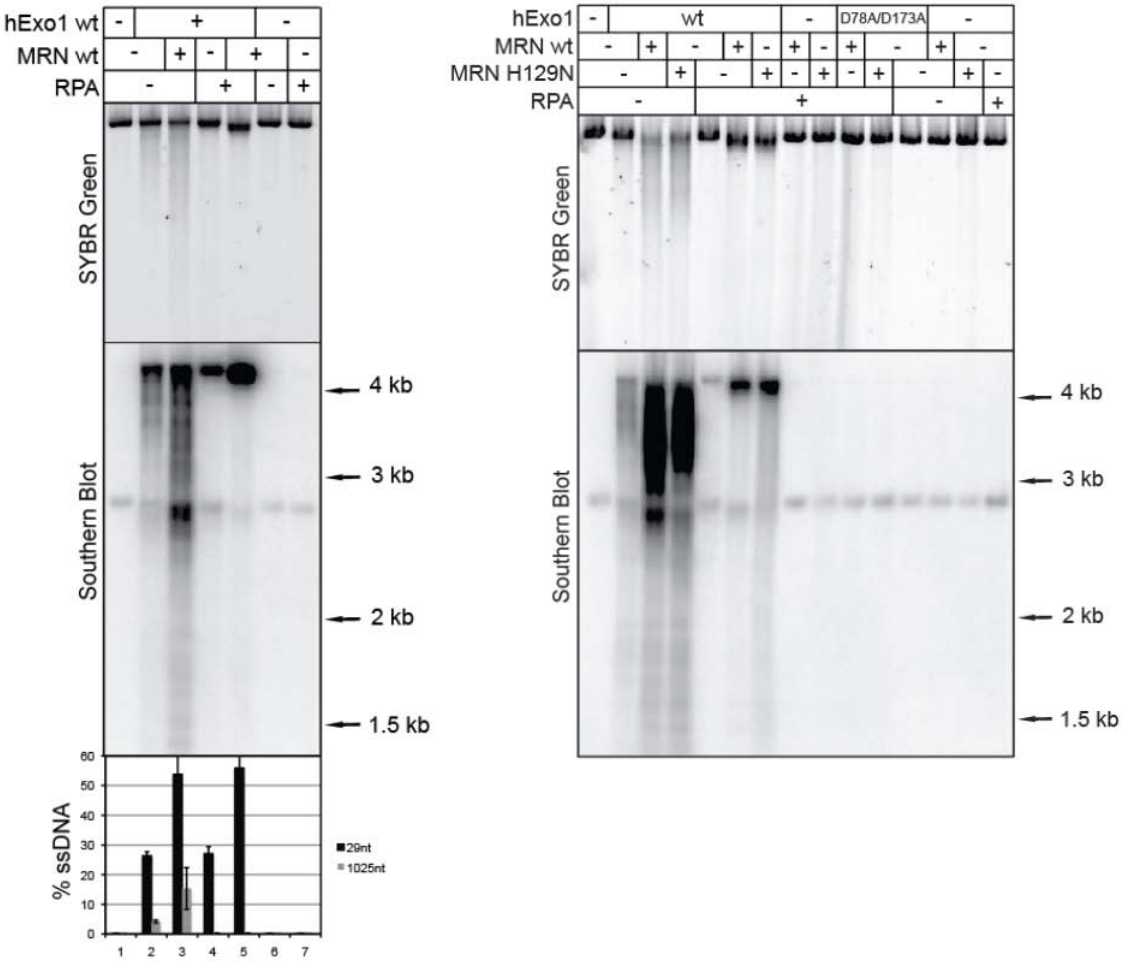

Figure 3.6 RPA limits long-range resection by Exo1.
Resection assays were performed in vitro with RPA (200 nM), Exo1 (1.0 nM), MRN (5 nM), and 0.67 nM linearized plasmid DNA substrate in the presence of 25mM MOPS, 2mM ATP, 0.1 mM DRR, 100 µl/mL BSA, 0.05% Triton X-100, and 5 mM $MgCl_2$ (similar to previously published conditions (Nimonkar et al, 2011)). Reaction products were analyzed by SYBR green staining (top), by non-denaturing southern blot using a probe for the 3' strand (middle), or by qPCR (bottom). Right panel: Resection assays were performed as in the left panel except with nuclease-deficient MRN and Exo1 as indicated. Reaction products were analyzed by SYBR green staining (top) and by non-denaturing southern blot using a probe for the 3' strand (bottom).

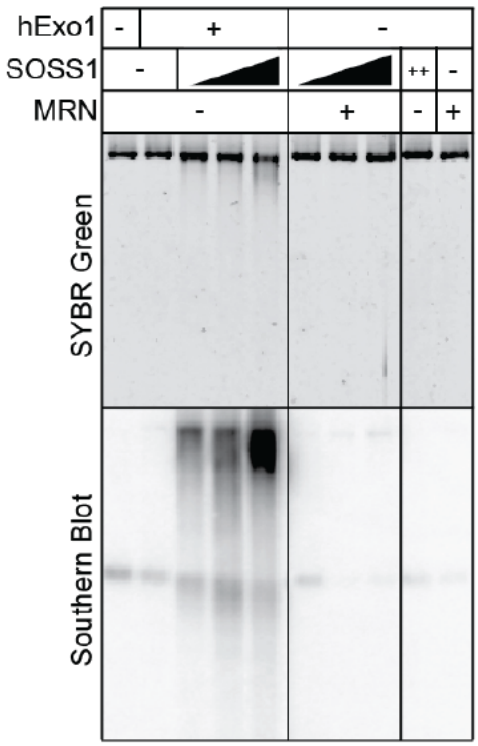

Figure 3.7 SOSS1 does not stimulate MRN nuclease acitivity.
Resection assays were performed in vitro as in Figure 3.5 with SOSS1 (14, 42, and 126 nM), Exo1 (0.4 nM), and MRN (4.5 nM). Reaction products were analyzed by SYBR green staining (top) or by non-denaturing southern blot using a probe for the 3' strand. "++" indicates highest level of SOSS1 used in this reaction.

SOSS1 overcomes RPA inhibition of Exo1-mediated resection

As RPA exhibits higher affinity for ssDNA and binds more stably compared to SOSS1, we then asked how SOSS1 would function in resection in the presence of RPA. Consistent with the results in Figure 3.5, SOSS1 stimulated 5' strand processing in the presence of hExo1 (0.374 to 1.5 nM), while RPA limited the extent of Exo1 activity (Figure 3.8A, B). Notably, in reactions where SOSS1 and RPA were incubated together with Exo1, the presence of SOSS1 was sufficient to overcome the limiting effect of RPA on Exo1 to a comparable extent as MRN (Figure 3.8A, compare lanes 12-13 with lanes 14-15). However, addition of SOSS1 and MRN together to reactions containing Exo1 and RPA did not have any further stimulatory effect on resection (Figure 3.8A, lane 16 and 17). These results suggest that SOSS1 and MRN can independently stimulate Exo1 activity and overcome the limiting effect of RPA on Exo1 to promote 5' strand resection.

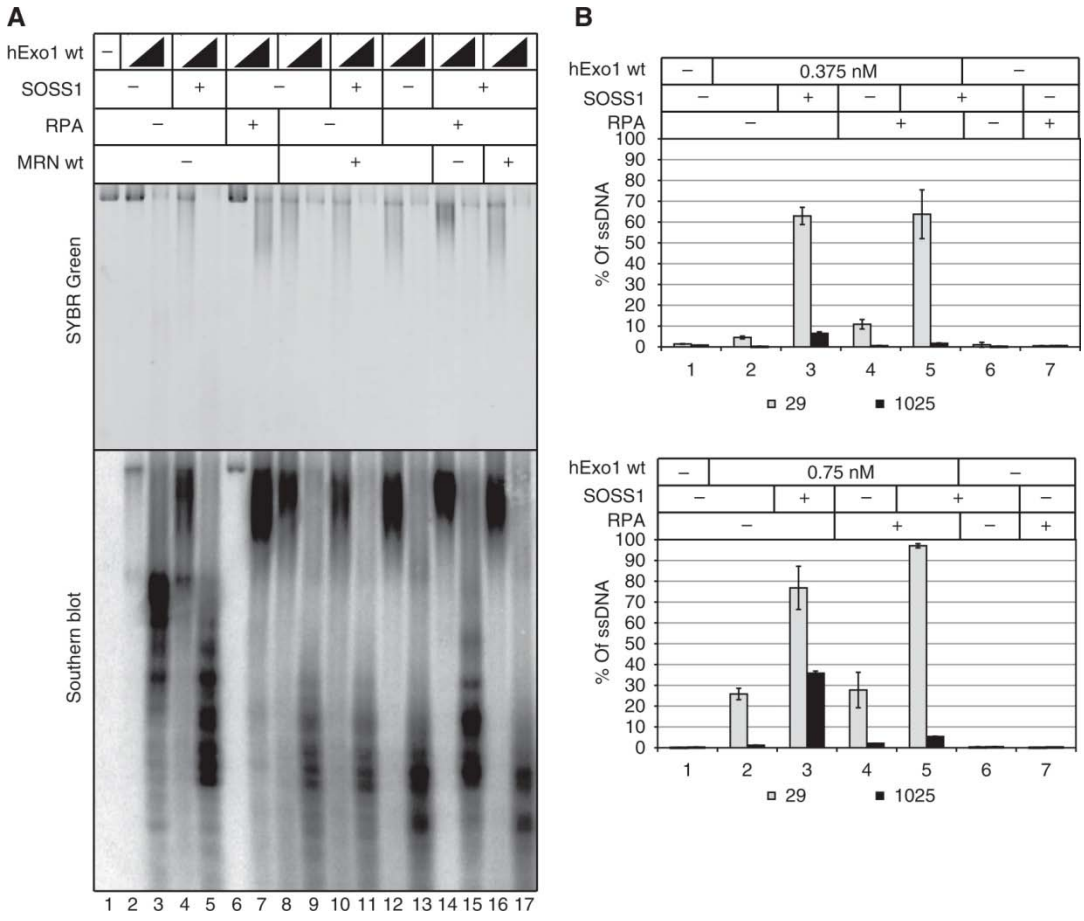

Figure 3.8 The SOSS1 complex can block RPA inhibition of Exo1.
(A) Resection assays were performed as in Fig. 3.5 except that each reaction contained 0.375 or 1.5 nM hExo1. (B) Resection assays as in (A) with either 0.375 nM (top) or 0.75 nM (bottom) Exo1, quantified as in Fig. 3 by qPCR.

SOSS1 stimulates the exo- and endonuclease activities of hExo1

Human Exo1 has been reported to exhibit both 5' to 3' exonuclease activity as well as 5' flap structure-specific endonuclease activity (Lee & Wilson, 1999). To characterize the resection products generated by Exo1 in the presence of SOSS1, RPA, and MRN, resection assays were performed using a 1.7 kb DNA substrate internally labeled with [^{32}P]. The exonuclease activity of Exo1 was measured by quantifying the amount of radiolabeled NMP released using thin layer chromatography. Exo1 by itself exhibits low exonuclease activity in this assay (Figure 3.9A, lane 2 and Figure 3.10). However, addition of SOSS1 stimulated the exonuclease activity of Exo1 by approximately 6-fold while RPA did not increase its activity (Figure 3.9A, compare lane 2 with lanes 7-8; also the quantitation of triplicate experiments in Figure 3.9B). Similar to the results shown in Fig. 3 and 4, MRN stimulated the exonuclease activity of Exo1 to a comparable level as that observed with SOSS1 (Figure 3.9A, compare lane 2 with lanes 6-7). Both SOSS1 and MRN increased the exonuclease activity of Exo1 in the presence of RPA but their stimulatory activity was not cooperative (Figure 3.9A, lanes 9-12).

To examine the effects of SOSS1 and RPA on the endonucleolytic activity of Exo1, nuclease assays were performed using a 5' Cy3/Cy5-labeled 717 bp DNA substrate and analyzed using a denaturing sequencing gel (Figure 3.9B). In reactions containing both SOSS1 and Exo1, 5' labeled products ranging from 8-46 nt were formed (Figure 3.9B, lane 3) whereas RPA with Exo1 did not produce any oligonucleotide products (Figure 3.9B, lane 4). These products were dependent on the presence of Exo1 and can

only be generated by endonucleolytic activity. Taken together, these results suggest that SOSS1 complex participates in DNA DSB resection through a functional interaction with Exo1, and promotes both the exonuclease and endonuclease activities of this enzyme.

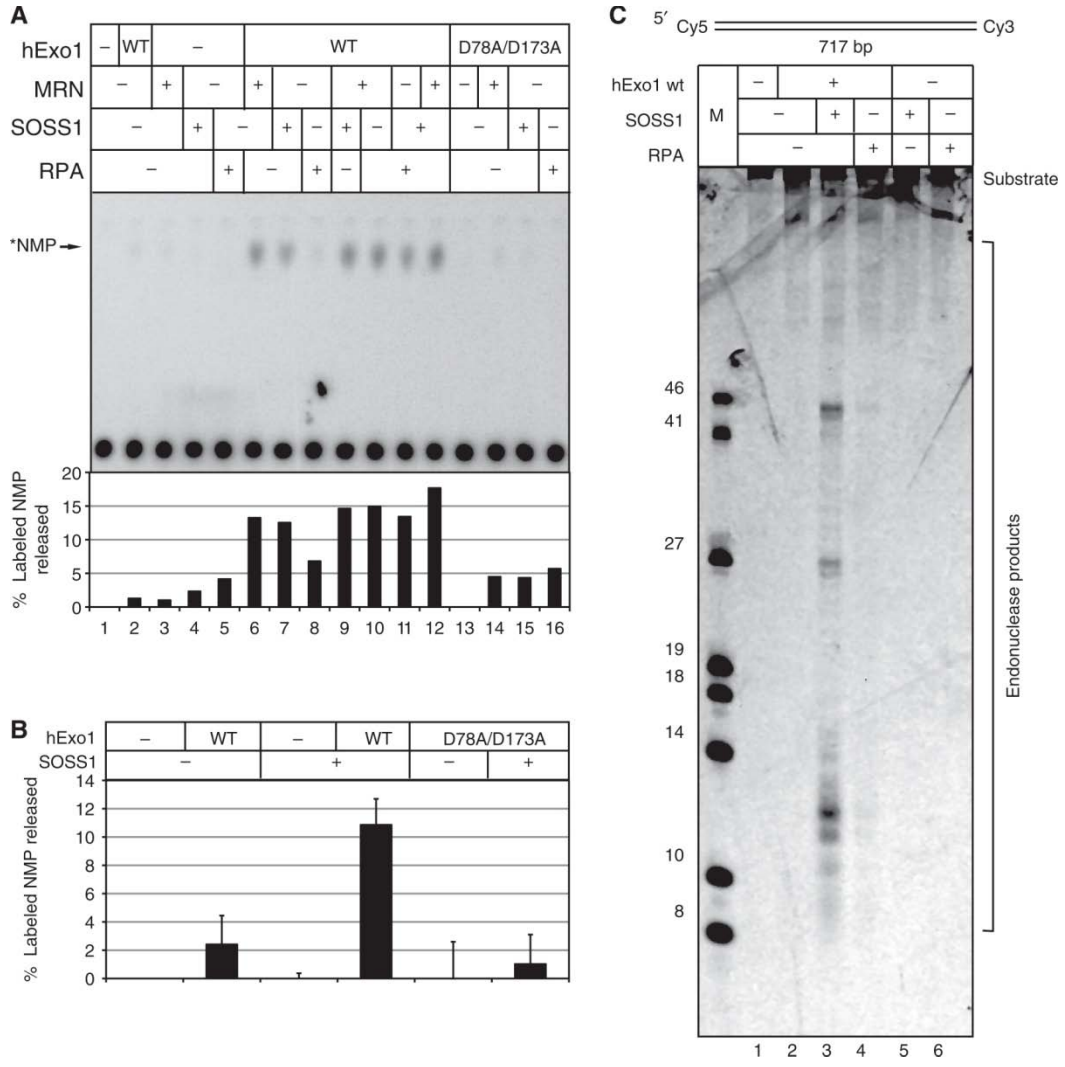

Figure 3.9 The SOSS1 complex promotes both exo- and endonucleolytic cleavage of DNA by Exo1.

(A) Resection assays were performed using a 1.7 Kb DNA substrate internally labeled with [^{32}P], containing 0.16 nM wild-type Exo1 and Exo1(D78A/D173A), 15 nM MRN, 36 nM SOSS1(T117E) complex, and 36nM RPA. Reaction products were separated by thin layer chromatography, analyzed by phosphorimager, and the amount of labeled NMP released by the exonuclease activity of hExo1 was quantified by the ImageQuant software. (B) Resection assays as in (A) with 0.16 nM wild-type Exo1 and 36 nM SOSS1 (T117E) complex; the average of three experiments is shown, with standard deviation. (C) Resection assays were performed using a Cy3/Cy5 labelled 717bp DNA substrate, incubated with wild-type Exo1 (5 nM), SOSS1 (T117E) complex (72 nM) and RPA (72 nM). Reaction products were analyzed on a 20% denaturing sequencing gel and scanned for Cy5 emission.

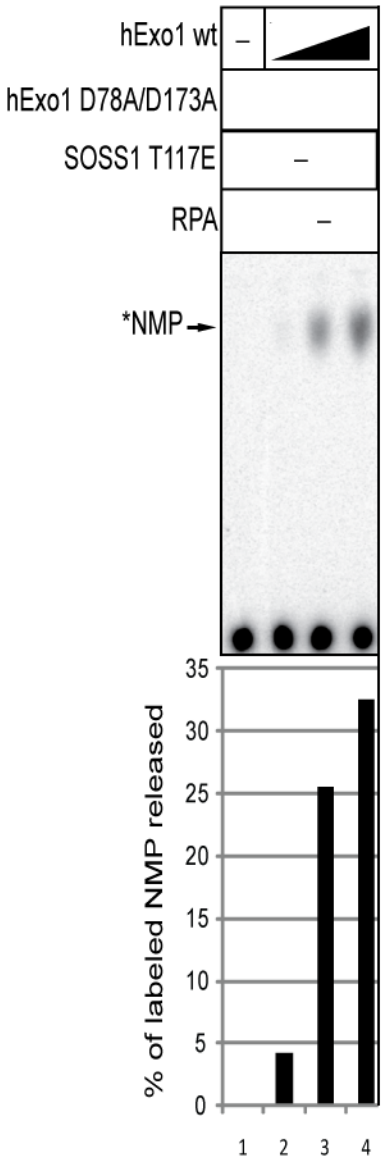

Figure 3.10 Exo1 generates single nucleotide products.
Wild-type human Exo1 (4, 12, and 36 nM) was incubated with a 700 bp duplex, internally labeled with ^{32}[P]. Products from the reaction were separated by thin-layer chromatography (top panel) and quantified (bottom panel).

SOSS1 stimulates the recruitment of Exo1 to DNA ends

SOSS1 complexes promote a dramatic increase in the enzymatic activity of Exo1, but the functional mechanism underlying this process remains to be determined. Previous studies with the bacterial SSB protein have shown that SSB protein stimulates RecJ by increasing the affinity of RecJ to DNA substrates (Han et al, 2006a; Handa et al, 2009; Sharma & Rao, 2009), and we previously found that MRX and Sae2 promote the binding of yeast Exo1 to DNA ends (Nicolette et al, 2010). Therefore we examined whether SOSS1 could affect Exo1 recruitment to DNA ends by performing gel mobility shift assays using a 717 bp double-stranded PCR product internally labeled with [^{32}P] and also containing three azide groups at each 5' end. The substrate was incubated with nuclease-deficient Exo1 (D78A/D173A) in the presence or absence of SOSS1 or RPA to examine DNA binding by Exo1 in the absence of catalysis. After incubation, the reaction was exposed to 254nm UV light to induce cross-linking of the azide groups with amino acids located close to the azide moieties, separated in a non-denaturing agarose gel, and visualized by phosphorimager analysis. Neither Exo1 nor SOSS1 bound to this substrate efficiently when incubated alone, but incubation of the complexes together produced a distinct gel shift product that was dependent on the concentration of hExo1 (Figure 3.11A, lanes 3 and 6). In contrast, RPA did not form a gel shift product with Exo1 D78A/D173A (Figure 3.11A, lane 4 and 7), suggesting that a specific interaction between SOSS1 and Exo1 forms on double-stranded DNA ends.

To further confirm the formation of a cooperative complex containing Exo1 and SOSS1, a DNA binding assay was performed using a 717 bp PCR product containing biotin on the end of one 5’ strand and three azide groups on the opposite 5’ strand of the DNA as shown in Figure 3.11B. The substrate was initially incubated with magnetic streptavidin beads for immobilization, and was then incubated with Exo1 D78A/D173A and SOSS1 proteins and cross-linked with UV as in Figure 3.11A. The immobilized DNA and bound protein were released from the magnetic beads under denaturing conditions, separated by SDS-PAGE, and analyzed by western blot using an antibody specific for Exo1. This assay showed that Exo1 alone did not bind efficiently to DNA but SOSS1 promoted the recruitment of hExo1 to the substrate (Figure 3.11B). Overall, we conclude that SOSS1 stimulates DSB processing by promoting Exo1 recruitment to DNA ends, leading to an increased activity of Exo1 at DSBs.

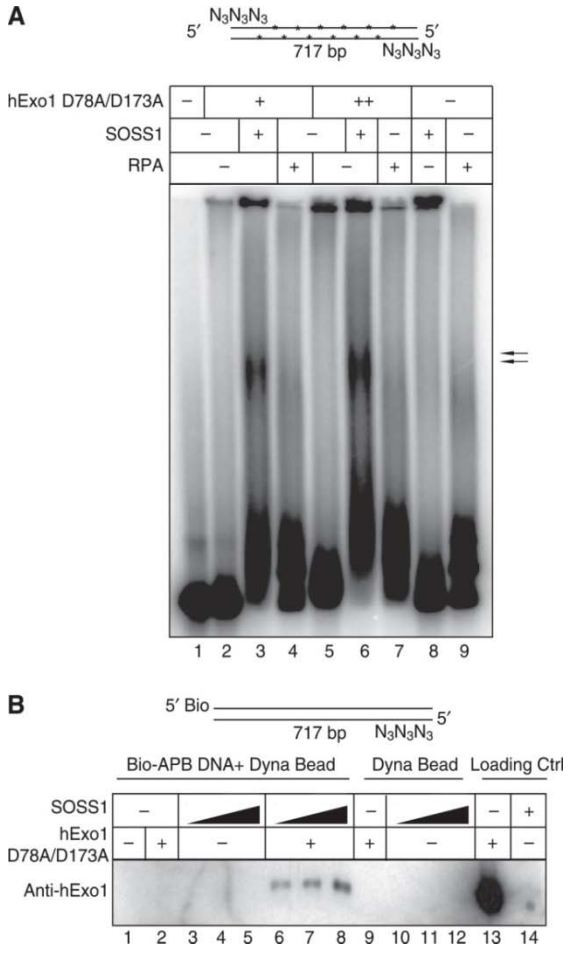

Figure 3.11 The SOSS1 complex recruits Exo1 to DNA ends.

(A) hExo1 D78A/D173A binding to DNA 5' ends was tested through DNA crosslink and gel shift assays using a 717 bp double-stranded DNA substrate internally labeled with [^{32}P](asterisks) and containing three azide groups (N_3) at the 5' end. The substrate was incubated with 10 nM or 20 nM hExo1(D78A/D173A) protein in the presence of 208 nM SOSS1(T117E) complex or 208 nM RPA as indicated. After incubation, each reaction was UV crosslinked (254 nm) then loaded and separated in a 1% native agarose gel. The gel was dried and visualized by exposure to phosphorimager. Arrows indicate position of crosslinked protein-DNA species formed in the presence of both Exo1 and SOSS1. (B) DNA binding of hExo1(D78A/D173A) was measured using a 717 bp PCR product containing biotin and three azide groups as shown. The substrate was pre-incubated with magnetic streptavidin beads for immobilization. The immobilized substrate was incubated with 80 nM Exo1(D78A/D173A) and 21.6 nM, 86.4 nM, or 345.6 nM SOSS1(T117E) complex as indicated, the beads were washed, and bound protein was separated on a 6% SDS-PAGE and visualized by western blot using an antibody against Exo1.

SOSS1 does not stimulate the resection activity of Dna2 in vitro

RPA has been shown in previous studies to stimulate DSB resection through its interaction with Dna2 and BLM (Cejka et al, 2010a; Niu et al, 2010). However, we have found that RPA generally limits both yeast and human Exo1 activity in vitro (Figures 3.5 and 3.8) (Nicolette et al, 2010). To confirm that our preparation of human RPA is active in resection, we tested the protein in a resection reaction containing human Dna2 and BLM (Dna2/BLM) and found that, consistent with previous reports, RPA strongly stimulated Dna2/BLM in 5' strand processing together with MRN (Figure 3.12). As we have established the stimulatory role of SOSS1 in DSB resection through Exo1, we further examined the effect of SOSS1 on Dna2/BLM in the presence or absence of MRN. Unlike RPA, SOSS1 was completely unable to stimulate DSB resection mediated by Dna2/BLM (Figure 3.12), suggesting that SOSS1 and RPA promote DSB resection in a manner that is specific to each nuclease.

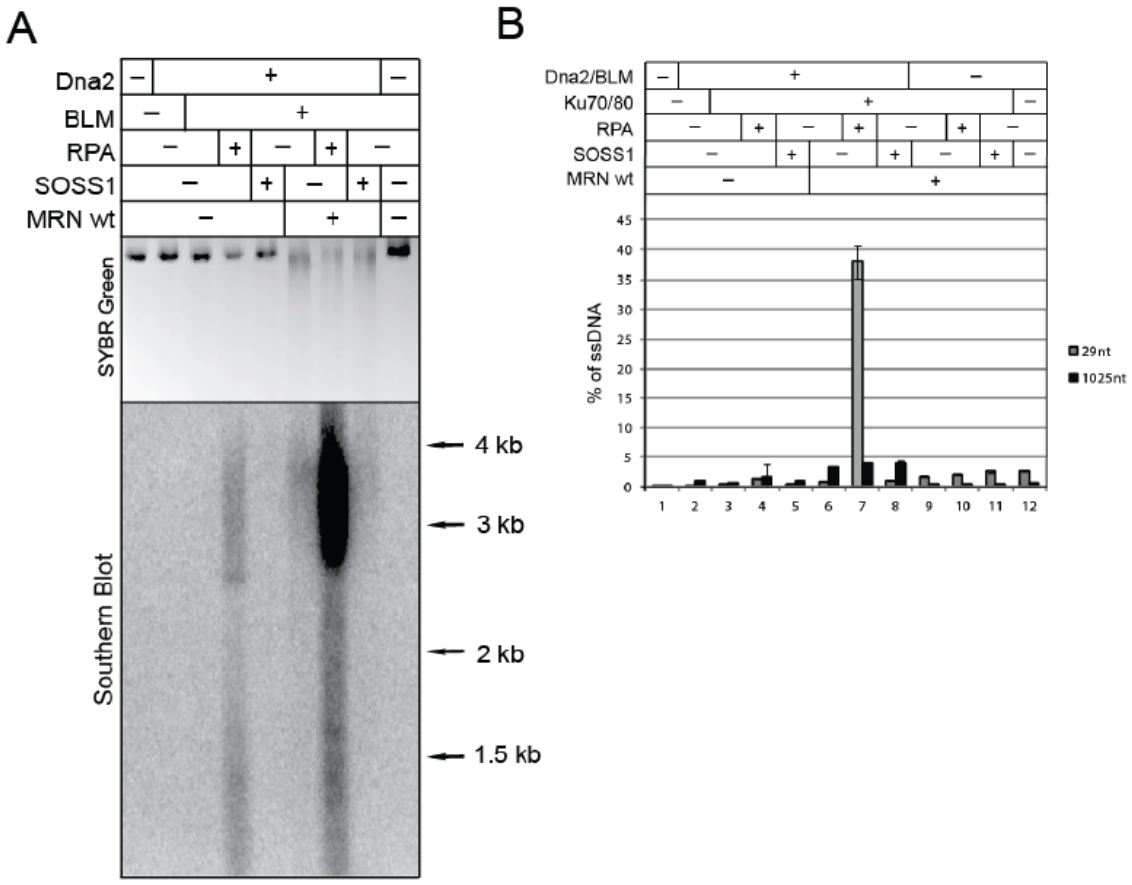

Figure 3.12 Dna2/BLM is stimulated by MRN and RPA.
(A) Resection assays were performed in vitro as in Figure 3.5 except with Dna2 (2 nM), BLM helicase (10 nM) in addition to RPA (80 nM), MRN (30 nM), and SOSS1 (80 nM). (B) Resection assays were performed as in (A) except with 16 nM Dna2, 10 nM BLM, 100 nM Ku, 80 nM RPA, 80 nM SOSS1, and 32 nM MRN with 0.067 nM DNA and analyzed by qPCR.

MRN overcomes Ku inhibition of Exo1 and Dna2-mediated resection

Processing DSB ends to generate 3' ssDNA overhangs is one of the initial steps required for repair of DSBs via homologous recombination. In vivo studies from yeast cells have shown that the yeast Ku70/80 heterodimer, which is required for canonical nonhomologous end joining (NHEJ), inhibits yeast Exo1 recruitment to DSB sites and generally blocks resection of DSBs in the G1 phase of the cell cycle (Barlow et al, 2008; Clerici et al, 2008; Shim et al, 2010; Zierhut & Diffley, 2008). Results in vitro and in vivo show that MRX complexes perform the unique role of displacing Ku from DNA ends and promoting resection by yExo1 (Langerak et al, 2011; Shim et al, 2010). To examine whether human Ku70/80 complexes inhibit Exo1 activity and if MRN is required to overcome the inhibitory effect of Ku on hExo1, we performed in vitro resection assays as described above. Even with high concentrations of Exo1 (such that Exo1 by itself shows robust resection activity), Ku exhibited strong inhibition of Exo1 (Figure 3.13A, lane 3). However, MRN largely recovered Exo1 resection activity, increasing resection at 29 nt and 1025 nt from the DNA end 4-fold when compared to Exo1 + Ku in the absence of MRN (Figure 3.13A, compare lanes 3-4).

As SOSS1 and RPA promote DSB resection with Exo1 and Dna2/BLM, respectively, we also tested the abilities of each ssDNA-binding complex to overcome Ku inhibition of Exo1 and Dna2 activity. Both SOSS1 and RPA partially recovered Exo1 resection activity in the presence of Ku at 29 nt from the DNA end (1.6- and 1.8-fold, respectively) but had less effect on longer distance resection measured 1025 nt from the end (Figure 3.13A, compare lane 3 with lanes 7-8). As described above, MRN complex has the most dramatic stimulatory effect on Exo1 in the presence of Ku, but RPA did

stimulate Exo1 activity further under these conditions (Figure 3.13A, lanes 4-6). As Dna2/BLM functions redundantly with Exo1 in promoting resection in eukaryotic cells (Nimonkar et al, 2011; Zhu et al, 2008), we further examined whether MRN, RPA, and SOSS1 could stimulate recombinant Dna2/BLM activity in resection in the presence of Ku. These results indicate that RPA together with MRN can overcome the inhibitory effects of Ku and stimulate the short range resection activity of Dna2/BLM while SOSS1 does not have any effect on this reaction (Figure 3.13B, lanes 5-9). RPA alone could not overcome the block to Dna2/BLM-mediated resection imposed by Ku, but was dramatically stimulated by the presence of MRN. Overall, these data suggests that the presence of Ku is strongly inhibitory of both Exo1 and Dna2, and that MRN plays the most critical role in the removal of this inhibition.

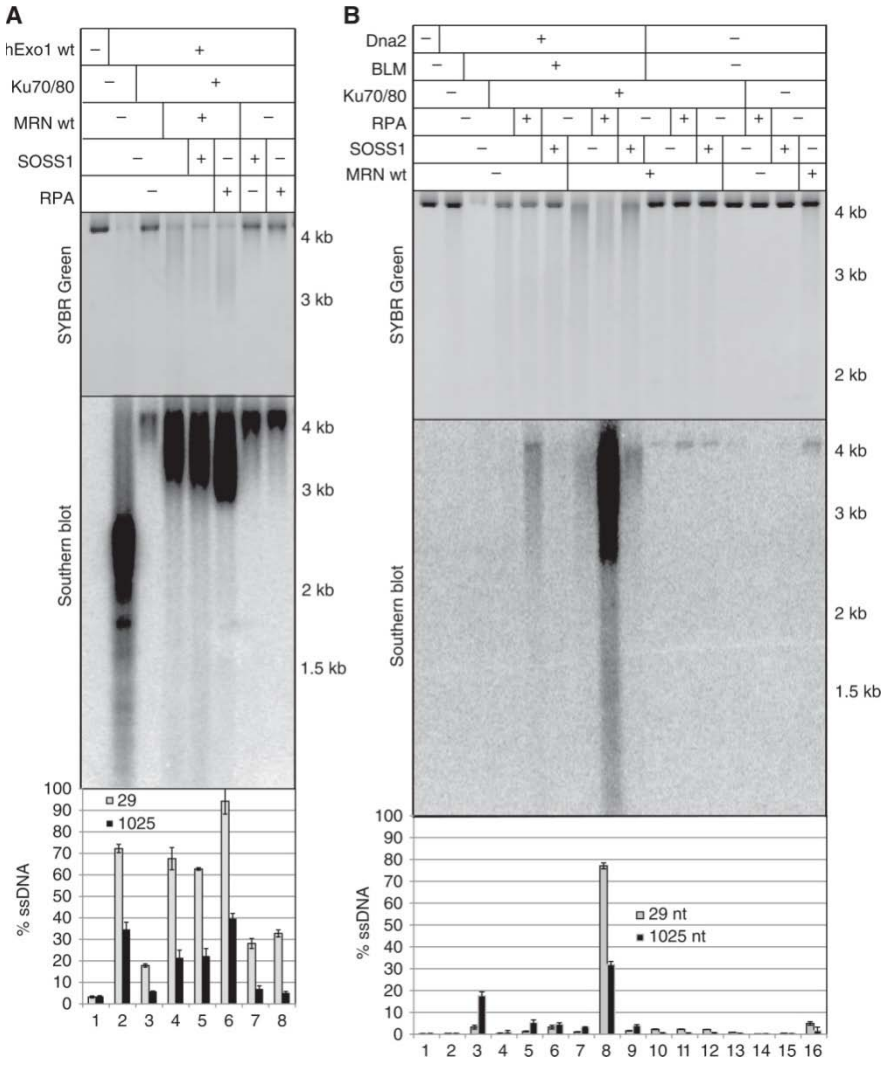

Figure 3.13 MRN promotes Exo1-mediated resection of DNA ends in the presence of Ku70/80.

(A) Resection assays were performed as in Fig. 3.5, except that the reactions contained higher levels of Exo1 (1.5 nM), 40 nM SOSS1(T117E) complex, 40 nM RPA, 32 nM MRN, and 100 nM Ku. (B) Reaction assays were performed as in (A) except with 16 nM Dna2, 10 nM BLM, 100 nM Ku, 80 nM RPA, 80 nM SOSS1, and 32 nM MRN.

DISCUSSION

Recent studies have identified SOSS1 as a novel SSB complex in human cells that exists in addition to the well-known heterotrimeric complex RPA. Both RPA and SOSS1 are critical for the regulation of DNA damage checkpoint activation as well as for DSB resection prior to homologous recombination (Huang et al, 2009; Oakley & Patrick, 2010; Richard et al, 2008; Richard et al, 2010). However, RPA is essential for replication and co-localizes with replication centers during S phase, while SOSS1 does not exhibit this specificity. SOSS1 co-localizes with γ-H2AX rapidly after DSB formation (Huang et al, 2009; Richard et al, 2008), while RPA is seen in foci only at later time points where it can be seen largely co-localized with Rad51 (Golub et al, 1998; Raderschall et al, 1999). Foci of SSB1, the OB-fold-containing SSB protein within the SOSS1 complex, also do not overlap with foci of RPA after ionizing radiation (Richard et al, 2008), further suggesting a diversification of roles for these single-stranded DNA-binding complexes. In this study, we have characterized the DNA-binding properties of SOSS1 in comparison to RPA, as well as their abilities to promote resection catalyzed by Exo1 and Dna2. Overall we find that the DNA binding properties of RPA and SOSS1 are such that RPA is likely to be dominant over SOSS1 at sites of single-stranded DNA, but that SOSS1 has unique DNA binding cooperativity with Exo1 that promotes its activity on double-stranded DNA ends.

SOSS1 and RPA display distinct modes of ssDNA binding

RPA has been shown to bind to single-stranded DNA in several different conformations, depending on the length of the DNA, the number of OB fold domains available for binding, and the reaction conditions (Fanning et al, 2006). An initial unstable interaction is formed between the A and B domain of the RPA70 subunit and 8 to 10 nt of DNA, followed by association of the C domain of RPA70 and the D domain of RPA32, leading to a more stable binding conformation that covers ~30 nt of single-stranded DNA (Bochkareva et al, 2002; Gomes et al, 1996). The single-molecule FRET characterization of RPA binding to single-stranded DNA shown here indicates two molecules of RPA binding to a 40 nt ssDNA tail, where one of the molecules is extremely stable while the other dissociates within one to two hours. On longer ssDNA substrates (58 and 71 nt), no dissociation is observed even after several hours (Figure 3.4 and data not shown). The affinity of RPA as measured in these experiments is ~1 nM, similar to previous reports (Gomes et al, 1996). In contrast, the affinity of SOSS1 for ssDNA is ~10-fold lower than that of RPA (~10 nM measured in single-molecule FRET) and only one complex appears to bind to the ssDNA substrates used in this study, consistent with a larger length of ssDNA required for binding of SOSS1 (~35 nt). The presence of six OB fold domains in RPA vs. only one in SOSS1 likely accounts for the greater affinity of RPA for ssDNA, although it is remarkable that SOSS1 binds with such high affinity considering this is the case. The affinity for ssDNA measured here in gel mobility shift assays and the FRET experiments with SOSS1 is 30 to 160-fold higher

than the affinity reported for SSB1 alone (Richard et al, 2008), thus the SOSSA(INTS3) and SOSSC(C9orf80) components of the complex contribute significantly to the DNA binding ability of the complex and may contain DNA-binding domains that are not recognizable by their sequence.

An important difference between RPA and SOSS1 is the observed fluctuation in FRET correlation values in the presence of SOSS1, indicating a more dynamic binding mode of SOSS1 on ssDNA, whereas RPA did not exhibit any movement on the DNA with either the first or second complex binding. Overall, the binding experiments suggest that RPA has a higher affinity for ssDNA sites and forms complexes that are higher in stability compared to SOSS1. In addition, quantitation of protein levels in human cells (HCT116 and a normal human fibroblast line) suggests that levels of the SSB1 protein are approximately 20 to 40-fold lower than RPA (data not shown). Considering this difference in concentration along with the cooperative nature of SOSS1 binding to ssDNA, RPA would be expected to predominate over SOSS1 at sites of ssDNA in vivo. Taken together, these observations likely explain why SOSS1 is not observed at replication sites with RPA and does not colocalize with RPA foci at ssDNA sites in human cells.

Functional interactions between SOSS1 and Exo1 in DSB resection

Despite the low affinity of SOSS1 for double-stranded DNA, we observed cooperative binding of SOSS1 and human Exo1 to double-stranded DNA substrates in vitro. This binding correlates with a striking increase in Exo1 activity on linear DNA substrates in the presence of SOSS1, similar to the effect observed with MRN. An analogous effect was previously shown with the budding yeast MRX and Sae2 complexes and yeast Exo1 in vitro (Nicolette et al, 2010). Since we did not observe any direct protein-protein interactions between yeast Exo1 and the complexes that promote its activity, our working hypothesis is that these factors contribute to the binding of Exo1 by stabilizing an opened duplex, or "Y" structure, at the end of the DNA. In support of this idea, analysis of the degradation products of Exo1 in this study demonstrated oligonucleotide-sized initial products containing the 5' end of the 5' strand of the double-stranded substrate. These were dependent on Exo1 catalytic activity as well as the presence of SOSS1, directly demonstrating that Exo1-mediated endonucleolytic activity generates the first 5' strand product. In addition, when a Y structure is directly used as a substrate, SOSS1 exhibits relatively little stimulation of Exo1 (Figure 3.14), consistent with the idea that this DNA mimics the conformation that SOSS1 stabilizes. The structure of human Exo1 shows that the enzyme binds at a junction between single-stranded and double-stranded DNA and stabilizes a sharp bend in the helix (Orans et al, 2011). The frayed-end arrangement observed in the crystal structures of Exo1 on DNA could be

promoted by another protein binding stably to the 3' strand, a role previously shown for yeast Sae2(Nicolette et al, 2010); here we suggest that this role is played by SOSS1.

The functional cooperativity demonstrated here for SOSS1 and Exo1 appears to be specific for Exo1 since we observed no stimulation of Dna2, another flap endonuclease that functions in lagging strand replication as well as in DSB end processing (Fortini et al, 2011; Kang et al, 2010). Budding yeast Dna2/Sgs1 complexes have been shown to remove the 5' strand of DNA at double-strand breaks in conjunction with Rmi1/Top3 in vivo (Gravel et al, 2008; Mimitou & Symington, 2008; Zhu et al, 2008). Yeast RPA is critical for the strand specificity of this reaction in vitro and for the stimulation of Dna2/Sgs1 (Cejka et al, 2010a; Niu et al, 2010). We confirm in this study with human proteins that RPA is strongly stimulatory of Dna2 in a reaction with the BLM helicase (Nimonkar et al, 2008), yet RPA blocks Exo1 activity similar to previous findings with yeast RPA and yeast Exo1 (Nicolette et al, 2010). This difference also points to distinct modes of single-stranded DNA binding by these complexes that dictates the exo-/endonuclease that each complex stimulates.

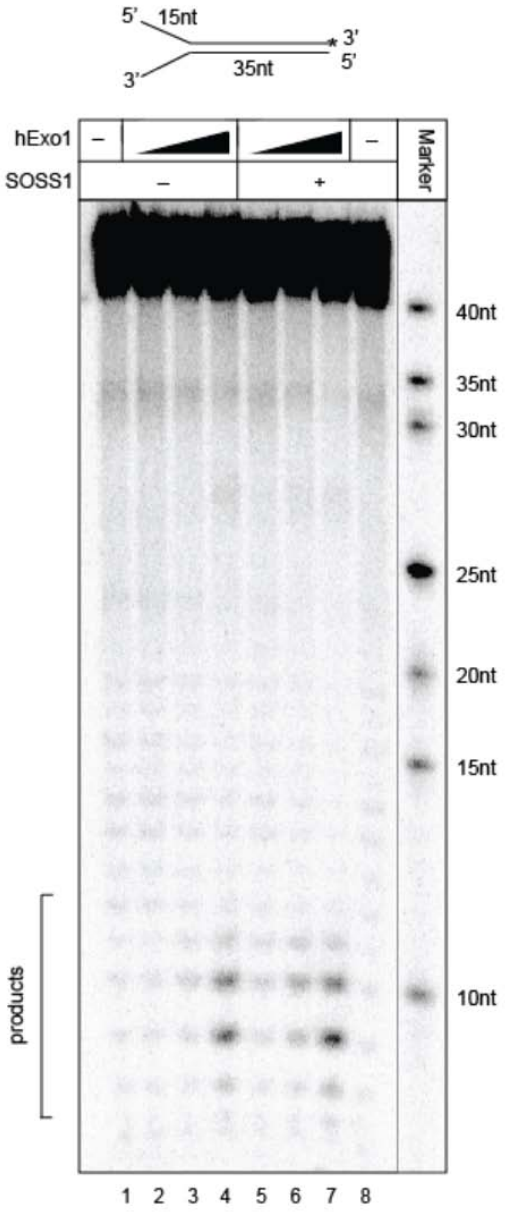

Figure 3.14 SOSS1 does not stimulate Exo1 cleavage of a branched DNA structure. Nuclease assays were performed using a double-stranded DNA substrate composed of two oligonucleotides (TP2622 annealed to TP1152) with a flap region of 15 nt and a duplex of 35 bp, as shown (top) with TP2622 labeled on the 3' end with [^{32}P]. Reactions included 0.45, 1.35, or 4 nM Exo1 and 42 nM SOSS1 and were analyzed by denaturing polyacrylamide gel electrophoresis followed by phosphorimager analysis.

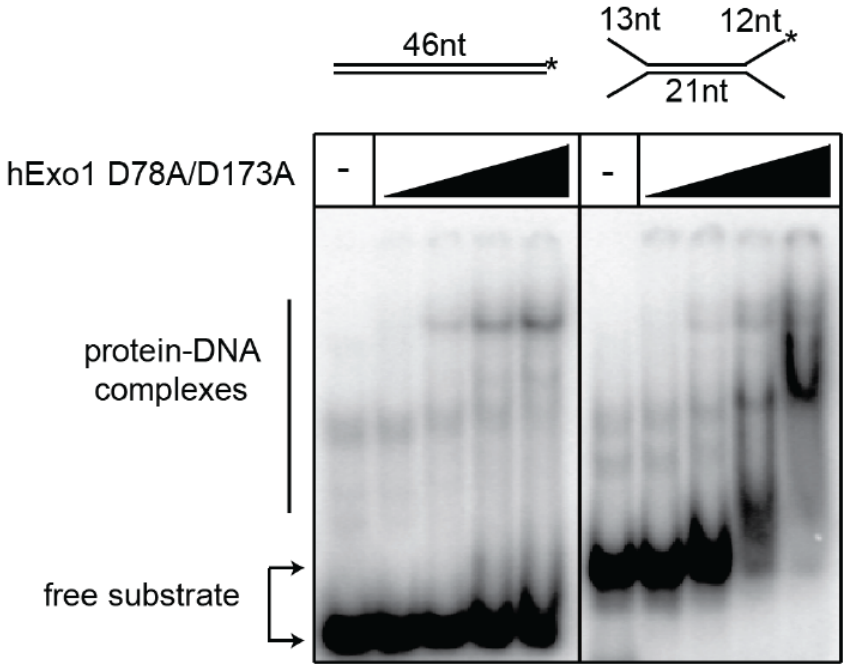

Figure 3.15 Exo1 binds preferentially to a branched DNA structure.
DNA-binding assays were performed with 3' [^{32}P]-labeled oligonucleotide DNA substrates (TP74/TP504 and TP74/TP2681, respectively) as shown. DNA (2 nM) was incubated with Exo1 (8, 16, 32, or 64 nM) complexes as indicated and analyzed by native gel mobility shift assay as in Figure 3.2.

Ku inhibition of resection and release by MRN

Previous studies in budding yeast have demonstrated that the Ku heterodimer inhibits 5' strand resection in vivo and in vitro (Barlow et al, 2008; Clerici et al, 2008; Langerak et al, 2011; Shim et al, 2010; Zierhut & Diffley, 2008). Here we show with human components that Ku70/80 complexes show a similar repression of both Exo1 and Dna2 in vitro and that the MRN complex facilitates the activity of both these enzymes in the presence of Ku. Although SOSS1 and RPA still stimulate Exo1 and Dna2, respectively, it is clear that MRN plays a primary role in removing or displacing Ku from DNA ends to allow resection to take place.

The MRN complex interacts directly with SOSS1 through the SOSSA(INTS3) component (Huang et al, 2009) and the SOSSB(hSSB1) component (Richard et al, 2011), and is important for the formation of SOSS1 foci, particularly in S and G_2 phases of the cell cycle (Huang et al, 2009). From the experiments shown here it is not clear if these interactions are required for resection, since MRN and SOSS1 appear to act independently on Exo1 to stimulate its activity in vitro. MRN interacts with several proteins that localize at DSB sites, most significantly the ATM protein kinase that phosphorylates histone H2AX and many DNA damage response factors. Phosphorylation of SSB1 by ATM on threonine 117 was shown to prevent proteosome-mediated degradation of SSB1 (Richard et al, 2008), thus ATM phosphorylation of SSB1 is a direct mechanism by which the kinase promotes homologous recombination. All of the

experiments shown in this work were performed with SOSS1 containing the phosphomimic mutation T117E, since the wild-type protein was not sufficiently stable during expression to allow for efficient purification of the complex. Interaction of ATM with SSB1 phosphorylated on T117 also was found to stimulate ATM kinase activity on other substrates (Richard et al, 2008), thus SOSS1 can promote the DNA damage response through this positive feedback loop.

In conclusion, the experiments presented here illustrate the similarities between the SOSS1 and RPA complexes in their specificity for ssDNA, but also indicate why the complexes have very different biological functions in cells. We propose that these differences are based on the affinity of the complexes for their substrates, and also on the specific conformations of double-stranded DNA substrates when bound simultaneously by SOSS1/RPA and endonuclease enzymes. The functional cooperativity between SOSS1 and Exo1 is likely an important part of the role of SOSS1 in DNA repair, along with its stimulatory effects on ATM and Rad51 in signaling and homologous recombination, respectively (Richard et al, 2008).

CHAPTER 4: MECHANISM OF MRN AND CTIP STIMULATION OF RESECTION

INTRODUCTION

The Mre11/Rad50 complex is conserved in all organisms and plays a global role in the DNA damage response. Mre11 exhibits exo- and endonuclease activity in vitro (Paull & Gellert, 1998; Trujillo & Sung, 2001; Trujillo et al, 1998) while Rad50 possesses ATP binding, hydrolysis, adenylate kinase activity, and ATP-dependent DNA unwinding activity (Bhaskara et al, 2007; Cannon et al, 2013; Chen et al, 2005; Hopfner et al, 2000; Paull & Gellert, 1999). The eukaryotic MR complex specifically associates with another component called Nbs1 (or Xrs2 in *S. cerevisiae*) (Stracker et al, 2004), which regulates the enzymatic activities of Mre11 and Rad50 in addition to their functional interaction with the ATM(Tel1) kinase (Falck et al, 2005; Lee & Paull, 2004; Lee & Paull, 2005; Nakada et al, 2003; Paull & Gellert, 1999). MRN(X) localizes to DSB sites shortly after DNA damage (Berkovich et al, 2007; Lisby et al, 2004; Mirzoeva & Petrini, 2001; Shroff et al, 2004) and stimulates efficient DSB repair as well as DNA damage signaling through activation of the ATM(Tel1) kinase (D'Amours & Jackson, 2002; Lee & Paull, 2007).

Several groups have studied how the activities of MRN contribute to ATM stimulation. MRN recruits the ATM kinase to DSB sites and facilitates ATM monomerization and activation (Lee & Paull, 2004; Lee & Paull, 2005). MRN also exhibits Mre11-dependent nuclease activity as well as Rad50-ATP-dependent DNA binding and unwinding activities (Cannon et al, 2013; Paull & Gellert, 1998; Paull & Gellert, 1999). An in vivo study using mouse embryonic fibroblasts and an in vitro study using recombinant human proteins have proposed that Mre11 nuclease activity is not required for ATM activation (Buis et al, 2008; Lee et al, 2013). In constrast, the ATP-dependent activities of Rad50 were proposed to be responsible for activating ATM, possibly by opening duplex DNA ends to increase ATM recruitment (Lee & Paull, 2005).

MRN(X) also promotes HR repair by regulating DSB resection. MRN(X) stimulates Exo1- or Dna2/BLM(Sgs1-Top3-Rmi1)-dependent resection pathways by promoting the recruitment and activity of the processing enzymes on DSB ends (Cejka et al, 2010a; Nicolette et al, 2010; Nimonkar et al, 2011; Niu et al, 2010; Yang et al, 2013). In addition, MRN(X) promotes the resection activity of Exo1 and Dna2/BLM(Sgs1-Top3-Rmi1) by inhibiting the activity of the NHEJ factor, Ku, on DNA ends (Langerak et al, 2011; Shim et al, 2010; Yang et al, 2013). Despite the critical role of MRN in DSB resection, the molecular mechanisms linking MRN nuclease activity or ATP-dependent DNA unwinding activity to this process are not fully understood.

In yeast, MRX(N) acts with the Sae2(Ctp1) endonuclease to promote resection activity at both meiotic and mitotic DSBs. During meiosis, MRX(N) and Sae2(Ctp1) stimulate the removal of covalent Spo11(Rec12) conjugates from the 5' strand of the break site (Clerici et al, 2005; Hartsuiker et al, 2009; Moreau et al, 1999; Rothenberg et al, 2009). In mitotic cells, MRX and Sae2 are not essential in processing endonuclease-induced DSBs (Clerici et al, 2005; Ivanov et al, 1994). However, MRX and Sae2 accelerate the rate of short-range resection at endonuclease-induced DSB sites, as deletion of MRX or Sae2 delays processing of the 5' strand adjacent to the DSB site *in vivo* (Mimitou & Symington, 2008; Zhu et al, 2008).

CtIP, a transcriptional regulator and tumor suppressor protein (Fusco et al, 1998; Schaeper et al, 1998; Wong et al, 1998; Yu et al, 1998), is also known as the functional ortholog of Sae2, containing a short sequence homology region at the C-terminus (Sartori et al, 2007). Like Sae2, CtIP appears to function in DNA repair. For example, human CtIP recruits to DNA damage sites and promotes DSB resection as well as DNA damage signaling (Sartori et al, 2007). In addition, CtIP is essential for conferring resistance against DSBs induced by IR or topoisomerase inhibitors in human cells (Huertas & Jackson, 2009; Sartori et al, 2007). Moreover, CtIP functions in cooperation with MRN to regulate DSB resection for HR repair (Chen et al, 2008; Lloyd et al, 2009; Sartori et al, 2007; Williams et al, 2009), although the precise mechanism of this interaction is unclear.

In this study, I investigate how the nuclease activity and the ATP-driven DNA unwinding activity of MRN function in processing DSBs. Further, I examine how MRN and CtIP interaction contributes to DSB resection using a reconstituted in vitro system. Here I show that the ATP-dependent activity of MRN is required for overcoming Ku inhibition of Exo1 on linear DNA ends. Based on work from our laboratory and collaboration with the Russell laboratory demonstrates that MRN opens duplex DNA in an ATP-dependent manner (Cannon et al, 2013). In this study, I propose that the ATP-dependent DNA unwinding activity of MRN is required to repress Ku inhibition in resection by pushing Ku away from the DNA ends. In addition, I show that the nuclease activity of MRN is required for processing covalent protein-DNA intermediates. Moreover, I demonstrate that CtIP increases MRN activity in overcoming Ku inhibition of Dna2/BLM-dependent resection activity in vitro. Overall, this study reveals the mechanism of how the enzymatic properties of MRN as well as the activity of CtIP contribute to DSB resection.

RESULTS

Mre11 nuclease activity is not required for Exo1 stimulation on DNA ends

The MR(N/X) complex stimulates DSB resection by regulating the activities of DNA processing exonucleases and helicases at DSB ends (Cejka et al, 2010a; Hopkins &

Paull, 2008; Nimonkar et al, 2011; Niu et al, 2010; Yang et al, 2013). Mre11 exhibits manganese-dependent 3' to 5' exo- and endonuclease activity and weak 5' to 3' endonuclease activity with magnesium in vitro (Hopkins & Paull, 2008; Nicolette et al, 2010; Paull & Gellert, 1998; Trujillo & Sung, 2001; Trujillo et al, 1998). Mre11 nuclease activity is required for processing DSBs generated by Spo11 during meiosis but not for DSBs generated by site-specific endonucleases in vegetative cells in yeast (Moreau et al, 1999). To examine whether Mre11 nuclease activity is required to promote human Exonuclease 1 (hExo1) resection activity in vitro, human MRN complexes containing Mre11 nuclease deficient mutants were expressed and purified. Human Mre11 (H129N) and Mre11 (H129L/D130V) are equivalent to the yeast Mre11 nuclease deficient mutants Mre11 (H125N) and *mre11-3* allele, respectively (Bressan et al, 1998; Moreau et al, 1999). As shown in Figure 4.1, both human M(H129N)RN and M(H129L/D130V)RN mutants are completely deficient in nuclease activity compared to wild-type MRN complex under conditions with manganese where the nuclease activity of MRN is most detectable.

To examine whether Mre11 nuclease activity is required for hExo1 stimulation at DNA ends, in vitro resection assays were performed with purified recombinant hExo1 in the presence of wild-type MRN or M(H129N)RN using either 4.5 kb linear plasmid DNA (Figure 4.2A) or 5' Cy3/Cy5 labelled DNA (Figure 4.2B). All of the in vitro resection reactions contained magnesium as it is the more physiologically relevant metal ion. Resection products were analyzed by using SYBR Green stain (top panel) and non-

denaturing southern hybridization with an RNA probe that hybridizes to a 1 kb region on the 3' end of the DNA substrate (bottom panel) as described previously (Nicolette et al, 2010) (Figure 4.2A). Resection assays performed with 5' Cy3/Cy5 labelled DNA were analyzed using denaturing sequencing gels as described previously (Yang et al, 2013) (Figure 4.2B). Both wild-type and Mre11 (H129N) containing MRN complexes were able to stimulate hExo1 resection activity on linear DNA ends. However, no stimulatory effect of the MRN complexes was observed when using the nuclease-deficient hExo1 (D78A/D173A) (Figure 4.2A, compare lanes 2-4 and 5-7), suggesting that resection depends on the nuclease activity of hExo1. The resection activity of hExo1 on Cy3/Cy5 labelled DNA was also stimulated by wild-type MRN and M(H129N)RN to a comparable level (Figure 4.2B, lanes 2-4). Overall, these results suggest that human Mre11 nuclease activity is not required for hExo1 stimulation on DNA ends. This is consistent with the results with yeast MRX(N) and Exo1 as well as with archaeal MR and the nuclease/helicase complex NurA/HerA (Hopkins & Paull, 2008; Langerak et al, 2011; Nicolette et al, 2010; Shim et al, 2010).

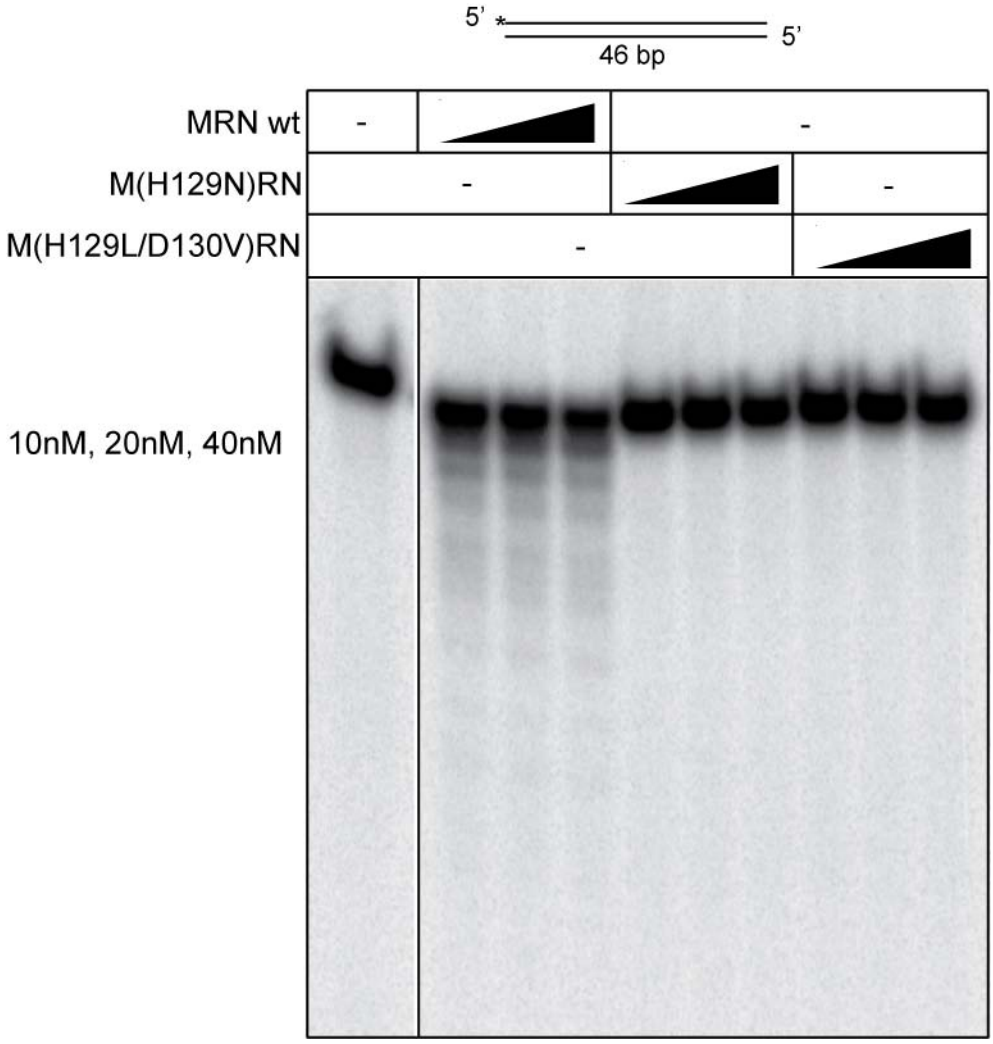

Figure 4.1 Nuclease assays with wild-type MRN (wt), M(H129N)RN, and M(H129L/D130V)N.

Nuclease assays were performed in manganese chloride using a [^{32}P]-labeled double-stranded oligonucleotide substrate as indicated. Concentrations of each complex were 10 nM, 20 nM, and 40 nM, respectively. Reaction products were separated with a denaturing polyacrylamide sequencing gel and analyzed by phosphorimager.

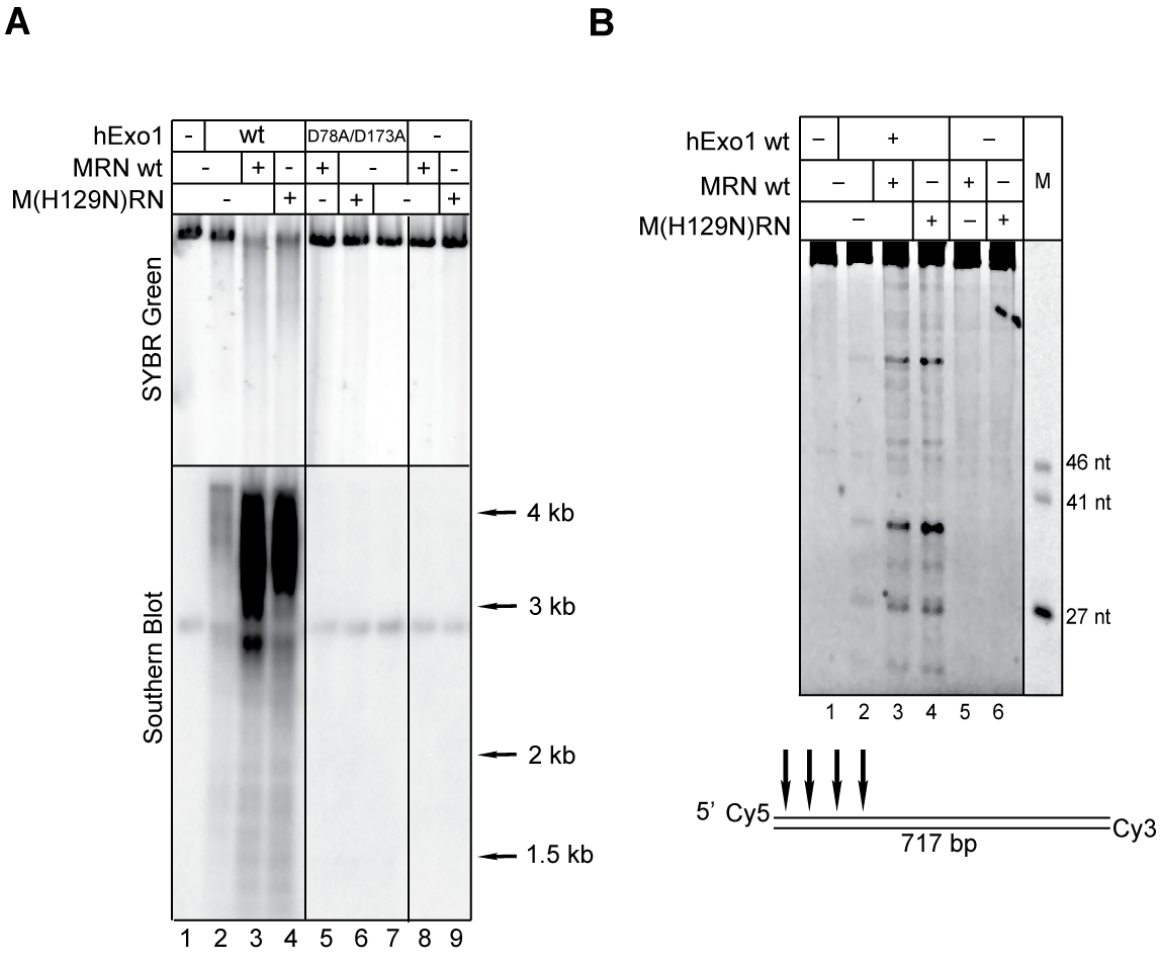

Figure 4.2 Mre11 nuclease activity is not required for hExo1 stimulation on DNA ends. (A) In vitro resection assay was performed with Exo1 (1.0 nM) and MRN (5 nM) on 0.67 nM linearized plasmid DNA substrate in the presence of 25mM MOPS, 2mM ATP, 0.1 mM DTT, 100 μl/mL BSA, 0.05% Triton X-100, and 5 mM $MgCl_2$. Reaction products were analyzed by SYBR green staining (top) and by non-denaturing southern blot using a probe for the 3' strand (bottom). (B) Resection assay was performed in vitro with Exo1 (5nM) and MRN (62.5nM) using 39 nM 5' Cy3/Cy5 labelled DNA in the presence of 25mM MOPS, 1mM DTT, 5mM $MgCl_2$, 1mM ATP. Reaction products were analyzed on a 20% denaturing sequencing gel and scanned for Cy5 emission.

Mre11 nuclease activity is not required to block Ku inhibition of hExo1 on DNA ends

Previous studies from yeast have shown that the MRX(N) complex suppresses Ku accumulation at DSB ends and stimulates Exo1 recruitment to the ends, promoting DSB resection (Langerak et al, 2011; Shim et al, 2010). In *S. cerevisiae*, Mre11 nuclease activity is dispensable for MRN inhibition of Ku, blocking resection activity in vivo (Shim et al, 2010) whereas in *S. pombe* Mre11 nuclease activity was shown to be required for releasing MRN and Ku from DNA ends (Langerak et al, 2011). Previously, I have shown that human MRN overcomes Ku inhibition of hExo1- and Dna2/BLM-resection activities on DNA ends in reconstituted resection reactions in vitro (Yang et al, 2013). To further examine the effect of Mre11 nuclease activity on Ku inhibition of hExo1, I performed in vitro resection assays as described above with MRN wt and M(H129N)RN in the presence of hExo1 and Ku (Figure 4.3). As shown in my previous study, Ku strongly inhibits the resection activity of hExo1 on DNA ends (Figure 4.3, compare lanes 2 and 3). However, the presence of either wild-type MRN or M(H129N)RN completely rescues hExo1 resection activity at 29nt and 1025nt from the DNA end (Figure 4.3, compare lanes 3-5). This suggests that MRN overcomes the inhibitory effect of Ku in resection through a mechanism that does not require the endonucleolytic removal of Ku from DNA ends.

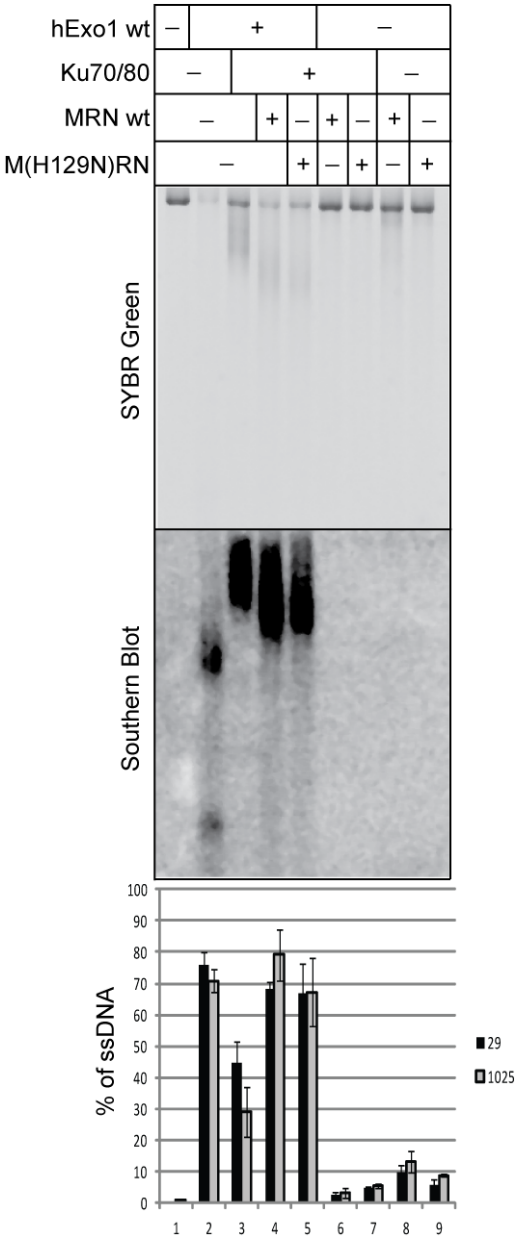

Figure 4.3 Mre11 endonucleolytic activity is not required to overcome Ku inhibition of hExo1-mediated resection.

Resection reactions were performed on linearized plasmid DNA as described previously (Nicolette et al, 2010) with 1.5 nM hExo1, 105 nM Ku, and 47.5 nM MRN (wt) or nuclease-deficient (M(H129N)RN) .

The Rad50 signature motif in MRN is required to overcome the inhibitory effect of Ku on hExo1 resection activity at DNA ends

Ku exists as a heterodimer and forms a ring-shaped structure that threads onto the DNA ends (Walker et al, 2001). Once bound, Ku can slide into the DNA strand, allowing more Ku molecules to thread onto the DNA (Doherty & Jackson, 2001). As the resection assay was performed on a 4.5-kb linear plasmid DNA, it is possible that MRN displaces Ku from DNA ends by pushing Ku down the DNA strand, exposing the ends for hExo1 recruitment. In previous studies, the ATP-dependent activities of the MRN complex was proposed to be responsible for unwinding duplex DNA ends and activating ATM in the presence of DSB ends (Lee & Paull, 2005; Paull & Gellert, 1999). Further, it was shown that MRN containing a serine to arginine mutation in the signature motif of Rad50 (Rad50 S1202R) compromises these ATP-stimulated activities of MRN (Bhaskara et al, 2007; Moncalian et al, 2004). Recently, a smFRET assay was established to demonstrate an ATP-dependent DNA unwinding activity of MRN in vitro (Cannon et al, 2013). Further, the study shows that the S1202R mutation compromises the DNA unwinding activity of MRN (Cannon et al, 2013). In this study I examined whether the ATP-dependent DNA unwinding activity of MRN is responsible for displacing Ku from DNA ends to stimulate hExo1 resection activity on DNA ends. I performed in vitro resection assay as described above with hExo1 and Ku in the presence of either wild-type MRN or MR(S1202R)N (Figure 4.4). I found that the resection activity of hExo1 is blocked by Ku and relieved by wild-type MRN, but that the MR(S1202R)N had no effect

in this process (Figure 4.4, lanes 4, 5, 7, and 8). I observed 2 to 3-fold and 7 to 8-fold increased levels of resection with wild-type MRN than MR(S1202R)N mutant complex at sites located 29 nt and 229 nt from DNA ends, respectively. In constrast, with limiting concentrations of hExo1 (0.67 nM under these conditions) in the absence of Ku, both MRN wt and MR(S1202R)N were able to stimulate hExo1-mediated resection of the 5' end of DNA to a comparable level (Figure 4.4, compare lanes 9-11). In vivo results have also shown that the expression of Rad50 S1202R mutant in human cells depleted of endogenous Rad50 results in deficient resection at DSB ends (Cannon et al, 2013). Taken together, these results suggest that the ATP-dependent DNA unwinding activity of the MRN complex plays a critical role in regulating DNA DSB resection.

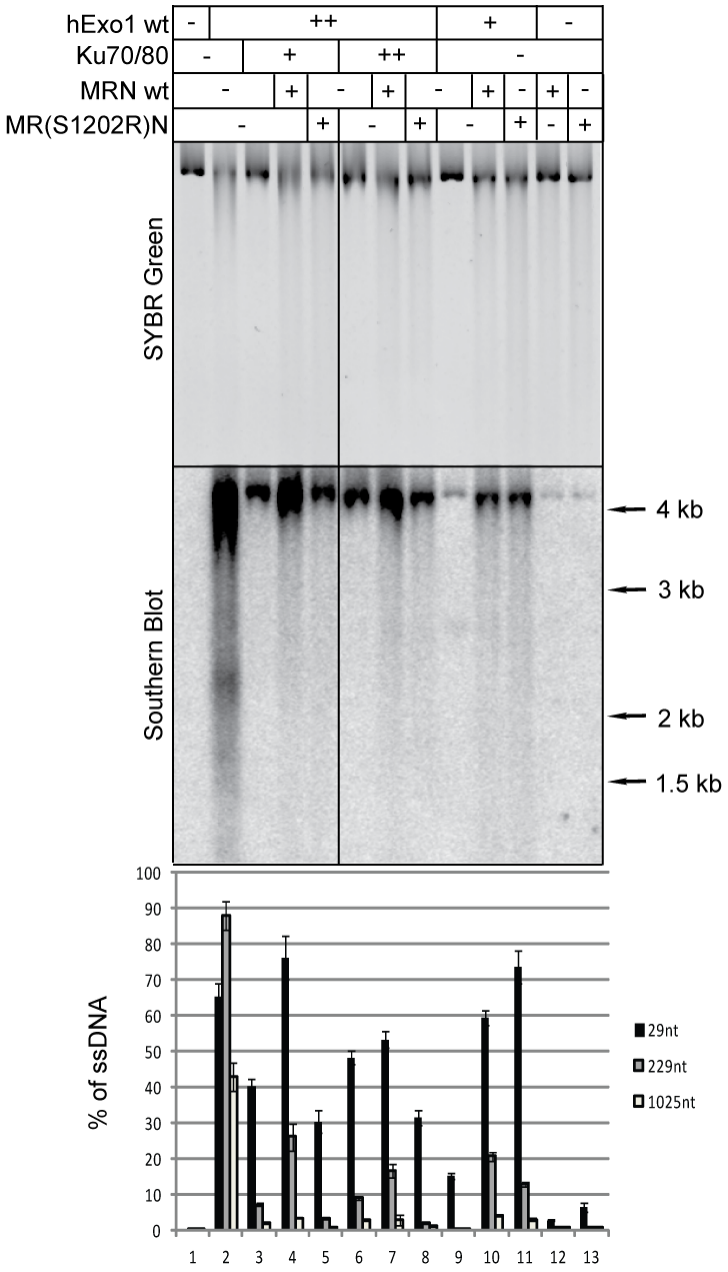

Figure 4.4 ATP-catalytic activity of Rad50 is required to block Ku inhibition of hExo1 on DNA ends.

Resection assays were performed as in Figure 4.3 except with 0.67 or 2 nM hExo1, 160 or 200 nM Ku, and 44.4 nM wild-type MRN and MR(S1202R)N.

Mre11 nuclease activity is required for processing protein covalent conjugates from DNA ends

In yeast cells, Mre11 nuclease activity is required for removing the type II topoisomerase-like protein Spo11 (Rec12) from the 5' strand of DNA at the break sites to proceed in meiotic DSB resection (Hartsuiker et al, 2009; Milman et al, 2009; Moreau et al, 1999; Rothenberg et al, 2009; Usui et al, 1998). In addition, the nuclease-deficient MRX complex exhibits weak sensitivity to ionizing radiation (IR) and delays the rate of resection at IR-induced DSBs in budding yeast (Budd & Campbell, 2009; Moreau et al, 1999). IR-induced DSBs are present with modified bases, covalent protein adducts, and altered structures at the ends (Barker et al, 2005; Henner et al, 1983). Therefore, I examined whether the nuclease activity of human MRN is required to stimulate resection on DSB ends containing protein adducts in vitro. I reconstituted an in vitro resection assay using a DNA substrate with protein covalently conjugated to the ends. In this assay I used Ku as the conjugated protein due to its high affinity to DNA ends as well as its large molecular weight. The reaction was performed under conditions where hExo1 processes the unconjugated DNA substrate efficiently while it exhibits limited activity on the protein-conjugated DNA substrate (data not shown). As shown in Figure 4.5, hExo1 with wild-type MRN degrades approximately 30-40% of the protein-conjugated DNA while hExo1 with M(H129L/D130V)RN degrades approximately 16-28% of the substrate (compare lanes 3-4 with 5-6). This result demonstrates that Mre11 nuclease activity is required for the removal of protein adducts from DNA ends to promote efficient resection by hExo1. As I have shown that Mre11 nuclease activity is dispensable for processing

DNA with free ends, overall these results suggest that Mre11 nuclease activity in human cells is likely to be required for processing DSBs covalently modified at the ends. Whether the nuclease activity of MRN is required for the resection of DSB ends with modified structures or bases remains further study.

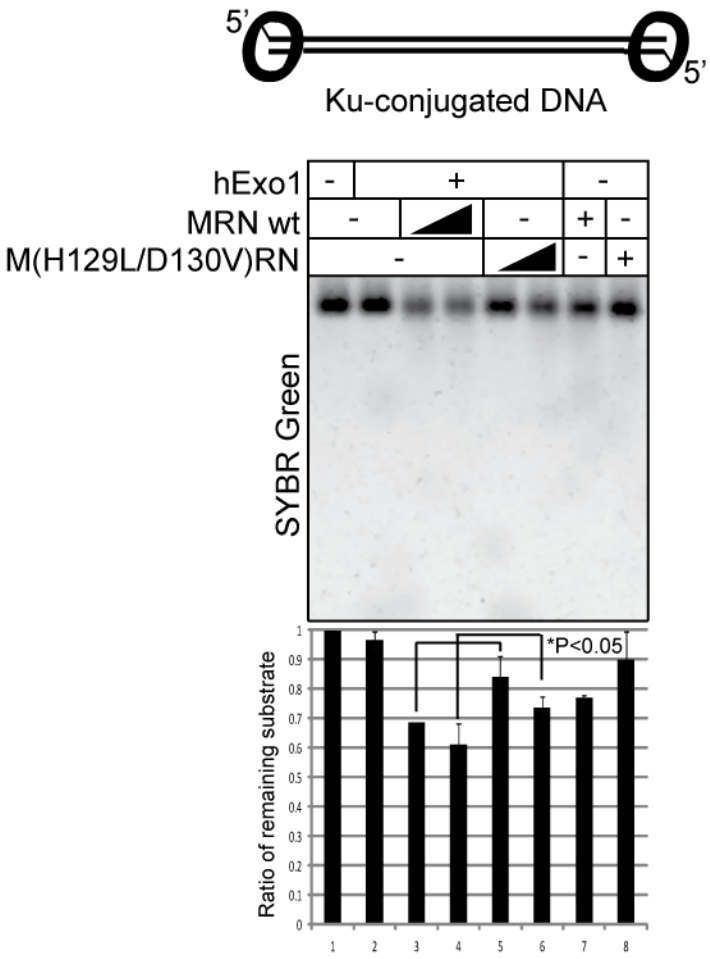

Figure 4.5 Mre11 nuclease activity promotes resection on DNA ends with protein adducts.

Resection assays were performed at 37°C for 2 hours using a 700 bp double-stranded DNA conjugated with Ku on either the 5' or 3' end with 50 nM or 150 nM wild-type MRN or M(H129L/D130V)RN and 5.5 nM hExo1. Reactions were separated in a 1.5% non-denaturing agarose gel, analyzed by SYBR Green staining (top panel), and quantified by the ImageQuant software (bottom panel). The graph shows the percent substrate remaining with the average from 3 experiments; error bars indicate standard deviation.

CtIP and MRN overcome Ku inhibition of Dna2/BLM

MRN(X) has been shown to promote the initiation of resection and stimulate Exo1- and Dna2/BLM(Sgs1-Top3-Rmi1)-dependent resection activity at DSB ends (Cejka et al, 2010a; Nicolette et al, 2010; Nimonkar et al, 2011; Niu et al, 2010; Yang et al, 2013). In budding yeast, MRX represses Ku accumulation at DNA ends and stimulates Exo1 recruitment and resection activity on DNA ends in vivo (Shim et al, 2010). In addition, MRX and Sae2 cooperate to promote Exo1 resection activity on DNA ends in vitro, possibly by creating end structures that are preferable for Exo1 binding (Nicolette et al, 2010). Moreover, in fission yeast, Ctp1 interacts with MRN to promote Ku removal from DSB ends and to stimulate the resection activity of the DNA processing enzymes (Langerak et al, 2011). In this study, I examined the contribution of CtIP to the ability of MRN to overcome Ku inhibition of Dna2/BLM in resection assays as described previously (Yang et al, 2013). Notably, CtIP together with MRN can further overcome the inhibitory effect of Ku and stimulate the short range resection activity of Dna2/BLM by approximately 3-fold (Figure 4.6, compare lanes 6 and 9). CtIP alone could not block Ku inhibition of Dna2/BLM (Figure 4.6, lane 7), suggesting that CtIP requires MRN for its effect on Dna2/BLM in the presence of Ku at DNA ends. Recently, two conserved amino acid residues, N289 and H290, were identified to be responsible for CtIP nuclease activity (Makharashivili et al, 2013). However, when I performed the resection assay with the nuclease deficient CtIP mutant (CtIP N289A/H290A) there was no significant difference compared to the wild-type CtIP (Figure 4.6, compare lanes 9 and 10). This

suggests that repression of Ku inhibition of Dna2/BLM at DNA ends does not require endonucleolytic cleavage by CtIP.

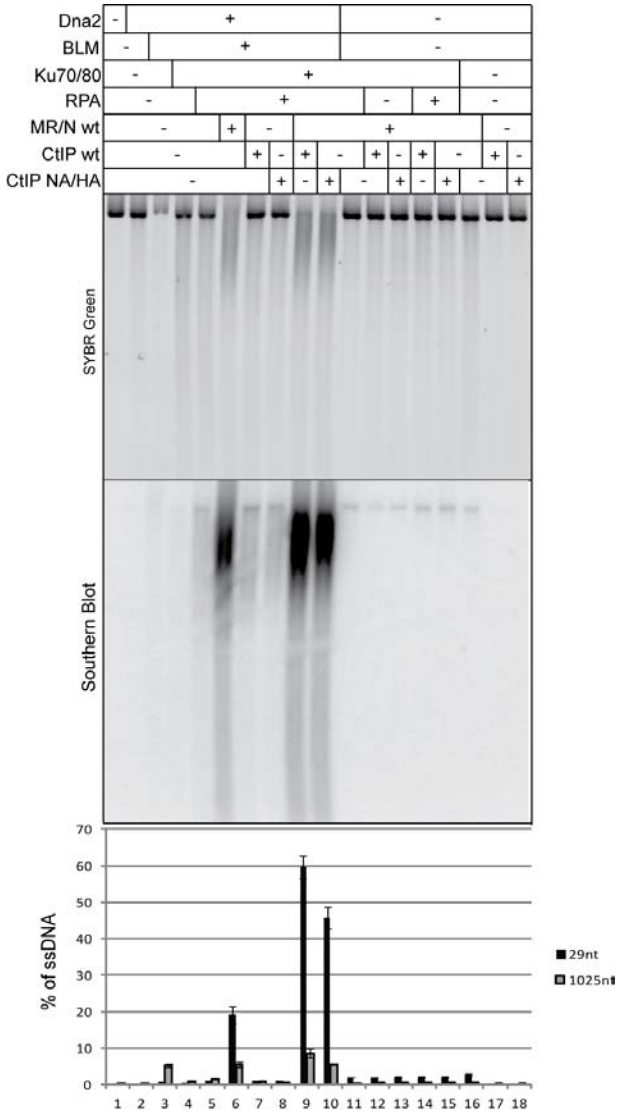

Figure 4.6 CtIP promotes Dna2/BLM resection activity over Ku with MRN in vitro. Resection assay was performed as in Figure 4.3 except in a reaction containing 30 mM NaCl with 16 nM Dna2, 10 nm BLM, 100 nM Ku, 80 nM RPA, 10 nM MRN, and 50 nM CtIP wild-type or nuclease-deficient (N289A/H290A) proteins as indicated.

CONCLUSION

The Mre11/Rad50 complex is a multifunctional protein unit that exhibits endo- and exonuclease activity through Mre11 and ATP-dependent DNA unwinding activity through Rad50 (Bhaskara et al, 2007; Cannon et al, 2013; Paull & Gellert, 1998; Trujillo & Sung, 2001; Trujillo et al, 1998). The MR complex in archaea and the MRN(X) complex in eukaryotes promote HR repair by regulating 5' strand resection (Mimitou & Symington, 2009; Paull, 2010). In this study, I have investigated the biochemical roles of the human MRN complex in regulating DSB resection in vitro. Here I show that Mre11 nuclease activity is not required for antagonizing the effect of Ku in hExo1-dependent resection activity at DSB ends. However, I found that the ATP-dependent activity of Rad50 in the MRN complex is responsible to overcome Ku inhibition of hExo1. As studies have indicated that the ATP-dependent activity of Rad50 is responsible for unwinding duplex DNA ends (Cannon et al, 2013; Lee & Paull, 2005; Paull & Gellert, 1999), these results suggest that MRN inhibits Ku activity on DNA ends by pushing Ku away from the DNA ends rather than by removing Ku through endonucleolytic cleavage. On the other hand, I show that Mre11 nuclease activity is required for efficient resection on DNA substrates with protein covalent conjugates on the ends with hExo1, supporting the role of MRN(X) nuclease activity in processing meiotic DSBs and IR-induced DSBs in vegetative cells.

Human CtIP physically interacts with MRN to stimulate resection at DSB sites (Sartori et al, 2007; Yuan & Chen, 2009). However, the biochemical mechanism of how MRN and CtIP contribute to resection remains unclear. In this study, I show that CtIP stimulates MRN activity to overcome Ku inhibition of Dna2/BLM in resection. In a recent study, CtIP was found to exhibit 5' flap endonuclease activity and two conserved amino acid residues, N289 and H290, were responsible for this catalytic activity (Makharashivili et al, 2013). However, in my in vitro system CtIP nuclease activity did not have an effect on stimulating Dna2/BLM activity over Ku with MRN. In human cells, CtIP is required for resistance against camptothecin or etoposide treatment, which generate DSBs with topoisomerase I or II covalently attached to the ends, respectively (Huertas & Jackson, 2009; Sartori et al, 2007). Notably, CtIP nuclease activity was shown to be required for resistance against topoisomerase inhibitors in cells (Makharashivili et al, 2013), suggesting that CtIP nuclease activity is required for the efficient removal of topoisomerase-DNA intermediates.

MRN and CtIP are required for repairing DSBs generated by IR in vivo (Sartori et al, 2007). IR treatment generates DSBs with modified bases or structures in addition to covalent protein conjugates at the ends (Barker et al, 2005; Henner et al, 1983). Previous studies in budding yeast have shown Sae2 to be responsible for processing hairpin DNA structures (Lengsfeld et al, 2007; Rattray et al, 2001; Rattray et al, 2005). Further, Sae2 cooperates with MRX to exhibit efficient processing activity on hairpin DNAs (Lengsfeld et al, 2007; Lobachev et al, 2002). Therefore, it is possible that MRN and CtIP cooperates

in processing DSBs with modified structures, including hairpin DNAs, although further biochemical studies are required to elucidate this mechanism.

CHAPTER 5: DISCUSSION

DNA double strand breaks (DSBs) are one of the most deleterious forms of DNA lesions and failure to repair DSBs can lead to human disorders including developmental, immunological, and neurological diseases as well as cancer (Hoeijmakers, 2001; Khanna & Jackson, 2001). Therefore, proper repair of these DSBs is critical for cell survival and the maintenance of genome integrity. Cells use primarily two pathways to repair DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR). The NHEJ repair pathway mediates direct religation of broken ends whereas the HR repair pathway uses information from the intact sister chromatid to repair the break (Kanaar et al, 1998; Lieber, 2010). One of the critical determinants of repair pathway choice is the initiation of 5' strand resection of DNA ends, which commits cells to HR repair and prevents repair by NHEJ (Chapman et al, 2012).

Recent studies have identified a novel function of RPA in HR repair by stimulating the resection activity of Dna2/BLM (Sgs1) at the 5' strand of DNA ends (Cejka et al, 2010a; Nimonkar et al, 2011; Niu et al, 2010). In addition to RPA, SOSS1 has been identified as a new ssDNA binding protein complex in human cells (Huang et al, 2009; Richard et al, 2010). SOSS1 has been shown to promote HR repair by regulating the generation of ssDNA at DSB sites in vivo (Huang et al, 2009; Richard et al, 2008), although whether SOSS1 functions directly in DSB resection is unknown. In

this study, I have characterized the DNA binding properties of SOSS1 as well as its effect on hExo1 or Dna2/BLM resection activity in comparison to RPA in vitro.

The MRN complex is one of the initial proteins to detect DSBs in cells and is responsible for regulating DSB resection for HR repair (Paull, 2010). MRN interacts with CtIP in human cells to process DNA ends and to promote efficient HR repair (Chen et al, 2008; Sartori et al, 2007). However, the biochemical understanding of how MRN functions in resection and how CtIP participates in DSB resection with MRN is unclear. In this study, I have reconstituted in vitro resection assays using purified recombinant human proteins to understand the mechanistic role of MRN and CtIP in processing DSBs.

HUMAN SINGLE STRANDED DNA BINDING PROTEINS PROMOTE HR REPAIR BY STIMULATING DSB RESECTION

Characterization of the DNA binding properties of SOSS1 and RPA

RPA and SOSS1 are the major ssDNA binding complexes in human cells. The work described in this study characterizes the DNA binding specificities of RPA and SOSS1 in vitro. Using ensemble and single-molecule assays, this study demonstrates that RPA binds with higher affinity and stability to ssDNA compared to SOSS1. In our system, RPA had approximately 10-fold higher affinity for ssDNA compared to SOSS1.

It is known that RPA displays two distinct modes in binding ssDNA: an initial unstable binding mode that involves two OB domains of RPA1 and a more stable binding mode that involves the association of OB domains from RPA1 and RPA2 (Bochkarev et al, 1997; Brill & Bastin-Shanower, 1998; Wold, 1997). In constrast, I found that SOSS1 displays only a single binding mode that is much more dynamic compared to RPA. These results are conceivable considering that SOSS1 contains a single OB domain to bind ssDNA while RPA contains six OB domains, four of which bind DNA (Huang et al, 2009; Oakley & Patrick, 2010; Richard et al, 2008). As expected, RPA and SOSS1 bound poorly to duplex DNA ends. However, weak binding activities of these proteins to duplex DNA ends were observed in my in vitro assays. This is likely the result of denaturation of duplex DNA by these proteins, as RPA was previously shown to disrupt the hydrogen bonds between complementary DNA molecules to generate ssDNA for binding (Lao et al, 1999; Patrick & Turchi, 1999; Treuner et al, 1996). Unlike RPA, SOSS1 does not localize to DNA replication sites and does not affect cell progression to S phase in human cells (Huang et al, 2009; Richard et al, 2008). However, no definitive explaination for this has been proposed. Results from this study suggests that RPA outcompetes SOSS1 at DNA replication sites due to its high affinity and stability in binding ssDNA as well as its high concentration compared to SOSS1 in human cells (HCT116 and fibroblasts). Overall, this study describes the DNA binding affinity, stability, and kinetics of RPA and SOSS1 at both ensemble and single-molecule levels.

The biochemical function of SOSS1 in DNA DSB repair

In this study, I have reconstituted DNA end resection using purified recombinant SOSS1, RPA, hExo1, Dna2, BLM, and MRN. I have found that SOSS1 stimultates hExo1-dependent resection by promoting the binding of hExo1 to duplex DNA ends in an independent manner to MRN in vitro. This result was striking as both hExo1 and SOSS1 are poor in binding duplex DNA. A structural study of the catalytic domain of hExo1 with DNA has shown that hExo1 binds and induces a sharp bend of the 5' end of DNA, generating a frayed 5' end structure to perform nucleolytic activities (Orans et al, 2011). Notably, in this study, hExo1 resection activity was independent of SOSS1 on Y-ended DNA substrates. In addition, hExo1 displayed increased binding activity on Y-ended DNA compared to nearly blunt-ended DNA. Similar to what was observed with RPA in binding duplex DNA (Lao et al, 1999; Patrick & Turchi, 1999; Treuner et al, 1996), it is possible that SOSS1 is denaturing the duplex DNA ends to generate open-ended structures to increase hExo1 recruitment and activity on DNA ends. Recently, a single-molecule FRET system was well established to visualize DNA end unwinding activity by MRN (Cannon et al, 2013). Applying this system to SOSS1 will be a good approach to examine whether SOSS1 can directly open/unwind duplex DNA ends. On the other hand, unlike its activity on Dna2 and BLM, RPA was inhibitory to hExo1 resection activity in our in vitro system. Previously in fission yeast, dissociation of MRN from DNA was shown to be required for efficient DSB resection performed by Exo1 in vivo (Langerak et al, 2011). Considering the stable binding mode of RPA compared to

the dynamic binding mode of SOSS1 to DNA, it is possible that once resection is initiated, RPA persists on the ssDNA and impedes hExo1 progression, thus inhibiting extensive resection by hExo1.

In human cells, SOSS1 forms foci shortly after DNA damage at sites adjacent to the DSB ends (Richard et al, 2008), suggesting that SOSS1 may function in the early steps in DSB repair. Co-depletion of the component of SOSS1 and CtIP decreases HR efficiency further than that achieved by single depletion in human cells (Huang et al, 2009), indicating that SOSS1 and CtIP play non-redundant roles in regulating HR repair. In this study, SOSS1 stimulation of hExo1 was independent of MRN or CtIP and was able to stimulate hExo1 to a comparable level to MRN. Therefore, it is possible that SOSS1 functions as an additional factor to MRN/CtIP to promote the initiation of resection by hExo1.

SOSS1 has a unique ability to stimulate hExo1-dependent resection independently of MRN in vitro. However, protein-protein interactions between the components of MRN and SOSS1 were observed in human cells (Huang et al, 2009; Richard et al, 2011). SOSS1 foci formation at DSBs also was shown to require MRN during the S/G2 phase of the cell cycle (Huang et al, 2009), although it is unclear whether the physical interaction between SOSS1 and MRN is required for this process. MRN is known to stimulate ATM kinase activity in response to DSBs to amplify DNA damage signaling in cells (Lee & Paull, 2007). Once activated, ATM phosphorylates the Nbs1 component of MRN,

although the function of Nbs1 phosphorylation is not fully understood (Lee & Paull, 2007). The hSSB1 component of SOSS1 is phosphorylated by ATM at T117 in response to DSB damage and this phosphorylation event is required to further potentiate ATM kinase activity (Richard et al, 2008). Notably, silencing of the components of SOSS1 reduces ATM kinase activity in response to DSB damage (Li et al, 2009; Richard et al, 2008), suggesting a role of SOSS1 in DSB signaling. Considering the timing of localization and distribution pattern of SOSS1 and RPA at DSBs, it is possible that SOSS1 acts in the initial steps in DSB damage signaling by activating ATM prior to resection whereas RPA acts in the later steps in DSB damage signaling by activating ATR after extensive resection. As RPA stimulation of the ATR kinase occurs through its interaction with the ATR interacting protein (ATRIP) (Ball et al, 2007; Kim et al, 2005; Zou & Elledge, 2003), it is also possible that SOSS1 regulates ATM activation through its interaction with MRN, which is distinct from its activity in resection.

Characterization of RPA and SOSS1 phosphorylation in DSB repair

Post-translational modifications modulate the activity of most proteins in eukaryotes. Such modification includes proteolytic cleavage or addition of a modifying group to one or more amino acids in the protein. Post-translational phosphorylation is one of the most common and important modifications that occurs in eukaryotic cells. Phosphorylation can increase or decrease the biological activity of an enzyme, regulate

protein localization between subcellular compartments, allow interactions between proteins or with DNA, and also label proteins for degradation (Secko, 2003).

RPA is a multifunctional protein complex that is involved in DNA replication, DNA damage signaling, and repair (Oakley & Patrick, 2010). Studies in eukaryotes have shown that RPA is phosphorylated at the RPA2 subunit by cyclin-dependent kinases (CDKs) in a cell cycle dependent manner and by ATM, ATR, and DNA-PKcs in response to DNA damage (Oakley & Patrick, 2010). In fact, phosphorylation of RPA was shown to affect cell localization and distribution, protein-protein interactions, and DNA binding affinity of the complex (Oakley & Patrick, 2010). For example, RPA2 phosphorylation induces a conformation change of the complex to alter the ssDNA binding mode of RPA1 and increase RPA1 interaction with DNA repair proteins such as Mre11, p53, ATRIP, and Rad9 in response to DNA damage (Ball et al, 2007; Bochkareva et al, 2005; Lin et al, 1996; Olson et al, 2007; Xu et al, 2008). Moreover, RPA distribution within mitotic cells changes in response to DNA damage-dependent phosphorylation (Stephan et al, 2009). This suggests that phosphorylation of RPA is a critical modulator in organizing its activity in response to DNA damage. RPA has a multifaceted role in HR repair: stimulation of 5' strand resection and sequestration of ssDNA generated by resection (Cejka et al, 2010a; Nimonkar et al, 2011; Niu et al, 2010). However, it is unclear whether phosphorylation has a direct effect in regulating RPA activity in these processes. Therefore, development of an in vitro system that

includes physiologically relevant RPA phosphorylation may help us better understand the biochemical mechanisms underlying this dual role of RPA in HR repair.

The recruitment mode of SOSS1 to DSB sites differs depending on the cell cycle phase. SOSS1 foci formation requires MRN in S/G2-arrested cells but is independent of MRN and CtIP in G1 cells (Huang et al, 2009). Although a direct protein-protein interaction between SOSSA and Nbs1 was suggested to affect SOSS1 DSB foci formation in S/G2 cells (Huang et al, 2009), it does not clarify why this interaction is effective only during the S/G2 phase for SOSS1 recruitment to DSB sites. CDKs play a critical role in regulating the decision of DSB repair pathway in different cell cycle phases (Wohlbold & Fisher, 2009). Sae2 and its functional orthologs Ctp1 and CtIP are one of the primary targets of CDKs, in which CDK-dependent phosphorylation of the proteins primes S/G2 cells to go under HR repair (Huertas & Jackson, 2009; Wang et al, 2013; Williams et al, 2009). Notably, CDK-dependent phosphorylation of fission yeast Ctp1 and human CtIP affects their interaction with Nbs1, which affects their activity in DNA damage signaling and resection (Wang et al, 2013; Williams et al, 2009). I have found multiple potential CDK target sites in SOSSA (INTS3), all that are within the CDK consensus sequence [S/T]PX[R/K]X. It is possible that SOSS1 serves a novel CDK target that also plays a critical role in the decision of DSB repair in cells. If SOSSA (INTS3) is a true target of CDK, CDK-dependent phosphorylation of SOSSA could be the determinant factor that regulates its interaction with Nbs1 to enable localization of SOSS1 to DSBs sites in S/G2 cells, where it can participate to promote HR repair.

MRN AND CTIP PROMOTE DNA DSB RESECTION IN VITRO

Biochemical activities of MRN in processing DSBs

Regulation of DSB resection dictates the choice between HR repair versus NHEJ (Symington & Gautier, 2011). Genetic studies in yeast suggest a competition between MRX(N), which promotes resection, and Ku, which promotes NHEJ, underlies this process (Langerak et al, 2011; Shim et al, 2010). In this study, I have examined the biochemical mechanisms underlying the regulation of DNA end resection through a reconstituted in vitro resection assay. I demonstrated that Ku represses hExo1- and Dna2/BLM-mediated DNA end resection and that MRN relieves this Ku inhibitory effect of hExo1 and Dna2/BLM activity in vitro. Further, I showed that the nuclease-deficient mutant MRN (H129N or H129L/D130V) does not affect its activity in overcoming Ku inhibition of hExo1 resection activity in vitro, consistent with the result observed in budding yeast with MRX and Ku (Shim et al, 2010). However, when I tested the MR(S1202R)N mutant in resection, which is deficient in ATP-dependent activities of MRN (Moncalian et al, 2004), it failed to overcome the Ku inhibitory effect of hExo1-dependent resection in vitro. The MR(S1202R)N mutant was also shown in a previous study to be deficient in ATM activation in vitro (Lee & Paull, 2005). This defect was suppressed when using DNA molecules with unwound ends (Lee & Paull, 2005), suggesting that the deficiency is in part from its inability to unwind DNA ends. A recent

study from our group in collaboration with the Russell's group established a single-molecule system to visualize MRN binding activity on DNA (Cannon et al, 2013). The study demonstrates that MRN exhibits DNA unwinding activity in an ATP-dependent manner and that the S1202R mutation compromises this activity (Cannon et al, 2013). Considering these results, it is possible that MRN represses Ku inhibition of resection by pushing Ku away from the DNA ends through its ATP-dependent DNA unwinding activity than by removing Ku directly from DNA ends through Mre11-dependent nucleolytic cleavage. This hypothesis is currently being pursued by other members of the laboratory using single-molecule techniques.

In my experiments, I found that Mre11 nuclease activity was dispensable for hExo1 stimulation of resection in vitro, where the DNA substrates had ends generated by restriction enzyme cleavage. Studies in budding yeast have also demonstrated that Mre11 nuclease activity is not essential for 5' strand resection of endonuclease-induced DSBs (Llorente & Symington, 2004; Nicolette et al, 2010; Shim et al, 2010). While the physical absence of MRX delays the rate of resection on endonuclease-induced DSB ends (Mimitou & Symington, 2008), the Mre11 nuclease-deficient yeast strain has no effect (Llorente & Symington, 2004). Moreoever, yeast Dna2 can substitute for MRX nuclease activity in DSB repair (Budd & Campbell, 2009). An in vitro study with the archaeal MR complex also demonstrated that Mre11 nuclease activity is dispensable for activating NerA/HerA-dependent resection (Hopkins & Paull, 2008). DSBs in these studies are considered to have "clean" ends, where the break site is not chemically altered on the

ends. However, in contrast to these results, repair of DSBs with “dirty” ends, where the break sites have modified bases, structures, or covalent adducts on the ends, appears to require the nuclease activity of Mre11. In yeast, strains expressing Mre11 nuclease-deficient mutant display a delay in resection on IR-induced DSBs (Moreau et al, 1999). Moreover, Mre11 activity is essential for processing DSBs induced by Spo11 cleavage (Milman et al, 2009; Moreau et al, 1999). Overall, these results suggest that the nuclease activity of MRN(X) is not essential when the broken DNA ends are chemically normal but becomes critical when the ends are modified or blocked with covalent adducts.

Roles of MRN nuclease activity and CtIP in DSB resection

To examine whether the nuclease activity of MRN plays a direct role in processing DSBs with “dirty” ends, for example breaks with protein adducts attached to the ends, DNA substrates with Ku conjugated to either the 5’ or 3’ ends were constructed. Notably, in contrast to wild-type MRN, the nuclease deficient mutant M(H129L/D130V)RN was inefficient in stimulating hExo1 resection activity on these substrates, supporting the idea that MRN utilizes its nuclease activity to process DSBs with blocked ends. MRN interacts with the human Sae2 ortholog, CtIP, to perform limited processing at DNA ends (Chen et al, 2008; Sartori et al, 2007). In yeast, the nuclease activity of MRX(N) and Sae2(Ctp1) are critical for processing Spo11(Rec12)-DNA intermediates during meiosis (Neale et al, 2005; Prinz et al, 1997). Human cells

depleted of MRN or CtIP are sensitive to topoisomerase inhibitors, which create DNA breaks with topoisomerases covalently linked to the ends (Sartori et al, 2007). It is unclear whether the nuclease activity of Mre11 and CtIP take part in the process of removing topoisomerase covalent complexes from DNA, as a novel phosphodiesterase called Tdp1/TTRAP was identified as the primary enzyme to remove 5' tyrosyl linkages in human cells (Cortes Ledesma et al, 2009). However, it is possible that MRN and CtIP may be involved in an alternative pathway of the nucleolytic removal of topoisomerase covalent complexes in cells.

The effect of CtIP nuclease activity in resection

CtIP exhibits 5' flap endonuclease activity in vitro (Makharashivili et al, 2013). However, in my in vitro system the nuclease activity of CtIP did not affect hExo1- or Dna2/BLM-dependent resection activity. Genetic studies in yeast have suggested the requirement of Sae2 to remove the topoisomerase-like protein Spo11 from DNA break sites during meiosis (McKee & Kleckner, 1997; Prinz et al, 1997). In addition, genetic and biochemical assays have described a role for Sae2 in processing hairpin-capped DNA ends by exhibiting endonuclease activity (Lengsfeld et al, 2007; Lobachev et al, 2002). In vivo results with CtIP have shown that the nuclease deficient mutant CtIP (N289A/H290A) only partially complements CtIP deletion in response to topoisomerase inhibitors in human cells, whereas the mutant shows full activity when restriction

enzyme-induced DSBs are required (Makharashivili et al, 2013). In addition, expression of the CtIP N289A/H290A mutant in mouse pre-B cells depleted of endogenous CtIP displayed defective activity in processing hairpin-sealed coding ends (Makharashivili et al, 2013). Altogether, this suggests that the nuclease activity of CtIP is likely involved in processing topoisomerase-intermediates in cells or in processing DSB ends with modified structures. Therefore, reconstitution of resection with topoisomerase-conjugated DNA intermediates or with hairpin DNAs in vitro will help us understand the mechanistic role of CtIP nuclease activity in DSB resection.

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