BIOCHEMICAL AND GENETIC STUDIES OF GENOMIC STABILITY

Thesis by

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For Mom. I know you would have been proud.

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ABSTRACT

Genomes face a constant barrage of threats from endogenous and exogenous sources. The need to maintain fidelity while replicating the entire genome during each cell division necessitates a dynamic cadre of proteins and protein complexes that participate in the DNA replication process. Furthermore, DNA can be damaged during all phases of the cell's life and that damage must be recognized and repaired in a way that preserves genetic information. These studies focus on one enzyme at the nexus of DNA replication and DNA repair, the helicase/nuclease Dna2. We show that Dna2 possesses a novel ATP/Mn^{2+} dependent flap endo/exonuclease activity and a DNA end-independent endonuclease activity that is inhibited by Replication Protein A. The regulation of Dna2 activity in the context of the global DNA damage response is of great interest. To that end, we explored the relationship of Dna2 and the DNA damage sensor kinase Mec1. We find that Dna2 is phosphorylated by Mec1 following DNA damage in its N-terminal domain. We then extended these studies from yeast to higher eukaryotes utilizing the *Xenopus* cell free extract system. Using simulated double strand breaks (DSBs), we constructed a timeline of protein processing steps required for homologous recombination mediated repair. This strategy using *Xenopus* extracts also place Dna2 on chromatin during DNA replication, physically interacting with other proteins involved in lagging strand replication. Taken together, these biochemical and genetic studies elucidate the multiple roles the Dna2 enzyme plays in order to ensure genomic stability.

Table of Contents

List of Figures and tables

Page

Nomenclature

And-1 Acidic nucleoplasmic DNA-binding protein 1

ATM Ataxia telangiectasia mutated

ATP Adenosine triphosphate

ATR ATM and Rad3-related

ChIP Chromatin immunoprecipitation

Ctf4 Chromosome transmission fidelity 4

DNA Deoxyribonucleic Acid

DSB Double strand break of DNA

dsDNA Double-stranded DNA

FEN1 Flap Endonuclease 1

HR Homologous recombination

HU Hydroxyurea

MMS Methyl methanesulphonate

MRN Mre11-Rad50-Nbs1 protein complex

MRX Mre11-Rad50-Xrs2 protein complex

MS Mass spectrometry

NHEJ Non-homologous end joining

nt nucleotides

OFP Okazaki fragment processing

PCNA Proliferating cell nuclear antigen

PIKK Phosphoinositol-3 kinase-related kinase

Pol α Polymerase alpha

Pol δ DNA polymerase delta

Pre-RC Pre-replication complex

RFC Replication factor C

RNA Ribonucleic acid

RPA Replication Protein A

S.c. *Saccharomyces cerevisiae*

SSA Single-strand annealing

ssDNA Single stranded DNA

CHAPTER 1

INTRODUCTION

Maintaining genomic stability is vital to the fitness of all organisms. The consequences of genomic instability, chromosomal rearrangements, chromosome loss or duplications, are all hallmarks of cancer. Cells have developed many ways to prevent and repair DNA damage, although many of these pathways remain to be comprehensively characterized. Each cell division presents the challenge of replicating an entire genome with high fidelity, both on the continuous leading strand and the discontinuous lagging strand. Single strand lesions during DNA replication can become double strand breaks if replication forks pass the damage. Even the act of creating one continuous DNA molecule from Okazaki fragments on the lagging strand requires the coordinated efforts of many proteins.

LAGGING STRAND REPLICATION

Due to the anti-parallel nature of DNA and the fact that DNA is replicated only in the 5' to 3' direction, moving replication forks can only synthesize one of the two strands continuously. The other strand, known as the lagging strand, is synthesized in small sections from the template strand (Alberts et al., 2008; Ayyagari et al., 2003; Balakrishnan and Bambara, 2011; Jin et al., 2003). These segments of replicated DNA, called Okazaki fragments are approximately 150 nucleotides longs, resulting in upwards

of 10^5 fragments of DNA that must be joined in each cell division in *Saccharomyces cerevisiae.*

Each Okazaki fragment originates from a RNA-DNA primer synthesized by the polymerase α/primase complex. It is important to note that pol α is a low fidelity polymerase lacking a proofreading function to correct any mistakes in nucleotide incorporation. The RNA-DNA primer is recognized by the Replication Factor C (RFC) complex which results in the loading of the PCNA ring and polymerase switching from pol α to pol δ. Polymerase δ extends the nascent Okazaki fragment until it encounters the RNA-DNA primer of the previous Okazaki fragment. While polymerase δ does not possess the ability to remove the RNA primer, it can displace the 5' end of the previous Okazaki fragment and continue to synthesize DNA along the template. This strand displacement synthesis results in a 5' single stranded RNA-DNA flap that must be removed in order to create a ligatable nick that can be processed into a continuous DNA strand.

The nuclease responsible to digesting the majority of the displaced flaps is Flap Endonuclease 1 (FEN1). DNA ligase I then seals the nick left by FEN1 resulting in a mature Okazaki fragment that is part of the continuous DNA duplex. However, the gene that encodes FEN1, *RAD27*, is not an essential gene in yeast implying additional pathways of Okazaki fragment processing. Further, FEN1 cannot remove long single stranded DNA flaps coated in Replication Protein A (RPA). Therefore, further characterization of lagging strand DNA replication required the identification of other proteins involved in the processing of Okazaki fragments.

THE ROLE OF DNA2 IN DNA REPLICATION

The enzyme Dna2, discovered in a screen for DNA replication genes using permeabilized nuclei (Kuo et al., 1983), was the first helicase identified as required for chromosomal replication (Budd and Campbell, 1995; Budd et al., 1995). The Dna2 protein was found to have single stranded DNA-dependent ATPase and helicase activity. The first indications of Dna2's specific role in DNA replication came from the physical and genetic interaction between itself and *RAD27* (FEN1), the flap endonuclease responsible for processing most 5' ssDNA flaps created by polymerase δ during stand displacement synthesis of Okazaki fragments (Budd and Campbell, 1997). As mentioned above, *rad27*Δ is viable, albeit with high rates of recombination, temperature sensitivity, and MMS sensitivity (Xie et al., 2001). This mutant, however, cannot survive without Dna2 function, as *dna2-1* and *rad27*Δ are synthetically lethal (Budd and Campbell, 1997).

DNA2 mutants are also synthetically lethal with *ctf4*Δ and *pol1*Δ, members of the pol α/primase complex (Formosa and Nittis, 1999), and cannot fully replicate their DNA (Braguglia et al., 1998; Fiorentino and Crabtree, 1997). Further studies of Dna2 found the enzyme also possessed nuclease activity leading to a model of Dna2 and FEN1 acting together in Okazaki fragment processing (Ayyagari et al., 2003; Bae and Seo, 2000; Bae et al., 1998; Budd et al., 2000). The activity of Dna2 endonuclease was found to be highest on long 5' ssDNA flaps coated with RPA protein, a substrate that is incompatible for processing by FEN1 (Bae and Seo, 2000; Bae et al., 2001). However, the importance

of this function has always been questioned as most models suggest that long flaps are not routine and FEN1 is sufficient to process the majority of Okazaki fragments (Garg and Burgers, 2005; Jin et al., 2003; Kao and Bambara, 2003; Kao et al., 2004).

Recently a new model has been developed that proposes in hard to replicate regions, a long stretch of strand displacement synthesis by polymerase δ is used to remove the entire portion of the previous Okazaki fragment replicated by the lower fidelity polymerase α (Balakrishnan and Bambara, 2011). In this model, the helicase PIF1 stimulates polymerase δ strand displacement synthesis, creating a long flap that Dna2 cleaves, removing the DNA with potential mismatches and preserving genomic stability. Evidence for this model comes from the fact that *pif1* mutants can suppress the lethality of *dna2*Δ (Budd et al., 2006), implying that without *PIF1* stimulating the formation of long flaps, FEN1 is sufficient for Okazaki fragment processing. Additional evidence is found in the effects of acetylation, an increasingly prominent post-translational modification, that has opposing effects on FEN1 and Dna2 enzymatic activity, creating a potential short/long flap pathway switch (Balakrishnan et al., 2010c). While the intricate dance at Okazaki fragments continues to be characterized to greater detail (Balakrishnan et al., 2010a; Burgers, 2009; Henry et al., 2010; Kang et al., 2010; Pike et al., 2009; Pike et al., 2010; Stewart et al., 2009), many of the regulation or coordination mechanisms remain to be elucidated.

THE DNA DAMAGE RESPONSE

Even if DNA is replicated with perfect fidelity, genotoxins, either endogenous or exogenous, threaten the genetic material during all phases of the cell cycle. DNA damage is addressed by checkpoints, sophisticated control mechanisms that slow progression through the cell cycle while the DNA damage is repaired (Friedel et al., 2009; Navadgi-Patil and Burgers, 2009). In S phase, slowing or stalling of replication forks can trigger a checkpoint, even in the absence of damage. In yeast, this response is coordinated by the kinase Mec1, a member of the PIKK family of DNA damage response kinases (see Table 1.2 for nomenclature). Mec1 activation results in stabilization of replication forks, transcription of DNA damage induced genes, DNA repair, and prevention of late origin firing and entry into mitosis.

Activation of Mec1, or its human homologue ATR, depends on the accumulation of RPA coated ssDNA, either at stalled replication forks or due to nucleolytic processing of DSBs. The 9-1-1 clamp (Rad9-Rad1-Hus1 in humans and Rad17-Mec3-Ddc1 in yeast) is loaded onto to 5' junctions between single and double stranded DNA, Dpb11/TopBP1 is recruited, and the Mec1/ATR kinase is activated. Mec1 then phosphorylates numerous targets including effector kinases Rad53 and Chk1, and mediator proteins Rad9/53BP1 and Mrc1/Claspin. When the DNA damage or fork arrest is handled properly, these pathways allow the replication fork to restart and the cell cycle to continue with repaired DNA.

The most deleterious form of DNA damage are double strand breaks (DSB), as they can lead to gross chromosomal changes, duplications, deletions and translocations.

Two separate pathways, non-homologous end joining (NHEJ) and homologous recombination (HR), provide the cell with an opportunity to repair the broken chromosome. When double strand breaks occur, two other kinases become activated. Tel1/ATM is a member of the PIKK family. As the name ATM (Ataxia Telangiectasia Mutated) implies, mutations in ATM lead to the human disorder ataxia telangiectasia, whose cells are extremely sensitive to ionizing radiation and exhibit chromosomal instability. Following phleomycin-induced DSBs in S phase, Tel1 phosphorylates Xrs2 and is required for Rad53 phosphorylation. While *tel1*Δ mutants show some defects in preventing cell cycle progression after DSBs in S phase, *TEL1* is not required of the DSB response in G1, S or G2/M phase and the signaling contributions of Tel1 remain an open question.

Cdk1 (*CDC28*) is a also a critical partner in genomic stability (Enserink et al., 2009). *CDC28* is responsible for transcriptional programs at each stage in the cell cycle, budding and cell morphology, and formation of the mitotic spindle (Mendenhall and Hodge, 1998). Cdk1 determines whether DSBs are repaired by NHEJ or HR because the first step of HR depends on activity Cdk1 which is low in G1 phase (Ira et al., 2004). In S and G2/M phase when Cdk1 is active, homologous recombination is preferred due to the presence of a repair template in the sister chromatid.

The first step of HR is resection of the 5' end of the double strand break to create 3' single stranded DNA tails. This ssDNA is first bound by RPA, which activates Mec1/ATR, and later coated by Rad51. The resection step can be divided into two phases, the slower initial processing for the region near the break and long range resection. The initial response is instigated by the Mre11-Rad50-Xrs2 complex (Mre11-

Rad50-Nbs1 in human cells) and Sae2 (Paull, 2010). Mre11 is a nuclease, but displays manganese-dependent 3'-5' endonuclease activity meaning it is not the nuclease responsible for long range 5'-3' resection. One important role of the MRX/N complex is to oppose Ku, a heterodimer that competes with MRX/N to bind to DSB ends and initiate NHEJ. While the nuclease responsible for the bulk of DSB resection was unknown until recently, the creation of the Rad51 filament was clearly essential in the search for homology, strand invasion, and eventual synthesis of DNA using the sister chromatid template (Holthausen et al., 2010).

DNA2 IN THE DNA DAMAGE RESPONSE

Early on in the study of Dna2, two classes of mutants were identified; those with temperature sensitivity did not correlate with mutants that were sensitive to DNA damaging agent MMS (Formosa and Nittis, 1999). While both the nuclease and helicase domains of Dna2 are essential (Budd et al., 2000; Budd et al., 1995; Lee et al., 2000), surprisingly the essential function could be suppressed with the deletion of *RAD9*, a key player in the DNA damage checkpoint (Budd et al., 2011; Fiorentino and Crabtree, 1997; Formosa and Nittis, 1999). Growth defects in some *dna2* mutants are also suppressed by the disruption of *MEC1* (Budd et al., 2005). Moreover, Dna2 is important for preventing DSBs at replication fork barriers in ribosomal DNA, sites where replication fork stability is required for avoid DSBs (Weitao et al., 2003a; Weitao et al., 2003b).

A synthetic genetic array, used to identify pathways outside of Okazaki Fragment Processing that require *DNA2*, found connections to DSB repair, mismatch repair, the

replication stress checkpoint, and osmotic and oxidative stress responses (Budd et al., 2005). Some Dna2 mutants are also sensitive to DSBs caused by X-rays and bleomycin (Budd and Campbell, 2000a, b). In 2008, several labs discovered that the major nuclease involved in DSB resection was Dna2 (Budd and Campbell, 2009; Liao et al., 2008; Mimitou and Symington, 2009a, b; Zhu et al., 2008), and that either Dna2 or Mre11 nuclease activity is required to survive DSBs.

STUDIES OF DNA2 AND GENOMIC STABILITY

The contributions of Dna2 to genomic stability are numerous, both in avoiding DNA breaks and errors during DNA replication and in order to properly repair DNA damage that arises from exogenous sources. In these studies, we further characterize the Dna2 enzyme biochemically, uncovering an ATP/Mn^{2+} dependent flap endo/exonuclease activity. Dna2 is a structure-specific enzyme whose flap endo/exonuclease and helicase functions require a free, unblocked ssDNA end for activity (Balakrishnan et al., 2010b; Stewart et al., 2010). Here, we describe an endonuclease activity that does not require, but can in fact create, DNA ends. The endonuclease function is inhibited by RPA, highlighting the important of studying Dna2 enzymatically in the context of other proteins involved in DNA metabolism.

We also explore the relationship between Dna2 and multiple kinases involved in maintaining genomic stability. We find that Dna2 can be purified from yeast cells in a phosphorylated form, and treatment of the protein with phosphatase inhibits both ATPase and flap endo/exonuclease *in vitro.* We further demonstrate that Dna2 is phosphorylated

by both Cdk1 and Mec1 *in vitro*, and that Dna2 is phosphorylated *in vivo* following DNA damage in its N-terminal regulatory domain. Individual phosphorylation events, however, are dispensable for survival of DNA damage, and their regulatory effects are yet to be characterized.

In order to assess the role of Dna2 in genomic stability in a more comprehensive way, we used the *Xenopus* cell free extract system to reconstruct the initial response to DSBs and establish a timeline of DSB end processing steps. Protein-protein interactions between Dna2 and DSB response proteins ATM and NBS1 were discovered, and we found that Dna2 was required for timely recruitment of RPA to simulated DSBs. This *Xenopus* extract system was also useful for uncovering interactions between Dna2 and DNA replication proteins And-1 and Mcm10, and demonstrating the recruitment of Dna2 to chromatin during DNA replication. With a combination of these biochemical and genetic approaches, we find that Dna2 is a player in the two important aspects of maintaining genomic stability, DNA replication fidelity and DNA damage repair. Importantly, we find that these roles are conserved from yeast to human Dna2 proteins.

Table 1.1 Key Proteins Involved in Okazaki Fragment Processing

Protein	Step	Function
RPA	Multiple	ssDNA binding protein, prevents secondary structures, coordinates replication associated proteins
Primase	Priming	Synthesis of 8-12 nt RNA primer
Pol α		Extends RNA primer with ~20 nt DNA
Ctf4		Stabilizes pol α
RFC	Extension	Triggers polymerase switching
Polδ		Lagging strand polymerase that extends the RNA-DNA primer, proofreading polymerase
Dna2	Flap processing	Removes long 5' ssDNA flaps bound by RPA leaving a short ssDNA flap
Fen1		Removes short ssDNA flaps leaving a ligatable nick
Pif1		Helicase that can promote strand displacement synthesis, especially on fold back flaps*
Exo1		Nuclease that can function as a back-up in removing short flaps to create a nick in the absence of Fen1
Ligase	Nick ligation	Ligates nick to create continuous strand

*fold back flaps are 5' ssDNA flaps created by displacement synthesis of tandem repeat sequences that can form hairpins

Table 1.2 Proteins Involved in DNA Damage Response

S. cerevisiae	*S. pombe*	Human/*Xenopus*	Function
RPA	RPA	**RPA**	ssDNA binding protein
Rad24	Rad17	**Rad17**	RFC-like clamp loader subunit
Ddc1	Rad9	**Rad9**	Sensor, 9-1-1 checkpoint clamp
Rad17	Rad1	**Rad1**	Sensor, 9-1-1 checkpoint clamp
Mec3	Hus1	**Hus1**	Sensor, 9-1-1 checkpoint clamp
Dpb11	Cut5	**TopBP1**	Sensor, activates Mec1/ATR
Mec1	Rad3	**ATR**	PIKK sensor kinase
Ddc2	Rad26	**ATRIP**	Mec1/ATR regulatory subunit
Tel1	Tel1	**ATM**	PIKK sensor kinase
Cdc28	Cdc2	**Cdk1**	Cdk, DSB resection initiation
Rad9	Crb2	**53BP1/BRCA1**	Mediator, scaffold protein
Mrc1	Mrc1	**Claspin**	Mediator, scaffold protein
Rad53	Cds1	**Chk2**	Effector kinase
Chk1	Chk1	**Chk1**	Effector kinase
Mre11	Rad32	**Mre11**	MRX/N DSB sensor/processing
Rad50	Rad50	**Rad50**	MRX/N DSB sensor/processing
Xrs2	Nbs1	**Nbs1**	MRX/N DSB sensor/processing
Sae2	Ctp1	**CtIP**	Nuclease, promotes resection
Sgs1	Rqh1	**BLM/WRN**	RecQ helicase

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CHAPTER 2

CHARACTERIZATION OF THE ENDONUCLEASE AND ATP-DEPENDENT FLAP ENDO/EXONUCLEASE OF DNA2

ABSTRACT

Two processes, DNA replication and DNA damage repair, are key to maintaining genomic fidelity. The Dna2 enzyme lies at the heart of both of these processes, acting in conjunction with FEN1 and RPA in DNA lagging strand replication and with BLM/Sgs1 and MRN/X in double strand break repair. *In vitro*, Dna2 helicase and flap endo/exonuclease activities require an unblocked 5' ssDNA end in order to unwind or

cleave DNA. In this study we characterize a Dna2 nuclease activity that does not require, and in fact can create, 5' ssDNA ends. Both endonuclease and flap endo/exonuclease are abolished by the Dna2-K677R mutation, implicating the same active site in catalysis. In addition we define a novel ATP-dependent flap endo/exonuclease activity, which is observed only in the presence of Mn^{2+}. The endonuclease is blocked by ATP and is thus experimentally distinguishable from the flap endo/exonuclease function. Thus, Dna2 activities resemble those of RecB and AddAB nucleases even more closely than previously appreciated. This work has important implications for understanding the mechanism of action of Dna2 in multi-protein complexes, where dissection of enzymatic activities and cofactor requirements of individual components contributing to orderly and precise execution of multi-step replication/repair processes depends on detailed characterization of each individual activity.

INTRODUCTION

The Dna2 protein is an essential enzyme involved in both Okazaki fragment processing and double strand break repair (Bae and Seo, 2000; Budd and Campbell, 1997, 2000; Budd et al., 2005; Weitao et al., 2003). The conserved Dna2 enzymatic activities, single-stranded DNA (ssDNA) dependent ATPase, 5'-3' helicase, 5'-3' endo/exonuclease, 3'-5' exonuclease, single-strand annealing, and strand exchange, function in DNA replication and DSB repair in both the nucleus and mitochondria of yeast and human cells (Copeland and Longley, 2008; Duxin et al., 2009; Kim et al., 2006; Liao et al., 2008; Masuda-Sasa et al., 2006a; Masuda-Sasa et al., 2006b; Masuda-Sasa et

al., 2008; Nimonkar et al., 2011; Wawrousek et al., 2010; Zheng et al., 2008; Zhu et al., 2008). Genetic and biochemical studies have shown yeast, *Xenopus,* and human Dna2 nuclease to function with Sgs1/BLM helicase and the MRX/MRN complex in the 5' end resection of double strand breaks and with Flap endonuclease 1 (FEN1), RPA, and Ctf4 in lagging strand DNA replication (Bae et al., 2003; Budd and Campbell, 2009; Burgers, 2009; Formosa and Nittis, 1999; Garg and Burgers, 2005; Mimitou and Symington, 2009a, b; Nimonkar et al., 2011; Niu et al., 2010; Stewart et al., 2009; Stith et al., 2008; Tsutsui et al., 2005; Wawrousek et al., 2010). Given its key roles in these two important pathways and its multiplicity of biochemical functions, it is of interest to further characterize the unique enzymatic functions and substrate preferences of the Dna2 enzyme that underlie these physiological processes.

The preference of Dna2 for ssDNA flaps, such as 5' terminated tails generated during strand displacement synthesis by pol δ during Okazaki fragment processing, is evident in binding and enzymatic studies (Stewart et al., 2006, 2010). The helicase activity of Dna2 requires a free 5' ssDNA end in order to unwind DNA, and the flap endo/exonuclease activity requires an unblocked 5' end in order to cleave flap structures (Balakrishnan et al., 2010; Kao et al., 2004a). While the DNA end is necessary to stimulate the flap nuclease activity, cleavage occurs within the flap up to 6-7 nucleotides from the ssDNA/dsDNA junction making the reaction technically endonuclease (Bae and Seo, 2000; Budd et al., 2000). This function is termed flap endo/exonuclease in this study consistent with other proteins that have similar modes of action. DNA fork structures or ssDNA flap structures (single-stranded tails at a nick or gap in duplex DNA) compete for Dna2 binding more effectively than nicked duplex or ssDNA gap regions,

showing that Dna2 is a structure-specific enzyme (Stewart et al., 2010). This binding is independent of the free end required for nucleolytic cleavage, since Dna2 can bind to a DNA fork or flap when the 5' end is blocked, even though its nuclease activity cannot cleave the structure (Stewart et al., 2006, 2009).

The purpose of the DNA end is of considerable interest in understanding how Dna2 functions in the cell. The Dna2 enzyme is proposed to initially bind to the ssDNA/dsDNA junction and then track down the flap from the free end. Evidence for this model comes from experiments with a blockage in the middle of the flap that results in cleavage between the end and the blockage point but not beyond the block toward the base of the flap (Kao et al., 2004a). One unique role of Dna2 is to remove long flaps that are coated by the ssDNA binding protein RPA, as these structures cannot be removed by FEN1 during Okazaki fragment processing (Pike et al., 2009; Stewart et al., 2009; Stewart et al., 2008). Interestingly, yeast Dna2 can displace RPA from flaps with blocked 5' ends, even though Dna2 cannot track down the length of the blocked flap (Stewart et al., 2008).

These observations have led us to investigate further the requirement for free ends in Dna2 activity. In this study, we characterize Dna2 endonuclease activity in the absence of DNA ends (Budd et al., 2000). We show that Dna2 can bind circular ssDNA and cleave it, but that, in contrast to previous results, circular DNA is not an effector of the ATPase activity (Bae et al., 2002). We also find that the endonuclease and the flap endo/exonuclease are catalyzed by the same active site, but that, nevertheless, the activities are distinguishable based on their cofactor requirements. The presence of this endonuclease function allows the Dna2 enzyme to create free ends for further helicase

and flap endo/exonuclease processing, which might be encountered during repair of DSBs with ends modified by proteins or terminating in modified nucleotides.

EXPERIMENTAL PROCEDURES

Materials and Substrates- M13mp18 and ΦX174 phage ssDNA was purchased from New England Biolabs. All synthetic oligonucleotides were purchased from Integrated DNA Technologies. Oligonucleotide sequences are listed in Table 1. [γ-^{32}P]ATP, 10 mCi/ml, was purchased from MP Biomedicals. The 5' oligonucleotide labeling reactions were performed as described previously with T4 polynucleotide kinase and [γ-^{32}P]ATP (Kao et al., 2004b). For annealing to M13mp18, oligonucleotides were placed in TE (1 mM EDTA and 10 mM Tris–HCl, pH 8.0), heated to 100°C for 5 min, and slowly cooled to room temperature. Yeast RPA protein was purified as described (Alani et al., 1992).

Dna2 protein purification- Human Dna2 was purified as described previously (Masuda-Sasa et al., 2006a). Wild-type and nuclease-dead (K677R) *S. cerevisiae* Dna2 proteins were expressed in yeast grown in 2 liters minimal media without uracil supplemented with 2% glycerol, 3% lactic acid, and 3% galactose. Cells were lysed using a CryoMill (Retsch) with continuous liquid nitrogen cooling. Pellets were resuspended in 50 ml of 20 mM Tris-HCl (pH 8.0), 35 mM imidazole, 750 mM NaCl, 5% glycerol (v/v), 1 mM 2-mercaptoethanol, 0.01% Triton X-100, and Complete EDTA-free protease inhibitor cocktail (Roche). Extracts were clarified by ultracentrifugation at 29000 RPM for 20 minutes in a Beckman TI45 rotor. The supernatant was mixed with 2 ml Ni-NTA agarose (Qiagen) for 1 hour at 4°C. Resin was collected by gravity flow and washed

three times with 20 ml of 20 mM Tris-HCl (pH 8.0), 35 mM imidazole, 5% glycerol, 1 mM 2-mercaptoethanol buffer containing decreasing levels of NaCl (750 mM, 300 mM, and 100 mM NaCl, respectively). Dna2 was eluted in 1 ml fractions of 20 mM Tris-HCl (pH 8.0), 400 mM imidazole, 5% glycerol, 1 mM 2-mercaptoethanol, and 100 mM NaCl. Fractions containing Dna2 were pooled and loaded onto a 1 ml MonoQ FPLC column (GE Healthcare) equilibrated with MonoQ buffer (25 mM Tris-HCl (pH 7.5), 10% glycerol, 100 mM NaCl, 1 mM EDTA). The column was washed with 10 ml MonoQ buffer and eluted over a 10 ml gradient of 100–600 mM NaCl. Fractions containing Dna2 were pooled and dialyzed into storage buffer containing 25 mM Tris-HCl (pH 7.5), 500 mM NaCl, 25% glycerol, and 1 mM EDTA. Aliquots were stored at -80°C.

Endonuclease Assay with circular ssDNA- Nuclease reactions containing 50 fmol Dna2 protein, 500 ng M13mp18 or ΦX174 circular ssDNA, and $MgCl_2$, $MnCl_2$, and ATP as noted in the figures and figure legends in 20 µl reaction buffer (50 mM Tris-HCl pH 7.5, 25 mM NaCl, 2 mM dithiothreitol (DTT), 0.25 mg/ml bovine serum albumin (BSA)) were incubated at 37° for 15 minutes. Reactions were stopped by the addition 5x buffer (60 mM EDTA, 40% sucrose, 0.6% SDS, 0.25% bromophenol blue and 0.25% xylene cyanole FF) and electrophoresed on a 1% agarose gel containing ethidium bromide. Reactions including RPA, 0.75 µg or 1.5 µg, were first incubated in reaction buffer (25 mM Tris-HCl pH 7.5, 25 mM NaCl, 2 mM dithiothreitol (DTT), 0.25 mg/ml bovine serum albumin (BSA), 1mM $MgCl_2$ or $MnCl_2$, and 100 ng M13mp18) minus Dna2 for 10 minutes at room temperature to facilitate RPA-ssDNA binding. 50 fmol of Dna2 was then added and reactions were incubated at 37° for 15 minutes.

Electrophoretic Mobility Shift Assay with competitor- Reactions, mixed on ice, contained 1 pmol nuclease-dead Dna2-K677R and 20 fmol of ^{32}P-labeled 5' flap substrate in 20 µl reaction buffer (25 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.5 mg/ml BSA, 2 mM ATP, 5% glycerol, and 1 mM or 2 mM $MgCl_2$ as indicated). Unlabeled competitors, M13mp18 circular ssDNA and 5' flap substrate were added as indicated and reactions were incubated at 30° for 20 minutes. Samples were electrophoresed on a 12% polyacrylamide gel at 4°, 100V for 3 hours with 0.5X TBE running buffer and products were detected by PhosphoImager.

ATPase Assay- ATPase reactions containing 1 pmol wild-type or nuclease-dead Dna2 protein in 20 µl reaction buffer (40mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT, 0.5 mg/ml BSA, 0.2 mM ATP, 10% glycerol, and 3µCi [γ-^{32}P]ATP) were supplemented with 15.625 ng, 62.5 ng. 250 ng, or 1µg ssDNA (M13mp18 ssDNA circle and 5'-flap substrate as indicated) and incubated at 30° for 1 hour. The reactions were stopped by adding EDTA to a final concentration of 4 mM. 0.8 µl of each reaction was spotted onto a polyethyleneimine cellulose TLC plate (Selecto Scientific) and developed in 0.5 M LiCl, 1 M formic acid solution. Products were detected by PhosphoImager.

Exonuclease Assay with Radiolabeled Substrate- Nuclease reactions containing 50 fmol of Dna2 protein, 20 fmol of radiolabeled substrate, and $MgCl_2$, $MnCl_2$, and ATP as noted in 20 µl reaction buffer (50 mM Tris-HCl (pH 7.5), 25 mM NaCl, 2 mM DTT, 0.25 mg/ml BSA) were incubated at 37° for 15 minutes. Reactions were stopped using 2x denaturing termination dye (95% deionized formamide, 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol), and boiled for 5 min. The cleavage products

were separated on a 12% sequencing gel (SequaGel, National Diagnostics) and detected by PhosphoImager.

Endonuclease and Helicase Assays with Radiolabeled Substrate- Standard reactions contained 300 fmol of Dna2 or Dna2-K677R and 10 fmol of helicase substrates (^{32}P-labeled H1 (5' flap) or H2 (fully annealed) oligonucleotides annealed to M13mp18) in 20 µl reaction buffer (25 mM Tris–HCl (pH 7.5), 25 mM NaCl, 2 mM DTT, 0.25 mg/ml BSA) with $MgCl_2$, $MnCl_2$, and ATP as indicated. Reaction buffers with increasing NaCl contained from 0 to 200 mM NaCl as noted. After incubation at 37° for 30 minutes, reactions were stopped with 5× stop solution (60 mM EDTA, 40% sucrose, 0.6% SDS, 0.25% bromophenol blue and 0.25% xylene cyanole FF). Reaction products were then separated using 8% native polyacrylamide gels containing 0.1% SDS, and detected by PhosphoImager.

For endonuclease reactions with yeast RPA using ^{32}P-labeled H2 oligonucleotide annealed to M13mp18 as the substrate, 2.5 pmol RPA protein per reaction was incubated with 10 fmol of the substrate in 1x reaction buffer for 10 minutes at room temperature prior to the addition of yDna2 protein and 1 mM $MgCl_2$ or 1 mM $MnCl_2$ as indicated.

RESULTS

Dna2 has Endonuclease Activity. Several recent biochemical studies have demonstrated the requirement for a free 5' DNA end for Dna2 helicase and flap endo/exonuclease activity on synthetic oligonucleotide substrates (Balakrishnan et al., 2010; Kao et al., 2004a). However, prior studies showed that Dna2 has nuclease activity

on circular ssDNA, i.e. DNA entirely without ends (Bae and Seo, 2000; Budd et al., 2000). To reconcile these observations, we revisited the endonuclease function of Dna2. We first asked whether yeast Dna2 could cut a long single-stranded DNA (ssDNA) substrate without an end. For this, we used the single-stranded circular DNA of phage M13mp18 (M13). Under standard Dna2 nuclease assay conditions, as shown in Fig. 2.1A, lane 2, the phage DNA was degraded by Dna2. ATP is known to inhibit the flap endo/exonuclease activity of Dna2. As shown in Fig. 2.1A, lane 3, ATP also protects the circular ssDNA substrate from Dna2 endonuclease activity. A ratio of 1:1 Mg^{2+} to ATP significantly reduces the endonuclease activity of Dna2 (Fig. 2.1A, lane 4).

Recently, Dna2 has been shown to function in conjunction with Mre11 and Sgs1/Rmi1/Top3 in resection of a DSB break. It is not clear whether the Mre11 is performing a preprocessing event, such as preliminary resection, or if its primary role is in recruiting the Dna2/Sgs1 proteins to the unprocessed break. The nuclease activity of Mre11 is dependent on Mn^{2+} and is not supported by Mg^{2+} under most conditions. However, Mn^{2+}, even in the presence of Mg^{2+}, inhibits the DSB resection reaction reconstituted from the human counterparts to the yeast proteins: MRN, BLM, and DNA2, raising the question of which protein in the resection reaction is inhibited by Mn^{2+} (Nimonkar et al., 2011). We found that M13mp18 is completely degraded by yeast Dna2 in the presence of Mn^{2+} alone as a cofactor (Fig. 2.1A, lane 5). Addition of ATP at a ratio of 2 ATP : 1 Mn^{2+} inhibits the endonuclease activity (Fig. 2.1A, lane 6), as it does with Mg^{2+}, but at a 1 ATP : 1 Mn^{2+} ratio, ATP is not sufficient to protect the M13 circular DNA (lane 7). Endonuclease activity is somewhat greater with Mn^{2+} than Mg^{2+} with a circular substrate. A mixture of Mg^{2+} and Mn^{2+} also supported endonuclease activity

(Fig. 2.1, lane 8) and was not inhibited by a 1:1 ratio of ATP to Mg^{2+} and Mn^{2+} (lane 9), unlike the reaction with Mg^{2+} alone, suggesting that Mn^{2+} is dominant over Mg^{2+} in supporting Dna2 endonuclease.

Since Mn^{2+} does not inhibit, but stimulates Dna2, it is not Dna2 in the Dna2/BLM/MRN reconstituted resection reaction that is inhibited by Mn^{2+}, though the effects of Mn^{2+} could be different in the presence of the additional proteins. Similar endonuclease properties were observed with ΦX174 circular ssDNA (Fig. 2.1B). In this case it is clear that the ΦX174 DNA preparation contained a significant fraction of linear as well as circular DNA. Both are degraded by Dna2 in the presence of either Mg^{2+} or Mn^{2+}. The contamination of the ΦX174 circular DNA preparation with linear DNA, documented by the provider, can explain a previous observation suggesting that the helicase activity of Dna2 could function in the absence of a free end for Dna2 loading (Bae et al., 2002). No endonuclease is observed with Dna2-K677R, which is endo/exonuclease defective in the presence of either Mg^{2+} or Mn^{2+} (Fig. 2.1C). We conclude that, unlike Dna2 helicase and flap endo/exonuclease functions, Dna2 can act as an endonuclease in the absence of a free DNA end and that the same active site catalyzes both nucleolytic activities.

Association of Dna2 with ssDNA does not require an End. We next investigated whether association of Dna2 with ssDNA required a DNA end. Previous studies have shown that Dna2 can bind to ssDNA flaps with blocked ends, but those substrates contained ssDNA/dsDNA junctions and other secondary structures that are strong binding sites for Dna2 (Stewart et al., 2010). We asked whether circular ssDNA could compete with a radiolabeled flap substrate for Dna2 binding as measured by EMSA

(electrophoretic mobility shift assay). In order to protect the labeled flap substrate from Dna2 nuclease activity, the nuclease deficient Dna2-K677R mutant was used. In Fig. 2.2, lane 2, it is clear that Dna2 binds to the flap substrate. Competition with increasing amounts of unlabeled flap substrate reduces the amount shifted (Fig. 2.2, lanes 6-8 and 13-15). When unlabeled circular ssDNA was added as a competitor, the amount of flap substrate shifted by Dna2 was also reduced (Fig. 2.2, lanes 3-5 and 10-12). Therefore, Dna2 does not require a free DNA end or a flap/fork junction to bind ssDNA. As in the case of the flap substrate, the binding to the ssDNA circle was not inhibited by ATP, even though these conditions protect the circle from Dna2 endonuclease degradation (see Fig. 2.1). We conclude that Dna2 can bind to DNA without ends and that the inhibitory effect of high levels of ATP on nuclease activity is not due to interference with DNA binding.

DNA Ends are required to Stimulate Dna2 ATPase Activity. Dna2 is a single-stranded DNA-dependent ATPase. With wild-type Dna2 protein, the circular ssDNA and linear ssDNA promote ATPase activity equally (Fig. 2.3, lanes 10-13 and 14-17). With the nuclease-dead Dna2-K677R protein, however, only linear DNA serves as an ATPase effector (Fig. 2.3, lanes 1-4), even though we demonstrated that Dna2-K677R does bind to the circular ssDNA (Fig. 2.2). We infer that wild-type Dna2 first linearizes the circular DNA, which then serves as a Dna2 cofactor. This verifies that DNA ends, not ssDNA alone, are required to stimulate ATPase activity. This also establishes the fact that the K677R mutation prevents the generation of these ends, confirming that the K677R mutation abolishes both endonuclease and flap endo/exonuclease activities.

Endo- and Exo-nuclease Activities can be Separated. The strong Dna2 endonuclease activity in the presence of Mn^{2+} prompted us to investigate flap

endo/exonuclease activity in the presence of Mn^{2+}. As shown in Fig. 2.4A, lane 2, yeast Dna2 flap endo/exonuclease, in the presence of Mg^{2+}, removes the 5' ssDNA flap from 20 fmols of radiolabeled substrate. This activity is reduced in the presence of ATP as evidenced by remaining uncut substrate (Fig. 2.4A, lane 3). Mn^{2+} cannot substitute for Mg^{2+} in cutting the 5' flap (Fig. 2.4A, lane 4). Surprisingly, however, when Mn^{2+} is supplemented with ATP, the flap endo/exonuclease activity is restored (Fig. 2.4A, lane 5). This ATP-dependent exonuclease activity in the presence of Mn^{2+} stands in direct contrast to the Mg^{2+}-dependent endonuclease activity that is inhibited by the presence of ATP.

When Mg^{2+} and Mn^{2+} are present in equal concentrations, no flap endo/exonuclease is detected, similar to Mn^{2+} alone (compare Fig. 2.4A lanes 4 and 6). When ATP is added to the reaction with Mg^{2+} and Mn^{2+} (Fig. 2.4A, lane 7), the substrate is cleaved. The inhibition of Mg^{2+}-dependent flap endo/exonuclease activity by Mn^{2+} is puzzling, considering both cofactors support robust endonuclease activity alone or together. However, this observation demonstrates that the endonuclease and flap endo/exonuclease functions are experimentally separable, even though they are both abolished with a mutation at the same residue in the nuclease active site. We analyzed three different 5' flap substrates and found that this Mn^{2+} dependent behavior was consistent regardless of the substrate structure at the ssDNA/dsDNA junction (Fig. 2.4A).

Comparison of the reaction products in Fig. 2.4A, especially between lanes 9 and 12 for the 5' overhang substrate and lanes 16 and 19 for the 5' flap substrate adjacent to an upstream oligonucleotide, shows the Mn^{2+}/ATP stimulated nuclease products to be shorter than those observed with Mg^{2+} alone. It is unknown whether this is due to lower

processivity along the flap or secondary cutting after the initial flap removal with Mn^{2+} and ATP. However, similar nuclease assays with 3' labels on the 5' fork substrate show the final products after both Mg^{2+} and Mn^{2+}/ATP mediated cutting to be the same length (data not shown). The presence of Mn^{2+} does not enable Dna2 to cut or degrade linear double stranded DNA, with or without ATP, consistent with behavior with cofactor Mg^{2+} (data not shown).

All of these activities, including the ATP-dependent, Mn^{2+} stimulated flap removal activity were conserved in the human Dna2 protein (Fig. 2.4B). As previously reported, hDna2 does not show nuclease when Mn^{2+} is substituted for Mg^{2+} (Fig. 2.4B, lanes 4 and 6). However, we now show that Mn^{2+} does exhibit nuclease activity if ATP is added (Fig. 2.4B, lanes 5 and 7).

Endonuclease can Create DNA end for Helicase or Exonuclease Activity. It has been recently established that the Dna2 helicase requires a free DNA end in order to unwind DNA (Balakrishnan et al., 2010). Considering the end independent endonuclease activity characterized above, we wondered whether Dna2 could create a free end suitable for its helicase activity. We first measured helicase activity using the Dna2-K677R mutant and a 5'-^{32}P labeled oligonucleotide with an 18 nucleotide 5' non-complementary flap annealed to the M13mp18 circular ssDNA. Helicase products are generated in the presence of ATP with either Mg^{2+} or Mn^{2+} but not in the absence of ATP (Fig. 2.5A, compare lanes 4 and 5 with lanes 2 and 3). Note that the small amount of label migrating ahead of the oligonucleotide in lane 2 is due to minor residual exonuclease seen in Dna2-K677R preparations after extended incubation, but that no endonuclease products, such

as are seen in Fig. 2.5B, lane 4, are apparent. While not as potent as Mg^{2+}, we find Mn^{2+} is a functional cofactor for Dna2 helicase activity.

In keeping with the demonstration that substrates without a ssDNA flap or with a flap whose 5' end is blocked by a bulky adduct cannot be unwound by Dna2, when we annealed an oligonucleotide lacking a non-complementary 5' flap to M13mp18 and incubated with wild-type Dna2 under conditions that inhibit the endonuclease, 1 mM Mg^{2+} and 2 mM ATP, the substrate is not unwound (Fig. 2.5B, lane 3). This is likely because there is no free 5' ssDNA tail on the oligonucleotide and the circle is protected from degradation by the excess ATP and cannot provide an alternative end. As shown in Fig. 2.5B, lane 4, when this assay is repeated in conditions that allow some endonuclease activity, 1mM Mg^{2+} and 1mM ATP as established in Fig. 2.1, both endonuclease products and helicase products are seen. Increasing amounts of NaCl have been shown previously to inhibit the flap endo/exonuclease activity of Dna2 (Bae et al., 2002; Kim et al., 2006; Masuda-Sasa et al., 2006a). The endonuclease activity was also clearly inhibited by increasing NaCl (Fig. 2.5B, lanes 5-8), and this resulted in a corresponding reduction of unwound helicase product. These results lead to the conclusion that Dna2 endonuclease can create a substrate for its helicase activity.

Replication Protein A Inhibits Dna2 Endonuclease Activity. Several studies have shown that Dna2 enzymatic activity is modulated by RPA; 5' flap endo/exonuclease activity is stimulated while 3' exonuclease activity is inhibited (Bae et al., 2003; Masuda-Sasa et al., 2008; Stewart et al., 2008). This regulation is thought to give Dna2 specificity to process the correct strand of DSB ends and nascent Okazaki fragments. We were interested to see how RPA affected the Dna2 endonuclease function on the

M13mp18 circular ssDNA lacking dsDNA/ssDNA junctions. As shown in Fig. 2.6A, RPA inhibited endonucleolytic cleavage of M13 DNA by Dna2 protein in the presence of either Mg^{2+} (Fig. 2.6A, lanes 4-6) or Mn^{2+} (Fig. 2.6A, lanes 8-10). This result is consistent with the inferred inhibition of Dna2 endonuclease by RPA on a substrate comprised of a radiolabeled oligonucleotide (52mer) annealed to ΦX174, a molecule with a single-strand/double- strand DNA junction (Bae and Seo, 2000). In Fig. 2.6B, we show a similar experiment using M13 annealed to the shorter H2 (24mer) oligonucleotide. Dna2 alone cleaves the circular substrate, but does not displace the oligonucleotide, as expected (Fig. 2.6B, lanes 4 and 6). In the presence of RPA, however, we could not assess endonuclease activity, because, surprisingly, in the presence of both RPA and high levels of Dna2 (Fig. 2.6B, lanes 5 and 7), the entire labeled oligonucleotide was displaced. This displacement is due to yet another form of interaction between RPA and Dna2, since neither RPA (Fig. 2.6B, lane 3) nor Dna2 alone (Fig. 2.6B, lanes 4 and 6) displace the oligonucleotide. Therefore, we conclude that RPA inhibits the endonuclease activity of Dna2, and that RPA and Dna2 can together destabilize partial duplex DNA in the absence of ATP.

DISCUSSION

In this study, we have established that the Dna2 enzyme exhibits true endonuclease activity. Unlike the well-characterized flap endo/exonuclease function and helicase activity, the Dna2 endonuclease does not require a free ssDNA end. While the

endonuclease and flap endo/exonuclease appear to share an active site, assays with the cofactor Mn^{2+} show these two functions are distinguishable.

The physiological significance of the endonuclease activity is unclear. The ability to cut ssDNA internally could conceivably result in a DSB at sites of DNA damage or between nascent Okazaki fragments. Therefore, the endonuclease activity must be somehow regulated. In the cell, long stretches of ssDNA would be bound by RPA, and we have shown that RPA inhibits the endonuclease on single-stranded circular DNA in addition to single-stranded circular DNA with an annealed oligonucleotide. While RPA stimulates the Dna2 flap endo/exonuclease and RPA can be removed from DNA by Dna2 without threading from an end (Stewart et al., 2008), RPA shows inhibitory effects on multiple Dna2 functions in addition to the endonuclease (Cejka et al., 2010; Masuda-Sasa et al., 2008; Nimonkar et al., 2011). Other structural features or participation of additional proteins in a complex at the replication fork may also modulate the endonuclease activity, like the endonuclease of RecB in the RecBCD complex (Sun et al., 2006). The need for regulation of this activity, however, should not obscure the fact that endonuclease may, at times, be beneficial, as it could allow Dna2 to process intermediate structures into a form compatible with its helicase and flap endo/exonuclease requirements. The helicase function of Dna2 as studied *in vitro* would imply it is not important to cells since the substrates are more likely to be cut by the flap endo/exonuclease than unwound. However, genetic experiments show the helicase activity does play a role in the survival of damage *in vivo* (Weitao et al., 2003).

Roles for the endonuclease may be hard to discern as mutants are also defective in flap endo/exonuclease activity. The observation that the two activities are distinguishable

using the co-factor Mn^{2+} provides an approach to elucidate the contributions of the endonuclease function to Okazaki fragment processing or DBS resection *in vitro.* Future studies must also address the role of post-translational modifications and other proteins, especially Sgs1/BLM, in stimulating or inhibiting the endonuclease function of Dna2 on both DNA repair intermediates and Okazaki fragment processing steps.

In reactions with Mn^{2+}, Dna2 exhibits helicase activity and endonuclease activity as with Mg^{2+}. What is surprising is that addition of Mn^{2+} reveals an ATP-dependent exonuclease activity, like that of the bacterial enzymes RecBCD and AddAB (Kooistra et al., 1993; Niu et al., 2009; Sun et al., 2006). The nuclease domain of Dna2 shares some sequence homology with RecB (Budd et al., 2000). The AddAB enzyme, like RecBCD, is involved in DNA repair and homologous recombination with double- and single-stranded DNA dependent ATPase, helicase, and ATP-dependent endo- and exo-nuclease activities. Dna2 and AddAB have also been proposed to share structural similarities, in the nuclease active site and in an iron staple domain spanning the active site (Yeeles et al., 2009). Perhaps conformational changes in the nuclease active site of Dna2 while bound to Mn^{2+} cause Dna2 to behave more like these related enzymes. Weak ATP-dependent nuclease has been seen previously with Dna2 in the presence of Mg^{2+}, but was most likely 3' exonuclease dependent on ATP-driven helicase activity exposing a 3' ssDNA tail (Bae et al., 1998; Bae et al., 2002; Budd et al., 2000).

Functionally, the use of Mn^{2+} as a co-factor *in vitro* will allow Dna2 to be studied under conditions that promote both helicase and flap endo/exonuclease strongly. Use has been made of the ratio of cofactor to ATP in priming the Dna2 enzyme for preferentially observing either helicase or nuclease activity, respectively (Kao et al., 2004a). However,

the activity of the two domains is likely to be coupled (Bae et al., 2002). In fact, genetic experiments show that a mutant with Dna2 helicase activity but not nuclease activity is more sensitive to DSBs than a double helicase and nuclease dead mutant (Budd and Campbell, 2009). Understanding how the two domains work in concert with each other and with other enzymes present at sites of DNA replication and repair, will be important, however, to understanding the role of Dna2 in genomic stability in a comprehensive fashion.

While Mn^{2+} is not as abundant in the cell as Mg^{2+}, several enzymes are manganese-dependent (Paull and Gellert, 1998; Schneider et al., 2009; Supek et al., 1996). Prominent among these is double strand break processing nuclease Mre11. Recently, purified Mre11-Rad50 complex from bacteriophage T4, which also uses Mn^{2+} as a divalent cation, was found to exhibit some nuclease activity in Mg^{2+} when assayed with proteins UvsY (RAD52) and gp32 (RPA) (Herdendorf et al., 2011). As with Dna2, the products from the Mg^{2+} and Mn^{2+} catalyzed reactions were slightly different. Another example of a manganese-dependent repair enzyme is the latent endonuclease of human MutLα, which is critical in human mismatch repair (Kadyrov et al., 2006).

Dna2 is one of the key players in eukaryotic double strand break repair (Cejka et al., 2010; Liao et al., 2008; Wawrousek et al., 2010; Zhu et al., 2008). It is important to point out that human Mre11, one of the stimulatory components of the DNA end resection machinery, exhibits strictly Mn^{2+} dependent endo- and exo-nuclease activity *in vitro* (Paull and Gellert, 1998). Substitution of Mg^{2+} for Mn^{2+} in DNA end resection assays did not generate resection products (Nimonkar et al., 2011). Furthermore, addition of Mn^{2+} to the Mg^{2+} driven reactions inhibited resection. It was suggested that this is due

to inability of one or more of the proteins in the complex to function in the presence of Mn^{2+}. Based on results presented here, the presence of Mn^{2+} in the resection reactions should not have an adverse effect with respect to Dna2 activity.

Changes in Dna2 active site conformation, and thus activity, caused by Mn^{2+} instead of Mg^{2+} binding *in vitro* may mimic a change in conformation when bound to other proteins in the cell or when post-translationally modified *in vivo*. These results may have importance in studying higher order DNA/protein complexes involving Dna2, a snapshot of which is provided by the studies of DSB resection, referred to above, by Nimonkar et al (Nimonkar et al., 2011). Detailed biochemical characterization of Dna2 will be important for the design of *in vitro* reconstitution experiments involving Dna2 in multi-protein complexes and dissection of enzymatic activities and cofactor requirements of individual components contributing to orderly and precise execution of multi-step replication/repair processes.

FOOTNOTES

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FIGURES AND TABLES

Table 2.1 Oligonucleotides used in this study.

Oligo	Length (nt)	Sequence
5’ Flap	98	TTCACGAGATTTACTTATTTCACTGCGGCTACATGATGCATCGTTAGGCGATTCCGCCTAACGATGCATCATGTCGCGAACCCTATTTAGGGTTCGCG
5’ Tail	74	TTCACGAGATTTACTTATTTCACTGCGGCTACATGATGCATCGTTAGGCGATTCCGCCTAACGATGCATCATGT
H1 (5’ Flap)	42	AGCTAGCTCTTGATCGTAGACGTTGTAAAACGACGGCCAGTG
H2 (Annealed)	24	GACGTTGTAAAACGACGGCCAGTG

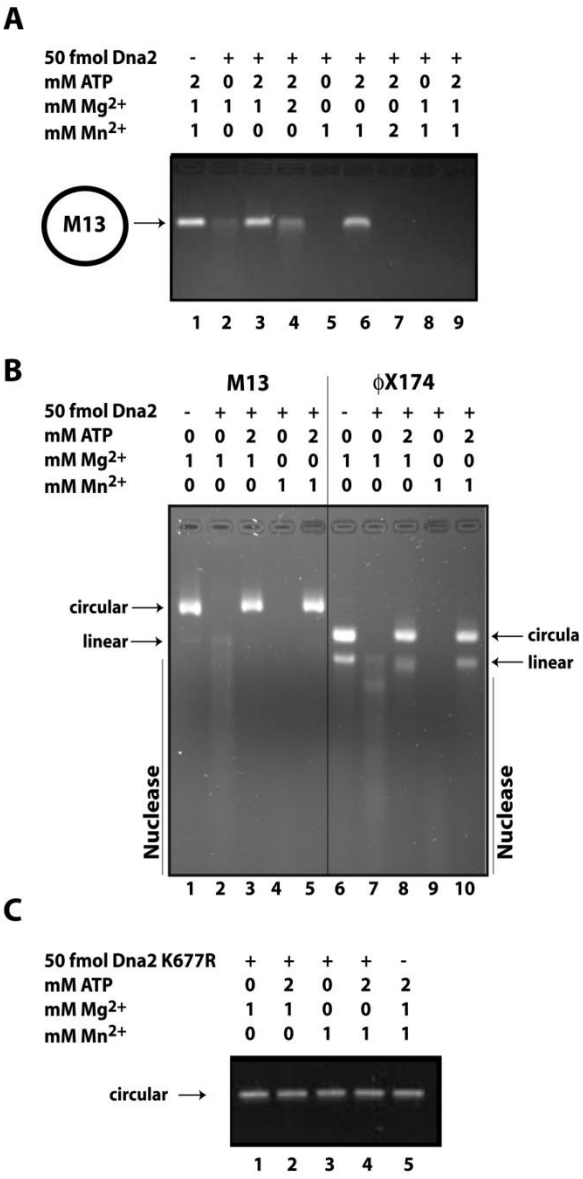

Figure 2.1 Dna2 has endonuclease activity. *A.* Nuclease assays were performed as described in Experimental Procedures. Briefly, 50 fmol of yeast Dna2 were incubated with 500 ng of M13mp18 phage ssDNA and $MgCl_2$, $MnCl_2$, and ATP as indicated for 15 minutes at 37°. Reaction products were subjected to electrophoresis on a 1% agarose gel, and DNA was stained with ethidium bromide. *B.* Nuclease reactions were performed as above with M13mp18 and ΦX174 ssDNA as indicated. *C.* Nuclease assay with M13mp18 ssDNA and Dna2-K677R, nuclease defective.

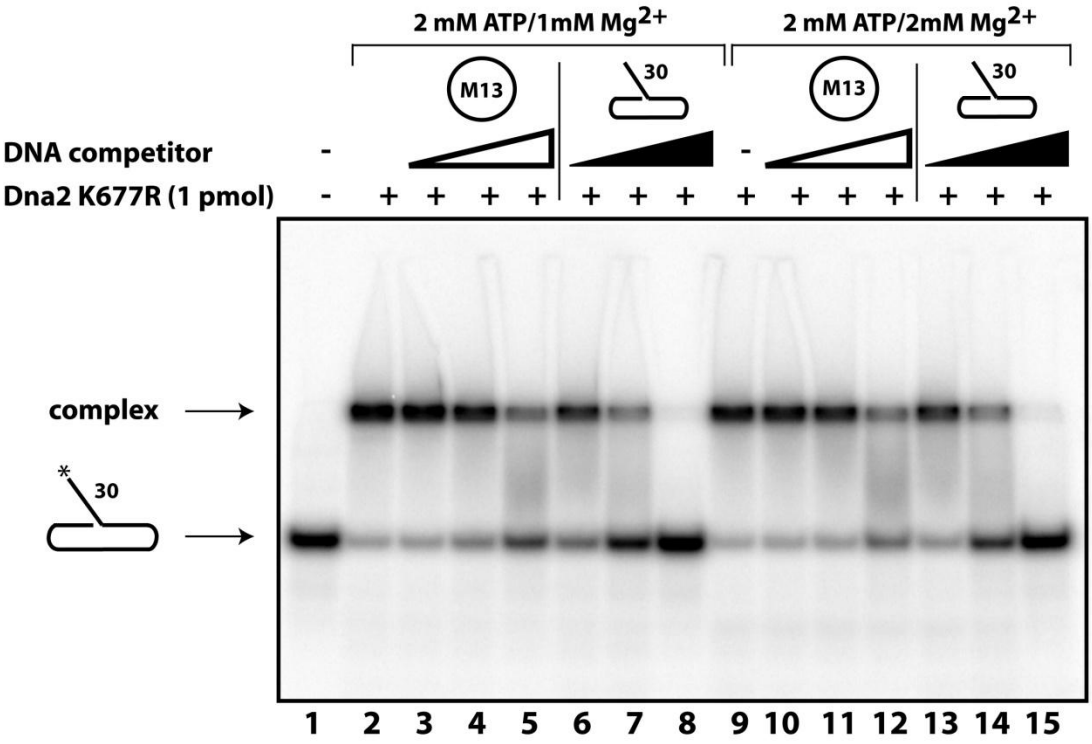

Figure 2.2 Association of Dna2 with ssDNA does not require an end. EMSA was performed as described in Experimental Procedures. ^{32}P-labeled 5' flap substrate was mixed on ice with 1 pmol Dna2-K677R protein in reaction buffer supplemented with Mg_2Cl and ATP as indicated. Lane 1 contains the substrate in the absence of Dna2 protein. Lanes 2 and 9 indicate the electrophoretic mobility shift of the substrate with Dna2 protein in the absence of DNA competitor. Lanes 3-5 and 10-12 show binding of the labeled substrate in the presence of 2.5 ng, 10 ng, and 100 ng of M13mp18 circular ssDNA competitor as noted by the white triangles. Lanes 6-8 and 13-15 show binding of the labeled substrate in the presence of 2.5 ng, 10 ng, and 100 ng of unlabeled 5' flap substrate competitor, as noted by black triangles.

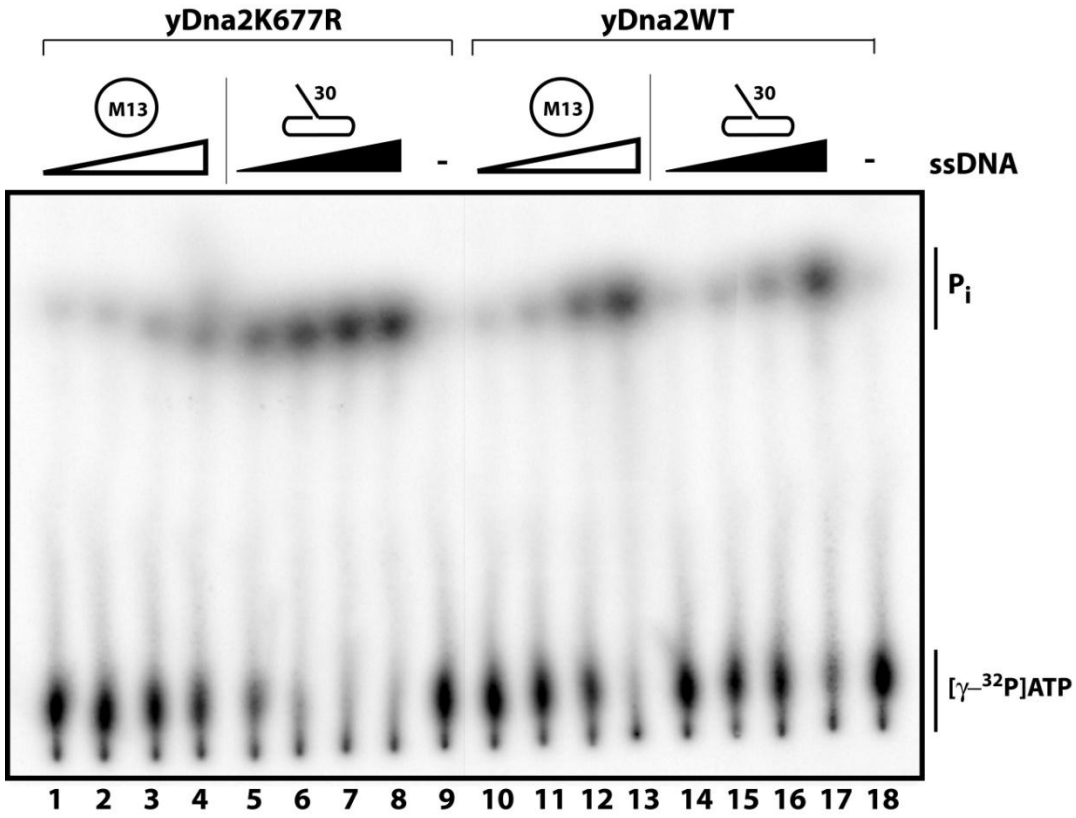

Figure 2.3 DNA ends are required for ATPase Activity. ATPase assays were performed as described in Experimental Procedures. Reactions in lanes 1-9 were performed with 1 pmol nuclease-dead Dna2-K677R. Reactions in lanes 10-18 were performed with 1 pmol wild-type Dna2 protein. Lanes 1-4 and 10-13 were supplemented with 15.625 ng, 62.5 ng, 250 ng, and 1µg M13mp18 circular ssDNA as denoted by the white triangles. Lanes 5-8 and 14-17 were supplemented with 15.625 ng, 62.5 ng, 250 ng, and 1µg 5' flap substrate as denoted by the black triangles. Reactions in lanes 9 and 18 lacked ssDNA.

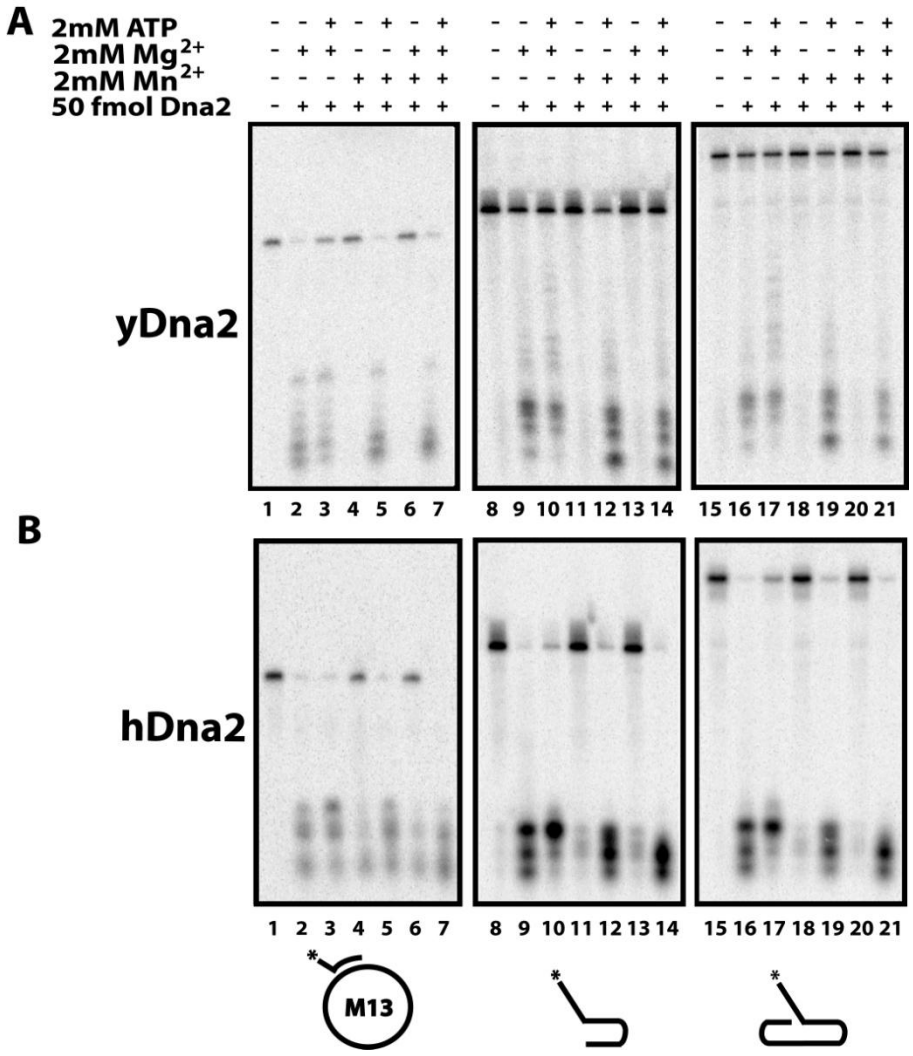

Figure 2.4. Endonuclease and Exonuclease functions can be separated. *A & B.* Nuclease assays were performed as described in Experimental Procedures with 50 fmol of yeast Dna2 (A) or human Dna2 (B) protein for 15 minutes at 37°. The radiolabeled substrates are depicted under the respective panels with an asterisk denoting the position of the label: H1 (5' flap) annealed to M13 phage ssDNA (lanes 1-7), 5' tail with dsDNA hairpin (lanes 8-14) and 5' flap with dsDNA hairpin (lanes 15-21). Addition of 2mM ATP, 2mM $MgCl_2$ and 2mM $MnCl_2$ are denoted by +/- marks.

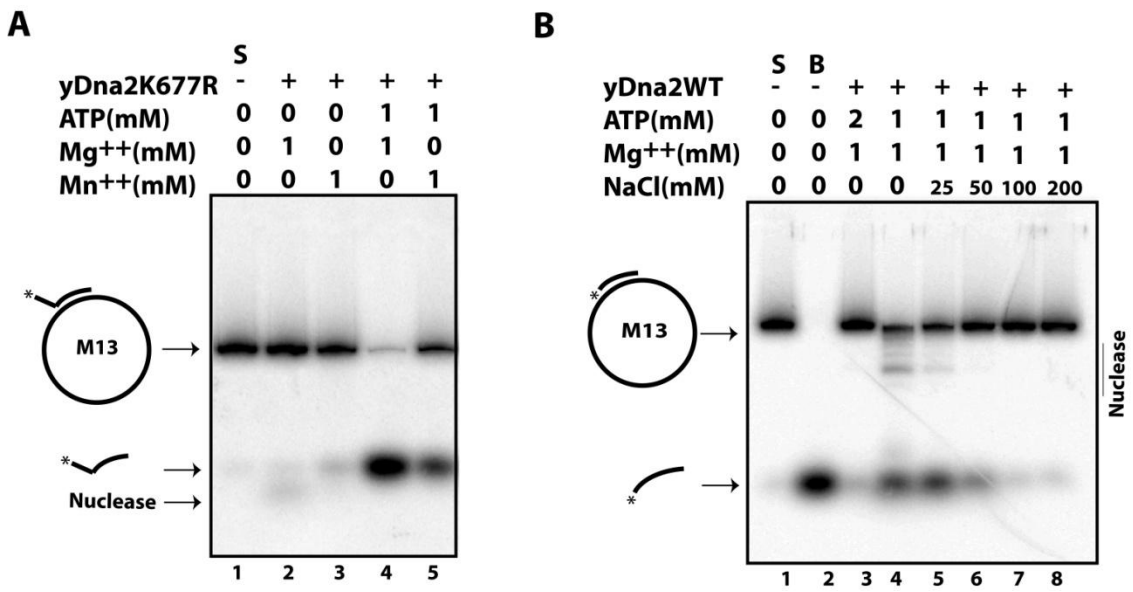

Figure 2.5 Endonuclease activity can generate helicase substrate. *A.* Helicase assays were performed as described in Experimental Procedures using nuclease-dead Dna2-K677R and 1mM $MgCl_2$, 1mM $MnCl_2$, and 2mM ATP as indicated with radiolabeled H1 (5' flap) oligonucleotide annealed to M13mp18 ssDNA. Reactions were incubated at 37° for 30 minutes and products were resolved on non-denaturing PAGE. Lane 1 represents the substrate alone. Helicase reaction products are indicated by the arrow corresponding to the oligonucleotide alone. Products of residual Dna2 flap endo/exonuclease activity in lane 2 are indicated by the nuclease arrow. *B.* Helicase reactions were performed as above using wild-type Dna2 and a radiolabeled H2 (fully annealed) oligonucleotide annealed to M13mp18 ssDNA. Lane 1 contains the substrate alone and lane 2 the substrate after boiling. Helicase products are noted by the arrow corresponding to the labeled oligonucleotide after boiling. Lanes 4-8 contain increasing concentrations of NaCl from 0 to 200 mM as indicated.

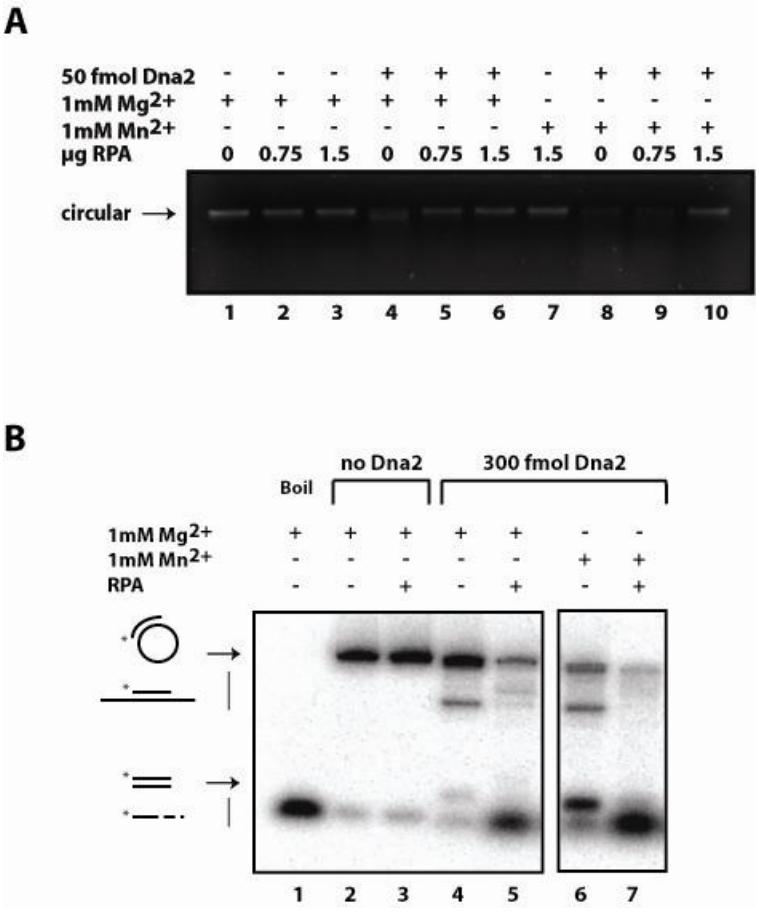

Figure 2.6 Replication Protein A Inhibits Dna2 Endonuclease. *A.* Endonuclease reactions were performed as described in Experimental Procedures. Briefly, RPA protein, 0, 0.75 µg, or 1.5 µg, was incubated with 100 ng M13mp18 ssDNA in endonuclease buffer containing 1mM $MgCl_2$ or 1mM $MnCl_2$ as indicated, for 10 minutes at room temperature prior to the addition of wild type Dna2. Reactions were then incubated at 37° for 15 minutes and products were resolved using electrophoresis on a 1% agarose gel, and DNA was stained with ethidium bromide. *B.* Dna2 and RPA together, but neither alone, can displace short oligonucleotides annealed to M13mp18. 2.5 pmol of RPA protein was incubated with radiolabeled H2 (fully annealed) oligonucleotide annealed to M13mp18 ssDNA in 1x endonuclease/helicase buffer containing no NaCl for 10 minutes at room temperature prior to the addition of wild type Dna2, 1mM $MgCl_2$, and 1mM $MnCl_2$ as indicated. Reactions were incubated at 37° for 30 minutes and products were resolved by non-denaturing PAGE. Lane 1 shows the substrate after boiling to displace the oligonucleotide.

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CHAPTER 3

DNA2 IS A SUBSTRATE OF DNA DAMAGE RESPONSE KINASES MEC1 AND CDK1

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ABSTRACT

Saccharomyces cerevisiae Dna2 is an essential enzyme with helicase, nuclease, ATPase, ssDNA annealing and strand exchange enzymatic activities. In addition to an established role in Okazaki fragment processing and telomere maintenance, both genetic and biochemical evidence implicate Dna2 in the survival of DNA damage. It has been recently established that Dna2 is the primary nuclease involved in the resection of 5' double strand break ends. In this study, we find that Dna2 can be purified as a phosphorylated protein and treatment with phosphatase inhibits Dna2 enzymatic activity. Further, we show that Dna2 can be phosphorylated *in vitro* by both Cdk1 and Mec1, two kinases responsible for the cellular DNA damage response. Mec1 phosphorylates Dna2 at Serine 287 after multiple forms of DNA damage *in vivo*, but this post-translational modification appears dispensable for Dna2 survival after double strand breaks.

INTRODUCTION

Maintaining genomic integrity is vital to the fitness of all organisms. *Saccharomyces cerevisiae* makes use of an elaborate network of proteins to maintain genomic fidelity at various stages of its cell cycle. These proteins must work in an orchestrated manner in order to detect and repair DNA replication errors and DNA damage. One important method for coordinating the DNA damage response is post-translational modification, namely phosphorylation.

Mec1, a member of the PI-3 kinase-related kinase (PIKK) family, is activated upon DNA damage including double strand breaks (DSBs), single-stranded DNA lesions, and stalled replication forks (Navadgi-Patil and Burgers, 2009; Segurado and Tercero, 2009). The activation of Mec1 leads to phosphorylation of many targets, such as Rad53, Mrc1, Rad9, and RPA, and is responsible for halting cell cycle transitions, stabilizing stalled replication forks, and affecting DNA repair (Cobb et al., 2003; Gasch et al., 2001; Mallory and Petes, 2000; Tercero and Diffley, 2001). Mutations in Mec1 lead to sensitivity to exogenous DNA damaging agents and spontaneous recombination events. During normal S phase, Mec1 is important for replication fork progression, especially in replication slow zones in order to prevent chromosomal breaks at fragile sites (Cha and Kleckner, 2002). The other PIKK family member in yeast, Tel1, is responsible for telomere maintenance and functions in the DSB response, although Mec1 also functions independently in this pathway (Grenon et al., 2006).

Dna2 is an essential nuclease, helicase, and ATPase with single-strand exchange and single-strand annealing activities (Bae et al., 1998; Bae et al., 2002; Budd et al.,

2000; Budd et al., 1995; Masuda-Sasa et al., 2006). The most well defined role for Dna2 is in Okazaki fragment processing. During lagging strand synthesis, polymerase δ displaces the newly synthesized DNA strand from the previous Okazaki fragment, leading to a 5' flap that is typically cleaved by FEN1 generating a nick that is sealed by ligase. In the event the flap becomes long enough to be coated with RPA, Dna2 is required remove the 5' flap *in vitro* (Henry et al., 2010; Kang et al., 2010; Kao et al., 2004; Pike et al., 2010; Stewart et al., 2008). In addition, however, *DNA2* mutants in both the helicase and nuclease domains are sensitive to a variety of DNA damaging agents and are synthetically lethal with genes involved in DSB repair, mismatch repair, and the replication stress checkpoint (Budd and Campbell, 2000; Budd et al., 2000; Fiorentino and Crabtree, 1997; Formosa and Nittis, 1999; Weitao et al., 2003a; Weitao et al., 2003b).

Recently, several labs have reported that Dna2 is a critical nuclease for the initial processing of DSB ends in the homologous recombination pathway (Budd and Campbell, 2009; Mimitou and Symington, 2009; Nimonkar et al., 2011; Niu et al., 2010; Shim et al., 2010; Zhu et al., 2008). After the induction of a single DSB by the HO endonuclease, the Mre11-Rad50-Xrs2 (MRX) complex is required to initiate DSB processing, while Sgs1 and Dna2 in concert extend the region of ssDNA using Dna2's nuclease activity. This activity is conserved in *Xenopus laevis* where Dna2 can be detected at DNA ends that mimic DSBs within 15 minutes of introduction into cell free extracts (Wawrousek et al., 2010). The arrival of Dna2 follows ATM, a Mec1 homologue, and the MRN complex, and coincides with the accumulation of RPA. In extracts depleted of Dna2, RPA accumulation on the DSB end is substantially delayed. ATM protein is also detected in

Dna2 immunoprecipitates. Taken together these studies identify DNA2 as a major component in the nuclear response to DNA damage in multiple eukaryotes, and points to a relationship between Dna2 and PIKK family of kinases.

In 2002, Gavin et al discovered Dna2 in a complex with Ddc2 (Lcd1), the putative homolog of human ATRIP and binding partner to Mec1 (Gavin et al., 2002). Since that time, the hypomorphoic *mec1-21* mutant was discovered to become often disomic for chromosome VIII, which includes the *DNA2* gene (Vernon et al., 2008). This spontaneous chromosome duplication was suppressed by expressing an extra copy *DNA2* on a plasmid, meaning that *mec1* dysfunction preferentially increased *DNA2* levels, indicating a genetic relationship between Dna2 and Mec1. While the viability of *dna2*-2 mutant is reduced with *tel1*Δ, the *mec1*Δ mutation suppressed the *dna2-2* slow growth phenotype (Budd et al., 2005). This increase in viability in the presence of *mec1* mutation was also seen in the *dna2-20* and *dna2-21* helicase mutants (Fiorentino and Crabtree, 1997).

The essential function of *DNA2* is linked to the DNA damage response. Rad9 is a scaffolding protein required for the complete activation of Mec1 and the resulting checkpoint after DNA damage (Navadgi-Patil and Burgers, 2009). Deletion of *RAD9* and the *rad9-320* mutation suppress *dna2* mutants including *dna2*Δ *(Budd et al., 2011; Fiorentino and Crabtree, 1997; Formosa and Nittis, 1999).* A strain with further disruption of the DNA damage checkpoint, *dna2-1 rad9*Δ *mrc1AQ* even grows at 34° (Budd et al., 2011). We know that Dna2 is regulated following DSBs, as the protein leaves telomeres upon treatment with bleomycin (Choe et al., 2002), but how this contributes to overall genomic stability is unclear.

Mec1 is not the only kinase that could potentially regulate the activity of Dna2 in the DNA damage response. Yeast Dna2 has been shown to be phosphorylated by cyclin-dependent kinase Cdk1 (*CDC28*) (Holt et al., 2009; Kosugi et al., 2009; Smolka et al., 2007; Ubersax et al., 2003). Cdk1 is known to be required for activation of the DNA damage checkpoint in response to DSBs, and recruitment of homologous recombination proteins and efficient 5' to 3' resection at DSB ends (Barlow and Rothstein, 2009; Ira et al., 2004). This regulatory pathway is independent of the PIKK signaling pathways.

In this study, we show that Dna2 is phosphorylated in yeast cells, and that removing this phosphorylation results in the inhibition of enzymatic activity *in vitro.* We further show that Dna2 is phosphorylated *in vitro* by both Mec1 and Cdk1. Using a phospho-specific antibody, we find that Dna2 is phosphorylated in its N-terminal domain following DNA damage by Mec1, but this phosphorylation event is dispensable for survival of DNA damage.

MATERIALS AND METHODS

Strains and plasmids - The plasmid pSEY18GAL-HA-DNA2, referred to here as pGAL-DNA2 was described previously (Budd and Campbell, 2009). The plasmid pRS314-DNA2 has the 6kb EcoRI fragment containing *DNA2* cloned into the pRS314-TRP1 (CEN) plasmid at the EcoRI site as described (Budd and Campbell, 2009). Mutations were made using QuickChange mutagenesis for the phenotype screen. The pRS314-dna2-S287A plasmid was made from pRS314-DNA2 using the primers 5'-CGCAAGAGTGAGGCTCAGATAAC-3' and 5'-GTTATCTGAGCCTCACTCTTGCG-

3’. The pRS314-dna2-S287E mutation was made using the primers 5’-CGCAAGAGTGAGGAACAGATAAC-3’ and 5’-GTTATCTGTTCCTCACTCTTGCG-3’. The pRS314-dna2-S1189A mutation was made using primers 5’-GGACGAGGCAGCTCAAATTTCAATGCC-3’ and 5’-GGCATTGAAATTTGAGCTGCCTCGTCC-3’. Other mutants listed in Table 3.1 were constructed similarly. Non-phosphorylatable mutant and wild type strains were constructed from MB110 (*MATα dna2Δ::kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trp1*Δ (pSEY18GALDNA2) by transforming with pRS314-DNA2-TRP or mutant plasmid and plating on 5’FOA to select against the pSEY18GALDNA2-URA plasmid. The pGAL-CLB2-TAP strain was a gift of Dr. David Morgan.

Antibodies - Dna2 S287 phospho-specific antibody was produced in rabbits to peptide CYVEKRKSEpSQITQ and purified by AnaSpec Corp. α-HA antibody (12CA5) was purchased from AbCam. α-Rad53 antibody (sc-6749) was purchased from Santa Cruz Biotechnology. α-pRad53 antibody was a gift of Dr. John Diffley.

Protein purification - Dna2 protein was purified as described in Chapter 2. The Clb2/CDC28 (Cdk1) kinase complex was purified as described (Ubersax et al., 2003) with the exception of cell lysis, which was performed by manual grinding in liquid nitrogen.

Nuclease Assay - Dna2Δ105 protein was incubated in λ PPA buffer (50 mM HEPES pH 7.5, 100mM NaCl, 2 mM DTT, 0.01% Brij 35, 1 mM $MnCl_2$) with or without (mock) 200U λ phosphatase at 30° for 40 minutes. Proteins were then diluted in PPA buffer as noted in the figure legend and added to nuclease reaction buffer (50 mM Tris pH 7.5, 25

mM NaCl, 2 mM DTT, 0.25 µg/ml BSA, 2 mM $MgCl_2$, and 30 fmol radiolabeled 5' flap substrate) for incubation at 37° for 15 minutes. Reactions were stopped using 2x denaturing termination dye (95% deionized formamide, 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol), and boiled for 5 min. The cleavage products were separated on a 12% sequencing gel (SequaGel, National Diagnostics) and detected by PhosphoImager.

ATPase Assay - Dna2Δ105 protein was incubated in λ PPA buffer (50 mM HEPES pH 7.5, 100mM NaCl, 2 mM DTT, 0.01% Brij 35, 1 mM $MnCl_2$) with or without (mock) 200U λ phosphatase at 30° for 40 minutes. 130 fmol of protein was then added to ATPase reactions in 20 µl reaction buffer (40mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT, 0.5 µg/ml BSA, 0.2 mM ATP, 10% glycerol, and 3µCi [γ-^{32}P]ATP) were supplemented with 1 µg ssDNA as noted and incubated at 37° for 30 minutes. The reactions were stopped by adding EDTA to a final concentration of 4 mM. 0.8 µl of each reaction was spotted onto a polyethyleneimine cellulose TLC plate (Selecto Scientific) and developed in 0.5 M LiCl, 1 M formic acid solution. Products were detected by PhosphoImager.

In vitro kinase assays. Cdk1 kinase assays were performed using 1.3 pmol yDna2Δ105 and 0.5µl purified Clb2/Cdc28 complex in buffer containing 25 mM HEPES pH 7.5, 1mM EGTA, 0.1mM ATP, 2mM MgCl2, and 6.7 µCi γ-P32-ATP for 30 minutes at 30°. Reactions were stopped by boiling with 2X SDS-PAGE sample buffer for 5 minutes. Mec1 kinase assay was performed using HA-Mec1 (wt) and HA-Mec1 kinase dead (kd) immunoprecipitated using as α-HA as described (Mallory and Petes, 2000) and Dna2 protein purified from baculovirus-infected SF9 cells as described (Budd et al., 2000).

Dna2 S287 phosphorylation. Cells carrying pGal-DNA2 plasmid were induced with 2% galactose prior to the addition of the damaging agent. Cells were lysed as described (Budd et al., 2006) in buffer supplemented with phosphatase inhibitors NaF and $NaVO_4$, and proteins were separated by SDS-PAGE and blotted with indicated antibodies.

Survival Assays. Assays were performed by spotting 10-fold serial dilutions on YPD plates containing the indicated damaging agents. Plates were incubated at 30° for 3-5 days prior to imaging and scoring. Cells were treated with X-rays as previously described using a Pantak MKII X-ray machine operated at 20mA and 70 keV (Budd and Campbell, 2009). For the HU fork stability assay, cells were synchronized for 90 minutes in 10 µg/ml nocodazole and washed with water. Cells were then resuspended in YPD prior to addition of 200mM HU. At the indicated time points, samples were washed with water and re-suspended in YPD and diluted for plating. Survival percentage was calculated based on colonies counted after 3 days relative to 0 time point.

Synthetic Lethality Screen. Strains *dna2*Δ *rad27*Δ pSEY18-DNA2-URA3 and *dna2*Δ *exo1*Δ pSEY18-DNA2-URA3 were transformed with pRS314-dna2-TRP1 plasmids containing the indicated mutation and selected on SC-Trp plates. Strains were then spotted in 10 fold serial dilutions on SC-Trp +5-FOA to assess survival in the absence of wild-type *DNA2* (pSEY18-DNA2-URA3).

RESULTS

Dna2 protein with a 105 amino acid amino-terminal deletion was purified from yeast cells. While this small truncation does not result in the loss of enzymatic activity

(Budd et al., 1995), the protein was found to migrate as a doublet of two distinct forms after PAGE when stained with Coomassie blue (Figure 3.1A). Subjecting the protein to λ phosphatase treatment resulted in the protein migrating as a single band. Because the gel shift seen in Figure 3.1A was likely the result of phosphorylation, we were interested to determine the relative enzymatic activity of the two forms of Dna2. To assess Dna2 nuclease activity after phosphatase treatment, the protein was incubated with λ phosphatase for 40 minutes at 30° prior to dilution in nuclease reaction buffer. The ability to cleave a radiolabeled 5' ssDNA flap was assessed with decreasing amounts of Dna2 protein. As shown in Figure 3.1B lanes 1-5, Dna2 as purified from yeast cells efficiently removes the labeled single-stranded DNA flap from 10 fmol of substrate with as little as 65 fmols of Dna2 protein. However, after treatment with λ phosphatase, 260 fmols of Dna2 is required to process the same amount of substrate as 65 fmols of phosphorylated Dna2 (compare Figure 3.1B lanes 6 and 3).

Next, we assessed the ATPase activity of Dna2 as purified and after phosphatase treatment. Dna2 has ssDNA-dependent ATPase activity, thus in the absence of ssDNA, approximately 95% of the radioactive phosphate remains as [γ-^{32}P]ATP (Figure 3.1C). When ssDNA is added to the reaction with untreated Dna2, most of the γ-^{32}P is released to migrate as inorganic phosphate. With Dna2 that had been subjected to λ phosphatase, however, the amount of phosphate released dropped from 73.4% to 29.8%. Thus, both nuclease and ATPase activities are inhibited by phosphatase treatment.

Dna2 is phosphorylated by Cdk1 and Mec1. The effect of phosphorylation on the Dna2 nuclease and ATPase prompted us to investigate which kinases could be modulating Dna2 enzymatic activity. Previous studies have identified phosphorylation events that

meet the consensus site for the Cdk1 (*CDC28*) kinase, SP or TP motifs (Holt et al., 2009; Kosugi et al., 2009; Smolka et al., 2007; Ubersax et al., 2003). We first asked if Cdk1 could phosphorylate Dna2 protein *in vitro*. To this end, the Cdc28/Clb2 complex (Cdk1) was purified from yeast cells and incubated with Dna2. As shown in Figure 3.2A, Dna2 is phosphorylated *in vitro* by Cdk1.

We were especially interested in the DNA damage sensor kinase Mec1 because of Dna2's role in DNA damage repair and the genetic relationships between *MEC1*, *RAD9* and *DNA2*. Mec1 and a kinase-dead mutant of the kinase were immunoprecipitated (IP) from yeast cells and used for *in vitro* kinase reaction along with mock immunoprecipitations. As shown in Figure 3.2B, Dna2 is also phosphorylated *in vitro* by the Mec1 kinase, but not by the kinase-dead Mec1 mutant nor the mock IPs.

In order to determine the role of Dna2 phosphorylation *in vivo*, we first needed to identify putative residues to investigate. Potential phosphorylation sites which match the consensus sequence of both kinases are spread throughout the Dna2 protein (Figures 3.2C and 3.6B). Serines 17 and 237, both in SP motifs, were identified by previous studies aimed at finding Cdk1 phosphorylation sites (Holt et al., 2009; Kosugi et al., 2009; Smolka et al., 2007). Both lie in the N-terminal third of the protein, but the function of this domain is not well understood (Figure 3.2C). Deletion of this region causes an increase in all Dna2 enzymatic activities in vitro (Bae et al., 2001). *In vivo*, deletion of the N-terminal 405 amino acids results in a reduction in cellular levels of Dna2 but is not lethal or sensitive to MMS, though the mutant exhibits a temperature sensitive phenotype under conditions that support rapid growth (Bae et al., 2003; Liu et al., 2000). Over-expression of the N-terminal domain leads to de-repression of telomeric transcription

units (Singer et al., 1998). Protein-protein interactions within the Dna2 protein itself and with other proteins, such as RPA, have been proposed to contribute to the regulatory properties of this domain (Bae et al., 2003; Bae et al., 2001).

Also in the N-terminal region is Serine 287, a potential Mec1 phosphorylation site identified using a MS screen for DNA damage dependent phosphorylation events (J. Villin and S. Gygi, personal communication). Because of the unknown function of the N terminus of Dna2, and the importance of Dna2 enzymatic activity following DSBs, we decided to generate a phospho-specific antibody against the Serine 287 residue of Dna2 and use the antibody to monitor when S287 is phosphorylated *in vivo*. Due to the low abundance of Dna2 protein in the nucleus, in order to detect the phosphorylated form, it was necessary to over-express *DNA2* and its respective mutant forms using a galactose-inducible promoter prior to applying various DNA damaging agents. Wild-type pGAL-DNA2 (and the mutants described below) supported growth of a *dna2Δ* strain at 30°C when expressed from the *GAL1* promoter, even when grown on glucose, as previously observed. Western blotting of extracts reveals that Dna2 is not strongly phosphorylated in the absence of DNA damage but becomes phosphorylated at S287 after 120 minutes in 0.05% MMS, 40µg/ml camptothecin, which inhibits topoisomerase leading to protein bound double strand breaks, and 25µg/ml phleomycin, which induces double strand breaks (Figure 3.3A). Quantification of the phosphorylation signal reveals a similar level, ~3 fold increase, of modification in response to those three drugs relative to protein level (Figure 3.3B). Note that treatment with MMS leads to a reduction in total Dna2 protein levels. However, Dna2 does not show an increase in phosphorylation at S287 in response to hydroxyurea, an agent which causes stalling of replication forks and

activation of the Mec1 dependent S phase checkpoint, but not direct DNA damage (Figure 3.3A and 3.3B).

In yeast, two PIKK family kinases, Mec1 and Tel1 phosphorylate SQ/TQ motifs following DNA damage. To determine which kinase was responsible for phosphorylation of S287 in vivo, *mec1* and a *tel1* deficient strains were treated with phleomycin. Clearly, S287 phosphorylation is greatly reduced in *mec1* strain compared to wild type (Figure 3.3C). However, S287 phosphorylation is also reduced after phleomycin treatment in a *tel1* deficient strain (Figure 3.3C). Because Tel1 appears to be required for an early step in the processing of some DSBs (Mantiero et al., 2007), the reduction in phosphorylation of Dna2 at S287 could be due to a delay or reduction in activation of Mec1. A second interpretation is that *tel1* loss has slowed DSB resection and Dna2 is not phosphorylated by 120 minutes because some particular resection step remains to be completed before Dna2 can be modified.

In a control experiment, non-phosphorylatable Dna2-S287E protein was not recognized by the phospho-specific antibody after treatment with phleomycin demonstrating that the antibody is specific to phosphorylated S287 of Dna2 (Figure 3.3D). We also asked whether S287 became phosphorylated on a helicase domain mutant, Dna2-S1189A. We were interested in this mutant, not only because it lies in an important helicase motif, but also because S1189 is the only SQ/TQ motif conserved from yeast to human Dna2. As shown in Figure 3.3D, this mutation did not abrogate phosphorylation in response to phleomycin. Notably, over-expression of the helicase motif mutant Dna2-S1189A led to phosphorylation of S287 even in the absence of exogenous DNA damage (Figure 3.3D). We conclude that over-expression of the

helicase deficient Dna2 leads to DNA damage, activating Mec1, and resulting in phosphorylation of S287. This raises the question of whether other Dna2 mutants are constitutively phosphorylated. In these experiments, a small amount of pS287 signal is detected in the absence of exogenous damage. Since it is known that overproduction of Dna2 is deleterious to cells (Parenteau and Wellinger, 1999), a low level of DNA damage may be accumulating due to the Dna2 overproduction.

Phosphorylation of Dna2 after DSB induction does not correlate with survival of damage. Because Dna2 is involved in the resection of 5' DNA ends in homologous recombination mediated repair of DSBs and is phosphorylated after phleomycin and camptothecin treatment, we hypothesized that the phosphorylation may be involved in either recruiting Dna2 to the DSB or activating the nuclease to initiate DSB resection. In order determine the importance of the phosphorylation, we studied survival in a strain with non-phosphorylatable amino acid substitutions at the S287 Dna2 phosphorylation site following DNA damage. Because *DNA2* is an essential gene, Serine 287 was mutated to Alanine on a pRS314-HA-DNA2 single-copy, Trp+ plasmid expressing *DNA2* under its natural promoter. This plasmid was introduced by plasmid shuffling into a *dna2*Δ strain carrying the Ura+ plasmid pSEY18GAL-DNA2 (MB110) and plating on 5-FOA. Surprisingly, the *dna2-S287A* strain is not sensitive to DSB damage induced using X-rays when compared to the wild type *DNA2* strain (Figure 3.4A).

One possibility to explain the X-ray resistance of *dna2-S287A* was that Mre11 nuclease could compensate for the absence of Dna2 activity. Previously, it has been shown that Dna2 and Mre11 nucleases can play interchangeable roles in the processing of DSBs induced by ionizing radiation since plasmids containing either *DNA2* or *MRE11*

can rescue the X-ray sensitivity of a *dna2*Δ*pif1*Δ*mre11-D56N* (mre11 nuclease deficient) strain (Budd and Campbell, 2009). To determine if phosphorylation of Dna2 S287 was required for X-ray resistance in a strain lacking Mre11 nuclease, the ability of *dna2-S287A* plasmid to complement the *dna2*Δ*pif1*Δ*mre11-D56N* strain was compared to that of the wild type *DNA2* (Figure 3.4B). Deletion of *PIF1* is required for the viability of the *dna2*Δ strain, but the mechanism of suppressing the loss of Dna2's essential function appears unrelated to the ability of cells to survive X-ray damage (Budd et al., 2006). Clearly, the *dna2-S287A* mutant complements efficiently. Therefore, phosphorylation at S287 does not appear essential for Dna2 nuclease activity in response to DSBs induced by ionizing radiation, at least in the absence of Pif1. This conclusion is supported by the lack of defect in the *dna2-S287A* mutant in resection of an HO-induced double-strand break in G2 phase cells (G. Ira, personal communication). In order to determine whether the lack of a DNA damage sensitive phenotype was due to an effect of the mutant *DNA2* gene being introduced on a plasmid, the S287A mutation was integrated into the yeast genome, but this strain was also not sensitive to DNA damage (data not shown).

Mec1 checkpoint is active in dna2-S287 mutants. Resection of 5' ends of DSBs by Dna2 generates ssDNA which becomes bound by RPA. This ssDNA coated in RPA protein loads the Ddc1-Rad17-Mec3 (Rad9-Rad1-Hus1) checkpoint clamp that activates Mec1 to mediate the DNA damage checkpoint (Navadgi-Patil and Burgers, 2009; Zou et al., 2003). Thus, we were interested in if the Mec1 mediate checkpoint was intact in the *dna2-S287A* and *dna2-S287E* mutants. The *dna2-S287E* mutation was made to potentially mimic phosphorylation at Serine 287. One marker of Mec1 checkpoint activation is the phosphorylation of downstream effector kinase Rad53. As shown in

Figure 3.5A, Rad53 is shifted to slower migrating forms after both MMS which causes both single and double stranded DNA damage and HU which stalls replication forks, indicating checkpoint activation. The phosphorylation shift is similar in wild-type *DNA2* and both *S287* mutants. We also analyzed the ability of the mutant strains to recover from 120 or 180 minutes of 200mM HU, a classic test of the Mec1 mediated stability of stalled replication forks. We see no difference in the ability of the mutants *dna2-S287A* or *dna2-S287E* to return to growth compared to the wild type, meaning that the phosphorylation of S287 is not required for the replication fork stabilization (Figure 3.5B). Thus we conclude that activation of the Mec1 mediated checkpoint is not affected by the phosphorylation status of S287 of Dna2, although some steps in the DSB response may be affected.

DNA Damage Sensitivity Screen of Potential Phosphorylation Sites. The lack of sensitivity of the *dna2-S287A* mutant to DSBs prompted us to investigate other potential phosphorylation sites. While *in vitro* phosphorylation of Dna2 by Mec1 only labeled Dna2 with about 10% efficiency, we nevertheless used mass spectrometry to search for phosphorylated residues before and after treatment with Mec1. Two residues, T492 and T962 were found to be modified in a Mec1 dependent manner.

Because phosphorylation was inefficient *in vitro* and peptide coverage was only approximately 50%, we considered that the mass spectrometry results might miss an important functional phosphorylation site. Therefore, we altered many potential Mec1 phosphorylation sites in Dna2 and determined the phenotype of resulting mutants. Dna2 contains 17 SQ and TQ motifs including S287, the consensus sites for Mec1 serine and threonine kinase activity. The conservation of these sites in the senso stricto yeasts and

higher eukaryotes is shown in Figure 3.6, but mutants were made regardless of conservation status. SQ/TQ motifs were changed to AQ using pRS314-HA-DNA2 as described above. Single mutants, as well as several combined mutants, were assessed for growth defects and DNA damage sensitivity. Two of the SQ/TQ mutants showed a defective phenotype: *dna2-T962A* and *dna2-S1189A* (Table 3.2). The *dna2-T962A* strain showed reduced growth at 37°, and the *dna2-S1189A* strain was sensitive to MMS and HU and failed to growth at 37°.

While S1189 is of potential interest because it has the strongest phenotype and because it is the only SQ/TQ motif conserved from yeast to human Dna2, it falls in an essential catalytic region of Dna2, residing in a key helicase motif involved in ATP hydrolysis. This makes it difficult to distinguish potential phosphorylation effects from a generic helicase defect. To try to distinguish these possibilities, Q1190A/N mutants, which should reduce Mec1-dependent phosphorylation were made (Kim et al., 1999; Lees-Miller et al., 1992). Unlike S1189A, neither of these mutants showed a defect in MMS survival and both grew normally at 37° (Table 3.2). We also combined T492A, T962A, the two sites found by MS to be phosphorylated in vitro, with Q1190A, but saw no further reduction in growth at 37° or increase in MMS sensitivity compared to the single mutants. Therefore, we propose that the defect in S1189A is more likely due to a helicase defect rather than lack of phosphorylation, though we note that mutating the Q residue may not be sufficient to completely abrogate phosphorylation *in vivo* (Kim et al., 1999), nor rule out the possibility that phosphorylation at S1189 exists and modulates helicase activity to some extent in response to DNA damage but that lack of phosphorylation does not lead to cell death.

We also constructed several Cdk1 phosphorylation site mutants in the same manner as the Mec1 potential sites. Phosphorylated Serine 647, in a SP motif, was identified by MS analysis in the Mec1 phosphorylation screen. This site, however, also conforms to the controversial consensus sequence for the Rad53 kinase (Sidorova and Breeden, 2003; Sweeney et al., 2005), and it has not been found to be phosphorylated by Cdk1 in other screens (G. Ira, personal communication). We find that *dna2-S237A* is somewhat temperature sensitive, but like the Mec1 mutants, disruption of a single phosphorylation site does not lead to major defects in the ability to tolerate DNA damaging agents (Table 3.2).

Many of the important steps in the response to DNA damage can be performed redundantly by multiple enzymes. This provides the cell with resiliency to survive many different kinds of lesions and under a wide array of conditions. This redundancy however may obscure the effects of phosphorylation events. Therefore, we decided to assess the ability of certain mutants to survive when combined with another gene deletion. The inability to survive two mutations that are alone non-lethal is call synthetic lethality. We first asked whether the mutants would be synthetically lethal with *rad27*Δ. *RAD27* (FEN1) functions with Dna2 in Okazaki fragment processing and when overexpressed can suppress the lethality of a *dna2*Δ (Budd and Campbell, 1997). Neither mutations at S237 nor S287 were synthetically lethal with *rad27*Δ (Table 3.3). The S1189 mutants were, however, synthetically lethal with *rad27*Δ. This was not unexpected because other helicase domain mutants are also synthetically lethal with *rad27*Δ *(Formosa and Nittis, 1999).* The *dna2-647A* mutant was not synthetically lethal with *rad27*Δ, as we could see colonies when plated on 5-FOA. However, these colonies

were much smaller than in control strains indicating a possible defect resulting from the combined mutations.

We next investigated synthetic lethality of the selected mutants with *exo1*Δ. Exo1 is a nuclease that also functions in the DSB response and can resect the 5' end of double strand breaks, albeit less efficiently than Dna2. We did not find synthetic lethality with *dna2-S237A, dna2-S287A, dna2-S287E, dna2-S647A,* or *dna2-S237A, S647A.* We did see synthetic lethality with *dna2-S1189D,* but surprisingly not with *dna2-S1189A.* This result raises interesting questions about the function of the helicase domain in the DSB response. The S1189D mutant was constructed in order to mimic phosphorylation on Serine 1189. However, the placement of the charged Aspartic Acid (D) in the key helicase domain motif, the Walker B box, makes the amino acid sequence more similar to other helicases with DEAD motifs, perhaps resulting in increased or undisturbed helicase activity compared to the S1189A mutation. It is of potential interest that increased Dna2 helicase activity would necessitate the nuclease activity of Exo1 to assist the Dna2 nuclease domain in processing the resulting DNA replication or repair intermediates, because a helicase$^+$ nuclease$^-$ *DNA2* plasmid is more toxic to the *dna2Δ pif1Δ mre11-H125N* strain than the helicase$^-$ nuclease$^-$ form of *DNA2*.

DISCUSSION

In this study, we show that Dna2 is phosphorylated following DNA damage, but these individual phosphorylation events do not appear to be required for the survival of that DNA damage. The function of individual phosphorylation sites is difficult to assess

due to redundancy, both in multiple processing pathways for similar forms of DNA damage, as well as potential secondary phosphorylation sites in the N-terminal region of Dna2.

How the activity of Dna2 is regulated during various DNA damage responses is a topic of great interest. In Figure 3.3C, we find that Dna2 phosphorylation levels are lower in *tel1*Δ cells compared to the wild type strain. It is known that Tel1 contributes to efficient 5' resection at DSBs. In Mantiero et al., maximal Rad53 phosphorylation by Mec1 is delayed from 120 minutes to 180 minutes in the *tel1*Δ strain (Mantiero et al., 2007). This effect is due to the reduction of 5' DSB resection in *tel1*Δ as it is suppressed by the over-expression of Exo1. We speculate that Dna2, like Rad53, is phosphorylated by the Mec1 kinase in response to the DSBs, but that activation of Mec1 is delayed due to inefficient DSB processing when Tel1 is absent, as opposed to Tel1 kinase activity contributing directly to the phosphorylation level. That said, the *dna2-2* mutant is synthetically sick with *tel1sml1* mutation and *dna2-2* helicase deficiency causes spontaneous DSBs at the site of stalled replication forks. The negative synergy between *dna2* and *tel1* mutants appears to be related to the processing of double strand breaks, a function that does not seem to be regulated by the S287 phosphorylation site. Whether Tel1 can phosphorylate Dna2 at another site remains an open question, as both proteins function in DSB resection and telomere maintenance.

As the primary nuclease responsible for 5' DSB end resection, these results imply that Dna2 is phosphorylated by Mec1 at S287 after Dna2 has participated in the resection processing of the DSB end, perhaps explaining why *dna2-S287A* is not sensitive to X-ray damage while other *dna2* nuclease defective mutants are. This raises the question of

whether the creation of ssDNA by Dna2 is the signal that causes Mec1 to phosphorylate Dna2 at S287 following DNA damage. It has been suggested that Rad9 activation of Mec1 after the accumulation of ssDNA at breaks serves to inhibit further DNA degradation allowing recombination to proceed (Lazzaro et al., 2008). If that is the case, removal of this regulation step in *rad9*Δ may explain why *dna2* mutants are able to grow under conditions of replication stress as it would allow slower, alternate pathways to address the DNA damage before DSB end resection was attenuated. The N terminal domain of Dna2 has previously been characterized as having an inhibitory role towards the enzymatic activity of Dna2 *in vitro* (Bae et al., 2001).

The role of other potential phosphorylation sites. The activity of the Dna2 helicase has been hard to demonstrate *in vitro*, even though the domain is essential in yeast cells (Budd et al., 1995; Kim et al., 2006; Zheng et al., 2008). While the *dna2-S1189A* mutant has dramatic DNA damage and temperature sensitivity, the location of the SQ site in the heart of the helicase motif II makes it impossible to conclude that Mec1 phosphorylation is responsible for this phenotype. However, the importance of the helicase domain on Dna2 function after DNA damage is clear even though helicase activity of Dna2 *in vitro* is minor compared to nuclease activity. Strains with Dna2 helicase activity but no nuclease activity are more sensitive to DSBs caused by X-rays than when both helicase and nuclease activity are impaired (Budd and Campbell, 2009). Thus, the helicase domain is a compelling candidate for regulation by post-translational modification.

Mass spectrometry screens have identified several sites of phosphorylation, and thus potential regulation of Dna2 activity, by both Mec1 and Cdc28/Cdk1 kinases. While mutating the other Mec1 consensus phosphorylation sites listed in Table 3.2 individually

did not result in a damage sensitive phenotype, combining mutations may uncover other roles of Mec1 modulation of Dna2 activity. Rad9, Srs2 and Sae2, for example, are phosphorylated by both Mec1 and Cdc28/Cdk1. Both kinases appear to control extensive DNA damage response pathways which maintain genomic stability. While the target substrates may overlap, the function of Mec1 and Cdc28 after damage is independent.

Dna2 clearly functions in multiple, distinct roles for maintaining genomic stability by both participating in DSB repair via resecting of the 5' ends and by preventing the formation of DSBs at stalled replication forks and single stranded DNA lesions between nascent Okazaki fragments. Future studies must continue to address the mechanism of Dna2 regulation through additional post-translational modifications in response to other DNA damage agents, other modes of DNA repair, and in other stages of the DNA damage response. While Serine 287 is not conserved, even among the closely related yeasts, every Dna2 protein contains SQ/TQ motifs. Further, *Xenopus* Dna2 immunoprecipitates the PIKK ATM, and like yDna2 participates in resection of DSB ends. We speculate that the scheme of Dna2 regulation by members of the PIKK family will be conserved to higher eukaryotes, even if specific phosphorylation sites are not.

Table 3.1 Strains used in this study.

Strain	Genotype	Source
W303	*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ssd1*	
JMY312-6d	*sml::HIS3 tel1::ura3 MEC1-HA*	T. Petes
JMY313-9a	*mec1-HA(KD) sml1::HIS3 tel1::ura3*	T. Petes
W4133-2D	*MATa trp1-1 mec1::TRP1 sml1::HIS3*	R. Rothstein
W4133-2D	(pSEY18*GAL-DNA2*)	this study
SPY40	*MATa tel1::URA3 trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	T. Petes
SPY40	(pSEY18*GAL-DNA2*)	this study
BJ5459	*a ura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1*	
BJ5459	(pSEY18*GAL-DNA2*)	(Budd et al., 1995)
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Invitrogen
BY4742	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Invitrogen
MB110	*MATα dna2Δ::kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trp1*Δ (pSEY18*GAL-DNA2*)	(Budd et al., 2005)
BKF001	*MATα dna2Δ::kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trp1*Δ (pRS314-*DNA2*)	this study
BKF002	*MATα dna2Δ::kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trp1*Δ (pRS314-*dna2-S287A*)	this study
BKF003	*MATα dna2Δ::kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trp1*Δ (pRS314-*dna2-S287E*)	this study
BKF004	*MATα dna2Δ::kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trp1*Δ (pRS314-*dna2-S1189A*)	this study
BKF005	*MATα dna2Δ::kanMX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trp1*Δ (pRS314-*dna2-S1189D*)	this study
MB161B-56	*4741 MATα dna2*Δ*::kanMX pif1*Δ*::HIS3 trp1*Δ *bar1*Δ*::kanMX mre11-D56N*	(Budd and Campbell, 2009)
MB161B-56	(pRS314-*DNA2*)	(Budd and Campbell, 2009)
MB161B-56	(pRS314-*dna2-S287A*)	This study
BKF101	MB110 *rad27*Δ*::LEU2*	This study
BKF102	*4741 dna2*Δ*::natR exo1*Δ*::G418*	This study

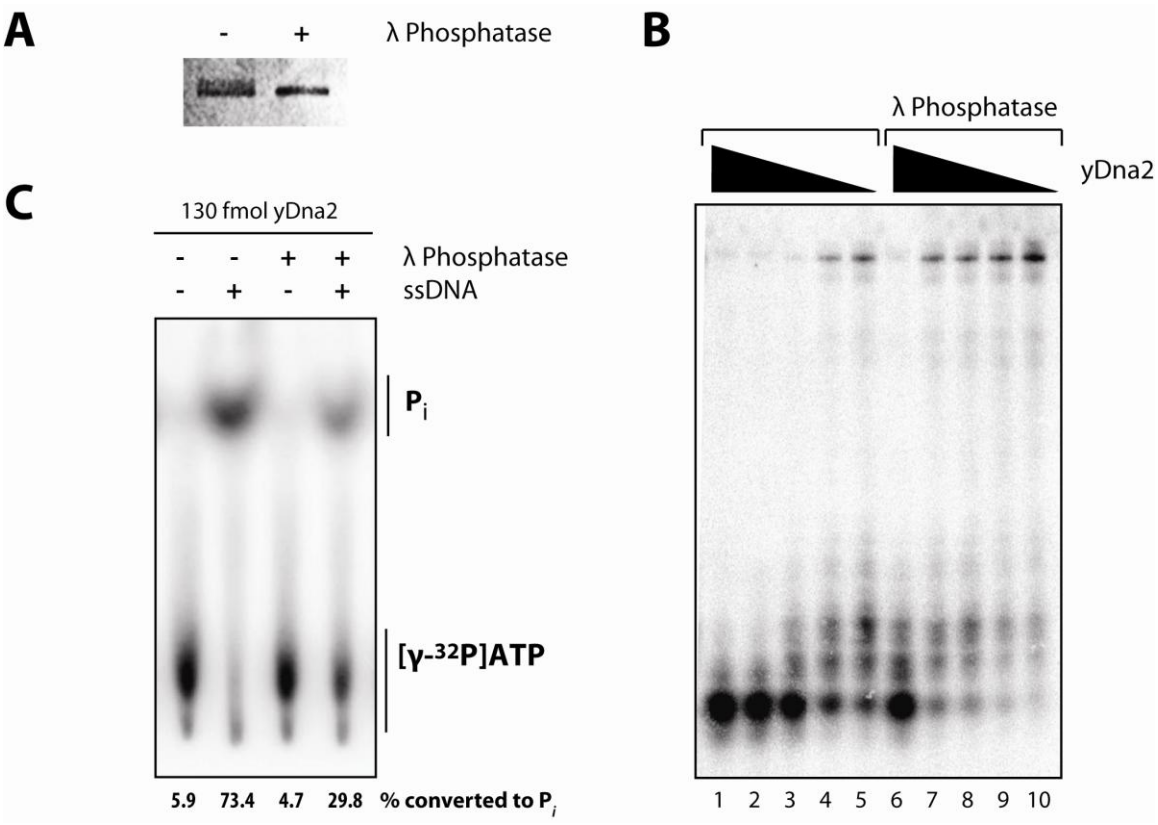


Figure 3.1 Phosphatase treatment of purified Dna2 inhibits enzymatic activity. (A) Dna2Δ105 migrates as two bands on PAGE. Treatment with λ phosphatase for 40 minutes reduces Dna2 to one form. (B) Nuclease assay following treatment with λ phosphatase (lanes 6-10) is reduced compared to mock phosphatase treatment (lanes 1-5). Dna2Δ105 protein was added in the following dilutions: 260 fmol (lanes 1 and 6), 130 fmol (lanes 2 and 7), 65 fmol (lanes 3 and 8), 32.5 fmol (lanes 4 and 9), 16.25 fmol (lanes 5 and 10) as described in Materials and Methods. (C) ATPase assay with 130 fmols of Dna2Δ105 protein after treatment with λ phosphatase or mock treatment with 1μg of ssDNA as indicated.

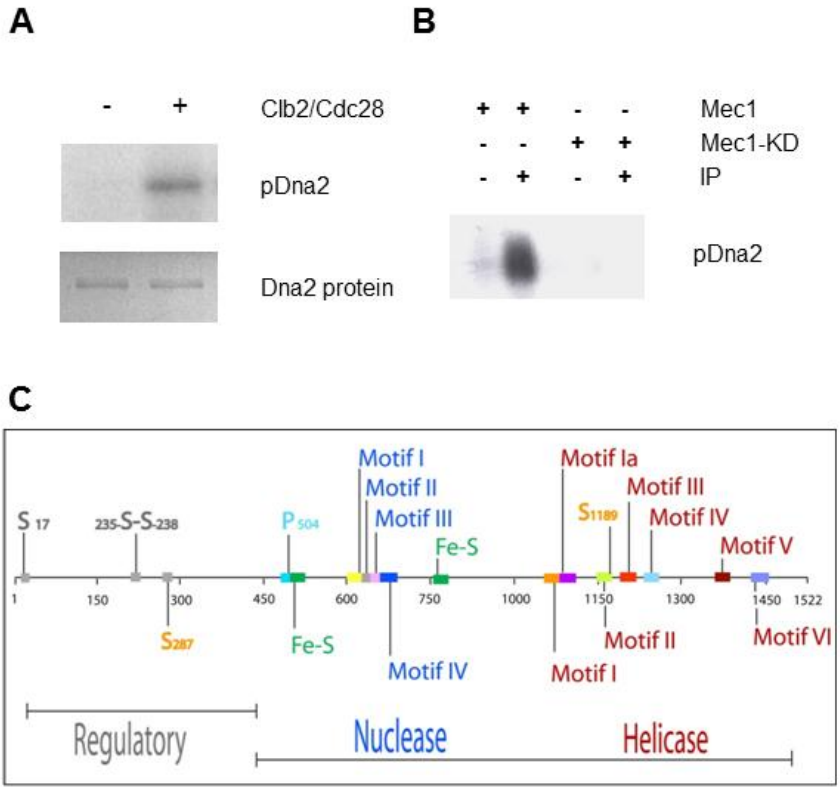


Figure 3.2 Yeast Dna2 is phosphorylated *in vitro*. (A) 1.3 pmol of Dna2 was incubated with TAP purified Clb2/CDC28 kinase complex in 25 mM HEPES pH 7.5, 1mM EGTA, 0.1mM ATP, 2mM MgCl2, and 6.7 µCi γ-P32-ATP for 30 minutes at 30°. Proteins were separated using SDS-PAGE and exposed to phosphoimager screen (top panel) before staining with Coomaisse Blue to show equal protein levels (bottom panel). Phospho-image shows incorporation of radioactive phosphate. (B) Immunoprecipitated Mec1 kinase and Mec1 kinase dead, or mock immunoprecipitations (- IP), were used in *in vitro* kinases assays as described in Materials and Methods. Dna2 is labeled with radioactive phosphate by Mec1 immunoprecipitates. (C) Schematic of Dna2 showing enzymatic motifs and domains and putative phosphorylation sites.

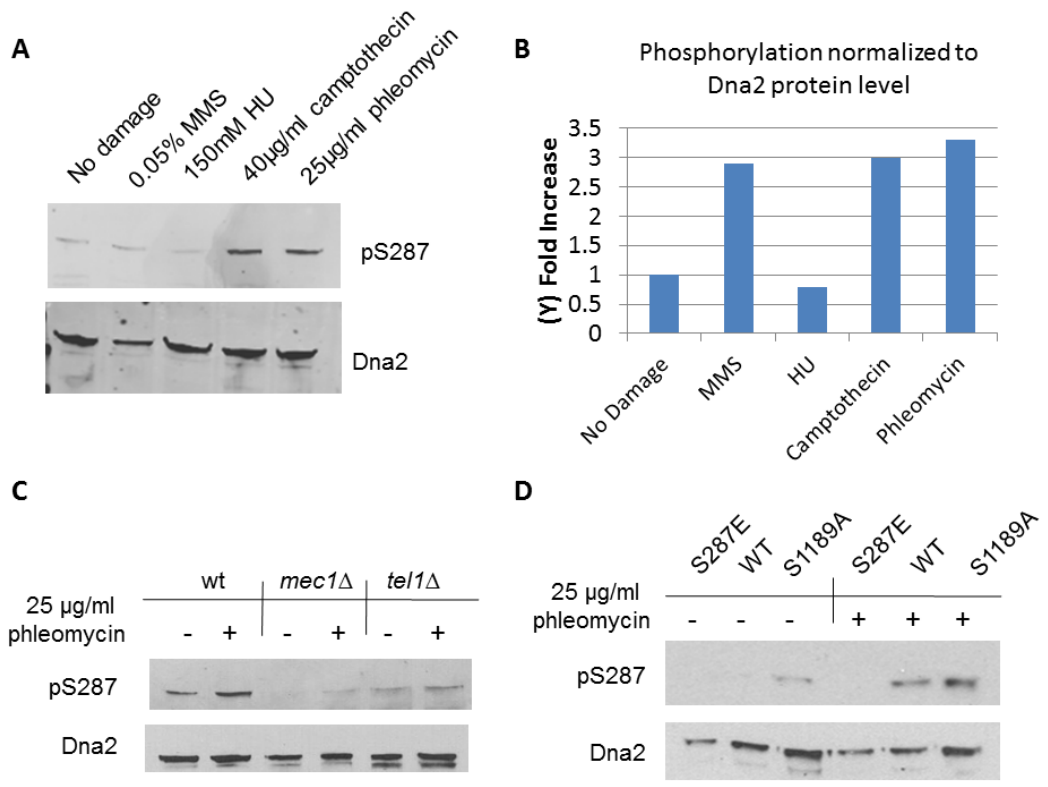


Figure 3.3 Dna2 is phosphorylated at Serine 287 by Mec1 *in vivo* following multiple forms of damage. (A) Cells containing the pGAL-DNA2 plasmid were induced with 2% galactose for 2 hours prior to addition of 0.05% MMS, 150mM hydroxyurea (HU), 40µg/ml camptothecin, and 25µg/ml phleomycin. Cells were harvested at 120 minutes, whole cell lysates were separated by SDS-PAGE, and blotted with α-pS287-Dna2 antibody (top panel) or α-HA to show total Dna2 levels (bottom panel). (B) The fold increase in phosphorylation over background for the experiment in panel (A) was plotted as a ratio of phospho-Dna2 signal to Dna2 protein level relative to undamaged sample. (C) Dna2 phosphorylation is absent in *mec1*Δ cells and reduced in *tel1*Δ cells. Wild type (wt), *mec1*Δ, or *tel1*Δ strains carrying the pGAL-DNA2 plasmid were induced with 2% galactose and treated with 25ug/ml phleomycin for 2 hours. (D) Antibody is

specific for phosphorylation at S287 and over-expression of the S1189 helicase mutant causes constitutive phosphorylation at S287. Cells carrying pGAL-DNA2, pGAL-dna2-S287E, or pGAL-dna2-S1189A plasmids were induced with 2% galactose for 20 minutes prior to the addition of 25μg/ml phleomycin for 2 hours.

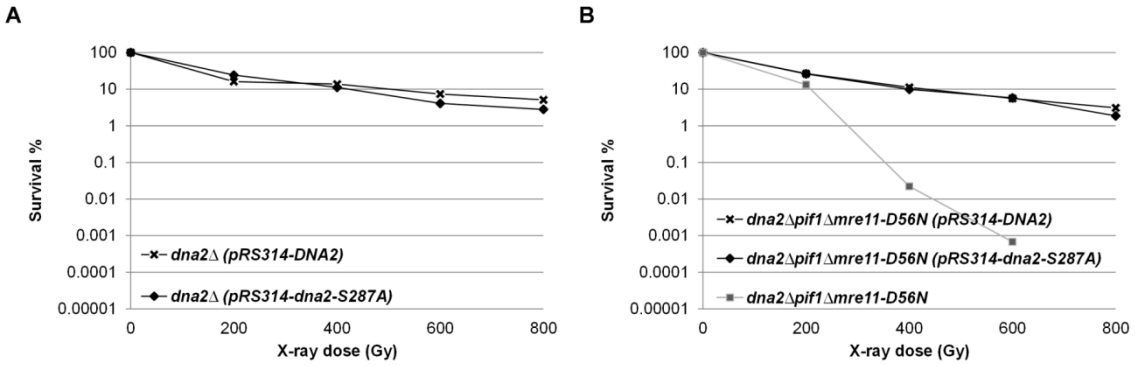


Figure 3.4 Dna2 phosphorylation is not required for survival after DSBs caused by X-rays. **(**A) Survival curve of *dna2*Δ strain with pRS314-DNA2 (×) or pRS314-dna2-S287A (♦) treated with 0, 200, 400, 600, and 800 Gy of X-ray and plated on YPD for 3 days. (B) Survival after X-rays of *dna2-S287A* mutant in the absence of Mre11 nuclease activity. Survival curve of *dna2*Δ*pif1*Δ*mre11-D56N* (pRS314-DNA2*)* (×), *dna2*Δ*pif1*Δ*mre11-D56N* (pRS314-dna2-S287A*)* (♦), and *dna2*Δ*pif1*Δ*mre11-D56N* (■) treated with 0, 200, 400, 600, and 800 Gy of X-ray. All dilutions of *dna2*Δ*pif1*Δ*mre11-D56N* cells had 0 colonies after 800 Gy dose; thus no survival % is plotted.

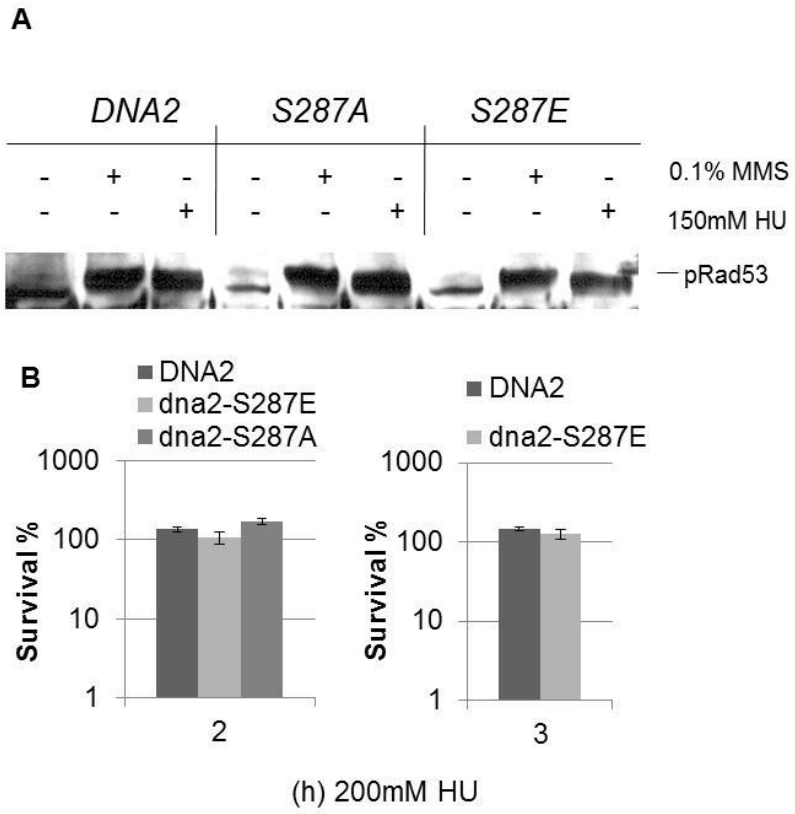


Figure 3.5 The Mec1 mediated checkpoint is intact in *dna2-S287* mutants. (A) Cells expressing endogenous levels of *DNA2*, *dna2-S287A* or *dna2-S287E* were treated with 0.1% MMS or 150mM HU for 75 minutes. Whole cell lysates were blotted with α-phospho-Rad53 antibody to determine Mec1 activation. (B) *dna2-S287A* and *dna2-S287E* show no defect in recovery from HU treatment, a test of stalled replication fork stability. Cells expressing endogenous levels of *DNA2*, *dna2-S287A* or *dna2-S287E* mutant were incubated in 200mM HU for either 120 or 180 minutes prior to plating on YPD. Survival was calculated relative to undamaged culture.

Table 3.2 DNA damage sensitivity of phosphorylation site mutants.

DNA2 mutation	Kinase	37°	X rays	MMS	Phleomycin	Camptothecin	HU
T116A	Mec1	-		-			-
S237A	Cdk1	+	-		-	-	
S276A	Mec1	-		-			-
S287A	Mec1	-	-	-	-	-	-
S287E	Mec1	-	-	-	-	-	-
T371A, T373A	Mec1	-		-			-
T437A	Mec1	-		-			-
T492A	Mec1	-		-			-
T630A	Mec1	-		-			
S647A	Unknown		-				
S237A, S647A	Cdk1	-	-	-	-	-	-
S807A	Mec1	-		-			-
S885A, S891A	Mec1	-		-			-
T962A	Mec1	+		-			-
T1135A	Mec1	-		-			-
S1189A	Mec1	++		+			+
Q1190N	Mec1	-		-			
S1411A	Mec1	-		-			-

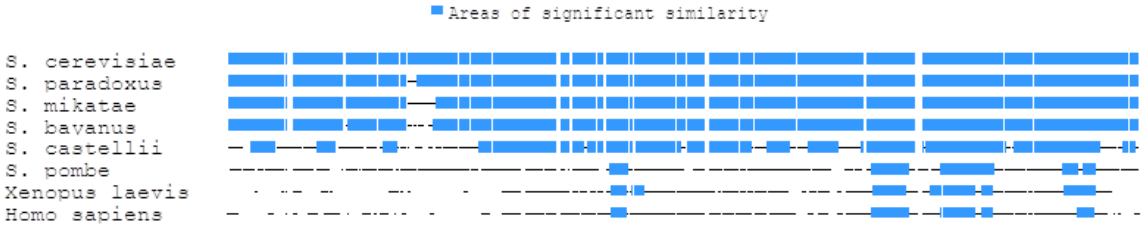
The *dna2*Δ pRS314-dna2 mutant strains were created using plasmid shuffle and plated on YPD with the indicated damaging agents in 10 fold serial dilutions. After 3 days of growth, mutants were scored for sensitivity. Indicated kinase is based on consensus sequence. Blank fields indicate the mutant's sensitivity to the damaging agent was not determined.

Table 3.3 Synthetic lethal screen of phosphorylation site mutants with *rad27*Δ and *exo1*Δ.

DNA2 Mutation	Kinase	*rad27*Δ	*exo1*Δ
S17A	Cdk1	-	
S237A	Cdk1	-	-
S287A	Mec1	-	-
S287E	Mec1	-	-
S647A	Unknown	+/-	-
S237A,S647A	Cdk1, unknown	-	-
S1189A	Mec1	+	-
S1189D	Mec1	+	+

Strains *dna2*Δ *rad27*Δ pSEY18-DNA2-URA3 and *dna2*Δ *exo1*Δ pSEY18-DNA2-URA3 were transformed with pRS314-dna2-TRP1 plasmids containing the indicated mutation and selected on SC-Trp plates. Strains were then spotted in 10 fold serial dilutions on SC-Trp +5-FOA to assess survival in the absence of wild-type *DNA2* (pSEY18-DNA2-URA3). (+) indicates synthetic lethality and (-) indicates survival of dna2 mutants with the noted deletion. (+/-) indicates a possible growth defect in surviving colonies. Blank fields indicate the synthetic lethality was not tested.

A. Sequence Similarity Map



B. Sequence Alignment

Figure 3.6 Sequence Alignment of Dna2. (A) Sequence similarity map highlights areas of similarity with black line and high similarity with blue box. Note high level of protein conservation among yeast species and high level of similarity in enzymatic domains from *S. cerevisiae* to higher eukaryotes. (B) Global Protein Alignment with *S. cerevisiae DNA2* as reference sequence using Blosum 62 scoring matrix. Amino acids are coded as identical (black), similar (grey) or dissimilar (red). Potential Mec1 (yellow) and Cdk1 (green) phosphorylation sites highlighted. Similarity map and sequence alignment produced using Sci Ed Central Clone Manager Professional Suite version 6.0. Accession numbers for sequences are *Saccharomyces cerevisiae* (P38859), *Schizosaccharomyces pombe* (NP_596499), *Xenopus laevis* (NP_001079231), *Homo sapiens* (NP_001073918) from the NCBI Protein database and *Saccharomyces mikatae* (smik663-g18.1), *Saccharomyces paradoxus* (spar31-g17.1), *Saccharomyces bayanus* (sbayc599-g16.1), *Saccharomyces castellii* (Scas546.2) from the Fungal Orthogroup Repository.

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CHAPTER 4

XENOPUS DNA2 IS A HELICASE/NUCLEASE THAT IS FOUND IN COMPLEXES WITH REPLICATION PROTEINS AND-1/CTF4 AND MCM10 AND DSB RESPONSE PROTEINS NBS1 AND ATM

This chapter characterizes the role of Dna2 in DNA replication and DNA repair using the *Xenopus* egg extract system. BF's contribution to this work includes preparation of proteins for MS identification (see Figure 4.4A) and preparation of biotinylated DNA substrates for replicating double strand breaks (see Figures 4.6 and 4.7). The copyright for the presented material, published as:

> Karen E. Wawrousek, Barbara K. Fortini, Piotr Polaczek, Lu Chen, Qingquan Liu, William G. Dunphy and Judith L. Campbell. (2010). Xenopus DNA2 is a helicase/nuclease that is found in complexes with replication proteins And-1/Ctf4 and Mcm10 and DSB response proteins Nbs1 and ATM. Cell Cycle 9(6), 1156-1166

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ABSTRACT

We have used the versatile *Xenopus laevis* egg extract system to study the roles of vertebrate Dna2 in DNA replication and double-strand-break (DSB) repair. We first establish that *Xenopus* Dna2 is a helicase, as well as a nuclease. We further show that Dna2 is a nuclear protein that is actively recruited to DNA only after replication origin licensing. Dna2 co-localizes in foci with RPA and is found in a complex with replication fork components And-1 and Mcm10. Dna2 interacts with the DSB repair and checkpoint proteins Nbs1 and ATM. We also determine the order of arrival of ATM, MRN, Dna2, TopBP1, and RPA to duplex DNA ends and show that it is the same both in S phase and M phase extracts. Interestingly, Dna2 can bind to DNA ends independently of MRN, but efficient nucleolytic resection, as measured by RPA recruitment, requires both MRN and Dna2. The nuclease activity of Mre11 is required, since its inhibition delays both full Dna2 recruitment and resection. Dna2 depletion inhibits but does not block resection, and Chk1 and Chk2 induction occurs in the absence of Dna2.

INTRODUCTION

Yeast Dna2 is an essential helicase/nuclease involved in removing RNA/DNA primers during Okazaki fragment processing (OFP). It is also required for mitochondrial genome stability. Recently, yeast Dna2 has been recognized as a nuclease critical for resection of 5′ ends during the early steps of homology dependent repair of double-strand breaks (DSBs) (Budd et al., 2009). Dna2 provides a previously missing link in the early steps of DSB repair, and the discovery of its participation now allows biochemical

analysis of this stage of repair. Functional genomic screens indicate additional but to date poorly characterized roles for Dna2 in maintenance of chromatin, nuclear structure, and telomere biogenesis (Budd et al., 2005). Thus, Dna2 is a major contributor to genomic stability in yeast.

While the Dna2 nuclease and helicase active sites are well conserved in evolution, the regulatory sequences are divergent between yeast and vertebrates. Furthermore, there is controversy as to whether Dna2 from *Xenopus laevi*s and human cells contain functional helicase activity (Kim et al., 2006; Liu et al., 2000; Masuda-Sasa et al., 2006; Zheng et al., 2008). Finally, the extent to which the physiological functions are conserved is a matter of debate. In *C. elegans*, Dna2 is dispensable for embryogenesis, and in human cells, it has been proposed that Dna2 is exclusively mitochondrial (Lee et al., 2003b; Zheng et al., 2008). Recently, nuclear localization has been established in human cells and shRNA knockdown of Dna2 has been shown to lead to cell cycle delay and aberrant cell division (Duxin et al., 2009). However, many fundamental questions remain about the nuclear function of Dna2 in vertebrates.

Xenopus egg extracts offer an attractive system in which to determine the steps of nuclear DNA replication and DSB repair that require Dna2 in vertebrates. Egg extracts contain abundant maternal replication protein stores, providing a rich source of these activities compared to normal S phase extracts. *Xenopus* extracts are prepared from eggs arrested in metaphase of meiosis II, which resemble M phase extracts. Addition of calcium stimulates the extract to enter interphase, or S phase, and further addition of chromosomal DNA and an ATP-regeneration system results in the formation of nuclei that initiate replication. This DNA replication is both regulated and synchronized

occurring at replication origins spaced almost every 4 kb along the DNA. In addition, cellular responses to DNA damage can be successfully recapitulated in this extract (Blow and Laskey, 1986; Garner and Costanzo, 2009). Furthermore, the powerful immunodepletion technique allows biochemical analysis of these pathways. We previously showed that depletion of *Xenopus* Dna2 from such extracts led to a reduction in replication of sperm chromatin, but not a complete absence of DNA synthesis (Liu et al., 2000). In addition, recent elegant studies demonstrated that Dna2 is required for single-strand annealing (SSA), a reaction that can occur in extracts and that mimics the early steps of homology-dependent repair of a DSB (Budd and Campbell, 2009; Liao et al., 2008; Zhu et al., 2008). In *Xenopus*, SSA is a two step process in which the duplex DNA ends are unwound by a helicase, primarily WRN/FFA-1, and then the free 5′ single-stranded DNA (ssDNA) is degraded by a nuclease, primarily Dna2, revealing a free 3′ strand that can anneal to a complementary sequence (Liao et al., 2008). Thus, *Xenopus* extracts offers an attractive system in which to study both the DNA replication and DSB functions of Dna2.

In this work, we use *Xenopus* to explore in further depth the S phase activities of nuclear Dna2 in vertebrates. We first establish biochemically that *Xenopus* Dna2 is a helicase as well as a nuclease. We then show that Dna2 fails to bind to undamaged chromatin in S phase if replication initiation is inhibited. Interestingly, proteomic analysis reveals that a major Dna2-interacting protein detectable in S phase is And-1, a protein involved in stabilizing lagging strand replication complexes and in establishing sister chromatid cohesion. In addition we show that Dna2 interacts with DSB checkpoint and repair proteins ATM and Nbs1, a component of the MRN complex. Dna2 is

recruited to DSBs after ATM and MRN, and with similar timing to RPA. Depletion of Dna2 from the extracts leads to a reduction in resection. We specifically analyze the interplay between Dna2 and MRN in the resection reaction. Finally, we find that depletion of Dna2 from extracts does not prevent induction of the DNA damage checkpoint response. Together, our results suggest that the main roles of Dna2 are in DNA replication and repair machinery rather than in signaling.

RESULTS

Xenopus Dna2 has both helicase and nuclease activities. Due to robust nuclease activity, previous attempts to detect helicase activity in purified wild-type *Xenopus* Dna2 protein were unsuccessful. We reinvestigated helicase activity by changing a key aspartate residue, D278, in the putative Dna2 nuclease active site to alanine. We expressed and purified the mutant protein from insect cells, as previously described for the wild-type protein (Liu et al., 2000), and demonstrated loss of nuclease activity (Fig. 1). We measured the ability of the D278A mutant to unwind a labeled oligonucleotide with 22 bp of complementary sequence and a non-complementary 30 nt 5′ tail annealed to M13mp18. As shown in Fig. 4.1, there is significant accumulation of the free 52 nucleotide product. Thus, like yeast and human Dna2, *Xenopus* Dna2 is a combined helicase-nuclease.

Dna2 associates with S-phase chromatin in a regulated manner. In this text, interphase extract refers to S phase extract with no DNA added. CSF extract refers to extract that has not been stimulated by addition of calcium, a state resembling M phase

extracts. For DNA replication, *Xenopus* demembranated sperm chromatin was used as the source of chromosomal DNA. We first examined the association of Dna2 with chromatin in S phase extract. We see that Dna2 does bind chromatin and accumulates on chromatin during DNA replication with a time course consistent with the dynamics of proteins that participate in DNA replication, such as RPA70, the large subunit of the replicative single-stranded DNA binding protein, and Cdc45, a protein required for activation of the MCM helicase complex (Fig. 4.2A).

We next determined whether DNA replication initiation was required for the association of Dna2 with chromatin. Initiation of DNA replication requires the formation of a pre-replication complex (pre-RC), which consists of ORC, the MCM2-7 helicase complex, Cdc6, and Cdt1. The pre-RC is thought to serve as a "landing pad" for the remaining components of the replisome, or as a "licensing" complex for the initiation of DNA replication. As shown in Fig. 4.2B, depletion of Mcm3, a component of the MCM2-7 helicase, reduced Dna2 binding to chromatin, suggesting that licensing of the replication fork is required for Dna2 association with chromatin in S phase and indicating that the binding we see is specific to replicating chromatin. To reinforce this conclusion, we took advantage of the fact that Cdt1 is required for stable association of the MCM helicase with pre-RC components, and that geminin, an inhibitor of Cdt1, inhibits pre-RC formation and thus initiation of DNA replication. As shown in Fig. 4.2C, geminin inhibits the accumulation of Dna2 on chromatin. Thus, we conclude that pre-RC formation is required for Dna2 loading. Some residual binding is observed in the presence of geminin, which may be due to a low level of insoluble Dna2 in the extract, since the same amount is seen in control extracts to which no DNA was added.

Alternatively, if *Xenopus* Dna2 is also both a nuclear and mitochondrial protein, as in yeast and human, this assay could also be detecting small amounts of residual mitochondrial Dna2 contaminating the chromatin fraction.

The pre-RC is activated for replication by binding of additional proteins, some of which require the active Cdk2 cyclin dependent kinase, to form the pre-IC (preinitiation complex). The binding of Cdc45, which is dependent on active Cdk2, marks the transition from the pre-RC to pre-IC. p27 is an inhibitor of Cdk2 and inhibits origin firing by preventing the loading of some replication proteins, including Cdc45, in *Xenopus* extracts. As shown in Fig. 4.2D, Dna2 still associates with chromatin in the presence of p27. The recruitment of Dna2 to chromatin after pre-RC formation but in the presence of p27 is similar to loading of integral replication fork proteins such as Mcm10 and distinguishes Dna2 from pre-IC proteins such as Cdc45 (Wohlschlegel et al., 2002). We conclude that pre-RC formation is specifically required for the loading of Dna2 at the beginning of S phase, but Cdk2 activity is not required.

Immunofluorescence was used to track the localization of Dna2 during DNA replication. As sperm chromatin is being replicated in the extract, Dna2 co-localizes with RPA in numerous foci within the single nucleus shown in Fig. 4.3. Co-localization of Dna2 and RPA in the absence of DNA damaging agents suggests that the foci observed are due to DNA replication complexes. Although a small fraction of the RPA foci may represent DNA repair at sites of endogenous damage, the large number of foci we observe in the absence of exogenous DNA damage is most consistent with DNA replication foci.

Dna2 interacts with And-1 and Mcm10. In yeast, Dna2 has been shown to interact

physically with RPA and FEN1. Because of the abundance of replication proteins in egg extracts, *Xenopus* seemed a good system in which to extend our knowledge of the subassemblies of replication proteins with which Dna2 interacts during the vertebrate S phase. We wished to use an unbiased approach to identify such proteins, and therefore Dna2 was immunoprecipitated from *Xenopus* extracts, individual bands larger than 80 kD were isolated from a protein preparative gel, and the proteins in these bands were identified by tandem mass spectrometry. A summary of results is provided in Fig. 4.4A. Unexpectedly, the major DNA replication protein identified was the replication and sister chromatid cohesion protein And-1, the *Xenopus* ortholog of yeast Ctf4 (chromosome transmission fidelity 4), found in the 130-140 kDa band. A variety of additional interesting proteins were identified, including importin beta and nucleoporin 205. Importin beta is likely responsible for the nuclear import of Dna2, and nucleoporin 205 may also affect the nuclear localization of the Dna2 protein. We consider this significant because Loeillet and colleagues have shown synthetic-lethal interactions with nuclear pore proteins and DNA replication and repair proteins (Loeillet et al., 2005). TPR (Translocated Promoter Region) is also a constitutive component of the nuclear pore complex (Byrd et al., 1994). Pcm-1 (pericentriolar material 1) is essential for the radial organization of microtubules and recruitment of proteins to the centrosome (Dammermann and Merdes, 2002; Hames et al., 2005), and is necessary for preventing cells from exiting the cell cycle (Balczon et al., 2002; Srsen et al., 2006).

To rule out that the putative And-1 interaction was due to cross reaction with the Dna2 antibody, rather than to interaction with Dna2, we carried out the reciprocal immunoprecipitation; we immunoprecipitated And-1 from interphase extracts. Western

blotting of the And-1 immunoprecipitate showed that Dna2 co-immunoprecipitates with And-1, confirming that And-1 and Dna2 are in the same complex (Fig. 4.4B). Conversely, And-1 was also sufficiently abundant to be easily detectable in Western blots of Dna2 immunoprecipitates (Fig. 4.4C). Since And-1 is known to interact with the lagging strand DNA polymerase, DNA polymerase α, the And-1 immunoprecipitate was also probed for pol α. Interestingly, pol α was also found in the same immunoprecipitate with Dna2 (Fig. 4.4B). Note that sperm chromatin was not added to these extracts, and therefore And-1 interacts with Dna2 and pol α even in the absence of DNA,

Yeast Ctf4 is the most abundant DNA polymerase α binding protein and a component of the RPC, (Replication Progression Complex, consisting of Mcm2-7, Cdc45, GINS, Mcm10, Mrc1, Tof1-Csm3, and FACT), and therefore it plays an important role at replication forks (Gambus et al., 2006; Gambus et al., 2009; Miles and Formosa, 1992; Tanaka et al., 2009a). Similar interactions have been recently documented in human cells and *Xenopus* extracts (Im et al., 2009; Tanaka et al., 2009b; Xu et al., 2009; Zhu et al., 2007). However, yeast and *Xenopus* Ctf4 also participate in recombination and establishment of sister chromatid cohesion, and the mechanistic contribution of Ctf4 to DNA replication is not yet clear. *Xenopus* Mcm10, a well-studied replication initiation and elongation protein, has been shown to interact with *Xenopus* And-1 and together with And-1 may be required for recruitment of DNA polymerase α and lagging strand DNA replication (Zhu et al., 2007). If Dna2 is interacting with And-1 during DNA replication in *Xenopus* extracts, we would expect to be able to detect complexes containing both Dna2 and Mcm10. As shown in Fig. 4.4D and E, we find that Mcm10 and Dna2 co-immunoprecipitate. Taken together, these interaction studies

suggest that Dna2, And-1, and Mcm10 are present in the same DNA replication complex.

In *Xenopus*, Mcm10 is required for chromatin loading of And-1 in S phase, and And-1 is required for stable loading of pol α (Zhu et al., 2007). We wished to determine if the Dna2 interactions with And-1 and Mcm10 are required for the association of Dna2 with chromatin in S phase. Zhu et al. elegantly demonstrated that addition of anti-And-1 antibodies to the extract block pol α recruitment to chromatin (Zhu et al., 2007). Unfortunately, we were not able to replicate inhibition of pol α loading using And-1 antibody (unpublished data). Instead, we were able to deplete Mcm10 from the extracts (Wohlschlegel et al., 2002), and to ask if Dna2 was still able to associate with chromatin. Interestingly, the association of Dna2 with chromatin does not appear to be dependent on Mcm10 (Fig. 4.4F). Taken together, our results indicate that Dna2 associates with chromatin after pre-RC formation, independently of Cdk2 activation, and independently of Mcm10. This could occur if Dna2 and Mcm10 interact with a common scaffolding protein or common intermediate structure.

Dna2 interacts with DSB response proteins and with DNA ends in both S phase and M phase. Biochemical experiments have recently shown that Dna2 is a major 5′-3′ nuclease in *Xenopus* extract and that depletion of Dna2 results in inhibition of the completion of SSA in *Xenopus* egg extracts (Liao et al., 2008). However, the mechanism of Dna2 participation has not been fully investigated. To further characterize the participation of Dna2 in events at DSBs, we investigated the interaction of Dna2 with other proteins involved in DSB repair and checkpoint pathways in S phase extracts. We speculate that Dna2 acts in the early steps of recombination, so we asked if Dna2 interacts with ATM or the MRN (Mre11/Rad50/Nbs1) complex. As shown in Fig. 4.5A,

Dna2 co-immunoprecipitates both ATM and Nbs1. Thus, we conclude that Dna2 interacts with proteins that participate in the early events of DSB signaling and repair. These interactions are observed in egg extract and thus do not require DNA damage and are not mediated by DNA.

When DSBs are induced, proteins involved in checkpoint signaling and DNA repair accumulate transiently on chromatin (Andegeko et al., 2001; Lee et al., 2003a; Lisby et al., 2004). To address this, PflM1, a restriction enzyme that produces a 3′ overhang, was used to induce DSBs in sperm chromatin in S phase extracts, and the amount of Dna2 on chromatin was analyzed. While some insoluble Dna2 is detected in samples that do not contain DNA, Dna2 levels on PflM1 treated chromatin are clearly increased compared to undamaged chromatin. We also observed further accumulation of Dna2 on chromatin containing DSBs when caffeine or wortmannin, inhibitors of checkpoint kinases, were present (Fig. 4.5B). As expected, we see a corresponding increase in ATM on damaged chromatin in the presence of checkpoint inhibitors. Similar results were seen when using EcoRI, which cleaves to reveal a 5′ overhang, to induce DSBs (unpublished data). Inhibition of the checkpoint may retard the release of Dna2 or may lead to the formation of inactive complexes on chromatin.

Dna2 likely associates with and dissociates from DNA ends to allow for downstream processing events. In yeast and human, ATM and the MRN complex (Mre11/Rad50/Nbs1) are among the first proteins to be detected at a DSB, and RPA is subsequently recruited, presumably through the production of ssDNA by 5′ resection (Budd and Campbell, 2009; Lisby et al., 2004; Shiotani and Zou, 2009; Shroff et al., 2004; Zhu et al., 2008; Zou and Elledge, 2003). ATR is recruited after the generation of

RPA-coated ssDNA, and TopBP1 is involved in the ATM-dependent activation of ATR (Shiotani and Zou, 2009; Yoo et al., 2009). To investigate the likely transient association of Dna2 with DSBs, we compared the kinetics of association of Dna2, MRN, ATM, and other proteins with DNA ends. To look directly at DNA ends, we examined the binding of Dna2 to linear DNA in a manner similar to previous experiments to examine Ku binding to DNA ends (Postow et al., 2008). For these experiments, we biotinylated either 1 or 2 ends of linear pBluescript with a fill-in reaction to produce blunt ends, and bound the biotinylated DNA to streptavidin beads, generating beads with DNA resembling either unbroken DNA (2X, both ends of pBluescript bound to beads) or DNA with a DSB (1X, one free end) (Fig. 4.6A). Protein binding to the DNA ends was then monitored over time in interphase extract, with 3×10^{11} DNA ends/μl of extract (Fig. 6B). Controls show that there is background binding of DSB-response proteins to the "unbroken" (2X biotin) DNA control beads, which we propose derives from incomplete binding of the biotinylated DNA ends to the beads. However, binding to the "broken" DNA beads (1X) is clearly greater than to controls, especially at the earlier time points. With the "broken" DNA beads (1X), MRN and ATM associate at the earliest time points, and Dna2 associates with a slight delay compared to MRN. Dna2 accumulates to peak levels with similar timing to RPA, consistent with the role of Dna2 in producing single-stranded DNA overhangs that may recruit RPA. ATR then accumulates on the RPA-coated DNA ends, consistent with the requirement of RPA-coated ssDNA for the switch from ATM to ATR in human DNA end resection (Shiotani and Zou, 2009). The kinetics of binding and release of Dna2 at the DNA ends is consistent with a role in DSB resection, since Dna2 accumulates after ATM and with similar timing to RPA70. MRX, ortholog of

MRN, appears to dissociate from the DNA ends slightly before Dna2 in yeast as well, consistent with our results (Lisby et al., 2004; Shroff et al., 2004; Zhu et al., 2008).

The role of Dna2 in DSB processing may be cell cycle regulated. Since Dna2 is required in S phase for DNA replication, the role of Dna2 in DSB repair may be limited to S phase, with a main function potentially in the response to collapsed replication forks. To determine the effect of cell cycle stage on association of Dna2 at DNA ends, we performed the same DNA end binding assay in M phase, CSF, extracts (Fig. 4.6C). Under these conditions, Dna2 associates with DNA ends after ATM and Nbs1, and with similar timing to RPA, consistent with results from interphase extract. We also monitored TopBP1, required for activation of ATR, and found that it associated with similar timing to RPA. While the timing of each step varies between interphase and CSF extracts, the general temporal program of binding to DNA ends is consistent. Therefore, we conclude that the role of Dna2 in DNA end resection is not limited to one phase of the cell cycle.

Interplay of nucleases: Dna2 and MRN. To establish that Dna2 participates in DNA end resection/processing in these extracts, we used accumulation of RPA at DNA ends in the bead-based assay as a measure of successful resection/processing of a DSB. We depleted Dna2 from interphase extracts by immunodepletion and monitored RPA accumulation on DNA ends. When Dna2 is lacking, both ATM and Nbs1 can associate with DNA ends. However, RPA does not accumulate on DNA ends to the same level as it does in the presence of Dna2 (Fig. 4.7A), indicating that Dna2 is necessary for efficient DNA end processing, though some residual processing may occur in its absence. In yeast, Dna2 can compensate for the loss of Mre11 nuclease activity, but not for complete

lack of Mre11 protein, in repair of X-ray-induced DNA damage in vivo (Budd and Campbell, 2009). This raises the question of what the additional role of the MRN complex may be. One plausible role for the MRN complex, given the Dna2/Nbs1 interaction shown in Fig. 4.5 and the fact that Dna2 binds duplex ends after Nbs1 as shown in Fig. 4.6, is to recruit Dna2 to breaks. To test this idea, extracts were depleted of Nbs1, which efficiently depletes the MRN complex (Yoo et al., 2009), and association of repair proteins was re-assessed. Dna2 still accumulates on DNA ends in the absence of Nbs1, though there is a reproducibly lower accumulation than in the presence of MRN (Fig. 4.7B). Without MRN, however, RPA does not accumulate on DNA ends. Therefore, although Dna2 is recruited, there is not enough ssDNA generated to recruit RPA. We conclude that MRN is not absolutely required for the recruitment of Dna2 to DNA ends, but is required for DNA end processing and resection.

To investigate if Mre11 nuclease activity is required for resection, Dna2 and RPA recruitment to DNA ends was monitored in the presence and absence of Mre11 nuclease activity. Mirin, a small molecule inhibitor of the Mre11 nuclease, was used to block the nuclease activity of endogenous Mre11 in extracts (Dupre et al., 2008; Garner et al., 2009), and the association of Dna2 and RPA with DNA ends was again assessed. Care was taken to use functionally validated mirin (see Materials and Methods). In the presence of mirin, Dna2 binds DNA ends, although binding is reduced compared to extracts without mirin (Fig. 4.7C). RPA accumulation is retarded but not abolished in the presence of mirin. We propose that RPA accumulation on chromatin is delayed upon inhibition of Mre11 nuclease, implying a partial defect in resection. We conclude that either mirin only partially inhibits Mre11 nuclease, or other nucleases can compensate

when Mre11 nuclease activity is compromised. Dna2 may be one of these nucleases, since it binds to DNA ends in the absence of Mre11 nuclease activity (Fig. 4.7, B and C).

Dna2 is not required for induction or signaling of checkpoints. In the DSB checkpoint, the ATM kinase is first activated, and active ATM subsequently activates the ATR kinase (Yoo et al., 2007). Recognition of RPA-ssDNA complexes is thought to be part of the ATR-activation process (Shiotani and Zou, 2009; Zou and Elledge, 2003). Since Dna2 is involved in resection to produce ssDNA, we asked if Dna2 is also involved in activation or signaling of the DSB checkpoint. To do this, we depleted Dna2, added known checkpoint inducers, and monitored phosphorylation of Chk1, an effector kinase and target of ATR, and Chk2, an effector kinase and target of ATM, as indicators of checkpoint activation and signaling. First, the checkpoint was induced with pA/T70 oligonucleotides, which activate both ATM and ATR, resulting in phosphorylation of Chk1. In Dna2-depleted extract with added pA/T70 oligonucleotides, Chk1 was efficiently phosphorylated (Fig. 4.8A). Since the pA/T70 oligonucleotides can form a variety of structures, we also examined the checkpoint response to linear DNA in the Dna2-depleted extract. Linear pBluescript was added to extract to activate the DSB checkpoint response, and we see that in the absence of Dna2, Chk2 is well phosphorylated in response to linear DNA (Fig. 4.8B). The weaker signal for phospho-Chk2 is due to a weaker interaction with the antibody in the immunoblot (Guo and Dunphy, 2000). To eliminate the possibility that Dna2 has a specific role in the checkpoint response that was overcome in these assays with synthetic checkpoint activators, we studied the activation of checkpoints in nuclei during S phase. We observed that nuclear Chk1 was also phosphorylated in the presence of stalled replication

forks induced by aphidicolin and in the presence of DSBs induced by PflM1, regardless of the presence of Dna2 (Fig. 4.8C). We conclude that the Dna2 protein itself is not necessary for checkpoint signaling. We have shown that Dna2 plays a role in 5′-3′ resection, as measured by RPA recruitment; activation of checkpoints in the absence of Dna2 implies that another nuclease(s) can compensate for the lack of Dna2 in producing sufficient single-stranded DNA to activate ATR.

DISCUSSION

Dna2 is a DNA replication protein in vertebrates. In this work, we present evidence supporting our previous results suggesting that *Xenopus* Dna2 participates in chromosomal DNA replication. *Xenopus* Dna2 is recruited to chromatin in a regulated manner and binds chromatin with similar timing to other DNA replication proteins. Efficient Dna2 recruitment to chromatin requires formation of the pre-RC and origin licensing, as Dna2 is not efficiently recruited to chromatin in the absence of the MCM replicative helicase or in the presence of geminin. Dna2 appears to participate in the elongation of replicating DNA; Dna2 and RPA co-localize during replication, consistent with Dna2 being present at DNA replication forks and traveling with forks throughout DNA replication. Interestingly, And-1 and Mcm10, components of the replication fork, associate with Dna2 in interphase extracts, indicating that Dna2, like And-1 and Mcm10, may function on the lagging strand (Pacek et al., 2006; Yoshizawa-Sugata and Masai, 2009; Zhu et al., 2007).

Although formation of the pre-RC is a pre-requisite for *Xenopus* Dna2 binding to chromatin in S phase, Dna2 binding does not require activation of the pre-RC by Cdk2 activity, as is also the case for the Mcm10 protein (Figs. 4.1 and 4.3). Like Dna2, Mcm10 binds chromatin after the MCM2-7 helicase complex and independently of Cdk2 activity (Wohlschlegel et al., 2002). Mcm10 is, in turn, required for the binding of Cdc45, which allows unwinding of the origin of replication. Despite their similar requirements for chromatin binding, the association of Dna2 with chromatin is independent of Mcm10. Therefore, Dna2 associates with chromatin early in the formation of the replication fork, after the MCM helicase complex but independent of the binding of Mcm10 and Cdc45. These findings may indicate that *Xenopus* Mcm10 and Dna2 interact with a similar intermediate in the formation of the replisome. Since this stage of replication, the conversion of the pre-RC into an active replication fork, is poorly understood in eukaryotic DNA replication, the exact role of these proteins in the transition is difficult to predict at the moment.

Dna2 is likely involved in lagging strand replication. The interaction of Dna2 with And-1 and Mcm10 correlate with genetic interactions seen in yeast, but for which no underlying mechanism had been discovered. Ctf4, the yeast ortholog of human And-1, is the most abundant DNA polymerase α-interacting protein in yeast, and *dna2-2* shows synthetic lethality with *ctf4Δ* (Formosa and Nittis, 1999; Miles and Formosa, 1992). Additionally, yeast *dna2* is synthetically lethal with *mcm10*, and the same *mcm10* mutant is synthetically lethal with both *dna2* and *ctf4* (Araki et al., 2003; Budd et al., 2005). The presence of these three proteins in the same complex could account for the observed synthetic lethality, since mutation of either protein might destabilize complexes

containing them. The direct protein-protein contacts in the putative complex, if any, remain to be determined, however.

The accepted role of Dna2 in yeast DNA replication is to assist the major Okazaki fragment processing nuclease, Fen1, in removal of RNA/DNA primers on the lagging strand (Budd et al., 2009; Burgers, 2009). The best single piece of evidence for this is that *DNA2* is an essential gene, yet deletion of *DNA2* can be complemented by overproduction of *FEN1* (Budd and Campbell, 2000). Numerous biochemical and genetic interactions support this model (Budd et al., 2005; Stewart et al., 2008). In both yeast and *Xenopus*, And-1, Mcm10, and DNA polymerase α are all implicated in replication of the lagging strand. DNA polymerase α is necessary for RNA/DNA primer synthesis, and Mcm10 is responsible for preventing premature degradation of DNA polymerase α in both yeast and human cells (Chattopadhyay and Bielinsky, 2007; Ricke and Bielinsky, 2004). Yeast Ctf4, Mcm10, and DNA polymerase α are part the replication progression complex along with the MCM helicase, and it has been proposed that Ctf4 and Mcm10 serve to couple the lagging strand polymerase with the replicative MCM helicase (Ricke and Bielinsky, 2004; Tanaka et al., 2009a; Zhu et al., 2007). The occurrence of these proteins in complexes that also contain Dna2 is both consistent with the idea that Dna2 is involved in lagging strand events in *Xenopus* and, in turn, supports the previous findings suggesting lagging strand roles Mcm10 and And-1.

It has been claimed that in human cells, Dna2 is solely a mitochondrial protein (Zheng et al., 2008). While other work has revealed that human Dna2 does reside in both nuclei and mitochondria (Duxin et al., 2009), the role of human Dna2 in nuclei has yet to

be thoroughly studied. Our results show that *Xenopus* Dna2 clearly participates in nuclear genomic DNA replication, and the protein-protein interactions demonstrated here with And-1 and Mcm10 indicate an important role for nuclear Dna2. It is likely that these mechanisms are conserved in human cells, where depletion of Dna2 leads to cell cycle delay in G2 and aberrant nuclear division (Duxin et al., 2009).

Dna2 in DSB Repair. In addition to its role during lagging strand DNA replication, yeast Dna2 has been shown to play a major role in 5′ to 3′ resection during the early steps of DSB repair during G2 phase of the cell cycle (Budd and Campbell, 2009; Zhu et al., 2008). Previous evidence for a similar role in *Xenopus* is also strong (Liao et al., 2008). Our new findings add to this evidence. We show that Dna2 physically interacts with ATM and Nbs1 (Fig. 4.5A), which are both recruited to and accumulate at DSBs. We found that Dna2 hyper-loads on chromatin containing induced DSBs, similar to the hyper-loading of pol α seen on chromatin in aphidicolin-inhibited replicating chromatin(Yan and Michael, 2009). In our studies, Dna2 accumulates to an even greater extent on DNA ends when checkpoint kinase inhibitors such as caffeine and wortmannin are present. This may be due to either retention of DSB processing proteins on chromatin or the generation of non-functional DNA replication and repair complexes on chromatin.

DSB repair and checkpoint proteins associate with and dissociate from DSBs in a specific temporal order (Shiotani and Zou, 2009). Our finding that Dna2 accumulates slightly after ATM and Nbs1, that Dna2 peak levels of binding occur after ATM and Nbs1 have already begun to dissociate, and that RPA accumulates with similar timing to Dna2 suggests that resection may be initiated by MRN but continues due to the activity

of Dna2 nuclease (Liao et al., 2008). Like MRN, Dna2 also binds transiently, presumably allowing for the downstream strand invasion events, though we did not study those events here. These kinetics are similar to the ordered binding and dissociation of MRN and Dna2 that is observed in *S. cerevisiae* (Lisby et al., 2004; Shroff et al., 2004; Zhu et al., 2008). We also show that these events occur in both S phase and M phase. These data place Dna2 early in the timeline of the double-strand break response, and we speculate that the nuclease activity of Dna2 participates in DSB resection.

Resection of DSBs in yeast involves both Dna2 and the MRX complex. MRX appears to initiate strand displacement and Dna2 further degrades the 5′ strand, revealing an elongated 3′ ssDNA strand to be used for strand exchange (Zhu et al., 2008). The MRX complex itself must be present for resection, but resection still occurs with a complex containing nuclease-dead Mre11 (Budd and Campbell, 2009; Llorente and Symington, 2004). The ability to bypass the requirement for the Mre11 nuclease activity relies on compensation by Dna2 for the nuclease-dead Mre11. However, Dna2 can not compensate for the complete absence of the Mre11 protein (Budd and Campbell, 2009; Zhu et al., 2008). The non-nucleolytic role of Mre11 is matter of interest. One possible explanation is that another protein, such as Ku, may compete in the resection reaction in the absence of MRN (Wasko et al., 2009). Another possibility is that the MRN protein complex is required at DSBs to recruit additional proteins necessary for DNA end resection, or perhaps the real requirement for successful DNA end resection has more to do with Rad50 or Nbs1 in the MRN complex, as opposed to the Mre11 nuclease activity. The MRN complex, regardless of Mre11 nuclease activity, may be necessary to process the DNA and create a substrate for Dna2. The *Xenopus* extract system used here allows

us to begin to discriminate among such possibilities. We found that the MRN complex was not necessary for recruitment of Dna2, but even though Dna2 was recruited to DNA ends, resection was not efficient. Further study is warranted, however, since the level of Dna2 on DNA ends was reproducibly lower in the absence of MRN.

Mirin is an inhibitor of the Mre11 nuclease that does not prevent the binding of MRN to a DSB (Dupre et al., 2008; Garner et al., 2009). Thus, mirin can be used to distinguish whether it is the presence of the MRN complex or the Mre11 nuclease activity that is required for bound Dna2 to create a substrate for RPA. Mirin, as expected, does not inhibit the recruitment of Dna2 to the DSB. Unlike the MRN depletion, however, RPA did accumulate at the DNA ends, although with a significant delay. We speculate this delayed RPA accumulation is due to other nucleases, possibly Dna2, compensating for the lack of Mre11 nuclease activity, as this appears to happen in yeast. Alternatively, we cannot rule out that mirin may not fully inhibit Mre11 nuclease activity, and we are detecting residual activity.

DNA damage checkpoint activation. The DSB checkpoint first activates the ATM kinase, which subsequently activates the ATR kinase (Shiotani and Zou, 2009; Yoo et al., 2007). A possible role for Dna2 in checkpoint activation and signaling was assessed by monitoring phosphorylation and activation of Chk1 and Chk2, downstream targets of the DNA damage checkpoint pathway, in the absence of Dna2. When checkpoint inducers, pA/T70 oligonucleotides and linear DNA, are added to Dna2-depleted extracts, Chk1 and Chk2 are well phosphorylated. We also observed checkpoint activation in the absence of Dna2 in nuclei during DNA replication stress, i.e., in the presence of stalled replication forks induced by aphidicolin, or DSBs induced by the addition of PflM1 restriction

endonuclease (Fig. 4.8). Therefore, neither the Dna2 protein itself nor the enzymatic activities of Dna2 appear to be necessary for the checkpoint response, indicating that the role of Dna2 in replication fidelity does not rest with activation of checkpoints, but with allowing efficient DNA replication and repair of damaged DNA. We speculate that another nuclease may compensate for the lack of Dna2, so ssDNA will still be generated at DSBs and the checkpoint will be functional. Redundancy in resection is consistent with the observation that processing is not completely defective in the SSA assay in *Xenopus* nuclear extracts (Liao et al., 2008). We observed minimal RPA binding to DNA ends in Dna2-depleted extracts in our bead-based assay (Fig. 4.7A), but limitations of this assay restrict its usage for early time-points. However, the checkpoint assays in which aphidicolin or PflM1 is added to induce checkpoint activation observe a much later time-point (100 min). Compensating nucleases may be slower than Dna2 to resect the DSB, but 100 min. may be sufficient for compensation. It is also possible that compensating nucleases are more concentrated in nuclei than in interphase extract, allowing a more efficient nuclease compensation than in interphase extract. A likely candidate nuclease is the homolog of yeast Exo1, and it will be valuable to test *Xenopus* Exo1.

In conclusion, our studies have used biochemistry, depletion, and protein-protein interaction studies to probe the physiological roles of Dna2. This study is the first to show that *Xenopus* Dna2 is a helicase/nuclease, that And-1 and Mcm10 interact with Dna2, and that there is an interplay between Dna2 and MRN at DNA ends resembling double-strand breaks. At this stage of our studies, we find many similarities between yeast Dna2 and vertebrate Dna2. While future detailed studies can be expected to reveal differences between metazoan and yeast Dna2, given divergent regulatory sequences, the

general roles of Dna2 in DNA appear to be evolutionarily conserved.

MATERIALS AND METHODS

Helicase assay. Helicase activity of recombinant Dna2 was measured using the nuclease-deficient mutant of Dna2 (human Dna2 D294A or *Xenopus* Dna2 D278A) in a 20 μl standard reaction mixture containing 50 mM Tris-HCl (pH 7.5), 25 mM NaCl, 2 mM DTT, 0.25 mg/ml bovine serum albumin, 4 mM $MgCl_2$, 4 mM ATP and ^{32}P-labeled helicase substrate. After incubation at 37°C for 1 h, reactions were stopped with 5x stop solution, which consisted of 60 mM EDTA, 40% sucrose, 0.6% SDS, 0.25% bromophenol blue and 0.25% xylene cyanole FF. Reaction products were then separated using 8% native polyacrylamide gels containing 0.1% SDS and detected using a Storm 860 PhosphorImager.

Xenopus egg extracts. *Xenopus* cell-free extract was prepared as described previously (Murray, 1991). To elicit a checkpoint response, extracts were treated with either 50 μg/ml pA/T70 oligonucleotides or 25 μg/ml linear pBluescript (Guo and Dunphy, 2000; Kumagai and Dunphy, 2000). For reactions involving nuclei, demembranated sperm chromatin was incubated at 3,000 sperm/μl in extract for 100 min. Inhibitors (5 mM caffeine, 0.1 mM wortmannin, 0.3 mM geminin, 0.1 mM p27) were incubated in extract for 20 min on ice before addition of sperm chromatin. Double-strand breaks were

induced by addition of 0.1 units/μl PflM1. Nuclei and chromatin were isolated as described (Lee et al., 2003a). Chromatin isolation in Mcm10-depleted extracts was performed as previously described (Zhu et al., 2007).

Antibodies and recombinant proteins. Anti-Dna2 antibodies were affinity-purified with the N-terminal 712 aa of Dna2 as previously described (Liu et al., 2000). Antibodies recognizing DNA polymerase α p70 subunit, RPA70, Cdc45, Claspin, Orc2, ATM, Nbs1, Chk2, ATR, and TopBP1 were previously described (Guo and Dunphy, 2000; Kumagai et al., 2006; Lee et al., 2003a; Yoo et al., 2009; Yoo et al., 2004). Anti-human BM28 monoclonal antibody, which recognizes *Xenopus* Mcm2, was purchased from Cell Signaling Technology (Beverly, MA), and control rabbit IgG was purchased from Zymed Laboratories (South San Francisco, CA). Anti-And-1 antibodies were a gift of A. Dutta (Univ. of Virginia), anti-Mcm10 antibodies were a gift of J. Walter (Harvard Medical School), and anti-Mcm3, anti-Cdc6, and anti-RPA70 antibodies used for immunofluorescence were a gift of P. Jackson (Genentech). Production of recombinant *Xenopus* Dna2 is described in Liu et al, 2000 (Liu et al., 2000). ^{35}S-Labeled Chk1 was generated using the TnT system (Promega, Madison, WI).

Immunological methods. For immunoprecipitations, 2.5 μg antibodies were pre-incubated with 5 μl Protein A Support (BioRad) and subsequently incubated with 50 μl interphase extract for 1hr at 4°C. Beads were washed 4 times with 10 mM HEPES-KOH [pH 7.6], 150 mM NaCl, 0.1% CHAPS, 2.5 mM EGTA and analyzed by SDS-PAGE.

Mcm3 was depleted with 30 μl of antibodies per 100 μl extract, using 2 rounds of depletion that were 45 minutes each. Immunofluorescence on sperm nuclei was performed as described, using 30 μl anti-RPA70 antibodies raised in chicken and 2.5 μl anti-Dna2 antibodies raised in rabbit per sample (Carpenter et al., 1996). Dna2 and Nbs1 depletions were performed as described (Liu et al., 2000; Yoo et al., 2009).

Mass Spectrometry. Dna2 interphase IPs were performed as described above but from 400 μl extract, subjected to SDS-PAGE and stained with Coomassie Blue. Bands were excised, an in-gel trypsin digest was performed, peptides were extracted and subjected to electrospray ionization tandem mass spectrometry, and samples were identified with the *Xenopus* Mascot Search database. Hits with an ion score >500 were used for analysis. Mass spectrometry work was done by Sonja Hess at the Proteome Exploration Laboratory at Caltech.

DNA end binding experiments. DNA binding experiments were modified from previously published assays using DNA immobilized on magnetic beads (Nishiyama et al., 2006; Postow et al., 2008). Briefly, pBluescript II KS– was linearized using either NotI (for biotinylation of both ends) or NotI and EcoRI (for biotinylation of one end). Klenow was used for fill-in reactions in the presence of biotin-[C^{14}]dCTP. The 2.9 kb DNA fragments were then purified and bound to M-280 Streptavidin Dynabeads (Invitrogen, Carlsbad, CA) at a concentration of 0.5 μg DNA/5 μg beads, following the manufacturer's protocol. Beads were incubated in extract for the indicated times, washed

2 times with 5 volumes of 20 mM HEPES-KOH [pH 7.6], 80 mM KCl, 2.5 mM K-gluconate, 10 mM Mg-gluconate, 1% NP-40, and 1 mM DTT, and subjected to SDS-PAGE and immunoblotting. For experiments involving mirin, 100 μM mirin was added to extracts. Experiments were conducted using both validated mirin that was a kind gift of Dr. Alan Eastman and mirin purchased from Enzo Life Sciences (Plymouth Meeting, PA). The two mirin preparations yielded similar results, and were thus determined equivalent

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FIGURES

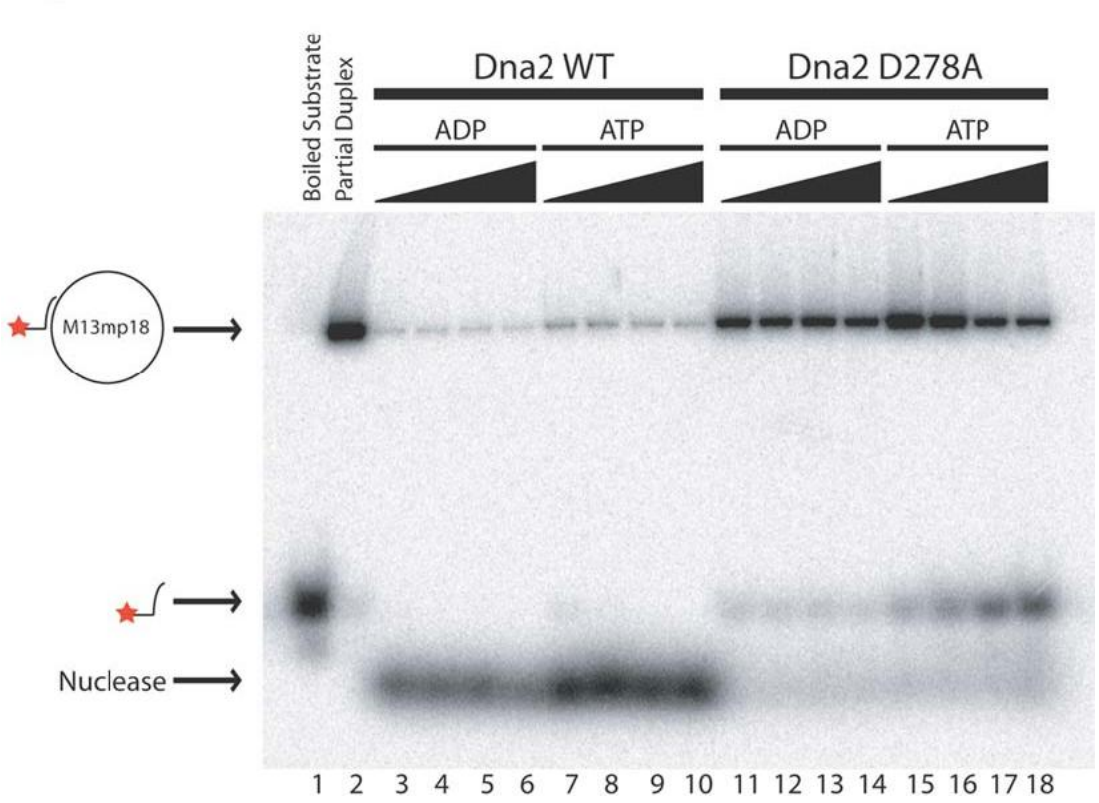

Figure 4.1 Helicase activity of *Xenopus* Dna2. Wild-type *Xenopus* Dna2 (lanes 3 to 10) and nuclease deficient *Xenopus* Dna2 D278A (lanes 11 to 18) were incubated in helicase assay conditions with approximately 1 fmol of annealed M13-HPR substrate at 37°C for 15 min, either in the presence of ADP (lanes 3 to 6 and 11 to 14) or ATP (lanes 7 to 10 and 15 to 18). Solid triangles represent increasing amount of Dna2 protein: 43 (lanes 3 and 7), 86 (lanes 4 and 8), 172 (lanes 5 and 9), and 344 fmol (lanes 6 and 10) of wild-type *Xenopus* Dna2 and 43 (lanes 3 and 7), 86 (lanes 4 and 8), 172 (lanes 5 and 9), and 344 fmol (lanes 6 and 10) of *Xenopus* Dna2 D278A were used. No protein was added in lanes 1 and 2. The reaction in lane 1 was boiled for 4 min. All products were separated using native gel electrophoresis and detected by autoradiography. Positions of the substrate, helicase products, and nuclease products are indicated on the left of the figure.

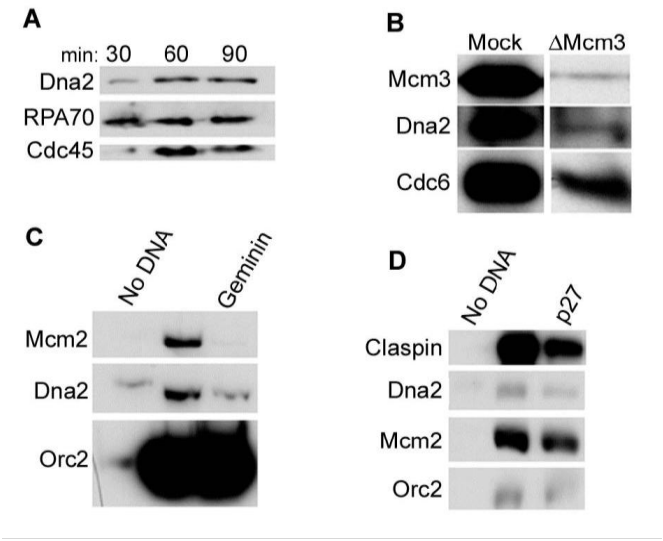

Figure 4.2 Dna2 associates with S-phase chromatin. A) Dna2 accumulation on sperm chromatin throughout S phase. Sperm chromatin was incubated in cell-free extract at 3,000 sperm/μl, isolated at indicated time-points throughout DNA replication as previously described (Lee et al., 2003a) and analyzed by immunblotting. DNA replication begins at 30 min; 60 min is representative of mid-S phase; and DNA is fully replicated by 90 min. Cdc45 and RPA both associate with replicating chromatin. B) Dna2 accumulation on chromatin requires the MCM helicase complex. Extracts were mock-depleted or Mcm3-depleted, preventing formation of the MCM helicase complex, and sperm chromatin was incubated in these extracts. Chromatin was then isolated as described (Lee et al., 2003a), and protein association with chromatin was assayed by immunoblotting. C) Pre-RC formation is necessary for Dna2 binding to chromatin. Sperm chromatin was incubated without or with 300 nM geminin, which prevents formation of the pre-RC, in extract for 100 min., and a sample containing no sperm chromatin was used as a negative control. Chromatin was then isolated and analyzed by immunoblotting as described (Lee et al., 2003a). D) Pre-IC formation is not required for Dna2 binding to chromatin. Chromatin was incubated in untreated extracts or extracts

containing 0.1 mM p27, an inhibitor of pre-IC formation, for 100 min. The negative control was a sample containing no chromatin. Chromatin fractions were isolated and analyzed by immunoblotting as described (Lee et al., 2003a).

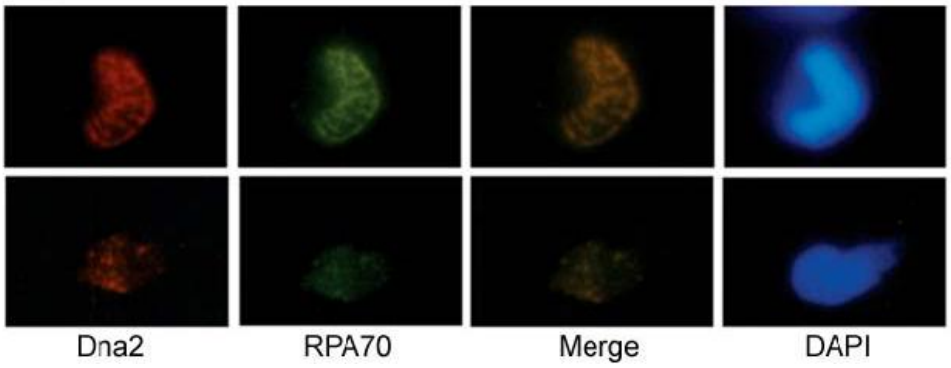

Figure 4.3 Dna2 in DNA replication. Dna2 foci during DNA replication. Sperm chromatin was incubated in extract, fixed, centrifuged onto coverslips, and subjected to immunofluorescence as previously described (Carpenter et al., 1996). Antibodies for immunfluorescence recognized Dna2 and RPA, and DNA was stained with DAPI.

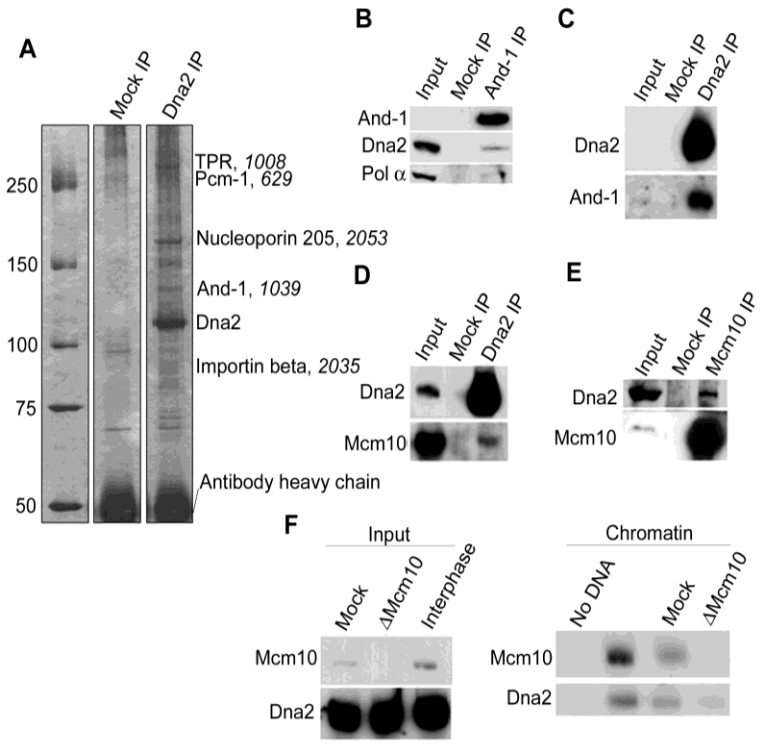

Figure 4.4 Dna2 interacts with DNA replication fork proteins. A) Silver-stained gel of a Dna2 immunoprecipitation from interphase extract. Results from electrospray ionization tandem mass spectrometry analysis of Dna2 immunoprecipitates are labeled, with the score from mass spectrometry analysis in italics. Only hits with an ion score above 500 are listed. B) Immunoprecipitations from interphase extract were performed with control or anti-And-1 antibodies, and immunoprecipitates were analyzed by immunoblotting. C) Control IgG (Mock) and anti-Dna2 antibodies were used for immunoprecipitations from interphase extracts, and samples were analyzed by immunoblotting. D) Dna2 was immunoprecipitated in interphase extract using anti-Dna2 antibodies, and isolates were analyzed by immunoblotting. E) Control and anti-Mcm10 antibodies were used to immunoprecipitate proteins from interphase extract. Reactions were analyzed by immunoblotting. F) The ability of Dna2 to bind chromatin was assessed in the presence

and absence of Mcm10. Interphase extracts were mock or Mcm10-depleted; 0.5 μl of this extract was analyzed by immunoblotting to confirm depletion of Mcm10. Sperm chromatin was added to the mock or Mcm10-depleted extracts, incubated for 100 min., chromatin fractions were isolated as previously described (Zhu et al., 2007), and chromatin-associated proteins were analyzed by immunoblotting.

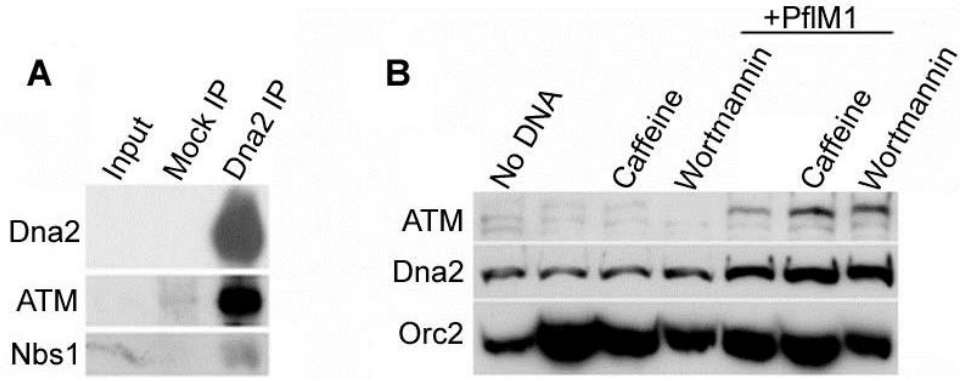

Figure 4.5 Dna2 and double-strand breaks. A) Dna2 interacts with DSB proteins. Anti-Dna2 antibodies were used to immunoprecipitate Dna2 from interphase extract, and immunblots were performed for Dna2, ATM, and Nbs1. B) Dna2 accumulates on damaged chromatin. Sperm chromatin was added to interphase extracts in the presence or absence of 0.1 units/μl PflM1 and/or chekpoint inhibitors (5 mM caffeine or 0.1mM wortmannin) as indicated. After a 100 min. incubation, chromatin fractions were isolated from extract as previously described (Lee et al., 2003a) and protein levels on chromatin were analyzed by immunoblotting.

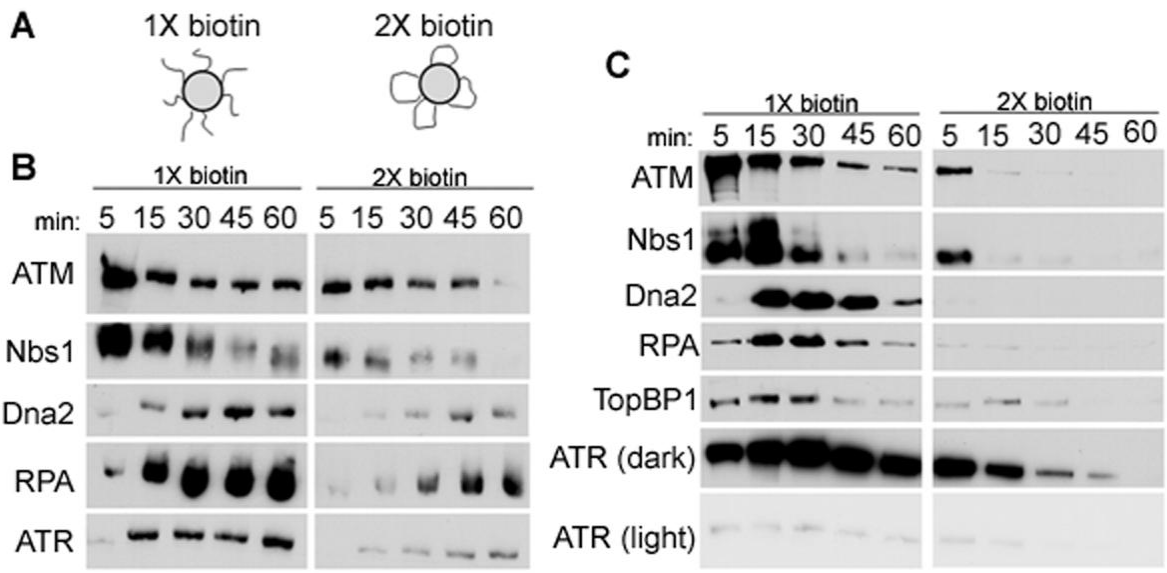

Figure 4.6 Dna2 at DNA ends. A) Schematic of beads used for experiments. pBluescriptIIKS- was linearized and biotinylated on one or both ends, and bound to streptavidin beads. These beads simulated unbroken DNA or DNA with a DSB. B) Time-course of binding of DSB repair and checkpoint proteins to DNA ends. Beads were incubated in interphase extract, isolated at indicated time-points, and the relative amounts of Dna2, ATM, Nbs1, RPA70, and ATR bound to the beads were analyzed by immunoblotting. C) Time-course of binding of DSB proteins to DNA ends in CSF extract. Experiment was performed as described for panel B of this figure, except in CSF, not interphase, extract.

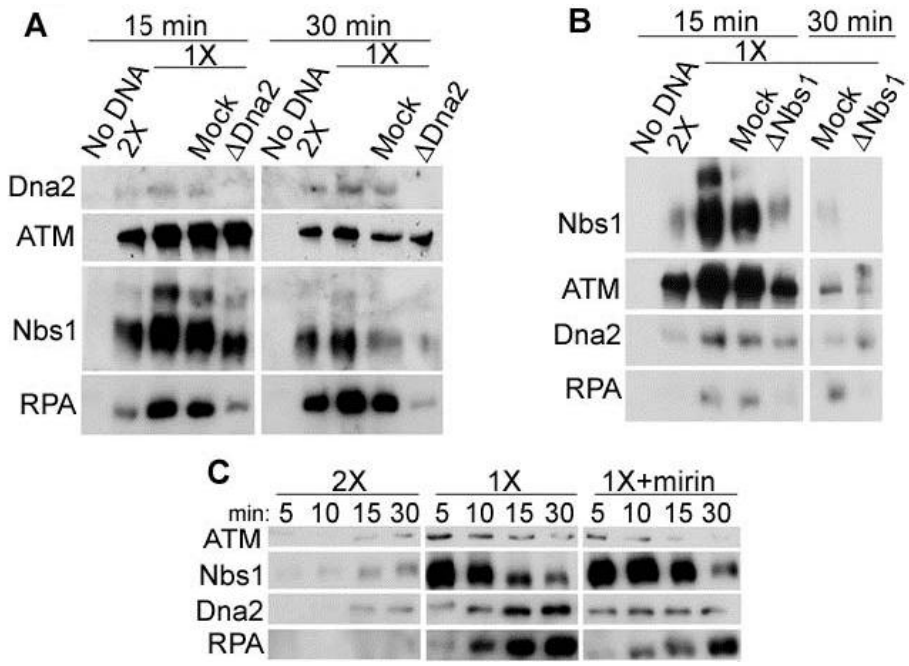

Figure 4.7 Dna2 and MRN at DNA ends. A) Effect of Dna2 depletion on processing of DNA ends. Interphase extracts were untreated, mock, or Dna2-depleted, and incubated with the appropriate beads for 15 or 30 min. Beads were isolated and protein binding was assessed by immunoblotting. B) DNA end binding of proteins in Nbs1-depleted extract. Extracts were untreated, mock-depleted, or Nbs1-depleted, which depletes the whole MRN complex, and incubated with the appropriate beads for 15 or 30 min. Beads were isolated, and protein binding to the beads was analyzed by immunoblotting. C) Mirin was used to inhibit the nuclease activity of Mre11. Mirin or DMSO was incubated in extracts with the appropriate beads. Beads were isolated at the indicated times and protein levels were analyzed by immunoblotting.

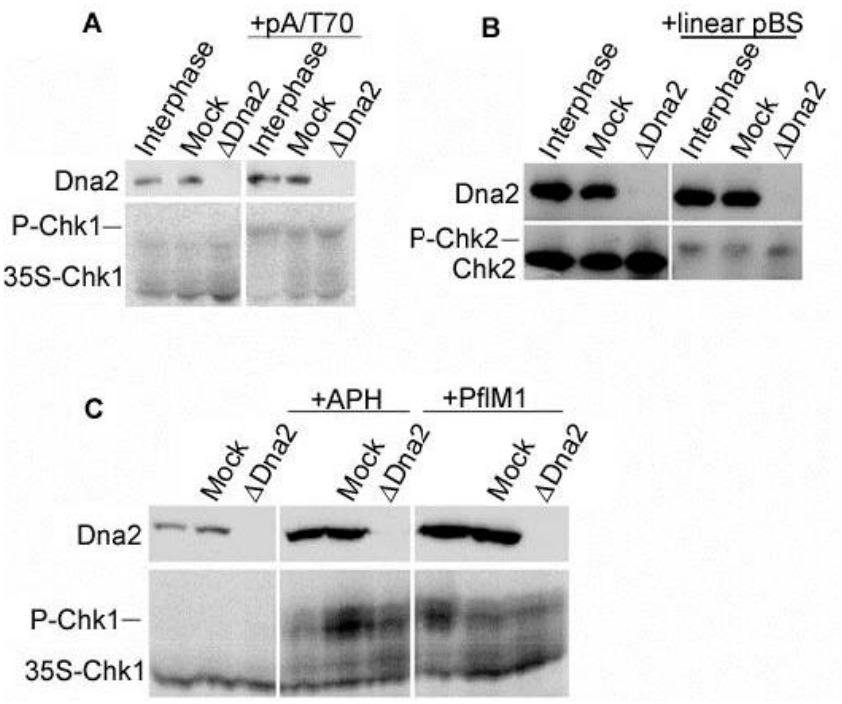

Figure 4.8 Assessment of the DNA replication checkpoint in Dna2-depleted extracts. For all panels in this figure, interphase extracts were untreated, mock, or Dna2-depleted. A) Phospho-Chk1 in Dna2-depleted extracts. pA/T70 oligos were added to interphase extract to elicit a checkpoint response, as previously described (Kumagai and Dunphy, 2000). The electrophoretic mobility of ^{35}S-Chk1 was monitored by autoradiography after a 100 min incubation in extract containing pA/T70, and ^{35}S -Chk1 is well phosphorylated, as indicated by the arrow. B) Phospho-Chk2 in extracts lacking Dna2. Linear pBluescript was added to extract to elicit the DSB checkpoint response, as previously described (Guo and Dunphy, 2000), and immunoblotting was used to assess activation of the checkpoint by monitoring Chk2 phosphorylation after 100 min in extract containing linear pBluescript. C) The checkpoint response to stalled replication forks and DSBs was assessed in nuclei using aphidicolin (APH) and PflM1, respectively. Sperm chromatin was incubated in extracts for 100 minutes without or with APH, or

without or with PflM1 to induce replication fork stalling or DSBs, respectively. Nuclei were isolated as previously described(Lee et al., 2003a). Dna2 levels in nuclei were assessed by immunoblotting, while ^{35}S -Chk1 electrophoretic mobility was assessed by SDS-PAGE and autoradiography.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

In these studies, we explored mechanisms that preserve genomic stability through the enzymatic functions and protein interactions of the DNA replication and DNA repair enzyme Dna2. We identified a novel ATP and Mn^{2+} dependent flap endo/exonuclease activity conserved from yeast to human Dna2 proteins. This observation is important for understanding the activity of Dna2 together with the DSB repair nuclease Mre11 *in vitro* which requires Mn^{2+}. The presence of a novel ATP-dependent nuclease activity is also of note due to the structural similarity of Dna2 to bacterial enzymes RecBCD and AddAB (Budd et al., 2000; Yeeles et al., 2009). Both of these helicase-nuclease enzyme complexes are involved in recombinational repair and possess ATP-dependent nuclease activity (Kooistra et al., 1997; Sun et al., 2006).

Detailed biochemical analysis of the endonuclease activity of Dna2 showed that, in addition to the well characterized helicase and flap endo/exonuclease activities which require unblocked 5' single-stranded DNA tails in order cleave or unwind, Dna2 can cut ssDNA lacking ends and use the resulting DNA ends as a substrate for further enzymatic processing. This endonuclease function is inhibited by the addition of ATP and by the presence of RPA protein bound to the ssDNA. Through these studies we also discovered a new activity, the disruption of partial duplex DNA, by Dna2 and RPA acting in concert.

This insight highlights the importance of studying the enzymes involved in DNA replication and DNA repair in the context of multi-protein complexes working in concert and not in isolation.

To this end, we investigated post-translational modification, specifically phosphorylation, of Dna2 by kinases involved in genomic stability. λ phosphatase treatment of Dna2 purified from yeast cells resulted in the inhibition of Dna2 ATPase and nuclease activity *in vitro*. We, in addition to other laboratories, find that Dna2 is phosphorylated by Cdk1 (Holt et al., 2009; Kosugi et al., 2009; Smolka et al., 2007). Further, we found that Dna2 was phosphorylated *in vitro* and *in vivo* by Mec1, the PIKK DNA damage sensor kinase. Phosphorylation at serine 287 by Mec1 was detected after induction of double strand breaks. While this phosphorylation event was dispensable to survival of double strand breaks, the Dna2 protein is important for the survival of DNA damage.

The relationship between Dna2 and the DNA damage sensor kinases is not limited to yeast cells. Using the *Xenopus* cell free extract system, a physical interaction between Dna2 and ATM, a member of the PIKK family, was detected (Wawrousek et al., 2010). Using *Xenopus* cell free extracts and linear DNA bound to beads simulating DSBs, we constructed a timeline of protein interactions and processing steps needed for the successful repair of double strand breaks. In *Xenopus* extracts, Dna2 is required for efficient resection of DSBs to create ssDNA, a necessary step in homologous recombination. This system also provided clear evidence for Dna2 at replication forks. Dna2 associates with S phase chromatin after pre-RC formation but regardless of pre-IC formation, and the Dna2 protein physically interacts with proteins involved in lagging

strand replication. The physical interaction of *Xenopus* Dna2 with And-1 shows that the genetic interaction between its yeast ortholog Cft4 and yeast Dna2 is relevant to higher eukaryotes (Formosa and Nittis, 1999).

Recent studies with both human and yeast proteins have extend this approach of studying genomic stability by reconstituting DSB repair processing with combinations of purified proteins, including Dna2 (Cejka et al., 2010; Nimonkar et al., 2011; Niu et al., 2010). Future studies will continue this approach and move beyond the individual DNA replication and repair steps and focus on the regulation and interactions that make the process both efficient and accurate.

One area of particular interest is the physical interaction between Dna2 and RecQ helicases BLM and Sgs1. This physical interaction has not been mapped in relation to the domains of the protein. Also of interest are the circumstances that control the interaction. Clearly, the two proteins are not in a stable complex at all times. When and where the two proteins come together in relation to DNA damage may elucidate other signaling steps in the DNA damage response.

While it has been known for some time that RPA and Dna2 physically interact, this protein interaction has not been well characterized (Bae et al., 2003). RPA controls many facets of the DNA damage response including activating checkpoints and ensuring the proper strand is resected for homologous recombination. RPA modulates the endonuclease, 5' flap endo/exonuclease, and 3' exonuclease of Dna2 *in vitro*, and some RPA mutants genetically interact with Dna2 (Bae and Seo, 2000; Bae et al., 2001; Masuda-Sasa et al., 2008). As a target of post-translational modification, RPA is a

dynamic protein that has many possible modes of interacting with Dna2. The parameters for the complex regulation of Dna2 activity by RPA remains on open question.

One significant and exciting avenue of investigation for the future is the use of single molecule studies (Bustamante et al., 2000). While the biochemical assays described in this study show differences in total product processed in a given time frame using a mix of proteins, single molecule studies can unlock the movement of single Dna2 enzymes along various DNA replication or repair intermediates. The interaction of Dna2 with DNA structures is of great interest due to the observation that Dna2 can bind to ss/dsDNA junctions, but cannot cleave them, if the 5' end of the ssDNA is blocked (Stewart et al., 2010). How the interaction or stimulation with the DNA end works on the Dna2 protein would be quite mechanistically enlightening. These types of studies could also show whether increased Dna2 activity under certain circumstances correlates with faster translocation along DNA, more stable associations with DNA, or higher affinity for individual substrate molecules. These experiments can also be extended to include Sgs1, FEN1, and especially RPA to asses Dna2 action on both DNA repair and DNA replication intermediates in the context of companion proteins.

The contributions of the nuclease function of Dna2 to genomic stability have been characterized in ever greater detail recently. However, Dna2 possesses several other enzymatic activities *in vitro* that have not be investigated in the context of genomic stability of cells. One such function is the ability to process G4 DNA (Masuda-Sasa et al., 2008). G4 DNA is controversial, but could potentially arise in telomeres and rDNA, locations that Dna2 is known to contribute to genomic stability (Maizels, 2006). Other functions of Dna2 that has not been extensively analyzed *in vivo* are single-strand

annealing and strand exchange activities (Masuda-Sasa et al., 2006). A potential place for this function is in the later steps of homologous recombination in resolving Holliday junctions and other recombination intermediates. Two genes identified to participate in this stage of HR in yeast are *SGS1*, the DSB resection partner of Dna2, and *YEN1*, a gene synthetically sick with *dna2-2* (Mimitou and Symington, 2009).

Many of the important functions of Dna2 were first discovered in the yeast model system, but these activities are proving to be well conserved in the human Dna2 protein. As a member of the DSB response, Dna2 function is important to avoiding chromosomal instability and thus cancer. By building on this knowledge of Dna2 activity *in vitro*, potential intervention points in the DNA damage response for cancer drug development can be identified. Further characterization of Dna2 acting in human cells is needed to determine what other protein complexes and pathways Dna2 participates in as human cells have even more elaborate networks to respond to DNA damage, but techniques such as immunofluorescence and RNA interference make it possible to visualize and investigate the Dna2 protein acting in real time in response to DNA damage. These studies, in the context of the global approach to genomic stability in cells, will lead to a deeper understanding of basic processes such as DNA replication and provide a toe-hold to rational drug design for treating or even preventing cancer in the future.

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