Contributions of Dna2 and the Tim/Tipin Complex to Genomic Stability

Thesis by

Karen E. Wawrousek

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2010

(Defended November 20, 2009)

To My Family

Acknowledgements

I was only able to complete the work in this thesis with the help and support of many people, including both scientific colleagues and personal friends. I would like to thank my advisor, Bill Dunphy, for his support, for allowing me to pursue a variety of projects, and for always insisting upon the utmost scientific rigor. Akiko Kumagai has been a wonderful source of ideas and a great reference over the years. I am grateful for the support and advice of fellow past and present members of the Dunphy lab: Esther Bae, Jason DeHart, Dan Gold, Seong-Yun Jeong, Soo-Mi Kim, Sanjay Kumar, Joon Lee, Wenhui Li, Zheng Meng, Timur Pogodin, Juanse Ramirez-Lugo, and Hae Yong Yoo.

I am indebted to members of my thesis committee: Judy Campbell, Doug Rees, and Paul Sternberg. I deeply appreciate the time that Doug and Paul have spent in various discussions with me and the encouragement that they have provided along the way. Judy was an advisor to me for the Dna2 project; through this work, I learned much about "doing science" within the scientific community and the training of scientists. I would also like to thank members of the Campbell lab for helpful discussions regarding Dna2, including Barbara Kraatz Fortini, Martin Budd, and Piotr Polaczek.

Throughout my years at Caltech, I have been pleased and proud to be a member of the Advisory Board of the Women Mentoring Women Program at the Caltech Women's Center. I truly enjoyed participating in stimulating discussions with fellow board members on how to better conditions for women at Caltech and in science. I must give special credit to both Dr. Felicia Hunt and Ornah Becker, who both gave me guidance, encouragement, and support. It was through this program that I met Dr. Katy McCabe, who became a comrade, confidant, and kindred soul.

Many friends at Caltech have helped me along the way. I became friends with Jenn within my first year at Caltech, and I will always remember our late nights at coffee shops and all the times we were "recognized" in public. My conversations with Beth, regarding science or otherwise, are always entertaining, and I have truly enjoyed the time I've spent with both Catherine and Anna. Tamara has certainly helped me to push my own personal limits, always in a good way. I must recognize my running buddies, who were all great to talk to on our long runs and who also pushed me more than I would have liked sometimes. All of my friends at Special Olympics have been a source of inspiration to me, from the athletes to the coaches. Bob Wilson is not only a great fellow-volunteer, but also made sure that my gastronomic experiences were expanded to include foods from almost every continent.

I am very grateful for the support of my family. My Dad basically taught me high school chemistry and physics, with occasional help from Eric and Hans Sr. Without my father's patience and interest in science, I likely would not have developed the great interest in science that led me to my graduate studies at Caltech. Eric encouraged me to try laboratory research, and even allowed me to live with him for three summers when I was an undergraduate summer-student at NIH. Eric is also an excellent scientist and a great source of advice. My mother and sisters have also been supportive over the years, and, more recently, the Murphys have been very understanding and encouraging as I finish my degree. While at Caltech, I met my fiancé Shane. Shane encourages me, challenges me, and supports me in whatever I choose to pursue. Words cannot express how grateful I am to have him in my life.

Abstract

This thesis describes the essential roles of Dna2 and the Tim/Tipin complex in the maintenance of genomic stability. Dna2 participates in DNA replication and double-strand break repair by homologous recombination. Meanwhile, the Tim/Tipin complex is required for efficient checkpoint activation upon replication stress, which can be caused by stalled DNA replication forks.

While yeast genetics and experiments with purified proteins have revealed much about yeast Dna2, we chose to pursue characterization of metazoan Dna2 using *Xenopus* cell-free extracts. We show that binding of Dna2 to origins of replication is dependent upon formation of pre-replication complexes but independent of CDK2 activity. Upon initiation of DNA replication, Dna2 travels with replication forks. Physical interactions with Mcm10 and And-1, proteins involved in lagging strand DNA replication, are indicative of a role in replication of the lagging strand; this result is consistent with genetic results in yeast and *in vitro* biochemical experiments.

Dna2 also participates in the response to double-strand breaks and accumulates on chromatin containing double-strand breaks. We show that Dna2 binds to free DNA ends after the Mre11-Rad50-Nbs1 complex and ATM, but before RPA. Dna2-depleted extracts exhibit delayed processing of DNA ends, indicating that other nucleases do not easily compensate for the lack of Dna2. Consistent with genetic results in yeast, we find that the Mre11-Rad50-Nbs1 protein complex is essential for the processing of free DNA ends, but inhibition of Mre11 nuclease activity only slows processing. This observation indicates that other nucleases, possibly Dna2, can compensate for loss of Mre11 nuclease

activity. Despite the role of Dna2 in double-strand break processing, Dna2 is not required for checkpoint activation.

Timeless (Tim) and Tipin participate in the checkpoint response to stalled replication forks. We demonstrate here that Tim and Tipin form a complex, associate with chromatin in S phase, and physically interact with many proteins at the replication fork. Human cells lacking the Tim/Tipin complex do not exhibit robust checkpoint activation in response to stalled replication forks. Finally, we show that Tipin is also a target of both the ATR and Cdc7 kinases, which respond to stalled replication forks.

Table of Contents

Table of Figures

1 Introduction

As cells grow and divide, their DNA content is monitored to prevent accumulation of mutations which can lead to the development of cancer (Hartwell & Kastan, 1994). Mutations are the result of DNA damage caused by external sources such as UV light, X-rays, radiation, and chemical carcinogens, or they can be caused by cellular events including stalled DNA replication forks, incompletely replicated DNA, and programmed DNA double-strand breaks. Damaged DNA can be repaired, but mutations are made permanent when the damaged DNA is not repaired accurately or cells divide without fixing the damage. To prevent accumulation of mutations, cells have developed checkpoints to delay cellular growth in the presence of DNA damage or incomplete DNA replication, giving the cell time to repair the DNA before making mutations permanent upon cell division (Weinert, 1998). Abrogation of these checkpoints leads to increased susceptibility to cancer, neurodegeneration, and accelerated aging (Cimprich & Cortez, 2008; Czornak et al, 2008).

Cells can suffer DNA damage at all points in the cell cycle. It is both the type of DNA damage and the stage of the cell cycle that determines which checkpoints are activated as well as the method of repair. During DNA replication in S phase of the cell cycle, cells monitor for both stalled replication forks, which activates the ATR-dependent checkpoint response, and DNA double-strand breaks, which activate the ATM- and DNA-PK dependent checkpoints. The ATM-dependent checkpoint and repair mechanism results in a more accurate repair of double-strand breaks, while the DNA-PK-dependent pathway can be accurate or error-prone repair. The ATM-dependent pathway is the prominent repair pathway for DNA double-strand breaks (DSBs) during S or G2

phase, since this pathway relies on the presence of a sister chromatid which is only present in S and G2 phases, while the DNA-PK pathway is the primary DSB repair pathway in G1 and M phase when a sister chromatid is not available.

The ATR-dependent pathway can be activated in S or G2 phases, while DNA is being replicated and incomplete DNA replication is detected. Some of the same proteins that are necessary for these checkpoint responses are also involved in DNA replication. In S phase, the process of DNA replication is physically coupled to checkpoint mechanisms. Some proteins that are required for checkpoint activation that travel with the replication fork are TopBP1, Claspin, and the Timeless/Tipin complex (Chini & Chen, 2003; Errico et al, 2007; Gotter et al, 2007; Hashimoto & Takisawa, 2003; Kumagai & Dunphy, 2000; Lee et al, 2003; Makiniemi et al, 2001; Unsal-Kacmaz et al, 2007; Van Hatten et al, 2002; Yoshizawa-Sugata & Masai, 2007). Recent work in yeast also highlights this coupling of DNA replication and checkpoints in S phase; Mrc1, the yeast ortholog of Claspin, is required for activation of the S-phase checkpoint and physically interacts with Pol 2, a catalytic subunit of DNA polymerase ε (Alcasabas et al, 2001; Lou et al, 2008). Upon checkpoint activation by stalled replication forks, this interaction is altered but not abolished, potentially altering polymerase activity in response to the replication stress. Alternatively, the change in Mrc1 binding may provide a docking site for additional checkpoint response proteins (Lou et al, 2008). Physically coupling these mechanisms ensures an immediate response when problems arise in DNA replication, so any stalled replication forks can be stabilized quickly to prevent dissociation of replication fork proteins, a signal of fork collapse from which the replication fork can not recover. The coupling of DNA replication and checkpoint

activation is exemplified by the fact that the ATR-dependent DNA replication checkpoint requires the initiation of DNA replication (Stokes et al, 2002). Since these mechanisms are intertwined, both DNA replication and checkpoints are discussed below.

1.1 DNA Replication Initiation

The replication of genomic DNA is a well-conserved and highly regulated process. All chromatin must be replicated once and only once per cell cycle with a minimal rate of mutation. The activity of cell cycle-dependent kinases regulates the process of DNA replication; origins of replication are "marked" for replication in late M phase and early G1 phase, when S-phase CDK (S-CDK) activity is low, and DNA is replicated when S-CDK levels are high. While there are many points of regulation and feedback, this overarching model guides DNA replication in the cell cycle (Diffley, 1996).

When S-CDK levels are low, the Origin Recognition Complex (ORC) first binds chromatin and marks the origins of replication during early G1 phase for the upcoming S phase (Diffley, 2004; Nguyen et al, 2001). Origins are interspersed throughout chromosomes, and not every origin is used every S phase (for review see Natsume & Tanaka, 2009). Cdc6 and Cdt1 subsequently bind origins of replication during G1 phase. Recent results revealed that the Cdt1 that binds origins is bound to Mcm9, a protein with ATPase activity and a helicase domain (Lutzmann & Méchali, 2008). The MCM2-7 complex, which is the replicative helicase, subsequently binds origins of replication, and the binding of this set of proteins signals the formation of the pre-replication complex (pre-RC) and "licensing" origins of replication (Bell & Dutta, 2002; Nishitani &

Lygerou, 2002). Pre-RC complexes must be formed in G1 phase, when S-CDK levels are low, because they can not form when S-CDK levels are high in S phase (Diffley, 2004; Nguyen et al, 2001). Also, at the onset of DNA replication, Cdt1 is bound by geminin, not Mcm9, and is subsequently degraded in a PCNA- and Cul4-dependent manner, preventing the licensing of additional origins of replication (Arias & Walter, 2005; Arias & Walter, 2006; Lutzmann & Méchali, 2008). Cdc6 is also regulated to prevent licensing of additional forks. Upon initiation of DNA replication, yeast Cdc6 is degraded, while human Cdc6 is exported from the nucleus, to prevent additional origin firing (Elsasser et al, 1999; Jallepalli et al, 1998; Jiang et al, 1999; Perkins et al, 2001; Petersen et al, 1999; Saha et al, 1998). These are some of the many points of regulation in which DNA replication can be limited to one round of DNA replication per cell cycle (Arias & Walter, 2007).

It is at this pre-RC stage of replication fork maturation that proteins such as Dna2, required for efficient DNA replication, and Mcm10, responsible for retention of DNA polymerase α at the fork, bind origins of replication (this work and Liu et al, 2000b; Wohlschlegel et al, 2002). TopBP1, which is necessary for the ATR-dependent checkpoint, also binds the developing replication fork at this point (Van Hatten et al, 2002). Binding of the above proteins at this stage of replication fork development is essential; in the absence of Mcm10 and TopBP1, replication forks do not develop further. The presence of Mcm10 at the fork is also required for binding of Cdc45 and formation of the CMG complex, mentioned below. TopBP1 is necessary for both Cdc45 binding and association of the GINS complex, also described below (Im et al, 2009; Kubota et al,

2003; Van Hatten et al, 2002). This ordered binding of proteins is highly conserved from yeast to human, with only minor variations.

Upon entry into S phase, marked by activation of S-CDK, pre-RC complexes are activated by binding of additional proteins, transforming it into what is known as the pre-initiation complex (pre-IC). Activation of both the Cdc7 and CDK2 kinases are required for pre-IC formation, and this step is also well-conserved from yeast to metazoans (Aparicio et al, 1999; Mimura et al, 2000; Walter & Newport, 2000; Zou & Stillman, 2000). Notable pre-IC proteins include Cdc45 and the GINS (Go, Ichi, Nii, and San) complex, and the chromatin binding of Cdc45 and GINS are mutually dependent (Kubota et al, 2003). Cdc45 recruits DNA polymerase α/primase, necessary for initiation of replication, and GINS activates it (De Falco et al, 2007; Mimura et al, 2000). It also may be that both Cdc45 and the GINS complex activate the MCM replicative helicase, as the MCM2-7 helicase can be isolated in the Cdc45-MCM-GINS (CMG) complex and is bound to the same region of DNA as Cdc45 and GINS upon uncoupling of the replicative helicase and polymerase activities in both *Xenopus* and yeast (Gambus et al, 2006; Kubota et al, 2003; Pacek et al, 2006).

DNA can be unwound by the MCM replicative helicase and subsequently bound by RPA, a single-stranded DNA binding protein. It is also at the pre-IC stage of replication fork maturation that additional DNA replication proteins bind the fork, including notable proteins involved in DNA replication, such as the DNA polymerases, Claspin, the Tim/Tipin complex, and the ssDNA-binding protein RPA (Lee et al, 2003; Mimura et al, 2000; Tanaka et al, 2009; Walter & Newport, 2000).

Once replication has initiated, DNA replication forks proceed away from the replication origins with coupled leading and lagging strand replication. Data from yeast suggests that DNA polymerase ε is the leading strand polymerase and polymerases α and δ participate in lagging strand replication (McElhinny et al, 2008; Pavlov et al, 2006; Pursell et al, 2007). Claspin, Timeless (Tim), and Tipin are not essential for DNA replication but serve to stabilize replication forks and prevent fork stalling in both metazoans and yeast (Chou & Elledge, 2006; Errico et al, 2007; Katou et al, 2003; Liu et al, 2006b; Noguchi et al, 2003; Noguchi et al, 2004; Petermann et al, 2008; Szyjka et al, 2005; Tourriere et al, 2005; Unsal-Kacmaz et al, 2007; Yoshizawa-Sugata & Masai, 2007).

The termination of DNA replication is less understood than the initiation of replication. In eukaryotic cells, replication forks terminate when two replication forks meet or when one replication fork approaches the end of a telomere. However, most data on the termination of replication are from studies in prokaryotes; the current theory is that as two forks approach each other from opposite directions, one fork stops while the other proceeds (Kaplan, 2006). This process is regulated so forks do not collide and all DNA is replicated.

1.2 Checkpoints

Checkpoints are cellular mechanisms that pause the cell cycle to prevent pre-mature entry into the next phase of the cell cycle. DNA damage and the subsequent repair of that damage activate checkpoints to allow the cell additional time to repair the damage before it is made permanent by cell division. When DNA is not completely replicated in S phase or DNA damage is encountered, cells delay the cell cycle, maintain

the replication fork structure so replication can re-start upon repair of the damage, repair the DNA, inhibit replication from other origins of DNA replication, and alter the transcriptional program of the cell. If checkpoint mechanisms fail, cells suffer from higher mutation rates and potentially cell death, are more likely to become cancerous, and can suffer from accelerated aging (Czornak et al, 2008; Erkko et al, 2008; Hartwell & Kastan, 1994; Karppinen et al, 2006; Kerzendorfer & O'Driscoll, 2009; Singh et al, 2009).

Like DNA replication, the initial steps of checkpoints are better understood than the termination of the checkpoint signal and the recovery from checkpoints. It is likely that checkpoints are down-regulated upon removal of the activating signal once the damaged DNA has been repaired. If DNA damage can not be repaired, an adaptation response, observed in yeast, *Xenopus*, and human cells, can occur in which cells enter mitosis with the DNA damage still present (Leroy et al, 2003; Pellicioli et al, 2001; Sandell & Zakian, 1993; Syljuasen et al, 2006; Toczyski et al, 1997; Vaze et al, 2002; Yoo et al, 2004a).

The type of DNA damage present and the stage of the cell cycle specifies which checkpoint mechanism and method of repair will be utilized by the cell. Checkpoint responses to DNA replication stress and DNA double-strand breaks, both active in S and G2 phases, are described below; other checkpoint responses are present in G1 and M phases. The main checkpoint mechanisms each have a main, apical kinase regulating many aspects of the checkpoint response. It is the ATR kinase that responds to replication stress, while ATM is the prominent kinase that responds to DSBs in S and G2 phases.

1.3 Replication Stress

As cells replicate their DNA, replication stress occurs when replication forks stall upon encountering damaged DNA, protein bound to DNA, abnormal DNA structures, or when the helicase and polymerase activities of the fork become uncoupled. Replication stress triggers the activation of the ATR-dependent checkpoint, and signaling through this pathway retards cell cycle progression, keeping the cell in S phase and providing additional time to complete the replication of genomic DNA. In addition to replication stress, the ATR-dependent checkpoint is also activated by ATM in the presence of DNA double-strand breaks (DSBs), discussed below (Jazayeri et al, 2006; Yoo et al, 2007).

Upon occurrence of replication stress, tracts of single-stranded DNA (ssDNA) are formed at the replication fork, likely due to the activity of the MCM replicative helicase. As with most ssDNA in the cell, this is coated in the ssDNA-binding protein RPA to protect the ssDNA from degradation. RPA-coated ssDNA is believed to be the signal for activation of the ATR-dependent DNA replication checkpoint (Costanzo et al, 2003; Zou & Elledge, 2003). The extent of checkpoint activation depends upon both the length of RPA-coated ssDNA tracts and the type of DNA end exposed, with maximal checkpoint activation when a 5′ end is exposed at a ssDNA-dsDNA (double-stranded DNA) junction (MacDougall et al, 2007; Shiotani & Zou, 2009).

The ATR kinase and its partner ATRIP, henceforth referred to as the ATR-ATRIP complex, are recruited to RPA-coated ssDNA through an RPA-binding domain in ATRIP (Ball et al, 2005; Kim et al, 2005; Zou & Elledge, 2003). Other proteins recruited to the RPA-coated ssDNA are TopBP1, a BRCT-containing protein, and Rad17. Rad17 forms a DNA damage response alternative RFC complex, containing the same small RFC

subunits as the replicative RFC complex but the large subunit is replaced by Rad17. Whereas the RFC complex participates in loading of the PCNA sliding clamp onto DNA for DNA replication fidelity, the Rad17 complex loads the 9-1-1 (Rad9-Rad1-Hus1) clamp onto damaged DNA. The Rad17 complex preferentially loads the 9-1-1 complex onto DNA containing a free 5′ end at a ssDNA-dsDNA junction, the same DNA structure that elicits maximal checkpoint activation (Ellison & Stillman, 2003; MacDougall et al, 2007; Shiotani & Zou, 2009). Once the 9-1-1 complex is loaded onto chromatin, the C-terminal tail of Rad9 in the 9-1-1 complex interacts with both ATRIP in the ATR-ATRIP complex and with TopBP1 (Lee et al, 2007; Yan & Michael, 2009). Both TopBP1 and Rad9 stimulate ATR kinase activity (Delacroix et al, 2007; Kumagai et al, 2006; Mordes et al, 2008). While the signal to activate the checkpoint appears to be RPA-coated ssDNA, its real purpose may be to simply bring these three proteins in close proximity, increasing the local concentration of these proteins. Elegant experiments in both yeast and human cells reveal that simply increasing the local concentration of checkpoint proteins is sufficient to activate the checkpoint response in the absence of DNA damage or replication stress (Bonilla et al, 2008; Soutoglou & Misteli, 2008; Stucki & Jackson, 2006).

Interestingly, ATR-ATRIP binding to RPA-coated ssDNA is not strictly essential for ATR kinase activity stimulation, since ATR can still be activated when bound to ATRIP mutants lacking the ability to bind RPA (Ball et al, 2005; Kim et al, 2005). The reciprocal result was shown in yeast; mutants of Rfa1, the yeast homolog of RPA, that are unable to bind Ddc2, the ATRIP homolog, only suffer minor checkpoint activation defects (Lee et al, 1998; Umezu et al, 1998). Additionally, overexpression of the ATR-

activating protein TopBP1 in human cells results in ATR-dependent phosphorylation of proteins in the absence of RPA-bound ssDNA (Ball et al, 2007; Kumagai & Dunphy, 2006). This also supports the theory that simply increasing the local concentration of checkpoint proteins is sufficient to activate checkpoints, as previously seen in both yeast and human cells (Bonilla et al, 2008; Soutoglou & Misteli, 2008; Stucki & Jackson, 2006).

Active ATR phosphorylates a myriad of proteins, some of which have recently been identified through large-scale screens (Matsuoka et al, 2007; Mu et al, 2007; Smith et al, 2009; Smolka et al, 2007; Stokes et al, 2007). One of the most studied ATR substrates is Chk1, a serine/threonine kinase. Human Chk1 is activated upon ATR-dependent phosphorylation of residues S317 and S345 (Liu et al, 2000a; Zhao & Piwnica-Worms, 2001). Active Chk1 then inhibits the Cdc25 phosphatases, thus preventing CDK activation and entry into mitosis (Furnari et al, 1997; Peng et al, 1997; Sanchez et al, 1997).

Interestingly, TopBP1 and Rad17, proteins responsible for the activation of ATR kinase activity, are also targets of ATR phosphorylation (Bao et al, 2001; Kumagai et al, 2006). ATR-dependent Rad17 phosphorylation is required for 9-1-1 foci formation and checkpoint activation (Bao et al, 2001; Medhurst et al, 2008). Rad9, of the 9-1-1 complex, binds the same basic cleft in RPA that ATRIP binds, and the Rad9 C-terminal tail bridges TopBP1 and the ATR-ATRIP complex (Lee et al, 2007; Xu et al, 2008). This data again suggests that without Rad17 phosphorylation and 9-1-1 accumulation, TopBP1 alone would not activate ATR as efficiently. Therefore, the checkpoint signal would not be amplified and perpetuated, so the checkpoint signal would not be sufficiently activated

in response to ssDNA. Alternatively, the ATR-dependent phosphorylation of Rad17 may serve another purpose in checkpoint signaling.

In addition to slowing cell cycle progression, as mentioned above, activate ATR kinase also affects other pathways. Upon checkpoint activation, the further firing of DNA replication origins is inhibited until the DNA damage is repaired and the checkpoint mechanism is down-regulated (Costanzo et al, 2003; Maya-Mendoza et al, 2007; Shechter et al, 2004). ATR also participates in the recovery from stalled replication forks, as illustrated by recent work on the ATR-dependent phosphorylation of Mcm2. Mcm2, a member of the MCM replicative helicase complex, can be phosphorylated by either ATM or ATR upon checkpoint activation (Trenz et al, 2008; Yoo et al, 2004b). During checkpoint activating conditions, ATR phosphorylates Mcm2 on S92 of the *Xenopus* protein, and this modification promotes Plx1, the *Xenopus* orthologue of Plk1 (Polo-like kinase 1), binding to Mcm2. This interaction in turn promotes recovery from the checkpoint response by promoting origin firing (Trenz et al, 2008). Plx1 also binds and phosphorylates Claspin, causing it to release from chromatin and thus down-regulate the checkpoint response (Yoo et al, 2004a). In human cells, Plk1-dependent degradation of Claspin in G2 phase of the cell cycle also downregulates the checkpoint mechanism by physically removing proteins that can keep the checkpoint activated (Gewurz & Harper, 2006).

ATR phosphorylates many proteins at the replication fork, including RPA, the MCM2-7 complex, Mcm10, various DNA polymerases, TopBP1, Rad9 of the 9-1-1 complex, Tipin, WRN, BLM, and BRCA1 (Bao et al, 2001; Cortez et al, 2004; Liu et al, 2006a; Matsuoka et al, 2007; Oakley et al, 2001; Yoo et al, 2004b). The function of

many of these phosphorylation events is as yet unknown; they may be related to maintenance of replication fork integrity, DNA repair, checkpoint recovery, protein degradation, or the transcription of genes. Understanding the purpose of these ATR-mediated modifications will shed light on the role of the ATR-dependent checkpoint pathway.

Other unresolved questions surround the topic of replication fork stability upon stalling. Both Claspin and the Tim/Tipin complex are required to maintain replication fork integrity, but results in yeast reveal that they are not equivalent, and forks lacking the respective homologs of these proteins respond differently when encountering a replication fork barrier (Chou & Elledge, 2006; Errico et al, 2007; Katou et al, 2003; Mohanty et al, 2006; Petermann et al, 2008; Unsal-Kacmaz et al, 2007; Yoshizawa-Sugata & Masai, 2007). Many questions remain regarding the similarities and differences between these fork-stabilizing proteins. Also, the effect of ATR-dependent phosphorylation of Tipin is as yet unknown; however, Claspin phosphorylation by ATR is necessary for ATR-dependent checkpoint signaling in response to DSBs, but not in response to replication stress (Yoo et al, 2006).

It is worth noting that there are also ATR-independent responses to stalled replication forks, although these mechanisms are not as well described. One example of an ATR-independent response is the ubiquitylation of PCNA upon induction of stalled replication forks (Chang et al, 2006; Yang et al, 2008). Ubiquitylation of PCNA promotes recruitment of translesion DNA polymerases, believed to be necessary for efficient replication in the presence of damaged DNA (Lopes et al, 2006). This response

is dependent upon both Claspin and the Tim/Tipin complex, but independent of ATR (Yang et al, 2008).

The Cdc7 kinase has been detected in a complex with the Claspin and Tim/Tipin homologs in yeast, may be a target of the ATR-dependent checkpoint, and these proteins participate in checkpoint mechanisms in yeast (Jares et al, 2000; Matsumoto et al, 2005; Shimmoto et al, 2009; Sommariva et al, 2005; Yanow et al, 2003). In yeast, the Cdc7 homolog participates in both the response to stalled replication forks and the response to alkylated DNA (Shimmoto et al, 2009; Sommariva et al, 2005). The human Cdc7/Dbf4 complex is a target of Chk1, and Dbf4 overexpression reverses the checkpoint response (Heffernan et al, 2007). Cdc7 is involved in the metazoan checkpoint response, but the extent of conservation is unresolved. Further research is needed to examine the role of Cdc7 in the response to replication stress and, potentially, the ATR-dependent checkpoint.

1.4 DSB Resection through Homologous Recombination

Unlike DNA replication stress, DNA double-strand breaks (DSBs) can occur at any point during the cell cycle. DSBs can be caused by exogenous sources, such as exposure to ionizing radiation, radiomimetic drugs, or UV light, or by endogenous cellular processes, including meiosis, V(D)J recombination, and immunoglobulin class-switch recombination. Unlike the replication stress checkpoint, there are two kinases that may regulate the checkpoint response to and repair of DSBs. The ATM-dependent checkpoint pathway is coupled to repair by homologous recombination (HR), while the DNA-PK-dependent checkpoint pathway is associated with repair of DSBs by non-

homologous end joining (NHEJ). The repair method of choice is also cell-cycle dependent; homologous recombination relies on the presence of a sister chromatid, only present in S or G2 phases, for use as a template for repair, so HR is the dominant repair mechanism in S and G2. Meanwhile, NHEJ is the predominant DSB repair mechanism in G1 phase, since cells have a 1N DNA content and there is no sister chromatid template available. Repair by HR is an accurate repair, since there is a DNA template for repair, while repair by NHEJ is not necessarily accurate, as DNA ends are joined irrespective of homology (for review see Hartlerode & Scully, 2009). The HR mechanism and ATM-dependent checkpoint response will be reviewed here.

Upon DSB formation, free DNA ends are rapidly bound by the MRN (Mre11-Rad50-Nbs1) complex. Mre11 is a nuclease, Rad50 is a member of the SMC family proposed to hold sister chromatids in close proximity, and Nbs1 physically interacts with the ATM kinase (for review see Lavin, 2007). MRN is also bound to CtIP (CtBP interacting protein), which interacts with BRCA1 and is essential for DNA end joining through homologous recombination (Sartori et al, 2007; Takeda et al, 2007; Yu et al, 2006). Mre11 in the MRN complex first processes the break by degrading the 5′ ends, and this processing releases short single-stranded DNA oligomers capable of activating the ATM kinase (Jazayeri et al, 2008). Inactive ATM dimers are recruited to the DSB ends and activated by the MRN activity. ATM dimers autophosphorylate and dissociate, and the active ATM monomers subsequently phosphorylate proteins involved in the checkpoint response and repair of DSBs (Bakkenist & Kastan, 2003). One protein of interest that is quickly phosphorylated by ATM is the histone variant H2AX. Phosphorylated H2AX, together with the MDC1 adapter protein, recruit DSB checkpoint

and repair proteins to sites of damage and mediate repair by homologous recombination (Hartlerode & Scully, 2009). ATM is also responsible for phosphorylating and activating Chk2, a serine/threonine effector kinase that further phosphorylates proteins in response to DSBs (Matsuoka et al, 1998; Matsuoka et al, 2000).

While the checkpoint signal is perpetuated and amplified by the ATM and Chk2 kinases, the break is also being processed for repair. DSBs are first processed by Mre11 in the MRN/CtIP complex, as mentioned above. This Mre11 processing is both the initial step of repair and an activating step for the checkpoint pathway. DNA ends are further processed by a helicase and nuclease, either WRN and Dna2 or BLM and Exo1 (Liao et al, 2008; Nimonkar et al, 2008). This mechanism of processing is well-conserved and consistent with genetic results in yeast (Budd & Campbell, 2009; Zhu et al, 2008). If the break is processed by the WRN helicase and the Dna2 helicase-nuclease, WRN unwinds the duplex DNA and Dna2 degrades the 5′ strand, lengthening the 3′ ssDNA tail (Liao et al, 2008). Yeast genetics indicate that it is the nuclease, not the helicase, activity of Dna2 that participates in DSB processing (Budd & Campbell, 2009). Exo1 is also capable of processing this 5′ end, thus extending the 3′ ssDNA tail, and the BLM protein stimulates Exo1 activity without requiring BLM helicase activity (Nimonkar et al, 2008). While these two parallel pathways exist, the factors that drive the cell to use one pathway versus another are as yet unknown.

The nuclease degradation of the 5′ strand to reveal a longer 3′ ssDNA tail allows the accumulation of RPA on the ssDNA. Eventually, the bound RPA is replaced with Rad51 recombinase with the help of BRCA1, BARD1, and BRCA2. It is the Rad51-coated ssDNA that is competent for strand invasion of the sister chromatid to search for a

homologous sequence. The cell can then synthesize the remaining missing DNA sequence, and this DNA structure is resolved by one of many enzymes capable of resolving the structure, including BLM and Topoisomerase IIIα, GEN1, SLX4, and the MUS81-EME1 complex (for review see Hartlerode & Scully, 2009).

Checkpoint signaling in response to DSBs is not independent of the repair process. Many DSB repair proteins are substrates of the ATM kinase, including Mre11. It has only recently been discovered that phosphorylation of Mre11 by ATM triggers the dissociation of Mre11 from DNA ends, likely releasing these ends for further processing for repair (Di Virgilio et al, 2009). ATM phosphorylates many additional targets that participate in DSB repair, although the function of many of these modifications is as yet unknown (for review see Czornak et al, 2008). These ATM-dependent phosphorylations may be involved in the recruitment of proteins to DSBs, in processing the DSBs, or in the down-regulation of the checkpoint signal.

Processing of a DSB by HR also activates the ATR-dependent checkpoint pathway. The extended RPA-coated 3′ ssDNA tails generated during processing of DNA ends may serve to recruit ATR-ATRIP (Shiotani & Zou, 2009). Active ATM kinase phosphorylates TopBP1 with MRN as a mediator, which in turn activates the ATR kinase (Yoo et al, 2007; Yoo et al, 2009). Therefore, DSBs activate both the ATM- and ATR-dependent pathways, thus expanding the cellular response to DNA damage. There is also cross-talk between the ATR- and ATM-dependent responses. Chk1, a downstream target of the ATR kinase activated in response to replication stress, is essential for repair of DSBs by HR (Sorensen et al, 2005). Additionally, Exo1, one of the nucleases that participates in the resection of DSBs by HR, was recently found to be a target of the ATR

under conditions of replication stress, with phosphorylation leading to degradation of Exo1 (El-Shemerly et al, 2008). It is as yet unknown if ATM-mediated ATR activation by the DSB checkpoint also causes degradation of Exo1, or if this effect is limited to replication stress. Degradation of Exo1 may signal completion of a given step of repair, or it may function in down-regulation of the checkpoint response. Cross-talk between cellular pathways is a point of interest as we strive to understand the cellular response to DNA damage.

The importance of resolving DSBs is underscored by the multiple redundancies in the DSB processing pathway detected through yeast genetics. In yeast, the nuclease activity of the Mre11 is not essential, although the MRN protein complex itself is necessary for DSB processing. This is likely due to other nucleases, potentially Dna2 and Exo1, compensating for the lack of Mre11 nuclease activity. Yeast lacking Dna2 or Exo1 can still repair DSBs, suggesting that cells can compensate for the lack of either of these proteins and that Dna2 and Exo1 may be able to compensate for each other (Budd et al, 2009; Zhu et al, 2008). Supporting this theory, yeast genetic interactions revealed that it is the nuclease activity of the Dna2 helicase-nuclease that participates in DSB repair; a helicase-dead mutant had no affect on repair efficiencies (Budd & Campbell, 2009). Yeast genetic interactions has revealed much about these functional redundancies, and a current point of interest is understanding these functional redundancies in metazoans.

While much has also been learnt about the response to DSBs, many questions remain regarding the DSB checkpoint and repair. Recent data suggests how the cell cycle may influence the prevalence of DSB repair by HR versus NHEJ at different cell cycle stages. CtIP, which participates in HR, is regulated by CDK-dependent

phosphorylation events, and this may promote repair by HR over NHEJ in S and G2 phases (Chen et al, 2008; Huertas & Jackson, 2009; Yun & Hiom, 2009). There are likely many more points of cell cycle regulation that are not yet understood, including as yet unidentified proteins and modifications to proteins involved in DSB repair.

Cross-talk between checkpoint pathways is also a point of interest. The ATM-dependent activation of ATR was only recently identified, and the extent of cross-talk between other PIKKs, particularly ATR and DNA-PK, is an area of current research. ATM does phosphorylate DNA-PK in irradiated cells, and this ATM-dependent modification acts cooperatively with DNA-PK autophosphoryalation for cooperative activation of the DNA-PK kinase activity (Chen et al, 2007). ATR-dependent activation of ATM upon DNA replication fork stalling or UV has been reported in human cells; however, this effect was not observed in *Xenopus* extract treated ssDNA or dsDNA to induce checkpoint activation (Jazayeri et al, 2008; Stiff et al, 2006).

Finally, the role of MRN in DSB repair is intriguing. Mre11 nuclease activity is not required for DSB resection, but the intact MRN protein complex is required. MRN may serve as a scaffold for binding of other repair proteins, or it may be a domain in Rad50 or Nbs1 that is essential for DSB repair. Understanding the role of MRN past the role of the nuclease, the non-essential role, should yield a greater understanding of DSB repair. Interestingly, MRN also participates in DSB repair by NHEJ (non-homologous end joining). Removal of the MRN complex, or any protein within this complex, greatly reduces the efficiency of NHEJ by both classical and alternative pathways in human cells (Deng et al, 2009; Dimitrova & de Lange, 2009; Dinkelmann et al, 2009; Rass et al, 2009; Xie et al, 2009). The role of MRN in repair of DSBs by both HR and NHEJ is

interesting, and is likely to be one of multiple points in which these pathways are intertwined.

1.5 *Xenopus* Cell-Free System

This work examines the vertebrate homologs of the well-studied *S. cerevisiae* proteins, Dna2, Tof1, and Csm3, in an effort to further the understanding of these proteins in metazoans. Most experiments were performed in *Xenopus laevis* cell-free extract, derived from *X. laevis* eggs arrested in metaphase of meiosis II that were crushed by centrifugation. The addition of calcium stimulates the extract to enter interphase, or S phase, and the further addition of chromosomal DNA and an ATP-regeneration system results in the formation of nuclei and DNA replication in this cell-free extract. The maternal stores of protein from the eggs are sufficient for DNA replication without the need for transcription. This DNA replication is both regulated and synchronized, and cellular responses to DNA damage can be successfully recapitulated in this extract (Blow & Laskey, 1986; Garner & Costanzo, 2009).

In this text, interphase extract refers to S phase extract with no DNA added, and CSF extract is extract that has not been stimulated by addition of calcium, so it is arrested in metaphase II of meiosis. CSF extract is commonly referred to as “M phase” extract, due to its resemblance to M phase extracts. For all experiments involving the addition of DNA to these extracts, *Xenopus laevis* demembranated sperm chromatin was used as the source of chromosomal DNA.

1.6 References

Alcasabas AA, Osborn AJ, Bachant J, Hu F, Werler PJH, Bousset K, Furuya K, Diffley JFX, Carr AM, Elledge SJ (2001) Mrc1 transduces signals of DNA replication stress to activate Rad53. *Nat Cell Biol* **3**(11)**:** 958-965

Aparicio OM, Stout AM, Bell SP (1999) Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. *Proceedings of the National Academy of Sciences of the United States of America* **96**(16)**:** 9130-9135

Arias EE, Walter JC (2005) Replication-dependent destruction of Cdt1 limits DNA replication to a single round per cell cycle in *Xenopus* egg extracts. *Genes & Development* **19**(1)**:** 114-126

Arias EE, Walter JC (2006) PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication. *Nat Cell Biol* **8**(1)**:** 84-90

Arias EE, Walter JC (2007) Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells. *Genes & Development* **21**(5)**:** 497-518

Bakkenist CJ, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421**(6922)**:** 499-506

Ball HL, Ehrhardt MR, Mordes DA, Glick GG, Chazin WJ, Cortez D (2007) Function of a conserved checkpoint recruitment domain in ATRIP proteins. *Molecular and Cellular Biology* **27**(9)**:** 3367-3377

Ball HL, Myers JS, Cortez D (2005) ATRIP binding to replication protein A-single-stranded DNA promotes ATR-ATRIP localization but is dispensable for Chk1 phosphorylation. *Molecular Biology of the Cell* **16**(5)**:** 2372-2381

Bao S, Tibbetts RS, Brumbaugh KM, Fang Y, Richardson DA, Ali A, Chen SM, Abraham RT, Wang X-F (2001) ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses. *Nature* **411**(6840)**:** 969-974

Bell SP, Dutta A (2002) DNA replication in Eukaryotic cells. *Annual Review of Biochemistry* **71**(1)**:** 333-374

Blow JJ, Laskey RA (1986) Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of Xenopus eggs. *Cell* **47**(4)**:** 577-587

Bonilla CY, Melo JA, Toczyski DP (2008) Colocalization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage. *Molecular Cell* **30**(3)**:** 267-276

Budd M, Campbell J (2009) Interplay of Mre11 nuclease with Dna2 plus Sgs1 in Rad51-dependent recombinational repair. *Plos One* **4**(1)**:** e4267

Budd ME, Cox LS, Campbell JL (2009) Coordination of Nucleases and Helicases during DNA Replication and Double-strand Break Repair. In *Molecular Themes in DNA Replication*, Cox LS (ed). London: RSC Publishing

Chang DJ, Lupardus PJ, Cimprich KA (2006) Monoubiquitination of proliferating cell nuclear antigen induced by stalled replication requires uncoupling of DNA polymerase and mini-chromosome maintenance helicase activities. *Journal of Biological Chemistry* **281:** 32081-32088

Chen BPC, Uematsu N, Kobayashi J, Lerenthal Y, Krempler A, Yajima H, Lobrich M, Shiloh Y, Chen DJ (2007) Ataxia telangiectasia mutated (ATM) is essential for DNA-PKcs phosphorylations at the Thr-2609 cluster upon DNA double strand break. *Journal of Biological Chemistry* **282**(9)**:** 6582-6587

Chen LC, Nievera CJ, Lee AYL, Wu XH (2008) Cell cycle-dependent complex formation of BRCA1.CtIP.MRN is important for DNA double-strand break repair. *Journal of Biological Chemistry* **283**(12)**:** 7713-7720

Chini CCS, Chen J (2003) Human Claspin is required for replication checkpoint control. *Journal of Biological Chemistry* **278**(32)**:** 30057-30062

Chou DM, Elledge SJ (2006) Tipin and Timeless form a mutually protective complex required for genotoxic stress resistance and checkpoint function. *Proceedings of the National Academy of Sciences of the United States of America* **103**(48)**:** 18143-18147

Cimprich KA, Cortez D (2008) ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol* **9**(8)**:** 616-627

Cortez D, Glick G, Elledge SJ (2004) Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. *Proceedings of the National Academy of Sciences of the United States of America* **101**(27)**:** 10078-10083

Costanzo V, Shechter D, Lupardus PJ, Cimprich KA, Gottesman M, Gautier J (2003) An ATR- and Cdc7-Dependent DNA Damage Checkpoint that Inhibits Initiation of DNA Replication. *Molecular Cell* **11**(1)**:** 203-213

Czornak K, Chughtai S, Chrzanowska KH (2008) Mystery of DNA repair: the role of the MRN complex and ATM kinase in DNA damage repair *Journal of Applied Genetics* **49**(4)**:** 383-396

De Falco M, Ferrari E, De Felice M, Rossi M, Hubscher U, Pisani FM (2007) The human GINS complex binds to and specifically stimulates human DNA polymerase alpha-primase. *Embo Reports* **8**(1)**:** 99-103

Delacroix S, Wagner JM, Kobayashi M, Yamamoto K, L.M. K (2007) The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1. *Genes & Development* **21**(12)**:** 1472-1477

Deng YB, Guo XL, Ferguson DO, Chang S (2009) Multiple roles for MRE11 at uncapped telomeres. *Nature* **460**(7257)**:** 914-U157

Di Virgilio M, Ying CY, Gautier J (2009) PIKK-dependent phosphorylation of Mre11 induces MRN complex inactivation by disassembly from chromatin. *DNA Repair* **In Press, Corrected Proof**

Diffley JF (1996) Once and only once upon a time: specifying and regulating origins of DNA replication in eukaryotic cells. *Genes & Development* **10**(22)**:** 2819-2830

Diffley JFX (2004) Regulation of early events in chromosome replication. *Current Biology* **14**(18)**:** R778-R786

Dimitrova N, de Lange T (2009) Cell cycle-dependent role of MRN at dysfunctional telomeres: ATM signaling-dependent induction of nonhomologous end joining (NHEJ) in G(1) and resection-mediated inhibition of NHEJ in G(2). *Molecular and Cellular Biology* **29**(20)**:** 5552-5563

Dinkelmann M, Spehalski E, Stoneham T, Buis J, Wu YP, Sekiguchi JM, Ferguson DO (2009) Multiple functions of MRN in end-joining pathways during isotype class switching. *Nature Structural & Molecular Biology* **16**(8)**:** 808-U825

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

Ellison V, Stillman B (2003) Biochemical Characterization of DNA Damage Checkpoint Complexes: Clamp Loader and Clamp Complexes with Specificity for 5' Recessed DNA. *PLoS Biol* **1**(2)**:** e33

Elsasser S, Chi Y, Yang P, Campbell JL (1999) Phosphorylation controls timing of Cdc6p destruction: A biochemical analysis. *Molecular Biology of the Cell* **10**(10)**:** 3263-3277

Erkko H, Pylkas K, Karppinen SM, Winqvist R (2008) Germline alterations in the CLSPN gene in breast cancer families. *Cancer Letters* **261**(1)**:** 93-97

Errico A, Costanzo V, Hunt T (2007) Tipin is required for stalled replication forks to resume DNA replication after removal of aphidicolin in *Xenopus* egg extracts. *Proceedings of the National Academy of Sciences of the United States of America* **104**(38)**:** 14929-14934

Furnari B, Rhind N, Russell P (1997) Cdc25 mitotic inducer targeted by Chk1 DNA damage checkpoint kinase. *Science* **277**(5331)**:** 1495-1497

Gambus A, Jones RC, Sanchez-Diaz A, Kanemaki M, van Deursen F, Edmondson RD, Labib K (2006) GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. *Nature Cell Biology* **8**(4)**:** 358-U341

Garner E, Costanzo V (2009) Studying the DNA damage response using in vitro model systems. *DNA Repair* **8**(9)**:** 1025-1037

Gewurz BE, Harper JW (2006) DNA-damage control: Claspin destruction turns off the checkpoint. *Current Biology* **16**(21)**:** R932-R934

Gotter AL, Suppa C, Emanuel BS (2007) Mammalian TIMELESS and Tipin are evolutionarily conserved replication fork-associated factors. *Journal of Molecular Biology* **366**(1)**:** 36-52

Hartlerode AJ, Scully R (2009) Mechanisms of double-strand break repair in somatic mammalian cells. *Biochemical Journal* **423**(2)**:** 157-168

Hartwell LH, Kastan MB (1994) Cell-cycle control and cancer. *Science* **266**(5192)**:** 1821-1828

Hashimoto Y, Takisawa H (2003) *Xenopus* Cut5 is essential for a CDK-dependent process in the initiation of DNA replication. *EMBO Journal* **22**(10)**:** 2526-2535

Heffernan TP, Unsal-Kacmaz K, Heinloth AN, Simpson DA, Paules RS, Sancar A, Cordeiro-Stone M, Kaufmann WK (2007) Cdc7-dbf4 and the human S checkpoint response to UVC. *Journal of Biological Chemistry* **282**(13)**:** 9458-9468

Huertas P, Jackson SP (2009) Human CtIP Mediates Cell Cycle Control of DNA End Resection and Double Strand Break Repair. *Journal of Biological Chemistry* **284**(14)**:** 9558-9565

Im JS, Ki SH, Farina A, Jung S, Hurwitz J, Lee JK (2009) Assembly of the Cdc45-Mcm2-7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins. *Proceedings of the National Academy of Sciences of the United States of America* **106**(37)**:** 15628-15632

Jallepalli PV, Tien D, Kelly TJ (1998) sud1(+) Targets cyclin-dependent kinase-phosphorylated Cdc18 and Rum1 proteins for degradation and stops unwanted diploidization in fission yeast. *Proceedings of the National Academy of Sciences of the United States of America* **95**(14)**:** 8159-8164

Jares P, Donaldson A, Blow JJ (2000) The Cdc7/Dbf4 protein kinase: target of the S phase checkpoint? *EMBO Reports* **1**(4)**:** 319-322

Jazayeri A, Balestrini A, Garner E, Haber JE, Costanzo V (2008) Mre11-Rad50-Nbs1-dependent processing of DNA breaks generates oligonucleotides that stimulate ATM activity. *EMBO J* **27**(14)**:** 1953-1962

Jazayeri A, Falck J, Lukas C, Bartek J, Smith GCM, Lukas J, Jackson SP (2006) ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nature Cell Biology* **8**(1)**:** 37-U13

Jiang W, Wells NJ, Hunter T (1999) Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. *Proceedings of the National Academy of Sciences of the United States of America* **96**(11)**:** 6193-6198

Kaplan DL (2006) Replication termination: mechanism of polar arrest revealed. *Current Biology* **16**(17)**:** R684-686

Karppinen SM, Erkko H, Reini K, Pospiech H, Heikkinen K, Rapakko K, Syvaoja JE, Winqvist R (2006) Identification of a common polymorphism in the TopBP1 gene associated with hereditary susceptibility to breast and ovarian cancer. *European Journal of Cancer* **42**(15)**:** 2647-2652

Katou Y, Kanoh Y, Bando M, Noguchi H, Tanaka H, Ashikari T, Sugimoto K, Shirahige K (2003) S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature* **424**(6952)**:** 1078-1083

Kerzendorfer C, O'Driscoll M (2009) Human DNA damage response and repair deficiency syndromes: Linking genomic instability and cell cycle checkpoint proficiency. *DNA Repair* **8**(9)**:** 1139-1152

Kim SM, Kumagai A, Lee J, Dunphy WG (2005) Phosphorylation of Chk1 by ATM- and Rad3-related (ATR) in *Xenopus* egg extracts requires binding of ATRIP to ATR but not the stable DNA-binding or coiled-coil domains of ATRIP. *Journal of Biological Chemistry* **280**(46)**:** 38355-38364

Kubota Y, Takase Y, Komori Y, Hashimoto Y, Arata T, Kamimura Y, Araki H, Takisawa H (2003) A novel ring-like complex of *Xenopus* proteins essential for the initiation of DNA replication. *Genes & Development* **17**(9)**:** 1141-1152

Kumagai A, Dunphy WG (2000) Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in *Xenopus* egg extracts. *Molecular Cell* **6**(4)**:** 839-849

Kumagai A, Dunphy WG (2006) How cells activate ATR. *Cell Cycle* **5**(12)**:** 1265-1268

Kumagai A, Lee J, Yoo HY, Dunphy WG (2006) TopBP1 activates the ATR-ATRIP complex. *Cell* **124**(5)**:** 943-955

Lavin MF (2007) ATM and the Mre11 complex combine to recognize and signal DNA double-strand breaks. *Oncogene* **26**(56)**:** 7749-7758

Lee J, Kumagai A, Dunphy WG (2003) Claspin, a Chk1-regulatory protein, monitors DNA replication on chromatin independently of RPA, ATR, and Rad17. *Molecular Cell* **11**(2)**:** 329-340

Lee J, Kumagai A, Dunphy WG (2007) The Rad9-Hus1-Rad1 checkpoint interaction of TopBP1 with ATR. *Journal of Biological Chemistry* **282**(38)**:** 28036-28044

Lee SE, Moore JK, Holmes A, Umezu K, Kolodner RD, Haber JE (1998) *Saccharomyces* Ku70, Mre11/Rad50, and RPA proteins regulate adaptation to G2/M arrest after DNA damage. *Cell* **94**(3)**:** 399-409

Leroy C, Lee SE, Vaze MB, Ochsenbein F, Guerois R, Haber JE, Marsolier-Kergoat MC (2003) PP2C Phosphatases Ptc2 and Ptc3 are required for DNA checkpoint inactivation after a double-strand break. *Molecular Cell* **11**(3)**:** 827-835

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

Liu JS, Kuo SR, Melendy T (2006a) Phosphorylation of replication protein A by S-phase checkpoint kinases. *DNA Repair* **5**(3)**:** 369-380

Liu Q, Guntuku S, Cui X-S, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA, Elledge SJ (2000a) Chk1 is an essential kinase that is regulated by Atr and required for the G2/M DNA damage checkpoint. *Genes & Development* **14**(12)**:** 1448-1459

Liu QQ, Choe WC, Campbell JL (2000b) Identification of the *Xenopus laevis* homolog of *Saccharomyces cerevisiae* DNA2 and its role in DNA replication. *Journal of Biological Chemistry* **275**(3)**:** 1615-1624

Liu S, Bekker-Jensen S, Mailand N, Lukas C, Bartek J, Lukas J (2006b) Claspin operates downstream of TopBP1 to direct ATR signaling towards Chk1 activation. *Mol Cell Biol* **26**(16)**:** 6056-6064

Lopes M, Foiani M, Sogo JM (2006) Multiple mechanisms control chromosome integrity after replication fork uncoupling and restart at irreparable UV lesions. *Molecular Cell* **21**(1)**:** 15-27

Lou H, Komata M, Katou Y, Guan Z, Reis CC, Budd M, Shirahige K, Campbell JL (2008) Mrc1 and DNA Polymerase epsilon function together in linking DNA replication and the S phase checkpoint. *Molecular Cell* **32**(1)**:** 106-117

Lutzmann M, Méchali M (2008) MCM9 Binds Cdt1 and is required for the assembly of prereplication complexes. *Molecular Cell* **31**(2)**:** 190-200

MacDougall CA, Byun TS, Van C, Yee MC, Cimprich KA (2007) The structural determinants of checkpoint activation. *Genes & Development* **21**(8)**:** 898-903

Makiniemi M, Hillukkala T, Tuusa J, Reini K, Vaara M, Huang DQ, Pospiech H, Majuri I, Westerling T, Makela TP, Syvaoja JE (2001) BRCT domain-containing protein TopBP1 functions in DNA replication and damage response. *Journal of Biological Chemistry* **276**(32)**:** 30399-30406

Matsumoto S, Ogino K, Noguchi E, Russell P, Masai H (2005) Hsk1-Dfp1/Him1, the Cdc7-Dbf4 kinase in *Schizosaccharomyces pombe*, associates with Swi1, a component of the replication fork protection complex. *Journal of Biological Chemistry* **280**(52)**:** 42536-42542

Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, III, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP, Elledge SJ (2007)

ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316**(5828)**:** 1160-1166

Matsuoka S, Huang MX, Elledge SJ (1998) Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science* **282**(5395)**:** 1893-1897

Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K, Elledge SJ (2000) Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **97**(19)**:** 10389-10394

Maya-Mendoza A, Petermann E, Gillespie DAF, Caldecott KW, Jackson DA (2007) Chk1 regulates the density of active replication origins during the vertebrate S phase. *EMBO Journal* **26**(11)**:** 2719-2731

McElhinny SAN, Gordenin DA, Stith CM, Burgers PMJ, Kunkel TA (2008) Division of labor at the eukaryotic replication fork. *Molecular Cell* **30**(2)**:** 137-144

Medhurst AL, Warmerdam DO, Akerman I, Verwayen EH, Kanaar R, Smits VAJ, Lakin ND (2008) ATR and Rad17 collaborate in modulating Rad9 localisation at sites of DNA damage. *J Cell Sci* **121**(23)**:** 3933-3940

Mimura S, Masuda T, Matsui T, Takisawa H (2000) Central role for Cdc45 in establishing an initiation complex of DNA replication in *Xenopus* egg extracts. *Genes to Cells* **5**(6)**:** 439-452

Mohanty BK, Bairwa NK, Bastia D (2006) The Tof1p-Csm3p protein complex counteracts the Rrm3p helicase to control replication termination of *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America* **103**(4)**:** 897-902

Mordes DA, Glick GG, Zhao RX, Cortez D (2008) TopBP1 activates ATR through ATRIP and a PIKK regulatory domain. *Genes & Development* **22**(11)**:** 1478-1489

Mu JJ, Wang Y, Luo H, Leng M, Zhang JL, Yang T, Besusso D, Jung SY, Qin J (2007) A proteomic analysis of ataxia telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) substrates identifies the ubiquitin-proteasome system as a regulator for DNA damage checkpoints. *Journal of Biological Chemistry* **282**(24)**:** 17330-17334

Natsume T, Tanaka T (2009) Spatial regulation and organization of DNA replication within the nucleus. *Chromosome Research*

Nguyen VQ, Co C, Li JJ (2001) Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. *Nature* **411**(6841)**:** 1068-1073

Nimonkar AV, Ozsoy AZ, Genschel J, Modrich P, Kowalczykowski SC (2008) Human exonuclease 1 and BLM helicase interact to resect DNA and initiate DNA repair. *Proceedings of the National Academy of Sciences of the United States of America* **105**(44)**:** 16906-16911

Nishitani H, Lygerou Z (2002) Control of DNA replication licensing in a cell cycle. *Genes to Cells* **7**(6)**:** 523-534

Noguchi E, Noguchi C, Du LL, Russell P (2003) Swi1 prevents replication fork collapse and controls checkpoint kinase Cds1. *Molecular and Cellular Biology* **23**(21)**:** 7861-7874

Noguchi E, Noguchi C, McDonald WH, Yates JR, Russell P (2004) Swi1 and Swi3 are components of a replication fork protection complex in fission yeast. *Molecular and Cellular Biology* **24**(19)**:** 8342-8355

Oakley GG, Loberg LI, Yao JQ, Risinger MA, Yunker RL, Zernik-Kobak M, Khanna KK, Lavin MF, Carty MP, Dixon K (2001) UV-induced hyperphosphorylation of replication protein A depends on DNA replication and expression of ATM protein. *Molecular Biology of the Cell* **12**(5)**:** 1199-1213

Pacek M, Tutter AV, Kubota Y, Takisawa H, Walter JC (2006) Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. *Molecular Cell* **21**(4)**:** 581-587

Pavlov YI, Frahm C, McElhinny SAN, Niimi A, Suzuki M, Kunkel TA (2006) Evidence that errors made by DNA polymerase alpha are corrected by DNA polymerase delta. *Current Biology* **16**(2)**:** 202-207

Pellicioli A, Lee SB, Lucca C, Foiani M, Haber JE (2001) Regulation of *Saccharomyces* Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest. *Molecular Cell* **7**(2)**:** 293-300

Peng CY, Graves PR, Thoma RS, Wu ZQ, Shaw AS, PiwnicaWorms H (1997) Mitotic and G(2) checkpoint control: Regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science* **277**(5331)**:** 1501-1505

Perkins G, Drury LS, Diffley JFX (2001) Separate SCFCDC4 recognition elements target Cdc6 for proteolysis in S phase and mitosis. *EMBO Journal* **20**(17)**:** 4836-4845

Petermann E, Helleday T, Caldecott KW (2008) Claspin promotes normal replication fork rates in human cells. *Mol Biol Cell* **19**(6)**:** 2373-2378

Petersen BO, Lukas J, Sorensen CS, Bartek J, Helin K (1999) Phosphorylation of mammalian CDC6 by Cyclin A/CDK2 regulates its subcellular localization. *EMBO Journal* **18**(2)**:** 396-410

Pursell ZF, Isoz I, Lundstrom EB, Johansson E, Kunkel TA (2007) Yeast DNA polymerase epsilon participates in leading-strand DNA replication. *Science* **317**(5834)**:** 127-130

Rass E, Grabarz A, Plo I, Gautier J, Bertrand P, Lopez BS (2009) Role of Mre11 in chromosomal nonhomologous end joining in mammalian cells. *Nature Structural & Molecular Biology* **16**(8)**:** 819-U838

Saha P, Chen JJ, Thome KC, Lawlis SJ, Hou ZH, Hendricks M, Parvin JD, Dutta A (1998) Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. *Molecular and Cellular Biology* **18**(5)**:** 2758-2767

Sanchez Y, Wong C, Thoma RS, Richman R, Wu RQ, PiwnicaWorms H, Elledge SJ (1997) Conservation of the Chk1 checkpoint pathway in mammals: Linkage of DNA damage to Cdk regulation through Cdc25. *Science* **277**(5331)**:** 1497-1501

Sandell LL, Zakian VA (1993) Loss of a yeast telomere- arrest, recovery, and chromosome loss. *Cell* **75**(4)**:** 729-739

Sartori AA, Lukas C, Coates J, Mistrik M, Fu S, Bartek J, Baer R, Lukas J, Jackson SP (2007) Human CtIP promotes DNA end resection. *Nature* **450**(7169)**:** 509-U506

Shechter D, Costanzo V, Gautier J (2004) ATR and ATM regulate the timing of DNA replication origin firing. *Nature Cell Biology* **6**(7)**:** 648-655

Shimmoto M, Matsumoto S, Odagiri Y, Noguchi E, Russell P, Masai H (2009) Interactions between Swi1-Swi3, Mrc1 and S phase kinase, Hsk1 may regulate cellular responses to stalled replication forks in fission yeast. *Genes to Cells* **14**(6)**:** 669-682

Shiotani B, Zou L (2009) Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. *Molecular Cell* **33**(5)**:** 547-558

Singh DK, Ahn B, Bohr VA (2009) Roles of RECQ helicases in recombination based DNA repair, genomic stability and aging. *Biogerontology* **10**(3)**:** 235-252

Smith E, Dejsuphong D, Balestrini A, Hampel M, Lenz C, Takeda S, Vindigni A, Costanzo V (2009) An ATM- and ATR-dependent checkpoint inactivates spindle assembly by targeting CEP63. *Nat Cell Biol* **11**(3)**:** 278-285

Smolka MB, Albuquerque CP, Chen SH, Zhou H (2007) Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases. *Proceedings of the National Academy of Sciences of the United States of America* **104**(25)**:** 10364-10369

Sommariva E, Pellny TK, Karahan N, Kumar S, Huberman JA, Dalgaard JZ (2005) *Schizosaccharomyces pombe* Swi1, Swi3, and hskl are components of a novel S-phase

response pathway to alkylation damage. *Molecular and Cellular Biology* **25**(7)**:** 2770-2784

Sorensen CS, Hansen LT, Dziegielewski J, Syljuasen RG, Lundin C, Bartek J, Helleday T (2005) The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. *Nature Cell Biology* **7**(2)**:** 195-U121

Soutoglou E, Misteli T (2008) Activation of the cellular DNA damage response in the absence of DNA lesions. *Science* **320**(5882)**:** 1507-1510

Stiff T, Walker SA, Cerosaletti K, Goodarzi AA, Petermann E, Concannon P, O'Driscoll M, Jeggo PA (2006) ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. *EMBO Journal* **25**(24)**:** 5775-5782

Stokes MP, Rush J, MacNeill J, Ren JM, Sprott K, Nardone J, Yang V, Beausoleil SA, Gygi SP, Livingstone M, Zhang H, Polakiewicz RD, Comb MJ (2007) Profiling of UV-induced ATM/ATR signaling pathways. *Proceedings of the National Academy of Sciences of the United States of America* **104**(50)**:** 19855-19860

Stokes MP, Van Hatten R, Lindsay HD, Michael WM (2002) DNA replication is required for the checkpoint response to damaged DNA in *Xenopus* egg extracts. *J Cell Biol* **158**(5)**:** 863-872

Stucki M, Jackson SP (2006) gamma H2AX and MDC1: Anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair* **5**(5)**:** 534-543

Sung P, Klein H (2006) Mechanism of homologous recombination: mediators and helicases take on regulatory functions. *Nature Reviews Molecular Cell Biology* **7**(10)**:** 739-750

Syljuasen RG, Jensen S, Bartek J, Lukas J (2006) Adaptation to the ionizing radiation-induced G(2) checkpoint occurs in human cells and depends on checkpoint kinase 1 and Polo-like kinase 1 kinases. *Cancer Research* **66**(21)**:** 10253-10257

Szyjka SJ, Viggiani CJ, Aparicio OM (2005) Mrc1 is required for normal progression of replication forks throughout chromatin in *S. cerevisiae*. *Molecular Cell* **19**(5)**:** 691-697

Takeda S, Nakamura K, Taniguchi Y, Paull TT (2007) Ctp1/CtIP and the MRN complex collaborate in the initial steps of homologous recombination. *Molecular Cell* **28**(3)**:** 351-352

Tanaka H, Kubota Y, Tsujimura T, Kumano M, Masai H, Takisawa H (2009) Replisome progression complex links DNA replication to sister chromatid cohesion in *Xenopus* egg extracts. *Genes Cells* **14**(8)**:** 949-963

Toczyski DP, Galgoczy DJ, Hartwell LH (1997) CDC5 and CKII control adaptation to the yeast DNA damage checkpoint. *Cell* **90**(6)**:** 1097-1106

Tourriere H, Versini G, Cordon-Preciado V, Alabert C, Pasero P (2005) Mrc1 and Tof1 promote replication fork progression and recovery independently of Rad53. *Molecular Cell* **19**(5)**:** 699-706

Trenz K, Errico A, Costanzo V (2008) Plx1 is required for chromosomal DNA replication under stressful conditions. *EMBO Journal* **27**(6)**:** 876-885

Umezu K, Sugawara N, Chen C, Haber JE, Kolodner RD (1998) Genetic analysis of yeast RPA1 reveals its multiple functions in DNA metabolism. *Genetics* **148**(3)**:** 989-1005

Unsal-Kacmaz K, Chastain PD, Qu PP, Minoo P, Cordeiro-Stone M, Sancar A, Kaufmann WK (2007) The human Tim/Tipin complex coordinates an intra-S checkpoint response to UV that slows replication fork displacement. *Molecular and Cellular Biology* **27**(8)**:** 3131-3142

Van Hatten RA, Tutter AV, Holway AH, Khederian AM, Walter JC, Michael WM (2002) The *Xenopus* Xmus101 protein is required for the recruitment of Cdc45 to origins of DNA replication. *Journal of Cell Biology* **159**(4)**:** 541-547

Vaze MB, Pellicioli A, Lee SE, Ira G, Liberi G, Arbel-Eden A, Foiani M, Haber JE (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. *Molecular Cell* **10**(2)**:** 373-385

Walter J, Newport J (2000) Initiation of eukaryotic DNA replication: Origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. *Molecular Cell* **5**(4)**:** 617-627

Weinert T (1998) DNA damage and checkpoint pathways: Molecular anatomy and interactions with repair. *Cell* **94**(5)**:** 555-558

Wohlschlegel JA, Dhar SK, Prokhorova TA, Dutta A, Walter JC (2002) *Xenopus* Mcm10 binds to origins of DNA replication after Mcm2-7 and stimulates origin binding of Cdc45. *Molecular Cell* **9**(2)**:** 233-240

Xie AY, Kwok A, Scully R (2009) Role of mammalian Mre11 in classical and alternative nonhomologous end joining. *Nature Structural & Molecular Biology* **16**(8)**:** 814-U831

Xu X, Vaithiyalingam S, Glick GG, Mordes DA, Chazin WJ, Cortez D (2008) The basic cleft of RPA70N binds multiple checkpoint proteins, including RAD9, to regulate ATR signaling. *Molecular and Cellular Biology* **28**(24)**:** 7345-7353

Yan S, Michael WM (2009) TopBP1 and DNA polymerase-alpha directly recruit the 9-1-1 complex to stalled DNA replication forks. *Journal of Cell Biology* **184**(6)**:** 793-804

Yang XH, Shiotani B, Classon M, Zou L (2008) Chk1 and Claspin potentiate PCNA ubiquitination. *Genes & Development* **22**(9)**:** 1147-1152

Yanow SK, Gold DA, Yoo HY, Dunphy WG (2003) *Xenopus* Drf1, a regulator of Cdc7, displays checkpoint-dependent accumulation on chromatin during an S-phase arrest. *Journal of Biological Chemistry* **278**(42)**:** 41083-41092

Yoo HY, Jeong SY, Dunphy WG (2006) Site-specific phosphorylation of a checkpoint mediator protein controls its responses to different DNA structures. *Genes & Development* **20**(7)**:** 772-783

Yoo HY, Kumagai A, Shevchenko A, Dunphy WG (2004a) Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase. *Cell* **117**(5)**:** 575-588

Yoo HY, Kumagai A, Shevchenko A, Dunphy WG (2007) Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM. *Journal of Biological Chemistry* **282**(24)**:** 17501-17506

Yoo HY, Kumagai A, Shevchenko A, Dunphy WG (2009) The Mre11-Rad50-Nbs1 complex mediates activation of TopBP1 by ATM. *Molecular Biology of the Cell* **20**(9)**:** 2351-2360

Yoo HY, Shevchenko A, Dunphy WG (2004b) Mcm2 is a direct substrate of ATM and ATR during DNA damage and DNA replication checkpoint responses. *Journal of Biological Chemistry* **279**(51)**:** 53353-53364

Yoshizawa-Sugata N, Masai H (2007) Human Tim/Timeless-interacting protein, Tipin, is required for efficient progression of S phase and DNA replication checkpoint. *Journal of Biological Chemistry* **282**(4)**:** 2729-2740

Yu XC, Fu SA, Lai MY, Baer R, Chen JJ (2006) BRCA1 ubiquitinates its phosphorylation-dependent binding partners CtIP. *Genes & Development* **20**(13)**:** 1721-1726

Yun MH, Hiom K (2009) CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. *Nature* **459**(7245)**:** 460-U184

Zhao H, Piwnica-Worms H (2001) ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Mol Cell Biol* **21**(13)**:** 4129-4139

Zhu Z, Chung WH, Shim EY, Lee SE, Ira G (2008) Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell* **134**(6)**:** 981-994

Zou L, Elledge SJ (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* **300**(5625)**:** 1542-1548

Zou L, Stillman B (2000) Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. *Molecular and Cellular Biology* **20**(9)**:** 3086-3096

2 *Xenopus* Dna2 is a Helicase/Nuclease with Roles in DNA Replication and Double-Strand Break Processing

Karen E. Wawrousek, Barbara K. Fortini, Piotr Polaczek, Lu Chen, Qingquan Liu, William G. Dunphy, Judith L.Campbell

2.1 Abstract

Xenopus laevis egg extracts provide experimental approaches to study DNA replication and double-strand-break (DSB) repair that complement other systems. Here, we define specific mechanisms by which Dna2 participates in these processes. We establish that *Xenopus* Dna2 is a helicase, as well as a nuclease. We further show that Dna2 is actively recruited to DNA only after replication origin licensing and that Dna2 levels on chromatin increase after induction of DSBs. Dna2 co-localizes with RPA and binds the lagging strand replication proteins And-1 and Mcm10. Dna2 also interacts with the DSB repair and checkpoint proteins Nbs1 and ATM. In yeast, Dna2 nuclease is required for initial steps of 5′ resection at DSBs, and the order of arrival of proteins at DSBs suggests the same is true in *Xenopus*. Dna2 binds to DNA ends independently of MRN, but resection requires MRN. Mre11 nuclease inhibition delays both full Dna2 recruitment and resection. Although Dna2 is necessary for RPA loading at early time points, resection by Dna2 is not required for checkpoint induction, likely due to functionally redundant nucleases.

2.2 Introduction

Dna2 is a structurally well-conserved helicase and nuclease. Yeast Dna2 is an essential protein involved in removing RNA/DNA primers during Okazaki fragment processing (OFP), as well as resection of 5′ ends during the early steps of homology dependent repair of double-strand breaks (DSBs) (Budd et al, 2009). Functional genomic screens indicate additional roles in the maintenance of chromatin, nuclear structure, and telomere biogenesis (Budd et al, 2005). Thus, Dna2 is a major contributor to genomic stability in yeast, and an important remaining question is the extent to which its physiological functions are conserved in metazoans.

Work in *Xenopus, C. elegans*, and human cells suggests a conserved role for Dna2 in nuclear DNA replication (Duxin et al, 2009; Kim et al, 2005; Lee et al, 2003b; Liu et al, 2000). Yeast and human Dna2 (hDna2) both have the unusual feature of functioning as a DNA replication and repair protein in both the nucleus and mitochondrion (Copeland & Longley, 2008; Duxin et al, 2009; Zheng et al, 2008). Knockdown of hDna2 leads to growth arrest, accumulation of cells in G2, accumulation of tetraploid cells, and aberrant cell division resulting in the formation of inter-nuclear chromatin bridges, suggesting that hDna2 is necessary for the completion of nuclear DNA replication (Duxin et al, 2009). Like yeast Dna2, hDna2 may be involved in replication and repair of telomeres, since both human and *S. cerevisiae* Dna2 can bind telomeric G4 structures and unwind telomeric DNA structures (Masuda-Sasa et al, 2008). Consistent with a role in mitochondrial DNA replication and repair, hDna2 knockdown also results in delayed base excision repair in mitochondria (Duxin et al, 2009; Zheng et al, 2008).

We previously showed that depletion of *Xenopus* Dna2 from cell-free extracts led to a 90% reduction in replication of sperm chromatin, but not a complete absence of replication (Liu et al, 2000). This effect is consistent with yeast *dna2* hypomorphic mutant phenotypes, in which DNA replication is incomplete and low molecular weight nascent DNA intermediates, not full-length DNA, accumulate (Budd et al, 1995). *Xenopus* Dna2 may be required for DNA replication, but further evidence is required to determine whether or not it plays the same roles as in yeast.

Elegant studies with purified proteins and in nuclear extracts of *Xenopus* recently demonstrated that Dna2 is required for processing of DNA double-strand ends by 5′ resection, another function conserved between yeast and *Xenopus* (Budd & Campbell, 2009; Liao et al, 2008; Zhu et al, 2008). Dna2-dependent processing is required for single-strand annealing (SSA), which mimics the early steps of homology-dependent repair of a DSB. 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).

In this work, we extend our studies of the role of Dna2 in DNA replication and repair. We first demonstrate that Dna2 is a helicase; therefore, *Xenopus* Dna2 is a helicase-nuclease as in yeast and human. We confirm that Dna2 is a DNA replication protein; Dna2 binds chromatin during S phase in a regulated manner, and Dna2 interacts with proteins involved in lagging strand DNA replication. We also confirm and extend the data on the role of Dna2 in early steps of the response to DSBs. Namely, we find that Dna2 is recruited to DSBs after ATM and MRN, and with similar timing to RPA. Both

Dna2 and MRN are necessary for efficient resection of DSBs, but when Mre11 nuclease is inhibited and Dna2 is present, resection is delayed but not completely inhibited. Importantly, depletion of Dna2 from extracts does not prevent induction of the DNA damage checkpoint response, indicating that the main roles of Dna2 are in DNA replication and repair.

2.3 Results

2.3.1 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, raising the question of whether the helicase activity had been lost during evolution. Since Dna2 is a potent nuclease that degrades both standard helicase substrates and products, we increased the sensitivity of the helicase assay by mutating a key aspartate residue, D278, in the Dna2 nuclease domain 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 assayed for helicase activity. The helicase assay measures unwinding of a labeled oligonucleotide; this 52 nucleotide sequence has 22 bp of complementary sequence that is annealed to M13mp18 and has a 30 nucleotide noncomplementary tail. As shown in Figure 2.1, there is little if any residual nuclease activity in the protein, but there is significant accumulation of the free 52 nucleotide product. Thus, like yeast and human Dna2, *Xenopus* Dna2 is a combined helicase-nuclease.

2.3.2 Dna2 Associates with S-phase Chromatin

To clarify the role of Dna2 in DNA replication, we used the *Xenopus* cell-free extract system. We first examined the association of Dna2 with chromatin in S phase. We see that Dna2 does bind chromatin and accumulates on chromatin during DNA replication, 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 (Figure 2.2A).

We next determined the stage of replication fork assembly which is required for Dna2 binding. 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 Figure 2.2B, 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 strengthen 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. As shown in Figure 2.2C, 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 a 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 what is referred to as the pre-IC (pre-initiation 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 Figure 2.2D, Dna2 still associates with chromatin in the presence of p27. The ability to load after pre-RC formation but in the presence of p27 suggests that loading of Dna2 is similar to loading of other replication fork proteins such as Mcm10 and different from that of pre-IC proteins such as Cdc45. 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 DNA is being replicated, Dna2 co-localizes with RPA in numerous foci (Figure 2.3). Although RPA is known to form foci at sites of both DNA replication and DNA repair, co-localization of Dna2 and RPA on chromatin in the absence of DNA damage suggests that the foci observed are due to DNA replication complexes. The co-localization of Dna2 with RPA during DNA replication suggests that Dna2 travels with replication forks during DNA replication.

2.3.3 Dna2 Interacts with And-1 and Mcm10

We wished to define the subassemblies of replication proteins with which Dna2 interacts during S phase. To do so, we immunoprecipitated Dna2 from *Xenopus* extracts, isolated individual bands larger than 80 kD from a protein preparative gel, and identified the proteins by tandem mass spectrometry. A summary of results is provided in Figure 2.4A. The protein of greatest interest to us was And-1, the *Xenopus* ortholog of Ctf4 (chromosome transmission fidelity 4). In both yeast and *Xenopus*, And-1 (acidic nucleoplasmic DNA-binding protein 1) is necessary for both DNA replication and the establishment of sister chromatid cohesion and is known to physically interact with Mcm10 and DNA polymerase α (Tanaka et al, 2009b; Tsutsui et al, 2005; Zhu et al, 2007). In the yeast replication progression complex (RPC), it is thought to be part of the assembly that links the polymerase on the lagging strand to the replicative helicase, the MCM complex (Gambus et al, 2006; Tanaka et al, 2009a). In addition, *dna2 ctf4* double mutants display synthetic lethality in yeast (Budd et al, 2005; Formosa & Nittis, 1999). We therefore chose to further pursue this interaction (see below).

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. In yeast, 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). Pcm1 (pericentriolar material 1) is essential for the radial organization of microtubules and recruitment of proteins to the centrosome (Dammermann & 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). Other proteins involved in DNA replication, repair, and sister chromatid cohesion were identified in our analysis, but the scores of these proteins were below our cutoff. Some notable proteins were DNA polymerase κ, DNA polymerase α catalytic subunit, Smc5, Scc2-1a, Securin, and FFA-1/WRN. We cannot determine at this time whether their low abundance was due to migration in the gel between the bands that were actually excised or to non-specific and substoichiometric association with Dna2, so they will not be discussed further.

To verify the Dna2 and And-1 interaction, we carried out immunoprecipitations from interphase extracts. We see that Dna2 co-immunoprecipitates And-1 (Figure 2.4A and B). Conversely, anti-And-1 antibodies also co-immunoprecipitate Dna2 with And-1 from interphase extract (Figure 2.4C). DNA polymerase α, a lagging strand DNA polymerase, was also observed in the And-1 immunoprecipitate along with Dna2, consistent with the reported interaction between And-1 and DNA polymerase α (Zhu et al, 2007). Thus, these proteins interact even in the absence of DNA.

Although yeast Ctf4 is a component of the RPC (Gambus et al, 2006; Tanaka et al, 2009b), its precise roles in DNA replication, sister chromatid cohesion, and recombination are not clear. *Xenopus* Mcm10, an 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 (Zhu et al, 2007). As shown in Figure 2.4D and E, we find that Mcm10 and Dna2 co-immunoprecipitate, suggesting that Dna2 interacts with both Mcm10 and And-1 during DNA replication. The interaction of Dna2 with both

And-1 and Mcm10 indicates that Dna2 is a member of the complex of proteins involved in lagging strand replication.

In *Xenopus*, Mcm10 is required for chromatin loading of And-1 in S phase (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 anti-And-1 antibodies can be used for an immunoblock by simply adding anti-And-1 antibodies to the extract. Unfortunately, we were not able to replicate this And-1 immunoblock, since we still detected DNA polymerase α on chromatin in the And-1 immunoblock samples (unpublished data and Zhu et al, 2007). However, we were able to deplete Mcm10 from the extracts (Wohlschlegel et al, 2002). Interestingly, the association of Dna2 with chromatin does not appear to be dependent on Mcm10 (Figure 2.4F). We conclude that Dna2 interacts with an early intermediate in replication fork establishment, given that Mcm10 is loaded onto the pre-RC before Cdc45 (Wohlschlegel et al, 2002), and Dna2 associates with chromatin after pre-RC formation, independently of CDK2 phosphorylation and independently of Mcm10.

2.3.4 Dna2 Interacts with DSB Repair and Checkpoint Proteins

In response to DSBs, cells induce both specific repair pathways and checkpoints that inhibit cell cycle progression to allow time for repair to proceed. Biochemical experiments have shown that Dna2 is a major 5′-3′ nuclease in *Xenopus* cell-free extract and is required for 5′-3′ resection during homology dependent repair of DSBs by SSA in *Xenopus*, as it is in yeast (Budd & Campbell, 2009; Liao et al, 2008; Zhu et al, 2008). 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. We speculate that Dna2 acts in the early steps of recombination, so we asked if Dna2 interacts with ATM and Nbs1. As shown in Figure 2.5A, 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 extracts and therefore do not require DNA.

As mentioned earlier, when DSBs are induced, proteins involved in checkpoint signaling and DNA repair accumulate transiently on chromatin, and we were interested to see if Dna2 behaves similarly. To address this, PflM1, a restriction enzyme that produces a 3′ overhang, was used to induce DSBs in sperm chromatin, and the amount of Dna2 on chromatin was analyzed. Dna2 is found on PflM1 treated chromatin, with further accumulation on chromatin containing DSBs when the checkpoint is inhibited by caffeine or wortmannin (Figure 2.5B). 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, and we see a corresponding increase in ATM on damaged chromatin in the presence of checkpoint inhibitors.

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 & Campbell, 2009; Lisby et al, 2004; Shiotani & Zou, 2009; Shroff et al, 2004;

Zhu et al, 2008; Zou & Elledge, 2003). ATR is recruited after the generation of RPA-coated ssDNA, and TopBP1 is involved in the ATM-dependent activation of ATR (Shiotani & 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) (Figure 2.6A). Protein binding to the DNA ends was then monitored over time in interphase extract, with 3×10^{11} DNA ends/μl of extract (Figure 2.6B). 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 & Zou, 2009). MRN dissociation from breaks correlates with activation of the ATM kinase and progression of

DSB repair (Di Virgilio et al, 2009). MRN is expected to dissociate from the DNA ends slightly before Dna2 in yeast, consistent with our results (Lisby et al, 2004; Shroff et al, 2004; Zhu et al, 2008). 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.

The role of Dna2 in DSB processing may be cell cycle regulated. To determine the effect of cell cycle stage on association of Dna2 at DNA ends, we performed the same DNA end binding assay in CSF extracts, which mimic M phase, since the previous experiment was carried out in interphase extracts, which are in S phase (Figure 2.6C). Under these conditions, Dna2 associates with DNA ends after ATM and Nbs1, and with similar timing to RPA, consistent with results from interphase extract. 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.

2.3.5 Interplay of Nucleases: Dna2 and MRN

To confirm that Dna2 participates in DNA end processing, we used the accumulation of RPA at DNA ends in the bead-based assay as a measure of successful resection/processing of a DSB. When Dna2 is removed from the extract by immunodepletion, both ATM and Nbs1 can still associate with DNA ends. However, RPA does not accumulate on DNA ends to the same level as it does in the presence of Dna2 (Figure 2.7A), confirming that Dna2 is necessary for efficient DNA end processing,

though some residual processing may occur. This is consistent with the role of Dna2 in SSA (Liao et al, 2008).

In yeast, Dna2 can compensate for the loss of Mre11 nuclease activity, but not for the complete lack of Mre11 protein, in the repair of X-ray-induced DNA damage (Budd & Campbell, 2009). One possible role for the MRN complex is to recruit Dna2 to breaks, consistent with our observation that MRN seems to associate with DSBs before Dna2. To test this idea, extracts were depleted of Nbs1, which efficiently depletes the MRN complex (Yoo et al, 2009), and the 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 (Figure 2.7B). 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 (Figure 2.7C). 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, as in yeast, 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 (Figure 2.7B and C).

2.3.6 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 & Zou, 2009; Zou & 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. 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 oligos, Chk1 was efficiently phosphorylated (Figure 2.8A). Since the pA/T70 oligos 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 (Figure 2.8B), with the weaker signal for phospho-Chk2 due to a

weaker interaction with the antibody in the immunoblot (Guo & 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 (Figure 2.8C). Thus, 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, and activation of checkpoints in the absence of Dna2 implies that another nuclease(s) can compensate for the lack of Dna2.

2.4 Discussion

2.4.1 Dna2 is a DNA Replication Protein

In this work, we present evidence supporting our previous results suggesting that *Xenopus* Dna2 participates in chromosomal DNA replication, as does yeast and human Dna2. *Xenopus* Dna2 is recruited to chromatin in a regulated manner and binds chromatin in egg extracts with similar timing as 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. Dna2 associates with And-1 and Mcm10, proteins associated with

lagging strand replication, that also travel with the replication fork (Pacek et al, 2006; Yoshizawa-Sugata & 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 the case for the Mcm10 protein (Figures 2.1 and 2.3). 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 Cdc45. These findings may indicate that *Xenopus* Mcm10 and Dna2 interact with a similar intermediate in the formation of the replisome.

2.4.2 Dna2 is Likely Involved in Lagging Strand Replication

The interaction of Dna2 with And-1 and Mcm10 correlate with genetic interactions seen in yeast. 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 & Nittis, 1999; Miles & 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). We believe that the physical interactions that we have detected in *Xenopus* between Dna2, And-1, and Mcm10 explain genetic interactions seen in yeast, where such physical interactions

cannot be studied easily. 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 accepted role of Dna2 in yeast DNA replication is to assist the major Okazaki fragment processing nuclease, Fen1, in the removal of RNA/DNA primers on the lagging strand. 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 & 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 & Bielinsky, 2007; Ricke & Bielinsky, 2004). Yeast Ctf4, Mcm10, and DNA polymerase α are part of 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 & Bielinsky, 2004; Tanaka et al, 2009a; Zhu et al, 2007). The occurrence of these proteins in complexes that also contain Dna2 is consistent with the idea that Dna2 is involved in lagging strand events in *Xenopus*.

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

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.

2.4.3 Dna2 in DSB Repair

In addition to its role during lagging strand DNA replication, yeast Dna2 has been shown to play a role in 5′ to 3′ resection during the early steps of DSB repair (Budd & Campbell, 2009; Zhu et al, 2008). Evidence for a similar role in *Xenopus* is also strong (Liao et al, 2008). DSBs activate homologous recombination pathways and the DNA damage checkpoint. Our finding that Dna2 physically interacts with ATM and Nbs1, (Figure 2.5A), which are both recruited to and accumulate at DSBs, stimulated us to investigate the role of Dna2 in the homologous recombination and DNA damage checkpoint pathways. DSB repair and checkpoint proteins associate with and dissociate from DSBs in a specific temporal order (Shiotani & Zou, 2009). Our finding that Dna2 accumulates slightly after ATM and Nbs1 and with similar timing to RPA is consistent with the documented role of Dna2 in resection (Liao et al, 2008). This is similar to the ordered binding that is observed in *S. cerevisiae* (Lisby et al, 2004; Shroff et al, 2004; Zhu et al, 2008). 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. 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 & Campbell, 2009; Llorente & 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 & Campbell, 2009; Zhu et al, 2008). The non-nucleolytic role of Mre11 is a 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 it was significantly delayed. We speculate this delayed RPA accumulation is due to other nucleases, possibly Dna2, compensating for the lack of Mre11 nuclease activity, as this clearly happens in yeast. Alternatively, we cannot rule out that mirin may not fully inhibit Mre11 nuclease activity, and we are detecting residual activity.

2.4.4. DNA Damage Checkpoint Activation

The DSB checkpoint first activates the ATM kinase, which subsequently activates the ATR kinase (Shiotani & 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 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 (Figure 2.8). Therefore, neither the Dna2 protein itself nor the enzymatic activities of Dna2 are 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), which also suggests functional redundancy in the nucleases participating in resection. We observed minimal RPA binding to DNA ends in Dna2-depleted extracts in our bead-based assay (Figure 2.7A), but limitations of this assay restrict its usage for early time-points. However, in the checkpoint assays in which aphidicolin or PflM1 is added to induce checkpoint activation, we observed 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. We find that both the DNA replication and DSB repair activities are similar to those in yeast. While there may potentially be some differences between metazoan and yeast Dna2, such as the timing of association with origins of replication and the nature of the interaction with the Mre11 nuclease, the diverse roles of Dna2 in DNA metabolism are evolutionarily conserved.

2.5 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 oligos or 25 μg/ml linear pBluescript (Guo & Dunphy, 2000; Kumagai & 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 (Liu et al, 2000). Antibodies recognizing DNA polymerase α p70 subunit, RPA70, Cdc45, Claspin, Orc2, ATM, BLM, Nbs1, Chk2, ATR, and TopBP1 were previously described (Guo & Dunphy, 2000; Kumagai & Dunphy, 2006; Lee et al, 2003a; Li et al, 2004; 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, anti-Mcm10 antibodies were a gift of J. Walter, and anti-Mcm3, anti-Cdc6, and anti-RPA70 antibodies used for immunofluorescence were a gift of P. Jackson. Production of recombinant *Xenopus* Dna2 is described in 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, 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.

Bead experiments. DNA-bead binding experiments were modified from previously published assays (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-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.

2.6 Acknowledgements

We thank Dr. A. Eastman (Dartmouth College) for the gift of validated mirin and helpful discussions, Dr. A. Dutta (University of Virginia) for the gift of antibodies recognizing *Xenopus* And-1, Dr. J. Walter (Harvard University) for the gift of antibodies recognizing *Xenopus* Mcm10, and Dr. P. Jackson for the gift of anti-Mcm3, anti-RPA70, and anti-Cdc6 antibodies. We thank Sonja Hess of the Proteome Exploration Laboratory at Caltech for performing our mass spectrometry work, and we also thank Jules Chen for the immunofluorescence work in Figure 2.3. This work was supported by NIH grants GM043974 and GM070891 to W.G.D. and GM078666 to J.L.C.

2.7 Figures

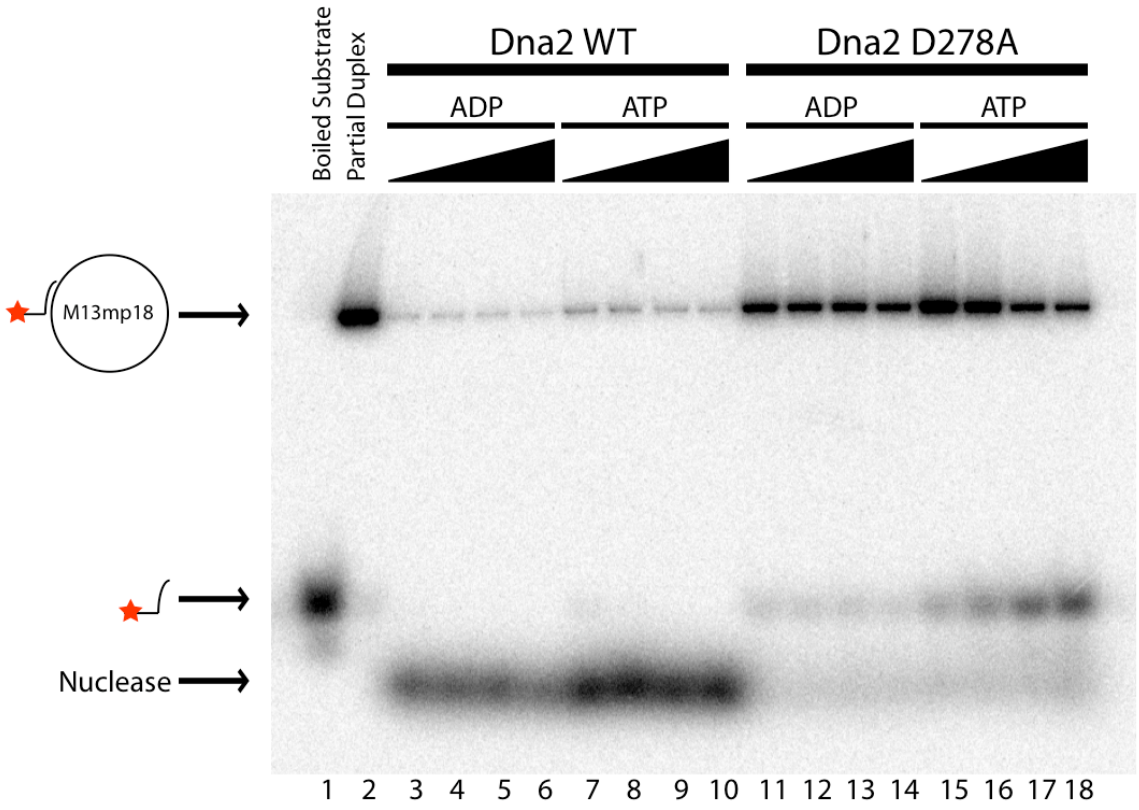

Figure 2.1: Helicase activity of *Xenopus* Dna2. Wild-type *Xenopus* Dna2 (lanes 3 to 10) and nuclease-deficient *Xenopus* Dna2 D278A (lanes 11 to 18) were incubated in helicase assay conditions with approximately 1 fmol of annealed M13-HPR substrate at 37°C for 15 min, either in the presence of ADP (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: approximately 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 proteins were added in lanes 1 and 2. The reaction in lane 1 was boiled for 4 min, and 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.

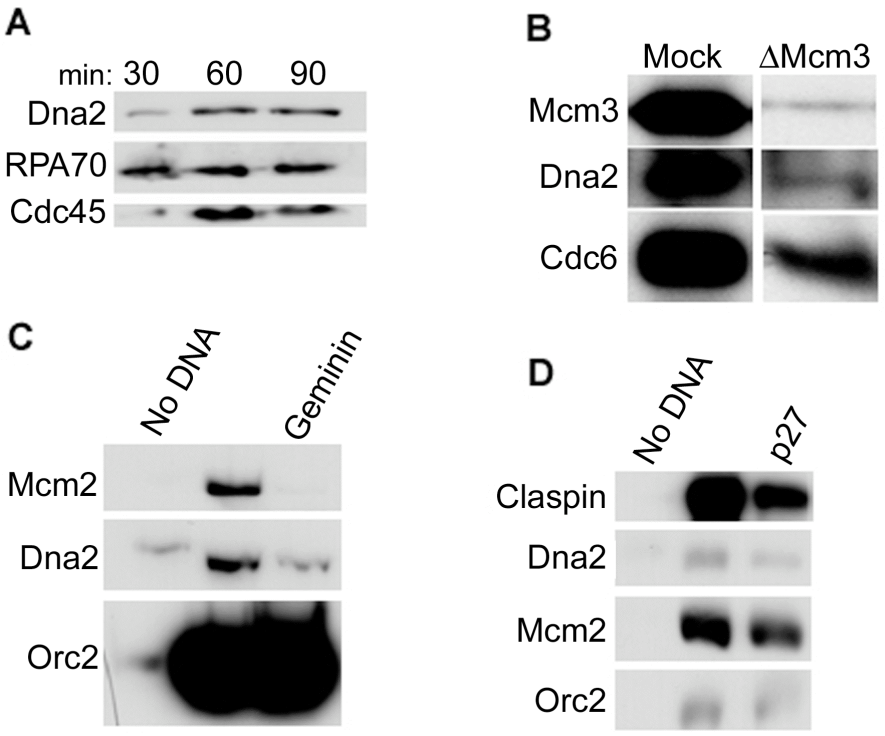

Figure 2.2: Dna2 associates with S-phase chromatin. A) Dna2 accumulation on sperm chromatin throughout S phase. Sperm chromatin was incubated in cell-free extract at 3,000 sperm/μl, isolated at indicated time-points throughout DNA replication and analyzed by immunblotting. DNA replication begins at 30 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, 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. 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.

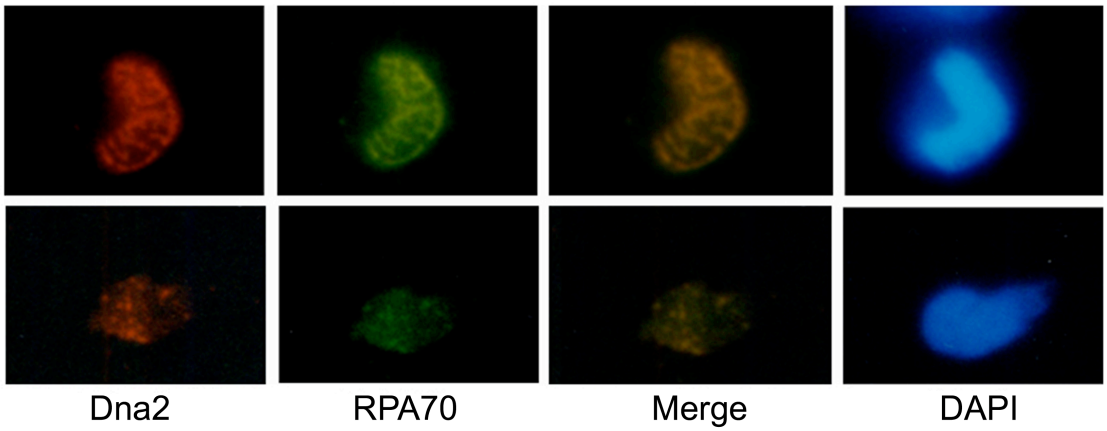

Figure 2.3: Dna2 foci during DNA replication. Sperm chromatin was incubated in extract, fixed, centrifuged onto coverslips, and subjected to immunofluorescence with antibodies to Dna2 and RPA. DNA was stained with DAPI.

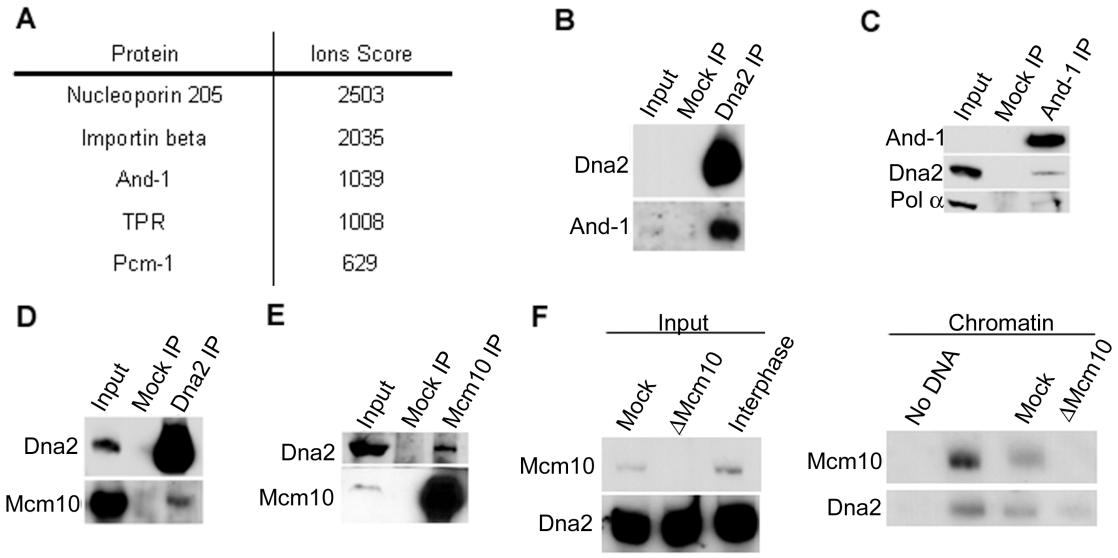

Figure 2.4: Dna2 interacts with DNA replication fork proteins. A) Results from electrospray ionization tandem mass spectrometry analysis of Dna2 immunoprecipitates from interphase extract. Only hits with an ion score above 500 are listed. B) Control IgG (Mock) and anti-Dna2 antibodies were used for immunoprecipitations from interphase extracts, and samples were analyzed by immunoblotting. C) Immunoprecipitations from interphase extract were performed with control or anti-And-1 antibodies, and immunoprecipitates 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, and chromatin-associated proteins were analyzed by immunoblotting.

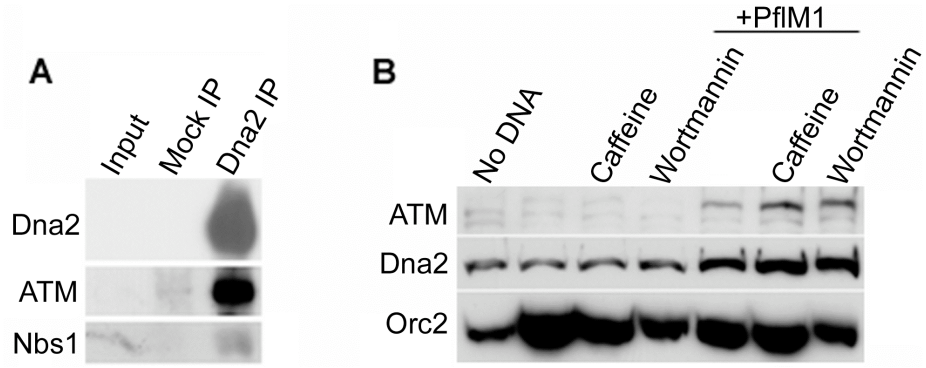

Figure 2.5: Dna2 and double-strand breaks. A) Dna2 interacts with DSB proteins. Anti-Dna2 antibodies were used to immunoprecipitate Dna2 from interphase extract, and immunoblots were performed for Dna2, ATM, and Nbs1. B) Dna2 accumulates on damaged chromatin. Chromatin-association of proteins was analyzed in the absence or presence of induced double-strand breaks (0.1 units/μl PflM1) and inhibited checkpoint (5mM caffeine, 0.1mM wortmannin). Chromatin fractions were isolated from extract and protein levels on chromatin were analyzed by immunoblotting.

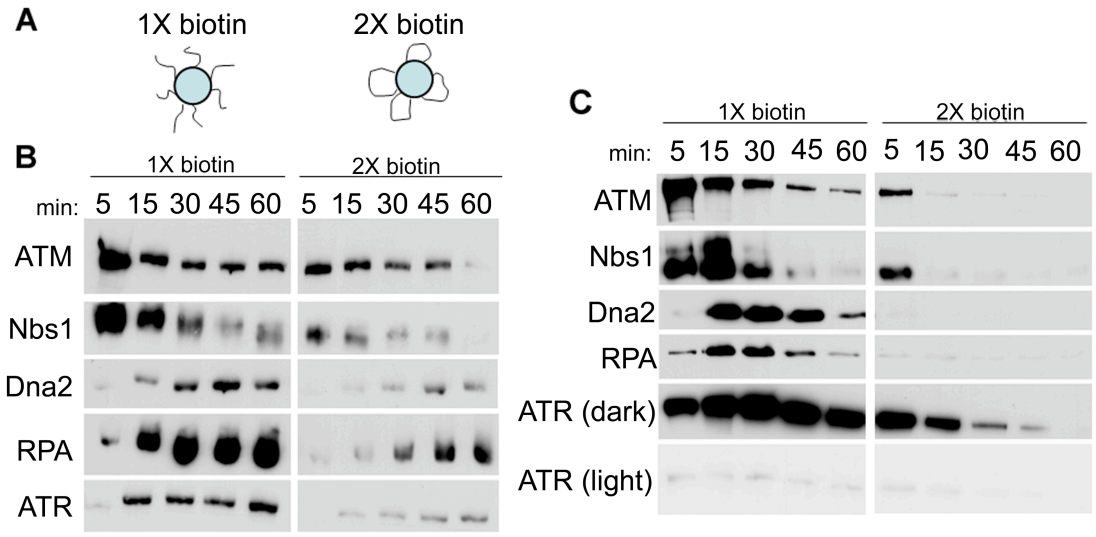

Figure 2.6: Dna2 at DNA ends. A) Schematic of beads used for experiments. pBluescriptIIKS- was linearized and biotinylated on one or both ends, and bound to streptavidin beads. These beads simulated unbroken DNA or DNA with a DSB. B) Time-course of binding of DSB repair and checkpoint proteins to DNA 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, except in CSF, not interphase, extract.

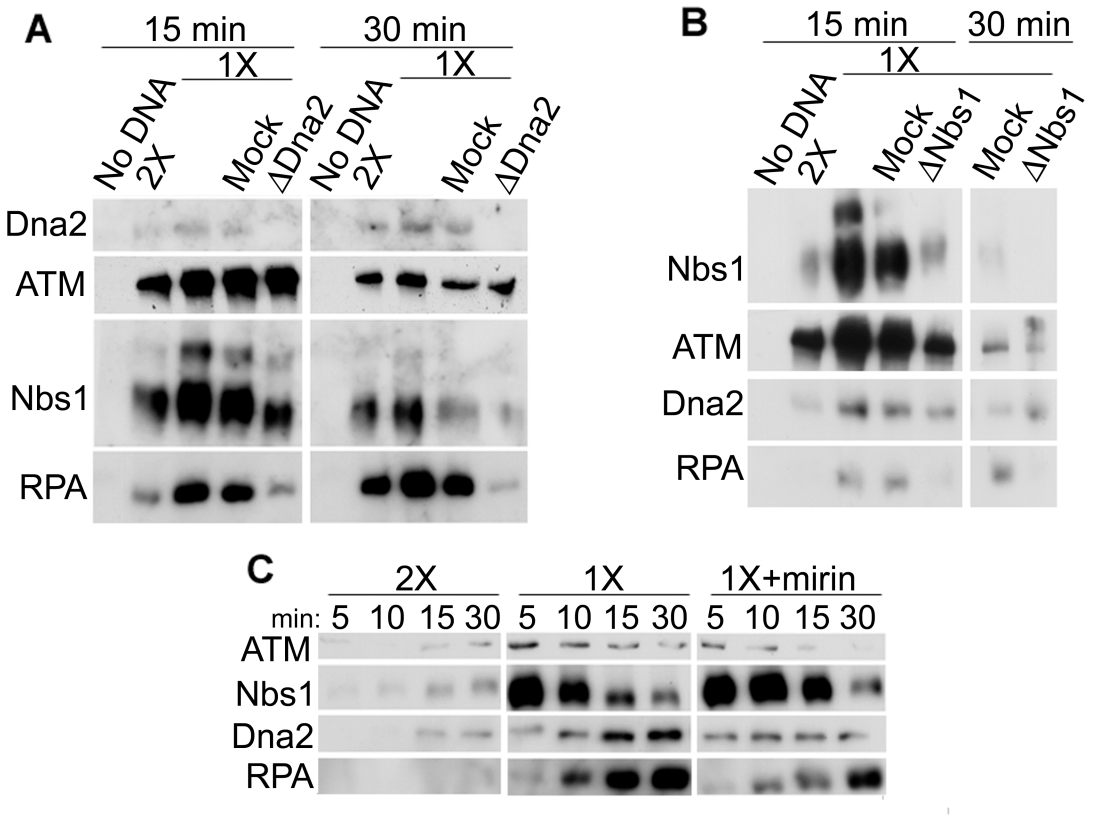

Figure 2.7: Dna2 and MRN at DNA ends. A) Effect of Dna2 depletion on processing of DNA ends. Interphase extracts were untreated, mock, or Dna2-depleted, and incubated with the appropriate beads for 15 or 30 min. Beads were isolated and protein binding was assessed by immunoblotting. B) DNA end binding 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.

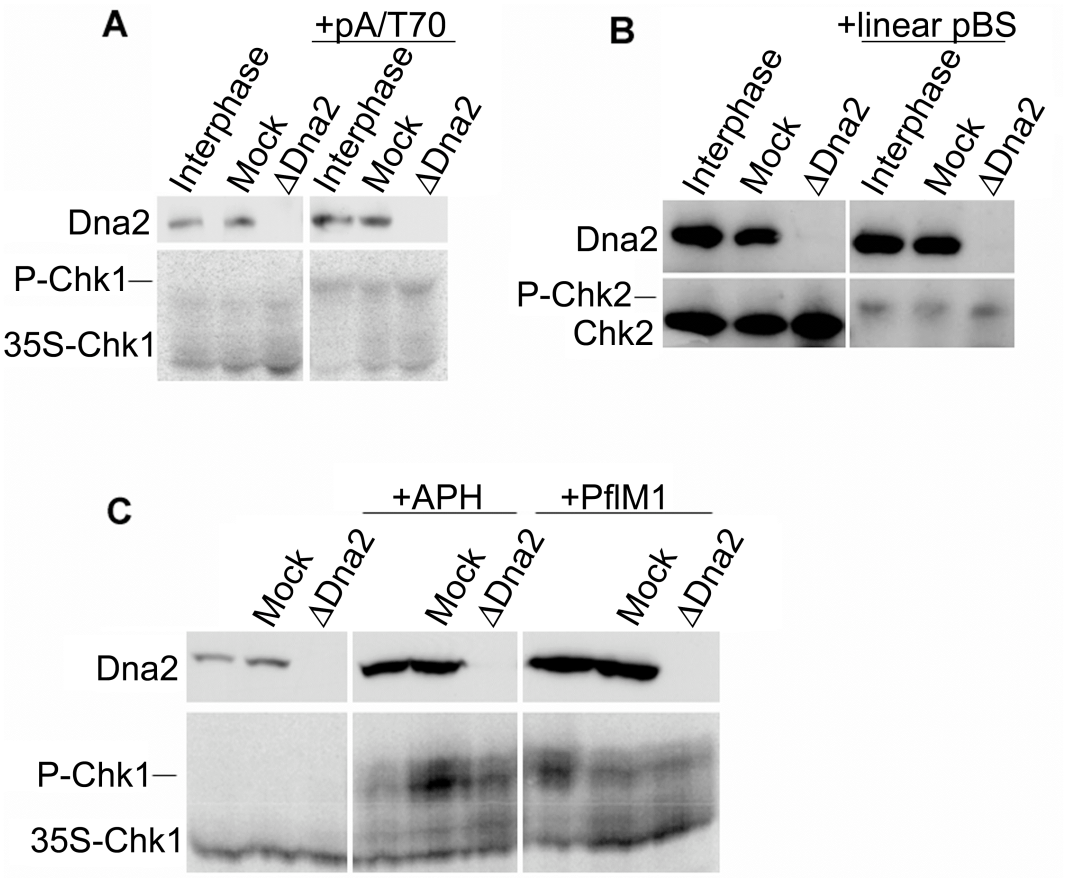

Figure 2.8: Assessment of the DNA replication checkpoint in Dna2-depleted extracts. For all panels in this figure, interphase extracts were untreated, mock, or Dna2-depleted. A) Phospho-Chk1 in Dna2-depleted extracts. pA/T70 oligos were added to interphase extract to elicit a checkpoint response. The electrophoretic mobility of ^{35}S-Chk1 was monitored by autoradiography, and ^{35}S-Chk1 is well phosphorylated, as indicated by the arrow. B) Phospho-Chk2 in extracts lacking Dna2. Linear pBluescript was added to the extract to elicit the DSB checkpoint response, and immunoblotting was used to assess activation of the checkpoint by monitoring Chk2 phosphorylation. C) The checkpoint response to stalled replication forks and DSBs was assessed in nuclei using APH and PflM1, respectively. Sperm chromatin was incubated in extracts without or with APH, or without or with PflM1 to induce replication fork stalling or DSBs, respectively. Nuclei were isolated. Dna2 levels in nuclei were assessed by immunoblotting, while ^{35}S-Chk1 electrophoretic mobility was assessed by SDS-PAGE and autoradiography.

2.8 References

Araki Y, Kawasaki Y, Sasanuma H, Tye BK, Sugino A (2003) Budding yeast mcm10 /dna43 mutant requires a novel repair pathway for viability. *Genes to Cells* **8**(5)**:** 465-480

Balczon R, Simerly C, Takahashi D, Schatten G (2002) Arrest of cell cycle progression during first interphase in murine zygotes microinjected with anti-PCM-1 antibodies. *Cell Motility and the Cytoskeleton* **52**(3)**:** 183-192

Budd M, Campbell J (2009) Interplay of Mre11 nuclease with Dna2 plus Sgs1 in Rad51-dependent recombinational repair. *Plos One* **4**(1)**:** e4267

Budd ME, Campbell JL (2000) The pattern of sensitivity of yeast dna2 mutants to DNA damaging agents suggests a role in DSB and postreplication repair pathways. *Mutation Research-DNA Repair* **459**(3)**:** 173-186

Budd ME, Choe W-C, Campbell JL (1995) DNA2 encodes a DNA helicase essential for replication of eukaryotic chromosomes. *Journal of Biological Chemistry* **270**(45)**:** 26766-26769

Budd ME, Cox LS, Campbell JL (2009) Coordination of nucleases and helicases during DNA replication and double-strand break repair. In *Molecular Themes in DNA Replication*, Cox LS (ed). London: RSC Publishing

Budd ME, Tong AHY, Polaczek P, Peng X, Boone C, Campbell JL (2005) A network of multi-tasking proteins at the DNA replication fork preserves genome stability. *Plos Genetics* **1**(6)**:** 634-650

Byrd DA, Sweet DJ, Pante N, Konstantinov KN, Guan T, Saphire AC, Mitchell PJ, Cooper CS, Aebi U, Gerace L (1994) Tpr, a large coiled coil protein whose amino terminus is involved in activation of oncogenic kinases, is localized to the cytoplasmic surface of the nuclear pore complex. *J Cell Biol* **127**(6)**:** 1515-1526

Carpenter PB, Mueller PR, Dunphy WG (1996) Role for a Xenopus Orc2-related protein in controlling DNA replication. *Nature* **379**(6563)**:** 357-360

Chattopadhyay S, Bielinsky AK (2007) Human Mcm10 regulates the catalytic subunit of DNA polymerase-alpha and prevents DNA damage during replication. *Molecular Biology of the Cell* **18**(10)**:** 4085-4095

Copeland WC, Longley MJ (2008) DNA2 Resolves Expanding Flap in Mitochondrial Base Excision Repair. *Molecular Cell* **32**(4)**:** 457-458

Dammermann A, Merdes A (2002) Assembly of centrosomal proteins and microtubule organization depends on PCM-1. *J Cell Biol* **159**(2)**:** 255-266

Di Virgilio M, Ying CY, Gautier J (2009) PIKK-dependent phosphorylation of Mre11 induces MRN complex inactivation by disassembly from chromatin. *DNA Repair* **In Press, Corrected Proof**

Dupre A, Boyer-Chatenet L, Sattler RM, Modi AP, Lee JH, Nicolette ML, Kopelovich L, Jasin M, Baer R, Paull TT, Gautier J (2008) A forward chemical genetic screen reveals an inhibitor of the Mre11-Rad50-Nbs1 complex. *Nature Chemical Biology* **4**(2)**:** 119-125

Duxin JP, Dao B, Martinsson P, Rajala N, Guittat L, Campbell JL, Spelbrink JN, Stewart SA (2009) Human Dna2 is a nuclear and mitochondrial DNA maintenance protein. *Molecular and Cellular Biology* **29**(15)**:** 4274-4282

Formosa T, Nittis T (1999) Dna2 mutants reveal interactions with Dna polymerase alpha and Ctf4, a Pol alpha accessory factor, and show that full Dna2 helicase activity is not essential for growth. *Genetics* **151**(4)**:** 1459-1470

Gambus A, Jones RC, Sanchez-Diaz A, Kanemaki M, van Deursen F, Edmondson RD, Labib K (2006) GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. *Nature Cell Biology* **8**(4)**:** 358-341

Garner KM, Pletnev AA, Eastman A (2009) Corrected structure of mirin, a small-molecule inhibitor of the Mre11-Rad50-Nbs1 complex. *Nature Chemical Biology* **5**(3)**:** 129-130

Guo ZJ, Dunphy WG (2000) Response of *Xenopus* Cds1 in cell-free extracts to DNA templates with double-stranded ends. *Molecular Biology of the Cell* **11**(5)**:** 1535-1546

Hames RS, Crookes RE, Straatman KR, Merdes A, Hayes MJ, Faragher AJ, Fry AM (2005) Dynamic recruitment of Nek2 kinase to the centrosome involves microtubules, PCM-1, and localized proteasomal degradation. *Molecular Biology of the Cell* **16**(4)**:** 1711-1724

Kim DH, Lee KH, Kim JH, Ryu GH, Bae SH, Lee BC, Moon KY, Byun SM, Koo HS, Seo YS (2005) Enzymatic properties of the *Caenorhabditis elegans* Dna2 endonuclease/helicase and a species-specific interaction between RPA and Dna2. *Nucleic Acids Research* **33**(4)**:** 1372-1383

Kumagai A, Dunphy WG (2000) Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in *Xenopus* egg extracts. *Molecular Cell* **6**(4)**:** 839-849

Kumagai A, Dunphy WG (2006) How cells activate ATR. *Cell Cycle* **5**(12)**:** 1265-1268

Lee J, Kumagai A, Dunphy WG (2003a) Claspin, a Chk1-Regulatory Protein, Monitors DNA Replication on Chromatin Independently of RPA, ATR, and Rad17. *Molecular Cell* **11**(2)**:** 329-340

Lee K-H, Lee M, Lee T, Han J, Park Y, Ahnn J, Seo Y-S, Koo H-S (2003b) Dna2 requirement for normal reproduction of *Caenorhabditis elegans* is temperature-dependent. *Molecules and Cells* **15**(1)**:** 81-86

Li W, Kim S-M, Lee J, Dunphy WG (2004) Absence of BLM leads to accumulation of chromosomal DNA breaks during both unperturbed and disrupted S phases. *J Cell Biol* **165**(6)**:** 801-812

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

Lisby M, Barlow JH, Burgess RC, Rothstein R (2004) Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins. *Cell* **118**(6)**:** 699-713

Liu QQ, Choe WC, Campbell JL (2000) Identification of the *Xenopus laevis* homolog of *Saccharomyces cerevisiae* DNA2 and its role in DNA replication. *Journal of Biological Chemistry* **275**(3)**:** 1615-1624

Llorente B, Symington LS (2004) The Mre11 nuclease is not required for 5' to 3' resection at multiple HO-induced double-strand breaks. *Mol Cell Biol* **24**(21)**:** 9682-9694

Loeillet S, Palancade B, Cartron M, Thierry A, Richard G-F, Dujon B, Doye V, Nicolas A (2005) Genetic network interactions among replication, repair and nuclear pore deficiencies in yeast. *DNA Repair* **4**(4)**:** 459-468

Masuda-Sasa T, Polaczek P, Peng XP, Chen L, Campbell JL (2008) Processing of G4 DNA by Dna2 helicase/nuclease and replication protein a (RPA) provides insights into the mechanism of Dna2/RPA substrate recognition. *Journal of Biological Chemistry* **283**(36)**:** 24359-24373

Miles J, Formosa T (1992) Protein affinity chromatography with purified yeast DNA polymerase alpha detects proteins that bind to DNA polymerase. *PNAS* **89**(4)**:** 1276-1280

Murray AW (1991) Cell cycle extracts. *Methods Cell Biol* **36:** 581-605

Nishiyama A, Muraki K, Saito M, Ohsumi K, Kishimoto T, Ishikawa F (2006) Cell-cycle-dependent Xenopus TRF1 recruitment to telomere chromatin regulated by Polo-like kinase. *EMBO J* **25**(3)**:** 575-584

Pacek M, Tutter AV, Kubota Y, Takisawa H, Walter JC (2006) Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. *Molecular Cell* **21**(4)**:** 581-587

Postow L, Ghenoiu C, Woo EM, Krutchinsky AN, Chait BT, Funabiki H (2008) Ku80 removal from DNA through double strand break-induced ubiquitylation. *J Cell Biol* **182**(3)**:** 467-479

Ricke RM, Bielinsky AK (2004) Mcm10 regulates the stability and chromatin association of DNA polymerase-alpha. *Molecular Cell* **16**(2)**:** 173-185

Shiotani B, Zou L (2009) Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. *Molecular Cell* **33**(5)**:** 547-558

Shroff R, Arbel-Eden A, Pilch D, Ira G, Bonner WM, Petrini JH, Haber JE, Lichten M (2004) Distribution and dynamics of chromatin modification induced by a defined DNA double-strand break. *Current Biology* **14**(19)**:** 1703-1711

Srsen V, Gnadt N, Dammermann A, Merdes A (2006) Inhibition of centrosome protein assembly leads to p53-dependent exit from the cell cycle. *J Cell Biol* **174**(5)**:** 625-630

Stewart JA, Miller AS, Campbell JL, Bambara RA (2008) Dynamic removal of replication protein A by Dna2 facilitates primer cleavage during Okazaki fragment

processing in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **283**(46)**:** 31356-31365

Tanaka H, Katou Y, Yagura M, Saitoh K, Itoh T, Araki H, Bando M, Shirahige K (2009a) Ctf4 coordinates the progression of helicase and DNA polymerase alpha. *Genes to Cells* **14**(7)**:** 807-820

Tanaka H, Kubota Y, Tsujimura T, Kumano M, Masai H, Takisawa H (2009b) Replisome progression complex links DNA replication to sister chromatid cohesion in *Xenopus* egg extracts. *Genes Cells* **14**(8)**:** 949-963

Tsutsui Y, Morishita T, Natsume T, Yamashita K, Iwasaki H, Yamao F, Shinagawa H (2005) Genetic and physical interactions between *Schizosaccharomyces pombe* Mcl1 and Rad2, Dna2 and DNA polymerase α: evidence for a multifunctional role of Mcl1 in DNA replication and repair. *Current Genetics* **48**(1)**:** 34-43

Wasko BM, Holland CL, Resnick MA, Lewis LK (2009) Inhibition of DNA double-strand break repair by the Ku heterodimer in mrx mutants of *Saccharomyces cerevisiae*. *DNA Repair* **8**(2)**:** 162-169

Wohlschlegel JA, Dhar SK, Prokhorova TA, Dutta A, Walter JC (2002) *Xenopus* Mcm10 binds to origins of DNA replication after Mcm2-7 and stimulates origin binding of Cdc45. *Molecular Cell* **9**(2)**:** 233-240

Yoo HY, Kumagai A, Shevchenko A, Dunphy WG (2007) Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM. *Journal of Biological Chemistry* **282**(24)**:** 17501-17506

Yoo HY, Kumagai A, Shevchenko A, Dunphy WG (2009) The Mre11-Rad50-Nbs1 complex mediates activation of TopBP1 by ATM. *Molecular Biology of the Cell* **20**(9)**:** 2351-2360

Yoo HY, Shevchenko A, Shevchenko A, Dunphy WG (2004) Mcm2 is a direct substrate of ATM and ATR during DNA damage and DNA replication checkpoint responses. *Journal of Biological Chemistry* **279**(51)**:** 53353-53364

Yoshizawa-Sugata N, Masai H (2009) Roles of human AND-1 in chromosome transactions in S phase. *Journal of Biological Chemistry* **284**(31)**:** 20718-20728

Zheng L, Zhou MA, Guo ZG, Lu HM, Qian LM, Dai HF, Qiu JZ, Yakubovskaya E, Bogenhagen DF, Demple B, Shen BH (2008) Human DNA2 is a mitochondrial nuclease/helicase for efficient processing of DNA replication and repair intermediates. *Molecular Cell* **32**(3)**:** 325-336

Zhu W, Ukomadu C, Jha S, Senga T, Dhar SK, Wohlschlegel JA, Nutt LK, Kornbluth S, Dutta A (2007) Mcm10 and And-1/CTF4 recruit DNA polymerase alpha to chromatin for initiation of DNA replication. *Genes & Development* **21**(18)**:** 2288-2299

Zhu Z, Chung WH, Shim EY, Lee SE, Ira G (2008) Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell* **134**(6)**:** 981-994

Zou L, Elledge SJ (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* **300**(5625)**:** 1542-1548

3 The Tim/Tipin Complex Participates in the DNA Replication Checkpoint and is a Target of Multiple Checkpoint Kinases

3.1 Abstract

Tim and Tipin participate in DNA replication, checkpoints, and sister chromatid cohesion. In this work, we characterize *Xenopus* Tim and Tipin; like the yeast homologs Tof1 and Csm3, respectively, Tim and Tipin form a complex and associate with DNA during S phase. Tim and Tipin interact with various DNA replication and checkpoint proteins, including Orc2, Claspin, ATR, ATRIP, RFC40, Rad17, Cdc7, and Drf1. Efficient phosphorylation of Chk1 upon replication fork stress requires Tipin, and it is Tipin that is a target of the ATR kinase upon checkpoint activation. We also find that Tipin is additionally a target of the Cdc7 kinase, which is active during the early stages of DNA replication and recently reported to be involved in the checkpoint response to stalled replication forks in yeast. We conclude that many roles of Tim and Tipin are well-conserved from yeast, and that Tim/Tipin both participates in and is a target of the DNA replication checkpoint.

3.2 Introduction

Faithful replication of the genome is essential to prevent cells from acquiring deleterious mutations, and the interplay of many proteins is required to maintain this fidelity. Studies in yeast indicate that Mrc1 (Mediator of Replication Checkpoint 1), Tof1 (Topoisomerase 1-associated Factor 1), and Csm3 (Chromosome segregation in meiosis 3) form a replication fork protection complex (RPC); these proteins are not essential for cell viability, but they do contribute to replication fork stability and the maintenance of genomic integrity. In the absence of any of these three proteins, there is increased DNA replication fork stalling and collapse, decreased sister chromatid cohesion, and cells no longer pause the cell cycle in response to DNA replication stress (Alcasabas et al, 2001; Foss, 2001; Katou et al, 2003; Krings & Bastia, 2004; Mayer et al, 2004; Noguchi et al, 2003; Noguchi et al, 2004; Tanaka & Russell, 2001; Warren et al, 2004; Xu et al, 2004). The Tof1 and Csm3 proteins form a complex, explaining the similar phenotypes when either is deleted, and this Tof1/Csm3 complex interacts with Mrc1. Mrc1 does interact directly with both Tof1 and Csm3, but judging by the stoichiometry of the interactions, Mrc1 is clearly an interacting protein and not a member of the Tof1/Csm3 complex. (Bando et al, 2009; Mayer et al, 2004; Nedelcheva et al, 2005; Noguchi et al, 2004).

Loss of Swi1 or Swi3, the *S. pombe* orthologs of Tof1 and Csm3, respectively, results in increased spontaneous DNA damage in an otherwise unchallenged S phase, as evidenced by increased DNA repair foci (Noguchi et al, 2003; Noguchi et al, 2004). Also, like Mrc1-deficient cells, yeast cells lacking either member of the Tof1/Csm3 (or Swi1/Swi3) complex are sensitive to agents that cause DNA replication fork stalling,

such as UV and HU (hydroxyurea) (Alcasabas et al, 2001; Foss, 2001; Noguchi et al, 2003; Noguchi et al, 2004; Szyjka et al, 2005; Tanaka & Russell, 2001). One main difference between the Tof1/Csm3 complex and Mrc1 is apparent when replication forks encounter RFBs (replication fork barriers), such as proteins tightly bound to the DNA at pause sites surrounding the DNA-encoding ribosomes. Replication forks lacking Mrc1 do pause at RFB sites like wild-type cells, but fail to restart replication after release of the pausing agent. However, in the absence of Tof1/Csm3, replication forks fail to pause at RFBs, unlike forks in wild-type or Mrc1-deficient cells. It is possible that Tof1/Csm3 inhibit Rrm3, a helicase that removes RFBs, and that without this inhibition Rrm3 is free to clear RFBs prematurely (Calzada et al, 2005; Mohanty et al, 2006; Tourriere et al, 2005). Therefore, it appears that the main role of Tof1/Csm3 involves replication fork stability.

Initial studies in *Xenopus* extracts and human cells indicate that the role of Tim and Tipin, the Tof1 and Csm3 homologs respectively, is conserved from yeast. Tim/Tipin are involved in the maintenance of genomic stability, namely through activation of the DNA replication checkpoint response to stalled replication forks, and are also involved in the recovery of stalled replication forks (Chou & Elledge, 2006; Errico et al, 2007; Gotter et al, 2007; Unsal-Kacmaz et al, 2007; Yoshizawa-Sugata & Masai, 2007). Claspin, the *Xenopus* and human ortholog of Mrc1, also functions during DNA replication and is a mediator protein necessary for the DNA replication checkpoint response to replication stress (Chini & Chen, 2003; Kumagai & Dunphy, 2000; Lee et al, 2003), indicating that the roles of Claspin are also well-conserved from yeast to metazoans.

The Tof1/Csm3 complex (and the Swi1/Swi3 complex) interact both physically and genetically with Hsk1, the yeast homolog of Cdc7. In the absence of Tof1/Csm3 or Hsk1, the cell cycle is not paused in response to DNA damage by alkylating agents. These effects are independent of the downstream effector checkpoint kinases Cds1 and Chk1; therefore, Tof1/Csm3 and Hsk1 are implicated in a Chk1, Cds1-independent checkpoint pathway (Matsumoto et al, 2005; Sommariva et al, 2005). Interestingly, Mrc1 also interacts with Tof1/Csm3 and Hsk1, and these proteins form a complex involved in the cellular response to stalled DNA replication forks (Shimmoto et al, 2009).

In this work, we further studies on *Xenopus* Tim/Tipin in an effort to understand the effect of these proteins at the DNA replication fork. Both the data presented in this work and published data on Tim/Tipin suggest conserved roles for the Tim/Tipin complex. Our results confirm and expand upon current data on Tim/Tipin, including the Tim/Tipin association with chromatin during S phase, interactions with DNA replication and checkpoint proteins, and the necessity of Tim/Tipin for the checkpoint response to stalled forks (Chou & Elledge, 2006; Errico et al, 2007; Gotter et al, 2007; Unsal-Kacmaz et al, 2007; Unsal-Kacmaz et al, 2005; Yoshizawa-Sugata & Masai, 2007). We further show that Tipin is phosphorylated upon checkpoint activation and is a target of the ATR kinase. Finally, we show that Tim/Tipin interacts with Cdc7, Drf1, and Claspin in *Xenopus* extracts, and that Tipin is a target of the Cdc7 kinase.

3.3 Results

3.3.1 Tim and Tipin Form a Complex

To investigate the role of Timeless (Tim) and Tipin in vertebrates, antibodies were raised to the *Xenopus* homologs of Tim and Tipin and subsequently used to characterize Tim and Tipin in a *Xenopus* cell-free system. Antibodies to either Tim or Tipin efficiently isolate both Tim and Tipin, confirming that these proteins interact and form a complex in interphase extract (Figure 3.1A), consistent with their homologs in yeast (Bando et al, 2009; Mayer et al, 2004; Noguchi et al, 2004). Immunoprecipitation of either Tim or Tipin from interphase extracts reveals that Tim and Tipin are the main proteins in the complex that they form, and either antibody pulls down a complex with similar stoichiometric ratios of Tim and Tipin as visualized by Coomassie stain (Figure 3.1B). Therefore, most of the Tim and Tipin in interphase extract are present in the Tim/Tipin complex.

3.3.2 Nuclear Tim/Tipin

Yeast Tof1 and Csm3 are nuclear proteins. It was next confirmed that *Xenopus* Tim and Tipin are nuclear proteins and the Tim/Tipin complex also exists in nuclei. The Tim antibody immunopreciptated the Tim/Tipin complex from nuclear extracts, and the reciprocal Tipin immunoprecipitation also isolated this complex (Figure 3.1C). Interestingly, the stoichiometry of the interacting partner seems to have changed slightly in these immunoprecipations compared to those done in interphase extract. The Tipin immunoprecipitation consistently pulled down more Tipin than the Tim immunoprecipitation, and the reverse was true for the Tim pull-down. While some of the Tim/Tipin complex remains intact, this result indicates that some of the Tim/Tipin

complexes dissociate in the nucleus. Thus, we are detecting free Tim and free Tipin in our immunoprecipitations. This dissocation of the complex in nuclei has not yet been reported, and it is not known what function such a dissociation would serve. However, yeast Tof1 and Csm3 do have slightly different phenotypes when deleted (Bando et al, 2009; Noguchi et al, 2004), so the individual proteins are likely acting when not in the Tof1/Csm3 complex.

Tof1/Csm3 associates with DNA replication forks, so the potential presence of Tim/Tipin at the DNA replication fork was evaluated in order to address conservation with yeast (Bando et al, 2009; Calzada et al, 2005; Gambus et al, 2006; Katou et al, 2003; Krings & Bastia, 2004; Mohanty et al, 2006; Noguchi et al, 2004). Tim and Tipin associate with chromatin in S phase, although not all nuclear Tim and Tipin is chromatin-bound (Figure 3.1D). To determine if this is a regulated association with chromatin in S phase, the binding of Tim and Tipin to chromatin was assessed in the presence of geminin, an inhibitor of pre-RC formation. When geminin is added, the Origin Recognition Complex (ORC) complex still binds chromatin, but binding of Cdt1, Cdc6, and the Mcm2-7 complex is disrupted. The association of Tim and Tipin with chromatin is dependent on pre-RC formation, as binding is inhibited with geminin (Figure 3.1E). Therefore, the association of Tim and Tipin with chromatin during S phase is regulated and specific, and it requires the early steps of formation of the replication fork, namely the association of ORC, the MCM replicative helicase complex, Cdc6, and Cdt1 with origins of replication.

Tim and Tipin could mainly be necessary for the initiation of replication, or the proteins could travel with the replication forks during replication of the DNA strands. To

differentiate between these models, levels of Tim and Tipin on chromatin were monitored during DNA replication. Both Tim and Tipin protein levels increase during DNA replication and decrease at time-points consistent with the completion of DNA replication (Figure 3.1F). This binding pattern is consistent with other DNA replication proteins that travel with the replication fork, so we propose that Tim and Tipin travel with the replication fork, like Tof1 and Csm3 in yeast (Bando et al, 2009; Gambus et al, 2006; Katou et al, 2003; Mohanty et al, 2006). The behavior of Tim and Tipin on chromatin was also examined when DNA replication forks were stalled by the addition of aphidicolin. Aphidicolin inhibits DNA polymerases and leads to the uncoupling of DNA polymerases and the MCM helicase, resulting in larger regions of single-stranded DNA (ssDNA) that promote activation of the DNA replication checkpoint (Byun et al, 2005). In the presence of aphidicolin, chromatin-bound Tim and Tipin increase as in S phase with no treatment, except these proteins continue to accumulate on chromatin past the normal peak levels in mid-S phase at 40-60 minutes (Figure 3.1F). This behavior is consistent with many other proteins involved in DNA replication and the DNA replication checkpoint, including Claspin (Kumagai & Dunphy, 2000).

3.3.3 Tim/Tipin Interactions in Interphase

To elucidate the cellular pathways in which Tim and Tipin participate, Tim/Tipin interacting proteins were identified. Tim and Tipin were individually immunoprecipitated from interphase extract, and interacting proteins by detected by immunoblot. Very few proteins interact with the Tim/Tipin complex in interphase

extract, but both Tim and Tipin antibodies detected a physical interaction with Orc2 (Figure 3.2A), a member of ORC and part of the pre-RC required for Tim/Tipin loading onto chromatin in S phase. In addition to its role at the origins of replication, human Orc2 also impacts upon both centrosome copy number and chromosome structure, with Orc2 knock-down cells arresting with abnormally condensed chromosomes (Prasanth et al, 2004). From this single interaction, we can not conclusively determine within which of these pathways Tim/Tipin contributes.

Interestingly, we also detected BLM, a helicase involved in DNA replication and repair (Li et al, 2004; Wu & Hickson, 2006), in the Tipin immunoprecipitate from interphase extract, but we did not see this interaction with Tim (Figure 3.2B). This may represent a non-specific interaction, since Tipin was not detected in a BLM immunoprecipitation from interphase extract, or it may be that a small amount of Tipin that is not in a complex with Tim interacts with BLM. Alternatively, BLM association with Tim/Tipin could occupy the Tim antibody binding site, eliminating the possibility of immunoprecipitating this complex with the Tim antibody. Both BLM and FFA-1, the *Xenopus* homolog of the WRN helicase, are RecQ helicases and BLM can physically interact with FFA-1 (von Kobbe et al, 2002), so the potential for FFA-1 and Tim/Tipin interactions was assessed. FFA-1 does interact with BLM, but not Tim or Tipin (Figure 3.2B). Therefore, Tipin is not binding indiscriminately to RecQ helicases. It is worth noting that the potential interaction between Tipin and BLM is one of the few differences detected regarding protein-protein interactions with Tim and Tipin.

3.3.4 Nuclear Tim/Tipin Interactions

Novel protein-protein interactions of Tim and Tipin were examined in nuclei, also using immunoprecipitations. For these experiments, Tim antibody was used to immunoprecipitate the Tim/Tipin complex from extracts of nuclear proteins, not the weaker Tipin antibody. Interacting proteins were identified both through immunoblotting and mass spectrometry (data not shown), and many of the detected interactions listed below were confirmed by Tipin immunoprecipitation follow by immunoblotting (data not shown). For these immunoprecipitations, sperm chromatin was incubated in extract for 50 minutes, when Tim and Tipin levels on chromatin peak during S phase (Figure 3.1F). Chromatin was then isolated and proteins were eluted off chromatin with buffer containing 250 mM NaCl. Tim was then immunoprecipitated from the eluate after salt concentrations were adjusted to 150 mM. Tim immunoprecipitations pulled down various DNA replication and checkpoint proteins as shown in Figure 3.2C, a compilation of Tim immunoprecipitation experiments. As in interphase extract, Orc2 interacts with Tim. This interaction has been detected using a variety of antibodies both to Tim and Tipin, indicating that this is a specific interaction (data not shown). However, due to the reasons listed above, it can not be conclusively determined that this interaction is pertinent to DNA replication. Additionally, Tim also interacts with Claspin, Cdc7, Drf1 (Dbf4-related factor 1), RFC40 (replication factor C 40), Rad17, ATR (ataxia telangiectasia and Rad-3 related), and ATRIP (ATR-interacting protein). Cdc7 and Drf1 are necessary for DNA replication in *Xenopus* extract and participate in both the DNA replication checkpoint and the establishment of sister chromatid cohesion (Takahashi et

al, 2008; Takahashi & Walter, 2005; Yanow et al, 2003). ATRIP and the ATR kinase form a complex and are necessary for the activation of the DNA replication checkpoint, and Claspin is a checkpoint mediator protein, necessary for the ATR phosphorylation of downstream targets including Chk1 (Cortez et al, 2001; Kumagai & Dunphy, 2000). RFC40 is a subunit of the RFC and alternative RFC complexes and is involved in DNA replication, checkpoint responses, and sister chromatid cohesion (Mossi & Hubscher, 1998). Rad17, a large subunit of one of the alternative RFC complexes, is also present in the Tim isolate. The Rad17 complex is involved in both the DNA replication checkpoint and its initial activation (Bao et al, 2001; Delacroix et al, 2007; Enoch et al, 1992; Lee et al, 2007), yet Tim interacts with Rad17 even in the absence of DNA damage.

Since Tim/Tipin interacts with DNA replication checkpoint proteins, and the Tim/Tipin homologs in yeast are involved in S phase checkpoint activation (Foss, 2001; Noguchi et al, 2003), the possibility that checkpoint activation may alter the protein complexes with which Tim/Tipin interacts was investigated. To assess this, demembranated sperm was pre-treated with UV light to generate lesions in the DNA. The DNA damage caused by UV light results in activation of the checkpoint due to the stalling of DNA replication forks, providing time to repair the lesions and resume DNA synthesis (Kumagai et al, 1998). Under these conditions of checkpoint activation, Tim/Tipin interacts with similar proteins as during unchallenged DNA replication, namely Orc2, Claspin, ATR, ATRIP, RFC40, and Rad17. Rad17 forms an alternative RFC complex with four small RFC subunits, one of which is RFC40, and loads the Rad9-Rad1-Hus1 (9-1-1) clamp onto damaged DNA (Bermudez et al, 2003; Ellison & Stillman, 2003; Majka & Burgers, 2003). Interestingly, Hus1 was not detected in our

Tim immunoprecipitation, indicating that Tim/Tipin interacts with Rad17 but not the protein complex that it loads onto damaged DNA. Given the checkpoint defects of yeast Tof1/Csm3 mutants and of Tim/Tipin knockdown cells, it is tempting to speculate that the interaction of Tim/Tipin with Rad17 may be involved in activation of the checkpoint, since Rad17 can activate the ATR kinase through TopBP1 (Chou & Elledge, 2006; Errico et al, 2007; Foss, 2001; Gotter et al, 2007; Lee et al, 2007; Yoshizawa-Sugata & Masai, 2007). Alternatively, this interaction may also influence downstream events including maintenance or down-regulation of the checkpoint signal.

Given the newly identified protein-protein interactions, the role of Tim in the loading of these proteins onto chromatin was assessed. Chromatin was isolated from either mock or Tim/Tipin-immunodepleted extracts, and chromatin-associated proteins were analyzed by immunoblotting. Orc2, Claspin, and ATRIP protein levels on chromatin were largely unchanged (Figure 3.3). It is important to note that the Tim/Tipin depletion is incomplete. Previously published work reveals that Claspin association with chromatin is compromised in the absence of the Tim/Tipin complex (Errico et al, 2007; Tanaka et al, 2009; Yoshizawa-Sugata & Masai, 2007), but this is not apparent in our results. This inconsistency is likely due to residual Tim/Tipin remaining in the depleted extracts, which may be sufficient to elicit wild-type conditions.

3.3.5 Tim/Tipin is Not Essential for DNA Replication

Because the Tim/Tipin complex physically interacts with proteins involved in DNA replication and checkpoints, we sought to determine whether or not this complex is

necessary for DNA replication. To assess this, incorporation of ^{32}P from radiolabeled dATP into chromatin during DNA replication was monitored in both mock and Tim/Tipin-depleted extracts (Figure 3.4A and B). There was no significant change in ^{32}P incorporation in extracts without the full complement of Tim/Tipin, indicating that the Tim/Tipin complex is not essential for DNA replication. Again, there was likely a small amount of Tim/Tipin remaining in the immunodepleted extract, so we cannot rule out the possibility that this small amount was sufficient to suppress any defects. However, when Tipin was knocked down in human cells, the cells grew and divided with similar timing to the control cells, indicating that Tipin is not essential for DNA replication and cells were proceeding normally through the cell cycle (data not shown).

3.3.6 Tipin is Involved in Activation of the DNA Replication Checkpoint

The necessity of Tof1/Csm3 for activation of the S-phase checkpoint in yeast (Bando et al, 2009; Foss, 2001; Noguchi et al, 2003; Noguchi et al, 2004) compelled us to investigate the conservation of this role in *Xenopus*. Activation of the DNA replication checkpoint in response to stalled replication forks was assessed in human cells; HeLa cells with or without siRNA knock-down of human Tipin were monitored for checkpoint activation in the presence of hydroxyurea (HU). When HU, a ribonucleotide reductase inhibitor that leads to inhibition of DNA synthesis (Koc et al, 2004), is used to induce replication fork stalling, the ATR kinase is activated. Active ATR phosphorylates downstream proteins involved in the checkpoint response, such as Chk1, and activation of the ATR kinase is used as a marker for stalled replication forks and DNA damage.

Checkpoint activation was assessed by monitoring Chk1 phosphorylation at S317, a known target of the ATR checkpoint kinase (Zhao & Piwnica-Worms, 2001). When Tipin knock-down cells were treated with HU, the Chk1-S317 phosphorylation was not as complete as phosphorylation in control cells (Figure 3.4C). Therefore, Tipin is necessary for full activation of the DNA replication checkpoint in response to stalled replication forks. This is consistent with results published subsequent to the initiation of this project (Chou & Elledge, 2006; Errico et al, 2007; Gotter et al, 2007; Unsal-Kacmaz et al, 2007; Yoshizawa-Sugata & Masai, 2007).

3.3.7 Tipin is a Target of the ATR Checkpoint Kinase and Cdc7 kinase

Many proteins involved in the DNA replication checkpoint are also targets of checkpoint kinases, including Claspin and Chk1 (Guo & Dunphy, 2000; Kumagai & Dunphy, 2000; Zhao & Piwnica-Worms, 2001). To assess if Tim and/or Tipin are also targets of checkpoint kinases and potentially regulated by the checkpoint, phosphorylation of Tim and Tipin was monitored in interphase extract containing a synthetic checkpoint activator, pA/T70, which activates both the ATR and ATM kinases (Kumagai & Dunphy, 2000). Upon checkpoint activation, neither Tim nor Tipin undergo an observable mobility shift on an SDS-PAGE gel. However, when ^{32}P-ATP is added to the extract, Tipin is clearly phosphorylated in a checkpoint-dependent manner (Figure 3.5A). Both ATR and ATM kinases primarily phosphorylate proteins at SQ/TQ (Kim et al, 1999). Therefore, Tipin protein sequences from *Xenopus*, human, mouse, and chicken were compared. Analysis of these sequences revealed only 1 conserved SQ sequence,

S222 in human Tipin, which is not in a particularly well-conserved sequence of the protein (Figure 3.5B). Interestingly, this serine in human Tipin was also identified as a target of checkpoint kinases in a large-scale proteomic screen (Matsuoka et al, 2007).

To determine if Tipin is a substrate of ATR, ATM, both, or neither of these kinases, human Tipin and Tipin-S222A were expressed as GST fusion proteins in bacteria, purified, and used as substrates for *in vitro* ATM and ATR kinase assays using ^{32}P-ATP. Incorporation of radiolabeled phosphate into GST-Tipin or GST-Tipin-S222A indicates that the protein is indeed a substrate of the kinase. Incubation of GST-Tipin or GST-TipinS222A with active ATM yielded no increase in radiolabeled GST-Tipin or GST-TipinS222A compared to these proteins incubated with inactive ATM (Figure 3.5C). However, PHAS-I, a known substrate of ATM and ATR, was well-phosphorylated by ATM, as evidenced by the increase of radiolabel in the presence of active ATM kinase. Therefore, we do not believe that Tipin is a substrate of the ATM kinase.

GST-Tipin and GST-Tipin-S222A were also used as substrates in an ATR kinase assay in which a TopBP1 fragment was used to activate ATR (Kumagai et al, 2006). The radiolabel on GST-Tipin increases significantly upon stimulation of ATR kinase activity with the TopBP1 fragment (Figure 3.5D). However, this increase is not seen with GST-Tipin-S222A, indicating that S222 is the one and only ATR phosphorylation site in human Tipin. We are confident that the ATR kinase is active in all reactions containing both the kinase and the TopBP1 fragment, as this fragment is also a target of ATR phosphorylation and is phosphorylated in all reactions in which it is present.

In addition to the ATR-dependent checkpoint pathway involving Chk1, yeast Tof1/Csm3 has been reported to participate in a Chk1 independent checkpoint pathway in response to DNA damaged by alkylation (Sommariva et al, 2005). This pathway is dependent on Hsk1, the yeast homolog of Cdc7. In *Xenopus*, the Cdc7 kinase and Drf1, the Cdc7 regulatory subunit in *Xenopus* extracts, are necessary for initiation of DNA replication and may be a target of the DNA replication checkpoint (Takahashi & Walter, 2005; Yanow et al, 2003). To determine if this relationship between Tim/Tipin and Cdc7 may be conserved in *Xenopus*, Tim was immunoprecipitated from egg extracts. Both Cdc7 and Drf1 were present in the pull-down, and this interaction was not dependent on DNA damage (Figure 3.2C). Since Cdc7 is a kinase, we were also interested to see if either Tim or Tipin is a substrate of Cdc7 and may be regulated by this kinase. GST-Tipin is phosphorylated by Cdc7 in an *in vitro* kinase assay, as is the positive control protein Mcm2 (Figure 3.6). Tim was also evaluated as a substrate of Cdc7, but did not appear to be a target of the kinase (data not shown). Therefore, it is possible that Cdc7 regulates Tim/Tipin through phosphorylation of Tipin.

3.4 Discussion

Initial observations in yeast regarding the participation of Tof1 and Csm3 in the maintenance of genomic integrity motivated us to pursue characterization of these proteins in a metazoan system to determine the extent of conservation of their functions. Subsequent to the initiation of this project, there has been a fair amount of literature published on the roles of *Xenopus* and human Tim and Tipin.

Consistent with yeast Tof1/Csm3 and human Tim/Tipin, *Xenopus* Tim and Tipin form a complex, are the major proteins in that complex, and associate with DNA replication forks (Figure 3.1) (Bando et al, 2009; Calzada et al, 2005; Chou & Elledge, 2006; Gotter, 2003; Gotter et al, 2007; Katou et al, 2003; Mayer et al, 2004; Nedelcheva et al, 2005; Tourriere et al, 2005; Unsal-Kacmaz et al, 2007; Yoshizawa-Sugata & Masai, 2007). The protein-protein interactions of *Xenopus* Tim/Tipin indicate roles in DNA replication and the checkpoint response to stalled DNA replication forks, again consistent with roles in yeast and metazoans (Chou & Elledge, 2006; Errico et al, 2007; Foss, 2001; Gotter, 2006; Gotter et al, 2007; Noguchi et al, 2004; Smith et al, 2009; Tanaka et al, 2009; Unsal-Kacmaz et al, 2007; Urtishak et al, 2009; Yoshizawa-Sugata & Masai, 2007).

Through this work, we have identified additional proteins that interact with Tim/Tipin in interphase extract and in nuclei. The reason for the physical interaction between Tim/Tipin and Orc2 in both interphase extract and nuclei is puzzling, as it is yet unclear if this interaction is related to DNA replication or an alternate function of Orc2. In addition to its role in DNA replication, Orc2 participates in replication of centromeres, and other proteins in the Orc1-6 complex, which contains Orc2, are involved in chromosome segregation, cytokinesis, and centriole and centromere copy number (Hemerly et al, 2009; Prasanth et al, 2004; Prasanth et al, 2002). Meanwhile, in addition to their roles in DNA replication and checkpoints, Tim/Tipin is also involved in sister chromatid cohesion and potentially in circadian rhythms (Chan et al, 2003; Mayer et al, 2004; Tanaka et al, 2009; Unsal-Kacmaz et al, 2005; Warren et al, 2004; Xu et al, 2004). Further study is needed to examine the nature of the interaction between Orc2 and

Tim/Tipin and determine if this relationship is related to DNA replication or another cellular process.

When proteins are isolated from chromatin, it was found that Tim/Tipin interacts with ATR, ATRIP, Claspin, RFC40, Rad17, Cdc7, and Drf1, all components of the DNA replication fork that travel with it throughout S phase. Previously published work has identified additional Tim/Tipin interacting proteins, including RPA, the MCM helicase complex, DNA polymerases δ and ε, PCNA, and Claspin (Chou & Elledge, 2006; Errico et al, 2007; Gotter et al, 2007; Unsal-Kacmaz et al, 2007; Yang et al, 2008). These interactions all support the data that Tim/Tipin associate with chromatin in S phase and travel with the DNA replication fork.

Some proposed roles for Tof1/Csm3 and Swi1/Swi3 in yeast are the stabilization of replication forks and the recovery of stalled replication forks (Calzada et al, 2005; Katou et al, 2003; Mohanty et al, 2006; Noguchi et al, 2003; Tourriere et al, 2005). The many Tim/Tipin protein-protein interactions also support the idea that Tim/Tipin may be a fork stabilizer, as its partners contact many parts of the fork. In human cells lacking Tim or Tipin, low levels of H2AX phosphorylation are present during otherwise unchallenged DNA replication, indicative of low levels of endogenous DNA damage (Chou & Elledge, 2006; Smith et al, 2009; Urtishak et al, 2009). These results are consistent with ours, in that Tipin knock-down cells do not exhibit obvious growth defects and the rate of DNA replication is largely unaffected in the absence of Tim/Tipin.

The Tim/Tipin-interacting proteins ATR, ATRIP, Claspin, RFC40, Rad17, Cdc7, and Drf1 are all involved in the checkpoint response to stalled DNA replication forks, indicating that Tim/Tipin may also be involved in this response. This was confirmed

when Tipin was knocked down in human cells, and Chk1 was not well phosphorylated upon activation of the ATR-dependent checkpoint by exposure to HU. Tipin is phosphorylated in a checkpoint-dependent manner, and human Tipin is only phosphorylated upon one residue by the ATR kinase (Figure 3.5D). It is tempting to speculate that this phosphorylation by ATR may be necessary for a fully functional ATR-dependent checkpoint response. Experiments to test this hypothesis yielded results that were inconclusive, leaving us unable to resolve this issue (data not shown). However, it is also possible that this phosphorylation is involved in recovery from the checkpoint, sister chromatid cohesion, or the regulation of protein-protein interactions. The conserved S222 that is phosphorylated by ATR is adjacent to a conserved RPA-interacting domain in Tipin, stretching from amino acids 185-218 (Unsal-Kacmaz et al, 2007). Gotter and colleagues have reported that the Tim/Tipin interaction with RPA is weakened upon UV treatment in human cells, although the other protein-protein interactions of Tim/Tipin appear unaffected by UV exposure (Gotter et al, 2007). ATR is activated upon cellular exposure to UV, so it is possible that ATR phosphorylation of Tipin diminishes the ability of Tim/Tipin to bind RPA, allowing other proteins potentially involved in DNA repair or checkpoint recovery to bind the RPA-coated ssDNA. To test this, it would be interesting to compare the RPA-binding capabilities of ATR-phosphorylated Tipin and non-phosphorylated Tipin. Alternatively, this phosphorylation could also alter the dynamics between Tim and Tipin, although a significant change in association of Tim and Tipin has not been detected upon checkpoint activation (Gotter et al, 2007). It has been reported that murine Tipin disrupts association

of Tim complexes (Gotter, 2003), and it would be interesting to see if Tim forms higher order complexes when Tipin is phosphorylated by ATR.

Tipin is the target of ATR, the checkpoint kinase activated by incomplete DNA replication and stalled replication forks, and not ATM, the checkpoint kinase activated by DNA double-strand breaks (Figure 3.5), suggesting that the role of Tim/Tipin is related to DNA replication fork stability and the cellular response to stalled replication forks. In *Xenopus* and human, Tim and Tipin are necessary for the recovery of stalled replication forks, maintenance of replication fork progression rate, and prevention of endogenous damage during unchallenged DNA replication (Chou & Elledge, 2006; Errico et al, 2007; Gotter et al, 2007; Smith et al, 2009; Tanaka et al, 2009; Unsal-Kacmaz et al, 2007; Urtishak et al, 2009; Yoshizawa-Sugata & Masai, 2007). All of these functions are consistent with the involvement of Tim/Tipin in the ATR-dependent checkpoint pathway.

Tim/Tipin also interacts with Cdc7 and its regulatory subunit in *Xenopus* extracts, Drf1 (Figure 3.2C). Cdc7 activity is necessary for the maturation of replication forks for DNA replication, and in yeast this protein participates in checkpoint mechanisms (Matsumoto et al, 2005; Shimmoto et al, 2009; Sommariva et al, 2005). Given the role of Tim/Tipin in the DNA replication checkpoint, this relationship with Cdc7/Drf1 was further investigated in *Xenopus*. Tim/Tipin physically interacts with Cdc7/Drf1 in nuclei, potentially explaining genetic interactions seen in yeast. Tipin is also a target of the Cdc7 kinase, and this phosphorylation may serve to regulate the Tim/Tipin complex. Interestingly, like Tim/Tipin, *Xenopus* Cdc7/Drf1 is also involved in the establishment of sister chromatid cohesion at the DNA replication fork (Chan et al, 2003; Takahashi et al,

2008; Tanaka et al, 2009). Further studies are required to determine if the role of Tim/Tipin in sister chromatid cohesion involves Cdc7.

ATM, ATR, and DNA-PK, three PIKKs that regulate the cellular response to damaged DNA, are all proposed to have a smaller regulatory subunit (Falck et al, 2005). Tim and Tipin have extremely similar phenotypes when either knocked-down in human cells or when deleted in yeast (Bando et al, 2009; Gotter et al, 2007; Katou et al, 2003; Noguchi et al, 2004; Yoshizawa-Sugata & Masai, 2007), leaving us to wonder about the roles that the individual Tim and Tipin proteins play. It is tempting to speculate that Tipin is the smaller, regulatory protein in this complex, like ATRIP in the ATR/ATRIP complex, but in this case Tim has no known enzymatic activity. It is interesting, though, that it is the smaller protein in this complex that is the target of both the Cdc7 kinase and the ATR checkpoint kinase, yet there was no observable change in Tim phosphorylation upon DNA damage checkpoint induction or incubation with the active Cdc7 kinase (Figures 3.5 and 3.6). *Xenopus* Tipin is also a target of the Cdk2 kinase as well as other CDK kinases, although the function of these modifications is as yet unknown (Errico et al, 2007). Additionally, it is Tipin that contains a conserved RPA-binding domain, similar to those found in XPA and SMARCAL1, proteins involved in checkpoints and DNA repair (Bansbach et al, 2009; Unsal-Kacmaz et al, 2007). The Tim/Tipin complex is involved in various cellular processes, many of which could be regulated through Tipin. These individual pieces of data collectively imply a regulatory function for Tipin, but direct evidence for this is lacking. The functional rationale for these post-translational modifications to Tipin should prove revealing for both the roles of Tim and Tipin and the cellular processes as a whole.

3.5 Materials and Methods

Xenopus Egg Extracts. The preparation of *Xenopus* cell-free extracts was performed as previously described (Murray, 1991). For reactions in which a checkpoint response was induced in interphase extracts, 50 μg/ml pA/T70 oligos was added to extracts (Kumagai & Dunphy, 2000). For reactions requiring isolation of nuclei or chromatin, demembranated sperm chromatin was incubated at 3,000 sperm/μl in extract for 100 min or the indicated time. Nuclei and chromatin were isolated as described (Lee et al, 2003). When geminin was used to inhibit pre-RC formation, it was added at a concentration of 0.3 mM to the extract prior to addition of sperm chromatin.

Antibodies. Polyclonal anti-Tipin antibodies were generated by injecting rabbits with bacterially-expressed *Xenopus* Tipin amino acids 11-181. Antibodies to *Xenopus* Tim were also generated in rabbits, with bacterially expressed *Xenopus* Tim amino acids 1109-1286 serving as the antigen. Both antibodies were affinity purified with their respective antigens according to standard protocols. Antibodies to Claspin, Orc2, ATR, ATRIP, Drf1, Rad17, Hus1, and BLM were previously described (Kumagai & Dunphy, 2000; Lee et al, 2003; Li et al, 2004; Yanow et al, 2003). Antibodies recognizing FFA-1 were generated by S.K. Kim and W.G. Dunphy (unpublished). Anti-RFC40 antibodies were a kind gift of J. Hurwitz (Uhlmann et al, 1996), and anti-Cdc7 antibodies were kindly provided by J. Walter (Takahashi & Walter, 2005). The BM28 monoclonal antibody, which recognizes *Xenopus* Mcm2, and antibodies to Ser317-phosphorylated Chk1 were purchased from Cell Signaling Technology (Beverly, MA). Anti-human

Tipin antibodies were purchased from Bethyl Laboratories (Montgomery, TX), and control rabbit IgG was purchased from Zymed (South San Francisco, CA).

Recombinant Proteins. The cDNA for human Tipin, a kind gift of A. Gotter, was cloned into pGEX4T-3 with BamH1 and Xho1. The GST-Tipin-S222A mutant was created using site-directed mutagenesis, and a HindIII site was also inserted to verify successful cloning. Primers for site-directed mutagenesis were hTipin_g654t_a664g_g665c_t666c and hTip_g654t_a664g_g665c_t666c_R, with sequences of 5'-GGA AAG AAG GCA GGC AAA GCT TCT GAG TAA TGC CCA GAC CCT AGG AAA TGA TAT G -3' and 5'- CAT ATC ATT TCC TAG GGT CTG GGC ATT ACT CAG AAG CTT TGC CTG CCT TCT TTC C -3', respectively. Mutagenesis was performed with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Proteins were expressed in *E. coli* BL21 CodonPlus RIL cells at 30°C for 3 hours after induction with 1 mM IPTG. Recombinant proteins were isolated using Glutathione Sepharose (Pharmacia), eluted with glutathione, dialyzed, frozen, and subsequently used as substrate in kinase assays.

Immunological Methods. To immunoprecipitate Tim or Tipin from interphase extracts, 2.5 μg purified antibody was pre-bound to 5 μl Protein A Support (BioRad), beads were incubated in interphase extract for 90 min at 4°C, subsequently washed four times with 10mM HEPES-KOH [pH 7.6], 150 mM NaCl, 0.5% NP-40, 2.5 mM EGTA, and analyzed by SDS-PAGE. For immunoprecipitations involving radiolabeled ATP, ^{32}P-ATP was added to the extract to a concentration of 0.35 μCi/μl, and the immunoprecipitation was performed as described above. Samples were analyzed by

SDS-PAGE, and the gel was stained, dried, and imaged on a Phosphorimager 445 SI. For immunoprecipitations from chromatin fractions, demembranated sperm chromatin was added to a concentration of 3,000 sperm/μl in activated extract and incubated at room temperature for 50 min. Extracts (250 μl) were spun through 1ml sucrose cushion (20 mM HEPES-KOH [pH 7.6], 1 M sucrose, 80 mM KCl, 2.5 mM K-gluconate, and 10 mM Mg-gluconate) at 6,100xg for 5 minutes at 4°C twice. Four pellets were combined, resuspended in an additional 1ml sucrose cushion, and spun down as before. Pellets were then resuspended in 1 ml sucrose cushion + 0.5% NP-40 and spun down as before. Proteins were eluted from chromatin with 125 μl 10mM HEPES-KOH [pH 7.6], 250 mM NaCl, 0.5% NP-40, and insoluble chromatin was pelleted by spinning at 6,100xg, 5 min, 4°C. This step was repeated, supernatants containing eluted proteins were pooled, salt concentrations were adjusted to 10 mM HEPES-KOH [pH 7.6], 150 mM NaCl, 0.5% NP-40, and antibody pre-bound to Protein A beads, as for interphase extract immunoprecipitations, was added to this extract. Immunoprecipitations were rotated at 4°C for 2 hours, beads were washed twice with 10 mM HEPES-KOH [pH 7.6], 150 mM NaCl, 0.5% NP-40, and bead-bound proteins were analyzed by SDS-PAGE and immunoblotting. Tim/Tipin was immunodepleted from 100 μl interphase extract using 100 μg anti-Tim antibodies and 60 μg anti-Tipin antibodies pre-bound to Protein A support (BioRad), and each of 2 rounds of depletion were performed at 4°C, rotating for 90 min.

Replication Assay. Replication assays were performed as described previously (Yanow et al, 2003).

Human cell culture. HeLa cells were maintained in DMEM containing 10% fetal bovine serum at 37°C, 5% CO_2, according to standard protocols. siRNA oligos to human Tipin were from Qiagen (Valencia, CA), and sequences were as follows: Tipin 1: 5'-AAGCTTGGCGTTACTATGTAT-3'; Tipin 2: 5'-CTGAGTTAAGTAGAAGCCTAA-3'; Tipin 3: 5'-ACCCTAGGAAATGATATGTTA-3'. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used to transfect siRNA oligos, according to the manufacturer's protocol. Cells were transfected with either Qiagen AllStars Negative Control or a mixture of siRNA oligos; A was a 1:1 mix of siRNA oligos Tipin 1 and Tipin 2, B was a 1:1 mix of Tipin 2 and Tipin 3, and C was a 1:1 mix of Tipin 1 and Tipin 3. Cells were transfected twice with siRNA oligos, on Day 1 and Day 2, and on Day 4 cells were treated with 10 mM hydroxyurea (HU) for 2 hours. Cells were then harvested and analyzed by SDS-PAGE.

Kinase assays. ATR and ATM kinase assays have been described previously (Kumagai et al, 2006; Yoo et al, 2004). For the Cdc7 kinase assay, bacteria expressing His6-FLAG-Cdc7-Dbf4 were grown at 30°C, and 8ml of bacterial culture was processed for each batch of kinase. Bacteria were pelleted and suspended in lysis buffer (10 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml chymostatin, 10 μg/ml pepstatin) + 0.1 mg/ml lysozyme on ice for 15 min. Samples were sonicated and again pelleted. His6-FLAG-Cdc7-Dbf4 was isolated first by pull-down with Ni^{+2} beads. Ni^{+2} resin was incubated with the soluble fraction for 1 hour at 4°C, the beads were subsequently washed 3 times with lysis buffer,

and bound proteins were eluted with 250 mM imidazole in lysis buffer. Eluted proteins were subsequently FLAG immunoprecipitated at 4°C for 1 hour, and purified Cdc7-Drf1 was eluted from beads with FLAG peptide suspended in 10 mM HEPES-KOH [pH 7.6], 150 mM NaCl, and used in Cdc7 kinase assays. Cdc7 kinase assays were performed as described for ATR kinase assays, except the composition of the kinase buffer was 50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT, and 50 μM ATP.

3.6 Figures

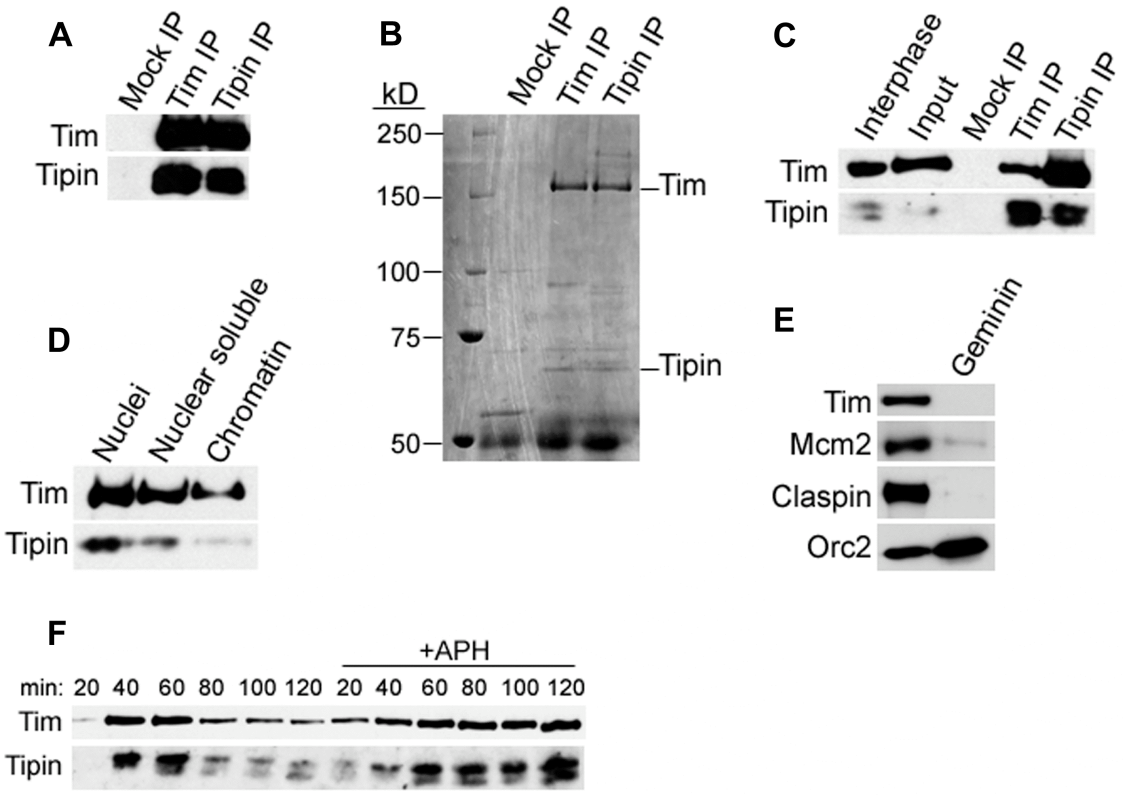

Figure 3.1: Tim and Tipin associate with replicating DNA. A) Tim and Tipin antibodies were used to immunoprecipitate the Tim/Tipin complex from interphase extract. Reactions were analyzed by immunoblotting. B) Tim and Tipin immunoprecipitates from interphase extract were subjected to SDS-PAGE and proteins were detected by silver stain. C) Anti-Tim antibodies and anti-Tipin antibodies were used to immunoprecipitate Tim and Tipin, respectively, from extracts containing chromatin-associated proteins. Tim and Tipin were detected in the pull-down by immunoblotting. D) Nuclei were isolated from extract and fractionated into soluble and chromatin-bound fractions. Equal amounts of fractions were analyzed by SDS-PAGE and immunoblotting. E) Chromatin was isolated from extract in the absence or presence of geminin, a pre-RC inhibitor, and protein binding to chromatin was assessed by immunoblotting. F) Tim and Tipin binding to chromatin was monitored over time during DNA replication, with the addition of sperm nuclei at 0 minutes. Chromatin was isolated from cell-free extracts at the indicated time-points, and Tim and Tipin levels were analyzed by immunoblotting. For samples in which aphidicolin (APH) was added to stall DNA replication forks, aphidicolin was added at 0 minutes.

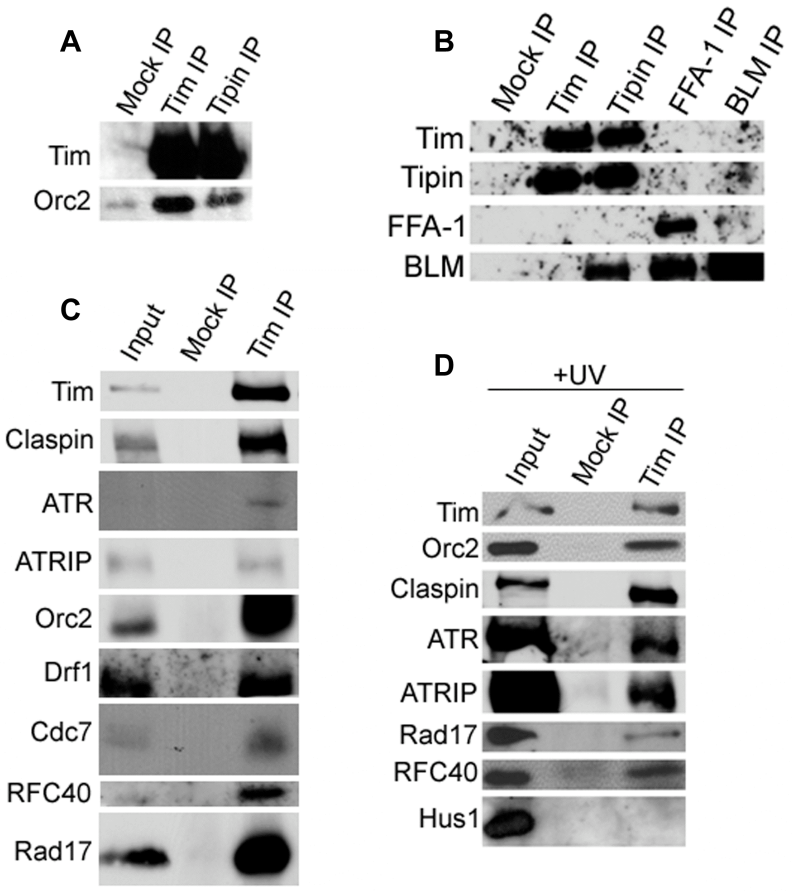

Figure 3.2: Tim/Tipin interactions. A) Mock, anti-Tim, and anti-Tipin immunoprecipitates from interphase extract were subjected to SDS-PAGE, and interacting Tim and Orc2 were detected in immunoprecipitates by immunoblotting. Rabbit IgG was used for Mock IP. B) Interactions between Tim, Tipin, FFA-1, and BLM in interphase extract were analyzed. Each of these proteins was immunoprecipitated, as well as a mock sample. Samples were analyzed by SDS-PAGE and immunobloting. Blots were probed for Tim, Tipin, FFA-1, and BLM. C) Extracts with nuclei were incubated for 50 min, which corresponds to mid-S phase, nuclei were pelleted, and chromatin-associated proteins were isolated through salt-washes of chromatin. Control or anti-Tim antibodies were used for immunoprecipitation of proteins from this fraction of chromatin-associated proteins, and interacting proteins were detected by immunoblotting. Input is 20 μl of the fraction of chromatin-associated proteins prior to immunoprecipitations. D) Experiment was performed as in part C, except sperm DNA was pre-treated with UV to induce checkpoint activation upon DNA replication, and extracts containing nuclei were incubated for 100 min to allow activation and amplification of checkpoint signaling.

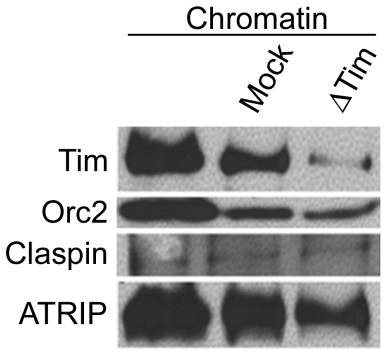

Figure 3.3: Characterization of Tim-depleted chromatin. Interphase extracts were mock or Tim-immunodepleted. Sperm chromatin was added to extract (3,000 nuclei/μl), incubated for 100 min, chromatin was isolated, and chromatin-associated proteins were analyzed by immunoblotting. Unlike published results, Claspin levels are not significantly decreased in the absence of Tim, likely due to the small amount of remaining Tim in Tim-depleted extracts that associates with chromatin.

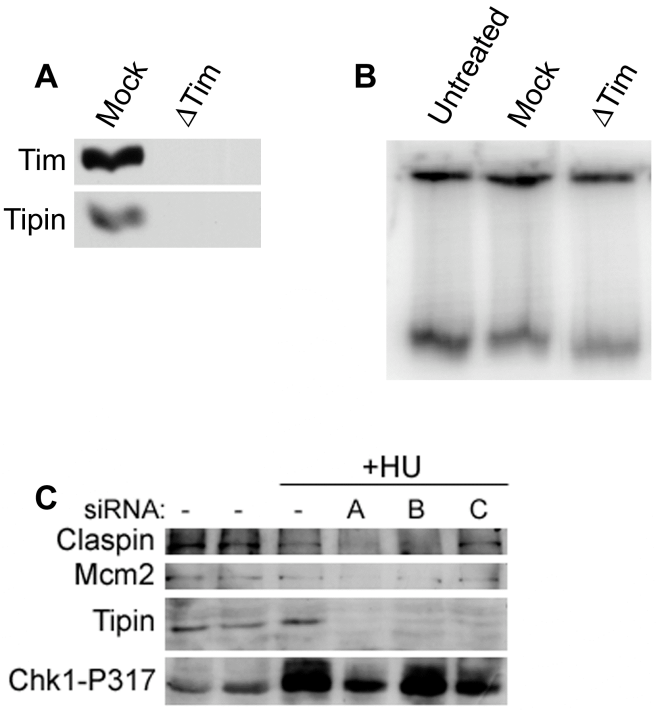

Figure 3.4: Tim/Tipin in DNA replication and the DNA replication checkpoint. A) The amount of Tim and Tipin in 1 μl of mock or Tim-immunodepleted extracts was analyzed by immunoblotting. These extracts were subsequently used for the DNA replication assay in panel B. B) Incorporation from ^{32}P-dATP was used to monitor DNA replication in extracts that were untreated, mock depleted, or Tim-depleted. DNA (3,000 sperm/μl) was incubated in extracts for 100 minutes before reactions were stopped. Samples were analyzed on a 1% agarose gel and imaged by Phosphorimager. C) HeLa cells were transfected twice with either control or Tipin siRNA oligos. 48 hours after the second transfection, cells were treated with 10 mM HU for 2 hours, harvested, and analyzed by immunoblotting. siRNA oligo sequences are listed in *Materials and Methods*.

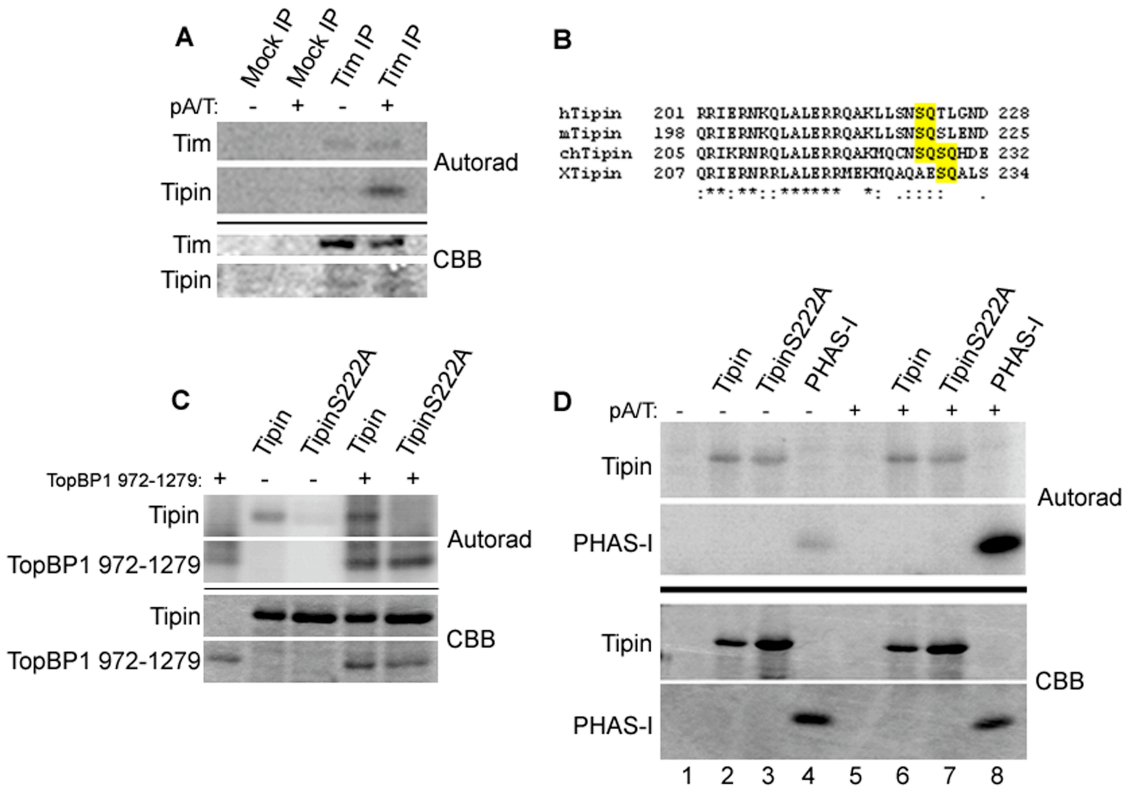

Figure 3.5: ATR phosphorylation of Tipin. A) Interphase extracts containing ^{32}P-ATP were incubated without or with pA/T70 oligos to stimulate checkpoint activation. Mock or anti-Tipin immunoprecipitates were isolated from these extracts, separated by SDS-PAGE, visualized by Coomassie staining, and exposed to a Phosphorimager screen for autoradiography. B) Alignment of human, mouse, chicken, and *Xenopus* Tipin sequences near the best conserved SQ residue, S222 in human Tipin. C) GST-Tipin and GST-Tipin-S222A were used as substrates in an *in vitro* ATR kinase assay. All reactions contained ATR kinase and ^{32}P-ATP, and TopBP1 972-1279 was added to select reactions to activate ATR kinase activity. GST-Tipin and GST-Tipin-S222A were incubated with inactive and active ATR, and SDS-PAGE followed by Coomassie staining was used to visualize proteins. The gel was then subjected to autoradiography. D) Inactive ATM and active ATM were isolated from interphase extracts lacking or containing pA/T70 oligomers, respectively, and used for *in vitro* kinase assays. Substrates for the kinase assays were GST-Tipin, GST-Tipin-S222A, and PHAS-I, a documented ATM substrate. Substrates were incubated with inactive (lanes 1-4) or active (lanes 5-8) ATM, proteins were separated by SDS-PAGE, visualized by Coomassie stain, and the gel was subsequently analyzed by autoradiography. While PHAS-I protein levels are equivalent in lanes 4 and 8, ATM activation by the pA/T70 oligos is evidenced by the increased signal in the autoradiograph from phosphorylated PHAS-I (lane 8) compared to that incubated with inactive ATM (lane 4).

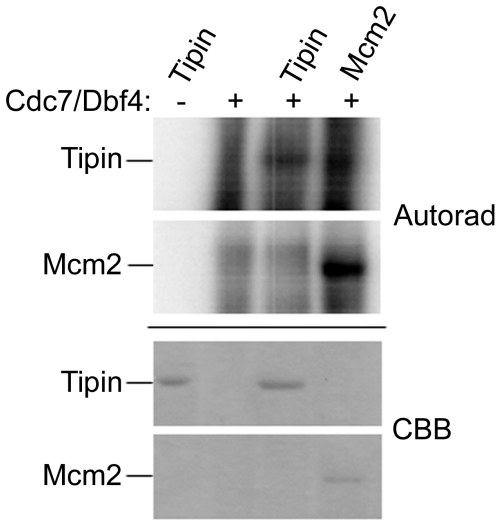

Figure 3.6: Tipin is phosphorylated by Cdc7. Bacterially expressed Cdc7/Dbf4 complex was isolated and used in an *in vitro* kinase assay, with substrates of Cdc7 being Tipin and Mcm2 as a positive control. Substrates were incubated with the kinase complex and ^{32}P-ATP, analyzed by SDS-PAGE, visualized by Coomassie stain, and subsequently analyzed by autoradiography.

3.7 References

Alcasabas AA, Osborn AJ, Bachant J, Hu F, Werler PJH, Bousset K, Furuya K, Diffley JFX, Carr AM, Elledge SJ (2001) Mrc1 transduces signals of DNA replication stress to activate Rad53. *Nat Cell Biol* **3**(11)**:** 958-965

Bando M, Katou YM, Komata M, Tanaka H, Itoh T, Sutani T, Shirahige K (2009) Csm3, Tof1, and Mrc1 form a heterotrimeric mediator complex that associates with DNA replication forks. *Journal of Biological Chemistry* **284**(49): 34355-65

Bansbach CE, Bétous R, Lovejoy CA, Glick GG, Cortez D (2009) The annealing helicase SMARCAL1 maintains genome integrity at stalled replication forks. *Genes & Development* **23**(20): 2405-14

Bao S, Tibbetts RS, Brumbaugh KM, Fang Y, Richardson DA, Ali A, Chen SM, Abraham RT, Wang X-F (2001) ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses. *Nature* **411**(6840)**:** 969-974

Bermudez VP, Lindsey-Boltz LA, Cesare AJ, Maniwa Y, Griffith JD, Hurwitz J, Sancar A (2003) Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **100**(4)**:** 1633-1638

Byun TS, Pacek M, Yee M-c, Walter JC, Cimprich KA (2005) Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. *Genes & Development* **19**(9)**:** 1040-1052

Calzada A, Hodgson B, Kanemaki M, Bueno A, Labib K (2005) Molecular anatomy and regulation of a stable replisome eukaryotic DNA at a paused replication fork. *Genes & Development* **19**(16)**:** 1905-1919

Chan RC, Chan A, Jeon M, Wu TF, Pasqualone D, Rougvie AE, Meyer BJ (2003) Chromosome cohesion is regulated by a clock gene paralogue TIM-1. *Nature* **423**(6943)**:** 1002-1009

Chini CCS, Chen J (2003) Human Claspin is required for replication checkpoint control. *Journal of Biological Chemistry* **278**(32)**:** 30057-30062

Chou DM, Elledge SJ (2006) Tipin and Timeless form a mutually protective complex required for genotoxic stress resistance and checkpoint function. *Proceedings of the National Academy of Sciences of the United States of America* **103**(48)**:** 18143-18147

Cortez D, Guntuku S, Qin J, Elledge SJ (2001) ATR and ATRIP: Partners in checkpoint signaling. *Science* **294**(5547)**:** 1713-1716

Delacroix S, Wagner JM, Kobayashi M, Yamamoto K, L.M. K (2007) The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1. *Genes & Development* **21**(12)**:** 1472-1477

Ellison V, Stillman B (2003) Biochemical characterization of DNA damage checkpoint complexes: clamp loader and clamp complexes with specificity for 5′ recessed DNA. *PLoS Biol* **1**(2)**:** e33

Enoch T, Carr AM, Nurse P (1992) Fission yeast genes involved in coupling mitosis to completion of DNA replication. *Genes & Development* **6**(11)**:** 2035-2046

Errico A, Costanzo V, Hunt T (2007) Tipin is required for stalled replication forks to resume DNA replication after removal of aphidicolin in *Xenopus* egg extracts. *Proceedings of the National Academy of Sciences of the United States of America* **104**(38)**:** 14929-14934

Falck J, Coates J, Jackson SP (2005) Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature* **434**(7033)**:** 605-611

Foss EJ (2001) Toflp regulates DNA damage responses during S phase in *Saccharomyces cerevisiae*. *Genetics* **157**(2)**:** 567-577

Gambus A, Jones RC, Sanchez-Diaz A, Kanemaki M, van Deursen F, Edmondson RD, Labib K (2006) GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. *Nature Cell Biology* **8**(4)**:** 358-U341

Gotter AL (2003) Tipin, a novel Timeless-interacting protein, is developmentally co-expressed with Timeless and disrupts its self-association. *Journal of Molecular Biology* **331**(1)**:** 167-176

Gotter AL (2006) A Timeless debate: resolving TIM's noncircadian roles with possible clock function. *Neuroreport* **17**(12)**:** 1229-1233

Gotter AL, Suppa C, Emanuel BS (2007) Mammalian TIMELESS and Tipin are evolutionarily conserved replication fork-associated factors. *Journal of Molecular Biology* **366**(1)**:** 36-52

Guo ZJ, Dunphy WG (2000) Response of *Xenopus* Cds1 in cell-free extracts to DNA templates with double-stranded ends. *Molecular Biology of the Cell* **11**(5)**:** 1535-1546

Hemerly AS, Prasanth SG, Siddiqui K, Stillman B (2009) Orc1 controls centriole and centrosome copy number in human cells. *Science* **323**(5915)**:** 789-793

Katou Y, Kanoh Y, Bando M, Noguchi H, Tanaka H, Ashikari T, Sugimoto K, Shirahige K (2003) S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature* **424**(6952)**:** 1078-1083

Kim S-T, Lim D-S, Canman CE, Kastan MB (1999) Substrate specificities and identification of putative substrates of ATM kinase family members. *Journal of Biological Chemistry* **274**(53)**:** 37538-37543

Koc A, Wheeler LJ, Mathews CK, Merrill GF (2004) Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. *Journal of Biological Chemistry* **279**(1)**:** 223-230

Krings G, Bastia D (2004) swi1- and swi3-dependent and independent replication fork arrest at the ribosomal DNA of Schizosaccharomyces pombe. *Proceedings of the National Academy of Sciences of the United States of America* **101**(39)**:** 14085-14090

Kumagai A, Dunphy WG (2000) Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in *Xenopus* egg extracts. *Molecular Cell* **6**(4)**:** 839-849

Kumagai A, Guo Z, Emami KH, Wang SX, Dunphy WG (1998) The *Xenopus* Chk1 protein kinase mediates a caffeine-sensitive pathway of checkpoint control in cell-free extracts. *J Cell Biol* **142**(6)**:** 1559-1569

Kumagai A, Lee J, Yoo HY, Dunphy WG (2006) TopBP1 activates the ATR-ATRIP complex. *Cell* **124**(5)**:** 943-955

Lee J, Kumagai A, Dunphy WG (2003) Claspin, a Chk1-regulatory protein, monitors DNA replication on chromatin independently of RPA, ATR, and Rad17. *Molecular Cell* **11**(2)**:** 329-340

Lee J, Kumagai A, Dunphy WG (2007) The Rad9-Hus1-Rad1 checkpoint interaction of TopBP1 with ATR. *Journal of Biological Chemistry* **282**(38)**:** 28036-28044

Li W, Kim S-M, Lee J, Dunphy WG (2004) Absence of BLM leads to accumulation of chromosomal DNA breaks during both unperturbed and disrupted S phases. *J Cell Biol* **165**(6)**:** 801-812

Majka J, Burgers PMJ (2003) Yeast Rad17/Mec3/Ddc1: A sliding clamp for the DNA damage checkpoint. *Proceedings of the National Academy of Sciences of the United States of America* **100**(5)**:** 2249-2254

Matsumoto S, Ogino K, Noguchi E, Russell P, Masai H (2005) Hsk1-Dfp1/Him1, the Cdc7-Dbf4 kinase in *Schizosaccharomyces pombe*, associates with Swi1, a component of the replication fork protection complex. *Journal of Biological Chemistry* **280**(52)**:** 42536-42542

Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, III, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP, Elledge SJ (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316**(5828)**:** 1160-1166

Mayer ML, Pot I, Chang M, Xu H, Aneliunas V, Kwok T, Newitt R, Aebersold R, Boone C, Brown GW, Hieter P (2004) Identification of protein complexes required for efficient sister chromatid cohesion. *Molecular Biology of the Cell* **15**(4)**:** 1736-1745

Mohanty BK, Bairwa NK, Bastia D (2006) The Tof1p-Csm3p protein complex counteracts the Rrm3p helicase to control replication termination of Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences of the United States of America* **103**(4)**:** 897-902

Mossi R, Hubscher U (1998) Clamping down on clamps and clamp loaders - The eukaryotic replication factor C. *European Journal of Biochemistry* **254**(2)**:** 209-216

Murray AW (1991) Cell cycle extracts. *Methods Cell Biol* **36:** 581-605

Nedelcheva MN, Roguev A, Dolapchiev LB, Shevchenko A, Taskov HB, Shevchenko A, Francis Stewart A, Stoynov SS (2005) Uncoupling of unwinding from DNA synthesis

implies regulation of MCM helicase by Tof1/Mrc1/Csm3 checkpoint complex. *Journal of Molecular Biology* **347**(3)**:** 509-521

Noguchi E, Noguchi C, Du LL, Russell P (2003) Swi1 prevents replication fork collapse and controls checkpoint kinase Cds1. *Molecular and Cellular Biology* **23**(21)**:** 7861-7874

Noguchi E, Noguchi C, McDonald WH, Yates JR, Russell P (2004) Swi1 and Swi3 are components of a replication fork protection complex in fission yeast. *Molecular and Cellular Biology* **24**(19)**:** 8342-8355

Prasanth SG, Prasanth KV, Siddiqui K, Spector DL, Stillman B (2004) Human Orc2 localizes to centrosomes, centromeres and heterochromatin during chromosome inheritance. *EMBO J* **23**(13)**:** 2651-2663

Prasanth SG, Prasanth KV, Stillman B (2002) Orc6 involved in DNA replication, chromosome segregation, and cytokinesis. *Science* **297**(5583)**:** 1026-1031

Shimmoto M, Matsumoto S, Odagiri Y, Noguchi E, Russell P, Masai H (2009) Interactions between Swi1-Swi3, Mrc1 and S phase kinase, Hsk1 may regulate cellular responses to stalled replication forks in fission yeast. *Genes to Cells* **14**(6)**:** 669-682

Smith KD, Fu MA, Brown EJ (2009) Tim-Tipin dysfunction creates an indispensible reliance on the ATR-Chk1 pathway for continued DNA synthesis. *J Cell Biol* **187**(1)**:** 15-23

Sommariva E, Pellny TK, Karahan N, Kumar S, Huberman JA, Dalgaard JZ (2005) *Schizosaccharomyces pombe* Swi1, Swi3, and hskl are components of a novel S-phase response pathway to alkylation damage. *Molecular and Cellular Biology* **25**(7)**:** 2770-2784

Szyjka SJ, Viggiani CJ, Aparicio OM (2005) Mrc1 is required for normal progression of replication forks throughout chromatin in *S. cerevisiae*. *Molecular Cell* **19**(5)**:** 691-697

Takahashi TS, Basu A, Bermudez V, Hurwitz J, Walter JC (2008) Cdc7-Drf1 kinase links chromosome cohesion to the initiation of DNA replication in *Xenopus* egg extracts. *Genes & Development* **22**(14)**:** 1894-1905

Takahashi TS, Walter JC (2005) Cdc7-Drf1 is a developmentally regulated protein kinase required for the initiation of vertebrate DNA replication. *Genes & Development* **19**(19)**:** 2295-2300

Tanaka H, Kubota Y, Tsujimura T, Kumano M, Masai H, Takisawa H (2009) Replisome progression complex links DNA replication to sister chromatid cohesion in *Xenopus* egg extracts. *Genes Cells* **14**(8)**:** 949-963

Tanaka K, Russell P (2001) Mrc1 channels the DNA replication arrest signal to checkpoint kinase Cds1. *Nature Cell Biol* **3**(11)**:** 966-972

Tourriere H, Versini G, Cordon-Preciado V, Alabert C, Pasero P (2005) Mrc1 and Tof1 promote replication fork progression and recovery independently of Rad53. *Molecular Cell* **19**(5)**:** 699-706

Uhlmann F, Cai J, Flores-Rozas H, Dean FB, Finkelstein J, O'Donnell M, Hurwitz J (1996) In vitro reconstitution of human replication factor C from its five subunits. *PNAS* **93**(13)**:** 6521-6526

Unsal-Kacmaz K, Chastain PD, Qu PP, Minoo P, Cordeiro-Stone M, Sancar A, Kaufmann WK (2007) The human Tim/Tipin complex coordinates an intra-S checkpoint response to UV that slows replication fork displacement. *Molecular and Cellular Biology* **27**(8)**:** 3131-3142

Unsal-Kacmaz K, Mullen TE, Kaufmann WK, Sancar A (2005) Coupling of human circadian and cell cycles by the Timeless protein. *Mol Cell Biol* **25**(8)**:** 3109-3116

Urtishak KA, Smith KD, Chanoux RA, Greenberg RA, Johnson FB, Brown EJ (2009) Timeless maintains genomic stability and suppresses sister chromatid exchange during unperturbed DNA replication. *Journal of Biological Chemistry* **284**(13)**:** 8777-8785

von Kobbe C, Karmakar P, Dawut L, Opresko P, Zeng XM, Brosh RM, Hickson ID, Bohr VA (2002) Colocalization, physical, and functional interaction between Werner and Bloom syndrome proteins. *Journal of Biological Chemistry* **277**(24)**:** 22035-22044

Warren CD, Eckley DM, Lee MS, Hanna JS, Hughes A, Peyser B, Jie C, Irizarry R, Spencer FA (2004) S-phase checkpoint genes safeguard high-fidelity sister chromatid cohesion. *Molecular Biology of the Cell* **15**(4)**:** 1724-1735

Wu L, Hickson ID (2006) DNA helicases required for homologous recombination and repair of damaged replication forks. *Annual Review of Genetics* **40**(1)**:** 279-306

Xu H, Boone C, Klein HL (2004) Mrc1 is required for sister chromatid cohesion to aid in recombination repair of spontaneous damage. *Molecular and Cellular Biology* **24**(16)**:** 7082-7090

Yang XH, Shiotani B, Classon M, Zou L (2008) Chk1 and Claspin potentiate PCNA ubiquitination. *Genes & Development* **22**(9)**:** 1147-1152

Yanow SK, Gold DA, Yoo HY, Dunphy WG (2003) *Xenopus* Drf1, a regulator of Cdc7, displays checkpoint-dependent accumulation on chromatin during an S-phase arrest. *Journal of Biological Chemistry* **278**(42)**:** 41083-41092

Yoo HY, Shevchenko A, Dunphy WG (2004) Mcm2 is a direct substrate of ATM and ATR during DNA damage and DNA replication checkpoint responses. *Journal of Biological Chemistry* **279**(51)**:** 53353-53364

Yoshizawa-Sugata N, Masai H (2007) Human Tim/Timeless-interacting protein, Tipin, is required for efficient progression of S phase and DNA replication checkpoint. *Journal of Biological Chemistry* **282**(4)**:** 2729-2740

Zhao H, Piwnica-Worms H (2001) ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Mol Cell Biol* **21**(13)**:** 4129-4139

4 Conclusions

4.1 Dna2

4.1.1 Summary of Results

Dna2 is a well-conserved protein from yeast to human, and we first demonstrate that *Xenopus* Dna2 is indeed a helicase-nuclease, as in yeast and human. Since previous work indicated a role for Dna2 in *Xenopus* DNA replication (Liu et al, 2000), the association of Dna2 with chromatin in S phase was first examined. Dna2 associates with chromatin in S phase and does so in a regulated manner; Dna2 binding to chromatin requires formation of the pre-replication complex (pre-RC) but is independent of both CDK2 activity and the presence of Mcm10 at the forming replication fork, indicating that Dna2 interacts with an early intermediate during replication fork formation. Co-localization with RPA, a single-stranded DNA-binding protein that concentrates at DNA replication forks, reveals that Dna2 moves with the replication fork throughout S phase. And-1, the *Xenopus* ortholog of yeast Ctf4, and Mcm10 were identified as Dna2 interacting proteins; both of these proteins participate in replication of the lagging strand, and Mcm10 is necessary for stable association of DNA polymerase α with the lagging strand during DNA replication (Chattopadhyay & Bielinsky, 2007; Ricke & Bielinsky, 2004). These novel protein-protein interactions are indicative of a role in lagging strand replication, consistent with the role of Dna2 in yeast (Budd & Campbell, 1997; Formosa & Nittis, 1999).

In addition to involvement in DNA replication, Dna2 is also a DNA repair protein that participates in the repair of DNA double-strand breaks (DSBs). Dna2 physically interacts with ATM and Nbs1, proteins involved in the early steps of the cellular response to DSBs when repaired by homologous recombination (HR). Upon DSB induction in chromosomal DNA, Dna2 accumulates on the broken DNA, consistent with other repair proteins, and accumulates on broken DNA to an even greater extent upon checkpoint inhibition. To clarify the role of Dna2 in the processing of a DSB for repair by HR, the temporal association of HR proteins with broken DNA ends was assessed. ATM and Nbs1 associate with DNA ends quickly, with subsequent Dna2 and RPA binding. ATR accumulation is also late, corresponding to the established model that ATR is recruited by RPA-coated ssDNA. This pattern of binding was observed in both interphase (S phase) and CSF (M phase) extracts, revealing that the ability of Dna2 to bind free DNA ends is not limited to a single phase of the cell cycle. Depletion of various proteins reveal that both the Dna2 protein and the MRN complex are necessary for processing of a DSB by HR, but the nuclease activity of Mre11 is not essential for DSB processing. Finally, we see that even though Dna2 participates in DNA replication and repair of DSBs by HR, neither the Dna2 protein itself nor its enzymatic activities are essential for checkpoint signaling in response to stalled DNA replication forks or DSBs.

4.1.2 Significance of Results and Future Directions

While *Xenopus* Dna2 is known to be a potent nuclease (Liao et al, 2008; Liu et al, 2000), this work is the first to show that the helicase activity of *Xenopus* Dna2 has also been evolutionarily conserved. Since the enzymatic activities of Dna2 are well-

conserved, the biochemical roles of Dna2 in cellular functions are also expected to be conserved. We find that this is true of the roles of Dna2 in both DNA replication and DSB repair by HR.

As to whether Dna2 is a genomic DNA replication protein, this work clearly shows that the DNA replication observed is replication of nuclear DNA, not just mitochondrial DNA as suggested previously (Zheng et al, 2008). The regulated binding of Dna2 to chromatin during S phase provides insight into the role of Dna2 in DNA replication, since Dna2 binding is dependent on pre-RC formation but independent of either CDK2 activation or Mcm10 association with the replication fork. As to further studies regarding the role of Dna2 at the replication fork, interesting experiments could include depleting Dna2 and assessing the binding of DNA replication proteins that associate with the fork downstream of Dna2, such as Cdc45, And-1, and DNA polymerase α.

The novel protein-protein interactions detected between And-1, Mcm10, and Dna2 physically link Dna2 to proteins that participate in lagging strand replication. This role in lagging strand replication is consistent with the role of yeast Dna2 in Okazaki fragment processing, as indicated by genetic interactions and assays with purified proteins (Budd et al, 1995; Budd et al, 2005). These physical interactions also suggest potential roles for Dna2 at the replication fork; And-1 is proposed to link the MCM replicative helicase to the DNA polymerases, and Mcm10 stabilizes DNA polymerase α at the replication fork (Chattopadhyay & Bielinsky, 2007; Ricke & Bielinsky, 2004; Tanaka et al, 2009a). Further studies are required to determine if Dna2 contributes to either of these activities. Disruption of either of these processes would lead to replication

stress and increased DNA damage in an otherwise unchallenged S phase. While DNA replication is not efficient in the absence of Dna2, replication does initiate. Understanding how replication fails in the absence of Dna2 would provide insight into the necessity of Dna2 in replication.

This work is the first to demonstrate that Dna2 accumulates on chromatin containing DSBs and is necessary for the processing of DSBs by the HR pathway in *Xenopus* extracts. We show that Dna2 physically interacts with proteins involved in HR and the DSB checkpoint response, namely ATM and Nbs1. The temporal association of these proteins with DNA ends that we see reflects the order in which these enzymes process the DNA ends (Liao et al, 2008). Analyses of depletion experiments allow us to discriminate between the roles of multiple, possibly redundant, nucleases involved in HR. This work is the first to examine relevant genetic results in yeast, presenting biochemical evidence that this phenomenon observed in yeast HR is conserved in *Xenopus,* and providing novel physical interactions of proteins that both confirm and explain known yeast genetic interactions. Mre11 and Dna2 were the main nucleases studied in this work, but future work with CtIP, Exo1, and other proteins involved in HR will continue to provide insight into this DNA repair mechanism, as will exploring the expected redundancies in the Dna2 and Exo1 nucleases in repair by HR.

The ATM-dependent DSB repair checkpoint can be activated in the absence of Dna2. A likely reason for this result is that Exo1, a nuclease, may compensate for lack of Dna2, consistent with yeast Exo1 and Dna2 (Budd & Campbell, 2009; Zhu et al, 2008). To continue to understand the HR mechanism in *Xenopus*, exploring the functional interactions of Dna2 and Exo1 should be pursued.

The biophysical data presented in this work furthers knowledge of the role of Dna2 in both lagging strand DNA replication and DSB repair by HR. Much future work is needed to comprehend the details of the participation of Dna2 in both processes, for which this work lays a foundation.

4.2 Tim/Tipin

4.2.1 Summary of Results

Timeless (Tim) and Tipin form a complex in both interphase extract and in nuclei, and these two proteins are the main members of this complex. The Tim/Tipin complex binds chromatin in S phase in a regulated manner and accumulates on chromatin when replication forks stall. Immunoprecipitation analyses identified a range of Tim/Tipin-interacting proteins that participate in DNA replication and the DNA replication checkpoint, including ATR, ATRIP, Rad17, RFC40, Claspin, Cdc7, Drf1, and BLM. Absence of the Tim/Tipin complex does not greatly affect bulk DNA replication, but full activation of the DNA replication checkpoint is dependent upon the presence of the Tim/Tipin complex. Consistent with participation in the DNA replication checkpoint, Tipin is also a target of the ATR kinase on residue S222 of human Tipin. This appears to be the only ATR phosphorylation site on Tipin, as detected in an *in vitro* kinase assay. Tipin is not phosphorylated by the ATM kinase, indicating that the Tim/Tipin complex is mainly responsible for checkpoint activation due to stalled replication forks and incomplete DNA replication, not DNA double-strand breaks. Interestingly, Tim was not phosphorylated upon induction of the ATM and ATR-dependent checkpoints. Tipin, but

not Tim, was also found to be a target of the Cdc7 kinase. While the Cdc7 phosphorylation site(s) on Tipin has not been found, potential regulation of Tim/Tipin by Cdc7 has interesting implications for DNA replication and checkpoint activation.

4.2.2 Significance of Results and Future Directions

The findings that Tim and Tipin form a complex, associate with replicating chromatin in a regulated manner, accumulate on chromatin with stalled replication forks, interact with Claspin, are not necessary for DNA replication, and are involved in activation of the DNA replication checkpoint have all been confirmed by published papers subsequent to the initiation of this project (Chou & Elledge, 2006; Errico et al, 2007; Gotter et al, 2007; Smith et al, 2009; Tanaka et al, 2009b; Unsal-Kacmaz et al, 2007; Urtishak et al, 2009; Yoshizawa-Sugata & Masai, 2007). We confirm that Tipin is phosphorylated upon checkpoint induction (Matsuoka et al, 2007) and determine that Tipin is a target of the ATR, not ATM, kinase. Tipin contains one conserved SQ motif, and it is this serine, S222 of human Tipin, that is the target of ATR. Mutation of this residue reveals that this is the only ATR phosphorylation site in Tipin. Interestingly, S222 resides at the edge of a conserved RPA binding site in Tipin, and it has been previously reported that the interaction between Tipin and RPA is lessened upon UV treatment (Gotter et al, 2007; Unsal-Kacmaz et al, 2007). Given these results, we speculate that ATR phosphorylation of Tipin on residue S222 may weaken the RPA-binding activity of Tipin, promoting dissociation of Tim/Tipin from RPA-coated ssDNA and allowing the binding of other proteins necessary for replication fork re-start or DNA repair. However, ATR phosphorylation of Tipin could affect many other processes in

which Tim/Tipin are involved, including checkpoint activation, recovery or adaptation, DNA replication, or sister chromatid cohesion.

Cdc7 and Drf1 are necessary for efficient DNA replication in *Xenopus*, and like Tim/Tipin, accumulate on chromatin when DNA replication forks stall (Yanow et al, 2003). The yeast homolog of Cdc7, Hsk1, participates in DNA replication checkpoints with the yeast homolog of Tim (Matsumoto et al, 2005; Shimmoto et al, 2009; Sommariva et al, 2005), and this work shows that *Xenopus* Tim interacts with both Cdc7 and Drf1. The Cdc7 kinase also phosphorylates Tipin, but not Tim. This data may indicate that Tim/Tipin and Cdc7 cooperate in the DNA replication checkpoint. Examination of impacts on cellular processes in the presence of either disrupted Cdc7-Tim/Tipin binding or a Tipin mutant that is not phosphorylated by Cdc7 would further understanding of this interaction.

Regulatory kinases tend to phosphorylate Tipin, not the larger Tim protein; Tipin is a target of ATR, Cdc7, and CyclinE/CDK2 (this work and Errico et al, 2007). Neither Tim nor Tipin has any known enzymatic function, so current theory revolves around the Tim/Tipin complex as scaffolding proteins. Since it is Tipin that is the target of regulatory kinases and disrupts Tim self-association (Gotter, 2003), data indicates that Tipin may be serving as the regulatory member of the Tim/Tipin complex. However, knockdown of either Tim or Tipin destabilizes its partner (Chou & Elledge, 2006; Unsal-Kacmaz et al, 2007; Yoshizawa-Sugata & Masai, 2007), so it has been difficult to determine the roles of the individual proteins. Tipin may regulate binding of the Tim/Tipin complex to RPA-coated ssDNA, since it is Tipin that contains a conserved RPA binding site (Unsal-Kacmaz et al, 2007), and the post-translational modifications to

Tipin may affect binding of the complex to the replication fork and ssDNA. These modifications may also affect stability of the replication fork, since replication forks do not extend as far as wild-type in the absence of Tipin (Unsal-Kacmaz et al, 2007).

Further studies should be conducted regarding the Tim/Tipin interaction with Orc2, the regulation of Tipin by regulatory kinases, and the roles of the individual Tim and Tipin proteins. Additionally, when Tim is immunoprecipitated from interphase extract, there is caffeine-sensitive kinase activity in the immunoprecipitate that phosphorylates Tim. This activity is present even when washes are sufficiently harsh to dissociate Tipin from Tim (unpublished data). Identification of the protein responsible for this kinase activity may also help delineate the role of the Tim/Tipin complex in cellular processes.

In conclusion, both Dna2 and Tim/Tipin contribute to the maintenance of genomic integrity in different ways. These proteins travel with the DNA replication forks during S phase, but they likely have very different roles at the fork, since they also respond to different forms of DNA damage. Dna2 participates in the response to DNA double-strand breaks, while Tim/Tipin respond to replication forks. These mechanisms represent part of the cadre of cellular responses to DNA damage, yet impairment of just one of these processes produces significant effects. Through understanding each of these processes, research can progress to develop better treatments for diseases caused by these failed cellular mechanisms.

4.3 References

Budd M, Campbell J (2009) Interplay of Mre11 nuclease with Dna2 plus Sgs1 in Rad51-dependent recombinational repair. *PLoS One* **4**(1)**:** e4267

Budd ME, Campbell JL (1997) A yeast replicative helicase, Dna2 helicase, interacts with yeast FEN-1 nuclease in carrying out its essential function. *Mol Cell Biol* **17**(4)**:** 2136-2142

Budd ME, Choe W-C, Campbell JL (1995) DNA2 encodes a DNA helicase essential for replication of eukaryotic chromosomes. *Journal of Biological Chemistry* **270**(45)**:** 26766-26769

Budd ME, Tong AHY, Polaczek P, Peng X, Boone C, Campbell JL (2005) A network of multi-tasking proteins at the DNA replication fork preserves genome stability. *PLoS Genetics* **1**(6)**:** 634-650

Chattopadhyay S, Bielinsky AK (2007) Human Mcm10 regulates the catalytic subunit of DNA polymerase-alpha and prevents DNA damage during replication. *Molecular Biology of the Cell* **18**(10)**:** 4085-4095

Chou DM, Elledge SJ (2006) Tipin and Timeless form a mutually protective complex required for genotoxic stress resistance and checkpoint function. *Proceedings of the National Academy of Sciences of the United States of America* **103**(48)**:** 18143-18147

Errico A, Costanzo V, Hunt T (2007) Tipin is required for stalled replication forks to resume DNA replication after removal of aphidicolin in *Xenopus* egg extracts. *Proceedings of the National Academy of Sciences of the United States of America* **104**(38)**:** 14929-14934

Formosa T, Nittis T (1999) Dna2 mutants reveal interactions with Dna polymerase alpha and Ctf4, a Pol alpha accessory factor, and show that full Dna2 helicase activity is not essential for growth. *Genetics* **151**(4)**:** 1459-1470

Gotter AL (2003) Tipin, a novel Timeless-interacting protein, is developmentally co-expressed with Timeless and disrupts its self-association. *Journal of Molecular Biology* **331**(1)**:** 167-176

Gotter AL, Suppa C, Emanuel BS (2007) Mammalian TIMELESS and Tipin are evolutionarily conserved replication fork-associated factors. *Journal of Molecular Biology* **366**(1)**:** 36-52

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

Liu QQ, Choe WC, Campbell JL (2000) Identification of the *Xenopus laevis* homolog of *Saccharomyces cerevisiae* DNA2 and its role in DNA replication. *Journal of Biological Chemistry* **275**(3)**:** 1615-1624

Matsumoto S, Ogino K, Noguchi E, Russell P, Masai H (2005) Hsk1-Dfp1/Him1, the Cdc7-Dbf4 kinase in *Schizosaccharomyces pombe*, associates with Swi1, a component of the replication fork protection complex. *Journal of Biological Chemistry* **280**(52)**:** 42536-42542

Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, III, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP, Elledge SJ (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316**(5828)**:** 1160-1166

Ricke RM, Bielinsky AK (2004) Mcm10 regulates the stability and chromatin association of DNA polymerase-alpha. *Molecular Cell* **16**(2)**:** 173-185

Shimmoto M, Matsumoto S, Odagiri Y, Noguchi E, Russell P, Masai H (2009) Interactions between Swi1-Swi3, Mrc1 and S phase kinase, Hsk1 may regulate cellular responses to stalled replication forks in fission yeast. *Genes to Cells* **14**(6)**:** 669-682

Smith KD, Fu MA, Brown EJ (2009) Tim-Tipin dysfunction creates an indispensible reliance on the ATR-Chk1 pathway for continued DNA synthesis. *J Cell Biol* **187**(1)**:** 15-23

Sommariva E, Pellny TK, Karahan N, Kumar S, Huberman JA, Dalgaard JZ (2005) *Schizosaccharomyces pombe* Swi1, Swi3, and hskl are components of a novel S-phase response pathway to alkylation damage. *Molecular and Cellular Biology* **25**(7)**:** 2770-2784

Tanaka H, Katou Y, Yagura M, Saitoh K, Itoh T, Araki H, Bando M, Shirahige K (2009a) Ctf4 coordinates the progression of helicase and DNA polymerase alpha. *Genes to Cells* **14**(7)**:** 807-820

Tanaka H, Kubota Y, Tsujimura T, Kumano M, Masai H, Takisawa H (2009b) Replisome progression complex links DNA replication to sister chromatid cohesion in *Xenopus* egg extracts. *Genes Cells* **14**(8)**:** 949-963

Unsal-Kacmaz K, Chastain PD, Qu PP, Minoo P, Cordeiro-Stone M, Sancar A, Kaufmann WK (2007) The human Tim/Tipin complex coordinates an intra-S checkpoint response to UV that slows replication fork displacement. *Molecular and Cellular Biology* **27**(8)**:** 3131-3142

Urtishak KA, Smith KD, Chanoux RA, Greenberg RA, Johnson FB, Brown EJ (2009) Timeless maintains genomic stability and suppresses sister chromatid exchange during unperturbed DNA replication. *Journal of Biological Chemistry* **284**(13)**:** 8777-8785

Yanow SK, Gold DA, Yoo HY, Dunphy WG (2003) *Xenopus* Drf1, a regulator of Cdc7, displays checkpoint-dependent accumulation on chromatin during an S-phase arrest. *Journal of Biological Chemistry* **278**(42)**:** 41083-41092

Yoshizawa-Sugata N, Masai H (2007) Human Tim/Timeless-interacting protein, Tipin, is required for efficient progression of S phase and DNA replication checkpoint. *Journal of Biological Chemistry* **282**(4)**:** 2729-2740

Zheng L, Zhou MA, Guo ZG, Lu HM, Qian LM, Dai HF, Qiu JZ, Yakubovskaya E, Bogenhagen DF, Demple B, Shen BH (2008) Human DNA2 is a mitochondrial nuclease/helicase for efficient processing of DNA replication and repair intermediates. *Molecular Cell* **32**(3)**:** 325-336

Zhu Z, Chung WH, Shim EY, Lee SE, Ira G (2008) Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell* **134**(6)**:** 981-994