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

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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

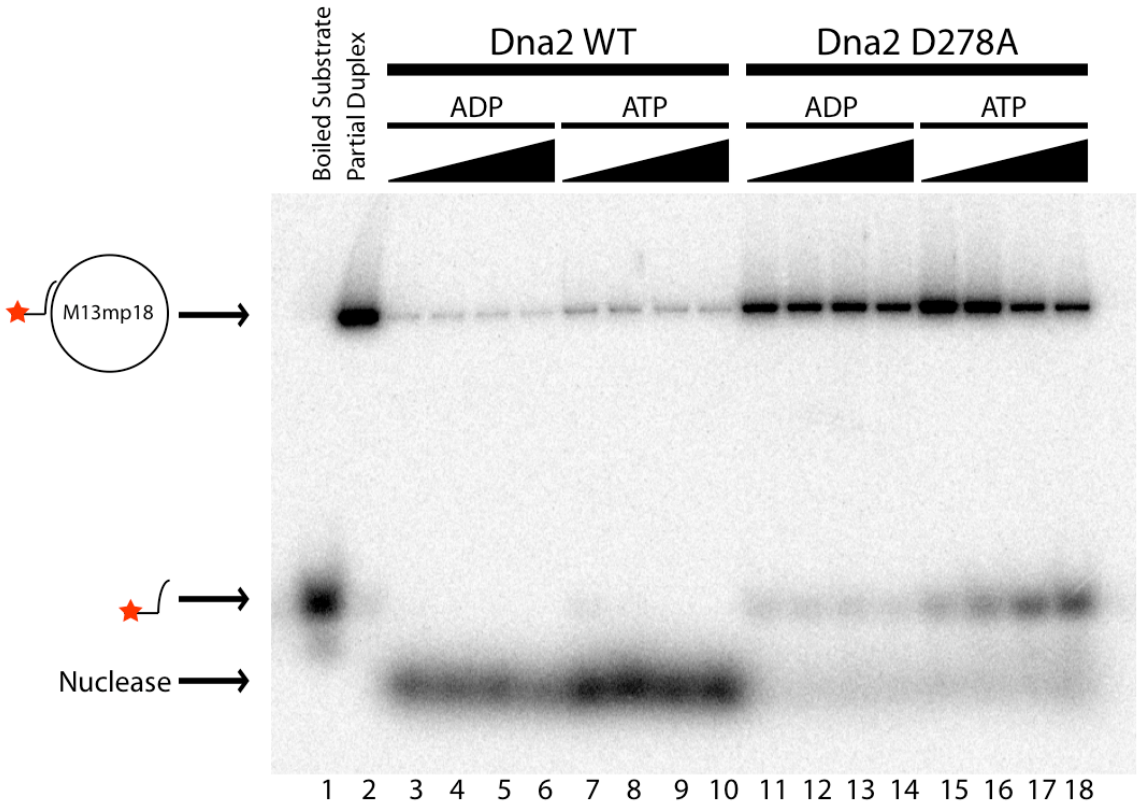

Figure 2.1: Helicase activity of *Xenopus* Dna2. Wild-type *Xenopus* Dna2 (lanes 3 to 10) and nuclease-deficient *Xenopus* Dna2 D278A (lanes 11 to 18) were incubated in helicase assay conditions with approximately 1 fmol of annealed M13-HPR substrate at 37°C for 15 min, either in the presence of ADP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

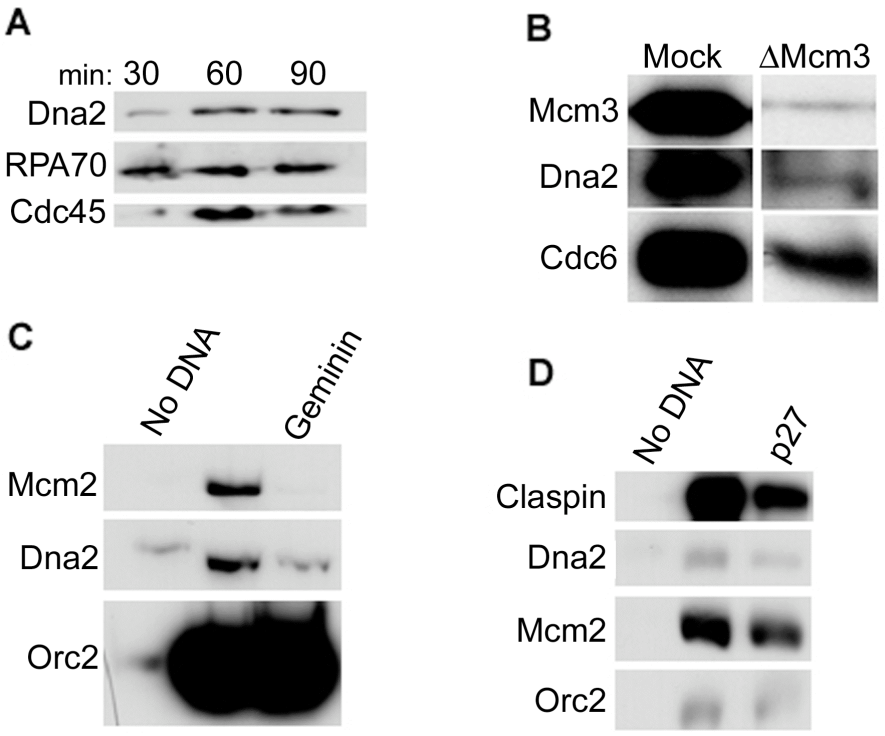

Figure 2.2: Dna2 associates with S-phase chromatin. A) Dna2 accumulation on sperm chromatin throughout S phase. Sperm chromatin was incubated in cell-free extract at 3,000 sperm/μl, isolated at indicated time-points throughout DNA replication and analyzed by immunblotting. DNA replication begins at 30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

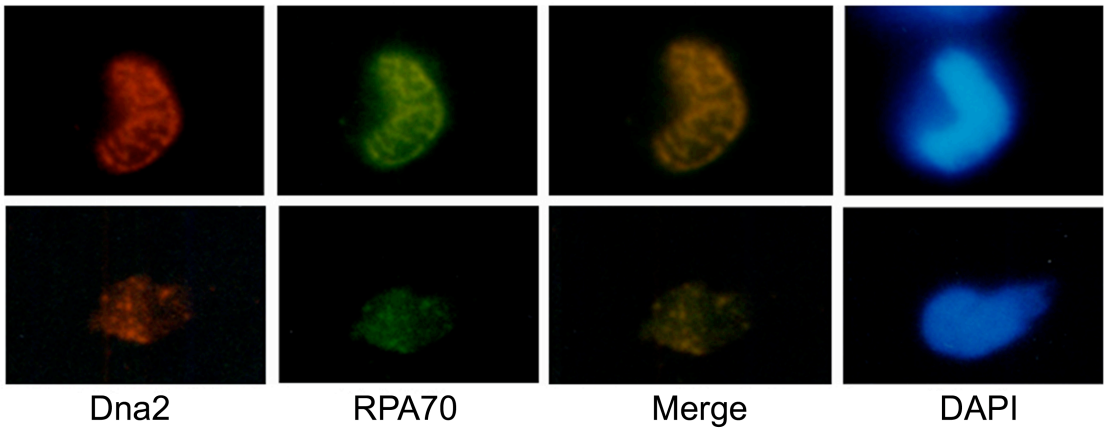

Figure 2.3: Dna2 foci during DNA replication. Sperm chromatin was incubated in extract, fixed, centrifuged onto coverslips, and subjected to immunofluorescence with antibodies to Dna2 and RPA. DNA was stained with DAPI.

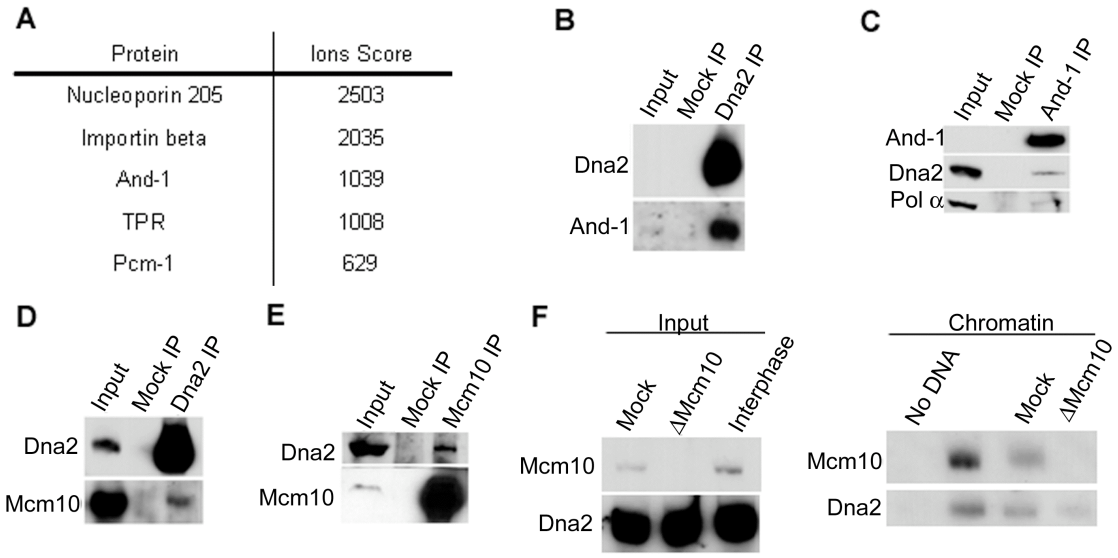

Figure 2.4: Dna2 interacts with DNA replication fork proteins. A) Results from electrospray ionization tandem mass spectrometry analysis of Dna2 immunoprecipitates from interphase extract. Only hits with an ion score above 500 are listed. B) Control IgG (Mock) and anti-Dna2 antibodies were used for immunoprecipitations 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.

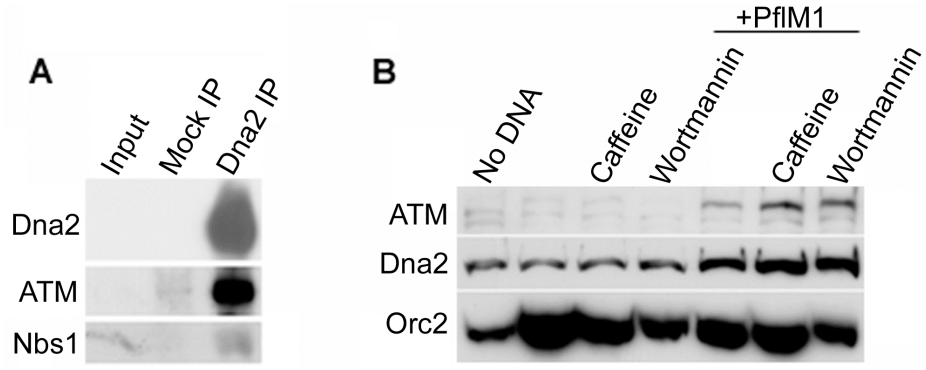

Figure 2.5: Dna2 and double-strand breaks. A) Dna2 interacts with DSB proteins. Anti-Dna2 antibodies were used to immunoprecipitate Dna2 from interphase extract, and immunoblots were performed for Dna2, ATM, and Nbs1. B) Dna2 accumulates on damaged chromatin. Chromatin-association of proteins was analyzed 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.

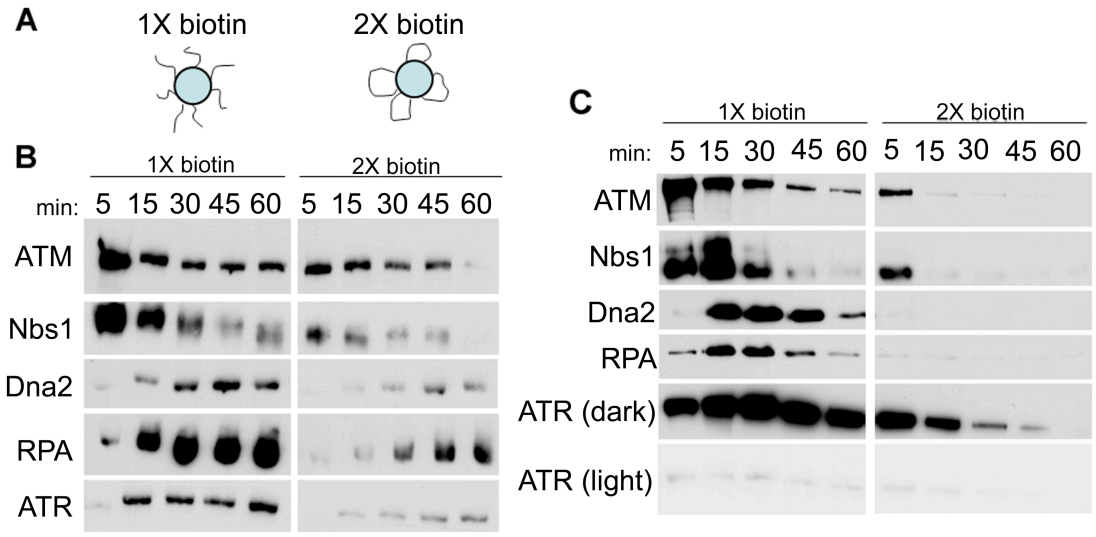

Figure 2.6: Dna2 at DNA ends. A) Schematic of beads used for experiments. pBluescriptIIKS- was linearized and biotinylated on one or both ends, and bound to streptavidin beads. These beads simulated unbroken DNA or DNA with a DSB. B) Time-course of binding of DSB repair and checkpoint proteins to DNA 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.

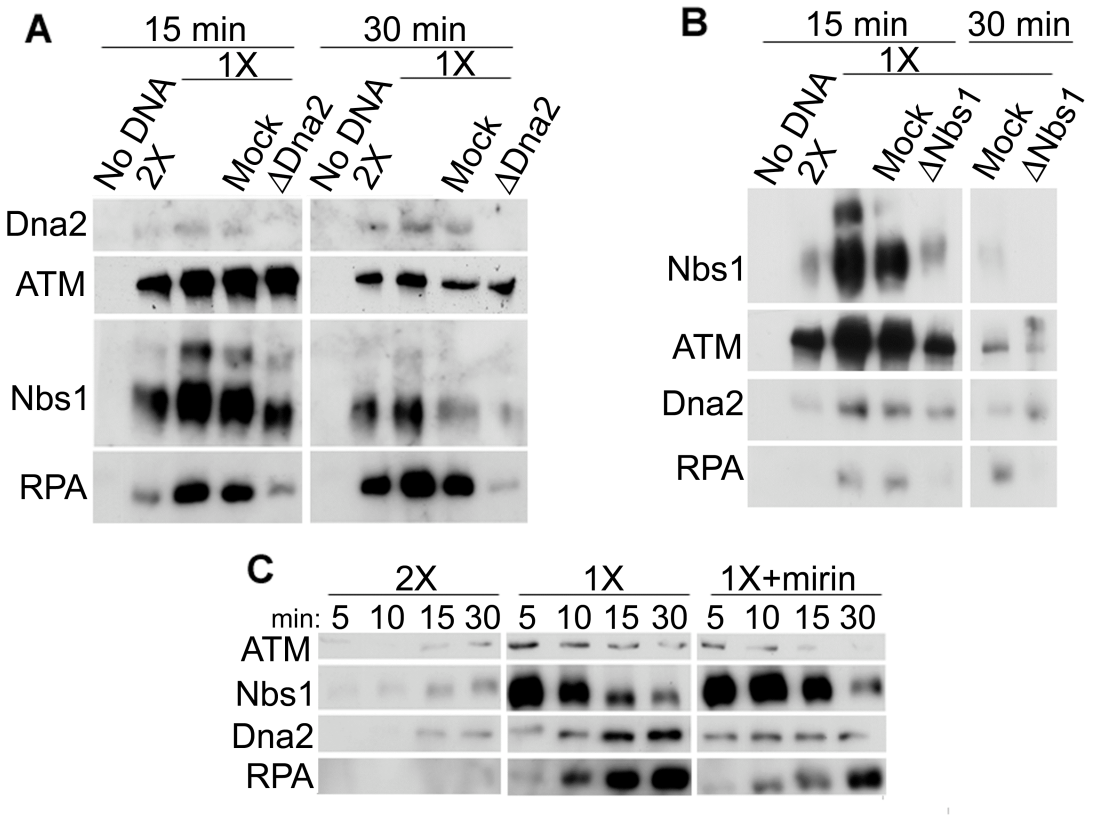

Figure 2.7: Dna2 and MRN at DNA ends. A) Effect of Dna2 depletion on processing of DNA ends. Interphase extracts were untreated, mock, or Dna2-depleted, and incubated with the appropriate beads for 15 or 30 min. Beads were isolated and protein binding was assessed by immunoblotting. B) DNA end binding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

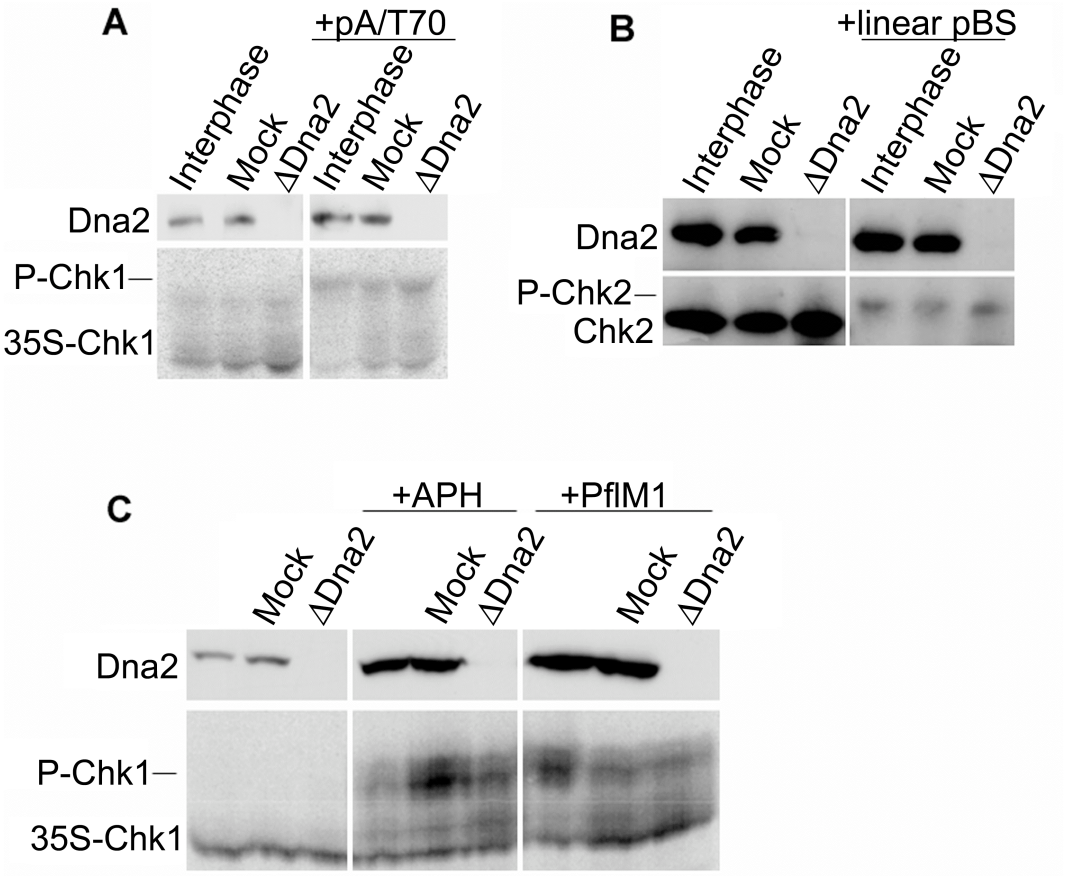

Figure 2.8: Assessment of the DNA replication checkpoint in Dna2-depleted extracts. For all panels in this figure, interphase extracts were untreated, mock, or Dna2-depleted. A) Phospho-Chk1 in Dna2-depleted extracts. pA/T70 oligos were added to interphase extract to elicit a checkpoint response. The 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