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.

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