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

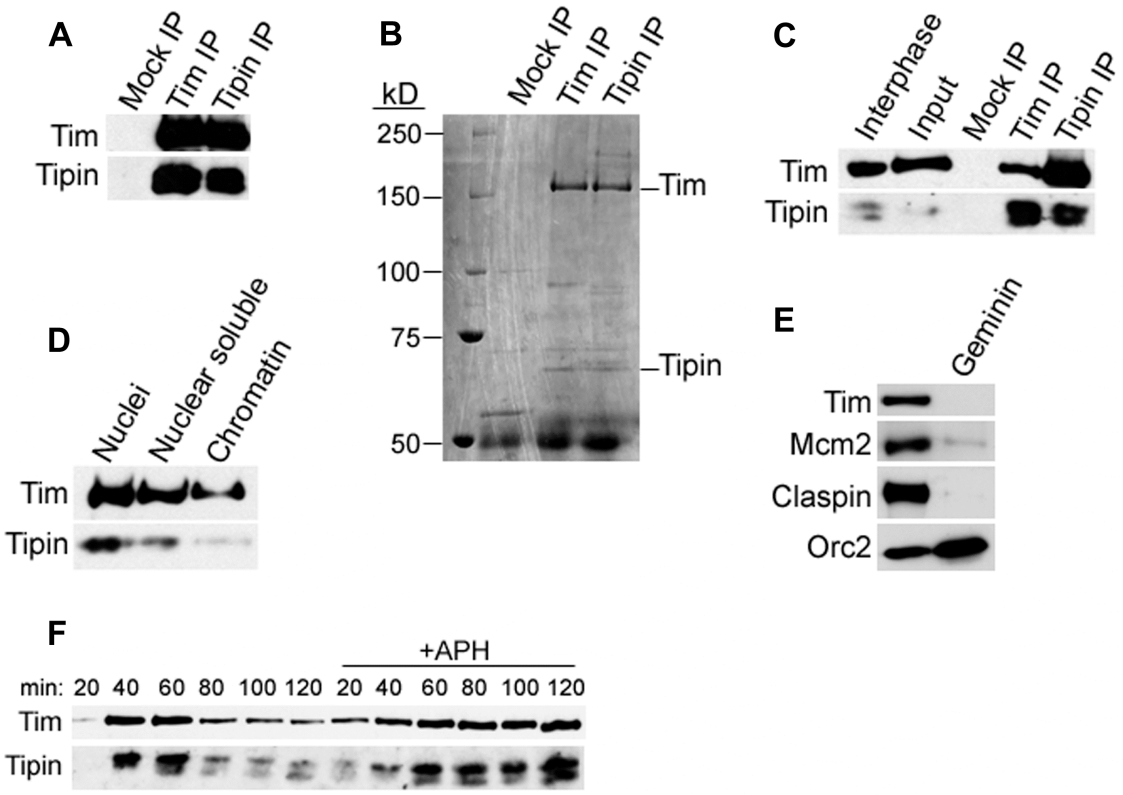

Figure 3.1: Tim and Tipin associate with replicating DNA. A) Tim and Tipin antibodies were used to immunoprecipitate the Tim/Tipin complex from interphase extract. Reactions were analyzed by immunoblotting. B) Tim and Tipin immunoprecipitates from interphase extract were subjected to SDS-PAGE and proteins 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.

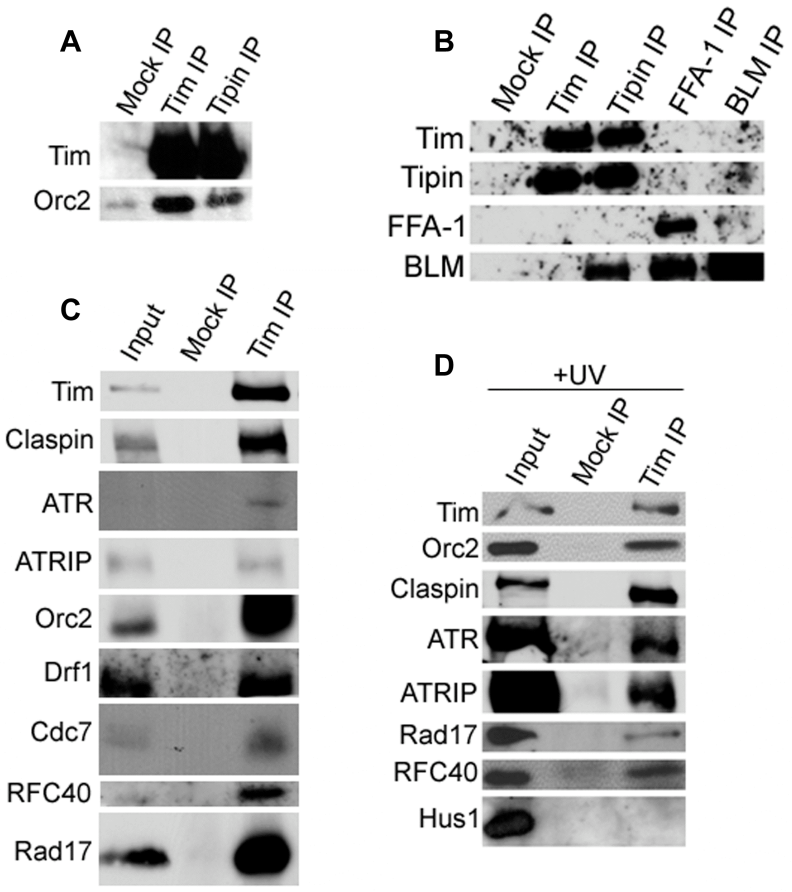

Figure 3.2: Tim/Tipin interactions. A) Mock, anti-Tim, and anti-Tipin immunoprecipitates from interphase extract were subjected to SDS-PAGE, and interacting Tim and Orc2 were detected in immunoprecipitates by immunoblotting. Rabbit IgG was used for Mock IP. B) Interactions between Tim, Tipin, FFA-1, 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.

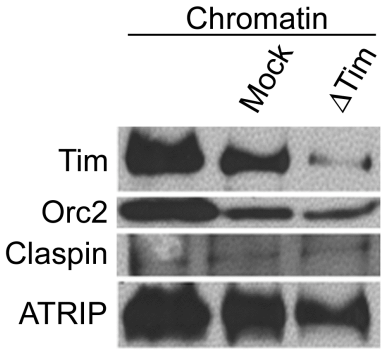

Figure 3.3: Characterization of Tim-depleted chromatin. Interphase extracts were mock or Tim-immunodepleted. Sperm chromatin was added to extract (3,000 nuclei/μl), incubated for 100 min, chromatin was isolated, and chromatin-associated proteins were analyzed by immunoblotting. Unlike published results, 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.

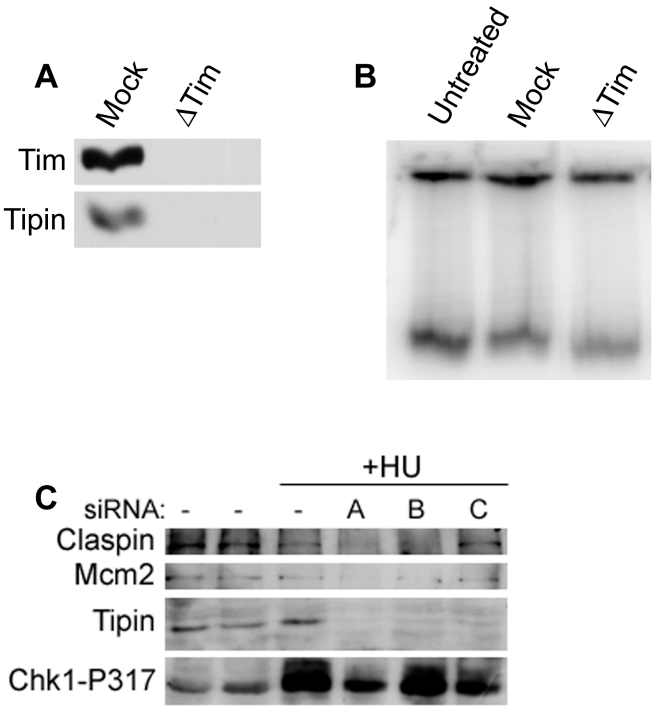

Figure 3.4: Tim/Tipin in DNA replication and the DNA replication checkpoint. A) The amount of Tim and Tipin in 1 μl of mock or Tim-immunodepleted extracts was analyzed by immunoblotting. These extracts were subsequently used for the DNA replication assay in panel B. B) Incorporation from ^{32}P-dATP 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*.

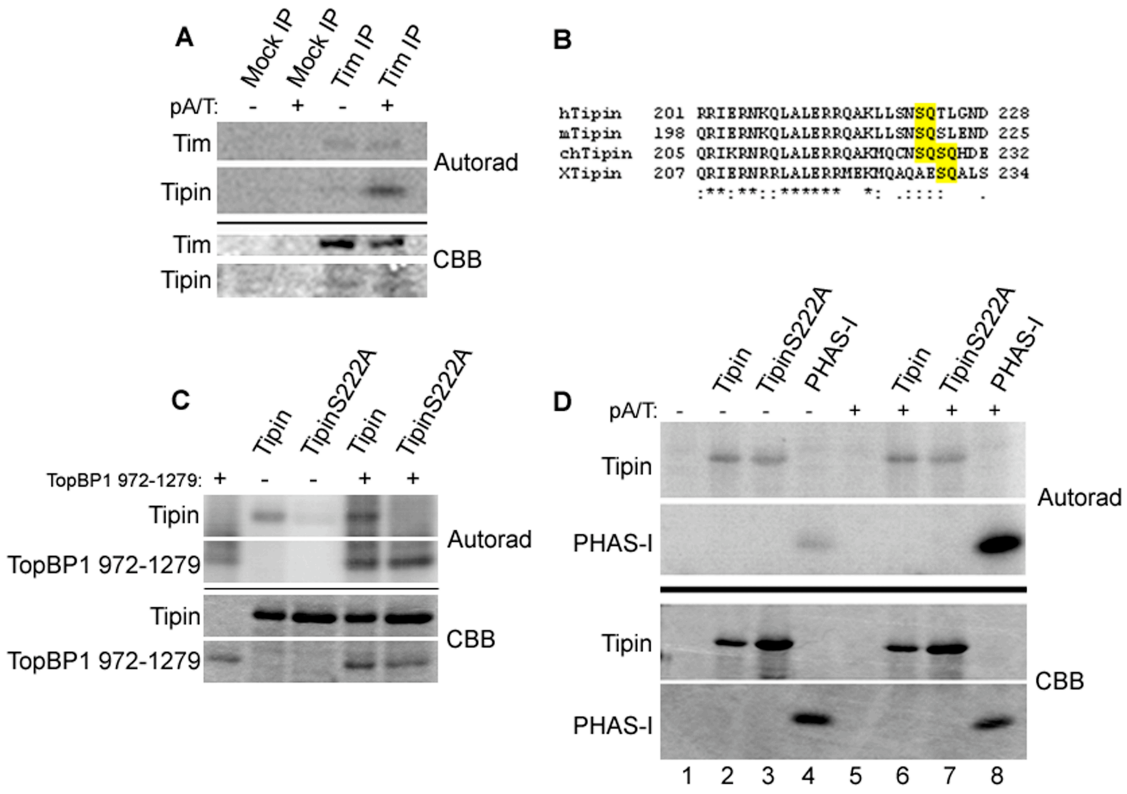

Figure 3.5: ATR phosphorylation of Tipin. A) Interphase extracts containing ^{32}P-ATP were incubated without or with pA/T70 oligos to stimulate checkpoint activation. Mock or anti-Tipin immunoprecipitates were isolated from these extracts, separated by SDS-PAGE, visualized by Coomassie staining, and 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).

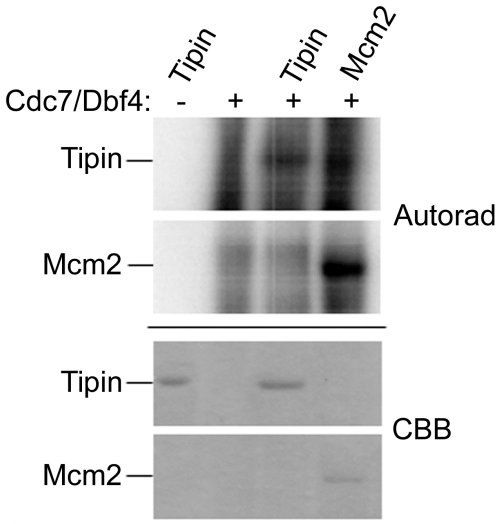

Figure 3.6: Tipin is phosphorylated by Cdc7. Bacterially expressed Cdc7/Dbf4 complex was isolated and used in an *in vitro* kinase assay, with substrates of Cdc7 being Tipin and Mcm2 as a positive control. Substrates were incubated with the kinase complex and ^{32}P-ATP, analyzed by SDS-PAGE, visualized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

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