

Molecular Characterization of the Dbf4/Drf1-  
Dependent Kinase (DDK) and the DNA Replication  
Checkpoint Mediator Claspin in *Xenopus* Egg  
Extracts

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, CA

2010

(Defended September 23, 2009)

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*To my family.*

# Acknowledgements

I would like to thank my thesis advisor, Bill Dunphy, for his unwavering commitment to performing the most rigorous science possible; a commitment that I hope has been instilled in me. I am also thankful for his support over the years that I have been in graduate school. I would also like to thank the members of my thesis committee who have been supportive of me over a long period: Judy Campbell, Bruce Hay, David Chan and Paul Sternberg. In particular, I want to thank David Chan for standing behind me under adverse circumstances that arose when I taught a discussion section for his course. I would like to acknowledge Akiko Kumagai for her endless technical and strategic advice, as well as for providing an excellent scientific role model for me. I am indebted to my labmates, past and present, who have provided advice and comradeship: Hae Yong Yoo, Stephanie Yanow, Esther Bae, Soo-Mi Kim, Seong-Yun Jeong, Jianghai Wang, Wenhui Li, Jason DeHart, Joon Lee, Sanjay Kumar, and Zheng Meng. I want to extend a gratitude that I truly do not have the appropriate words to express to Juanse Ramírez-Lugo, without whom I doubt I would have finished my degree with any measure of sanity (not to mention his scientific and technical advice over the years).

I am also grateful for many personal relationships that have been invaluable for a multitude of reasons over my graduate career: Johannes Graumann for being both my fiercest critic and strongest ally, Anna Maria Salazar for giving me a much finer appreciation of the gradations of the color black not to mention being

a kindred soul, David Walker for career, relationship, and cultural advice, Scott Detmer for his contagious prideful exuberance, Ofer Mazor for teaching me how to be strong as an Ox, Ben Rubin for his soundness of advice and endless ideas, most of which I wish I could have thought of, Melanie Lee for her sharp wit, Tim Lebestky for his irreverent humor and ambitious drive, Ernie, Sumi, and Tai for caloric and soulful sustenance, Nina Tang Sherwood for her sly humor and good cheer, Allan Wong for his passion for science, Baris Bingol for his singular style, Wulf Haubensack for being, well, Wulf, Michael Reiser for being a friend and co-conspirator since I was 15 years old, and Stijn Cassanaer who has made me look forward to the end of nearly every week I have been at Caltech. Special mention should be also paid to Kate Chabarek, Pankaj Mehta, Matt Levin, Nina Gutin and Yan Michael Wang who have given me balance in very different ways. Kate also provided unexpected but most welcome help with proofreading and formatting this document.

Lastly, I thank my parents for their love, support, and encouragement.

# Abstract

Maintaining the integrity of DNA replication control and checkpoint mechanisms is essential for cellular viability and preventing tumorigenesis. Replication initiation is catalyzed by the S-phase Cyclin-dependent kinase (S-CDK) and the Drf1/Dbf4-dependent kinase (DDK) that mediate the unwinding of the replication fork and loading of DNA polymerases onto chromatin. Genotoxic stress that affects cells specifically in S-phase is detected by a discrete replication checkpoint pathway. Replication blockages activate the ATR kinase which, in turn, activates the downstream effector kinase Chk1 through the mediator protein, Claspin. Chk1 facilitates downstream checkpoint functions including arresting cell cycle progression and inhibiting replication firing.

Claspin has two broadly defined roles, one to mediate Chk1 activation and the other as a component of the replication fork. Our studies have attempted to address the linkage between these two facets of Claspin function. Here, we show that Claspin associates with several core replication fork proteins in *Xenopus* egg extracts. We identified a replication fork-interacting domain on Claspin that associates with these replication fork proteins and is required for Claspin association with chromatin. However, chromatin binding-deficient Claspin proteins can still mediate Chk1 activation in Claspin-depleted extracts, albeit with reduced efficiency. Thus, the localization of Claspin at the replication fork is not required for its role in mediation of Chk1 activation but it does potentiate this process.

Another focus of this study, DDK, is composed of the catalytic subunit Cdc7 and one of two distinct adaptor proteins, Drf1 or Dbf4. Drf1 forms a stable, active complex with Cdc7, even after replication arrest in egg extracts. Accumulation of Drf1 on chromatin in the presence of replication blocks is dependent upon ATR and Claspin but not Chk1. We also show that, in the presence of the DNA polymerase inhibitor aphidicolin, inhibition of the replication checkpoint by caffeine leads to modest DNA replication initiation in both somatic cells and egg extracts. This coincides with our observation that DDK remains active in the presence of aphidicolin, and thus may be responsible for initiating origins or stabilizing stalled replication forks against collapse even under conditions of replication stress.

Based on our finding that Drf1 accumulation on chromatin was Claspin-dependent, we further investigated the relationship between these proteins. We characterized *Xenopus* Claspin as a kinase substrate of DDK which forms a stable nuclear complex with Cdc7 and Drf1 under both arrested and unperturbed replication conditions. Moreover, we identified a region of Claspin required for association with DDK that lies within the Chk1-binding domain, which contains a series of repeat sequences. This DDK-associating region is the first, but not the second of these repeat sequences. Furthermore, we have identified two evolutionarily conserved residues within this region required for DDK interaction. Claspin mutant proteins unable to interact with DDK are still able to bind to Chk1 and rescue Chk1 activation in Claspin-depleted extracts. Therefore, we conclude that DDK regulates a largely checkpoint-independent role of Claspin function.

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# Chapter 1

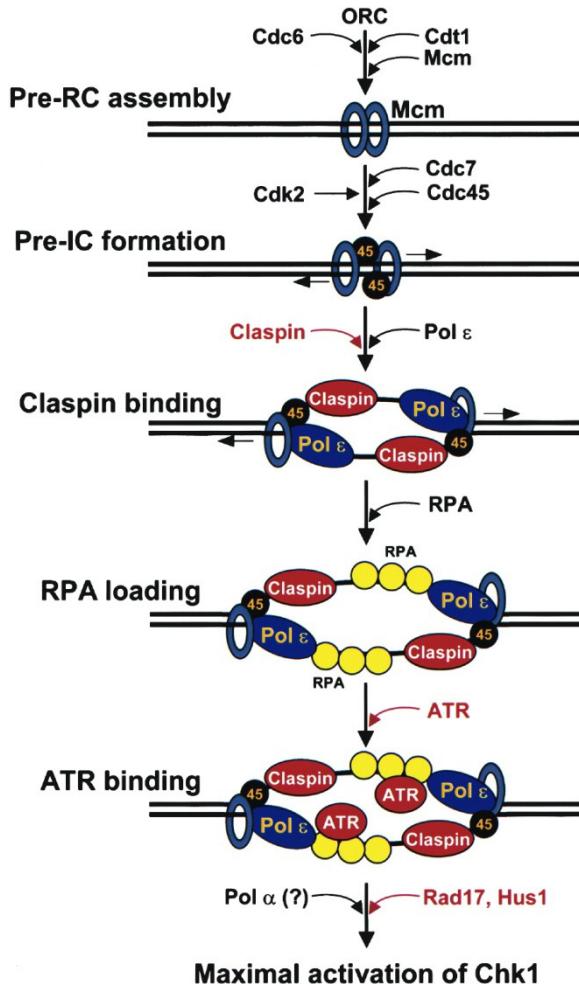
# Introduction

Accurately and completely replicating the genome is of fundamental importance to every organism. To this end, eukaryotes have evolved conserved mechanisms to promote the orderly assembly of replication components that span the exit of M-phase through the completion of S-phase. Besides the proper control of replication initiation and elongation, cells also have processes to ensure protection against both intrinsic and extrinsic genotoxic stress in order to maintain the integrity of the genome. This is accomplished through a set of checkpoints that prevent continuing replication and entry into mitosis in the presence of DNA damage and stalled replication forks, allowing time for repair or a commitment to enter into apoptosis. The breakdown of these checkpoints, either through accrued mutation in the genome or inheritance can lead to unchecked proliferation of cells, a hallmark of cancer.

## **1.1 Replication Initiation**

The mechanisms underlying eukaryotic DNA replication initiation are becoming increasingly well understood (reviewed in (Arias and Walter, 2007; Bell and Dutta, 2002; Takeda and Dutta, 2005)). Briefly, the assembly of replication factors begins with the loading of a set of proteins known as the prereplicative complex (pre-RC) onto chromatin in late M-phase (figure 1.1). These factors assemble in a stepwise fashion starting with the binding of the origin recognition

complex (ORC) onto DNA replication origins. ORC then recruits the initiation factors Cdc6 and Cdt1 to replication origins and in turn, Cdc6 and Cdt1 are required for the loading of the minichromosome maintenance complex, MCM2-7. The MCM2-7 complex is thought to be the eukaryotic replicative helicase, but it is inactive at this point in the cell cycle. Together, ORC, Cdt1, Cdc6, and MCM2-7 comprise the pre-RC and are distributed at potential replication origins throughout the chromosome in M-phase. Only a subset of chromatin-loaded pre-RCs is actually converted into active replication forks during S-phase. The conversion of pre-RCs into active replication forks is catalyzed by the concerted actions of two S-phase kinase complexes, S-phase Cyclin-dependent kinase (S-CDK) and the Drf1/Dbf4-dependent kinase (DDK). These kinases recruit a set of initiation factors including Cdc45, GINS, and Dpb11/TopBP11 which lead to the activation of the MCM2-7 helicase and subsequent unwinding of the replication fork, allowing the association of DNA polymerases and other replication elongation factors.



**Figure 1.1.** Summary of requirements for binding of Claspin to chromatin.

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The molecular details regarding DDK will be extensively reviewed later in this introduction. The role of S-CDK in replication initiation has only recently become apparent in yeast. The essential step in replication initiation involving this kinase that has been described is the S-CDK mediated phosphorylation of two proteins, Sld2 and Sld3, leading to their association with a third protein, Dpb11, and their subsequent loading onto chromatin (Masumoto et al., 2000; Tanaka et al., 2007b; Zegerman and Diffley, 2007). Yeast strains containing suppressing mutations that bypass the requirement of S-CDK in budding yeast for replication initiation still require DDK activity, demonstrating that S-CDK and DDK regulate parallel pathways (Tanaka et al., 2007b; Zegerman and Diffley, 2007). The role of S-CDK in higher eukaryotes is still under investigation. TopBP11 has been identified as the homologue of Dpb11 in higher eukaryotes but homologues of Sld2 and Sld3 have yet to be identified (Tanaka et al., 2007a).

### *1.1.1 Characterization of DDK as an essential S-phase kinase*

The *cdc7* allele was originally identified in the Hartwell screen for temperature-sensitive cell division mutants in *S. cerevisiae* (budding yeast) and was found to be required for the initiation of S-phase (Hartwell, 1971, 1973). The Cdc7 gene was subsequently cloned and predicted to be a serine/threonine kinase based on sequence analysis and was thereafter demonstrated to be an active kinase (Patterson et al., 1986; Yoon and Campbell, 1991). Subsequently, Cdc7 homologues in the fission yeast, *S. pombe* (Hsk1), human and *Xenopus* (both

called Cdc7) were also identified (Jiang and Hunter, 1997; Masai et al., 1995; Sato et al., 1997). The kinase domains of Cdc7 share approximately 60% identity between fission and budding yeasts and mammalian Cdc7 kinase domain shares nearly 45% identity with yeast Cdc7 (Masai and Arai, 2002).

Genetic analyses in a variety of systems also provided evidence that Cdc7 is a key regulator of DNA replication initiation. Firstly, the phenotype for the null mutant of *S. pombe* Hsk1 is premature entry into mitosis and an inability to initiate DNA replication (Masai et al., 1995). Secondly, several studies revealed that antibody competition against or immunodepletion of Cdc7 from *Xenopus* egg extracts prevents DNA replication from occurring (Jares et al., 2000; Roberts et al., 1999; Walter, 2000). Additionally, Cdc7 null knockout mice were found to be embryonic lethal at E3.5–6.5 (Kim et al., 2002). Further investigation revealed that conditional Cdc7 allele knockout mouse ES (embryonic stem) cells display an arrest of DNA replication that leads to activation of the replication checkpoint and entry into apoptosis (Kim et al., 2002; Kim et al., 2003). Interestingly, despite its role at the beginning of S-phase, the Cdc7 protein is stable and expressed in a constitutive manner throughout the cell cycle (Sato et al., 1997; Takeda et al., 1999). These data supported the assertion that Cdc7 was an evolutionarily conserved kinase essential for initiation of replication in studied eukaryotes.

Investigations concerning another gene in yeast, Dbf4, gave further insight into the regulation of Cdc7. The *dbf4* (dumbbell former) mutant was isolated in a screen to identify factors that would arrest *S. cerevisiae* strains in a characteristic

dumbbell shape, which was considered to be a hallmark of failure to initiate DNA replication (Johnston and Thomas, 1982). Genetic linkage to Cdc7 was demonstrated when multiple copies of *DBF4* were later found capable of suppressing the temperature sensitive *cdc7* allele in budding yeast (Kitada et al., 1992). Additionally, the kinase activity of Cdc7 was determined to be dependent upon the presence of Dbf4 (Yoon et al., 1993). It was also observed that Cdc7 and Dbf4 directly interact with each other (Dowell et al., 1994; Hardy and Pautz, 1996). A model then emerged where Dbf4 acts as a Cdc7 adaptor protein, required for activating the Cdc7 kinase. Supporting this, the *S. pombe* homologue of Dbf4 (Dfp1 or Him1) was identified and found to be defective in DNA replication (Brown and Kelly, 1998, 1999; Takeda et al., 1999). In contrast to the steady cell cycle expression of Cdc7, the expression profile of the DBF4 protein in budding yeast varies in a cell cycle-dependent fashion where protein abundance increases in G1, peaks in S-phase and decreases in G2/M (Chapman and Johnston, 1989). The human homologue of Dbf4 was identified by two separate yeast 2-hybrid screens using human Cdc7 as the bait and was named ASK (activator of S-phase kinase) and Dbf4 respectively (Jiang et al., 1999; Kumagai et al., 1999). ASK/Dbf4 was subsequently determined to be necessary for human Cdc7 activation *in vitro* (Masai et al., 2000). The *Xenopus* homologue of Dbf4 was cloned several years later (Jares et al., 2004). ASK/Dbf4 conditional knockout ES cells displayed a phenotype similar to Cdc7 conditional knockout cells where DNA replication is compromised and the cells eventually enter into apoptosis (Yamashita et al., 2005).

There are three conserved sequence motifs of Dbf4 that are called N, M, and C in order from the N-termini to C-termini of the protein (Masai and Arai, 2002).

The M and C motifs comprise a bipartite domain required for binding to and activating the Cdc7 kinase (Masai and Arai, 2002; Ogino et al., 2001). The N motif contains sequences loosely related to BRCA1 C-terminal (BRCT) repeats which, in tandem, are involved in binding phosphopeptides involved in DNA damage repair and checkpoint signaling (Manke et al., 2003; Masai and Arai, 2002). In budding yeast, the Dbf4 N motif contains additional sequences besides the BRCT motif that are conserved amongst Dbf4 homologues but distinct from BRCT-containing proteins and called the BRDF (BRCT and Dbf4 similarity) motif (Gabrielse et al., 2006). The BRDF sequence is required for budding yeast to respond normally to DNA damage (Gabrielse et al., 2006). This coincides with the data that N motif deletions in fission yeast are sensitive to DNA damaging agents (Fung et al., 2002; Ogino et al., 2001).

A second adaptor protein for Cdc7 was identified using a bioinformatic approach to find genes similar to Dbf4 in the human genome and was named Drf1 (Dbf4-related factor 1) (Montagnoli et al., 2002). Human Drf1 physically interacts with Cdc7 and stimulates its kinase activity *in vitro* in a similar fashion to Dbf4 (Montagnoli et al., 2002). Moreover, Drf1 is expressed in mammalian somatic cells along with Dbf4 and exhibits a similar cell cycle-dependent regulation of expression where the protein levels increase in G1, peak in S-phase and decrease in G2/M (Montagnoli et al., 2002). Using overexpressed epitope-tagged recombinant proteins in immunoprecipitation experiments, it was

determined that Cdc7 forms distinct complexes with Drf1 and Dbf4 (Montagnoli et al., 2002). Shortly thereafter, the *Xenopus* homologue of Drf1 was isolated and subsequent immunoprecipitation experiments in *Xenopus* egg extracts with anti-Dbf4 and anti-Drf1 antibodies confirmed that Cdc7 exists in distinct complexes with each adaptor protein (chapter 2) (Takahashi and Walter, 2005; Yanow et al., 2003). Additional studies using *Xenopus* egg extracts showed that the Drf1/Cdc7 complex was in greater abundance than Dbf4/Cdc7 and that Drf1 but not Dbf4 was required for replication initiation in the extract (Silva et al., 2006; Takahashi and Walter, 2005). This situation persists until gastrulation in the embryo when Drf1 protein levels decline and Dbf4 levels increase, demonstrating that Drf1 drives Cdc7 activity in early embryonic cell cycles (Silva et al., 2006; Takahashi and Walter, 2005). Both human and *Xenopus* Drf1 homologues have the characteristic N, M, and C motifs in common with Dbf4, suggesting they are in the same gene family (chapter 2) (Montagnoli et al., 2002; Yanow et al., 2003).

There are several similarities between Cyclin-dependent kinases (Cdk(s)) and Cdc7, since like Cdk(s), Cdc7 is stable and expressed throughout the cell cycle and its activity is regulated by the binding of an adaptor protein that is expressed in a cell cycle-dependent manner. Therefore, the Cdc7 is referred to as DDK (Dbf4-dependent kinase or sometimes Drf1/Dbf-dependent kinase) by analogy to Cdk(s) (Johnston et al., 1999). The major physiological substrate for DDK is thought to be the minichromosome maintenance complex, MCM2-7. Other known substrates of DDK include the replication fork unwinding factor, Cdc45, in yeast (Nougarede et al., 2000), DNA polymerase  $\alpha$  in yeast (Weinreich and

Stillman, 1999), the polo box domain (PBD) of Cdc5 in yeast (Miller et al., 2009), the cohesin loading protein Scc2 in *Xenopus* (Takahashi et al., 2008), the p150 subunit of the histone deposition protein, CAF-1 (Gerard et al., 2006), and the checkpoint mediator protein Claspin in mammalian cells (Kim et al., 2008).

### *1.1.2 The role of DDK in replication initiation*

Several subunits of the MCM2-7 complex including MCM2, MCM4, and MCM6 are substrates of Cdc7 (Kihara et al., 2000; Sato et al., 1997; Sheu and Stillman, 2006; Weinreich and Stillman, 1999). Cdc7 and MCM2 interact genetically and MCM2 is phosphorylated in a Cdc7-dependent manner *in vivo* in several systems (Jares et al., 2000; Lei et al., 1997; Snaith et al., 2000; Takeda et al., 2001).

Several physiologically relevant phosphorylation sites have been identified on mammalian MCM2, however a consensus target sequence has yet to be elucidated (Montagnoli et al., 2006). The most important piece of initial data linking the MCM complex as the key target of Cdc7 came from genetic analysis in budding yeast. A genetic screen for suppressors that mediated the DNA replication bypass of the temperature sensitive allele of Cdc7 in *S. cerevisiae* identified *bob1* as one such allele (Jackson et al., 1993). The *bob1* suppressor was subsequently revealed to be a mutation of MCM5 that can suppress null mutants of both Cdc7 and Dbf4, therefore suggesting that the MCM complex plays an important role in DDK function (Hardy et al., 1997; Jackson et al., 1993).

There is extensive research supporting the idea that the MCM2-7 complex is the main replicative helicase (reviewed in detail in (Arias and Walter, 2007; Bell and

Dutta, 2002; Takahashi et al., 2005; Takeda and Dutta, 2005)). Briefly, MCM2-7 proteins form a hexameric ring complex in which each MCM molecule contains a characteristic AAA<sup>+</sup> helicase motif. The MCM2-7 complex has ATP-dependent helicase activity and travels with the replisome. Further insight into the role of the MCM2-7 complex comes from studies involving several mutants and inhibitors of the helicase activity that block DNA replication at the replication origin unwinding step. More recent work has suggested that the actual helicase activity of MCM2-7 is thought to be stimulated by the binding of the unwinding factor Cdc45 and the GINS (Go, Ichi, Nii, and San) complex which has been referred to as the CMG (Cdc45, MCM, and GINS) complex (Gambus et al., 2006; Kubota et al., 2003; Masuda et al., 2003; Moyer et al., 2006). Cdc45 and GINS lack ATPase motifs but they associate with active replication forks and are required for fork progression (Aparicio et al., 1997; Pacek et al., 2006; Takayama et al., 2003; Tercero et al., 2000). Importantly, DDK activity is required for Cdc45 association with the replication fork (Walter, 2000; Zou and Stillman, 2000). Further investigations revealed that phosphorylation of MCM2 and MCM4 by DDK stimulate binding of Cdc45 to chromatin (Masai et al., 2006; Sheu and Stillman, 2006; Tsuji et al., 2006). Thus, the prevailing model is that DDK activity helps convert inactive pre-RC complexes into active DNA replication forks by phosphorylating components of the MCM2-7 complex, thus stimulating MCM helicase activity by promoting the formation of the Cdc45, MCM, and GINS complex on chromatin (figure 1.1).

Besides enzymatic regulation of Cdc7 kinase activity, subnuclear localization is thought to provide a key layer of regulation of the DDK complex. There are several pieces of data that suggest that the DDK complex must be localized to replication origins in order to effectively initiate DNA replication. First, Cdc7 activity is required throughout S-phase for initiation of both early and late firing origins (Bousset and Diffley, 1998; Donaldson et al., 1998). Second, DDK is ratelimiting for origin firing in budding yeast, and tethering Hsk1-Dfp1 to DNA sequences near an origin makes firing at that origin more efficient (Patel et al., 2008). Third, DDK phosphorylates a stable MCM2-7/Cdc45 complex on chromatin exclusively in S-phase (Sheu and Stillman, 2006). Fourth, DDK associates with replication origins in a pre-RC-dependent manner in yeast and *Xenopus* egg extracts (Dowell et al., 1994; Edwards et al., 2002; Jares et al., 2000; Jares et al., 2004). Finally, efficient phosphorylation, *in vitro*, of MCM2-7 requires a stable interaction of those factors and DDK with preferentially phosphorylated MCM complexes that were associated with origin DNA (Francis et al., 2009). These reports suggest that DDK phosphorylates MCM2-7 after association with chromatin and directly at origins of replication. Thus, chromatin interaction of DDK is essential for its activity.

## 1.2 The replication checkpoint

Different types of genotoxic stress can adversely affect cell viability through fundamentally distinct mechanisms. In order to mount an appropriate response to varied classes of stress, cells have evolved multiple signaling pathways that are activated according to the type of challenge encountered (reviewed in (Bartek

and Lukas, 2007; Kastan and Bartek, 2004; Melo and Toczyski, 2002; Sancar et al., 2004)). Damage induced by insults such as ionizing radiation (IR) or free radicals leads to the formation of DNA double-strand breaks (DSBs) which activate the DNA damage checkpoint pathway. Stress that affects cells specifically during S-phase and culminates in the arrest of replication forks, such as ultraviolet radiation, is responded to by a pathway known as the replication checkpoint. These checkpoints respond to genotoxic stress by ensuring that cell cycle progression is arrested until the damage can be repaired and failing that, enter the cell into the programmed cell death pathway. Both the DNA damage and replication checkpoints are organized in similar fashion, with sensor molecules that detect damage and activate a sensor phosphatidylinositol 3-kinase (PI3K)-like protein kinase that controls the initial activation of the pathway. Ataxia telangiectasia mutated (ATM) is the apical (PI3K)-like kinase atop the DNA damage checkpoint pathway and ATM- and Rad3-related (ATR) is its counterpart kinase regulating the replication checkpoint pathway. The next class of organization is made up of checkpoint mediators or adaptor proteins, which cooperate with the sensor kinases to activate downstream effector kinases that regulate the cell cycle arrest, DNA repair, and apoptotic pathways. For the purpose of brevity, the main focus of the below discussion will be on the replication checkpoint in higher eukaryotes.

The prevailing model for the mechanism of activation of the replication checkpoint is that abnormal stretches of single-stranded DNA (ssDNA) are generated by stalled replication forks and this is the key signal recognized by the

checkpoint sensor machinery (reviewed in (Bartek and Lukas, 2007; Kastan and Bartek, 2004; Sancar et al., 2004)). RPA (replication protein A), a single-stranded DNA binding protein, coats the ssDNA generated by the arrested fork and recruits ATR and its binding partner ATRIP to that site. In addition to ATR/ATRIP, the sliding clamp complex of Rad9-Rad1-Hus1 (9-1-1) is loaded by the clamp-loading Rad17 complex onto the ssDNA site. The chromatin-loaded 9-1-1 complex is required for the activation of ATR. The main target for ATR in this context is the downstream effector kinase, Chk1, whose phosphorylation by ATR is mediated by the adaptor protein Claspin. ATR-activated Chk1 kinase transduces the replication checkpoint signal to various factors that effect the arrest of cell cycle progression, inhibit DNA synthesis, late origin firing and if necessary, activation of the programmed cell death pathway.

#### *1.2.1 Claspin as a mediator of the replication checkpoint*

Claspin was first identified in our laboratory as a novel protein that bound to recombinant Chk1 protein isolated from checkpoint-stimulated *Xenopus* egg extracts and was subsequently determined to be required for the activation of Chk1 in response to arrested replication forks (Kumagai and Dunphy, 2000). Claspin is evolutionarily conserved amongst higher eukaryotes and the human homologue shares 49% identity with *Xenopus* Claspin (Kumagai and Dunphy, 2000). Functional yeast homologues were identified and named MRC1 (mediator of replication checkpoint) but these proteins contain little sequence similarity to Claspin (Alcasabas et al., 2001; Tanaka and Russell, 2001). Claspin is a large, acidic (pI = 4.5) protein which contains no shared protein motifs except

between other Claspin homologues in other organisms (Kumagai and Dunphy, 2000). Human Claspin is also required for activation of Chk1 in response to replication stress (Chini and Chen, 2004; Lin et al., 2004). Further investigation into the mechanism of Chk1 activation mediated by *Xenopus* Claspin revealed that an evolutionarily conserved 57 amino acid region of Claspin, called the CKBD (Chk1 binding domain), is required for Chk1 binding and activation (Kumagai and Dunphy, 2003). However a somewhat larger, 129 amino acid region (called the CKAD, Chk1 activating domain) is needed to rescue Chk1 activation after the depletion of Claspin in egg extracts (chapter 3) (Lee et al., 2005). The CKBD protein region is composed of two amino acid repeat sequences, each containing conserved serines that are phosphorylated by an as yet unidentified kinase in response to replication arrest and these phosphoserines are essential for binding to and activating Chk1 (Kumagai and Dunphy, 2003). The human Claspin CKBD is phosphorylated in a similar fashion to the *Xenopus* homologue. However, it contains three repeat sequences (as do several Claspin homologues in other organisms) but only two are required for Chk1 activation (Chini and Chen, 2006; Clarke and Clarke, 2005). These checkpoint-induced phosphoserines of Claspin bind the catalytic domain of Chk1, mediating Chk1 activation by ATR/ATRIP, and culminate in the release of activated Chk1 (Guo et al., 2000; Jeong et al., 2003; Kumagai et al., 2004).

### *1.2.2 The role of Claspin on the replication fork*

In addition to its role in mediating Chk1 activation in response to replication stress, a number of studies have demonstrated that Claspin also has a role in

replication. Claspin was found to associate with the replication fork during S-phase in *Xenopus* egg extracts (Lee et al., 2003). This association was dependent upon Cdc45 and S-CDK, but not RPA, implying that Claspin loads onto chromatin at the DNA unwinding step of replication initiation, which is similar to the time that DNA polymerase  $\epsilon$  (Pol  $\epsilon$ ) is loaded onto chromatin (figure 1.1) (Lee et al., 2003). Claspin interacts with replication fork proteins such as Cdc45, RFC (replication factor C), RPA, and Pol  $\epsilon$  (Lee et al., 2005; Lee et al., 2003; Lin et al., 2004; Sar et al., 2004). Moreover, Claspin is required for a normal rate of DNA replication (Lee et al., 2003; Lin et al., 2004; Petermann et al., 2008). The budding yeast homologue of Claspin, Mrc1, also was found to associate with the replication fork, is required for a normal rate of replication, and interacts with replication fork proteins (Hodgson et al., 2007; Katou et al., 2003; Lou et al., 2008; Osborn and Elledge, 2003; Szyjka et al., 2005; Tourriere et al., 2005).

Subnuclear spatial dynamics are thought to be important for many aspects of replication checkpoint signaling. Claspin is involved in stabilizing replication forks after replication arrest and Claspin accumulates onto replication-arrested chromatin (Lee et al., 2005; Lee et al., 2003; Lin et al., 2004). The subcellular localization of Claspin is exclusively nuclear (Chini and Chen, 2003; Liu et al., 2006). However, despite its accumulation onto chromatin in response to replication stress, no subnuclear change is observed by immunofluorescence in cells under those conditions (Chini and Chen, 2003; Liu et al., 2006). Chk1 is nucleoplasmic and very mobile, which is thought to be important for its role in mediating signaling between the site of a replication fork arrest and its targets of

checkpoint arrest such as preventing the firing of other replication origins (Bekker-Jensen et al., 2006). Claspin displays spatial properties and dynamics similar to Chk1 in response to damage (Liu et al., 2006). One question that arises from these observations is whether Claspin is required to be associated with the replication fork in order to mediate Chk1 activation. To answer this question, a truncated form of *Xenopus* Claspin that has a compromised ability to bind to replication forks was generated and was found capable of activating Chk1 in response to replication arrest in Claspin depleted extracts but with a significantly reduced efficiency (chapter 3) (Lee et al., 2005). This suggests that Claspin association with stalled replication forks is not strictly required to mediate Chk1 activation but the stalled fork binding does increase the efficiency of the process.

Recent investigations have implicated Claspin with another complex of proteins on the replication fork. A study in mammalian cells found that the protein TIM (timeless) interacts with Chk1, ATR, and ATRIP in an replication checkpoint-stimulated manner (Unsal-Kacmaz et al., 2005). Furthermore, siRNA-mediated knockdown of TIM led to reduced activation of Chk1 after replication arrest (Unsal-Kacmaz et al., 2005). TIM has a stable binding partner named TIPIN, which was found to interact with Claspin in mammalian cells (Gotter et al., 2007). TIPIN also interacts with Claspin in *Xenopus* egg extracts and is required for Chk1 activation and chromatin accumulation of Claspin in response to arrested replication in the extract (Errico et al., 2007). Moreover, TIM and TIPIN interact with several replication fork proteins such as RPA and components of the MCM2-7 complex (Chou and Elledge, 2006; Gotter et al., 2007; Unsal-Kacmaz et al.,

2007). Depletion of TIM or TIPIN by siRNA in mammalian cells leads to an increased sensitivity to genotoxic stress in the cells as well as down-regulation of the intra-S-phase checkpoint that results in damage resistant DNA synthesis (Chou and Elledge, 2006; Gotter et al., 2007; Unsal-Kacmaz et al., 2007; Unsal-Kacmaz et al., 2005; Yoshizawa-Sugata and Masai, 2007). Interestingly, depletion of TIM in mammalian cells leads to a reduced rate of replication fork progression in unperturbed cells but depletion of TIPIN in mammalian cells and *Xenopus* egg extracts had no such effect (Errico et al., 2007; Unsal-Kacmaz et al., 2007). However, depletion of TIPIN in the egg extract abated replication fork recovery after removal of the DNA polymerase inhibitor aphidicolin, perhaps due to the inability of Claspin to load onto chromatin under these conditions (Errico et al., 2007). Finally, depletion of Chk1, Claspin, or TIM prevents the monoubiquitylation of PCNA in response to replication stress, which is thought to recruit translesion DNA polymerases to repair damaged chromatin (Yang et al., 2008). Taken together, the data demonstrate that Claspin exists in a replication monitoring complex consisting of TIM, TIPIN, ATR, and ATRIP that is involved in ensuring fork stability and restart after replication arrest.

#### *1.2.3 Claspin is required for deactivation of the replication checkpoint*

Other recent studies have characterized the involvement of Claspin in turning off the replication checkpoint response. In mammalian cells, Claspin levels are high in S-phase but low in G2, M-phase and G1, suggesting possible regulation by ubiquitin-mediated proteolysis (Mailand et al., 2006; Mamely et al., 2006). Using a candidate approach, depletion of  $\beta$ -TRCP ( $\beta$ -transducin repeat containing

protein), the substrate adaptor for the E3 ubiquitin ligase  $SCF^{\beta\text{-TRCP}}$  (Skp1/Cul1/F-box protein), led to a increase of Claspin protein levels in G2, when it is normally present at low levels (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Also, Claspin was found to interact with  $\beta\text{-TRCP}$  (Peschiaroli et al., 2006). In G1, Claspin is degraded by a distinct E3 ubiquitin ligase, the APC (anaphase promoting complex) and siRNA knockdown of  $SCF^{\beta\text{-TRCP}}$  had no effect on Claspin stability in this cell cycle phase (Fastrup et al., 2009; Gao et al., 2009). The deubiquitylating enzyme USP7 was found to counteract  $SCF^{\beta\text{-TRCP}}$  to maintain Claspin stability but had no effect on APC-mediated ubiquitylation (Fastrup et al., 2009). Plk1 (pololike kinase) was found to be required for Claspin degredation in G2 and promoted Claspin ubiquitylation by  $SCF^{\beta\text{-TRCP}}$  *in vitro* (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Cells expressing nondegradable Claspin, lacking the  $SCF^{\beta\text{-TRCP}}$  ubiquitylation site, exhibit delayed entry into the cell cycle after replication stress due to prolonged activation of Chk1 (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). This coincides with data demonstrating that Plk1 is required for recovery after DNA damage and its kinase activity is inhibited in response to DNA damage (Smits et al., 2000; van Vugt et al., 2004). The finding also complements earlier work showing that Plx1 (the *Xenopus* homologue of Plk1) was found to interact with and phosphorylate Claspin in *Xenopus* egg extracts (Yoo et al., 2004). Extracts where Claspin has been depleted and replaced by a recombinant Claspin protein incapable of interacting with or being phosphorylated by Plx1 are defective in their ability to adapt to aphidicolin-

induced replication stress (Yoo et al., 2004). Specifically, under these conditions Chk1 is not down-regulated and Claspin remains associated with chromatin at high levels (Yoo et al., 2004). Thus, Claspin plays key roles in mediating both activation and deactivation of the replication checkpoint.

### **1.3 The role of DDK in the replication and DNA damage checkpoints**

#### *1.3.1 Evidence in yeast for the involvement of DDK in replication checkpoint*

Experiments in budding and fission yeasts suggest a role for DDK in the replication and DNA damage checkpoints. Several laboratories have demonstrated that budding yeast Dbf4, fission yeast Dfp1/Him1, and Hsk1 are hyperphosphorylated in a Rad53/Cds1-dependent manner in response to the S-phase inhibitor hydroxyurea (HU) (Brown and Kelly, 1999; Snaith et al., 2000; Takeda et al., 1999; Weinreich and Stillman, 1999). There was also a reduction in budding yeast Cdc7 kinase activity observed in response to HU treatment, which reduces the cellular pool of dNTPs (Weinreich and Stillman, 1999). Additionally, the *S. cerevisiae* Cdc7/Dbf4 complex dissociates from replication origins after treatment with HU (Duncker et al., 2002; Pasero et al., 1999). This coincides with the finding that *in vitro* phosphorylation of budding yeast Dbf4 by the Rad53 checkpoint effector kinase inactivates Cdc7-Dbf4 kinase activity (Kihara et al., 2000). However, another report showed that the budding yeast Cdc7 kinase remained active even after HU treatment (Oshiro et al., 1999). As mentioned previously, the N-terminal domain of Dbf4, which is not required for

replication, is required for the viability of budding yeast cells in response to HU (Gabrielse et al., 2006; Varrin et al., 2005).

In fission yeast, a temperature sensitive allele of Hsk1 (*hsk1-89*) is hypersensitive to HU and the DNA damaging alkylating agent methyl methanesulfate (MMS) (Takeda et al., 2001). Moreover, the normally HU-activated kinase Cds1 has its activity compromised after HU treatment in the *hsk1-89* genetic background in both permissive and non-permissive temperature regimes (Takeda et al., 2001). It was also found that N motif deletions in fission yeast Dfp1 conferred sensitivity to UV irradiation, HU, ionizing radiation (IR) and MMS (Fung et al., 2002). Further work has defined that Hsk1 acts in a complex with the replication mediator MRC1 (Claspin) and replication fork stability proteins Swi1 (TIM) and Swi3 (TIPIN) in response to a variety of replication stresses in *S. pombe* (Matsumoto et al., 2005; Shimmoto et al., 2009; Sommariva et al., 2005).

### *1.3.2 The role of DDK in replication checkpoint in higher eukaryotes*

There are several conflicting reports regarding the regulation of DDK complex dynamics under conditions of genotoxic stress in higher eukaryotes. A challenge in characterizing the role of DDK under these conditions is differences that have been observed between the Cdc7-Dbf4 and the Cdc7-Drf1 complexes. Drf1-Cdc7 kinase activity was found to be invariant in response to the DNA polymerase inhibitor aphidicolin, nor to induced double-strand breaks (DSBs) in *Xenopus* egg extracts (chapter 2) (Petersen et al., 2006; Yanow et al., 2003).

Similar results were seen in mammalian cells in response to HU or etoposide treatment (Tenca et al., 2007; Tsuji et al., 2008). However, the topoisomerase II inhibitor, etoposide, which induces double-stranded DNA breaks in S-phase causes the dissociation of Dbf4 from Cdc7 and inhibition of the Cdc7 kinase in *Xenopus* egg extracts (Costanzo and Gautier, 2003). This Dbf4-Cdc7 dissociation was also observed in response to etoposide in leukemia cells (Dierov et al., 2004). In contrast, later investigations by two laboratories working with *Xenopus* egg extracts disputed the earlier *Xenopus* report by showing that Cdc7 remains active and in a stable complex with both Dbf4 and Drf1 in response to aphidicolin and etoposide (Silva et al., 2006; Tsuji et al., 2008). Also in contradiction to this earlier report, other laboratories found that Drf1-Cdc7 and Dbf4-Cdc7 complexes were stable in response to HU or etoposide treatment in HeLa cells and in response to etoposide treatment in two different hematopoietic cell lines, a leukemia cell line and a colon cancer cell line (Tenca et al., 2007; Tsuji et al., 2008). It was suggested by the authors of this study that the Cdc7 antibody used by Dierov et al., (2004) failed to immunoprecipitate native Cdc7-Dbf4 in their laboratory (Tenca et al., 2007). It would seem that an accumulating body of evidence indicates that DDK remains stable and active under conditions of genotoxic stress although there may be some cell-type-specific conditions where the kinase is inhibited. Thus, there seems to be a divergence between checkpoint regulation of DDK between yeasts and higher eukaryotes.

There are reports that DDK association with chromatin may be a mode of regulation for DDK since this interaction is crucial for its role in activating

replication origins. Treatment with aphidicolin leads to the accumulation of Drf1 on stalled replication forks in an ATR- and Claspin-dependent manner in *Xenopus* egg extracts (chapter 2) (Yanow et al., 2003). Aphidicolin treatment as well as depletion of Drf1 (in both unperturbed and aphidicolin-treated extracts) results in a drastic reduction of Cdc45 binding to chromatin (chapter 2) (Yanow et al., 2003). The aphidicolin-stimulated Drf1 chromatin accumulation and the Cdc45 chromatin suppression were also counteracted by treatment with caffeine, which is a PI3K-like kinase inhibitor (chapter 2) (Yanow et al., 2003). This coincides with additional data that caffeine-mediated inhibition of the intra-S-phase checkpoint results in some nascent DNA replication initiation in both *Xenopus* tissue culture (XTC) cells and egg extracts (chapter 2) (Yanow et al., 2003). More detailed analysis has shown that caffeine can lead to increased early origin firing even under unperturbed conditions as well as with low amounts of aphidicolin in *Xenopus* egg extracts (Marheineke and Hyrien, 2004; Shechter et al., 2004). However, the aphidicolin-induced Drf1 accumulation was not observed in a later study with *Xenopus* egg extracts (Silva et al., 2006). However, my inspection of the published data that the authors claim supports their conclusion indicates that there is, in fact, a modest increase in chromatin-bound Drf1 after aphidicolin treatment (Silva et al., 2006). The role of regulation of DDK chromatin association in replication and DNA damage checkpoints remains murky but the more recent investigations seem to suggest that DDK remains bound to replication forks under these conditions. Taken together, the

body of evidence suggests that DDK is not a target for down-regulation by the DNA damage and replication checkpoints in higher eukaryotes.

There are several preliminary indications that DDK may be an upstream regulator of the DNA damage and replication checkpoints in higher eukaryotes.

Overexpression of Dbf4 in mammalian cells or ectopic addition of excess recombinant Dbf4-Cdc7 complexes in *Xenopus* egg extracts suppresses the activation of the ATR-Chk1 pathway, inhibition of late replication, and cell cycle progression in response to etoposide and other DNA damaging agents (Tsuij et al., 2008). Phosphorylation of mammalian MCM4 induced by HU and etoposide was abated by siRNA-mediated knockdown of Cdc7 in HeLa cells (Tenca et al., 2007). Additionally, conditional null Cdc7 knockout mouse ES cells display a reduction in Chk1 activation in response to UV- and HU-induced replication stress but have no effect on Chk2 activation in response to IR treatment (Kim et al., 2008). Similar results were observed in human cancer cells where Cdc7 was depleted by siRNA-mediated knockdown (Kim et al., 2008). These cells displayed other defects in checkpoint activation such as HU sensitivity and a loss of HU-induced phosphorylation and chromatin accumulation of Claspin (Kim et al., 2008). One caveat to these experiments is that replication initiation is required for Chk1 activation in response to replication stress causing agents such as aphidicolin and HU (Lupardus et al., 2002; Michael et al., 2000; Stokes et al., 2002). By eliminating Cdc7 activity, the diminished Chk1 activation in response to HU in the mammalian Cdc7 depletion experiments may be a result of an overall reduction in the number of active replication forks rather than a direct

modulation of the ATR-Chk1 pathway by DDK. However, the authors do observe that ATR still localizes to HU-induced foci under the Cdc7 depleted conditions, suggesting that some upstream replication checkpoint activity remains intact (Kim et al., 2008). One candidate for direct modulation of the ATR-Chk1 pathway is the replication checkpoint mediator, Claspin. Adding credence to this hypothesis is the fact that Cdc7 interacts with and phosphorylates Claspin in mammalian cells (Kim et al., 2008). However, this association and phosphorylation occurs independently of HU treatment (Kim et al., 2008). We have confirmed the Claspin interaction with and phosphorylation by DDK in *Xenopus* egg extracts. This interaction and phosphorylation is independent of aphidicolin and DNA damage checkpoint-inducing DNA templates in the extract (Gold, D.A., et al. unpublished data). One possible model for the role of DDK in replication checkpoint signaling is that it may be required to maintain replication fork stability when the fork is stalled, allowing for possible replication restart if the damage is overcome.

## Chapter 2

# *Xenopus* Drf1, a Regulator of Cdc7, Displays Checkpoint-Dependent Accumulation on Chromatin during an S-phase Arrest

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We have cloned a *Xenopus* Dbf4-related factor named Drf1 and characterized this protein by using *Xenopus* egg extracts. Drf1 forms an active complex with the kinase Cdc7. However, most of the Cdc7 in egg extracts is not associated with Drf1, which raises the possibility that some or all of the remaining Cdc7 is bound to another Dbf4-related protein. Immunodepletion of Drf1 does not prevent DNA replication in egg extracts. Consistent with this observation, Cdc45 can still associate with chromatin in Drf1-depleted extracts, albeit at significantly reduced levels. Nonetheless, Drf1 displays highly regulated binding to replicating chromatin. Treatment of egg extracts with aphidicolin results in a substantial accumulation of Drf1 on chromatin. This accumulation is blocked by addition of caffeine and by immunodepletion of either ATR or Claspin. These observations suggest that the increased binding of Drf1 to aphidicolin-treated chromatin is an active process that is mediated by a caffeine-sensitive checkpoint pathway containing ATR and Claspin. Abrogation of this pathway also leads to a large

increase in the binding of Cdc45 to chromatin. This increase is substantially reduced in the absence of Drf1, which suggests that regulation of Drf1 might be involved in the suppression of Cdc45 loading during replication arrest. We also provide evidence that elimination of this checkpoint causes resumed initiation of DNA replication in both *Xenopus* tissue culture cells and egg extracts. Taken together, these observations argue that Drf1 is regulated by an intra-S-phase checkpoint mechanism that down-regulates the loading of Cdc45 onto chromatin containing DNA replication blocks.

## 2.1 Introduction

In eukaryotes, DNA replication is initiated by a multistep process. Early in the G<sub>1</sub>-phase, replication initiation factors are sequentially assembled onto replication origins to form prereplicative complexes (pre-RCs). At the core of the pre-RC is the origin recognition complex, a six-subunit protein assembly which is required for the subsequent loading of other pre-RC components, including Cdc6/Cdc18, Cdt1, and the Mcms (Nishitani and Lygerou, 2002). The second phase of the initiation process involves the transition of the pre-RC to a pre-initiation complex (Zou and Stillman, 1998). A key determinant for this step is the loading of Cdc45 onto pre-RCs (Aparicio et al., 1999; Zou and Stillman, 2000). This binding requires the concerted actions of the Mcm10 and Mus101 proteins, and the activities of two types of kinases: the S-phase cyclin-dependent kinases (primarily cyclin E-Cdk2) and Dbf4-Cdc7 (Wohlschlegel et al., 2002; Zou and Stillman, 1998, 2000) (Costanzo et al., 2000; Jares and Blow, 2000; Mimura and Takisawa, 1998; Van Hatten et al., 2002; Walter and Newport, 2000). It has been

proposed that the S-phase cyclin-dependent kinases act as global activators of S-phase onset, whereas Dbf4-Cdc7 functions at the level of the individual origins and is therefore required throughout S-phase (Bousset and Diffley, 1998; Donaldson et al., 1998).

The Dbf4-Cdc7 kinase consists of a regulatory subunit, Dbf4, and a catalytic subunit, Cdc7. In yeast, the Cdc7 subunit is present at constant levels throughout the cell cycle, whereas the Dbf4 subunit accumulates only during the G<sub>1</sub>/S-phase and is then degraded by ubiquitin-mediated proteolysis (Brown and Kelly, 1998; Pasero et al., 1999; Takeda et al., 1999; Weinreich and Stillman, 1999). The activation of Cdc7 by Dbf4 leads to the phosphorylation of key substrates within the pre-RC. The best characterized substrate both *in vitro* and *in vivo* is the Mcm2 subunit of the Mcm complex (Brown and Kelly, 1998; Jiang et al., 1999; Lei et al., 1997; Masai et al., 2000). It has been suggested that phosphorylation of Mcm2 alters the structure of the pre-initiation complex to induce DNA unwinding (Geraghty et al., 2000). Mcm subcomplexes possess DNA helicase activity *in vitro* and may be involved in DNA unwinding during origin firing (Ishimi, 1997; Lee and Hurwitz, 2001)

In S-phase cells, the presence of stalled replication forks or damaged DNA invokes a checkpoint response that delays entry into mitosis until the defect has been repaired (Melo and Toczyski, 2002; O'Connell et al., 2000; Zhou and Elledge, 2000). These regulatory mechanisms are known as S-M checkpoints. The signal transduction cascades that underlie S-M checkpoints have been well

characterized in yeast model systems. A defect is recognized by various sensor proteins, which elicit activation of the effector kinases Chk1 or Cds1 (Rad53 in budding yeast), depending on the nature of the checkpoint-inducing DNA signal. These kinases then phosphorylate key targets within the cell cycle machinery to delay mitosis (O'Connell et al., 2000). In vertebrates, homologues of the Rad3 protein include ATR and ATM, both members of the family of phosphatidylinositol 3-kinase-like kinases (Abraham, 2001). Evidence suggests that ATR is involved in the detection of replication blocks, whereas ATM is activated in response to DNA damage, leading to activation of Chk1 and Chk2, respectively (Bartek et al., 2001; Guo and Dunphy, 2000; Guo et al., 2000; Hekmat-Nejad et al., 2000; Liu et al., 2000; Zhao and Piwnica-Worms, 2001). Another protein that has been implicated in the signaling in response to replication blocks is Claspin, which was discovered in *Xenopus* as a Chk1-binding protein that is essential for activation of Chk1 following induction of the replication checkpoint (Kumagai and Dunphy, 2000).

Recent studies in vertebrate systems have also identified a checkpoint pathway that prevents the onset of DNA replication in the presence of damaged DNA. In *Xenopus*, one of the targets of this checkpoint pathway is the Cdc45 protein. Reconstitution of the DNA damage checkpoint in a *Xenopus* cell-free system identified a pathway that activates ATM in response to DNA containing double-strand breaks (Costanzo et al., 2000). ATM, in turn, down-regulates cyclin E-Cdk2 activity, thus preventing the loading of Cdc45 onto chromatin and inhibiting DNA replication. Similarly, Cdc45 is a target of a checkpoint induced by treatment

with etoposide, a topoisomerase II inhibitor (Costanzo et al., 2003). Activation of this checkpoint, which is mediated by ATR, leads to the down-regulation of Cdc7-associated kinase activity and inhibition of the binding of Cdc45 to chromatin.

In yeast, similar pathways are involved in signaling a third checkpoint, the intra-replication checkpoint. This checkpoint inhibits the firing of late replication origins in the presence of stalled replication forks or DNA damage that occurs during S-phase. In budding yeast, both subunits of the Dbf4-Cdc7 kinase undergo Rad53-dependent phosphorylation in response to treatment with hydroxyurea. This phosphorylation releases Dbf4-Cdc7 from chromatin, inhibits its kinase activity, and thereby prevents the loading of Cdc45 (Aparicio et al., 1999; Kihara et al., 2000; Pasero et al., 1999; Weinreich and Stillman, 1999). In mammalian cells, a recent study showed that mice lacking Cdc7 are embryonic lethal, demonstrating that this kinase is essential for embryonic development (Kim et al., 2002). Furthermore, when Cdc7 was conditionally removed from an embryonic stem cell line, cells arrested within S-phase with partially replicated DNA. These results support a critical role for the Dbf4-Cdc7 kinase in ensuring the integrity of the DNA throughout the process of replication.

In this study we have cloned a *Xenopus* member of the Dbf4 family, which we have named *Xenopus* Dbf4-related factor 1 (Drf1). The Drf1 protein is not essential for DNA replication or the recruitment of Cdc45 to chromatin during normal S-phase. However, Drf1 displays highly regulated binding to replicating chromatin. Unlike the scenario in yeast and the etoposide-induced pathway in

*Xenopus*, we have observed that, following replication fork arrest, Drf1 and Cdc7 accumulate on chromatin. This binding is dependent upon ATR and Claspin, and is abrogated by treatment with caffeine. The loss of Drf1 from chromatin in the presence of caffeine correlates with an increase in Cdc45 loading, which is also observed in aphidicolin-treated extracts lacking ATR or Claspin. Caffeine is also capable of overriding an intra-S checkpoint that prevents further initiation events in the presence of aphidicolin, both in XTC cells and in egg extracts. We have established a biochemical assay for this checkpoint by using alkaline agarose gels and have observed a significant increase in the synthesis of small DNA fragments when extracts are treated with aphidicolin and caffeine. We hypothesize that activation of ATR by stalled replication forks leads to the accumulation of Drf1 on chromatin in a checkpoint-dependent manner. This checkpoint-dependent association of Drf1 with chromatin may play a role in preventing the binding of Cdc45 to chromatin during a replication arrest.

## 2.2 Experimental Procedures

2.2.1 *Cloning of a Xenopus Drf1 Homologue*—The following oligonucleotides were designed corresponding to sequences in a *Xenopus* expressed sequence tag (accession no. BG408573 [GenBank] ) from the EMBL sequence library: sense, 5'-gcagcaggacgtgaaccccccattggcc-3'; antisense, 5'-cctccgttccgcagcctggattggac-3'. A polymerase chain reaction (PCR) using a *Xenopus* cDNA library generated with the Marathon RACE kit (Clontech) served as the template. The 270bp PCR product was subsequently biotinylated and

used as a probe to screen a *Xenopus* oocyte cDNA library in the pAX-NMT vector (Mueller et al., 1995) using the ClonCapture cDNA selection kit (Clontech). The same PCR fragment was radiolabeled and used to isolate the full-length cDNA from the pool of clones enriched for Drf1. Positive clones were verified by PCR, and sequencing of both strands was performed with an ABI model 373 automated sequencer. The GenBank accession no. for Drf1 is AY328889 [GenBank].

**2.2.2 Antibodies**—*Xenopus* Cdc7 was amplified by PCR with the following oligonucleotides: 5'-gggaattccatatgagttcgggcataattcagg-3' and 5'-actgggaaattcctaccgcatgttttaaacagagc-3' (Roberts et al., 1999). The RACE *Xenopus* cDNA library described above served as the template. The full-length Cdc7 coding sequence was cloned into the *Nde*I and *Eco*RI sites of the pET3-His6X vector, and the resulting plasmid was transformed into Codon Plus *Escherichia coli* cells for expression and purification as described (Roberts et al., 1999). Antibodies were affinity purified using standard methods with the antigen described above conjugated to CNBr-activated Sepharose 4B (Amersham Biosciences).

Antibodies were raised against an N-terminal fragment of Drf1. The fragment was amplified by PCR using the following oligonucleotides: sense, 5'-gggaattccatatgcagcaggacgtgaacc-3'; antisense, 5'-gccaggtgaattcctatgtgggctcac-3'. The 1270bp fragment was cloned into the *Nde*I and *Eco*RI sites of the pET3-His6X vector and expressed in Codon Plus cells.

The fusion protein was purified from inclusion bodies as described above for Cdc7. Antibodies were affinity-purified as described above. Affinity-purified antibodies against *Xenopus* Claspin, Chk1, and ATR, and antisera against Orc2 were described previously (Carpenter et al., 1996; Kumagai and Dunphy, 2000; Lee et al., 2003). Antisera against *Xenopus* Cdc45 were generously provided by J. Lee. Monoclonal antibodies against human Mcm2 were obtained commercially (BM28, BD Biosciences). Antibodies recognizing *Xenopus* Mcm4 were kindly provided by J. Blow. Control rabbit IgG was obtained from Zymed Laboratories.

**2.2.3 Recombinant proteins-** *Xenopus* GST-Mcm2 was cloned into pGEX4T2 vector (Amersham Biosciences), expressed in Codon Plus cells, and purified with GST-agarose. Recombinant geminin was purified as described (Carpenter et al., 1996; Kumagai and Dunphy, 2000; Lee et al., 2003).

**2.2.4 Egg Extracts**—*Xenopus* egg extracts were prepared as described (Murray, 1991). Egg extracts arrested in interphase because of the presence of unreplicated DNA routinely contained 3000 demembranated *Xenopus* sperm nuclei/ $\mu$ l of extract and 100  $\mu$ g/ml aphidicolin. Caffeine was added to a final concentration of 5 mM from a 100 mM solution freshly dissolved in 10 mM PIPES-KOH (pH 7.5).

**2.2.5 Immunodepletion**—Immunodepletions of Chk1 and Claspin from egg extracts were carried out with Affiprep-protein A beads (Bio-Rad) as described previously (Kumagai and Dunphy, 2000). For immunodepletion of Cdc7 and Drf1, 100  $\mu$ l of serum coupled to Affiprep-protein A beads was used per 100  $\mu$ l of CSF-

arrested extract, during two rounds of depletion. Pre-immune serum served as a control. For immunodepletion of ATR, 25 µg of antibody coated on protein A-magnetic beads (Dynal) was incubated with extracts on ice for 1 h. The beads were removed with a magnet, and the procedure was repeated.

**2.2.6 Chromatin Isolation**—Egg extracts (100 µl) containing 3,000 sperm nuclei/µl were overlaid on a 1-ml sucrose cushion containing chromatin isolation buffer (CIB; 20 mM HEPES-KOH (pH 7.6), 1 M sucrose, 80 mM KCl, 25 mM potassium gluconate, and 10 mM magnesium gluconate), and centrifuged at 6,100 × g for 5 min. The pellets were washed twice with CIB + 0.5% Nonidet P-40, and centrifuged as above. The chromatin pellets were boiled in 2× SDS sample buffer and subjected to SDS-PAGE. To elute chromatin-associated proteins, chromatin pellets were prepared from 400 µl of extract by overlaying 200 µl on a 1ml sucrose cushion in duplicate. Samples were centrifuged at 6,100 × g for 5 min. Chromatin pellets were washed twice with CIB and then once with CIB + 0.5% Nonidet P-40. The supernatants were removed, and the chromatin pellets were resuspended in 100 µl of 1 M NaCl, 10 mM HEPES-KOH (pH 7.6). Samples were incubated on ice for 10 min and then centrifuged at 11,700 × g for 5 min. The supernatant contained the proteins eluted from chromatin.

**2.2.7 Replication Assays**—To monitor DNA replication, egg extracts were incubated with sperm nuclei (1,000–3,000 sperm nuclei/µl), 0.4 mM CaCl<sub>2</sub>, 100 µg/ml cycloheximide, and 1 µl of [ $\alpha$  - <sup>32</sup>P]dATP for 90 min at room temperature. The reaction was terminated upon addition of an equal volume of 2× replication

stop buffer (80 mM Tris-HCl (pH 8), 8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS, and 0.2% bromophenol blue), and 1 mg/ml proteinase K, followed by incubation at 55°C for 1 h. Samples were run on 1% agarose gels, dried, and detected by a PhosphorImager (Amersham Biosciences).

**2.2.8 Immunoprecipitations and Kinase Assays**—For immunoprecipitation of Drf1 and Cdc7 from egg extracts, 100  $\mu$ l of extract was diluted 3-fold with 10 mM HEPES-KOH (pH 7.6), 150 mM NaCl, 2.5 mM EGTA, 20 mM  $\beta$ -glycerophosphate, and 0.5% Nonidet P-40, and centrifuged at 11,700  $\times g$  for 10 min to pellet nuclei. Supernatants were removed and incubated with 5  $\mu$ g of affinity-purified antibody bound to Affiprep-protein A beads for 1 h at 4°C with rotation. The beads were washed three times with buffer X (10 mM HEPES-KOH (pH 7.6), 80 mM NaCl, 2.5 mM EGTA, 20 mM  $\beta$ -glycerolphosphate, and 0.1% Nonidet P-40), then once with HBS (150 mM NaCl, 10 mM HEPES-KOH (pH 7.6)). For immunoprecipitation of proteins eluted from chromatin, the supernatant was removed and diluted 8-fold with 50 mM NaCl, 10 mM HEPES-KOH (pH 7.6) and incubated with 5  $\mu$ g of antibody bound to Affiprep-protein A beads for 1 h at 4°C with rotation. The beads were washed three times with buffer X, and then once with HBS. For *in vitro* kinase assays, the beads were resuspended in HBS. One-half was boiled in 2 $\times$  SDS sample buffer, subjected to SDS-PAGE, and immunoblotted for Drf1 and Cdc7. The other half was incubated with kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 50  $\mu$ M ATP) containing [ $\gamma$ -<sup>32</sup>P]ATP and 1  $\mu$ g of GST-Mcm2. Samples were incubated at

room temperature for 15 min with rotation, then boiled in 2× SDS sample buffer, subjected to SDS-PAGE, and detected by a PhosphorImager.

**2.2.9 Cell Culture and Immunofluorescence**—XTC-2 cells were grown on poly-D-lysine-coated coverslips in 61% Leibovitz's (L-15) medium supplemented with 10% fetal calf serum and antibiotics. Caffeine-treated cells were incubated with 5 mM caffeine 90 min prior to aphidicolin treatment, where applicable. The cells were then incubated with 100  $\mu$ M BrdUrd and 5  $\mu$ g/ml aphidicolin where indicated. At 5.5 h, cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and subjected to indirect immunofluorescence. For BrdUrd visualization, cells were refixed with 0.1% formaldehyde and incubated for 10 min in 2 M HCl and 0.1% Triton X-100 at room temperature. The acid was washed away, and the immunofluorescence procedure was repeated with anti-BrdUrd (Roche) as the primary antibody and Texas Red conjugated anti-mouse IgG (Jackson Laboratories) at a 1:500 dilution as the secondary antibody. The coverslips were washed (with 1  $\mu$ g/ml Hoechst 33258 in the last wash) and mounted onto glass slides with Vectamount (Vector Laboratories). The samples were imaged with a SpotRT CCD camera (Diagnostic Instruments) and analyzed with Adobe Photoshop.

**2.2.10 Alkaline Gel Electrophoresis**—Replication reactions (typically 40  $\mu$ l of egg extract) were resuspended in 300  $\mu$ l of StopN (20 mM Tris-HCl (pH 8), 200 mM NaCl, 5 mM EDTA, and 0.5% SDS) containing 2  $\mu$ g/ml RNase, and digested for 10 min at 37°C. Proteinase K (200  $\mu$ g/ml) was added, and samples were

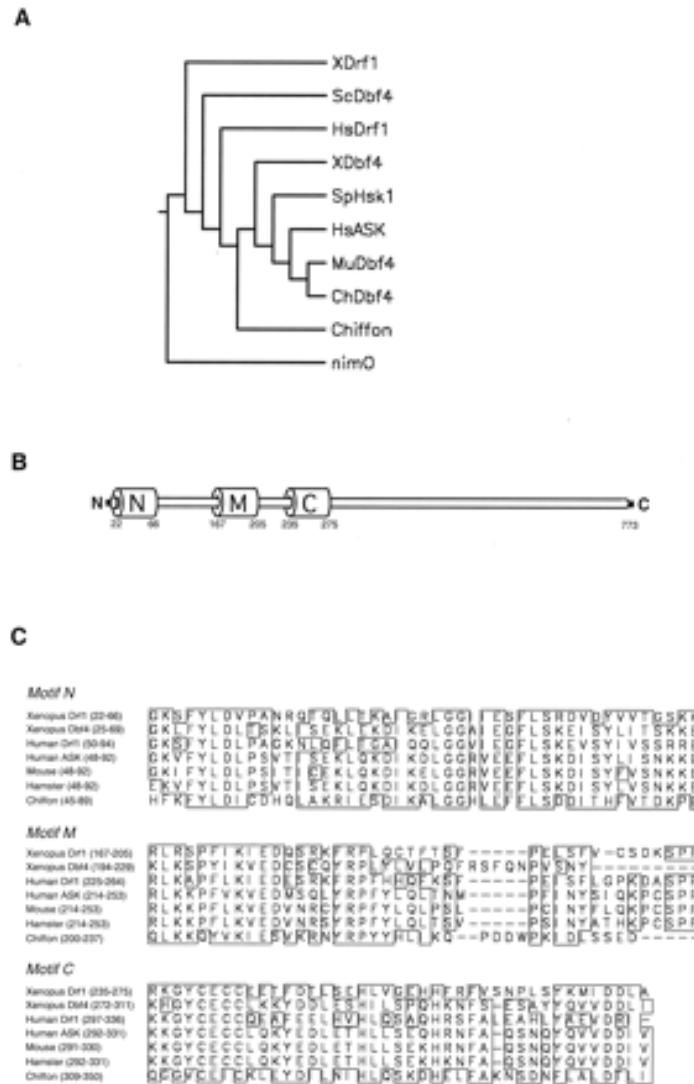
incubated for another 30 min at 37°C. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (Sigma) and then precipitated with ethanol. Pellets were resuspended in 10  $\mu$ l of 10 mM EDTA, and then diluted with an equal volume of 2 $\times$  alkaline loading buffer (100 mM NaOH, 2 mM EDTA, 2.5% Ficoll, and 0.025% bromcresol green). Samples were loaded onto gels containing 1% agarose in 50 mM NaCl and 1 mM EDTA, and run overnight in alkaline gel running buffer (50 mM NaOH, 1 mM EDTA). Gels were fixed with 7% trichloroacetic acid, dried, and autoradiographed.

### 2.3 Results

2.3.1 *Identification of a cDNA Encoding Xenopus Drf1*—A *Xenopus* homologue from the Dbf4 family was cloned by first amplifying a 270bp sequence with oligonucleotides designed to recognize a *Xenopus* expressed sequence tag with homology to Dbf4 proteins in other species. This fragment was then used to screen a *Xenopus* cDNA library to isolate the full-length open reading frame. Recently, a *Xenopus* Dbf4 sequence has been entered in the GenBank database (accession no. AB095983 [GenBank]). Our cloned protein shares significant sequence homology with this protein (28% identical) but represents a second, distinct Dbf4-like polypeptide (figure 2.1A). In humans, two Dbf4-like proteins have been identified, both of which activate Cdc7 (Jiang et al., 1999; Montagnoli et al., 2002). The cDNA that we have identified in *Xenopus* encodes a 772-amino acid protein that is homologous to both human proteins (32% and 26% identical to human Drf1 and ASK, respectively) but shares a higher identity with Drf1 (figure 2.1A). In comparison with other vertebrate Dbf4 proteins, Xdrf1 shares

29% identity with the hamster Dbf4 and 30% identity with the mouse Dbf4. Based on the sequence and the experimental evidence presented below, we believe that we have cloned a Dbf4-related factor, and have named this protein Xdrf1 (referred to hereafter as Drf1).

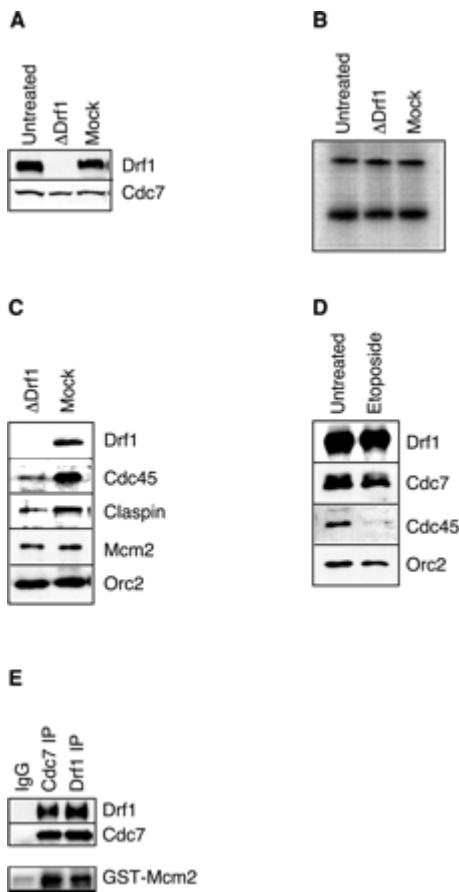
Three distinct amino acid motifs have been described for the Dbf4 gene family: motifs N, M, and C (Masai and Arai, 2000). Motif N shares similarity to a BRCT-like domain and may be functionally important for the replication and DNA damage checkpoints; mutations in this region of the fission yeast homologue, Dfp1/Him1, do not affect the replication functions of the kinase but cause hypersensitivity to drugs that block DNA replication or damage DNA (Ogino et al., 2001). Motif M consists of a proline-rich region, and motif C resembles a CCHH-type zinc finger motif (Masai and Arai, 2000). These two regions of Dfp1/Him1 are both necessary and sufficient for full activation of the kinase (Ogino et al., 2001). All three of these motifs are highly conserved in the *Xenopus* Drf1 protein (figure 2.1B and C).



**Figure 2.1** Sequence analysis of *Xenopus* Drf1.

(A) phylogenetic tree displaying the evolutionary relationships among the following Dbf4-like family members: *Xenopus* (XDrf1 and XDbf4), *Saccharomyces cerevisiae* (ScDbf4), human (HsDrf1 and HsASK), *Schizosaccharomyces pombe* (SpHsk1), mouse (MuDbf4), Chinese hamster (ChDbf4), *Drosophila* (Chiffon), and *Aspergillus* (nimO). (B) schematic of the *Xenopus* Drf1 protein illustrating the three motifs conserved in Dbf4-related proteins. Numbers correspond to amino acid position in the sequence of Drf1. (C) alignment of the motifs in B with those in other species. Numbers in parentheses correspond to amino acid positions in each respective protein.

**2.3.2 Drf1 Is Dispensable for DNA Replication and Cdc45 Loading**—We raised antibodies against an N-terminal fragment of the *Xenopus* Drf1 protein. This antibody efficiently detects the endogenous Drf1 protein by immunoblotting (figure 2.2A). Although the predicted molecular mass of Drf1 is 85 kDa, we found that the protein detected by two different antibodies raised against Drf1 migrated more slowly, at ~150 kDa. Upon removal of Drf1 with anti-Drf1 antibodies, we observed that a significant amount of Cdc7 remained in the Drf1-depleted extract. Possible explanations are that there is a pool of free Cdc7 or that a second Dbf4-like protein may also form a complex with Cdc7, as is the case in human cells. To determine whether Drf1 is required for DNA synthesis, we depleted Drf1 from egg extracts and monitored the extent of DNA replication. We observed no defect in DNA replication in the absence of Drf1 compared with the mock-depleted or untreated extracts (figure 2.2B). This result contrasts with the nearly complete inhibition of replication that has been reported for depletion of the *Xenopus* Cdc7 protein (Jares and Blow, 2000; Roberts et al., 1999). It is possible that our antibodies failed to completely deplete Drf1 from the extracts, thereby allowing some residual kinase to promote DNA replication. However, immunoprecipitation of Drf1 from a Drf1-depleted extract failed to detect any Drf1 protein (data not shown). Hence, our results suggest that either Drf1 is not the regulatory subunit of Cdc7 that is essential for DNA replication or, alternatively, that when Drf1 is depleted from extracts, the replication function of the kinase is performed by a Dbf4-Cdc7 complex.



**Figure 2.2** Drf1 forms an active kinase with Cdc7 but is not essential for DNA replication.

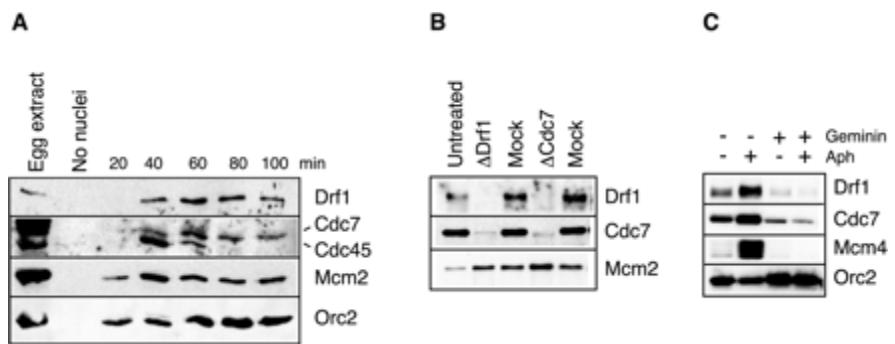
(A) Western blot analysis of Drf1 and Cdc7 in untreated, Drf1-depleted, and mock-depleted extracts. (B) sperm nuclei were incubated in interphase extracts from A in the presence of [ $\gamma$ -<sup>32</sup>P]dATP for 90 min. DNA replication was determined as described under "Experimental Procedures." (C) sperm nuclei were incubated for 60 min in mock-depleted and Drf1-depleted extracts. Chromatin fractions were isolated and immunoblotted with antibodies against Drf1, Cdc45, Claspin, Mcm2, and Orc2. (D) sperm nuclei were incubated in interphase extracts for 100 min in the absence or presence of 30  $\mu$ M etoposide. Chromatin fractions were isolated and immunoblotted with antibodies against Drf1, Cdc7, Cdc45, and Orc2. (E) immunoprecipitation of Cdc7 and Drf1 from interphase extracts. Half of the immunoprecipitate was immunoblotted with antibodies against Drf1 and Cdc7. The other half was incubated with GST-Mcm2 and [ $\gamma$ -<sup>32</sup>P]ATP for *in vitro* kinase assays.

In yeast, both the Dbf4 and Cdc7 subunits are required for the loading of Cdc45 onto chromatin. In *Xenopus*, a similar requirement has been shown for Cdc7 (Walter, 2000). To determine whether depletion of Drf1 affects the ability of Cdc45 to bind to chromatin, we analyzed the chromatin fraction of Drf1-depleted extracts that had been incubated with sperm nuclei for 60 min (figure 2.2C). Although we observed a significant reduction in the levels of chromatin-bound Cdc45 in the Drf1-depleted extracts compared with the control extract, Cdc45 was nonetheless still capable of binding to chromatin. Consistent with this observation, Claspin, which is dependent on Cdc45 for its recruitment to chromatin during interphase (Lee et al., 2003), was also bound to chromatin in the absence of Drf1, albeit at lower levels. As expected, the binding of components of the pre-RC, including Orc2 and Mcm2, was not dependent on Drf1. These results are consistent with the hypothesis that another member of the Dbf4 family must contribute to the replication function of the Cdc7 kinase.

A recent report identified a *Xenopus* Dbf4 protein as the target of an etoposide-induced checkpoint (Costanzo et al., 2003). In the presence of etoposide, it was observed that Dbf4 failed to bind to chromatin, resulting in the loss of Cdc45 loading. We tested whether we could also detect the loss of chromatin binding of Drf1 in the presence of etoposide (figure 2.2D). Although etoposide efficiently inhibited DNA replication (data not shown), we failed to observe any change in the chromatin binding of Drf1. However, the chromatin binding of Cdc45 was

inhibited by etoposide, consistent with the published results. Collectively, these results support the conclusion that Drf1 constitutes a second member of the Dbf4 family in *Xenopus laevis*.

Given the different properties of *Xenopus* Dbf4 and Drf1, we verified that Drf1 is indeed a regulatory subunit of the Cdc7 kinase. Using Drf1 and Cdc7 antibodies, we tested whether these proteins could be coimmunoprecipitated with each other from *Xenopus* egg extracts (figure 2.2E, upper panel). We found that Drf1 and Cdc7 formed a stable complex that could be immunoprecipitated with antibodies against either subunit. This complex also formed an active kinase that readily phosphorylated GST-Mcm2 *in vitro* (figure 2.2E, bottom panel). These results confirm that the Drf1-Cdc7 complex has kinase activity.



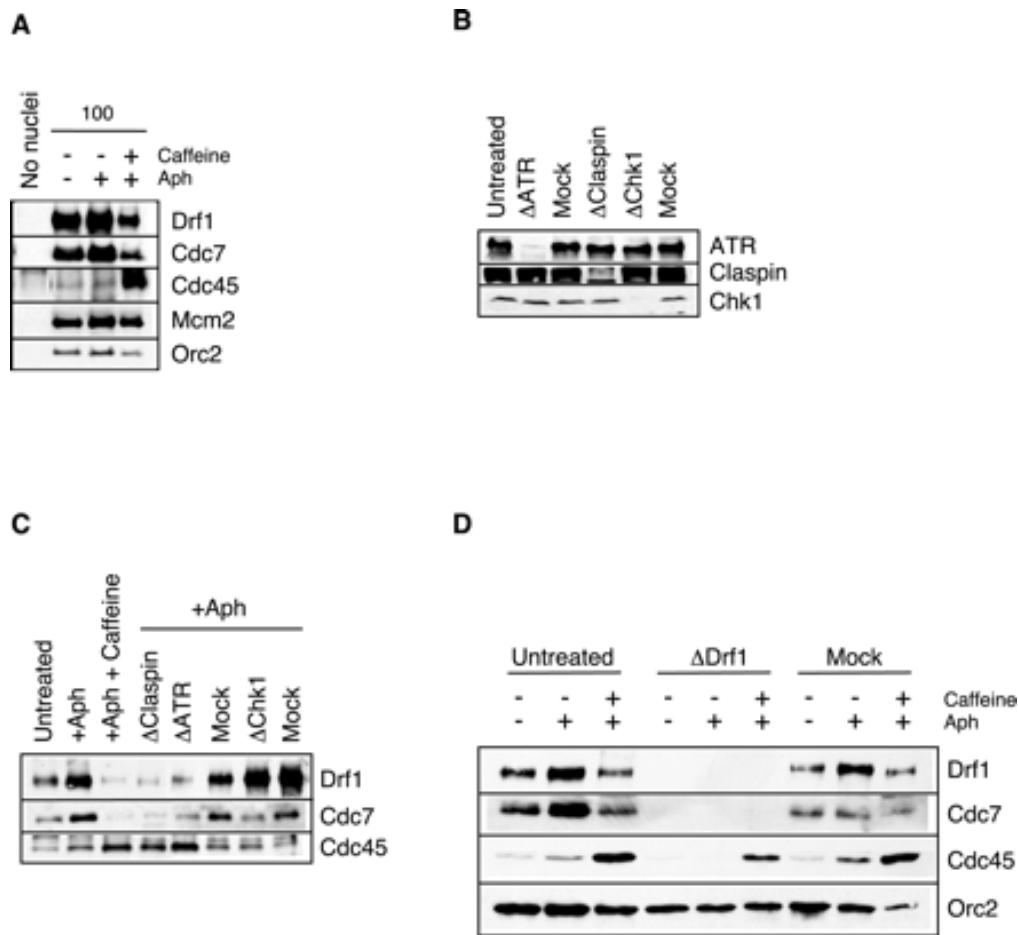
**Figure 2.3** Drf1 is a chromatin-binding protein.

(A) whole-egg extract and chromatin fractions isolated at various times (minutes) following addition of sperm chromatin to interphase extracts were immunoblotted with antibodies against Drf1, Cdc7, Cdc45, Mcm2, and Orc2. The second lane depicts a mock chromatin fraction from an extract lacking DNA. (B) mock-depleted, Drf1-depleted, and Cdc7-depleted extracts were incubated with sperm nuclei for 100 min. Subsequently, chromatin fractions were immunoblotted with antibodies against Drf1, Cdc7, and Mcm2. (C) sperm nuclei were incubated in egg extracts in the absence and presence of aphidicolin (Aph) and geminin, as indicated. After 100 min, chromatin fractions were immunoblotted with antibodies against Drf1, Cdc7, Mcm4, and Orc2.

**2.3.3 Drf1 Binds to Chromatin in a Regulated Manner**—We next characterized the binding of Drf1 to chromatin. As was reported for Cdc7 (Jares and Blow, 2000), Drf1 binds to chromatin after Mcm2 and around the same time as Cdc45 (figure 2.3A). Because both Drf1 and Cdc7 bind to chromatin at the onset of S-phase, we tested whether each subunit requires the other to bind to chromatin (figure 2.3B). In Drf1-depleted extracts, Cdc7 binding was barely detectable. Likewise, in Cdc7-depleted extracts, we failed to observe any chromatin-bound Drf1. These observations suggest that Drf1 and Cdc7 bind to chromatin as a complex or indirectly stabilize each other on chromatin. Furthermore, when extracts were treated with geminin, an inhibitor of Cdt1-dependent Mcm loading (McGarry, 2002), the binding of both Drf1 and Cdc7 to chromatin was strongly inhibited (figure 2.3C). This finding indicates that binding of Drf1 and Cdc7 is dependent upon the pre-RC.

**2.3.4 Chromatin Binding of Drf1 and Cdc7 following Aphidicolin Treatment Is Checkpoint regulated**—Because Cdc7 and Dbf4 are regulated by the DNA replication checkpoint in *Xenopus* and in yeast, we tested whether Drf1 may also have a checkpoint-specific function. In the presence of aphidicolin, which induces a block to replication, it has been reported that the binding of Cdc7 to chromatin is increased (McGarry, 2002). We observed a similar increase in the levels of Drf1 following incubation of sperm nuclei in extracts in which the replication checkpoint was activated by aphidicolin (figure 2.4A). This increase is also sensitive to geminin, which is consistent with a requirement for initiation to occur to establish the replication checkpoint induced by aphidicolin (figure 2.3C).

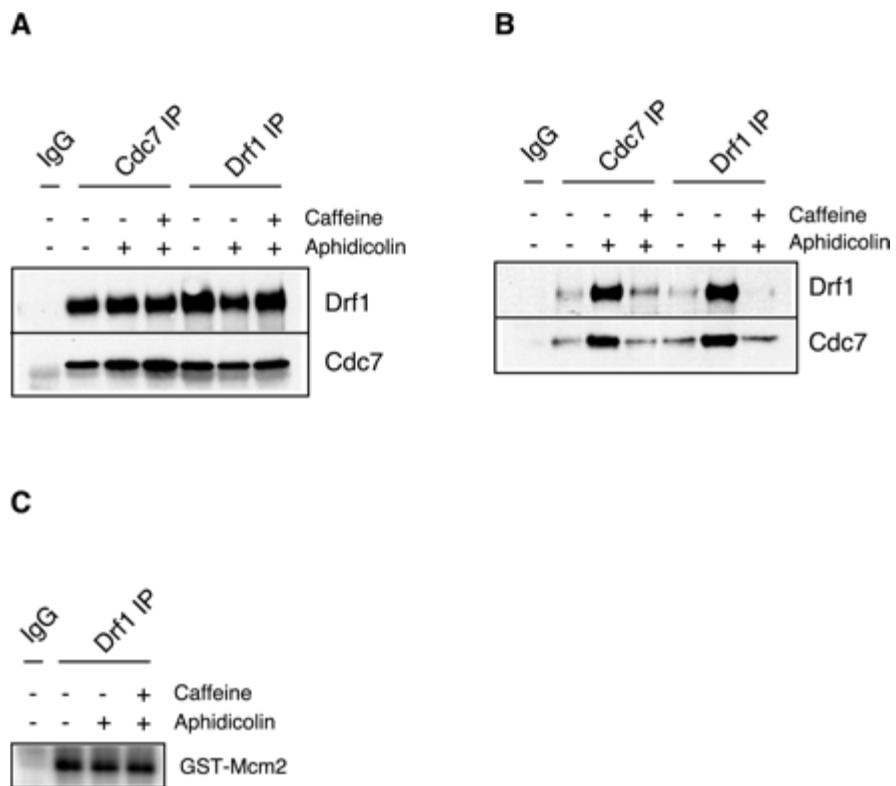
However, the increase in Drf1 and Cdc7 binding to chromatin could simply be a consequence of the increased number of replication forks that stall in response to aphidicolin. In this case, we would predict that the chromatin binding of Drf1 and Cdc7 would be independent of the checkpoint-signaling pathways. To test this possibility, we treated extracts with aphidicolin and caffeine, an inhibitor of ATM and ATR, to determine whether inhibition of checkpoint-signaling affects the levels of Drf1 and Cdc7 on chromatin. We found that the levels of both Drf1 and Cdc7 on chromatin were significantly reduced in the presence of caffeine (figure 2.4A). This behavior is in stark contrast to the regulation of Cdc45. A slight increase in chromatin binding of Cdc45 was observed in the presence of aphidicolin, but the association of Cdc45 with chromatin was dramatically up-regulated upon addition of caffeine (figure 2.4A). This observation suggests that Cdc45 loading may be suppressed by a checkpoint pathway that is activated by a replication block.



**Figure 2.4** Regulation of the binding of Drf1 and Cdc7 to chromatin.

(A) sperm nuclei were incubated for 100 min in interphase extracts with no drug, aphidicolin (Aph), or aphidicolin plus caffeine. Chromatin fractions were immunoblotted for Drf1, Cdc7, Cdc45, Mcm2, and Orc2. (B) untreated, mock-depleted, ATR-depleted, Claspin-depleted, and Chk1-depleted extracts were immunoblotted with anti-ATR, anti-Claspin, and anti-Chk1 antibodies. (C) the extracts described in B were incubated with sperm nuclei in the presence of no drug, aphidicolin, or aphidicolin plus caffeine for 100 min. Chromatin was isolated and immunoblotted with antibodies against *Xenopus* Drf1, Cdc7, and Cdc45. (D) interphase, Drf1-depleted, and mock-depleted extracts were incubated with sperm nuclei and either no drug, aphidicolin, or aphidicolin plus caffeine for 100 min. Chromatin fractions were immunoblotted with antibodies against Drf1, Cdc7, Cdc45, and Orc2.

To characterize further the checkpoint-dependent chromatin binding of Drf1 and Cdc7, we tested whether this binding requires specific components of the checkpoint-signaling pathway. For this purpose, we depleted ATR, Claspin, or Chk1 from egg extracts (figure 2.4B). Sperm nuclei and aphidicolin were added to these depleted extracts, and chromatin fractions were immunoblotted for Drf1 and Cdc7. In both the ATR-depleted and Claspin-depleted extracts, we found that Drf1 and Cdc7 levels were reduced as compared with the mock-depleted controls and were very similar to the levels observed in extracts treated with both aphidicolin and caffeine (figure 2.4C). In contrast, when Chk1 was depleted, there was no effect on the chromatin binding of either Drf1 or Cdc7. These results suggest that upstream components of the replication checkpoint pathway are required for the recruitment or stability of Drf1 and Cdc7 on chromatin following aphidicolin treatment, yet the chromatin binding of Drf1 and Cdc7 is not dependent on the effector kinase Chk1. Another significant finding involves the effect of ATR and Claspin depletion on Cdc45. When extracts were treated with aphidicolin in the absence of ATR or Claspin, Cdc45 levels on chromatin increased significantly, similar to those observed in the presence of both aphidicolin and caffeine (figure 2.4A and C). In contrast, depletion of Chk1 had no effect on Cdc45. From these results we conclude that the regulated chromatin binding of Drf1, Cdc7, and Cdc45 during a replication block is dependent upon ATR and Claspin.



**Figure 2.5** The kinase activity of Drf1-Cdc7 is not affected by aphidicolin treatment.

Interphase extracts were incubated without aphidicolin, with aphidicolin, and with aphidicolin plus caffeine. The extracts (A) and chromatin fractions isolated from these extracts (B) were immunoprecipitated (IP) with control, anti-Cdc7, and anti-Drf1 antibodies. The immunoprecipitates were then probed for Cdc7 and Drf1 by immunoblotting. (C) the kinase activity of immunoprecipitates from A was tested *in vitro* using GST-Mcm2 as a substrate.

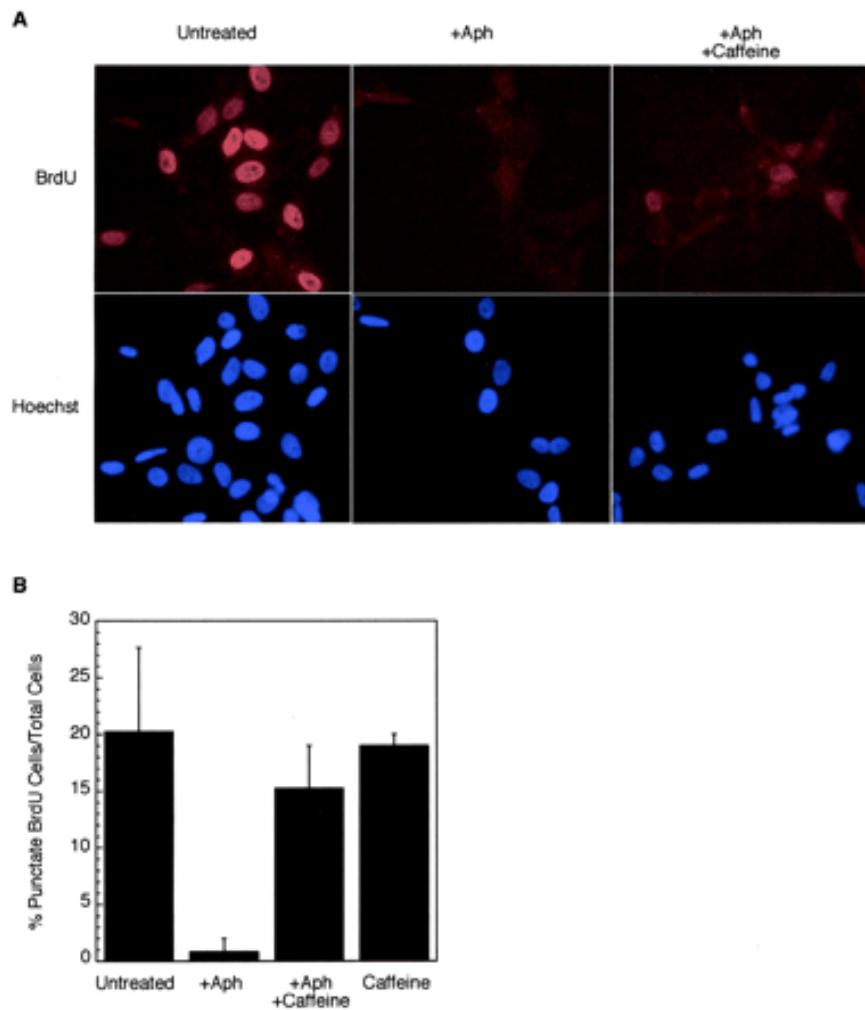
These findings suggest that a replication checkpoint pathway may respond to stalled replication forks by suppressing further loading of Cdc45 at these forks or at other origins. To test whether Drf1 plays a role in preventing additional Cdc45 from binding to chromatin, we depleted Drf1 from egg extracts and then examined the levels of chromatin-bound Cdc45 in these extracts in the presence of no drug, aphidicolin, or aphidicolin and caffeine (figure 2.4D). In mock-depleted extract, low levels of Cdc45 were detected in the absence and presence of aphidicolin, whereas a significant increase in Cdc45 loading was observed in the presence of both aphidicolin and caffeine. However, only a small increase in the binding of Cdc45 occurred in Drf1-depleted extracts that were treated with both aphidicolin and caffeine. These findings imply that Drf1 is involved in regulating Cdc45 loading during a replication block.

*2.3.5 Regulation of Drf1-Cdc7 Kinase Activity during the Replication Block*—In *Xenopus* egg extracts, treatment with the DNA damaging agent etoposide disrupts the interaction between Dbf4 and Cdc7. This treatment therefore inactivates the kinase and prevents the binding of Cdc45 to chromatin (Costanzo et al., 2003). To determine whether Drf1 and Cdc7 are regulated in a similar fashion in response to aphidicolin, we tested whether both subunits could be coimmunoprecipitated with each other in the extract and on chromatin. In both fractions, Drf1 and Cdc7 coimmunoprecipitated with each other in the presence of aphidicolin (figure 2.5A and B). This observation suggests that the Drf1-Cdc7 complex is maintained during a replication block.

We next measured the kinase activity associated with Drf1 that had been immunoprecipitated from either the extract or chromatin fractions, using GST-Mcm2 as a substrate. In the extract, no obvious change in the kinase activity was observed in response to aphidicolin or aphidicolin plus caffeine (figure 2.5C). Similar results were observed when the kinase activity of the chromatin-eluted Drf1-Cdc7 complex was measured (data not shown). Taken together, these results indicate that the aphidicolin-induced checkpoint does not lead to a readily discernible inhibition of the kinase activity of Drf1-Cdc7 under these assay conditions.

**2.3.6 A Caffeine-sensitive Checkpoint Inhibits Replication in XTC Cells during Aphidicolin Arrest**—Our chromatin binding data suggest that an aphidicolin-induced, caffeine-sensitive checkpoint regulates the binding of Drf1 and Cdc7 to chromatin. The aphidicolin-dependent increase in the chromatin binding correlates with a suppression of Cdc45 that is reversed upon abrogation of the checkpoint with caffeine. These observations suggest that Drf1 is a target of an intra-S checkpoint that regulates the loading of Cdc45. To investigate whether an intra-S checkpoint exists in *Xenopus* cells, we treated *Xenopus* tadpole cells (XTC cells) with BrdUrd and monitored its incorporation in asynchronous cells after a 5.5 hour incubation with aphidicolin or with aphidicolin and caffeine. Treatment with caffeine alone or Me<sub>2</sub>SO served as controls. We then counted the percentage of the total number of cells that exhibited punctate BrdUrd staining, an indicator that these cells were in S-phase. As expected, we observed a dramatic decrease in the number of BrdUrd-positive S-phase cells after treatment

with aphidicolin (figure 2.6A and B). When caffeine was added together with aphidicolin, the percentage of cells increased significantly ( $p < 0.01$ ), approximately to levels observed in untreated cells or cells treated with caffeine alone. Similar results were recorded when we calculated the percentage of punctate BrdUrd cells over the total number of BrdUrd-positive cells (data not shown). However, the intensity of the BrdUrd labeling in the nuclei of cells treated with both caffeine and aphidicolin was strongly reduced compared with untreated S-phase cells (figure 2.6A, compare left and right panels). These observations indicate that caffeine mostly does not reverse the inhibitory effect of aphidicolin on DNA polymerase activity to allow extensive elongation and completion of DNA synthesis. Instead, the enhanced incorporation of BrdUrd in the presence of caffeine may reflect the abrogation of a checkpoint that suppresses origin unwinding and early events associated with the initiation of DNA replication.

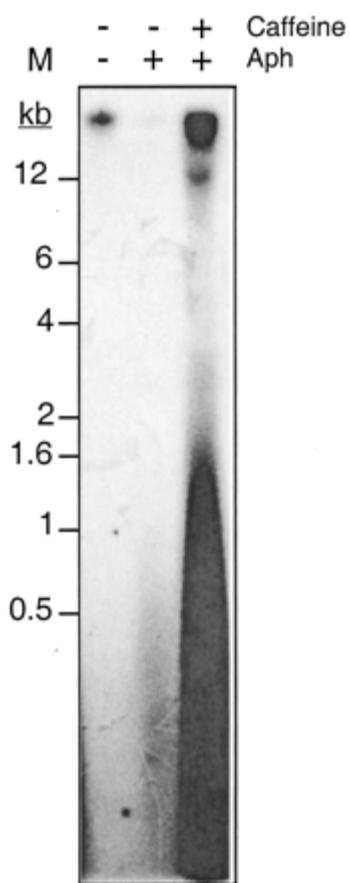


**Figure 2.6** Caffeine induces some BrdUrd (BrdU) incorporation in aphidicolin (Aph)-treated XTC cells.

(A) immunofluorescence of BrdUrd-labeled XTC cells treated with  $\text{Me}_2\text{SO}$ , aphidicolin alone, or aphidicolin and caffeine for 5.5 h. BrdUrd incorporation was visualized using an anti-BrdUrd antibody (upper panels), and Hoechst dye was used for nuclear DNA staining (lower panels). (B) quantification of cells in A expressed as the percentage of cells with punctate BrdUrd staining over the total number of cells.

### 2.3.7 Caffeine-sensitive Inhibition of Nascent Strand DNA Synthesis in *Xenopus*

*Extracts*—We next sought a biochemical assay to determine whether an analogous intra-S checkpoint may operate in *Xenopus* egg extracts. It has been reported that in the presence of lower doses of aphidicolin (10 µg/ml), small DNA fragments ranging in size from 0.1 to 1 kb accumulate during a replication block. These fragments can be detected in alkaline agarose gels (Mahbubani et al., 1997). Using this dose of aphidicolin, we were able to detect a low level of DNA fragments that accumulated within this same size range (figure 2.7A). We hypothesized that, if a checkpoint functions to suppress origin firing in the presence of aphidicolin, perhaps this checkpoint may be reversed by caffeine, thereby allowing more synthesis of these small DNA fragments. Indeed, in extracts treated with both aphidicolin and caffeine, we observed a significant increase in the accumulation of these small fragments compared with treatment with aphidicolin alone (figure 2.7A). Although some of the fragments appeared longer than in the aphidicolin-blocked sample, the bulk of DNA synthesized in the presence of caffeine remained within the same size range. This observation suggests that aphidicolin still prevents elongation in the presence of caffeine, but some aspect of initiation is under checkpoint control.



**Figure 2.7** Caffeine-sensitive accumulation of nascent DNA during replication arrest.

DNA from untreated extracts (control, lane 1) or extracts treated for 100 min with 10 $\mu$ g/ml aphidicolin (Aph, lane 2) or aphidicolin and 5 mM caffeine (lane 3) was isolated and run on a 1% agarose gel under alkaline conditions. M,  $^{32}$ P-labeled 1-kb DNA marker. For the first lane, one tenth as much sample was electrophoresed as in the other lanes.

## 2.4 Discussion

In this work, we have cloned and characterized *Xenopus* Drf1, a member of the Dbf4 family of proteins. Based on published results that describe another Dbf4-related homologue in *Xenopus* (Costanzo et al., 2003) and its reported sequence, Drf1 represents a second, distinct protein. Thus, *Xenopus* is the second vertebrate species, after humans, to have two Dbf4 homologues. In fission yeast, two Dbf4-like complexes have also been identified: Dfp1-Hsk1 and Spo6-Spo4. Both complexes form active kinases, but their functions are quite divergent. Dfp1-Hsk1, which is most analogous to mammalian Dbf4-Cdc7, is essential for DNA replication (Jares and Blow, 2000; Masai et al., 1995; Takeda et al., 1999), whereas Spo6-Spo4 is involved in progression through the second meiotic division (Nakamura et al., 2002). In humans, the two Dbf4-like proteins (ASK and Drf1) regulate the same catalytic partner, Cdc7. Both complexes are activated during S-phase. Although ASK-Cdc7 is essential for DNA replication (Jares and Blow, 2000; Kumagai et al., 1999), it is not known what role human Drf1-Cdc7 plays during this phase of the cell cycle.

We have found that the *Xenopus* Drf1 homologue is dispensable for DNA replication and loading of Cdc45 onto chromatin. It is possible that both Drf1-Cdc7 and Dbf4-Cdc7 complexes contribute to Cdc45 loading, and therefore a defect in replication would be observed only by depleting both regulatory subunits. This possibility would be consistent with our findings that Cdc45 levels on chromatin are reduced in Drf1-depleted extracts, and published results that

depletion of the Cdc7 subunit is sufficient to block DNA replication and Cdc45 loading (Jares and Blow, 2000; Roberts et al., 1999). Although Drf1 may not be essential for DNA replication in *Xenopus*, it does bind to chromatin in a highly regulated manner. In particular, it associates with chromatin at S-phase onset at around the same time as Cdc7 and Cdc45. The binding of Drf1 to chromatin is sensitive to geminin, as was shown previously for Cdc7 (McGarry, 2002), implying that binding of Drf1 and Cdc7 occurs once the Mcm complex has been recruited to the pre-RC. Although the exact function of chromatin-bound Drf1 and Cdc7 is not known, the two subunits form a complex on chromatin that exhibits kinase activity toward Mcm2 *in vitro*.

Upon further characterization of Drf1, we found that the chromatin binding properties of this protein are regulated during a replication block. Whereas treatment with hydroxyurea causes the budding yeast Dbf4-Cdc7 complex to dissociate from chromatin (Pasero et al., 1999), a replication block induced by aphidicolin results in the accumulation of both *Xenopus* Drf1 and Cdc7 on chromatin. This binding is sensitive to caffeine and diminished in extracts lacking ATR or Claspin. These observations argue against the model that Drf1 and Cdc7 remain on chromatin simply because of the presence of stalled replication forks. Instead, these results suggest that Drf1 and Cdc7 are either actively recruited to or stabilized on chromatin by signaling components of the caffeine-sensitive replication checkpoint. Importantly, we found that depletion of Drf1 substantially reduced the effect of caffeine on Cdc45 loading, suggesting that, although Cdc45 can bind to chromatin in a Drf1-depleted extract to allow normal replication to

proceed, the suppression of Cdc45 by checkpoint activation involves Drf1. One possible model is that the Drf1-Cdc7 kinase on chromatin phosphorylates different targets under checkpoint conditions than during DNA replication, resulting in the inhibition of further Cdc45 loading. Alternatively, Drf1 and Cdc7 may simply be sequestered on chromatin in a manner that contributes to the lack of Cdc45 loading at sites of initiation. This concept is consistent with the model proposed by Edwards et al. 2002, in which Cdc7 can interact with MCMs throughout broad regions of chromatin outside of the initiation sites.

The activation of a caffeine-sensitive replication checkpoint that targets the chromatin binding of Cdc45 shares certain similarities with the DNA damage pathways previously reported in *Xenopus* cell-free extracts. Treatment with etoposide, an inhibitor of topoisomerase II, results in activation of ATR, which leads to disruption of the Dbf4-Cdc7 complex and inhibition of its kinase activity. This inhibition prevents the loading of Cdc45 and entry into S-phase (Costanzo et al., 2003). Interestingly, Dbf4 dissociates from chromatin under these conditions, but Cdc7 remains bound. In contrast, Drf1 remains associated with chromatin in the presence of etoposide, suggesting that its regulation in response to DNA damage differs from that of Dbf4.

In the presence of aphidicolin, limited origin firing and elongation can occur before replication forks stall (Mahbubani et al., 1997; Walter and Newport, 2000). These arrested replication structures are known to activate a checkpoint pathway that activates ATR, Claspin, and Chk1 to inhibit entry into mitosis (Guo et al.,

2000; Hekmat-Nejad et al., 2000; Kumagai and Dunphy, 2000; Liu et al., 2000; Zhao and Piwnica-Worms, 2001). However, results from yeast and mammalian systems have also identified an intra-S checkpoint pathway that functions to prevent the initiation of replication at late origins in the presence of stalled replication forks (Dimitrova and Gilbert, 2000; Santocanale and Diffley, 1998; Shirahige et al., 1998). This pathway is also caffeine sensitive and involves several components of the S-M checkpoint, suggesting that activation of the replication checkpoint has multiple cell cycle targets. Our data from XTC cells identified a similar caffeine-sensitive intra-S checkpoint that is activated by aphidicolin. Only very low levels of BrdUrd labeling could be detected when cells were treated with aphidicolin, but distinctly punctate BrdUrd labeling was observed when caffeine was also added. It is important to note that the overall intensity of the BrdUrd labeling was not nearly as high as in the untreated nuclei, consistent with continued inhibition of DNA polymerases. Nevertheless, these results suggest that some aspect of DNA unwinding is inhibited by the replication checkpoint pathway in the presence of incompletely replicated DNA.

Using alkaline agarose gels, we have established a biochemical assay for the checkpoint-dependent block to replication in response to aphidicolin in egg extracts. Consistent with our observations in XTC cells, treatment with aphidicolin and caffeine allowed the synthesis of short nascent DNA strands. These results support the hypothesis that an intra-S checkpoint operates in egg extracts to prevent further initiation events in the presence of stalled replication forks. One attractive hypothesis is that, in response to aphidicolin, ATR and Claspin are

activated, leading to bifurcating signaling events. One pathway leads to activation of Chk1 and inhibition of Cdc2. The other pathway would lead to the accumulation of Drf1-Cdc7 kinase on chromatin. Abrogation of this pathway by caffeine would then allow Drf1-Cdc7 to participate in the loading of Cdc45 onto chromatin. This process could involve either new origin firing at unreplicated sites or resumption of replication within regions in which origins have already fired. Montagnoli et al. 2002 have proposed that the human Drf1 might be a specific activator of Cdc7 to fire late origins selectively. Although late origins have not been characterized in the *Xenopus* egg extract system, our results nonetheless are consistent with the possibility that Drf1 plays some role in origin utilization during a DNA replication block.

Another related possibility is that Drf1-Cdc7 acts to ensure proper resumption of DNA replication upon recovery from the arrest. In fission yeast, *dfp1* and *hsk1* mutants exhibit severe defects in recovery from hydroxyurea arrest, consistent with a role in restarting stalled replication forks (Snaith et al., 2000; Takeda et al., 1999). In *Xenopus*, ATR and Claspin may participate in the recovery process by recruiting or stabilizing Drf1 and Cdc7 on chromatin. Chromatin-bound Cdc7 has been shown to be capable of supporting DNA replication (Jares and Blow, 2000). Thus, when the block to replication is removed, the kinase is poised to load significant amounts of Cdc45 either at origins or at stalled forks to quickly re-initiate and complete DNA synthesis. In both egg extracts and XTC cells, abrogation of this checkpoint could lead to premature re-starting of replication forks and DNA unwinding even in the presence of aphidicolin.

## 2.5 Acknowledgements

This chapter is reprinted with permission from the *Journal of Biological Chemistry*, Stephanie K. Yanow, Daniel A. Gold, Hae Yong Yoo, and William G. Dunphy, “*Xenopus* Drf1, a regulator of Cdc7, displays checkpoint-dependent accumulation on chromatin during an S-phase arrest”, volume 278, issue 42, p. 41083–41092, October 17, 2003. H. Yoo provided recombinant GST-Mcm2. D. Gold performed the studies investigating caffeine sensitivity of the intra-S-phase checkpoint in XTC cells (figure 2.6). S. Yanow performed all other work in this study.

## Chapter 3

# Roles of Replication Fork-interacting and Chk1-activating Domains from Claspin in a DNA Replication Checkpoint Response

Joon Lee, Daniel A. Gold, Anna Shevchenko, Andrej Shevchenko, and William G. Dunphy

Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. Claspin associates with replication forks upon origin unwinding. We show that Claspin contains a replication fork-interacting domain (RFID, residues 265–605) that associates with Cdc45, DNA polymerase  $\epsilon$ , replication protein A, and two replication factor C complexes on chromatin. The RFID contains two basic patches (BP1 and BP2) at amino acids 265–331 and 470–600, respectively. Deletion of either BP1 or BP2 compromises optimal binding of Claspin to chromatin. Absence of BP1 has no effect on the ability of Claspin to mediate activation of Chk1. By contrast, removal of BP2 causes a large reduction in the Chk1-activating potency of Claspin. We also find that Claspin contains a small Chk1-activating domain (residues 776–905) that does not bind stably to chromatin, but it is fully effective at high

concentrations for mediating activation of Chk1. These results indicate that stable retention of Claspin on chromatin is not necessary for activation of Chk1. Instead, our findings suggest that only transient interaction of Claspin with replication forks potentiates its Chk1-activating function. Another implication of this work is that stable binding of Claspin to chromatin may play a role in other functions besides the activation of Chk1.

### **3.1 Introduction**

Checkpoint control mechanisms ensure the integrity of the genome by preventing the transmission of incompletely replicated or damaged DNA to progeny cells (McGowan and Russell, 2004; O'Connell and Cimprich, 2005; Osborn et al., 2002; Sancar et al., 2004). For example, the DNA replication checkpoint monitors whether DNA synthesis occurs normally throughout S phase. When problems become evident, this regulatory network forestalls premature entry into mitosis and stabilizes aberrant replication forks until normal replication can resume and subsequently reach completion. The manifestation of this checkpoint is most obvious when DNA replication forks stall at sites of exogenously inflicted DNA damage. However, this pathway also operates when spontaneous errors arise during replication or the replication apparatus encounters difficult-to-replicate sequences.

Checkpoint pathways contain a variety of regulatory proteins that detect the status of the genome and that relay this information to effector enzymes that regulate downstream processes (Melo and Toczyski, 2002; Osborn et al., 2002;

Sancar et al., 2004). In the DNA replication checkpoint, ATR functions at or near the top of this regulatory hierarchy (Abraham, 2001). ATR is a member of the phosphoinositide kinase-related family of protein kinases that also includes ATM. One key function of ATR involves activation of the checkpoint effector kinase Chk1 (Guo et al., 2000; Hekmat-Nejad et al., 2000; Liu et al., 2000). Significantly, ATR cannot carry out this function alone but must cooperate with numerous other proteins. For example, ATR possesses a conserved binding partner called ATRIP (Cortez et al., 2001). Other collaborating factors include the checkpoint clamp assembly of Rad9, Rad1, and Hus1 (the 9-1-1 complex) (Sancar et al., 2004). A checkpoint clamp loader consisting of Rad17 and the four small subunits of replication factor C (RFC) is responsible for deposition of the 9-1-1 complex onto DNA. The checkpoint clamp loader and clamp proteins most likely interact with boundaries between single-stranded and double-stranded regions of DNA that would be present in incompletely replicated or damaged DNA (Ellison and Stillman, 2003; Lee et al., 2003; You et al., 2002; Zou et al., 2003).

ATR and ATM also work together with a class of proteins known as mediators (McGowan and Russell, 2004; O'Connell and Cimprich, 2005). In vertebrates, these proteins consist of Claspin and various BRCA1 C-terminal repeat (BRCT)-containing proteins, including TopBP1, 53BP1, Mdc1, and BRCA1 itself (Canman, 2003; Chini and Chen, 2003; Kumagai and Dunphy, 2000). These mediators either have been shown to or are thought to serve as adaptors between ATR/ATM and the downstream kinases Chk1 and Chk2. In addition,

accumulating evidence has indicated that mediator proteins may function as sensors of chromatin structures. For example, in response to double-stranded DNA breaks, mammalian 53BP1 and its fission yeast relative Crb2 recognize methylated forms of lysine 79 in histone H3 and lysine 20 in histone H4, respectively (Huyen et al., 2004; Sanders et al., 2004). These histone methylations do not vary in response to DNA damage. Instead, modified histones may become inappropriately exposed at sites of damage. Furthermore, 53BP1, Crb2, and other BRCT-containing proteins respond to the phosphorylation of histone H2AX and H2A in mammals and fission yeast, respectively (Celeste et al., 2003; Nakamura et al., 2004). This type of histone phosphorylation is not required for initial recruitment of mediator proteins to sites of damage, but it is necessary for their stable incorporation into damage-induced foci. These observations suggest that the interaction of mediator proteins with chromatin is multifaceted and may fulfill multiple functions.

We have been studying the DNA replication checkpoint in *Xenopus* egg extracts. In this system, the DNA replication inhibitor aphidicolin elicits the formation of stalled replication forks, which in turn trigger the activation of *Xenopus* Chk1 (Xchk1) (Kumagai et al., 1998; Michael et al., 2000). The *Xenopus* homologue of ATR (Xatr) is responsible for the phosphorylation-dependent activation of Xchk1 (Guo et al., 2000; Hekmat-Nejad et al., 2000). This process also requires the mediator protein Claspin in *Xenopus* egg extracts and human cells (Chini and Chen, 2003; Kumagai and Dunphy, 2000; Lin et al., 2004). Claspin associates directly with Xchk1 and thereupon strongly enhances the ability of Xatr to

phosphorylate Xchk1 (Kumagai and Dunphy, 2003; Kumagai et al., 2004). In addition, Claspin displays dynamic spatial localization by associating with replication forks during S phase (Lee et al., 2003). This binding requires Xcdc45 and Cdk2 but not replication protein A (RPA), suggesting that Claspin associates with incipient replication forks at around the time of DNA unwinding (Lee et al., 2003). The initial binding of Claspin occurs before Xatr-Xatrip and the replication factor C (RFC) proteins and therefore must involve, at least in part, chromatin structures that are distinct from those recognized by these proteins. The budding yeast homologue of Claspin called Mrc1 likewise associates specifically with replication forks (Katou et al., 2003; Osborn and Elledge, 2003). These observations imply that Claspin and its homologues may also function as checkpoint sensor proteins.

In this study, we have explored the mechanism by which Claspin associates with the DNA replication apparatus to understand the purpose of this interaction. Our results indicate that Claspin uses a conserved domain to interact with key replication and checkpoint proteins, including Cdc45, DNA polymerase  $\epsilon$  (Pol  $\epsilon$ ), RPA, and both the replicative and Rad17-containing RFC complexes. Interestingly, although a portion of this domain is required for optimal activation of Chk1, stable retention of Claspin on chromatin is not essential for its Chk1-activating function.

### 3.2 Materials and Methods

#### 3.2.1 *Xenopus Egg Extracts*

Extracts from *Xenopus* eggs were prepared as described previously (Lee et al., 2003). The DNA replication checkpoint was induced by the addition of demembranated *Xenopus* sperm nuclei (3,000/μl) and aphidicolin (100 μg/ml). Caffeine (5 mM) was used to override this checkpoint response. Isolation of nuclear and chromatin fractions from egg extracts was described previously (Lee et al., 2003).

#### 3.2.2 *Antibodies*

Antibodies against *Xenopus* Claspin, RPA70, Xorc2, phospho-Ser864 of Claspin, Xatr, Xatrip, Xchk1, Xrad17, and Xhus1 were described previously (Kumagai and Dunphy, 2003; Kumagai et al., 2004; Lee et al., 2003). Antibodies against *Xenopus* RFC40 and proliferating cell nuclear antigen (PCNA) were raised against bacterially expressed His6-tagged proteins containing either residues 1–320 of RFC40 or full-length PCNA, respectively. The sequence of *Xenopus* RFC40 (expressed sequence tag database XGI TC34364) showed 89.6% identity at the amino acid level with human RFC40. Anti-Xcdc45 and anti-p60 of Pol ε antibodies were raised as described previously (Mimura and Takisawa, 1998; Waga et al., 2001). These antibodies were all affinity purified with their antigens. Antisera against *Xenopus* importin α, *Xenopus* Pol δ (p125 subunit), Xsld5, *Xenopus* cyclin E, and Xmcm7 were generously supplied by D. Görlich (Universität Heidelberg, Heidelberg, Germany), S. Waga (Osaka University,

Osaka, Japan), H. Takisawa (Osaka University, Osaka, Japan), P. Jackson (Stanford University, Stanford, CA), and J. Blow (University of Dundee, Dundee, Scotland, United Kingdom), respectively. Antisera against human RFC37 and *Xenopus* RFC140 were the kind gifts of J. Hurwitz (Memorial Sloan-Kettering Cancer Center, New York, NY) and S. Waga, respectively. Anti-human Chk1 phospho-Ser345 antibody was purchased from Cell Signaling Technology (Beverly, MA). Purified control rabbit IgG and anti-FLAG monoclonal antibodies were obtained from Zymed Laboratories (South San Francisco, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Immunodepletion procedures for Claspin, Xcdc45, and RPA were described previously (Lee et al., 2003).

### 3.2.3 Recombinant Proteins

The pBluescript vector was engineered to encode a nuclear localization signal (NLS) (TPPKKKRKVEDP) (Moore et al., 2002) fused upstream of Claspin fragments for *in vitro* protein synthesis. The same NLS was inserted into pGEX-4T-3 (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) to make glutathione S-transferase (GST)-NLS fusion constructs of Claspin. Baculoviruses encoding full-length or truncated His6-Claspin-FLAG proteins were generated with the Bac-to-Bac system with a His6 tag and FLAG epitope (DYKDDDDK) at the N-terminal and C-terminal ends, respectively. Various Claspin mutants (internal deletions or amino acid substitutions) were produced through PCR-based mutagenesis by standard methods. Recombinant Claspin proteins were expressed and purified from baculovirus-infected insect cells or bacteria as described previously (Kumagai and Dunphy, 2003). <sup>35</sup>S-labeled proteins were

synthesized in vitro with the TnT system (Promega, Madison, WI). Human GST-p27 (gift from T. Hunter, Salk Institute, La Jolla, CA) was expressed in bacteria and purified with glutathione agarose.

### *3.2.4 Identification of Claspin-binding Proteins from Egg Extracts*

Claspin was immunoprecipitated from 5 ml of interphase egg extract with 200 µg of anti-Claspin antibodies cross-linked to Affiprep protein-A beads (Bio-Rad, Hercules, CA). The beads were washed four times with immunoprecipitation (IP) buffer B (10 mM HEPES-KOH, pH 7.6, 0.5 M NaCl, 0.1% NP-40, and 20 mM β-glycerolphosphate). Samples were boiled in SDS buffer, concentrated, separated by SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau S. Protein bands containing p55 and p90 were excised and subjected to chemical sequencing and nanoelectrospray tandem mass spectrometry in the Howard Hughes Medical Institute protein sequencing facility at University of California (Berkeley, CA). Sequences from p55 (KXTQH/AP and KYFXGEEA) and p90 (KFYAK and KTLATWATK) corresponded to importin  $\alpha$  and  $\beta$ , respectively.

### *3.2.5 Identification of Claspin-binding Proteins in Chromatin Eluates*

For analytical experiments, sperm nuclei reconstituted in interphase egg extract (1 ml) were collected through a sucrose cushion (Lee et al., 2003). Nuclei were resuspended in 100 µl of HEPES-buffered saline (10 mM HEPES-KOH, pH 7.6, and 150 mM NaCl) supplemented with 10% dimethyl sulfoxide and 5 mM caffeine, incubated at room temperature for 30 min, and cooled on ice for 20 min. An equal volume of elution buffer (10 mM HEPES-KOH, pH 7.6, 1 M NaCl, and

1% NP-40) was added, and the incubation was continued on ice for 20 min. After dilution with 200  $\mu$ l of 10 mM HEPES-KOH, pH 7.6, soluble proteins were recovered by centrifugation at 14,000 rpm in an Eppendorf centrifuge for 10 min. Antibodies (2.5  $\mu$ g) cross-linked to protein A beads were incubated with these preparations for 1 h at 4 °C, washed twice with IP buffer C (10 mM HEPES-KOH, pH 7.6, 150 mM NaCl, 0.1% CHAPS, 2.5 mM EGTA, and 20 mM  $\beta$ -glycerolphosphate), and subjected to SDS-PAGE.

For identification by protein sequencing, four batches of egg extract totaling 50 ml were used to reconstitute nuclei (3,000/ $\mu$ l) in the presence of aphidicolin and caffeine. Proteins were removed from chromatin by elution with 0.5 M NaCl and immunoprecipitated with anti-Claspin antibodies as described above. Bound proteins were concentrated, separated by SDS-PAGE, stained with Coomassie Blue, and subjected to in-gel trypsin digestion as described previously (Shevchenko et al., 1996). Methods for nanoelectrospray tandem mass spectrometry of tryptic peptides and analysis of the sequencing data were carried out exactly as described previously (Shevchenko et al., 2003; Shevchenko et al., 2001; Yoo et al., 2004). The following protein identifications were obtained: p180 (Claspin, 10 peptides), p150 (RFC140, 3 peptides), and p65 (RPA70, 3 peptides). p36-40 contained RFC36 (2 peptides), RFC37 (5 peptides), RFC38 (2 peptides), and RFC40 (5 peptides).

### *3.2.6 Oligonucleotide Binding Assay*

Preparation of magnetic beads coated with DNA oligonucleotides and assay

conditions for binding of proteins in egg extracts to these beads were described previously (Lee et al., 2003). For the double-stranded template, annealed (dA)<sub>70</sub>-(dT)<sub>70</sub> was prepared as described previously (Kumagai and Dunphy, 2000). For the 30d-40s branched DNA, the following oligonucleotides were annealed: 5'-ACTGATTACGGTGCTGCTTATCGATGGTTGCAGTGCTCGCATGGAGCTGGTTCCGGCCTGCTAATGG and 5'-biotin-CCATTAGCAAGGCCGGAAACCAGCTCCATGATCATTGGCAATCATTGGCAC AACGATCAGCCAACTAAC. Oligonucleotides were added to egg extracts at a final concentration of 5.5 nM.

### 3.3 Results

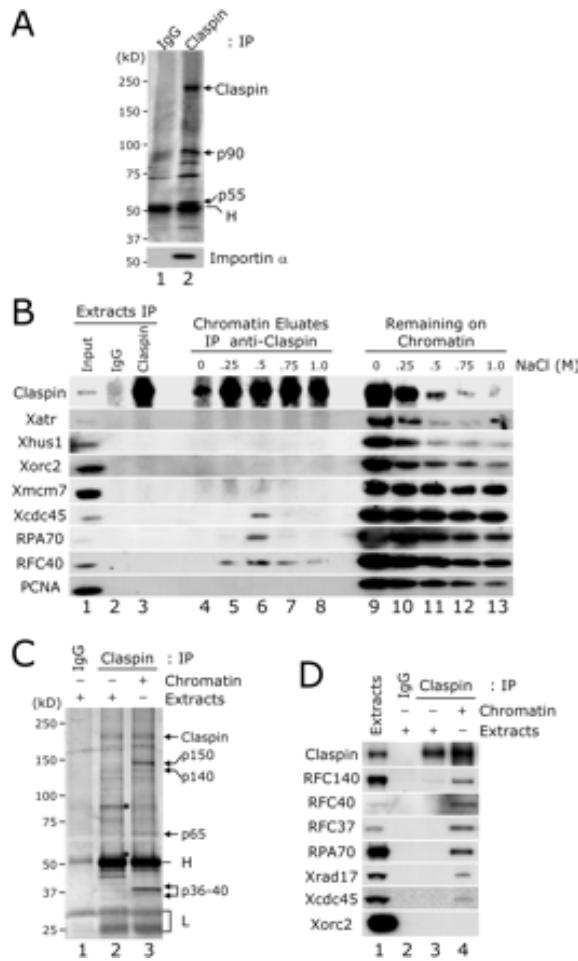
#### 3.3.1 Search for Claspin-interacting Proteins in Cytoplasmic Egg Extracts

To probe the mechanism by which Claspin promotes the phosphorylation of Xchk1 in *Xenopus* egg extracts that contain incompletely replicated DNA, we attempted to find Claspin-interacting proteins. In particular, we wished to evaluate whether Claspin could recognize specific components of the DNA replication apparatus. We first searched for Claspin-interacting proteins by immunoprecipitating Claspin from whole cytoplasmic egg extracts (figure 3.1A). We identified two prominent binding proteins at 55 and 90 kDa as *Xenopus* importins  $\alpha$  and  $\beta$ , respectively (see Materials and Methods). The binding of importin  $\alpha$  was verified by immunoblotting with anti-importin  $\alpha$  antibodies (figure 3.1A). These proteins could not be found in anti-Claspin immunoprecipitates from nuclear extracts (see below), which is consistent with the fact that importins

dissociate from their cargo upon nuclear entry. In further studies, we found that importin  $\alpha$  binds well to a GST peptide containing the C-terminal 56 amino acids of Claspin (residues 1230–1285), which contains sequences that closely resemble a NLS (our unpublished data).

### *3.3.2 Claspin Interacts with Key Replication Proteins on Chromatin*

Because we could not identify any additional Claspin-binding proteins in cytoplasmic extracts, we examined whether Claspin associates specifically with other proteins upon binding to chromatin. To pursue these experiments, we first considered the richest potential source of Claspin-interacting, chromatin-binding proteins. Previously, we observed that Claspin accumulates on chromatin in aphidicolin-treated extracts. The binding of Claspin increases dramatically upon the further addition of caffeine. Recent studies have indicated that caffeine stimulates the firing of inhibited replication origins in aphidicolin-treated chromatin (Marheineke and Hyrien, 2004; Shechter et al., 2004; Yanow et al., 2003). Therefore, the increased binding of Claspin to chromatin in such extracts seems to reflect binding to numerous additional origins of replication. Under this condition, >80% of the nuclear Claspin is bound to chromatin. Hence, for the experiments described below, we have searched for Claspin-binding proteins on chromatin in extracts containing both aphidicolin and caffeine.



**Figure 3.1.** Identification of Claspin-interacting proteins in cytoplasmic and chromatin fractions from *Xenopus* egg extracts.

(A) Claspin-binding proteins from egg extracts. IP was performed with control (lane 1) or anti-Claspin antibodies (lane 2) from interphase egg extracts. Samples were analyzed by silver staining (top) and immunoblotted for *Xenopus* importin  $\alpha$  (bottom). H, IgG heavy chain. (B) Interaction of Claspin with proteins on chromatin. Chromatin-binding proteins were eluted from reconstituted nuclei (derived from 250  $\mu$ l of egg extract) with various concentrations of NaCl and immunoprecipitated with anti-Claspin antibodies (lanes 4–8). Chromatin fractions after salt elution were loaded in lanes 9–13. In parallel, whole egg extracts (50  $\mu$ l) were immunoprecipitated with control (lane 2) and anti-Claspin antibodies (lane 3). Lane 1 depicts 1  $\mu$ l of egg extract. Samples were immunoblotted for the indicated proteins. (C) Silver staining of Claspin-binding proteins on chromatin. Interphase egg extracts were immunoprecipitated with control (lane 1) and anti-Claspin antibodies (lane 2). For lane 3, chromatin proteins from nuclei reconstituted in 1 ml of egg extract were eluted with 0.5 M NaCl and immunoprecipitated with anti-Claspin antibodies. Samples were subjected to SDS-PAGE and silver staining. Bands designated as Claspin (p180), p150, p140, p65, and p36–40 were prepared on a large scale for identification by mass spectrometry (see Materials and Methods). Asterisks in lane 2 denote importin  $\alpha$  and  $\beta$ . H and L, IgG heavy and light chains. (D) Confirmation of Claspin-binding proteins by immunoblotting. Samples from C were immunoblotted for the indicated proteins (lanes 2–4). Lane 1 depicts 1  $\mu$ l of egg extract.

Next, we assessed different methods for immunoprecipitation of the chromatin-derived form of Claspin. Some commonly used techniques, such as nuclease digestion or sonication of the chromatin before immunoprecipitation, resulted in anti-Claspin immunoprecipitates that were contaminated by general DNA binding proteins. We concluded that these methods yielded chromatin fragments that were large enough to bridge Claspin nonspecifically with other proteins during immunoprecipitation. As an alternative, we attempted to extract Claspin from chromatin by mild salt treatment under conditions that would maintain certain protein–protein interactions that had initially formed on the DNA. For this purpose, we exposed chromatin to increasing concentrations of NaCl (i.e., 0.25, 0.5, 0.75, and 1 M). As shown in figure 3.1B, treatment with 0.25 M NaCl led to a substantial decrease in chromatin-bound Claspin, and 0.5 M NaCl removed Claspin almost completely. Various replication and checkpoint regulatory proteins, including Xorc2, Xmcm7, Xcdc45, RPA70, RFC40, PCNA, Xatr, and Xhus1, each displayed their own characteristic salt elution profile.

We immunoprecipitated Claspin from the salt eluates and immunoblotted for various key replication and checkpoint proteins. We could readily detect Xcdc45, RPA70, and RFC40 in the anti-Claspin immunoprecipitates from the 0.5 M NaCl eluate, and, to a lesser extent, in the 0.75 M eluate (figure 3.1B). The interaction between Claspin and RFC40 was very strong, remaining even in the presence of 1 M NaCl. It has been established that Xcdc45, Claspin, and Pol ε load onto replication origins around the same time (Lee et al., 2003; Mimura et al., 2000). Therefore, we also immunoblotted the anti-Claspin immunoprecipitates with anti-

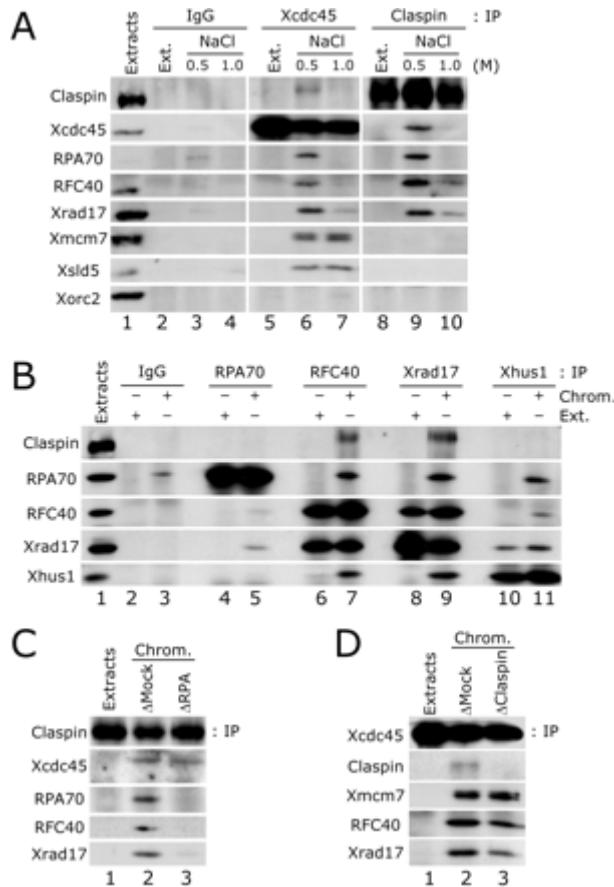
Pol  $\varepsilon$  antibodies, but we could detect only a very faint signal for Pol  $\varepsilon$  in the 0.5 and 0.75 M NaCl eluates (our unpublished data). However, we were able to demonstrate an interaction between Claspin and Pol  $\varepsilon$  by a different method (see below). We could not detect the specific presence of Pol  $\alpha$  or Pol  $\delta$  in anti-Claspin immunoprecipitates by immunoblotting with anti-Pol  $\alpha$  or anti-Pol  $\delta$  antibodies (our unpublished data). Finally, we could not find Xatr, Xhus1, Xorc2, Xmcm7, or PCNA in anti-Claspin immunoprecipitates from any of the salt eluates. These observations argue that our procedure detects specific protein–protein interactions that become established on chromatin. Furthermore, the absence of abundant DNA binding proteins such as Xorc2 indicates that these immunoprecipitates do not contain DNA fragments with contaminating proteins.

To identify Claspin-binding proteins in a more general manner, we analyzed anti-Claspin immunoprecipitates by silver staining (figure 3.1C). We observed specifically associated bands at 150, 140, and 65 kDa as well as a cluster of bands at 36–40 kDa. Analysis by nanoelectrospray tandem mass spectrometry indicated that p150 and p65 correspond to the largest subunits of RFC (RFC140) and RPA (RPA70), respectively. In addition, p36-40 contained all four small subunits of RFC (e.g., RFC36, RFC37, RFC38, and RFC40). p140 is still under investigation. We verified these associations by immunoblotting anti-Claspin immunoprecipitates with specific antibodies that recognize the *Xenopus* versions of RFC140 and RPA70 (figure 3.1D). We could also detect the presence of RFC37 and RFC40 by immunoblotting with antibodies against these proteins. Because the small RFC subunits are also present in other clamp loader

complexes, such as the one containing Rad17, we asked whether Claspin could also associate with Rad17. As shown in figure 3.1D, Xrad17 could also be found in anti-Claspin immunoprecipitates from chromatin by immunoblotting with anti-Xrad17 antibodies.

### *3.3.3 Claspin Interacts Successively with Xcdc45 and RFC Complexes*

To corroborate these results, we carried out various reciprocal immunoprecipitation experiments. First, we asked whether complexes containing Xcdc45 and Claspin could also be identified by immunoprecipitation with anti-Xcdc45 antibodies. As shown in figure 3.2A, we could clearly detect Claspin as well as RPA70, RFC40, and Xrad17 in anti-Xcdc45 immunoprecipitates from 0.5 M NaCl eluates of chromatin. In addition, we could find Xmcm7 and Xsld5, components of the MCM and GINS complexes, respectively, in anti-Xcdc45 immunoprecipitates of both 0.5 and 1 M NaCl chromatin eluates. Consistent with the results described above, we found Xcdc45, RPA70, RFC40, and Xrad17 in anti-Claspin immunoprecipitates that were prepared in parallel. However, we could not find either Xmcm7 or Xsld5 in anti-Claspin immunoprecipitates. Therefore, Claspin does not associate with Xcdc45 indirectly through either the MCM or GINS complexes.



**Figure 3.2.** Binding relationships between Claspin and other proteins on chromatin.

(A) Immunoprecipitation with anti-Xcdc45 antibodies. Control (lanes 2–4), anti-Xcdc45 (lanes 5–7), and anti-Claspin antibodies (lanes 8–10) were used for immunoprecipitation from egg extracts (lanes 2, 5, and 8) or chromatin eluates prepared with either 0.5 M (lanes 3, 6, and 9) or 1 M NaCl (lanes 4, 7, and 10). The samples were immunoblotted for the indicated proteins. Lane 1 depicts whole egg extract. (B) Association of Claspin with RFC40 and Xrad17. Egg extracts (lanes 2, 4, 6, 8, and 10) or 0.5 M NaCl chromatin eluates (lanes 3, 5, 7, 9, and 11) were immunoprecipitated with control (lanes 2 and 3), anti-*Xenopus* RPA70 (lanes 4 and 5), anti-*Xenopus* RFC40 (lanes 6 and 7), anti-Xrad17 (lanes 8 and 9), and anti-Xhus1 antibodies (lanes 10 and 11). Samples were immunoblotted for various proteins as indicated. (C) RPA-dependent association of RFC complexes with Claspin. Claspin was immunoprecipitated from egg extracts (lane 1) and from chromatin eluates obtained from either mock-depleted (lane 2) or RPA-depleted extracts (lane 3). Samples were immunoblotted for the indicated proteins. (D) Xcdc45 interacts with RFC independently of Claspin. Xcdc45 was immunoprecipitated from either egg extracts (lane 1) or chromatin eluates prepared from either mock-depleted (lane 2) or Claspin-depleted extracts (lane 3). Samples were immunoblotted for the indicated proteins.

Next, we carried out immunoprecipitations with anti-RPA70, anti-RFC40, and anti-Xrad17 antibodies. We could find Claspin in anti-RFC40 and anti-Xrad17, but not in anti-RPA70 immunoprecipitates (figure 3.2B). We also performed immunoprecipitations of the chromatin eluates with antibodies against Xhus1, a component of the 9-1-1 complex. Consistent with the results obtained in the anti-Claspin immunoprecipitation studies, we could not detect any Claspin in anti-Xhus1 immunoprecipitates from chromatin eluates (figure 3.2B). On the other hand, we could observe RPA70, RFC40, and Xrad17 in these immunoprecipitates. It should be noted that these results do not rule out an interaction of Claspin with the 9-1-1 complex, because it is possible that the chromatin elution procedure could disrupt such an association.

Previously, we reported that binding of Claspin to chromatin requires Xcdc45 but not RPA (Lee et al., 2003). Moreover, it is well established that the replicative and Rad17-containing RFC complexes associate with replication forks after RPA-stabilized unwinding of the DNA (Ellison and Stillman, 2003; Lee et al., 2003; You et al., 2002; Zou et al., 2003). Therefore, we examined whether the association of Claspin with RFC proteins also requires RPA. We immunodepleted RPA from egg extracts, prepared salt eluates of chromatin fractions from these extracts, and then immunoprecipitated Claspin from these eluates. As depicted in figure 3.2C, we could detect Xcdc45 but not RFC40 or Xrad17 in anti-Claspin immunoprecipitates from RPA-depleted chromatin.

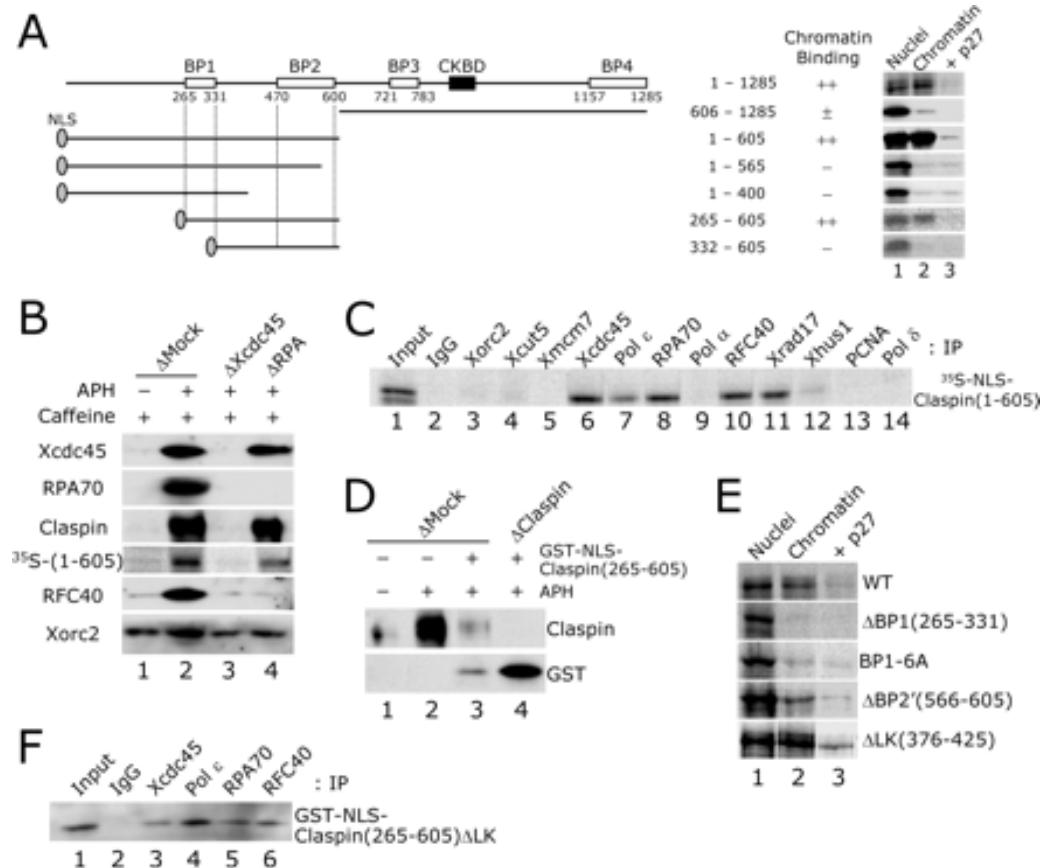


Figure 3.3

**Figure 3.3.** Identification and characterization of an RFID from Claspin.

(A) Chromatin-binding fragments of Claspin. Various  $^{35}\text{S}$ -labeled fragments from the N-terminal half of Claspin were incubated for 100 min in egg extracts containing sperm chromatin, aphidicolin, and caffeine. In this experiment, His6-Claspin(606-1285)-FLAG protein was used to represent the C-terminal half of the protein. Separate nuclear (lane 1) and chromatin fractions were prepared (lanes 2 and 3). To assess dependency of chromatin binding on S-phase cyclin-dependent kinase activity, extracts were treated with p27 (lane 3). Fragments were visualized by SDS-PAGE and phosphorimaging, except for the 606-1285 fragment, which was detected by immunoblotting with anti-FLAG antibodies. (B) The N-terminal domain of Claspin has very similar chromatin binding properties as full-length Claspin. Extracts were subjected to an immunodepletion procedure with control (lanes 1 and 2), anti-Xcdc45 (lane 3), or anti-RPA antibodies (lane 4). Extracts were incubated with  $^{35}\text{S}$ -NLS-Claspin(1-605) and the indicated drugs. Binding to chromatin was assessed by phosphorimaging or immunoblotting for endogenous proteins as indicated. (C) Interaction of N-terminal domain of Claspin with various proteins on chromatin.  $^{35}\text{S}$ -NLS-Claspin(1-605) was incubated in egg extracts as in A. Aliquots of chromatin eluates were immunoprecipitated with antibodies against the indicated proteins. The amount of bound  $^{35}\text{S}$ -fragment was detected by SDS-PAGE and phosphorimaging. Lane 1 depicts 15% of the input chromatin eluate for each lane. (D) Interaction of bacterially expressed RFID with chromatin. Mock-depleted (lanes 1–3) and Claspin-depleted extracts (lane 4) were incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of GST-NLS-Claspin(265-605). Chromatin fractions were prepared and immunoblotted with anti-Claspin (top) and anti-GST antibodies (bottom). (E) Contribution of the RFID to the binding of full-length Claspin to chromatin in undepleted extracts.  $^{35}\text{S}$ -Labeled versions of full-length Claspin containing no deletion or deletions of residues 265–331 ( $\Delta\text{BP1}$ ), 566–605 ( $\Delta\text{BP2}'$ ), or 376–425 ( $\Delta\text{LK}$ ) were prepared. For the 6A mutant, the highly conserved residues Gln287, Arg288, Leu289, Pro298, Tyr299, and His300 were all changed to alanine. Nuclear accumulation (lane 1), chromatin binding (lane 2), and p27 sensitivity of chromatin binding (lane 3) were determined as described in A. (F) Interaction of the RFID with replication proteins on chromatin. GST-NLS-Claspin(265-605)  $\Delta\text{LK}$  was incubated with egg extracts as in A. Aliquots of chromatin eluates were immunoprecipitated with the indicated antibodies and immunoblotted with anti-GST antibodies. Lane 1 depicts 15% of the input chromatin eluate for each sample.

To characterize these interactions further, we analyzed the binding partners of Xcdc45 in the absence of Claspin. As shown in figure 3.2D, Xcdc45 could bind well to RFC40 and Xrad17 even in Claspin-depleted extracts. From these results, we conclude that Claspin interacts first with Xcdc45 at replication forks and later associates with RFC complexes after RPA-stabilized unwinding of the DNA. At this juncture, Xcdc45 also forms connections with RFC proteins, but these interactions do not require the presence of Claspin. These results, along with the fact that the interactions of Claspin with Xcdc45 versus RFC40 display different salt sensitivities, imply that Xcdc45 and RFC complexes associate with Claspin independently.

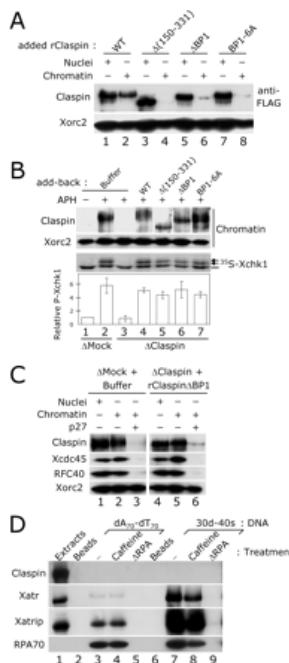
#### *3.3.4 A Conserved N-Terminal Domain Mediates Interaction of Claspin with Chromatin*

To assess the functional significance of the interactions with other proteins on chromatin, we attempted to map the chromatin-binding region of Claspin. Because *in vitro* translated  $^{35}\text{S}$ -Claspin bound to chromatin as efficiently as endogenous Claspin, we used  $^{35}\text{S}$ -labeled fragments of Claspin for the initial phase of these experiments. We incubated various truncated forms of Claspin in extracts containing aphidicolin and caffeine and compared their ability to associate with chromatin. For any fragment that did not contain the last 56 amino acids of Claspin, which are essential for nuclear uptake, we incorporated an ectopic NLS into the polypeptide chain. Because expression of individual fragments was variable, we normalized the data by examining what percentage

of each fragment in nuclear fractions from the extracts could associate with chromatin.

These studies indicated that a fragment containing residues 1–605 of Claspin binds exceptionally well to chromatin (figure 3.3A). By contrast, various fragments from the C-terminal end of Claspin (e.g., 606–1285) showed little, if any, stable interaction with chromatin. The binding of the 1–605 fragment to chromatin was sensitive to the Cdk inhibitor p27, which inhibits firing of replication origins and thus prevents binding of full-length Claspin to replication forks. Furthermore, the interaction of this fragment with chromatin depended on Xcdc45 but not on RPA (figure 3.3B), as for full-length Claspin.

Next, we asked whether the 1–605 fragment could associate with the same replication and checkpoint proteins as full-length Claspin. As shown in figure 3.3C, we could immunoprecipitate the 1–605 fragment from chromatin fractions with antibodies against Xcdc45, RPA70, RFC40, and Xrad17. For these experiments, we also examined binding to three major eukaryotic DNA polymerases, namely, Pol  $\alpha$ , Pol  $\beta$ , and Pol  $\epsilon$ . We could observe strong interaction of the 1–605 fragment with Pol  $\epsilon$ , but no binding to either Pol  $\alpha$  or Pol  $\delta$ . Finally, we could detect little or no binding of this fragment to Xorc2, Xcut5, Xmcm7, PCNA, and Xhus1 in immunoprecipitations with antibodies against these respective proteins. From these observations, we conclude that the ability of Claspin to interact stably with chromatin resides in the 1–605 fragment.



**Figure 3.4.** Role of the BP1 region from the RFID in binding of Claspin to chromatin and activation of Xchk1 in extracts lacking endogenous Claspin.

(A) Various His6-Claspin-FLAG proteins were prepared from baculovirus-infected insect cells and added directly into undepleted egg extracts containing sperm chromatin, aphidicolin, and caffeine. The proteins included full-length Claspin (WT) (lanes 1 and 2), a deletion of residues 150–331 (lanes 3 and 4), and a deletion of residues 265–331 (ΔBP1, lanes 5 and 6), and the 6A mutant (lanes 7 and 8). Nuclear (lanes 1, 3, 5, and 7) and chromatin fractions (lanes 2, 4, 6, and 8) were immunoblotted with anti-FLAG antibodies (top). Samples were also immunoblotted for Xorc2 as a loading control (bottom). (B) Mock-depleted (lanes 1–3) or Claspin-depleted extracts (lanes 3–7) containing control buffer (lanes 1–3) or equivalent amounts of the indicated forms of His6-Claspin-FLAG (lanes 4–7) were incubated in the absence (lane 1) or presence (lanes 2–7) of aphidicolin. Chromatin fractions were immunoblotted for Claspin and Xorc2 (top two panels). Phosphorylation of <sup>35</sup>S-Xchk1 in nuclear fractions was assessed by phosphorimaging (third panel from top). For quantitation (bottom), the amount of shifted <sup>35</sup>S-Xchk1 was divided by the total <sup>35</sup>S-Xchk1 in each lane and normalized to the signal in the absence of aphidicolin (lane 1). Results are the mean  $\pm$  SD for two experiments. (C) Binding of the ΔBP1 mutant to chromatin is sensitive to p27. Mock-depleted (lanes 1–3) and Claspin-depleted extracts (lanes 4–6) containing control buffer (lanes 1–3) or the ΔBP1 mutant of His6-Claspin-FLAG were incubated with aphidicolin and caffeine. p27 was also added in lanes 3 and 6. Nuclear (lanes 1 and 4) and chromatin fractions (lanes 2, 3, 5, and 6) were immunoblotted for the indicated proteins. (D) Claspin does not bind detectably to branched DNA in egg extracts. Magnetic beads coated with no DNA (lanes 2 and 6), (dA)<sub>70</sub>-(dT)<sub>70</sub> (lanes 3–5), or 30d–40s DNA (a branched structure that contains a 30-mer double-stranded region and two 40-mer single-stranded regions) (lanes 7–9) were incubated in mock-depleted (lanes 3, 4, 7, and 8) or RPA-depleted egg extracts (lanes 5 and 9). Caffeine was included in some incubations (lanes 4 and 8). The beads were recovered, washed, and immunoblotted for the indicated proteins. Lane 1 shows 1  $\mu$ l of egg extract.

In further mapping studies, we found that removal of the N-terminal 264 residues from the 1–605 fragment had no effect on chromatin-binding efficiency (figure 3.3A). However, additional deletion of residues 265–331 abrogated binding. Similarly, removal of residues 566–605 from the opposite C-terminal end of this fragment also abolished interaction with chromatin. From these studies, we conclude that a region of Claspin stretching from residues 265–605 is involved in the interaction with chromatin. To corroborate these findings, we prepared a GST fusion protein containing residues 265–605 of Claspin as well as an ectopic NLS. The resulting GST-NLS-Claspin(265–605) protein could bind well to chromatin (figure 3.3D). The binding was much higher in Claspin-depleted extracts. However, binding also occurred in mock-depleted extracts, where inclusion of the fragment also reduced the binding of endogenous Claspin to chromatin. Therefore, the isolated 265–605 fragment of Claspin fragment seems to compete with endogenous Claspin for a finite number of binding sites in chromatin. We named the 265–605 region of Claspin the replication fork-interacting domain (RFID). Two recent studies have shown that human Claspin and its fission yeast homologue Mrc1 possess an *in vitro* DNA binding activity that is mediated by a DNA binding domain (DBD) (Sar et al., 2004; Zhao and Russell, 2004). The DBD in human Claspin (residues 149–340) corresponds closely to residues 150–331 of *Xenopus* Claspin, which overlap partially with the RFID. During our studies, we noticed an interesting structural feature of Claspin. Overall, Claspin is a very acidic protein ( $pI = 4.5$ ), but it does contain four patches of basic amino acids (residues 265–331, 470–600, 721–783, and 1157–1285) with a  $pI$  value  $>10$ .

(figure 3.3A and supplemental figure S1). These segments, which we denoted BP1, BP2, BP3, and BP4, are highly conserved in metazoan Claspin, and the yeast Mrc1 proteins also possess similar basic segments. Notably, BP1 and BP2 define the boundaries of the RFID.

To evaluate directly whether the RFID can account for the chromatin-binding ability of full-length Claspin, we prepared various forms of full-length  $^{35}\text{S}$ -labeled Claspin with mutations in this domain. We first examined relatively large deletions in the protein. Initially, we deleted BP1 ( BP1, residues 265–331) and 40 amino acids encompassing the C-terminal end of BP2 ( BP2', residues 566–605). Moreover, we also produced a mutant (6A) in which six residues within BP1 that are highly conserved in metazoan Claspin proteins and conserved to some extent in the yeast Mrc1 proteins were changed to alanine. As shown in figure 3.3E, when we added  $^{35}\text{S}$ -labeled forms of the BP1, BP2', and 6A mutants to undpleted egg extracts that contain their full complement of endogenous Claspin, binding to chromatin was abrogated or greatly reduced. By contrast, deletion of a poorly conserved segment (residues 376–425) from the center of the RFID, which we named the linker region (LK), had negligible effect on binding to chromatin (figure 3.3E). Consistent with this observation, a bacterially expressed form of GST-NLS-Claspin(265–605) LK bound well to chromatin (our unpublished data) and associated specifically with Xcdc45, Pol  $\epsilon$ , RPA, and RFC40 in chromatin immunoprecipitation experiments (figure 3.3F).

### 3.3.5 The BP1 Region of Claspin Is Not Required for Activation of Chk1

Next, we sought to replace endogenous Claspin in egg extracts with various RFDI mutants to assess their abilities to function in checkpoint regulation. Toward this end, we created mutations in a baculovirus-expressed version of Claspin that contains His6 and FLAG tags at the N- and C-terminal ends, respectively (His6-Claspin-FLAG). First, we focused on the BP1 region. We produced BP1 and 6A mutants of the baculovirus-expressed protein. We also prepared a larger deletion (residues 150–331) that removes all of BP1 and a block of upstream conserved sequences. The deleted region in this latter mutant corresponds to the whole DBD that was identified in human Claspin (Sar et al., 2004). When we examined binding to chromatin in undepleted egg extracts, we observed that none of the BP1, 6A, and (150-331) mutants of baculovirus-expressed His6-Claspin-FLAG could bind to chromatin (figure 3.4A).

We proceeded to immunodeplete endogenous Claspin from the extracts and to replace it with equivalent amounts of these three mutants. First, we monitored the binding of these mutants to chromatin in extracts that now lacked endogenous Claspin. Unexpectedly, we observed that these three mutants could now bind very well to chromatin (figure 3.4B). This observation indicates that, in the absence of competition from endogenous Claspin, these three mutants retain significant ability to interact with chromatin. Furthermore, binding of the BP1 mutant to chromatin displayed the same sensitivity to p27 as wild-type Claspin (figure 3.4C), which argues this mutant binds specifically to replication forks. We next examined the ability of these mutants to mediate the activation of Xchk1 in

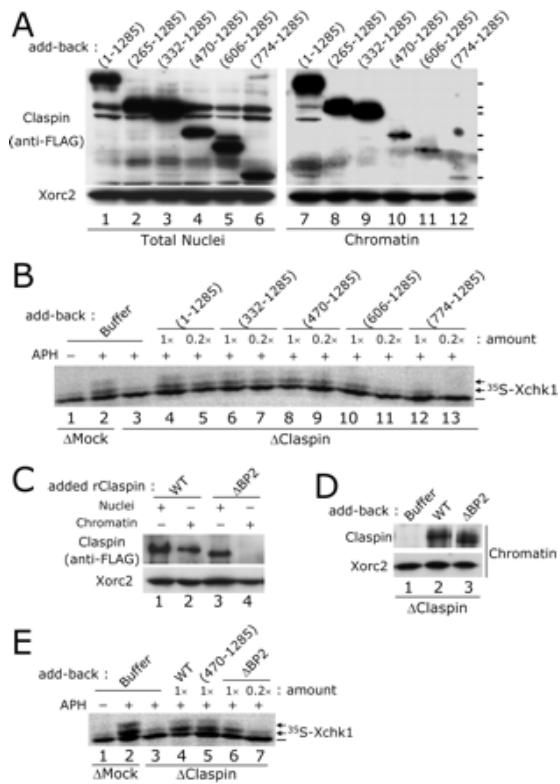
response to treatment with aphidicolin. As shown in figure 3.4B, all three mutants did not show any obvious difference from wild-type Claspin in the ability to promote the phosphorylation of Xchk1. Together, these results indicate that neither BP1 nor the whole DBD region is required for activation of Xchk1. Deletion of BP1 or the DBD region does weaken the interaction of Claspin with chromatin. However, without competition from endogenous full-length Claspin, mutants lacking these sequences bind very well to chromatin, which implies that the remaining sequences of Claspin are sufficient for chromatin binding.

We have previously shown that Claspin does not bind detectably to either single-stranded or double-stranded DNA in egg extracts (Kumagai et al., 2004; Lee et al., 2003). More recently, it was reported that recombinant human Claspin seems to have a stronger *in vitro* binding activity toward branched DNA than single-stranded or double-stranded DNA (Sar et al., 2004). Therefore, we tested directly whether endogenous Claspin in egg extracts can associate stably with branched DNA. As the template (30d-40s DNA), we annealed two 70-mers that are complementary for 30 nucleotides at one end. As shown in figure 3.4D, we could not detect any binding of Claspin to either the branched 30d–40s DNA or double-stranded (dA)<sub>70</sub>-(dT)<sub>70</sub>. On the other hand, we could readily observe binding of RPA, Xatr, and Xatrip to both templates. Furthermore, addition of caffeine or immunodepletion of RPA, both of which greatly increase the binding of Claspin to chromatin in aphidicolin-containing egg extracts (Lee et al., 2003), did not result in any binding of Claspin to either template. The concentration of Claspin in egg extracts (240 nM) (Kumagai and Dunphy, 2000) is well over the observed  $K_d$  for

*in vitro* binding of recombinant human Claspin to branched DNA and thus should not be a limiting factor. Together, these results suggest that endogenous Claspin in egg extracts cannot bind stably to DNA alone in egg extracts and that interaction with proteins at the replication fork is necessary for stable association with chromatin.

### *3.3.6 The BP2 Region of Claspin Potentiates Its Chk1-activating Function*

To study the role of the RFD more systematically, we prepared various mutant versions of Claspin containing serial N-terminal deletions and compared their chromatin binding properties in the absence of endogenous Claspin. We performed these experiments in the presence of aphidicolin and caffeine to maximize binding, but we obtained qualitatively similar binding in the presence of aphidicolin alone (our unpublished data). As shown in figure 3.5A, both Claspin(265–1285) and Claspin(332–1285), which lack part of or the entire DBD region, bind as well as full-length Claspin to chromatin. On the other hand, further deletions from the N-terminal end resulted in a dramatic drop in association with chromatin. In particular, binding of the Claspin(470–1285), Claspin(606–1285), and Claspin(774–1285) fragments was markedly reduced, although the 470–1285 fragment did reproducibly bind somewhat better than the 606–1285 and 774–1285 fragments.



**Figure 3.5. C-terminal fragments of Claspin display reduced potency in mediating activation of Xchk1.**

(A) Full-length (residues 1–1285) and N-terminally truncated forms of Claspin (residues 265–1285, 332–1285, 470–1285, 606–1285, and 774–1285) were prepared as His6-Claspin-FLAG proteins in insect cells and added into Claspin-depleted extracts containing sperm chromatin, aphidicolin, and caffeine. Nuclear (lanes 1–6) and chromatin fractions (lanes 7–12) were prepared and immunoblotted with anti-FLAG (top) and anti-Xorc2 antibodies (bottom). The bars on right denote positions of the different recombinant Claspin proteins. (B) Mock-depleted (lanes 1 and 2) and Claspin-depleted extracts (lanes 3–13) containing control buffer (lanes 1–3) or the indicated forms of His6-Claspin-FLAG (lanes 4–13) were incubated in the absence (lane 1) or presence (lanes 2–13) of aphidicolin. Recombinant Claspin proteins were added at a molar concentration equal to (1×) or one-fifth (0.2×) that of endogenous Claspin. Phosphorylation of <sup>35</sup>S-Xchk1 in nuclear fractions from the extracts was assessed by phosphorimaging. (C) Wild-type His6-Claspin-FLAG (lanes 1 and 2) and a deletion mutant lacking residues 470–605 (ΔBP2, lanes 3 and 4) were added into undepleted egg extracts containing sperm chromatin, aphidicolin, and caffeine. Nuclear (lanes 1 and 3) and chromatin fractions (lanes 2 and 4) were immunoblotted with anti-FLAG (top) and anti-Xorc2 antibodies (bottom). (D) Claspin-depleted extracts were prepared and incubated in the presence of aphidicolin with buffer alone (lane 1), wild-type His6-Claspin-FLAG (lane 2), or the ΔBP2 mutant (lane 3). Chromatin fractions were immunoblotted for Claspin (top) and Xorc2 (bottom). (E) Phosphorylation of <sup>35</sup>S-Xchk1 was compared in extracts containing the full-length, 470–1285, and ΔBP2 versions of Claspin at the indicated concentrations as described in B.

We proceeded to compare the ability of the various truncation mutants to mediate the activation of Xchk1. For these experiments, we immunodepleted endogenous Claspin from egg extracts and then added back the various recombinant Claspin proteins at two different concentrations to compare their relative potencies. In particular, we used molar concentrations corresponding to the amount of endogenous Claspin (1×) and a fivefold dilution of this amount (0.2×), respectively. Finally, we added sperm chromatin and aphidicolin to the extracts and then monitored phosphorylation of Xchk1. We observed that the full-length, 332–1285, and 470–1285 polypeptides all could restore phosphorylation of Xchk1 in Claspin-depleted extracts at the both 1× and 0.2× concentrations (figure 3.5B). However, the shorter fragments of Claspin, namely, 606–1285 and 774–1285 were significantly less potent at promoting the phosphorylation of Xchk1. This effect was most evident at the diluted concentration of these fragments. For example, an extract containing the 774–1285 mutant at the 0.2× concentration displayed little or no phosphorylation of Xchk1. These observations indicate that the C-terminal region of Claspin, which does not bind strongly to chromatin, shows reduced capacity to mediate the activation of Xchk1. However, the 470–1285 fragment seemed similar to full-length Claspin in the ability to mediate phosphorylation of Xchk1, even though it did not display the stable chromatin-binding capacity of full-length Claspin.

These observations suggested that all or part of the BP2 region in Claspin (residues 470–600) might be important for checkpoint signaling. To pursue this possibility, we prepared a mutant of full-length His6-Claspin-FLAG with a deletion

of residues 470–605 ( BP2). Consistent with the results observed for the smaller deletion of residues 566–605, the BP2 mutant could bind to chromatin in Claspin-depleted extracts but not in undepleted extracts containing endogenous Claspin (figure 3.5C and D). When we examined checkpoint regulation, we observed that aphidicolin-treated extracts containing a fivefold dilution of the BP2 mutant showed significantly reduced phosphorylation of Xchk1 (figure 3.5E). Therefore, deletion of BP2 seems to compromise the checkpoint-signaling potency of Claspin.

### *3.3.7 The C-terminal Region of Claspin Contains a Small Chk1-activating Domain*

These observations suggested that the C-terminal domain of Claspin has a significantly reduced potency for activation of Xchk1. To pursue this issue, we systematically compared the potencies of full-length Claspin and the C-terminal 774–1285 fragment for mediating the activation of Xchk1 (figure 3.6A and B). For this purpose, we immunodepleted endogenous Claspin from egg extracts and added back different dilutions of full-length Claspin and the 774–1285 fragment. This procedure enabled us to compare the relative potencies of the two proteins. Significantly, full-length Claspin was highly resistant to dilution, indicating that there is a large functional surplus of Claspin in egg extracts. For example, we observed half-maximal phosphorylation of Xchk1 in extracts containing a concentration of recombinant full-length Claspin  $\sim 0.055 \times$  the level of endogenous Claspin (figure 3.6B). By contrast, a molar concentration of the 774–1285 fragment  $\sim 1.8$ -fold greater than that of endogenous full-length Claspin was

required to elicit a similar extent of phosphorylation. Therefore, the 774–1285 fragment seems to be ~33-fold less potent than full-length Claspin. To evaluate whether the 774–1285 fragment acts by a similar mechanism as full-length Claspin, we examined phosphorylation of Claspin on Ser864, which is required for phosphorylation of Xchk1 (Kumagai and Dunphy, 2003). As shown in figure 3.6C, the 774–1285 fragment became very efficiently phosphorylated on Ser864 in aphidicolin-treated extracts.

These results imply that the C-terminal region of Claspin should contain the minimal sequences necessary to carry out the biochemical process required for the ATR-dependent phosphorylation of Xchk1. To identify these sequences, we prepared various C-terminal fragments of Claspin as fusion proteins containing GST and an ectopic NLS. The fusion constructs were designed to contain the previously identified Chk1-binding domain of Claspin (CKBD, residues 847–903), in which serines 864 and 895 must be phosphorylated for binding to Xchk1 (Kumagai and Dunphy, 2003). We could observe phosphorylation of Xchk1 in the presence of a fragment from Claspin containing residues 776–956 but not an overlapping fragment containing residues 844–1170 (figure 3.6D). Further analysis indicated that a 130-amino acid fragment containing amino acids 776–905 is sufficient for restoring phosphorylation of Xchk1 to Claspin-depleted extracts (figure 3.6E and F). We named this fragment the Chk1-activating domain (CKAD). The CKAD, as well as the longer 776–956 and 774–1285 fragments, displayed little or no binding to chromatin (figure 3.6G). Importantly, however, the CKAD is considerably less potent than full-length Claspin. For example, in figure

3.6E, we needed to add the CKAD fragment at a six-fold molar excess over the amount of endogenous Claspin, which is already in surplus, to observe a similar extent of Xchk1 phosphorylation.

Overall, these findings indicate that the capacity of Claspin to mediate the activation of Xchk1 resides in a small C-terminal region containing no more than 130 amino acids. However, sequences from the BP2 region greatly increase the overall potency of Claspin for activation of Xchk1. Deletion of BP2 weakens the interaction of Claspin with chromatin. On the other hand, a truncated form of Claspin (residues 470–1285) that contains BP2 does not bind stably to chromatin and nonetheless induces phosphorylation of Xchk1 as efficiently as full-length Claspin. These observations indicate that stable retention of Claspin on chromatin is not necessary for activation of Xchk1.

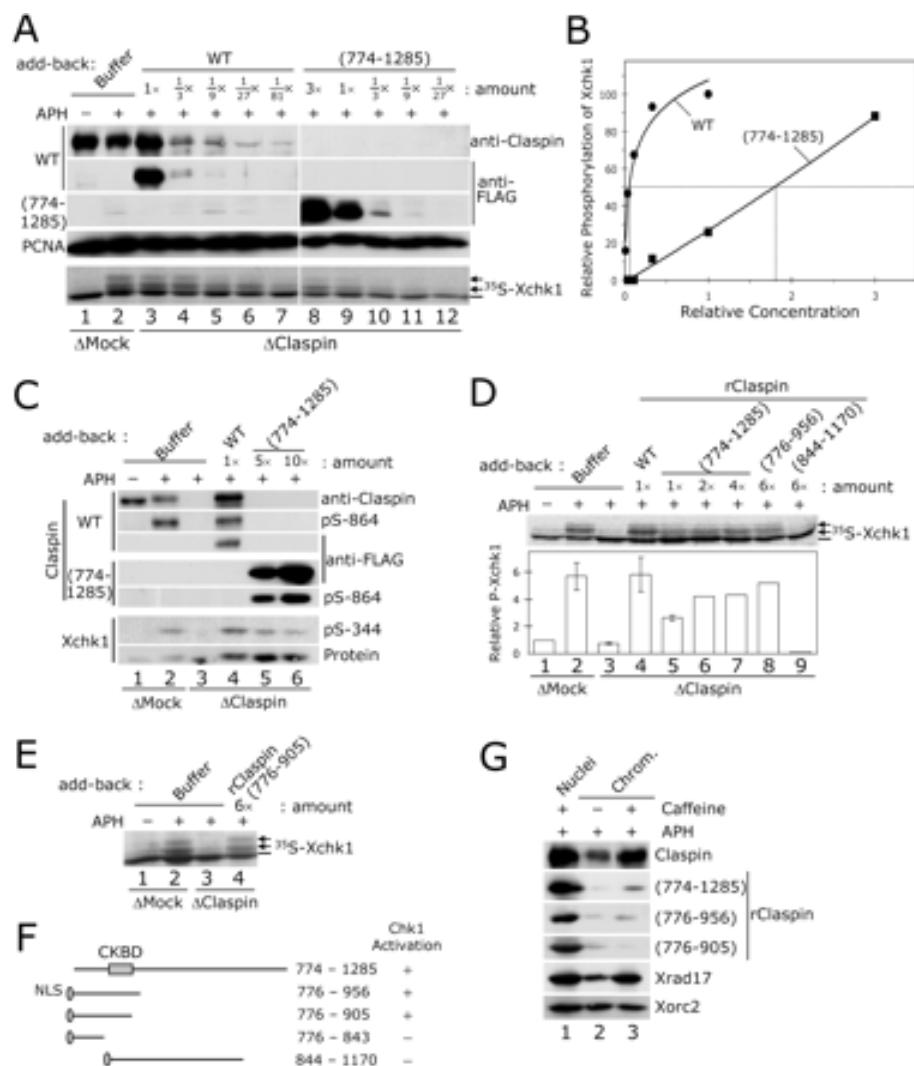


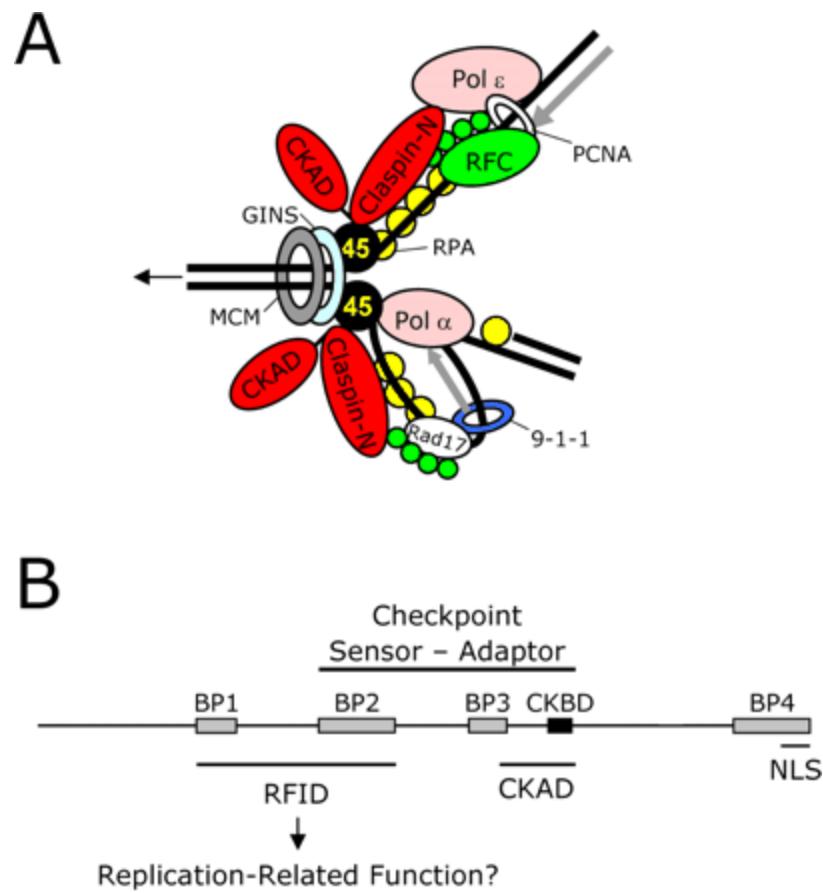
Figure 3.6

**Figure 3.6.** A small fragment from the C-terminal end of Claspin is sufficient at high concentrations for mediating phosphorylation of Xchk1.

(A) Mock-depleted (lanes 1 and 2) and Claspin-depleted extracts (lanes 3–12) were incubated with control buffer (lanes 1 and 2) and the indicated dilutions of full-length His6-Claspin-FLAG (lanes 3–7) or the His6-Claspin(774–1285)-FLAG fragment (lanes 8–12) in the absence (lane 1) or presence (lanes 2–12) of aphidicolin. Nuclear fractions were prepared and immunoblotted with anti-Claspin antibodies (top), anti-FLAG antibodies to detect both full-length Claspin (second panel from top) and the 774–1285 fragment (third panel from the top), and anti-PCNA antibodies (fourth panel from top). Note that the anti-Claspin antibodies do not detect the 774–1285 fragment. Phosphorylation of  $^{35}\text{S}$ -Xchk1 was assessed by phosphorimaging (bottom). (B) Quantitation of results from A for full-length Claspin (closed circles) and the 774–1285 fragment (closed squares). (C) Phosphorylation of the 774–1285 fragment on Ser864. Mock-depleted (lanes 1 and 2) and Claspin-depleted extracts (lanes 3–6) containing control buffer (lanes 1–3), full-length His6-Claspin-FLAG (lane 4), or His6-Claspin(774–1285)-FLAG (lanes 5 and 6) at the indicated amounts relative to endogenous Claspin were incubated in the absence (lane 1) or presence (lanes 2–6) of aphidicolin. Nuclear fractions were immunoblotted with anti-Claspin, anti-P-Ser864 of Claspin, and anti-FLAG antibodies as indicated. To examine phosphorylation of Xchk1, samples were immunoblotted with anti-P-Ser344 of Xchk1 and anti-Xchk1 antibodies (bottom two panels). (D) C-terminal fragments of Claspin fully rescue phosphorylation of Xchk1 at high concentrations. Mock-depleted (lanes 1 and 2) and Claspin-depleted extracts (lanes 3–9) containing control buffer (lanes 1–3), full-length His6-Claspin-FLAG (lane 4), His6-Claspin(774–1285)-FLAG (lanes 5–7), GST-NLS-Claspin(776–956) (lane 8), and GST-NLS-Claspin(844–1170) (lane 9) at the indicated concentrations relative to endogenous Claspin were incubated in the absence (lane 1) or presence (lanes 2–9) of aphidicolin. Phosphorylation of  $^{35}\text{S}$ -Xchk1 was detected with a phosphorimager (top) and quantitated (bottom). Results are mean  $\pm$  SD for two experiments. (E) The 776–905 fragment of Claspin is sufficient at a high concentration for full phosphorylation of Xchk1. Mock-depleted (lanes 1 and 2) and Claspin-depleted extracts (lanes 3 and 4) were incubated with control buffer (lanes 1–3) or GST-NLS-Claspin(776–905) (lane 4) at a molar concentration sixfold higher than that of endogenous Claspin and examined for phosphorylation of  $^{35}\text{S}$ -Xchk1. (F) Summary for identification of the CKAD from Claspin. The CKBD is denoted with the shaded box. (G) Fragments from C-terminal half of Claspin display little or no stable binding to chromatin. Extracts were incubated with His6-Claspin(774–1285)-FLAG, GST-NLS-Claspin(776–956), or GST-NLS-Claspin(776–905) in the presence of aphidicolin (lane 2) or aphidicolin plus caffeine (lanes 1 and 3). Polypeptides were added at 4–6 $\times$  the molar concentration of endogenous Claspin. Nuclear (lane 1) and chromatin fractions (lanes 2 and 3) were immunoblotted with anti-FLAG or anti-GST antibodies to detect recombinant Claspin proteins as appropriate. Samples were also immunoblotted for endogenous Claspin, Xrad17, and Xorc2.

### 3.4 Discussion

One characteristic of the checkpoint mediator protein Claspin is that it interacts specifically with both normal and stalled DNA replication forks during S phase. In this report, we have investigated the mechanism and functional significance of this interaction. To this end, we have mapped the regions of Claspin that enable binding to chromatin and searched for chromatin-bound proteins that mediate this interaction. Our studies have indicated that Claspin contains a relatively large RFD that is responsible for interaction with chromatin. This domain associates with Xcdc45, RPA, and both the replicative and Rad17-containing RFC complexes (figure 3.7). We have also established that there is specific binding of Claspin to one of the major replicative DNA polymerases, namely, Pol  $\epsilon$ . Therefore, Claspin interacts with both essential replication proteins and a key checkpoint regulator on chromatin. Consistent with these observations, it has been reported that budding yeast Mrc1 and Cdc45 can be coimmunoprecipitated from nuclease digests of chromatin (Katou et al., 2003). However, these studies did not resolve whether Mrc1 and Cdc45 associate by protein–protein interactions or simply bind near one another on the same digested DNA fragments.



**Figure 3.7.** Model for the interaction of Claspin with stalled replication forks.

(A) Diagram summarizes the protein–protein interactions of Claspin at replication forks. See text for discussion. (B) Summary of the different functional domains within Claspin.

### *3.4.1 Claspin Interacts Successively with Replication Fork Proteins*

We have found that Xcdc45 forms a complex with Claspin without loading of RPA or RFC onto chromatin. By contrast, RFC depends upon the loading of RPA to interact with Claspin. These dependencies reflect the hierarchy in which these proteins associate with chromatin. Therefore, our results indicate that upon binding to chromatin Claspin interacts first with Xcdc45. After unwinding of the DNA, loading of RPA, and synthesis of initiating primers, the replicative RFC complex would recognize the 3' recessed ends of nascent DNA strands. At this point, RFC would also become incorporated into a complex with Claspin. We also find that Claspin interacts with the Rad17-containing RFC complex. Recent evidence has indicated that the Rad17-containing complex interacts with the 5' recessed ends of DNA strands (Ellison and Stillman, 2003; Zou et al., 2003). Thus, Claspin would be in a position to participate in the detection of an array of different DNA replication intermediates (figure 3.7A).

### *3.4.2 Role of Claspin as a Checkpoint Sensor Protein*

One important question in the cell cycle checkpoint field involves the issue of how cells detect the presence of incompletely replicated or damaged DNA. Numerous studies in recent years have suggested that cells use a combinatorial mechanism to detect and discriminate between arrays of checkpoint-triggering signals (Sancar et al., 2004). Mediator proteins such as Claspin and the BRCT-containing proteins can provide additional modes of discrimination to checkpoint-sensing mechanisms, and indeed they may be crucial for cells to distinguish between different checkpoint-inducing signals. The fact that Claspin associates

strongly with replication forks raised the possibility that this binding would be closely related with the ability of Claspin to mediate the activation of Xchk1. Interestingly, however, we find that certain fragments of Claspin retain the ability to mediate the activation of Xchk1 without being able to associate stably with chromatin. These fragments fall into two classes. Fragments from the C-terminal half of Claspin retain full efficacy for activation of Xchk1 but are significantly less potent. For example, the 774–1285 fragment is ~33-fold less potent than full-length Claspin for half-maximal activation of Xchk1. By contrast, a fragment containing residues 470–1285 does not bind to chromatin stably but is nonetheless comparable to full-length Claspin in its potency for activation of Xchk1. Significantly, this fragment retains a substantial portion of the RFID, namely, BP2 (residues 470–600) (figure 3.7B). The presence of the BP2 region may allow the 470–1285 fragment to interact transiently with replication forks and thereby enhance the ability of Claspin to mediate the activation of Xchk1. Alternatively, it is possible that the BP2 region increases the activity of Claspin as a mediator by a mechanism that does not involve any interaction with chromatin. However, our mapping studies have clearly indicated that this region is important for optimal interaction with chromatin. For example, deletion of BP2 (residues 470–600) from full-length Claspin impaired chromatin binding in egg extracts containing all of its endogenous Claspin, indicating that this mutant cannot compete effectively with normal Claspin. Furthermore, in Claspin-depleted extracts, the BP2 mutant can bind to chromatin, but it displays significantly reduced ability to mediate the activation of Xchk1. Therefore, this mutant seems

to associate with chromatin in an aberrant manner that is not compatible with normal activation of Xchk1.

Recently, it has been shown that human Claspin and its fission yeast relative Mrc1 possess a DBD (Sar et al., 2004; Zhao and Russell, 2004). The DBD in human Claspin (residues 149–340) is highly homologous to residues 150–331 in *Xenopus* Claspin and contains the BP1 region of the RFID that we have identified in this study. Our results indicate that the DBD region is not essential either for interaction with chromatin or for activation of Xchk1. We found that versions of Claspin that lack this region, such as the (150–331) and 332–1285 constructs, bind very well to chromatin in Claspin-depleted extracts and display a comparable potency with full-length Claspin for mediating the activation of Xchk1. Nonetheless, these mutants do seem to have a lower affinity for replication forks in that they are unable to bind to chromatin in undepleted extracts containing the full complement of endogenous Claspin. The physiological role of the DBD in human Claspin is not known. Fission yeast harboring a version of Mrc1 with a mutated DBD displayed a modest increase in sensitivity to hydroxyurea and a partial defect in the DNA replication checkpoint. However, the abilities of this mutant to associate with chromatin in fission yeast cells and to mediate the activation of Cds1, the checkpoint effector kinase downstream of Mrc1, have not yet been described. We suspect that the DBD/BP1 region may have a role in some other function of Claspin besides mediating the activation of Xchk1.

### *3.4.3 Identification of a Minimal Chk1-activating Domain*

Another finding of this work is that a very small fragment of Claspin (the CKAD), only 130 amino acids long, is fully sufficient at high concentrations to sustain the Xatr-dependent activation of Xchk1 in aphidicolin-treated extracts. We can detect little or no association of the CKAD with chromatin, which reinforces the concept that stable binding of Claspin to chromatin is not obligatory for checkpoint activation. Nonetheless, even this small fragment presumably interacts transiently with sites of replication to collaborate with replication fork-associated Xatr in the phosphorylation of Xchk1. Similarly, we can never detect binding of Xchk1 to chromatin (our unpublished data), which implies that Xchk1 would also interact only transiently with Xatr-Xatrip and Claspin at replication forks. Consistent with this idea, Claspin has a low affinity for the fully activated form of Xchk1, which presumably dissociates from Claspin immediately upon kinase activation (Jeong et al., 2003). Together, these observations indicate that Xatr-Xatrip, Claspin, and Xchk1 associate evanescently with one another during the process that results in activation of Xchk1.

### *3.4.4 Stable Chromatin Binding of Claspin May Reflect an Additional Function*

Our observations support the possibility that the RFID has another function in addition to initial activation of Xchk1. In the absence of Claspin, DNA replication in egg extracts occurs somewhat more slowly than normal (Lee et al., 2003). Interestingly, overexpression of Claspin in human cells enhances the rate of cell proliferation (Lin et al., 2004). Furthermore, it is well established that the budding yeast Mrc1 protein, the apparent functional counterpart of Claspin, has a role in

DNA replication. Yeast mutants lacking Mrc1 replicate their DNA more slowly, accumulate DNA damage in S phase, and exhibit defects in sister chromatid cohesion (Alcasabas et al., 2001; Osborn and Elledge, 2003; Xu et al., 2004). Although the exact role that Claspin/Mrc1 plays in S-phase regulation remains to be established, our data raise the possibility that the stable retention of Claspin on chromatin may be related to this function.

### **3.5 Acknowledgements**

This chapter is reprinted with permission from the Molecular Biology of the Cell by the American Society for Cell Biology, MBC Online, <http://www.molbiolcell.org>, Joon Lee, Daniel A. Gold, Anna Shevchenko, Andrej Shevchenko, and William G. Dunphy “Roles of replication fork-interacting and Chk1-activating domains from Claspin in a DNA replication checkpoint response”, volume 16, issue 11, p. 5269–5282, November 2005. A. Shevchenko and A. Shevchenko provided mass spectrophotometric identification of Claspin interacting proteins. D. Gold performed initial structure-function investigations to find regions of Claspin required for association with chromatin. D. Gold created the 1-605 Claspin fragment and characterized the role of the fragment in chromatin binding. J. Lee performed all other work in this study.

## Chapter 4

# Drf1-Dependent Kinase (DDK) Interacts with Claspin through a Conserved Protein Motif

Daniel A. Gold and William G. Dunphy

The Drf1/Dbf4-dependent kinase (DDK) is required for the initiation of DNA replication in eukaryotes. Another protein, Claspin, mediates the activation of a cellular checkpoint response to stalled replication forks and is also a regulator of replication. In this study, we found that DDK phosphorylates Claspin *in vitro* and forms a nuclear complex containing Cdc7, Drf1, and Claspin in *Xenopus* egg extracts. We identified a conserved binding site on Claspin required for its interaction with DDK. This site corresponds to the first of two sequence repeats in the Chk1-binding domain (CKBD) of Claspin. Furthermore, we have established that two amino acids in this motif, D861 and Q866, are essential for the interaction between Claspin and DDK. Finally, we found that a mutant form of Claspin incapable of interacting with DDK is still able to associate with and activate Chk1 in response to replication blocks. These findings suggest that the

interaction of DDK with Claspin mediates a checkpoint-independent function of Claspin, most likely related to DNA replication.

#### 4.1 Introduction

The maintenance of genomic stability is of paramount importance to living organisms. For example, proliferating cells must ensure the fidelity of chromosomal DNA replication. To accomplish this task, eukaryotic organisms have evolved a highly ordered series of steps to carry out DNA replication as well as a system of checkpoints to monitor the integrity of this process.

The stepwise process of eukaryotic DNA replication begins with the formation of pre-replication complexes (pre-RCs) at replication origins (Arias and Walter, 2007; Takeda and Dutta, 2005). The conversion of the pre-RCs into active replication forks is promoted by the action of two kinases, the S-phase cyclin-dependent kinase (S-CDK) and the Drf1/Dbf4-dependent kinase Cdc7 (DDK). In higher eukaryotes, the kinase activity of Cdc7 is dependent on the association with either of the regulatory subunits, Dbf4 or Drf1 (Jiang et al., 1999; Kumagai et al., 1999; Montagnoli et al., 2002). However, Drf1 is the primary Cdc7-binding partner that drives replication in early *Xenopus* embryos (Takahashi and Walter, 2005) (Silva et al., 2006). The most well-established target of DDK activity is the MCM2-7 complex, the putative replicative helicase. DDK phosphorylates multiple components of the MCM complex (Masai et al., 2006; Sheu and Stillman, 2006; Tsuji et al., 2006). Moreover, the action of DDK is required for the recruitment of Cdc45 to chromatin (Walter, 2000; Zou and Stillman, 2000). It

is thought that Cdc45 stimulates replicative helicase activity, which allows binding of DNA polymerases and other DNA replication elongation factors to origins (Aparicio et al., 2009; Gambus et al., 2006; Moyer et al., 2006; Pacek et al., 2006).

Cells frequently encounter barriers during the course of DNA replication. Cells respond to the resulting stalled replication forks mainly by employing the ATR-Chk1 checkpoint pathway (Kastan and Bartek, 2004; Melo and Toczyski, 2002). ATR is a sensor kinase that becomes activated in response to stalled replication forks and thereby transduces signals through the effector kinase Chk1. These signaling steps lead to the prevention of the inappropriate entry into mitosis and the inhibition of late replication origin firing (Kastan and Bartek, 2004; Melo and Toczyski, 2002). ATR directly phosphorylates Chk1 by a mechanism that depends on the checkpoint mediator protein Claspin (Chini and Chen, 2003; Kumagai and Dunphy, 2000; Kumagai et al., 2004). Besides its well-established function in mediating the activation of Chk1 in response to replication stress, Claspin also has roles in the regulation of unperturbed DNA replication and in the stabilization of disrupted replication forks (Lee et al., 2005; Lee et al., 2003; Lin et al., 2004). Claspin is a component of both normal and stalled replication forks. It interacts with a number of replication factors, including Cdc45, RFC, RPA, and DNA polymerase  $\epsilon$  (Pol  $\epsilon$ ), and is necessary for a normal rate of fork progression (Lee et al., 2005; Lee et al., 2003; Lin et al., 2004; Petermann et al., 2008; Sar et al., 2004). The association of Claspin with chromatin depends on Cdc45 and occurs at about the same time as Pol  $\epsilon$  (Lee et al., 2003). Likewise, the yeast

ortholog of Claspin, Mrc1, moves with the replication fork and is necessary for a normal rate of DNA replication (Hodgson et al., 2007; Katou et al., 2003; Lou et al., 2008; Osborn and Elledge, 2003; Szyjka et al., 2005; Tourriere et al., 2005).

Despite the inhibition of late origin firing under conditions of checkpoint-inducing replication stress, the Cdc7/Drf1 kinase remains active (Tenca et al., 2007; Tsuji et al., 2008; Yanow et al., 2003). Several lines of evidence indicate that DDK may play a role in the regulation of checkpoint responses in metazoan systems. Drf1 accumulates on replication-arrested chromatin in an ATR- and Claspin-dependent manner in *Xenopus* egg extracts (Yanow et al., 2003). Also, human Cdc7 has been shown to phosphorylate and interact with human Claspin in HeLa cells (Kim et al., 2008). Moreover, when Cdc7 is knocked down by siRNA, mammalian cells are more sensitive to agents that elicit replication stresses such as hydroxyurea (HU). These Cdc7-ablated cells are defective in the checkpoint-associated phosphorylation of Chk1 and Claspin (Kim et al., 2008). Finally, ectopic addition of excess Dbf4-Cdc7 to *Xenopus* egg extracts or overexpression of Dbf4 in HeLa cells is able to down-regulate the ATR-Chk1 pathway signaling elicited by agents that induce replication stress (Tsuji et al., 2008).

For these reasons, we sought to examine the potential role of DDK in response to replication stress by further examining the relationship between DDK and the replication-monitoring protein, Claspin. In this study, we established that DDK phosphorylates and interacts with Claspin in *Xenopus* egg extracts. Furthermore, we precisely mapped the site of this interaction to an evolutionarily conserved protein motif on Claspin. We found that two amino acids located

within the region of Claspin shown to be essential for binding and activating Chk1 were required for the interaction with DDK. Interestingly, however, we demonstrate that this interaction is not required for Claspin to mediate activation of Chk1. These findings suggest that interaction with DDK reflects a checkpoint-independent function of Claspin.

## 4.2 Experimental Procedures

**4.2.1 *Xenopus* egg extracts-** Interphase egg extracts were treated with 50 µg/ml annealed (dA)<sub>70</sub>-(dT)<sub>70</sub> (pA-pT) to activate checkpoint responses as described previously (Kumagai and Dunphy, 2000). To produce chromatin containing DNA replication blocks, extracts were incubated with demembranated sperm nuclei (3000–4000/µL) and 50 µg/ml aphidicolin.

**4.2.2 Antibodies-** Immunodepletion and immunoblotting of Claspin were performed with antibodies raised against residues 232–606 of Claspin (Yoo et al., 2006). The anti-Claspin immunoprecipitations (IPs) in this chapter were carried out with antibodies generated against residues 1–464 of Claspin (Kumagai and Dunphy, 2000). Anti-Drf1, anti-Cdc7, anti-Plx1, anti-RPA70, and anti-Chk1 antibodies were described previously (Kumagai and Dunphy, 1996; Kumagai et al., 1998; Lee et al., 2003; Yanow et al., 2003). Anti-GST (Santa Cruz Biotechnology), anti-FLAG M2 (Sigma), and antibodies that detect phosphorylated Ser344 of *Xenopus* Chk1 (Cell Signaling Technology) were purchased from commercial sources. Anti-Scc2 serum (Takahashi et al., 2008) was a generous gift of T. Takahashi (Osaka University).

**4.2.3 Recombinant Proteins-** Glutathione-S-transferase (GST)-fused Claspin fragments 847–903 and 847–903(2AG) were described previously (Kumagai and Dunphy, 2003). DNA sequences for additional GST-fused Claspin fragments (amino acids 1–258, 258–518, 518–775 776–851, 847–962, 961–1078, 1076–1285, 776–877, 776–867, 776–856, 847–877, 856–903, 868–903, 878–903, 902–961, 776–1078, and 878–961) were cloned into the pGEX-2T expression vector using PCR-based methods. Sequences encoding the minimal DDK binding site (residues 856–867) and point mutants thereof (Q866K, D861E, and Q866K/D861E) were cloned into pGEX-2T by producing oligonucleotide linkers that create 5' BamHI and 3' EcoRI cohesive ends upon annealing. These oligonucleotides were subsequently ligated into the linearized vector prepared with the appropriate restriction sites. Constructs for fragments of human Claspin (HuCKBD1, residues 908–919; HuCKBD2, residues 937–948; and HuCKBD3, residues 974–985) were prepared in a similar manner. The above GST-fused peptides were expressed in *E. coli* BL21 CodonPlus (RIL) cells and purified with glutathione-agarose beads. Site-directed mutagenesis using the QuikChange kit (Stratagene) was performed to create the D861E, Q866K, and D861E/Q866K mutants with the pFastBac-His6-Claspin-FLAG (full-length) and pGEX-2T(776–1078) as templates. Recombinant baculoviruses were created using the Bac-to-Bac method (Invitrogen). Wild-type Chk1-GST-His6 protein was expressed and purified in Sf9 insect cells as previously described (Kumagai and Dunphy, 2000). Full-length Claspin proteins were expressed in Sf9 insect cells and purified with

M2 anti-FLAG agarose beads (Sigma) and eluted with 3×FLAG peptide (Sigma) in HEPES-buffered saline (HBS).

*4.2.4 Immunoprecipitations, immunodepletions, and GST pulldowns-* Interphase immunoprecipitations were carried out according to previously defined methods (Yanow et al., 2003). Immunodepletions of Claspin from extracts as well as GST pulldowns from extracts were performed as in earlier work (Kumagai and Dunphy, 2000).

*4.2.5 Immunoprecipitation from nuclear lysates and isolation of soluble nuclear lysates-* Nuclear immunoprecipitations (IPs) were performed by incubating 100  $\mu$ L (per IP reaction) of extracts lacking or containing aphidicolin (4,000 nuclei/ $\mu$ L) for 65 minutes at room temperature and subsequently centrifuging them through 1 ml of ice-cold sucrose cushion (20 mM HEPES-KOH, pH 7.5, 1 M sucrose, 80 mM KCl, 2.5 mM K-gluconate, and 10 mM Mg-gluconate) at 6,100  $\times$  g for 5 minutes at 4°C. The supernatant was removed, leaving a loose pellet, to which another 500  $\mu$ L of sucrose cushion was added. The sample was centrifuged again as above. The supernatant was removed and 25  $\mu$ L of high-salt lysis buffer (10 mM HEPES-KOH, pH 7.5, 300 mM NaCl, 2.5 mM EGTA, 0.1% CHAPS, and 0.5% NP-40) was added to resuspend the pellets. The samples were incubated for 10 minutes at 4°C, diluted 4-fold with 75  $\mu$ L of 20 mM HEPES-KOH (pH 7.5) to reduce the salt concentration, and subsequently centrifuged at 10,000 rpm for 10 minutes at 4°C. For immunoprecipitations, the supernatant was incubated with agitation at 4°C for 1 hour with Affiprep Protein A beads (BioRad) that had been coated with the appropriate antibodies. The

beads were then washed 4 times with a buffer containing 10 mM HEPES-KOH (pH 7.5), 80 mM NaCl, 2.5 mM EGTA, 20 mM  $\beta$ -glycerolphosphate, and 0.1% NP-40 and finally boiled in SDS-PAGE sample buffer.

The nuclear soluble fraction was obtained by centrifuging 25  $\mu$ L of extracts (mock or Claspin-immunodepleted) twice through a 500  $\mu$ L sucrose cushion as described above and then resuspending the pellets in 30  $\mu$ L of chromatin isolation buffer (Lee et al., 2003) on ice for 10 minutes. The lysates were then centrifuged at 6,100  $\times g$  for 5 minutes at 4°C. The supernatant was collected and boiled in SDS-PAGE sample buffer.

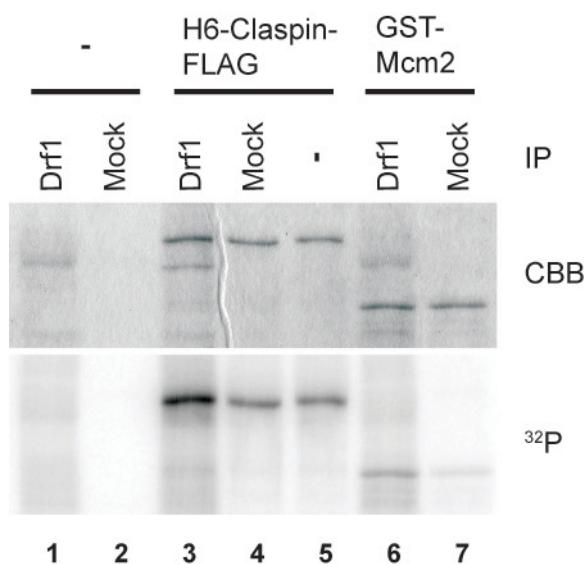
### 4.3 Results

**4.3.1 DDK forms a stable complex with Claspin in S-phase egg extracts.** An active form of the Cdc7 kinase can be isolated from interphase *Xenopus* egg extracts by immunoprecipitating the Drf1 protein (Yanow et al., 2003). In the course of testing substrates for this kinase activity, we observed that Cdc7 was able to phosphorylate recombinant full-length His6-Claspin-FLAG protein *in vitro* (figure 4.S.1, lane 3). This result led us to ask whether Drf1 and Claspin can interact with one another in egg extracts. To this end, we subjected interphase egg extracts to immunoprecipitation with either anti-Drf1 or anti-Claspin antibodies and then immunoblotted the immunoprecipitates for Claspin and Drf1, respectively. These extracts were also incubated in the absence or presence of the checkpoint-inducing DNA oligonucleotides (dA)<sub>70</sub>-(dT)<sub>70</sub>. We could easily detect Claspin in anti-Drf1 immunoprecipitates and vice versa. These

interactions were not affected by the presence of (dA)<sub>70</sub>-(dT)<sub>70</sub> (figure 4.1A).

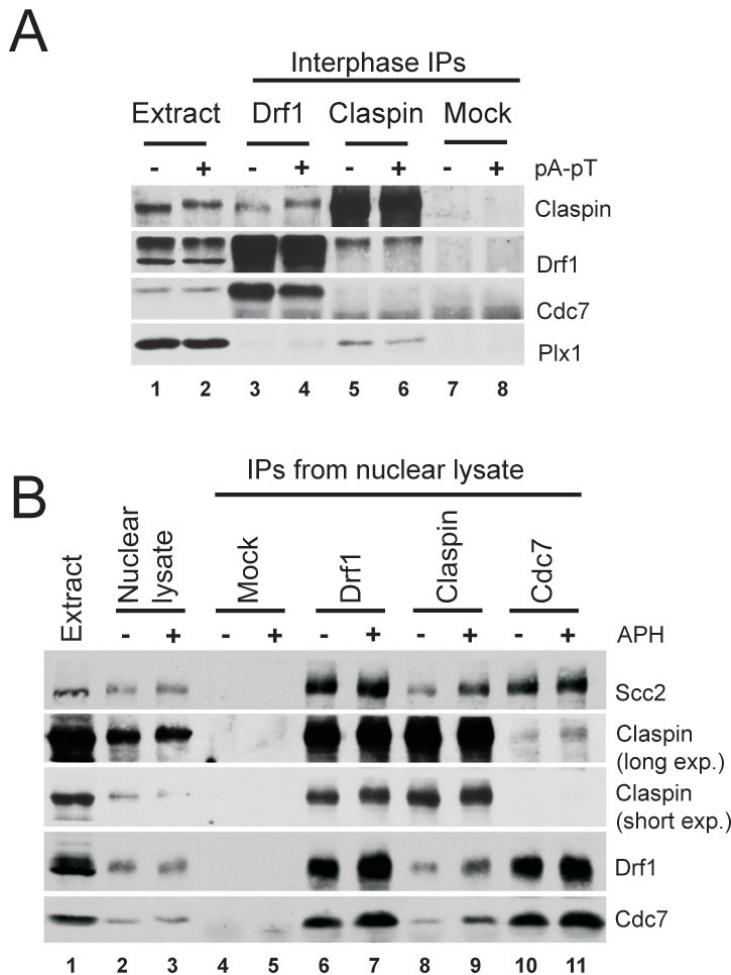
These results are consistent with studies on human Claspin (Kim et al., 2008).

To examine this finding in greater detail, we also prepared lysates of replicating nuclei from interphase egg extracts in which we had eluted proteins from chromatin by salt treatment. We immunoprecipitated these lysates with anti-Claspin, anti-Drf1, and anti-Cdc7 antibodies and then immunoblotted for all three proteins. In these experiments, we also found that Claspin, Cdc7, and Drf1 formed a specific complex (figure 4.1B). In addition, we observed that the formation of this complex was not affected by aphidicolin, a drug that causes the formation of stalled DNA replication forks in egg extracts. Recently, Cdc7 and Drf1 have been shown to form a stable complex with the cohesin loading protein Scc2 in *Xenopus* egg extracts. Moreover, this interaction is required for the loading of the cohesin complex onto chromatin during replication (Takahashi et al., 2008). In our experiments, we observed that Scc2 and Claspin also associate with each other in egg extracts in both the absence and presence of aphidicolin (figure 4.1B).



**Figure 4.S.1** DDK isolated from egg extracts can phosphorylate Claspin *in vitro*.

Interphase egg extracts were immunoprecipitated with either anti-Drf1 or mock control antibodies. The immunoprecipitates were added to *in vitro* kinase reactions containing bacterially expressed GST-Mcm2 (lanes 6 and 7), His6-Claspin-FLAG (lanes 3 and 4), or no recombinant protein (lanes 1 and 2) in the presence of [ $-^{32}\text{P}$ ]ATP. The reaction in lane 5 contains His6-Claspin-FLAG and [ $-^{32}\text{P}$ ]ATP but no immunoprecipitates samples. The reactions were subjected to SDS-PAGE, stained with Coomassie Brilliant Blue (CBB) (top panel), and analyzed by autoradiography (bottom panel). Kinase assays were carried out as described (Yanow et al., 2003).



**Figure 4.1** Claspin forms a nuclear interphase complex with Cdc7 and Drf1 (DDK) in *Xenopus* egg extracts.

(A) Mock, anti-Drf1 and anti-Claspin immunoprecipitates (IPs) were prepared from nuclei-free interphase egg extracts lacking or containing (dA)<sub>70</sub>-(dT)<sub>70</sub> (pA-pT) and immunoblotted for Claspin, Drf1, Cdc7, and Plx1. Lanes 1 and 2 depict aliquots of pre-IP extracts.

(B) Nuclear lysates were prepared by isolating nuclei from with and without aphidicolin (APH) treated interphase extracts supplemented with 4,000/ $\mu$ L demembranated sperm DNA. The nuclei were lysed and salt-extracted with 300 mM NaCl diluted back to physiological salt levels after centrifugation. The nuclear lysates were immunoprecipitated with control (mock), anti-Drf1, anti-Claspin, and anti-Cdc7 antibodies and immunoblotted for Scc2, Claspin, Drf1, and Cdc7. The Claspin immunoblots are presented as both long and short film exposures. Lane 1 is an aliquot of interphase egg extract, and lanes 2 and 3 are aliquots of pre-IP nuclear lysate lacking or containing aphidicolin.

**4.3.2 Mapping a DDK-binding site on Claspin.** In order to characterize further the interaction between DDK and Claspin, we sought to identify the minimal region of Claspin that is required for this binding. For this purpose, we first constructed a series of seven GST-fused Claspin fragments that spanned the entirety of the protein. Immunoprecipitations of Drf1 were carried out with interphase extracts that had been supplemented with these GST-Claspin protein fragments. We found that Drf1 strongly interacted with the Claspin fragment containing amino acids 847–962 (figure 4.2A, lane 11, and figure 4.S.2A). There was also a weak interaction of Drf1 with the fragment containing amino acids 258–518 (figure 4.2A, lane 5).

Subsequently, we focused on the 847–962 fragment both because this interaction was very strong and because this fragment contains the Chk1-binding domain (CKBD), a region required for the binding and activation of Chk1 (Kumagai and Dunphy, 2003). The CKBD of Claspin is composed of two repeat sequences, each containing serines that are phosphorylated during a replication checkpoint response. In order to map more finely the sequence that is required for Claspin to associate with Drf1, we prepared a series of overlapping GST-fused Claspin peptide fragments ranging from amino acids 776 to 903. We found that peptide fragments starting at position 776 and ending at or before amino acid 856 could not interact with Drf1 (figure 4.2B, D, and figure 4.S.2B). Conversely, Claspin fragments starting at before residue 857 could associate with Drf1, while there was no binding of the 868–903 and 878–903 fragments (figure 4.2B, D, and figure 4.S.3). Thus, the region comprising amino acids 856

through 867 of Claspin is required for this interaction (figure 4.2D). Interestingly, this region corresponds to the first repeat of the CKBD (856–867). Notably, fragments containing the second CKBD repeat (887–898) failed to bind to Drf1. In order to ascertain whether this minimal region was sufficient to for the interaction with DDK, we constructed a GST fusion protein containing only residues 856–867 from Claspin. When we tested this fragment in the binding assay, we could readily observe binding to Drf1 (figure 4.2C, lane 7). Thus, residues 856–867 of Claspin are both necessary and sufficient for recognition of Drf1.

It is striking that only the first CKBD repeat and not the second one could interact with Drf1, given that only two amino acids (namely, aspartate 861 and glutamine 866) are different between the repeats (figure 4.3C). Some Claspin homologs, including human Claspin, have three repeats instead of the two found in *Xenopus laevis* (Chini and Chen, 2006; Clarke and Clarke, 2005). When we compared CKBD repeat sequences across a selection of metazoan Claspin homologs, we noticed that these two positions displayed the most variability (figure 4.3C). We initially focused on the position containing glutamine 866, which changes from a glutamine to a lysine in the second *Xenopus* repeat sequence. This variation represents a difference in charge. By contrast, the position corresponding aspartate 861 contains a similar acid residue (glutamate) in the second CKBD repeat.

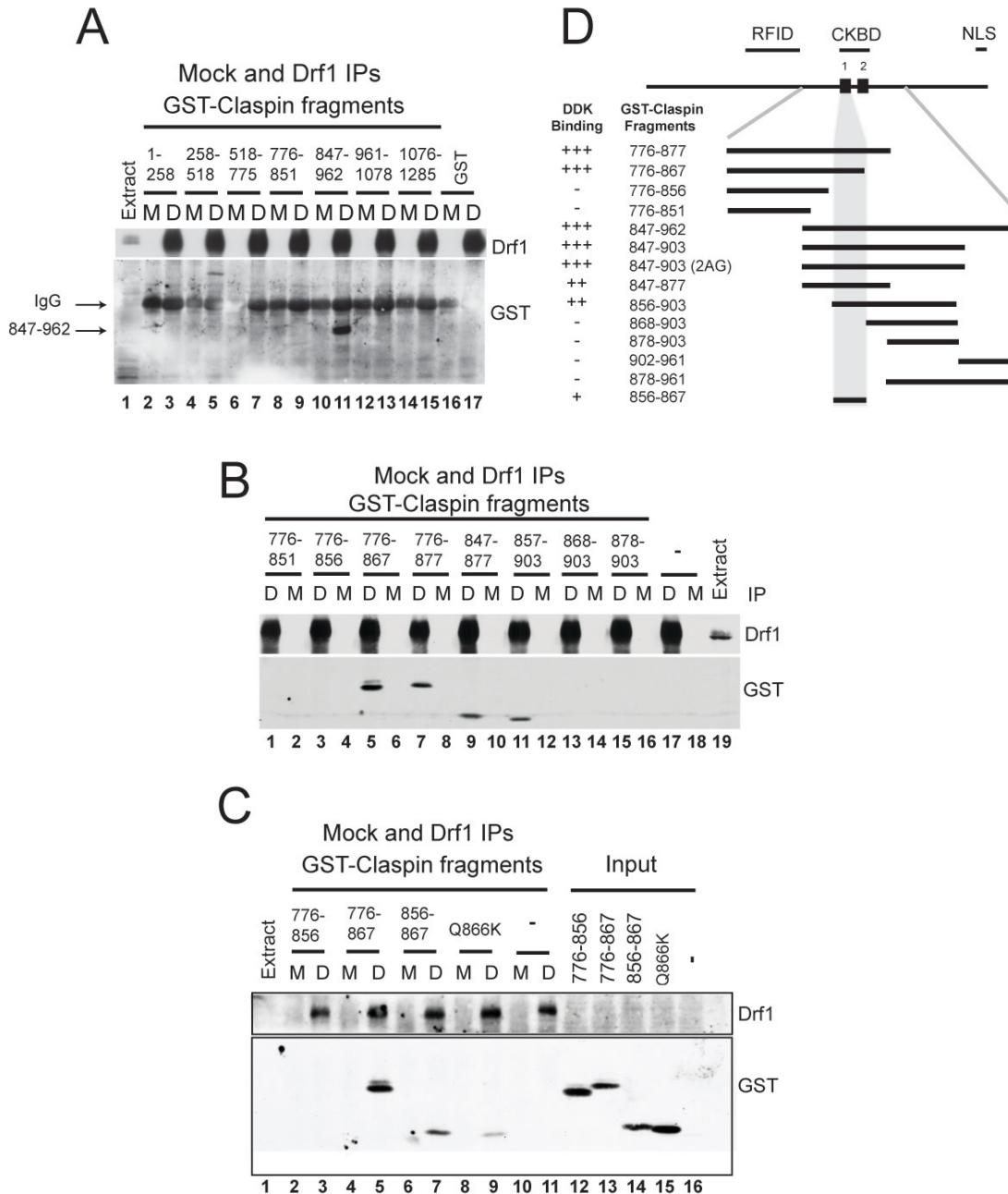


Figure 4.2

**Figure 4.2** The region of Claspin required for interaction with DDK

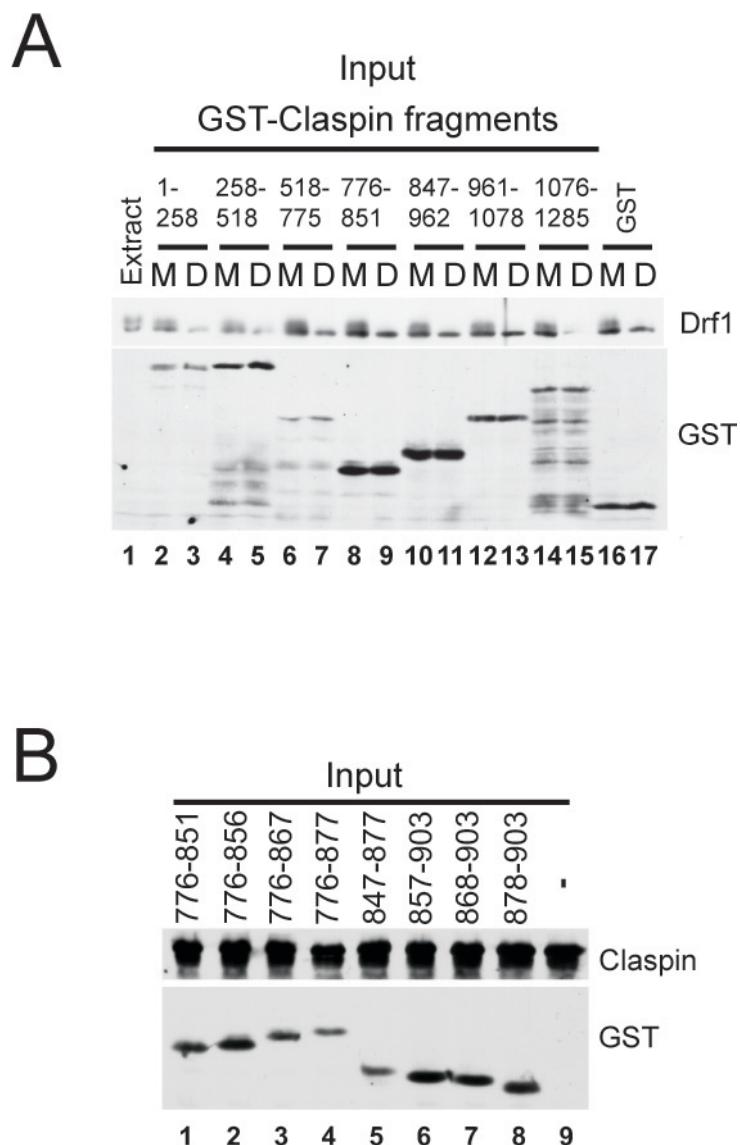
maps to the first repeat sequence of the CKBD.

(A) Interphase egg extracts containing the indicated GST-Claspin fragments or GST alone were immunoprecipitated with anti-Drf1 (D) or mock (M) control antibodies and immunoblotted for Drf1 and GST. The arrow denotes a cross-reacting IgG band. Lane 1 depicts an aliquot of pre-IP extract. Input levels for the recombinant peptides are shown in figure 4.S.2A.

(B) Interphase nuclei-free egg extracts containing the indicated GST-Claspin fragments or no recombinant protein were immunoprecipitated with anti-Drf1 (D) or mock (M) control antibodies and immunoblotted for Drf1 and GST. Lane 19 depicts an aliquot of pre-IP extract. Input levels for the recombinant peptides are shown in figure 4.S.2B.

(C) Interphase nuclei-free egg extracts containing GST-Claspin(776–856), GST-Claspin(776–867), GST-Claspin(856–867), GST-Claspin(856–867)-Q866K or no recombinant protein were immunoprecipitated with anti-Drf1 (D) or mock (M) control antibodies and immunoblotted for Drf1 and GST (lanes 2–11). Lane 1 is depicts an aliquot of the initial extract of cytostatic factor. Lanes 12–16 represent aliquots of pre-IP extracts containing the recombinant peptides listed above.

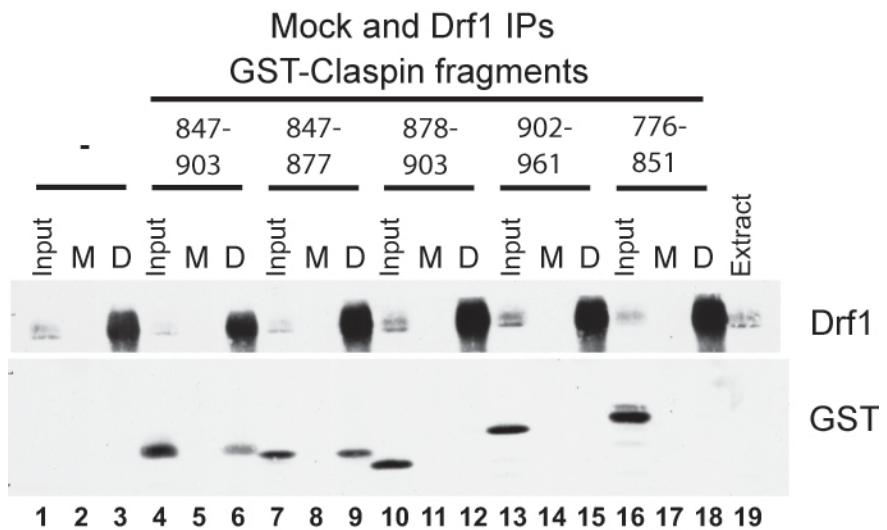
(D) (Top) A schematic depiction of several known domains of Claspin. The domains are the replication-fork interacting domain (RFID), the first and second repeats of the Chk1-binding domain (CKBD), and the nuclear localization sequence (NLS). (Bottom) A summary of the abilities of Claspin peptide fragments' ability to bind DDK.



**Figure 4.S.2** Loading controls for figure 4.2A and B.

(A) Aliquots of extracts containing recombinant proteins from figure 2A (lanes 2–17) were taken prior to immunoprecipitation. Lane 1 is an aliquot of extract with no recombinant proteins. Samples were immunoblotted with Drf1 and GST.

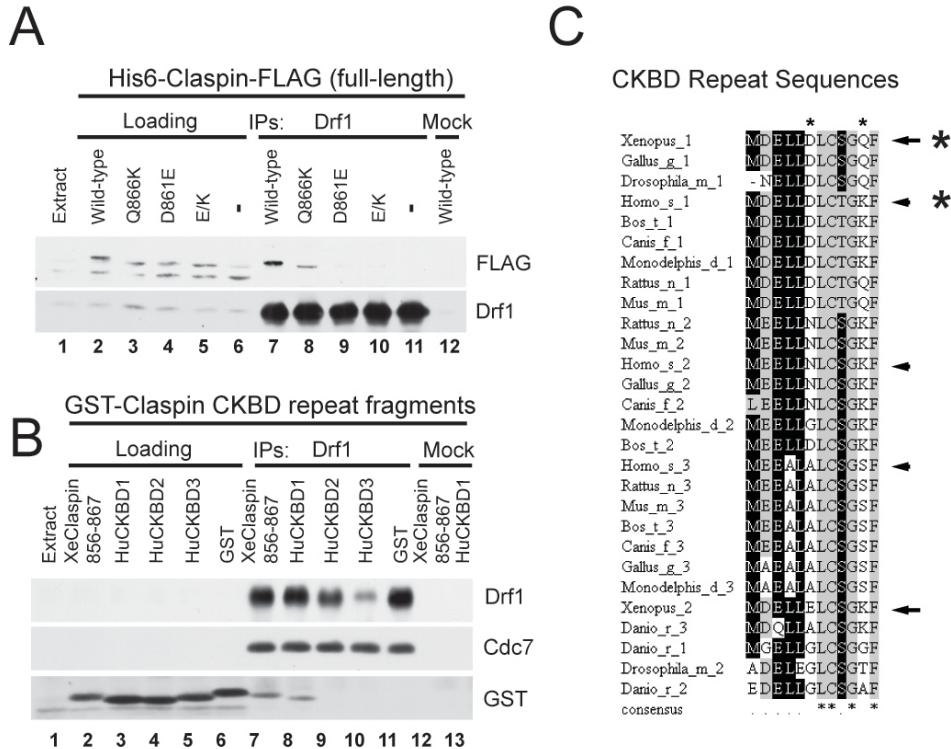
(B) Aliquots of extracts with the recombinant proteins from figure 4.2B (lanes 1–8) were taken prior to immunoprecipitation. Lane 9 is an aliquot of extract with no recombinant proteins. Samples were immunoblotted for Claspin and GST.



**Figure 4.S.3** Additional structure-function studies of the DDK-Claspin interaction.

Interphase, nuclei-free egg extracts containing GST-Claspin(847–903), GST-Claspin(847–877), GST-Claspin(878–903), GST-Claspin(902–961), GST-Claspin(776–851), or no recombinant protein (lanes 2 and 3) were immunoprecipitated with anti-Drf1 (D) or mock (M) control antibodies and immunoblotted for Drf1 and GST. Lanes 1, 4, 7, 10, 13, and 16 are aliquots (taken before immunoprecipitation) of extracts containing recombinant proteins, and lane 19 is an aliquot of the initial extract.

We constructed a version of the GST-fused 856–867 Claspin in which glutamine 866 was changed to lysine (hereafter referred to as the Q866K mutant). Upon testing this mutated fragment, we observed that binding to Drf1 was significantly reduced but not completely abolished relative to the original fragment (figure 4.2C, lane 9, and figure 4.S.4A). Next, we proceeded to change aspartate 861 to glutamate (to generate the D861E mutant). We also prepared the D861E /Q866K double mutant (referred to as E/K). In the context of the 856–867 fragment, the D861E mutant displayed a more marked reduction of binding to Drf1 in comparison with Q866K mutant. Moreover, the double E/K mutation essentially abolished the interaction with Drf1 (figure 4.S.4A). We observed similar results in a larger Claspin GST-fused fragment (776–1078) that contains the entire CKBD and is by itself sufficient to rescue Chk1 activation in a Claspin-depleted extracts (figure 4.S.4B) (Lee et al., 2005). Finally, we decided to test the capacity of these mutants to interact with DDK in the context of the full-length Claspin protein. We expressed and purified FLAG-tagged recombinant versions of wild-type, D861E, Q866K, and E/K full-length Claspin proteins from insect cells using a baculovirus protein expression system. The full-length proteins followed the same pattern as the smaller Claspin fragments in that binding of the Q866K mutant to Drf1 was reduced, while binding of the D861E single mutant and the double E/K binding was nearly eliminated (figure 4.3A).



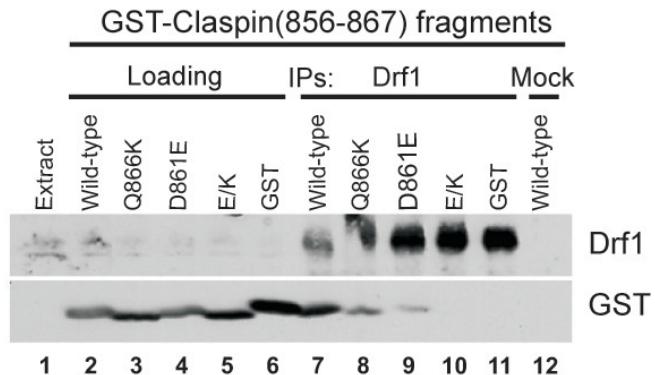
**Figure 4.3** Identification of conserved sites in Claspin required for interaction with DDK.

(A) Interphase, nuclei-free egg extracts containing full-length, wild-type His6-Claspin-FLAG, His6-Claspin-FLAG-Q866K, His6-Claspin-FLAG-D861E, His6-Claspin-FLAG-E/K, or no recombinant protein were immunoprecipitated with anti-Drf1 antibodies (lanes 7–11). Extracts containing wild-type His6-Claspin-FLAG were mock immunoprecipitated with control antibodies (lane 12). Lanes 2–6 depict pre-IP aliquots of extracts supplemented with the recombinant proteins listed above. Lane 1 shows an aliquot of interphase extract alone. The immunoprecipitates were immunoblotted for the FLAG epitope and Drf1.

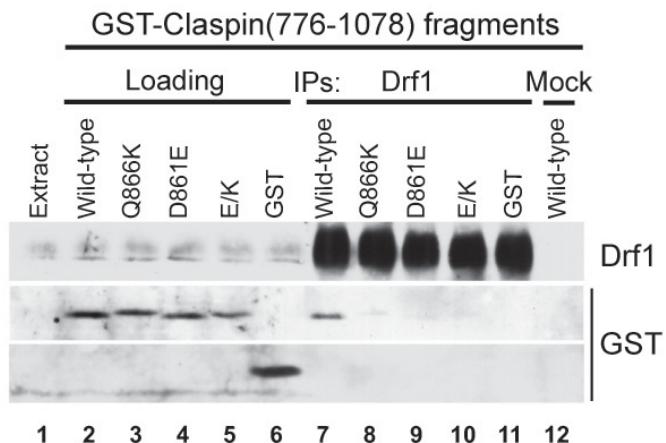
(B) Interphase, nuclei-free egg extracts containing GST-XeClaspin(856–867), GST-HuClaspin-CKBD1, GST-HuClaspin-CKBD2, GST-HuClaspin-CKBD3, and GST were immunoprecipitated with anti-Drf1 antibodies (lanes 7–11). Extracts containing GST-Claspin(856–867) and GST-HuCKBD1 were mock immunoprecipitated with control antibodies (lanes 12 and 13). Lane 1 contains an aliquot of pre-IP interphase extract, and lanes 2–6 contain pre-IP aliquots of extracts supplemented with the recombinant proteins listed above. The immunoprecipitates were immunoblotted for Drf1, Cdc7, and GST.

(C) A sequence alignment of the Chk1-binding domain (CKBD) repeat sequences across a selection of metazoan Claspin homologs. The organisms represented here are *Xenopus laevis* (South African claw-toed frog), *Gallus gallus* (domestic chicken), *Drosophila melanogaster* (fruit fly), *Homo sapiens* (human), *Bos taurus* (domestic cow), *Canis familiaris* (domestic dog), *Monodelphis domestica* (gray short-tailed opossum), *Rattus norvegicus* (Norway rat), *Mus musculus* (house mouse), and *Danio rerio* (zebrafish). The dark arrows point to the *X. laevis* Claspin CKBD repeats and the light arrows point to the *H. sapiens* CKBD repeats. Asterisks (\*) indicate the peptides that were immunoprecipitated by anti-Drf1 antibodies.

A



B

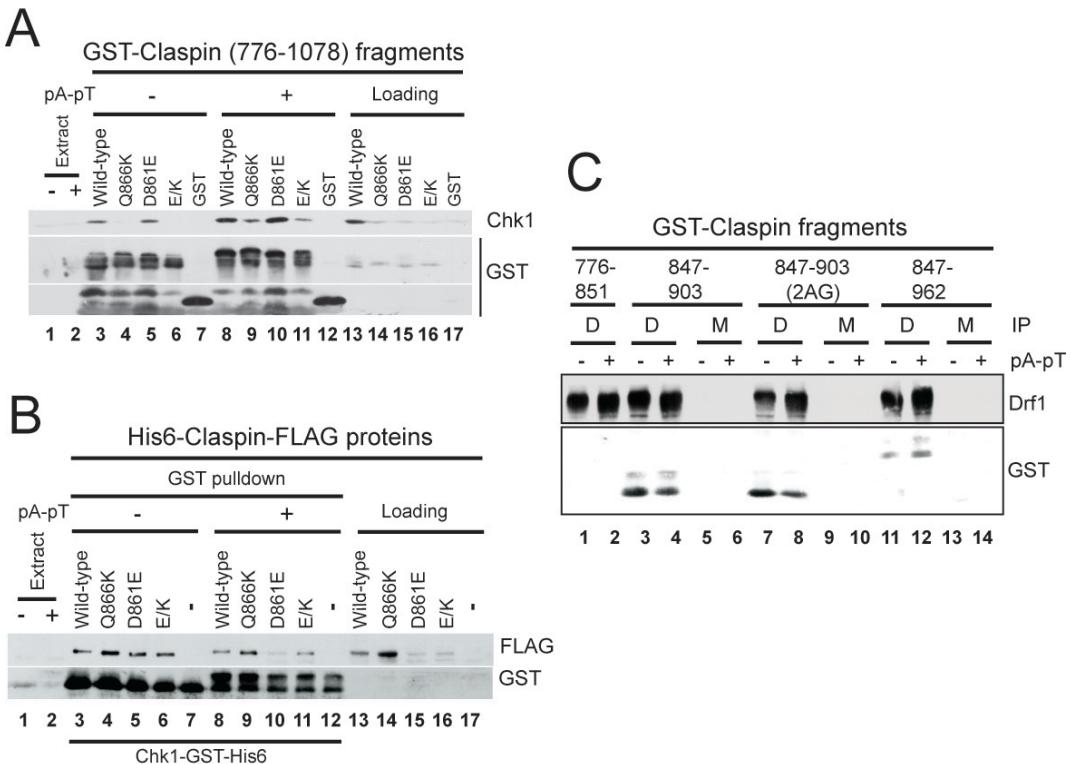


**Figure 4.S.4** Characterization of point mutations in the DDK-interacting region of Claspin.

(A) Interphase, nuclei-free egg extracts containing wild-type GST-Claspin(856–867), GST-Claspin(856–867)-Q866K, GST-Claspin(856–867)-D861E, GST-Claspin(856–867)-E/K, and GST were immunoprecipitated with anti-Drf1 antibodies (lanes 7–11). Extract containing wild-type GST-Claspin(856–867)-wild type was also mock immunoprecipitated with control antibodies (lane 12). Lane 1 contains an aliquot of pre-IP interphase extract prior to immunoprecipitation, and lanes 2–6 contain pre-IP aliquots of extracts supplemented with the recombinant proteins listed above. The immunoprecipitates were immunoblotted for Drf1 and GST.

(B) Interphase nuclei-free egg extracts containing wild-type GST-Claspin(776–1078), GST-Claspin(776–1078)-Q866K, GST-Claspin(776–1078)-D861E, GST-Claspin(776–1078)-E/K, and GST were immunoprecipitated with anti-Drf1 antibody (lanes 7–11). Extract containing GST-Claspin(776–1078)-wild type was mock immunoprecipitated with control antibodies (lane 12). Lane 1 contains an aliquot of pre-IP interphase extract, and lanes 2–6 contain pre-IP aliquots of extracts supplemented with the recombinant proteins listed above. The immunoprecipitates were immunoblotted for Drf1 and GST.

Since the Claspin CKBD sequences are well conserved across metazoans, we decided to test whether these amino acid sites were important in the human ortholog of Claspin. At the amino acid position equivalent to aspartate 861 in *Xenopus* Claspin, the human Claspin CKBD repeats contain the residues aspartate, asparagine, and alanine in the first, second, and third repeat, respectively. Accordingly, we expressed and purified the three human Claspin CKBD repeats as GST-fused peptides, added them to *Xenopus* egg extracts, and assayed binding to Drf1 by immunoprecipitating with anti-Drf1 antibodies as above. We observed that the first human Claspin CKBD repeat (HuCKBD1) bound efficiently to Drf1, whereas the second and third repeats (HuCKBD2 and HuCKBD3) did not bind at all (figure 4.3B, lanes 8–10). These findings suggest that the DDK-binding domain of Claspin may be conserved across metazoan species.

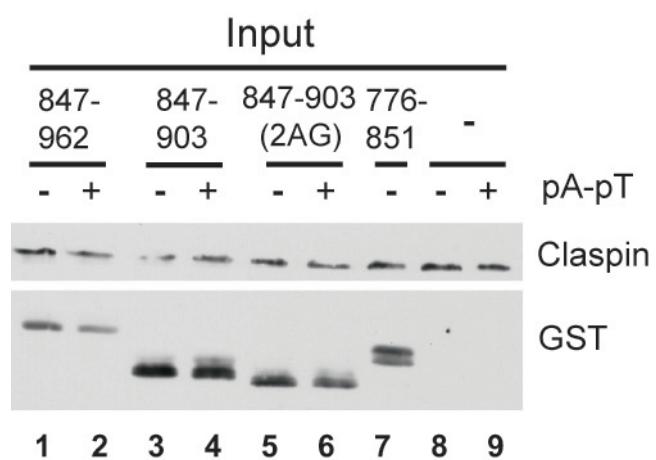


**Figure 4.4** Interactions of Claspin with DDK and Chk1 have distinct requirements.

(A) Interphase extracts, lacking or containing  $(dA)_{70}-(dT)_{70}$  (pA-pT), were supplemented with wild-type GST-Claspin(776–1078), GST-Claspin(776–1078)-Q866K, GST-Claspin(776–1078)-D861E, GST-Claspin(776–1078)-E/K, and GST. Extracts were subjected to pulldowns with glutathione agarose beads (lanes 3–12). Lanes 13–17 are aliquots of pre-pulldown extracts supplemented with the above recombinant peptides. Lanes 1 and 2 depict extracts without and with oligonucleotides, but with no added recombinant proteins. The pulldown fractions were immunoblotted for Claspin, Chk1, and GST.

(B) Interphase extracts, lacking or containing  $(dA)_{70}-(dT)_{70}$  (pA-pT), were supplemented with recombinant Chk1-GST-His6. Extracts were also supplemented with the indicated versions of recombinant His6-Claspin-FLAG or no recombinant Claspin. Extracts were subjected to pulldowns with glutathione agarose beads (lanes 3–12). Lanes 13–17 depict aliquots of extracts prior to the pulldowns. Lanes 1 and 2 depict extracts without and with oligonucleotides, but with no added recombinant proteins. The samples were immunoblotted for the FLAG epitope and GST.

(C) Interphase extracts containing GST-Claspin(776–851), wild-type GST-Claspin(847–903), GST-Claspin(847–903)-2AG, and GST-Claspin(847–962) were incubated in the absence or presence of checkpoint-inducing oligonucleotides and immunoprecipitated with anti-Drf1 antibodies (D) or mock (M) control antibodies. The immunoprecipitates were immunoblotted for Drf1 and GST.



**Figure 4.S.5** Loading control for figure 4.4C.

Aliquots of extracts with the recombinant proteins from figure 4.4C (lanes 1–7) prior to immunoprecipitation. Lanes 8 and 9 are aliquots of extract with no recombinant proteins. Samples were immunoblotted for Claspin and GST.

*4.3.3 The interaction of Claspin with DDK is separable from its role in mediating the activation of Chk1.* Since we have determined that the region of Claspin that interacts with DDK also encompasses a key portion of its binding domain for Chk1, we decided to test whether the interaction with DDK affects the role of Claspin in binding to and activating Chk1. We first tested whether or not Chk1 could bind to mutants that were defective for the interaction with DDK. To this end, we performed GST pulldowns with our array of DDK-interaction mutations in the context of the GST-fused 776–1078 Claspin peptide fragment mentioned above. This peptide fragment of Claspin contains both the CKBD and sequences capable of rescuing Chk1 activation in Claspin-depleted extracts (Lee et al., 2005).

When we used glutathione beads to isolate the wild type, D861E, Q866K, and E/K mutant Claspin (776–1078) fragments from nuclei-free interphase extracts, we found that all of these proteins could bind Chk1 in extracts that had been treated those extracts that were treated with the checkpoint-inducing (dA)<sub>70</sub>-(dT)<sub>70</sub> oligonucleotides (figure 4.4A). Notably, the mutant Claspin fragments displayed an equivalent reduction in electrophoretic mobility as the wild-type fragment in extracts treated with (dA)<sub>70</sub>-(dT)<sub>70</sub> (figure 4.4A). This electrophoretic shift is characteristic of Claspin peptides containing the CKBD and is mediated by phosphorylation in response to checkpoint activation (Kumagai and Dunphy, 2003; Lee et al., 2005).

Next, we tested the Claspin-Chk1 interaction in a reciprocal manner by incubating glutathione beads in extracts supplemented with both a GST-tagged

version of Chk1 (Chk1-GST-His6) and the different versions of the full-length His6-Claspin-FLAG protein. By this methodology, we also observed that D861E, Q866K, and E/K mutants could associate normally with Chk1 (figure 4.4B). Taken together, these results indicate that elimination of the ability of Claspin to associate with DDK has virtually no effect on binding to Chk1.

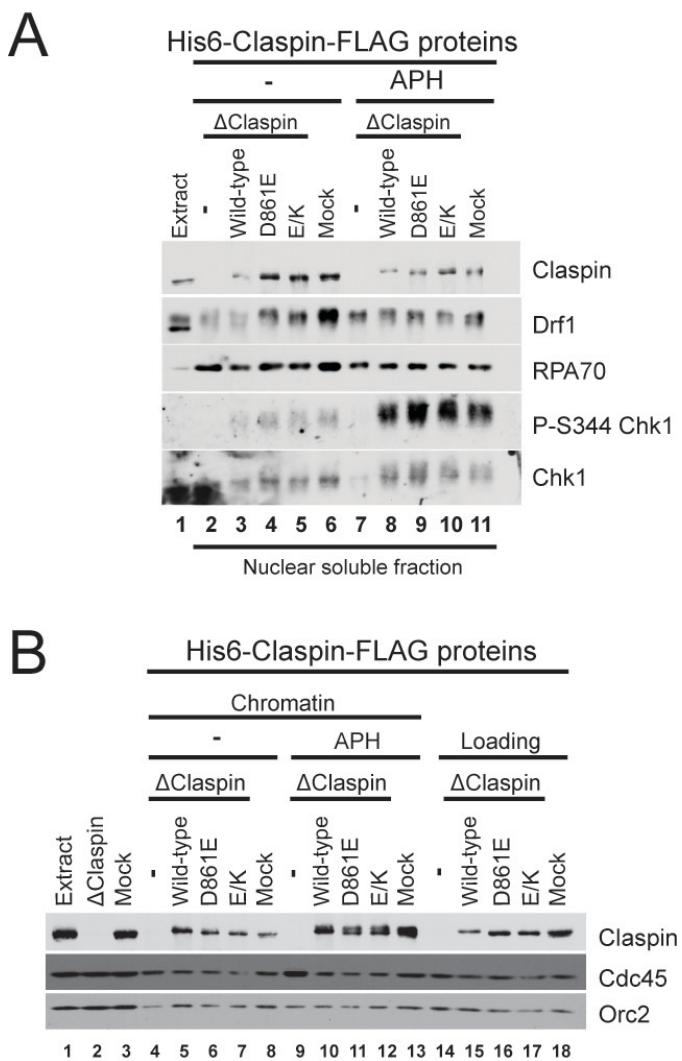
Subsequently, we endeavored to test whether Claspin fragments that are unable to bind to Chk1 were still competent to interact with DDK. Chk1 interacts with Claspin through two phosphoserines (S864 and S895) that become phosphorylated in response to the activation of the DNA replication checkpoint (Kumagai and Dunphy, 2003). We performed anti-Drf1 immunoprecipitations with interphase extracts supplemented with CKBD-containing GST-fused Claspin fragments (847–903) that were either wild type or had the ser864 and ser895 mutated to alanines (2AG). The wild-type and 2AG fragments were found at equivalent levels in the Drf1 immunoprecipitates, regardless of whether the extracts contained checkpoint inducing (dA)<sub>70</sub>-(dT)<sub>70</sub> oligonucleotides (figure 4.4C and figure 4.S.5). Thus, the binding of Chk1 to Claspin is distinct from the interaction of Claspin with DDK.

Next, we tested whether the DDK-binding compromised Claspin mutants were competent to activate Chk1 in response to replication arrest. To address this question, we immunodepleted Claspin from extracts and then added back wild-type or mutant full-length His6-Claspin-FLAG proteins to approximately endogenous levels. The extracts were incubated with demembranated sperm chromatin in the absence or presence of aphidicolin. The activation of Chk1 was

monitored with anti-phosphopeptide antibodies that detect phosphorylation of Chk1 on Ser344, a well established marker for activation of this kinase. As expected, activation of Chk1 activation in response to aphidicolin was ablated in the absence of Claspin, and recombinant wild-type Claspin could reverse this defect (figure 4.5A, lane 7). Significantly, the both the D861E and the E/K mutant Claspin proteins were also fully able to rescue the activation of Chk1 (figure 4.5A). These results are fully consistent with the fact that these mutants can still bind Chk1 normally.

Claspin loads onto chromatin during S-phase in a manner that depends on both the pre-RC and Cdc45 (Lee et al., 2003). The most important known kinase target of DDK is the MCM2-7 complex, and this phosphorylation leads to the recruitment of Cdc45 (Masai et al., 2006; Sheu and Stillman, 2006; Tsuji et al., 2006). Accordingly, we examined the ability of the DDK-interaction mutants of Claspin to associate with chromatin that is undergoing replication in egg extracts. For this purpose, we isolated chromatin from extracts in which we had immunodepleted the endogenous Claspin and replaced it with wild-type recombinant Claspin or various DDK-interaction mutants. For these experiments, we also incubated the various extracts in the absence or presence of aphidicolin. We observed that, in the absence of aphidicolin, both the D861E and E/K mutants, bound to chromatin with an efficiency similar to that of wild-type Claspin (figure 4.5B). In the presence of aphidicolin, Claspin binds in elevated amounts to chromatin and undergoes an electrophoretic shift due to checkpoint-dependent phosphorylation. We found that both the D861E and E/K mutants,

like wild-type Claspin, associated in higher amounts with aphidicolin-treated chromatin and displayed a checkpoint-dependent shift in electrophoretic mobility (figure 4.5B). Finally, our laboratory observed previously that Cdc45 accumulates in higher amounts on chromatin in the absence of Claspin (Lee et al., 2003; Yanow et al., 2003). Addition of recombinant wild-type Claspin reduced the binding of Cdc45 to normal levels (figure 4.5.B). Likewise, both the D861E and E/K mutant Claspin proteins could elicit the normal binding pattern of Cdc45. Taken together, these results indicate that Claspin can associate normally with chromatin under a variety of different conditions even if it has lost the ability to interact with DDK.



**Figure 4.5** DDK-interaction mutants of Claspin can still mediate activation of Chk1

and associate with replicating chromatin.

(A) Nuclear soluble fractions were obtained from interphase egg extracts that were either immunodepleted with anti-Claspin antibodies (lanes 2–5 and 7–10) or mock control antibodies (lanes 6 and 11). The extracts were supplemented with full-length versions of wild-type His6-Claspin-FLAG (lanes 3 and 8), His6-Claspin-FLAG-D861E (lanes 4 and 9), His6-Claspin-FLAG-E/K (lanes 5 and 10), or no recombinant protein (lanes 2 and 7). Lane 1 contains an aliquot extract with no recombinant protein. The fractions were immunoblotted for Claspin, Drf1, RPA70, phosphoserine-344 of Chk1, and Chk1.

(B) Chromatin was isolated from interphase extracts supplemented with 3,000 sperm nuclei/ $\mu$ l and incubated in the absence (lanes 4–8) or presence of aphidicolin (lanes 9–13). Extracts were immunodepleted with either anti-Claspin (lanes 4–7 and 9–12) or mock control antibodies (lanes 8 and 13). Extracts were also supplemented with the indicated versions of the His6-Claspin-FLAG protein (lanes 5–7 and 10–12) or buffer alone (lanes 4, 8, 9, and 13). Lanes 14–18 depict aliquots of extract taken before isolation of chromatin. Aliquots of extract were taken before (lane 1) and after immunodepletion (lanes 2 and 3). Samples were immunoblotted for Claspin, Cdc45, and Orc2.

#### 4.4 Discussion

The role of Claspin as a mediator in the activation of Chk1 in response to replication stress has been well studied. On the other hand, the role of Claspin as a regulator of replication is still being elucidated. In this study, we have confirmed that Claspin associates specifically with the replication initiating kinase DDK in *Xenopus* egg extracts. A similar finding has been made in human cells (Kim et al., 2008). Moreover, this complex includes Claspin, Cdc7, Drf1, and the cohesin-loading protein Scc2. The discovery that Claspin can be co-immunoprecipitated with Scc2 coincides with the finding that the Drf1-Cdc7 complex interacts with Scc2 and is necessary for the loading of Scc2 onto chromatin during S-phase (Takahashi et al., 2008). Even though we did not see a defect in the loading of Scc2 onto chromatin in Claspin-depleted extracts with no perturbation of replication (data not shown), it remains possible that Claspin may play a role in regulating the increased loading of the cohesin complex onto chromatin in the presence of stalled replication forks (Strom et al., 2007).

We found that the most minimal sequence of Claspin required for binding to DDK was the first repeat of the CKBD. By comparing CKBD repeat sequences both within *Xenopus* Claspin and between other metazoan Claspin homologs, we identified two amino acid positions of maximum variability within the repeats. By changing aspartate 861 to glutamate and glutamine 866 to lysine (thereby converting the first *Xenopus* CKBD repeat into the second), we were able to abolish almost completely the ability of Claspin to interact with Drf1. We

examined the evolutionary conservation of this binding by testing the ability of GST-fused human CKBD minimal repeat peptides to interact with *Xenopus* Drf1. There are three human CKBD repeats, but only the first two are required for activation of Chk1 (Chini and Chen, 2006; Clarke and Clarke, 2005). The first human CKBD repeat, which has an aspartate at the position equivalent to aspartate 861 of *Xenopus* Claspin, was the only fragment that could interact with *Xenopus* Drf1 in egg extracts. While this is an admittedly heterologous interaction, there is a high degree of sequence identity between CKBD repeats across metazoan species. The first repeat seems to contain aspartic acid at this position in nearly every case (except zebrafish), whereas the other repeats have a higher variability of residues and none contain aspartic acid. Therefore, it seems reasonable to speculate that there is a high degree of conservation across metazoan Claspin molecules in order for this site to interact with DDK.

The mechanism by which Claspin activates Chk1 has been well described in *Xenopus* egg extracts. In response to replication stress, serines 864 and 895 are phosphorylated in an ATR-dependent manner by an unidentified kinase. These phosphoserines associate with the kinase domain of Chk1 during a process that ultimately leads to the activation of Chk1 (Jeong et al., 2003; Kumagai and Dunphy, 2003; Kumagai et al., 2004). The proximity of the aspartate 861 and glutamine 866 to serine 864 was striking and led us to test whether the D861E or E/K mutants would affect the ability of Claspin to activate Chk1. Subsequently, we found that these mutants were fully competent to associate with Chk1 and mediate its activation of Chk1 in response to replication stress. Additionally, a

2AG mutant version of Claspin fragment that is incapable of binding to Chk1 (owing to mutations in serines 864 and 895) was still able to interact with Drf1, suggesting that the role of DDK interaction with Claspin is distinct from that of activating Chk1. It has been reported that the ablation of Cdc7 by siRNA leads to reduced activation of the ATR-Chk1 pathway in mammalian cells (Kim et al., 2008). However, this effect may be the result of a reduced number of replication forks the absence of Cdc7 rather than a direct modulation of Claspin. Other work has shown that overexpression or ectopic addition of excess Dbf4-Cdc7 can lead to an attenuation of Chk1 signaling upon the addition of etoposide in mammalian cells and *Xenopus* egg extracts (Tsuji et al., 2008). While we observed no defect in the ability of the DDK-interaction mutants to activate Chk1 in response to aphidicolin, it remains possible that there could be a defect in recovery from damage as a result of the loss of interaction with DDK. Additionally, there could be differences between the Dbf4-Cdc7 and Drf1-Cdc7 complexes or in responses to different types of damage.

Our preliminary experiments suggest that addition of the D861E and E/K mutants of Claspin to Claspin-depleted extracts may result in a modestly slower rate of DNA replication in comparison with Claspin-depleted extracts (Lee et al., 2003). This preliminary work also hints that there is a modest delay in mitosis in the presence of these mutants, which would be consistent with delayed replication. However, these effects were relatively subtle. Nonetheless, DDK is an important regulator of replication. Although Claspin and its yeast homolog Mrc1 are not absolutely required for replication, these proteins are involved in the control of

replication-related processes. Hence, it seems likely the Claspin-DDK interaction may be important for some aspect of DNA replication in metazoans.

## Chapter 5

# Concluding Remarks

### **5.1 Checkpoint-regulated Drf1 chromatin binding dynamics**

DDK (Drf1/Dbf4-dependent kinase) is one of two evolutionarily conserved kinases required to facilitate the conversion of inactive replication origins into initiated replication forks in S-phase (Arias and Walter, 2007; Bell and Dutta, 2002; Takeda and Dutta, 2005). Drf1 and Dbf4 are cell-cycle-regulated proteins that are required for the activation of the kinase Cdc7. Dbf4 and Drf1 exist in distinct complexes with Cdc7. DDK phosphorylates the MCM2-7 complex, recruiting the replication fork unwinding complex of Cdc45 and GINS to the MCM2-7 complex, leading to the activation of the MCM2-7 helicase, fork unwinding and recruitment of replication fork elongation factors (e.g., DNA Polymerases) and replication monitoring proteins (e.g., Claspin).

#### *5.1.1 Summary of results*

The *Xenopus* homologue of the Cdc7 adaptor protein, Drf1, was cloned. Drf1 forms an active, stable complex that is recruited to replicating chromatin in *Xenopus* egg extracts. Both Drf1 and Cdc7 remain stably bound to chromatin in the presence of the replication inhibitor etoposide. The DDK kinase activity is unaffected by the DNA polymerase inhibitor aphidicolin, which activates the replication checkpoint. Immunodepletions of either Cdc7 or Drf1 from the extract

demonstrate that each protein is required for the other to associate with chromatin. Drf1 hyper-accumulates on chromatin treated with aphidicolin and this hyper-accumulation is dependent on the replication checkpoint proteins Claspin and ATR and it is sensitive to the ATR inhibitor caffeine. However, the effect is independent of the downstream replication checkpoint effector kinase Chk1. In contrast to the effect on Drf1, treatment of the extract with caffeine and aphidicolin leads to chromatin hyper-accumulation of Cdc45. Depletion of Claspin or ATR in the presence of caffeine also leads to Cdc45 hyper-accumulation on chromatin. A small amount DNA replication was found to occur after treatment of aphidicolin and caffeine in *Xenopus* tissue culture (XTC) cells. Likewise, nascent DNA replication was also detected under the same conditions in the egg extract. In keeping with this, DDK was found to be active in the presence of aphidicolin and caffeine in the extract.

### *5.1.2 Significance of results*

These results are of relevance to both the fields of DNA replication initiation and replication checkpoint control. Providing evidence that Drf1-Cdc7 is an active kinase that remains stably associated to both each other and chromatin in the presence of arrested replication has been an important contribution to a controversial subject. Additionally, describing the dependency of Drf1 hyper-accumulation on ATR and Claspin and not Chk1 demonstrates regulation by the replication checkpoint but it is not a downstream effect of the checkpoint. Similarly, the presence of some limited replication initiation in the presence of aphidicolin when the replication checkpoint has been down-regulated by caffeine

suggests an underlying activity of some replication machinery, such as DDK, despite the blockage to replication.

## 5.2 Investigating the role of Claspin at the replication fork

Claspin is a protein required to mediate activation of the replication checkpoint effector kinase, Chk1, in response to arrested replication (Kumagai and Dunphy, 2000). Previous studies of Claspin in *Xenopus* egg extracts have described the mechanism by which Claspin activates Chk1. Claspin binds to Chk1 through a conserved motif comprised of repeat sequences where a key serine in each repeat is phosphorylated in response to arrested replication by an unknown kinase in an ATR-dependent manner (Kumagai and Dunphy, 2003). These phosphoserines bind to the catalytic domain of Chk1, coordinating activation by ATR/ATRIP, and culminates in the release of activated Chk1 from this complex (Jeong et al., 2003; Kumagai et al., 2004). Claspin was also identified as a component of the replication fork, loading onto chromatin in a Cdc45 and S-CDK-dependent manner (Lee et al., 2003).

### 5.2.1 Summary of Results

Claspin associates with the replication fork proteins Cdc45, DNA polymerase  $\epsilon$  (Pol  $\epsilon$ ), Replication Protein A (RPA), and two Replication Factor C complexes (RFCs) on chromatin in *Xenopus* egg extracts. Using a differential immunoprecipitation approach, we verified that Claspin does not interact with Cdc45 indirectly through other members of the CMG complex, GINS and MCM2-7. Claspin interaction with the RFC is dependent on the presence of RPA but the

Cdc45 interaction is independent of RPA, suggesting that Claspin interacts with Cdc45 before origin unwinding and the RFC after this event. An N-terminal region of Claspin comprising amino acids 265–605 was found to be required for the association of Claspin with chromatin and the replication fork proteins Xcdc45, RPA70, RFC40, Xrad17, and Pol ε. This region was named the replication fork-interacting domain (RFID) as it is essential for Claspin association with the replication fork. This region is composed of two well-conserved basic patches in what is an otherwise very acidic protein that are required for efficient association of Claspin with chromatin. The first basic patch is not required for Claspin to mediate Chk1 activation but the second basic patch is required for efficient activation. A small fragment of Claspin (amino acids 776-905) that contains the previously characterized CKBD is the most minimal fragment found to be able to complement Claspin-depleted *Xenopus* egg extracts (Kumagai and Dunphy, 2003). This fragment, termed the Chk1 activating domain (CKAD), is unable to associate with chromatin and is significantly less efficient in mediating Chk1 activation than endogenous Claspin, which is already in excess abundance in the egg extract. Therefore, Claspin association with chromatin is not strictly required for its ability to mediate Chk1 activation but the association is required for full efficiency of this process.

### 5.2.2 Significance of Results

This work is of significance to the fields of both replication control and replication checkpoint regulation. A key question that this work answers is the spatial requirement for the activation of the replication checkpoint in response to

replication arrest. Chk1 is a dynamic, nucleoplasmic protein whereas Claspin is both nucleoplasmic and associated with replication forks, where it accumulates upon replication arrest (Bekker-Jensen et al., 2006; Liu et al., 2006). These studies show that Claspin is not strictly required to be localized to the replication fork in order to mediate Chk1 activation. However, the spatial localization of Claspin to the replication fork is required for a high efficiency of Chk1 activation relative to non-chromatin associated Claspin molecules.

### **5.3 Identification of a DDK interaction motif on Claspin**

Claspin has been shown to have two broad functions, one as a mediator of Chk1 activation and the other as a component of the replication fork (Kumagai and Dunphy, 2000; Lee et al., 2003). Claspin mediates Chk1 activation through a conserved motif comprised of amino acid repeat sequences (Kumagai and Dunphy, 2003). Several studies have provided evidence that Claspin is required for normal replication fork progression (Lee et al., 2003; Lin et al., 2004; Petermann et al., 2008). A recent study has demonstrated that Cdc7 interacts with and phosphorylates Claspin in mammalian cells but neither the mechanism nor physiological implications of these findings are clear (Kim et al., 2008).

#### *5.3.1 Summary of results*

We confirmed that *Xenopus* Claspin is phosphorylated by DDK *in vitro*. We found that Claspin forms a nuclear complex with Cdc7 and Drf1, irrespective of the activation state of the replication checkpoint. Claspin also coimmunoprecipitated the cohesin loading protein Scc2, a finding that coincides

with a recent study demonstrating that DDK binds to and is required for Scc2 chromatin loading (Takahashi et al., 2008). We prepared a series of Claspin peptide fragments and identified the most minimal amino acid sequence in Claspin required for interacting with DDK. Surprisingly, this region turned out to be the first, but not the second, repeat sequence of the CKBD. There are only two amino acid positions that vary between the two repeats and we mutated the first repeat both singly and combinatorially at those positions (residues D861E, Q866K and E/K). We found that the D861 and E/K mutations conferred the largest reduction of interaction with DDK when these mutations were expressed in successively larger Claspin peptide fragments and eventually the full-length protein. The CKBD sequence repeats are well conserved in higher eukaryotes and the presence of an aspartate residue in the first repeat — as well as its absence in the downstream repeat(s) — is almost completely conserved as well. We were able to confirm that human CKBD fragments follow the same pattern of interaction when tested for interaction with DDK in *Xenopus* egg extracts. Since the main described function of the CKBD is binding to and mediating activation of Chk1, we tested whether the loss of interaction with DDK would impact this role of Claspin. CKBD peptide fragments mutated to be incapable of binding Chk1 were still able to interact with DDK. CKAD peptide fragments containing the D861E and E/K mutations were capable of reciprocally binding to Chk1 in replication checkpoint stimulated *Xenopus* egg extracts. Moreover, both the D861E and E/K full-length recombinant mutants of Claspin were capable of successfully rescuing Chk1 activation in replication arrested Claspin-depleted

extracts. The mutant full-length Claspin proteins interact with chromatin under both unperturbed and replication-arrested conditions in an equivalent manner to wild-type and endogenous Claspin molecules. The mutant Claspin proteins were also capable of rescuing a chromatin binding defect of Cdc45 from replication-arrested Claspin-depleted extracts.

### *5.3.2 Significance of results*

This work provides a molecular characterization of the interaction between the essential replication kinase DDK and the replication checkpoint mediator Claspin. The most intriguing aspect of this study is the finding that the Claspin interacts with DDK through part of the highly conserved region previously identified as the Chk1 binding domain (CKBD). Based on both the pattern of sequence conservation of the key amino acid position mediating DDK binding and the binding pattern of the human CKBD peptides, the DDK interaction motif on Claspin may be a highly conserved sequence element. There are two other highly significant results from this study. One is that the Claspin mutants incapable of binding DDK are still able to mediate Chk1 activation in response to replication arrest. The other is that interaction with DDK is not required for Claspin chromatin loading.

## Appendix A

# Physiological effects of Claspin DDK interaction mutants

### A.1 Introduction

In chapter 4, we demonstrated that the S-phase kinase DDK (Drf1/Dbf4-dependent kinase) interacts with the replication checkpoint protein Claspin through a conserved protein motif. In that study, we identified the first repeat sequence of the Chk1 binding domain (CKBD) on Claspin as being essential for DDK interaction and furthermore showed that the amino acid positions D861 and Q866 in particular were required for this interaction (Figs. 4.2C and 4.3A) . In addition, we demonstrated that the Claspin interaction with DDK was distinct from the ability of Claspin to bind to Chk1 and mediate Chk1 activation in response to replication blocks (Figs. 4.4A, 4.4B and 4.5A). We also provided evidence that the Claspin mutants deficient in DDK binding (D861E and E/K) displayed a normal ability to load onto replicating chromatin as well as a typical hyper-accumulation on replication arrested chromatin. Here we describe preliminary data providing several physiological implications resulting from adding the Claspin mutants that are deficient in DDK interaction to Claspin-depleted extracts.

## A.2 Results

Claspin is a regulator of replication in mammalian cells and is required for a normal rate of DNA replication, even under unperturbed conditions (Lee et al., 2005; Lee et al., 2003; Petermann et al., 2008; Sar et al., 2004). However, in *Xenopus* egg extracts, immunodepletion of Claspin yields only a very modest decrease in DNA replication (Lee et al., 2003). Since the primary role established for DDK is as an S-phase kinase required for the initiation of replication forks, we wanted to see if Claspin mutant proteins with a reduced ability to interact with DDK (described in chapter 4) would have an equal or greater impairment of DNA replication in extracts. To this end, we immunodepleted Claspin from interphase extracts and added back either buffer alone or recombinant insect cell-expressed His6-Claspin-FLAG proteins. We measured DNA replication by adding  $^{32}\text{P}$ -dATP before the onset of replication and took samples at selected time points until replication is typically completed in interphase extracts. Replication characteristically starts at approximately 45 minutes after entry into interphase in *Xenopus* egg extracts. We found that Claspin-depleted extracts with no recombinant protein added did not result in a consistent decrease in replication compared to mock-depleted or recombinant wild-type Claspin rescued extracts (figure A.1A). In contrast, when the D861E and E/K mutants were added back to Claspin depleted extracts, there was a ~30% decrease in replication as compared to the wild type or mock-depleted conditions (Fig A.1A). The different rates of replication are apparent at the second time point, 60 minutes, implying that there is an early role for Claspin in

DNA replication. These results are consistent with Claspin involvement in maintaining a normal basal replication rate in other systems (Lee et al., 2003; Lin et al., 2004; Petermann et al., 2008) and suggest that DDK may play a part in this regulation.

The S- and M-phases of *Xenopus* early extracts are tightly linked as there is no G2 phase in this system (reviewed in (Arias and Walter, 2007)). Since the Claspin mutants D861E and E/K seem to result in slower replication when complementing Claspin-depleted conditions in interphase extracts, we wondered whether there might be a concomitant delay in mitosis. Previous work has not established a delay in entry into mitosis upon the depletion of Claspin in *Xenopus* egg extracts. We decided to complement Claspin-depleted extracts with mutant and wild-type recombinant proteins or buffer alone as above. We used the super gel-shifted form of the protein Cdc25, which is a key positive regulator of the M-phase Cdk, as a marker for M-phase in these extracts (Kumagai and Dunphy, 1992). Typically, extracts enter M-phase around 90 minutes after entry into interphase following fertilization signals. As such, the mock-depleted extract and Claspin-depleted extract rescued with wild-type Claspin displayed a characteristic supershift of Cdc25 at the 90 minute time point (figure A.1B, lanes 10 and 17). Surprisingly, the Claspin-depleted extract containing buffer alone had a slightly accelerated mitosis with a super-shifted Cdc25 band visible at 75 minutes but still present at 90 minutes (figure A.1B, lanes 4 and 5). However, the Claspin-depleted extracts rescued with the D861E or E/K Claspin recombinant proteins resulted in the super-shifted form of Cdc25 appearing at 105 minutes, a

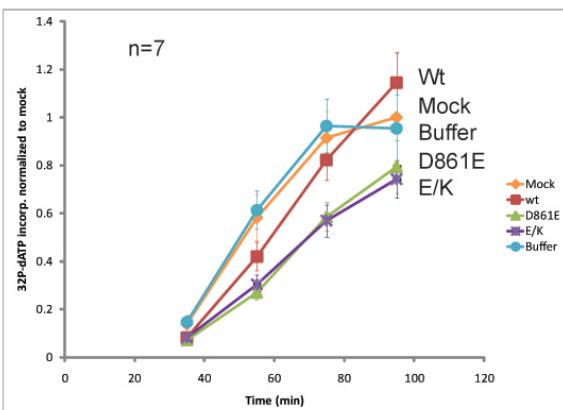
15 minute delay in mitosis (figure A.1B, lanes 23 and 28). This suggests that the delay in replication may be linked to a longer S-phase in the usually rapidly oscillating system of the *Xenopus* early embryo.

Claspin is loaded onto chromatin during S-phase in a pre-RC and Cdc45 dependent manner (Lee et al., 2003). The most important known kinase target of DDK is the MCM2-7 complex and this phosphorylation leads to the recruitment of the replication fork unwinding factor, Cdc45 (Masai et al., 2006; Sheu and Stillman, 2006; Tsuji et al., 2006). Given this information we wondered whether the DDK interaction mutants would be deficient in chromatin loading. We isolated chromatin from extracts where we immunodepleted Claspin and added back various Claspin recombinant proteins. In both the cases of unperturbed and aphidicolin-treated extracts, the mutant Claspin proteins, D861E and E/K, were loaded onto chromatin in a fashion comparable to wild-type rescued and mock-depleted Claspin (figure A.2, lanes 5–8 and 10–13). In the case of the aphidicolin-treated extracts, the mutant Claspin proteins hyper-accumulated on chromatin and displayed a characteristic gel shift, both hallmarks of a replication checkpoint response (figure A.2, lanes 10–13). The replication fork protein Cdc45 hyper-accumulates onto aphidicolin-treated chromatin after Claspin has been immunodepleted and this can be reversed by wild-type recombinant Claspin (chapter 2) (Yanow et al., 2003). Here we found that the D861E and E/K Claspin mutants can reverse the hyper-accumulation of Cdc45, Pol  $\epsilon$ , Pol  $\alpha$ , and RFC40 seen in the Claspin-depleted extract with no recombinant protein added-back equally as well as wild-type (Fig A.2, lanes 9–13) (J.Lee and W.G. Dunphy,

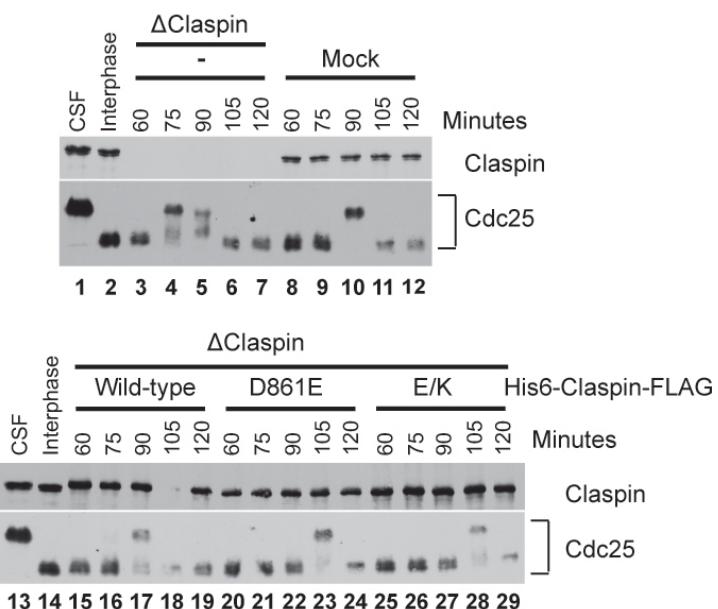
unpublished data). Orc2 serves here as a loading control since the loading onto chromatin is unchanged in these conditions.

Previous work in our lab found that Drf1 accumulated onto chromatin in aphidicolin treated extracts and that this increase was dependent upon Claspin (Yanow et al., 2003). We have confirmed that finding, and have also found that the D861E and E/K Claspin mutants have lesser potency in rescuing this accumulation defect relative to wild-type and mock-depleted extracts (figure A.2, lanes 9–13). The fact that the accumulation of Drf1 is not completely abrogated as in the buffer-only extract (Fig A.2) implies that either the D861E and E/K mutants still retain some DDK binding capacity or that there are other proteins that can compensate for this defect.

A

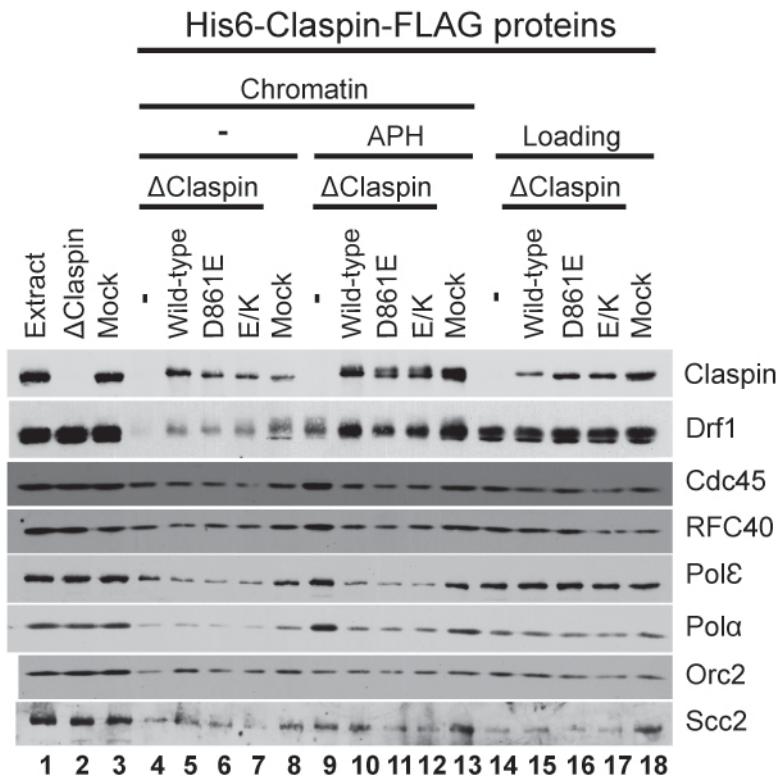


B



**Figure A.1** Claspin mutant proteins result in delayed replication progression and mitosis when complementing Claspin-depleted extracts.

- (A) Replication assays were carried out by incubating  $^{32}\text{P}$ -dATP in either mock (Mock) or Claspin-immunodepleted *Xenopus* egg extracts. The extracts were complemented with recombinant Wild-type (Wt), D861E, or E/K His6-Claspin-FLAG proteins or buffer alone. Aliquots were taken at 35, 55, 75, and 95 minutes, treated with replication stop buffer, digested with proteinase K for 60 minutes, the products were subjected to electrophoresis on an agarose gel and analyzed by autoradiography. The data was detected by Phosphorimage analysis and were normalized to the Mock (95 minute) sample.
- (B) Mitosis assays were performed by taking aliquots from either mock (Mock) (lanes 8-12) or Claspin-immunodepleted extracts. The extracts were complemented with recombinant Wild-type (Wt) (lanes 15-19), D861E (lanes 20-24), or E/K His6-Claspin-FLAG (lanes 25-29) proteins or buffer alone (lanes 3-7). Lanes 1 and 13 are aliquots of CSF (cytostatic factor) arrested extracts and lanes 2 and 14 are aliquots of interphase extracts. The extracts were western blotted with anti-Claspin and anti-Cdc25 antibodies.



**Figure A.2** Claspin mutant proteins partially rescue replication chromatin loading defects

Chromatin was isolated from interphase extracts supplemented with 3,000 sperm nuclei/ $\mu$ l and incubated in the absence (lanes 4–8) or presence of aphidicolin (lanes 9–13). Extracts were immunodepleted with either anti-Claspin (lanes 4–7 and 9–12) or mock control antibodies (lanes 8 and 13). Extracts were also supplemented with the indicated versions of the His6-Claspin-FLAG protein (lanes 5–7 and 10–12) or buffer alone (lanes 4, 8, 9, and 13). Lanes 14–18 depict aliquots of extract taken before isolation of chromatin. Aliquots of extract were taken before (lane 1) and after immunodepletion (lanes 2 and 3). Samples were immunoblotted for Claspin, Drf1, Cdc45, RFC40, Pol  $\epsilon$ , Pol  $\alpha$ , Orc2, and Scc2.

### A.3 Discussion

We have described several physiological effects resulting from the complementation of Claspin-depleted extracts with Claspin mutant proteins deficient in their ability to interact with DDK. To begin, there is a slower rate of replication in extracts containing the D861E and E/K Claspin mutants relative to wild-type Claspin-rescued or mock-depleted extracts. There could be several explanations for this reduction in replication. One possibility could be the inappropriate activation of the intra-S-phase replication checkpoint where the mutant Claspin proteins would behave in a hyperactive manner with respect to mediating Chk1 activation. However, this is unlikely based on the evidence in figure 4.5A where neither of the Claspin mutants display higher levels of the phospho-serine 344 Chk1 signal nor a slower gel-migrating form of Chk1 relative to wild-type recombinant Claspin when complementing Claspin-depleted extracts during unperturbed replication. If the slower replication rate is the result of a replication initiation defect, there would be either a delay in the firing of replication origins or an overall reduced number of origins firing. Alternatively, this delay could be the result of a slower rate in the elongation phase of DNA synthesis. We see differences between the rates of DNA replication between wild-type and mutant-complemented Claspin-depleted extracts as early as the 60-minute time point (figure A.1.A), suggesting that perhaps there is a defect in initiation. However, this is by no means conclusive. Considering that DDK is required for replication origin firing through its phosphorylation of the MCM2-7 complex, which leads to the recruitment of the unwinding factor Cdc45, it is

possible that Claspin may have a role in the substrate targeting of DDK or the efficiency of DDK activity in this role (Masai et al., 2006; Sheu and Stillman, 2006; Tsuji et al., 2008). Also supporting the initiation hypothesis, Claspin is known to bind to Cdc45, although Claspin is not required for Cdc45 recruitment to chromatin (Lee et al., 2005; Lee et al., 2003; Yanow et al., 2003). However, Claspin also interacts with proteins involved in DNA replication elongation such as Pol  $\epsilon$  and the RFC complex as has the yeast Claspin homologue MRC1 so a defect in elongation cannot be completely ruled out (Lee et al., 2005; Lee et al., 2003; Lou et al., 2008). Whereas in mammalian systems the deletion or knockdown of Claspin has had a significant reduction in the rate of replication (Lin et al., 2004; Petermann et al., 2008; Sar et al., 2004), in *Xenopus* egg extracts the reduction in replication rate has been decidedly modest (Lee et al., 2003). The reduction in replication rate seen when complementing Claspin-depleted extracts with Claspin D861E and E/K is more similar to the phenotype of Claspin-depleted mammalian cells (Lin et al., 2004; Petermann et al., 2008; Sar et al., 2004). Further investigation will be required to determine the cause of the Claspin mutant replication defect from amongst the scenarios we have depicted.

The second physiological effect of adding the mutant Claspin proteins to Claspin-depleted extracts was a subtle delay in mitosis (Fig A.1B). This approximately 15-minute delay could, like the replication defect, result from the inappropriate activation of the Claspin-mediated G2/M checkpoint. However, as detailed above, we did not observe abnormal activation of Chk1 under unperturbed

conditions (Fig 3.5A). Another possibility is that the mitotic delay may be the direct result of the delayed replication observed with the same mutant Claspin proteins and that the onset of entry into M-phase is concomitant with the later end of replication. We view this as the most likely possibility but further work in dissecting the mutant Claspin proteins role in delaying replication will also help to elucidate the mitotic delay.

The final physiological effect of the Claspin D861E and E/K mutant complementation experiments that we observed was the incomplete rescue of Drf1 aphidicolin-induced chromatin hyper-accumulation. Claspin loading onto chromatin is S-CDK (as determined by use of the S-Cdk inhibitor p27) and Cdc45-dependent (Lee et al., 2003). We noted in chapter 4 that the mutant Claspin proteins associate with unperturbed and replication-arrested chromatin to an equivalent extent and dynamics as wild-type and endogenous Claspin proteins. This provides evidence that a strong DDK interaction is not required for loading Claspin onto chromatin. We have described in chapter 2 that the Drf1 hyper-accumulation onto aphidicolin-treated chromatin is ATR, Claspin, and caffeine-dependent (Yanow et al., 2003). Therefore, the incomplete rescue of the Claspin-depleted Drf1 hyper-accumulation defect by the Claspin DDK association mutants fits well with a model of Claspin regulating the recruitment or retention of DDK at stalled replication forks. The physiological implication of the DDK hyper-accumulation at stalled forks is not currently known. Moreover, there is still significant controversy in the field as to not only whether Drf1 hyper-accumulates on replication-arrested chromatin but the regulation of DDK as a

whole under these conditions (extensively discussed in chapter 1.3.2). The most recent work on this subject suggests that Drf1-Cdc7 remains in a stable, active complex in response to replication fork arrest and is localized to the arrested chromatin (Kim et al., 2008; Silva et al., 2006; Tenca et al., 2007; Tsuji et al., 2008). Assuming that this is the case, one result of DDK accumulation on stalled replication forks may be to stabilize these damaged forks against fork collapse and possibly prepare them for replication restart after adaptation or repair. Some modest replication initiation is observed after replication arrest following treatment of caffeine, which inhibits the replication checkpoint, suggesting the presence of active DDK at these structures (chapter 2) (Yanow et al., 2003). A possible scenario is that DDK attenuates the replication checkpoint at certain late time points after replication arrest. One piece of data supporting this model is that the DDK interaction mutants do not impinge on the ability of Claspin to mediate Chk1 activation under these conditions, suggesting that the DDK association with Claspin is not a stimulatory one (chapter 4). Another argument to support this is that the overexpression or addition of ectopic excess of DDK inhibits the replication checkpoint (Tsuji et al., 2008). Replication is required for the activation of the replication checkpoint in response to replication stresses such as UV and HU (Lupardus et al., 2002; Michael et al., 2000; Stokes et al., 2002). The inhibition of the replication checkpoint in response to Cdc7 depletion in mammalian cells (Kim et al., 2008) may be a result of the reduction in the number of initiated replication forks as a consequence of the loss of DDK activity rather than direct inhibition of Claspin by DDK. Clearly, more study into the

effects of DDK phosphorylation and interaction with Claspin, especially at later time-points following replication arrest.

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