I, Jennifer Searle, hereby submit this work as part of the requirements for the degree of:

**Doctor of Philosophy (Ph.D.)**

in:

**Molecular Genetics, Biochemistry and Microbiology**

It is entitled:

**The Role of PKA in the DNA Damage Checkpoint**

This work and its defense approved by:

Chair: Yolanda Sanchez
David J Robbins
Mark E. Olah
Carolyn Price
James Stringer
The Role of PKA in the DNA Damage Checkpoint

A dissertation submitted to the
Division of Research and Advanced Studies
of the University of Cincinnati
in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY (Ph.D.)

in the Department of Molecular Genetics, Biochemistry
and Microbiology
College of Medicine
2005

By
Jennifer Searle

B.S., Ohio State University, 1999

Committee Chair: Yolanda Sanchez, Ph.D.
Abstract

Checkpoint proteins block progression through the cell cycle in order to prevent cell division when a cell has damaged or incompletely replicated DNA. This dissertation will focus on the DNA damage checkpoint signal transduction pathways which prevent mitotic progression following DNA damage. Mutations in the genes that encode for checkpoint proteins can lead to genomic instability, uncontrolled cell growth and cancer. Checkpoint proteins are being considered as drug targets for treatment of cancers, based on the hypothesis that the inactivation of a checkpoint protein enhances the rate at which cells fail to stop division with damaged DNA, which leads to accumulation of damaged DNA, and increases the probability of death for the cancer cell. Therefore, identifying novel checkpoint proteins could lead to the identification of new drug targets. The checkpoint pathways are conserved in the genetically amenable *Saccharomyces cerevisiae*, making it a good model system in which to identify novel proteins involved in regulating mitosis. The conserved checkpoint kinases Chk1 and Rad53 prevent mitotic progression by blocking the degradation of the securin, Pds1, thus preventing separation of the sister chromatids, and by inhibiting activation of the mitotic exit network (MEN), respectively. Chk1 and Rad53 play supporting roles in preventing mitosis following DNA damage. Although Chk1 prevents the degradation of Pds1 following DNA damage, Pds1 is degraded faster in cells containing a mutation in an upstream checkpoint kinase than in a *chk1* mutant, suggesting that another pathway can regulate the destruction of Pds1. We show that the cAMP dependent protein kinase (PKA) pathway has a supporting role to Chk1 in preventing the degradation of the mitotic inhibitors Pds1 and Clb2 via regulation of the mitotic inducer Cdc20. We also found that proteins that
regulated the levels of cAMP, and the checkpoint dependent phosphorylation of the PKA regulatory subunit were required to support the DNA damage checkpoint in blocking mitotic progression. These results suggested that there is cross-talk between the DNA damage checkpoint and PKA pathways, and that the DNA damage checkpoint may recruit the PKA pathway to reinforce the mitotic block following DNA damage.
Acknowledgements

I would first like to thank my mentor Yolanda Sanchez for teaching me how to be a scientist and then allowing me the independence to learn to do science on my own. Yoli’s support and friendship has encouraged me to do my best and never give up throughout my graduate school experience.

I would also like to thank my committee members: Mark Olah, Carolyn Price, David Robbins, and Jim Stringer for their helpful suggestions and advice.

A special thank you to the members of the Sanchez Lab, past and present, for their help and friendship and making the work environment a fun environment.

I would also like to thank my family and friends for all of their support especially my parents and my sister who have supported me in so many ways, and my fiancé, Jerome who has always been there for me.
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Chapter 1

1. Introduction

Maintenance of genomic stability requires that a cell be able to transfer a complete and unaltered genome to its daughter cell. Passing on an incomplete or altered genome can lead to either cell death or, unregulated cell growth and cancer. Throughout the life of a cell, it is constantly assaulted with internal and external sources of DNA damage (Kastan and Bartek, 2004). The DNA damage must be fixed before the cell divides its genome and passes on the damage to the daughter cell. Failure to inhibit mitotic progression following DNA damage can cause a cell to divide with damaged DNA or incompletely replicated DNA, which can lead to genomic instability. To facilitate the repair of the damaged DNA, a cell undergoes three important functions. First, it stops cell division so that the DNA can be repaired. Second, the DNA repair proteins must be activated and localized to the source of the damage, Third, the cell must up-regulate the gene expression or protein expression of DNA repair or cell cycle arrest genes (Zhou and Elledge, 2000), (Clarke and Gimenez-Abian, 2000). This dissertation will focus on how cells stop mitotic progression in response to DNA damage, and more specifically, how the DNA damage checkpoint utilizes the cAMP dependent protein kinase (PKA) pathway to reinforce the mitotic block in response to DNA damage. Because the DNA damage checkpoint regulates proteins that mediate mitotic progression, I will first introduce cell cycle and mitotic progression, followed by what is known about the checkpoint and how it regulates mitotic progression. Finally, I will discuss what is known about how the PKA pathway regulates mitotic progression, and the components that are known to regulate the PKA pathways.
1.1 Cell Cycle and Mitotic progression

1.1.1 Cell Cycle

The cell cycle can be divided into four stages, synthesis (S) phase where the DNA is replicated, and mitosis (M) phase, in which the DNA is divided into two daughter cells. These two stages are separated by two gap phases (G1 and G2). The budding yeast Saccharomyces cerevisiae is an excellent model system for studying the cell cycle as they are genetically amenable and the size of the bud provides a visual indication of the cell cycle stage of the cell. Many years of research on the cell cycle in budding yeast has helped establish the mechanisms that regulate cell cycle progression. These mechanisms are conserved from yeast to mammals.

Progression though the cell cycle is driven by a kinase called the cyclin dependent kinase (CDK), which phosphorylates specific substrates to drive the cell from one cell cycle stage to the next (Fig. 1.1)(Gautier et al., 1988), (Lohka et al., 1988). The cyclin dependent kinase is activated via interaction with proteins called cyclins (Labbe et al., 1989). The levels of the cyclins fluctuate throughout the cell cycle. High levels of a cyclin activate the CDK and drive the cell from one cell cycle stage to the next. The high Cdk activity is also inhibitory for the entry into a new cell cycle (Murray et al., 1989), (Evans et al., 1983). Therefore, progression through consecutive cell cycles also requires the inactivation of the Cdk, which is carried out by ubiquitination and destruction of the cyclins and/or post-translational modification of the Cdk (King et al., 1995). For example, Cyclin B/Cdk activity promotes entry into mitosis. However, exit from mitosis requires that the Cyclin B/Cdk activity be downregulated (Fig 1.2). In order to
Figure 1.1 The cell cycle is divided into four stages: S-phase when the DNA is replicated, M-phase when the replicated DNA is divided and the daughter cells separate and two gap phases, G1 and G2. The transitions between G1 and S phase, and G2 and M phase are regulated by high Cdk activity regulated by the cyclin partner. Exit from mitosis and entry into the next G1 requires a down regulation of Cdk activity.

Figure 1.2 Mitotic Progression. High cyclinB/Cdk levels promote entry into mitosis. At metaphase the sister chromatids align and in anaphase the sister chromatids separate. Inactivation of the cyclinB/Cdk complex allows mitotic exit, cytokinesis and entry into the next G1. See text for more detail.
downregulate cyclin B, the cyclin B protein levels are decreased by ubiquitin mediated destruction and downregulation of gene expression. In addition, phosphatases are activated which dephosphorylate the CDK substrates (Visintin et al., 1998).

1.1.2 Mitotic progression

Mitosis is divided into several stages including, metaphase, anaphase, telophase, mitotic exit, and cytokinesis, which are defined by phenotypic and molecular characteristics (Fig. 1.2). In metaphase the condensed chromatids are associated with their sister chromatid via centromeres and other chromatid associated proteins called cohesins (Haering et al., 2002), (Hirano, 2002). Also attached to the centromeres is the spindle, which will provide the mechanical force to pull the sisters apart in the next stage of mitosis, anaphase. In anaphase, the cohesins are cleaved and the force exerted by the spindle causes the sister chromatids to separate (Koshland and Guacci, 2000), (Ciosk et al., 1998). The sister chromatids then move to opposite ends of the cell. This stage is called telophase. Mitotic exit is defined as the point at which the cyclin/Cdk complexes are inactivated, which allows pre-replication complexes to form on the chromatin in order to prepare the cell for the next round of replication if the cell commits to a new cell division cycle (Yeong et al., 2002). The physical separation of the cytoplasm and formation of two separate daughter cells is called cytokinesis (Fig1.2).

1.1.3 Regulation of Mitotic Progression

At the molecular level, progression through mitosis is regulated at two stages, the metaphase to anaphase transition and mitotic exit, by the ordered destruction of mitotic inhibitors. The mitotic inhibitor, Pds1 (securin), regulates the metaphase to anaphase
Figure 1.3 Regulation of Mitotic Progression. The mitotic inhibitors, Pds1, blocks the metaphase to anaphase transition by preventing the separation of the sister chromatids. The B-type cyclin, Clb2 inhibits the mitotic exit network (MEN) by activation of the Cdk. Progression through mitosis is regulated by the ordered destruction of Pds1 and Clb2. The destruction of the inhibitors is carried out by ubiquitination and degradation. The E3 ubiquitin ligase, APC, with its specificity factor Cdc20 targets both Pds1 and Clb2 for ubiquitination and destruction. The APC with its specificity factor Hct1 regulates the ubiquitination and destruction of the second wave of Clb2 destruction.
transition by inhibiting cleavage of the proteins that hold the sister chromatids together, (cohesins) thereby blocking separation of the sister chromatids (Yamamoto et al., 1996). In order to separate the sister chromatids, the cohesins must be cleaved by the separase, Esp1 (Ciosk et al., 1998). Pds1 prevents separation of the sister chromatids by binding to and inhibiting the activation of the separase. In an unperturbed cell cycle, upon entry into mitosis, Pds1 is ubiquitinated and targeted for destruction via the proteosome, thus freeing the separase so that the sister chromatids can separate (Fig. 1.3) (Cohen-Fix et al., 1996).

Exit from mitosis and entry into the next G1 require inactivation of the cyclin dependent kinase. Mitotic exit is carried out both by the ordered destruction of B-type cyclins, particularly Clb5 and Clb2 (Jacobson et al., 2000), and dephosphorylation of CDK substrates. The B-type cyclin, Clb2, undergoes two waves of destruction (Yeong et al., 2000). The first wave of destruction of Clb2 begins the activation of a complex regulatory pathway called the mitotic exit network (MEN) (Fig. 1.3) (Bardin and Amon, 2001), (Morgan, 1999). One of the core components of the MEN is a phosphatase, Cdc14, which dephosphorylates CDK substrates (Visintin et al., 1998). Following the initial wave of destruction of Clb2, a GTPase activating protein (consisting of Bfa1 and Bub2) is inactivated, leading to the activation of a Ras family GTP binding protein called Tem1 (Ro et al., 2002), (Lee et al., 2001). Activation of Tem1 leads to the release of Cdc14 from the nucleolus (activation). Cdc14 then dephosphorylates Cdk substrates, including Hct1/Cdh1, which leads to the final wave of destruction of Clb2. Cdc14 also dephosphorylates and activates the CDK inhibitor Sic1, thus allowing the cell to both exit
mitosis and set up pre-replication complexes vital for the next cell cycle (Wasch and Cross, 2002).

The ordered destruction of the mitotic inhibitors is carried out by proteosomal degradation of the ubiquitinated inhibitors. An E3 ubiquitin ligase made up of at least 11 proteins called the anaphase promoting complex or cyclosome (APC/C) catalyzes the ubiquitination of the mitotic inhibitors (Sudakin et al., 1995), (Peters, 2002). In addition to the proteins that compose the core APC/C, there are also two specificity factors called Cdc20 and Cdh1/Hct1 that help mediate the specificity and order of substrate ubiquitination (Visintin et al., 1997). The APC\textsuperscript{Cdc20} targets both inhibitors, Pds1 and Clb2, for ubiquitination and destruction (Fig. 1.3) (Lim et al., 1998); however, since Pds1 blocks the ubiquitination and destruction of Clb2, this ensures that Clb2 will not be targeted for destruction until Pds1 protein levels are sufficiently downregulated and anaphase has taken place (Cohen-Fix and Koshland, 1999). Clb2 ubiquitination and degradation is carried out in two phases. Cdc20 is responsible for mediating the first wave of ubiquitination and destruction which may be enough to allow exit from mitosis (Fig.1.2)(Wasch and Cross, 2002). The ubiquitination of the remaining Clb2 protein is mediated by Cdh1/Hct1 (Fig 1.3). APC\textsuperscript{Cdh1/Hct1} is not activated until the end of mitosis as it is inhibited via phosphorylation mediated by the cyclin dependent kinase, Cdk1. Thus, down-regulation of the Cdk1 activity by destruction of the cyclin activates Cdc14 and the dephosphorylation of Cdh1/Hct1 (Visintin et al., 1997). The APC\textsuperscript{Cdh1/Hct1} is activated by dephosphorylation of Cdh1 causing the final wave of destruction of Clb2.

Because the correct ordered destruction of mitotic inhibitors is crucial for maintaining genomic stability, the APC and its specificity factors are highly regulated
throughout the cell cycle. *CDC20* is transcribed only during late S-phase and mitosis, and the protein is very unstable throughout the cell cycle, especially in G1 (Prinz et al., 1998). Cdc20 contains a destruction box (DB), which is required for its destruction in G1. The destruction of Cdc20 in G1 is likely mediated by APC$^{Cdh1/Hct1}$, but there may also be another mechanism to regulate the destruction of Cdc20 in G1. Cdh1/Hct1 is not required for cell viability, which suggests that Cdc20 can at least partially compensate for the loss of Cdh1/Hct1. Cdh1/Hct1 is more stable than Cdc20 throughout the cell cycle. In addition the proper timing of Cdc20 expression in late S phase is dependent on APC$^{Cdh1Hct1}$ activity in G1 (Huang et al., 2001). Cdc20 is a crucial component for regulating mitotic progression, and is regulated by both the spindle checkpoint, via interaction with a spindle checkpoint protein, and DNA damage checkpoint via phosphorylation. (Hwang et al., 1998), (Agarwal et al., 2003), (Searle et al., 2004).

### 1.2 DNA Damage Checkpoint

The DNA damage checkpoint inhibits cell cycle progression by influencing activities of proteins that mediate cell cycle progression. In order to inhibit mitotic progression, checkpoint proteins are organized in to a signal transduction network. These proteins can be divided into three categories, sensors, transducers and effectors (Table 1.1). The sensors can detect the DNA damage and send a signal to transducers, which conveys the signal to the effector kinases. Effector kinases regulate the phosphorylation of proteins needed for mitotic progression.
1.2.1 Upstream signal transducers

Two of the most upstream transducers of the signal transduction network that inhibits mitosis in response to DNA damage are the kinase, Mec1, and the signal amplifier, Rad9 (Weinert, 1992). Deletion of either of the genes encoding these proteins will result in a complete checkpoint defect in mitotic arrest. Thus, a mec1 or rad9 mutant challenged with a DNA damage signal will not arrest and the cells will progress through mitosis with similar kinetics as wild type cells with no DNA damage (Table 1.1) (Clarke and Gimenez-Abian, 2000). Mec1 is a phospho-inositide like kinase that phosphorylates (S/T)Q residues. The Mec1 homolog in mammals is ATR (ataxia-telangiectasia- and Rad3-related) kinase. ATR plays a similar role to Mec1 in checkpoint signaling in response to DNA damage (Kastan and Bartek, 2004). Although Rad9 does not have a homolog in mammalian cells, the C-terminus of Rad9 has homology to BRCA1 (breast cancer associated protein 1), and Rad9 function is dependent on the conserved BRCT domain (BRCA1 C-terminus) (Bork et al., 1997). Rad9 is hyper-phosphorylated in a Mec1 dependent manner in response to DNA damage. This phosphorylation is required for the mitotic checkpoint activity of Rad9 (Vialard et al., 1998). Both Mec1 and Rad9 are required for the phosphorylation and activation of the downstream effector kinases.

1.2.2 Effector Kinases

The checkpoint pathway bifurcates to regulate mitotic progression through activation of Chk1 and Rad53, the effector kinases. Chk1 inhibits the metaphase to anaphase transition and Rad53 inhibits mitotic exit (Fig.1.4) (Sanchez et al., 1999). Mutation of either CHK1 or RAD53 results in failure to stop mitotic progression after
Figure 1.4 Regulation of cell cycle progression by the DNA damage checkpoint. Following DNA damage a signal transduction cascade is activated in which the upstream kinase Mec1, and the signal amplifier Rad9, activate the effector kinases, Chk1 and Rad53. Chk1 blocks the metaphase to anaphase transition by phosphorylation and stabilization of Pds1. Rad53 activates a downstream checkpoint kinase, Dun1 to block the activation of the MEN.

Table 1.1 The categories of DNA damage checkpoint genes and the phenotype associated with loss of the genes.
DNA damage. However, the phenotypes of *chk1Δ* and *rad53* mutants are different from those of a *mec1* or *rad9* mutant, which supports the model that Chk1 and Rad53 act downstream of Mec1 and Rad9, and that the two kinases act in separate pathways (Sanchez et al., 1999), (Sanchez et al., 1996). Rad53 is a kinase containing FHA domains, which are known to interact with hyper-phosphorylated residues on proteins. Indeed, Rad53 interacts with the hyper-phosphorylated form of Rad9, and this interaction is required for the Mec1 dependent phosphorylation and activation of Rad53 (Sun et al., 1998). Chk1 phosphorylation and activation also requires interaction of Chk1 with Rad9, but not the hyper-phosphorylated form of Rad9. The exact role of Rad9 in Chk1 activation has not yet been determined.

### 1.2.3 Inhibition of Mitotic Progression

Chk1 blocks the metaphase to anaphase transition by regulating the mitotic inhibitor, Pds1. In response to DNA damage, Pds1 is phosphorylated in a Chk1 dependent fashion. This DNA damage induced phosphorylation of Pds1 prevents its ubiquitination and degradation, thereby preventing the separation of the sister chromatids (Fig. 1.4) (Sanchez et al., 1999), (Cohen-Fix and Koshland, 1997). Rad53 activates another checkpoint kinase called Dun1. The Rad53 pathway helps prevent mitotic exit by activating the GTPase activating protein, Bfa1 (Fig. 1.4) (Hu et al., 2001). The activation of Bfa1 prevents Cdc14 release from the nucleolus and prevents activation of the mitotic exit network.

Although Chk1 and Rad53 act in two independent pathways, they also help reinforce each other in a somewhat redundant fashion. This phenomenon is due mostly
to the interdependence of anaphase and mitotic exit mediated by Pds1 and the separase. Pds1 and the separase, Esp1, have a role in preventing the release of Cdc14 from the nucleolus and therefore preventing mitotic exit (Cohen-Fix and Koshland, 1999), (Tinker-Kulberg and Morgan, 1999). Therefore, Chk1, by stabilizing Pds1 and preventing activation of the separase, also helps prevent mitotic exit.

Pds1 is degraded with faster kinetics in a mec1 mutant than in a chk1Δ mutant suggesting that another pathway besides Chk1 is regulating the stability of Pds1 (Sanchez et al., 1999). Recent results provide evidence that the Rad53 pathway also has a role in the delay of anaphase specifically by preventing the interaction of Pds1 and the APC specificity factor Cdc20, thus presumably helping prevent the degradation of Pds1 (Agarwal et al., 2003). These results do not rule out the possibility that another pathway may also be regulating the stability of Pds1 in response to DNA damage.

1.3 PKA Pathway

1.3.1 PKA in mitotic progression

Studies carried out in many organisms including yeast, frog, and mammalian cells suggested that PKA may have a role in regulating mitotic progression. The first evidence was established from work carried out in the fission yeast, *Schizosaccharomyces pombe*, which demonstrated that deletion of the catalytic subunit of PKA resulted in decreased phosphorylation of an APC subunit and restored growth of an APC mutant (Yamada et al., 1997), (Yamashita et al., 1999). In *S. cerevisiae*, work done by several labs has suggested that PKA inhibits mitotic progression by regulating the APC. Overexpression of Pde2 which is a phosphodiesterase, the enzyme which degrades cAMP, was able to
restore the growth of an APC temperature sensitive mutant at its restrictive temperature, suggesting that upregulation of PKA signaling inhibits progression through mitosis (Heo et al., 1999). Also, the addition of exogenous cAMP (which presumably activates PKA) resulted in mitotic arrest with accumulation of the APC substrates, Pds1 and Clb2 (Anghileri et al., 1999). Further studies implicated the APC specificity factor, Cdc20, as a potential target of PKA (Bolte et al., 2003). Work in *Xenopus* egg extracts revealed that PKA phosphorylates the APC and that this phosphorylation was inhibitory to progression through mitosis (Kotani et al., 1998). These results suggest that PKA has a role in regulating mitosis. Additionally, in mammalian cells, changes in PKA activity have been associated with chromosomal instability in different types of cancers, suggesting PKA has a role in maintaining chromosomal stability (Bossis et al., 2004), (Matyakhina et al., 2002).

### 1.3.2 PKA Tetramer

PKA (protein kinase A) is a cAMP dependent protein kinase, which is also called cAPK. PKA in its inactive form consists of 2 regulatory subunits (R) and two catalytic subunits (C). In *S. cerevisiae*, there is only one regulatory subunit encoded by the gene *BCY1*, but there are three catalytic subunits encoded by *TPK1, TPK2, and TPK3*. Each regulatory subunit of the tetramer co-operatively binds to two molecules of the second messenger cAMP, causing release and activation of the catalytic subunits. The PKA pathway is involved in many functions of the cell including nutrient sensing, heat shock response pathway, cell wall integrity, and nitrogen starvation in *S. cerevisiae* (Thevelein and de Winde, 1999), and has diverse roles in metazoan cells including the insulin
response pathway, neuronal signaling, development and the immunological response (Matyakhina et al., 2002).

Because PKA plays such a pleotropic role in the cell, it has many levels of regulation. Like other kinases, PKA phosphorylates substrates on a specific serine or threonine within a defined consensus site. The consensus sequence for PKA can be found at least once in most proteins, suggesting that this is not the only factor to determine which proteins are phosphorylated by PKA (Griffioen and Thevelein, 2002). In addition, there are also different cellular responses to activation of PKA depending on what signal stimulates PKA activity. For example, addition of glucose to cells growing in a non-fermentable carbon source activates PKA and results in the accumulation of trehalose (Thevelein, 1994). In contrast, depriving cells of nitrogen also activates PKA, but trehalose does not accumulate. Instead, cells exhibit a rearrangement of the cytoskeleton and invasive growth (Pan and Heitman, 1999). The substrates phosphorylated by PKA, in cells with basal levels of cAMP are different than the substrates phosphorylated when there is a transient increase of cAMP signaling. Even within the cell cycle, activation of PKA can lead to different responses (Yanagida et al., 1999), (Thevelein, 1992). Additional levels of regulation are clearly needed to generate the required specificity of kinase and substrate.

Studies in both *S. cerevisiae* and higher eukaryotes have identified mechanisms by which PKA is regulated. One mechanism of regulation is by regulation of the second messenger, cAMP. By controlling the levels of cAMP, PKA catalytic activity can be regulated. Proteins which compose PKA, the catalytic subunits and the R subunit are also highly regulated. Each of the molecules, either in the holoenzyme form or as
independent molecules, can be regulated by either sub-cellular localization, post-translational modification, or protein-protein interactions (Griffioen and Thevelein, 2002).

**1.3.3 Regulation of cAMP**

Many of the components required for regulation of cAMP levels, and hence cAMP induced signaling, have been identified. cAMP levels are essentially regulated by two enzymes, adenylate cyclase, which catalyzes the conversion of ATP to cAMP and phosphodiesterases (Pde1 and Pde2), which catalyze the hydrolysis of the cAMP to AMP. Thus, these two proteins regulate, the amount of intracellular cAMP through production and destruction of cAMP, respectively. (Thevelein and de Winde, 1999). Therefore, the expression and regulation of these proteins is crucial to regulate cAMP signaling (Fig.1.5).

**1.3.3.1 Adenylate Cyclase**

In mammalian cells there are 10 known adenylate cyclases, nine of which are membrane bound proteins and one of which is not membrane bound. The number of different adenyl cyclases in the cell suggest that activation of different adenylate cyclases will lead at least in part to the specificity of cAMP signaling. (Tesmer and Sprang, 1998). However, in *S. cerevisiae* only one adenyl cyclase has been identified. *S. cerevisiae* adenyl cyclase is regulated similarly to metazoan cyclases in other ways. For example, another mechanism of regulation of the adenyl cyclase in mammalian cells is through the interaction with heterotrimeric G proteins. Structural analysis has shown that adenyl cyclase interaction with G proteins can lead to a structural change in the adenyl
cyclase rendering the catalytic region more open or closed to the ATP (Tesmer and Sprang, 1998). In addition, there are several G protein coupled receptors associated with different adenylate cyclases, so this is likely an important mechanism of regulation of the cyclase (Tesmer and Sprang, 1998). In *S. cerevisiae*, a G protein coupled receptor has been linked with cAMP signaling (Xue et al., 1998). Phosphorylation is another mechanism which can regulate adenyl cyclase activity. In mammalian cells, PKC and calmodulin kinase have been associated with phosphorylation and regulation of activity of adenyl cyclases (Tesmer and Sprang, 1998). No phosphorylation of the cyclase has yet been identified in *S. cerevisiae*.

### 1.3.3.2 Phosphodiesterases

In *S. cerevisiae* there are two genes which encode phosphodiesterases, *PDE1* and *PDE2*. Pde1 is a low affinity and Pde2 is a high affinity phosphodiesterase. In *S. cerevisiae*, the high affinity Pde2, seems to regulate the basal level of cAMP, while the low affinity phosphodiesterase, Pde1, regulates cAMP levels in response to agonist induced cAMP signaling (Ma et al., 1999). Both phosphorylation of Pdes, and localization have been shown to be important regulatory mechanisms for Pdes in mammalian cells. In *S. cerevisiae*, Pde1 has been shown to be phosphorylated and the phosphorylation was important for the agonist-induced decrease in cAMP levels (Ma et al., 1999).
**1.3.3.3 Ras Pathway**

In addition to adenylate cyclase and the phosphodiesterases regulating the levels of cAMP, there is another level of regulation which involves proteins that regulate these two key proteins. The first components of cAMP signaling identified in *S. cerevisiae* besides the adenyl cyclase and the phosphodiesterase were the Ras proteins, Ras1 and Ras2 and a guanine nucleotide exchange factor Cdc25. Deletion of both *RAS* genes rendered the cell unviable, but a mutation in *RAS2* (*RAS2*VAL19) that resulted in a hyperactive Ras protein led to an increase in cAMP levels (Toda et al., 1985). Ras was also found to be required for the increase in cAMP when glucose was added to cells growing in a non-fermentable carbon source, and in response to intracellular acidification (Colombo et al., 1998). Due to the lethality of a *RAS* double mutant, it was difficult to determine whether the signal was actually transmitted though Ras, or whether Ras had a more structural role in cAMP signaling. Later, activation of Ras was determined by measuring the amount of GTP bound Ras compared to GDP bound Ras. These experiments demonstrated that there was no increase in GTP bound Ras in response to addition of glucose; however, there was an increase in bound GTP in response to intracellular acidification. These results suggested that signaling to upregulate cAMP levels was going though Ras in response to intracellular acidification, but not in response to glucose (Colombo et al., 1998). It was also shown that a mutant Ras protein that was not farnesylated (a post-translational modification which facilitates membrane association) and localized to the membrane inhibited cAMP signaling in response to glucose (Bhattacharya et al., 1995), which suggested that the role of Ras in glucose signaling is not through signal transduction, but that Ras is either required for the
localization of adenylate cyclase to the plasma membrane or a conformational change of the adenyl cyclase when bound to Ras.

1.3.3.4 Glucose signaling

Unlike *S. cerevisiae* where the Ras pathway seemed to predominate in regulating adenylate cyclase, G-protein coupled receptors (GPCRs) are the main mechanism for activating adenylate cyclase in metazoans. However, a GPCR system was also found to be utilized in cAMP signaling in *S. cerevisiae* as well (Xue et al., 1998). Addition of glucose to cells growing in a non-fermentable carbon source activated cAMP signaling and this activation required the GPCR, Gpr1, and its associated G protein, Gpa2 (Fig 1.5) (Yun et al., 1998).

In addition to a requirement for the Gpr1, activation of PKA signaling also requires intracellular glucose kinase. *S. cerevisiae* has three glucokinases, hexokinase 1 and 2 (Hxk1 and Hxk2) and glucokinase Glk1. Hxk2 is by far the most efficient glucose kinase, and Hxk2 is required for the increase in cAMP levels following glucose addition to cells growing in a non-fermentable carbon source. Surprisingly, Hxk2 is also required for the increase in cAMP levels due to intracellular acidification or activation of the Ras pathway. Hxk2 also plays a critical role in glucose signaling that leads to catabolite repression (Hohmann et al., 1999). Indeed, Hxk2 is phosphorylated in non-fermentable carbon sources {Randez-Gil, 1998 #25} and localized to the nucleus when the cells are grown in glucose {Ahuatzi, 2004 #26}. This led to the question: is the hexokinase activity of Hxk2 required for its function in signaling? Structure function studies suggested that although the hexokinase activity was not required for its role in catabolite
repression, the catalytic activity is required for its role in cAMP signaling (Fig 1.5) (Kraakman et al., 1999).

**Figure 1.5 Regulation of PKA.** The R subunit of PKA binds to cAMP which causes dissociation of the inactive tetramer and activation of PKA. Intracellular cAMP is generated by Adenylate Cyclase and cAMP is hydrolyzed by the Phosphodiesterases (Pde2). cAMP levels can be regulated by addition of glucose to cells growing in a non-fermentable carbon source, or by activation of the Ras pathway by intracellular acidification. In either situation, Hxk2 is required for the increase in cAMP.
1.3.4 Regulation of PKA tetramer and subunits

Because PKA has so many different functions in the cell, this suggests that there are many layers of regulation of PKA in addition to regulation by cAMP signaling. Indeed, in both higher eukaryotes and *S. cerevisiae*, phosphorylation of either the PKA catalytic and/or regulatory subunits has been identified as a mechanism of regulating PKA. Sub-cellular localization also seems to play a key role in mediating a specific PKA response. In both metazoans and *S. cerevisiae* the phosphorylation of the R subunit in response to some stimuli may regulate its localization (Griffioen, 2002 #27).

In metazoans, proteins called A kinase anchoring proteins (AKAPs) have been identified. These proteins by definition, are required to mediate the interaction between the R subunit of PKA and specific sub-cellular components. The AKAPs can also bind to other proteins including kinases and phosphatases, which implies that AKAPs may act as a platform for signaling to activate or inactivate PKA activity, and/or mediate the interaction of PKA and its specific substrate(s) (Colledge and Scott, 1999). Phosphorylation of the R subunit has also been shown to mediate the interaction of the R subunit with a specific AKAP. For example, RII localized to the centrosome by an AKAP can be phosphorylated by cyclinB-CDK which increases its specificity for an AKAP localized to the chromatin (Carlson et al., 2001). Therefore, in some cases, phosphorylation of the R subunit can influence the localization of the PKA holoenzyme.
1.3.4.1 Post translational modifications of PKA

At least some of the mechanisms that regulate phosphorylation or localization of PKA in multi-cellular organisms are conserved in *S. cerevisiae*. Bcy1 is phosphorylated in cells grown to stationary phase, cells growing in a non-fermentable carbon source and when cells undergo heat shock (Griffioen et al., 2001), (Griffioen et al., 2003). There are two serine-rich regions in the amino terminal region of Bcy1 which are required for phosphorylation of Bcy1 (Griffioen et al., 2001). Two of the kinases required for phosphorylation of Bcy1 have been identified. A protein kinase, Yak1, is required for phosphorylation of Bcy1 in conditions of limiting glucose (Griffioen et al., 2001), and glycogen synthase kinases (GSK3) are required for phosphorylation in response to heat shock and addition of extra-cellular calcium (Griffioen et al., 2003). Mammalian GSK3 has also been shown to phosphorylate the RII subunit *in vitro*, suggesting that this mechanism may be conserved (Werner-Washburne et al., 1991).

GSK3 kinases have many roles in the cell including the one they are named for, phosphorylation of glycogen synthase. One of the GSK3 kinases, Mck1 was shown to negatively regulate pyruvate kinase. Interestingly, further studies showed that Mck1 does not directly phosphorylate pyruvate kinase but instead regulates the function of PKA in order to negatively regulate pyruvate kinase (Rayner et al., 2002). Mck1 has been shown to have a role in meiosis and very weakly restores viability to *rad53Δ* or *mec1Δ* cells, suggesting Mck1 may have a function in the DNA damage checkpoint (Desany et al., 1998).
1.3.4.2 Localization of PKA in *S. cerevisiae*

There are no known AKAPs in *S. cerevisiae*; however, both the catalytic and regulatory subunits have been shown to differentially localize in response to certain stimuli. Cells growing in glucose rich conditions display almost complete nuclear localization of Bcy1 while cells growing in a non-fermentable carbon source have nearly even distribution of Bcy1 throughout the nucleus and cytoplasm (Griffioen et al., 2001). Heat shock also results in the nuclear-cytoplasmic distribution of Bcy1 (Griffioen et al., 2003). Nuclear/cytoplasmic localization of Bcy1 correlated with its phosphorylation, suggesting that, phosphorylation of the regulatory subunit (Bcy1) was required for its cytoplasmic localization, in a manner analogous to that in higher eukaryotes. Therefore, not surprisingly, cells expressing only phosphorylation defective Bcy1 did not display nuclear/cytoplasmic localization in conditions in which the localization of wild type Bcy1 was nuclear-cytoplasmic (Griffioen et al., 2003), (Griffioen et al., 2001).

Although no AKAPs have been identified in *S. cerevisiae*, one protein called Zds1 was identified in a yeast two hybrid screen which interacted with the amino terminal region of Bcy1 (Griffioen et al., 2001). Zds1 was found to be required for the cytoplasmic distribution of Bcy1 in cells growing in non-fermentable carbon sources (Griffioen et al., 2001). Further studies showed that either Zds1 or its paralog Zds2, were required for the cytoplasmic localization of Bcy1 in cells that had undergone heat shock (Griffioen et al., 2003). ZDS1 was identified in many different screens therefore it was given the name *zillion different screens* (ZDS). Many of the functions of Zds1 and Zds2 have been identified and further characterized. The Zds proteins have been shown to have functions in telomere silencing, aging, bud polarity, and cell cycle (Roy and Runge,
Deletion of both \textit{ZDS1} and \textit{ZDS2} results in a G2 delay of the cell cycle however the single mutants seem to have a normal cell cycle (Yu et al., 1996). The \textit{zds1}Δ mutant may have a slightly more severe defect as the cells have an elongated cell shape (polarity defect) and exhibit a slight delay in reaching anaphase when released synchronously from a G1 block (Mizunuma et al., 2001). These results indicated that Zds1 and Zds2 have at least some overlapping function. Further studies have shown that overexpression of Zds1 or Zds2 can restore growth to a cohesin mutant, suggesting that Zds1 and Zds2 may also play a role in regulating the transition from metaphase to anaphase (Heo et al., 1999).

\subsection*{1.4 Conclusions}

In conditions which lead to phosphorylation of the R subunit and/or cytoplasmic localization of the R subunit, cellular responses typically associated with PKA signaling such as trehalose mobilization and repression of STRE-controlled genes are not activated. This suggests that phosphorylation and or localization may regulate specific PKA functions that have not been identified yet. Activation of the PKA pathway can lead to inhibition of mitotic progression, therefore, we postulated that PKA could be activated by the DNA damage checkpoint in order to help prevent mitosis. Here I have described a complex regulatory network in place to regulate the activation of PKA. Using a model system like \textit{S. cerevisiae} will provide an excellent genetic tool to dissect the components that regulate PKA in response to DNA damage.
Chapter 2

2. Goal of Dissertation

2.1 Hypothesis

Because the kinetics of Pds1 degradation were faster in \textit{mec1} mutants than in \textit{chk1} mutants we wanted to determine what other pathways were regulating Pds1 stability in response to DNA damage. The PKA pathway was a strong candidate as activation of the PKA pathway led to inhibition of mitotic progression. Therefore, I hypothesized that PKA has a role in restraining mitosis in response to DNA damage.

2.2 Overview

After I determined that PKA had a role in the DNA damage checkpoint we set out to define the role of PKA in the checkpoint and determine how the DNA damage checkpoint signaled to PKA, I asked the following questions:

1) What is the mechanism by which PKA inhibits Pds1 destruction and progression through mitosis?
2) How does the DNA damage checkpoint regulate PKA in order to help inhibit mitotic progression?

Since the DNA damage checkpoint pathways are conserved in \textit{S. cerevisiae} and mammals, we used the genetically amenable \textit{S. cerevisiae} model system to address these questions.

Chapter 3 shows results from genetic experiments establishing that PKA has a role in the DNA damage checkpoint and that it acts in a separate pathway from Chk1 and
Rad53. To determine the mechanism by which PKA blocks the degradation of Pds1 and Clb2, I used a candidate approach to test whether Cdc20, which is required for the degradation of both Pds1 and Clb2, was the target of PKA following DNA damage. I found that the APC specificity factor, Cdc20, was phosphorylated in response to DNA damage, and that this phosphorylation was regulated by PKA. This phosphorylation prevented the interaction of Cdc20 with its substrate Clb2. From these data, a model was constructed in which PKA is regulated by the DNA damage checkpoint.

In Chapter 4 I test the hypothesis that PKA is regulated by the DNA damage checkpoint. In order to test this hypothesis, I had to first identify the mechanism by which PKA was regulated. Since phosphorylation of the R subunit was shown in *S. cerevisiae* and mammalian systems to regulate the sub-cellular localization and/or to provide specificity of PKA signaling, I investigated the role of the DNA damage checkpoint in phosphorylation of the R subunit. To further elucidate the mechanism by which the DNA damage checkpoint could regulate PKA, I used genetic techniques and found that three other proteins previously implicated in the regulation of PKA also had a role in the checkpoint including Hxk2, which links nutrient sensing to cAMP signaling. My results support the model that both R subunit phosphorylation and cAMP signaling are required for the role of PKA in restraining mitosis following DNA damage.
Chapter 3

3. The DNA damage checkpoint and PKA pathways converge on APC substrates and Cdc20 to regulate mitotic progression

3.1 Summary

In response to a DNA damage signal a signal transduction cascade is activated to prevent separation of the sister chromatids. The upstream kinase Mec1 is activated which in turn activates the effector kinases, Chk1 and Rad53. Chk1 stabilizes the mitotic inhibitor Pds1 in response to DNA damage; however, Pds1 is degraded faster in a \textit{mec1} mutant than in a \textit{chk1} mutant, suggesting that in WT cells following DNA damage there are at least two mechanisms to prevent Pds1 degradation, one of which proceeds through Chk1.

This chapter describes genetic experiments that show that the PKA pathway has a role in preventing Pds1 and Clb2 degradation, thus helping to restrain mitotic progression following DNA damage. In addition, we showed that PKA is acting in a separate pathway from either of the effector kinases, Chk1 or Rad53.

We also found that the APC specificity factor, Cdc20, which mediates the destruction of Pds1 and Clb2, was phosphorylated in response to DNA damage, and that this phosphorylation required a PKA catalytic subunit. Phosphorylation of Cdc20 in response to DNA damage prevented the interaction of Cdc20 with its substrate, Clb2, and phosphorylation-defective \textit{cdc20} mutants were defective in stabilizing Clb2 and mitotic arrest following DNA damage. These results suggested that the PKA and checkpoint mediated phosphorylation of Cdc20 had a role in preventing mitotic progression in response to DNA damage.
Our results support the model shown in Figure 3.1. In response to DNA damage, Cdc20 is phosphorylated in a checkpoint and PKA dependent fashion, and this phosphorylation prevents the interaction of Cdc20 with the APC substrates. Thus PKA mediated phosphorylation of Cdc20 helps prevent the ubiquitination and degradation of Pds1 and Clb2 and thus restrains mitotic progression.

**Figure 3.1** Our data supports a model in which PKA acts in a separate pathway from Chk1 and Rad53 to help inhibit mitotic progression. PKA helps stabilize Pds1 and Clb2 following DNA damage. Cdc20 is phosphorylated in a PKA dependent fashion following DNA damage and this phosphorylation blocks the interaction of Cdc20 and Clb2.
3.2 ABSTRACT

The conserved checkpoint kinases Chk1 and Rad53-Dun1 block the metaphase to anaphase transition by phosphorylation and stabilization of securin, and the mitotic exit network regulated by Bfa1/Bub2. However, both *chk1* and *rad53* mutants are able to exit mitosis and initiate a new cell cycle, suggesting that both pathways play supporting roles in restraining anaphase and in blocking inactivation of mitotic cyclin/Cdk1 complexes. We find that the PKA pathways play a supporting role with Chk1 in the regulation of mitosis by targeting the mitotic inducer Cdc20. Cdc20 is phosphorylated on PKA consensus sites following DNA damage and this phosphorylation requires the ATR orthologue, Mec1 and the PKA catalytic subunits Tpk1 and Tpk2. We show that inactivation of PKA or expression of phosphorylation-defective Cdc20 proteins in *chk1* mutants accelerates securin and Clb2 destruction and is sufficient to remove most of the DNA damage-induced delay. Mutation of the Cdc20 phosphorylation sites allowed interaction of Cdc20 with Clb2 under conditions that should halt cell cycle progression. These data show that PKA pathways regulate mitotic progression through Cdc20 and play a supporting role with the DNA damage checkpoint pathways to regulate Clb2 destruction and mitotic exit by stabilization of Pds1 and regulation of Cdc20.
3.3 INTRODUCTION

Ensuring genomic stability is of utmost importance to the viability of single cell organisms and necessary to prevent cancer in multi-cellular organisms. Accurate transmission of chromosomes to each daughter cell requires that cells restrain anaphase until all chromosomes have been completely replicated and correctly aligned on the spindle. Similarly, cells that have incurred DNA damage in late S phase or G2 delay progression through mitosis presumably to repair the damage. Genomic integrity is safeguarded in part by checkpoints, which are biochemical pathways that provide cells with a mechanism to detect DNA damage and respond by arresting the cell cycle to allow DNA repair (Zhou and Elledge, 2000). In addition, several stress-activated pathways have been identified in Saccharomyces cerevisiae that inhibit cell cycle progression by targeting proteins involved in mitotic progression (Clarke and Gimenez-Abian, 2000; Elledge, 1996).

Progression through mitosis is regulated by the sequential ubiquitin-mediated proteolysis of the anaphase inhibitor, Pds1 (securin), and the B type (S phase and mitotic) cyclins, Clb2 and Clb5. These steps are catalyzed by the multi-protein anaphase promoting complex or cyclosome (APC/C)(Cohen-Fix et al., 1996)(Irniger et al., 1995), which is an E3 ubiquitin ligase that is regulated, in part, by association with the Cdc20 and Hct1/Cdh1 proteins that act as specificity factors (Visintin et al., 1997). The APC^{Cdc20} mediates the ubiquitination and destruction of the anaphase inhibitor Pds1 (Shirayama et al., 1999)(Lim et al., 1998; Schott and Hoyt, 1998) that binds to and inhibits the caspase-like protease Esp1 (also known as separase)(Ciosk et al., 1998). Esp1 cleaves Scc1, a component of the cohesin complex that holds sister chromatids together, which leads to loss of cohesion and onset of anaphase (Uhlmann et al., 1999). Exit from mitosis and the establishment of pre-replication
complexes requires the inactivation of the mitotic cyclin dependent kinase (Cdk) complex, achieved in part by the APC-mediated proteolysis of the mitotic cyclins and by the increased levels of the Cdk inhibitor Sic1 (Verma et al., 1997).

Following a successful anaphase, the degradation of Clb5 and the initial degradation of the mitotic cyclin Clb2 (Shirayama et al., 1999) are also regulated by the APC\textsuperscript{Cdc20}. Clb2 ubiquitination and degradation leads to the activation of an intricate regulatory network that regulates mitotic exit (MEN)(Jaspersen et al., 1998). Activation of MEN involves the inactivation of the heteromeric Bfa/Bub2 GTPase-activating complex (GAP), a negative regulator of the Tem1 GTP-binding protein of the Ras superfamily that directs Clb2 destruction (Jaspersen et al., 1998; Pereira et al., 2000). One of the functions of MEN is the release of the Cdc14 phosphatase from the nucleolus, where it is sequestered by the regulator of nucleolar silencing and telophase (RENT) complex (Shou et al., 1999; Visintin et al., 1999). Activation of the polo-like kinase Cdc5 also promotes Cdc14 release from the nucleolus. Cdc14 catalyzes the reversal of Cdk phosphorylation events, which allows formation and full activation of the APC\textsuperscript{Hct/Cdh1} (Visintin et al., 1997) that directs the ubiquitination and second phase of degradation of the mitotic cyclin Clb2. Cdc14 also regulates accumulation of both Sic1 transcripts and protein (Visintin et al., 1998). Recent findings suggest that the first phase of Clb2 degradation, which is regulated by the APC\textsuperscript{Cdc20}, may be sufficient for mitotic exit. The destruction of Clb2 regulated by APC\textsuperscript{Hct/Cdh1} is necessary for further inactivation of Clb2/Cdk complexes (Baumer et al., 2000; Irniger et al., 1995; Lim et al., 1998)(Wasch and Cross, 2002), and is thought to allow the coordination of cell size and length of G1 in the following cell cycle (Wasch and Cross, 2002).
In *S. cerevisiae*, DNA damage or either a mono-oriented or unattached kinetochore triggers checkpoint pathways that bifurcate to regulate both the metaphase to anaphase transition and mitotic exit (Sanchez et al., 1999) (Wang et al., 2000) (Hu et al., 2001) (Alexandru et al., 1999; Li, 1999). Chk1 and Rad53, two checkpoint kinases activated by phosphorylation in response to DNA damage, form parallel branches downstream of the kinase Mec1 to regulate the metaphase to anaphase transition and mitotic exit, respectively (Gardner et al., 1999; Sanchez et al., 1999). Chk1 inhibits the metaphase to anaphase transition by phosphorylation and stabilization of Pds1 (Sanchez et al., 1999; Wang et al., 2001). Rad53 blocks Clb2 degradation and exit from mitosis by blocking the activation of the MEN. The latter is achieved by the Dun1-dependent activation or reversal of the inhibition of Bfa1 (Hu et al., 2001). Although Chk1 function is required for the stabilization of Pds1 following DNA damage, Pds1 is degraded with different kinetics in *chk1-*Δ and *mec1-*Δ cells in the presence of DNA damage (Sanchez et al., 1999), suggesting that proteins other than Chk1 may influence the rate of degradation of Pds1 in this response.

It has also been shown that mitotic progression is regulated by the cAMP-dependent protein kinase (PKA) pathway (Kishimoto and Yamashita, 2000). In *S. cerevisiae*, PKA consists of a complex containing any two of the three catalytic subunits, Tpk1, Tpk2, and Tpk3, and two Bcy1 regulatory (inhibitory) subunits (Cannon and Tatchell, 1987; Toda et al., 1987; Toda et al., 1987). Artificial activation of PKA by increasing the level of cAMP causes cells to arrest pre-anaphase with high levels of Pds1 and Clb2 proteins (Anghileri et al., 1999). In addition, conditional mutations in the subunits of the APC/C that result in a pre-anaphase arrest are suppressed when the PKA pathway is attenuated or inhibited, suggesting that PKA inhibits mitosis via the APC (Anghileri et al., 1999; Heo et al., 1999;
Yamada et al., 1997; Yamashita et al., 1996). In an *in vitro* system, mammalian PKA inhibits the APC, which correlates with phosphorylation of at least three of the APC components (Kotani et al., 1998).

To ensure the ordered progression through mitosis, the APC specificity factors are highly regulated. Due to its dual role in the induction of both anaphase and mitotic exit, *CDC20* transcript and protein levels are tightly regulated throughout the cell cycle (Goh et al., 2000; Prinz et al., 1998). Cells are highly sensitive to the concentration of Cdc20 protein, and overexpression of Cdc20 is lethal to the cell (Lim and Surana, 1996). In addition to being highly regulated in an unperturbed cell cycle, inhibition of Cdc20 by the checkpoint that monitors spindle attachment and orientation blocks sister chromatid separation (Hwang et al., 1998; Schott and Hoyt, 1998). Cdc20 may also play a role in the DNA damage checkpoint, as transient overexpression of Cdc20 renders cells more sensitive to DNA damage (Hwang et al., 1998; Lim and Surana, 1996). Cells overexpressing Cdc20 progress through and exit mitosis along with inactivation of Cdc28/Clb2 kinase activity in the presence of DNA damage (Lim and Surana, 1996), which is dependent on an active APC (Hwang et al., 1998).

In this study, we investigated 1) the interaction between the DNA damage checkpoint and PKA pathways in the regulation of mitotic progression and 2) the regulation of Cdc20 by these pathways. We show that deletion of *TPK1* or *TPK2* enhances the cell cycle arrest defect of *chk1*-Δ cells but does not enhance the defect in *rad53-21* cells, suggesting that PKA acts in a separate pathway from Chk1 and may be acting in the same or similar sub-pathway as Rad53. In addition, we show that PKA and DNA damage checkpoint pathways have a common target, Cdc20. Cdc20 can be phosphorylated by Tpk1 and Tpk2 and is phosphorylated on PKA consensus sites following DNA damage in a Tpk- and Mec1-
dependent manner. Cells expressing phosphorylation-defective mutated Cdc20 proteins are deficient in the checkpoint response and the $CDC20$ mutants enhance the rate at which $chk1$ and $rad53$ cells fail in the DNA damage induced arrest. The $CDC20$ phosphorylation mutants suppress the temperature sensitivity of the conditional APC mutant $cdc23-1$ in a similar manner to down-regulation of the PKA pathway, further supporting the model that Cdc20 acts downstream of PKA. Our results identify Cdc20 as a novel target of the DNA damage checkpoint and PKA pathways, which act cooperatively to regulate mitosis.

3.4 METHODS

**Plasmid and Strain construction:** All strains are congenic with Y300 except those derived from the B447411 strains as indicated and were derived using standard genetic techniques. Strains expressing HA-Pds1 were described before (Sanchez et al., 1999), FOA-resistant colonies of strains Y809 and Y811 were obtained for these studies. Mutagenesis of CDC20 was carried out by Quickchange PCR method (Stratagene) using pCM4 as a template, and the following primers:

S52A FOR: CGCTAAAGAGATCAAGTGCACTGAACATTAG
REV: CTAATGTTCAGTGCACTTGATCTCTTTAGCG

S88A FOR: GTTAATAAGACGAGATGCTTCATTTTTCAAAG
REV: CTTTGAAAAATGAAGCATCTCGTCTTATTAAC

Epitope tagging of Cdc20 was carried out by QuickChange method (Stratagene) of the constructs pCM4, pJS1, pJS2 and pJS7 with the following primers:
Cell cycle analyses and immunofluorescence were carried out as previously described (Sanchez et al., 1999).

Histidine tagged (His6) Cdc20 proteins were generated using the PCR and cloned into the pQE30 plasmid (Qiagen) for expression in M15 bacterial cells (Qiagen). Expression of proteins was induced at 37°C by addition of 1mM IPTG and the protein extracts prepared and bound to the nickel column under denaturing conditions following manufacturer’s instructions. After extensive washing the proteins were renatured, quantified by western analyses and used in vitro kinase reactions as previously described (Portela et al., 2001). For kinase assays with bacterially produced proteins, Gst-Tpk catalytic subunits isolated from yeast cells using glutathione sepharose (Pan and Heitman, 2002) were extensively washed with lysis buffer containing 1% deoxycholate and 200 mM NaCl. After analyses by SDS PAGE and coomassie staining for homogeneity, the proteins were eluted off the glutathione sepharose with 40 mM glutathione in 150 mM NaCl, 50 mM Tris-Cl, pH 7.5. The purified proteins were used to phosphorylate bacterially produced Cdc20 proteins in vitro.

### TABLE OF YEAST STRAINS USED IN THESE STUDIES

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- the tpk1-Δ and tpk2-Δ strains were generated by backcrossing strains from the deletion collection (B447411) 1261 and 1089 with Y300

**TABLE OF CONSTRUCTS USED IN THESE STUDIES**

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**Analyses of protein by Western, immunoprecipitation and pulldown assays.** Cells were grown to OD$_{600}$= 0.5-0.8 in YPD unless otherwise noted. When cells were subjected to the α-factor release/restore protocol in rich media, YPD at a pH of 3.9 was used to stabilize α-factor. Cells were synchronized in S phase by treatment with 200 mM HU. In cases where a gene was expressed under the control of the GAL promoter the cells were grown in YP raffinose as a carbon source. Galactose was added to 2 or 3 % to induce expression for 2 hours.

For western analyses cells were harvested and processed for protein extracts as described (Foiani et al., 1994) except that the proteins were separated on either 8% and 10% acrylamide gels or 10 and 12% acrylamide gels containing 0.067 % or 0.08% bisacrylamide, respectively unless otherwise indicated. The proteins were transferred to either nitrocellulose or PVDF
membranes and detected by using either anti-HA (16B12, COVANCE) anti-myc (9E10, COVANCE) or anti-Clb2, anti-GST (Santa Cruz Biotechnology) and Horseradish peroxidase conjugated secondary antibodies. The immunocomplexes were visualized by chemiluminescence (NEN). Pulldown assays/kinase assays were carried out as in (Sanchez et al., 1999) and (Pan and Heitman, 2002). Phosphatase treatment was carried out as in (Sanchez et al., 1999) except that the protein extracts were prepared following immunoprecipitation protocol (below). The immunoprecipitation was carried out with amount of extract that represented similar amount of HA-Cdc20 proteins as previously quantified by Western analyses. The protein extracts were diluted into 4 ml yeast lysis buffer for immunoprecipitation. Immunoprecipitation of Cdc20 to detect associated proteins was carried out as in (Hwang et al., 1998). Pulldown experiments were carried out in immunoprecipitation buffer as in (Hwang et al., 1998), except that they were carried out at room temperature and the last wash was carried out in 150mM KCl.

3.5 RESULTS

3.5.1 Deletion of TPK1 or TPK2 enhances the cell cycle arrest defect of chk1Δ cells.

In response to DNA damage, Pds1 is stabilized causing cells to delay the metaphase-anaphase transition (Cohen-Fix et al., 1996). Although Pds1 stabilization is mainly dependent on Chk1, Pds1 is degraded with different kinetics in chkl-Δ and mecl-Δ mutants, suggesting that Pds1 levels may be regulated by another mechanism in addition to Chk1 (Sanchez et al., 1999). Because activation of PKA also inhibits mitosis and cells arrest with high levels of Pds1 (Anghileri et al., 1999), we investigated whether the PKA pathway played a role in mitotic delay and Pds1 stability in response to DNA damage.
*cdc13-1* mutant cells grown at the restrictive temperature accumulate single-stranded DNA at the telomeres, inducing a DNA damage checkpoint-dependent mitotic arrest. *cdc13-1* cells with mutations in checkpoint genes such as *CHK1* or *RAD53* fail to arrest and form micro-colonies when cells are raised to the restrictive temperature (Weinert and Hartwell, 1993). Mutation of both *CHK1* and *RAD53* results in complete loss of mitotic delay that leads to micro-colonies with more cells than those of the single mutants (Fig. 3.2A). To determine the effect of a *tpk* deletion on the checkpoint defect of *cdc13-1chk1-Δ* and *cdc13-1rad53-21* cells, *TPK1* or *TPK2* deletions were introduced into strains containing the *cdc13-1* mutation in addition to *chk1-Δ* and/or *rad53-21*. The checkpoint defect of the mutant cells was quantified by determining the number of cells in the micro-colonies formed after plating the cells at the restrictive temperature.

After 8 hours at the non-permissive temperature, *chk1-Δ* and *rad53-21* mutants formed micro-colonies with an average of 13 or 10 cells each, respectively (Fig. 3.2B). The *cdc13-1tpk1-Δ* and *cdc13-1tpk2-Δ* cells did not form micro-colonies, and most cells arrested with 2 large-budded cells similar to the *cdc13-1* cells (Fig. 3.2B). *chk1-Δtpk1-Δ* or *chk1-Δtpk2-Δ* cells formed micro-colonies of greater than 20 cells on average, approximately the same number of cells per micro-colony as *chk1-Δrad53-21* cells. However, the *rad53-21tpk1-Δ* and *rad53-21tpk2-Δ* cells, on average, formed micro-colonies with the same number of cells as those formed by *rad53-21* cells (Fig. 3.2B). These results indicated that Tpk1 and Tpk2 have a role in the DNA damage checkpoint response. However, the *TPK1* and *TPK2* deletions did not cause a checkpoint defect on their own, indicating the existence
Figure 3.2: Deletion of TPK1 or TPK2 enhances the checkpoint defect of chk1-Δ cells. A and B. Y81t (cdc13-1) YJS7 (cdc13-1tpk1-Δ) YJS8 (cdc13-1tpk2-Δ) Y818 (cdc13-1chk1-Δ), YJS9 (cdc13-1chk1-Δtpk-Δ), YJS10 (cdc13-1chk1-Δtpk2-Δ) Y831 (cdc13-1rad53-21), YJS11 (cdc13-1rad53-21tpk1-Δ), YJS12 (cdc13-1rad53-21tpk2-Δ), Y836 (cdc13-1chk1-Δrad53-21), YJS13 (cdc13-1chk1-Δrad53-21tpk1-Δ) and YJS14 (cdc13-1chk1-Δrad53-21tpk2-Δ) were grown in YPD media at room temperature overnight, plated on pre-warmed plates and incubated at 30°C. Eight hours later the cells were examined for micro-colony formation and the number of cells per micro-colony were counted (n= 20). The graph represents the average number of cells per micro-colony for 3 different trials for each strain used. The error bars represent the standard deviation between the numbers obtained from the three different experiments.
Figure 3.2 (cont) C. cdc13-1 expressing HA-Pds1 (YJS41) and cdc13-1chk1-Δ cells expressing HA-Pds1 and containing either an empty vector (YJS34) or Bcy1 on a multi-copy plasmid (YJS35) were synchronized using HU in selection media shifted to the restrictive temperature of 32°C for cdc13-1 and released into YPD at 32°C. Cells were collected every 15 minutes and the cell lysates were separated on a 8% SDS-PAGE gel. The protein was transferred to a nitrocellulose membrane and probed with anti-HA to detect the HA-Pds1 or anti-Clb2. D. Y816 (cdc13-1) YJS7 (cdc13-1tpk1-Δ) YJS8 (cdc13-1tpk2-Δ) Y818 (cdc13-1chk1-Δ), YJS10 (cdc13-1chk1-Δtpk2-Δ) cells were synchronized in YPD containing HU and released into the cell cycle as in Fig. 1C. The cell lysates were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes and the membranes were probed with anti-Clb2 antibodies. E. TOP: Y816 (cdc13-1), Y818 (cdc13-1chk1-Δ), YJS9 (cdc13-1chk1-Δtpk1-Δ), YJS10 (cdc13-1chk1-Δtpk2-Δ), YJS47 (cdc13-1chk1-Δtpk1-Δtpk2-Δ) and YKS4 (cdc13-1chk1-Δrad9-Δ) cells were synchronized in YPD containing HU and released into the cell cycle and proteins analyzed as in Fig. 1D. BOTTOM: The same strains were used to monitor cells failing in the M-A checkpoint by scoring large budded cells with late nuclear division morphology (telophase) by DAPI staining.
of redundant pathways with PKA that function to regulate mitosis. In addition, these results suggested that Tpk1 and Tpk2 act in a separate pathway from Chk1, but could be a part of the Rad53 response or function in a pathway that overlaps with one of the Rad53 functions. The *chk1−Δtpk1−Δ* cells displayed checkpoint defect similar to *chk1−Δrad53-21* cells, suggesting that the PKA mutations were not only accelerating anaphase but also mitotic exit.

To determine whether inactivation of the PKA pathway had an effect on Pds1 and Clb2 levels in *chk1−Δ* cells following DNA damage, we examined Pds1 in *cdc13-1 chk1−Δ HA-PDS1* cells expressing the PKA regulatory subunit, Bcy1, from a multicopy plasmid. Cells synchronized in S-phase using the drug hydroxyurea (HU) were shifted to 32°C and released into the cell cycle at the restrictive temperature. Cells were removed every 15 minutes, and cell extracts were analyzed by Western analyses to visualize HA-Pds1 and Clb2. Both Pds1 and Clb2 were degraded with faster kinetics in the cells overexpressing Bcy1 than in cells containing an empty vector (Fig. 3.2C, compare time points 90 and 105 for Pds1 and 135 and 150 for Clb2).

To determine whether overexpression of Bcy1 had the same effect as deletion of *TPK1* or *TPK2* in enhancing the checkpoint defect of *chk1* mutants, we examined the Clb2 levels in *cdc13-1, cdc13-1tpk1−Δ, cdc13-1tpk2−Δ, cdc13-1chk1−Δ, cdc13-1chk1−Δtpk1−Δ* or *cdc13-1chk1−Δtpk2−Δ* cells. The experiment was carried out as in Fig. 3.2C with the exception that the cells were grown in YPD at all times. We found that Clb2 protein levels were maintained in the *cdc13tpk1−Δ, cdc13tpk2−Δ* (Fig. 3.2D) consistent with our findings the *cdc13tpk1−Δ, cdc13tpk2−Δ*, did not form microcolonies. However, the Clb2 levels decreased with faster kinetics in the *cdc13-1chk1−Δtpk1−Δ* and *cdc13-1chk1−Δtpk2−Δ* cells than in the *cdc13-1chk1−Δ* double mutants. Furthermore, the kinetics of Clb2 destruction in
cdc13-1chk1-Δtpk1-Δ and cdc13-1chk1-Δtpk2-Δ cells was identical to that of cdc13-1rad9Δ cells, which are completely defective in the M-A checkpoint (Fig. 3.2E, TOP). To determine whether the loss of Pds1 and Clb2 levels was concomitant with loss of pre-anaphase arrest in the cdc13-1chk1-Δtpk-Δ cells, we examined the kinetics by which the cells were failing in the pre-anaphase arrest by monitoring the percentage of cells exhibiting a late mitotic division phenotype in the presence of a DNA damage signal. We observed that the reduction in Pds1 and Clb2 levels correlated with the cells inability to delay anaphase (Fig. 3.2E BOTTOM panel). The cdc13-1chk1-Δtpk1-Δ and cdc13-1chk1-Δtpk2-Δ cells failed in the checkpoint with similar kinetics to cdc13-1 cells lacking the upstream checkpoint component Rad9, which indicates that the chk1tpk mutants had lost all checkpoint-induced delay. In addition, deletion of both TPK1 and TPK2 genes did not exacerbate the checkpoint defect of the cdc13-1chk1-Δtpk1-Δ mutants. These results support our findings from both the micro-colony assay as well as the Bcy1 overexpression experiment and indicate that the PKA pathway influences the stability of both Pds1 and Clb2 in response to DNA damage.

3.5.2 Cdc20 contains consensus sites for phosphorylation by PKA and is phosphorylated on these residues following DNA damage.

Inactivation of the PKA pathway enhances the rate of Pds1 and Clb2 degradation in chk1 mutants, and genetic and biochemical studies have shown that PKA inhibits mitosis by inhibiting the APC/C, leading to increased levels of Pds1 (Anghileri et al., 1999). Our data suggests that PKA could have a role in the regulation of mitotic progression following DNA damage by inhibiting the APC or its specificity factors. Of the two APC specificity factors, Cdc20 is necessary for the ubiquitin-mediated degradation of both Pds1 and Clb2; thus, we
hypothesized that PKA regulation of the APC could occur through inactivation of the specificity factor Cdc20. We first addressed this hypothesis by determining whether Cdc20 was modified or phosphorylated in response to DNA damage and whether the PKA catalytic subunits could interact with and phosphorylate Cdc20.

To analyze the protein levels of Cdc20 throughout the cell cycle and in response to DNA damage, wild-type and cdc13-1 cells expressing HA-Cdc20 under control of its own promoter were synchronized in G1 using α-factor and then grown at the restrictive temperature (31º C) to induce a DNA damage signal. The cells were released from α-factor-mediated arrest, and α-factor was added back to the cultures when the cells were in S phase in order to prevent the wild-type cells (no DNA damage signal) from progressing into a new cell cycle. Aliquots of cells were taken at indicated time points and cell extracts were examined by Western analysis to visualize HA-Cdc20. Cdc20 protein was first detected 60 minutes after release from α-factor and the levels were high in mitosis and rapidly declined as the cells completed mitosis and the protein was undetectable by the time the cells reached the G1 block, consistent with the reports of others (Goh et al., 2000; Hwang et al., 1998; Prinz et al., 1998)(Fig. 3.3A). However, in cells with a DNA damage signal, Cdc20 protein remained high throughout the pre-anaphase arrest.

Since the Cdc20 levels were the highest in mitosis and it is at this point that we would predict that a DNA damage-induced modification would occur, we compared the Cdc20 proteins from cells in mitosis, in the presence or absence of a DNA damage signal. For this, the same strains as in Figure 2A were synchronized in S-phase (HU), and then grown at the restrictive temperature (31º C) to induce a DNA damage signal. The cells were released from HU into media containing the microtubule destabilizing drug nocodazole to prevent the
wild-type cells (no DNA damage signal) from progressing through mitosis. Aliquots of the cells were taken every 15 minutes and the cell extracts were examined by western analysis to visualize HA-Cdc20. Cdc20 protein began to appear 45 minutes after release from HU (Fig. 3.3B). Cdc20 underwent an electrophoretic shift in mobility beginning 90 minutes after release from HU in cdc13-1 cells but not in the wild-type cells blocked in mitosis with nocodazole (Fig.3.3B, TOP). The modification of Cdc20 occurred only in response to DNA damage as it was observed in cells collected 120 min after release from HU in the presence (cdc13-1) but not in the absence (CDC13) of a DNA damage signal (Fig. 3.3B, BOTTOM).

In addition, the slower migrating forms of Cdc20 were restored to the faster migrating form by the addition of phosphatase but not by the addition of phosphatase and phosphatase inhibitors (Fig. 3.3C). Incubation of Cdc20 with phosphatase resulted in a faster migrating form of Cdc20 that migrated at the same position as Cdc20 protein from cells arrested in mitosis with nocodazole (Fig. 3.3C, compare first and third lanes). These results indicated that Cdc20 is phosphorylated in response to DNA damage.

To determine whether Cdc20 could form a complex with the yeast PKA catalytic subunits, GST-Tpk1 and GST-Tpk2 on beads were incubated with HA-Cdc20 from cdc13-1 cells that had been incubated at the restrictive temperature for 2h. The protein complexes were examined for the presence of Cdc20 by Western analyses. We observed that Cdc20 was pulled down by the beads bound with GST-Tpk1 and GST-Tpk2, but not by a control GST peptide (Fig. 3.3D). We then examined whether the association between Tpk1/Tpk2 and Cdc20 was regulated by a checkpoint signal by performing the pulldown experiment with extracts from MEC1 and mec1-Δ cells with a DNA damage signal or MEC1 cells arrested
Figure 3.3: Cdc20 is phosphorylated in response to DNA damage, interacts with Tpk1/Tpk2 and is phosphorylated by Tpk2 *in vitro*. A. YJS2 (wild-type) and YJS16 (cdc13-1) cells expressing an HA tagged Cdc20 were synchronized in G1 phase with α-factor, raised to the restrictive temperature and released into media at the restrictive temperature of 31°C. Alpha factor was added back to the cultures to prevent wild-type cells from entering another cell cycle. Cells were collected at indicated timepoints after release from, and proteins separated on a 10% acrylamide gel. HA-Cdc20 was visualized by Western analyses with anti-HA antibodies the bottom panels for each time course experiment shows a cross-reacting band as loading control. B. YJS2 (wild-type) and YJS16 (cdc13-1) cells expressing an HA tagged Cdc20 were synchronized in S phase with HU, raised to the restrictive temperature and released into media at the restrictive temperature. Nocodazole was added to the media that YJS2 cells were released into to block cells in mitosis. Cells were collected at indicated timepoints after release from HU, and proteins separated on a 10% acrylamide gel. HA-Cdc20 was visualized by Western analyses with anti-HA antibodies. Shown is the expression of HA-Cdc20 in cdc13-1 cells during the time course of the experiment. To compare modified and unmodified forms of Cdc20, proteins from cells collected 60 or 120 minutes after release from HU were separated side-by-side by SDS-PAGE and analyzed by western analysis using anti-HA antibodies. C. YJS2 (wild-type) and YJS16 (cdc13-1) cells were raised to the restrictive temperature and nocodazole was added to the YJS2 cells to prevent them from progressing through mitosis. After 120 min the cells were collected and protein extracts were prepared (Foiani et al., 1994). HA-Cdc20 was isolated by immunoprecipitation and treated with alkaline phosphatase buffer plus phosphatase inhibitors (first and second lanes), alkaline phosphatase (third lane) or alkaline phosphatase plus phosphatase inhibitors (fourth lane). D. Extracts from (YJS16) cdc13-1 cells expressing HA-Cdc20 at the restrictive temperature were incubated with glutathione beads, beads bound to a GST-control peptide (fragment of human p53) or GST-Tpk1/GST-Tpk2 beads. The protein complexes were washed in IP buffer containing 150 mM KCl (see methods), separated by SDS-PAGE and analyzed by western analysis using anti-HA and anti-GST antibodies. E. Extracts from YJS2 (wild-type) cells incubated in nocodazole and (YJS16) cdc13-1 and (YJS21) cdc13-1mec1-Δ cells grown at the restrictive temperature, all expressing HA-Cdc20, were incubated with GST-Tpk1/GST-Tpk2 beads. The protein complexes were washed in IP buffer containing 150 mM KCl (see methods), separated by SDS-PAGE and analyzed by western analysis using anti-HA and anti-GST antibodies.
with nocodazole. We found that the association of Cdc20 with Tpk1/Tpk2 was increased in response to DNA damage in a Mec1-dependent manner (Fig. 3.3E).

PKA phosphorylates substrates on serine or threonine residues, and has overlapping consensus sites with Chk1 and Dun1 kinases (O'Neill et al., 2002; Sanchez et al., 1997) (R-x-x-S/T), where R/K is preferred at position –2 with respect to S/T for PKA and Dun1. Cdc20 contains two such consensus sites at serines 52 and 88 (Fig. 3.4A), which were changed to alanine residues to prevent phosphorylation at these sites. The mutations in Cdc20 did not compromise the S phase checkpoint since \textit{S. cerevisiae} strains expressing wild-type, Cdc20-S52A, Cdc20-S88A and Cdc20-S52AS88A mutated Cdc20 proteins were not sensitive to 80mM hydroxyurea (data not shown), which allowed us to use HU to synchronize \textit{cdc13-1} cells expressing wild-type HA-\textit{CDC20} or HA-\textit{CDC20S52AS88A} in S-phase prior to inactivation of Cdc13 at the restrictive temperature and releasing cells to a DNA damage signal. The cells were collected 135 minutes after release from the S-phase block in order to examine the Cdc20 proteins. The wild-type Cdc20 protein exhibited slower electrophoretic mobility while the Cdc20-S52AS88A mutated protein did not (Fig. 3.4B). In order to determine that the DNA damage induced phosphorylation of Cdc20 occurred on Serines 52 and/or 88 we compared the migration of the mutated protein Cdc20-S52AS88A to that of wild-type protein in cells with a DNA damage signal (\textit{cdc13-1}) and to wild-type protein in cells with no DNA damage signal (\textit{CDC13}). Because the \textit{cdc13-1} signal causes a delay at M-A a stage in which Cdc20 protein accumulates, the wild-type cells were synchronized in mitosis with nocodazole. The phosphorylation defective mutated protein migrated at the same position as the wild-type protein in cells without DNA damage signal (Fig. 3.4B, Bottom right). In order to determine whether Cdc20 could be phosphorylated by the PKA
catalytic subunits, GST-Tpk1 and GST-Tpk2 proteins were isolated from *S. cerevisiae* cells (Pan and Heitman, 2002). A cocktail containing soluble catalytic subunits, cAMP and $\gamma^{32}\text{P}$ ATP was prepared and 0.2 pmol of each soluble subunit were incubated with or without bacterially expressed Cdc20 (Pan and Heitman, 2002; Portela et al., 2001). Labeled phosphate groups were incorporated into Cdc20 indicating that Tpk1/2 phosphorylated Cdc20 *in vitro* (Fig. 3.4C). In addition the *in vitro* phosphorylation of Cdc20 by Tpk1 and Tpk2 catalytic subunits was much reduced in the Cdc20-S88A and Cdc20-S52AS88A mutated proteins (Figure 3.4C). These results indicated that the PKA catalytic subunits phosphorylate Cdc20 on Ser88 and possibly Ser52, the same residues that are phosphorylated in response to DNA damage.

3.5.3 The DNA damage-induced phosphorylation of Cdc20 is regulated by the checkpoint and PKA pathways.

In order to determine the role of checkpoint kinases and PKA in the DNA damage-induced phosphorylation of Cdc20, we examined the phosphorylation of HA-Cdc20 in *cdc13*-1, *cdc13*-1chk1-*Δ*, *cdc13*-1rad53-21, *cdc13*-1mec1-*Δ*, *cdc13*-1tpk1-*Δ*, *cdc13*-1tpk2-*Δ* and *cdc13*-1chk1-tpk1-*Δ* cells after 2h of incubation at the restrictive temperature. The DNA damage-induced phosphorylation of Cdc20 was abrogated in cells lacking Tpk1 or Tpk2, supporting a role for these kinases in the regulation of Cdc20 following DNA damage (Fig. 3.5A). The form of Cdc20 in *tpk1*, *tpk2* and *chk1tpk1* cells migrated at the same position as the phosphorylation defective Cdc20-S52AS88A protein in *cdc13*-1 cells (Fig. 3.5A), further supporting the role of the PKA pathways in the phosphorylation of Cdc20 on Ser52 and
Figure 3.4: Cdc20 is phosphorylated on Ser52 and Ser88 in response to DNA damage.  

A. Cdc20 amino acid sequence was analyzed for consensus sites for phosphorylation by PKA.  Cdc20 contains two consensus sites for phosphorylation by protein kinase A, which also fit consensus sites for Chk1 and Dun1. $\phi =$ hydrophobic residue.  Each site was changed from a serine residue to an alanine.  

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<tr>
<td>Dun1:</td>
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Cdc20 sites: L K R S $S_2$ L I R R D $S_8$ A

B.  

**cdc13-1** cells expressing either wild-type HA-CDC20 (YJS16) or HA-CDC20-S52AS88A (YJS17) were arrested with HU and a DNA damage signal was induced by growing the cells at the non-permissive temperature for cdc13-1.  TOP: Proteins prepared from cells removed at the indicated timepoints were separated on a 10% acrylamide gel.  HA-Cdc20 was visualized as in Fig. 2A.  BOTTOM LEFT: To separate modified forms of Cdc20 the proteins from cells collected 135 minutes after release from HU and were separated on a 12% low bisacrylamide SDS-PAGE gel and analyzed by Western blotting as above.  RIGHT: Proteins from cdc13-1 cells expressing either wild-type HA-CDC20 (YJS16) or HA-CDC20-S52AS88A (YJS17) grown at the restrictive temperature were resolved next to proteins from YJS2 (wild-type) cells expressing wild-type HA-CDC20 that had been synchronized in mitosis with the drug nocodazole and the proteins were visualized as above.  

C. Tpk1 and Tpk2 (PKA catalytic subunits) were isolated from (XPY310 and XPY311) yeast overexpressing Gst-Tpk1 and Gst-Tpk2 (Pan and Heitman, 2002), using glutathione sepharose (Pan and Heitman, 2002) and were extensively washed with lysis buffer containing 1% deoxycholate and 200 mM NaCl.  LEFT: The washed Tpk subunits were analyzed by SDS PAGE and coomassie staining to determine homogeneity.  C= control peptide used in pulldown experiments (Fig. 2).  RIGHT: Soluble proteins from this preparation were incubated with bacterially produced wild-type and phosphorylation-defective mutated Cdc20-His$_6$ proteins purified on Nickel agarose, along with $\gamma^{32P}$ ATP (Portela et al., 2001).  Proteins were resolved by SDS-PAGE, fixed, stained with coomassie and analyzed by autoradiography.
Ser88 following DNA damage. The DNA damage-induced phosphorylation of Cdc20 was abrogated in cells lacking Mec1 and was reduced in \textit{chk1}Δ but not in \textit{rad53-21} cells (Fig. 3.5B), indicating that the phosphorylation of Cdc20 is regulated by the DNA damage checkpoint and that the PKA pathway was activated in \textit{rad53-21} cells. Because inactivation of PKA signaling did not enhance the checkpoint defect of \textit{rad53} cells (Fig. 3.2B), yet \textit{rad53} cells are proficient in the phosphorylation of Cdc20, we examined the levels of Cdc20 protein in \textit{cdc13-1RAD53} and \textit{cdc13-1rad53-21} cells following synchronization in G1 with \textit{α}-factor and release into the cell cycle at the restrictive temperature for \textit{cdc13-1}. \textit{α}-factor was added back to the culture after the cells entered S phase in order to prevent the \textit{rad53-21} cells (checkpoint defective), from initiating a new cell cycle after failing the DNA damage-induced block in mitosis. Cdc20 levels increased in both \textit{cdc13-1} and \textit{cdc13-1rad53-21} cells; however, the Cdc20 levels were maintained in \textit{cdc13-1} but not in the \textit{cdc13-1rad53-21} cells (Fig. 3.5C). In fact, the levels of Cdc20 dropped concomitantly with a drop in Clb2 levels (Fig. 3.5C), suggesting that inappropriate activation of the MEN in \textit{rad53} cells could lead to the activation of APC/C^{Hct/Cdh1}, which targets Cdc20 for ubiquitination.

\textbf{3.5.4 Activation of PKA restored Clb2 stability and checkpoint delay to \textit{mec1-21} cells.}

To determine whether PKA signaling functions downstream of the checkpoint kinase Mec1, we increased PKA signaling by overexpression of the catalytic subunits Tpk1 or Tpk2 and examined whether increased PKA signal could restore cell cycle delay and B-type cyclin (Clb2) stability in \textit{mec1-21} cells following DNA damage. Using the \textit{cdc13-1} mutation to induce DNA damage, wild-type and \textit{mec1-21} cells transformed with a high copy plasmid
Figure 3.5: The DNA damage-induced phosphorylation of Cdc20 is defective in cells lacking Tpk1, Tpk2 or Mec1; and overexpression of TPK1 or TPK2 restored Clb2 stability and checkpoint delay to mec1-21 cells. 

A. YJS16 (cdc13-1), YJS43 (cdc13-1tpk1-Δ), YJS44 (cdc13-1tpk2-Δ) YJS42 (cdc13-1tpk1-Δ chk1-Δ) cells expressing an HA tagged Cdc20 and YJS17 (cdc13-1) cells expressing HA-Cdc20-S52AS88A were grown to logarithmic phase and raised to the restrictive temperature for 2 h. Cells were collected, and proteins were prepared by TCA precipitation, separated on 8% PAGE gels and analyzed by Western analysis using anti-HA antibodies.

B. YJS16 (cdc13-1), YJS45 (cdc13-1chk1-Δ), YJS46 (cdc13-1rad53-21) YJS21 (cdc13-1mec1-Δ) cells expressing HA-Cdc20 and YJS17 (cdc13-1) cells expressing HA-Cdc20-S52AS88A were treated as above, and the proteins separated and analyzed by Western analyses using anti-HA antibodies.

C. YJS16 (cdc13-1) and YJS46 (cdc13-1rad53-21) expressing HA-Cdc20 were synchronized in α-factor and released at 32°C. α-factor was added back to the cultures 45 min after release to prevent cells from entering another cell cycle. Cells were collected at the indicated time points and processed and analyzed as above. D. cdc13-1 cells transformed with a vector and cdc13-1mec1-21 cells transformed with a vector (pRS426) or high copy plasmids encoding TPK1 (pXP2 (Pan et al., 2000)) or TPK2 (pXP3 (Pan et al., 2000)) were synchronized in G1 using the pheromone α-factor and released into the cell cycle at the restrictive temperature for cdc13-1. Aliquots of cells were removed at the indicated timepoints for analyses of Clb2 levels by western analyses. E. Aliquots were also removed for monitoring cell cycle progression of the cdc13-1mec1-21 cells transformed with a vector (pRS426) or high copy plasmids encoding TPK1 or TPK2 by analysis of nuclear morphology following staining with DAPI. All strains are cdc13-1mec1-21, except where indicated for wild-type (cdc13-1MECl).
encoding TPK1, TPK2 or the empty vector, were synchronized in G1 using α-factor and released into the cell cycle at the restrictive temperature for cdc13-1. Aliquots of cells were collected at the indicated timepoints and the levels of Clb2 protein were monitored using Western analyses. Failure in the M-A checkpoint was measured by scoring the cells that displayed a late mitotic division phenotype.

The wild-type cells maintained high levels of Clb2 as previously shown (Fig. 3.2 and Fig 3.5D) and mec1-21 cells transformed with a vector failed to maintain Clb2 levels consistent with a M-A checkpoint defect as evidenced by an increased number of cells with late mitotic division phenotype (Fig. 3.5D and E). However, the mec1-21 cells expressing high levels of Tpk1 or Tpk2 maintained levels of Clb2 similar to what was observed for the wild-type cells (Fig. 3.5D). Furthermore, mec1-21 cells expressing high levels of Tpk1 or Tpk2 had fewer cells with late mitotic division phenotype compared to the mec1-21 cells transformed with a vector, indicating that overexpression of Tpk1 or Tpk2 caused a reduction and delay in the number of cells failing in the checkpoint (Fig. 3.5E). In a second approach we increased PKA signaling by triggering intracellular acidification, which has been shown to increase cAMP levels via inhibition of the Ras GTPase activating proteins Ira1 and Ira2 (Colombo et al., 1998) (Fig. 3.5S). Increased levels of cAMP mediated by intracellular acidification also restored Clb2 stability in mec1-21 cells following DNA damage (Fig. 3.5S). These data support our hypothesis that PKA signaling functions downstream of the checkpoint kinase Mec1 to inhibit Clb2 destruction and halt progression through mitosis in the presence of DNA damage.
3.5.5 Expression of Cdc20-S88A enhances the rate at which cdc13-1 chk1-Δ cells enter telophase.

Cdc20 is an inducer of mitotic progression; thus, the phosphorylation of Cdc20 in response to DNA damage should be inhibitory. Phosphorylation-defective mutants should be refractory to checkpoint signal and should promote mitosis in the presence of damage; i.e., should act as dominant mutations. We set out to test the hypothesis that Cdc20 phosphorylation was required to down-regulate its activity and/or abundance in response to DNA damage. We wanted to determine the relationship between Cdc20 and the DNA damage checkpoint pathways. Our results suggested that Tpk1 and Tpk2 were acting in a separate pathway from Chk1; therefore, we predicted that expression of Cdc20-S88A (the major site phosphorylated in vitro by Tpk1 and Tpk2) would enhance the checkpoint defect of chk1-Δ cells, similar to the effect observed in chk1-Δ tpk1-Δ or chk1-Δ tpk2-Δ cells. To

figure 3.5S. Increased PKA signaling via intracellular acidification restored Clb2 stability to mec1-21 cells. cdc13-1 and cdc13-1mec1-21 cells were synchronized in G1 using the pheromone α-factor and released into the cell cycle in medium at pH 3.9 or 6.3 at the restrictive temperature for cdc13-1. DNP was added to the medium 40 min after release from the block. Aliquots of cells were removed at the indicated timepoints for analyses of Clb2 levels by western analyses.
test this, a synchrony experiment was carried out using strains expressing wild-type Cdc20 or Cdc20-S88A in a \textit{cdc13-1}, \textit{cdc13-1chk1-\Delta} or \textit{cdc13-1rad53-21} background.

Cells carrying an empty vector or a plasmid copy of either wild-type \textit{CDC20} or mutated \textit{CDC20} with an alanine at Ser88, each under the control of the endogenous promoter, were arrested in G1 with the pheromone \(\alpha\)-factor. Loss of Cdc13 protein function leads to a pre-anaphase DNA damage-induced arrest. The cells were released into the cell cycle at the restrictive temperature for \textit{cdc13-1} and analyzed at different time points to determine nuclear and bud morphology. The appearance of divided nuclei (telophase cells) was indicative of cells that had failed the checkpoint arrest and had progressed through mitosis. The Cdc20-S88A mutated proteins behaved in a dominant manner and were able to drive a small percentage of the cells through mitosis in the presence of a DNA damage signal (Fig. 3.6A), indicating that the Cdc20-S88A mutants are not responsive to the DNA damage and that expression of the mutated Cdc20 protein renders the cell checkpoint deficient.

Expression of Cdc20-S88A protein enhanced the checkpoint defect of a \textit{chk1} mutant (Fig. 3.6A). The \textit{cdc13chk1-\Delta} mutants expressing the \textit{CDC20-S88A} allele exhibited kinetics of mitotic progression similar to cells that contain mutations in both \textit{CHK1} and \textit{RAD53} (data not shown). The expression of Cdc20-S88A protein had the same effect as deletion of \textit{TPK1} or \textit{TPK2} in the \textit{chk1-\Delta} background (compare Fig. 3.6A with Fig. 3.2E); however, expression of Cdc20-S88A also enhanced the checkpoint defect of \textit{rad53-21} cells (Fig. 3.6A). These results suggested that Cdc20 acts in a separate pathway from Chk1. Since expression of the Cdc20-S88A also enhanced the checkpoint defect of \textit{rad53-21} cells, this suggested that more than one pathway converges on Cdc20. There are at least two models that could explain
these results. First, Cdc20 could be acting downstream of both the Chk1 and Rad53 pathways or second, Cdc20 could be targeted by a parallel pathway to both Chk1 and Rad53 branches.

In order to determine the mechanism by which the Cdc20-S88A accelerated mitosis in a \textit{chk1-}∆ background we compared the rates of Clb2 degradation in \textit{cdc13-1 chk1-}∆ cells expressing either Cdc20-S52AS88A mutant or Bcy1 following inactivation of \textit{cdc13-1}. \textit{cdc13-1} or \textit{cdc13-1chk1-}∆ cells with a tagged chromosomal copy of \textit{PDS1}, and containing a plasmid copy of \textit{CDC20} or \textit{CDC20S52AS88A} or an empty vector were synchronized in S-phase and released into the cell cycle with a DNA damage signal. Expression of Cdc20-S52AS88A enhanced the rate at which the Pds1 and Clb2 protein levels decreased in the \textit{chk1-}∆ cells and exhibited similar kinetics as the \textit{chk1-}∆ cells overexpressing Bcy1 (Fig. 3.6B). Mutation of the Ser88 and Ser52 residues on Cdc20 caused the same effect on Pds1 and Clb2 protein levels as inactivation of the PKA pathway (compare figures 3.6B with 3.2E), which supports the hypothesis that Cdc20 is a target of the PKA pathway(s), and that the regulation of Cdc20 by PKA occurs via phosphorylation of Ser52 and/or Ser88 residues.

3.5.6 Phosphorylation of Cdc20 is required to block interaction with Clb2 in the presence of a DNA damage signal.

We further investigated the mechanism by which phosphorylation regulated Cdc20 function. One possibility was that phosphorylation regulates Cdc20 interaction with its substrates and thus the phosphorylation defective mutated proteins would still bind and presumably promote ubiquitination of Pds1 and/or Clb2. To test this, wild-type and \textit{cdc13-1} cells expressing HA-Cdc20 under control of its own promoter were grown at the restrictive temperature (32°C) to induce a DNA damage signal. The Cdc20 proteins were
Figure 3.6: Phosphorylation-defective Cdc20 mutated proteins interact with Clb2 in the presence of a DNA damage signal and their expression accelerates Clb2 destruction and the checkpoint defect in chk1-Δ cells. An extra plasmid copy of CDC20 or CDC20-S88A enhances the checkpoint defect of chk1-Δ and rad53-21 cells. A. cdc13-1 expressing wild-type CDC20 (YJS18), CDC20-S88A (YJS20) or containing an empty vector (YJS15); cdc13-1chk1-Δ expressing wild-type CDC20 (YJS29), CDC20-S88A (YJS30) or containing an empty vector (YJS28) and cdc13-1rad53-21 cells expressing wild-type CDC20 (YJS39), CDC20-S88A (YJS40) or containing an empty vector (YJS38) were synchronized in G1 using α-factor and DNA damage signal was induced by incubation of the cells at the non-permissive temperature of 32°C for cdc13-1. Cells were released into the cell cycle, aliquots of the cells were taken every 15 minutes, and the cells were stained with DAPI and α-tubulin antibody to visualize the nuclei and spindles. The percentage of cells exhibiting late nuclear division (telophase) were calculated and graphed. B. cdc13-1 cells containing vector only (YJS41) and cdc13-1chk1-Δ cells containing vector only (YJS34), a centromeric plasmid encoding wild-type CDC20 (YJS36), CDC20-S52A88A (YJS37); or BCY1 on a multi-copy plasmid (YJS35) were synchronized and the proteins were detected and visualized as in Fig. 1D. C. TOP: YJS2 (wild-type) cells synchronized in mitosis with nocodazole (no damage) and cdc13-1 cells containing vector only (YJS15) or a centromeric plasmid encoding wild-type CDC20 (YJS16) were grown to log phase and shifted to 32°C for 2 hours. HA-Cdc20 proteins were isolated by immunoprecipitation, the immune complexes separated by SDS-PAGE and analyzed by western analysis using anti-HA and anti-Clb2 antibodies. BOTTOM: cdc13-1 cells containing vector only (YJS15) or a centromeric plasmid encoding wild-type CDC20 (YJS16) or CDC20-S52A88A (YJS17) were grown to log phase and shifted to 32°C for 2 hours. HA-Cdc20 proteins were isolated by immunoprecipitation and analyzed as above. D. The HA-CDC20 wild-type and mutants under the control of the endogenous promoter were transformed into cdc23-1 cells (YJS22, YJS23, YJS24, YJS25, YJS26). As a positive control a high copy plasmid encoding the PKA regulatory subunit Bcy1 was also transformed into the cdc23-1 cells (YJS27). Transformants were streaked out at the semi-permissive temperature of 22°C (Palmer et al., 1989). E. Model of interaction between checkpoint and PKA pathways, see text. NOTE: although we have drawn Clb2 as the cyclin partner of Cdk1 that inhibits Hct1/Cdh1, several Cyclin/Cdk1 complexes, including Clb5/Cdk1 have been shown to phosphorylate Hct1/Cdh1.
immunoprecipitated with anti-HA antibodies and the immune complexes were analyzed for the presence of Clb2. The Cdc20 wild-type protein interacted with its target, Clb2, in cells arrested with nocodazole but failed to interact with Clb2 in the presence of the cdc13-1-induced DNA damage signal (Fig. 3.6C, TOP); whereas, the phosphorylation defective mutated proteins were able to interact with Clb2 under conditions that should halt cell cycle progression (Fig. 3.6C, BOTTOM). These data suggest that phosphorylation of Cdc20 serves to inhibit binding to Clb2, which would result in the maintenance of high Clb2/Cdk1 kinase activity.

3.5.7. Down regulation of PKA signaling or expression of Cdc20S52-S88A restored growth to a temperature-sensitive mutant of the APC component Cdc23.

Inhibition of the PKA pathway has been shown to suppress the temperature sensitivity of several APC mutants, including cdc16-1, cdc23-1, cdc27-1 and apc10 (Anghileri et al., 1999; Heo et al., 1999; Irniger et al., 2000). Because deletion of TPK1 or TPK2 removes the DNA damage-induced phosphorylation of Cdc20 in vivo, we postulated that PKA signaling inhibits mitotic progression in APC mutants by phosphorylation and inactivation of Cdc20. If that were the case, then expression of Cdc20 proteins with mutations at Ser52 or Ser88 should rescue the temperature sensitivity of APC mutants in the same way as inhibition of PKA.

Wild-type Cdc20, Cdc20-S52A, Cdc20-S88A, or Cdc20-S52AS88A were expressed in cdc23-1 cells (Palmer et al., 1989). For a positive control, BCY1, the inhibitory subunit of PKA, was also expressed in the cdc23-1 cells from a multi-copy plasmid. The transformants
were selected on ura- media at 13°C for several days and colonies were streaked out and grown at 22°C, the semi-permissive temperature for this strain when grown on minimal media. \textit{cdc23-1} cells containing multiple copies of \textit{BCY1} were able to grow at 22°C, while cells containing the empty vector grew poorly at 22°C (Fig. 3.6E). Cells expressing Cdc20-S88A or Cdc20-S52AS88A also suppressed the temperature sensitivity of \textit{cdc23-1} mutants and allowed the \textit{cdc23-1} cells to form colonies at 22°C (Fig. 3.6E). These results suggested that phosphorylation on Ser88 and Ser52 of Cdc20, presumably by PKA, causes inhibition of the APC and hence inhibition of mitosis.

### 3.6 DISCUSSION

**The checkpoint pathways cooperate to regulate anaphase entry and mitotic exit.**

In its most simple form, the DNA damage checkpoint pathway bifurcates to regulate the checkpoint kinases Chk1 and Rad53. Chk1 inhibits the metaphase to anaphase transition via the stabilization of Pds1 and the Rad53/Dun1 pathway inhibits mitotic exit (Hu et al., 2001; Sanchez et al., 1999; Wang et al., 2000). Although the Rad53 and Chk1 branches are largely independent, they act in a partially redundant manner to reinforce each other. \textit{rad53} mutants eventually enter anaphase despite Chk1 phosphorylation of Pds1 and \textit{chk1} mutants eventually exit mitosis despite Rad53 inhibition of mitotic exit.

Recent work from several laboratories has begun to elucidate the mechanism that regulates the interdependence of anaphase and mitotic exit, which may explain why \textit{chk1} mutants are able to exit mitosis despite having an active Rad53 pathway (Sanchez et al., 1999). Pds1 is an inhibitor of sister chromatid separation through binding to and inhibition of Esp1. However, overproduction of Pds1 and the destruction box mutant (dbmPds1) cause
a longer delay of mitotic exit than inactivation of esp1 (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999) indicating a function for Pds1 distinct from its role in Esp1 inhibition, perhaps in preventing Cdc14 release from the nucleolus (Shirayama et al., 1999).

In addition to Pds1 having an alternative role in mitotic progression, Esp1 has also been implicated in the regulation of mitotic exit. Esp1, along with three other proteins, forms a regulatory network termed the Cdc14 early release network (FEAR) that allows initial release of Cdc14 from the nucleolus, which in turn can activate the mitotic exit network (MEN)(Shirayama et al., 1999). Thus, Chk1 through the stabilization of the Esp1 inhibitor, Pds1, mediates a delay both in anaphase entry and mitotic exit.

Rad53 has been proposed to have two separate functions in the regulation of mitotic progression, re-enforcement of the pre-anaphase delay and inhibition of mitotic exit (Hu et al., 2001; Sanchez et al., 1999). The Rad53/Dun1 checkpoint pathway mediates the activation or reverses the inhibition of Bfa1, which prevents the release of Cdc14 from the nucleolus and mitotic exit (Hu et al., 2001). It has been proposed that Cdc14 may also influence anaphase entry by reversing the CDK-dependent phosphorylation of Pds1, which would result in the release of Esp1 (Agarwal and Cohen-Fix, 2002). This would suggest that Bfa1 inhibition of anaphase is dependent on Cdc14. One could argue that loss of Rad53 function could influence anaphase entry by activation of the MEN, which allows Cdc14 to remain out of the nucleolus to promote both mitotic exit and anaphase. However, cdc14 mutants block mitotic exit, but have no effect on the anaphase entry defect of rad53 mutants (Sanchez et al., 1999). Thus, these studies indicate that there is another role of Rad53 in the
M-A checkpoint distinct from its role in mitotic exit and suggests perhaps the involvement of another pathway in controlling anaphase.

**Checkpoint and PKA pathways act cooperatively to regulate the interdependence of anaphase and mitotic exit.**

PKA pathways play many roles in the homeostasis of the cell (Thevelein et al., 2000)(Pan et al., 2000). In vertebrate cells, the association of the catalytic subunits with anchoring and scaffold proteins (AKAP) compartmentalizes the role of PKA in various cellular processes (Edwards and Scott, 2000). The mechanism by which yeast cells achieve specificity of PKA signaling is not fully understood. However, both the regulatory and catalytic subunits are regulated by sub-cellular localization (Griffioen et al., 2000; Griffioen et al., 2001; Griffioen et al., 2003). Several groups had observed genetic and biochemical interactions between the components that regulate mitosis and the PKA pathway (Anghileri et al., 1999; Heo et al., 1999; Kotani et al., 1998; Yanagida et al., 1999), and this role of PKA is conserved between yeast and mammals. Recently, it has been shown that PKA-mediated inhibition of the APC/C by carbon source and activated Ras proteins could be suppressed by overexpression of Cdc20 in *S. cerevisiae* (Bolte et al., 2003), further supporting a connection between PKA signaling and Cdc20. Our data show that the PKA pathway cooperates with the Chk1 pathway to block anaphase and mitotic exit. Loss of function of PKA enhanced the checkpoint defect of a *chk1* mutant and accelerated the destruction of Pds1 and, to a greater extent, Clb2 in *chk1* cells, even in the presence of a wild-type RAD53. Our results place the PKA and Rad53 pathways in the same epistasis group; however, our data suggests that PKA could be carrying out a role that is partially redundant with Rad53 and that its role
cannot be uncovered in cells lacking the Rad53-mediated regulation of MEN because in these cells Cdc20 levels were not maintained. The damage-induced phosphorylation of Cdc20 required the function of the upstream kinase Mec1, indicating that the phosphorylation is regulated by the DNA damage checkpoint. In our model, PKA functions in a parallel branch with Chk1 and Rad53; however, Chk1 function would be sufficient to block anaphase and reinforce the mitotic exit function of Rad53. It is also possible that the Rad53 pathway could help maintain PKA activation to regulate Cdc20 in order to reinforce Pds1 stabilization and block the first phase of Clb2 destruction. For example, if the PKA pathway requires high levels of Clb2 and inactivation of Cdh1, which controls Cdc20 levels, in order to maintain the signal, then Rad53 could indirectly impinge on the PKA pathway. Our data suggest that one of the latter explanations is possible since Cdc20 levels dropped along with Clb2 levels as \textit{rad53} cells failed in the DNA damage-induced arrest. In that case the contribution of PKA in the inactivation of Cdc20 would be difficult to measure. Due to the dual role of Pds1 in blocking anaphase and mitotic exit, stabilization of Pds1 by phosphorylation would serve to reinforce the Rad53-mediated block to mitotic exit by preventing the APC^{Cdc20} from targeting Clb2, even in the absence of a PKA signal. This coupled with our observation that Cdc20 levels are not maintained in \textit{rad53} cells would explain why the role of PKA in the DNA damage induced arrest could only be uncovered in mutants that fail to stabilize Pds1, i.e., \textit{chk1} mutants but not in mutants that fail to inactivate the MEN.

An interesting finding was that deletion of either \textit{TPK1} or \textit{TPK2} exhibited a synthetic phenotype with a \textit{chk1} mutation. One possible explanation for this observation is that the PKA complexes that mediate the pre-anaphase arrest are composed of Tpk1/Tpk2
heterodimers in a complex with 2 Bcy1 subunits. Absence of either catalytic subunit would prevent the formation of the mitotic-specific PKA complex. This explanation is supported by the finding that Tpk1 and Tpk2 were identified in the same protein complex by mass spectrometric protein complex identification (Ho et al., 2002) and that deletion of both TPK1 and TPK2 did not result in an enhanced phenotype. However, we did not test whether Tpk3 had a role in the DNA damage-induced cell cycle arrest. The possibility exists that reduction of the levels of catalytic subunits by deletion of any one of the TPK genes could result in the availability of additional inhibitory subunits (Bcy1) to bind and lower the activity of all catalytic subunits, which would result in a checkpoint phenotype.

**Regulation of Cdc20 by the DNA damage checkpoint.**

Cdc20 is a highly regulated protein both in the normal cell cycle and in response to misaligned chromosomes, and has been implicated as a target of the DNA damage checkpoint (Hwang et al., 1998; Lim and Surana, 1996; Prinz et al., 1998). Cdc20 overexpression has been shown to bypass the *cdc13-1* induced checkpoint arrest and promotes both anaphase entry (Hwang et al., 1998) and exit from mitosis (Lim and Surana, 1996). From our work, it is clear that cells are sensitive to Cdc20 levels at the DNA damage-induced arrest as addition of a plasmid copy of CDC20 enhanced the checkpoint defect of *chk1-*Δ cells. In addition, we show here that Cdc20 is regulated by phosphorylation in response to DNA damage.

The DNA damage-induced phosphorylation of Cdc20 requires Mec1, Tpk1/Tpk2 and is reduced in *chk1* mutants. Tpk1/Tpk2 can form a complex with and phosphorylate Cdc20. Mutation of the residues of Cdc20 that are phosphorylated in response to DNA damage enhanced the checkpoint defect of *chk1* mutants and suppressed the temperature sensitivity of
cdc23-1 mutants, providing evidence that PKA signaling regulates the APC\(^{\text{CDC20}}\) function and that the PKA and checkpoint pathways converge on Cdc20 to regulate both anaphase and mitotic exit. Furthermore, we showed that phosphorylation could serve to regulate the interaction between Cdc20 and one of the proteins that it targets for ubiquitination by the APC/C. It is interesting to note that previous studies had mapped the site of interaction of Cdc20 with its target Pds1 to amino acids 158-559 of the Cdc20 protein (Hilioti et al., 2001). Our results are not incompatible with those observations because it is possible that phosphorylation at amino acids 52 and/or 88 could serve to change the protein conformation in order to block the domain(s) on Cdc20 that interact with Clb2, alternatively phosphorylation could serve to create a docking site for a third protein that could physically block activation of Cdc20 or the interaction with Clb2.

Although we could not detect interaction between Cdc20 and Chk1 following DNA damage nor phosphorylation of Cdc20 by Chk1 \textit{in vitro} (Fig. S3 and data not shown), we observed reduced phosphorylation of Cdc20 in \textit{chk1} cells, indicating that Chk1 has a role in signaling to Cdc20. There are several possible explanations for our results. Chk1 could have a role, either direct or indirect, in maintaining the PKA response and this could be through phosphorylation and stabilization of Pds1. However, we cannot rule out that under certain physiological conditions activated Chk1 could also phosphorylate Cdc20. Nevertheless, the fact that down-regulation of PKA by mutation of catalytic subunits or expression of \textit{BCY1} or expression of Cdc20-S88A all exacerbate the checkpoint defect of \textit{chk1} cells, points to PKA as the major pathway regulating Cdc20.

Earlier studies along with these results suggest that the PKA pathway functions to restrain mitosis even in the absence of DNA damage. Our data also suggest that there is
cross-talk between the pathways that regulate the APC, APC regulators and the DNA damage checkpoint pathways operating at the M-A transition. This is not surprising, since the cell’s ability to monitor nutrient availability, spindle integrity and genomic integrity in order to regulate cell division implies that many pathways integrate signals into common effectors in order to regulate progression through mitosis.

One possible mechanism for this cross-talk could be through a mechanism that monitors the levels of Cdc20 and Clb2. During a pre-anaphase arrest with high levels of securin (DNA damage or APC mutant arrest) and mitotic cyclin/cdk1 kinase activity, the high levels of Cdc20 that accumulate in a situation where Cdh1 is inhibited activate a second level of regulation, i.e., phosphorylation, perhaps by the ubiquitin-mediated inactivation or destruction of an inhibitor of these pathways.

Our results are consistent with a model (Fig. 3.6C) in which the presence of DNA damage induces the conserved essential checkpoint kinase MEC1 to activate the Rad53, Chk1 and possibly PKA kinases, which in turn act to inhibit separate branches of the mitotic machinery. The first branch, controlled by Chk1, results in the phosphorylation of Pds1, maintenance of Pds1 levels, and prevents sister chromatid separation through inhibition of Esp1. Chk1 and PKA also act to help prevent activation of the mitotic exit branch by maintaining the levels of Pds1 and blocking activation of Cdc20. The other branch, controlled by RAD53 and DUN1, results in a block to the MEN via activation of Bfa1/Bub2. In the absence of phosphorylated Pds1 and inhibition of Cdc20, the first phase of Clb2 degradation will take place and through a second mechanism, perhaps by accumulation of Sic1, the levels of mitotic cyclin kinase activity drop sufficiently to allow mitotic exit. Thus,
each kinase acts primarily to inhibit a specific branch of the mitotic progression pathway and plays a supporting role in helping the longer-term maintenance of arrest of the other branch.

Most cancer cells are partially compromised for their ability to respond to DNA damage or replication blocks, which limits the effectiveness of current cancer treatments based on the use of DNA-damaging agents. Thus, cancer cells could be rendered more sensitive to genotoxic agents through inactivation of an additional branch or signaling point of the checkpoint. Given the large body of evidence that supports a conserved role for PKA signaling in the regulation of mitotic progression, the role of PKA in mammalian checkpoint control should be evaluated. By understanding the different levels of regulation of the checkpoint kinases, and their role in genomic stability, we will be able to design specific inhibitors of checkpoint signaling that are more effective as modifiers of therapeutic approaches in the treatment of cancer.
Chapter 4

4. Phosphorylation of the PKA Regulatory Subunit and cAMP signaling help restrain mitosis following DNA damage

4.1 Summary

The cAMP dependent protein kinase (PKA) pathway has a supporting role in restraining anaphase and blocking the inactivation of mitotic cyclin/Cdk1 complexes. PKA helps inhibit mitosis by regulating the phosphorylation of the mitotic inducer Cdc20. This phosphorylation of Cdc20 blocks the interaction of Cdc20 with its substrate, the B type cyclin Clb2, and prevents Clb2 destruction and progression through mitosis. The DNA damage induced phosphorylation of Cdc20 is dependent on both PKA and the upstream checkpoint kinase, Mec1; furthermore, overexpression of a catalytic subunit of PKA in a mec1 mutant partially restored the checkpoint defect. These results suggest that PKA is regulated by the DNA damage checkpoint downstream of Mec1, thus, we set out to determine how PKA is regulated in response to DNA damage. PKA activity is regulated by intracellular levels of cAMP, but can also be mediated by localization, protein-protein interactions, or modification of the R subunit. We found that the R subunit is phosphorylated in response to DNA damage. In addition, we found that two proteins which regulate the R subunit of PKA in S. cerevisiae, either by regulating its phosphorylation or its localization, also function to block mitotic progression in response to DNA damage. We also found that the adenylate cyclase and the glucose kinase, Hxk2, both of which have been shown to regulate intracellular cAMP levels, had a role in restraining mitosis following
DNA damage. Our results support a model in which the phosphorylation of the R subunit and cAMP signaling are required for the role of PKA in restraining mitosis following DNA damage.

4.2 Introduction

Maintenance of genomic stability requires that a cell be able to transfer a complete and unaltered genome to each daughter cell. Cell division with damaged DNA can lead to genomic instability and uncontrolled cell growth in single cell organisms and cancer in multi-cellular organisms. In order to maintain the integrity of the genome, DNA damage checkpoint proteins comprise a signal transduction network that prevents anaphase until damaged DNA has been repaired. Other stress activated pathways including the cAMP dependent protein kinase (PKA) pathway have been shown to inhibit mitotic progression (Yamada et al., 1997), (Anghileri et al., 1999), (Heo et al., 1999), and mutation or mis-regulation of PKA subunits has been associated with chromosomal instability in cancer cells (Matyakhina et al., 2002).

The PKA pathway has a supporting role in preventing Pds1 and Clb2 destruction following DNA damage (Searle et al., 2004). Since availability of cAMP triggers the activation of PKA, the activation of PKA can be regulated by mediating the intra-cellular levels of cAMP. Regulation of PKA can also occur via post-translational modification or protein interactions of the catalytic or R subunits.

cAMP levels are primarily controlled by Adenylate Cyclase (AC) which catalyzes the reaction to generate cAMP, and the phosphodiesterases (PDEs) that hydrolyze the cAMP into AMP. At least two other signaling pathways in S. cerevisiae regulate a
transient increase in cAMP levels. The Ras pathway, which can be stimulated by intracellular acidification, activates the production of cAMP. The glucose signaling pathway, which is stimulated by the addition of glucose to cells growing in non-fermentable carbon source, also activates the production of cAMP (reviewed in (Thevelein and de Winde, 1999)). The glucose kinase, Hxk2, is required for the transient increase in cAMP levels in both the Ras and glucose signaling pathways (Rolland et al., 2001).

Both the R subunit and catalytic subunits can be regulated directly through post-translational modification, sub-cellular localization and protein-protein interactions. In S. cerevisiae the R subunit is phosphorylated when cells are either deprived of glucose or exposed to heat shock or calcium. The Yak1 kinase is required for the phosphorylation of the R subunit when the cells are grown in a non-fermentable carbon source, i.e. deprived of glucose, (Griffioen et al., 2001), and Mck1 is a GSK-3 that is required for the phosphorylation of the R subunit in response to heat shock. Phosphorylation is required for localization of the R subunit to the cytoplasm (Griffioen et al., 2003). In addition, Zds1 and Zds2 have been implicated in the regulation of the localization of the R subunit to the cytoplasm in response to the same stresses that lead to phosphorylation of the R subunit (Griffioen et al., 2003).

Understanding how different regulatory networks coordinate to prevent separation of incompletely replicated or damaged DNA will provide greater insight into the cellular mechanisms that protect the cell from genetic instability. Here we show that the checkpoint regulated phosphorylation of the R subunit of PKA has a role in restraining mitosis following DNA damage, suggesting that there is cross-talk between the DNA
damage checkpoint and the PKA pathways. In addition, we found that proteins that regulate the phosphorylation of the R subunit, and proteins that regulate the levels of intracellular cAMP are both required for regulating mitotic progression during a checkpoint mediated arrest. Our results suggest that following DNA damage, PKA signaling is regulated by both phosphorylation of the R subunit and stimulated by cAMP.

4.3 Materials and methods

4.3.1 Strains and Plasmids

Strains used in this study are listed in Table 1. Yeast strains were generated using standard genetic techniques (Guthrie and Fink, 1991). To generate the $bcy1\Delta$ strains, a DNA fragment that contained the $URA3$ gene flanked by 50 bp of sequences homologous to the 5’ and 3’-UTRs of $BCY1$ was prepared by PCR. The DNA fragment was then transformed into Y816, Y818, and YJS52 resulting in the replacement of $BCY1$ with $URA3$ by homologous recombination. Gene disruption was confirmed by PCR. Strains containing the $cdc35-1$ mutation were generated by crossing $cdc35-1$ (CMY282) {Dubacq, 2002 #28} into the Y300 strain background.

Deletion of $ZDS1$, $MCK1$, and $HXK2$ was carried out by using a construct generated by PCR amplification of the genomic DNA surrounding the gene that had been replaced with $KanMX$ (deletion strain collection open biosystems).

Plasmids used in this study are listed in Table 2.

$MCK1$ including upstream and downstream sequences was amplified using the primers: CTGGATCCTCTTCCCTCTTTCCCAATT, and GCTCTAGATGACGCGATCA AAGG which contained a BamHI and XbaI site.
respectively. The amplified sequence was ligated into a pRS425 vector digested with the enzymes, BamHI and XbaI.

pJS11 was generated by subcloning BCY1 from pXP1 (Pan and Heitman, 1999) into pRS425 using BamHI and HindIII. pYCJ1 was generated as described in (Griffioen et al., 2001). pY CJ2 was generated using a three step PCR method using pJS11 and the primers:

Reverse: CTGGCTCGAGCTTGAGCTTGAGCTGCTTGAGGTCTGGAAAATGAC
Forward: CAAGCTCAAGCTCGAGCCAGAGCGGCTGTTATGTTCAAATCCCCC

which generated the serine to alanine mutations described in (Griffioen et al., 2001). The resulting PCR product was used to amplify the region upstream of the mutated sites using pJS11 as the template and the forward primer: CGTCCGACTTTCTTCAGTTC. The PCR product generated in the second PCR was used as the forward primer and the reverse primer: CGTCATACATGAGTCTCTTC were used to amplify the region between BspEI and BsrGI sites containing the nucleotide changes to generate the serine to alanine mutations. The resulting PCR product was digested with BspEI and BsrGI enzymes and replaced the fragment generated by digestion of pJS11 with BspEI and BsrGI.

4.3.2 Growth Conditions

Cells were grown in YPD rich medium or SC-Leu medium as described in Guthrie and Fink, 1991. When alpha-factor was added to synchronize cells, SC-Leu or YPD pH3.9 was used.
4.3.3 Visualization of nuclei

Cells were grown to OD$_{600}$=3.5-4.5 at 22°C. Cells were synchronized in G1 using alpha-factor (10µg/ml) or in late S-phase using 200mM Hydroxyurea (HU) as indicated. Unless indicated otherwise, the temperature was raised to 32°C for 60 min. prior to release. Cells were washed and resuspended in YPD pH3.9 at 32°C to release the cells into the cell cycle. To stop the cells from undergoing multiple cell cycles, alpha-factor was added back to the cells when cells released from a G1 block entered S-phase (re-budded) or immediately upon release for cells released from a late S-phase arrest. The cells were fixed and permeabilized using 70% ethanol. Cells were re-hydrated in PBS and stained with 0.1 mg/ml 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, St. Louis, MO). Cells were mounted on glass slides coated with 0.1% poly-L-lysine for microscopy.

4.3.4 Western Analysis

Protein extracts were prepared by trichloro-acetic acid (TCA) precipitation as previously described (Foiani et al., 1994). TCA precipitated proteins were solubilized by boiling in 100µL of 1M Tris-HCl pH 8.0 and 200µl loading buffer (1% SDS, 10mM Tris-HCl pH 7.5, 2% 2- Mercaptoethanol, and 20% Glycerol) Proteins were separated on 10% acrylamide/0.067% bis-acrylamide gels, and transferred to nitrocellulose membranes. Bcy1 was detected by Western analysis using anti-Bcy1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The immune complexes were detected by chemiluminescence (NEN).
4.3.5 Phosphatase Assay

Protein from cdc13-1 cells grown to OD$_{600}$=3.5 at 22°C and incubated at 32°C for 120 min. was isolated by TCA precipitation. TCA precipitated protein was resolubilized by boiling in buffer containing 1% SDS and 10mM Tris. Yeast lysis buffer with no SDS was used to dilute the solubilized protein solution so that the final SDS concentration was 0.1%. Bcy1 was immuno-precipitated using 0.2µg anti-Bcy1 antibody (Santa Cruz) and protein-A sepharose beads (Amersham). Bcy1 bound to the beads was treated with 20 units of alkaline phosphatase (Boehringer Mannheim) and/or phosphatase inhibitors (1mM sodium orthovanadate, and 1mM sodium fluoride) in alkaline phosphatase buffer (50mM Tris-HCl pH 8.5 and 0.1mM EDTA) for 1 hour at 22°C. Bcy1 was removed from the beads by boiling in loading buffer. Immuno-precipitated proteins were resolved and analyzed as described above.

Table 4.1

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<td>As Y300 cdc13-1</td>
<td>Searle, et al., 2004</td>
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<td>As Y300 cdc13-1 chk1-Δ::HIS3</td>
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4.4 Results

4.4.1 The R subunit is phosphorylated in response to DNA damage.

We recently showed that PKA had a role in the DNA damage checkpoint. PKA supported mitotic arrest by regulating the phosphorylation of Cdc20 and helped maintain high levels of the mitotic inhibitors, Pds1 and Clb2. In addition, we showed that phosphorylation of Cdc20 was both Mec1 and PKA dependent, and that overexpression of PKA catalytic subunits partially rescued the checkpoint defect of mec1-21 cells (Searle...
et al., 2004). These results suggested that Mec1 and PKA are in the same signaling pathway and that PKA was likely acting downstream of Mec1 in response to DNA damage. We set out to identify the mechanism by which PKA is regulated in response to DNA damage.

PKA can be regulated by many mechanisms including regulation of cAMP levels, localization of the PKA holoenzyme or catalytic subunits, interactions with other proteins, and phosphorylation of the catalytic subunits or the R subunit (Griffioen and Thevelein, 2002), (Thevelein and de Winde, 1999). Since Mec1 itself is a kinase and it activates a signal transduction cascade that regulates many downstream kinases, we wanted to determine whether the R subunit was phosphorylated in response to DNA damage, and whether this phosphorylation was dependent on Mec1. \textit{cdc13-1} cells were used to activate a DNA damage signal. Growth of \textit{cdc13-1} cells at a restrictive temperature results in the inactivation of the telomere binding protein Cdc13 which causes single stranded DNA at the telomeres. This single stranded DNA is recognized as DNA damage in G2/M, and the DNA damage checkpoint is activated blocking anaphase and mitotic exit (Garvik et al., 1995). In protein extracts from synchronized cells grown at the restrictive temperature for \textit{cdc13-1}, a slower migrating form of the R subunit was detected by western analysis at a time when the DNA damage signal was present. A similar slower migrating form of the R subunit was detected in protein extracts from cells growing in YP ethanol. (Fig. 4.1A) Because it was previously shown that the R subunit was phosphorylated in cells growing in YP ethanol (Griffioen et al., 2001), this suggested that the slower migrating form of the R subunit was due to phosphorylation.
In order to show that the slower migrating form of the R subunit was due to phosphorylation, the R subunit was isolated by immuno-precipitation and treated with phosphatase with and without phosphatase inhibitors. Treatment with phosphatase resulted in loss of the slower migrating form of the R subunit, while the slower migrating form of the R subunit was maintained in the treatment of the immuno-complexes with phosphatase and phosphatase inhibitors. (Fig. 4.1B) The results shown in Figure 1A indicated that the R subunit is modified by phosphorylation in response to activation of the DNA damage checkpoint.

The R subunit was also phosphorylated in cells treated with the DNA damaging agent, MMS, and hydroxyurea (HU), a drug which slows DNA replication which can result in collapsed replication forks and DNA damage. To demonstrate that the phosphorylation of the R subunit following treatment with HU was due to DNA damage and not stalled replication forks, a rad9 deletion mutant was also treated with HU. The phosphorylation of the R subunit in HU required the checkpoint protein Rad9 which suggested that the R subunit was phosphorylated in response to DNA damage induced by HU. (Fig. 4.1B) Cells that were arrested in mitosis with nocodazole but had no DNA damage did not have phosphorylated R subunit, indicating that the phosphorylation of the R subunit is not due to a cell cycle position effect (Fig. 4.1B).

Because the R subunit is phosphorylated in response to DNA damage we next examined whether Mec1 was required for the phosphorylation of the R subunit. In cells lacking Mec1, the R subunit was not phosphorylated (Fig. 4.1D). This result suggested that Mec1 is required for the phosphorylation of the R subunit in response to DNA
Figure 4.1 The R subunit is phosphorylated in response to DNA damage in a Mec1 dependent manner. 

A) WT (Y300) cells were synchronized in alpha-factor and released at 32°C. Cells were collected at indicated timepoints and lysed in TCA. For the YPE sample, WT cells were grown in YP media containing ethanol overnight and the cells were lysed in TCA. Proteins were separated on a 10% polyacrylamide 0.067% bisacrylamide gel. Western analysis was used to detect the R subunit using an anti-Bcy1 (R subunit) antibody.

B) WT (Y300), rad9Δ (YJS76) and cdc13-1 (Y816) cells were grown at 22°C and raised to 32°C for 2 hours. 0.1% MMS, 1µg/ml nocodazole, or 200mM hydroxyurea was added to WT (Y300) or rad9Δ (YJS76) cells as indicated for the 2 hours the cells were at 32°C. Cells were lysed and proteins detected as in Fig. 4.1A.

C) cdc13-1 (Y816) cells were grown at 22°C and the temperature was raised to 32°C for 120 min., cells were collected and TCA precipitated protein extracts were prepared. After re-solubilizing the TCA precipitated protein by boiling, the R subunit was isolated by immunoprecipitation and treated with alkaline phosphatase and/or phosphatase inhibitors (sodium orthovanadate and sodium fluoride) and as indicated. Separation and detection of the R subunit was as described above.

D) cdc13-1 (Y816) and cdc13-1mec1Δ (YJS51) cells were grown and treated as in Fig. 4.1B, and detection of R subunit was done as in Fig. 4.1A.
damage and supports the hypothesis that Mec1 regulates PKA when there is a DNA damage signal.

### 4.4.2 Mck1 is required for R subunit phosphorylation following DNA damage and has a role in the DNA damage checkpoint

The GSK3 kinase, Mck1 was found to be required for the phosphorylation of the R subunit in response to heat shock (Griffioen et al., 2003). To determine whether Mck1 has a role in the DNA damage checkpoint, we examined the ability of cells lacking *MCK1* to stop mitosis in response to DNA damage. Because PKA pathways also have a role in the regulation of the G1/S transition, we monitored cells released from an HU mediated arrest in late S-phase, as they proceeded through mitosis. The number of cells that had failed in checkpoint mediated arrest was determined by scoring the number of cells that exhibited a late mitotic phenotype (separated nuclei) in response to DNA damage. *cdc13-1* cells lacking *MCK1*, or *TPK2* were proficient in the checkpoint arrest and remained arrested throughout the experiment, however, *cdc13-1chk1Δ* cells lacking *MCK1* or *TPK2* exhibited more cells with a late mitotic phenotype at earlier time points than in the *cdc13-1chk1* cells (Fig. 4.2B). This phenotype was similar to that of a *chk1Δtpk2Δ* mutant. These results indicated that Mck1 had a role in the checkpoint mediated arrest.

To determine whether Mck1 was acting in the same pathway as PKA to restrain mitosis following DNA damage, we tested whether deletion of *MCK1* would further enhance the checkpoint defect of *cdc13-1chk1Δtpk2Δ* cells. Cells were released into the cell cycle from an alpha-factor induced G1 arrest. Only cells lacking *TPK2* were used in
this experiment so that all cells would have the same delay in the G1/S transition and therefore would enter S-phase with the same kinetics. \textit{cdc13-1chkl}Δ lacking \textit{TPK2} failed in the checkpoint with the same kinetics as \textit{cdc13-1chkl}Δ cells lacking both \textit{TPK2} and \textit{MCK1} (Fig.4.2C), suggesting that Mck1 and Tpk2 were acting in the same pathway following DNA damage.

To determine whether or not the role of Mck1 in the DNA damage checkpoint was to regulate the phosphorylation of the R subunit, we analyzed protein from \textit{cdc13-1} or \textit{cdc13-1mck1}Δ cells raised to the semi-permissive temperature of 32°C for \textit{cdc13-1}, by western analysis. The R subunit was phosphorylated in \textit{cdc13-1} cells; however, no phosphorylated R subunit was detected in cells lacking \textit{MCK1} (Fig.4.2A) suggesting that Mck1 is required for the phosphorylation of the R subunit in response to DNA damage.

We previously showed that overproduction of Tpk1 or Tpk2 restored the cell cycle delay in \textit{mec1-21} mutants after DNA damage (Searle et al., 2004). Because Mck1 and Mec1 were required for the phosphorylation of the R subunit following DNA damage, we used the same assay to determine whether Mck1 was acting downstream of Mec1 to restrain mitosis following DNA damage. \textit{cdc13-1} or \textit{cdc13-1mec1-21} cells containing an empty vector or \textit{MCK1} on a multi-copy vector were released from G1 (alpha-factor block) at 32°C and the cells were stained with DAPI to determine the number of cells exhibiting late mitotic phenotype at the indicated time points. Overexpression of Mck1 partially alleviated the checkpoint defect of \textit{mec1-21} cells following DNA damage (Fig. 4.2D), suggesting that Mck1 was acting downstream of Mec1 to restrain mitosis following DNA damage.
Figure 4.2. Mck1 is required for phosphorylation of the R subunit and has a role in restraining anaphase following DNA damage.  A) WT (Y300), cdc13-1(Y816), and cdc13-1mck1Δ (YJS52) cells were raised to 32°C and nocodazole was added to WT cells for 120 min. Cells were collected and lysed, and the R subunit was detected as described in Fig. 4.1.  B) cdc13-1 (Y816), cdc13-1chk1Δ (Y818), cdc13-1mck1Δ (YJS52), cdc13-1chk1Δmck1Δ (YJS56), and cdc13-1chk1Δtpk2Δ (YJS10), cells were arrested in S-phase by addition of HU then released into the cell cycle at 32°C. Cells were collected at the indicated time points, fixed in ethanol, and stained with DAPI to visualize the DNA and nuclear morphology. The number of cells that exhibited a late mitotic phenotype were counted.  C) cdc13-1tpk2Δ (YJS8), cdc13-1chk1Δtpk2Δ (YJS10), and cdc13-1chk1Δtpk2Δmck1Δ (YJS59) cells were arrested in G1 with alpha-factor and released into the cell cycle at 32°C. Samples were taken at indicated times and analyzed as in Fig. 4.2B.  D) cdc13-1 and cdc13-1mec1-21 cells transformed with either pRS425 vector or the same high copy vector expressing MCK1 (YJS62, YJS63, YJS60, YJS61) were grown at 22°C and arrested in G1 by addition of alpha-factor. The temperature was raised to 32°C and the cells were released from G1 into the cell cycle at 32°C. Cells were collected at the indicated time points and analyzed as in Fig. 4.2B. OP stands for over produced.
4.4.3 Phosphorylation of the R subunit is required for the role of PKA in the DNA damage checkpoint

Two serine rich clusters on the R subunit were shown to be required for the phosphorylation and cytoplasmic localization of the R subunit in response to heat shock and growth in non-fermentable carbon sources. To determine whether these sites were required for phosphorylation of the R subunit in response to DNA damage, protein from cells expressing wild type R subunit or the R subunit with serines in cluster I or cluster II (Griffioen et al., 2001) mutated to alanines, and given a DNA damage signal were analyzed by western blot. The wild type R subunit was phosphorylated under these conditions but the phosphorylation was greatly reduced when the serines in cluster I or II were mutated to alanine (Fig 4.3A). These results suggested that the R subunit is phosphorylated on serine residues located in both cluster I and cluster II.

Since Mck1 is both required for the R subunit phosphorylation and has a role the DNA damage checkpoint similar to PKA, we hypothesized that the role of PKA in the DNA damage checkpoint required phosphorylation of the R subunit on cluster I and cluster II serines. Therefore, phosphorylation defective mutants should have the same phenotype as deletion of a PKA catalytic subunit. cdc13-1bcy1Δ or cdc13-1chk1Δbcy1Δ cells expressing wild type BCY1 or bcy1CI or bcy1CII mutants were released into the cell cycle from an HU mediated arrest in late S-phase with a DNA damage signal, and the number of cells exhibiting a late mitotic phenotype were counted. Expression of the
Figure 4.3. The R subunit phosphorylation defective mutants enhanced the DNA damage checkpoint defect of chk1Δ cells. A) cdc13-1bcy1Δ + BCY1 (YJS65), cdc13-1bcy1Δ+bcy1CI (YJS66), and cdc13-1bcy1Δ+bcy1CII (YJS67) cells were grown at 22°C and the temperature was raised to 32°C for 120 min. Cells were collected, lysed and the proteins were analyzed as in Fig. 1A. B) cdc13-1bcy1Δ + BCY1 (YJS65), cdc13-1bcy1Δ+bcy1CI (YJS66), cdc13-1bcy1Δ+bcy1CII (YJS67), cdc13-1chk1Δbcy1Δ+BCY1 (YJS69), cdc13-1chk1Δbcy1Δ+bcy1CI (YJS70), and cdc13-1chk1Δbcy1Δ+bcy1CII (YJS71) cells were arrested in S-phase by addition of HU to the media. The temperature was raised to 32°C and the cells were released into the cell cycle at 32°C. Aliquots were taken at the indicated times and each sample was analyzed as in Fig. 4.2B. C) cdc13-1bcy1Δ+BCY1 (YJS65), cdc13-1chk1Δbcy1Δ +BCY1 (YJS69), cdc13-1chk1Δmck1Δ+BCY1 (YJS77), cdc13-1chk1Δmck1Δ+bcy1CI(YJS78), and cdc13-1chk1Δmck1Δ+bcy1CII (YJS79) cells were analyzed as in Fig. 4.3B
phosphorylation defective R subunit mutants enhanced the checkpoint defect of a *chk1Δ* cell to the same extent as deletion of a PKA catalytic subunit (Fig 4.3B). Furthermore, expression of the phosphorylation defective R subunit mutants did not further enhance the checkpoint defect of cells mutant for both *chk1* and *mck1* (Fig 4.3C). These results indicated that phosphorylation of the serines in cluster I and II play a role in the DNA damage checkpoint and suggest that the role of Mck1 in the DNA damage checkpoint is via regulation of the R subunit by phosphorylation on one or more of the serine residues in cluster I and cluster II.

### 4.4.4 Zsd2 has a role in the DNA damage checkpoint

Two proteins, Zds1 and Zds2, have been implicated in regulating the cytoplasmic localization of the R subunit under the same conditions that cause R subunit phosphorylation. Zds1 was required for the cytoplasmic localization of the R subunit in glucose restricting conditions (Griffioen et al., 2001), and Zds1 and its homologue, Zds2, were required for cytoplasmic localization in response to heat shock and addition of extra-cellular calcium (Griffioen et al., 2003). To test whether Zds1 or Zds2 had a role in the DNA damage checkpoint, we analyzed the rate at which *cdc13-1*, or *cdc13-1chk1Δ* cells lacking *ZDS1, ZDS2*, or *TPK2* failed in the checkpoint mediate arrest by scoring the number of cells exhibiting a late mitotic phenotype. *cdc13-1, cdc13-1zds1Δ* and *cdc13-1zds2Δ* cells were proficient in restraining mitosis, however *cdc13-1chk1Δ* lacking *ZDS2* or *TPK2* exhibited more cells with a late mitotic phenotype at earlier time points that *cdc13-1chk1Δ* or *cdc13-1chk1Δzds1Δ* cells (Fig 4.4A and B), indicating that Zds2 has a role in the checkpoint similar to the role of Tpk2. Neither Zds1 nor Zds2 was required
for the phosphorylation of the R subunit following DNA damage (Fig. 4.4C), indicating that Zds2 has a role in the checkpoint independent of the phosphorylation of the R subunit.

### 4.4.5 cAMP signaling and Hxk2 had a role in the DNA damage checkpoint

Our genetic experiments support the existence of a signaling complex that includes phosphorylated R subunit, PKA catalytic subunit(s), and Zds2. To examine whether cAMP is required to activate this complex following DNA damage, proteins involved in regulation of cAMP signaling were deleted or mutated in cdc13-1chk1Δ cells. In this way we could gain insight as to whether or not cAMP was required for the role of PKA in the DNA damage checkpoint. A cdc35-1 mutation, which causes inactivation of adenylate cyclase when the cells are grown at the restrictive temperature (Boutelet et al., 1985), was introduced into cdc13-1 and cdc13-1chk1Δ cells. The cells were released from a G1 arrest into the cell cycle. As the cells progressed through S phase, a DNA damage signal was generated and/or adenylate cyclase was inactivated as the cells progressed through S phase by raising the temperature to 35°C.

In a chk1Δ cell inactivation of adenylate cyclase enhanced the checkpoint defect of the chk1Δ cell (Fig. 4.5B), suggesting cAMP signaling has a role similar to the role of PKA in the DNA damage checkpoint.
Figure 4.4 Zds2 has a role in the DNA damage checkpoint. A) cdc13-1 (Y816), cdc13-1chk1Δ (Y818), cdc13-1zds1Δ (YJS53), cdc13-1chk1Δzds1Δ (YJS57) and cdc13-1chk1Δtpk2Δ (YJS10) cells were arrested in S-phase using HU and released into the cell cycle at 32°C. Aliquots from each culture were taken at indicated times following release into the cell cycle, fixed and analyzed as in Fig. 4.2B. B) Same as Fig. 4.4A except cdc13-1(Y816), cdc13-1chk1Δ (Y818), cdc13-1zds2Δ (YJS54), cdc13-1chk1Δzds2Δ (YJS58), and cdc13-1chk1Δtpk2Δ (YJS10) cells were analyzed. C) Proteins from WT cells treated with nocodazole for 120 minutes, and proteins from cdc13-1(Y816), cdc13-1zds1Δ (YJS53), and cdc13-1zds2Δ (YJS54) cells grown at 32°C for 120 minutes and were analyzed as in Fig. 4.1A.
Hexokinase 2 (Hxk2) is required for activation of cAMP signaling upon addition of glucose to cells growing in non-fermentable carbon source and in cells with intracellular acidification (Rolland et al., 2001). Since both of these mechanisms activate a transient increase in cAMP levels and require Hxk2 to regulate cAMP, we wanted to determine whether Hxk2 function was required for the DNA damage checkpoint. Deletion of HXK2 exacerbated the checkpoint defect of cdc13-1chk1 cells (Fig. 4.5A). The cdc13-1chk1Δhxk2Δ cells failed in the checkpoint with similar kinetics as a cdc13-1rad9Δ cell (Fig. 4.5A), indicating that the majority of the checkpoint response was gone, and that Hxk2 had a role in the DNA damage checkpoint. Deletion of HXK2 also enhanced the checkpoint defect of chk1Δ cells with similar kinetics as inactivation of the cyclase (Fig. 4.5B) suggesting that Hxk2 may have a role in regulating cAMP levels in the DNA damage checkpoint. Furthermore, deletion of HXK2 did not enhance the checkpoint defect of cdc13-1chk1Δtpk2Δ cells (Fig. 4.5C), suggesting that Hxk2 is acting in the same pathway as PKA to help restrain mitosis following DNA damage. These results support a model in which cAMP is required for regulation of PKA following DNA damage. In addition, Hxk2 is not required for the phosphorylation of the R subunit following DNA damage (Fig. 4.5D). These results support a model in which a signaling complex containing the phosphorylated R subunit and Zds2 is regulated by cAMP signaling in order to help restrain mitosis following DNA damage.

4.5 Discussion

How a tetramer such as PKA can interpret different stimuli to elicit a distinct cellular response is an area of intense investigation in the signaling field. PKA
Figure 4.5. cAMP and Hxk2 are required for the role of PKA in the DNA damage checkpoint. A) cdc13-1(Y816), cdc13-1chk1Δ (Y818), cdc13-1rad9Δ (YJS50), cdc13-1hxk2Δ (YJS55), and cdc13-1chk1Δhxk2Δ (YJS72) cells were arrested in G1 using alpha-factor and released into the cell cycle at 32°C. Aliquots were removed at indicated time points and analyzed as in Fig. 4.2B. B) cdc13-1(Y816), cdc13-1chk1Δ (Y818), cdc13-1cdc35-1 (YJS74), cdc13-1cdc35-1chk1Δ (YJS75), cdc13-1chk1Δhxk2Δ (YJS72) cells were arrested in G1 with alpha-factor and released into the cell cycle at 22°C. 30 minutes following release from G1 the temperature was gradually raised so that 45 minutes after release from G1 the temperature was 35°C. Aliquots of the cells were taken at the indicated timepoints and analyzed as in Fig. 4.2B. C) cdc13-1tpk2Δ (YJS8), cdc13-1chk1Δtpk2Δ (YJS10), and cdc13-1chk1Δtpk2Δhxk2Δ (YJS73) cells were released from a G1 block into the cell cycle at 32°C. Aliquots of the cells were taken at indicated timepoints and analyzed as in Fig. 4.2B. D) cdc13-1(Y816), cdc13-1hxk2Δ (YJS55) cells were grown at 22°C and the temperature was increased to 32°C for 120 minutes. The cells were lysed and the proteins analyzed as in Fig. 4.1A.
specificity of signaling in mammalian cells is regulated at least in part by phosphorylation of the catalytic or regulatory subunits, as well as interactions with specific proteins, and sub-cellular localization. Previous studies in *S. cerevisiae* have shown that the R subunit of PKA is phosphorylated in response to various conditions; however, the phosphorylation of the R subunit did not have a role in regulating responses previously associated with PKA activation (Griffioen et al., 2003). We previously showed that the catalytic subunits of PKA have a role in restraining mitosis after DNA damage. Our results presented here indicated that phosphorylation of the R subunit helps inhibit mitosis in response to DNA damage, suggesting that phosphorylation of the R subunit in response to DNA damage could contribute to phosphorylation of PKA substrates involved in restraining mitosis by PKA.

### 4.5.1 Model for activation of PKA by the DNA damage checkpoint

Previous studies showed that growing cells in non-fermentable carbon sources led to phosphorylation of the R subunit (Griffioen et al., 2001), and that addition of glucose to cells growing in a non-fermentable carbon source caused a transient increase of intracellular cAMP (Thevelein, 1994). From these results we postulated that activation of PKA in response to DNA damage is carried out by two steps: the phosphorylation of the R subunit, probably to provide the specificity of the signal, and cAMP activation of PKA catalytic activity. We showed that the R subunit was phosphorylated in response to DNA damage. We also showed that inactivation of adenylate cyclase caused a defect in DNA damage checkpoint mediated arrest, suggesting that cAMP is required for the role of PKA in the DNA damage checkpoint. Our data supports a model in which
Figure 4.6 Model for PKA regulation following DNA damage. Our data supports a model in which Mck1 and Mec1 regulate the phosphorylation of the R subunit following DNA damage. Zds2 regulates the phosphorylated R subunit to help inhibit mitosis. cAMP signaling which may be regulated by Hxk2 controls the final step in the regulation of PKA in order to help restrain mitosis.
the R subunit phosphorylation and cAMP signaling are required for PKA mediated restraint of mitosis following DNA damage.

We have identified three proteins that have a role in the DNA damage checkpoint. These proteins were previously implicated in regulation of PKA by regulating the phosphorylation of the R subunit (Mck1), regulating the cytoplasmic localization of the R subunit (Zds2) (Griffioen et al., 2003), and regulation of cAMP levels (Hxk2) (Rolland et al., 2001). We found that Mck1, but not Zds2 or Hxk2, was required for the phosphorylation of the R subunit. Based on the functions previously attributed to these proteins and the evidence presented, here we propose that in response to DNA damage the R subunit is phosphorylated in a checkpoint and Mck1 dependent fashion. The phosphorylated R subunit still associated with the catalytic subunits is further regulated by Zds2, possibly by protein-protein interaction or sub-cellular localization. In the final step of regulation, cAMP levels regulated by Hxk2 are required for activation of PKA signaling to help restrain mitosis. (Fig. 4.6)

4.5.2 DNA damage checkpoint uses a Mck1 dependent phosphorylation of the R subunit

Mck1 was previously identified as a regulator of R subunit phosphorylation in response to heat shock and extra-cellular calcium; however, the Mck1 dependent phosphorylation of the R subunit did not result in phenotypes previously associated with activation of PKA signaling (Griffioen et al., 2003). It is interesting to note that both calcium and heat shock cause defects in maintaining cell wall integrity, in which case a delay in cell division would benefit the cell. Furthermore, previous studies have shown
that addition of calcium does cause a slight delay in anaphase (Mizunuma et al., 2001).
Therefore, we can speculate that Mck1 mediated phosphorylation of the R subunit may
activate or target PKA specifically towards substrates that, when phosphorylated, would
lead to inhibition of anaphase.

4.5.3 Zds2 regulates PKA following DNA damage

Zds2 may also help provide specificity to PKA signaling. We found that Zds2,
not Zds1, enhanced the checkpoint defect of chk1Δ cells. In fact, deletion of ZDS1
delayed the rate at which chk1Δ cells failed in the checkpoint mediated arrest. This result
was not surprising as Zds1 has been shown to promote mitosis, and cells lacking ZDS1
have a delay in G2 and mitosis under normal growth conditions (Mizunuma et al., 2001).
These results suggests that Zds2, but not Zds1, is involved in the DNA damage
checkpoint; however, it was previously shown that both Zds1 and Zds2 were involved in
regulating the cytoplasmic localization of the phosphorylated R subunit under conditions
in which the phosphorylation of the R subunit was Mck1 dependent (Griffioen et al.,
2003). Based on this data we cannot rule out a role for Zds1 in restraining mitosis
following DNA damage. There are at least two possibilities for the role of Zds1 in the
checkpoint. First Zds1 may play a role in restraining anaphase that cannot be uncovered
due to the role of Zds1 in promoting mitosis. Second, both Zds1 and Zds2 may have a
role in regulating PKA signaling in response to DNA damage, but only Zds2 regulates
PKA signaling associated with restraining mitosis.

It is interesting to note that both Zds1 and Zds2 mediated the cytoplasmic
localization of the R subunit in conditions in which the R subunit was phosphorylated,
but here we show that Zds2 and phosphorylation of the R subunit are involved in restraining mitosis, which is an event that takes place primarily in the nucleus. Because there is little known about how Zds1 and Zds2 regulate R subunit localization we can only speculate. It is possible that Zds2 is required for the export of the phosphorylated R subunit from the nucleus, but not the PKA holoenzyme thereby maintaining active catalytic subunits to help restrain mitosis. Alternatively Zds2 could act to retain the holoenzyme in the cytoplasm so it can be activated and then the active catalytic subunits could re-enter the nucleus.

4.5.4 Regulation of cAMP

In *S. cerevisiae*, increases in cAMP due to either intracellular acidification, or addition of glucose, require Hxk2 (Rolland et al., 2001). In our studies, either deletion of *HXK2* or inactivation of adenylate cyclase enhanced the checkpoint defect of *chk1Δ* cells, suggesting that Hxk2 regulates cAMP levels following DNA damage. Since we do not know yet whether or not cAMP levels increase in response to DNA damage, there are at least two models by which Hxk2 could be regulating cAMP in the checkpoint response. In the first model we propose that a basal level of cAMP, which is maintained by Hxk2, is sufficient for the activation of PKA in the checkpoint response. Alternatively the checkpoint could lead to increased cAMP levels in the cell by regulating Hxk2.

Structure-function studies have suggested that the glucose phosphorylating catalytic activity of Hxk2 is required for the increase in cAMP levels (Kraakman et al., 1999). If this model is correct, we can predict that glucose may be required in order to activate Hxk2. We found, however, that the non-Chk1 dependent checkpoint was intact.
in cells growing with glycerol or raffinose as their only carbon source (Data not shown). This suggests that glucose is not required for the checkpoint, but, we cannot rule out that the cells maintain a sufficient level of glucose to activate Hxk2 by breaking down trehalose stores in the cell. It is also possible that Hxk2 has a role in the DNA damage checkpoint separate from its role in regulating levels of cAMP.

4.5.5 Conclusion

Our results support a model in which the DNA damage checkpoint can regulate the PKA pathway to induce specific PKA signaling in order to phosphorylate substrates that act to restrain mitosis. We identified three novel proteins, Mck1, Zds2 and Hxk2 that have a role in the DNA damage checkpoint via regulation of PKA. These and further studies into the proteins that regulate mitotic restraint in response to DNA damage could provide putative targets for therapeutic treatments of diseases such as cancer that can result from genomic instability.
5. Discussion and Future Directions

Investigation of the kinetics of Pds1 degradation in response to DNA damage prompted the discovery that PKA had a role in the DNA damage checkpoint. We have investigated the role of PKA in the DNA damage checkpoint, including the mechanism by which PKA inhibits mitotic progression and the mechanisms by which the components of the DNA damage checkpoint regulate PKA. In this discussion section, I will address what we and others have uncovered about how PKA is regulated by the DNA damage checkpoint and address what questions remain regarding the mechanism by which the crosstalk between the DNA damage checkpoint and PKA pathways occur.

5.1 Direct or Indirect Regulation of PKA by the DNA damage checkpoint

The DNA damage checkpoint could regulate PKA through a direct or indirect mechanism. I will define a direct mechanism as regulation of PKA or proteins that regulate PKA by DNA damage checkpoint proteins, whereas an indirect mechanism would be regulation of PKA by cell cycle regulators or cell cycle position effect. There is some evidence that supports both models, but ignorance of the exact point of crosstalk between the two pathways precludes direct testing of either model.

Evidence presented in this dissertation, that mutants defective for CDC20 phosphorylation could restore growth of APC mutants to a similar extent to inactivation of PKA signaling (Searle et al., 2004) (Fig. 5D), suggests that the APC mutants and the DNA damage checkpoint both regulate phosphorylation of Cdc20 via regulation of PKA signaling. Because both the DNA damage checkpoint and APC mutants arrest with high
levels of APC substrates (Murray, 1995), this evidence suggests that it could be a cell
cycle position effect (indirect regulation) not checkpoint signaling that regulates PKA in
response to DNA damage.

In APC mutants and upon activation of the DNA damage checkpoint, there is a
delay in mitotic progression and accumulation of APC substrates including Pds1, Clb2
and Cdc20. When there is accumulation of the B type cyclin (Clb2), there is an increase
in cyclin CDK activity (Sanchez et al., 1999). The increase in CDK activity could lead to
phosphorylation of a substrate that would activate the PKA pathway. Similarly,
accumulation of Cdc20 could activate the PKA pathway by Cdc20 mediated
ubiquitination and degradation of an inhibitor of PKA activation. If accumulation of
Cdc20 and/or Clb2 results in activation of PKA then we would expect that in nocodazole
arrested cells where there is accumulation of Clb2 and Cdc20 (Minshall et al., 1996)that
PKA would be activated. We found, however, that neither Cdc20 nor the R subunit was
phosphorylated in nocodazole arrested cells, suggesting that PKA is not regulated in the
same way in a nocodazole induced arrest as in the DNA damage checkpoint (Figure
S2(Searle et al., 2004)and figure 4.1). These results suggested that the role of PKA in
the checkpoint is due to a direct signal where the DNA damage checkpoint proteins
regulate PKA or proteins that regulate PKA activation.

5.2 Points of Regulation of PKA by the DNA damage checkpoint

Although our data supports a model that the DNA damage checkpoint directly
regulates PKA, we need to identify the point of crosstalk between the DNA damage
checkpoint and PKA pathways before we can directly test this hypothesis. We have
identified three proteins previously implicated in regulating PKA as components of the DNA damage checkpoint. In this discussion I will address what has been elucidated by us and others about these proteins and how they regulate PKA as well as how they are regulated by the DNA damage checkpoint.

5.2.1 The role of Hxk2, cAMP and Glucose in the checkpoint

We identified Hxk2 as a component of the DNA damage checkpoint. Hxk2 has been shown to be regulated by phosphorylation and localization (Randez-Gil et al., 1998), (Ahuatzi et al., 2004). Some of the functions of Hxk2 other than phosphorylating glucose require the glucose kinase catalytic activity of the protein (Kraakman et al., 1999). These data suggest that Hxk2 activity in the checkpoint could be regulated by either post-translational modification of Hxk2 or by mediating the amount of intracellular glucose, or it could be a combination of both mechanisms.

Phosphorylation of Hxk2 has been detected in cells growing in glucose, but is abolished when the cells are grown in a non-fermentable carbon source. This phosphorylation seems to be critical in down regulating transcription of genes that are involved in glucose metabolism (Ahuatzi et al., 2004). Based on the data that the cAMP in the cell is increased upon addition of glucose, and that this increase in cAMP levels is dependent on Hxk2 (Rolland et al., 2001), we can speculate that the phosphorylation of Hxk2 may be required for its role in the checkpoint. To test whether Hxk2 is regulated by a post-translational modification by the checkpoint we could determine whether there is a change of mobility of the protein from cells that had a DNA damage signal compared to those with no DNA damage in an SDS-PAGE gel. The phosphorylation of Hxk2 could
regulate a change in Hxk2 protein levels, a change in the sub-cellular localization of Hxk2, and/or a change in the catalytic activity of Hxk2. In addition, a different type of modification, such as ubiquitination, or modification of protein-protein interactions could regulate Hxk2 following DNA damage.

Hxk2 may also be regulated by the availability of intracellular glucose. The glucose kinase catalytic activity of Hxk2 has been shown to be required for the increase in cAMP after addition of glucose to glucose-depressed cells (Kraakman et al., 1999). From this result we can speculate that intracellular glucose could activate Hxk2 to perform its role in the checkpoint. However, our results that the role of Hxk2 in the checkpoint is still intact in cells growing in glycerol or raffinose (data not shown) suggests that glucose is not required for the DNA damage checkpoint. This experiment does not rule out the possibility that there is enough glucose released from trehalose stores to activate Hxk2. Determining whether or not the checkpoint requires glucose is very complicated as glucose and especially glucose-6-phosphate is required for many cellular functions. If we deleted Tps1, a protein involved in converting excess glucose-6-phosphate to trehalose (Petit and Francois, 1994), we may be able to deplete the trehalose stores so that no glucose could be released. In this mutant we may be able to determine the role of glucose in the DNA damage checkpoint. The experiment has many caveats as there are glucose-6-phosphatases that could generate glucose from the glucose-6-phosphate also Hxk2 itself is regulated by Tps1 (Hohmann et al., 1993). In addition, accumulation of glucose-6-phosphate also inhibits Hxk2 activity. Although the question of whether or not glucose is required for the checkpoint is both an important and
interesting question, the experiments and interpretation of the results from these experiments could be difficult.

5.2.2 The role of Hxk2 in restraining mitosis

Hxk2 is an interesting protein in that it is both a glucose kinase, and has been reported to have protein kinase activity (Herrero et al., 1989). Interestingly, Hxk2 was shown to be required for the increase in cAMP levels in response to an increase in glucose concentration, or activation of the Ras pathway (Thevelein and de Winde, 1999). Because of the diverse functions of Hxk2, there are at least two possibilities by which Hxk2 may help restrain mitosis in response to DNA damage. First Hxk2 could be regulating cAMP levels in the cell or Hxk2 may use its protein kinase activity to mediate signal transduction to restrain cell cycle progression.

Inactivation of adenylate cyclase enhanced the checkpoint defect of \( \text{chk1}^{-}\) cells (Fig. 4.5), suggesting that cAMP is required for the DNA damage checkpoint. This data does not address whether an increase in cAMP is required for the checkpoint response or whether basal levels of cAMP are sufficient for restraining mitosis. It would be interesting to measure the amount of cAMP in the cell to determine whether there is more cAMP after treatment with DNA damage; however, a lack of increase in the levels of cAMP would not rule out the possibility that there is an increase of cAMP only in a small region of the cell. Localized increases in cAMP have been found in mammalian cells (Rich and Karpen, 2002) and this may be a likely result in \( S. \text{cerevisiae} \) in response to DNA damage as treating cells with DNA damage does not lead to the same transcriptional response as activation of PKA. In order to determine localized PKA
signaling, we could perform loss of FRET (fluorescence resonance energy transfer) experiments in which dissociation of the catalytic and regulatory subunits would result in loss of FRET.

Based on the data that Bcy1 is phosphorylated in response to DNA damage, it is also possible that the phosphorylation of Bcy1 either lowers the affinity of Bcy1 for the catalytic subunit or increases the affinity of Bcy1 for cAMP resulting in a lower level, perhaps basal levels of cAMP, being able to activate PKA.

5.2.3 Regulation of Mck1 by the checkpoint

We showed that Mck1 was required for the phosphorylation of Bcy1 in response to DNA damage (Fig. 4.2A) suggesting that Mck1 may be a point of cross-talk between the DNA damage checkpoint and the PKA pathway. Since Mck1 has been shown to be phosphorylated on serine residues (Lim et al., 1993) we predict that the DNA damage checkpoint could regulate Mck1 via phosphorylation. We could detect modification of Mck1 by analyzing the change in mobility of Mck1 protein from cells treated or not with DNA damage.

5.2.4 Function of Zds2 in the checkpoint

We identified Zds2 as a component required for the mitotic delay of chk1Δ cells following DNA damage. We also showed that phosphorylation of the R subunit does not require Zds2 (Fig. 4.4). Because Zds2 was identified as a regulator of Bcy1 cytoplasmic localization under conditions in which the R subunit is phosphorylated (Griffioen et al., 2003), we can postulate that Zds2 regulates PKA via phosphorylated Bcy1 in response to
DNA damage. Zds2 could help inhibit mitotic progression in response to DNA damage by regulating the export of phosphorylated Bcy1 from the nucleus, thereby removing the regulatory subunit from the nucleus and rendering the catalytic subunits still in the nucleus active.

Zds2 could also act as a scaffolding protein, providing docking sites for the phosphorylated Bcy1 which would still be part of the PKA holoenzyme and substrates that when phosphorylated by PKA would inhibit mitotic progression. Thus, when PKA was activated, (presumably by cAMP) the substrates would be in close proximity of the catalytic subunits providing a specific response to the DNA damage checkpoint signal. Building on this model we could also speculate that interaction of Zds2 with the phosphorylated Bcy1 may allow PKA to be activated by basal levels of cAMP.

5.3 The role of PKA signaling in the DNA damage checkpoint in the mammalian system.

Checkpoint components have been shown to be mutated in a large percentage of cancer cells and it is generally understood that mutations in checkpoint proteins can lead to uncontrolled cell growth and cancer. Some cancer treatments use inhibitors of checkpoint proteins along with DNA damaging agents to promote the death of cancer cells (Kastan and Bartek, 2004). As we identify more components of the pathways that protect the integrity of the genome, we can also test these components as potential drug targets for the treatment of cancer. Therefore, it will be important to determine whether the role of the PKA pathway in the DNA damage checkpoint is conserved in mammalian cells.
It has been shown that perturbations in PKA signaling can lead to genomic instability and cancer (Matyakhina et al., 2002). In addition, it was shown that the APC is phosphorylated by PKA and that this phosphorylation causes inhibition of the APC (Kotani et al., 1998). Therefore, it would be interesting to determine whether the APC or Cdc20 was phosphorylated in response to DNA damage in mammalian cells, and whether this phosphorylation was dependent on PKA. Another way to determine whether PKA was activated in response to DNA damage is by a loss of FRET analysis in which the excitement of a fluorescent tag on the regulatory subunit could in turn excite the fluorescent tag on the catalytic subunit. Thus, when PKA is in its active form and the regulatory subunits are not bound to the catalytic subunits, there will be a loss of FRET (Evellin et al., 2004; Rich and Karpen, 2002). Because only a small fraction of PKA may be activated, a loss of FRET analysis in which activation of PKA would result in loss of FRET would be informative as to whether or not PKA is activated, and would also provide insight as to in which regions of the cell PKA is activated.

5.4 Final Conclusions

Our results support a model in which PKA has a role in restraining mitosis following DNA damage by regulating phosphorylation of Cdc20 and stabilization of the mitotic inhibitors, Pds1 and Clb2. Checkpoint regulated phosphorylation of the R subunit as well as cAMP signaling have a role in the checkpoint, suggesting that PKA is regulated by both phosphorylation of the R subunit and cAMP signaling following DNA damage. This model provides excellent groundwork for uncovering the mechanisms of crosstalk between the DNA damage checkpoint and PKA pathways. We have identified
two potential points of crosstalk. First, the checkpoint could regulate the phosphorylation of the R subunit via regulation of the GSK3 kinase, Mck1. Second, the checkpoint could regulate cAMP levels by signaling to Hxk2 and/or adenylate cyclase. Elucidation of this pathway has provided insight into the mechanisms which are in place to prevent genetic instability. It will be important to determine whether PKA has a role in the checkpoint pathway in mammalian cells so we can apply what we have learned using the *S. cerevisiae* model system to generate drug targets for the treatments of diseases such as cancer. Elucidation of this pathway will also provide insight into basic biological questions such as, how specificity of signaling is generated, and how different signaling pathways communicate to produce a single cellular response.
Chapter 6

6. References


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