AD NUMBER

ADB286264

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Sep 2002. Other requests shall be referred to US Army Medical Research and Materiel Comd., 504 Scott St., Fort Detrick, MD 21702-5012.

AUTHORITY

USAMRMC ltr, dtd 28 July 2003

AD_____

Award Number: DAMD17-99-1-9460

TITLE: Mechanisms and Components of the DNA Damage Checkpoint

PRINCIPAL INVESTIGATOR: Marc F. Schwartz, Ph.D.
David F. Stern, Ph.D.

CONTRACTING ORGANIZATION: Yale University
New Haven, Connecticut 06510

REPORT DATE: September 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-99-1-9460
Organization: Yale University

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Carol B Christian _____

1-21-03 _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Annual Summary (1 Sep 99 - 31 Aug 02)

4. TITLE AND SUBTITLE
Mechanisms and Components of the DNA Damage Checkpoint

5. FUNDING NUMBERS
DAMD17-99-1-9460

6. AUTHOR(S)
Marc F. Schwartz, Ph.D.
David F. Stern, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University
New Haven, Connecticut 06510

E-Mail: marc.f.schwartz@yale.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Sep 02). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. Abstract

Survival of genotoxic shock is essential to the survival of any organism. The DNA damage checkpoint (DDC) controls responses to DNA damage. Dysfunction of components of the mammalian DDC pathways, such as ATM, hChk2, p53, and BRCA1, correlate with increased cancer risk. DDC mechanisms are conserved; in *Saccharomyces cerevisiae*, *ATM*-family kinase *MEC1* is required for the DDC, as are hChk2-homolog Rad53 and BRCA1-like Rad9. The powerful genetic and biochemical techniques in the *S. cerevisiae* model present it as an ideal system in which to study conserved DDC mechanisms.

DNA damage induces the *MEC1*-dependent phosphorylation of Rad53. *MEC1* is also required for the damage-dependent phosphorylation of Rad9, and Rad53 interacts with phosphorylated Rad9, suggesting that Rad9 is an adaptor for the DDC. The goal of this work is to characterize physical and catalytic interactions between Mec1, Rad53, and Rad9. As summarized herein, I identified multiple Mec1 phosphorylation sites within Rad9 that are induced in response to DNA damage. I determined the contribution of these phosphorylation sites to Rad9 function, including the interaction of Rad9 with Rad53. Finally, I reconstructed the interaction of phosphorylated Rad9 with *in vitro*, demonstrating that the Rad53 FHA domains specifically bind the phosphorylated form of Rad9 peptides.

14. SUBJECT TERMS	15. NUMBER OF PAGES
DNA damage checkpoint	25
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Survival of genotoxic shock is essential to the survival of any organism. The DNA damage checkpoint signaling pathway controls the cellular response to DNA damage (10, 19, 23, 24). Dysfunction of components of the mammalian DNA damage checkpoint pathway, such as ATM, hChk2, p53, and BRCA1, correlates with accelerated tumorigenesis, increased cancer risk, and tumor chemotherapeutic resistance (2, 8, 11, 18, 20, 21). Eukaryotic DNA damage checkpoint pathway mechanisms are conserved; in *Saccharomyces cerevisiae*, *ATM*-family kinase *MEC1* is required for the DNA damage response, as are Rad53 and Rad9 (1, 5, 12, 14, 16, 19, 22, 25, 29, 30, 31). Rad53 is the founding member of a kinase family implicated in DNA checkpoints, including mammalian homolog hChk2 (3, 4, 12, 13, 15, 30, 37). Rad9 shares homology with the BRCA1 C-terminus (32). *S. cerevisiae* thus provides a powerful genetic system in which to study the conserved DNA damage checkpoint pathway mechanisms.

The Mec1-dependent phosphorylation and activation of Rad53 is a step in the propagation of the DNA damage signal (17, 26, 27). Mec1 is also required for the DNA damage-induced phosphorylation of Rad9 (26, 7, 28), which leads to the binding of Rad53 by phospho-Rad9 via the FHA domains of Rad53 (26, 6). The original objectives of this project were: first, to define the interactions between Mec1, Rad53, and Rad9 that lead to the activation of Rad53 by the DNA damage checkpoint; and second, to identify the mammalian homolog of *RAD53*. As reported after the first year of this grant, the second objective was completed and published by multiple independent laboratories shortly after the beginning of the funding period for this grant (3, 4, 15, 37). Therefore, the work summarized herein focused entirely upon the first objective, a detailed characterization of the mechanism of Rad53 activation by the DNA checkpoint in *S. cerevisiae*.

Progress on Objective 1: Characterization of physical and catalytic interactions of Mec1

Task 1: Identification and characterization of Mec1

To determine if Mec1 phosphorylates Rad9 or Rad53, the first goal was to obtain catalytically active Mec1. As I reported after Year 1, the first route to study of Mec1 activity was to generate bacterially expressed Mec1 kinase domain fusion proteins. A similar approach was previously employed for the study of the catalytic activity of ATM (2). The first step in generating Mec1 constructs was to clone *MEC1* from a wildtype yeast strain. I cloned *MEC1* from diploid yeast strain NY882 by recombination repair. The clone fully rescues the checkpoint deficiencies of a *mec1*Δ strain, suggesting that the clone is fully functional. A bacterial GST-kinase domain fusion was constructed and expressed in protease deficient bacteria. While the fusion protein expressed well, and was easily purified, the construct lacked detectable kinase activity as measured by either autophosphorylation or phosphorylation of a test substrate. Similar truncated fusion proteins expressed in mammalian tissue culture cells also lacked activity, suggesting that the lack of activity is inherent to the construct and not the expression system.

Turning to *S. cerevisiae* as an expression system, I generated a full-length galactose-induced expression construct of Mec1 with an N-terminal V5 epitope tag. This construct also rescues the checkpoint defects of a *mec1*Δ strain, confirming that the epitope tag does not disrupt the function of the protein. Mec1 proved to be an unstable protein; Mec1 rapidly degrades when stored in the freezer. After repeated attempts, I succeeded in observing V5Mec1 in both cell lysates and immunoprecipitations. V5Mec1 is also catalytically active, as measured by autophosphorylation and test substrate phosphorylation in *in vitro* kinase assays. Phosphorylation of test substrates revealed that in the proper context, Mec1 phosphorylates threonine. Other possible substrate residues, such as serine or tyrosine, were not ruled out by these experiments. The V5Mec1 reagent generated in this Task was subsequently used to demonstrate that Mec1 can phosphorylate Rad9 *in vitro* at physiologically relevant sites.

Task 2: Determination of the physiological relevance of Mec1 activity

Rad9 is phosphorylated in response to DNA damage in a *MEC1*-dependent manner. To confirm that Mec1 is indeed the kinase that phosphorylates Rad9 in response to DNA damage, I plan to identify Rad9 sites of *in vivo* phosphorylation, and determine if Mec1 phosphorylates these residues *in vitro*. As I reported in Year 1, and described below in Task 3, we identified Rad9 T603 as a putative Mec1 phosphorylation site involved in Rad53 binding. Using a bacterially produced Rad9 fusion protein containing either T603 or a T603A substitution, I performed a series of *in vitro* immune complex kinase assays with V5Mec1. V5Mec1 specifically phosphorylated the wildtype T603 fusion protein, and not the T603A mutant version, demonstrating that Mec1 indeed phosphorylates Rad9 *in vitro*. As I also reported in Year 1, described below in Task 3, subsequent experiments on this site within Rad9 demonstrated that loss of T603 does not significantly reduce the phosphorylation of Rad9 *in vivo*. One possible interpretation of this data is that Rad9 T603 is simply one of several Mec1 phosphorylation sites that occur within Rad9. As described in Task 3, I undertook during Years 1 and 2 a series of experiments that determined that Rad9 T603, as well as a cluster of putative Mec1 phosphorylation sites ([S/T]Q cluster domain, SCD) within Rad9 are sites of physiological Rad9 phosphorylation and Rad53 binding. Therefore, in Year 3, I developed evidence that directly confirmed that at least a subset of these sites are indeed phosphorylated *in vivo*.

To directly identify sites of *in vivo* phosphorylation on Rad9, I planned to use tryptic phosphopeptide mapping (TPM). This experiment requires the ability to obtain a large amount of *in vivo* ^{32}P-labeled Rad9, which is a very challenging task as it involved handling large amounts (mCi) of radioactive ^{32}P. Furthermore, TPM is most successful on smaller proteins that yield a simpler tryptic peptide map. Nonetheless, as reported in Year 1, I performed pilot experiments to determine the feasibility of this method. Previously in our laboratory, Rad9 was detected and purified via a C-terminal FLAG epitope tag. Unfortunately, shortly before the initiation of these studies, the anti-FLAG antibody we used was discontinued. Altering our protocols to incorporate other anti-FLAG reagents proved intractable. Hence, I replaced the C-terminal FLAG tag on Rad9 with a triple HA epitope (3xHA). This epitope, like the FLAG epitope, does not compromise the function of Rad9. Using Rad9-3xHA, I was able to labeled and purify small amounts of ^{32}P-labeled Rad9. However, this method only generated a small amount of labeled material. Therefore, we instead sought other approaches to generate direct evidence for Rad9 phosphorylation sites.

During Year 3 of this grant, I sought to biochemically purify phosphorylated Rad9 from yeast cell lysates via the use of tandem epitope tags. This material was then to be used to identify Rad9 phosphorylation sites by mass spectrometry. In this approach, I made use of two published multiply-tagged *RAD9* overexpression plasmids. The first plasmid expressed Rad9 tagged with HA and 6xHis tags (7) and the second expressed Rad9 with GST and 6xHis tags (40). The first form of Rad9 was readily expressed and properly phosphorylated in response to DNA damage, yet we found that it very poorly bound to either immunoaffinity or IMAC matrix, suggesting that the epitope tags were inaccessible to the matrix. On the other hand, the GST/His-tagged Rad9 both expressed well and was easily purified to a coomassie-stainable band. However, the GST/His tagged form of Rad9 was not detectable phosphorylated in response to DNA damage, suggesting that the tandem affinity tags were interfering with the *in vivo* function of Rad9. As neither version of overexpressed, tagged Rad9 was acceptable, we explored other methods for directly identifying Rad9 phosphorylation sites.

By Year 3, the mutagenesis screen described in Task 3 had identified a number of putative Mec1-consensus phosphorylation sites within Rad9 (the Rad9 SCD and T603) that were required for the phosphorylation of Rad9 and interaction of Rad9 with Rad53. To generate direct evidence that supported the identification of these sites a *bona fide* Rad9 phosphorylation sites induced by DNA damage, in Year 3 I explored the use of phosphorylation specific antibodies to detect these sites by Western blot. Cell Signaling Technologies (CST) markets a rabbit polyclonal antibody that recognizes ATM/ATR/Mec1 family consensus phosphorylation sites (phospho-[S/T]Q), especially when preceded by a hydrophobic residue). In immunoblots of yeast lysates from cells expressing various forms of Rad9, I observed that this antibody strongly recognized the phosphorylated form of Rad9 induced by DNA damage. Furthermore, mutation of Rad9 T603 strongly reduced this immunoreactivity, suggesting that Rad9 T603 is indeed a physiological DNA checkpoint-induced

Mec1 phosphorylation site within Rad9. The additional mutation of the Rad9 SCD abrogated the remaining phospho-[S/T]Q immunoreactivity observed with the CST antibody , suggesting that the Rad9 SCD indeed contains additional Mec1 phosphorylation sites.

Also in Year 3, to generate further direct evidence for a Mec1 phosphorylation site within the Rad9 SCD, I generated a polyclonal antibody that recognized one of the Rad9 SCD sites when phosphorylated. The antibodies were generated against phosphopeptides synthesized by the Keck Facility at Yale University School of Medicine. I initially attempted to generate a phosphospecific antibody using the Rad9 SCD site T390 peptide TENNSNRSpTQIVNNPR. Although antibodies were purified both through positive and negative selection on phospho- and nonphosphopeptide columns, the antibodies purified in this attempt, while peptide specific, were not phosphorylation specific, as determined by ELISA and immunoblot. We reasoned that shortening the peptide surrounding the phospho-site would increase the chances of developing a phosphospecific antibody against Rad9 T390. Therefore, we retried this protocol using the Rad9 T390 phosphopeptide KSNRSpTQIVN. This peptide contained only four residues on either side of the phosphorylation site, in addition to a lysine residue added at the N-terminus to facilitate crosslinking of the peptide to carrier. This modification of the antigenic peptide successfully allowed the purification of antibody that specifically recognized Rad9 T390 when phosphorylated, directly demonstrating that the Rad9 SCD is indeed a target of phosphorylation in response to DNA damage.

Task 3: identification of physical interactions between Mec1, Rad53, and Rad9

To characterize the formation of a complex including Mec1, Rad53, and Rad9, I sought to identify by immunoprecipitation a complex containing Mec1 and Rad9 or Rad53. As I reported in Year 1, working with Damon Banks, a rotation student in the lab, we sought two-hybrid evidence for an interaction between these proteins. We constructed three overlapping Mec1 constructs, and tested them in combination with Rad9 and Rad53 constructs. Unfortunately, we did not observe a significant two-hybrid interaction between these constructs. During Years 1 and 2, unsuccessful attempts were also made to coimmunoprecipitate Mec1 with either Rad9 or Rad53. Thus, to date, we have been unable to directly identify a stable interaction between Mec1 and either Rad9 or Rad53. However, we continued our analysis of the robust DNA damage-induced interaction between Rad53 and phosphorylated Rad9.

As I discussed after Year 1, Durocher *et al* (6) demonstrated *in vitro* phospho-specific binding of FHA domains to artificial substrates. Surprisingly, they were also able to use a GST-Rad53 FHA1 fusion protein to precipitate Rad9-containing complexes from cell lysates. This was unexpected given our two-hybrid analysis that suggested that Rad53 FHA1 is not able to substitute for Rad53 FHA2 (26). This data suggest that Rad53 FHA1 may participate in the Rad53/Rad9 interaction; alternatively, Rad53 FHA1 may also be capable of interacting with other components of a Rad9-containing complex. Therefore, during Year 3, I studied the *in vivo* contribution of both of the Rad53 FHA domains to its interaction with phosphorylated Rad9 in the DNA damage checkpoint pathway. To this end, I used a series of yeast expression constructs that express endogenous levels of Rad53 with mutations in conserved residues of FHA1, FHA2, or both. Mutation of Rad53 FHA1 or FHA2 did not alter Rad9 phosphorylation profiles induced by DNA damage, consistent with the lack of a requirement for RAD53 for Rad9 phosphorylation. Mutation of either Rad53 FHA1 or FHA2 strongly reduced the ability of Rad53 to coimmunoprecipitate with phosphorylated Rad9, and similarly reduced the DNA damage-induced phosphorylation of Rad53. Notably, mutation of Rad53 FHA2 impaired the coimmunoprecipitation of Rad53 with Rad9 and Rad53 phosphorylation even more severely than mutation of Rad53 FHA1. Finally, mutation of both Rad53 FHA domains completely eliminated the coimmunoprecipitation of Rad9 with Rad53 prevented the DNA damage-induced phosphorylation of Rad53. These results indicate that the Rad53 FHA domains are both required for the stable interaction of Rad53 with phosphorylated Rad9, although there is a slight preference for Rad53 FHA2.

Genetic and biochemical data indicates that Rad9 is likely a direct substrate of Mec1 *in vivo*, and, as I reported in Year 1 Task 2, Mec1 phosphorylates an ATM-family consensus [S/T]Q site within Rad9 *in vitro*. To expand our understanding of the Rad53-Rad9 interaction first discovered in our laboratory, I studied the

possibility that the putative Mec1 phosphorylation [S/T]Q sites within Rad9 are *bona fide* DNA damage-induced phosphorylation sites that serve as Rad53 FHA domain docking sites *in vivo*. This is especially exiting as, though the FHA domain is a novel phosphorylation-dependent protein binding domain with several literature reports of *in vitro* binding specificities, not one *in vivo* FHA domain binding site has yet been described. As I reported in Year 1, two-hybrid analysis of Rad9 deletion constructs identified a 78 amino acid sequence within Rad9 that is sufficient for the two-hybrid interaction with Rad53 FHA2. Supporting the hypothesis that Mec1 creates the binding site for Rad53, a putative ATM-family phosphorylation site ([S/T]Q) was observed within this sequence at Rad9 T603. Mutation of the threonine to alanine largely abrogated the two-hybrid interaction, and mutation to a positively charged arginine abolished the interaction. However, substitution of a negatively charged glutamate for the threonine, mimicking the negative charge of a phosphorylated threonine, largely rescued the two-hybrid interaction.

To confirm the identification of a Rad53 phospho-binding site within Rad9 *in vivo*, during Year 1, I generated Rad9-3xHA expression constructs containing either a threonine-to-alanine substitution at T603, or a 28 amino acid deletion encompassing T603. Surprisingly, these mutations failed to impair the Rad9 phosphorylation observed in response to DNA damage. These data suggested that Rad9 T603 either is not a major phosphorylation site within Rad9, or phosphorylation of T603 does not have a major affect on the electrophoretic mobility shift of Rad9. If Rad53 FHA2 binds Rad9 primarily at T603, these mutations should prevent this interaction, detaching Rad53 from DNA damage checkpoint regulation. However, these mutations fail to both alter the activation of Rad53, and disrupt the normal action of the G_2/M checkpoint-dependent arrest after DNA damage. These data suggest that Rad9 T603 is either redundant with other Rad53 binding sites, or was a false positive identified in the potentially artificial environment of the two-hybrid system.

As I reported in Year 1, to identify other putative Mec1 phosphorylation sites that contribute to the interaction of Rad53 with Rad9, I constructed a set of strains containing combinations of mutations these residues. To eliminate the possibility of redundant contribution of T603, these mutations were generated in Rad9 already containing the 28 amino acid deletion surrounding T603. While most of these additional mutations had no effect, the deletion of 69 amino acids containing a cluster of six of these sites (the Rad9 SCD) both abrogated the DNA damage induced phosphorylation of Rad9, and impaired the interaction of Rad53 with Rad9.

To confirm that the disruptions in Rad53/Rad9 behavior observed are due to the loss of the phosphorylation sites, and not due to a nonspecific disruption of Rad9 from the deletion, in Year 1 I also generated a mutant with alanine substitutions at the six putative phosphorylated residues ($rad9^{6xA}$). Like the cluster deletion, this six-alanine mutation (6xA) impairs both the DNA damage dependent phosphorylation of Rad9, and its subsequent interaction with Rad53. These data suggest that this cluster is a *bona fide* binding site for Rad53. Building on the 6xA mutation, I alanine-substituted T603 to generate the 7xA mutant. $rad9^{7xA}$ biochemically appears similar to the $rad9^{6xA}$.

The Rad9 SCD contains a total of six [S/T]Q sites. As I reported in Year 2, to determine if a single site has a dominant contribution to Rad9 phosphorylation and interaction with Rad53, I tested a series of *rad9* mutants containing single alanine substitutions of individual SCD sites. Mutation of single [S/T]Q sites within the SCD had little apparent effect on the DNA damage induced phosphorylation of Rad9, immunoprecipitation of Rad9 with Rad53, or Rad53 phosphorylation . Since simultaneous substitution of all six sites does inactivate these functions, these data suggest that there are multiple phosphorylation sites within this group, and that Rad53 interacts redundantly with some or all of these sites.

As I reported in Year 2, to characterize the ability of a single Rad9 SCD site to support the interaction of Rad9 with Rad53, I examined the rescue of Rad9 and Rad53 DNA damage checkpoint regulation after restoration of single [S/T]Q residues into an otherwise mutant SCD. Reintroduction of a wildtype residue at Rad9 S435 best restored the DNA damage-induced slower mobility forms of Rad9. However, reintroduction of Rad9 S435 only modestly rescued the DNA damage-induced Rad9 coimmunoprecipitation with Rad53, and the phosphorylation of Rad53. By contrast, reintroduction of Rad9 T390 moderately restored the DNA damage-induced slower mobility forms of both Rad9 and Rad53. However, even this add-back mutant only partially

rescued coimmunoprecipitation of Rad9 with Rad53, and no single Rad9 cluster add-back allele fully restored the checkpoint-induced phosphorylation or coimmunoprecipitation of Rad9 and Rad53. These data indicate that the Rad9 [S/T]Q cluster sites may act in an additive or cooperative manner to recruit Rad53. Moreover, these results extend the absolute correlation between the ability of Rad9 to coimmunoprecipitate with Rad53 and the damage-regulated phosphorylation of Rad53, supporting the model wherein Rad9 functions as an adaptor for the DNA damage checkpoint signaling pathway.

To determine if the Rad9 SCD and T603 contribute to the function of Rad9 in the checkpoint response to DNA damage, I analyzed the ability of these mutants cells to prevent mitosis in the face of a strong DNA damage signal. In this assay, *rad53* cells initiate arrest, but fail to maintain the G_2 arrest as single nucleated cells, instead proceeding with nuclear division to yield cells with two nuclei (9, 26). *rad9*Δ cells demonstrate a complete failure of the G_2/M checkpoint arrest, proceeding through nuclear division without pause (9). If the rad9^{6xA} mutant fails to activate Rad53 due to a general defect in Rad9 function, then *rad9*^{6xA} cells will behave similar to *rad9*Δ. Alternatively, if rad9^{6xA} is functionally deficient for Rad53 activation as it is biochemically, then *rad9*^{6xA} cells will behave similar to *rad53* cells. As I reported in Year 1, my analysis of the G_2/M DNA damage checkpoint in wildtype, *rad9*^{6xA}, *rad9*Δ, and *rad53*$^{FHA2\text{-}NVS}$ demonstrated that *rad9*^{6xA} has defects similar to those of the *rad53* cells .

In Year 2, I expanded this functional analysis to include a subset of the partial SCD site mutants described above. Consistent with the ability of the T390 add-back mutant to significantly recover the DNA damage-induced interaction of Rad9 with Rad53, reintroduction of Rad9 T390 to *rad9*^{6xA} also partially recovered the *RAD53*-dependent G_2/M arrest. However, this rescue was weak, suggesting that the regulation of Rad53 via an intact Rad9 SCD is required for the full action of the *RAD53*-dependent G_2/M DNA damage checkpoint arrest. Readdition of S435 to the *rad9*^{6xA} mutant only weakly recovering the coimmunoprecipitation of Rad9 with Rad53, was less able to recover the G_2/M arrest.

Both Rad53 and another DNA damage checkpoint kinase, Chk1, act to enforce the G_2/M checkpoint arrest in response to DNA damage (9, 26, 38, 39). To confirm that the G_2/M arrest defect in the *rad9*^{7xA} cells reflects the failure of the DNA damage checkpoint pathway in coupling to Rad53 rather than Chk1, in Year 3 I determined the effect of the *rad9*^{7xA} mutation in *chk1*Δ and *rad53*Δ cells. *rad53*Δ *rad9*^{7xA} cells were as defective in the G_2/M DNA damage checkpoint arrest as *rad53*Δ cells, suggesting that the two mutations disrupt the same pathway. By contrast, *chk1*Δ *rad9*^{7xA} cells were less competent for the function of the G_2/M checkpoint arrest than *chk1*Δ cells, suggesting that Chk1 and the Rad9 phosphorylation sites function in separate pathways.

RAD9 is required for the DNA damage-induced phosphorylation of Chk1, and Rad9 interacts with Chk1 in a yeast two-hybrid assay (38), suggesting that Rad9 may also directly recruit Chk1 to the DNA damage checkpoint pathway. Therefore, In Year 3, I determined whether the DNA damage-induced phosphorylation of Chk1 is intact in *rad9*^{7xA} cells. As expected, the DNA damage-dependent phosphorylation of Rad53 and Chk1 was absent in a *rad9Δ* strain, and Rad53 phosphorylation was abrogated in *rad9*^{7xA} cells. However, the damage-dependent phosphorylation of Chk1 was not lost in the *rad9*^{7xA} strain. These data indicate that the major DNA damage-induced Rad9 phosphorylation sites are not essential for the regulation of Chk1 by Rad9.

Task 4: in vitro reconstruction of the Rad53-Rad9 interaction

One of the outstanding questions about the Rad53-Rad9 interaction is whether the interaction is direct, or via another polypeptide or other intermediary. To distinguish these possibilities, I sought to demonstrate Rad53 binding to Rad9 *in vitro* using components generated independently of *S. cerevisiae*. After identification of Rad9 T603 as a putative phospho-specific binding site for Rad53, I had synthesized two versions of the 28 mer tryptic peptide containing T603: a normal version, and a version with phosphothreonine at TQ#8. To demonstrate binding of Rad53 to this peptide, I developed the following assay: the synthetic Rad9 peptides were dot blotted to a membrane, and a soluble, bacterially expressed Rad53 FHA2 fusion protein was floated in. GST-Rad53 FHA2 binding was detected via chemiluminescence with antibodies to GST. This experiment

demonstrated that Rad53 FHA2 specifically binds the phosphorylated version of a Rad9 peptide containing an *in vivo* phosphorylation site induced by DNA damage.

During Year 2 of this project, I identified several Mec1 phosphorylation sites within Rad9 that are induced by DNA damage, including Rad9 T390. The ability of $Rad9^{6xA+T390}$ to enable a significant proportion of the Rad9 interaction with Rad53 implies that T390 is a major functional site for Rad53 binding *in vivo*. To determine if Rad53 binds directly to Rad9 T390 in a phosphorylation-dependent manner, I measured the increase of surface plasmon resonance caused by binding of soluble, bacterially produced Rad53 FHA domains to synthetic Rad9 T390 peptides immobilized on a BIAcore sensor chip. Rad53 FHA1 specifically interacted with the phosphorylated Rad9 T390 peptide (*P*-T390), and mutation of two conserved FHA domain residues abolished this binding. Similar to FHA1, GST-Rad53 FHA2 preferentially bound the phosphorylated Rad9 T390 peptide, demonstrating an affinity lost upon mutation of conserved FHA2 residues. On average, Rad53 FHA1 bound the phosphorylated Rad9 T390 peptide with a K_D of 2.5 μM (s=0.3, n=6), and GST-Rad53 FHA2 bound with a K_D of 1.4 μM (s=0.3, n=3), though the observed affinity of GST-FHA2 may have been artificially increased due to the ability of GST to homodimerize. Taken together, these results show that the Rad53 FHA domains can directly and specifically bind Rad9 peptides phosphorylated at ATM-family consensus [S/T]Q phosphorylation sites. Thus, we successfully reconstructed *in vitro* the DNA damage-induced binding of Rad53 FHA domains to Rad9 phosphorylation sites.

Progress on Objective 2: Identification of mammalian Rad53 homologs

Task 1: Initiation of screens for mammalian checkpoint homologs

As reported in Year 1 of the project:

At the outset of this project, there were no known mammalian homologs of Rad53. To identify these genes, I planned a degenerate-PCR strategy to isolate homologs based on their sequence similarity in FHA and kinase domains. Working with Damon Banks, we designed and had synthesized a set of highly degenerate primers for both the FHA and kinase domains. We selected a λ-gt11 library constructed from growing Jurkat cells, lymphocytes we reasoned were likely to express DNA damage checkpoint genes. We tested a range of PCR amplification parameters, including annealing and extension temperatures, primer and template concentration, and divalent cation content. In a pilot experiment, we amplified and cloned several products. Of eight isolates, in two the primers had amplified a genuine target sequence; one of the kinase domain primers successfully amplified a kinase. Surprisingly, both independently amplified kinase clones were Chk1, a known DNA damage checkpoint component. While the pilot experiment did not identify a novel checkpoint gene, this result suggested that this was a viable approach.

Unfortunately, shortly after the pilot experiments in developing the screen for mammalian homologs for Rad53, several laboratories published their independent isolation and characterization of Rad53 homologs (3, 4, 15, 37). The identified gene, named Chk2, is a component of the mammalian ATM/ATR DNA damage checkpoints, validating the concept for this objective. However, between these and subsequent publications, much of the remaining experiments proposed for Objective 2 were reported. Therefore, based on he advice of my mentor, Dr. David Stern, and my thesis advisory committee, I am focusing my efforts on the development and expansion of the goals within the first objective. The work required to develop, as well as the results derived from my goals in Objective 1 are much more expansive than I originally anticipated. Indeed, the "overly ambitious" nature of my proposal was one of the few negative comments given by the proposal's reviewers.

Key Research Accomplishments

- Cloned, expressed, and purified ATM-homolog Mec1
- Developed *in vitro* Mec1 kinase assay

- Identified Rad9 as a Mec1 substrate *in vitro*
- Identified putative Mec1 phosphorylation sites (Rad9 SCD and T603) within Rad9 *in vivo* required for Rad53 interaction and function of the DNA damage checkpoint
- Characterized the contribution of the Rad9 SCD and T603 to:
    - Rad9 phosphorylation
    - Interaction of Rad9 with Rad53
    - Rad53 phosphorylation
    - Function of the G_2/M DNA damage checkpoint arrest
    - Survival of genotoxic stress
    - Interaction of Rad9 with Chk1
- Reconstructed *in vitro* the binding of Rad53 FHA2 to a phosphorylated Rad9 peptide
- Demonstrated *in vitro* the direct binding of Rad53 FHA domains to a phosphorylated Rad9 SCD peptide
- Confirmed Rad9 T603 and SCD site T390 as physiological phosphorylation sites induced by DNA damage
- Initiated a degenerate-PCR screen for Rad53 homologs

Reportable Outcomes

1. Manuscripts, abstracts, and presentations
    - Schwartz, M.F. (2002) FHA domain-mediated regulation of Rad53 by DNA checkpoint pathways in *Saccharomyces cerevisiae*. Ph.D. Thesis, Department of Cell Biology, Yale University.
    - Schwartz, M.F., Lee, S., Duong, J.K., and Stern, D.F. (2002) Regulation of Rad53 by its FHA domains. *In preparation.*
    - Schwartz, M.F., Duong, J.K., Sun, Z., Morrow, J.S., Pradhan, D., and Stern, D.F. (2002) Rad9 phosphorylation sites couple Rad53 to the *Saccharomyces cerevisiae* DNA damage checkpoint. Molecular Cell 9: 1055-1065. *(reprint included as Appendix 2)*
    - Schwartz, M.F., Duong, J.K., Sun, Z., Pradhan, D., and Stern, D.F. (2002) Rad9 phosphorylation sites directly couple Rad53 to regulation by the DNA damage checkpoint pathway. Keystone Symposium, Molecular Mechanisms of DNA Replication and Recombination.
    - Schwartz, M.F., Sun, Z., Hsiao, J., and Stern, D.F. (2000) Interaction of Rad53 with Rad9 in the DNA Damage Checkpoint of *S. cerevisiae*. Department of Defense Breast Cancer Research Program Era of Hope Meeting.
    - Schwartz, M.F., Sun, Z., Hsiao, J., and Stern, D.F. (2000) Phosphorylation-dependent Interaction of Rad53 with Rad9 in the DNA Damage Checkpoint of *S. cerevisiae*. 65th Cold Spring Harbor Laboratory Symposium, Biological Responses to DNA Damage.

2. Patents and licenses applied for and/or issued
3. Degrees obtained
   - Doctor of Philosophy (Cell Biology), 2002, Yale University
4. Development of cell lines, tissue or serum repositories
5. Informatics
6. Funding applied for
7. Employment or research opportunities applied for

Conclusions

All of the goals of the first objective of this proposal have been met and exceeded. I successfully developed Mec1 as a biochemical reagent. I identified and confirmed *in vivo* Mec1 phosphorylation sites within Rad9 that are required for both the phosphorylation of Rad9 and the interaction of Rad53 with Rad9 in response to DNA damage. I determined the contribution of individual sites within the Rad9 SCD to the function of Rad9 in regulating Rad53 and the G_2/M DNA damage checkpoint. I demonstrated that Mec1 can phosphorylate Rad9 *in vitro*, and that both Rad53 FHA domains bind Rad9 phosphopeptides *in vitro*. I found that both Rad53 FHA domains are required *in vivo* for the interaction of Rad53 with Rad9, while the Rad9 phosphorylation sites bound by Rad53 are not required for the *RAD9*-dependent DNA damage checkpoint regulation of another conserved checkpoint kinase, Chk1.

Over the course of this grant, these results have been presented at several meetings, namely a Cold Spring Harbor Symposium, a BCRP Era of Hope Meeting, and a Keystone Symposium. In addition, a significant portion of Years 2 and 3 were spent successfully preparing and publishing these results in the form of a paper in *Molecular Cell*, and a Doctoral thesis. In addition, another paper manuscript is under preparation, and will be submitted shortly after the end of the grant period.

References

1. Allen, J. B., Zhou, Z., Siede, W., Friedberg, E. C., and Elledge, S. J. (1994). The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev. *8*, 2416-2428.

2. Baskaran, R., Wood, L. D., Whitaker, L. L., Canman, C. E., Morgan, S. E., Xu, Y., Barlow, C., Baltimore, D., Wynshaw-Boris, A., Kastan, M. B., and Wang, J. Y. J. (1997). Ataxia telangiectasia mutant protein activates c-Abl tyrosine kinase in response to ionizing radiation. Nature *387*, 516-519.

3. Blasina, A., Weyer, de I. V., Laus, M. C., Luyten, W. H., Parker, A. E., and McGowan, C. H. (1999) A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. Curr. Biol. *9*, 1-10.

4. Brown, A. L., Lee, C. H., Schwarz, J. K., Mitiku, N., Piwnica-Worms, H., and Chung, J.H. (1999) A human Cds1-related kinase that functions downstream of ATM protein in the cellular response to DNA damage. Proc. Natl. Acad. Sci. *96*, 3745-3750.

5. de la Torre-Ruiz, M. A., Green, C. M., and Lowndes, N. F. (1998). Rad9 and Rad24 define two additive, interacting branches of the DNA damage checkpoint pathway in budding yeast normally required for Rad53 modification and activation. EMBO J. *17*, 2687-2698.

6. Durocher, D., Henckel, J., Fersht, A. R., and Jackson, S. P. (1999) The FHA domain is a modular phosphopeptide recognition motif. Mol. Cell *4*, 387-394.

7. Emili, A., and Hartwell, L. H. (1998). MEC1-dependent phosphorylation of Rad9p in response to DNA damage. Mol Cell *2*, 183-189.

8. Ford, D., Easton, D. F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D. T., Weber, B., Lenoir, G., Changclaude, J., Sobol, H., Teare, M. D., Struewing, J., Arason, A., Scherneck, S., Peto, J.,

Rebbeck, T. R., Tonin, P., Neuhausen, S., Barkardottir, R., Eyfjord, J., Lynch, H., Ponder, B. A. J., Gayther, S. A., Birch, J. M., Lindblom, A., *et al.* (1998). Genetic heterogeneity and penetrance analysis of the *BRCA1* and *BRCA2* genes in breast cancer families. Am. J. Hum. Genet. *62*, 676-689.

9. Gardner, R., Putnam, C.W., and Weinert, T. (1999) RAD53, DUN1 and PDS1 define two parallel G2/M checkpoint pathways in budding yeast. EMBO J *18*, 3173-85.

10. Hartwell, L. H., and Weinert, T. A. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science *246*, 629-634.

11. Hawn, M. T., Umar, A., Carethers, J. M., Marra, G., Kunkel, T. A., Boland, C. R., and Koi, M. (1995). Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. Cancer Res. *55*, 3721-3725.

12. Kato, R., and Ogawa, H. (1994). An essential gene, *ESR1,* is required for mitotic cell growth, DNA repair, and meiotic recombination in *Saccharomyces cerevisiae*. Nucleic Acids Res. *22*, 3104-3112.

13. Kiser, G. L., and Weinert, T. A. (1996). Distinct roles of yeast *MEC* and *RAD* checkpoint genes in transcriptional induction after DNA damage and implications for function. Mol. Biol. Cell *7*, 703-718.

14. Lydall, D., and Weinert, T. (1995). Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. Science *270*, 1488-1491.

15. Matsuoka, S., Huang, M., and Elledge, S. J. (1998) Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science *282*, 1893-1897.

16. Paulovich, A. G., Margulies, R. U., Garvik, B. M., and Hartwell, L. H. (1997). *RAD9*, *RAD17*, and *RAD24* are required for S phase regulation in *Saccharomyces cerevisiae* in response to DNA damage. Genetics *145*, 45-62.

17. Sanchez, Y., Desany, B. A., Jones, W. J., Liu, Q., Wang, B., and Elledge, S. J. (1996). Regulation of *RAD53* by the *ATM*-like kinases *MEC1* and *TEL1* in yeast cell cycle checkpoint pathways. Science *271*, 357-359.

18. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S. R., Simmons, A., Clines, G. A., Sartiel, A., Gatti, R. A., Chessa, L., Sanal, O., Lavin, M. F., Jaspers, N. G. J., Malcolm, A., Taylor, R., Arlett, C. F., Miki, T., Weissman, S. M., Lovett, M., Collins, F. S., and Shiloh, Y. (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science *268*, 1749-1753.

19. Schiestl, R. H., Reynolds, P., Prakash, S., and Prakash, L. (1989). Cloning and sequence analysis of the *Saccharomyces cerevisiae RAD9* gene and further evidence that its product is required for cell cycle arrest induced by DNA damage. Mol. Cell. Biol. *9*, 1882-1896.

20. Scully, R., Chen, J., Ochs, R. L., Keegan, K., Hoekstra, M., Feunteun, J., and Livingston, D. M. (1997). Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. Cell *90*, 425-435.

21. Sherr, C. J. (1996). Cancer cell cycles. Science *274*, 1672-1677.

22. Siede, W., Friedberg, A. S., Dianova, I., and Friedberg, E. C. (1994). Characterization of the G_1 checkpoint control in yeast *Saccharomyces cerevisiae* following exposure to DNA-damaging agents. Genetics *138*, 271-281.

23. Siede, W., Friedberg, A. S., and Friedberg, E. C. (1993). *RAD9* dependent G1 arrest defines a second checkpoint for damaged DNA in the cell cycle of *Saccharomyces cerevisiae*. Proc.Natl.Acad.Sci. *90*, 7985-7989.

24. Slater, M. L. (1973). Effect of reversible inhibition of deoxyribonucleic acid synthesis on the yeast cell cycle. J. Bacteriol. *113*, 263-270.

25. Stern, D. F., Zheng, P., Beidler, D. R., and Zerillo, C. (1991). Spk1, a new kinase from *S. cerevisiae,* phosphorylates proteins on serine, threonine, and tyrosine. Mol. Cell. Biol. *11*, 987-1001.

26. Sun, Z., Hsiao, J., Fay, D. S., and Stern, D. F. (1998). Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. Science *281*, 272-274.

27. Sun, Z., Fay, D. S., Marini, F., Foiani, M., and Stern, D. F. (1996). Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. Genes & Dev. *10*, 395-406.
28. Vialard, J. E., Gilbert, C. S., Green, C. M., and Lowndes, N. F. (1998) The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. EMBO J. *17*, 5679-5688.
29. Weinert, T. A., and Hartwell, L. H. (1988). The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science *241*, 317-322.
30. Weinert, T. A., Kiser, F. L., and Hartwell, L. H. (1994). Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. *8*, 652-665.
31. Zhou, Z., and Elledge, S. J. (1993). *DUN1* encodes a protein kinase that controls the DNA damage response in yeast. Cell *75*, 1119-1127.
32. Koonin, E. V., Altschul, S. F., and Bork, P. (1996). BRCA1 protein products: functional motifs. Nature Genetics *13*, 266-268.
33. Park, H. and Sternglanz, R. (1999) Identification and characterization of the genes for two topoisomerase I-interacting proteins from *Saccharomyces cerevisiae*. Yeast *15*, 35-41.
34. Foss, E. (2001). Tof1p Regulates DNA Damage Responses During S Phase in *Saccharomyces cerevisiae*. Genetics *157*, 567-77.
35. Tercero, J.A. and Diffley, J.F. (2001) Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. Nature *412*, 553-7.
36. Lopes, M., Cotta-Ramusino, C., Pellicoli, A., Liberi, G., Plevani, P., Muzi-Falconi, M., Newlon, C. S., and Foiani, M. (2001) The DNA replication checkpoint response stabilizes stalled replication forks. Nature *412*, 557-61.
37. Chaturvedi, P., Eng, W.K., Zhu, Y., Mattern, M.R., Mishra, R., Hurle, M.R., Zhang, X., Annan, R.S., Lu, Q., Faucette, L.F., Scott, G.F., Li, X., Carr, S.A., Johnson, R.K., Winkler, J.D., and Zhou, B.B. (1999). Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. Oncogene *18*, 4047-54.
38. Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., and Elledge, S.J. (1999). Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. Science *286*, 1166-71.
39. Liu, Q., Guntuku, S., Cui, X.S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., Donehower, L.A., and Elledge, S.J. (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev *14*, 1448-59.
40. Zhu, H., M. Bilgin, R. Bangham, D. Hall, A. Casamayor, P. Bertone, N. Lan, R. Jansen, S. Bidlingmaier, T. Houfek, T. Mitchell, P. Miller, R. A. Dean, M. Gerstein, and M. Snyder. (2001). Global analysis of protein activities using proteome chips. Science *293*, 2101.

Appendix 1 – Paper Reprint

In lieu of figures, a reprint of the following publication is included to illustrate the data described above.

Schwartz, M.F., Duong, J.K., Sun, Z., Morrow, J.S., Pradhan, D., and Stern, D.F. (2002) Rad9 phosphorylation sites couple Rad53 to the *Saccharomyces cerevisiae* DNA damage checkpoint. Molecular Cell *9*, 1055-1065.

Molecular Cell, Vol. 9, 1055–1065, May, 2002, Copyright ©2002 by Cell Press

Rad9 Phosphorylation Sites Couple Rad53 to the *Saccharomyces cerevisiae* DNA Damage Checkpoint

Marc F. Schwartz,[1,2] Jimmy K. Duong,[2] Zhaoxia Sun,[2,3,5] Jon S. Morrow,[2] Deepti Pradhan,[2] and David F. Stern[2,4]
Departments of Cell Biology[1] and Pathology[2]
Yale University School of Medicine
[3]Department of Molecular, Cellular, and Developmental Biology
Yale University
New Haven, Connecticut 06510

Summary

Rad9 is required for the *MEC1/TEL1*-dependent activation of *Saccharomyces cerevisiae* DNA damage checkpoint pathways mediated by Rad53 and Chk1. DNA damage induces Rad9 phosphorylation, and Rad53 specifically associates with phosphorylated Rad9. We report here that multiple Mec1/Tel1 consensus [S/T]Q sites within Rad9 are phosphorylated in response to DNA damage. These Rad9 phosphorylation sites are selectively required for activation of the Rad53 branch of the checkpoint pathway. Consistent with the in vivo function in recruiting Rad53, Rad9 phosphopeptides are bound by Rad53 forkhead-associated (FHA) domains in vitro. These data suggest that functionally independent domains within Rad9 regulate Rad53 and Chk1, and support the model that FHA domain-mediated recognition of Rad9 phosphopeptides couples Rad53 to the DNA damage checkpoint pathway.

Introduction

DNA checkpoints attenuate the mutagenicity, genomic instability, and cell lethality resulting from DNA damage. In response to DNA damage, these pathways delay cell cycle progression, increase the transcription of DNA checkpoint, replication, and repair genes, and can activate apoptosis. In addition, DNA checkpoint pathways may directly regulate the DNA repair process. Several checkpoint pathway components are recognized as tumor suppressors, and dysfunction of checkpoint signaling pathways is frequently associated with cancers (reviewed in Zhou and Elledge, 2000).

DNA checkpoint pathways amplify and transmit the checkpoint signal to the effector pathways through an evolutionarily conserved kinase cascade. The first tier of this cascade involves members of a family of phosphatidylinositol-(3′) kinase-like protein kinases (PIKKs) that includes mammalian *ATM*, *ATR*, and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). These PIKKs regulate the activation of two unrelated effector checkpoint kinases (Chks), represented by mammalian Chk1 and Chk2 (reviewed in Rhind and Russell, 2000; Durocher and Jackson, 2001; Abraham, 2001).

In *Saccharomyces cerevisiae*, the PIKK to Chk kinase cascade is an obligatory component of two DNA checkpoint signaling pathways. The DNA damage checkpoint pathway is sensitive to various forms of DNA damage throughout the cell cycle. The DNA replication checkpoint pathway functions in S phase and is activated in response to both inhibition of DNA synthesis and DNA damage (reviewed in Elledge, 1996). Although both of these pathways involve the PIKKs Mec1 or Tel1, the DNA replication checkpoint pathway activates the Chk2 homolog Rad53 but not Chk1. By contrast, the DNA damage checkpoint pathway activates both Rad53 and Chk1 (Sun et al., 1996; Sanchez et al., 1996, 1999). This differential regulation is partially mirrored in fission yeast, where the PIKK Rad3 is required for all DNA checkpoint pathways, and DNA damage activates Chk1 while replication inhibition activates the Chk2 homolog Cds1 (Walworth et al., 1993; al-Khodairy et al., 1994; Bentley et al., 1996; Lindsay et al., 1998).

S. cerevisiae RAD9 is the prototype DNA damage checkpoint gene (Weinert and Hartwell, 1988) and is required for the DNA damage checkpoint pathway throughout the cell cycle (Weinert and Hartwell, 1988; Siede et al., 1993; Paulovich et al., 1997). Rad9 shares localized homology with the mammalian tumor suppressor *BRCA1* (Koonin et al., 1996; Bork et al., 1997). Loss of *RAD9* impairs checkpoint-induced cell cycle arrest and increases genomic instability (Weinert and Hartwell, 1988, 1990). *RAD9* is required for the activation of Rad53 and Chk1 by the DNA damage checkpoint pathway (Navas et al., 1996; Sanchez et al., 1999). Moreover, DNA damage but not replication block induces Rad9 phosphorylation in a Mec1/Tel1-dependent manner, and Rad53 specifically interacts with phosphorylated Rad9 in vivo (Sun et al., 1998; Emili, 1998; Vialard et al., 1998). These data suggested that Rad9 acts as a pathway-specific adaptor that recruits Rad53 and Chk1 to DNA damage-dependent activation complexes (Sun et al., 1998; Sanchez et al., 1999). This model is supported by the recent observation that immunoprecipitated phosphorylated Rad9 facilitates the activation of exogenous Rad53 in vitro (Gilbert et al., 2001).

Rad53 and other Chk2 orthologs are coupled to DNA checkpoint pathways through their FHA domains (Sun et al., 1998; Bell et al., 1999; Chehab et al., 2000; Lee and Chung, 2001). FHA domains are conserved modular domains that bind specific phosphopeptides (Hofmann and Bucher, 1995; Sun et al., 1998; Durocher et al., 1999; Li et al., 1999; Liao et al., 1999). FHA domains were first implicated as phosphorylation-specific protein-protein binding domains in a study of the binding of *Arabidopsis thaliana* kinase-associated protein phosphatase (KAPP) to a receptor-like kinase (RLK) (Stone et al., 1994). Rad53 contains two FHA domains, which are hypothesized to couple Rad53 to the DNA checkpoint pathways (Sun et al., 1998). Both Rad53 FHA domains can bind phosphorylated Rad9 in vitro (Sun et al., 1998; Durocher et al., 1999), and mutation of conserved amino acids in the

[4]Correspondence: df.stern@yale.edu
[5]Present address: Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

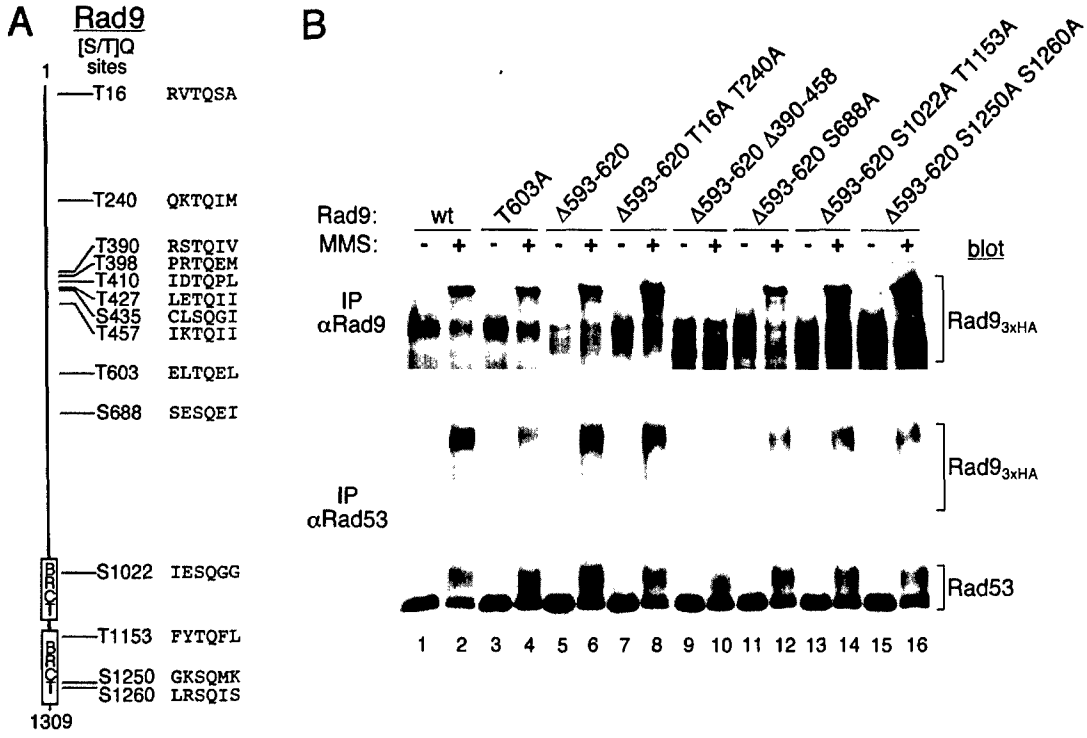

Figure 1. Mutagenesis of Rad9 PIKK Phosphorylation Sites

(A) Schematic diagram of Rad9: the tandem BRCT domains are boxed, hash marks indicate the locations of the fourteen [S/T]Q consensus PIKK phosphorylation sites, and the sequence describes ± two residues surrounding each [S/T]Q site.

(B) Anti-HA (top and middle) and anti-Rad53 (bottom) immunoblot analyses of anti-Rad9 (top) and anti-Rad53 (middle and bottom) immunoprecipitates from the indicated *RAD9* or *rad9* DLY408-derived strains. Asynchronous cultures were either mock treated (−) or treated with 0.1% MMS (+) for 1 hr.

second Rad53 FHA domain (FHA2) disrupts the DNA damage checkpoint pathway activation of Rad53 in vivo (Sun et al., 1998).

Phosphopeptide binding preferences of FHA domains have been identified in vitro using peptide libraries (Durocher et al., 2000; Liao et al., 2000; Wang et al., 2000; Byeon et al., 2001). Despite the requirement for FHA domains in multiple signaling pathways, including the Rad9/Rad53 interaction, the specific phosphopeptides recognized by FHA domains in vivo are not known. We report here the identification of multiple PIKK sites within Rad9 that are phosphorylated in response to DNA damage and are required for the *RAD9*-dependent regulation of Rad53.

Results

Identification of the Rad53 Binding Sites within Rad9

We had previously identified an interaction between Rad53 FHA2 and Rad9 in a two-hybrid assay (Sun et al., 1998). Deletion analysis revealed that Rad9 residues 542–620 are minimally required for this interaction (data not shown). This domain contains a single consensus PIKK phosphorylation site, [S/T]Q (Anderson and Lees-Miller, 1992; Kim et al., 1999), at Rad9 T603 (Figure 1A). Mec1 phosphorylates this site in immune-complex kinase assays (data not shown). Alanine substitution or deletion of Rad9 T603 did not eliminate the DNA damage-induced Rad9 phosphorylation, coimmunoprecipitation of Rad9 with Rad53, or the *RAD9*-dependent function of the G_2/M DNA damage checkpoint arrest (Figure 1B, lanes 1–6, and data not shown). Hence, Rad9 T603 is not essential for Rad9 phosphorylation and recruitment of Rad53.

To determine whether other Rad9 [S/T]Q sites are required for DNA damage-dependent Rad9 phosphorylation and its interaction with Rad53, we generated a series of strains lacking Rad9 T603 in combination with either alanine substitutions or deletion of these sites (Figure 1B, lanes 7–16). Of these mutations, only the additional deletion of the [S/T]Q cluster domain (SCD), amino acids 390–458, impaired the DNA damage-induced phosphorylation of Rad9 (Figure 1B, lanes 9–10). Furthermore, Rad9 and Rad53 did not coimmunoprecipitate in this strain, and Rad53 phosphorylation was partially impaired (Figure 1B, lanes 9–10). As this experiment used asynchronous cultures, the residual Rad53 phosphorylation observed was probably mediated by the *RAD9*-independent DNA replication checkpoint pathway. The single deletion of Rad9 amino acids 390–458 also abrogated the DNA damage-induced phosphorylation of Rad9 (Figure 2A).

Since deletions within Rad9 may nonspecifically disrupt Rad9 function, we examined the effects of alanine substitution at the [S/T]Q sites within the SCD and at T603. The *rad9*^{6xA} allele has alanine substitutions at each [S/T]Q within the SCD (T390, T398, T410, T427, S435, and T457), and the *rad9*^{7xA} allele is the *rad9*^{6xA} allele with

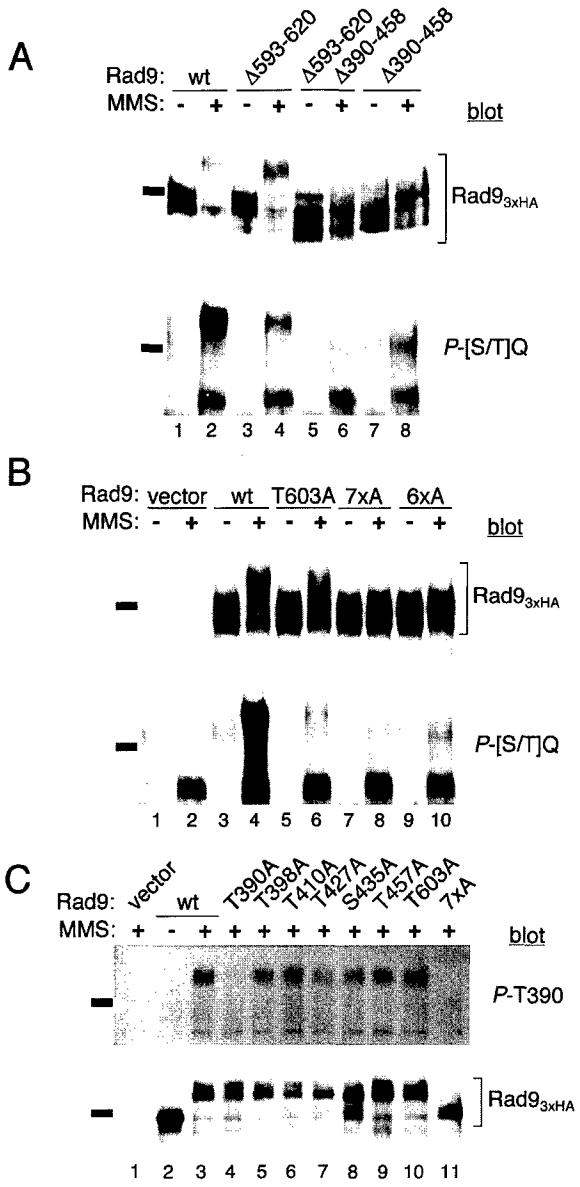

Figure 2. DNA Damage Induces Rad9 Phosphorylation at Multiple [S/T]Q Sites

In (A) and (B), duplicate blots of TCA lysates from asynchronous cultures of (A) DLY408-derived and (B) DLY418-derived strains were immunoblotted with anti-HA (top panels) and anti-phospho-[S/T]Q (bottom). In (C), a blot of TCA lysates from *cdc15-2* synchronized DLY418-derived strains was immunoblotted with anti-phospho-T390 (top panel) and anti-HA (bottom panel). The bar to the left of the blots indicates the position of the 207 kDa marker. Cells were either mock treated (−) or treated with 0.1% MMS (+) for 1 hr.

the additional alanine substitution of T603. Compared to wild-type Rad9, both Rad9^{7xA} and Rad9^{6xA} displayed severely reduced DNA damage-dependent electrophoretic mobility shift (Figure 2B), indicating that these mutations eliminate the majority of Rad9 phosphorylation sites or otherwise impair the damage-dependent Rad9 phosphorylation.

Multiple forms of DNA damage induce the *MEC1/TEL1*-dependent phosphorylation of Rad9 (Emili, 1998). Since it is not known whether these forms of phosphorylated Rad9 involve different combinations of phosphorylation sites, we examined the effect of Rad9 SCD mutations on the mobility shift of Rad9 induced by different types of DNA damage. Rad9^{6xA} was similarly impaired for phosphorylation and Rad53 coimmunoprecipitation induced by DNA damage from MMS, 4NQO, and UV and ionizing radiation (data not shown). Hence, the Rad9 SCD is a major locus for both Rad9 phosphorylation and interaction with Rad53 induced by DNA damage.

Detection of Phosphorylated Rad9 [S/T]Q Sites, Including T603 and SCD Site T390

DNA damage-dependent Rad9 phosphorylation requires the PIKKs Mec1 or Tel1 (Sun et al., 1998; Emili, 1998; Vialard et al., 1998). Rad9 contains a total of fourteen [S/T]Q consensus PIKK phosphorylation sites, including T603 (Figure 1A). Immunoblot analysis with an antibody that recognizes phosphorylated [S/T]Q sites revealed a strong immunoreactive band that is induced by DNA damage, is eliminated by deletion of *RAD9*, and comigrates with phosphorylated Rad9 (Figure 2B, lanes 1–4). According to the manufacturer, this antibody preferentially recognizes phospho-[S/T]Q when preceded by a leucine or other hydrophobic residue. Thus, given its context, Rad9 T603 would be an ideal binding substrate if phosphorylated. Indeed, although Rad9$^{\Delta 593-620}$ and Rad9^{T603A} undergo apparently normal phosphorylation-dependent electrophoretic mobility shifts, their phospho-[S/T]Q immunoreactivity was significantly reduced (Figure 2A, lanes 3–4; Figure 2B, lanes 5–6). This residual phospho-[S/T]Q immunoreactivity was eliminated with the additional loss of the Rad9 SCD, but was partially recovered in the Rad9 mutant only for the SCD (Figure 2A, lanes 5–8; Figure 2B, lanes 7–10). Together, these data imply that DNA damage induces Rad9 phosphorylation on multiple [S/T]Q sites, including T603 and other [S/T]Q sites within the Rad9 SCD.

To further directly identify Rad9 phosphorylation sites, we generated and purified antibodies that specifically recognize phosphorylated Rad9 T390, the first [S/T]Q site within the SCD. This site is preceded by a serine and thus is not predicted to be strongly recognized by the phospho-[S/T]Q antibody. Anti-phospho-T390 recognized the slowest mobility form of phosphorylated Rad9 induced by DNA damage (Figure 2C, lanes 1–3). Immunoreactivity was abolished by alanine substitution of T390 but unaffected by substitution of other SCD [S/T]Q sites or T603 (Figure 2C, lanes 4–10). Preincubation of the phospho-T390 antibody with phosphorylated-T390 peptide (but not with the nonphosphorylated peptide) completely eliminated the immunoreactivity of phosphorylated Rad9 (data not shown). Thus, Rad9 T390 is another of the Rad9 [S/T]Q sites that are phosphorylated in response to DNA damage.

Redundant Function of Individual [S/T]Q Sites within the Rad9 SCD

We evaluated the effects of single alanine substitutions within the Rad9 SCD to determine whether a single site within the Rad9 SCD has a dominant role in the phosphorylation of Rad9 and the interaction of Rad9 with Rad53. In these and subsequent experiments, activation of the DNA replication checkpoint pathway was avoided

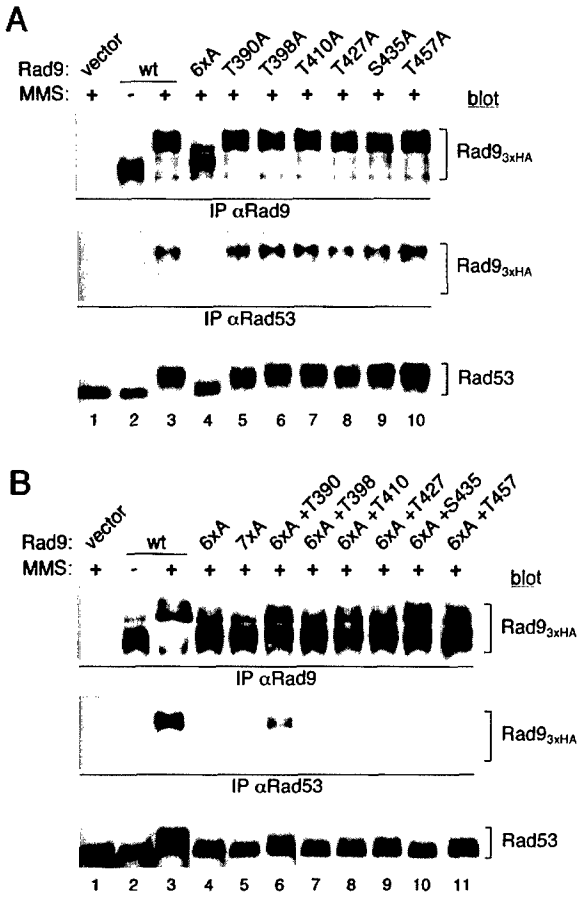

Figure 3. The Rad9 SCD Is Required for the Phosphorylation and Interaction of Rad9 and Rad53

In (A) and (B), anti-HA (top and middle panels) and anti-Rad53 (bottom panel) immunoblot analyses of anti-Rad9 (top panel) and anti-Rad53 (middle panel) immunoprecipitates and corresponding lysates (bottom panel) from the indicated *RAD9* or *rad9* strains. Cells were synchronized in telophase at the *cdc15-2* arrest point and either mock treated (−) or treated with 0.1% MMS (+) for 1 hr. The DLY418-derived strains carry a plasmid encoding *RAD9*, *rad9*^{6xA}, (A) alanine substitutions of individual [S/T]Q sites within the Rad9 SCD, or (B) *rad9*^{7xA}, and *rad9*^{6xA} strains with single wild-type residues restored.

by synchronization of cells in telophase using the conditional *cdc15-2* allele (Futcher, 1999), thus allowing evaluation of Rad53 phosphorylation solely dependent on *RAD9*. Similar to the alanine substitution of Rad9 T603, substitution of single [S/T]Q sites within the SCD had little impact on the DNA damage-induced phosphorylation of Rad9, coimmunoprecipitation of Rad9 with Rad53, or Rad53 phosphorylation (Figure 3A). Single mutation of the Rad9 SCD sites also had little impact on the phospho-[S/T]Q immunoreactivity of phosphorylated Rad9 (data not shown). Since simultaneous substitution of all six SCD sites does inactivate these functions (Figure 3A, lane 4), these data suggest that multiple SCD sites are phosphorylated and that Rad53 interacts redundantly with some or all of these sites.

Rad53 specifically interacts with phosphorylated Rad9 (Sun et al., 1998; Emili, 1998). To characterize the ability of single Rad9 SCD sites to support this interaction, we restored single [S/T]Q residues in the Rad9^{6xA} SCD. Reintroduction of a wild-type residue at Rad9 S435 best, although incompletely, restored the DNA damage-induced slower mobility forms of Rad9 but only modestly rescued coimmunoprecipitation with Rad53 and Rad53 phosphorylation (Figure 3B, lane 10). By contrast, reintroduction of Rad9 T390 moderately restored the DNA damage-induced slower mobility forms and also the interaction of Rad9 and Rad53 (Figure 3B, lane 6). No single Rad9 cluster add-back allele fully restored the checkpoint-induced phosphorylation or coimmunoprecipitation of Rad9 and Rad53. Thus, multiple Rad9 SCD phosphorylation sites act in an additive or cooperative manner to recruit Rad53.

Rad53 FHA Domains Bind Phosphorylated Rad9 T390 In Vitro

The ability of Rad9^{6xA} T390 to restore a significant proportion of the Rad9 interaction with Rad53 implies that T390 is a major in vivo functional site for Rad53 binding. To confirm that Rad53 binds directly to Rad9 T390 in a phosphorylation-dependent manner, we measured the increase of surface plasmon resonance (SPR) caused by binding of soluble, bacterially produced Rad53 FHA domains to synthetic Rad9 T390 peptides immobilized on a BIAcore sensor chip. Rad53 FHA1 specifically interacted with the phosphorylated Rad9 T390 peptide (*P*-T390) (Figure 4A, middle versus left panel). Mutation of two conserved FHA domain residues abolished the binding of the Rad53 FHA1 protein to the phosphorylated T390 peptide (Figure 4A, right panel). Similar to FHA1, GST-Rad53 FHA2 preferentially bound the phosphorylated Rad9 T390 (Figure 4B, middle versus left panel), demonstrating an affinity lost upon substitution of conserved FHA2 residues (Figure 4B, right panel). On average, Rad53 FHA1 bound the phosphorylated Rad9 T390 peptide with an apparent K_D of 2.5 μM (s = 0.3, n = six experiments), and GST-Rad53 FHA2 bound with an apparent K_D of 1.4 μM (s = 0.3, n = three experiments). These affinities are within the general range of those reported for FHA domain-phosphopeptide binding (Durocher et al., 1999; Li et al., 1999; Liao et al., 1999, 2000; Durocher et al., 2000; Wang et al., 2000; Byeon et al., 2001). However, the observed affinity of GST-FHA2 may have been artificially increased due to the ability of GST to homodimerize (Ladbury et al., 1995). In addition, SPR-based determination of FHA domain binding affinity has been demonstrated to overestimate K_D by as much as 20-fold, as compared to binding affinity determined by isothermal calorimetry (Durocher et al., 1999).

In a filter binding assay with immobilized synthetic peptides, GST-Rad53 FHA2 also preferentially bound the phosphorylated form of a Rad9 T603 peptide (data not shown). In a recent study of Rad53 FHA2 binding Rad9 phosphothreonine peptides that fit a Txx[I/L] consensus, Rad53 FHA2 bound a phospho-T603 peptide with a K_D of 12.9 μM (Byeon et al., 2001). Taken together, these results show that the Rad53 FHA domains can directly and specifically bind Rad9 phosphopeptides containing physiological PIKK phosphorylation sites.

Rad9 Phosphorylation Sites Are Required for the Survival of Genotoxic Stress

Survival of genotoxic stress requires the activities of the DNA checkpoint pathways, as well as the DNA repair

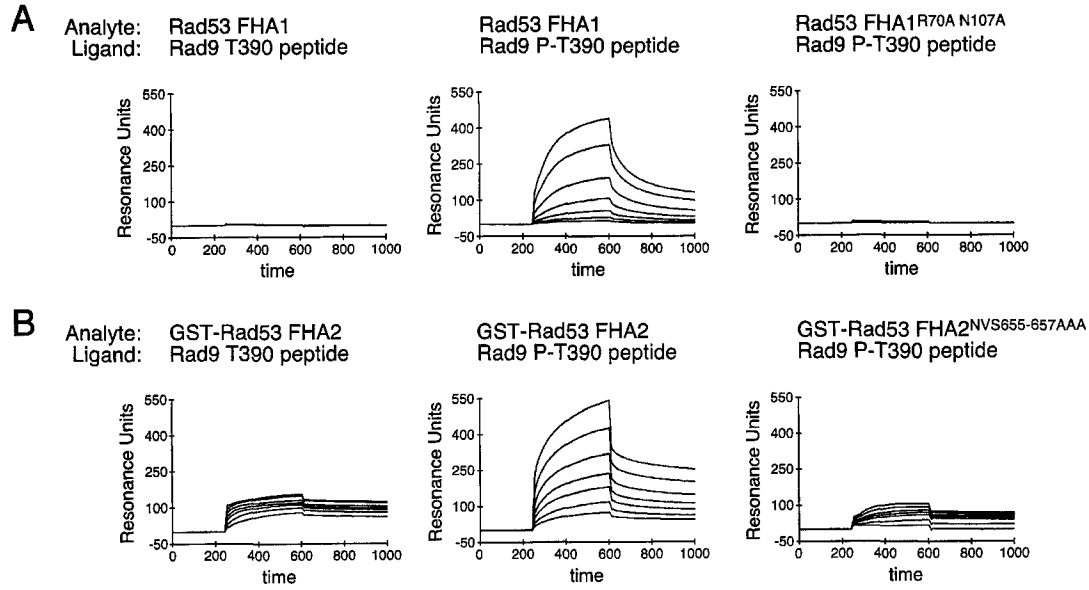

Figure 4. Phosphorylation-Dependent Binding of Rad53 FHA Domains to Rad9 T390 In Vitro

Synthetic peptides containing Rad9 amino acids 382–397, either nonphosphorylated (T390) or phosphorylated (*P*-T390) at T390, were biotinylated and coated onto a BIAcore sensor chip. The sensorgrams indicate binding measured by an increase in surface plasmon resonance during analyte flow over the chip surface, with the nonspecific signal from the biotin-only surface subtracted.

(A) Concentrations of 0.1–4.8 μM of bacterially produced Rad53 FHA1 (left and middle) or Rad53 FHA1 with mutations in conserved FHA domain residues (right) were flowed across the nonphosphorylated (left) and phosphorylated (middle and right) Rad9 T390 peptide surfaces.

(B) Concentrations of 0.19–4.9 μM of bacterially produced GST-Rad53 FHA2 (left and middle) or GST-Rad53 FHA2 with mutations in conserved FHA domain residues (right) were flowed across the nonphosphorylated (left) and phosphorylated (middle and right) Rad9 T390 peptide surfaces. The low level of peptide- and FHA domain-independent resonance observed in (B) was specific to that sensor chip, since it was not seen when the same GST-FHA2 preparation was flowed over the sensor chip used in (A).

systems. Rad9 is involved in both signaling through the DNA damage checkpoint pathway and DNA repair, such that loss of *RAD9* decreases the viability of cells challenged with DNA damage (Weinert and Hartwell, 1988, 1990; Terleth et al., 1990; de la Torre-Ruiz and Lowndes, 2000). Therefore, we determined whether DNA damage-induced Rad9 phosphorylation sites are required for Rad9 function in response to a variety of genotoxic stresses.

Cdc13 is a telomere-associated protein required for the maintenance of chromosome ends (Garvik et al., 1995; Nugent et al., 1996; Lin and Zakian, 1996). At elevated temperatures, cells bearing the temperature-sensitive *cdc13-1* allele exhibit a rapid loss of viability (Weinert et al., 1994). Cells bearing *rad9*^{7xA} were compromised for survival at 37°C in a *cdc13-1* background, nearly as severely as a *rad9*Δ strain (Figure 5A). The *cdc13-1 rad9*^{6xA} strain demonstrated an intermediate loss of viability, suggesting that Rad9 T603 supports a moderate level of Rad9 function in vivo. Consistent with their partial recovery of Rad53 phosphorylation, *cdc13-1 rad9*$^{6xA+T390}$ cells are more viable at 37°C than the *cdc13-1 rad9*^{6xA} strain (Figure 5A).

The absence of Rad9 phosphorylation sites in *rad9*^{7xA} cells also reduced survival of UV irradiation, but this loss of viability was less than that associated with the loss of *RAD9* (Figure 5B, right panels). Interestingly, the *rad9*^{7xA} strain demonstrated a loss of viability approaching that of a *rad9*Δ strain when grown on media containing MMS (Figure 5B, left panels), suggesting that the Rad9 [S/T]Q phosphorylation sites play a greater role in the *RAD9*-dependent survival of MMS-derived DNA damage than in the survival of UV irradiation.

Survival of replication block due to hydroxyurea (HU) treatment is normally *RAD9* independent and involves the activation of Rad53 through the DNA replication checkpoint pathway (Weinert et al., 1994; Allen et al., 1994; Navas et al., 1996). However, if the replication checkpoint pathway is compromised, such as in a *tof1*Δ mutant, Rad53 regulation and HU survival involves signaling through Rad9 (Navas et al., 1996; Foss, 2001). Therefore, we determined the effect of the loss of Rad9 [S/T]Q phosphorylation sites on HU survival in a *tof1*Δ strain. As expected, wild-type, *rad9*Δ, and *rad9*^{7xA} strains were similarly viable when grown on HU-containing media (Figure 5C). The *tof1*Δ mutant was slightly impaired for viability, and the *tof1*Δ *rad9*Δ double mutant was very sensitive to HU (Figure 5C). The *tof1*Δ *rad9*^{7xA} strain was nearly as sensitive as the *tof1*Δ *rad9*Δ strain, indicating a strong requirement for Rad9 [S/T]Q phosphorylation sites for the survival of HU treatment when the replication checkpoint pathway is impaired.

G_2/M DNA Damage Checkpoint Arrest in Rad9 [S/T]Q Mutants

At elevated temperatures, *cdc13-1* strains accumulate telomeric single-stranded DNA that induces a strong *RAD9*-dependent cell cycle arrest at G_2/M (Weinert and Hartwell, 1993; Lydall and Weinert, 1995; Garvik et al., 1995). Rad53 and Chk1 act in parallel downstream of Rad9 to enforce this arrest, such that *rad53* or *chk1* cells have a partial delay and are unable to maintain the

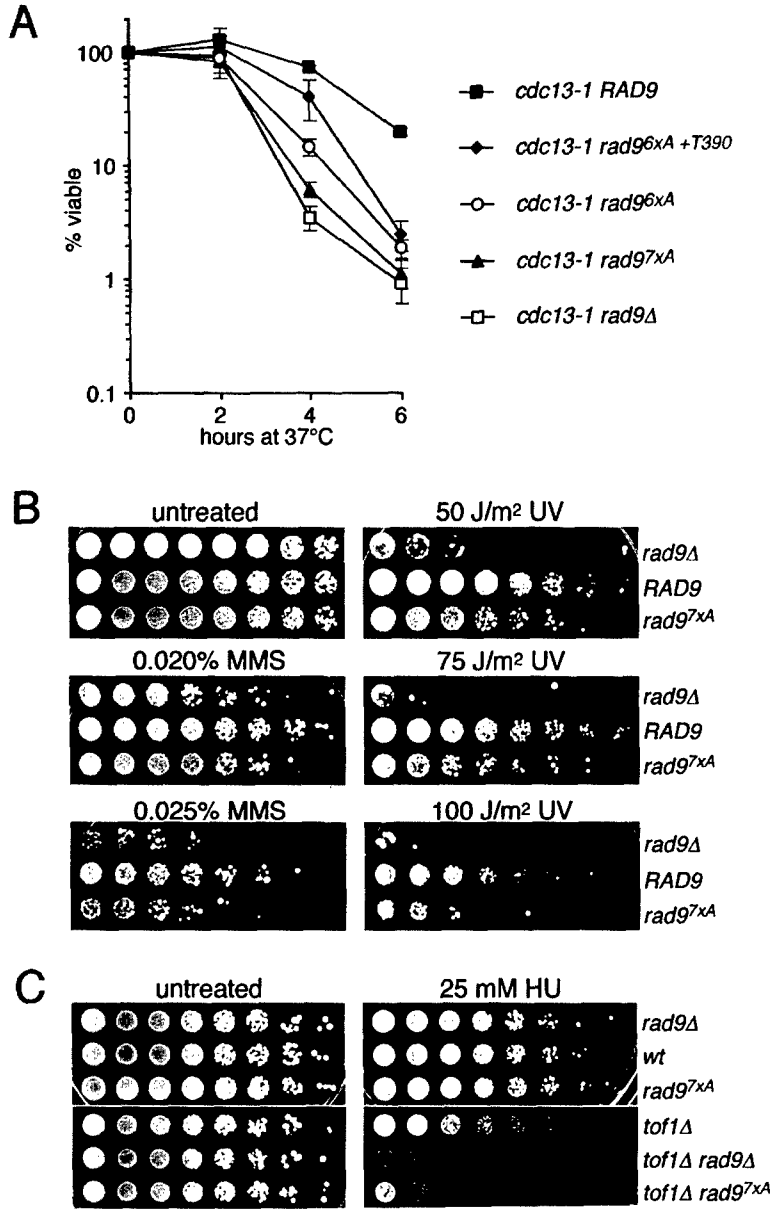

Figure 5. Genotoxin Sensitivity of *rad9* [S/T]Q Mutants

(A) Log-phase cultures of 1588-4C-derived *cdc13-1* strains expressing the indicated forms of Rad9 were shifted to 37°C, and their viability was measured as the number of colony-forming units when plated at permissive temperature. Error bars indicate ± the standard deviation between two matched strains. (B and C) Serial 4-fold dilutions of the (B) 1588-4C-derived *rad9Δ* or (C) U960-5C-derived *rad9Δ* and *rad9Δ tof1Δ* strains carrying plasmids to generate the illustrated genotypes were spotted onto plates either untreated, UV irradiated, containing MMS, or containing HU as indicated. The lethality of *rad53Δ* in U960-5C-derived strains is suppressed by *sml1-1*.

G_2/M cell cycle arrest (Sun et al., 1998; Gardner et al., 1999; Sanchez et al., 1999; Liu et al., 2000). To determine the relationship of Rad9 phosphorylation sites required for Rad53 activation with the functionality of the DNA damage checkpoint pathway, we ascertained the effect of Rad9 [S/T]Q mutations on the G_2/M arrest in *cdc13-1* cells. Similar to *rad53* cells, *rad9*^{6xA} and *rad9*^{7xA} cells transiently delayed prior to anaphase, but eventually escaped into telophase (Figure 6A). The *rad9*^{7xA} defect was consistently slightly more severe than the *rad9*^{6xA} defect, demonstrating that Rad9 T603 partially contributes to the function of Rad9 in maintaining the G_2/M DNA damage checkpoint arrest. Consistent with the ability of the T390 add-back mutant to significantly restore the DNA damage-induced interaction of Rad9 with Rad53, reintroduction of Rad9 T390, or, less effectively, S435, to *rad9*^{6xA} partially restored G_2/M arrest (Figure 6B). These rescues were intermediate, suggesting that the regulation of Rad53 by multiply phosphorylated Rad9 is required for the full action of the *RAD53*-dependent G_2/M DNA damage checkpoint.

Rad9 [S/T]Q Mutants Retain Regulation of Chk1

RAD9 is required for the DNA damage-induced phosphorylation of Chk1, and Rad9 interacts with Chk1 in a yeast two-hybrid assay (Sanchez et al., 1999), suggesting that Rad9 may also directly recruit Chk1 to the DNA damage checkpoint pathway. Therefore, we determined whether the DNA damage-induced phosphorylation of Chk1 is intact in *rad9*^{7xA} cells. As expected, the DNA damage-dependent phosphorylation of Rad53 and Chk1 was absent in a *rad9Δ* strain, and Rad53 phosphorylation was abrogated in *rad9*^{7xA} cells (Figure 7). However, the damage-dependent phosphorylation of Chk1 was not lost in the *rad9*^{7xA} strain (Figure 7, lanes 5–6). These data indicate that the major DNA damage-induced Rad9 phosphorylation sites are not essential for the regulation of Chk1 by Rad9.

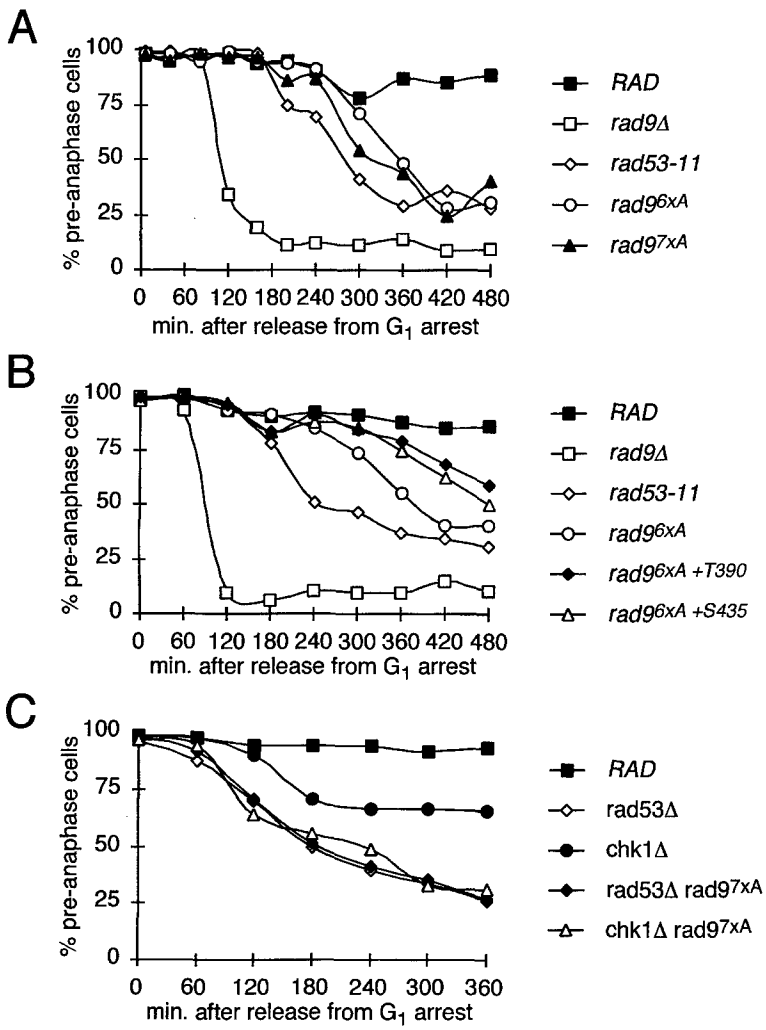

Figure 6. Rad9 Phosphorylation Sites Are Required for the G_2/M DNA Damage Checkpoint Arrest

The ability of the DNA damage checkpoint pathway to enforce the G_2/M arrest as pre-anaphase cell in the face of DNA damage from *cdc13-1* was assessed in the indicated DLY408-derived strains.

(A) Comparison of Rad9 phosphorylation site mutants to wild-type, *rad9Δ*, and *rad53-11*.

(B) Comparison of Rad9 SCD mutants bearing single intact SCD sites at T390 or S435 to *rad9*^{6xA}.

(C) Comparison of *rad53Δ* or *chk1Δ* to the double *rad53Δ rad9*^{7xA} or *chk1Δ rad9*^{7xA} mutants. The lethality of *rad53Δ* in these cells was suppressed by deletion of *SML1*.

Rad53 and Chk1 both act to enforce the G_2/M checkpoint arrest (Sun et al., 1998; Gardner et al., 1999; Sanchez et al., 1999; Liu et al., 2000). Thus, the intermediate G_2/M checkpoint arrest defect in the *rad9*^{7xA} cells (Figure 6A) could be due to disruption of either Rad53 or Chk1 function. To confirm that the G_2/M arrest defect in the *rad9*^{7xA} cells reflects the failure of the DNA damage checkpoint pathway in coupling to Rad53 rather than Chk1, we determined the effect of the rad9^{7xA} mutation in *chk1Δ* and *rad53Δ* cells. *rad53Δ rad9*^{7xA} cells were as defective in the G_2/M DNA damage checkpoint arrest as *rad53Δ* cells, suggesting that the two mutations disrupt the same pathway (Figure 6C). By contrast, *chk1Δ rad9*^{7xA} cells were less competent for the function of the G_2/M checkpoint arrest than *chk1Δ* cells (Figure 6C), suggesting that Chk1 and the Rad9 phosphorylation sites function in separate pathways. This is consistent with the observation that loss of the Rad9 phosphorylation sites disrupts the *RAD9*-dependent phosphorylation of Rad53 and not Chk1.

Discussion

Site-directed mutagenesis identified a requirement for Rad9 [S/T]Q sites in the DNA damage-induced Rad9 phosphorylation and interaction with Rad53. Western blot analysis with phosphorylation-specific antibodies directly implicated specific Rad9 [S/T]Q residues as physiological DNA damage-induced phosphorylation sites, and these Rad9 [S/T]Q phosphopeptides are bound by Rad53 FHA domains in vitro. Rad9 phosphorylation site mutants are partially defective for genotoxin survival and function of the G_2/M checkpoint arrest in a manner consistent with dysfunction of the Rad53 effector pathway. These defects caused by Rad9 mutation are specific for its role in Rad53 regulation, since they have little effect on the phosphorylation and function of Chk1.

Rad53 FHA Domain Binding Sites within Rad9

Prior analyses of the phosphopeptide binding properties of FHA domains were performed exclusively in vitro using phosphopeptides of unknown physiological relevance (Durocher et al., 1999, 2000; Liao et al., 2000; Byeon et al., 2001). Studies of Rad53 FHA domains binding to peptide libraries determined that Rad53 FHA1 prefers phosphothreonine followed by aspartate at the +3 position, while Rad53 FHA2 prefers phosphothreonine followed by isoleucine or leucine at +3 (Durocher et al., 1999, 2000; Liao et al., 2000; Byeon et al., 2001). Rad9 T390, the individual Rad9 SCD site which best supports the Rad9 interaction with Rad53 in vivo,

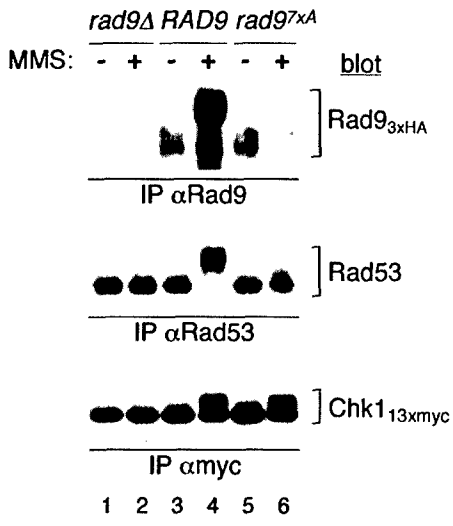

Figure 7. Chk1 Phosphorylation Is Not Disrupted in a Rad9 SCD Mutant

Immunoprecipitation and immunoblot analyses of anti-Rad9 (top), anti-Rad53 (middle), and anti-myc (bottom) from DLY418-derived strains expressing the indicated forms of Rad9. Cells were synchronized in telophase at the *cdc15-2* arrest point and either mock treated (−) or treated with 0.1% MMS (+) for 1 hr.

is followed by valine at +3 and thus precisely fits neither of these preferred patterns. However, both Rad53 FHA domains have a weaker general selectivity for a hydrophobic residue at +3 (Durocher et al., 2000; Byeon et al., 2001), which is consistent with Rad9 T390. Four of the five TQ sites within the Rad9 SCD and six of the nine total TQ sites within Rad9, including T603, have isoleucine, leucine, or valine residues at the +3 position. The identification of phosphorylated Rad9 [S/T]Q sites as in vivo binding targets for the Rad53 FHA domains is in agreement with the prediction that Mec1/Tel1 phosphorylation sites within Rad9 are required for its interaction with Rad53.

Rad9 [S/T]Q Phosphorylation Sites Regulate Rad53 Activation

Single mutation of Rad9 [S/T]Q sites did not have a significant effect on the mobility shift of phosphorylated Rad9 or on the interaction of phosphorylated Rad9 with Rad53. Multiple substitutions of these sites, however, revealed their requirement for Rad9 phosphorylation and regulation of Rad53. Moreover, individual sites within the Rad9 SCD are able to support partial levels of Rad9 phosphorylation and interaction with Rad53, but no single site accounts for all of either function. These data imply that multiple Rad9 [S/T]Q phosphorylation sites together mediate the interaction of Rad9 with Rad53.

The CDK inhibitor Sic1 is phosphorylated at multiple CDK consensus sites. Individually, these sites constitute only moderate-affinity Cdc4 binding sites, but these phosphorylation sites act in concert to target Sic1 for Cdc4-mediated degradation once a threshold of Sic1 phosphorylation is achieved (Nash et al., 2001). Similarly, multiple phosphorylation of [S/T]Q sites within Rad9 may together act to achieve a threshold of Rad53 binding required to induce its phosphorylation and activation. This activation could be achieved both through the transphosphorylation of Rad53 by Mec1/Tel1 and through the concentration-dependent transphosphorylation of Rad53 by Rad53 (Gilbert et al., 2001).

Rad53 is itself potentially bivalent for interactions with Rad9, since both Rad53 FHA domains bind phosphorylated Rad9 (Sun et al., 1998; Durocher et al., 1999), and both FHA domains bind Rad9 phosphopeptides in vitro. Hence, multivalent interactions between multiply phosphorylated Rad9 and bivalent Rad53 may stabilize the Rad9-Rad53 interaction. The contact of an FHA domain to a phosphopeptide directly involves at least six residues encompassing the phosphorylation site (Durocher et al., 2000). Considering that the six Rad9 SCD sites are spread over 69 amino acids, a single Rad9 SCD may engage with multiple Rad53 FHA domains. Furthermore, both Rad9 (Soulier and Lowndes, 1999) and Rad53 (Z.S. and D.F.S., unpublished data) are capable of homotypic interactions. Taken together, these intricate interactions may contribute to aggregation of oligomeric checkpoint complexes. Through the accumulation of these complexes, the ability of Rad53 and similarly its mammalian homolog, Chk2, to self-associate and activate may accelerate a positive feedback loop promoting the activation of DNA damage checkpoint effectors (Gilbert et al., 2001; Xu et al., 2002; Ahn et al., 2002).

Rad9 participates in a number of DNA damage checkpoint pathway-mediated responses to DNA damage (reviewed in Lowndes and Murguia, 2000), including the regulation of Chk1 phosphorylation (Sanchez et al., 1999). Mutation of the Rad9 [S/T]Q phosphorylation sites does not abolish the phosphorylation or function of Chk1. Thus, the domains within Rad9 essential for the regulation of Rad53 and Chk1 are functionally separable, and the mechanism of regulation of Chk1 by Rad9 remains to be determined. The fact that the major damage-induced Rad9 phosphorylation sites are dispensable for the regulation of Chk1 correlates with the lack of recognizable phosphorylation-dependent interaction domains, such as an FHA domain, within Chk1.

SCDs as Targets of DNA Checkpoint Phosphorylation and Interaction

Individual SQ or TQ dipeptides are fairly common in polypeptide sequences, theoretically occurring approximately once every 200 or so residues. However, clusters of [S/T]Q sites are readily recognizable in a handful of known DNA checkpoint or replication proteins. A pattern-based search for SCD of at least five [S/T]Q sites separated by no more than 35 amino acids identified roughly 1% of the human, murine, or budding yeast PROSITE database. Of these, 10% are readily recognizable as DNA checkpoint proteins, including human and/or murine Atm, DNA-PKcs, Brca1, Brca2, Chk2, and Mdm2, and yeast Rad9, Rad53, and Chk1. Experimental evidence already suggests that the SCDs in Brca1, Chk2, and Mdm2 are targets for phosphorylation by PIKKs (Cortez et al., 1999; Sanchez et al., 1999; de Toledo et al., 2000; Matsuoka et al., 2000; Melchionna et al., 2000; Wakayama et al., 2001). Within the group of proteins identified by the PROSITE pattern search, the mammalian DNA-PKcs and yeast Rad9, Rad53, and

Chk1 SCDs are notably rich in TQ residues. Since FHA domains have a strong binding preference for phosphothreonine over phosphoserine (Durocher et al., 1999) and since the threonine-rich Rad9 SCD is a binding site for Rad53 FHA domains, TQ-rich clusters may often be PIKK-dependent FHA domain binding sites. It remains to be determined whether phosphorylated SCDs in proteins other than Rad9 similarly mediate FHA domain interactions or serve other regulatory functions.

Interactions between phosphorylated Rad9 and Rad53 are a prototype for assembly of DNA damage-dependent checkpoint complexes. Two important, unanticipated features of these complexes are the involvement of an intermediary adaptor protein for functional connection of a PIKK with a substrate and the PIKK phosphorylation dependence of that interaction. These features, and the potential multivalency of these interactions, may be common vehicles for the rapid assembly of focal eukaryotic checkpoint complexes at sites of DNA damage.

Experimental Procedures

Plasmids

For pRS306 *rad9*^{T603A} and pRS306 *rad9*$^{\Delta 593-620}$, the mutations were introduced by PCR into the SpeI-HindIII internal *RAD9* fragment cloned into the same sites of pRS306. To construct pRS306 *RAD9*^{3xHA}, the FLAG-tag of pRS314G *RAD9*^{FLAG} (Sun et al., 1998) was replaced with a 3xHA tag followed by ~500 bp of *RAD9* 3′ UTR, and the MunI-SacII fragment was cloned into pRS306. pRS316 *RAD9*^{3xHA} was constructed by replacing the MunI-SacII fragment of pRS316 *RAD9*^{FLAG} (Sun et al., 1998) with the MunI-SacII from pRS306 *RAD9*^{3xHA}. pRS306 *rad9*Δ was created by replacing the *RAD9* coding sequence in the XhoI-SmaI fragment of pRS306 *RAD9*^{3xHA} with ~1.17 kb of *RAD9* 5′ UTR. To create *CHK1*^{13xmyc}, the *CHK1* ORF and surrounding UTR was isolated by PCR and cloned into pBSKII^{+}. The SmaI-SpeI fragment encoding the 13xmyc tag from pFA6a-13myc-His3MX6 (Longtine et al., 1998) was inserted into equivalent sites created by PCR at the 3′ end of the *CHK1* ORF. The entire *CHK1*^{13xmyc} locus was then cloned into pRS315. For the GST-FHA1 expression construct, *RAD53* encoding amino acids 1–197 were amplified by PCR and cloned into pGEX4T3. The alanine substitutions of the conserved FHA domain residues at R70 and N107 were introduced by PCR. The GST-Rad53 FHA2 fusion proteins were described previously (Sun et al., 1998).

Strains

DLY408 (*cdc13-1 cdc15-2*), DLY409 (*cdc13-1 cdc15-2 rad9::HIS3*), DLY418 (*cdc15-2*), and DLY554 (*cdc13-1 cdc15-2 rad53-21*) are from the Weinert laboratory (Gardner et al., 1999; Lydall and Weinert, 1997). 1588-4C and U960-5C (*sml1-1 rad53-XB::HIS3*) are from the Rothstein laboratory (Zhao et al., 1998). These strains are in a W303 background.

The initial *rad9*^{T603A} and *rad9*$^{\Delta 593-620}$ mutations were engineered into DLY408 by two-step allele replacement with the appropriate pRS306 constructs. pRS306 *RAD9*^{3xHA} was used to epitope tag *RAD9* in DLY408. For the site-directed mutagenesis of Rad9 [S/T]Q sites, PCR cassettes containing the different mutations were used to replace the corresponding sequence in a *rad9*$^{\Delta 593-620}$ strain by two-step allele replacement (Erdeniz et al., 1997). pRS306 *rad9*Δ was used to delete *RAD9* in 1588-4C and DLY408, and *cdc13-1* was subsequently introduced into 1588-4C *rad9*Δ with the plasmid pDL420 (Weinert laboratory). To construct DLY408 *rad53*Δ strains, *SML1* was first replaced with a *sml1::TRP1* cassette PCR amplified from U973 (*sml1::TRP1 esr1-1*, Rothstein laboratory). *RAD53* was then replaced with a *rad53::HIS3* PCR cassette that targets the precise removal of the *RAD53* ORF. *CHK1* and *TOF1* ORFs in DLY408 and U960-5C, respectively, were replaced with kan^{R} cassettes PCR amplified from the corresponding deletion strains (Research Genetics).

Drop-out media was purchased from Bufferad. Synthetic and rich media were typically supplemented with adenine to 47.5 mg/l. For immunoblot analyses, cultures were grown to early/mid-log phase. For *cdc15-2* synchronization, early-log cultures were shifted to 37°C for 3 hr. Cultures were mock treated or treated with 0.1% MMS (Sigma) for 1 hr, washed, and either lysed immediately or frozen in liquid nitrogen and stored at −80°C.

Immunoprecipitations and Immunoblotting

Immunoprecipitations and anti-Rad53 Western blotting were as previously described (Sun et al., 1998). In brief, lysates were prepared by mechanical disruption in TG (PBS + 1% Triton X-100, 10% glycerol, and phosphatase and protease inhibitors [Roche and Sigma]). 1–2.5 mg of lysate was used per immunoprecipitation with rabbit anti-Rad53 serum, rabbit anti-Rad9 serum (G. Liu, M.F.S., and D.F.S., unpublished data), mouse anti-myc monoclonal 9E10 (Covance), or control antibodies. TCA lysates were prepared as described (Foiani et al., 1999), with minor volume adjustments. Lysates and immunoprecipitations were resolved on 6% or 7.5% polyacrylamide gels prior to transfer to Immobilon-P (Millipore).

Rad9^{3xHA} was detected in immunoblot analysis with HRP-conjugated rat anti-HA monoclonal 3F10 (Roche), Rad53 with rabbit anti-Rad53 serum or a goat anti-Rad53 antibody (Santa Cruz), and Chk1^{13xmyc} with HRP-conjugated 9E10 (Santa Cruz). The rabbit anti-phospho-[S/T]Q antibody was used as per manufacturer's suggestions (Cell Signaling Technology). Polyclonal rabbit antibody against Rad9 phospho-T390 was generated and purified as described (DiGiovanna et al., 1998) using the T390 phosphopeptide KSNRST*QIVN as antigen and nonphosphorylated and phosphorylated forms of the peptide TENNSNRST*QIVNNPR for purification.

GST Fusion Protein Purification for BIAcore

Bacterial cultures expressing GST-Rad53 and Rad9 fusion proteins were collected by centrifugation and lysed by sonication in TG with 5 mM DTT. Clarified lysates were rotated with glutathione-sepharose beads at 4°C. The beads were then washed in batch format once with >100× bead bed volume lysis buffer and twice with PBS or HS-t (10 mM HEPES [pH 7.6], 150 mM NaCl, 0.005% Tween-20). For the FHA1 fusions, the latter two washes lacked protease inhibitors, and thrombin cleavage was performed as per manufacturer's suggestions (Amersham). Thrombin was removed by incubation with benzamidine-agarose beads (Sigma). The GST-Rad53 FHA2 construct, a noncleavable GST fusion protein, was eluted with glutathione, and the glutathione was removed by dialysis.

Synthetic Peptides

Rad9 peptides were synthesized and purified by the Small Scale Peptide Synthesis facility at the W.M. Keck Biotechnology Resource Center at the Yale School of Medicine.

BIAcore Peptide Binding Assay

The 16-mer Rad9 T390 peptides were combined with biotin-LC-NHS (Pierce) at a molar ratio of 1.5:1 peptide:biotin overnight at 4°C. Remaining NHS groups were blocked with 20 mM Tris. Biotinylated peptides and a similar biotin-only mixture were diluted and flowed over the surface of a streptavidin-coated sensor chip in a BIAcore 2000 as per manufacturer's instructions (BIAcore). The peptide surfaces typically yielded 70–200 RUs over the biotin-only surface. Analytes were dialyzed into HBS-Tw (10 mM HEPES [pH 7.6], 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween-20). For the SPR measurements, 60 μl of various concentrations of analyte at 10 μl/min or 125 μl at 25 μl/min were flowed over each surface at 15°C. Surface regeneration was accomplished with a pulse of 1 M NaCl, a pulse of 2 M $MgCl_2$, and extensive washing with HBS-Tw.

Genotoxin Sensitivity Assays

For the *cdc13-1* assay, log-phase cultures of 1588-4C *cdc13-1 rad9*Δ strains bearing appropriate plasmids were shifted to 37°C, and aliquots plated in duplicate on YPAD at the indicated times. Plates were incubated at 23°C for 3 days. For the remaining assays, cultures of 1588-4C- or U960-5C-derived strains bearing appropriate plasmids were brought to similar densities and serially diluted 4-fold in a 96-well plate. A 48-pin inoculator was used to spot the diluted cultures onto YPAD, YPAD+HU, or YPAD+MMS plates. Sets

of YPAD plates were UV irradiated in a prewarmed Stratalinker (Stratagene). The plate cultures were grown at 30°C for 2–3 days.

Assay for the G_2/M DNA Damage Checkpoint Function

This assay was performed largely as described (Lydall and Weinert, 1995, 1997). In brief, strains derived from DLY408, DLY409, or DLY554 bearing the appropriate plasmids were grown to early-log phase at 23°C in synthetic media, shifted into YPAD, and synchronized with α factor. Cells were washed into prewarmed YPAD and grown at 37°C. 70% ethanol fixed samples were sonicated and spotted onto poly-L-lysine (Sigma)-coated slides, dried, and sealed under a coverslip with DAPI mounting media (Vector Laboratories). For each sample, at least 100 cells were scored. Cells with gross nuclear or morphological defects and cells terminally G_1 arrested as determined by size and morphology were not scored.

Acknowledgments

We are grateful for the assistance of J. Falato and G. Bellinger, and for strains and reagents from the Weinert, Hartwell, Rothstein, and McAlear laboratories. We thank D. Durocher and members of the Stern laboratory for discussions and critical reading of the manuscript. This work was supported by grants from the A-T Children's Project (Z.S. and D.F.S.), USPHS R01CA82257 (D.F.S.), USAMRMC DAMD17-98-1-8272 (J.K.D. and D.F.S.), and NIDDK P01DK55389 (J.S.M. and D.P.). M.F.S. was supported by NIH NRSA T32GM07223 from the NIGMS and by USAMRMC DAMD 17-99-1-9460.

Received: April 30, 2001
Revised: April 12, 2002

References

Abraham, R.T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. *15*, 2177–2196.

Ahn, J.Y., Li, X., Davis, H.L., and Canman, C.E. (2002). Phosphorylation of threonine 68 promotes oligomerization and autophosphorylation of the Chk2 protein kinase via the forkhead-associated (FHA) domain. J. Biol. Chem., in press.

al-Khodairy, F., Fotou, E., Sheldrick, K.S., Griffiths, D.J., Lehmann, A.R., and Carr, A.M. (1994). Identification and characterization of new elements involved in checkpoint and feedback controls in fission yeast. Mol. Biol. Cell *5*, 147–160.

Allen, J.B., Zhou, Z., Siede, W., Friedberg, E.C., and Elledge, S.J. (1994). The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev. *8*, 2401–2415.

Anderson, C.W., and Lees-Miller, S.P. (1992). The nuclear serine/threonine protein kinase DNA-PK. Crit. Rev. Eukaryot. Gene Expr. *2*, 283–314.

Bell, D.W., Varley, J.M., Szydlo, T.E., Kang, D.H., Wahrer, D.C., Shannon, K.E., Lubratovich, M., Verselis, S.J., Isselbacher, K.J., Fraumeni, J.F., et al. (1999). Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science *286*, 2528–2531.

Bentley, N.J., Holtzman, D.A., Flaggs, G., Keegan, K.S., DeMaggio, A., Ford, J.C., Hoekstra, M., and Carr, A.M. (1996). The *Schizosaccharomyces pombe* rad3 checkpoint gene. EMBO J. *15*, 6641–6651.

Bork, P., Hofmann, K., Bucher, P., Neuwald, A.F., Altschul, S.F., and Koonin, E.V. (1997). A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. FASEB J. *11*, 68–76.

Byeon, I.J., Yongkiettrakul, S., and Tsai, M.D. (2001). Solution structure of the yeast Rad53 FHA2 complexed with a phosphothreonine peptide pTXXL: comparison with the structures of FHA2-pYXL and FHA1-pTXXD complexes. J. Mol. Biol. *314*, 577–588.

Chehab, N.H., Malikzay, A., Appel, M., and Halazonetis, T.D. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev. *14*, 278–288.

Cortez, D., Wang, Y., Qin, J., and Elledge, S.J. (1999). Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science *286*, 1162–1166.

de la Torre-Ruiz, M., and Lowndes, N.F. (2000). The *Saccharomyces cerevisiae* DNA damage checkpoint is required for efficient repair of double strand breaks by non-homologous end joining. FEBS Lett. *467*, 311–315.

de Toledo, S.M., Azzam, E.I., Dahlberg, W.K., Gooding, T.B., and Little, J.B. (2000). ATM complexes with HDM2 and promotes its rapid phosphorylation in a p53-independent manner in normal and tumor human cells exposed to ionizing radiation. Oncogene *19*, 6185–6193.

DiGiovanna, M.P., Roussel, R.R., and Stern, D.F. (1998). Production of antibodies that recognize specific Tyrosine-phosphorylated peptides. In Current Protocols in Molecular Biology, F.M. Ausubel, et al., eds. (New York: John Wiley & Sons), pp. 18.6.1–18.6.19.

Durocher, D., and Jackson, S.P. (2001). DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? Curr. Opin. Cell Biol. *13*, 225–231.

Durocher, D., Henckel, J., Fersht, A.R., and Jackson, S.P. (1999). The FHA domain is a modular phosphopeptide recognition motif. Mol. Cell *4*, 387–394.

Durocher, D., Taylor, I.A., Sarbassova, D., Haire, L.F., Westcott, S.L., Jackson, S.P., Smerdon, S.J., and Yaffe, M.B. (2000). The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. Mol. Cell *6*, 1169–1182.

Elledge, S.J. (1996). Cell cycle checkpoints: preventing an identity crisis. Science *274*, 1664–1672.

Emili, A. (1998). MEC1-dependent phosphorylation of Rad9p in response to DNA damage. Mol. Cell *2*, 183–189.

Erdeniz, N., Mortensen, U.H., and Rothstein, R. (1997). Cloning-free PCR-based allele replacement methods. Genome Res. *7*, 1174–1183.

Foiani, M., Liberi, G., Piatti, S., and Plevani, P. (1999). *Saccharomyces cerevisiae* as a model system to study DNA replication. In Eukaryotic DNA Replication: A Practical Approach, S. Cotterill, ed. (Oxford, UK: Oxford University Press), pp. 185–200.

Foss, E. (2001). Tof1p regulates DNA damage responses during S phase in *Saccharomyces cerevisiae*. Genetics *157*, 567–577.

Futcher, B. (1999). Cell cycle synchronization. Methods Cell Sci. *21*, 79–86.

Gardner, R., Putnam, C.W., and Weinert, T. (1999). RAD53, DUN1 and PDS1 define two parallel G2/M checkpoint pathways in budding yeast. EMBO J. *18*, 3173–3185.

Garvik, B., Carson, M., and Hartwell, L. (1995). Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol. Cell. Biol. *15*, 6128–6138.

Gilbert, C.S., Green, C.M., and Lowndes, N.F. (2001). Budding yeast Rad9 is an ATP-dependent Rad53 activating machine. Mol. Cell *8*, 129–136.

Hofmann, K., and Bucher, P. (1995). The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors. Trends Biochem. Sci. *20*, 347–349.

Kim, S.T., Lim, D.S., Canman, C.E., and Kastan, M.B. (1999). Substrate specificities and identification of putative substrates of ATM kinase family members. J. Biol. Chem. *274*, 37538–37543.

Koonin, E.V., Altschul, S.F., and Bork, P. (1996). BRCA1 protein products. Functional motifs. Nat. Genet. *13*, 266–268.

Ladbury, J.E., Lemmon, M.A., Zhou, M., Green, J., Botfield, M.C., and Schlessinger, J. (1995). Measurement of the binding of tyrosyl phosphopeptides to SH2 domains: a reappraisal. Proc. Natl. Acad. Sci. USA *92*, 3199–3203.

Lee, C.H., and Chung, J.H. (2001). The hCds1 (Chk2)-FHA domain is essential for a chain of phosphorylation events on hCds1 that is induced by ionizing radiation. J. Biol. Chem. *276*, 30537–30541.

Li, J., Smith, G.P., and Walker, J.C. (1999). Kinase interaction domain of kinase-associated protein phosphatase, a phosphoprotein-binding domain. Proc. Natl. Acad. Sci. USA *96*, 7821–7826.

Liao, H., Byeon, I.J., and Tsai, M.D. (1999). Structure and function of a new phosphopeptide-binding domain containing the FHA2 of Rad53. J. Mol. Biol. *294*, 1041–1049.

Liao, H., Yuan, C., Su, M.I., Yongkiettrakul, S., Qin, D., Li, H., Byeon, I.J., Pei, D., and Tsai, M.D. (2000). Structure of the FHA1 domain of yeast Rad53 and identification of binding sites for both FHA1 and its target protein Rad9. J. Mol. Biol. *304*, 941–951.

Lin, J.J., and Zakian, V.A. (1996). The Saccharomyces CDC13 protein is a single-strand TG1-3 telomeric DNA-binding protein in vitro that affects telomere behavior in vivo. Proc. Natl. Acad. Sci. USA *93*, 13760–13765.

Lindsay, H.D., Griffiths, D.J., Edwards, R.J., Christensen, P.U., Murray, J.M., Osman, F., Walworth, N., and Carr, A.M. (1998). S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in *Schizosaccharomyces pombe*. Genes Dev. *12*, 382–395.

Liu, Y., Vidanes, G., Lin, Y.C., Mori, S., and Siede, W. (2000). Characterization of a *Saccharomyces cerevisiae* homologue of *Schizosaccharomyces pombe* Chk1 involved in DNA-damage-induced M-phase arrest. Mol. Gen. Genet. *262*, 1132–1146.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast *14*, 953–961.

Lowndes, N.F., and Murguia, J.R. (2000). Sensing and responding to DNA damage. Curr. Opin. Genet. Dev. *10*, 17–25.

Lydall, D., and Weinert, T. (1995). Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. Science *270*, 1488–1491.

Lydall, D., and Weinert, T. (1997). Use of cdc13-1-induced DNA damage to study effects of checkpoint genes on DNA damage processing. Methods Enzymol. *283*, 410–424.

Matsuoka, S., Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., and Elledge, S.J. (2000). Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc. Natl. Acad. Sci. USA *97*, 10389–10394.

Melchionna, R., Chen, X.B., Blasina, A., and McGowan, C.H. (2000). Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. Nat. Cell Biol. *2*, 762–765.

Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F.B., Mendenhall, M.D., Sicheri, F., Pawson, T., and Tyers, M. (2001). Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. Nature *414*, 514–521.

Navas, T.A., Sanchez, Y., and Elledge, S.J. (1996). RAD9 and DNA polymerase epsilon form parallel sensory branches for transducing the DNA damage checkpoint signal in *Saccharomyces cerevisiae*. Genes Dev. *10*, 2632–2643.

Nugent, C.I., Hughes, T.R., Lue, N.F., and Lundblad, V. (1996). Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. Science *274*, 249–252.

Paulovich, A.G., Margulies, R.U., Garvik, B.M., and Hartwell, L.H. (1997). RAD9, RAD17, and RAD24 are required for S phase regulation in *Saccharomyces cerevisiae* in response to DNA damage. Genetics *145*, 45–62.

Rhind, N., and Russell, P. (2000). Chk1 and Cds1: linchpins of the DNA damage and replication checkpoint pathways. J. Cell Sci. *113*, 3889–3896.

Sanchez, Y., Desany, B.A., Jones, W.J., Liu, Q., Wang, B., and Elledge, S.J. (1996). Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. Science *271*, 357–360.

Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., and Elledge, S.J. (1999). Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. Science *286*, 1166–1171.

Siede, W., Friedberg, A.S., and Friedberg, E.C. (1993). RAD9-dependent G1 arrest defines a second checkpoint for damaged DNA in the cell cycle of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *90*, 7985–7989.

Soulier, J., and Lowndes, N.F. (1999). The BRCT domain of the *S. cerevisiae* checkpoint protein Rad9 mediates a Rad9-Rad9 interaction after DNA damage. Curr. Biol. *9*, 551–554.

Stone, J.M., Collinge, M.A., Smith, R.D., Horn, M.A., and Walker, J.C. (1994). Interaction of a protein phosphatase with an Arabidopsis serine-threonine receptor kinase. Science *266*, 793–795.

Sun, Z., Fay, D.S., Marini, F., Foiani, M., and Stern, D.F. (1996). Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. Genes Dev. *10*, 395–406.

Sun, Z., Hsiao, J., Fay, D.S., and Stern, D.F. (1998). Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. Science *281*, 272–274.

Terleth, C., Schenk, P., Poot, R., Brouwer, J., and van de Putte, P. (1990). Differential repair of UV damage in rad mutants of *Saccharomyces cerevisiae*: a possible function of G2 arrest upon UV irradiation. Mol. Cell. Biol. *10*, 4678–4684.

Vialard, J.E., Gilbert, C.S., Green, C.M., and Lowndes, N.F. (1998). The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. EMBO J. *17*, 5679–5688.

Wakayama, T., Kondo, T., Ando, S., Matsumoto, K., and Sugimoto, K. (2001). Pie1, a protein interacting with Mec1, controls cell growth and checkpoint responses in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *21*, 755–764.

Walworth, N., Davey, S., and Beach, D. (1993). Fission yeast chk1 protein kinase links the rad checkpoint pathway to cdc2. Nature *363*, 368–371.

Wang, P., Byeon, I.J., Liao, H., Beebe, K.D., Yongkiettrakul, S., Pei, D., and Tsai, M.D. (2000). II. Structure and specificity of the interaction between the FHA2 domain of Rad53 and phosphotyrosyl peptides. J. Mol. Biol. *302*, 927–940.

Weinert, T.A., and Hartwell, L.H. (1988). The RAD9 gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science *241*, 317–322.

Weinert, T.A., and Hartwell, L.H. (1990). Characterization of RAD9 of *Saccharomyces cerevisiae* and evidence that its function acts posttranslationally in cell cycle arrest after DNA damage. Mol. Cell. Biol. *10*, 6554–6564.

Weinert, T.A., and Hartwell, L.H. (1993). Cell cycle arrest of cdc mutants and specificity of the RAD9 checkpoint. Genetics *134*, 63–80.

Weinert, T.A., Kiser, G.L., and Hartwell, L.H. (1994). Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. *8*, 652–665.

Xu, X., Tsvetkov, L.M., and Stern, D.F. (2002). Chk2 activation and phosphorylation-dependent oligomerization. Mol. Cell. Biol. *21*, 4419–4432.

Zhao, X., Muller, E.G., and Rothstein, R. (1998). A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Mol. Cell *2*, 329–340.

Zhou, B.B., and Elledge, S.J. (2000). The DNA damage response: putting checkpoints in perspective. Nature *408*, 433–439.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

Phyllis Rinehart
PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB233865
ADB265530
ADB244706
ADB285843
ADB240902
ADB264038
ADB285885
ADB274458
ADB285735
ADB286597
ADB285707
ADB274521
ADB259955
ADB274793
ADB285914
ADB260288
ADB254419
ADB282347
ADB286860
ADB262052
ADB286348
ADB264839
ADB275123
ADB286590
ADB264002
ADB281670
ADB281622
ADB263720
ADB285876
ADB262660
ADB282191
ADB283518
ADB285797
ADB269339
ADB264584
ADB282777
ADB286185
ADB262261
ADB282896
ADB286247
ADB286127
ADB274629
ADB284370
ADB264652
ADB281790
ADB286578
ADB264750
ADB282776
ADB286264
ADB260563
ADB277918
ADB286365
ADB275327
ADB286736
ADB286137
ADB286146
ADB286100
ADB286266
ADB286308
ADB285832