# A domain of Rad9 specifically required for activation of Chk1 in budding yeast

**Richard T. Blankley and David Lydall*,‡**

School of Biological Sciences, University of Manchester, G38 Stopford Building, Oxford Rd, Manchester, M13 9PT, UK

*Present address: School of Clinical Medical Sciences – Gerontology, University of Newcastle, Henry Wellcome Laboratory for Biogerontology Research, Newcastle General Hospital, Newcastle Upon Tyne, NE4 6BE, UK

‡Author for correspondence (e-mail: d.a.lydall@ncl.ac.uk)

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## Summary

**The Rad9 protein is a key adaptor protein in *Saccharomyces cerevisiae* DNA damage checkpoint pathways. Its adaptor function is to link the activity of the Mec1 kinase to the activation of two parallel signalling pathways dependent on the Rad53 and Chk1 kinases. The mechanisms by which Rad9 interacts with, and activates, Rad53 are well understood. However, little was known about how Rad9 facilitates the activation of Chk1. We show here that the N-terminus of Rad9 is specifically important for phosphorylation and activation of the Chk1 kinase but not for the phosphorylation and activation of the Rad53 kinase. The Chk1 activation domain (CAD) of Rad9 is specifically important for signalling cell-cycle arrest after *cdc13-1*- and *yku70*Δ-induced telomere damage but not for tolerating ultraviolet-induced damage or inhibiting nuclease activity at telomeres. This work extends data showing that separable domains within the Rad9 adaptor protein allow it to activate two distinct kinase signalling pathways independently of each other.**

Key words: RAD9, CHK1, Checkpoint, Yeast, Adaptor

## Introduction

DNA damage checkpoints are mechanisms utilized by eukaryotic cells to monitor the integrity of their genomes and they play an important role in maintaining genomic stability. Checkpoints are signalling pathways built around the regulated activity of kinases. The kinases at the heart of DNA damage checkpoint signalling pathways are conserved from yeast to mammals, as are the accessory proteins that localize to DNA lesions and that regulate the activation of the kinases (Melo and Toczyski, 2002; Nyberg et al., 2002; Rouse and Jackson, 2002a).

In budding yeast, the major kinase involved in DNA damage checkpoint pathways is encoded by the essential gene *MEC1*. Mec1 is a phosphoinositide 3-kinase-like kinase (PIKK), and its homologues in fission yeast and vertebrates are Rad3 and ATR (for 'ATM and Rad3-related'), respectively (reviewed by Abraham, 2001; Nyberg et al., 2002). Two protein kinases, Rad53 and Chk1, act downstream of Mec1 in distinct pathways (Gardner et al., 1999; Sanchez et al., 1999). Rad53 and Chk1 are also conserved in fission yeast as Cds1 and Chk1, and in vertebrates as Chk2 and Chk1, respectively (Abraham, 2001; Rhind and Russell, 2000). Mec1 appears to be activated in virtually all pathways of DNA damage checkpoint signalling. Rad53 is activated following DNA damage and replication block (Allen et al., 1994; Navas et al., 1996; Sanchez et al., 1996; Weinert et al., 1994), whereas Chk1 is only required for arrest following a subset of DNA damage lesions and does not appear to be required following replication block (Liu et al., 2000; Sanchez et al., 1999). In *Schizosaccharomyces pombe*, there is a clearer distinction in function: Cds1 (Rad53) is activated following replication block (Lindsay et al., 1998) and Chk1 is activated following DNA damage (Walworth et al., 1993).

*RAD9* was the first checkpoint gene to be defined (Weinert and Hartwell, 1988) but its biochemical function has only recently become apparent. Current models are that Rad9 is an 'adaptor' checkpoint protein whose function is to couple the activation of upstream kinases (Mec1) with downstream effector kinases (Rad53 and Chk1) (Melo and Toczyski, 2002). The role of adaptor proteins such as Rad9 seems to be both in ensuring specificity in checkpoint activation and in amplification of the damage signal (Gilbert et al., 2001). Other examples of adaptor or mediator proteins include Mrc1 (yeast), Crb2 (*S. pombe*), Claspin (*Xenopus*) and BRCA1 (human) (Melo and Toczyski, 2002) and a new mammalian mediator protein, MDC1, has recently been described (Goldberg et al., 2003; Lou et al., 2003a; Lou et al., 2003b; Stewart et al., 2003).

In both yeast and human cells, at least two checkpoint protein complexes appear to be recruited to sites of DNA lesions. One complex contains a PIKK kinase and its interacting partner (Mec1 and Ddc2 in budding yeast), whereas a second hetero-trimeric complex is recruited independently (Mec3-Rad17-Ddc1 in budding yeast) (Kondo et al., 2001; Melo et al., 2001; Rouse and Jackson, 2002b; Zou and Elledge, 2003). The presence of both intact complexes is required for efficient checkpoint signalling, possibly via an amplification loop. A candidate structure for the recruitment of these checkpoint protein complexes (and thus checkpoint activation) is extended regions of single-stranded DNA (ssDNA) (Lydall and Weinert, 1995; Zou and Elledge, 2003). The function of adaptor proteins such as Rad9 appears to be the physical linking of the activity of the checkpoint protein complexes at sites of DNA lesions (Mec1) to the activation of downstream checkpoint kinases that transmit the signal throughout the

nucleus (Rad53 and Chk1). This is consistent with the finding that Mec1 binds to DNA lesions, Rad9 is weakly recruited and Rad53 is not recruited (Kondo et al., 2001; Melo et al., 2001; Rouse and Jackson, 2002b; Zou and Elledge, 2003).

The adaptor role that Rad9 plays in signal transduction from Mec1 to Rad53 following DNA damage is well understood. Rad9 is hyper-phosphorylated in a *MEC1*-dependent manner after DNA damage (Emili, 1998; Sun et al., 1998; Vialard et al., 1998), and the phosphorylation of S/T[Q] consensus sites in Rad9 facilitates the recruitment and binding of Rad53 through its FHA (forkhead-associated) domains (Schwartz et al., 2002; Sun et al., 1998). Large phosphorylated Rad9 complexes are believed to act as 'solid surface catalysts' that facilitate a trans-autophosphorylation activity of Rad53 molecules (Gilbert et al., 2001). Phosphorylated (and activated) Rad53 molecules are then released by Rad9 to diffuse away and transmit the checkpoint signal throughout the nucleus. In budding yeast, some of the Rad53 downstream targets include the Dun1 protein kinase (de la Torre Ruiz and Lowndes, 2000; Gardner et al., 1999), the Cdc5 polo-like kinase (Sanchez et al., 1999) and Asf1, which is involved in chromatin remodelling (Emili et al., 2001; Hu et al., 2001).

The in vivo activation of Chk1 is also dependent on *MEC1* and *RAD9* (Sanchez et al., 1999). The role that Rad9 plays in the signal transduction from Mec1 to Chk1 is not well understood. Two reports have described a Rad9-Chk1 interaction by two-hybrid analysis in undamaged cells (Sanchez et al., 1999; Uetz et al., 2000) but other biochemical evidence for a physical interaction between Rad9 and Chk1 is lacking. Chk1 does not contain FHA domains and disruption of Mec1 phosphorylation sites in Rad9 does not abrogate the activation of Chk1 (Schwartz et al., 2002).

In fission yeast and mammalian cells, the mechanism by which Chk1 activity enforces a G2/M arrest are better characterized. Chk1 regulates Cdk (Cdc2) activity directly by inhibitory phosphorylation and also by targeting Cdc25, a negative regulator of Cdc2 (Rhind and Russell, 2000). In budding yeast, activation of the DNA damage checkpoint arrests cells at the metaphase-to-anaphase transition with high Cdk activity. The only downstream component of the Chk1 signalling pathway identified in *S. cerevisiae* to date is Pds1 (Securin) (Gardner et al., 1999; Sanchez et al., 1999; Wang et al., 2001).

Our aim was to understand more about how Rad9 functions in response to DNA damage. In this paper we describe a genetic screen to identify separation of function alleles in *RAD9*. We identified a series of alleles defective in checkpoint signalling but proficient at maintaining the viability of *cdc13-1* mutants. By further characterizing these alleles we show that an N-terminal domain of Rad9 is required specifically for activation of the Chk1 kinase but not the Rad53 (Cds1/Chk2) kinase following DNA damage.

## Materials and Methods

### Plasmid constructions

A library of mutant *RAD9* alleles was generated using the GPS-LS Linker Scanning Kit (New England Biolabs). The procedure involved integrating a 1706 bp transposon into *RAD9* centromeric plasmids, and then removal of the transposon by digestion with the *Pme*I restriction enzyme to leave behind a 15 bp insertion. In the case of the *rad9-s1* allele, the *Pme*I digestion was unsuccessful and the transposon remained in the plasmid. A library of >24,300 linker insertions into a pRS416 *RAD9* plasmid was constructed (pDL767). The N-terminal truncation and internal deletion alleles were constructed by PCR cloning by introducing *Nde*I sites to generate alternative start sites. The *rad9Δ1-231* allele was cloned into pRS406 as a *Nde*I-*Bam*HI fragment (pDL848).

### Strains

All strains are in the W303 genetic background and are *RAD5*$^{+}$. The *rad9Δ1-231* allele was subcloned from pDL848 into pRS406 as a *Not*I-*Xho*I fragment to create pDL901, and was integrated at the *URA3* locus of a *rad9::LEU2 cdc13-1 cdc15-2* strain to generate DLY1908. The *rad9Δ1-231* allele was then crossed to other *rad9Δ* mutant strains.

### Drop testing

Yeast strains were grown up to mid-log phase before a 1:5 dilution series was performed in either YEPD or SC-uracil media. Small drops of each series were spotted onto solid media plates using a 48-prong replica plating device (Sigma). Plates were incubated at appropriate temperatures for 4-5 days. In the rapid death assay, plates were subjected to a protocol of 36°C for 4 hours followed by 23°C for 4 hours, and this was repeated a further two times. After the completion of three cycles, the plates were incubated at 23°C.

### *cdc13-1* arrest assay

All strains carry *cdc13-1*, *cdc15-2* and *bar1Δ* mutations. Strains were grown to early log phase at 23°C before the addition of α-factor for 2.5 hours. After this period, the G1 arrest was observed by the presence of 'schmoos' by microscopy. Cultures were spun down and the cell pellets washed twice in YEPD at room temperature. Cells were then resuspended in 50 ml YEPD pre-warmed to 36°C and cultured at 36°C. Samples were removed at intervals of 20 minutes, fixed in ethanol, stained with DAPI (4′,6′-diamidino-2-phenylindole) and at least 100 cells scored by fluorescence microscopy (Lydall and Weinert, 1997). Cells with abnormal morphology were not scored.

### Immunoblotting

Protein extracts were performed by glass bead breakage into TCA as previously described (Foiani et al., 1999) with minor adjustments. A sheep anti-Rad9 polyclonal antibody was generated using a His-tagged (pET22b) N-terminal half of Rad9 (*Nde*I-*Sac*I fragment) as an antigen and the sera was affinity purified before use. The rabbit anti-Rad53 polyclonal antibody was a kind gift from Noel Lowndes. Mouse monoclonal 12CA5 (anti-HA) and 9E10 (anti-Myc) antibodies were used to detect epitope-tagged Chk1.

### Methylmethanesulphate (MMS) insult

Cells harbouring the *cdc15-2* allele were grown to mid-log phase at 23°C before being shifted to 37°C for three hours. After three hours, MMS was added to a final concentration of 0.1% (v/v) and cells were cultured for a further 1 hour at 37°C. MMS was not added to mock-treated cells but they were otherwise cultured identically. Strains are DLY2191 (*RAD9 cdc15-2*), DLY2192 (*rad9::LEU2 cdc15-2*) and DLY2193 (*ura3*:*rad9Δ1-231:URA3 rad9::LEU2 cdc15-2*)

## Results

### A *RAD9* separation of function screen

*RAD9* has at least two functions in response to *cdc13-1*-induced telomeric DNA damage. First, *RAD9* is required to signal a cell-cycle arrest of *cdc13-1* cells at non-permissive temperatures (Weinert and Hartwell, 1993). Second, *RAD9* is

required to maintain the viability of *cdc13-1* mutants at 36°C (Lydall and Weinert, 1995). To understand better the role of Rad9 in the DNA damage response, we sought to identify alleles of *RAD9* that were defective in one or other of these functions but not defective in both.

*CDC13* encodes an essential telomere-binding protein; in *cdc13-1* mutants at non-permissive temperatures, the end-capping of telomeres appears to be compromised (Nugent et al., 1996). As a result, *cdc13-1* cells accumulate ssDNA at their telomeres (Booth et al., 2001; Garvik et al., 1995; Lydall and Weinert, 1995). *RAD9* is required for cell-cycle arrest of *cdc13-1* mutants presumably because it mediates interactions between upstream and downstream checkpoint kinases. *RAD9* also helps maintain the viability of *cdc13-1* cells at 36°C by inhibiting nuclease activity at telomeres, whereas other checkpoint genes, such as *RAD24*, promote nuclease activity at telomeres (Booth et al., 2001; Lydall and Weinert, 1995).

Fig. 1A outlines the logic underlying two simple growth assays that measure different activities of *RAD9*. The 'checkpoint arrest' assay measures the ability of *RAD9* alleles to inhibit growth of *cdc13-1* mutants at the semi-permissive temperature of 27.3°C. *cdc13-1 RAD9*$^+$ cells enter a checkpoint arrest and cannot form colonies at 27.3°C. By contrast, *cdc13-1 rad9*Δ mutants are defective in checkpoint signalling and can form colonies at 27.3°C when low, presumably nonlethal, levels of DNA damage are present (Fig. 1B, rows 3,4). The 27.3°C temperature was determined empirically to maximize the difference in growth between *cdc13-1 RAD9* and *cdc13-1 rad9*Δ cells (the temperature was set at 27.3°C but fluctuated between approximately 26.5 and 28°C). Therefore, by measuring growth of *cdc13-1* mutants containing a library of *RAD9* mutations at 27.3°C, we measure the ability of each *RAD9* allele to activate a checkpoint-dependent cell-cycle arrest.

The 'rapid death' assay measures the ability of *RAD9* alleles to maintain viability of *cdc13-1* mutants incubated at 36°C for 4 hours. *cdc13-1 RAD9* cells maintain good viability when incubated at 36°C for 4 hours (Fig. 1B, rows 1,2). By contrast, *cdc13-1 rad9*Δ mutants rapidly lose viability at 36°C, presumably as a result of the high levels of ssDNA induced under these conditions (Fig. 1B, rows 3,4). Therefore, by measuring viability of *cdc13-1* mutants containing a library of *RAD9* alleles after short incubations at 36°C, we presumably measure the ability of each *RAD9* allele to inhibit nucleases at telomeres. Rapid loss of viability is not a result of failing to arrest because *cdc13-1 rad24*Δ cells are checkpoint defective but maintain viability as well as *cdc13-1 RAD*$^+$ mutants (Lydall and Weinert, 1995).

## Identification of potential separation of function alleles

To identify mutations in *RAD9* that were specifically defective in checkpoint signalling or nuclease inhibition, we generated a library of >23,000 *rad9* alleles by linker scanning mutagenesis. This library was transformed into a *cdc13-1 rad9*Δ strain and ~3,600 transformants were individually tested in the

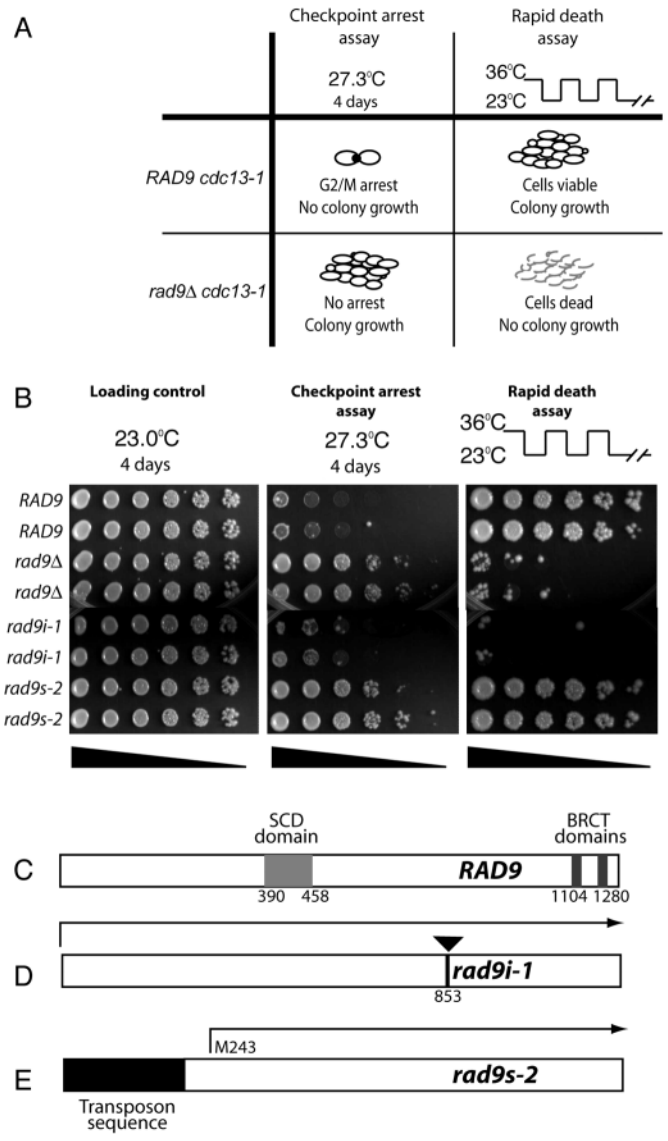



**Fig. 1.** Genetic separation of function screen. (A) The 'checkpoint arrest' assay measures colony growth at the semi-permissive temperature of 27.3°C. In this assay, *RAD9 cdc13-1* cells do not form colonies because they enter a G2/M arrest. By contrast, *rad9*Δ *cdc13-1* cells cannot arrest and do form colonies. The rapid death assay measures colony growth after three periods of growth at the restrictive temperature of 36°C for 4 hours and finally a return to growth at 23°C. In this assay *RAD9 cdc13-1* cells retain viability and form colonies, whereas *rad9*Δ *cdc13-1* cells lose viability in this assay. In practice, to distinguish better between the growth of *RAD9*$^+$ and *rad9*Δ cells, plates were subjected to three 4-hour periods at the restrictive temperature of 36°C, separated by 4-hour periods of recovery at the permissive temperature of 23°C. Colonies were then allowed to form at 23°C. (B) Novel alleles *rad9i-1* and *rad9s-2* have opposite phenotypes in the two assays. pRS416 plasmids containing the *RAD9* (lanes 1,2), *rad9i-1* (lanes 5,6) and *rad9s-2* (lanes 7,8) alleles were transformed into a *rad9*Δ *cdc13-1* strain. An empty vector was transformed into lanes 3 and 4. A 1:5 dilution series was prepared and spotted onto SC-uracil (left two) or YEPD (right) plates, which were incubated as shown. (C) Wild-type *RAD9* is 1309 amino acids, contains an [S/T]Q cluster domain (SCD) and two BRCT domains. (D) The *rad9i-1* allele contains an in-frame 5 amino acid insertion of GMFKH after C853. (E) The *rad9s-2* allele contains a transposon insertion into the 5′ half of the *RAD9* gene. The first 711 bp of the *RAD9* coding sequence have been removed and replaced by 335 bp of transposon sequence (shown in black). The longest open reading frame remaining encodes an N-terminal truncation of Rad9 using the ATG at methionine M243 as a start codon.

checkpoint arrest and rapid death assays (Fig. 1A,B). Two classes of allele with phenotypes different to that of *RAD9* and *rad9*Δ alleles were chosen for further analysis (Fig. 1B). One class permitted no colony growth in either the checkpoint arrest or rapid death assays (Fig. 1B, lanes 5,6). These alleles might encode Rad9 proteins that are proficient in checkpoint signalling but defective in nuclease inhibition and therefore we termed them *rad9i* (inhibition defective). We identified three weak and one strong *rad9i* alleles that we named *rad9i-1* (Fig. 1B, lanes 5,6). A second class of allele permitted colony growth in both the checkpoint arrest and rapid death assays (Fig. 1B, lanes 7,8). These alleles might encode Rad9 proteins that are defective in checkpoint signalling but proficient in nuclease inhibition, and therefore we termed them *rad9s* (signalling defective). Approximately 10 *rad9s* alleles with differing strength phenotypes were identified.

Sequence analysis showed that the *rad9i-1* allele encodes a full-length Rad9 with a 5 amino acid insertion after cysteine 853 (Fig. 1D). However, subsequent analysis of the *rad9i-1* allele has determined that it encodes a temperature-sensitive allele of *RAD9* that appears to be defective at both cell-cycle arrest and nuclease inhibition functions at 36°C (data not shown).

The *rad9s-1* allele contained a 1.7 kb transposon insertion 711 bp into the *RAD9* coding sequence (data not shown). The *rad9s-2* allele shown is a derivative of *rad9s-1* with the first 711 bp of *RAD9* coding sequence and the majority of the transposon sequence removed (Fig. 1E). Sequence analysis demonstrated that no fusion proteins could be generated between the transposon and *RAD9* sequences (data not shown). This suggested that the transposon sequences were activating expression of an N-terminal truncation of *RAD9*. The longest open reading frame (ORF) that remains in *rad9s-2* uses the ATG encoding M243 as an alternative start codon.

## N-terminal truncations of Rad9 have a checkpoint signalling defect

The phenotypes of the *rad9s-1* and *rad9s-2* alleles suggested that N-terminal truncations of Rad9 resulted in a loss of checkpoint signalling activity but retention of nuclease inhibition activity. To confirm this, we constructed a series of de novo N-terminal truncations of Rad9 on single copy plasmids. Three constructs that used the ATG codons encoding methionines M232, M243 and M358 of wild-type Rad9 as alternative start codons were generated. All three truncated Rad9 proteins were the predicted sizes by western blotting and had a clear *rad9s* phenotype (data not shown). This shows that the first 358 amino acids of Rad9 are not required for preventing rapid death of *cdc13-1* mutants but contribute to signalling cell-cycle arrest in these mutants.

## Deletion analysis of *RAD9*

To determine further which N-terminal amino acids of Rad9 are important for signalling cell-cycle arrest, we performed a deletion mapping analysis to identify the smallest deletion of *RAD9* that retained the checkpoint-defective *rad9s* phenotype (Fig. 2A). Deletion of amino acids 1-39, or 201-231, did not affect Rad9 function in either the checkpoint arrest or the rapid death assays (Fig. 2A). However, deletion of amino acids in the 40-200 region resulted in weak *rad9s* phenotypes

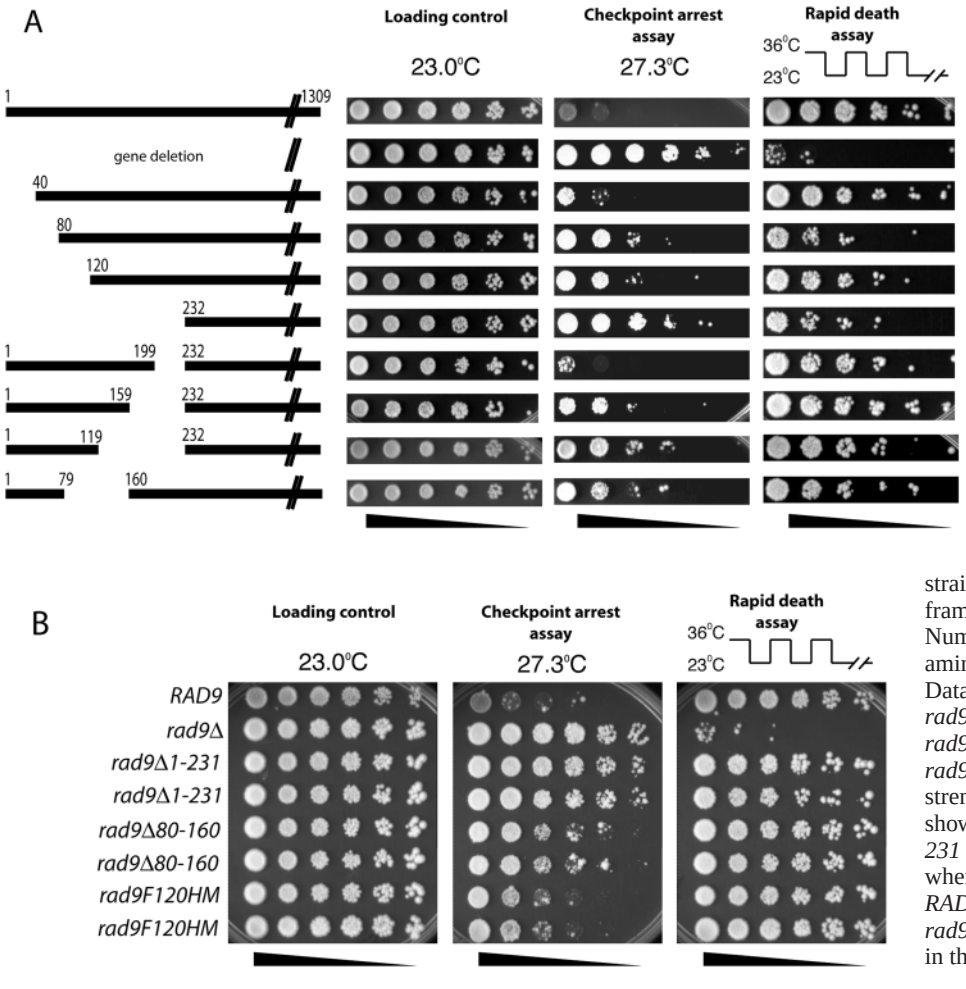



**Fig. 2.** Deletion analysis of the Rad9 N-terminus. (A) Phenotypes of truncation and internal deletion *RAD9* alleles in two growth assays. A series of N-terminal truncations and internal deletions on single copy plasmids transformed into a *rad9*Δ *cdc13-1* strain. The gaps in the black bars represent in-frame deletions of the coding sequence. Numbers over the black bars correspond to the amino acids that remain flanking the deletions. Data is not shown for constructs *rad9*Δ1-159, *rad9*Δ1-199, *rad9*Δ1-239, *rad9*Δ40-159, *rad9*Δ80-119, *rad9*Δ80-159, *rad9*Δ80-231 or *rad9*Δ120-159, all of which had intermediate-strength *rad9s* phenotypes similar to those shown in rows 4-6 and 8-10. (B) The *rad9*Δ*1-231* allele has the strongest *rad9s* phenotype when integrated; de novo deletion alleles of *RAD9* were integrated at the *URA3* locus of a *rad9::LEU2 cdc13-1 cdc15-2* strain and tested in the two growth assays.

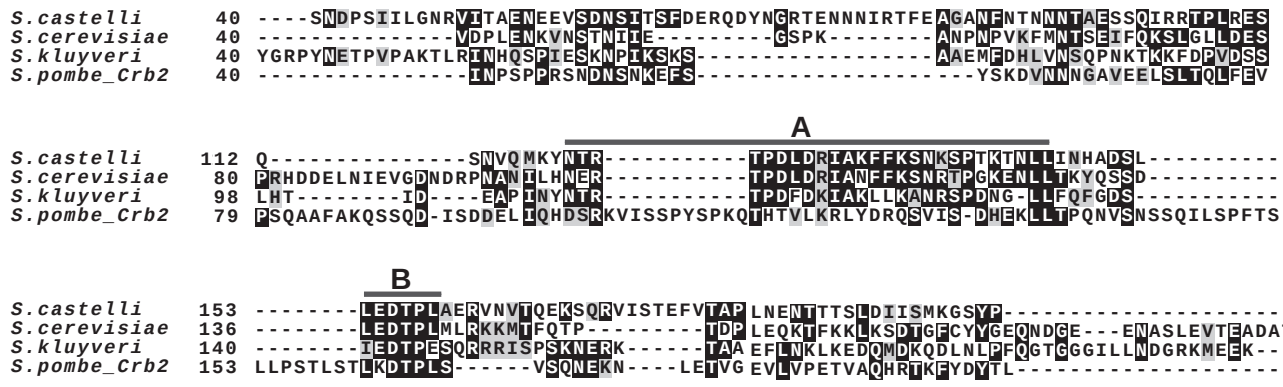



**Fig. 3.** N-terminal domains of Rad9 are comparatively well conserved in other yeast species. Rad9 orthologues in two divergent budding yeasts (*Saccharomyces castelli* and *Saccharomyces kluyveri*) were obtained from the *Saccharomyces* Genome Database. Amino acids 40-200 of the three Rad9 orthologues were aligned with amino acids 40-200 from *S. pombe* Crb2 using the ClustalW algorithm. Perfectly conserved residues are highlighted in black, structurally similar residues are highlighted in grey.

(Fig. 2A). Other small deletions within the 40-200 region also resulted in weak *rad9s* phenotypes, leading us to conclude that a comparatively large region of the first 200 amino acids of Rad9 was important for signalling cell-cycle arrest. We did not measure expression levels of the various deletions so cannot exclude the possibility that some of the *rad9s* phenotypes are a result of altered protein abundance.

We had previously noted that our *rad9s* alleles had stronger phenotypes when integrated compared with when expressed from plasmids. We therefore integrated several novel *rad9* alleles at the *URA3* locus of a *cdc13-1* strain. The *rad9Δ1-231* allele had a consistently stronger *rad9s* (signalling-defective) phenotype than deletions of 80 amino acids or a small F120HM substitution (Fig. 2B), so the integrated *rad9Δ1-231* allele was used for further experiments.

To help determine which amino acids within the N-terminus of Rad9 are important for signalling cell-cycle arrest, we first examined an alignment between *S. cerevisiae* Rad9 and its putative orthologues in two divergent budding yeasts, *S. castelli* and *S. kluyveri* [sequences from the *Saccharomyces* Genome Database (SGD) website (Cliften et al., 2003)]. The primary amino acid sequence was not highly conserved between these three species but some patches of sequence showed high identity in the three proteins (Fig. 3, domains A and B). We then took amino acids 40-200 from the three *Saccharomyces* proteins and aligned them with Crb2, the *S. pombe* homologue. Although the overall level of homology was relatively poor, the alignment did reveal a $_{140}$LEDTPL$_{145}$ motif in *S. cerevisiae* Rad9 that is very well conserved in the two other budding yeasts and in *S. pombe* Crb2 (Fig. 3, domain B). However, when we repeated this alignment process with other ‘adaptor’ proteins, we did not find any significant alignment to Mrc1 (*S. cerevisiae*), xClaspin (*Xenopus*), BRCA1 (human) or p53BP1 (human) (data not shown).

### *rad9Δ1-231* mutants have a *chk1Δ*-like phenotype

The checkpoint signalling function of Rad9 in response to *cdc13-1*-induced damage is mediated through the downstream kinases Rad53 and Chk1, acting in parallel pathways (Gardner et al., 1999; Sanchez et al., 1999). We hypothesized that *rad9Δ1-231* mutants might be defective in one or both of these parallel signalling branches. To test this, we examined phenotypes that distinguish between *chk1* and *rad53* mutants.

To test whether *rad9Δ1-231* mutants were defective in *RAD53*- and/or *CHK1*-dependent signalling pathways, we first measured viability after ultraviolet (UV) irradiation. In budding yeast, *chk1Δ* mutants are not UV sensitive but *rad53* mutants are UV sensitive (Allen et al., 1994; Liu et al., 2000; Sanchez et al., 1996).

Fig. 4A shows that *rad9Δ1-231* cells are as UV resistant as wild-type and *chk1Δ* cells. By contrast, *rad53Δ* cells are UV sensitive, but not as sensitive as *rad9Δ* cells. In our strain background, we consistently did not observe any enhanced UV sensitivity of *rad53Δ chk1Δ* double mutants compared with *rad53Δ* single mutants (Fig. 4A).

The lack of UV sensitivity of *rad9Δ1-231* mutants suggested that the *RAD53* pathway was intact in these cells; however, this experiment did not provide any information on whether the *CHK1* pathway was also intact. To address this issue we combined the *rad9Δ1-231* allele with a *yku70Δ* mutation.

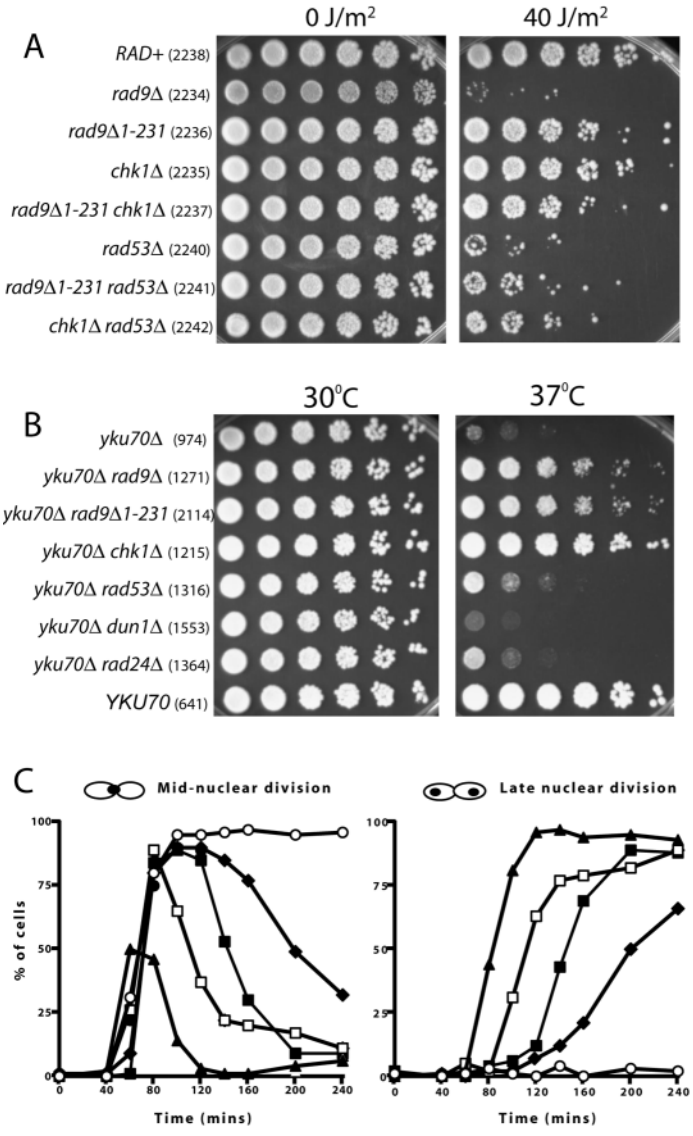
Ku is a conserved hetero-dimeric protein involved in non-homologous end-joining repair and also binds to telomeres in yeast and mammalian cells (Baumann and Cech, 2000; Haber, 1999; Polotnianka et al., 1998; Porter et al., 1996). Deletion of the yKu70 protein makes cells temperature sensitive. At 37°C, *yku70Δ* cells accumulate ssDNA at their telomeres and enter a G2/M arrest dependent on *RAD9* and *CHK1* but independent of *RAD53* and *DUN1* (Maringele and Lydall, 2002) (Fig. 4B). If *rad9Δ1-231* mutants were unable to activate the *CHK1*-dependent pathway to arrest cell division in *yku70Δ* mutants, they should, like *chk1Δ* mutants, permit growth of *yku70Δ* mutants at 37°C. Fig. 4B shows that *yku70Δ rad9Δ1-231* double mutants grew as well at 37°C as *yku70Δ rad9Δ* mutants, and grew similarly to *yku70Δ chk1Δ* mutants. By contrast, *yku70Δ rad53Δ* and *yku70Δ dun1Δ* strains grew poorly at 37°C. These data strongly suggested that the *CHK1* signalling pathway was abrogated in *rad9Δ1-231* mutants.

To gain further evidence that the *CHK1* signalling pathway was specifically defective in *rad9Δ1-231* mutants, we measured the cell-cycle arrest kinetics of *cdc13-1* mutants. At 36°C, *rad9Δ cdc13-1* cells have a complete checkpoint defect, whereas *dun1Δ cdc13-1*, *rad53Δ cdc13-1* or *chk1Δ cdc13-1* are only partially checkpoint defective (Gardner et al., 1999;

Sanchez et al., 1999; Schwartz et al., 2002). To measure the checkpoint defect of *rad9Δ1-231* strains, *bar1* and *cdc15-2* mutations were used to quantify the fraction of *cdc13-1* mutant cells that had failed to arrest during a single cell cycle (Lydall and Weinert, 1997).

When released from α-factor at 36°C, *rad9Δ cdc13-1 cdc15-2* cells begin to accumulate at telophase (the checkpoint-independent *cdc15-2* arrest point) within 80-100 minutes (Fig. 4C). By contrast, *chk1Δ cdc13-1 cdc15-2* cells reach telophase later, at 120-140 minutes, and *dun1Δ cdc13-1 cdc15-2* later still at 200-240 minutes (Fig. 4C). *rad9Δ1-231 cdc13-1 cdc15-2* strains are not as checkpoint defective as *rad9Δ* strains, but they reach late nuclear division with faster kinetics than *chk1Δ* mutants. Thus, the phenotype of *rad9Δ1-231* mutation is most similar to *chk1Δ* rather than *dun1Δ* mutations in causing partial checkpoint arrest in response to *cdc13-1*-induced damage.

In summary, analysis of the UV sensitivity, and cell-cycle arrest after *yku70Δ*- and *cdc13-1*-induced damage strongly suggests that *rad9Δ1-231* cells are specifically defective in the *CHK1-PDS1* branch of checkpoint control rather than the *RAD53-DUN1* pathway.




### MMS-induced Chk1 phosphorylation is defective in *rad9Δ1-231* cells

To gain biochemical support for the idea that *rad9Δ1-231* cells are specifically defective in activating the Chk1-dependent checkpoint signalling pathway, we examined phosphorylation of Rad9, Chk1 and Rad53 following genotoxic insult with the alkylating agent methylmethanesulphate (MMS). To avoid the complication that *RAD9*-independent phosphorylation of Rad53 is observed in asynchronous cultures (Pellicioli et al., 1999), we arrested the cultures in telophase using the *cdc15-2* conditional allele prior to MMS treatment. At the *cdc15-2* arrest point, MMS-induced phosphorylation of Rad53 and Chk1 is totally dependent on *RAD9* (Schwartz et al., 2002).

Following treatment with the DNA-alkylating agent MMS, the truncated Rad9Δ1-231 protein was phosphorylated to apparently the same extent as the wild-type Rad9 protein (Fig. 5). MMS-induced phosphorylation of Rad53 occurred in both *RAD9* and *rad9Δ1-231* cells but not in *rad9Δ* mutants (Fig. 5). We noted that the extent of the Rad53 phosphorylation is slightly greater in *RAD9* cells compared with *rad9Δ1-231* cells. By contrast, epitope-tagged Chk1 was clearly phosphorylated in *RAD9* cells but this phosphorylation is defective in *rad9Δ1-231* cells and *rad9Δ* cells (Fig. 5). Therefore, *rad9Δ1-231* cells are specifically defective at phosphorylating Chk1 but not Rad53 in response to MMS-induced DNA damage.

## Discussion

DNA damage responsive checkpoint pathways are exquisitely sensitive signal transduction cascades that can inhibit cell division in response to single double-strand breaks. As in many other signal transduction cascades, kinases lie at the heart of checkpoint pathways. Adaptor/mediator proteins, which facilitate interactions between upstream and downstream checkpoint kinases, are also essential for checkpoint signal transduction. In this paper, we have sought to understand the role of the budding yeast Rad9 adaptor protein in facilitating interactions between upstream and downstream checkpoint kinases.

Rad9 mediates interactions between the upstream kinase Mec1 and two parallel downstream kinases Rad53 and Chk1. The mechanism by which Rad9 facilitates interactions between

**Fig. 4.** *rad9Δ1-231* mutants have similar phenotypes to *chk1Δ* mutants. Strains of the indicated genotypes were grown in liquid culture before a fivefold dilution series was prepared and spotted to YEPD plates. Strain numbers are in parenthesis. (A) *rad9Δ1-231* mutants are not UV sensitive. Plates were untreated or exposed to 40 J/m$^2$ UV before incubation for three days at 30°C. (B) *yku70 rad9Δ1-231* cells are checkpoint arrest defective at restrictive temperatures. Growth was measured at the permissive temperature of 30°C and the restrictive temperature of 37°C. (C) Arrest kinetics of *cdc13-1* mutants in synchronous cultures. Checkpoint mutations were combined with the *cdc13-1* and *cdc15-2* mutations. Cultures were arrested with α-factor at 23°C then released into fresh media at 36°C. Accumulation of cells at mid-nuclear division (*cdc13-1* arrest point) and late nuclear division (*cdc15-2* arrest point) was measured by fluorescence microscopy at intervals of 20 minutes.

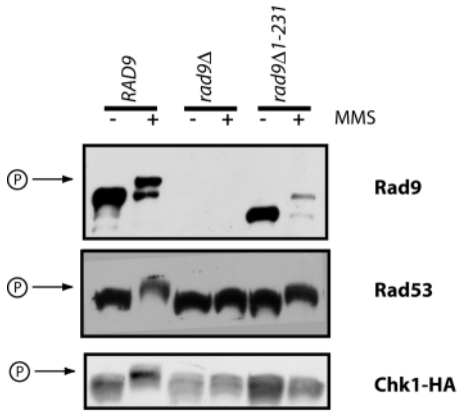



**Fig. 5.** MMS-induced Chk1 phosphorylation is abrogated in *rad9Δ1-231* mutants. Indicated strains containing the *cdc15-2* allele were transformed with a *CHK1*-HA plasmid and cultured in selective media. Cultures were arrested in telophase by incubation for 3 hours at 37°C before being mock treated or treated with 0.1% MMS (v/v) for 1 hour. Whole cell extracts were prepared and immunoblotted with α-Rad9, α-Rad53 or α-HA antibodies. The same phosphorylation patterns were observed with two separate extracts and Chk1 phosphorylation was detected with both HA- and Myc-tagged proteins.

the upstream checkpoint kinase Mec1 (orthologue of human ATR) and the downstream checkpoint kinase Rad53 (orthologue of human Chk2) is well understood (Gilbert et al., 2001; Schwartz et al., 2002). However, little was understood about how Rad9 mediates interactions between Mec1 and Chk1.

We show here that an N-terminal truncation of Rad9, missing the first 231 amino acids, has a signalling-defective (*rad9s*) phenotype. Our experiments demonstrate that amino acids 1-231 are required for efficient checkpoint signalling, but not for maintaining viability, in *cdc13-1* mutants at non-permissive temperatures. Further analysis has demonstrated that *rad9Δ1-231* mutants are specifically defective at activating the Chk1 pathway after DNA damage. In four different assays, *rad9Δ1-231* mutants behaved more like *chk1*-defective strains than *rad53*-defective strains. First, *rad9Δ1-231* and *chk1Δ* mutants are not UV sensitive, unlike *rad9Δ* and *rad53Δ* mutants (Fig. 4A). Second, *rad9Δ1-231* and *chk1Δ* mutants are defective at inducing arrest of *yku70Δ* mutants at 37°C, unlike *dun1Δ* and *rad53Δ* mutants (Fig. 4B). Third, *rad9Δ1-231* mutants have a partial checkpoint defect in response to *cdc13-1*-induced damage that is most like a *chk1Δ* rather than a *dun1Δ* phenotype (Fig. 4C). Fourth, *rad9Δ1-231* cells are defective at phosphorylating Chk1 after a genotoxic insult but are proficient at phosphorylating Rad53 (Fig. 5).

We mapped the Chk1 activation domain by deletion analysis. When expressed from plasmids, Rad9 proteins with amino acids 1-40 or 200-232 deleted had no phenotype, implying these residues are not required for the Chk1 activation domain. Various-sized deletions in the 40-200 amino acid region had partial *rad9s* phenotypes, but the magnitude of the signalling defect (measured by growth at 27.3°C) differed. The strongest *rad9s* phenotype was observed with the *rad9Δ1-231* N-terminal truncation allele, and the implication is that more than one residue or motif within the 40-200 amino acid region are required for Chk1 activation. This is supported by the fact that several motifs seem to be well conserved across three *Saccharomyces* species against a background of reasonably weak sequence identity (Fig. 3). The 40-200 amino acid region does not contain any Mec1 phosphorylation target sites but interestingly does contain three potential Cdk (cyclin-dependent kinase) phosphorylation sites (Toh and Lowndes, 2003). Rad9 phosphorylation in undamaged cells is cell-cycle regulated (Vialard et al., 1998); in addition, the activity of the fission yeast Crb2 protein is also regulated by Cdk phosphorylation (Caspari et al., 2002; Esashi and Yanagida, 1999). The question of whether Cdk (Cdc28)-dependent phosphorylation somehow regulates the Rad9-Chk1 checkpoint signalling pathway is therefore an interesting line of further enquiry. We note that there is a conserved potential phosphorylation site (DTP) in motif B of Rad9 and its orthologues (Fig. 3).

In *Xenopus*, the Claspin protein is believed to play an adaptor role similar to Rad9 and couples the activation of xATR to the activation of xChk1 in the S-phase checkpoint. Biochemical analysis has recently defined a minimal xChk1-binding domain in Claspin (Kumagai and Dunphy, 2003). This 57 amino acid domain contains two conserved repeated motifs, each of which contains a serine whose phosphorylation is required for the binding of xChk1. The kinase responsible for phosphorylating these SG motifs is not identified. These features are not conserved in the Chk1 activation domain we have genetically identified in Rad9.

In summary, the data presented here supports the view that Rad9 activates Rad53 and Chk1 by distinct and independent mechanisms (Schwartz et al., 2002). This is consistent with the fact that phosphopeptide-binding FHA domains in Rad53 mediate its interaction with Rad9 and that these domains are absent in Chk1.

The Chk1 activation domain (CAD) we have identified is distinct from two other previously described domains in the protein: BRCT domains, which facilitate Rad9 oligomerization following DNA damage (Soulier and Lowndes, 1999); and the SCD ([S/T]Q cluster domain), which is required for the interaction and activation of Rad53 (Schwartz et al., 2002). The CAD in Rad9 might facilitate a direct physical interaction between Rad9 and Chk1 but our attempts to demonstrate physical interaction have so far been unsuccessful. This suggests that any interactions between the Rad9 CAD and Chk1 may be transient. Irrespective of the nature of the interaction between Rad9 and Chk1, the CAD domain we have identified is clearly important for activating Chk1 in response to diverse types of DNA damage in budding yeast. Similar domains may be identified in adaptor proteins from other eukaryotes.

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