Delineating the position of *rad4+/cut5+* within the DNA-structure checkpoint pathways in *Schizosaccharomyces pombe*

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Summary

The fission yeast BRCT domain protein Rad4/Cut5 is required for genome integrity checkpoint responses and DNA replication. Here we address the position at which Rad4/Cut5 acts within the checkpoint response pathways. Rad4 is shown to act upstream of the effector kinases Chk1 and Cds1, as both Chk1 phosphorylation and Cds1 kinase activity require functional Rad4. Phosphorylation of Rad9, Rad26 and Hus1 in response to either DNA damage or inhibition of DNA replication are independent of Rad4/Cut5 checkpoint function. Further we show that a novel, epitope-tagged allele of *rad4+/cut5+* acts as a dominant suppressor of the checkpoint deficiencies of *rad3–*, *rad26–* and *rad17–* mutants. Suppression results in the restoration of mitotic arrest and is dependent upon the remaining checkpoint Rad proteins and the two effector kinases. High-level expression of the *rad4+/cut5+* allele in *rad17* mutant cells restores the nuclear localization of Rad9, but this does not fully account for the observed suppression. We conclude from these data that Rad4/Cut5 acts with Rad3, Rad26 and Rad17 to effect the checkpoint response, and a model for its function is discussed.

Supplemental figure available online

Key words: Rad4, Chk1, Checkpoint control, Genome integrity, DNA damage, DNA replication

Introduction

During cell growth and division the maintenance of genome integrity is aided by surveillance mechanisms that have been termed DNA structure checkpoint controls. These control mechanisms act to block cell-cycle progression in response to both DNA damage and the inhibition of DNA replication. The importance of DNA structure checkpoint mechanisms in normal growth control is best highlighted by the finding that mutations in certain mammalian checkpoint control genes (e.g. p53, ATM and CHK2) can lead to a predisposition to cancer and to other cellular pathologies (Hartwell and Kastan, 1994; Elledge, 1996; Carr, 2000). The G2/M or DNA damage checkpoint arrests cell-cycle progression at the G2/M transition in the presence of damaged DNA, whereas the DNA replication checkpoint prevents entry into mitosis in the presence of stalled DNA replication forks and is referred to as the S-M checkpoint. Checkpoint-mediated responses to DNA damage are, to a large extent, conserved. Studies in the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), in which these two major checkpoint pathways have been defined, have made a significant contribution to the understanding of the organization of the checkpoint pathways.

Genetic and physiological experiments have revealed a core set of six proteins, collectively termed the ‘checkpoint Rad’ proteins (Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1). These proteins are fundamental to both pathways and appear necessary for the receipt and transmission of the checkpoint signal (Al-Khodairy and Carr, 1992; Enoch et al., 1992; O’Connell et al., 2000). As yet, the precise mechanism(s) by which these proteins act to achieve this goal is unclear, although recent studies have indicated the presence of discrete intracellular complexes between Rad3 and Rad26, between Rad17 and the four small subunits of replication factor C (RFC) and between Rad9, Rad1 and Hus1 (Edwards et al., 1999; Shimada et al., 1999; Kostrub et al., 1998; Caspari et al., 2000a). The Rad3-Rad26 complex functions as a PI3-related protein kinase and the Rad1-dependent complex (known as the 9-1-1 complex) is related to the proliferating cell nuclear antigen (PCNA) sliding clamp. Rad17 associates with RFC subunits and, by analogy with the mechanism of eukaryotic DNA replication, may act to load the PCNA-like 9-1-1 complex onto chromatin (for a review, see O’Connell et al., 2000). The regulated assembly of specific protein complexes onto the DNA in response to different checkpoint cues may prove a general and important feature of checkpoint regulation. Homologs of Rad3-Rad26 and Rad9-Rad1-Hus1 have been

shown in *S. cerevisiae* and human systems to load independently at sites of DNA damage. Loading of the 9-1-1 complex is dependent on Rad17, consistent with the homology to replication factors RFC and PCNA (Kondo et al., 2001; Melo et al., 2001; Zou et al., 2002).

Downstream of the checkpoint Rad proteins lie two conserved effector kinases, Chk1 and Cds1, which act in the G2/M and S-M checkpoint pathways, respectively (Walworth et al., 1993; Murakami and Okayama, 1995). Chk1 is phosphorylated in response to DNA damage and recent work demonstrates a direct link between Rad3-dependent serine 345 phosphorylation and Chk1 function (Walworth and Bernards, 1996; Lopez-Girano et al., 2001; Capasso et al., 2002). Chk1 phosphorylation therefore serves as a useful biochemical marker for the activation of the DNA damage checkpoint pathway. Activation and phosphorylation of Cds1 is S-phase-specific in response to either inhibition of DNA replication or DNA damage, and kinase activation correlates with phosphorylation of the protein (Lindsay et al., 1998), providing a useful marker for the S-M checkpoint. Rad9 and Rad26 are also phosphorylated in response to radiation-induced DNA damage and Hus1 is phosphorylated in response to both DNA damage and prolonged inhibition of DNA replication (Kostrub et al., 1998; Edwards et al., 1999; Caspari et al., 2000a; Caspari et al., 2000b). Ultimately, all of these phosphorylation events have been demonstrated to be Rad3-dependent and this has enabled hierarchical relationships between fission yeast checkpoint proteins to be determined. The current model predicts that at least two complexes are loaded independently at the sites of DNA damage, Rad3-Rad26 and the 9-1-1 complex that is loaded in a Rad17-dependent manner. Rad3-Rad26 kinase complex appears to be at the head of a phosphorylation cascade immediately upstream of the PCNA-related 9-1-1 complex. This organization may be maintained for both the G2/M and S-M pathways. In the event of DNA damage occurring in S phase, the Rad17 and 9-1-1 complexes appear to be required for Rad3 phosphorylation of Rad26, suggesting that the loading or activation of Rad3 requires a function of the RFC- and PCNA-related proteins. Thus under these circumstances they may formally act above the Rad3 kinase (Edwards et al., 1999; O'Connell et al., 2000).

In addition to the proteins discussed above, Crb2/Rhp9 and Rad4/Cut5 (two BRCT-domain-containing proteins) and Mrc1 (Mediator of the Replication Checkpoint) are also required for the checkpoint responses (Willson et al., 1997; Saka et al., 1997; Alcasabas et al., 2001). Cells deleted for the *crb2*$^{+}$ gene fail to arrest the cell cycle in response to UV and ionizing radiation-mediated DNA damage but are functional for the replication checkpoint. Crb2 has been shown to interact with Chk1 and the second BRCT-domain protein Rad4/Cut5, hereafter termed Rad4, in response to the DNA damage (Saka et al., 1997). Phenotypic analysis of conditional mutations of *rad4* reveal that, at the restrictive temperature, these mutants fail to complete DNA replication yet continue to divide (Saka and Yanagida, 1993). This results in cells undergoing a lethal mitosis and the accumulation of 'cut' cells (cell untimely torn) in which the nucleus is bisected by the septum. Hence, *rad4*$^{+}$ defines a gene that as well as being essential for DNA replication appears to play a positive role in the S-M checkpoint. Subsequent work established that, under conditions permissive for DNA replication, *rad4* mutants lack checkpoint responses to both DNA damage and to the inhibition of DNA replication (Saka et al., 1994; McFarlane et al., 1997). This formally established independent functional requirements for Rad4 during DNA replication and for DNA structure checkpoint control. Despite this, the position of Rad4 within the checkpoint control pathways remains to be determined. Work in budding yeast has demonstrated that the Rad4 homologue, Dpb11, is required for activation of the Rad53 checkpoint kinase following engagement of the S-phase checkpoint (Wang and Elledge, 1999). More recently, Dpb11 has been shown to interact with Ddc1, *S. pombe rad9* homologue, a result that implies a role for Dpb11 in the DNA damage checkpoint as well as the S-phase checkpoint (Wang and Elledge, 2002).

In this study we present a combined genetic and biochemical approach to delineating the position of Rad4 within the checkpoint pathways. We demonstrate that the phosphorylation of Chk1 in the presence of DNA damage is Rad4-dependent. Similarly, Cds1 kinase activity requires functional Rad4. In contrast, phosphorylation of Rad9 and Rad26 in response to DNA damage and of Hus1 in the presence of either DNA damage or a DNA replication inhibitor are independent of Rad4 activity. During our investigations we found that a novel allele of *Rad4* acts as a dominant suppressor of the checkpoint deficiencies of certain *rad3*$^{-}$, *rad26*$^{-}$ and *rad17*$^{-}$ mutants. Suppression resulted in the restoration of mitotic arrest and was dependent upon the remaining checkpoint Rad proteins and the two effector kinases. We conclude from our data that Rad4 acts with Rad3, Rad26 and Rad17 to effect the checkpoint response, and suggest that Rad4 defines a requirement for a bifurcated checkpoint response to converge on the regulatory checkpoint kinases Cds1 and Chk1 in either the S-M or G2/M checkpoint pathways, respectively.

Materials and Methods

Strains and media

All strains used in this study are listed in Table 1 and were constructed by standard genetic techniques (Moreno et al., 1991). Briefly, strain SpSc382, representing *rad4-116* cells carrying HA-tagged Chk1 was generated by crossing SpSc372 with SpSc122. Strains carrying Rad9 (SpSc412) and Hus1 (SpSc411) genes C-terminally tagged with, respectively, three tandem copies of the haemagglutinin (HA) epitope recognized by the 12CA5 monoclonal antibody (MAb) (11) or 13 copies of the c-myc (myc) epitope recognized by the 9E10 MAb (Evan et al., 1985) were generated using a PCR-based approach described previously (Bahler et al., 1998). Each of these strains was then crossed with SpSc122 to generate *rad4-116*-tagged strains (SpSc384, SpSc385). Cells were routinely cultured in YES medium or EMM medium lacking thiamine, solid media contained 2% agar (Moreno et al., 1991). YEP medium (0.5% yeast extract, 2% peptone, 3% glucose, supplemented with 100 mg each of adenine, leucine, histidine and uracil) was used in all lactose-gradient synchronization experiments.

Plasmid construction

A full-length *rad4*$^{+}$ cDNA flanked by a Nde1 restriction endonuclease site at the ATG and a 3′ *Bam*HI was isolated by PCR from a *S. pombe* cDNA library (gift of C. Norbury) and inserted into pREP41HAN (Craven et al., 1998). The sequence of the cDNA was confirmed and the plasmid was tested for complementation of the conditional growth

Table 1. Strains used in this study

Strain	Genotype
SpSc 29	*rad17-w*, *leu1-32*, *ade6-704 h*⁻
SpSc 55	*ura4-D18*, *leu1-32*, *ade6-M210*, *h*⁻
SpSc 57	*ura4-D18*, *leu1-32*, *ade6-M210*, *h*⁺
SpSc 29	*rad17-w*, *leu1-32*, *ade6-704 h*⁻
SpSc 72	*hus1-4, leu1-32 h*⁻
SpSc 103	*rad3-56*, *ura4-D18*, *leu1-32*, *ade6-M210 h*⁻
SpSc 122	*rad4-116*, *ura4-D18*, *leu1-32*, *ade6-M210* h⁺
SpSc 143	*rad1-1, ura4-D18, leu1-32*
SpSc 169	*rad9*::*ura4*⁺, *ura4-D18, leu1-32, ade6-704 h*⁻
SpSc 171	*rad26*::*ura4*⁺, *ura4-D18, leu1-32, ade6-M210, h*⁻
SpSc 176	*chk1*::*ura4*⁺, *ura4-D18, leu1-32, ade6-704*
SpSc 186	*rad1*::*ura4*⁺, *ura4-D18, leu1-32, his3*⁻
SpSc 372	*chk1::3HA*, *ura4-D18*, *leu1-32*, *ade6-704*
SpSc 382	*rad4-116*, *chk1-3HA, ura4-D18*, *leu1-32*, *ade6-704*
SpSc 384	*rad4-116*, *hus1::13myc*, *ura4-D18*, *leu1-32*, *ade6-704*
SpSc 385	*rad4-116*, *rad9::3HA, ura4-D18*, *leu1-32*, *ade6-704*
SpSc 411	*hus1::13myc*, *ura4-D18*, *leu1-32*, *ade6-704*
SpSc 412	*rad9::3HA*, *leu1-32*, *ade6-704*
SpSc 419	*rhp9*::*ura4*⁺, *ura4-D18, leu1-32, ade6-704*
SpSc 420	*rad3*::*ura4*⁺, *chk1-3HA, ura4-D18, leu1-32, ade6-704*
SpSc 434	*rad17-w, rad26::ura4, leu1-32, ade6-M210*
SpSc 456	*rad3-KD*, *ura4-D18*, *leu1-32*, *ade6-704*
SpSc 457	*rad9-192, ura4-D18, leu1-32*
SpSc 459	*rad17*::*ura4*⁺, *ura4-D18*, *leu1-32*, *ade6-704* h⁺
SpSc 463	*cds1*::*ura4*⁺, *ura4-D18, leu1-32,*
SpSc 464	*crb2-1, leu1-32, ura4-D18, ade6-M210*
SpSc 465	*rad3-56, rad1*::*ura4*⁺, *ura4-D18, leu1-32*
SpSc 466	*rad3-56, rad9*::*ura4*⁺, *ura4-D18, leu1-32, ade6-704*
SpSc 467	*rad3-56, rad17-w, leu1-32, ade6-704*
SpSc 468	*rad3-56, rad26*::*ura4*⁺, *ura4-D18, leu1-32, ade6-M210*
SpSc 469	*rad3-56, hus1-4, leu1-32*
SpSc 470	*rad3-56, chk1*::*ura4*⁺, *ura4-D18, leu1-32, ade6-704*
SpSc 471	*rad3-56, cds1*::*ura4*⁺, *ura4-D18, leu1-32, ade6-704*
SpSc 473	*rad3-56, rhp9*::*ura4*⁺, *ura4-D18, leu1-32, ade6-704*
SpSc 529	*rad9::13myc ura4-D18, leu1-32, ade6-704*
SpSc 530	*rad9::13myc rad17w ura4-D18, leu1-32, ade6-704*

and checkpoint deficiencies of the *rad4-116* allele. The *rad4*⁺ cDNA was also inserted into pREP41 (Basi et al., 1993) and tested for its ability to complement *rad4-116*.

Biochemical checkpoint cell culture experiments

One-hundred ml cultures were grown at 26°C overnight to OD 0.5-1.0. For each required sample, a volume of culture representing 5 OD units was harvested for 5 minutes (1650 ***g***) and resuspended in preheated YES medium. Cells were preheated at the temperatures indicated for 90 minutes and then grown in the presence or absence of either 20 μg/ml bleomycin for 3 hours or 10 mM hydroxyurea (HU) for 3 hours (5 hours for Hus1 experiments). In the case of the Chk1 dose dependency experiment, ionizing radiation was delivered by a Gammacell 1000 ^{37}Caesium source at 12 Gy/min. For the UV experiments, after preincubation, cells were centrifuged at 1650 ***g*** for 5 minutes, resuspended in 150 μl of YES and plated as a lawn of cells on YES plates. The plates were then irradiated (or mock irradiated) with 150 J/m^2 UV in a Spectrolinker™ XL-1000 UV crosslinker (Spectronics Corporation) and the cells collected and recovered in 5 ml of fresh YES at the temperatures indicated for 30 minutes. Cell extracts were prepared as described.

Genetic suppression checkpoint analyses

Typically, 30-40 ml cultures were grown at 26°C to mid-log phase in YES (Figs 1 and 2) or EMM – thiamine (Figs 3-5). Cultures were then split and to one half, 10 mM HU or 20 μg/ml bleomycin was added. One-hundred μl samples were then removed every 2 hours over an 8-hour period and a dilution series plated out onto YES. Plates were incubated at 26°C for 3-4 days, the number of colonies formed counted and the viability of each culture at each time point determined. Samples were also fixed for nuclear staining as described. In the case of the UV irradiation dose response experiments, the cell densities of mid-log phase cultures were determined and a dilution series set up to give a final density of 5×10^3 cells cm^{-2}. Ten μl of each dilution (500 cells) were then spotted onto a series of YES plates and exposed to 0, 25, 50, 75, 100 and 150 J/m^2 UV irradiation as indicated.

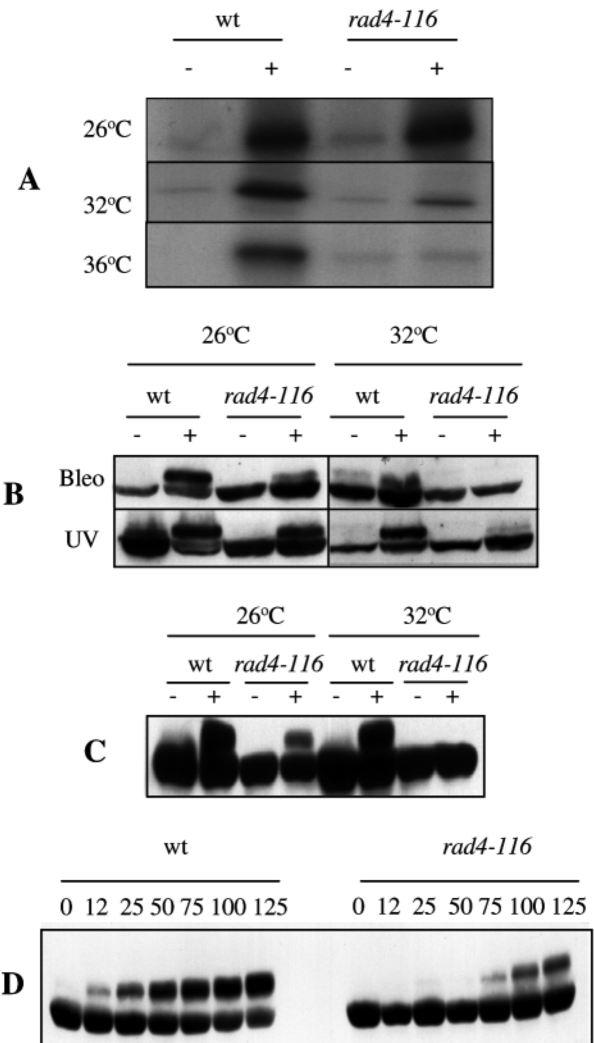

Fig. 1. Rad4 is required for activation of Cds1 and Chk1 kinases. (A) Wild-type and *rad4-116* cells were synchronized in G2, released into pre-warmed media and grown in the absence (–) or presence (+) of 10 mM HU at the temperatures indicated. Whole cell extracts from S-phase cells were prepared and assayed for Cds1p kinase activity. The data show that Cds1p kinase activity is severely reduced or absent in *rad4-116* cells at 32°C and 36°C, respectively, indicating that Rad4p is required for the activation of Cds1p kinase. (B) Wild-type and *rad4-116* cells carrying an integrated HA-*chk1*⁺ allele were grown overnight to mid-log and then released into pre-warmed media and grown in the absence (–) or presence (+) of 20 μg/ml bleomycin sulphate or irradiated with 150 J/m^2 UV. Cells were also exposed to either a fixed dose of γ-irradiation 125 Gy (C) or doses in the range 0-125 Gy (D) at the temperatures indicated. Total protein was prepared from all cells and Chk1 phosphorylation analysed by immunoblot using anti-HA antibody (B-D).

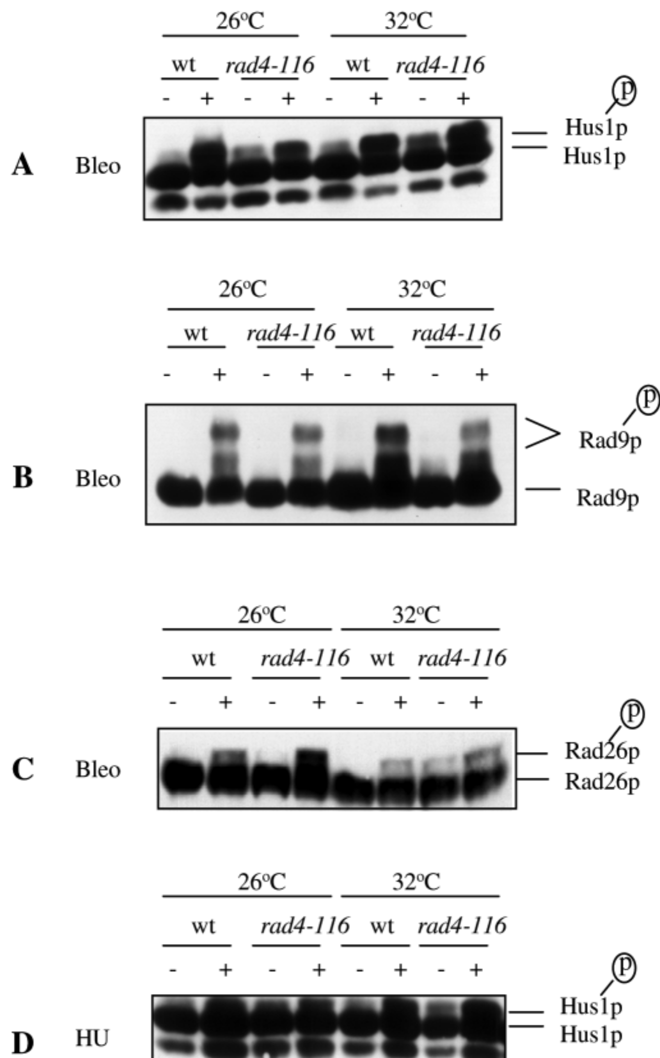

Fig. 2. Phosphorylation of Rad9, Hus1 and Rad26 in response to DNA damage and/or inhibition of DNA replication is independent of Rad4. Wild-type and *rad4-116* cells carrying integrated, epitope-tagged alleles of *hus1*, *rad9* or *rad26* were used in these experiments. Cells were grown overnight to mid-log, harvested and inoculated into fresh pre-warmed media in the absence (–) or presence (+) of 20 μg/ml bleomycin sulphate; (A) Hus1-myc, (B) Rad9-HA, (C) Rad26-myc. (D) Exponentially growing Hus1-myc cells were treated with 10 mM HU for 5 hours. Total protein was prepared from all cells and phosphorylation analysed by immunoblot using either anti-HA or anti-myc antibodies.

Plates were incubated for 3-4 days at 26°C until colonies formed and the relative viability of each strain at varying doses was determined. Where necessary, cells were synchronized in G2 following lactose gradient centrifugation (Mitchison and Carter, 1975).

Cds1 kinase assays

One l cultures were grown overnight at 26°C to mid-log phase. G2 cells were isolated by lactose gradient centrifugation (Mitchison and Carter, 1975) and released into pre-warmed YEP medium in the presence or absence of 10 mM HU at the temperatures indicated. Samples were removed every 20 minutes and stained with 4,6 diaminidino-2-phenylindole (DAPI) and Calcofluor white to monitor progression into S-phase. S-phase whole cell extracts were prepared and Cds1p kinase activity assayed as described previously (Lindsay et al., 1998).

Preparation of protein extracts

Initially, soluble whole cell extracts were prepared using lysis buffer [50 mM Tris (7.5), 80 mM β-glycerophosphate, 250 mM NaCl, 15 mM nitophenylphosphate, 50 mM NaF, 5 mM EDTA, 1 mM DTT, 1% tergitol-NP40] supplemented with protease inhibitors (40 μg/ml pepstatin A, 40 μg/ml aprotinin, 20 μg/ml leupeptin, 200 μg/ml PMSF) as described previously (Lindsay et al., 1998). However, it became necessary to prepare total cell extracts using tri-chloroacetic acid (TCA) precipitation (see Caspari et al., 2000a), as we found that the checkpoint proteins invariably associated with the insoluble fraction.

Polyacrylamide gel electrophoresis and western blotting

In the case of initial Chk1 Westerns, soluble protein extracts were boiled for 3 minutes in SDS sample buffer and then 60 μg of protein loaded onto small (BioRad) 8% SDS-polyacrylamide gels. For all subsequent checkpoint Westerns, TCA extracts were boiled for 5 minutes and then 50 μl volumes loaded onto large 8% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose (Hybond-C, Amersham), via wet blotting. The membranes were blocked in 1xPBS, 0.1% Tween 20, 5% dried milk for 1 hour (or overnight), incubated in PBS/Tween containing the appropriate primary antibody [Monoclonal HA.11 (Babco) used at 1:2000; monoclonal 9E10 (Babco), used at 1:2000], washed in PBS/Tween (3×10 minutes) and incubated with horse radish peroxidase-conjugated secondary antibody [Goat Anti-Mouse (DAKO), used at 1:5000]. Detection was by chemiluminescence using the Amersham ECL kit. All antibody incubations were for 1 hour.

Microscopy and immunofluorescence

Nuclear staining was performed as described in Moreno et al. (Moreno et al., 1991), using mounting medium comprising 1 μg/ml DAPI, 1 mg/ml p-Phenylenediamine/Antifade and 50% glycerol. Immunofluorescence experiments examining Rad9-myc localization were performed using a standard protocol (Hagan and Hyams, 1988). Briefly, 50 ml cultures were grown overnight to mid-exponential phase (OD 0.5-1.0). Ten OD units of cell were harvested by centrifugation at 1650 ***g*** and resuspended in 40 ml of YES to which 5.5 ml of fresh 30% paraformaldehyde were added. After incubation at 26°C for 10 minutes, cells were then washed twice with PBS, once with PEM (100 mM PIPES, 1 mM EGTA and 1 mM $MgSO_4$, pH 6.9) and then once with PEMS (1.2 M sorbitol in PEM). Following this, the cells were resuspended in PEMS containing Zymolyase 20000 (ICN) at 1.25 mg/ml and then incubated at 37°C for 70 minutes to aid cell-wall digestion. After harvesting (1650 ***g***), cells were washed once with PEMS and once with 1% Triton-X100 in PEMS, and then twice with PEM. The cells were then blocked with PEMBAL (1% BSA, 0.1% sodium azide and 100 mM L-lysine in PEM, pH 6.8) by incubating on a rotating wheel for at least 1 hour at room temperature. The cells were then incubated in primary antibody [monoclonal 9E10 (Babco), used at 1:200] overnight at room temperature on a rotating wheel. The next day, cells were washed three times with PEMBAL before further incubation for 20 minutes on a rotating wheel at room temperature. The cells were then incubated with the FITC-conjugated secondary antibody [Goat Anti-Mouse, (Sigma), used at 1:100] for at least 6 hours in the dark. The stained samples were then washed twice with PEM and once with PBS before further incubation with PBS on the rotating wheel for 10 minutes. The cells were washed with PBS followed by PBS containing 100 mM sodium azide prior to microscopic examination.

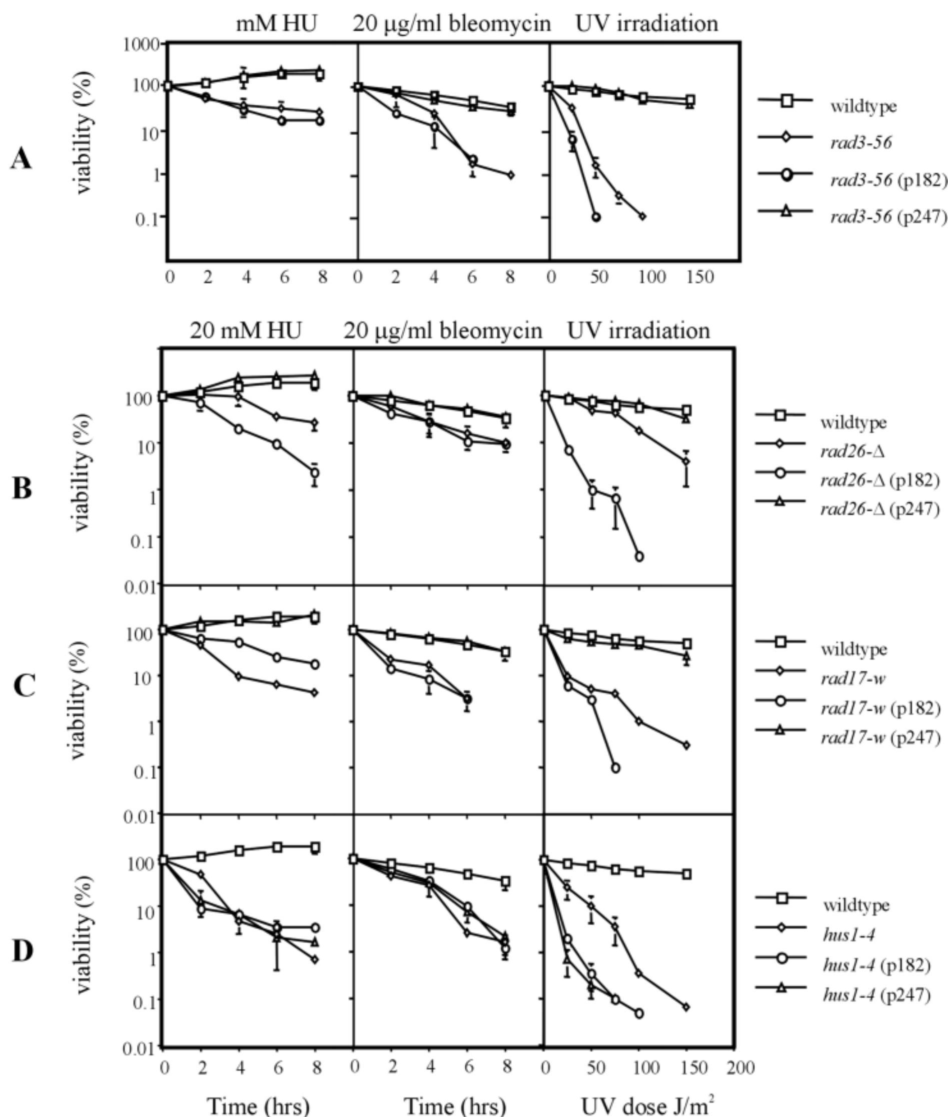

Fig. 3. A dominant allele of *rad4*$^+$ suppresses the DNA damage and HU-sensitive phenotypes of checkpoint mutants. (A). Survival rates are shown for wild-type and *rad3-56* cells as well as *rad3-56* cells carrying either pREP41-HA (p182) or pREP41-HA-*rad4*$^+$ (p247) following exposure to 10 mM HU, 20 μg/ml bleomycin sulphate and UV irradiation (0-150 J/m^2). The presence of multi-copy HA-*rad4*$^+$ suppresses the sensitivity of *rad3-56* cells to all three agents, restoring viability to wild-type levels. (B-D) Equivalent experiments for *rad26Δ* (B), *rad17-w* (C) and *hus1-4* (C). Wild-type and the respective mutant strains carrying either pREP41-HA (p182) or pREP41-HA-*rad4*$^+$ (p247) were assayed for survival following exposure to 20 μg/ml bleomycin sulphate, 10 mM HU or UV radiation (0-150 J/m^2). Comparison of B and C with D shows that the presence of the HA-Rad4-expressing plasmid suppresses sensitivity to all three agents in *rad26Δ* and *rad17-w* but not *hus1-4* cells when compared with control cells carrying the vector alone.

Results

Rad4 lies upstream of Cds1 in the DNA replication checkpoint

Previous physiological studies have implicated Rad4 in S-M checkpoint function (Saka and Yanagida, 1993; McFarlane et al., 1997). The S-M checkpoint requires Cds1 kinase activity to be activated in S phase in response to the inhibition of DNA replication by the checkpoint proteins (Lindsay et al., 1998). We therefore assayed Cds1 kinase activity in synchronized wild-type and *rad4-116* S-phase cells following growth in the presence of the DNA replication inhibitor HU. As expected, Cds1 kinase activity was seen in both wild-type and *rad4-116* cells at the permissive temperature of 26°C when cells were blocked in S phase with HU, but not when cells were allowed to proceed through the cycle unchallenged (Fig. 1A). In contrast, Cds1 kinase activity was either dramatically reduced or completely absent in the *rad4-116* cells exposed to HU at the semi-permissive temperature (32°C) and the restrictive temperature (36°C) (Fig. 1A). At 32°C, the Rad4p checkpoint function is abrogated but the replication function remains intact. At 36°C both the checkpoint and DNA replication functions are lost. These data confirm that S phase-specific Cds1 kinase activity is dependent upon Rad4 checkpoint function in response to the inhibition of DNA replication.

Rad4 lies upstream of Chk1 in the DNA damage checkpoint

Chk1 phosphorylation in response to DNA damage is dependent upon the checkpoint Rad proteins, correlates with G2/M checkpoint activation and is limited to the G2 phase of the cell cycle (Walworth and Bernards, 1996; Martinho et al., 1998). In order to establish the requirement for Rad4 in this response, Chk1 modification was monitored in *rad4-116* cells following exposure to DNA-damaging agents. Asynchronous cultures of wild-type and *rad4-116* cells were treated with either UV irradiation or bleomycin, a widely used γ-irradiation mimetic. In both strains Chk1 was phosphorylated at 26°C, but phosphorylation was absent (bleomycin) or severely reduced (UV-irradiation) in *rad4-116* cells at 32°C (Fig. 1B). During the course of these experiments we noted that the level of Chk1 phosphorylation in the presence of bleomycin was consistently

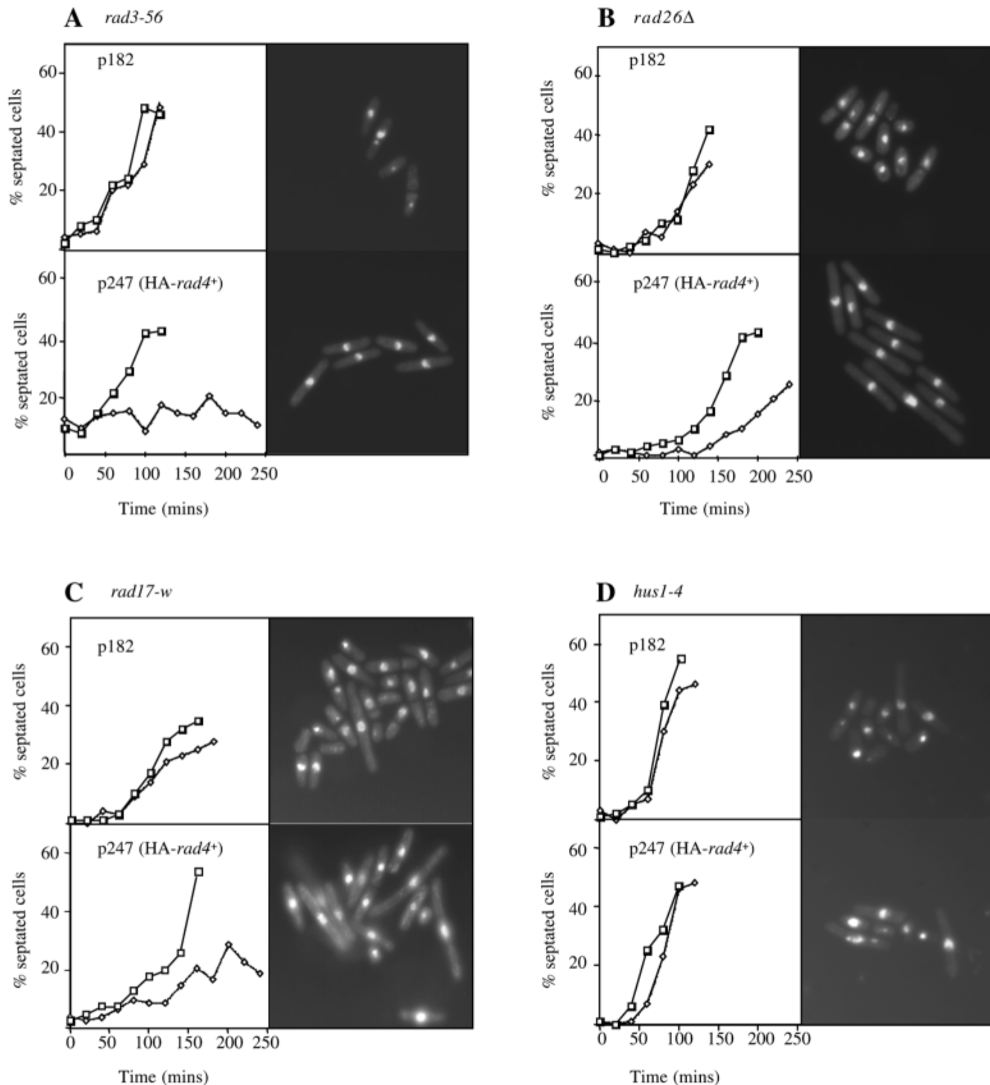

Fig. 4. Multi-copy HA-*rad4*+-mediated suppression of *rad3-56*, *rad26Δ* and *rad17-w* correlates with restoration of a checkpoint. Checkpoint-deficient strains *rad3-56* (A), *rad26Δ* (B), *rad17-w* (C) and *hus1-4* (D) carrying either pREP41-HA (p182) or pREP41-HA-*rad4*+ (p247) were synchronized in G2, incubated in the absence (□) or presence (✧) of 20 μg/ml bleomycin and the septation index scored at 20-minute intervals to quantify the number of cells passing through mitosis. A mitotic delay can be seen in *rad3-56*, *rad26Δ* and *rad17-w* cells expressing multi-copy HA-*rad4*+ (p247) following exposure to bleomycin, whereas *hus1-4* cells undergo mitosis with similar kinetics regardless of the presence or absence of multi-copy HA-*rad4*+ and in the presence or absence of damage. Microscopic analysis of the same cells (stained with DAPI) shows that all mutants exhibit cut cells in the presence of damage (top-right panel), whereas those mutants in which suppression was observed exhibit an elongated cell phenotype (lower-right panel) indicative of a checkpoint delay.

lower in *rad4-116* mutants relative to wild-type cells, even at 26°C. In order to confirm this, we tested Chk1 phosphorylation in response to γ-irradiation. Again, lower levels of Chk1 phosphorylation were seen in *rad4-116* cells at 26°C compared with wild-type and phosphorylation was completely absent in the mutant strain at 32°C (Fig. 1C). These data suggested that the DNA damage checkpoint response to DNA double-strand breaks was impaired in *rad4-116* cells at the permissive temperature. To examine this further we performed a dose-dependency experiment at 26°C, in which Chk1 phosphorylation was measured in *rad4-116* and wild-type cells exposed to a range of γ-irradiation doses. The results show that Chk1 phosphorylation is clearly reduced in *rad4-116* cells relative to wild-type for all doses tested (Fig. 1D). Taken together, these data demonstrate that Chk1 phosphorylation in the presence of both UV-mediated DNA damage and DNA double-strand breaks is dependent upon Rad4 function, consistent with a loss of G2/M checkpoint function at 32°C. The results further show that the response to ionizing radiation is compromised in *rad4-116* cells at the permissive temperature, consistent with a mild DNA damage sensitivity at 26°C.

Rad4 is not required for phosphorylation of Rad9, Hus1 or Rad26 in response to DNA damage

In addition to Chk1, *S. pombe* Rad9, Hus1 and Rad26 are known to be phosphorylated in response to DNA damage. Rad9 and Rad26 have been shown to be phosphorylated in response to ionizing radiation, whereas Hus1 has been shown to be phosphorylated in response to both ionizing radiation and UV. We assayed Rad9 and Hus1 phosphorylation in *rad4-116* and wild-type cells following exposure to UV and/or bleomycin (see Materials and Methods) by band shift on SDS-PAGE. In contrast to the results obtained with Chk1, we found that both Hus1 and Rad9 were phosphorylated in *rad4-116* cells following DNA damage at both 26°C and 32°C (Fig. 2A,B). This result clearly demonstrates that Rad4 checkpoint function is not required for phosphorylation of either Rad9 or Hus1, and suggests that Rad4 acts downstream of, or parallel to, these proteins within the DNA damage checkpoint pathway. Similarly, Rad26 is phosphorylated in *rad4-116* cells at 32°C, although this may be reduced slightly (Fig. 2C). A small amount of Rad26 phosphorylation is also detectable in the absence of DNA damage at 32°C. This is consistent with earlier data that demonstrated that Rad26 is phosphorylated in

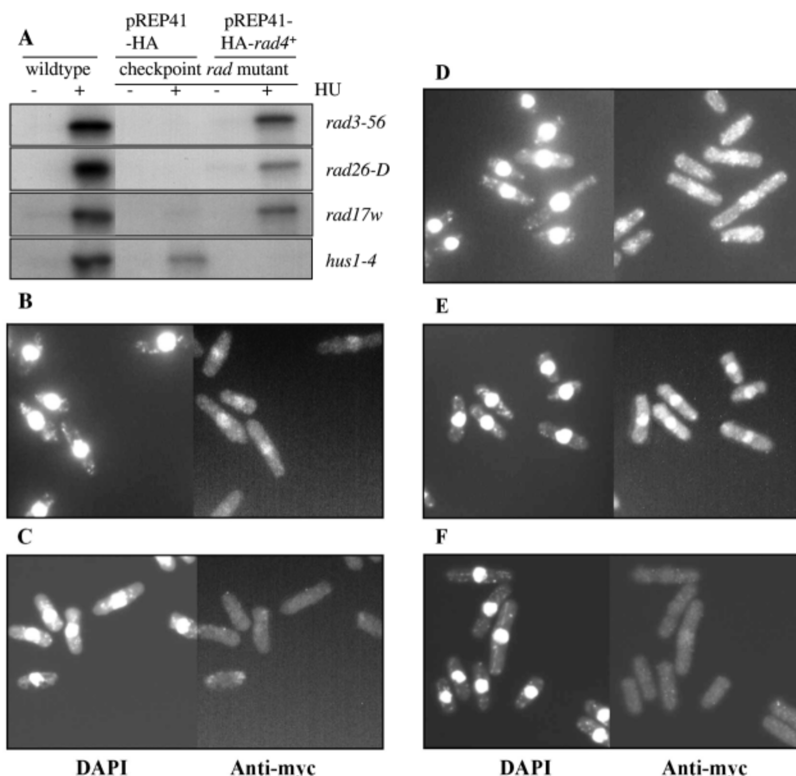

Fig. 5. HA-Rad4p-mediated suppression restores Cds1 kinase activity in response to the inhibition of DNA replication and correlates with nuclear localization of Rad9 in *rad17-w* mutants. (A) Cds1 kinase activity was assayed in asynchronous cultures of wild-type and *rad3-56*, *rad26Δ*, *rad17-w* and *hus1-4* cells carrying either pREP41-HA (p182) or pREP41-HA-*rad4*$^+$ (p247) following growth in the absence (–) or presence (+) of 10 mM HU for 3 hours. As shown, Cds1p kinase activity can be detected in all strains in which suppression was observed. (B-F) Nuclear localization of Rad9p in *rad17-w* mutants. The effects of multi-copy Rad4p and HA-Rad4p on Rad9p localization are compared in wild-type and *rad17-w* cells. Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI), whereas Rad9p was visualized using an anti-myc monoclonal antibody. (B) Wild-type cells (SpSc 529) and Rad9p residing in the nucleus. In contrast, Rad9p locates to the cytoplasm in *rad17-w* cells (SpSc 530) carrying pREP41-HA. (C) The presence of the HA-Rad4p plasmid in *rad17-w* cells causes Rad9p to relocate to the nucleus (D), however, the same was true for *rad17-w* cells expressing the untagged version of *rad4*$^+$, which remain checkpoint deficient (E). (F) Rad9p nuclear staining is absent in *rad17-w* cells containing the HA-Rad4p plasmid and deleted for *hus1*$^+$, implying that nuclear localization of Rad9p is Hus1p-dependent.

several Rad checkpoint-deficient backgrounds (Edwards et al., 1999).

Checkpoint-dependent phosphorylation of Hus1 has also been reported after several hours' exposure to HU. In order to establish the dependency relationship between Rad4 and Hus1 within the DNA replication checkpoint pathway, we examined Hus1 phosphorylation in wild-type and *rad4-116* cells following exposure to HU at 26°C and 32°C. Hus1 is phosphorylated in *rad4-116* cells, irrespective of the growth temperature (Fig. 2D). Hence, Hus1 phosphorylation is also independent of Rad4 in the event of DNA replication inhibition.

A dominant allele of *rad4* suppresses DNA damage and HU sensitivity of *rad3*$^-$, *rad26*$^-$ and *rad17*$^-$ mutations

In parallel to the experiments described above, we investigated the effects of increased Rad4p expression. Two different plasmids, pREP41-HA-*rad4*$^+$ (p247) and pREP41-HA (empty vector) were used in the initial experiment. The plasmid expressing the epitope-tagged *rad4* allele fully complemented the *rad4-116* mutation and no phenotypic effects were observed in wild-type cells carrying the plasmid. Comparison of protein levels between a single integrated copy of rad4-HA and the plasmid-borne copy indicate a significant increase in the levels of protein in the latter case, although proteolytic degradation prevented accurate quantification (C. Kemplen, Delineating the position of rad4$^+$ within the DNA damage and DNA replication checkpoints in the fission yeast *Schizosaccharomyces pombe*, PhD thesis, University of Sheffield, 2002). Cell-doubling time, cell size, DNA structure checkpoint function and sensitivity to DNA-damaging agents were as for *rad4*$^+$ control cells carrying empty vector (C. Kemplen, PhD thesis).

The sensitivity to HU, bleomycin and UV irradiation of exponentially growing *rad3-56* mutant cells carrying pREP41-HA-*rad4*$^+$ was tested. The data show that the presence of the HA-Rad4-expressing plasmid suppresses the sensitivity of *rad3-56* cells to all three agents (Fig. 3A), restoring viability over the course of the experiment to wild-type levels. Subsequent experiments demonstrated that a pREP41 plasmid expressing the wild-type *rad4*$^+$ cDNA was unable to suppress the *rad3-56* phenotype (data not shown) and this is confirmed below. The tagged, plasmid-borne *rad4*$^+$ cDNA was expressed from the *nmt41* promoter, although suppression was observed in both the presence and absence of thiamine.

We next tested the ability of HA-Rad4 to suppress *rad26Δ*, *rad17-w* and *hus1-4* mutations. The sensitivity to HU, bleomycin and UV irradiation of exponentially growing mutant strains carrying pREP41-HA-*rad4*$^+$ was assessed. The data show the presence of the HA-Rad4-expressing plasmid suppresses sensitivity to all three agents in *rad26Δ* and *rad17-w* cells when compared with control cells carrying the vector alone (Fig. 3B,C). In contrast, Hus1p-deficient cells carrying the plasmid remain sensitive to HU and both forms of DNA damage (Fig. 3D). We note that the presence of the vector alone increases the sensitivity of *rad26Δ* cells to HU and of all three mutants to UV irradiation. Conversely, it slightly reduces the sensitivity of *rad17-w* cells to HU. We can offer no convincing explanation for this, although they are robust observations in that all of the above suppression experiments were repeated three times and the mean values plotted. In the remaining suppression experiments the *hus1-4* mutant strain expressing increased levels of HA-Rad4 was employed as a negative control.

Suppression correlates with restoration of checkpoints

We then investigated whether suppression correlated with

restoration of a checkpoint response. Mutant strains carrying pREP41-HA-Rad4 were synchronized in G2, incubated in the presence of bleomycin and the septation index scored at 20-minute intervals to quantify the number of cells passing through mitosis. The data demonstrate that those strains in which suppression was observed exhibit a mitotic delay relative to control cells (Fig. 4A). These data are consistent with the presence of the Rad4p-expressing plasmid causing a restoration of checkpoint function in *rad3-56*, *rad26Δ* and *rad17-w* mutants. Conversely, *hus1-4* cells carrying the plasmid undergo mitosis with similar kinetics in the presence and absence of the DNA-damaging agent, a characteristic of cells lacking the G2/M checkpoint. Microscopic examination of the cells demonstrated that in the presence of bleomycin, *rad3-56*, *rad26Δ* and *rad17-w* cells, containing pREP41-HA-*rad4*$^+$, exhibit an increase in cell length, consistent with a checkpoint-induced mitotic delay (Fig. 4A-C). Control *hus1-4* cells carrying pREP41-HA-*rad4*$^+$ fail to elongate and exhibit the classic 'cut' (cell untimely torn) phenotype of checkpoint-deficient cells (Fig. 4D). These results indicate that expression of HA-Rad4p suppresses the G2/M checkpoint deficiency conferred by the *rad3-56*, *rad26Δ* and *rad17-w* mutations. All the experiments were repeated three times and data from a representative experiment are shown. We then performed similar experiments in asynchronous cultures in the presence of HU with similar results. In all three cases in which suppression of HU sensitivity was observed, cells exhibited a clear mitotic arrest, whereas control cells had a cut phenotype (data not shown).

Suppression is allele-specific for *rad17* and *rad3*, but not *rad26*

We assessed the ability of pREP41-HA-*rad4*$^+$ to suppress mutations in several genes implicated in DNA structure checkpoint control. The results of these experiments are summarized in Table 2. Suppression was restricted to those genes discussed above, and all other strains retained sensitivity to the genotoxic stresses applied. It is important to note that HA-*rad4*$^+$-mediated suppression was allele-specific with respect to the *rad3* locus, the plasmid failing to suppress either a deletion of *rad3* or a kinase-deficient mutant (Table 2). This result implies that the *rad3-56* mutant retains some aspect of its function, which is required for HA-*rad4*$^+$-mediated suppression and that this is probably its kinase activity. Similarly, there was no suppression of a *rad17* deletion, perhaps indicating a physical requirement for the mutant Rad17. Next, the dependency relationships of suppression were examined using the double mutant strains listed in Table 1. Taking the *rad3-56* mutation as the exemplar, we tested the sensitivity to HU and DNA damage of strains carrying this mutation in combination with mutations in all the other known *S. pombe* checkpoint genes in addition to a *rad26Δ rad17-w* double mutant. The data, presented in Table 3, demonstrate that suppression requires functional copies of all of the other checkpoint genes.

Biochemical characterization of *rad4*$^+$-mediated suppression

The physiological data presented above indicate that high-level expression of Rad4p is able to compensate for mutation of *rad3*, *rad26* and *rad17* as a result of restoration of checkpoint function. In order to further explore the nature of suppression we examined the biochemical events described above in mutant strains carrying either pREP41-HA-*rad4*$^+$ or pREP41-HA. Cds1p kinase activity was assayed in synchronous cultures in the presence of HU. In all cells in which pREP41-HA-*rad4*$^+$-mediated suppression of HU sensitivity was observed, significant levels of Cds1p kinase activity were present (Fig. 5A). These results are consistent with the physiological data indicating a restoration of checkpoint function. In contrast, the *hus1-4* mutant expressing increased levels of Rad4 completely lacked Cds1p kinase activity. It should be noted that in control *hus1-4* cells carrying the pREP41-HA vector, a small amount of Cds1 kinase activity was detectable in the presence of HU. This presumably reflects a partial activation of the S-M checkpoint, although this was insufficient to rescue HU sensitivity.

Table 2. Summary of HA-Rad4 suppression of checkpoint-deficient mutations

Strain	UV suppression	Bleomycin suppression	HU suppression
rad3-56	+	+	+
rad3-KD	–	–	–
rad3::ura4$^+$	–	–	–
rad17::ura4$^+$	–	–	–
rad17-w	+	+	+
rad26::ura4$^+$	+	+	+
rad1-1	–	–	–
rad1::ura4$^+$	–	–	–
rad9-192	–	–	–
rad9::ura4$^+$	–	–	–
hus1-4	–	–	–
cds1::ura4$^+$	na	na	–
chk1::ura4$^+$	–	–	na
crb2-1	–	–	–
rhp9::ura4$^+$	–	–	–

na, not applicable.

Nuclear localization of Rad9p in *rad17-w* mutants

It had previously been demonstrated that Rad9 failed to localize to the nucleus in *rad17* null mutant cells (Caspari et al., 2000a). One possible mechanism for HA-*rad4*$^+$ suppression might be restoration of nuclear localization of the 9-1-1 PCNA-like complex. The results of experiments to test

Table 3. Suppression requires the presence of all other checkpoint Rad proteins

Strain	UV suppression	Bleomycin suppression	HU suppression
rad3-56, rad17-w	–	–	–
rad3-56 rad26::ura4$^+$	–	–	–
rad3-56 rad1::ura4$^+$	–	–	–
rad3-56, rad9::ura4$^+$	–	–	–
rad3-56, hus1-4	–	–	–
rad3-56, cds1::ura4$^+$	na	na	–
rad3-56, chk1::ura4$^+$	–	–	na
rad3-56, rhp9::ura4$^+$	–	–	–
rad26::ura4, rad17-w	–	–	–

na, not applicable.

this possibility are presented in Fig. 5A-F. We first examined Rad9 localization in the *rad17-w* mutant and found the protein to reside mainly in the cytoplasm (Fig. 5C). The presence of the HA-*rad4*+ plasmid caused Rad9 to relocate to the nucleus (Fig. 5D). However, in *rad17-w* cells expressing the untagged version of *rad4*+, which remain checkpoint-deficient, Rad9 was also found in the nucleus (Fig. 5E). Nuclear localization was dependent upon Hus1, as would be predicted by a requirement for complex formation (Fig. 5F). We conclude from these data that, although restoration of the nuclear localization of this complex may offer a partial explanation for the observed suppression, it is not sufficient for restoration of checkpoint function, at least in a *rad17-w* mutant.

Discussion

Previous work had demonstrated a role for Rad4 in both DNA replication and DNA structure checkpoint control (Saka and Yanagida, 1993). Subsequent analysis showed that these roles were distinct, and that Rad4 functioned in several distinct checkpoint sub-pathways, including the S-M checkpoint and the G2/M DNA damage checkpoint in response to both ionizing and UV irradiation (McFarlane et al., 1997; Verkade and O'Connell, 1998). In this study we have focused on the checkpoint function of Rad4, employing a combined biochemical and genetic approach to map the position of the protein within the S-M and G2/M pathways relative to the other checkpoint gene products. Activation and/or modification of the Cds1 and Chk1 checkpoint-effector kinases is dependent upon all of the checkpoint Rad proteins tested to date. The data presented here demonstrate that these events are also dependent upon Rad4 checkpoint function, placing Rad4 upstream of these two kinases and grouping it with the other 'checkpoint Rad' proteins.

Hus1 is phosphorylated in response to prolonged inhibition of DNA replication and in the presence of DNA damage. Rad9 forms a complex with Hus1 and Rad1 and is itself phosphorylated in response to DNA damage. Both of these post-translational modification events are dependent on the remaining checkpoint Rad proteins but independent of Crb2 (also termed Rhp9) and the effector kinases (Kostrub et al., 1998; Caspari et al., 2000a; Caspari et al., 2000b). Fig. 2 demonstrates that phosphorylation of Hus1 and Rad9 is independent of Rad4. Formally, these data are consistent with Rad4 acting downstream of the 9-1-1 complex, perhaps in conjunction with Crb2 as has been previously suggested (Saka et al., 1997). However, for the reasons discussed below, we believe this interpretation is probably incomplete.

We have shown that a modified version of Rad4, carrying three copies of the 12CA5 HA epitope at its N-terminus, acts to suppress mutation of each of *rad3*, *rad26* and *rad17*. These proteins are thought to act upstream of the PCNA-like checkpoint complex. Moreover, the observed suppression is dependent upon the presence of the PCNA-like complex for the S-M and G2/M pathways (see Table 3). These data are inconsistent with a model in which Rad4 acts downstream of the PCNA-like complex. Suppression is also dependent upon Cds1 if DNA replication is compromised, and on Crb2 and Chk1 in the presence of DNA damage. Suppression is allele-specific with respect to *rad3* and *rad17* (Table 2). HA-Rad4 is unable suppress deletion of either of these genes, but is able to suppress point mutations of both genes. This suggests that a physical interaction between HA-Rad4, Rad3 and Rad17 is required for suppression. One of the most surprising results is that deletion of *rad26* is suppressed by HA-Rad4 expression. Rad26 and its homologues are widely thought to provide a chromatin-loading function for Rad3-related kinases. It may be that high levels of the tagged Rad4 provide this function in the absence of Rad26. Alternatively, this might be explained if Rad26 is auxiliary to the main complex, but normally acts to provide additional stability to that complex – an activity that would be dispensable in the presence of the modified Rad4. Further, Rad3 requires an active kinase domain for the observed suppression.

The most parsimonious interpretation of the above data places Rad4 downstream of Rad3, Rad26 and Rad17, but parallel to the 9-1-1 complex, thus defining a bifurcation of both the S-M and G2/M checkpoint pathways. The bifurcated pathways then converge to activate the respective effector kinases. In the case of the response to DNA damage, Crb2 will probably act downstream of Rad4 as it is required for Chk1 phosphorylation and for the HA-Rad4-mediated suppression described here. It remains possible that Rad4 and Crb2 act in concert and there is evidence to suggest that they may form a heteromeric complex (Saka et al., 1997). Rad3 and Rad26 are known to form an active protein kinase complex and are shown to act as a complex at the head of the phosphorylation cascade associated with the S-M and G2-M checkpoint pathways (Edwards et al., 1999). Rad17 is not required for Rad3/Rad26 activity, as measured by phosphorylation of Rad26, but is required for Hus1 and Rad9 modification (Edwards et al., 1999; Kosrub et al., 1998; Caspari et al., 2000b) as well as for suppression. This positions Rad17 upstream of both Rad4 and the 9-1-1 complex.

As suggested above, in order to fully explain all of these observations we hypothesize the existence of a multimeric complex between Rad4 and Rad3/Rad26/Rad1-Rad9-Hus1. Given the independent loading of Rad3-Rad26 and 9-1-1 complexes on the DNA at sites of damage, we suggest this interaction occurs at the time of assembly onto the DNA. Our genetic interaction between *rad17* and *rad4* may reflect biochemical interactions between Rad4 with the 9-1-1 complex (and possibly Rad17 as well), because Rad17 is required to load 9-1-1 at sites of damage and a physical interaction has been identified between the Dpb11 and Ddc1, the homologs in *S. cerevisiae* of Rad4 and Rad9, respectively (Wang and Elledge, 2002). We speculate that Rad4 interacts with these complexes to integrate the bifurcation of the pathway (evident at the point of chromatin loading). This integration then allows the Rad3-dependent phosphorylation cascade to link to Chk1. A requirement of this argument is that both branches must be active for a functional checkpoint response as evidenced by the dependency of suppression. Thus physically, these data reflect the fact that activation of the effector kinase requires two independent steps. This model is summarized in Fig. 6. We imagine that Rad4 plays a similar role in recruiting Cds1 to the appropriate complexes in the presence of stalled replication forks. Once present at the correct site, the kinase is activated and proceeds to interact with the relevant target proteins, which are probably distinct from the Chk1 targets. It is not yet clear why only the epitope-tagged form of Rad4 is able to suppress the checkpoint mutations. One possibility is that it alters the

stability of the protein such that it confers an increased stability to the mutant complexes, thus allowing them to function. However, it will be necessary to develop further reagents before this question can be properly addressed.

One remaining question relates to the nature of the observed suppression. In an attempt to address this we examined the nuclear localization of Rad9 that had previously been shown to be dependent upon Rad17 (Caspari et al., 2000a). The data indicated that high-level expression of either Rad4 or HA-Rad4 restores the nuclear localization of Rad9 in *rad17-w* cells (Fig. 5). High-level expression of wild-type Rad4 is unable to rescue the checkpoint deficiency of a *rad17-w* mutant, and therefore the restoration of Rad9 nuclear localization cannot fully explain HA-Rad4-mediated suppression. However, it may explain this suppression in part and offer an insight into the function of Rad4. It could be that Rad4, in forming the multimeric complex hypothesized above, also acts as an accessory factor with the Rad17-Rfc complex for chromatin loading of the 9-1-1 PCNA-related complex, perhaps at sites of unusual DNA structure. If this were so, then it might also explain the role of Rad4 during DNA replication, in which it could interact with RFC to facilitate PCNA loading. This interpretation would be consistent with data implicating the *S. cerevisiae* homologue, Dpb11, in processive DNA replication and perhaps in the loading of processive DNA polymerases (Reid et al., 1999). Dpb11 has also been shown to be required for the association of DNA polymerases α and ε with an origin of DNA replication (Masumoto et al., 2000).

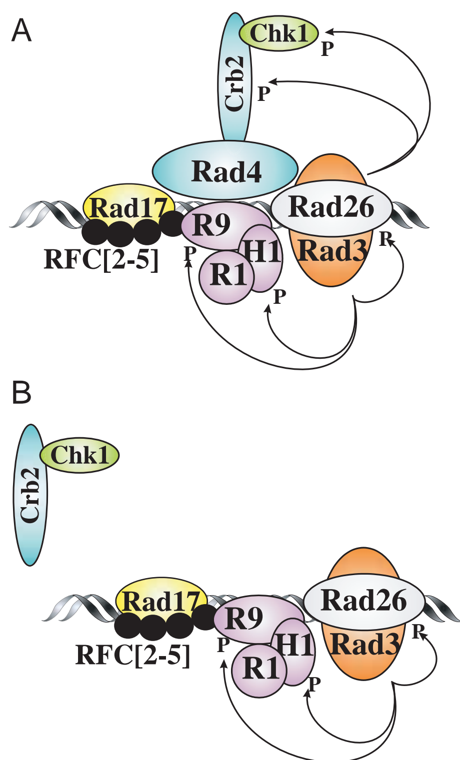

Fig. 6. (A) In response to DNA damage (or replication inhibition – not shown), Rad3-Rad26 is loaded onto the DNA. The 9-1-1 complex is loaded independently of Rad3-Rad26 by Rad17, which is itself constitutively DNA associated. Rad4, which may be constitutively chromatin associated or loaded in response to DNA damage, makes contact with Rad3-Rad26 (possibly by associating with both proteins) and Rad17. Rad4 anchors Crb2 and, indirectly, Chk1 to the region. It is anticipated that Rad4 plays a similar role in tethering Cds1 to the checkpoint complex in the event of an inhibition of DNA replication. (B) In the absence of Rad4, Rad3 and 9-1-1 are loaded normally, but Chk1 (or Cds1 in the case of replication inhibition) is not available for phosphorylation or activation, whereas Rad9 and Hus1 are.

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