The *SAD1/RAD53* protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast

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Inhibition of DNA synthesis prevents mitotic entry through the action of the S-phase checkpoint. We have isolated S-phase arrest-defective (*sad1*) mutants that show lethality in the presence of the DNA synthesis inhibitor hydroxyurea (HU). Several of these mutants show phenotypes consistent with inappropriate mitotic entry in the presence of unreplicated DNA, indicating a defect in the S-phase checkpoint. *sad1* mutants are additionally defective for the G₁ and G₂ DNA damage checkpoints, and for DNA damage-induced transcription of *RNR2* and *RNR3*. The transcriptional response to DNA damage requires activation of the Dun1 protein kinase. Activation of Dun1 in response to replication blocks or DNA damage is blocked in *sad1* mutants. The HU sensitivity of *sad1* mutants is suppressed by mutations in *CKS1*, a subunit of the p34^{CDC28} kinase, further establishing a link between cell cycle progression and lethality. *sad1* mutants are allelic to *rad53*, a radiation-sensitive mutant. *SAD1* encodes an essential protein kinase. The observation that *SAD1* controls three distinct checkpoints suggests a common mechanism for cell cycle arrest at these points. Together, these observations implicate protein phosphorylation in the cellular response to DNA damage and replication blocks.

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A successful eukaryotic cell division requires that certain cellular processes occur in a defined order and are coupled so that the initiation of one event is dependent on the completion of another. In most cell types, entry into mitosis is dependent on the completion of DNA synthesis. Cells blocked for DNA replication arrest in S phase and delay mitotic initiation. Cell cycle arrest in response to S-phase inhibition is attributable to the presence of a feedback control or checkpoint mechanism (for reviews on cell cycle checkpoints, see Hartwell and Weinert 1989; Enoch and Nurse 1991; Murray 1992; Roberge 1992; Li and Deshaies 1993). Checkpoint controls also provide regulation of cell cycle progression in response to environmental challenges such as DNA damage. Cellular responses to DNA damage and blocks in DNA replication are similar: Cells arrest progression through the cell cycle at distinct points and induce the transcription of genes whose products facilitate DNA repair. In response to DNA damage, eukaryotic cells arrest either in G₁ to prevent replication of damaged genetic templates or in G₂ to avoid segregation of defective chromosomes. A current point of interest is whether feedback controls also order events in a normal cell cycle in the absence of DNA damage or replication blocks. In this regard it has been proposed that relative timing mecha-

nisms may order events in a normal cell cycle and that cell cycle arrest checkpoints are activated to ensure dependencies in cases when relative timing is insufficient (Murray 1992; Li and Deshaies 1993).

Defects in cell cycle checkpoints can result in genomic instability and increase the rate that cells accumulate heritable genetic damage. In budding yeast, mutations exist that exhibit elevated levels of chromosome loss and are unable to arrest the cell cycle in G₁ (Siede et al. 1993) or G₂ (Weinert and Hartwell 1988; Weinert et al. 1994) in response to DNA damage. Among these, the *mec1* and *mec2* mutants have been implicated in the S-phase checkpoint because of sensitivity to conditions resulting in replication blocks and abnormal spindle structures in the presence of hydroxyurea (HU) (Weinert et al. 1994). However, a definitive role in the S-phase checkpoint has not yet been established for these mutants because cells can leak through a HU block, and it was not demonstrated that these mutants entered mitosis with DNA that was less than fully replicated. Strains lacking feedback controls that couple spindle assembly and exit from mitosis also lose chromosomes at a higher rate than wild-type cells (Hoyt et al. 1991; Li and Murray 1991). Furthermore, it has been suggested that checkpoint control deficiency in mammalian cells may play a

role in cellular transformation (Hartwell 1992; Livingstone et al. 1992; Kastan et al. 1992; Murray 1992; Yin et al. 1992). Ataxia-telangiectasia (AT) is a human recessive disorder characterized by a number of phenotypes including a high incidence of cancer. Cell lines derived from AT patients are defective for both the G₁ and G₂ DNA damage checkpoints (Painter and Young 1980; Zambetti-Bosseler and Scott 1981; Nagasawa et al. 1985; Cohen and Levy 1989; Rudolph and Latt 1989). In addition, primary murine fibroblasts that carry null or mutant alleles of the tumor suppressor gene p53 fail to arrest the cell cycle in G₁ in response to ionizing radiation (Kuerbitz et al. 1992; Yin et al. 1992) and are defective for the DNA damage-induced transcription of CIP1/WAF1 (El Diery et al. 1993, 1994), an inhibitor of Cdk2 and Cdk4 (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993). Taken together, these observations suggest that the inability to respond to DNA damage by cell cycle arrest or to induce transcription of genes that facilitate DNA repair may potentiate cellular transformation.

A number of checkpoint mutants have been identified in a variety of species; however, the biochemical nature of the cell's ability to coordinate cell cycle progression with the replicational status and integrity of the genome remains unclear. The eukaryotic cell cycle is composed of two major transitions, one at the G₁/S boundary and another at G₂/M. These transitions are controlled by the p34 protein kinase and associated regulatory subunits called cyclins. The p34/cyclin complex is an attractive target for checkpoint inhibition. In *Schizosaccharomyces pombe*, cells treated with the chemical HU are blocked for DNA replication and maintain high levels of inhibitory Tyr-15 phosphorylation on p34^{cdc2} (Gould and Nurse 1989; Nurse 1990). Cells that contain an unphosphorylatable mutant of p34^{cdc2}, Y15F, bypass the cell cycle arrest imposed by unreplicated DNA and enter a lethal mitosis (Gould and Nurse 1989). It is not yet clear whether the dominant cdc2:Y15F mutant functions indirectly by bypassing the checkpoint, or directly by blocking its target, phosphorylation of Tyr-15. In contrast, *Saccharomyces cerevisiae* strains that contain an analogous mutation on p34^{CDC28} are competent to respond to S-phase inhibition and progress normally through the cell cycle (Amon et al. 1992; Sorger and Murray 1992). This suggests that budding yeast possess a mode of checkpoint control distinct from inhibitory tyrosine phosphorylation of p34.

Although progress has been made in understanding cell cycle checkpoints and the cellular response to DNA damage, several outstanding questions remain. First, what is the molecular nature of the S-phase checkpoint? Second, does it operate during an unperturbed cell cycle or only in response to stress? Third, is there overlap between the DNA damage checkpoint pathways in G₁ and G₂ and the S-phase checkpoint pathway? Fourth, to what extent do the cell cycle arrest and DNA damage transcriptional induction pathways overlap? To address these questions and elucidate the signaling pathway coordinating S phase and mitosis, we have focused on the isolation of S-phase arrest-defective (*sad*) mutants that

disrupt cell cycle arrest in response to blocks in DNA replication.

Results

Isolation of *sad* mutants

HU inhibits the enzyme ribonucleotide reductase (Rnr) by quenching the tyrosyl free radical required for catalysis. Inhibition of Rnr results in the inability to synthesize deoxyribonucleotides, which blocks DNA synthesis. Wild-type cells respond to HU treatment by reversibly arresting cell cycle progression through activation of the S-phase checkpoint. Mutations in the S-phase checkpoint pathway should confer sensitivity to HU because the inability to delay cell cycle progression may result in mitosis in the absence of DNA replication. EMS-mutagenized colonies (250,000) were replica-plated to plates containing 100 mM HU, and ~700 HU-sensitive mutants were isolated. To specifically identify *sad* mutants, two secondary screens were employed. First, HU-sensitive mutants were screened for loss of viability in the presence of HU (HU reversibility) by replica-plating and liquid survival assays, and second, for rescue by prior arrest with α -factor as described in Materials and methods. *sad* mutants should exhibit rapid loss of viability in the presence of HU because of their inability to delay cell cycle progression when DNA synthesis is inhibited. In addition, HU-induced lethality should be suppressed by prior treatment with α -factor, which arrests cells in G₁ and prevents inappropriate cell cycle progression. Six recessive mutants representing five different complementation groups, *sad1*–*sad5*, were isolated that exhibit rapid loss of viability with HU that is α -factor suppressible. *sad* mutants display significant killing after 6 hr of HU treatment compared with wild-type controls (Fig. 1A). In addition, *sad* mutants are significantly more sensitive to UV-irradiation than wild-type strains (Fig. 1B). This suggests that *sad* mutants may be additionally defective for DNA repair or the DNA damage cell cycle checkpoints. *sad1-1* mutants exhibit the most rapid lethality and were selected for further analysis. α -Factor suppression of *sad1-1* HU lethality is shown in Figure 1C.

Screen of *rad* mutants for S-phase feedback control deficiency

Mutants defective for DNA damage-induced cell cycle arrest have been identified among preexisting collections of mutations that confer radiation sensitivity in budding yeast and fission yeast (*rad* mutants) (Weinert and Hartwell 1988; Al-Khodairy and Carr 1992; Enoch et al. 1992; Rowley et al. 1992; Weinert 1992). It is also likely that the DNA damage and the S-phase checkpoint pathways share common components (Al-Khodairy and Carr 1992; Enoch et al. 1992; Weinert 1992). In this regard, a bank of budding yeast *rad* mutants were screened for the *sad* phenotype. As shown in Table 1, *rad50*, *rad51*, and *rad53* mutants exhibit some *sad* phenotypes. *sad* mutants

Figure 1. Checkpoint deficiency and radiation sensitivity of *sad* mutants. (A) HU lethality of wild-type and *sad* strains. Wild-type strain Y300 (■) and *sad* mutant cells (Y301, *sad1-1*, ●; Y305, *sad2-1*, □; Y306, *sad3-1*, ○; Y307, *sad4-1*, ▲; and Y308, *sad5-1*, △) were grown to log phase in YPD (pH 3.9), and HU was added to 0.2 M. Aliquots were removed at timed intervals to determine cell number and to score for viable colony-forming units on YPD plates. (B) Radiation sensitivity of *sad* mutants. Wild-type (Y300, *SAD1*, ■) and *sad* mutant cells (Y301, *sad1-1*, ●; Y305, *sad2-1*, □; Y306, *sad3-1*, ○; Y307, *sad4-1*, ▲; and Y308, *sad5-1*, △) were irradiated with the indicated doses of UV light, and percent survival relative to unirradiated controls was determined. (C) α -Factor rescue of *sad1-1* cells. Log-phase cultures were treated with 10 μ g/ml of α -factor for 1 hr. Samples were washed with YPD, split, and resuspended in YPD containing 0.2 M HU (Y300, wild-type, ■; Y301, *sad1-1*, ●) or 0.2 M HU plus 10 μ g/ml of α -factor (Y300, wild-type, □; Y301, *sad1-1*, ○). α -Factor (2 μ g/ml) was subsequently added every 2 hr to α -factor-treated samples. (D) DNA damage-induced G_1 cell cycle delay in *SAD* and *sad1-1*. Wild-type Y300 (left) and *sad1-1* Y301 (right) strains were synchronized with α -factor and UV-irradiated. Unirradiated (–) and irradiated (+) samples were resuspended into fresh YPD media, and cell cycle progression was monitored by FACS analysis. Numbers in the center refer to the time in minutes after release from α -factor block. (E) Survival of *cdc9 sad1* double mutants at the restrictive temperature. Wild-type strain Y300 (■), Y301 *sad1-1* (●), Y309 *cdc9* (□), and Y310 *cdc9 sad1* double mutants (○) were grown to log phase at 23°C and shifted to 37°C for 4 hr. Dilutions were plated at timed intervals and incubated at 23°C to determine cell viability. (F) DNA damage-induced G_2 cell cycle delay in wild-type and *sad1-1* strains. Wild-type strain Y300 (□, ■) and Y301 *sad1-1* cells (○, ●) were arrested with 100 μ g/ml of benomyl and UV-irradiated. Unirradiated (■, ●) and UV-irradiated (40 J/m^2 , ○, □) samples were resuspended into fresh media, and cell cycle progression was monitored by direct visualization of nuclear division using DAPI staining.

were crossed with *rad50*, *rad51*, and *rad53* to test for complementation and linkage. *rad53* is allelic to *sad1*

while *rad50* and *rad51* are not allelic to any of the *sad* mutants.

Table 1. Screen of *rad* mutants for *sad* phenotype

Mutant	HU		HU lethality suppressed by α -factor
	sensitive	reversible	
<i>rad5</i>	no		
<i>rad8</i>	no		
<i>rad14</i>	no		
<i>rad16</i>	no		
<i>rad23</i>	no		
<i>rad24</i>	no		
<i>rad50</i>	yes	no	yes
<i>rad51</i>	yes	no	yes
<i>rad52</i>	yes	yes	
<i>rad53</i>	yes	no	yes
<i>rad54</i>	yes	yes	
<i>rad55</i>	yes	yes	
<i>rad56</i>	yes	yes	
<i>rad57</i>	yes	yes	

A collection of preexisting *rad* mutants were screened for HU sensitivity, HU reversibility, and α -factor suppression of HU lethality as described in Materials and methods.

sad1 is deficient for DNA damage-induced cell cycle delay in G_1 and G_2

RAD9 is required for cell cycle arrest in response to DNA damage in G_1 (Siede et al. 1993) and G_2 (Weinert and Hartwell 1988). In addition, several *cdc* mutants are dependent on *RAD9* for their *cdc* phenotypes and arrest reversibilities. For example, temperature-sensitive mutants in *cdc9*, encoding DNA ligase, arrest in G_2 with the bulk of DNA synthesis completed but with unligated Okazaki fragments (Johnston and Nasmyth 1978). *cdc9* mutants arrest reversibly at high temperatures, whereas *rad9 cdc9* double mutants exhibit rapid loss of viability at the restrictive temperature because of entry into mitosis with damaged DNA.

Although *RAD9* is not required for the S-phase checkpoint (Elledge and Davis 1990; Weinert and Hartwell 1993), the UV-sensitivity of *sad1-1* suggests that *SAD1* may have a role in the *RAD9*-dependent G_1 and G_2 DNA damage checkpoints. To examine the G_1 DNA damage checkpoint, wild-type and *sad1-1* mutant strains were arrested in G_1 with α -factor, UV-irradiated, and released from α -factor. Samples were removed at timed intervals to monitor cell cycle progression by measuring DNA content and bud formation. Unirradiated wild-type cells progress normally through the cell cycle following release from α -factor while irradiation produces a G_1 delay (Fig. 1D). In contrast, both untreated and irradiated *sad1-1* cells progress through the cell cycle in a similar fashion after release from α -factor as measured by both FACS analysis (Fig. 1D) and bud formation (data not shown). These results demonstrate that *SAD1* is required for the G_1 DNA damage cell cycle checkpoint.

To examine the G_2 DNA damage checkpoint as defined by Weinert and Hartwell (1988), *cdc9 sad1* double mutant strains were constructed. As shown in Figure 1E, *cdc9 sad1* mutants lose viability at high temperature

compared with *cdc9* strains, consistent with a defect in DNA damage-induced G_2 delay. To address this point further, wild-type and mutant strains were synchronized with the microtubule inhibitor benomyl and irradiated. Previous studies have demonstrated that the *RAD9*-dependent checkpoint can also function at the benomyl block, which is likely to be post- G_2 in early M phase by analogy to other organisms. Because clear cytological criteria are not available in *S. cerevisiae* to distinguish G_2 from early M phase, we refer to this point as G_2 /M to facilitate discussion. Cells treated with benomyl complete DNA replication but are unable to assemble a mitotic spindle and arrest the cell cycle through the *BUB*- and *MAD*-dependent checkpoint pathways (Li and Murray 1991; Hoyt et al. 1991). After release from benomyl arrest, the kinetics of visible nuclear division are followed (Fig. 1F). Unirradiated wild-type and *sad1-1* mutant cells normally progress through mitosis with similar kinetics, whereas irradiated wild-type cells exhibit a UV-induced cell cycle delay relative to unirradiated cells. In contrast, *sad1-1* mutants are largely defective for UV-induced cell cycle delay at G_2 /M. The residual delay observed in UV-treated *sad1-1* mutants may be attributed to the hypomorphic nature of the *sad1-1* allele (deletion of *SAD1* is lethal). These results show that *sad1-1* mutants are also unable to arrest the cell cycle in G_2 or G_2 /M in response to DNA damage. Because the phenotypes of *rad9* represent a subset of the *sad1-1* phenotypes, it is likely that *SAD1* and *RAD9* function in the same pathway.

Characterization of the S-phase feedback control deficiency in *sad1*

If *sad1-1* strains are completely deficient for HU-induced cell cycle arrest, then the kinetics of spindle elongation in *sad1-1* cells after G_1 release should be similar in the presence or absence of HU. To further understand the S-phase arrest deficiency of *sad1-1* mutants, we examined the rate and extent of entry into mitosis (anaphase) in synchronized wild-type and *sad1-1* mutant strains. Strains were arrested in G_1 with α -factor and released either into YPD or YPD containing 200 mM HU. Samples were removed at 15-min intervals to determine viability, DNA content, and to assess cellular, nuclear, and microtubule morphologies.

When released from G_1 into media containing HU, both wild-type and *sad1-1* mutant cells maintain a G_1 DNA content (Fig. 2A). Wild-type cells released from α -factor into media without HU progress normally through the cell cycle, initially exhibiting spindle elongation and nuclear division ~60 min after G_1 release (Fig. 2B). *sad1-1* cells released from G_1 in the absence of HU also progress normally through the cell cycle, although the rate of mitotic entry is slower than wild type. Wild-type cells released into media containing HU are prevented from entering mitosis and arrest as budded, uninucleate cells with short spindles (Fig. 2B,D). However, in contrast, a significant proportion of *sad1-1* cells released from G_1 into media containing HU enter mito-

Figure 2. *sad1-1* cells exhibit premature spindle elongation in the presence of HU. Wild-type strain (Y300) and *sad1-1* (Y301) cells were synchronized in G₁ with α -factor and released into YPD in the presence and absence of HU. (A) DNA content of HU-arrested wild-type and *sad1-1* cells measured by FACS analysis. (Asy) Asynchronous cells. Numbers at right represent the time in minutes after release from α -factor arrest into YPD containing 200 mM HU. (B) Kinetics of spindle elongation in wild-type (■, □) and *sad1-1* mutant cells (●, ○) released from G₁ arrest in the presence (○, □) or absence (■, ●) of HU. Samples were taken at timed intervals as above and stained with antitubulin antibodies, and the percentage of cells that exhibit elongated spindles was determined using indirect immunofluorescence. A typical data set is shown. A statistical analysis of the data gathered from four independent experiments is presented in the text. (C) *sad1-1* cells lose viability in late G₁ and S phase upon G₁ release in the presence of HU. Dilutions of G₁-synchronized *sad1-1* cells were plated onto YPD plates at different times after release into 0.2 M HU, and cell viability was determined with respect to G₁-arrested samples (□). Samples were also scored for bud emergence (○). (D) Photomicrographs of wild-type and *sad1-1* cells 60 min after G₁ release into HU-containing media. Nuclear morphology was visualized with DAPI (center panels) and microtubule morphology was visualized with anti- α -tubulin (right panels) in wild-type (top panels) and *sad1-1* cells (middle and bottom rows). *sad1* mutants exhibit partially and fully elongated spindles in the presence of HU.

sis as judged by nuclear division and spindle elongation (Fig. 2B,D). Entry into mitosis with incompletely replicated DNA is the defining characteristic of S-phase

checkpoint-defective mutants. *sad1-1* mutants exhibit a mixture of partially (two- to threefold longer than in HU-arrested wild-type) and fully elongated spindles in HU.

Interestingly, HU-treated *sad1-1* cultures accumulate cells with elongated spindles at a rate faster than *sad1-1* cells released from G₁ in the absence of HU (Fig. 2B). The mean time for 30% of the cells to exhibit spindle elongation in *sad1* mutants in HU was 51.2 ± 2.4 min, compared with 61.8 ± 2.5 min without HU (averaged over four data sets). Thus, HU accelerates mitotic onset by 10.5 ± 2.4 min. In addition, *sad1-1* cells released in the presence of HU contain a significant proportion of unbudded and small budded cells that possess mitotic spindles (data not shown). Unbudded or small budded cells that contain elongated spindles were not observed in wild-type cultures and were seen only rarely in *sad1-1* strains not treated with HU.

sad1-1 cells released from G₁ in the presence of HU quickly lose viability following G₁ release (Fig. 2C). Correlation of *sad1-1* lethality with cellular morphology indicates that *sad1-1* cells lose viability coincident with entry and progression through S phase. These kinetics are consistent with a commitment to lethality once replication is delayed for a short period of time.

SAD1 is required for the DNA damage inducibility of RNR2 and RNR3

Both prokaryotic and eukaryotic cells respond to DNA damage by arresting the cell cycle and inducing transcription of genes whose products facilitate DNA repair. We were interested in whether these pathways share common components. To address this, *sad1-1* mutants were examined for regulation of the DNA damage-inducible *RNR2*, *RNR3*, and *UBI4* genes. *RNR2* and *RNR3* encode the catalytic and an alternative regulatory subunit of ribonucleotide reductase, respectively. *UBI4* encodes polyubiquitin. As shown in Figure 3, *sad1-1* mutants are defective for methylmethane sulfonate (MMS)-induced transcription of *RNR2* and *RNR3* but are competent for induction of *UBI4*. This phenotype is similar to that observed for *dun1* mutants and supports the observation that multiple pathways exist for transcription induced by DNA damaging agents (Zhou and Elledge 1993).

Sad1 controls the kinase activity of Dun1 in response to DNA damage

The Dun1 protein kinase is required for the DNA damage-induced expression of *RNR2* and *RNR3* (Zhou and Elledge 1993). Dun1 is activated in response to DNA damage as evidenced by the DNA damage-dependent generation of slower migrating forms of Dun1 in in vitro autophosphorylation assays and an increase in *DUN1*-dependent autophosphorylation in vivo. While *DUN1* is required for the transcriptional response to DNA damage, it is not required for the G₁-, G₂-, or S-phase checkpoints. *sad1* mutants are defective for both DNA damage-induced cell cycle arrest and transcription of *RNR2* and *RNR3*. It is therefore likely that *SAD1* functions in the same pathway as *DUN1*. Because *SAD1* also controls cell cycle checkpoints and *DUN1* does not, it is possible

Figure 3. *sad1-1* is defective for DNA damage inducibility of *RNR2* and *RNR3*. Cultures at OD₆₀₀ of 0.05 in YPD were treated with or without 0.1% MMS for 1 hr at 30°C. RNA was extracted, fractionated on a formaldehyde–1% agarose gel, transferred to nitrocellulose, and probed with ³²P-labeled DNA probes derived from *RNR2*, *RNR3*, *UBI4*, and *URA3* as described in Materials and methods.

that *SAD1* is upstream of *DUN1* and regulates its activity in response to DNA damage. To test this hypothesis, DNA damage-induced activation of Dun1 was assayed in *sad1* mutant strains. Wild-type cells treated with either HU or MMS exhibit slower migrating forms of Dun1 (Fig. 4A). Neither treatment in *sad1* mutants resulted in the Dun1 mobility shift observed in wild-type cells, indicating that activation of Dun1 by DNA damaging agents is *SAD1*-dependent.

To further investigate the relationship between *SAD1* and *DUN1*, the DNA damage-induced hyperphosphorylation of Dun1 in vivo was examined in wild-type and *sad1* mutant cells. As shown in Figure 4B, MMS treatment of wild-type cells overexpressing Dun1 results in an increase of the phosphorylation state of Dun1. However, this response is absent in *sad1* mutants, further strengthening the correlation of the mobility shift and autophosphorylation in response to DNA damage. These results suggest further that *SAD1* functions upstream of *DUN1* in the signaling pathway that activates DNA damage-induced transcription.

Cloning and disruption of SAD1

A wild-type *SAD1* genomic clone was isolated by complementation of *sad1-1* HU sensitivity and sequenced. This clone complements all of the checkpoint and transcriptional defects of the *sad1-1* mutant (data not shown). A GenBank data base search revealed that the open reading frame encodes a previously cloned gene, *SPK1*, a serine/threonine protein kinase. *SPK1* was isolated in a screen to identify yeast protein kinases (Stern et al. 1991) using anti-phosphotyrosine antibodies, and was shown to possess kinase activity in vitro. Further-

Figure 4. Dun1 is not activated by DNA damage or replication blocks in *sad1-1* mutants. (A) DNA damage-induced slow-migrating forms of Dun1 are not generated in *sad1-1* mutants. Wild-type (Y300) and *sad1-1* (Y301) strains were grown in YPD to mid-log phase at 30°C and cultured for 3 hr in the presence or absence of 150 mM HU or 0.1% MMS. Endogenous Dun1 was immunoprecipitated, and in vitro kinase assays were performed. Kinase reaction products were fractionated on 8% SDS-polyacrylamide gels and subjected to autoradiography. Dun1 from DNA-damaged wild-type cells migrates more slowly than that from undamaged cells. The production of slower migrating forms of Dun1 by DNA damage is abolished in a *sad1-1* genetic background. Arrows indicate slow- and fast-migrating forms of Dun1. (B) MMS-induced hyperphosphorylation of Dun1 is absent in *sad1-1* mutants. Wild-type (Y300) and *sad1-1* (Y301) strains containing a plasmid, pZZ74 (Zhou and Elledge 1993) that overproduce Dun1 under control of the *GAP* promoter were grown in YPD to mid-log phase and labeled with [32 P]H $_3$ PO $_4$ for 2 hr at 30°C in the presence or absence of 0.1% MMS. Dun1 was immunoprecipitated from protein extracts, fractionated by SDS-PAGE, and transferred to nitrocellulose. Autoradiography was performed to determine the phosphorylation status of Dun1, and the filter was immunoblotted with anti-Dun1 to show that equal amounts of Dun1 were precipitated in each sample.

more, Spk1 was shown to be nuclear localized and to display cell cycle-regulated transcription (Zheng et al. 1993).

A null allele for *SAD1* was generated (Fig. 5A) and used to create a diploid heterozygous for *SAD1*. Sporulation of the *SAD1*/ Δ *sad1*::*HIS3* heterozygous diploid revealed that *SAD1* is essential for cell viability (see Materials and methods) as has also been observed for *SPK1* (Zheng et al. 1993). Inclusion of *SAD1* on a centromeric *URA3* plasmid allows recovery of haploids with chromosomal *sad1*::*HIS3* alleles. These strains were unable to grow in the absence of the plasmid as evidenced by lethality on media containing 5-fluoro-orotic acid (5-FOA) (data not shown), demonstrating further that *SAD1* is essential for mitotic viability.

Identification of *SAD1* as a protein kinase suggests that protein phosphorylation is important for checkpoint function and for coupling S phase and mitosis. To test this hypothesis, a mutant allele in which a conserved lysine in the ATP-binding site is changed to an alanine was constructed, *sad1-11* (K227A), and transformed into *sad1-1* and Δ *sad1*::*HIS3* null mutants. The identical mutation in the Dun1 kinase was found to diminish its kinase activity severely without affecting its stability (Zhou and Elledge 1993). *sad1-11* (K227A) fails to complement the HU sensitivity of *sad1-1* (Fig. 5B) or lethality of the Δ *sad1*::*HIS3* mutation (Fig. 5C) using a plasmid shuffle assay, suggesting that Sad1 kinase activity is required for both the cell cycle checkpoint and essential functions.

cks1 Mutants suppress the HU sensitivity of *sad1*

The mechanism through which the DNA damage and S-phase checkpoint pathways inhibit cell cycle progression is not known. An attractive target for inhibition of the G $_1$ /S and G $_2$ /M cell cycle transitions is the p34^{CDC28} protein kinase, as both transitions are regulated by p34 as well as the *SAD1*-dependent checkpoints. There is evidence to suggest that HU treatment of fission yeast cells results in persistent inhibitory phosphorylation of Tyr-15 on p34^{cdc2}. However, mutational inhibition of tyrosine phosphorylation on p34^{CDC28} has no effect on the budding yeast S-phase checkpoint, and double mutant combinations of *cdc28*:Y19F and *sad1-1* do not exacerbate *sad1-1* HU lethality (data not shown). An equally likely target is *CKS1* (Hadwiger et al. 1989), the budding yeast homolog of *S. pombe* *suc1*⁺. p13^{suc1} binds p34/cyclin complexes and has the ability to alter the substrate specificity of p34^{cdc2} (Kusubata et al. 1992). *CKS1* is also required for both the G $_1$ /S and G $_2$ /M transitions (Tang and Reed 1993) and is found associated with p34^{CDC28} in HU-treated cells (Stueland et al. 1993). Although the biochemical significance of p18^{CKS1} binding to p34^{CDC28} is not clear, a possible mechanism for *SAD1*-dependent inhibition of cell cycle progression is negative regulation of *CKS1*. If this were the case, it might be possible to suppress the HU sensitivity of *sad1-1* by a partial loss of *CKS1* function. To test this possibility, *sad1 cks1* double mutants were constructed

Figure 5. *SAD1* kinase activity is required to complement *sad1-1* and $\Delta sad1$ mutants; and *cks1* mutants can suppress the HU sensitivity of *sad1-1*. (A) Position of *SAD1* on chromosome XVI. Arrows indicate the direction of transcription. Restriction sites: (B) *Bam*HI; (M) *Mlu*I; (S) *Sty*I; (X) *Xho*I. The position of the replacement of the *SAD1*-coding sequences with the *HIS3*-*Km^r* cassette is indicated. Also shown is the position of the K227A site-directed mutation. (B) Yeast strains were streaked upon an SC-uracil plate containing 18 mM HU and incubated at 23°C. From the top quadrant and clockwise: (Y303) *sad1-1* *CKS1* + pJA92 (*SAD1*); (Y302) *sad1-1* *CKS1* + pUN70; (Y314) *SAD1* *cks1-ts38* + pUN70; (Y316) *sad1-1* *cks1-ts38* + pUN70; (Y317) *sad1-1* *cks1-ts38* + pJA103 (*CKS1*); and (Y304) *sad1-1* *CKS1* + pJA107 (*sad1-11* K227A). All strains were grown on SC-uracil in the absence of HU (data not shown). (C) To test for complementation of the *sad1::HIS3* deletion, a plasmid shuffle assay was employed using Y324 [*ura3-1* *trp1-1* $\Delta sad1::HIS3$ + pJA92 (*URA3*, *SAD1*)]. Y324 containing pUN40 (*TRP1*), pJA121 (*SAD1* *TRP1*), or pJA101 (*TRP1* *sad1-11*) and Y300 containing pUN40 (*TRP1*) were streaked onto SC plates containing 5-FOA and incubated at 30°C. Only cells capable of growing in the absence of pJA92 can survive on this media.

and assayed for growth on HU. As shown in Figure 5B, the *cks-ts38* temperature-sensitive allele suppresses the HU-sensitivity of *sad1-1*. Suppression of *sad1-1* is attributable to the presence of the *cks1-ts38* mutation, because a plasmid-borne copy of wild-type *CKS1* abolishes the ability of the double mutant to grow. This result suggests that the *SAD1*-dependent checkpoint pathway might inhibit cell cycle progression through negative regulation of *CKS1*. Alternatively, *cks1* mutants may suppress *sad1* through nonspecific elongation of a critical cell cycle transition. Regardless of the involvement of *CKS1* in checkpoint function, these experiments provide further evidence supporting the notion that it is inappropriate cell cycle progression that leads to death in *sad1* mutants.

Overproduction of *SAD1* can suppress the UV sensitivity of *rad9* mutants

sad1 and *rad9* mutants are both unable to arrest the cell cycle in *G*₁ or *G*₂ in response to DNA damage. It is possible that they are components in a common genetic pathway. To investigate this relationship further, we examined the effects of *SAD1* overproduction on UV sensitivity in a *rad9* mutant. If *RAD9* acts downstream of *SAD1* in a linear genetic pathway, then overexpression of *SAD1* in a *rad9* null mutant should have no effect on the *rad9* phenotype. However, overproduction of *SAD1* from the inducible *GAL* promoter strongly suppresses the UV sensitivity of *rad9* mutants (Fig. 6). This result suggests that it is unlikely that *RAD9* is downstream of

Figure 6. Overexpression of *SAD1* suppresses the UV sensitivity of *rad9* mutants. Y318 (*rad9::LEU2*), containing pUN70 (*URA3*, ■, □), or pJA98 (*URA3 GAL:SAD1*, ●, ○), were grown to log phase in SC-uracil media containing either glucose (□, ●), or galactose (□, ○) as a carbon source. Dilutions were plated onto SC-uracil medium containing the appropriate carbon source and were UV-irradiated at the indicated doses. After 3–4 days growth at 30°C, colony-forming units were counted.

SAD1 in a linear pathway, although alternative interpretations of this experiment exist and are discussed below.

Discussion

Inhibition of DNA synthesis results in cell cycle arrest prior to mitosis through the action of a cell cycle checkpoint. A genetic screen coupled with an analysis of pre-existing *rad* mutants has identified *SAD* genes controlling this checkpoint. Some of these *sad* and *rad* mutants allow yeast cells to continue cell cycle progression in the presence of the DNA synthesis inhibitor HU. We have focused on *SAD1*, an essential gene that we found to encode a protein kinase. *sad1* and *sad3* (data not shown) mutants are defective in the S-phase checkpoint, as well as the G_1 and G_2 DNA damage checkpoints. In addition, *sad1* mutants are also defective for transcriptional activation of DNA damage-inducible genes. During the course of this work, *sad1* was also isolated independently as *mec2* in a screen for checkpoint mutants showing synthetic lethality with *cdc13* mutations (Weinert et al. 1994). As mentioned in the introductory section, *mec2* mutants were found to display properties consistent with a role in the S-phase and G_2 checkpoints and were shown to be sensitive to a variety of DNA damaging treatments.

SAD1-dependent cell cycle arrest

SAD1 controls the G_1 , G_2 , and S-phase checkpoints. The S-phase and G_2 checkpoints may actually represent the same arrest point in the cell cycle, but G_1 is clearly distinct. The fact that all three checkpoints are controlled by a single gene suggests a common mechanism of cell cycle arrest at these different points. Because *SAD1* controls both G_1 and G_2 checkpoints, it is possible that the

Sad1 target also has a role during both transitions. It is well established that both the G_1/S and the G_2/M transitions in budding yeast are controlled by the p34^{CDC28} protein kinase, associated cyclin regulatory subunits, and the *CKS1* gene, a Cdk-binding protein of unknown biochemical function. The observation that *cks1-ts38* suppresses the HU sensitivity of *sad1-1* suggests a possible role for *CKS1* in *SAD1*-dependent checkpoints. *Sad1* could negatively regulate *Cks1* and prevent its transition-promoting activity. To this end, it will be of interest to determine whether *Cks1* association with p34^{CDC28} is cell cycle or DNA damage regulated as well as whether *Sad1* can phosphorylate *Cks1* in vitro. However, it is equally plausible that hypomorphic alleles of *CKS1* suppress *sad1* by providing a synthetic delay at key cell cycle transitions. If *CKS1* is not involved in the *SAD1*-dependent checkpoint, it must define the existence of an independent checkpoint because *sad1* mutants do not alter its *cdc* phenotype. Furthermore, the *CKS1* checkpoint is distinct from the checkpoint activated by nocodazole because nocodazole treatment cannot suppress *sad1*.

Cyclins are another possible inhibitory target. Cyclin overexpression fails to show checkpoint deficiencies (Stueland et al. 1993), although other aspects of cyclin function have not been explored and remain as potential effectors. An alternative explanation is the existence of a control system that can alter p34^{CDC28} activity in a manner independent of tyrosine phosphorylation, such as by synthesis of a Cdk inhibitor analogous to the mammalian *CIP1* gene, an inhibitor of cyclin-dependent kinases. However, yeast homologs have not yet been identified. Finally, it is also possible that *SAD1* delays the cell cycle in a p34-independent manner, perhaps through negative regulation of an unidentified cell cycle regulator such as the NimA kinase in *Aspergillus* (Osmani et al. 1988). Identification of in vivo *Sad1* substrates will be a critical step toward understanding how this cell cycle arrest is mediated.

Nature of HU-induced lethality

sad1 mutants commit to inviability soon after entering S phase in the presence of HU. A similar observation has been reported recently for a class of mutants in *S. pombe* (Enoch et al. 1992), and it has been suggested that these mutants are defective for recovery from S-phase arrest in addition to being deficient in the S-phase feedback control. We have examined DNA synthesis in *sad1* cells released from an α -factor block into HU and nocodazole for 30 min and then placed into nocodazole alone and have observed no difference between wild-type and *sad1* cells by FACS (J. Allen and S. Elledge, unpubl.). This suggests that the majority of DNA synthesis can resume after HU arrest. Whereas *sad1* mutants may also be defective for some aspect of S-phase arrest recovery, an alternative explanation is that following release from G_1 in the presence of HU, *sad1* cells become prematurely and irreversibly committed to a series of biochemical reactions that result in initiation of mitosis. Although

segregation of unreplicated chromosomes would certainly be lethal, it is unlikely to be the sole explanation of lethality in these cells because nocodazole, which blocks anaphase, is unable to suppress killing when present during and after HU treatment (data not shown). This is based on the assumption that cells are still competent to replicate DNA at the nocodazole block, which has not been tested. Thus, the nature of HU-induced lethality is not clear but may result from other aspects of premature mitotic commitment such as chromosome condensation, maturation of kinetochores, or alterations in nuclear architecture.

Does SAD1 coordinate S phase and mitosis in a normal cell cycle?

The S-phase and DNA damage checkpoints allow cells to integrate cell cycle progression with the replicational status and integrity of the genome. However, it is not known whether these checkpoints also order events in a normal cell cycle in the absence of DNA damage or replication blocks, or whether S-phase and mitotic coordination is normally ensured by relative timing. The interpretation of an active mitotic inhibitory signal produced from S-phase nuclei is supported by the experiments of Rao and Johnson (1970), who observed that fusion of unperturbed mammalian S-phase cells to G₂ cells caused the G₂ nucleus to arrest entry into mitosis until the S-phase nucleus completed DNA replication. The data presented here do not provide a definitive answer for yeast, although several properties of *sad1* mutants are germane to this issue. First, *SAD1* is a member of the *MluI* cell cycle box (MCB) group of cell cycle-regulated genes expressed in S phase that encode many enzymes essential for S phase (Zheng et al. 1993; J. Allen and S. Elledge, unpubl.). Second, unlike *RAD9*, *SAD1* is essential for viability, suggesting a role in every cell cycle. It is likely that this essential function is during S phase, although *SAD1* could be essential for reasons unrelated to feedback control. Finally, *sad1* mutants accelerate mitotic entry in the presence of HU, although not significantly faster than wild-type cells. It is difficult to imagine why a treatment that normally blocks cell cycle progression would have the opposite effect in a *sad1* mutant. Perhaps the signal to inhibit mitosis emanates from active replication forks that are diminished in the presence of HU. Normally, a dampened but persistent signal would be sufficient to activate the checkpoint, but in a *sad1-1* mutant the reduced signal and diminished transducer together would be insufficient and cells would commit to mitosis faster. Without a conditional lethal allele of *SAD1*, it is not possible to determine definitively whether Sad1 controls normal timing of mitosis.

SAD1 controls the cell cycle arrest response to DNA damage

sad1 mutants are defective for the *RAD9*-dependent G₁ and G₂ DNA damage checkpoints. If *RAD9* functions downstream of *SAD1*, then overexpression of *SAD1* in a

Δrad9 background should have no effect with respect to the damage sensitivity of *Δrad9* mutants. However, overexpression of *SAD1* partially suppresses the UV sensitivity of *Δrad9* strains. If *SAD1* overproduction enhances its role in checkpoint-mediated cell cycle arrest, then *RAD9* cannot function after *SAD1* in a linear pathway. However, overexpression of *SAD1* slightly slows down the cell cycle in wild-type cells. If this slow growth is not a manifestation of the checkpoint function of *SAD1*, suppression of *rad9* mutants may be attributable to nonspecifically lengthening the cycle. Because we cannot distinguish between these alternatives, we cannot definitively order *SAD1* and *RAD9*.

SAD1 controls the transcriptional response to DNA damage

In addition to multiple checkpoint deficiencies, *sad1* mutants also exhibit a DNA damage-uninducible phenotype similar to that of the *dun* mutants (Zhou and Elledge 1993), defective for *RNR2* and *RNR3* induction but proficient for *UBI4* induction. Mutants in *DUN1*, a protein kinase whose activity is increased in response to DNA damage, are proficient in their cell cycle response to DNA damage and have only a subset of *sad1* phenotypes, suggesting that *DUN1* is downstream of *SAD1*. In addition, Dun1 is not activated in *sad1* mutants. Based on these observations coupled with the DNA damage checkpoint role, it is likely that the Sad1 kinase is positioned very close to the signal sensor in the DNA damage signaling pathway.

The relationship between the checkpoint and transcriptional roles of *SAD1* is not clear. The absence of *RNR3* expression during a normal S phase in which *SAD1* is likely to function presents a puzzle. If Sad1 is functioning during a normal S phase to activate the checkpoint, why does it not also activate expression of *RNR3* at that time? The presence of DNA damage or a replication block must provide information distinct from normal DNA replication to *SAD1* or its effectors. Sad1 does contain potential regulatory domains on either side of the kinase domain that could respond to or send different signals. Activation of *RNR3* transcription could require higher levels of Sad1 function than is necessary to activate the checkpoint. Alternatively, the activation of a checkpoint might lengthen the period of the cell cycle during which a factor accumulates that facilitates transcriptional activation. This factor could be active *SAD1* or a substrate. Another possibility is that a *SAD1*-independent pathway is additionally required to provide DNA damage information necessary for *RNR3* induction. These complexities can only be resolved by rigorous biochemical and genetic analysis of the *SAD1* kinase.

A model depicting the genetic pathway regulated by Sad1 is shown in Figure 7. *SAD1* is placed downstream from the direct sensors for DNA damage and DNA replication blocks. Although no formal evidence has been presented that Sad1 is regulated in response to these stimuli, the fact that Sad1 is a protein kinase makes it

Figure 7. A model for the role of *SAD1* in cell cycle control and the cellular response to DNA damage. *SAD1* is required to delay the cell cycle in response to DNA damage in G_1 and G_2 and for arrest induced by replication blocks. An arrow is placed between DNA damage and replication blocks, as some agents are likely to activate both pathways. *RAD9* is tentatively placed upstream of *SAD1*. Although shown schematically, it is not clear whether *SAD1* arrests the cell cycle in response to replication blocks by the same mechanism that *SAD1* prevents mitosis in response to DNA damage at G_2/M . *SAD1* is also required for the DNA damage-induced expression of *RNR2* and *RNR3*. *Dun1* is placed downstream of *SAD1* because *Dun1* kinase activity is not activated in response to DNA damage in *sad1-1* mutants.

highly likely that it will function as a signal transducer. An arrow is placed between DNA damage and replication blocks because some agents may activate both pathways. *DUN1* is placed downstream of *SAD1* because *SAD1* is necessary for activation of *Dun1* kinase activity in response to DNA damage and replication blocks. This epistatic relationship suggests the possibility that *Dun1* may be a direct substrate for the *Sad1* kinase. Because *Dun1* has intact cell cycle checkpoints, a separate branch leads from *SAD1* to the cell cycle arrest. It should be noted that although *Dun1* mutants are not checkpoint defective, *Dun1* may play a redundant role in the checkpoint pathway. *RAD9* is placed upstream of *SAD1* because of the suppression data, although as mentioned earlier, this positioning is tentative.

A number of experiments in fission yeast have suggested that active replication is necessary to prevent mitotic entry. *cdc18* and *cut5* mutants fail to initiate DNA replication and allow cells to enter mitosis with a G_1 content of DNA (Kelly et al. 1993; Saka and Yanagida 1993). If they are allowed to first enter S phase before a shift to the nonpermissive temperature, they must finish replication before mitosis can begin. Thus, active replication is implicated in checkpoint function. These mutants are unlike *sad1* mutants in that they are not HU sensitive. An alternative model for *Sad1* function might be in maintaining the integrity of the replication com-

plexes needed for signaling during disruptive events that might promote complex disassembly. Complex disassembly would signal S-phase completion. In this model, *SAD1* would play an indirect role in checkpoint function. Current information cannot distinguish between these models. The model that *Sad1* is a direct transducer of the checkpoint signal predicts that *Sad1* activity will vary in response to checkpoint activating signal. Analysis of *Sad1* kinase activity regulation in response to DNA damage or isolation of constitutive cell cycle arrest mutants of *SAD1* would facilitate resolution of these issues.

Feedback controls and cancer

The consequences of checkpoint control deficiency in unicellular organisms include chromosome loss, genetic instability, and cell death. There is an accumulating body of evidence that suggests that lesions in feedback controls, such as p53 and AT, play a pivotal role in tumorigenesis in mammals. It is probable that checkpoint control mechanisms will be conserved throughout evolution as is the basic cell cycle regulatory machinery and that further molecular analysis of the *SAD* and *RAD* genes will provide a general framework for understanding feedback controls and checkpoint function in all eukaryotes.

Materials and methods

Yeast strains, genetic procedures, and growth conditions

Yeast strains used in this study are listed in Table 2. Standard yeast genetic procedures were used (Rose et al. 1990). Yeast cultures were grown in YPD (1% yeast extract, 2% Bacto-peptone, supplemented with 2% glucose or galactose, as indicated). Synthetic complete (SC) medium is minimal medium containing various amino acids as described (Sherman et al. 1979) and was used when plasmid selection was required. Yeast transformation was performed by the lithium acetate procedure (Ito et al. 1983). The DNA synthesis inhibitor HU (Sigma) was added to media at 0.2 M unless stated otherwise. Benomyl, which hydrolyzes in solution to the microtubule inhibitor methyl benzimidazole-2-yl-carbamate, was supplied by DuPont. G_1 cell cycle arrest was achieved by incubating log-phase cultures grown in YPD (pH 3.9) with 5 μ g/ml of α -factor (Peninsula Laboratories) for 1 hr at 30°C and subsequent addition of 5 μ g/ml of α -factor for 1 hr prior to release. Yeast strains were grown at 30°C unless stated otherwise. Killing curves for HU and UV treatment were performed in duplicate, and the average of two points is shown.

Staining of cells for microscopy and flow cytometry

Cells were fixed by the addition of 3.7% formaldehyde to growing cultures that were incubated at 30°C for 1 hr. Cells were washed twice in phosphate-buffered saline (PBS) and resuspended in PBS + 1.2 M sorbitol. Cells were stained for DNA with 1 μ g/ml of DAPI, and microtubules were visualized with the antitubulin monoclonal antibody YOL1/34 and a FITC-conjugated secondary antibody as described (Kilmartin and Adams 1984). To categorize cellular morphologies, 200 cells were scored for each time point. To measure DNA content, cells were fixed with 70% ethanol and stained with propidium iodide as

Table 2. Strain list

Name	Relevant genotypes	Source
Y300	<i>MATa; can1-100; ade2-1; his3-11,15; leu2-3,112; trp1-1; ura3-1</i>	this study
Y301	<i>MATa; sad1-1; can1-100; ade2-1; his3-11,15; leu2-3,112; trp1-1; ura3-1</i>	this study
Y302	Y301 + pUN70 (<i>URA3</i>)	this study
Y303	Y301 + pJA92 (<i>URA3 SAD1</i>)	this study
Y304	Y301 + pJA107 (<i>URA3 sad1-11 K227A</i>)	this study
Y305	<i>MATa; sad2-1; can1-100; ade2-1; his3-11,15; leu2-3,112; trp1-1; ura3-1</i>	this study
Y306	<i>MATa; sad3-1; can1-100; ade2-1; his3-11,15; leu2-3,112; trp1-1; ura3-1</i>	this study
Y307	<i>MATa; sad4-1; can1-100; ade2-1; his3-11,15; leu2-3,112; trp1-1; ura3-1</i>	this study
Y308	<i>MATa; sad5-1; can1-100; ade2-1; his3-11,15; leu2-3,112; trp1-1; ura3-1</i>	this study
Y309	<i>MATa; cdc9-1; his7-1; ura1-1</i>	this study
Y310	<i>MATa; cdc9-1; sad1-1</i>	this study
Y311	<i>MATa; cdc9-1; sad1-1; ade2-1</i>	this study
Y312	<i>MATa/α; SAD1/sad1::HIS3; can1-100/can1-100; ade2-1/ade2-1; his3-11,15/his3-11,15; leu2-3,112/leu2-3,112; trp1-1/trp1-1; ura3-1/ura3-1</i>	this study
Y313	<i>MATa; cks1::LEU2; ade1; his2; leu2-3,112; trp1-1; +pCKS1 (TRP1 CKS1)</i>	Tang and Reed (1993)
Y314	<i>MATa; cks1::LEU2; ade1; his2; leu2-3,112; trp1-1; +pcks1-ts38 (TRP1 cks1-ts38)</i>	Tang and Reed (1993)
Y315	<i>MATa; sad1-1; cks1::LEU2; leu2-3,112; trp1-1; +pcks1-ts38 (TRP1 cks1-ts38)</i>	this study
Y316	Y315 + pUN70 (<i>URA3</i>)	this study
Y317	Y315 + pJA103 (<i>URA3 CKS1</i>)	this study
Y318	<i>MATa; rad9::LEU2; ade2-1; his3-11,15; leu2-3,112; trp1-1; ura3-1</i>	this study
Y319	Y318 + pUN70 (<i>URA3</i>)	this study
Y320	Y318 + pJA98 (<i>URA3 GAL-SAD1</i>)	this study
Y321	Y300 + pUN70 (<i>URA3</i>)	this study
Y322	Y300 + pJA98 (<i>URA3 GAL-SAD1</i>)	this study
Y323	<i>MATa/α; can1-100/can1-100; ade2-1/ade2-1; his3-11,15/his3-11,15; leu2-3,112/leu2-3,112; trp1-1/trp1-1; ura3-1/ura3-1</i>	this study
Y324	<i>MATa; can1-100; ade2-1; his3-11,15; leu2-3,112; trp1-1; ura3-1 Δsad1::HIS3 + pJA92 (URA3, SAD1)</i>	this study

described (Hutter and Eipel 1979). The DNA content of at least 10,000 cells was determined for each sample with an EPICS model 753 Laser System.

sad mutant isolation

Y300 was mutagenized with ethyl methanesulfonate to 20% survival and plated on YPD plates at 30°C. Mutagenized colonies were replica-plated to YPD plates, and YPD plates containing 100 mM HU and ~700 HU-sensitive strains were isolated. HU-sensitive mutants were assayed for HU reversibility by replica-plating to YPD plates containing 200 mM HU, incubation for 12 hr at 30°C, and replica-plating back to YPD plates. Forty-five strains that failed to recover were chosen for further study. These were assayed further for HU reversibility by treating log-phase cultures in YPD with 200 mM HU for 6 hr at 30°C. Aliquots were removed at 2-hr intervals, and dilutions were plated onto YPD plates and incubated at 30°C for 3–4 days to determine percent survival. Nine mutants were isolated that showed a rapid loss of viability in the presence of HU. α -Factor suppression of HU-induced lethality was tested by arresting mutant strains in G₁ with α -factor prior to release into YPD that contained 200 mM HU or media containing α -factor and HU. At 2-hr intervals, dilutions were plated on YPD plates to determine percent survival, and 2 μ g/ml of α -factor was added to α -factor-treated samples. Six *sad* mutants representing five complementation groups were isolated that exhibit α -factor-suppressible HU lethality. Mutants were backcrossed six times to nonmutagenized parental strains to rid the cell background of unlinked mutation arising during mutagenesis.

Determination of DNA damage-induced cell cycle delay

To measure G₁ arrest in response to DNA damage, log-phase cultures were synchronized in G₁ with α -factor as described above, plated onto YPD plates, and UV-irradiated with 40 J/m². Cells were washed from plates, rinsed to remove pheromone, and resuspended in fresh YPD. At timed intervals, samples were removed for analysis of DNA content and determination of budding kinetics. Two hundred cells were scored for each sample.

Y310 and Y311 (*cdc9-1 sad1-1*) were derived by crossing Y301 and Y309. The presence of both mutations was verified by temperature sensitivity and failure to complement *cdc9* or *sad1* strains and by complementation of *cdc9-1 sad1-1* HU sensitivity by pJA90 (*SAD1*). For reversibility analysis, wild-type (Y300), *cdc9-1* (Y309), and *cdc9-1 sad1-1* (Y310 and Y311) strains were grown to log phase at 23°C and shifted to 37°C for 4 hr. Samples were removed at timed intervals and dilutions were plated onto YPD plates at 23°C. After 5 days growth, colony-forming units were counted to determine cell survival. G₂/M DNA damage-induced cell cycle delay was also assayed by UV-irradiating benomyl-arrested cells and following the rate of nuclear division following benomyl release as described (Weinert and Hartwell 1993).

DNA damage-induced transcription in sad1 mutants

Y300 and Y301 were grown in YPD to OD₆₀₀ at 0.05 at 23°C. Low temperature and density were used to maintain low *UBI4* basal levels. Cultures were split into two, and experimental samples were treated with 0.1% MMS for 1 hr. Total RNA was prepared and subjected to Northern analysis. Probes corre-

sponded to the 0.9-kb *HindIII* fragment of *RNR2* from pSE310, the 2.5-kb *MluI*–*HindIII* fragment of *RNR3* from pSE734, and the 2.2-kb *EcoRI* fragment of *UBI4* from pUB200.

Immunoprecipitations and kinase assays

Ueast extract (100 μ g) was diluted in 500 μ l of DB buffer (20 mM Tris-HCl at pH 7.9, 100 mM NaCl, 2 mM EDTA, 0.05% Tween 20, 1 mM PMSF, 0.1 μ g/ml of leupeptin, 0.1 mM benzimidazole, 1 μ M aprotinin, 0.1 μ g/ml of pepstatin, 0.1 mM Na_3VO_4 , and 30 mM NaF) and incubated with 10–20 μ l of affinity-purified anti-Dun1 antibodies for 1 hr at 4°C. Protein A–Sepharose CL-4B beads were used to precipitate immunocomplexes as described (Zhou and Elledge 1993). Immunocomplexes were incubated in 25 μ l of kinase buffer with 2 μ M cold ATP and 2.5 μ Ci of [γ - 32 P]ATP for 30 min at 30°C. Reactions were terminated by the addition of 25 μ l of 2 $\times$ SDS sample buffer. Phosphorylated products were visualized by 8% SDS-polyacrylamide gels and autoradiography.

Determination of the *in vivo* phosphorylation state of Dun1

Y300 and Y301 were grown to OD₆₀₀ of 0.2 in 100 ml of YPD, without inorganic phosphate (YPD-PO₄) (Salah-ud-Din et al. 1990). Cells were pelleted, resuspended in 10 ml of YPD-PO₄ containing 1 mCi of [32 P]H₃PO₄, and cultured for 2 hr at 30°C in the presence or absence of 0.1% MMS. Protein extraction and immunoprecipitation of Dun1 were performed as described above except that 100 μ g of RNase A was added to each immunoprecipitation reaction prior to electrophoresis. Samples were subjected to Western blot analysis and autoradiography.

Cloning of SAD1

A yeast genomic centromeric library carrying the *URA3*-selectable marker (Ramer et al. 1993) was transformed into *sad1-1*. *Ura*⁺ transformants (5×10^5) were plated onto SC plates lacking uracil containing 100 mM HU. Three plasmids, pJA90, pJA91, and pJA92, complemented *sad1-1* upon retransformation and contained overlapping genomic inserts. A common 1.5-kb *XhoI* fragment was subcloned into pBS KSII+ (Stratagene) and sequenced (Sanger et al. 1977). A null allele for *SAD1* was generated by transplacement of the 3.9-kb *HIS3*-containing *XhoI* fragment from pJA100 into Y323 and selection for histidine prototrophy. Diploids (Y312) heterozygous for *SAD1* were confirmed by Southern blotting. pJA93 (*URA3 SAD1*) rescues viability of His⁺ haploids from tetrads. His⁺ *Ura*⁺ spores are also 5-FOA sensitive, demonstrating the requirement for wild-type *SAD1*.

Plasmid constructions

The 4.2-kb *XhoI* partial fragment from pJA90 was subcloned into *XhoI*-digested pBS KSII+ (Stratagene) to give pJA93. The *SAD1* open reading frame was amplified from pJA93 by polymerase chain reaction (PCR) with the 5' primer, 5'-CAGGCATATCGGAAATATTACACAACCC-3', and the 3' primer, 5'-TCTTAGCGGCCGCCCATGGGCGAAATTGCAATTCGCGG-3'. *NdeI* and *NotI* sites are underlined, respectively. The PCR-derived product was digested with *BglII*, and the resulting 2.0-kb fragment representing the 3' region of *SAD1* was subcloned as a *BglII* blunt-ended fragment into *BglII*–*SmaI*-digested pSE386 to give pJA95.

To place *SAD1* under T7 control, a three-way ligation was performed using the 0.5-kb fragment from the *NdeI*–*BglII*-digested *SAD1* PCR-derived product described above, the 3.2-kb

BglII–*NotI* fragment from pJA95, and the *NdeI*–*NotI*-digested pETHAX vector to give pJA97. The PCR-derived fragment was sequenced after cloning and was not mutant. To place *SAD1* under control of the *GAL* promoter on a *CEN* plasmid, the 2.7-kb *NcoI*(blunt)–*NotI* fragment from pJA97 was cloned into *XbaI*(blunt)–*NotI*-digested pSE556 to give pJA98.

A transplacement vector, pJA100, containing a *SAD1* null allele was constructed by replacing the 1.7-kb *StyI* fragment of pJA93 with the 2.3-kb *SmaI* fragment from pJA50 (Allen and Elledge 1994), which contains the *HIS3* and Tn5 *neo*-selectable markers.

Site-directed mutagenesis was performed to generate a kinase-deficient mutant of *sad1*. pJA93 was used as a template for PCR to mutate nucleotides 712–714 from AAG (Lys) to GCC (Ala). First, two PCR reactions were carried out to produce overlapping amplified fragments. In reaction 1, wild-type forward primer (5') CATGCCATGGGTAGAAACCCAGCCTGTGAC (3') and mutagenic reverse primer (5') ACTTATAATGGCCACCGCGAATGTTTTTC (3') were used. In reaction 2, mutagenic forward primer (5') GGGAAAACATTCCGGTGGCCATTATAAGTAAA (3') and wild-type reverse primer (5') GCTCTAGATTATTGAGCATCGTCCATATTTTC (3') were used. PCR-derived products were recovered from agarose gels, and 10 ng of each were combined and amplified using wild-type forward and reverse primers. The full length PCR-derived fragment was digested with *XhoI* and *BamHI*, and the resulting 1.2-kb fragment was subcloned into *XhoI*–*BamHI*-cleaved pBS KSII+ for sequencing to confirm the presence of the mutant codon and the absence of other PCR-induced mutations. The 1.2-kb *BglII*–*BamHI* fragment from PCR-mutagenized product was subcloned into *BglII*–*BamHI*-digested pJA93 to give pJA104. The 1.7-kb *StyI* fragment from pJA93 was subcloned into the *StyI* site of pJA104 to give pJA105. In addition, the 2.4-kb *SacI* fragment from pJA93 was subcloned into *SacI*-digested pJA105 to give pJA106. A site-directed mutagenized *sad1* allele (*sad1-11* K227A) under control of the endogenous *SAD1* promoter was generated in a three-way ligation using the 0.5-kb *KpnI*–*BglII* fragment from pJA93, the 3.2-kb *BglII*–*XbaI* fragment from pJA106, and *KpnI*–*XbaI*-digested pUN70 (Elledge and Davis 1988).

pJA103 (*CKS1 URA3*) was made by ligating the 2.2-kb *SmaI* *URA3 neo* fragment from pJA53 (Allen and Elledge 1994) into *EcoRV*-cleaved pSE271:CKS1 (Tang and Reed 1993).

Southern and Northern blot analysis

DNA probes were labeled by the hexamer primer method (Feinberg and Vogelstein 1983). Hybridizations for Southern blots were carried out as described previously (Elledge and Davis 1987). Yeast RNA was isolated using the hot phenol method (Kohrer and Domdey 1991). RNA was resolved on formaldehyde–1.2% agarose gels (Sambrook et al. 1989), and hybridizations were carried out as described for Southern analysis.

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