Suppressors of Cdc25p Overexpression Identify Two Pathways That Influence the G2/M Checkpoint in Fission Yeast

Kristi Chrispell Forbes, Timothy Humphrey[1] and Tamar Enoch

Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115

Manuscript received July 8, 1998
Accepted for publication August 24, 1998

ABSTRACT

Checkpoints maintain the order of cell-cycle events. At G2/M, a checkpoint blocks mitosis in response to damaged or unreplicated DNA. There are significant differences in the checkpoint responses to damaged DNA and unreplicated DNA, although many of the same genes are involved in both responses. To identify new genes that function specifically in the DNA replication checkpoint pathway, we searched for high-copy suppressors of overproducer of Cdc25p (*OPcdc25*$^+$), which lacks a DNA replication checkpoint. Two classes of suppressors were isolated. One class includes a new gene encoding a putative DEAD box helicase, *s*uppressor of *u*ncontrolled *m*itosis (*sum3*$^+$). This gene negatively regulates the cell-cycle response to stress when overexpressed and restores the checkpoint response by a mechanism that is independent of Cdc2p tyrosine phosphorylation. The second class includes *chk1*$^+$ and the two *Schizosaccharomyces pombe* 14-3-3 genes, *rad24*$^+$ and *rad25*$^+$, which appear to suppress the checkpoint defect by inhibiting Cdc25p. We show that *rad24*Δ mutants are defective in the checkpoint response to the DNA replication inhibitor hydroxyurea at 37° and that *cds1*Δ *rad24*Δ mutants, like *cds1*Δ *chk1*Δ mutants, are entirely checkpoint deficient at 29°. These results suggest that *chk1*$^+$ and *rad24*$^+$ may function redundantly with *cds1*$^+$ in the checkpoint response to unreplicated DNA.

CONTROL mechanisms called checkpoints help to maintain the correct order of cell-cycle events (Hartwell and Weinert 1989). In wild-type cells, checkpoints ensure that later events are dependent upon the completion of earlier events. The G2/M checkpoint ensures that mitosis does not take place when DNA replication is incomplete or chromosomes are damaged. As a result of this checkpoint, wild-type fission yeast cells with damaged or incompletely replicated DNA undergo cell-cycle arrest at G2/M. The cells continue to grow while arrested, becoming highly elongated. In contrast, checkpoint mutants are unable to delay their cell cycle in response to incomplete DNA replication or DNA damage (Enoch and Nurse 1990). They enter mitosis and form a spindle, but the unreplicated chromosomes remain in the center of the cell. Ultimately the nucleus is cleaved by the septum resulting in inviable cells. Such aberrant mitoses are referred to as "cuts" because they resemble the phenotype of *cut*$^-$ mutants (Hirano *et al.* 1986).

In fission yeast, inhibitory tyrosine phosphorylation of Cdc2p, the catalytic subunit of cyclin-dependent kinase, is required for the G2/M checkpoint (Enoch and Nurse 1990; Enoch *et al.* 1991; Rhind *et al.* 1997). The balance between the activities of the Cdc2p inhibitory kinases, Wee1p and Mik1p, and the Cdc2p activating phosphatases, Cdc25p and Pyp3p, determines whether a cell will pass the G2/M boundary (Dunphy 1994). Cells in which this balance is altered are checkpoint defective. For example, a strain that constitutively overproduces Cdc25p (*OPcdc25*$^+$) lacks the DNA replication checkpoint. A *cdc2-3w* strain, which renders Cdc2p activation independent of Cdc25p, is also defective in this checkpoint (Enoch and Nurse 1990). A *wee1-50 mik1*Δ strain, which has greatly decreased tyrosine kinase activity because of a temperature-sensitive allele of *wee1*$^+$ and the deletion of the *mik1*$^+$ gene, is checkpoint defective even at the permissive temperature for *wee1-50*. At the nonpermissive temperature, these cells are inviable (Sheldrick and Carr 1993).

A picture of the molecular events that may underlie the checkpoint response to damaged DNA is beginning to emerge. It is hypothesized that Rad3p kinase is activated in the presence of DNA damage. The products of five other genes termed the "*checkpoint rad*" genes, *rad1*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$, and *hus1*$^+$, are also required for the early phase of the checkpoint response (Humphrey and Enoch 1995). Many of these genes are evolutionarily conserved (Stewart and Enoch 1996); for example, the *rad3*$^+$ gene is functionally and structurally similar to the *MEC1* and *TEL1* genes from *Saccharomyces cerevisiae* and the human *ATM* gene, which is mutated in the severe cancer prone syndrome, ataxia-telangiectasia (Lavin *et al.* 1995). Another protein kinase, Chk1p, is phosphorylated in a Rad3p-dependent

Corresponding author: Kristi Chrispell Forbes, Department of Genetics, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115.

[1]*Present address:* Radiation and Genome Stability Unit, Medical Research Council, Harwell, Didcot, Oxfordshire OX11 ORD, United Kingdom.

manner (Walworth and Bernards 1996). In mammalian cells, and presumably also in fission yeast (Furnari *et al.* 1997), Chk1p phosphorylates Cdc25p on serine residues, creating binding sites for 14-3-3 proteins (Peng *et al.* 1997; Sanchez *et al.* 1997). Binding of 14-3-3p inhibits Cdc25p activity by a mechanism that is not clear (Peng *et al.* 1997). In the absence of active Cdc25p, Cdc2p remains in a tyrosine phosphorylated, inactive conformation. Chk1p may also positively regulate Wee1p (O'Connell *et al.* 1997).

The checkpoint response to unreplicated DNA is less well understood. The response requires *rad3*$^+$ and the other *checkpoint rad* genes, because mutations in these genes abolish cell-cycle arrest in the presence of the DNA synthesis inhibitor, hydroxyurea (HU). The response also requires tyrosine phosphorylation of Cdc2p (Enoch *et al.* 1991). However, the mechanisms linking Rad3p to tyrosine phosphorylation of Cdc2p in response to the unreplicated DNA are not known. Although mutations in *chk1*$^+$ or *rad24*$^+$ abolish the response to damaged DNA, they do not ordinarily disrupt the response to unreplicated DNA (Walworth *et al.* 1993; Al-Khodairy *et al.* 1994), though a recent study shows that *chk1*Δ mutants have a partial replication checkpoint defect at high temperatures (Francesconi *et al.* 1997). Other effectors may link Rad3p to the cell-cycle machinery when DNA replication is blocked. A possible effector is the kinase encoded by *cds1*$^+$; however, *cds1*$^-$ mutants arrest normally in HU although they lose viability rapidly (Murakami and Okayama 1995). Recently, Boddy *et al.* (1998) have shown that Cds1p phosphorylates Wee1p *in vitro.* However, the role of this interaction in checkpoint control has not been established.

To identify transducers of the incomplete DNA replication checkpoint signal in *Schizosaccharomyces pombe*, we overexpressed known checkpoint genes and evaluated their ability to suppress the checkpoint defect of *OPcdc25*$^+$ in the presence of HU. We find that overexpression of *chk1*$^+$ is able to suppress the HU sensitivity of *OPcdc25*$^+$. To identify new genes specifically involved in the DNA replication checkpoint, we performed a screen for high-copy plasmid suppressors of the checkpoint defect of *OPcdc25*$^+$. Two classes of suppressors were isolated, which appear to suppress *OPcdc25*$^+$ by distinct mechanisms. One class includes a new gene encoding a putative DEAD box helicase, *s*uppressor of *u*ncontrolled *m*itosis (*sum3*$^+$). This gene negatively regulates the cell-cycle response to stress when overexpressed and restores checkpoint response by a mechanism that is independent of Cdc2p tyrosine phosphorylation. The second class includes *chk1*$^+$ and two genes encoding 14-3-3 proteins, *rad24*$^+$ and *rad25*$^+$, which appear to suppress the checkpoint defect by inhibiting Cdc25p. The finding of this class of genes was unexpected, as they were previously thought to be involved only in the response to damaged DNA, and indeed *chk1*$^-$, *rad24*$^-$, and *rad25*$^-$ mutants arrest normally in HU at 29° (Walworth *et al.* 1993; Al-Khodairy *et al.* 1994). We show that *rad24*Δ mutants are defective in the checkpoint response to HU at 37° and that *cds1*Δ *rad24*Δ mutants, like *cds1*Δ *chk1*Δ mutants, are entirely checkpoint deficient at 29°. These results suggest that *chk1*$^+$ and *rad24*$^+$ may function redundantly with *cds1*$^+$ in the checkpoint response to unreplicated DNA.

MATERIALS AND METHODS

Growth of *S. pombe*: Standard media and growth conditions were used as described (Moreno *et al.* 1991). Cells were transformed by electroporation as described (Prentice 1992). Phase-contrast micrographs were obtained using an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) and a Photonic Microscope Image Processor C1966 (Hamamatsu Photonic Sys. Corp., Bridgewater, NJ). Cells were counted using a Coulter counter. All strains and plasmids are listed in Table 1. Cells were grown at 29° unless otherwise noted.

Screening for high-copy plasmid suppressors of *OPcdc25*$^+$: TE387 (*OPcdc25*$^+$) was transformed with a *LEU2 S. pombe* cDNA library in which cDNA expression is regulated by the thiamine-repressible *nmt1*$^+$ promoter (B. Edgar and C. Norbury, unpublished results; Maundrell 1993). Leu$^+$ transformants were selected on Edinburgh minimal media (EMM) plates with 2 μm thiamine, and colonies were replica plated to EMM plates without thiamine overnight to induce the *nmt1*$^+$ promoter. Colonies were then replica plated to EMM plates with the vital dye, phloxine B (Fisher Scientific, Pittsburgh, PA) with and without 5 mm HU, and grown for 2 days. Colonies that grew in both the presence and absence of HU were examined microscopically. Out of the 89,000 transformants screened, 150 formed colonies in the presence and absence of HU. Plasmids were recovered from most of these transformants and were retested by a new transformation into the TE387 strain and replica plating as described. Twenty-nine transformants that were normal in size in the absence of HU and elongated in the presence of HU were analyzed further. The cDNA inserts of 19 plasmids (Table 2), which had consistently strong suppressor phenotypes, were sequenced.

Testing for suppression of *OPcdc25*$^+$, *cdc2-3w*, or *wee1-50 mik1*Δ: Strains TE387, TE361, or TE386 (Table 1) were transformed with *LEU2* REP plasmids containing the thiamine-repressible *nmt1*$^+$ promoter (B. Edgar and C. Norbury, unpublished results; Maundrell 1993). Leu$^+$ transformants were selected on EMM plates with 2 μm thiamine, and colonies were replica plated to EMM plates without thiamine overnight to induce the *nmt1*$^+$ promoter. Colonies were then replica plated to EMM plates with phloxine B, with and without 5 mm or 10 mm HU, and grown for 2 days at 29° (or 25° in the case of TE386). Cells were examined microscopically for viability and elongation phenotypes. To investigate cell number increase, TE 387 transformants were grown in liquid EMM with thiamine, washed once with EMM lacking thiamine, and inoculated in EMM lacking thiamine. Cells were fixed every 2 hr in formal saline and the cell number was determined using a Coulter counter. To demonstrate colony growth in the presence or absence of HU, Leu$^+$ transformants were grown on EMM without thiamine for 24 hr to induce the *nmt1*$^+$ promoter, then streaked on EMM plates with or without 5 mm HU, and grown for 5 days. Images of petri plates were captured using the Stratagene (La Jolla, CA) Eagle Eye II.

Description of *sum2*$^+$ subclones: To examine the "*sum2N*Δ" and the 40S ribosomal p40 protein portions of the "*sum2N*Δ+*p40*" fusion (pTE306) separately, each portion of the fusion was subcloned into a REP3X plasmid (Maundrell

1993). Plasmid pTE452 (REP3X *sum2NΔ*) contains nucleotides 1 to 661 of the fusion, including 224 amino acids of the *sum2*$^+$ sequence, followed by an *Sph*I-*Bam*HI linker used to aid in cloning. Plasmid pTE458 (REP3X *sum2p40*) contains nucleotides 661 to 1660 (the 3′ end) of the fusion, preceded by a *Xho*I-*Sph*I linker used to aid in cloning. Plasmid pTE458 includes the entire open reading frame of the 40S ribosomal p40 protein gene. A complete clone of *sum2*$^+$ on a REP3X vector was obtained (a gift from J. Bähler and J. Pringle, which we have called pTE462). The complete *sum2*$^+$ was subcloned on a *Bam*HI-*Sal*I fragment into the vectors REP41X (pTE490), and REP81X (pTE491; Maundrell 1993).

Nucleotide sequence accession number: The cDNA sequence of *sum3*$^+$ has been deposited with GenBank under accession number AF025536. The complete sequence of *sum2*$^+$ can be found under accession number D89169.

RNA analysis: Strain TE235 (wild type) transformed with plasmids pTE101, pTE301, and pTE304 (Table 1) and strain TE640 (*sty1*$^-$/*spc1*$^-$) transformed with pTE101 were grown to midlog phase in EMM media. KCl was added to a final concentration of 1 m to half of each culture 60 min before harvesting. The pelleted cells were lysed with glass beads in 1 ml of solution containing 0.32 m sucrose, 20 mm Tris-HCl (pH 7.5), and 10 mm EDTA and diluted with 4 ml of the above solution containing 1% SDS. Phenol extraction was performed at 60° for 3 min followed by further phenol/chloroform extraction at 22°, and total RNA was ethanol precipitated. For Northern hybridizations, 7 μg of total RNA was separated on a denaturing formaldehyde agarose gel (Rave *et al.* 1979). Following transfer to nitrocellulose (GeneScreen; New Life Science Products, Boston), the bound RNA was hybridized to either *gpd1*$^+$ (Pidoux *et al.* 1990) or *act1*$^+$ (Mertins and Gallwitz 1987) probes that were generated as described (Humphrey and Enoch 1998). Probes were labeled as described (Feinberg and Vogelstein 1984).

Analysis of phosphotyrosine levels of Cdc2 protein: Strain TE235 (wild type) and TE22 (*hus1-14*) were grown to midlog phase in EMM at 29°. HU was added to a final concentration of 10 mm at $t = 0$, and cells were harvested at $t = 0$, 2, 4, and 6 hr. Pelleted cells were lysed with glass beads (Sigma Chemical Co., St. Louis) into lysis buffer H containing 0.1% NP-40, 10% glycerol, 50 mm Tris-HCl (pH 7.5), 15 mm EDTA, 100 mm sodium chloride, 0.1 mm NaF, 2 mm sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 20 μm TPCK, 1 mm PMSF, 60 mm β-glycerophosphate, 15 mm paranitrophenol phosphate, and 1 μm okadaic acid. The Cdc2 protein was isolated by affinity purification using p13^{suc1} beads (Brizuela *et al.* 1987). Protein was resolved by 12% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane. The membrane was immunoblotted with anti-Cdc2p (PN24) and anti-pTyr (4G10; Upstate Biotechnology, Lake Placid, NY) antibodies, which were detected by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Between primary antibodies, the membrane was stripped according to the manufacturer's instructions (Amersham). Bands were quantitated using densitometry [Molecular Dynamics (Sunnyvale, CA) ImageQuant program].

To investigate the effects of overexpressing suppressor genes on Cdc2p tyrosine phosphorylation, strain TE235 was transformed with plasmids pTE101, pTE301, pTE302, pTE303, and pTE304 (Table 1). These strains were grown to midlog phase in EMM media for 22 hr in media with 2 μm thiamine to repress the *nmt1*$^+$ promoter, or without thiamine to derepress the *nmt1*$^+$ promoter. Tyrosine phosphorylated Cdc2p was measured as described above.

Western blot analysis of Cdc25p: Strain TE235 (wild type) was transformed with plasmids pTE102, pTE170, pTE301, pTE303, pTE304, and pTE413 (Table 1). These strains were grown to midlog phase in EMM media for 22 hr with 2 μm thiamine to repress the *nmt1*$^+$ promoter, or without thiamine to derepress the *nmt1*$^+$ promoter. Strain TE79 (*cdc25Δ cdc2-3w*) was grown to midlog phase in YE5S media. HU was added to all the cultures to a final concentration of 10 mm 3 hr before harvesting. Pelleted cells were lysed with glass beads (Sigma) in 2× Laemmli buffer and boiled immediately. The proteins were resolved by 10% SDS-PAGE and transferred electrophoretically to an Immobilon P membrane (Millipore Corp., Bedford, MA). The membrane was immunoblotted with anti-Cdc25p antibody (BP2 serum, gift of Sergio Moreno), and then blotted with anti-rabbit secondary antibody (Amersham), which was detected by enhanced chemiluminescence (ECL; Amersham) according to the manufacturer's instructions.

Analysis of HU and ultraviolet (UV) response: Wild-type (TE235), *rad24Δ* (TE465), *cds1Δ* (TE700), *chk1Δ* (TE548), *rad3Δ* (TE890), *chk1Δ rad24Δ* (TE 922), *cds1Δ rad24Δ* (TE919), and *cds1Δ chk1Δ* (TE856) cells were grown to midlog phase in rich media and HU was added to a final concentration of 10 mm. The culture was divided in half, with half the cells remaining at 29° and half the cells being shifted to 37°. Samples from each half were collected at 2-hr intervals. Cells were fixed for microscopy and analyzed for ''cut'' formation as previously described (Enoch *et al.* 1992). At least 100 cells were counted at each time point. For analysis of the response to UV radiation, the same strains were grown to early log phase, plated on YE5S, and irradiated with increasing doses of UV. The number of colonies growing on two plates after 5 days was counted for each viability measurement.

RESULTS

Overexpression of *chk1*$^+$ restores *OPcdc25*$^+$ checkpoint function during HU treatment: To learn more about the transducers of the incomplete DNA replication checkpoint signal in *S. pombe*, high-copy suppressors of the checkpoint defect of strains overexpressing Cdc25p (*OPcdc25*$^+$) were sought. *OPcdc25*$^+$ mutants are unable to delay cell-cycle progression in the presence of unreplicated DNA and therefore attempt to undergo mitosis and segregate a single set of chromosomes when treated with HU, an inhibitor of ribonucleotide reductase (Enoch and Nurse 1990). In these inappropriately dividing cells, the septum bisects the single nucleus. This results in aneuploid or anucleate cells with phenotypes resembling the morphology of *cut*$^-$ mutants (Hirano *et al.* 1986). Consequently, *OPcdc25*$^+$ cells fail to form colonies in the presence of HU. In contrast, wild-type cells initially undergo cell-cycle arrest in the presence of HU, and then resume the cell cycle with a longer S-phase, forming slowly growing colonies consisting of highly elongated cells.

Overexpression of upstream components of the G2/M checkpoint pathway or positive regulators of the checkpoint response might be expected to amplify the checkpoint signal and thus suppress this defect. Negative regulators of Cdc25p or positive regulators of Wee1p or Mik1p might also suppress *OPcdc25*$^+$. Overexpression of such suppressors may allow growth of *OPcdc25*$^+$ on HU as elongated cells. These can be distinguished from

genes that counteract the effects of HU, such as the catalytic subunit of ribonucleotide reductase, because cells overexpressing genes that counteract the effects of HU divide at a normal length on HU. Suppressors can also be distinguished from general negative regulators of the cell cycle, because those genes block cell division both in the presence and absence of HU (Humphrey and Enoch 1998).

Before performing a screen for new genes, we overexpressed some known checkpoint genes and evaluated their ability to suppress the checkpoint defect of *OPcdc25*$^+$ in the presence of HU. *OPcdc25*$^+$ mutant cells were transformed with the *rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$, *chk1*$^+$, *cds1*$^+$, and *hus1*$^+$ genes under the control of the thiamine-repressible *nmt1*$^+$ promoter on a vector carrying the *LEU2* gene (see materials and methods; Table 1). Leu$^+$ transformants were selected in the presence of thiamine. High-level expression of each gene was then activated by replica plating cells to media lacking thiamine for at least 18 hr, after which cells were replica plated to media without thiamine and with or without 10 mm HU. Cells were examined microscopically for viability and elongation phenotypes.

Overexpression of the *rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$, *cds1*$^+$, or *hus1*$^+$ genes did not allow *OPcdc25*$^+$ transformants to survive in the presence of HU better than the vector control (Figure 1). Overexpression of *rad26*$^+$ was somewhat difficult to evaluate as cells overexpressing this gene did not grow well. Only the overexpression of *chk1*$^+$ permitted growth of *OPcdc25*$^+$ in the presence of HU (Figure 1). This is surprising because Chk1p is thought to be specifically responsible for trans-

TABLE 1

***S. pombe* strains and plasmids**

Strain no.	Genotype	Source
TE22	*hus1-14 leu1-32 h*$^-$	
TE79	*cdc25::ura4 cdc2-3w leu1-32 ura4 D18 ade6-M?*	
TE235	*leu1-32 h*$^-$	
TE361	*cdc2-3w ade6-M210 leu1-32 h*$^-$	
TE386	*mik1::LEU2 wee1-50 leu1-32 h*$^-$	Gift of A. Bueno
TE387	*adh:cdc25*$^+$ *cdc25::ura4*$^+$ *leu1-32 ura4-D18 h*$^-$	Gift of P. Russell
TE465	*rad24::ura4*$^+$ *ade6- leu1-32 ura4-D18 h*$^+$	Gift of A. Carr
TE548	*chk1::ura4*$^+$ *ura4-d18 h*$^-$	Gift of A. Carr
TE640	*spc1-M13 leu1-32 ura4-D18 h*$^+$	Gift of P. Shiozaki and P. Russell
TE700	*cds1::ura4*$^+$ *leu1-32 ura4-D18 h*$^-$	Gift of H. Murakami and H. Okayama
TE790	*chk1-T15 ade6-704 leu1-32 ura4-D18 h*$^+$	This study
TE856	*cds1::ura4*$^+$ *chk1::ura4*$^+$ *ade6-704 leu1-32 ura4-D18 h*$^+$	This study
TE890	*rad3::ura4*$^+$ *leu1-32 ura4-D18 h*$^-$	C. R. Chapman
TE919	*cds1::ura4*$^+$ *rad24::ura4*$^+$ *leu1-32 ura4-D18 h*$^-$	This study
TE922	*chk1::ura4*$^+$ *rad24::ura4*$^+$ *leu1-32 ura4-D18 h*$^+$	This study

Plasmid no.	Construct	Source
pTE32	*REP1 hus1*$^+$	
pTE101	*REP3X*	
pTE102	*REP1*	
pTE157	*REP1 rad3*$^+$	Gift of A. Carr
pTE169	*REP1 rad26*$^+$	Gift of A. Carr
pTE170	*REP1 chk1*$^+$	Gift of A. Carr
pTE301	*REP1 pyp1*$^+$	Gift of J. Millar
pTE302	*REP3X rad25*$^+$	This study
pTE303	*REP3X rad24*$^+$	This study
pTE304	*REP3X sum3*$^+$	This study
pTE306	*REP3X sum2NΔ+p40*	This study
pTE413	*REP1 cdc25*$^+$	
pTE452	*REP3X sum2NΔ*	This study
pTE458	*REP3X sum2p40*	This study
pTE462	*REP3X sum2*$^+$	Gift of J. Bähler and J. Pringle
pTE478	*REP1 rad17*$^+$	C. R. Chapman
pTE479	*REP1 rad9*$^+$	C. R. Chapman
pTE490	*REP41X sum2*$^+$	This study
pTE491	*REP81X sum2*$^+$	This study
pTE531	*REP1 cds1*$^+$	This study
pTE567	*REP1 rad1*$^+$	C. Kostrub

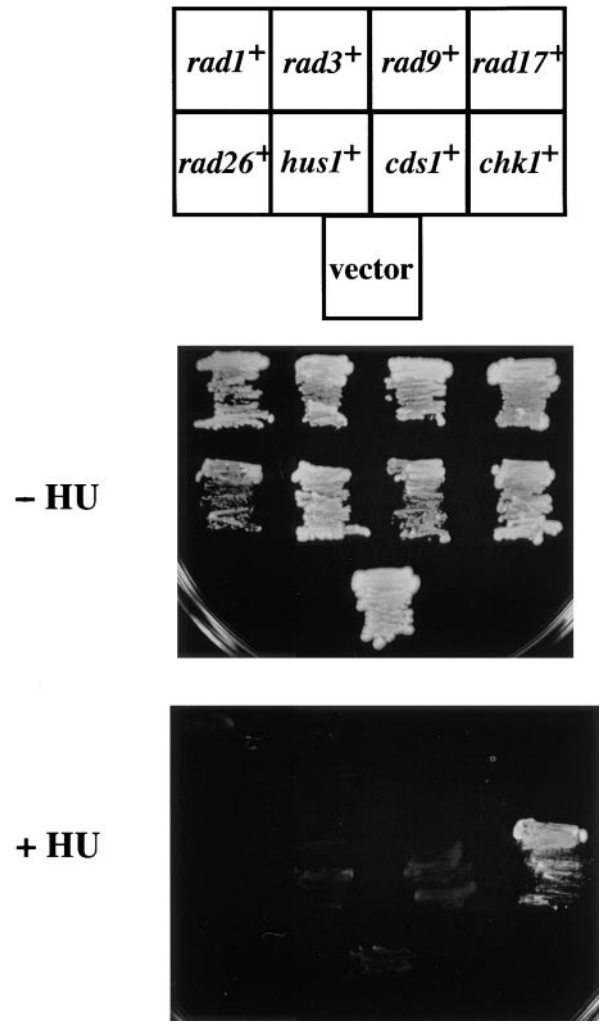

Figure 1.—Overexpression of *chk1*$^+$ rescues the checkpoint defect of *OPcdc25*$^+$. *OPcdc25*$^+$ (TE387) was transformed with the plasmids *rad1*$^+$ (pTE567), *rad3*$^+$ (pTE157), *rad9*$^+$ (pTE479), *rad17*$^+$ (pTE478), *rad26*$^+$ (pTE169), *hus1*$^+$ (pTE32), *cds1*$^+$ (pTE531), *chk1*$^+$ (pTE170), or vector control (pTE102, pREP1), where expression of each gene was controlled by the thiamine-repressible *nmt1*$^+$ promoter (Maundrell 1993). Each transformant was grown in the absence of thiamine for 24 hr to induce the *nmt1*$^+$ promoter, and then patched on EMM in the absence (top) or presence (bottom) of 5 mm HU and grown for 5 days at 29°.

mitting the checkpoint signal for DNA damage, and *chk1*$^-$ cells arrest normally in HU (Walworth *et al.* 1993; Al-Khodairy *et al.* 1994; Walworth and Bernards 1996). Because examination of some known checkpoint genes detected only a gene involved in the DNA damage checkpoint pathway, but not genes specifically involved in the DNA replication checkpoint, a screen for new genes was undertaken.

Isolation of suppressor genes that restore *OPcdc25*$^+$ checkpoint function during hydroxyurea treatment: To identify new genes specifically involved in the DNA replication checkpoint, *OPcdc25*$^+$ was transformed with an *S. pombe* cDNA library under the control of the thiamine-repressible *nmt1*$^+$ promoter on a vector encoding the *S. cerevisiae LEU2* gene (B. Edgar and C. Norbury, unpublished results; Maundrell 1993). Plasmids that allowed the cells to form colonies on minimal media plates in the presence of HU following derepression of the *nmt1*$^+$ promoter (see materials and methods) were identified. Colonies were examined microscopically, and transformants that showed an elongated phenotype in the presence of HU were selected. Genes that counteract the effects of HU, such as the catalytic subunit of ribonucleotide reductase, should not be identified by this screening method because cells overexpressing those genes divide at a normal length on HU rather than as elongated cells (K. Chrispell Forbes, T. Humphrey and T. Enoch, unpublished observations). Plasmids that caused cell-cycle arrest in the absence of HU, or plasmids that suppressed by inhibiting septation, were not examined further. A total of 89,000 *OPcdc25*$^+$ transformants were screened, and 29 plasmids were chosen for further study.

TABLE 2

Genes isolated in the screen for suppressors of *OPcdc25*

Name	No. of isolates	Gene family
rad24$^+$	4	14-3-3
rad25$^+$	10	14-3-3
sum2NΔ+p40	2	(Fusion)
sum3$^+$	3	"DEAD" helicase

Further analysis of the cDNA inserts of the suppressors showed that the most frequently isolated suppressor had been identified 10 times, and the second most common suppressor was identified 4 times (Table 2). While this study was in progress, these two suppressor genes were independently identified as the two *S. pombe* 14-3-3 genes, *rad25*$^+$ and *rad24*$^+$, respectively (Ford *et al.* 1994).

Disrupting *rad24*$^+$ activity reduces the DNA damage checkpoint response, making the cells radiation sensitive (Ford *et al.* 1994), but was not reported to affect the response to unreplicated DNA. It is intriguing that the 14-3-3 proteins, which were hypothesized to be involved in the damage checkpoint response but not in the DNA replication checkpoint, were isolated in a screen for checkpoint genes responding to incomplete replication. Because the results of this screen indicate that overexpression of *rad24*$^+$ and *rad25*$^+$ enhances the checkpoint signal, while other studies have shown that lack of *rad24*$^+$ causes a loss of the DNA damage checkpoint (Ford *et al.* 1994), it seems possible that these proteins are directly involved in transducing the checkpoint signal.

In addition to the plasmids containing the 14-3-3 genes, two other sets of plasmids were found to allow *OPcdc25*$^+$ to grow in the presence of HU (Table 2). Se-

quencing revealed that one set of plasmids contains an identical artifactual fusion, which we call *sum2NΔ+p40*. The N-terminal portion of each clone contained the first 372 nucleotides of a 1113-nucleotide open reading frame (ORF) similar to the *S. cerevisiae SCD6* gene, a multicopy suppressor of clathrin deficiency (D. Gelperin and S. Lemmon, personal communication). We have named this *S. pombe SCD6*-related gene *sum2*$^+$. The C terminus of the fusion contains the complete coding sequence of a gene closely related to 40S ribosomal p40 proteins, in a different reading frame from the truncated *sum2*$^+$ gene. To determine which ORF was responsible for the suppressor phenotype, the N- and C-terminal ORFs were subcloned into REP vectors (materials and methods; Table 1; Maundrell 1993). In addition, a complete clone of *sum2*$^+$ (pTE462, gift of Jurg Bähler and John Pringle) was studied. Transformation of these plasmids into checkpoint-deficient yeast showed that the truncated *sum2*$^+$, *sum2NΔ*, gave a poor rescue of *OPcdc25*$^+$ in the presence of HU (data not shown). The 40S ribosomal p40 gene did not allow *OPcdc25*$^+$ to survive treatment with HU (pTE452, data not shown). The complete clone of *sum2*$^+$ was lethal when highly overexpressed (pTE462, REP3X vector), and was unable to allow *OPcdc25*$^+$ to survive treatment with HU when overexpressed at lower levels (pTE490, REP41X vector, and pTE491, REP81X vector, data not shown). We conclude that the N-terminal fragment of *sum2*$^+$, *sum2NΔ*, has some capacity to rescue the *OPcdc25*$^+$ checkpoint defect, which is enhanced by fusion to the 40S ribosomal p40 protein for unknown reasons. Because the full-length gene does not appear to have suppressing activity, the biological significance of suppression by *sum2NΔ* is not clear.

Surprisingly, *chk1*$^+$ was not isolated in this screen. This may be because it is not sufficiently represented in the library that was used. Alternatively, it may have been discarded because *OPcdc25*$^+$ cells overexpressing *chk1*$^+$ are not as elongated in HU as cells overexpressing *rad24*$^+$, *rad25*$^+$, or *sum3*$^+$.

***sum3*$^+$ encodes a member of the DEAD box helicase family:** Three other plasmids isolated in the screen contained a novel ORF that we have called *sum3*$^+$ (Table 2). Sequencing of the cDNA inserts of these *sum3*$^+$ plasmids revealed a 1908-nucleotide ORF encoding a 636-amino-acid protein, with a predicted molecular weight of ~70 kD. Comparisons of *sum3*$^+$ with GenBank sequences show that *sum3*$^+$ encodes a member of the DEAD box family of ATP-dependent RNA helicases. Members of this family share nine regions of amino acid conservation, including the Asp-Glu-Ala-Asp (DEAD) motif (Fuller-Pace 1994). The predicted protein product of *sum3*$^+$ contains these motifs. The predicted protein product of *sum3*$^+$ shows 74% amino acid similarity and 61% identity to *DED1*, its closest *S. cerevisiae* homologue (Figure 2). Like *DED1*, the *sum3*$^+$ gene is essential (Struhl 1985; B. Grallert and K. Labib, personal communication).

The suppressors enhance the DNA replication checkpoint response of *OPcdc25*$^+$: To determine whether the suppressors isolated in the screen acted as general negative regulators of cell division or if they were specifically restoring the checkpoint response, the phenotypes of *OPcdc25*$^+$ cells overexpressing the suppressors were studied. If the suppressors were acting as general inhibitors of the cell cycle, they should have caused the cells to become elongated and should have blocked division even in the absence of HU. *OPcdc25*$^+$ cells were transformed with a vector control, *rad24*$^+$ or *sum3*$^+$, where expression of each gene was controlled by the thiamine-repressible *nmt1*$^+$ promoter. Cells were grown in the absence of thiamine to induce the *nmt1*$^+$ promoter, and the cell number was counted every 2 hr. The number of cells in the samples overexpressing *rad24*$^+$ or *sum3*$^+$

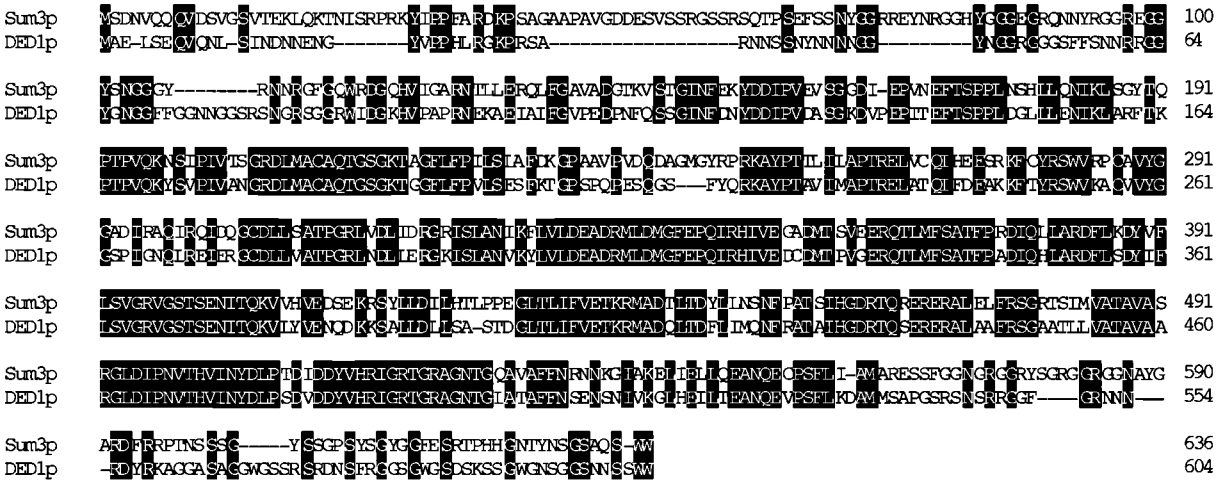

Figure 2.—Alignment of the amino acid sequence of *S. pombe sum3*$^+$ predicted protein product with that of DED1p from *S. cerevisiae*. Identical residues are indicated by white lettering on a black background. The sequence alignment was performed using the DNASTAR Megalign program. The cDNA sequence of *sum3*$^+$ has been deposited with GenBank under accession number AF025536.

continued to increase as rapidly as the number of cells containing the vector control, indicating that the cells overexpressing the suppressors were not arrested or significantly delayed in their cell cycles (Figure 3A; data for the first 12 hr after the removal of thiamine is not shown). In general, the length of the cells overexpressing *rad24*$^+$ or *sum3*$^+$ was comparable to the length of cells containing the vector control (Figure 3B, −HU), demonstrating that the cell cycle was not being delayed in these cells. Results for cells overexpressing *rad25*$^+$ or *chk1*$^+$ were similar (data not shown). *OPcdc25*$^+$ cells overexpressing *rad24*$^+$, *rad25*$^+$, or *sum3*$^+$ are able to form colonies (Figure 4A, −HU), which also suggests they are not cell-cycle arrested.

To determine whether overexpression of the suppressors isolated in the screen was enhancing the checkpoint response of *OPcdc25*$^+$, we studied the phenotypes of *OPcdc25*$^+$ cells overexpressing *rad24*$^+$ or *sum3*$^+$ in the presence and absence of HU. Overexpression of vector, *rad24*$^+$ or *sum3*$^+$ was induced by the removal of thiamine, and HU was added to half the cells for 6 hr. As shown in Figure 3B, *OPcdc25*$^+$ cells containing a vector control do not elongate, and many form cuts in the presence of HU because the cells lack the unreplicated DNA checkpoint (Figure 3B, +HU; arrows indicate cuts). In contrast, *OPcdc25*$^+$ cells overexpressing *rad24*$^+$ or *sum3*$^+$ in the presence of HU did not cut and show an elongated phenotype indicative of cell-cycle arrest (Figure 3B, +HU). Cells overexpressing *rad25*$^+$ are indistinguishable from those overexpressing *rad24*$^+$ (data not shown). These results show that the suppressors enhance the checkpoint response of *OPcdc25*$^+$ in the presence of HU, but do not affect cell-cycle progression under normal conditions. However, all of the suppressors caused significant cell-cycle delays when overex-

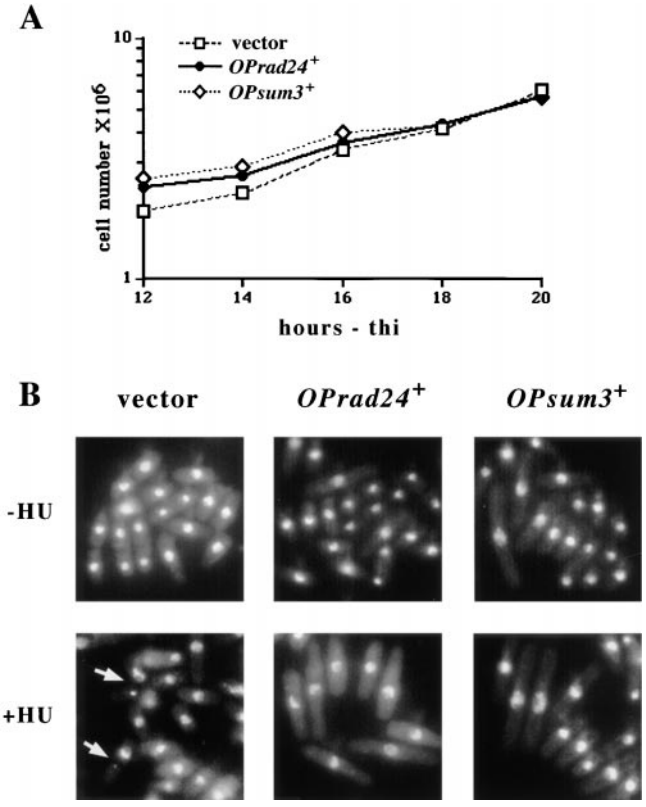

Figure 3.—Suppressors rescue the checkpoint defect of *OPcdc25*$^+$ but do not affect proliferation in the absence of HU. (A) *OPcdc25*$^+$ cells overexpressing *rad24*$^+$ or *sum3*$^+$ continue to divide in the absence of HU. *OPcdc25*$^+$ (TE387) was transformed with the vector (pTE102), *rad24*$^+$ (pTE303), or *sum3*$^+$ (pTE304), where expression of each gene was controlled by the thiamine-repressible *nmt1*$^+$ promoter (Maundrell 1993). Cells were grown in the absence of thiamine to induce the *nmt1*$^+$ promoter. Samples were fixed every 2 hr and the number of cells was counted using a Coulter counter. (B) Overexpression of *rad24*$^+$ or *sum3*$^+$ restores *OPcdc25*$^+$ checkpoint function during hydroxyurea treatment. The transformants in A were grown in the absence of thiamine for 12 hr to induce the *nmt1*$^+$ promoter. HU (10 mm) was added to half of the cells. Cells in the presence or absence of HU were fixed 6 hr later, stained with DAPI, and photographed. Arrows indicate cuts.

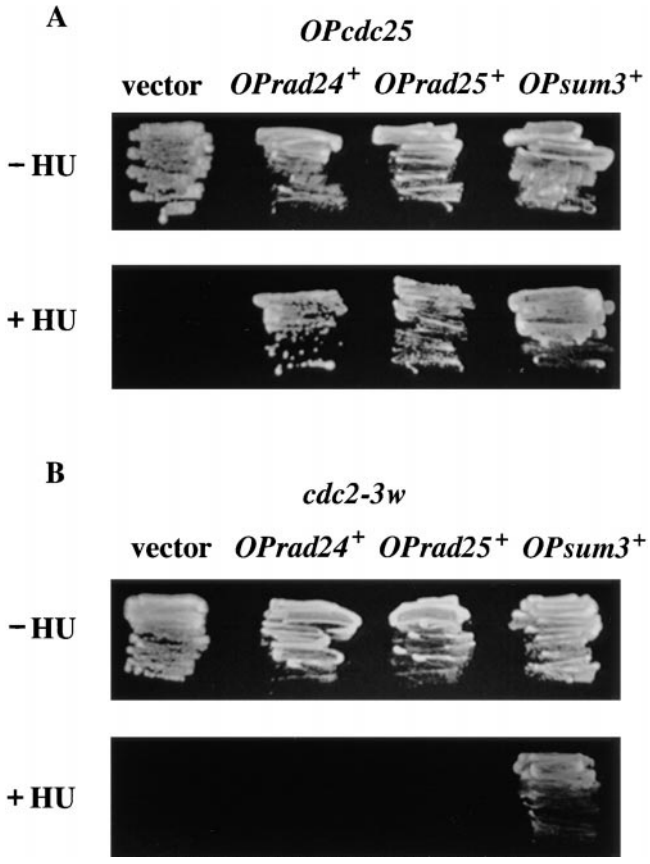

Figure 4.—*rad24*$^+$ and *rad25*$^+$ interact specifically with *cdc25*$^+$. (A) Overexpression of *rad24*$^+$, *rad25*$^+$, or *sum3*$^+$ restores *OPcdc25*$^+$ checkpoint function during HU treatment. *OPcdc25*$^+$ (TE387) transformed with vector (pTE102), *rad24*$^+$ (pTE303), *rad25*$^+$ (pTE302), or *sum3*$^+$ (pTE304) was grown in the absence of thiamine for 24 hr to induce the *nmt1*$^+$ promoter, then patched on EMM in the absence (top) or presence (bottom) of 5 mm HU, and grown for 5 days at 29°. (B) Overexpression of *sum3*$^+$ restores *cdc2-3w* checkpoint function during HU treatment, but overexpression of *rad24*$^+$ or *rad25*$^+$ does not. *cdc2-3w* (TE361) was transformed with the same plasmids and grown as described in A.

pressed in wild-type cells, indicating that the suppressors inhibit cell-cycle progression when not counteracted by mutations in cell-cycle regulators (data not shown).

***rad24*$^+$ and *rad25*$^+$ restore checkpoint control through a specific interaction with *cdc25*$^+$:** To investigate the mechanism of action of the suppressors, it was of interest to determine whether they could restore checkpoint control when expressed in other checkpoint mutants. Like *OPcdc25*$^+$, *cdc2-3w* or *wee1-50 mik1*Δ cells lack a checkpoint response to unreplicated DNA and are inviable when replica plated onto media containing HU, even at the permissive temperature for *wee1-50 mik1*Δ. As we have stated, overexpression of *rad24*$^+$, *rad25*$^+$, or *sum3*$^+$ restores checkpoint function in *OPcdc25*$^+$ (Figure 4A, +HU). To determine whether the suppressors could also rescue *cdc2-3w* or *wee1-50 mik1*Δ in the presence of HU, plasmids containing *rad24*$^+$, *rad25*$^+$, *chk1*$^+$, *sum3*$^+$, and *sum2N*Δ+*p40* were transformed into these strains.

cdc2-3w or *wee1-50 mik1*Δ cells overexpressing *rad24*$^+$, *rad25*$^+$, or *chk1*$^+$ did not form colonies in the presence of HU (Figure 4B and data not shown). In contrast, overexpression of *sum2N*Δ+*p40* or *sum3*$^+$ allowed *cdc2-3w* or *wee1-50 mik1*Δ to survive in the presence of HU (Figure 4B and data not shown). Therefore, we conclude that the suppressors we have identified restore checkpoint control by two different mechanisms. The finding that *rad24*$^+$, *rad25*$^+$, and *chk1*$^+$ worked specifically to rescue *OPcdc25*$^+$ suggests that these genes may act to negatively regulate Cdc25p. In contrast, *sum2N*Δ+*p40* and *sum3*$^+$ apparently suppress checkpoint defects by a more global mechanism as they are able to suppress several checkpoint mutants.

Overexpression of *sum3*$^+$ negatively regulates the cell-cycle response to osmotic stress: When wild-type fission yeast are exposed to conditions of high osmolarity, their entry into mitosis is stimulated (Millar *et al.* 1995). This has two beneficial effects: the cells enter G1, so they can mate or sporulate, and cell size is reduced, reducing the metabolic requirements for doubling. This response requires the stress-activated *sty1*$^+$/*spc1*$^+$ MAPK pathway (Figure 5A), but the molecular mechanism of the stress-activated MAPK's effects on the cell cycle are not understood. Loss-of-function mutants for the kinases in this pathway or strains overexpressing either of the two phosphatases (*pyp1*$^+$ and *pyp2*$^+$) that negatively regulate *sty1*$^+$/*spc1*$^+$ MAPK cannot stimulate mitosis, and become abnormally long under conditions of high osmolarity (Millar *et al.* 1992; Shiozaki and Russell 1995). We have recently identified a gene, *sum1*$^+$, that suppresses the checkpoint defect of *cdc2-3w* and also inhibits the osmotic cell-cycle response (Humphrey and Enoch 1998). Like overexpression of *sum3*$^+$, overexpression of *sum1*$^+$ allows survival of *cdc2-3w*, *OPcdc25*$^+$, or *wee1-50 mik1*Δ in the presence of HU. Moreover, we have shown that overexpression of *pyp1*$^+$, or mutation of *sty1*$^+$/*spc1*$^+$, suppresses the checkpoint defect of *cdc2-3w* (Humphrey and Enoch 1998). These results suggest

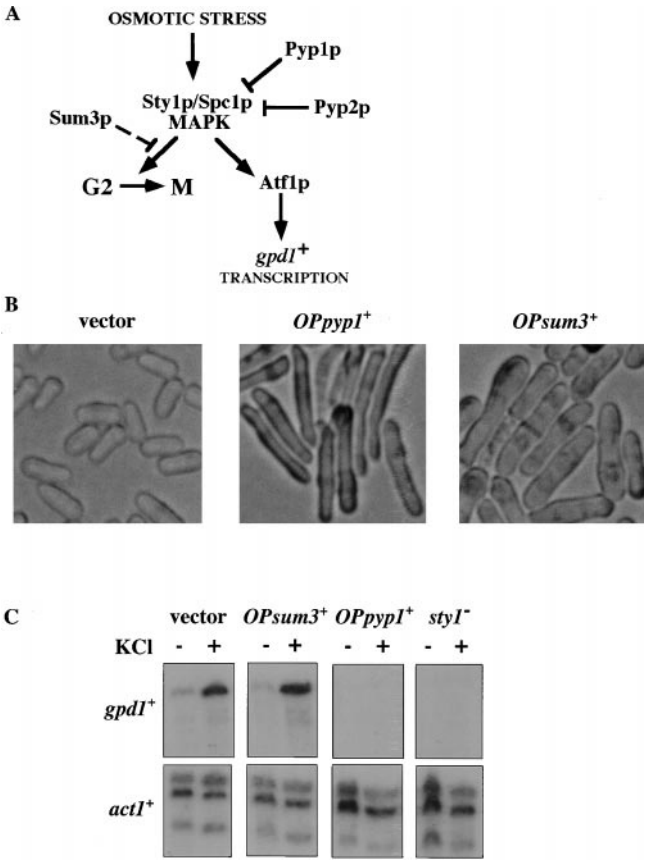

Figure 5.—*sum3*$^+$ negatively regulates the stress response. (A) The stress-activated MAPK pathway in fission yeast. Stress activates a kinase cascade (not shown) leading to activation of the MAPK, Sty1p/Spc1p. Active Sty1p/Spc1p phosphorylates Atf1p, which promotes the transcription of genes including *gpd1*$^+$ and advances mitosis through an unidentified mechanism (G2 → M). A plausible position for Sum3p, downstream of Sty1p/Spc1p on the branch of the pathway that leads to activation of mitosis, is shown (dashed ⊥). (B) Overexpression of *sum3*$^+$ disrupts the cell-cycle response to osmotic stress. Wild-type cells (strain TE235) with vector (pTE101) or overexpressing *sum3*$^+$ (pTE304) or *pyp1*$^+$ (pTE304) were grown on EMM plates containing thiamine, replica plated onto EMM plates for 24 hr to derepress the *nmt1*$^+$ promoter, and then replica plated to EMM plates containing 1 m KCl. Cells were photographed after 24 hr. (C) Northern blot analysis of *gpd1*$^+$ transcription levels in wild-type cells (strain TE235) overexpressing vector (pTE101), *sum3*$^+$ (pTE304), or *pyp1*$^+$ (pTE301) and *sty1*$^-$/*spc1*$^-$ (strain TE640) cells with vector (pTE101). Overexpression of *sum3*$^+$ does not inhibit the transcriptional response of the MAPK pathway. KCl was added to a final concentration of 1 m for 60 min where indicated (KCl, + lanes). Total RNA generated from the above strains was separated on a Northern gel, blotted to nitrocellulose, and transcripts visualized with *gpd1*$^+$ and *act1*$^+$ probes as indicated.

that the stress response pathway may counteract checkpoint controls. To investigate whether any of the suppressors we had identified restored checkpoint control through a similar mechanism, we examined wild-type cells overexpressing *rad24*$^+$, *rad25*$^+$, *sum2N*Δ+*p40*, and *sum3*$^+$ under conditions of osmotic stress. Like mutants lacking *sty1*$^+$/*spc1*$^+$ activity, *sum3*$^+$ overexpressors were

found to be elongated on 1 m KCl (Figure 5B) or 1.5 m sorbitol (data not shown). The phenotype of *sum2NΔ+p40* is similar (data not shown). This phenotype is similar to that of cells overexpressing *pyp1*$^+$ (Figure 5B, middle; Shiozaki and Russell 1995), although the cells in which *sum3*$^+$ is overexpressed look somewhat wider. Thus, like *sum1*$^+$ (Humphrey and Enoch 1998), overexpression of *sum3*$^+$ and *sum2NΔ+p40* apparently inhibits the stress response to high-osmolarity conditions. Overexpression of *rad24*$^+$, *rad25*$^+$, and *chk1*$^+$ did not cause elongation under these conditions, which confirms that they are likely to restore checkpoint control by a different mechanism (data not shown).

Overexpression of *sum3*$^+$ does not inhibit the stress-induced transcriptional response: The osmotic stress phenotype of overexpressed *sum3*$^+$ suggests that Sum3p could be negatively regulating the stress response pathway. For example, Sum3p could negatively regulate one of the MAP kinases, or it could positively regulate *pyp1*$^+$ or *pyp2*$^+$. As shown in Figure 5A, the stress response pathway bifurcates after activation of the Sty1p/Spc1p MAPK. One branch, requiring the transcription factor Atf1p, leads to transcriptional activation of genes required for the stress response (Degols *et al.* 1996; Shiozaki and Russell 1996; Wilkinson *et al.* 1996). A second branch leads to stimulation of cell division (Millar *et al.* 1995), although the components of this branch have not yet been identified. In *atf1*$^-$ mutants, the transcriptional response to stress is abolished, but the cell-cycle response is not affected (Shiozaki and Russell 1996; Wilkinson *et al.* 1996). Loss-of-function mutations in any of the MAPK genes, or overexpression of *pyp1*$^+$ or *pyp2*$^+$, causes Atf1p-dependent transcription to be uninducible (Degols *et al.* 1996; Shiozaki and Russell 1996; Wilkinson *et al.* 1996). If Sum3p were negatively regulating one of the kinases or positively regulating *pyp1*$^+$ or *pyp2*$^+$, then overexpression of *sum3*$^+$ should prevent expression of the genes downstream of Atf1p, such as *gpd1*$^+$, even in the presence of stress.

A Northern blot of total RNA from wild-type cells grown in the presence or absence of osmotic stress was probed with *gpd1*$^+$ (Figure 5C, top) and reprobed with actin as a loading control (Figure 5C, bottom). In wild-type cells carrying a vector control, transcription of *gpd1*$^+$ was strongly induced by exposure to 1 m KCl, whereas wild-type cells overexpressing *pyp1*$^+$, or *sty1*$^-$/*spc1*$^-$ cells, showed no increase in *gpd1*$^+$ transcription. Overexpression of *sum3*$^+$ did not reduce the induction of *gpd1*$^+$ transcription (Figure 5C). This result suggests that overexpression of *sum3*$^+$ interferes with the stress response at a point downstream of MAP kinase activation, on the branch of the pathway that leads to activation of mitosis (Figure 5A). Similar results were found for *sum1*$^+$ (Humphrey and Enoch 1998). Overexpression of *rad24*$^+$, *rad25*$^+$, or *chk1*$^+$ allowed normal induction of *gpd1*$^+$ transcription (data not shown).

Activation of the G2/M checkpoint leads to tyrosine phosphorylation of Cdc2p in wild-type cells, but not in *hus1*$^-$ mutants: *rad24*$^+$, *rad25*$^+$, and *chk1*$^+$ interact genetically with *cdc25*$^+$. Cdc25p is required for removing an inhibitory tyrosine phosphate from Cdc2p. We conjecture that in the presence of unreplicated DNA, these gene products and others inhibit Cdc25p, thus resulting in an accumulation of tyrosine-phosphorylated Cdc2p. Genetic studies are consistent with this model (Enoch and Nurse 1990; Rhind *et al.* 1997); however, biochemical studies have challenged this view with the finding that Cdc2p kinase activity is maintained in G2/M-arrested cells after treatment with HU (Knudsen *et al.* 1996). To test whether Cdc2p tyrosine phosphorylation increases in response to unreplicated DNA, we examined Cdc2p tyrosine phosphorylation in wild-type cells and in the checkpoint-deficient mutant *hus1*$^-$ (Figure 6A). In wild-type cells, a fourfold increase in tyrosine-phosphorylated Cdc2p can be seen at 4 and 6 hr after the addition of HU, while in the checkpoint-defective *hus1*$^-$ cells there is no increase in tyrosine-phosphorylated Cdc2p relative to the total amount of Cdc2p (Figure 6A). These data support the model that incompletely replicated DNA activates a checkpoint in wild-type cells that causes Cdc2p to become phosphorylated on tyrosine. Similar results were reported by others while this article was under review (Rhind and Russell 1998).

The two classes of suppressors have different effects on tyrosine phosphorylation of Cdc2p in wild-type cells: One mechanism by which suppressors could restore the G2/M checkpoint would be to increase tyrosine phosphorylation of Cdc2p. To see if this was the case, plasmids overexpressing *rad24*$^+$, *rad25*$^+$, *chk1*$^+$, *pyp1*$^+$, and *sum3*$^+$ were transformed into wild-type cells, and the effects on Cdc2p tyrosine phosphorylation were evaluated. Unlike *OPcdc25*$^+$ cells overexpressing these genes, wild-type cells overexpressing these genes become elongated in the absence of HU (data not shown). Cdc2p was affinity purified from cellular lysates using p13^{suc1} beads (Brizuela *et al.* 1987), resolved by SDS-PAGE, and transferred to an Immobilon membrane that was sequentially probed with an antibody to phosphotyrosine and an antibody to Cdc2p. The amount of tyrosine-phosphorylated Cdc2p and total Cdc2p was quantitated using a densitometer, and the amount of tyrosine-phosphorylated Cdc2p was divided by the amount of total Cdc2p. To normalize the results, the ratio of tyrosine-phosphorylated Cdc2p to total Cdc2p with the promoter off was defined as equal to one. Typical results are shown in Figure 6B. Qualitatively similar results were obtained in several independent experiments.

Induction of a vector control has no effect on Cdc2p tyrosine phosphorylation (Figure 6B). When *rad24*$^+$ or *rad25*$^+$ is overexpressed in wild-type cells, the amount of tyrosine-phosphorylated Cdc2p increases; similar results are seen for *chk1*$^+$ (data not shown). Thus, these

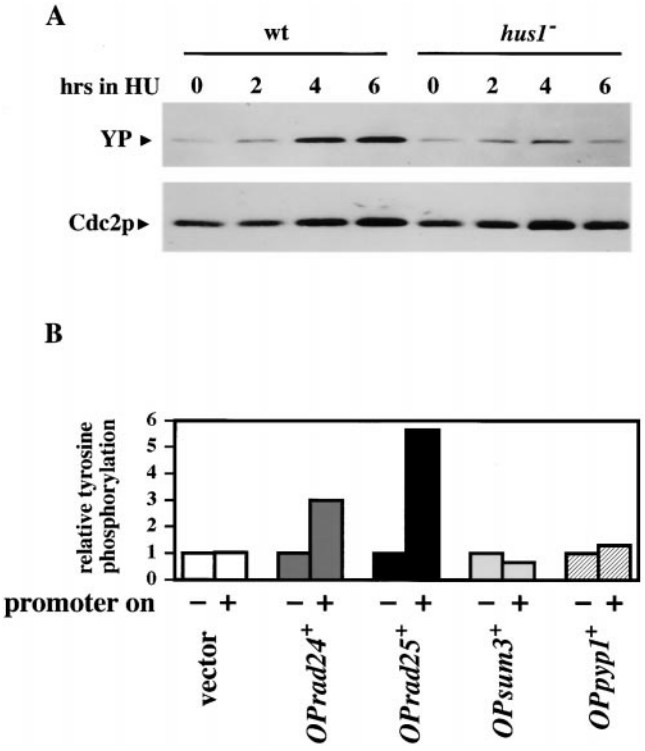

Figure 6.—Stimulation of Cdc2 tyrosine phosphorylation by *rad24*$^+$, *rad25*$^+$, and *chk1*$^+$. (A) Activation of the G2/M checkpoint by HU increases tyrosine phosphorylation of Cdc2p in wild-type cells, but not in checkpoint mutants. Wild-type cells (TE235) and *hus1-14* (TE22) mutant cells were grown to midlog phase in EMM at 29°. HU was added to a final concentration of 10 mm, and cells were harvested every 2 hr. Cdc2p was isolated by affinity purification, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. The blot was probed sequentially with a phosphotyrosine antibody and a Cdc2p antibody. (B) Wild-type cells (TE235) were transformed with vector (pTE102), *rad24*$^+$ (pTE303), *sum3*$^+$ (pTE304), and *pyp1*$^+$ (pTE301). These strains were grown to midlog phase with thiamine (promoter off, − lanes) or without thiamine (promoter on, + lanes). Cdc2p was analyzed as described above. Phosphotyrosine and total Cdc2p were detected by sequential immunoblotting, and the bands were quantitated using a densitometer (Molecular Dynamics ImageQuant program). The amount of the phosphotyrosine signal was normalized to the amount of total Cdc2p, and the ratio of phosphotyrosine to total Cdc2p with the promoter repressed was set equal to one.

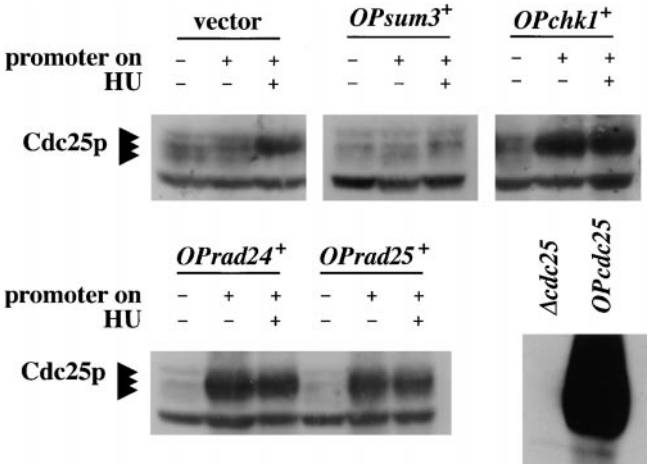

Figure 7.—Overexpression of *rad24*$^+$, *rad25*$^+$, or *chk1*$^+$ increases levels of Cdc25p. Wild-type cells (TE235) overexpressing vector (pTE102), *chk1*$^+$ (pTE170), *rad24*$^+$ (pTE303), *rad25*$^+$ (pTE302), and *sum3*$^+$ (pTE304) were grown in media with thiamine (promoter off, − lanes) or without thiamine (promoter on, + lanes). HU was added to the cultures to a final concentration of 10 mm 3 hr before harvesting (HU, + lanes). Total proteins were resolved by 10% SDS-PAGE and transferred to an Immobilon P membrane, stained with Ponceau S to ensure equal loading, and then immunoblotted with anti-Cdc25p antibody. *cdc25*Δ (TE79) and wild-type cells overexpressing Cdc25p (pTE413) are shown as controls.

suppressors can stimulate tyrosine phosphorylation of Cdc2p. This is consistent with the hypothesis that this class of suppressors negatively regulates Cdc25p. Stimulation of Cdc2p tyrosine phosphorylation by *rad24*$^+$ and *rad25*$^+$ in *OPcdc25*$^+$ cells was much weaker (data not shown). This is not surprising because the excess of Cdc25p in these strains should make the activity of *rad24*$^+$ and *rad25*$^+$ more difficult to detect. We also did not see consistent differences in Cdc2p tyrosine phosphorylation after HU treatment in cells overexpressing any of the suppressors compared to control cells (data not shown). This is probably because HU treatment alone stimulates Cdc2p tyrosine phosphorylation substantially, making it difficult to detect any further enhancement due to overexpression of suppressors.

Overexpression of *sum3*$^+$ does not cause an increase in Cdc2p tyrosine phosphorylation (Figure 6B). This suggests that this class of suppressors regulates cell-cycle progression by a mechanism independent of tyrosine phosphorylation of Cdc2p. Overexpression of *pyp1*$^+$ also does not increase in Cdc2p phosphorylation (Figure 6B), suggesting that inactivation of the stress-activated MAPK pathway arrests cells at G2/M by a mechanism independent of tyrosine phosphorylation of Cdc2p.

Overexpression of *rad24*$^+$, *rad25*$^+$, or *chk1*$^+$ increases levels of Cdc25p: To investigate the effects of *rad24*$^+$, *rad25*$^+$, *chk1*$^+$, and *sum3*$^+$ on the biochemical status of Cdc25p, cell lysates of wild-type cells overexpressing each of these genes were prepared and analyzed by Western blotting for Cdc25p. Inducing overexpression of a vector control or *sum3*$^+$ has no major effect on the level or mobility of Cdc25p (Figure 7). In contrast, inducing overexpression of *rad24*$^+$, *rad25*$^+$, or *chk1*$^+$ leads to a marked increase in the levels of Cdc25p. No further increase is observed in response to HU (Figure 7). Overexpression of *rad24*$^+$ or *chk1*$^+$ also results in increased Cdc25p accumulation in *OPcdc25*$^+$ cells (data not shown). Cdc25p appears to shift to a slower migrating form in the presence of HU in cells containing a vector control or overexpressing *sum3*$^+$ (Figure 7, +HU). In cells overexpressing *rad24*$^+$, *rad25*$^+$, or *chk1*$^+$, Cdc25p accumulates to such high levels that it is not

possible to determine if its mobility is altered in response to HU.

The accumulation of Cdc25p that was observed in wild-type cells overexpressing *rad24*$^+$, *rad25*$^+$, or *chk1*$^+$ could be the result of increased transcription or translation of Cdc25p, or perhaps the stabilization or sequestration of the Cdc25p, so that it is not degraded at normal rates. The accumulation of Cdc25p cannot explain the suppression of the checkpoint defect by these suppressors, because increased Cdc25p levels would be predicted to stimulate mitosis and reduce the efficiency of checkpoint control. Therefore, we believe that overexpression of *rad24*$^+$, *rad25*$^+$, or *chk1*$^+$ may cause Cdc25p to be sequestered in an inactive state where it is less susceptible to proteolysis, which is a normal part of its regulation (Moreno *et al.* 1990; Kovelman and Russell 1996; Nefsky and Beach 1996).

Loss of *chk1*$^+$ or *rad24*$^+$ function compromises the checkpoint response to unreplicated DNA: Our results suggest that Chk1p and 14-3-3 proteins could function in the checkpoint response to unreplicated DNA by inhibiting Cdc25p activity, which prevents Cdc2p tyrosine dephosphorylation and thus blocks mitosis. However, previous studies have shown that *chk1*Δ and *rad24*Δ mutants arrest normally in response to unreplicated DNA, although they lack a checkpoint response to damaged DNA (Walworth *et al.* 1993; Al-Khodairy *et al.* 1994; Ford *et al.* 1994). While this study was in progress, Francesconi *et al.* (1997) demonstrated that *chk1*Δ mutants have a partial replication checkpoint defect at high temperatures. Given that overexpression of *rad24*$^+$, *rad25*$^+$, or *chk1*$^+$ seems to enhance the checkpoint signal in HU, we speculated that *rad24*Δ might show a similar replication checkpoint defect at high temperatures. We did not examine *rad25*Δ because it has a much weaker defect in the DNA damage checkpoint (Ford *et al.* 1994). The *rad24*Δ *rad25*Δ double mutant is inviable (Ford *et al.* 1994), so its checkpoint responses cannot be evaluated.

The response of *rad24*Δ and *chk1*Δ cells to HU at high temperature was evaluated. Wild-type, *rad3*Δ, *rad24*Δ, and *chk1*Δ cells were shifted to 37° and HU was added. Cell phenotypes were examined at 2-hr intervals. As reported by Francesconi *et al.* (1997), under these conditions *chk1*Δ cells show a partial replication checkpoint defect (Figure 8A). *rad24*Δ cells are as defective as *chk1*Δ cells, with 27% cuts after 8 hr in the presence of HU at 37° (Figure 8A), indicating that under these conditions, *rad24*$^+$ is also required for the checkpoint response to unreplicated DNA. However, neither strain is fully defective in comparison to *rad3*Δ, in which cuts accumulate sooner and reach higher levels (Figure 8A).

***cds1*Δ *rad24*Δ, like the *cds1*Δ *chk1*Δ double mutant, is completely checkpoint defective:** Our results suggest that Chk1p and 14-3-3 proteins could be directly involved in the checkpoint response to unreplicated DNA. It has also been proposed that Cds1p, a protein kinase

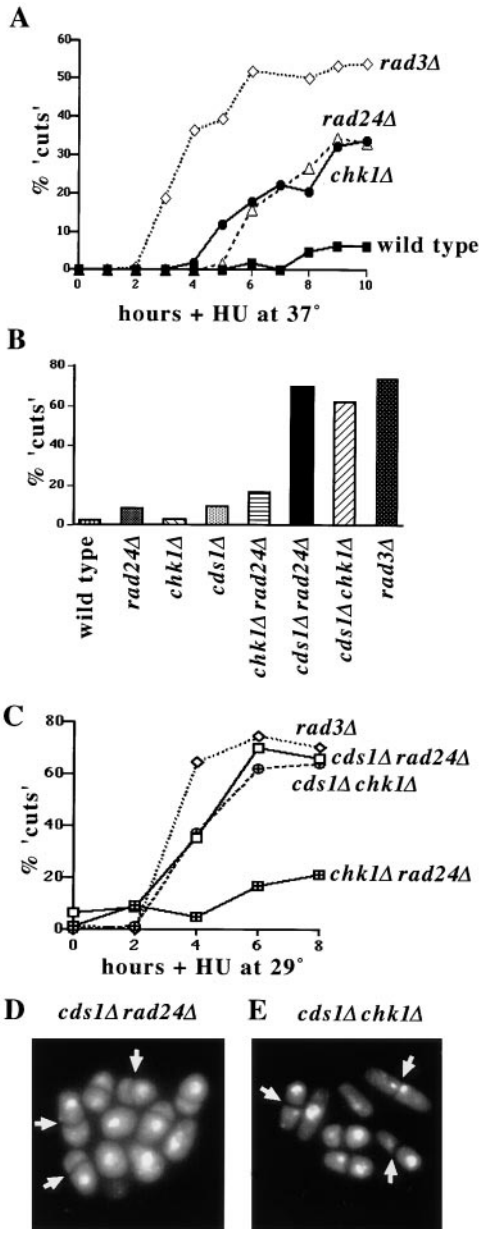

Figure 8.—*rad24*$^+$ and *chk1*$^+$ are required for the checkpoint response to unreplicated DNA. (A) Wild-type (TE235), *rad24*Δ (TE465), *chk1*Δ (TE548), and *rad3*Δ (TE890) cells were grown to midlog phase. HU was added to a final concentration of 10 mm and cells were shifted to 37° at $t = 0$. Samples were collected at 2-hr intervals, fixed, and stained with DAPI, and cuts were counted. At least 100 cells were counted for each data point. (B) The strains listed in A along with *cds1*Δ (TE700), *chk1*Δ *rad24*Δ (TE 922), *cds1*Δ *rad24*Δ (TE919), and *cds1*Δ *chk1*Δ (TE856) were grown and HU was added as described in A, except that cells were at 29° throughout the experiment. The percentage of cuts at 6 hr is shown. (C) Cells from the same experiment as B were examined at 2-hr intervals. (D) *cds1*Δ *rad24*Δ (TE919) cells and (E) *cds1*Δ *chk1*Δ (TE856) cells after 6 hr in 10 mm HU at 29°, stained with DAPI. Arrows indicate cut cells.

with significant similarity to Rad53p, which is required for the checkpoint response to unreplicated DNA in *S. cerevisiae* (Allen *et al.* 1994; Weinert *et al.* 1994), is involved in the checkpoint response to unreplicated DNA. *cds1*$^+$ cannot be the only gene necessary for checkpoint arrest, because *cds1*$^-$ mutants arrest normally in response to HU, though they quickly lose viability (Murakami and Okayama 1995). To explain why single mutants in *rad24*$^+$, *cds1*$^+$, and *chk1*$^+$ are only modestly checkpoint defective, we considered the possibility that redundant pathways are involved in the checkpoint response to unreplicated DNA. To investigate further the roles of *rad24*$^+$, *cds1*$^+$, and *chk1*$^+$ in the checkpoint response to unreplicated DNA, we constructed double mutants among these genes and studied their checkpoint responses by examining their phenotypes in the presence of HU at 29°. Like *rad24*Δ single mutants (Ford *et al.* 1994), *cds1*Δ *rad24*Δ and *chk1*Δ *rad24*Δ were semi-wee and had a round morphology under normal conditions (data not shown).

In the presence of HU, *chk1*Δ *rad24*Δ cells were only slightly more checkpoint defective than the *chk1*Δ or *rad24*Δ single mutants, showing 17% cuts after 6 hr (Figure 8, B and C). As the double mutant is not more checkpoint defective than the single mutants, *rad24*$^+$ and *chk1*$^+$ may function together in the checkpoint response to unreplicated DNA. In contrast, *cds1*Δ *rad24*Δ double-mutant cells were much more checkpoint defective than *rad24*Δ or *cds1*Δ single mutants, showing 70% cuts after 6 hr in the presence of HU (Figure 8, B and D). As has been reported elsewhere (Boddy *et al.* 1998; Lindsay *et al.* 1998; Zeng *et al.* 1998), the *cds1*Δ *chk1*Δ cells were also more severely checkpoint defective than *chk1*Δ or *cds1*Δ cells, with 62% cuts at 6 hr in the presence of HU (Figure 8, B and E). As shown in Figure 8C, the kinetics and extent of cut formation in *cds1*Δ *rad24*Δ and *cds1*Δ *chk1*Δ cells suggest that these mutants are completely defective in the checkpoint response to unreplicated DNA (compare to *rad3*Δ). These results suggest that *rad24*$^+$ and *chk1*$^+$ function in parallel with *cds1*$^+$ in the checkpoint response to unreplicated DNA, as the checkpoint response can be fully eliminated by combining mutations in either *rad24*$^+$ or *chk1*$^+$ with *cds1*$^+$.

We also examined the DNA damage checkpoint response of the double mutants by evaluating their survival at increasing doses of UV irradiation. Again, the *chk1*Δ *rad24*Δ cells were somewhat checkpoint defective, showing UV-sensitivity comparable to *chk1*Δ, while *cds1*Δ *rad24*Δ and *cds1*Δ *chk1*Δ cells were severely UV-sensitive like *rad3*Δ cells (data not shown). These results suggest that *cds1*$^+$ may function in parallel with *rad24*$^+$ and *chk1*$^+$ in the checkpoint response to damaged DNA, as the checkpoint response can be fully eliminated by combining mutations in either *rad24*$^+$ or *chk1*$^+$ with *cds1*$^+$.

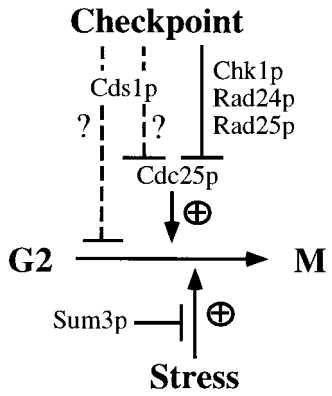

Figure 9.—Model of genetic interactions influencing checkpoint control. The checkpoint response to unreplicated DNA uses parallel pathways, one requiring Cds1p and the other requiring Chk1p and 14-3-3 proteins. The targets of Cds1p are not known. The stress response counteracts the checkpoint response, and inhibition of this pathway indirectly enhances the checkpoint response. Sum3p inhibits the cell-cycle response to stress through unknown effectors. For further discussion, see the text.

DISCUSSION

We have conducted a screen for genes involved in the checkpoint response to unreplicated DNA, by searching for high-copy suppressors of the replication checkpoint defect of cells overexpressing *cdc25*$^+$ (*OPcdc25*$^+$). None of the suppressors that were identified affect the cell-cycle progression of *OPcdc25*$^+$ under normal conditions, so they are not simply negative regulators of the G2/M transition. Rather, they suppress by somehow enhancing the ability of *OPcdc25*$^+$ cells to respond to checkpoint signals. As summarized in Figure 9, the suppressors fall into two classes. One group of suppressors includes *rad24*$^+$, *rad25*$^+$, and *chk1*$^+$, three genes known to be involved in the DNA damage checkpoint. Our analysis suggests that these genes negatively regulate Cdc25p, thus rescuing the checkpoint defect caused by Cdc25p overexpression. We have shown that the same interactions are important during the checkpoint response to unreplicated DNA in wild-type cells, as *rad24*$^-$ and *chk1*$^-$ mutants have replication checkpoint defects, particularly when combined with mutations in *cds1*$^+$. Thus, we believe that the damage checkpoint proteins Rad24p, Rad25p, and Chk1p function in concert with the protein kinase Cds1p in the checkpoint response to unreplicated DNA. Another group of suppressors includes *sum3*$^+$. Overexpression of *sum3*$^+$ may block the stress response pathway that inhibits cell-cycle progression by a mechanism that is independent of Cdc2 tyrosine phosphorylation.

***sum3*$^+$ regulates the osmotic stress and checkpoint responses:** *sum3*$^+$ encodes a member of the DEAD box family of ATP-dependent RNA helicases. DEAD box proteins have been found to have roles in many cellular processes including RNA splicing, RNA degradation,

ribosome biogenesis, ribosome assembly, translation, and regulation of maternally expressed RNAs or developmentally regulated mRNAs (Schmid and Linder 1992; Py *et al.* 1996). Members of this family include mouse eIF-4A and *PL10*, numerous *S. cerevisiae* genes, and Drosophila *WM6* (Warbrick and Glover 1994). The latter was identified in a screen for Drosophila cDNAs able to suppress the mitotic catastrophe phenotypes of *wee1-50 OPcdc25*$^+$ or *wee1-50 mik1*Δ at the restrictive temperature (Schmid and Linder 1992; Warbrick and Glover 1994). Compared to many other DEAD helicases, the protein encoded by *WM6* is relatively distantly related to Sum3p with 29% identity and 52% similarity, and it is not known whether it can rescue checkpoint mutants in the presence of HU. The closest known homologue of *sum3*$^+$ is the *S. cerevisiae* gene, *DED1* (Figure 2). Ded1p was previously believed to function in splicing, but recent work implicates it in the initiation step of translation (Chuang *et al.* 1997; de la Cruz *et al.* 1997). Both *DED1* and *sum3*$^+$ are essential for viability (Struhl 1985; B. Grallert and K. Labib, personal communication). Intriguingly, we have recently isolated another essential gene potentially implicated in translation initiation, *sum1*$^+$, in a screen for suppressors of the checkpoint mutant *cdc2-3w* (Humphrey and Enoch 1998). *sum1*$^+$ is homologous to the *S. cerevisiae* WD repeat protein *TIF34*, which is associated with an essential multiprotein complex, eIF3, required for translational initiation (Naranda *et al.* 1997). We believe that these suppressors are not working by stimulating translation of ribonucleotide reductase protein (the target of HU), because cells overexpressing the *sum* genes in the presence of HU are elongated, while cells overexpressing ribonucleotide reductase are not.

Given that the cell-cycle effectors of the Sty1p/Spc1p stress-activated MAPK pathway are not known, at least two models can be drawn to account for Sum3p's negative regulation of the cell-cycle response to osmotic stress. In one model, overexpression of *sum3*$^+$ inhibits phosphorylation of a MAPK target. For example, overexpression of Sum3p could disrupt the formation of a complex involved in translation initiation, such that the complex could not act as a substrate for Sty1p/Spc1p. In another model, the *sum* genes and the MAPK pathway share a common target, which is involved in regulation of the G2 to M transition. For example, it is possible that MAP kinase inactivation indirectly causes cell-cycle arrest by inhibiting translation of a cell-cycle regulator. Overexpression of *sum1*$^+$ or *sum3*$^+$ may also inhibit translation of this regulator, and thus cause a similar cell-cycle response.

The molecular mechanism by which mitosis is inhibited by the negative regulators of the stress response is not clear. As shown here, overexpression of *pyp1*$^+$ or *sum3*$^+$ does not stimulate tyrosine phosphorylation of Cdc2p (Figure 6B), suggesting that a novel mode of G2/M regulation is being employed. Possible mechanisms could include effects on Cdc2p threonine 167 phosphorylation, cyclin B stability, or interactions with other proteins such as cyclin-dependent kinase inhibitors.

Chk1p, Rad24p, and Rad25p may negatively regulate Cdc25p: Overexpression of *rad24*$^+$, *rad25*$^+$, or *chk1*$^+$ restores *OPcdc25*$^+$ checkpoint function during hydroxyurea treatment (Figures 1, 3, and 4). Because overexpression of these genes is not able to restore checkpoint function in other checkpoint-deficient strains, we hypothesize that Chk1p, Rad24p, and Rad25p may negatively regulate Cdc25p. This idea is supported by the observation that overexpression of these genes induces accumulation of Cdc25p. A model to describe the negative regulation of Cdc25p by Chk1p and 14-3-3p has been proposed: Chk1p phosphorylates Cdc25p on serine residues, creating binding sites for 14-3-3 proteins (Peng *et al.* 1997; Sanchez *et al.* 1997). Binding of 14-3-3p inhibits Cdc25p activity by an unknown mechanism (Peng *et al.* 1997). It is attractive to speculate that overexpression of either 14-3-3p or Chk1p leads to sequestration of Cdc25p in an inactive complex. In this form Cdc25p might also be less susceptible to ubiquitination and proteolysis (Moreno *et al.* 1990; Kovelman and Russell 1996; Nefsky and Beach 1996), and thus accumulate to high levels. Perhaps sequestration of the excess Cdc25p rescues the checkpoint defect in these strains by restoring the balance between positive and negative regulators of Cdc2p tyrosine phosphorylation.

A role for *rad24*$^+$ and *chk1*$^+$ in the checkpoint response to unreplicated DNA? *rad24*$^+$, *rad25*$^+$, and *chk1*$^+$ have been identified in this study as high-copy overexpression suppressors of the replication checkpoint defect of *OPcdc25*$^+$ mutants. The loss of function of *rad24*$^+$ or *chk1*$^+$ is known to compromise the DNA damage checkpoint response (Walworth *et al.* 1993; Al-Khodairy *et al.* 1994; Ford *et al.* 1994). A mutation in the Drosophila homologue of *chk1*$^+$, *grapes*, has been shown to disrupt the replication checkpoint in developing embryos (Fogarty *et al.* 1997; Sibon *et al.* 1997). The replication checkpoint is defective in *chk1*Δ yeast cells at high temperatures (Francesconi *et al.* 1997), and we have shown that this is the case for *rad24*Δ cells as well (Figure 8A). In addition, we have constructed the double mutants *cds1*Δ *rad24*Δ and *cds1*Δ *chk1*Δ. These double mutants show complete loss of both the DNA damage and unreplicated DNA checkpoints (Figure 8 and our unpublished data). Thus, both overexpression and loss of function of Chk1p and Rad24p can affect the checkpoint response to unreplicated DNA. Together, these results suggest that *rad24*$^+$ and *chk1*$^+$ may be directly involved in the checkpoint response to unreplicated DNA.

Cds1p may act in parallel with Chk1p and 14-3-3 proteins: The protein kinase Cds1p has been considered a possible candidate effector of the replication checkpoint. However, *cds1*$^-$ mutants arrest normally in re-

sponse to HU (Murakami and Okayama 1995). We have observed that double mutants *cds1*Δ *rad24*Δ and *cds1*Δ *chk1*Δ are completely defective in both the DNA damage and unreplicated DNA checkpoints (Figure 8 and our unpublished data; Boddy *et al.* 1998; Lindsay *et al.* 1998; Zeng *et al.* 1998).

A possible model to explain these results is that Chk1p and Rad24p are acting in a common pathway to arrest the cell cycle in response to checkpoint signals, while Cds1p is acting in a parallel pathway (Figure 9). When both pathways are compromised the checkpoint is abolished. However, if only one pathway is compromised its function can be replaced by the other pathway. Interestingly, while Chk1p is not normally phosphorylated in response to HU, Lindsay *et al.* (1998) have observed significant phosphorylation of Chk1p in *cds1*$^-$ mutants in response to HU. One interpretation of this finding is that Cds1p normally prevents Chk1p phosphorylation, possibly because the Cds1p pathway is preferred in wild-type cells. We favor a model that Cds1p, like Chk1p, can directly regulate cell-cycle progression by phosphorylating cell-cycle control proteins. Like overexpression of *chk1*$^+$, overexpression of *cds1*$^+$ blocks cell-cycle progression and stabilizes Cdc25p in wild-type cells (data not shown). Surprisingly, however, overexpression of *cds1*$^+$ does not rescue the checkpoint defect of *OPcdc25*$^+$ (Figure 1). This could be because additional regulators are required to activate Cds1p in response to unreplicated DNA. For example, *rad26*$^+$ interacts with *cds1*$^+$ genetically and biochemically (Lindsay *et al.* 1998) and is likely to be involved in the replication checkpoint because *rad26*$^-$ alleles specifically defective in only the replication checkpoint have been identified (Uchiyama *et al.* 1997). Alternatively, Cds1p could mediate the checkpoint response by interacting with other regulators of Cdc2p such as Wee1p or Mik1p, as proposed by Boddy *et al.* (1998).

A different model for the role of *cds1*$^+$ in checkpoint control has been proposed by Lindsay *et al.* (1998), who have independently noticed the severe defect in the checkpoint response of the *cds1*Δ *chk1*Δ double mutant. They propose that Cds1p performs a repair function specifically required when S-phase is blocked. According to their model, in the absence of *cds1*$^+$, HU treatment causes DNA damage and thus *chk1*$^+$ becomes essential for cell-cycle arrest. At this point, both models explain the experimental observations. To distinguish between them it will be necessary to determine whether Cds1p interacts directly with cell-cycle regulators *in vivo*.

We thank S. Moreno and P. Nurse for antibodies, R. Daga for advice on Cdc25p detection, and A. Carr, J. Millar, P. Shiozaki, P. Russell, A. Bueno, J. Bähler, J. Pringle, H. Murakami, and H. Okayama for providing strains or plasmids. A. Carr, H. Lindsay, B. Grallert, and K. Labib are thanked for discussing unpublished data. We thank Hamid Ghazizadeh, Gladys Reimundo, and Maria Sanchez for technical support and Fred Winston, Carolyn Chapman, Sarah Evans, Elissa Lei, Musetta Leung, Elizabeth Moynihan, and Elspeth Stewart for valuable advice and comments on this manuscript. This work was supported by a grant from the National Institutes of Health (GM50015). T.H. was supported by a European Molecular Biology Organization long-term fellowship, a Human Frontier Science Program Organization fellowship, and a Charles A. King Trust fellowship.

LITERATURE CITED

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

Allen, J. B., Z. Zhou, W. Seide, E. C. Freidberg and S. J. Elledge, 1994 The SAD1 protein kinase controls multiple checkpoints and DNA-damage induced transcription in yeast. Genes Dev. **8:** 2401–2415.

Boddy, M., B. Furnari, O. Mondesert and P. Russell, 1998 Replication checkpoint enforced by kinases Cds1 and Chk1. Science **280:** 909–912.

Brizuela, L., G. Draetta and D. Beach, 1987 p13*suc1* acts in the fission yeast cell division cycle as a component of the p34cdc2 protein kinase. EMBO J. **6:** 3507–3514.

Chuang, R.-Y., P. L. Weaver, Z. Liu and T.-H. Chang, 1997 Requirement of the DEAD-box protein Ded1p for messenger RNA translation. Science **275:** 1468–1471.

de la Cruz, J., I. Iost, D. Kressler and P. Linder, 1997 The p20 and Ded1 proteins have antagonistic roles in eIF4E-dependent translation in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **94:** 5201–5206.

Degols, G., K. Shiozaki and P. Russell, 1996 Activation and regulation of the Spc1 stress-activated kinase in *Schizosaccharomyces pombe*. Mol. Cell. Biol. **16:** 2870–2877.

Dunphy, W., 1994 The decision to enter mitosis. Trends Cell Biol. **4:** 202–207.

Enoch, T., and P. Nurse, 1990 Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. Cell **60:** 665–673.

Enoch, T., K. L. Gould and P. Nurse, 1991 Mitotic checkpoint control in fission yeast. Cold Spring Harbor Symp. Quant. Biol. **56:** 409–416.

Enoch, T., A. M. Carr and P. Nurse, 1992 Fission yeast genes involved in coupling mitosis to completion of DNA replication. Genes Dev. **6:** 2035–2046.

Feinberg, A. P., and B. Vogelstein, 1984 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137:** 266–267.

Fogarty, P., S. Campbell, R. Abu-Shumays, B. de Saint Phalle, K. Yu *et al.*, 1997 The *Drosophila grapes* gene is related to checkpoint gene *chk1/rad27* and is required for the late syncytial division fidelity. Curr. Biol. **7:** 418–426.

Ford, J. C., F. Al-Khodairy, E. Fotou, K. S. Sheldrick, D. J. F. Griffiths *et al.*, 1994 S. pombe 14-3-3 homologues encode an essential function required for the DNA damage checkpoint. Science **265:** 533–535.

Francesconi, S., M. Grenon, D. Bouvier and G. Baldacci, 1997 p56^{chk1} protein kinase is required for replication checkpoint at 37°C in fission yeast. EMBO J. **16(6):** 1332–1341.

Fuller-Pace, F., 1994 RNA helicases: modulators of RNA structure. Trends Cell Biol. **4:** 271–274.

Furnari, B., N. Rhind and P. Russell, 1997 Cdc25 mitotic inducer targeted by Chk1 DNA damage checkpoint kinase. Science **277:** 1501–1505.

Hartwell, L., and T. Weinert, 1989 Checkpoints: Controls that ensure the order of cell cycle events. Science **246:** 629–634.

Hirano, T., S. Funahashi, T. Uemura and M. Yanagida, 1986 Isolation and characterization of *Schizosaccharomyces pombe cut* mutants that block nuclear division but not cytokinesis. EMBO J. **5:** 2973–2979.

Humphrey, T., and T. Enoch, 1995 Keeping mitosis in check. Curr. Biol. **5:** 376–379.

Humphrey, T., and T. Enoch, 1998 Sum1, a highly conserved WD-repeat protein, suppresses S-M checkpoint mutants and inhibits the osmotic stress response in fission yeast. Genetics **148:** 1731–1742.

Knudsen, K. E., E. S. Knudsen, J. Y. Wang and S. Subramani, 1996 p34cdc2 kinase activity is maintained upon activation of the replication checkpoint in Schizosaccharomyces pombe. Proc. Natl. Acad. Sci. USA **93:** 8278–8283.

Kovelman, R., and P. Russell, 1996 Stockpiling of Cdc25 during a DNA replication checkpoint arrest in *Schizosaccharomyces pombe*. Mol. Cell. Biol. **16:** 86–93.

Lavin, M., K. Khanna, H. Beamish, K. Spring, D. Watters *et al.*, 1995 Relationship of ataxia-telangiectasia protein ATM to phosphoinositide 3-kinase. Trends Biochem. Sci. **20:** 382–383.

Lindsay, H. D., D. J. F. Griffiths, R. Edwards, J. M. Murray, P. U. Christense *et al.*, 1998 S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in Schizosaccharomyces pombe. Genes Dev. **12:** 382–395.

Maundrell, K., 1993 Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene **123:** 127–130.

Mertins, P., and D. Gallwitz, 1987 A single intronless actin gene in the fission yeast *Schizosaccharomyces pombe*: nucleotide sequence and transcripts formed in homologous and heterologous yeast. Nucleic Acids Res. **15:** 7369–7379.

Millar, J. B. A., P. Russell, J. E. Dixon and K. L. Guan, 1992 Negative regulation of mitosis by two functionally overlapping PTPases in fission yeast. EMBO J. **11:** 4943–4952.

Millar, J. B. A., V. Buck and M. G. Wilkinson, 1995 Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. Genes Dev. **9:** 2117–2130.

Moreno, S., P. Nurse and P. Russell, 1990 Regulation of mitosis by cyclic accumulation of $p80^{cdc25}$ mitotic inducer in fission yeast. Nature **344:** 549–552.

Moreno, S., A. Klar and P. Nurse, 1991 Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*, pp. 795–823 in *Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology*, edited by C. Guthrie and G. R. Fink. Academic Press, New York.

Murakami, H., and H. Okayama, 1995 A kinase from fission yeast responsible for blocking mitosis in S phase. Nature **374:** 817–819.

Naranda, T., M. Kainuma, S. E. MacMillan and J. W. B. Hershey, 1997 The 39-kilodalton subunit of eukaryotic translation initiation factor 3 is essential for the complex's integrity and for cell viability in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **17:** 145–153.

Nefsky, B., and D. Beach, 1996 Pub1 acts as an E6-AP-like protein ubiquitin ligase in the degradation of Cdc25. EMBO J. **15:** 1301–1312.

O'Connell, M. J., J. M. Raleigh, H. M. Verkade and P. Nurse, 1997 Chk1 is a wee1 kinase in the G2 DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation. EMBO J. **16:** 545–554.

Peng, C.-Y., P. R. Graves, R. S. Thoma, Z. Wu, A. Shaw *et al.*, 1997 Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine 216. Science **277:** 1501–1505.

Pidoux, A. L., E. H. Fawell and J. Armstrong, 1990 Glycerol-3-phosphate dehydrogenase homologue from *Schizosaccharomyces pombe*. Nucleic Acids Res. **18:** 7145.

Prentice, H. L., 1992 High efficiency transformation of *Schizosaccharomyces pombe* by electroporation. Nucleic Acids Res. **20:** 621.

Py, B., C. Higgins, H. Krisch and A. J. Carpousis, 1996 A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. Nature **381:** 169–172.

Rave, N., R. Crkvenjakov and H. Boedtker, 1979 Identification of procollagen mRNAs transferred to diazobenzyloxymethyl paper from formaldehyde agarose gels. Nucleic Acids Res. **6:** 3559–3567.

Rhind, N., and P. Russell, 1998 Tyrosine phosphorylation of Cdc2 is required for the replication checkpoint in *Schizosaccharomyces pombe*. Mol. Cell. Biol. **18:** 3782–3787.

Rhind, N., B. Furnari and P. Russell, 1997 Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast. Genes Dev. **11:** 504–511.

Sanchez, Y., C. Wong, R. S. Thoma, R. Richman, Z. Wu *et al.*, 1997 Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science **277:** 1497–1501.

Schmid, S., and P. Linder, 1992 DEAD protein family of putative RNA helicases. Mol. Microbiol. **6(3):** 283–292.

Sheldrick, K., and A. M. Carr, 1993 Feedback controls and G2 checkpoints: fission yeast as a model system. Bioessays **15:** 775–781.

Shiozaki, K., and P. Russell, 1995 Cell cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature **378:** 739–743.

Shiozaki, P., and P. Russell, 1996 Conjugation, meiosis, and osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. Genes Dev. **10:** 2276–2288.

Sibon, O. C., V. A. Stevenson and W. E. Theurkauf, 1997 DNA-replication checkpoint at the *Drosophila* midblastula transition. Nature **388:** 93–97.

Stewart, E., and T. Enoch, 1996 S-phase and DNA damage checkpoints: a tale of two yeasts. Curr. Opin. Cell Biol. **8:** 781–787.

Struhl, K., 1985 Nucleotide sequence and transcriptional mapping of the yeast pet56-his3-ded1 gene region. Nucleic Acids Res. **13:** 8587–8601.

Uchiyama, M., I. Galli, D. J. Griffiths and T. S. Wang, 1997 A novel mutant allele of *Schizosaccharomyces pombe rad26* defective in monitoring S-phase progression to prevent premature mitosis. Mol. Cell. Biol. **17:** 3103–3115.

Walworth, N., and R. Bernards, 1996 *rad*-dependent response of the *chk1*-encoded protein kinase at the DNA damage checkpoint. Science **271:** 353–356.

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

Warbrick, E., and D. M. Glover, 1994 A *Drosophila* gene encoding a DEAD box RNA helicase can suppress the loss of *wee1*/*mik1* function in *Schizosaccharomyces pombe*. Mol. Gen. Genet. **245:** 654–657.

Weinert, T. A., G. L. Kiser and L. H. Hartwell, 1994 Mitotic checkpoint genes in budding yeast and dependence of mitosis on DNA replication and repair. Genes Dev. **8:** 652–656.

Wilkinson, M. G., M. Samuels, T. Takeda, W. M. Toone, J. Shieh *et al.*, 1996 The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. Genes Dev. **10:** 2289–2301.

Zeng, Y., K. L. Chrispell Forbes, Z. Wu, S. Moreno, H. Piwnica-Worms *et al.*, 1998 Phosphorylation of Cdc25p by Cds1p or Chk1p is required for the checkpoint response to unreplicated DNA in fission yeast. Nature **395:** 507–510.

Communicating editor: M. D. Rose