Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways

Zhaoxia Sun,[1,2] David S. Fay,[1] Federica Marini,[3] Marco Foiani,[3] and David F. Stern[1,4]

[1]Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520-8023; [2]Department of Biology, Yale University, New Haven, Connecticut 06520-8103 USA; [3]Dipartimento di Genetica e di Biologia dei Microrganismi, Universita' degli Studi di Milano, Milan, Italy

***SPK1/RAD53/MEC2/SAD1* of *Saccharomyces cerevisiae* encodes an essential protein kinase that is required for activation of replication-sensitive and DNA damage-sensitive checkpoint arrest. We have investigated the regulation of phosphorylation and kinase activity of Spk1p during the cell cycle and by conditions that activate checkpoint pathways. Phosphorylation of Spk1p is induced by treatment of cells with agents that damage DNA or interfere with DNA synthesis. Although only S- and G_2-phase *cdc* mutants arrest with hyperphosphorylated Spk1p, damage-induced phosphorylation of Spk1p can occur in G_1 and M as well. Hydroxyurea (HU) induces phosphorylation of kinase-defective forms of Spk1p, demonstrating that this regulated phosphorylation of Spk1p occurs in *trans*. HU-induced phosphorylation is associated with increased catalytic activity of Spk1p. Furthermore, overexpression of wild-type *SPK1*, but not checkpoint-defective alleles, delays progression through the G_1/S boundary. Damage-dependent phosphorylation of Spk1p requires both *MEC1* and *MEC3*, whereas *MEC1* but not *MEC3*, is required for replication block-induced phosphorylation. These data support the model that Spk1p is an essential intermediate component in a signal transduction pathway coupling damage and checkpoint functions to cell cycle arrest. This regulation is mediated through a protein kinase cascade that potentially includes Mec1p and Tel1p as the upstream kinases.**

[*Key Words*: *Saccharomyces cerevisiae*; DNA damage; checkpoint pathway; *SPK1/RAD53/MEC2/SAD1*; *MEC1*]

Received October 27, 1995; revised version accepted December 29, 1995.

The integrity of the genome is under constant assault through errors in replication and chromosome mechanics, and through the action of extrinsic mutagens. Prokaryotic and eukaryotic cells have evolved a network of mechanisms for minimizing the consequences of DNA damage. Basal and damage-inducible DNA repair systems are called into play by an array of DNA lesions. Damage-regulated checkpoint controls are important elements of the cellular response to DNA lesions and presumably work by providing enough time for the operation of DNA repair systems. In parallel, replication-sensitive checkpoint controls may be important to ensure that cells remain in S until replication is complete. Failure of these cell cycle restraints can increase mutation rates and genomic instability, which can lead to cancer in humans (Hartwell and Kastan 1994).

p53, the most frequently mutated tumor suppressor gene, is required for damage-regulated arrest at the G_1/S checkpoint (Lane 1992). A second human checkpoint gene, *ATM*, is also a tumor suppressor gene. *ATM* mutations cause the ataxia telangiectasia (AT) syndrome, which is characterized by a number of defects including greatly elevated cancer risk (for review, see Meyn 1995). Cells from AT individuals are hypersensitive to ionizing radiation and fail to show normal damage-induced cell cycle arrest. The *ATM* gene seems to operate upstream from p53 and is homologous to a gene family including lipid and protein kinases (Kastan et al. 1992; Lehmann and Carr 1995; Savitsky et al. 1995). Significantly, *ATM* is structurally homologous to budding yeast *TEL1* and *MEC1*, and fission yeast *rad3+* (Kato and Ogawa 1994; Greenwell et al. 1995; Savitsky et al. 1995; Zakian 1995). The latter two have DNA checkpoint functions, indicating a functional homology. Hence, the identification of the human damage checkpoint gene *ATM* forged a link with the genetic analysis of checkpoint control pathways in yeasts.

Multiple DNA-related checkpoint pathways have been identified in *Saccharomyces cerevisiae*. When DNA is damaged, these controls prevent cells in G_1 from entering S phase, cells in late S or G_2 phase from undergoing mitosis, and they also retard S phase progression (Weinert and Hartwell 1988; Hartwell and Weinert 1989;

[4]Corresponding author.

Siede et al. 1993; Paulovich and Hartwell 1995). Another checkpoint prevents cells with incompletely replicated DNA from exiting S phase (Hartwell and Weinert 1989; Murray 1992). Three distinct sets of DNA checkpoint genes have been identified in *S. cerevisiae*. One set, including *MEC3*, *RAD9*, and *RAD24*, is required for function of the damage-regulated G_1/S and late S/G_2 checkpoints, and is dispensable for the S-phase replication checkpoint (Siede et al. 1993; Weinert et al. 1994). A second set, defined by *dun* alleles of *POL2*, is required for the S-phase checkpoint but is unnecessary for the damage checkpoints (Navas et al. 1995). Finally, the two essential genes *MEC1* and *SPK1/RAD53/SAD1/MEC2* are necessary for both damage-regulated and replication checkpoints (Allen et al. 1994; Weinert et al. 1994).

SPK1 encodes a protein kinase that is located primarily in the nucleus (Stern et al. 1991; Zheng et al. 1993). *SPK1* is activated transcriptionally near the G_1/S boundary. This regulation is probably conferred by a pair of consensus *Mlu1* cell cycle box (MCB) regulatory elements such as those found in a number of genes transcribed in S phase including DNA synthetic enzymes and cyclins Clb5 and Clb6. Other upstream sequences similar to DNA damage response elements suggested that *SPK1* is involved in the DNA damage response (Zheng et al. 1993). *SPK1*, like *MEC1*, is required for the activation of DNA damage-induced checkpoints in G_1 and G_2 and for the induction of the S phase checkpoint, as partial loss-of-function mutants are defective in these processes (Allen et al. 1994; Weinert et al. 1994). In addition, *SPK1* is required for the transcriptional stimulation of DNA repair genes *RNR2* and *RNR3* (Allen et al. 1994). This is genetically distinguishable from the checkpoint response, as *dun1* cells are checkpoint competent (Zhou and Elledge 1993). Finally, the nuclear localization, transcriptional regulation, and terminal arrest phenotypes of *spk1*Δ null mutants raised the possibility that the essential function of Spk1p is associated with DNA replication (Zheng et al. 1993). Hence, Spk1p may be a pleiotropic regulator of both DNA replication and the damage response.

The distinct phenotypes of *RAD9*-like genes and *POL2 (dun2)*-like genes has led to the model that each set defines an independent signaling pathway activated by either DNA damage or partly replicated DNA. The common phenotypes of *spk1* and *mec1* and the fact that they are required for both sets of checkpoints might mean that these genes are involved in both sets of recognition processes or, alternatively, that they function in a common pathway downstream from the recognition pathways and couple those pathways to cell cycle arrest (Allen et al. 1994; Weinert et al. 1994). Although these models are consistent with current data, there is virtually no direct evidence to verify them. For the most part, the existence of these pathways has been inferred from common mutant phenotypes, rather than genetic or biochemical epistasis experiments. For example, there is no direct evidence that Spk1p and Mec1p act in the same pathway or that either is regulated by DNA replication or DNA damage.

As a first step toward biochemically defining these checkpoint pathways, we have investigated the regulation of Spk1p in the cell cycle and in response to induction of checkpoint arrest. We show here that Spk1p phosphorylation is regulated by damage and replication checkpoints. Activation of Spk1p through overexpression is sufficient to inhibit cell cycle progression at the G_1/S boundary. The results suggest that Mec1p and Spk1p participate serially in a linear damage- and replication-regulated pathway in which Spk1p is regulated in a protein kinase cascade.

Results

Cell cycle regulation of Spk1p abundance and modification

Initially, *SPK1* transcripts are very low in G_1 arrested cells and increase to maximal levels as cells enter S phase (Zheng et al. 1993). This apparent cell cycle regulation may be misleading, however, as the majority of MCB gene products give rise to stable proteins that do not fluctuate much during the cell cycle (McIntosh 1993). Therefore, we examined Spk1p abundance in a population of cells synchronized at Start and then released into G_1 (Fig. 1). Growth of strain 1608-21C (*cln1*Δ, *cln2*Δ, *cln3*Δ, *GAL1–CLN3*) (Table 1) is strictly regulated by galactose induction of Cln3p. These cells arrest uniformly at Start in raffinose and proceed synchronously through the cell cycle after induction with galactose. Spk1p was present at Start and increased substantially 30 min after release from Start arrest, coincident with the emergence of small budded cells (Fig. 1A). Spk1p levels were sustained until 70 min, when large budded cells first appear (Fig. 1A). This timing is consistent with an S phase-specific increase in Spk1p levels and is consonant with RNA analysis (Zheng et al. 1993). In contrast to the mRNA, however, Spk1p was present in significant quantities at Start, suggesting that Spk1p is more stable than *SPK1* mRNA.

In subsequent experiments with a different antibody preparation and different electrophoresis conditions, Spk1p resolved into multiple species (Fig. 1B). A lower mobility form appeared in parallel with small budded cells. [The sample for the 40 min time point was evidently lost, as Spk1p persisted and remained shifted in other experiments (Fig. 1C).] After 60 min Spk1p reverted to the lower molecular weight singlet form in parallel with the appearance of large budded cells (Fig. 1B). Spk1p remained in singlet form as cells divided and entered the next G_1 (data not shown). Because cell cycle progression driven by *CLN3* overexpression in a triple *cln* disruption background may be abnormal, we carried out similar experiments in which isogenic wild-type cells (1255–5C) were synchronized with α-factor. Under these conditions, the Spk1p mobility shift was not detected (data not shown). This negative result may have resulted from poorer synchrony or may mean that the

Table 1. *Plasmids and strains used in this study*

Plasmid/Strain[a]	Description/Genotype	Source
pNB187	YCp50 (*URA3, ARS1, CEN4*) with *GAL1* promoter	P. Novick[b]
pNB187–*SPK1*	*SPK1* coding sequences behind *GAL1* promoter in pNB187	[c]
pNB187–*spk1*K227A	*spk1* K227A coding sequences behind *GAL1* promoter in pNB187	[c]
pNB187–*spk1-1*	*spk1-1* coding sequences behind *GAL1* promoter in pNB187	[d]
316*SPK1*	*SPK1* (*Eco*RI fragment) in pRS316	[e]
316*spk1*K227A	*spk1*K227A (*Eco*RI fragment) in pRS316	[d]
316*spk1*A208P	*spk1*A208P (*Eco*RI fragment) in pRS316	[d]
316*spk1*D339A	*spk1*D339A(*Eco*RI fragment) in pRS316	[d]
316*spk1*D339A/K227A	*spk1*D339A/K227A (*Eco*RI fragment) in pRS316	[d]
1608-21C	*MATa bar1 cln1::TRP1 cln2Δ cln3Δ leu2::LEU2::GAL1::CLN3*	F. Cross[e]
1255-5C	*MATa bar1 CLN1 CLN2 CLN3*	F. Cross[e]
NY605	*MATa leu2-3,112/+ ura3-52/ura3-52 his4-619/+*	P. Novick (pers. comm.)
NY882	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3-Δ200/his-Δ200 LYS2-801/+ ade4/+*	P. Novick (pers. comm.)
DZ6	*MATa/MATα spk1ΔXB::HIS3/+*; disruption of NY882	[d]
DZ413	*MATα spk1ΔXB::HIS3*; segregant from sporulation of DZ6-1	[d]
h9cla1	*MATa his7 ura1 cdc9-1*	L. Hartwell[f]
h5clb1	*MATa his7 ura1 cdc5-1*	L. Hartwell[f]
h28c4a1	*MATa cdc28-4 his7 ura1*	L. Hartwell[f]
h4cla1	*MATa cdc4-1 his7 ura1*	L. Hartwell[f]
h7c4a1	*MATa cdc7-4 his7 ura1*	L. Hartwell[f]
h2c2a1	*MATa cdc2-2 his7 ura1*	L. Hartwell[f]
h8cla1	*MATa cdc8-1 his7 ura1*	L. Hartwell[f]
h17cla1	*MATa cdc17-1 his7 ura1*	L. Hartwell[f]
h16cla1	*MATa cdc16-1 his7 ura1*	L. Hartwell[f]
2593	*MATa ade1 ade2 gal1 his1 lys2 tyr1 ura1 cdc20-2*	K. Nasmyth and L. Hartwell
TWY146	*MATα cdc13-1 ura3 his7*	T. Weinert[g]
TWY148	*MATα cdc13-1 mec2-1 ura3 his7*	T. Weinert[g]
TWY158	*MATα cdc13-1 mec1-1 ura3 his7*	T. Weinert[g]
TWY159	*MATα cdc13-1 mec3-1 ura3 his7*	T. Weinert[g]
TWY446	*MATa cdc17-1 his7*	T. Weinert[g]
TWY447	*MATα cdc17-1 mec1-1 trp1*	T. Weinert[g]
TWY448	*MATa cdc17-1 mec2-1 his7*	T. Weinert[g]
TWY449	*MATα cdc17-1 mec3-1 ura1 ura3 his7 trp1*	T. Weinert[g]
TWY397	*MATa ura3 his7 leu2 trp1*	T. Weinert[h]
K699	*MATa ura3-52 leu2-3,112 trp1-1 ade2-1 can1-100 his3-11,15 GAL psi*$^+$	K. Nasmyth (Research Institute of Molecular Pathology, Vienna, Austria)
1082	*MATα trp ade leu ura lys tyr cdc14-3*	K. Nasmyth
1081	*MATa ade1 ade2 ura1 tyr1 his7 lys2 gal1 cdc15-4*	K. Nasmyth
ZY35-34A	*MATa ade2 ura3 leu2 trp1 his3 cdc34-2*	D. Gonda (Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT)
Y203	*MATa ade2-1 his3 leu2-3,112 lys2 trp1 ura3-Δ100, rnr3::RNA3–URA3–LEU2*	S. Elledge[i]

[a]1608-21C and 1255-5C are isogenic derivatives of BF264-15D (*trp1-1a leu2-3,112 ura3 ade1 his2*). 2593 originated from an A364a background. Stains from L. Hartwell or T. Weinert are isogenic with A364a. K699 is the same as W303. 1082 and 1081 have been crossed to W303 background and are from K. Nasmyth. ZY35-34A is isogenic with YMW1. Y203 is isogenic with XS955-36B.
[b]Salminen and Novick (1989).
[c]Zheng et al. (1993).
[d]D.S. Fay and D.F. Stern (in prep.).
[e]Lambertus and Cross (1994).
[f]Weinert and Hartwell (1993).
[g]Weinert et al. (1994).
[h]Weinert and Hartwell (1990).
[i]Zhou and Elledge (1993).

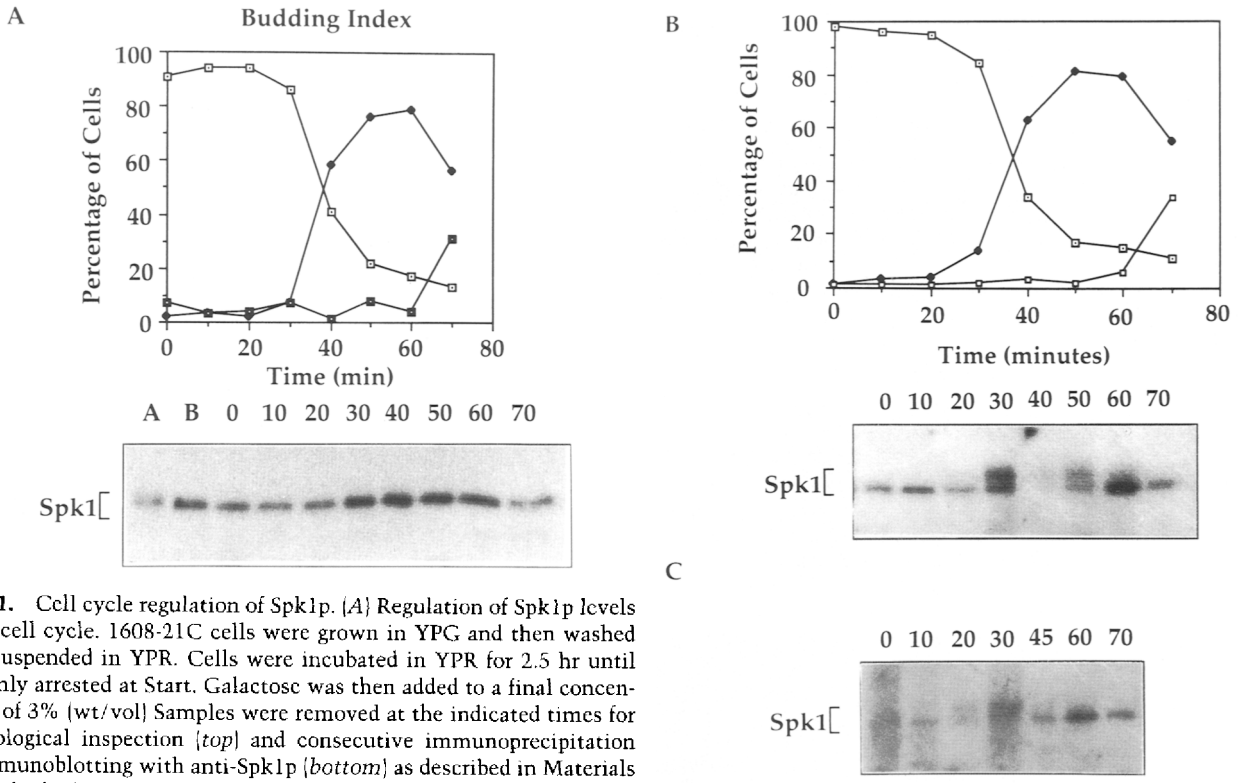

Figure 1. Cell cycle regulation of Spk1p. (*A*) Regulation of Spk1p levels during cell cycle. 1608-21C cells were grown in YPG and then washed and resuspended in YPR. Cells were incubated in YPR for 2.5 hr until uniformly arrested at Start. Galactose was then added to a final concentration of 3% (wt/vol) Samples were removed at the indicated times for morphological inspection (*top*) and consecutive immunoprecipitation and immunoblotting with anti-Spk1p (*bottom*) as described in Materials and methods. (Lane A) Immunoprecipitated overexpressed Spk1p from NY605 *SPK1* cells; (lane B) immunoprecipitated Spk1p from log-phase 1608-21C cells. Other lanes are Spk1p at the indicated times after addition of galactose. (□) Nonbudded cells; (◆) small budded cells; (■) large budded cells. (*B*) Modification of Spk1p during cell cycle progression. This is similar to the experiment in *A*, but Spk1p was immunoprecipitated and immunoblotted with a different polyclonal antibody preparation, and electrophoresis was substantially slowed down to 5 mA constant current. (*Top*) Budding indices; (*bottom*) Spk1p immunoprecipitation and immunoblot, at indicated times after galactose addition. (□) Nonbudded cells; (◆) small budded cells; (□) large budded cells. (*C*) Similar to the experiment in *B*.

apparent S-phase regulation of Spk1p in *cln1*Δ, *cln2*Δ, *cln3*Δ, *GAL1–CLN3* cells may have been induced or enhanced by using an atypical Cln3-driven cell cycle. A number of functional differences have been described between Cln3 and Cln1 and Cln2, although they are functionally redundant in passage through Start (Vallen and Cross 1995).

Modification of Spk1p in cell cycle-arrested cdc^{ts} *mutants*

We then used a set of temperature-sensitive cell division cycle (*cdc*^{ts}) mutant strains under nonpermissive conditions to define more precisely the timing of the mobility shift. At 37°C, *cdc28* (cyclin-dependent kinase) cells arrest at Start, whereas *cdc34*, *cdc4*, and *cdc7* strains arrest after Start but before the onset of DNA synthesis (Hereford and Hartwell 1974; Kolman et al. 1992; Smith et al. 1992; Sorger and Murray 1992). Cdc34p is a ubiquitin-conjugating enzyme apparently required to activate G_1/S Clb/Cdc28 complexes through destruction of the inhibitor Sic1p (Schwob et al. 1994). *CDC4* interacts genetically with *CDC34* (Smith et al. 1992). Cdc7 is a protein kinase with a terminal arrest point genetically downstream of the *cdc4* arrest point (Hereford and Hartwell 1974; Bahman et al. 1988). Spk1p migrated as a singlet in *cdc28-*, *cdc34-*, and *cdc4*-arrested cells but was shifted partially in *cdc7*-arrested cells (Fig. 2, lanes 0). Thus, Spk1p begins to undergo modification near the G_1/S boundary.

We then examined Spk1p in cells arrested in S phase. *cdc2* (DNA polymerase δ); *cdc17* (DNA polymerase α), and *cdc8* (thymidylate kinase) strains arrest with stalled DNA replication (Sclafani and Fangman 1984; Lucchini et al. 1990; Budd and Campbell 1993). Consistent with results derived from the *GAL–CLN3* arrest–release experiment (see Fig. 1B), Spk1p underwent modification in S phase-arrested *cdc* mutants (*cdc2*, *cdc8*, and *cdc17*) (Fig. 3). Under these conditions, Spk1p was converted almost completely to the shifted form. We also used hydroxyurea (HU) as another method for accumulating cells arrested in S phase. HU inhibits ribonucleotide reductase, depleting deoxyribonucleotide pools so that cells accumulate at the S-phase checkpoint. Spk1p shifts were induced by treatment of non-*cdc* strains with HU (Fig. 2).

Spk1p was also analyzed in *cdc* mutants that arrest in G_2 and M. Mutants in *cdc9* (DNA ligase) and *cdc13* arrest in G_2 (Tomkinson et al. 1992; Weber and Byers 1992). Spk1p was converted to the shifted form in *cdc9*

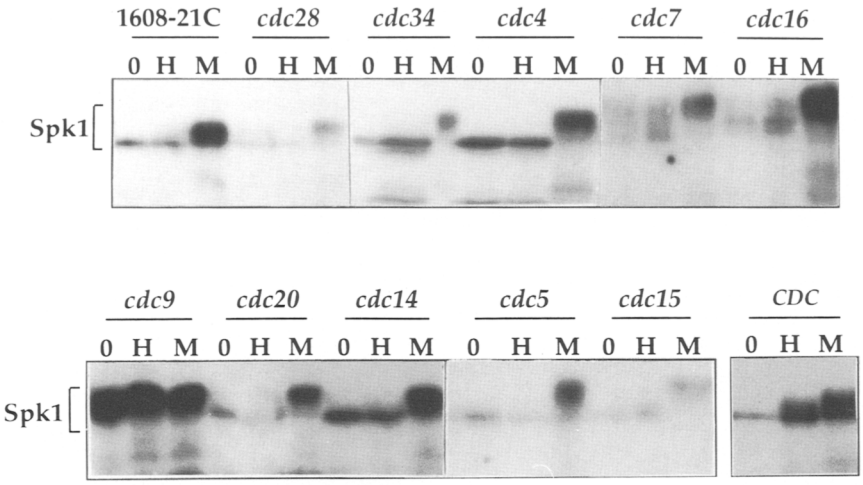

Figure 2. MMS- and HU-regulated Spk1p modification in *cdc* mutants. *cdc*^{ts} mutants were arrested by incubation at the nonpermissive temperature (37°C) for 4 hr. 1608-21C cells were arrested by incubation in YPD at 30°C for 4 hr. Cells were then incubated in YPD (lanes 0), YPD–100 mM HU (lanes H), or YPD–0.1% MMS (lanes M) for an additional 4 hr at 30°C, or 37°C (*cdc*^{ts}), followed by immunoprecipitation and immunoblotting with anti-Spk1p. In this composite, 1608-21C and h28cla1 (*cdc28*) lanes were from a single gel; ZY35-34A (*cdc34*) and h4cla1 (*cdc4*) lanes were from a single gel; h7c4a1 (*cdc7*) and h16cla1 (*cdc16*) samples were from a single gel; h9cla1 (*cdc9*), 2593 (*cdc20*), 1082 (*cdc14*) samples were from a single gel; hclb1 (*cdc5*) and 1081 (*cdc15*) samples were from a single gel; and Y203 (*CDC*) lanes were from a single gel. Indicated strains were arrested and incubated in YPD (lanes 0), in YPD–HU (lanes H), or YPD–MMS (lanes M), and Spk1p was detected by immunoprecipitation/immunoblotting.

(Fig. 2, lane 0) and *cdc13* (Fig. 4A, lane *cdc13*/+) cells, whereas in *cdc16* cells, arrested at the metaphase/anaphase boundary, Spk1p was shifted only partially (Fig. 2, lane 0). Spk1p migrated as a singlet in all other M phase-arrested *cdc* mutants (Fig. 2, *cdc20, cdc14, cdc5, cdc15*, lanes 0).

Phosphatase treatment of Spk1p

To determine whether the mobility shift of Spk1p results from phosphorylation, Spk1p was immunoprecipitated from lysates of *cdc8*^{ts} cells arrested at the nonpermissive temperature and incubated with calf intestine alkaline phosphatase (CIP). Much of Spk1p was converted to the basal mobility form (Fig. 5, cf. 20-min time point to Gal–Spk1 basal form). This conversion did not occur in the presence of β-glycerophosphate, a phosphatase inhibitor (Fig. 5). The remainder was converted to an intermediate form. Although it is likely that this component is partially CIP resistant for technical reasons (e.g., the precipitating antibody may block access to phosphorylation sites), it is possible that there is an additional modification (e.g., ubiquitination).

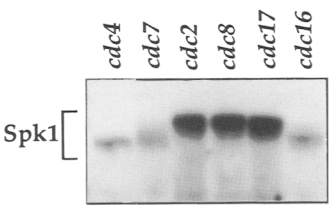

Figure 3. Spk1p modification in *cdc*^{ts} strains. h4cla1, h7c4a1, h2c2a1, h8cla1, h17cla1, and h16cla1 (*cdc4, cdc7, cdc2, cdc8, cdc17*, and *cdc16*, respectively) strains as indicated were arrested by incubation at the nonpermissive temperature for 4 hr. Lysates were immunoprecipitated and immunoblotted with anti-Spk1p.

Trans-*phosphorylation of Spk1p*

The regulated phosphorylation of Spk1p could arise from autophosphorylation or from phosphorylation by another protein kinase. To distinguish between these two possibilities, Spk1p phosphorylation was analyzed in *spk1*Δ strains expressing only kinase-defective versions of Spk1p [*spk1-1* (A208P), *spk1 D339A*, and *spk1 K227A/D339A*] after treatment with HU. *spk1-1* is a viable checkpoint allele that contains a single point mutation

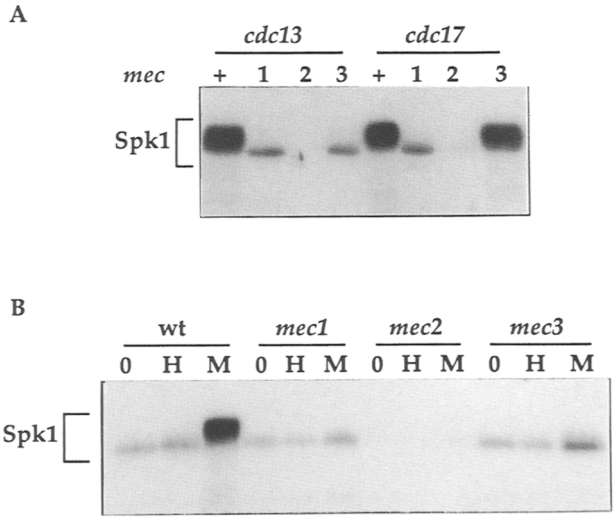

Figure 4. Regulation of Spk1p in *mec* strains. (*A*) TWY146 (*cdc13*), TWY158 (*cdc13 mec1*), TWY148 (*cdc13 mec2*), TWY159 (*cdc13 mec3*), TWY446 (*cdc17*), TWY447 (*cdc17 mec1*), TWY448 (*cdc17 mec2*), and TWY449 (*cdc17 mec3*) cells were incubated at 37°C, the nonpermissive temperature for 4 hr, and analyzed by anti-Spk1p immunoprecipitation and immunoblotting. (*B*) TWY397 (*MEC*), TWY308 (*mec1*), TWY312 (*mec2*), and TWY316 (*mec3*) cells were arrested by incubation in nocodazole for 150 min (Jacobs et al. 1988) and treated with 100 mM HU or 0.1% MMS for 2 hr. Lysates were immunoprecipitated and immunoblotted with anti-Spk1p.

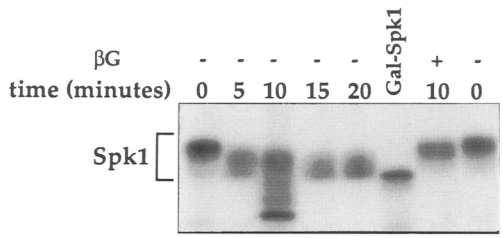

Figure 5. Phosphatase treatment of Spk1p. Spk1p was immunoprecipitated from lysates of h8cla1 (*cdc8*) cells arrested by incubation at the nonpermissive temperature for 4 hr. Immune complexes bound to protein A–Sepharose were incubated in 100 μl HEPES buffer [20 mM HEPES–NaOH (pH 7.3), 20 mM $MgCl_2$, 40 mM KCl, 0.2 mM PMSF] containing 100 units of CIP at 37°C for the indicated periods of time (Rice et al. 1994). Gal–Spk1 is a marker for the nonshifted form. The low molecular weight material at the 10-min time point is smaller than the Spk1p primary translation product and probably arose through proteolysis. β-Glycerophosphate (5 mM), an inhibitor of CIP, was included in the reaction marked. Shown is the time course of Spk1p incubation with CIP in the absence (−) or presence (+) of β-glycerophosphate (βG).

(A208P) and has ~10% of the catalytic activity of wild-type Spk1p (D.S. Fay and D.F. Stern, in prep.). [Although *SPK1* is an essential gene, *spk1Δ* strains can be derived. These strains presumably harbor second-site suppressors, but their growth, checkpoint, and damage sensitivity phenotypes are all reverted by reintroduction of *SPK1* (Zheng et al. 1993; D.S. Fay and D.F. Stern, in prep.)]. Both *spk1 D339A* and *spk1 K227A/D339A* mutants have substitutions at conserved residues within the catalytic domain of Spk1p, have no biological activity, and are likely to be devoid of phosphotransferase activity (D.S. Fay and D.F. Stern, in prep.) All three kinase-defective proteins shifted to mobilities comparable to those of wild-type Spk1p after treatment with HU (Fig. 6, lanes H). Thus, HU induces Spk1p phosphorylation in *trans*.

DNA damage induces Spk1p phosphorylation in a cell cycle-independent manner

Spk1p is postulated to be an intermediary in a signal transduction cascade linking incompletely replicated or damaged DNA to cell cycle arrest (Zhou and Elledge 1993; Allen et al. 1994). If so, then induction of checkpoint arrest should activate Spk1p functionally. Hence, we sought to determine whether Spk1p is regulated by phosphorylation during the damage response. After treatment of log-phase cultures with the alkylating agent methyl methanesulfonate (MMS), Spk1p shifted to the low mobility form (see Fig. 2). There are at least two possible explanations for this result. One is that treatment with MMS leads to the accumulation of cells in cell cycle phases wherein Spk1p is normally phosphorylated. An alternative is that exposure to MMS induces Spk1p phosphorylation independently, through pathways associated with the DNA damage response. To distinguish between these two possibilities, cdc^{ts} strains, prearrested in either G_1 or M phases by temperature shift, were exposed to MMS or HU. Similarly, 1608-21C cells (*cln1Δ, cln2Δ, cln3Δ, GAL–CLN3*) were prearrested in G_1 by switching to glucose medium before treatment with MMS or HU. As discussed above, Spk1p migrated either as a single band in untreated cells (1608-21C, *cdc28, cdc34, cdc4, cdc20, cdc14, cdc5,* and *cdc15*) or was shifted partially (*cdc7*) (see Fig. 2, lanes 0). When these strains were treated with MMS, Spk1p was converted to the shifted form (see Fig. 2, lanes M). This result demonstrates that the induction of Spk1p phosphorylation in response to DNA damage is independent of position within the cell cycle and that none of these *CDC* and *CLN* genes are required for the induction of Spk1p phosphorylation. In contrast to MMS, HU did not affect Spk1p mobility in G_1 and M phase-arrested cells (see Fig. 2, lanes H). This result is not surprising, as the effects of HU, which depletes nucleotide precursors, may only be sensed in the face of ongoing replication.

Some checkpoint-defective mutants are defective in the induction of Spk1p phosphorylation

The above results suggest a direct correlation between Spk1p phosphorylation and the activation of checkpoint pathways. If Spk1p phosphorylation is required for these pathways, it should be possible to identify checkpoint mutants that act upstream of Spk1p by measuring the extent of Spk1p phosphorylation in checkpoint-defective strains treated with agents that induce checkpoint arrest (MMS and HU). *MEC1, MEC2/SPK1,* and *MEC3* were identified in a screen for mutants in the DNA damage checkpoint that is synthetically lethal with $cdc13^{ts}$ (Weinert et al. 1994). *MEC1* is related to human *ATM* (Savitsky et al. 1995), and *MEC2* is allelic to *SPK1*. *MEC1* and *MEC2* are both essential genes that are necessary for DNA damage-induced and S phase checkpoint pathways, whereas *MEC3* is involved exclusively in DNA damage-induced checkpoint arrest (Weinert et al. 1994). $cdc17^{ts}$ and $cdc13^{ts}$ were used to activate replication and damage checkpoints, respectively, in MEC^+, *mec1, mec2,* and *mec3* backgrounds (see Fig. 4A).

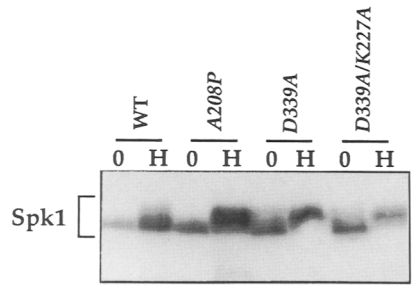

Figure 6. Phosphorylation of kinase-defective Spk1p. DZ413 (*spk1Δ*) cells expressing wild type (WT) and kinase-defective *spk1* mutants were incubated in YPD (lanes 0) or YPD–HU (lanes H) for 4 hr at 30°C. Spk1p was detected by immunoprecipitation and immunoblotting.

Spk1p was converted to the phosphorylated form after temperature shift in *cdc13^{ts} MEC* and *cdc17^{ts} MEC* cells (Fig. 4A, lanes *cdc13/+* and *cdc17/+*). In contrast, Spk1p remained unshifted in *cdc13^{ts} mec1* and *cdc17^{ts} mec1* strains (Fig. 4A, lanes *cdc13/1* and *cdc17/1*). Mobility shift of Spk1p was observed in *cdc17 mec3* cells but not in *cdc13 mec3* cells.

To verify that the *MEC-1*-dependent shift of Spk1p is not a result of cell cycle synchronization, we prearrested cells in M phase with nocodazole and then treated them with MMS (Fig. 4B). In nocodazole-arrested wild-type cells, Spk1p shifted in response to MMS, whereas in *mec1*- and *mec3*- arrested cells, Spk1p remained at the unshifted position (Fig. 4B). These results placed Mec1p upstream from Spk1p in both damage- and replication-regulated pathways, and Mec3p upstream from Spk1p in the damage-regulated pathway where Mec3p functions.

Mec2-1p accumulated at low levels and remained at the singlet position in both *cdc13^{ts} mec2* and *cdc17^{ts} mec2* cells at the nonpermissive temperature (Fig. 4A, lane *cdc17/2* (for *cdc13*, Z. Sun, unpubl.) and in nocodazole-arrested cells treated with MMS (Fig. 4B). Hence, the *mec2-1* defect may prevent interaction with upstream regulators. Both the failure of Mec2-1p to undergo phosphorylation in response to either DNA damage or DNA replication blocks and the low protein levels may account for the checkpoint defect of *mec2-1* strains.

Kinase activity of Spk1p increases with phosphorylation

The results described above demonstrate that Spk1p phosphorylation is regulated during cell cycle progression and in response to DNA damage or blocks to DNA replication, and suggest that this phosphorylation links upstream regulation of damage-response pathways to Spk1p. If this phosphorylation is significant, it would be expected to regulate Spk1p function. Therefore, we compared the kinase activities of shifted and basal forms of Spk1p. We used an exogenous substrate (histone H1) in immune complex kinase assays to eliminate uncertainties regarding basal occupancy of Spk1p autophosphorylation sites. *GAL1–SPK1*-overexpressed Spk1p was immunoprecipitated from cells treated with HU, and immune complexes were incubated with [γ-^{32}P]ATP and histone H1. The intensities of histone phosphorylation relative to Spk1p levels (determined by immunoblotting) were normalized after quantification by PhosphorImager analysis. A representative experiment is shown in Figure 7. Histone H1 kinase activity was not associated with the kinase-impaired mutant Spk1-1p (Fig. 7), verifying that the assay measures Spk1p activity and not that of a contaminating H1 kinase. (H1 kinase activity was not recovered with immune complexes from HU-treated *spk1-1* cells either; data not shown.) In data from seven independent trials, the H1 kinase activity of Spk1p from HU-treated cells normalized to Spk1p protein level was 2.4 times that of unshifted Spk1p (standard error = 0.5). A two-tail *t*-test verified that this difference is statistically significant ($P<0.05$). Thus, Spk1p catalytic activity is increased modestly after exposure of Spk1p to agents that induce checkpoint arrest.

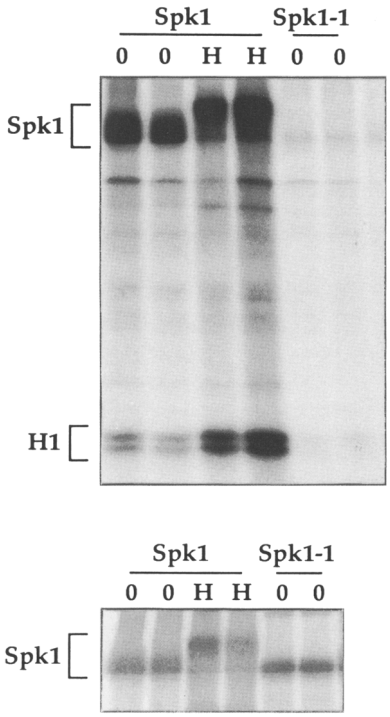

Figure 7. Kinase activity of shifted Spk1p. NY605 *SPK1* or NY605 *spk1-1* cells were incubated in YPG for 3 hr at 30°C, harvested (lanes 0), or followed by additional overnight incubation in YPG–100 mM HU (lanes H). Anti-Spk1p immunoprecipitates were prepared and apportioned for parallel assay of histone H1 kinase activity (*top*) and for Spk1p protein level (by immunoblotting) (*bottom*) as described in Materials and methods. Phosphorylated histone H1 and immunoreactive Spk1p (detected with ^{125}I-labeled protein A) were quantified using a General Dynamics PhosphorImager. Data shown are representative of seven similar experiments that were subjected to statistical analysis as discussed in the text. Anti-Spk1p immune complexes containing Spk1p or Spk1-1p (as marked) were divided and incubated with [γ^{32}P]ATP in the presence of histone H1 (*top*) or immunoblotted with anti-Spk1p (*bottom*). Samples were prepared from mock-treated (lanes 0) or HU-treated cells (lanes H).

Overexpression of Spk1p leads to cell cycle delay

If Spk1p is a member of a pathway connecting DNA damage to checkpoint arrest, then functional activation of Spk1p should be sufficient to induce arrest. To test this possibility, cells carrying galactose-inducible *SPK1* and checkpoint-defective *spk1* kinase mutants (Zheng et al. 1993; D.S. Fay and D.F. Stern, in prep.) were arrested with α factor and induced with galactose. After release from Start arrest, cells were analyzed by flow microfluorimetry. Cells overexpressing *spk1-1*, *spk1 K227A*, and empty vector entered S phase after 45 min, coordinate with appearance of budded cells (Fig. 8). However, overexpression of wild-type Spk1p delayed DNA synthesis

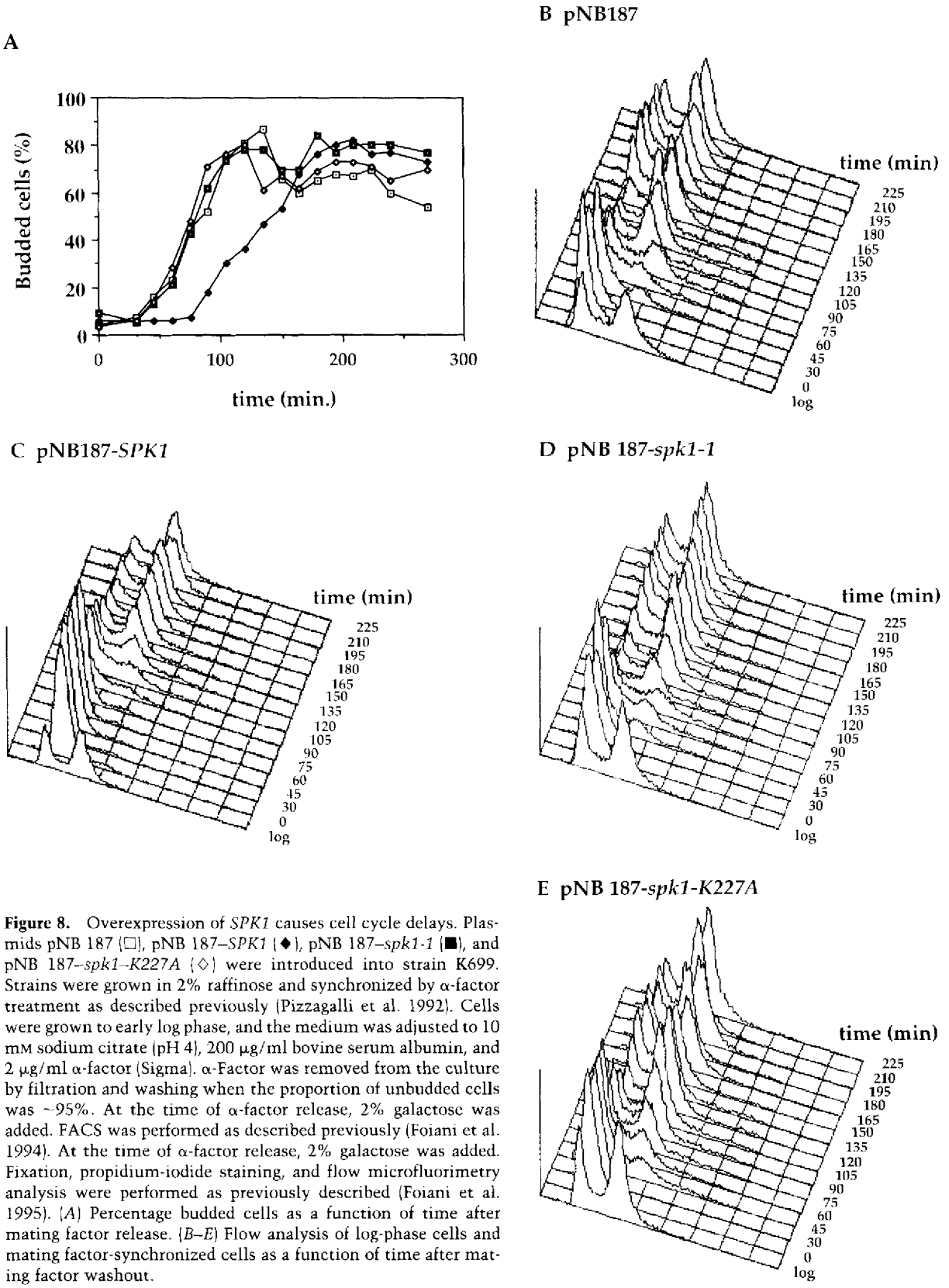

Figure 8. Overexpression of *SPK1* causes cell cycle delays. Plasmids pNB 187 (□), pNB 187–*SPK1* (◆), pNB 187–*spk1-1* (■), and pNB 187–*spk1–K227A* (◇) were introduced into strain K699. Strains were grown in 2% raffinose and synchronized by α-factor treatment as described previously (Pizzagalli et al. 1992). Cells were grown to early log phase, and the medium was adjusted to 10 mM sodium citrate (pH 4), 200 μg/ml bovine serum albumin, and 2 μg/ml α-factor (Sigma). α-Factor was removed from the culture by filtration and washing when the proportion of unbudded cells was ~95%. At the time of α-factor release, 2% galactose was added. FACS was performed as described previously (Foiani et al. 1994). At the time of α-factor release, 2% galactose was added. Fixation, propidium-iodide staining, and flow microfluorimetry analysis were performed as previously described (Foiani et al. 1995). (*A*) Percentage budded cells as a function of time after mating factor release. (*B–E*) Flow analysis of log-phase cells and mating factor-synchronized cells as a function of time after mating factor washout.

and budding until 90 min (Fig. 8, *GAL1–SPK1*). Thus, Spk1p overexpression delays the G_1/S transition (or prevents substantial replication from occurring within S). The inability of checkpoint-defective mutants to induce

the delay suggests that overexpressed Spk1p acts directly on checkpoint-specific targets.

Discussion

Spk1p is a common signal transducer in multiple checkpoint pathways

Previous evidence suggested that Spk1p is a node for integration and transmission of the damage and replication checkpoint signals. This model predicts that (1) Spk1p is required for these pathways; (2) intrinsic and extrinsic activation of these pathways should regulate Spk1p; (3) Spk1p regulation should be associated with altered Spk1p function; and (4) artificial activation of Spk1p should induce checkpoint arrest. With the present data, these criteria have now been met: (1) *spk1* mutants are checkpoint defective; (2) Spk1p phosphorylation is increased by inducers of damage and replication checkpoints; (3) Spk1p phosphorylation correlates with increased kinase activity; and (4) overexpression of Spk1p, but not a checkpoint mutant, induces cell cycle delay.

Checkpoint regulation of Spk1p

Spk1p was mobility-shifted in *cdc9* and *cdc13* cells, MMS-treated, and HU-treated cells. Thus, conditions that activate DNA damage-dependent checkpoint pathways, as well as the S phase (HU) checkpoint pathway, also up-regulate Spk1p phosphorylation. Phosphorylation of Spk1p after treatment with HU moderately increased the in vitro kinase activity of Spk1p. Use of a more physiological substrate (once identified) or soluble enzyme rather than immune complexes might reveal a more profound catalytic regulation. Although it is possible that other phosphorylation-regulated processes (localization, stability, or binding to other proteins) are important for checkpoint functions, our finding that a kinase domain mutation that weakens, but does not ablate, Spk1p catalytic activity leads to checkpoint defects suggests that catalytic regulation of Spk1p is a limiting component of Spk1p signaling (D.S. Fay and D.F. Stern, in prep.).

MEC1 *and* MEC3 *may act upstream of* SPK1

In an effort to define further the signaling cascade leading to checkpoint activation, we examined cells containing checkpoint-defective alleles of *MEC1* and *MEC3* for the ability to shift Spk1p in response to DNA damage and blocks to DNA replication. Spk1p remained in the basally phosphorylated state after temperature shift of both *mec1cdc13*^{ts} and *mec3cdc13*^{ts} cells and MMS treatment of nocodazole-arrested *mec1* and *mec3* cells. These results suggest that *MEC1* and *MEC3* lie upstream in the pathway leading to checkpoint arrest after DNA damage (Fig. 9). It should be noted, however, that MMS treatment and *cdc13* arrest generate different lesions and may exert different effects on checkpoint pathways. Spk1p was unshifted in *mec1cdc17*^{ts} strains at the nonpermissive temperature but was shifted in *mec3cdc17*^{ts} cells. Because *MEC3* is not required for activation of the S-phase checkpoint, it appears that *MEC3* acts upstream of *SPK1* only in the DNA damage-induced checkpoint pathway, whereas *MEC1* functions upstream in both pathways. The phosphorylation data establish a strong correlation of Spk1p mobility shift with the functional activation of these two checkpoint pathways and provide direct biochemical evidence corroborating the hypothesis that multiple checkpoint pathways converge on Spk1p.

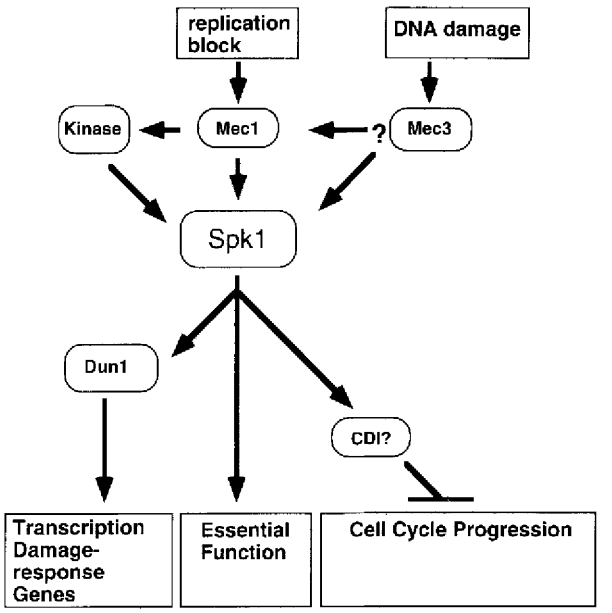

Figure 9. Model for *SPK1* function. Spk1p is regulated by inhibition of replication, by DNA damage, and probably by an S-phase function during normal growth. *MEC1* and *MEC3* are required for Spk1p phosphorylation and for operation of damage (*MEC3*) or damage plus replication (*MEC1*) checkpoints. Mec1 is a likely candidate for the kinase that phosphorylates Spk1p. *SPK1* is required for an essential function (probably linked to replication), for induction of certain DNA damage-induced transcripts (requires *DUN1*), and for checkpoint arrest (*DUN1* independent). See text for further details.

Spk1p in a protein kinase cascade

Spk1p functions within a protein kinase cascade because kinase-defective Spk1p shows HU-dependent phosphorylation and because Spk1p phosphorylation correlates with Spk1p activity. This cascade may extend one level deeper, as the protein kinase Dun1p itself requires *SPK1* for damage-regulated phosphorylation (Zhou and Elledge 1993). The strongest candidates for protein kinases that regulate Spk1p directly are Mec1p and Tel1p. Structurally, they belong to a subgroup of the lipid kinase family including *Schizosaccharomyces pombe rad3*, *Drosophila melanogaster mei-41*, mammalian *ATM*, and DNA–protein kinase catalytic subunit ($DNA–PK_{CS}$) (Al-Khodairy and Carr 1992; Hari et al. 1995; Morrow et al.

1995; Savitsky et al. 1995). Of these, only DNA–PK_{CS} has been tested for catalytic activity, and it is a protein kinase with no detectable lipid kinase activity (Hartley et al. 1995). Currently, it is not known whether Mec1 can act as a protein kinase or whether the catalytic activity of Mec1 is stimulated by DNA damage. Nevertheless, it is logical to suppose that Spk1p not only interacts directly with Mec1p but that Mec1p and its relative Tel1p are the protein kinases that regulate Spk1p. Intriguingly, Spk1p contains multiple consensus sites for phosphorylation by DNA–PK (Hartley et al. 1995). The other obvious candidates, Cdc28p and Cdc7p, cannot be required exclusively, as MMS regulates Spk1p phosphorylation in *cdc28-* and *cdc7-* arrested cells (Fig. 2). However, it is quite possible that multiple protein kinases act on Spk1p.

DNA replication and checkpoint controls

In contrast to other checkpoint control genes, Mec1p and Spk1p share the property of being essential for viability. Little is known about the essential functions of these proteins, but based on circumstantial evidence (coregulation with DNA synthetic enzymes, terminal arrest phenotype, nuclear localization) we have hypothesized that the essential function of Spk1p is associated with DNA replication (Zheng et al. 1993). The *cln1Δ*, *cln2Δ*, *cln3Δ*, *GAL1–CLN3* experiment may indicate that Spk1p is modified post-translationally in S-phase cells and that this modification parallels activation through the induction of checkpoint pathways. Three models might explain such a function. The most likely model is that Spk1p has dual functions: to regulate replication positively and to regulate cell cycle progression negatively. For example, it would be advantageous for damage-dependent checkpoint pathways to coordinately inhibit cell cycle progression and activate DNA replication and repair enzymes, which could be accomplished through phosphorylation of different proteins by Spk1p. A similar function in S phase would link activation of normal DNA replication to inhibition of progression out of S-phase. This model predicts that replication and checkpoint functions are separable, that checkpoint functions are dispensable for viability, and that Spk1p normally functions in S phase. Although these predictions are met, there is no direct evidence regarding a requirement of Spk1p for replication. In a variant of this model, the checkpoint functions are secondary to induction of "replication structures" by Spk1p. Such structures could themselves be the signal recognized by the checkpoint apparatus. A problem with this model is that it does not fit well with the damage checkpoint function, as it would require a second, independent mechanism to identify DNA damage outside of S phase. (Such a mechanism could involve an analogous "transcription structure" created by processing RNA polymerase complexes.) A final model is that the chronic function of Spk1p is a checkpoint function, that, for example, curtails G_2 activities during S phase. This is consistent with the checkpoint function of Spk1p, and with S-phase phosphorylation. Thus *cdc18* and *cut5* of fission yeast have been proposed to play a role in both the initiation of DNA replication and the restraint of mitosis (Kelly et al. 1993; Saka and Yanagida 1993). However, this model is difficult to reconcile with the viability and robust growth of checkpoint mutants.

Conservation of checkpoint pathways

The pathways regulating the response to DNA damage seem to be conserved among all eukaryotes. A fission yeast gene, $cds1^+$, has considerable structural homology to the amino-terminal and catalytic domains of Spk1p. $cds1^+$ may be required for activation of the S phase but not for the DNA damage-regulated checkpoint (Murakami and Okayama 1995). Similarly, $rad3^+$ is similar to *MEC1* and may operate upstream of $cds1^+$, as $cds1^+$ overexpression suppresses *rad3*–HU sensitivity (Murakami and Okayama 1995). Thus, fission yeast genes and the *SPK1* homolog $cds1^+$ are both structurally and functionally similar to *MEC1* and *SPK1* and are both necessary for the HU-sensitive checkpoint (Al-Khodairy and Carr 1992; Murakami and Okayama 1995). The further structural and functional conservation of Mec1-related genes in humans and the vast genetic distance between *S. cerevisiae* and *S. pombe* make it likely that a human homolog of *SPK1* exists. An increasing number of human tumor suppressor genes have functions connected directly to the DNA damage–response network. The conservation of these pathways in all eukaryotes means that further elucidation of the Mec1p/Spk1p pathway in budding yeast is certain to further our understanding of the role that checkpoint genes play in human cancer.

Materials and methods

Immunoprecipitation of Spk1p

Immunoprecipitations were carried out in antibody excess as determined by titration. For Figures 1A and B, 2, 3, 4B and C, 5, and 6, cells from 500 ml of culture per sample were collected by centrifugation and suspended in 2 ml of glass bead disruption buffer [20 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 0.3 M ammonium sulfate, with freshly added 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mix (Ausubel et al. 1991), 1 mM sodium orthovanadate, and 100 μM sodium orthovanadate]. Samples were vortexed five times for 45 sec in the presence of glass beads interspersed with cooling on ice for 1–2 min. Two milliliters of TG–VO_4 solution [1% Triton X-100, 10% glycerol, 0.198 trypsin inhibitor units (TIU) of aprotinin per milliliter of Dulbecco's phosphate–buffered saline lacking divalent cations with fresh 100 μM sodium orthovanadate] was added, and samples were vortexed for an additional minute. Samples were cleared by centrifugation at 2500 rpm for 10 min at 4°C in an IEC DPR-6000 centrifuge. Supernatants were transferred, diluted with 40 ml of denaturation solution [10 mM Tris-HCl (pH 7.5), SDS 0.5%], heated to 100°C for 10 min, and cooled on ice. Triton X-100 was added to 1.0%, 100 μl of rabbit anti-Spk1p serum (Zheng et al. 1993) was added, and incubation was carried out at 4°C overnight with shaking. One hundred microliters of protein

A–Sepharose (50% suspension in PBS without divalent cations) was added, and incubations were continued for 3 hr at 4°C with shaking. Beads were collected by centrifugation, transferred to 1.5-ml Eppendorf tubes, and washed three times in 0.5 ml of TG–VO_4 buffer. For Figure 4A, immunoprecipitation was carried out as described for the immunoprecipitation step in histone H1 kinase assays.

Immunoblotting

Samples were heated to 100°C for 5 min in 1× SDS sample buffer and fractionated on a 10% acrylamide–0.13% bisacrylamide gel. Proteins were transferred to nitrocellulose (Bio-Rad) in transfer buffer (72.25 grams of glycine, 15 grams of Tris base, 3.75 grams of SDS, 0.46 grams of sodium orthovanadate, 1 liter of methanol, H_2O to 4 liters) at 4°C for 1 hr and 45 min at 500 mA. Filters were then blocked in Blotto (5% Carnation Instant Milk in rinsing buffer (RB: 10 mM Tris-HCl at pH 7.4, 0.9% NaCl, 0.01% sodium azide) overnight. Anti-Spk1p antibody (1:100) in Blotto was incubated with the filter for 2 hr at room temperature with shaking followed by two 10-min washes in RB, one 10-min wash in RB containing 0.05% Triton X-100, and two 10-min washes in RB. The filter was then incubated with ^{125}I-labeled protein A in RB/Blotto (30 μCi in 30 ml) for 30 min at room temperature with shaking and washed five times as above. Filters were autoradiographed for 2 days using preflashed film at −70°C.

Histone H1 kinase assay

NY605 *SPK1* cells were grown overnight in synthetic complete medium lacking uracil (SC − Ura), diluted, and grown to log phase in YPR. *SPK1* overexpression was induced by addition of galactose to a final concentration of 2% (wt/vol). Three hours later, 100 mM HU was added and cells were incubated at 30°C overnight. For HU-untreated cells, galactose was added on day 2 to a fresh log-phase culture of NY605 *SPK1* and NY605 *spk1-1* cells (grown as described as above). Cells were incubated for 3 hr and harvested in parallel with HU-treated cells. One hundred fifty milliliters of cells was collected for each sample.

Cells were lysed in 0.5 ml of glass bead disruption buffer by vortexing (as above), diluted with 0.5 ml of TG–VO_4, and 80 μl of anti-Spk1p serum was added. After incubation for 1.5 hr at 4°C, each lysate was divided into four equal parts. Fifty microliters of protein A–Sepharose (50% in TG–VO_4 solution) was added to each portion followed by a further incubation with rotation for 30 min at 4°C . Sepharose beads were collected by centrifugation and were washed twice with TG–VO_4 solution and twice with kinase buffer [20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$]. Duplicate portions from each treatment were boiled immediately in 2× sample buffer for Western blot analysis. The other two were used for kinase assays. The washed complexes were incubated with 20 μCi of [γ-^{32}P]ATP (Amersham; sp. act. >5000 Ci/mmole), 2 μg of histone H1 (Sigma), and 1 μM cold ATP in 25 μl of kinase buffer for 7 min on ice. Reactions were terminated with 25 μl of 2× sample buffer and boiling for 5 min. The eluted proteins were analyzed by SDS PAGE. The intensities of phosphorylated histone H1 (kinase assays) and Spk1p (immunoblots) were determined by PhosphorImager analysis.

Acknowledgments

We are grateful to Mike Snyder for several helpful discussions. We thank Dave Riese II, Mark Solomon, and David Gonda for evaluating the manuscript, and Fred Cross, Steve Elledge and Ted Weinert for providing strains. This work was supported by grant VM-70 to D.F.S. from the American Cancer Society (Z.S., D.S.F., and D.F.S.) and by Progetto Bilaterale 95.00840.CT04 from Centro Nazionale Ricerche Italy to M.F.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Al-Khodairy, F. and A.M. Carr. 1992. DNA repair mutants defining G2 checkpoint pathways in *Schizosaccharomyces pombe*. *EMBO J.* **11:** 1343–1350.

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

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1991. *Current protocols in molecular biology* (ed. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman, and K. Struhl), pp. 13.13. Wiley Interscience, New York.

Bahman, M., V. Buck, A. White, and J. Rosamond. 1988. Characterisation of the *CDC7* gene product of *Saccharomyces cerevisiae* as a protein kinase needed for the initiation of mitotic DNA synthesis. *Biochim. Biophys. Acta* **951:** 335–343.

Budd, M.E. and J.L. Campbell. 1993. DNA polymerase δ and ε are required for chromosomal replication in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13:** 496–505.

Foiani, M., F. Marini, D. Gamba, G. Lucchini, and P. Plevani. 1994. The B subunit of the DNA polymerase α–primase complex in *Saccharomyces cerevisiae* executes an essential function at the initial stage of DNA replication. *Mol. Cell. Biol.* **14:** 923–933.

Foiani, M., G. Liberi, G. Lucchini, and P. Plevani. 1995. Cell cycle-dependent phosphorylation and dephosphorylation of the yeast DNA polymerase α-primase B subunit. *Mol. Cell. Biol.* **15:** 883–891.

Greenwell, P.W., S.L. Kronmal, S.E. Porter, J. Gassenhuber, B. Obermaier, and T.D. Petes. 1995. *TEL1*, a gene involved in controlling telomere length in *S. cerevisiae*, is homologous to the human Ataxia Telangiectasia gene. *Cell* **82:** 823–829.

Hari, K.L., A. Santerre, J.J. Sekelsky, K.S. Mckim, J.B. Boyd, and R.S. Hawley. 1995. The *mei-41* gene of *D. melanogaster* is a structural and functional homolog of the human Ataxia Telangiectasia gene *Cell* **82:** 815–821.

Hartley, K.O., D. Gell, G.C.M. Smith, H. Zhang, N. Divecha, M.A. Connelly, A. Admon, S.P. Lees-Miller, C.W. Anderson, and S.P. Jackson. 1995. DNA-dependent protein kinase catalytic subunit: A relative of phosphatidylinositol 3-kinase and the Ataxia Telangiectasia gene product. *Cell* **82:** 849–856.

Hartwell, L.H. and M.B. Kastan. 1994. Cell cycle control and cancer. *Science* **266:** 1821–1827.

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

Hereford, L.M. and L.H. Hartwell. 1974. Sequential gene function in the initiation of *Saccharomyces cerevisiae* DNA synthesis *J. Mol. Biol.* **84:** 445–461.

Jacobs, C.W., A.E. M. Adams, P.J. Szaniszlo, and J.R. Pringle. 1988. Functions of microtubules in the *Saccharomyces cer-*

evisiae cell cycle. *J. Cell Biol.* **107:** 1409–1426.
Kastan, M.B., Q. Zhan, W.S. El-Deiry, F. Carrier, T. Jacks, W.V. Walsh, B.S. Plunkett, B. Vogelstein, and J.A.J. Fornace. 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in Ataxia-Telangiectasia. *Cell* **71:** 587–597.
Kato, R. and H. Ogawa. 1994. An essential gene, *ESR1*, is required for mitotic cell growth, DNA repair, and meiotic recombination in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **22:** 3104–3112.
Kelly, T.J., G.S. Martin, S.L. Forsburg, R.J. Stephen, A. Russo, and P. Nurse. 1993. The fission yeast *cdc18* gene product couples S phase to START and mitosis. *Cell* **74:** 371–382.
Kolman, C.J., J. Toth, and D.K. Gonda. 1992. Identification of a portable determinant of cell cycle function within the carboxyl-terminal domain of the yeast *CDC34 (UBC3)* ubiquitin conjugating (E2) enzyme. *EMBO J.* **11:** 3081–3090.
Lambertus, J.W.M.O. and F.R. Cross. 1994. G1 cyclins *CLN1* and *CLN2* repress the mating factor response pathway at Start in the yeast cell cycle. *Genes & Dev.* **8:** 1058–1070.
Lane, D.P. 1992. p53, guardian of the genome. *Nature* **358:** 15–16.
Lehmann, A.R. and A.M. Carr. 1995. The ataxia-telangiectasia gene: A link between checkpoint controls, neurodegeneration and cancer. *Trends Genet.* **11:** 375–377.
Lucchini, G., M.M. Falconi, A. Pizzagalli, A. Aguilera, H.L. Klein, and P. Plevani. 1990. Nucleotide sequence and characterization of temperature-sensitive *pol1* mutants of *Saccharomyces cerevisiae*. *Gene* **90:** 99–104.
McIntosh, E.M. 1993. MCB elements and the regulation of DNA replication genes in yeast. *Curr. Genet.* **24:** 185–192.
Meyn, M.S. 1995. Ataxia-telangiectasia and cellular responses to DNA damage. *Cancer Res.* **55:** 5991–6001
Morrow, D.M., D.A. Tagle, Y. Shiloh, F.S. Collins, and P. Hieter. 1995. *TEL1*, an *S. cerevisiae* homolog of the human gene mutated in ataxia telangiectasia, is functionally related to the yeast checkpoint gene *MEC1*. *Cell* **82:** 831–840.
Murakami, H. and H. Okayama. 1995. A kinase from fission yeast responsible for blocking mitosis in S phase. *Nature* **374:** 817–819.
Murray, A. 1992. Creative blocks: Cell cycle checkpoints and feedback controls. *Nature* **359:** 599–604.
Navas, T.A., Z. Zhou, and S.J. Elledge. 1995. DNA polymerase ε links the DNA replication machinery to the S phase checkpoint. *Cell* **80:** 29–39.
Paulovich, A.G. and L.H. Hartwell. 1995. A checkpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. *Cell* **82:** 841–847.
Pizzagalli, A., S. Piatti, D. Derossi, I. Gander, P. Plevani, and G. Lucchini. 1992. Positive cis-acting regulatory sequences mediate proper control of *POL1* transcription in *Saccharomyces cerevisiae*. *Curr. Genet.* **21:** 183–189.
Rice, S.A., M.C. Long, V. Lam, and C.A. Spencer. 1994. RNA polymerase II is aberrantly phosphorylated and localized to viral replication compartments following herpes simplex virus infection. *J. Virol.* **68:** 988–1001.
Saka, Y. and M. Yanagida. 1993. Fission yeast *cut5+*, required for S phase onset and M Phase restraint, is identical to the radiation-damage repair gene *rad4+*. *Cell* **74:** 383–393.
Salminen, A and P.J. Novick. 1989. The Sec15 protein responds to the function of the GTP binding protein, Sec4, to control vesicular traffic in yeast. *J. Cell. Biol.* **109:** 1023–1036.
Savitsky, K., A. Bar-Shira, S. Gilad, G. Rotman, Y. Ziv, L. Vanagaite, D.A. Tagle, S. Smith, T. Uziel, S. Sfez, M. Ashkenazi, I. Pecker, M. Frydman, R. Harnik, S.R. Patanjali, A. Simmons, G.A. Clines, A. Sartiel, R.A. Gatti, L. Chessa, O. Sanal, M.F. Lavin, N.G.J. Jaspers, A. Malcolm, R. Taylor, C.F. Arlett, T. Miki, S.M. Weissman, M. Lovett, F.S. Collins, and Y. Shiloh. 1995. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* **268:** 1749–1753.
Schwob, E., T. Bohm, M.D. Mendenshall, and K. Nasmyth. 1994. The B-type cyclin kinase inhibitor $p40^{SIC1}$ controls the G1 to S transition in *S. cerevisiae*. *Cell* **79:** 233–244.
Sclafani, R.A. and W.L. Fangman. 1984. Yeast gene *CDC8* encodes thymidylate kinase and is complemented by herpes thymidine kinase gene *TK*. *Proc. Natl. Acad. Sci.* **81:** 5821–5825.
Siede, W., A.S. Friedberg, and E.C. Friedberg. 1993. *RAD9* dependent G1 arrest defines a second checkpoint for damaged DNA in the cell cycle of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **90:** 7985–7989.
Smith, S.A., P. Kumar, I. Johnston, and J. Rosamond. 1992. *SCM4*, a gene that suppresses mutant *cdc4* function in budding yeast. *Mol. & Gen. Genet.* **235:** 285–291.
Sorger, P.K. and A.W. Murray. 1992. S-phase feedback control in budding yeast independent of tyrosine phosphorylation of $p34^{cdc28}$. *Nature* **355:** 365–368.
Stern, D.F., P. Zheng, D.R. Beidler, and C. Zerillo. 1991. Spk1, a new kinase from *S. cerevisiae*, phosphorylates proteins on serine, threonine, and tyrosine. *Mol. Cell. Biol.* **11:** 987–1001.
Tomkinson, A.E., N.J. Tappe, and E.C. Friedberg. 1992. DNA ligase I from *Saccharomyces cerevisiae*: Physical and biochemical characterization of the *CDC9* gene product. *Biochem.* **31:** 11762–11771.
Vallen, E.A. and F.R. Cross. 1995. Mutations in *RAD27* define a potential link between G1 cyclins and DNA replication. *Mol. Cell. Biol.* **15:** 4291–4302.
Weber, L. and B. Byers. 1992. A *RAD9* dependent checkpoint blocks meiosis of *cdc13* yeast cells. *Genetics* **131:** 55–63.
Weinert, T.A. and L.H. Hartwell. 1988. The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science* **241:** 317–322.
———. 1990. Characterization of *RAD9* of *Saccharomyces cerevisiae* and evidence that its function acts posttranslationally in cell cycle arrest after DNA damage. *Mol. Cell. Biol.* **10:** 6554–6564.
———. 1993. Cell cycle arrest of *cdc* mutants and specificity of the RAD9 checkpoint. *Genetics* **134:** 63–80.
Weinert, T.A., F.L. Kiser, and L.H. Hartwell. 1994. Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. *Genes & Dev.* **8:** 652–665.
Zakian, V.A. 1995. ATM-related genes: What do they tell us about functions of the human gene. *Cell* **82:** 685–687.
Zheng, P., D.S. Fay, J. Burton, H. Xiao, J.L. Pinkham, and D.F. Stern. 1993. *SPK1* is an essential S-phase-specific gene of *Saccharomyces cerevisiae* that encodes a nuclear serine/threonine/tyrosine kinase. *Mol. Cell. Biol.* **13:** 5829–5842.
Zhou, Z. and S.J. Elledge. 1993. *DUN1* encodes a protein kinase that controls the DNA damage response in yeast. *Cell* **75:** 1119–1127.