The EMBO Journal Vol.16 No.17 pp.5216–5226, 1997

The novel DNA damage checkpoint protein Ddc1p is phosphorylated periodically during the cell cycle and in response to DNA damage in budding yeast

Maria Pia Longhese, Vera Paciotti, Roberta Fraschini, Raffaella Zaccarini, Paolo Plevani and Giovanna Lucchini[1]

Dipartimento di Genetica e di Biologia dei Microrganismi,
Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

[1]Corresponding author
e-mail: lucchini@imiucca.csi.unimi.it

The *DDC1* gene was identified, together with *MEC3* and other checkpoint genes, during a screening for mutations causing synthetic lethality when combined with a conditional allele altering DNA primase. Deletion of *DDC1* causes sensitivity to UV radiation, methyl methanesulfonate (MMS) and hydroxyurea (HU). *ddc1Δ* mutants are defective in delaying G_1–S and G_2–M transition and in slowing down the rate of DNA synthesis when DNA is damaged during G_1, G_2 or S phase, respectively. Therefore, *DDC1* is involved in all the known DNA damage checkpoints. Conversely, Ddc1p is not required for delaying entry into mitosis when DNA synthesis is inhibited. *ddc1* and *mec3* mutants belong to the same epistasis group, and *DDC1* overexpression can partially suppress MMS and HU sensitivity of *mec3Δ* strains, as well as their checkpoint defects. Moreover, Ddc1p is phosphorylated periodically during a normal cell cycle and becomes hyperphosphorylated in response to DNA damage. Both phosphorylation events are at least partially dependent on a functional *MEC3* gene.

Keywords: budding yeast/checkpoints/DNA damage/phosphorylation

Introduction

Cell proliferation is dependent on the ordered completion of two key events during the mitotic cell cycle: genome replication during S phase and segregation of the duplicated genomes during mitosis. A complex network of surveillance mechanisms, called checkpoints, delays cell cycle progression when DNA is damaged or incompletely replicated, or when the mitotic spindle is not assembled properly, probably allowing time for DNA repair and replication before entry into mitosis and for the alignment of chromosomes on the spindle before initiation of anaphase (for reviews, see Hartwell and Weinert, 1989; Murray, 1994, 1995; Friedberg *et al*., 1995; Elledge, 1996; Paulovich *et al*., 1997b). Defective checkpoint controls may play an important role in the genesis of cancer cells, allowing rapid accumulation of genetic changes (Hartwell and Kastan, 1994). For example, mutations in the p53 tumor suppressor gene or in the ataxia telangiectasia *ATM* gene, both involved in the response to DNA damage in human cells, are often associated with cancer, probably due to increased genomic instability and mutagenesis (reviewed in Enoch and Norbury, 1995). Several data indicate that the basic mechanisms controlling the response to DNA damage are conserved in all eukaryotic cell types, even if different organisms seem to have adapted them in different ways (reviewed in Carr and Hoekstra, 1995; Elledge, 1996; Lydall and Weinert, 1996; Carr, 1997). Studies in simple model systems, such as the evolutionarily distant yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have allowed the identification of many checkpoint proteins, several of which have structural and functional equivalents in man, and provide an important contribution to the understanding of the biochemical basis of checkpoint controls in all eukaryotes.

In the budding yeast *S.cerevisiae*, at least three DNA damage checkpoints have been identified, which inhibit the G_1–S transition (G_1/S checkpoint) (Siede *et al*., 1993, 1994), slow down progression through S phase (intra-S checkpoint) (Paulovich and Hartwell, 1995) and delay the G_2–M transition (G_2/M checkpoint) (Weinert and Hartwell, 1988), when DNA is damaged during G_1, S or G_2 phase, respectively. Several genes are known to be involved in these control mechanisms. The *MEC1* and *RAD53* essential gene products must play pivotal roles in different signal transduction pathways, since they are required not only for proper response to DNA damage, but also for the S/M checkpoint preventing entry into mitosis when S phase is inhibited by the ribonucleotide reductase inhibitor hydroxyurea (HU) (Zheng *et al*., 1993; Allen *et al*., 1994; Weinert *et al*., 1994; Sanchez *et al*., 1996; Siede *et al*., 1996; Sun *et al*., 1996). The non-essential genes *RAD9*, *RAD17*, *RAD24* and *MEC3* are required for all the known DNA damage checkpoints, but not for delaying entry into mitosis when S phase is inhibited (Siede *et al*., 1993, 1994; Weinert and Hartwell, 1993; Weinert *et al*., 1994; Longhese *et al*., 1996a; Paulovich *et al*., 1997a). Moreover, both the large subunit of replication protein A (RPA) and the catalytic subunit of DNA primase are involved in a subset of DNA damage checkpoints, i.e. the G_1/S and intra-S checkpoints (Longhese *et al*., 1996b; Marini *et al*., 1997), while the S/M checkpoint requires DNA polymerase ε (pol ε), the large subunit of replication factor C (RF-C) and the *DPB11* gene product (a protein interacting with pol ε) (Araki *et al*., 1995; Navas *et al*., 1995; Sugimoto *et al*., 1996). Finally, several checkpoint genes have different roles in transcriptional induction following DNA damage (Aboussekhra *et al*., 1996; Kiser and Weinert, 1996; Navas *et al*., 1996).

Recent data indicate that the *RAD9* and *RAD24* gene products are both required for processing single-stranded subtelomeric DNA regions, which accumulate in *cdc13* temperature-sensitive mutant cells at non-permissive temperature (Garvik *et al*., 1995; Lydall and Weinert, 1995, 1996). *MEC3*, *RAD17* and *RAD24* belong to the same epistasis group, while *RAD9* is in a class on its own and acts in opposition to *RAD24* after *cdc13*-induced damage (Lydall and Weinert, 1995). Since Rad17p shows similarity to a 3′–5′ DNA exonuclease, it has been proposed that Rad17p, Rad24p and Mec3p control degradation of DNA after Cdc13p inactivation, and that the role of Rad9p is to inhibit this degradation (Lydall and Weinert, 1995, 1996). The involvement of these four checkpoint proteins in DNA metabolism suggests that they might act close to the primary DNA damage event, but the molecular mechanisms linking DNA damage recognition, processing and repair to cell cycle arrest are still obscure, and other factors and interactions are likely to be involved.

Here we describe a new gene, *DDC1*, whose deletion causes sensitivity to UV radiation, methyl methanesulfonate (MMS) and HU comparable with that observed in *mec3*Δ strains. We show that *DDC1* and *MEC3* genes belong to the same epistasis group and *DDC1* function is required to delay cell cycle progression when DNA is damaged during G_1, S or G_2 phase, but not to block S–M transition when S phase is inhibited by HU. Furthermore, Ddc1p is phosphorylated periodically during a normal cell cycle and hyperphosphorylated in response to DNA damage. *MEC3* is required for proper phosphorylation of Ddc1p, and *DDC1* overexpression partially compensates the checkpoint defects of *mec3*Δ strains.

Results

Cloning and disruption of the DDC1 gene

A genetic screening for mutations causing synthetic lethality when combined with the *pri1-2* cold-sensitive allele, altering the catalytic subunit of DNA primase, allowed the identification of a number of independent mutations belonging to seven complementation groups, possibly corresponding to seven different genes, that we named *PIP1–7* (Longhese *et al*., 1996a). Some of these mutations caused additional phenotypes, like hypersensitivity to UV radiation, MMS and HU, suggesting some function of the corresponding gene products in DNA repair and/or checkpoint mechanisms. Cloning of the *PIP3* gene allowed the establishment that it is in fact the *MEC3* DNA damage checkpoint gene (Longhese *et al*., 1996a). Transformation of the remaining *pri1-2 pip* double mutants with centromeric plasmids carrying the *MEC1*, *RAD53*, *RAD17* and *RAD24* genes showed that synthetic lethality due to *pip1 pri1-2* and *pip7 pri1-2* combinations was fully compensated by *MEC1* and *RAD24*, respectively. The identity of *PIP1* with *MEC1* was confirmed by an allelism test (data not shown). The synthetic lethal effect due to combination of the *pri1-2* allele with the *pip2*, *pip4*, *pip5* and *pip6* mutations could not be complemented by any of the checkpoint genes analyzed.

Cloning of the *PIP5* gene was achieved by screening a yeast genomic DNA library constructed in a *LEU2* centromeric plasmid (Jansen *et al*., 1993) for complementation of the *pri1-2 pip5-1* synthetic lethal phenotype (see Materials and methods). Sequencing of ~300 nucleotides from both ends of the smallest yeast DNA insert identified by this screening and a search of the yeast genome database revealed that the cloned fragment was located on *S.cerevisiae* chromosome XVI, between positions 175 452 and 186 891. Further analysis allowed us to establish that a 2772 bp *Nsi*I–*Bst*BI DNA fragment (Figure 1A) was sufficient to complement the *pri1-2 pip5-1* synthetic lethal phenotype. This fragment contained only one complete open reading frame (ORF), YPL194w (nucleotides 179 276–181 111; accession No. U212C1), 1836 bp long, which had not been characterized previously and which we renamed *DDC1* (DNA damage checkpoint). The identity between *PIP5* and *DDC1* was confirmed further by complementation and allelism tests (see Materials and methods). The *DDC1* ORF encodes a highly hydrophilic protein of 612 amino acid residues, with a predicted mol. wt of 69 685 Da. BESTFIT analysis indicates that the amino acid sequence of Ddc1p is 20.6% identical and 45.9% similar to the *S.pombe rad9*$^+$ checkpoint gene product (Murray *et al*., 1991; Al-Khodairy and Carr, 1992; Enoch *et al*., 1992; Lieberman *et al*., 1992) and 23.5% identical, 48.6% similar to the *Schizosaccharomyces octosporus rad9*$^+$ gene product (Lieberman and Hopkins, 1994).

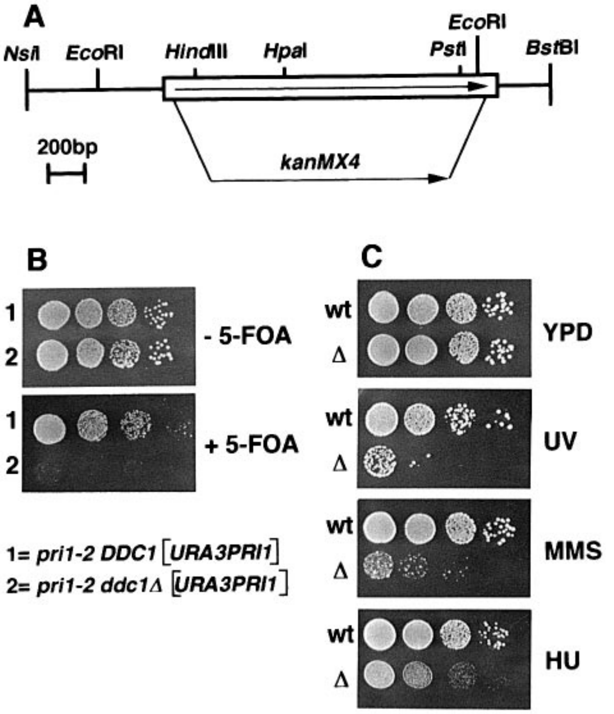

Fig. 1. *DDC1* disruption is lethal in *pri1-2* cells and causes sensitivity to UV, MMS and HU. (**A**) Restriction map of the *DDC1* locus: the box delimits the *DDC1* ORF, with an arrow indicating the direction of translation. Also shown is a schematic representation of replacement of the region between positions +33 and +1785 from the translation initiation codon, giving rise to the *ddc1*Δ allele. (**B**) Serial dilutions of YPD-saturated cell cultures of strains YLL231 (1) and YLL245 (2) were spotted on SC plates without (–) or with (+) 5-FOA, to assay the ability of the two strains to lose the *URA3* centromeric plasmid carrying the *PRI1* allele [*URA3 PRI1*]. (**C**) Serial dilutions of YPD-saturated cell cultures of strains K699 (wt) and YLL244 (Δ) were spotted on YPD plates without (YPD) or with MMS (0.01%) or HU (150 mM). YPD plates were made in duplicate and one of them was UV irradiated (30 J/m^2) (UV).

Substitution of most of the *DDC1* chromosomal ORF with the heterologous *KanMX4* cassette gave rise to the *ddc1*Δ allele (see Figure 1A and Materials and methods) that was not lethal in *PRI1* cells, while *pri1-2 ddc1*Δ strains were inviable (Figure 1B). The cell viability of the

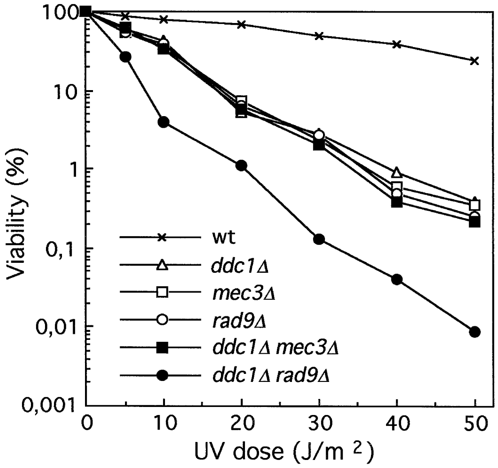

Fig. 2. The *DDC1* gene belongs to the *MEC3* epistasis group. Strains were K699 (wt), YLL244 (*ddc1*Δ), YLL134 (*mec3*Δ), YLL157 (*rad9*Δ), YLL271 (*ddc1*Δ *mec3*Δ) and YLL301 (*ddc1*Δ *rad9*Δ). One hundred and 1000 cells from overnight saturated YPD cultures were spread on YPD plates, which were then exposed to the indicated dosages of UV radiation. Plates were incubated at 28°C and colonies were counted after 3 days.

pol1-1 and *rfa1-M2* DNA replication mutants (Longhese *et al*., 1996a) was also severely affected by the *ddc1*Δ mutation (data not shown). Finally, like the original *pip5-1* mutant (Longhese *et al*., 1996a), *ddc1*Δ strains were more sensitive than wild-type to UV, MMS and HU (Figure 1C).

The DDC1 gene belongs to the MEC3 epistasis group and is involved in all the known DNA damage checkpoints

As shown in Figure 2, strains carrying the single *ddc1*Δ or *mec3*Δ alleles showed very similar sensitivity to UV radiation, which was indistinguishable from that of a *ddc1*Δ *mec3*Δ double mutant, indicating that the *DDC1* and *MEC3* genes belong to the same epistasis group. The *ddc1*Δ *rad9*Δ strain was instead more sensitive to UV than was each single mutant (Figure 2), similarly to what was observed previously for *mec3*Δ *rad9*Δ double mutants (Lydall and Weinert, 1995; Longhese *et al*., 1996a). Therefore, *DDC1* belongs to the *RAD24* epistasis group, that also includes the *MEC3* and *RAD17* genes (Lydall and Weinert, 1995), while *RAD9* represents a different group.

As shown in Figure 3A, *ddc1*Δ cells are defective in delaying G_1–S transition after UV irradiation in G_1. In fact, when *ddc1*Δ α-factor-arrested cell cultures were UV irradiated and then released from G_1 block, both progression through S phase (Figure 3A, top) and budding kinetics (Figure 3A, bottom) were much faster than in wild-type cell cultures under the same conditions. Cell survival after UV treatment was lower in *ddc1*Δ cell cultures than in wild-type (12 and 58%, respectively). *ddc1*Δ cell viability did not increase when cell cycle progression was delayed by holding the UV-irradiated cultures in G_1 by α-factor for 120 min (data not shown). A similar behavior was also observed previously in strains carrying null alleles of *RAD9* (Siede *et al*., 1993) and *MEC3* (our unpublished observation) checkpoint genes, for which a direct involvement in DNA repair has been suggested (Lydall and Weinert, 1995).

Slowing down the rate of DNA synthesis when DNA is damaged during S phase is a genetically controlled process (Paulovich and Hartwell, 1995), and the data in Figure 3B show that Ddc1p is involved in this checkpoint mechanism. In fact, α-factor-synchronized *ddc1*Δ cells, when released from G_1 arrest in the presence of MMS, mostly reached a 2C DNA content within 45 min (Figure 3B), while wild-type cell cultures under the same conditions progressed through S phase very slowly, reaching a 2C DNA content only after 180 min. MMS-treated *ddc1*Δ cells progressively lost viability during the experiment (25, 12 and 0.4% cell survival after 30, 60 and 180 min of MMS treatment, respectively), while the MMS concentration used did not substantially affect wild-type cell survival throughout the experiment. Therefore, Ddc1p is needed for the intra-S control mechanism that requires all the checkpoint proteins analyzed so far (Paulovich and Hartwell, 1995; Longhese *et al*., 1996a,b; Navas *et al*., 1996; Marini *et al*., 1997; Paulovich *et al*., 1997a).

A functional *DDC1* gene product is also essential for properly delaying the G_2/M transition when DNA is damaged in G_2 (Figure 3C). In fact, when cell cultures were released from nocodazole arrest after UV irradiation, the appearence of binucleate cells in wild-type cultures was appreciably delayed compared with the unirradiated control, while *ddc1*Δ cells went through nuclear division much faster than wild-type. *ddc1*Δ cell survival after UV treatment was much lower than that of wild-type cells under the same conditions (15 and 82%, respectively). As already observed when cells were irradiated in G_1, *ddc1*Δ cell viability was not increased by holding the cultures in nocodazole for 120 min after UV irradiation in G_2 (data not shown), again suggesting a direct involvement of Ddc1p in DNA repair.

Based on the above results, the *DDC1* gene product is required for all the known DNA damage checkpoints. Conversely, Ddc1p does not appear to be involved in the control mechanism coupling completion of S phase to entry into mitosis, since *ddc1*Δ cells properly arrest with a single nucleus and short spindles after S phase block by HU treatment (data not shown). The small, but significant, increase in HU sensitivity of *ddc1*Δ strains compared with wild-type (Figure 1C), which is similar to that observed for other DNA damage checkpoint mutants with a proficient S/M checkpoint (Weinert *et al*., 1994; Longhese *et al*., 1996a), must therefore be related to something other than defective cell cycle arrest in response to incomplete DNA replication.

As previously observed for other DNA damage checkpoint mutants (Longhese *et al*., 1996; Paulovich *et al*., 1997a), *ddc1*Δ cells still show some delay in cell cycle progression after DNA damage in G_1, G_2 or S phase compared with untreated cells, suggesting that an as yet unidentified *DDC1*-independent pathway(s) might contribute to these responses.

DDC1 overexpression can partially suppress sensitivity to MMS and HU of mec3Δ mutants, as well as their intra-S checkpoint defect

Since *ddc1*Δ and *mec3*Δ mutants belong to the same epistasis group and exhibit very similar phenotypes, we examined the effect of overexpressing *DDC1* in a *mec3*Δ background. For this purpose, the *DDC1* ORF was fused to the galactose-inducible *GAL1* promoter and a single

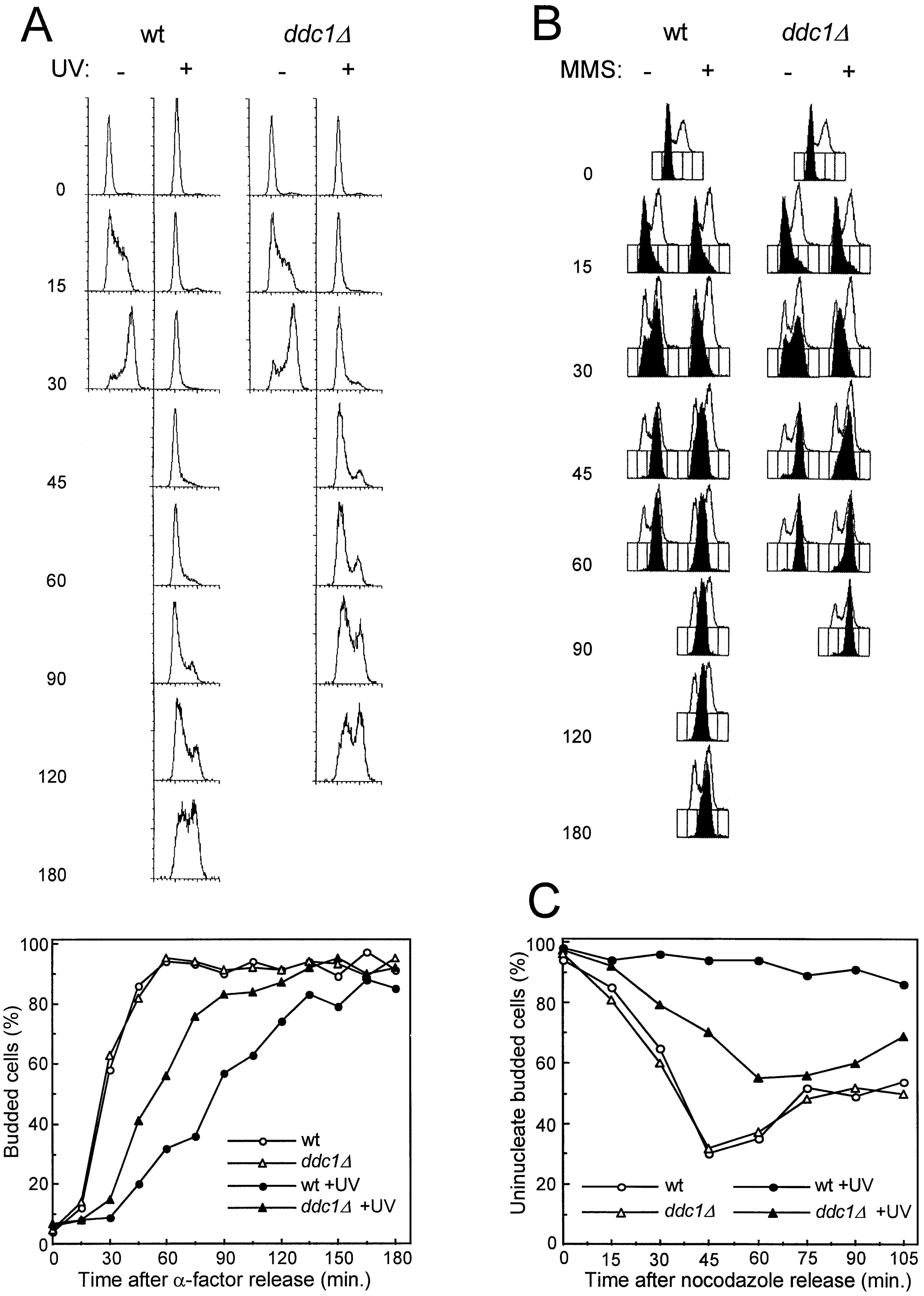

Fig. 3. A functional *DDC1* gene is required for all the known DNA damage checkpoints. Strains were K699 (wt) and YLL244 (*ddc1*Δ) and times are given in minutes. (**A**) α-Factor-synchronized cultures were UV irradiated (40 J/m^2) and released from α-factor at time zero. FACS analysis of unirradiated (–) and irradiated (+) cultures at the indicated times after α-factor release (time zero) is shown in the top part of the panel, while the bottom part shows the percentage of budded cells in both unirradiated (open symbols) and irradiated (closed symbols) cultures. (**B**) α-Factor-synchronized cultures were released from α-factor at time zero, either in YPD or in YPD containing 0.02% MMS. Untreated (–) or MMS-treated (+) samples were taken at the indicated times after α-factor release (time zero) and analyzed by FACS (black histograms). Overlayed histograms represent the cell cycle distributions of the asynchronous cultures. (**C**) Cell cultures were arrested with nocodazole and were UV irradiated (50 J/m^2). Cell cycle progression was monitored at the indicated times in unirradiated (open symbols) and UV-irradiated (closed symbols) cultures after release from nocodazole, by direct visualization of nuclear division using DAPI staining.

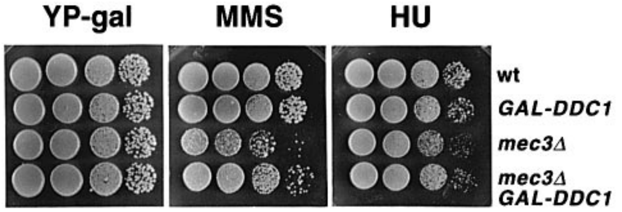

Fig. 4. Sensitivity to HU and MMS of *DDC1*-overexpressing strains. Serial dilutions of overnight YP-raffinose-saturated cell cultures of strains K699 (wt), YLL280 (*GAL-DDC1*), YLL134 (*mec3*Δ) and YLL288 (*mec3*Δ *GAL-DDC1*) were spotted on YP-gal plates without (YP-gal) or with MMS (0.01%) or HU (150 mM). No difference was observed between strains YLL134 and YLL288 when the carbon source in the media was glucose instead of galactose (not shown).

copy of the *GAL1–DDC1* gene fusion was integrated at the *LEU2* locus of otherwise isogenic wild-type and *mec3*Δ strains (see Materials and methods). *DDC1* overexpression, which did not cause any detectable phenotype in the wild-type background, partially suppressed MMS sensitivity and, to a lower extent, HU sensitivity of the *mec3*Δ strain (Figure 4).

As shown in Figure 5A, *mec3*Δ *GAL1-DDC1* cell cultures, synchronized with α-factor and then released from the G_1 block in the presence of MMS under galactose-induced conditions, progressed through S phase more slowly than similarly treated *mec3*Δ cell cultures. Furthermore, cell survival following MMS treatment was higher in *mec3*Δ *GAL1-DDC1* than in *mec3*Δ cell cultures (Figure 5B). Therefore, high levels of Ddc1p can partially suppress the intra-S checkpoint defect of the *mec3*Δ mutant. *DDC1* overexpression in wild-type cells did not cause any significant effect on response to MMS treatment during S phase (Figure 5A and B). When a similar experiment was carried out by comparing wild-type, *GAL1-MEC3*, *ddc1*Δ and *ddc1*Δ *GAL1-MEC3* cell cultures (see Table I and Materials and methods), *MEC3* overexpression did not suppress the MMS sensitivity of the *ddc1*Δ strain and had no effect on the rate of DNA synthesis in any genetic background, neither in the absence nor in the presence of MMS (data not shown).

Ddc1p is phosphorylated periodically during the cell cycle and in response to DNA damage

In order to characterize the *DDC1* gene product, we constructed a fully functional copy of the gene, expressing a 2HA-tagged Ddc1p, that was used to generate strain YLL334, carrying the *2HA-DDC1* allele at the *DDC1* chromosomal locus (see Materials and methods). As shown in Figure 6B, when anti-HA antibodies were used on Western blots of crude extracts prepared from exponentially growing YLL334 cells, they specifically detected two major bands that did not appear in extracts prepared from the isogenic strain carrying the untagged *DDC1* allele, therefore identifying Ddc1p. While the faster migrating band was present throughout the whole cell cycle, the slower migrating band was not present in α-factor-arrested wild-type cells and accumulated periodically during the cell cycle, increasing in level throughout S phase (Figure 6B and C) and decreasing concomitantly with the appearance of binucleate cells (Figure 6A). Therefore, Ddc1p is subject to cell cycle-dependent post-translational modification(s). When a similar experiment was performed in

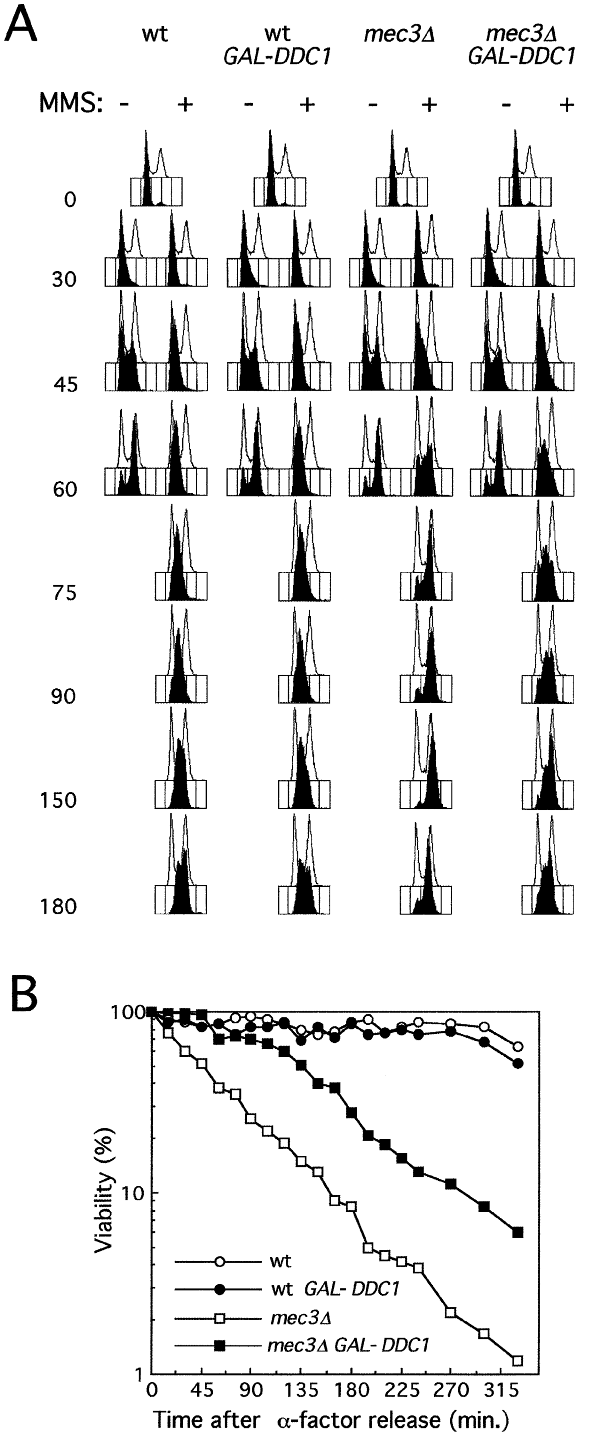

Fig. 5. Overexpression of *DDC1* partially counteracts the intra-S DNA damage checkpoint defect of a *mec3*Δ mutant. Cultures of strains K699 (wt), YLL280 (wt *GAL-DDC1*), YLL134 (*mec3*Δ) and YLL288 (*mec3*Δ *GAL-DDC1*), logarithmically growing in YP-raffinose, were synchronized with α-factor. Galactose to 2% was added 20 min before release from α-factor, that was performed at time zero by transferring the cultures to YP medium containing both raffinose and galactose, with or without 0.015% MMS. The same experiment was repeated independently three times with reproducible results. (**A**) Untreated (–) or MMS-treated (+) samples were taken at the indicated times (minutes) after α-factor release (time 0) and analyzed by FACS (black histograms). Overlayed histograms represent the cell cycle distributions of the asynchronous cultures. (**B**) Aliquots were removed from the MMS-treated cultures at timed intervals to determine cell number and to score for colony-forming units on YPD plates at 28°C.

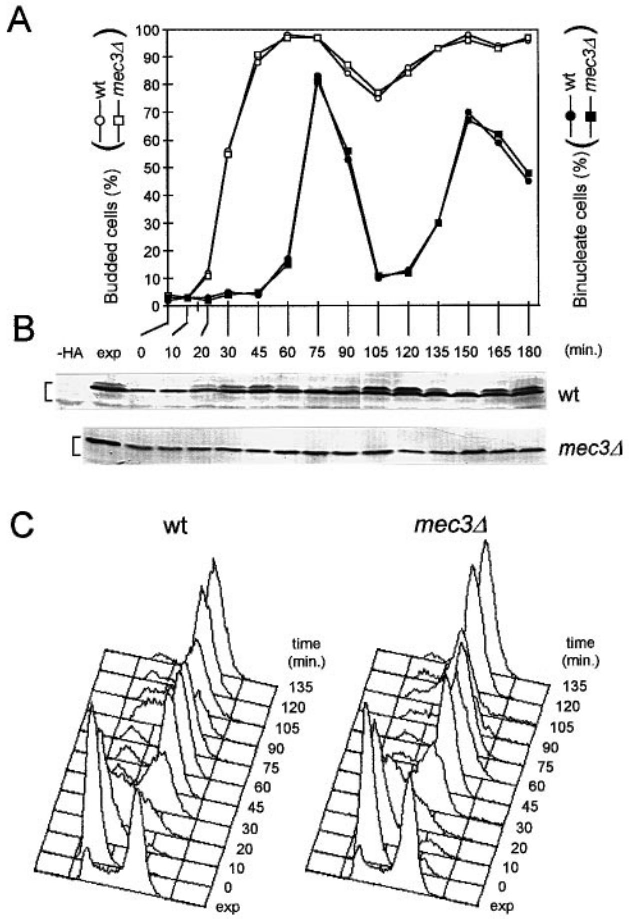

Fig. 6. Ddc1p is modified periodically during the cell cycle in wild-type, but not in *mec3*Δ cells. Exponentially growing (exp) YLL334 (wt) and YLL335 (*mec3*Δ) cells, expressing 2HA-Ddc1p from the *DDC1* promoter, were synchronized with α-factor and released at time zero. (**A**) The percentage of budded (open symbols) and binucleate cells (closed symbols) was monitored at the indicated times. (**B**) At the same times, protein extracts were prepared and analyzed by Western blot with 12CA5 antibody, together with K699 cell extract containing only untagged Ddc1p (–HA). Protein bands corresponding to Ddc1p are indicated by brackets. (**C**) FACS analysis at the indicated times after α-factor release.

a *mec3*Δ strain carrying the *2HA-DDC1* allele, only the faster migrating Ddc1p form could be detected throughout the whole cell cycle (Figure 6B), indicating that post-translational modification of Ddc1p depends on functional Mec3p.

As shown in Figure 7A, treatment of wild-type cells with UV and MMS caused accumulation of a modified form of Ddc1p, which migrated more slowly than the retarded protein species observed in untreated S phase cells. The observed Ddc1p modification was at least partially *MEC3* dependent. In fact, Ddc1p was predominantly unmodified in UV- and MMS-treated *mec3*Δ cells, and the small amount of modified protein observed in *mec3*Δ protein extracts migrated faster than the form detected in extracts prepared from similarly treated wild-type cells (Figure 7A). The observed changes in Ddc1p electrophoretic mobility were shown to be due to phosphorylation events (Figure 7B). In fact, the slower migrating protein species in both MMS-treated and S phase cell extracts was converted to the fastest migrating form by treatment with bacteriophage λ phosphatase.

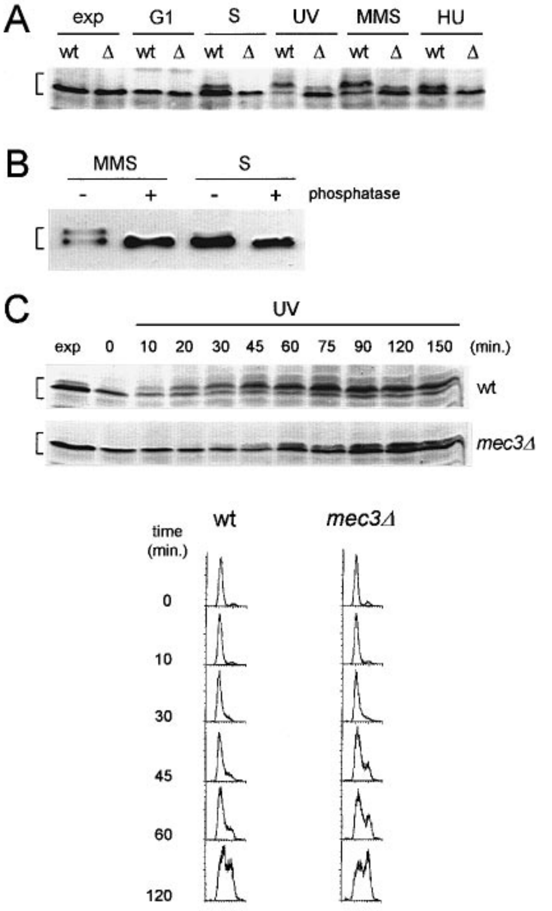

Fig. 7. Ddc1p phosphorylation during S phase and hyperphosphorylation in response to UV and MMS treatment are dependent on *MEC3*. Strains were YLL334 (wt) and YLL335 *mec3*Δ (Δ). Ddc1p is indicated by brackets. (**A**) Western blot analysis of protein extracts prepared from exponentially growing cell cultures that were either untreated (exp) or treated with UV (40 J/m^2), MMS (0.02% for 2 h) or HU (100 mM for 2 h). Protein extracts were also prepared from α-factor-arrested cells (G$_1$), or cells progressing through S phase 30 min after release from α-factor (S). (**B**) Protein extracts from MMS-treated exponentially growing (MMS) or S phase untreated wild-type cells (S) were immunoprecipitated with 12CA5 antibody. Immunoprecipitates were then incubated at 30°C without (–) or with (+) λ phosphatase, before electrophoresis and Western blot analysis using 12CA5 antibody. (**C**) Cultures were synchronized with α-factor, UV irradiated (40 J/m^2) and released from α-factor. The top part of the panel shows a Western blot analysis with 12CA5 antibody of protein extracts prepared from exponentially growing (exp) and, at the indicated times after α-factor release, from UV-irradiated cells. The bottom part of the panel shows FACS analysis of the irradiated cultures. Time zero for UV-treated cultures corresponds to cell samples taken immediately before UV irradiation and release from α-factor.

HU treatment of wild-type cells caused the accumulation of a Ddc1p form with electrophoretic mobility indistinguishable from that accumulated during normal S phase (Figure 7A). By considering that Ddc1p is not required for HU-induced cell division arrest, while it is required for DNA damage response, Ddc1p hyperphosphorylation appears to correlate with Ddc1p checkpoint function.

In order to better define the kinetics of Ddc1p phosphorylation in response to DNA damage and its dependence on *MEC3*, α-factor-arrested cells were UV irradiated and Ddc1p was analyzed by Western blot after release from α-factor block. As shown in Figure 7C, hyperphosphorylated Ddc1p in UV-treated wild-type cells appeared immediately after release from α-factor, it became the most abundant form in ~45 min, when most cells were unbudded (data not shown) with a 1C DNA content (Figure 7C, bottom), and it was then maintained until the end of the experiment. Both the kinetics and extent of Ddc1p phosphorylation in response to DNA damage in G_1 were at least partially dependent on *MEC3*. In fact, when *mec3*Δ cells were UV irradiated in G_1, Ddc1p phosphorylation was delayed by 20–30 min compared with wild-type (Figure 7C), although *mec3*Δ cells progressed through the cell cycle after α-factor release much faster than did wild-type cells under the same conditions (Figure 7C, bottom). Moreover, both the total amount of modified protein and the extent of modification, as judged by the changes in electrophoretic mobility, were reduced in UV-irradiated *mec3*Δ cells compared with wild-type (Figure 7C). No Ddc1p phosphorylation was observed in extracts prepared from wild-type cells that were kept in the presence of α-factor for 2 h after UV treatment in G_1 (data not shown).

Similarly to what was observed after UV irradiation in G_1, Ddc1p was also hyperphosphorylated in response to DNA damage in G_2 (Figure 8). In fact, while nocodazole-arrested unirradiated cells contained only unphosphorylated Ddc1p, the hyperphosphorylated form of Ddc1p was detectable immediately after release from nocodazole arrest of UV-treated wild-type cells (Figure 8C), when most cells still contained undivided nuclei (Figure 8B). This response to UV-induced damage does not require cell cycle progression, since an identical extent of Ddc1p phosphorylation was observed in wild-type cells either released from nocodazole or kept for 2 h in the presence of the drug after UV treatment in G_2 (Figure 8D). As expected, Ddc1p was instead phosphorylated only during S phase when cells were released from nocodazole arrest in the absence of DNA damage, and again this modification was *MEC3* dependent (Figure 8A and C).

Ddc1p phosphorylation in response to DNA damage in G_2 is also at least partially dependent on *MEC3*. In fact, only a small amount of partially modified Ddc1p was detectable in *mec3*Δ cells either released from nocodazole or kept in the presence of the drug after UV irradiation in G_2 (Figure 8C and D). Therefore, the difference in Ddc1p phosphorylation between UV-treated wild-type and *mec3*Δ cells was not due to different kinetics of cell cycle progression.

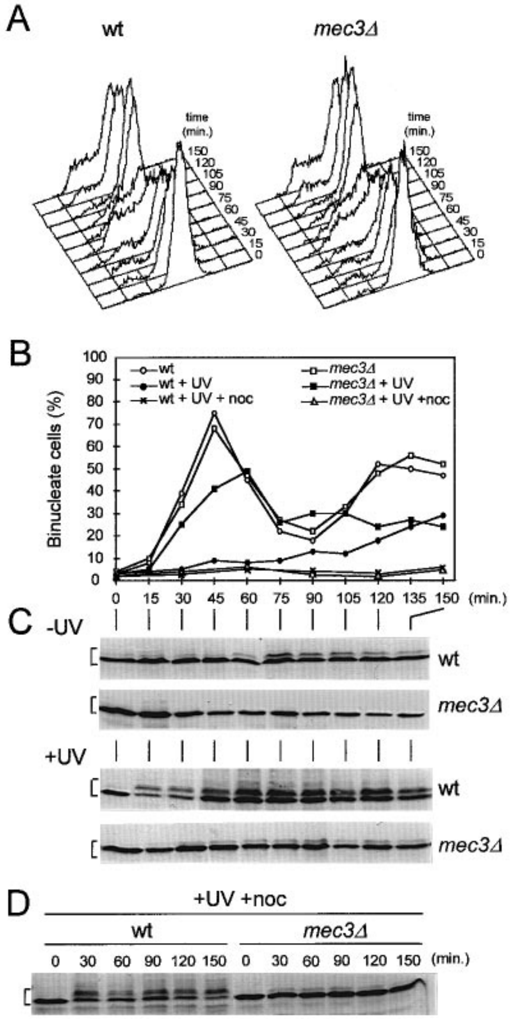

Fig. 8. Phosphorylation of Ddc1p after UV treatment in G_2. Cell cultures of strains YLL334 (wt) and YLL335 (*mec3*Δ) were arrested with nocodazole and UV-irradiated (50 J/m^2). Unirradiated (–UV) and UV-irradiated (+UV) samples were resuspended into YPD medium or into YPD medium containing 15 μg/ml nocodazole (+UV +noc). (**A**) FACS analysis of untreated wild-type and *mec3*Δ cells at the indicated times after release from nocodazole. (**B**) Cell cycle progression was monitored by direct visualization of nuclear division using propidium iodide. (**C**) Western blot analysis using 12CA5 antibody of protein extracts from untreated (–UV) and treated (+UV) wild-type and *mec3*Δ cultures after release from nocodazole. (**D**) Western blot analysis of protein extracts from wild-type and *mec3*Δ cultures held in nocodazole for the indicated times after UV treatment in G_2. Ddc1p is indicated by brackets. Time zero for UV-treated cultures corresponds to cell samples taken immediately before UV irradiation and release from nocodazole, while time zero for untreated cultures corresponds to cell samples taken immediately before release from nocodazole.

Discussion

Response to DNA damage in eukaryotic cells involves specific surveillance mechanisms, which are genetically controlled and are essential for accurate transmission of genetic information during cell proliferation. In *S.cerevisiae*, the *RAD9*, *RAD17*, *RAD24* and *MEC3* gene products are all required for these processes and are proposed to act in concert, although with different roles, in processing DNA lesions, thus generating signals that arrest or slow down cell cycle progression in the presence of DNA damage (reviewed in Lydall and Weinert, 1996).

Role of DDC1 in checkpoint and DNA repair mechanisms

The previously uncharacterized *DDC1* gene product is involved in all the known surveillance mechanisms controlling cell response to DNA damage. In fact, null *ddc1*Δ mutants, besides being more sensitive than wild-type to UV, MMS and HU, are defective in delaying G_1–S and G_2–M transition and in slowing down the rate of DNA synthesis when DNA is damaged during G_1, G_2 or S phase, respectively. Conversely, Ddc1p is not required for delaying entry into mitosis when DNA synthesis is inhibited by HU. As previously suggested for other DNA damage checkpoint genes (Siede *et al*., 1993; Lydall and Weinert, 1995), *DDC1* function is likely to be required for DNA damage processing/repair events, since lethality of G_1 or G_2 UV-irradiated *ddc1*Δ cells is not rescued by artificially arresting the cell cycle with α-factor or nocodazole, respectively.

Why *ddc1* mutants, as well as *mec3*, *rad24* and *rad17* mutants, are more sensitive to HU than wild-type, even though these factors are not involved directly in cell cycle arrest in response to HU treatment (Weinert and Hartwell, 1993; Weinert *et al*., 1994; Longhese *et al*., 1996a; this work), is still an open question. Since *ddc1* and *mec3* mutations are synthetic lethal with *pri1* mutations and severely affect cell viability of other DNA replication mutants at the permissive temperature (Longhese *et al*., 1996a; this work), Ddc1p and Mec3p might be involved specifically in sensing/processing altered DNA molecules arising from defective DNA replication, and HU might cause similar effects by interfering with DNA synthesis.

The Ddc1p amino acid sequence shows some homology with the product of the *S.pombe* $rad9^+$ gene, which is also involved in DNA damage checkpoints (Al-Khodairy and Carr, 1992; Enoch *et al*., 1992). Since the two yeast proteins are structurally related and functionally share similarities, they might have diverged from the same protein. The recently identified human Rad9 protein (Lieberman *et al*., 1996) is also related, but seems to have more similarities to the *S.pombe* gene product. As there is no obvious enzymatic activity associated with these proteins, it is possible that this reflects more flexibility of structural divergence.

Ddc1p is phosphorylated periodically during the cell cycle and is hyperphosphorylated in response to DNA damage

A phosphorylated form of Ddc1p appears periodically during the cell cycle, reaching the maximum level when most cells are in S phase and decreasing concomitantly with nuclear division. The event(s) leading to dephosphorylation or/and degradation of the phosphorylated form do not require nuclear division, since the phosphorylated Ddc1p is not detectable in nocodazole-arrested cells. The correlation between Ddc1p phosphorylation and progression through S phase suggests that the signal leading to this modification might be intrinsic to the DNA replication process. Since *DDC1* is likely to be involved, together with the other *RAD24* group genes, in processing single-stranded DNA lesions, its S phase-dependent phosphorylation might result from sensing single-stranded replication intermediates and/or spontaneous errors arising during DNA replication. This, in turn, might result in potentially active Ddc1p, that would then be required if accumulation of DNA lesions rises above the physiological level. Thus, Ddc1p phosphorylation is not expected to take place in undamaged nocodazole-arrested cells since, once DNA replication has been completed properly, there should be no more signals leading to Ddc1p phosphorylation.

When wild-type cells are UV irradiated in either G_1 or G_2, Ddc1p is hyperphosphorylated. This modification takes place in G_2 cells even if they are held in nocodazole after irradiation, and in unbudded cells with 1C DNA content following DNA damage in G_1. Therefore, Ddc1p hyperphosphorylation correlates with DNA damage response and does not require ongoing DNA synthesis.

Functional interactions between DDC1 and MEC3

The *RAD9*, *RAD17*, *RAD24* and *MEC3* genes have all been implicated in processing *cdc13*-induced lesions near to the telomeres (Garvik *et al*., 1995; Lydall and Weinert, 1995). The properties of the four genes suggest that they might have a role as modifiers or sensors of DNA lesions (reviewed in Lydall and Weinert, 1996). However, the biochemical activities of the corresponding proteins, their reciprocal interactions and their interactions with other factors are still under investigation.

We have observed that the effect of deleting the *MEC3* gene can be partially suppressed by overexpressing *DDC1*, since sensitivity to MMS and HU and checkpoint defects of *mec3*Δ strains are diminished when Ddc1p is overproduced, indicating that high levels of Ddc1p can partially mimic Mec3p function. Several models can be envisaged to explain these results. For example, the two gene products might have partially overlapping functions. In this case, since *MEC3* overexpression cannot suppress MMS sensitivity or checkpoint defects of *ddc1*Δ strains, overproduced Mec3p should require some limiting step(s) to be able to substitute for Ddc1p. Ddc1p and Mec3p might also perform subsequent functions, the Mec3p-dependent reaction preceding that involving Ddc1p. In this case, partial suppression of the *mec3*Δ phenotypes by *DDC1* overexpression might result from a reduced requirement for upstream functions, including Mec3p. The observation that *DDC1* overexpression also partially suppresses the sensivity to MMS of *rad9*Δ mutants (our unpublished data) supports this ordering. Finally, phosphorylation of Ddc1p both during the normal cell cycle and in response to DNA damage is at least partially dependent on the presence of Mec3p. This finding not only provides insights into the relationships between *MEC3* and *DDC1*, but also correlates Ddc1p phosphorylation with activation of DNA damage checkpoint pathways.

Taken together, our data indicate that Ddc1p participates together with Mec3p and, possibly, Rad17p and Rad24p in DNA damage recognition/processing events at an early step in the DNA damage response process, and it might be involved in the signal sensing and transducing branch of the pathway. Future work will be focused on understanding how this part of the pathway is integrated into the cascade of events leading to cell cycle arrest. To this end, it will be crucial to establish the functional role(s) of Ddc1p phosphorylation and its connections with the Mec1p and Rad53p general transducers, as well as to identify the kinase(s) responsible for Ddc1p modification.

Materials and methods

Oligonucleotides used for PCR amplifications

The following oligonucleotides were used: PRP33, 5′ GGCTGATGTTAGCTCACGCTCTGT 3′; PRP34, 5′ CGCGGATCCATATGTCATTTAAGGCAACTATCACCGAG 3′; PRP46, 5′ GGAATTCCATATGTACCCATACGATGTTCCT 3′; PRP49, 5′ CCTTAAGCATATGGGATCCTGCATAGTCCGG 3′; PRP21, 5′ GCTTAGACATATATGTCATTTAAGGCAACTATCACCGAGTCGGGGCGTACGCTGCAGGTCGAC 3′; PRP22, 5′ TATACCCCTTGGCTTTTCTACTTGTGTTAGACCCAGCCCATCTTCATCGATGAATTCGAGCTCG 3′.

Plasmids

Plasmid pML80.1 is the original pUN100 derivative plasmid (Jansen *et al*., 1993), carrying a *S.cerevisiae* chromosome XVI fragment located

Table I. *S.cerevisiae* strains used in this study

Strain	Genotype[a]	Reference/Source
K2346	*MAT***a** *ade2-1 ade3 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100*	K.Nasmyth
K2348	*MATα ade2-1 ade3 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100*	K.Nasmyth
K699	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100*	K.Nasmyth
K2346CS33	*MAT***a** *ade2-1 ade3 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 pri1-2*	Longhese *et al*. (1996a)
K2348CS33	*MATα ade2-1 ade3 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 pri1-2*	Longhese *et al*. (1996a)
YLL231	*MAT***a** *ade2-1 ade3 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 pri1-2* [pML9 ADE3 URA3 PRI1]	this study
DMP1777/4D	*MAT***a** *ade2-1 ade3 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 pri1-2 pip5-1* [pML9 *ADE3 URA3 PRI1*]	this study
DMP1813/1A	*MAT***a** *ade2-1 ade3 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 pip5-1*	this study
DMP262/2C	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 ddc1Δ::KanMX4*	this study
YLL244	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 ddc1Δ::KanMX4*	this study
YLL245	*MAT***a** *ade2-1 ade3 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 ddc1Δ::KanMX4 pri1-2* [pML9 *ADE3 URA3 PRI1*]	this study
YLL134	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 mec3Δ1::TRP1*	Longhese *et al*. (1996a)
YLL271	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 mec3Δ1::TRP1 ddc1Δ::KanMX4*	this study
YLL157	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad9Δ::URA3*	Longhese *et al*. (1996a)
YLL301	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad9Δ::URA3ddc1Δ::KanMX4*	this study
YLL280	*MAT***a** *ade2-1 trp1-1 leu2-3,112::GAL1-DDC1::LEU2 his3-11,15 ura3 can1-100*	this study
YLL288	*MAT***a** *ade2-1 trp1-1 leu2-3,112::GAL1-DDC1::LEU2 his3-11,15 ura3 can1-100 mec3Δ1::TRP1*	this study
YLL302	*MAT***a** *ade2-1 trp1-1 leu2-3,112::GAL1-MEC3::LEU2 his3-11,15 ura3 can1-100*	this study
YLL303	*MAT***a** *ade2-1 trp1-1 leu2-3,112::GAL1-MEC3::LEU2 his3-11,15 ura3 can1-100 ddc1Δ::KanMX4*	this study
YLL334	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1*	this study
YLL335	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 mec3Δ1::TRP1 HA2-DDC1::LEU2::ddc1*	this study

[a] Plasmids are indicated within brackets

between positions 175 452 and 186 891. Plasmid pML89 was obtained by replacing the *Xba*I–*Acc*I fragment of plasmid YCplac111 (Gietz and Sugino, 1988) with the 2772 bp *Nsi*I–*Bst*BI DNA fragment, containing the *DDC1* gene. To construct plasmid pML109, where the 2467 bp fragment, spanning from the *DDC1* ATG to the *Eco*47III site and containing the whole *DDC1* coding region, is fused to the *GAL1* promoter, an *Eco*RI–*Bam*HI fragment containing the *GAL1-10* promoter was first used to replace the *Eco*RI–*Bam*HI fragment within the YIplac128 polylinker region (Gietz and Sugino, 1988), giving rise to plasmid pML95; a *DDC1* fragment spanning from position +1 to position +173 from the translation initiation codon was then amplified by PCR using plasmid pML89 as a template and oligonucleotides PRP33 and PRP34 as primers and then cloned into the *Bam*HI–*Hin*dIII sites of plasmid pML95, to give rise to plasmid pML101. The 4182 bp *Hin*dIII fragment from plasmid pML80.1 was then cloned into the *Hin*dIII site of plasmid pML101, followed by excision of the *Eco*47III–*Bgl*II DNA fragment from the derivative plasmid, to give rise to plasmid pML109. To construct plasmid pML118, carrying a 2HA-tagged *GAL1–DDC1* fusion (*GAL1-HA2-DDC1*), plasmid B2385 (Kolodziej and Young, 1991) was used as a template for PCR amplification with oligonucleotides PRP49 and PRP46 as primers. The amplification product, containing two copies of the HA epitope-coding sequence, was cloned into the *Nde*I site at position +1716 from the *DDC1* translation initiation codon in plasmid pML109, giving rise to plasmid pML118, whose 1664 bp *Xmn*I–*Hin*dIII *DDC1* fragment was then cloned into the *Sma*I–*Hind*III sites of YIplac128, and the derivative plasmid pML119, carrying the *2HA-DDC1* allele, was used to construct strains YLL334 and YLL335 (see below). pML113, whose construction will be described elsewhere, is a YIplac128 derivative plasmid carrying the whole *MEC3*-coding sequence fused to the *GAL1* promoter.

All the PCR reactions were carried out using Vent DNA polymerase (Biolabs). The fidelity of PCR amplification was controlled by nucleotide sequence analysis of the *GAL1–DDC1* and *GAL1–MEC3* fusions. Both the *GAL1–DDC1* and the *GAL1–MEC3* fusions were shown to complement the defects of the cognate null alleles. The centromeric plasmids pDL179 and pDL214, carrying respectively the *RAD17* and *RAD24* genes, were a kind gift from D.Lydall (Tucson University, AZ). Plasmids pML78 and pML79 were constructed by cloning the *RAD53 Eco*RI–*Eco*RI fragment from plasmid pRS316-SPK1 (gift from D.Stern, Yale University, CT) and the *MEC1 Spe*I–*Spe*I fragment from plasmid pRK900 (gift from I.Ogawa, Osaka University), respectively, into the *Eco*RI and the *Spe*I sites of YCplac111.

Yeast strains and media

The genotypes of all the yeast strains used in this study are listed in Table I. All the strains are derivatives of W303 (*MAT***a**/*MATα ade2-1/ade2-1 trp1-1/trp1-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ura3/ura3*). Strain YLL231 was derived from strain K2346CS33 by transformation with plasmid pML9 (Longhese *et al*, 1996a). Strain DMP1777/4D is a meiotic segregant from a cross of the original *pip5-1* mutant (Longhese *et al*., 1996a) with strain K2348CS33. Strain DMP1813/1A is a meiotic segregant from a cross between strains DMP1777/4D and K2348. One-step replacement of 1752 bp of the *DDC1* coding region with the *kanMX4* cassette (*ddc1Δ::kanMX4*) was carried out by transforming strains K699, YLL231, YLL134 and YLL157 with a PCR-amplified *kanMX4* cassette (see below) to give rise to strains YLL244, YLL245, YLL271 and YLL301, respectively. Strain DMP262/2C is a meiotic segregant from a W303 derivative heterozygous for the *ddc1Δ::kanMX4* allele (see below). Strains YLL280 and YLL288, carrying a single copy of a *GAL1–DDC1* fusion integrated at the *LEU2* locus, were obtained by transforming, respectively, strains K699 and YLL134 with *Bst*XI-digested plasmid pML109. Strains YLL302 and YLL303, carrying a single copy of a *GAL1–MEC3* fusion integrated at the *LEU2* locus, were obtained from strains K699 and YLL244, respectively, by transformation with *Bst*XI-digested plasmid pML113. Strains YLL334 and YLL335, carrying the *2HA-DDC1* allele at the *DDC1* chromosomal locus, were obtained by trasforming, respectively, strains K699 and YLL134 with *Pst*I-digested plasmid pML119. The *2HA-DDC1* allele is fully functional, since strains K699 and YLL334 were indistinguishable from one another.

The accuracy of all gene replacements and integrations was verified by Southern blot analysis. Standard yeast genetic techniques and media were according to Rose *et al*. (1990). YP media contained either 2% glucose (YPD), 2% galactose (YP-gal), 2% raffinose (YP-raffinose) or both galactose and raffinose (2% each) as the carbon source. Transformants carrying the *kanMX4* cassette were selected on YPD plates containing 400 µg/ml G418 (450 µg/mg, US Biological).

Cloning and disruption of the PIP5/DDC1 gene

To clone the gene identified by the *pip5-1* mutation, strain DMP1777/4D was transformed with a yeast genomic DNA library constructed in the pUN100 *LEU2* centromeric plasmid (Jansen *et al*., 1993), and transformants were screened for the presence of recombinant plasmids able to restore a Sect$^+$ 5-FOA$^+$ phenotype, and therefore possibly complementing synthetic lethality (Longhese *et al*., 1996a). Five different plasmids carrying partially overlapping yeast DNA inserts were identified by this screening. The minimal region complementing synthetic lethality was within an *Nsi*I–*Bst*BI fragment (Figure 1A), and contained the *PIP5* gene, which we renamed *DDC1* (see Results). To construct a *DDC1* chromosomal deletion (*ddc1Δ*; Figure 1A), the heterologous *kanMX4* cassette was amplified by PCR using plasmid pFA6a-*kanMX4* (Wach *et al*., 1994) as a template and oligonucleotides PRP21 and PRP22 as primers. The amplification product contained the *kanMX4* cassette flanked by *DDC1* sequences (underlined in the oligonucleotide sequences) and was used to transform the diploid strain W303. G418-resistant transformants were shown by PCR analysis to be heterozygous for the replacement of most of the *DDC1* chromosomal ORF with the *kanMX4* cassette. By sporulation and tetrad analysis of one of these transformants, *ddc1*Δ segregants were shown to be viable and to grow as wild-type on different media at different temperatures. A *ddc1*Δ/*pip5-1* diploid strain obtained by crossing strain DMP262/2C to strain DMP1813/1A (see Table I) was as sensitive to UV, MMS and HU as the parent strains (data not shown). Although spore viability was severely affected, we could test 50 viable meiotic segregants from 30 tetrads of this diploid strain, and they were all sensitive to UV, MMS and HU, thus confirming that the *ddc1*Δ and *pip5-1* mutations are allelic.

UV, MMS and HU synchrony experiments

Cell synchronization in G_1 was obtained by treatment of exponentially growing YPD cell cultures with 2 μg/ml of α-factor, followed by release in YPD. G_2 arrest was obtained by treating exponentially growing YPD cell cultures with 5 μg/ml of nocodazole and 1% dimethylsulfoxide (DMSO) until 90–95% of cells were large budded. α-Factor- and nocodazole-arrested cells were collected by centrifugation, and 5×10^8 cells were spread on 14 cm diameter YPD plates (Allen *et al*., 1994), followed by UV irradiation with 40 and 50 J/m^2, respectively. When required, cell cultures were held in G_1 or G_2 after UV irradiation by treatment with 2 μg/ml of α-factor or 15 of μg/ml nocodazole and 1% DMSO, respectively. MMS synchrony experiments were carried out as previously described (Paulovich and Hartwell, 1995) using respectively 0.02% MMS in YPD medium, and 0.015% MMS in galactose- and raffinose-containing YP medium. HU synchrony experiments were according to Allen *et al*. (1994), using 200 mM HU.

Protein extracts and Western blot analysis

Protein extracts for Western blot analysis were prepared from trichloroacetic acid-treated yeast cells as previously described (Foiani *et al*., 1994). Protein extracts were resolved by electrophoresis on 12.5% SDS–polyacrylamide gels and proteins were transferred to nitrocellulose membranes, which were then incubated for 2 h at room temperature with anti-HA monoclonal antibody 12CA5 (1:5000 dilution in Tris-buffered saline with 0.2% Triton X-100 and 4% non-fat milk), followed by incubation with peroxidase-labeled anti-mouse antibody (Amersham).

Immunoprecipitation and phosphatase treatment

Protein extracts for immunoprecipitation were prepared from exponentially growing cells collected by centrifugation and resuspended in an equal volume (w/v) of lysis buffer [0.1% SDS, 1% Triton X-100, 1% Na deoxycholate, 0.05 M Tris–HCl pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 20 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 60 mM β-glycerophosphate and 1 mM sodium orthovanadate]. After addition of 1:1 volume of acid-washed glass beads, 2.5 mg of clarified protein extracts were incubated for 1 h at 4°C with 75 μl of a 50% (v/v) protein A–Sepharose CL-6B, covalently linked to 12CA5 monoclonal antibody. Immunoprecipitates were washed twice with 1 ml of phosphate-buffered saline, resuspended in 60 μl of phage λ phosphatase buffer (50 mM Tris–HCl pH 7.8, 2 mM $MnCl_2$, 5 mM dithiothreitol, 100 μg/ml acetylated bovine serum albumin) and divided into two samples, which were incubated at 30°C for 30 min with or without the addition of 150 U of λ phosphatase (Biolabs). Phosphatase was removed by two washes with PBS, the resin was resuspended in 20 μl of SDS-gel loading buffer and bound proteins were resolved by electrophoresis on a 12.5% SDS–polyacrylamide gel and visualized by Western blotting.

Acknowledgements

We wish to thank A.Carr, D.Lydall, K.Nasmyth, I.Ogawa, D.Stern, A.Wach and T.Weinert for gifts of strains and plasmids, L.Mizzi for help in computer analysis, G.Liberi for help in immunoprecipitation experiments, M.Foiani and S.Piatti for critical reading of the manuscript, and A.Carr and all the members of our laboratory for useful discussions and criticisms. This work was partially supported by grants from Progetto Strategico Ciclo Cellulare e Apoptosi, Associazione Italiana per la Ricerca sul Cancro, CNR grant 96.03101.CT04, and EU contracts CHRX-CT93-0248 and ERB CHRX-CT94-O685. M.P.L. and V.P. were supported by fellowships from Fondazione Adriano Buzzati-Traverso and Fondazione Confalonieri, respectively.

References

Aboussekhra,A., Vialard,J.E., Morrison,D.E., de la Torre-Ruiz,M.A., Cernàkovà,L., Fabre,F. and Lowndes,N.F. (1996) A novel role for the budding yeast *RAD9* checkpoint gene in DNA damage-dependent transcription. *EMBO J.*, **15**, 3912–3922.

Al-Khodairy,F. and Carr,A.M. (1992) DNA repair mutants defining G_2 checkpoint pathways in *Schizosaccharomyces pombe*. *EMBO J.*, **11**, 1343–1350.

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

Araki,H., Leem,S.H., Phongdara,A. and Sugino,A. (1995) Dpb11, which interacts with DNA polymerase II (ε) in *Saccharomyces cerevisiae*, has a dual role in S-phase progression and at a cell cycle checkpoint. *Proc. Natl Acad. Sci. USA*, **92**, 11791–11795.

Carr,A.M. (1997) Control of cell cycle arrest by the Mec1^{sc}/Rad3^{sp} DNA structure checkpoint pathway. *Curr. Opin. Genet. Dev.*, **7**, 93–98.

Carr,A.M. and Hoekstra,M.F. (1995) The cellular responses to DNA damage. *Trends Cell Biol.*, **5**, 32–40.

Elledge,S.J. (1996) Cell cycle checkpoints: preventing an identity crisis. *Science*, **274**, 1664–1672.

Enoch,T. and Norbury,C. (1995) Cellular responses to DNA damage: cell cycle checkpoints, apoptosis and the roles of p53 and ATM. *Trends Biochem. Sci.*, **20**, 426–430.

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

Foiani,M., Marini,F., Gamba,D., Lucchini,G. and Plevani,P. (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.

Friedberg,E.C., Walker,G.C. and Siede,W.D. (1995) *DNA Repair and Mutagenesis*. ASM Press, Washington, DC.

Garvik,B., Carson,M. and Hartwell,L. (1995) Single-stranded DNA arising at telomeres in *cdc13* mutants may constitute a specific signal for the *RAD9* checkpoint. *Mol. Cell. Biol.*, **15**, 6128–6138.

Gietz,R.D. and Sugino,A. (1988) New yeast–*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six base-pair restriction sites. *Gene*, **74**, 527–534.

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

Hartwell,L.H. and Kastan,M.B. (1994) Cell cycle control and cancer. *Science*, **266**, 1821–1828.

Jansen,R., Tollervey,D. and Hurt,E.C. (1993) A U3 snoRNP protein with homology to splicing factor PRP4 and Gβ domains is required for ribosomal RNA processing. *EMBO J.*, **12**, 2549–2558.

Kiser,G.L. and Weinert,T.A. (1996) Distinct roles of yeast *MEC* and *RAD* checkpoint genes in transcriptional induction after DNA damage and implications for function. *Mol. Biol. Cell*, **7**, 703–718.

Kolodziej,P.A. and Young,R.A. (1991) Epitope tagging and protein surveillance. *Methods Enzymol.*, **194**, 508–510.

Lieberman,H.B. and Hopkins,K.M. (1994) *Schizosaccharomyces malidevorans* and *Sz.octosporus* homologues of *Sz.pombe rad9*, a gene that mediates radioresistance and cell-cycle progression. *Gene*, **150**, 281–286.

Lieberman,H.B., Hopkins,K.M., Laverty,M. and Chu,H.M. (1992) Molecular cloning and analysis of *Schizosaccharomyces pombe rad9*, a gene involved in DNA repair and mutagenesis. *Mol. Gen. Genet.*, **232**, 367–376.

Lieberman,H.B., Hopkins,K.M., Nass,M., Demetrick,D. and Davey,S. (1996) A human homolog of the *Schizosaccharomyces pombe rad9*$^+$ checkpoint control gene. *Proc. Natl Acad. Sci. USA*, **93**, 13890–13895.

Longhese,M.P., Fraschini,R., Plevani,P. and Lucchini,G. (1996a) Yeast *pip3/mec3* mutants fail to delay entry into S phase and to slow DNA replication in response to DNA damage, and they define a functional link between Mec3 and DNA primase. *Mol. Cell. Biol.*, **16**, 3235–3244

Longhese,M.P., Neecke,H., Paciotti,V., Lucchini,G. and Plevani,P. (1996b) The 70 kDa subunit of replication protein A is required for the G_1/S and intra-S DNA damage checkpoints in budding yeast. *Nucleic Acids Res.*, **24**, 3533–3537

Lydall,D. and Weinert,T. (1995) Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. *Science*, **270**, 1488–1491.

Lydall,D. and Weinert,T. (1996) From DNA damage to cell cycle arrest and suicide: a budding yeast perspective. *Curr. Opin. Genet. Dev.*, **6**, 4–11

Marini,F., Pellicioli,A., Paciotti,V., Lucchini,G., Plevani,P., Stern,D.F. and Foiani,M. (1997) A role for DNA primase in coupling DNA replication to DNA damage response. *EMBO J.*, **16**, 639–650.

Murray,A. (1994) Cell cycle checkpoints. *Curr. Opin. Cell Biol.*, **6**, 872–876

Murray,A. (1995) The genetics of cell cycle checkpoints. *Curr. Opin. Genet. Dev.*, **5**, 5–11.

Murray,J.M., Carr,A.M., Lehman,A.R. and Watts,F.Z. (1991) Cloning and characterization of the DNA repair gene, *rad9*, from *Schizosaccharomyces pombe*. *Nucleic Acids Res.*, **19**, 3525–3531.

Navas,T.A., Zhou,Z. and Elledge,S.J. (1995) DNA polymerase ε links the DNA replication machinery to the S phase checkpoint. *Cell*, **80**, 29–39.

Navas,T.A., Sanchez,Y. and Elledge,S.J. (1996) *RAD9* and DNA polymerase ε form parallel sensory branches for transducing the DNA damage checkpoint signal in *Saccharomyces cerevisiae*. *Genes Dev.*, **10**, 2632–2643.

Paulovich,A.G. and Hartwell,L.H. (1995) A checkpoint regulates the rate of progression through S phase in *S.cerevisiae* in response to DNA damage. *Cell*, **82**, 841–847.

Paulovich,A.G., Margulies,R.U., Garvik,B.M. and Hartwell,L.H. (1997a) *RAD9*, *RAD17*, and *RAD24* are required for S phase regulation in *Saccharomyces cerevisiae* in response to DNA damage. *Genetics*, **145**, 45–62.

Paulovich,A.G., Toczyski,D.P. and Hartwell,L.H. (1997b) When checkpoints fail. *Cell*, **88**, 315–321.

Rose,M.D., Winston,F. and Hieter,P. (1990) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanchez,Y., Desnay,B.A., Jones,W.J., Liu,Q., Wang,B. and Elledge,S.J. (1996) Regulation of *RAD53* by the *ATM*-like kinases *MEC1* and *TEL1* in yeast cell cycle checkpoint pathways. *Science*, **271**, 357–360.

Siede,W., Friedberg,A.S. and Friedberg,E.C. (1993) *RAD9*-dependent G_1 arrest defines a second checkpoint for damaged DNA in the cell cycle of *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **90**, 7985–7989.

Siede,W., Friedberg,A.S., Dianova,I. and Friedberg,E.C. (1994) Characterization of G_1 checkpoint control in the yeast *Saccharomyces cerevisiae* following exposure to DNA damaging agents. *Genetics*, **138**, 271–281.

Siede,W., Allen,J.B., Elledge,S.J. and Friedberg,E.C. (1996) The *Saccharomyces cerevisiae MEC1* gene, which encodes a homolog of the human ATM gene product, is required for G_1 arrest following radiation treatment. *J. Bacteriol.*, **178**, 5841–5843.

Sugimoto,K., Shimomura,T., Hashimoto,K., Araki,H., Sugino,A. and Matsumoto,K. (1996) Rfc5, a small subunit of replication factor C complex, couples DNA replication and mitosis in budding yeast. *Proc. Natl Acad. Sci. USA*, **93**, 7048–7052.

Sun,Z., Fay,D.S., Marini,F., Foiani,M. and Stern,D.F. (1996) Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and DNA damage checkpoint pathways. *Genes Dev.*, **10**, 395–406.

Wach,A., Brachat,A., Pohlmann,R. and Philippsen,P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*, **10**, 1793–1808.

Weinert,T.A. and Hartwell,L.H. (1988) The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science*, **241**, 317–322.

Weinert,T.A. and Hartwell,L.H. (1993) Cell cycle arrest of *cdc* mutants and specificity of the *RAD9* checkpoint. *Genetics*, **134**, 63–80.

Weinert,T.A., Kiser,G.L. and Hartwell,L.H. (1994) Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. *Genes Dev.*, **8**, 652–665.

Zheng,P., Fayn,D.S., Burton,J., Xiao,H., Pinkham,J.L. and Stern,D.F. (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.

Received on April 10, 1997; revised on June 13, 1997