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Mec1p is essential for phosphorylation of the yeast DNA damage checkpoint protein Ddc1p, which physically interacts with Mec3p

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Checkpoints prevent DNA replication or nuclear division when chromosomes are damaged. The *Saccharomyces cerevisiae DDC1* gene belongs to the *RAD17*, *MEC3* and *RAD24* epistasis group which, together with *RAD9*, is proposed to act at the beginning of the DNA damage checkpoint pathway. Ddc1p is periodically phosphorylated during unperturbed cell cycle and hyperphosphorylated in response to DNA damage. We demonstrate that Ddc1p interacts physically *in vivo* with Mec3p, and this interaction requires Rad17p. We also show that phosphorylation of Ddc1p depends on the key checkpoint protein Mec1p and also on Rad24p, Rad17p and Mec3p. This suggests that Mec1p might act together with the Rad24 group of proteins at an early step of the DNA damage checkpoint response. On the other hand, Ddc1p phosphorylation is independent of Rad53p and Rad9p. Moreover, while Ddc1p is required for Rad53p phosphorylation, it does not play any major role in the phosphorylation of the anaphase inhibitor Pds1p, which requires *RAD9* and *MEC1*. We suggest that Rad9p and Ddc1p might function in separated branches of the DNA damage checkpoint pathway, playing different roles in determining Mec1p activity and/or substrate specificity.
Keywords: budding yeast/checkpoints/*DDC1*/DNA damage/*MEC1*

Introduction

Alterations of DNA structure caused by genotoxic agents induce a number of cellular responses, including DNA repair and cell-cycle arrest mediated by surveillance mechanisms known as checkpoints (for reviews, see Elledge, 1996; Paulovich *et al.*, 1997b). These two protective mechanisms are tightly linked to each other, since some checkpoint proteins may participate in processing DNA lesions (Lydall and Weinert, 1995) and are required to induce the expression of DNA repair genes (Zhou and Elledge, 1993; Aboussekhra *et al.*, 1996; Kiser and Weinert, 1996). The balanced and concerted action between cell-cycle controls and DNA repair minimizes the potentially harmful effects of DNA alterations and provides the cells with the capacity to survive genotoxic insults. Failure to respond properly to DNA damage allows the cell to replicate and segregate damaged DNA molecules. This might result in hypersensitivity to DNA-damaging agents and increased genetic instability that, in multicellular organisms, may lead to cancer (Hartwell and Kastan, 1994). In fact, if DNA damage is not repaired as soon as it arises, some options for repair may be lost when cells undergo the next cell-cycle transition, and primary DNA lesions may be processed, thus generating secondary lesions (for reviews, see Carr and Hoekstra, 1995; Paulovich *et al.*, 1997b).

The DNA damage checkpoint pathway is a complex signal transduction system, expected to have specialized sensors of perturbations in DNA structure, including DNA damage and incomplete DNA replication, and specific transducers transmitting the signal to the cell-cycle machinery. In eukaryotes, following DNA perturbations, cell-cycle progression is halted at different stages, depending on the type of damage and the cell-cycle phase at which the damage occurs (Weinert and Hartwell, 1988, 1993; Siede *et al.*, 1993, 1994; Allen *et al.*, 1994; Weinert *et al.*, 1994; Paulovich and Hartwell, 1995). Checkpoint genes have been isolated in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The identification of several structural and functional homologues in the two evolutionarily distant yeasts and in human cells underscores conservation throughout evolution.

Since there are many different types of DNA damage, there are likely to be multiple sensors for specific DNA perturbations at different stages of the cell cycle. In budding yeast, the checkpoints operating in response to DNA damage or to incomplete DNA replication depend on different factors. In fact, the DNA replication proteins Polε, Rfc5p and Dpb11p are proposed specifically to sense DNA damage and replication blocks during DNA synthesis, thus linking entry into mitosis to proper completion of S phase (Araki *et al.*, 1995; Navas *et al.*, 1995, 1996; Sugimoto *et al.*, 1996, 1997). On the other hand, Rad9p, Rad17p, Rad24p and Mec3p are required for response to DNA damage, but not to incomplete DNA replication (Weinert and Hartwell, 1988; Siede *et al.*, 1993; Weinert *et al.*, 1994; Longhese *et al.*, 1996a; Paulovich *et al.*, 1997a). This subfamily of checkpoint proteins is thought to act at the beginning of the checkpoint pathway, by recognizing changes in DNA structure and initiating the signal transduction cascade (Lydall and Weinert, 1995, 1996). The *RAD17*, *RAD24* and *MEC3* genes belong to the same epistasis group, while *RAD9* is in a group on its own (Lydall and Weinert, 1995; Longhese *et al.*, 1996a). We have recently identified the *DDC1* gene (Longhese *et al.*, 1997), which belongs to the *RAD24* epistasis group and whose gene product shows some amino acid sequence homology with *S.pombe rad9* checkpoint gene product, as well as with its human homologue (Murray *et al.*, 1991; Al-Khodairy and Carr, 1992; Lieberman *et al.*, 1992, 1996). Ddc1p, which is required

for all known DNA damage checkpoints, probably acts together with Mec3p, Rad17p and Rad24p at an early step of the DNA damage recognition process. Furthermore, *DDC1* overexpression partially suppresses both DNA damage sensitivity and checkpoint defects of *mec3*Δ cells, suggesting that Ddc1p may act in a subsequent step with respect to Mec3p (Longhese *et al*., 1997).

Once DNA perturbations are sensed, the checkpoint signals are propagated through the two protein kinases Mec1p and Rad53p which, being involved in both the DNA damage and the DNA replication checkpoints, play a central role in the checkpoint signal transduction cascade (Allen *et al*., 1994; Weinert *et al*., 1994; Paulovich and Hartwell, 1995; Siede *et al*., 1996). Rad53p is a protein kinase, which becomes phosphorylated in response to DNA damage, and this modification has been shown to be dependent on Rad9p, Mec3p, Polε and Mec1p (Zheng *et al*., 1993; Navas *et al*., 1996; Sanchez *et al*., 1996; Sun *et al*., 1996). Mec1p is a member of the large lipid kinase motif family, which includes *S.pombe* Rad3p (Bentley *et al.*, 1996; Cimprich *et al.*, 1996; for review, see Carr, 1997), as well as mammalian Ataxia-Telangiectasia Mutated (ATM) gene (Savitsky *et al.*, 1995), ATR (AT and rad-related; Bentley *et al.*, 1996) and the catalytic subunit of DNA-dependent protein kinase (DNA-PK; Jeggo *et al.*, 1995). It has been shown that DNA-PK can bind by itself to DNA and this activates it for phosphorylation of protein targets (Yaneva *et al*., 1997).

Although multiple and different impairments to DNA metabolism merge into common pathways, these signal transduction cascades may cause cell-cycle delay by using different effectors depending on the cell-cycle stage at which the damage occurs. For example, the RAD53p-dependent inhibition of *CLN1-2* transcription has been proposed to be one of the mechanisms required for delaying bud emergence and entry into S phase after DNA damage in G_1 (Sidorova and Breeden, 1997). Moreover, DNA replication proteins themselves could be effectors of the checkpoint pathways operating specifically during S phase, since 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 (Longhese *et al.*, 1996b; Marini *et al.*, 1997). Finally, the anaphase inhibitor Pds1p, whose inactivation is necessary for sister chromatid separation (Cohen-Fix *et al.*, 1996; Yamamoto *et al.*, 1996), is a component of the DNA damage checkpoint specifically acting in G_2 (Yamamoto *et al.*, 1996).

The current model of the checkpoint pathways in budding yeast predicts that Mec1p acts upstream of Rad53p, since it is required for Rad53p DNA damage-induced phosphorylation. Moreover, the observation that Mec1p is required for both DNA damage and S phase checkpoint controls suggests that Mec1p might act downstream of Rad9p and the Rad24p group of proteins. How Rad9p, Ddc1p, Rad17p, Rad24p and Mec3p connect to Mec1p and how the function of these proteins is positioned in the signal transduction pathway is not known.

Ddc1p is phosphorylated both during an unperturbed S phase and in response to DNA damage, and this phosphorylation is at least partially dependent on Mec3p (Longhese *et al.*, 1997). In order to gain insights into the organization of the genetic pathway controlling the DNA damage response, we have been studying the dependence of Ddc1p phosphorylation on different checkpoint proteins. Since the overexpression of *DDC1* partially suppressed the checkpoint defect of *mec3*Δ cells, we have also been looking for interactions between the *DDC1* and *MEC3* gene products. We show that Mec1p is essential for periodic phosphorylation of Ddc1p under unperturbed conditions, as well as for DNA damage-induced Ddc1p hyperphosphorylation. This last modification is at least partially dependent on the Rad17p and Rad24p checkpoint proteins, while it does not require Rad9p or Rad53p. Moreover, Ddc1p physically interacts with Mec3p, and Rad17p is required for this interaction.

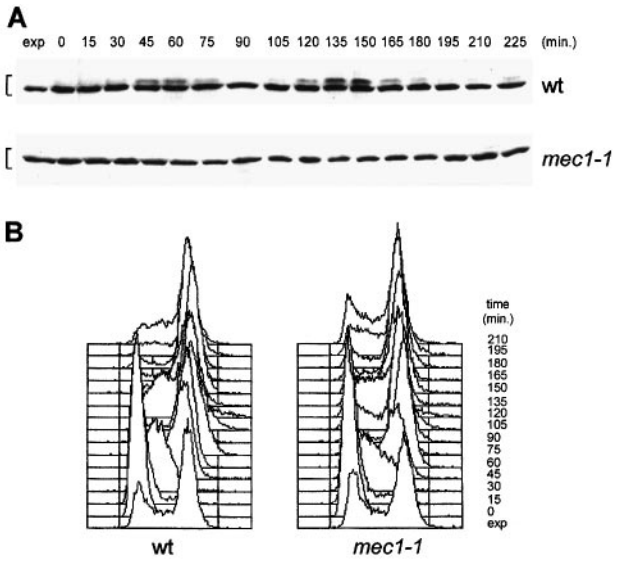

Fig. 1. Periodic modification of Ddc1p during an unperturbed cell cycle depends on Mec1p. Exponentially growing (exp) wild-type (YLL334) and *mec1-1* (DMP2541/8A) cells, expressing Ddc1p–HA2 from the *DDC1* promoter, were synchronized with α-factor and released at time zero. (**A**) Western blot analysis with 12CA5 antibody of protein extracts prepared at the indicated times after release from α-factor. Protein bands corresponding to Ddc1p are indicated by brackets. (**B**) FACS analysis of the synchronized wild-type and *mec1-1* cultures at the indicated times after α-factor release.

Results

Ddc1p phosphorylation during unperturbed S phase depends on Mec1p

A phosphorylated form of Ddc1p appears periodically during a normal cell cycle, reaching its maximum level when most cells are in S phase and decreasing concomitantly with nuclear division (Longhese *et al.*, 1997) (Figure 1). Our previous studies showed that this cell-cycle-dependent phosphorylation requires Mec3p (Longhese *et al.*, 1997), suggesting that checkpoint pathways could be also activated during a normal cell cycle. This could imply that single-stranded DNA or other replication intermediates, which normally arise during DNA synthesis, may be signals for the checkpoint pathways (Garvik *et al.*, 1995). Since Mec1p is a central regulator of checkpoint responses, we tested whether Mec1p is required for Ddc1p phosphorylation during unperturbed S phase, by measuring the extent of Ddc1p

phosphorylation in the *mec1-1* mutant (Weinert *et al.*, 1994; see Materials and methods). As judged by electrophoretic mobility changes (Figure 1), Ddc1p phosphorylation was completely abolished in undamaged, synchronously dividing *mec1-1* mutant cells, demonstrating that Mec1p is required for Ddc1p phosphorylation during unperturbed S phase.

Mec1p is required for Ddc1p phosphorylation in response to DNA damage

Ddc1p becomes hyperphosphorylated in response to DNA damage and this phosphorylation is partially dependent on Mec3p (Longhese *et al.*, 1997), suggesting a direct correlation between Ddc1p modification and the activation of the DNA damage checkpoints. If Ddc1p phosphorylation is required for response to DNA damage, it should be possible to identify checkpoint proteins other than Mec3p that are required for this modification. To this end, since Mec1p is required for Ddc1p phosphorylation during unperturbed S phase, we first measured the extent of Ddc1p phosphorylation after UV irradiation in *mec1* mutants. We tested the previously characterized *mec1-1* allele (Weinert *et al.*, 1994) and a new checkpoint-defective *mec1* allele, which we call *mec1-14*, identified during a screening for mutations causing synthetic lethality with a mutation affecting DNA primase, and previously named *pip1-14* (Longhese *et al.*, 1996a, 1997). As shown in Figure 2, phosphorylated forms of Ddc1p in G_1 UV-treated wild-type cells appeared immediately after α-factor release (see also Longhese *et al.*, 1997), and this response was dependent on Mec1p. In fact, as judged by changes in electrophoretic mobility, Ddc1p was not phosphorylated in *mec1-1* cells, and both the amount of phosphorylated Ddc1p and the extent of phosphorylation were dramatically reduced in *mec1-14* cells after UV irradiation in G_1 (Figure 2A). The differences in the behaviour of the two *mec1* mutants correlate with differences in their DNA damage checkpoint defects. In fact, after UV irradiation in G_1, both entry into S phase and budding kinetics were much faster in *mec1-1* than *mec1-14* cells (Figure 2B and C), indicating that the *mec1-14* mutant is only partially defective both in delaying cell-cycle progression in response to DNA damage and in promoting Ddc1p phosphorylation. Under unperturbed conditions, budding kinetics (Figure 2C) and FACS profiles (not shown) of wild-type and the *mec1* strains were indistinguishable one from another.

In order to prove that differences in Ddc1p phosphorylation between wild-type and UV-treated *mec1* mutant cells were not due to different kinetics of cell-cycle progression, we analysed Ddc1p DNA damage-induced phosphorylation in G_2-arrested cells, which were maintained in G_2 after UV irradiation (Figure 3A). Similarly to what was observed after UV irradiation in G_1, Ddc1p phosphorylation in response to DNA damage in G_2 was also dependent on Mec1p. In fact, when cells were kept in nocodazole for 1 h after UV irradiation in G_2, Ddc1p was unmodified in *mec1-1* cells and a very small amount of partially modified Ddc1p was present in *mec1-14* cells (Figure 3A), while a consistent amount of hyperphosphorylated Ddc1p was already detectable in wild-type cells 30 min after UV treatment of G_2-blocked cells (Figure 3A).

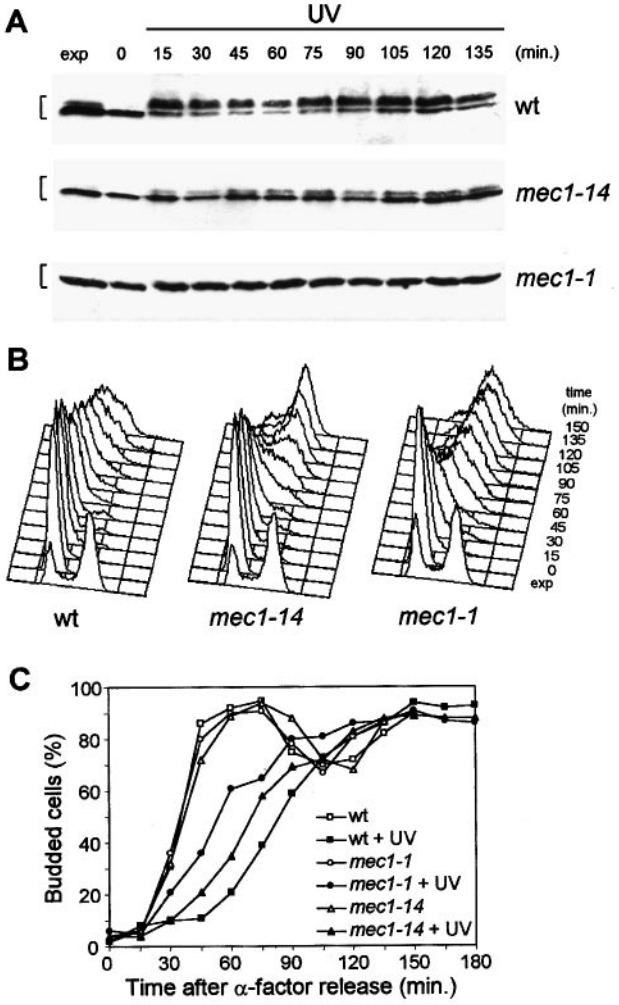

Fig. 2. Ddc1p phosphorylation in response to DNA damage in G_1 is completely dependent on Mec1p. Cultures of wild-type (YLL334), *mec1-1* (DMP2541/8A) and *mec1-14* (DMP2145/31D) cells, expressing Ddc1p–HA2 from the *DDC1* promoter, were synchronized with α-factor and UV-irradiated (40 J/m^2) just before release from the α-factor block. Cells were collected at the indicated times after α-factor release. Time zero corresponds to cell samples withdrawn immediately before UV irradiation and release from α-factor. (**A**) Western blot analysis with 12CA5 antibody of protein extracts prepared from exponentially growing (exp) and from UV-irradiated cells. Ddc1p is indicated by brackets. (**B**) FACS analysis of the irradiated cultures. (**C**) Percentage of budded cells of unirradiated and irradiated cultures.

UV-induced Ddc1p phosphorylation partially depends on Rad17p, Rad24p and Mec3p, but not on Rad9p and Rad53p

DNA damage-induced phosphorylation of Ddc1p was greatly reduced in *rad17*Δ and *rad24*Δ single mutants (Figure 3B), as it was previously reported for *mec3*Δ cells (Longhese *et al*., 1997). The small amount of partially phosphorylated Ddc1p detectable in these mutants after UV irradiation in G_2 did not depend on the activity of the remaining Rad24 group of proteins, since Ddc1p phosphorylation in UV-treated *rad17*Δ *rad24*Δ *mec3*Δ triple mutant cells was indistinguishable from that found in any single mutant (Figure 3B). As shown in Figure 3C, Rad9p was not required for Ddc1p hyperphosphorylation in response to DNA damage in G_2. Rad9p had no role in the residual Ddc1p phosphorylation in *mec3*Δ cells, since

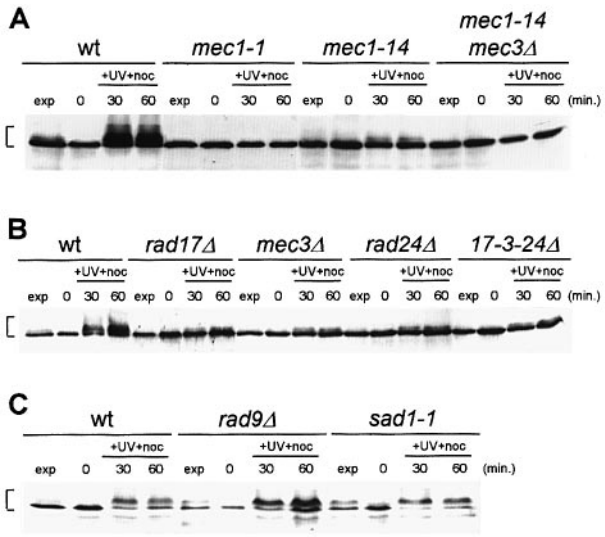

Fig. 3. Phosphorylation of Ddc1p after UV treatment in G_2 depends on Mec1p, Mec3p, Rad17p and Rad24p, but not on Rad9p and Rad53p. Cell cultures, expressing Ddc1p–HA2 from the *DDC1* promoter, were arrested with nocodazole, UV-irradiated (50 J/m^2) and resuspended into YPD medium containing 15 μg/ml nocodazole (+UV+noc). In all panels the Ddc1p–HA2 protein was visualized with 12CA5 antibody on Western blots of protein extracts prepared at the indicated times. Ddc1p is indicated by brackets. Time zero corresponds to cell samples taken immediately before UV irradiation. (**A**) Western blot analysis of protein extracts from wild-type (YLL334), *mec1-1* (DMP2541/8A), *mec1-14* (DMP2145/31D) and *mec1-14 mec3*Δ (DMP2145/16C) cells. (**B**) Western blot analysis of protein extracts from wild-type (YLL334), *rad17*Δ (DMP2141/1A), *mec3*Δ (YLL335), *rad24*Δ (DMP2149/1D) and *rad17*Δ *mec3*Δ *rad24*Δ (DMP2161/25B) cells. (**C**) Western blot analysis of protein extracts from wild-type (YLL334), *rad9*Δ (DMP2137/3A) and *sad1-1* (*rad53*) (DMP2163/2A) cells.

Ddc1p was phosphorylated at the same level in UV-irradiated *mec3*Δ and in *mec3*Δ *rad9*Δ double mutant cells (data not shown). Furthermore, Ddc1p phosphorylation after UV irradiation in G_2 of cells carrying the *sad1-1* allele of the *RAD53* gene was indistinguishable from that observed in wild-type cells under the same conditions (Figure 3C), indicating that this modification does not require Rad53p.

Rad53p and Pds1p phosphorylation show different requirements for DDC1

DNA damage induced by UV or γ radiations leads to phosphorylation not only of Rad53p, but also of Pds1p, which is proposed to be a downstream target of the DNA damage checkpoint pathway blocking the metaphase/anaphase transition (Cohen-Fix and Koshland, 1997). As shown in Figure 4A, a functional *DDC1* gene product is absolutely required to promote Rad53p phosphorylation in response to DNA damage, since we failed to detect any DNA damage-induced phosphorylation of Rad53p in *ddc1*Δ cells. Phosphorylation of Pds1p in response to DNA damage was previously shown to be completely dependent on Mec1p and Rad9p (Cohen-Fix and Koshland, 1997). As shown in Figure 4B, Pds1p was phosphorylated in *ddc1*Δ cells after UV irradiation in G_2, as judged by mobility shift, although some subtle changes in Pds1p electrophoretic mobility could not be excluded in *ddc1*Δ cells compared with wild-type. As expected, Pds1p was unmodified in *rad9*Δ cells under the same conditions

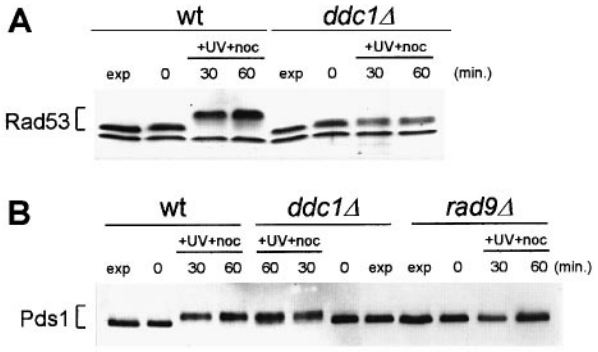

Fig. 4. Phosphorylation of Rad53p and Pds1p in the absence of *DDC1*. Cell cultures were arrested with nocodazole, UV-irradiated (50 J/m^2) and resuspended into YPD medium containing 15 μg/ml nocodazole (+UV+noc). Time zero corresponds to cell samples taken immediately before UV irradiation. (**A**) Western blot analysis with anti-Rad53p polyclonal antibodies of protein extracts from wild-type (K699) and *ddc1*Δ (YLL244) cells. (**B**) Western blot analysis with anti-MYC (9E10) monoclonal antibodies of protein extracts from wild-type (DMP2625/2D), *ddc1*Δ (DMP2625/7D) and *rad9*Δ (DMP2625/7C) cells.

(Figure 4B). Therefore, Ddc1p appears to play a minor role, if any, in UV-induced Pds1p phosphorylation. This modification did not depend on the presence of the other Rad24 group of proteins, since the level of phosphorylated Pds1p in a *rad17*Δ *mec3*Δ *rad24*Δ *ddc1*Δ quadruple mutant after UV irradiation in G_2 was indistinguishable from that found in the *ddc1*Δ single mutant strain under the same conditions (data not shown).

The MEC3-independent Ddc1p phosphorylation in response to DNA damage in G_1 requires S phase entry

The partial DNA damage-induced phosphorylation of Ddc1p observed in *mec3*Δ cells (Longhese *et al*., 1997) (Figure 3B) required Mec1p function. In fact, Ddc1p was completely unmodified in UV-treated *mec3*Δ *mec1-14* double mutants, although the *mec1-14* single mutant displayed residual Ddc1p phosphorylation under the same conditions (Figure 3A). We observed previously that the residual modification of Ddc1p in *mec3*Δ cells in response to DNA damage in G_1 (*MEC3*-independent Ddc1p phosphorylation) was delayed compared with the wild-type, becoming detectable concomitantly with S phase entry (Longhese *et al.*, 1997). It is possible that, in the absence of Mec3p—and thus of a fully functional checkpoint—DNA replication of a damaged template leads to formation of secondary lesions (single- or double-strand breaks). These might in turn partially activate Mec1p. If this were the case, we would expect to abolish Ddc1p modification by preventing *mec3*Δ cells from entering S phase after UV treatment in G_1. To explore this possibility, we used a strain carrying a temperature-sensitive allele of the *CDC4* gene, which is required for initiation of S phase by promoting proteolysis of the specific inhibitor of cyclin B-dependent kinases p40^{SIC1} (Schowb *et al.*, 1994).

As shown in Figure 5, the residual DNA damage-induced phosphorylation of Ddc1p in *mec3*Δ mutant cells depended on passage through S phase, since it was completely abolished in a *cdc4 mec3*Δ double mutant shifted to non-permissive temperature after UV treatment.

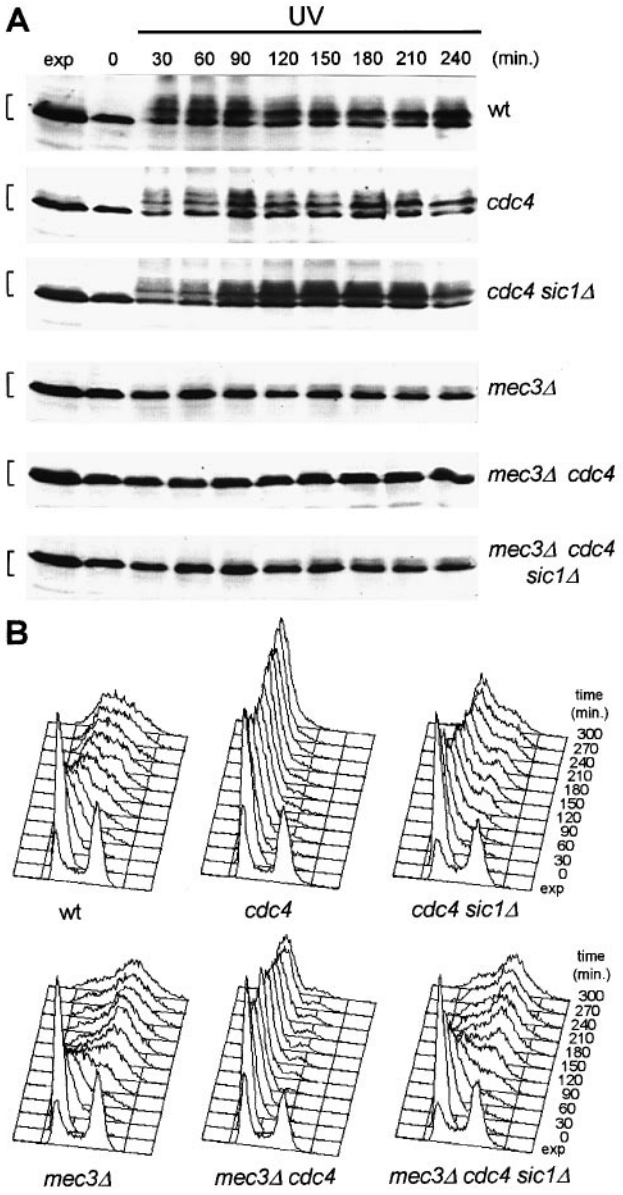

Fig. 5. Entry into S phase is required for *MEC3*-independent Ddc1p partial phosphorylation in response to DNA damage. Cultures of wild-type (YLL334), *cdc4-1* (DMP2371/5A), *cdc4-1 sic1*Δ (DMP2371/9C), *mec3*Δ (YLL335), *mec3*Δ *cdc4-1* (DMP2571/6B) and *mec3*Δ *cdc4-1 sic1*Δ (DMP2571/5B) cells, expressing Ddc1p–HA2 from the *DDC1* promoter, were synchronized with α-factor at 25°C and UV-irradiated (40 J/m^2) just before release from the α-factor block at 37°C. Samples were taken at the indicated times after α-factor release. Time zero corresponds to cell samples withdrawn immediately before UV irradiation and release from α-factor. (**A**) Western blot analysis with 12CA5 antibody of protein extracts prepared from exponentially growing (exp) and from UV-irradiated cells. Ddc1p is indicated by brackets. (**B**) FACS analysis of the irradiated cultures.

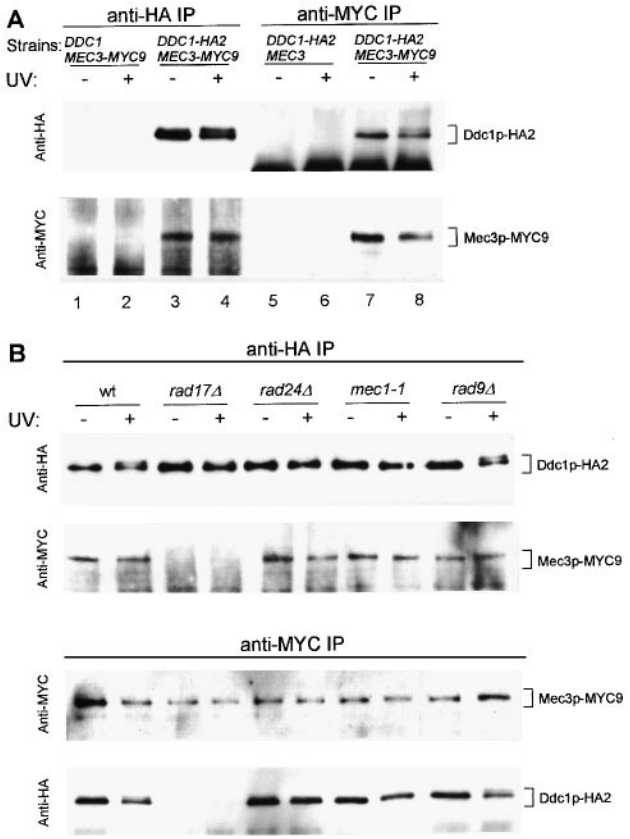

Fig. 6. Ddc1p–Mec3p physical interaction. (**A**) Extracts were prepared from exponentially growing untreated (–) or UV-treated (+) (50 J/m^2) cells expressing Mec3p–MYC9 (YLL352, lanes 1, 2) or Ddc1p–HA2 (YLL334, lanes 5, 6) or both (YLL354, lanes 3, 4, 7, 8), as indicated in the top part of the panel, and subjected to immunoprecipitation with anti-HA (12CA5) (anti-HA IP, lanes 1–4) or anti-MYC (9E10) (anti-MYC IP, lanes 5–8) antibodies. Ddc1p and Mec3p were then detected by Western blot analysis of the immunoprecipitates probed with the antibodies indicated on the left side of the panel. (**B**) Western blot analysis of the immunoprecipitates prepared with anti-HA or anti-MYC antibodies of protein extracts from untreated (–) or UV-treated (+) *rad17*Δ (DMP2612/5B), *rad24*Δ (DMP2612/5A), *mec1-1* (DMP2609/7C) and *rad9*Δ (DMP2613/13C) cells, expressing both Ddc1p–HA2 and Mec3p–MYC9 tagged proteins.

Consistent with this hypothesis, inactivation of Sic1p, which allowed *cdc4 mec3*Δ cells to replicate DNA at restrictive temperature, also restored the ability of the same cells to phosphorylate partially Ddc1p after DNA damage in G_1 (Figure 5A and B). As expected, in UV-treated *MEC3* cells, where the checkpoint response was fully functional, phosphorylated Ddc1p was detectable immediately after UV irradiation and α-factor release at restrictive temperature in both *CDC4* and *cdc4* mutant cells, although the last were unable to enter into S phase (Figure 5A and B).

Ddc1p and Mec3p form a stable complex

Previous genetic data suggested a direct link between Ddc1p and Mec3p (Longhese *et al.*, 1997). To investigate this point further, we generated a strain concomitantly expressing fully functional Ddc1p–HA2- and Mec3p–MYC9-tagged proteins from their own promoters. Western blots on crude protein extracts from this strain showed that the HA-tag and MYC-tag antibodies specifically recognized Ddc1p–HA2 and Mec3p–MYC9, respectively (data not shown). These antibodies were then used to immunoprecipitate independently Ddc1p–HA2 and Mec3p–MYC9 from unirradiated and UV-irradiated cells. A protein species with the same electrophoretic mobility as Mec3p–MYC9 was recognized by the anti-MYC antibody in Ddc1p–HA2 immunoprecipitates (Figure 6A, lanes 3 and 4). The reciprocal experiment showed that Mec3p–MYC9 immunoprecipitates contained Ddc1p–HA2, as revealed by immunoblotting using anti-HA anti-

body (Figure 6A, lanes 7 and 8). This indicates an *in vivo* physical interaction between Ddc1p and Mec3p. This interaction does not depend on DNA damage, nor does it change in its presence, since Ddc1p and Mec3p can be co-immunoprecipitated at the same levels both in untreated and in UV-treated cells. The observed Mec3p–Ddc1p interaction was specific, since we failed to detect Mec3p–MYC9 in anti-HA immunoprecipitates from cell extracts lacking the Ddc1p–HA2 protein (Figure 6A, lanes 1 and 2) or to detect Ddc1p–HA2 in anti-MYC immunoprecipitates from cell extracts lacking the Mec3p–MYC9 protein (Figure 6A, lanes 5 and 6). We found that both phosphorylated and unphosphorylated Ddc1p forms were associated with Mec3p, suggesting that Ddc1p phosphorylation is not required for the maintenance of this complex.

Rad17 is required for Ddc1p–Mec3p complex formation

Based on the genetic interactions between Ddc1p and the other components of the DNA damage checkpoint pathway and on the above-described phosphorylation dependencies, we asked whether other checkpoint proteins were required for Ddc1p–Mec3p physical interaction. As shown in Figure 6B, Mec3p–MYC9 failed to co-immunoprecipitate with Ddc1p–HA2 in both unirradiated and UV-irradiated *rad17*Δ cell extracts treated with anti-HA antibody. In the reciprocal experiment, Ddc1p–HA2 did not co-precipitate with Mec3p–MYC9 when *rad17*Δ cell extracts were treated with anti-MYC antibody. Conversely, Ddc1p–Mec3p interaction was not affected in *rad24*Δ, *mec1-1* or *rad9*Δ cells. Since the levels of Mec3p–MYC9 or Ddc1p–HA2 were the same in all the checkpoint mutants, these results indicate that, among the analysed checkpoint proteins, only Rad17p is necessary for a stable Ddc1p–Mec3p interaction. The finding that the Ddc1p–Mec3p complex is present in *mec1-1* cells, which are completely defective in Ddc1p phosphorylation, further confirms the observation that Ddc1p phosphorylation is not needed for Ddc1p–Mec3p interaction.

Discussion

DNA is prone to chemical and structural alterations, which may arise as a consequence of errors during replication and repair or be induced by genotoxic agents. Living cells respond to DNA damage either by restoring the correct DNA structure through DNA repair mechanisms or by tolerating the damage. The DNA damage tolerance mechanisms allow cells to deal with the consequences of unrepaired lesions in their chromosomes, frequently at the cost of decreased genomic stability, since translesion DNA synthesis seems to be the major cause of UV-induced mutagenesis (Walker, 1995). In eukaryotic cells, the balance between these mechanisms is probably modulated through surveillance mechanisms that delay cell-cycle progression when DNA is damaged, providing additional time for repair.

The *S.cerevisiae* checkpoint proteins Rad9p, Rad24p, Rad17p and Mec3p are thought to monitor and possibly process DNA damage, thus generating a signal that, through the sequential action of the Mec1p and Rad53p transmitters, causes a delay in cell-cycle progression (for review, see Elledge, 1996). The relationships between Mec1p and the other checkpoint proteins have not been tested and no direct biochemical data about the function and regulation of the *RAD9*, *RAD17*, *RAD24* and *MEC3* gene products are available. The *DDC1* gene is a recently discovered component of the *RAD24* epistasis group, which also includes *RAD17* and *MEC3*. Ddc1p is the only protein of this group that has been shown to be phosphorylated periodically in a normal cell cycle and to be hyperphosphorylated in response to DNA damage (Longhese *et al.*, 1997). This allowed us to use Ddc1p modification as a means to better define the order of functions in the DNA damage response pathway. To this end, we tested several checkpoint mutants for differences in the phosphorylation pattern of Ddc1p, by following changes in its electrophoretic mobility.

Ddc1p phosphorylation depends on a complex interplay between Mec1p and the Rad24 group of proteins

Ddc1p is a phosphoprotein that becomes hyperphosphorylated in response to DNA damage. This Ddc1p modification correlates with the activation of DNA damage checkpoint pathways. In fact, phosphorylation of Ddc1p occurs immediately after DNA damage and, based on electrophoretic mobility, it appears to be totally dependent on Mec1p and, at least partially, depends on the Rad24 group of proteins. Rad53p does not seem to be required for Ddc1p modification, while its own phosphorylation in response to DNA damage is dependent on Ddc1p, indicating that Ddc1p as well as Rad17p, Rad24p Mec3p, Rad9p and Mec1p acts upstream of Rad53p. Our observation that Rad9p is not required for Ddc1p phosphorylation, together with the previous epistasis data (Lydall and Weinert, 1995; Longhese *et al.*, 1997), support the hypothesis that Rad9p probably participates in aspects of DNA damage sensing, processing or signalling which are different from those involving the Rad24 group of proteins.

As previously mentioned, current models of the DNA damage checkpoint pathway place Mec1p upstream of Rad53p, but downstream of Rad9p and the Rad24 group of proteins. Our observation that Mec1p is absolutely required for Ddc1p phosphorylation in response to DNA damage strongly indicates that Mec1p might participate with Ddc1p and possibly with the checkpoint proteins Rad17p, Rad24p and Mec3p in sensing and signalling altered DNA structures. Whether the Mec1p-dependent phosphorylation of Ddc1p contributes to modulate Mec1p function and/or to determine its effector specificity has not yet been clarified.

In the absence of either one or all of the other Rad24 group of proteins, Ddc1p modification in response to DNA damage is dramatically reduced, although some residual Ddc1p phosphorylation is still observed and requires a functional *MEC1*. These data may suggest that Mec1p might still be partially active in the absence of Rad17p, Rad24p and Mec3p or that the Rad24 group of proteins may be required downstream of Mec1p for proper Ddc1p phosphorylation. The residual Mec1p-dependent phosphorylation of Ddc1p observed in *rad17*Δ *rad24*Δ *mec3*Δ cells after UV irradiation in G_1 does not immediately follow DNA damage, but can be detected only when cells enter S phase. After UV treatment in G_1, *rad17*Δ *rad24*Δ *mec3*Δ cells progress through S phase with damaged DNA

molecules. We propose that these unprocessed DNA lesions cannot activate Mec1p by themselves, while DNA replication of a damaged template could spontaneously generate some checkpoint signals (single- or double-strand breaks) (Michel *et al*., 1997). These might in turn partially activate Mec1p, either directly or through other DNA damage sensors, therefore allowing some Ddc1p phosphorylation.

We showed previously that Ddc1p undergoes Mec3p-dependent phosphorylation during an unperturbed S phase and becomes dephosphorylated concomitantly with nuclear division (Longhese *et al*., 1997). Here, we show that Mec1p is also required for this S phase-dependent Ddc1p phosphorylation. This finding further supports the hypothesis that the DNA replication process by itself could generate signals that activate the checkpoint response. Single-stranded DNA regions, which are proposed to be checkpoint signals, are likely to be produced by the action of Rad24p, Rad17p and Mec3p in processing damaged DNA (Garvik *et al*., 1995; Lydall and Weinert, 1995). Analogously, ssDNA regions, produced spontaneously during DNA replication, might be sensed and induce Ddc1p phosphorylation via the checkpoint pathway. This would imply that an intrinsic checkpoint signal is present during an unperturbed S phase, where the checkpoint response might be in a pre-activated state since replication intermediates arising during DNA synthesis are continuously processed and correctly resolved when S phase is unperturbed.

Physical interactions in the RAD24 epistasis group

Epistasis analysis and genetic interactions suggest that the *DDC1* gene product acts together with Mec3p, Rad17p and Rad24p (Longhese *et al*., 1997), but no evidence for physical interactions among the proteins of the *RAD24* epistasis group have been provided until now. Here, we show that Ddc1p is associated with Mec3p *in vivo* independently of DNA damage and that Rad17p is needed for a stable interaction. Whether Rad17p and possibly Rad24p contribute to the formation of a larger complex has not been directly tested, but the essential role of Rad17p in Mec3p–Ddc1p complex formation/stabilization indicates that this protein might directly interact with Mec3p and/or Ddc1p. The observation that Mec3p–Ddc1p interaction is detectable in the absence of Rad24p does not exclude the possibility that Rad24p may be part of the same complex.

Physical interactions between checkpoint proteins have been recently observed in *S.pombe*, where Rad1p forms a stable complex with Hus1p and this interaction depends on Rad9p (Kostrub *et al*., 1998). Interestingly, *S.pombe* Rad1p and Rad9p share different degrees of homology with *S.cerevisiae* Rad17p and Ddc1p, respectively (Lydall and Weinert, 1995; Longhese *et al*., 1997). This finding provides further evidence that the molecular bases of the checkpoint mechanisms have been conserved throughout evolution, although different organisms have adapted them in different ways. The functional consequences of these interactions are still obscure, and further work will be required to clarify them.

Since Ddc1p and Mec3p respond to DNA alterations induced by different DNA-damaging agents and cause cell-cycle arrest at different stages, the Ddc1p–Mec3p complex is likely to be capable of sensing common DNA structure intermediates, which are generated by the processing of different types of DNA damage. The observation that the Ddc1p–Mec3p complex is present in undamaged cells suggests that these proteins might also act together during an unperturbed cell cycle. In this respect, the Ddc1p–Mec3p complex might be involved in the constant monitoring of the DNA structure and in sensing possible DNA alterations. This ‘guardian’ role may represent an important mechanism in living cells, whereby the integrity of the genome could be constantly sensed.

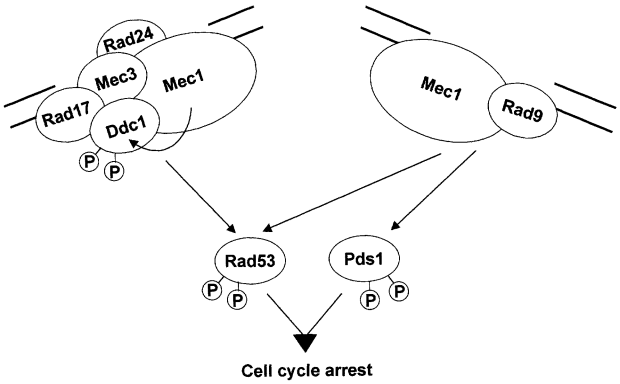

Fig. 7. A model for the DNA damage signal transduction pathway. DNA-damaged molecules are represented by single-stranded DNA. The proximity of Mec1p with the Rad24 group of proteins or with Rad9p does not imply direct interaction between the proteins, and the model does not imply that Ddc1p, Rad53p and Pds1p are direct substrates of Mec1p. See text for details.

A working model for the DNA damage checkpoints

It has been suggested previously that Rad24p, Rad17p and Mec3p act at the beginning of the checkpoint pathways. The finding that Ddc1p interacts physically with Mec3p and that Rad17p is required for this interaction strongly indicates that Ddc1p acts together with Mec3p, Rad17p and possibly Rad24p. Moreover, the observation that Mec1p is absolutely required for Ddc1p phosphorylation indicates that Mec1p could be involved together with the Rad24 group of proteins at an early step of the DNA damage recognition process, and suggests that the checkpoint response may be more complex than a simple linear pathway. Given its structural similarity with DNA-PK, Mec1p might be able to interact with specific DNA or protein–DNA structures. The recruitment of Mec1p into a catalytically active complex and the choice of downstream effectors might then be modulated by its interaction with regulatory subunits, whose function is in turn influenced by their phosphorylation state.

In the model depicted in Figure 7, Rad9p and the Rad24 group of proteins play different roles in determining Mec1p activity and/or substrate specificity, possibly sensing different DNA lesions or protein–DNA structures in different chromosome regions. We have in fact shown that Ddc1p phosphorylation does not require the *RAD9* gene product, although it requires a functional *MEC1* gene. Moreover, *RAD9* and *DDC1* appear to have different roles in promoting phosphorylation of the metaphase/anaphase inhibitor Pds1p and, possibly, in activating other

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	*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100*	K.Nasmyth
K700	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100*	K.Nasmyth
YLL244	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 ddc1Δ::KanMX4*	Longhese *et al*. (1997)
YLL262/2C	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 ddc1Δ::KanMX4*	Longhese *et al*. (1997)
YLL334	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1*	Longhese *et al*. (1997)
YLL335	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 mec3Δ::TRP1*	Longhese *et al*. (1997)
YLL352	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 MYC9-MEC3::TRP1::mec3*	this study
YLL354	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 MYC9-MEC3::TRP1::mec3 HA2-DDC1::LEU2::ddc1*	this study
Y301	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 sad1-1*	Allen *et al*. (1994)
DMP1497/2B	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 sad1-1*	this study
TWY308	*MATα ura3 trp1 mec1-1 sml1*	Weinert *et al*. (1994)
DMP2394/18B	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 mec1-1 sml1*	this study
DMP2541/8A	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 mec1-1 sml1*	this study
pip1-14	*MATα ade2-1 ade3 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 pri1Δ::HIS3* (pML9 *ADE3URA3 PRI1*) (pLAN33 *TRP1 pri1-2*) *pip1-14*	Longhese *et al*. (1996a)
DMP2145/31D	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 mec1-14*	this study
DMP2145/16C	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 mec3Δ::TRP1 mec1-14*	this study
DLY282	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 mec3ΔG::URA3 rad17Δ::LEU2 rad9Δ::HIS3 rad24Δ::TRP1*	D.Lydall
DMP1913/11D	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad17Δ::LEU2*	Longhese *et al*. (1997)
DMP1913/15B	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad24Δ::TRP1*	Longhese *et al*. (1997)
DMP2141/1A	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 rad17Δ::LEU2*	this study
DMP2149/1D	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 rad24Δ::TRP1*	this study
DMP2149/7A	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 mec3Δ::TRP1 rad24Δ::TRP1*	this study
DMP2161/25B	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 rad17Δ::LEU2 rad24Δ::TRP1 mec3Δ::TRP1*	this study
YLL157	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad9Δ::URA3*	Longhese *et al*. (1996a)
DMP2137/2A	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 rad9Δ::URA3*	this study
DMP2137/3A	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 rad9Δ::URA3*	this study
DMP2163/2A	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 sad1-1*	this study
SP527	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 cdc4-1 sic1Δ::HIS3*	S.Piatti
DMP2371/5A	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 cdc4-1*	this study
DMP2371/9C	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 cdc4-1 sic1Δ::HIS3*	this study
DMP2571/6B	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 cdc4-1 mec3Δ::TRP1*	this study
DMP2571/5B	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 cdc4-1 mec3Δ::TRP1 sic1Δ::HIS3*	this study
DMP2612/5A	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 MYC9-MEC3::TRP1::mec3 HA2-DDC1::LEU2::ddc1 rad24Δ::TRP1*	this study
DMP2161/14B	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA2-DDC1::LEU2::ddc1 rad24Δ::TRP1 rad17Δ::LEU2*	this study
DMP2612/5B	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 MYC9-MEC3::TRP1::mec3 HA2-DDC1::LEU2::ddc1 rad17Δ::LEU2*	this study
DMP2613/13C	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 MYC9-MEC3::TRP1::mec3 HA2-DDC1::LEU2::ddc1 rad9Δ::URA3*	this study
DMP2609/7C	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 MYC9-MEC3::TRP1::mec3 HA2-DDC1::LEU2::ddc1 mec1-1 sml1*	this study
K6445	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 MYC18-PDS1::LEU2::pds1*	Shirayama *et al*. (1998)
DMP2155/4B	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 MYC18-PDS1::LEU2::pds1 ddc1Δ::KanMX4*	this study
DMP2625/2D	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 MYC18-PDS1::LEU2::pds1*	this study
DMP2625/7C	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 MYC18-PDS1::LEU2::pds1 rad9Δ::URA3*	this study
DMP2625/7D	*MAT**a** ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 MYC18-PDS1::LEU2::pds1ddc1Δ::KanMX4*	this study

[a] Plasmids are indicated within brackets.

targets. While Rad9p seems to be required specifically for Mec1p-dependent Pds1p modification, Ddc1p does not appear to play any major role in the pathway leading to UV-induced Pds1p phosphorylation. On the other hand, the *MEC1*-dependent signal transduction pathway leading to Rad53p phosphorylation in response to DNA damage requires both Rad9p and the Rad24 group of proteins (Navas *et al.*, 1996; Sanchez *et al.*, 1996; Sun *et al.*, 1996; this work), suggesting that all these proteins are essential for full Mec1p activity within this pathway. In this respect, Mec1p should be able to integrate incoming signals by interacting with different and specific subunits, thus

defining which effectors will be activated depending on the nature of the DNA lesion and on the cell-cycle stage at which the genotoxic insults occur.

Materials and methods

Plasmids

To construct plasmid pML128, carrying a *MYC9-MEC3* allele, a *MEC3* fragment spanning from position –851 to +1 from the translational initiation codon was amplified by PCR using plasmid pML46 (Longhese *et al.*, 1997) as a template and oligonucleotides PRP57 (5′-CGG GGT ACC GCG GCC GCT CAT TTA ACT GAA TTA AGA CAC ACC-3′) and PRP58 (5′-CGA TCA ATT ATA GCC GGT AGT AAC AG-3′) as primers, while the whole *MEC3* ORF was amplified using oligonucleotides PRP59 (5′-CGG GGT ACC GCG GCC GCA AAT TAA AAT TGA TAG TAA ATG GTT-3′) and PRP61 (5′-CGG GGG CCC CGG GAT CCA GAA AAG CAG TTA GTA TGT AAA GCA-3′) as primers and pML46 as a template. Both the amplification products were then cloned into the *Sac*I–*Pst*I sites within the Yiplac211 polylinker region (Gietz and Sugino, 1988), giving rise to plasmid pML126, where a *Not*I site was present at the translational initiation codon of the *MEC3* coding sequence. A *Not*I DNA fragment containing nine copies of the MYC epitope-coding sequence was cloned into the *Not*I site of the pML126 plasmid, giving rise to plasmid pML127. The fragment *Xmn*I–*Eco*RI from plasmid pML127 was then cloned into the *Sma*I–*Eco*RI sites within the Yiplac211 polylinker region, giving rise to plasmid pML128. All the PCR reactions were carried out using Pfu DNA polymerase (Stratagene).

Yeast strains and media

The genotypes of all the yeast strains used in this study are listed in Table I. All yeast strains were in W303 background. Strains Y301 and TWY308, kindly provided respectively by S.Elledge and T.Weinert, were backcrossed four times with strains K700 and K699 to generate the meiotic segregants DMP1497/2B and DMP2394/18B, carrying respectively the *sad1-1* and *mec1-1* alleles in W303 background. As previously reported, the *mec1-1* allele was lethal in the A364a background and its essential function, but not the checkpoint defect, was shown to be bypassed by a second site suppressor (called *sml1*) which did not cause *per se* any sensitivity to DNA-damaging agents and did not show any checkpoint defects (Paulovich *et al.*, 1997a). Every time we crossed strain TWY308 or its *mec1-1* derivatives with W303 derivative strains, we obtained a preponderance of tetrads showing 3:1 segregation for viability, indicating that *mec1-1* was lethal also in W303 background and that all the viable *mec1-1* segregants carried the *sml1* allele. All the *mec1-1 sml1* segregants in W303 background were as sensitive to methyl methane sulfonate hydroxyurea and UV as the TWY308 parental strain. Furthermore, Ddc1p phosphorylation was not affected in *sml1 MEC1* strain, while it was impaired to the same extent in *mec1-14* single mutant and in *mec1-14 sml1* double mutant strains (data not shown). Therefore, the presence of *sml1* mutation did not influence Ddc1p phosphorylation in any background.

Strains YLL352 and YLL354, carrying the *MYC9-MEC3* allele at the *MEC3* chromosomal locus, were obtained by transforming, respectively, strains K699 and YLL334 with *Nru*I-digested plasmid pML128. The *MYC9-MEC3* allele was fully functional, since strains K699 and YLL352 were indistinguishable one from another. Strain DMP2541/8A was a meiotic segregant from a cross between strains YLL334 and DMP2394/18B, and strains DMP2145/31D and DMP2145/16C were derived from a cross between strains YLL335 and pip1-14. Strain DLY282, kindly provided by D.Lydall, was backcrossed with strain K700 to generate strains DMP1913/11D and DMP1913/15B. Strain DMP2141/1A was a meiotic segregants from a cross between strains DMP1913/11D and YLL334, strains DMP2149/1D and DMP2149/7A were meiotic segregants from a cross between strains YLL335 and DMP1913/15B, and strains DMP2161/14B and DMP2161/25B were derived from a cross between strains DMP2141/1A and DMP2149/7A. Strains DMP2137/2A and DMP2137/3A were meiotic segregants from a cross between strains DMP2149/7A and YLL157, while strain DMP2163/2A was a meiotic segregant from a cross between strains DMP1497/2B and YLL334. Strains DMP2371/5A, DMP2371/9C, DMP2571/6B and DMP2571/5B were meiotic segregants from a cross between strains YLL335 and SP527. Strains DMP2612/5A and DMP2612/5B were derived from a cross between strains YLL354 and DMP2161/14B. Strains DMP2613/13C and DMP2609/7C originated from a cross between strains DMP2137/2A and YLL354, and between strains YLL354 and DMP2394/18B, respectively. Strains DMP2625/2D, DMP2625/7C and DMP2625/7D are meiotic segregants from a cross between strains YLL157 and DMP2155/4B, that was obtained from a cross between strains YLL262/2C and K6445, kindly provided by M.Shirayama. In all the meiotic segregants, the presence of the appropriate null alleles has been assessed both by the presence of the disruption markers and by allelism tests.

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).

Synchronization 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. Yeast cells were synchronized in G_2 by treating exponentially growing YPD cell cultures with 5 μg/ml of nocodazole in 1% dimethylsulfoxide (DMSO). α-factor- and nocodazole-arrested cells were collected by centrifugation, and 2.5×10^8 cells were spread on 14 cm diameter YPD plates, followed by UV irradiation with 40 and 50 J/m^2, respectively. When required, cell cultures were held in G_2 after UV irradiation by resuspension in YPD medium containing 15 μg/ml nocodazole.

Protein extracts and Western blot analysis

Protein extracts for Western blot analysis were prepared from trichloroacetic acid (TCA) -treated yeast cells. Briefly, 10 ml of exponentially growing cells were collected by centrifugation, washed with 2 ml of 20% TCA and resuspended in 50 μl of 20% TCA. Samples were then vortexed with glass beads and centrifuged at 3000 r.p.m. for 10 min. The pellets were then resuspended in 100 μl of SDS-gel loading buffer, boiled for 3 min, followed by centrifugation at 3000 r.p.m.. Protein extracts were resolved by electrophoresis on a 12.5% SDS–polyacrylamide gel and proteins were transferred to nitrocellulose membranes, which were then incubated for 2 h with anti-HA monoclonal antibody 12CA5 (1:5000 dilution in Tris-buffered saline with 0.2% Triton X-100 and 4% non-fat milk), or anti-MYC monoclonal antibody 9E10 (1:300 dilution in Tris-buffered saline with 0.2% Triton X-100 and 4% non-fat milk), followed by incubation with peroxidase-labelled anti-mouse antibody (Amersham). Rad53p was visualized using anti-Rad53 polyclonal antibodies provided by D.Stern and peroxidase-labelled anti-rabbit antibodies (Amersham).

Immunoprecipitation experiments

Protein extracts for immunoprecipitation were prepared from exponentially growing cells collected by centrifugation and resuspended in an equal volume (w/v) of buffer containing 50 mM HEPES pH 7.5, 500 mM NaCl, 20% glycerol, 1 mM sodium orthovanadate, 60 mM β-glycerophosphate and protease inhibitor cocktail (Boehringer Mannheim). After addition of 1:1 volume of acid-washed glass beads and breakage, 300 μl of clarified protein extracts were incubated for 2 h at 4°C with 30 μl of a 50% (v/v) Protein A–Sepharose, covalently linked to 12CA5 monoclonal antibodies or with 9E10 Agarose-conjugates monoclonal antibodies (Santacruz Biotechnology), supplemented with 800 μl of phosphate buffer containing 500 mM NaCl. The resins were then washed twice with 1 ml of phosphate buffer containing 500 mM NaCl and resuspended in 30 μl of SDS-gel loading buffer. Bound proteins were visualized by Western blotting with 12CA5 or 9E10 monoclonal antibodies after electrophoresis on a 10% SDS–polyacrylamide gel.

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