Cell cycle progression in the presence of irreparable DNA damage is controlled by a Mec1- and Rad53-dependent checkpoint in budding yeast

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We studied the response of nucleotide excision repair (NER)-defective *rad14Δ* cells to UV irradiation in G₁ followed by release into the cell cycle. Only a subset of checkpoint proteins appears to mediate cell cycle arrest and regulate the timely activation of replication origins in the presence of unrepaired UV-induced lesions. In fact, Mec1 and Rad53, but not Rad9 and the Rad24 group of checkpoint proteins, are required to delay cell cycle progression in *rad14Δ* cells after UV damage in G₁. Consistently, Mec1-dependent Rad53 phosphorylation after UV irradiation takes place in *rad14Δ* cells also in the absence of Rad9, Rad17, Rad24, Mec3 and Ddc1, and correlates with entry into S phase. Two-dimensional gel analysis indicates that late replication origins are not fired in *rad14Δ* cells UV-irradiated in G₁ and released into the cell cycle, which instead initiate DNA replication from early origins and accumulate replication and recombination intermediates. Progression through S phase of UV-treated NER-deficient *mec1* and *rad53* mutants correlates with late origin firing, suggesting that unregulated DNA replication in the presence of irreparable UV-induced lesions might result from a failure to prevent initiation at late origins.

Keywords: budding yeast/checkpoints/DNA damage/
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Introduction

Maintaining the integrity of genetic information is fundamental for the life of cells and the survival of species. DNA damage, whether it results from spontaneous hydrolytic events, radiation or chemical mutagens, must be recognized and repaired efficiently. Unrepaired DNA or inaccurate repair of DNA damage can lead to genomic instability, mutations and ultimately cancer (for a review see Hartwell and Kastan, 1994). Since DNA is constantly suffering damage, eukaryotic cells respond to DNA insults by restoring the correct DNA structure through several repair systems and by delaying cell cycle progression through surveillance mechanisms known as checkpoints. The balanced and concerted action of DNA repair and checkpoint mechanisms prevents accumulation of mutations and ensures genetic integrity (for reviews see Carr and Hoekstra, 1995; Paulovich *et al.*, 1997b; Longhese *et al.*, 1998; Weinert, 1998).

The cell cycle can be transiently arrested at different stages, depending on the phase at which DNA alterations occur. In budding yeast, at least three DNA damage checkpoints have been identified, which inhibit the G₁–S transition (Siede *et al.*, 1993, 1994), slow down progression through S phase (Paulovich and Hartwell, 1995) and delay entry into mitosis (Weinert and Hartwell, 1988, 1993; Weinert *et al.*, 1994), when DNA is damaged during G₁, S or G₂ phases, respectively. Due to the vast array of DNA insults, multiple sensors may be required to recognize specific DNA perturbations at different cell cycle stages. Proteins encoded by the *RAD9* gene and by the genes of the *RAD24* epistasis group, including *RAD17*, *RAD24*, *MEC3* and *DDC1*, are required specifically for proper DNA damage response, and are proposed to act at an early step of DNA damage recognition at any stage of the cell cycle (Weinert and Hartwell, 1988; Siede *et al.*, 1993; Weinert *et al.*, 1994; Longhese *et al.*, 1996a, 1997; Paulovich *et al.*, 1997a; Paciotti *et al.*, 1998). The finding that Ddc1, Mec3 and Rad17 interact physically with each other provides the evidence that these putative sensor proteins, inferred from genetic studies to operate in one pathway, do indeed interact biochemically (Paciotti *et al.*, 1998; Kondo *et al.*, 1999). Conversely, the DNA replication proteins Pole, Dpb11 and Rfc5 appear to sense both replication blocks and DNA damage specifically during DNA synthesis (Araki *et al.*, 1995; Navas *et al.*, 1995, 1996; Sugimoto *et al.*, 1996, 1997). Once DNA perturbations are sensed, the signalling process involves a protein phosphorylation cascade propagated through the two protein kinases Mec1 and Rad53 (Allen *et al.*, 1994; Weinert *et al.*, 1994; Paulovich and Hartwell, 1995). Mec1 is a member of a large lipid kinase family, which includes *Schizosaccharomyces pombe* Rad3 (Bentley *et al.*, 1996; Cimprich *et al.*, 1996; for review see Carr, 1997), as well as mammalian ATM (ataxia-telangiectasia mutated; 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). After DNA damage, the Mec1 protein kinase, whose activity might be regulated by sensor proteins, is required to phosphorylate and activate several substrates, including the protein kinase Rad53 (Zheng *et al.*, 1993; Sanchez *et al.*, 1996; Sun *et al.*, 1996). Since both Rad9 and Ddc1 are phosphorylated (directly or indirectly) by Mec1, these proteins are suggested to modulate the recruitment of Mec1 into a catalytically active complex and its specificity towards downstream effectors (Emili, 1998; Paciotti *et al.*, 1998; Sun *et al.*, 1998; Vialard *et al.*, 1998). Rad53 and Mec1 are necessary for phosphorylation of target proteins in response to checkpoint activation. For example, the Rad53-dependent inhibition of *CLN1-2* transcription through phosphorylation of the transcriptional regulator Swi6 has been proposed to account for delaying G₁–S transition

after DNA damage in G₁ (Sidorova and Breeden, 1997). Moreover, Mec1, but not Rad53, is required to phosphorylate the anaphase inhibitor Pds1, which is a component of the DNA damage checkpoint acting specifically in G₂ (Yamamoto *et al.*, 1996; Cohen-Fix and Koshland, 1997). Finally, components of the DNA replication apparatus could be effectors of the DNA damage checkpoint operating specifically during DNA synthesis. Indeed, mutations affecting the large subunit of RP-A and DNA primase impair the cellular response to DNA alterations during S phase (Longhese *et al.*, 1996b; Marini *et al.*, 1997). The reduced rate of ongoing DNA synthesis in the presence of DNA damage may reflect controls at the level of origin initiation and/or a decrease in the rate of elongation of nascent strands. Recent studies indicate that both treatment with hydroxyurea, which blocks the progression of DNA replication, and chronically damaging DNA molecules during S phase with methyl-methane sulfonate (MMS) selectively inhibit the firing of late replication origins in a *RAD53*-dependent manner (Santocanale and Diffley, 1998; Shirahige *et al.*, 1998).

We have limited knowledge about the signals that elicit DNA damage checkpoints. Indeed, the DNA damage checkpoint activation probably requires the recognition of particular DNA structures or alterations in the genome at any stage of the cell cycle. For example, genotoxic agents cause many types of primary damage that can be converted to secondary lesions during DNA replication. In fact, a replication fork encountering a bulk in the template may stop, and resuming replication downstream of the damage might generate a daughter strand gap. Moreover, DNA replication across a single-strand nick is likely to cause replication fork collapse and to produce double-strand breaks (Kuzminov, 1995). Therefore, primary DNA lesions by themselves, secondary structures generated during DNA replication and/or the actual process of repair could be sensed as checkpoint triggering signals. In order to study the influence of DNA damage processing on checkpoint activation, we analysed the response of NER-defective cells to DNA damage in G₁ followed by release into the cell cycle. *Saccharomyces cerevisiae* cells lacking the *RAD14* gene product, which encodes the structural and functional homologue of human XPA, are defective in NER. Irradiation of *rad14Δ* cells arrested in G₁ by α -factor treatment with low doses of UV results in the introduction of irreparable covalent changes in the genome and slows down cell cycle progression after release from α -factor arrest (Siede *et al.*, 1994). The cell cycle delay caused by UV damage in G₁, which is *RAD9* dependent in a NER-proficient background, was shown to be *RAD9* independent in NER-defective cells (Siede *et al.*, 1994). This suggests that NER-processed UV photoproducts, but not unexcised dimers, might be able to activate the *RAD9*-dependent checkpoint response. In this view, the inhibition of cell cycle progression observed after release from α -factor block of NER mutants UV-irradiated in G₁ could be due to physical interference of unrepaired DNA damage with enzymes involved in DNA metabolism. Alternatively, checkpoint genes other than *RAD9* might be able to recognize lesions and slow down cell cycle progression in the absence of DNA repair.

Here we provide evidence that a DNA damage checkpoint mechanism acts during DNA replication of irrepar-

ably UV-damaged templates and we try to address some of the several unanswered questions about the molecular basis of this control. We show that *RAD53* and *MEC1* mediate DNA damage-induced cell cycle arrest of *rad14Δ* mutants, whereas neither *RAD9* nor the *RAD24* group of genes are required for this arrest. The finding that Mec1-dependent Rad53 phosphorylation in response to UV irradiation takes place in *rad14Δ* cells, in either the presence or the absence of Rad9, Rad17, Rad24, Mec3 and Ddc1, further supports the notion that a Mec1- and Rad53-dependent checkpoint is active in the absence of a functional excision repair pathway. In addition, we show by two-dimensional gel analysis that *rad14Δ* cells, after UV irradiation in G₁ and release into the cell cycle, do not immediately arrest cell cycle progression but initiate DNA replication from early origins and, in the attempt to replicate damaged DNA templates, accumulate replication and recombination intermediates. Conversely, initiation of DNA replication from late origins is inhibited in a *MEC1*- and *RAD53*-dependent manner, suggesting that the firing of late origins in *mec1* and *rad53* mutants might allow DNA replication in the presence of irreparable UV-induced lesions.

Results

***RAD53* and *MEC1*, but not *RAD9* and the *RAD24* group of genes, are required for DNA damage-induced cell cycle arrest in *rad14Δ* mutants**

Saccharomyces cerevisiae cells missing the *RAD14* gene product, which is essential for NER, after UV treatment in G₁ and release into the cell cycle show slow cell cycle progression compared with wild type (Siede *et al.*, 1994). It has been shown previously that in contrast to excision repair-proficient cells, the UV-induced delay of cell cycle progression in *rad14Δ* mutants takes place also in the absence of Rad9 (Siede *et al.*, 1994). This cell cycle delay might be due to physical interference of unrepaired photoproducts with replication or, alternatively, might be controlled by checkpoint genes other than *RAD9*. To determine whether the slowing of cell cycle progression in *rad14Δ* mutants was an actively regulated process, we introduced mutant or null alleles of several checkpoint genes in the *rad14Δ* background and analysed cell cycle progression after UV irradiation in G₁ followed by release into the cell cycle. ρ^0 strains were derived from each double mutants to avoid mitochondrial DNA contribution to cell DNA content measured by flow cytometry. These strains were synchronized in G₁ by α -factor treatment, UV-irradiated with 5 J/m² and released from the pheromone block. This low UV dose was sufficient to induce an apparent G₁ or early S phase arrest in the *rad14Δ* mutant as determined by FACS analysis (Figure 1), whereas it was not able to delay bud emergence in this strain (data not shown). Under the same conditions, cell cycle progression of the wild-type strain was not significantly affected compared with the unirradiated control. As shown in Figure 1, both the *rad14Δ rad9Δ* and *rad14Δ ddc1Δ* double mutants showed a delay of cell cycle progression after UV irradiation in G₁ and release into the cell cycle which was indistinguishable from that observed for the *rad14Δ* single mutant. Similar results

Fig. 1. FACS analysis of *rad14Δ* strains defective in different checkpoint genes after UV irradiation in G_1 . Exponentially growing wild-type (K699p^o), *rad14Δ* (YHN156), *rad14Δ rad9Δ* (YHN160), *rad14Δ ddc1Δ* (YHN159), *rad14Δ rad9Δ ddc1Δ* (YHN161), *rad14Δ mec1-1* (YHN169) and *rad14Δ rad53* (YHN158) cells were synchronized in G_1 phase with α -factor, UV-irradiated with 5 J/m² and released from the pheromone block. Aliquots were taken at the indicated times and analysed by FACS. Time zero corresponds to cell samples withdrawn immediately after UV irradiation and release from α -factor. Plating efficiency was measured by removing two independent samples from each culture to determine cell number and to score for c.f.u. on YEPD plates at 25°C. Plating efficiencies (% $\pm$ SD) were as follows: wt (-UV), 78.3 $\pm$ 6.1; wt (+UV), 76.3 $\pm$ 5.5; *rad14Δ*, 2.08 $\pm$ 0.03; *rad14Δ rad9Δ*, 1.11 $\pm$ 0.48; *rad14Δ ddc1Δ*, 0.23 $\pm$ 0.01; *rad14Δ rad9Δ ddc1Δ*, 0.11 $\pm$ 0.01; *rad14Δ mec1-1*, 0.12 $\pm$ 0.03; *rad14Δ rad53*, 0.10 $\pm$ 0.02.

were obtained when the same analysis was performed for *rad14Δ rad17Δ*, *rad14Δ rad24Δ* and *rad14Δ mec3Δ* double mutants (data not shown). Therefore, not only *RAD9*, but also the genes of the *RAD24* epistasis group were dispensable for UV-induced cell cycle arrest in the *rad14Δ* mutant. Since *RAD9* and the *RAD24* group of genes are believed to act in different branches of the DNA damage checkpoint response (Lydall and Weinert, 1995; de la Torre-Ruiz *et al.*, 1998; Paciotti *et al.*, 1998), we further analysed whether the *rad14Δ rad9Δ ddc1Δ* triple mutant was still able to delay cell cycle progression in response to UV irradiation in G_1 (Figure 1). The behaviour of the *rad14Δ rad9Δ ddc1Δ* triple mutant was the same as that of the *rad14Δ* single mutant, thus excluding the possibility that the *RAD9* gene could compensate for the lack of the *RAD24* group of genes, and vice versa, in the absence of NER. In contrast, as shown in Figure 1, the *rad14Δ mec1-1* and *rad14Δ rad53* double mutants entered S phase between 30 and 60 min after UV irradiation and replicated most if not all of their DNA during the subsequent 60–90 min. Therefore, UV-induced cell cycle arrest in the absence of NER is controlled by a mechanism depending on the *MEC1* and *RAD53* genes, whereas neither *RAD9* nor the *RAD24* group of genes appear to

have a role in slowing down cell cycle progression in the presence of irreparable lesions. These data indicate that *Mec1*, which is thought to act upstream of *Rad53*, might become activated even in the absence of *Rad9* and the *Rad24* group of proteins. In this view, in NER-deficient strains, either the high concentration of unrepaired UV photoproducts or the secondary lesions arising during replication of damaged templates might spontaneously generate checkpoint signals that in turn directly or indirectly activate *Mec1*.

Rad53 is phosphorylated in rad14Δ mutants in response to UV damage in G_1 followed by release into the cell cycle

Rad53 is phosphorylated in response to DNA damage and its phosphorylation correlates with the activation of DNA damage checkpoints (Sanchez *et al.*, 1996; Sun *et al.*, 1996). Since the UV-induced cell cycle arrest in *rad14Δ* mutants is dependent on *RAD53* and *MEC1*, we would expect UV damage in G_1 to cause *Rad53* phosphorylation in both wild-type and *rad14Δ* cells. To explore this possibility, exponentially growing wild-type and *rad14Δ* cells were synchronized in G_1 by α -factor treatment, UV-irradiated and released from the pheromone arrest. As

Fig. 2. UV-induced phosphorylation of Rad53 in the absence of *RAD14*. Exponentially growing wild-type (YLL334) and *rad14Δ* (DMP2539/3D) cells were synchronized in G₁ phase with α -factor, UV-irradiated with 5 J/m² and released from the pheromone block at time zero. Plating efficiencies (% $\pm$ SD) were as follows: wt, 78.1 $\pm$ 5.4; *rad14Δ*, 3.02 $\pm$ 0.04. Aliquots were taken from exponentially growing cells (exp), G₁-synchronized cells (α F) and at the indicated times (min) after release from α -factor. Time zero corresponds to samples withdrawn immediately after UV irradiation and release from α -factor. (A) FACS analysis of wild-type and *rad14Δ* cultures. (B) The top part of the panel shows the percentage of bud emergence (circles) and nuclei division (triangles) of wild-type (closed symbols) and *rad14Δ* (open symbols) cultures at the indicated times after α -factor release. The bottom part of the panel shows Western blot analysis with anti-Rad53 polyclonal antibodies of protein extracts from the samples withdrawn at the times indicated. Rad53 is indicated by square parentheses.

shown in Figure 2A and B (top), low doses of UV irradiation caused *rad14Δ* cells to arrest with an approximately 1C DNA content, but did not consistently delay bud emergence compared with wild-type cells under the same conditions. Phosphorylated Rad53 was detectable immediately after release from α -factor in both wild-type and *rad14Δ* UV-irradiated cell cultures (Figure 2B, bottom), further supporting the notion that a Rad53-dependent checkpoint pathway is operating in excision repair-deficient cells. The persistence of Rad53 phosphorylated forms reflected differences in DNA damage-induced cell cycle delay in wild-type and *rad14Δ* cells. In fact, 135 min after UV irradiation and release from the α -factor block, the amount of Rad53 phosphorylated forms in wild-type cells progressively decreased concomitantly with nuclear division (Figure 2B). Conversely, in *rad14Δ* cells that arrested cell cycle progression after UV treatment in G₁, the phosphorylated forms of Rad53 persisted until the end of the experiment (210 min; Figure 2B).

Fig. 3. UV-induced phosphorylation of Rad53 in checkpoint mutants in the absence of *RAD14*. Exponentially growing cell cultures were synchronized in G₁ phase with α -factor, UV-irradiated with 5 J/m² and released from the pheromone block. Aliquots were taken from exponentially growing cells (exp), G₁-synchronized cells (α F) and at the indicated times (min) after UV irradiation and protein extracts were analysed by Western blot with anti-Rad53 antibodies. Rad53 is indicated by square parentheses. (A) Wild type (YLL334), *rad14Δ* (DMP2539/3D), *rad9Δ* (DMP2137/3A), *rad14Δ rad9Δ* (DHN133/5C), *ddc1Δ* (YLL244), *rad14Δ ddc1Δ* (DMP2057/10A), *rad9Δ ddc1Δ* (DHN200/11D), *rad14Δ rad9Δ ddc1Δ* (DHN179/1C), *rad17Δ* (DMP2141/1A), *rad14Δ rad17Δ* (DHN205/2C), *rad24Δ* (DMP2149/1D), *rad14Δ rad24Δ* (DHN206/1A), *mec3Δ* (YLL335) and *rad14Δ mec3Δ* (DHN207/2D). (B) *rad14Δ mec1-1* (DHN198/6D) and *rad14Δ rad9Δ ddc1Δ mec1-1* (DHN201/8A).

Irreparable DNA lesions promote Rad53 phosphorylation in different checkpoint mutants

Rad53 phosphorylation has been shown to be dependent on Mec1, Rad9, Rad17, Rad24, Mec3 and Ddc1 (Zheng *et al.*, 1993; Navas *et al.*, 1996; Sanchez *et al.*, 1996; Sun *et al.*, 1996; Paciotti *et al.*, 1998; Figure 3). If checkpoint signals could activate Mec1 in NER-deficient mutants even in the absence of *RAD9* and the *RAD24* group of genes, we would expect deletion of *RAD14* to be able to promote UV-induced phosphorylation of Rad53 in cells

lacking any of the above checkpoint proteins but Mec1. We therefore analysed Rad53 phosphorylation in protein extracts from different *rad14Δ* checkpoint mutants after synchronization in G₁ by α -factor, UV irradiation and release from the pheromone arrest. As shown in Figure 3A, deletion of *RAD14* gene promotes phosphorylation of Rad53 in strains lacking the above listed proteins. In fact, Rad53 became phosphorylated after UV damage in G₁ in *rad9Δ rad14Δ*, *ddc1Δ rad14Δ*, *mec3Δ rad14Δ*, *rad24Δ rad14Δ*, *rad17Δ rad14Δ* and *ddc1Δ rad9Δ rad14Δ* cells. As expected, Rad53 phosphorylation was instead dramatically reduced in *rad9Δ* and, to an even greater extent, in *ddc1Δ*, *rad17Δ*, *mec3Δ* and *rad24Δ* single mutants, and it was completely eliminated in the *rad9Δ ddc1Δ* double mutant after UV irradiation in G₁ (Figure 3A). Since Mec1 is necessary together with Rad53 to arrest cell cycle progression in *rad14Δ* cells, the UV-induced Rad53 phosphorylation in *rad14Δ* cells was expected to depend on Mec1. Indeed, we failed to detect any DNA damage-induced phosphorylation of Rad53 in *rad14Δ mec1-1* double mutants as well as in *rad14Δ rad9Δ ddc1Δ mec1-1* quadruple mutants after UV irradiation in G₁ (Figure 3B). Since Rad53 phosphorylation correlates with checkpoint activation, the above data are consistent with the activation of a *RAD53*- and *MEC1*-dependent DNA damage checkpoint response in NER-deficient background despite the lack of *RAD9* and the *RAD24* group of genes.

Early origin firing is not inhibited in *rad14Δ* mutants released into the cell cycle after UV damage in G₁

Since the delay of cell cycle progression in the presence of irreparable covalent changes in the DNA molecules is mediated through the activation of a *MEC1*- and *RAD53*-dependent checkpoint response, the accumulation of un-repaired pyrimidine dimers and/or secondary DNA lesions generated during the attempt to replicate a damaged template might activate the checkpoint pathway. To explore these possibilities further, we decided to determine whether *rad14Δ* mutant cells, incapable of dimer excision, immediately arrested the cell cycle or were able to initiate replication of a damaged template after UV irradiation in G₁ followed by release into the cell cycle. Since FACS analysis was not sensitive enough to distinguish whether cell cycle progression of UV-treated *rad14Δ* was blocked in G₁ or in early S phase, we analysed the firing of the early-replicating *ARS305* origin by 2-D gel electrophoresis (Friedman and Brewer, 1995). To this end, *rad14Δ* cells were synchronized in G₁ phase by α -factor treatment and one half of the culture was released from the pheromone block, whereas the other half was irradiated prior to release. After release into the cell cycle, samples from unirradiated or UV-irradiated cells were collected at different times and pooled together (see legend to Figure 4 and Materials and methods). Digested chromosomal DNA was separated by 2-D gel electrophoresis and hybridized to an *ARS305* probe (see Materials and methods). As shown in Figure 4, both UV-irradiated and unirradiated *rad14Δ* cells showed hybridization signals for the bubble arc, indicating that DNA replication started from the *ARS305* origin independently of DNA damage. Therefore, the presence of irreparable UV photoproducts in G₁ does not prevent the initiation of DNA replication from an

Fig. 4. 2-D gel analysis of the early *ARS305* replication origin in *rad14Δ* cells after UV damage in G₁. *rad14Δ* (DMP2539/3D) and *rad14Δ rad52Δ* (DHN219/8C) cells were synchronized in G₁ by α -factor treatment, and half of each culture was UV irradiated before release. Samples were collected from 10 to 50 min for unirradiated (–UV) and from 10 to 100 min for irradiated (+UV) cells after release from the pheromone block and pooled. The same amounts of *EcoRV*-digested chromosomal DNA prepared from the different pools were separated by 2-D agarose gel electrophoresis, transferred to membrane and hybridized to an *ARS305*-specific probe. The scheme in the top right depicts a typical migration pattern of a restriction fragment analysed by 2-D gel electrophoresis (Friedman and Brewer, 1995). The upper and the lower arc signals represent, respectively, bubble intermediates generated from bi-directional replication starting from the analysed origin, and a series of Y-shaped replication forks. The spike signal represents a series of fully replicated X-shaped DNA molecules with 2*N* molecular weight. The directions of the first and the second dimension gel electrophoresis are indicated.

early origin of replication. The signals for the Y-arc, normally generated when the analysed origin is also passively replicated by forks initiated from origins outside, was reproducibly increased in the irradiated *rad14Δ* cells compared with the unirradiated culture (Figure 4). This accumulation of simple Y-DNA intermediates might arise from the blockage of replication fork progression at the unexcised UV photoproducts. Moreover, a strong spike signal was detected specifically in the *rad14Δ* irradiated culture, indicating that fully replicated but connected X-DNA molecules were being accumulated during the attempt to replicate UV-damaged DNA template (Figure 4). These UV-induced X-shaped molecules might represent recombination intermediates. In fact, it is thought that newly synthesized DNA following UV irradiation in NER-defective cells contains gaps opposite dimers, which might be cured by sister chromatid recombination (Lehmann, 1972; Wolff *et al.*, 1974, 1977; Clark and Hanawalt, 1984; Berger and Edenberg, 1986; Kadyk and Hartwell, 1992, 1993; Paulovich *et al.*, 1998). To investigate this possibility further, we analysed whether the *RAD52* gene, which encodes a central recombination protein (for review see Petes *et al.*, 1991), was required to generate the X-shaped

molecules in *rad14Δ* cells. To this end, *rad14Δ rad52Δ* double mutants were synchronized in G₁ with α-factor, UV-irradiated before release from the pheromone block, and 2-D gel analysis was performed as described above. As shown in Figure 4, hybridization signals for both bubble and Y-arcs were detectable with the *ARS305* probe in the absence of Rad52, whereas the spike representing X-shaped DNA molecules was absent. Therefore, the DNA damage-induced X-shaped molecules accumulated in *rad14Δ* cells are likely recombination intermediates whose formation is dependent on Rad52.

Mec1 and Rad53, but neither Rad9 nor Ddc1, mediate inhibition of late origin firing in rad14Δ mutants after UV irradiation in G₁

FACS analysis data showed that the absence of Rad53 and Mec1 allows *rad14Δ* cells to replicate DNA in the presence of UV-induced lesions, suggesting that these checkpoint proteins might regulate the initiation and/or the elongation step of DNA replication of UV-damaged templates. As shown in Figure 5, 2-D gel analysis did not show significant differences for the early origin *ARS305* between the *rad14Δ* single, the *rad14Δ rad53*, *rad14Δ mec1-1* double and the *rad14Δ ddc1Δ rad9Δ* triple mutants after UV irradiation in G₁ followed by release into the cell cycle, thus indicating that none of the checkpoint mutations analysed affects initiation from early origins. Since Rad53 regulates the timing of replication initiation during normal cell growth and blocks initiation of replication from late origins in MMS-treated cells (Shirahige *et al.*, 1998), we investigated whether this was the case also in the presence of irreparable UV-induced dimers in the genome by analysing the activation of the efficient late-firing origin *ARS501*. We found that the presence of UV-induced damage selectively blocked initiation of DNA replication from this late origin in *rad14Δ* cells. In fact, as shown in Figure 5, 2-D gel analysis of *rad14Δ* DNA after UV irradiation in G₁ did not show any bubble arc with the *ARS501* probe, whereas this was detectable in the unirradiated *rad14Δ* cells. A similar behaviour was observed also for irradiated *ddc1Δ rad9Δ rad14Δ* triple mutants, indicating that the *DDC1* and *RAD9* genes are not required to inhibit late origin firing in the presence of UV-induced lesions (Figure 5). In contrast, 2-D gel analysis of irradiated *rad14Δ rad53* and *rad14Δ mec1-1* double mutants clearly showed the bubble arc *ARS501* hybridization signal, similar to that found for this late origin in the unirradiated cultures and for the early *ARS305* origin in both unirradiated and irradiated cells (Figure 5). Therefore, both Mec1 and Rad53 are necessary to block initiation of DNA replication from late origins in the presence of UV-induced lesions. Moreover, an increase of X- and Y-shaped DNA molecules was detected with the *ARS501* probe in irradiated *rad14Δ rad53* and *rad14Δ mec1-1* double mutants compared with the irradiated *rad14Δ* cells (Figure 5). The accumulation of such DNA molecules, similar to that detected for the early *ARS305* origin, might be induced by replication forks starting from the late *ARS501* origin in the absence of *MEC1* and *RAD53*. In this view, activation of DNA replication from the late *ARS501* origin or from origins elsewhere in the genome, which may be fired in *rad53* and *mec1* mutants, might increase the substrates for recombination events and the number of

Fig. 5. Activation of the late *ARS501* replication origin is inhibited in a Mec1- and Rad53-dependent manner in response to UV irradiation of *rad14Δ* cells. The *rad14Δ* (DMP2539/3D), *rad14Δ rad53* (DMP2538/11D), *rad14Δ mec1-1* (DHN198/6D) and *rad14Δ ddc1Δ rad9Δ* (DHN179/1C) cells were synchronized in G₁ by α-factor treatment, and half of each culture was UV irradiated before release. Samples were collected from 10 to 50 min for unirradiated (–UV) and from 10 to 100 min for irradiated (+UV) cells after release from the pheromone block and pooled. The same amounts of *EcoRV*-(*ARS305*) or *XbaI*-(*ARS501*) digested chromosomal DNA prepared from the different pools were separated by 2-D agarose gel electrophoresis, transferred to membrane and hybridized to *ARS305*- or *ARS501*-specific probes.

Fig. 6. Entry into S phase is required for Rad53 phosphorylation in *rad14Δ* cells UV-irradiated in G_1 . Exponentially growing wild-type (YLL334), *cdc4-1* (DMP2371/5A), *rad14Δ* (DMP2539/3D), *rad14Δ cdc4-1* (DHN176/10B) and *rad14Δ cdc4-1 sic1Δ* (DHN176/11C) cells were synchronized in G_1 with α -factor at 25°C, UV-irradiated (5 J/m²) and released from the α -factor block at 37°C. Aliquots were taken from exponentially growing cells (exp), G_1 -synchronized cells (α F) and at the indicated times (min) after UV irradiation, and protein extracts were analysed by Western blot with anti-Rad53 antibodies. Time zero corresponds to samples withdrawn immediately after UV irradiation and release from α -factor. Rad53 is indicated by square parentheses.

replication forks that become blocked at the UV photo-products.

Rad53 phosphorylation of *rad14Δ* cells UV-irradiated in G_1 requires entry into S phase

The observation that *rad14Δ* cells UV-irradiated in G_1 initiate DNA replication from early origins when released into the cell cycle suggested that unprocessed DNA damage in NER-defective cells might require DNA replication to be sensed and to activate the Mec1- and Rad53-dependent checkpoint response. If this were the case, we would expect to abolish Rad53 modification by preventing *rad14Δ* cells from entering S phase after UV treatment in G_1 . We therefore analysed Rad53 phosphorylation after UV irradiation in G_1 and release from α -factor at restrictive temperature in strains 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} (Schwob *et al.*, 1994). As shown in Figure 6, DNA damage-induced phosphorylation of Rad53 in *rad14Δ* cells after UV treatment in G_1 depended on entry into S phase. In fact, it was abolished in *cdc4 rad14Δ* double mutant cells released from α -factor at 37°C, and it was restored by further deletion of the *SIC1* gene (Figure 6), which allows *cdc4* cells to enter S phase at restrictive temperature (Schwob *et al.*, 1994). Conversely, Rad53 was readily phosphorylated in NER-proficient wild-type and *cdc4* mutants UV-irradiated in G_1 and released from α -factor at 37°C.

Discussion

Genotoxic insults induce many different forms of DNA damage that may undergo structural transformations and

metabolic processing during the cell cycle, thus generating secondary lesions that further challenge checkpoint and DNA repair pathways. It is still unknown whether DNA damage by itself is sufficient to activate a checkpoint response or whether DNA damage must be processed in order to induce a checkpoint-mediated cell cycle arrest. DNA intermediates and/or DNA-protein complexes, which can form during different stages of DNA repair, recombination or replication, are good candidates for structures monitored by checkpoints. For example, single-stranded DNA regions could be sensed as checkpoint signals (Garvik *et al.*, 1995) and checkpoint components may be directly involved in processing damaged DNA to its single-stranded form (Lydall and Weinert, 1995).

Some evidence suggests that DNA damage *per se* might not be sufficient for checkpoint activation and that the primary damage must be processed to yield DNA intermediates. For example, in *Escherichia coli*, the presence of UV-induced DNA lesions is not sufficient to cause SOS induction. Rather, the SOS-inducing signal is generated when cells attempt to replicate the damaged DNA, leading to the accumulation of single-stranded regions (Sassanfar and Roberts, 1990). Moreover, if NER-deficient human XPA⁻ cells are UV irradiated, an increase in p53 protein level is not detectable under conditions in which cells cannot replicate DNA, whereas it is induced if DNA replication is allowed, suggesting that the primary damage must be modified by DNA replication in order to activate the checkpoint response (Nelson and Kastan, 1994).

Genes from the different DNA repair pathways do not appear to be required to slow down DNA synthesis in the presence of DNA lesions (Paulovich *et al.*, 1997a). Conversely, mutations in NER genes cause further slowing of cell cycle progression after UV irradiation in G_1 compared with excision repair-proficient cells (Siede *et al.*, 1994). A fraction of excision repair-defective mutants can survive UV irradiation due to highly efficient mechanisms that enable them to tolerate large amounts of unrepaired damage during DNA replication. This accommodation of lesions might account for the slowing of S phase and involve mutagenic and/or recombinogenic pathways that may be regulated by checkpoint proteins.

A Mec1- and Rad53-dependent checkpoint arrests cell cycle progression of UV-damaged *rad14Δ* cells in early S phase

We found that early origins are fired in *rad14Δ* cells after UV irradiation in G_1 followed by release into the cell cycle, suggesting that the presence of unexcised UV photoproducts does not prevent cells from entering S phase. In fact, we observe replication bubbles in 2-D gels of *rad14Δ* DNA which was prepared from cells released into the cell cycle after UV irradiation in G_1 , and probed for the early *ARS305* replication origin. Altogether, our data indicate that UV-induced DNA damage in G_1 in NER-defective cells causes an early S phase arrest. We have investigated whether this arrest was due to genetically controlled mechanisms, and our analysis shows that Rad53 and Mec1 are necessary to slow down DNA synthesis in *rad14Δ* mutants after UV irradiation in G_1 . The activation of a Mec1- and Rad53-dependent checkpoint in the presence of irreparable lesions is further confirmed by the finding that Rad53 is phosphorylated in *rad14Δ* cells

released into the cell cycle after UV irradiation in G₁ and that Mec1 is absolutely required for this phosphorylation. Therefore, in the absence of NER, a DNA damage checkpoint is acting specifically during DNA synthesis and is responsible for the S phase arrest. In this view, cells lacking the possibility of processing UV photo-products might not be able to activate the checkpoint pathway until they enter S phase, suggesting that unprocessed DNA damage might require DNA replication to be sensed and to activate the *Mec1*- and *RAD53*-dependent checkpoint response. This hypothesis is supported by the finding that Rad53 phosphorylation in *rad14Δ* cells UV-irradiated in G₁ does not take place when entry into S phase is prevented by Cdc4 inactivation. Neither *RAD9* nor the *RAD24* group of genes, which are needed to slow down S phase progression in excision repair-proficient cells, seem to have any role in regulating the rate of ongoing S phase in cells that are unable to remove UV-induced DNA damage. The persistence of damage-induced Rad53 phosphorylated forms in *rad14Δ* mutants also carrying the *rad9Δ*, *rad24Δ*, *rad17Δ*, *mec3Δ* or *ddc1Δ* mutation is consistent with the hypothesis that the lack of Rad9 and the Rad24 group of proteins does not impair the activation of a Mec1- and Rad53-dependent checkpoint in the absence of NER. Therefore, Rad9 and the Rad24 group of proteins appear to be dispensable for checkpoint response when chromosomes containing irreparable UV-induced dimers experience DNA replication. Since the checkpoint-mediated S phase arrest in mutants incapable of dimer excision requires *Mec1* and *RAD53*, we propose that DNA replication of a damaged template might spontaneously generate some checkpoint signals able to activate Mec1, either directly or through other DNA damage sensors, therefore allowing Rad53 phosphorylation. Since replication proteins like DNA polymerase ϵ , Rfc5, Dpb11 and Rpa1 have been implicated in DNA damage checkpoint response specifically acting during S phase (Araki *et al.*, 1995; Navas *et al.*, 1995, 1996; Longhese *et al.*, 1996b; Sugimoto *et al.*, 1997), it is possible that once DNA replication is initiated, the DNA damage signalling system works through these replication factors in sensing and transducing signals to Mec1.

The consequences of unregulated progression into S phase in the presence of DNA damage are largely unknown. For example, DNA strand breaks, single-stranded DNA regions and/or DNA recombination intermediates might result from replicating UV-damaged DNA and might be recognized as checkpoint signals. Indeed, after UV irradiation of *rad14Δ* cells, we observe an enormous accumulation of Y- and X-shaped molecules at the *ARS305* origin-containing fragment. Y-DNA intermediates could form if replication forks initiated at the early *ARS305* origin or at origins elsewhere in the chromosome are blocked at the damaged sites. Inhibition of DNA replication by DNA damage had previously been observed in *E.coli* as well as in mammalian cells (Lerner *et al.*, 1994). When *E.coli* cells are exposed to DNA-damaging agents, DNA synthesis is transiently inhibited (Livneh *et al.*, 1993) and this inhibition is mostly due to effects of the lesions on the progress of the replication fork (Rupp and Howard-Flanders, 1968). Moreover, the capacity of lesions such as cyclobutane pyrimidine dimers to inhibit the progression of DNA polymerases is well established

in vitro (for review see Friedberg *et al.*, 1995). On the other hand, the UV-induced accumulation of X-shaped DNA molecules at the *ARS305* origin-containing fragment, which we detected as a spike signal on a 2-D genomic DNA blot, could represent Holliday junctions (Zou and Rothstein, 1997). For example, recombination intermediates could be generated when stalling of the replication fork is caused by DNA polymerase encountering a lesion and DNA synthesis is resumed downstream of the damage. This results in the formation of a daughter strand gap that could be repaired by strand exchange with the undamaged sister molecule, thus generating a Holliday junction (Friedberg *et al.*, 1995). The induction of recombination in cells incapable of excising UV-induced pyrimidine dimers might increase the ability to circumvent DNA synthesis blockage, and therefore complete DNA replication in the presence of irreparable lesions. Several data in different organisms indicate that recombination mechanisms might enable cells to replicate their DNA after UV irradiation. *Escherichia coli* mutants, incapable of excising UV-induced pyrimidine dimers, revealed a recombination mechanism involving sister strand exchange (Rupp and Howard-Flanders, 1968; Rupp *et al.*, 1971; Ganesan, 1974; Smith and Sharma, 1987). Moreover, in NER-defective mammalian cells, newly synthesized DNA following UV irradiation is thought to contain gaps opposite dimers, which are then resolved by sister chromatid recombination (Lehmann, 1972; Wolff *et al.*, 1974, 1977; Clark and Hanawalt, 1984; Berger and Edenberg, 1986). Finally, when NER yeast mutants encounter DNA damage, they experience the induction of replication-dependent sister chromatid exchange, which requires Rad52 (Kadyk and Hartwell, 1993; Paulovich *et al.*, 1998). The finding that the formation of these molecules is dependent upon the function of the recombination protein Rad52 in UV-irradiated *rad14Δ* cells provides evidence that the observed X-DNA forms are recombination intermediates, thus suggesting that UV-induced lesions are channelled into the recombination repair pathway during DNA replication of a damaged template.

Role of Mec1 and Rad53 in regulating DNA synthesis in the presence of irreparable lesions

The replication of damaged DNA represents a major mechanism of genetic instability since the rate of DNA synthesis might determine the frequency by which DNA lesions are converted to permanent changes (for a review see Naegeli, 1994). The checkpoint control decreases the rate of ongoing DNA replication, avoiding the consequences of replicating a damaged template (Paulovich and Hartwell, 1995). Since cells entering S phase in the presence of DNA damage show an increased dependence on checkpoint function for survival, the slowing down of DNA replication probably allows cells to survive DNA damage better either by removing the damage more efficiently or by tolerating it better than would be possible during an unrestrained S phase. The regulated decrease in the rate of DNA replication on a damaged template could be due to a control of initiation and/or elongation of DNA replication, and/or to induction of alternative modes of replication slower than normal DNA synthesis and possibly dependent on recombinogenic mechanisms. One question

is how the lack of *MEC1* and *RAD53* genes allows *rad14Δ* cells to succeed in replicating their chromosomes in the presence of irreparable damage. Although DNA lesions are thought to induce inhibition of replicon initiation (Larner *et al.*, 1994) and chain elongation (Sarasin and Hanawalt, 1980), several data suggest that cells have the ability to replicate damaged chromosomes. Different mechanisms that allow DNA synthesis on templates containing blocking lesions have been hypothesized (for reviews see Echols and Goodman, 1991; Naegeli, 1994). One possibility is by continuing DNA replication across the template lesion (so-called trans-lesion synthesis). This process involves error-prone DNA replication often resulting in mutagenesis (Rajagopalan *et al.*, 1992). Since in *mec1 rad14Δ* and *rad53 rad14Δ* double mutants, UV-induced mutation rates are reduced compared with those observed in *rad14Δ* single mutants under the same conditions (our unpublished observation), trans-lesion DNA synthesis is not likely to be responsible for damage-resistant DNA replication in the absence of *MEC1* and *RAD53*.

Cells might also reinitiate DNA replication downstream of the block, thus producing a daughter-strand gap opposite the site of damage, which subsequently might become filled in by recombinational repair. If the induction of replication-dependent recombination facilitates the bypass of a bulge in the template and could account for the slow DNA synthesis in the presence of DNA damage, Rad53 and Mec1 might regulate the formation of recombination intermediates. However, we observe accumulation of X-shaped DNA molecules in both the *rad14Δ* single mutant and the *rad14Δ mec1* and *rad14Δ rad53* double mutants, suggesting that Rad53 and Mec1 are not required for their formation. Nevertheless, we cannot exclude the possibility that Mec1 and Rad53 might regulate the stability, processing and/or resolution of the DNA recombination intermediates that arise during replication of a damaged template. If this were the case, the regulation of replication-dependent recombination by the checkpoint pathway might allow cells to accommodate unrepaired DNA damage and may partially account for the checkpoint-dependent slowing of DNA replication in the presence of DNA damage, as suggested previously by Paulovich *et al.* (1998).

Finally, uncontrolled firing of replication origins might account for the damage-resistant DNA replication in the absence of *MEC1* and *RAD53*. Our finding that unrepaired UV damage in G₁ selectively blocks initiation of DNA replication from the late *ARS501* origin in a *MEC1*- and *RAD53*-dependent manner suggests that the unregulated initiation of DNA synthesis occurring in *mec1* and *rad53* mutants at late origins may be one of the mechanisms allowing DNA replication in the presence of irreparable UV photoproducts. Consistently, after UV irradiation in G₁ and release into the cell cycle, the *ddc1Δ rad9Δ rad14Δ* triple mutant neither fires late origins nor progresses through S phase, further strengthening the correlation between unregulated activation of replication origins and progression through S phase. Therefore, when unrepaired UV-induced dimers block progression of replication forks started at early origins, DNA replication may be further supported by initiation at late origins on the same chromosome. Since deletion of the B-type cyclin gene *CLB5* has

been shown to reduce the efficiency of replication initiation at late chromosomal origins (Donaldson *et al.*, 1998) and to cause a lengthened S phase (Epstein and Cross, 1992; Schwob and Nasmyth, 1993), we speculate that Clb5/Cdk1 may be one of the targets of the DNA damage checkpoint acting during DNA synthesis.

We cannot exclude the possibility that the DNA damage checkpoint pathway may control not only the initiation but also the elongation step of DNA synthesis, and the control of these two processes might have different requirements for checkpoint proteins. In contrast to what was observed in excision repair-deficient cells, both *ddc1Δ* and *rad9Δ* NER-proficient cells are partially defective in slowing down DNA synthesis in the presence of DNA damage, suggesting that Ddc1 and Rad9 might regulate only a subset of functions of the DNA damage checkpoint acting during replication. In this view, we propose that the checkpoint proteins Mec1, Rad53, Ddc1 and Rad9 are all involved in controlling the rate of fork progression, stopping the advancing replication forks until lesions are repaired, but only Mec1 and Rad53 might regulate replication initiation in the presence of DNA damage. In *ddc1Δ* and *rad9Δ* checkpoint mutants, unrestrained fork movement in excision repair-proficient cells may be sufficient to promote DNA replication on damaged DNA, while in NER-deficient strains the presence of large amounts of irreparable bulge in the template might obstruct ongoing replication forks. In this case, only *rad53* and *mec1* mutants, which are allowed to fire origins that in wild-type cells are inhibited by DNA damage, may succeed in replicating DNA in the absence of NER.

The inability of *rad9Δ* and *ddc1Δ* mutants to promote DNA replication on irreparable UV-damaged DNA might also be explained by the fact that Rad9 and Ddc1 do not have any role in sensing unrepaired UV dimers, which instead might be recognized, directly or indirectly, by Mec1 and Rad53. For example, Rad9 and the Rad24 group of proteins might detect DNA or protein–DNA structures generated during the repair process. Since in NER-proficient strains these intermediates are likely to be processed quickly during repair, Rad9 and the Rad24 group of proteins could be required to sustain and/or amplify these transient signals, thus facilitating their recognition by Mec1.

Altogether, our data strongly support the notion that Mec1 and Rad53 play a primary role in the S phase damage-sensing pathway. Given its structural similarity to DNA-PK, Mec1 might directly recognize specific DNA or protein–DNA structures generated during DNA synthesis on damaged templates and/or the replication apparatus might be engaged in promoting Mec1 activation. It will be interesting to determine whether replication proteins such as DNA polymerase ϵ , Dpb11, Rfc5 and Rpa1 are required for the Mec1-dependent inhibition of late origin firing, thus implying complex relationships between checkpoint pathways and replication machinery.

Materials and methods

Strains and media

Strains used in this study are listed in Table I. All yeast strains were derivatives of or were backcrossed at least three times to W303 (*MATa* or *MAT α* , *ade2-1*, *trp1-1*, *leu2-3,112*, *his3-11,15*, *ura3*). To construct

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

Strain	Genotype	Reference/source
K699	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3</i>	Longhese <i>et al.</i> (1997)
YLL334	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1</i>	Longhese <i>et al.</i> (1997)
YLL355	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad14Δ::KanMX4</i>	this study
DMP2133/2D	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 sad1-1</i>	this study
DMP2538/3A	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4</i>	this study
DMP2538/11D	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 sad1-1</i>	this study
DMP2132/5A	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1</i>	this study
DMP2539/3D	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4</i>	this study
DMP2137/3A	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad9Δ::URA3</i>	Paciotti <i>et al.</i> (1998)
YLL157	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad9Δ::URA3</i>	Longhese <i>et al.</i> (1996a)
YLL244	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ddc1Δ::KanMX4</i>	Longhese <i>et al.</i> (1997)
DMP2141/1A	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad17Δ::LEU2</i>	Paciotti <i>et al.</i> (1998)
DMP2149/1D	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad24Δ::TRP1</i>	Paciotti <i>et al.</i> (1998)
YLL335	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 mec3Δ::TRP1</i>	Longhese <i>et al.</i> (1997)
DMP2541/8A	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 mec1-1 sml1</i>	Paciotti <i>et al.</i> (1998)
YHN133	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad52Δ::TRP1</i>	this study
DHN133/5C	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 rad9Δ::URA3</i>	this study
DMP2057/10A	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad14Δ::KanMX4 ddc1Δ::KanMX4</i>	this study
DHN205/2C	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 rad17Δ::LEU2</i>	this study
DHN206/1A	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 rad24Δ::TRP1</i>	this study
DHN207/2D	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 mec3Δ::TRP1</i>	this study
DHN198/6D	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 mec1-1 sml1</i>	this study
DHN196/11C	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 rad52Δ::TRP1</i>	this study
SP1056	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 bar1Δ::KanMX4</i>	S.Piatti
DHN219/8C	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 rad52Δ::TRP1 bar1Δ::KanMX4</i>	this study
YLL262/2C	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ddc1Δ::KanMX4</i>	Longhese <i>et al.</i> (1997)
DHN179/1C	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad14Δ::KanMX4 rad9Δ::URA3 ddc1Δ::KanMX4</i>	this study
DHN179/4A	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad14Δ::KanMX4 rad9Δ::URA3 ddc1Δ::KanMX4</i>	this study
DHN200/11D	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad9Δ::URA3 ddc1Δ::KanMX4</i>	this study
DHN201/8A	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad14Δ::KanMX4 rad9Δ::URA3 ddc1Δ::KanMX4 mec1-1 sml1</i>	this study
DMP2371/5A	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 cdc4-1</i>	Paciotti <i>et al.</i> (1998)
DMP2371/9C	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 cdc4-1 sic1Δ::HIS3</i>	Paciotti <i>et al.</i> (1998)
DHN176/10B	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 cdc4-1</i>	this study
DHN176/11C	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 cdc4-1 sic1Δ::HIS3</i>	this study
K699p°	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 p°</i>	this study
YHN156	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 p°</i>	this study
YHN160	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 rad9Δ::URA3 p°</i>	this study
YHN159	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad14Δ::KanMX4 ddc1Δ::KanMX4 p°</i>	this study
YHN161	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad14Δ::KanMX4 rad9Δ::URA3 ddc1Δ::KanMX4 p°</i>	this study
YHN158	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 sad1-1 p°</i>	this study
YHN169	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 HA2-DDC1::LEU2::ddc1 rad14Δ::KanMX4 mec1-1 sml1 p°</i>	this study

a *RAD14* chromosomal deletion, the *rad14Δ::kanMX4* cassette was constructed by PCR using plasmid pFA6a-kanMX4 (Wach *et al.*, 1994) as a template and oligonucleotides PRP11 (3'-ttg cca cca tgc aga act tga atg gtg gtt ata tca acc cta agg aca agc gta cgc tgc agg tgc ac-5') and PRP12 (3'-ttc ttc agt tct tag ccc gca gtc tgt aca tct tct tct ttg aat ttg ata tgc atg aat tgc agc acg-5') as primers. One-step replacement of 638 bp of the *RAD14* coding region with the *KanMX4* cassette was carried out by transforming strain K699 with the *rad14Δ::KanMX4* PCR product to give rise to strain YLL355. To construct strain YHN133, carrying the *RAD52* chromosomal deletion, we transformed strain K699 with the *Bam*HI linearized plasmid pSM21 (a gift from M.Fasullo, Loyola University, Chicago, IL), which carries a *rad52Δ::TRP1* cassette cloned

into the *Bam*HI site of plasmid pBR322. The accuracy of the *RAD14* and *RAD52* gene replacements was verified by Southern blot analysis. Strains DMP2538/3A and DMP2538/11D are meiotic segregants of a cross between strains YLL355 and DMP2133/2D. Strain DMP2539/3D is a meiotic segregant of a cross of strains YLL355 and DMP2132/5A. Strain DMP2538/3A was crossed with strains YLL157, YLL244, DMP2141/1A, DMP2149/1D, YLL335, DMP2541/8A and YHN133 to generate meiotic segregants DHN133/5C, DMP2057/10A, DHN205/2C, DHN206/1A, DHN207/2D, DHN198/6D and DHN196/11C, respectively. Strain DHN219/8C was derived from a cross between strains DHN196/11C and SP1056, kindly provided by S.Piatti. Strain DHN133/5C was crossed with strain YLL262/2C to generate the meiotic segregants

DHN179/1C, DHN179/4A and DHN200/11D. Strain DHN201/8A is a meiotic segregant of a cross between strains DHN198/6D and DHN179/4A. Strains DHN176/10B and DHN176/11C were obtained from a cross of strains DMP2371/9C and DMP2538/3A. Strains K699 ρ^+ , YHN156, YHN158, YHN160, YHN159, YHN161 and YHN169 are ρ^+ derivatives of strains K699, DMP2539/3D, DMP2538/11D, DHN133/5C, DMP2057/10A, DHN179/1C and DHN198/6D, respectively. To generate ρ^+ strains, cells were plated on YEPD plates and 10 μ l ethidium bromide (10 mg/ml) was spotted at the centre of the plate. After 3 days incubation at 25°C, small colonies from the middle of the zone between border and centre of the Petri dish were picked up and tested for growth on YEP-ethanol plates (1% yeast extract, 2% bactopectone, 50 mg/l adenine, 2% ethanol). Clones not growing on ethanol-containing medium were tested for loss of mitochondria by DAPI staining and fluorescence microscopy.

Cells were grown at 25°C in YEPD medium (1% yeast extract, 2% bactopectone, 50 mg/l adenine, 2% glucose). Transformants carrying the *KanMX4* cassette were selected on YEPD plates containing 400 μ g/ml G418 (US Biological).

Synchronization experiments

Cell synchronization in G₁ was obtained by treatment of exponentially growing YEPD cell cultures with 2 μ g/ml of α -factor, followed by release in YEPD. α -factor-arrested cells were collected by centrifugation and 2.5×10^8 cells were spread on 14-cm diameter YEPD plates, followed by UV irradiation with 5 J/m² using a Stratilinker 2400 (Stratagene). Aliquots were taken and flow cytometric DNA quantitation was determined on a Becton-Dickinson FACScan. Survival was measured as plating efficiency of appropriate dilutions of the cultures on YEPD plates. Cell density was determined by counting aliquots fixed in 3.7% formaldehyde.

Western blot analysis

For Western blot analysis, protein extracts were prepared by TCA precipitation as described previously (Longhese *et al.*, 1997). Protein extracts were resolved by electrophoresis on 10% SDS-polyacrylamide gels and proteins were transferred to Protran membranes (Schleicher and Schuell). Rad53 was detected using anti-Rad53 polyclonal antibodies kindly provided by C.Santocanale (ICRF, Clare Hall Laboratories, UK). Secondary antibodies were purchased from Amersham and proteins were visualized by an enhanced chemiluminescence system according to the manufacturer's instructions.

Two-dimensional agarose gel electrophoresis analysis

Cell cultures (2 l; 1×10^7 cells/ml) were grown in YEPD medium, synchronized with 2 μ g/ml (0.66 μ g/ml for the *bar1* strain DHN219/8C) of α -factor and split in two. One half was not irradiated, released in 500 ml YEPD medium and samples for DNA preparation (10 ml) were removed every minute from 10 to 50 min after release from the pheromone block, supplied with 100 μ l of 10% NaN₃, pooled and chilled with 100 ml of frozen 0.2 M EDTA pH 8, 0.1% NaN₃. The other half was centrifuged and cells were spread on 10 14-cm diameter YEPD plates (1×10^9 cells/plate), UV-irradiated with 16 J/m², resuspended and pooled in 500 ml YEPD medium. The UV dose used in these experiments was adjusted to 16 J/m² in order to obtain the same survival as in the experiments where only 2.5×10^8 cells were spread on 14-cm diameter plates. Samples for DNA preparation were removed every 2 min from 10 to 100 min after UV irradiation and pooled as described above. DNA from pooled S phase cells was prepared as previously described (Hubermann *et al.*, 1987; Brewer *et al.*, 1992). DNA samples (5 μ g) were analysed by 2-D gel electrophoresis as described by Friedman and Brewer (1995). First dimension gels were 0.4% agarose and second dimension gels were 1.1% agarose. Fragments probed were a 4.2-kb *EcoRV* *ARS305* fragment of chromosome III and a 3.2-kb *XbaI* *ARS501* fragment of chromosome V (Donaldson *et al.*, 1998). The *ARS305* probe was PCR-amplified from genomic DNA using primers *ARS305F* (3'-att cgc ctg aca gga cg-5') and *ARS305R* (3'-ata acg gag gcc gaa cc-5'). The *ARS501* probe was obtained by *XbaI* digestion of plasmid pUC-*ARS501* and gel-purification. Primers *ARS305F* and *ARS305R* and plasmid pUC-*ARS501* were kindly provided by B.Brewer (University of Washington, Seattle, WA). Blots were exposed for 10–14 days as required.

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