The *set1* Δ mutation unveils a novel signaling pathway relayed by the Rad53-dependent hyperphosphorylation of replication protein A that leads to transcriptional activation of repair genes

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SET domain proteins are present in chromosomal proteins involved in epigenetic control of transcription. The yeast SET domain protein Set1p regulates chromatin structure, DNA repair, and telomeric functions. We investigated the mechanism by which the absence of Set1p increases DNA repair capacities of checkpoint mutants. We show that deletion of *SET1* induces a response relayed by the signaling kinase Rad53p that leads to the *MEC1*/*TEL1*-independent hyperphosphorylation of replication protein A middle subunit (Rfa2p). Consequently, the binding of Rfa2p to upstream repressing sequences (URS) of repair genes is decreased, thereby leading to their derepression. Our results correlate the *set1* Δ -dependent phosphorylation of Rfa2p with the transcriptional induction of repair genes. Moreover, we show that the deletion of the amino-terminal region of Rfa2p suppresses the sensitivity to ultraviolet radiation of a *mec3* Δ checkpoint mutant, abolishes the URS-mediated repression, and increases the expression of repair genes. This work provides an additional link for the role of Rfa2p in the regulation of the repair capacity of the cell and reveals a role for the phosphorylation of Rfa2p and unveils unsuspected connections between chromatin, signaling pathways, telomeres, and DNA repair.

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Several chromatin regulators involved in epigenetic control of transcription contain a characteristic motif of unknown function called the SET domain that takes its name from Su(v)ar 3–9, E(z) and trithorax (for review, see Jenuwein et al. 1998). The SET domain has been now identified in >40 proteins from fungi, plant, and mammalian species.

The 1081-residue Set1p protein of *Saccharomyces cerevisiae* contains a carboxy-terminal SET domain of 130 residues. Inactivation of the full-length Set1p or of its SET domain alleviates telomeric position effect (TPE) (Nislow et al. 1997; Corda et al. 1999). The SET domain of Set1p interacts with the carboxy-terminal region of the checkpoint protein Mec3p, which is required for efficient DNA damage-dependent checkpoints at G₁/S, in-

tra S, and G₂/M (Longhese et al. 1996; Corda et al. 1999). Mec3p belongs to the sensor class of the DNA checkpoint machinery, which along with Mec3p includes Rad9p, Rad17p, Rad24p, and Ddc1p (for review, see Lowndes and Murguia 2000). The sensor proteins are thought to recognize DNA damage and generate a signal relayed by the central transducer Mec1p to the protein kinases Rad53p and Dun1p and the metaphase–anaphase regulator Pds1p (Allen et al. 1994; Sanchez et al. 1996; Sun et al. 1996; Paciotti et al. 1998; Gardner et al. 1999; Pellicoli et al. 1999). The checkpoint genes required for cell cycle delay also regulate the transcriptional induction of DNA repair and DNA metabolism genes, thereby increasing the DNA repair capacities of the cell (Aboussekhra et al. 1996; Huang et al. 1998). We have shown previously that the deletion of *SET1* increases the viability after DNA damage of a *mec3* null mutant and also of other checkpoint mutants (*rad9*, *rad17*, and *rad24*), but not of cells devoid of the signaling kinase Rad53p. *SET1* deletion does not restore any of the three

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DNA damage checkpoints regulated by Mec3p. Finally, Set1p and Mec3p were found to modulate in opposite directions TPE and telomere length (Corda et al. 1999).

Replication protein A (RPA) is an eukaryotic single-stranded DNA-binding protein essential for replication, recombination, and DNA repair (Wold 1997). Human as well as yeast RPA is composed of three subunits of 70, 32/34, and 14 kD (Brill and Stillman 1991; Philipova et al. 1996). It has been proposed that RPA plays a role in DNA damage sensing mainly on the basis of the fact that RPA 32/34 (in yeast Rfa2p) becomes phosphorylated in response to DNA damage and in the presence of ssDNA (for review, see Iftode et al. 1999). Brush et al. (1996) showed that the yeast checkpoint Mec1p, but not Rad53p, was required for the phosphorylation of Rfa2p during normal cell cycle and after DNA damage. It has been shown recently that Rfa1p is also phosphorylated in response to various forms of genotoxic stress, including radiation and hydroxyurea exposure (Brush and Kelly 2000). Interestingly, phosphorylation of Rfa1p after radiation depends on both checkpoint regulators, Mec1p and Rad53p (Brush and Kelly 2000). RPA has been shown to bind specifically to double-stranded oligonucleotides containing upstream repressing sequences (URS) found upstream of several DNA repair genes (Singh and Samson 1995). It has been hypothesized from these results that RPA may be involved in the transcriptional regulation of repair genes. Finally, it has been shown that in vivo hyperphosphorylation of RPA 32/34 after irradiation was concordant with a decreased ability of RPA to bind ssDNA in mouse cell extracts (Fried et al. 1996).

In this study, we document the *set1Δ*-induced cellular response. We show that inactivation of Set1p leads to the constitutive *MEC1/TEL1*-independent hyperphosphorylation of Rfa2p in a process that requires Rad53p. The Set1-dependent phosphorylation of Rfa2p leads to the transcriptional activation of repair genes but not to a cell cycle arrest. This pathway explains why Set1p inactivation increases the repair capacities of the cell when checkpoints fail. This response differs from the Mec1-dependent DNA damage induction pathway.

Results

Rad53-dependent hyperphosphorylation of Rfa2p in set1Δ mutants

Phosphorylation of Rfa2p appears during the G₁/S transition and persists through the S phase and in response to genotoxic insult (Din et al. 1990; Brush et al. 1996). Because the deletion of *SET1* results in decrease of sensitivity to ionizing radiation in checkpoint mutants, we asked whether the *set1Δ* mutation was associated with the phosphorylation of Rfa2p. Rfa2p phosphorylation was determined by immunoblot analysis with anti-Rfa2p polyclonal antibodies (Din et al. 1990) of protein extracts prepared from synchronized cells that have been released from α -factor at the indicated times (Fig. 1). Phosphorylated forms of Rfa2p exhibited reduced mobility in SDS/PAGE (Fig. 1A). The two bands that appear in

Figure 1A were converted into a single species upon treatment of crude extracts with phosphatase, indicating that the reduced mobility of Rfa2p was due to phosphorylation (data not shown). Flow cytometry analysis was carried out in parallel to monitor cell progression through S phase (Fig. 1A). As expected, cell cycle-regulated Rfa2p phosphorylation was compromised in *sml1 mec1-1* cells (Brush et al. 1996) (see Materials and Methods for the *sml1 mec1-1* allele). In contrast, in *set1Δ* cells, the level of Rfa2p phosphorylation was enhanced. The ratio of phosphorylated to unphosphorylated Rfa2p increased as compared with wild type in *set1Δ* exponentially growing cells. Interestingly, in exponential growing *sml1 mec1-1* cells, the absence of *SET1* maintained a high level of Rfa2p phosphorylation; >80% of Rfa2p was found under its phosphorylated form in exponentially growing cells and there was only a slight increase of Rfa2p phosphorylation during S phase. We concluded that Rfa2p is hyperphosphorylated in *set1Δ* cells in a manner independent of *MEC1*.

Noteworthy, we found that the deletion of *MEC3* did not modify the phosphorylation pattern of Rfa2p in wild-type and in *set1Δ* cells.

To identify a kinase involved in the phosphorylation of Rfa2p in *set1Δ* cells, we analyzed the phosphorylation of Rfa2p in *rad53* mutants. We introduced in our mutant strains a *rad53* allele (*rad53K227A*) carrying a substitution within the conserved kinase domain of Rad53p (Fay et al. 1997; Pelliccioli et al. 1999). We found that inactivation of the kinase activity of Rad53p did not alter the phosphorylation of Rfa2p in wild-type cells. In contrast, introduction of the *rad53K227A* allele in *set1Δ* cells abolished the *set1Δ*-dependent hyperphosphorylation of Rfa2p (Fig. 1A). This observation was confirmed by the analysis of Rfa2p phosphorylation in *sml1 mec1-1 rad53K227A* and *sml1 mec1-1 rad53K227A set1Δ* mutants. We failed to detect any significant phosphorylation of Rfa2p when the *rad53K227A* allele was introduced in *sml1 mec1-1* and *sml1 mec1-1 set1Δ* mutants.

We observed that G₁ cells, which normally do not exhibit phosphorylation of Rfa2p, showed such a modification in *set1Δ* cells. This *set1Δ*-dependent extra phosphorylation of Rfa2p, which seems to be cell cycle independent, disappeared in *set1Δ rad53K227A* cells, whereas the cell cycle-dependent phosphorylation of Rfa2p, which is apparently Mec1p dependent, remained unaffected (Fig. 1A).

We concluded that Rfa2p is hyperphosphorylated in *set1Δ* cells by both Rad53p- and Mec1p-dependent mechanisms. The Rad53p-dependent phosphorylation of Rfa2p, evidenced in *set1Δ* cells, seems to be cell cycle independent.

Hyperphosphorylation of Rfa2p in set1Δ mutants occurs in the absence of both ATM homologs, Mec1p and Tel1p

The response resulting from the inactivation of *SET1* appears different from the one resulting from DNA damage. We therefore asked whether the high level of phos-

Figure 1. *set1Δ*-induced hyperphosphorylation of Rfa2p. (A, top) Exponentially growing cells (exp) of K699 derivative strains with the indicated relevant genotypes were synchronized with α -factor and released at time zero. FACS analysis of the synchronized cultures at the indicated times after α -factor release. (Bottom) Protein extracts prepared at the indicated times after release from α -factor were analyzed by SDS-PAGE and immunoblotting with anti-pRfa2p polyclonal antibodies. The upper band corresponds to the phosphorylated form of Rfa2p. (B) *set1Δ*-dependent phosphorylation of Rfa2p after MMS treatment in *sml1 mec1-1* and *sml1 mec1-1 tel1Δ* mutants. Protein extracts from exponentially growing cultures (exp), from G_1 -arrested cultures (α -F), and MMS treated G_1 -arrested cultures (MMS), were analyzed by Western blot with anti-pRfa2p polyclonal antibodies.

phorylation of Rfa2p in *set1Δ* cells can be further increased after DNA damage (Brush et al. 1996). As over-

expression of *TEL1* can restore some responses in checkpoint mutant cells, we also asked whether hyper-

phosphorylation of Rfa2p in *set1Δ* mutants could occur in the absence of both ATM homologs, Mec1p and Tel1p. To distinguish DNA damage-induced Rfa2p phosphorylation from the cell cycle-regulated reaction, we blocked cells in G₁ with α -factor and compared methyl methane sulfonate (MMS)-treated and untreated cells. Phosphorylation of Rfa2p was analyzed 30 min after MMS addition. The level of Rfa2p phosphorylation was increased by roughly the same level in wild-type and *set1Δ* cells (Fig. 1B). This result indicates that the level of phosphorylation of Rfa2p in *set1Δ* cells is not maximal and can still be increased by DNA damage. We then investigated whether this DNA damage-induced phosphorylation in *set1Δ* cells was depending on both ATM homologs *MEC1* and *TEL1*. In a *sml1 mec1-1* mutant, DNA damage-induced phosphorylation of Rfa2p was weak and totally absent in a *sml1 mec1-1 tel1* mutant in agreement with the study of Brush et al. (1996). As above, we observed that Rfa2p was hyperphosphorylated in *set1Δ* cells even in the absence of *MEC1* and *TEL1*. However, no significant increase in Rfa2p phosphorylation after MMS treatment was detected in *sml1 mec1-1 set1Δ* and *sml1 mec1-1 tel1 set1Δ* (Fig. 1B). Therefore, the MMS-induced Rfa2p phosphorylation is impaired in the absence of *MEC1* and *TEL1* in *set1Δ* cells. Our findings indicate that Rfa2p phosphorylation occurs in the absence of *MEC1* and *TEL1* in *set1Δ* cells and can still be increased by DNA damage in *set1Δ* cells in the presence of *MEC1* and *TEL1*.

set1Δ-dependent phosphorylation of Rfa2p requires the amino terminus of Rfa2p

To further characterize the Set1-depending Rfa2p phosphorylation, we expressed a chromosomally encoded truncated form of Rfa2p, lacking 38 amino acids from the amino terminus (Rfa2p Δ 40). In the human counterpart of the yeast Rfa2p, this region has been shown to contain the major phosphorylation sites (Niu et al. 1997; Zernik-Kobak 1997). In yeast, the *rfa2 Δ40* allele corresponds to the largest viable amino-terminal truncation of Rfa2p (Philipova et al. 1996).

Interestingly, the yeast-made Rfa2p Δ 40 protein is phosphorylated, indicating the presence of phosphorylation sites outside of the amino-terminal region (Fig. 2A). When we treated native extracts from the exponentially growing cells producing Rfa2p Δ 40 with phosphatase, we observed that the upper band was converted to the lower one, indicating that the reduced mobility of Rfa2p Δ 40 is due to phosphorylation (Fig. 2B). We also observed that Rfa2p Δ 40 was neither phosphorylated in *sml1 mec1-1* nor in *sml1 mec1-1 set1Δ* mutants (Fig. 2A). This latter result suggests that the Mec1-dependent phosphorylation of Rfa2p may occur outside of the conserved amino-terminal region of Rfa2p, whereas the *set1Δ*-dependent phosphorylation of Rfa2p, revealed in *set1Δ* cells appears to depend on the presence of the amino-terminal region of Rfa2p.

Figure 2. Phosphorylation of Rfa2p Δ 40. (A) Exponentially growing cells of K699 derivative strains with the indicated relevant genotypes were synchronized with α -factor and released at time zero. Protein extracts prepared at the indicated times after release from α -factor were analyzed by Western blot with anti-pRfa2p polyclonal antibodies. The upper band corresponds to the phosphorylated form of Rfa2p Δ 40. (B) Native extracts of exponentially growing *rfa2 Δ40* and *rfa2 Δ40 set1Δ*. Cells were divided in two samples and treated (+) or not (–) with λ -phosphatase.

The set1Δ mutation does not induce a DNA damage checkpoint signal

We report above that the deletion of *SET1* induces the constitutive phosphorylation of Rfa2p. Because phosphorylation of Rfa2p occurs after DNA damage, we investigated whether the *set1Δ* mutation induces a DNA damage checkpoint signal. Thus, we examined the phosphorylation of proteins involved in DNA damage checkpoints in *set1Δ* cells. As Rfa2p, the checkpoint protein Ddc1p is phosphorylated periodically during the cell cycle and in response to DNA damage (Longhese et al. 1997). Moreover, Ddc1p phosphorylation during an unperturbed cell cycle and after DNA damage depends on Mec1p (Paciotti et al. 1998). Ddc1p phosphorylation was analyzed under the same conditions as for Rfa2p phosphorylation in Figure 1A. We found that in *set1Δ* cells, Ddc1p is phosphorylated during S phase and its phosphorylation is very similar to the one of wild-type cells (data not shown). We also observed by immunoblot analysis that the effector kinase Rad53p was apparently not phosphorylated in *set1Δ* cells (data not shown). We concluded that *set1Δ* does not induce a constitutive hyperphosphorylation of checkpoint proteins.

set1Δ-dependent hyperphosphorylation of Rfa2p correlates with the enhancing of viability of checkpoint mutants after DNA damage

We have reported previously that the deletion of *SET1* increases the viability of *rad9*, *rad17*, *rad24*, and *mec3*

mutants but not the viability of cells carrying the *sad1-1* allele of *RAD53* (Corda et al. 1999). In agreement with our previous work, we also observed that the *set1Δ* mutation did not increase the viability after DNA damage of the *rad53K227A* mutant (Fig. 3A). Because Rfa2p is not phosphorylated in *set1Δ rad53K227A* cells, we considered the possibility that the *set1Δ*-dependent phosphorylation of Rfa2p was linked to the increase of the DNA repair capacities of the cell.

We first asked whether inactivation of Set1p partially rescued the ultraviolet sensitivity of a *sml1 mec1-1* mutant (Fig. 3A). We showed that the viability after irradiation of a *sml1 mec1-1* mutant was increased in the absence of *SET1* (Fig. 3A). Similarly, the *set1Δ* mutation increased the viability after UV of a *sml1 mec1-1 tel1* mutant (Fig. 3A).

To examine whether the increase of viability of *sml1 mec1-1 set1Δ* cells with respect to *sml1 mec1-1* cells after ultraviolet treatment requires the kinase activity of Rad53p, we compared the ultraviolet sensitivity of a

sml1 mec1-1 rad53K227A with the isogenic *sml1 mec1-1 rad53K227A set1Δ*. Both mutants had the same sensitivity to UV irradiation (Fig. 3A). Moreover, the deletion of *SET1* did not increase the viability after UV irradiation of a *mec3Δ rad53Δ* double mutant (data not shown). These results indicate that the *set1Δ*-dependent increase of viability of *sml1 mec1-1* and *mec3Δ* mutants after DNA damage is mediated by kinase activity of Rad53p.

Surprisingly and yet unexplained, we observed repeatedly that the *sml1 mec1-1 rad53K227A* (or *rad53Δ*) mutant was less sensitive to DNA damage than the *sml1 mec1-1* mutant. Thus, the inactivation of *RAD53* increased the viability after DNA damage of a *sml1 mec1-1* mutant. This explained the fact that the *sml1 mec1-1 rad53K227A set1Δ* exhibited increased survival after UV irradiation with respect to the *sml1 mec1-1 set1Δ*, contrary to what one could have expected, as Rfa2p is not hyperphosphorylated in the *sml1 mec1-1 rad53K227A set1Δ* strain.

Figure 3. Viability after UV irradiation. Approximately 1000 cells of K699 derivative strains with the indicated relevant genotypes were spread on YPD plates and exposed to ultraviolet irradiation. Cells were grown at 30°C for 5 d before we counted the number of colonies. (A) The *set1Δ* mutation partially rescues ultraviolet sensitivity of *sml1 mec1-1* but not of *rad53K227A* and *sml1 mec1-1 rad53K227A* mutants; (B) the deletion of *SET1* suppresses the growth defect of a *sml1 mec1-1 tel1* double mutant; (C) the *set1Δ* mutation increases the viability after ultraviolet of *mec3Δ dun1Δ* and *sml1 mec1-1 dun1Δ* cells.

We also observed that the deletion of *SET1* suppressed the growth defect of the *sml1 mec1-1tel1* mutant (Fig. 3B), strengthening evidence for the existence of pathways activated by *SET1* inactivation, but independent of *MEC1* and *TEL1*. Interestingly, we noticed that the deletion of *SET1* in a *sml1 mec1Δ tel1Δ* strain increased telomere length (data not shown). This could account for the rescue of the poor viability of the *sml1 mec1Δ tel1Δ* mutant by the deletion of *SET1*. From these data and the data in Figure 1B, we ruled out that the *set1Δ* mutation exerts its action via *MEC1* and *TEL1*.

The protein kinase Dun1p has been shown to act downstream of Rad53p and to be involved in the induction of the expression of many repair genes after DNA damage (Zhou and Elledge 1993). We asked whether Dun1p relays the increase of the DNA repair capacities in *set1Δ* cells. We introduced the *set1Δ* mutation in *dun1Δ mec3Δ* and *dun1 mec1-1 sml1* mutants. We found that the deletion of *SET1* increased the viability of both mutants, *dun1Δ mec3Δ* and *dun1 mec1-1 sml1* after ultraviolet treatment (Fig. 3C).

These results suggest that in *set1Δ* cells, the Rad53 kinase-dependent phosphorylation of Rfa2p induces a Dun1-independent increase of the DNA repair capacities of the cell.

set1Δ-dependent phosphorylation of Rfa2p decreases its binding to URS elements of repair genes, providing a mechanism for transcriptional induction

Our results indicate that phosphorylation of Rfa2p correlates with the increase of the DNA repair capacity of the cell. We therefore investigated whether the *set1Δ*-dependent phosphorylation of Rfa2p could modulate the induction of repair genes. It has been reported by Singh and Samson (1995) that RPA binds in vitro to the URS of 3-methyladenine DNA glycosylase repair gene (*MAG*) and to *MAG* URS1-like sequences of other repair genes, some of them being inducible by DNA-damaging agents such as *RAD51* and *RNR2*.

We first investigated the association of Rfa2p to URS of *RNR2* and *RAD51* in vivo by performing chromatin immunoprecipitation experiments (ChIP) (Strahl-Bolsinger et al. 1997). We fused a 18-mer Myc epitope to the caboxyl terminus of the genomic copy of *RFA2* and also of *HDF2* (*yKU80*). We used *yKu80* as a control for the specific association of Rfa2p with the URS. Chromatin from cells (UCC1001) expressing either Rfa2p–Myc18 or *yKu80*–Myc18 was cross-linked, immunoprecipitated, and analyzed by PCR. We performed PCR on input and on immunoprecipitated DNA with primers flanking the URS elements of *RAD51* and *RNR2* identified by Singh and Samson (1995) (Fig. 4A). Immunoprecipitation of Rfa2p–Myc18 resulted in the retention of PCR products corresponding to a region flanking the URS elements of *RAD51* and *RNR2*, whereas the URS PCR fragments were not retained in untagged or *yKu80*–Myc18 strains (Fig. 4B). To show that the interaction between Rfa2p and URS2 is specific, we examined, as an additional control, the binding of Rfa2p to DNA regions adjacent to the

URS2 of *RAD51* and *RNR2*. These regions start in the coding sequences of *RAD51* and *RNR2* and are localized at about 1400 bp of the URS2 sequences of *RNR2* and *RAD51*. As shown in Figure 4B, Rfa2p does not bind to these regions.

These cross-linking experiments indicate that Rfa2p binds to URS of *RNR2* and *RAD51* in vivo.

As *set1Δ*-dependent phosphorylation of Rfa2p correlates with the enhancing of the DNA repair capacities, we compared the binding of Rfa2p with the URS of *RAD51* in wild-type and *set1Δ* cells. We carried out ChIP experiments on chromatin from wild-type and *set1Δ* cells expressing Rfa2p–Myc18. We performed quantitative PCR with a Light Cycler on input DNA and on immunoprecipitates with URS-specific primers allowing the amplification of a PCR fragment flanking the URS of *RAD51*. PCR amounts in the immunoprecipitates were calculated by comparing the signal in the immunoprecipitates with a standard curve obtained by performing PCRs with the same primers on corresponding serially diluted input DNAs. The PCR quantitation analysis indicated that the amount of DNA corresponding to URS of *RAD51* bound to Rfa2p was decreased by fourfold in a *set1Δ* mutant (Fig. 4C). As above, no URS was retained in wild-type or *yKu80*–Myc18 strains. These results may reflect the fact that the *set1Δ*-dependent hyperphosphorylation of Rfa2p decreases the binding of RPA to URS of *RAD51*. We further tested the effect of the *SET1* deletion on the Rfa2p URS-binding activity by analyzing the in vivo association of Rfa2p to *RAD51* URS in *set1Δ* cells in the presence or absence of *MEC1* and *RAD53*. The ChIP assays indicate that the amount of Rfa2p-associated URS in *sml1Δ mec1Δ set1Δ* cells (hyperphosphorylation of Rfa2p) was 3.7-fold lower than the one in *sml1Δ mec1Δ* cells (no phosphorylation) (Fig. 4C). In contrast, the amount of Rfa2p associated with the URS remains approximately the same in *sml1Δ rad53Δ mec1Δ* cells (no phosphorylation of Rfa2p) and in *sml1Δ rad53Δ mec1Δ set1Δ* cells (no phosphorylation of Rfa2p) (Fig. 4C). Together these results indicate that the URS-binding activity of Rfa2p is reduced in *set1Δ* cells in a Rad53-dependent manner. The binding activity of Rfa2p to URS is thus likely to depend on its phosphorylation.

Such regulation may account for the transcriptional derepression of *RNR2* and *RAD51* genes in *set1Δ* cells. To confirm this hypothesis, we used a *CYC1-lacZ* vector (pNG22) containing the *MAG* URS1 region cloned 3' of the *CYC1* UAS (pNG22+URS1) (Singh and Samson 1995). We transformed pNG22 and pNG22+URS1 in wild-type and *set1Δ* strains and measured the β-galactosidase activity of equivalent amounts of cells. As reported by Singh and Samson (1995), we observed a threefold decrease of *CYC1* UAS function in the presence of the *MAG* URS1 in a wild-type strain (Fig. 5A). In contrast, we observed that the repressive effect of the *MAG* URS1 region was abolished in *set1Δ* cells (Fig. 5A). Moreover, we found that the repressing activity of the *MAG* URS1 region was restored in *set1Δ* cells in the absence of the Rad53p kinase (Fig. 5A), which links the phosphorylation of Rfa2p with transcriptional induction. To fur-

Figure 4. *set1Δ*-dependent phosphorylation of Rfa2p decreases its binding to URS elements and leads to transcriptional induction of repair genes. (A) Scheme of the DNA regions flanking the URS elements of *RNR2* (pURS2-Rnr2) and *RAD51* (pURS2-Rad51) that were used to analyze the binding of Rfa2p to the URS elements. The pairs of primers used to amplify pURS2-Rnr2, pURS2-Rad51, and the control fragments pRnr2, pRad51, and pBdf1 are indicated. (B, top) Rfa2p binds to URS elements in vivo. The ChIP assay was performed as described in Materials and Methods. Rpa2myc and Ku80myc indicate that the parental strain UCC1001 bears a 18-mer Myc epitope fused to the carboxyl terminus of the genomic copy of *RFA2* and *HDF2*, respectively. Immunoprecipitation was performed with anti-myc antibodies (9 E10). After DNA purification, PCR was performed with primers for the sequences flanking the URS2 of *RNR2* (pURS2-Rnr2) and *RAD51* (pURS2-Rad51), and for the control sequence in *BDF1* (pBdf1). PCR reactions were performed on immunoprecipitated DNA and on the corresponding input DNA. PCR products (indicated by arrows) were resolved on 2% agarose gels. (Bottom) Rfa2p binds to URS2-Rnr2 and URS2-Rad51 but not to the adjacent sequences, pRnr2 and pRad51. DNA regions pRnr2 and pRad51 are localized at ~1400 bp of the URS2 sequences of *RNR2* and *RAD51*, respectively. (C) In vivo association of Rfa2p with the URS2 of *RAD51* is diminished in *set1Δ* cells. Chromatin solutions from wild-type and *set1Δ* cells expressing the Rfa2-myc were subjected to ChIP. The amount of precipitated DNA was analyzed by quantitative PCR (see Materials and Methods). The signals obtained by PCR in the Rfa2-myc immunoprecipitates were quantitated by referring them to a standard curve. This standard curve was obtained by plotting the signals obtained from PCRs performed with the same primers on serial dilutions of the corresponding input DNA. The effect of the *set1Δ* mutation was analyzed in different parental strains. For each couple of strains (UCC1001 (wt), UCC1001 *set1Δ*), (UCC1001 *mec1Δ sml1Δ*, UCC1001 *mec1Δ sml1Δ set1Δ*), and (UCC1001 *mec1Δ rad53Δ sml1Δ*, UCC1001 *mec1Δ rad53Δ sml1Δ set1Δ*), amplification of the input DNA samples with the specific and control primers produced similar amount of pBdf1 and pURS2-Rad51 PCR DNA fragments. For each graph, the amount of DNA corresponding to pURS2-Rad51 in the precipitated DNA from the parental strain is arbitrary plotted as 1. The experiments shown were done in triplicate.

ther investigate the effect of the *set1Δ* mutation on the induction of repair genes, wild-type and *set1Δ* cells containing a chromosomally encoded *RAD51-lacZ* gene fusion were assayed for β -galactosidase activity. We observed that *RAD51-lacZ* expression was increased three-fold in exponentially growing *set1Δ* cells relative to wild-type cells (Fig. 5B, left). This result suggests that the

level of Rad51p and probably of other repair proteins is increased in *set1Δ* cells. Finally, results of Figure 3C indicate that Dun1p was not required for the effect of the *set1Δ* mutation on the increase of the DNA repair capacities of the cell. We further investigated this point by measuring β -galactosidase activities of cells bearing the β -galactosidase-reporter plasmid pZZ20. This plasmid

Figure 5. (A) The *set1Δ* mutation abolishes the repression mediated by the MAG URS1 element. We compared for each strain the β-galactosidase activity associated with the *CYC1-lacZ* vector (pNG22) and with pNG22 containing the MAG URS1 element (pNG22+URS1). The enzymatic activity associated with pNG22 was taken as control. (B, left) The *set1Δ* mutation increases the *RAD51-lacZ* expression; (right) the *set1Δ* mutation does not act via the D1 element of the *RNR2* promoter. (C) protein levels of Rad51p and Rnr2p increase in *set1Δ* cells. A 13-mer Myc epitope was fused to the carboxyl terminus of the genomic copy of *RAD51* and *RNR2*. Ku80myc was used as an internal control. Identical amounts of total protein extracts were analyzed by SDS-10% PAGE and immunoblotting. The presence of Rnr2p, Rad51p, and Ku80p was revealed with 9 E10 (anti-myc) antibodies. Relative amounts of proteins were determined by analyzing the chemiluminescence signals.

contains a 42-bp element (D1) from the *RNR2* promoter inserted between the α1 gene *UAS* and the *CYC1* basal promoter. The D1 element has been shown to be critical for the Mec1–Rad53–Dun1-dependent transcriptional response to DNA damage (Huang et al. 1998). We found that wild-type and *set1Δ* cells bearing this plasmid had a similar *lacZ* activity suggesting that the absence of *SET1* did not mediate any changes in the expression of *RNR2* through sequences within D1 (Fig. 5B, right).

The use of *lacZ* reporter constructs suggests that *RAD51* expression is increased threefold in exponentially growing *set1Δ* cells relative to wild-type cells. To

confirm that the protein levels of Rad51p and Rnr2p increase in *set1Δ* cells, we compared the amounts of Rnr2p, Rad51p, and Ku80p in *set1Δ* cells with wild-type cells. We analyzed by immunoblotting in wild-type and *set1Δ* cells identical amounts of total protein extracts for the presence of Myc-tagged Rnr2p, Rad51p, and Ku80p (Fig. 5C). Quantification analysis indicates that there is a three- to fourfold induction of the expression of Rnr2p and Rad51p in *set1Δ* cells compared with wild-type, whereas the amount of Ku80p remains unchanged.

Taken together, these results indicate that the *set1Δ*-dependent phosphorylation of Rfa2p decreases its binding to URS, resulting in a derepression of repair genes.

Rfa2 regulates the transcriptional induction of repair genes

To directly test the role of Rfa2p in the induction of the expression of repair genes, we analyzed the ultraviolet sensitivity of a *mec3Δ* strain carrying the *rfa2 Δ40* allele. The *mec3Δ rfa2 Δ40* strain produces Rfa2p Δ40 as a sole form of Rfa2p. Strikingly, the *rfa2 Δ40* allele strongly suppressed the sensitivity to ultraviolet of the checkpoint mutant *mec3Δ* mutant strain (Fig. 6A). These data indicate that the sensitivity to DNA damage of the checkpoint mutant *mec3Δ* is suppressed by the deletion of the amino-terminal region of Rfa2p. Moreover, we found that the *set1Δ* mutation had no additive effect on the ultraviolet sensitivity of the *mec3Δ rfa2 Δ40* (Fig. 6A), indicating that the deletion of the amino-terminal region of Rfa2p abolishes the effect of the *set1Δ* mutation. Overall, these data are in full agreement with the idea that the amino terminus of Rfa2p is a target of a phosphorylation regulated by *SET1*.

Because the sensitivity to ultraviolet radiation of a *mec3Δ* checkpoint mutant was suppressed by the *rfa2 Δ40* mutation, we determined whether the *rfa2 Δ40* mutation modified cell-cycle progression after DNA damage and whether this mutation restored the ability of *mec3Δ* cells to arrest cell cycle progression. We released cells synchronized in G₁ into the cell cycle in either the presence or absence of a sublethal dose of MMS and measured the DNA content of cell cultures every 15 min. (Fig. 6B). In the absence of DNA damage, both *rfa2 Δ40* and *rfa2 Δ40 mec3Δ* mutants appear to replicate their DNA slightly more slowly than wild-type or *mec3Δ* cells (Fig. 6B). In the presence of MMS, wild-type and *rfa2 Δ40* cells have still not completed replication 150 min after release from α-factor, whereas most *mec3Δ* cells had a 2C DNA content 75 min after α-factor release. This indicates that the *rfa2 Δ40* mutation does not alter the ability of cells to delay replication after DNA damage. During MMS treatment, DNA replication in *mec3Δ rfa2 Δ40* cells is completed 120 min after release, whereas *rfa2 Δ40* cells still have a 1C DNA content 150 min after release. Thus, the *rfa2 Δ40* mutation does not seem to suppress the checkpoint defect of *mec3Δ* cells. We also measured cell lethality during the MMS treatment (Fig. 6B). In agreement with the results described in Figure 6A, the deletion of the amino-terminal domain of Rfa2p

Figure 6. (A) The sensitivity to ultraviolet radiation of the *mec3* Δ mutant is suppressed by the deletion of the amino-terminal region of Rfa2p. Approximately 1000 cells of K699 derivative strains with the indicated relevant genotypes were spread on YPD plates and exposed to ultraviolet irradiation. Cells were grown at 30°C for 5 d before we counted the number of colonies. (B) Analysis of intraS checkpoint of the *rfa2* Δ 40 mutants. Exponentially growing (exp) wild-type, *mec3* Δ *rfa2* Δ 40 and *mec3* Δ *rfa2* Δ 40 cells were synchronized with α -factor and released in YPD medium with (+MMS) or without 0.02% MMS. (Left) Untreated or MMS-treated samples were taken at the indicated times after α -factor release and analyzed. (Right) Aliquots were removed from the MMS-treated cultures to determine cell number and to score for colony-forming units on YPD plates at 25°C. (C) The *rfa2* Δ 40 mutation abolishes the repression mediated by the MAG URS2 element. We measured the β -galactosidase activity associated with the *CYC1-lacZ* vector (pNG22) and with pNG22 containing the MAG URS1 element (pNG22+URS1). (D) The *rfa2* Δ 40 mutation increases the *RAD51-lacZ* expression.

suppressed the sensitivity to DNA damage of the *mec3* Δ mutant.

We then asked whether enhancing of the DNA repair capacities in the *rfa2* Δ 40 mutant was associated with

transcriptional derepression of repair genes as is the case in *set1Δ* cells. We first transformed pNG22 (*CYC1-lacZ* vector) and pNG22+URS1 in the *rfa2 Δ40* mutant and measured the β -galactosidase activity. As for the *set1Δ* mutation, we observed that the repressive effect of the MAG URS1 region was abolished in *rfa2 Δ40* cells (Fig. 6C). We further investigated the effect of the *rfa2 Δ40* mutation on the induction of repair genes by fusing *lacZ* to *RAD51* in *rfa2 Δ40* cells. We observed that expression of the chromosomally encoded *RAD51-lacZ* was increased about threefold in exponentially growing *rfa2 Δ40* cells relative to wild-type cells (Fig. 6D). This result suggests that the level of Rad51p and probably of other repair proteins is increased in *rfa2 Δ40* cells as is the case in *set1Δ* cells.

Discussion

The absence of SET1 activates a Rad53p-signaling pathway

We have shown previously that the deletion of *SET1* abolishes TPE, reduces telomere length, and increases the viability after DNA damage of a *mec3* null mutant and also of other checkpoint mutants (*rad9*, *rad17*, *rad24*). The major contribution of this work is the finding that, in response to the loss of *SET1*, a novel Rad53-dependent pathway leading to Rfa2p hyperphosphorylation is activated and this results in activation of repair genes.

The proteins containing a SET domain are considered to behave as global chromatin regulators, a concept strengthened by the recent finding of a SET-dependent histone H3-specific methyltransferase activity (Rea et al. 2000). The first interpretation of the Rad53-dependent pathway induction upon the loss of *SET1* is that this response results from an alteration in the chromatin organization. It is tempting to speculate that this pathway is initiated by changes in the posttranslational modification of chromatin components. This suggests the existence of chromatin damages that can be relayed by signaling pathways.

Another, not exclusive, interpretation for the *set1Δ*-induced cellular response is that *SET1* regulates phosphorylation pathways by modulating the activity of kinases or phosphatases. For instance, phosphatases that dephosphorylate Rfa2p could be potential targets for Set1p. Cui et al. (1998) proposed that interaction between the SET domain and SET interacting domains (SID) in mammalian cells might regulate the activity of phosphatases or so-called antiphosphatases, thereby engaging cells through growth or differentiation. Interestingly, *sml1 mec1-1 set1Δ* cells exhibit elongated buds (V. Schramke and V. Gelí, unpubl.) similar to those displayed by *hsl1* and *hsl7* (histone synthetic lethal) or *cdc55* mutants (Ma et al. 1996; Jiang and Broach 1999). *hsl1* and *hsl7* were identified in a screen for mutations that are lethal in combination with a deletion of the amino terminus of histone H3, whereas Cdc55p is a regulatory subunit of phosphatase 2A. Strikingly, we

have also observed that the deletion of *SET1* suppresses the growth defect of a *sml1 mec1-1 tel1* double mutant. *sml1 mec1-1 tel1* double-mutant strains have very short telomeres and a senescent phenotype in *S. cerevisiae* and lose all telomeric sequences in *Schizosaccharomyces pombe* (Naito et al. 1998; Ritchie et al. 1999). As a model for the functions of Tel1p and Mec1p, it has been proposed that Tel1p and Mec1p affect the accessibility of telomeres to telomerase and exonucleases by phosphorylation of target proteins located at telomeres (Ritchie et al. 1999). Taken together, these observations suggest further links between *SET1*, chromatin, telomeres, and signaling phosphorylation.

The set1Δ pathway overlaps with but is distinct from the DNA damage pathway

Our work establishes that the *set1Δ*-induced response is different from the response produced by a DNA damage, although some components are shared and although both pathways result in an increased repair potency. In particular, we showed that (1) the hyperphosphorylation of Rfa2p is ATM homolog independent; (2) although the Rad53 kinase domain is required, no apparent phosphorylation of Rad53p can be visualized in *set1Δ* cells; (3) the activation of the repair genes in *set1Δ* cells does not require *DUN1*; (4) the checkpoint functions and cell cycle progression are not altered in *set1Δ* cells. On the basis of these findings, a model for the signal transduction pathway in response to *set1Δ* can be suggested (Fig. 7; see below). We do not think that the effects of *set1Δ* are simply due to the *set1Δ*-induced loss of TPE or to the alteration in expression of genes near telomeres, because a *sas2* mutant, which also has a clear TPE defect (Reifsnnyder et al. 1996), does not exhibit the phenotypes associated with the *set1Δ* mutant.

Rfa2p hyperphosphorylation in response to the loss of SET1

Phosphorylation of yeast Rfa2p has been shown to depend on Mec1p and Tel1p (Brush et al. 1996). Nevertheless, it has not been determined whether *MEC1* and *TEL1* encode RPA kinases, a role that has been mainly suggested for DNA-PK, ATM, and cyclin-dependent kinases (CDK) in human cells (Liu and Weaver 1993; Brush et al. 1996; Henricksen et al. 1996; Niu et al. 1997). Our results suggest that the amino-terminal region of Rfa2p is required for the Set1p-regulated phosphorylation of Rfa2p in a Mec1-independent manner. Importantly, we show that Rad53p is required for this *set1Δ*-dependent phosphorylation. Our data also indicate that either Mec1p is involved in the phosphorylation of a region outside of the first 40 residues of Rfa2p, or that truncation of the amino-terminal region of Rfa2p unveils a cryptic site phosphorylated in a Mec1-dependent way. Additional work will be required to identify the Mec1- and Set1-dependent phosphorylation sites of Rfa2p.

Figure 7. A schematic representation of the *set1Δ*-induced cellular response that leads to the transcriptional activation of repair genes. We propose that phosphorylation of Rfa2p can result either from (1) the activation of Rad53p (Rad53*) in response to a *set1Δ*-induced chromatin damage, or (2) from the deregulation of a phosphorylation pathway controlled by Set1p. Both models are not exclusive. The *set1Δ* cellular response is shown in parallel with the DNA damage response. N-Pi and C-Pi indicated phosphorylation in the amino and carboxyl terminus of Rfa2p, respectively.

set1Δ alleviates the repression of repair genes

We observed previously that the deletion of *SET1* increased the viability after DNA damage of DNA damage sensor mutants (*rad9*, *rad17*, *rad24*, *mec3*) without restoring any of the three DNA damage checkpoints (Corda et al. 1999). We now establish that this holds true for *sml1 mec1-1* and *sml1 mec1-1 tel1* double mutants and that the increased repair capacity of *set1Δ* cells can be, at least to some extent, explained by an enhanced expression of repair genes. The transcription of many repair genes is under the negative control of URS elements that are recognized in vitro by RPA (Singh and Samson 1995). We show here that these sequences are efficiently bound by Rfa2p in vivo, but are no longer associated with Rfa2p in *set1Δ* cells. Furthermore, the loss of *SET1* relieves the repressive effect of an URS element located upstream of a heterologous promoter in a Rad53-dependent fashion. Altogether, these results suggest that the loss of *SET1* alleviates the repressive effect of RPA at the promoter of some repair genes. Whether the Mec1-dependent phosphorylation of Rfa2p contributes to the derepression of the repair genes remains to be determined.

In addition to an increased expression of repair genes, *set1Δ* cells could improve their repair capacity by other means. For instance, the phosphorylated Rfa2p proteins could participate in the processing of DNA lesions. It has been proposed that *RAD9*, *RAD17*, *RAD24*, and *MEC3* process some forms of DNA damage to a single strand by DNA degradation (Lydall and Weinert 1995).

Rfa2p regulates in vivo the expression of repair genes

Our results indicate a strong correlation between the Rad53-dependent phosphorylation of Rfa2p in *set1Δ* cells and the partial suppression of checkpoint mutant sensitivity to radiation. The role of Rfa2p in this process is strengthened by the observation that the deletion of the amino terminus of Rfa2p suppresses the sensitivity of *mec3Δ* cells to ultraviolet and increases the expression of the *Rad51-lacZ* reporter protein. We explain the similarity between the phenotypes displayed by the *rfa2 Δ40* mutant and those displayed by *set1Δ* mutant by the fact that both the Rfa2p $\Delta 40$ and the hyperphosphorylated Rfa2p have a reduced ability to bind the URS elements. In agreement with this proposal, the deletion of *SET1* does not further increase the ultraviolet resistance of *rfa2Δ 40 mec3Δ* cells. This shows that the forty-first amino acids of Rfa2p are required for *set1Δ* to rescue the ultraviolet sensitivity of *mec3Δ*. Because *rfa2 Δ40* appears to be epistatic to *set1Δ*, this further links hyperphosphorylation of Rfa2p with the increased ultraviolet resistance of a *mec3Δ set1Δ* strain as compared with a *mec3Δ* strain.

Suppression of UV and MMS sensitivity of *mec3Δ* cells by the *rfa2 Δ40* allele does not appear to imply suppression of *mec3Δ* checkpoint defect. These data are in agreement with previous observations (Lydall and Weinert 1995; Corda et al. 1999), suggesting that the loss of viability after DNA damage of the checkpoint mutants is not solely due to their inability to arrest cell cycle progression after DNA damage. The delay in DNA

replication that we observed in the *mec3Δ rfa2 Δ40* MMS-treated cells compared with the *mec3Δ* single mutant is likely due to the fact that the *rfa2 Δ40* mutation causes, per se, a delay in DNA replication even in the absence of DNA damage. The effect of this replication defect might be amplified when DNA synthesis occurs on a damaged template.

New connections between chromatin and repair

A remarkable result of this work is that the same mutation (*set1Δ*) increased repair potency of cells and lead to chromatin injuries. The *set1Δ* cellular response may correspond to the derepression of at least 12 DNA repair and metabolism genes carrying MAG URS-like sequences (Xiao et al. 1993), namely *MAG* and *MGT1* (DNA alkylation repair genes), *PHR1* (involved in light-dependent repair of pyrimidine dimers), *RAD1*, *RAD2*, *RAD4*, *RAD10*, and *RAD16* (components of the nucleotide excision repairosome), *RAD51* (a central component of the recombinational repair), *DDR48* (a stress protein induced by DNA damage), and *RNR2* and *RNR3* (subunits of the ribonucleotide reductase). Understanding the *raison d'être* of this response is likely to provide new insights into the connections between chromatin and repair. For instance, one can speculate that the increased repair capacity counteracts an enhanced susceptibility of DNA to damage in the context of a dysfunctional chromatin. Also, the repair genes can have a function in chromatin organization, even in the absence of DNA damage. This could occur in wild-type cells when the chromatin has to be transiently reconstructed, for example, during cell cycle or when large chromatin domains are reorganized in response to modifications of the cellular program.

In light of the conservation through evolution of the proteins involved in the *set1Δ* pathway, the relationships between chromatin-associated SET domain proteins and signaling kinases established here in yeast are likely to be shared by higher eukaryotes.

Materials and methods

Yeast strains and plasmids

Most yeast strains used in this study are isogenic either to K699 (W303 background) or UCC1001 (YPH250 background). Chromosomal deletions of *SET1* and *MEC3* were performed as described (Corda et al. 1999). The *sm11 mec1-1* strain (DMP2394/18B) is virtually isogenic to K699 (Paciotti et al. 1998). The *sm11 mec1-1* allele that we used eliminates the essential functions of *MEC1* (Weinert et al. 1994; Zhao et al. 1998). To disrupt the 5' end of *TEL1* with *URA3*, we linearized plasmid pPG47 with *SacI* and transformed appropriate yeast strains (Greenwell et al. 1995). The *rad53K227A* mutant strain is isogenic with W303 (Pelliccioli et al. 1999). To introduce the null allele of *MEC1* and *RAD53*, we first disrupted *SML1* by PCR amplifying a *sm11::TRP1* disruption cassette from plasmid pWJ700 (kindly provided by X. Zhao, Columbia University, New York). The *set1Δ::KAN*, *mec1Δ::KAN*, *mec1Δ::LYS2* null mutations were introduced as described (Fairhead et al. 1996). *RAD53* was dis-

rupted by integration of a PCR product containing the nourseothricin (*nat*) resistance gene (Goldstein and McCusker 1998). *yKu80* and *Rfa2p* were tagged at their carboxyl termini with 18 MYC epitopes with the help of pVL1001 (from Connie Nugent, Baylor College of Medicine, Houston, TX) and pGG1 (from Jérôme Tonnelier, CNRS, Marseille, France), respectively. To analyze *Ddc1p* phosphorylation in *set1Δ* cells, *SET1* has been disrupted in YLL334 that carries a chromosomal *DDC1-HA2*-tagged allele.

lacZ plasmids to assay the URS function, pNG22 and pNG22+URS1, were described by Singh and Samson (1995). The *RAD51-lacZ* gene fusion has been described previously (Aboussekhra et al. 1992). The *RAD51-lacZ* gene fusion containing the entire *RAD51* gene has been integrated into the *RAD51* locus by homologous recombination. Plasmid pZZ20 that contains the $\alpha 1$ -*UAS-D1-CYC1-lacZ* region has been described by Huang et al. (1998).

The deletion removing the *RFA2* intron and the region extending from codon 3 to codon 40 of *RFA2* was performed by PCR site-directed mutagenesis. By replacing the chromosomally encoded *RFA2* with the *rfa2 Δ40* mutant allele, we generated an allele encoding a truncated protein extending from residue 1 to 165 (*rfa2 165*) that is coexpressed with *Rfa2p Δ40*. To eliminate potential effects of *rfa2 165*, this nonviable truncated allele was fully disrupted with a *kanMX4* marker. The resulting strain produces *Rfa2p Δ40* as the sole form of *Rfa2p*. As a control, we also constructed a strain producing the truncated *Rfa2p 165* with the full-length *Rfa2p* (data not shown). This control was done to ensure that the phenotypes associated with the allele *rfa2 Δ40* are due to the deletion of the amino terminus region.

Protein extracts and Western blot analysis

Protein extracts for Western blot analysis were prepared from trichloroacetic acid (TCA)-treated yeast cells. Protein extracts were resolved by electrophoresis on a 15% SDS-polyacrylamide gel (80:1 acrylamide:bis-acrylamide). Immunoblots were developed with rabbit anti-serum directed against *Rfa2p* (kindly provided by S. Brill, Rutgers University, NJ) and B. Stillman, Cold Spring Harbor Laboratory, NY) or with anti-HA monoclonal antibodies (for the analysis of *Ddc1p*).

Native extract preparation and phosphatase treatment

Exponentially growing cells (15 mL) were harvested by centrifugation at 4°C. The pellet was washed with 10 mL of ice-cold water and the cells were collected by centrifugation. The pellet was resuspended in 0.2 mL of lysis buffer (50 mM HEPES at pH 7.5, 2 mM EDTA, 2 mM DTT, 20% glycerol, 0.2 mM PMSF). An equal volume of acid-washed glass beads was added and the cells were disrupted by vortexing. Native extracts were divided into two samples, and 15 μ L of native extract were incubated for 30 min at 30°C with 2 μ L of 10 $\times$ λ -phosphatase buffer (Biolabs) supplemented with 2 mM $MnCl_2$ with or without 150 units of λ -phosphatase (Biolabs). *Rfa2p* was analyzed by Western blot analysis as described previously.

Chromatin immunoprecipitation

The extraction and sonication of formaldehyde-fixed yeast cells were done as described (Strahl-Bolsinger et al. 1997). The average size of sonicated DNA was between 500 and 1000 bp. Immunoprecipitation of cross-linked DNA was performed with 9 E10 (anti-myc) agarose-conjugate monoclonal antibodies (Santa-cruz Biotechnology) for 3 h at 4°C. An aliquot of each sample was not immunoprecipitated (input). PCR reaction parameters were as described (Strahl-Bolsinger et al. 1997) except for the annealing temperature (62°C). Primers used to amplify the 500-

bp *RNR2 URS2* (pURS2Rnr2) (5'-GCAACGGGCAACCGTTTGGGG-3'; 5'-GGAAGGGGTCTCTTTAGGC-3') and the 250-bp *RAD51 URS2* (pURS2Rad51) (5'-CGCGTCATTCCGCTATTC-3'; 5'-CGAACCGTTCCCGTACTGAAGC-3') are specific for the upstream regions of *RNR2* and *RAD51*, respectively. Both PCR DNA fragments flank the *URS2* sequence. Control primers used to amplify the 520-bp *RNR2* (pRnr2) (5'-CATCTCCTTGCCCGTAAGACC-3'; 5'-CCGCAGATGGGACCTCTTATG-3') and the 230-bp *RAD51* (pRad51) (5'-CTCACCTTGCTTACCAGAGGC-3'; 5'-CCGCAGTAGGGTTGCAGGTA-3') control sequences are specific for regions starting 120 and 159-bp upstream of the stop codons of *RNR2* and *RAD51*, respectively. Control primers specific for *BDF1* (5'-GCCCGCGCACTTTGTGTGTGC-3'; 5'-GGGAAGGAGCGACCAGTAGGC-3') produce a 100-bp PCR fragment.

For DNA quantitation, amplifications were done by use of a LightCycler (Roche Biochemicals) in a standard PCR reaction containing 0.5 μ M of primers specific for the pURS2Rad51 and SYBR Green I Dye (Roche Biochemicals). Template concentration was determined by absorbance at 260 nm. The reactions were cycled 45 times with the following parameters: denaturation at 95°C, annealing at 62°C for 8 sec followed by a 16-sec extension at 72°C. Fluorescence was acquired at the end of the extension phase. The melting curves were obtained at the end of amplification by cooling the sample to 70°C (20°C/sec) and then increasing the temperature to 95°C at 0.1°C/sec. Fluorescence was acquired every 0.1°C. Quantitation data was analyzed by use of the Light Cycler analysis software.

Miscellaneous

Radiation sensitivity and MMS synchrony were carried out as described (Paulovich and Hartwell 1995; Corda et al. 1999). β -galactosidase activity was assayed as described previously (Kovari et al. 1990). Protein concentration was determined with the BCA protein assay reagent. Total protein extracts were TCA precipitated and analyzed by SDS-10% PAGE and immunoblotting. The presence of Rnr2p-myc13, Rad51p-myc13, and Ku80p-myc18 was revealed with 9 E10 (anti-myc) antibodies. Relative amounts of proteins were determined with a Kodak image station 440 CF.

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