The set1Δ 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 set1Δ-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 mec3Δ 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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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; Filipova 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.

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 smll mec1-1 cells [Brush et al. 1996] (see Materials and Methods for the smll 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 smll 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; Pellicioli 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 smll mec1-1 rad53K227A and smll mec1-1 rad53K227A set1Δ mutants. We failed to detect any significant phosphorylation of Rfa2p when the rad53K227A allele was introduced in smll mec1-1 and smll 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-
Transcriptional activation of repair genes by RPA

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 tel1 mutants. Protein extracts from exponentially growing cultures (exp), from G1-arrested cultures (α-F), and MMS treated G1-arrested cultures (MMS), were analyzed by Western blot with anti-pRfa2p polyclonal antibodies.

Phosphorylation of Rfa2p in set1Δ cells can be further increased after DNA damage (Brush et al. 1996). As overexpression 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 Δ40]. 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 correlates to the largest viable amino-terminal truncation of Rfa2p [Philipova et al. 1996].

Interestingly, the yeast-made Rfa2p Δ40 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 Δ40 with phosphatase, we observed that the upper band was converted to the lower one, indicating that the reduced mobility of Rfa2p Δ40 is due to phosphorylation [Fig. 2B]. We also observed that Rfa2p Δ40 was neither phosphorylated in sml1 mec1-1 nor in sml1 mec1-1 set1Δ mutants [Fig. 2A]. This latter result suggests that the Mec1p-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 Δ40. [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 Δ40. [B] Native extracts of exponentially growing rfa2Δ40 and rfa2Δ40 set1Δ cells were divided in two samples and treated [+ or -] 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 mecl-1 mutant [Fig. 3A]. We showed that the viability after irradiation of a sml1 mecl-1 mutant was increased in the absence of SET1 [Fig. 3A]. Similarly, the set1Δ mutation increased the viability after UV of a sml1 mecl-1 tel1 mutant [Fig. 3A].

To examine whether the increase of viability of sml1 mecl-1 set1Δ cells with respect to sml1 mecl-1 cells after ultraviolet treatment requires the kinase activity of Rad53p, we compared the ultraviolet sensitivity of a sml1 mecl-1 rad53K227A with the isogenic sml1 mecl-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 mecl-1 and mec3Δ mutants after DNA damage is mediated by kinase activity of Rad53p.

Surprisingly and yet unexplained, we observed repeatedly that the sml1 mecl-1 rad53K227A [or rad53Δ] mutant was less sensitive to DNA damage than the sml1 mecl-1 mutant. Thus, the inactivation of RAD53 increased the viability after DNA damage of a sml1 mecl-1 mutant. This explained the fact that the sml1 mecl-1 rad53K227A set1Δ exhibited increased survival after UV irradiation with respect to the sml1 mecl-1 set1Δ, contrary to what one could have expected, as Rfa2p is not hyperphosphorylated in the sml1 mecl-1 rad53K227A set1Δ strain.
We also observed that the deletion of SET1 suppressed the growth defect of the sml1 mec1-1 tel1Δ 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 carboxyl 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 URSs 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-
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 threefold 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
Schramke et al.

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 Rfa2 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 Rfa2 in the induction of the expression of repair genes, we analyzed the ultraviolet sensitivity of a mec3Δ strain carrying the rfa2Δ40 allele. The mec3Δ rfa2Δ40 strain produces Rfa2pΔ40 as a sole form of Rfa2p. Strikingly, the rfa2Δ40 allele strongly suppressed the sensitivity to ultraviolet of the checkpoint 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 rfa2Δ40 (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 rfa2Δ40 mutation, we determined whether the rfa2Δ40 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 rfa2Δ40 and rfa2Δ40 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 rfa2Δ40 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 rfa2Δ40 mutation does not alter the ability of cells to delay replication after DNA damage. During MMS treatment, DNA replication in mec3Δ rfa2Δ40 cells is completed 120 min after release, whereas rfa2Δ40 cells still have a 1C DNA content 150 min after release. Thus, the rfa2Δ40 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
suppressed the sensitivity to DNA damage of the mec3Δ mutant. We then asked whether enhancing of the DNA repair capacities in the rfa2 Δ40 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. Gélí, 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.

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; Henrickson 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.

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 [Reifsnyder et al. 1996], does not exhibit the phenotypes associated with the set1Δ mutant.

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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 smI1 mec1-1 and smI1 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 rfa2Δ mutant and those displayed by set1Δ mutant by the fact that both the Rfa2p Δ40 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 mec3Δ cells. This shows that the forty-first amino acids of Rfa2p are required for set1Δ to rescue the ultraviolet sensitivity of mec3Δ. Because rfa2Δ40 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 rfa2Δ40 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 sml1 mec1-1 strain [DMP2394/18B] is virtually isogenic to K699 (Paciotti et al. 1998). The sml1 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 [Pelliccioni et al. 1999]. To introduce the null allele of MEC1 and RAD53, we first disrupted SML1 by PCR amplifying a sml1::TRP1 disruption cassette from plasmid pW700 [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 disrupted 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 pG11 [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 UR5 function, pNG22 and pNG22+URS1, were described by Singh and Samson (1995). The RAD51–lacZ gene fusion has been described previously (Aboussouskha 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 a1–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 μL of native extract were incubated for 30 min at 30°C with 2 μL of 10× λ-phosphatase buffer [Biolabs] supplemented with 2 mM MnCl2 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-
Transcriptional activation of repair genes by RPA

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The set1Δ 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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