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# Srs2 DNA helicase is involved in checkpoint response and its regulation requires a functional Mec1-dependent pathway and Cdk1 activity

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**In *Saccharomyces cerevisiae* the rate of DNA replication is slowed down in response to DNA damage as a result of checkpoint activation, which is mediated by the Mec1 and Rad53 protein kinases. We found that the Srs2 DNA helicase, which is involved in DNA repair and recombination, is phosphorylated in response to intra-S DNA damage in a checkpoint-dependent manner. DNA damage-induced Srs2 phosphorylation also requires the activity of the cyclin-dependent kinase Cdk1, suggesting that the checkpoint pathway might modulate Cdk1 activity in response to DNA damage. Moreover, *srs2* mutants fail to activate Rad53 properly and to slow down DNA replication in response to intra-S DNA damage. The residual Rad53 activity observed in *srs2* cells depends upon the checkpoint proteins Rad17 and Rad24. Moreover, DNA damage-induced lethality in *rad17* mutants depends partially upon Srs2, suggesting that a functional Srs2 helicase causes accumulation of lethal events in a checkpoint-defective context. Altogether, our data implicate Srs2 in the Mec1 and Rad53 pathway and connect the checkpoint response to DNA repair and recombination.**

*Keywords*: checkpoint/DNA recombination/DNA repair/DNA replication/Srs2

## Introduction

All living cells respond to DNA damage by promoting the transcription of several DNA metabolism genes and by coordinating chromosome replication and segregation with DNA repair and recombination (for reviews see Carr and Hoekstra, 1995; Paulovich *et al*., 1997; Weinert, 1998; Lowndes and Murguia, 2000). Many of the genes involved in the DNA damage checkpoint pathway, which is specifically responsible for delaying cell cycle progression in response to DNA damage in eukaryotic cells, have been identified in budding and in fission yeast (Foiani *et al*., 2000). Most of these genes have been highly conserved throughout evolution and their function is important for preventing genome instability and cancer in mammalian cells (Hartwell and Kastan, 1994; Weinert, 1997).

It is generally believed that the DNA damage checkpoint pathway is necessary to delay the cell cycle in response to DNA damage in order to provide the cell with enough time to repair the lesions prior to chromosome replication or segregation. Once the DNA damage has been removed, the cell can re-establish a normal cell cycle through a process known as recovery (Sandell and Zakian, 1993). Recent evidence indicates that the recovery process is associated with checkpoint inactivation (Pellicioli *et al*., 1999), although the nature of this mechanism is still unclear. Moreover, in the presence of an irreparable DNA lesion, the cells do not remain permanently arrested and they resume cell cycle progression through a process known as adaptation (Sandell and Zakian, 1993; Toczyski *et al*., 1997; Lee *et al*., 1998).

In the yeast *Saccharomyces cerevisiae* the DNA damage checkpoint pathway is controlled by a cascade of phosphorylation events mediated principally by the *MEC1*, *RAD53* and *DUN1* gene products (Foiani *et al*., 2000). Mec1 is a member of the evolutionarily conserved subfamily of phosphatidylinositol 3-kinase (PI3-kinase) that includes budding yeast Tel1, fission yeast Rad3, mammalian ATM and ATR and DNA-dependent protein kinase (DNA-PK) (Elledge, 1996). It is generally assumed that Mec1 is a protein kinase, but in the absence of direct biochemical evidence the physiological targets of Mec1 remain speculative. The Rad53 protein kinase is highly homologous to human Chk2 and *Schizosaccharomyces pombe* Cds1 (Lowndes and Murguia, 2000), and it is phosphorylated and activated in response to DNA damage through a process that requires a functional Mec1 (Sanchez *et al*., 1996; Sun *et al*., 1996). The C-terminal of Rad53 contains a forkhead-associated domain that mediates the interaction with Rad9, another checkpoint protein (Sun *et al*., 1998). Rad53 is also required for phosphorylation of Dun1, another protein kinase involved in the checkpoint response (Zhou and Elledge, 1993; Gardner *et al*., 1999). Dun1 plays a major role in the transcriptional induction of several DNA metabolism genes in response to genotoxic treatments (Zhou and Elledge, 1993) and in channelling DNA repair into a non-recombinational pathway (Fasullo *et al*., 1999).

Other factors involved in the DNA damage response include Mec3, Ddc1, Rad17 and Rad24. These proteins are absolutely required for checkpoint activation in $G_1$, while they are only partially needed in response to DNA damage during S phase (Pellicioli *et al*., 1999). Even though the role of these checkpoint proteins is still unknown, it has been suggested that they might participate in DNA damage recognition and/or processing (Lydall and Weinert, 1997; Foiani *et al*., 2000; Lowndes and Murguia, 2000). DNA polymerase ε, replication factor C (RF-C) and the DNA helicase Sgs1 have also been implicated in the checkpoint response and in Rad53 activation (Navas *et al*., 1995;

Sugimoto *et al*., 1997; Frei and Gasser, 2000; Pellicioli *et al.*, 1999). DNA polymerase ε and RF-C are required for DNA replication (Jonsson and Hubscher, 1997), although the catalytic domain of DNA polymerase ε appears to be dispensable (Kesti *et al*., 1999). The role of Sgs1 seems to be more complex since its function has been invoked not only in checkpoint activation (Frei and Gasser, 2000), but also in DNA recombination, replication and transcription (Chakraverty and Hickson, 1999; Lee *et al*., 1999; Gangloff *et al*., 2000).

While significant progress has been made in identifying some of the factors acting in the DNA damage checkpoint pathway, very little is known about the physiological targets of this signal transduction cascade. The DNA replication machinery seems to be one of the final targets of DNA damage checkpoint, which expands the length of S phase in the presence of genotoxic agents (Paulovich and Hartwell, 1995). In fact, two essential replication factors, replication protein A (RP-A) and the DNA polymerase α–primase complex (pol–prim), are modulated by the checkpoint response (Brush *et al*., 1996; Pellicioli *et al*., 1999). RP-A is involved in replication, recombination and repair (Wold, 1997) and, in response to DNA damage, it is phosphorylated through a mechanism dependent upon Mec1, but not on Rad53 (Brush *et al*., 1996). The pol–prim complex, which is required for initiation of DNA synthesis at origins of replication and for lagging strand DNA synthesis (Foiani *et al*., 1997), is also involved in the checkpoint pathway (Marini *et al*., 1997) and is kept unphosphorylated under damaging conditions (Pellicioli *et al*., 1999). Moreover, it has been shown that the cyclin-dependent kinases Cdc7/Dbf4 and Cdc28/Cyclin B (Cdk1), which control DNA synthesis under normal conditions, are also regulated in response to DNA damage checkpoint activation (Cheng *et al*., 1999; Dohrmann *et al*., 1999; Pellicioli *et al*., 1999). However, it is not yet clear whether the delay in S phase progression caused by checkpoint activation in response to genotoxic treatments is simply due to negative regulation of the replication process or, instead, results from intrinsically slow replication mechanisms that couple replication to recombination and repair (Foiani *et al*., 2000). In fact, several pieces of data indicate that prokaryotic and eukaryotic cells use specialized replication mechanisms such as template switching or break-induced replication (BIR) to replicate a damaged template (Higgins *et al*., 1976; Malkova *et al*., 1996; Kogoma, 1997; Holmes and Haber, 1999). These two processes need the function of proteins involved in leading and lagging strand synthesis and, therefore, require a reprogramming of the replication machinery since most of the factors involved are the same as those used under normal conditions. Strand switching models, leading to the formation of Holliday junctions by annealing of the two newly synthesized strands upon encounter of replication forks with DNA lesions, have been proposed to explain translesion synthesis in *Escherichia coli* and mammalian cells (Higgins *et al*., 1976; Seigneur *et al*., 1998) and to account for the accumulation of recombination intermediates in certain yeast replication mutants (Zou and Rothstein, 1997). BIR has been demonstrated in prokaryotes (Kogoma, 1997) and yeast cells (Malkova *et al*., 1996). Although these replication-coupled recombination processes may be responsible for the increase in the length of S phase as a consequence of genotoxic treatments, so far there are no indications that they are regulated by the checkpoint response.

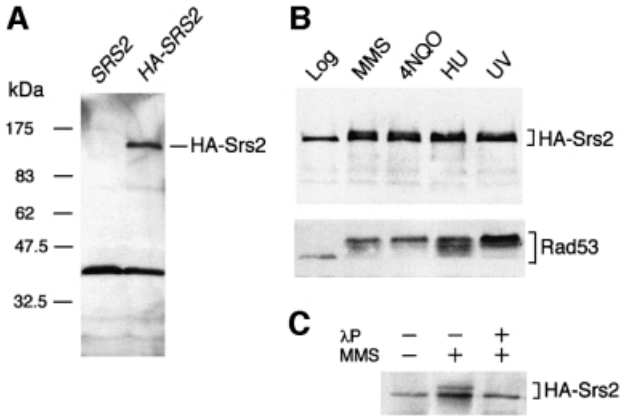


**Fig. 1.** HA-SRS2 is phosphorylated in response to DNA damage. (**A**) Aliquots of total protein extracts prepared from strains K699 (*SRS2*) and CY2715 (*HA-SRS2*), as described in Materials and methods, were separated by SDS–PAGE and analysed by western blotting with the 12CA5 monoclonal antibody (mAb) directed against the HA epitope tag. (**B**) Western blot analysis with 12CA5 mAb and anti-Rad53 polyclonal antibodies (Ab) was performed on aliquots of total protein extracts prepared from CY2715 cells after treatment with 0.02% MMS for 3 h, 2 μg/ml 4-NQO for 1 h, 0.2 M HU for 3 h or 100 J/m$^2$ UV. (**C**) HA-Srs2 immunoprecipitates, obtained from CY2715 cells grown in the presence or absence of MMS, were analysed by western blotting with the 12CA5 mAb. Where indicated, the HA-Srs2 immunoprecipitate was treated with λ-phosphatase (λP) prior to gel loading.

In this paper we show that the *SRS2* gene product is a regulatory target of the checkpoint response. Srs2 is a DNA helicase with 3′–5′ polarity (Rong and Klein, 1993) and mutations in the *SRS2* gene result in an increased rate of gene conversion (Rong *et al*., 1991). Srs2 has been implicated in DNA repair (Aboussekhra *et al*., 1989) and recombination (Pâques and Haber, 1997). Here we show that Srs2 is phosphorylated in response to DNA damage and that this modification is dependent upon a functional checkpoint pathway and on Cdk1 activity. Moreover, we provide evidence that *srs2* mutants are unable to activate Rad53 properly in response to intra-S DNA damage and consequently are defective in slowing down the DNA replication process. Furthermore, our findings suggest that a functional Srs2 helicase causes lethal events in a *rad17* mutant background in response to intra-S DNA damage. Altogether our data implicate Srs2 helicase in the DNA damage checkpoint response.

## Results

We have produced a HA-tagged version of the *S.cerevisiae SRS2* gene to analyse the level and modifications of the corresponding gene product under normal growth conditions and in response to DNA damage. The HA-tagged *SRS2* gene behaves like wild type both under normal growing conditions and in response to DNA damage (data not shown). Western blot analysis performed on a crude extract, prepared from logarithmically growing cells carrying the *HA-SRS2* gene, revealed a major polypeptide with an apparent mol. wt of 140 kDa (Figure 1A). This immunoreactive polypeptide was not present in extracts

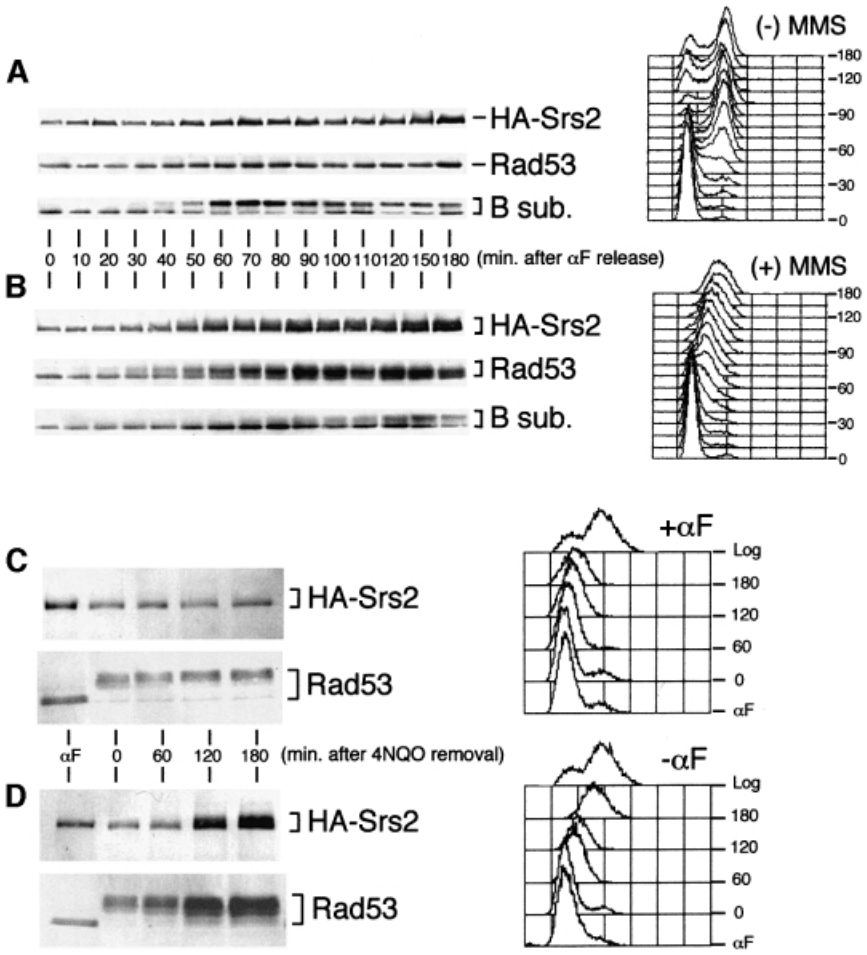


**Fig. 2.** HA-Srs2 phosphorylation requires checkpoint activation and entry into S phase. (**A** and **B**) Aliquots of protein extracts, prepared from CY2715 cells taken at the indicated time after α-factor (αF) release in the absence (A) or presence (B) of 0.02% MMS, were analysed by western blotting with 12CA5 mAb, anti-Rad53 Ab and the 6D2 mAb recognizing the pol–prim B subunit. Aliquots of cells taken at the indicated time were also processed for FACS analysis as described in Materials and methods. (**C** and **D**) CY2715 cells were blocked in $G_1$ by αF treatment, as described in Materials and methods. $G_1$-arrested cells were treated for 15 min with 0.25 μg/ml 4-NQO; the drug was then removed by washing the cells with YPD containing (C) or not containing (D) αF to maintain the $G_1$ block or to allow cell cycle progression. At the indicated times after 4-NQO removal, aliquots of protein extracts were analysed by western blotting with 12CA5 mAb and anti-Rad53 Ab. At the same time points, cell samples were also processed for FACS analysis.

prepared from untagged cells and its size is that predicted for a fusion protein carrying three copies of the HA epitope.

Since Srs2 is involved in DNA repair, we tested whether the level of the protein increased in cells treated with a variety of DNA damaging agents, and/or whether the protein was post-translationally modified under these conditions. As shown in Figure 1B, the amount of HA-Srs2 increased slightly (2- to 4-fold) in response to DNA damaging treatments, similar to that found by measuring *SRS2-lacZ* expression after UV irradiation (Heude *et al*., 1995). Moreover, an additional immunoreactive band migrating more slowly than the HA-Srs2 polypeptide found in extracts from untreated cells was clearly visible after genotoxic treatments. This modified polypeptide was no longer detectable after phosphatase treatment (Figure 1C), indicating that it represents a HA-Srs2 phosphorylated isoform.

We then analysed the HA-Srs2 phosphorylation state in synchronized cells during an unperturbed cell cycle and in response to DNA damage. As shown in Figure 2A, under normal conditions, both HA-Srs2 and the checkpoint protein kinase Rad53 were present in their unphosphorylated forms at any stage of the cell cycle, whereas the B subunit of the pol–prim complex was clearly modified during S phase (Foiani *et al*., 1995). The level of the three proteins approximately doubled when cells were entering S phase, a finding that is likely to be related to the presence of an MCB box in the promoter of the corresponding genes (Johnston and Lowndes, 1992). When wild-type cells were released from the $G_1$ block in the presence of sublethal concentrations of methyl methane sulfonate (MMS) the intra-S DNA damage checkpoint was induced, leading to phosphorylation and activation of the Rad53 protein kinase (Figure 2B; Pellicioli *et al*., 1999) and to a delay in pol–prim B subunit phosphorylation (compare western blots in Figure 2A and B and Pellicioli *et al*., 1999). As a consequence of checkpoint activation, the kinetics of S phase progression was dramatically slowed (Figure 2A and B). In response to MMS treatment, HA-Srs2 became phosphorylated and this modification occurred slightly later than Rad53 activation, raising the possibility that

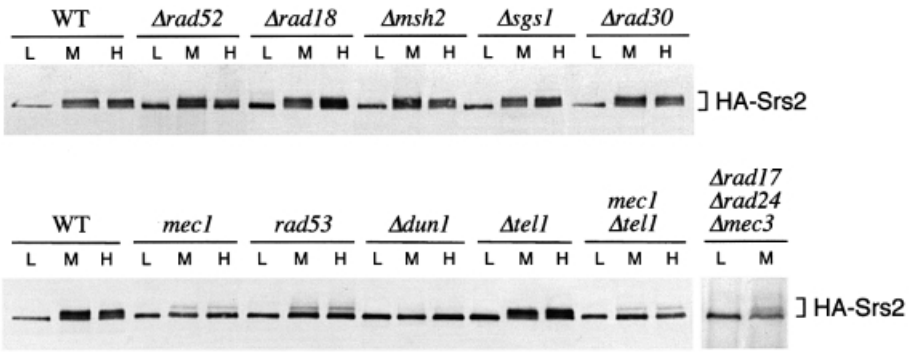


**Fig. 3.** The checkpoint kinases Mec1, Rad53 and Dun1 are required for HA-Srs2 phosphorylation in response to DNA damage. Cultures of strains CY2715 (WT), CY2823 (Δ*rad52*), CY2822 (Δ*rad18*), CY2884 (Δ*msh2*), CY3137 (Δ*sgs1*), CY3135 (Δ*rad30*), CY2835 (*mec1*), CY2837 (*rad53*), CY3138 (Δ*dun1*), CY2885 (Δ*tel1*), CY2888 (*mec1*Δ*tel1*) and CY2827 (Δ*rad17*Δ*rad24*Δ*mec3*) were collected in log phase (L) or treated for 3 h with 0.02% MMS (M) or 0.2 M HU (H). Aliquots of protein extracts were analysed by western blotting with the 12CA5 mAb.

HA-Srs2 phosphorylation might be mediated by the checkpoint response. However, when treatment with the DNA damaging agent 4-nitroquinoline-*N*-oxide (4NQO) was carried out on α-factor-arrested cells that were kept in $G_1$ by maintaining the α-factor block, cells were able to activate Rad53, but failed to phosphorylate HA-Srs2 (Figure 2C). Conversely, when α-factor-arrested cells were treated with 4NQO and then allowed to proceed through the cell cycle after removing the $G_1$ block and the DNA damaging agent, Rad53 remained active throughout the experiment, while HA-Srs2 became phosphorylated at the approximate time of S phase entry and remained phosphorylated for at least 3 h (Figure 2D). This finding indicates that HA-Srs2 cannot be modified in response to DNA damage in $G_1$, but phosphorylation can occur only when cells are allowed to proceed further in the cell cycle. It is possible that in $G_1$ cells either the kinase responsible for HA-Srs2 phosphorylation is limiting, or HA-Srs2 is not available for modification.

To identify genetically the pathway required for HA-Srs2 phosphorylation, we tested whether this modification was impaired in certain genetic backgrounds defective in DNA repair or in the DNA damage checkpoint. As shown in Figure 3, *Δrad52*, *Δmsh2*, *Δrad18*, *Δsgs1* and *Δrad30* mutant cells, which are defective in different repair pathways (Friedberg *et al*., 1995), were still able to phosphorylate HA-Srs2 in response to hydroxyurea (HU) and MMS treatments. Conversely, DNA damage-induced HA-Srs2 phosphorylation was prevented or strongly reduced in *mec1*, *rad53* and *Δdun1* mutant cells, which are defective in three protein kinases required for a proper DNA damage response (Figure 3). This finding indicates that a functional checkpoint is required for HA-Srs2 phosphorylation in response to MMS and HU treatments. The observation that HA-Srs2 was still modified in *Δtel1* cells and that *mec1* and *Δtel1 mec1* mutants exhibited the same level of residual phosphorylation of Srs2 suggests that Tel1 is not required for Srs2 phosphorylation. Several factors including Rad17, Mec3, Ddc1, Rad24 and Rad9 are required for proper Rad53 activation in response to MMS treatment, while they appear to be dispensable in the presence of HU (Pellicioli *et al*., 1999). The finding that in a triple *Δrad17 Δrad24 Δmec3* mutant strain HA-Srs2 phosphorylation was reduced in response to MMS treatment (Figure 3) further confirms that HA-Srs2 phosphorylation is dependent upon a functional checkpoint pathway.

We then analysed the Srs2 phosphorylation state during the recovery process, which allows cells to restore cell cycle progression once the genotoxic stress has been removed. Wild-type cells were treated with HU to activate the checkpoint fully and then released from the HU block to allow recovery. Under these conditions, Rad53 is rapidly inactivated (Pellicioli *et al*., 1999). As shown in Figure 4A, HA-Srs2 was still phosphorylated at 40–50 min after HU removal, while, at the same time, Rad53 was completely dephosphorylated and its kinase activity strongly reduced (data not shown).

We have recently found that Rad53 probably modulates the activity of the Cdc28 protein kinase in response to DNA damage (Pellicioli *et al*., 1999). We thus tested whether Cdk1 was playing a role in HA-Srs2 phosphorylation by overexpressing the Cdk1 inhibitor Sic1 when the cells were recovering from the HU block. Cdk1 inhibition caused a faster dephosphorylation of HA-Srs2, while the kinetics of Rad53 dephosphorylation was unaffected (Figure 4B). Analogous results on the timing of HA-Srs2 dephosphorylation were obtained by overexpressing the Cdc14 phosphatase, another Cdk1 inhibitor (data not shown). Cdk1 inhibition by Sic1 overexpression also caused a slower progression through S phase (Figure 4B), probably because the timing of late origin firing was altered under these conditions. To test directly the effect of Cdk1 inhibition on HA-Srs2 dephosphorylation in the absence of any cell cycle effect, Sic1 was overexpressed in HU-arrested cells. As shown in Figure 4C, HA-Srs2 became dephosphorylated in HU-blocked cells, while the Rad53 kinase remained fully phosphorylated and active (Figure 4C and data not shown). Again, identical results were obtained by overexpressing the Cdc14 phosphatase (data not shown). To further confirm the involvement of Cdk1 in Srs2 modification, we tested whether Srs2 phosphorylation was dependent upon the Cdk1-associated protein Cks1, which is required for kinase function of Cdk1 (Hadwiger *et al*., 1989). *cks1* temperature-sensitive mutant cells were treated with MMS at the permissive temperature to allow checkpoint activation, and then the culture was shifted to the restrictive temperature to inactivate Cks1. As

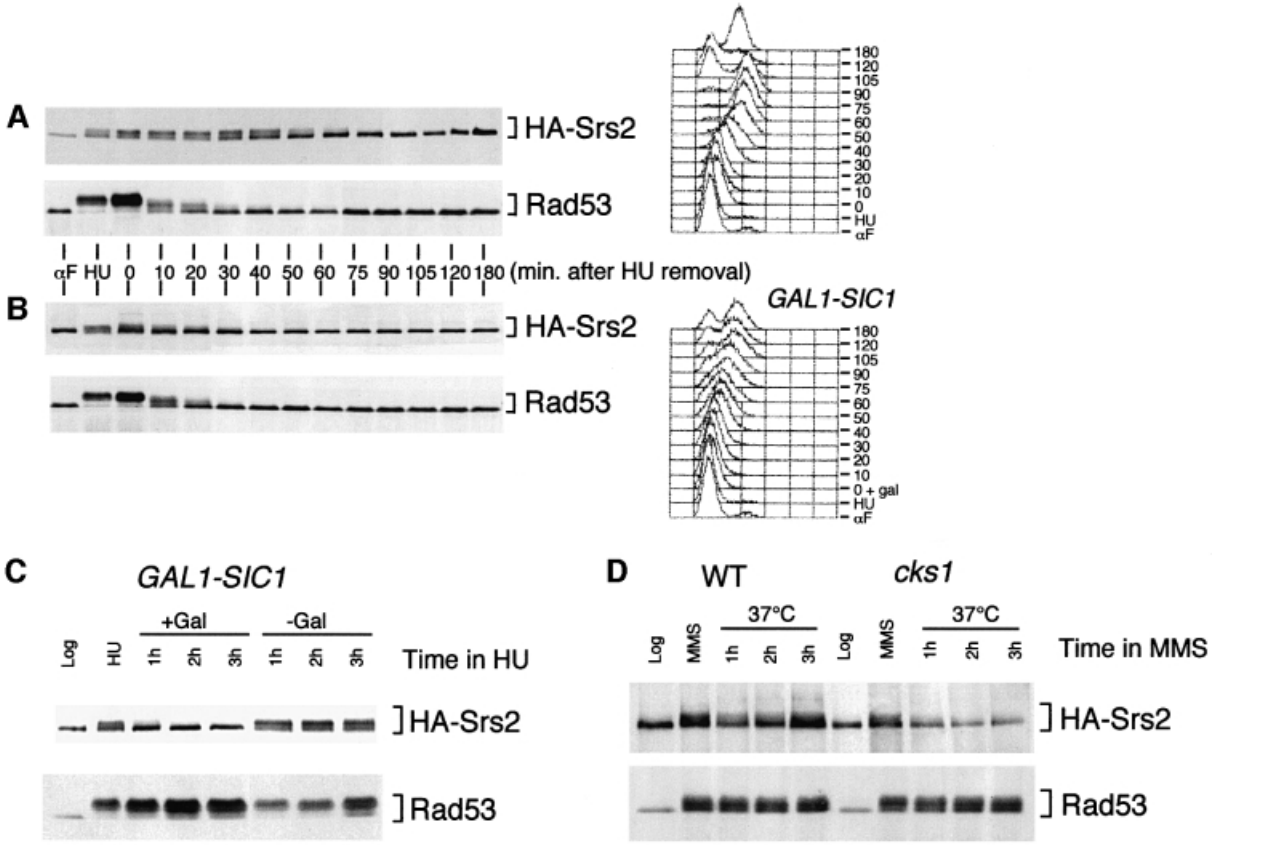


**Fig. 4.** HA-Srs2 is phosphorylated during recovery from HU and its phosphorylation requires a functional Cdk1. (A and B) A log-phase culture of strain CY2735 (*GAL1-SIC1*) was grown in raffinose, presynchronized by αF treatment and released from the $G_1$ block in 0.2 M HU-containing media. Half of the culture was maintained in raffinose (**A**), while galactose was added to the other half of the culture to overexpress Sic1 (**B**). The HU block was then removed by washing the cells with YPD containing either raffinose (A) or galactose (B). Samples were taken at the time points indicated and processed for FACS or for protein extraction. Aliquots of protein extracts were analysed by western blotting performed with the 12CA5 mAb and with anti-Rad53 Ab. (**C**) Log-phase cultures (Log) of strain CY2735 (*GAL1-SIC1*) were grown in raffinose and treated with 0.2 M HU for 3 h. The culture was then divided into two parts, which were maintained in HU with (+Gal) or without (–Gal) galactose addition to induce Sic1 expression. Aliquots of protein extracts were analysed by western blotting with the 12CA5 mAb and anti-Rad53 Ab. (**D**) Log-phase cultures of strains CY2829 (WT) and CY2830 (*cks1*) were grown at 25°C and treated for 3 h with 0.02% MMS. The cultures were then shifted to 37°C in the presence of the drug. Aliquots of protein extracts were analysed by western blot analysis performed with the 12CA5 mAb and anti-Rad53 Ab.

shown in Figure 4D, we found that Cks1 inactivation causes Srs2 dephosphorylation without interfering with the Rad53 phosphorylation state. This result suggests that a functional Cks1 is required to maintain Srs2 phosphorylation in response to DNA damage.

All the data presented until now indicate that HA-Srs2 modification depends upon a functional checkpoint and is mediated by Cdk1, whose activity is in turn modulated by the checkpoint response. To test more directly a possible role of *SRS2* in the checkpoint response, *Δsrs2* cells were arrested in $G_1$ by α-factor treatment and released from the $G_1$ block in the presence of MMS. As shown in Figure 5A and B, the timing of Rad53 activation is not significantly altered in *Δsrs2* cells compared with wild-type cells. However, the level of Rad53 kinase activity was reduced in MMS-treated *Δsrs2* cells, although the level of the protein was the same in wild-type and *Δsrs2* cells (Figure 5A and B). We have previously shown that the defective intra-S DNA damage checkpoint response of a *rad53* mutant allele correlates with premature phosphorylation of the pol–prim B subunit (Pellicioli *et al*., 1999). We found that the inability to activate Rad53 properly in *Δsrs2* cells also correlates with premature phosphorylation of the B subunit, faster cell cycle progression and increased cell lethality (Figure 5A, B and E). However, Rad53 was properly activated in *Δsrs2* cells treated with 4NQO or UV radiation in $G_1$-blocked cells (data not shown). Furthermore, under unperturbed conditions, progression through the cell cycle was indistinguishable in wild-type and *Δsrs2* cells, and Rad53 remained unphosphorylated and maintained the same basal level of kinase activity (Figure 5C and D and data not shown).

We then tested whether the residual Rad53 activity observed in MMS-treated *Δsrs2* cells was dependent upon the checkpoint activating factor Rad17 or Rad24. We found that, in response to MMS treatment, the decrease in the level of Rad53 activity and the rate of S phase progression were indistinguishable in *Δrad17* and *Δrad17Δsrs2* cells, which were both more defective than *Δsrs2* cells (Figure 6A). Therefore, at least for the checkpoint function, *RAD17* seems to be epistatic to *SRS2*. However, cell viability in *Δsrs2* and *Δsrs2Δrad17* mutant cells was higher than in *Δrad17* mutants (Figure 6B), suggesting that cell lethality caused by intra-S DNA damage in a *Δrad17* mutant background is due partially to a functional Srs2 protein.

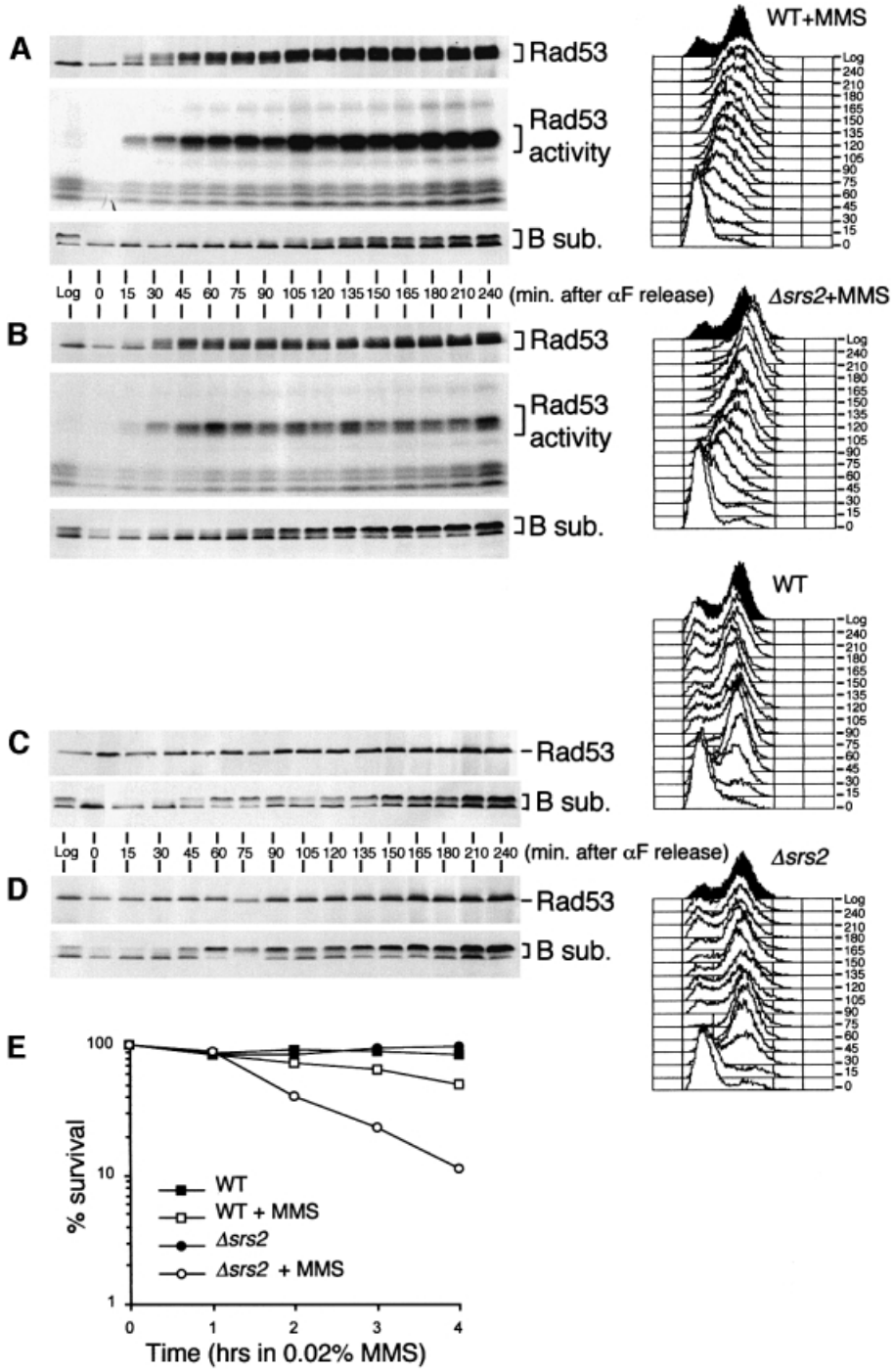


**Fig. 5.** Srs2 is required for proper Rad53 activation in response to DNA damage and it is dispensable for cell cycle progression under unperturbed conditions. (A–E) Log-phase cultures (Log) of strain K699 (WT) and CY2643 (*Δsrs2*) were synchronized by αF treatment and released from the $G_1$ block in the presence (**A** and **B**) or absence (**C** and **D**) of 0.02% MMS. At the indicate time points, aliquots of cells were taken for FACS analysis and protein extraction. Aliquots of the protein exctracts were analysed by western blotting using the 12CA5 and 6D2 mAbs and anti-Rad53 Ab and tested using the ISA assay (A and B). Cell survival (**E**) was determined as plating efficiency on YPD plates.

Analogous results were obtained when we tested the epistasis relationship between *RAD24* and *SRS2* (data not shown).

## Discussion

The cellular response to DNA damage, which is mediated by the Mec1, Rad53 and Dun1 protein kinases, allows cell survival by delaying cell cycle progression and by promoting transcription of several genes involved in DNA metabolism. Thus, the repair machinery is able to remove the DNA lesions and the cell can recover by restoring a normal cell cycle progression.

Although an intimate relationship between DNA repair and DNA damage checkpoint is somehow expected, it is still unclear whether the DNA damage checkpoint is

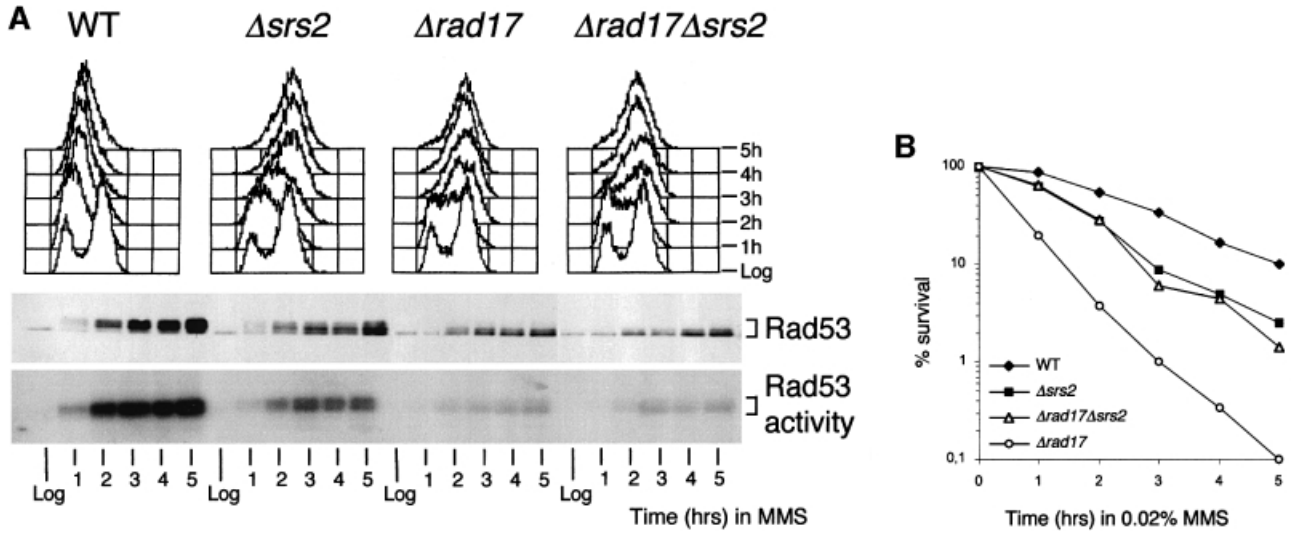


**Fig. 6.** *SRS2* deletion partially rescues the MMS sensitivity but not the checkpoint defect of *Δrad17* mutant cells. (A and B) Log-phase cultures (Log) of strains K699 (WT), CY2643 (*Δsrs2*), DMP1913/11C (*Δrad17*), CY3221(*Δrad17Δsrs2*) were treated with 0.02% MMS. Samples were taken at the times indicated for FACS analysis. Aliquots of protein extracts were analysed by western blotting performed using anti-Rad53 Ab and were used for the ISA assay (**A**). Cell survival (**B**) was determined as plating efficiency on YPD plates.

directly involved in promoting specific repair pathways. Similarly, it is presently unknown whether the recovery process is dependent upon DNA repair or, instead, the checkpoint is turned off in a time-dependent manner.

We have recently suggested a role for Mec1 and Rad53 in triggering a replication-coupled repair pathway requiring the lagging strand DNA replication apparatus (Foiani *et al*., 2000), but direct experimental evidence connecting the checkpoint to the repair machinery is still lacking.

The Srs2 DNA helicase is a likely candidate to connect several DNA transactions. In fact, Srs2 seems to be involved in channelling damaged DNA towards specific repair pathways (Aboussekhra *et al*., 1989; Schiestl *et al*., 1990; Rong *et al*., 1991) and in stabilizing some recombination intermediates (Pâques and Haber, 1997). *srs2* mutants are also defective in DNA replication and transcription (Lee *et al*., 1999), although this phenotype is probably caused by an indirect effect due to unrestrained recombination (Gangloff *et al*., 2000). Here we have shown that the Srs2 DNA helicase is phosphorylated in response to DNA damage and that this modification requires a functional checkpoint pathway, thus suggesting that Srs2 may directly connect the checkpoint pathway to the repair process.

Our data indicate that damage-induced Srs2 phosphorylation is mediated by the Mec1, Rad53 and Dun1 protein kinases, but it does not occur in $G_1$. Several observations suggest that Srs2 is not a Rad53 substrate: (i) Rad53 is activated in $G_1$-arrested cells in response to DNA damage, but Srs2 remains unphosphorylated; (ii) Rad53 is fully functional in *Δdun1* cells treated with genotoxic agents (data not shown), but Srs2 is not phosphorylated; (iii) Rad53 is rapidly inactivated during recovery from HU, while Srs2 remains phosphorylated; (iv) Cdk1 inactivation drives Srs2 dephosphorylation even though Rad53 remains active. Dun1 has been placed downstream of Rad53 (Zhou and Elledge, 1993) and our data indicate that Srs2 phosphorylation is abolished in *Δdun1* mutant cells. Dun1-dependent Srs2 phosphorylation might be explained in view of recent results that have pointed out a role for Dun1 in channelling DNA repair into a non-recombinogenic pathway (Fasullo *et al*., 1999). Since Srs2 plays a similar role (Aboussekhra *et al*., 1989), it has been suggested that the higher rate of mitotic recombination observed in *Δdun1* mutants might result from the inability to phosphorylate specific repair proteins, such as the Srs2 helicase (Fasullo *et al*., 1999). Although we cannot exclude the possibility that Srs2 is a direct substrate of Dun1, we would like to suggest that the pathway dependent upon Mec1, Rad53 and possibly Dun1 acts to modulate Cdk1, which, either directly or indirectly, would cause Srs2 phosphorylation. Srs2 contains seven putative consensus sites for Cdk1 and their mutagenesis is under way to test directly the role of Cdk1 in Srs2 phosphorylation.

Interestingly, it has been shown recently that the Rad55 protein, which is required for double strand break repair, is also phosphorylated in response to checkpoint activation through a process that requires a functional checkpoint. However, differently from Srs2, Rad55 protein can also be phosphorylated in $G_1$ and does not seem to play any role in Rad53 activation in response to DNA damage (Bashkirov *et al*., 2000).

There is an apparent paradox between the observation that the phosphorylation of the pol–prim B subunit, which requires Cdk1 activity under normal conditions, is negatively regulated by the checkpoint response (Pellicioli *et al*., 1999), while the checkpoint-dependent Srs2 phosphorylation requires a functional Cdk1. A possible explanation could be that activation of the DNA damage checkpoint changes the substrate specificity of Cdk1 towards targets required for cell survival under damaging conditions (Figure 7A). Hence this checkpoint-dependent Cdk1 (Cdk1*) will not be able to phosphorylate the usual targets (i.e. pol–prim), but would promote the phosphorylation of specific repair proteins (i.e. Srs2). This hypothesis is in agreement with previous observations indicating that Cdc28 activity is high in HU-treated cells (Amon *et al*.,

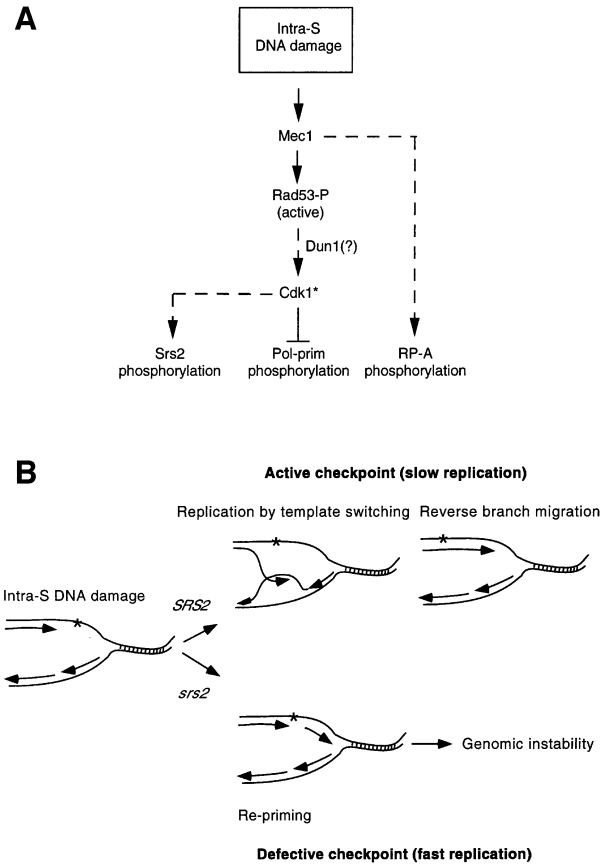


**Fig. 7.** Model for the intra-S DNA damage checkpoint. (**A**) DNA damage during S phase leads to phosphorylation of RP-A and Rad53 mediated by Mec1. Active Rad53 modulates the target specificity of Cdk1*, causing inhibition of pol–prim phosphorylation and modification of Srs2. (**B**) Replication forks encountering DNA damage have the option, in the presence of a functional *SRS2* gene product, to switch the template copying the newly synthesized lagging strand, thus bypassing the DNA lesion. The re-establishment of a conventional replication fork can occur by reverse branch migration. This mechanism allows duplication of a damaged template without leaving gaps opposite the lesions. In the absence of Srs2, a faster replication mechanism, involving repriming downstream of the damage, is favoured. This generates gapped DNA molecules that will be highly recombinogenic, likely to lead to increased genomic instability (see text for details).

1992; Sorger and Murray, 1992) and might account for the finding that any attempt to override the checkpoint by ectopic activation of Cdk1 has so far been unsuccessful (Pellicioli *et al*., 1999).

Although at the moment we do not know the functional significance of Srs2 phosphorylation, the finding that cells overexpressing Srs2 exhibit enhanced DNA damage sensitivity (Kaytor *et al*., 1995), together with the observation that Srs2 plays a crucial role in channelling DNA damage to specific repair pathways (Aboussekhra *et al*., 1989), strongly suggest that the cell has to tightly regulate the Srs2 helicase and that its phosphorylation might be relevant to allow cell survival under damaging conditions. Since Srs2 phosphorylation is concomitant with the modulation of the replication machinery, i.e. the lagging strand DNA polymerase and RF-A (Brush *et al*., 1996; Pellicioli *et al*., 1999), it is tempting to speculate that, in response to intra-S DNA damage, the cell promotes a specialized replication-coupled repair process dependent upon phosphorylated Srs2 and the lagging strand replication apparatus.

However, it should be pointed out that Srs2 phosphorylation might be related to other cellular processes, such as recovery or adaptation. In fact, we have recently shown that, when the checkpoint is activated by HU treatment, the cell has already synthesized all the protein factors required to execute recovery (Pellicioli *et al*., 1999). Moreover, recent results implicate Srs2 in the adaptation response (S.E.Lee and J.E.Haber, personal communication) and, in this view, Srs2 phosphorylation might resemble Cdc5 phosphorylation, which is dependent upon Rad53 and is also required for adaptation (Toczyski *et al*., 1997; Sanchez *et al*., 1999).

The finding that *Δsrs2* exhibits a reduced level of Rad53 activity under damaging conditions further enforces the connections between the checkpoint response and the Srs2 helicase, suggesting that Srs2 is indeed a relevant component of the pathway. In an attempt to elucidate the functional relationships between Srs2 and other components of the pathway, the phenotypes of a number of double mutant cells were tested. We found that in *Δsrs2Δrad17* double mutant cells, the level of residual Rad53 activity and the checkpoint defect in response to MMS treatment were indistinguishable from those found in a *Δrad17* single mutant. However, *SRS2* deletion partially rescues the cell lethality of *Δrad17* cells under damaging conditions, and the cell viability of the *Δsrs2Δrad17* double mutant was comparable to that of *Δsrs2* cells. This finding suggests that, in a *Δrad17* background, a functional *SRS2* gene product may cause lethal events. Hence, cell viability and cell cycle progression in *Δrad17Δsrs2* cells seem to be uncoupled. The picture is further complicated by the observation that the DNA damage-induced cell lethality in *Δsrs2* mutant cells is caused by unrestrained recombination (Milne *et al*., 1995; Schild, 1995; Chanet *et al*., 1996). Moreover, we have shown previously that Rad53 activation in response to MMS treatment depends on Rad17 completely in $G_1$, but only partially in S phase (Pellicioli *et al*., 1999). Conversely, Srs2 seems to be relevant specifically in response to intra-S DNA damage.

In an attempt to reconcile our results with a role for Srs2 in preventing certain lethal recombination pathways (Aboussekhra *et al*., 1989) and, at the same time, in promoting the formation of other recombination intermediates (Pâques and Haber, 1997), we suggest the following model (Figure 7B).

Cells experiencing DNA damage while replicating DNA have different options (Friedberg *et al*., 1995). Among them, cells can restart replication downstream of the lesion through a process that requires repriming and therefore DNA primase. Such an option would inevitably lead to the formation of highly recombinogenic gaps and, therefore, massive intra-S recombination would jeopardize genome integrity. An alternative option would be to promote the formation of other recombination intermediates by annealing newly synthesized leading and lagging strands through template switching. Such intermediates (Holliday junctions) might occur spontaneously to relieve the superhelical tension that accumulates ahead of the replication fork due to the replication pausing caused by

DNA damage (Doe *et al*., 2000). These DNA structures could then migrate through replication by allowing the leading strand to copy the newly synthesized lagging strand (Figure 7B). This mechanism of replication would prevent genotoxic recombination, allowing error-free DNA synthesis. Furthermore, once the damage has been bypassed, the cell could resolve the Holliday junctions, resetting a normal replication fork through a process that is better known as reverse branch migration (Figure 7B). Indeed, it is possible to envisage a role for a 3′–5′ DNA helicase either in promoting replication by template switching by stabilizing the Holliday junctions during branch migration, or in the subsequent reverse branch migration step. This mechanism has been proposed in mammalian cells to describe DNA replication in response to MMS treatment (Higgins *et al*., 1976) and to explain the role of Rqh1 helicase in *S.pombe* (Doe *et al*., 2000).

We would therefore like to suggest that the Srs2 helicase may channel intra-S DNA damage into a template switching mode of replication. In *srs2* mutants, instead, replication would be achieved by extensive repriming causing accumulation of recombinogenic gaps, similar to what has already been suggested in the case of the checkpoint-defective DNA primase mutant *pri1-M4* (Marini *et al*., 1997). This hypothesis is in accordance with a role of Srs2 during S phase and with the finding that Srs2 stabilizes certain recombination intermediates (Pâques and Haber, 1997). Moreover, this model might explain the observation that *srs2* mutants exhibit unrestrained recombination in response to damaged DNA and that *srs2* DNA damage sensitivity can be rescued by preventing certain recombination pathways dependent upon Rad52 and Rad51 (Milne *et al*., 1995; Chanet *et al*., 1996).

*Δsrs2* cells are defective in maintaining Rad53 activity in response to intra-S DNA damage. This can be explained by assuming that Srs2 might generate checkpoint signals during DNA replication perhaps by processing the primary damage or by accumulating DNA structures that contribute to activate the pathway leading to Rad53 activation. Alternatively, since *Δsrs2* cells are unable to prevent the recruitment of certain recombination pathways, some recombination factors might actively mask the checkpoint signals by binding to damaged DNA, thus causing a defect in Rad53 activation. On the other hand, it is also possible that unscheduled error-prone recombination might accelerate the processing of DNA lesions, causing premature inactivation of Rad53.

The functional relationship between Rad17 and Srs2 still remains unclear. It is possible that Rad17 plays an indirect role during S phase in restraining specific repair processes that might be dangerous in a replication context, and this function is relevant if Srs2 is functional. Indeed, it has been suggested recently that Rad17 and other checkpoint genes may play a role in accommodating DNA damage during replication by antagonizing certain recombination pathways (Paulovich *et al*., 1998). Hence, Rad17 and Srs2 might act in the same direction in channelling damage towards a replication-coupled repair process such as template switching. However, in a *Δrad17* background the cell may not be able to prevent certain repair events, which, in the presence of a functional Srs2, might cause accumulation of abortive or abnormal repair intermediates. The finding that Srs2 triggers lethal events in a *Δrad17* background could also be ascribed to an excessive amount of unphosphorylated Srs2 under damaging conditions, since in *Δrad17* Srs2 does not get properly phosphorylated (Figure 3 and data not shown).

Another DNA helicase, Sgs1, has recently been implicated in the checkpoint response. Interestingly, *Δsgs1* and *Δsrs2* mutants exhibit intriguing analogies since they are both required to activate Rad53 properly. However, Sgs1 that has been placed upstream of Rad53 (Frei and Gasser, 2000) is not required for Srs2 phosphorylation. Moreover, we found that although *Δsgs1*, like *Δsrs2* cells, are unable to activate Rad53 properly in response to intra-S DNA damage, *Δsgs1* does not rescue the cell viability of *Δrad17* mutants (our unpublished observations). It has been found recently that *Δsrs2Δsgs1* double mutants show a synthetic slow growth phenotype associated with enhanced genomic instability, which can be rescued by *RAD51* deletion (Gangloff *et al*., 2000), and it has been suggested that the *S.pombe* Sgs1 homologue might be involved in catalysing reverse branch migration of Holliday junctions arising from template switching (Doe *et al*., 2000). It will then be relevant to establish the functional relationship between Sgs1 and Srs2 within the DNA damage response pathway.

## Materials and methods

### Plasmids

The HA-tagged version of *SRS2* gene was produced as follows. The N-terminal region of *SRS2* (from –536 to +180) was amplified by PCR using the primers 5′-CCCAAGCTTCTCACGATCTACGAGATGC-3′ (Srs-*Hin*dIII#3) and 5′-CGGGATCCGGGATGAATATGGTGGTGCT-3′ (Srs-*Bam*HI#4), and it was cloned into the *Hin*dIII–*Bam*HI sites of plasmid YIplac211 (Gietz and Sugino, 1998) to generate plasmid pG34. pG34 was then amplified by PCR using the primers 5′-GCGGCCGCCCATTTGCTATCCCTAAGTAC-3′ (Srs-*Not*I#1) and 5′-GGCGGCCGCTCGTCGAACAATGATCTTTG-3′ (Srs-*Not*I#2) to generate plasmid pG35, which contains a *Not*I site at the ATG codon of *SRS2* ORF. Three tandem copies of the HA epitope were cloned into the *Not*I site of pG35 in-frame with *SRS2* to generate plasmid pG36. All the PCRs were performed using the *Pfu* Turbo DNA polymerase (Stratagene) and the final products of amplification were controlled by sequence analysis.

Plasmids pMHT (*GAL1-SIC1*) and p100 (*GAL1-CDC14*) were provided by J.Diffley and A.Amon, respectively.

### Yeast strains

The genotypes of the strains used are listed in Table I. Strains CY2715, CY2835, CY2827, CY2837, CY3138, CY2829 and CY2830 were originated by integrating a *Pst*I-linearized pG36 plasmid at the *SRS2* locus in strains K699, DMP2541/8A (Paciotti *et al*., 1998), DMP2161/25B (Paciotti *et al*., 1998), CY2034 (Pellicioli *et al*., 1999), YA145 (Fasullo *et al*., 1999), K5247 and K5248 (kindly provided by K.Nasmyth), respectively, and by selecting for 5-FOA-resistant cells. Correct *HA-SRS2* integration was controlled by Southern analysis.

Since strains CY2715 and K699 exhibited the same DNA damage sensitivity in response to UV and MMS treatment, we concluded that the *HA-SRS2*-tagged gene is fully functional. Gene disruptions were produced according to Wach *et al*. (1994, 1997) using either the KanMX4 or HIS3MX6 cassettes. The strains originated were controlled by genomic PCR. Strains CY2823, CY2822, CY2884, CY3135 and CY3137 were obtained by deleting the *RAD52*, *RAD18*, *MSH2*, *RAD30* and *SGS1* genes, respectively, in strain CY2715. Strains CY2888 and CY2885 were obtained by deleting the *TEL1* gene in strains CY2835 and CY2715, respectively. Strains CY2643, CY3221 and CY3223 were obtained by deleting the *SRS2* gene in strains K699, DMP1913/11C (kindly provided by M.P.Longhese) and DMP1913/20B (kindly provided by M.P.Longhese), respectively. Strains CY2735 and CY2904 were obtained by integrating plasmids pMHT or p100, respectively, at the *URA3* locus.

**Table I.** Strains used in this study

| Strain | Genotype | Reference/source |
|---|---|---|
| K699 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100* | K.Nasmyth |
| CY2715 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2* | this study |
| CY2735 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 GAL1-SIC1::URA3* | this study |
| CY2904 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 GAL1-CDC14::URA3* | this study |
| CY2823 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 rad52Δ::HIS3MX6* | this study |
| CY2822 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 rad18Δ::HIS3MX6* | this study |
| CY2884 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 msh2Δ::HIS3MX6* | this study |
| CY3135 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 rad30Δ::KanMX4* | this study |
| CY3137 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 sgs1Δ::KanMX4* | this study |
| CY2835 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 HA2DDC1::LEU2:: ddc1 mec1-1 sml1-1* | this study |
| CY2837 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 rad53-K227A::KanMX4::rad53* | this study |
| CY3138 | *MAT*α *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100 HA3SRS2::srs2 dun1-Δ100::HIS3* | this study |
| CY2885 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 tel1Δ::HIS3MX6* | this study |
| CY2888 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 HA2DDC1::LEU2::ddc1 tel1Δ::HIS3MX6 mec1-1 sml1-1* | this study |
| CY2827 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 HA2DDC1::LEU2::ddc1 rad17Δ::LEU2 rad24Δ::TRP1 mec3Δ::TRP1* | this study |
| CY2643 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 srs2Δ::KanMX4* | this study |
| CY2829 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 cks1::LEU2 [CKS1 TRP1 ARS1 CEN4]* | this study |
| CY2830 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 HA3SRS2::srs2 cks1::LEU2 [cks1-ts38 TRP1 ARS1 CEN4]* | this study |
| DMP1913/11C | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad17Δ::LEU2* | M.P. Longhese |
| CY3221 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad17Δ::LEU2 srs2Δ::KanMX4* | this study |
| DMP1913/20B | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad24Δ::TRP1* | M.P. Longhese |
| CY3223 | *MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 rad24Δ::TRP1 srs2Δ::KanMX4* | this study |

Plasmids are indicated by brackets.

### *Media and growth conditions*

Unless otherwise indicated, strains were grown at 28°C in YP (1% yeast extract, 2% Bacto-peptone; Oxoid) containing glucose, galactose or raffinose at 2% w/v.

$G_1$ cells synchronization was achieved by adding 2 μg/ml α-factor to the cultures, except for the experiment described in Figure 2C in which we used 20 μg/ml α-factor.

### *Western blot analysis and immunological reagents*

Crude extract was prepared as described in Foiani *et al*. (1999). Total protein extract (25 μg) was used for western blotting or *in situ* autophosphorylation assay (ISA) (see below). The western blot procedure has already been described (Foiani *et al*., 1995), except that the secondary antibodies were peroxidase labelled (Amersham). The monoclonal antibody against the pol–prim B subunit has been described previously (Foiani *et al*., 1995) and the polyclonal antibodies against Rad53 were a generous gift from C.Santocanale and J.Diffley.

### *Immunoprecipitation and phosphatase treatment*

Cells ($2 \times \sim 10^9$) grown under normal conditions or in the presence of 0.02% MMS, were resuspended in 1 ml of lysis buffer (0.4 M sorbitol, 150 mM potassium acetate, 20 mM PIPES–KOH pH 6.8, 2 mM magnesium acetate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, supplemented with protease inhibitor cocktail; Roche). Cells were broken in the presence of glass beads in a mini beadbeater (Biospec) and the extract was clarified in a microfuge. Lysis buffer (800 μl) was added to 200 μl of crude extract and incubated for 2 h at 4°C in the presence of $1 \times \sim 10^7$ magnetic beads (Dynal), which were pre-incubated with 12CA5 monoclonal antibody. The beads were then washed three times with 1 ml of lysis buffer and treated with 4000 U of λ-phosphatase (Biolabs). An identical aliquot of beads was treated under the same conditions without λ-phosphatase addition. Samples were incubated for 10 min at 30°C. The beads were then washed with 1 ml of lysis buffer and resuspended in 20 μl of Laemmli buffer.

### *ISA*

The procedure to measure Rad53 activity *in situ* has already been described (Pellicioli *et al*., 1999).

### *FACS analysis*

FACS analysis was performed as described in Foiani *et al*. (1999).

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