# Asf1 links Rad53 to control of chromatin assembly

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**Yeast defective in the checkpoint kinase Rad53 fail to recover from transient DNA replication blocks and synthesize intact chromosomes. The effectors of Rad53 relevant to this recovery process are unknown. Here we report that overproduction of the chromatin assembly factor Asf1 can suppress the Ts phenotype of *mrc1rad53* double mutants and the HU sensitivity of *rad53* mutants. Eliminating silencing also suppresses this lethality, further implicating chromatin structure in checkpoint function. We find that Asf1 and Rad53 exist in a dynamic complex that dissociates in response to replication blocks and DNA damage. Thus, checkpoint pathways directly regulate chromatin assembly to promote survival in response to DNA damage and replication blocks.**

DNA damage and DNA replication interference represent life threatening situations to which cells have evolved sophisticated responses (Lindahl and Wood 1999). The repair of DNA damage requires not only recognition of the altered DNA structure, but also the recruitment of repair factors to the site of damage. Furthermore, it may be necessary to reorganize the chromatin to optimally initiate repair. After repair, the chromatin structure of the repaired DNA must be reassembled. It is not understood how chromatin reorganization takes place during repair, however, a number of chromatin remodeling complexes such as the Swi/Snf and Rsc complexes exist that can facilitate chromatin reorganization (Cairns 1998). Likewise, chromatin assembly factors such as the CAF1 complex and RCAF (Asf1) can act to deposit chromatin onto newly synthesized DNA during DNA replication and possibly during repair and replication fork reorganization. Subunits of CAF1 have been shown to bind to PCNA and localize to DNA in response to DNA damage, presumably during resynthesis following excision repair (Shibahara and Stillman 1999).

This response to replication interference and DNA damage is orchestrated through checkpoint pathways that carry out a number of important functions to promote survival (Zhou and Elledge 2000). These include cell cycle arrest, inhibition of late origins of replication, transcriptional induction of DNA repair genes, post-translational modification of repair proteins, and alteration of chromatin structure. In budding yeast, central among the proteins involved in this response are the checkpoint kinases Mec1, Rad53, Chk1, and Dun1.

Mec1 is the central transducer of these stress-response signals and it acts to turn on the other three kinases (Zhou and Elledge 2000). Both Rad53 and Mec1 are key proteins involved in the response to replication blocks and they act together with a novel regulator of Rad53, Mrc1, that is also required for a subset of Rad53 responses (A. Alcasabas et al., in prep.). Cells lacking Rad53 or Mec1 die rapidly when treated with hydroxyurea, an inhibitor of dNTP synthesis, due primarily to an inability to recover and properly synthesize complete chromosomes after the replication block is removed (Desany et al. 1998). How Rad53 promotes the synthesis of complete chromosomes after replication insults is unclear, but the DNA damage response pathway has been linked to the control of chromatin organization. In response to DNA damage, certain proteins that are normally localized to silent telomeric chromatin, such as the Sir3 silencing factor, relocalize to sites of DNA damage (Martin et al. 1999; Mills et al. 1999). This relocalization is dependent on Mec1. In *rad53* mutants, telomeric silencing is enhanced, whereas in *mec1* mutants it is disrupted (Craven and Petes 2000; Longhese et al. 2000).

Another connection to silencing comes from checkpoint studies in meiotic cells. Mutants in *dmc1* or *zip1* result in pachytene arrest that is dependent on the DNA damage checkpoint (Grushcow et al. 1999). In addition, mutations in three genes involved in silencing, *PCH1/DOT1*, *PCH2*, or *SIR2* bypass the pachytene arrest, thereby linking chromatin structure to checkpoint control (San-Segundo and Roeder 1999; Roeder and Bailis 2000). How chromatin structure controls checkpoints and how checkpoints control chromatin structure is not known. In this work we discover that the chromatin assembly factor *ASF1* resides in a complex with Rad53 that dissociates in the presence of replication blocks or DNA damage. We propose that in response to DNA replication blocks and DNA damage, Rad53 directs Asf1 to regulate nucleosome deposition and thereby links Rad53 to chromatin assembly.

## Results

### mrc1-1 rad53-21 *mutants have a temperature-sensitive lethal phenotype*

Mrc1 is a new component of the S-phase checkpoint pathway that is required to prevent spindle elongation in response to HU treatment. Although epistasis studies suggest that Mrc1 and Rad53 function in the same pathway in the S-phase checkpoint pathway with respect to spindle elongation in HU-arrested cells, the relationship between *MRC1* and *RAD53* is complex (Alcasabas et al., in prep.). For example, *mrc1Δrad53-21* double mutants are lethal, and the lethality cannot be suppressed by *RNR1* overexpression, suggesting Mrc1 has at least one function distinct from Rad53. *mrc1-1 rad53-21* double mutants grow very slowly at room temperature and they

are temperature sensitive for growth at 37°C, whereas the single mutants are not (Fig. 1A). The double mutants lose viability after growth at 37.5°C (Fig. 1B).

To investigate the lethality of *mrc1 rad53* double mutants, we analyzed the cell cycle progression of *mrc1-1 rad53-21* mutants at the nonpermissive temperature. Asynchronous log phase cultures at 24°C were shifted to 37.5°C and samples were taken every 2.5 h. From FACS analysis, we observed that the majority of *mrc1-1 rad53-21* mutants delay in S phase. After 5 h at 37.5°C, they became arrested with a $G_2$ DNA content (Fig. 1C) and short spindles (data not shown). To determine whether this arrest was in S phase or in $G_2$, we examined whether the *mrc1-1 rad53-21* mutant had finished DNA replication using pulsed-field gel electrophoresis (PFGE). Replicating chromosomes cannot enter the gel because of secondary structures caused by replication intermediates and, therefore, remain in the well. However, fully replicated chromosomes will migrate into the gel. We can detect fully replicated chromosome signals in the gel from wild-type cells at all time points tested. However, *mrc1-1 rad53-21* double mutants fail to enter the gel within 2.5 h after the shift (Fig. 1D) and show an accumulation of S phase cells (Fig. 1C). Thus, *mrc1-1 rad53-21* double mutants have a severe defect in DNA replication and arrest with incompletely replicated chromosomes at the nonpermissive temperature. The failure of chromosomes to migrate could also be caused by unresolved recombination intermediates. However, given the accumulation of cells with an S-phase DNA content at 2.5 h after the shift, we feel incomplete replication is the simplest explanation.
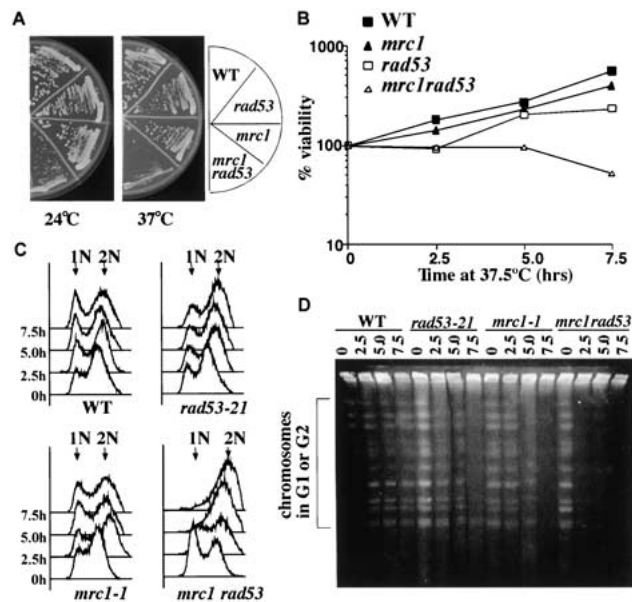


**Figure 1.** *mrc1-1 rad53-21* double mutants arrest at the nonpermissive temperature with incompletely replicated chromosomes. (*A*) Temperature sensitivity of *mrc1-1 rad53-21* double mutants. Yeast strains of the indicated genotypes were streaked out on YPD plates and were either grown at room temperature for 4 d or grown at 37°C for 2 d. (*B–D*) After growth at 37.5°C, *mrc1-1 rad53-21* double mutants lose viability and arrest with incompletely replicated chromosomes. Asynchronous log-phase culture at 24°C were shifted to 37.5°C and samples were taken every 2.5 h to examine viability (*B*), to analyze DNA content (*C*), or to prepare DNA for PFGE analysis (*D*).
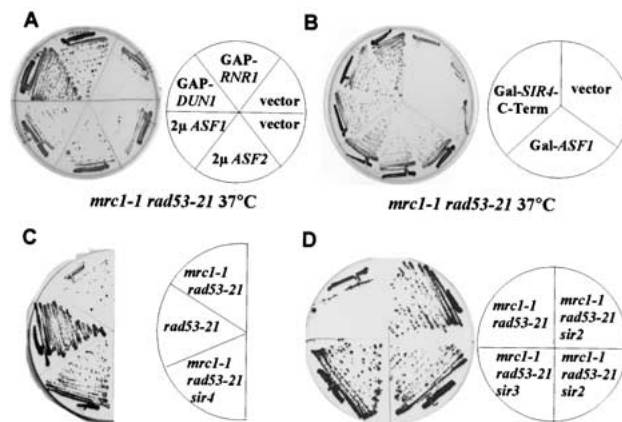


**Figure 2.** Suppression of the *mrc1-1 rad53-21* Ts phenotype by *RNR1* and *DUN1* overexpression and anti-silencing. (*A,B*) *mrc1-1 rad53-21* mutants containing the indicated plasmids were streaked out on YPD plates (*A*) or YP-GAL plates (*B*) and grown at 37°C for 2 d. (*C,D*) Yeast strains of indicated genotypes were streaked out on YPD plates and grown at 37°C for 2 d.

## Dosage suppressors of the mrc1-1 rad53-21 Ts phenotype

To understand the synthetic lethality of the *mrc1 rad53* double mutant, we isolated high-copy suppressors of their Ts phenotype. Four suppressors, *SLT2*, *PCL1*, *YER033C*, and *ASF1* were found in this screen. *SLT2* encodes a MAP kinase. *PCL1* encodes a $G_1$ cyclin. Slt2 and Pcl1 are involved in activating the SWI transcription complex (Igual et al. 1996; Madden et al. 1997), which controls *RNR1* and *RNR3* expression. Because the phenotype of *mrc1-1 rad53-21* is similar to a *rad53* null mutation and the lethality of *rad53Δ* can be suppressed by *RNR1* overexpression (Desany et al. 1998), we asked whether *RNR1* could suppress *mrc1-1 rad53-21* mutants. Both *RNR1* and its positive regulator *DUN1* suppress *mrc1-1 rad53-21* mutants when overproduced (Fig. 2A), suggesting that *SLT2* and *PCL1* may work through *RNR1* regulation. Unlike *RNR1*, overexpression of *ASF1* does not suppress the lethality of *rad53Δ*. Therefore, in this respect, *mrc1-1 rad53-21* does not fully mimic the *rad53Δ* mutation.

## Disrupting silencing can suppress the lethality of mrc1-1 rad53-21 mutants

*ASF1* has been implicated in histone deposition during DNA replication (Tyler et al. 1999). *asf1* mutants are sensitive to HU and DNA-damaging agents (Le et al. 1997; Tyler et al. 1999). Both overproduction and deletion of *ASF1* lead to silencing defects at telomere and mating-type loci (Le et al. 1997; Singer et al. 1998). In addition, Rad53 has been implicated in regulation of chromatin adjacent to telomeres (Craven and Petes 2000; Longhese et al. 2000). Therefore, we asked whether the suppression of the *mrc1 rad53* double mutants might reflect the anti-silencing property of *ASF1* by expressing other anti-silencing factors in *mrc1-1 rad53-21* mutants, such as *ASF2* and the carboxyl terminus of *SIR4*. Overexpression of *ASF2* causes silencing defects at the *HML* and *HMR* loci (Le et al. 1997) and overexpression of carboxyl termini of *SIR4* disrupts Sir complex assembly, thereby interfering with silencing (Marshall et al. 1987;

Cockell et al. 1995). Surprisingly, overexpression of either *ASF2* or of the carboxyl terminus of *SIR4* suppresses *mrc1-1 rad53-21*, but the vector alone cannot (Fig. 2A,B). Furthermore, *sir2*, *sir3*, or *sir4* null mutants, which cause loss of silencing, can also suppress *mrc1-1 rad53-21* (Fig. 2C,D). Thus, anti-silencing can suppress *mrc1-1 rad53-21*. We have also checked the suppression of *mrc1-1 rad53-21* Ts phenotype by overexpressing other *DOT* genes identified as dosage disruptors of telomeric silencing (Singer et al. 1998). Unlike *SIR4* carboxyl terminus or *ASF1*, overexpression of all other *DOT* genes fail to suppress (data not shown). This suggests that either the degree of anti-silencing must be beyond a threshold level to suppress, or that *ASF1* may possess a special property relevant to Rad53 function. The suppression of the Ts phenotype of the *mrc1 rad53* double mutant by *ASF1* overproduction is not due to the psuedo-diploid nature of these mutants resulting from *ASF1*'s anti-silencing properties because the homozygous *mrc1 rad53* diploid is still Ts, although less so than the haploid. Overproduction of ASF1 still suppresses the Ts phenotype.

*Mrc1 is required for silencing*

The involvement of silencing in the temperature sensitivity of *mrc1 rad53* mutants led us to examine whether Mrc1 or Rad53 has a role in silencing. It has been reported that *rad53* mutants have a modest increase of telomeric silencing (Craven and Petes 2000; Longhese et al. 2000), implicating Rad53 in control of telomeric chromatin. To test whether Mrc1 also has a role, the *mrc1* mutation was introduced into test strains that have a *URA3* reporter at either telomeric or *HMR* loci. *mrc1* mutants have partial loss of silencing at both loci, which is reflected by increased growth on SC-ura plates and reduced growth on 5-FOA plates (Fig. 3A,B). This silencing defect is not as dramatic as in *sir* mutants. Overexpression of *RAD53* also causes partial loss of silencing (Fig. 3C), whereas overexpression of either *DUN1* or *RNR1* does not (data not shown). Thus, both Rad53 and Mrc1 can affect silencing and may be involved in chromatin regulation.

*Connections between silencing and checkpoint control*

Due to the connection between anti-silencing and the suppression of the *mrc1-1 rad53-21* Ts phenotype, we next asked whether anti-silencing could also suppress the sensitivity of *rad53* mutants to HU. Interestingly, overexpression of *ASF1* can strongly suppress the HU sensitivity of both *rad53-21* and *rad53Δ* mutants on 10 mM HU, whereas the vector control cannot (Fig. 4A). Overexpression of *ASF2* (Fig. 4A) or mutation of *SIR3* (Fig. 4B) can also weakly suppress the HU sensitivity of *rad53* mutants. The weak suppression by *sir3* mutants indicates that the suppression by *ASF1* cannot be attributed solely to its anti-silencing property.

The strong suppression of *rad53* HU sensitivity by Asf1 overproduction suggests that Asf1 may have a role in the *RAD53*-dependent DNA replication stress-response pathway. We examined the viability of *asf1Δ* mutants in response to HU treatment. Compared with wild-type cells, *asf1* mutants lose viability after prolonged exposure to HU (Fig. 4C).
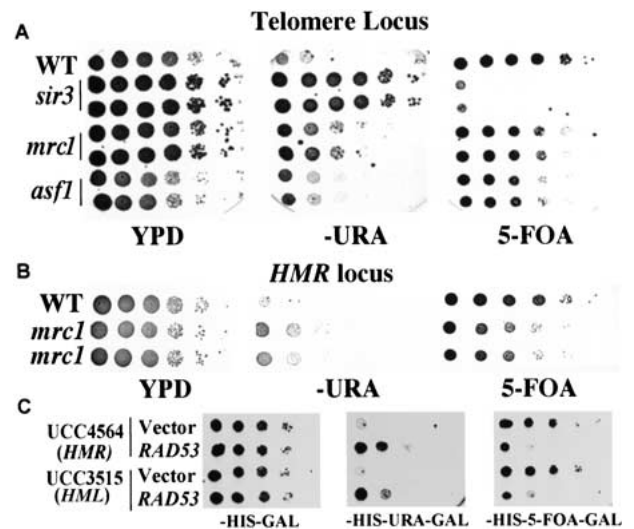
We also examined the activation of Rad53 in response



**Figure 3.** Role of Mrc1 and Rad53 in silencing. (*A*) *mrc1* mutants have a telomere silencing defect. Wild-type (UCC3505), isogenic *sir3Δ*, or *mrc1Δ* mutants were grown to saturation. Serial 10X dilutions of these cultures were made and spotted onto YPD, SC-ura, or SC 5-FOA plates and were grown for 4–5 d at 30°C. (*B*) *mrc1* mutants have a *HMR*-silencing defect. The test strain used is UCC4564. (*C*) Overproduction of Rad53 causes a silencing defect at both *HML* and *HMR* loci. Saturated cultures of *URA3* reporter strains UCC4564 or UCC3515 containing either vector alone or *HIS3–GAL–RAD53* plasmids were diluted and spotted onto SC-his and SC-his,ura plates or SC-his plates containing 5-FOA as indicated, and grown as in *A*.

to HU and MMS in *asf1* mutants. Asynchronous cultures of wild-type and *asf1* mutant cells were treated with HU and MMS and Rad53 modification status was examined by Western blotting. Regulation of Rad53 by MMS was unaffected, but there was a significant reduction in the accumulation of the hyperphosphorylated form of Rad53 in response to HU in *asf1* mutants (Fig. 4D). To evaluate the kinetics of Rad53 phosphorylation, we examined synchronized cultures. Due to their silencing defects, *asf1* mutants cannot be synchronized with α-factor. Instead, we used the *cdc15* mutation to synchronize cells in telophase and released the cells into 200 mM HU. Rad53p shows a partial mobility shift during an unperturbed cell cycle in *asf1* mutants, suggesting that *asf1* mutants may be defective in DNA replication and accumulate DNA damage that activates Rad53. In response to HU, Rad53 gets further activated in *asf1* mutants, but the kinetics and extent of hyperphosphorylation are delayed compared with wild-type cells (Fig. 4D). This could be a result of the slow cell cycle progression of *asf1* mutants or may mean that Asf1 plays a more direct role in activating Rad53. Consistent with a delay in Rad53 activation, we find that *asf1* mutants treated with HU have a partial checkpoint defect with more cells displaying partially elongated spindles (Fig. 4E). This defect is not as severe as seen in *rad53* mutants with respect to either percentage of elongated spindles or the length of spindle elongation (Fig. 4E).

*Physical interaction between Asf1 and Rad53 modulated by DNA damage*

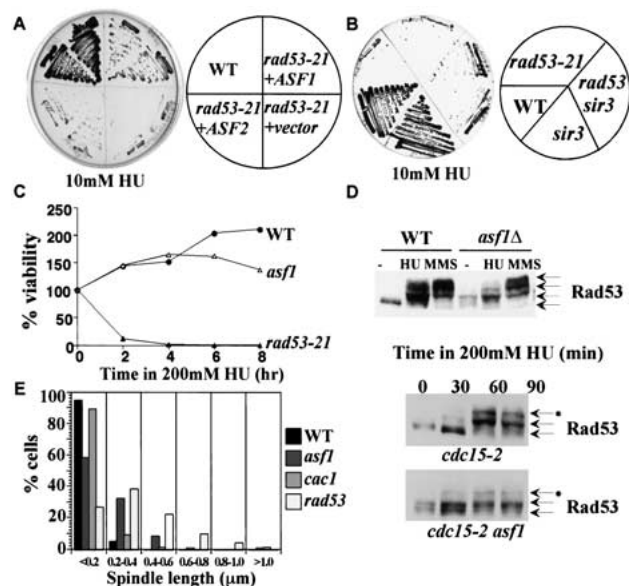Although ASF1 overexpression suppresses *rad53*'s HU sensitivity, it cannot suppress *mec1-21*, *dun1Δ*, or

**Figure 4.** Connection between silencing and checkpoint control. (*A*) Suppression of the HU sensitivity of *rad53* mutants by *ASF1* or *ASF2* overexpression. Yeast strains of indicated genotypes were streaked out on YPD plates containing 10 mM HU and grown for 2 d at 30°C. (*B*) *sir3Δ* can weakly suppress the HU sensitivity of *rad53* mutants on 10 mM HU. (*C*) *asf1* mutants lose viability after prolonged exposure to HU. A total of 200 mM HU was added to asynchronous cultures at 30°C and samples were taken every 2 h to examine viability. (*D*) Activation of Rad53 in *asf1* mutants. (*Top*) Wild-type (WT) or *asf1* mutant cells were grown to log phase at 30°C. Protein extracts were made from cells untreated (−), treated with 200 mM HU for 1.5 h (HU), or treated with 0.05% MMS for 1 h (MMS). (*Bottom*) Log phase culture of *cdc15-2* or *cdc15-2 asf1* mutant cells were shifted to 37°C for 2 h. Those telophase-arrested cells were then shifted back to room temperature in YPD medium containing 200 mM HU. Samples were taken every half-hour and protein extracts were made. Proteins were resolved on 8% SDS-PAGE. Anti-Rad53 antibody (1:250 dilution) was used to detect Rad53p. (*E*) Spindle phenotypes of *asf1* mutants in HU. Asynchronous culture of wild-type, *asf1*, *cac1*, or *rad53-21* mutant cells were treated with 200 mM HU for 3 h at 30°C and spindle lengths were measured.

*mrc1Δ* mutants (data not shown). This suggests that Asf1 overproduction is supplying a particular function normally carried out specifically by Rad53 as opposed to the pathway in general. The suppression of HU sensitivity of *rad53* mutants by increased Asf1 levels cannot be attributed solely to the anti-silencing effect of Asf1 overproduction because direct abrogation of silencing by *sir* mutations provide only a marginal suppression. Together, these observations suggest that this suppression may reflect specific interactions between Asf1 and Rad53.

To explore this hypothesis, we tested for a physical interaction. We tagged the genomic *ASF1* gene with a triple HA tag and immunoprecipitated Asf1 with anti-HA antibodies. We detected Rad53 in the IP product, but not in the control IP in which Asf1 is not tagged (Fig. 5A). Moreover, the amount of Rad53 pulled down by HA antibodies is much less in the presence of the DNA damage agent, MMS (Fig. 5A) or in the presence of HU (Fig. 5B). We observed only the unphosphorylated form of Rad53 in the IP, although the majority of Rad53 is phosphorylated in the presence of MMS. We can also detect Asf1 in the reciprocal immunoprecipitations using anti-Rad53

antibodies (Fig. 5A). We hypothesize that in the presence of DNA damage, activation of the damage checkpoint leads to phosphorylation of Rad53 and dissociation of Asf1 and Rad53.

### Dissociation of Rad53 and Asf1 is regulated by Mec1

It is not clear whether the dissociation of Asf1 and Rad53 occurs because of Rad53 activation or merely correlates with it. Therefore, we examined the association of Rad53 and Asf1 in *mec1Δ* mutants, in which the phosphorylation of Rad53 is significantly reduced. *mec1Δ* cells show reduced dissociation of the complex (Fig. 5B), indicating that this response is partially Mec1-dependent in asynchronous cells. However, there was still a significant amount of dissociation of Asf1 and Rad53 in *mec1* mutants upon MMS treatment (Fig. 5B). We also examined the dissociation of Asf1 and Rad53 in *mec1* mutants in response to HU. Whereas in wild-type cells treated with HU there is very little association of Asf1 and Rad53, significant association is maintained in *mec1* mutants treated with HU (Fig. 5B).

The differences in wild-type and *mec1* mutants could be due to the fact that *MEC1* function is required for dissociation or could result from the fact that wild-type cells treated with HU or DNA damage are arrested in the cell cycle, whereas *mec1* mutants progress to different stages of the cycle. To control for cell cycle differences, we first arrested cells at $G_2$/M using nocodazole and then introduced DNA damage using γ-irradiation while maintaining the $G_2$/M block. In this experiment, the dissociation of Asf1 and Rad53 is totally abolished in *mec1* mutants. We conclude that in response to checkpoint signals during replication blocks and DNA damage, Mec1 activation leads to activation of Rad53 and dissociation of Asf1.
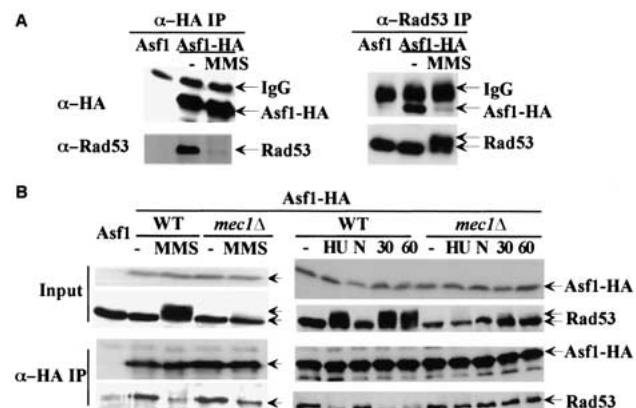
**Figure 5.** Interaction between Asf1p and Rad53p. (*A*) Coimmunoprecipitation of Asf1–HA3 and Rad53p. Protein extracts from *ASF1*, *ASF1–HA3*, or *ASF1–HA3* cells treated with 0.05% MMS for 1 h were immunoprecipitated with either anti-HA or anti-Rad53 antibodies. The immunoprecipitates were resolved by SDS-PAGE and probed with either anti-HA or anti-Rad53 antibodies as indicated. (*B*) Coimmunoprecipitation of Asf1–HA3 and Rad53p in *mec1Δ* mutants in response to DNA damage and replication blocks. Asynchronous cultures of wild-type or *mec1Δ* mutants were untreated (−), treated with 0.05% MMS for 1 h, or 200 mM HU for 1.5 h (HU). Separate cultures were arrested with nocodazole and either untreated (N) or γ-irradiated (6 Krad), while maintaining the nocodazole arrest. Samples were taken either 30 min (30) or 60 min (60) after irradiation and immunoprecipitation studies were performed as in *A*.

## Discussion

In this study we provide evidence for several connections between checkpoint pathway and chromatin assembly. First, Mrc1 is required for efficient silencing. We have also found that, unlike the silencing defect of *mec1* mutants (Craven and Petes 2000; Longhese et al. 2000), the defect in *mrc1* mutants is not suppressed by overproducing *RNR1* (data not shown) and is likely to be more direct than that in *mec1* mutants. Secondly, we identified *ASF1* as a dosage suppressor of *rad53 mrc1* mutants. This lethality can also be suppressed by interfering with silencing by mutations in Sir2, Sir3, and Sir4. This suggests that heterochromatic structures may be incompatible with these mutants. We can only speculate as to the nature of this incompatibility. One possibility is that heterochromatic regions are difficult to replicate through and provide a significant challenge to checkpoint defective mutants. By eliminating silencing, these regions are converted to euchromatic regions that present less of a problem. A second possibility is that certain repair factors are sequestered at telomeric chromatin through the silencing machinery (Martin et al. 1999; Mills et al. 1999) and these are released in response to damage and may be important for overcoming the DNA replication problems experienced by the *mrc1 rad53* double mutants. By interfering with silencing, these factors may be released to facilitate repair of the replication defect. Whereas *mrc1* mutants themselves interfere with silencing, they do so to a lesser degree than the mutants capable of suppressing the lethality of the double mutants. A third possibility is that without silencing, simultaneous expression of *MAT a/MAT α* genes could change the efficiency of repair pathways (Astrom et al. 1999) and thus affect the viability of *mrc1-1 rad53-21* double mutants. The *mrc1-1 rad53-21* homozygous mutant diploids are still Ts and the Ts can be suppressed by overexpression of *ASF1*. It should be noted that the diploid is not as Ts as the haploid, and interfering with Sir function does not enhance growth (data not shown), therefore, diploid status might have a partial effect on the temperature sensitivity but cannot explain the suppression by *ASF1*.

The final connection between checkpoints and chromatin is the physical association between Rad53 and the chromatin assembly factor Asf1. Whereas eliminating silencing was found to provide weak suppression to *rad53* mutants, overproduction of *ASF1* provided a very strong suppression of the lethality of *rad53* mutants experiencing replication blocks. This suppression is likely to be direct because Asf1 and Rad53 exist together in a dynamic complex that dissociates in the presence of DNA damage or replication blocks. The dissociation is dependent on the activation of the checkpoint pathway, because the interaction of Asf1 and Rad53 is maintained in *mec1* mutants in response to replication blocks or DNA damage. The phosphorylation of Rad53 by Mec1 is correlated with the dissociation, but it is unclear whether Rad53 phosphorylation is the critical factor.

*ASF1* overproduction suppresses *rad53-21* mutants and, although there is a physical interaction between Rad53 and Asf1, we did not see restoration of Rad53-21 kinase activity by *ASF1* overproduction, nor increase in its abundance (data not shown). We have also not detected phosphorylation of Asf1 by Rad53 in vivo or in vitro. However, as *ASF1* overproduction is such a strong suppressor of the *rad53* mutants's HU sensitivity, it suggests that *ASF1* is an effector of Rad53, carrying out part of Rad53's function in response to DNA replication blocks. This hypothesis is supported by the fact that *asf1* mutants are sensitive to HU and DNA damage as are *rad53* mutants, suggesting a similar function for these proteins. This leads to the question of the role of Rad53 in Asf1 regulation. The most plausible explanation for the relationship between Asf1 and Rad53 is that they work together to repair damage and alleviate DNA replication problems. In response to DNA damage, Rad53 may activate Asf1 or confer special properties on it, causing it to leave its complex and contribute to repair. Rad53 is a chromatin-associated protein (Frei and Gasser 2000), and it is possible that Rad53 localizes Asf1 to sites of damage, where it is released to facilitate chromatin assembly. This might explain why Asf1 overproduction suppresses *rad53* mutants, because when overexpressed, Asf1 now can locate these sites without the aid of Rad53. Alternatively, Asf1 release in response to replication blocks and damage may allow it to sequester free nucleosomes whose excess might interfere with repair processes by binding to DNA. Although this is a plausible explanation, if this were true, one might expect *ASF1* overproduction to suppress *mec1* mutants, which it does not.

The above model represents the simplest explanation for the relationships between Asf1 and Rad53, but more complex models exist. For example, Asf1 by virtue of its ability to interact with nucleosomes could help localize Rad53 to replication forks and help it find substrates or facilitate its activation. This would be consistent with the delayed kinetics of Rad53 hyperphosphorylation and the partial checkpoint defect in *asf1* mutants in response to HU treatment. In this model, the suppression of the HU sensitivity of *rad53* mutants by *ASF1* overproduction would be indirect. It is also possible that both models could be partially correct and these proteins could mutually regulate one another. Whereas the precise biochemical relationship between these proteins remains to be fully elucidated, their genetic and physical interactions strongly suggest that they function together to facilitate DNA repair and replication. Future investigation of their common functions should provide significant insights into the connection between chromosome structure and checkpoint function.

## Materials and methods

*Yeast strains and cytological techniques*

All strains used in this study are isogenic with the W303-derived Y300 strain (Zhou and Elledge 1993) except for the silencing test strains UCC3505, UCC3515, and UCC4564 (Singer et al. 1998). *SIR3* and carboxy-terminal 2561 bp of *MRC1* were deleted in UCC3505 or UCC4564 by use of a PCR-based gene disruption method (Longtine et al. 1998) and confirmed by PCR analysis. *mrc1-1 rad53-21 sir* mutants were constructed by deleting *SIR* genes in *mrc1-1 rad53-21* mutants by use of the same method (Longtine et al. 1998). Genomic HA3-tagged *ASF1* and *asf1Δ* mutants were constructed according to the same method (Longtine et al. 1998). All other stains are constructed by standard genetic cross.

*Plasmids*

The plasmid used for *GAP–RNR1* was pBAD70 (Desany et al. 1998). The plasmid used for *GAP–DUN1* was pZZ74-2 (Zhou and Elledge 1993). *GAL*-carboxy-terminal *SIR4* and *GAL–ASF1* were kindly given as pTRP10 and pTRP53 (Singer et al. 1998) by Dr. Gottschling (Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA). pARF24 containing 2µ ASF2 (Le et al. 1997) was kindly

given by Dr. Sternglanz (Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York). pFH10 was used as 2μ ASF1 in this study. PCR reactions using primers 5′-CAGGATAT TGTCTTGGTGGCGTC and 5′-ACGTAGCTGTTGCCCTAACCCG were performed to amplify the *ASF1* gene. This genomic fragment was then cloned into PCR2.1-TOPO (Invitrogen) to give pFH8. The *Eco*RI fragment of pFH8 containing *ASF1* was cloned into pRS426 to give pFH10.

*Protein Techniques*

Protein extracts for Western blots were made by use of the TCA precipitation method as described (Paciotti et al. 1998). For immunoprecipitations, log phase cultures of yeast cells were pelleted, resuspended in buffer containing 50 mM Tris7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, and proteinase inhibitors and broken open by bead beating. A total of 5 mg of protein extract was diluted to 1 mL with the same buffer and precleared by incubating with Pro-G beads for 1 h at 4°C. For α-Rad53 immunoprecipitations, 30 μL of anti-Rad53 antibodies were added to precleared supernatant and rotated at 4°C overnight. After spinning for 10 min at 14 K, 40 μL of Protein-A beads were added to the supernatant and rotated for another hour. For α-HA immunoprecipitation, 40 μL of anti-HA matrix was added to precleared supernatant and rotated at 4°C overnight. Beads were then washed with 1 mL buffer four times. SDS-loading dye was added and samples were boiled and resolved on SDS-PAGE.

## Acknowledgments

## References

Astrom, S.U., Okamura, S.M., and Rine, J. 1999. Yeast cell-type regulation of DNA repair. *Nature* **397:** 310.

Cairns, B.R. 1998. Chromatin remodeling machines: Similar motors, ulterior motives. *Trends Biochem. Sci.* **23:** 20–25.

Cockell, M., Palladino, F., Laroche, T., Kyrion, G., Liu, C., Lustig, A.J., and Gasser, S.M. 1995. The carboxy termini of Sir4 and Rap1 affect Sir3 localization: Evidence for a multicomponent complex required for yeast telomeric silencing. *J. Cell Biol.* **129:** 909–924.

Craven, R.J. and Petes, T.D. 2000. Involvement of the checkpoint protein Mec1p in silencing of gene expression at telomeres in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **20:** 2378–2384.

Desany, B.A., Alcasabas, A.A., Bachant, J.B., and Elledge, S.J. 1998. Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. *Genes & Dev.* **12:** 2956–2970.

Frei, C. and Gasser, S.M. 2000. The yeast Sgs1p helicase acts upstream of Rad53p in the DNA replication checkpoint and colocalizes with Rad53p in S-phase-specific foci. *Genes & Dev.* **14:** 81–96.

Grushcow, J.M., Holzen, T.M., Park, K.J., Weinert, T., Lichten, M., and Bishop, D.K. 1999. Saccharomyces cerevisiae checkpoint genes MEC1, RAD17 and RAD24 are required for normal meiotic recombination partner choice. *Genetics* **153:** 607–620.

Igual, J.C., Johnson, A.L., and Johnston, L.H. 1996. Coordinated regulation of gene expression by the cell cycle transcription factor Swi4 and the protein kinase C MAP kinase pathway for yeast cell integrity. *EMBO J.* **15:** 5001–5013.

Le, S., Davis, C., Konopka, J.B., and Sternglanz, R. 1997. Two new S-phase-specific genes from Saccharomyces cerevisiae. *Yeast* **13:** 1029–1042.

Lindahl, T. and Wood, R.D. 1999. Quality control by DNA repair. *Science* **286:** 1897–1905.

Longhese, M.P., Paciotti, V., Neecke, H., and Lucchini, G. 2000. Check-

point proteins influence telomeric silencing and length maintenance in budding yeast. *Genetics* **155:** 1577–1591.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast* **14:** 953–961.

Madden, K., Sheu, Y.J., Baetz, K., Andrews, B., and Snyder, M. 1997. SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway. *Science* **275:** 1781–1784.

Marshall, M., Mahoney, D., Rose, A., Hicks, J.B., and Broach, J.R. 1987. Functional domains of SIR4, a gene required for position effect regulation in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **7:** 4441–4452.

Martin, S.G., Laroche, T., Suka, N., Grunstein, M., and Gasser, S.M. 1999. Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast. *Cell* **97:** 621–633.

Mills, K.D., Sinclair, D.A., and Guarente, L. 1999. MEC1-dependent redistribution of the Sir3 silencing protein from telomeres to DNA double-strand breaks. *Cell* **97:** 609–620.

Paciotti, V., Lucchini, G., Plevani, P., and Longhese, M.P. 1998. Mec1p is essential for phosphorylation of the yeast DNA damage checkpoint protein Ddc1p, which physically interacts with Mec3p. *EMBO J.* **17:** 4199–4209.

Roeder, G.S. and Bailis, J.M. 2000. The pachytene checkpoint. *Trends Genet.* **16:** 395–403.

San-Segundo, P.A. and Roeder, G.S. 1999. Pch2 links chromatin silencing to meiotic checkpoint control. *Cell* **97:** 313–324.

Shibahara, K. and Stillman, B. 1999. Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. *Cell* **96:** 575–585.

Singer, M.S., Kahana, A., Wolf, A.J., Meisinger, L.L., Peterson, S.E., Goggin, C., Mahowald, M., and Gottschling, D.E. 1998. Identification of high-copy disruptors of telomeric silencing in Saccharomyces cerevisiae. *Genetics* **150:** 613–632.

Tyler, J.K., Adams, C.R., Chen, S.R., Kobayashi, R., Kamakaka, R.T., and Kadonaga, J.T. 1999. The RCAF complex mediates chromatin assembly during DNA replication and repair. *Nature* **402:** 555–560.

Zhou, B.B. and Elledge, S.J. 2000. The DNA damage response: Putting checkpoints in perspective. *Nature* **408:** 433–439.

Zhou, Z. and Elledge, S.J. 1993. DUN1 encodes a protein kinase that controls the DNA damage response in yeast. *Cell* **75:** 1119–1127.