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.

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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, translational 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 (Densy 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, relocate 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, PCH2, 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, mrc1 rad53-21 double mutants are lethal, and the lethality cannot be suppressed by RNK1 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 G2 DNA content (Fig. 1C) and show an accumulated chromosomes will migrate into the gel. We can therefore, remain in the well. However, fully replicating chromosomes cannot enter the gel because of secondary structures caused by replication 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](image)

**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 (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, 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.

![Figure 2](image)

**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.

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 Pets 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 ei-
ther 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 sup-
pression 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 homozy-
gous 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 involvment of silencing in the temperature sensi-
tivity of mrc1 rad53 mutants led us to examine whether 
Mrc1 or Rad53 has a role in silencing. It has been re-
ported that rad53 mutants have a modest increase of 
telomeric silencing (Craven and Petes 2000; Longhese et 
alone 2000), implicating Rad53 in controlof telomeric chro-
mosomal silencing (Craven and Petes 2000; Longhese et al.
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mosomal silencing (Craven and Petes 2000; Longhese et 
alone 2000). We have also checked the suppression of 
mrc1 rad53 mutations in reporter strains UCC4564 or 
UCC3515 containing either vector alone or HIS-GAL–RAD53 
plasmids were diluted and spotted onto SC-his and SC-his,ura plates 
with SC-his plates containing 5-FOA as indicated, and grown as in A.

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 attrib-
uted 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-res-
ponse pathway. We examined the viability of asf1Δ mu-
tants 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 dilu-
tions of these cultures were made and spotted onto YPD, SC-ura, or 
SC-5-FOA plates and were grown for 4-5 days 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 HIS-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.

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
The reciprocal immunoprecipitations using anti-Rad53 in the IP, although the majority of Rad53 is phosphorylated by MMS (Fig. 5A) or in the presence of HU (Fig. 5B). We observed only the unphosphorylated form of Rad53 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 G2/M using nocodazole and then introduced DNA damage using γ-irradiation while maintaining the G2/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 mec1 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 mec1 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 mec1 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 a 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 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’ 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 in this study are isogenic with the W303-derived Y300 strain [Zhou and Elledge 1993] except for the cloning 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 asf1Delta mutants were constructed according to the same method [Longtine et al. 1998]. All other strains are constructed by standard genetic crosses.

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 pTRPS3 [Singer et al. 1998] by Dr. Gotschlich (Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA). pARF2 containing 2μ ASF2 [Le et al. 1997] was kindly
given by Dr. Sternfanz (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 were performed with primers 5'-CAGGATAT TGTCTTGGTGCGCTC and 5'-ACGTAGCTTGGCCTAAACCGC were performed to amplify the ASF1 gene. This genomic fragment was then cloned into PCR2.1-TOPO (Invitrogen) to give pFH8. The EcoRI 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 monoclonal antibody (12CA5) 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.

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