Saccharomyces cerevisiae chromatin-assembly factors that act during DNA replication function in the maintenance of genome stability

Kyungjae Myung*, Vincent Pennaneach, Ellen S. Kats, and Richard D. Kolodner†

Ludwig Institute for Cancer Research, Cancer Center and Department of Medicine, University of California at San Diego School of Medicine, La Jolla, CA 92093

Contributed by Richard D. Kolodner, April 15, 2003

Some spontaneous gross chromosomal rearrangements (GCRs) seem to result from DNA-replication errors. The chromatin-assembly factor I (CAF-I) and replication-coupling assembly factor (RCAF) complexes function in chromatin assembly during DNA replication and repair and could play a role in maintaining genome stability. Inactivation of CAF-I or RCAF increased the rate of accumulating different types of GCRs including translocations and deletion of chromosome arms with associated *de novo* telomere addition. Inactivation of CAF-I seems to cause damage that activates the DNA-damage checkpoints, whereas inactivation of RCAF seems to cause damage that activates the DNA-damage and replication checkpoints. Both defects result in increased genome instability that is normally suppressed by these checkpoints, RAD52-dependent recombination, and PIF1-dependent inhibition of *de novo* telomere addition. Treatment of CAF-I- or RCAF-defective cells with methyl methanesulfonate increased the induction of GCRs compared with that seen for a wild-type strain. These results indicate that coupling of chromatin assembly to DNA replication and DNA repair is critical to maintaining genome stability.

Maintaining the stability of the genome is crucial for cell survival and normal cell growth. The presence of specific genome rearrangements and the ongoing accumulation of genome rearrangements are seen in many types of cancer cells (1–5). Similarly, the inheritance of genome rearrangements underlies other human genetic diseases (6, 7). Studies with model organisms have identified multiple mechanisms by which genome rearrangements arise and multiple pathways that act to maintain genome stability (8). A number of studies have suggested that spontaneous genome rearrangements result from errors during DNA replication that possibly lead to stalled or broken replication forks (9–15). A number of pathways seem to act on these errors to promote their correct repair and prevent their conversion into genome rearrangements (8, 16). Among these pathways are (*i*) at least three different checkpoints that act during S phase, (*ii*) recombination pathways similar to those that promote break-induced replication, (*iii*) a pathway that prevents *de novo* addition of telomeres to broken DNAs, and (*iv*) possibly mismatch repair that prevents recombination between divergent DNA sequences (8). An alternative source of DNA damage that can lead to genome instability is degradation of telomeres in the absence of telomerase (8, 16, 17); these degraded chromosomes seem to be acted on by many of the same pathways that have been suggested to act on DNA-replication errors. Interestingly, the human homologs of many of the *Saccharomyces cerevisiae* genes that function in suppression of genome instability and human genes encoding interacting proteins have been implicated in pathways that suppress the development of cancer (18–28).

DNA replication and chromatin assembly are coordinated, and in human cells S phase is also not completed in the absence of chromatin assembly (29–32). At least two chromatin-assembly complexes (CACs), chromatin-assembly factor I (CAF-I) and replication-coupling assembly factor (RCAF), function in the assembly of chromatin linked to DNA synthesis (33–39). CAF-I is a three-subunit complex, consisting of CAC1–CAC3, in *S. cerevisiae*, that interacts with histones H3 and H4 and targets them to DNA through an interaction between CAC1 and proliferating cell nuclear antigen (PCNA) (34, 40–42). CAF-I can promote the assembly of nucleosomes *in vitro* (33, 36, 40). Mutations that inactivate CAF-I cause defects in silencing at telomeres and mating-type loci as well as mild sensitivity to UV irradiation but do not effect viability or generally cause sensitivity to other DNA-damaging agents or hydroxyurea (36, 40). Recently it was shown that expression of a dominant-negative CAC1 protein in mammalian cells seems to induce DNA damage during S phase and activation of S-phase checkpoints (31). Interestingly, several genetic studies have suggested that CAC1–CAC3 may also have independent functions (43–47). RCAF consists of antisilencing function 1 (ASF1) and histones H3 and H4 and can also promote assembly of nucleosomes *in vitro* (36). RCAF interacts with CAF-I through an interaction between ASF1 and CAC2, and biochemical studies have indicated that RCAF and CAF-I cooperate in *in vitro* chromatin-assembly assays (36, 48, 49). Mutations in *ASF1* cause weak defects in silencing as well as a broader range of defects than CAF-I defects including slow growth and sensitivity to a broader range of DNA-damaging agents and hydroxyurea (36, 50). The slow-growth phenotype of *asf1* mutants is associated with a defect in transiting S phase, and *asf1* mutants show a defect in response to hydroxyurea treatment similar to that seen in checkpoint-defective mutants (36, 51, 52). Consistent with this phenotype, ASF1 physically interacts with the checkpoint protein RAD53, and *asf1* mutations cause partial defects in some checkpoint responses to hydroxyurea (51, 53). Inactivation of both CAF-I and RCAF results in stronger defects than inactivation of either complex alone (36, 50, 54), which suggests that CAF-I and RCAF have distinct roles in chromatin assembly as well as likely a joint role implied by the physical interaction between the two complexes.

Because chromatin assembly is important for DNA replication and repair, correct chromatin assembly may also be important for maintaining genome stability. Because defects in CAF-I and RCAF cause defects in S-phase and/or DNA-repair defects, we tested whether mutations in the genes encoding these two chromatin-assembly factors cause genome instability. The results reported here indicate that CAF-I and RCAF defects cause the accumulation of DNA damage resulting in increased rates of accumulating genome rearrangements. Our results also support the view that CAF-I and RCAF play different roles in suppression of genome

Abbreviations: CAC, chromatin-assembly complex; CAF-I, chromatin-assembly factor I; RCAF, replication-coupling assembly factor; ASF1, antisilencing function 1; GCR, gross chromosomal rearrangement; MMS, methyl methanesulfonate; DSB, double-strand break; NHEJ, nonhomologous end joining.

*Present address: Genome Instability Section, Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Building 49, Room 4A22, Bethesda, MD 20892.

†To whom correspondence should be addressed at: Ludwig Institute for Cancer Research, University of California at San Diego School of Medicine, CMME 3058, 9500 Gilman Drive, La Jolla, CA 92093-0669. E-mail: rkolodner@ucsd.edu.

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Table 1. Effect of CAF-I and RCAF defects on the rate of accumulating GCRs

	Wild type		*asf1*Δ	
Relevant genotype	Strain no.	Mutation rate (Can^r 5-FOA^r)	Strain no.	Mutation rate (Can^r 5-FOA^r)
Wild type	3615	3.5×10^{-10} (1)	4755	2.5×10^{-8} (71)
*cac1*Δ	4753	1.2×10^{-7} (343)	4779	3.9×10^{-8} (111)
*cac2*Δ	5003	3.0×10^{-8} (87)	5011	1.5×10^{-8} (43)
*cac3*Δ	5005	1.1×10^{-8} (32)	5013	1.2×10^{-8} (35)
*cac1*Δ *cac2*Δ	5001	5.1×10^{-8} (145)		ND
*cac1*Δ *cac3*Δ	5009	6.9×10^{-7} (1,997)		ND
*cac2*Δ *cac3*Δ	5007	3.4×10^{-8} (97)		ND

The numbers in parentheses are the fold increases in rate relative to that of the wild-type strain. The GCR rate with RDKY5075 (*cac1*Δ *cac2*Δ *cac3*Δ) was 1×10^{-8} (29). ND, not determined.

instability, consistent with the view that these complexes have both distinct and common roles in chromatin assembly.

Experimental Procedures

General Genetic Methods. Media for propagation of strains and determining gross chromosomal rearrangement (GCR) rates were as described (11, 55). All *S. cerevisiae* strains were propagated at 30°C except for *rfc5-1* and *dpb11-1* mutants, which were grown at 23°C. Gene disruptions all were made by standard PCR-based gene-disruption methods, and correct gene disruptions were verified by PCR as described (10). The *hta1S129STOP* and *hta2S129STOP* mutations were inserted by using pop-in pop-out plasmids and verified by sequence analysis (56). The sequences of primers used to generate disruption cassettes and confirm disruption of indicated genes are available on request. All strains were derived from the S288c parental strain RDKY3023 (*MATa*, *ura3-52*, *leu2Δ1*, *trp1Δ63*, *his3Δ200*, *lys2ΔBgl*, *hom3-10*, *ade2Δ1*, *ade8*) and in addition contained the *hxt13*::*URA3* insertion used in the GCR assay. Relevant genotypes of these strains are RDKY3615 wild type; RDKY3733 *sml1*::*KAN*; RDKY4753 *cac1*::*TRP1*; RDKY5003 *cac2*::*HIS3*; RDKY5005 *cac3*::*HIS3*; RDKY5001 *cac1*::*TRP1 cac2*::*HIS3*; RDKY5009 *cac1*::*TRP1 cac3*::*HIS3*; RDKY5007 *cac2*::*TRP1 cac3*::*HIS3*; RDKY4755 *asf1*::*HIS3*; RDKY4779 *cac1*::*TRP1 asf1*::*HIS3*; RDKY5011 *cac2*::*TRP1 asf1*::*HIS3*; RDKY5013 *cac3*::*HIS3 asf1*::*TRP1*; RDKY3719 *rad9*::*HIS3*; RDKY3723 *rad24*::*HIS3*; RDKY3814 *sgs1*::*HIS3*; RDKY3727 *rfc5-1*; RDKY4538 *dpb11-1*; RDKY4757 *rad9*::*HIS3 cac1*::*TRP1*; RDKY4761 *rad24*::*HIS3 cac1*::*TRP1*; RDKY4765 *cac1*::*TRP1 sgs1*::*HIS3*; RDKY4769 *rfc5-1 cac1*::*HIS3*; RDKY4773 *dpb11-1 cac1*::*HIS3*; RDKY4759 *rad9*::*HIS3 asf1*::*TRP1*; RDKY4763 *rad24*::*HIS3 asf1*::*TRP1*; RDKY4767 *asf1*::*HIS3 sgs1*::*TRP1*; RDKY4771 *rfc5-1 asf1*::*HIS3*; RDKY4775 *dpb11-1 asf1*::*HIS3*; RDKY4343 *pif1-m2*; RDKY4224 *tlc1*::*TRP1*; RDKY3735 *sml1*::*KAN mec1*::*HIS3*; RDKY3731 *tel1*::*HIS3*; RDKY3739 *dun1*::*HIS3*; RDKY3745 *chk1*::*HIS3*; RDKY4781 *pif1-m2 cac1*::*TRP1*; RDKY4785 *cac1*::*TRP1 tlc1*::*HIS3*; RDKY4789 *sml1*::*KAN mec1*::*HIS3 cac1*::*TRP1*; RDKY4793 *tel1*::*HIS3 cac1*::*TRP1*; RDKY4801 *dun1*::*HIS3 cac1*::*TRP1*; RDKY4807 *chk1*::*HIS3 cac1*::*TRP1*; RDKY4783 *pif1-m2 asf1*::*HIS3*; RDKY4787 *asf1*::*HIS3 tlc1*::*TRP1*; RDKY4791 *sml1*::*KAN mec1*::*TRP1 asf1*::*HIS3*; RDKY4795 *tel1*::*HIS3 asf1*::*TRP1*; RDKY4803 *asf1*::*HIS3 dun1*::*TRP1*; RDKY4809 *asf1*::*HIS3 chk1*::*TRP1*; RDKY4857 *hta1 S129STOP hta2 S129STOP*; RDKY3641 *lig4*::*HIS3*; RDKY3640 *yKU80*::*HIS3*; RDKY4421 *rad52*::*HIS3*; RDKY4823 *hta1 S129STOP hta2 S129STOP cac1*::*TRP1*; RDKY4827 *lig4*::*HIS3 cac1*::*TRP1*; RDKY4831 *cac1*::*TRP1 yKU80*::*HIS3*; RDKY4843 *cac1*::*TRP1 rad52*::*HIS3*; RDKY4825 *hta1 S129STOP hta2 S129STOP asf1*::*HIS3*; RDKY4829 *lig4*::*HIS3 asf1*::*TRP1*; RDKY4833 *yKU80*::*HIS3 asf1*::*TRP1*; RDKY4855 *asf1*::*HIS3 rad52*::*TRP1*; RDKY4853 *asf1*::*HIS3 rad59*::*TRP1*; RDKY4861 *sml1*::*KAN cac1*::*TRP1*; and RDKY5075 *cac1*::*TRP1 cac2*::*HYG cac3*::*HIS3*. We were unable to construct a *cac1 cac3 asf1* triple mutant. In selected cases, we tested whether *ARS CEN* plasmids expressing either ASF1 or CAC1 under control of their native promoters complemented *asf1*- and *cac1*-induced GCR rates, respectively, to ensure that observed increased GCR rates were not due to accumulation of second-site mutations.

Characterization of GCR Rates and Breakpoints. All GCR rates were determined independently by fluctuation analysis two or more times by using either 5 or 11 cultures, and the average value is reported (11, 55). Statistical significance was evaluated by the Mann–Whitney test by using programs available at http://faculty.vassar.edu/lowry/vshome.html. The effect of methyl methanesulfonate (MMS) treatment on cell survival and GCR frequency was determined as described (57). The sequences of independent rearrangement breakpoints were determined and classified as described (11, 55).

Results

Chromatin-Assembly Factors Suppress Genome Instability. Mutations in each of the three genes encoding components of CAF-I were tested for their effect on the GCR rate (Table 1). A *cac1* mutation increased the GCR rate by 340-fold, and the resulting GCRs were a mixture of *de novo* telomere additions and translocations with microhomology or nonhomology breakpoints (Table 2). In contrast, mutations in the *CAC2* and *CAC3* genes encoding the other two subunits of CAF-I increased the GCR rate but not to the same level as that caused by a *cac1* mutation ($P = 0.0003$ and 0.0005, respectively). Interestingly, compared with the increased GCR rate caused by a *cac1* mutation, the *cac1 cac2* double-mutant strain had a decreased GCR rate ($P = 0.0036$), and the *cac1 cac3* double-

Table 2. Structure of observed rearrangement breakpoints generated from strains defective in CAF-I and RCAF

Relevant genotypes	Strain no.	Telomere addition	Translocation/deletion, non, micro
Wild type*	3615	5	1, 0
*cac1*Δ *sml1*Δ	4861	6	2, 2
*cac1*Δ	4753	7	2, 1
*asf1*Δ	4755	9	0, 2
*cac1*Δ *asf1*Δ	4779	4	2, 4
*tel1*Δ*	3731	0	0, 6
*mec1*Δ *sml1*Δ*	3735	9	0, 0
*tel1*Δ *cac1*Δ	4793	2	3†, 4
*mec1*Δ *sml1*Δ *cac1*Δ	4789	9	0, 0
*lig4*Δ‡	3641	6	0, 0
*cac1*Δ *lig4*Δ	4827	20	0, 0

''non, micro'' indicates the number of nonhomology and microhomology breakpoints observed, respectively.

*Data are from Myung *et al.* (55).

†One case was a deletion that had 143 bp of non-chromosome V sequences inserted at the breakpoint.

‡Data are from Myung *et al.* (16).

Table 3. Interaction between CAF-I or RCAF defects and checkpoint defects

	Wild type		*cac1*Δ		*asf1*Δ	
Relevant genotype	Strain no.	Mutation rate (Can^r 5-FOA^r)	Strain no.	Mutation rate (Can^r 5-FOA^r)	Strain no.	Mutation rate (Can^r 5-FOA^r)
Wild type	3615	3.5×10^{-10} (1)*	4753	1.2×10^{-7} (343)	4755	2.5×10^{-8} (71)
*rad9*Δ	3719	2.0×10^{-9} (6)*	4757	4.0×10^{-7} (1,142)	4759	1.0×10^{-7} (285)
*rad24*Δ	3723	4.0×10^{-9} (11)*	4761	4.8×10^{-7} (1,371)	4763	2.0×10^{-7} (571)
*sgs1*Δ	3814	7.7×10^{-9} (22)†	4765	6.3×10^{-7} (1,800)	4767	9.7×10^{-8} (278)
rfc5-1	3727	6.6×10^{-8} (189)*	4769	1.3×10^{-7} (371)	4771	8.6×10^{-7} (2,457)
dpb11-1	4538	9.0×10^{-8} (257)*	4773	1.2×10^{-7} (342)	4775	2.3×10^{-7} (657)
*mec1*Δ *sml1*Δ‡	3735	6.8×10^{-8} (194)*	4789	5.2×10^{-7} (1,486)	4791	1.7×10^{-7} (486)
*dun1*Δ	3739	7.3×10^{-8} (208)*	4801	5.6×10^{-7} (1,600)	4803	6.9×10^{-8} (197)
*chk1*Δ	3745	1.3×10^{-8} (37)*	4807	8.0×10^{-7} (2,285)	4809	6.9×10^{-8} (197)

The numbers in parentheses are the fold increases in rate relative to that of the wild-type strain.
*Data are from Myung *et al.* (55).
†Data are from Myung *et al.* (57).
‡GCR rate of the *sml1* strain was 3.1×10^{-10} (1).

mutant strain had an increased GCR rate ($P = 0.0001$). The *cac2 cac3* double-mutant strain had a GCR rate that was not different from that caused by a *cac2* mutation ($P = 0.42$). The *cac1 cac2 cac3* triple mutant had a GCR rate that was similar to the GCR rate of the *cac2* and *cac3* single mutants ($P = 0.43$ and 0.25, respectively) and the *asf1* (see below; $P = 0.1$) mutant; this GCR rate was reduced compared with the GCR rate of either the *cac1* or *cac1 cac3* mutant strains. One interpretation of these results is that the increased GCR rate of the *cac1* single mutant and the *cac1 cac3* double mutant may in part be due to the aberrant activity of subcomplexes of CAF-I containing CAC2 and possibly ASF1 (see below). It should be noted that other studies have also suggested that the different CAF-I subunits may also have distinct functions in addition to a shared function implied by their presence in the CAF-I complex (43–47), and defects in these different functions could also contribute to the differences between the effects of the *cac1*, *cac2*, and *cac3* mutations seen here.

To analyze the role of the RCAF complex in suppression of genome instability, a mutation in the *ASF1* gene was tested for its effect on the GCR rate. An *asf1* mutation increased the GCR rate by 70-fold (Table 1), and the resulting GCRs were a mixture of mainly *de novo* telomere-addition GCRs and a low proportion of translocations with microhomology breakpoints (Table 2). The GCR rate of the *asf1 cac1* double-mutant strain was reduced relative to that observed in the *cac1* single-mutant strain ($P = 0.012$) but was not significantly different than the GCR rate of the *cac1 cac2* double mutant ($P = 0.32$). The GCR rate of the *asf1 cac2* double mutant was somewhat less than but not significantly different than that of either the *asf1* or *cac2* single mutants ($P = 0.13$ and 0.5, respectively); these results are consistent with the observation that ASF1 and CAC2 interact and may function together (48, 49). These results further support the idea that the increased GCR rate of the *cac1* single mutant may in part be due to the aberrant activity of ASF1 as well as CAC2 (see above). The GCR rate of the *asf1 cac3* double mutant was not different than that of the *cac3* mutant ($P = 0.41$) and somewhat less than that of the *asf1* mutant ($P = 0.028$).

***cac1* and *asf1* Mutations Seem to Activate Different Checkpoints.** The increased GCR rate caused by *cac1* and *asf1* mutations could occur if defects in chromatin assembly due to the *cac1* and *asf1* defects cause either replication defects or damage to the newly replicated DNA. If this is true, *cac1* and *asf1* mutations might show synergistic interactions with mutations that cause defects in the replication and DNA-damage checkpoints that act during S phase to suppress spontaneous GCRs (55, 57). To investigate this possibility, the effect of combining *cac1* and *asf1* mutations with mutations that inactivate different checkpoint sensor functions was tested (Table 3). Combining a *cac1* mutation with mutations that inactivate different DNA-damage and intra-S DNA-damage checkpoint functions (*rad9*, *rad24*, and *sgs1*) resulted in a synergistic increase in the GCR rate. However, combining a *cac1* mutation with either *rfc5-1* or *dpb11-1* mutations that cause defects in the replication checkpoint did not increase the GCR rate above that caused by the *cac1* mutation ($P = 0.13$ and 0.1, respectively). In contrast, when an *asf1* mutation was combined with *rad9*, *rad24*, *sgs1*, *rfc5-1*, or *dpb11-1* mutations, a synergistic increase in the GCR rate was observed. We do not know why the *rfc5-1* and *dpb11-1* mutations showed somewhat different effects when combined with the *asf1* mutation, although we note that neither of these mutations is a complete loss-of-function mutation. These results suggest that the errors that lead to increased GCR rates in a *cac1* mutant are recognized by the DNA-damage checkpoints, whereas the errors caused by an *asf1* mutation are recognized by both the DNA-damage and replication checkpoints.

***cac1* and *asf1* Mutations Interact Differently with Mutations in Genes Encoding Components of the Checkpoint Signal Transduction Cascade.** Signals generated by activation of different cell-cycle checkpoints are transduced to effector proteins by a signal transduction cascade (52). To determine which checkpoint transducer functions play a role in suppressing *cac1*-induced GCRs, double mutants containing mutations in the *CAC1* gene and genes encoding transducer proteins were analyzed (Table 3). When a *cac1* mutation was combined with *mec1*, *dun1*, or *chk1* mutations, a synergistic increase in the GCR rate was observed. The rearrangement breakpoints formed in the *cac1 mec1* strain were all *de novo* telomere-addition events (Table 2) consistent with previous observations that *mec1* mutations result in a large increase in the rate of *de novo* telomere additions (55). This result is consistent with the idea that a checkpoint involving MEC1, DUN1, and CHK1 acts in suppressing *cac1*-induced GCRs. This role of MEC1, DUN1, and CHK1 parallels their role in the DNA-damage checkpoints and the observed interaction between *cac1* mutations and intra-S DNA-damage checkpoint sensor-defective mutations.

When an *asf1* mutation was combined with either a *mec1* or *chk1* mutation, a modest synergistic increase in the GCR rate was observed (Table 3). In contrast, the GCR rate of the *dun1 asf1* strain was not increased compared with that of the *dun1* strain ($P = 0.07$). This suggests that the MEC1 CHK1 signal transduction cascade branch is most important in suppression of *asf1*-induced GCRs. The lack of an interaction between *asf1* and *dun1* mutations could suggest that this signal transduction cascade branch is less important for suppressing *asf1*-induced GCRs. However, it is known that *asf1* mutations cause a partial checkpoint defect in response to hydroxyurea including a defect in activating the RAD53 kinase and, by inference, potentially a defect in activating DUN1

Table 4. Interaction between CAF-I or RCAF defects and defects in telomere maintenance functions or DSB repair

	Wild type		*cac1*Δ		*asf1*Δ	
Relevant genotype	Strain no.	Mutation rate (Can^r 5-FOA^r)	Strain no.	Mutation rate (Can^r 5-FOA^r)	Strain no.	Mutation rate (Can^r 5-FOA^r)
Wild type	3615	3.5×10^{-10} (1)†	4753	1.2×10^{-7} (343)	4755	2.5×10^{-8} (71)
*tel1*Δ	3731	2.0×10^{-10} (0.6)†	4793	6.4×10^{-8} (183)	4795	2.0×10^{-9} (5.6)
pif1-m2	4343	8.3×10^{-8} (237)‡	4781	3.5×10^{-7} (1,000)	4783	2.7×10^{-7} (771)
*tlc1*Δ	4224	3.1×10^{-10} (0.9)‡	4785	2.0×10^{-9} (6)	4787	1.3×10^{-6} (3,700)
*hta1 S129*hta2 S129**	4857	4.4×10^{-10} (1.3)	4823	9.0×10^{-9} (26)	4825	2.6×10^{-8} (74)
*lig4*Δ	3641	1.6×10^{-9} (5)‡	4827	2.1×10^{-7} (600)	4829	2.4×10^{-8} (69)
*yKu80*Δ	3640	7.8×10^{-10} (2)‡	4831	9.3×10^{-10} (2.7)	4833	1.0×10^{-9} (2.9)
*rad52*Δ	4421	4.4×10^{-8} (126)‡	4843	3.0×10^{-7} (857)	4855	4.4×10^{-7} (1,257)

The numbers in parentheses are the fold increases in rate relative to that of the wild-type strain.
†Data are from Myung *et al.* (55).
‡Data are from Myung *et al.* (16).

because it lies downstream of RAD53 in regard to some checkpoint responses (36, 51, 58). Thus, an alternative explanation for the lack of an interaction between *asf1* and *dun1* mutations is that *asf1* mutations significantly inactivate the RAD53 DUN1 checkpoint branch. We previously suggested that three different checkpoints function to suppress genome instability and that each functions through distinct but partially overlapping signal transduction cascade components (55, 57). Thus, an alternative explanation for the limited interaction between an *asf1* mutation and *mec1*, *chk1*, and *dun1* mutations is that an *asf1* defect may result in damage that activates multiple checkpoints and hence multiple signal transduction cascades. Consequently, inactivating only one signal transduction cascade may cause little defect in suppressing the genome instability induced by an *asf1* mutation.

***cac1* and *asf1* Mutations Interact Differently with Mutations in Genes Encoding Telomere Maintenance Functions.** Combining a *tel1* mutation with a *cac1* mutation resulted in an ≈2- to 3-fold reduction of the GCR rate compared with a *cac1* single mutant (Table 4). Analysis of GCR breakpoints formed in the *cac1 tel1* strain revealed a decrease in the proportion of *de novo* telomere-addition events, although the number of events analyzed was small (Table 2). Calculating the rate of accumulating *de novo* telomere additions and translocations in the *cac1 tel1* strain suggested that inactivation of TEL1 exclusively reduced the rate of *de novo* telomere additions. This result indicates that a TEL1-dependent pathway is important for the formation of *de novo* telomere-addition GCRs, consistent with the role of TEL1 in telomere maintenance (16, 55, 59). As predicted by this observation, combining a *pif1-m2* mutation (16, 60) with a *cac1* mutation resulted in a synergistic increase in the GCR rate, whereas combining a *tlc1* mutation (61) with a *cac1* mutation reduced the GCR rate, which indicates that GCR formation in the *cac1* strain by *de novo* telomere addition is suppressed by PIF1 and requires telomerase.

Combining an *asf1* mutation with a *tel1* mutation resulted in a large decrease in the GCR rate, indicating that a major proportion of the GCRs that occur in an *asf1* mutant are TEL1-dependent. Because most of the GCRs in an *asf1* mutant are telomere additions, this could reflect the role of TEL1 in telomere maintenance. Consistent with this hypothesis, combining an *asf1* mutation with a *pif1-m2* mutation resulted in a large increase in the GCR rate, indicating that most of the GCRs in an *asf1* mutant are suppressed by PIF1 (16). Surprisingly, combining an *asf1* mutation with a *tlc1* mutation resulted in a large increase in the GCR rate rather than the predicted decrease in GCR rate if *asf1*-induced telomere additions require telomerase. It seems unlikely that the *de novo* telomere additions that occur in an *asf1* mutant do not require the activity of telomerase. A possible explanation for this effect is that, in the absence of ASF1, chromatin structure may be altered near telomeres, resulting in increased disruption of telomere integrity in a *tlc1* mutant leading to increased genome instability (16, 17). Consistent with this idea, it has been shown that an *asf1* mutation enhances the growth defects caused by a *cdc13* mutation that causes a defect in telomere maintenance (50). Alternatively, because this effect is similar to the increase in the GCR rate observed in a *tel1 tlc1* double mutant compared with the respective single mutants (16), it is possible that *asf1* and *tel1* mutations may cause similar checkpoint defects in response to telomere damage.

Double-Strand Break (DSB)-Repair Pathways Are Required for Suppression and Formation of GCRs in *cac1* and *asf1* Strains. Previous studies have suggested that the break-induced replication pathway of recombination, presumably by promoting recombination with sister chromatids, plays a role in suppressing GCRs and that nonhomologous end joining (NHEJ) can act on damage that leads to GCRs and sometimes results in the formation of translocations (11, 16). The role of these pathways in the formation and suppression of GCRs observed in *cac1* and *asf1* strains was investigated, combining mutations that cause defects in different DSB-repair pathways with *cac1* and *asf1* mutations (Table 4). Break-induced replication is highly dependent on *RAD52* but shows less dependence on other *RAD50* epistasis group genes because of redundancy between different gene products or only a partial requirement at normal growth temperatures (62, 63). Combining a *rad52* mutation with either a *cac1* or *asf1* mutation resulted in a synergistic increase in the GCR rate. Synergistic effects were also seen with *rad51*, *rad54*, *rad55*, *rad57*, and *rad59* mutations (data not shown), which suggests that the GCRs induced by *cac1* and *asf1* mutations are suppressed partially by the homologous recombination. Inactivation of NHEJ by a *lig4* mutation (64) in combination with *cac1* mutation caused a significant increase in the GCR rate compared with that caused by a *cac1* mutation ($P = 0.028$). The rearrangement breakpoints that occurred in a *cac1 lig4* double mutant were all *de novo* telomere additions (Tables 2 and 4), indicating that *cac1*-induced translocation events require NHEJ. In contrast, combining a *lig4* mutation with an *asf1* mutation did not change the GCR rate compared with that seen in an *asf1* single-mutant strain ($P = 0.46$). We did not analyze the GCRs formed in an *asf1 lig4* double mutant because of the low rate of formation of translocations induced by an *asf1* mutation (Table 2). These results suggest that ligase 4 plays a role in suppressing some of the *cac1*-induced GCRs but not in suppressing *asf1*-induced GCRs. Inactivation of the *yKU80* gene in both *cac1* and *asf1* strains reduced the GCR rates observed to almost wild-type levels, which is consistent with previous observations that *yKU80* is required for both efficient NHEJ and efficient *de novo* telomere addition (16), although it is also possible that *asf1*- and *cac1*-induced GCRs are lethal in the absence of Ku. Although our favored hypothesis is that recombination and NHEJ act to suppress GCRs induced by *asf1* and *cac1* mutations, it is also possible that in the absence of recombination or NHEJ,

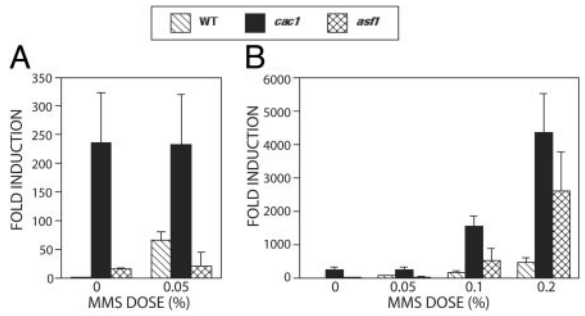

Fig. 1. Induction of GCRs by DNA damage in CAF-I- and RCAF-defective strains. Log-phase cells of the wild-type (RDKY3615) and *cac1* (RDKY4753) or *asf1* (RDKY4755) mutant strains were treated with the indicated concentration of MMS in water for 2 h, washed, diluted into 10 times the starting volume of yeast extract/peptone/dextrose, and grown to saturation. The cells then were plated on appropriate medium to determine the frequency of Can^r 5-FOA^r cells present. Three to five independent cultures of each strain were used in each experiment, and each experiment was performed at least twice. The average fold increase in the frequency of GCRs relative to no-MMS treatment is reported. Results obtained at either 0% or 0.5% MMS (*A*) are plotted with a fold-induction scale (*y* axis) different from the complete data set for 0%, 0.05%, 0.1%, and 0.2% MMS (*B*).

there are increased levels of broken DNAs, the repair of which is less efficient in the absence of ASF1 or CAC1.

Histone H2A is phosphorylated in response to DNA damage and becomes localized to the site of DSBs in DNA, suggesting an involvement in DSB repair (56, 65). Mutations that eliminate the phosphorylation sites in histone H2A cause increased sensitivity to agents that cause DSBs (56). The effects of *cac1* and *asf1* mutations were tested in combination with *hta1* and *hta2* mutations that eliminate the histone H2A phosphorylation sites (Table 4). The *hta1* and *hta2* mutations had no effect on the GCR rate by themselves. When these mutations were combined with a *cac1* mutation, the GCR rate was reduced significantly ($P = 0.0001$), yet they had no effect on the GCR rate when combined with an *asf1* mutation ($P = 0.1$). These results suggest that phosphorylation of histone H2A in response to DNA damage contributes to the formation of GCRs when CAC1 is not functional.

***cac1* and *asf1* Mutations Increase the Frequency of DNA-Damage-Induced GCRs.** Treatment of *cac1* or *asf1* strains with 0.05% MMS, an MMS concentration that only activates the intra-S checkpoint (66, 67), did not result in a further increase in the GCR frequency, whereas treatment of a wild-type control strain with 0.05% MMS significantly increased the GCR frequency (Fig. 1*A*) (68). However, when *cac1* or *asf1* strains were treated with higher doses of MMS (0.1% or 0.2%), which are concentrations that activate both the intra-S checkpoint and G_1 and G_2 DNA-damage checkpoints (66, 67), a large increase in GCR frequency was observed (Fig. 1*B*) (68). The *cac1* mutant was no more sensitive to killing by all doses of MMS tested than the wild-type strain, whereas the *asf1* mutant was 10- to 20-fold more sensitive to killing by MMS (data not shown), consistent with other studies (36, 50). These results suggest that chromatin assembly involving CAC1 and ASF1 plays an important role in the repair of high-dose but not low-dose MMS-induced DNA damage. Alternatively, it is possible that the intra-S checkpoint is already activated in *cac1* and *asf1* strains, resulting in suppression of low-dose MMS-induced GCRs. As a result, induction of GCRs is only observed in response to treatment with higher concentrations of MMS.

Discussion

Previous studies have led to the hypothesis that errors during S phase can result in spontaneous genome rearrangements (9–15). To investigate this hypothesis further, we tested whether the chromatin-assembly factors CAF-I and RCAF, which are thought to function in the assembly of chromatin during DNA replication (33–39), are important for suppression of GCRs. Consistent with this idea, mutations in genes encoding components of these two chromatin-assembly factors resulted in increased spontaneous genome instability. In each case, the GCRs that resulted were translocations/deletions or terminal deletions of chromosome arms associated with *de novo* telomere addition driven by telomere maintenance functions. The *cac1*-induced (CAF-I) translocations/deletions seemed to be formed by NHEJ of broken chromosomes. Genetic analysis indicated that *cac1*-induced (CAF-I) GCRs were suppressed by DNA-damage checkpoints but not the replication checkpoint, which is consistent with recent observations that expression of a dominant-negative human CAC1 (p150) induced DNA-damage foci and altered chromatin structure during S phase (31). In contrast, *asf1*-induced (RCAF) GCRs were suppressed by both DNA-damage checkpoints and the replication checkpoint, consistent with previous observations that *asf1* mutations seem to cause damage during S phase and that ASF1 alleviates histone-mediated inhibition of DNA replication *in vitro* (36, 51, 69). In both cases, the GCRs seem to be suppressed by RAD52-dependent recombination and PIF1-dependent suppression of *de novo* telomere additions similar to that observed for spontaneous GCRs (16). Defects in CAF-I and RCAF also resulted in increased MMS-induced GCRs, consistent with the observation that *asf1* mutations cause sensitivity to agents that induce DSBs and that CAF-I promotes chromatin assembly during DNA repair (35, 36, 42, 50).

Our observations suggest that CAF-I and RCAF promote different chromatin-assembly functions. This conclusion is based on the observation that mutations in *CAC1* and *ASF1* each resulted in different GCR rates and showed different interactions with checkpoint defects, NHEJ defects, and defects in histone H2A phosphorylation. These conclusions are consistent with the results of other genetic studies that indicate that CAF-I and RCAF may have distinct functions and cooperate with each other (36–39, 50). We observed that *cac2* and *asf1* single mutants and the *cac2 asf1* double mutant had similar GCR rates, which suggests a role for CAC2 in RCAF function consistent with the observed physical interaction between CAC2 and ASF1 (48, 49). Interestingly, *asf1* and *cac2* mutations reduced the GCR rate of a *cac1* mutant strain, and a *cac2* mutation reduced the GCR rate of the *cac1 cac3* double mutant. This observation raises the possibility that some of the GCRs that occur in a *cac1* mutant or a *cac1 cac3* double mutant may result from aberrant reactions promoted by RCAF in the absence of a functional CAF-I complex and by aberrant reactions promoted by CAF-I subcomplexes. This idea is consistent with the hypothesis that CAF-I and RCAF are codependent, which is based on biochemical studies (36, 49, 69). An alternate possibility is that *asf1* and *cac2* mutations activate a checkpoint that partially suppresses the GCRs induced in *cac1* and *cac1 cac3* mutants. The *hta1 S129STOP hta2 S129STOP* mutations also reduced the GCR rate of a *cac1* mutant strain, suggesting that phosphorylation of improperly assembled chromatin may also contribute to genome instability. Interestingly, induction of a dominant-negative CAC1 in mammalian cells induced phosphorylated histone H2Ax foci (31). It should also be noted that other studies have suggested that CAC1–CAC3 all seem to have distinct functions in addition to a common function (43–47), and it is known that a portion of CAC3 does not copurify with the CAF-I complex (49, 70). Thus, it is also possible that these differences in function could contribute to the differences in the effects of the *cac1*, *cac2*, and *cac3* mutations seen here.

A model describing our observations is presented in Fig. 2. Our results suggest that failure to assemble chromatin properly during DNA replication results in spontaneous DNA damage that can be processed to yield GCRs. The differences in the interaction be-

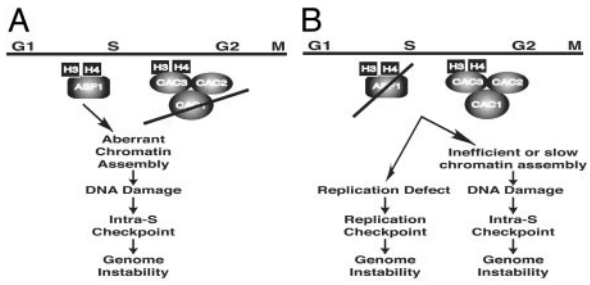

Fig. 2. Model for the induction of GCRs by *CAC1* and *ASF1* defects. (*A*) In the absence of CAC1, aberrant chromatin assembly occurs on newly replicated DNA and results in DNA damage, possibly including DSBs. This damage may be mediated in part by the action of ASF1 (and CAC2) and histone H2A phosphorylation in the absence of normal CAF-I function. This DNA damage results in GCRs, which are suppressed in part by the DNA-damage checkpoints. (*B*) Induction of GCRs by *ASF1* defects. In the absence of ASF1, aberrant chromatin assembly occurs during DNA replication and results in DNA damage, possibly including DSBs. Replication defects and checkpoint defects likely also occur. The resulting DNA damage and replication and checkpoint defects result in GCRs, which are suppressed in part by both the DNA-damage and replication checkpoints.

tween *cac1* (Fig. 2*A*) and *asf1* (Fig. 2*B*) with mutations that cause defects in the different checkpoints that act in suppressing genome instability may reflect the induction of different types of damage by *asf1* and *cac1*. The checkpoint and replication (S-phase) defects caused by *ASF1* but not *CAC1* mutations may also contribute to the differences in genetic instability caused by *ASF1* and *CAC1* mutations. Our genetic data also suggest that *cac1*-induced GCRs (and *cac1 cac3*-induced GCRs, where tested) may involve ASF1 and CAC2 action and histone H2A phosphorylation in the absence of normal CAF-I function. There are several possible explanations for this result: (*i*) aberrant reactions promoted by CAF-I subcomplexes or by ASF1, CAC2, and histone H2A phosphorylation in the absence of normal CAF-I function may result in increased damage or decreased repair; or (*ii*) possibly the absence of ASF1, CAC2, or histone H2A phosphorylation could activate a pathway (such as a checkpoint pathway) that then promotes suppression of *cac1*-induced GCRs. In all cases, the formation and suppression of the resulting GCRs seem to involve the same pathways as have been implicated in the formation and suppression of spontaneous GCRs (8, 16). These results suggest that some spontaneous GCRs may result from spontaneous errors in chromatin assembly and also raise the possibility that genetic defects in chromatin-assembly genes could promote genome instability in mammalian cells. A remaining question of interest is that of what roles other chromatin assembly and modification functions play in maintaining genome stability.

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