

Chromatin assembly factors Asf1 and CAF-1 have overlapping roles in deactivating the DNA damage checkpoint when DNA repair is complete

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In response to a DNA double-strand break (DSB), chromatin is rapidly modified by the damage dependent checkpoint kinases. Also, disassembly of chromatin occurs at the break site. The damage-induced modification of chromatin structure is involved in the maintenance of the checkpoint. However, it has not been determined how chromatin is restored to its undamaged state when DSB repair is complete. Here, we show the involvement of two chromatin assembly factors (CAFs), Asf1 and CAF-1, in turning off the DNA damage checkpoint in budding yeast. DSB repair or formation of γ -H2AX does not depend on either the CAF-1 protein, Cac1, or Asf1. Absence of these proteins does not impair the ability of cells to resume cell cycle progression in the presence of an unrepaired DSB (adaptation). However, recovery from cell cycle checkpoint arrest when the DSB is repaired by gene conversion is substantially defective in the absence of both CAF-1 and Asf1, whereas deleting *CAC1* or *ASF1* individually had little effect. We suggest that CAF-1 and Asf1 function redundantly to deactivate the checkpoint by restoring chromatin structure on the completion of DSB repair.

To maintain genomic integrity, cells have to perform accurate and efficient DNA repair in response to different kinds of DNA damage. In eukaryotic cells, DNA repair is intimately connected to chromatin structure. Originally, chromatin structure was considered merely as a physical obstacle to the access of DNA repair machinery (1). However, a number of studies provide evidence that modifications of chromatin structure have active roles in the DNA damage-dependent response (2). Modification of chromatin structure by the DNA damage checkpoint kinases at the early stages of DNA damage response has been extensively studied (3–7). Also, the disassembly of chromatin at DNA double-strand breaks (DSBs) has been detected (8–10), implying that histones are removed before or during DSB repair. However, it is still unknown how the modified and disassembled chromatin is restored to its original status when DSB repair is complete.

In budding yeast, the induction of even a single DSB activates the DNA damage checkpoint, leading to the cell cycle arrest until the repair of the break is complete (11). By arresting the cell cycle at the G₂/M phase, the checkpoint provides enough time for cells to repair DSBs before cells enter into mitosis. After the formation of a DSB, Mec1 and Tel1 (yeast orthologs of mammalian ATR and ATM, respectively) are initially activated. Mec1 is more critical for the activation of the DNA damage checkpoint, whereas Tel1 has a less important role (12). Both Mec1 and Tel1 can phosphorylate histone H2A on serine 129 (3), whereas Mec1 principally phosphorylates the checkpoint signal transducer kinases, Chk1 and Rad53 (yeast's homolog of mammalian Chk2), as well as the checkpoint mediator protein, Rad9 (13, 14). Budding yeast histone H2A contains the same SQE motif at its C'-terminus that is found in mammalian histone H2AX and is a consensus target sequence of Mec1 and Tel1 kinases. Hereafter, we will refer to phosphorylated yeast H2A-S129 as γ -H2AX. The Mec1/Tel1-dependent phosphorylation of Rad53 is required for autophosphorylation of Rad53 (11). Hyperphosphorylated Rad53 is then able to modulate downstream effectors of cell cycle progression.

Hyperphosphorylated Rad53 is maintained during the persistence of the checkpoint (11). As DNA damage is repaired, cells deactivate the checkpoint to reenter the cell cycle; this process is called recovery. Alternatively, cells can resume the cell cycle even in the presence of an unrepaired DNA break, a process termed adaptation (11). In both processes, a prominent phenomenon is the disappearance of hyperphosphorylated Rad53, which indicates inactivation of the checkpoint (11, 15). Deletions of DNA metabolism proteins such as yKu70, yKu80, and Tid1 (Rdh54), as well as the *cdc5-ad* mutation, are adaptation defective; deletions of the PP2C phosphatases Ptc2 and Ptc3, casein kinase II, and the helicase Srs2 are defective both in adaptation and recovery (12, 15–18).

Chromatin assembly factor (CAF)-1 and Asf1 are cooperatively involved in replication-coupled nucleosome assembly via the interaction with histone H3/H4 (19–21). Also, studies in mammalian cells have shown that CAF-1 is linked to nucleotide excision repair (NER) as well as DNA replication (22, 23). In an in vitro assay, CAF-1 mediates the assembly of nucleosomes onto naked DNA templates in a PCNA-dependent manner (24, 25). Recently, by visualizing transiently expressed tagged histone H3.1 with a specific antibody in human cells, Polo *et al.* (26) demonstrated that CAF-1 is involved in the deposition of histones onto repaired UV lesions in vivo. In budding yeast, deletions of the genes encoding the subunits of CAF-1, *CAC1*, *CAC2*, and *CAC3* result in cellular sensitivity to UV (27). Deletion of Asf1, a histone H3/H4 chaperone, also leads to the UV sensitivity of mutant cells (28). Also, *asf1* mutants are defective in completion of DNA replication when exposed to hydroxyurea (29). Asf1 has other important roles in regulating gene silencing (30) and in facilitating the acetylation of lysine 56 residue on nascent histone H3, which is synthesized mostly during S phase (31, 32). Also, the fact that mutant cells lacking either a subunit of CAF-1 or Asf1 are sensitive to DSB-inducing agents (33, 34) suggests that CAF-1 and Asf1 are involved in the DSB-mediated response. Yeast cells lacking Asf1 accumulate in G₂/M phase because the DNA damage checkpoint is activated by spontaneous DNA damage during replication, although DNA DSB repair is competent in the cells (35). Double deletion of Asf1 and 1 of the subunits of CAF-1, *Cac1*, *Cac2*, or *Cac3*, enhances the G₂/M accumulation of the cells (36); however, DNA replication itself is proficient in the mutant cells, and the transient checkpoint arrest phenotype of these mutants is rescued by deletion of checkpoint proteins such as Mec1 and Rad53 (35, 36). These results raise the possibility that the CAFs are related to the DNA damage-activated checkpoint.

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In this study, we provide evidence that CAF-1 and Asf1 have redundant roles in regulating deactivation of the damage-activated checkpoint after DSB repair is complete. When the DNA damage checkpoint is robustly activated in response to the induction of a DSB, *asf1Δ cac1Δ* double mutants fail to recover from the checkpoint-mediated arrest. However, the absence of these factors affects neither repair of a DSB nor the removal of γ -H2AX from regions surrounding the repaired locus. These results indicate that the failure of the checkpoint deactivation is not caused by persistent DNA damage or modified histones around the site or by defects in γ -H2AX disassembly. However, adaptation, which occurs in the absence of repair, is not defective in *asf1Δ cac1Δ* cells. Therefore, we suggest that when DSBs are repaired, CAF-1 and Asf1 cooperatively restore chromatin at the repair sites and mediate efficient deactivation of the checkpoint to terminate DSB response.

Results

The Absence of Both Functional CAF-1 and Asf1 Leads to a Defect in the Viability of the Cells Experiencing a Repairable DSB. To determine the involvement of CAFs in the DSB-response process, we first examined whether the absence of CAFs affects the viability of cells in response to an HO endonuclease-induced DSB. In strain YJK17 (37), HO endonuclease induced by addition of galactose to the medium generates a DSB at the *MAT α* locus on chromosome III. Because the cells lack both *HML* and *HMR*, which contain sequences homologous to *MAT*, the DSB cannot be repaired by intrachromosomal homologous recombination (HR), which normally leads to mating type switching (38). Instead, the strain contains *MATa-inc* sequences on chromosome V, so that the DSB at *MAT α* can be repaired by ectopic recombination by using the *MATa-inc* sequence as a homologous donor (Fig. 1A). The *MATa-inc* sequence carries a mutated HO recognition site (a-inc) that cannot be cleaved by HO endonuclease (39). Therefore, once the HO-induced DSB at the *MAT* locus is repaired by ectopic HR, cells can proliferate without enduring additional HO-induced DSBs in the medium containing galactose; $\approx 80\%$ wild-type cells are viable on galactose plates (Fig. 1B), whereas isogenic cells lacking either Asf1 or Cac1 generated a small but significant reduction in viability, 63% ($P = 0.038$) and 64% ($P = 0.047$), respectively. Deletion of both *ASF1* and *CAC1* led to only $\approx 26\%$ of viability, a result statistically significantly different from the viability of wild-type cells ($P < 0.001$), as well as from either *cac1Δ* or *asf1Δ* cells ($P = 0.001$ and $P < 0.001$, respectively). Because CAFs could affect gene expression and prevent cells from growing on galactose-containing medium, we tested whether isogenic cells lacking an HO cleavage site (a cell that carried *MATa-inc* instead of *MAT α*) were unable to proliferate on galactose medium. However, there was no reduction in the viability of the cells in the absence of Asf1 and Cac1 (Fig. 1B). Therefore, we concluded that only on induction of a DSB, do the cells lacking both Asf1 and Cac1 have a defect in viability. We note that both *asf1Δ* and *asf1Δ cac1Δ* cells, which were derived from the strain YJK17 and used for this study, exhibit the expected sensitivity to different DNA damaging agents as previously reported [supporting information (SI) Fig. S1] (28), suggesting that strain differences do not affect the phenotype of cells lacking Asf1 and Cac1 in our assay.

DSB Repair in *asf1Δ cac1Δ* Is As Efficient As that in Wild-Type Cells.

After induction of a DSB, cells could fail to proliferate for 2 different reasons: defects in repairing DNA damage or defects in resuming cell cycle by failing to turn off the damage-mediated checkpoint. We first monitored the HR-mediated DSB repair in mutants lacking Asf1 and/or Cac1. By using a probe specific to the *MAT* distal region (Fig. 1A), formation of an HO-induced DSB and the repair of the break were detected; 1 h after the induction of HO endonuclease, all of the mutant as well as wild-type cells exhibited efficient HO cleavage at the *MAT* locus on chromosome III (Fig. 1A). In wild-type cells, the appearance of repair product was seen

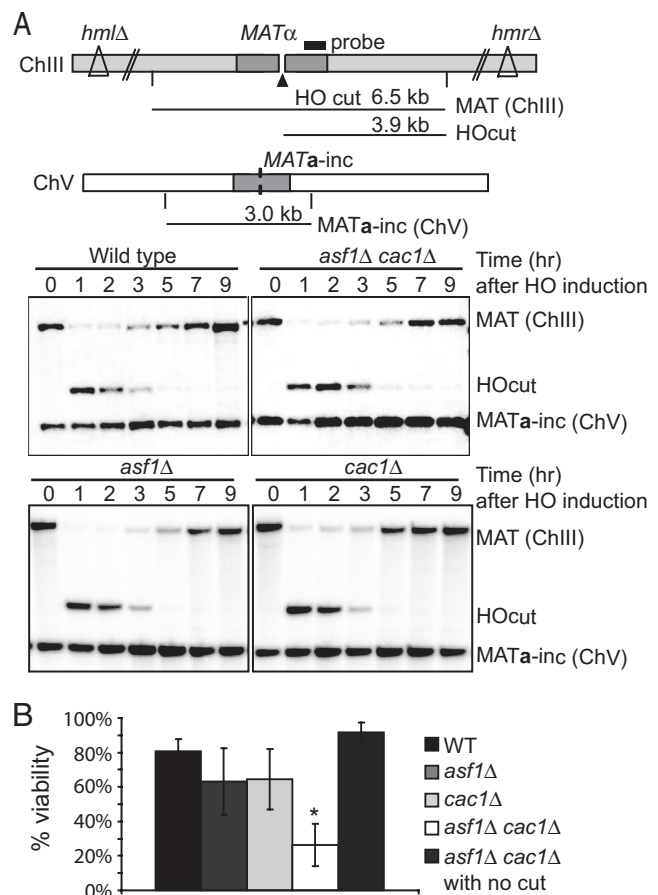


Fig. 1. An HO endonuclease-induced DSB leads to the reduced viability of *asf1Δ cac1Δ*. (A, Upper) diagram of *MAT α* in chromosome III (ChIII) and *MATa-inc* in ChV in strain YJK17. Vertical lines indicate *EcoRI* cleavage sites. The black bar denotes the probe specific to the *MAT* distal region (for PCR primers, see Table S1). Prior to HO-induced DSB formation, a 6.5 kb *EcoRI* fragment [MAT (ChIII)] is detected by the probe. After HO cleavage, a 3.9-kb fragment (HOcut) is generated. In addition, a 3.0-kb fragment surrounding *MATa-inc* [MATa-inc (ChV)] is also detected. (A, Lower) Exponentially growing cells were collected at different time points after HO induction. DNA from each time point was digested with *EcoRI*, and then run on a 0.8% agarose gel for the Southern analysis using the probe described above. The HO-induced DSB is repaired by interchromosomal homologous recombination by using *MATa-inc* on ChV as a homologous donor resulting in the replacement of *MAT α* on ChIII is replaced by *MATa-inc* sequences. (B) Percentage viability of a wild type (YJK17) and its derivatives lacking Asf1, Cac1, or both of them on galactose plates. Wild-type and mutant cells were grown in YEP/Lactose media to the exponential phase and then ≈ 100 –200 cells were plated either on YEP/Dextrose plates or YEP/Galactose plates. After 5 days, colonies were counted. Percent viability was calculated by dividing colony numbers on galactose with that on dextrose plates. “*asf1Δcac1Δ* with no cut” indicates *asf1Δcac1Δ* containing *MATa-inc* sequence at *MAT* on chromosome III prior to HO induction. The graph shows the average of 5 experiments with error bar representing SDs. * indicates the value of *asf1Δcac1Δ*, which is significantly different wild type as well as either *asf1Δ* or *cac1Δ*.

as early as 3 h after DSB induction and by 9 h, $\approx 80\%$ of the wild-type cells completed the repair. DSB repair in all of the mutant cells were as efficient as that in wild-type cells (Fig. 1A). Therefore, it is unlikely that the reduced viability of *asf1Δ cac1Δ* cells after the induction of a DSB is caused by a defect in DSB repair.

The *asf1Δ cac1Δ* Cells Do Not Resume Cell Cycle As Efficiently As Wild-Type Cells Due to a Defect in Turning Off the Damage Checkpoint.

Next, we determined whether the DNA damage checkpoint activated in response to a DSB is involved in the viability of *asf1Δ cac1Δ*

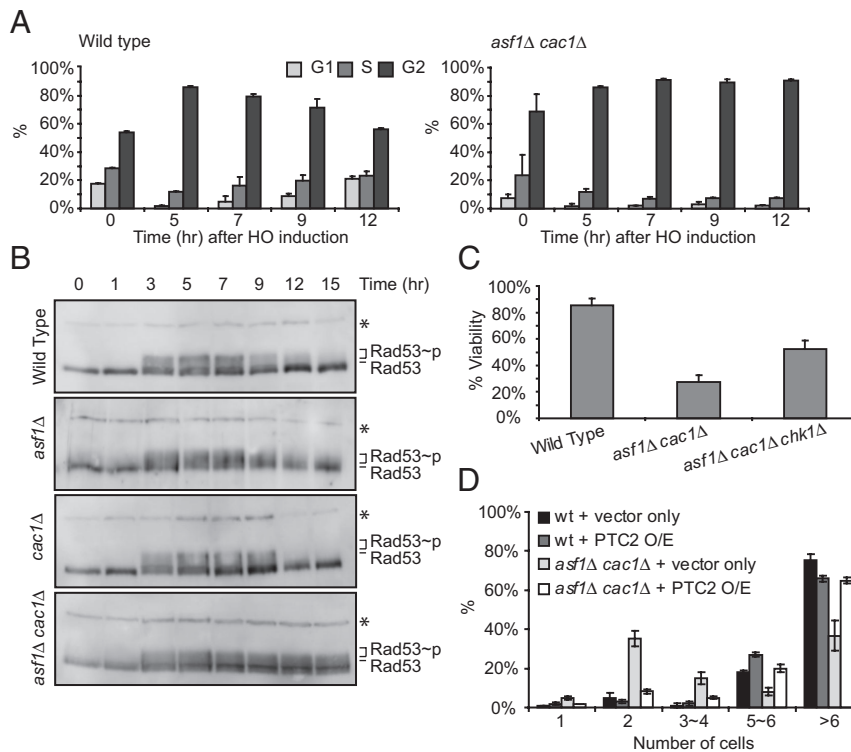


Fig. 2. *asf1Δcac1Δ* cells are deficient in recovering from the DNA damage checkpoint-mediated arrest. (A) Wild-type and *asf1Δ cac1Δ MATα* cells lacking *HML* and *HMR* were grown in YEP/Lactate medium to the exponential phase and then 2% galactose was added to induce HO endonuclease. Cells were observed microscopically. Unbudded cells (G1), small budded cells (S), and large budded cells (G2/M) were counted. Three different experiments counting 100–200 cells were performed to obtain the average shown in this figure. Error bars indicate SDs. (B) Western analysis with anti-Rad53 antibody determined the extent of phosphorylated Rad53 in whole cell extracts of wild-type and mutant cells during DSB repair. Phosphorylated Rad53 (Rad53~p) migrates slowly compared to unphosphorylated Rad53 (Rad53). * indicates a nonspecific band detected by cross-reactivity of the Rad53 antibody, which was used as a loading control. (C) Percent viabilities were measured as described in Fig. 1. (D) Wild-type (YJK17) and its derivatives lacking *Asf1* and/or *Cac1* were grown in YEP/Lactate media to the exponential phase and then the unbudded cells (G1) were micromanipulated on galactose plates. Overexpression (O/E) of *Ptc2* was driven by galactose-inducible promoter. One day after plating, the number of cells derived from a single cell was counted under the microscope. The graph shows the average of 3 different experiments with error bars representing SDs.

cells. We monitored cell cycle progression microscopically; 5 h after HO induction in cycling cells, ~80% wild-type cells were arrested at the G₂/M phase and, subsequently, the population of G₂/M declined (Fig. 2A). In contrast, the majority of *asf1Δ cac1Δ* cells (>80%) persisted at the G₂/M phase as late as 12 h after DSB induction. Although *asf1Δ cac1Δ* cells tend to be constitutively delayed in the G₂/M stage than wild-type cells (Fig. 2A, 0 hr time point), the induction of a DSB was definitely required for the complete arrest of the *asf1Δ cac1Δ* cells, which remained as late as 12 h after the break induction. These results suggest that the DNA damage checkpoint is not efficiently deactivated in the absence of *Asf1* and *Cac1*.

To examine the persistence of the DNA damage checkpoint more directly, we also monitored phosphorylation of Rad53. In wild-type cells, phosphorylated Rad53 appears ≤3 h after DSB induction and then disappears at the same time that repair of the DSB is complete, 9–12 h after HO induction (Fig. 2B). In *asf1Δ* or *cac1Δ* cells, the appearance of phosphorylated Rad53 occurred almost at the same time as wild-type cells, whereas its disappearance occurred either earlier or at the same time as in wild type (Fig. 2B). By contrast, in *asf1Δ cac1Δ* cells, phosphorylated Rad53 appears by 3 h, but persists as late as 15 h after the formation of an HO-induced DSB (Fig. 2B), even though DSB repair is almost complete by 9 h (Fig. 1A). These results indicate that deactivation of the DNA damage-mediated checkpoint is strikingly defective in cells lacking both functional CAF-1 and *Asf1*.

Consistent with the idea that the CAFs engage in cross-talk with the DNA damage checkpoint, we found that deletion of the

checkpoint kinase, *Chk1*, which is required for maintenance of checkpoint-mediated arrest beyond ~8 h (40), partially suppressed the failure of *asf1Δ cac1Δ* cells to resume growth after ectopic recombination in strain YJK17 (Fig. 2C). PP2C protein phosphatases *Ptc2* and *Ptc3* are involved in deactivation of the damage checkpoint (16, 41). Although cells lacking *Ptc2* and *Ptc3* are defective both in recovery and adaptation and fail to dephosphorylate Rad53 (16, 41), overexpression of *Ptc2* rescues the adaptation defect by dephosphorylating Rad53 (16). Recovery from the DNA damage-activated checkpoint can be monitored by observing division of individual cells on galactose plates (15). When we overexpressed *Ptc2* in *asf1Δ cac1Δ* cells on galactose plates, by 24 h, 70% the cells generated >6 cells, which were as many as their wild-type counterpart. In contrast, ~60% *asf1Δ cac1Δ* cells containing empty vector were still arrested either at G₂/M phase or at 4 cell stage (Fig. 2D). These results indicate that the defect in the recovery from the damage-activated checkpoint in *asf1Δ cac1Δ* cells is suppressed by turning off the damage checkpoint. We note that overexpressed *Ptc2* results in the lethality of even wild-type cells, so we could not determine the effect of overexpressed *Ptc2* on the viability of cells.

Although *Asf1* and *Cac1* Are Not Involved in Removing γ -H2AX from Chromatin, the Nucleosome Assembly Function of *Asf1* Is Required for the Viability of *asf1Δ cac1Δ* Cells After DSB Repair. Coincident with time that the DSB is repaired in strain YJK17, γ -H2AX begins to be removed from the 50-kb region around the DSB (37). We compared the kinetics of γ -H2AX appearance and disappearance in wild-type and mutant cells by using a ChIP assay (3); 1 h after HO

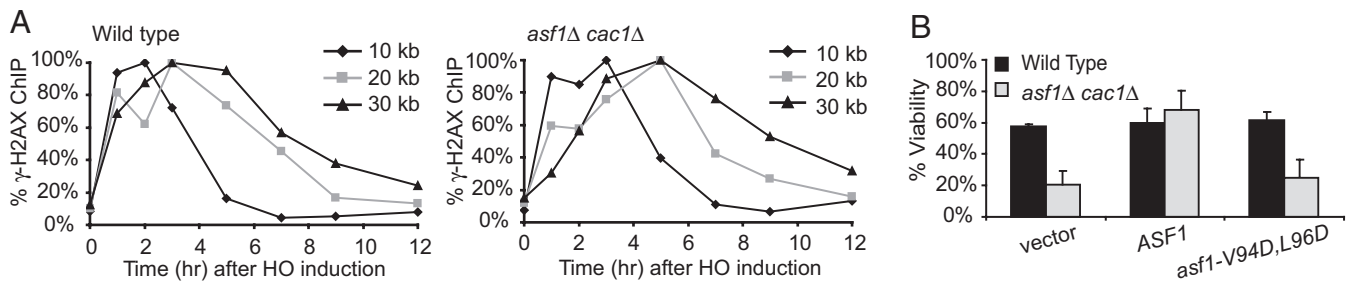


Fig. 3. Although appearance and disappearance of γ -H2AX is not affected by the absence of both Asf1 and Cac1, the nucleosome assembly function of Asf1 is required for the viability of *asf1* Δ *cac1* Δ cells after DSB repair. (A) γ -H2AX ChIP values at 10 kb (black bars), 20 kb (gray bars) or 30 kb (white bars) to the right of the DSB at *MAT* in wild-type and *asf1* Δ *cac1* Δ cells were measured several time points (1, 2, 3, 5, 7, 9, and 12 hrs) after HO induction (see Fig. S2) and the percent maximum value of γ -H2AX ChIP is shown. (C) Percent viabilities of wild-type (black bars) and *asf1* Δ *cac1* Δ (gray bars) cells with an empty plasmid, a plasmid with *ASF1*, or a plasmid with *asf1-V94D, L96D*. Cells grown in SC-Ura/Raffinose (synthetic complete medium containing raffinose instead of dextrose but lacking uracil) were plated either on YEP/Dextrose or on YEP/Galactose plates. Viability was measured as described in Fig. 1.

induction, γ -H2AX was enriched 10, 20, and 30 kb away from the DSB in *asf1* Δ *cac1* Δ as well as in wild-type cells and reached its maximum between 2 and 3 h (Fig. 3A and Fig. S2). In wild-type cells, the ChIP signal then decreased when the break is repaired. The decrease of γ -H2AX occurred progressively from the break site (Fig. 3A). However, there was no substantial difference in the disappearance of γ -H2AX between wild-type and *asf1* Δ *cac1* Δ cells, or in the 2 single mutants (data not shown). These results suggest that removal of γ -H2AX from chromatin during DSB repair does not depend on either Asf1 or CAF-1.

To relate the defect in checkpoint deactivation after DSB repair in *asf1* Δ *cac1* Δ directly to the cooperative nucleosome assembly functions of Asf1 and CAF-1, we tested whether introduction of a plasmid-borne *asf1-V94D, L96D* mutant that is defective in histone binding (42) would affect the viability of *asf1* Δ *cac1* Δ cells. Unlike the wild-type counterpart, the mutant *asf1-V94D, L96D* did not suppress the viability defect in *asf1* Δ *cac1* Δ cells (Fig. 3B). This result suggests that the histone binding and presumably the chromatin assembly activity of Asf1 is required for the cells experiencing an HO-induced DSB repair to deactivate the damage-activated checkpoint on the completion of DSB repair.

Asf1 and CAF-1 Do Not Affect Recovery When DSB Repair Occurs Without Strongly Activating the DNA Damage Checkpoint. When there is a homologous donor either at *HML* or at *HMR*, an HO-induced DSB at the *MAT* locus is rapidly repaired by intrachromosomal HR. In this circumstance, the repair of the HO-induced DSB is completed as early as 2 h, and the Rad53 checkpoint kinase does not become hyperphosphorylated; however, Mec1 and/or Tel1 carry out the early checkpoint responses and phosphorylate γ -H2AX around the *MAT* locus (Fig. S3) (11). Consistent with previous reports (37, 43), more γ -H2AX is detected at a region 10 kb away than 5 kb away from the break. To monitor the viability of the cell experiencing intrachromosomal HR on galactose plates, we constructed *MATa* strain YJK139, deleted for *HMR* and carrying *HML* α -inc sequences. Thus, cells switch from *MATa* to *MAT* α -inc, and cannot be again cleaved by HO on galactose medium. In this background, deletion of both *ASF1* and *CAC1* did not reduce viability of the cells (Fig. 4), even though ≥ 700 bp of new DNA must be synthesized to replace the *MAT* *Ya* sequence with *Y* α -inc copied from *HML* α -inc (and packaged into nucleosomes). Therefore, we conclude that only when the DNA damage checkpoint is strongly activated, which is indicated by hyperphosphorylation of Rad53, is the cooperative function of Asf1 and CAF-1 required for cell proliferation after DSB repair.

Asf1 and CAF-1 Are Not Required for Adaptation in the Absence of DSB Repair. To determine whether repair per se was required before the loss of Asf1 and Cac1 displayed an arrest phenotype, we examined

the ability of cells to resume cell cycle progression in the presence of an unreparable DSB, a process termed adaptation (12). A DSB was induced in strains carrying *MAT* α but deleted for both *HML* and *HMR* donors; 8 h after DSB induction, wild-type cells and an isogenic derivative lacking Asf1 only or both Asf1 and Cac1 arrest as large dumbbell-shaped cells characteristic of *G*₂/M arrest, and by 48 h, > 80% of both wild-type and mutant cells had adapted by dividing 1 or more times (Fig. 5). On the contrary, 95% *tid1* Δ cells were persistent as a dumbbell-shaped cell as late as 48 h, which is a characteristic of adaptation-defective (17). These results show that the CAFs are not required to silence the checkpoint during adaptation, but only when the DSB has been repaired, presumably, because in repaired cells, chromatin must be reestablished at the repaired, intact DNA. We note that, although $\approx 50\%$ of wild-type cells reach >6 cells by 48 h after a DSB induction, only 20% *asf1* Δ and none of *asf1* Δ *cac1* Δ cells have >6 cells by then (Fig. 5). By observing growth of wild-type and *asf1* Δ *cac1* Δ cells, both of which lack the HO cut site (inc), on galactose plates, we found that *asf1* Δ *cac1* Δ cells grow approximately twice as slowly as wild-type cells (Fig. S4). This result indicates that the smaller number of *asf1* Δ *cac1* Δ cells reached >6 cells by 48 h because of the slow division of the cells, compared with wild-type cells. In the adaptation assay we use, cells eventually die because the broken chromosome III is not repaired (44); because degradation of the DSB ends continues even in cells arrested in *G*₂/M (11), they will reach a point of inviability

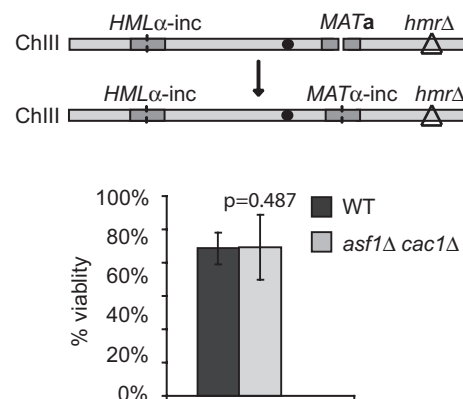


Fig. 4. Asf1 and CAF-1 do not affect recovery when DSB repair occurs without strongly activating the DNA damage checkpoint. Percentage viabilities of YJK139 wild type and its derivatives lacking both Asf1 and Cac1 were measured as described in Fig. 1A. The diagram shows the HO-induced DSB at the *MAT* locus on ChIII and the consequence of the DSB repair by intrachromosomal HR by using the *HML* α -inc donor located at the left arm of chromosome III. The *CEN3* locus is marked as a black dot.

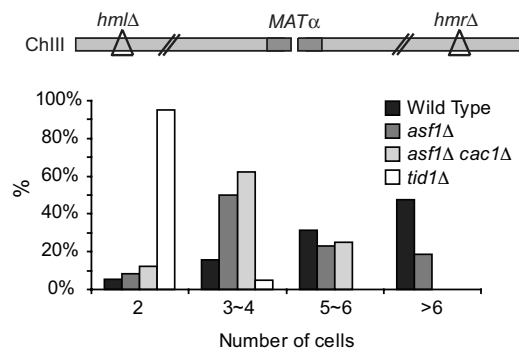


Fig. 5. *Asf1* and *CAF-1* are not required for the adaptation in the absence of DSB repair. The diagram shows ChIII containing *MATα* but lacking both *HML* and *HMR*. Wild-type, *asf1Δ*, *asf1Δ cac1Δ*, and *tid1Δ* cells were synchronized to G1 by growth to saturation and unbudded cells were spread onto galactose plates. The number of buds or cells was counted at 48 h after spreading (HO induction), and the number of each population with different bud/cell numbers was converted to a percentage value.

with fewer cell divisions when the cells grow substantially slower. Given that most cells progress beyond the 2-cell arrest seen for *tid1Δ*, we conclude that *asf1Δ* and *asf1Δ cac1Δ* cells are competent for the checkpoint adaptation.

Discussion

As DSB repair is complete, cells must extinguish the checkpoint signal to resume cell division. Previous studies have shown that turning off the checkpoint requires the PP2C phosphatases, *Ptc2* and *Ptc3*, as well as casein kinase II; these factors appear to be involved in the dephosphorylation of *Rad53* and perhaps *Chk1* (16, 18, 40, 45). *Srs2* DNA helicase is also required, possibly to remove a checkpoint signaling protein from the repaired region (15). Also, the presence of γ -H2AX delays efficient termination of the damage-activated checkpoint (37). We have previously shown that γ -H2AX is only dephosphorylated after it is removed from the chromatin surrounding the repaired site (37). It is not known how the modified histones are removed, or whether chromatin is restored to its original undamaged state or to a novel configuration. Specifically for repair of a DSB during ectopic recombination, our previous studies showed that 5' to 3' resection of DSB ends, which is required for efficient *Rad51*-mediated repair, removes γ -H2AX from the sites near DSBs (37, 43); however, its displacement occurs in a resection-independent manner (37). We initially hypothesized that *Asf1* might be involved in the disassembly of γ -H2AX from the chromatin surrounding DSBs, given the fact that *Asf1* is involved in the histone eviction for transcriptional activation (46). However, we found that both *Asf1* and *CAF-1* are dispensable for the removal of γ -H2AX during the repair of an HO-induced DSB. Also, although a previous report argued that *asf1 cac1* double mutants were defective in DSB repair pathways (33), the absence of either *Asf1* or *Cac1*, a subunit of *CAF-1* did not affect DSB repair itself in our assay system. Also, in our hands, γ -H2AX removal also does not depend on *Ino80* or *Swr1* chromatin remodeling complexes or on the histone chaperone *Nap1*, even in multiple mutant combinations (data not shown).

However, when the damage dependent checkpoint is robustly activated, the *asf1Δ cac1Δ* cells are defective in the deactivation of the checkpoint that is coupled to DSB repair. Neither *asf1Δ* nor *cac1Δ* alone exhibited this defect. These results suggest that *Asf1* and *CAF-1* have cooperative roles in the regulation of the damage checkpoint. Importantly, we did not detect a recovery defect in *asf1Δ cac1Δ* cells when a DSB is repaired by intrachromosomal gene conversion that does not cause *Rad53*-mediated cell-cycle arrest. This result suggests the involvement of the CAFs in the DNA damage checkpoint pathway only when it is robustly activated.

When the checkpoint is not maintained, in a *chk1Δ* mutant that releases cells from arrest after ≈ 8 h, the defect in *asf1Δ cac1Δ* cells is partially suppressed; the fact that some cells remain arrested could imply that the constitutive problems of the double mutant to traverse G₂/M augment the intensity of the checkpoint signal and is not fully suppressed by *chk1Δ*. *Asf1* and *CAF-1* also do not affect the ability of cells that fail to repair a DSB from recovering by adaptation. The result is important, because it suggests that cells in which a DSB cannot be repaired (and in which there is no reestablishment of chromatin) turn off the checkpoint in a way that is fundamentally different from what happens when there is repair.

How could *Asf1* and *CAF-1* mediate the termination of the damage checkpoint? *Asf1* and *CAF-1* were originally identified as replication-dependent CAFs (19, 47). Also, when UV-damage is repaired by NER, human *Asf1* and *CAF-1* synergistically reassemble chromatin in NER-dependent manner (24), during which ≈ 30 –50 nt of new DNA is synthesized (48). When a DSB is repaired by HR in the system we study, ≥ 700 base pairs must be synthesized to repair the break (49). We speculate that the newly synthesized DNA by HR-mediated repair must undergo chromatin assembly to establish and reorganize intact chromatin. If this idea is correct, our study suggests that *Asf1* and *CAF-1* can be strong candidates to connect the repair-mediated chromatin assembly with DSB repair and the checkpoint. Although the removal of γ -H2AX occurs even without *Asf1* and *CAF-1*, the repaired DNA may not have a full complement of nucleosomes in the absence of these CAFs, so that proteins bound to naked DNA might maintain the damage checkpoint, which is indicated by the persistence of phosphorylated *Rad53*. Our result that the ectopic mutant *Asf1* lacking the histone H3/H4 binding ability fails to suppress the viability defect in *asf1Δ cac1Δ* cells supports the idea that chromatin assembly function is required for the termination of the damage checkpoint. Alternatively, a specific histone mark might be incorporated into the chromatin at repair sites in an *Asf1* and *CAF-1* dependent manner. This repair mark might trigger the signal to deactivate the checkpoint. In either case, the cells without the restored chromatin could fail to turn off the checkpoint efficiently. Interestingly, *Asf1* physically interacts with the checkpoint kinase, *Rad53* (50). This association is weakened when *Rad53* is hyperphosphorylated by the activation of the DNA damage checkpoint (50, 51). Consequently, the *Asf1* that completes its chromatin assembly function might facilitate dephosphorylation of *Rad53*, leading to the reassociation of *Asf1* with *Rad53*.

While we were preparing this manuscript, J. Tyler and co-workers (52) reported the involvement of *Asf1* in the checkpoint recovery. However, our results (many using the same strains created in our laboratory) are different in many ways. In their study, *Asf1* alone is defective in cell viability after repair of a DSB. However, the viability defect in *asf1Δ* cells by our ectopic HR assay is moderate, whereas the defect in *asf1Δ cac1Δ* cells is more striking. Also, we did not detect a viability defect of any mutant cells lacking *Asf1* and/or *Cac1* after intrachromosomal HR. Also, although Chen *et al.* (52) showed that chromatin assembly is defective during intrachromosomal *MAT* switching, we did not see a defect in viability of either an *asf1Δ* or an *asf1Δ cac1Δ* strain undergoing *MAT* switching. Last, Chen *et al.* (52) interpreted that *asf1Δ* is adaptation-defective. However, we present the evidence that both the *asf1Δ* and the *asf1Δ cac1Δ* double mutant are not adaptation defective. Some of our differences could be explained by the duration or intensity of the checkpoint signal in different repair assays. For example, we found that *ptc2Δ ptc3Δ* cells undergoing the same ectopic recombination event we have studied here were not permanently arrested, whereas cells undergoing a long-delayed single-strand annealing event (strain YMV002 used by Tyler's group) were permanently arrested (16). It is possible that the different duration or intensity of the checkpoint signal, generated by different repair assays, determines the degree of *Asf1* depen-

dency for turning off the checkpoint. Chen *et al.* (52) proposed that the Asf1- and Rtt109-dependent incorporation of H3K56Ac is involved in the recovery from the checkpoint. Whether Rtt109 affects CAF-1 activity is unknown. Further study should reveal the mechanism by which Asf1 and CAF-1 regulate the DSB repair-mediated chromatin assembly to signal checkpoint termination.

Materials and Methods

Yeast Strains. The strains used in this study were derived either from JKM179 (*MAT α ho Δ hml Δ ::ADE1 hmr Δ ::ADE1 ade1-100 leu2-3,112 trp::hisG lys5 ura3-52 ade3::GAL::HO*) (53), or YJK17 (*MAT α ho Δ hml Δ ::ADE1 hmr Δ ::ADE1 ade1-100 leu2-3,112 trp::hisG lys5 ura3-52 ade3::GAL::HO arg5,6::MATa-inc::HPH1*) (37). *ASF1* and *CAC1* were deleted by KanMX and NatMX, respectively. YXW2 (*MAT α ho Δ HML α HMRa leu2-3,112 his3-11,15 ade2-1 can1-100 ura3-1 trp1-1 bar1::hisG*

ade3::GAL::HO) is used to determine γ -H2AX enrichment around the *MAT* locus during normal mating type switching.

Western Blot Analysis. Cell extracts were prepared by trichloroacetic acid (TCA) extraction and then subjected to the Western blot analysis. Rad53 was detected by polyclonal anti-Rad53 antibody (Santa Cruz, 1:200).

ChIP of γ -H2AX. ChIP of γ -H2AX was carried out as previously described (3, 43), by using an antibody against yeast γ -H2AX, generously provided by C. Redon and W. Bonner (National Institutes of Health). The primers used for ChIP analysis are listed in Table S1.

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