Histone deposition protein Asf1 maintains DNA replisome integrity and interacts with replication factor C

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Chromatin assembly and DNA replication are temporally coupled, and DNA replication in the absence of histone synthesis causes inviability. Here we demonstrate that chromatin assembly factor Asf1 also affects DNA replication. In budding yeast cells lacking Asf1, the amounts of several DNA replication proteins, including replication factor C (RFC), proliferating cell nuclear antigen (PCNA), and DNA polymerase ϵ (Pol ϵ), are reduced at stalled replication forks. In contrast, DNA polymerase α (Pol α) accumulates to higher than normal levels at stalled forks in *asf1 Δ* cells. Using purified, recombinant proteins, we demonstrate that RFC directly binds Asf1 and can recruit Asf1 to DNA molecules in vitro. We conclude that histone chaperone protein Asf1 maintains a subset of replication elongation factors at stalled replication forks and directly interacts with the replication machinery.

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In eukaryotes, DNA is assembled into a nucleoprotein complex called chromatin. The fundamental repeating unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around an octamer of core histone proteins, comprised of two H2A/H2B dimers flanking an inner (H3/H4)₂ tetramer. In vivo, histone deposition can occur by DNA replication-coupled or replication-independent mechanisms (for review, see Franco and Kaufman 2004). In either case, histone deposition is mediated by specialized assembly proteins. The best characterized replication-coupled chromatin assembly factor is chromatin assembly factor-1 (CAF-1), an evolutionarily conserved protein complex that binds histone H3 and H4 and delivers them to replicating DNA through an interaction with proliferating cell nuclear antigen (PCNA), the DNA polymerase processivity protein (for review, see Franco and Kaufman 2004). Inhibition of CAF-1 blocks S-phase progression in human cells, suggesting that replication-coupled nucleosome assembly is

essential (Hoek and Stillman 2003; Ye et al. 2003). Replication-independent histone deposition is carried out by a number of histone chaperones including the Hir proteins (Ray-Gallet et al. 2002; Tagami et al. 2004; for review, see Franco and Kaufman 2004).

Asf1, another evolutionarily conserved histone chaperone, functions during both replication-coupled and replication-independent chromatin assembly. Asf1 bound to histones H3/H4 stimulates histone deposition by CAF-1 in vitro (Tyler et al. 1999; Sharp et al. 2001), and Asf1 physically interacts with the p60/Cac2 subunit of CAF-1 (Tyler et al. 2001; Krawitz et al. 2002; Mello et al. 2002). In yeast, Asf1 and the Hir proteins directly interact and function together to promote heterochromatic gene silencing (Kaufman et al. 1998; Sharp et al. 2001; Sutton et al. 2001; Krawitz et al. 2002). Consistent with a role in multiple deposition pathways, Asf1 copurifies with both CAF-1 and Hir protein complexes in human cell extracts (Tagami et al. 2004).

Asf1 also contributes to genome stability during S phase in a manner distinct from both CAF-1 and the Hir proteins. Unlike yeast cells lacking CAF-1 or the Hir proteins, cells lacking Asf1 are sensitive to the DNA synthesis inhibitor hydroxyurea (HU) and the DNA topoisomerase I inhibitor camptothecin (CPT), and display synthetic genetic interactions with DNA synthesis genes including the initiation/elongation factor *CDC45* and *CDC17* (polymerase α ; Pol α) (Tyler et al. 1999;

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Ramey et al. 2004; Tong et al. 2004). Although Asf1 is not required for activation of the S-phase checkpoint (Emili et al. 2001), Asf1 directly interacts with the DNA damage/replication checkpoint kinase Rad53 in a manner regulated by checkpoint activation (Emili et al. 2001; Hu et al. 2001). Also, *asf1Δ* cells display multiple phenotypes suggesting elevated levels of spontaneous DNA damage, including increased phosphorylation of Rad53, Rad9, Mrc1, and H2A (Hu et al. 2001; Schwartz et al. 2003; Prado et al. 2004; Ramey et al. 2004); accumulation of Ddc2-GFP foci; and increased rates of gross chromosomal rearrangements and sister chromatid exchanges (Myung et al. 2003; Prado et al. 2004; Ramey et al. 2004). However, the cause of this spontaneous DNA damage and the molecular nature of the link between Asf1 and the DNA replication machinery had been unknown.

DNA synthesis is an intrinsically hazardous process because it requires the generation of broken DNA strands that are recognized as damaged DNA if not properly processed. Thus, agents that disrupt DNA synthesis often promote accumulation of dangerous chromosomal abnormalities. The DNA replication checkpoint is a surveillance mechanism that protects genome integrity by regulating origin firing, maintaining dNTP pools, maintaining stalled replication fork integrity, and ensuring that mitosis does not occur prior to the completion of DNA synthesis (for review, see Osborn et al. 2002; Cobb et al. 2004). The replication checkpoint pathway is highly conserved in eukaryotic organisms. In mammalian cells, initial defects are sensed by PI3-kinase-like protein kinases termed ATM and ATR, which transmit signals to the Chk1 and Chk2 protein kinases. Defects in these proteins are associated with inherited predispositions to genome instability and cancer (Shiloh 2001). In budding yeast, the replication checkpoint requires the ATR protein kinase ortholog Mec1 and its target, the Chk2 protein kinase ortholog Rad53.

Chromatin immunoprecipitation (ChIP) assays have demonstrated that inactivation of the DNA replication checkpoint leads to changes in replication fork architecture at stalled forks. In *mec1* cells, these changes include loss of Cdc45 and polymerase ϵ (Pol ϵ) from chromatin (Cobb et al. 2003; Katou et al. 2003). In *rad53Δ* cells, losses of DNA polymerases α , δ , and ϵ from stalled forks have been reported (Lucca et al. 2004). Additionally, electron microscopic analysis of replication intermediates from *rad53Δ* cells reveals an increased amount of single-stranded DNA and four-branched structures (reversed replication forks) (Sogo et al. 2002). However, the mechanisms by which the S-phase checkpoint preserves replication fork integrity remain poorly understood.

Here we show that Asf1 is required for the completion of DNA synthesis when the replication fork is perturbed either by DNA damaging agents or by mutation of *RFC1*, the large subunit of replication factor C (RFC). RFC is the heteropentameric ATPase that topologically loads PCNA onto DNA and is therefore essential for processive bidirectional DNA replication (for review, see Waga and Stillman 1998b). Further, we present a molecular explanation for the role of Asf1 during DNA replication.

In the absence of Asf1, levels of replication proteins including Rfc3, PCNA, and Pol ϵ are reduced at stalled replication forks. These deficits likely contribute to the observed spontaneous DNA damage that occurs in the absence of Asf1. Additionally, in *asf1Δ* cells, Mcm4 is more broadly dispersed, and Pol α accumulates to higher levels at stalled forks. These data reveal a novel form of replisome dissociation suggesting that Asf1 acts to maintain a subset of replisome proteins during replication stress. Additionally, we show that RFC directly interacts with Asf1 and can recruit Asf1 to DNA in vitro. We conclude that chromatin assembly protein Asf1 interacts with the DNA replication machinery and promotes genome stability by maintaining replication elongation proteins at sites of stalled DNA synthesis.

Results

Asf1 and RFC function together to promote S-phase progression

Previous genetic data suggested a specialized role for Asf1 during S phase. For example, deletion of the *ASF1* gene causes synthetic slow growth in combination with mutations affecting DNA replication genes such as *CDC45* (Tong et al. 2004), *CDC17* (Pol α) (Ramey et al. 2004), and the CAF-1 subunit *CAC1* (Tyler et al. 1999). During our investigation of Asf1 function during S phase, we constructed double-mutant cells combining an *ASF1* gene deletion with a cold-sensitive allele of the large subunit of RFC, *rfc1-1*. Strong synthetic growth defects were observed in these double-mutant cells (Fig. 1A). Consistent with previous data, *asf1Δ* cells displayed a mild growth defect at 30°C (Tyler et al. 1999) while *rfc1-1* cells did not (Howell et al. 1994). In contrast, the *asf1Δ rfc1-1* double-mutant cells grew more slowly than either single mutant. The *rfc1-1* allele did not impair the growth of all cells with defects in histone deposition, because *rfc1-1* did not cause slow growth when CAF-1 function was disrupted by deletion of *CAC1* (Kaufman et al. 1997), or Hir protein function was disrupted by deletion of *HIR1* (Kaufman et al. 1998). Additionally, *asf1Δ* did not cause slow growth when combined with mutations in genes encoding alternative RFC subunits implicated in DNA damage recognition and sister chromatid cohesion (Supplementary Fig. 1). These data suggest that cells lacking Asf1 are particularly sensitive to perturbation of the S-phase DNA replication fork.

To better assess the growth defect in *asf1Δ rfc1-1* double-mutant cells, flow cytometric analysis (FACS) was used to measure DNA content per cell in asynchronous cultures grown at 30°C (Fig. 1B). Previous studies reported that *asf1Δ* cells display mild delays in S phase and G2/M (Tyler et al. 1999; Ramey et al. 2004). Consistent with these data, we did not observe dramatic cell cycle delays in the *asf1Δ* single-mutant cells, although the peaks were mildly broadened because of cell size heterogeneity. In contrast, *rfc1-1* cells accumulated in the G2/M phase of the cell cycle, as previously reported

Figure 1. Asf1 promotes chromosome replication when RFC function is altered. (A) Growth defects in *asf1Δ rfc1-1* cells. Tenfold serial dilutions of exponentially growing yeast strains of the indicated genotypes were plated on rich media for 3 d at 30°C. (B) *asf1Δ rfc1-1* cells accumulate with 2N DNA content. The indicated strains were grown to log phase at 30°C and DNA content was measured by flow cytometry. (C) *asf1Δ rfc1-1* cells display bipolar spindles and chromosome segregation defects. Mitotic spindles were visualized in exponentially growing cells by immunostaining with anti-tubulin antibodies and nuclei were visualized by DAPI staining. Inset shows chromosome missegregation events visible in ~1% of *asf1Δ rfc1-1* cells. (D) *asf1Δ rfc1-1* cells have an increased proportion of incompletely replicated chromosomes. Indicated yeast strains were grown to log phase at 30°C, and their isolated chromosomes were separated by pulse field gel electrophoresis. Total DNA was visualized by ethidium bromide (top), and Chromosome III was visualized by Southern blotting (bottom).

(Adams and Holm 1996). The poorly growing *asf1Δ rfc1-1* mutant cells displayed a much more dramatic delay than the single-mutant cells, and the vast majority of cells existed in a single broad peak with approximately 2N DNA content. These data suggested that *asf1Δ rfc1-1* cells delay cell cycle progression after the onset of DNA replication and before cytokinesis.

To distinguish aspects of the cell cycle delay in *asf1Δ rfc1-1* cells, indirect immunofluorescence was performed using anti-tubulin antibodies to visualize mitotic spindles and DAPI staining to visualize nuclear morphology. If cells were delayed during S phase or at the metaphase-to-anaphase transition, an increase in the proportion of cells with short bipolar spindles with a single nucleus at the bud neck would be expected. In contrast, if the delay occurred during anaphase, an increase in the frequency of cells with long spindles and two nuclei would be expected. Consistent with the flow cytometry data and previous reports (Howell et al. 1994; Tyler et al. 1999; Ramey et al. 2004), an increase in the proportion of cells with short spindles and nuclei at the bud neck in the single-mutant cells was observed (Fig. 1C; Supplementary Table 2). In the *asf1Δ rfc1-1* mutant cells, the percentage of cells with short bipolar spindles and a single nucleus was greater than in either single mutant (68% vs. 41% or 42%) (Supplementary Table 2). Additionally, we observed a small percentage of binucleate double-mutant cells that were not present in the single-mutant populations. We conclude that *asf1Δ rfc1-1* double-mutant cells are delayed between the onset of S phase and the metaphase-to-anaphase transition.

Because RFC function is critical to DNA replication, we hypothesized that S-phase progression is delayed in the *asf1Δ rfc1-1* double-mutant cells. Therefore, pulsed-field gel electrophoresis was used to detect the presence of chromosomes undergoing replication or that have stalled replication forks, because incompletely replicated chromosomes do not enter these gels but remain trapped in the well (Hennessy et al. 1991). Total DNA

was visualized using ethidium bromide staining and Chromosome III and Chromosome IV were visualized by DNA hybridization (Fig. 1D; data not shown). The amount of DNA entering the gel from wild-type *asf1Δ* and *rfc1-1* cells was approximately equivalent (Fig. 1D, lanes 1,3,4). As a positive control for chromosomes with replication intermediates, we treated wild-type cells with HU. As expected, the amount of DNA entering the gel was greatly reduced by HU treatment (Fig. 1D, lane 2). Compared with the untreated wild-type and single-mutant samples, fewer chromosomes isolated from the *asf1Δ rfc1-1* double-mutant cells entered the gel (Fig. 1D, lane 5), suggesting the accumulation of incompletely replicated chromosomes.

rfc1-1 cells have elongated telomeres (Adams and Holm 1996), suggesting poor coordination of telomeric C-rich strand synthesis by the lagging strand machinery and G-rich strand synthesis by telomerase in these cells (Diede and Gottschling 1999). To rule out the possibility that hyperelongated telomeres were the cause of the S-phase delay in *asf1Δ rfc1-1* cells, telomere lengths were assayed. Deletion of *ASF1* did not further elongate telomeres in *rfc1-1* cells (Supplementary Fig. 2A). Additionally, deletion of *SML1*, which encodes an inhibitor of ribonucleotide reductase, had no effect on the slow growth phenotype of *asf1Δ rfc1-1* cells (Supplementary Fig. 2B), suggesting that the replication defects in *asf1Δ rfc1-1* mutant cells were not caused by limited dNTP pools. We conclude that Asf1 is required for efficient completion of chromosome synthesis when RFC function is impaired and that the resulting defects in DNA replication are unrelated to telomere length or dNTP levels.

Asf1 promotes S-phase progression in the presence of DNA damaging agents

The *rfc1-1* mutation activates the DNA damage checkpoint even at the permissive temperature (Howell et al.

Franco et al.

1994). Because *asf1Δ* mutant cells are sensitive to exogenous DNA damaging agents (Le et al. 1997; Tyler et al. 1999; Ramey et al. 2004), we hypothesized that *asf1Δ rfc1-1* double-mutant cells delayed in S phase (Fig. 1) because the endogenous damage caused by the *rfc1-1* mutation was particularly deleterious in the absence of Asf1. We therefore predicted that Asf1 would be required for efficient completion of DNA synthesis when chromosomes were damaged. To test this idea, the ability of *asf1Δ* cells to progress through S phase in the presence of exogenous DNA damaging agents was measured by FACS analysis. Wild-type and *asf1Δ* mutant cells were synchronized in G1 phase and released into media containing either no drug or various concentrations of the DNA alkylating agent methyl-methanesulfonate (MMS). After 1 h, cell cycle progression was monitored by FACS (Fig. 2A). High concentrations (0.05%) of MMS inhibited S-phase progression in wild-type cells, reflecting the checkpoint-mediated block to S-phase progression (Paulovich and Hartwell 1995; Tercero and Diffley 2001). However, at lower doses of MMS, wild-type cells replicated most of their DNA. Because *asf1Δ* mutant cells are not checkpoint defective, they also arrested DNA synthesis at high doses of MMS as expected (Fig. 2A; Emili et al. 2001; Hu et al. 2001; Ramey et al. 2004). However, unlike wild-type cells, *asf1Δ* mutant cells were unable to complete S phase in 1 h in the presence of low doses of MMS. Similar defects were observed in the presence of low doses of bleomycin (BLM), which generates both nicks and double-strand breaks (Fig. 2B; Povirk 1996). Like MMS, exposure to BLM in S phase causes replication stress and activation of the S-phase checkpoint (D'Amours and Jackson 2001). Furthermore, *asf1Δ* cells remained similarly arrested after incubation for an additional hour (data not shown). Together, these data suggest that the sensitivity of *asf1Δ* mutant cells to DNA damaging agents (Le et al. 1997; Tyler et al. 1999) results at least partially from defects in completing DNA synthesis in the presence of lesions that cause replication stress.

Stalled replisomes are unstable in *asf1Δ* cells

The S-phase checkpoint allows cells to survive in the presence of replication stress by maintaining the stability of replisome proteins at stalled replication forks (Cobb et al. 2003; Katou et al. 2003; Tercero et al. 2003; Lucca et al. 2004). Because *asf1Δ* cells display spontaneous DNA damage phenotypes (see introduction), and because Asf1 is required to complete DNA synthesis in the presence of DNA damage (Fig. 2), we hypothesized that Asf1 would have a role in maintaining replisome stability. To test this idea, the localization of an epitope-tagged version of the catalytic subunit of Pol ϵ (Pol ϵ -HA) at two early DNA replication origins (ARS607 and ARS305) was measured in wild-type and *asf1Δ* cells (Fig. 3A,B). Consistent with previous data, ChIP experiments in cells synchronized by α -factor and released into HU demonstrated that Pol ϵ -HA was enriched at early origins (Aparicio et al. 1999; Cobb et al. 2003; Katou et al.

Figure 2. Asf1 is required for S-phase progression in the presence of DNA damaging agents. (A) Asf1 is required to complete DNA synthesis in the presence of low concentrations of MMS. Wild-type (WT) and *asf1Δ* mutant cells were arrested in G1 with α -factor and released into rich media containing the indicated concentration of MMS. Samples were harvested after 1 h and DNA content was measured by flow cytometry. (B) Asf1 promotes DNA synthesis in the presence of BLM. Cultures were treated as in A except that they were released into the indicated concentration of BLM.

2003; Lucca et al. 2004). As expected, Pol ϵ -HA was not enriched in wild-type cells at sequences 14 kb from ARS607 or 8 kb from the ARS 305, indicating that HU had stalled forks before they reached distal positions. Notably, Asf1 was required to detect significant enrichment of Pol ϵ -HA at the stalled forks. FACS analyses of the cells used for the ChIP experiments confirmed that cells were properly synchronized (Fig. 3C). Another possible explanation for the loss of replication proteins from

Figure 3. Pol ϵ is displaced from stalled replication forks in *asf1Δ* mutant cells. (A) Pol ϵ -HA association with the origins in synchronized cells arrested with HU was assayed by ChIP followed by PCR analysis. Products were analyzed by native PAGE and ethidium bromide staining. (B) Quantitation of real-time PCR data. The amount of DNA recovered from wild-type (WT) and *asf1Δ* extracts is graphed relative to the untagged control for the early firing origins ARS607 and ARS305, as well as unreplicated regions 14 kb away from ARS607 (ARS607 + 14 kb) and 8 kb away from ARS305 (ARS305 + 8 kb). (C) *asf1Δ* mutant cells arrest in response to HU treatment. The strains used in the above ChIP experiment were synchronized in α -factor and released into HU. Samples were harvested every 30 min and DNA content measured by FACS. (D) Early origins replicate in *asf1Δ* cells. PCR products from an early (ARS305) and a late (ARS501) origin were quantified from cells arrested in α -factor (DNA α -factor) or HU (DNA HU) and expressed as a ratio (DNA HU/DNA α -factor). For all strains, twice as much ARS305 DNA was recovered from cells arrested in HU, indicating that early origin DNA duplicated. In contrast, the amount of ARS501 DNA did not double as expected because late origins do not fire in HU-arrested cells (Santocane and Diffley 1998; Shirahige et al. 1998).

early origins could have been that Asf1 was important for replication initiation. To address this, we quantified total DNA isolated from cells arrested in either α -factor or HU (Fig. 3D). In all strains, the amount of DNA at an early origin, but not a late origin, doubled upon release into HU. Therefore, the decrease in Pol ϵ -HA association at early origins in *asf1Δ* cells is not due to failure to initiate DNA replication. We conclude that Asf1 is required for normal levels of Pol ϵ association with stalled replication forks.

Not all mutations that affect the stability of stalled replisomes cause identical patterns of protein displacement (Zegerman and Diffley 2003). We therefore tested the extent of replisome instability in the absence of Asf1 by determining the localization of additional replisome proteins at stalled forks (Fig. 4). Because of the genetic interaction between Asf1 and RFC, we first explored how DNA replication proteins were affected, starting with RFC itself.

Biochemical experiments indicate that after Pol δ binds PCNA, RFC can remain associated with the replisome in vitro (Yuzhakov et al. 1999). However, the fate

of RFC at stalled forks in vivo had not been explored. Using an anti-Rfc3 polyclonal antibody, we observed that RFC indeed remains associated with stalled replication forks in vivo in wild-type cells (Fig. 4A). As observed for Pol ϵ , the association of Rfc3 at the fork was greatly reduced in the absence of Asf1. We next tested the association of PCNA with stalled replication forks under identical conditions. Like Pol ϵ and Rfc3, PCNA associated with stalled replication forks in wild-type cells, but this association was reduced in *asf1Δ* mutant cells (Fig. 4B). Additionally, the absence of Asf1 did not alter the levels of any of these replisome proteins in whole cell lysates made from either untreated or HU-treated cells (Supplementary Fig. 3). We conclude that Pol ϵ , RFC, and PCNA all require Asf1 for normal association with stalled forks. Notably, these three replisome protein complexes are all involved in the elongation phase of DNA synthesis (for review, see Waga and Stillman 1998b).

These data suggested that either the entire set of replication proteins was poorly associated with stalled forks in *asf1Δ* cells, or instead, that a subset of replication

Figure 4. Differential replication fork protein stability at stalled forks in *asf1Δ* mutant cells. (A) Rfc3 localization to stalled replication forks requires Asf1. ChIP experiments were performed as described in Figure 3 except that the amount of Rfc3 associated with early origins was assayed using a polyclonal antibody. The amount of DNA recovered from wild-type (WT) and *asf1Δ* extracts is graphed relative to the amount recovered in the normal rabbit sera control for the early firing origins ARS607 and ARS305, as well as two unreplicated regions 14 kb away from ARS607 (ARS607 + 14 kb) and 8 kb away from ARS305 (ARS305 + 8). (B) PCNA localization to stalled replication forks is reduced in *asf1Δ* mutant cells. ChIP experiments were performed and analyzed as described in A except that a polyclonal antibody raised against yeast PCNA was used. (C) Mcm4 localizes to stalled replication forks in the absence of Asf1. ChIP experiments were performed as described above and the amount of origin DNA that coprecipitated with HA-tagged Mcm4 (Mcm4-HA) is graphed relative to the untagged control. (D) Pol α association with stalled replication forks increases in the absence of Asf1. ChIP experiments were performed as described above and the amount of origin DNA that coprecipitated with HA-tagged Pol α (Pol α -HA) is graphed relative to the untagged control.

protein complexes might be more severely affected than others. To distinguish these possibilities, we examined two additional protein complexes. First, we examined the localization of a subunit of the Minichromosome maintenance (MCM) protein complex. The MCM proteins are loaded at origins prior to initiation, travel with elongating forks, and are prime candidates for the role of a replicative helicase (for review, see Bell and Dutta 2002). We measured the presence of the Mcm4 subunit of the complex at stalled replication forks and observed that Asf1 had no significant effect on its localization at the early origin sequences (Fig. 4C). In contrast, at 8 kb distal from ARS305, there were elevated levels of Mcm4 in the *asf1Δ* cells; this differential accumulation was not significantly observed at 14 kb from ARS607. These data suggest that a subset of MCM complexes were decoupled from polymerases in *asf1Δ* cells; similar observations have been observed in HU-treated cells lacking S-phase checkpoint proteins Mrc1 or Tof1 (Katou et al. 2003; Zegerman and Diffley 2003). These data demonstrate that the entire replisome is not lost from DNA in the absence of Asf1, but that uncoupling of the MCMs from the replication machinery may occur.

We then examined Cdc17, the catalytic polymerase subunit of the DNA Pol α -primase complex required for initiation of DNA synthesis on the leading strand and at each Okazaki fragment on the lagging strand. Asf1 was not required for the association of Pol α -HA with stalled replication forks (Fig. 4D). Instead, Pol α -HA levels at early origins increased in the absence of Asf1. These data are consistent with the observation that early origin firing occurs in *asf1Δ* cells (Fig. 3C). The increased Pol α -HA levels at early origins in the absence of Asf1 could result from increased exposure of single-stranded DNA upon MCM uncoupling. Alternatively, these data could result if Asf1 regulates polymerase switching, the process by which RFC/PCNA complexes assist the replacement of Pol α with a processive polymerase after initiation (for review, see Waga and Stillman 1998b).

Because Asf1 regulates replisome structure at stalled forks, we expected Asf1 should itself be a component of replicating chromatin. ChIP experiments using anti-Asf1 sera demonstrated that Asf1 could be detected not only at early origins in HU-treated cells, but also at all loci tested (Supplementary Fig. 4; data not shown). Asf1 is a very abundant nuclear protein (~10,000 copies per yeast cell; data not shown) and its presence at multiple loci would allow it to contribute to multiple processes, including heterochromatic gene silencing (Le et al. 1997; Singer et al. 1998; Tyler et al. 1999; Sharp et al. 2001) and replication fork stability (Figs. 3, 4).

Asf1 interacts with RFC

Asf1's role in fork stability and its genetic interactions with replication proteins suggested that Asf1 would directly interact with fork components. Previous genetic data demonstrated that mutations in PCNA affect Asf1 function (Sharp et al. 2001), suggesting that Asf1 might be directly recruited to DNA by binding PCNA. How-

ever, no physical interaction between Asf1 and PCNA was detected in solution (data not shown). Because PCNA can utilize different interaction surfaces once loaded onto DNA (Gomes and Burgers 2000), we tested whether Asf1 could bind PCNA when loaded onto nicked DNA substrates by purified recombinant yeast RFC. Surprisingly, RFC alone was sufficient for recovery of Asf1 on the DNA (Fig. 5).

In these experiments, PCNA loading by RFC was measured using an immobilized DNA template containing a 3'OH primer-template junction in the presence of the eukaryotic single-strand binding protein Replication Protein A (RPA) (Waga and Stillman 1998a). In the absence of Asf1, 0.5 pmol RFC efficiently loaded PCNA in the presence of ATP or ATP γ S, confirming that this preparation of RFC was active. Notably, RFC recruited Asf1 to the DNA template under conditions where Asf1 would not remain on DNA in the absence of RFC (Fig. 5, cf. lanes 4 and 7). This occurred in the presence of either ATP or ATP γ S, suggesting that ATP hydrolysis is not required for the Asf1-RFC interaction. Asf1 also reduced the amount of PCNA loaded by RFC; this effect is under investigation and may reflect alteration of the RFC catalytic cycle by Asf1 (data not shown). We conclude that the interaction with RFC targets Asf1 to DNA molecules in vitro.

To test for a direct interaction between Asf1 and RFC in the absence of DNA, purified recombinant epitope-tagged yeast Asf1 (Asf1-Flag) was combined with purified recombinant RFC. After incubation, protein complexes were immunoprecipitated via the epitope tag on Asf1 and detected by Coomassie staining. All five subunits of RFC coprecipitated in an Asf1-dependent manner (Fig. 6A, lanes 1,2), demonstrating that Asf1 interacts directly with RFC and that Asf1 binding does not cause detectable dissociation of the RFC complex. To test the stability of the Asf1-RFC interaction, similar coimmunoprecipitations were washed with increasing amounts of NaCl. The interaction between Asf1 and RFC was

Figure 5. RFC recruits Asf1 to DNA. RFC (0.5 pmol) was incubated with 0.5–5.0 pmol of Asf1 or buffer as indicated and then combined with 1 pmol of primed, biotinylated DNA attached to streptavidin-agarose beads, 5 pmol PCNA, and the indicated nucleotide. Proteins associated with the DNA beads were separated by SDS-PAGE and visualized by immunoblotting. “SA” indicates streptavidin detected by Ponceau S staining, which serves as an internal control for protein recovery from the beads.

Figure 6. RFC interacts with Asf1 in solution. (A) RFC and Rfc2-5 coprecipitate with Asf1. Fifteen picomoles of Flag-tagged Asf1 were mixed with 15 pmol of either RFC or the Rfc2-5 subcomplex prior to precipitation with anti-Flag conjugated beads. After washing with buffer + 0.1 M NaCl, precipitated polypeptides were visualized by SDS-PAGE and Coomassie staining. (B) The interaction between Asf1 and RFC is resistant to 0.5 M NaCl. One picomole of Asf1-Flag and RFC were combined in buffer with 0.1 M NaCl and precipitated as above. Precipitates were washed with buffer containing either 0.1, 0.25 or 0.5 M NaCl as indicated and recovered proteins were detected by immunoblotting. The percentage of Rfc3 recovered in each lane relative to the amount in lane 2 is presented at the bottom. (C) Both full-length and the N terminus of Asf1 interact with RFC. Immunoprecipitates were washed with either 0.1 M NaCl (lanes 1–3) or 0.5 M NaCl (lanes 4–6) and detected by immunoblotting as above. (D) Nucleotide binding partially reverses the Asf1–RFC interaction. One picomole of RFC and 1 pmol of Asf1 were combined prior to the addition of the indicated nucleotides. The amount of Rfc3 that coprecipitated with Asf1-Flag was assayed by immunoblotting. Graphed are the averages of immunoprecipitations performed in triplicate.

largely stable to washes with 500 mM NaCl (Fig. 6B, lanes 2,6). To further characterize the interaction, the stable Rfc2-5 subcomplex (Gerik et al. 1997) was purified and tested for association with Asf1 to determine whether the large subunit of RFC (Rfc1) was necessary for the interaction. Like RFC, Rfc2-5 coprecipitated in an Asf1-dependent manner (Fig. 6A, lanes 3,4). These data demonstrate that Asf1 strongly interacts with RFC and that Rfc1 is not required for Asf1 binding.

The first 155 of the 279 residues of yeast Asf1 are highly conserved in all eukaryotes, and are sufficient for Asf1 functions in vitro and in vivo (Daganzo et al. 2003). The C-terminal half of Asf1 is less well conserved and in yeast species is composed of long poly-acidic tracts. To determine whether RFC binds to the conserved region of Asf1, coprecipitation experiments were performed with RFC and epitope-tagged full-length or C-terminally truncated Asf1 (Asf1N). RFC coprecipitated with both Asf1 and Asf1N (Fig. 6C, lanes 2,3). However, the interaction between Asf1N and RFC was more salt sensitive (Fig. 6C, cf. lanes 5 and 6). RFC therefore interacts with the conserved N terminus of Asf1, and the acidic tail of Asf1 contributes to the avidity of the interaction.

We sought to determine whether the Asf1–RFC inter-

action was modulated by nucleotides, which alter the catalytic state and structure of the RFC complex (Jeruzalmi et al. 2002; Bowman et al. 2004). To test this, RFC was prebound to Asf1-Flag and that preformed complex was then challenged with either ATP or ATPγS. The amount of RFC that coprecipitated with Asf1-Flag was measured by immunoblot detection of Rfc3 (Fig. 6D). The addition of nucleotide reduced the interaction between Asf1 and RFC by ~50%, demonstrating that the Asf1–RFC interaction is reversible. These data suggest that Asf1 may regulate replication forks via transient interactions with RFC in vivo. Consistent with this idea, the interaction between Asf1 and RFC was detected in yeast cell extracts, but only upon overexpression of the RFC complex (data not shown).

Discussion

New roles for Asf1 at the DNA replication fork

Asf1 has been implicated in both replication-coupled and replication-independent histone deposition (Tyler et al. 1999; Sharp et al. 2001; Tagami et al. 2004). However, deletion of *ASF1* in budding yeast results in phenotypes not associated with loss of other histone deposition proteins including sensitivity to DNA damaging agents (Le et al. 1997; Tyler et al. 1999; Ramey et al. 2004) and activation of the DNA damage checkpoint in the absence of exogenous DNA damage (Schwartz et al. 2003; Prado et al. 2004; Ramey et al. 2004). Here, we observed that Asf1 is required to efficiently complete DNA replication in the presence of DNA damaging agents or defective replication machinery (Figs. 1, 2). Furthermore, we demonstrated that Asf1 is required to maintain replisome proteins at stalled forks, providing a molecular mechanism for the previously observed phenotypes. We have also discovered a direct physical interaction between the conserved core of Asf1 and the small subunits of the PCNA-loading complex RFC. Although the alternative clamp loaders containing Rad24, Ctf18, or Elg1 share this Rfc2-5 subcomplex, our double-mutant data suggest that binding of Asf1 primarily imposes its regulatory function upon RFC and not the other clamp loaders (Supplementary Fig. 1). Additionally, we show that RFC stimulates recruitment of Asf1 to primed DNA in vitro (Fig. 5). Together, these data suggest direct functional roles for Asf1 at the replication fork, including coordination of replisome architecture upon fork stalling.

Changes in stalled replisome architecture in the absence of Asf1

Asf1 promotes replisome stability in a manner distinct from S-phase checkpoint proteins. The S-phase checkpoint promotes survival in the presence of DNA damage by maintaining the integrity of stalled replication forks (Desany et al. 1998), stabilizing DNA replication proteins at stalled forks (Cobb et al. 2003; Katou et al. 2003; Lucca et al. 2004), and preventing the generation of aberrant DNA structures that can lead to chromosomal

rearrangements (Lopes et al. 2001; Tercero and Diffley 2001; Sogo et al. 2002; Tercero et al. 2003). Upon deletion of the protein kinases Mec1 and Tel1 required for sensing DNA damage, Pol α , Pol ϵ , and Mcm4 are all lost from stalled forks (Katou et al. 2003); likewise, a mutant allele of Mec1 alone has been shown to reduce DNA Pol ϵ association (Cobb et al. 2003). Thus, although checkpoint signaling appears crucial for fork stability, Pol α had not been observed to behave differently from other DNA polymerases previously. Here, we discovered that deletion of Asf1 decreased the amount of Pol ϵ , Rfc3, and PCNA at early origins in HU-arrested cells, but Mcm4 remained and Pol α was enriched at stalled forks (Figs. 3, 4). We conclude that Asf1 is required for localization of a subset of replication proteins to stalled forks, consistent with earlier genetic data suggesting that Asf1 is regulated by Rad53 as an effector of the S-phase checkpoint (Emili et al. 2001; Hu et al. 2001).

How might Asf1 promote replication fork stability? One possibility could have been that *asf1* Δ mutants were defective in activating the S-phase checkpoint. Asf1 binds the essential checkpoint kinase, Rad53, and in the absence of Asf1, Rad53 phosphorylation in response to HU is slightly reduced (Hu et al. 2001). However, work from multiple laboratories has demonstrated that the DNA damage and S-phase checkpoints are largely intact and can indeed be activated in *asf1* Δ mutant cells (Emili et al. 2001; Hu et al. 2001; Schwartz et al. 2003; Prado et al. 2004; Ramey et al. 2004). Therefore, *asf1* Δ cells do not display defects in fork stability because of defects in checkpoint signaling. An alternative possibility could have been that Asf1 directly promotes repair of DNA lesions. However, recent data suggest that Asf1 is not required for DNA double-strand break repair (Ramey et al. 2004) and *asf1* Δ mutants are not sensitive to ultraviolet radiation (Tyler et al. 1999). Therefore, evidence presented here (Figs. 1, 2) and elsewhere (Tyler et al. 1999; Myung et al. 2003; Prado et al. 2004; Ramey et al. 2004) are all consistent with a role for Asf1 in genome stability that is restricted to S phase.

There are at least two models to explain the replisome defects in *asf1* Δ cells. First, Asf1 may regulate RFC-dependent loading of PCNA onto primed DNA during the switch from replication initiation to elongation. Specifically, RFC facilitates displacement of Pol α and recruitment of the replicative DNA polymerases δ and ϵ (for review, see Waga and Stillman 1998b). In the absence of RFC, abnormal initiation of DNA synthesis by Pol α occurs in vitro (Tsurimoto and Stillman 1991) and depletion of Pol ϵ from *Xenopus* egg extracts results in increased Pol α chromatin association (Waga et al. 2001; Fukui et al. 2004). Therefore, the increased levels of Pol α and decreased levels of Rfc3, PCNA, and Pol ϵ at stalled forks in *asf1* Δ mutant cells could result from inefficient or reversed polymerase switching (Fig. 7). Because *asf1* Δ mutant cells are not defective in traversing S phase in the absence of DNA damage (Ramey et al. 2004), it is unlikely that Asf1 is required for the switch from replication initiation to elongation in unperturbed cells. In this model, we propose instead that at stalled

Figure 7. Asf1 interacts with RFC and maintains the replication elongation machinery at stalled forks. In wild-type cells, the integrity of the stalled replisome is maintained. In the absence of Asf1, components of the replication elongation machinery are lost from the replisome, including RFC, PCNA, and Pol ϵ . Note that the association of DNA Pol α with the stalled fork increases in the absence of Asf1. This may result from defects in the polymerase switch event or from uncoupling of the MCM complex from the replisome resulting in excess single-stranded DNA.

forks Asf1 may be required to prevent reversal of the polymerase switch event via regulation of RFC.

Alternatively, our ChIP data suggest that there may be some uncoupling of MCM proteins from stalled forks in *asf1* Δ cells (Fig. 4C). If so, this would be consistent with the inability of *asf1* Δ cells to finish DNA replication in the presence of DNA damage (Fig. 2). Loading the MCM complex is a key step in the cell cycle-regulated formation of "pre-RC" complexes on replication origins, such that if MCM proteins are lost from replisomes after replication initiation, they cannot be functionally reloaded until the next G1 phase of cell cycle (for review, see Bell and Dutta 2002). The exposure of excess single-stranded DNA upon movement of MCM proteins away from a stalled fork would provide a recruitment platform for Pol α and also for checkpoint signaling complexes Mec1/Ddc2 (Zou and Elledge 2003) and the alternative RFC complex that loads the alternative PCNA-like 9-1-1 complex (for review, see Melo and Toczyski 2002). This possibility is consistent with our observation of Pol α enrichment at stalled forks in *asf1* Δ cells; other aspects of this model are under investigation. Because MCM displacement has also been observed in cells lacking the S-phase checkpoint kinase adapter proteins Mrc1 or Tof1 (Katou et al. 2003), our data suggest that multiple defects can lead to this result. However, in those cases, Pol α and Pol ϵ also became similarly uncoupled. Therefore, the pattern of protein displacement at stalled forks in *asf1* Δ cells is unique to date.

We note that our ChIP data derived from HU-arrested *asf1* Δ cells cannot distinguish between displacement of RFC, PCNA, and Pol ϵ from forks and inefficient loading of these factors. Indeed, these possibilities are not mutually exclusive, and in either case would indicate that regulation of polymerase switching events involving Pol α are a likely target for Asf1 regulation. We note that Pol α is already known to be a key downstream target of S-phase checkpoint regulation, as a phosphorylation substrate of the Rad53 kinase (Pelliccioli et al. 1999).

In conclusion, we describe here a new connection between histone deposition protein Asf1 and the DNA synthesis apparatus. Asf1 preserves replication elongation proteins at stalled forks, and its absence results in increased Pol α loading. Asf1 also interacts directly with RFC. These new functions are in addition to the known histone binding and deposition activities of Asf1 (for review, see Franco and Kaufman 2004). We are currently investigating how histone binding by Asf1 relates to its role in maintaining replisome proteins at stalled forks. Given the extremely high conservation of the core domain of this protein (Daganzo et al. 2003) it is likely that Asf1's role in maintaining stalled replisomes will also be conserved throughout eukaryotic organisms. We note that in human cells, replication-coupled chromatin assembly is required for completion of S phase (Nelson et al. 2002; Hoek and Stillman 2003; Ye et al. 2003). Furthermore, one human isoform of Asf1 is required for formation of the facultative heterochromatin that forms during cellular senescence (Zhang et al. 2005) and for progression through S phase (Groth et al. 2005). Future experiments will be important for determining how Asf1 isoforms in human cells contribute to S-phase progression.

Materials and methods

See Supplemental Material for information on growth conditions, flow cytometry, and pulsed-field gel analyses. Yeast strains are listed in Supplementary Table 2.

ChIP

ChIP assays were performed essentially as described (Strahl-Bolsinger et al. 1997; Cobb et al. 2003), with details provided in the Supplemental Material.

Protein purification

Overproduction and purification of recombinant scPCNA (Ayyagari et al. 1995), scRPA (He et al. 1996; Bastin-Shanower and Brill 2001), scRfc2-5 (Gomes et al. 2000), scAsf1-Flag, and scAsf1N-Flag (Daganzo et al. 2003) have been described previously. Yeast RFC bearing a seven-histidine tag in place of the ligase homology domain in Rfc1 (amino acids 2–273) was purified from *Escherichia coli* as described (Gomes et al. 2000), with modifications in the Supplemental Material.

PCNA loading/DNA association assay

The PCNA loading/DNA association assay was performed as described (Waga and Stillman 1998a), with details presented in the Supplemental Material.

In vitro Asf1–RFC binding experiments

Binding reactions were carried out in 20 μ L of binding buffer (BB; 30 mM HEPES NaOH at pH 7.5, 10% glycerol, 0.1% Triton X-100, 100 mM NaCl, 0.1% bovine serum albumin [BSA]) for 30 min and 30°C. When nucleotides were included (1 mM ATP or 0.1 mM ATP γ S), 8 mM MgOAc was also present. After binding, the volume was increased to 200 μ L with BB and 5 μ L of anti-Flag agarose (Sigma) was added. Antibody binding proceeded at

room temperature (RT) for 1 h. Immunoprecipitates were washed three times for 3 min each with 1 mL of wash buffer (WB) containing 30 mM HEPES NaOH (pH 7.5), 10% glycerol, 0.1% Triton X-100, and 500 mM NaCl (unless stated otherwise). Beads were then transferred to fresh tubes and eluted in SDS-PAGE sample buffer. Proteins were separated on 12.5% SDS-PAGE gels and visualized either by Coomassie staining or immunoblotting. PCNA was detected using polyclonal antibodies raised against recombinant scPCNA (Daganzo et al. 2003). Polyclonal antibodies recognizing the conserved N terminus of Asf1 (Daganzo et al. 2003) and Rfc3 (Li and Burgers 1994) were generated as described.

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Histone deposition protein Asf1 maintains DNA replisome integrity and interacts with replication factor C

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