

# The absence of the yeast chromatin assembly factor Asf1 increases genomic instability and sister chromatid exchange

Félix Prado, Felipe Cortés-Ledesma & Andrés Aguilera<sup>+</sup>

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain

**Histone chaperone Asf1 participates in heterochromatin silencing, DNA repair and regulation of gene expression, and promotes the assembly of DNA into chromatin *in vitro*. To determine the influence of Asf1 on genetic stability, we have analysed the effect of *asf1Δ* on homologous recombination. In accordance with a defect in nucleosome assembly, *asf1Δ* leads to a loss of negative supercoiling in plasmids. Importantly, *asf1Δ* increases spontaneous recombination between inverted DNA sequences. This increase correlates with an accumulation of double-strand breaks (DSBs) as determined by immunodetection of phosphorylated histone H2A and fluorescent detection of Rad52–YFP foci during S and G2/M phases. In addition, *asf1Δ* shows high levels of sister chromatid exchange (SCE) and is proficient in DSB-induced SCE as determined by physical analysis. Our results suggest that defective chromatin assembly caused by *asf1Δ* leads to DSBs that can be repaired by SCE, affecting genetic stability.**

Keywords: chromatin assembly; homologous recombination; sister chromatid exchange; DNA replication

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## INTRODUCTION

Nucleosome assembly is tightly connected to DNA-associated processes such as DNA replication and repair, which can influence genome integrity (Kolodner *et al*, 2002). Newly synthesized DNA is assembled into nucleosomes in a stepwise manner, with the deposition of a histone H3/H4 tetramer followed by the binding of two histone H2A/H2B dimers (Tyler, 2002). Asf1 is a highly conserved histone chaperone that co-purifies with histones H3 and H4 carrying lysine acetylation patterns characteristic of newly synthesized histones (Tyler *et al*, 1999). Asf1 interacts physically with the chromatin assembly factor CAF-1 (Cac1–Cac3 in *Saccharomyces cerevisiae*) (Tyler *et al*, 2001;

Krawitz *et al*, 2002; Mello *et al*, 2002), with which it cooperates in the assembly of nucleosomes on newly replicated DNA *in vitro* (Tyler *et al*, 1999). Consistent with defects in nucleosome assembly during DNA replication, yeast *asf1* and *cac* mutants are delayed in the S and G2/M phases and are defective in heterochromatin silencing mediated by the replication clamp PCNA (Tyler *et al*, 1999; Sharp *et al*, 2001). These defects increase synergistically in *asf1 cac* double mutants, indicating that Asf1 and CAF-1 have redundant chromatin assembly functions (Tyler *et al*, 1999).

Asf1 also participates in DNA repair in response to DNA damage and replication blocks. Thus, Asf1 cooperates with CAF-1 in the assembly of nucleosomes during nucleotide excision repair *in vitro* (Mello *et al*, 2002) and, consistently, *asf1Δ cac* double mutants are highly sensitive to ultraviolet light compared with either single mutant (Tyler *et al*, 1999). Also, *asf1Δ* cells are sensitive to the DNA-damaging agents methylmethanesulphonate (MMS), phleomycin and 4-nitroquinoline-*N*-oxide (4-NQO), and to the replication inhibitor hydroxyurea (Le *et al*, 1997; Tyler *et al*, 1999; Emili *et al*, 2001), and are defective in the repair of double-strand breaks (DSBs) induced by the endonuclease (Qin & Parthun, 2002). Asf1 forms with the checkpoint Rad53 protein a dynamic complex that dissociates in response to DNA damage and replication blocks, connecting the role of Asf1 in DNA repair with S-phase replication (Emili *et al*, 2001). In addition, *asf1Δ* affects histone gene transcription and is lethal in combination with mutations in transcription elongation factors, suggesting that Asf1 participates in the formation of chromatin structures that influence gene expression (Sutton *et al*, 2001; Formosa *et al*, 2002).

The association of Asf1 with a number of processes related to genetic instability, particularly replication and repair, led us to determine its role in homologous recombination. Homologous recombination in vegetatively growing cells participates in the repair of a number of DNA lesions (Kupiec & Steinlauf, 1997; Aguilera *et al*, 2000). Many of these lesions occur during DNA replication, suggesting that homologous recombination is involved in the rescue of stalled replication forks (Rothstein *et al*, 2000). If homologous recombination occurs between sister chromatids (sister chromatid exchange, SCE) genome integrity is preserved, but if it takes place between ectopic DNA sequences it can be a source of genetic instability.

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes 6, 41012 Sevilla, Spain

<sup>+</sup>Corresponding author. Tel: +34 954557107; Fax: +34 954557104; E-mail: aguilo@us.es

In this study, we show that *asf1Δ* mutants present chromatin assembly defects that are related to an increase in spontaneous recombination. This increase correlates with an accumulation of DSBs and with high levels of SCE. In accordance, we show that *asf1Δ* cells are proficient in DSB-induced SCE. We propose that *asf1Δ* accumulates DSBs that can be repaired by SCE, affecting genetic stability.

**RESULTS**

**A role of Asf1 in nucleosome assembly *in vivo***

As histone chaperones are redundant in yeast, we considered the determination of the effect of *asf1Δ* on nucleosome assembly to be important. For this purpose, we analysed plasmid superhelical density and chromatin accessibility to micrococcal nuclease (MNase), which cuts linker DNA between nucleosomes. As one nucleosome introduces one negative superhelical turn in a circular DNA molecule (Wang, 1982), a defect in nucleosome assembly should lead to a loss of negative supercoiling of plasmid pRS316-SU, which was used later for recombination analysis (see below). As shown in Fig 1A, the absence of Asf1 shifted the distribution of plasmid topoisomers relative to the wild type, indicating a loss of negative superhelical density.

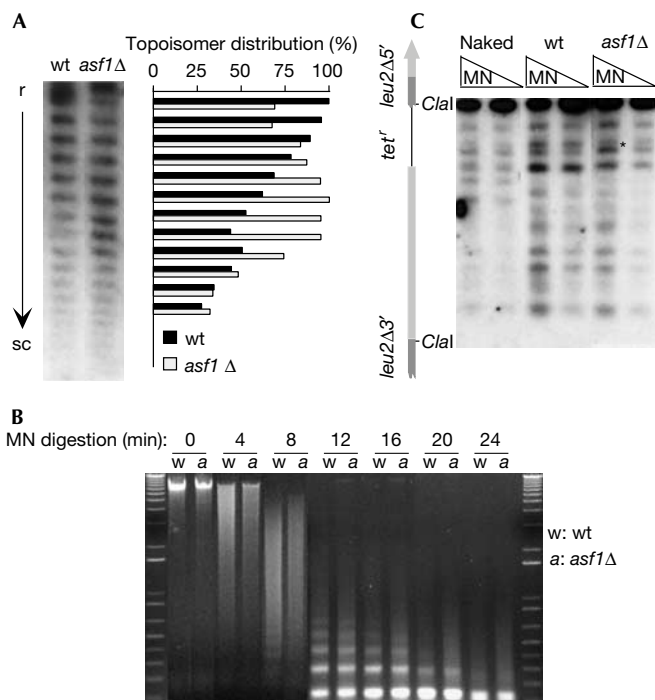
To determine the effect of *asf1Δ* on nucleosome organization, the accessibility of MNase to bulk chromatin and the nucleosome positioning at the SU inverted repeat region were analysed. As can be seen in Fig 1B, bulk chromatin accessibility was not altered,

indicating that *asf1Δ* does not lead to a major loss of nucleosome density. As shown in Fig 1C, only a minor modification in the chromatin structure of the SU system was detected. Altogether, our results are consistent with an impairment of nucleosome assembly in *asf1Δ* cells that affects DNA topology without important alterations in nucleosome density and positioning.

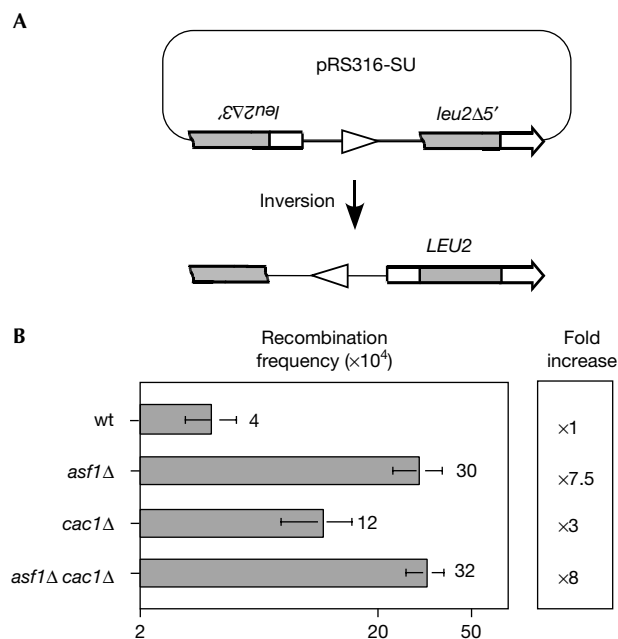
***asf1Δ* increases recombination and accumulates DSBs**

To determine the effect of *asf1Δ* on spontaneous ectopic recombination, we used the plasmidic inverted repeat system SU, which is based on two truncated *leu2* alleles that share a 0.6-kb internal fragment. Homologous recombination between the repeats leads to the inversion of the intervening sequence and the formation of a *LEU2* wild-type copy (Prado & Aguilera, 1995) (Fig 2A). As shown in Fig 2B, *asf1Δ* increased the frequency of inversions eightfold above the wild type. Therefore, the absence of Asf1 leads to genetic instability mediated by ectopic recombination.

Given the physical and genetic interactions between Asf1 and CAF-1, we analysed the possibility that the effect of *asf1Δ* on recombination was mediated by CAF-1. We determined the frequency of inversions in a *cac1Δ* mutant, which lacks the largest subunit of the CAF-1 complex. As shown in Fig 2B, *cac1Δ* increased inversions threefold above wild-type levels, whereas the *asf1Δ cac1Δ* double mutant showed the same level of recombination as *asf1Δ* (eightfold above the wild type). These results indicate that Asf1 and CAF-1 act in the same pathway of nucleosome assembly to prevent hyper-recombinogenicity, with a more prominent role of Asf1.



**Fig 1** | Effect of *asf1Δ* on plasmid topology and chromatin structure in strains BY4741 (wt) and Y11310 (*asf1Δ*). (A) Topoisomer distribution of plasmid pRS316-SU. (B) MNase accessibility of bulk chromatin and (C) nucleosome positioning in the SU recombination system. r and sc, relaxed and supercoiled plasmids, respectively; MN, MNaseI. A scheme of the SU region analysed is shown. The asterisk indicates an MNase cleavage site protected in *asf1Δ* versus wild type.



**Fig 2** | Frequency of homologous recombination in chromatin assembly mutants. (A) Plasmid pRS316-SU carrying the inverted repeat system SU and its recombination product. Grey boxes indicate the inverted repeats. (B) Frequency of Leu<sup>+</sup> recombinants in strains BY4741 (wt), Y11310 (*asf1Δ*), Y05437 (*cac1Δ*) and BYac1-1B (*asf1Δ cac1Δ*).

As DSBs efficiently stimulate homologous recombination in yeast (Paques & Haber, 1999), we wondered whether the increase in spontaneous recombination in *asf1Δ* cells was related to an accumulation of DSBs. For this purpose, we studied the accumulation of phosphorylated histone H2A and Rad52–yellow fluorescent protein (YFP) foci. It has been previously shown that phosphorylation of Ser 129 of histone H2A (P-H2A) occurs at DSB sites via the Mec1 kinase (Downs et al, 2000) and that Rad52–YFP foci are formed and colocalize *in situ* with DSBs (Lisby et al, 2003). Rad52–YFP foci appear both spontaneously and in response to DSBs during S and G2/M phases, providing a specific role of the recombination Rad52 protein during DNA replication (Lisby et al, 2001).

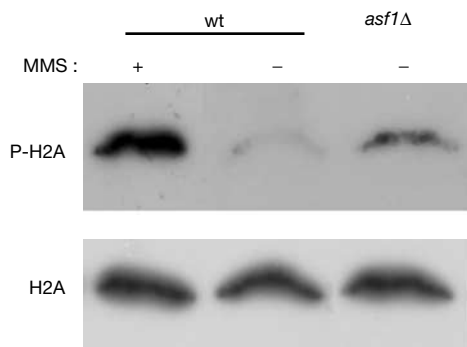
As expected, an accumulation of P-H2A was observed in wild-type cells treated with the DSB inducer agent MMS but not in untreated cells, as determined by western analysis (Fig 3). However, P-H2A was clearly detected in the absence of induced DNA damage in *asf1Δ* cells. Consistent with previous results, Rad52–YFP foci were detected in 3% of unbudded cells (G1) and in 24% of budded cells (S and G2/M) in the wild type (Fig 4) (Lisby et al, 2001). In *asf1Δ*, the Rad52–YFP foci occurred at wild-type levels (5%) in unbudded cells, but increased to 53% in budded

cells (Fig 4). This increase was observed in both small (S phase) (from 18% in wild type to 30% in *asf1Δ*) and large budded cells (mostly G2/M) (from 24 to 53%), as expected for events that originated in S phase (Lisby et al, 2003). *asf1Δ* cells contained on average one Rad52–YFP focus per cell, which has led to the interpretation that each focus represents a centre of recombinational repair of multiple DSBs (Lisby et al, 2001, 2003). The extensive phosphorylation of histone H2A relative to the twofold increase in Rad52–YFP foci is, indeed, consistent with this interpretation. Altogether, these results indicate that the absence of Asf1 leads to an accumulation of DSBs, preferentially during DNA replication.

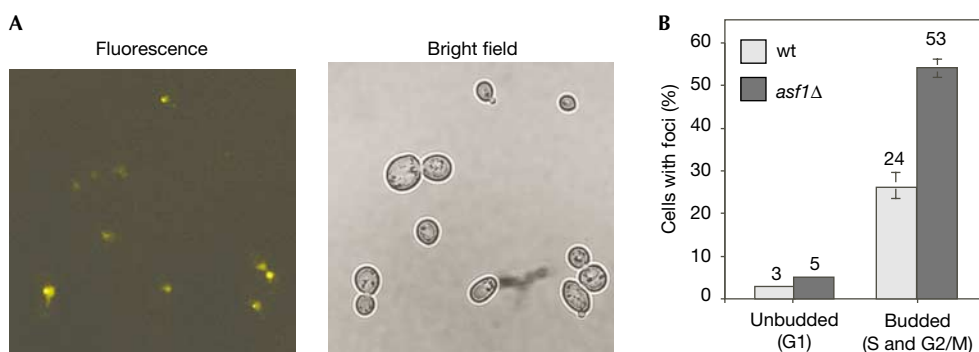
### Spontaneous and DSB-induced SCE in *asf1Δ* cells

The hyper-recombination phenotype and the accumulation of DSBs in *asf1Δ* suggest that DSBs are repaired by SCE, a recombination mechanism that occurs during the S and G2 phases. To test this, we used a recently developed recombination system (TINV) for the molecular analysis of SCE (Fig 5A; González-Barrera et al, 2003). In this system, spontaneous unequal SCE between the *leu2* inverted repeat copies yields a head-to-head dimer that is detected after *SpeI*–*XhoI* digestion by the appearance of two specific recombinant bands (2.9 and 4.7 kb). Southern analysis revealed that spontaneously occurring dimers, identified by the 2.9-kb and 4.7-kb bands, were undetectable in wild-type cells but could be detected in *asf1Δ* cells, regardless of whether cells were grown in 3% glycerol-lactate (Fig 5B; *t* = 0) or 2% glucose (data not shown). These dimers were unlikely to have been the result of recombination between two copies of the plasmid accumulated by missegregation, because no dimers were detected in cells carrying two plasmids that differed only in the selection marker (data not shown). These results indicate that *asf1Δ* increases spontaneous SCE. In this sense, it is worth noticing that the proportion of dimers is in the range of spontaneous *Leu*<sup>+</sup> recombinants observed for the TINV system in *asf1Δ* ( $2 \times 10^{-3}$  in *asf1Δ* versus  $3 \times 10^{-4}$  in the wild type).

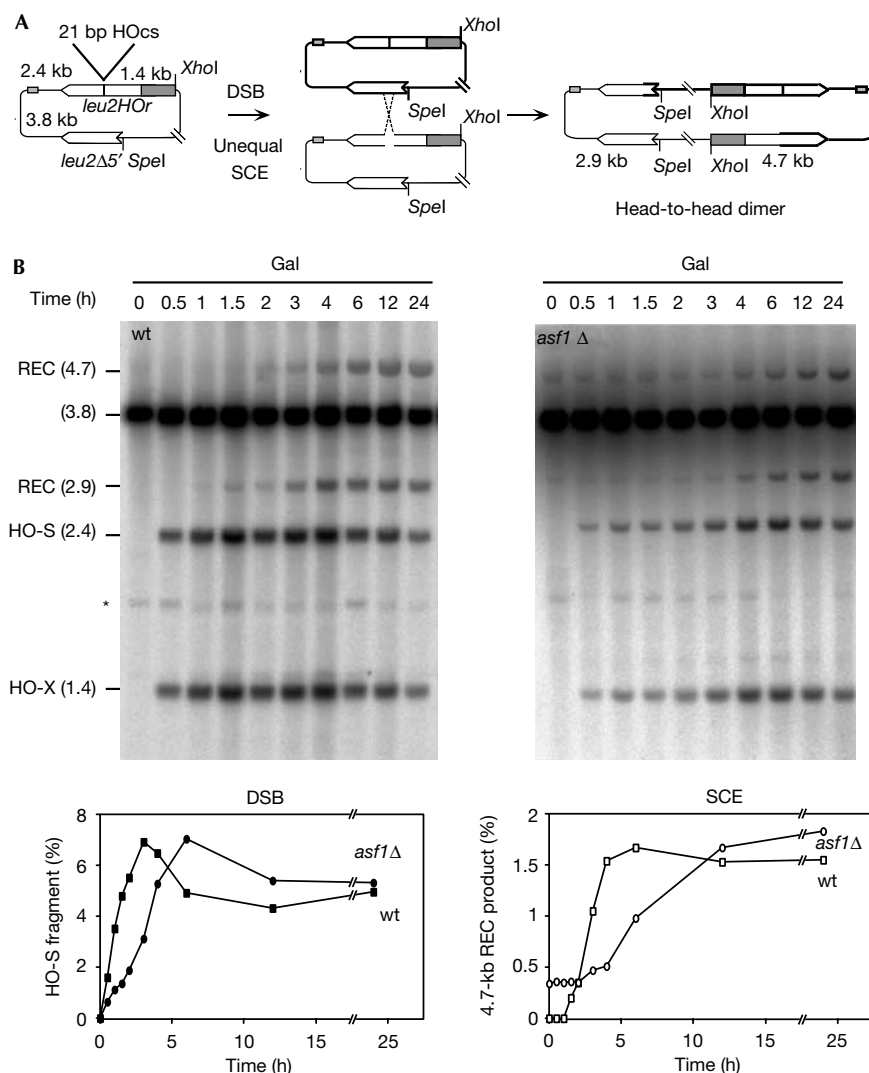
These results suggest that the DSBs accumulated in *asf1Δ* cells are repaired by SCE. However, Asf1 has recently been shown to be required for efficient repair of DSBs induced by the endonuclease



**Fig 3** | Accumulation of P-H2A in BY4741 (wt) and Y11310 (*asf1Δ*) strains grown with 0.05% (+) or without (–) MMS for 2 h. Total and Ser 129-phosphorylated histone H2A were detected by western blot as described previously (Downs et al, 2000).



**Fig 4** | Accumulation of Rad52–YFP foci in *asf1Δ* cells. Rad52–YFP foci were visualized by fluorescence microscopy in asynchronous cultures of BY4741 (wt) and Y11310 (*asf1Δ*) cells transformed with plasmid pWJ1344. (A) Fluorescence and brightfield microscopy of *asf1Δ* cells expressing the Rad52–YFP fusion protein. (B) Percentage of unbudded (G1) and budded (S and G2/M) wild-type and *asf1Δ* cells containing Rad52–YFP foci. The total numbers of analysed cells were 55 and 199, and 62 and 396 for unbudded and budded wild-type and *asf1Δ* cells, respectively.



**Fig 5** | Physical analysis of spontaneous and HO-induced SCE in WS (wt) and WS-*asf1* (*asf1*Δ) strains. (A) Plasmid pRS316-TINV carrying the inverted repeat system TINV and the dimer resulting from unequal SCE between the inverted repeats. (B) Kinetics of HO-induced SCE. Mid-log-phase cells from SC-3% glycerol/2% lactate plus 5 μg/ml doxycycline were grown for different times in SC-2% galactose (Gal). Spontaneous SCE in the absence of HO breaks is observed as the accumulation of the 2.9-kb and 4.7-kb bands in *asf1*Δ cells in glycerol-lactate (*t* = 0). The quantification of the HO-induced DSB (HO-S 2.4 kb) and the 4.7-kb recombination product (REC 4.7) is shown. The average of two experiments performed with independent transformants is plotted. Variation for each point was < 30%. DNA was isolated, digested with *XhoI*-*SpeI* and analysed by Southern blot with the *Clal*-*EcoRV* *LEU2* internal fragment for spontaneous and HO-induced SCE. Bands resulting from HO cleavage (HO-S and HO-X) and recombination intermediates (REC bands) were quantified in a Fuji FLA3000 and normalized relative to the total plasmid DNA. The asterisk shows unspecific hybridization.

HO during mating-type switching (Qin & Parthun, 2002). Therefore, we decided to determine whether SCE could repair HO-induced DSBs in *asf1*Δ cells. For this purpose, DSBs were induced at the 21-bp HO site of the *leu2HOr* allele in the TINV system. As the 21-bp HO site is cut by HO at low efficiency (7%), most HO-induced DSBs are limited to one chromatid and can be repaired using as template any of the *leu2* alleles of the intact chromatid (González-Barrera et al, 2003). If the donor copy is the *leu2*Δ5' allele located in the sister chromatid, the head-to-head dimer could be detected (Fig 5A). As the HO endonuclease was under the control of the *GAL1* promoter, the kinetics of DSB repair can

be analysed by Southern analysis after galactose induction. As can be seen in Fig 5B, the kinetics of DSBs accumulation, determined by the accumulation of the 1.4-kb and 2.4-kb bands, was similar in wild-type and *asf1*Δ cells, although it was delayed 2–3 h in the mutant. Compared with the wild type, the efficiency of SCE, determined by the accumulation of the 2.9-kb and 4.7-kb bands, was not reduced in the absence of *Asf1*. Although dimer accumulation was also delayed in *asf1*Δ, it is worth noticing that the maximum level of dimers appeared 3 and 6 h after the peaks of DSBs in wild-type and mutant cells, respectively (Fig 5B).

## DISCUSSION

In this study, we provide evidence that the absence of the chromatin assembly factor Asf1 influences genetic stability by the accumulation of DSBs that are repaired via SCE. *asf1Δ* and *cac1Δ* increase the frequency of inversions between homologous DNA sequences eightfold and threefold above wild-type levels, respectively. The hyper-recombinogenic effect of *asf1Δ* is epistatic over *cac1Δ*, suggesting that Asf1 and CAF-1 participate in the same pathway in the maintenance of genome integrity. Therefore, the absence of chromatin assembly factors, particularly Asf1, causes genetic instability. This is consistent with recent reports showing that a dominant-negative mutant of p150CAF-1 causes DNA breaks in human cells (Ye *et al*, 2003), and that yeast *asf1Δ* and *cac1Δ* mutants strongly increase gross chromosomal rearrangements (GCRs) such as telomere addition, translocations and deletions (Myung *et al*, 2003). These GCRs involve microhomology or nonhomology breakpoints and increase synergistically in *rad52Δ* cells, indicating that they do not occur by homologous recombination; instead, homologous recombination prevents their formation (Myung *et al*, 2003). Our results extend the effect of defective chromatin assembly to rearrangements between DNA homologous sequences. This observation is in accordance with the increase in homologous recombination caused by either partial depletion of histone H4 (F. Prado and A. Aguilera, unpublished data) or a thermosensitive mutation in the histone chaperone Spt6 (Malagon & Aguilera, 2001).

Chromatin assembly is tightly associated with DNA synthesis during replication (Shibahara & Stillman, 1999; Sharp *et al*, 2001). Defective nucleosome assembly in *asf1Δ* could uncouple this connection and impair DNA synthesis, leading to the accumulation of DNA damage. We provide molecular evidence consistent with defective nucleosome assembly in *asf1Δ*. Thus, *asf1Δ* changes the superhelical density of a plasmid without a major loss of nucleosome density or positioning. This could be explained by a change in the path of the DNA around an altered histone octamer in *asf1Δ*. This possibility is consistent with the proposed role for Asf1 in H3/H4 tetramer deposition on the DNA (Tyler *et al*, 1999), as the tetramer is a major determinant in the organization of DNA in the nucleosome (Ramakrishnan, 1997). In accordance with impaired DNA synthesis and accumulation of DNA damage, *asf1Δ* accumulates DSBs during S and G2/M phases of the cell cycle—as determined by Rad52–YFP foci and P-H2A detection (Figs 3 and 4)—and shows high levels of spontaneous SCE (Fig 4), and is delayed in the S and G2/M phases (Tyler *et al*, 1999; data not shown). Indeed, the high accumulation of Rad52–YFP foci in *asf1Δ* is similar to that previously observed with mutants affected in the replicative DNA polymerase  $\alpha$  complex (Lisby *et al*, 2001). Consistently, expression of a dominant-negative mutant of p150CAF-1 in human cells causes accumulation of P-H2A and S-phase arrest (Ye *et al*, 2003). Therefore, a tight regulation of DNA synthesis and histone deposition is essential to prevent genetic instability during DNA replication.

The observation that SCE-mediated dimers accumulate spontaneously at high levels in *asf1Δ* cells suggests that SCE could be responsible for the increase in homologous recombination observed in *asf1Δ*. Indeed, *asf1Δ* cells are proficient in DSB repair by SCE, as determined by physical analysis of HO-induced dimers. This result contrasts with the requirement of Asf1 in the repair of HO-induced DSBs by intramolecular gene conversion in

mating-type switching (Qin & Parthun, 2002). Whether or not this is due to the existence of redundant nucleosome assembly functions associated with SCE or due to the formation of DSBs at the 21-bp HO site specifically during the S phase remains to be determined.

In summary, our results support the importance of chromatin assembly in the maintenance of genetic stability, and establish a new link between DNA replication and homologous recombination mediated by chromatin assembly factors. The molecular evidence that SCE is a major mechanism of DSB repair in *asf1Δ* supports the idea that the hyper-recombinogenicity of *asf1Δ* cells is linked to replication impairment.

## METHODS

**Yeast strains and plasmids.** Yeast strains used in this study were BY4741 (*MAT $\alpha$  his3Δ0 leu2Δ0 ura3Δ0 met15Δ0*) and its isogenic derivatives Y11310 (*MAT $\alpha$  asf1Δ::KAN*), Y05437 (*MAT $\alpha$  cac1Δ::KAN*) (Euroscarf) and BYac1-1B (*MAT $\alpha$  asf1Δ::KAN cac1Δ::KAN*), as well as WS (*MAT $\alpha$ -inc ade3::GAL-HO leu2Δ::SFA1 ade2-1 can1-100 his3-11,15 trp1-1 ura3-1*) (González-Barrera *et al*, 2003) and its isogenic WS-*asf1* (*asf1Δ::KAN*). Yeast cells were grown at 30 °C in synthetic complete (SC) medium as described (Kaiser *et al*, 1994). pRS316-SU—identical to pRS314-SU (Prado & Aguilera, 1995) but in vector pRS316—and pRS316-TINV (González-Barrera *et al*, 2003) are centromeric plasmids containing the recombination systems used in this study. pWJ1344 is a centromeric plasmid containing the Rad52–YFP construct (by R. Rothstein, Columbia University).

**Genetic and physical analysis of recombination.** Spontaneous recombination frequencies were obtained by a fluctuation test as the median value of six independent colonies as described previously (Prado & Aguilera, 1995). The average value of three of four independent assays performed with two or three independent transformants is shown. Physical analysis of SCE was performed as described (González-Barrera *et al*, 2003).

**Chromatin analysis by MNase digestion.** Mapping of MNase cleavage sites was performed as previously published (Chavez *et al*, 1995). For bulk chromatin accessibility to MNase, chromatin was digested with 400 mU of MNase and DNA samples were taken at different times and resolved in 1% agarose gel. For nucleosome positioning by indirect end labelling, chromatin was digested with different amounts of MNase during 20 min. DNA was extracted and restricted with *Clal*, resolved in 1.5% agarose gel and probed with the 221-bp fragment located immediately downstream of the *LEU2 Clal* site.

**Plasmid supercoiling.** Total DNA from mid-log-phase cultures was extracted and resolved in chloroquine gels according to Brill & Sternglanz (1988). Topoisomer distribution of plasmid pRS316-SU was detected with the *Clal*–*EcoRV* *LEU2* probe, quantified in a Fuji FLA3000 and normalized relative to the most abundant topoisomer.

**Detection of Rad52–YFP.** Rad52–YFP foci from mid-log-phase cells transformed with plasmid pWJ1344 were visualized by a confocal microscope Leica TCS SL (excitation 514 nm; emission 535 nm), according to Lisby *et al* (2001).

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