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Regulation of Histone Deposition Proteins Asf1/Hir1 by Multiple DNA Damage Checkpoint Kinases in *Saccharomyces cerevisiae*

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ABSTRACT

CAF-1, Hir proteins, and Asf1 are histone H3/H4 binding proteins important for chromatin-mediated transcriptional silencing. We explored genetic and physical interactions between these proteins and S-phase/DNA damage checkpoint kinases in the budding yeast *Saccharomyces cerevisiae*. Although cells lacking checkpoint kinase Mec1 do not display defects in telomeric gene silencing, silencing was dramatically reduced in cells lacking both Mec1 and the Cac1 subunit of CAF-1. Silencing was restored in *cac1*Δ and *cac1*Δ *mec1*Δ cells upon deletion of Rad53, the kinase downstream of Mec1. Restoration of silencing to *cac1*Δ cells required both Hir1 and Asf1, suggesting that Mec1 counteracts functional sequestration of the Asf1/Hir1 complex by Rad53. Consistent with this idea, the degree of suppression of silencing defects by *rad53* alleles correlated with effects on Asf1 binding. Furthermore, deletion of the Dun1 kinase, a downstream target of Rad53, also suppressed the silencing defects of *cac1*Δ cells and reduced the levels of Asf1 associated with Rad53 *in vivo*. Loss of Mec1 and Rad53 did not alter telomere lengths or Asf1 protein levels, nuclear localization, or chromosome association. We conclude that the Mec1 and Dun1 checkpoint kinases regulate the Asf1-Rad53 interaction and therefore affect the activity of the Asf1/Hir complex *in vivo*.

THE DNA of all eukaryotic genomes is packaged into a nucleoprotein complex called chromatin. Chromatin is essential for compacting genomic DNA and plays a primary role in governing accessibility for transcription, replication, and recombination. The fundamental repeating unit of chromatin is the nucleosome, containing an octamer of histone proteins, two each of H2A, H2B, H3, and H4, around which 146 bp of DNA wraps 1.7 times (Luger *et al.* 1997). Nucleosome assembly during S phase occurs in a manner that is tightly linked to DNA replication (Lucchini and Sogo 1995). However, replication-independent nucleosome assembly mechanisms also exist to ensure replacement of histones outside of S phase during gene transcription and DNA repair (Ahmad and Henikoff 2002; McKittrick *et al.* 2004). These processes are mediated by multiple specialized histone chaperones (reviewed in Franco and Kaufman 2004).

The best-characterized DNA replication-linked histone deposition complex is chromatin assembly factor-1 (CAF-1). CAF-1 is a heterotrimeric protein complex that is both structurally and functionally conserved among all eukaryotes (Kaufman *et al.* 1995, 1997; Tyler *et al.* 1996, 2001; Kaya *et al.* 2001; Quivy *et al.* 2001). In human cells, CAF-1 localizes to sites of DNA synthesis during S phase and also at sites of DNA repair outside of S phase (Krude 1995; Martini *et al.* 1998; Green and Almouzni 2003). Inhibition or degradation of human CAF-1 results in impaired S-phase progression, suggesting that CAF-1 helps to coordinate DNA synthesis and chromatin formation (Hoek and Stillman 2003; Ye *et al.* 2003). Consistent with this idea, the large subunit of CAF-1 from all organisms binds to PCNA, the processivity factor for DNA polymerases that is required for both DNA replication and repair (Shibahara and Stillman 1999; Moggs *et al.* 2000; Zhang *et al.* 2000; Krawitz *et al.* 2002).

In *Saccharomyces cerevisiae*, the *CAC1-3* genes encode the subunits of CAF-1. Budding yeast cells lacking either one or all of *CAC* genes display normal kinetics of cell cycle progression (Sharp *et al.* 2002), yet have reduced chromatin-mediated gene silencing at the telomeres, at the silent mating loci, and at ribosomal DNA (Enomoto *et al.* 1997; Kaufman *et al.* 1997; Monson *et al.* 1997; Enomoto and Berman 1998; Smith *et al.* 1999). CAF-1 also contributes to the proper structure and function of centromeric chromatin in budding yeast (Sharp *et al.* 2002, 2003). Together, these data indicate a conserved role for CAF-1 in chromatin formation.

In yeast, the histone regulatory (*HIR*) genes, *HIR1*, *HIR2*, *HIR3*, and *HPC2* (Osley and Lycan 1987; Xu

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et al. 1992), encode proteins that compose a histone deposition pathway that functionally overlaps CAF-1 (Kaufman *et al.* 1998). Although mutations in yeast *HIR* genes alone do not alter silencing at telomeres and the silent mating loci, *cac*Δ *hir*Δ double-mutant cells display a synergistic reduction of position-dependent gene silencing at both these loci (Kaufman *et al.* 1998; Qian *et al.* 1998). Consistent with these genetic data, biochemical analyses of vertebrate Hir protein homologs also indicate a role in histone deposition. HIRA, the Xenopus homolog of the *HIR1/HIR2* genes, exhibits replication-independent histone deposition activity (Ray-Gallet *et al.* 2002), and the human HIRA protein is associated with the constitutively expressed histone H3.3 isoform, which is deposited into chromatin in times outside of S phase (Ahmad and Henikoff 2002; Tagami *et al.* 2004). Thus, all eukaryotes have multiple histone deposition proteins, some linked to DNA synthesis (CAF-1) and others that operate in a non-replication-linked manner (Hir proteins).

Genetic and biochemical data from multiple organisms indicate that the contribution of Hir proteins to chromatin assembly requires the highly conserved histone H3/H4-binding protein Asf1 (Sharp *et al.* 2001; Sutton *et al.* 2001; Daganzo *et al.* 2003). Asf1 binds to the Hir1 and Hir2 proteins in yeast and to the HIRA protein in vertebrates (Sharp *et al.* 2001; Sutton *et al.* 2001; Daganzo *et al.* 2003; Tagami *et al.* 2004; Zhang *et al.* 2005). The interaction site between Asf1 and Hir proteins is required for formation of silent chromatin in yeast (Daganzo *et al.* 2003) and for formation of heterochromatin during cellular senescence in human cells (Zhang *et al.* 2005). Therefore, the Asf1/Hir protein complex is an evolutionarily conserved histone deposition factor.

Both yeast and metazoan organisms have signal transduction mechanisms that modulate Asf1 in response to DNA damage checkpoint activation (Emili *et al.* 2001; Hu *et al.* 2001; Sillje and Nigg 2001; Groth *et al.* 2003). The DNA damage checkpoint is a surveillance mechanism responsible for sensing DNA damage, pausing the cell cycle to allow time for repair of the damaged DNA and activating damage response/repair pathways (reviewed in Melo and Toczyski 2002) (see Figure 1). In yeast, DNA damage triggers a protein phosphorylation cascade through Mec1, a protein kinase related to phosphoinositide kinases (Abraham 2001). Downstream of Mec1 is the protein kinase Rad53, which is activated via Mec1-dependent phosphorylation in response to DNA damage and replication blocks (Sanchez *et al.* 1996; Sun *et al.* 1996). Crosstalk between the DNA damage checkpoint and chromatin assembly in yeast was suggested when Asf1 was shown to physically interact with Rad53 (Emili *et al.* 2001; Hu *et al.* 2001). This interaction was shown to inhibit histone deposition by Asf1 *in vitro*, but the biological consequences were undetermined.

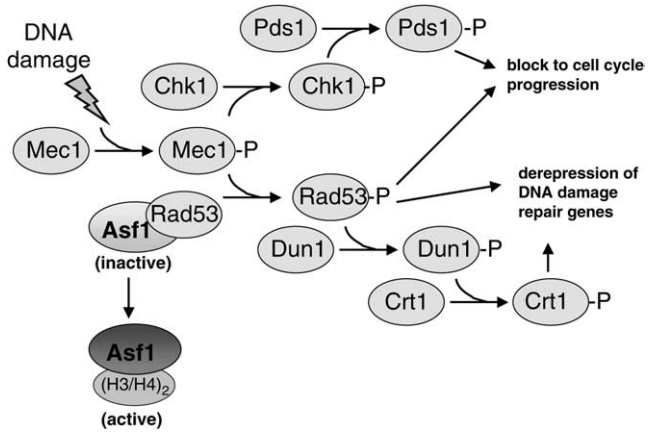

Figure 1.—Model for the cellular response to DNA damage. The Mec1 kinase is a central coordinator of protective mechanisms that are activated in the presence of DNA damage. Two major pathways have been described (Zhou and Elledge 1993; Allen *et al.* 1994; Weinert *et al.* 1994; Sanchez *et al.* 1996, 1999; Sun *et al.* 1996; Cohen-Fix and Koshland 1997; Huang *et al.* 1998; Gardner *et al.* 1999; Clarke *et al.* 2001; Wang *et al.* 2001; Agarwal *et al.* 2003):

1. Cells must halt cell cycle progression until DNA damage can be repaired. Activation of the Chk2 kinase by Mec1 promotes the phosphorylation of Pds1. The phosphorylated form of Pds1 is refractory to destruction by the anaphase-promoting complex, thus causing accumulation of cells at the G2/M phase of the cell cycle in the presence of DNA damage.
2. Increased transcription of genes required for DNA damage repair depends on Mec1. Mec1 activation causes Rad53 phosphorylation and the dissociation of the Asf1/Rad53 complex. Rad53-dependent phosphorylation of Dun1 leads to the phosphorylation of Crt1, a cofactor required for Tup1/Ssn6-mediated transcriptional repression of DNA damage repair genes. The Rad53 branch of the checkpoint also acts to block cell cycle progression in the presence of DNA damage in a manner that is independent of Pds1 phosphorylation. Further, because some genes induced by DNA damage do not depend on Dun1 function, alternative targets of Rad53-mediated transcriptional activation are likely to exist.

We present data here demonstrating that the Rad53-Asf1 interaction is an important regulator of heterochromatin assembly in yeast. Although previous work demonstrated that the Rad53 and Mec1 kinases regulate chromatin-mediated silencing of yeast telomere-proximal genes (Craven and Petes 2000; Longhese *et al.* 2000), the downstream effectors remained unknown. Here, we demonstrate that Mec1 affects telomeric silencing via Rad53-mediated regulation of Asf1/Hir protein activity. Furthermore, we show that the Dun1 kinase, a downstream target of Rad53 phosphorylation, positively regulates the Rad53-Asf1 interaction *in vivo*. Therefore, the Rad53-Asf1 interaction is critical for heterochromatin formation and is regulated by multiple DNA damage checkpoint kinases.

MATERIALS AND METHODS

Yeast strains: Strain genotypes are listed in Table 1. The *cac1*Δ::*hisG*, *cac1*Δ::*LEU2*, *hir1*Δ::*HIS3*, *asf1*Δ::*TRP1*, and

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
PKY090	*MAT***a**; *URA3*-VIIL	Kaufman *et al.* (1997)
PKY638	*MAT***a**; *cac1*Δ::*hisG*; *URA3*-VIIL	Sharp *et al.* (2001)
PKY1766	*MAT***a**; *sml1*Δ::*HIS3*; *URA3*-VIIL	This study
PKY1769	*MAT***a**; *cac1*Δ::*hisG*; *sml1*Δ::*HIS3*; *URA3*-VIIL	This study
PKY1768	*MAT***a**; *mec1*Δ::*TRP1*; *sml1*Δ_:*HIS3*; *URA3*-VIIL	This study
PKY1771	*MAT***a**; *cac1*Δ::*hisG*; *mec1*Δ::*TRP1*; *sml1*Δ::*HIS3*; *URA3*-VIIL	This study
PKY2704	*MAT***a**; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY2706	*MAT***a**; *cac1*Δ::*LEU2*; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY2702	*MAT***a**; *rad53*Δ::*HIS3*; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY2710	*MAT***a**; *cac1*Δ::*LEU2*; *rad53*Δ::*HIS3*; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY3611	*MAT***a**; *pds1*Δ::*LEU2*; *URA3*-VIIL	This study
PKY3616	*MAT***a**; *cac1*Δ::*hisG*; *pds1*Δ::*LEU2*; *URA3*-VIIL	This study
PKY2758	*MAT***a**; *asf1*Δ::*TRP1*; *rad53*Δ::*HIS3*; *sml1*Δ::*kanMX6*; *HMR*wt::*ADE2*; *URA3*-VIIL	This study
PKY3564	*MAT*α; *hir1*Δ::*HIS3*; *rad53*Δ::*HIS3*; *sml1*Δ::*kanMX6*; *URA3*-VIIL	This study
PKY2763	*MAT*α; *cac1*Δ::*LEU2*; *rad53*Δ::*HIS3*; *asf1*Δ::*TRP1*; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY3566	*MAT*α; *cac1*Δ::*hisG*; *rad53*Δ::*HIS3*; *hir1*Δ::*HIS3*;*sml1*Δ::*kanMX6*; *URA3*-VIIL	This study
PKY3045	*MAT*α; *asf1*Δ::*TRP1*; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY3676	*MAT***a**; *hir1*Δ::*HIS3; sml1*Δ::*kanMX6*; *URA3*-VIIL	This study
PKY2755	*MAT*α; *cac1*Δ::*LEU2*; *asf1*Δ::*TRP1*; *sml1*Δ::*kanMX6*; *HMR*wt::*ADE2*; *URA3*-VIIL	This study
PKY3680	*MAT*α; *cac1*Δ::*LEU2*; *hir1*Δ::*HIS3*; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY2776	*MAT***a**; *mec1*Δ::*TRP1*; *rad53*Δ::*HIS3*; *sml1*Δ::*kanMX6*; *URA3*-VIIL	This study
PKY2779	*MAT***a**; *cac1*Δ::*LEU2*; *mec1*Δ::*TRP1*; *rad53*Δ::*HIS3*; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY2503	*MAT***a**; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY2719	*MAT***a**; *mec1*Δ::*TRP1*; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY2711	*MAT***a**; *cac1*Δ::*LEU2*; *rad53*Δ::*HIS3*; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY2723	*MAT***a**; *cac1*Δ::*LEU2*; *mec1*Δ::*TRP1*; *sml1*Δ::*kanMX6*; *URA3*-VIIL	This study
PKY408	*MAT***a**; (*hht1-hhf1*Δ)::*LEU2*; *URA3*-VIIL	Kaufman *et al.* (1998)
PKY993	*MAT***a** *asf1*Δ::*TRP1*; *URA3*-VIIL	Sharp *et al.* (2001)
PKY1027	*MAT***a** *asf1*Δ::*HIS3*; (*hht1-hhf1)*Δ::*LEU2*; *URA3*-VIIL	This study
PKY3583	*MAT*α; *dun1*Δ::*HIS3*; *URA3*-VIIL	This study
PKY3584	*MAT***a**; *dun1*Δ::*HIS3*; *cac1*Δ::*LEU2*; *URA3*-VIIL	This study
PKY3586	*MAT*α; *dun1*Δ::*HIS3*; *asf1*Δ::*TRP1*; *URA3*-VIIL	This study
PKY3591	*MAT*α; *dun1*Δ::*HIS3*; *hir1*Δ::*HIS3*; *URA3*-VIIL	This study
PKY3588	*MAT***a**; *dun1*Δ::*HIS3*; *asf1*Δ::*TRP1*; *cac1*Δ::*LEU2*; *URA3*-VIIL	This study
PKY3592	*MAT***a**; *dun1*Δ::*HIS3*; *hir1*Δ::*HIS3*; *cac1*Δ::*LEU2*; *URA3*-VIIL	This study
PKY2256	*MAT*α; *ADE2*-VR; *URA3*-VIIL + pBAD54	This study
PKY2259	*MAT*α; *ADE2*-VR; *URA3*-VIIL + pBAD70	This study
PKY2262	*MAT*α; *ADE2*-VR; *URA3*-VIIL + pBAD79	This study
PKY2258	*MAT*α; *cac1*Δ::*hisG*; *ADE2*-VR; *URA3*-VIIL + pBAD54	This study
PKY2261	*MAT*α; *cac1*Δ::*hisG*; *ADE2*-VR; *URA3*-VIIL + pBAD70	This study
PKY2264	*MAT*α; *cac1*Δ::*hisG*; *ADE2*-VR; *URA3*-VIIL + pBAD79	This study
PKY2735	*MAT***a**; *ASF1-HA*::*his5*$^+$; *URA3*-VIIL	This study
PKY3607	*MAT***a**; *dun1*Δ::*HIS3*; *ASF1-HA*::*his5*$^+$; *URA3*-VIIL	This study
PKY2747	*MAT*α; *ASF1-HA*::*his5*$^+$; *rad53*Δ::*HIS3*; *sml1*Δ::*kanMX6*; *URA3*-VIIL	This study
PKY2703	*MAT*α; *rad53*Δ::*HIS3*; *sml1*Δ::*kanMX6*; *URA3*-VIIL; *HMR*wt::*ADE2*	This study
PKY3748	*MAT***a**; *cac1*Δ::*hisG*; *rad53*Δ::*HIS3*; *sml1*Δ::*kanMX6*; *URA3*-VIIL	This study

All strains were in the W303 background and contained the *leu2-3, 112*; *his3-11, 15*; *trp1-1*; *ade2-1* and *can1-100* mutations. Strains are listed in the order in which they are listed in the figure legends.

URA3-VIIL alleles have been previously described (Gottschling *et al.* 1990; Sherwood *et al.* 1993; Kaufman *et al.* 1997; Sharp *et al.* 2001). Strains containing the *dun1*Δ::*HIS3* and *pds1*Δ::*LEU2* deletions were provided by T. Weinert (Gardner *et al.* 1999). The *mec1*Δ::*TRP1*, *rad53*Δ::*HIS3*, and *sml1*Δ::*HIS3* deletions were provided by R. Rothstein (Zhao *et al.* 1998). The *sml1*Δ::*kanMX6* deletion and *ASF1-HA*::*his5*$^+$ cassette were introduced into the W303 genetic background by single-step gene replacement and checked by PCR for correct insertion into the genome (Longtine *et al.* 1998). In contrast to *asf1*Δ cells, which are sensitive to hydroxyurea (HU) (Tyler *et al.* 1999), *ASF1-HA*::*his5*$^+$ cells displayed wild-type levels of growth on HU-containing media, thus demonstrating functionality of the tagged allele. Genetic crosses and tetrad analysis were performed following standard procedures (Kaiser *et al.* 1994).

Plasmids: Plasmids are listed in Table 2. To construct plasmids pPK196 and pPK197, a 4-kb *Pst*I-*Nhe*I genomic fragment containing the *ASF1* locus was first introduced into *Pst*I-*Xba*I-digested pBluescript. A 2.67-kb *Bam*HI-*Sac*I fragment containing only the *ASF1* ORF was cloned into similarly digested

TABLE 2

Plasmids used in this study

Plasmid	Description	Reference
pRS414	ARS-CEN-*TRP1*	Sikorski and Hieter (1989)
pRS415	ARS-CEN-*LEU2*	Sikorski and Hieter (1989)
pPK196	ARS-CEN-*LEU2-ASF1*	This study
YEP35l	2μ-*LEU2*	Lee *et al.* (2003)
pPK197	2μ-*LEU2-ASF1*	This study
pMS383	ARS-CEN-*TRP1*-GAL-*HHT1*	Mitch Smith (University of Virginia)
pPK128	2μ-*LEU2-HHT1-HHF1 HTA1-HTB1*	Kaufman *et al.* (1998)
pRS423	2μ-*HIS3*	Sikorski and Hieter (1989)
pMH151-7	2μ-*HIS3-CRT1*	Huang *et al.* (1998)
pBAD54	2μ-*TRP1* + GAP promoter	Desany *et al.* (1998)
pBAD70	2μ-*TRP1* + GAP-*RNR1*	Desany *et al.* (1998)
pBAD79	2μ-*TRP1* + GAP-*RNR3*	Desany *et al.* (1998)
p*RAD53*	ARS-CEN-*LEU2-RAD53*	Lee *et al.* (2003); Schwartz *et al.* (2003)
p*rad53*^{fha1}	ARS-CEN-*LEU2-rad53* R70A N107A	Lee *et al.* (2003); Schwartz *et al.* (2003)
p*rad53*^{fha2}	ARS-CEN-*LEU2-rad53* N655A V666A S657A	Lee *et al.* (2003); Schwartz *et al.* (2003)
p*rad53*^{kd}	ARS-CEN-*LEU2-rad53* K227A D339A	Lee *et al.* (2003); Schwartz *et al.* (2003)

pRS415 and pRS425 to yield plasmids pPK196 and pPK197, respectively.

Telomeric silencing assays: Strains containing the telomere-proximal *URA3*-VIIL reporter gene were grown to log phase. Cell density was adjusted to OD 1.0. A 10-fold dilution series was performed for each culture, and 5 μl of each dilution was spotted onto media containing 5′-fluoroorotic acid (5′-FOA). As a control for growth, 5 μl of the same dilution series was also spotted onto rich media (YPD) or synthetic media when plasmid selection was required. Plates with the temperature-sensitive *pds1*Δ::*LEU2*-containing strains were incubated at 25°. All other plates were incubated at 30° and photographed after 3 days (growth-control media) or 7 days (5′-FOA media). Strains containing the combinations of gene deletions described in Table 1, but without the *URA3*-VIIL cassette, grew similarly on 5′-FOA media, indicating that these mutations caused no intrinsic 5′-FOA sensitivity or resistance (data not shown).

Antibodies: A rabbit polyclonal antibody raised against the conserved core of Asf1 (Daganzo *et al.* 2003) was used for immunoblot analysis (1:20,000 dilution), immunofluorescence (1:5000 dilution), and chromatin immunoprecipitation (1:1000 dilution). A rat anti-tubulin antibody (Accurate Scientific, Westbury, NY) was used for immunoblot analysis (1:1000 dilution). The 12CA5 anti-HA monoclonal antibody (gift from D. Rio) was partially purified by ammonium sulfate precipitation and used for immunoprecipitation (10 μg/ml) and immunoblot analysis (1 μg/ml). Goat polyclonal anti-Rad53 sera (Santa Cruz Biotechnologies) was used for immunoprecipitation (1:500 dilution) and immunoblot analysis (1:1000 dilution). Secondary antibodies were: Cy3-conjugated anti-rabbit (2 μg/ml; Jackson ImmunoResearch, West Grove, PA), HRP-conjugated anti-rabbit and anti-mouse (1:10,000; Amersham, Arlington Heights, IL), HRP-conjugated anti-rat (1:1000; Jackson ImmunoResearch), and HRP-conjugated anti-goat (1:2000; Santa Cruz Biotechnologies).

Immunofluorescence: Log-phase yeast cultures were fixed with 5% formaldehyde for 1 hr and then sonicated briefly. Cells were washed twice with 0.1 M KPO_4 pH 7.5 and spheroplasted with zymolyase for 30 min at 37°. Spheroplasts were pelleted, resuspended in 0.1 M KPO_4 pH 7.5, and then adhered to slides coated with poly-L-lysine (Sigma, St. Louis). Spheroplasts were blocked with PBS containing 0.1% BSA for 45 min. Antibody incubations and DAPI staining were performed as described (Pringle *et al.* 1991).

Immunoprecipitation: Cell extracts from log-phase yeast cultures (OD_{600} 0.6–0.8) were prepared as described previously (Sharp *et al.* 2001), except that phosphatase inhibitors (Sigma phosphatase inhibitor cocktails I and II) were also included in the lysis buffer. Extracts were normalized for protein concentration and incubated with the appropriate antibody and Protein G sepharose beads (Amersham) at 4°. Beads were washed three times in the lysis buffer prior to elution in SDS-PAGE loading buffer containing 5% β-mercaptoethanol. Trichloroacetic acid (TCA)-precipitated extracts were prepared for immunoblot analysis as described (Marsolier *et al.* 2000).

Telomere length analysis: Ten micrograms of genomic DNA was digested with *Xho*I, separated on a 0.8% agarose gel, and hybridized overnight with a radioactive synthetic poly-d(GT) probe (Sigma). Standard hybridization conditions were used (Longhese *et al.* 2000). Blots were washed three times at 65° for 15 min in 1× SSC, 0.5% SDS prior to exposure to film.

Chromatin immunoprecipitation: Chromatin crosslinking and immunoprecipitation protocols were used as described (Meluh and Broach 1999; Sharp *et al.* 2002). The protein concentration of crosslinked chromatin lysates was measured using a detergent-compatible Bradford assay (Bio-Rad, Hercules, CA). All samples were then normalized for a protein concentration of 0.5 mg/ml in a 7.5-ml volume for each immunoprecipitation. PCR analysis was performed, using primers to amplify the regions: *ACT1*, 0.5 kb from telomere VI-R, 7.5 kb from telomere VI-R as described (Sharp *et al.* 2003).

RESULTS

Mec1 and Rad53 have opposite effects on silencing in *cac1*Δ cells: Both *MEC1* and *RAD53* have been implicated in the regulation of telomeric silencing (Craven and Petes 2000; Longhese *et al.* 2000). Because histone deposition proteins also contribute to silencing, we determined whether there is an epistatic relationship between these genes and CAF-1. We constructed yeast strains containing combinations of *CAC1*, *MEC1*, and *RAD53* gene deletions and a telomere-proximal *URA3* reporter gene on the left arm of chromosome VII to

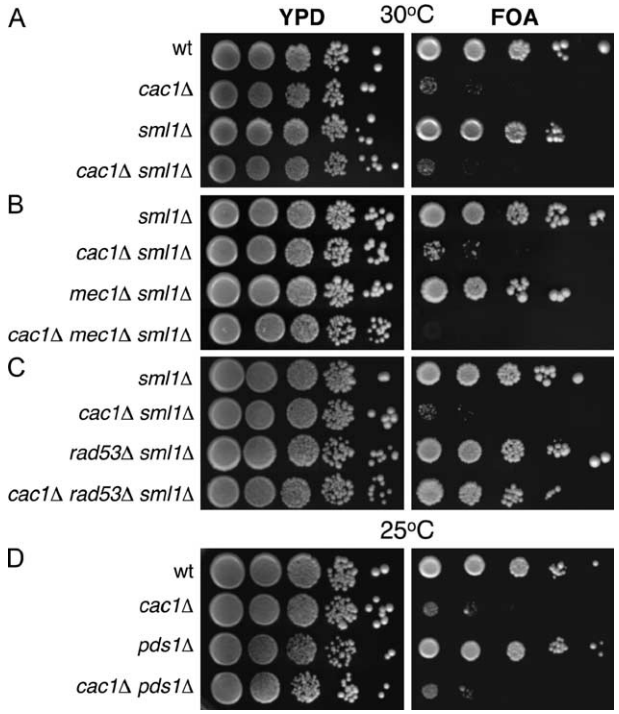

FIGURE 2.—Mec1 and Rad53 have opposing roles in regulating the strength of telomeric silencing. Log-phase cultures of the indicated genotypes were plated onto rich media (YPD) or synthetic media containing 5′-FOA. All strains contained *URA3*-VIIL (GOTTSCHLING *et al.* 1990). (A) Deletion of *SML1* has no effect on telomeric silencing. Strains were PKY090 (wild type), PKY638 (*cac1*Δ), PKY1766 (*sml1*Δ), and PKY1769 (*cac1*Δ *sml1*Δ). (B) Synergistic loss of telomeric silencing in *cac1*Δ *mec1*Δ double-mutant cells. Strains were PKY1766 (*sml1*Δ), PKY1769 (*cac1*Δ *sml1*Δ), PKY1768 (*mec1*Δ *sml1*Δ), and PKY1771 (*cac1*Δ *mec1*Δ *sml1*Δ). (C) Deletion of *RAD53* suppresses the telomeric silencing defect in *cac1*Δ cells. Strains were PKY2704 (*sml1*Δ), PKY2706 (*cac1*Δ *sml1*Δ), PKY2702 (*rad53*Δ *sml1*Δ), and PKY2710 (*cac1*Δ *rad53*Δ *sml1*Δ). (D) Deletion of *PDS1* has no effect on telomeric silencing. Strains were PKY090 (wild type), PKY638 (*cac1*Δ), PKY3611 (*pds1*Δ), and PKY3616 (*cac1*Δ *pds1*Δ).

assay chromatin-mediated telomeric transcriptional silencing (Figure 2A) (GOTTSCHLING *et al.* 1990). Serial dilutions of strains were plated on rich YPD media as an internal control for cell number and on media containing the uracil analog 5′-FOA, which is toxic to cells expressing *URA3* (BOEKE *et al.* 1987), to measure *URA3*-VIIL silencing. None of the genetic combinations tested proved to be sensitive to FOA in the absence of the *URA3*-VIIL reporter gene, ruling out the possibility that the strains were intrinsically sensitive to this compound (data not shown).

Consistent with previous findings, both wild-type and *cac1*Δ cells grew equivalently on rich YPD media, confirming that CAF-1 is not required for viability under normal growth conditions and that equivalent numbers of cells were present in the different strains analyzed (Figure 2A) (ENOMOTO *et al.* 1997; KAUFMAN *et al.* 1997). As expected, the growth of *cac1*Δ mutant cells on 5′-FOA media was markedly less efficient than that of wild-type cells, reflecting defective silencing of the *URA3* reporter gene in the absence of CAF-1 activity (ENOMOTO *et al.* 1997; KAUFMAN *et al.* 1997; MONSON *et al.* 1997). We also analyzed effects of deleting the *SML1* gene, which encodes a ribonucleotide reductase inhibitor, because this gene must be deleted to maintain viability of cells lacking the *MEC1* or *RAD53* genes (ZHAO *et al.* 1998). Deletion of *SML1* had no significant effects on growth or telomeric silencing in *cac1*Δ cells (Figure 2A).

Although semidominant alleles of *MEC1* can cause telomeric silencing defects, deletion of *MEC1* resulted in levels of silencing comparable to that of wild-type cells (Figure 2B), as has been previously reported (CRAVEN and PETES 2000; LONGHESE *et al.* 2000). In contrast, *cac1*Δ *mec1*Δ double-mutant cells failed to grow on 5′-FOA media, indicating that *cac1*Δ *mec1*Δ cells are extremely defective for telomeric silencing (Figure 2B). These data suggest that the DNA damage checkpoint machinery is essential for residual telomeric silencing when CAF-1 is absent. To test this idea, we determined whether a *rad53*Δ gene deletion also caused a synergistic loss of silencing when combined with *cac1*Δ. However, in contrast to *cac1*Δ *mec1*Δ cells, *cac1*Δ *rad53*Δ cells displayed levels of telomeric silencing nearly as robust as that observed for wild-type cells (Figure 2C). Therefore, although the Mec1 and Rad53 kinases cooperate in the same signaling pathway in response to DNA damage, these proteins have opposing effects on the strength of telomeric silencing in cells lacking CAF-1.

We sought to determine whether any perturbation of the DNA damage checkpoint pathway would affect silencing in *cac1*Δ cells. To test this, we examined an important downstream effector of the DNA damage checkpoint pathway, the securin protein Pds1 that holds sister chromatids together and that therefore must be proteolyzed prior to chromosome segregation during mitosis (Figure 1) (COHEN-FIX *et al.* 1996). Pds1 is phosphorylated and protected from proteolysis upon activation of the checkpoint, serving to prevent mitosis in the presence of DNA damage (COHEN-FIX and KOSHLAND 1997; GARDNER *et al.* 1999; SANCHEZ *et al.* 1999; TINKER-KULBERG and MORGAN 1999; CLARKE *et al.* 2001; WANG *et al.* 2001). We found that deletion of the *PDS1* gene had no effect on telomeric silencing and did not significantly alter silencing in *cac1*Δ cells (Figure 2D). These results implicate the Mec1 and Rad53 checkpoint kinases, but not all checkpoint pathway components, in chromatin-mediated gene silencing.

Because Mec1 and Rad53 had opposing effects on silencing, we tested their epistatic relationship regarding this phenotype by examining all possible combinations of deletions of the *CAC1*, *RAD53*, and *MEC1* genes (Figure 3A). As expected, deletion of both *RAD53* and *MEC1* had no effect on silencing when the *CAC1* gene was intact. We also observed that *cac1*Δ *mec1*Δ *rad53*Δ triple-mutant cells displayed nearly wild-type levels of

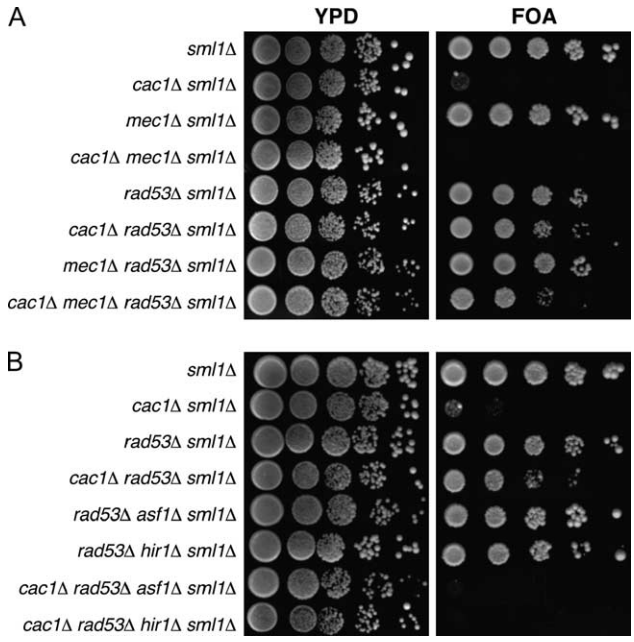

FIGURE 3.—Rad53 modulates telomeric silencing strength in an Asf1- and Hir1-dependent manner. Log-phase cultures of the indicated genotypes were plated onto rich media (YPD) or synthetic media containing 5′-FOA. (A) Deletion of *RAD53* reverses the synergistic telomeric silencing defect of *cac1*Δ *mec1*Δ cells. Strains were PKY1766 (*sml1*Δ), PKY1769 (*cac1*Δ *sml1*Δ), PKY1768 (*mec1*Δ *sml1*Δ), PKY1771 (*cac1*Δ *mec1*Δ *sml1*Δ), PKY2702 (*rad53*Δ *sml1*Δ), PKY2710 (*cac1*Δ *rad53*Δ *sml1*Δ), PKY2776 (*mec1*Δ *rad53*Δ *sml1*Δ), and PKY2779 (*cac1*Δ *mec1*Δ *rad53*Δ *sml1*Δ). (B) Deletion of *RAD53* failed to suppress the *cac1*Δ telomeric silencing defect in strains that lacked either *ASF1* or *HIR1* genes. Strains were PKY2704 (*sml1*Δ), PKY2706 (*cac1*Δ *sml1*Δ), PKY2702 (*rad53*Δ *sml1*Δ), PKY2710 (*cac1*Δ *rad53*Δ *sml1*Δ), PKY2758 (*asf1*Δ *rad53*Δ *sml1*Δ), PKY3564 (*rad53*Δ *hir1*Δ *sml1*Δ), PKY2763 (*cac1*Δ *rad53*Δ *asf1*Δ *sml1*Δ), and PKY3566 (*cac1*Δ *rad53*Δ *hir1*Δ *sml1*Δ). Consistent with previously published data, strains PKY3045 (*asf1*Δ *sml1*Δ) and PKY3676 (*hir1*Δ *sml1*Δ) exhibited wild-type levels of telomeric silencing, whereas strains PKY2755 (*cac1*Δ *asf1*Δ *sml1*Δ) and PKY3680 (*cac1*Δ *hir1*Δ *sml1*Δ) showed no growth on 5′-FOA media (data not shown) (KAUFMAN *et al.* 1998; TYLER *et al.* 1999).

telomeric silencing, as had been observed in *cac1*Δ *rad53*Δ cells. Therefore, deletion of *RAD53* suppresses the severe silencing defect observed in *cac1*Δ *mec1*Δ cells. Together, these data suggest that Mec1 and Rad53 have opposite roles in regulating a factor that contributes to residual telomeric silencing when CAF-1 function is absent.

Restoration of silencing in *cac1*Δ *rad53*Δ cells requires Asf1 and Hir1: Rad53 binds Asf1 in a manner regulated by the DNA damage checkpoint (EMILI *et al.* 2001; HU *et al.* 2001; SCHWARTZ *et al.* 2003). Our silencing data supported the idea that Asf1 is sequestered by Rad53, but can be released by active Mec1 during a normal cell cycle (Figure 10). In this case, the suppression of silencing defects by deletion of *RAD53* would depend on Asf1. We therefore tested the effects of an *asf1*Δ deletion on silencing phenotypes. Previous data

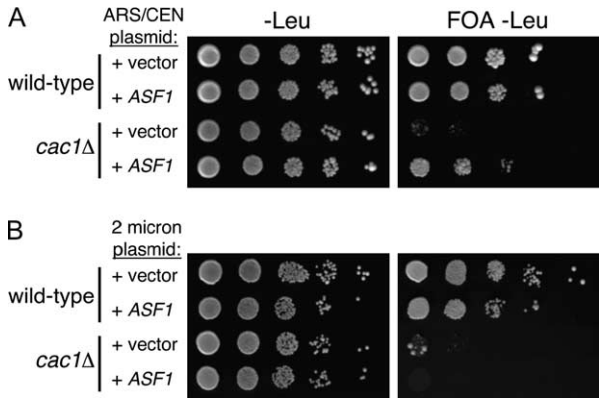

FIGURE 4.—Gene dosage-dependent effects of ASF1 on telomeric silencing. TPE assays: (A) Suppression of the telomeric silencing defect in *cac1*Δ cells by low-level overexpression of *ASF1*. Wild-type (PKY090) and *cac1*Δ (PKY638) strains isogenic for *URA3*-VIIL were transformed with empty vector (pRS415; ARS-CEN-*LEU2* plasmid) or a plasmid containing the *ASF1* gene (pPK196; ARS-CEN-*LEU2-ASF1*). Transformants were plated onto synthetic media lacking leucine (−Leu) with and without 5′-FOA. (B) 2μ-level *ASF1* overexpression disrupts silencing in *cac1*Δ cells. Wild-type and *cac1*Δ strains were transformed with empty vector (YEP35l; 2μ-*LEU2* plasmid) or a 2μ-plasmid containing the *ASF1* gene. (pPK197; 2μ-*LEU2-ASF1*). Transformants were plated onto synthetic media lacking leucine (−Leu) with and without 5′-FOA.

showed that deletion of *ASF1* alone has little effect on telomeric silencing, but exacerbates silencing defects in *cac1*Δ cells (TYLER *et al.* 1999; SHARP *et al.* 2001). We observed no silencing defects in *rad53*Δ *asf1*Δ double-mutant cells (Figure 3B), but *cac1*Δ *rad53*Δ *asf1*Δ cells displayed a strong loss of telomeric silencing. We conclude that Asf1 is required for the suppression of silencing defects by a *rad53*Δ deletion.

The contribution of Asf1 to chromatin-mediated gene silencing requires the Hir proteins, in a manner dependent on the Asf1-Hir protein interaction (SHARP *et al.* 2001; SUTTON *et al.* 2001; DAGANZO *et al.* 2003). We therefore predicted that the restoration of silencing to *cac1*Δ cells caused by deletion of *RAD53* would also require Hir proteins. We observed that *cac1*Δ *rad53*Δ *hir1*Δ cells displayed a strong loss of telomeric silencing, similar to the defect in *cac1*Δ *rad53*Δ *asf1*Δ cells (Figure 3B). These data demonstrate that both the Asf1 and Hir1 histone deposition proteins are required for the suppression of silencing defects by *rad53*Δ.

To test the prediction that the concentration of active Asf1 protein affects the efficiency of telomeric silencing in *cac1*Δ cells, we deliberately increased the dosage of the *ASF1* gene using a low-copy centromeric plasmid and determined the effect on silencing. We observed that this modest increase in *ASF1* gene dosage partially suppressed the silencing defect of *cac1*Δ cells (Figure 4A). However, we also observed that a much larger increase in gene dosage provided by a high-copy 2-μm vector impaired silencing in both wild-type and *cac1*Δ cells (Figure 4B). These latter observations are consistent with the isolation

of the *ASF1* gene as a dominant disruptor of silencing upon high levels of overexpression (Le *et al.* 1997; Singer *et al.* 1998) and are thought to result from extensive sequestration of histones by Asf1. We conclude that the levels of active Asf1 are critical for silencing.

Mec1 and Rad53 do not affect telomere length, localization, expression levels, or chromatin association of Asf1: To understand how Mec1 and Rad53 might affect Asf1 function, we tested whether the cellular localization of Asf1 protein was altered in *mec1*Δ and *rad53*Δ mutant cells. We also examined *cac1*Δ *mec1*Δ and *cac1*Δ *rad53*Δ strains that display opposite silencing efficiencies. All genotypes analyzed displayed proper nuclear localization of Asf1 as judged by overlap with DAPI staining (Figure 5A), except in the *asf1*Δ negative control cells that demonstrate the specificity of the antisera.

We then considered the possibility that the cellular levels of Asf1 protein might be altered in *mec1*Δ and *rad53*Δ mutant cells. However, except in the *asf1*Δ negative control, immunoblot analysis of whole-cell extracts from all genotypes tested failed to detect different amounts of Asf1 relative to tubulin, which served as an internal loading control (Figure 5B). To test for changes in chromatin association of Asf1, we examined the relative abundance of the chromatin-associated pool of nuclear Asf1, using chromatin immunoprecipitation (ChIP; Figure 5C). In this assay, Asf1 associated with all loci with equal efficiency in all strains, regardless of the genotypic status of *RAD53*, *MEC1*, or *CAC1* (Figure 5C). For example, the amount of Asf1 associated with the euchromatic locus *ACT1* as well as with two sequences proximal to the telomere of chromosome VI was approximately equal among the different strains tested. Similar conclusions have been made using a different chromatin immunoprecipitation protocol (Franco *et al.* 2005). We also note that our attempts to study the chromatin association of Asf1 in noncrosslinked samples (Donovan *et al.* 1997; Liang and Stillman 1997) have been inconclusive because Asf1 is loosely associated with chromatin and can be released by washing without nuclease treatment (data not shown).

Previous experiments have shown that telomere shortening is associated with reduced telomeric silencing (Kyrion *et al.* 1992). Because some alleles of *MEC1* and *RAD53* have been implicated in telomere length maintenance (Longhese *et al.* 2000), we determined whether the observed telomeric silencing phenotypes in *cac1*Δ *rad53*Δ and *cac1*Δ *mec1*Δ cells correlated with alterations in telomere length. Telomeric DNA was analyzed by Southern blot hybridization with a synthetic poly(dGT) probe, which hybridizes to the yeast telomeric repeats (Figure 5D). The telomere lengths of all strains analyzed were comparable to that of the wild-type strain, demonstrating that altered telomeric silencing in *cac1*Δ *rad53*Δ and *cac1*Δ *mec1*Δ cells does not result from telomere length changes. Together, our data indicate that Mec1 and Rad53 regulate the silencing function of Asf1/Hir proteins, but not through modulation of Asf1 protein levels, nuclear localization, chromatin association, or changes in telomere length.

Effects of histone gene dosage and expression: As highly basic proteins, histones pose a problem for cells when not in their nucleosomal form, because they can interact strongly with anionic molecules without biological specificity. To cope with this problem, chaperone proteins regulate the pool of nascent histones. In yeast, an Asf1-independent role for Rad53 in regulating histone levels *in vivo* has been described (Gunjan and Verreault 2003). Specifically, *rad53*Δ cells are sensitive to elevated histone gene dosage, and the growth defects and DNA damage sensitivity of *rad53*Δ cells are ameliorated by reduction of histone gene copy number. Because these phenotypes could contribute to the restoration of silencing that we had observed in *cac1*Δ *rad53*Δ cells, we tested the generality of the published findings. First, we tested whether the observed sensitivity of *rad53*Δ *sml1*Δ cells to elevated histone H3 gene expression was affected by the *sml1*Δ gene deletion required to maintain the viability of *rad53*Δ cells. We observed that *sml1*Δ and *rad53*Δ *sml1*Δ cells displayed similar sensitivity to overexpression of histone H3 (Figure 6A), indicating that the sensitivity of *rad53*Δ *sml1*Δ cells to histone H3 overexpression is at least partially due to the absence of Sml1. Because the *sml1*Δ deletion does not alter our silencing assay (Figure 2), and because the silencing phenotypes depended on the Asf1 and Hir1 proteins (Figures 3 and 8), our data suggest that Rad53's primary role in silencing is directly related to Asf1/Hir protein activity.

Second, in human cells the Asf1-histone interaction itself appears to be a major target of regulation governing chromatin assembly activity (Groth *et al.* 2005). Although not detected previously (Gunjan and Verreault 2003), we observed that *asf1*Δ cells indeed were sensitive to overexpression of core histones (Figure 6B) or to reduction of histone gene dosage (Figure 6C). These data support the idea that Asf1 is a significant mediator of nascent histone interactions in yeast, consistent with its roles described in other eukaryotes. Furthermore, these data demonstrate that cellular resistance to histone overexpression involves multiple proteins in addition to Rad53.

The FHA domains of Rad53 affect Asf1 activity *in vivo*: To test whether the Rad53-Asf1 interaction is critical for regulating the silencing activity of Asf1, we sought to specifically perturb this interaction and determine the effects on silencing. Prominent Asf1 binding sites on Rad53 have been mapped to the Rad53 FHA domains (Schwartz *et al.* 2003). FHA domains are phosphopeptide recognition motifs (Sun *et al.* 1998; Durocher *et al.* 1999; Schwartz *et al.* 2002), and Rad53 is unusual among Chk2 kinase family member in having two such motifs (Figure 7A). Coprecipitation

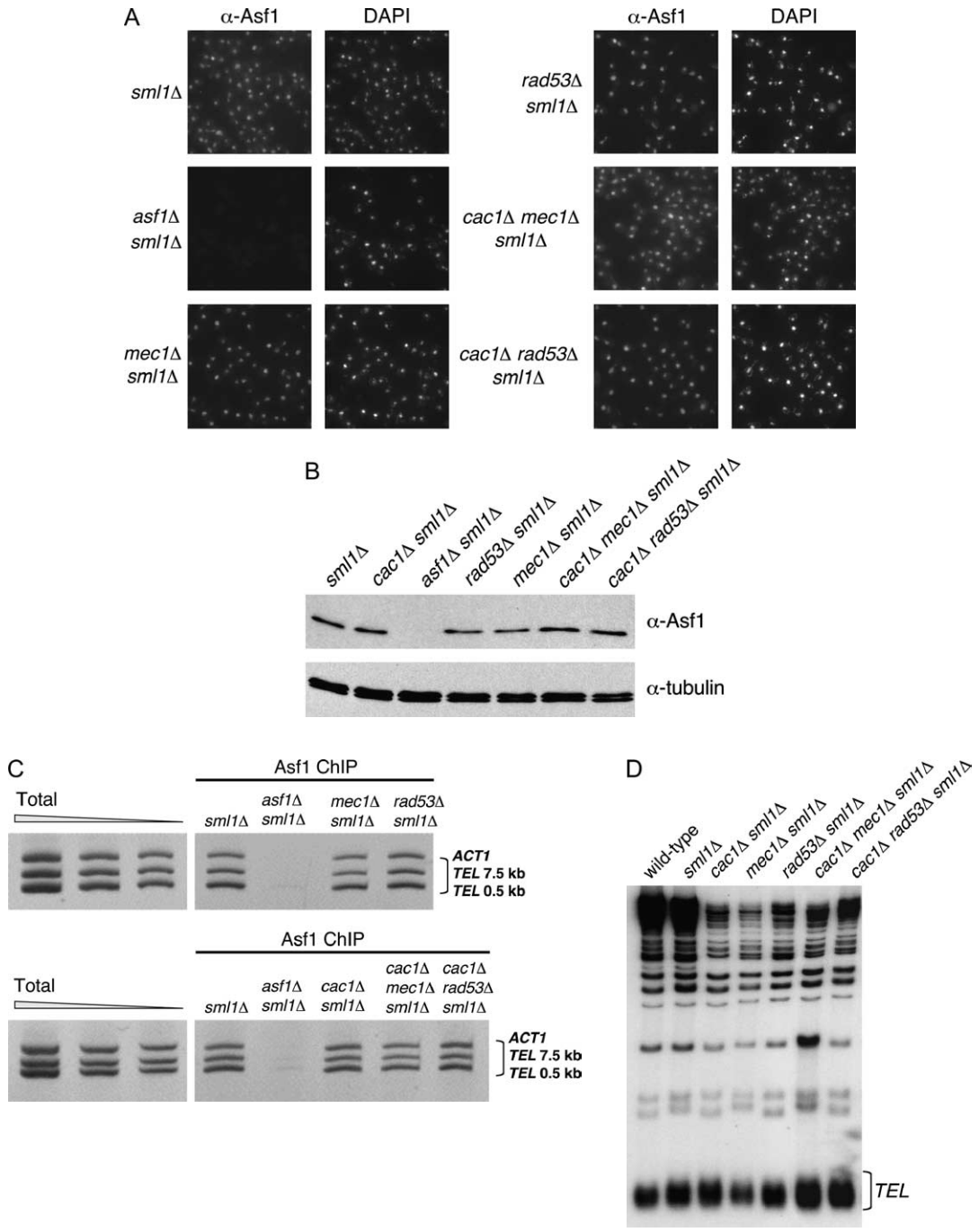

FIGURE 5.—Ablation of *MEC1* or *RAD53* gene function does not interfere with Asf1 nuclear localization, expression, or chromatin association and does not cause gross changes in average telomere length. (A) Asf1 immunofluorescence. Strains of the indicated genotypes were spheroplasted briefly and incubated with a rabbit polyclonal antibody raised against the conserved core of Asf1 (DAGANZO *et al.* 2003). A Cy3-conjugated anti-rabbit antibody was used for secondary detection. Cells were incubated with DAPI to detect nuclear staining. (B) Immunoblot analysis of Asf1 protein levels. Crude cell extracts prepared from strains of the indicated genotypes were normalized for total protein and resolved on a 10% SDS-PAGE gel. After transfer of proteins to nitrocellulose membrane, the blot was probed with rabbit anti-Asf1 and rat anti-tubulin. (C) Chromatin immunoprecipitation of Asf1. Crosslinked chromatin was immunoprecipitated with rabbit anti-Asf1 sera. PCR analysis of ChIP eluates was performed with oligonucleotides specific for *ACT1*, 0.5 kb from telomere VI-R and 7.5 kb from telomere VI-R. Total chromatin was titrated to determine the linear range of the PCR; the 1:64, 1:128, and 1:256 dilutions that fall within this range are shown. For immunoprecipitations, one-fiftieth of the eluates were used for PCR analysis. (D) DNA blot analysis of telomere length. Average telomere length is indicated by the bracket. Strains in A–D were: wild type (PKY090), *sml1*Δ (PKY2503), cac1Δ *sml1*Δ (PKY1769), *asf1*Δ *sml1*Δ (PKY3045), *mec1*Δ *sml1*Δ (PKY2719), *rad53*Δ *sml1*Δ (PKY2702), *cac1*Δ *mec1*Δ *sml1*Δ (PKY2723), and *cac1*Δ *rad53*Δ (PKY2711).

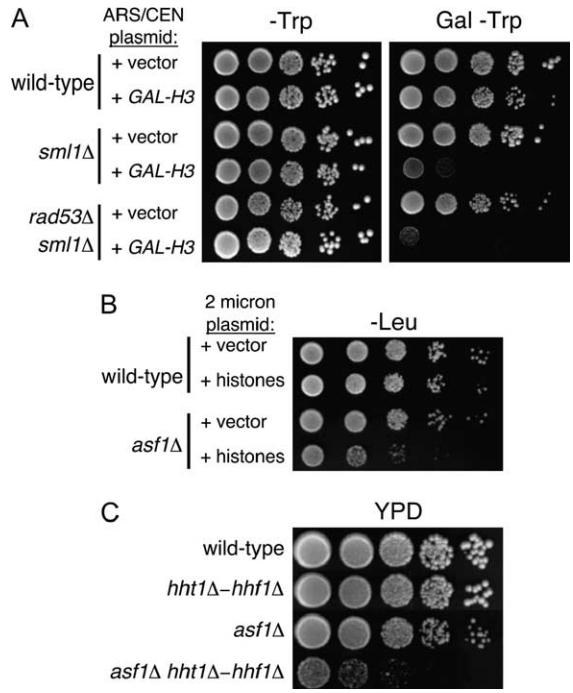

FIGURE 6.—Histone gene dosage phenotypes. (A) Sensitivity of *sml1*Δ cells to histone overexpression. pMS383 (ARS-CEN-*TRP1*-GAL-*HHT1*) and pRS414 (ARS-CEN-*TRP1*) were transformed into wild-type (PKY090), *sml1*Δ (PKY2503), and *rad53*Δ *sml1*Δ (PKY2703) strains. Cells were grown to log phase and plated onto synthetic glucose media lacking tryptophan (−Trp) or synthetic galactose media lacking tryptophan (Gal − Trp) to induce overexpression of histone H3. Plates were incubated at 30°. (B) *asf1*Δ cells are sensitive to increased histone gene dosage. Wild-type (PKY090) and *asf1*Δ (PKY993) cells were transformed with either a 2μ-plasmid containing all four histone genes (+histones, pPK128) or an empty 2μ-plasmid (+vector, yEP351). Transformants were then grown to log phase, and 10-fold serial dilutions were plated onto media lacking leucine (−Leu) and incubated at 30°. (C) *asf1*Δ cells are sensitive to reduced histone gene dosage. Cells lacking both Asf1 and one of the two gene pairs encoding histones H3 and H4 (*HHT1-HHF1*) grow slowly. Log-phase cultures of the indicated genotypes were plated onto rich media (YPD) and incubated at 30°. Strains were PKY090 (wild type), PKY408 (*hht1-hhf1*Δ), PKY993 (*asf1*Δ), and PKY1027 (*asf1*Δ *hht1-hhf1*Δ).

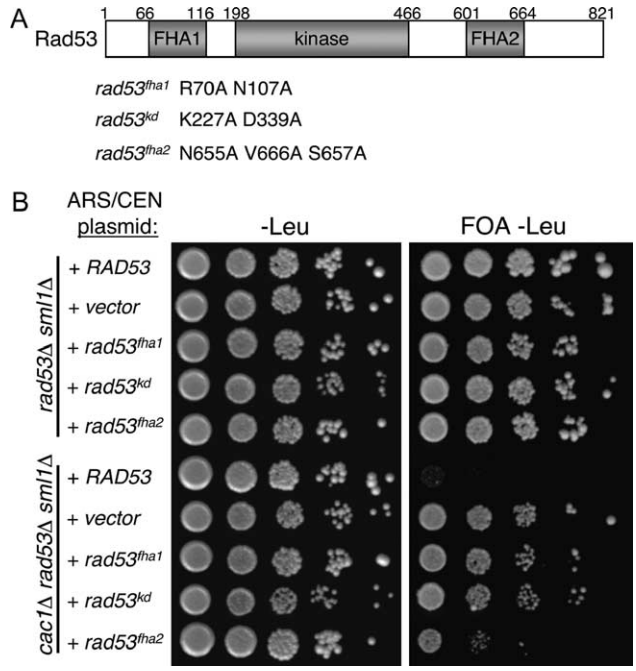

FIGURE 7.—Alleles of Rad53 defective for Asf1 binding cause elevated TPE in *cac1*Δ cells. (A) Schematic of protein domain structure of Rad53. Numbers indicate amino acid position in the Rad53 protein. Shown below the schematic are mutations resulting in impaired function of the FHA1, kinase, and FHA2 domains (after SCHWARTZ *et al.* 2003). (B) Telomeric silencing assay of *RAD53* alleles. ARS/CEN plasmids containing the indicated *RAD53* alleles were transformed into PKY2703 (*rad53*Δ, *sml1*Δ, *URA3*-VIIL) and PKY3748 (*cac1*Δ, *rad53*Δ, *sml1*Δ, *URA3*-VIIL). Transformants were grown to log phase, serially diluted, and plated onto synthetic media lacking leucine (−Leu) as well as −Leu plates containing 5′-FOA.

experiments using anti-Rad53 sera or recombinant GST-Rad53 FHA domain fusion proteins have shown that a high-affinity Asf1 binding site resides in the FHA1 domain (SCHWARTZ *et al.* 2003). Additionally, weak but detectable Asf1 binding was observed for the FHA2 domain. Furthermore, the kinase activity of Rad53 is important for formation of a stable Rad53-Asf1 interaction *in vivo*, because the catalytically inactive *rad53-kd* allele strongly reduces coprecipitation (SCHWARTZ *et al.* 2003).

We hypothesized that mutation of the FHA domains or active site residues would affect Rad53-mediated regulation of Asf1 *in vivo*. In the telomeric silencing assay, we tested low-copy plasmid-borne *RAD53* alleles, including clustered point mutations in FHA1 (R70A N107A), FHA2 (N665A V666A S657A), and a catalytically inactive version (K227A D339A). All Rad53 proteins tested are expressed at levels comparable to that of the wild-type protein (SCHWARTZ *et al.* 2003). We first demonstrated that none of these *rad53* alleles had dominant effects on silencing in *CAC1* cells (Figure 7B, top). In *cac1*Δ *rad53*Δ cells, we observed that the plasmid-borne wild-type *RAD53* allele fully complemented the chromosomal *rad53*Δ deletion, generating cells with poor telomeric silencing because of the lack of CAF-1. As expected, an empty vector in the *cac1*Δ *rad53*Δ cells resulted in efficient silencing, indicating suppression of the *cac1*Δ silencing defect by the absence of Rad53. Notably, the silencing phenotypes of the mutant *rad53* alleles correlated with their Asf1-binding properties (Figure 7B) (SCHWARTZ *et al.* 2003). Both the *rad53*^{fha1} and the *rad53*^{kd} alleles strongly restored silencing to the *cac1*Δ cells, consistent with poor binding of Asf1 by these Rad53 mutants. The *rad53*^{fha2} allele only modestly suppressed silencing, consistent with the weaker Asf1 binding by the FHA2 domain. We conclude that Rad53 affects silencing via the strength of its interaction with Asf1.

The Dun1 kinase also affects silencing in *cac1*Δ cells: To further characterize how checkpoint proteins affect

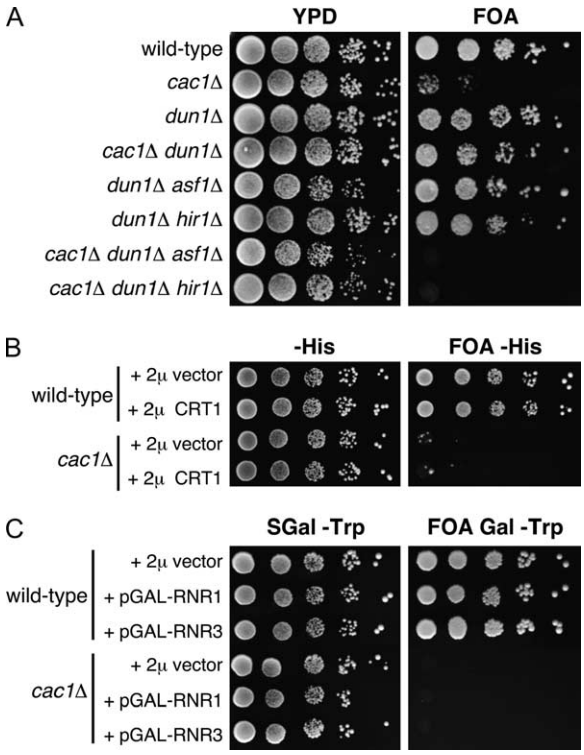

FIGURE 8.—Dun1 modulates telomeric silencing strength in an Asf1- and Hir1-dependent manner. (A) *dun1*Δ suppresses the *cac1*Δ telomeric silencing defect, but only when *ASF1* and *HIR1* genes are intact. Strains were (top to bottom): PKY090, PKY638, PKY3583, PKY3584, PKY3586, PKY3591, PKY3588, and PKY3592. (B) Overexpression of the *CRT1* transcriptional repressor has no effect on telomeric silencing. Wild-type (PKY090) and *cac1*Δ strains (PKY638) were transformed with pRS423 (2μ-*HIS3* vector) or pMH151-7 (2μ-*HIS3*-*CRT1*). Transformants were plated onto histidine-deficient synthetic media with and without 5′-FOA. (C) Overexpression of *RNR* genes has no effect on telomeric silencing. Wild-type and *cac1*Δ strains containing pBAD54 (2μ-*TRP1* plasmid + galactose-inducible GAP promoter), pBAD70 (2μ-*TRP1* + GAP-*RNR1*), or pBAD79 (2μ-*TRP1* + GAP-*RNR3*) were plated on tryptophan-deficient synthetic media (with and without 5′-FOA) containing galactose to induce overexpression. Strains were PKY2256, PKY2259, PKY2262, PKY2258, PKY2261, and PKY2264.

Asf1 function, we examined Dun1, a direct downstream substrate and effector of Rad53 kinase signaling. Dun1 is a serine/threonine kinase that coordinates the transcriptional response to DNA damage in part through phosphorylation-mediated inhibition of the Crt1 repressor (ZHOU and ELLEDGE 1993; HUANG *et al.* 1998).

To test whether checkpoint genes downstream of Rad53 affect Asf1 function, we assayed the effect of a *dun1*Δ gene deletion on silencing (Figure 8A). As observed for *rad53*Δ, a *dun1*Δ deletion had little effect on silencing in wild-type cells or in cells lacking Asf1 or Hir1. In contrast, a *dun1*Δ deletion substantially suppressed the silencing defects in *cac1*Δ cells. Furthermore, the suppression of silencing defects by the *dun1*Δ deletion required the presence of Asf1 and Hir1. We conclude that both Rad53 and Dun1 affect the silencing activity of the Asf1/Hir protein complex.

To understand if the role of Dun1 in regulating transcription was involved in modulating silencing, we tested whether overexpression of the *CRT1* repressor improved silencing in *cac1*Δ cells. Overexpression of *CRT1* mimics a *dun1*Δ mutation, in that some *RNR* genes become uninducible in the presence of DNA damage (HUANG *et al.* 1998). In contrast to the *dun1*Δ mutation, we observed that 2μ-based high-copy-number overexpression of *CRT1* had no effect on silencing in wild-type or *cac1*Δ cells (Figure 8B). Conversely, we also examined whether deliberate overexpression of damage-inducible genes could affect silencing. However, galactose-mediated overexpression of the ribonucleotide reductase subunits Rnr1 or Rnr3 had no effect on silencing (Figure 8C), consistent with the fact that deletion of the Rnr inhibitor Sml1 also does not affect silencing (Figure 1) (LONGHESE *et al.* 2000). Therefore, we propose that Dun1 itself, but not damage-inducible transcription *per se*, affects Asf1 silencing activity.

One possibility raised by these data was that Dun1 directly interacted with Asf1. However, co-immunoprecipitation experiments using cell extracts or a recombinant GST-Asf1 fusion protein and *in vitro*-translated Dun1 detected no direct interaction between these proteins (data not shown). We therefore tested the idea that Dun1 affected the Asf1-Rad53 interaction by examining the amount of Rad53 coprecipitated from cell extracts with an epitope-tagged Asf1-HA fusion protein. Consistent with previous results (EMILI *et al.* 2001; HU *et al.* 2001), we observed coprecipitation of Rad53 with Asf1-HA in an epitope-dependent manner in wild-type cells (Figure 9A). As expected, the Asf1-Rad53 interaction was abolished upon addition of HU, which activates the DNA replication checkpoint by depleting cellular dNTP pools. Notably, in *dun1*Δ cells, the amount of Rad53 stably associated with Asf1 was reduced ~2.5-fold (Figure 9, A and B). These data suggested that a *dun1*Δ deletion suppresses silencing defects in *cac1*Δ cells by releasing a subset of Asf1 from Rad53.

Because Asf1 release occurs concomitantly with Rad53 phosphorylation, we examined the phosphorylation status of Rad53 in *dun1*Δ cells. Rad53 becomes heavily phosphorylated after checkpoint activation (SANCHEZ *et al.* 1996; SUN *et al.* 1996), as can be detected by a mobility shift on protein gels. In wild-type cells, immunoprecipitated Rad53 was detected with a single mobility, whereas treatment with HU produced the expected widening and reduced mobility of the band, reflecting checkpoint-mediated phosphorylation (Figure 9C, left). In *dun1*Δ cells, a subset of Rad53 molecules became slower migrating, consistent with previous observations (MARSOLIER *et al.* 2000). Similar results were observed upon examination of Rad53 in TCA-precipitated cell extracts (Figure 9C, right). This latter analysis also demonstrated that the reduced levels of Rad53-Asf1

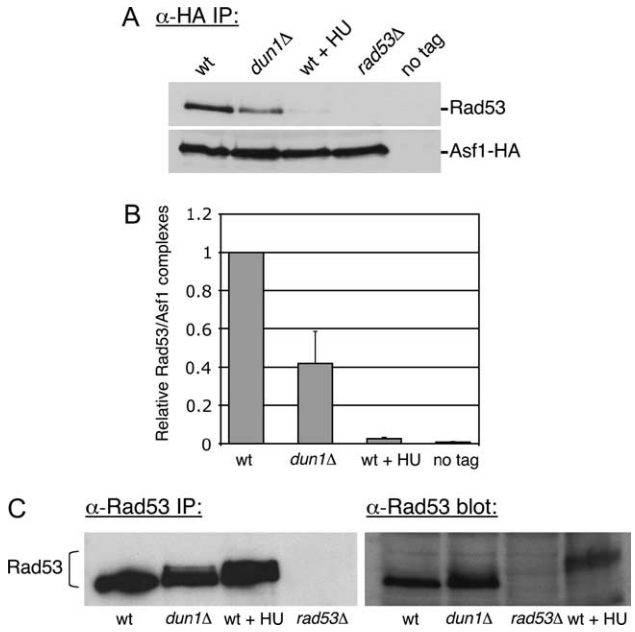

FIGURE 9.—Asf1/Rad53 complexes are less abundant in *dun1*Δ mutant cells. (A) Immunoblot analysis of Asf1-HA immunoprecipitates. Cell extracts prepared from *ASF1-HA* (PKY2735), *dun1*Δ *ASF1-HA* (PKY3607), *ASF1-HA* + 0.2 M HU (PKY2735), *rad53*Δ *sml1*Δ *ASF1-HA* (PKY2747), and *ASF1* (PKY090) strains were subjected to immunoprecipitation with an anti-HA antibody. Eluates were probed with Rad53 and HA antibodies to compare relative recovery of Asf1-HA/Rad53 complexes. (B) Quantitation of Rad53 coprecipitation with Asf1-HA from wild-type and *dun1*Δ cell extracts. The efficiency of Rad53 coprecipitation with Asf1-HA was measured from three independent experiments using Quantity One software (Bio-Rad). In each experiment, recovery of Rad53 from the wild-type extract was normalized to 1.0. Average recovery of Rad53 and the standard deviation for each genotype are plotted on the graph. (C) Immunoblot analysis of phosphorylated Rad53: (Left) Cell extracts from the same strains as in A were subjected to immunoprecipitation with an anti-Rad53 antibody. Eluates were analyzed on a 7.5% SDS-PAGE gel to detect slower-migrating forms of Rad53. (Right) TCA-precipitated cell extracts from the same strains were analyzed in parallel.

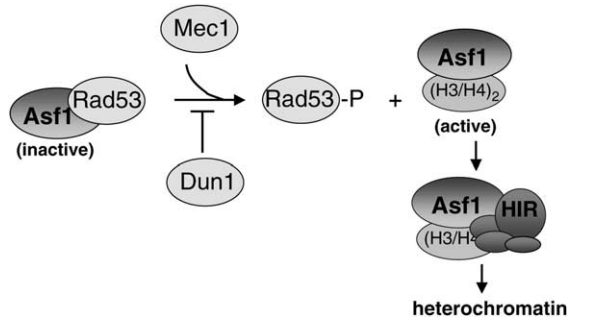

FIGURE 10.—Model for the regulation of Asf1/HIR complex heterochromatin function. In the absence of DNA damage, Mec1 and Dun1 have opposing roles in balancing the cellular concentration of active Asf1. Mec1 promotes the dissociation of the Rad53-Asf1 complex, whereas Dun1 blocks ectopic activation of Rad53. In the proposed model, Hir protein association with Asf1 requires dissociation of the Rad53-Asf1 complex. As a result, Mec1 and Dun1 would influence the levels of Asf1-Hir complex formation that is critical for heterochromatin-mediated gene silencing.

interaction in *dun1*Δ cells cannot be attributed to reduced Rad53 protein levels. Together, these data support the idea that deletion of *DUN1* increases the concentration of active Asf1 by increasing the steady-state level of phosphorylated Rad53.

DISCUSSION

Mec1 and Dun1 regulate silencing via Asf1: Multiple lines of evidence presented here indicate that DNA damage checkpoint kinases regulate the ability of Asf1 to contribute to chromatin-medicated telomeric gene silencing. A modest increase of *ASF1* gene dosage causes more robust telomeric silencing in *cac1*Δ mutant cells, as does disruption of the Asf1 inhibitor Rad53. Further, both Mec1 and Asf1 are required for the residual levels of telomeric silencing in *cac1*Δ mutant cells, suggesting that Mec1 promotes silencing by Asf1. We propose that even in the absence of exogenous DNA damage, Mec1 facilitates the release of Asf1 from Rad53, thereby freeing Asf1 to participate in chromatin-mediated gene silencing (Figure 10). Mec1's activity in dissociating Asf1 from Rad53 could occur transiently during a normal S phase and could modulate the local concentration of Rad53/Asf1 complexes in a locus-specific manner.

Like Rad53, the absence of Dun1 restores silencing to cells lacking CAF-1 in a manner that requires the Asf1/Hir1 proteins (Figure 8). Furthermore, the absence of Dun1 results in constitutive phosphorylation of Rad53 (MARSOLIER *et al.* 2000) and a reduction in the steady-state level of association between Rad53 and Asf1 (Figure 8). Thus, the Dun1 kinase functions to restrict Rad53 phosphorylation and maintain its association with Asf1, thereby limiting the free pool of Asf1 (Figure 10).

To understand how Dun1 restricts Rad53 phosphorylation, we pursued several hypotheses. First, we detected no direct interaction between Dun1 and Asf1, thereby excluding the possibility that Dun1 participated in a ternary complex with Rad53-Asf1. Next, we attempted to distinguish whether the effect of Dun1 occurred via its role in transcriptional control or instead via feedback control on Rad53 activity. Overexpression of Crt1, which mimics loss of Dun1 signaling, had no effect on silencing. We also tested whether Dun1 was exerting feedback control onto Rad53 through the Ptc2/Ptc3 protein phosphatases that act to turn off phosphorylation-mediated checkpoint signaling (LEROY *et al.* 2003). However, deletion of either Ptc2 or Ptc3, or both together, had no effect on the silencing function of Asf1 (data not shown). Finally, another possibility is that *dun1*Δ cells experience constitutive DNA damage in a manner resulting in Rad53 phosphorylation. However,

we do not favor this explanation for the restoration of silencing in *dun1*Δ *cac1*Δ cells, because deliberate treatment of cells with DNA damaging agents impairs, not strengthens, telomeric silencing due to loss of silencing proteins from telomeres under these conditions (Martin *et al.* 1999; Mills *et al.* 1999). Furthermore, our data suggest that any DNA damage induced by low dNTP levels is unable to significantly affect silencing, because Crt1 overexpression, which represses Rnr gene transcription, had no effect on silencing (Figure 8B). Future studies will be required to determine what proteins are required for the effects of Dun1 on the Rad53-Asf1 interaction.

The Asf1-Hir1 silencing complex: The highly conserved histone-binding protein Asf1 has multiple protein partners in addition to the histones. Previous data demonstrated that Asf1 is sequestered from histones by Rad53 in a manner relieved by HU treatment (Emili *et al.* 2001; Hu *et al.* 2001). Here, we demonstrate for the first time that regulation of Asf1 and Hir1 by checkpoint kinases is critical for their silencing function *in vivo*, even in undamaged cells. Both Hir1 and Asf1 were required for Rad53- and Dun1-mediated effects on silencing, reinforcing the data that these histone deposition proteins act together to build heterochromatin (Figures 3 and 8) (Sharp *et al.* 2001; Daganzo *et al.* 2003; Zhang *et al.* 2005). We propose that Rad53 and the Hir proteins may be in separate complexes with Asf1 (Figure 10), with the former representing an inactive, sequestered form, and the latter representing an active species. Subtle fluctuations in the cellular concentration of Rad53-Asf1 complexes would thereby result in reciprocal changes in Asf1-Hir complexes, altering the histone deposition activity of Asf1. This hypothesis is consistent with the observed effects of *rad53* alleles on silencing (Figure 7).

Our functional assay to detect genetic interactions between the DNA damage checkpoint and histone deposition pathways is based on position-dependent gene silencing at a yeast telomere. We note, however, that the partnership between Asf1 and Hir proteins is highly conserved in eukaryotic organisms (Daganzo *et al.* 2003; Zhang *et al.* 2005). In human cells, the interaction between the homologous Asf1a and HIRA proteins is required for senescence-associated heterochromatin formation (SAHF) (Zhang *et al.* 2005). SAHF is a phenomenon in which large regions of human chromosomes become visibly compacted and acquire histone modifications such as H3-K9 methylation associated with heterochromatic gene silencing (Narita *et al.* 2003). Therefore, despite the evolutionary distance between budding yeast and humans, the Asf1/Hir protein pathway for histone deposition is maintained as a regulatory target. Whether the mammalian DNA damage checkpoint regulates the contribution of Asf1a and HIRA to SAHF remains an outstanding question.

The Asf1-Rad53 interaction: The highest-affinity Asf1-binding site on Rad53 resides within the FHA1 domain (Schwartz *et al.* 2003). FHA domains are often phosphopeptide-binding motifs (Sun *et al.* 1998; Durocher *et al.* 1999; Schwartz *et al.* 2002), and λ-phosphatase treatment reduces the affinity of Asf1 in cell extracts for a GST-Rad53-FHA1 fusion protein (Schwartz *et al.* 2003). These data suggested that phosphorylation of Asf1 or another protein stimulates the Asf1-Rad53 interaction. However, Asf1 from either wild-type or *rad53*Δ cell extracts interacts similarly with GST-FHA1, suggesting that Rad53 itself does not generate a phosphoepitope essential for the Asf1-Rad53 interaction. Nevertheless, Rad53 kinase activity is required for efficient Rad53-Asf1 interaction *in vivo* (Schwartz *et al.* 2003). Thus, either a Rad53 autophosphorylation event or transphosphorylation of another protein during checkpoint signaling is important for dynamic regulation of the Rad53-Asf1 interaction. We note that Rad53 autophosphorylates itself (Gilbert *et al.* 2001) and in doing so may alter the structure of the full-length Rad53 kinase to promote Asf1 binding. Such automodification may not be required for the isolated FHA1 domain.

We showed here that Dun1 kinase stimulates the Asf1-Rad53 interaction, raising the possibility that it modified Asf1. However, directed *in vitro* experiments revealed no phosphorylation or binding of Asf1 by Dun1 (data not shown). Future experiments will be required to determine whether modification of Asf1 is directly related to the Rad53 interaction.

Regulation of Asf1 in other organisms: Rad53 is unusual among checkpoint kinases in that it has two FHA domains. Its mammalian orthologs, the Chk1 and Chk2 proteins that function immediately downstream of the PIKK-family ATM/ATR kinases, contain a single FHA domain. Notably, although in human cells the protein associations of Asf1 are regulated by HU-mediated checkpoint signaling, the Chk1 and Chk2 proteins appear not to be involved in sequestration of Asf1 (Groth *et al.* 2005). Therefore, Asf1 is a highly conserved central regulator of histone metabolism, but the partner proteins and mechanisms used to regulate Asf1 appear to be more diverged in distant eukaryotic species.

In metazoans, the two homologous protein kinases termed Tlk1 and Tlk2 phosphorylate Asf1 proteins (Sillje and Nigg 2001). These kinases are maximally active during S phase and are negatively regulated by DNA damage checkpoint signaling (Groth *et al.* 2003). It has therefore been proposed that Asf1 phosphorylation by Tlks modulates Asf1 activity in a manner regulated by the DNA damage checkpoint. However, phosphorylation of Asf1 by Tlks does not affect histone binding, and it remains to be determined what the functional consequences of these modifications are. Yeast do not have Tlk homologs. Therefore, although regulation of Asf1 by DNA damage checkpoints is an important, conserved aspect of coordinating DNA synthesis and chromatin assembly in all eukaryotes, this goal is achieved in different ways. In yeast, it involves direct sequestration of

Asf1 by Rad53, and in metazoans it involves Tlk-mediated signaling. Future biochemical experiments will be required to determine how these different mechanisms of activation and repression are achieved.

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LITERATURE CITED

Abraham, R. T., 2001 Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. **15:** 2177–2196.

Agarwal, R., Z. Tang, H. Yu and O. Cohen-Fix, 2003 Two distinct pathways for inhibiting pds1 ubiquitination in response to DNA damage. J. Biol. Chem. **278:** 45027–45033.

Ahmad, K., and S. Henikoff, 2002 The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. Mol. Cell **9:** 1191–1200.

Allen, J. B., Z. Zhou, W. Siede, E. C. Friedberg and S. J. Elledge, 1994 The *SAD1/RAD53* protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev. **8:** 2416–2428.

Boeke, J. D., J. Trueheart, G. Natsoulis and G. R. Fink, 1987 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154:** 164–175.

Clarke, D. J., M. Segal, S. Jensen and S. I. Reed, 2001 Mec1p regulates Pds1p levels in S phase: complex coordination of DNA replication and mitosis. Nat. Cell Biol. **3:** 619–627.

Cohen-Fix, O., and D. Koshland, 1997 The anaphase inhibitor of Saccharomyces cerevisiae Pds1p is a target of the DNA damage checkpoint pathway. Proc. Natl. Acad. Sci. USA **94:** 14361–14366.

Cohen-Fix, O., J. M. Peters, M. W. Kirschner and D. Koshland, 1996 Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev. **10:** 3081–3093.

Craven, R. J., and T. D. Petes, 2000 Involvement of the checkpoint protein Mec1p in silencing of gene expression at telomeres in Saccharomyces cerevisiae. Mol. Cell. Biol. **20:** 2378–2384.

Daganzo, S. M., J. P. Erzberger, W. M. Lam, E. Skordalakes, R. Zhang *et al.*, 2003 Structure and function of the conserved core of histone deposition protein Asf1. Curr. Biol. **13:** 2148–2158.

Desany, B. A., A. A. Alcasabas, J. B. Bachant and S. J. Elledge, 1998 Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev. **12:** 2956–2970.

Donovan, S., J. Harwood, L. S. Drury and J. F. Diffley, 1997 Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. Proc. Natl. Acad. Sci. USA **94:** 5611–5616.

Durocher, D., J. Henckel, A. R. Fersht and S. P. Jackson, 1999 The FHA domain is a modular phosphopeptide recognition motif. Mol. Cell **4:** 387–394.

Emili, A., D. M. Schieltz, J. R. Yates and L. Hartwell, 2001 Dynamic interaction of DNA damage checkpoint protein Rad53 with chromatin assembly factor Asf1. Mol. Cell **7:** 13–20.

Enomoto, S., and J. Berman, 1998 Chromatin assembly factor I contributes to the maintenance, but not the reestablishment, of silencing at the yeast silent mating loci. Genes Dev. **12:** 219–232.

Enomoto, S., P. D. McCune-Zierath, M. Gerami-Nejad, M. Sanders and J. Berman, 1997 *RLF2*, a subunit of yeast chromatin assembly factor I, is required for telomeric chromatin function *in vivo*. Genes Dev. **11:** 358–370.

Franco, A. A., and P. D. Kaufman, 2004 Histone deposition proteins: links between the DNA replication machinery and epigenetic gene silencing. Cold Spring Harbor Symp. Quant. Biol. **69:** 1–8.

Franco, A. A., W. M. Lam, P. M. Burgers and P. D. Kaufman, 2005 Histone deposition protein Asf1 maintains DNA replisome integrity and interacts with replication factor C. Genes Dev. **19:** 1365–1375.

Gardner, R., C. W. Putnam and T. Weinert, 1999 RAD53, DUN1 and PDS1 define two parallel G2/M checkpoint pathways in budding yeast. EMBO J. **18:** 3173–3185.

Gilbert, C. S., C. M. Green and N. F. Lowndes, 2001 Budding yeast Rad9 is an ATP-dependent Rad53 activating machine. Mol. Cell **8:** 129–136.

Gottschling, D. E., O. M. Aparicio, B. L. Billington and V. A. Zakian, 1990 Position effect at *S. cerevisiae* telomeres: reversible repression of PolII transcription. Cell **63:** 751–762.

Green, C. M., and G. Almouzni, 2003 Local action of the chromatin assembly factor CAF-1 at sites of nucleotide excision repair in vivo. EMBO J. **22:** 5163–5174.

Groth, A., J. Lukas, E. A. Nigg, H. H. Sillje, C. Wernstedt *et al.*, 2003 Human Tousled like kinases are targeted by an ATM- and Chk1-dependent DNA damage checkpoint. EMBO J. **22:** 1676–1687.

Groth, A., D. Ray-Gallet, J. P. Quivy, J. Lukas, J. Bartek *et al.*, 2005 Human Asf1 regulates the flow of S phase histones during replicational stress. Mol. Cell **17:** 301–311.

Gunjan, A., and A. Verreault, 2003 A Rad53 kinase-dependent surveillance mechanism that regulates histone protein levels in S. cerevisiae. Cell **115:** 537–549.

Hoek, M., and B. Stillman, 2003 Chromatin assembly factor 1 is essential and couples chromatin assembly to DNA replication in vivo. Proc. Natl. Acad. Sci. USA **100:** 12183–12188.

Hu, F., A. A. Alcasabas and S. J. Elledge, 2001 Asf1 links Rad53 to control of chromatin assembly. Genes Dev. **15:** 1061–1066.

Huang, M., Z. Zhou and S. J. Elledge, 1998 The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. Cell **94:** 595–605.

Kaiser, C., S. Michaelis and A. Mitchell, 1994 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kaufman, P. D., R. Kobayashi, N. Kessler and B. Stillman, 1995 The p150 and p60 subunits of chromatin assembly factor 1: a molecular link between newly synthesized histones and DNA replication. Cell **81:** 1105–1114.

Kaufman, P. D., R. Kobayashi and B. Stillman, 1997 Ultraviolet radiation sensitivity and reduction of telomeric silencing in *Saccharomyces cerevisiae* cell lacking chromatin assembly factor-I. Genes Dev. **11:** 345–357.

Kaufman, P. D., J. L. Cohen and M. A. Osley, 1998 Hir proteins are required for position-dependent gene silencing in *Saccharomyces cerevisiae* in the absence of chromatin assembly factor I. Mol. Cell. Biol. **18:** 4793–4806.

Kaya, H., K. I. Shibahara, K. I. Taoka, M. Iwabuchi, B. Stillman *et al.*, 2001 FASCIATA genes for chromatin assembly factor-1 in arabidopsis maintain the cellular organization of apical meristems. Cell **104:** 131–142.

Krawitz, D. C., T. Kama and P. D. Kaufman, 2002 Chromatin assembly factor-I mutants defective for PCNA binding require Asf1/Hir proteins for silencing. Mol. Cell. Biol. **22:** 614–625.

Krude, T., 1995 Chromatin assembly factor 1 (CAF-1) colocalizes with replication foci in HeLa cell nuclei. Exp. Cell Res. **220:** 304–311.

Kyrion, G., K. A. Boakye and A. J. Lustig, 1992 C-terminal truncation of *RAP1* results in the deregulation of telomere size, stability, and function in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:** 5159–5173.

Le, S., C. Davis, J. B. Konopka and R. Sternglanz, 1997 Two new S-phase-specific genes from *Saccharomyces cerevisiae*. Yeast **13:** 1029–1042.

Lee, S. J., M. F. Schwartz, J. K. Duong and D. F. Stern, 2003 Rad53 phosphorylation site clusters are important for Rad53 regulation and signaling. Mol. Cell. Biol. **23:** 6300–6314.

Leroy, C., S. E. Lee, M. B. Vaze, F. Ochsenbien, R. Guerois *et al.*, 2003 PP2C phosphatases Ptc2 and Ptc3 are required for DNA checkpoint inactivation after a double-strand break. Mol. Cell **11:** 827–835.

Liang, C., and B. Stillman, 1997 Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. Genes Dev. **11:** 3375–3386.

Longhese, M. P., V. Paciotti, H. Neecke and G. Lucchini, 2000 Checkpoint proteins influence telomeric silencing and length maintenance in budding yeast. Genetics **155:** 1577–1591.

Longtine, M. S., A. McKenzie, 3rd, D. J. Demarini, N. G. Shah, A. Wach *et al.*, 1998 Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast **14:** 953–961.

Lucchini, R., and J. M. Sogo, 1995 Replication of transcriptionally active chromatin. Nature **274:** 276–280.

Luger, K., A. W. Mäder, R. K. Richmond, D. F. Sargent and T. J. Richmond, 1997 Crystal structure of the nucleosome core particle at 2.8Å resolution. Nature **389:** 251–260.

Marsolier, M. C., P. Roussel, C. Leroy and C. Mann, 2000 Involvement of the PP2C-like phosphatase Ptc2p in the DNA checkpoint pathways of *Saccharomyces cerevisiae*. Genetics **154:** 1523–1532.

Martin, S. G., T. Laroche, N. Suka, M. Grunstein and S. M. Gasser, 1999 Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast. Cell **97:** 621–633.

Martini, E., D. M. Roche, K. Marheineke, A. Verreault and G. Almouzni, 1998 Recruitment of phosphorylated chromatin assembly factor 1 to chromatin after UV irradiation of human cells. J. Cell Biol. **143:** 563–575.

McKittrick, E., P. R. Gafken, K. Ahmad and S. Henikoff, 2004 Histone H3.3 is enriched in covalent modifications associated with active chromatin. Proc. Natl. Acad. Sci. USA **101:** 1525–1530.

Melo, J., and D. Toczyski, 2002 A unified view of the DNA-damage checkpoint. Curr. Opin. Cell Biol. **14:** 237–245.

Meluh, P. B., and J. R. Broach, 1999 Immunological analysis of yeast chromatin. Methods Enzymol. **304:** 414–430.

Mills, K. D., D. A. Sinclair and L. Guarente, 1999 MEC1-dependent redistribution of the Sir3 silencing protein from telomeres to DNA double-strand breaks. Cell **97:** 609–620.

Moggs, J. G., P. Grandi, J. P. Quivy, Z. O. Jonsson, U. Hubscher *et al.*, 2000 A CAF-1-PCNA-mediated chromatin assembly pathway triggered by sensing DNA damage. Mol. Cell. Biol. **20:** 1206–1218.

Monson, E. K., D. de Bruin and V. A. Zakian, 1997 The yeast Cac1 protein is required for the stable inheritance of transcriptionally repressed chromatin at telomeres. Proc. Natl. Acad. Sci. USA **94:** 13081–13086.

Narita, M., S. Nunez, E. Heard, A. W. Lin, S. A. Hearn *et al.*, 2003 Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell **113:** 703–716.

Osley, M. A., and D. Lycan, 1987 Trans-acting regulatory mutations that alter transcription of *Saccharomyces cerevisiae* histone genes. Mol. Cell. Biol. **7:** 4204–4210.

Pringle, J. R., A. E. M. Adams, D. G. Drubin and B. K. Haarer, 1991 Immunofluorescence methods for yeast. Methods Enzymol. **194:** 565–602.

Qian, Z., H. Huang, J. Y. Hong, C. L. Burck, S. D. Johnston *et al.*, 1998 Yeast Ty1 retrotransposition is stimulated by a synergistic interaction between mutations in chromatin assembly factor-I and histone regulatory (Hir) proteins. Mol. Cell. Biol. **18:** 4783–4792.

Quivy, J. P., P. Grandi and G. Almouzni, 2001 Dimerization of the largest subunit of chromatin assembly factor 1: importance in vitro and during Xenopus early development. EMBO J. **20:** 2015–2027.

Ray-Gallet, D., J. P. Quivy, C. Scamps, E. M. Martini, M. Lipinski *et al.*, 2002 HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis. Mol. Cell **9:** 1091–1100.

Sanchez, Y., B. A. Desany, W. J. Jones, Q. Liu, B. Wang *et al.*, 1996 Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. Science **271:** 357–360.

Sanchez, Y., J. Bachant, H. Wang, F. Hu, D. Liu *et al.*, 1999 Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. Science **286:** 1166–1171.

Schwartz, M. F., J. K. Duong, Z. Sun, J. S. Morrow, D. Pradhan *et al.*, 2002 Rad9 phosphorylation sites couple Rad53 to the Saccharomyces cerevisiae DNA damage checkpoint. Mol. Cell **9:** 1055–1065.

Schwartz, M. F., S. Lee, J. K. Duong, S. Eminaga and D. F. Stern, 2003 FHA domain-mediated DNA checkpoint regulation of Rad53. Cell Cycle **2:** 384–396.

Sharp, J. A., E. T. Fouts, D. C. Krawitz and P. D. Kaufman, 2001 Yeast histone deposition protein Asf1p requires Hir proteins and PCNA for heterochromatic silencing. Curr. Biol. **11:** 463–473.

Sharp, J. A., A. A. Franco, M. A. Osley and P. D. Kaufman, 2002 Chromatin assembly factor-I and Hir proteins contribute to building functional kinetochores in *Saccharomyces cerevisiae*. Genes Dev. **16:** 85–100.

Sharp, J. A., D. C. Krawitz, K. A. Gardner, C. A. Fox and P. D. Kaufman, 2003 The budding yeast silencing protein Sir1 is a functional component of centromeric chromatin. Genes Dev. **17:** 2356–2361.

Sherwood, P. W., S. V. Tsang and M. A. Osley, 1993 Characterization of *HIR1* and *HIR2*, two genes required for regulation of histone gene transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:** 28–38.

Shibahara, K., and B. Stillman, 1999 Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. Cell **96:** 575–585.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Sillje, H. H., and E. A. Nigg, 2001 Identification of human Asf1 chromatin assembly factors as substrates of Tousled-like kinases. Curr. Biol. **11:** 1068–1073.

Singer, M. S., A. Kahana, A. J. Wolf, L. L. Meisinger, S. E. Peterson *et al.*, 1998 Identification of high-copy disruptors of telomeric silencing in *Saccharomyces cerevisiae*. Genetics **150:** 613–632.

Smith, J., E. Caputo and J. Boeke, 1999 A genetic screen for ribosomal DNA silencing defects identifies multiple DNA replication and chromatin-modulating factors. Mol. Cell. Biol. **19:** 3184–3197.

Sun, Z., D. S. Fay, F. Marini, M. Foiani and D. F. Stern, 1996 Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. Genes Dev. **10:** 395–406.

Sun, Z., J. Hsiao, D. S. Fay and D. F. Stern, 1998 Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. Science **281:** 272–274.

Sutton, A., J. Bucaria, M. A. Osley and R. Sternglanz, 2001 Yeast ASF1 protein is required for cell-cycle regulation of histone gene transcription. Genetics **158:** 587–596.

Tagami, H., D. Ray-Gallet, G. Almouzni and Y. Nakatani, 2004 Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. Cell **116:** 51–61.

Tinker-Kulberg, R. L., and D. O. Morgan, 1999 Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage. Genes Dev. **13:** 1936–1949.

Tyler, J. K., M. Bulger, R. T. Kamakaka, R. Kobayashi and J. T. Kadonaga, 1996 The p55 subunit of Drosophila chromatin assembly factor-I is homologous to a histone deacetylase-associated protein. Mol. Cell. Biol. **16:** 6149–6159.

Tyler, J. K., C. R. Adams, S. R. Chen, R. Kobayashi, R. T. Kamakaka *et al.*, 1999 The RCAF complex mediates chromatin assembly during DNA replication and repair. Nature **402:** 555–560.

Tyler, J. K., K. A. Collins, J. Prasad-Sinha, E. Amiott, M. Bulger *et al.*, 2001 Interaction between the Drosophila CAF-1 and ASF1 chromatin assembly factors. Mol. Cell. Biol. **21:** 6574–6584.

Wang, H., D. Liu, Y. Wang, J. Qin and S. J. Elledge, 2001 Pds1 phosphorylation in response to DNA damage is essential for its DNA damage checkpoint function. Genes Dev. **15:** 1361–1372.

Weinert, T. A., G. L. Kiser and L. H. Hartwell, 1994 Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. **8:** 652–665.

Xu, H., U. J. Kim, T. Schuster and M. Grunstein, 1992 Identification of a new set of cell cycle-regulatory genes that regulate

S-phase transcription of histone genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:** 5249–5259.

Ye, X., A. A. Franco, H. Santos, P. D. Kaufman and P. D. Adams, 2003 Inhibition of S-phase chromatin assembly causes DNA damage, activation of the S-phase checkpoint and S-phase arrest. Mol. Cell **11:** 341–351.

Zhang, Z., K. Shibahara and B. Stillman, 2000 PCNA connects DNA replication to epigenetic inheritance in yeast. Nature **408:** 221–225.

Zhang, R., M. V. Poustovoitov, X. Ye, H. A. Santos, W. Chen *et al.*, 2005 Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Dev. Cell **8:** 19–30.

Zhao, X., E. G. Muller and R. Rothstein, 1998 A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Mol. Cell **2:** 329–340.

Zhou, Z., and S. J. Elledge, 1993 *DUN1* encodes a protein kinase that controls the DNA damage response in yeast. Cell **75:** 1119–1127.

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