Modulation of Gene Silencing by Cdc7p via H4 K16 Acetylation and Phosphorylation of Chromatin Assembly Factor CAF-1 in *Saccharomyces cerevisiae*

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ABSTRACT CAF-1 is an evolutionarily conserved H3/H4 histone chaperone that plays a key role in replication-coupled chromatin assembly and is targeted to the replication fork via interactions with PCNA, which, if disrupted, leads to epigenetic defects. In *Saccharomyces cerevisiae*, when the silent mating-type locus *HMR* contains point mutations within the *E* silencer, Sir protein association and silencing is lost. However, mutation of *CDC7*, encoding an S-phase-specific kinase, or subunits of the H4 K16-specific acetyltransferase complex SAS-I, restore silencing to this crippled *HMR*, *HMRae***. Here, we observed that loss of *Cac1p*, the largest subunit of CAF-1, also restores silencing at *HMRae***, and silencing in both *cac1Δ* and *cdc7* mutants is suppressed by overexpression of *SAS2*. We demonstrate *Cdc7p* and *Cac1p* interact *in vivo* in S phase, but not in G1, consistent with observed cell cycle-dependent phosphorylation of *Cac1p*, and hypoacetylation of chromatin at H4 K16 in both *cdc7* and *cac1Δ* mutants. Moreover, silencing at *HMRae*** is restored in cells expressing *cac1p* mutants lacking *Cdc7p* phosphorylation sites. We also discovered that *cac1Δ* and *cdc7-90* synthetically interact negatively in the presence of DNA damage, but that *Cdc7p* phosphorylation sites on *Cac1p* are not required for responses to DNA damage. Combined, our results support a model in which *Cdc7p* regulates replication-coupled histone modification via a *CAC1*-dependent mechanism involving H4 K16ac deposition, and thereby silencing, while CAF-1-dependent replication- and repair-coupled chromatin assembly *per se* are functional in the absence of phosphorylation of *Cdc7p* consensus sites on CAF-1.

KEYWORDS *CDC7*; *CAC1*; *SAS2*; histone H4 K16; silencing; CAF-1

DURING DNA replication, nucleosomes must be disassembled from in front of the replication fork and reassembled behind the replication fork. When the fork travels through loci, reassembly must be coordinated to ensure that locus-specific patterns of histone modifications on nucleosomes and specialized chromatin are maintained on nucleosomes containing parental histones, as well as recreated on nucleosomes containing newly synthesized histones. This reconstruction is essential to ensure that epigenetic states are inherited (Burgess and Zhang 2010; Margueron and Rein-

berg 2010; Alabert and Groth 2012). Nucleosome assembly associated with DNA replication is thought to primarily involve deposition of a histone (H3/H4)₂ tetramer, which is then followed by the addition of H2A/H2B dimers (Smith and Stillman 1991; Ransom *et al.* 2010; Xu *et al.* 2010; Mattioli *et al.* 2017). *Asf1p*, CAF-1, and *Rtt106p* are histone chaperones that play predominant roles in transporting histones H3/H4 and chromatin assembly during DNA replication in *Saccharomyces cerevisiae*, although yeast lacking these assembly factors singly or in combination remain viable. The interaction between H3/H4 and *Rtt106p* or CAF-1 is promoted by H3 K56ac (Li *et al.* 2008), which requires *Asf1p* to present H3/H4 as a substrate for the sole H3 K56-specific acetyltransferase *Rtt109p* (Driscoll *et al.* 2007; Han *et al.* 2007; Tsubota *et al.* 2007). This effect, in combination with the ability of CAF-1 and *Rtt106p* to bind H3/H4 tetramers (Liu *et al.* 2010; Fazly *et al.* 2012; Mattioli *et al.* 2017), and *Asf1p* to bind exclusively to H3/H4 dimers in a manner that prevents H3/H4 tetramer formation (English *et al.* 2006;

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Natsume *et al.* 2007), supports a model in which *Asf1p* functions upstream of CAF-1 and *Rtt106p* during replication-coupled chromatin assembly, and transfers H3/H4 to CAF-1 or *Rtt106p*. In this model, CAF-1 or *Rtt106p* then promotes H3/H4 tetramer formation onto newly synthesized DNA, with CAF-1 likely being recruited to the replication fork through an interaction with the DNA processivity factor *PCNA* (Shibahara and Stillman 1999; Winkler *et al.* 2012). As *Rtt106p* can also bind *Cac1p* independent of H3/H4 *in vitro* as well as co-immunoprecipitate *Cac2p* in a *CAC1*-dependent manner *in vivo* (Huang *et al.* 2005), both chaperones potentially function together, or directly exchange H3/H4 during chromatin assembly.

The fidelity of replication-coupled chromatin assembly impacts gene silencing in *S. cerevisiae*, as well as epigenetic processes in organisms ranging from *Schizosaccharomyces pombe* to *Drosophila* to humans [see Dohke *et al.* (2008), Huang *et al.* (2010), Alabert and Groth (2012), and Young and Kirchmaier (2013), and references within]. In budding yeast, silent chromatin is transcriptionally inactive and is found in telomeric regions, the rDNA locus, and the silent mating-type loci. Silent chromatin is formed at the silent mating-type loci *HMR* and *HML* by the initial recruitment of Silent Information Regulator proteins *Sir1p*, *Sir2p*, *Sir3p*, and *Sir4p* to regulatory silencer regions, *E* and *I*, of these *HM* loci. Upon being recruited to the *HMR-E* silencer, *Sir2p*, *Sir3p*, and *Sir4p* then propagate throughout the region to form silent chromatin (Hoppe *et al.* 2002; Rusché *et al.* 2002; Bose *et al.* 2004). Sir propagation requires the deacetylation of H4 K16 by activity of the NAD⁺-dependent histone deacetylase *Sir2p*, which generates a higher-affinity binding site for *Sir3p* (Hecht *et al.* 1995; Braunstein *et al.* 1996; Carmen *et al.* 2002; Hoppe *et al.* 2002; Rusché *et al.* 2002; Buchberger *et al.* 2008). Once established, silent chromatin is efficiently maintained throughout the cell cycle and inherited upon DNA replication, thereby ensuring a transcriptionally silenced epigenetic state in cells and their progeny [see Pillus and Rine (1989), Gottschling *et al.* (1990), and Young and Kirchmaier (2013), and references within].

Screens designed to uncover mutations that restore silencing at a *HMR* locus containing a crippled *E* silencer, *e***, have identified several factors involved in DNA replication or chromatin modification (Axelrod and Rine 1991; Ehrenhofer-Murray *et al.* 1999; Miller *et al.* 2008, 2010). *HMRae*** contains point mutations in the *Rap1p*- and *Abf1p*-binding sites within the *E* silencer (Figure 1A), which lead to loss of silencing of *a1-a2* at *HMRae*** by preventing Sir proteins from being recruited (Miller *et al.* 2008), and results in the inability of *MATα* cells to mate due to their *a/α* pseudodiploid expression state (Kimmerly *et al.* 1988). Thus, *HMRae*** serves as a model locus for analyzing mutations that cause off-target silencing at normally transcriptionally active loci. Previous studies by others and our laboratory have demonstrated that loss of acetylation at H4 K16, either by deleting genes encoding subunits of SAS-I acetyltransferase complex

(*SAS2*, *SAS4*, and *SAS5*) or by mutating H4 K16 to R, results in restoration of Sir protein recruitment (Yang *et al.* 2008) and silencing at *HMRae*** (Reifsnyder *et al.* 1996; Ehrenhofer-Murray *et al.* 1997; Meijsing and Ehrenhofer-Murray 2001; Miller *et al.* 2008; Yang *et al.* 2008), indicating the importance of this modification in promoting the formation of silent chromatin. CAF-1 and *Asf1p* both interact with SAS-I, and loss of *CAC1* or *ASF1* results in a delay in the accumulation of H4 K16ac in S phase and an overall decrease in chromatin-associated H4 K16ac levels (Meijsing and Ehrenhofer-Murray 2001; Osada *et al.* 2001; Miller *et al.* 2010; Reiter *et al.* 2015; Heise *et al.* 2012). Consistent with SAS-I acting prior to or during deposition of (H3/H4)₂ onto DNA by CAF-1, H3/H4 copurifying with *Cac1p* contains H4 K16ac (Zhou *et al.* 2006). Mutations in *PCNA* that disrupt or weaken *PCNA*'s interaction with *Cac1p* also exhibit decreased chromatin-associated H4 K16ac levels (Miller *et al.* 2010). Like *asf1Δ* and *sas1Δ* mutants, these *PCNA* mutants can rescue silencing at *HMRae*** by restoring Sir recruitment to this locus (Miller *et al.* 2008; Yang *et al.* 2008).

Mutants of *CDC7*, which encodes a serine/threonine replication kinase, restore silencing at *HMRae*** as well (Axelrod and Rine 1991). *CDC7* is essential, as *Cdc7p* phosphorylates several subunits of the mini-chromosome maintenance, MCM, helicase to initiate unwinding of DNA at origins of DNA replication [see Labib (2010) and Randell *et al.* (2010), and references within]. Although *Cdc7p* is expressed throughout the cell cycle, *Cdc7p*'s kinase function is restricted to S phase as its activity requires binding to the cyclin *Dbf4p*, which is only expressed in late G1 through S phase (Chapman and Johnston 1989; Jackson *et al.* 1993; Weinreich and Stillman 1999). In human cells, Cdc7 phosphorylates p150, the largest subunit of CAF-1, which stabilizes p150 as a monomer (Gerard *et al.* 2006). This monomeric form of p150 has a more stable interaction with PCNA (Gerard *et al.* 2006). In yeast, *Cac1p*, the homolog of p150, is also phosphorylated by *Cdc7p* *in vitro* (Jeffery *et al.* 2015), but the significance of *Cdc7p*-mediated phosphorylation of *Cac1p* *in vivo* has remained unclear.

In addition to CAF-1 being involved in chromatin assembly during DNA replication, CAF-1 promotes histone deposition after repair of UV-damaged DNA (Kaufman *et al.* 1997; Game and Kaufman 1999; Polo *et al.* 2006). In yeast, CAF-1 mutants are sensitive to multiple types of DNA damage and CAF-1, together with *Asf1p*, promotes checkpoint deactivation after DNA repair [e.g., Kaufman *et al.* (1997), Linger and Tyler (2005), Li *et al.* (2008), and Kim and Haber (2009)]. Exposure to DNA-damaging agents also alters *Cac1p*'s phosphorylation states (Zhou *et al.* 2016), but the impact of these phosphorylation events has not been assessed previously. Here, we provide evidence that *Cdc7p* influences silencing, in part, via a CAF-1-mediated pathway involving SAS-I and H4 K16ac, and describe interactions between *CAC1* and *CDC7*, or repair pathway mutants of *PCNA*, in response to DNA damage.

Materials and Methods

Strain/plasmid construction

Yeast strains used for this study (Supplemental Material, Table S1) were generated using conventional methods, including genetic crosses and homologous recombination. One-step gene conversion was used to delete open reading frames and plasmid shuffling was utilized to generate yeast strains expressing histone mutants (Adams *et al.* 1998). Generally, at least two independent clones for each genotype were analyzed in experiments described throughout this study.

Plasmids used for this study are described in Table S2. Plasmids expressing *SAS2-M1*, *cac1*, or histone mutants were generated by site-directed mutagenesis. PCR to introduce the mutations was performed using Phusion polymerase (catalog number M0530S; New England Biolabs, Beverly, MA) using primers described below and in Table S3. PCR reactions were then treated with *DpnI*, DNA was isolated using a QIAquick PCR Purification Kit (catalog number 28104; QIAGEN, Valencia, CA), and transformed into competent DH5 α cells by electroporation. pAK1347, which expresses a catalytically inactive form of *SAS2*, *SAS2-M1*, was derived from the template plasmid AE778 (Meijnsing and Ehrenhofer-Murray 2001) using oALK1687 and oALK1688. pAK1225, pAK1226, pAK1228, pAK1229, pAK1308, pAK1309, pAK1328, and pAK1329, which express *cac1* mutants, were derived from the template plasmid pAK1223, which encodes *CAC1* expressed from its endogenous promoter on an *ARS/CEN* plasmid. pAK1225 was generated using oligos oALK1361 and oALK1362; pAK1226 was generated using oALK1359 and oALK1360; pAK1228 was made using oALK1355 and oALK1356; pAK1229 was generated using oALK1357 and oALK1358; pAK1308 was generated from pAK1226 using oALK1355 and oALK1356; pAK1309 was generated from pAK1225 using oALK1357 and oALK1358; pAK1328 was made from pAK1226 using oALK1624 and oALK1625; and pAK1329 was generated from pAK1225 using oALK1626 and oALK1627. pAK1333, pAK1334, pAK1335, and pAK1337 were derived from pAK1224, which encodes *CAC1*-3XFLAG expressed from its endogenous promoter on an *ARS/CEN* plasmid, by site-directed mutagenesis as above. pAK1156, which expresses histone H3 T45A and H4, was derived from pMP3 (Kelly *et al.* 2000) by site-directed mutagenesis using oALK1242 and oALK1244.

Patch mating assays

Patch mating assays were conducted as described previously (Miller and Kirchmaier 2014). Briefly, yeast were patched onto YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% D-glucose, and 2% Bacto agar) medium or YM (0.67% yeast nitrogen base without amino acids, 2% D-glucose, and 2% Bacto agar) supplemented with adenine, histidine, tryptophan, and uracil (for strains with vector, *SAS2*, or *SAS-M1* expression plasmids), and grown overnight at 23 (for *cdc7* mutants) or 30°. Cells were then replica plated onto YPD medium as a control for growth and onto a *MATa his4*, or a

BY4741 (*MATa* S288C), tester lawn on YM medium, or on YM medium supplemented with histidine and leucine, respectively, to test for silencing at *HMR* or *HMRae***. Yeast were then incubated at 23 or 30° for 1–3 days prior to imaging.

Quantitative mating assays

Quantitative mating assays were conducted as described previously (Yang and Kirchmaier 2006; Miller and Kirchmaier 2014). Data were analyzed by the Wilcoxon rank-sum test or the Jonckheere–Terpstra trend test with MSTAT v.6.3 (<http://mcardle.oncology.wisc.edu/mstat>).

Fluorescence lifetime imaging microscopy-fluorescence resonance energy transfer

Fluorescence lifetime imaging microscopy-fluorescence resonance energy transfer (FLIM-FRET) was performed with a Microtime200 scanning confocal time-resolved inverted microscope system (PicoQuant). A 465-nm psec pulsed laser was used to excite the GFP tag. The excitation beam was delivered to the sample stage through an apochromatic water immersion objective lens (60 $\times$, NA 1.2; Olympus) and the emitted fluorescence was collected by the same objective lens. For an 80 $\times$ 80 μm^2 FLIM image, the laser dwelling time at each pixel was set to $\sim$ 0.8 msec. A dual-band dichroic (z467/638rpc; Chroma) was used to filter the excitation light from the emission and a 50- μm pinhole was applied to block the off-focus photons. The final signal was further filtered by a band-pass filter (520 $\pm$ 20 nm; Chroma) before reaching the single-photon avalanche photodiode detector (SPCM-AQR; Perkin-Elmer [Perkin Elmer-Cetus], Norwalk, CT). Collected photons were registered as a time-correlated single-photon counting (TCSPC) format for time-domain fluorescence lifetime calculation (Damayanti *et al.* 2017). Fluorescence lifetime (τ) was determined by fitting the decay rate of a TCSPC histogram as the photon frequency decreased to 1/e of the original: $F(t) = F_0 \exp(-t/\tau)$.

When FRET occurs, the nonradiative energy transfer between the donor and acceptor fluorophore (e.g., GFP-mCherry) causes a reduction in the donor fluorescence lifetime, and the FRET efficiency has an inverse sixth power relation to the intermolecule distance: $E_{\text{FRET}} = [1/(1 + (r/R_0)^6)] = 1 - (\tau_{\text{DA}}/\tau_{\text{D}})$, where R_0 is the Förster distance at which 50% energy is transferred, and τ_{DA} and τ_{D} are the fluorescence lifetimes of the donor in the presence and absence of acceptor, respectively. For the GFP-mCherry pair, R_0 is $\sim$ 5.24 nm. Live cells, either in logarithmic growth or arrested in G1 phase, were placed on a coverslip for FLIM measurements. The output laser power was set to 8 μW and a repetition rate of 40 MHz to prevent photo-damage. Ten images were collected from each sample and $\sim$ 100 cells with characteristic morphologies were analyzed. A 15% intensity threshold was applied in analysis to eliminate background noises and nonspecific signals from the cytoplasm. In a typical TCSPC fitting, $> 10^4$ photons were acquired from the region of interest (e.g., nucleus) to enhance the signal-to-noise ratio.

The lifetimes of GFP calculated from each image of the same strain were averaged and used to calculate the SD. To calculate the FRET efficiency, the average GFP lifetime measurement from ~30 to 40 cells that expressed both a GFP-tagged protein and a mCherry-tagged protein was divided by the average GFP lifetime measurement of ~30–40 cells expressing only the GFP-tagged protein [see also Miller *et al.* (2010)].

Color colony assays

Cells were grown overnight in complete supplement mixture (CSM)-URA medium [0.77 gm/liter CSM minus uracil (Sunrise Science), 0.67% yeast nitrogen base, and 2% glucose]. Next, 3 μ l of 10-fold serial dilutions were spotted onto CSM-URA agar (CSM-URA medium with 2% Bacto agar), starting with an initial concentration of 1 OD₆₀₀. Cells were grown at 30° for 2 days and then incubated for 4 days at 4°. Images were taken with a Leica MZ125 microscope and SPOT 4.1.1 imaging software [see Miller and Kirchmaier (2014)].

Growth assays of sensitivity to DNA-damaging agents

Wild-type and mutant yeast were grown logarithmically overnight in CSM-URA medium, diluted to 1×10^4 cells/ μ l, then 2.5 μ l of 10-fold serial dilutions were spotted onto YPD medium with or without noted amounts of hydroxyurea (HU), methyl methanesulfonate (MMS), or Zeocin, or treatment with UV as described previously [see Miller *et al.* (2008)].

Cell fractionation

In YPD medium, 200-ml yeast cultures were grown logarithmically to 0.8–1 OD₆₀₀, then yeast were collected by centrifugation at $910 \times g$ for 5 min. Cell pellets were resuspended in spheroplasting buffer (50 mM potassium phosphate buffer pH 6.5, 1.0 M sorbitol, 14 mM β -mercaptoethanol, plus 81 kU/gm yeast lytic enzyme) and incubated for 30–45 min at 30°. Spheroplasted cells were then pelleted by centrifugation at $5000 \times g$ for 5 min at 4° and washed once with spheroplasting buffer. Spheroplasts were then resuspended in 5 ml lysis buffer (20 mM potassium phosphate buffer pH 6.8, 18% Ficoll 400, 1.0 mM MgCl₂, 0.5 mM EDTA pH 8.0, 1.0 μ g/ml of leupeptin and pepstatin, and 1.0 mM PMSF), and lysed using a dounce homogenizer. Cell debris was pelleted by centrifugation at $5000 \times g$ for 20 min and the supernatant was collected. The pellet was resuspended in 5 ml of lysis buffer, rehomogenized, and cell debris was cleared by centrifugation as above. The supernatant was combined with the previously collected supernatant and centrifuged at $50,000 \times g$ for 30 min to separate the nuclei from the soluble cytoplasmic fraction.

The pelleted nuclei were resuspended and lysed in 500 μ l radioimmunoprecipitation assay, RIPA, buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP-40, 0.5% Na-Deoxycholate, and 0.1% SDS) by incubating the nuclei on ice with intermittent vortexing. The chromatin fraction was pelleted and separated from the soluble nuclear fraction by centrifugation at $14,000 \times g$ for 10 min at 4°. The chro-

matin fraction was washed twice with Buffer A (10 mM Tris pH 8.0, 0.5% NP-40, and 75 mM NaCl) and twice with Buffer B (10 mM Tris pH 8.0 and 300 mM NaCl). Chromatin was resuspended in 25 μ l 2 $\times$ SDS-PAGE sample buffer (25 mM Tris-HCl pH 6.8, 2.5% SDS, 2.5% glycerol, 0.01% bromophenol blue, and 1.25% β -mercaptoethanol) for protein blot analyses [see also Chen *et al.* (2012)].

Protein blot analyses

Chromatin fraction analyses: Chromatin fractions were separated on a 15% SDS-PAGE gel and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked with a 5% nonfat milk solution [5% nonfat dry milk, 0.1% Tween 20, and 1 $\times$ phosphate-buffered saline (PBS) (0.127 M NaCl, 2.7 mM KCl, 1.4 mM KH₂PO₄, and 0.01 M Na₂HPO₄, pH 7.4) for 1 hr at room temperature, and then incubated overnight with anti-acetyl H4 K16 antibodies (catalog number 07-329; Millipore, Bedford, MA) [1:5000 in PBS-T (PBS and 0.1% Tween 20)] at 4°. Membranes were then incubated in HRP-conjugated anti-rabbit antibodies (catalog number NA934; GE Healthcare Life Sciences) (1:10,000 in PBS-T). Membranes were stripped with 0.2 M NaOH at room temperature, then reprobed with 1:6000 anti-H3 antibodies (catalog number ab1791; Abcam). Blots were visualized using Luminata Crescendo Western HRP Substrate (Millipore) and imaged using a ChemiDoc XRS+ System. Blots were then quantified using Image Lab Software 5.1. Data were calculated by using the following equation $\frac{H4K16ac_{mut}/H4K16ac_{WT}}{H3_{mut}/H3_{WT}}$, where mut = indicated strain, mean $\pm$ SD, and $n = 3$. Data were analyzed by the Wilcoxon rank-sum test with MSTAT v.6.3.

Rad53 phosphorylation analyses: Yeast were grown logarithmically in YPD medium or in 200 mM HU for 3 hr, and then were collected by centrifugation at $910 \times g$ for 5 min. Whole-cell extracts were prepared by resuspending cells in 2.0 M NaOH with 8% β -mercaptoethanol and incubating for 5 min on ice. Cells were then washed in high-salt extraction buffer (40 mM HEPES pH 7.5, 350 mM NaCl, 0.1% Tween 20, and 10% glycerol) and resuspended in 2 $\times$ SDS-PAGE sample buffer. Whole-cell extracts were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 5% BSA in PBS overnight at 4° and incubated in anti-Rad53 antibodies (catalog number ab104232; Abcam) (1:2000 in PBS-T) for 2 hr at room temperature, followed by Alexa Fluor 680 anti-rabbit antibodies (catalog number A21109; Invitrogen, Carlsbad, CA) (1:5000 in PBS-T) for 2 hr at room temperature. Blots were imaged using Odyssey infrared imager and Odyssey software v1.2.

CAC1-3XFLAG expression analyses: Yeast were grown logarithmically in YPD medium and then were collected by centrifugation at $910 \times g$ for 5 min. Whole-cell extracts were prepared from each aliquot as follows: yeast were collected by centrifugation and washed with cold 20% trichloroacetic acid (TCA), resuspended in 250 μ l with 20% TCA, and lysed

by adding 250 μ l of zirconia/silica beads (catalog number 11079105z; BioSpec) and vortexing for ~20 min at 4°. Lysates were transferred to fresh tubes. Beads were washed with 750 μ l 20% TCA, washes were combined with previously collected lysates for each aliquot, and aliquots were centrifuged at $14,000 \times g$ for 10 min at 4°. Supernatants were discarded, pellets were washed with 1 ml cold 100% EtOH, and aliquots were centrifuged at $14,000 \times g$ for 5 min at 4°. Supernatants were discarded, and pellets were resuspended to a final concentration of 0.05 OD₆₀₀/ml in 1 $\times$ SDS-PAGE sample buffer. Whole-cell extracts were then resolved on 10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in blocking buffer (PBS-T and 5% milk) at 4°, then incubated with mouse monoclonal anti-FLAG antibodies (catalog number ab45766; Abcam) (1:1000 in PBS-T and 2% milk). Membranes were washed with PBS-T and incubated with HRP-conjugated sheep anti-mouse secondary antibodies (catalog number NA931VS; GE Healthcare Life Sciences) (1:10,000 in PBS-T containing 2% milk). Membranes were stripped by incubating in 0.1 M NaOH for 5 min, washed with water, and reprobed as above using anti-PGK1 antibodies (catalog number A-6457, Molecular Probes, Eugene, OR) (1:10,000 in PBS-T, 2% milk) and anti-mouse HRP-conjugated secondary antibodies as above. Images of the membranes were collected using a ChemiDoc XRS+ System.

Chromatin immunoprecipitation

Yeast were grown logarithmically in synthetic complete medium with 2% D-glucose and lacking uracil, then chromatin immunoprecipitation (ChIP) experiments were conducted using IgG (catalog number C15410206; Diagenode), anti-H4 K16ac (catalog number 07-329; Millipore) or anti-H3 (catalog number ab1791; Abcam) antibodies. Coprecipitated DNAs were analyzed as described previously by real-time PCR with an ABI Prism 7000 using oligonucleotides to monitor *e*** and *a1* (Kirchmaier and Rine 2006; Yang and Kirchmaier 2006; Miller *et al.* 2008). Data were analyzed by the Wilcoxon rank-sum test with MSTAT v.6.3 (<http://mcardle.oncology.wisc.edu/mstat>).

Flow cytometry analyses

Yeast were grown logarithmically in YPD to ~0.4 OD₆₀₀/ml, and were then incubated in YPD containing 200 mM HU for 3 hr to arrest cells in S phase. Cells were then washed 3 $\times$ with YPD, resuspended in 20 ml of YPD, and incubated at 23°. Next, 1 ml of cells was collected prior to HU treatment, after 3 hr in HU, and every 15 min after release from HU for 3 hr. Harvested cells were pelleted by centrifugation, resuspended in 70% EtOH, and incubated at room temperature for 1 hr before being stored overnight at 4°. Cells were then washed 2 $\times$ in flow cytometry, FACS, buffer (200 mM Tris-HCl, 20 mM EDTA, and 0.001% NaN₃), resuspended in 100 μ l of 0.1% RNase in FACS buffer solution, and incubated for 2 hr at 37°. Cells were washed once with 1 $\times$ PBS, then incubated in 100 μ l propidium iodide solution (0.05 mg/ml propidium iodide in 1 $\times$ PBS) for $\geq$ 1 hr in the dark at 4°. The sample volume was then increased to 1 ml with 1 $\times$ PBS and samples were briefly sonicated (Branson

Sonifier 450; VWR Scientific) before analysis by flow cytometry (BD Accuri C6).

Phos-tag gels

Yeast were grown to ~0.4 OD₆₀₀/ml and then incubated with 10 μ g/ml α -factor for 3 hr. Cells were then washed 2 $\times$ with YPD, and resuspended in YPD containing 10 μ g/ml Pronase from *Streptomyces griseus* (Sigma [Sigma Chemical], St. Louis, MO). Aliquots were collected from logarithmic cultures, from cultures arrested in G1, and then every 15 min for 1 hr upon release. Whole-cell extracts were prepared from each aliquot as follows. Yeast were collected by centrifugation and washed once with cold 20% TCA. Yeast were resuspended in 250 μ l with 20% TCA, and lysed by adding 250 μ l of zirconia/silica beads (catalog number 11079105z; BioSpec) and vortexing for ~20 min at 4°. Lysates were transferred to fresh tubes. Beads were washed with 300 μ l 20% TCA and washes were combined for each aliquot. Next, 700 μ l of 20% TCA was added to lysates and aliquots were centrifuged at $14,000 \times g$ for 10 min at 4°. Supernatants were discarded, pellets were washed with cold 750 μ l 100% EtOH, and aliquots were centrifuged at $14,000 \times g$ for 10 min at 4°. Supernatants were discarded and pellets were resuspended to a final concentration of 0.05 OD₆₀₀/ml in 2 $\times$ SDS-PAGE sample buffer that had been diluted from 4 $\times$ with 1 M Tris-HCl, pH 8.0. Whole-cell extracts were then resolved on 7% SDS-PAGE gels containing 30 μ M Phos-tag reagent (catalog number 304-93521; Wako Pure Chemical Industries) and 30 μ M MnCl₂. Prior to transferring proteins to PVDF membranes, the SDS-PAGE gel was washed in transfer buffer (25 mM Tris and 192 mM glycine) containing 2 mM EDTA for 10 min, and then washed in transfer buffer for 10 min. After transfer, the membranes were blocked overnight in blocking buffer (5% milk and PBS-T) at 4°, then incubated with anti-FLAG antibodies (catalog number ab45766; Abcam) (1:2500 in PBS-T). Membranes were then washed with PBS-T and incubated with anti-mouse HRP-conjugated secondary antibodies (catalog number NA931VS; GE Healthcare Life Sciences) (1:10,000 in PBS-T). Images of the membranes were collected using a ChemiDoc XRS+ System.

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the findings of the article are present within the article, figures, and tables. Supplemental material available at Figshare: <https://doi.org/10.25386/genetics.7427468>.

Results

Loss of CAF-1 restores silencing at HMRae**, which is suppressed by overexpression of SAS2

Loss of genes encoding H4 K16-specific acetyltransferase SAS-I subunits *SAS2*, *SAS4*, or *SAS5*, loss of H4 K16 acetylation via the H4 K16R mutation, or catalytic inactivation of

Figure 1 *cdc7* and *cac1* mutants restore silencing at *HMRae***. (A) *HMRae*** contains point mutations (asterisks) within the Rap1p- and Abf1p-binding sites of the *HMR* *E* silencer. (B) Silencing of *HMRae*** in *cac1Δ* mutants requires *SIR2*. (C) Silencing at *HMRae*** in *cac1Δ* and *asf1Δ* mutants is disrupted by overexpression of *SAS2*. (D) *cdc7-90* and *cac1Δ* mutants restore silencing at *HMRae***. (E) Silencing of *HMRae*** in *cdc7-90* mutants requires *SIR2*. (F) Overexpression of *SAS2* disrupts silencing at *HMRae*** in *cdc7-90* mutants. (G) Overexpression of *SAS2*, but not catalytically inactive *SAS2-M1*, disrupts silencing at *HMRae*** in *cac1Δ* and *cac1Δ cdc7-90* mutants. Cells with the indicated genotypes were grown on YPD or YM (0.67% yeast nitrogen base without amino acids, 2% D-glucose, and 2% Bacto agar) with supplements, at 23° (for *cdc7-90* mutants) or 30° overnight, then replica plated onto either YM plates lacking supplements and containing a lawn of *MATa* cells or YPD plates, and were grown at 23° or 30° for 1–2 days. Only cells that were able to mate grew on YM plates lacking supplements.

Sas2p restores silencing at *HMRae*** (Reifsnyder *et al.* 1996; Ehrenhofer-Murray *et al.* 1997; Xu *et al.* 1999; Meijsing and Ehrenhofer-Murray 2001; Miller *et al.* 2008; Yang *et al.* 2008). *Sas4p* interacts with the chromatin assembly factor *Asf1p* (Meijsing and Ehrenhofer-Murray 2001; Osada *et al.* 2001; Sutton *et al.* 2001) and loss of *ASF1* also restores silencing at *HMRae*** (Meijsing and Ehrenhofer-Murray 2001; Miller *et al.* 2008). As SAS-I also immunoprecipitates with CAF-1 (Meijsing and Ehrenhofer-Murray 2001), and *Asf1p* and CAF-1 interact *in vitro* and *in vivo* (Krawitz *et al.* 2002; Liu *et al.* 2012) (T. Young and A. L. Kirchmaier, unpublished data), we tested if inactivation of CAF-1 via loss of *CAC1* also led to restoration of silencing at *HMRae***. Like in *asf1Δ* mutants, silencing was restored at *HMRae*** in *cac1Δ* mutants relative to wild-type in a *SIR2*-dependent manner (Figure 1B) in patch mating assays [see also Miller and Kirchmaier (2014)]. Consistent with restoration of silencing occurring through defects in SAS-I-mediated H4 K16ac during chromatin assembly, overexpression of *SAS2* disrupted both *cac1Δ*- and *asf1Δ*-mediated silencing at *HMRae*** (Figure 1C).

cdc7 mutants have defects in H4 K16 acetylation

As Cdc7-dependent phosphorylation of the human ortholog of *Cac1p*, p150, promotes monomerization of p150 *in vitro*, and interaction between p150 and PCNA (Gerard *et al.* 2006), we considered whether *Cdc7p* could regulate CAF-1-dependent

functions in yeast. Consistent with this model, *cdc7-90* mutants also restored silencing at *HMRae*** in a *SIR2*-dependent manner [Figure 1, D and E; see also Axelrod and Rine (1991)]. Similar to *cac1Δ* and *asf1Δ* mutants (Figure 1C), overexpression of *SAS2* also disrupted both *cdc7-90*- and *cac1Δ cdc7-90*-mediated silencing at *HMRae*** (Figure 1, F and G). In contrast, overexpression of a catalytically inactive mutant of *Sas2p*, *Sas2-M1p* [aa 219–221 GYG to AAA (Osada *et al.* 2001; Sutton *et al.* 2003)] did not disrupt silencing at *HMRae*** in *cac1Δ* and *cac1Δ cdc7-90* mutants (Figure 1G). These observations were consistent with mutation of *CDC7* having created defects in a pathway involving CAF-1 and SAS-I.

We have previously demonstrated that the PCNA mutant *pol30-8*, which has defects in CAF-1-dependent pathways (Zhang *et al.* 2000; Sharp *et al.* 2001; Miller *et al.* 2008, 2010), also restores silencing at *HMRae***, and both *cac1Δ* and *pol30-8* mutants have hypoacetylated H4 K16 in chromatin (Miller *et al.* 2008, 2010) (see also Figure 2A and Table 1). To test whether *cdc7* mutants had similar histone-modification defects, we analyzed the levels of H4 K16ac in chromatin fractions by quantitative protein blots. H4 K16ac levels in chromatin were reduced in *cdc7-90* mutants compared to wild-type yeast, as well as in *cac1Δ* and *sas2Δ* mutants (Figure 2A and Table 1, $P = 0.03$ for all mutants relative to wild-type), supporting a model in which *CDC7* promoted SAS-I-dependent H4 K16ac by modulating CAF-1 activity.

The *mcm5/bob1-1* mutant of the replicative helicase acts as a bypass suppressor of an essential function of *CDC7* during DNA replication (Jackson *et al.* 1993). To assess whether *bob1-1* could also suppress *cdc7*-dependent histone H4 acetylation defects, we evaluated the levels of chromatin-associated H4 K16ac in *cdc7-90 bob1-1*, *cdc7Δ bob1-1*, and *bob1-1* mutants relative to wild-type. However, although *bob1-1* could restore viability to *cdc7Δ* mutants and logarithmic *cdc7Δ bob1-1* mutants have a similar cell cycle distribution as wild-type (Hardy *et al.* 1997), H4 K16ac acetylation levels were not similarly restored in *cdc7-90 bob1-1* or *cdc7Δ bob1-1* mutants (Figure 2A and Table 1, $P = 0.03$, relative to wild-type). Surprisingly, chromatin-associated H4 K16 was also hypoacetylated in *bob1-1* mutants relative to wild-type (Figure 2A and Table 1, $P = 0.03$). This hypoacetylation was not likely related to cell cycle defects, as logarithmic *bob1-1* mutants exhibit similar cell cycle distribution profiles as wild-type (Weinreich and Stillman 1999; Sclafani *et al.* 2002), and many origins in *bob1-1* mutants exhibit the same initiation kinetics as wild-type (Hoang *et al.* 2007). Instead, these results implied that the MCM helicase plays a previously unrecognized role in promoting H4 K16ac. Whether an interaction between the MCM helicase and Asf1 that has been observed in humans (Groth *et al.* 2007) is conserved in yeast, and whether the hypoacetylation phenotype could be related to defects in this interaction, remains to be determined. Consistent with their defect in H4 K16ac, *bob1-1* mutants also restored silencing at *HMRae*** relative to wild-type (Figure 2B). In contrast, silencing was lost at *HMRae*** in *cdc7-90 bob1-1* mutants (Figure 2B), indicating that hypoacetylation of H4 K16 was not sufficient to restore silencing in this context.

As the above observations implied that *CDC7* may contribute to silencing at *HMRae*** by more than one mechanism, we monitored silencing at *HMRae*** in *cac1Δ*, *cdc7-90*, and *sas2Δ* cells plus double- and triple-mutant combinations relative to wild-type yeast via quantitative mating (Table 2). In these analyses, *cac1Δ* and *cdc7-90* mutants interacted synthetically to restore silencing at this locus ($P = 0.000097$, Jonckheere–Terpstra trend test), as did *cdc7-90* and *sas2Δ* mutants ($P = 0.0027$, Jonckheere–Terpstra trend test). However, a major difference in mating efficiency between *sas2Δ* and *cac1Δ sas2Δ* mutants was not observed ($P = 0.1$, Wilcoxon rank-sum test), consistent with our observations demonstrating that *sas2Δ* mutants have a more severe defect in H4 K16ac than do *cac1Δ* mutants (Figure 2A and Table 1; Miller *et al.* 2010). In contrast, *cac1Δ cdc7-90 sas2Δ* mutants mated more efficiently than did any single mutant ($P = 0.05$, Wilcoxon rank-sum test). Together these results, and those outlined below, implied that *CDC7* could affect silencing by multiple mechanisms, at least one of which was independent of *CAC1* or *SAS2*.

cdc7-90 functions in a H3 T45 phosphorylation-independent manner to restore silencing at *HMRae***

In budding yeast, *Cdc7p* phosphorylates H3 T45 *in vitro* and *in vivo* in a cell cycle-dependent manner (Baker *et al.* 2010).

Figure 2 *bob1-1* and *Cdc7p* substrate H3 T45 affect silencing. (A) *cdc7* and *bob1-1* mutants have defects in chromatin-associated H4 K16ac. Logarithmically growing yeast with indicated genotypes were all grown at 30°, except for *cdc7-90* mutants, which were grown at 23°. Chromatin fractions were generated, and analyzed by immunoblotting with anti-H4 K16ac and anti-H3 antibodies. Representative protein blot for data quantified in Table 1 is shown. (B) *bob1-1* mutants restore silencing at *HMRae*** but suppress silencing in *cdc7* mutants. Patch mating assays were conducted with the indicated *MATα HMRae*** strains at 30° (left) or 23° (right), as outlined in Figure 1. (C) *H4 K16R*, but not *H3 T45A*, restores silencing at *HMRae***. Patch mating assays were conducted with the indicated *MATα HMRae*** strains as above. (D) Map of *HMR::ADE2*. (E) Colony color assay. *cac1Δ* and *H3 T45A* interact synthetically in silencing *HMR::ADE2*. Yeast with indicated genotypes were grown overnight in YPD, spotted onto a CSM-URA plate, incubated for 2 days at 30°, and then incubated for 4 days at 4° prior to imaging. CSM, complete supplement mixture; WT, wild-type.

As defects in certain histone modifications (H3 K56ac and H4 K16ac) restore silencing at *HMRae*** (Meijsing and Ehrenhofer-Murray 2001; Miller *et al.* 2008) and *cdc7* influenced silencing by more than one mechanism (see above), we tested whether loss of H3 T45 phosphorylation could also

Table 1 *cdc7*, *cac1*, and *bob1-1* mutants have defects in chromatin-associated H4 K16ac

Genotype	Relative levels of H4 K16ac ^a
Wild-Type	1.0
<i>sas2Δ</i>	0.32 ± 0.081
<i>cac1Δ</i>	0.54 ± 0.24
<i>cdc7-90</i>	0.64 ± 0.041
<i>bob1-1</i>	0.63 ± 0.10
<i>cdc7-90 bob1-1</i>	0.44 ± 0.24
<i>cdc7Δ bob1-1</i>	0.23 ± 0.10

^a Chromatin fractions were analyzed by immunoblotting using anti-H4 K16ac and anti-H3 antibodies (see *Materials and Methods* and Figure 2A). H4 K16ac was normalized to H3 and expressed as a percentage of H4 K16ac levels in wild-type cells. Average ± SD, *n* = 3. Each mutant was hypoacetylated relative to wild-type (*P* = 0.030; Wilcoxon rank-sum test).

restore silencing at *HMRae***. Silencing at *HMRae*** in H3 T45A mutants was compared to H4 K16R mutants, or wild-type histones H3 and H4, in patch mating assays. In contrast to *cdc7-90* (Figure 1, D–F) and H4 K16R mutants (Figure 2C), H3 T45A mutants did not restore silencing at *HMRae*** (Figure 2C). Together, these results are consistent with *CDC7* acting via a H3 T45-independent mechanism to restore silencing at *HMRae***.

To further explore potential impacts of *Cdc7p*-dependent phosphorylation of H3 T45 on silencing, we monitored silencing at the *HMR::ADE2* reporter gene (Figure 2D) in H3 T45A, *cac1Δ*, and H3 T45A *cac1Δ* mutants relative to wild-type yeast by monitoring the color of colonies. When silent chromatin is formed at *HMR::ADE2* in wild-type cells, colonies appear red in color, which is caused by an accumulation of a pigment when *ADE2* is not expressed. However, if silencing is lost, then *ADE2* will be expressed and colonies will be white. If a defect in the establishment of silencing occurs, then colonies will have a sectorized appearance, with some sectors being pink/red (originating from a cell that is silenced at *HMR::ADE2* plus its descendants) and others being white (originating from a genetically identical cell that is transcriptionally active at *HMR::ADE2* plus its descendants). Defects in maintenance or inheritance of silent chromatin can result in pink colonies due to an unstable silenced state (van Leeuwen and Gottschling 2002; Miller and Kirchmaier 2014). Thus, *HMR::ADE2* reports on different attributes of epigenetic processes compared to *HMRae***, which only requires that the silenced state of a single cell exists in G1 phase to enabling mating. In the colony color assay, *cac1Δ* mutants grew as pink- and white-sectorized colonies, and H3 T45A mutants were a mix of red, white, and pink colonies. When the H3 T45A mutant was expressed in *cac1Δ* mutants, more pink and white colonies grew, and fewer red colonies were present (Figure 2E). The impact of constitutive phosphorylation at H3 T45 on silencing could not be assessed as the phosphomimic H3 T45D was lethal [data not shown, see also Baker *et al.* (2010) for lethality of H3 T45E]. Thus, although modulating the phosphorylation state of H3 T45 does not restore silencing at *HMRae***, loss of *Cdc7p*-dependent phosphorylation of H3 T45 can affect silencing in other contexts.

Table 2 Synthetic interaction analyses measuring silencing at *HMRae***

Genotype	Relative efficiency of mating ^a
Wild-type <i>HMRae**</i>	1
<i>HMRae** cac1Δ</i>	118 ± 35.1
<i>HMRae** cdc7-90</i>	172 ± 48.5
<i>HMRae** sas2Δ</i>	1460 ± 513
<i>HMRae** cac1Δ sas2Δ</i>	905 ± 188
<i>HMRae** cdc7-90 sas2Δ</i>	3140 ± 1250
<i>HMRae** cac1Δ cdc7-90</i>	2390 ± 788
<i>HMRae** cac1Δ sas2Δ cdc7-90</i>	2850 ± 863

^a The efficiency of mating of the *MATα HMRae*** strain (wild-type) to tester strain BY4741 (*MATa*) was determined relative to its plating efficiency on minimal (YM: 0.67% yeast nitrogen base without amino acids, 2% D-glucose, and 2% Bacto agar) medium containing supplements (0.0202 ± 0.00440%, *n* = 3), and was set to 1. The mating efficiency of each strain with the indicated genotype relative to this strain is shown. Average ± SD, *n* = 3.

Cac1p and *Cdc7p* associate in a cell cycle-dependent manner

The vertebrate ortholog of *Cac1p*, p150, interacts with Dbf4, the regulatory subunit of the cell cycle-dependent kinase *Cdc7* *in vitro*, and Dbf4/*Cdc7* coprecipitates with p150 in mammalian cell extracts (Gerard *et al.* 2006). To determine if interactions between *Cdc7p* and *Cac1p* were conserved in yeast, we applied FLIM-FRET (see *Materials and Methods*). The fluorescence lifetime of GFP was measured in live cells expressing either *Cdc7*-GFPp (donor of FRET) only, or *Cdc7*-GFPp plus *Cac1*-mCherry (acceptor of FRET) or *Spc29*-mCherry (negative control acceptor) in small-budded, logarithmically growing cells. In these analyses, the fluorescence lifetime of GFP decreased in budded cells expressing both *Cdc7*-GFPp and *Cac1*-mCherry relative to cells expressing *Cdc7*-GFPp alone, indicating that *Cdc7p* and *Cac1p* were colocalized within 10 nm of each other in the nuclei of live small-budded cells (Figure 3, A and B). To quantitate these protein–protein interactions, FRET efficiency between *Cdc7*-GFPp and *Cac1*-mCherry was calculated from the fluorescence lifetimes obtained from the TCSPC decay histograms that were fitted with a double exponential function. The FRET efficiency of *Cdc7*-GFPp with *Cac1*-mCherry was 16 and 10% in independent analyses of two clones. In contrast, no such interaction was observed in cells expressing *Cdc7*-GFPp and the control *Spc29*-mCherry (Figure 3B). To test if this interaction between *Cdc7p* and *Cac1p* was cell cycle-dependent, we performed the same experiment with live cells arrested in G1 phase with α -factor. Under these conditions, the lifetime of GFP in cells expressing both *Cdc7*-GFPp and *Cac1*-mCherry did not change compared with negative controls in G1 phase (Figure 3, A and B). As yeast *Cdc7p* is expressed throughout the cell cycle, but *Cdc7p*'s regulatory subunit *Dbf4p* is degraded outside of S phase (Chapman and Johnston 1989; Weinreich and Stillman 1999), these results indicated that *Cac1p* and *Cdc7p* interacted in the nucleus in a cell cycle-dependent manner *in vivo*. This interaction potentially occurred via targeting of *Cdc7p* to *Cac1p* by *Dbf4p*, although a *Dbf4p*-*Cac1p* interface has not yet been defined.

Figure 3 Cac1p and Cdc7p interact in a cell cycle-dependent manner. (A) Confocal fluorescence lifetime images of GFP in strains expressing the indicated fluorescently tagged proteins. FLIM Bar, 2 ns, blue; 3 ns, red. (B) FRET efficiency of the indicated strains. Live cells were analyzed by FLIM-FRET, with S phase cells reflecting small-budded cells from logarithmic cultures and G1 phase cells reflecting yeast that had been arrested using α -factor. FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer.

Cac1p is phosphorylated in a cell cycle-dependent manner

To determine if this cell cycle-dependent interaction between Cdc7p and Cac1p correlated with phosphorylation of Cac1p, we monitored phosphorylation of Cac1p in synchronized cells when transitioning from G1 to S phase using Phos-tag gels. In this experiment, logarithmic *CAC1-3X FLAG* cells were arrested in G1 phase using α -factor, and then released into S phase. Samples were collected from logarithmic growing cells, G1-arrested cells, and every 15 min for 1 hr after release into S phase. Cac1p was then analyzed by resolving whole-cell extracts on a Phos-tag gel, which reduces the mobility of phosphorylated proteins through an SDS-PAGE gel, resulting in a shift to a greater molecular weight for phosphorylated proteins (Kinoshita *et al.* 2006). A larger-molecular weight band, indicating that Cac1p was phosphorylated, was present in the logarithmic growing samples, but disappeared once the cells are arrested in G1 phase (Figure 4A). However, upon release into S phase, the larger molecular weight band reappeared, indicating that Cac1p was phosphorylated in a cell cycle-dependent manner. To test if the phosphorylated form of Cac1p disappeared upon the deactivation of CDC7, we performed the same experiment using a *CAC1-3X FLAG cdc7-90 bob1-1* strain, thereby bypassing the requirement for Cdc7p to initiate DNA replication (Hardy *et al.* 1997) and allowing cells to grow at 30°. However, a

similar phosphorylation pattern of Cac1p was observed in the *CAC1-3X FLAG cdc7-90 bob1-1* cells (Figure S1), indicating that Cdc7p was not the only kinase that phosphorylated Cac1p in S phase. These results were consistent with work that was published during the course of this study, which observed both CDC28-dependent and CDC28-independent cell cycle-regulated phosphorylation of Cac1p, and also identified Cac1p as an *in vitro* substrate for Cdc7p at S503 (Jeffery *et al.* 2015). However, in contrast to *cdc7* mutants, *cdc28* mutants are unable to restore silencing at *HMRae*** (Ehrenhofer-Murray *et al.* 1999), implying that phosphorylation of Cac1p by Cdc28p does not regulate CAF-1/SAS-I-dependent H4 K16ac.

cac1 phospho-mutants affect silencing at HMRae**

To evaluate the impact of Cdc7p-dependent phosphorylation of Cac1p on silencing at *HMRae***, we examined potential Cdc7p targets on Cac1p. Cdc7p preferentially phosphorylates serines or threonines within the consensus site (S/T-D/E) (Masai *et al.* 2000; Cho *et al.* 2006; Sasanuma *et al.* 2008). One potential target of Cdc7p on Cac1p is S238, which is adjacent to the sole PIP box (a PCNA-interaction protein motif) on Cac1p and is phosphorylated in budding yeast *in vivo* (Swaney *et al.* 2013; Zhou *et al.* 2016). S238 in Cac1p was mutated to A or D to mimic constitutively unphosphorylated

Figure 4 *cac1* mutants with defects in phosphorylation promote silencing at *HMRae***. (A) *Cac1p* is phosphorylated in a cell cycle-dependent manner. Whole-cell extracts prepared from *CAC1-3X FLAG* cells collected during logarithmic growth, G1 arrest, and every 15 min for 1 hr after release into S phase. Samples were separated on a 30 μ M Phos-tag gel, a 7% SDS-PAGE gel, and analyzed by immunoblotting with anti-FLAG antibodies followed by incubating with anti-mouse HRP-conjugated antibodies (See *Materials and Methods*). (B and C) *cac1 S238A* and *S503A* mutants silence *HMRae***. Patch mating assays were performed as in Figure 1 with *MAT α HMRae** cac1 Δ ::KanMX* strains expressing *CAC1*, *cac1 S238* (B), or *S503* (C) mutants, or vector alone, except yeast were grown on CSM-URA, the *MATa* tester strain was BY4741, and yeast were grown for 3 days at 23° prior to imaging. CSM, complete supplement mixture.

or phosphorylated states, respectively, at this residue, and *cac1* mutants were expressed from an *ARS/CEN* plasmid in *HMRae** cac1 Δ* strains. Silencing at *HMRae*** was then evaluated by patch mating assays. Expression of *CAC1* or *cac1 S238D*, but not *S238A* or vector alone, in *MAT α HMRae** cac1 Δ* mutants disrupted silencing at *HMRae*** (Figure 4B and Table 3), consistent with phosphorylation at this *Cdc7p* consensus site being important for *Cac1p* function.

S503 of yeast *Cac1p* also lies within a *Cdc7p* consensus site, is phosphorylated by *Cdc7p* *in vitro* (Jeffery *et al.* 2015), and has been verified to be phosphorylated *in vivo* by mass spectrometry in high-throughput studies as well as during the course of this work [data not shown; see also Albuquerque *et al.* (2008), Gnad *et al.* (2009), Swaney *et al.* (2013), Jeffery *et al.* (2015), and Zhou *et al.* (2016)]. To assess effects of phosphorylation of this residue on *Cac1p* function, *CAC1*, *cac1 S503A*, *cac1 S503D*, or vector alone were introduced into *MAT α HMRae** cac1 Δ* mutants, and silencing was evaluated by patch mating assays. *cac1 S503D* partially disrupted silencing at *HMRae*** in *cac1 Δ* mutants, similar to wild-type *CAC1*. In contrast, *cac1 S503A* mutants, like vector alone, supported silencing at *HMRae*** (Figure 4C and Table 3), indicating that the charge state at this residue also affected *Cac1p* function. In addition, the combined *cac1 S238A S503A*

mutant correlated with enhanced silencing at *HMRae*** relative to single mutants and wild-type in quantitative mating assays (Table 3, $P = 0.001$), consistent with the charge state of both residues contributing to function.

To confirm that the above observations were not due to a lack of protein expression, the above experiments were repeated in analogous strains expressing *CAC1-3XFLAG* or *cac1-3XFLAG* phospho-mutants. The *cac1-3XFLAGp* phospho-mutants were expressed efficiently, with some clone-to-clone variation for tagged genotypes observed relative to wild-type [Figure S2A and data not shown, see Jeffery *et al.* (2015)] for telomeric variegation). Overall, similar patterns in silencing at *HMRae*** were observed for both the untagged and tagged strains (Figure 4, B and C, Figure S2, B and C, and Table 3, and data not shown). Moreover, *cac1p* with mutations at these residues could rescue defects in CAF-1-dependent responses to DNA damage (see below), indicating that expression was sufficient to restore other functions of CAF-1.

H4 K16 is hypoacetylated at HMRae** in cac1 phospho-mutants

The above results supported our hypothesis that silencing at *HMRae*** had been restored in the *cac1* and *cdc7* mutants via rescue of Sir recruitment due to hypoacetylation of H4 K16 at the *e*** silencer due to defects in CAF-1-dependent H4 K16ac by SAS-I. To test this hypothesis further, we monitored H4 K16ac levels at *HMRae*** by ChIP in *MAT α HMRae** cac1 Δ sir2 Δ* strains containing plasmids expressing *CAC1*, *cac1 S238A*, *cac1 S503A*, or *cac1 S238A S503A* mutants, or vector. Conducting this experiment in cells lacking *SIR2* enabled separation of CAF-1–SAS-I pathway-dependent effects from *Sir2p*-dependent effects on histone acetylation. Both *e*** and *a1* were hypoacetylated in strains expressing each *cac1* single or double mutant, or vector relative to those expressing *CAC1* (Table 4; $P = 0.030$), consistent with restoration of silencing at *HMRae*** being facilitated by hypoacetylation of histone H4 K16 at this locus.

cac1 S238 and S503 mutants support silencing at HMR::ADE2

As loss of *CAC1* causes defects in silencing at *HMR::ADE2* [see Enomoto and Berman (1998), Meijsing and Ehrenhofer-Murray (2001), and Miller *et al.* (2008)], we also assessed silencing in *cac1* double-phosphorylation mutants by expressing either wild-type *CAC1*, *cac1 S238*, *S503A*, *cac1 S238*, *S503D*, *cac1 S501*, *S503A*, *cac1 S501*, *S503D*, or vector alone in *HMR::ADE2 cac1 Δ* strains and monitoring colony color. Like *S238* and *S503*, *S501* of *Cac1p* is phosphorylated, but the kinase targeting this residue and the function of this phosphorylation event are unknown (Zhou *et al.* 2016). Yeast expressing *CAC1* were red, indicating that *HMR* was silenced, and *cac1 Δ* mutants with the empty vector were sectorized, indicating a silencing defect (Figure S3). *HMR::ADE2 cac1 Δ* strains expressing any of the *CAC1* mutants were also red, indicating that the changes in phosphorylation status of *Cac1p* at S238 plus S503, or S501 plus S503, could support

Table 3 *cac1* phospho-mutants rescue silencing at *HMRae***

Genotype	Relative efficiency of mating ^a
<i>HMRae</i> ** <i>cac1Δ</i> + <i>CAC1</i>	1
<i>HMRae</i> ** <i>cac1Δ</i> + <i>cac1</i> S238A	4.3 ± 1.6
<i>HMRae</i> ** <i>cac1Δ</i> + <i>cac1</i> S503A	2.3 ± 0.42
<i>HMRae</i> ** <i>cac1Δ</i> + <i>cac1</i> S238,503A	8.9 ± 1.9

^a The efficiency of mating of the indicated *MATα* *HMRae*** *cac1Δ* yeast expressing *CAC1* exogenously from a low-copy plasmid (pAK1223) to tester strain BY4741 (*MATα*) was determined relative to their plating efficiency on minimal (YM: 0.67% yeast nitrogen base without amino acids, 2% D-glucose, and 2% Bacto agar) medium containing supplements (0.43 ± 0.11%, *n* = 3), and was set to 1. The mating efficiency of each strain with the indicated genotype relative to this strain is shown. Avg. ± SD, *n* = 3. Combining single mutations correlated with enhanced silencing at *HMRae*** (*P* = 0.001; Jonckheere–Terpstra trend test).

silencing at *HMR::ADE2* (Figure S3), consistent with hypoacetylation of H4 K16 being permissive for silencing.

To further explore the role of phosphorylation of these *Cac1p* residues, we expressed either *CAC1*, *cac1* S238, 503A, *cac1* S238, 503D, or vector alone in several *pol30* mutant backgrounds with defects in responses to DNA damage (Kirchmaier 2011). Expression of the *cac1* phospho-mutants in *HMR::ADE2* *cac1Δ* strains containing the *pol30* mutations (see below) restored silencing to a similar extent as *CAC1* [Figure S3, see also Kirchmaier (2011)]. Together, these results indicated that the *cac1* phospho-mutants were largely competent for silencing a *HMR* locus containing a wild-type *E* silencer, implying that the mutants could function in replication-coupled chromatin assembly.

***cdc7-90* and *cac1Δ* synthetically interact in the presence of DNA damage**

The above analyses collectively implied *Cdc7p*-regulated silencing through both *Cac1p*-mediated and *Cac1p*-independent processes, and vice versa. *cdc7* and *cac1Δ* mutants are also sensitive to DNA-damaging reagents (Kaufman *et al.* 1997; Game and Kaufman 1999; Pessoa-Brandao and Sclafani 2004; Linger and Tyler 2005; Baker *et al.* 2010), and changes in the phosphorylation status of *Cac1p* at S501 and S503 occurs upon exposure to DNA damage (Zhou *et al.* 2016). To assess whether *Cdc7p* and *Cac1p* were also involved in responses to DNA damage through interdependent (e.g., promoting H4 K16ac) or independent processes, we initially conducted synthetic interaction analyses between *cac1Δ* and *cdc7-90* mutants in the presence or absence of HU [an inhibitor of ribonucleotide reductase, which slows replication forks (Krakoff *et al.* 1968)], MMS [an alkylating agent causing single- and double-stranded DNA breaks (Schwartz 1989)], or Zeocin [causes double-stranded DNA breaks (Chankova *et al.* 2007)] using serial dilution growth assays. *cdc7-90* *cac1Δ* mutants had greater defects in growth relative to either single mutant or wild-type cells on rich media, and this defect was further enhanced in the presence of HU, MMS, or Zeocin (Figure 5A), indicating that at least one of these proteins functioned in a second pathway during responses to DNA damage. As *sas2Δ* mutants did not exhibit growth defects relative to wild-type upon exposure to MMS,

bleomycin, HU, or UV (data not shown), these damage sensitivity phenotypes of *cac1Δ*, *cdc7-90*, and *cac1Δ* *cdc7-90* mutants were likely independent of their defects in H4 K16ac.

As phosphorylated H3 T45 accumulates in the presence of HU or camptothecin, but not MMS (Baker *et al.* 2010), and H3 T45A mutants are sensitive to HU and camptothecin (Baker *et al.* 2010), the above synthetic interaction could have been related, in part, to defects in *Cdc7p*-dependent phosphorylation of H3 T45. To test this possibility, we monitored damage sensitivity in wild-type cells relative to *cac1Δ*, *H3 T45A*, and *cac1Δ* *H3T45A* mutants. A minor decrease in growth of the double mutant relative to the *H3 T45A* mutant in HU was observed, and implied that phosphorylation defects at H3 T45 may have contributed to the synthetic interaction observed between *cdc7-90* and *cac1Δ* mutants (Figure 5B) (see Discussion).

As *Cdc7* participates in the activation and attenuation of DNA damage checkpoint responses (Takeda *et al.* 2001; Kim *et al.* 2008; Tsuji *et al.* 2008; Sheu and Stillman 2010), the synthetic defect in *cac1Δ* *cdc7-90* mutants in response to DNA damage (Figure 5A) raised the possibility that the *cac1* *cdc7-90* mutants had a defect in checkpoint activation. Therefore, we analyzed the phosphorylation status of *Rad53p* in wild-type, *cac1Δ*, *cdc7-90*, and *cac1Δ* *cdc7-90* mutants, which were grown in rich media in the presence or absence of HU, for 3 hr at permissive temperature prior to harvesting cells for analysis of extracts by protein blotting. Under these conditions, *Rad53p* became phosphorylated efficiently when treated with HU in both wild-type and the mutants tested, indicating that the increase in sensitivity to DNA damage for the *cac1Δ* *cdc7-90* mutant was not caused by the failure to activate *Rad53p* *per se* (Figure 6A).

We next tested if the increased sensitivity to DNA damage in *cdc7-90* *cac1Δ* mutants was instead related to a defect in cell cycle progression upon release from DNA damage-induced arrest. To do so, we monitored the progression of *cac1Δ* *cdc7-90* mutants through the cell cycle after release from HU relative to wild-type cells or single mutants by flow cytometry. Logarithmically growing cells were treated with HU for 3 hr, washed, and then released into fresh YPD. Samples were collected before the arrest (asynchronous), during the arrest (0 min), and every 15 min for 3 hr after release from arrest. In this experiment, *cac1Δ* *cdc7-90* cells reentered the cell cycle more rapidly relative to single mutants or wild-type cells (Figure 6B). Further studies will be required to clarify whether the altered cell cycle profile in these mutants reflects impaired recovery from the DNA damage response or a defect in another cellular process having contributed to the negative synthetic interaction observed in Figure 5A.

***cac1* phospho-mutants suppressed synthetic interactions between *pol30* and *cac1Δ* mutants**

Recently, Elledge and colleagues quantified global changes in the phosphorylation of proteins in budding yeast upon exposure to DNA damage (Zhou *et al.* 2016), and identified *Cac1p* as being differentially phosphorylated upon treatment with

Table 4 H4 K16 is hypoacetylated at *HMRae* in *cac1* phospho-mutants**

Genotype	Relative levels of H4 K16ac ^a	
	e**	a1
<i>CAC1</i>	1	1
<i>cac1 S238A</i>	0.051 ± 0.022	0.045 ± 0.019
<i>cac1 S503A</i>	0.27 ± 0.22	0.14 ± 0.10
<i>cac1 S238,503A</i>	0.11 ± 0.014	0.079 ± 0.020
Vector	0.072 ± 0.036	0.037 ± 0.010

^a H4 K16ac levels at the e** silencer and a1 in *MATα HMRae** sir2Δ cac1Δ* strains with plasmids expressing *CAC1*, the indicated *cac1* mutants, or vector were monitored by chromatin immunoprecipitation. The efficiency of coprecipitation of DNA with H4 K16ac antibodies was determined relative to that with anti-H3 antibodies, and normalized to yeast expressing *CAC1*, which was set to 1. Data were calculated as $2^{[(H4\ K16ac\ C_T - H3\ C_T)_{CAC1} - (H4\ K16ac\ C_T - H3\ C_T)_{genotype}]}$. Mean ± SEM, *n* = 3. Each mutant and vector were hypoacetylated relative to *CAC1* at both e** and a1 (*P* = 0.030; Wilcoxon rank-sum test).

MMS, HU, or IR, irradiation. In this analysis, the number of phosphopeptides identified with phosphorylation at S501 or S503 on *Cac1p* decreased or increased, respectively. As *CDC7* functions in DNA repair (Ogino *et al.* 2006; Sasanuma *et al.* 2008; Wan *et al.* 2008; Vaziri and Masai 2010; Princz *et al.* 2017) and can phosphorylate S503 on *Cac1p* *in vitro* (Jeffery *et al.* 2015), we evaluated effects of phosphorylation mimics at these residues on responses to DNA damage. To do so, we expressed *cac1 S501, 503A* or *cac1 S501, 503D* mutants in *POL30 cac1Δ* cells, as well as in sensitized genetic backgrounds of *pol30* mutants exhibiting defects in several repair pathways (Figure S4, A and B), including translesion synthesis DNA repair and error-free DNA repair (loss of mono- or polyubiquitination, respectively, of *PCNA* in *pol30 K164R* mutants), and homologous recombination (loss of sumoylation of *PCNA* in *pol30 K127R* and *pol30 K127, 164R* mutants) [see Hofmann and Pickart (1999), Hoege *et al.* (2002), Haracska *et al.* (2004), Chiu *et al.* (2006), Frampton *et al.* (2006), Motegi *et al.* (2006), Unk *et al.* (2006, 2008), Branzei *et al.* (2008), Windecker and Ulrich (2008), Parker and Ulrich (2009), and Kirchmaier (2011), and references within]. Like *CAC1*, expression of *cac1 S501, 503A* or *cac1 S501, 503D* mutants in *POL30 cac1Δ* cells suppressed DNA damage sensitivity relative to vector alone (Figure S4, A and B), implying that the *cac1* phospho-mutants were competent for DNA repair-coupled chromatin assembly. *pol30 K164R cac1Δ* mutants containing vector alone were hypersensitive to MMS, HU, and UV relative to either single mutant (Figure S5, A and B). Expression of *cac1* phosphorylation mutants in *pol30 K164R cac1Δ* cells suppressed this hypersensitivity, similar to *CAC1*. In contrast, strong negative synthetic interactions between *cac1Δ* and *pol30 K127R* or *pol30 K127R K164R* mutants were not observed, although *pol30 K127R* partially suppressed hypersensitivity of *cac1Δ* mutants to MMS (Figure S4A), revealing that altering a key binding surface on *PCNA* and/or preventing sumoylation of *PCNA* on K127 in response to DNA damage by an alkylating agent partially overcame a DNA repair defect related to CAF-1-dependent chromatin assembly.

We next conducted similar analyses for *cac1 S238, 503A* or *cac1 S238, 503D* mutants (Figure S5) to target both *Cdc7p* consensus sites on *Cac1p*. Expression of *cac1 S238, 503A* or *cac1 S238, 503D* mutants, but not vector alone, in *POL30 cac1Δ* cells similarly suppressed DNA damage sensitivity (Figure S5). DNA damage sensitivity was also suppressed in the *pol30 cac1Δ* mutants (Figure S5). Thus, the charge states of these residues on *Cac1p* were not critical for repair-related role(s) of *Cac1p* under the conditions tested. Collectively, the above results implied that *Cdc7p*-mediated regulation of H4 K16ac via phosphorylation of *Cac1p* was not required for either *Cdc7p*'s or *Cac1p*'s functions during responses to multiple types of DNA damage.

Discussion

In this study, we provide evidence that *CDC7* can influence silencing through a *Cac1p*-mediated pathway that affects H4 K16ac. We demonstrated that defects in *CDC7* or *CAC1* restored silencing to *HMRae***, and that overexpression of *SAS2* partially suppressed this phenotype (Figure 1), likely by modulating H4 K16ac levels as chromatin-associated H4 K16 levels were decreased in *cdc7* and *cac1* mutants (Figure 2A and Table 1, and model in Figure 7A). We also showed that *Cdc7p* interacted with *Cac1p* during S phase, but not in G1 phase (Figure 3), which correlated with cell cycle-dependent phosphorylation of *Cac1p* (Figure 4A). In addition, we showed that *CDC7* and *CAC1* could function in independent pathways in response to DNA damage (Figure 5A and Figure 6), but that phosphorylation of *Cac1p* at *Cdc7p* consensus sites was not required for function in the presence of DNA damage (Figures S4 and S5). Together, these observations support a model in which the deposition of H4, which is acetylated on K16 by SAS-I, is mediated by CAF-1 during S phase, and that *Cdc7p* affects silencing by regulating this function of CAF-1, potentially through affecting CAF-1-SAS-I interactions, and/or CAF-1-*PCNA* or CAF-1-DNA interactions (Figure 7B) (discussed below). Consistent with this model, H4 K16 was hypoacetylated at *HMRae*** in *cac1* mutants mimicking the hypoacetylated state at consensus sites for *Cdc7p* (Table 4).

Phosphorylation, CAF-1, and silencing

One potential way in which phosphorylation of *Cac1p* may be regulating CAF-1 function is by influencing CAF-1's interactions with other proteins. Human p150 is phosphorylated by the kinase Cdc7, and phosphorylation of p150 regulates the activity of p150 by stabilizing the monomeric (active) over the dimeric (inactive) form of p150 (Gerard *et al.* 2006). In yeast, *Cac1p* can dimerize *in vitro*, but whether *Cac1p*'s function is regulated by oligomerization or if phosphorylation regulates oligomerization in yeast has not been reported (Liu *et al.* 2016). However, in *cdc7-1* mutants, less *PCNA* coprecipitates with *Cac1p* than in wild-type cells (Jeffery *et al.* 2015), indicating that *Cdc7p* somehow promotes the interaction between *Cac1p* and *PCNA*. *Cdc7p* could regulate

Figure 5 *cac1Δ cdc7-90* mutants are hypersensitive to DNA damage. (A) *cac1Δ* synthetically interacts with *cdc7-90*. (B) Synthetic interaction between *cdc7-90* and *cac1Δ* is not due to the loss of H3 T45 phosphorylation. Yeast were grown overnight in YPD, and 2.5 μ l of 10-fold serial dilutions were spotted onto either YPD or YPD plates containing the indicated DNA-damaging agent, and incubated for 2 days at 23° (A) or 30° (B) prior to imaging.

silencing in this manner as *pol30* mutants with defects in interacting with CAF-1 restore silencing at *HMRae*** (Zhang *et al.* 2000; Miller *et al.* 2010). As the phosphorylation site S238 lies close to the PIP box in *Cac1p* (Rolef Ben-Shahar *et al.* 2009) and the S238A mutation results in a modest decrease in co-immunoprecipitation of PCNA with *cac1p* (Jeffery *et al.* 2015), it is possible that phosphorylation of S238 by *Cdc7p* modulates *Cac1p*'s interaction with PCNA and, in turn, silencing. Consistent with this model, like *cdc7* mutants, expression of *cac1* S238A, but not *cac1* S238D, restored silencing at *HMRae*** in *cac1Δ* mutants (Figure 4B). Alternatively, phosphorylation of this residue by *Cdc7p* may influence the stability of *Cac1p* (Jeffery *et al.* 2015) (Figure S2). However, *cac1* mutants that lack phosphorylation at *Cdc7p* consensus sites could support silencing at *HMR::ADE2* (Figure S3) and suppressed defects in the DNA damage sensitivity of *cac1Δ* mutants (Figure 5 and Figure S4). In combination, these data support a model in which *Cdc7p* influences silencing by regulating the interaction between *Cac1p* and PCNA via phosphorylation of S238 on *Cac1p* (Figure 7B).

Phosphorylation of *Cac1p* may also regulate interactions between CAF-1 and other factors such as DNA (Zhang *et al.* 2016; Sauer *et al.* 2017), SAS-I (Meijsing and Ehrenhofer-Murray 2001; Osada *et al.* 2001; Sutton *et al.* 2001), or *Sir1p* (Sharp *et al.* 2003), and phosphorylation of S503 may influence silencing through a mechanism that is different from that of S238 (Figure 7B) as *cac1* S238A S503A restored silencing at *HMRae*** more efficiently than did single mutants (Table 3). S503 falls within a region near the C-terminus of *Cac1p*, between the *Cac2p*-binding region and the winged helix domain, that promotes CAF-1's binding to DNA (Zhang *et al.* 2016; Sauer *et al.* 2017), raising the possibility that *Cdc7p* modulates interactions between *Cac1p* and DNA (Figure 7B). However, *cac1* S503Ap mutants are enriched in chromatin like *Cac1p* cells (Jeffery *et al.* 2015). Although S503 is near, but not part of, the *Cac1p*-*Cac2p*-binding interface, this residue might influence protein-protein interactions within the CAF-1 complex itself (Liu *et al.* 2016).

Phosphorylation of *Cac1p* by *Cdc7p* may also affect the interactions between *Cac1p* and SAS-I (Figure 7B). Members of the SAS-I complex coprecipitate with *Cac1p*, but not *Cac2p* or *Cac3p*, and full-length *Sas2p* interacts with aa 80–517 of *Cac1p* in yeast two-hybrid assays (Meijsing and Ehrenhofer-Murray 2001). Additionally, *cac1* S238, 503A mutants were resistant to DNA damage relative to *cac1Δ* mutants (Figures S4 and S5) and *sas2Δ* mutants did not exhibit growth defects relative to wild-type upon exposure to MMS, bleomycin, HU, or UV (data not shown). Therefore, our data best fit the model in which *Cdc7p* modulates CAF-1-SAS-I binding or function, as opposed to CAF-1-PCNA and CAF-1-DNA interactions (Figure 7), as these latter interactions likely directly contribute to DNA repair-coupled chromatin assembly. Our data also leave open the possibility that *Cdc7p* may also modulate interactions between *Cac1p* and other binding partners affiliated with epigenetic processes such as *Sir1p* (Sharp *et al.* 2003). Future analyses on cell cycle-dependent interactions with *Cac1p* should reveal regulatory role(s) of *Cdc7p* during chromatin assembly.

CDC7, MCM5, DNA replication, and silencing

Synthetic interactions between *cdc7-90* and *cac1Δ* mutants (Table 2), as well as the loss of silencing at *HMRae*** in *cdc7-90 bob1-1* mutants despite hypoacetylation of chromatin-associated H4 K16 (Figure 2A and Table 1), are consistent with *cdc7* affecting silencing by more than one mechanism. In contrast to H4 K16 hypoacetylation, loss of *Cdc7p*-dependent phosphorylation of H3 T45 did not restore silencing at *HMRae*** (Figure 2C), but instead lead to silencing defects at *HMR::ADE2* that became more severe in the absence of *CAC1* (Figure 2E). Another possible mechanism for *cdc7* to affect silencing is through origin firing. The *HMR-E* silencer can function as an inefficient origin of replication (Hurst and Rivier 1999; Raghuraman *et al.* 2001), and a slight increase in origin firing at the *e*** silencer relative to the *E* silencer at *HMR* has been observed previously (Ehrenhofer-Murray *et al.* 1999). Therefore, mutants such as *cdc7* or *cdc45-1* (Axelrod and Rine 1991; Ehrenhofer-Murray *et al.* 1999) that have

Figure 6 DNA damage checkpoint response in *cdc7-90 cac1Δ* mutants. (A) Rad53p is activated upon DNA damage in *cdc7-90 cac1Δ* mutants. (B) *cdc7-90 cac1Δ* mutants exhibit premature release from checkpoint arrest. Whole-cell extracts were prepared from yeast that were grown logarithmically to ~0.4 OD₆₀₀/ml, then incubated in YPD containing 200 mM HU for 3 hr. Extracts were separated on 10% SDS-PAGE gels and analyzed by immunoblotting with anti-Rad53p antibodies, followed by incubation with Alexa Fluor 680 anti-rabbit antibodies. Async, asynchronized.

defects in origin firing could help restore silencing at *HMRae***, and those that increase origin firing may cause defects in silencing. However, this possibility is inconsistent with the observations that *cdc7 bob1-1* mutants have an origin-firing deficiency (Hoang *et al.* 2007). Several replication factor mutants with defects in initiating replication are also unable to restore silencing to *HMRae*** (Ehrenhofer-Murray *et al.* 1999), and *cdc7-1* mutants display ARS Consensus Sequence, ACS-independent defects in telomeric silencing (Rehman *et al.* 2006). *Cdc7p* promotes DNA replication, in part, by phosphorylating MCM proteins (Lei *et al.* 1997; Weinreich and Stillman 1999; Labib 2010; Randell *et al.* 2010), which, in turn, facilitates loading of *Cdc45p* onto MCM2-7 (Tanaka and Nasmyth 1998; Geraghty *et al.* 2000; Sclafani *et al.* 2002; Sheu and Stillman 2006; Heller *et al.* 2011), whereas the *bob1-1/mcm5* mutant bypasses this phosphorylation requirement (Fletcher *et al.* 2003; Hoang *et al.* 2007; Simon *et al.* 2016). The combination of *cdc7-90* and *bob1-1* may result in unique changes to the replication fork that adversely affect silencing even though H4 K16ac is hypoacetylated in the double mutants (Figure 2A and Table 1). In this scenario, hypoacetylation of H4 K16 would promote Sir recruitment to *HMRae*** in the *cdc7-90 bob1-1* mutants, but silent chromatin would be inefficiently maintained or inherited during replication, resulting in expression of both α and α mating-type information, and a failure to mate. One possible explanation is that *bob1-1* mutants may compromise interactions between *Sir2p* and *Mcm3p* or *Mcm7p* proteins, which are mediated by *Mcm10p* and are important for silenc-

ing (Liachko and Tye 2009). In *Drosophila*, depletion of *Mcm2* or *Mcm5* proteins leads to a defect in chromatin condensation, and *Mcm10* interacts with heterochromatin protein 1 (HP1) (Christensen and Tye 2003). Perturbations of MCM structure in the *bob1-1* mutants could also potentially lead to a defect in nucleosome disassembly during replication as *Mcm2* interacts with *Asf1* in humans (Groth *et al.* 2007), and with the FAcilitates Chromatin Transactions (FACT) complex in yeast and humans via a histone bridge (Foltman *et al.* 2013). As defects in *Mcm2p* binding to histones cause silencing defects (Foltman *et al.* 2013), defects in *Mcm2p* association with one or more of its interacting partners could also have affected silencing in our experiments. For example, if the *Asf1p-Mcm2p* interaction is conserved, hypoacetylation of H4 K16 in *bob1-1* mutants could reflect a defect in SAS-I/*Asf1p* interactions with the MCM complex during nucleosome disassembly at the replication fork. However, in contrast to *bob1-1*, four other *mcm* mutants (*mcm2*, *mcm4*, *mcm7*, and another mutant of *mcm5*) do not restore silencing at *HMRae*** (Ehrenhofer-Murray *et al.* 1999).

CAC1, CDC7, and DNA damage

Cac1p is differentially phosphorylated at *Cdc7p* consensus sites upon exposure to different DNA-damaging agents (Zhou *et al.* 2016). However, although *cac1Δ* plus *cdc7-90*, *pol30K164R*, *pol30-K127R*, or *pol30 K127, 164R* mutants genetically interacted in response to DNA damage (Figure 5A, and Figures S4 and S5), the *cac1* phospho-mutants behaved largely similar to *CAC1* in the presence of DNA-damaging agents (Figures S4 and S5). Thus, our findings are consistent with *CAC1* and *CDC7* having largely independent functions during DNA damage responses, and phosphorylation of *Cac1p* at S501 and S503 may, in part, reflect a checkpoint-driven accumulation of cells within S phase in experiments by Zhou *et al.* (2016). However, our observations do not preclude *Cdc7p* regulating CAF-1 function in response to DNA damage in a manner that was not detected in the above growth assays.

Although H3 T45 is phosphorylated by *Cdc7p* and accumulates in the presence of DNA damage (Baker *et al.* 2010), loss of H3 T45 phosphorylation did not phenocopy *cdc7-90* or *cac1Δ* mutants upon exposure to DNA damage (Figure 5B). Thus, defects in H3 T45 phosphorylation alone did not explain the synthetic interaction between *cdc7-90* and *cac1Δ*, or that H3 T45 phosphorylation and *Cac1p* functioned independently. Consistent with this observation, H3 T45 phosphorylation and *Rtt109p*-mediated H3 K56ac are not dependent on each other to occur (Baker *et al.* 2010), whereas H3 K56ac is required for efficient interaction between CAF-1 and H3-H4 (Li *et al.* 2008).

Although loss of *CDC7* in yeast results in a failure to hyperphosphorylate *Rad53p* in the presence of HU (Ogi *et al.* 2008), wild-type, *cac1Δ*, *cdc7-90*, and *cdc7-90 cac1Δ* mutants all activated the DNA damage checkpoint in response to HU at permissive temperature (Figure 6A). However, *cdc7-90*

Figure 7 Model: Cdc7p modulates SAS-I-dependent acetylation of H4 K16 via phosphorylation of CAF-1. (A) ORC, Rap1p, and Abf1p are required for recruitment of Sir proteins to the wild-type *HMR E* silencer and silencing (left). Point mutations in the binding sites for Rap1p and Abf1p at the *e^{**}* silencer disrupt recruitment of Sir proteins to the silencer, and silencing is lost (middle). In *cac1Δ* or *cdc7* mutants, hypoacetylated H4 K16 is assembled into nucleosomes during replication through *HMRae^{**}*. This creates high-affinity binding sites for Sir proteins on these nucleosomes, which, in combination with ORC, can restore recruitment of Sir proteins to *e^{**}* and silencing (right). (B) Cdc7p-dependent phosphorylation of CAF-1 may regulate one, or possibly multiple, cell cycle-dependent interactions between CAF-1 and SAS-I, PCNA, DNA, or another factor(s) during replication-coupled chromatin assembly. *cdc7* mutants exhibit defects in this pathway, resulting in hypoacetylation of H4 K16, which restores silencing at *HMRae^{**}*. WHD, winged helix domain.

cac1Δ mutants reentered the cell cycle more rapidly than wild-type and single mutants (Figure 6B), which could account for their damage-dependent synthetic growth defects (Figure 5A) if S or M phase had not completed properly. Consistent with our observations, *cac1Δ* mutants lose Rad53p phosphorylation earlier than wild-type after the induction of a double-stranded break by HO (Ogi *et al.* 2008; Kim and Haber 2009). Similar mechanisms may occur in other organisms as, like in yeast (Kim and Haber 2009), human CAF-1 is involved in chromatin assembly after DNA repair (Green and Almouzni 2003) and human Cdc7 promotes activation of the ATR-Chk1 DNA damage checkpoint (Kim *et al.* 2008). Cdc7 is also involved in multiple additional responses to DNA damage across organisms (see Ogi *et al.* (2008), Vaziri and Masai (2010), and Yamada *et al.* (2014), and references within].

Conclusions

This study provides insights into how epigenetic processes are influenced by cell cycle-dependent regulation of histone modification through chromatin assembly, and support a model in which Cdc7p can influence silencing at *HMRae^{**}* by regulating CAF-1. Our data are consistent with phosphorylation of Caf1p by Cdc7p promoting interaction between Caf1p and SAS-I, or regulating interactions between other proteins and Caf1p, thereby affecting H4 K16ac during replication-coupled chromatin assembly. Our observations provide a general model for how protein-protein interactions with, and dynamic modification of, assembly factors may regulate post-translational modifications present on newly deposited histones to ensure the maintenance of appropriate epigenetic states and chromatin composition throughout the genome.

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