Proliferating Cell Nuclear Antigen and ASF1 Modulate Silent Chromatin in *Saccharomyces cerevisiae* via Lysine 56 on Histone H3

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ABSTRACT

The formation and stability of epigenetically regulated chromatin is influenced by DNA replication and factors that modulate post-translational modifications on histones. Here we describe evidence that PCNA can affect silencing in *Saccharomyces cerevisiae* by facilitating deposition of H3 K56ac onto chromosomes. We propose that PCNA participates in this process through a pathway that includes replication factor C, the chromatin assembly factor Asf1p, and the K56-specific acetyltransferase Rtt109p. We show that mutation of *POL30* or loss of K56-acetylation in *rtt109* and histone H3 mutants enhances silencing at the crippled *HMR* locus *HMRae*** via restoring Sir binding and that *pol30* mutants with silencing phenotypes have reduced levels of H3 K56ac. Although loss of acetylation on H3 K56 was generally compatible with silencing, mutations at this residue also led to defects in silencing an *ADE2* reporter at *HMR* and abolished silencing when combined with *cac1* or *pol30-8*. These silencing phenotypes are analogous to those in *asf1* mutants or *pol30-6* and *pol30-79* mutants with defects in *ASF1*-dependent pathways. On the basis of these findings, we propose that mutations in DNA replication factors alter acetylation of H3 K56. We show that this defect, in turn, contributes to misregulation of epigenetic processes as well as of cellular responses to DNA damage.

THE foundation supporting epigenetically regulated chromatin is nucleosomal DNA. In *Saccharomyces cerevisiae*, the strength and stability of silent chromatin depends on the characteristics of histones present not only at silenced loci but also elsewhere throughout the genome. In yeast, transcribed loci are enriched in both acetylated and methylated histones whereas histones in silenced loci are hypoacetylated and hypomethylated (BRAUNSTEIN *et al.* 1996; SUKA *et al.* 2001; BERNSTEIN *et al.* 2002; BRYK *et al.* 2002; HOPPE *et al.* 2002; RUSCHÉ *et al.* 2002; NG *et al.* 2003; SANTOS-ROSA *et al.* 2004; KATAN-KHAYKOVICH and STRUHL 2005; RUDNER *et al.* 2005; LI *et al.* 2006). Loss of acetylation in silent chromatin is mediated by the NAD⁺-dependent histone deacetylase Sir2p (RUSCHÉ *et al.* 2003 and references therein). Sir2p enables the major structural components of silent chromatin, Sir2, -3, and -4 proteins, to bind preferentially to hypoacetylated histones and spread across the chromosome (HOPPE *et al.* 2002; LUO *et al.* 2002; RUSCHÉ *et al.* 2002). The resulting silent chromatin then blocks transcription and prevents histone-modifying enzymes from reaching their targets. Silencing defects at the *HM* loci and telomeres in yeast can occur upon overexpression or loss of histone-modifying enzymes. In these instances, inappropriately modified histones may prevent Sir proteins

from interacting stably with silent loci. In addition, global loss of histone modifications is thought to result in the redistribution of Sir protein binding throughout the genome, thereby limiting the pool of Sir proteins available for forming silent chromatin at appropriate sites (SINGER *et al.* 1998; VAN LEEUWEN *et al.* 2002; RUSCHÉ *et al.* 2003).

Silencing defects are also observed in cells with altered expression of proteins that load histones onto DNA. Cells lacking the chromatin assembly factors CAF-I (composed of Cac1p, Cac2p, and Cac3p) and Asf1p exhibit silencing defects (KAUFMAN *et al.* 1997; MONSON *et al.* 1997; ENOMOTO and BERMAN 1998; TYLER *et al.* 1999). These defects are more severe in *asf1 cac1* and *asf1 cac2* mutants relative to single mutants (TYLER *et al.* 1999; SUTTON *et al.* 2001). Overexpression of Asf1p also leads to defects in silencing at both the *HM* loci and telomeres (LE *et al.* 1997; SINGER *et al.* 1998). The silencing defects associated with *cac1* and *asf1* mutants may reflect both improper chromatin assembly and altered post-translational modifications on histones. Cells lacking *CAC1* have reduced levels of histone H3 as well as Sir proteins at *HMR* and telomere *VIR* (TAMBURINI *et al.* 2006; HUANG *et al.* 2007) and loss of *CAC1* or *ASF1* alters the topology of plasmids *in vivo* (PRADO *et al.* 2004; DRISCOLL *et al.* 2007). Both CAF-I and Asf1p physically interact with the histone H4-K16-specific acetyltransferase complex SAS-I (MEIJSING and EHRENHOFFER-MURRAY 2001; OSADA *et al.* 2001). Deletion of genes encoding the SAS-I subunits *SAS2*, *SAS4*, or *SAS5* alters

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the distribution of Sir proteins on chromosomes and results in silencing defects (REIFSNEYDER *et al.* 1996; EHRENHOFER-MURRAY *et al.* 1997; XU *et al.* 1999a,b; MEIJSING and EHRENHOFER-MURRAY 2001; OSADA *et al.* 2001; KIMURA *et al.* 2002; SUKA *et al.* 2002).

Recently, a second histone acetyltransferase that interacts with Asf1p has been identified. This acetyltransferase, Rtt109p, acetylates lysine 56 on histone H3 (DRISCOLL *et al.* 2007; HAN *et al.* 2007a; TSUBOTA *et al.* 2007). Acetylation of K56 on histone H3 by Rtt109p is cell cycle regulated and peaks during S phase (MASUMOTO *et al.* 2005; XU *et al.* 2005; CELIC *et al.* 2006; MAAS *et al.* 2006; RECHT *et al.* 2006; ZHOU *et al.* 2006; DRISCOLL *et al.* 2007), implying that this modification may be linked to DNA replication. Acetylation of K56 on histone H3 by Rtt109p is stimulated by Asf1p and by a second Rtt109p-binding partner, Vps75p, *in vitro* (HAN *et al.* 2007b,c; TSUBOTA *et al.* 2007). *In vivo*, both *RTT109* and *ASF1* are required for H3-K56 acetylation, whereas H3 K56 remains acetylated in *vps75* mutants (CELIC *et al.* 2006; RECHT *et al.* 2006; SCHNEIDER *et al.* 2006; ADKINS *et al.* 2007; DRISCOLL *et al.* 2007; HAN *et al.* 2007a,c). Recent reports also indicate that modulation of H3 K56 acetylation can lead to defects in silencing (HYLAND *et al.* 2005; XU *et al.* 2007), suggesting that *ASF1*-dependent silencing phenotypes may be related to acetylation of K56 on histone H3 in addition to acetylation of K16 on histone H4. In addition, work from several groups has led to the identification of a pathway involving H3 K56 acetylation, *RTT109*, and *ASF1* in maintaining genome integrity in response to DNA damage (MASUMOTO *et al.* 2005; OZDEMIR *et al.* 2005; CELIC *et al.* 2006; MAAS *et al.* 2006; RECHT *et al.* 2006; SCHNEIDER *et al.* 2006; AGEZ *et al.* 2007; DRISCOLL *et al.* 2007; HAN *et al.* 2007a,b,c; TSUBOTA *et al.* 2007; JESSULAT *et al.* 2008). The findings that (1) acetylation of K56 on histone H3 affects DNA damage sensitivity and silencing, (2) a DNA-damaging agent prevents silent chromatin formation (KIRCHMAIER and RINE 2006; see also MILLER and NASMYTH 1984; FOX *et al.* 1997), and (3) checkpoint activation in response to DNA damage alters Sir localization and silencing (MARTIN *et al.* 1999; MCAINSH *et al.* 1999; MILLS *et al.* 1999; SHARP *et al.* 2005) have motivated us to explore the relationship between histone modification, silencing, DNA replication, and responses to DNA damage.

To understand the contribution of DNA replication to H3 K56 acetylation and silencing, we examined whether the DNA polymerase processivity factor PCNA could influence H3 K56 acetylation through its role in directing the activity of chromatin assembly factors. Defects in telomeric silencing in *pol30-8* mutants have previously been linked to a CAF-1-dependent pathway (ZHANG *et al.* 2000; SHARP *et al.* 2001), whereas silencing defects in *pol30-6* and *pol30-79* mutants are primarily defective in an Asf1p-mediated pathway (SHARP *et al.* 2001). Here, we describe evidence for a pathway linking PCNA to silencing via Asf1p, Rtt109p, and lysine 56 on

histone H3. PCNA mutants have defects in H3 K56 acetylation, and mutations in H3 K56 or *RTT109* result in silencing and DNA repair phenotypes that overlap with those of *asf1* and *pol30* mutants. Our experiments argue that acetylation of H3 K56 is associated with DNA replication.

MATERIALS AND METHODS

Plasmids and strains: Strains used in this study are listed in Table 1. Yeast strains were generated using standard genetic techniques, including homologous recombination and genetic crosses. Deletions of noted open reading frames were generated by one-step gene conversion, and strains expressing histone or *pol30* mutants were generated by plasmid shuffling (STEARNS *et al.* 1990; WACH *et al.* 1994; ADAMS *et al.* 1997; GOLDSTEIN and MCCUSKER 1999).

Plasmids used in this study are listed in Table 2. Plasmids containing histone mutants were generated by site-directed mutagenesis according to the Quick Change site-directed mutagenesis kit protocol (Stratagene, La Jolla, CA) using oligonucleotides listed in supplemental Table 1 and then confirmed by sequencing. Plasmid AK965 (H3 K56R H4) was generated from pMP3 (KELLY *et al.* 2000) using oligonucleotides oALK642 and oALK643; plasmid AK972 (H4 K16R) was generated from pMP3 using oALK593 and oALK594; plasmid AK973 (H3 K56Q H4) was generated from pMP3 using oALK691 and oALK692; plasmid AK981 (H3 K56R H4 K16R) was generated from AK965 using oALK593 and oALK594; and plasmid AK982 (H3 K56Q H4 K16R) was generated from AK973 using oALK593 and oALK594.

RNA analyses: Total RNA was isolated from logarithmically growing cells, and *yFR057w* mRNA levels relative to control *SCR1* levels were analyzed by quantitative real-time PCR as described previously and in Figure 3 and supplemental Figure 1 legends (SCHMITT *et al.* 1990; KIRCHMAIER and RINE 2001; YANG and KIRCHMAIER 2006).

Extraction of chromatin-associated histones: Histones were isolated from the chromatin fraction of yeast nuclei on the basis of previous methods (EDMONDSON *et al.* 1996; STRAHL *et al.* 1999). To isolate chromatin-associated histones, 200-ml cultures of yeast were grown in rich medium (YPD) to an OD₆₀₀ of 0.8–1.5. Cells were harvested and resuspended in 3 ml of spheroplasting buffer [1 M sorbitol, 50 mM potassium phosphate, pH 6.5, 14 mM β-mercaptoethanol plus 5 mg/ml final concentration yeast lytic enzyme (ICN)] and incubated at 30° for ~30 min. Spheroplasted cells were washed with spheroplasting buffer, lysed in 5 ml of lysis buffer (18% Ficoll 400, 20 mM potassium phosphate, pH 6.8, 1.0 mM MgCl₂, 0.5 mM EDTA, 1.0 mM PMSF, and 1.0 μg/ml each of leupeptin and pepstatin) using a Dounce homogenizer, and then nuclei were isolated by ultracentrifugation at 50,000 × *g* for 30 min. Nuclei were resuspended and lysed in 0.3 ml RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% NP-40, 0.5% Na deoxycholate, and 0.1% SDS) and vortexed vigorously. The chromatin fraction was isolated by centrifugation at 16,000 × *g* for 10 min, washed twice with buffer A (10 mM Tris-HCl, pH 8.0, 0.5% NP-40, 75 mM NaCl) and twice with buffer B (10 mM Tris-HCl, pH 8.0, 400 mM NaCl), and then stored at –80°.

Protein blot analyses: For analysis of histone modifications, chromatin fractions from each strain (Figure 1E and Table 3) or whole-cell extracts (Figure 1G and Table 4) (RUSCHÉ *et al.* 2002) were separated on 15% SDS-polyacrylamide gels and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked with a 1:1 dilution of Odyssey blocking buffer (Li-Cor Biosciences) and 1× phosphate-

TABLE 1
Yeast strains used in this study

Strain	Genotype	Source
JRY2726	<i>MATa his4</i>	P. Schatz
JRY2728	<i>MATα his4</i>	P. Schatz
W303	<i>MATa</i> or <i>α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100</i>	R. Rothstein
JRY4012	W303 <i>MATa ADE2 lys2Δ::hisG</i>	J. Rine
AKY1968	W303 <i>MATα hht1-hhf1Δ::LEU2 hht2 hhf2Δ::HIS3</i> plus PK189	YANG and KIRCHMAIER (2006) ^a
AKY3857	AKY1968 <i>rtt109Δ::KanMX</i>	This study ^a
AKY1692	W303 <i>MATa HMR::ADE2 hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3</i> plus PK189	This study ^a
AKY2811	AKY1692 <i>asf1Δ::KanMX</i>	This study ^a
AKY2809	AKY1692 <i>cac1Δ::KanMX</i>	This study ^a
AKY3706	AKY1692 <i>rtt109Δ::KanMX</i>	This study ^a
AKY1721	AKY1692 <i>pol30Δ::hisG</i> plus PK169	This study ^a
AKY1785	W303 <i>MATa HMR::ADE2 POL30Δ::hisG</i> plus PK169	This study ^a
AKY3659	AKY1785 <i>rtt109Δ::KanMX</i>	This study ^a
JRY5273	W303 <i>MATα HMRae**</i>	EHRENHOFER-MURRAY <i>et al.</i> (1999)
AEY2363	W303 <i>MATα HMRae** asf1Δ::KanMX</i>	MEIJSING and EHRENHOFER-MURRAY (2001)
AEY474	W303 <i>MATα HMRae** sas2Δ::KanMX</i>	MEIJSING and EHRENHOFER-MURRAY (2001)
AKY4221	W303 <i>MATα HMRae** hir1Δ::KanMX</i>	This study
JRY1485	<i>MATα HMRae** cdc44-5::URA3 ade2 trp1 leu2 ura3</i>	EHRENHOFER-MURRAY <i>et al.</i> (1999)
AKY1744	W303 <i>MATα HMRae** hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3</i> plus PK189	This study ^{a,b}
AKY3861	AKY1744 <i>rtt109Δ::KanMX</i>	This study ^a
AKY1785	W303 <i>MATα HMRae** pol30Δ::hisG</i> plus PK169	This study ^a

^a Parental strains used in this study. See Table 2 for plasmids that were introduced into these strains for experiments described in text.

^b Derived from AEY1976 (MEIJSING and EHRENHOFER-MURRAY 2001).

buffered saline (137 mM NaCl, 2.7 mM KCl, 5.4 mM NaH₂PO₄, 1.47 mM KH₂PO₄). Protein blots were probed with anti-acetyl-histone H3 (K56) antibodies (1:10,000; Upstate) using Alexa Fluor anti-rabbit IgG (H + L) as the secondary antibody (1:20,000; Molecular Probes, Eugene, OR). Membranes were stripped with 0.2 M NaOH at room temperature as described previously (GALLAGHER *et al.* 2004) prior to reprobing with

anti-acetyl histone H4 (K16) antibodies (1:10,000; Upstate) or anti-histone H3 antibodies (1:20,000; Abcam). Antibodies were diluted in 50% Odyssey blocking buffer, 0.5× PBS, and 0.1% Tween 20 prior to use. Blots were analyzed using an Odyssey infrared imager and Odyssey software v1.2 according to the manufacturer's instructions (Li-Cor Biosciences, Odyssey User Guide, version 1.2). Data were analyzed by the

TABLE 2
Plasmids used in this study

Plasmid	Description	Source
pPK189	<i>HHT2 HHF2 ARS/CEN/URA3</i>	P. Kaufman
pMP3	<i>HHT2 HHF2 ARS/CEN/TRP1</i>	KELLY <i>et al.</i> (2000)
pAK965	H3 K56R H4 <i>ARS/CEN/TRP1</i>	This study
pAK973	H3 K56Q H4 <i>ARS/CEN/TRP1</i>	This study
pAK972	H3 H4 K16R <i>ARS/CEN/TRP1</i>	This study
pAK981	H3 K56R H4 K16R <i>ARS/CEN/TRP1</i>	This study
pAK982	H3 K56Q H4 K16R <i>ARS/CEN/TRP1</i>	This study
pFA6::KanMX4		WACH <i>et al.</i> (1994)
pFA6::NatMX4		GOLDSTEIN and McCUSKER (1999)
pPK169	<i>POL30/ARS/CEN/URA3</i>	P. Kaufman
pBL243	<i>POL30Δ::hisGURA3hisG</i>	AYYAGARI <i>et al.</i> (1995)
pBL230	<i>POL30/ARS/CEN/TRP1</i>	AYYAGARI <i>et al.</i> (1995)
pBL230-8	<i>pol30-8/ARS/CEN/TRP1</i>	AYYAGARI <i>et al.</i> (1995)
pBL230-6	<i>pol30-6/ARS/CEN/TRP1</i>	AYYAGARI <i>et al.</i> (1995)
pBL230-79	<i>pol30-79/ARS/CEN/TRP1</i>	EISSENBERG <i>et al.</i> (1997)
pPK260	<i>ASF1 YEp24-ADE3</i>	P. Kaufman

Wilcoxon rank-sum test with MSTAT v2.6. (<http://mcardle.oncology.wisc.edu/mstat>).

Chromatin immunoprecipitation: Chromatin immunoprecipitation (ChIP) experiments were performed on yeast that had been grown logarithmically in rich medium (YPD), and samples were analyzed by real-time PCR using an ABI Prism 7000 as described previously (RUSCHÉ *et al.* 2002; KIRCHMAIER and RINE 2006; YANG and KIRCHMAIER 2006). Anti-Sir3 and anti-Sir2 protein antibodies and oligonucleotides used for real-time PCR have been described previously (RUSCHÉ *et al.* 2002; KIRCHMAIER and RINE 2006; YANG and KIRCHMAIER 2006).

Mating and colony color assays: Patch mating assays were performed in duplicate and quantitative mating assays were performed in triplicate as described in VAN LEEUWEN and GOTTSCHLING (2002) and in YANG and KIRCHMAIER (2006) and in Figure 1, Table 5, and supplemental Tables 2 and 3. Quantitative data were analyzed by the Wilcoxon rank-sum test. Images were taken with an Alpha Innotech imager and ChemiImager 5500 v2.02 software. Colony color assays were conducted using two independent yeast strains for each genotype and were performed as outlined in Figure 4 and as described previously (VAN LEEUWEN and GOTTSCHLING 2002). To monitor silencing, logarithmically growing yeast containing *ADE2* integrated between the *E* and *I* silencers at *HMR* were plated on YPD plates, incubated at 30° for 2 days, and stored at 4° for 3 days before acquiring images using a Leica MZ125 microscope and SPOT 4.1.1 imaging software. Red colonies indicate that *ADE2* at *HMR* is silenced, white colonies indicate that *ADE2* is expressed, pink colonies indicate a defect in maintaining or inheriting silencing of *ADE2* at *HMR*, and sectorized colonies indicate a defect in establishing silencing.

Analysis of sensitivity to DNA-damaging agents: Yeast were grown logarithmically in rich medium (YPD) and diluted to $\sim 1 \times 10^4$ cells/ μ l. Then 3 μ l of 10-fold serial dilutions of cultures were plated onto YPD, YPD containing either 0.02 or 0.005% methyl methanesulfonate (MMS), 50 mM hydroxyurea, or 1.5 million units/ml bleomycin or onto YPD and exposed to 50 J/m² UV and then wrapped in foil and maintained in darkness. Yeast were incubated for 2 days at 30° prior to photographing.

RESULTS

Loss of histone H3 K56 acetylation restores silencing at *HMRae*:** Several studies have recently implicated Rtt109p and its binding partner Asf1p in the acetylation of lysine 56 on histone H3 (CELIC *et al.* 2006; RECHT *et al.* 2006; SCHNEIDER *et al.* 2006; ADKINS *et al.* 2007; DRISCOLL *et al.* 2007; HAN *et al.* 2007a,b,c; TSUBOTA *et al.* 2007). Here we tested for links between acetylation of K56 on histone H3 and silencing by asking if loss of acetylation at this residue could mimic several *ASF1*-dependent silencing phenotypes.

We first examined silencing at *HMRae***. *HMRae*** has a mutated *E* silencer that contains an ORC-binding site and point mutations in the Rap1p- and Abf1p-binding sites (Figure 1A) (KIMMERLY *et al.* 1988). This crippled *e*** silencer was defective in recruiting Sir proteins to *HMR* (Figure 2A) and the intact *HMR-I* silencer does not serve as a site for Sir protein loading (RUSCHÉ *et al.* 2002). This silencing defect at *HMRae*** was suppressed in *asf1Δ* cells (Figure 1B and supple-

mental Table 2; see also EHRENHOFER-MURRAY *et al.* 1999; MEIJSSING and EHRENHOFER-MURRAY 2001). Similarly, *cdc44-5* and the PCNA mutants *pol30-6* (Figure 1B and supplemental Table 2; see also EHRENHOFER-MURRAY *et al.* 1999), *pol30-8*, and *pol30-79* (data not shown) also restored silencing at *HMRae***. Significantly, the proteins encoded by these genes are all known to interact with one another. *CDC44* encodes Rfc1p, the large subunit of the PCNA clamp loading complex RF-C. RF-C physically interacts with Asf1p (FRANCO *et al.* 2005). And *pol30-6* and *pol30-79* have silencing defects that overlap with an *ASF1*-dependent pathway (SHARP *et al.* 2001). These results implied that a common mechanism(s) was responsible for restoring silencing at *HMRae*** in these mutants.

Previously, *ASF1*- and *POL30*-dependent effects on silencing have been linked to *HIR1* (SHARP *et al.* 2001; DAGANZO *et al.* 2003). The Hir1/HIRA family of proteins bind to Asf1p and function in replication-independent chromatin assembly (SHARP *et al.* 2001; SUTTON *et al.* 2001; RAY-GALLET *et al.* 2002; DAGANZO *et al.* 2003; TAGAMI *et al.* 2004). To determine whether loss of *HIR1* also restored silencing at *HMRae***, we monitored silencing at *HMRae*** in *hir1Δ* mutants by patch and quantitative mating assays (Figure 1B and supplemental Table 2). In contrast to *asf1Δ* and *pol30* mutants, the silencing defect at *HMRae*** was not suppressed in *hir1Δ* mutants. These results implied that a *HIR1*-independent pathway had restored silencing at *HMRae*** in the *asf1Δ* and *pol30* mutants. Therefore, we next monitored silencing in *MATα HMRae*** cells lacking *RTT109* as acetylation of histone H3 on K56 occurs through a pathway requiring *ASF1* and *RTT109*, but not *HIR1* (RECHT *et al.* 2006). As with *asf1Δ*, *cdc44-5*, and *pol30* mutants, silencing was restored in *rtt109Δ* mutants (Figure 1C) that were defective in acetylating K56 on histone H3 (Figure 1G). Mutation of lysine 56 on histone H3 to arginine to mimic the hypoacetylated form of this residue similarly rescued silencing at *HMRae*** (Figure 1D). In contrast, mutation of lysine 56 to glutamine to mimic the hyperacetylated state could not rescue silencing (supplemental Table 3).

To assess whether mutations in *POL30* that restored silencing at *HMRae*** could also affect acetylation levels of K56 on histone H3, we isolated chromatin-associated histones from *POL30* and *pol30* cells and monitored acetylation of K56 on histone H3 in these strains by quantitative protein blot analyses (Figure 1E and Table 3). H3 K56 acetylation levels were significantly reduced in *pol30-8*, *pol30-6*, and *pol30-79* mutants relative to *POL30* cells ($P = 0.018$ for each *pol30* mutant relative to wild type). This reduction in acetylation was not likely caused by a smaller fraction of the population of *pol30* cells being in S phase in the cultures. *pol30-8* and *pol30-79* mutants have similar flow cytometry profiles as wild type and the fraction in S phase in *pol30-6* mutants is increased relative to wild type (ZHANG *et al.*

FIGURE 1.—Loss of histone H3 K56 acetylation restores silencing at *HMRAE***. (A) Map of chromosome III with *HMRAE***. The Rap1p- and Abf1p-binding sites at the *E*silencer contain point mutations. (B) Silencing at *HMRAE*** is restored in *asf1*, *cdc44*, or *pol30* mutants but not in *hir1* mutants. (C and D) Silencing at *HMRAE*** is restored in *rtt109* (C) and in histone H3 K56R mutants (D). (E) *pol30* mutants have defects in acetylation of K56 on histone H3. Chromatin fractions containing histones were analyzed by immunoblots with anti-histone H3 K56ac (top) and anti-histone H3 antibodies (bottom) (see MATERIALS AND METHODS for details). (F) Silencing at *HMRAE*** is restored in *sas2* mutants. (G) Histone H3 K56 and histone H4 K16 are acetylated independently. Protein blot analyses of whole-cell extracts of the indicated strains were conducted using anti-histone H3 K56ac (top), anti-histone H4 K16ac (middle), or anti-histone H3 antibodies (bottom, loading control) (see MATERIALS AND METHODS). (H) H3 K56Q hyperacetylation mutants disrupt *rtt109Δ*-dependent silencing at *HMRAE***. For patch mating analyses, the indicated *MATα*

strains were grown on minimal medium with supplements overnight and then replicated to rich medium (YPD) and to *MATα* lawns (a; JRY2726) on minimal medium and grown for 2 days at 30°. Restoration of silencing at *HMRAE*** results in loss of *a1* expression, thereby enabling the *MATα* cells to mate. Mating results in growth of diploids on minimal medium.

2000). The level of acetylation of K56 on chromatin-associated histone H3 was also reduced in cells lacking *CAC1* (Table 3) and H3 K56 acetylation was not detected in *asf1Δ* mutants in control experiments (Table 3; see also CELIC *et al.* 2006; RECHT *et al.* 2006; SCHNEIDER *et al.* 2006; ADKINS *et al.* 2007; DRISCOLL *et al.* 2007; HAN *et al.* 2007a). As transcription-coupled chromatin assembly of H3 K56ac by Asf1p occurs readily (RUFANGE *et al.* 2007), this pathway was likely responsible for most of the chromatin-associated H3 K56ac remaining in the *pol30* mutants.

We next examined histone H3 K56 acetylation in whole-cell extracts from wild-type cells *vs.* *pol30*, *cac1Δ*, and *asf1Δ* mutants (Table 4). Total H3 K56 acetylation levels were similar in both *POL30* and *pol30* cells. This observation, together with the data in Table 3, implied

that histone H3 could be acetylated efficiently in the *pol30* mutants, but the *pol30* mutants had a defect in incorporating H3 K56ac into chromatin. In contrast, total H3 K56 acetylation levels were reduced in *cac1Δ* relative to wild-type cells ($P = 0.018$), consistent with a previous report that histone H3 copurifying in a CAF-1-histone complex is acetylated on K56 (ZHOU *et al.* 2006) (see DISCUSSION). In the past, the use of a qualitative rather than a quantitative approach to monitor H3 K56 acetylation likely prevented the detection of a defect in acetylation in *cac1* mutants (RECHT *et al.* 2006). Finally, H3 K56 acetylation was not detected whole-cell extracts from *asf1Δ* mutants (Table 4; see also CELIC *et al.* 2006; RECHT *et al.* 2006; SCHNEIDER *et al.* 2006; ADKINS *et al.* 2007; DRISCOLL *et al.* 2007; HAN *et al.* 2007a). Together, these results implied that mutations in *POL30* could

FIGURE 2.—Sir protein recruitment to *HMRAE***. (A) *HMRAE*** disrupts Sir protein recruitment to *HMR*. Sir3 protein association at *HMR-E* or *HMR e***, *HMRa1*, *HMR-I*, and *HML-E* was monitored in *MAT* α *HMR* and *HMRAE*** cells by ChIP. (B) Loss of H3 K56 acetylation restores Sir protein recruitment to *HMRAE***. Sir2 (top) and Sir3 (bottom) protein association at *HMR e***, and *HMRa1* was monitored in the indicated mutant strains by ChIP. ChIPs were performed using anti-Sir2 or anti-Sir3 antibodies and unimmunized rabbit serum, IgG. Coprecipitating DNA was examined by quantitative real-time PCR using primers that amplified *HMR-E* or *HMR e***, *HMR a1*, *HMR-I*, *HML-E*, or *MAT* (see MATERIALS AND METHODS). The efficiency of the coprecipitation of each locus was compared to *MAT* as follows: locus IP/*MAT* IP = $2^{(MAT C_T - \text{locus } C_T)}$; $n = 2$ for A or $n = 3$ for B.

TABLE 3

Acetylation of K56 on chromatin-associated histone H3

Strain	Relative levels of H3K56ac ^a
<i>POL30</i>	100
<i>pol30-8</i>	79 ± 15
<i>pol30-6</i>	85 ± 17
<i>pol30-79</i>	67 ± 14
<i>cac1Δ</i>	65 ± 35
<i>asf1Δ</i>	ND

^a The level of chromatin-associated histone H3 K56ac relative to chromatin-associated H3 in each strain was determined by quantitative Western blot analysis as described in MATERIALS AND METHODS and is expressed as a percentage of that observed in *POL30* cells. Data were calculated as follows: $(\text{H3K56ac}/\text{H3})_{\text{mutant}}/(\text{H3K56ac}/\text{H3})_{\text{POL30}} \times 100$, where mutant is the indicated strain. Average ± SD; *n* = 3; ND, not detected.

lead to a defect in replication-coupled chromatin assembly of H3 K56ac.

Restoration of silencing at *HMRae*** has previously been linked to loss of acetylation at K16 of histone H4 in the absence of the H4 K16-specific histone acetyltransferase Sas2p (Figure 1F; see also REIFSNYDER *et al.* 1996; EHRENHOFER-MURRAY *et al.* 1997; MEIJSING and EHRENHOFER-MURRAY 2001). As both CAF-I and Asf1p physically interact with SASI (MEIJSING and EHRENHOFER-MURRAY 2001; OSADA *et al.* 2001), it was possible that acetylation of K16 on histone H4 could either influence or be influenced by the acetylation status of K56 on histone H3. Therefore, we compared the acetylation levels of H3 K56 and H4 K16 in wild-type cells to cells expressing either H4 K16R or H3 K56R and to cells lacking *RTT109* using protein blots (Figure 1G). This analysis indicated that neither acetylation of K56 on histone H3 nor acetylation of K16 on histone H4 was required for acetylation of the other lysine residue.

TABLE 4

Acetylation of K56 on histone H3 from whole-cell extracts

Strain	Relative levels of H3K56ac ^a
<i>POL30</i>	100
<i>pol30-8</i>	90 ± 17
<i>pol30-6</i>	100 ± 28
<i>pol30-79</i>	99 ± 25
<i>cac1Δ</i>	66 ± 16
<i>asf1Δ</i>	ND

^a The level of histone H3 K56ac relative to H3 in whole-cell extracts in each strain was determined by quantitative Western blot analysis as described in MATERIALS AND METHODS and is expressed as a percentage of that observed in *POL30* cells. Data were calculated as follows: $(\text{H3K56ac}/\text{H3})_{\text{mutant}}/(\text{H3K56ac}/\text{H3})_{\text{POL30}} \times 100$, where mutant is the indicated strain. Average ± SD; *n* = 3; ND, not detected.

TABLE 5

Quantitative mating analysis

Strain	<i>HHT2-HHF2</i>	Relative efficiency of mating ^a
<i>RTT109</i>	H3/H4	1
<i>RTT109</i>	H3 K56R/H4	6.6 ± 0.27
<i>RTT109</i>	H3/H4 K16R	7.2 ± 3.0
<i>RTT109</i>	H3K56R/H4 K16R	9.4 ± 0.68
<i>rtt109Δ</i>	H3 K56R/H4	8.0 ± 3.2
<i>rtt109Δ</i>	H3/H4 K16R	12 ± 1.6

^a The efficiency of mating of *MATα HMRae*** cells expressing wild-type histones H3 and H4 to tester strain JRY2726 (*MATa*) was determined relative to their plating efficiency ($5.0 \pm 0.58\%$, *n* = 3), and was set to 1. The mating efficiencies of each strain relative to *MATα HMRae*** cells expressing wild-type H3 and H4 was determined as indicated in MATERIALS AND METHODS. Average ± SD; *n* = 3.

The above observations implied that *sas2* and *rtt109* might be functioning separately to restore silencing. To test this possibility, we examined silencing at *HMR* and *HMRae*** in *rtt109Δ* cells expressing either wild-type histones H3 and H4 or several mutants of K56 on histone H3 and of K16 on histone H4 (Figure 1H and Table 5). Neither H3 K56R nor H4 K16R disrupted silencing in *rtt109Δ* cells. In contrast, H3 K56Q disrupted *rtt109Δ*-dependent silencing at *HMRae*** (Figure 1H; see also Figure 2B). Combining H4 K16R with H3 K56Q could not restore silencing at *HMRae*** in *rtt109Δ* mutants (Figure 1H). Quantitative mating assays indicated that H3 K56R and H4 K16R mutants could restore silencing at *HMRae*** to a similar degree and that mating was enhanced in H3 K56R H4 K16R relative to H3 K56R mutants (*P* = 0.025). Mating was also more efficient in *rtt109Δ* H4 K16R relative to H4 K16R mutants (*P* = 0.063), whereas no difference in mating was observed in *rtt109Δ* H3 K56R relative to H3 K56R mutants (*P* = 0.25) (Table 5; see also supplemental Table 3). Together, these results implied that restoration of silencing at *HMRae*** in cells lacking *RTT109* occurred through loss of acetylation of K56 on histone H3.

Loss of histone H3 K56 acetylation restores Sir recruitment to *HMRae*:** In the above experiments, mating would have been restored to *MATα HMRae*** cells if *rtt109Δ*, *asf1Δ*, and *pol30-6* disrupted transcription from the *a1* promoter at *HMR* in a Sir-independent manner or if the recruitment of Sir proteins to the crippled *HMR* locus had been rescued. In separate analyses, we have found that *a1* mRNA was expressed efficiently in the mutants in the absence of Sir proteins (our unpublished observations). Here, we monitored Sir2p and Sir3p association at *HMRae*** in cells expressing H3 K56R or in *rtt109Δ*, *asf1Δ*, and *pol30-6* mutants by chromatin immunoprecipitation (Figure 2B *vs.* Figure 2A). Expression of H3 K56R or deletion of *RTT109* restored the recruitment of Sir proteins to *HMRae***.

FIGURE 3.—Histone H3 K56 mutants disrupt telomeric silencing. (A and B) H3 K56 hyperacetylation disrupts telomeric silencing, but is not the sole cause of silencing defects upon overexpression of Asf1p. (C and D) Telomeric silencing in *asf1Δ* and *cac1Δ* (C) or *rtt109Δ* mutants (D) expressing wild-type histone H3 or H3 hyper- and hypoacetylation mutants. *yFR057w* (A, C, and D) or *ASF1* (B) mRNA levels relative to *SCR1* (internal control) were determined for each strain by

In contrast, expression of H3 K56Q in *rtt109Δ* cells disrupted Sir protein association. This was consistent with loss of acetylation of K56 on histone H3 playing a key role in Sir protein recruitment (Figure 2B; see also Figure 1H). Sir protein recruitment to the crippled *e*** silencer was similarly restored in *asf1Δ* and *pol30-6* mutants. However, Sir protein spreading across *HMR* was compromised in *pol30-6* relative to *rtt109Δ* mutants. This result indicated that the *pol30-6* mutation likely led to additional defect(s) that adversely affected silencing, such as a defect in a *HIR1*-related function of *ASF1* (see SHARP *et al.* 2001; DAGANZO *et al.* 2003; HUANG *et al.* 2007). Together, our results revealed that the common mechanism by which these mutants restored mating to *MATα HMRac*** cells was through facilitating the localization of Sir proteins to *HMR*.

Histone H3 K56 mutants disrupt telomeric silencing:

Asf1p was originally identified by its ability to disrupt silencing when overexpressed (LE *et al.* 1997; SINGER *et al.* 1998). To assess whether the Asf1p-overexpression phenotype was linked to the acetylation status of K56 on histone H3, we compared silencing of *yFR057w* at telomere *VIR* in cells expressing wild-type histone H3, H3 K56R, or H3 K56Q in the absence or presence of overexpression of *ASF1* (Figure 3, A and B). In this analysis, *yFR057w* was partially derepressed in cells expressing H3 K56Q relative to cells expressing either wild-type histone H3 or H3 K56R, similar to the telomeric and rDNA silencing phenotypes observed in H3 K56Q, but not in H3 K56R mutants (HYLAND *et al.* 2005). While this article was in preparation, XU *et al.* (2007) reported telomeric silencing defects in H3 K56Q mutants and, in contrast to Figure 3A, also observed telomeric silencing defects in H3 K56R mutants. The reason for this difference between these studies is not clear. As expected, overexpression of *ASF1* in cells expressing wild-type histones H3 and H4 partially disrupted silencing of *yFR057w* (Figure 3, A and B). *yFR057w* was further derepressed in cells overexpressing *ASF1* in the presence of either H3 K56R or H3 K56Q. These results implied that, although K56 on histone H3 influenced telomeric silencing, loss of silencing upon overexpression of *ASF1* was not exclusively due to neutralization of a positive charge at residue 56 on histone H3 and may have also been linked to Sas2p-dependent acetylation of K16 on histone H4 or to another function of *ASF1*.

Mutations in histone H3 K56 result in silencing defects at *HMR::ADE2*: To test the effects of mutations in K56 on histone H3 on silencing of an *ADE2* reporter

quantitative real-time PCR (see MATERIALS AND METHODS and YANG and KIRCHMAIER 2006) and expressed relative to mRNA levels in yeast expressing wild-type histones H3 and H4, which have been set to 1. (Wild type in C and D are identical.) Data were calculated as follows: $2^{[(\text{locus } C_T - \text{SCR1 } C_T)_{H3/H4} - (\text{locus } C_T - \text{SCR1 } C_T)_a]} \times 100$, where “a” is the indicated genotype. Data were obtained from two independent clones for each genotype. Average $\pm$ SD; $n = 3$.

FIGURE 4.—Synthetic interaction analyses of silencing defects at *HMR::ADE2* in yeast with altered histone H3 K56 acetylation. (A) Colony color assays monitoring silencing at *HMR::ADE2* in yeast lacking chromatin assembly factors or *rtt109Δ* and expressing wild-type histone H3, H3 K56R, or H3 K56Q. (B and C) Colony color assays monitoring silencing at *HMR::ADE2* in *POL30* and *pol30* cells expressing wild-type histone H3, H3 K56R, or H3 K56Q (B) or expressing or lacking Rtt109p (C) (see MATERIALS AND METHODS).

gene at *HMR*, we generated yeast lacking chromosomal copies of histone H3 and H4 genes and expressing either wild-type or mutant histones H3 and H4 from a plasmid. Cells expressing wild-type histone H3 and H4 were silenced at *HMR::ADE2* and grew as red colonies whereas cells expressing either H3 K56R or H3 K56Q formed pink colonies, indicating that these mutants had mild defects in maintaining silencing (Figure 4A). We then examined silencing in analogous strains lacking *ASF1* or *CAC1*. *asf1Δ* cells expressing wild-type histones H3 and H4 were defective in maintaining silencing and grew as light pink colonies. *cac1Δ* cells expressing wild-type histone H3 and H4 also exhibited silencing defects and grew as red and white sector colonies. In contrast to the genetic background analyzed here, *asf1 HMR::ADE2* yeast with two chromosomal copies of genes encoding histones H3 and H4 grow as dark pink colonies and those lacking *cac1* grow as pink colonies (TYLER *et al.* 1999). The more severe silencing defects observed here are likely due to the reduced copy number of histone H3 and H4 genes in our strains. Mutations in lysine 56 on histone H3 to either arginine or glutamine did not further disrupt silencing in *asf1Δ* cells. In contrast, *cac1Δ* cells expressing either H3 K56R or H3 K56Q grew as white colonies as silencing of *ADE2* at *HMR* was lost (Figure 4A). The silencing defects of *cac1* cells expressing H3 K56 mutants were similar to those reported for *asf1 cac1* mutants (TYLER *et al.* 1999) and implied that loss of *CAC1* and mutation of K56 on histone H3 affected silencing of *HMR::ADE2* through different pathways.

We then examined synthetic interactions between histone H3 K56 mutants and *pol30* mutants with silencing phenotypes. The *pol30*p mutants, encoded by *pol30-6*, *pol30-8*, and *pol30-79*, all have defects in both physically interacting with Cac1p and silencing *HMR::ADE2*. But, the silencing defects at telomeres in *pol30-6* and *pol30-79* mutants have been primarily linked to an *ASF1*-dependent

pathway whereas those of *pol30-8* mutants are associated with a *CAC1*-dependent pathway (ZHANG *et al.* 2000; SHARP *et al.* 2001). To determine whether the silencing defects at *HMR::ADE2* in the *pol30* mutants were related to K56 on histone H3, we combined histone H3 K56 mutations with *pol30-6*, *pol30-8*, or *pol30-79* and monitored silencing of *ADE2* integrated at *HMR* (Figure 4B). As had been observed in Figure 4A, *HMR::ADE2* was partially derepressed in *POL30* cells expressing either H3 K56R or H3 K56Q (Figure 4B). *pol30-6* mutants expressing wild-type histone H3 formed uniform light pink colonies, indicating a defect in maintaining silencing. Yeast colonies remained light pink in *pol30-6* cells expressing either H3 K56R or H3 K56Q, but *pol30-6* cells expressing H3 K56R exhibited severe growth defects relative to either single mutant (see also Figure 5A). The colony colors of *pol30-79* mutants expressing wild-type histone H3 varied from light pink to dark pink as did those of *pol30-79* mutants expressing either H3 K56R or H3 K56Q. Like *pol30-6*, *pol30-79* cells expressing H3 K56R also exhibited growth defects relative to *pol30-79* mutants (see also Figure 5A). *pol30-8* mutants expressing wild-type histone H3 grew as red and white sector colonies, indicating that *pol30-8* mutants existed in two semistable populations in which *HMR* was either silenced or expressed (see also ZHANG *et al.* 2000). In contrast to *pol30-6* and *pol30-79* mutants, when *pol30-8* was combined with either H3 K56R or H3 K56Q, yeast colonies were white, indicating that silencing of *HMR::ADE2* had been disrupted (Figure 4B). This observation was similar to previous reports of synergistic silencing defects observed in *pol30-8 asf1Δ* cells relative to *pol30-8* mutants (SHARP *et al.* 2001) as well as those seen for the *cac1Δ* double mutants (Figure 4A).

We also monitored silencing at *HMR::ADE2* in cells lacking *RTT109*. We did not observe dramatic defects in silencing in *rtt109Δ* cells expressing wild-type or mutant

FIGURE 5.—PCNA silencing mutants, histone H3 K56 modification, and response to DNA damage. Synthetic interaction analyses of responses to DNA-damaging agents in *POL30* and *pol30* cells expressing histone H3, H3 K56R, or H3 K56Q (A) in *RTT109* *POL30* or *pol30* cells and in *rtt109Δ* *POL30* or *pol30* mutants (B). Ten-fold serial dilutions of yeast with the indicated genotypes expressing wild-type histone H3, H3 K56R, or H3 K56Q were plated onto YPD or YPD containing the indicated amount of MMS (0.02 or 0.005%), 50 mM HU, 1.5 milliunits/ml BLM or YPD and then exposed to 50 J/m² UV. Yeast were grown for 48 hr at 30° prior to photographing.

histone H3 (Figure 4A). In contrast to *pol30-8* cells expressing histone H3 mutants, silencing was similar in *rtt109Δ pol30-8* mutants and in *rtt109Δ POL30* cells. *rtt109Δ pol30-6* cells, however, displayed more severe silencing and growth defects (Figure 4C; see also Figure 5B). Together, these results indicated that mutation of K56 on histone H3 directly or indirectly led to subtle structural changes to chromatin that were not compatible with silencing *HMR::ADE2*, whereas identical alterations in chromatin structure may not have occurred in *rtt109Δ* mutants.

Effects of histone H3 K56 on silencing vs. sensitivity to DNA damage: In addition to restoring silencing at *HMRae***, deletion of *ASF1* can lead to silencing defects (TYLER *et al.* 1999; DAGANZO *et al.* 2003; MOUSSON *et al.* 2005; Figures 3C and 4) as well as hypersensitivity to DNA-damaging agents (*e.g.*, TYLER *et al.* 1999). Previously, silencing phenotypes in *asf1* mutants have been attributed to both *SAS2*- and *HIR1*-dependent pathways (MEIJSSING and EHRENHOFER-MURRAY 2001; OSADA *et al.* 2001; SHARP *et al.* 2001; KRAWITZ *et al.* 2002; DAGANZO

et al. 2003). However, *asf1*-dependent defects in silencing do not always correlate with loss of H4 K16 acetylation (HUANG *et al.* 2007) and, under some conditions, can be genetically separated from *HIR1* (SHARP *et al.* 2001; KRAWITZ *et al.* 2002; DAGANZO *et al.* 2003; MOUSSON *et al.* 2005; see also Figure 1B). *HIR1*-independent, *ASF1*-dependent acetylation of K56 on histone H3 K56 has been tied to cellular responses to DNA damage (*e.g.*, RECHT *et al.* 2006) and H3 K56 acetylation has been linked to silencing (HYLAND *et al.* 2005; XU *et al.* 2007; Figures 1–4). To assess whether the silencing phenotypes at *HMRae*** were related to those at telomeres or to sensitivity to DNA damage, we examined the effects of H3 K56 on telomeric silencing and DNA damage sensitivity in the *asf1*, *cac1*, *rtt109*, and *pol30* mutants.

To assess whether K56 on histone H3 contributed to telomeric silencing defects in *asf1Δ* mutants, we compared silencing of *yFR057w* at telomere *VIR* in wild-type cells to *asf1Δ*, *cac1Δ*, and *rtt109Δ* mutants expressing wild-type histone H3, H3 K56R, or H3 K56Q (Figure 3, C

FIGURE 6.—Chromatin assembly, histone H3 K56 modification, and response to DNA damage. Synthetic interaction analyses of responses to DNA-damaging agents in wild-type cells or in cells lacking *ASF1* or *CAC1* (A) and *RTT109* (B) and expressing wild-type histone H3, H3 K56R, or H3 K56Q. Ten-fold serial dilutions of yeast with the indicated genotypes were assayed as described in Figure 5 and MATERIALS AND METHODS.

and D). Both *asf1*Δ and *cac1*Δ mutants expressing wild-type histone H3 had mild silencing defects at *yFR057w*. Expression of H3 K56R in the *asf1*Δ mutants did not further disrupt silencing. In contrast, as at *HMR::ADE2*, the defect in silencing *yFR057w* was more severe in *cac1*Δ mutants expressing H3 K56R relative to wild-type histone H3 (Figure 3C), analogous to the silencing defects of *asf1 cac1* mutants (TYLER *et al.* 1999). Loss of *RTT109* did not disrupt silencing at *yFR057w* in cells expressing wild-type histone H3 and did not alter the telomeric silencing defects of H3 K56Q mutants (Figure 3D). Thus, in the absence of other defects, the lack of *RTT109*-mediated acetylation on K56 of histone H3 was compatible with telomeric silencing as well as with silencing at *HMRac***.

We then tested the sensitivity of the above strains to the DNA-damaging agents MMS, hydroxyurea (HU), bleomycin (BLM), and ultraviolet light (UV) (Figure 6A). MMS is an alkylating agent that induces single- and double-stranded breaks (SCHWARTZ 1989), HU is an inhibitor of ribonucleotide reductase that leads to defects in replication fork progression (KRAKOFF *et al.* 1968), BLM induces double-strand breaks (POVIRK 1996), and exposure to UV results in the formation of thymidine dimers (BEUKERS and BERENDS 1960). Confirming previous reports, mutation of histone H3 to either K56R or K56Q resulted in sensitivity to MMS, HU, and BLM, with H3 K56R being far more sensitive than H3 K56Q (MASUMOTO *et al.* 2005; OZDEMIR *et al.* 2005; MAAS *et al.* 2006; RECHT *et al.* 2006; SCHNEIDER *et al.*

2006; DRISCOLL *et al.* 2007; HAN *et al.* 2007a,c). Histone H3 K56R mutants also exhibited a mild growth defect upon exposure to UV (Figure 6A; see also MASUMOTO *et al.* 2005; OZDEMIR *et al.* 2005; HAN *et al.* 2007c).

asf1Δ mutants expressing either wild-type or H3 K56R exhibited growth defects on rich media and were hypersensitive to all forms of DNA damage tested. In contrast, H3 K56Q mutants could largely suppress *asf1Δ*-dependent growth defects on rich medium as well as sensitivity to MMS, HU, BLM, and UV (Figure 6A; see also RECHT *et al.* 2006; AGEZ *et al.* 2007). H3 K56Q mutants also partially suppressed *rtt109Δ*-dependent growth defects in rich medium as well as sensitivity to MMS, HU, BLM, and UV (Figure 6B). Thus, whereas the restoration of Sir protein recruitment and silencing at *HMRae*** (Figures 1 and 2) and hypersensitivity to DNA-damaging agents (Figure 6) correlated well with defects in acetylation of K56 (Tables 4 and 5) in these mutants, the loss of telomeric silencing (Figure 3) did not.

We next assessed telomeric silencing and DNA damage sensitivity in the *pol30* mutants. Relative to *POL30* cells, *pol30* mutants had mild telomeric silencing defects that could generally be enhanced by mutating H3 K56 (supplemental Figure 1, A and B). The telomeric silencing defect of *pol30-6* and *pol30-79* mutants, which have defects in an *ASF1*-dependent pathway (SHARP *et al.* 2001), could not be suppressed by expression of histone H3 K56Q (supplemental Figure 1B). And, similar to *HMR::ADE2* (Figure 4), the telomeric silencing defects of *pol30* mutants were also not enhanced by deletion of *RTT109* (supplemental Figure 1A).

The *pol30* mutants were sensitive to DNA-damaging agents to varying degrees relative to *POL30* cells (Figure 5, A and B; see also AYYAGARI *et al.* 1995; EISSENBERG *et al.* 1997; ZHANG *et al.* 2000). This sensitivity was enhanced when combined with either histone or *rtt109* mutations. *pol30-6* mutants were more sensitive to MMS, HU, BLM, and UV than *POL30* cells whereas *pol30-8* and *pol30-79* were primarily sensitive to high levels of MMS (Figure 5, A and B). Both *pol30* mutants were, however, somewhat less sensitive to DNA-damaging agents than *asf1* mutants (Figure 5 *vs.* Figure 6A). This may, in part, reflect residual activity of the *ASF1*-dependent pathway in *pol30* mutants or continued replication-independent chromatin assembly of acetylated H3 K56 by Asf1p in these mutants (Figure 1E and Tables 3 and 4). Supporting this notion, deletion of *RTT109*, like expression of H3 K56R, in *pol30-6* or *pol30-79* mutants increased their sensitivity to MMS, HU, and BLM (Figure 5, B and A, respectively). *pol30-6* and, especially, *pol30-79* mutants expressing H3 K56Q were also more sensitive to MMS, HU, and BLM than either single mutant (Figure 5A). Like *cac1Δ* mutants (Figure 6A), *pol30-8* cells expressing H3 K56Q were less sensitive to DNA-damaging agents than *pol30-8* cells expressing H3 K56R (Figure 5A). However, under most conditions, the sensitivity to DNA-damaging agents could not be completely suppressed by

mimicking the hyperacetylated state of K56 on histone H3. This implied an alteration in chromatin structure caused by the histone mutation or an inability to reverse the acetylated state may have interfered with normal repair processes. In summary, as in *asf1Δ* and *rtt109Δ* mutants (Figure 6), hypersensitivity to DNA-damaging agents in *pol30* mutants (Figure 5) correlated with defects in K56 acetylation (Tables 4 and 5) and with the restoration of Sir protein recruitment and silencing at *HMRae*** (Figures 1 and 2), but not with telomeric silencing defects (supplemental Figure 1).

DISCUSSION

In this study, we provide evidence that PCNA can affect silencing by influencing the acetylation status of lysine 56 on histone H3. Maintaining a positive charge at this residue by mutation to arginine, deletion of *RTT109*, or mutation of *POL30* rescued silencing at *HMRae*** (Figure 1, C and D; Table 5; supplemental Tables 2 and 3), revealing that the unacetylated state of K56 on histone H3 enhances Sir protein interactions with the silencer. However, mutations of K56 on histone H3 also led to defects in maintaining silent chromatin (Figure 3; Figure 4, A and B; supplemental Figure 1), indicating that this residue also plays an important role in the structural changes to chromatin that occur when silent chromatin is formed. By analyzing genetic interactions between histone H3 K56 mutants, *pol30*, and chromatin assembly factor mutants, we have defined a mechanism by which factors involved in DNA replication contribute to silencing (Figures 1 and 2) in addition to the cell's ability to respond to DNA damage (Figures 5 and 6). On the basis of these findings, we propose that DNA replication factors can differentially contribute to these processes by affecting acetylation of lysine 56 on histone H3.

Lysine 56 lies within the α N helix region of histone H3 at the junction between the N terminal tail and the histone fold domain (LUGER *et al.* 1997; WHITE *et al.* 2001). In the nucleosome core particle, neutralization of this positively charged residue likely influences interactions with DNA. Consistent with this function, the *in vivo* superhelical density of plasmids is altered in *asf1* and *rtt109* mutants that lack H3 K56 acetylation (PRADO *et al.* 2004; DRISCOLL *et al.* 2007). Superhelical density of plasmids is also altered in histone H3 K56R and K56Q mutants (MASUMOTO *et al.* 2005). Our observations support a role of lysine 56 on histone H3 in determining chromatin structure at silenced loci.

Other studies have also recently reported a link between lysine 56 on histone H3 and silencing. Histone H3 is hypoacetylated at K56 in silent chromatin (XU *et al.* 2007; B. YANG and A. L. KIRCHMAIER, unpublished results) and Sir2p can deacetylate this residue *in vitro* (XU *et al.* 2007). As K56 on histone H3 is hypoacetylated in cells lacking *RTT109*, the nucleosomes in *rtt109Δ*

mutants were largely compatible with silencing (Figure 1, C and H; Figure 3D; Figure 4, A and C; supplemental Figure 1). The side chain of arginine retains a positive charge as is found on lysine and, in our experiments, the H3 K56R mutation was also generally tolerated in silent chromatin (Figure 1H, Figure 3, and supplemental Figure 1). Silent chromatin was, however, sensitive to the subtle structural differences between arginine and lysine at this residue in the sensitized *cac1Δ* and *pol30-8* genetic backgrounds (Figure 3C; Figure 4, A and B). Repair of DNA damage caused by BLM was also compromised in *pol30-8* mutants expressing H3 K56R relative to *pol30-8 rtt109Δ* mutants (Figure 5, A and B). One prediction from these observations is that *rtt109Δ* or H3 K56R mutants will also have severe defects in silencing when combined with *cac1* mutants that are defective in interacting with PCNA (KRAWITZ *et al.* 2002).

Mutation of K56 to Q on histone H3 can lead to defects in silencing at the *HM* loci, telomeres, and rDNA locus (Figures 3 and 4; supplemental Figure 1; HYLAND *et al.* 2005; XU *et al.* 2007). Although H3 K56Q does not disrupt Sir protein association at telomere *VIR* (XU *et al.* 2007; B. YANG and A. L. KIRCHMAIER, unpublished results), H3 K56Q can alter both transcription activity and sensitivity to DAM methylase in this region (Figure 3, A and D; supplemental Figure 1; XU *et al.* 2007). Interestingly, H3 K56 normally becomes highly acetylated in response to DNA damage (CELIC *et al.* 2006; MAAS *et al.* 2006; THAMINY *et al.* 2007). Together, our results support a link between H3 K56 acetylation, Rtt109p, Asf1p, and PCNA that is important for repair of DNA damage but is antagonistic to silencing. Future studies should reveal whether hyperacetylation of K56 on histone H3 during response to DNA damage directly leads to the disruption of telomeric silencing (MARTIN *et al.* 1999; MCAINSH *et al.* 1999; MILLS *et al.* 1999; SHARP *et al.* 2005).

Acetylation of K56 on histone H3 is also regulated in a cell-cycle-dependent manner. This occurs primarily by the opposing actions of cell-cycle-regulated expression of Rtt109p and the NAD⁺-dependent deacetylases Hst3p and Hst4p (MASUMOTO *et al.* 2005; XU *et al.* 2005; CELIC *et al.* 2006; MAAS *et al.* 2006; RECHT *et al.* 2006; ZHOU *et al.* 2006). Although newly synthesized histone H3 can be acetylated on K56 and loaded onto chromosomes in G₁ (RUFANGE *et al.* 2007), bulk H3 K56 acetylation levels remain low in G₁ and then rise and peak in S phase before decreasing again in G₂/M via deacetylation by Hst3p and Hst4p. In addition to this pattern of regulation, several other observations argue that incorporation of H3 K56ac is, in part, coupled to DNA replication. These findings include (1) the reduction of the levels of H3 K56 acetylation in chromatin in *pol30* mutants (Figure 1 and Table 3), (2) the requirement of a chromatin assembly factor for acetylation of K56 on histone H3, and (3) the localization of Asf1p and CAF-I to DNA via RF-C or PCNA and to DNA replication foci (KRUEDE 1995; SHIBAHARA and STILLMAN

1999; ZHANG *et al.* 2000; FRANCO *et al.* 2005; SCHULZ and TYLER 2006).

A pathway linking *POL30* to acetylation of K56 on histone H3 may function in two different ways. Rtt109p may acetylate histone H3 on lysine 56 when (H3/H4) is bound by Asf1p, but prior to Asf1p being recruited to the replication fork during DNA replication. *In vitro*, Rtt109p can acetylate histone H3 on K56 while bound by Asf1p in the absence of DNA replication, but not while H3 is in nucleosomes or in tetramers bound to DNA (DRISCOLL *et al.* 2007; HAN *et al.* 2007b; TSUBOTA *et al.* 2007). In one scenario, defects in recruiting Asf1p bound to H3 K56ac to the replication fork in *pol30* mutants would lead to a failure to load H3 K56ac onto DNA during replication-coupled chromatin assembly. Alternatively, Rtt109p may also acetylate K56 on histone H3 while Asf1p-(H3/H4) is associated with RF-C (and, potentially, CAF-I) at the replication fork, but before Asf1p unloads (H3/H4) onto DNA. In this case, the defect in acetylation of K56 on histone H3 in *pol30* (and, potentially, *cac1*) mutants would have been caused by a defect in targeting Rtt109p to the replication fork during replication-coupled chromatin assembly.

Both mechanisms may ultimately contribute to the reduction in chromatin-associated H3 K56ac in *pol30* mutants (Figure 1E and Table 3). Acetylation events analogous to the first mechanism described above likely permitted transcription-coupled, Asf1p-dependent incorporation of H3 K56ac into chromatin to continue in *pol30* mutants. However, reduced levels of H3 K56ac in chromatin in *pol30* mutants would not have been expected if transcription-coupled chromatin assembly was the only mechanism for loading H3 K56ac onto the chromosome. We are unaware of reports of functions of *POL30* in transcription in yeast. We have found that acetylation of K56 on histone H3 continues to oscillate during the cell cycle in *pol30* mutants in α -factor synchronization-release experiments (data not shown). Thus, Asf1p was still weakly associated with the replication fork in *pol30* mutants, Asf1p-dependent chromatin assembly of H3 K56ac during transcription was subject to deacetylation by Hst3p and Hst4p during G₂/M, or CAF-I also incorporated H3 K56ac during replication. The observations that histone H3 K56 acetylation levels in *cac1Δ* mutants were reduced (Tables 3 and 4) and that H3 K56ac copurifies with Cac1p support a role of CAF-I in this process as well (ZHOU *et al.* 2006).

Our observations reveal a pathway linking the restoration of silencing at *HMRae*** in replication factor mutants to acetylation of K56 on histone H3 (Figure 7). In this pathway (Figure 7A), Rtt109p acetylates K56 on histone H3 in the context of (H3/H4) bound to Asf1p either prior to or upon association with the replication fork. Asf1p bound to acetylated (H3/H4) localizes to the replication fork through interactions between Asf1p and the Rfc1p subunit of RF-C. Additional interactions between Asf1p and the Cac2p subunit of CAF-I likely

FIGURE 7.—Model linking DNA replication factors to histone modifications and silencing. (A) Replication-coupled histone modifications. Rtt109p binds to Asf1p in a complex with histones H3 and H4 and acetylates K56 on histone H3. H3 bound to CAF-I is also acetylated on K56 (ZHOU *et al.* 2006). Whether Rtt109p acetylates K56 on H3 while H3 is bound by CAF-I is not known (light gray arrow). SAS-I associates with CAF-I or Asf1p bound to histones H3 and H4 and the Sas2p subunit of SAS-I acetylates K16 on histone H4. Chromatin assembly factors CAF-I and PCNA or between Asf1p and the Rfc1p subunit of RF-C, and then histones H3 and H4 are loaded onto DNA. Additional interactions between Asf1p and the Cac2p subunit

stabilize the association of chromatin assembly factors with PCNA and RF-C at the replication fork. Consistent with this notion, Asf1p binds Cac2p *in vitro*, and cac1p mutants with defects in binding to PCNA have severe silencing defects in the absence of *ASF1* (KRAWITZ *et al.* 2002). Analogous pathways linking acetylation of K16 on histone H4 by Sas2p to CAF-I and Asf1p at the replication fork may also contribute to the silencing phenotypes of *pol30* mutants. In the case of *HMRae***, the normal route for Sir protein recruitment to the *HMR* locus (Figure 7B) has been disrupted through mutation of Rap1p- and Abf1p-binding sites at the *E* silencer (Figure 7C). We propose that mutations in factors associated with acetylation of K56 on histone H3 and the replication fork restore silencing by rescuing Sir protein recruitment (Figure 7D). This could occur through the creation of high-affinity binding sites for Sir proteins on nucleosomes at *HMR*, as has been proposed for mutants with defects in H4 K16 acetylation. Alternatively, defects in histone acetylation in these replication mutants may have altered ORC's interactions with the chromosome, thereby enhancing the binding of Sir proteins to ORC. Either possibility has the potential to result in the mislocalization of Sir proteins to other chromosomal loci throughout the genome. Future studies characterizing how chromatin is regulated during cellular responses to DNA damage should clarify how signals during DNA repair also influence epigenetic processes (MARTIN *et al.* 1999; McAINSH *et al.* 1999; MILLS *et al.* 1999; SHARP *et al.* 2005; KIRCHMAIER and RINE 2006).

Together, the work presented here implies that nucleosomal DNA that serves as the foundation for epigenetically regulated chromatin can contain inappropriately modified histones when defects in replication-coupled chromatin assembly occur. These observations explain how defects in DNA replication factors can affect epigenetic processes when DNA replication itself is not required to form silent chromatin in yeast (FOX *et al.* 1997; KIRCHMAIER and RINE 2001; LI *et al.* 2001). These findings also raise the possibility that DNA damage phenotypes associated with other replication fork mutants may be related to perturbations in replication-coupled modifications on histones.

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of CAF-I (dashed line) facilitate chromatin assembly factor association with the replication fork. Other proteins that can associate with the above factors or with the replication fork are not shown for simplicity. (B) Sir protein recruitment to the *HMR E* silencer requires ORC, Rap1p, and Abf1p. (C) Loss of silencing at *HMRae*** is the result of a defect in Sir protein recruitment. Point mutations in the binding sites for Rap1p and Abf1p at the *E* silencer disrupt Sir protein recruitment to the silencer. (D) Loss of histone acetylation in *cac1*, *asf1*, *rtt109*, *pol30*, and H3 K56R strains as well as *sas2* and H4 K16R mutants restore Sir protein recruitment and silencing at *HMRae***.

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LITERATURE CITED

- ADAMS, A., D. E. GOTTSCHLING, C. A. KAISER and T. STEARNS, 1997 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Plainview, NY.
- ADKINS, M. W., J. J. CARSON, C. M. ENGLISH, C. J. RAMEY and J. K. TYLER, 2007 The histone chaperone anti-silencing function 1 stimulates the acetylation of newly synthesized histone H3 in S-phase. *J. Biol. Chem.* **282**: 1334–1340.
- AGEZ, M., J. CHEN, R. GUEROIS, C. VAN HEIJENOORT, J. Y. THURET *et al.*, 2007 Structure of the histone chaperone ASF1 bound to the histone H3 C-terminal helix and functional insights. *Structure* **15**: 191–199.
- APPAGARI, R., K. J. IMPELLIZZERI, B. L. YODER, S. L. GARY and P. M. BURGERS, 1995 A mutational analysis of the yeast proliferating cell nuclear antigen indicates distinct roles in DNA replication and DNA repair. *Mol. Cell. Biol.* **15**: 4420–4429.
- BERNSTEIN, B. E., E. L. HUMPHREY, R. L. ERLICH, R. SCHNEIDER, P. BOUMAN *et al.*, 2002 Methylation of histone H3 Lys 4 in coding regions of active genes. *Proc. Natl. Acad. Sci. USA* **99**: 8695–8700.
- BEUKERS, R., and W. BERENDS, 1960 Isolation and identification of the irradiation product of thymine. *Biochim. Biophys. Acta* **41**: 550–551.
- BRAUNSTEIN, M., R. E. SOBEL, C. D. ALLIS, B. M. TURNER and J. R. BROACH, 1996 Efficient transcriptional silencing in *Saccharomyces cerevisiae* requires a heterochromatin histone acetylation pattern. *Mol. Cell. Biol.* **16**: 4349–4356.
- BRYK, M., S. D. BRIGGS, B. D. STRAHL, M. J. CURCIO, C. D. ALLIS *et al.*, 2002 Evidence that Set1, a factor required for methylation of histone H3, regulates rDNA silencing in *S. cerevisiae* by a Sir2-independent mechanism. *Curr. Biol.* **12**: 165–170.
- CELIC, I., H. MASUMOTO, W. P. GRIFFITH, P. MELUH, R. J. COTTER *et al.*, 2006 The sirtuins hst3 and hst4p preserve genome integrity by controlling histone h3 lysine 56 deacetylation. *Curr. Biol.* **16**: 1280–1289.
- 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.
- DRISCOLL, R., A. HUDSON and S. P. JACKSON, 2007 Yeast Rtt109 promotes genome stability by acetylating histone H3 on lysine 56. *Science* **315**: 649–652.
- EDMONDSON, D. G., M. M. SMITH and S. Y. ROTH, 1996 Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. *Genes Dev.* **10**: 1247–1259.
- EHRENHOFER-MURRAY, A. E., D. H. RIVIER and J. RINE, 1997 The role of Sas2, an acetyltransferase homologue of *Saccharomyces cerevisiae*, in silencing and ORC function. *Genetics* **145**: 923–934.
- EHRENHOFER-MURRAY, A. E., R. T. KAMAKAKA and J. RINE, 1999 A role for the replication proteins PCNA, RF-C, polymerase ϵ and Cdc 45 in transcriptional silencing in *Saccharomyces cerevisiae*. *Genetics* **153**: 1171–1182.
- EISENBERG, J. C., R. APPAGARI, X. V. GOMES and P. M. BURGERS, 1997 Mutations in yeast proliferating cell nuclear antigen define distinct sites for interaction with DNA polymerase delta and DNA polymerase epsilon. *Mol. Cell. Biol.* **17**: 6367–6378.
- ENOMOTO, S., and J. BERMAN, 1998 Chromatin assembly factor I contributes to the maintenance, but not the re-establishment, of silencing at the yeast silent mating loci. *Genes Dev.* **12**: 219–232.
- FOX, C. A., A. E. EHRENHOFER-MURRAY, S. LOO and J. RINE, 1997 The origin recognition complex, SIR1, and the S phase requirement for silencing. *Science* **276**: 1547–1551.
- FRANCO, A. A., W. M. LAM, P. M. BURGERS and P. D. KAUFMAN, 2005 Histone deposition protein Asf1 maintains DNA replication integrity and interacts with replication factor C. *Genes Dev.* **19**: 1365–1375.
- GALLAGHER, S., S. E. WINSTON, S. A. FULLER and J. G. R. HURRELL, 2004 Immunoblotting and immunodetection, pp. 10.18.16–10.18.17 in *Current Protocols in Molecular Biology*, edited by F. M. AUSUBEL, R. BRENT, R. E. KINGSTON, D. D. MOORE, J. G. SEIDMAN *et al.* John Wiley & Sons, New York.
- GOLDSTEIN, A. L., and J. H. MCCUSKER, 1999 Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15**: 1541–1553.
- HAN, J., H. ZHOU, B. HORAZDOVSKY, K. ZHANG, R. M. XU *et al.*, 2007a Rtt109 acetylates histone H3 lysine 56 and functions in DNA replication. *Science* **315**: 653–655.
- HAN, J., H. ZHOU, Z. LI, R. M. XU and Z. ZHANG, 2007b Acetylation of lysine 56 of histone H3 catalyzed by Rtt109 and regulated by ASF1 is required for replisome integrity. *J. Biol. Chem.* **282**: 28587–28596.
- HAN, J., H. ZHOU, Z. LI, R. M. XU and Z. ZHANG, 2007c The Rtt109-Vps75 histone acetyltransferase complex acetylates non-nucleosomal histone H3. *J. Biol. Chem.* **282**: 14158–14164.
- HOPPE, G., J. TANNY, A. RUDNER, S. GERBER, S. DANAIE *et al.*, 2002 Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation. *Mol. Cell. Biol.* **22**: 4167–4180.
- HUANG, S., H. ZHOU, J. TARARA and Z. ZHANG, 2007 A novel role for histone chaperones CAF-1 and Rtt106p in heterochromatin silencing. *EMBO J.* **26**: 2274–2283.
- HYLAND, E. M., M. S. COSGROVE, H. MOLINA, D. WANG, A. PANDEY *et al.*, 2005 Insights into the role of histone H3 and histone H4 core modifiable residues in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **25**: 10060–10070.
- JESSULAT, M., M. ALAMGIR, H. SALSALI, J. GREENBLATT, J. XU *et al.*, 2008 Interacting proteins Rtt109 and Vps75 affect the efficiency of non-homologous end-joining in *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **469**: 157–164.
- KATAN-KHAYKOVICH, Y., and K. STRUHL, 2005 Heterochromatin formation involves changes in histone modifications over multiple cell generations. *EMBO J.* **24**: 2138–2149.
- KAUFMAN, P. D., R. KOBAYASHI and B. STILLMAN, 1997 Ultraviolet radiation sensitivity and reduction of telomeric silencing in *Saccharomyces cerevisiae* cells lacking chromatin assembly factor-I. *Genes Dev.* **11**: 345–357.
- KELLY, T. J., S. QIN, D. E. GOTTSCHLING and M. R. PARTHUN, 2000 Type B histone acetyltransferase Hat1p participates in telomeric silencing. *Mol. Cell. Biol.* **20**: 7051–7058.
- KIMMERLY, W., A. BUCHMAN, R. KORNBERG and J. RINE, 1988 Roles of two DNA-binding factors in replication, segregation and transcriptional repression mediated by a yeast silencer. *EMBO J.* **7**: 2241–2253.
- KIMURA, A., T. UMEHARA and M. HORIKOSHI, 2002 Chromosomal gradient of histone acetylation established by Sas2p and Sir2p functions as a shield against gene silencing. *Nat. Genet.* **32**: 370–377.
- KIRCHMAIER, A. L., and J. RINE, 2001 DNA replication-independent silencing in *S. cerevisiae*. *Science* **291**: 646–650.
- KIRCHMAIER, A. L., and J. RINE, 2006 Cell-cycle requirements in assembling silent chromatin in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **26**: 852–862.
- KRAKOFF, I. H., N. C. BROWN and P. REICHARD, 1968 Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. *Cancer Res.* **28**: 1559–1565.
- 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 I (CAF-1) colocalizes with replication foci in HeLa cell nuclei. *Exp. Cell Res.* **220**: 304–311.
- LE, S., C. DAVIS, J. B. KONOPKA and R. STERNGLANZ, 1997 Two new S-phase-specific genes from *Saccharomyces cerevisiae*. *Yeast* **13**: 1029–1042.
- LI, C., J. E. MUELLER and M. BRYK, 2006 Sir2 represses endogenous polymerase II transcription units in the ribosomal DNA nontranscribed spacer. *Mol. Biol. Cell* **17**: 3848–3859.
- LI, Y. C., T. H. CHENG and M. GARTENBERG, 2001 Establishment of transcriptional silencing in the absence of DNA replication. *Science* **291**: 650–653.

- LUGER, K., A. W. MADER, R. K. RICHMOND, D. F. SARGENT and T. J. RICHMOND, 1997 Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389**: 251–260.
- LUO, K., M. A. VEGA-PALAS and M. GRUNSTEIN, 2002 Rap1-Sir4 binding independent of other Sir, yKu or histone interactions initiates the assembly of telomeric heterochromatin in yeast. *Genes Dev.* **16**: 1528–1539.
- MAAS, N. L., K. M. MILLER, L. G. DEFazio and D. P. TOCZYSKI, 2006 Cell cycle and checkpoint regulation of histone h3 k56 acetylation by hst3 and hst4. *Mol. Cell* **23**: 109–119.
- 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.
- MASUMOTO, H., D. HAWKE, R. KOBAYASHI and A. VERREAULT, 2005 A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. *Nature* **436**: 294–298.
- McAINSH, A. D., S. SCOTT-DREW, J. A. MURRAY and S. P. JACKSON, 1999 DNA damage triggers disruption of telomeric silencing and Mec1p-dependent relocation of Sir3p. *Curr. Biol.* **9**: 963–966.
- MEIJING, S. H., and A. E. EHRENHOFER-MURRAY, 2001 The silencing complex SAS-I links histone acetylation to the assembly of repressed chromatin by CAF-I and Asf1 in *Saccharomyces cerevisiae*. *Genes Dev.* **15**: 3169–3182.
- MILLER, A. M., and K. A. NASMYTH, 1984 Role of DNA replication in the repression of silent mating type loci in yeast. *Nature* **312**: 247–251.
- 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.
- 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.
- MOUSSON, F., A. LAUTRETTE, J. Y. THURET, M. AGEZ, R. COURBEYRETTE *et al.*, 2005 Structural basis for the interaction of Asf1 with histone H3 and its functional implications. *Proc. Natl. Acad. Sci. USA* **102**: 5975–5980.
- NG, H. H., D. N. CICCONI, K. B. MORSHEAD, M. A. OETTINGER and K. STRUHL, 2003 Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation. *Proc. Natl. Acad. Sci. USA* **100**: 1820–1825.
- OSADA, S., A. SUTTON, N. MUSTER, C. E. BROWN, J. R. YATES, III *et al.*, 2001 The yeast SAS (something about silencing) protein complex contains a MYST-type putative acetyltransferase and functions with chromatin assembly factor ASF1. *Genes Dev.* **15**: 3155–3168.
- OZDEMIR, A., S. SPICUGLIA, E. LASONDER, M. VERMEULEN, C. CAMPSTEIJN *et al.*, 2005 Characterization of lysine 56 of histone H3 as an acetylation site in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280**: 25949–25952.
- POVIRK, L. F., 1996 DNA damage and mutagenesis by radiomimetic DNA-cleaving agents: bleomycin, neocarzinostatin and other enediynes. *Mutat. Res.* **355**: 71–89.
- PRADO, F., F. CORTES-LEDESMA and A. AGUILERA, 2004 The absence of the yeast chromatin assembly factor Asf1 increases genomic instability and sister chromatid exchange. *EMBO Rep.* **5**: 497–502.
- 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.
- RECHT, J., T. TSUBOTA, J. C. TANNY, R. L. DIAZ, J. M. BERGER *et al.*, 2006 Histone chaperone Asf1 is required for histone H3 lysine 56 acetylation, a modification associated with S phase in mitosis and meiosis. *Proc. Natl. Acad. Sci. USA* **103**: 6988–6993.
- REIFSNYDER, C., J. LOWELL, A. CLARKE and L. PILLUS, 1996 Yeast SAS silencing genes and human genes associated with AML and HIV-1 Tat interactions are homologous with acetyltransferases. *Nat. Genet.* **14**: 42–49.
- RUDNER, A. D., B. E. HALL, T. ELLENBERGER and D. MOAZED, 2005 A nonhistone protein-protein interaction required for assembly of the SIR complex and silent chromatin. *Mol. Cell. Biol.* **25**: 4514–4528.
- RUFIANGE, A., P. E. JACQUES, W. BHAT, F. ROBERT and A. NOURANI, 2007 Genome-wide replication-independent histone H3 exchange occurs predominantly at promoters and implicates H3 K56 acetylation and Asf1. *Mol. Cell* **27**: 393–405.
- RUSCHÉ, L. N., A. L. KIRCHMAIER and J. RINE, 2002 Ordered nucleation and spreading of silenced chromatin in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **13**: 2207–2222.
- RUSCHÉ, L. N., A. L. KIRCHMAIER and J. RINE, 2003 The establishment, inheritance, and function of silenced chromatin in *Saccharomyces cerevisiae*. *Annu. Rev. Biochem.* **72**: 481–516.
- SANTOS-ROSA, H., A. J. BANNISTER, P. M. DEHE, V. GELI and T. KOUZARIDES, 2004 Methylation of H3 lysine 4 at euchromatin promotes Sir3p association with heterochromatin. *J. Biol. Chem.* **279**: 47506–47512.
- SCHMITT, M. E., T. A. BROWN and B. L. TRUMPOWER, 1990 A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **18**: 3091–3092.
- SCHNEIDER, J., P. BAJWA, F. C. JOHNSON, S. R. BHAUMIK and A. SHILATIFARD, 2006 Rtt109 is required for proper H3K56 acetylation: a chromatin mark associated with the elongating RNA polymerase II. *J. Biol. Chem.* **281**: 37270–37274.
- SCHULZ, L. L., and J. K. TYLER, 2006 The histone chaperone ASF1 localizes to active DNA replication forks to mediate efficient DNA replication. *FASEB J.* **20**: 488–490.
- SCHWARTZ, J. L., 1989 Monofunctional alkylating agent-induced S-phase-dependent DNA damage. *Mutat. Res.* **216**: 111–118.
- 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., G. RIZKI and P. D. KAUFMAN, 2005 Regulation of histone deposition proteins Asf1/Hir1 by multiple DNA damage checkpoint kinases in *Saccharomyces cerevisiae*. *Genetics* **171**: 885–899.
- SHIBAHARA, K.-I., and B. STILLMAN, 1999 Replication-dependent marking of DNA by PCNA facilitates CAF-I-coupled inheritance of chromatin. *Cell* **96**: 575–585.
- SINGER, M. S., A. KAHANA, A. J. WOLF, L. L. MEISINGER, S. E. PETERSON *et al.*, 1998 Identification of high-copy disrupters of telomeric silencing in *Saccharomyces cerevisiae*. *Genetics* **150**: 613–632.
- STEARNS, T., H. MA and D. BOTSTEIN, 1990 Manipulating yeast genome using plasmid vectors. *Methods Enzymol.* **185**: 280–297.
- STRAHL, B. D., R. OHBA, R. G. COOK and C. D. ALLIS, 1999 Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in Tetrahymena. *Proc. Natl. Acad. Sci. USA* **96**: 14967–14972.
- SUKA, N., Y. SUKA, A. A. CARMEN, J. WU and M. GRUNSTEIN, 2001 Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin. *Mol. Cell* **8**: 473–479.
- SUKA, N., K. LUO and M. GRUNSTEIN, 2002 Sir2p and Sas2p oppositely regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. *Nat. Genet.* **32**: 378–383.
- 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. ALMOUZZI 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.
- TAMBURINI, B. A., J. J. CARSON, J. G. LINGER and J. K. TYLER, 2006 Dominant mutants of the *Saccharomyces cerevisiae* ASF1 histone chaperone bypass the need for CAF-I in transcriptional silencing by altering histone and Sir protein recruitment. *Genetics* **173**: 599–610.
- THAMINY, S., B. NEWCOMB, J. KIM, T. GATBONTON, E. FOSS *et al.*, 2007 Hst3 is regulated by Mec1-dependent proteolysis and controls the S phase checkpoint and sister chromatid cohesion by deacetylating histone H3 at lysine 56. *J. Biol. Chem.* **282**: 37805–37814.
- TSUBOTA, T., C. E. BERNDSEN, J. A. ERKMANN, C. L. SMITH, L. YANG *et al.*, 2007 Histone H3-K56 acetylation is catalyzed by histone chaperone-dependent complexes. *Mol. Cell* **25**: 703–712.
- 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.
- VAN LEEUWEN, F., and D. E. GOTTSCHLING, 2002 Assays for gene silencing in yeast. *Methods Enzymol.* **350**: 165–186.

- VAN LEEUWEN, F., P. R. GAFKEN and D. E. GOTTSCHLING, 2002 Dot1p modulates silencing in yeast by methylation of the nucleosome core. *Cell* **109**: 745–756.
- WACH, A., A. BRACHAT, R. POHLMANN and P. PHILIPPSEN, 1994 New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**: 1793–1808.
- WHITE, C. L., R. K. SUTO and K. LUGER, 2001 Structure of the yeast nucleosome core particle reveals fundamental changes in internucleosome interactions. *EMBO J.* **20**: 5207–5218.
- XU, E. Y., S. KIM, K. REPLOGLÉ, J. RINE and D. H. RIVIER, 1999a Identification of SAS4 and SAS5, two genes that regulate silencing in *Saccharomyces cerevisiae*. *Genetics* **153**: 13–23.
- XU, E. Y., S. KIM and D. H. RIVIER, 1999b SAS4 and SAS5 are locus-specific regulators of silencing in *Saccharomyces cerevisiae*. *Genetics* **153**: 25–33.
- XU, F., K. ZHANG and M. GRUNSTEIN, 2005 Acetylation in histone H3 globular domain regulates gene expression in yeast. *Cell* **121**: 375–385.
- XU, F., Q. ZHANG, K. ZHANG, W. XIE and M. GRUNSTEIN, 2007 Sir2 deacetylates histone H3 lysine 56 to regulate telomeric heterochromatin structure in yeast. *Mol. Cell* **27**: 890–900.
- YANG, B., and A. L. KIRCHMAIER, 2006 Bypassing the catalytic activity of SIR2 for SIR protein spreading in *S. cerevisiae*. *Mol. Biol. Cell* **17**: 5287–5297.
- ZHANG, Z., K. SHIBAHARA and B. STILLMAN, 2000 PCNA connects DNA replication to epigenetic inheritance in *S. cerevisiae*. *Nature* **408**: 221–225.
- ZHOU, H., B. J. MADDEN, D. C. MUDDIMAN and Z. ZHANG, 2006 Chromatin assembly factor 1 interacts with histone H3 methylated at lysine 79 in the processes of epigenetic silencing and DNA repair. *Biochemistry* **45**: 2852–2861.

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