HST3/HST4-dependent Deacetylation of Lysine 56 of Histone H3 in Silent Chromatin

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The composition of posttranslational modifications on newly synthesized histones must be altered upon their incorporation into chromatin. These changes are necessary to maintain the same gene expression state at individual chromosomal loci before and after DNA replication. We have examined how one modification that occurs on newly synthesized histone H3, acetylation of K56, influences gene expression at epigenetically regulated loci in *Saccharomyces cerevisiae*. H3 K56 is acetylated by Rtt109p before its incorporation into chromatin during S phase, and this modification is then removed by the NAD⁺-dependent deacetylases Hst3p and Hst4p during G2/M phase. We found silenced loci maintain H3 K56 in a hypoacetylated state, and the absence of this modification in *rtt109* mutants was compatible with HM and telomeric silencing. In contrast, loss of *HST3* and *HST4* resulted in hyperacetylation of H3 K56 within silent loci and telomeric silencing defects, despite the continued presence of Sir2p throughout these loci. These silencing defects in *hst3Δ hst4Δ* mutants could be suppressed by deletion of *RTT109*. In contrast, overexpression of Sir2p could not restore silencing in *hst3Δ hst4Δ* mutants. Together, our findings argue that *HST3 HST4* play critical roles in maintaining the hypoacetylated state of K56 on histone H3 within silent chromatin.

INTRODUCTION

Newly synthesized histones are decorated with several post-translational modifications that are altered upon their incorporation into chromatin after DNA replication. The changes acquired after chromatin assembly enable individual chromosomal loci to retain specific patterns of modifications on histones that are compatible with their preexisting states of gene expression. One such evolutionarily conserved modification that is subject to alterations is acetylation of K56 on histone H3.

H3 K56ac is found in diverse eukaryotes, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila*, and *Tetrahymena* (Hyland *et al.*, 2005; Masumoto *et al.*, 2005; Ozdemir *et al.*, 2005; Xu *et al.*, 2005; Recht *et al.*, 2006; Schneider *et al.*, 2006; Zhou *et al.*, 2006; Garcia *et al.*, 2007; Xhemalce *et al.*, 2007; Haldar and Kamakaka, 2008). H3 K56 is also conserved in mammals but only low levels of the acetylated form of this residue may be present in human cells (Xu *et al.*, 2005; Garcia *et al.*, 2007). In budding and fission yeast, H3 K56 is acetylated by the recently discovered acetyltransferase Rtt109p (Schneider *et al.*, 2006; Collins *et al.*, 2007; Driscoll *et al.*, 2007; Han *et al.*, 2007a,b,c; Tsubota *et al.*, 2007; Xhemalce *et al.*, 2007). In both organisms, H3 K56ac is regulated throughout the cell cycle by the combined actions of Rtt109p and the NAD⁺-dependent deacetylases Hst3p and Hst4p in *S. cerevisiae* and their orthologues encoded by *rtt109⁺* and *hst4⁺* in *S. pombe*. In budding yeast, H3 K56ac and expression of *RTT109* peak during S phase. This modi-

fication is later removed in an *HST3 HST4*-dependent manner during G2 and M 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). *HST3* and *HST4* are also cell cycle regulated; expression of *HST3* and *HST4* peaks during G2/M, and M/G1, respectively (Maas *et al.*, 2006). Expression of *hst4⁺* and H3 K56ac in *S. pombe* during the cell cycle is similar (Recht *et al.*, 2006; Xhemalce *et al.*, 2007; Haldar and Kamakaka, 2008).

Rtt109p acetylates K56 on newly synthesized H3, while H3/H4 dimers are bound by the chromatin assembly factor Asf1p (Han *et al.*, 2007b; Tsubota *et al.*, 2007). Once assembled into nucleosomes, H3 K56 becomes positioned near the “entry-exit point” of the DNA double helix on the nucleosome (Luger *et al.*, 1997), making this residue a potential regulatory site for changes in chromatin structure. Consistent with this notion, different charge states at this residue coincide with structural alterations in chromatin. Chromatin isolated from cells expressing H3 K56Q mutants, mimicking the acetylated state at this residue, is more sensitive to digestion by micrococcal nuclease than is chromatin from cells expressing wild-type histones or H3 K56R mutants mimicking the deacetylated state. And, plasmids from cells expressing H3 K56Q are less supercoiled in topology assays than those from cells expressing wild-type histones or H3 K56R (Masumoto *et al.*, 2005), whereas plasmids from *rtt109* mutants are more supercoiled than those from wild-type cells (Driscoll *et al.*, 2007). These observations imply the acetylated form of this residue reflects a more “relaxed” state of chromatin.

Also, consistent with the model that this modification may be regulatory in nature, multiple studies have recently reported mutations leading to either a constitutively hypo- or hyperacetylated state at H3 K56 through either mutation of K56 on H3 or deletion of *RTT109* or *HST3* and *HST4* result in defects in chromatin integrity and hypersensitivity to DNA damaging agents (Brachmann *et al.*, 1995; Hyland *et al.*, 2005;

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Abbreviations used: ChIP, chromatin immunoprecipitation.

Masumoto *et al.*, 2005; Ozdemir *et al.*, 2005; Celic *et al.*, 2006; Maas *et al.*, 2006; Recht *et al.*, 2006; Schneider *et al.*, 2006; Collins *et al.*, 2007; Driscoll *et al.*, 2007; Han *et al.*, 2007a,b,c; Thamyat *et al.*, 2007; Xhemalce *et al.*, 2007; Fillingham *et al.*, 2008; Jessulat *et al.*, 2008; Miller *et al.*, 2008). In this study, we have explored the relationship between H3 K56 and epigenetic gene regulation in *S. cerevisiae*.

Across phyla, epigenetically silent chromatin is formed through varying processes sharing a common series of steps. These steps include recruitment of the structural components of silent chromatin to specific chromosomal sites, alterations in chromatin modifications to permit propagation of those structural components across large chromosomal regions, plus additional, often poorly understood, events that ultimately result in the heritable inactivation of gene expression. In budding yeast, genes adjacent to telomeres and at the silent mating-type loci are regulated epigenetically through a process called silencing. The structural components of silent chromatin found at the silent mating-type loci, *HML* and *HMR*, and at telomeres are the Sir proteins, Sir1-4p. When silent chromatin forms at the *HM* loci, the Sir proteins are recruited to silencers where they physically interact with DNA binding proteins, including Rap1p, Abf1p, and ORC. Sir proteins are recruited to telomeres in a similar manner, but primarily via multiple Rap1p binding sites (Strahl-Bolsinger *et al.*, 1997; Lieb *et al.*, 2001, and references within). Once recruited to a silencer or the end of a telomere, Sir proteins will propagate along the chromosome by binding nucleosomes (Hoppe *et al.*, 2002; Luo *et al.*, 2002; Rusché *et al.*, 2002, 2003). During Sir spreading, histones H3 and H4 are deacetylated through the enzymatic action of Sir2p, a NAD⁺-dependent histone deacetylase that preferentially removes acetyl groups from K9 and K14 on histone H3 and K16 on histone H4 (Imai *et al.*, 2000; Tanny and Moazed, 2001). Histone deacetylation is critical for silencing and cells expressing catalytically inactive mutants of Sir2p are defective in both Sir spreading and silencing (Imai *et al.*, 2000; Hoppe *et al.*, 2002; Luo *et al.*, 2002; Rusché *et al.*, 2002; Kirchmaier and Rine, 2006; Yang and Kirchmaier, 2006).

Several groups have reported links between H3 K56ac and silencing. In both *S. cerevisiae* and *S. pombe*, cells lacking *HST3* and *HST4* or *hst4*⁺, respectively, have silencing defects (Brachmann *et al.*, 1995; Freeman-Cook *et al.*, 1999; Grunweller and Ehrenhofer-Murray, 2002; Durand-Dubief *et al.*, 2007). Mutations affecting the charge of H3 K56 also influence silencing (Hyland *et al.*, 2005; Xu *et al.*, 2007; Miller *et al.*, 2008) and Sir2p can deacetylate K56 on histone H3 in vitro (Xu *et al.*, 2007). In this study, we have explored the relationship between acetylation of K56 on histone H3 and silencing. Our findings revealed that *HST3* *HST4* are required for telomeric silencing and efficient deacetylation of K56 on H3 within silent chromatin.

MATERIALS AND METHODS

Yeast Strains and Plasmids

Yeast strains (Table 1) and plasmids (Table 2) used in this study. Yeast strains AKY4454, AKY4456, AKY4460, AKY4470, and AKY4472 containing *URA3* and *TRP1* at *Tel VIII* were generated by integration of *Sall* to *EcoRI* fragments of pVII-L *URA3*-TEL or pADTRP1(+) at *ADH4* as described previously (Gottschling *et al.*, 1990) and were confirmed by DNA blots (data not shown). pAK966 encoding histone H3 K9,14,56R/H4 K16R was generated from pAK923 (Yang and Kirchmaier, 2006) by site-directed mutagenesis using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) following the QuikChange site-directed mutagenesis kit protocol (Stratagene) using oALK642 5'-GAA GAT TCC AAA GAT CTA CTG AAC T-3' and oALK643 5'-AGT TCA GTA

GAT CTT TGG AAT CTT C-3', then confirmed by sequencing. pAK974 was generated by digesting pAR14 (Holmes *et al.*, 1997) with *EcoRI*/*HindIII*, and ligating the 6-kbp fragment from pAR14 containing *SIR2* expressed from the *GAL10* promoter to *EcoRI*/*HindIII* of pRS426 (Christianson *et al.*, 1992). pAK975 was generated by cleavage of pAK974 with *Sall* and religation of the vector to remove *SIR2*. pAK996 was generated by ligating a 3-kbp *Sall*/*SacI* fragment of ICe72 (*pCEN-URA3-HST3*) (Celic *et al.*, 2006) containing *HST3* into *Sall*/*SacI* of pRS415 (Christianson *et al.*, 1992). pAK998 was generated by ligating the same 3-kbp *Sall*/*SacI* fragment of ICe72 into *XhoI*/*SacI* of pRS426.

Chromatin Immunoprecipitation (ChIP)

ChIP experiments were performed as described previously (Rusché *et al.*, 2002; Yang and Kirchmaier, 2006) by using anti-Acetyl-H4 (K16) antibodies (07-329; Millipore, Billerica, MA), anti-Acetyl-H3 (K56) antibodies (07-677; Millipore), anti-H3 antibodies (Ab1791-100; Abcam, Cambridge, MA), anti-Sir2p and anti-Sir3p antibodies (Axelrod, 1991), or normal rabbit serum (100 853; Roche Diagnostics, IN). ChIPs were analyzed by real-time polymerase chain reaction (PCR) by using an ABI Prism 7000 (Applied Biosystems, Foster City, CA) as described previously and in figure legends (Kirchmaier and Rine, 2006; Yang and Kirchmaier, 2006). Oligonucleotides used during analyses have been described previously (Yang and Kirchmaier, 2006). Statistical analyses were performed using the Wilcoxon rank sum test with MSTAT version 2.6 (<http://mcards.oncology.wisc.edu/mstat>), and ChIP data are expressed as average $\pm$ SD, $n = 3$. The level of H3 K56ac (normalized to H3) at *SSC1* was similar to those at three other control loci, *ACT1*, *MAT*, and 7.5 Kb from *Tel VIR*, in *hst3 hst4* mutants, validating the use of *SSC1* as an internal control for this study (data not shown).

RNA Analysis

Total RNA was extracted from logarithmically growing cells as described previously (Schmitt *et al.*, 1990; Kirchmaier and Rine, 2001), and levels of *a1*, *yFR057w*, *HST3*, and *SCR1* transcripts were analyzed as described previously by quantitative real-time PCR using an ABI Prism 7000 (Applied Biosystems) (Kirchmaier and Rine, 2006; Yang and Kirchmaier, 2006). Oligonucleotides used for the analyses of *HST3* expression were oALK825 5'-CAGTC-GATCGGGCTCAATGT-3' and oALK826 5'-TTCATCGTCGGCATCAAGAC-3'. Statistical analyses were performed using the Wilcoxon Rank sum test with MSTAT version 2.6, and transcription data are expressed as average $\pm$ SD, $n = 3$.

Immunoblot Analyses

Immunoblot analyses were performed as described previously (Rusché *et al.*, 2002; Miller *et al.*, 2008). We separated 0.33 OD cell equivalents of whole cell lysates from logarithmically growing cells in 15% (for analysis of histone H3) or 7.5% (for Sir2p) SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to polyvinylidene difluoride membranes (162-0177; Bio-Rad). Immunoblots in Figure 4 were probed with anti-acetyl-H3 (K56) antibodies (1:10,000; 07-677, Millipore) or anti-Sir2p (1:10,000; Axelrod, 1991) and with the secondary antibody Alexa Flour anti-rabbit IgG (H+L) (1:20,000; A21109, Invitrogen, Carlsbad, CA). Membranes were stripped (Gallagher *et al.*, 2004) and reprobed with anti-H3 antibodies (1:10,000; 06-755, Millipore) or anti-PGK1 antibodies (1:10,000; A-6457, Invitrogen), respectively, and with the secondary antibodies, Alexa Flour anti-rabbit immunoglobulin G (IgG) (H+L) (as described above) or Alexa Flour anti-mouse IgG (H+L) (1:20,000; A21058, Invitrogen), respectively. Immunoblots were analyzed using an Odyssey Infrared Imager (Li-Cor Biosciences, Lincoln, NE) according to manufacturer's instructions.

Mating Assays

Patch mating assays were performed on two independent yeast strains for each genotype as described previously (van Leeuwen and Gottschling, 2002; Yang and Kirchmaier, 2006) and as outlined in Figure 3. In quantitative mating assays, the mating efficiency of each strain relative to wild-type was determined as follows: (colonies on YM plate with indicated tester strain/colonies on YM plate with supplements)_{indicated strain}/(colonies on YM plate with indicated tester strain/colonies on YM plate with supplements)_{wild type}.

Telomeric Silencing Assays

Yeast were grown logarithmically in minimal medium containing adenine, histidine, tryptophan, and uracil and containing or lacking leucine, and then they were diluted to $\sim 1 \times 10^4$ cells/ μ l. Three microliters of 10-fold serial dilutions of yeast were plated onto complete synthetic medium; synthetic medium lacking leucine, uracil, leucine, and uracil, or leucine and tryptophan; or medium containing 5-fluorotic acid (5-FOA). Cells were incubated for 2 d at 30°C and then photographed using an Alpha Innotech (San Leandro, CA) imager and Chemiluminescence 5500 version 2.02 software. In wild-type cell populations, telomeric reporter genes are expressed or silenced in different fractions of the population. Depressed subpopulations will express telomeric *TRP1* or *URA3* reporter genes and grow in the absence of tryptophan or uracil, respectively. Expression of

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
AKY1968	W303 <i>MATα hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3</i> plus pPK189	Yang and Kirchmaier (2006) ^a
AKY944	W303 <i>MATα hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3</i> plus pPK189	Yang and Kirchmaier (2006) ^a
AKY1101	W303 <i>MATα LEU2::sir2-345 sir2Δ::KanMX hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3</i> plus pPK189	Yang and Kirchmaier (2006) ^a
AKY1103	W303 <i>MATa LEU2::sir2-345 sir2Δ::KanMX hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3</i> plus pPK189	Yang and Kirchmaier (2006) ^a
AKY3857	AKY1968 <i>rtt109Δ::KanMX</i>	Miller <i>et al.</i> (2008)
AKY3991	AKY944 <i>rtt109Δ::KanMX</i>	This study ^a
AKY3855	AKY1101 <i>rtt109Δ::KanMX</i>	This study ^a
AKY3995	AKY1103 <i>rtt109Δ::KanMX</i>	This study ^a
PKY4220	W303 <i>MATa bar1Δ::his5⁺ hst3Δ::NatMX hst4Δ::KanMX</i>	P. Kaufman
PKY4222	PKY4220 <i>MATα</i>	P. Kaufman
AKY4250	PKY4222 <i>rtt109Δ::HphMX</i>	This study
AKY4534	W303 <i>MATα hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3 hst3Δ::NatMX hst4Δ::HphMX</i> plus pPK189	This study ^a
AKY4496	W303 <i>MATα LEU2::sir2-345 sir2Δ::KanMX hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3 hst3Δ::NatMX hst4Δ::HphMX</i> plus pPK189	This study ^a
AKY4454	W303 <i>MATα URA3-TELVII-L</i>	This study ^a
AKY4456	W303 <i>MATα sir2Δ::TRP1 URA3-TELVII-L</i>	This study ^a
AKY4470	PKY4222 <i>URA3-TELVII-L</i>	This study ^a
AKY4460	W303 <i>MATα URA3-TRP1-TELVII-L</i>	This study ^a
AKY4472	PKY4222 <i>URA3-TRP1-TELVII-L</i>	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.

URA3 prevents growth in the presence of 5-FOA due to the conversion of 5-FOA into toxic 5-fluorouracil by *Ura3p*.

RESULTS

Acetylation of K56 on Histone H3 Disrupts Telomeric Silencing

Recently, we have found that mutating K56 to Q on histone H3, which neutralizes the positive charge at this residue, disrupts telomeric silencing, whereas mutating H3 K56 to

R, which maintains a positive charge at this residue, is largely compatible with silencing (Miller *et al.*, 2008; see also Hyland *et al.*, 2005; Xu *et al.*, 2007). Consistent with acetylation of K56 on H3 influencing silencing, cells lacking the H3 K56-specific deacetylases encoded by *HST3* and *HST4* are defective in silencing a telomeric *URA3* reporter gene adjacent to *Tel IV* (Brachmann *et al.*, 1995). Similarly, we found that silencing of a *URA3* reporter at *Tel VIII* was disrupted in *hst3Δ hst4Δ* mutants by at least

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>	Miller <i>et al.</i> (2008)
pAK972	H3 H4 K16R <i>ARS/CEN/TRP1</i>	Miller <i>et al.</i> (2008)
pAK981	H3 K56R H4 K16R <i>ARS/CEN/TRP1</i>	Miller <i>et al.</i> (2008)
pAK923	H3 K9,14R H4 K16R <i>ARS/CEN/TRP1</i>	Yang and Kirchmaier, (2006)
pAK966	H3 K9,14,56R H4 K16R <i>ARS/CEN/TRP1</i>	This study
pFA6::kanMX4		Wach <i>et al.</i> (1994)
pAG32	pFA6::HphMX4	Goldstein and McCusker (1999)
pAG25	pFA6::NatMX4	Goldstein and McCusker (1999)
pRS415	<i>ARS/CEN/LEU2</i>	Christianson <i>et al.</i> (1992)
pRS426	2 μ m/ <i>URA3</i>	Christianson <i>et al.</i> (1992)
pAK975	<i>GAL10p</i> in pRS426	This study
pAK974	<i>GAL10p SIR2</i> in pRS426	This study
pAK996	<i>HST3</i> in pRS415	This study
pAK998	<i>HST3</i> in pRS426	This study
ICe72	<i>HST3 ARS/CEN/URA3</i>	Celic <i>et al.</i> (2006)
pAR14	<i>GAL10p SIR2</i> in YEp51	Holmes <i>et al.</i> (1997)
YEp351	2 μ m/ <i>LEU2</i>	Hill <i>et al.</i> (1986)
LP349	<i>SIR2</i> in YEp351	Sherman <i>et al.</i> (1999)
pVII-L <i>URA3-TEL</i>	<i>URA3-TELVII-L</i>	Gottschling <i>et al.</i> (1990)
pADTRP1(+)	<i>URA3-TRP1-TELVII-L</i>	Gottschling <i>et al.</i> (1990)

Figure 1. Overexpression of *SIR2* does not restore telomeric silencing in *hst3Δ hst4Δ* mutants. (A and B) Silencing of *URA3* at *Tel VII-L*. For *URA3-TRP1-VII-L*, transcription of *URA3* occurs toward the telomere. (C) Silencing of *TRP1* and *URA3* at *Tel VII-L*. For *URA3-TRP1-VII-L*, transcription of *URA3* and *TRP1* occur toward the telomere. Plasmids in indicated strains include YEp351 or pLP349 (A and C) and pRS415 or pAK996 (B). Ten-fold serial dilutions of logarithmically growing yeast with the indicated genotypes were plated onto the indicated media and were incubated for 2 d at 30°C before photographing (see *Materials and Methods*).

1×10^4 -fold as measured by growth on 5-FOA plates (Figure 1). This silencing defect was similar in magnitude to defects observed in cells lacking *SIR2* (Figure 1A). The silencing defect in *hst3Δ hst4Δ* mutants could be suppressed by ectopic expression of *HST3* (Figure 1B) but not by overexpression of *SIR2* (Figure 1, A and C). Sir2p has

been reported to modulate silencing via deacetylation of H3 K56 (Xu *et al.*, 2007). So, to clarify the role of *HST3 HST4* in silencing, we assessed whether *HST3 HST4*-dependent silencing defects were also related to the acetylation status of H3 K56.

To test the effects of H3 K56 acetylation on silencing, we first examined expression of *yFR057w*, a gene adjacent to *Tel*

Figure 2. Loss of *HST3* and *HST4* disrupts telomeric silencing. (A) Map of *Tel VIR*. The regions amplified at *Tel VIR* are noted. (B) Transcription from *yFR057w* at *Tel VIR* in *hst3Δ hst4Δ* mutants. Transcript levels of *yFR057w* relative to *SCR1* (internal control) were determined by quantitative real-time PCR for each strain and then expressed relative to wild type, which was set to 1. Data were calculated as $2^{[(\text{locus } C_T - \text{SCR1 } C_T)_{\text{wild-type}} - (\text{locus } C_T - \text{SCR1 } C_T)_a]}$, where “a” is the indicated genotype. (C–E) *HST3* and *HST4* are required for H3 K56 hypoacetylation at *Tel VIR*. H3 K56ac (C), Sir2p (D), and H4 K16ac (E) levels at *Tel VIR* in wild-type and *hst3Δ hst4Δ* strains were monitored by ChIP. The efficiency of coprecipitation of DNA from 0.6 and 1.2 kb from the end of *Tel VIR* or *SSC1* (internal control) with anti-H3 K56ac (C) or H4 K16ac (E) antibodies was determined relative to that with anti-H3 antibodies, and then each locus was normalized to *SSC1* which was set to 1. Data were calculated as $2^{[(\text{H3 K56ac or H4 K16ac } C_T - \text{H3 } C_T)_{\text{SSC1}} - (\text{H3 K56ac or H4 K16ac } C_T - \text{H3 } C_T)_a]}$, where “a” is the indicated locus. For anti-Sir2p ChIPs (D), the efficiency of coprecipitation of each locus with anti-Sir2p antibodies was determined relative to Input and then each locus was normalized to *SSC1*, which was set to 1. Data were calculated as $2^{[(\text{Sir2p } C_T - \text{Input } C_T)_{\text{SSC1}} - (\text{Sir2p } C_T - \text{Input } C_T)_a]}$, where “a” is the indicated locus (see *Materials and Methods*).

Figure 3. Loss of *HST3* and *HST4* leads to hyperacetylation of H3 K56 but does not disrupt silencing at *HMR*. (A) Map of *HMR*. The regions amplified at *HMR* are noted. (B) Mating analyses of silencing at *HM* loci in *hst3Δ hst4Δ* mutants. Haploid *MATα* or *MATa hst3Δ hst4Δ* mutants were grown for 1 d at 30°C on rich medium (YPD) and then tested for silencing at *HML* and *HMR* by replica plating to α or *a* mating-type tester lawns on minimal medium and incubated for 2 d at 30°C. Growth of diploids on minimal medium indicates that *HML* or *HMR* was silenced. Relative mating efficiencies from quantitative mating assays are noted on right. The efficiencies of mating of the *MATa* or *MATα HST3 HST4* strains to tester strains JRY2728 (*MATα*) or JRY2726 (*MATa*), respectively, was determined relative to their plating efficiency on minimal medium plus supplements (for *MATa*, $100 \pm 6.7\%$, $n = 3$; for *MATα*, $87 \pm 17\%$, $n = 3$) and were set to 1. The mating efficiencies of *hst3Δ hst4Δ* mutants were determined similarly and are expressed relative to *HST3 HST4* strains (see *Materials and Methods*), average $\pm$ SD, $n = 3$. (C–E) *HST3* and *HST4* are required for deacetylation of H3 K56 at *HMR*. H3 K56ac (C), Sir2p (D), and H4 K16ac (E) levels at *HMR* in wild-type and *hst3Δ hst4Δ* strains were monitored by ChIP. The efficiency of coprecipitation of DNA from the *HMR E* silencer, *HMR a1* or *SSC1* with anti-H3 K56ac, H4 K16ac, or anti-Sir2p antibodies was determined and data were calculated as shown in Figure 2.

VIR (Figure 2A), in *hst3Δ hst4Δ* mutants by quantitative real-time PCR (Figure 2). *yFR057w* was derepressed in *hst3Δ hst4Δ* mutants relative to wild-type strains (Figure 2B), and this silencing defect in the *hst3Δ hst4Δ* mutants could be suppressed by expression of *HST3* from a low copy plasmid (Figure 2B and Supplemental Figure 1). The severity of the silencing defect in *hst3Δ hst4Δ* mutants was similar to that found in cells expressing a catalytically inactive mutant of

Sir2p (see below). These findings indicated *HST3* and *HST4* were required for silencing *yFR057w*.

Because deacetylation of K56 on histone H3 is regulated by Hst3p and Hst4p (Celic *et al.*, 2006; Maas *et al.*, 2006) and the silencing defect of the *hst3Δ hst4Δ* mutants could be suppressed by deletion of *RTT109* encoding the H3 K56ac-specific acetyltransferase (Figure 2B), we reasoned that this silencing defect must have been caused by hyperacetylation

Figure 4. Overexpression of *SIR2* does not reduce H3 K56ac or restore silencing in *hst3Δ hst4Δ* mutants. (A) Immunoblot analyses of Sir2p and H3 K56ac. Whole cell extracts of *hst3Δ hst4Δ* mutants containing a vector (pAK975) or a *SIR2* overexpression plasmid (pAK974) were analyzed by immunoblotting using anti-H3 K56ac or anti-Sir2p antibodies plus anti-H3 or anti-Pgk1p antibodies (internal controls), respectively (see *Materials and Methods*). Cells were grown to OD 0.5 in SC-Ura with 2% glucose, washed, diluted in SC-Ura with 2% galactose, and incubated at 30°C for 4 h to induce *SIR2*. Two independent clones for each genotype are shown. (B and C) Overexpression of *SIR2* does not reduce H3 K56ac at *Tel VIR* in *hst3Δ hst4Δ* mutants. H3 K56ac levels at 0.6 kb (B) and 1.2 kb (C) from the end of *Tel VIR* were monitored by ChIP in *MATα hst3Δ hst4Δ* yeast containing a vector or a *SIR2* overexpression plasmid. The efficiency of coprecipitation of each locus with anti-H3 K56ac antibodies was determined relative to that with anti-H3 antibodies, then each locus was normalized to *hst3Δ hst4Δ* mutants containing the vector control, which was set to 1. Data were calculated as $2^{[(H3\ K56ac\ C_T - H3\ C_T)_{vector} - (H3\ K56ac\ C_T - H3\ C_T)_{SIR2\ O/E}]} - 1$. (D) Overexpression of *SIR2* in *hst3Δ hst4Δ* mutants does not restore silencing at *Tel VIR*. Transcript levels of *yFR057w* relative to *SCR1* were determined by quantitative real-time PCR for each strain and then expressed relative to wild type, which was set to 1. Data were calculated as shown in Figure 2. (E and F) Overexpression of *SIR2* in *hst3Δ hst4Δ* mutants does not reduce H3 K56ac at *HMR*. H3 K56ac levels at the *HMR E* silencer (E) and *a1* (F) were monitored by ChIP as described above.

of H3 K56 at *Tel VIR* in the *hst3Δ hst4Δ* mutants. To test this possibility, we examined the acetylation status of K56 on histone H3 at *Tel VIR* in *hst3Δ hst4Δ* mutants by ChIP. H3 K56ac levels at *Tel VIR* were increased in *hst3Δ hst4Δ* mutants relative to wild-type cells (Figure 2C; see also Xu *et al.*, 2007). Because telomeric silencing requires Sir proteins (Aparicio *et al.*, 1991), it was possible that hyperacetylation of H3 K56 at *Tel VIR* had disrupted Sir localization in the above-mentioned experiments. To determine whether the silencing defect in *hst3Δ hst4Δ* mutants resulted from the loss Sir protein binding at *Tel VIR*, we compared Sir2p association at *Tel VIR* in wild-type and *hst3Δ hst4Δ* strains by ChIP (Figure 2D). Surprisingly, Sir2p binding at *Tel VIR* was similar in both wild-type and *hst3Δ hst4Δ* strains. And, in contrast to H3 K56ac, the Sir2p-specific substrate H4 K16 remained hypoacetylated at *Tel VIR* in both wild-type and *hst3Δ hst4Δ* strains (Figure 2E), despite the loss of silencing of *yFR057w* (Figure 2B).

hst3 and *hst4* mutants have mild defects in silencing a *URA3* reporter at *HMR* (Grunweller and Ehrenhofer-Murray, 2002). Therefore, we next examined silencing at the native *HM* loci in *hst3Δ hst4Δ* mutants (Figure 3). In contrast to telomeric silencing, silencing was maintained at the native *HM* loci in cells lacking *HST3* and *HST4*, as measured by both patch and quantitative mating assays (Figure 3B). However, like at *Tel VIR*, H3 K56ac levels at *HMR* increased in *hst3Δ hst4Δ* mutants relative to wild-type cells (Figure 3C), despite the continued presence of Sir2p (Figure 3D), and H4 K16ac (Figure 3E) levels at *HMR* having remained similar in both *hst3Δ hst4Δ* mutants and wild-type cells. Together, these findings indicated *HST3* and *HST4* were required for efficient deacetylation of H3 K56 at both *HMR* and *Tel VIR*, and hyperacetylation of K56 on histone H3 resulted in the disruption of telomeric silencing. The differences in sensitivity of *a1* and *URA3* at *HMR* to loss of *HST3* and *HST4* argue sensitivity to disruption of silencing via hyperacetylation of H3 K56 may be promoter-dependent.

Overexpression of *SIR2* Neither Restores Silencing Nor Decreases H3 K56ac in *hst3 hst4* Mutants

Our data indicated that deletion of *HST3* and *HST4* increased the levels of H3 K56ac at both *yFR057w* and *HMR*, implying Hst3p and Hst4p were required to maintain the deacetylated state of H3 K56 within silent chromatin. This result was somewhat surprising as Sir2p remained physically present at both loci in the vicinity of H3 K56ac and was able to deacetylate H4 K16 at these sites efficiently (Figures 2 and 3). A possible explanation for this observation was that the amount of Sir2p present at *HMR* and *yFR057w* might somehow be limiting in the *hst3Δ hst4Δ* mutants, and this had adversely affected Sir2p's ability to deacetylate H3 K56. To test this possibility, we overexpressed *SIR2* from the galactose inducible promoter *GAL10* in *hst3Δ hst4Δ* mutants and monitored H3 K56ac. Overexpression of Sir2p did not alter the levels of H3 K56ac in whole cell extracts from *hst3Δ hst4Δ* mutants (Figure 4A). We then examined the acetylation status of H3 K56 at *Tel VIR* and *HMR* by ChIP. Overexpression of *SIR2* in *hst3Δ hst4Δ* mutants did not lead to the deacetylation of H3 K56 at *Tel VIR* (Figure 4, B and C) and could not restore silencing at *yFR057w* (Figure 4D), despite the ability of Sir2p to deacetylate H3 K56 efficiently *in vitro* (Xu *et al.*, 2007). Similarly, overexpression of *SIR2* in *hst3Δ hst4Δ* mutants did not reduce H3 K56ac at the *E* silencer (Figure 4E) or *a1* (Figure 4F) at *HMR* relative to control strains. In contrast, overexpression of either *HST3* or *HST4* from the galactose inducible promoter *GAL1* in *hst3Δ hst4Δ* mutants resulted in hypoacetylation of H3 K56 at *Tel VIR*

(data not shown). Together, these results plus the observation that loss of *RTT109* had restored telomeric silencing in

Figure 5. *SIR* protein spreading in *sir2-345* H4 K16R mutants does not disrupt H3 K56ac at *Tel VIR*. (A–C) Sir2p association and H4 K16ac and H3 K56ac levels at *Tel VIR* in *SIR2* or *sir2-345* strains expressing wild-type histones or H4 K16R were monitored by ChIP. For each of the indicated genotypes, the efficiency of coprecipitation of DNA from 0.6 and 1.2 kb from the end of *Tel VIR* or *SSC1* with anti-Sir2p (A), H4 K16ac (B), or H3 K56ac (C) antibodies was determined and data were calculated as shown in Figure 2.

hst3Δ hst4Δ mutants (Figure 2B) argued that, although Sir2p can deacetylate H3 K56 (Xu *et al.*, 2007), Hst3p/Hst4p were playing critical roles in deacetylating this residue within silent chromatin.

Hypoacetylation of H3 K56 at HMR Requires Silencing

The above-mentioned experiments established that *HST3* *HST4* were required for efficient deacetylation of H3 K56 within silent chromatin. We next assessed whether hypoacetylation of K56 on histone H3 required silencing per se or simply the presence of Sir proteins by using *SIR2* and *sir2-345* yeast. *sir2-345* is a catalytically inactive mutant of Sir2p that is stably expressed, can interact with other Sir proteins and is readily recruited to silencers in the presence of other Sir proteins (Imai *et al.*, 2000; Rusché *et al.*, 2002; Kirchmaier and Rine, 2006). However, Sir protein propagation along chromatin is defective in *sir2-345* mutants (Rusché *et al.*, 2002; Kirchmaier and Rine, 2006; see also (Hoppe *et al.*, 2002; Luo *et al.*, 2002). Sir spreading, but not silencing, can be restored in *sir2-345* cells expressing histone mutants that mimic the hypoacetylated state of substrates of Sir2p (Yang and Kirchmaier, 2006; Yang *et al.*, 2008). Because loss of acetylation of H4 K16 is both necessary and sufficient for Sir protein spreading (Yang *et al.*, 2008), we monitored H3 K56ac levels in *SIR2* or *sir2-345* yeast expressing either wild-type histones or H4 K16R mutants. ChIP assays using anti-Sir2p, anti-H4 K16ac or anti-H3 K56ac antibodies indicated Sir2p was present at *Tel VIR* (Figure 5A), and both H4 K16 (Figure 5B) and H3 K56 (Figure 5C) were hypoacetylated in *SIR2* cells expressing wild-type histones. In contrast, Sir2p was absent from *Tel VIR* (Figure 5A) and both H4 K16 (Figure 5B) and H3 K56 (Figure 5C) were hyperacetylated in *sir2-345* mutants expressing wild-type histones. In *SIR2* and *sir2-345* cells expressing H4 K16R mutants, Sir2p and *sir2-345* spread throughout *Tel VIR* (Figure 5A). And, although H3 K56 was hypoacetylated in *SIR2* cells, H3 K56 remained hyperacetylated in *sir2-345* mutants (Figure 5C), implying deacetylation of this residue was not critical for Sir spreading (see also Figures 2 and 3). Similar results were observed

at *HMR* (Supplemental Figure 2). Thus, *HMR* and *Tel VIR* remained transcriptionally active (see Figures 6 and 7 and Supplemental Figures 3 and 4 below) and hyperacetylated at H3 K56 in *sir2-345* mutants expressing H4 K16R in which Sir spreading had been restored. This hyperacetylated state of H3 K56 in *sir2-345* mutants was consistent with the above-mentioned experiments, which collectively indicated Hst3p/Hst4p-dependent loss of H3 K56ac at *HMR* and *Tel VIR* was also silencing dependent.

H3 K56ac Levels Are Similar in *hst3Δ hst4Δ*, *sir2-345*, and *hst3Δ hst4Δ sir2-345* Mutants

To assess the relative contributions of Sir2p and Hst3p/Hst4p to the deacetylation of H3 K56, we next combined *hst3Δ hst4Δ* and *sir2-345* mutants and assessed silencing, H3 K56ac and Sir3p association at *yFR057w* adjacent to *Tel VIR* and at *HMR* (Figure 6 and Supplemental Figure 3, respectively). *yFR057w* was derepressed in both *sir2-345* mutants and *hst3Δ hst4Δ* mutants expressing wild-type histones relative to wild-type cells. However, *yFR057w* transcript levels were slightly lower in the *hst3Δ hst4Δ* mutants than in the *sir2-345* mutants (Figure 6A). This reduction correlated with the presence of Sir3p adjacent to *Tel VIR* in *hst3Δ hst4Δ* mutants versus their absence in *sir2-345* mutants (Figure 6B). Because a mild reduction in *yFR057w* transcript levels was also observed in *hst3Δ hst4Δ sir2-345* mutants expressing H4 K16R in which Sir spreading, but not silencing had been restored (Figure 6, B and A, respectively), Sir protein spreading across *yFR057w* may have interfered with transcription in the absence of the formation of heritable silent chromatin in these mutants rather than residual *HST3 HST4*-independent telomeric silencing having occurred. Similar decreases in transcript levels occurred in *sir2-345* mutants expressing hypoacetylated histone mutants that support Sir protein spreading (e.g., Figure 7B; see also Yang and Kirchmaier, 2006; Yang *et al.*, 2008). Also consistent with this notion, silencing of a telomeric *URA3* reporter gene had been disrupted to a similar degree in both *hst3Δ hst4Δ*

Figure 6. Derepression of *yFR057w* near *Tel VIR* in *hst3Δ hst4Δ* and *hst3Δ hst4Δ sir2-345* mutants. (A) Loss of *HST3* and *HST4* disrupts telomeric silencing. Transcript levels of *yFR057w* relative to *SCR1* were determined for each strain by quantitative real-time PCR and then normalized to *sir2-345* mutants expressing wild-type histones, which was set to 100%. Data were calculated as $2^{-(\text{locus } C_T - \text{SCR1 } C_T)_{\text{H3/H4}}}$ for *SIR2* and $2^{-(\text{locus } C_T - \text{SCR1 } C_T)_{\text{H3/H4 K16R}}}$ for *sir2-345* mutants. (B) Sir3p localization to *Tel VIR*. The efficiency of Sir3p association at *Tel VIR* in strains with the indicated genotypes was monitored by ChIP using anti-Sir3p antibodies and data were calculated as shown in Figure 2. (C and D) Loss of *HST3* and *HST4* results in hyperacetylation of H3 K56 at *Tel VIR*. For each strain, the efficiency of coprecipitation of DNA from 0.6 kb (C) and 1.2 kb (D) from the end of *Tel VIR* with anti-H3 K56ac antibodies was determined relative to that with anti-H3 antibodies, and then each locus was normalized to *HST3 HST4 sir2-345* mutants expressing wild-type histones, which was set to 1. Data were calculated as $2^{-(\text{H3 K56ac } C_T - \text{H3 } C_T)_{\text{H3/H4}}}$ for *SIR2* and $2^{-(\text{H3 K56ac } C_T - \text{H3 } C_T)_{\text{H3/H4 K16R}}}$ for *sir2-345* mutants. (E) Similar results were observed at *HMR*.

Figure 7. Loss of H3 K56ac in *rtt109* mutants does not disrupt silencing at *Tel VIR*. (A) Loss of histone H3 K56ac in *rtt109* mutants does not disrupt Sir spreading. The efficiency of Sir2p association at *Tel VIR* in *RTT109* and *rtt109Δ* strains with the indicated genotypes were monitored by ChIP using anti-Sir2p antibodies and data were calculated as shown in Figure 2. (B) Deletion of *RTT109* neither disrupts telomeric silencing in *SIR2* cells nor rescues telomeric silencing in *sir2-345* cells expressing H4 K16R. Transcript levels of *yFR057w* relative to *SCR1* were determined for each strain by quantitative real-time PCR and then normalized to *sir2-345* mutants expressing wild-type histones, which were set to 100%. Data were calculated as $2^{[(\text{locus } C_T - \text{SCR1 } C_T)_{\text{mutant}} - (\text{locus } C_T - \text{SCR1 } C_T)_{\text{wild-type}}]} \times 100$, where “a” is the indicated genotype. (C and D) Deletion of *RTT109* disrupts H3 K56ac at *Tel VIR*. H3 K56ac levels at *Tel VIR* in each strain were monitored by ChIP. For each strain, the efficiency of coprecipitation of DNA from 0.6 kb (C) and 1.2 kb (D) from the end of *Tel VIR* with anti-H3 K56ac antibodies was determined relative to that with anti-H3 antibodies, and data were calculated as shown in Figure 6.

and *sir2Δ* mutants (Figure 1). On loss of silencing, the increase in H3 K56ac adjacent to *Tel VIR* was similar in *sir2-345*, *hst3Δ hst4Δ*, and *hst3Δ hst4Δ sir2-345* mutants expressing wild-type histones and in *hst3Δ hst4Δ sir2-345* mutants expressing H4 K16R mutants relative to wild-type cells (Figure 6, C and D). Thus, the absence of *HST3* *HST4* in the context of the catalytically inactive *sir2-345p* did not lead to further increases H3 K56ac adjacent to *Tel VIR*. Similar patterns of Sir association and levels of H3 K56ac were observed at *HMR*, but, in contrast to *yFR057w*, *HMR* remained silenced in *hst3Δ hst4Δ* mutants (Supplemental Figure 3). In contrast to these findings, Xu *et al.* (2007) reported reduced telomeric H3 K56ac in *hst3Δ*

hst4Δ sir2Δ mutants relative to *hst3Δ hst4Δ* mutants (Xu *et al.*, 2007). The reason for this difference between the two studies is not clear. Together, our results indicate *SIR2* and *HST3/HST4* play distinct roles in defining telomeric silent chromatin.

Loss of H3 K56ac Does Not Disrupt Sir Protein Spreading and Silencing

Silencing has been proposed to occur when Sir2p deacetylates H3 K56, thereby facilitating the compaction of silent chromatin (Xu *et al.*, 2007). In this scenario, deacetylation of H3 K56 might reflect a late step in silent chromatin forma-

tion that influences the efficiency of Sir spreading or silencing. To test the effect of the absence of H3 K56ac on silent chromatin formation, we compared Sir protein localization and silencing in *SIR2* and *sir2-345* strains containing or lacking *RTT109* and expressing either wild-type histones or H4 K16R mutants (Figure 7 and Supplemental Figure 4). We first tested whether loss of H3 K56ac in *rtt109Δ* mutants affected Sir protein association with *Tel VIR* or *HMR* by ChIP. Similar patterns of Sir2p spreading were observed at both *Tel VIR* and *HMR* in *SIR2* and *sir2-345* cells containing or lacking *RTT109* and expressing wild-type histones or H4 K16R (Figure 7A and Supplemental Figure 4A, respectively). Thus, the absence of H3 K56ac neither facilitated nor compromised Sir2p spreading. We next compared silencing in the same yeast strains. Deletion of *RTT109* did not alter silencing at *yFR057w* adjacent to *Tel VIR* or at *HMR* in *SIR2* cells expressing either wild-type or mutant histones (Figure 7B and Supplemental Figure 4B, respectively; see also Figure 8A). Therefore, the constitutively hypoacetylated state of K56 on H3 was compatible with silencing (see also Miller *et al.*, 2008). Deletion of *RTT109* does not alter the levels of *yFR057w* or *a1* mRNA (Figure 7B and Supplemental Figure 4B, respectively) in *sir2-345* cells expressing wild-type histones. Thus, the acetylated state of K56 on H3 was not required for transcription from the *yFR057w* and *a1* promoters. Finally, deletion of *RTT109* also did not rescue silencing at *yFR057w* or *HMR* (Figure 7B and Supplemental Figure 4B, respectively; see also Figure 8) in *sir2-345* cells expressing H4 K16R mutants, despite the lack of H3 K56ac at *Tel VIR* or *HMR* in these mutants (Figures 7, C and D, and Supplemental Figure 4, C and D, respectively). The reduction in transcript levels seen in *sir2-345* mutants expressing H4 K16R relative to wild-type histones is due to Sir protein spreading interfering with transcription rather than the formation of heritable silent chromatin in a subset of the population. Transcript levels of *a1* in *sir4Δ* mutants expressing wild-type histones or H4 K16R is similar (Yang *et al.*, 2008), and, as described below, mating was not restored in *sir2-345* mutants expressing H4 K16R (see also Yang and Kirchmaier, 2006; Yang *et al.*, 2008).

To examine further the impact of H3 K56 on silencing at the *HM* loci, we tested whether loss of H3 K56ac by deletion of *RTT109* or mutation of H3 K56 to R could restore mating in *sir2-345* yeast expressing histone mutants that supported Sir protein spreading (Figure 8). Deletion of *RTT109* did not restore *HM* silencing in *sir2-345* mutants expressing either wild-type or mutant histones (Figure 8A). In contrast, *MATα SIR2* cells lacking *RTT109* and expressing either wild-type histones or various histone mutant combinations mated efficiently (Figure 8A), indicating silencing was intact at *HMR*. However, unlike *MATα* cells, *MATa SIR2 rtt109Δ* cells expressing H3 K9,14R H4 K16R were defective in mating (Figure 8A; compare also with *RTT109* cells expressing H3 K9,14R H4 K16R in Figure 8C). Similarly, silencing at *HML* was lost in *MATa SIR2* cells expressing H3 K9,14,56R H4 K16R (Figure 8C). Together, these results implied that the absence of H3 K56ac is largely compatible with silencing in *SIR2* cells, except at *HML* under conditions in which the N-terminal tail of histone H3 is also hypoacetylated (e.g., H3 K56R H4 K16R vs. H3 K9,14,56R H4 K16R in Figure 8, B and C). Silencing at the native *HML* locus is more sensitive to disruption than is silencing at *HMR* under a variety of conditions (e.g., Ehrenhofer-Murray *et al.*, 1997; Huang *et al.*, 1997; Xu *et al.*, 1999; Yang *et al.*, 2008). Relocalization of Sir proteins to other loci throughout the genome due to the global hypoacetylated state of histone H3 and H4 may contribute

Figure 8. Loss of H3 K56ac does not rescue *HM* silencing in *sir2-345* mutants. (A) Patch mating analyses of *HM* silencing in *SIR2* or *sir2-345 rtt109Δ* cells expressing the indicated histone mutants. (B and C) Patch mating analyses of *HM* silencing in *SIR2* or *sir2-345 rtt109Δ* cells expressing the indicated histone mutants. Patch-mating assays were conducted as shown in Figure 3.

to the observed silencing defects at *HML*. Loss of H4 K16ac causes Sir spreading beyond normally silenced loci (Kimura *et al.*, 2002; Suka *et al.*, 2002) and loss of acetylation at H3 K56 permits Sir recruitment to loci not normally bound by Sir proteins (Miller *et al.*, 2008).

The absence of silencing in *sir2-345* mutants in which Sir spreading has been restored (Yang and Kirchmaier, 2006; Figures 7 and 8 and Supplemental Figure 4) implies additional unknown substrates of Sir2p are normally deacetylated during silent chromatin formation. As defects in biological pathways can often be suppressed by overexpression of genes in overlapping or related pathways, we examined whether overexpression of *HST3*

Figure 9. Overexpression of *HST3* does not rescue silencing in *sir2-345* mutants. (A) *HST3* overexpression. Transcript levels of *HST3* relative to *SCR1* in yeast containing a vector (pRS426) or *HST3* expressed from its endogenous promoter on a 2 μ plasmid (pAK998) were determined by quantitative real-time PCR and then expressed relative to *SIR2* yeast containing a vector, which was set to 1. Data were calculated as $2^{[(HST3)_{C_T} - (SCR1)_{C_T}]_{vector} - [(HST3)_{C_T} - (SCR1)_{C_T}]_{HST3\ overexpression}}$. (B) HM silencing. Patch mating analyses of *SIR2* or *sir2-345* cells containing a vector or an *HST3* overexpression plasmid were conducted as shown in Figure 3. (C) Telomeric silencing. Transcription from *yFR057w* at *Tel VIR* in MAT α *SIR2* or *sir2-345* cells expressing the indicated histones and containing a vector or an *HST3* overexpression plasmid were determined as in Figure 2. Data were calculated as $2^{[(locus)_{C_T} - (SCR1)_{C_T}]_{vector} - [(locus)_{C_T} - (SCR1)_{C_T}]_a} \times 100$, where “a” is the indicated genotype.

could restore silencing in *sir2-345* cells expressing wild-type or histone hypoacetylation mutants. Unlike overexpression of *SIR2* (Yang and Kirchmaier, 2006), overexpression of *HST3* (Figure 9A) did not restore silencing in *sir2-345* mutants at HMR or HML (Figure 9B) or at *yFR057w* at *Tel VIR* (Figure 9C), even in the context of histone hypoacetylation mutants that support Sir spreading. Together, these results imply *HST3/HST4* and *SIR2* primarily regulate the acetylation status of different histone residues within silent chromatin and they generally cannot substitute for one another.

DISCUSSION

We previously assessed the impact of mutating H3 K56 on silencing in *S. cerevisiae* and found that telomeric silencing is disrupted in H3 K56Q mutants, but not in H3 K56R mutants (Miller et al., 2008; see also Hyland et al., 2005; Xu et al., 2007). Here, we show that Hst3p and Hst4p are required for efficient deacetylation of H3 K56 within silent loci (Figures 2, 3, 6, and Supplemental Figure 3). In the absence of Hst3p and Hst4p, hyperacetylation of H3 K56 at *Tel VIR* disrupted silencing without resulting in the dissociation of Sir proteins or an increase in H4 K16ac (Figure 2). This telomeric silencing defect in *hst3 Δ hst4 Δ* mutants could be suppressed and hypoacetylation of H3 K56 at *Tel VIR* could be restored by ectopic expression of *HST3* or by deletion of *RTT109* (Figures 1 and 2) but not by overexpression of *SIR2* (Figure 4; see also Figure 1).

These findings revealed the mechanism by which *HST3* and *HST4* contribute to silencing in budding yeast is through regulating the acetylation status of H3 K56.

Like in *S. cerevisiae*, the single *HST3* *HST4* orthologue in *S. pombe*, *hst4⁺*, is required for deacetylation of H3 K56 in vivo (Halder and Kamakaka, 2008). Fission yeast lacking *hst4⁺* also have telomeric silencing defects, are sensitive to multiple DNA-damaging agents, and are defective in chromosome maintenance (Freeman-Cook et al., 1999; Durand-Dubief et al., 2007; Halder and Kamakaka, 2008). And, overexpression of *S. pombe hst4⁺* suppresses telomeric silencing defects in *S. cerevisiae hst3 Δ hst4 Δ* mutants (Freeman-Cook et al., 1999), implying the role of these orthologues in silent chromatin is similar. Hst4⁺ has been localized to silent chromatin in fission yeast (Durand-Dubief et al., 2007). However, we have not observed preferential enrichment of Hst3p at silenced loci in budding yeast by ChIP (data not shown; see also Grunweller and Ehrenhofer-Murray, 2002) despite Hst3p/Hst4p-dependent hypoacetylation of H3 K56 at HMR and *Tel VIR* being silent chromatin dependent (Figures 5–7 and Supplemental Figures 2–4). It is possible that the epitope on Hst3p for our ChIP analysis was inaccessible in silent chromatin or that Hst3/Hst4p is present at silent loci only transiently during the cell cycle. In contrast to Sir proteins, which are relatively stable, Hst3p is rapidly turned over by cells (Dasgupta et al., 2004; Belle et al., 2006; Thaminy et al., 2007). This instability may result in short-lived interactions with other factors.

H3 K56ac and the Formation and Stability of Silent Chromatin

Both the stability of telomeric silent chromatin and the levels of H3 K56ac vary during the cell cycle, raising the possibility that these events are related. Sir proteins partially delocalize from telomeric foci during G2/M (Laroche *et al.*, 2000), and the transcriptional activator Ppr1p can activate a telomeric *URA3* reporter within silent chromatin during G2/M, but not during G0, G1, or early S phase (Aparicio and Gottschling, 1994). The presence of H3 K56ac at telomeres may contribute to this reduced stability of telomeric silent chromatin during G2/M. Consistent with this notion, mutations in H3 K56 increase the sensitivity of telomeric DNA to modification by *dam* methylase and of cleavage of DNA by enzymes (Masumoto *et al.*, 2005; Xu *et al.*, 2007). Mutation of H3 K56 also can alter the superhelicity of plasmids (Masumoto *et al.*, 2005) and result in the derepression of silent chromatin (Hyland *et al.*, 2005; Xu *et al.*, 2007; Miller *et al.*, 2008). Because replication-coupled assembly of newly synthesized H3 K56ac would occur near the end of S phase in late replicating regions such as the telomeres (Raghuraman *et al.*, 2001), these regions likely still contain high levels of H3 K56ac upon entry into G2/M. Our ChIP analyses hint that minor defects in telomeric localization of Sir2p may occur in hyperacetylated *hst3Δ hst4Δ* mutants (e.g., Figure 2). Future studies using microscopy should clarify whether partial Sir delocalization and derepression of silenced genes during G2/M are a function of temporary structural changes at silenced regions caused by incorporation of H3 K56ac.

It will also be of interest to determine whether *HST3* *HST4*-dependent deacetylation of H3 K56ac reflects a cell cycle requirement for establishing silencing under certain conditions. Passage through mitosis facilitates the assembly of silent chromatin at both telomeres and *HM* loci, but the mechanism involved is not known (Lau *et al.*, 2002; Martins-Taylor *et al.*, 2004; Katan-Khaykovich and Struhl, 2005; Maticic *et al.*, 2006). Ultimately, however, the establishment of silencing will likely turn out to be more complex as H3 K56ac does not disrupt silencing at the *HM* loci except under sensitized conditions (Figures 3 and 8), and passage through S phase is sufficient to establish silencing at *HMR*, despite low levels of *HST3* and *HST4* during this period of the cell cycle (Miller and Nasmyth, 1984; Fox *et al.*, 1997; Kirchmaier and Rine, 2001; Li *et al.*, 2001; Lau *et al.*, 2002).

Deacetylation by Sir2p, Hst3p/Hst4p, and Silencing

Sir2p has multiple critical functions in silencing. One key function is to deacetylate histones to permit Sir2-4p to bind to nucleosomes and spread along the chromosome. Sir proteins do not readily spread away from silencers along chromatin in cells expressing catalytically inactive sir2-345p unless histones residues normally deacetylated by Sir2p maintain a positive charge (Yang and Kirchmaier, 2006). However, Sir spreading alone is not sufficient for silencing (Figure 7 and Supplemental Figure 4), (Kirchmaier and Rine, 2006; Yang and Kirchmaier, 2006), implying Sir2p mediates one or more additional critical events during silent chromatin formation. Such events may include the deacetylation of novel key residues on histones to promote structural changes in chromatin needed for inactivating transcription or to generate 2'-O-acetyl-ADP ribose, a product of the deacetylation reaction that alters the conformation and stoichiometry of Sir2-4p complexes and Sir-nucleosome interactions in vitro (Liou *et al.*, 2005; Onishi *et al.*, 2007). Our findings indicate the production of 2'-O-acetyl-ADP ribose specifically through the deacetylation of H3 K56 by Sir2p or

Hst3p/Hst4p was not required for silencing at the *HM* loci. Rather, deacetylation of a different unknown substrate by Sir2p may be needed for silencing. Consistent with this, hypoacetylation of H3 K56 by mutation or deletion of *RTT109* did not restore silencing in *sir2-345* mutants or disrupt silencing at *HMR* in *SIR2* cells (Figures 7 and 8 and Supplemental Figure 4). The changes required for silencing are *SIR2*-dependent as overexpression of *SIR2* (Yang and Kirchmaier, 2006) but not *HST3* (Figure 9), in *sir2-345* mutants restores silencing at both *HMR* and *HML*.

H3 K56ac is assembled into chromatin through both replication- and transcription-coupled pathways (Ozdemir *et al.*, 2006; Schneider *et al.*, 2006; Rufiange *et al.*, 2007; Miller *et al.*, 2008, and references within). Our data are consistent with a model in which hypoacetylation of H3 K56 at silenced loci is maintained by at least two methods. In the first method, Sir2p-mediated silencing prevents transcription, thereby ensuring H3 K56ac is not loaded onto DNA at silenced loci via transcription-coupled chromatin assembly (Figure 5) (Xu *et al.*, 2007). In the second method, when H3 K56ac is loaded onto DNA at silenced loci each cell cycle via replication-coupled chromatin assembly, deacetylation of this residue is primarily regulated by the NAD⁺-dependent deacetylases Hst3p/Hst4p, although Sir2p may also assist with maintaining the hypoacetylated state of H3 K56 throughout the cell cycle (Xu *et al.*, 2007). Future studies should clarify how each of these histone deacetylases control H3 K56ac in silent chromatin.

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REFERENCES

- Aparicio, O. M., Billington, B. L., and Gottschling, D. E. (1991). Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. *Cell* 66, 1279–1287.
- Aparicio, O. M., and Gottschling, D. E. (1994). Overcoming telomeric silencing-A *trans*-activator competes to establish gene expression in a cell cycle-dependent way. *Genes Dev.* 8, 1133–1146.
- Axelrod, A. (1991). Role of a Cell-Cycle Gene in Transcriptional Silencing. Ph.D. Thesis. Berkeley, CA: University of California.
- Belle, A., Tanay, A., Bitincka, L., Shamir, R., and O'Shea, E. K. (2006). Quantification of protein half-lives in the budding yeast proteome. *Proc. Natl. Acad. Sci. USA* 103, 13004–13009.
- Brachmann, C. B., Sherman, J. M., Devine, S. E., Cameron, E. E., Pillus, L., and Boeke, J. D. (1995). The *SIR2* gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. *Genes Dev.* 9, 2888–2902.
- Celic, I., Masumoto, H., Griffith, W. P., Meluh, P., Cotter, R. J., Boeke, J. D., and Verreault, A. (2006). The sirtuins hst3 and hst4p preserve genome integrity by controlling histone h3 lysine 56 deacetylation. *Curr. Biol.* 16, 1280–1289.
- Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. *Gene* 110, 119–122.
- Collins, S. R. *et al.* (2007). Functional dissection of protein complexes involved in yeast chromosome biology using a genetic interaction map. *Nature* 446, 806–810.
- Dasgupta, A., Ramsey, K. L., Smith, J. S., and Auble, D. T. (2004). Sir Antagonist 1 (San1) is a ubiquitin ligase. *J. Biol. Chem.* 279, 26830–26838.
- Driscoll, R., Hudson, A., and Jackson, S. P. (2007). Yeast Rtt109 promotes genome stability by acetylating histone H3 on lysine 56. *Science* 315, 649–652.

- Durand-Dubief, M., Sinha, I., Fagerstrom-Billai, F., Bonilla, C., Wright, A., Grunstein, M., and Ekwall, K. (2007). Specific functions for the fission yeast Sirtuins Hst2 and Hst4 in gene regulation and retrotransposon silencing. *EMBO J.* 26, 2477–2488.
- Ehrenhofer-Murray, A. E., Rivier, D. H., and Rine, J. (1997). The role of Sas2, an acetyltransferase homologue of *Saccharomyces cerevisiae*, in silencing and ORC function. *Genetics* 145, 923–934.
- Fillingham, J., Recht, J., Silva, A. C., Suter, B., Emili, A., Staglar, I., Krogan, N. J., Allis, C. D., Keogh, M. C., and Greenblatt, J. F. (2008). Chaperone Control of the Activity and Specificity of the Histone H3 Acetyltransferase Rtt109. *Mol. Cell Biol.* 28, 4342–4353.
- Fox, C. A., Ehrenhofer-Murray, A. E., Loo, S., and Rine, J. (1997). The origin recognition complex, SIR1, and the S phase requirement for silencing. *Science* 276, 1547–1551.
- Freeman-Cook, L. L., Sherman, J. M., Brachmann, C. B., Allshire, R. C., Boeke, J. D., and Pillus, L. (1999). The *Schizosaccharomyces pombe* hst4(+) gene is a SIR2 homologue with silencing and centromeric functions. *Mol. Biol. Cell* 10, 3171–3186.
- Gallagher, S., Winston, S. E., Fuller, S. A., and Hurrell, J.G.R. (2004). Immunoblotting and Immunodetection. In: *Current Protocols in Molecular Biology*, ed. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, New York: John Wiley & Sons, 10.18.16–10.18.17.
- Garcia, B. A., Hake, S. B., Diaz, R. L., Kauer, M., Morris, S. A., Recht, J., Shabanowitz, J., Mishra, N., Strahl, B. D., Allis, C. D., and Hunt, D. F. (2007). Organismal differences in post-translational modifications in histones H3 and H4. *J. Biol. Chem.* 282, 7641–7655.
- Goldstein, A. L., and McCusker, J. H. (1999). Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 15, 1541–1553.
- Gottschling, D. E., Aparicio, O. M., Billington, B. L., and Zakian, V. A. (1990). Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. *Cell* 63, 751–762.
- Grunweller, A., and Ehrenhofer-Murray, A. E. (2002). A novel yeast silencer. the 2 μ m origin of *Saccharomyces cerevisiae* has HST3-, MIG1- and SIR-dependent silencing activity. *Genetics* 162, 59–71.
- Haldar, D., and Kamakaka, R. T. (2008). *Schizosaccharomyces pombe* Hst4 functions in DNA damage response by regulating histone H3 K56 acetylation. *Eukaryotic Cell* 7, 800–813.
- Han, J., Zhou, H., Horazdovsky, B., Zhang, K., Xu, R. M., and Zhang, Z. (2007a). Rtt109 acetylates histone H3 lysine 56 and functions in DNA replication. *Science* 315, 653–655.
- Han, J., Zhou, H., Li, Z., Xu, R. M., and Zhang, Z. (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., Zhou, H., Li, Z., Xu, R. M., and Zhang, Z. (2007c). The Rtt109-Vps75 histone acetyltransferase complex acetylates non-nucleosomal histone H3. *J. Biol. Chem.* 282, 14158–14164.
- Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986). Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* 2, 163–167.
- Holmes, S. G., Rose, A. B., Steuerle, K., Saez, E., Sayegh, S., Lee, Y. M., and Broach, J. R. (1997). Hyperactivation of the silencing proteins, Sir2p and Sir3p, causes chromosome loss. *Genetics* 145, 605–614.
- Hoppe, G., Tanny, J., Rudner, A., Gerber, S., Danaie, S., Gygi, S., and Moazed, D. (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, H., Kahana, A., Gottschling, D. E., and Prakash, L. (1997). The ubiquitin-conjugating enzyme Rad6 (Ubc2) is required for silencing in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 17, 6693–6699.
- Hyland, E. M., Cosgrove, M. S., Molina, H., Wang, D., Pandey, A., Cottee, R. J., and Boeke, J. D. (2005). Insights into the role of histone H3 and histone H4 core modifiable residues in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 25, 10060–10070.
- Imai, S., Armstrong, C. M., Kaerberlein, M., and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* 403, 795–800.
- Jessulat, M., Alamgir, M., Salsali, H., Greenblatt, J., Xu, J., and Golshani, A. (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 Struhl, K. (2005). Heterochromatin formation involves changes in histone modifications over multiple cell generations. *EMBO J.* 24, 2138–2149.
- Kelly, T. J., Qin, S., Gottschling, D. E., and Parthun, M. R. (2000). Type B histone acetyltransferase Hat1p participates in telomeric silencing. *Mol. Cell Biol.* 20, 7051–7058.
- Kimura, A., Umehara, T., and Horikoshi, M. (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 Rine, J. (2001). DNA replication-independent silencing in *S. cerevisiae*. *Science* 291, 646–650.
- Kirchmaier, A. L., and Rine, J. (2006). Cell-cycle requirements in assembling silent chromatin in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 26, 852–862.
- Laroche, T., Martin, S. G., Tsai-Pflugfelder, M., and Gasser, S. M. (2000). The dynamics of yeast telomeres and silencing proteins through the cell cycle. *J. Struct. Biol.* 129, 159–174.
- Lau, A., Blitzblau, H., and Bell, S. P. (2002). Cell-cycle control of the establishment of mating-type silencing in *S. cerevisiae*. *Genes Dev.* 16, 2935–2945.
- Li, Y. C., Cheng, T. H., and Gartenberg, M. (2001). Establishment of transcriptional silencing in the absence of DNA replication. *Science* 291, 650–653.
- Lieb, J. D., Liu, X., Botstein, D., and Brown, P. O. (2001). Promoter-specific binding of Rap1 revealed by genome-wide maps of protein-DNA association. *Nat. Genet.* 28, 327–334.
- Liou, G. G., Tanny, J. C., Kruger, R. G., Walz, T., and Moazed, D. (2005). Assembly of the SIR complex and its regulation by O-acetyl-ADP-ribose, a product of NAD-dependent histone deacetylation. *Cell* 121, 515–527.
- Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 389, 251–260.
- Luo, K., Vega-Palas, M. A., and Grunstein, M. (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., Miller, K. M., Defazio, L. G., and Toczyski, D. P. (2006). Cell cycle and checkpoint regulation of histone H3 K56 acetylation by Hst3 and Hst4. *Mol. Cell* 23, 109–119.
- Martins-Taylor, K., Dula, M. L., and Holmes, S. G. (2004). Heterochromatin spreading at yeast telomeres occurs in M phase. *Genetics* 168, 65–75.
- Masumoto, H., Hawke, D., Kobayashi, R., and Verreault, A. (2005). A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. *Nature* 436, 294–298.
- Matecic, M., Martins-Taylor, K., Hickman, M., Tanny, J., Moazed, D., and Holmes, S. G. (2006). New alleles of SIR2 define cell cycle specific silencing functions. *Genetics* 173, 1939–1950.
- Miller, A. M., and Nasmyth, K. A. (1984). Role of DNA replication in the repression of silent mating type loci in yeast. *Nature* 312, 247–251.
- Miller, A., Yang, B., Foster, T., and Kirchmaier, A. L. (2008). Proliferating cell nuclear antigen and ASF1 modulate silent chromatin in *Saccharomyces cerevisiae* via lysine 56 on histone H3. *Genetics* 179, 793–809.
- Onishi, M., Liou, G. G., Buchberger, J. R., Walz, T., and Moazed, D. (2007). Role of the conserved Sir3-BAH domain in nucleosome binding and silent chromatin assembly. *Mol. Cell* 28, 1015–1028.
- Ozdemir, A., Masumoto, H., Fitzjohn, P., Verreault, A., and Logie, C. (2006). Histone H3 lysine 56 acetylation: a new twist in the chromosome cycle. *Cell Cycle* 5, 2602–2608.
- Ozdemir, A., Spicuglia, S., Lasonder, E., Vermeulen, M., Campsteijn, C., Stunnenberg, H. G., and Logie, C. (2005). Characterization of lysine 56 of histone H3 as an acetylation site in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 280, 25949–25952.
- Raghuraman, M. K., Winzler, E. A., Collingwood, D., Hunt, S., Wodicka, L., Conway, A., Lockhart, D. J., Davis, R. W., Brewer, B. J., and Fangman, W. L. (2001). Replication dynamics of the yeast genome. *Science* 294, 115–121.
- Recht, J., Tsubota, T., Tanny, J. C., Diaz, R. L., Berger, J. M., Zhang, X., Garcia, B. A., Shabanowitz, J., Burlingame, A. L., Hunt, D. F., Kaufman, P. D., and Allis, C. D. (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.
- Rufiange, A., Jacques, P. E., Bhat, W., Robert, F., and Nourani, A. (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., Kirchmaier, A. L., and Rine, J. (2002). Ordered nucleation and spreading of silenced chromatin in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 13, 2207–2222.

- Rusché, L. N., Kirchmaier, A. L., and Rine, J. (2003). The establishment, inheritance, and function of silenced chromatin in *Saccharomyces cerevisiae*. *Annu. Rev. Biochem.* 72, 481–516.
- Schmitt, M. E., Brown, T. A., and Trumppower, B. L. (1990). A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 18, 3091–3092.
- Schneider, J., Bajwa, P., Johnson, F. C., Bhaumik, S. R., and Shilatifard, A. (2006). Rtt109 is required for proper H3K56 acetylation: a chromatin mark associated with the elongating RNA polymerase II. *J. Biol. Chem.* 281, 37270–37274.
- Sherman, J. M., Stone, E. M., Freeman-Cook, L. L., Brachmann, C. B., Boeke, J. D., and Pillus, L. (1999). The conserved core of a human SIR2 homologue functions in yeast silencing. *Mol. Biol. Cell* 10, 3045–3059.
- Strahl-Bolsinger, S., Hecht, A., Luo, K., and Grunstein, M. (1997). SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev.* 11, 83–93.
- Suka, N., Luo, K., and Grunstein, M. (2002). Sir2p and Sas2p oppositely regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. *Nat. Genet.* 32, 378–383.
- Tanny, J. C., and Moazed, D. (2001). Coupling of histone deacetylation to NAD breakdown by the yeast silencing protein Sir2, Evidence for acetyl transfer from substrate to an NAD breakdown product. *Proc. Natl. Acad. Sci. USA* 98, 415–420.
- Thaminy, S., Newcomb, B., Kim, J., Gatbonton, T., Foss, E., Simon, J., and Bedalov, A. (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., Berndsen, C. E., Erkmann, J. A., Smith, C. L., Yang, L., Freitas, M. A., Denu, J. M., and Kaufman, P. D. (2007). Histone H3–K56 acetylation is catalyzed by histone chaperone-dependent complexes. *Mol. Cell* 25, 703–712.
- van Leeuwen, F., and Gottschling, D. E. (2002). Assays for gene silencing in yeast. *Methods Enzymol.* 350, 165–186.
- Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* 10, 1793–1808.
- Xhemalce, B., Miller, K. M., Driscoll, R., Masumoto, H., Jackson, S. P., Kouzarides, T., Verreault, A., and Arcangioli, B. (2007). Regulation of histone H3 lysine 56 acetylation in *Schizosaccharomyces pombe*. *J. Biol. Chem.* 282, 15040–15047.
- Xu, E. Y., Kim, S., and Rivier, D. H. (1999). SAS4 and SAS5 are locus-specific regulators of silencing in *Saccharomyces cerevisiae*. *Genetics* 153, 25–33.
- Xu, F., Zhang, K., and Grunstein, M. (2005). Acetylation in histone H3 globular domain regulates gene expression in yeast. *Cell* 121, 375–385.
- Xu, F., Zhang, Q., Zhang, K., Xie, W., and Grunstein, M. (2007). Sir2 deacetylates histone H3 lysine 56 to regulate telomeric heterochromatin structure in yeast. *Mol. Cell* 27, 890–900.
- Yang, B., Britton, J., and Kirchmaier, A. L. (2008). Insights into the impact of histone acetylation and methylation on Sir protein spreading and silencing in *Saccharomyces cerevisiae*. *J. Mol. Biol.* 381, 826–844.
- Yang, B., and Kirchmaier, A. L. (2006). Bypassing the catalytic activity of SIR2 for SIR protein spreading in *S. cerevisiae*. *Mol. Biol. Cell* 17, 5287–5297.
- Zhou, H., Madden, B. J., Muddiman, D. C., and Zhang, Z. (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.