Collaboration between the essential Esa1 acetyltransferase and the Rpd3 deacetylase is mediated by H4K12 histone acetylation in *Saccharomyces cerevisiae*

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**ABSTRACT**

Histone modifications that regulate chromatin-dependent processes are catalyzed by multi-subunit complexes. These can function in both targeting activities to specific genes and in regulating genome-wide levels of modifications. In *Saccharomyces cerevisiae*, Esa1 and Rpd3 have opposing enzymatic activities and are catalytic subunits of multiple chromatin modifying complexes with key roles in processes such as transcriptional regulation and DNA repair. Esa1 is an essential histone acetyltransferase that belongs to the highly conserved MYST family. This study presents evidence that the yeast histone deacetylase gene, *RPD3*, when deleted, suppressed *esa1* conditional mutant phenotypes. Deletion of *RPD3* reversed rDNA and telomeric silencing defects and restored global H4 acetylation levels, in addition to rescuing the growth defect of a temperature-sensitive *esa1* mutant. This functional genetic interaction between *ESA1* and *RPD3* was mediated through the Rpd3L complex. The suppression of *esa1*’s growth defect by disruption of Rpd3L was dependent on lysine 12 of histone H4. We propose a model whereby Esa1 and Rpd3L act coordinately to control the acetylation of H4 lysine 12 to regulate transcription, thereby emphasizing the importance of dynamic acetylation and deacetylation of this particular histone residue in maintaining cell viability.

**INTRODUCTION**

The genome of eukaryotic cells is packaged in chromatin, where the DNA is organized into a nucleosomal subunit structure. Nucleosomes consist of DNA wrapped around a histone octamer that contains two copies of each of the four core histones (H2A, H2B, H3, and H4), each of which can be post-translationally modified with multiple
types of chemical and protein additions. The addition and removal of these modifications are catalyzed by histone modifying enzymes that function in a wide range of nuclear processes.

One dynamic histone modification is the acetylation and deacetylation of lysine residues. Enzymes that add an acetyl group to a lysine residue are known as histone acetyltransferases (HATs), and the enzymes that remove acetyl groups are called histone deacetylases (HDACs). The opposing activities of these two types of enzymes control the status of histone acetylation in the cell. For example, in the budding yeast *S. cerevisiae*, regulation of H4 lysine 16 (H4K16) acetylation is critical in maintaining transcriptionally silent chromatin at the telomeres and regulating replicative life span, via activities of the HAT Sas2 and the HDAC Sir2 (Kimura et al. 2002; Suka et al. 2002; Dang et al. 2009).

Roles for the other acetylated lysines on H4 are less clearly defined. Some information has come from studying these modifications on a genome-wide level. Through one microarray expression study, it became apparent that H4K5, H4K8, and H4K12 might contribute non-specifically but jointly to transcription. Each lysine, when individually mutated to an unacetylatable amino acid, results in minimal changes in genome-wide transcription. However, when combined to make double or triple lysine mutants, they display additive effects on transcription (Dion et al. 2005). In addition to participating in transcriptional control, H4 acetylation is critical for other nuclear processes, including DNA replication and repair (Megee et al. 1995; Bird et al. 2002; Choy and Kron 2002). Esa1 and Rpd3 are yeast enzymes with opposing activities.
toward H4 lysine acetylation, and are also members of two highly conserved families of histone modifying enzymes (reviewed in DOYON and CÔTÉ 2004; YANG and SETO 2008).

Rpd3 is one of the founding members of class I HDACs, which include the human proteins HDAC1, -2, -3, and -8 that are often overexpressed in human cancer cells. Indeed, HDAC inhibitors are being actively used and studied as therapeutic agents for multiple types of cancer (reviewed in YANG and SETO 2008). In yeast, Rpd3 deacetylates lysines on both H3 and H4 (RUNDLETT et al. 1996) and is involved in a wide-range of nuclear processes. On a global scale, Rpd3 is responsible for transcriptional regulation of a large number of genes (BERNSTEIN et al. 2000; SABET et al. 2004; ALEJANDRO-OSORIO et al. 2009). For many, but not all of these genes, transcriptional regulation occurs through modification of the lysines on the H3 and H4 N-termini (SABET et al. 2004). When examined at specific loci, Rpd3 represses transcription of INO1 and IME2 by deacetylating histones at the promoters of these genes (KADOSH and STRUHL 1998b; RUNDLETT et al. 1998). In contrast, there are several transcripts that require Rpd3 for their activation (DE NADAL et al. 2004; SERTIL et al. 2007). For example, Rpd3 is required for expression of the DNA damage inducible genes HUG1 and RNR3 (SHARMA et al. 2007). In line with Rpd3 having a role in DNA repair, rpd3 mutants are defective in nonhomologous end-joining (JAZAYERI et al. 2004). Mutants of rpd3 increase silencing at the telomeres, ribosomal DNA (rDNA) repeats, and HM cryptic mating-type loci (DE RUBERTIS et al. 1996; RUNDLETT et al. 1996; VANNIER et al. 1996; SUN and HAMPSEY 1999), although the mechanism for this is unknown. Rpd3 also contributes to cell cycle control, in that rpd3 mutants undergo early DNA replication origin firing (VOGELAUER et al. 2002; APARICIO et al. 2004).
Rpd3 exists in two biochemically defined complexes, named Rpd3S (small) and Rpd3L (large) to reflect their relative sizes (CARROZZA et al. 2005b; KEOGH et al. 2005). The identification of the subunits in each of the two Rpd3-containing complexes has begun to reveal a separation in Rpd3 complex functions. Rpd3L is likely responsible for Rpd3’s role at gene promoters, as Rpd3 is recruited to chromatin via the Rpd3L-specific subunit Ume6, which recognizes specific upstream promoter sequences (KADOSH and STRUHL 1997; CARROZZA et al. 2005a). Rpd3L-specific mutants also display increased rDNA, telomeric, and HM loci silencing (VANNIER et al. 1996; ZHANG et al. 1998; SUN and HAMPSEY 1999; LOEWITH et al. 2001; CARROZZA et al. 2005a; KEOGH et al. 2005), and replication timing defects (KNOTT et al. 2009) similar to rpd3Δ itself, indicating that Rpd3L is responsible for Rpd3’s role in silencing and regulation of replication initiation. The smaller Rpd3S complex is recruited to methylated H3K36 within coding sequences to repress intragenic transcription initiation (CARROZZA et al. 2005b; KEOGH et al. 2005), a role not shared by Rpd3L.

Whereas Rpd3 is a class I HDAC, Esa1 is part of the evolutionarily conserved MYST family of HATs. Tip60, the human homolog of Esa1, is associated with many human diseases, including HIV, Alzheimer’s and multiple cancers. Tip60 acetylates the tumor suppressor p53 and acts as a transcriptional co-activator for c-Myc and Nf-κB (reviewed in AVVAKUMOV and CÔTÉ 2007). In yeast, Esa1 is essential for viability (SMITH et al. 1998; CLARKE et al. 1999), although Esa1’s essential function may not be limited to its catalytic activity (DECKER et al. 2008). Esa1 acetylates specific lysine residues on histones H2A, H3, H4 (SMITH et al. 1998; CLARKE et al. 1999), and the histone variant H2A.Z (BABIARZ et al. 2006; KEOGH et al. 2006; MILLAR et al. 2006).
Similar to Rpd3, Esa1 is the catalytic subunit of two chromatin modifying complexes that have shared subunits. The larger of these is known as NuA4, whereas the smaller is piccolo (ALLARD et al. 1999; BOUDREAULT et al. 2003). In vitro activity assays indicate that piccolo is the more active acetyltransferase complex on chromatin, but separate roles for the two complexes have not been established in vivo (BOUDREAULT et al. 2003).

Through the study of conditional esa1 mutants, Esa1 has been discovered to play an important role in many nuclear processes. Esa1 functions in cell cycle progression (CLARKE et al. 1999) and DNA repair (BIRD et al. 2002). Esa1 also contributes both to transcriptional activation and repression. Esa1 binding has been observed at the promoters of many transcriptionally active genes (ROBERT et al. 2004), and specifically at ribosomal protein genes, where Esa1 is needed for their transcriptional activation (REID et al. 2000). In a somewhat opposing role, Esa1 is required for transcriptional silencing at the telomeres and the rDNA. Specifically, Pol II reporter genes that are normally repressed when inserted at the telomeres and the rDNA display increased expression in esa1 mutants (CLARKE et al. 2006).

In this study, we defined an in vivo collaboration between the histone acetyltransferase Esa1 and the histone deacetylase Rpd3. Genetic dissection of the functional interactions revealed that the collaboration is mediated specifically through the Rpd3L complex. Deletion of RPD3 suppressed multiple phenotypes of esa1 mutants, including temperature-sensitivity, rDNA and telomeric silencing defects, and restored global H4 acetylation defects. Deletion of genes encoding Rpd3L-specific subunits Pho23 or Sds3, likewise promoted suppression of esa1 phenotypes, suggesting that Esa1 coordinates acetylation specifically with Rpd3L. Consistent with this interpretation,
deletion of the Rpd3S subunit encoded by RCO1 did not suppress esa1 phenotypes.
Finally, suppression of the growth defect in the esa1 mutant by deletion of Rpd3L
subunits was specifically dependent on acetylation of lysine 12 on histone H4, thereby
pointing to a crucial yet previously unsuspected role for this specific residue.

**MATERIALS AND METHODS**

**Yeast strains and plasmids:** All strains and plasmids are listed in Tables S1 and
S2. Except where noted with specific allele designations, all mutations used are null
alleles constructed using standard molecular methods. The rpd3::kanMX (LPY12154),
hdal::kanMX (LPY13472), hos1::kanMX (LPY13706), and hos2::KanMX3 (LPY13583)
mutants were constructed using marker swap plasmids M3926, M3929, and M3925 as
described in (VOTH et al. 2003) on rpd3::LEU2 (DY1539) (KASTEN et al. 1997),
hdal::TRP1 (DY4891), hos1::HIS3 (DY6073), hos2::TRP1 (DY4549) (all generous gifts
from D. Stillman) and then backcrossed prior to use. The pho23Δ::kanMX (LPY12732),
sds3Δ::kanMX (LPY12958), and rco1Δ::kanMX (LPY12645) mutants were constructed
by amplification (oligonucleotides listed in Table S3) of the kanMX cassettes from
Saccharomyces Genome Deletion Project strains, transformed into W303-1a (LPY5) and
backcrossed prior to use. All double mutants and the silencing markers rDNA::ADE2-
CAN1 (Fritze et al. 1997) and TELVR::URA3 (Renauld et al. 1993) were introduced
through standard genetic crosses. Construction of the RPD3 catalytic mutant plasmid,
rdp3-H150A-H151A, is described in (RUAULT and PILLUS 2006). Histone mutant strains,
derived from MSY1905 (a generous gift from M. M. Smith) (RUAULT and PILLUS 2006)
are chromosomally deleted for both HHF-HHT loci, and initially contained wild-type
histones on the plasmid pJH33 (CEN URA3 HTA1 HTB1 HHF2 HHT2) (Ahn et al. 2005). For mutant construction, strains were transformed with a TRP1 plasmid containing the relevant H4 (HHF2) mutation, and then subjected to a plasmid shuffle by counterselection on 5-FOA to remove pJH33. Histone mutant plasmids were constructed by site-specific mutagenesis using oligonucleotides listed in Table S3.

**Growth dilution assays and silencing assays:** Unless otherwise noted, all dilution assays represent five-fold serial dilutions, starting from an A_{600} of 1.0 after growth to saturation in 3 mL of liquid synthetic complete (SC) medium. Growth and silencing plates were incubated at 30°. Suppression of esa1’s growth defect on SC plates was assayed at the restrictive temperature of 35°. The rDNA silencing assays were performed with strains containing the rDNA::ADE2-CAN1 reporter as described (Van Leeuwen and Gottschling 2002). Strains were grown in SC-Ade-Arg medium to saturation, normalized as above, and plated on SC-Ade-Arg (growth control) and SC-Ade-Arg plates containing 32 µg/mL of canavanine (to assay rDNA silencing). Telomeric silencing assays were performed with the TELVR::URA3 reporter as described (Renauld et al. 1993; Van Leeuwen and Gottschling 2002). Strains were grown in SC medium and plated on both SC (growth control) and SC with 0.1% 5-FOA (to assay telomeric silencing). Camptothecin (CPT) sensitivity was assayed using CPT dissolved in DMSO added to plates buffered with 100 mM potassium phosphate (pH 7.5) to maintain maximal drug activity (Nitiiss and Wang 1988). Growth control plates contained equal concentrations of DMSO and phosphate buffer. All images were captured after 2-4 days of growth.
**Protein immunoblots:** Whole cell extracts were prepared from cells grown to A₆₀₀ of 0.6-1.0 at 30°C. In the temperature shift experiment, cells were grown first at 30°C and then shifted to 34°C for six hours. Extracts were prepared by bead-beating as described previously (CLARKE *et al.* 1999). Briefly, cells were resuspended in phosphate buffered saline with protease inhibitors and lysed by vortexing with glass beads. Whole cell extracts were then denatured by boiling in sample loading buffer and separated from the insoluble pellet by centrifugation. Proteins were separated on 18% SDS-polyacrylamide gels and transferred to nitrocellulose (0.2µm). Primary antisera used were anti-H4K5Ac (1:5000 dilution, Serotec), anti-H4K8Ac (1:2500 dilution, Serotec), anti-H4K12Ac (1:2500 dilution, Serotec), anti-H4K16Ac (1:2500 dilution, Upstate) and anti-H3 CT (1:10,000 dilution, Upstate). Goat anti-rabbit conjugated to horseradish peroxidase (Promega) was used as a secondary antibody, and signal was detected with Western Lightning® Chemiluminescence Reagent (Perkin Elmer) on Kodak™ X-Omat™ film.

**RESULTS**

**Deletion of the histone deacetylase gene RPD3 suppressed the growth defect of esal:** Histone acetylation and deacetylation are opposing chemical modifications that must be balanced for transcriptional regulation. The interplay between HATs and HDACs is complicated by the presence of numerous enzymes, some of which have very specific substrates, whereas others share overlapping histone targets. In attempting to dissect these relationships, large-scale studies have uncovered an intricate network of genetic
interactions between multiple HATs and HDACs (Collins et al. 2007; Lin et al. 2008; Mitchell et al. 2008).

In a study directly examining Esa1’s functions in rDNA silencing, a surprising relationship was discovered between Esa1 and the Class III HDAC Sir2 (Clarke et al. 2006). When either ESA1 or SIR2 is overexpressed, each suppresses the rDNA silencing defects of the other mutant. For example, overexpression of ESA1 rescues the rDNA silencing defect of the sir2 mutant (Clarke et al. 2006).

Neither increased dosage nor deletion of SIR2, however, had any effect on the growth defect of the esal-414 temperature-sensitive mutant at elevated temperatures. In searching for other genes encoding chromatin modifying enzymes that might functionally interact with esal mutants, deletion of RPD3 was discovered to specifically suppress this growth defect (Figure 1).

To ask if deletion of genes encoding other HDACs could also support viability of the temperature-sensitive esal-414 allele at restrictive temperatures, double mutants of esal-414 were constructed in combination with a series of HDAC mutants. Along with the Class III family deacetylase Sir2, deletion of genes encoding Class I and II HDACs were tested. Rpd3, Hos1, Hos2, and Hda1, are all Class I and II yeast HDACs that share 25-50% protein sequence identity. Of these, only RPD3 deletion supported growth of esal mutants at elevated temperatures (Figure 1). Mutation of the other genes either had no effect or in the case of hos2, exacerbated the severity of the esal growth defect. Some of these results are parallel to interactions reported in a genome-wide study (Lin et al. 2008).
The *esa1-414* temperature-sensitive mutant contains a frameshift mutation that results in an early truncation of the protein, and displays reduced HAT activity both *in vitro* and *in vivo* (Clarke et al. 1999). To test the allele-specificity of the suppression, *RPD3* was deleted in another *esa1* temperature-sensitive allele, *esa1-L254P*, and was also found to suppress the *esa1* growth defect at restrictive temperature (Figure S1A). The *esa1-L254P* allele contains a point mutation that resides near the HAT domain, and similar to *esa1-414*, is temperature-sensitive and lacks *in vitro* and *in vivo* HAT activity (Clarke et al. 1999). Thus, *rpd3* suppression of the *esa1* growth defect is not allele-specific and may be a general property for catalytically compromised Esa1. Furthermore, using the *RPD3* catalytically-dead allele, *rpd3-H150A-H151A* (Kadosh and Struhl 1998a) in combination with the *esa1* mutant showed results consistent with the *rpd3Δ* (Figure S1B). Therefore, the growth rescue observed is due to loss of histone deacetylase activity by Rpd3, and not some other function of Rpd3.

To test if *RPD3* deletion could bypass the non-viable *esa1Δ* phenotype, two tests were conducted. In the first, a plasmid shuffle was performed with a wild-type *ESA1* plasmid in the *esa1Δ rpd3Δ* double mutant. This strain was unable to grow under conditions that select for loss of the wild-type *ESA1* plasmid (Figure S1C). In the second test, an *esa1Δ/ESA1 rpd3Δ/rpd3Δ* diploid was sporulated, dissected, and examined for viability. All genotypically *esa1Δ rpd3Δ* double mutants were inviable. Some double mutants germinated and were able to undergo a small number of divisions, but none continued dividing to form colonies (data not shown), similar to *esa1Δ* itself (Clarke et al. 1999). This analysis confirmed the plasmid shuffle result, demonstrating that *rpd3* did not bypass suppress the inviable *esa1Δ*. 
Suppression of esa1’s growth defect was mediated exclusively by the Rpd3L complex: Rpd3S and Rpd3L, the two Rpd3-containing HDAC complexes, each have shared subunits as well as a number of distinct subunits (CARROZZA et al. 2005a; CARROZZA et al. 2005b; KEOGH et al. 2005) (Figure 2A). Both Rpd3S and Rpd3L also contain proteins that function in additional nuclear complexes. By evaluating subunits that are unique to Rpd3S and Rpd3L, the complexes were dissected genetically to determine if deletion of both is required, or if instead the suppression observed in the esa1 rpd3 double mutant is mediated through one specific complex. Double mutants were constructed with esa1 and genes specific to each of the two complexes. These double mutants were then tested for suppression of esa1.

Rpd3S, the smaller of the two complexes, has only two subunits (Eaf3, Rco1) that distinguish it from Rpd3L (CARROZZA et al. 2005b; KEOGH et al. 2005). However, Eaf3 is not unique to Rpd3S since it is also a component of NuA4 (EISEN et al. 2001), an Esa1-containing complex. Loss of EAF3 disrupts both NuA4 and Rpd3S, thus RCO1 was chosen instead to disrupt Rpd3S. The Rco1 protein contains a PHD finger, and is required for the complex integrity of Rpd3S (CARROZZA et al. 2005b). As shown in Figure 2B (top panel), deletion of RCO1 in an esa1 mutant did not suppress the esa1 growth defect. In fact, the esa1 rco1 double mutant displayed a slightly exacerbated growth defect compared to that of esa1. Therefore, the suppression of esa1’s growth defect is not mediated through Rpd3S.

Rpd3L contains several subunits distinct from those in Rpd3S. Some are involved in the function of other transcriptional complexes, such as Cti6, which recruits the SAGA
HAT complex to chromatin for transcriptional activation (PAPAMICHTOS-CHRONAKIS et al. 2002). In contrast, Sds3 is a subunit unique to Rpd3L. Sds3 is essential for the integrity of the Rpd3L complex, and Rpd3L dissociates in sds3 mutants, thereby resulting in a loss of all Rpd3L histone deacetylase activity (LECHNER et al. 2000; CARROZZA et al. 2005a). Deletion of SDS3 in an esa1 mutant mimicked the suppression seen in esa1 rpd3 (Figure 2B, bottom), providing evidence that suppression of esa1 is mediated through Rpd3L.

Pho23 is another Rpd3L-specific protein with a PHD finger and is one of three yeast proteins that belong to the ING tumor suppressor family (LOEWITH et al. 2001). In contrast to the sds3Δ mutant, the Rpd3L complex is structurally intact in pho23Δ cells and has normal levels of in vitro histone deacetylase activity (CARROZZA et al. 2005a). Deletion of PHO23 in the esa1 mutant mimicked the suppression seen in both the esa1 rpd3 and esa1 sds3 double mutants (Figure 2B, bottom). This minor disruption in the Rpd3L complex is able to suppress esa1’s growth defect and supports the idea that Pho23 may have a key targeting function that works in opposition to other PHD finger proteins that exist in NuA4 and piccolo.

Comparing the growth at elevated temperatures of esa1 rco1 mutants to the esa1 sds3 and esa1 pho23 strains thus demonstrates that the rescue of esa1’s growth defect by deletion of RPD3 is mediated by the Rpd3L complex and not by Rpd3S. This specificity of suppression further establishes functional and not merely structural distinctions between the two Rpd3 complexes. To determine if the specificity of suppression extended to the diverse biological roles of Esa1, a broader analysis of defective esa1 functions was evaluated.
Disruption of Rpd3L suppressed silencing phenotypes of the *esa1* mutant:

Mutants of *ESA1* have a wide range of phenotypes, including defects in cell cycle control, transcriptional silencing, and the DNA damage response (Clarke *et al.* 1999; Bird *et al.* 2002; Clarke *et al.* 2006). To determine the involvement of Rpd3L in contributing to these phenotypes, the *esa1 rpd3* double mutants along with the complex-specific double mutants were examined for the integrity of these functions using *in vivo* assays.

First, rDNA silencing was assayed in the *esa1 rpd3* double mutant. Rpd3 has a previously reported increase in rDNA silencing (Sun and Hampsey 1999), confirmed here with the observation that *rpd3* increases repression of a *CAN1* reporter integrated at a single 25S rDNA locus (Figure 3A). In contrast, *esa1* mutants are defective in silencing at the rDNA (Clarke *et al.* 2006) (Figure 3A). Deletion of *RPD3* in combination with *esa1* suppressed this rDNA silencing defect. Deletion of *RPD3* in the *esa1* mutant not only rescued the rDNA silencing defect, but increased silencing in the double mutant to that seen in an *rpd3* single mutant (Figure 3A, top). This same trend was observed when the catalytic residues of *RPD3* were mutated in combination with *esa1* (Figure S2A). The complex-specific double mutants were next tested for rDNA silencing. The previous observations that Rpd3L-specific mutants display increased rDNA silencing (Sun and Hampsey 1999; Loewith *et al.* 2001; Keogh *et al.* 2005) were confirmed. Consistent with the suppression of *esa1*'s growth defect, rDNA silencing was suppressed only when Rpd3L was disrupted in *esa1* and not when Rpd3S was disrupted (Figure 3A, compare *sds3* and *pho23* to *rco1* mutants).

Telomeric silencing was next assayed and revealed suppression patterns parallel to those for rDNA silencing. The *esa1* mutant is defective in silencing a *URA3* reporter
gene integrated at telomere VR (Clarke et al. 2006) (Figure 3B). The rpd3 and Rpd3L-specific mutants displayed increased silencing (Figure 3B), confirming previous reports (Vannier et al. 1996; Zhang et al. 1998; Loewith et al. 2001; Carrozza et al. 2005a; Keogh et al. 2005). When genes encoding Rpd3L subunits were deleted in combination with esal, they all restored telomeric silencing to esal mutants, whereas deletion of the Rpd3S-specific RCO1 had no effect on esal’s reduced telomeric silencing (Figure 3B, Figure S2B). Therefore, Esa1 and Rpd3L share a critical opposing role in silencing at both the rDNA and telomeres.

Rpd3L disruption did not suppress the DNA damage phenotype of the esal mutant: Whereas the growth and silencing phenotypes of esal are consistent with Esa1’s transcriptional functions, Esa1 also has a role in DNA double-strand break repair. This is readily observed in that esal mutants are sensitive to camptothecin (Bird et al. 2002), a phenotype associated with defects in DNA repair and genome integrity. Camptothecin causes double-strand breaks by inhibiting topoisomerase I (Hsiang et al. 1985). Rpd3 also contributes to double-strand break repair, and rpd3 mutants are sensitive to phleomycin, another DNA damaging agent (Jazayeri et al. 2004).

When tested in plate assays, an rpd3 single mutant displayed increased sensitivity to camptothecin, and rpd3 did not suppress esal’s sensitivity to camptothecin (Figure 4). In fact, the esal rpd3 double mutant had increased sensitivity to camptothecin compared to esal alone. Deletion of Rpd3L- and Rpd3S-specific subunits either exacerbated or had a minimal effect on camptothecin sensitivity in the esal mutant (Figure 4).
Esa1 and Rpd3 are among several chromatin modifiers that are recruited to the repair of double-strand breaks resulting from DNA damage (Downs et al. 2004; Tamburini and Tyler 2005; Lin et al. 2008). Camptothecin sensitivity in esal cells is thought to result from a failure of Esa1 and NuA4 recruitment to double-strand breaks. Therefore, rpd3 as a suppressor of esal is unlikely to involve Esa1’s role in acetylation at sites of DNA damage.

In addition to the silencing and DNA damage phenotypes, rpd3 mutants have reduced mating efficiency and are cycloheximide sensitive (Vidal and Gaber 1991). To examine if mutation of ESA1 could reciprocally suppress rpd3 phenotypes, the esal rpd3 and the complex-specific double mutants were examined for changes in mating efficiency and cycloheximide sensitivity. Mutation of ESA1 in rpd3 had no effect on the reduced mating efficiency of rpd3, and the same was seen for the Rpd3L-specific mutants pho23 and sds3 (Figure S3A). The mating defect of rpd3 mutants appeared specific to Rpd3L, shown by the reduced mating of pho23 and sds3 compared to wild-type mating in rco1 (Figure S3A). Reduced mating efficiency has been observed previously for sap30, another Rpd3L-specific mutant (Zhang et al. 1998).

To determine if mutation of ESA1 could suppress rpd3’s cycloheximide sensitivity, growth of the esal rpd3 double mutant was examined on cycloheximide-containing plates. No suppression was observed; in fact the esal mutant displayed a previously unreported modest cycloheximide resistance (Figure S3B). Together, rpd3 in the context of Rpd3L can suppress many but not all esal mutant phenotypes. The nature of this functional interaction is not, however, reciprocal because esal mutants do not suppress the rpd3 defects tested.
Deletion of *RPD3* restored global histone acetylation levels of shared target residues in the *esa1* mutant: To test whether the genetic relationship discovered between *esa1* and *rpd3* was observed at the molecular level of histone modification, global histone acetylation was evaluated using isoform-specific antisera for lysines targeted by Esa1 and Rpd3. The enzymatic activities of Esa1 and Rpd3 both target specific lysines on the N-terminal tail of histone H4 (Figure 5A). Esa1 has global effects *in vivo* on H4K5 acetylation (CLARKE *et al.* 1999) and also acetylates multiple lysines on H4 at sites within the rDNA (CLARKE *et al.* 2006) and at specific gene promoters (SUKA *et al.* 2001). Rpd3 globally deacetylates H4K5 and H4K12 (RUNDLETT *et al.* 1996) and is responsible for deacetylation of most histone tail lysine residues at specific gene promoters (SUKA *et al.* 2001).

Lysates were collected from cells grown at permissive and slightly elevated temperatures that maintained viability. Immunoblots were performed on these lysates and probed with antisera specific for each acetylated lysine. As a control, histone levels were assayed and found comparable among all strains, as shown by probing for the C-terminus of H3 (Figure 5B, top panel). As expected, *esa1* mutants displayed decreased bulk histone acetylation, most dramatic for H4K5 (CLARKE *et al.* 1999) and H4K12, and *rpd3* mutants had slightly increased acetylation of H4K5 and H4K12 compared to wild-type, consistent with earlier reports (RUNDLETT *et al.* 1996) (Figure 5B). In the non-catalytic mutants (*rcol, sds3*, and *pho23*), global histone acetylation changes were not observed (Figure S4). This result might be expected since an Rpd3 complex is still present in each of these mutants. Thus, it was not surprising that there were only very subtle changes in
acetylation in these mutants when combined with *esa1* (Figure S4). Additionally, at two H4 lysines that are not shared targets of Esa1 and Rpd3, H4K8 and H4K16, acetylation was not changed in the *esa1 rpd3* double mutant.

Finally, in the *esa1 rpd3* double mutant, there was almost a complete restoration of the *esa1* global acetylation defect at both permissive and elevated temperatures that was strongest for H4K12 (Figure 5B). There was also an intermediate effect on acetylation at H4K5. These results provide a molecular basis for the growth defect and silencing suppression observed in the *esa1 rpd3* double mutant (Figure 1, 3).

**Suppression of *esa1*’s growth defect by deletion of *RPD3* is mediated through H4K12 acetylation:** Because the most dramatic change in global histone acetylation in *esa1 rpd3* was at H4K12 (Figure 5B), it seemed likely that this particular residue was most critical for the functional interaction between the two enzymes. To evaluate the possibility, mutants were constructed in which each target lysine was changed to alanine, an amino acid residue that cannot be acetylated. The ability of *rpd3* to suppress *esa1*’s growth defect was then tested with each histone lysine mutant. In wild-type cells, the H4K12A mutant by itself did not display any growth defects, nor did it affect growth in the *esa1* or *rpd3* single mutant. However, H4K12A in combination with the *esa1 rpd3* double mutant displayed a dramatic reduction in growth at elevated temperature compared to the *esa1 rpd3* double mutant (Figure 6A, top). The other H4 lysine mutants (H4K5A, H4K8A, and H4K16A) had minimal effects on the growth of the *esa1 rpd3* double mutant (Figure 6A, bottom). The dependence on H4K12 was also observed in the *esa1 rpd3-H150A-H151A* catalytic mutant (Figure S5). Therefore, the suppression
observed in the *esa1 rpd3* double mutant is specifically dependent on H4K12, and not H4K5, K8, or K16.

H4K12 was also found to be the key acetylated lysine in suppression of *esa1* by disruption of the Rpd3L complex. As shown in Figure 6B, when each lysine was individually mutated in the *esa1 sds3* and *esa1 pho23* double mutants, only the H4K12A substitution resulted in a loss of suppression, although to a more modest degree than in *esa1 rpd3*. Although the dependence on H4K12 appears subtle in the *esa1 pho23* double mutant, this slight effect was observed reproducibly. Notably, in the protein immunoblots H4K5 showed a moderate acetylation increase in the *esa1 rpd3* double mutant compared to *esa1* (Figure 5B), yet the H4K5A mutant had little impact on the growth of *esa1 rpd3*, *esa1 sds3*, or *esa1 pho23* (Figure 6).

Thus H4K5 and H4K12 are common targets of global acetylation and deacetylation by both Esa1 and Rpd3. However, the distinction observed here between the growth of *esa1 rpd3* in H4K5A versus H4K12A mutants points to H4K12 as the critical shared target of Esa1 and the Rpd3L complex for regulating growth and viability (Figure 7A).

**DISCUSSION**

The findings presented here tightly link Esa1’s acetyltransferase activity and Rpd3’s deacetylase activity in critical cellular processes. Loss of Rpd3L specifically alleviated many of the cell’s needs for fully functional Esa1 activity, a property not shared by the Rpd3S complex, nor by other Class I-III deacetylases (summarized in Table S4). This exclusive relationship between Esa1 and Rpd3L centers on their shared histone
target H4K12. In addition, Esa1 works specifically with the Rpd3L complex in maintaining silencing at the rDNA and telomeres, but not in repairing camptothecin-induced double-strand breaks (Figure 7, Table S4).

Esa1 and Rpd3 have both previously been shown to be required for rDNA and telomeric silencing. Esa1 is enriched at the rDNA by chromatin immunoprecipitation and Esa1-dependent changes in H4 acetylation are seen at the rDNA (Clarke et al. 2006). Unlike its role in growth, the rpd3-mediated suppression of esa1’s rDNA and telomeric silencing defects was not dependent on H4K12 acetylation (Figure S6). Hence suppression at these loci is mechanistically distinct. Rpd3L’s role in rDNA and telomeric silencing involves boundary formation (Zhou et al. 2009) and is dependent on the histone deacetylase Sir2 that targets H4K16 (Sun and Hampsey 1999; Raisner and Madhani 2008). Therefore the observed dependence on H4K16 acetylation (Figure S6) was not surprising. This dependence on Sir2 and H4K16 deacetylation has led to the idea that Rpd3 has an indirect effect in silencing, possibly through altering Sir2 activity (Sun and Hampsey 1999) or the expression of other genes involved in silencing. Because esa1’s rDNA and telomeric silencing defects were suppressed by disruption of Rpd3L (Figure 3), and were dependent on H4K16 acetylation (Figure S6), it is likely that Esa1 and Rpd3L’s role in silencing is upstream of Sir2 (Figure 7B).

It is becoming evident that histone modifying enzymes also target many non-histone substrates (reviewed in Sterner and Berger 2000). Indeed, recent data indicate that such non-histone substrates exist for NuA4 (Lin et al. 2009), including Yng2, which is also a substrate of Rpd3 (Lin et al. 2008). Further studies should provide additional insight into the range and roles of non-histone substrates in Esa1 and Rpd3 functions,
perhaps revealing a more direct link for their influence on rDNA and telomeric silencing. In this case however, we have shown that H4K12 is a key shared target for the contributions of Esa1 and Rpd3 to cell growth and viability.

**A critical role for dynamic acetylation and deacetylation of H4K12 by Esa1 and Rpd3L:** The cell contains numerous HATs and HDACs that together acetylate many lysine residues on histones. The intricacies of histone acetylation and deacetylation result from several features: each HAT and HDAC often targets multiple lysine residues, different HATs and HDACs have overlapping acetylation targets, and other post-translational modifications may influence activity or substrate recognition. For example, a simple mutation of H4K12 did not suppress *esa1* defects (Figure 6A), even though it is a key target of Esa1. This is because Esa1 has many other histone targets, including other lysines on H4, H2A, and H2A.Z, and lack of acetylation of these also contributes to *esa1*’s growth defect.

Defining roles for specific histone acetylation sites is further complicated by the genome-wide data that acetylation of H4K5, H4K8, and H4K12 are redundant in transcription (DION et al. 2005). One support of this idea is that the H4K12A single mutant displayed no obvious growth defects (Figure 6A). A previous study defined the H3 and H4 N-termini as the functional targets of Rpd3 in regulation of transcription (SABET et al. 2004). The connections between Esa1, Rpd3L, and H4K12 presented here strengthen this functional importance through further identification of a key specific lysine (H4K12) in the H4 N-terminus, and the acetyltransferase responsible (Esa1). Since in the absence of Esa1 and Rpd3L, H4K12 acetylation became particularly important for cell viability (Figure 6), these specific links define a model whereby control of H4K12...
acetylation is essential for transcriptional regulation of a subset of genes by Esa1 and Rpd3L for cell viability (Figure 7A).

Among the several genome-wide ChIP datasets that define Esa1 and Rpd3 binding (Reid et al. 2000; Kurdistani et al. 2002; Robert et al. 2004), little overlap has been observed between regions strongly enriched for Esa1 and those enriched for Rpd3. This may be due to the fact that Esa1 and Rpd3 both exist in multiple complexes in the cell, creating noise in the datasets. Esa1-bound loci would include both NuA4 and piccolo, thereby conflating their occupancy sites. Likewise, genome-wide ChIP that has been performed does not allow discrimination between sites of Rpd3L versus Rpd3S occupancy. When analyzed at specific loci, Rpd3S functions at downstream regions (Carrozza et al. 2005b; Keogh et al. 2005), thus it is likely that the genome-wide binding of Rpd3 found at downstream regions can be attributed to Rpd3S and binding at promoters can be attributed to Rpd3L. However, because the genomic binding maps were generated with non-tiling arrays, resolving the differences in Rpd3L and Rpd3S binding with available datasets is not possible. The differences in function between Rpd3L and Rpd3S in relation to Esa1 provide a new tool for refining understanding of the two complexes.

**Distinguishing complexes and their functional interactions:** Esa1 and Rpd3 each act as the catalytic subunit of two multiprotein histone modifying complexes. The two Rpd3 complexes, Rpd3S and Rpd3L, are composed of distinct subunits that allow them to be genetically dissected. Several recent papers have examined different roles for Rpd3S and Rpd3L (Carrozza et al. 2005a; Carrozza et al. 2005b; Keogh et al. 2005; Biswas et al. 2008; Knott et al. 2009). We have established that disruption of Rpd3L
function is specifically responsible for the genetic suppression of \textit{esa1} mutants for cell viability and silencing phenotypes (Figure 2, 3).

Two different roles for histone modifying enzymes in the DNA damage response have been uncovered. One role is to participate in the transcriptional response through the activation of DNA repair genes. For example, Esa1 and Rpd3 are both required for transcriptional activation of the damage-inducible genes \textit{HUG1} and \textit{RNR3} (Sharma et al. 2007). The identification of these as shared targets for activation raises a useful distinction because \textit{rpd3} cannot suppress \textit{esa1’s} sensitivity to DNA damage. It seems likely therefore that Esa1 and Rpd3 target genes relevant to changes in H4K12 acetylation are those that Esa1 activates and Rpd3 represses. Identification of these genes should prove of great interest.

The other function for histone modifying enzymes in DNA repair is more direct: chromatin modification targeted to the site of DNA damage. Along with several other HATs and HDACs, Esa1, some members of NuA4, and Rpd3 itself all bind at double-strand breaks, followed by changes in acetylation of nearby chromatin (Bird et al. 2002; Downs et al. 2004; Tamburini and Tyler 2005; Lin et al. 2008). Our observations showing that deletion of Rpd3L-specific subunits does not suppress repair defects of \textit{esa1} mutants make it unlikely that Rpd3L functions together with Esa1 at sites of DNA damage. However, since Rpd3 is present at double-strand breaks and is required for nonhomologous end-joining (Jazayeri et al. 2004; Tamburini and Tyler 2005), perhaps Rpd3S and Esa1 coordinate acetylation at sites of DNA damage (Figure 7C).

By constructing specific double deletion mutants, it was possible to refine understanding of Rpd3S and Rpd3L functions beyond earlier reports. In contrast, Esa1
exists in the NuA4 and piccolo complexes, yet because piccolo is a subcomplex of NuA4, it has not yet been possible to disrupt piccolo without also disrupting NuA4 function. Therefore, it remains to be determined whether \textit{rpd3} suppression of \textit{esa1} is mediated through NuA4 or piccolo.

Future studies should provide additional insight into distinctions between NuA4 and piccolo that may allow this question to be answered. One idea comes from studies examining the chromatin modifying complexes SLIK/SALSA and SAGA. These complexes share most subunits, including the histone acetyltransferase Gcn5. It was found that the shared subunit Spt7, exists in a C-terminally truncated form in the smaller SLIK/SALSA complex, allowing for construction of specific \textit{SPT7} alleles that favor a specific complex (STERNER \textit{et al.} 2002; WU and WINSTON 2002). Analogous to this shift between SLIK/SALSA and SAGA, the discovery of specific alleles of piccolo components that favor activity of one complex over another may allow for future dissection of Esa1’s interactions with Rpd3.

One possibility is that NuA4 and Rpd3S, which share the chromodomain protein Eaf3, work together, whereas piccolo and Rpd3L are also a functional pair. Some data supporting this idea can be extrapolated from genome-wide studies. For example, double mutants of \textit{RPD3} and genes encoding NuA4-specific subunits show reduced fitness, whereas a double deletion mutant of \textit{RPD3} and \textit{EPL1}, which is in both piccolo and NuA4, shows synthetic rescue (LIN \textit{et al.} 2008). However, because deletion of \textit{EPL1} disrupts both piccolo and NuA4, it is difficult to make a clear distinction between the two.
The composition of NuA4, piccolo, Rpd3L, and Rpd3S is evolutionarily conserved (reviewed in DOYON and CÔTÉ 2004; YANG and SETO 2008). One particular class of proteins in both is the PHD finger-containing ING family of tumor suppressors. Yng2 is a yeast ING protein that is a subunit of both piccolo and NuA4 (LOEWITH et al. 2000), whereas Pho23 is another yeast ING protein that is a subunit of Rpd3L (LOEWITH et al. 2001; CARROZZA et al. 2005a). The precise functions of Yng2 and Pho23 in their complexes are unknown, but analogous to the opposing activities between Esa1 and Rpd3, Yng2 and Pho23 have opposite effects on p53-dependent transcriptional activation, shown in an experiment where human p53 was expressed in yeast to drive transcription (NOURANI et al. 2003). This opposing effect on activity of a human protein in transcription emphasizes the conserved nature underlying the partnership between the Esa1 and Rpd3 complexes reported here. In addition, the identification of H4K12 as a critical shared acetylation target uncovers the importance of the dynamic acetylation and deacetylation of a particular histone residue in the context of Esa1 and Rpd3L function.

Dynamic and reciprocal histone modifications are increasingly recognized as key regulatory switches. This principle was highlighted in a recent study investigating histone ubiquitination in metazoan development. Coordinate control of H2B ubiquitination in Drosophila by the ubiquitin ligase dBRE1 and the ubiquitin protease Scravy was found to be essential for regulating gene silencing to promote cellular differentiation (BUSZCZAK et al. 2009). Our studies identify links between Esa1 and Rpd3L specifically in the acetylation and deacetylation of H4K12. Further, they reveal a critical distinct characteristic of the Rpd3L complex in relation to Esa1, and identify roles for specific histone residues in promoting cell viability. Future functional dissection of Rpd3 and
Esal multiprotein complexes will deepen understanding of how such chromatin modifiers control important and diverse cellular processes.

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**FIGURE LEGENDS**

**FIGURE 1.**–Deletion of the histone deacetylase gene *RPD3* suppresses the growth defect of *esa1*. Deletion of *RPD3* suppressed the temperature-sensitivity of the *esa1-414* allele, whereas deletion of other genes encoding histone deacetylases did not. Top panel: serial dilutions of wild-type (LPY5), *esa1* (LPY4774), *esa1 rpd3* (LPY12156), *rpd3* (LPY12154), *esa1 sir2* (LPY11160), and *sir2* (LPY11) are shown on SC at the restrictive temperature for *esa1* (35°C), compared to growth at the permissive temperature (30°C). Bottom panel: serial dilutions of wild-type (LPY5), *esa1* (LPY4774), *esa1 hos1* (LPY13712), *esa1 hos2* (LPY13585), *esa1 hda1* (LPY13478), *hos1* (LPY13706), *hos2* (LPY13583), and *hda1* (LPY13472) on SC at restrictive and permissive temperatures. Note that some of these interactions overlap with published results from a genome-wide study (Lin et al. 2008), yet others are distinct. The differences may be due to strain background- or allele-specific effects (personal communication, Y. Lin and J. Boeke).
**FIGURE 2.**—Rpd3L is the Rpd3-containing complex responsible for suppression of the growth defect in the *esa1* mutant. (A) Cartoon highlighting the unique and shared members of the Rpd3S and Rpd3L complexes. (B) Deletion of *RCO1*, specific to Rpd3S, did not suppress *esa1*’s growth defect at restrictive temperature. Deletion of *PHO23* and *SDS3*, both specific to Rpd3L, mimicked the suppression seen in *esa1 rpd3*. Serial dilutions of wild-type (LPY5), *esa1* (LPY4774), *esa1 rco1* (LPY12652), *rco1* (LPY12645), *esa1 sds3* (LPY12956), *sds3* (LPY12958), *esa1 pho23* (LPY12729), and *pho23* (LPY12732), were plated on SC at permissive (30˚) and restrictive temperatures (35˚). Cartoon of complexes is modified from (ROGUEV and KROGAN 2007).

**FIGURE 3.**—Disruption of Rpd3L suppresses *esa1* silencing phenotypes. (A) Disruption of Rpd3L suppressed the rDNA silencing defect of an *esa1* mutant. Wild-type (LPY4909), *esa1* (LPY4911), *esa1 rpd3* (LPY12147), *rpd3* (LPY12145), *esa1 sds3* (LPY13517), *sds3* (LPY13513), *esa1 pho23* (LPY13859), *pho23* (LPY13854), *esa1 rco1* (LPY13505), and *rco1* (LPY13501) all have the rDNA::ADE2-CAN1 reporter to test for rDNA silencing on plates containing canavanine. Decreased growth on canavanine indicates a defect in rDNA silencing. (B) Disruption of Rpd3L suppressed the telomeric silencing defect of an *esa1* mutant. Wild-type (LPY4917), *esa1* (LPY4919), *esa1 rpd3* (LPY12211), *rpd3* (LPY12093), *esa1 sds3* (LPY13540), *sds3* (LPY13536), *esa1 pho23* (LPY13769), *pho23* (LPY13765), *esa1 rco1* (LPY13528), and *rco1* (LPY13524) all have the TELVR::URA3 silencing marker to test for telomeric silencing on plates containing 5-FOA. Decreased growth on 5-FOA indicates a defect in telomeric silencing.
FIGURE 4.—Rpd3L disruption does not suppress esa1’s camptothecin sensitivity.
The same strains tested in Figure 1 and Figure 2B were plated on a control YPD plate containing DMSO and a plate containing 20 µg/ml of camptothecin in DMSO.

FIGURE 5.—Deletion of RPD3 restores global acetylation levels of specific histone H4 residues in esa1 mutants. (A) Diagram of the histone H4 N-terminal tail highlighting sites of acetylation modifications. (B) Deletion of RPD3 restored global acetylation of H4K5 and H4K12, but not H4K8 and H4K16. Whole cell protein extracts from wild-type (LPY5), esa1 (LPY4774), esa1 rpd3 (LPY12156), and rpd3 (LPY12154) cells at both permissive (30˚) and restrictive (34˚) temperature were immunoblotted with an antiserum specific to the C-terminus of H3 to control for histone levels, and with H4 antisera to detect the amount of bulk histone acetylation at each lysine residue.

FIGURE 6.—Suppression of esa1’s growth defect by rpd3 is dependent on H4K12.
Strains are deleted for all copies of H3 and H4 and carry a TRP1 plasmid with either wild-type H4 or H4 with one mutated lysine residue. Plasmid retention was required for survival. (A) Serial dilutions of the following strains were plated at permissive (30˚) and restrictive temperature (35˚) on SC. Top panel: growth of H4K12A mutants in combination with esa1 rpd3. Wild-type (LPY12383), H4K12A (LPY12394), esa1 (LPY12384), esa1 H4K12A (LPY12071), esa1 rpd3 (LPY12707), esa1 rpd3 H4K12A (LPY12714), rpd3 (LPY12695), rpd3 H4K12A (LPY12702). Bottom panel: growth of esa1 rpd3 mutants in combination with each lysine individually mutated to alanine. esa1 rpd3 (LPY12707), esa1 rpd3 H4K5A (LPY12708), esa1 rpd3 H4K8A (LPY12711), esa1
rpd3 H4K12A (LPY12714), esa1 rpd3 H4K16A (LPY12717). (B) Suppression of esa1’s growth defect through deletion of non-catalytic Rpd3L subunits was also dependent on H4K12. Top panel: two-fold dilutions, starting at an A600 of 0.1, were plated on SC-Trp for assaying growth of esa1 sds3 in combination with each lysine individually mutated to alanine. esa1 sds3 (LPY14175), esa1 sds3 H4K5A (LPY14176), esa1 sds3 H4K8A (LPY14177), esa1 sds3 H4K12A (LPY14178), esa1 sds3 H4K16A (LPY14179). Bottom panel: as above except in esa1 pho23 mutant. Strains are esa1 pho23 (LPY14165), esa1 pho23 H4K5A (LPY14166), esa1 pho23 H4K8A (LPY14167), esa1 pho23 H4K12A (LPY14168), esa1 pho23 H4K16A (LPY14169).

FIGURE 7.—A model depicting a critical role for Esa1 and Rpd3L in coordinating the dynamic acetylation of H4K12. (A) Esa1 and Rpd3L control H4K12Ac for general transcriptional targets contributing to cell viability and growth. (B) Esa1 and Rpd3L contribute to rDNA and telomeric silencing. This relationship is not mediated specifically through H4K12 acetylation, but likely through a number of other targets. Sir2 deacetylation of H4K16 appears downstream of the role for Esa1 and Rpd3L. (C) Esa1 and Rpd3S, but not Rpd3L, may control acetylation at sites of DNA damage.

LITERATURE CITED

AHN, S. H., W. L. CHEUNG, J. Y. HSU, R. L. DIAZ, M. M. SMITH et al., 2005 Sterile 20 kinase phosphorylates histone H2B at serine 10 during hydrogen peroxide-induced apoptosis in S. cerevisiae. Cell 120: 25-36.
ALEJANDRO-OSORIO, A. L., D. J. HUEBERT, D. T. PORCARO, M. E. SONNTAG, S.
NILLASITHANUKROH et al., 2009 The histone deacetylase Rpd3p is required for transient changes in genomic expression in response to stress. Genome Biol 10: R57.

ALLARD, S., R. T. UTLEY, J. SAVARD, A. CLARKE, P. GRANT et al., 1999 NuA4, an essential transcription adaptor/histone H4 acetyltransferase complex containing Esa1p and the ATM-related cofactor Tra1p. EMBO J. 18: 5108-5119.

APARICIO, J. G., C. J. VIGGIANI, D. G. GIBSON and O. M. APARICIO, 2004 The Rpd3-Sin3 histone deacetylase regulates replication timing and enables intra-S origin control in Saccharomyces cerevisiae. Mol. Cell. Biol. 24: 4769-4780.

AVVAKUMOV, N., and J. CÔTÉ, 2007 The MYST family of histone acetyltransferases and their intimate links to cancer. Oncogene 26: 5395-5407.

BABIAZ, J. E., J. E. HALLEY and J. RINE, 2006 Telomeric heterochromatin boundaries require NuA4-dependent acetylation of histone variant H2A.Z in Saccharomyces cerevisiae. Genes Dev. 20: 700-710.

BERNSTEIN, B. E., J. K. TONG and S. L. SCHREIBER, 2000 Genomewide studies of histone deacetylase function in yeast. Proc. Natl. Acad. Sci. USA 97: 13708-13713.

BIRD, A. W., D. Y. YU, M. G. PRAY-GRANT, Q. QIU, K. E. HARMON et al., 2002 Acetylation of histone H4 by Esa1 is required for DNA double-strand break repair. Nature 419: 411-415.

BISWAS, D., S. TAKAHATA and D. J. STILLMAN, 2008 Different genetic functions for the Rpd3(L) and Rpd3(S) complexes suggest competition between NuA4 and Rpd3(S). Mol. Cell. Biol. 28: 4445-4458.
BOUDREULT, A. A., D. CRONIER, W. SELLECK, N. LACOSTE, R. T. UTLEY et al., 2003
Yeast enhancer of polycomb defines global Esa1-dependent acetylation of chromatin. Genes Dev. 17: 1415-1428.

BUSZCZAK, M., S. PATERNO and A. C. SPRADLING, 2009 Drosophila stem cells share a common requirement for the histone H2B ubiquitin protease scrawny. Science 323: 248-251.

CARROZZA, M. J., L. FLORENS, S. K. SWANSON, W. J. SHIA, S. ANDERSON et al., 2005a
Stable incorporation of sequence specific repressors Ash1 and Ume6 into the Rpd3L complex. Biochim. Biophys. Acta 1731: 77-87; discussion 75-76.

CARROZZA, M. J., B. LI, L. FLORENS, T. SUGANUMA, S. K. SWANSON et al., 2005b
Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell 123: 581-592.

CHOY, J. S., and S. J. KRON, 2002 NuA4 subunit Yng2 function in intra-S-phase DNA damage response. Mol. Cell. Biol. 22: 8215-8225.

CLARKE, A. S., J. E. LOWELL, S. J. JACOBSON and L. PILLUS, 1999 Esa1p is an essential histone acetyltransferase required for cell cycle progression. Mol. Cell. Biol. 19: 2515-2526.

CLARKE, A. S., E. SAMAL and L. PILLUS, 2006 Distinct Roles for the Essential MYST Family HAT Esa1p in Transcriptional Silencing. Mol. Biol. Cell 17: 1744-1757.

COLLINS, S. R., K. M. MILLER, N. L. MAAS, A. ROGUEV, J. FILLINGHAM et al., 2007
Functional dissection of protein complexes involved in yeast chromosome biology using a genetic interaction map. Nature 446: 806-810.
DANG, W., K. K. STEFFEN, R. PERRY, J. A. DORSEY, F. B. JOHNSON et al., 2009 Histone H4 lysine 16 acetylation regulates cellular lifespan. Nature 459: 802-807.

DE NADAL, E., M. ZAPATER, P. M. ALEPUZ, L. SUMOY, G. MAS et al., 2004 The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoreponsive genes. Nature 427: 370-374.

DE RUBERTIS, F., D. KADOWSH, S. HENCHOZ, D. PAULI, G. REUTER et al., 1996 The histone deacetylase RPD3 counteracts genomic silencing in Drosophila and yeast. Nature 384: 589-591.

DECKER, P. V., D. Y. YU, M. IIZUKA, Q. QIU and M. M. SMITH, 2008 Catalytic-site mutations in the MYST family histone acetyltransferase Esa1. Genetics 178: 1209-1220.

DION, M. F., S. J. ALTSCHULER, L. F. WU and O. J. RANDO, 2005 Genomic characterization reveals a simple histone H4 acetylation code. Proc. Natl. Acad. Sci. USA 102: 5501-5506.

DOWNS, J. A., S. ALLARD, O. JOBIN-ROBITAILLE, A. JAVAHERI, A. AUGER et al., 2004 Binding of chromatin-modifying activities to phosphorylated histone H2A at DNA damage sites. Mol. Cell 16: 979-990.

DOYON, Y., and J. CÔTÉ, 2004 The highly conserved and multifunctional NuA4 HAT complex. Curr. Opin. Genet. Dev. 14: 147-154.

EISEN, A., R. T. UTLEY, A. NOURANI, S. ALLARD, P. SCHMIDT et al., 2001 The yeast NuA4 and Drosophila MSL complexes contain homologous subunits important for transcription regulation. J. Biol. Chem. 276: 3484-3491.
FRITZE, C. E., K. VERSCHUEREN, R. STRICH and R. EASTON ESPOSITO, 1997 Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA. EMBO J. 16: 6495-6509.

HSIANG, Y. H., R. HERTZBERG, S. HECHT and L. F. LIU, 1985 Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J. Biol. Chem. 260: 14873-14878.

JAZAYERI, A., A. D. MCAINSH and S. P. JACKSON, 2004 Saccharomyces cerevisiae Sin3p facilitates DNA double-strand break repair. Proc. Natl. Acad. Sci. USA 101: 1644-1649.

KADOSH, D., and K. STRUHL, 1997 Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. Cell 89: 365-371.

KADOSH, D., and K. STRUHL, 1998a Histone deacetylase activity of Rpd3 is important for transcriptional repression in vivo. Genes Dev. 12: 797-805.

KADOSH, D., and K. STRUHL, 1998b Targeted recruitment of the Sin3-Rpd3 histone deacetylase complex generates a highly localized domain of repressed chromatin in vivo. Mol. Cell. Biol. 18: 5121-5127.

KASTEN, M. M., S. DORLAND and D. J. STILLMAN, 1997 A large protein complex containing the yeast Sin3p and Rpd3p transcriptional regulators. Mol. Cell. Biol. 17: 4852-4858.

KEOGH, M. C., S. K. KURDISTANI, S. A. MORRIS, S. H. AHN, V. PODOLNY et al., 2005 Cotranscriptional Set2 methylation of histone H3 lysine 36 recruits a repressive Rpd3 complex. Cell 123: 593-605.
KEOGH, M. C., T. A. MENNELLA, C. SAWA, S. BERTHELET, N. J. KROGAN et al., 2006 The *Saccharomyces cerevisiae* histone H2A variant Htz1 is acetylated by NuA4. Genes Dev. **20:** 660-665.

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.

KNOTT, S. R., C. J. VIGGIANI, S. TAVARE and O. M. APARICIO, 2009 Genome-wide replication profiles indicate an expansive role for Rpd3L in regulating replication initiation timing or efficiency, and reveal genomic loci of Rpd3 function in *Saccharomyces cerevisiae*. Genes Dev. **23:** 1077-1090.

KURDISTANI, S. K., D. ROBYR, S. TAVAZOIE and M. GRUNSTEIN, 2002 Genome-wide binding map of the histone deacetylase Rpd3 in yeast. Nat. Genet. **31:** 248-254.

LECHNER, T., M. J. CARROZZA, Y. YU, P. A. GRANT, A. EBERHARTER et al., 2000 Sds3 (suppressor of defective silencing 3) is an integral component of the yeast Sin3·Rpd3 histone deacetylase complex and is required for histone deacetylase activity. J. Biol. Chem. **275:** 40961-40966.

LIN, Y. Y., J. Y. LU, J. ZHANG, W. WALTER, W. DANG et al., 2009 Protein acetylation microarray reveals that NuA4 controls key metabolic target regulating gluconeogenesis. Cell **136:** 1073-1084.

LIN, Y. Y., Y. QI, J. Y. LU, X. PAN, D. S. YUAN et al., 2008 A comprehensive synthetic genetic interaction network governing yeast histone acetylation and deacetylation. Genes Dev. **22:** 2062-2074.
LOEWITH, R., M. MEIJER, S. P. LEES-MILLER, K. RIABOWOL and D. YOUNG, 2000 Three yeast proteins related to the human candidate tumor suppressor p33(ING1) are associated with histone acetyltransferase activities. Mol. Cell. Biol. 20: 3807-3816.

LOEWITH, R., J. S. SMITH, M. MEIJER, T. J. WILLIAMS, N. BACHMAN et al., 2001 Pho23 is associated with the Rpd3 histone deacetylase and is required for its normal function in regulation of gene expression and silencing in Saccharomyces cerevisiae. J. Biol. Chem. 276: 24068-24074.

MEGEE, P. C., B. A. MORGAN and M. M. SMITH, 1995 Histone H4 and the maintenance of genome integrity. Genes Dev. 9: 1716-1727.

MILLAR, C. B., F. XU, K. ZHANG and M. GRUNSTEIN, 2006 Acetylation of H2AZ Lys 14 is associated with genome-wide gene activity in yeast. Genes Dev. 20: 711-722.

MITCHELL, L., J. P. LAMBERT, M. GERDES, A. S. AL-MADHOUN, I. S. SKERJANC et al., 2008 Functional dissection of the NuA4 histone acetyltransferase reveals its role as a genetic hub and that Eaf1 is essential for complex integrity. Mol. Cell. Biol. 28: 2244-2256.

NITISS, J., and J. C. WANG, 1988 DNA topoisomerase-targeting antitumor drugs can be studied in yeast. Proc. Natl. Acad. Sci. USA 85: 7501-7505.

NOURANI, A., L. HOWE, M. G. PRAY-GRANT, J. L. WORKMAN, P. A. GRANT et al., 2003 Opposite role of yeast ING family members in p53-dependent transcriptional activation. J. Biol. Chem. 278: 19171-19175.

PAPAMICHOS-CHRONAKIS, M., T. PETRAKIS, E. KTISTAKI, I. TOPALIDOU and D. TZAMARIAS, 2002 Cti6, a PHD domain protein, bridges the Cyc8-Tup1
corepressor and the SAGA coactivator to overcome repression at GAL1. Mol. Cell 9: 1297-1305.

RAISNER, R. M., and H. D. MADHANI, 2008 Genomewide screen for negative regulators of sirtuin activity in Saccharomyces cerevisiae reveals 40 loci and links to metabolism. Genetics 179: 1933-1944.

REID, J. L., V. R. IYER, P. O. BROWN and K. STRUHL, 2000 Coordinate regulation of yeast ribosomal protein genes is associated with targeted recruitment of Esa1 histone acetylase. Mol. Cell 6: 1297-1307.

RENAULD, H., O. M. APARICIO, P. D. ZIERATH, B. L. BILLINGTON, S. K. CHHABLANI et al., 1993 Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. Genes Dev. 7: 1133-1145.

ROBERT, F., D. K. POKHOLOK, N. M. HANNETT, N. J. RINALDI, M. CHANDY et al., 2004 Global position and recruitment of HATs and HDACs in the yeast genome. Mol. Cell 16: 199-209.

ROGUEV, A., and N. J. KROGAN, 2007 SIN-fully silent: HDAC complexes in fission yeast. Nat. Struct. Mol. Biol. 14: 358-359.

RUAULT, M., and L. PILLUS, 2006 Chromatin-modifying enzymes are essential when the Saccharomyces cerevisiae morphogenesis checkpoint is constitutively activated. Genetics 174: 1135-1149.

RUNDLETT, S. E., A. A. CARMEN, R. KOBAYASHI, S. BAVYKIN, B. M. TURNER et al., 1996 HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes
that regulate silencing and transcription. Proc. Natl. Acad. Sci. USA 93: 14503-14508.

RUNDLETT, S. E., A. A. CARMEN, N. SUKA, B. M. TURNER and M. GRUNSTEIN, 1998
Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. Nature 392: 831-835.

SABET, N., S. VOLO, C. YU, J. P. MADIGAN and R. H. MORSE, 2004 Genome-wide analysis of the relationship between transcriptional regulation by Rpd3p and the histone H3 and H4 amino termini in budding yeast. Mol. Cell. Biol. 24: 8823-8833.

SERTIL, O., A. VEMULA, S. L. SALMON, R. H. MORSE and C. V. LOWRY, 2007 Direct role for the Rpd3 complex in transcriptional induction of the anaerobic DAN/TIR genes in yeast. Mol. Cell. Biol. 27: 2037-2047.

SHARMA, V. M., R. S. TOMAR, A. E. DEMPSEY and J. C. REESE, 2007 Histone deacetylases RPD3 and HOS2 regulate the transcriptional activation of DNA damage-inducible genes. Mol. Cell. Biol. 27: 3199-3210.

SMITH, E. R., A. EISEN, W. GU, M. SATTAH, A. PANNUTI et al., 1998 ESA1 is a histone acetyltransferase that is essential for growth in yeast. Proc. Natl. Acad. Sci. USA 95: 3561-3565.

STERNER, D. E., R. BELOTSEKOVSKAYA and S. L. BERGER, 2002 SALSA, a variant of yeast SAGA, contains truncated Spt7, which correlates with activated transcription. Proc. Natl. Acad. Sci. USA 99: 11622-11627.

STERNER, D. E., and S. L. BERGER, 2000 Acetylation of histones and transcription-related factors. Microbiol. Mol. Biol. Rev. 64: 435-459.
SUKA, N., K. LUO and M. GRUNSTEIN, 2002 Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. Nat. Genet. 32: 378-383.

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.

SUN, Z. W., and M. HAMPSEY, 1999 A general requirement for the Sin3-Rpd3 histone deacetylase complex in regulating silencing in Saccharomyces cerevisiae. Genetics 152: 921-932.

TAMBURINI, B. A., and J. K. TYLER, 2005 Localized histone acetylation and deacetylation triggered by the homologous recombination pathway of double-strand DNA repair. Mol. Cell. Biol. 25: 4903-4913.

VAN LEEUWEN, F., and D. E. GOTT SCHLING, 2002 Assays for gene silencing in yeast. Methods Enzymol. 350: 165-186.

VANNIER, D., D. BALDERES and D. SHORE, 1996 Evidence that the transcriptional regulators SIN3 and RPD3, and a novel gene (SDS3) with similar functions, are involved in transcriptional silencing in S. cerevisiae. Genetics 144: 1343-1353.

VIDAL, M., and R. F. GABER, 1991 RPD3 encodes a second factor required to achieve maximum positive and negative transcriptional states in Saccharomyces cerevisiae. Mol. Cell. Biol. 11: 6317-6327.

VOGELAUER, M., L. RUBBI, I. LUCAS, B. J. BREWER and M. GRUNSTEIN, 2002 Histone acetylation regulates the time of replication origin firing. Mol. Cell 10: 1223-1233.
VOTH, W. P., Y. W. JIANG and D. J. STILLMAN, 2003 New 'marker swap' plasmids for converting selectable markers on budding yeast gene disruptions and plasmids. *Yeast* 20: 985-993.

WU, P. Y., and F. WINSTON, 2002 Analysis of Spt7 function in the *Saccharomyces cerevisiae* SAGA coactivator complex. *Mol. Cell. Biol.* 22: 5367-5379.

YANG, X. J., and E. SETO, 2008 The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. *Nat. Rev. Mol. Cell Biol.* 9: 206-218.

ZHANG, Y., Z. W. SUN, R. IRATNI, H. ERDJUMENT-BROMAGE, P. TEMPST et al., 1998 SAP30, a novel protein conserved between human and yeast, is a component of a histone deacetylase complex. *Mol. Cell* 1: 1021-1031.

ZHOU, J., B. O. ZHOU, B. A. LENZMEIER and J. Q. ZHOU, 2009 Histone deacetylase Rpd3 antagonizes Sir2-dependent silent chromatin propagation. *Nucleic Acids Res* 37: 3699-3713.
Figure 1
Figure 2
**Figure 3**

Panel A: WT, esa1, esa1 rpd3, rpd3, WT, esa1, esa1 sds3, sds3, esa1 pho23, pho23, esa1 rco1, rco1.

Panel B: control, rDNA silencing, SC-Ade-Arg, canavanine, SC, 5-FOA.
Figure 4
Figure 5

A

B

|          | WT | esa1 | esa1 rpd3 | rpd3 | rpd3 | esa1 rpd3 | esa1 | WT |
|----------|----|------|-----------|------|------|-----------|------|----|
| H3-CT    |    |      |           |      |      |           |      |    |
| H4K5Ac   |    |      |           |      |      |           |      |    |
| H4K8Ac   |    |      |           |      |      |           |      |    |
| H4K12Ac  |    |      |           |      |      |           |      |    |
| H4K16Ac  |    |      |           |      |      |           |      |    |

30° 34°
### Figure 6

**A**

| strain   | plasmid  | control | growth |
|----------|----------|---------|--------|
| WT       | WT       | ![Image](A.png) | ![Image](A.png) |
| WT       | H4K12A   | ![Image](A.png) | ![Image](A.png) |
| esa1     | WT       | ![Image](A.png) | ![Image](A.png) |
| esa1     | H4K12A   | ![Image](A.png) | ![Image](A.png) |
| esa1 rpd3| WT       | ![Image](A.png) | ![Image](A.png) |
| esa1 rpd3| H4K12A   | ![Image](A.png) | ![Image](A.png) |
| rpd3     | WT       | ![Image](A.png) | ![Image](A.png) |
| rpd3     | H4K12A   | ![Image](A.png) | ![Image](A.png) |
| esa1 rpd3| WT       | ![Image](A.png) | ![Image](A.png) |
| esa1 rpd3| H4K5A    | ![Image](A.png) | ![Image](A.png) |
| esa1 rpd3| H4K8A    | ![Image](A.png) | ![Image](A.png) |
| esa1 rpd3| H4K12A   | ![Image](A.png) | ![Image](A.png) |
| esa1 rpd3| H4K16A   | ![Image](A.png) | ![Image](A.png) |

**B**

| strain   | plasmid  | control | growth |
|----------|----------|---------|--------|
| esa1 sds3| WT       | ![Image](B.png) | ![Image](B.png) |
| esa1 sds3| H4K5A    | ![Image](B.png) | ![Image](B.png) |
| esa1 sds3| H4K8A    | ![Image](B.png) | ![Image](B.png) |
| esa1 sds3| H4K12A   | ![Image](B.png) | ![Image](B.png) |
| esa1 sds3| H4K16A   | ![Image](B.png) | ![Image](B.png) |
| esa1 pho23| WT    | ![Image](B.png) | ![Image](B.png) |
| esa1 pho23| H4K5A    | ![Image](B.png) | ![Image](B.png) |
| esa1 pho23| H4K8A    | ![Image](B.png) | ![Image](B.png) |
| esa1 pho23| H4K12A   | ![Image](B.png) | ![Image](B.png) |
| esa1 pho23| H4K16A   | ![Image](B.png) | ![Image](B.png) |
Figure 7

A  growth

B  silencing

C  damage