Suppression Analysis of esa1 Mutants in Saccharomyces cerevisiae Links NAB3 to Transcriptional Silencing and Nucleolar Functions

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ABSTRACT The acetyltransferase Esa1 is essential in the yeast Saccharomyces cerevisiae and plays a critical role in multiple cellular processes. The most well-defined targets for Esa1 are lysine residues on histones. However, an increasing number of nonhistone proteins have recently been identified as substrates of Esa1. In this study, four genes (LYS20, LEU2, VAP1, and NAB3) were identified in a genetic screen as high-copy suppressors of the conditional temperature-sensitive lethality of an esa1 mutant. When expressed from a high-copy plasmid, each of these suppressors rescued the temperature-sensitivity of an esa1 mutant. Only NAB3 overexpression also rescued the rDNA-silencing defects of an esa1 mutant. Strengthening the connections between NAB3 and ESA1, mutants of nab3 displayed several phenotypes similar to those of esa1 mutants, including increased sensitivity to the topoisomerase I inhibitor camptothecin and defects in rDNA silencing and cell-cycle progression. In addition, nuclear localization of Nab3 was altered in the esa1 mutant. Finally, posttranslational acetylation of Nab3 was detected in vivo and found to be influenced by ESA1.

Nucleosomes containing the core histones (H2A, H2B, H3, and H4) form the basic packaging unit of DNA that organizes chromatin into higher-order structures. The N-terminal tails of histones are subject to multiple covalent modifications that can influence gene expression locally at specific promoters or within large regions of chromatin. Increased histone acetylation is associated with both transcriptional activation and repression. Lysine acetyltransferases (KAT), the enzymes that catalyze the acetylation reaction on histones, have been ascribed multiple cellular functions. Recently, nonhistone targets have also been identified for many KATs, including Esa1 (Lin et al. 2009) [reviewed in Yang and Seto (2008)].

The Esa1 KAT of Saccharomyces cerevisiae is a member of the deeply conserved MYST family of acetyltransferases and is essential in yeast (Smith et al. 1998a; Clarke et al. 1999). Esa1 is the catalytic component of the NuA4 and piccolo complexes that acetylate histone H4, H2A, and its variant H2A.Z (Allard et al. 1999; Babiart et al. 2006; Keogh et al. 2006; Millar et al. 2006). Many of the NuA4 subunits, including Esa1, are essential (Galarneau et al. 2000; Loewith et al. 2000; Eisen et al. 2001), indicating that this complex has critical cellular roles.

Esa1 has a role in regulating expression of ribosomal protein genes (Reid et al. 2000). Further, genome-wide expression analysis reveals widespread transcriptional changes in esa1 mutants (Durant and Pugh 2006), and genome-wide binding profiles show Esa1 bound to the promoters of actively transcribed genes (Robert et al. 2004). Esa1 also functions in transcriptional silencing of the rDNA and at telomeres (Clarke et al. 2006). The variety of genomic targets identified thus far suggests Esa1 activity regulates transcription at many loci, indicative of its function in multiple cellular processes.

Genetic analysis further defines Esa1’s role in diverse cellular functions. Temperature-sensitive mutants of esa1 display a G2/M cell-cycle arrest at the restrictive temperature that is dependent upon the RAD9 DNA damage checkpoint (Clarke et al. 1999) and are hypersensitive to the topoisomerase I inhibitor camptothecin (Bird et al. 2002). Esa1 localizes to double-strand breaks where it functions in repair of DNA damage (Downs et al. 2004). Together, these results suggest Esa1 activity is required for cell-cycle regulation and genomic integrity.
Table 1 Yeast strains used in this study

| Strain | Genotype | Reference |
|--------|----------|-----------|
| LPY5 (W303-1a) | MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 | Thomas and Rothstein 1989 |
| LPY3291 | MATa his3Δ200 leu2-3,112 trp1Δ1 ura3-52 esa1Δ::HIS3 + pLP863 (esa1-414) | Clarke et al. 1999 |
| LPY4774 | W303 MATa esa1-414 | Clarke et al. 2006 |
| LPY4909 | W303 MATa rDNA::ADE2-CAN1 | Clarke et al. 2006 |
| LPY4911 | W303 MATa esa1-414 rDNA::ADE2-CAN1 | Clarke et al. 2006 |
| LPY4917 | W303 MATa TELVR::URA3 | Clarke et al. 2006 |
| LPY4919 | W303 MATa esa1-414 TELVR::URA3 | Clarke et al. 2006 |
| LPY5406 | W303 MATa nab3-10 rDNA::ADE2-CAN1 | Clarke et al. 2006 |
| LPY5407 | W303 MATa nab3-10 TELVR::URA3 | Clarke et al. 2006 |
| LPY10622 | W303 MATa nab3-10 | Chang and Pillus 2009 |
| LPY11286 | W303 MATa nab3-10 adh4::ADE2 TELVIIL | Chang and Pillus 2009 |
| LPY11300 | W303 MATa adh4::ADE2 TELVIIL | Chang and Pillus 2009 |
| LPY12154 | W303 MATa rpd3::kanMX | Chang and Pillus 2009 |
| LPY15000 | W303 MATa NAB3-2Flag::kanMX | Chang and Pillus 2009 |
| LPY15004 | W303 MATa esa1-414 NAB3-2Flag::kanMX | Chang and Pillus 2009 |

Except where noted, strains were constructed during the course of this study or are part of the standard lab collection.

although Esa1’s catalytic activity may not be its only essential role (Decker et al. 2008).

Suppression analyses have linked ESA1 to the deacetylase Sir2, a key silencing protein. Overexpression of Sir2 was found to suppress esal rDNA-silencing defects, thereby suggesting that Sir2 and Esa1 may function coordinate to silence the rDNA array (Clarke et al. 2006). Several other studies have identified additional suppressors of conditional alleles of esal (Biswa et al. 2008; Lin et al. 2008; Chang and Pillus 2009; Scott and Pillus 2010).

To pursue genetic interactors of ESA1, a dosage suppression screen was performed on an esal mutant. Of the four high-copy suppressors identified, NAB3 became a focus for two primary reasons. First, only NAB3 overexpression rescued both the temperature-sensitivity and the silencing defects of esal mutants. Second, NAB3 has known roles in RNA processing, and this functional connection to Esa1 may establish a novel link between two nuclear processes. Numerous studies have characterized roles for Nab3 and its binding partner Nrd1 in 3’-end processing of several classes of small noncoding RNAs [reviewed in Lykke-Andersen and Jensen (2007)]. These classes of RNAs include small-nuclear (sn) RNAs, small-nucleolar (sno) RNAs, and cryptic unstable transcripts (CUT). Nab3 and Nrd1 each recognize specific RNA sequences for 3’-end formation and transcription termination (Carroll et al. 2004).

This study reports new mutant phenotypes of nab3, revealing roles for Nab3 in rDNA silencing, the DNA damage response, and cell-cycle progression. Further, Nab3 was found to be posttranslationally modified by acetylation. This acetylation was reduced in an esal conditional mutant that displays reduced Esa1 acetyltransferase activity, providing evidence that Nab3 is a nonhistone substrate of Esa1 whose function may be influenced by acetylation.

MATERIALS AND METHODS

Dosage suppressor screen

A URA3-marked 2μ genomic library (generously provided by P. Hieter) was transformed into two isolates of the esa1-414 strain LPY3291 in six independent experiments, yielding a total of 130,000 transformants with an approximate 70-fold coverage of the genome. Transformants were grown under permissive conditions on SC-Trp-Ura plates, and then replica-plated and incubated at 28°, 35°, and 37°. Two hundred colonies were able to grow at 35° but not 37° (this was a secondary screen used to avoid recovering wild-type ESA1). These candidates were tested for plasmid dependence by growing original transformants on 5-fluoroorotic acid (5-FOA) plates. The resulting resistant strains, which had lost the URA3-marked plasmid, were tested for temperature sensitivity at 35°. This resulted in 34 suppressor strains being classified as plasmid-dependent. Suppressing plasmids were rescued from yeast, and inserts were sequenced using T3 and T7 primers. Of the 34 plasmids, 22 were WT ESA1, 3 were unidentified, and the remaining 9 comprised six independent clones containing one of the four following genes: LYS20, NAB3, VAP1, or LEU2. Library fragments that contained multiple ORFs were dissected by subcloning to identify the gene responsible for suppression. Strategy for identification of the four suppressors is described in detail (Clarke 2001). The plasmid subclones were retransformed into LPY3291 to confirm the suppressing phenotype.

Yeast methods and strain and plasmid construction

All yeast strains and plasmids used in this study are listed in Tables 1 and 2. The silencing markers rDNA::ADE2-CAN1 (Fritze et al. 1997) and TELVR::URA3 (Renauld et al. 1993) were introduced through standard genetic crosses. All nab3-10 strains originate from YPN100 (provided by M. Swanson) (Conrad et al. 2000). Nab3 Flag-tagging was carried out by amplification of pFA6a-2FLAG-kanMX6 and transformation into LPY5 (W303-1a) using the method described (Longtine et al. 1998) to make LPY15000. All library plasmids are in the pRS202 (pLP1402) backbone. pLP1238 (NAB3 in pRS202) and pLP2018 (NAB3 in pRS426) were subcloned from pLP1419 (NAB3 library construct) using EcoRI and XhoI. pLP1310 (NAB3 in pLP271) was subcloned from pLP1419 using EcoRI. Dilution assays for growth, silencing, and drug sensitivity were performed as described (Chang and Pillus 2009) and represent 5-fold serial dilutions starting from an A600 of 1.0. Images were captured after 2–4 days of growth at the indicated temperatures.

Northern analysis, protein immunoblots, and immunoprecipitations

RNA was isolated using the hot acid phenol protocol as described (Collart and Oliviero 2001). Northern blotting was performed as described (Cox and Walter 1996), and results were obtained by phosphorimager (Storm, GE Healthcare). Yeast extracts were prepared by
and Rpd3), and transferred to nitrocellulose (0.2 m). Primary antisera were anti-H4K12Ac (Serotec), anti-H4K16Ac (Upstate), anti-Sir2 (Garcia and Pillus 2002), anti-FLAG (Sigma-Aldrich, #9681), and anti-acetyl-lysine (Cell Signaling, #9681). Secondary antibodies conjugated to horseradish peroxidase in combination with chemiluminescence reagents were used for detection on film. FLAG-Nab3 was immunoprecipitated with anti-FLAG M2 Affinity Gel (Sigma-Aldrich, A2220), eluted in SDS sample buffer, separated on a SDS-polyacrylamide gel, and immunoblotted with either anti-FLAG or anti-acetyl lysine. All experiments were performed in triplicate or more and a representative blot was chosen for quantification. Quantification of all immunoblots was performed with ImageQuant software.

**Nab3 and Sir2 immunofluorescence**

Immunofluorescence was performed as described (Gotta et al. 1997; Stone et al. 2000). WT and esa1 strains were grown in YPD for four hours at either 28° or 37°. Cells were fixed by adding paraformaldehyde to the cultures at a final concentration of 3.3% at 30° for 10 min. Samples were washed twice in YPD, resuspended at 1 ml per 0.1 g of cells in 0.1 M EDTA, KOH pH 8.0, and 19 mM DTT, and then incubated at 30° for 10 min with gentle agitation. The primary antibodies used were anti-Nab3 (mouse monoclonal 2F12) (Wilson et al. 1994) and anti-Sir2 (Garcia and Pillus 2002). Texas Red-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit were used as secondary antibodies. Staining was visualized with an Applied Precision Delvaxis optical sectioning deconvolution microscope.

**Flow cytometry**

Cell-cycle profiles were obtained by flow cytometry of propidium iodide stained cells on a FACSCalibur machine (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson). Cells were grown to an A600 of between 0.6 and 1.0, fixed in ethanol overnight, and stained with propidium iodide. Stained cells were sonicated and then analyzed by flow cytometer. For each sample, 100,000 cells were counted and analyzed.

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### Table 2 Plasmids used in this study

| Plasmid (Alias) | Description | Source/Reference |
|-----------------|-------------|-----------------|
| pLP362 (pRS426) | Vector URA3 2μ | Sikorski and Hieter 1989 |
| pLP1402 (pRS202) | Library vector URA3 2μ | P. Hieter |
| pLP37 | SIR2 URA3 2μ | Clarke et al. 2006 |
| pLP271 | Vector TRP1 2μ | Clarke et al. 1999 |
| pLP796 | ESA1 URA3 2μ | Clarke et al. 1999 |
| pLP798 | ESA1 TRP1 2μ | Clarke et al. 1999 |
| pLP843 | esa1-414 TRP1 CEN | Clarke et al. 1999 |
| pLP1238 | NAB3 URA3 2μ | Clarke et al. 1999 |
| pLP1259 | VAP1 URA3 2μ | Clarke et al. 1999 |
| pLP1310 | NAB3 TRP1 2μ | Clarke et al. 1999 |
| pLP1412 | LYS20 URA3 2μ | Clarke et al. 1999 |
| pLP1405 | LYS20-library clone URA3 2μ | Clarke et al. 1999 |
| pLP1406 | VAP1-library clone URA3 2μ | Clarke et al. 1999 |
| pLP1417 | LEU2-library clone URA3 2μ | Clarke et al. 1999 |
| pLP1419 | NAB3-library clone URA3 2μ | Clarke et al. 1999 |
| pLP2018 | NAB3 URA3 2μ | Clarke et al. 1999 |
| pLP2054 | NRD1 URA3 2μ | Clarke et al. 1999 |

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### RESULTS

#### Four suppressors of the esa1 temperature-sensitive phenotype

To identify genes that interact functionally with ESA1, a dosage-suppressor screen was performed utilizing a genomic 2μ plasmid library. The esa1-414 temperature-sensitive strain was transformed with the library, and transformants were tested for growth at both permissive and restrictive temperatures. Plasmids were rescued from transformants that grew at the restrictive temperature to determine the identity of suppressing genomic fragments. The results of this analysis revealed four esa1 dosage suppressors: LEU2, LYS20, NAB3, and VAP1 (Figure 1). None of these suppressors bypassed the inviable esa1Δ. When tested with other previously characterized esa1 alleles (Clarke et al. 1999), some allele-specificity was observed (supplementing information, Table S1). The series of alleles was also tested for suppression of other esa1 phenotypes (see below).

**LEU2 and LYS20** are nonessential genes required for the biosynthesis of leucine and lysine [reviewed in Kohlhaw (2003) and Xu et al. (2006), respectively]. **VAP1** is also involved in amino acid metabolism, encoding a transporter of several amino acids, including tyrosine, treptophan, valine, and leucine (Schmidt et al. 1994). Characterization of **LYS20** as a suppressor of **esa1** revealed additional roles for this metabolic gene in DNA damage repair (Scott and Pillus 2010). **NAB3**, as noted, is an essential gene critical for 3’-end processing of nonpolyadenylated transcripts [reviewed in Lykke-Andersen and Jensen (2007)].

#### Increased dosage of NAB3 suppresses multiple esa1 mutant phenotypes

To understand the connection between the suppressors and **Esa1** function, overexpression of the four genes was tested for suppression of esa1 mutant defects other than temperature sensitivity. One phenotype of esa1 mutants is a strong rDNA-silencing defect and a slight increase in mitotic rDNA recombination (Clarke et al. 2006). Previously, it was shown that increased gene dosage of **SIR2** suppresses the esa1 rDNA-silencing defect (Clarke et al. 2006). Using an esa1 strain with the ADE2-CAN1 dual reporter integrated at a single 25S rDNA repeat (Fritze et al. 1997) (Figure 2A), the suppressors were
tested for their effect on silencing of the rDNA locus. Only increased dosage of NAB3 robustly suppressed the esa1 rDNA-silencing defect, restoring silencing to near wild-type levels (Figure 2B). By contrast, LYS20 slightly exacerbated esa1’s silencing defect, whereas LEU2 and VAP1 had little to no effect (Figure 2B). Unlike increased dosage of SIR2 in a wild-type strain (Smith et al. 1998b), NAB3 did not enhance wild-type rDNA silencing (Figure 2C). None of the suppressors had significant effects on rDNA recombination. As there appeared to be a link between NAB3 and ESA1 for both silencing and growth, we chose to characterize NAB3 in greater detail.

In addition to their rDNA-silencing defects, esa1 mutants are defective in telomeric silencing (Clarke et al. 2006) (Figure 3A), as shown by diminished growth on 5-FOA when using a URA3 reporter gene on the right arm of chromosome V (TELVR) (Renauld et al. 1993). Increased dosage of NAB3 in esa1 mutants allowed for increased growth on 5-FOA, thereby rescuing the sensitivity shown by the esa1 mutant (Figure 3A). Recent studies have shown that readout of this reporter-based assay for some genes may reflect changes in nucleotide metabolism instead of telomeric-silencing defects (Rossmann et al. 2011; Takahashi et al. 2011). Thus, based on these new studies, rescue of esa1’s 5-FOA sensitivity by NAB3 in strains carrying the URA3 telomeric reporter gene can be interpreted as the ability of NAB3 overexpression to suppress telomeric-silencing defects or nucleotide metabolism changes in an esa1 mutant. Because ESA1 has no known defects in HM silencing or mating efficiency (Clarke et al. 2006), NAB3 dosage was not tested for effects on mating efficiency.

Another phenotype of esa1 mutants is sensitivity to DNA damage induced by camptothecin, a topoisomerase I inhibitor that triggers double-strand breaks (Bird et al. 2002). NAB3 overexpression was tested for its ability to suppress this esa1 mutant defect in the DNA damage response and was found to exacerbate esa1’s camptothecin sensitivity (Figure 3B). This result is in contrast to NAB3-mediated suppression of esa1’s silencing defects, highlighting a difference between Nab3 and Esa1’s functions in transcriptional silencing and DNA damage repair.

At a molecular level, global H4 acetylation is dramatically reduced in esa1 mutants when grown at restrictive temperatures (Clarke et al. 1999). To determine whether increased dosage of NAB3 restores wild-type levels of histone acetylation to esa1 mutants, a series of protein immunoblots with isoform-specific antibodies was performed to define the global acetylation state in esa1 strains overexpressing NAB3.
All the histone H4 lysine residues that Esa1 is known to acetylate (K5, K8, K12, and K16) (Clarke et al. 1999) were tested in these experiments (Figure 3C). Total histone levels were determined by probing with a control antibody specific to the C-terminus of histone H3. This series of immunoblots shows that increased dosage of NAB3 in esa1 strains did not restore H4 acetylation. Therefore, NAB3 overexpression does not rescue esa1 mutants by restoring global acetylation defects at substrate residues in the H4 N-terminal tail.

A distinct potential mechanism for NAB3 suppression is through Esa1’s role in the cell cycle. Since Esa1 is required for cell-cycle progression through G2/M, cell-cycle profiles of esa1 mutant strains with increased dosage of NAB3 were examined by flow cytometry to distinguish cellular DNA content before (1C) and after (2C) replication. The esa1 mutants at restrictive temperature have a well-defined G2/M cell-cycle block, visualized as a decrease in the 1C peak and an accumulation of the 2C peak (Clarke et al. 1999). With NAB3 overexpression, no change in the esa1 cell-cycle profile was observed (Figure 3D), indicating that NAB3 overexpression does not bypass the G2/M cell-cycle block of esa1 mutants. Thus, increased dosage of NAB3 suppresses a defined subset of esa1 mutant phenotypes, which includes silencing defects and temperature sensitivity.

Nab3 does not affect protein or transcript levels of histone-modifying enzymes

In addition to their function for termination of noncoding RNAs, there is evidence that Nab3 and its partner Nrd1 participate in 3’-end formation of protein-coding transcripts (Sugimoto et al. 1995; Arigo et al. 2006a; Darby et al. 2012). We considered the possibility that Nab3 might bind to Esa1 mRNA to direct its 3’-end formation. Nab3 binding sites have the simple UCUU consensus sequence (Carroll et al. 2004) that is found at several positions within the Esa1 transcript. Northern blotting was performed to determine whether there were any NAB3-dependent changes in the Esa1 transcript. NAB3 is an essential gene (Wilson et al. 1994) and, thus, the temperature-sensitive 

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**Figure 3 Overexpression of NAB3 affects multiple esa1 mutant phenotypes.** (A) Top: Diagram of TELVR::URA3 telomeric silencing marker on the right arm of chromosome V. Bottom: Increased gene dosage of NAB3 suppresses the esa1 5-FOA sensitivity in this assay. An esa1 strain with the TELVR::URA3 reporter (LPY4919) was transformed with vector (pLP271), ESA1 (pLP798), or NAB3 (pLP1310), and plated on SC-Trp (growth) with and without 5-FOA (telomeric silencing) at 33°C. (B) Increased gene dosage of NAB3 exacerbates esa1’s sensitivity to the DNA damaging agent camptothecin. An esa1 strain (LPY4774) was transformed with vector (pLP326), ESA1 (pLP796), or NAB3 (pLP2018), and plated on SC-Ura with DMSO (growth) and 20 μg/ml camptothecin (DNA damage). (C) Overexpression of NAB3 does not increase global acetylation levels of H4K5, H4K8, H4K12, or H4K16 in esa1 mutants. Whole-cell extracts were made from wild-type (LPY5) and esa1 (LPY4774) strains with vector (pLP362) or 2μ NAB3 (pLP2018) grown in SC-Ura media at both permissive (28°C) and elevated (34°C) temperatures. These were immunoblotted for amounts of isospecific H4 acetylation and total H3. An H3 reprobe was performed for each individual H4 acetylation blot. Quantification data shown are normalized for H3 loading. (D) Overexpression of NAB3 does not influence esa1’s G2/M cell-cycle block. The same strains as in (C) were grown at 28° and shifted to 37° for 4 hr before fixing and staining with propidium iodide. Cell-cycle profiles were analyzed by flow cytometry.
Nab3 overexpression rescued esa1’s 5-FOA sensitivity (Figure 3A) likely results through an indirect mechanism. In contrast, when assayed for rDNA-silencing defects, nab3 mutants displayed a strong defect, similar to that observed in esa1 (Figure 5B). Together, these data suggest that Nab3 functions directly in rDNA silencing but not telomeric silencing.

NAB3 overexpression did not suppress the DNA damage and cell-cycle phenotypes of esa1 mutants (Figure 3, B and D). However, when nab3 mutants were examined for defects in DNA damage repair and cell-cycle progression, the results revealed a role for NAB3 in these processes. As seen in Figure 5C, nab3 mutants are sensitive to the topoisomerase I inhibitor camptothecin, although less so than esa1. Cell-cycle profiles of nab3 mutants also showed a G2/M block resembling that of esa1 mutants (Figure 5D). In addition to the defective rDNA silencing of nab3, the identification of these phenotypes for nab3 mutants reveals a more extensive functional overlap with esa1 mutants.

We earlier considered the possibility that a molecular link for Nab3 and Esa1 functions would be that Nab3 influences histone acetylation (Figure 3C). When tested for changes in acetylation of H4K5, the primary in vivo target of Esa1 (Clarke et al. 1999), global acetylation in nab3 mutants was maintained at wild-type levels (Figure 5E). Therefore, NAB3 does not directly influence the global histone acetylation activity of Esa1’s primary target.

Localization and posttranslational acetylation of Nab3 are altered in esa1 mutants

The nucleolus is a key compartment for RNA processing in the nucleus. Ultrastructural analysis has shown esa1 mutants to have aberrant nucleoli (Clarke et al. 1999), and esa1 mutants display strong rDNA-silencing defects and rDNA chromatin structure defects (Clarke et al. 2006). Because of these connections of Esa1 to nucleolar function and Nab3’s influence on rDNA silencing (Figure 5B), Nab3 localization was visualized in esa1 mutants. Immunofluorescence was performed using an antibody directed against Nab3 in wild-type and esa1 strains. In addition, Sir2 staining was used to demarcate the nucleolus.

Nab3 localization has been previously described as dispersed throughout the nucleus but distinct from nucleolar structure proteins (Wilson et al. 1994) (Figure 6A, top). At permissive temperatures, Nab3 localization appeared normal in both wild-type and esa1 cells. However, at restrictive temperature, Nab3 localization in esa1 became diffuse and no longer confined to the nucleolus as defined by DAPI staining (Figure 6A, middle), indicating that Nab3 localization is altered in the esa1 mutant. Sir2 staining was also affected in the esa1 mutant and no longer found in discrete nucleolar and telomeric foci, although Sir2 protein expression appeared essentially normal at elevated temperature (Figure 4C). Nab3 protein levels were also found to be equal by immunoblot between wild-type and esa1 cells at both permissive and restrictive temperatures (Figure 6A, bottom).

Because WT levels of Nab3 were observed in esa1 mutants, whereas simple overexpression suppressed esa1 phenotypes, we considered the possibility that the mutants, Nab3 protein differs not quantitatively but qualitatively. One such qualitative difference could be at the level of its posttranslational modification. We tested the idea that Nab3 might itself be an in vivo substrate for Esa1, a possibility first raised by a proteomics survey suggesting that Esa1 could acetylate Nab3 in vitro (Lin et al. 2009). To examine whether this modification
and the Sen1 helicase. This Nab3 complex ensures proper termination and 3'-end interactions, nuclear localization and posttranslational acetylation of mutants, displaying defects in rDNA silencing, cell-cycle progression, and defects in rDNA silencing. Thus, suppression of these defects is expected if Esa1 acetylates Nab3 directly or indirectly (Figure 6B) to provide a more likely explanation for the dosage suppression observed between NAB3 and NRD1. In accordance with these tightly linked functions, Nab3 and Nrd1 form a heterodimer (Carroll et al. 2000). In accordance with these tightly linked functions of Nab3 and Nrd1, we found that overexpression of NRD1 also suppresses some esa1 mutant phenotypes (Figure S2). Because genetic suppression by NRD1 was less dramatic than that by NAB3, our focus in this study was on NAB3's genetic interaction with Esa1, but our observations with NRD1 support the idea that suppression is mediated by Nab3 in the context of the Nab3-Nrd1-Sen1 complex, and not via an independent role of Nab3 alone.

Because the ESA1 transcript was unchanged in the nab3-10 mutant (Figure 4A), this implies that the Nab3-Nrd1-Sen1 complex does not direct 3'-end termination of the ESA1 transcript. It should be noted that our study was restricted to this loss-of-function nab3-10 mutation. Thus, considering the genetic limitations of studying essential genes such as NAB3, we cannot fully eliminate the possibility that the Nab3 complex processes the ESA1 transcript, as we have not studied multiple mutant alleles of NAB3. However, we consider our in vivo data showing that Nab3 acetylation is influenced by Esa1 either directly or indirectly (Figure 6B) to provide a more likely explanation for the dosage suppression observed between Esa1 and Nab3. Consistent with these data, one potential model for the suppression is that Esa1 acetylation of Nab3 influences its function such that the reduced Nab3 acetylation in esa1 mutants results in its reduced cell viability and defects in rDNA silencing. Thus, suppression of these defects is
obtained in the esa1 mutant by overexpressing NAB3 to compensate for the decreased pools of acetylated Nab3.

In S. cerevisiae, the rDNA is a repetitive array in the genome that is mainly transcribed by Pol I and Pol III. Reporter genes that are transcribed by Pol II and inserted in the array are known to undergo Sir2-mediated transcriptional silencing. An endogenous Pol II transcript has been detected in the “nontranscribed” spacer region (NTS1) of the rDNA. This transcript is a CUT that is processed by the Nab3 complex and degraded by the exosome (Housey et al. 2007; Vasiljeva et al. 2008). In addition to uncovering an rDNA-silencing defect for nab3 mutants (Figure 5B), we observed that overexpression of NAB3 rescued the rDNA-silencing defects of esa1 mutants (Figure 2). Esa1 binding is enriched at the rDNA, and histone acetylation at the rDNA is reduced in the esa1 mutant (Clarke et al. 2006). Although Nab3 does not appear nucleolar by immunofluorescence (Wilson et al. 1994) (Figure 6A), a recent study found that Nab3 localizes to the rDNA via chromatin immunoprecipitation (Lepore and Lafontaine 2011). Thus, one possibility is that Nab3 recruitment to the CUTs within the rDNA is regulated by its acetylation status through Esa1 activity. Future studies will establish how Esa1 functions with the Nab3-Nrd1 complex in contributing to transcriptional silencing at the rDNA.

The number of nonhistone proteins known to be acetylated by Esa1 and the MYST family of KATs has expanded in recent years. Several schools of thought exist about the function of this posttranslational modification. In parallel with the models for histone acetylation, acetylation of nonhistone proteins may change the activity of these proteins or may serve as a recruitment platform for physical binding of other proteins [reviewed in Sapountzi and Côté (2011)]. Our finding that Nab3 is acetylated in vivo raises several possibilities regarding the function of this posttranslational modification. Whereas Nab3 acetylation is reduced in an esa1 mutant, overall levels of Nab3 remain constant (Figure 6). Therefore, it is unlikely that acetylation affects Nab3 stability but, rather, that it influences its activity or function. Knowing that Nab3 is aberrantly localized in the esa1 mutant, one possible scenario is that acetylation of Nab3 by Esa1 promotes proper Nab3 nuclear localization.

In contrast to NAB3, the other three suppressors identified in our dosage-suppression screen (LEU2, LYS20, and VAP1) are all involved in amino acid metabolism. A separate study defined the connections between LYS20 and ESA1 through DNA repair that could be distinguished from Lys20’s role in amino acid biosynthesis, potentially through a noncanonical role in acetylation (Scott and Pillus 2010). Recent findings report the prevalence of lysine acetylation as a posttranslational modification in the regulation of metabolic proteins in mammals (Zhao et al. 2010). In light of these studies and ours, it is possible that Esa1 acetylates the protein products of the genes we identified as dosage suppressors. Only Nab3, and not the other suppressors, was identified as a substrate in the in vitro proteomics study (Lin et al. 2009). However, a number of other metabolic enzymes were found, including the gluconeogenic enzyme Pck1 that is reciprocally deacetylated by Sir2, providing a link to our earlier suppression studies between ESA1 and SIR2 (Clarke et al. 2006). One potential explanation for our current findings of dosage suppression of esa1 by LEU2 and VAPI is that Leu2 and Vap1 are acetylated by Esa1 in vivo. Future studies to determine in vivo Esa1 targets of nonhistone proteins will shed light on additional substrates and their functions.

Although it has been assumed that Esa1’s catalytic activity is its essential activity, it is unclear exactly why esa1Δ strains are inviable. One recent study found that an Esa1 strain bearing a mutation in a residue important for catalysis retained viability, proposing that there may be more to the essential nature of Esa1 than its histone acetyltransferase activity (Decker et al. 2008). Given that our screen highlights a strong genetic interaction between ESA1 and the essential gene NAB3, along with several genes encoding metabolic proteins (LEU2, VAPI, LYS20), one of Esa1’s essential functions may be the recognition and acetylation of important nonhistone substrates.

Suppressor analysis is a widely used strategy that facilitates the identification of functional relationships between different proteins. A recent investigation of hundreds of dosage suppressors in yeast revealed that dosage suppression provides functional links between two genes (Magtanong et al. 2011). In addition, dosage suppression

Figure 6 Nab3 localization and acetylation is altered in esa1 cells. (A) Nab3 localization is aberrant in the esa1 mutant. Top: At a permissive temperature (28°C), Nab3 staining in wild type (LPY4909) and esa1 (LPY4911) cells appears as punctate nuclear foci interspersed with diffuse nuclear staining. Sir2 localization demarcates the nucleolus in a crescent shape (inset, green) and is normal. At a restrictive temperature (37°C), Nab3 staining is diffuse in the esa1 mutant but appears normal in the wild-type strain. No Sir2 foci are observed in the esa1 mutant. Bottom: WT and esa1 strains used above were grown at permissive and elevated temperatures and used for immunoblotting to detect total Nab3 levels using anti-Nab3. Anti-PGK1 (phosphoglycerate kinase) was used as a loading control. (B) Nab3 is acetylated in vivo in an ESA1-dependent manner. To detect posttranslational acetylation of Nab3, a WT (LPY15000) and esa1 (LPY15004) strain containing a chromosomal FLAG-tagged version of Nab3 were grown at an elevated temperature (37°C) and used in an anti-FLAG immunoprecipitation followed by an immunoblot with anti-acetyl lysine. Decreased levels of Nab3 acetylation is observed in the esa1 mutant. Quantiﬁcation of ﬁlms from independent experiments shows a 48% decrease in Nab3 acetylation in esa1 compared with wild-type. An untagged WT strain (LPY5) is used as a negative control. Nab3-FLAG levels are not them-
can identify unique interactions that are not discovered through other types of genome-wide studies, such as protein-protein and synthetic sickness interactions. In our study, genetic suppression has provided an effective platform for identifying and characterizing potential new substrates for an enzyme primarily studied as an acetyltransferase targeting histones.

ACKNOWLEDGMENTS

We thank M. Swanson for the nab3-10 strain and the Nab3 antibody, and E. Steinmetz, D. Brow, and J. Corden for nrd1 strains and inNBR1 plasmids. M. Grunstein provided the Rpd3 antibody, and T. Johnson, for the 2μ genomic library; J. DuRose and M. Niwa for assistance with northern analysis; M. Busse for help with Sir2 westerns; J. Feramisco and S. McMullen for assistance with deconvolution microscopy; A. Castillo and M. Winey for help with the dosage suppressor screen; the David Lab for access to its FACS instrument; and L. Clark, R. Garza, R. Otsuka, and A. L. Torres Machorro for technical assistance. We thank members of the Pillus lab for helpful advice throughout the course of this study, and A. L. Torres Machorro for critical reading of the manuscript. This work was supported by National Institutes of Health grants GM-56469, GM-90177, and T32-GM-007240.

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Communicating editor: J. Rine