Eaf3 Chromodomain Interaction with Methylated H3-K36 Links Histone Deacetylation to Pol II Elongation

Short Article

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Summary

Eaf3, a component of the NuA4 histone acetylase and Rpd3 histone deacetylase complexes, is important for the global pattern of histone acetylation in *Saccharomyces cerevisiae*. Preferential deacetylation of coding regions requires the Eaf3 chromodomain and H3-K36 methylation by Set2. The Eaf3 chromodomain interacts with methylated H3-K36 peptides, suggesting that this interaction leads to preferential association and histone deacetylation of the 3′ portions of coding regions by the Rpd3 complex. However, the Eaf3 chromodomain and H3-K36 methylation do not significantly affect acetylation at promoters, suggesting that Eaf3 has a distinct function, presumably in the NuA4 complex. Lastly, Eaf3 inhibits internal initiation within mRNA coding regions in a manner similar to FACT and Spt6. Our results link the pattern of preferential deacetylation at coding regions to the underlying patterns of H3-K36 methylation and phosphorylation of the RNA polymerase II C-terminal domain, and ultimately to the mechanism by which repressive chromatin structure is restored after transcriptional elongation.

Introduction

Histone modifications do not occur randomly throughout eukaryotic genomes but rather occur in patterns that represent underlying molecular mechanisms and are used to decode the information in DNA in the context of chromatin. In the yeast *Saccharomyces cerevisiae*, H3-K4, -K36, and -K79 methylation are mediated, respectively, by the Set1, Set2, and Dot1 histone methylases, and all of these methylation events are mechanistically connected to the process of transcriptional elongation by RNA polymerase II (Pol II) (Sims et al., 2004). The Paf1 complex, which travels with elongating Pol II, recruits Set1 and Set2 to active coding regions. The Paf1 complex is required for genome-wide H3-K4 and H3-K79 methylation and for elevated levels of H3-K36 methylation in coding regions. Recruitment of Set1 and Set2 to active mRNA coding regions depends on the specific phosphorylation status of the C-terminal domain of Pol II at serines 5 and 2, respectively. As the phosphorylation pattern changes as Pol II transcribes along the gene, H3-K4 methylation (particularly trimethylation) is restricted to the 5′ portion of coding regions, whereas H3-K36 methylation is biased toward the 3′ portion.

Histone acetylation across the *S. cerevisiae* genome is also not uniform but rather occurs in patterns with underlying mechanisms (Pokholok et al., 2005; Reid et al., 2004; Robyr et al., 2002). Histone acetylases and deacetylases can be recruited to specific promoters by interaction with DNA bound transcriptional activators or repressors, thereby causing promoter-localized regions of acetylation or deacetylation. In addition, histone acetylases and deacetylases also act in an untargeted manner. Wild-type yeast cells have a distinct pattern of global H3 and H4 acetylation in which promoter regions are typically more acetylated than their corresponding coding regions.

Eaf3, a subunit of the NuA4 histone acetylase and Rpd3 histone deacetylase complexes, plays a critical role in controlling the pattern of histone acetylation that distinguishes promoters from coding regions (Reid et al., 2004). In the absence of Eaf3, H3 and H4 acetylation increases at coding regions and decreases at promoters such that histone acetylation levels become evenly distributed across the genome. In *S. cerevisiae*, Eaf3 is not required for cell growth, and its role in transcription is quantitatively modest and limited to a small subset of genes. Eaf3 contains several structural motifs, the most notable of which is chromodomain, a structural domain that can bind methylated lysines in histones (Brehm et al., 2004).

The mechanism by which Eaf3 affects the global pattern of histone acetylation is unknown. We previously suggested the possibility that the Eaf3 chromodomain, through an interaction with H3-K36, might lead to preferential association of Rpd3 histone deacetylase with coding regions, leading to preferential deacetylation (Reid et al., 2004). This suggestion was based on the observation that histone deacetylation, like H3-K36 methylation, is biased toward the 3′ portion of coding regions. Alternatively, we also suggested that Eaf3 might be important for preferential association of the NuA4 complex with promoters. However, there is no experimental evidence for either of these suggestions, and it is also unknown if Eaf3-mediated control of the histone acetylation pattern occurs by a unifying mechanism or is a combination of multiple mechanisms.

Here, we provide genetic and biochemical evidence that Eaf3 mediates preferential deacetylation of coding regions by virtue of an interaction between the Eaf3 chromodomain and methylated H3-K36 that presumably results in preferential association of the Rpd3 complex. Loss of Eaf3 results in internal initiation within mRNA coding regions in a manner similar to loss of FACT or Spt6 (Kaplan et al., 2003; Mason and Struhl, 2003). The pattern of preferential deacetylation at coding regions is determined by the underlying pattern of H3-K36 methylation, which in turn is determined by the underlying pattern of phosphorylation of the Pol II C-terminal domain. As such, Eaf3 is ultimately linked to the mechanism by which repressive chromatin structure is restored after transcriptional elongation.
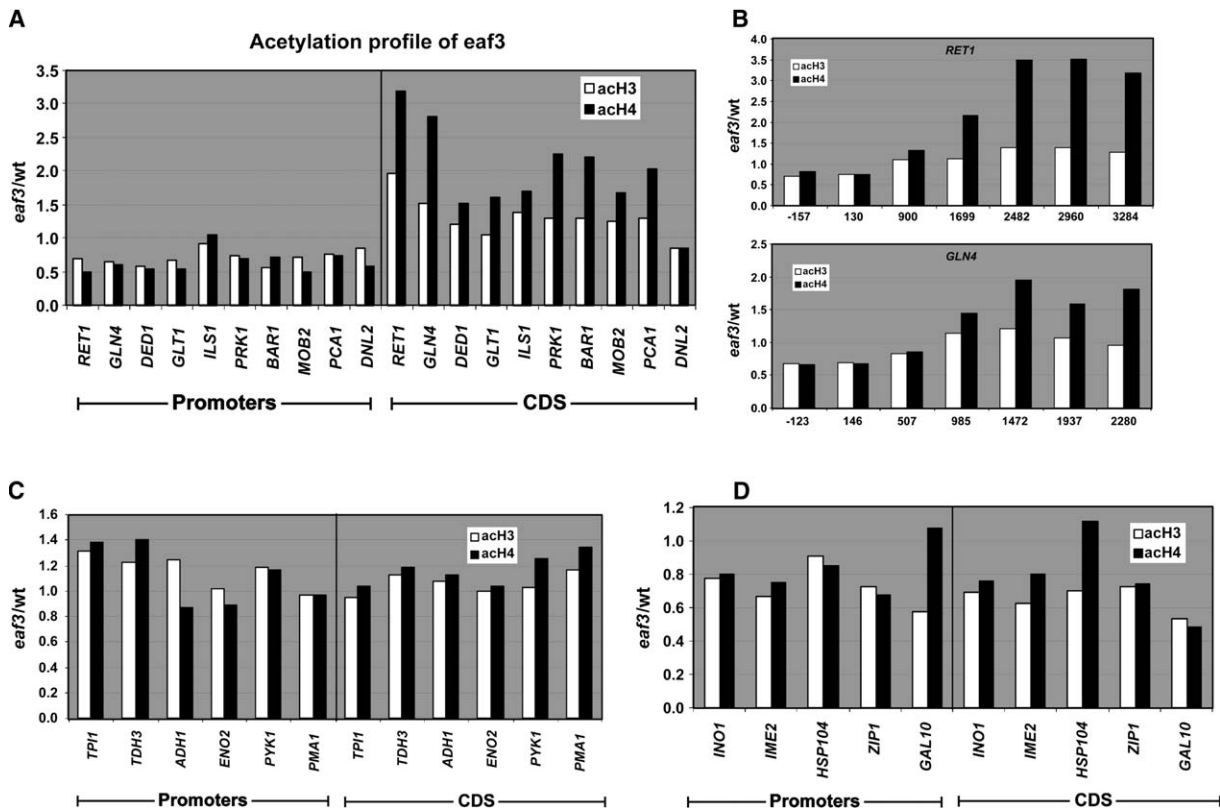
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A

Acetylation profile of eaf3

B

C

D

Figure 1. Effect of Eaf3 on Histone H3 and H4 Acetylation at Promoters and Coding Regions

Data are expressed as the ratios of H3 (white bars) and H4 (black bars) acetylation levels in the *eaf3* deletion strain versus the wild-type strain at the indicated locations. (A) Promoters and coding sequences of randomly selected genes. (B) Positions (with respect to the ATG codon) within the *RET1* and *GLN4* genes. (C) Promoters and coding sequences of very highly expressed genes. (D) Promoters and coding sequences of inactive or repressed genes.

Results

The Eaf3-Dependent Effect on Histone Acetylation Occurs on Many Genes, but Not Those that Are Very Highly Transcribed or Strongly Repressed

We extended our previous observation that Eaf3 is important for the pattern of histone acetylation (Reid et al., 2004) by examining more genomic regions and by normalizing histone acetylation levels to histone density. Consistent with previous results, the *eaf3* deletion strain shows a modest increase in acetylation at the coding sequences of most randomly selected genes (Figure 1A), with this increase being more pronounced for H4 (1.5- to 3-fold) than for H3 (up to 2-fold). Also as expected, mapping experiments on *RET1* and *GLN4* show that increased acetylation in the *eaf3* strain is significantly biased toward the 3′ end of the coding region (Figure 1B). Conversely, most promoter regions show reduced H3 and H4 acetylation in the *eaf3* strain.

Our previous analysis suggested that the Eaf3-dependent effect was largely independent of transcriptional activity, but it did not investigate many genes that were either very highly expressed or strongly repressed. Analysis of six of the most highly active genes indicates that loss of Eaf3 did not result in increased acetylation of coding regions (Figure 1C). In the wild-type strain, these highly active genes do not exhibit the typical pattern of histone acetylation but rather display high levels of acetylation throughout the entire gene. Conversely, Eaf3 does not affect the pattern of histone acetylation of five repressed/inactive genes (Figure 1D). For these repressed/inactive genes, levels of histone acetylation were extremely low at the promoters and coding regions when compared to typical genes. Taken together, our results indicate that the Eaf3-dependent effect occurs at most promoters over a wide range of transcriptional activities but that it requires a minimal level of transcriptional activity and is overridden by very high levels of transcription.

Set2-Mediated Methylation of H3-K36 Is Required for Preferential Deacetylation of Coding Regions

We previously noted that the pattern of histone acetylation (high at promoters and low at coding regions with a 3′ bias) is inversely related to the pattern of H3-K36 methylation, and we suggested that Eaf3 might mediate preferential Rpd3-dependent deacetylation through an interaction with H3-K36 (Reid et al., 2004). Strikingly, the pattern of histone acetylation in the strain lacking Set2 histone methylase is similar to the pattern observed in the *eaf3* strain. Specifically, the *set2* mutant exhibits increased acetylation of H4 (Figure 2A) and H3 (Figure 2B) in the coding regions, with a larger effect on H4, and the pattern of increased H4 acetylation closely resembled that shown by the *eaf3* mutant. Unlike the *eaf3* deletion strain, the *set2* strain does show
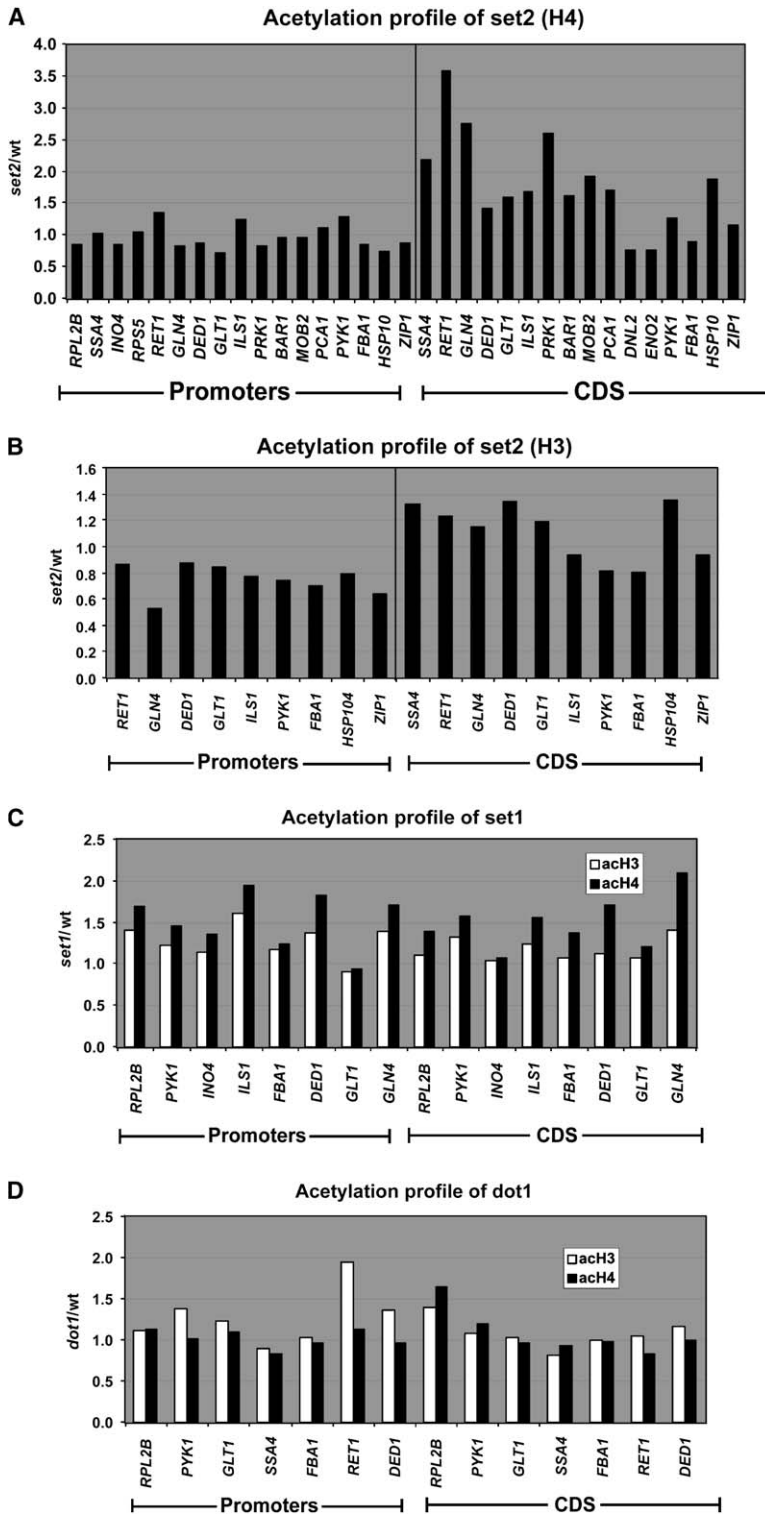
A

B

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Figure 2. Set2, but Not Set1 or Dot1, Is Required for Preferential Histone Deacetylation at Coding Regions

Data are expressed as the ratios of H3 and H4 acetylation levels in the indicated deletion strain versus the wild-type strain at the indicated promoters and coding sequences. (A) Effect of Set2 on H4 acetylation levels. (B) Effect of Set2 on H3 acetylation levels. (C) Effect of Set1 on H3 and H4 acetylation levels. (D) Effect of Dot1 on H3 and H4 acetylation levels.

decreased H3 or H4 acetylation of the promoters tested. In contrast to the *set2* deletion strain, histone acetylation of the promoter and coding regions tested is unaffected by loss of Set1 (Figure 2C) or Dot1 (Figure 2D). Thus, Set2 histone methylase, and presumably H3-K36, is important to mediate the global pattern of histone acetylation in a manner related to Eaf3.

The Eaf3 Chromodomain Is Important for Preferential Deacetylation of Coding Regions

Eaf3 contains both a chromodomain, a structural motif that interacts with methylated lysine residues on histones and RNA (Brehm et al., 2004), and a chromoshadow domain (Bertram and Pereira-Smith, 2000). To address the functional role of these domains, we analyzed Eaf3

A

Eaf3 structure diagram with Chromo domain (77-120) and CSD (322-377)
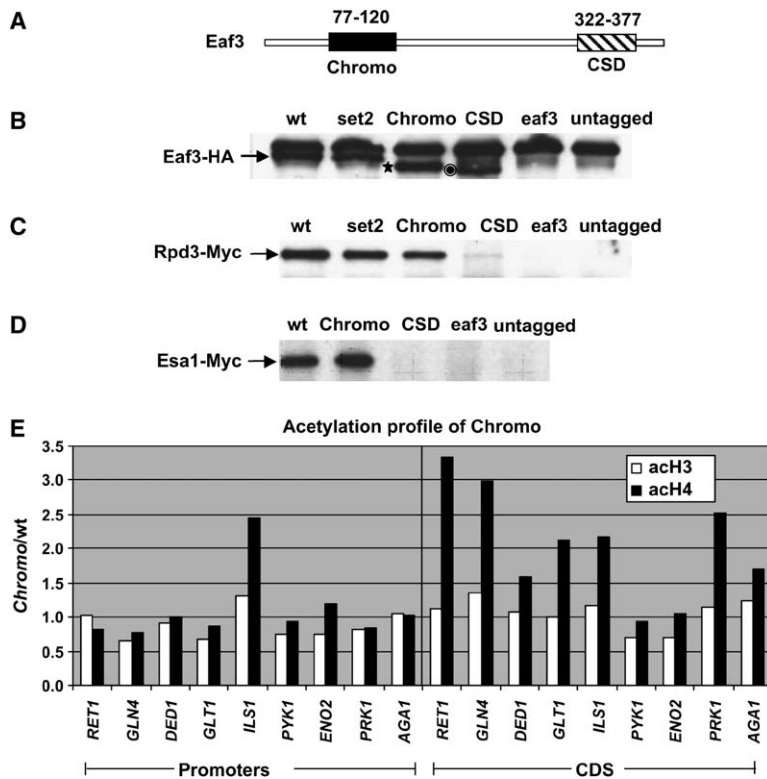
B

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E

Figure 3. The Eaf3 Chromodomain Is Required for Preferential Histone Deacetylation at Coding Regions

(A) Structure of Eaf3 and mutant derivatives with the chromodomain and chromoshadow domains indicated.

(B) Cell extracts from a wild-type (wt) or *set2* deletion strain expressing wild-type Eaf3-HA or derivatives lacking the chromodomain (chromo), chromoshadow domain (CSD), or HA tag (untagged) or an *eaf3* deletion strain and also expressing Rpd3-Myc were immunoprecipitated with an antibody against the HA epitope. The resulting immunoprecipitates were analyzed by Western blotting with the antibody against the HA epitope.

(C) Same as (B) except that the Western blot was analyzed with antibodies against the Myc epitope.

(D) Same as (C) except that the strains contained Esa1-Myc instead of Rpd3-Myc.

(E) Ratios of H3 and H4 acetylation levels in the strain deleted for the Eaf3 chromodomain versus the wt strain at the indicated promoters and coding sequences.

derivatives that cleanly delete either of these domains (Figure 3A). The Eaf3 derivative lacking the chromodomain is expressed at wild-type levels, whereas the derivative lacking the chromoshadow domain is expressed at only 10% of the wild-type level (Figure 3B). Coimmunoprecipitation experiments show that both Rpd3 (Figure 3C) and Esa1 (Figure 3D) associate with wild-type HA-Eaf3 to comparable extents in a wild-type and *set2* mutant strain. Furthermore, wild-type Eaf3 and the derivative lacking the chromodomain behave similarly with respect to their ability to coimmunoprecipitate either Esa1 or Rpd3. Thus, Set2 and the Eaf3 chromodomain do not affect the structural integrity of the NuA4 or Rpd3 complexes, even though they play a critical role in Eaf3 function.

The strain containing the Eaf3 derivative lacking the chromodomain has a similar but not identical acetylation profile as *eaf3* and *set2* deletion strains (Figure 3E). Specifically, the coding regions of most genes tested exhibit an increase in H4 acetylation (range 1.5- to 3-fold), whereas the very highly transcribed *PYK1* and *ENO2* genes are unaffected. Thus, the Eaf3 chromodomain and Set2 are required for the Eaf3-mediated effect on histone acetylation at coding regions. In contrast, the strain lacking the chromodomain does not display a significant increase in H3 acetylation, and the decrease in H3 and H4 acetylation levels at promoters (12% of which is at the margin of significance) is less apparent than the reduction in the *eaf3* strain (ranges between 30% and 60%).

Chromodomain-Dependent Binding of Eaf3 to Methylated H3-K36 Peptides

The experiments above indicate that Eaf3 and Set2 function in the same pathway that results in deacetylation of coding regions and that the Eaf3 chromodomain is important for this function. As chromodomains bind to methylated lysines in histones, the simplest model is that Eaf3 (in the context of the Rpd3 complex) associates with H3-K36, thereby causing preferential histone deacetylation of 3′ portions of coding regions. To test this hypothesis, we coupled a variety of modified and unmodified histone peptides to beads and incubated the resulting material with a mixed cell-free extract that was generated by mixing equal numbers of cells containing the wild-type and chromodomain-deleted Eaf3 derivative. This approach permits an internally controlled comparison between the wild-type and chromodomain-deleted Eaf3 derivatives and is much more suited for detecting small differences in association.

The wild-type Eaf3 derivative interacts detectably with mono-, di-, and trimethylated H3-K36 peptides and with the trimethylated H3-K4 peptide (Figure 4A). Importantly, beads containing these methylated peptides preferentially associate with complexes containing full-length Eaf3 as compared to the derivative lacking the chromodomain. Preferential Eaf3 chromodomain-dependent association with these methylated peptides is quantitatively modest. In contrast, the wild-type and chromodomain-deleted Eaf3 complexes interact poorly with the unmodified H3 peptide as well as other modified H3 peptides, including those trimethylated at lysine 9 or dimethylated at lysine 79 (Figure 4A). As removal of the chromodomain does not compromise the integrity of Eaf3-containing complexes, this result suggests that the Eaf3 chromodomain interacts directly with methylated H3-K36 and methylated H3-K4 but not with methylated H3-K9 or H3-K79.
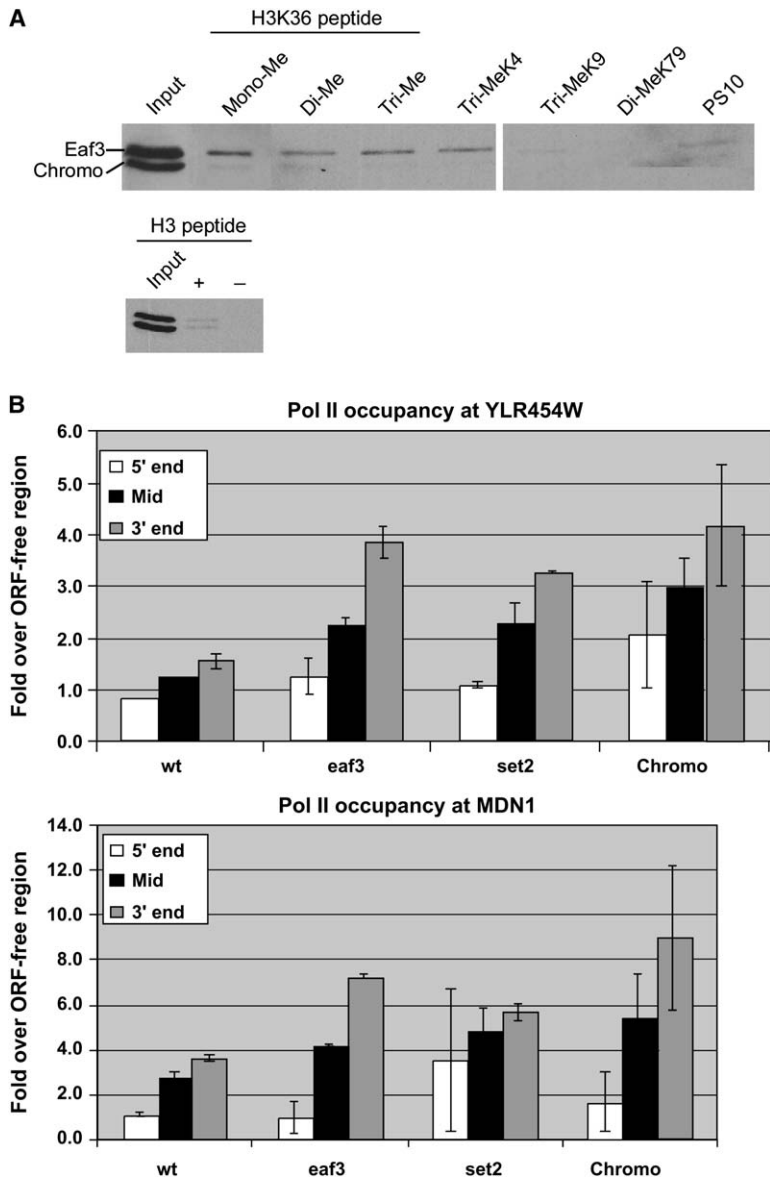
A

Figure 4. Eaf3 Chromodomain Interacts with Methylated H3-K36 Peptides

(A) A mixed cell-free extract containing equal numbers of cells expressing the wt and chromodomain-deleted Eaf3 derivative were incubated with beads containing the indicated H3 peptides. The input samples and associated products were analyzed using anti-HA antibody to simultaneously detect full-length and mutant Eaf3 protein (positions indicated) in each sample.

(B) Pol II association at the indicated regions (defined with respect to the transcriptional initiation site) of *YLR454* and *MDN1* in wild-type and indicated mutant strains. Data are presented as the average of three independent experiments along with the SEM.

B

Eaf3 Is Important to Inhibit Transcriptional Initiation within mRNA Coding Regions

H3-K36 methylation is linked to phosphorylation of the C-terminal domain of Pol II and the process of transcriptional elongation. Although the connection of histone deacetylation within coding regions to transcriptional elongation was unexpected, it is critical that cells have a mechanism to restore normal chromatin structure after disruption upon passage of Pol II. In this regard, the FACT and Spt6 complexes inhibit inappropriate initiation within protein-coding regions, presumably as part of restoring normal chromatin structure upon transcriptional elongation (Kaplan et al., 2003; Mason and Struhl, 2003). To address whether the Eaf3 chromodomain and Set2 play a related role in restoring repressive chromatin structure after the passage of elongating Pol II, we examined Pol II density at two genes (*YLR454* and *MDN1*) that show internal initiation in the absence of FACT (Mason and Struhl, 2003) and presumably Spt6. As observed for FACT-deleted cells, loss of Eaf3, Set2, or the Eaf3

chromodomain does not significantly affect Pol II occupancy at the *YLR454* and *MDN1* promoter regions, but it does confer increased Pol II occupancy at the coding regions, with a stronger effect at a more downstream position (Figure 4B). These observations indicate that the Eaf3 chromodomain, Set2-mediated methylation of H3-K36, and preferential deacetylation of coding regions are important to inhibit inappropriate initiation within these (and presumably other) coding regions.

Discussion

Interaction between the Eaf3 Chromodomain and Methylated H3-K36 Mediates Preferential Histone Deacetylation of Coding Regions

In principle, Eaf3 could control the pattern of histone acetylation in the context of the NuA4 histone acetylase complex and/or the Rpd3 histone deacetylase complex. Here, we show that loss of Set2 histone methylase, and hence H3-K36 methylation, or deletion of the Eaf3

chromodomain results in increased histone acetylation at many coding regions in a manner indistinguishable from that observed in an *eaf3* deletion strain. In addition, we show a chromodomain-dependent interaction of Eaf3 with methylated H3-K36 peptides in vitro. Thus, the Eaf3 chromodomain and methylated H3-K36, and presumably the interaction between them, are critical for preferential histone deacetylation of many coding regions.

Taken together, our observations suggest a simple model in which Rpd3 histone deacetylase complex preferentially associates with coding regions via an interaction between the Eaf3 chromodomain and methylated H3-K36. As a consequence of this preferential association of the Rpd3 complex, coding regions become relatively deacetylated with respect to promoter regions. This model is strongly supported by the observation here (Figure 1B) and elsewhere (Reid et al., 2004) that Eaf3-mediated histone deacetylation is strongly biased to the 3′ portion of coding regions, because H3-K36 methylation is similarly biased to the 3′ portion of coding regions (Kizer et al., 2005; Krogan et al., 2003). This model also explains why coding regions of very poorly transcribed genes are not subjected to Eaf3-dependent deacetylation (Figure 1D), because H3-K36 methylation is linked to transcriptional elongation. Lastly, as targeted recruitment of the Rpd3 complex to promoters represses transcription (Kadosh and Struhl, 1997; Rundlett et al., 1998), recruitment of an Rpd3 complex via Set2-mediated methylation of H3-K36 can explain why artificial targeting of Set2 to promoters represses transcription (Strahl et al., 2002). This model has been experimentally supported by independent investigations, and a specific Rpd3 complex containing Eaf3 has been implicated in the process (Carrozza et al., 2005; Keogh et al., 2005).

Three experimental observations, though not inconsistent with the model, require additional explanation. First, although histone deacetylation by Rpd3 occurs with comparable efficiency on all four histones (Suka et al., 2001), Eaf3-dependent deacetylation at coding regions is more pronounced on H4 than on H3. One possibility is that this is related to the specificity of the antibodies (which were raised against tetraacetylated H4 and diacetylated H3) such that the relationship between quantitative ChIP results and quantitative histone acetylation status is not linear. Alternatively, the presence of an H3-specific acetylase in coding regions might partially mask the effects of Rpd3 on H3 but not on H4. Second, the coding regions of the most highly transcribed genes appear immune to Eaf3-dependent deacetylation. This observation might be explained by the rapid histone exchange that occurs at highly transcribed yeast genes (Lee et al., 2004; Schwabish and Struhl, 2004; Zhang et al., 2005) or, hypothetically, by high levels of histone acetylases associated with elongating Pol II that override the effect of Eaf3-dependent deacetylation. Third, as the Eaf3 chromodomain can interact with both methylated H3-K4 and H3-K36, it is unclear whether or how the Rpd3 and NuA4 complexes distinguish between these residues in vivo. Whatever the ultimate explanations for these observations, our results strongly suggest that interaction of the Eaf3 chromodomain with methylated H3-K36 represents an important mechanism for preferentially deacetylation of histones at many coding regions.

The Eaf3-Dependent Effect on Histone Acetylation at Promoters Occurs by a Distinct Mechanism

Although the above mechanism nicely explains preferential deacetylation of coding regions, it does not easily account for why Eaf3 is important for high levels of acetylation at promoters. In contrast to strains lacking Eaf3, strains lacking Set2 or the Eaf3 chromodomain have a limited (and perhaps no) effect on histone acetylation at promoters, suggesting that Eaf3-dependent effects at promoters and coding regions are mechanistically distinct. As Eaf3 positively affects histone acetylation at promoters, it seems likely that its function at promoters occurs in the context of the NuA4 acetylase complex rather than the Rpd3 histone deacetylase complex. Eaf3 might facilitate the interaction of NuA4 with DNA, which is preferentially accessible in many promoter regions due to low histone density (Bernstein et al., 2004; Lee et al., 2004; Sekinger et al., 2005; Yuan et al., 2005), or it might permit NuA4 to recognize some feature of chromatin that is distinct between promoters and coding regions. It is tempting to speculate that the Eaf3 chromodomain in the context of NuA4 preferentially associations with trimethylated H3-K4 because this modification is strongly biased to promoters and 5′ portions of coding regions (Ng et al., 2003; Santos-Rosa et al., 2002), but Eaf3-dependent acetylation of promoter regions is largely independent of the Eaf3 chromodomain. Nevertheless, the existence of Eaf3 in both the Rpd3 and NuA4 complexes provides a means to coordinately or independently regulate the patterns of histone acetylation at promoters and coding regions throughout the genome.

Coupling of Histone Deacetylation to Transcriptional Elongation

Our results indicate that histone deacetylation at coding regions is linked to H3-K36 methylation, which in turn is linked to phosphorylation of the C-terminal domain of Pol II and the process of transcriptional elongation. As such, the mechanistic coupling of histone deacetylation within coding regions to transcriptional elongation is in striking contrast to the usual relationship of histone acetylation, transcriptional activity, and active chromatin structure. However, transcriptional elongation is a dynamic process that involves histone eviction (or gross alteration of histone-DNA interactions) and subsequent histone deposition (Kristjuhan and Svejstrup, 2004; Lee et al., 2004; Schwabish and Struhl, 2004). The FACT and Spt6 complexes are important to restore normal chromatin structure upon transcriptional elongation, thereby preventing inappropriate initiation within protein-coding regions (Kaplan et al., 2003; Mason and Struhl, 2003). Here, we show that Eaf3, via its chromodomain, and Set2-mediated methylation of H3-K36 play a related role in restoring repressive chromatin structure after the passage of elongating Pol II. This role of Eaf3 and Set2-mediated methylation of H3-K36 in inhibiting internal initiation within coding regions is presumably less significant than the role of FACT and Spt6, because loss of Eaf3 or Set2 has a minimal effect on cell viability, whereas FACT and Spt6 are essential for cell growth. This role for Eaf3 and Set2 is unlikely to involve histone deposition or positioning but rather reduced accessibility of the genomic DNA in coding regions due to histone deacetylation.

Experimental Procedures

Yeast Strains
The wild-type (BY4741) and isogenic *eaf3*, *set2*, *set1*, and *dot1* deletion strains were obtained from Research Genetics. Eaf3 derivatives lacking the chromodomain (deletion of amino acid residues 77–120) and chromoshadow domain (deletion of amino acid residues 322–377) were generated by PCR mutagenesis and used to replace the wild-type *EAF3* gene in BY4741. Eaf3 from the wild-type and the chromodomain and chromoshadow domain mutation-bearing strains was tagged with the HA3 epitope. Rpd3 and Esa1 were tagged with the Myc3 epitope in separate wild-type and mutant strains to give doubly tagged strains. All epitope tagging was at the 3′ end of the genes and was done using plasmids described previously (Longtine et al., 1998).

Chromatin Immunoprecipitation
Formaldehyde-crosslinked chromatin was immunoprecipitated with antibodies against tetraacetylated (lysines 5, 8, 12, and 16) H4 (Upstate Biotechnology), diacetylated (lysines 9 and 14) H3 (Upstate Biotechnology), or Pol II (8WG16; Covance) and the resulting material analyzed by quantitative PCR analysis in real time (Aparicio et al., 2004; Reid et al., 2004). For each genomic region, the apparent immunoprecipitation efficiency was calculated by dividing the amount of PCR product obtained with the immunoprecipitated sample by the amount of PCR product obtained with the corresponding input sample. All immunoprecipitation efficiencies measured here were normalized to histone density, as determined by the H3 immunoprecipitation efficiency at the particular genomic location. All measurements of histone acetylation levels represent the average of at least three independent experiments, and the experimental error for individual determinations is ±25%.

Coimmunoprecipitation Experiments
Extracts from 10 ml cells containing an HA-tagged Eaf3 derivative and either Myc-tagged Esa1 or Myc-tagged Rpd3 were obtained by vortexing with glass beads four times for 30 s. Cell extracts in lysis buffer (50 mM Tris HCl [pH 7.4], 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin) were incubated with antibody against the HA epitope (Santa Cruz Biotechnology) for 1–2 hr at 4°C. The resulting immune complexes were collected by incubating with protein A Sepharose beads for 1 hr. Immunoprecipitates were washed five times for 5 min each with wash buffer (same as lysis buffer, but with 0.1% Triton X-100) and one time with phosphate-buffered saline at room temperature and then boiled with SDS-PAGE buffer.

Interaction of Eaf3 Derivatives with Histone Peptides
Histone peptides used for these analyses were obtained from Abcam, except for the unmodified H3 peptide (Upstate). The peptides were covalently coupled to a Sulfolink gel (Pierce) through their C-terminal cysteine residue, with the exception of the unmodified H3 peptide that was coupled to streptavidin Sepharose (Amersham Biosciences) via a biotin tag. Coupling of the histone peptides to respective resins were performed as per manufacturers' protocols. Cell extracts obtained from a mixed 25 ml culture (obtained by mixing equal amounts of cells from the wild-type and *eaf3* chromodomain deletions strains) were incubated with the peptide-coupled and control resins for 1–2 hr at room temperature, and bound complexes were washed as described above for coimmunoprecipitation experiments. Proteins interacting with histone peptides (or control resins) were then boiled with SDS-PAGE buffer.

Western Blotting
Immune complexes and peptide pulldown products obtained from equivalent amounts of cell extracts (except for the chromoshadow domain mutant) were eluted in 100 μl of SDS-PAGE buffer. One percent of the input and 25% of the immunoprecipitate/pulldown product were electrophoretically separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose filters. The filters were probed with antibodies against HA epitope (Santa Cruz Biotechnology) and Myc epitope (Upstate Biotechnology).

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