HDAC-mediated suppression of histone turnover promotes epigenetic stability of heterochromatin

Ozan Aygün, Sameet Mehta & Shiv I S Grewal

Heterochromatin causes epigenetic repression that can be transmitted through multiple cell divisions. However, the mechanisms underlying silencing and stability of heterochromatin are not fully understood. We show that heterochromatin differs from euchromatin in histone turnover and identify histone deacetylase (HDAC) Clr3 as a factor required for inhibiting histone turnover across heterochromatin domains in Schizosaccharomyces pombe. Loss of RNA-interference factors, Clr4 methyltransferase or HP1 proteins involved in HDAC localization causes increased histone turnover across pericentromeric domains. Clr3 also affects histone turnover at the silent mating-type region, where it can be recruited by alternative mechanisms acting in parallel to H3K9me–HP1. Notably, the JmjC-domain protein Epe1 promotes histone exchange, and loss of Epe1 suppresses both histone turnover and defects in heterochromatic silencing. Our results suggest that heterochromatin-silencing factors preclude histone turnover to promote silencing and inheritance of repressive chromatin.

In eukaryotic cells, genomic DNA is folded with histone and non-histone proteins to form discrete structural and functional chromatin domains. The genome is organized into euchromatin and heterochromatin domains by mechanisms that involve post-translational modifications of histones and remodeling of nucleosomes. Whereas heterochromatin is typically condensed and is generally inhibitory to the transcriptional machinery, euchromatin is less condensed and more readily transcribed. Hypoacetylation of histones is one hallmark that distinguishes heterochromatin from euchromatin, and it is believed to be a critical feature required for the assembly of repressive chromatin. Moreover, histone H3 lysine 9 (H3K9) is specifically methylated (H3K9me) in heterochromatic regions of the genome. H3K9me serves to recruit conserved HP1 protein family members. Heterochromatin plays an important part in the regulation of gene expression and also protects genome integrity by inhibiting unwanted recombination between repetitive DNA elements and facilitating proper segregation of chromosomes.

Studies using the fission yeast S. pombe have contributed greatly to the understanding of heterochromatin assembly and its biological significance. Whereas H3K9me and associated factors can be detected at several sites across the S. pombe genome, heterochromatin is preferentially enriched across large domains at centromeres, subtelomeres and the silent mating-type locus. At centromeres, pericentromeric regions containing tandem and inverted arrays of repeat and dh repeats are assembled into heterochromatin. Similarly, heterochromatin coats extended domains at subtelomeres as well as at a 20-kilobase (kb) domain at the mating-type region that includes the silent mating-type cassettes mat2 and mat3 and a centH element that shares strong homology with dg and dh repeats.

The dg and dh repeats and their homologous sequences are transcribed by RNA polymerase II (RNAPII), producing transcripts that are processed into short interfering RNAs (siRNAs) by RNA-interference (RNAi) factors, including Argonaute (Ago1), Dicer (Dcr1) and RNA-dependent RNA polymerase (Rdp1). The processing of repeat-derived transcripts by RNAi is coupled to the loading of heterochromatin proteins. siRNAs are bound by Ago1, a subunit of the RNA-induced transcriptional silencing (RITS) complex that is composed of two additional proteins: Chp1 and Tas3. The RITS-bound Ago1 assists in the localization of RITS to chromatin, which in turn is believed to target Clr4, a homolog of mammalian SUV39h that methylates H3K9 to nucleate heterochromatin. Additional RNAi-independent mechanisms nucleate heterochromatin at the mat locus and at centromeres. Once nucleated, heterochromatin spreads across domains defined by boundary DNA elements.

H3K9me facilitates the localization of chromodomain proteins required for diverse heterochromatin functions. Binding of the RITS subunit Chp1 to H3K9me is critical for the processing of heterochromatic-repeat transcripts. H3K9me also mediates recruitment of the HP1-family proteins Chp2 and Swi6, which in turn associate with factors involved in transcriptional gene silencing (TGS), such as the Snf2–HDAC repressor complex (SHREC) containing Clr3 HDAC, Asf1–HIRA histone chaperone and Clr6 HDAC complexes. The antisilencing factor Epe1 also associates with Swi6 and facilitates transcription of heterochromatic repeats. Although heterochromatin precludes RNAi accessibility at target loci, Epe1 counteracts these effects to allow RNAi transcription of centromeric repeats that is required to generate siRNA precursors. The exact mechanisms by which TGS effectors and Epe1 modulate heterochromatin are not fully understood.
The accessibility of DNA in eukaryotic genomes is largely determined by nucleosome stability\(^\text{34,35}\). In euchromatin, histone exchange at regulatory elements provides access to factors involved in transcription and other chromosomal processes\(^\text{36–39}\). However, heterochromatic sequences are generally inaccessible to trans-acting factors. TGS effectors such as SHREC and Asf1–HIRA have been shown to influence nucleosome occupancy at discrete sites within heterochromatin domains including pericentromeric regions and the silent mating-type region\(^\text{27,30,40}\). However, these localized changes in nucleosome occupancy that are restricted to specific sites cannot fully explain changes across extended domains that affect global expression patterns. A previous study reported that Clr4 and Swi6 affect histone dynamics at heterochromatic loci\(^\text{41}\). However, the relationship between nucleosome turnover and the epigenetic stability of heterochromatin was not explored. Moreover, whether heterochromatin-associated TGS effectors such as SHREC, which localize throughout heterochromatin domains, can preclude turnover of nucleosomes to assemble repressive chromatin domains was not examined.

To gain insight into the mechanisms by which heterochromatin factors mediate assembly and propagation of repressive chromatin, we investigated the relationship between the epigenetic stability of heterochromatin and replication-independent nucleosome turnover. Our detailed analyses using chromatin immunoprecipitation combined with microarray (ChIP-chip) show that heterochromatinsilencing machinery prevents histone exchange across large chromosomal domains at both centromeres and the silent mating-type region. Clr3 HDAC, a component of a TGS effector, which is targeted to heterochromatic loci by H3K9me–H1P1 or by alternative recruitment mechanisms, is required to inhibit histone turnover. We also demonstrate that Epe1 counteracts heterochromatic silencing by promoting nucleosome turnover. These results suggest that histone deacetylation, one of the most conserved features of silenced chromatin domains in eukaryotes from yeast to humans, promotes heterochromatin stability by inhibiting turnover of histones.

RESULTS

Hetero- and euchromatin domains differ in histone turnover

Nucleosome stability affects the accessibility of underlying DNA sequences\(^\text{34}\). We therefore wondered whether heterochromatin and euchromatin differ in the dynamic properties of nucleosomes within these domains. To measure histone turnover, we used S. pombe cells expressing C-terminal Flag-tagged histone H3 (H3-Flag) under the control of the inv1 promoter, which can be rapidly induced by shifting the growth-medium carbon source from glucose to sucrose\(^\text{42}\) (Fig. 1a). H3-Flag expression was induced after DNA replication was blocked by hydroxyurea (Fig. 1b,c). We found that the tagged histone H3 was incorporated into chromatin, as indicated by methylation of tagged H3 at lysine 4 (Supplementary Fig. 1b). Cross-linked chromatin was digested to mononucleosomes by micrococcal nuclease (MNase), followed by immunoprecipitation of Flag-tagged H3. DNA isolated from immunoprecipitated chromatin was subjected to microarray analyses using a custom tiling array that covers heterochromatic regions at 10-base-pair (bp) resolution and includes pericentromeric repeats, the silent mating-type region and \(\sim 225\) kb of a euchromatic region from chromosome 2. We observed widespread exchange of histones in euchromatic regions (Fig. 1d,e), consistent with results from budding yeast and Drosophila\(^\text{36–39}\). In particular, histone exchange at gene promoters was more prominent, whereas we measured relatively modest levels of H3 replacement across the bodies of genes (Supplementary Fig. 1a). Therefore, the low nucleosome occupancy at S. pombe promoters\(^\text{43}\) might reflect the intrinsic dynamic behavior of histones at these sites.

In contrast to euchromatin, the pericentromeric and silent mating-type regions are enriched in heterochromatin\(^\text{10}\) and showed lower histone-H3 replacement (Fig. 1d,e). These heterochromatin domains are largely occupied by nucleosomes, as indicated by the relatively few MNase-hypersensitive sites compared to those of euchromatic...
regions (Fig. 1d,e). We also detected H3 turnover at tRNA clusters and inverted-repeat heterochromatin boundaries at centromeres and the mat locus, respectively (Fig. 1d,e). The histone exchange at these sites might directly delimit the spread of heterochromatin, as suggested in other systems, or help to expose binding sites for trans-acting factors. Indeed, tRNAs and inverted repeats, which are refractory to heterochromatin, are bound by the transcription factor TFIIIC.

RNAi and Clr4 regulate histone exchange at centromeres

The marked differences in histone replacement across heterochromatin domains compared with euchromatic regions prompted us to ask whether the heterochromatin machinery prevents nucleosome turnover. As a first step, we investigated the effects of loss of Dcr1 and Clr4, which causes severe defects in heterochromatin assembly at centromeres. Deletion of either dcr1 or clr4 caused an increase in histone-H3 turnover across pericentromeric regions (Fig. 2a). The increase in histone turnover in mutants as compared to wild type was not due to variation between microarrays and could be reproduced in biological replicates. To quantify these differences, we compared normalized H3 turnover in each mutant to that in wild type by calculating the fold-enrichment values across pericentromeric heterochromatin relative to a euchromatic region (Supplementary Fig. 2). These analyses revealed a more than two-fold increase in histone turnover in dcr1 or clr4 mutants as compared to wild type. The observed changes extended beyond the dg and dh repeats and included the entire heterochromatin-coated domain (Fig. 2a). Notably, the elevated histone exchange throughout pericentromeric regions in clr4Δ cells could be detected readily within 15 min after H3-Flag induction (Supplementary Fig. 3b). Because H3-Flag is barely detectable at this time point (Supplementary Fig. 3a), the increased H3 exchange in heterochromatin-defective mutants was probably not due to H3-Flag overexpression. The observed differences in histone turnover in wild-type and mutant cells were not attributable to gross changes in histone occupancy in hydroxyurea-treated cells, as revealed by ChIP-chip analyses of endogenous H3 distributed across pericentromeric regions (Supplementary Fig. 3c). Moreover, the normalization of H3 turnover to total H3 measured under conditions used to induce H3-Flag expression revealed that the increased histone exchange in dcr1Δ and clr4Δ is not due to increased H3 occupancy (Supplementary Fig. 4a,b). As expected, neither dcr1Δ nor clr4Δ showed a major increase in H3 replacement at euchromatic regions (Supplementary Fig. 5).

Because S. pombe cells spend the majority of their vegetative life cycle in the G2 phase of the cell cycle, we wondered whether the impact of clr4Δ on H3 exchange could be recapitulated in G2-arrested cells. Loss of Clr4 also resulted in a marked increase in H3 turnover when H3-Flag was expressed in cells blocked at the G2-M boundary (Supplementary Fig. 6). Together, these results suggest involvement of heterochromatin-assembly factors in the suppression of histone replacement across pericentromeric domains.

Heterochromatin and histone exchange at the mat locus

Heterochromatin at the mat region is nucleated by redundant mechanisms that, in addition to RNAi, involve sequence-specific DNA-binding factors. We asked whether RNAi plays a part in the control of histone replacement across the silent mating-type region. Consistent with previous results showing that RNAi is dispensable for the maintenance of heterochromatin at the mat locus, the loss of Dcr1 had little or no effect on nucleosome replacement at this region (Fig. 2b).

Figure 2 Clr4 and RNAi are required to suppress H3 replacement at centromeres but not at the silent mating-type locus. (a) H3 replacement across the right pericentromeric region of cen2 in clr4Δ, dcr1Δ or wild-type (WT) cells, measured by MNase-ChIP-chip as in Figure 1. (b) H3 replacement across the silent mating-type region in clr4Δ or dcr1Δ, as in a.
Figure 4 Clr3 HDAC is required for suppression of histone-H3 exchange across heterochromatin domains. (a,b) Histone-H3 replacement, measured across the silent mating-type region (a) and pericentromeric heterochromatin (b) in clr3Δ or wild-type cells, as in Figure 1.

We next investigated the effect of loss of Clr4, which is essential for H3K9 methylation across the silent mating-type region. In contrast to a previous observation that Clr4 affects histone replacement across the entire silent mating-type region, our analyses did not detect a domain-wide increase in nucleosome turnover across this region in clr4Δ cells (Fig. 2b). This may be due to differences in the resolution of the techniques used in the two studies. Whereas previous work compared the levels of histone deposition at heterochromatic regions with those at a reference euchromatic region by using conventional ChIP, we measured H3 turnover by using high-resolution tiling microarrays. Notably, loss of Clr4 also did not cause a major increase in H3 exchange in G2-arrested cells at the mat region (Supplementary Fig. 6c). These observations are consistent with the finding that loss of Clr4 alone has only a minor effect on the silencing of endogenous mat2 and mat3 loci but is required for the spreading of repressive chromatin and the silencing of reporter genes artificially inserted in this region.

HP1 proteins cooperate to control histone exchange

The HP1-family proteins Chp2 and Swi6, which bind methylated H3K9, are enriched across heterochromatin domains and perform overlapping functions in transcriptional gene silencing. Whereas the chp2Δ or swi6Δ single mutants display modestly affected expression of target loci, the chp2Δ swi6Δ double mutant exhibits a severe loss of heterochromatic silencing at centromeres. To explore whether HP1 proteins have a role in preventing histone turnover, we analyzed histone-H3 exchange in the single- and double-HP1-mutant cells (Fig. 3). The loss of Chp2 or Swi6 alone slightly increased histone replacement at pericentromeric regions (Fig. 3a). However, the double mutant that lacks both of these factors showed a more than two-fold increase in H3 replacement, comparable with that observed in clr4Δ or dcr1Δ cells (Supplementary Fig. 2 and Fig. 3a). In contrast, loss of either or both of the HP1 proteins only modestly altered H3 replacement at the silent mating-type region (Fig. 3b). Together, these observations suggest that HP1 proteins mediate the downstream effects of the Clr4 methyltransferase to suppress histone exchange at pericentromeric regions. However, as with Clr4 loss, loss of these proteins has little effect at the mat locus, which raises the possibility that factors working independently of the H3K9me–HP1 pathway compensate to preclude histone turnover at this domain.

Clr3 HDAC is required for suppression of histone turnover

HP1 proteins might directly suppress histone exchange at heterochromatic loci, but H3K9me–HP1 also serves as a platform for recruitment as well as for spreading of effectors involved in transcriptional silencing. In light of the observations that Clr4 and HP1 proteins have modest effects on histone turnover at the silent mating-type region (Figs. 2b and 3b), we wondered whether TGS effectors targeted by HP1-dependent or HP1-independent mechanisms are critical for preventing histone turnover. Indeed, although Clr4 and HP1 are essential for localization of SHREC across pericentromeric regions, the components of SHREC, including Clr3 HDAC, are targeted to the silent mat region by additional recruitment mechanisms. Therefore tested whether Clr3 is involved in precluding changes in RNAPII transcription. (a) Histone-H3 replacement (blue) measured across the mating-type locus by MNase-ChIP-Chip analysis as described in Figure 1. RNAPII occupancy (ChIP versus input), measured by ChIP-Chip in clr3Δ, clr4Δ or wild-type cells, is shown plotted in alignment with the map (red). Values presented are relative enrichments (linear scale). (b) Reverse-transcription PCR (RT-PCR) analysis, performed on total RNA samples isolated from clr3Δ, clr4Δ or wild-type cells. Genomic DNA (gDNA) is shown as a control. The locations amplified by primer pairs (49, 51, 65 and 70 (labeled in parentheses) are highlighted with red shading (primer references are in Online Methods). Heterochromatin and euchromatin portions of the mating-type region are indicated at top.
Histone turnover, which may also explain the differential effects of Clr4 and HP1 on nucleosome replacement at centromeres and the mat locus. Notably, clr3Δ cells showed a domain-wide increase in histone turnover both at the silent mating-type region and the pericentromeric domains (Fig. 4a,b). The increase in H3 turnover correlated with defective silencing (Supplementary Fig. 7)\(^2\)\(^6\)\(^2\)\(^7\) and supports a functional connection between histone replacement and heterochromatic silencing.

**Heterochromatin and cotranscriptional histone exchange**

Given the potential impact of RNA Pol II transcription on histone replacement\(^4\)\(^6\)\(^4\)\(^–\)\(^8\)\(^4\), we considered that the increase in H3 turnover in the clr3Δ mutant might be linked to elevated RNA Pol II transcription of heterochromatic sequences. However, we found that high histone-H3 turnover within the silent mating-type region were observed in clr3Δ even at sites that show no detectable increase in RNA Pol II transcription (Fig. 5a,b). This result suggests that changes in H3 replacement across the heterochromatin domain cannot solely be explained by transcription-coupled turnover of nucleosomes.

**Epe1 stimulates histone turnover across heterochromatin**

As mentioned previously, the JmjC domain–containing antisilencing factor Epe1 binds Swi6 and counteracts heterochromatic silencing by Clr3 (refs. 31–33,49). Because JmjC proteins have been shown to demethylate histones\(^49\), Epe1 could function as a histone demethylase. However, no such activity has been detected\(^49\). Considering that the Clr3 HDAC is required for inhibiting turnover of nucleosomes at heterochromatic loci (this study) and that Epe1 genetically interacts with Clr3 (ref. 31), we wondered whether Epe1 promotes nucleosome turnover. To test this hypothesis, we first investigated the effects of epe1Δ on histone replacement in ago1Δ cells. Similar to dcr1Δ, ago1Δ cells showed an approximately two-fold increase in H3 replacement across the pericentromeric heterochromatin domain.

**Histone turnover and epigenetic stability of heterochromatin**

A remarkable feature of heterochromatin is that the silenced chromatin state can be propagated through multiple cell divisions\(^2\)\(^1\). Previous analyses have shown that replacement of a portion of the region between the silent mating-type cassettes (referred to as the K region), which includes the cenH heterochromatin nucleation center, with ura4\(^+\) (KA:: ura4\(^+\)) results in a metastable locus\(^5\)\(^2\). Cells containing KA:: ura4\(^+\) display alternate silenced (ura4\(^-\)) and expressed (ura4\(^+\)) epigenetic states. This variegation is due to defects in de novo heterochromatin assembly in KA:: ura4\(^+\) (ref. 21). Once established, however, the heterochromatic state is stably inherited in cis\(^2\)\(^1\). Notably, the cis inheritance of heterochromatin requires binding of Clr4 to H3K9me4 through its chromodomain\(^1\(^7\). These studies suggest that pre-existing methylated H3K9 provides the initial binding site for Clr4 to establish a feedback loop for the clonal propagation of heterochromatin. This model predicts that factors that prevent the loss of methylated histones would be essential for heterochromatin maintenance. We therefore investigated whether cells carrying ura4\(^-\)off and ura4\(^+\)on epigenetic states differ in the Clr3 association and turnover of histones at this locus (Fig. 7). ChIP experiments revealed that ura4\(^-\)off cells show considerable enrichment of Clr3 at the KA:: ura4\(^+\) locus (Fig. 7a). More importantly, nucleosome turnover was suppressed at the silent mating-type region of ura4\(^-\)off cells (Fig. 7b). In contrast, the levels of Clr3 associated with this region were lower in ura4\(^+\)on cells, and these cells showed higher histone replacement (Fig. 7a,b). The increase in histone replacement in ura4\(^-\)on cells, as compared with ura4\(^-\)off cells, correlated with the reduction in H3K9me at KA:: ura4\(^+\) (Fig. 7c). Together with the observations that loss of Clr3 or the transient treatment of cells with an HDAC inhibitor affects the maintenance of preassembled heterochromatin at the mat locus\(^2\)\(^6\)\(^5\)\(^3\), these results suggest that the intrinsic nature of heterochromatin to prevent nucleosome turnover through activities such as Clr3 might be linked to the stable propagation of these structures.
DISCUSSION

The heterochromatin assembly pathway is highly conserved in eukaryotes from fission yeast to humans. The repressive heterochromatin defined by methylation of H3K9 and associated HP1 proteins possesses the ability to spread across chromosomes and to be epigenetically transmitted through mitotic and meiotic cell divisions. HP1 proteins recruit various effectors including chromatin-modifying activities that modulate chromatin structure. In S. pombe, the loss of HP1 proteins and their associated activities causes defects in nucleosome occupancy and defective heterochromatic silencing. However, changes in steady-state nucleosome patterns are restricted to a few discrete sites, and the mechanism for global repression across heterochromatin domains has remained unclear.

Our results define a critical feature that differentiates heterochromatin from euchromatin and also provide important insights into transcriptional repression and the propagation of heterochromatin domains. We found that the Ctr3 HDAC, which can be targeted by multiple dependencies, depending upon the chromosomal context, is required to suppress histone turnover across heterochromatin. Loss of Ctr3 or factors involved in its localization, such as Ctr4, affect acetylation of histones at heterochromatic loci (Supplementary Fig. 8). Therefore, deacetylation of a combination of histone residues may prevent histone turnover by nucleosome-remodeling factors that require acetylated histones. In addition, Ctr3 affects the subnuclear localization of certain target loci, and this spatial reorganization correlates with changes in chromatin structure. Therefore, it is possible that Ctr3 may also affect nucleosome dynamics by modulating genome organization.

RNAi components and Ctr4, which recruit HP1 proteins, also affected histone turnover (Fig. 2). Oligomerization of HP1 bound to H3K9me may help bridge nucleosomes to prevent histone exchange. Alternatively, the effects of H3K9me–HP1 might be mediated through associated effectors such as SHREC. Notably, the loss of Ctr4 or HP1 has little effect on histone turnover at the mat region, where HDACs can be recruited by alternative mechanisms. Other factors are also likely to affect histone turnover, such as Sp6, a chromatin-assembly factor that affects heterochromatic silencing. Asf1–HIRA facilitates histone deacetylation by HDAC Clr6, which together with Ctr3 is essential for hypoacetylation of histones at heterochromatic loci. Because Asf1–HIRA also associates with HP1, the nucleosome turnover observed upon loss of Ctr4 or RNAi most probably reflects the cumulative effects of defective localization of SHREC and other effectors. Indeed, histone exchange levels observed at the centromeres of ctr4Δ cells are higher than those of ctr3Δ cells (Figs. 2a and 4b and Supplementary Fig. 2), which suggests additional functions for Ctr4 and/or its associated factors in this process.

How does histone turnover affect epigenetic stability? The retention of histones decorated with H3K9me is predicted to be critical for the recruitment of Clr4 through its chromodomain to modify newly assembled nucleosomes, thus promoting the parental histone-modification pattern and clonal propagation of heterochromatin in cis. We note that Ctr3 is essential for suppression of nucleosome turnover as well as for transmission of epigenetic information and propagation of heterochromatin. Nucleosome stability also affects the accessibility of the underlying DNA sequences to the transcription machinery, and the increased histone exchange in heterochromatin mutants may provide access to trans-acting factors, including transcriptional machinery, that manifest in loss of silencing. In this respect, heterochromatin partially resembles the chromatin at open reading frames of genes in which the suppression of histone exchange prevents activation.

Figure 7 Ctr3-dependent suppression of histone turnover correlates with epigenetic stability of heterochromatin. (a) ChIP analysis of Ctr3 localization at the silent mat region. Left, ChIP DNA from strains expressing myc-tagged Ctr3 in KΔ:ura4D+ ura4+ or ura4-off state, analyzed by semiquantitative competitive PCR using primers that amplify both full-length KΔ:ura4D+ and endogenous mini-ura4D+ (ura4D/E) as internal control. Right, relative enrichments determined by calculating the ratio of the band intensities of [ChIP/ChIP/ChIP][Input ChIP/ChIP/ChIP]. Results were confirmed by quantitative real-time PCR (qPCR). Relative enrichment of KΔ:ura4D+ was normalized against untagged negative control, and the mean enrichment is shown. Error bars, s.e.m. (n = 3). (b) H3 replacement measured in KΔ:ura4D+ ura4+ or ura4-off cells. The endogenous ura4D+ was deleted in the strains used. (c) ChIP analysis of H3K9me2 levels at KΔ:ura4D+ ura4-on cells. Experiments were performed with the same strains as in a. H3K9me2 levels were confirmed by qPCR and the mean enrichment is shown (right). Error bars, s.e.m. (n = 4).

Figure 8 Model showing effects of factors that affect the epigenetic stability of heterochromatin. HDAC recruited by HP1 or other mechanisms is required to suppress nucleosome turnover and promote epigenetic stability of heterochromatin. In contrast, Epe1, which also associates with Swi6 (HP1), stimulates histone exchange. The balance between these opposing activities that affect nucleosome turnover may underlie the epigenetic switch between ‘off’ and ‘on’ states.
of transcription from cryptic promoters. Indeed, factors such as Asf1–HIRA and Ctr6 HDAC affect both heterochromatic silencing and suppress cryptic promoters within gene

Our analyses also revealed that the JmjC protein Epe1, which counteracts the silencing effects of HDACs, promotes histone turnover. The loss of Epe1 suppressed not only the elevated histone H3 exchange present in RNAi mutants but also the pericentromeric silencing (Fig. 6). In contrast, overexpression of Epe1 increased histone replacement across heterochromatin domains and impaired silencing at these loci. These observations provide a notable functional link between histone turnover and heterochromatin silencing and argue that JmjC proteins, in addition to their roles in demethylation of histones, may remodel chromatin by affecting histone turnover

The increased turnover of histones promoted by Epe1 may facilitate low-level transcription of heterochromatic repeats, which is necessary to produce precursors for siRNAs required for heterochromatin assembly. Epe1 has also been shown to remodel heterochromatin at specific loci in response to signals that induce sexual differentiation, such as Polycomb, have been found to associate with HDACs, and notably, these activities map to regions exhibiting low histone turnover. Therefore, it is possible that the inhibition of histone turnover by distinct silencing effectors will emerge as a unifying theme for maintaining epigenetic memory in all systems. Future investigations into the mechanisms that regulate histone turnover are expected to shed light on the dynamic control of heterochromatin and the organization of the genome into distinct chromatin domains.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession code. Microarray data have been deposited in the GEO database under accession code GSE45378.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

We are thankful to J. Barrowman for editing the manuscript, K. Zhang, K. Yamane and E. Luk for discussions, members of the Grewal laboratory for their help and P. Russell (Scripps Research Institute, La Jolla, California, USA) for the gift of pINV1 plasmid. This work is supported by the Intramural Research Program of the US National Institutes of Health, National Cancer Institute. O.A. was supported by a European Molecular Biology Organization long-term post-doctoral fellowship.

AUTHOR CONTRIBUTIONS

O.A. and S.I.S.G. designed research, O.A. performed all experiments, S.M. helped with microarray probe design, and O.A. and S.I.S.G. analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
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ONLINE METHODS

Strains and plasmids. To express C-terminal Flag-tagged histone H3 (H3-Flag), the hht2 gene open reading frame was cloned between NdeI and NotI sites of the pFN1 plasmid. The Flag epo sequence was added to the C terminus of hht2 by inclusion on the reverse primer during the PCR amplification. The resulting pFN1-H3-2-Flag plasmid was transformed into strains with the indicated genetic backgrounds. The strain overexpressing epe1 contains the full strength nmt1 promoter integrated immediately upstream of the endogenous Epe1 promoter. All strains are MatM-Smt0 mating type except for KA::ura4+, which are h6 mating type. The endogenous 1.8-kb ura4+ locus is deleted in the strains containing KA::ura4+, KA::ura4- cells were recovered in medium containing 5-FOA.

MNaSe-ChiP. Each experiment presented was reproduced with at least two biological replicates. For each MNaSe-ChiP experiment, fresh cells were grown on EMM-LEU agar plates for 3 d at 30 °C. Cells were inoculated into 330 ml EMM-LEU + 8% glucose at a density of OD600 ~0.02 and grown overnight at 30 °C with shaking at 250 r.p.m. Cell synchronization by DNA replication arrest was started when cells reached OD600 ~0.1–0.15 (mid-log phase) by adding sterile hydroxyurea (HU) at a final concentration of 15 mM. Cells were synchronized by growing for 4 h at 30 °C with shaking at 250 r.p.m. (except for KA::ura4+, clr3Δ, chp2Δ and swi6Δ chp2Δ cells, which were synchronized by adding 20 mM HU and growing for 5 h at 30 °C). For nmt-epe1 cells, synchronization was optimized by incubating cells in 15 mM HU for 4.5 h. Cells were pelleted at 24 °C and washed twice with 25 ml of EMM-LEU-glucose + 4% sucrose containing 15 mM HU (except for KA::ura4+, clr3Δ, chp2Δ and swi6Δ chp2Δ cells, where the concentration of HU was kept at 20 mM throughout the experiment). After the last wash, cells were inoculated into 315 ml EMM-LEU-glucose + 4% sucrose containing 15 mM HU and grown for 2 h at 30 °C with shaking at 250 r.p.m. to induce the expression of H3-Flag. Finally, cells were cross-linked for 20 min at room temperature by adding 1% formaldehyde.

For MNaSe-ChiP experiments, cells were resuspended in 400 µl of ChIP cell lysis buffer (50 mM HEPES KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton and 0.1% deoxycholate) supplemented with EDTA-free protease-inhibitor cocktail (Roche). Cell lysis was performed with a Mini-BeadBeater-8 (BioSpec) with lysis buffer (50 mM HEPES KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton, 1% deoxycholate, 0.5% NP-40, 0.5% deoxycholate and 1 mM EDTA) and once with TE buffer (50 mM Tris-HCl, pH 8.0, and 10 mM EDTA) at 4 °C. ChIP-DNA was eluted in 200 µl elution buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 100 mM NaHCO3 and 1% SDS) by incubating at 65 °C for 30 min with constant agitation. RNase A (40 µg; Invitrogen) was added to each eluate, and cross-linking was reversed in input samples and DNA by incubating for 12 h at 65 °C. Finally, samples were treated with 20 µg Proteinase K (Invitrogen) for 1 h at 37 °C, and DNA was purified by using Qiagen PCR purification kits and spin columns.

Microarray analysis of MNaSe-ChiP and ChIP experiments. MNaSe-ChiP and ChIP samples were competitively hybridized with their respective input samples. Amplification, labeling, hybridization and analysis of the microarray experiments were performed as described in ref. 10, by using the high-resolution tiling microarray platform as described in ref. 30. Microarray data analysis was performed by using standard median normalization as described in ref. 10.

Nucleosome occupancy. Nucleosome occupancy was measured as described in ref. 30, and the data from the indicated study were plotted across centromere 2 and the mating-type region. For histone H3 occupancy after HU synchronization, a standard ChIP procedure was performed using anti–histone H3 antibody (Active Motif, cat. no. 39163 and Abcam, cat. no. ab1791). Three microliters of each antibody were added per ChIP experiment.

RNAP II occupancy. RNAP II occupancy was measured by using ChIP-chip analysis as described28 with 8WG6 antibody (Covance, cat. no. MMS-126R). Five microliters of antibody was used per ChIP experiment.

Fluorescence-activated cell-sorting analysis. Cells were fixed with 70% ethanol at the indicated time points of the experiment (Fig. 1a and data not shown). Cells were washed twice with 50 mM sodium citrate (dibasic) solution and incubated with 0.1 µg/µl RNase A in 50 mM sodium citrate solution for 8 h at 37 °C. Cells were stained with SytoxGreen (2 mM final concentration in 50 mM sodium citrate), and DNA content was analyzed by using a BD FACSCalibur flow cytometer. BD Cell Quest Pro software was used for raw data acquisition, and the Flowjo program was used for final data analysis. FACS analysis was performed for confirmation of all G1-arrested strains (data not shown).

RNA isolation and RT-PCR analysis. Total RNA was isolated from exponentially growing cultures of the indicated strains by using Epicentre MasterPure Yeast RNA purification kit. RT-PCR experiments were performed with 100 ng of total RNA and 28 amplification cycles, using Qiagen one-step RT-PCR kit and following the manufacturer’s instructions. The primer set PROA13: GAAAACACATCGTGTTCCTGAGAGG and PROA14: CGTTCTGTAGCTGCTAGTGAAG was used for the amplification of centromeric repeats (dh-centi), and the primer set PROA7: GAAAATCCCATGGACGGCAG and PROA8: CAATTTCAGCTTCGCGGTAG was used for the amplification of the act1 locus. The primer sets 49, 51, 53, 65 and 70 used for the silent mating-type region (Fig. 7b and Supplementary Fig. 7c) are described in ref. 12.

Western blotting. A total of 15 µl of cells (OD600 ~0.2–0.28) grown in medium containing either glucose or sucrose were collected and frozen in liquid nitrogen. Proteins were extracted by using the trichloroacetic acid (TCA) precipitation method. Briefly, cells were resuspended in 200 µl of 20% TCA and mixed with 400 µl acid-washed glass disruption beads. After bead-beating for 3 min with the Mini-Bead-Beater-8 (BioSpec), lysates were collected by washing the beads with 400 µl of 5% TCA. The resulting 600 µl lysate was centrifuged at 16,060g for 10 min at 4 °C, and supernatant was discarded. Precipitated proteins were dissolved in 2× NuPAGE sample buffer (Invitrogen) and analyzed by NuPAGE 12% protein gels, followed by western blotting with anti-Flag antibody (Sigma, cat. no. F7425) at 1:1,000 dilution.

To investigate H3K4 methylation of ectopically expressed H3-Flag protein, cells were resuspended in 500 µl of ChIP cell lysis buffer (50 mM HEPES KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton and 0.1% deoxycholate) supplemented by protease-inhibitor cocktail (Roche). Cell lysis was performed with the Mini-Bead-Beater-8 (BioSpec), using 1.25 µl of zirconia-silica beads (0.5-mm diameter, BioSpec) and beating at full power once for 3 min at 4 °C. Cell lysate was filtered into fresh tubes by washing the disruption beads with an additional 500 µl of ChIP cell lysis buffer. After a brief sonication at 4 °C, the lysate was cleared by centrifugation at 16,060g for 10 min at 4 °C, and supernatant was discarded. Precipitated proteins were dissolved in 2× NuPAGE sample buffer (Invitrogen) and analyzed by NuPAGE 12% protein gels, followed by western blotting with anti-Flag antibody (Sigma, cat. no. F7425) at 1:1,000 dilution.