The 19S proteasome is directly involved in the regulation of heterochromatin spreading in fission yeast

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Cumulative evidence suggests that non-proteolytic functions of the proteasome are involved in transcriptional regulation, mRNA export, and ubiquitin-dependent histone modification and thereby modulate the intracellular levels of regulatory proteins implicated in controlling key cellular functions. To date, the non-proteolytic roles of the proteasome have been mainly investigated in euchromatin; their effects on heterochromatin are largely unknown. Here, using fission yeast as a model, we investigated in euchromatin, that disrupts a non-proteolytic function of the proteasome in heterochromatin regulation. We identified a mutant allele, rpt4-1, that disrupts a non-proteolytic function of the proteasome, also known as a non-proteolytic allele. Experiments performed using rpt4-1 cells revealed that the proteasome is involved in the regulation of heterochromatin spreading to prevent its uncontrolled invasion into neighboring euchromatin regions. Intriguingly, the phenotype of the non-proteolytic rpt4-1 mutant resembled that of epe1Δ cells, which lack the Epe1 protein that counteracts heterochromatin spreading. Both mutants exhibited variegated gene-silencing phenotypes across yeast colonies, spreading of heterochromatin, bypassing of the requirement for RNAi in heterochromatin formation at the outer repeat region (otr), and up-regulation of RNA polymerase II. Further analysis revealed Mst2, another factor that antagonizes heterochromatin spreading, may function redundantly with Rpt4. These observations suggest that the 19S proteasome may be involved in modulating the activities of Epe1 and Mst2. In conclusion, our findings indicate that the proteasome appears to have a heterochromatin-regulating function that is independent of its canonical function in proteolysis.

The proteasome is a highly conserved multiprotein complex that engages in various cellular processes (1). The most well-known function of the proteasome is the degradation of poly-ubiquitylated proteins; this occurs via the collaborative efforts of two subcomplexes, the 19S regulatory particle (19S RP)2 and the 20S core particle (20S CP). The 19S RP may be subdivided into the lid and base subcomplexes (2, 3). The lid recognizes ubiquitylated proteins, and the base deubiquitylates, unfolds, and translocates the substrate into the 20S CP, which is the site of protein degradation (4, 5). Intriguingly, the 19S RP appears to exhibit a protein chaperone activity (6, 7) that is independent of its proteolytic function (8, 9). Other non-proteolytic functions of the proteasome have been reported, covering a range of cellular processes as follows: transcription initiation by regulating activators and co-activators (10–15); transcription elongation by remodeling/stabilizing the stalled polymerase complex (16); ubiquitin-dependent histone modification (17); and mRNA export (18). Furthermore, accumulating evidence suggests that the respective 19S RP subunits have distinct non-proteolytic roles. In particular, the six ATPases of the 19S RP (Rpt1–6) have been reported to play non-proteolytic roles, both as monomers and in combination. In the budding yeast, Saccharomyces cerevisiae, Rpt6p mediates the gene-promoter targeting and stimulation of the co-activator, SAGA (12). Rpt4p, together with Rpt6p, is recruited directly to the yeast GAL gene, where it binds to the Gal4p activation domain to strip the activator off the chromatin (14). Rpt2p was recently reported to engage in ejecting the H2Bub1-deubiquitylating module (Sgf73-DUBm) from the SAGA complex to facilitate mRNA export (18). In the fission yeast, Schizosaccharomyces pombe, Rpt1 contacts the PAF complex via Cks1 to facilitate efficient transcription elongation (19). In humans, heterodimers of Sug1, S7, and S6a (corresponding to fission yeast Rpt6, Rpt1, and Rpt5, respectively) are recruited to induced CIITA plV promoters (13). To date, however, the non-proteolytic functions of the proteasome have been mainly investigated with respect to euchromatin; their effects on heterochromatin remain poorly understood. Moreover, each 19S ATPase has a unique set of interaction partners, and the same subunit may act upon multiple processes (20), suggesting that there may be additional yet undiscovered non-proteolytic roles of the proteasome.

In fission yeast, constitutive heterochromatin is formed at centromeres, telomeres, and the mating-type locus. RNA inter-

2 The abbreviations used are: 19S RP, 19S regulatory particle; 20S CP, 20S catalytic particle; pol II, RNA polymerase II; TBZ, thibendazole; FOA, 5-fluoroorotic acid; HDAC, histone deacetylase; RITS, RNA-induced transcriptional silencing; NM, nuclear membrane; TEV, tobacco etch virus; -seq, -sequence; TAP, tandem affinity purification.
ference (RNAi) is the dominant mechanism through which heterochromatin is formed at centromeres, and it also contributes partly to forming heterochromatin at telomeres and the mating-type locus (21). Antiparallel transcription of the outer repeat by RNA polymerase II (pol II) produces non-coding RNA transcripts that are processed into small interfering RNAs (siRNAs) by the ribonuclease, Dicer (Dcr1). These siRNAs are subsequently loaded onto the RNA-induced transcriptional silencing (RITS) complex, which consists of Chp1, Argonaute ( Ago1), and Tas3, and uses the siRNAs to target it to homologous chromatin for silencing (22–24). The RITS complex recruits the cryptic loci regulator complex to chromatin via the bridging protein, Stc1 (25), resulting in a targeted H3K9 methylation (H3K9me) that is mediated by the Clr4/Suv39h methyltransferase (26–28). The H3K9me mark provides a binding site for the heterochromatin protein 1 (HP1) orthologs, Swi6, Chp1, and Chp2 (29).

Once established, heterochromatin is tightly confined to a defined domain to prevent unwanted invasion of heterochromatin into neighboring euchromatin. The borders of the heterochromatin domains in the centromeres are characterized by sharp transitions in histone modification profiles that coincide with specific boundary elements called IRCs; the exception to this is seen at centromere 2, where clusters of tRNA genes act in place of IRCs (30). The IRCs are enriched for Epe1, which has been shown to counteract heterochromatin spreading (31). Epe1 is recruited throughout the heterochromatin via Swi6, but it is maintained only at the boundary regions; elsewhere, it undergoes Cul4-Ddb1 E3 ligase-dependent ubiquitination and subsequent proteasome-mediated proteolysis (32). The possibility that the proteasome could have a function in heterochromatin other than conventional proteolytic degradation has long been suggested (33), but its roles in numerous aspects of protein homeostasis have obscured researchers from pinpointing such a function.

Recent reports have identified several other factors that are involved in maintaining the heterochromatin boundary. Leo1, which is a component of the Pafl complex, was identified as an antagonizing factor of heterochromatin spreading; several groups made this discovery but proposed different mechanisms (34–36). Mst1 and Mst2, which act as acetyltransferases of histone H4 lysine 16 (H4K16) and histone H3 lysine 14 (H3K14), respectively, have also been shown to antagonize heterochromatin spreading (31). Epe1 is recruited throughout the heterochromatin via Swi6, but it is maintained only at the boundary regions; elsewhere, it undergoes Cul4-Ddb1 E3 ligase-dependent ubiquitination and subsequent proteasome-mediated proteolysis (32). The possibility that the proteasome could have a function in heterochromatin other than conventional proteolytic degradation has long been suggested (33), but its roles in numerous aspects of protein homeostasis have obscured researchers from pinpointing such a function.

Here, we show for the first time that the proteasome functions at centromeric heterochromatin regions in a direct, non-proteolytic way. The non-proteolytic allele, rpt4-1, disrupts heterochromatin integrity and is associated with variegated heterochromatin spreading. Moreover, rpt4-1 takes a regulatory pathway similar to that of Epe1 and Mst2, but not Leo1 or Bdf2, suggesting that the proteasome could possibly act as a protein chaperone in the regulation of Epe1 and Mst2.

**Results**

**Proteasomes are localized at centromeres and involved in heterochromatin regulation**

The ATPase ring of the 19S base is composed of six AAA-type ATPases that effect the conformational change of the ATPase ring (41). As mentioned earlier, most of the non-proteolytic proteasome alleles reported in fission yeast and other organisms are mutations in one of these ATPases (4, 8, 9). We therefore selected three of the ATPases, Rpt3, Rpt4, and Rpt6, as our targets for mutagenesis in our effort to screen for non-proteolytic proteasome mutants that affect heterochromatin (Fig. 1A).

We performed random mutagenesis of all three subunits and successfully isolated a pool of mutations that affected the integrity of heterochromatin by examining the expression of an ade6+ reporter inserted in the outer repeat region (otr). The expression of ade6+ was examined in low-adenine medium (YES-Ade) and adenine-deficient medium (PMG-Ade) (Fig. 1C). To our surprise, all three subunits produced mutants that affected the heterochromatin to varying degrees. Although we do not know whether the three subunits share the same mechanism for regulating heterochromatin, our results clearly indicate that the proteasome plays a general role in heterochromatin regulation.

The proteasome has been reported to be localized at pericentric heterochromatin in fission yeast, yet only the components of 19S RP appear to be recruited (42). As the ChIP efficiency of the proteasome has been shown to be highly dependent on the utilized antibody or target subunit (43), we speculated that targeting a different subunit of the 20S CP might improve its ChIP efficiency and possibly reveal the physical residence of the intact 26S proteasome. To this end, we FLAG-tagged the 20S CP component, Pre1, and the 19S RP components, Rpt3, Rpt4, and Rpt6, respectively, to examine the localization of the intact 26S proteasome. To our surprise, all three subunits produced mutants that affected the heterochromatin to varying degrees. Although we do not know whether the three subunits share the same mechanism for regulating heterochromatin, our results clearly indicate that the proteasome plays a general role in heterochromatin regulation.

**Rpt4 mutant allele, rpt4-1, is non-proteolytic**

From the generated mutant alleles, we selected rpt4-1 for further investigation because it showed the most severe derepression of heterochromatin (Fig. 1C). We first tested whether there was any defect in the proteolytic function of rpt4-1 cells, by using a poly-ubiquitin antibody to test cell lysates for the accumulation of poly-ubiquitylated species (18). No accumula-
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A

B

C

YES

PMG

YES-Ade

PMG-Ade

Degree of de-repression

Degree of de-repression
tion of a poly-ubiquitylated product was observed in \textit{rpt4-1} cells, indicating that \textit{rpt4-1} is a non-proteolytic allele (Fig. 2A, lanes 1 and 3 versus lanes 2 and 4). To verify this observation, we examined the levels of FLAG-tagged Rum1, which is rapidly degraded by the proteasome (44, 45). No accumulation of Rum1 was observed in \textit{rpt4-1} cells (Fig. 2B, compare lanes 1–6 with lanes 7–9), which is consistent with the results of our poly-ubiquitination assays and further confirmed the non-proteolytic nature of the \textit{rpt4-1} allele. Silver staining of proteasomes purified from \textit{rpt4-1} cells showed that all subunits were intact (Fig. 2C, compare lanes 1 and 3).

Sequencing of \textit{rpt4-1} revealed a single aspartic acid to valine mutation at position 249 (D249V), which is highly conserved throughout all eukaryotes (Fig. 2D). The mutation is not located within the key ATPase domains, such as the Walker A, Walker B, Sensor, and R finger domains, where any mutation would significantly affect the ATPase function and subsequent proteolysis (46). Rather, the mutation is located adjacent to the first \(/H9251\)-helix domain after the Walker B motif. We speculate that it may induce a conformational change that is subtle enough to disturb only the non-proteolytic functions of the proteasome.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Regulation of heterochromatin by proteasome is non-proteolytic. A, poly-ubiquitylated proteins do not accumulate in \textit{rpt4-1} cells. Wild-type, \textit{rpt4-1}, \textit{mts2-1}, and \textit{rpt3-1} cells were collected, and whole-cell extracts were subjected to Western blotting with FK2 antibodies against poly-ubiquitin. Rpt2 was used as a loading control. B, Rum1-FLAG proteins do not accumulate in \textit{rpt4-1} cells. Wild-type, \textit{rpt4-1}, and \textit{mts3-1} cells were grown to log phase in YES at 30 °C and then shifted to 37 °C. The cells were collected at the indicated times, and whole-cell extracts were subjected to Western blotting with anti-FLAG antibodies. Rpt2 was used as a loading control. C, Rpn1-TAP-tagged 26S proteasomes were purified and visualized using silver staining. Protein molecular weight standards are indicated. Asterisk denotes the FLAG-tagged Rpn2 subunit in the \textit{rpt4-1} proteasome. D, schematic representation of the \textit{rpt4-1} mutation (D249V). Domains present in Rpt4p are shown (top) and the partial amino acid sequences of Rpt4 from eight species are aligned. The mutation site is highlighted in \textit{pink}.}
\end{figure}
**19S proteasome involvement in heterochromatin spreading**

Figure 3. *rpt4-1* cells show variegated silencing at pericentromeric regions. A and B, schematic diagrams of the *otr1::ade6+* and *otr1::ura4+* reporters (top). *rpt4-1* causes variegated silencing at the centromere *otr*. 5-Fold serial dilutions of single *rpt4-1* colonies harboring *ade6+* (A) or *ura+* (B) at the *otr* were spotted onto the indicated plates (bottom). C, *rpt4-1 epe1Δ* cells retain the variegated silencing at the centromere. 5-Fold serial dilutions of single *rpt4-1 epe1Δ* colonies harboring *ade6+* at the *otr* were spotted onto the indicated plates. D, *rpt4-1* causes chromosome segregation defects. 5-Fold serial dilutions of a population (top) or single colonies (bottom) of *rpt4-1* cells harboring *ade6+* at the *otr* were spotted onto low-adenine (low ade) medium or medium containing 10 μg/ml TBZ. N/S, non-selective.

**rpt4-1** cells exhibit variegated silencing at pericentromeric heterochromatin

The repression status of the pericentromere can be easily identified by colors using the *ade6+* reporter inserted at the *otr* region (Fig. 3, A, C, and D) (47). When cells are grown in a low-adenine medium (YE), the repressed state is reflected by a red color, although the de-repressed state is white. Unlike other mutant alleles that showed a uniform color change, *rpt4-1* cells exhibited a mixture of red and white colonies when plated on YE medium, indicating the co-existence of both repressed and de-repressed cells (Fig. 1C). To verify this observation, we randomly selected *rpt4-1* colonies grown on rich medium (YES) and spotted them onto low-adenine medium (YE). Indeed, the colonies differed in their degrees of repression, ranging from fully repressed (red) to fully de-repressed (white) colonies (Fig. 3A). To exclude the possibility that this variegation phenotype was an indirect effect of the *ade6+* reporter, we used a *ura4+* reporter inserted at the outer repeat region of *rpt4-1* cells (Fig. 3B, top), and we tested whether the variegation phenotype persisted. The same variegation of silencing was observed with the *ura4+* reporter, indicating that the variegation is a *bona fide* phenotype of *rpt4-1* (Fig. 3B, bottom).

The variegation phenotype of *rpt4-1* is reminiscent of the null mutation of Epe1, *epe1Δ*, which is well-known to exhibit variegation in heterochromatin silencing because of the oscillation of heterochromatin domains (48). Here, we found that this variegation status persisted in the double mutant of *rpt4-1 epe1Δ* (Fig. 3C), suggesting that Rpt4 and Epe1 may share the same pathway to prevent the stochastic dysregulation of heterochromatin. The stochastic nature of the *rpt4-1* mutant was also apparent when we tried to analyze its sensitivity to the microtubule-destabilizing agent, thiabendazole (TBZ). Although a population of *rpt4-1* cells seemed insensitive to TBZ (Fig. 3D, top), single colonies exhibiting full *ade6+* de-repression (white colonies) showed TBZ sensitivity (Fig. 3D, bottom). Varying TBZ sensitivity is another reported phenotype of *epe1Δ* (48), which further supports the possible linkage between Rpt4 and Epe1.

**rpt4-1** cells show heterochromatin spreading

Epe1 has been identified as an anti-silencing factor, as its inactivation stimulates the continuous spreading of heterochromatin beyond its natural boundaries (31). Because *rpt4-1* showed a variegation phenotype similar to that of *epe1Δ*, we tested whether *rpt4-1* also exhibited heterochromatin spreading. To this end, we generated *rpt4-1* mutant cells in which the *ura4+* reporter gene was inserted immediately outside the *IRC1L:ura4* mutant was showing poor growth in FOA medium. In cells with impaired boundary function, such as in the *epe1Δ* and *leolΔ* mutants (34, 36), heterochromatin spreads beyond the boundary to
silence the ura4+ reporter, conferring resistance to FOA. As anticipated, the rpt4-1 mutant showed growth on FOA, which is indicative of heterochromatin spreading (Fig. 4B, top). The mild overexpression of wild-type rpt4+ suppressed this evidence of heterochromatin spreading in the rpt4-1 mutant (supplemental Fig. S1), indicating that the observed heterochromatin spreading is a direct consequence of the rpt4-1 mutation. The spreading in the rpt4-1 mutant seemed marginal compared with those of the epe1Δ or leo1Δ mutants, but the spreading of FOA-resistant colony was just as robust as the known boundary regulator mutants (Fig. 4B, bottom). This indicates that heterochromatin spreads in a stochastic fashion in the rpt4-1 mutant, as reported previously in epe1Δ cells (48).

Beyond the epe1Δ mutant, the null mutations of Mst2, Bdf2, and Leo1 (mst2Δ, bdf2Δ and leo1Δ, respectively) also reportedly exhibit heterochromatin spreading (36–38). Our genetic analysis revealed that rpt4-1 showed synergistic enhancement of heterochromatin spreading with the bdf2Δ and leo1Δ mutations (Fig. 4C) but not with the mst2Δ or epe1Δ mutations (Fig. 4D). These results suggest that, for heterochromatin spreading, Rpt4 functions redundantly with Bdf2 and Leo1 via a different pathway, although it shares a pathway with Epe1 and Mst2. Our RNA-seq analysis of the rpt4-1, epe1Δ, and mst2Δ mutants further revealed that there is functional redundancy among Rpt4, Epe1, and Mst2. The gene clusters that exhibited differential expression in the epe1Δ and mst2Δ mutants (>1.5-fold change relative to wild-type) showed significant overlaps with those exhibiting differential expression in rpt4-1 cells (p = 9 × 10−31 and p = 7.3 × 10−29 for epe1Δ and mst2Δ, respectively), indicating that molecular functions are shared among Rpt4, Epe1, and Mst2 (supplemental Fig. S2A). Gene ontology analysis revealed that the three proteins are all involved in regulating the gene groups related to the stress response and protein synthesis, suggesting that they share a role in the cellular response to environmental stimuli (supplemental Fig. S2, B and C).

Because rpt4-1 is a non-proteolytic mutant and therefore does not affect the level of Epe1 or Mst2, we questioned whether it might affect the localizations of Epe1 and/or Mst2, particularly at the IRC boundary regions. However, Mst2 was recently reported to be absent from centromeric regions (40), making it most logical to examine the localization of Epe1 alone. We found that the localization of Epe1 was unaffected, or marginally increased, if any, in rpt4-1 cells (supplemental Fig. S3). Together, our results suggest that the functions of Epe1 and/or Mst2 may be impaired in the rpt4-1 mutant. These possibilities are discussed further below.

**rpt4-1 bypasses the requirement of RNAi in heterochromatin formation**

In epe1Δ and mst2Δ mutants, heterochromatin formation is restored in the absence of RNAi, implying that an RNAi-independent heterochromatin formation pathway is activated (37, 48). We tested whether the rpt4-1 mutant could bypass the requirement of RNAi for heterochromatin formation, and indeed we found that rpt4-1 cells bypassed the requirement of active RNAi. Compared with the ago1Δ and dcr1Δ mutants, rpt4-1 ago1Δ and rpt4-1 dcr1Δ mutants showed increased silencing of the ade6+ reporter, indicating that heterochromatin was restored in the double mutants (Fig. 5A). Likewise, rpt4-1 ago1Δ cells exhibited recovery of the poor growth seen in...
ago1Δ cells (Fig. 5B). Concordantly, the level of H3K9me2 was restored in rpt4-1 ago1Δ cells compared with that in ago1Δ cells, but not to the full wild-type level (Fig. 5C). The Swi6 level in the rpt4-1 ago1Δ mutant was lower than that in the ago1Δ mutant (Fig. 5D), indicating Swi6-independent silencing in rpt4-1 ago1Δ cells.

rpt4-1 reduces transcription at pericentromeric heterochromatin in the absence of RNAi

Pericentromeric heterochromatin is readily transcribed by pol II during S phase of the cell cycle to produce nascent RNAs that RNAi machineries eventually transform into siRNAs (49, 50). However, the accessibility of pol II is tightly regulated by the balance between the histone deacetylase protein, Clr3, and the anti-silencing protein, Epe1. Clr3 restricts the accessibility of pol II, whereas Epe1 counteracts this by promoting the accessibility of pol II (51). A previous study showed that the level of pol II was increased in dcr1Δ cells due to the de-condensation of heterochromatin; however, this was suppressed in dcr1Δ epe1Δ cells, demonstrating that Epe1 can promote pol II accessibility in pericentromeric heterochromatin (51). Similar to the dcr1Δ epe1Δ mutant, the dcr1Δ mst2Δ mutant also reportedly exhibits suppression of the increased pol II seen at the centromeres of dcr1Δ cells (37). We therefore anticipated that the rpt4-1 mutation would reduce the level of pol II in situations that are typically characterized by abnormally high levels of pol II. To test this hypothesis, we examined whether the rpt4-1 ago1Δ mutant exhibited suppression of the increased pol II level seen in the ago1Δ mutant (Fig. 6A). Consistent with this finding, the transcript level in the otr was reduced in rpt4-1 ago1Δ cells (Fig. 6B). We also failed to observe siRNAs in rpt4-1 ago1Δ cells, indicating that the restoration of silencing (i.e. the decrease in pol II) does not reflect the activation of an alternative small RNA-producing pathway (Fig. 6C). Together, these results indicate that a non-proteolytic function of the proteasome promotes the pericentromeric level of pol II.

Discussion

In this study, we elucidated a new, non-proteolytic function of the proteasome in regulating heterochromatin via a mechanism similar to that mediated by Epe1 and Mst2 (Fig. 7). Our observation that the non-proteolytic allele, rpt4-1, showed variegation of heterochromatin silencing (Fig. 3, A and B) and TBZ sensitivity (Fig. 3D) led us to investigate the potential involvement of Epe1, which was previously shown to prevent the spreading of heterochromatin beyond its natural borders (31).
Epe1-enriched a physical interaction (38), and we found that the proteasome proteins. In the case of Epe1, this hypothesis is feasible to some extent because 19S RP can remodel the accessibility of pol II (Fig. 6), which are two phenotypes also reportedly involved in both regulating heterochromatin and heterochromatin mutants, we also acquired the recruitment of the proteasome at the centromere. In fact, NM proteins are reportedly involved in both regulating heterochromatin and anchoring the proteasome to the NM (55, 56). Thus, it may be plausible that NM proteins are involved in recruiting the proteasome to the centromere.

As in epe1Δ cells, heterochromatin spreading was observed in rpt4-1 cells (Fig. 4A). Genetic analysis revealed that the rpt4-1 regulates heterochromatin spreading via a pathway that is distinct from those involving Bdf2 and Leo1 (Fig. 4C) but is shared by Epe1 and Mst2. Notably, rpt4-1 bypassed the requirement of RNAi for heterochromatin maintenance (Fig. 5) and reduced the accessibility of pol II (Fig. 6), which are two phenotypes also reported for epe1Δ and mst2Δ cells (48, 51).

As our group previously showed that 19S RP can remodel protein complexes via chaperone activity (18), a simple hypothesis would be that the proteasome regulates the recruitment of Epe1 and/or Mst2. However, our ChIP analysis showed that the recruitment of Epe1 is not affected in rpt4-1 cells (supplemental Fig. S3), and Mst2 is either absent from centromeric regions, as reported (40), or its action at centromeric regions may be too transient to be detected by the current ChIP technique. This leaves the possibility that the proteasome may directly remodel Epe1 and/or Mst2 to alter the activity of one or both of these proteins. In the case of Epe1, this hypothesis is feasible to some extent because 19S RP and Epe1 have been reported to undergo a physical interaction (38), and we found that the proteasome was highly enriched at heterochromatin regions, including the Epe1-enriched IRC boundaries (Fig. 1B). However, a detailed biochemical study is needed to fully examine the potential proteasome-directed remodeling of Epe1. Although Epe1 contains a JmjC domain, it seems to lack any demethylase activity (48, 51). It has been reported to promote histone turnover in vivo (52), but there is no conclusive biochemical evidence showing that histone turnover is a genuine function of Epe1. In contrast, Mst2 has well-characterized enzymatic activity as a histone H3K14 acetyltransferase. Like Epe1, Mst2 has also been reported to promote histone turnover in vivo, although the underlying mechanism remains unknown (39). The global histone H3K14ac level was not altered in the rpt4-1 mutant (data not shown), indicating that the histone acetylation activity of Mst2 is not affected by the rpt4-1 mutation. However, Mst2 was recently shown to acetylate BrI1 (40), a component of the histone H2B ubiquitin-ligase complex, but it might be possible that Rpt4 is involved in regulating the H2B mono-ubiquitylation pathway (17).

The recruitment of the proteasome to pericentromeric heterochromatin may arise through poly-ubiquitinated substrates, such as Epe1 (32). However, the enrichment of the proteasome at heterochromatin is above the genomic average, suggesting that there may be different modes of proteasome recruitment. Because the proteasome is enriched in NM (33) and both the proteasome and the centromere are localized at the inner nuclear membrane (NM) in fission yeast (53, 54), it could be the same nuclear compartment that accounts for the high enrichment of the proteasome at the centromere. In fact, NM proteins are reportedly involved in both regulating heterochromatin and anchoring the proteasome to the NM (55, 56). Thus, it may be plausible that NM proteins are involved in recruiting the proteasome to the centromere.

A previous study identified cep (centromere enhancer of position effect) mutants in a genetic screen for mutants that affect centromeric silencing within the central core region. The authors found that mutations in the 19S RP subunit, rpt2+ and rpn11+ (cep2-12 and cep1-1, respectively), were responsible for the enhancement of heterochromatin (33). The cep mutants were distinguished from the conventional proteolytic mts2-1 or mts3-1 mutants in that they did not accumulate short spindles. The authors hypothesized that substrate-specific degradation may have caused the phenotypes in cep mutants, but we propose that these phenotypes may actually have reflected the non-proteolytic activity we observed in this study. In fact, the rpt4-1 mutation enhanced the centromere core silencing (data not shown), suggesting the tantalizing possibility that there may be functional redundancy between the cep mutants and rpt4-1.

A recent study found that the 19S proteasome subunit, Rpt3, regulates the distribution of CENP-A (42). The C-terminal truncation mutant, rpt3-1, showed defective regulation of CENP-A, wherein CENP-A crossed the borders of the centromere core and spread to the otr. In our screening for heterochromatin mutants, we also acquired the rpt3-1 mutant. However, the rpt4-1 and rpt3-1 mutants were clearly distinct from one another. Besides the distinction that they are alleles of Rpt4 and Rpt3, respectively, the rpt4-1 mutant did not show temperature sensitivity in Cep1-overexpressing cells (supplemental Fig. S4), which is a key phenotype of the rpt3-1 mutant. We also failed to observe the spreading of CENP-A (characteristic of rpt3-1) to the otr in rpt4-1 mutant cells (data not shown). Finally, the rpt3-1 mutant was found to be significantly proteo-
lytic (Fig. 2A), which is another important distinction from the non-proteolytic \textit{rpt4-1} mutant. Our findings suggest that the phenotypes of \textit{rpt3-1} cells might be an indirect effect of defective proteolysis, whereas those of \textit{rpt4-1} cells appear to be the direct consequence of a non-proteolytic function of the proteasome.

In conclusion, the proteasome is a highly conserved protein complex from yeast to humans. Our study demonstrates for the first time that the proteasome plays a role in heterochromatin regulation that is independent of its canonical proteolytic function. In the future, it would be intriguing to investigate whether this non-proteolytic function is conserved in higher eukaryotes, such as humans.

**Experimental procedures**

**Yeast strains and plasmids**

The fission yeast strains used in this study are listed in supplemental Table S1. Standard procedures were used for growth...
and genetic manipulations. All strains were grown at 30 °C unless otherwise stated. The deletion strains and tagged strain were generated using a PCR-based method. The wild-type and mutated Rpt4 ORFs were cloned into an *S. pombe* expression plasmid under the control of the *nmt41* promoter. The wild-type and point-mutated Rpt4 ORFs were confirmed by sequencing.

**TAP purification**

Strains expressing TAP-tagged proteins were grown in YES at 30 °C to an OD_{600} of about 1.0 and harvested. Each harvested cell pellet was frozen in liquid nitrogen and ground to a fine powder using a Retch grinder (MM400). The ground powder was resuspended in lysis buffer (5 mM ATP, 50 mM Tris-Cl (pH 7.5), 150 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, 10% glycerol, supplemented with protease inhibitors), homogenized using a homogenizer, and centrifuged. The supernatant was directly applied to IgG-Sepharose (GE Healthcare) and subjected to the TEV cleavage reaction in cleavage buffer (10 mM Tris-Cl (pH 8.0), 5 mM ATP, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, 1 mM MgCl₂, 0.2 mM CaCl₂, and 10% glycerol) supplemented with appropriate nucleases and a protease inhibitor mixture (Roche Applied Science), for 1.5 h at room temperature. The TEV supernatant was mixed with calmodulin-binding buffer (2 mM ATP, 10 mM Tris-Cl (pH 8.0), 1 mM MgAc, 1 mM imidazole, 2 mM CaCl₂, 150 mM NaCl, 0.1% Nonidet P-40, 10 mM β-mercaptoethanol, and 10% glycerol), and the mixture was incubated overnight at 4 °C with calmodulin-Sepharose (Stratagene). The proteins were eluted with elution buffer (2 mM ATP, 10 mM Tris-Cl (pH 8.0), 1 mM MgAc, 1 mM imidazole, 3 mM EGTA, 150 mM NaCl, 0.1% Nonidet P-40, 10 mM β-mercaptoethanol, and 10% glycerol), resolved by 4–15% PAGE, and visualized using silver staining.

**Screening of proteasome mutants**

The wild-type ORFs encoding Rpt3, Rpt4, and Rpt6 were subjected to random mutagenesis by error-prone PCR using GeneMorph II random mutagenesis kits (Stratagene) according to the manufacturer’s protocol. The mutated ORFs were fused with the 3′- and 5′-UTRs of their respective genes and the Kan<sup>R</sup> cassette, and the generated constructs were transformed into an *otr1::ade6*+ reporter strain. Colonies showing both a white color when grown on YE plates and survival on PMG-Ade plates were selected. gDNAs of the selected colonies were sequenced to exclude false positives, and only colonies with mutation(s) in the respective ORF were selected. The proteasome mutants were then re-spotted on YE and PMG-Ade plates to test the effect of each mutation on heterochromatin silencing. The mutant with the strongest phenotype (*rpt4-1*) was selected. The effect of the *rpt4-1* mutation (D249V) was further verified by introducing it into the wild-type strain.

**ChIP and ChIP-seq analysis**

ChIP was performed as reported previously (18), with minor modifications. Briefly, 2.4 × 10⁶ cells were fixed in 1% formaldehyde for 15 min at room temperature, and cell extracts were prepared using the standard bead-beating method. Immunoprecipitation was performed overnight at 4 °C using the following antibodies: anti-FLAG (F3165, Sigma), anti-H3K9me2 (ab1220, Abcam), anti-H4K16ac (made in-house), and anti-Swi6 (made in-house). Immunoprecipitated DNA was recovered using the Chelex-100 resin (Bio-Rad) and quantified by quantitative PCR using the primers listed in supplemental Table S2. For Epe1-FLAG ChIP, a quantity of *S. cerevisiae* cells corresponding to 1/9 of the original input cells was added prior to the cell lysis step as an add-in control. ChIP-seq analysis was performed as described previously (57).

**Serial dilution assays**

Strains or single colonies were spotted in 5-fold dilutions onto the appropriate plates and incubated for 3–4 days at 30 °C. To assess the sensitivity to TBZ, serial dilutions were spotted onto YES containing 10 µg/ml TBZ.

**Western blot analysis**

Whole-cell extracts were prepared from logarithmically growing cells. Cells were harvested and resuspended in either trichloroacetic acid (for the blotting of Epe1) or 2 × SDS-PAGE loading buffer containing 1 mM PMSF (for all other blots). The resuspended cells were vortexed with beads, boiled in SDS-PAGE loading buffer, and used for immunoblotting.

**RNA-seq, small RNA purification, and library preparation for small RNA sequencing**

RNAs were purified using the previously described hot-phenol method (58) and subjected to library preparation using a NEXTflex® Illumina RNA-seq library preparation kit version 2 (BIOO) according to the manufacturer’s instructions. Small RNAs were purified as described previously (48), and the obtained small RNAs were subjected to library preparation using a NEXTflex™ small RNA-seq kit version 3 (BIOO) according to the manufacturer’s instructions. Each library was sequenced on a HiSeq2500 using the single-end method (50-bp reads). The adaptor sequences were automatically trimmed, and the processed reads were aligned to the *S. pombe* genome (ASM294v2) using the STAR (for RNA-seq) or Novoalign software packages. The bam2wig.py Python script from RSeQC (59) was used to further analyze the aligned read data. RNA-seq data of *mst2Δ* cells was adopted from GEO, accession number GSE93432.

**Antibodies**

The anti-H3K9me2 (ab1220, Abcam), anti-ubiquitylated proteins (04-263, Millipore), anti-FLAG M2 (F1804, Sigma), and anti-RNA pol II (8WG16, Abcam) antibodies were purchased as indicated. The rabbit polyclonal anti-Rpt2, anti-Swi6, and anti-H4K16ac antibodies were produced in-house as described previously (60).

**Data availability**

The sRNA and ChIP-seq data reported in this paper are available from GEO under accession number GSE97865.
19S proteasome involvement in heterochromatin spreading

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References

1. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Structure and functions of the 20S and 26S proteasomes. Annu. Rev. Biochem. 65, 801—847
2. Baumeister, W., Walz, J., Zühl, F., and Seemüller, E. (1998) The proteasome: paradigm of a self-compartmentalizing protease. Cell 92, 367—380
3. Glickman, M. H., Rubin, D. M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V. A., and Finley, D. (1998) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and elf3. Cell 94, 615—623
4. Kwak, J., Workman, J. L., and Lee, D. (2011) The proteasome and its regulatory roles in gene expression. Biochim. Biophys. Acta 1809, 88—96
5. Pickart, C. M., and Cohen, R. E. (2004) Proteasomes and their kin: proteases in the machine age. Nat. Rev. Mol. Cell Biol. 5, 177—187
6. Braun, B. C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P. M., Finley, D., and Schmidt, M. (1999) The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat. Cell Biol. 1, 221—226
7. Ferrell, K., Wilkinson, C. R., Dubiel, W., and Gordon, C. (2000) Regulatory subunit interactions of the 26S proteasome, a complex problem. Trends Biochem. Sci. 25, 83—88
8. McCann, T. S., and Tansey, W. P. (2014) Functions of the proteasome on chromatin. Biomolecules 4, 1026—1044
9. Bhat, K. P., and Greer, S. F. (2011) Proteolytic and non-proteolytic roles of ubiquitin and the ubiquitin proteasome system in transcriptional regulation. Biochim. Biophys. Acta 1809, 150—155
10. Ferdous, A., Kodadek, T., and Johnston, S. A. (2002) A nonproteolytic function of the 19S regulatory subunit of the 26S proteasome is required for efficient activated transcription by human RNA polymerase II. Biochemistry 41, 12798—12805
11. Gonzalez, F., Delahodde, A., Kodadek, T., and Johnston, S. A. (2002) Recruitment of a 19S proteasome subcomplex to an activated promoter. Science 296, 548—550
12. Lee, D., Ezhkova, E., Ll. B., Pattenden, S. G., Tansey, W. P., and Workman, J. L. (2005) The proteasome regulatory particle alters the SAGA coactivator to enhance its interactions with transcriptional activators. Cell 123, 423—436
13. Maganti, N., Moody, T. D., Truax, A. D., Thakkar, M., Spring, A. M., Germann, M. W., and Greer, S. F. (2014) Nonproteolytic roles of 19S ATPases in transcription of CIITApIV genes. PLoS ONE 9, e91200
14. Ferdous, A., Sikder, D., Gillette, T., Nalley, K., Kodadek, T., and Johnston, S. A. (2007) The role of the proteasomal ATPases and activator monoubiquitination in regulating Gal4 binding to promoters. Genes Dev. 21, 112—123
15. Chang, C., Gonzalez, F., Rothermel, B., Sun, L., Johnston, S. A., and Kodadek, T. (2001) The Gal4 activation domain binds Sug2 protein, a proteasome component, in vivo and in vitro. J. Biol. Chem. 276, 30956—30963
16. Ferdous, A., Gonzalez, F., Sun, L., Kodadek, T., and Johnston, S. A. (2001) The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II. Mol. Cell 7, 981—991
17. Ezhkova, E., and Tansey, W. P. (2004) Proteasomal ATPases link ubiquitylation of histone H2B to methylation of histone H3. Mol. Cell 13, 435—442
18. Lim, S., Kwak, J., Kim, M., and Lee, D. (2013) Separation of a functional deubiquitylating module from the SAGA complex by the proteasome regulatory particle. Nat. Commun. 4, 2641
19. Chaves, S., Baskerville, C., Yu, V., and Reed, S. I. (2010) Cks1, Cdk1, and the 19S proteasome collaborate to regulate gene induction-dependent nucleosome eviction in yeast. Mol. Cell. Biol. 30, 5284—5294
20. Collins, G. A., and Tansey, W. P. (2006) The proteasome: a utility tool for transcription? Curr. Opin. Genet. Dev. 16, 197—202
21. Grewal, S. I., and Jia, S. (2007) Heterochromatin revisited. Nat. Rev. Genet. 8, 35—46
22. Motamedi, M. R., Verdel, A., Colmenares, S. U., Gerber, S. A., Gygi, S. P., and Moazed, D. (2004) Two RNAi complexes, RITS and RDR2, physically interact and localize to non-coding centromeric RNAs. Cell 119, 789—802
23. Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zoffal, M., Jia, S., Moazed, D., and Grewal, S. I. (2004) RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. Nat. Genet. 36, 1174—1180
24. Ferdous, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S. I., and Moazed, D. (2004) RNAi-mediated targeting of heterochromatin by the RITS complex. Science 303, 672—676
25. Bayne, E. H., White, S. A., Kagansky, A., Bijos, D. A., Sanchez-Pulido, L., Hoe, K. L., Kim, D. U., Park, H. O., Ponting, C. P., Rappisbiler, J., and Allshire, R. C. (2010) Sctl: a critical link between RNAi and chromatin modification required for heterochromatin integrity. Cell 140, 666—677
26. Ewalt, K., and Ruussala, T. (1994) Mutations in rik1, ch2, ch3 and ch4 genes asymmetrically derepress the silent mating-type loci in fission yeast. Genetics 136, 53—64
27. Thon, G., Cohen, A., and Klar, A. J. (1994) Three additional linkage groups that repress transcription and meiotic recombination in the mating-type region of Schizosaccharomyces pombe. Genetics 138, 29—38
28. Rea, S., Eisenhaber, F., O’Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D., and Jenuwein, T. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406, 593—599
29. Huisenga, K. L., Brower-Toland, B., and Elgin, S. C. (2006) The contradictory definitions of heterochromatin: transcription and silencing. Chromosoma 115, 110—122
30. Cam, H. P., Sugiyama, T., Chen, E. S., Chen, X., FitzGerald, P. C., and Grewal, S. I. (2005) Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. Nat. Genet. 37, 809—819
31. Ayoub, N., Noma, K., Isaac, S., Kahan, T., Grewal, S. I., and Cohen, A. (2003) A novel jmjC domain protein modulates heterochromatization in fission yeast. Mol. Cell. Biol. 23, 4356—4370
32. Braun, S., Garcia, J. F., Rowley, M., Rougemaille, M., Shankar, S., and Madhani, H. D. (2011) The Cul4-Ddb1(Cdt2) ubiquitin ligase inhibits invasion of a boundary-associated antisilencing factor into heterochromatin. Cell 144, 41—54
33. Javerzat, J. P., McGurk, G., Cranston, G., Barreau, C., Bernard, P., Gordon, C., and Allshire, R. (1999) Defects in components of the proteasome enhance transcriptional silencing at fission yeast centromeres and impair chromosome segregation. Mol. Cell. Biol. 19, 5155—5165
34. Kovalek, K. M., Shimada, Y., Fluury, V., Stadler, M. B., Batki, J., and Bühler, M. (2015) The Paf1 complex represses small-RNA-mediated epigenetic gene silencing. Nature 520, 248—252
35. Sadeghi, L., Prasad, P., Ewalt, K., Cohen, A., and Svensson, J. P. (2015) The Paf1 complex factors Leol and Paf1 promote local histone turnover to modulate chromatin states in fission yeast. EMBO Rep 16, 1673—1687
36. Verrier, L., Taglini, F., Barrales, R. R., Webb, S., Urano, T., Braun, S., and Bayne, E. H. (2015) Global regulation of heterochromatin spreading by Leol. Open Biol. 5, 150045
37. Reddy, B. D., Wang, Y., Niu, L., Higuchi, E. C., Marguerat, S. B., Bähler, J., Smith, G. R., and Jia, S. (2011) Elimination of a specific histone H3K14 acetyltransferase complex bypasses the RNAi pathway to regulate pericentric heterochromatin functions. Genes Dev. 25, 214—219
38. Wang, J., Tadeo, X., Hou, H., Tu, P. G., Thompson, J., Yates, J. R., 3rd, and Jia, S. (2013) Epe1 recruits BET family bromodomain protein Bdf2 to establish heterochromatin boundaries. *Genes Dev.* **27**, 1886–1902

39. Wang, J., Reddy, B. D., and Jia, S. (2015) Rapid epigenetic adaptation to uncontrolled heterochromatin spreading. *Elife* **015 Mar 16;4**

40. Flury, V., Georgescu, P. R., Iesmantavicius, V., Shimada, Y., Kuzdere, T., Braun, S., and Bühler, M. (2017) The histone acetyltransferase Mst2 protects active chromatin from epigenetic silencing by acetylating the ubiquitin ligase Brl1. *Mol. Cell* **67**, 294–307

41. Sauer, R. T., and Baker, T. A. (2011) AAA+/H11001 proteases: ATP-fueled machines of protein destruction. *Annu. Rev. Biochem.* **80**, 587–612

42. Kitagawa, T., Ishii, K., Takeda, K., and Matsumoto, T. (2014) The 19S proteasome subunit Rpt3 regulates distribution of CENP-A by associating with centromeric chromatin. *Nat. Commun.* **5**, 3597

43. Geng, F., and Tansey, W. P. (2012) Similar temporal and spatial recruitment of native 19S and 20S proteasome subunits to transcriptionally active chromatin. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 6060–6065

44. Kominami, K., and Toda, T. (1997) Fission yeast WD-repeat protein pop1 regulates genome ploidy through ubiquitin-proteasome-mediated degradation of the CDK inhibitor Rum1 and the S-phase initiator Cdc18. *Genes Dev.* **11**, 1548–1560

45. Correa-Bordes, J., and Nurse, P. (1995) p25rum1 orders S phase and mitosis by acting as an inhibitor of the p34cdc2 mitotic kinase. *Cell* **83**, 1001–1009

46. Kim, H. M., Yu, Y., and Cheng, Y. (2011) Structure characterization of the 26S proteasome. *Biochim. Biophys. Acta* **1809**, 67–79

47. Ekwall, K., Cranston, G., and Allshire, R. C. (1999) Fission yeast mutants that alleviate transcriptional silencing in centromeric flanking repeats and disrupt chromosome segregation. *Genetics* **153**, 1153–1169

48. Trewick, S. C., Minc, E., Antonelli, R., Urano, T., and Allshire, R. C. (2007) The JmjC domain protein Epe1 prevents unregulated assembly and disassembly of heterochromatin. *EMBO J.* **26**, 4670–4682

49. Djupedal, I., Portoso, M., Spahr, H., Bonilla, C., Gustafsson, C. M., Allshire, R. C., and Ekwall, K. (2005) RNA pol II subunit Rpb7 promotes centromeric transcription and RNAi-directed chromatin silencing. *Genes Dev.* **19**, 2301–2306

50. Kato, H., Goto, D. B., Martienssen, R. A., Urano, T., Furukawa, K., and Murakami, Y. (2005) RNA polymerase II is required for RNAi-dependent heterochromatin assembly. *Science* **309**, 467–469

51. Zofall, M., and Grewal, S. I. (2006) Swi6/HP1 recruits a JmjC domain protein to facilitate transcription of heterochromatic repeats. *Mol. Cell* **22**, 681–692

52. Aygün, O., Mehta, S., and Grewal, S. I. (2013) HDAC-mediated suppression of histone turnover promotes epigenetic stability of heterochromatin. *Nat. Struct. Mol. Biol.* **20**, 547–554

53. Wilkinson, C. R., Wallace, M., Morpwh, M., Perry, P., Allshire, R., Javerzat, J. P., McIntosh, J. R., and Gordon, C. (1998) Localization of the 26S proteasome during mitosis and meiosis in fission yeast. *EMBO J.* **17**, 6465–6476

54. Funabiki, H., Hagan, I., Uzawa, S., and Yanagida, M. (1993) Cell cycle-dependent specific positioning and clustering of centromeres and telomeres in fission yeast. *J. Cell Biol.* **121**, 961–976

55. Tatebe, H., and Yanagida, M. (2000) Cut8, essential for anaphase, controls localization of 26S proteasome, facilitating destruction of cyclin and Cut2. *Curr. Biol.* **10**, 1329–1338

56. Barrales, R. R., Forn, M., Georgescu, P. R., Sarkadi, Z., and Braun, S. (2016) Control of heterochromatin localization and silencing by the nuclear membrane protein Lem2. *Genes Dev.* **30**, 133–148

57. Lee, J., Choi, E. S., Seo, H. D., Kang, K., Gilmore, J. M., Florens, L., Washburn, M. P., Choe, J., Workman, J. L., and Lee, D. (2017) Chromatin remodeler Fun30Fft3 induces nucleosome disassembly to facilitate RNA polymerase II elongation. *Nat. Commun.* **8**, 14527

58. Shim, Y. S., Choi, Y., Kang, K., Cho, K., Oh, S., Lee, J., Grewal, S. I., and Lee, D. (2012) Hrp3 controls nucleosome positioning to suppress non-coding transcription in Eu- and heterochromatin. *EMBO J.* **31**, 4375–4387

59. Wang, L., Wang, S., and Li, W. (2012) RSeQC: quality control of RNA-seq experiments. *Bioinformatics* **28**, 2184–2185

60. Oh, S., Jeong, K., Kim, H., Kwon, C. S., and Lee, D. (2010) A lysine-rich region in Dot1p is crucial for direct interaction with H2B ubiquitylation and high level methylation of H3K79. *Biochem. Biophys. Res. Commun.* **399**, 512–517