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Defects in RNA quality control factors reveal RNAi-independent nucleation of heterochromatin

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Abstract

Heterochromatin assembly at fission yeast centromeres involves a self-reinforcing loop mechanism wherein chromatin-bound RNAi factors facilitate targeting of Clr4–Rik1 methyltransferase. However, the initial nucleation of heterochromatin has remained elusive. We show that cells lacking Mlo3, a protein involved in mRNP biogenesis and RNA quality control, assemble functional heterochromatin capable of promoting chromosome segregation in RNAi deficient cells. Heterochromatin restoration is linked to RNA surveillance because loss of Mlo3-associated TRAMP also rescues heterochromatin defects of RNAi mutants. Remarkably, *mlo3*Δ, which causes accumulation of bidirectional repeat-transcripts, restores Rik1 enrichment at repeats, and triggers *de novo* heterochromatin formation in the absence of RNAi. RNAi-independent heterochromatin nucleation occurs at selected euchromatic loci that show upregulation of antisense RNAs in *mlo3*Δ cells. We find that the exosome RNA degradation machinery acts parallel to RNAi to promote heterochromatin formation. These results suggest that RNAi-independent mechanisms exploit transcription and non-coding RNAs to nucleate heterochromatin.

Heterochromatin is linked to numerous cellular functions including transcriptional regulation, chromosome segregation, and suppression of recombination[1,2]. Heterochromatic regions show a distinctive pattern of histone modifications. In addition to deacetylation of histones, heterochromatin in many eukaryotes including the fission yeast *Schizosaccharomyces pombe* is marked by methylation of histone H3 at lysine 9 (H3K9me)[3]. These modifications are critical for recruitment of heterochromatin factors including HP1 proteins and for the assembly of repressive chromatin structures[1,4].

In *S. pombe*, heterochromatin is preferentially enriched across large chromosomal domains at centromeres, subtelomeres and the mating type locus[3]. These heterochromatin domains contain *dg* and/or *dh* repeats that are transcribed by RNA polymerase II (RNAPII)[1,2].

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AUTHOR CONTRIBUTIONS

F.R., K.Z. and S.G designed research. K.Z., F.R. and M.Z. performed experiments. E.C. contributed reagents. F.R., K.Z. and S.I.S.G analyzed data. F.R. and S.G. wrote the paper. F.R., K.Z. and S.G edited the paper.

Transcripts generated from *dg*/*dh* repeats are processed into siRNAs by the RNAi machinery including Argonaute (*ago1*), Dicer (*dcr1*) or RNA-dependent RNA polymerase (*rdp1*)[1,2]. Mutations in RNAi factors cause defects in H3K9me at centromeres and loss of siRNAs[1]. 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[1]. siRNA-bound Ago1, together with binding of the H3K9me by Chp1, assist in the localization of RITS to heterochromatin[5,6]. RITS facilitates targeting of Clr4, a homolog of mammalian SUV39h, which methylates H3K9 at heterochromatic loci[7,8]. Clr4 exists in a multisubunit complex, ClrC, which among other factors contains Rik1 that is critical for RNAi-dependent loading of the complex onto transcribed repeats[8]. The results showing the involvement of RNAi in targeting H3K9me and the requirement of heterochromatin factors for generation of siRNA have revealed the existence of a positive feedback loop in the assembly of heterochromatin[5,9].

H3K9me also recruits HP1 family proteins Chp2 and Swi6, which in turn promote the localization of various effectors including factors involved in chromosome dynamics and gene silencing[1]. Swi6 is required for the localization of cohesin-loading complex involved in proper segregation of chromosomes[10]. Chp2 and Swi6 also associate with Snf2–HDAC Repressor Complex (SHREC), Asf1–HIRA histone chaperone and Clr6 histone deacetylase (HDAC) complexes involved in transcriptional silencing[10–13]. Heterochromatin precludes RNAPII accessibility at target loci, but paradoxically, RNAPII transcription of *dg/dh* repeats is required to generate siRNA precursors. Centromeric repeats are transcribed preferentially during the S phase of the cell cycle when heterochromatin is more amenable to transcription[14,15]. Apart from generating siRNA precursors, RNAPII transcription may have more direct roles in heterochromatin formation. Indeed, mutations in RNAPII subunits and RNA splicing factors impair heterochromatic silencing[16–18].

RNAPII transcription has been shown to integrate multiple aspects of nuclear metabolism. Elongating RNAPII recruits chromatin-modifying activities to help remodel chromatin[19]. RNAPII machinery also recruits RNA processing factors, including factors which promote mRNP biogenesis and RNA quality control[20,21]. RNA quality control in the nucleus is monitored by multiple factors such as the TRAMP complex. TRAMP containing Cid14, a member of the Trf4 family of polyA polymerases, channels RNAs into degradation pathways including the exosome with 3′–5′ exonucleolytic activity[22]. Both the exosome and TRAMP function to degrade centromeric transcripts in *S. pombe*[23,24].

Despite major advances, the exact cascade of events that leads to the initial nucleation of heterochromatin at centromeric repeats has remained unclear. It has been argued that a new class of small RNAs, termed primal RNAs, which require Ago1 for their biogenesis, nucleate heterochromatin, and that *ago1*Δ shows H3K9me levels comparable to *clr4*Δ cells[25]. In this study, we sought to explore mechanisms that nucleate heterochromatin structures. Our analyses have uncovered an RNAi-independent pathway that exploits RNAPII transcription and non-coding RNAs (ncRNAs) to nucleate heterochromatin at centromeres and other parts of the genome. Factors involved in co-transcriptional processes including that affect RNAPII processivity influence RNAi-independent heterochromatin

assembly. We provide evidence that the exosome RNA degradation machinery acts parallel to the RNAi to mediate heterochromatin formation.

RESULTS

Loss of Mlo3 restores centromeric silencing in RNAi mutants

Mutations in RNAPII lead to defective heterochromatic silencing at centromeres[16,17]. Given that RNAPII is linked to heterochromatin modifications, we investigated if RNAPII coupled processes affect heterochromatin assembly. Loss of Mlo3, a homolog of *S. cerevisiae* Yra1 and metazoan Ref or Aly that acts at the interface of RNAPII transcription and RNA metabolism[21,26], restores centromeric silencing in RNAi deficient cells. Whereas *ago1*Δ alleviated silencing of a *ura4*$^+$ reporter inserted at an outer centromeric repeat region (*otr1R::ura4*$^+$), simultaneous deletions of *mlo3* and *ago1* restored centromeric silencing (**Fig. 1a**). The observed suppression required heterochromatin machinery because *mlo3*Δ failed to suppress the silencing defect in *clr4*Δ mutant (**Supplementary Fig. 1**).

These results suggested that loss of Mlo3 promotes heterochromatic silencing in RNAi deficient cells. We next investigated whether *mlo3*Δ restores transcriptional repression and localization of heterochromatin factors at centromeres. Chromatin immunoprecipitation (ChIP) analyses across centromere 2 (*cen2*) showed that the observed changes in silencing correlated with marked reduction in RNAPII occupancy in *mlo3*Δ *ago1*Δ cells, as compared to *ago1*Δ (**Fig. 1b**). More importantly, *mlo3*Δ restored H3K9me and Swi6 localization at *otr1R::ura4*$^+$ and endogenous centromeric repeats in *ago1*Δ mutant (**Fig. 1 c–d**). *mlo3*Δ also restored silencing and heterochromatin formation at centromeres in *dcr1*Δ mutant (**Supplementary Fig. 2a**). The rescue of H3K9me in RNAi mutants was not due to changes in histone H3 occupancy (**Supplemental Fig. 3a**) or restoration of siRNAs (**Supplementary Fig. 3b**). Together, these results demonstrate that *mlo3*Δ suppresses heterochromatin defects of RNAi mutants.

*mlo3*Δ restores functional heterochromatin in RNAi mutants

Heterochromatin facilitates the loading of cohesin essential for sister chromatid cohesion[10,27]. Defective heterochromatin in RNAi mutants causes impaired centromere cohesion, resulting in chromosome missegregation and sensitivity to the microtubule destabilizing drug thiabendazole (TBZ)[28,29]. To test whether loss of Mlo3 in RNAi mutant cells restores functional heterochromatin, we measured the TBZ sensitivity of single and double mutants. As expected, cells carrying *ago1*Δ or *dcr1*Δ showed severe sensitivity to TBZ, consistent with defective chromosome segregation in these mutants[28,29]. Combining *mlo3*Δ with *ago1*Δ or *dcr1*Δ suppressed the TBZ sensitivity of RNAi mutants (**Fig. 1e, Supplementary Fig. 2a**). ChIP analyses showed that loss of TBZ sensitivity correlates with a partial restoration of cohesin localization at centromeres in *mlo3*Δ *ago1*Δ mutant (**Fig. 1e**). Therefore, heterochromatin assembled upon loss of Mlo3 is functional, capable of supporting proper segregation of chromosomes in RNAi deficient cells.

Mlo3 interacts with centromeric transcripts

To determine whether Mlo3 directly functions at centromeres, we tested if it interacts with centromeric transcripts. As expected for a factor involved in mRNP formation[26,30], Mlo3 interacted with a euchromatic gene (*fbp1*) transcript (**Fig. 1f**). Importantly, Mlo3 also interacted with *dh* transcript (**Fig. 1f**), consistent with results of ChIP analyses showing Mlo3 enrichment at transcribing centromeric repeats[30]. This interaction was greatly enhanced in *ago1*Δ cells. Therefore, in addition to euchromatic genes, Mlo3 targets heterochromatic repeat transcripts. These data argue that Mlo3 functions at centromeric repeats and that restoration of heterochromatin in RNAi mutant cells may be coupled to its role in co-transcriptional processing of centromeric transcripts[30].

TFIIS modulates RNAi-independent heterochromatin assembly

We wondered whether loss of Mlo3, like Yra1, causes defective RNAPII transcription. To test this, we constructed *mlo3*Δ *clr3*Δ double mutant. Mutant cells lacking SHREC subunit Clr3 show marked increase in RNAPII occupancy at centromeric repeats[10,11,31]. Combining *mlo3*Δ with *clr3*Δ resulted in small decrease in RNAPII as compared to *clr3*Δ, albeit levels of H3K9me or Swi6 at repeat elements were comparable in *clr3*Δ and *clr3*Δ *mlo3*Δ (**Supplementary Fig. 4a–c**). The change in RNAPII levels led us to wonder if defective RNAPII transcription is partially responsible for restoration of heterochromatin in RNAi mutants. We tested this possibility by deleting *tfs1* gene encoding the *S. pombe* homolog of TFIIS, a factor known to affect RNAPII processivity[32]. Deletion of *tfs1*, which led to 6-azauracil (6-AU) sensitivity (**Fig. 2a**)[33] and changes in the distribution of RNAPII at body of genes (**Supplementary Fig. 3c**), resulted in variegated suppression of silencing defects in *ago1*Δ and *dcr1*Δ mutants (**Fig. 2b** and **Supplementary Fig. 2b**). This suppression was more pronounced in *M* mating-type (*mat1-Msmt0*) cells and resulted in decreased RNAPII occupancy at centromeric repeats (**Fig. 2c**). *tfs1*Δ-dependent silencing required Clr4 (**Supplementary Fig. 1**) and was accompanied by an increase in levels of H3K9me and Swi6 at both *otr1R::ura4*$^{+}$ and centromeric repeats (**Fig. 2d–e** and **Supplementary Fig. 2b)**. The increase in H3K9me was not due to changes in histone H3 occupancy or restoration of siRNAs (**Supplementary Fig. 3a–b**). *tfs1*Δ suppressesed TBZ sensitivity and partially restored cohesin localization at centromeres in RNAi mutants (**Fig. 2f–g** and **Supplementary Fig. 2b**).

These results suggest that cells with compromised RNAPII transcription rescue heterochromatin defects caused by loss of RNAi machinery. Consistently, we found that loss of deubiquitylating enzyme Ubp3, which causes elevated levels of RNAPII ubiquitylation and degradation[34], partially suppressed silencing and heterochromatin defects of *ago1*Δ mutant (**Supplementary Fig. 5a–b).**

mlo3 and *tfs1* differentially affect heterochromatin assembly

Loss of Clr3 and RNAi factors causes a dramatic loss of H3K9me across pericentromeric domains (**Fig. 3a–d** and **Supplementary Fig. 6a–b**)[31]. To gain more insight into effects of *mlo3*Δ and *tfs1*Δ on heterochromatin formation in the absence of RNAi, we investigated their effects on heterochromatin modifications in *clr3*Δ *ago1*Δ double mutant cells. *tfs1*Δ

failed to restore H3K9me at *otr1R::ura4*$^{+}$ in *clr3*Δ *ago1*Δ cells (**Fig. 3a**). In contrast, *mlo3*Δ resulted in considerable restoration of H3K9me at centromeres in *clr3*Δ *ago1*Δ cells (**Fig. 3b**). These differences in H3K9me were not limited to *otr1R::ura4*$^{+}$. ChIP-chip analyses across centromere 2 showed that *mlo3*Δ, but not *tfs1*Δ, restored H3K9me across the entire pericentromeric region in *clr3*Δ *ago1*Δ cells (**Fig. 3c–d**, **Supplementary Fig. 6a–b**). *mlo3*Δ also decreased in RNAPII occupancy across pericentromeric domains in *clr3*Δ *ago1*Δ cells (**Supplementary Fig. 6c**), while *tfs1*Δ had only a minor effect (**Supplementary Fig. 6d**).

Differences in *mlo3*Δ and *tfs1*Δ mutants were also evident in their effects on hairpin RNA-triggered heterochromatin formation. Despite the prominent role of RNAi in silencing of centromeric repeats, this pathway is constrained and rarely targets detectable levels of heterochromatin *in trans*[35,36]. Expression of hairpin complementary to the *trp1*$^{+}$ (*trp1-HP*) failed to elicit H3K9me in *tfs1*Δ cells but induced H3K9me both *in cis* and *in trans* in *mlo3*Δ cells, dependent on RNAi (**Supplementary Fig. 7a–c**). Treating cells with 6-AU, which affects RNAPII transcription, caused increased H3K9me both in *mlo3*Δ and *tfs1*Δ cells but again the effect was stronger in *mlo3*Δ (**Supplementary Fig.7a–c**). Together, these data highlight differential effects of *tfs1*Δ and *mlo3*Δ on heterochromatin formation at centromeres and at an ectopic site. Moreover, these analyses suggest that additional RNAi and SHREC independent mechanism(s) nucleate heterochromatin at centromeres.

*cid14*Δ rescues heterochromatin defects of *ago1*Δ mutant

Mlo3 forms a complex with TRAMP, which is involved in RNA surveillance and degradation of centromeric transcripts[22,24,30]. We therefore investigated whether *mlo3*Δ-mediated suppression of heterochromatin defects in RNAi mutants is linked to defects in RNA surveillance. Remarkably, loss of Cid14 subunit of TRAMP affects RNAi-independent heterochromatin formation in a manner similar to *mlo3*Δ. Loss of Cid14 restored H3K9me at *otr1R::ura4*$^{+}$ and centromeric repeats in *ago1*Δ mutant (**Fig. 4a–b**). Moreover, *cid14*Δ suppressed the silencing defect caused by *ago1*Δ, as indicated by reduction in the levels of *dg/dh* transcript in *cid14*Δ *ago1*Δ as compared to *ago1*Δ (**Fig. 4c**). These results suggest that defects in RNA surveillance mechanisms involving Mlo3-associated TRAMP, promote heterochromatin formation independent of RNAi.

Exosome acts parallel to RNAi to nucleate heterochromatin

As mentioned above, Mlo3–TRAMP channels centromeric transcripts to downstream-acting RNA degradation factors including the exosome[24,30]. However, these factors share a complex genetic relationship indicative of their diversified functions. Unlike single mutants, double mutants containing null alleles of Mlo3–TRAMP and the exosome subunit *rrp6* show synthetic lethality (our unpublished data). In light of these observations and previous results showing that aberrant RNAs sequestered near site of transcription are degraded by the exosome[37,38], it was of interest to investigate whether the loss of Rrp6 affects centromeric heterochromatin. Northern and RT-PCR analyses using single and double mutants showed a large increase in centromeric repeat transcripts in *rrp6*Δ *ago1*Δ mutant as indicated by both Northern blot and RT-PCR analyses (**Fig. 5a–b**). This result is in marked contrast to the restoration of silencing observed in *cid14*Δ *ago1*Δ mutant (**Fig. 4**). We next investigated whether *rrp6*Δ alone or in combination with *ago1*Δ affects centromeric

heterochromatin assembly. Unlike *mlo3*Δ and *cid14*Δ, combining *rrp6*Δ with *ago1*Δ largely abolished H3K9me levels at *otr1R::ura4*$^{+}$ and *dg* repeats (**Fig. 5c**). Furthermore, ChIP-chip showed severe cumulative loss of H3K9me across the entire pericentromeric domain in *rrp6*Δ *ago1*Δ mutant, as compared to *rrp6*Δ or *ago1*Δ (**Fig. 5d**). Together, these data reveal the nuclear exosome acts parallel to RNAi to mediate heterochromatin assembly at centromeres.

De novo heterochromatin assembly in *mlo3*Δ cells

*mlo3*Δ restored H3K9me both in *ago1*Δ and *clr3*Δ *ago1*Δ cells. We next tested whether *mlo3*Δ, which causes accumulation of transcripts in the nucleus (**Supplementary Fig. 8**), can trigger *de novo* heterochromatin assembly in the absence of RNAi. For this purpose, we employed strains that carry either *clr4*Δ alone or in combination with *ago1*Δ or *ago1*Δ *mlo3*Δ. Since Clr4 is the sole H3K9 methyltransferase in *S. pombe*, these mutant strains lack H3K9me (**Fig. 6a**). We transformed the mutant strains with a plasmid containing *clr4*$^{+}$ and monitored H3K9me levels at centromeres by ChIP. Introduction of *clr4*$^{+}$ led to restoration of H3K9me at centromeres in *clr4*Δ single mutant but not in *clr4*Δ *ago1*Δ double mutant (**Fig. 6a**). Remarkably, H3K9me could be readily detected at centromeres in *clr4*Δ *ago1*Δ *mlo3*Δ cells upon introduction of the *clr4*$^{+}$ (**Fig. 6a**). These data demonstrate that loss of Mlo3, involved in processing of centromeric transcripts[30], triggers *de novo* heterochromatin assembly at centromeres, independent of RNAi.

We next tested if *mlo3*Δ affects localization of ClrC, which requires RNAi for its targeting to centromeric repeats[7,8,14]. In particular, ClrC subunit Rik1 is recruited to centromeres during S-phase, when both forward and reverse strands of centromeric repeats are transcribed[14]. We probed the effect of *mlo3*Δ on Rik1 enrichment at *cenH*, which is homologous to *dg/dh* and serves as an RNAi-dependent heterochromatin nucleation center at *mat* locus[39,40]. *cenH* was selected because defects in RNAi abolish Rik1 ChIP enrichment at this site without affecting heterochromatin structure nucleated by redundant mechanisms[8]. This regime ensures that Rik1 enrichment is not indirectly altered by changes in heterochromatin modifications in *mlo3*Δ. As expected, *ago1*Δ abolished Rik1 ChIP enrichment at *cenH* (**Fig. 6b**). However, we found that simultaneous deletion of *mlo3* and *ago1* restored Rik1 enrichment at *cenH* (**Fig. 6b**). Given the connection between transcript levels and Rik1 localization[8], we tested if *mlo3*Δ affects *cenH* transcripts. Levels of forward and reverse transcripts originating from *cenH* were elevated considerably in *mlo3*Δ cells (**Fig. 6b**). Together, these results demonstrate that *mlo3*Δ, which causes accumulation of bidirectional *cenH* transcripts, bypasses the RNAi requirement for targeting Rik1.

*mlo3*Δ induces H3K9me at euchromatic loci

Since cells lacking Mlo3 accumulate antisense RNAs at euchromatic loci[30], we wondered whether heterochromatin could be assembled at euchromatic loci in *mlo3*Δ cells. Our analyses showed that loss of Mlo3 induces H3K9me at selected euchromatic genes (**Fig. 7a**), which show increased accumulation of antisense transcripts in mutant cells (**Fig. 7b**). Notably, H3K9me at these loci was not abolished when *mlo3*Δ cells was combined with *ago1*Δ (**Fig. 7a**). Therefore, the targeting of H3K9me occurs even in the absence of RNAi (**Fig. 7a**). We also analyzed a locus showing overlapping sense and antisense transcripts that

were unaffected by *mlo3*Δ. However, H3K9me could not be detected at this site (**Fig. 7b**). One possibility is that the retention of transcripts at transcribed loci such as observed in RNA surveillance mutants[37,38], is necessary to generate signals for RNAi-independent heterochromatin formation. Regardless, it is interesting that *mlo3*Δ, which causes accumulation of antisense transcripts, results in heterochromatin modifications at euchromatic loci.

DISCUSSION

Heterochromatin assembly is a complex multistep process that involves a variety of factors[1]. Studies from diverse systems have suggested a prominent role for transcription and ncRNAs in heterochromatin assembly[1,41,42]. In *S. pombe*, RNAPII transcription of centromeric repeats provides scaffolds for heterochromatin formation. This process involves RNAi, which not only processes repeat transcripts but also mediates the loading of heterochromatin factors[7,8]. Despite the prominent role for RNAi in heterochromatin assembly, loss of RNAi factors does not completely abolish heterochromatin modifications such as H3K9me at centromeric repeats[31,43,44]. Similarly, evidence from other systems suggest that defects in RNAi has no major effects on heterochromatin modifications[45,46], although in some of these cases transcription and ncRNAs could be involved[42,47]. Our analyses suggest that RNAPII transcription and ncRNAs function to target heterochromatin via an RNAi-independent mechanism.

We demonstrate that defects in cotranscriptional RNA surveillance or factors that affect RNAPII processivity bypass the RNAi requirement to assemble functional heterochromatin. Besides loss of Mlo3 or Cid14, *tfs1*Δ restores heterochromatin in cells lacking the RNAi machinery. Distinct mechanisms are likely responsible for *mlo3*Δ- or *tfs1*Δ-mediated suppression of heterochromatin defect in RNAi mutants, despite similarities in phenotypes. We note that *mlo3*Δ, but not *tfs1*Δ, rescues H3K9me in *clr3*Δ *ago1*Δ double mutant deficient in heterochromatin modifications at centromeres[31]. Also, mutations in these factors differentially affect hairpin-induced H3K9me at an ectopic site. Given that the RNAi machinery interacts with RNAPII and modulates transcription in other organisms[48,49], defective RNAPII elongation could directly contribute to bypassing of RNAi. Tfs1 may affect heterochromatin by influencing processing of transcripts and/or release of RNAPII. Indeed, cells lacking Ubp3, which affects RNAPII stability[34], show partial rescue heterochromatin defects in *ago1*Δ cells. Another possibility is that impaired transcription affects chromatin dynamics by precluding elongation-coupled turnover of histones and/or their modification state[50,51]. The binding of factors, such as Clr4[8], to residual histones decorated with H3K9me could shift the dynamic equilibrium and stabilize and/or propagate heterochromatin *in cis*[40].

Mlo3 interacts with TRAMP[30], and these factors are required for processing centromeric transcripts and antisense RNAs[23,24,30]. It is therefore interesting that loss of either Mlo3 or TRAMP suppresses the heterochromatin defects in *ago1*Δ or *dcr1*Δ mutants which are deficient in siRNA production. *cid14*Δ causes severe reduction in siRNAs without altering H3K9me levels, leading to suggestion that low level of siRNAs are sufficient to nucleate heterochromatin[24]. Our results suggest that *cid14*Δ activates RNAi-independent

heterochromatin assembly pathway(s), which might also be triggered by the accumulation of RNAs produced by multiple copy sequences in other systems[52]. We show that the targeting of ClrC subunit Rik1 to *dg/dh* repeats, which normally requires RNAi[8], is restored in *mlo3*Δ cells showing elevated levels of forward and reverse repeat transcripts. Furthermore, *mlo3*Δ triggers *de novo* targeting of heterochromatin to centromeric repeats independent of RNAi. Remarkably, loss of Mlo3 also causes RNAi-independent targeting of H3K9me at selected euchromatic loci, which show accumulation of antisense transcripts in *mlo3*Δ mutant. These results argue that ncRNAs generated by centromeric repeats and certain euchromatic loci assemble heterochromatin by mechanism(s) independent of RNAi. Consistent with the existence of such pathway(s) that relies of accumulation of RNAs, we have found that the exosome involved in degradation of aberrant RNAs[22], acts in a pathway parallel to RNAi to mediate heterochromatin formation at centromeres.

How do accumulations of transcripts caused by loss of Mlo3–TRAMP impact heterochromatin assembly in RNAi mutants? One possibility is that ncRNAs accumulated at the sites of transcription recruit the exosome degradation machinery that in turn facilitates loading of heterochromatin factors in a manner similar to the RNAi, in which targeting of ClrC is linked to the processing of repeat transcripts *in cis*[5]. RNAi-dependent nucleation of heterochromatin involves RITS, which interacts with ClrC[7,8]. Indeed, RITS tethering to transcripts can induce heterochromatin formation[53]. However, this process still requires Dcr1, suggesting that additional siRNA-dependent steps, perhaps the generation of double stranded RNA (dsRNA) or other RNA structures, is necessary for nucleating heterochromatin. In this regard, loss of Mlo3–TRAMP, acting cotranscriptionally, could bypass RNAi requirement by generating dsRNA or yet undefined RNA signals capable of loading Clr4–Rik1. Such signals would be distinct from the recently described primal RNAs that require Ago1 for their biogenesis[25]. It is possible that mechanism(s) that trigger H3K9me in *mlo3*Δ or *cid14*Δ mutant are activated during S-phase when both strands of *dg/dh* repeat are transcribed, correlating with targeting of Rik1[14]. Regardless of the mechanism, it is clear that an RNAi-independent pathway(s) exists that relies on ncRNAs to target heterochromatin.

Since transcription and ncRNAs have been linked to epigenetic chromatin modifications in multiple organisms including mammals[42,47,54–56], our results may have general significance. In several instances, transcription and ncRNAs can modify chromatin independent of RNAi. For example, RNAPII transcription and ncRNAs trigger chromatin modifications and parental imprinting in mammals[54,57] In such cases, RNAs retained in the nucleus act largely *in cis*. The retention of bidirectional transcripts near their transcription sites might facilitate the localization of chromatin modifying activities. To this end, widespread transcription of eukaryotic genomes[41] might allow RNAPII and ncRNAs to function as a molecular sensors that specify certain genomic regions as preferential targets for repressive chromatin assembly. Such domains might include transposons that when uncontrolled can lead to genome instability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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METHODS

Yeast strains

Constructions of deletion or epitope tagged strains were done by standard PCR based methods. The *tfs1*Δ was a gift from N. Krogan (UCSF). Genetic crosses were used to construct double mutants. To test the requirement of RNAi in establishment of H3K9me in *mlo3*Δ strain, *clr4*$^+$ was subcloned into pWH5 (*pclr4*$^+$) and expressed under its endogenous promoter. Plasmids pREP3 (empty vector control) and *pclr4*$^+$ were introduced by transformation in the indicated strains. Most strains used contained *mat1-Msmt0* mating-type allele because these cells showed more pronounced suppression of RNAi mutants by *mlo3*Δ and *tfs1*Δ.

Chromatin immunoprecipitation (ChIP) and ChIP-chip

ChIP and ChIP-chip experiments were performed as previously described[3]. To test the requirement of RNAi in initiation of H3K9me at centromeres in *mlo3*Δ strains, cells were grown in EMM-Leu media. For the remaining ChIPs, cells were grown in rich media (YEA). Antibodies used were anti-Ser2phospho RNAPII (3E10) (ref. 58), anti-H3K9me (Abcam 1220), anti-Swi6 (ref. 3), anti-Flag (M2)-conjugated agarose (Sigma), anti-HA (12CA5, Covance), and anti-myc (A14, Santa Cruz).

RT-PCR and Northern blot analysis

For RT-PCR, total RNA was extracted from cells using the Master Pure™ Yeast RNA purification kit (Epicentre). One-hundred nanograms of RNA was amplified using the One-step RT-PCR kit (Qiagen) and strand specific primers. Northern blots were performed as described previously[59]

RNA immunoprecipitation (RIP)

RIP was performed as described previously[60] with following modifications. The final ethanol precipitated RNA pellets were resuspended in 100 μl of 1X DNaseI buffer (Amplification grade, Invitrogen) and treated with 3 units DNaseI (Amplification Grade) at 37°C for one hour. The treated RNA samples were phenol/chloroform extracted, ethanol precipitated, and resuspended in 20 μl DEPC treated H_2O. 1μl from each sample was subjected to RT-PCR analysis. RT-PCR was performed using the one-step RT-PCR kit from Qiagen with centromeric *dh* or *fbp1* primers.

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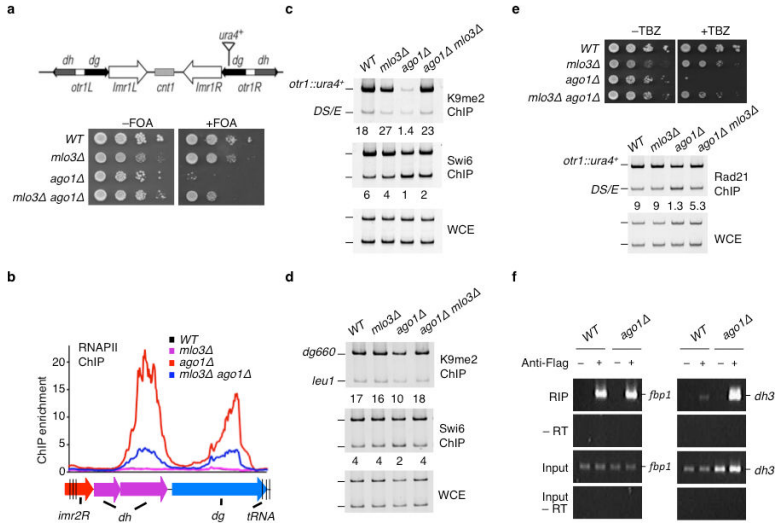

Figure 1. *mlo3*Δ restores functional heterochromatin at centromeres in *ago1*Δ mutant

(**a**) *mlo3*Δ suppresses the silencing defect at *otr1R::ura4*$^+$ in *ago1*Δ cells. Location of *ura4*$^+$ reporter inserted within pericentromeric region is shown. Serial dilutions of the indicated strains were spotted onto non-selective (-FOA) or counter-selective FOA-containing (+FOA) media to assay *ura4*$^+$ expression. FOA, 5-fluoroacetic acid. (**b**) *mlo3*Δ decreases RNAPII occupancy at centromeric repeats in *ago1*Δ cells. ChIP-chip using Ser2phospho RNAPII antibody was used to assay RNAPII occupancy. RNAPII levels are plotted in alignment with the right pericentromeric region of *cen2*. (**c**) *mlo3*Δ restores centrometric heterochromatin in *ago1*Δ cells. ChIP analysis of H3K9me and Swi6 at *otr1R::ura4*$^+$. DNA isolated from immunoprecipitated chromatin (ChIP) or whole-cell crude extracts (WCE) was analyzed by PCR. Relative fold enrichments depicting the ratios of signals at *otr1R::ura4*$^+$ locus relative to the euchromatic *ura4DS/E* (*DS/E*) locus, between ChIP and WCE, are shown underneath each lane. (**d**) *mlo3*Δ restores heterochromatin at *dg* repeats in *ago1*Δ cells. H3K9me2 and Swi6 enrichments at *dg* repeats relative to *leu1* were determined by ChIP. (**e**) *mlo3*Δ suppresses TBZ sensitivity and restores cohesin localization at centromeres in *ago1*Δ mutant. Localization of cohesin subunit Rad21 (Rad21-HA) at *otr1R::ura4*$^+$ was assessed by ChIP (bottom). **(f)** Mlo3 interacts with *dh* transcripts. Interaction of Mlo3 with the *fbp1* transcripts, used as a control, and *dh* transcripts was determined by RNA-IP. RNA isolated from immunoprecipitated Mlo3-Flag (Flag RIP) or whole cell extract (input) was analyzed by RT-PCR. –RT, no reverse transcription.

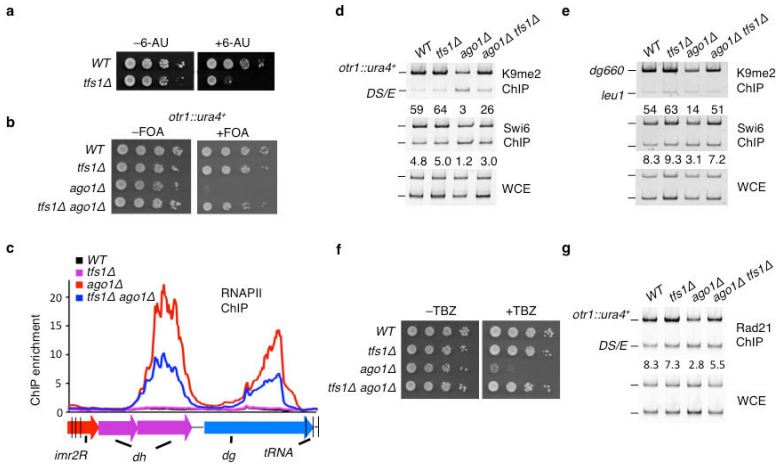

Figure 2. *tfs1*Δ restores centromeric heterochromatin in *ago1*Δ cells

(**a**) *tfs1*Δ renders cells sensitive to 6-AU. (**b**) *tfs1*Δ suppresses the silencing defect at *otr1R::ura4*$^+$ in *ago1*Δ cells. Serial dilutions of WT and mutant strains carrying *mat1-Msmt0* mating-type allele were spotted onto the indicated media to assay *otr1R::ura4*$^+$ expression. (**c**) *tfs1*Δ decreases RNAPII occupancy at centromeric repeats in *ago1*Δ cells. RNAPII occupancy at *dh*/*dg* repeats in centromere 2 was determined by ChIP-chip using Ser2 phospho RNAPII antibody. (**d**) *tfs1*Δ restores heterochromatin at *otr1R::ura4*$^+$ in *ago1*Δ cells. Relative fold enrichments of H3K9me and Swi6 at *otr1R::ura4*$^+$ were determined by ChIP. **e**, *tfs1*Δ restores heterochromatin at *dg* repeats in *ago1*Δ cells. H3K9me and Swi6 enrichments at *dg* repeats were determined by ChIP. (**f**) *tfs1*Δ suppresses TBZ sensitivity of *ago1*Δ cells. (**g**) *tfs1*Δ restores cohesin localization to centromeres. Localization of Rad21-HA at *otr1R::ura4*$^+$ was assessed by ChIP.

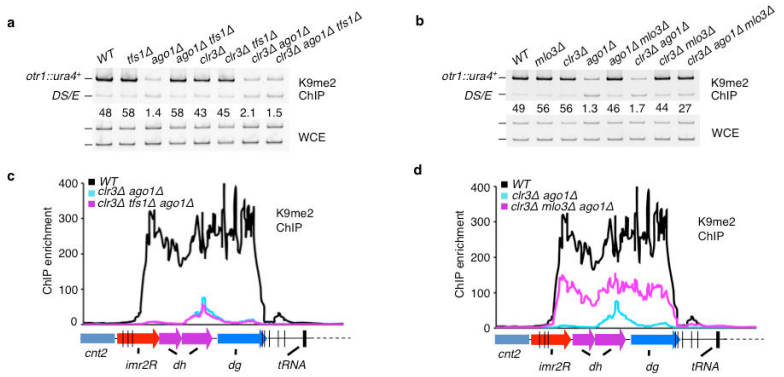

Figure 3. *tfs1*Δ and *mlo3*Δ differentially suppress heterochromatin defects in *clr3*Δ*ago1*Δ double mutant cells

(**a–d**) *mlo3*Δ, but not *tfs1*Δ, restores H3K9me at centromeres in *clr3*Δ *ago1*Δ cells. (**a–b)** Relative fold enrichments of H3K9me at *otr1R::ura4*$^{+}$ were determined by ChIP. (**c–d**) H3K9me levels across pericentromeric domains of *cen2* as determined by ChIP-chip.

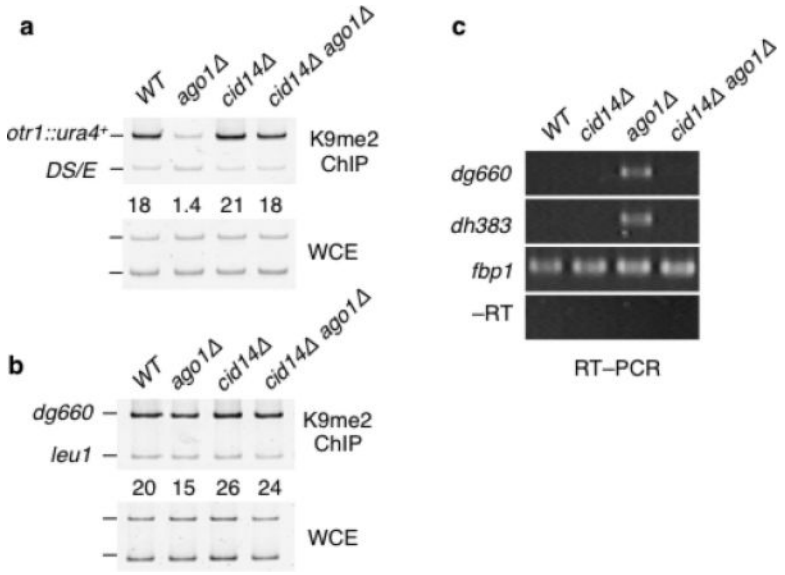

Figure 4. Loss of RNA surveillance factor TRAMP restores centromeric heterochromatin in *ago1*Δ cells

(**a–b**) *cid14*Δ restores heterochromatin at *otr1R::ura4*$^{+}$ and *dg* repeats in *ago1*Δ cells. H3K9me levels at *otr1R::ura4*$^{+}$ and *dg* were determined by ChIP. (**c**) *cid14*Δ suppresses centromeric silencing defect in *ago1*Δ cells. RT-PCR analysis of *dg* and *dh* transcripts in the indicated strains is shown. *fbp1* transcripts were assayed as an amplification control.

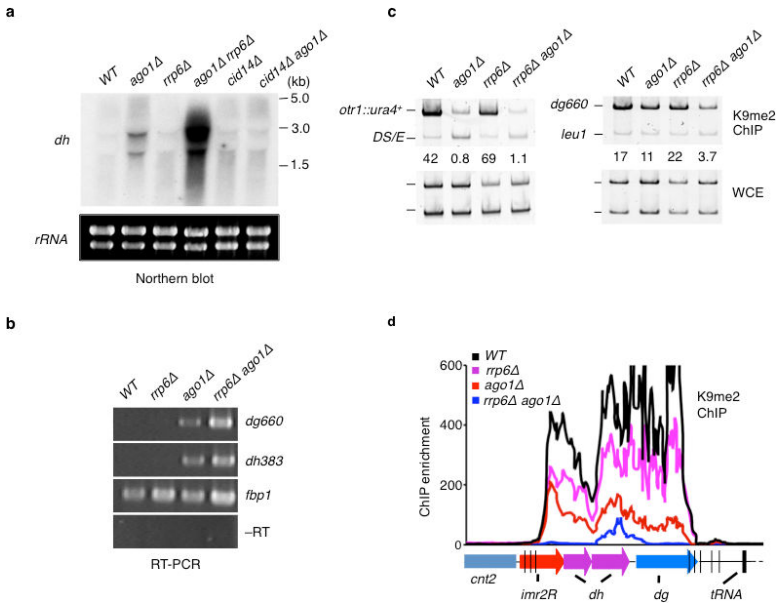

Figure 5. Rrp6 acts parallel to RNAi to mediate heterochromatin formation and silencing at centromeres

(**a**) Cid14 and Rrp6 differentially affect *dh* expression in *ago1*Δ cells. Northern blot analysis of *dh* transcripts in WT and mutant cells. rRNA was used as a loading control. (**b**) *rrp6*Δ and *ago1*Δ cause cumulative increase in *dg* and *dh* transcript levels. RT-PCR analysis of *dg* and *dh* transcript levels in the indicated strains is shown. *fbp1* transcripts were assayed as an amplification control. (**c**) Loss of Rrp6 in *ago1*Δ cells severely affects H3K9me at centromeres. H3K9me enrichment at *otr1R::ura4*$^{+}$ and *dg* repeats were determined by ChIP. (**d**) Deletions of *rrp6* and *ago1* cause cumulative loss in H3K9me across pericentromeric domains, as determined by ChIP-chip.

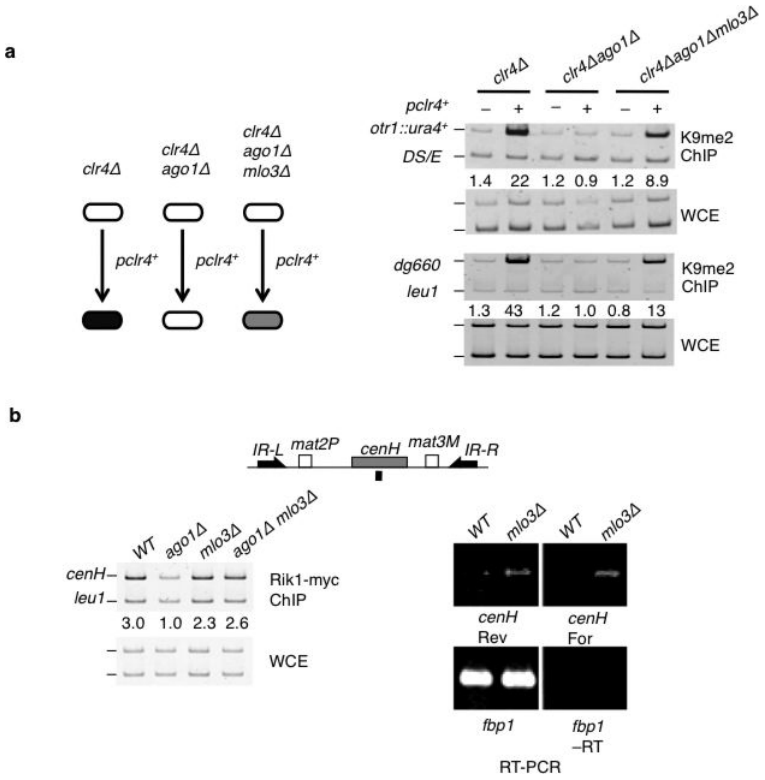

Figure 6. *mlo3*Δ restores Rik1 enrichment at centromeric repeats and triggers *de novo* heterochromatin formation in the absence of RNAi

(**a**) *mlo3*Δ induces establishment of heterochromatin at centromeres in the absence of Ago1. Indicated mutant strains were transformed with a plasmid containing *clr4*$^{+}$ gene. Levels of H3K9me were assayed by ChIP at *otr1R::ura4*$^{+}$ and *dg* repeats in the indicated strains. (**b**) *mlo3*Δ causes increased accumulation of bidirectional transcripts and restores Rik1 ChIP enrichment at *cenH* in *ago1*Δ mutant. Schematic representation indicating the location of *cenH* and primers (black bar) used is shown (upper). ChIP analysis of Rik1-myc at *cenH* (left). RT-PCR analysis of *cenH* transcripts (right). *fbp1* transcripts were assayed as an amplification control.

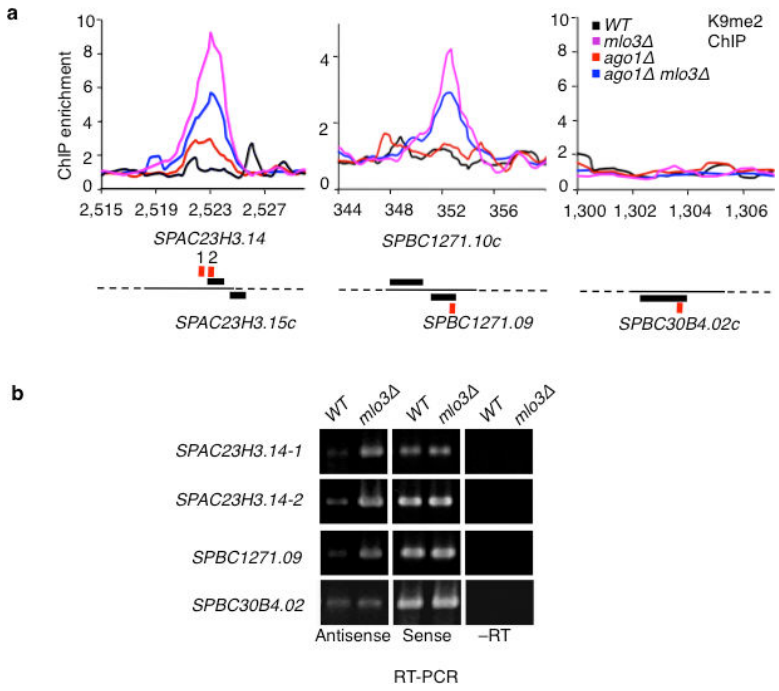

Figure 7. RNAi-independent heterochromatin formation occurs at euchromatic loci in *mlo3*Δ cells

(**a**) Appearance of H3K9me in *mlo3*Δ cells correlates with the accumulation of antisense transcripts. Relative fold enrichment of H3K9me was determined by ChIP-chip in WT and mutant strains. Red bars indicate locations of primers used for RT-PCR in '**b**'. (**b**) RT-PCR analysis of sense and antisense at indicated loci in WT and *mlo3*Δ cells.