

# Conserved factor Dhp1/Rat1/Xrn2 triggers premature transcription termination and nucleates heterochromatin to promote gene silencing

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**Cotranscriptional RNA processing and surveillance factors mediate heterochromatin formation in diverse eukaryotes. In fission yeast, RNAi machinery and RNA elimination factors including the Mtl1–Red1 core and the exosome are involved in facultative heterochromatin assembly; however, the exact mechanisms remain unclear. Here we show that RNA elimination factors cooperate with the conserved exoribonuclease Dhp1/Rat1/Xrn2, which couples pre-mRNA 3'-end processing to transcription termination, to promote premature termination and facultative heterochromatin formation at meiotic genes. We also find that Dhp1 is critical for RNAi-mediated heterochromatin assembly at retroelements and regulated gene loci and facilitates the formation of constitutive heterochromatin at centromeric and mating-type loci. Remarkably, our results reveal that Dhp1 interacts with the Clr4/Suv39h methyltransferase complex and acts directly to nucleate heterochromatin. Our work uncovers a previously unidentified role for 3'-end processing and transcription termination machinery in gene silencing through premature termination and suggests that noncanonical transcription termination by Dhp1 and RNA elimination factors is linked to heterochromatin assembly. These findings have important implications for understanding silencing mechanisms targeting genes and repeat elements in higher eukaryotes.**

heterochromatin | Dhp1/Xrn2 | *S. pombe* | transcription termination

Heterochromatin is a repressive form of chromatin that is critical for fundamental functions of eukaryotic genomes (1). Heterochromatin assembly pathways are highly conserved in eukaryotes and have been particularly well studied in the fission yeast *Schizosaccharomyces pombe* (1). Heterochromatin formation involves conserved histone-modifying enzymes. In addition to deacetylation of histones by histone deacetylases (HDACs), histone H3 is methylated on lysine 9 (H3K9me) by the Clr4/Suv39h family of methyltransferases (2). Methylated H3K9 is bound by members of the conserved family of HP1 proteins, which in turn associate with diverse effectors implicated in transcriptional and posttranscriptional silencing, proper segregation of chromosomes, and maintenance of genome stability (1, 3).

RNA polymerase II (RNAPII) transcriptional machinery and proteins involved in cotranscriptional processing of RNAs play important roles in targeting histone-modifying activities to nucleate heterochromatin at specific regions of the *S. pombe* genome (4–6). In particular, RNAi machinery is believed to function in the assembly of major constitutive heterochromatin domains coating centromeres, telomeres, and the mating type (*mat*) locus (7–9). RNAi-dependent mechanisms also assemble dynamically regulated facultative heterochromatin domains, hereafter referred to as “HOODs”, at discrete sites across the genome, including at developmental genes and retrotransposons (10). HOOD assembly is triggered by the specialized nuclear RNA processing and surveillance complex Mtl1–Red1 core (MTREC) and/or associated mRNA 3'-end processing activities, including the canonical poly (A) polymerase Pla1 and the poly(A)-binding protein Pab2, to

direct transcripts into RNA degradation pathways and target heterochromatin assembly (10, 11). Notably, HOODs are detected under certain growth conditions or when the 3'-to-5' exoribonuclease subunit Rrp6 of the nuclear exosome is compromised (10, 12).

RNA-processing factors such as MTREC also promote RNAi-independent assembly of facultative heterochromatin islands, which are modified in response to nutritional signals such as nitrogen starvation (11, 13–15). Heterochromatin islands preferentially target meiotic genes that are silenced during vegetative growth (15). Meiotic mRNAs that contain a determinant of selective removal (DSR) are recognized by the sequence-specific RNA-binding protein Mmi1 (16). Mmi1 in turn recruits MTREC and the Pir1/Iss10 protein to promote exosome-mediated elimination of transcripts and to recruit the Clr4 methyltransferase for heterochromatin island assembly (11, 13, 15, 17–20). MTREC also localizes to regions of the genome that do not assemble heterochromatin, suggesting that additional factors are needed to direct the formation of heterochromatin islands (11). Indeed, the exact mechanisms responsible for specifying MTREC-mediated heterochromatin assembly have remained unclear.

Here we report that RNA elimination factors promote premature transcription termination at facultative heterochromatin islands. This noncanonical transcription termination by RNA

## Significance

**RNA-mediated heterochromatin assembly requires transcription by RNA polymerases and a plethora of RNA-processing factors. However, the mechanisms that differentiate productive gene transcription from transcriptional activity linked to the assembly of repressive heterochromatin have remained elusive. Here we find that factors promoting noncanonical termination of RNA polymerase II transcription trigger heterochromatin assembly. We propose that termination and 3'-end processing factors serve as molecular sensors that, in addition to determining the fate of target transcripts, mediate heterochromatin assembly. Importantly, these findings provide insights into the paradoxical interdependence of heterochromatin assembly and transcription of target loci and suggest a mechanism for specifying certain genomic regions, including developmental genes and repeat elements, as targets for heterochromatin assembly.**

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elimination factors requires the conserved nuclear exoribonuclease, Dhp1. The functional connection between Dhp1 and RNA elimination factors extends beyond meiotic heterochromatin islands and also is required for the production of small RNAs and RNA-mediated assembly of HOODs at genes and retrotransposons. In addition, Dhp1 contributes to silencing and heterochromatin formation at constitutive heterochromatin domains such as at centromeres and the *mat* region. Our results show that Dhp1 associates with the Clr4 complex. These analyses suggest that the conserved termination factor Dhp1 plays a central role in transcription-dependent heterochromatin assembly pathways.

**Results**

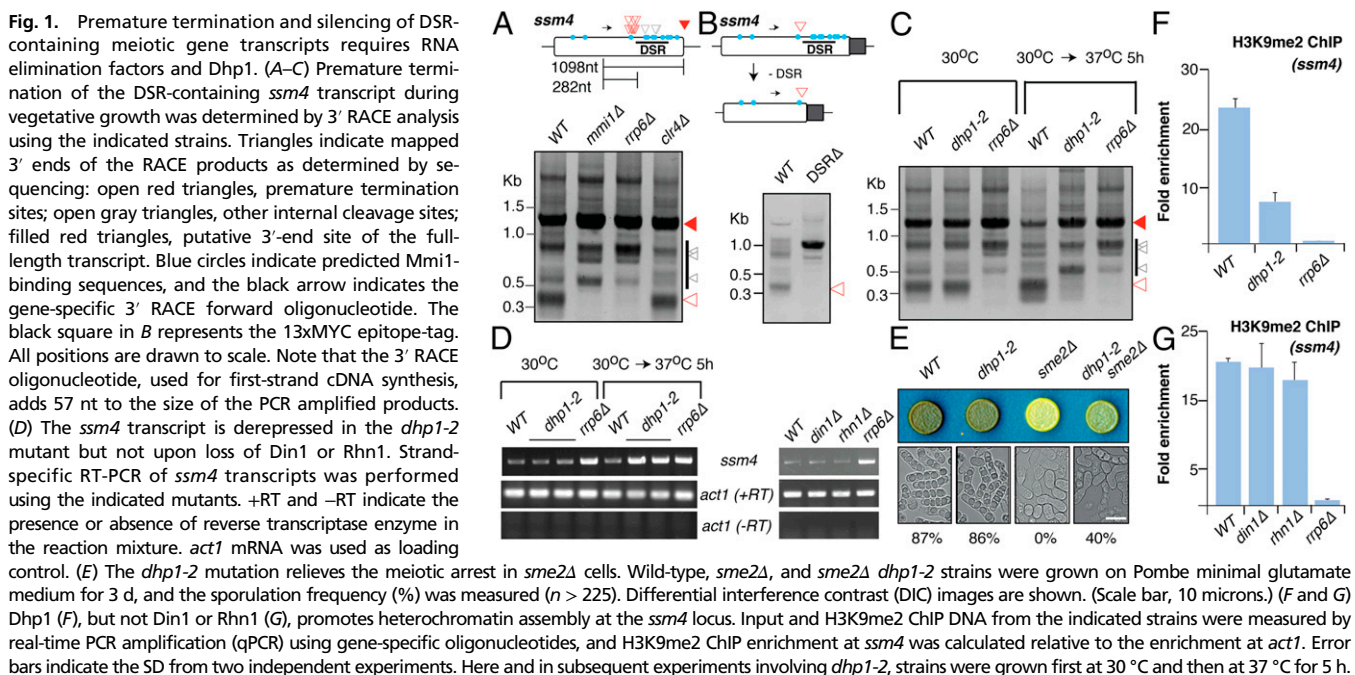
**DSR-Containing Meiotic Genes Are Targeted for Premature Transcription Termination.** RNA elimination factors required for meiotic gene silencing have been shown to colocalize with 3'-end processing factors (18). To investigate whether meiotic gene silencing involves inappropriate 3'-end formation, we mapped the 3' ends of *ssm4* meiotic gene transcripts in vegetative cells using 3' RACE. Our analyses of polyadenylated *ssm4* transcripts revealed short, unstable RNA species in addition to the full-length transcript (Fig. 1A). Sequencing of the 3' RACE products revealed that the 3' ends of these abbreviated transcripts mapped adjacent to Mmi1-binding sites in and around the DSR (denoted by open red and gray triangles in Fig. 1A), indicating internal cleavage events leading to premature transcription termination. Importantly, deletion of the DSR in *ssm4* abolished the premature termination that normally occurs upstream of the DSR (Fig. 1B). We also detected premature termination at other DSR-containing genes such as *mcp5* and, to a lesser extent, at *rec8* and *spo5* loci (Fig. S1A). In contrast, the *leu1* gene, which lacks a DSR, produced only a full-length transcript (Fig. S1B). These results suggest that DSR-containing meiotic genes are selectively targeted for premature termination.

**RNA Elimination Factors Promote Premature Transcription Termination.** Given the functional connection between 3'-end processing and degradation factors (18–21), we asked whether the elimination factors Mmi1 and Rrp6 are required for premature termination of DSR-containing gene transcripts. Remarkably, the loss of Mmi1 or Rrp6 prevented premature termination of *ssm4* and *mcp5*

transcripts upstream of the DSR (Fig. 1A and Fig. S1A). Because RNA elimination factors promote the assembly of facultative heterochromatin on meiotic genes (13–15), premature termination could be mediated through heterochromatin formation. Strikingly, we found that the loss of Clr4, the sole H3K9 methyltransferase in *S. pombe* that is essential for formation of heterochromatin islands (15), did not affect premature transcription termination (Fig. 1A and Fig. S1A). These results reveal that premature termination likely precedes Clr4-mediated heterochromatin assembly and show that RNA elimination factors, which are involved in heterochromatin assembly, are also required for premature termination.

**Dhp1 Promotes Premature Termination and Silencing of Meiotic Genes to Prevent Untimely Sexual Differentiation.** We identified Dhp1 as a factor that affects meiotic gene silencing (see below). Dhp1 is a conserved and essential protein related to *Saccharomyces cerevisiae* Rat1 and human Xrn2 that is implicated in coupling 3'-end processing to transcription termination (22–27). To test whether premature termination at meiotic genes requires Dhp1, we performed 3' RACE of *ssm4* in a mutant carrying the temperature-sensitive hypomorphic allele, *dhp1-2* (Materials and Methods and Fig. S1C). Strikingly, at the restrictive temperature the *dhp1-2* mutation prevented premature termination upstream of the *ssm4* DSR, in a manner similar to *mni1Δ* and *rrp6Δ* mutants (Fig. 1A and C). We also detected Dhp1-dependent premature transcription termination in the *mcp5* gene (Fig. S1A). Thus, premature termination at DSR-containing meiotic transcripts requires Dhp1.

We also observed stabilization of *ssm4* meiotic gene transcripts specifically when *dhp1-2* mutant cells were cultured at restrictive temperature (Fig. 1D). The accumulation of *ssm4* mRNAs in *dhp1-2* was comparable to that observed in *rrp6Δ* (Fig. 1D). In *S. cerevisiae*, the Dhp1 homolog collaborates with Rai1 and Rtt103 proteins, which are related to Din1 and Rhn1 in *S. pombe*, respectively (22). Rhn1 has been suggested to suppress the expression of certain meiotic loci (28). We found that, unlike *dhp1-2*, neither *din1Δ* nor *rhn1Δ* caused stabilization of the *ssm4* transcript (Fig. 1D) (28). Together, our results clearly show that Dhp1, but not Din1 or Rhn1, is a key conserved factor required for silencing several DSR-containing meiotic transcripts.



To determine whether Dhp1-mediated silencing of meiotic genes is physiologically important, we used a read-out assay based on the rescue of the meiotic arrest caused by the deletion of *sme2*; *sme2Δ* zygotes arrest in meiotic prophase because of the retention of Mmi1 activity, which consequently prevents activation of the genes required for the completion of meiosis. A general up-regulation of meiotic gene expression can suppress the *sme2Δ* meiotic arrest, as has been observed in MTREC and exosome mutants (16, 17). Remarkably, we observed a significant amount of sporulation in *dhp1-2 sme2Δ* zygotes (40%) relative to the control (0%) (Fig. 1E), indicating a strong up-regulation of Mmi1-targeted genes in *dhp1-2* cells. These results suggest an important role for Dhp1 in silencing meiotic loci to prevent untimely sexual differentiation.

**Dhp1 Affects the Assembly of Heterochromatin Islands at Meiotic Genes.** We wondered whether Dhp1, like RNA elimination factors, is also required for heterochromatin formation. Remarkably, ChIP analyses revealed that H3K9me levels were significantly reduced at the *ssm4* locus in the *dhp1-2* mutant (Fig. 1F). This effect was specific to the *dhp1-2* mutant, because neither *din1Δ* nor *rhn1Δ* affected H3K9me levels (Fig. 1G). Moreover, we found that Dhp1 also is required for the assembly of other Mmi1- and exosome-dependent heterochromatin islands on meiotic genes akin to *ssm4* (Fig. 2A). Together, these results clearly show that Dhp1 along with RNA elimination factors promotes premature transcription termination and also is required for heterochromatin assembly at meiotic gene loci during vegetative growth.

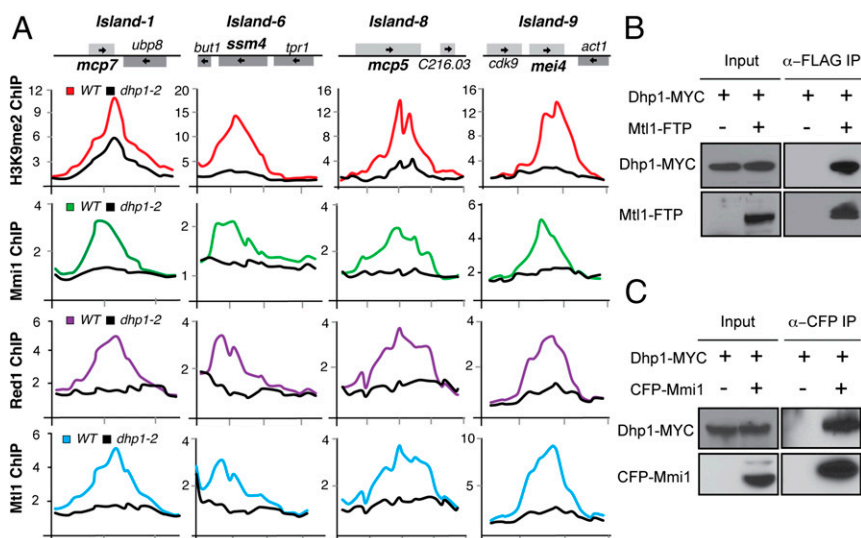
**Dhp1 Associates with RNA Elimination Factors.** Because Dhp1 and RNA elimination machinery are both functionally required for premature transcription termination and for heterochromatin assembly, we wondered whether Dhp1 and RNA elimination machinery are physically interconnected. We noticed that a previous purification of the MTREC subunit Mtl1 detected several peptides corresponding to Dhp1 (29). We therefore asked whether Dhp1 interacts with factors involved in RNA elimination. Indeed, we found that Dhp1 coimmunoprecipitates with Mtl1, even with Benzoylase treatment, indicating that the interactions with Dhp1 are not mediated by DNA or RNA (Fig. 2B and Fig. S2). We also detected an interaction between Dhp1 and Mmi1 (Fig. 2C). The interaction of Dhp1 with Mmi1 and the associated MTREC complex suggests that Dhp1 is part of a larger protein network that targets meiotic genes for premature transcription termination and

heterochromatin-mediated silencing. Supporting this idea, we found that *dhp1-2* showed severe defects in the localization of RNA elimination factors, including Mmi1, Red1, and Mtl1, at heterochromatin islands (Fig. 2A).

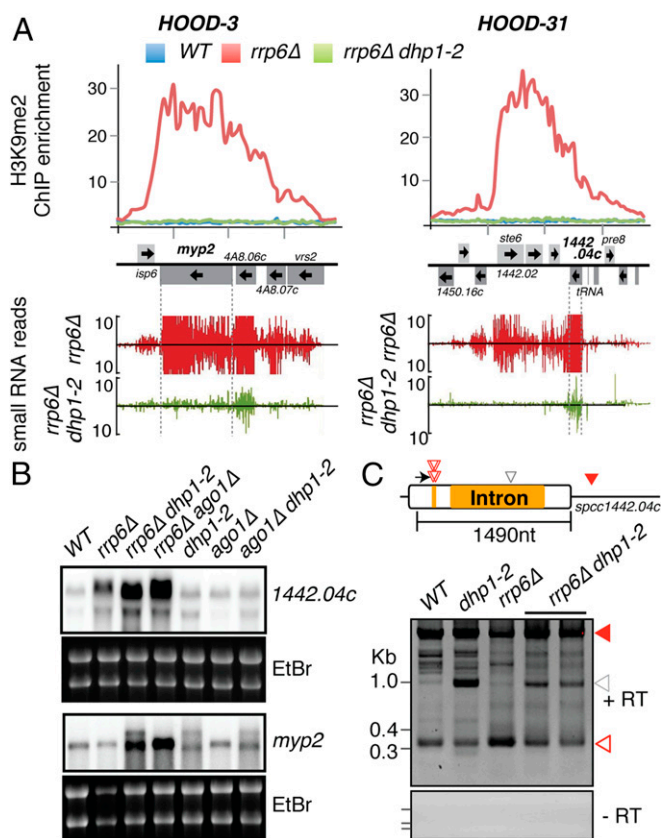
**RNAi-Dependent Heterochromatin Assembly at Genes and Retrotransposons Requires Dhp1.** MTREC along with associated RNA-processing factors also has been implicated in RNAi-dependent assembly of heterochromatin domains on developmental genes and retrotransposons (10, 11). To determine whether Dhp1 cooperates with MTREC to assemble RNAi-dependent HOODs, we introduced *dhp1-2* into a strain carrying *rrp6Δ* that allows detection of HOODs under standard growth conditions (10). Remarkably, ChIP analysis of H3K9me distribution in the *rrp6Δ dhp1-2* double mutant revealed defects in HOODs at several loci, including sexual differentiation genes such as *myp2* and *spcc1442.04c* as well as *Tf2* retrotransposons (Fig. 3A, Fig. S3, and Table S1).

We then examined small RNA production at HOODs in the *dhp1-2* mutant. High-throughput sequencing analyses showed that the production of small RNAs mapping to HOODs was severely reduced in the *dhp1-2* mutant (Fig. 3A, Fig. S3A, and Table S1). We also tested the effect of *dhp1-2* on the silencing of loci within HOODs that require both RNAi and the exosome for efficient silencing (10). Consistent with the reduction in H3K9me and siRNA levels, we found that *rrp6Δ dhp1-2* cells were severely defective in the silencing of genes within HOODs (Fig. 3B). Moreover, the up-regulation of transcripts observed in *rrp6Δ dhp1-2* was comparable to that in *rrp6Δ ago1Δ* (Fig. 3B). Together, these results show that Dhp1 is required for RNAi-dependent heterochromatin assembly and silencing of loci within HOODs.

**Dhp1 Promotes Premature Transcription Termination at a Gene Containing Cryptic Introns.** Because we found that Dhp1 along with RNA elimination machinery prematurely terminated meiotic mRNAs at heterochromatin islands, we wondered whether similar effects are observed at HOODs. To address this question, we performed 3' RACE of the *spcc1442.04c* transcript, which contains cryptic introns that promote RNAi-mediated generation of small RNAs and heterochromatin assembly (11). We observed a premature transcription termination product in the wild-type strain; however, in the *dhp1-2* mutant longer intermediate and full-length transcribed products were evident, indicating that Dhp1 is required for premature termination at HOODs, as it is at island loci (Fig. 3C). We also observed an accumulation of prematurely terminated transcripts in the absence of exosome activity, correlating with the



**Fig. 2.** Dhp1 is required for the localization of mRNA elimination factors to heterochromatin islands containing meiotic genes. (A) Dhp1 affects the localization of mRNA elimination factors that are required for the assembly of heterochromatin islands. H3K9me2, Mmi1, Red1, and Mtl1 enrichments at the indicated heterochromatin islands in wild-type and *dhp1-2* were determined by ChIP-chip analysis. The relative fold-enrichment of ChIP DNA (y axis) is plotted at the indicated chromosome position (x axis). (B) Coimmunoprecipitation analysis of Dhp1 and Mtl1 interaction. Flag-TEV-protein A (FTP) tagged Mtl1 was immunopurified using anti-FLAG M2 magnetic beads, and Dhp1-MYC was detected using anti-MYC antibody. (C) Coimmunoprecipitation analysis of Dhp1 and Mmi1 interaction. CFP-Mmi1 was immunopurified using anti-GFP agarose beads, and Dhp1-MYC was detected using anti-MYC antibody. IP, immunoprecipitation.



**Fig. 3.** Dhp1 is required for heterochromatin assembly and gene silencing at HOODs. (A) Dhp1 is crucial for small RNA production and heterochromatin assembly at HOODs. H3K9me2 distribution at the indicated HOODs in wild-type, *rrp6Δ*, and *rrp6Δ dhp1-2* strains was determined by ChIP-chip analysis. Normalized numbers of small RNA-sequencing (RNA-seq) reads mapping to the top and bottom DNA strands are represented by the signal above and below the line, respectively, and are plotted in alignment with HOOD loci. (B) Dhp1 is required for efficient silencing of target gene transcripts. The steady-state level of *myp2* and *spcc1442.04c* transcripts from the indicated strains was determined by Northern blot analysis. Ethidium bromide (EtBr) staining of ribosomal RNA was used as a loading control. (C) Dhp1 promotes premature termination of a cryptic intron-containing meiotic gene transcript. The schematic is as described in Fig. 1 A–C. Introns 1 and 2 in *spcc1442.04c* are shown in orange.

H3K9me enrichment in *rrp6Δ* cells (Fig. 3C). Premature transcription termination again was dependent on Dhp1, as confirmed by a corresponding decrease in abbreviated transcripts in the *rrp6Δ dhp1-2* double mutant (Fig. 3C). Taken together, these results indicate that Dhp1 promotes the premature termination of transcripts containing cryptic introns and is critical for channeling these RNAs into the RNAi pathway for small RNA production and heterochromatin assembly at developmental genes.

**Heterochromatic Silencing at Centromeres Requires Dhp1.** We found that Dhp1 acts together with MTREC to promote RNAi-independent and -dependent facultative heterochromatin domains at various loci. However, MTREC and its associated factors are dispensable for the production of small RNAs and heterochromatin assembly at centromeres (11). We wondered whether Dhp1 also is dispensable for RNAi-dependent heterochromatin assembly at centromeres. To test this possibility, we examined the generation of small RNAs produced from *dg/dh* repeats at pericentromeric regions in the wild-type and *dhp1-2* strains. We found that the level of small RNAs was higher in *dhp1-2* than in wild-type cells (Fig. 4A), suggesting that small RNA production at centromeres can be triggered by other mechanisms (30).

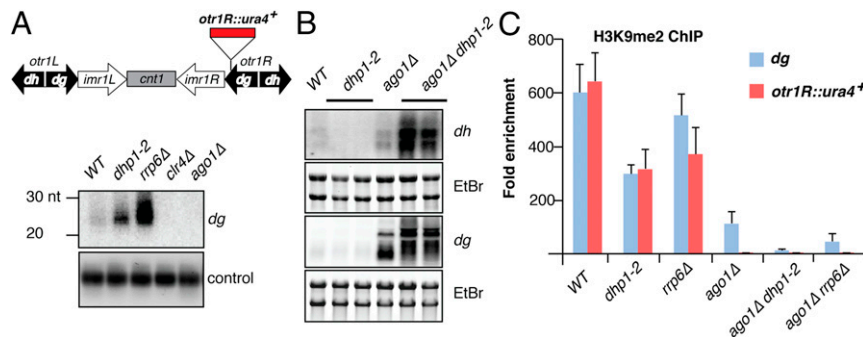
The increased production of siRNAs has been observed previously in mutants defective in either transcriptional silencing (*clr3* HDAC mutant) or degradation (*rrp6* exosome mutant) of transcripts in which RNAi processes extra primary *dg/dh* centromeric repeat transcripts into small RNAs (30, 31). Because we observed elevated levels of siRNAs in *dhp1-2*, we wondered whether mutant cells are defective in silencing. We observed that *dhp1-2* alone caused little or no change in levels of *dg/dh* transcripts (Fig. 4B). However, we observed a cumulative increase in *dg/dh* transcript levels when *dhp1-2* was combined with *ago1Δ* (Fig. 4B). Together with results showing elevated levels of *dg/dh* small RNAs in *dhp1-2* (Fig. 4A), these data suggest that the centromeric repeats are derepressed in *dhp1-2*, but degradation by RNAi prevents transcript accumulation.

We then asked whether Dhp1 contributes to heterochromatin formation at centromeres. ChIP analyses showed that the levels of H3K9me at native centromeric repeats or at *ura4+* inserted at a centromere were partially reduced in *dhp1-2* relative to wild type (Fig. 4C). The reduction in H3K9me levels at centromeres in *dhp1-2* was confirmed by ChIP-chip, which also revealed a reduction at subtelomere regions (Fig. S4 A and B). This result indicates that Dhp1 plays a more widespread role not only in facultative heterochromatin formation but also in the assembly of constitutive heterochromatin domains.

We next explored the relationship between Dhp1 and RNAi at centromeres by comparing H3K9me levels in single and double mutants. In contrast to the partial effect observed in *dhp1-2*, severe reduction of H3K9me was observed in *ago1Δ dhp1-2* (Fig. 4C). Because our previous work showed that *rrp6Δ* combined with *ago1Δ* causes a cumulative loss of heterochromatin at centromeres (32), we also assayed H3K9me levels in *dhp1-2 rrp6Δ*. However, no major reduction in H3K9me was observed in the double mutant compared with either the *rrp6Δ* or *dhp1-2* single mutant (Fig. S4C). Together, these results highlight the fact that Dhp1 does indeed affect heterochromatin assembly and silencing at centromeres, but its effects are masked by alternative mechanisms that trigger RNAi.

**Dhp1 Is Required for the Restoration of Constitutive Heterochromatin in RNAi Mutants.** A transcription-dependent but RNAi-independent mechanism of heterochromatin assembly has been shown at centromeres (32). Deletion of factors such as Tfs1 encoding TFIIIS, which promotes RNAPII processivity, can restore heterochromatin-mediated silencing in RNAi mutants (32), although restoration factors have not been identified. We found that the *dhp1-2* mutation inhibited the restoration of silencing of the *otr1R::ura4+* reporter gene in *ago1Δ tfs1Δ* (Fig. S5A). Although the loss of Tfs1 in the *ago1Δ* mutant restored heterochromatic silencing at pericentromeres, as previously reported (32), the *dhp1-2* mutation combined with *tfs1Δ* and *ago1Δ* affected the formation of functional heterochromatin as indicated by defective silencing, increased thiabendazole (TBZ) sensitivity, and the drastic reduction in H3K9me at *dg* repeats (Fig. S5B). Together, these results underscore the functionally important role of Dhp1 in RNAi-independent heterochromatin assembly at centromeres.

**Dhp1 and RNAi Function in Parallel to Assemble Heterochromatin at the *mat* Locus.** We further investigated whether Dhp1 affects heterochromatin assembly at the silent *mat* region. Heterochromatin formation at the *mat* locus involves redundant pathways (33, 34), which mask the effect of individual factors in this process. However, previous studies have described sensitized genetic backgrounds that can be used to study the effects of *trans*-acting factors on heterochromatin assembly. In particular, in a strain carrying a deletion of a local silencer adjacent to the *mat2* locus, defects in heterochromatin formation cause derepression of the silent *mat2P* cassette and the *ura4+* reporter (*mat2P::ura4*) located adjacent to this locus. Although *ura4+* derepression can be measured by plating



**Fig. 4.** Dhp1 promotes heterochromatin silencing at centromeres. (A, Upper) Schematic of the pericentromeric region showing the *dg/dh* inverted repeats and a *ura4+* (*otr1R::ura4+*; red filled rectangle) reporter gene inserted within the outer centromeric repeat. (Lower) Northern blot analysis of *dg* small RNA levels in the indicated strains. (B) Northern blot analysis of *dg* and *dh* transcript levels in the indicated strains. Note the cumulative derepression in the *ago1Δ dhp1-2* double mutant. (C) H3K9me2 enrichment at *dg*, *otr1R::ura4+* loci in the indicated strains. Note that cumulative loss of pericentromeric heterochromatin occurs in the *ago1Δ dhp1-2* double mutant. ChIP-qPCR and enrichment analysis at *dg* and *otr1R::ura4+* relative to the *act1* locus is as described in Fig. 1 F and G. Error bars indicate the SD from at least two independent experiments.

cells on medium lacking uracil or on counterselective 5-fluoroorotic acid (FOA) medium, defects in *mat2P* silencing in nonswitching *mat1M* cells causes simultaneous expression of *M* and *P* mating-type information, resulting in haploid meiosis (34). Haploid meiosis can be detected by exposing colonies to iodine vapors, which stain the starch-like compound in cells undergoing haploid meiosis.

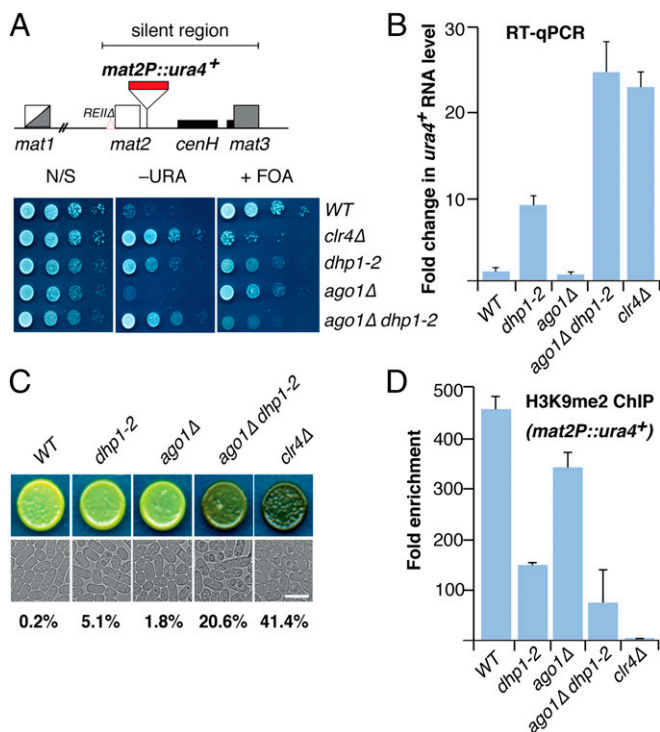
In this sensitized strain background, we found that *dhp1-2* cells are defective in silencing at the *mat* locus. Moreover, *ago1Δ dhp1-2* showed cumulative defects in the silencing of *mat2P::ura4+* (Fig. 5 A and B). The double mutant also showed elevated levels of haploid meiosis. The *dhp1-2* and *ago1Δ* single mutants stained yellow with exposure to iodine vapors, indicating little or no haploid meiosis, but the double mutant stained dark, indicating that haploid meiosis was induced as a result of a silencing defect (Fig. 5C). Microscopy analysis confirmed haploid meiosis in *ago1Δ dhp1-2* (Fig. 5C). Consistently, ChIP analysis revealed a considerable reduction in H3K9me at the *mat* locus, and the loss of heterochromatin was even more severe in the *ago1Δ dhp1-2* double mutant (Fig. 5D). The defects in heterochromatin formation and silencing also were detected in cells containing a *ura4+* reporter (*Kint2::ura4+*) inserted at the normally silent *mat2/3* interval (Fig. S6 A and B). Taken together, these results strongly suggest that Dhp1 plays an important role in heterochromatic silencing at the *mat* locus.

**Dhp1 Interacts with the Clr4 Methyltransferase Complex.** Our results indicate that Dhp1 is a central player in the assembly of all known transcription-dependent heterochromatin domains. To identify additional Dhp1-binding partners, we expressed Dhp1, which was tagged with a MYC epitope at its carboxyl terminus, under its native promoter. Mass spectrometry analysis specifically identified Dhp1, as well as the conserved Dhp1-interacting protein Din1, Mmi1, and many other heterochromatin assembly factors, in purified fractions from cells expressing tagged, but not untagged, Dhp1 (Fig. 6A and Fig. S7). In particular, we identified peptides derived from components of the Clr4-methyl transferase complex (ClrC), including Rik1 and Raf2, and confirmed the interactions (Fig. 6B and Fig. S8). We also detected other factors, including Rdp1 and Hrr1 of the RNA-dependent RNA polymerase complex (RDRC), Tas3 of the RNA-induced transcriptional silencing (RITS) complex, and Mms19, a transcription regulator (Fig. 6A–D and Fig. S8) implicated in heterochromatin assembly (35, 36). These exciting findings indicate that, in addition to collaborating with RNA processing factors such as MTREC, a small fraction of Dhp1 likely participates in heterochromatin assembly directly through its association with the ClrC and RNAi factors.

## Discussion

**Dhp1 Function in Developmental Gene Control.** Eukaryotic cells use diverse, often overlapping, strategies to prevent inappropriate expression of genes (37–39). In addition to their role in transcriptional control, RNA surveillance factors closely monitor the transcriptome. In *S. pombe*, RNA elimination factors selectively degrade meiotic mRNAs in vegetative cells (11, 14–16, 40). In this study, we uncover a mode of gene silencing that requires close cooperation between RNA degradation and transcription termination factors. We demonstrate that, in addition to its canonical role in transcription termination, the essential 3'-end processing and termination factor Dhp1/Rat1/Xrn2 plays an important role in premature termination to promote silencing of meiotic genes and prevent untimely activation of the meiotic pathway. Interestingly, this function of Dhp1 is independent of its putative cofactors, Din1 and Rhn1, but requires RNA degradation factors including the nuclear exosome. Our analyses show that Dhp1 associates with RNA elimination factors, including MTREC, which are involved in the degradation of meiotic mRNAs by the exosome. In light of emerging evidence showing that the nuclear exosome is required for transcription termination at certain loci (12, 41–43) and that human Xrn2 is involved in nuclear mRNA decay (44, 45), we envision that Dhp1 acts together with elimination factors (e.g., Mmi1 and MTREC) and the exosome as part of a specialized protein assembly that couples premature transcription termination to degradation and silencing of meiotic mRNAs. This process might require the catalytic 5'–3' exoribonuclease activity of Dhp1, in a manner similar to its role in termination of RNAPII transcripts at normal termination sites. However, another possibility is that Dhp1 serves as a scaffold for loading and/or stabilization of elimination factors that process meiotic mRNAs. Indeed, we find that mutant Dhp1 affects the localization of MTREC at meiotic genes. Regardless of the mechanism, this study implicates Dhp1 in the functional silencing of meiotic genes.

Dhp1 is also important for RNAi-mediated silencing of genes. Previously, we showed that Rrp6 and RNAi act in parallel to degrade transcripts containing cryptic introns via a mechanism that involves MTREC and its cofactors Pla1 and Pab2 (10, 11). In this study, we find that Dhp1 affects premature termination at a gene containing cryptic introns and is critical for processing transcripts into small RNAs. Consistent with its role in RNAi-mediated gene silencing, the *rrp6Δ dhp1-2* double mutant showed cumulative defects in gene silencing in a manner similar to *rrp6Δ ago1Δ*. These results highlighting the role of Dhp1 in small RNA production and gene silencing are highly relevant to gene-regulation studies in higher eukaryotes. In human cells, transcripts with defective splicing or impaired 3'-end formation are also



**Fig. 5.** Dhp1 acts in parallel to RNAi for heterochromatin assembly at the silent *mat* locus. (A) Dhp1 affects heterochromatin silencing at the *mat* locus. A serial dilution assay was used to measure expression of the *ura4<sup>+</sup>* reporter (*mat2P::ura4<sup>+</sup>*) inserted within the silent *mat* locus. The indicated strains were spotted onto medium with (nonselective; N/S) or without (selective; -URA) uracil or with counterselective medium containing FOA. (B) Dhp1 and RNAi simultaneously suppress a reporter gene in the silent *mat* locus. Strand-specific RT-qPCR amplification of *ura4<sup>+</sup>* inserted within the silent *mat* locus (*mat2P::ura4<sup>+</sup>*) was performed using the indicated strains. The relative fold-change (*ura4<sup>+</sup>*/*act1<sup>+</sup>*) in RNA expression was calculated. Error bars indicate the SD from two independent experiments. (C) Dhp1 and RNAi are required to prevent haploid meiosis. Strains were grown and stained with iodine vapors. Dark iodine staining indicates haploid meiosis and reflects *mat2P* derepression. DIC images and the percentage of azygotic spores formed by the indicated *mat1Msmt0* haploid strains are shown also ( $n > 492$ ). (Scale bar, 10 microns.) The *clr4Δ* strain is shown as a control. (D) Dhp1 acts parallel to RNAi for heterochromatin assembly at the *mat* locus. Note that cumulative loss of pericentromeric heterochromatin occurs in the *ago1Δ dhp1-2* double mutant. ChIP-qPCR and enrichment analysis at *mat2P::ura4<sup>+</sup>* locus relative to *act1* locus are as described in Fig. 1 F and G.

prematurely terminated and cotranscriptionally degraded by the Dhp1 human homolog, Xrn2 (44). Given that splicing mutants affect the generation of small RNAs at loci containing cryptic introns in *S. pombe* (11, 46) and that splicing is coupled to 3'-end formation in many cases (47–51), it is possible that the stalled spliceosome engages Dhp1/Xrn2 to trigger transcription termination and RNA degradation (44, 52). This Dhp1-mediated early termination of cryptic intron-containing transcripts with its associated cotranscriptional degradation activity perhaps provides an early and effective way to suppress aberrant or untimely gene expression and supports the emerging view that the fate of the transcripts is determined during 3'-end formation (53).

**Dhp1 and Heterochromatin Assembly.** RNA elimination machinery that participates in premature termination of meiotic gene transcripts also assembles heterochromatin at target gene loci (this study and ref. 15). We have previously reported that MTREC operates with both the nuclear exosome and RNAi and promotes the assembly of meiotic heterochromatin islands and HOODs (10, 11, 15). Here we show that Dhp1 also is required for assembly of meiotic islands and RNAi-dependent HOODs. Given the role of Dhp1 in transcription

elongation (54), it is possible that Dhp1 might regulate the rate of RNAPII elongation and indirectly affect heterochromatin assembly. However, our results showing that Dhp1 interacts with ClrC components and RNAi proteins, in addition to MTREC, suggest a more direct role for Dhp1 in facultative heterochromatin assembly.

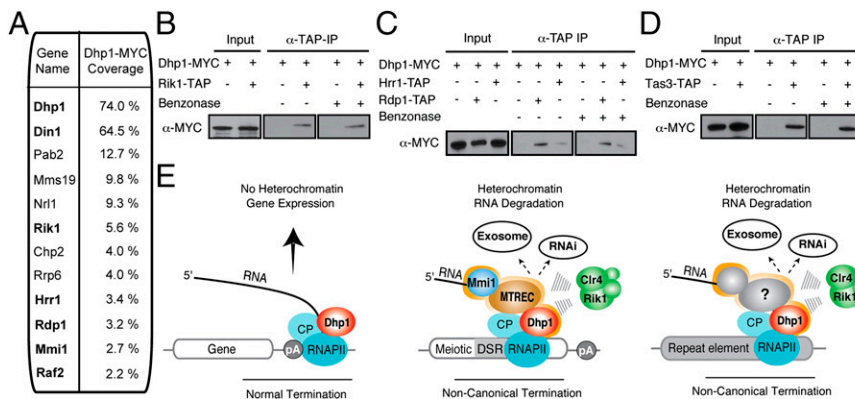
Although Dhp1 and MTREC mediate RNAi-mediated silencing at HOODs, both are dispensable for RNAi-mediated silencing at centromeres. Our analyses suggest that Dhp1 and RNAi act in parallel to assemble heterochromatin both at centromeres and at the *mat* locus. This result is reminiscent of previous work showing overlapping functions of the exosome and RNAi in centromeric heterochromatin assembly (32). Considering that Dhp1 and the exosome cooperate to assemble meiotic heterochromatin islands, it is conceivable that these factors also act together to assemble RNAi-independent heterochromatin at centromeres. Consistent with this possibility, we find that Dhp1 and the exosome have an epistatic relationship in constitutive heterochromatin formation. Together with data showing that Dhp1 is required for the restoration of centromeric heterochromatin in RNAi mutants, these observations suggest an RNAi-independent function for Dhp1 at constitutive heterochromatin loci. Nevertheless, we cannot rule out the possibility that other factors mask the effects of Dhp1 in the generation of centromeric siRNAs. Previous studies have implicated the Trf4/Air2/Mtr4p polyadenylation (TRAMP) complex in the production of centromeric siRNAs (30, 55), and another termination factor, Seb1 (Nrd1 in *S. cerevisiae*), affects heterochromatin assembly (56, 57).

How does Dhp1 affect MTREC- and RNAi-independent heterochromatin formation? Dhp1 may be a component of a specialized 3' pre-mRNA processing and termination complex that directly engages the ClrC complex. In this regard, we note that the Rik1 subunit of ClrC shares sequence similarity with the cleavage and polyadenylation specificity factor (CPSF)-A protein, which functions in 3'-end processing (58). The significance of Rik1 homology to a 3'-end processing factor has remained a long-standing mystery. Given that Rik1 acts upstream of other ClrC components (59), the association of Dhp1 with Rik1 suggests a critical role for 3'-end processing machinery in the recruitment of Clr4. Indeed, Rik1 and Clr4 act at the 3' end of convergent genes to suppress read-through transcription (60, 61). It is noteworthy, in this regard, that RNAi also has been implicated in transcription termination (62, 63).

In sum, this study reveals previously unrecognized direct cross-talk between 3'-end processing and heterochromatin machinery that hinges on the transcription termination factor Dhp1 (Fig. 6E). Dhp1 terminates RNAPII transcripts that do not target heterochromatin assembly, a function shown to require Dhp1 catalytic activity (22). Given the strong correlation between premature termination and heterochromatin assembly, it is possible that, in addition to the direct recruitment of assembly factors, the catalytic activity of Dhp1 also could be important in heterochromatic silencing. Current evidence suggests that Dhp1 forms a critical component of specialized machinery required for noncanonical termination and heterochromatin assembly (Fig. 6E). We envision that, in addition to determining the fate of coding and noncoding RNAs, 3'-end processing and termination factors serve to specify certain genomic regions including retroelements and repeat loci as preferential targets of heterochromatin assembly. Given that 3'-end processing factors leading to transcription termination also play a crucial role in the regulation of gene expression in *Arabidopsis*, *Caenorhabditis elegans*, and mammals (42, 44, 63–66), our results have important implications for understanding gene-regulatory mechanisms in metazoans.

**Materials and Methods**

All yeast strains and oligonucleotides used in this study are listed in Tables S2 and S3, respectively. Strains with gene deletions and strains expressing epitope-tagged proteins were constructed by standard procedures. The temperature-sensitive



**Fig. 6.** Dhp1 associates with ClrC components to facilitate heterochromatin assembly directly. (A) Dhp1-MYC copurified with components of the core Clr4 methyl transferase complex. Relevant proteins associating with Dhp1 are shown. Proteins with a putative association or that have been verified by coimmunoprecipitation analysis are highlighted in bold. (B–D) Dhp1 interacts with components of the ClrC (Rik1), RDRC (Hrr1 and Rdp1), and RITS (Tas3) complexes. The indicated tandem affinity purification (TAP)-tagged proteins were immunopurified with or without Benzonase treatment, and Dhp1 was detected using anti-MYC antibody. (E) Model for Dhp1-mediated silencing and heterochromatin assembly. Dhp1 promotes normal transcriptional termination at actively expressed genes and does not trigger heterochromatin formation. At sites of facultative heterochromatin formation, Dhp1 collaborates with RNA-binding factors (e.g., Mmi1) and processing factors including MTREC to promote noncanonical termination, degradation of transcripts by the exosome and RNAi, and loading of Clr4 methyltransferase complex to promote heterochromatin assembly. In addition, Dhp1 also plays a role in constitutive heterochromatin assembly at repeat elements that does not require MTREC but likely involves additional factors.

*dhp1-1* (*dhp1-1<<ura4<sup>+</sup>*) strain, MP102, was kindly provided by Kazuo Tatebayashi, University of Tokyo, Tokyo. To facilitate the use of *ura4<sup>+</sup>* as a reporter gene in our experiments, a modified version of the *dhp1-1* mutant strain, *dhp1-2* (*dhp1-1<<ura4-DS/E*), was generated in this work by converting the linked *ura4<sup>+</sup>* gene into a *ura4-DS/E* minigene. The *dhp1-2* mutant displayed a mild temperature-sensitive phenotype compared with the *dhp1-1* mutant (Fig. S1C). In all experiments, unless otherwise noted, the *dhp1-2* mutant phenotype was assayed after cells were first grown at 30 °C (permissive temperature) to early logarithmic phase and then were shifted to 37 °C (restrictive temperature) for 5 h. All strains analyzed in comparison with *dhp1-2* were also subjected to the same growth conditions. All experimental methods carried out in this work, including 3' RACE, reverse transcriptase PCR, ChIP, ChIP-

chip, coimmunoprecipitation, Western blotting, Northern blotting, and small RNA sequencing, are described in *SI Materials and Methods*.

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