**SUMMARY**

HP1 proteins are major components of heterochromatin, which is generally perceived to be an inert and transcriptionally inactive chromatin structure. Yet, HP1 binding to chromatin is highly dynamic and robust. Silencing of heterochromatin genes can involve RNA processing. Here, we demonstrate by a combination of in vivo and in vitro experiments that the fission yeast HP1 Swi6 protein guarantees tight repression of heterochromatic genes through RNA sequestration and degradation. Stimulated by positively charged residues in the hinge region, RNA competes with methylated histone H3K9 for binding to the chromodomain of HP1 Swi6. Hence, HP1 Swi6 binding to RNA is incompatible with stable heterochromatin association. We propose a model in which an ensemble of HP1 Swi6 proteins functions as a heterochromatin-specific checkpoint, capturing and priming heterochromatic RNAs for RNA degradation machinery. Sustaining a functional checkpoint requires continuous exchange of HP1 Swi6 within heterochromatin, which explains the dynamic localization of HP1 proteins on heterochromatin.

**INTRODUCTION**

Heterochromatin is a distinct chromatin structure that is late replicating, gene poor, and rich in transposons or other parasitic genomic elements. Heterochromatome structural requirements are necessary for proper centromere function, repression of recombination, sister chromatid cohesion, and the maintenance of telomere stability, and they also play an essential role in heritable gene silencing in a variety of organisms from yeast to humans (Grewal and Jia, 2007). One hallmark of heterochromatin is its association with members of the highly conserved heterochromatin protein 1 (HP1) family of proteins (James and Elgin, 1986). HP1 proteins consist of an N-terminal chromodomain (CD) and a structurally related C-terminal chromo shadow domain (CSD), separated by a hinge region. The CSD can mediate homodimerization of HP1 and binding to other proteins through a degenerate pentapeptide motif, PxVxL (Cowieson et al., 2000; Smothers and Henikoff, 2000). The CD binds the N-terminal tail of histone H3 when it is di- or trimethylated with high specificity but low affinity (Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002; Jacobs et al., 2001; Lachner et al., 2001; Nielsen et al., 2002) and the hinge region has been implicated in nucleic acid binding (Muchardt et al., 2002). The fission yeast Schizosaccharomyces pombe contains two HP1 homologs, HP1 Chp2 and HP1 Swi6, both of which bind to methylated lysine 9 of histone H3 (H3K9) and are involved in heterochromatin silencing (Grewal and Jia, 2007). In contrast to other eukaryotes, S. pombe contains only a single member of the SUV39 histone methyltransferase family of proteins, Clr4, which is responsible for the methylation of H3K9 (Nakayama et al., 2001).

Heterochromatin is generally perceived to be a structurally rigid and static chromatin compartment that is inaccessible to the transcription machinery, yet several findings challenge this view. For example, the H3K9 methyl-binding affinity of HP1 proteins can be rather low, and their association with heterochromatin is surprisingly dynamic (Cheutin et al., 2004, 2003; Festenstein et al., 2003; Schalch et al., 2009). Furthermore, recent work has revealed that both RNAi-dependent and -independent RNA turnover mechanisms are crucial for the quiescence of heterochromatic sequences in S. pombe, indicating that silencing of heterochromatin does not occur exclusively at the transcriptional level (Bühler et al., 2007). Repression of marker genes when inserted into heterochromatin depends on the noncanonical poly(A) polymerase Cid14, which is thought to target the heterochromatin RNA for degradation via the RNA exosome and/or the RNAi pathway. Similarly, silencing of subtelomeric genes marked by H3K9 methylation also depends on Cid14 (Keller et al., 2010; Wang et al., 2008). Importantly, heterochromatic gene silencing is impaired in Cid14 mutant strains, yet heterochromatin remains intact (Bühler et al., 2007). Thus, some level of transcription within heterochromatin is possible, and pathways to cope with the unwanted heterochromatic RNA do exist (Bühler, 2009). However, the mechanism of specific recognition of heterochromatic transcripts and thus their targeting for the Cid14-dependent degradation has remained elusive.

HP1 Swi6, one of the two S. pombe heterochromatin proteins, is best known for its critical role in proper centromere function. In swi6 mutant cells, centromeres lag on the spindle during anaphase, and chromosomes are lost at a high rate (Ekwall et al., 1995). This is associated with a failure in the recruitment of cohesin to pericentromeric heterochromatin (Bernard et al., 2007).
2001; Nonaka et al., 2002). Thus, one function of HP1^{Swi6} is the attraction of a high concentration of cohesin to S. pombe centromeres, which guarantees proper chromosome segregation. HP1^{Swi6} has also been implicated in the recruitment of cohesin outside constitutive heterochromatin, thus regulating transcription termination between convergent gene pairs (Gullerova and Proudfoot, 2008). Besides cohesin subunits, HP1^{Swi6} also co-purifies with a diverse set of other nuclear nonhistone proteins that are involved in a variety of nuclear functions such as chromatin remodelling and DNA replication (Fischer et al., 2009; Motamedi et al., 2008). Even though many of these interactions remain to be confirmed, HP1^{Swi6} may partner with many different factors and ensure genomic integrity. Apart from these functions, HP1^{Swi6} is also required for heterochromatin gene silencing, but on a mechanistic level this is poorly understood.

Here, we demonstrate that HP1^{Swi6} serves a general function linking transcription within heterochromatin to downstream RNA turnover. HP1^{Swi6} binds RNA via a molecular mechanism that involves the hinge region, the CD, and the N-terminal domain. Rather than tethering heterochromatic transcripts to chromatin, HP1^{Swi6} complexes with RNA dissociates from H3K9-methylated nucleosomes and escorts its associated RNAs to the RNA decay machinery. This detachment of HP1^{Swi6} from chromatin results from a competition mechanism that combines the interactions of RNA and methylated H3K9 to HP1^{Swi6} on the single-molecule level with dynamic exchange between the histone-bound and -unbound HP1^{Swi6} ensemble. Our results provide an explanation for the dynamic localization of HP1 proteins on heterochromatin and reveal insights into the role of RNA in the regulation of higher order chromatin structures.

RESULTS

Heterochromatic mRNA Transcripts Are Not Translated into Protein

Previous work revealed that the noncanonical polyA-polymerase Cid14 processes or eliminates a variety of RNA targets to control processes such as the maintenance of genomic integrity, meiotic differentiation, ribosomal RNA maturation, and heterochromatic gene silencing (Keller et al., 2010; Wang et al., 2008; Win et al., 2006). The effect of cid14+ mutations on heterochromatin silencing has previously been studied using the ura4+ reporter gene/5-FOA assay (Bühler et al., 2007). Because this assay does not allow a quantification of the resulting protein levels, and because it is also compromised by a general sensitivity of cid14+ mutant cells to 5-FOA (Figure S1), we created reporter strains carrying a gfp+ transgene inserted at the innermost centromeric repeat region (imr1R::gfp+) or at the mat3M locus (mat3M::gfp+) (Figure 1A). Consistent with previous results (Bühler et al., 2007), heterochromatic gfp+ mRNA levels from centromeric locations increased significantly in cid14Δ and dcr1Δ cells, but only modestly in cid14Δ cells (Figure 1B), with no corresponding increase in GFP protein levels upon cid14+ deletion (Figures 1C and S1A). Therefore, Cid14 plays a redundant role, if any at all, in the silencing of a reporter gene located in centromeric heterochromatin. In contrast, deleting the cid14+ gene resulted in strongly elevated gfp+ mRNA levels from the mating-type locus. Unexpectedly, however, this was not accompanied by a concomitant increase in GFP protein levels (Figures 1D and E).

To test whether mRNAs originating from heterochromatic genes engage in translation at all, we set out to profile their association with polyribosomes (Figure 1F). S. pombe cell lysates were separated on sucrose gradients and RNA was extracted from the individual fractions. The relative amount of a given mRNA in each fraction was then quantified by quantitative real-time RT-PCR (qRT-PCR). As expected, act1+ mRNA was highly enriched, whereas the nuclear U6 snRNA was absent from the polysomal fractions (Figure 1F). When transcribed from its endogenous locus, mRNA encoded by the ura4+ gene was also highly enriched in polysomes (data not shown). Similarly, ura4+ mRNA originating from a mat3M::ura4+ reporter was found in the polysomal fractions in the absence of the H3K9 methyltransferase Clr4. However, no considerable association with polysomes was observed for heterochromatic ura4+ reporter mRNA in wild-type or cid14Δ cells (Figure 1F).

Thus, although heterochromatic mRNAs can be over 10-fold more abundant in cid14Δ cells than in wild-type cells, they are not translated into protein effectively.

HP1^{Swi6} Functions as an H3K9 Methylation-Specific Checkpoint to Assemble Translationally Incompetent Ribonucleoprotein Particles

Atypical processing of 5’ or 3’ ends of heterochromatic mRNAs could explain why heterochromatic mRNAs do not engage in translation. However, our analysis of mRNA termini revealed no major differences between heterochromatic and euchromatic transcripts (Figure S2 and data not shown), suggesting that heterochromatic mRNAs per se do not contain aberrant features that would signal their destruction or render them translationally inactive. Rather, transcripts emerging from heterochromatin are more likely to be channeled into the RNA decay pathway by the assembly of a heterochromatin-specific ribonucleoprotein particle (hsRNP). Therefore, we postulate the existence of an H3K9 methylation-specific checkpoint that would function on chromatin and assemble emerging transcripts into hsRNPs that are translationally incompetent and prone for degradation (Figure 2A).

Obvious candidates for proteins that could function as such a checkpoint are HP1 proteins, because they have been reported to have affinity for both H3K9-methylated histone H3 tails and RNA. Therefore, HP1 proteins might capture heterochromatic RNAs in an H3K9 methylation-specific manner. The S. pombe genome contains two HP1 homologs, HP1^{Chp2} and HP1^{Swi6}. Interestingly, even though HP1^{Swi6} is essential for the full repression of heterochromatin, its contribution to transcriptional gene silencing is minimal. Furthermore, heterochromatic RNAs have been observed to copurify with HP1^{Swi6} but not HP1^{Chp2} (Motamedi et al., 2008).

Therefore, we tested whether heterochromatic mRNAs would become translated in cells lacking HP1^{Swi6}. Consistent with the checkpoint model, GFP protein expression from the mat3M::gfp+ allele was restored in swi6.1 and swi6.2 cid14Δ cells (Figure 2B). However, deletion of swi6.1 also resulted in a significant reduction in H3K9me2 at mat3M::gfp+ (Figure 2C),
not allowing us to definitely assign the checkpoint function to HP1\textsuperscript{Swi6}. In contrast, deletion of swi6 or cid14 or both did not significantly lower H3K9 methylation levels at the subtelomeric tlh1/2 genes, yet resulted in a strong upregulation of the respective mRNAs (Figures 2D and 2E). Importantly, association of tlh1/2 mRNA with polysomes was only observed in cells lacking swi6 but not cid14 (Figure 2F). These results place HP1\textsuperscript{Swi6} upstream of Cid14 and directly support a model in which HP1\textsuperscript{Swi6} acts on H3K9-methylated nucleosomes and promotes the assembly of translationally incompetent hsRNPs.

HP1\textsuperscript{Swi6} Binds RNA via the Hinge Region

The above results implicate HP1\textsuperscript{Swi6} in the checkpoint model as the H3K9 methylation “reader,” yet it was not clear whether HP1\textsuperscript{Swi6} itself or any of its interacting proteins could capture heterochromatic RNAs. Whereas RNA-binding affinity has been demonstrated for mammalian HP1\textsuperscript{a} (Muchardt et al., 2002), it was not known whether fission yeast HP1\textsuperscript{Swi6} can bind RNA directly. We purified recombinant HP1\textsuperscript{Swi6} and performed electrophoretic mobility shift assays (EMSA) using various RNA and DNA probes. In these assays, recombinant HP1\textsuperscript{Swi6} bound efficiently to the different RNAs but only weakly to DNA (Figure 3B). Furthermore, RNA binding could be competed with unlabeled RNA probes (Figure S3). HP1\textsuperscript{Swi6} consists of four domains: An N-terminal domain (NTD, residues 1–74), which is presumably flexibly disordered; a chromodomain (CD, residues 75–139), which binds K9-methylated histone tails (Bannister et al., 2001); a hinge region (H, residues 140–264); and a C-terminal chromo shadow domain (CSD, residues 265–328) (Cowieson et al., 2000). The hinge region of mammalian HP1\textsuperscript{a} has been implicated in RNA binding (Muchardt et al., 2002). To test whether the hinge region also confers RNA binding...
properties to HP1\(^{Swi6}\), we purified recombinant CD, hinge, and CSD. In contrast to the CD and the CSD, the isolated hinge region was sufficient for strong RNA binding (Figure 3B). By using NMR chemical shift titrations monitored on amide resonances in the flexible hinge region, we determined the binding constant of full-length HP1\(^{Swi6}\) to a 20-mer RNA as 38 ± 13 M\(^{-1}\) (Figure 3C). These results demonstrate that HP1\(^{Swi6}\) is able to bind RNA alone and that the hinge region is substantially involved in this binding interaction.

**Design of an HP1\(^{Swi6}\) Mutant that Affects RNA but Not H3K9me Binding**

Because heterochromatin at certain loci disintegrates upon removal of the \(swi6^+\) gene (Figure 2C), we aimed to develop an HP1\(^{Swi6}\) mutant with compromised RNA- but normal H3K9me-binding affinity. Therefore, we mutated the positively charged residues of the hinge region, 20 lysines and 5 arginines, to alamines (Figure 4A). For the resulting mutant protein, HP1\(^{Swi6}\)-KR25A, RNA binding was indeed drastically reduced when compared to the wild-type protein (Figure 4B). For the subsequent use of the protein in vivo, we assessed the impact of these 25 mutations on protein architecture by solution NMR spectroscopy using recombinant HP1\(^{Swi6}\) and HP1\(^{Swi6}\)-KR25A protein. Based on the full-length proteins and subconstructs thereof, we established complete sequence-specific resonance assignments for the isolated CD (residues 75–139) (Figure S4A), as well as domain-specific resonance assignments for the NTD, the hinge region, and the CSD of wild-type HP1\(^{Swi6}\). The chemical shift dispersion and intensities of the resonances in full-length HP1\(^{Swi6}\) indicated the CD and the CSD to be folded domains and the NTD and the hinge region to be flexibly unfolded polypeptide segments, as expected from predictions of the secondary structure. Analysis of the \(^{13}\)C\(^{a}\) and \(^{13}\)C\(^{\beta}\) secondary chemical shifts of the isolated CD indicates three \(\beta\)-strands and one large \(\alpha\)-helix at the C-terminal end of the domain (Figure S4E), which is well in agreement with the known
secondary structure elements in the homologous human chromobox homolog 3 (Kaustov et al., 2011). Importantly, 2D [15N,1H]-TROSY NMR spectra revealed the subspectra for the CD, the CSD, and the NTD, but not the hinge region of recombinant HP1Swi6-KR25A, to be essentially identical to wild-type HP1Swi6 (Figures 4D and 4E). Thus, the 25 Lys and Arg to Ala mutations in the hinge region abolish RNA binding without affecting the global fold of the CD and CSD domains or having a structural effect on the unfolded NTD. Binding to methylated H3K9 is, therefore, expected to be maintained in the HP1Swi6-KR25A mutant. We could confirm by surface plasmon resonance (SPR) measurements (Figure 4C). The binding constants of wild-type and HP1Swi6-KR25A to an immobilized peptide corresponding to residues 1–20 of a K9 trimethylated histone H3 tail (H3K9me3 peptide) (2.5 ± 0.5 μM and 7.8 ± 0.8 μM, respectively), were akin to and in correspondence with published values for the individual domains (Jacobs and Khorsanzadeh, 2002; Schalch et al., 2009).

Silencing but Not the Integrity of Heterochromatin Is Affected in the HP1Swi6 RNA-Binding Mutant

To study the functional relevance of RNA binding through the hinge region of HP1Swi6, we replaced the endogenous swi6+ open reading frame (ORF) with the HP1Swi6-KR25A mutant ORF. Consistent with previous results that assigned a nuclear localization signal (NLS) function to the hinge region (Wang et al., 2000), we observed that the HP1Swi6-KR25A protein localized mainly to the cytoplasm (Figure S5A and data not shown). Therefore, we added an N-terminal SV40 NLS to the wild-type and mutant HP1Swi6 alleles, which restored the characteristic heterochromatic foci in the nucleus and the specific association with RNA from heterochromatic regions (Figures 5A and S5B–S5F). Furthermore, in contrast to swi6Δ cells, neither NLS-HP1Swi6- nor NLS-HP1Swi6-KR25A-expressing cells were sensitive to thiabendazole (TBZ), showing that RNA binding to HP1Swi6 is not required for proper chromosome segregation (Figure 5B). Importantly, the H3K9 methylation defect observed at the mat3M::gfp+ locus in swi6Δ cells (Figure 2C) was rescued by the nls-swi6-KR25A allele (Figure 5D). Similarly, H3K9 methylation within telomeric heterochromatin remained unaffected in nls-swi6-KR25A cells (Figures 5E and 5F).

These results demonstrate that neither H3K9 methylation nor recruitment of HP1Swi6 to heterochromatin depend on RNA binding through the hinge region of HP1Swi6. However, silencing of heterochromatic genes was nonfunctional in nls-swi6-KR25A cells (Figures 5G–5J). Thus, RNA binding to HP1Swi6 is required for full repression of heterochromatic genes but dispensable for the integrity of heterochromatin. In summary, with nls-swi6-KR25A we created a separation-of-function allele of HP1Swi6 that fails to repress heterochromatic genes but still fulfills its architectural roles, with no impact on H3K9 methylation or chromosome segregation.
Consistent with published results (Cheutin et al., 2004), fluorescence recovery after photobleaching (FRAP) experiments revealed that HP1<sup>Swi6</sup> proteins are highly dynamic at the cellular ensemble level in vivo (Figure S5A). For proteins that are bound tightly to chromatin, recovery kinetics can be expected to be slow or not detectable, as observed for the telomere-binding
Figure 5. RNA Binding through the Hinge Region of HP1\textsuperscript{Swi6} Is Required for Silencing but Not Maintenance of Heterochromatin

(A) Microscopy of living S. pombe cells expressing C-terminally Dendra2-tagged HP1\textsuperscript{Swi6} variants driven from the endogenous promoter. Cells were grown in YES medium at 30 °C. To restore nuclear localization of the HP1\textsuperscript{Swi6-KR25A} mutant (Figure S4), a SV40 NLS was added N-terminally. Scale bar = 2 μm.

(B) In contrast to swi6\textsuperscript{Δ} cells, cells expressing the RNA-binding mutant NLS-HP1\textsuperscript{Swi6-KR25A} are not sensitive to thiabendazole (TBZ), indicating that chromosome segregation is normal. Cells were spotted on YES agar plates containing either 0 or 14 mg/l TBZ.

(C) Schematic diagram showing the location of three heterochromatic genes at the telomeres of chromosome I and II. tlh1\textsuperscript{+} and tlh2\textsuperscript{+} produce identical transcripts (Mandell et al., 2005). CEN, centromere; TEL, chromosome end.

(D–F) ChIP experiments demonstrating that H3K9me2 levels are not significantly reduced at mat3M::gfp\textsuperscript{+} (D), tlh1/2\textsuperscript{+} (E), and SPBCPT2R1.07c (F) in nls-swi6\textsuperscript{+} and nls-swi6-KR25A cells compared with wild-type cells. Mean values normalized to act1\textsuperscript{+} are shown (n = 4). Error bars represent SEM, p values were calculated using the Student’s t test.

(G) Western blot showing GFP protein levels in mat3M::gfp\textsuperscript{+} cells. Total protein from an equivalent number of cells was extracted by TCA. Tubulin serves as a loading control.

(H–J) Quantitative real-time RT-PCR showing mat3M::gfp\textsuperscript{+} (I), tlh1/2\textsuperscript{+} (K), or SPBCPT2R1.07c (L) transcript levels in the respective mutants. Mean values normalized to act1\textsuperscript{+} are shown (n = 5). Error bars represent SEM, p values were generated using the Student’s t test.
protein Taz1 (Figure S5B). This is not the case for HP1Swi6, for which fluorescence recovered rapidly after photobleaching with an exponential lifetime of 1.8 ± 0.1 s (Figure S5C). This dynamic exchange of the HP1Swi6 ensemble from chromatin in vivo is qualitatively consistent with the rapid exchange dynamics we observed in NMR peptide titration experiments in vitro. We found that the resonances of the CD involved in H3K9me3 peptide binding underwent line broadening due to intermediate chemical exchange. This indicates kinetic on/off rates for the exchange between bound and unbound forms of individual HP1Swi6 molecules in the range of about 0.01–1.0 ms⁻¹, corresponding to lifetimes of 1–100 ms. These in vivo and in vitro data thus demonstrate the highly dynamic behavior of HP1Swi6 and rule out the possibility that individual HP1Swi6 molecules remain tightly bound to heterochromatin for minutes or longer. Therefore, HP1Swi6 alone cannot tether heterochromatic RNAs to chromatin.

Localization of the HP1Swi6 Interaction Sites with RNA and H3K9me3

To obtain insight into the interactions of HP1Swi6 with RNA and methylated H3K9 at the atomic level, we used NMR chemical shift perturbation to identify residues structurally involved in these interactions. To this end, we monitored amide moiety chemical shifts, which are sensitive to structural changes of the polypeptide backbone. For the interaction of full-length HP1Swi6 with the H3K9me3 peptide, we observed chemical shift changes for 21 out of the 65 residues in the CD, as well as for one tryptophan side chain indole moiety (Figures 6A and 6B). The location of these residues in the amino acid sequence in HP1Swi6 corresponds to the location of the known binding pocket for the peptide in homologous domains (Jacobs and Khorasanizadeh, 2002; Kaustov et al., 2011; Nielsen et al., 2002). No significant chemical shift changes occurred for the backbone amide resonances of the CSD, but smaller chemical shift perturbations were observed for 8 residues of the N-terminal domain and 1 residue of the hinge region (Figure 6B). On the other hand, interaction with 20-mer-GFP RNA induced chemical shift changes for resonances of three different domains: 13 residues from the hinge region, 19 from the CD, and 10 from the N-terminal domain (Figures 6C and 6D). Furthermore, all resonances of the CD, 19 from the CD, and 10 from the N-terminal domain underwent line broadening at intermediate RNA concentrations due to intermediate exchange indicating kinetic on/off rate constants for RNA binding below about 1 ms⁻¹.

These data show that binding of RNA as well as binding of H3K9me3 peptide to HP1Swi6 occurs by a molecular mechanism that includes structural changes in three domains of HP1Swi6. The observation that these interaction sites partially overlap thereby points toward the intriguing possibility that histone tail and RNA binding are not independent. Rather, these could be competitive processes, meaning that HP1Swi6 dissociates from H3K9-methylated nucleosomes when complexed with RNA. Consistent with this idea, steady-state competition assays using SPR showed competitive behavior (Figure 6E). At substoichiometric RNA:HP1Swi6 ratios, the initial SPR response increased. This can be rationalized by the dimeric nature of HP1Swi6 caused by its CSD, which leads to complexes with 2 RNA and 2 peptide-binding sites. At concentrations above stoichiometry, however, the SPR response decreased with increasing RNA concentration, indicating competition for the peptide surface. Importantly, the 20-mer GFP-RNA did not bind to the immobilized peptide surface in a control experiment under the same buffer conditions (Figure S6D). Furthermore, binding of the HP1Swi6-KR25A mutant to H3K9me was insensitive and noncompetitive to the addition of RNA (Figures 6E and S6D).

In summary, our results implicate a mechanism by which RNA and methylated H3K9 compete for HP1Swi6 binding at the ensemble as well as the single-molecule level. Binding of RNA to HP1Swi6 structurally involves the hinge, the CD, and the NTD and impedes binding of HP1Swi6 to methylated H3K9. Thus, rather than tethering RNA to heterochromatin firmly, HP1Swi6 dynamically complexes with RNA and dissociates from H3K9-methylated nucleosomes.

Cid14 Functions in the Vicinity of Heterochromatin

The above results have established HP1Swi6 as a crucial constituent of hsRNPs, tagging RNAs as a result of their heterochromatic origin and priming them for degradation. Importantly, the dynamic properties of HP1Swi6 imply that the degradation of heterochromatric RNA originating from telomeres and the mating-type locus occurs off chromatin, but it is unclear whether Cid14 would join the hsRNP before or after dissociation from H3K9 methylated nucleosomes. If it would occur before dissociation from heterochromatin, it should be possible to crosslink Cid14 to telomers or the mating-type locus. However, ChiP experiments did not show enrichment of Cid14 at these loci (data not shown), suggesting that Cid14 joins the HP1Swi6/RNA complex only after dissociation from heterochromatin.

To test whether this still occurs in close proximity to heterochromatin, we employed the DNA adenine methyltransferase identification method (DamID, Figure 7A), a sensitive chromatin profiling technique that is suited to capture indirect or transient protein–chromatin interactions. We generated strains that express HP1Swi6 and Cid14 fused to the Dam DNA methyltransferase (Figure 7A; Woolcock et al., 2011) and assessed GATC methylation throughout the S. pombe genome using tiling arrays. As expected, HP1Swi6 was highly enriched at the mating-type locus, the centromeres, and the telomeric regions when compared to a Dam-only control (Figure 7B). Similarly, GATC methylation within the different heterochromatic regions was also observed for Dam–Cid14, demonstrating that Cid14 resides in close proximity to heterochromatin. Importantly, GATC methylation by Dam-Cid14 at the mating-type locus and telomeres is fully dependent on HP1Swi6 and not as strong as for Dam-HP1Swi6 (Figure 7C). This indicates that Cid14 joins hsRNPs after assembly and dissociation from heterochromatin at the mating-type region and the telomeres.

In conclusion, these results demonstrate that Cid14 resides in the vicinity of heterochromatin and that heterochromatic RNA originating from telomeres or the mating-type locus is delivered to Cid14 in a close spatial and temporal correlation to the dissociation of HP1Swi6 from H3K9-methylated nucleosomes. We speculate that the actual degradation of heterochromatic RNA might also occur near heterochromatin. The functional relevance of the HP1Swi6-independent association of Cid14 with centromeric heterochromatin remains unknown.
Figure 6. Localization and Competition of the HP1^{Sw6} Interactions

(A–D) Overlay of 2D [15N,1H]-TROSY correlation spectra of HP1^{Sw6}. The spectra are plotted in (A) and (C) at low base level, showing mainly resonance peaks from the two folded domains CD and CSD. The spectra are plotted in (B) and (D) at high base level, showing mainly resonances from the flexibly unfolded hinge and N-terminal domains. Residue type and number indicate sequence-specific resonance assignments for the CD. “H,” “N,” and “Trp” denote resonances from the hinge region, the NTD, and tryptophan side chains, respectively. (A and B) Black: HP1^{Sw6}; blue: 138 μM HP1^{Sw6} + 513 μM H3K9me3 peptide. (C and D) Black: HP1^{Sw6}; red: 95 μM HP1^{Sw6} + 560 μM RNA.

(E) SPR responses for competitive binding of H3K9me3 and RNA to HP1^{Sw6}. A constant concentration of 1 μM HP1^{Sw6} (black circles) or 5 μM HP1^{Sw6}-KR25A (red squares) with increasing concentrations of 20-mer GFP-RNA was injected to the H3K9me3 surface. The maximal SPR response after 200 s injection is plotted versus the RNA:protein concentration ratio. For each of the two proteins, the response in the absence of RNA was set to zero (raw data, see Figure S6D).
DISCUSSION

Association of HP1 Proteins with RNA

It was recognized earlier that proteins involved in chromatin regulation have the ability to bind RNA, although the functional relevance of this interaction has remained elusive. RNA binding was first demonstrated for the CDs of MOF and MSL-3, proteins involved in dosage compensation in *Drosophila* (Akhtar et al., 2000). For mammalian HP1α, the hinge region has been implicated in RNA binding (Muchardt et al., 2002). Here we demonstrate that HP1Swi6, the fission yeast homolog of HP1α, can also bind RNA directly. Importantly, we have found that the interaction of HP1Swi6 with RNA mechanistically includes the hinge region, the CD, and the NTD, a property that could be easily overlooked when working with isolated domains. Therefore, it will be interesting to revisit the RNA-binding properties of other HP1 proteins, such as mammalian HP1α, β, or γ, by approaches similar to those in this study. It might be that different HP1 isoforms display important differences in their interaction with RNA, which could reveal novel insights into their functional diversification. It will also be very interesting to elucidate the structural basis of the RNA and peptide binding of HP1Swi6 at the atomic level, which should give additional insights into the biophysical nature of their competitive binding mechanism.

Stable Repression of Heterochromatin through RNA Sequestration and Degradation

The results of our work reinforce previous findings that heterochromatin is not always refractory to transcription, yet is tightly repressed. We demonstrate here that HP1Swi6 mediates the recognition and destruction of heterochromatic RNA transcripts. HP1Swi6 proteins associate with H3K9-methylated nucleosomes (gray) only transiently and readily exchange from heterochromatin (dark blue). This continuous exchange of HP1Swi6 prevents saturation of heterochromatin with RNA. In case transcription within heterochromatin occurs, HP1Swi6 binds the newly synthesized RNA (red) and dissociates from H3K9 methylated nucleosomes as a result of competition between RNA and the histone tail for HP1Swi6 binding (light blue). Subsequently, the RNA is passed on to Cid14 (red), which in turn initiates RNA degradation.

It has been speculated that the functional relevance of the RNA affinities of HP1α or the dosage compensation complex might be the targeting to chromatin by major satellite or roX noncoding RNAs, respectively (Akhtar et al., 2000; Maison et al., 2002, 2011). In such a model, RNA is proposed to be involved structurally in the assembly of a higher order chromatin structure by serving as a recruitment platform. This is unlikely to apply to *S. pombe* HP1Swi6, as neither H3K9 methylation nor recruitment of HP1Swi6 to heterochromatin depends on RNA binding. In contrast, RNA bound to HP1Swi6 dissociates from heterochromatin as a result of exchange with the cellular HP1Swi6 ensemble and a decrease in affinity for methylated H3K9.

Figure 7. Cid14 Functions in the Vicinity of Heterochromatin

(A) In DamID, a Dam fusion protein is expressed at very low levels. On interaction of the fusion protein with chromatin (red), Dam methylates the adenine in the sequence context of GATC, which can be mapped by a methylation-specific PCR protocol.

(B and C) HP1Swi6 and Cid14 enrichments from DamID experiments (log2) at chromosomal regions.

(D) Model for HP1Swi6-mediated degradation of heterochromatin RNA. HP1Swi6 proteins associate with H3K9-methylated nucleosomes (gray) only transiently and readily exchange from heterochromatin (dark blue). This continuous exchange of HP1Swi6 prevents saturation of heterochromatin with RNA. In case transcription within heterochromatin occurs, HP1Swi6 binds the newly synthesized RNA (red) and dissociates from H3K9 methylated nucleosomes as a result of competition between RNA and the histone tail for HP1Swi6 binding (light blue). Subsequently, the RNA is passed on to Cid14 (red), which in turn initiates RNA degradation.

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satisfaction of heterochromatin with RNA. Competition between RNA and methylated H3K9 for HP1^{Swi6} binding at the ensemble level guarantees that RNA-free HP1^{Swi6} is preferably recruited to heterochromatin, thereby sustaining a functional checkpoint on the H3K9-methylated nucleosome and ensuring constant turnover of heterochromatic RNAs (Figure 7C).

In our model, HP1^{Swi6} functions on chromatin to bind to and assemble emerging heterochromatin transcripts into special RNPs, which we refer to as hsRNPs. Thereby, HP1^{Swi6} guarantees specific and tight repression of heterochromatic genes on at least two levels. First, HP1^{Swi6} prevents protein synthesis by sequestration of mRNAs from ribosomes, most likely through nuclear retention. Thus, a heterochromatic mRNA remains repressed even in the absence of RNA degradation. This explains why classical PEV screens failed to recover RNA decay factors such as Cid14. Notably, Cid14 itself is involved in the processing of ribosomal RNA and also associates with 60S ribosomal proteins (Keller et al., 2010; Win et al., 2006), raising the possibility that loss of Cid14 might result in a general defect in RNA synthesis. However, association of euchromatic mRNAs with polyribosomes, as well as protein expression levels, remain unaffected in cid14Δ cells (Figure 1 and data not shown), strongly arguing against such an indirect effect. Second, the HP1^{Swi6} ensemble ensures elimination of heterochromatic mRNAs by capturing the RNA at the site of transcription and escorting it to the degradation machinery. Rather than the classical features of an aberrant RNA, such as a truncated open reading frame or defective 5' or 3' ends, our data suggests that it is the physical association of a heterochromatic mRNA with HP1^{Swi6} that primes it for destruction. We note that artificial tethering of HP1^{Swi6} to a euchromatic mRNA does not result in RNA degradation (data not shown), suggesting that canonical mRNPs are immune to HP1^{Swi6}-mediated RNA turnover. Furthermore, since the kinetics of RNA binding to HP1^{Swi6} are fast, the hsRNPs may be stabilized by additional factors. However, at this point we can only speculate on such contributions by additional proteins or other molecules.

Concluding Remarks

In this study, we have discovered a function for one of the fission yeast HP1 proteins that provides the missing link between transcriptional origin and Cid14-dependent degradation of heterochromatic mRNAs. Our results highlight the role of RNA as a negative regulator of HP1^{Swi6} binding to chromatin and provide insights into the repression of heterochromatic domains at a posttranscriptional level. The high degree of conservation of HP1 proteins and heterochromatin-mediated gene silencing phenomena suggest that our findings may also apply to other eukaryotes.

Our work has revealed that HP1^{Swi6}, in addition to its role in proper centromere function, also guarantees tight repression of heterochromatic genes through RNA sequestration and degradation. Interestingly, the Drosophila HP1 protein Rhino has been linked recently to the piRNA pathway (Klattenhoff et al., 2009). In analogy to our checkpoint model, Rhino may bind the initial sense transcript at the heterochromatin transposon locus and subsequently escort it to the perinuclear “nuage” structure, where it can enter the ping-pong amplification cycle. Thus, rather than forming repressive chromatin, Rhino might specify the recognition and ensure efficient elimination of transposon RNA.

Finally, our results add another layer of complexity to the crosstalk between RNA and chromatin. In contrast to the emerging theme that RNA can serve as a scaffold to assemble, recruit, or guide chromatin-modifying complexes to their respective targets (Wang and Chang, 2011), we demonstrate that they may also function as “repellents.” RNA-mediated eviction might be a possible mechanism that counteracts HP1 spreading along the chromatin fiber or the formation of ectopic heterochromatin. Importantly, neither coding potential nor stability is important for an RNA to function as a repellent, offering a possible molecular function for the many short-lived, low-abundant noncoding RNAs that are present in the eukaryotic cell.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

Fission yeast strains and plasmids used in this study are described in Supplemental Information.

Western Blot and Polysome Profiling

Total proteins from exponentially growing cells were extracted using TCA and separated by SDS-PAGE. Antibodies for western blotting were used at the following concentrations: GFP (Roche; 1:3,000), tubulin (Woods et al., 1989; 1:5,000), Swi6 (Bioacademia; 1:10,000). Polysome profiling is described in Supplemental Information.

Chromatin Immunoprecipitation and Gene Expression Analysis

RNA isolation, cDNA synthesis, and quantitative RT-PCR was performed as described in Emmerth et al. (2010). Chromatin immunoprecipitation (ChIP) was performed as described in Bühl et al. (2006), using 2.5 μg of an antibody against dimethylated H3K9 (Kimura et al., 2008).

Electrophoretic Mobility Shift Assay (EMSA)

The desired amount of protein was diluted into 9 μl of 1 × electrophoretic mobility shift assay (EMSA) buffer (20 mM HEPES-KOH [pH 7.5], 100 mM KCl, 0.05% NP-40) and incubated for 10 min at RT. The substrate was added, incubated at 30°C for 30 min, and followed by gel electrophoresis (1.6% TBA agarose). Fluorescently labeled RNA was detected using a TyphoonTM 9400 Gel Scanner. RNA labeling is described in Supplemental Information.

Recombinant Protein Expression and Purification for NMR

Expression and purification was performed as described in Supplemental Information with the following modifications. Bacteria were grown in 6 l of M9 minimal medium containing 15N-NH4Cl as a nitrogen source. Induction was carried out using 0.5 mM IPTG. The lysate was incubated with 10 ml of glutathione-sepharose FF (GE). The protein was released from the glutathione-senharose by TEV-cleavage o/n at 4°C using acTEV (Invitrogen). This was followed by SourceIQ ion exchange chromatography (GE Healthcare). The purification was completed by size exclusion chromatography (Superdex 200; GE Healthcare) in 50 mM MES pH 6.5, 100 mM KCl, 5 mM DTT. The purified complex was concentrated to 100 μM by centrifugal filtration.

Solution NMR Spectroscopy and SPR

NMR experiments were performed on Bruker 800 MHz and 600 MHz spectrometers. The sequence-specific resonance assignments for the isolated HP1^{Swi6} CD (residues 75–139) were obtained from the two APSY-type experiments 4D APSY-HNCACB (15 projections) and 5D APSY-HNOCOCB (16 projections) (Gossert et al., 2011; Hiller et al., 2005, 2007) and subsequent automated backbone assignment by the algorithm MATCH (Volk et al., 2008). For SPR, samples were analyzed using a Biacore T-100 instrument (GE Healthcare). Further details are given in Supplemental Information.
DamID was carried out as previously published (Woolcock et al., 2011). Coordinates of heterochromatic regions are given in Supplemental Information.

ACCESSION NUMBERS

DamID data sets were deposited under accession number GSE36956 (NCBI Gene Expression Omnibus).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, Supplemental References, and five tables and can be found with this article online at doi:10.1016/j.molcel.2012.05.009.

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REFERENCES


