A conserved ncRNA-binding protein recruits silencing factors to heterochromatin through an RNAi-independent mechanism

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Long noncoding RNAs (lncRNAs) can trigger repressive chromatin, but how they recruit silencing factors remains unclear. In *Schizosaccharomyces pombe,* heterochromatin assembly on transcribed noncoding pericentromeric repeats requires both RNAi and RNAi-independent mechanisms. In *Saccharomyces cerevisiae*, which lacks a repressive chromatin mark (H3K9me [methylated Lys9 on histone H3]), unstable ncRNAs are recognized by the RNA-binding protein Nrd1. We show that the *S. pombe* ortholog Seb1 is associated with pericentromeric lncRNAs. Individual mutation of *dcr1*$^+$ (Dicer) or *seb1*$^+$ results in equivalent partial reductions of pericentromeric H3K9me levels, but a double mutation eliminates this mark. Seb1 functions independently of RNAi by recruiting the NuRD (nucleosome remodeling and deacetylase)-related chromatin-modifying complex SHREC (Snf2–HDAC [histone deacetylase] repressor complex).

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A major unsolved question in chromatin biology is how long intergenic noncoding RNAs (lincRNAs) trigger the formation of repressed chromatin. A large number of mammalian lincRNAs have been identified by systematic studies (Guttman et al. 2009). Many of these ncRNAs associate with chromatin-modifying complexes (Khalil et al. 2009). Multiple models have been proposed for how these ncRNAs are recognized and recruit chromatin-modifying factors, but little is understood mechanistically (Guttman and Rinn 2012).

In *Schizosaccharomyces pombe*, pericentromeric heterochromatin assembly is promoted by transcription of the *dg* and *dh* repeat sequences by RNA polymerase II (Pol II) (Djupedal et al. 2005; Kato et al. 2005). The corresponding long ncRNAs (lncRNAs) are converted into dsRNAs and processed into siRNAs by the combined action of RNA-directed RNA polymerase complex (RDRC) and Dicer (Dcr1) (Verdel et al. 2009; Lejeune and Allshire 2011). siRNAs produced by Dicer are bound by Argonaute (Ago1), a component of the RNA-induced transcriptional silencing (RITS) complex, and together they promote both degradation of pericentromeric ncRNAs and transcriptional silencing via repressive histone methylation (Verdel et al. 2004). These complexes in turn recruit the Clr4 methyltransferase complex (CLRC), which methylates Lys9 on histone H3 (H3K9me) (Nakayama et al. 2001; Zhang et al. 2008). The methyl mark serves as a binding platform for the repressive HP1 proteins Swi6 and Chp2 (Thon and Verhein-Hansen 2000; Bannister et al. 2001; Fischer et al. 2009). Both proteins promote the recruitment of SHREC (Snf2–HDAC [histone deacetylase] repressor complex) to pericentromeric heterochromatin (Sugiyama et al. 2007; Sadaie et al. 2008). Moreover, Chp2 has been found to associate with SHREC to form the SHREC2 complex (SHREC complex associated with Chp2) (Motamedi et al. 2008). The core of SHREC consists of silencing factors Clr1 and Clr2, the HDAC Clr3, and the putative chromatin-remodeling enzyme Mit1 (Sugiyama et al. 2007). SHREC and SHREC2 resemble the mammalian nucleosome remodeling and deacetylase (NuRD) complex (Sugiyama et al. 2007; Motamedi et al. 2008). Previous studies revealed that deletion of *clr3*$^+$ reduces the levels of pericentromeric H3K9me2 in cells lacking RNAi (Yamada et al. 2005; Reyes-Turcu et al. 2011), indicating that Clr3/SHREC can act independently of RNAi in pericentromeric heterochromatin assembly.

In addition to processing by the RNAi machinery, the pericentromeric lncRNAs are also recognized by several RNA quality control factors such as Mlo3 and Cid14 (Reyes-Turcu et al. 2011). Interestingly, mutations in these factors have been shown to suppress the silencing defect of RNAi mutants at pericentromeric repeats (Reyes-Turcu et al. 2011). This led to the suggestion that the ncRNAs might also act in the RNAi-independent pathway of heterochromatin assembly (Reyes-Turcu et al. 2011). Here we identify a conserved ncRNA-binding protein, Seb1/Nrd1, which binds pericentromeric ncRNAs and is required for H3K9me in cells deficient for RNAi. We demonstrate that this ncRNA-binding protein functions in the RNAi-independent pathway by recruiting the activities of SHREC.

Results and Discussion

To investigate how ncRNAs lead to the formation of repressed chromatin, we identified a candidate ncRNA recognition factor in *S. pombe* based on prior studies of ncRNAs in *Saccharomyces cerevisiae*. There, a class of unstable ncRNAs called cryptic unstable transcripts (CUTs) is recognized by an RNA-binding protein called Nrd1 (Arigo et al. 2006; Thiebaut et al. 2006). Nrd1 globally associates with Pol II via a C-terminal domain-interacting domain (CID) and recognizes a specific RNA oligonucleotide through its RNA recognition motif (RRM) domain (Steinmetz and Brow 1996, 1998; Conrad et al. 2000; Carroll et al. 2004; Meinhart and Cramer 2004; Vasiljeva et al. 2008a). Nrd1 also plays a role in recognizing precursors to stable ncRNAs such as nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs) to promote their 3′ end formation (Steinmetz et al. 2001; Kim et al.

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2006). Although the *S. cerevisiae* lineage lost both H3K9me and RNAi during its evolution, we hypothesized that the role of Nrd1 in ncRNA recognition might be conserved. To test this, we tagged the *S. pombe* Nrd1 ortholog Seb1 (Mitsuzawa et al. 2003) and used cross-linking and RNA immunoprecipitation (RIP) to assess its association with *dg* and *dh* ncRNAs as well as the snoRNA *snR30*. Seb1 displays a strong association with these ncRNAs but not with the *act1*$^+$ RNA (Fig. 1A). As expected, Hrr1, an RNAi factor, also associates with *dg* and *dh* ncRNAs but not with *snR30* or *act1*$^+$ RNA (Supplemental Fig. S1A). The association of Seb1 with *dg* and *dh* transcripts is maintained in a *clr4*Δ mutant that lacks H3K9me, indicating that this conserved heterochromatic methyl mark is not required for the association (Supplemental Fig. S1B). This is in contrast to RNAi factors that require H3K9me to display a RIP signal on the *dg* and *dh* transcripts (Rougemaille et al. 2012).

We next isolated a mutation in *seb1*$^+$ that was defective in heterochromatic silencing. Because *seb1*$^+$, like *NRD1*, is an essential gene (Mitsuzawa et al. 2003), we mutagenized a gene targeting construct for the endogenous *seb1*$^+$ locus (Supplemental Fig. S2) and transformed this library into a strain harboring an ectopic heterochromatic silencing reporter system that we had developed for other studies. This system involves the insertion of a 2811-base-pair (bp) fragment of *dh* repeat downstream from the endogenous *ura4*$^+$ gene such that the transcription of this fragment is driven by the *adh1*$^+$ promoter (Supplemental Fig. S3A). This fragment ("fragment 1") was identified as a highly potent inducer of *ura4*$^+$ silencing in a systematic study of the activities of *dg* and *dh* fragments (data not shown). Silencing by fragment 1 requires functional Clr4 and RNAi as well as the *adh1*$^+$ promoter; the latter observation indicates that transcription of fragment 1 is required for silencing (Supplemental Fig. S3B). Silencing by fragment 1 also causes a decrease of *ura4*$^+$ transcript level (Supplemental Fig. S3C) and an increase of H3K9me2 at the *ura4*$^+$ locus (Supplemental Fig. S3D). Screening of ~10,000 colonies produced by transformation of the *seb1*$^+$ mutant library yielded a single mutant that displays a defect in growth on 5-fluoroorotic acid (5-FOA) medium, which selects for strains with a silenced *ura4*$^+$ gene (Supplemental Fig. S2). This allele, *seb1-1*, has seven nucleotide substitutions in the *seb1*$^+$ coding sequence, three of which change the amino acid sequence. Replacement of *seb1*$^+$ with the *seb1-1* allele in the parental strain recapitulates the 5-FOA phenotype (Fig. 1B). This mutation also causes a silencing defect at endogenous heterochromatin: A strain harboring a *ura4*$^+$ reporter gene inserted into the innermost repeat (*imr*) region of centromere 1 displays reduced growth on 5-FOA when harboring the *seb1-1* allele (Fig. 1C). In the ectopic heterochromatic silencing reporter strain, the *seb1-1* mutation causes an accumulation of the *ura4*$^+$ transcript (Fig. 1D) and a strong defect in H3K9me2 at the *ura4*$^+$ gene (Fig. 1E), supporting the growth defect observed using the 5-FOA assay.

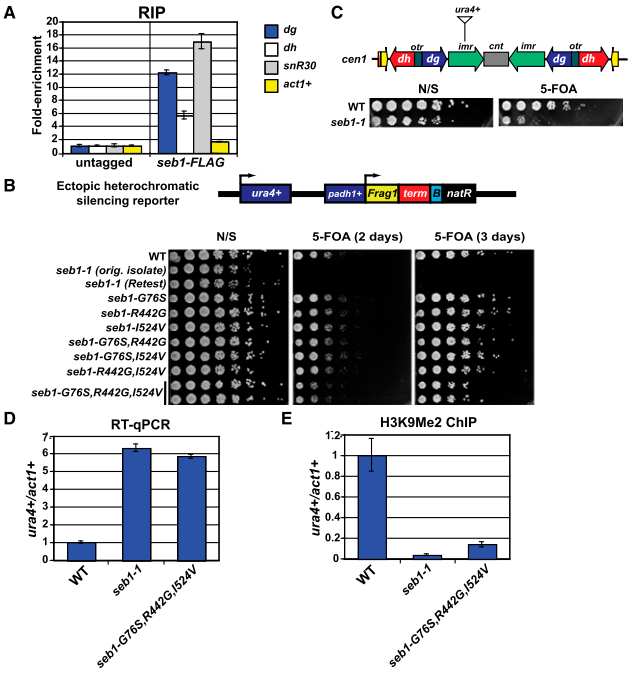

Figure 1. The *seb1-1* mutant is defective in heterochromatic silencing at pericentromeric repeats. (*A*) RIP experiments measuring the enrichment of Seb1-Flag at *dg*, *dh*, and *act1*$^+$ transcripts and *snR30* snoRNA in the wild-type strain. (*B*, *top*) Schematic of the ectopic heterochromatic silencing reporter construct used in the allele screen. (*Bottom*) Silencing assays of *seb1*$^+$ mutations in the ectopic heterochromatic silencing reporter strain background. Cells were plated on nonselective rich YS medium (N/S) and YS medium with 5-FOA (5-FOA). (*C*, *top*) Schematic of centromere 1 with the *ura4*$^+$ reporter gene inserted in the *innermost repeat* (*imr*) region. (*Bottom*) Silencing assay of *seb1-1* mutation in the pericentromeric *ura4*$^+$ reporter strain background. (*D*,*E*) RT-qPCR analysis of *ura4*$^+$ transcript levels (normalized to *act1*$^+$ transcript levels) (*D*) and ChIP analysis of H3K9me2 levels at the *ura4*$^+$ locus (normalized to H3K9me2 levels at the *act1*$^+$ locus) (*E*) in the wild-type strain, the *seb1-1* mutant, and the strain with three amino acid mutations in *seb1*$^+$.

We constructed all combinations of the three amino acid changes present in the *seb1-1* allele (G76S, R442G, and I524V) and used them to replace the wild-type *seb1*$^+$ sequence in the reporter strain. We found that all three mutations are required to produce a silencing defect on 5-FOA medium (Fig. 1B). However, the mutant with the three amino acid changes (triple mutant) has a milder silencing defect on 5-FOA when compared with the original *seb1-1* mutant. The silencing defect of the triple mutant is only obvious at 2 d of growth on 5-FOA, indicating that the silent mutations also contribute to the phenotype (Fig. 1B). Moreover, the triple mutant displays an intermediate defect in H3K9me2 at the *ura4*$^+$ locus when compared with the *seb1-1* allele (Fig. 1E). The triple mutant displays accumulation of *ura4*$^+$ transcript nearly comparable with that of the *seb1-1* mutant (Fig. 1D), likely reflecting distinct sensitivities and thresholds to gene function of the 5-FOA, RNA, and H3K9me2 assays. As each of the four silent mutations (A45G, T132A, T1194C, and T1260A) changes the wild-type codon to a rarer synonymous codon (Forsburg 1994), they could impact protein expression. Indeed, the level of Seb1 protein is lower in the *seb1-1* mutant when compared with its level in the wild-type *seb1*$^+$ strain (Supplemental Fig. S4). Since the *seb1-1* allele has a more robust silencing defect compared with the triple mutant, we used the *seb1-1* allele in our further analyses.

We examined the effect of *seb1-1* mutation on pericentromeric siRNA production using Northern hybridization and discovered that, unlike the *clr4Δ* mutant, *seb1-1* displays normal levels of pericentromeric siRNA accumulation (Fig. 2A). Consistent with the lack of a defect in

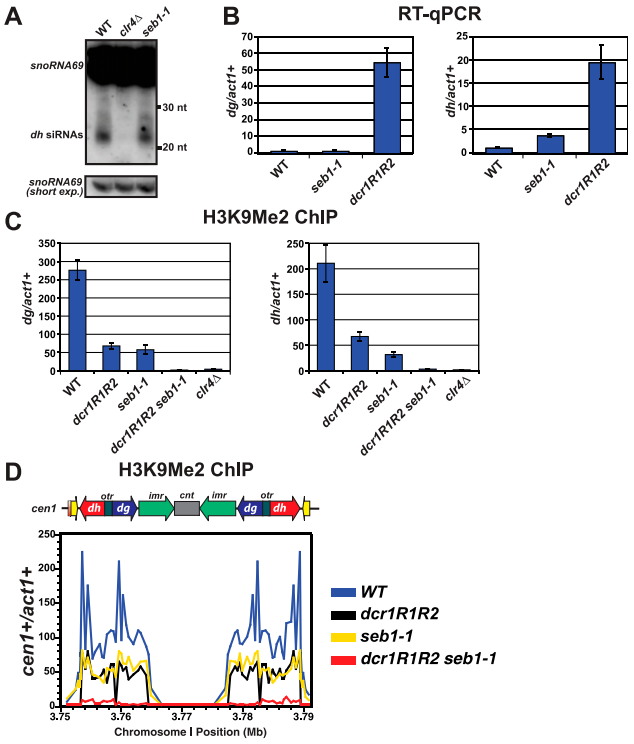

Figure 2. The *seb1-1* mutation does not affect RNAi. (*A*) Riboprobe siRNA Northern blot detecting *dh* siRNAs in the wild-type, *clr4Δ*, and *seb1-1* strains. *snoRNA69* was used as a loading control. (*B*) RT-qPCR analysis of *dg* and *dh* transcript levels (normalized to *act1*$^+$ transcript levels) in wild-type, *seb1-1*, and *dcr1-R1R2* strains. (*C*) ChIP analysis of H3K9me2 levels at *dg* and *dh* repeats (normalized to H3K9me2 levels at the *act1*$^+$ locus) in a wild-type strain, *seb1-1* and *dcr1-R1R2* single mutants, the *dcr1-R1R2 seb1-1* double mutant, and the *clr4Δ* mutant. (*D*) ChIP analysis of H3K9me2 levels at centromere 1 (normalized to H3K9me2 levels at the *act1*$^+$ locus) in wild-type strain, *seb1-1* and *dcr1-R1R2* single mutants, and the *dcr1-R1R2 seb1-1* double mutant.

siRNA production, the *seb1-1* mutation does not cause an accumulation of *dg* transcripts and only causes a slight increase in the level of *dh* transcripts (Fig. 2B). In contrast, a catalytically dead Dcr1 mutant (*dcr1-R1R2*) (Colmenares et al. 2007) displays a dramatic increase in *dg* and *dh* transcript levels (Fig. 2B). We serendipitously discovered that *seb1-1* confers a temperature-sensitive phenotype at 37°C (Supplemental Fig. S5A). As with the silencing phenotype, all seven mutations of *seb1*$^+$ are required to produce the temperature sensitivity (Supplemental Fig. S5A). Northern hybridization demonstrated no defect in siRNA levels in the *seb1-1* mutant, even at the nonpermissive temperature (Supplemental Fig. S5B). Taken together, these data argue that the silencing defect of the *seb1-1* allele cannot simply be explained by a defect in RNAi.

To test whether Seb1 functions in the RNAi-independent pathway of heterochromatin formation, we measured H3K9me2 levels at the *dg* and *dh* repeats in the single and double combinations of *seb1-1* and *dcr1-R1R2* alleles. Strikingly, while the levels of H3K9me2 are reduced by only threefold to fivefold in the single mutants, H3K9me2 is virtually abolished in the *dcr1-R1R2 seb1-1* double mutant to background levels comparable with those measured in the *clr4Δ* mutant (Fig. 2C). We observed similar results across the entire pericentromeric region in chromatin immunoprecipitation and quantitative PCR (ChIP-qPCR) experiments using tiled pairs of primers (Fig. 2D; Braun et al. 2011). Moreover, we found that the *seb1-1* mutation does not affect Chp1/RITS recruitment to *dg* and *dh* repeats (Supplemental Fig. S6). Together, these data demonstrate that Seb1 acts independently of RNAi to promote H3K9me at pericentromeric heterochromatin. To rule out the trivial possibility that the *dcr1-R1R2 seb1-1* double mutation reduces the transcription of components of the CLRC, thereby affecting H3K9me2 indirectly, we performed RT-qPCR analyses and found no reduction in them in the mutant (Supplemental Fig. S7).

Although classical heterochromatin mediated by H3K9me was ancestrally lost during the evolution of *S. cerevisiae*, Nrd1 has been reported to play a role in promoting a different type of silencing that occurs in the recombinant DNA (rDNA) repeats of budding yeast (Vasiljeva et al. 2008b). In this organism, cryptic unstable Pol II transcripts of unknown function are produced from rDNA, which is predominantly transcribed by Pol I. These Pol II transcripts are terminated and targeted for degradation by Nrd1 (Vasiljeva et al. 2008b). In Nrd1 mutants, the defect in termination and turnover leads to dramatic increases in the level of rDNA-derived Pol II transcripts and the accumulation of longer transcripts (Vasiljeva et al. 2008b). This transcriptional readthrough is associated with increased histone acetylation, reduced nucleosome occupancy, activation of inserted Pol II reporter genes, and increased recombination between rDNA repeats (Vasiljeva et al. 2008b). We tested whether Seb1 might act by such a mechanism in *S. pombe* pericentromeric regions by determining whether the *seb1-1* mutation causes an increase in pericentromeric transcript size and levels in cells mutated for Dcr1. Northern hybridization using a riboprobe complementary to the region of the *dh* repeats encoded by fragment 1 revealed heterogeneously sized transcripts produced from this region that accumulate in the *dcr1-R1R2* mutant (Supplemental Fig. S8A). This pattern is consistent with previous reports (Zaratiegui et al. 2011). No obvious increase in transcript size was apparent, and there was no significant increase in transcript level in the *seb1-1 dcr1-R1R2* double mutant relative to the *dcr1-R1R2* single mutant (Supplemental Fig. S8A). Careful quantification using RT-qPCR also yielded no increase in the abundance of *dh* transcript produced by the *seb1-1* mutation in a *dcr1-R1R2* genetic background (Supplemental Fig. S8B). These data indicate that mechanisms observed previously in *S. cerevisiae* cannot easily explain our observations in *S. pombe*.

Since it has been previously reported that the *clr3Δ* mutant, like the *seb1-1* mutant, further reduces H3K9me2 in the *dcr1Δ* mutant (Yamada et al. 2005; Reyes-Turcu et al. 2011), we tested whether Seb1 and SHREC function in the same pathway. We quantitatively measured the levels of H3K9me2 at *dg* and *dh* repeats in the *dcr1-R1R2 seb1-1* and *dcr1-R1R2 clr3Δ* double mutants. Consistent with published results (Yamada et al. 2005; Reyes-Turcu et al. 2011), we observed that while H3K9me2 levels at the *dg* and *dh* repeats are modestly reduced in the *dcr1-R1R2* single mutant, these levels are virtually eliminated in the *dcr1-R1R2 clr3Δ* double mutant, a phenotype strikingly similar to that of the *dcr1-R1R2 seb1-1* double mutant (Fig. 3A). Furthermore, the elimination of H3K9me2 at *dg*

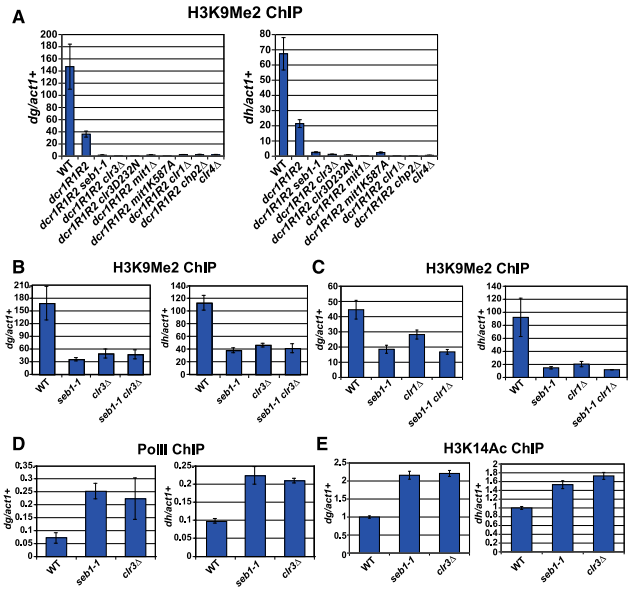

Figure 3. Seb1 functions in the same pathway as SHREC to promote H3K9me. (*A*) ChIP analysis of H3K9me2 levels at *dg* and *dh* repeats (normalized to H3K9me2 levels at the *act1*$^+$ locus) in wild-type, *dcr1-R1R2*, *dcr1-R1R2 seb1-1*, *dcr1-R1R2 clr3Δ*, *dcr1-R1R2 clr3D232N*, *dcr1-R1R2 mit1Δ*, *dcr1-R1R2 mit1K587A*, *dcr1-R1R2 clr1Δ*, *dcr1-R1R2 chp2Δ*, and *clr4Δ* strains. (*B*) ChIP analysis of H3K9me2 levels at *dg* and *dh* repeats (normalized to H3K9me2 levels at the *act1*$^+$ locus) in the wild-type strain, the *seb1-1* and *clr3Δ* single mutants, and the *seb1-1 clr3Δ* double mutant. (*C*) ChIP analysis of H3K9me2 levels at *dg* and *dh* repeats (normalized to H3K9me2 levels at the *act1*$^+$ locus) in the wild-type strain, the *seb1-1* and *clr1Δ* single mutants, and the *seb1-1 clr1Δ* double mutant. (*D*,*E*) ChIP analyses of Pol II (*D*) and H3K14ac (*E*) levels at *dg* and *dh* repeats (normalized to the levels at the *act1*$^+$ locus) in the wild-type strain and *seb1-1* and *clr3Δ* single mutants.

and *dh* repeats was also observed in strains that have the *dcr1-R1R2* mutation combined with a deletion mutation that eliminates the Clr1, Chp2, or Mit1 subunits of SHREC (Fig. 3A). Finally, a catalytically dead point mutation in the Clr3 HDAC or the Mit1 ATPase domain also eliminates H3K9me in *dcr1-R1R2* cells (Fig. 3A). These data demonstrate that all known activities of SHREC are required for the RNAi-independent pathway that promotes H3K9me at pericentromeric repeats.

To further test whether Seb1 functions in the same pathway as SHREC in promoting H3K9me, we compared H3K9me2 levels at *dg* and *dh* repeats of the *seb1-1 clr3Δ* double mutant with those of the corresponding single mutants. Significantly, the double and single mutants display very similar levels of H3K9me2, ~20%–30% of wild-type levels (Fig. 3B). Similar results were obtained when comparing H3K9me2 levels at *dg* and *dh* repeats of the *seb1-1 clr1Δ* double mutant with the levels of the corresponding single mutants (Fig. 3C). These data provide strong genetic support that Seb1 and SHREC proteins function in the same pathway in promoting H3K9me at pericentromeric repeats. Moreover, we found that the *seb1-1* mutation increases Pol II occupancy and H3K14 acetylation levels at *dg* and *dh* repeats (Figs. 3D,E), similar to that reported previously for the *clr3Δ* mutation (Sugiyama et al. 2007; Motamedi et al. 2008). To rule out the possibility that *seb1-1* mutation indirectly affects heterochromatin assembly by decreasing the levels of mRNAs that encode the subunits of SHREC, we measured their levels in the wild-type and *seb1-1* strains. We found that the *seb1-1* mutation does not reduce the levels of the mRNAs encoding any of the subunits of SHREC (Supplemental Fig. S9), further arguing for a direct role of Seb1 in heterochromatin formation.

Because Seb1 and SHREC function genetically in the same pathway, we hypothesized that Seb1 physically interacts with SHREC to recruit it to pericentromeric repeats. An observation that supports this hypothesis is that the purification of the Clr2 subunit of SHREC was reported to have yielded two peptides from Seb1 (Supplemental Table S1; Motamedi et al. 2008). However, Seb1 peptide coverage was low and was not obtained in other purifications reported. To test for a physical interaction between Seb1 and SHREC using a more sensitive assay, we performed coimmunoprecipitation (co-IP) immunoblotting experiments. We found that Flag-tagged Seb1 coimmunoprecipitated with Clr3-myc (Fig. 4A) but not with a control protein (Cdc2 detected by anti-PSTAIRE), indicating that these two proteins physically interact in vivo. This interaction is not bridged by RNA, since we could still detect the interaction in the presence of RNase A (Fig. 4B). Seb1 also coimmunoprecipitated with other components of SHREC, including Clr1 and Mit1 (Fig. 4C,D). These data provide strong evidence that Seb1 and SHREC not only function in the same pathway, but also physically interact in vivo.

To gain further insight into how Seb1 functions to promote H3K9me, we tested whether Seb1 acts upstream of SHREC, as might be expected for a recruitment factor. We performed ChIP experiments to measure the enrichment of SHREC in strains harboring an epitope-tagged version of the proteins. We found that every subunit of SHREC tested (Clr3, Clr1, Mit1, and Chp2) is enriched at

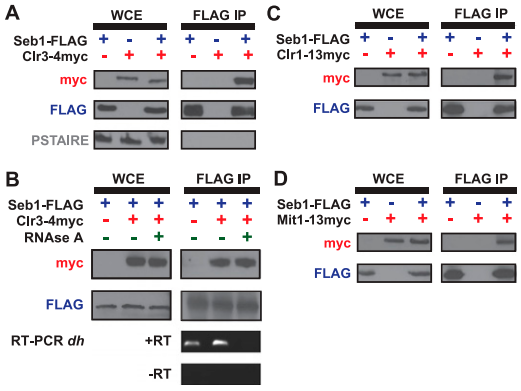

Figure 4. Seb1 physically associates with SHREC in vivo. (*A*) Co-IP of Seb1 with Clr3. Strains expressing endogenously tagged Seb1-CBP-2XFlag, Clr3-4myc, or both were subjected to anti-Flag immunoprecipitation. The whole-cell extract (WCE) and immunoprecipitated (IP) samples were detected by anti-myc (*top*), anti-Flag (*middle*), and anti-PSTAIRE (*bottom*) immunoblots; the latter serves as a loading and specificity control. (*B*) Co-IP of Seb1 with Clr3 in the presence of RNase A. The two *bottom* panels are images of agarose gels of RT-qPCR experiments to detect *dh* transcripts in the presence (+RT) or absence (−RT) of reverse transcriptase. (*C*,*D*) Co-IP of Seb1 with Clr1 and Mit1. Strains expressing endogenous levels of Seb1-CBP-2XFlag, Clr1-13myc, or both (*C*) and Seb1-CBP-2XFlag, Mit1-13myc, or both (*D*) were subjected to anti-Flag immunoprecipitation. The whole-cell extract (WCE) and immunoprecipitated (IP) samples were detected by anti-myc (*top*) and anti-Flag (*bottom*) immunoblots.

both *dg* and *dh* repeats. However, in the *seb1-1* mutant, this enrichment is abolished or strongly reduced (Figs. 5A–D). In contrast, RIP experiments demonstrate that Seb1 still associates with *dg* and *dh* transcripts in cells lacking SHREC, indicating that Seb1 recruitment to these ncRNAs is not downstream from SHREC (Fig. 5E). Interestingly, we observed a reproducible increase in Seb1 association with *dg* and *dh* ncRNAs in the *clr3*Δ mutant but not the *clr4*Δ mutant, suggesting negative regulation of Seb1 recruitment by Clr3 (Fig. 5E; Supplemental Fig. S1B). Together, these data support our hypothesis that Seb1 acts by recruiting SHREC to pericentromeric heterochromatin.

In *S. pombe,* heterochromatin is also found at the silent mating type locus and at subtelomeric regions. These loci contain DNA sequences that are similar to the pericentromeric *dg* and *dh* repeats. While Seb1 is also associated with the *dg*/*dh*-like transcripts originating from these nonpericentromeric heterochromatic loci (Supplemental Fig. S10), the *seb1-1* mutation does not affect SHREC recruitment to these loci (Supplemental Fig. S11). This is not completely unexpected, since there exists additional mechanisms of SHREC recruitment to the silent mating type locus and subtelomeric regions by DNA-binding proteins Atf1/Pcr1 and Taz1, respectively (Yamada et al. 2005; Sugiyama et al. 2007).

Our studies demonstrate an essential role for the ncRNA-binding protein Seb1 in RNAi-independent pericentromeric heterochromatin formation through recruitment of the chromatin-modifying complex SHREC. (Fig. 5F). SHREC may function to promote H3K9me by deacetylating the H3 tail, which is presumably necessary for its methylation by the CLRC. Alternatively, SHREC may have nonhistone substrates whose deacetylation promotes H3K9me. As HP1 proteins also promote the recruitment of SHREC, heterochromatin spread may be promoted by a positive feedback loop involving alternating cycles of histone deacetylation and methylation (Fig. 5F). Consequently, SHREC may be considered both an effector and a trigger of histone methylation.

Candidate recruiters of RNAi to heterochromatic regions in *S. pombe* include Pol II and the spliceosome (Djupedal et al. 2005; Kato et al. 2005; Bayne et al. 2008), which are essential for viability and operate at a very large number of genomic sites. Presumably, additional factors and signals determine where RNAi-related enzymes are recruited by these multiprotein complexes. Likewise, Seb1 also displays a strong association with nonheterochromatic *snR30* snoRNA (Fig. 1A). Interestingly, however, we did not detect recruitment of Clr3 to the *snR30* gene (Supplemental Fig. S12), suggesting that Seb1 may play SHREC-independent roles outside of heterochromatin. Our ongoing studies are aimed at defining the molecular cues (including potential RNA ligands) that distinguish Seb1's functions in heterochromatin versus euchromatin.

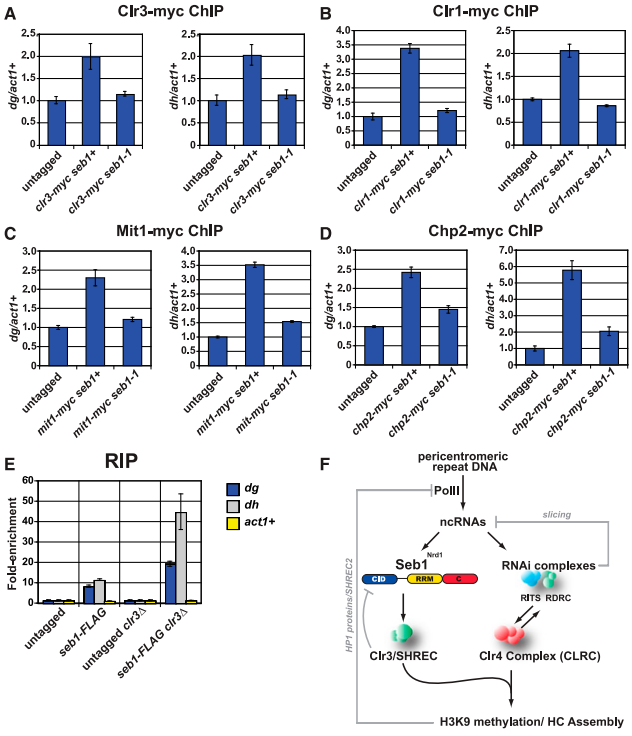

Figure 5. Seb1 recruits SHREC to pericentromeric heterochromatin. (*A–D*) ChIP analyses of Clr3-myc, Clr1-myc, Mit1-myc, and Chp2-myc levels at *dg* and *dh* repeats (normalized to their levels at the *act1*$^+$ locus) in the wild-type and *seb1-1* strains. (*E*) RIP experiments measuring the enrichment of Seb1-Flag at *dg*, *dh*, and *act1*$^+$ transcripts in the wild-type and *clr3Δ* strains. (*F*) Model for the RNAi-independent role of Seb1 in recruiting SHREC to pericentromeric heterochromatin.

Materials and methods

Strain construction and growth conditions

Strains were constructed and grown by standard fission yeast methods as described previously (Rougemaille et al. 2012), except that 2 g/L 5-FOA was used in silencing assays.

Immunoprecipitation experiments and RNA analyses

ChIP, RIP, co-IP, and RT-qPCR experiments and Northern analyses were performed using standard molecular biology techniques as described in detail in the Supplemental Material.

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