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Rex1BD and the 14-3-3 protein control heterochromatin organization at tandem repeats by linking RNAi and HDAC

Jinxin Gao[a,1], Wenqi Sun[b,1], Jie Li[c], Hyoju Ban[a], Tuokai Zhang[a], Junwei Liao[a], Namho Kim[a], Soon Hoo Lee[a], Qianhua Dong[a], Robert Madramootoo[a], Yong Chen[b,d,2], and Fei Li[a,2]

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Tandem DNA repeats are often organized into heterochromatin that is crucial for genome organization and stability. Recent studies revealed that individual repeats within tandem DNA repeats can behave very differently. How DNA repeats are assembled into distinct heterochromatin structures remains poorly understood. Here, we developed a genome-wide genetic screen using a reporter gene at different units in a repeat array. This screen led to identification of a conserved protein Rex1BD required for heterochromatin silencing. Our structural analysis revealed that Rex1BD forms a four-helix bundle structure with a distinct charged electrostatic surface. Mechanistically, Rex1BD facilitates the recruitment of Clr6 histone deacetylase (HDAC) by interacting with histones. Interestingly, Rex1BD also interacts with the 14-3-3 protein Rad25, which is responsible for recruiting the RITS (RNA-induced transcriptional silencing) complex to DNA repeats. Our results suggest that coordinated action of Rex1BD and Rad25 mediates formation of distinct heterochromatin structure at DNA repeats via linking RNAi and HDAC pathways.

heterochromatin | HDAC | Rex1BD | RNAi | *Schizosaccharomyces pombe*

Significance

Noncoding tandem repetitive DNA sequences are abundant in eukaryotic genomes and often organized into heterochromatin, highly packed chromatin regions that are inactive for transcription. Heterochromatin plays critical roles in gene expression and genome stability and has been linked to many diseases. Recent studies revealed that epigenetic silencing within heterochromatin repeats can vary significantly. How DNA repeats are assembled into distinct heterochromatin structures remains elusive. Using a genome-wide genetic screen, we identified a conserved protein Rex1BD required for heterochromatin silencing at tandem repeats. Further studies revealed that Rex1BD interacts with the 14-3-3 protein Rad25 to form a regulatory hub that promotes heterochromatin assembly at DNA repeats via linking RNAi and histone deacetylase (HDAC) pathways.

Noncoding tandem repetitive DNA sequences are widespread and constitute a significant proportion of the eukaryotic genome (1–3). Originally referred to as "junk DNA," now it is clear that the tandem DNA repeats play a key role in genome organization and gene expression. These repetitive regions also impose fundamental challenges for a functional genome. For example, repetitive DNA can lead to errors in homologous recombination-based DNA repair (4, 5). Tandem DNA repeats thus have been linked to many diseases, such as fragile X syndrome and Huntington disease (6, 7). Due to its repetitive nature, it has been challenging to understand how the individual units within tandem repeats behave.

Tandem repeats are often organized into heterochromatin, the densely packed and transcriptionally inactive chromatin domain. The histone 3 lysine 9 methylation (H3K9me) and histone hypoacetylation are two conserved epigenetic hallmarks in heterochromatic regions (8, 9). Exactly how DNA repeats are assembled into the repressive heterochromatin domain in vivo is still not well understood. In addition, though sharing the same sequence, epigenetic silencing between individual units in repeat tandem arrays can vary significantly. Recent studies using repeat-specific reporters have shown that tandem repeats can exhibit strikingly different levels of transcriptional silencing, and the unique epigenetic state within individual repeats is stably inherited (10–12). How the position-dependent heterochromatin silencing within DNA repeats is regulated and its biological significance remain elusive.

Fission yeast (*Schizosaccharomyces pombe*) has proven to be a valuable model for studying heterochromatin. Heterochromatin in *S. pombe* is mainly found in pericentromeres, telomeres, and the mating-type (*mat*) region. As in human cells, the heterochromatic regions in *S. pombe* are characterized by H3K9me and hypoacetylated histones (13, 14). H3K9me is mediated by the CLRC complex, which contains the H3K9 methyltransferase Clr4, Rik1, Dos1, Dos2, and Cul4 (15–20). H3K9me creates binding sites for the human HP1 homolog Swi6 that subsequently recruits effectors to promote heterochromatin formation (13, 21). RNA interference (RNAi) also plays an important role in H3K9me and heterochromatin silencing (21, 22). Fission yeast has a single copy of Argonaute (Ago1), Dicer (Dcr1), and the RNA-dependent RNA polymerase (Rdp1). Ago1, together with the chromodomain protein Chp1 and Tas3, forms the RITS (RNA-induced transcriptional silencing) complex, whereas Rdp1 interacts with Hir1 and Cid12, forming the RDRC (the RNA-dependent RNA polymerase complex) complex (23, 24). Heterochromatin can be lowly transcribed during the S phase of the cell cycle (25–28). The noncoding RNA transcripts are converted by RDRC into double-stranded RNAs for cleavage by

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[1]J.G. and W.S. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: fl43@nyu.edu or yongchen@sibcb.ac.cn.

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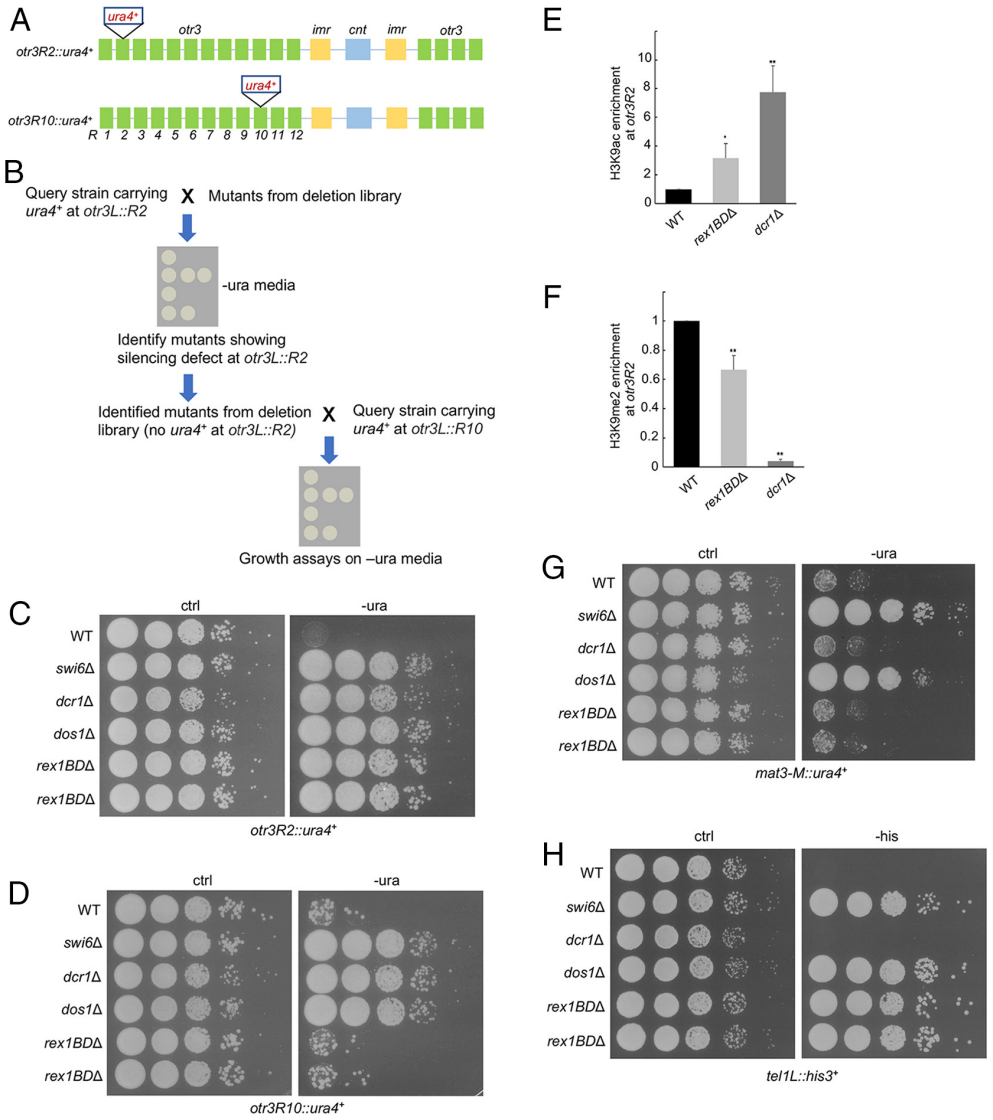

Fig. 1. A genetic screen identified Rex1BD required for heterochromatin silencing. (*A*) The schematic diagram of the *ura4*$^+$ reporter into *otr3R2* (*Top*) or *otr3R10* (*Lower*) at centromere 3. *cnt*, centromeric core. *imr*, innermost repeat. *otr*, outer repeats. *R*, repeat. (*B*) Schematic representation of the screening strategy. -ura, minimal medium without uracil. (*C*) Ten-fold serial dilutions of indicated cells harboring *ura4*$^+$ at *otr3R2* were plated on the minimal medium without uracil (-ura) and incubated at 30 °C for 4 d. The *swi6*Δ, *dcr1*Δ, and *dos1*Δ carrying *otr3R2::ura4*$^+$ were used as a control. Ctrl: control. (*D*) Ten-fold serial dilutions of indicated cells harboring *ura4*$^+$ at *otr3R2* were plated on -ura medium. (*E* and *F*) ChIP-qPCR analysis of H3K9 acetylation (*E*) and H3K9me2 (*F*) at the *otr3R2* region in the indicated strains. Actin was used as a control. Three independent experiments were performed. The level of WT was set to 1. Error bars indicate SD. **P* < 0.05, ***P* < 0.001. (*G*) Ten-fold serial dilutions of indicated cells carrying *ura4+* at the mating-type locus were plated on -ura4 medium. (*H*) Ten-fold serial dilutions of indicated cells carrying *his3*$^+$ at a subtelomeric region were plated on the minimal medium without histidine (-his). Biological replicates for *rex1BDΔ* in this figure are independently generated strains.

Dcr1 to generate siRNAs. RITS associates with heterochromatin transcripts to mediate the recruitment of the CLRC complex (21, 23, 29–31).

The hypoacetylation of histones in fission yeast heterochromatin is mediated by all three subtypes of histone deacetylases (HDACs), including Class I (Clr6), Class II (Clr3), and Class III (Sir2) (32–34). Clr6, a human HDAC1 homolog, is important for deacetylation of a variety of lysine residues in histone H3 and H4 (35). Clr6 associates with a WD40 repeat-containing protein Prw1 and other several conserved proteins to mediate both heterochromatin region and euchromatin (36–38). Sir2 is a conserved member of the Sirtuin family of HDACs that are dependent on NAD$^+$. Sir2 in fission yeast can also deacetylate multiple lysines in histone H3 and H4 (32, 34, 39, 40).

The pericentromeric heterochromatin in fission yeast is subdivided into an innermost repeat region (*imr*) and a larger outmost repeat region (*otr*). Each *otr* repeat is approximately 6.7 kb in length, consisting of *dg* and *dh* elements. The *otr* region at the left side of centromere 3 (*otr3*) has the largest number of repeats among the three centromeres, a total of 12 repeats (Fig. 1*A*) (12, 41). To probe the behavior of individual repeats in *otr* heterochromatin repeats, we recently developed a collection of strains carrying a *ura4*$^+$ reporter gene inserted in different *otr3* repeats at the left side of centromere 3 (11). Using these strains, we showed that different *otr* repeats exhibit dramatic differences in silencing: The *otr3* repeats distal to the centromere core, including repeats 2–8, are strongly silenced, whereas repeats close to the centromere display weaker silencing. We further showed that the position effect with the tandem repeat array depends on RNAi (11).

To gain further insight into the mechanism for how tandem repeats are assembled into distinct heterochromatin structures, here we developed a genome-wide genetic screen using *ura4*$^+$

reporter at different units in the *otr3* repeat array we created. This screen led to the identification of an uncharacterized protein Rex1BD required for heterochromatin assembly. We solved the high-resolution structure of Rex1BD and revealed that it forms a four-helix bundle structure with a distinct charged electrostatic surface. Our genetic analysis showed that Rex1BD regulates heterochromatin in an RNAi-independent manner, and overexpression of Rex1BD rescues the silencing defect in *sir2*Δ. Using mass spectrometry, we identified that Rex1BD interacts with histones and the HDAC Clr6. We further showed that Rex1BD facilitates the recruitment of Clr6 to heterochromatin. We also identified that the 14-3-3 protein Rad25 physically interacts with Rex1BD. Rad25 mediates heterochromatin silencing in DNA repeats by recruiting the RITS complex through interacting with RNAi effector Ago1. Together, our results showed that Rex1BD and Rad25 form a regulatory hub that defines heterochromatin silencing within tandem repeats via linking RNAi and HDAC. The study also sheds light on DNA repair in heterochromatin.

Results

A Genetic Screen Identified Rex1BD Important for Heterochromatin Silencing. To identify factors regulating heterochromatin silencing in different pericentromeric repeats, we developed a genetic screen using two query strains carrying the *ura4*$^+$ reporter at either 2nd (*otr3R2*) or 10th (*otr3R10*) repeat in the *otr3* repeat array at the left side of centromere 3 that we previously created (Fig. 1*A*) (11). We have shown that the *otr3R2* repeat displays the strongest silencing relative to other pericentromeric *otr* repeats examined, which results in substantially slow growth on minimal medium without uracil (-ura) (11), thus providing a sensitive readout for heterochromatin silencing in pericentromeric regions. We first crossed the query strain carrying *otr3R2::ura4*$^+$ with the mutant strains from the Bioneer *S. pombe* deletion library and analyzed heterochromatin silencing of the mutants using growth assays on the -ura medium. The mutant strains that disrupted silencing in *otr3R2* found by the first round of screening were then crossed with the query strain carrying *otr3R10::ura4*$^+$, which exhibits weaker silencing than *otr3R2::ura4*$^+$ (11). The resulting strains were analyzed by a growth assay on the -ura media (Fig. 1*B*). Through this screen, we identified that cells carrying *otr3R2::ura4*$^+$ but lacking an uncharacterized gene, SPAC4H3.06, showed strong growth on -ura medium, indicating that the gene is required for silencing at *otr3R2* (Fig. 1*C*). However, we found that the mutant displays little silencing defect in *otr3R10* (Fig. 1*D*). These results showed that SPAC4H3.06 is important for heterochromatin silencing in the *otr3* region. SPAC4H3.06 is highly conserved across eukaryotes, including mammals (*SI Appendix*, Fig. S1). Its human ortholog is predicted to be REX1BD. We thus name the gene *rex1BD*$^+$.

We next examined how histone acetylation and H3K9me at the pericentromeric region *otr3R2* in *rex1BD*Δ are affected by ChIP-qPCR. Correlated with the silencing defect, *rex1BD*Δ has a sharp increase in histone acetylation and a noticeable reduction in H3K9me2 in the silent pericentromere region (Fig. 1 *E* and *F*). These results indicate that Rex1BD is required for the heterochromatin integrity in pericentromeres.

We next tested heterochromatin silencing at the mating type locus using a strain carrying *ura4*$^+$ at the *mat3-M* region (Fig. 1*G*). The *rex1BD*Δ mutant grew similar to the wild type (WT) in the -ura media, indicating that deletion of *rex1BD*$^+$ results in little defect in heterochromatin silencing in the mating type locus. We also analyzed silencing in the subtelomeric region using the reporter *his3*$^+$ inserted in the region. The *rex1BD*Δ mutant grew much faster than WT in the minimal media without histidine. Rex1BD is thus also required for silencing in the telomeric region (Fig. 1*H*).

Rex1BD Is a Nuclear Protein that also Contributes to DNA Damage Repair. To investigate the localization of Rex1BD, we created GFP-tagged Rex1BD at its C-terminus under the control of its native promoter at the endogenous locus. Our growth assays using cells carrying *ura4*$^+$ at *otr3R2* showed that silencing in the pericentromeric region is not affected by Rex1BD-GFP, indicating that the tagged version of Rex1BD is functional (*SI Appendix*, Fig. S2). However, we were unable to detect the distribution pattern of Rex1BD-GFP since the GFP signal was too weak. To further analyze its localization, we constructed Rex1BD-GFP under the thiamin-repressible *nmt1* promoter, and overexpressed Rex1BD-GFP in WT cells also carrying mCherry-tagged Ish1, a nuclear membrane protein, in minimum media without thiamine. We observed that overexpressed Rex1BD-GFP was enriched within the nucleus, consistent with its role as a heterochromatin factor (*SI Appendix*, Fig. S3).

Since Rex1BD in *Chlamydomonas reinhardtii* has been implicated in DNA repair (42), we tested the sensitivity of the *rex1BD*Δ mutant to the DNA-damaging agent, methyl methanesulfonate (MMS), which covalently modifies DNA by adding methyl groups to a number of nucleophilic sites on the DNA bases (43). The mutant strain was plated on the minimal media containing 0, 0.005%, and 0.01% MMS. The deletion mutant of the repair protein Apn2 was used as a positive control. As expected, the *apn2*Δ mutant exhibits increased sensitivity to MMS compared with WT (*SI Appendix*, Fig. S4). We found that the *rex1BD*Δ cells were also sensitive to MMS, but relatively mild compared with *apn2*Δ (*SI Appendix*, Fig. S4). These results indicate that deletion of *rex1BD*$^+$ results in moderate defects in DNA damage repair.

Structure of Rex1BD. To reveal the structural feature of SpRex1BD, we purified the recombinant full-length SpRex1BD proteins from *Escherichia coli* and determined the crystal structure by single-wavelength anomalous diffraction at a resolution of 3.38 Å (*SI Appendix*, Table S1). Rex1BD folds into a compact helical bundle composed of four helices (Fig. 2*A*). The helical bundle is stabilized by the hydrophobic contacts through a series of hydrophobic residues (Fig. 2*B*), which are evolutionarily conserved from yeast to humans (*SI Appendix*, Fig. S1), suggesting that all the Rex1BD homologs should share the similar fold as SpRex1BD. The surface of Rex1BD is highly charged with distinct electrostatic-property patches which may be responsible for the interaction with different protein partners (e.g., histones) (Fig. 2*C*). The potential importance of these charged residues in mediating protein–protein interactions is partly supported by the existence of a crystallography Rex1BD tetramer mediated by the electrostatic interactions through α2 (*SI Appendix*, Fig. S5). The tetrameric Rex1BD might be a crystal-packing artifact because gel-filtration analysis shows that Rex1BD is a monomer in solution. However, we cannot rule out the possibility that Rex1BD could form a tetramer in vivo under certain circumstances, which merits further investigation.

N- and C-terminal Regions of Rex1BD Contribute to Its Role in Silencing. The N terminus of Rex1BD contains the α1 helix, whereas its C-terminus contains the α4 helix (Fig. 2*D*). Our structure analysis predicted that disruption of these helix motifs destabilizes the protein. To investigate the role of the α1 helix in heterochromatin silencing, we made partial deletion of the helix motif at the N terminus and replaced the endogenous *rex1BD*$^+$with the deleted version (*rex1BD-ND*) (Fig. 2*D*). Our growth assays

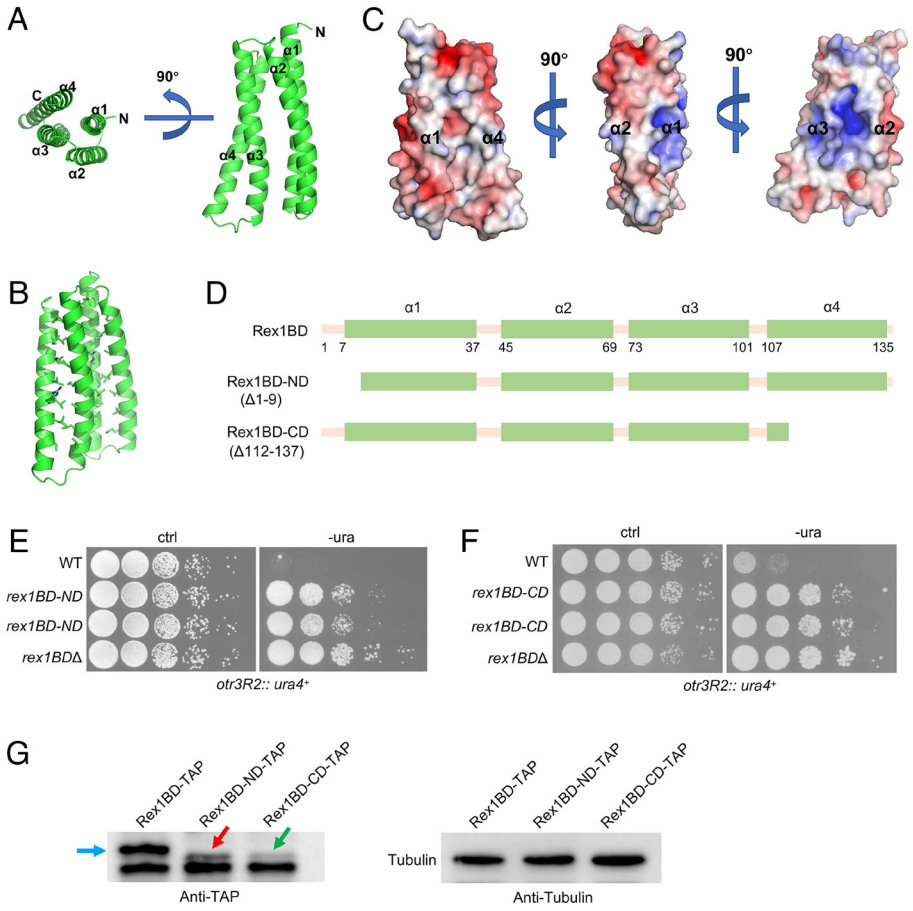

Fig. 2. Crystal structure and domain analysis of Rex1BD. (*A*) Ribbon drawing of the Rex1BD. Monomeric Rex1BD from fission yeast is a four-helical bundle. Right viewed as 90° rotation from Left. (*B*) A ladder of hydrophobic residues stabilizes the packing of four helices. (*C*) Surface electrostatic properties of Rex1BD. Monomeric Rex1BD shows a distinct charged surface. The contour level is at ±5 kT/e; red for negative potential and blue for positive potential. Right viewed as 90° rotation from Left. (*D*) A schematic diagram of Rex1BD domain-deletion mutants. The positions of the site of truncation are indicated. (*E*) Ten-fold serial dilutions of N-terminal-deleted Rex1BD mutant carrying *ura4*$^+$ at *otr3R2* were plated on -ura medium. Biological replicates for the *rex1BD-ND* mutant are independently generated strains. (*F*) Ten-fold serial dilutions of C-terminal-deleted Rex1BD mutant carrying *ura4*$^+$ at *otr3R2* were plated on -ura medium. Biological replicates for the *rex1BD-CD* mutant are independently generated strains. (*G*) Extracts from cells carrying Rex1BD-TAP, Rex1BD-ND-TAP, or Rex1BD-CD-TAP at its endogenous site were analyzed by western blotting with an anti-TAP antibody. Tubulin was used as a loading control. Blue arrow, Rex1BD-TAP protein band. Red arrow, Rex1BD-ND-TAP protein band. Green arrow, Rex1BD-CD-TAP protein band.

using the *ura4*$^+$ reporter at *otr3R2* showed that heterochromatin silencing in the pericentromeric region is partially lost compared with WT (Fig. 2*E*). We also partially deleted the α4 helix at the C-terminus of Rex1BD and created a C-terminal deleted version at its endogenous locus (*rex1BD-CD*) (Fig. 2*D*). Our growth assays showed that heterochromatin silencing in the pericentromeric region in *rex1BD-CD* is also partially lost (Fig. 2*F*). To test whether these partial-deleted mutants affect Rex1BD stability, we created the TAP-tagged version of *rex1BD-ND* and *rex1BD-CD* at its endogenous site. Our western blot analysis showed that the protein levels of Rex1BD-ND-TAP and Rex1BD-CD-TAP were significantly reduced compared to WT (Fig. 2*G*). Together, these data indicate that both N- and C-terminal regions of Rex1BD contribute to its function in heterochromatin assembly, supporting that both α1 helix and α4 helix are important for protein stability.

Rex1BD Regulates Heterochromatin Formation in an RNAi-Independent Pathway. RNA interference (RNAi) is required for H3K9me and heterochromatin silencing (21, 22). We next examined whether the Rex1BD acts in an RNAi-dependent manner. To test this, we took advantage of a hairpin structure of *ura4*$^+$ that can induce heterochromatin silencing in *trans* at a target locus near heterochromatin (44). The hairpin inserted on chromosome 1 contains a sequence complementary to 200 bp of *ura4*$^+$ under the *nmt1* promoter (U-HP) (Fig. 3*A*). siRNAs generated by the *ura4*$^+$ hairpin are sufficient to induce silencing of the *ura4*$^+$ reporter in *otr* repeats in *trans*. The U-HP-mediated silencing is lost in the *dcr1*Δ mutant, indicating that the hairpin-mediated *trans*-silencing requires RNAi (11). We crossed the U-HP construct into *rex1BD*Δ cells carrying *ura4*$^+$ at *otr3R2*. We found that silencing induced by U-HP is still intact in *rex1BD*Δ (Fig. 3*A*), suggesting that Rex1BD regulates heterochromatic gene silencing in an RNAi-independent pathway.

Overexpression of Rex1BD Rescues Silencing Defect in the *sir2*Δ Mutant. To further characterize the role of Rex1BD in heterochromatin pathways, we overexpressed Rex1BD from the plasmid under the control of the *nmt1* promoter in mutants defective in major heterochromatin pathways, including *dcr1*Δ, *sir2*Δ, the RITS mutant *chp1*Δ, and *dos1*Δ. Dos1/Raf1/Cmc1/Clr8 is part of the CLRC complex required for H3K9me (15–20). The mutant cells carrying *ura4*$^+$ at *otr3R2* grew slower than WT on the 5-fluoroorotic acid (FOA) counterselective media due to the loss of heterochromatin silencing. Remarkably, overexpressed Rex1BD restored heterochromatic silencing in the pericentromere region in the *sir2*Δ mutant (Fig. 3*B* and *SI Appendix*, Fig. S6).

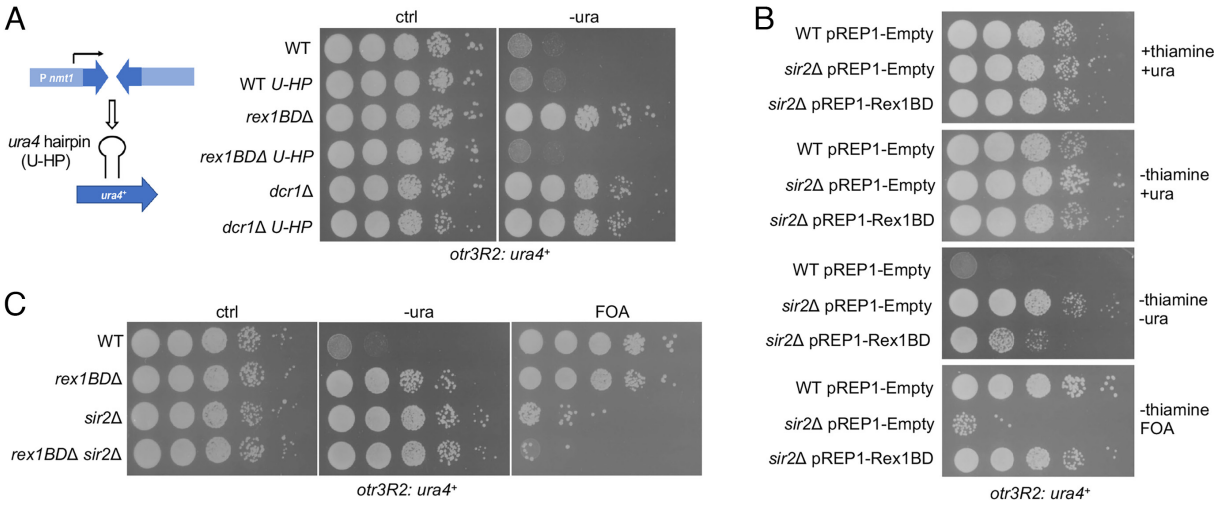

Fig. 3. Overexpression of Rex1BD rescues the silencing defect in *sir2*Δ. (*A*) Rex1BD acts in the RNAi-independent silencing pathway. Deletion of *rex1BD*$^{+}$ has little effect on the *ura4*$^{+}$ hairpin (U-HP)-induced silencing in *otr3R2* region. Indicated cells carrying U-HP and the *ura4*$^{+}$ reporter in the *otr3R2* region were analyzed by growth assays in -ura medium. (*B*) Overexpression of Rex1BD rescues the silencing defect in *sir2*Δ. The *rex1BD*$^{+}$ was constructed under the *nmt1* promoter. (*C*) Ten-fold serial dilutions of indicated cells carrying *ura4*$^{+}$ at *otr3R2* were plated on -ura or FOA medium.

However, defective silencing observed in *chp1*Δ, *dos1*Δ, and *dcr1*Δ cells could not be rescued by overexpressed Rex1BD (*SI Appendix*, Fig. S7). These data suggest that Rex1BD may regulate heterochromatin formation through an HDAC pathway parallel with Sir2. Consistent with the idea, we found that the *sir2*Δ *rex1BD*Δ double mutant displayed synthetic defects in heterochromatin silencing (Fig. 3*C*).

Rex1BD Associates with Histones, Clr6, and Rad25. To further probe the role of Rex1BD, we performed GST pull-down assays from cell lysates by using GST or GST-Rex1BD as baits and then used mass spectrometry to identify the potential binding partners in the pull-down samples. The proteins found in the GST-Rex1BD pull-down sample, not in the GST pull-down sample, were listed in Fig. 4*A*. Our mass spectrometry analysis identified histone $H2A^{hta1}$ and $H2B^{htb1}$ in the purified fraction (Fig. 4*A*). This is consistent with our prediction that Rex1BD may interact with histones due to its distinct charged surface revealed by its crystal structure. Furthermore, we found that Rex1BD associates with the histone deacetylase Clr6 complex subunit, Prw1 (Fig. 4*A*). Unlike Clr6, Prw1 is not essential for viability, but it is required for histone deacetylation activity of

A

Protein	Coverage	Peptide number	Function
Htb1	22%	3	Histone H2B
Hta1	7%	1	Histone H2A
Prw1	6%	3	Clr6C subunit
Nap1	19%	6	Histone chaperone
Rad25	50%	14	14-3-3 protein

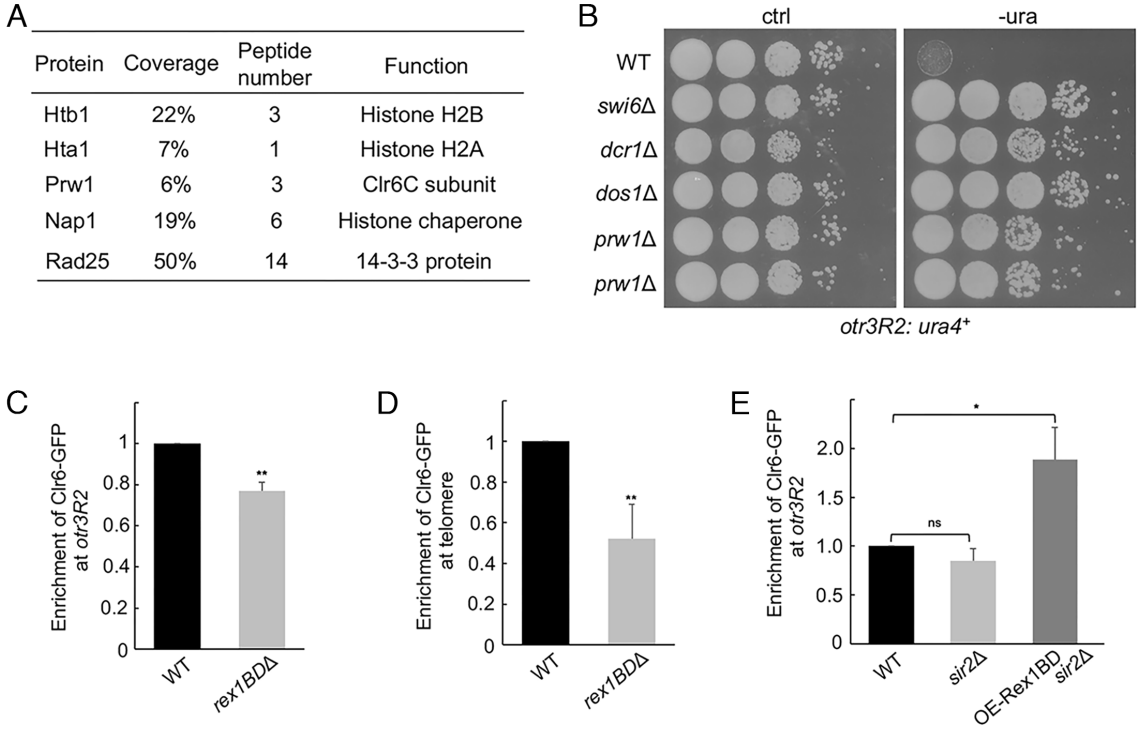

Fig. 4. Rex1BD mediates heterochromatic silencing by recruiting the HDAC Clr6. (*A*) Proteins identified by mass spectrometry analysis of purified Rex1BD. The number of peptides identified and sequence coverage (%) are shown. (*B*) Ten-fold serial dilutions of indicated cells carrying *ura4*$^{+}$ at *otr3R2* were plated on either -ura or FOA medium. Biological replicates for *prw1*Δ are independently generated strains. (*C* and *D*) ChIP-qPCR analysis of Clr6-GFP in the pericentromere (*C*) and the subtelomeric region (*D*) in the indicated strains. Actin was used as a control. Three independent experiments were performed. The level of WT was set to 1. Error bars indicate SD. $^{**}P < 0.001$. (*E*) ChIP-qPCR analysis of Clr6-GFP in the pericentromere in indicated strains. OE, overexpression. $^{*}P < 0.05$.

Clr6 (35). The *prw1*Δ mutant exhibited mild silencing defect in the *otr* region of chromosome 1 (*otr1*) (37). Using cells carrying *ura4*$^+$ at *otr3R2*, we showed that deletion of *prw1*$^+$ results in significant loss of silencing in the pericentromeric region (Fig. 4*B*), supporting that Prw1 is important for heterochromatic silencing. We also found the histone chaperone Nap1 in the complex (Fig. 4*A*). Nap1 has been shown to play a key role in chromatin assembly and histone storage (45). Intriguingly, the 14-3-3 protein Rad25 was one of the most abundant proteins exclusively associated with Rex1BD (Fig. 4*A*), which will be further characterized later in this work.

Rex1BD Facilitates the Recruitment of the Clr6 Complex to Heterochromatin. To determine how Rex1BD affects the association of the Clr6 complex with heterochromatin, we monitored the level of Clr6, a core component of the Clr6 HDAC complex, at the pericentromere region in the *rex1BD*Δ mutant by ChIP-qPCR. Our results indicated that Clr6-GFP was significantly reduced in both *otr3R2* and telomeres in the mutant (Fig. 4 *C* and *D*), suggesting that Rex1BD facilitates efficient recruitment of Clr6 to heterochromatin. We next examined the Clr6 level in *sir2*Δ cells overexpressing Rex1BD by ChIP. Our results showed that Clr6-GFP level in the pericentromeric region in the mutant was increased when Rex1BD was overexpressed (Fig. 4*E*). These data support that Rex1BD is important for the recruitment of Clr6 and may explain the observation that silencing defects in *sir2*Δ can be rescued by overexpressing Rex1BD.

Rad25 Is Important for Heterochromatin Silencing within Pericentromeric Repeats. Our mass spec data revealed that Rad25, a conserved member of the 14-3-3 family, physically associates with Rex1BD (Fig. 4*A*). To confirm the interaction, we conducted co-immunoprecipitation (Co-IP) using cells carrying Rad25-GFP and Rex1BD-TAP. We reproducibly detected the band corresponding to Rad25-GFP in the IP sample, confirming that Rad25 interacts with Rex1BD (Fig. 5*A*).

To determine how Rad25 regulates heterochromatin silencing in the pericentromeric repeats, we crossed the *rad25*Δ mutant into the background carrying *ura4*$^+$ at either *otr3R2* or *otr3R10*. Our growth assays showed that the mutant cells carrying *ura4*$^+$at *otr3R2* grew similarly to WT on the -ura media, indicating that silencing at *otr3R2* in *rad25*Δ is only mildly affected (Fig. 5*B*). However, the mutant displays a strong silencing defect in *otr3R10* (Fig. 5*C*). These results indicated that Rad25 is also important for heterochromatin silencing within pericentromeric silencing, and further suggested that coordinated action of Rad25 and Rex1BD may define epigenetic state within different pericentromeric repeats.

We also examined how the mutant contributes to silencing at the mating-type region and telomeres using strains carrying the *ura4*$^+$ or *his3*$^+$ reporter in these regions. Similar to the *rex1BD*Δ

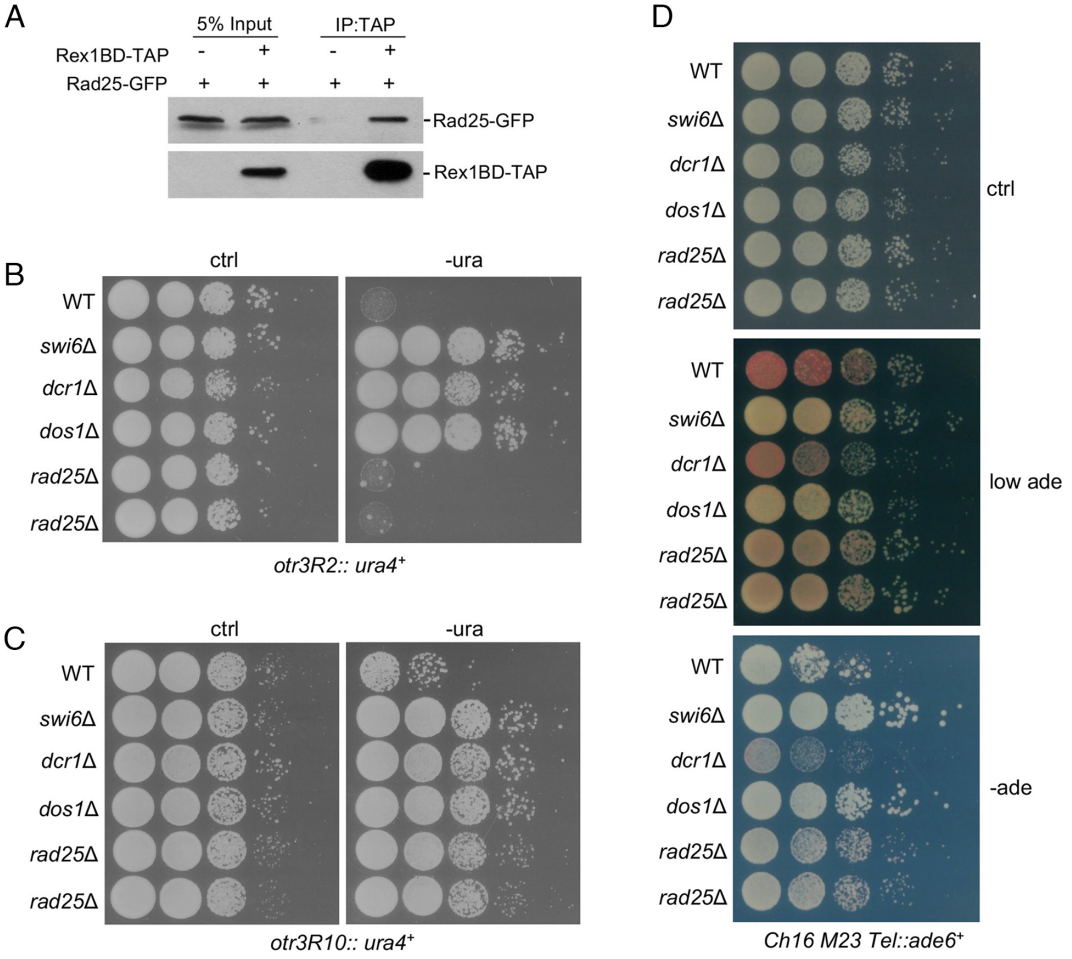

Fig. 5. Rad25 interacts with Rex1BD and is important for heterochromatin assembly. (*A*) Co-IP confirmed that Rad25 interacts with Rex1BD. Extracts from cells carrying Rex1BD-TAP and Rad25-GFP were immunoprecipitated with IgG beads. The input and immunoprecipitation were analyzed by western blotting using the antibody against the TAP or GFP tag. (*B*) Ten-fold serial dilutions of indicated cells with *ura4*$^+$ at *otr3R2* were plated on -ura medium. (*C*) Ten-fold serial dilutions of indicated cells with *ura4*$^+$ at *otr3R10* were plated on -ura medium. (*D*) Ten-fold serial dilutions of indicated cells with *ade6*$^+$ inserted at the subtelomeric region in minichromosome 16 were plated on YES medium supplemented with low adenine or minimal medium without adenine (-ade). Biological replicates for *rad25*Δ in this figure are independently generated strains.

mutant, we found that *rad25*Δ displays little silencing defect in the mating-type region (*SI Appendix*, Fig. S8). Silencing in telomeres in the *rad25*Δ cells did not show obvious changes, either (*SI Appendix*, Fig. S9). To further test the role of Rad25 in telomere silencing, we used a strain that carries the *ade6*$^+$ gene inserted in the subtelomere of the minichromosome Ch16-M23, in which the silencing level is decreased. WT cells with the minichromosome exhibit red colonies on low adenine media due to silencing of *ade6*$^+$. But *rad25*Δ cells carrying the minichromosome showed a light pink color, indicating that telomeric silencing in the minichromosome is disrupted. Consistent with this, *rad25*Δ cells with the *ade6*$^+$ reporter grew faster than WT on the minimum media without adenine (Fig. 5*D*). Although the silencing defect of *rad25*Δ is not as strong as *rex1BD*Δ, it contributes to the silencing in telomeres to a weaker extent.

Rad25 Regulates Heterochromatin Silencing by Recruiting the RITS Complex. To determine whether Rad25 acts in an RNAi-dependent manner, we crossed the *rad25*Δ mutant carrying *ura4*$^+$ reporter at *otr3R10* into the background of the *ura4*$^+$ hairpin (U-HP) inserted on chromosome 1. Unlike *rex1BD*Δ (Fig. 3*A*), *rad25*Δ cells showed a strong loss of silencing induced by U-HP (Fig. 6*A*), indicating that Rad25 mediates silencing in an RNAi-dependent manner. We further found that the double *rad25*Δ *rex1BD*Δ mutant carrying *otr3R10* shows synthetic silencing defect, consistent with the idea that Rad25 and Rex1BD act in different pathways in heterochromatin silencing regulation (Fig. 6*B*). Indeed, it has been previously demonstrated that Rad25 directly interacts with the RNAi effector protein Ago1 (46). But the effect of this interaction in heterochromatin silencing has not been studied. We next monitor the level of Chp1, a key component of the RITS complex, at the *otr3R10* repeat by ChIP-qPCR. Our data showed that the association of Chp1-GFP with *otr3R10* is significantly reduced in *rad25*Δ (Fig. 6*C*), indicating that Rad25 is important for the recruitment of the RITS complex to the heterochromatin repeat. Interestingly, we found that overexpression of *rad25*$^+$ could partially rescue the silencing defect of *rex1BD*Δ at pericentromere (Fig. 6*D* and *SI Appendix*, Fig. S10). It is likely that overexpression of Rad25 recruits more RITS to heterochromatin, which led to a higher level of CLRC and H3K9me that compensate for silencing loss in the *rex1BD*Δ mutant. Together, our data suggest that Rad25 and Rex1BD coordinate to mediate heterochromatin organization within pericentromeric repeats by connecting distinct heterochromatin pathways.

Discussion

Tandem DNA repeats are widespread and abundant in eukaryotic genomes (3, 47). They are often organized into heterochromatin, which acts as a key player in gene expression and genome stability (1, 6, 8, 9, 47, 48). Recent studies revealed that heterochromatin silencing between individual sequences within tandem repeats can

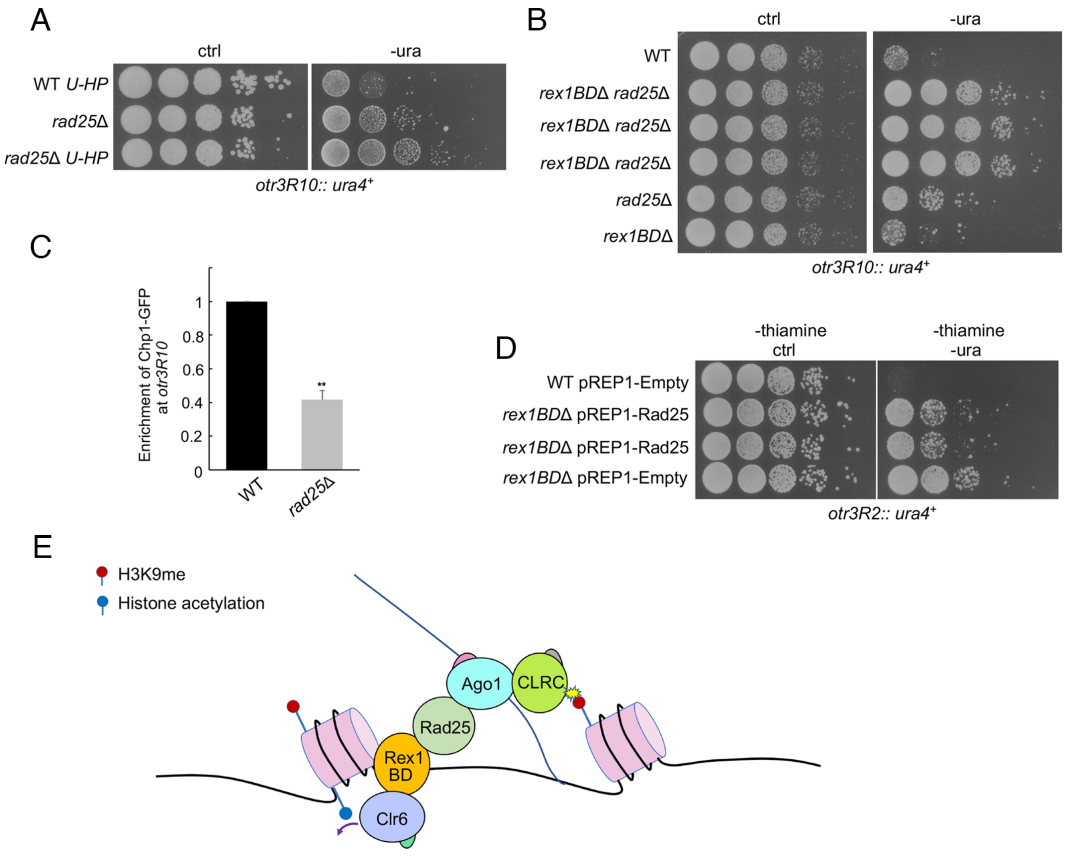

Fig. 6. Rex1BD regulates heterochromatin silencing by recruitment of the RITS complex. (*A*) Rad25 acts in RNAi-dependent heterochromatin pathway. Deletion of *rad25*$^+$ results in loss of the *ura4*$^+$ hairpin (U-HP)-induced silencing in the *otr3R10* region. Indicated cells carrying U-HP and the *ura4*$^+$ reporter in the *otr3R10* region were analyzed by growth assays in -ura medium. (*B*) Ten-fold serial dilution assay of the indicated strains carrying *otr3R2::ura4*$^+$on -ura medium. Biological replicates are independently generated strains. (*C*) ChIP-qPCR analysis of Chp1-GFP in the *otr3R2* in the indicated strains. Actin was used as a control. Three independent experiments were performed. The level of WT was set to 1. Error bars indicate SD. $^{**}P < 0.001$. (*D*) Overexpression of Rad25 partially rescues the silencing defect in *rex1BD*Δ. The *rad25*$^+$ was constructed under the *nmt1* promoter. Biological replicates are independently generated strains. (*E*) Model: Rex1BD promotes the recruitment of the Clr6 complex at heterochromatin repeats via interacting with histones, whereas Rad25 ensures the association of the RITS complex at the repetitive region. Coordinated action of Rex1BD and Rad25 mediates heterochromatin organization within tandem DNA repeats.

vary significantly (10–12). The molecular basis for the assembly of distinct heterochromatin structures at DNA repeats remains not well understood. In this study, we identified highly conserved Rex1BD together with the 14-3-3 protein Rad25 functions as a regulatory module regulating heterochromatin organization within tandem repeats by linking the HDAC pathway with RNAi. Our work also implicated heterochromatin assembly mediated by Rex1BD in heterochromatic DNA repair.

Using a reporter gene inserted at different units in a pericentromeric repeat array we created previously (11), we developed a genetic screen to identify factors important for the heterochromatin organization at tandem repeats. This unbiased genetic screen led to identification of a highly conserved but poorly characterized protein Rex1BD. Deletion of Rex1BD results in significant silencing loss at the strongly silenced repeat 2 in the *otr3* region but has little effect on repeat 10 in the repeat array, which exhibits weaker silencing. However, unlike Rex1BD, deletion of Dos1, a key component of the CLRC complex, and Dcr1 abolished the silencing in all the *otr* repeats (11). These suggest that Rex1BD may play a role in defining the position effect within the tandem repeat. Rex1BD is also important for telomere silencing, further highlighting its role in heterochromatin regulation. Our genetic assays revealed that Rex1BD acts in an RNAi-independent manner. Overexpression of *rex1BD*$^+$ could rescue the heterochromatic silencing defect of *sir2*Δ, suggesting that Rex1BD may act in an HDAC pathway parallel with Sir2. Indeed, our mass spec analysis of pull-downed Rex1BD showed that Rex1BD interacts with the HDAC Clr6 complex and also histones, including H2A and H2B. We further showed that Rex1BD is important for the recruitment of the Clr6 complex to heterochromatin regions.

Our mass spectrometry analysis also found that Rex1BD physically associates with the 14-3-3 protein Rad25. 14-3-3 proteins are a highly conserved family of regulatory hubs that function through a large network of protein–protein interactions. They modulate a variety of cellular processes, including protein localization, post-translational modification, and transcriptional activity (49, 50). But their role in chromatin regulation is largely unexplored. In this study, we found Rad25 is also important for heterochromatin assembly in pericentromeric repeats. But unlike *rex1BD*$^+$, deletion of *rad25*$^+$causes no obvious silencing defect at repeat 2 but severe silencing loss at repeat 10 in the *otr3* repeat array. The *rad25*Δ mutant also displays a moderate silencing defect in telomeres. Interestingly, our genetic assay revealed that Rad25 regulates heterochromatin silencing in the RNAi pathway. Consistent with the idea that Rad25 and Rex1BD act in different pathways, the *rex1BD*Δ *rad25*Δ double mutant exhibits synthetic silencing defect. In fact, it has been shown that Rad25 directly interacts with the Argonaute protein Ago1 (46). However, the importance of this interaction in heterochromatin silencing has not been explored. Our data indicated that Rad25 is critical for the recruitment of Ago1 to heterochromatin repeats, which may in turn promote the recruitment of the CLRC complex to mediate H3K9me.

HDAC-mediated histone deacetylation is a conserved mechanism used to regulate heterochromatin assembly (8, 14, 51). RNAi is also required for heterochromatin formation in many species (21). How the HDAC- and RNAi-mediated pathways are coordinated to ensure proper heterochromatin assembly remains poorly understood. This work established a physical connection between HDAC and RNAi pathways. Based on our data, we proposed the following model: Rex1BD facilitates efficient loading of Clr6 complex to heterochromatin repeats via interacting with histones, while Rad25 promotes the association of RITS with the tandem array. Rex1BD and Rad25 together form a regulatory hub that defines heterochromatin silencing within tandem repeats (Fig. 6*E*).

Due to its repetitive nature, heterochromatin presents unique challenges to DNA repair. Distinct mechanisms have been adopted to repair DNA damage in heterochromatin (4, 52). Rex1BD was originally found to be involved in DNA repair. The Rex1BD mutant in *C. reinhardtii* showed strong sensitivity to MMS and UV light (42). But the underlying mechanism remains unknown. We found that the deletion of *rex1BD*$^+$in fission yeast also results in moderate DNA repair defects. The mutants of the Clr6 subunits, including *prw1*Δ, are sensitive to DNA-damaging agents. It has been proposed that the defects could arise from the general relaxation of chromatin (35). Our observation that the association of Clr6 HDAC complex with chromatin depends on Rex1BD may explain the DNA repair defect observed in the *rex1BD*Δ mutant. Rad25 in fission yeast initially also was found to be involved with DNA damage repair (53). It is likely that the heterochromatin organization mediated by the Rex1BD-Rad25 module creates a local environment that facilitates DNA damage repair in heterochromatin.

Materials and Methods

Strains, Media, and Genetic Analysis. Standard media and genetic analysis for fission yeast were used (54). Yeast extract with supplements (YES) was used as a complete culture and pombe glutamate medium (PMG) as a minimum media. For silencing assays, a series of 10-fold dilutions with a starting concentration of 1 × 10^7 cells/mL were spotted on the designated media and incubated at 30 °C for 3–4 d. Domain-deleted *rex1BD* mutants were created by homologous recombination. The genetic screen will be described elsewhere. Fission yeast strains used in this study are listed in *SI Appendix*, Table S2.

ChIP-qPCR. Chromatin immunoprecipitation (ChIP) was performed as described (55). Briefly, 50 mL of log-phase yeast culture was cross-linked by adding 37% formaldehyde for 30 min. Cells were collected by centrifuge at 2,500 rpm for 2 min and sonicated by an Ultrasonic Processor. For immunoprecipitation, 1 μL of antibody was used. Immunoprecipitated DNA was purified using NucleoSpin® Gel and PCR Clean-Up kit (Takara Bio) and analyzed by quantitative PCR (qPCR). Antibodies used were anti-GFP (Abcam, ab290), anti-H3K9me2 (Abcam, ab1220), and anti-H3K9 acetylation (Millipore, 07-352).

qPCR was performed with SYBR Green on a Bio-Rad Optics Module CFX96 qPCR/RT PCR Thermal Cycler. Relative enrichments were calculated by comparative ΔCq and normalized to *act1*$^+$ in IP over input. Expression values are presented relative to the expression in the wild-type strain (56). Histograms represent three biological replicates; error bars represent one SD. Primers used in this study are listed in *SI Appendix*, Table S3.

Co-IP. Co-IP was performed as described previously (57). Briefly, 100 mL of log-phase yeast cells were collected and resuspended in 100 μL of lysis buffer with protease inhibitors prior to lysis by bead beating. Lysates were incubated with IgG Sepharose (GE Healthcare, No. 17096901) at 4 °C for 1 h. After washing three times with lysis buffer, proteins were eluted in the SDS loading buffer. Eluates were analyzed by western blotting using a commercial anti-GFP antibody (Abcam, ab290) and an anti-TAP antibody (P1291; Sigma).

Microscopic Analysis. Microscopy was performed as described (58). Cells were imaged using the Delta Vision System (Applied Precision, Issaquah, WA). Images were taken as z-stacks of 0.2-μm increments with an oil immersion objective (×100) and deconvolved using SoftWoRX2.50 software (Applied Precision).

Protein Expression and Purification. Rex1BD$_{2\text{-}137}$ (Uniprot: Q10214) was cloned into a modified pGEX-6P-1 vector with a GST-tag fused at the N terminus followed by a 3C protease site. Rex1BD was expressed in *E. coli* Rosetta cells. After induction at OD_{600} 0.8 for 16–18 h with 0.2 mM IPTG at 16 °C, cells were then collected and resuspended in lysis buffer containing 50 mM Tris-HCl pH 8.0, 400 mM NaCl, 10% glycerol, 2 mM β- mercaptoethanol, and protease inhibitor cocktail. After sonication and centrifugation, the supernatant was mixed with

glutathione Sepharose 4B beads (GE Healthcare) and rotated at 4 °C for 2 h. After extensive washing by lysis buffer, 3C protease was added to remove the GST tag, and the tag-free proteins were collected. For GST-Rex1BD$_{2\text{-}137}$, the bound proteins were eluted by the elution buffer containing 15 mM reduced glutathione. The eluted Rex1BD was further purified by size-exclusion chromatography with a Hiload Superdex 200 column (GE Healthcare). The purified Rex1BD was concentrated and stored at −80 °C. Se-Met Rex1BD was purified by the same procedure.

Crystallization, Data Collection, and Structure Determination. The crystals of the Se-Met Rex1BD$_{2\text{-}137}$ were obtained at 16 °C in the reservoir solution containing 8%v/v Tacsimate pH5.0, 20%w/v polyethylene glycol 3350. Se-Met Rex1BD diffraction data were collected at the beamline BL19U1 of the Shanghai Synchrotron Radiation Facility. The diffraction data were processed using XDS package and XSCALE (59), which was also used to merge and scale three data sets for Se-SAD phasing. The initial phasing and density modification were implemented using phenix.autosol at the resolution of 3.38 Å, and the initial model was built automatically by phenix.autobuild. The final structure model was obtained by iteratively manual building by Coot (60) with refinement using phenix.refine (61). There are eight Rex1BD molecules in one asymmetric unit, forming two tetramers. The monomer with the best density contains residues 4–137.

In Vitro Pull-down Assay and Mass Spectrum Analyses. For in vitro pull-down assay, fission yeast cells were grown in YES rich media to log phase at 30 °C and then collected by centrifugation. After cells were lysed using a BeadBeater, cell lysates were centrifuged for at least 20 min at 14,000 rpm at 4 °C. The supernatant was mixed with GST-Rex1BD and glutathione Sepharose 4B beads (GE Healthcare) and rotated at 4 °C for 4 h. After washing with lysis buffer three times, the bound proteins were eluted with 15 mM reduced glutathione. The eluted samples were then collected and sent out for mass spectrometry analysis (National Facility for Protein Science Shanghai).

Data, Materials, and Software Availability. Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Coordinates and structure factors have been deposited in the Protein Data Bank under accession code: 8J0H (62). All other data are included in the manuscript and/or *SI Appendix*.

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Author affiliations: [a]Department of Biology, New York University, New York, NY 10003; [b]Key Laboratory of Epigenetic Regulation and Intervention, State Key Laboratory of Molecular Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai 200031, China; [c]National Facility for Protein Science Shanghai, Zhangjiang Lab, Shanghai Advanced Research Institute, Chinese Academy of Science, Shanghai 201210, China; and [d]Key Laboratory of Systems Health Science of Zhejiang Province, School of Life Science, Hangzhou Institute for Advanced Study, University of Chinese Academy of Sciences, Hangzhou 310024, China

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