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Prospects & Overviews

Heterochromatin repeat organization at an individual level: Rex1BD and the 14-3-3 protein coordinate to shape the epigenetic landscape within heterochromatin repeats

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Abstract

In eukaryotic cells, heterochromatin is typically composed of tandem DNA repeats and plays crucial roles in gene expression and genome stability. It has been reported that silencing at individual units within tandem heterochromatin repeats exhibits a position-dependent variation. However, how the heterochromatin is organized at an individual repeat level remains poorly understood. Using a novel genetic approach, our recent study identified a conserved protein Rex1BD required for position-dependent silencing within heterochromatin repeats. We further revealed that Rex1BD interacts with the 14-3-3 protein to regulate heterochromatin silencing by linking RNAi and HDAC pathways. In this review, we discuss how Rex1BD and the 14-3-3 protein coordinate to modulate heterochromatin organization at the individual repeat level, and comment on the biological significance of the position-dependent effect in heterochromatin repeats. We also identify the knowledge gaps that still need to be unveiled in the field.

KEYWORDS
14-3-3 protein, DNA repeats, HDAC, heterochromatin, Rex1BD, RNAi, *Schizosaccharomyces pombe*

INTRODUCTION

The eukaryotic genomic DNA is wrapped around histone proteins, including histone H3, H4, H2A, and H2B, to form nucleosomes. Nucleosomes connected by the linker DNA are further packaged into chromatin. Chromatin has been divided into two structurally and functionally distinct domains: euchromatin and heterochromatin. Euchromatin is less compact and contains transcriptionally active genes, while heterochromatin is highly condensed and less accessible for transcription.[1,2] The highly condensed heterochromatin is typically composed of tandem repetitive DNA sequences, in which copies of DNA sequence are positioned one after another, such as seen in pericentromeres and telomeres in most eukaryotes[3–5] (Figure 1). Heterochromatin is preferentially localized toward the nuclear periphery. Repositioning of a gene from the interior of the nucleus to the nuclear periphery often correlates with an increase of transcriptional silencing. Nevertheless, heterochromatin can also be found in discrete bodies inside the nucleus.[6–8] Heterochromatin plays an important role in gene expression, genome stability, and centromere function. The silenced chromatin domain is also implicated in cell differentiation regulation during development.[1,9]

Heterochromatin is characterized by unique post-translational modifications (PTM) of histones, including histone H3K9 methylation and hypoacetylation of histones (Figure 1). These histone PTMs, considered as "epigenetic marks" for heterochromatin, are important for the establishment and maintenance of the chromatin domain. H3K9 methylation is catalyzed by the SUV39 class of histone methyltransferase enzymes (Figure 1). The modification then serves as a binding site for the chromodomain-containing protein HP1, which folds chromatin into higher-order structure. On the other hand, histone

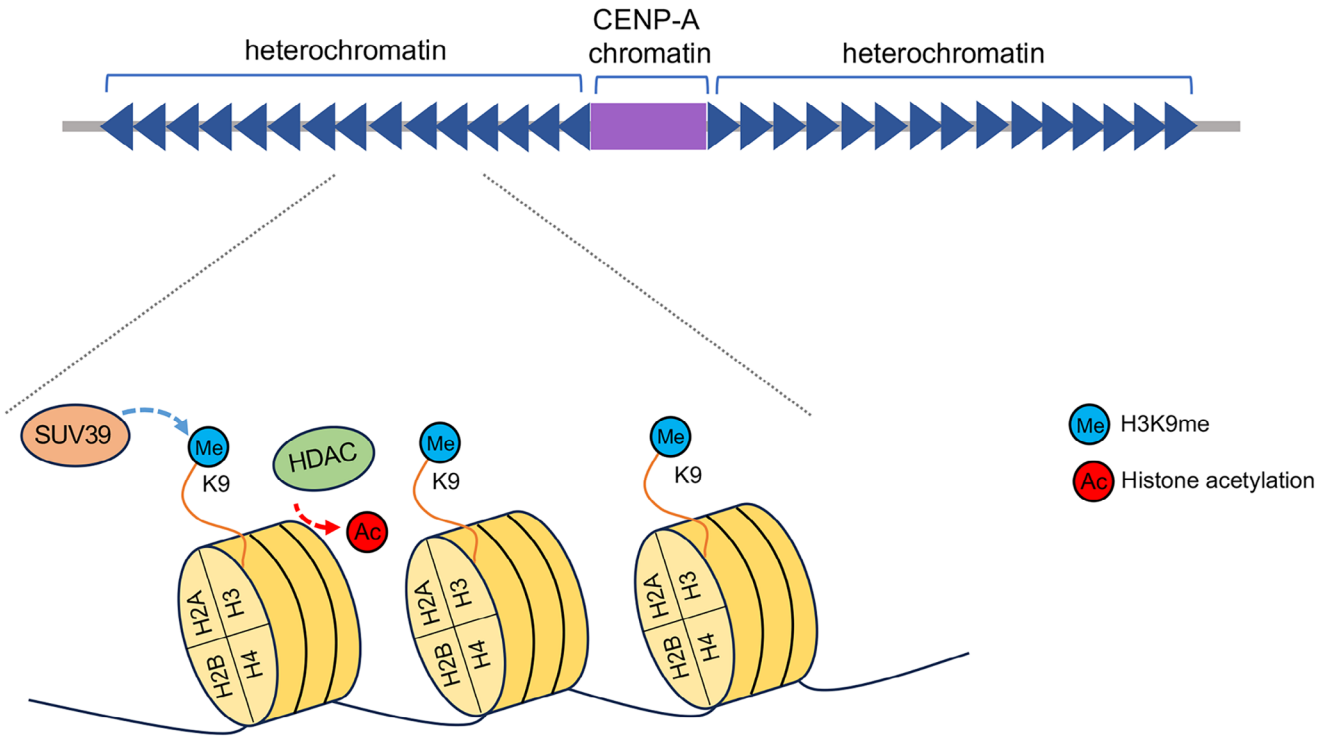

FIGURE 1 Heterochromatin generally consists of tandem repetitive DNA sequences and is characterized by histone H3K9 methylation and hypoacetylation of histones. The conserved SUV39-family histone methyltransferase catalyzes H3K9 methylation, while histone deacetylases (HDACs) are responsible for removing acetyl groups from lysine residues in histones. CENP-A chromatin in regional centromeres is typically flanked by pericentromeric heterochromatin repeats.

deacetylases (HDACs) are enzymes responsible for removing acetyl groups from lysine residues in histones[1,9] (Figure 1). It has also been shown that heterochromatin can also undergo low-level transcription to generate noncoding RNAs. These heterochromatin transcripts can be processed into small interference RNAs (siRNAs) by RNA interference (RNAi) machinery in a variety of organisms, including fission yeast, plants, and animals. The siRNAs generated from heterochromatin can in turn mediate H3K9 methylation and heterochromatin formation.[10,11] However, it seems that in flies and mammals, RNAi is only required for silencing DNA repeats in germline cells.[12] Recent studies have shown that noncoding RNAs from pericentromeric heterochromatin in mammalian cells can act in RNAi-independent manner to help recruit SUV39 at heterochromatin.[13–15]

Owing to their repetitive nature, it has been challenging to study the behavior of heterochromatin repeats at an individual level. It has been presumed that the silencing state in each DNA repeat is distributed uniformly. But recent studies have shown that transcription level and replication activity at different repeats in a repeat array can vary significantly.[16,17] How heterochromatin organization is regulated at individual levels and the biological significance of this position-dependent effect within the tandem repeats remain poorly understood. In this review, we highlight recent development in understanding mechanisms underlying heterochromatin organization at the individual level using fission yeast as a model organism, and point out the knowledge gaps that still need to be unveiled in the field.

Fission yeast as a model organism for studying heterochromatin repeat organization

The fission yeast is a unicellular eukaryote that offers unique advantages for studying heterochromatin assembly. In addition to being easily manipulated genetically, fission yeast has only three chromosomes and contains one of the smallest numbers of genes of a known eukaryotic genome.[18] In addition, heterochromatin in fission yeast, including pericentromeres, telomeres and the mating-type locus, are associated with conserved heterochromatin marks, including histone H3K9 methylation and histone hypoacetylation. However, heterochromatin in fission yeast lacks DNA methylation, which makes it simple to examine the relationship between histone modifications and chromatin epigenetic states.[19,20]

Fission yeast heterochromatin is also lowly transcribed into noncoding RNAs, especially during the S phase. The centromeric transcripts are processed into small interference RNAs (siRNAs) by RNAi, which promotes H3K9 methylation. In fact, the role of RNAi in heterochromatin formation was first demonstrated in fission yeast, and subsequently was also found in plants and animals. On the other hand, H3K9 methylation and RNAi are absent in the budding yeast *Saccharomyces cerevisiae*.[10,11,21] Also, unlike *S. cerevisiae* that contains genetically defined "point" centromeres-small regions of ~120 bp, fission yeast has large complex "regional" centromeres. Regional centromeres, which are attached to multiple microtubules during mitosis, are epigenetically

specified by the conserved histone H3 variant, CENP-A. CENP-A interacts with the CCAN (constitutive centromere-associated network) and forms a platform to recruit the outer kinetochore proteins.[22,23] Like in higher eukaryotes, the CENP-A-enriched centromere region in fission yeast is buried in the pericentromeric heterochromatin repeats (see details below). The peri-centromere heterochromatin in fission yeast comprises relatively small arrays of DNA repeats, which provides a simple framework to study the position-dependent heterochromatin organization.[24]

Mechanisms of heterochromatin assembly in fission yeast

H3K9 methylation

H3K9 methylation in fission yeast heterochromatin is mediated by ClrC (Clr4 methyltransferase complex), which consists of Clr4, Rik1, Raf1/Dos1, Raf2/Dos2, Cul4, and Lid2. Clr4, the homolog of the mammalian Suv39, is the sole H3K9 methyltransferase responsible for mono-, di-, and tri-methylation of H3K9 in *Schizosaccharomyces pombe*.[25,26] Rik1 is a WD-40 repeat protein that shares homology with the human E3 ligase adaptor DDB1.[27,28] Rik1 also contains an RNA-binding domain at its C-terminus.[29] Raf1, also called Dos1, Cmc1 and Clr8, is structurally similar to the human DDB2, the partner of DDB1.[27,30–33] Raf2/Dos2/Cmc2/Clr7 acts as a hub of the complex by interacting with all ClrC subunits.[34] Cul4 is the cullin scaffold protein serving as the central scaffold of the E3 ubiquitin ligase. The ClrC complex has the ubiquitin E3 ligase activity.[32,35] A recent work has further shown that ClrC ubiquitylates lysine 14 at histone H3 (H3K14ub), which further promotes the H3K9 methylation by Clr4.[36] Lid2 is an H3K4 demethylase also important for H3K9 methylation in heterochromatin.[37] Similar to higher eukaryotes, H3K9 methylation catalyzed by the ClrC complex serves as a binding site for the chromodomain reader proteins, including Swi6, Chp1, and Chp2, which recruit downstream effectors for the heterochromatin assembly.[25,38–41] During DNA replication, the Pol Epsilon complex interacts with the ClrC complex to mediate epigenetic inheritance of H3K9 methylation through the cell cycle.[42,43]

RNAi-mediated heterochromatin silencing

Fission yeast contains a single copy of Dicer (Dcr1), an RNase III enzyme, Argonaute (Ago1), and the RNA-dependent RNA polymerase (Rdp1). RNAi is required for H3K9 methylation and gene silencing in heterochromatin. The noncoding RNAs are convergently transcribed from heterochromatin by the RNA polymerase II (RNAPII) during the S phase of the cell cycle.[44–46] The noncoding transcripts are then converted into double-stranded RNAs (dsRNAs) by the RNA-directed RNA polymerase complex (RDRC), which consists of Rdp1, an RNA helicase Hrr1, and Cid12, a noncanonical Poly(A) polymerase.[47] The dsRNAs are cleaved into siRNAs by Dicer. The resulting siRNAs are incorporated into the RNA-induced transcriptional silencing (RITS) complex that comprises Ago1, the chromodomain protein Chp1, and Tas3.[48] The Argonaute protein Ago1 is responsible for the recognition and interaction with siRNAs. The siRNAs guide the localization of RITS into heterochromatin likely by sequence complementarity with heterochromatic RNAs.[48,49] Chromatin-associated RITS recruits the ClrC complex to heterochromatin through the interaction with the ClrC complex-Argonaute linker protein Stc1.[50]

Histone deacetylase (HDAC)-mediated silencing

The histone hypoacetylation in fission yeast is catalyzed by three subtypes of histone deacetylases (HDACs): Clr6 (Class I), Clr3 (Class II), and Sir2 (Class III). The Class I subtype Clr6 is a human HDAC1 homolog that forms a stable complex with the RbAp48-related histone-binding protein Prw1, the MRG-family protein Alp13, and the Sin3 homolog Pst2.[51] Clr6 is an essential gene that mediates both heterochromatin silencing and gene expression in euchromatin. Defects in the Clr6 complex also causes sensitivity to DNA-damaging agents and widespread antisense transcripts.[52,53] It has been shown that the conserved splicing factor RBM10 in fission yeast facilitates the recruitment of the Clr6 complex to heterochromatin.[54] The Class II subtype Clr3 is the key component of the SHREC complex. The SHREC complex, also including Clr1, Clr2 and the ATP-dependent chromatin remodeler Mit1, distributes throughout all major heterochromatin domains, and is important for heterochromatin assembly.[52,55] A recent study has shown that Clr3 is necessary and sufficient to support heterochromatin propagation in different chromosomal contexts.[56] The Class III subtype HDAC Sir2 is the Sirtuin family protein that can deacetylate a series of lysine of histone H3 and H4, and its catalytic activity requires NAD^+. Sir2 in fission yeast appears to be important for both the ClrC-mediated heterochromatin initiation and subsequent H3K9 methylation spreading steps.[57–59] Dpb4, a conserved DNA polymerase epsilon subunit, is important for the recruitment of Sir2 to heterochromatin during S phase.[60,61]

Centromere organization in *S. pombe*

In *S. pombe*, three centromeres are different in size but share a similar structure. Each centromere is composed of two distinct domains: the ~15 kb central region, and 20- to 100-kb surrounding outer repeat sequences (*otr*). The central region consists of the core region (cnt, centromere core domain) and the flanking innermost repeats (imr) that contain perfect repeats in an inverted orientation.[18,62] The central region is enriched with Cnp1, the *S. pombe* homolog of CENP-A, that functions as an epigenetic mark for specifying centromeres.[63,64] The pericentromeric *otr* region contains large repeats common to all centromeres. Each *otr* repeat is composed of *dg* and *dh* elements and spans ~6.7 kb. The number of *otr* repeats varies between different centromeres.[18,62] The *otr* repeat array in the left arm of the centromere in chromosome 3 (*otr3L*) is the largest, composed of 12

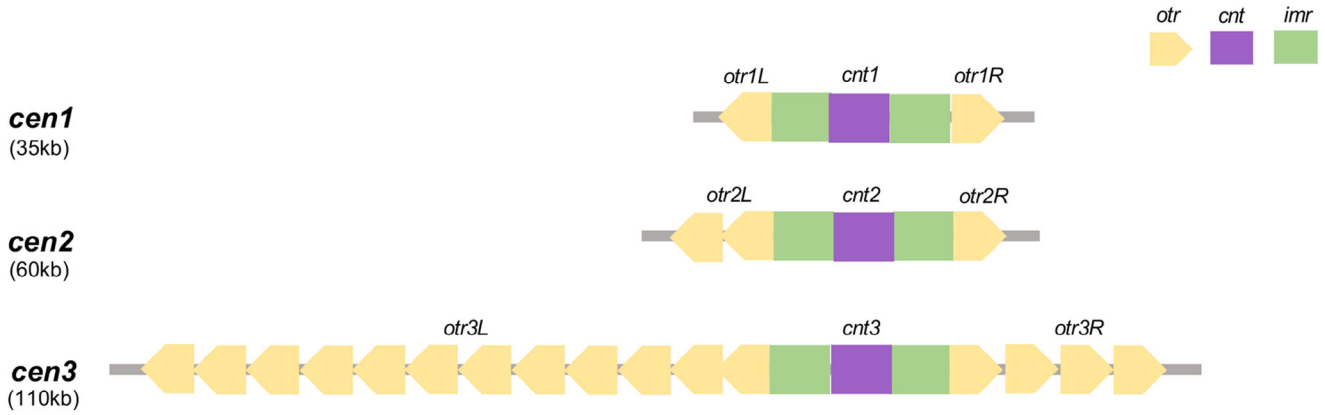

FIGURE 2 Scheme of fission yeast centromeres. The central core regions (*cnt*) are flanked by the innermost repeats (*imr*) and the outermost repeats (*otr*). Each of *otr* repeats is consisted of *dg* and *dh* elements.

repeats (Figure 2).[16] The pericentromeric *otr* region is organized into heterochromatin characterized by H3K9 methylation and histone hypoacetylation. This region can also be briefly transcribed during the S phase into noncoding transcripts, which are processed into siRNAs to mediate heterochromatin assembly, as discussed above.

Heterochromatin silencing at peri-centromeres in fission yeast can be detected using reporter genes inserted at the peri-centromeric repeats. This sensitive and reliable method has become one of the most powerful techniques used to decipher the molecular basis of heterochromatin structure and function in yeasts.[65,66] To investigate the heterochromatin organization at an individual DNA repeat level, our group recently constructed a series of strains in which the *ura4*$^+$ reporter gene is inserted in different individual repeats at the *otr3L* region.[16] Using this collection, we found that the transcriptional silencing and replication timing are significantly different among different repeats. As the repeats are close to the core centromere, the silencing of *ura4*$^+$ is decreased along with lower H3K9 methylation enrichment, whereas the silencing in the repeats close to the chromosome arm is stronger with higher level of H3K9 methylation.[16] This study reveals previously unknown position effects within tandem DNA repeats, but the mechanism for how DNA repeats at the individual level are regulated remains poorly understood.

A novel genetic screen identifies Rex1BD as a key regulator of heterochromatin repeat organization

To understand the mechanism underlying heterochromatin repeat organization at the individual level, we recently developed a novel genetic screen to identify factors important for the position-dependent silencing in pericentromeric repeats by taking advantage of the collection of strains with the repeat-specific reporter at the *otr3L* region.[67] The genetic screen includes two rounds of screening. In the first round, the query strain we used from the collection carrying the *ura4*$^+$ reporter in repeat 2 in the *otr3* repeat array (*otr3R2*) was crossed with the mutant strains from the Bioneer *S. pombe* gene deletion library (Figure 3). The *ort3R2* repeat has the strongest silencing level compared with other repeats in the *otr3* repeat array, and thus provides a sensitive readout for detecting the silencing change, even for mutants that exhibit weak silencing defects. The mutants showing silencing defects in *ort3R2* are then used for the second round of screening by crossing with the query stain carrying *otr3R10::ura4*$^+$, which displays weaker silencing than *ort3R2* (Figure 3).[67]

Through this screen, we identified an uncharacterized protein Rex1BD that is important for heterochromatin silencing for *otr3R2*, but deletion of the gene has little effect on silencing in *otr3R10*[67] (note: following new fission yeast gene nomenclature guidelines,[68] we recently renamed *rex1BD*$^+$ to *rss1*$^+$ (*r*epeat *s*pecific *s*ilencing factor 1)). In contrast, the deletion of *dcr1*$^+$ or the ClrC subunit *raf1*$^+$ leads to the absolute abolishment of silencing in all *otr* repeats.[16] Further analysis revealed that Rex1BD plays a key role in the site-specific regulation of heterochromatin silencing in the pericentromeric repeat region.[67] Rex1BD is highly conserved in eukaryotic organisms, including mammals. Rex1BD was implicated in DNA repair in *Chlamydomonas reinhardtii*,[69] and has also been linked with human diseases, including indeterminate leprosy and borderline leprosy.[70] But its molecular function remains little known. Our structural analysis demonstrated that Rex1BD is composed of four helices that fold into a compact helical bundle. The surface of Rex1BD is highly charged with distinct electrostatic-property patches which may account for the interaction with other proteins. Interestingly, although the gel-filtration analysis shows that Rex1BD is a monomer in solution, Rex1BD can form a tetramer mediated by the electrostatic interactions in vitro.[67] It will be interesting to investigate whether Rex1BD exists as a tetramer in vivo in future.

Rex1BD and the 14-3-3 protein coordinates to control repeat-specific heterochromatin silencing at pericentromeric repeats by linking RNAi and HDAC pathway

To determine whether Rex1BD regulates heterochromatin silencing in an RNAi-dependent manner, we took advantage of the strain carrying

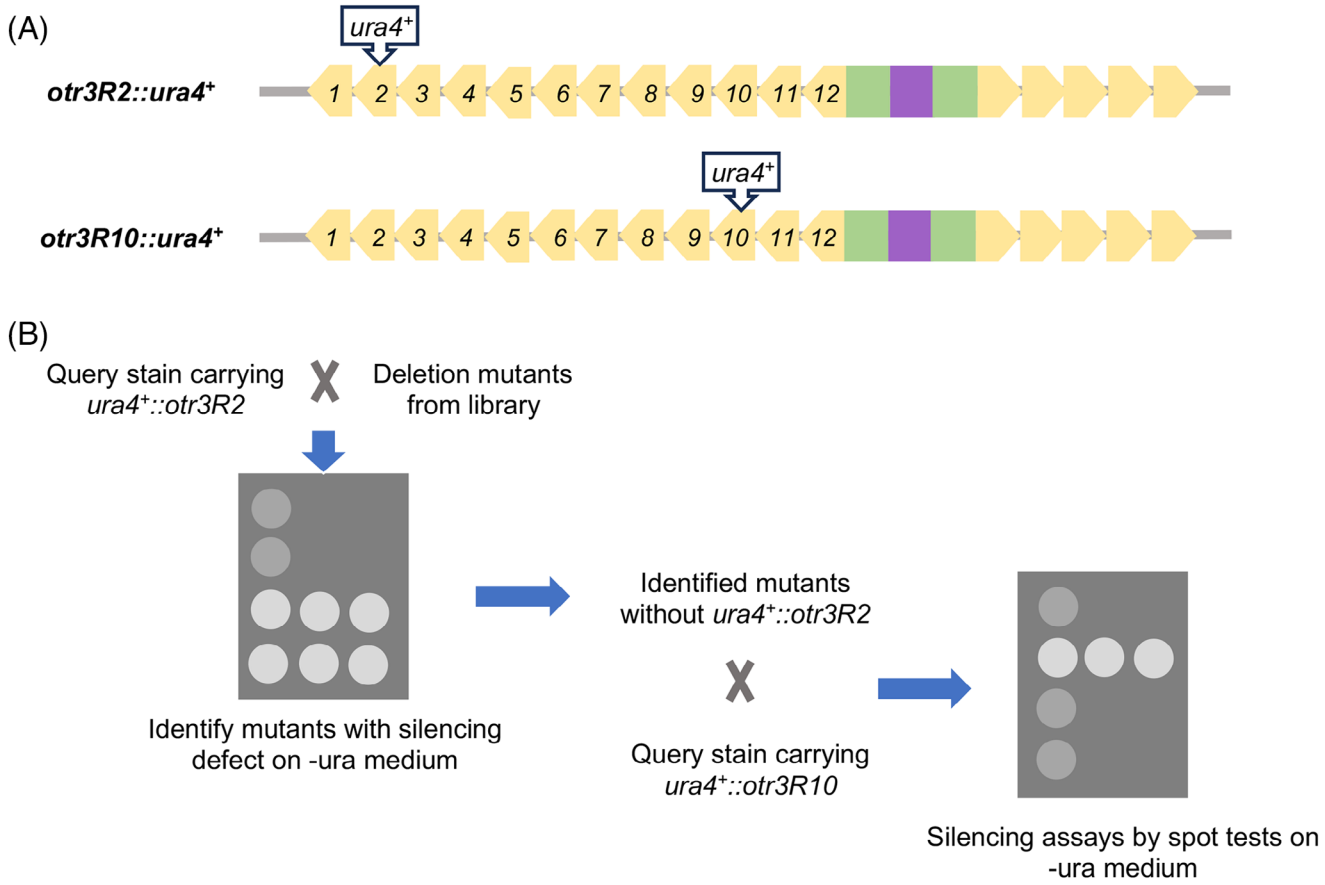

FIGURE 3 A novel genetic screen to identify factors involved in position effect in pericentromeric repeats. (A) Schematic structure of the query strains used in the genetic screen containing the *ura4*$^+$ reporter either at *otr3R2* or *otr3R10*. (B) Schematic representation of the screening strategy. -ura, no uracil.

a hairpin structure of *ura4*$^+$ (U-HP) that can induce heterochromatin silencing *in trans* at the *ura4*$^+$ target locus at heterochromatin via RNAi.[71] We found that the U-HP could rescue the silencing defect caused by the loss of Rex1BD, indicating the Rex1BD acts in an RNAi-independent manner.[67] Interestingly, overexpression of Rex1BD rescues the silencing defects in the HDAC *sir2*$^+$ deletion mutant, but does not restore the silencing level in other the key heterochromatin mutants, including *chp1*Δ, *raf1*Δ, and *dcr1*Δ. Furthermore, the double mutant *sir2*Δ *rex1BD*Δ showed a strong synthetic silencing defect compared to single mutants. These data suggest that Rex1BD may act in an HDAC pathway parallel with Sir2. Indeed, our mass spectrometry analysis of purified Rex1BD revealed that Rex1BD interacts with the key Clr6 complex subunit Prw1 as well as histone proteins. Deletion of Rex1BD results in significant loss of the Clr6 complex at the *otr3R2* region. These results indicate that Rex1BD regulates the heterochromatin silencing through the recruitment of the Clr6 HDAC complex to histones.[67]

Our mass spectrometry analysis also identified that Rex1BD interacts with the conserved 14-3-3 protein Rad25. Interestingly, silencing is lost in *otr3R10* in *rad25*Δ mutant, but has little change in *otr3R2*, indicating that Rad25 also participates in regulation of the position effect within the heterochromatin repeat array, but in a manner different from Rex1BD. Using the strain carrying the hairpin structure U-HP, we showed that Rad25 regulates heterochromatin in an RNAi-mediated pathway.[67] In fact, a previous study demonstrated that Rad25 directly interacts with Ago1.[72] Our ChIP assay further revealed that Rad25 recruits the RITS complex to heterochromatin through the interaction with Ago1. In addition, the *rad25*Δ *rex1BD*Δ mutant showed increased silencing defect compared to each single mutant. Together, our data suggest that Rex1BD and Rad25 coordinate to define the heterochromatin silencing at the individual level by linking HDAC and RNAi pathways (Figure 4).[67]

Although using the *ura4*$^+$ reporter is a powerful method to investigate the heterochromatin repeat organization, we cannot totally rule out that the position-dependent silencing we observed in heterochromatin repeats results from the artifact of *ura4*$^+$ insertion. Future experiments, such as replacing *ura4*$^+$ at *otr* repeats with a different reporter gene, including *ade6*$^+$, can further confirm it. Nevertheless, our genetic and biochemical characterization of Rex1BD and Rad25 as discussed above support that Rex1BD and Rad25 play a distinct role in pericentromeric heterochromatin repeat organization. Rex1BD and Rad25 are also important for silencing in telomeres.[67]

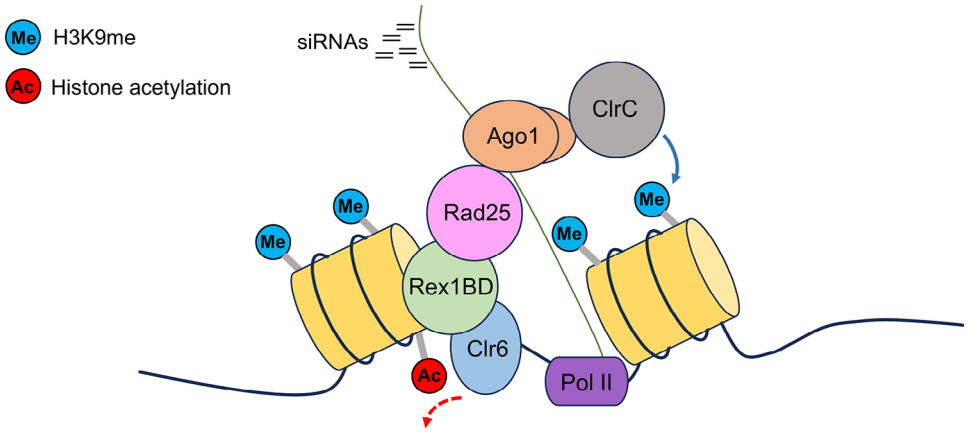

FIGURE 4 A model of Rex1BD-Rad25-mediated molecular regulation of heterochromatin repeat organization. Rex1BD interacts with histones to recruit the HDAC Clr6 complex to heterochromatin repeats, whereas Rad25 promotes the localization of the RITS complex at the repeats. Rex1BD and Rad25 coordinate to define heterochromatin silencing at individual repeats by linking the RNAi and HDAC pathways. H3K9me, H3K9 methylation.

The biological significance of position effect at pericentromeric repeats

The position-dependent effect within pericentromeric repeats suggests that these heterochromatin regions form distinct higher-order three-dimensional (3D) structures. What is the biological significance of the position effect? The centromere is vital for kinetochore assembly and accurate chromosome segregation during cell division.[22,73] CENP-A, the centromere epigenetic mark, must be properly positioned to centromeres. Mislocalization of CENP-A has been shown to result in kinetochore malfunction and chromosome missegregation in a variety of organisms.[74–80] The fact that both sides of centromeres contain the *otr* heterochromatin repeats in fission yeast has prompted the speculation several decades ago that the two sides of pericentromeric repeats interact to form the higher-order structure to stabilize the kinetochore assembly.[81,82] Recent works have demonstrated that heterochromatin is required for the establishment of CENP-A chromatin in the central region of centromeres.[83,84] Yang et al. have also shown that pericentromeric heterochromatin can protect CENP-A in centromeres from ubiquitin-mediated degradation.[85] The position-dependent effect within pericentromeric repeats thus may contribute to proper CENP-A positioning in centromeres.

Double strand break (DSB) repair is crucial for cell survival and genome integrity. DSB repair is particularly challenging in pericentromeric heterochromatin, due to the abundance of repeated sequences. Recent studies have shown that heterochromatin repeats have adopted unique mechanisms to repair DNA damage.[86–88] The Rex1BD mutant in *C. reinhardtii* was originally found to have severe DNA repair defects.[69] Rex1BD in fission yeast also showed moderate DNA repair defects.[67] Rad25 was initially reported to contribute to DNA damage repair.[89] In addition, mutants of *S. pombe* HDAC Clr6 subunits are sensitive to DNA-damaging agents, such as methyl methanesulfonate (MMS), likely because that the mutants lead to the general relaxation of chromatin.[51] These results suggest that the position effect in pericentromeric heterochromatin may also generate a local environment for proper DNA damage repair in heterochromatin. The severe DNA repair defects in *C. reinhardtii* may result from the fact that the green alga contains a large amount of DNA repeats in its genome.[90]

Concluding remarks

In eukaryotic cells, noncoding tandem DNA repeats are abundant in genomes and typically packed into highly condensed heterochromatin structures, which are important for regulating gene expression and maintaining genome stability.[1,91] Although recent studies revealed the epigenetic state among different repeats varies significantly, the mechanism for the position effect is still elusive. Taking advantage of the repeat-specific reporter system, we identified Rex1BD and Rad25 functions as a regulatory hub to control the heterochromatin organization at tandem DNA repeats by linking the RNAi and HDAC pathways (Figure 4). This work provides insight into the mechanism of how tandem DNA repeats are organized at an individual level. However, some interesting questions are still unaddressed. One of the key questions is how Rex1BD and Rad25 work together to mediate the position-dependent effect within heterochromatin repeats. Telomere position effect (TPE) is another well-known heterochromatin phenomenon that refers to the silencing of genes in nonrepetitive regions near telomeres. Mechanically, it is believed that TPE results from the continuous spread of heterochromatin silencing from the telomeres.[92,93] However, it is unlikely to be the case for the position effect within pericentromeric repeats since Rex1BD and Rad25 regulate silencing of distinct repeats. Rex1BD facilitates the recruitment of HDAC, whereas Rad25 is important for the association of the RITS complex with heterochromatin. One possibility is that different repeats may organize into distinct 3D architectures, which result in different levels of recruitment of Rex1BD and Rad25 to individual repeats. Also, it is well known that 14-3-3 proteins modulate the functions of other proteins primarily in a phosphorylation-dependent manner.[94,95] It is also possible

that 14-3-3 protein Rad25 mediates the activity of RITS and Rex1BD through phosphorylation of these proteins to ensure different epigenetic states at different repeats. It will also be interesting to know whether siRNA generated from individual repeats are different and how they contribute to the position effect.

In addition, how does the position effect affect the DNA repair at individual level? Are there any other factors mediating the position effect? How the position effect within DNA repeats is mediated through the cell cycle? DNA repeats are linked to many human diseases, such as Fragile X syndrome and Huntington's disease.[91,96] It will also be interesting to investigate the position effect within tandem repeats in humans and how it may contribute to human diseases. Answers to these questions will advance our understanding of the mechanisms underlying genome organization and shed light on the novel diagnostic and therapeutic strategies of some genetic diseases linked to DNA repeats.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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