Condensin Promotes Position Effects within Tandem DNA Repeats via the RITS Complex

Graphical Abstract

Highlights

- Silencing between individual units in a tandem repeat array can vary significantly
- ClrC complex and RNAi are essential for the position effect within the otr repeats
- Cut3 regulates the position effect in otr repeats and CENP-A localization
- Replication timing for individual otr repeats also differs significantly

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In Brief
How the individual sequences within tandem repeats behave remains poorly understood. He et al. show that the heterochromatin silencing between individual units within a peri-centromeric tandem array can vary significantly. They further show that condensin and RNAi components are important for the distinct silencing states in individual repeats.
**Condensin Promotes Position Effects within Tandem DNA Repeats via the RITS Complex**

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**SUMMARY**

Tandem repetitive DNA is highly abundant in eukaryotic genomes and contributes to transcription control and genome stability. However, how the individual sequences within tandem repeats behave remains largely unknown. Here we develop a collection of fission yeast strains with a reporter gene inserted at different units in a tandem repeat array. We show that, contrary to what is usually assumed, transcriptional silencing and replication timing among the individual repeats differ significantly. RNAi-mediated H3K9 methylation is essential for the silencing position effect. A short hairpin RNA of *ura4*+ induces silencing in *trans* within the tandem array in a position-dependent manner. Importantly, the position effect depends on the condensin subunit, *cut3*+. *Cut3* promotes the position effect via interaction with the RNA-induced transcriptional silencing (RITS) complex. This study reveals variations in silencing within tandem DNA repeats and provides mechanistic insights into how DNA repeats at the individual level are regulated.

**INTRODUCTION**

In eukaryotic cells, tandem repetitive DNA sequences occupy a substantial fraction of the genome. For example, tandem repetitive DNA arrays make up about 10% of mammalian genomes (Richard et al., 2008; Warburton et al., 2008). Initially considered “junk” DNA, repetitive DNA sequences now have been implicated in transcription control, genome stability, and cancer development (Martienssen et al., 2004; Richard et al., 2008; Shapiro and von Stemberg, 2005). However, due to their repetitive nature, it is difficult to study tandem DNA sequences. As a result, tandem repeat arrays remain among the most poorly understood structures in the genome.

DNA repeats often are organized into heterochromatin, the highly condensed and transcriptionally silenced chromatin domain (Martienssen et al., 2004). Heterochromatin structure and function have been extensively studied. Yet, very little is known about how heterochromatin at the individual repeat level is regulated. It has been assumed that the silenced heterochromatin state is uniformly distributed within a tandem repeat array.

In many eukaryotes, including humans, peri-centromeres are heterochromatic and consist of long arrays of tandem repetitive DNA (Allshire and Karpen, 2008; Martienssen et al., 2004). The peri-centromeric heterochromatin has been linked to centromere function and chromosome segregation (Boyarchuk et al., 2014; Folco et al., 2008; Gonzalez et al., 2014; Smith et al., 2011). Like many other eukaryotes, peri-centromeres in fission yeast (*Schizosaccharomyces pombe*) comprise arrays of large heterochromatic DNA repeats that have been used widely to study heterochromatin. Fission yeast has three chromosomes, each containing a single centromere, ranging from 35 kb to 110 kb (Wood et al., 2002). The core region of the centromeres (cnt, centromere core domain) is enriched with the CENP-A homolog Cnp1 (CNP-A<sup>crep1</sup>), a centromeric-specific histone H3 variant that defines centromere identity (Allshire and Karpen, 2008; Takahashi et al., 2000). Immediately flanking *cnt* are the innermost repeat regions (*imr*), which include imperfect inverted repeat elements. Outside of the *imr* region are the outermost repeat regions (*otr*). The *otr* regions contain large repeats, each of which consists of *dg* and *dh* elements and spans approximately 6.7 kb (Wood et al., 2002). Both *imr* and *otr* regions are heterochromatic (Allshire et al., 1994), and they are enriched for methylation at histone H3 lysine 9 (H3K9me), the conserved epigenetic mark of heterochromatin. H3K9me is regulated by the ClrC complex, which contains Clr4 (H3K9 methyltransferase), Rik1, Cui4, Dos1, and Dos2 (Hong et al., 2005; Hom et al., 2005; Jia et al., 2005; Li et al., 2005; Thon et al., 2005). RNA interference (RNAi) is also required for H3K9me and heterochromatin silencing. Fission yeast contains a single copy of Argonaute (*Ago1*), Dicer (*Dcr1*), and the RNA-dependent RNA polymerase (*Rdp1*). *Ago1*, together with the chromo-domain protein *Chp1* and *Tas3*, is assembled into the RITS (RNA-induced transcriptional silencing) complex (Mota-medi et al., 2004; Volpe et al., 2002). During the S phase of the cell cycle, the DNA Pol epsilon subunit Cdc20 promotes the transcription of peri-centromeric heterochromatin. RNAi subsequently processes the peri-centromeric transcripts into siRNAs, which in turn facilitate recruitment of the ClrC complex to the heterochromatin region (Chen et al., 2008; Kloc et al., 2008; Li et al., 2011a; Zaratiegui et al., 2011).

To directly probe the behavior of individual repeats in tandem repeat arrays, we developed a collection of strains carrying a reporter gene in different *otr* repeats. Our results demonstrate that heterochromatin silencing and replication timing between different *otr* repeats can vary significantly. The position effect is dependent on RNAi and *Cut3*, a subunit of the condensin complex. *Cut3* mutation also results in mislocalization of CENP-A<sup>crep1</sup>. This study
reveals previously unknown position effects within tandem DNA repeats, and it suggests a mechanism for how DNA repeats at the individual level are regulated. Our study also implicates the position effects at peri-centromeric regions as a possible contributor in the CENP-A positioning at the centromere.

RESULTS

Construction of Strains for Analyzing Silencing in Individual otr Repeats

To examine the level of silencing in each individual DNA repeat within the otr tandem array of chromosome 3 (Chr3), we created a collection of strains, in which the ura4+ reporter was inserted in a single otr repeat in each strain (Figure 1A). The otr region in Chr3 has the largest number of repeats among the three centromeres. The otr region at the right side of the Chr3 centromere contains four full-length 6.7-kb repeats. The number of otr repeats at the left side (otr3L) is not well defined, with estimated numbers ranging from 7 to 11 (Ellermeier et al., 2010; Wood et al., 2002). However, it is known that the first and last of the otr3L repeats contain only a partial otr sequence (Wood et al., 2002).

Using a recently described two-step process (Vader et al., 2011), we successfully obtained strains carrying ura4+ reporter inserted at each repeat in the otr3L region except repeats 6, 9, and 12 (Figures 1B and 1C). Based on this analysis, we concluded that the left arm of Chr3 contains a total of 12 otr repeats, including the first and last incomplete repeats (Figure 1C).

Further Southern blotting analysis indicated that, in a portion of the transformants, the total length of the otr3L array was different than the size of the otr region in wild type (Figure S1), likely resulting from aberrant, non-allelic recombination. In this study, we only focused on the transformants carrying the wild-type length of the otr region.

otr Tandem Repeats Exhibit Position-Dependent Silencing

To evaluate the level of silencing at each individual otr repeat, strains from our collection were analyzed by growth assays on medium lacking uracil (–ura). The growth rate of these strains differs on –ura medium (Figure 2A), indicating that different otr repeats exhibit variation in heterochromatin silencing. We found that cells carrying the ura4+ reporter inserted in the repeats distal to the centromere core, including repeats 2-8, have the slowest growth on –ura medium, indicating that these repeats are strongly silenced. Interestingly, repeat 1, which contains a partial otr repeat, also displays strong silencing. On the other hand, silencing of repeats close to the centromere is substantially reduced. Repeat 11, the rightmost repeat carrying the reporter, was successfully inserted at each repeat in otr3L region except repeats 6, 9, and 12. The blue lines mark the position of the predicted bands for digestion of DNA from cells carrying ura4+ in repeat 6 or 9 of the otr3L region. C, control strain without ura4+ at the otr region.

H3K9 Methylation and RNAi Are Essential for the Position Effect in otr Repeats

H3K9me plays an important role in heterochromatin silencing (Nakayama et al., 2001). Our chromatin immunoprecipitation (ChIP) assays showed that H3K9me was detected in all repeats (Figure 3A), indicating that the position-dependent regulation within tandem DNA repeats is stably inherited (Figures S2B and S2C).

Assembly of peri-centromeric heterochromatin requires the RNAi machinery (Volpe et al., 2002). Our growth assays showed...
that, similar to the dos1Δ background, position-dependent silencing in dcr1Δ strains with ura4Δ in different repeats is eliminated (Figure S3A). We also observed a similar loss of position effect in the deletion mutant of Chp1, a component of the RITS complex (Figure 3C). Furthermore, our ChIP analysis demonstrated that Chp1 is highly enriched in repeats 2 and 8, but decreased in repeats 10 and 11 (Figure 3D), a pattern consistent with the silencing state in these individual repeats.

A ura4Δ Hairpin Induces Silencing within otr Repeats In trans in a Position-Dependent Manner

A hairpin structure of ura4Δ in fission yeast can induce heterochromatin silencing in trans at a target locus near heterochromatin, but this has only minor effects on silencing within the single otr repeat in Chr1 (Iida et al., 2008; Simmer et al., 2010). To determine whether heterochromatin silencing can be induced by the ura4Δ hairpin in trans in the otr tandem repeats, we used a hairpin that contains a sequence complementary to 200bp of ura4Δ under the nmt1 promoter (U-HP) and was integrated on chromosome 1 (Simmer et al., 2010). We found that expression of U-HP can induce strong silencing in trans in repeat 11, but it has little effect on silencing in otr repeat 2 or 8 (Figure 3E). We also found that expression of U-HP in the dcr1Δ mutant cells did not result in silencing in the otr repeats, indicating that the hairpin-mediated trans-silencing requires RNAi (Figure S3B). Together, our result supports the idea that siRNAs generated by the ura4Δ hairpin induce silencing in otr repeats in trans, and that this construct can overcome the position-dependent regulation of heterochromatic silencing in the otr repeats. The weak effect of ura4Δ hairpin expression on silencing in repeats 2 and 8 may be due to the fact that these regions already form a highly condensed heterochromatin structure.

Replication Timing Varies between Different otr Repeats

DNA replication contributes to peri-centromeric heterochromatin formation in fission yeast (Li et al., 2011a). Our collection of repeat-specific reporters provides us the opportunity to analyze how replication timing differs between individual repeats. Using BrdU-IP, we observed that the incorporation of BrdU into repeats 10 and 11 is severely delayed compared to its incorporation into repeat 2 using BrdU-ChIP (Figures 3F and S3C). These results indicate that the strongly silenced repeats in otr region (i.e., repeat 2) replicate earlier than the weakly silenced repeats 10 and 11.

Cut3, a Condensin Subunit, Promotes the Position Effect in otr Repeats via the RITS Complex

Condensin, a protein complex that is essential for chromosome condensation during mitosis (Hudson et al., 2009), has been implicated in heterochromatin function (Chen et al., 2008; Oliveira et al., 2005). To determine whether condensin is involved in the position effect on silencing in the otr repeats, we analyzed the silencing states in otr repeats in a condensin mutant, cut3-477. cut3-477 is a temperature-sensitive mutant that is unable to grow at 37°C (Saka et al., 1994). We found that the position-dependent silencing pattern across the otr region is severely disrupted in cut3-477 mutant even at 32°C. However, unlike dos1Δ and RNAi mutants, which exhibit a total loss of silencing in all repeats tested, the effect of the cut3-477 mutation on silencing varied between repeats. Although silencing is only mildly reduced in the mutant in repeat 2, silencing in repeat 8 is significantly lost. In contrast, the mutation has little effect on the silencing in repeat 11 (Figure 4A). Consistent with this, H3K9me is drastically reduced in repeat 8 in the cut3-477 mutant (Figure 4B). We also observed that heterochromatin silencing is substantially decreased in repeats 1 and 5 (Figure S4A). Furthermore, we found that over-expression of Cut3 can enhance silencing in the repeat 11, but it has no obvious effect on silencing in repeats 2 and 8 (Figure 4C). These results indicate that Cut3 is a key regulator of the position effect in otr repeats. However, we found that the association of Cut3 with repeats 2, 8, and 11 is not significantly different (Figure S4B). In addition, disruption of centromere structure using a mutant of Mis6, an essential centromere protein, results in no obvious effect on Cut3 distribution between different repeats (Figure S4C). These data suggest that additional factors may be required for the position effect.

To determine how Cut3 contributes to the position-dependent silencing in the otr repeats, we investigated how the recruitment of the RITS complex to different otr repeats is affected in cut3-477 mutants by ChIP. We found that the level of Chp1 in repeat 8 is greatly reduced in the mutant, whereas its level in repeats 2 and 11 is only mildly affected (Figure 4D), indicating that Cut3 contributes to the position-dependent recruitment of RITS. Furthermore, our co-IP assays showed that Cut3-myc is
co-immunoprecipitated with Chp1-GFP (Figure 4E), demonstrating that Cut3 interacts with the RITS complex.

**DISCUSSION**

Despite the importance of tandem DNA repeats (Martienssen et al., 2004; Shapiro and von Sternberg, 2005), how the individual sequences within the tandem repeats behave and how their function is regulated remain poorly understood. Using a collection of repeat-specific reporters, Vader et al. (2011) have shown that the edges of a repetitive ribosomal DNA (rDNA) array in budding yeast are more susceptible to homologous recombination during meiosis. Here, using a similar approach, we demonstrated that transcriptional silencing in the different repeat units within the peri-centromeric tandem otr repeats can be strikingly different. An accompanying study in this issue of Cell Reports by Wang et al. (2016) also revealed a transcriptional position effect in the repetitive rDNA regions in budding yeast, suggesting that position effects in tandem repeat arrays are conserved.

We show that the otr tandem repeats in fission yeast Chr3 exhibit striking position-dependent silencing. These findings...
sugest that, although different repeats in the tandem array share the same sequence, each one is organized into a specific higher order structure. We demonstrated that the H3K9me and RNAi components are key effectors of the observed positional differences. Moreover, we found that condensin is essential for the formation of the position-dependent epigenetic states. In particular, the specific pattern of the RITS complex associated with different individual otr repeats is perturbed in the cut3 mutant. These results suggest that condensin acts as the upstream instructor to recruit the proper level of silencing effectors, including the RITS complex, to individual repeats, and to establish the unique heterochromatin state in the particular position. Nevertheless, we did not observe any significant difference in the level of Cut3 associated with different peri-centromeric repeats, suggesting that additional factor(s) may be required to function together with Cut3 to promote the position effect.

What is the biological relevance of the position effect at the peri-centromeric repeats? Centromeres are responsible for kinetochore assembly, and they play a key role in chromosome segregation (Allshire and Karpen, 2008). In most eukaryotes, centromere identity is believed to be predominantly epigenetically specified (Fukagawa and Earnshaw, 2014). CENP-A is the most likely candidate for the epigenetic mark used to define centromeres. How CENP-A is incorporated to centromeric regions remains poorly understood, but mis-regulation of CENP-A adversely affects chromosome segregation, resulting in aneuploidy and cancer (Allshire and Karpen, 2008). Our studies suggest that the distinct three-dimensional architecture created by the tandem arrays at peri-centromeres may contribute to proper positioning of CENP-A. Indeed, RNAI components and Cut4 have been shown to be required for establishment of CENP-A^{cre}I chromatin at centromere in fission yeast (Folco et al., 2008; Gonzalez et al., 2014). Previous studies also demonstrate that peri-centromeric heterochromatin is important for centromeric localization of CENP-A in Neurospora crassa and mouse cell lines (Boyarchuk et al., 2014; Smith et al., 2011). Here, we show that the key regulator of the position effect in peri-centromeric repeats, Cut3, is also required for the centromeric localization of CENP-A^{cre}I (Figure 4F). Condensin has been previously implicated in the assembly of CENP-A chromatin in Xenopus and human cells (Bernad et al., 2011; Samoshkin et al., 2009), but its precise role in the process is still unclear. We propose that condensin mediates the organization of peri-centromeric repeats into a specific higher-order structure, which in turn helps restrict CENP-A to centromeres.

We demonstrated that replication timing among the individual units in a tandem array can vary significantly. Replication of an otr repeat near the centromere is severely delayed compared to more strongly silenced repeats close to the chromosome arm. Although heterochromatin in general is associated with late replication, centromeric heterochromatin in fission yeast and a few of other organisms replicates early in the S phase (Kim et al., 2003). The reason for the early replication of these heterochromatic regions remains unclear. During DNA replication, CENP-A chromatin is disassembled and CENP-A must be faithfully reincorporated into centromeres after replication (Allshire et al., 2016).
and Karpen, 2008; Gonzalez et al., 2013). It has been shown that DNA replication is important for heterochromatin assembly at peri-centromeric regions in fission yeast (Chen et al., 2008; Kloc et al., 2008; Li et al., 2011a; Zaratiegui et al., 2011). We propose that early replication of strongly silenced peri-centromeric repeats allows the establishment of the position effect at an early stage so as to create a chromatin environment that can ensure the proper positioning of CENP-A. Consistent with this idea, the core region of centromeres, cnt, and the flanking imr regions, both of which are weakly silenced, replicate later than the highly heterochromatic otr regions (Li et al., 2011b).

Our findings reveal previously unrecognized position effects within tandem repeat arrays, and they support the concept that the position effects at peri-centromeric repeats promote the epigenetic specification of centromeres. In addition, our studies uncover condensin and RNAi components as key factors involved in position-dependent epigenetic regulation of tandem DNA repeats, and they provide insight into how DNA repeats at an individual level are controlled. It will be important in future studies to identify other key regulators of the position effects. It will also be interesting to explore how tandem repeats are spatially organized. These studies will shed light on regulation of this important but poorly studied part of the genome.

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic Analysis

The fission yeast strains used in this study are listed in Table S1. Standard media and genetic analysis for fission yeast were used (Moreno et al., 1991).

Strain Construction

The repeat-specific reporters were created using a recently described process with minor modifications (Vader et al., 2011), and further details are available in the Supplemental Information.

CHEF Gel Electrophoresis and Southern Blot

CHEF gel electrophoresis and Southern blotting were performed as described (Vader et al., 2011) with minor modifications, and further details are available in the Supplemental Information.

Co-immunoprecipitation and Western Blot Analysis

Immunoprecipitation was performed using an anti-GFP antibody (Abcam, ab290). Eluates were analyzed by standard western blotting protocols using an anti-γtubulin antibody (Sigma, C3956). For western blot analysis of the ab290). Eluates were analyzed by standard western blotting protocols using an anti-GFP antibody (Abcam, ab290). Blots were probed with anti-GFP (Roche, 11 814 460 001) or β-tubulin (Abcam, ab8160) antibodies.

ChiP

ChiP was performed as described (Li et al., 2008). The primers used are listed in Table S2. Quantifications were performed using ImageJ 1.46r software. All experiments were independently repeated three times. A two-tailed Student’s t test was used to determine the statistical significance between different experimental groups.

BrdU IP

BrdU IP was essentially performed as described previously (Li et al., 2011b). The primers used in this study are listed in Table S2.

Northern Blot

Northern blot was performed according to the standard protocol. Briefly, RNA samples were separated and transferred to an Amersham Hybond-N+ membrane in 2X SSC buffer. After UV cross-linking, the membranes were hybridized by 32P-labeled probe recognizing ura4+ or the 28S rRNA as a control and exposed to film for autoradiography.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.01.006.

AUTHOR CONTRIBUTIONS

H.H. performed the experiments with assistance from S.Z. and D.W.; F.L. designed the study and wrote the manuscript with input from A.H.

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