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REVIEW

Are all repeats created equal? Understanding DNA repeats at an individual level

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Abstract Repetitive DNA sequences, comprising up to 50 % of the genome in all eukaryotes, play important roles in a wide range of cellular functions, such as transcriptional regulation, genome stability, and cellular differentiation. However, due to technical difficulties in differentiating their sequences, DNA repeats remain one of the most mysterious parts of eukaryotic genomes. Key questions, such as how repetitive entities behave at individual level and how the internal architecture of these repeats is organized, are still poorly understood. Recent advances from our group reveal unexpected position-dependent variation within tandem DNA repeats in fission yeast. Despite sharing identical DNA sequences, the peri-centromeric repeats are organized into diverse epigenetic states and chromatin structures. We demonstrate that this position-dependent variation requires key heterochromatin factors and condensin. Our works further suggest that the peri-centromeric repeats are organized into distinct higher order structures that ensure a proper positioning of CENP-A, the centromere-specific histone H3 variant, to centromeres. These most recent developments offer insights into the mechanisms underlying the position effect within tandem DNA arrays, and have broad implications in the field of epigenetics and chromatin biology.

Introduction

One of the most conserved features in eukaryotic organisms is the presence of many repetitive DNA sequences. The sequence complexity of these DNA repeats varies from complete genes (such as the ribosomal DNA) to simple sequences of a few base pairs. These repeats can be organized into long tandem arrays (such as peri-centromeric repeats), or widely dispersed in the genome (such as tRNA and transposon elements) (Richard et al. 2008; Sutherland and Richards 1995). For a long time, researchers considered DNA repeats as "junk DNA". However, recent advances have implicated DNA repeats in many important cellular events, including transcriptional control, disease development, evolution, cellular differentiation, and genome stability (Armour 2006; Gemayel et al. 2012). Despite their importance, DNA repeats remain the least understood structures in the genome (Gemayel et al. 2012; Martienssen et al. 2004; Pearson et al. 2005). It is largely due to the technical challenges of analyzing repetitive DNA.

Repetitive DNA sequences are hotspots for meiotic crossover and other recombination events, which may cause genome instability (Jeffreys et al. 1999; Vader et al. 2011). To prevent this, DNA tandem arrays are often packaged into constitutive heterochromatin, such as centromeres and telomeres. Constitutive heterochromatin is the stable, compact chromatin structure containing low level of transcriptional activities (Bierhoff et al. 2014; Saksouk et al. 2015). Hypermethylation of histone 3 at lysine 9 (H3K9me) is a

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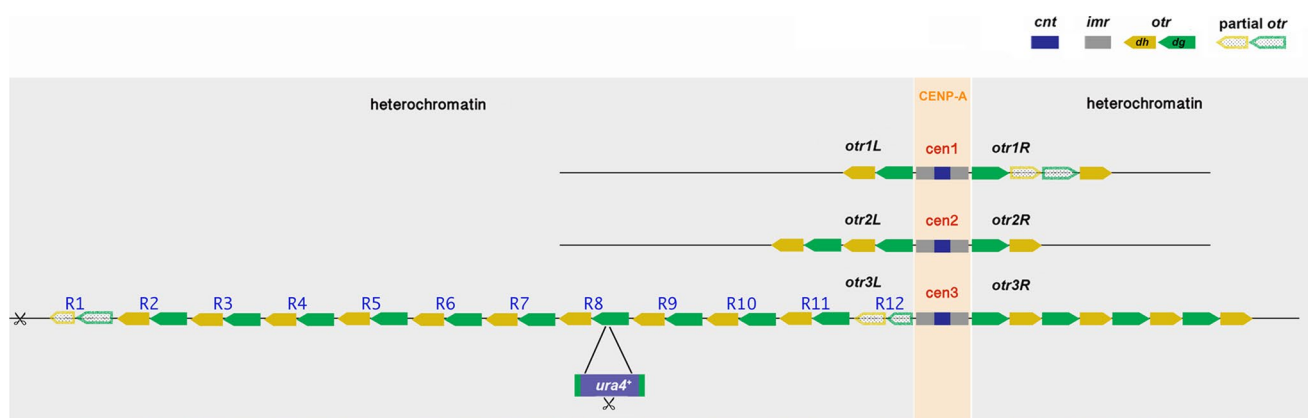1 Department of Biology, New York University, New York, NY 10003, USA

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conserved hallmark of constitutive heterochromatin, and plays a key role in heterochromatin assembly. It is generally assumed that epigenetic state is evenly distributed among the tandem repeats in constitutive heterochromatin. Notably, ribosomal DNA (rDNA), which encodes rRNA, also comprises of tandem arrays of rDNA genes in eukaryotic genomes. Since rRNA, an important component of ribosome, is essential for protein translation in all eukaryotes, rDNA has to be transcriptionally active. Interestingly, it has been shown that some fractions of rDNA repeats can be silenced and form heterochromatin-like structure (Guetg and Santoro 2012; Pasero and Marilley 1993). Moreover, the heterochromatic state in rDNA region is not static, and can be influenced upon stress, cell-cycle progression, and other stimuli (Benoit et al. 2013; Hamperl et al. 2013). Whether similar position-dependent silencing takes place within tandem repeats in constitutive heterochromatin, such as peri-centromeres, has not been explored. If that also is the case in constitutive heterochromatin, how specific chromatin states are maintained and regulated at the individual repeat units? More importantly, what is the functional significance of this interesting epigenetic phenomenon?

Fission yeast *Schizosaccharomyces pombe* is a superb model to study constitutive heterochromatin. *S. pombe* is a single-cell, genetically-tractable eukaryotic organism that is relatively easy to manipulate. Like many other eukaryotes, peri-centromeric regions in *S. pombe* contain tandem DNA repeats that are not found in the peri-centromeres in budding yeast *Saccharomyces cerevisiae* (He et al. 2014). Key mechanisms involved in heterochromatin regulation, such as methylation of histone H3 lysine 9 (H3K9me) and the RNAi interference (RNAi) machinery, are absent in budding yeast, but preserved in *S. pombe* (Nakayama et al. 2001; Verdel

et al. 2004). Heterochromatin of peri-centromeric repeats has been implicated in centromere positioning and chromosome segregation (Bernard et al. 2001; Folco et al. 2008; Gonzalez and Li 2012), providing an excellent opportunity to examine the functional relevance of position-dependent epigenetic events in the tandem arrays.

Fission yeast has three chromosomes, each chromosome harboring a single centromere surrounded by peri-centromeric tandem repeats. Like higher eukaryotic organisms, including humans, fission yeast has large regional centromere that is epigenetically defined. Specifically, fission yeast centromere contains a core region *cnt* (centromere core domain), which is flanked with imperfect repeat regions *imr* (innermost repeats). Both *cnt* and *imr* regions are marked with CENP-A[cnp1], the centromeric specific H3 variant. Beyond *imr* regions, there are tandem arrays of *otr* (outermost repeat regions) repeats, and 6.7 kb each consists of alternating *dg* and *dh* repeats. *otr* regions are heterochromatic and enriched with H3K9me (He et al. 2014; Takahashi et al. 2000). It has been challenging to assemble the exact copy numbers of *otr* repeats on each chromosome. By estimation, Chromosome I and II have 1–2 repeats on either side of *cnt*, while Chromosome III has 7–11 repeats on the left arm (*otr3L*) and 4 repeats on the right arm (*otr3R*). The considerable size of *otr3L* repeats makes it a favorable region to study heterochromatin structures in tandem array (Fig. 1) (Wood et al. 2002). However, powerful genetic tools, such as sequencing, chromatin immunoprecipitation (ChIP), and chromosome conformation capture (3C), which completely rely on DNA sequence specificities, fail to assemble large tandem arrays or differentiate among individual repeats bearing identical sequences.

Fig. 1 Schematic representation of centromeres in fission yeast and the development of repeat-specific reporter. Central core regions (*cnt*) are flanked by innermost repeats (*imr*) and outermost repeats (*otr*). Each of *otr* repeats consists of *dg* and *dh* repeat units as indicated. CENP-A[cnp1] is enriched in *cnt* and *imr* regions, while *otr* repeats are organized into peri-centromeric heterochromatin. The *ura4+* reporter flanked with part of *dg* sequence was randomly inserted into *otr* repeats by homologues recombination. The location of *ura4+* insertion is mapped by estimating the distance between two unique restriction sites in *ura4+* and a heterochromatin boundary region, respectively (indicated by two scissors)

A novel approach to study tandem repeats at individual level

To address how peri-centromeric repeats are regulated at the individual level, He et al. recently developed a collection of strains that mark individual repeats in peri-centromeric tandem array in *S. pombe* via the insertion of the *ura4*+ reporter gene (He et al. 2016). The exogenous *ura4*+ sets apart the repeat unit containing the reporter from other identical repeats. Therefore, for the first time, it is feasible to dissect structural and functional nature of heterochromatin states within peri-centromeric repeats at individual level.

To mark the individual repeat, the *ura4*+ reporter flanked with a section of *dg* repeats was constructed and transformed into wild-type *S. pombe* cells. The reporter was randomly inserted into the peri-centromeric repeats via homologous recombination. Strains that survive on the minimum medium without uracil were selected. The exact location of *ura4*+ insertion within the peri-centromeric repeats was mapped based on the distance between unique restriction enzyme sites in *ura4*+ and the boundary region of the repeat array by the Southern blot analysis. This experimental approach was adapted from a previous report in which a repeat-specific reporter was developed for rDNA tandem array in budding yeast (Vader et al. 2011). Mapping results by He et al. indicated that *otr3L* in *S. pombe* contains a total of 12 repeats. While the first repeat 1 (R1), which is distal to core centromeres, and the last repeat (R12) in *otr3L* have only partial repeat sequence, the rest of *otr3L* tandem array contains full-length 6.7 kb repeat DNA (Fig. 1). He et al. generated a collection of *ura4*+ insertion for every *otr3L* repeat with exception of R6, R9, and R12.

Not all repeats are created equal

Upon analyzing the transcriptional level of *ura4*+ by growth assay and Northern blotting, an apparent position-dependent effect is identified within the *otr3L* tandem array. Transcription of *ura4*+ is relatively active in repeats close to core centromere region, whereas *ura4*+ in distal repeats is more silenced. By taking advantage of repeat-specific *ura4*+ insertions, H3K9me enrichment in individual repeat unit can be assayed by ChIP. Repeats distal to the core centromere are enriched with H3K9me, while repeats close to the core centromere have less H3K9me. The distribution of H3K9 methylation is well correlated with the position-dependent gradient of transcriptional activity.

In fission yeast, H3K9me is mediated by Clr4 complex (ClrC), which contains Cul4, Rik1, Dos1, Dos2, Lid2, and the catalytic subunit histone methyltransferase Clr4 (Hong et al. 2005; Horn et al. 2005; Jia et al. 2005; Li et al. 2005, 2008; Thon et al. 2005). Loss of any of these subunits results in a significant reduction of silencing in peri-centromeric heterochromatin. In fission yeast, the assembly of peri-centromeric heterochromatin is also regulated by RNAi; a phenomenon found in many other organisms (Lejeune and Allshire 2011; Volpe and Martienssen 2011). Noncoding RNAs (ncRNAs) within peri-centromeric repeats are transcribed by RNA polymerase II (RNAPII) and later processed into siRNA by Dicer, RITS (RNA-induced transcriptional silencing), and RDRC (RNA-directed RNA polymerase complex). RITS complex, containing Ago1, Tas3, and the chromo-domain protein Chp1, is directed to peri-centromeric repeats via base-pairing interactions between Ago1-bound siRNAs and ncRNAs. This siRNA-containing complex in turn recruits ClrC for heterochromatin assembly (Kato et al. 2005; Motamedi et al. 2004). Similar to human HP1 (heterochromatin protein 1), the chromo-domain-containing Chp1 specifically binds to H3K9me (Schalch et al. 2009). Correspondingly, He et al. found that Chp1 distribution among *otr3L* repeats is correlated with the distribution of H3K9me, as Chp1 is enriched in repeats distal to centromeres, but not in repeats close to centromeres that are less silenced.

The collection of strains with the repeat-specific reporter also provides an opportunity for investigating the replication timing between different repeats. It is generally believed that euchromatin replicates earlier than heterochromatin (Gilbert 2002). However, peri-centromeric heterochromatin in fission yeast replicates at the early S phase. Similar phenomenon also was found in mammals (Kim et al. 2003). Using BrdU-ChIP, He et al. revealed that the replicating timing of the individual repeats in *otr3L* tandem array can vary considerably. In general, the repeats exhibiting strong silencing in *otr3L* tandem array appears to replicate earlier. The reason for the early replication of these repeats remains unclear.

These results indicate that although the DNA repeats with a tandem array contain the same sequence, the function and epigenetic state for each repeat can be very different, suggesting that each individual repeat unit can be organized into distinct higher order architecture. Consistent with this idea, siRNAs generated from an *ura4*+ hairpin can silence the *ura4*+ reporter inserted within *otr3L* tandem array *in trans* in a position-dependent manner. Overexpression of the *ura4*+ hairpin promotes heterochromatin formation in the *otr* repeats exhibiting weaker silencing, but it has little effect on the repeats containing strong silencing.

Mechanisms regulating position effect in tandem repeats

How this position-dependent heterochromatin differentiation is achieved in the repeats despite sharing identical sequences? Heterochromatin assembly required machineries, such as ClrC and RITS. Disruptions of these factors also lead to the total loss of the position effect within *otr3L* repeats, indicating that they are the key effectors for the position effect. He et al. also found that Cut3, a condensin subunit, is required for the position effect in the peri-centromeric region. An ancient but highly conserved family of proteins, condensin is important for chromosome compaction and organization. It is well established that condensin is recruited to peri-centromeric and rDNA tandem arrays, underscoring its role in higher order chromatin structures across repetitive sequences (Bloom 2014; Hirano 2016). He et al. found that the disruption of Cut3 alters the profile of position-dependent transcription in *otr3L* tandem array without total loss of silencing, suggesting that Cut3 functions as an upstream regulator of this position effect. Wang et al. also showed that the condensin is essential for the position effect in rDNA tandem repeat in budding yeast, indicating that the role of condensin in regulating the organization of DNA repeats is conserved (Wang et al. 2016).

He et al. further revealed that Cut3 physically interacts with the RITS complex, and is required for the association of the complex with individual repeats. We propose that condensin acts as the upstream instructor to recruit the proper amount of silencing effectors, such as the RITS complex, to individual repeats, which in turn establish the specific epigenetic state in these repeats (Fig. 2). However, among individual *otr3L* repeats, there is no apparent bias in Cut3 distribution, suggesting additional factors may be required to establish position-dependent heterochromatin organizations. Cohesins and other chromatin remodeling complexes are possible candidates to control the position effect within the peri-centromeric repeat array (Mizuguchi et al. 2014; Robellet et al. 2014).
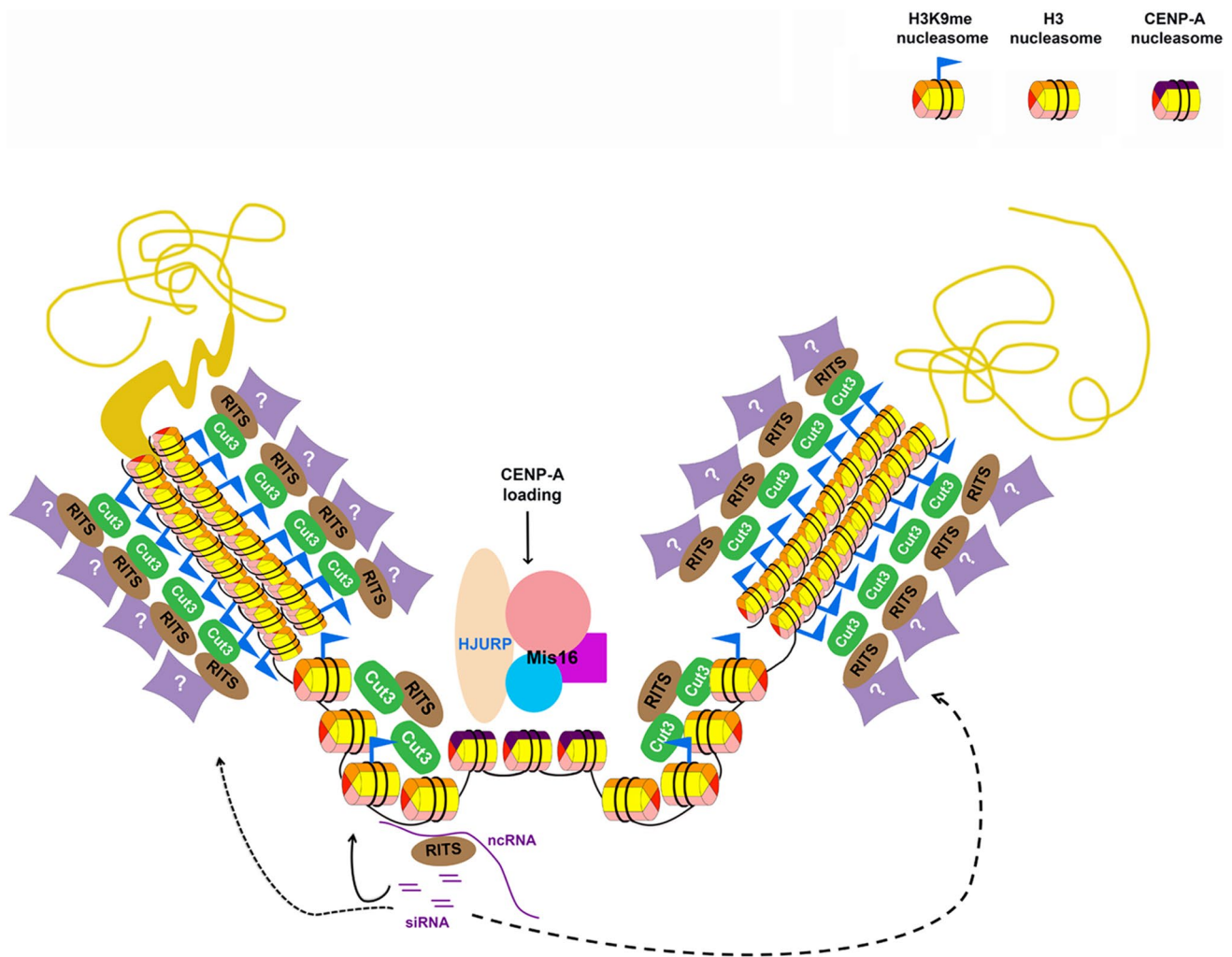
Biological significance of position effect in peri-centromeric repeats

Centromeres play an essential role in equal chromosome segregation during mitosis and meiosis. In most eukaryotes, the underlying DNA sequence in centromere is not sufficient to organize the assembly of centromeres, indicating that epigenetic mechanism is important for centromere specification. CENP-A, the histone H3 variant, is considered as the epigenetic mark to define the centromeres

(Allshire and Karpen 2008; Black and Cleveland 2011; Gonzalez et al. 2014). Multiple proteins, including HJURP and Mis16 complex, have been identified to be the key CENP-A loading factors (Gonzalez et al. 2013; Hayashi et al. 2004; Sanchez-Pulido et al. 2009; Takahashi et al. 2005). However, how CENP-A is precisely incorporated into centromeres remain unclear. The position effect in peri-centromereic *otr* repeats in *S. pombe* revealed by He et al. suggests that these repeats are organized into unique three-dimensional architecture. He et al. further showed that the disruption of the position effect led to the dissociation of CENP-A from centromeres and mis-targeting of CENP-A to ectopic regions. These results indicate that the higher order structure of peri-centromeric repeats may promote CENP-A positioning to centromeres (Fig. 2). Consistent with this, peri-centromeric heterochromatin has previously been implicated in centromeric localization of CENP-A in multiple organisms, including *Neurospora crassa*, *S. pombe*, and mouse cell lines (Boyarchuk et al. 2014; Folco et al. 2008; Smith et al. 2011).

Although heterochromatin is generally considered to be transcriptional inactive, transcription at peri-centromeric repeats was found in wide ranges of organisms and under various conditions, such as differentiation, heat-shock response, and tumorigenesis (Enukashvily and Ponomartsev 2013; Eymery et al. 2009; Hall et al. 2012; Saksouk et al. 2015). Paradoxically, the transcription in these regions can play a critical role in heterochromatin assembly. In fission yeast, peri-centromeric transcription peaks in early S phase, and subsequently processed into siRNAs. This cell-cycle-dependent transcription in peri-centromeric repeats has been proposed to explain the establishment of heterochromatin during S phase (Chen et al. 2008; Gonzalez and Li 2012; Kloc et al. 2008). The finding of position effect in *otr3L* indicated that under basal conditions, some repeats (R10 and R11) are relative open and susceptible to gene transcription. It is possible that siRNAs generated from these less condensed repeats may facilitate the establishment and maintenance of heterochromatin states in other *otr* repeats, in same or other chromosomes, throughout the cell cycle (Fig. 2). Supporting this idea, as mentioned above, siRNAs generated from artificial introduced *ura4+* hairpin is able to induce peri-centromeric *ura4+* silencing *in trans*. Further experiments need to be performed to address this hypothesis. In addition, tandem DNA repeats are hotspot for aberrant replication, repair, and recombination, which can lead to genome instability and diseases (Armour 2006; Jeffreys et al. 1999; Murray et al. 1999). The position effect in tandem DNA repeats may also reflect the higher order structure of these repeats that can be used to resolve the potential dysfunction of these DNA metabolic processes.

Fig. 2 Proposed model for the position effect in peri-centromeric tandem repeats. The condensin subunit Cut3 and other unknown upstream factors recruit key silencing effectors, such as RITS complex, to peri-centromeric repeat arrays, and mediate the level of the silencing at individual repeats, leading to the position-dependent silencing. The less silenced repeats may generate siRNAs that in turn facilitate heterochromatin assembly in other repeats *in trans*. Position effects in peri-centromeric repeat arrays indicate that these repeats are organized into distinct three-dimensional higher order structure; such spatial arrangement helps to define and position the CENP-A^{cnp1} enriched centromere core regions

Concluding remarks

Understanding tandem DNA repeats has been challenging due to its repetitive nature. Recent advances in the repeat-specific reporter approach allow us to study the behavior of tandem repeats at the individual level, revealing unexpected position effect within tandem repeat arrays. Using the peri-centromeric repeats as a model, we found that the position effect within the repeat array is regulated by key heterochromatin factors, such as RITS and CLRC complexes, and condensin. Out study further indicates that the internal position effect within tandem repeats may be involved in many aspects of array biology, including gene expression, chromatin structure, and DNA replication. Particularly, our works suggest that the peri-centromeric repeats are organized into specific higher order structures to ensure a proper targeting of CENP-A to centromeres. However, many important questions remain open. One of key questions is what are the factors responsible for regulating the position effects within tandem DNA repeat? In addition, how are the tandems repeats spatially organized? Is there important cross talk between individual repeats? It will also be interesting to know how the position effect in tandem repeats is regulated through the cell cycle and in response to environmental stresses. Answers to these questions will help us to understand this uncharted territory in eukaryotic genomes.

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