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REVIEW

Transcriptional silencing of centromere repeats by heterochromatin safeguards chromosome integrity

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Abstract

The centromere region of chromosomes consists of repetitive DNA sequences, and is, therefore, one of the fragile sites of chromosomes in many eukaryotes. In the core region, the histone H3 variant CENP-A forms centromere-specific nucleosomes that are required for kinetochore formation. In the pericentromeric region, histone H3 is methylated at lysine 9 (H3K9) and heterochromatin is formed. The transcription of pericentromeric repeats by RNA polymerase II is strictly repressed by heterochromatin. However, the role of the transcriptional silencing of the pericentromeric repeats remains largely unclear. Here, we focus on the chromosomal rearrangements that occur at the repetitive centromeres, and highlight our recent studies showing that transcriptional silencing by heterochromatin suppresses gross chromosomal rearrangements (GCRs) at centromeres in fission yeast. Inactivation of the Clr4 methyltransferase, which is essential for the H3K9 methylation, increased GCRs with breakpoints located in centromeric repeats. However, mutations in RNA polymerase II or the transcription factor Tfs1/TFIIS, which promotes restart of RNA polymerase II following its backtracking, reduced the GCRs that occur in the absence of Clr4, demonstrating that heterochromatin suppresses GCRs by repressing the Tfs1-dependent transcription. We also discuss how the transcriptional restart gives rise to chromosomal rearrangements at centromeres.

Keywords Pericentromere · Heterochromatin · Transcription · DNA repeat · Gross chromosomal rearrangement · Fission yeast

Introduction

Maintaining chromosome integrity is a crucial task for all living organisms. Centromeres play essential roles in the accurate segregation of chromosomes, and are divided into the centromere core and the pericentromeric regions. The histone H3 variant CENP-A specifically localizes to the core region and forms the CENP-A nucleosome (Allshire and Karpen 2008). The constitutive centromere-associated network (CCAN) proteins assemble onto the CENP-A chromatin throughout the cell cycle (McKinley and Cheeseman 2016; Nagpal and Fukagawa 2016). Prior to mitosis, the KNL1-Mis12-Ndc80 (KMN) network proteins are recruited to centromeres through the CCAN proteins, resulting in the formation of kinetochores, where the spindle microtubules attach (Dhatchinamoorthy et al. 2018). Heterochromatin is characterized by the di- and tri-methylation of histone H3 at lysine 9 (H3K9me2/3) and is formed in the pericentromeric region. The heterochromatin facilitates sister chromatid cohesion, to ensure faithful chromosome segregation (Allshire and Madhani 2018).

The pericentromeric region, as well as the centromere core, consists of repetitive DNA sequences in many eukaryotes (Fig. 1). Transcription of the centromere core occurs and is involved in the deposition of CENP-A nucleosomes (McNulty et al. 2017; Shukla et al. 2018). In contrast to the core, the transcription of pericentromeres is silenced by the formation of heterochromatin (Grewal and Jia 2007). The loss of the Suv39h1 and Suv39h2 methyltransferases, which are required for the H3K9 methylation, results in chromosome aneuploidy and predisposition to cancer (Peters et al. 2001), demonstrating the importance of heterochromatin. Interestingly, de-repression of the centromere repeats has been observed in a variety of human cancers, including

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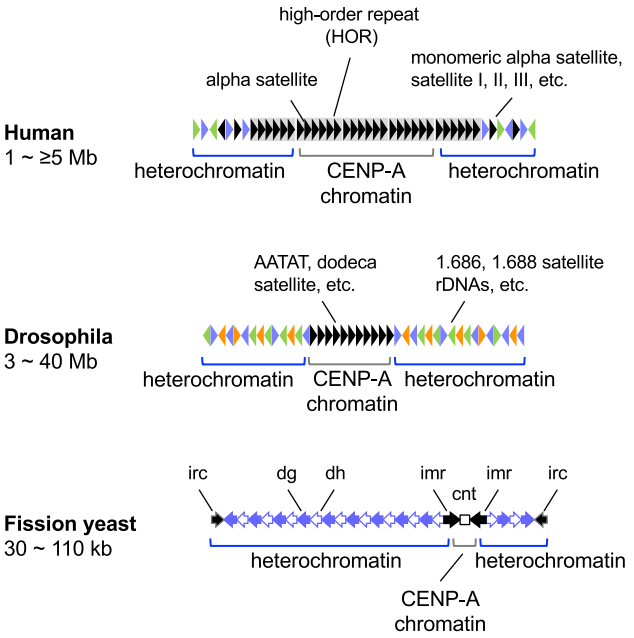


Fig. 1 Repetitive DNA sequences that are present in the centromere regions of human, Drosophila, and fission yeast chromosomes. Different types of DNA repeats are shown in different colors. The copy number of satellite repeats varies between different centromeres, even within the same organisms. In human and Drosophila, the copy numbers of satellite repeats are higher than those illustrated in this figure. In human, > 1000 copies of alpha satellite repeats are present at a centromere. The total lengths of the centromeres, including both the centromere core and the pericentromere regions, are indicated. CENP-A chromatin is assembled at the core region, whereas heterochromatin is formed at pericentromeres

BRCA1-mutated breast cancer (Ting et al. 2011; Zhu et al. 2011). A recent investigation in mice revealed that the forced transcription of centromere satellite RNAs in mammary glands is sufficient to induce tumor formation (Zhu et al. 2018). These studies suggest that the transcriptional silencing of centromere repeats is important for maintaining genome integrity and preventing tumorigenesis. However, the exact role of the pericentromere heterochromatin in the prevention of chromosomal rearrangements remains unclear. Here, we provide an overview of the chromosomal rearrangements that occur at repetitive centromeres, and highlight our recent findings, showing that heterochromatin suppresses the gross chromosomal rearrangements at centromeres through transcriptional silencing (Okita et al. 2019).

Repetitive DNA sequences in centromeres

In 1970, in situ hybridization experiments in mice revealed that centromeres consist of satellite DNA, which is an array of repeat sequences (Jones 1970; Pardue and Gall 1970). Figure 1 shows the centromere regions of human, the fly *Drosophila melanogaster*, and the fission yeast *Schizosaccharomyces pombe*. In the human centromere, the core region consists of a tandem array of alpha satellite sequences of ~ 171 bp in the form of high-order repeats (HORs), whereas the pericentromeric region consists of monomeric alpha satellites, as well as HORs, and other types of satellite repeats, such as satellite I, II, and III and transposable elements including LINEs (Lee et al. 1997; Plohl et al. 2014) (Fig. 1). It should be noted that, in the pericentromeric region, the repetitive sequences are present in either the tandem or inverted orientation. Alpha satellite DNA is estimated to represent up to 10% of the human genome, demonstrating the high prevalence of this repeat sequence (McNulty and Sullivan 2018). Approximately 35% of the alpha satellite DNA is located in CENP-A chromatin, and the rest is present in the pericentromeric heterochromatin. In Drosophila, arrays of short repeats, such as AATAT, are surrounded by other types of repeats, including the 1.686 satellite sequence in either the tandem or inverted orientation (Hoskins et al. 2007; Pimpinelli et al. 1995; Sun et al. 2003). In fission yeast, the centromeres consist of the central unique sequence (cnt) flanked by the imr, dg, dh, and irc repeats (Chikashige et al. 1989; Takahashi et al. 1992). As compared to those in other organisms, including human and fly, the total length of the centromere and the copy number of each centromere repeat are quite small in fission yeast. This simplicity makes fission yeast useful for detailed analyses of the centromere structure and function. Despite their important role in chromosome segregation, the nucleotide sequences of the centromere repeats have not been conserved during evolution, and they differ substantially between individual centromeres even within the same organism (Henikoff et al. 2001; Rosin and Mellone 2017). This is referred to as the centromere paradox.

Instability of the repetitive centromeres

Chromosomal rearrangements occur at the centromeres that consist of repetitive DNA sequences. The centromere is one of the chromosomal regions where chromosomal rearrangements are frequently detected in cancer cells (Barra and Fachinetti 2018). Chromosomal rearrangements at centromeres are not limited to cancer cells. The Robertsonian translocation is a translocation that occurs around the centromeres between two acrocentric chromosomes (human chromosomes 13, 14, 15, 21, and 22), and is the most prevalent chromosomal abnormality found in humans (1 in 1000 newborns) (Therman et al. 1989) (Fig. 2a). Carriers of the Robertsonian translocation are healthy and have a normal lifespan, but they are at increased risk of miscarriage (Song et al. 2016). Satellite III is one of the

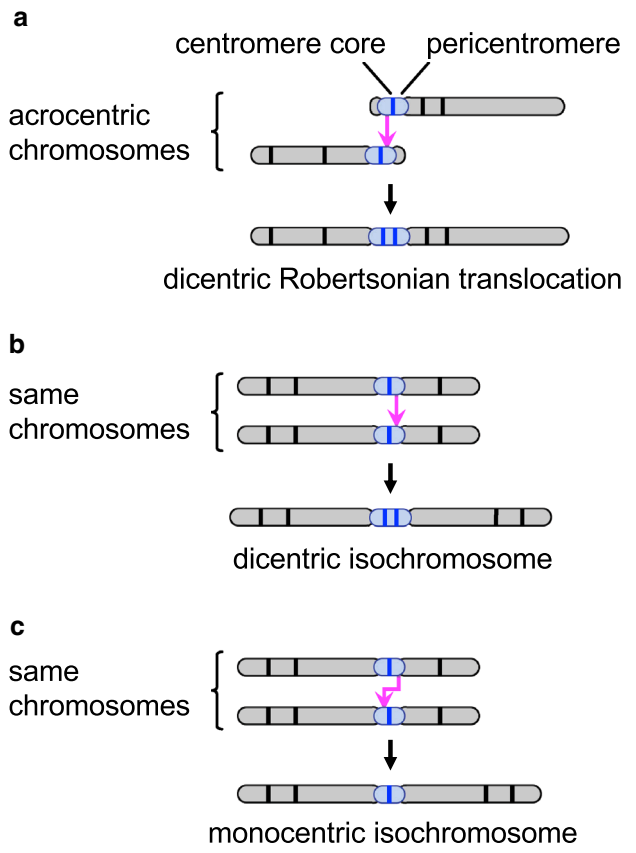


Fig. 2 Gross chromosomal rearrangements (GCRs) that occur around centromeres. **a** Chromosomal rearrangement at the pericentromeres of acrocentric chromosomes results in the dicentric Robertsonian translocation. **b** Chromosomal rearrangement on the same side of the pericentromeres of the same chromosomes results in dicentric isochromosomes. **c** Chromosomal rearrangement on different sides of the pericentromeres of the same chromosomes results in monocentric isochromosomes

satellite repeats that is present in the pericentromere, but not in the centromere core (Fig. 1). In some cases, satellite III has been found at breakpoints in dicentric Robertsonian translocations, which contain two centromere core regions in one chromosome (Gravholt et al. 1992) (Fig. 2a), suggesting that the chromosomal rearrangements at pericentromeres result in the Robertsonian translocation. Translocation between the same chromosomes, namely sister chromatids, can result in the formation of isochromosomes with arms that are the mirror images of each other (Fig. 2b). The isochromosome with the long arms of chromosome X, i(Xq), is the most common isochromosome in humans and also the most frequent chromosomal abnormality observed in Turner syndrome (Hook and Warburton 1983). A DNA microarray analysis of i(Xq) chromosomes from Turner syndrome patients demonstrated that they usually contain two domains of the centromere core, and therefore are dicentric isochromosomes (Koumbaris et al. 2011) (Fig. 2b). In some cases, i(Xq) lacks the entire euchromatin region of the short arm and has a breakpoint in the pericentromeric region. Chromosomal rearrangements between the same side of the pericentromeres lead to the formation of dicentric isochromosomes (Fig. 2b). The formation of monocentric isochromosomes has been observed in fission yeast and *Candida albicans* (Nakamura et al. 2008; Selmecki et al. 2006). Chromosomal rearrangements between opposite sides of the pericentromeres of sister chromatids give rise to monocentric isochromosomes (Fig. 2c). The chromosomal rearrangement that occurs at the pericentromeres, rather than the centromere cores, is one of the major sources of the Robertsonian translocation and isochromosome formation.

Recombination between inverted repeats can lead to gross chromosomal rearrangements (GCRs), including the Robertsonian translocation and isochromosome formation (Fig. 3). When DNA damage, such as DNA double-strand breaks (DSBs) and collapsed replication forks, is formed in repetitive sequences, recombinational repair of the damage occurs following the formation of single-stranded DNA tails with 3′-ends. These single-stranded DNAs can invade the inverted repeats at non-allelic as well as allelic positions. Resolution of the joint molecule by DNA endonucleases, such as the Mus81–Eme1 complex, in the crossover manner joins two different double-stranded DNAs (Fig. 3, the left pathway). Extensive DNA synthesis from the 3′ end of the invading strand by DNA polymerase delta (Pol δ), in break-induced replication (BIR), also results in GCRs (Fig. 3, the right pathway). These types of U-form recombination are the possible mechanisms by which isochromosome formation and Robertsonian translocation occur via inverted repeats. Indeed, in fission yeast, the spontaneous isochromosome formation that occurs in the absence of the canonical recombinase Rad51 requires Mus81 (Onaka et al. 2016), while the DSB-induced isochromosome formation requires Cdc27/Pol32, a subunit of DNA Pol δ (Tinline-Purvis et al. 2009). It remains unknown how these two pathways of GCRs are chosen in different situations. It is also possible that two broken chromosomes are joined around their centromeres by non-homologous end joining (NHEJ).

Heterochromatin might play an important role in maintaining pericentromeres. Abnormal centromere morphology (the juxtacentromeric heterochromatin) is the cytogenetic hallmark of the Immunodeficiency, Centromere instability, and Facial anomalies (ICF) syndrome (Jeanpierre et al. 1993; Tiepolo et al. 1979). In mammalian cells, cytosine methylation in the context of CpG dinucleotides is also required for heterochromatin formation (Rose and Klose 2014). Mutations in Dnmt3B, which is required for the DNA methylation, account for ~ 50% of the ICF patients (Hansen et al. 1999), suggesting the role of heterochromatin in maintaining centromere integrity.

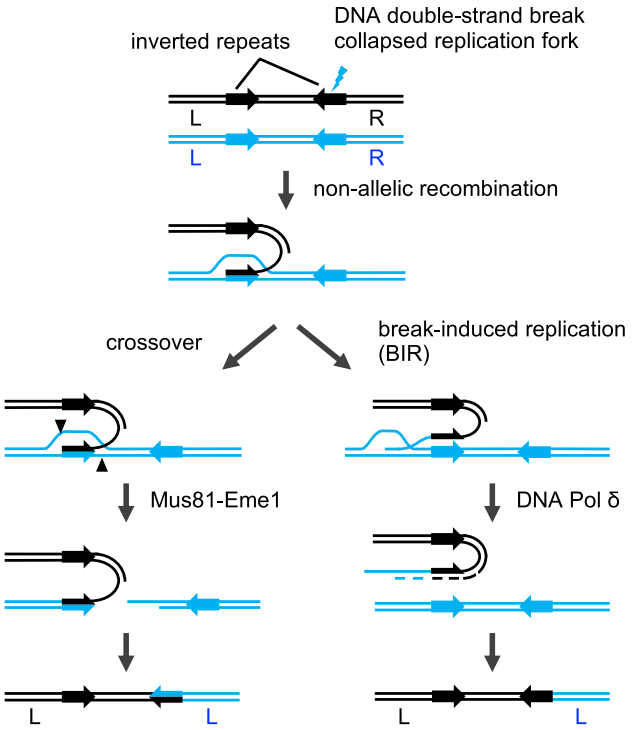
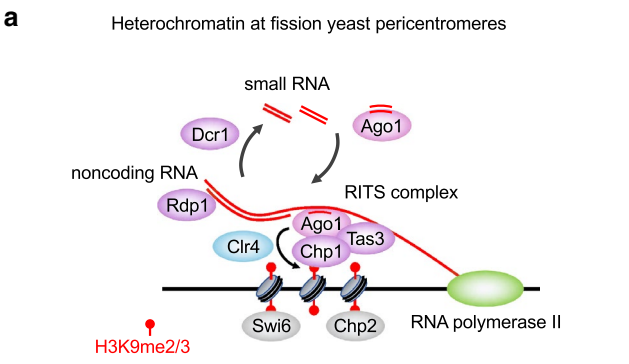


Fig. 3 A model that explains how recombination between inverted repeats results in gross chromosomal rearrangements (GCRs). DNA damage (blue lightning bolt), such as DNA double-strand breaks (DSBs) and collapsed replication forks, can be repaired by homologous recombination using inverted repeats. Recombination between allelic sequences does not result in GCRs. Even between non-allelic inverted repeats, non-crossover recombination does not result in GCRs. However, crossover or break-induced replication (BIR) between non-allelic inverted repeats gives rise to GCRs (the left and right pathways, respectively). In the case of a crossover, specific DNA endonucleases, such as Mus81–Eme1, digest the joint molecule at the positions indicated by the black arrowheads (Osman et al. 2003), resulting in the connection of the invading DNA (black) and the donor DNA (blue). In the case of BIR, DNA polymerase delta (Pol δ) performs DNA synthesis from the 3′-end of the invading strand (black) until the end of the donor DNA (blue) (Lydeard et al. 2007). Crossover or BIR explains how the isochromosome formation and the Robertsonian translocation occur using the inverted repeats that are present in pericentromeres



b

	Fission yeast	Drosophila	Human
H3K9 methyltransferase	Clr4	Su(var)3-9	Suv39h1, 2
histone H3 modification	H3K9me2/3	H3K9me2/3	H3K9me2/3
HP1 homologs	Swi6, Chp2	HP1a, b, c, d, e	HP1α, β, γ
RITS component	Chp1	n.d.	n.d.
RITS component	Tas3	GW182	TNRC6
Argonaute	Ago1	Ago1, 2, 3, Piwi Aubergine/Sting	Ago1, 2, 3, 4 Piwi1, 2, 3, 4
Dicer	Dcr1	Dcr1, 2	Dcr1
RNA-directed RNA polymerase	Rdp1	n.d.	n.d.

Fig. 4 Formation of heterochromatin at pericentromeres. **a** The heterochromatin formation at the pericentromeric region in fission yeast is depicted. Clr4 catalyzes the di- and tri-methylation of the ninth lysine of histone H3 (H3K9me2/3; red lollipops), which is recognized by Swi6 and Chp2, the fission yeast homologs of Heterochromatin Protein 1 (HP1). RNA polymerase II transiently produces noncoding RNA from pericentromeres. Rdp1 converts the noncoding RNA into double-stranded RNA, which is cleaved by Dcr1 to produce small RNAs. Ago1 captures the small RNAs and forms the RITS complex with Chp1 and Tas3. The RITS complex facilitates H3K9me2/3 by recruiting Clr4 to pericentromeres. **b** List of the factors that are required to form pericentromeric heterochromatin and their homologs in Drosophila and human

Heterochromatin formation at pericentromeres

The molecular mechanism of the heterochromatin assembly at pericentromeres has been extensively studied in fission yeast (Fig. 4). Clr4 is the lysine methyltransferase that catalyzes the H3K9 methylation at pericentromeres (Jih et al. 2017; Nakayama et al. 2001; Rea et al. 2000) (Fig. 4a, b). Clr4 is required to prevent the localization of RNA polymerase II (RNAPII) at pericentromeres (Chen et al. 2008). Swi6 and Chp2, the fission yeast homologs of heterochromatin protein 1 (HP1) (Fig. 4b), bind to the H3K9me2/3 marks via the chromodomain (Bannister et al. 2001; Lachner et al. 2001; Nakayama et al. 2001). Swi6 and Chp2 redundantly inhibit the pericentromeric localization of RNAPII (Fischer et al. 2009).

Paradoxically, the formation of pericentromeric heterochromatin requires the noncoding RNA from centromere repeats (Fig. 4a). Mutations in the subunits of RNAPII, including Rpb1, Rpb2, and Rpb7, reduce the levels of H3K9me2/3 and Swi6 localization at pericentromeres (Djupedal et al. 2005; Kajitani et al. 2017; Kato et al. 2005). In wild type, transcription transiently occurs at pericentromeres around the onset of S phase, followed by de novo methylation of H3K9 and Swi6 localization (Chen et al. 2008). The RNA interference (RNAi) machinery utilizes noncoding RNAs and facilitates heterochromatin assembly at pericentromeres (Martienssen and Moazed 2015) (Fig. 4a). The RNA-dependent RNA polymerase, Rdp1, creates double-stranded RNAs using the pericentromeric RNAs as templates. Dicer protein, Dcr1, a member of the RNase III

family, cleaves the double-stranded RNAs into small RNAs with predominant lengths of 22–23 bp. The Argonaute protein, Ago1, captures the small RNAs and forms the RNA-induced transcriptional silencing (RITS) complex with Chp1 and Tas3. The RITS complex localizes to pericentromeres through both the small RNAs that anneal to the nascent transcripts and Chp1, which contains the chromodomain that binds to H3K9me2/3. The RITS complex recruits the Clr4 complex to pericentromeres and facilitates the H3K9 methylation (Bayne et al. 2010). Most of these factors are evolutionally conserved in Drosophila and human (Fig. 4b).

Heterochromatin suppresses gross chromosomal rearrangements (GCRs) at pericentromeres

Heterochromatin plays important roles in maintaining genome integrity (Janssen et al. 2018), in addition to its role in chromosome segregation. When DNA double-strand breaks (DSBs) are formed in the heterochromatin domain in a nucleus, they relocalize to the periphery of the heterochromatin or the nuclear membrane during the process of DSB repair in Drosophila (Chiolo et al. 2011; Ryu et al. 2015; Tsouroula et al. 2016). Rad51 foci are formed at the DSB sites, but only after the relocation of the DSB sites from the heterochromatin domain. This spatial and temporal control of DSB repair is proposed to safeguard genome integrity, by preventing aberrant recombination between repetitive sequences. In mice, a chromosome orientation fluorescent in situ hybridization (CO-FISH) technique showed that the Dnmt3a and Dnmt3b DNA methyltransferases prevent sister chromatid exchange at centromeres (Jaco et al. 2008). In fission yeast, Rad51 promotes conservative non-crossover recombination, and thereby prevents the crossover recombination that results in isochromosome formation (Onaka et al. 2016; Zafar et al. 2017). Loss of an RNAi factor Dcr1 results in the localization of the Rad52 recombinase at pericentromeres during the S phase of the cell cycle (Zaratiegui et al. 2011), suggesting that the RNAi machinery prevents the replication fork collapse that induces DNA recombination. In *C. elegans*, loss of H3K9 methylation increases the transcription of repetitive sequences and accumulates RNA:DNA hybrids at the repetitive loci, resulting in the instability of tandem repeats, such as deletions and insertions (Zeller et al. 2016). These observations demonstrate the role of heterochromatin in suppressing aberrant recombination between repetitive sequences.

Heterochromatin affects not only the copy number of repetitive sequences, but also gross chromosomal rearrangements (GCRs). Abnormal chromosomes have been observed in the Suv39 knockout mice and the Su(var)3–9 mutant *Drosophila* (Peng and Karpen 2007; Peters et al. 2001). However, the sites of chromosomal rearrangements (i.e., breakpoints) and the mechanism of their formation remain unclear. Using the extra-chromosome ChL in fission yeast (Fig. 5a) (Nakamura et al. 2008; Niwa et al. 1986), our recent studies showed that heterochromatin plays an essential role to suppress GCRs at centromeres (Okita et al. 2019). Loss of the Clr4 methyltransferase increased the formation of isochromosomes, with breakpoints located in the centromere repeats (Fig. 5b) (Okita et al. 2019). Amino acid substitutions in the catalytic domain of the Clr4 methyltransferase (the SET domain) similarly increased GCRs (Okita et al. 2019). Finally, changing the H3K9 residue to alanine or arginine (i.e., H3K9A or H3K9R) also increased GCRs (Okita et al. 2019), demonstrating that the Clr4-dependent methylation of H3K9 is essential for suppressing GCRs at centromeres (Fig. 5c). The loss of Clr4 results in a ~ 100-fold increase in the rate of spontaneous isochromosome formation (Okita et al. 2019), whereas it only increases the rate of gene conversion between repetitive sequences by ~ twofold in the centromere core (Zafar et al. 2017). The marginal effect of Clr4 on the centromere core suggests that isochromosome breakpoints are present in the pericentromere, rather than the core region, in *clr4*Δ cells. The HP1 and RNAi systems are required to suppress GCRs in parallel. Deletion of both of the HP1 homologs, Swi6 and Chp2, only partially increased GCRs as compared to *clr4*Δ (Okita et al. 2019), showing that the HP1 proteins are not the only readers of H3K9me2/3 that are required for GCR suppression. This is also the case in maintaining H3K9me2/3 levels and in suppressing meiosis-specific DSBs at centromeres (Ellermeier et al. 2010; Sadaie et al. 2004). Loss of Chp1 in *swi6*Δ*chp2*Δ cells further increased GCRs to a level similar to that in *clr4*Δ cells (Okita et al. 2019), demonstrating that Chp1 has an HP1-independent role in GCR suppression. We propose that the Clr4-dependent histone methylation, H3K9me2/3, suppresses GCR formation at centromeres through the functions of both HP1 and the RNAi machinery (Fig. 5c).

Transcriptional restart after backtracking leads to gross chromosomal rearrangements (GCRs)

How does heterochromatin suppress gross chromosomal rearrangements (GCRs) at centromeres? The C-terminal domain (CTD) of the catalytic subunit of RNA polymerase II (RNAPII), Rbp1, consists of the YSPTSPS heptapeptide repeats and is the landing pad for the proteins involved in transcriptional regulation. The *rpb1-S7A* mutation, which changes all Ser7 residues to Ala in the YSPTSPS heptapeptide repeats, reduces the chromatin binding of RNAPII and increases the precocious termination of transcription in fission yeast (Kajitani et al. 2017; Okita et al. 2019;

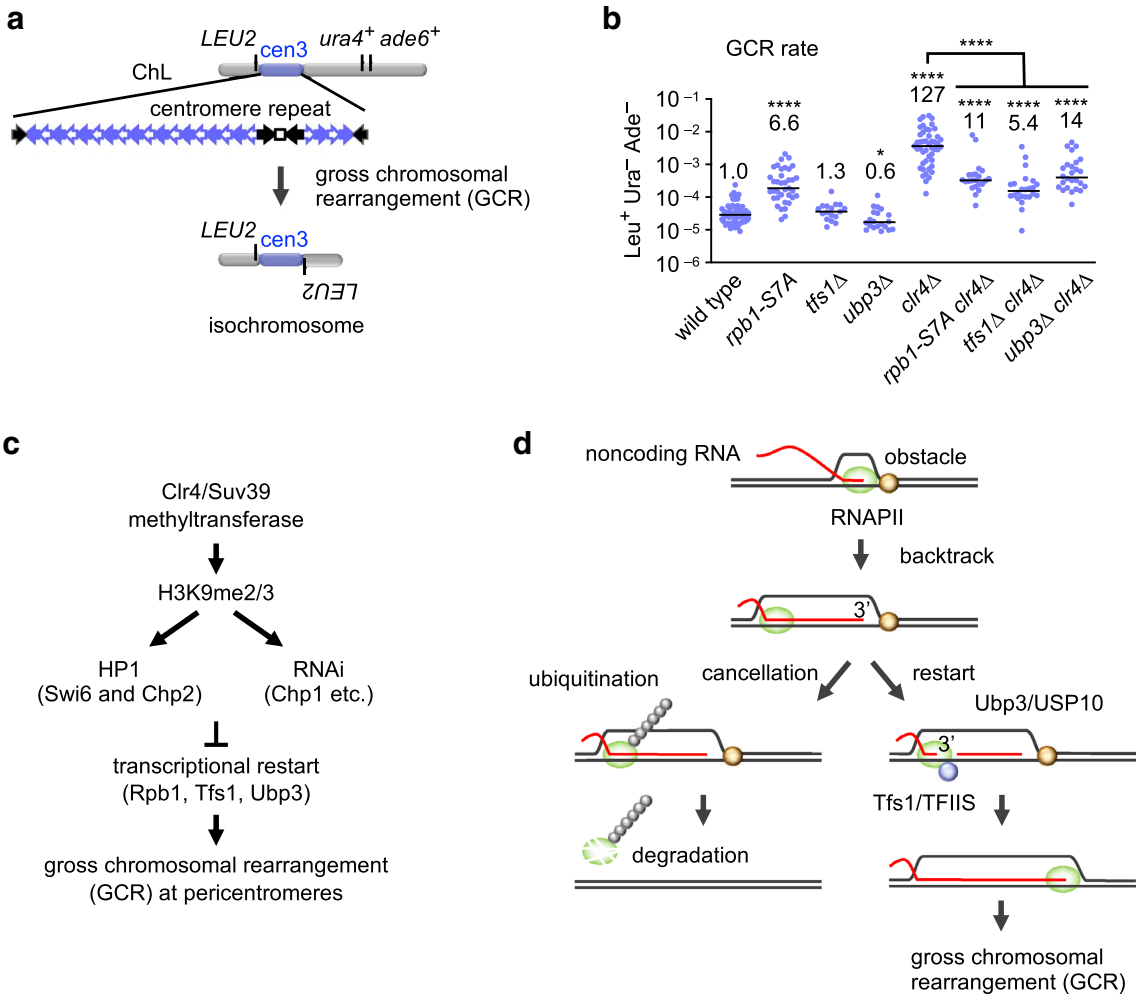


Fig. 5 The Clr4/Suv39 methyltransferase suppresses gross chromosomal rearrangements (GCRs) at centromeres by inhibiting transcriptional restart. **a** The extra-chromosome ChL, derived from the fission yeast chromosome 3 (Nakamura et al. 2008; Niwa et al. 1986). The positions of *LEU2*, *ura4*$^+$, *ade6*$^+$, and centromere 3 (cen3) are indicated. Clones that have spontaneously lost both the *ura4*$^+$ and *ade6*$^+$ markers were counted as GCR clones. Most of the GCR products detected in *clr4*Δ cells were the isochromosomes with breakpoints present at the centromere repeats (Okita et al. 2019). **b** GCR rates of wild type, *rpb1-S7A*, *tfs1*Δ, *ubp3*Δ, *clr4*Δ, *rpb1-S7A clr4*Δ, *tfs1*Δ *clr4*Δ, and *ubp3*Δ *clr4*Δ strains (TNF5676, 6848, 6688, 7456, 5702, 6850, 6726, and 7460, respectively). The wild type, *rpb1-S7A*, *tfs1*Δ, *clr4*Δ, *rpb1-S7A clr4*Δ, and *tfs1*Δ *clr4*Δ data were published previously (Okita et al. 2019). In the scatter plots, each dot represents the GCR rate, determined using a single colony formed on EMM + UA. Lines represent the median. The GCR rate relative to that of the wild type is indicated on the top of each column. Statistical significances of the differences relative to wild type (the top of each column), and those of the differences between pairs of strains were determined using the two-tailed Mann–Whitney test. *$P < 0.05$. ****$P < 0.0001$. **c** Depiction of how Clr4/Suv39 suppresses GCRs at centromeres. **d** Cancellation and restart of RNA polymerase II (RNAPII) after backtracking. After encountering an obstacle, RNAPII can backtrack on the template DNA, leaving the 3′ end of the nascent RNA behind. RNAPII can be subjected to polyubiquitin-dependent protein degradation, resulting in "cancellation" of transcription (the left pathway). The Ubp3/USP10 ubiquitin protease inhibits the RNAPII polyubiquitination. Tfs1/TFIIS stimulates RNAPII to cleave the nascent RNA to create a new 3′ end, resulting in "restart" of transcription (the right pathway)

Sanchez et al. 2018). In *clr4*Δ cells, *rpb1-S7A* reduced the centromeric localization of RNAPII and the GCRs at centromeres (Fig. 5b) (Okita et al. 2019), showing that RNAPII is involved in GCRs that occur at centromeres. During transcription, the progression of the RNAPII machinery pauses by different kinds of obstacles, including nucleosomes, other DNA-binding proteins, and some unique DNA sequences (Garcia-Muse and Aguilera 2016; Kireeva et al. 2005). When RNAPII backtracks on a template DNA strand after it encounters an obstacle, it will be polyubiquitinated and degraded by proteasomes (Somesh et al. 2005) (Fig. 5d, the left pathway). Alternatively, RNAPII resumes transcription through the cleavage of the nascent RNA by RNAPII itself, to create a new 3′ end of the RNA where ribonucleotides will be incorporated (Fig. 5d, the right pathway). Tfs1/TFIIS reportedly binds to Rpb1 and facilitates the RNA cleavage

to restart transcription (Izban and Luse 1992). Among the different transcription factors which we examined, only loss of Tfs1 dramatically reduced GCRs in *clr4*Δ cells (Okita et al. 2019) (Fig. 5b), suggesting the specific role of the transcriptional restart in GCRs. The ubiquitin protease (UBP), Ubp3/USP10, catalyzes the de-ubiquitination of RNAPII, and thereby prevents its polyubiquitin-dependent degradation (Kouranti et al. 2010; Kvint et al. 2008). The transcriptional restart model predicts that promoting RNAPII degradation should also reduce GCRs, by reducing the chance of transcriptional restart (Fig. 5d). In fact, we found that the loss of Ubp3, which results in an increase of polyubiquitin-dependent degradation of RNAPII, also reduced GCRs in *clr4*Δ cells (Fig. 5b). It is unlikely that the chromatin binding of RNAPII *per se* induces chromosomal rearrangements, as the steady-state binding of RNAPII to pericentromeres detected by chromatin immunoprecipitation (ChIP) was similar in *clr4*Δ and *tfs1*Δ*clr4*Δ cells (Okita et al. 2019). The loss of Tfs1 did not eliminate the pericentromeric RNAs in *clr4*Δ cells, showing that the transcription that occurs independently of Tfs1 is not as effective as the Tfs1-dependent transcription to induce chromosomal rearrangements. These results are consistent with the idea that heterochromatin suppresses chromosomal rearrangements at centromeres by inhibiting the transcriptional restart of RNAPII. The *rpb1*, *tfs1*, or *ubp3* mutation reduced the GCR rate to one-tenth in the absence of Clr4, but the double mutants still exhibited high GCR rates as compared to the wild type (Fig. 5b). Thus, the transcription restart is the major mechanism, but it may not be the only one that induces GCRs in the absence of heterochromatin.

How does the transcriptional restart lead to GCRs at pericentromeres? There are at least three possible ways for the transcription restart to cause GCRs (Fig. 6). First, the transcriptional restart may cause the dissociation of obstacles that impede transcriptional elongation, such as chromatin-binding proteins. Loss of Clr4 eliminates

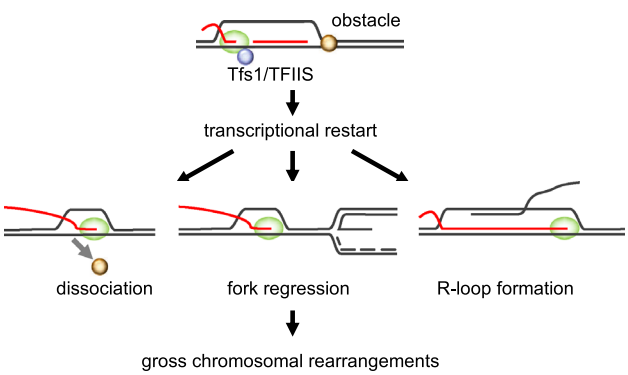


Fig. 6 A model that explains how the Tfs1/TFIIS-dependent transcriptional restart induces GCRs at pericentromeres

the S-phase-specific pericentromeric localization of the Smc5–Smc6 complex, which controls homologous recombination between repetitive sequences (Chiolo et al. 2011; Pebernard et al. 2008). Heterochromatin is required also for the accumulation of the Smc1–Smc3 cohesin complex at pericentromeres (Bernard et al. 2001; Litwin and Wysocki 2018; Villa-Hernandez and Bermejo 2018). The dissociation of such proteins may increase the aberrant recombination between inverted repeats that results in chromosomal rearrangements (Fig. 6). The transcriptional restart might also affect the localization of the Smc2–Smc4 condensin complex at pericentromeres (Chen et al. 2008; Robellet et al. 2017). Second, the transcription restart may interfere with the progression of replication forks and induce replication fork reversal or collapse. The single-stranded DNA tails created by the fork regression can anneal to homologous repetitive sequences and initiate chromosomal rearrangements. Third, the transcriptional restart might extend the region of the RNA:DNA hybrid and produce stable R-loop structures. The displaced single-stranded DNA in the R-loop can be used as a substrate for homologous pairing. One or more of these mechanisms may collectively cause a higher incidence of GCRs at centromeres. Further studies are needed to define the unexpected effects of the transcriptional restart on GCRs between repetitive sequences.

Closing remarks

It remains unclear why heterochromatin keeps pericentromeres transcriptionally silent. We propose that one of the reasons is to prevent gross chromosomal rearrangements (GCRs) using centromere repeats. The Clr4 methyltransferase suppresses isochromosome formation by H3K9 methylation (Okita et al. 2019). Heterochromatin suppresses GCRs by inhibiting transcription, as a mutation in RNA polymerase II (RNAPII) bypassed the requirement of Clr4 for GCR suppression (Okita et al. 2019). Loss of Tfs1/TFIIS or Ubp3/USP10, which both facilitate the RNAPII restart after backtracking, also reduced GCRs in *clr4*Δ cells (Okita et al. 2019) (in this study). Thus, the transcriptional restart may cause GCRs at centromeres. Interestingly, heterochromatin is not always formed at centromeres (Brown et al. 2014; Kapoor et al. 2015; Shang et al. 2013). In chicken DT40 cells, only a subset of centromeres consists of repetitive sequences, while others do not (Shang et al. 2013). Heterochromatin is formed at the centromeres that contain DNA repeats, but not at the centromeres that are devoid of DNA repeats, suggesting that transcriptional silencing is important for the repetitive centromeres. De-repression of the centromere repeats has been found in various kinds of cancers (Ting et al. 2011; Zhu et al. 2011). Gene amplification of TFIIS is observed in different kinds of cancer cells (Cerami

et al. 2012), suggesting that TFIIS facilitates tumorigenesis in human cells. The transcriptional restart may also cause chromosomal rearrangements at repetitive regions in the human genome.

Materials and methods

The *ubp3::kanMX6* and the *ubp3::kanMX6 clr4::hphMX6* strains (TNF7456 and TNF7460, respectively) were created in the same genetic background as the wild-type strain (TNF5676: *h–*, *smt0*, *mat2-3::natMX6*, *ade6Δ-D*, *ura4-D18*, *leu1-32*, ChL) (Okita et al. 2019). The rate of spontaneous GCRs was determined by counting the number of Leu+ cells and Leu+ Ura− Ade− cells, respectively, in 10-ml EMM + UA cultures, as described previously (Okita et al. 2019).

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