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The University of Osaka

Doctoral Thesis

**Transcription-induced
gross chromosomal rearrangements
at pericentromeric repeats in fission yeast**

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Abstract

The centromere is essential for faithful chromosome segregation. However, in many eukaryotes, the centromere is composed of repetitive DNA sequences. Recombination between centromeric repeats can lead to gross chromosomal rearrangements (GCRs), which cause cell death and genetic diseases. Heterochromatin, characterized by histone H3 lysine 9 methylation (H3K9me), assembles on pericentromeric DNA repeats and transcriptionally silences the pericentromeres. Importantly, heterochromatin suppresses GCRs by transcriptional silencing. However, how transcription causes GCRs at centromeres in the absence of heterochromatin remains unclear.

Here, I found that transcriptional pausing, backtracking, and restart (PBR) cycles at pericentromeres produce R-loops containing DNA-RNA hybrids and displaced single-stranded DNA (ssDNA), resulting in GCRs. DNA-RNA immunoprecipitation (DRIP) showed that loss of *Clr4*, the H3K9 methyltransferase essential for heterochromatin assembly, increased R-loops at centromeres. Overexpression of RNaseH1, which degrades R-loops, reduced R-loop levels and GCRs in *clr4Δ* cells, demonstrating that R-loops cause GCRs. A transcription termination factor, *Seb1*, pauses RNA polymerase II (RNAPII) at centromeres. A *seb1* mutation that abolishes RNAPII pausing reduced R-loops and GCRs, showing that transcriptional pausing is required for the formation of R-loops that cause GCRs. Moreover, loss of *Tfs1*/TFIIS transcription factor or *Ubp3* ubiquitin hydrolase, both of which facilitate transcriptional restart following pausing, decreased R-loops and GCRs in *clr4Δ* cells. Overall, these results establish that transcriptional PBR cycles create R-loops at pericentromeres.

Homology-mediated recombination essential for DNA damage repair can lead to GCRs at centromere repeats. Rad51- and Rad52-dependent recombination are the two major recombination pathways in fission yeast. Here, I found that R-loops cause GCRs in a manner dependent on Rad52-specific recombination activity but not Rad51. *rad52-R45K*, which impairs Rad52 single-strand annealing (SSA) activity, but not *rad51Δ*, reduced GCRs in *clr4Δ* cells. Microscope analysis showed that *clr4Δ* accumulated Rad52 foci. Chromatin immunoprecipitation (ChIP) revealed Rad52 localization at centromeres in a R-loop-dependent manner. An in vitro assay using purified proteins showed that the wild-type Rad52, but not

Rad51 or the mutant Rad52-R45K protein, facilitates the formation of an Annealing-induced DNA-RNA loop (ADR-loop) using the displaced ssDNA region of R-loops and ssDNA with complementary sequence, suggesting that Rad52 bridges centromeric repeats using R-loops. Break-induced replication (BIR) and crossover that resolve the joint DNA molecules can result in GCRs. The endonuclease Mus81, involved in crossover, is not required for GCRs in *clr4Δ* cells. In contrast, mutation in Cdc27/Pol32/POLD3, the subunit of DNA Pol δ that participates in BIR, reduced GCRs in *clr4Δ* cells. These data indicate that GCRs occur through BIR but not crossover in the absence of heterochromatin. Together, this study defined a transcription-induced GCR pathway in which transcriptional PBR cycles promote R-loop formation, and Rad52 converts R-loop into ADR-loop, initiating DNA repeat-mediated GCR events in the absence of heterochromatin.

1. Introduction

1.1. Maintenance of genome integrity is a challenging task for cells

Genome integrity is essential for all living cells because the genome encodes hereditary information. However, the genome is constantly exposed to damage from both endogenous and exogenous sources. It is estimated that each human cell is subjected to approximately 70,000 DNA lesions per day (Lindahl and Barnes 2000; Tubbs and Nussenzweig 2017). Many of these lesions arise from oxidative stress generated by cellular metabolism or from spontaneous hydrolysis of bases and can give rise to single-strand DNA breaks (SSBs). If not properly repaired, such lesions can be converted into double-strand DNA breaks (DSBs) during DNA replication. DSBs also occur during the conflict between transcription and replication, which share the genomic DNA as a common template (Garcia-Muse and Aguilera 2016). Although less frequent than SSBs, DSBs are particularly deleterious and pose a serious threat to genome stability. To preserve genome stability, cells have evolved multiple DNA repair pathways that recognize and repair DSBs. The two major DSB repair pathways are homologous recombination (HR) and non-homologous end joining (NHEJ). HR restores genetic information using an intact homologous DNA sequence as a template, whereas NHEJ directly rejoins broken DNA ends without the need of extensive homology. These pathways require precise temporal and spatial regulation. Dysregulation of repair factors or pathway choice can lead to mutations and chromosomal abnormalities that compromise genome integrity.

1.2. The chromatin structure and DNA repeats at centromeres

The centromere is a specialized chromosomal region essential for accurate chromosome segregation during cell division. It serves as the assembly platform for the kinetochore, a protein complex that links chromosomes to spindle microtubules and pulls sister chromatids toward opposite poles of the cell during mitosis (Fig. 1). Although centromeric DNA sequences vary greatly among species, centromere identity is established epigenetically rather than being defined by a conserved DNA sequence. A histone H3 variant, CENP-A,

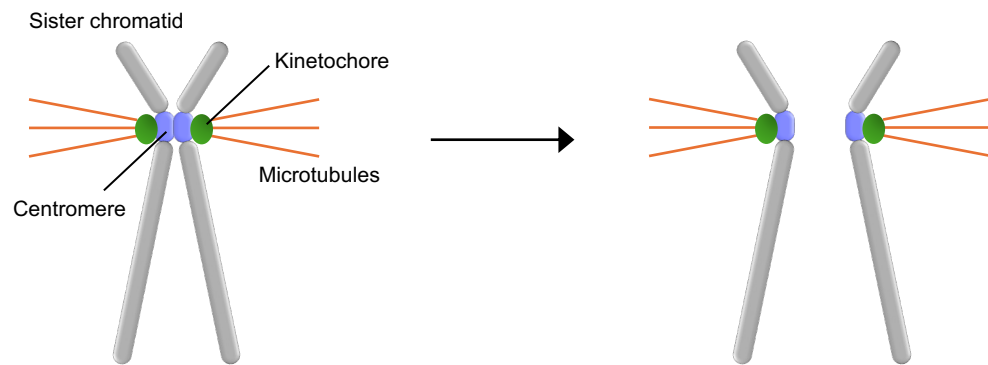


Fig. 1 Centromere is essential for chromosome segregation.

The centromere is the chromosome region where the protein complex kinetochore assembles. During mitosis, spindle microtubules attach to the kinetochore and force sister chromatid segregate.

replaces canonical histone H3 in nucleosomes and specifically localizes at the centromere. The number of CENP-A nucleosomes required for kinetochore assembly varies across organisms. In *Saccharomyces cerevisiae*, which has point centromeres of approximately 120-150 bp, a single CENP-A nucleosome is sufficient to nucleate kinetochore assembly. In contrast, in organisms with regional centromeres, multiple CENP-A nucleosomes are assembled to form a larger centromeric domain that promotes kinetochore formation (Furuyama and Biggins 2007; Fukagawa and Earnshaw 2014; Musacchio and Desai 2017). CENP-A nucleosomes intersperse with H3 nucleosomes at the core centromere region, called CENP-A chromatin. Surrounding CENP-A chromatin, constitutive heterochromatin characterized by histone H3 9th lysine di- and tri- methylation (H3K9me_{2,3}) is formed. Pericentromeric heterochromatin plays a role in kinetochore assembly, chromosome segregation, replication timing, and transcriptional silencing (Allshire and Madhani 2018; Grewal 2023).

Centromeres contain repetitive DNA sequences in many eukaryotes, even though the repeat sequences themselves are not conserved. In humans, centromeres contain alpha-satellite (α Sat) DNA repeats, which can form complex higher-order repeats (HORs). The α Sat HORs are surrounded by more divergent α Sat monomers, transposable elements, segmental duplications, and other satellite repeats, such as hSat1, 2, or 3 (Fig. 2A) (Altemose et al. 2022). CENP-A chromatin is usually formed within the HORs and transcriptionally active, while the pericentromeric repeats are heterochromatic and transcriptionally silenced. In *Schizosaccharomyces pombe*, centromeres include a unique core (cnt) sequence that is flanked by inverted repeats: the imr (inner repeat), otr (outer repeat) containing dg and dh repeats, and irc (outermost repeats) (Fig. 2B). CENP-A chromatin is formed at cnt, and the inner region of imr repeats. The otr, irc repeats, and the outer region of imr are heterochromatic. Interestingly, heterochromatin is not always formed at pericentromeric regions. In the *S. pombe* CBS2777 strain, centromeres that lack flanking repetitive DNA sequences do not form pericentromeric heterochromatin (Brown et al. 2014). In chicken DT40 cells, heterochromatin is formed only at centromeres that contain repetitive DNA sequences (Shang et al. 2013). These studies suggest that repetitive DNA sequences promote heterochromatin formation (Shang et al. 2010). Supporting this, a recent study using *S. pombe* has demonstrated that even tandemly repeated mRNA genes can actively enhance

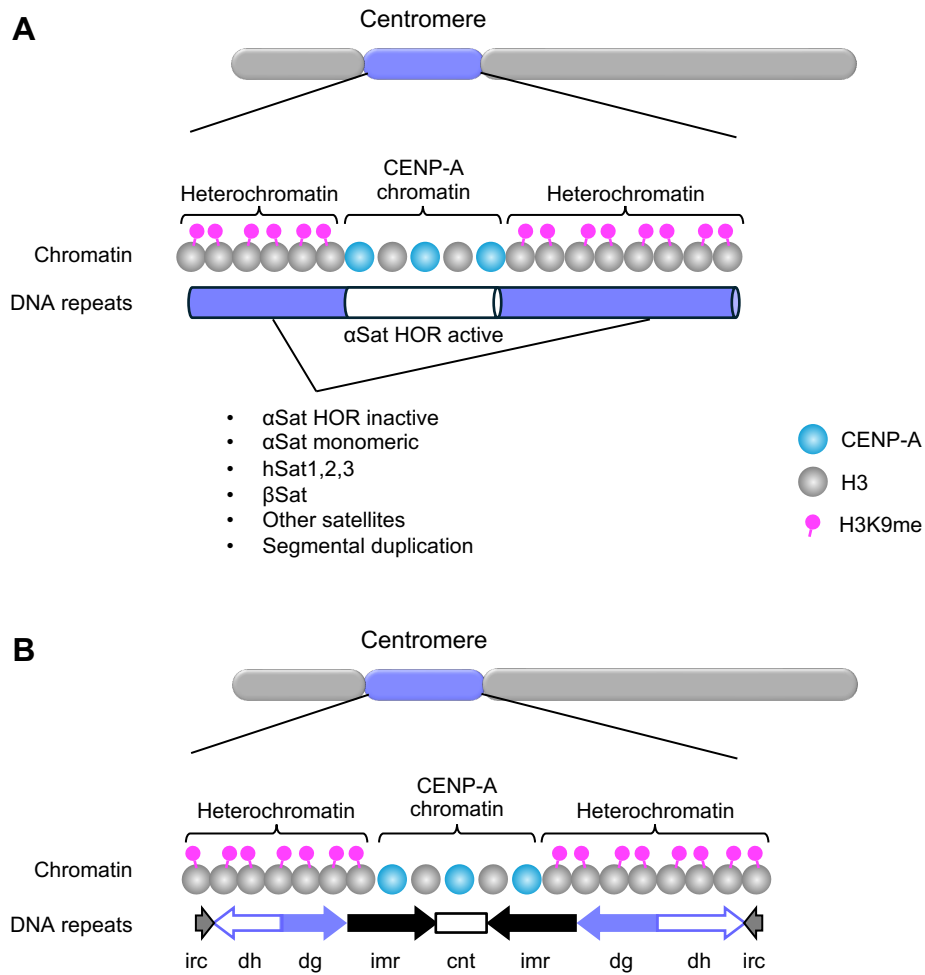


Fig. 2 Schematic of chromatin structure and DNA repeats at centromeres.

(A) Schematic of a human centromere region. The chromatin structure, including CENP-A chromatin and surrounding heterochromatin, and their underlying DNA repeats are shown.

(B) Schematic of a fission yeast centromere region.

heterochromatin assembly (Asanuma et al. 2022), reinforcing a functional link between DNA repetitive sequences and heterochromatin assembly.

1.3. Establishment of heterochromatin at pericentromeres

H3K9me_{2,3} is a defining epigenetic mark of heterochromatin. In higher eukaryotes, H3K9 methylation is deposited by multiple histone methyltransferases, including Suppressor of Variegation 3-9 homologue 1 (Suv39H1), Suv39H2, SET domain bifurcated 1 (SETDB1), and SETDB2 (Padeken et al. 2022). In contrast, *S. pombe* relies on a single enzyme, Cryptic Loci Regulator (Clr4), the ortholog of mammalian Suv39H1 and Suv39H2, to catalyze H3K9me_{2,3}. Clr4 contains a SET domain responsible for methyltransferase activity and a chromodomain that recognizes H3K9 methylation, thereby enabling a self-reinforcing positive feedback loop that promotes heterochromatin spreading (Muller et al. 2016). In addition to this intrinsic regulatory mechanism, post-translational modifications further refine Clr4 activity. Clr4 automethylation induces conformational changes that enhance its methyltransferase activity (Iglesias et al. 2018). In addition, monoubiquitination of Clr4 mediated by the E2 ubiquitin conjugating enzyme Ubc4 and the E3 ubiquitin ligase Cul4 facilitates the transition from H3K9 di-methylation (H3K9me₂) to tri-methylation (H3K9me₃), reinforcing transcriptional silencing (Kim et al. 2024). Together, these findings establish Clr4 as the central regulator of H3K9 methylation and heterochromatin formation in *S. pombe*.

The H3K9me_{2,3} mark is subsequently recognized by Heterochromatin Protein 1 (HP1) family members (HP1 α , HP1 β , and HP1 γ in mammals, and Swi6 and Chp2 in *S. pombe*) (Eissenberg and Elgin 2000). Unlike Clr4, HP1 proteins are largely dispensable for the establishment of H3K9 methylation but instead function as readers of H3K9me (Motamedi et al. 2008; Stunnenberg et al. 2015). Upon binding to H3K9me through their chromodomains, HP1 proteins multimerize and bridge neighboring nucleosomes, thereby promoting chromatin compaction. HP1 dimers can also undergo liquid-liquid phase separation, providing a physical mechanism for transcription silencing (Larson et al. 2017). In addition, HP1 proteins serve as scaffolds to recruit downstream effector proteins, including histone deacetylases (HDACs) and chromatin remodelers, thereby facilitating heterochromatin

organization (Eissenberg and Elgin 2000). Through these structural and regulatory functions, HP1 proteins play an important role in organizing higher-order heterochromatin structure downstream of H3K9 methylation.

Notably, DNA methylation is absent in *S. pombe*. In contrast, in mammals and plants, DNA methylation represents an additional player for heterochromatin regulation (Saksouk et al. 2015). Methylated DNA is recognized by methyl-CpG-binding domain (MBD) proteins, such as MeCP2, MBD1 and MBD2. These proteins recruit corepressor complexes that contain transcription repressor and HDACs, leading to histone deacetylation and transcription repression (Nan et al. 1998).

In *S. pombe*, pericentromeric repeats are transiently transcribed during S phase and processed to generate small interfering RNA (siRNA) for heterochromatin assembly (Chen et al. 2008). RNA polymerase II (RNAPII) transcribes non-coding RNAs (ncRNAs) from *dg* and *dh* repeat regions. These ncRNAs serve as a template for RNA-directed RNA polymerase complex (RDRC) and are converted into double-stranded RNA (dsRNA) (Motamedi et al. 2004). The Dicer protein Dcr1 cleaves dsRNAs into siRNAs, which are loaded onto the Argonaute protein 1, Ago1, to form the RNA-induced transcriptional silencing (RITS) complex. The RITS complex recognizes pericentromeric sequences through base pairing and recruits Clr4 H3K9 methyltransferase complex (CLRC) to establish H3K9me_{2,3}. Interestingly, cells lacking RNAi machinery factors still retain a residual level of H3K9 methylation (Volpe et al. 2002), suggesting that H3K9 methylation can be established independently of the RNAi pathway. A long non-coding RNA (lncRNA) of the pericentromeric region can induce Clr4 recruitment and H3K9me₃ at an ectopic chromosome site in the absence of Ago1 (Khanduja et al. 2024). Thus, transcription of pericentromeric repeats serves not only as a trigger for RNAi-mediated heterochromatin assembly but also as a foundation for RNAi-independent heterochromatin establishment. Similar transcription-coupled mechanisms have been observed in higher eukaryotes. In human cells, although the RNAi machinery is not central to constitutive heterochromatin formation, pericentromeric satellite repeats are transcribed under specific conditions, such as during S phase or cellular stress (Saksouk et al. 2015). These transcripts can form RNA-chromatin interaction that recruits methyltransferase SUV39H1 to reinforce H3K9 methylation (Johnson et al. 2017).

Together, these findings highlight a conserved mechanism in which transcription provides the foundation for heterochromatin establishment and maintenance across eukaryotes.

1.4. *S. pombe* is an ideal organism for studying centromere integrity

Centromeres are composed of repetitive DNA sequences, which make them fragile chromosome regions prone to mutations and chromosomal aberrations. Chromosomal abnormalities involving centromeres are frequently observed in various types of cancer cells (McKinley and Cheeseman 2016; Barra and Fachinetti 2018). To gain insights relevant to disease intervention, it is important to understand how centromere integrity is maintained and elucidate the mechanism underlying centromere instability.

S. pombe is an ideal organism for studying centromere functions and instability for several reasons. As discussed above, similar to humans, *S. pombe* centromeres are composed of repetitive DNA sequences and contain conserved epigenetic features such as CENP-A-containing nucleosomes and pericentromeric heterochromatin. However, *S. pombe* centromeres are much smaller (30-110 kb compared with ≥ 1 Mb in humans), and their repeat organization is less complex. Pericentromeric repeats are transcriptionally silenced both in *S. pombe* and humans. In *S. pombe*, DNA methylation is absent and Clr4 is the sole enzyme that catalyzes H3K9 methylation. Therefore, deletion of Clr4 eliminates heterochromatin, making it possible to understand the role of heterochromatin.

1.5. Heterochromatin maintains centromere integrity through transcription repression

Heterochromatin safeguards centromere integrity through transcription repression. DNA repetitive sequences pose a potential threat to genome integrity, as recombination between ectopic repeats can lead to alterations in repeat number and gross chromosomal rearrangements (GCRs) (Pascarella et al. 2022). Cells deficient in the DNA methyltransferases Dnmt3 exhibit increased recombination at centromeres and changes in centromeric repeat length, suggesting that heterochromatin suppresses recombination at centromeric repeats (Jaco et al. 2008). In patients with Immunodeficiency, Centromere

instability, and Facial anomalies (ICF) syndrome, they carry mutations in DNMT3b, ZBTB24, CDCA7, or HELLS genes that encode proteins for DNA methylation. On chromosomes 1, 9, and 16 of ICF cells, hypomethylation of pericentromeric satellite repeats is observed and multiradial chromosomes are fused via the pericentromeric region (Vukic and Daxinger 2019). Increased γ H2AX, which indicates DNA breaks, was found in ICF cells and cells disrupting DNMT3b (Unoki et al. 2020; Shih et al. 2022). These findings suggest that a defect in transcriptional silencing is linked with genome instability at pericentromeric regions. Using *S. pombe*, our lab previously showed that loss of H3K9 methyltransferase, Ctr4, greatly increases the frequency of centromeric GCRs that produce isochromosomes, whose arms are mirror images (Okita et al. 2019). Importantly, mutation on Rpb1, the catalytic subunit of RNAPII, reduced GCRs in *ctr4* Δ cells, indicating that transcription causes GCRs at centromeres. DNA sequences and DNA-binding proteins can block and pause RNAPII. After pausing and backtracking on DNA template, RNAPII restarts transcription with the aid of Tfs1/TFIIS, which stimulates Rpb1's RNA cleavage activity to create a nascent 3' end for ribonucleotide incorporation (Izban and Luse 1992). Paused RNAPII can be degraded in a ubiquitination-dependent manner. Ubiquitin protease Ubp3 removes ubiquitin from RNAPII to facilitate transcriptional restart (Kvint et al. 2008). Depletion of Tfs1 or Ubp3 reduced GCRs in *ctr4* Δ cells (Nakagawa and Okita 2019; Okita et al. 2019), showing that transcriptional restart induces GCRs. However, how transcription dynamics lead to GCRs in the absence of heterochromatin remains poorly understood.

1.6. The focus of this thesis

Centromeres are the fragile chromosome regions despite their essential role in ensuring accurate chromosome segregation during cell division. Heterochromatin, characterized by H3K9me_{2,3}, maintains centromere integrity through transcriptional silencing. However, how transcription triggers genome instability at centromeres remains poorly understood.

In this thesis, I investigate the molecular mechanism by which transcription induces GCRs at pericentromeric repeats when heterochromatin is lost. A particular focus is placed on R-loops, three-stranded nucleic acid structures composed of DNA-RNA hybrids and displaced ssDNA. R-loops are well recognized as the major source of genome instability. In part I, I provide experimental evidence demonstrating that R-loops are the cause of GCRs and that these R-loops are generated during transcriptional pausing, backtracking, and restart (PBR) cycles at pericentromeric repeats in the absence of heterochromatin. In part II, I show that R-loops induce a specific Rad52-dependent recombination pathway between pericentromeric repeats and examine how this aberrant recombination gives rise to GCRs. Based on these findings, I propose a model in which transcription, through the formation of R-loops and recombination intermediates termed Annealing-induced DNA-RNA loops (ADR-loop), drives genome instability at pericentromeres when heterochromatin is absent. Overall, this study identifies a previously unrecognized mechanism of R-loop formation and defines a distinct pathway by which R-loops induce genome instability.

2. Part I: Transcription dynamics form R-loops that lead to GCRs at pericentromeres

2.1. Introduction

2.1.1. R-loops are the major source of genome instability

During transcription, nascent RNA transcripts can rehybridize with the DNA template strand and displace the non-template strand as single-stranded DNA (ssDNA), forming a three-stranded structure, known as an R-loop. Genome-wide R-loop mapping studies have revealed that R-loops form across diverse genomic regions transcribed by RNA polymerases I, II, and III, with lengths ranging from 60 bp to several kilobases (Chedin et al. 2021). Although R-loops play essential physiological roles in DNA replication, transcriptional regulation, and immunoglobulin (Ig) class-switch recombination, numerous studies have demonstrated that unscheduled or persistent R-loops are a major source of spontaneous DNA damage (Aguilera and Garcia-Muse 2012; Crossley et al. 2019; Garcia-Muse and Aguilera 2019). For instance, R-loop accumulation, in the absence of the RNA-binding protein ASF/SF2, leads to elevated γ H2AX signals and fragmented chromosomal DNAs, suggesting that R-loops cause DSBs (Li and Manley 2005). ASF/SF2 is an RNA-binding protein involved in mRNA splicing. A genome-wide siRNA screen has revealed that downregulation of multiple mRNA processing proteins increases R-loop levels and γ H2AX and 53BP1 (Paulsen et al. 2009). Importantly, overexpression of RNaseH1, which degrades R-loops, decreases these DNA break signals, indicating that R-loops are a general source of spontaneous DNA damage.

The mechanisms by which R-loops lead to DNA damage have been extensively studied (Brickner et al. 2022). During the S phase, R-loops may block the replication fork, leading to replication stress and DNA breaks (Fig. I-1A). Using a bacterial replication system, a study showed that the replication fork can bypass a naked R-loop but not an RNAPII-bound R-loop, indicating that R-loop bound with RNAPII is a more severe threat to replication fork progression (Bruning and Marians 2020). Forks encounter R-loop-associated RNAPII in a head-on (HO) or co-directional (CO) orientation, resulting in different outcomes (Hamperl et al. 2017). The HO collision leads to replication fork stalling, while the CMG replicative helicase can unwind DNA-

RNA hybrids during a CO collision. The HO collision accumulates positive supercoils, slowing down replication progression and leading to replication fork collapse (Promonet et al. 2020). A recent study showed that upon a CO collision, CUL3-KCD10 E3 ubiquitin ligase senses and ubiquitinates RNAPII complex. This ubiquitination remodels the RNAPII complex and facilitates replisome bypass the RNAPII machinery (Kloeber et al. 2025). R-loops can lead to DNA breaks outside S phase. R-loops processed by endonucleases, XPG and XPF, lead to DNA break accumulation (Fig. I-1B) (Sollier et al. 2014; Stork et al. 2016). It was revealed that XPG and XPF can cleave the same strand upstream and downstream of DNA-RNA hybrids, potentially leaving a single-strand break (Crossley et al. 2023). Cooperate with TOP1 and FEN1, XPG and XPF cleave both sides of an R-loop, resulting in a double-strand break (Cristini et al. 2019). Furthermore, the ssDNA in R-loop might be attacked by cytidine deaminase, such as activation-induced cytidine deaminase (AID) and APOBEC proteins, which convert cytosines to uracil (Fig. I-1C) (Yu et al. 2003; Saini et al. 2017). The resulting uracil would be subsequently processed by glycosylase and base excision repair, leading to SSB. Together, these studies demonstrate that R-loops constitute a potent source of genome instability by inducing DNA damage through multiple mechanisms.

R-loops are formed in various mechanisms under different conditions. One well-characterized source of R-loops is RNAPII pausing (Petermann et al. 2022). Genome-wide mapping using catalytically inactive RNaseH1 has shown that R-loops accumulate at promoter-proximal pausing sites of protein-coding genes (Chen et al. 2017). Human Cobra1, also known as NELFB, which is a subunit of negative elongation factor (NELF) that pauses RNAPII at promoters, is required for R-loop accumulation at these regions (Zhang et al. 2017). In addition to normal pausing, artificial trapping of RNAPII in a backtracked state using a mutant version of TFIIS promotes R-loop formation anterior to RNAPII (Zatreanu et al. 2019). These findings show that RNAPII pausing is associated with R-loop accumulation, probably because it increases the chance of RNA rehybridization with the DNA template. R-loops are also formed at specific chromosome regions, such as telomeres and centromeres. At telomeres, telomeric repeat-containing RNA (TERRA) forms R-loops by hybridizing with the telomeric DNA. The recombinase Rad51 binds TERRA and mediates R-loop formation via strand invasion (Feretzaki et al. 2020). In the type of cells that alternative lengthening of telomeres (ALT) is activated, Rad51-associated protein 1 (Rad51AP1), instead of Rad51, binds TERRA and generates R-loops,

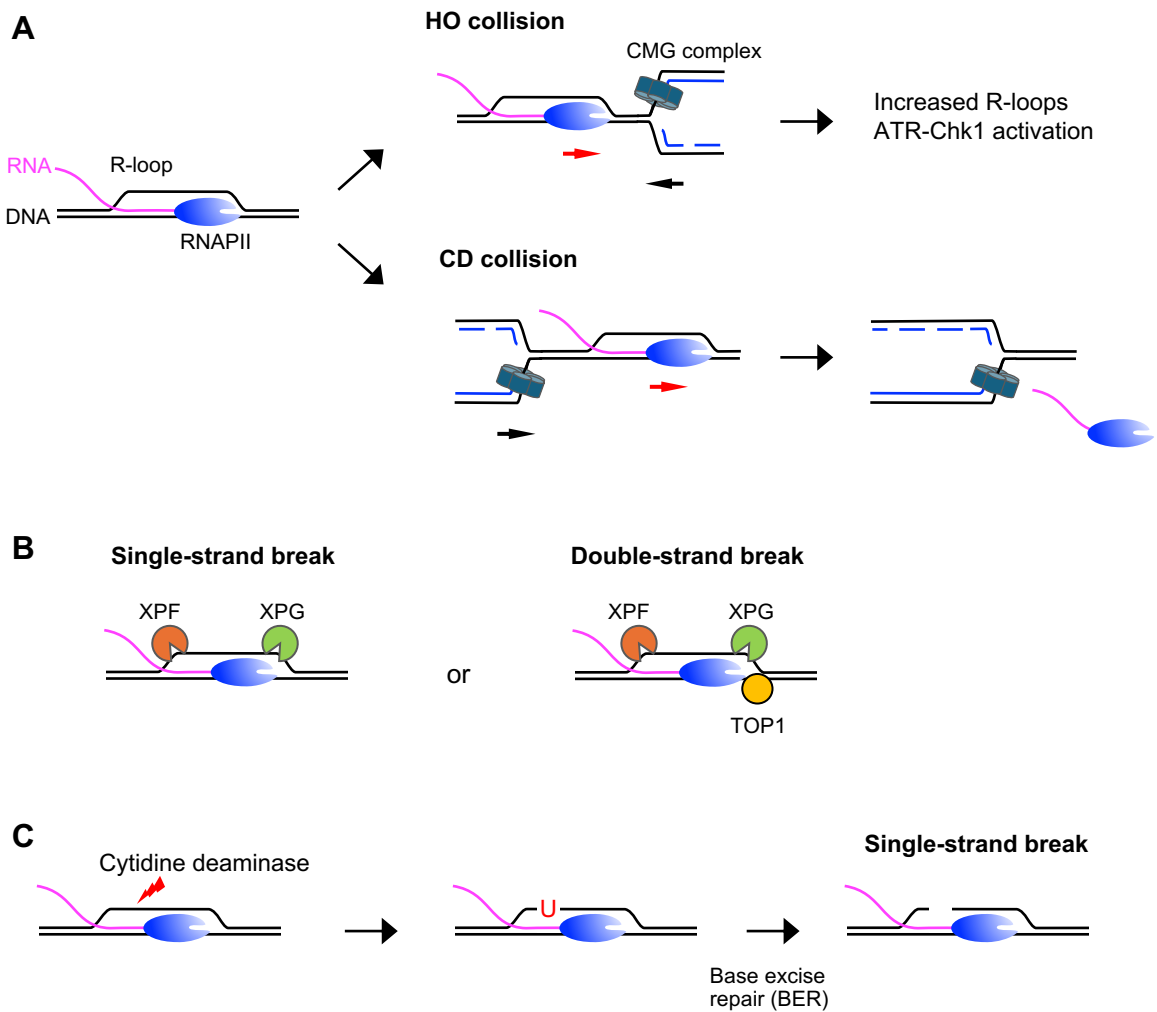


Fig. I-1 Schematic of R-loop-induced genome instability.

(A) R-loops encounter the replication fork in either head-on (HO) or co-directional (CD) orientation. HO collisions increase R-loop accumulation and stall the replication fork, leading to activation of ATR-Chk1 checkpoint. In contrast, during CD collisions, R-loops are resolved by the CMG complex and RNAPII is displaced from the DNA template, allowing the replication fork to bypass the co-directional R-loops.

(B) XPG and XPF cleave R-loops to generate single-strand breaks (SSBs), which are converted into double-strand breaks (DSBs) during DNA replication. Together with TOP1 and FEN1, XPG and XPF can also directly cause DSBs.

(C) The ssDNA within R-loop is exposed to cytosine deaminases, resulting in the formation of SSBs.

leading to telomeric replication stress (Yadav et al. 2022). At centromeres, R-loops formed during mitosis ensure accurate chromosome segregation (Kabeche et al. 2018). The RPA-coated R-loops activate ATR to stimulate Aurora B through Chk1 and prevent lagging chromosome formation. Pericentromeric R-loops also contribute to heterochromatin assembly in the presence of Ctr4 methyltransferase (Nakama et al. 2012; Velazquez Camacho et al. 2017). However, how R-loops are formed at centromeres remains unclear.

2.1.2. R-loop detection approaches

Precise detection of R-loops is essential for understanding their formation mechanism and biological consequences. Over the past decades, several experimental techniques have been developed to either map R-loops genome-wide or visualize them within cells. These methods can be broadly classified into antibody-based and enzyme-based approaches (Fig. I-2) (Chedin et al. 2021).

The most widely used technique employs the S9.6 monoclonal antibody, which has a high affinity for DNA-RNA hybrids (Fig. I-2A). This antibody enables DNA-RNA immunoprecipitation followed by high-throughput sequencing (DRIP-seq), a method that provides the global profiling of DNA-RNA hybrid landscapes in various cell types. However, the resolution of DRIP-seq is limited by the size of fragmented nucleic acids, since nearby DNA duplexes and DNA-RNA hybrids are co-immunoprecipitated and sequenced together. To overcome this limitation, DNA-RNA immunoprecipitation followed by cDNA conversion and sequencing (DRIPc-seq) was developed (Sanz and Chedin 2019). In DRIPc-seq, DNA is digested by S1 nuclease after immunoprecipitation, and RNA is reverse transcribed into cDNA, enabling strand-specific detection with nearly nucleotide-level resolution. Because the S9.6 antibody also exhibits an affinity for double-stranded RNA (dsRNA) (Hartono et al. 2018), it is essential to treat samples with RNase III, which digests dsRNA, before immunoprecipitation to prevent contamination in the DRIPc-seq assay. The catalytically inactive RNaseH1 (dRNaseH1), which lacks ribonuclease activity but retains high affinity for DNA-RNA hybrids, is also widely utilized for DNA-RNA hybrid detection. When fused with epitope tags such as Flag, HA, or GFP, dRNaseH1 can be used for chromatin immunoprecipitation (R-ChIP), live-cell imaging, or hybrid-specific pulldown assays. Furthermore, the MapR technique integrates dRNaseH1 with

micrococcal nuclease, achieving high-resolution DNA-RNA hybrid mapping without the need for nucleic acid fragmentation, thereby preserving the native chromatin context (Yan et al. 2019). Notably, neither S9.6- nor dRNaseH1- based methods distinguish DNA-RNA hybrids and the three-stranded R-loops that contain both displaced ssDNA and DNA-RNA hybrids. To specifically detect R-loops, bisDRIP-seq combines DRIP-seq with the bisulfite treatment (Fig. II-2A) (Dumelie and Jaffrey 2017), exploiting the fact that the ssDNA portion of R-loops is sensitive to cytidine deaminase. Upon bisulfite treatment, cytosine residues in ssDNA are converted to uracil, allowing strand-specific and near nucleotide-resolution mapping of R-loops. Overall, choosing the appropriate method for R-loop/DNA-RNA hybrid detection is critical, as each technique offers distinct advantages and limitations in terms of resolution, specificity, and compatibility with different experimental contexts.

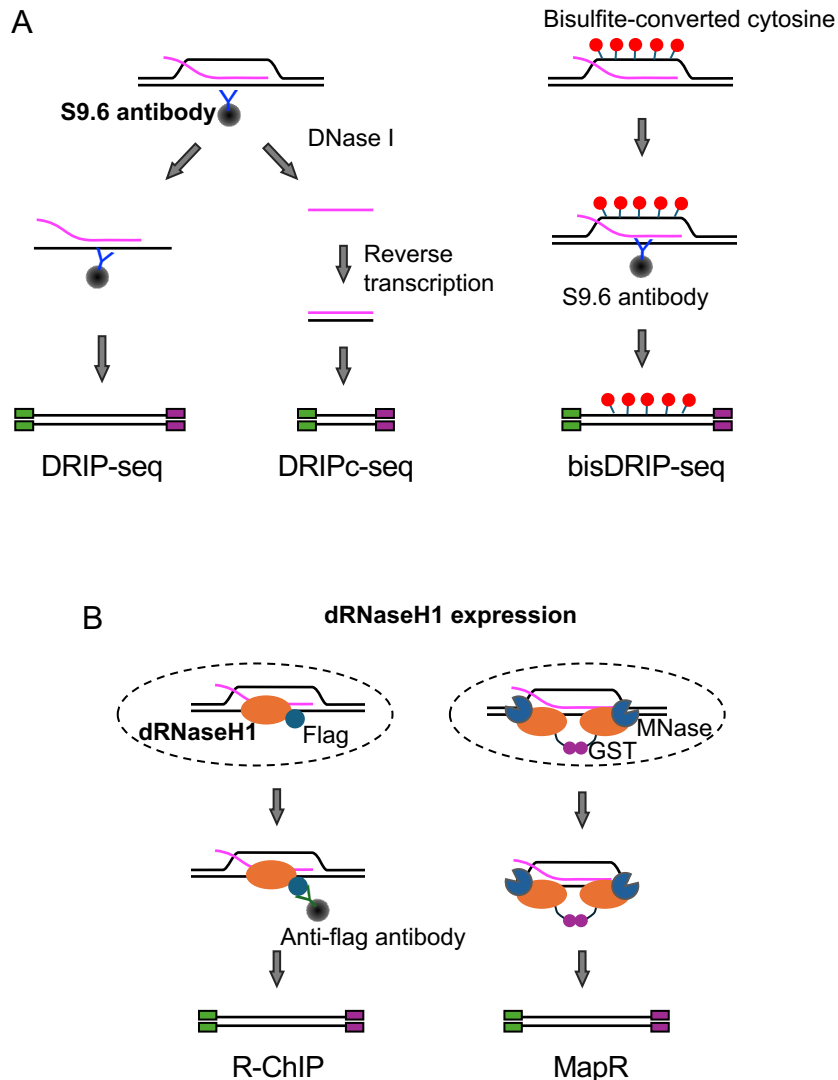


Fig. I-2 Schematic of R-loop detection approaches.

(A) S9.6-based R-loop detection methods. In DRIP-seq, fragmented nucleic acids are incubated with the S9.6 antibody, and the enriched DNA is subjected to library preparation followed by sequencing. In DRIPc-seq, RNA recovered from immunoprecipitated R-loops is purified and reverse-transcribed into cDNA for library preparation and sequencing. The method enables precise and strand-specific mapping of R-loops. In bisDRIP-seq, cytosines in single-stranded DNAs are converted by bisulfite before S9.6 immunoprecipitation and converted cytosines serve as high-resolution markers for R-loop regions.

(B) Catalytically dead RNaseH1 (dRNaseH1)-based R-loop detection methods. In R-ChIP, in vivo-expressed dRNaseH1 binds R-loops and the associated DNA is immunoprecipitated for library preparation and sequencing. In MapR, a construct encoding micrococcal nuclease (MNase)-tagged dRNaseH1 is expressed in vivo and used to recognize and purify R-loop structures.

2.2. Results

Clr4 methyltransferase complex suppresses DNA-RNA hybrids at pericentromeres

It is well known that R-loops can lead to genome instability. Therefore, I wondered whether transcription causes GCRs through forming R-loops in the absence of heterochromatin. To test this, I performed DRIP assays in a wild-type and a deletion strain of Clr4 methyltransferase. Nucleic acids extracted from yeast cells were fragmented by sonication. To verify the specificity of DNA-RNA hybrid detection, the samples were divided into two aliquots and treated with or without *E. coli* RNaseH before immunoprecipitation using the S9.6 antibody, which captures DNA-RNA hybrids. In collaboration with Crystal Tang and Dr. Daisuke Motooka, we analyzed DRIP samples by deep DNA sequencing (DRIP-seq). The core centromeric region containing *cnt* and inner parts of *imr* repeats is CENP-A chromatin, while the surrounding repeats are assembled into heterochromatin (Fig. I-3, top panels). tRNA genes are the boundaries between CENP-A chromatin and heterochromatin (Scott et al. 2006). As expected, DNA-RNA hybrid formation was observed at tRNA genes on all three centromeres of *S. pombe* (Fig. I-3, bottom panels) (Petermann et al. 2022). The L5 fragment can induce heterochromatin assembly at an ectopic site outside centromeres. Consistent with the role of DNA-RNA hybrids in heterochromatin assembly (Nakama et al. 2012), DNA-RNA hybrids were accumulated in L5. Surprisingly, overall levels of DNA-RNA hybrids were similar in wild-type and *clr4Δ* cells. To identify genomic regions enriched for DNA-RNA hybrids, we did Model-based Analysis for ChIP-Seq 2 (MACS2) (Zhang et al. 2008). MACS2 shows that *clr4Δ* increases DNA-RNA hybrids at two regions indicated as pericentromere repeat 1 (*cenR1*) and pericentromere repeat 2 (*cenR2*) (Fig. I-3). These data suggest that Clr4 methyltransferase suppresses DNA-RNA hybrids at the specific sites of pericentromeric repeats.

To confirm the increase of DNA-RNA hybrids in *clr4Δ* cells, I did real-time qPCR using the DRIP samples (DRIP-qPCR). Consistent with DRIP-seq, DNA-RNA hybrids were detected at *cenR1* in wild-type cells (Fig. I-4A). RNaseH treatment before S9.6 immunoprecipitation eliminated DRIP-qPCR signals, confirming the detection of DNA-RNA hybrids. Importantly, loss of Clr4 significantly increased DNA-RNA hybrids at heterochromatic *cenR1* and *cenR2* sites (Fig. I-4A). In contrast to pericentromeric sites, *clr4Δ* did not significantly change hybrid

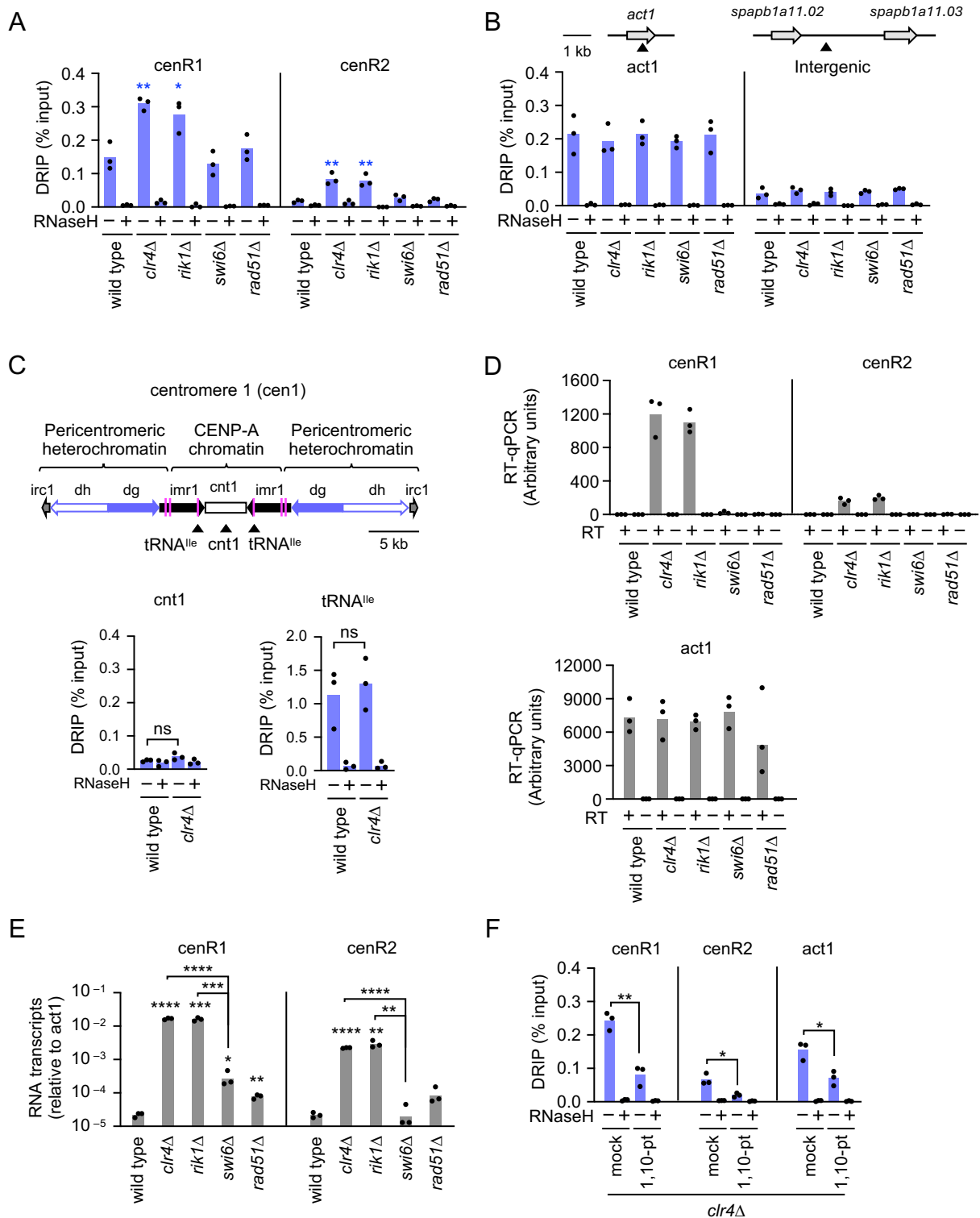


Fig. I-4 Transcription leads to R-loop accumulation in the absence of heterochromatin.

(A-B) DRIP-qPCR. DNA-RNA hybrid levels at (A) centromeric cenR1 and cenR2 sites and (B) non-centromeric act1 and Intergenic sites in wild-type, *clr4Δ*, *rik1Δ*, *swi6Δ*, and *rad51Δ* strains. DRIP-qPCR amplification sites of *act1* and Intergenic are indicated at the top of the graph. The percent recovery is shown. Each dot represents a biologically independent experiment ($n = 3$). Bars show the mean. (C) Top panel indicates DRIP-qPCR amplification sites of *cnt1* and *tRNA^{Ile}* in *cen1*. Bottom panel shows DNA-RNA hybrid levels at *cnt1* and *tRNA^{Ile}* in wild-type and *clr4Δ* strains. (D) RT-qPCR. RNA transcript levels of wild type, *clr4Δ*, *rik1Δ*, *swi6Δ*, and *rad51Δ* cells. RNA levels were expressed as arbitrary units based on the qPCR standard curve. RT, reverse transcription. (E) RT-qPCR. RNA transcript levels of cenR1 and cenR2 were normalized relative to act1 RNA levels. (F) Treatment of *clr4Δ* cells with a transcription inhibitor, 1,10-phenanthroline (1,10-pt) reduced DNA-RNA hybrids.

levels at the *act1* gene and an intergenic site outside the centromere (Fig. I-4B). At the CENP-A chromatin, *clr4Δ* did not significantly increase hybrids at *cnt1* or tRNA sites (Fig. I-4C). These results indicate that loss of Clr4 methyltransferase specifically increases DNA-RNA hybrids at pericentromeric repeats.

To further assess the role of heterochromatin in DNA-RNA hybrids, I tested additional factors involved in heterochromatin establishment. Rik1, a component of the CLRC complex, is required for Clr4 localization and H3K9me_{2,3} at pericentromeres (Hong et al. 2005; Horn et al. 2005; Jia et al. 2005). Swi6 is a homolog of HP1 that binds H3K9me_{2,3} (Lachner et al. 2001). In *swi6Δ* cells, H3K9me_{2,3} and transcriptional silencing are substantially maintained compared to *clr4Δ* cells (Motamedi et al. 2008; Stunnenberg et al. 2015). Like *clr4Δ*, *rik1Δ* increased DNA-RNA hybrids at *cenR1* and *cenR2*, but *swi6Δ* did not (Fig. I-4A and B), suggesting that H3K9me_{2,3} and transcriptional silencing suppress the DNA-RNA hybrid formation.

Rad51-mediated HR maintains centromere integrity. Like Clr4, Rad51 prevents isochromosome formation at centromeres (Nakamura et al. 2008; Onaka et al. 2016). Loss of Rad51 increases GCR rates by 100-fold. However, *rad51Δ* did not significantly change the hybrid levels (Fig. I-4A and B), suggesting that DNA-RNA hybrids are not byproducts of GCRs. Together, these results demonstrate that heterochromatin suppresses DNA-RNA hybrid formation at pericentromeric repeats.

DNA-RNA hybrids are formed in a transcription-dependent manner

The above data showed that loss of heterochromatin increased DNA-RNA hybrid formation at pericentromeric repeats, suggesting that transcription of pericentromeric repeats contributes to DNA-RNA hybrid formation. To test this, first, I determined transcription levels at pericentromeric repeats by performing reverse transcription followed by quantitative PCR (RT-qPCR). Total RNA extract was prepared by heating and freezing the cells in the presence of SDS and phenol, followed by DNase I treatment to remove contaminating DNA, and then reverse transcribed into cDNA. A no RT control was included to assess genomic DNA contamination. Primers specific to *cenR1*, *cenR2*, and the internal control gene *act1* were used for qPCR. No signals were detected in the no-RT control samples, confirming that detection of RNA transcripts

(Fig. I-4D). Deletion of *Clr4* caused more than a 100-fold increase in transcript levels at *cenR1* and *cenR2*, while it did not significantly change expression level at the *act1* locus. Consistent with DRIP-qPCR, *clr4Δ* and *rik1Δ*, but not *swi6Δ* or *rad51Δ*, greatly increased transcription levels at *cenR* sites (Fig. I-4D and E). To determine whether this transcriptional upregulation contributes to DNA-RNA hybrid accumulation, *clr4Δ* cells were treated with the transcription inhibitor 1,10-phenanthroline, followed by DRIP assays (Fig. I-4F). DRIP-qPCR showed that 1,10-phenanthroline treatment significantly reduced DNA-RNA hybrid levels in *clr4Δ* cells. Together, these results support the conclusion that increased transcription promotes DNA-RNA hybrid accumulation at pericentromeric repeats.

Rnh1 overexpression reduced DNA-RNA hybrid levels and GCRs in *clr4Δ* cells

It is possible that DNA-RNA hybrids accumulated in the absence of heterochromatin cause GCRs. To test this, I overexpressed the yeast RNaseH1 homolog Rnh1, which degrades RNA in DNA-RNA hybrids. Because DNA-RNA hybrids also play important physiological roles, Rnh1 was mildly overexpressed from the *adh1* promoter at an ectopic locus on chromosome 2 rather than from a multicopy vector. DRIP-qPCR showed that Rnh1 overexpression (*rnh1-OE*) did not significantly change DNA-RNA hybrid levels in wild-type cells (Fig. I-5A and B), indicating that the level of Rnh1 overexpression was not sufficient to globally eliminate DNA-RNA hybrids. However, *rnh1-OE* reduced DNA-RNA hybrids specifically at *cenR1* in *clr4Δ* cells, indicating that DNA-RNA hybrids produced at *cenR1* in *clr4Δ* cells are particularly sensitive to Rnh1-mediated degradation. I next examined the effect of *rnh1-OE* on spontaneous GCR rates using the extra-chromosome ChL derived from chromosome 3 (Fig. I-5C) (Nakamura et al. 2008). This assay detects GCR events in which ChL loses *ura4⁺* and *ade6⁺* markers. A fluctuation test showed that loss of *Clr4* greatly increased GCR rates, as expected (Fig. I-5D). A previous study showed that GCR products in *clr4Δ* cells are mainly isochromosomes, whose breakpoints are in centromere repeats (Okita et al. 2019). Importantly, *rnh1-OE* reduced GCR rates specifically in *clr4Δ* cells, consistent with the reduction in DNA-RNA hybrids. However, *rnh1-OE* did not significantly change GCR rates in *rad51Δ* cells, in which DNA-RNA hybrids do not accumulate at centromeres. Together, these results indicate that loss of heterochromatin accumulates DNA-RNA hybrids that cause GCRs at pericentromeric repeats.

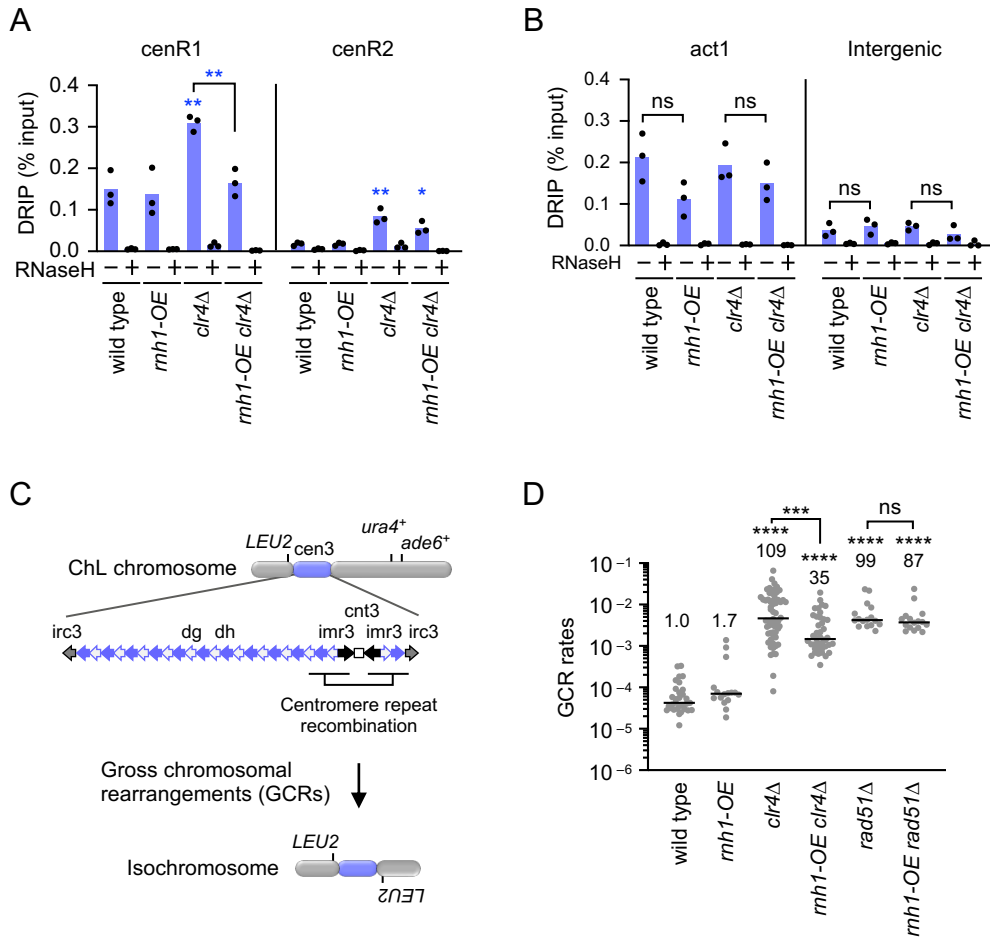


Fig. I-5 DNA-RNA hybrid accumulation at pericentromeric repeats causes GCRs.

(A-B) Rnh1 overexpression reduced DNA-RNA hybrids in *clr4Δ* cells. DNA-RNA hybrid levels at (A) cenR1 and cenR2 and (B) non-centromeric *act1* and Intergenic sites in wild-type, *mh1-OE*, *clr4Δ*, and *mh1-OE clr4Δ* strains. Each dot represents a biologically independent experiment ($n = 3$). Bars show the mean. (C) Illustrated are the extra-chromosome, ChL, and the centromere repeats in *cen3*. GCRs losing *ura4+* and *ade6+* markers were detected. Isochromosomes whose arms are mirror images are the major GCR products formed in *clr4Δ* or *rad51Δ* cells. (D) Rnh1 overexpression reduced GCRs in *clr4Δ* cells. GCR rates of wild-type, *mh1-OE*, *clr4Δ*, *mh1-OE clr4Δ*, *rad51Δ*, and *mh1-OE rad51Δ* strains. Each dot represents a biologically independent experiment. Lines show the median. GCR rates relative to wild type are shown at the top of each column.

Tfs1's transcriptional restart activity is required for DNA-RNA hybrids and GCRs in *clr4Δ* cells

A previous study from our laboratory showed that, among the transcription factors tested, Tfs1 is specifically required for GCR formation in *clr4Δ* cells (Okita et al. 2019). The paused and backtracked RNAPII is disengaged from the 3' end of the RNA transcript for ribonucleotide incorporation. Tfs1/TFIIS interacts with RNAPII active site through its conserved acidic residues Asp-Glu to cleave RNA strand and restart transcription by creating a new 3' end for RNA synthesis (Fig. I-6) (Kettenberger et al. 2003). Given that both Tfs1 and DNA-RNA hybrids are required for GCRs, transcriptional restart might cause GCRs through promoting DNA-RNA hybrid formation. Indeed, DRIP-qPCR showed that *tfs1Δ* significantly and specifically reduced DNA-RNA hybrids at cenR1 and cenR2, but not at act1 or intergenic site, in *clr4Δ* cells (Fig. I-7A and B). Importantly, *rnh1-OE* and *tfs1Δ* reduce GCRs in *clr4Δ* cells in an epistatic manner (Fig. I-7C), showing that Tfs1 and DNA-RNA hybrids cause GCRs in the same pathway. Tfs1's conserved acidic residues Asp-Glu are essential for its interaction with RNAPII and transcriptional restart activity (Jeon et al. 1994; Sigurdsson et al. 2010). Like *tfs1Δ*, *tfs1-DEΔ*, greatly reduced DNA-RNA hybrids at cenR1 and cenR2 sites and GCR rates in *clr4Δ* cells (Fig. I-7A, B, and D), showing that transcriptional restart is essential for DNA-RNA hybrids and GCR formation. Tfs1 has also been reported to facilitate transcription elongation through interaction with the PAF1 complex (Kim et al. 2010). However, in contrast to *tfs1* mutants, deletion of Leo1, a subunit of PAF1 complex, did not significantly change DNA-RNA hybrid levels nor GCRs in *clr4Δ* cells (Fig. I-7D). Overall, these data demonstrate that Tfs1-dependent transcriptional restart promotes formation of DNA-RNA hybrids that cause GCRs at pericentromeric repeats.

Ubiquitin protease Ubp3 antagonizes degradation factor Def1, facilitating DNA-RNA hybrid formation and GCRs

Paused RNAPII can be restated by Tfs1. Alternatively, RNAPII pausing can lead to ubiquitination and degradation of RNAPII (Fig. I-6). This degradation pathway is considered a “last resort” mechanism that acts when paused RNAPII cannot be efficiently restarted (Wilson et

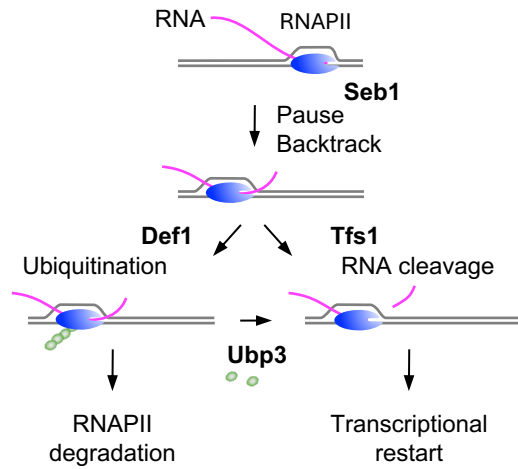


Fig. I-6 Schematic showing the roles of Tfs1, Ubp3, and Seb1 in transcription.

Tfs1 facilitates transcriptional restart by promoting RNA cleavage by RNAPII. Def1 promotes ubiquitin-dependent RNAPII degradation, whereas Ubp3 prevents RNAPII degradation by removing ubiquitin from RNAPII. Seb1 causes transcriptional pausing at pericentromeric repeats.

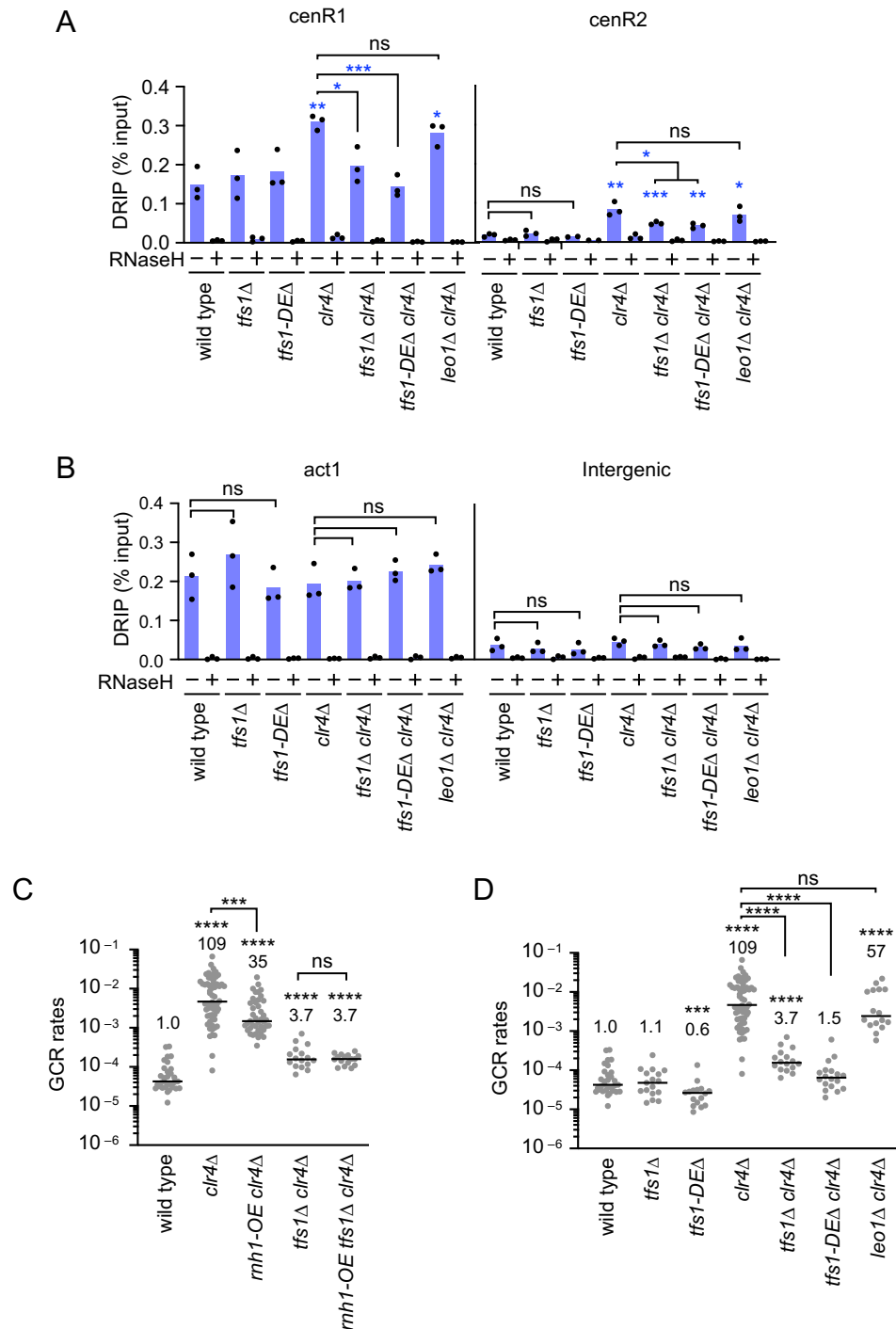


Fig. I-7 Tfs1 causes DNA-RNA hybrid accumulation and cause GCRs in *clr4* Δ cells.

(A-B) DNA-RNA hybrid levels at (A) cenR1 and cenR2 and (B) non-centromeric act1 and Intergenic sites in wild-type, *tfs1* Δ , *tfs1-DE* Δ , *clr4* Δ , *tfs1* Δ *clr4* Δ , *tfs1-DE* Δ *clr4* Δ , and *leo1* Δ *clr4* Δ strains. Each dot represents a biologically independent experiment ($n = 3$). Bars show the mean. (C) GCR rates of wild-type, *clr4* Δ , *mh1-OE clr4* Δ , *tfs1* Δ *clr4* Δ , and *mh1-OE tfs1* Δ *clr4* Δ strains. Each dot represents a biologically independent experiment. Lines show the median. GCR rates relative to wild type are shown at the top of each column. (D) GCR rates of wild-type, *tfs1* Δ , *tfs1-DE* Δ , *clr4* Δ , *tfs1* Δ *clr4* Δ , *tfs1-DE* Δ *clr4* Δ , and *leo1* Δ *clr4* Δ strains.

al. 2013). In this pathway, Def1 promotes polyubiquitination of stalled RNAPII, thereby targeting it for proteasomal degradation (Woudstra et al. 2002; Karakasili et al. 2014). Conversely, the deubiquitinating enzyme Ubp3/USP10 removes ubiquitin from RNAPII, preventing its degradation and allowing transcription to resume (Kvint et al. 2008). Previous work from our laboratory showed Ubp3 is involved in GCR formation in *clr4Δ* cells (Nakagawa and Okita 2019). To see whether Ubp3 promotes DNA-RNA hybrid formation, I performed DRIP-qPCR in *ubp3Δ* strains (Fig. I-8A and B). Loss of *ubp3* significantly reduced DNA-RNA hybrids at cenR1 in *clr4Δ* cells, indicating a role of Ubp3 in hybrid formation at this site. DNA-RNA hybrids at cenR2 were not significantly affected by *ubp3Δ*, suggesting that RNAPII pausing and restart dynamics differ between cenR1 and cenR2. Importantly, *ubp3Δ* and *rnh1-OE* epistatically reduced GCRs in *clr4Δ* cells (Fig. I-8C). These data show that Ubp3 promotes hybrid accumulation that causes GCRs in *clr4Δ* cells. Notably, *ubp3Δ* did not reduce DNA-RNA hybrids and GCRs in *def1Δ clr4Δ* cells (Fig. I-8A, B, and D), showing that Ubp3 promotes DNA-RNA hybrids and GCRs by antagonizing Def1-mediated RNAPII degradation. Deletion of *tfs1* reduced GCRs and DNA-RNA hybrids in *def1Δ clr4Δ* cells (Fig. I-8A, B, and D), reinforcing the essential role of transcriptional restart in DNA-RNA hybrids and GCRs at centromeres.

Distinct E3 ubiquitin ligases mediate RNAPII polyubiquitination under different conditions (Somesh et al. 2005). For example, in response to ultraviolet (UV) irradiation, the Ela1-Elc1-Cul3 E3 ligase complex is well known to polyubiquitylate RNAPII and facilitate its proteasomal degradation in *Saccharomyces cerevisiae* (Ribar et al. 2007). In addition, independent of DNA damage, the E3 ligase Asr1 targets RNAPII for ubiquitination to silence gene expression at subtelomeric regions (McCann et al. 2016). However, in contrast to Def1, deletion of *S. pombe* Ela1 homolog Pof4, Cul3, or Asr1 failed to restore the reduced GCR rates in *ubp3Δ clr4Δ* cells (Fig. I-9). These results suggest that spontaneous RNAPII ubiquitination at the centromere is mediated by an E3 ubiquitin ligase distinct from those previously characterized. Identifying the E3 ubiquitin ligase responsible for centromere-specific RNAPII degradation will be one of the important directions for future investigation.

RNAPII pausing factor, Seb1, is involved in DNA-RNA hybrid formation and GCRs

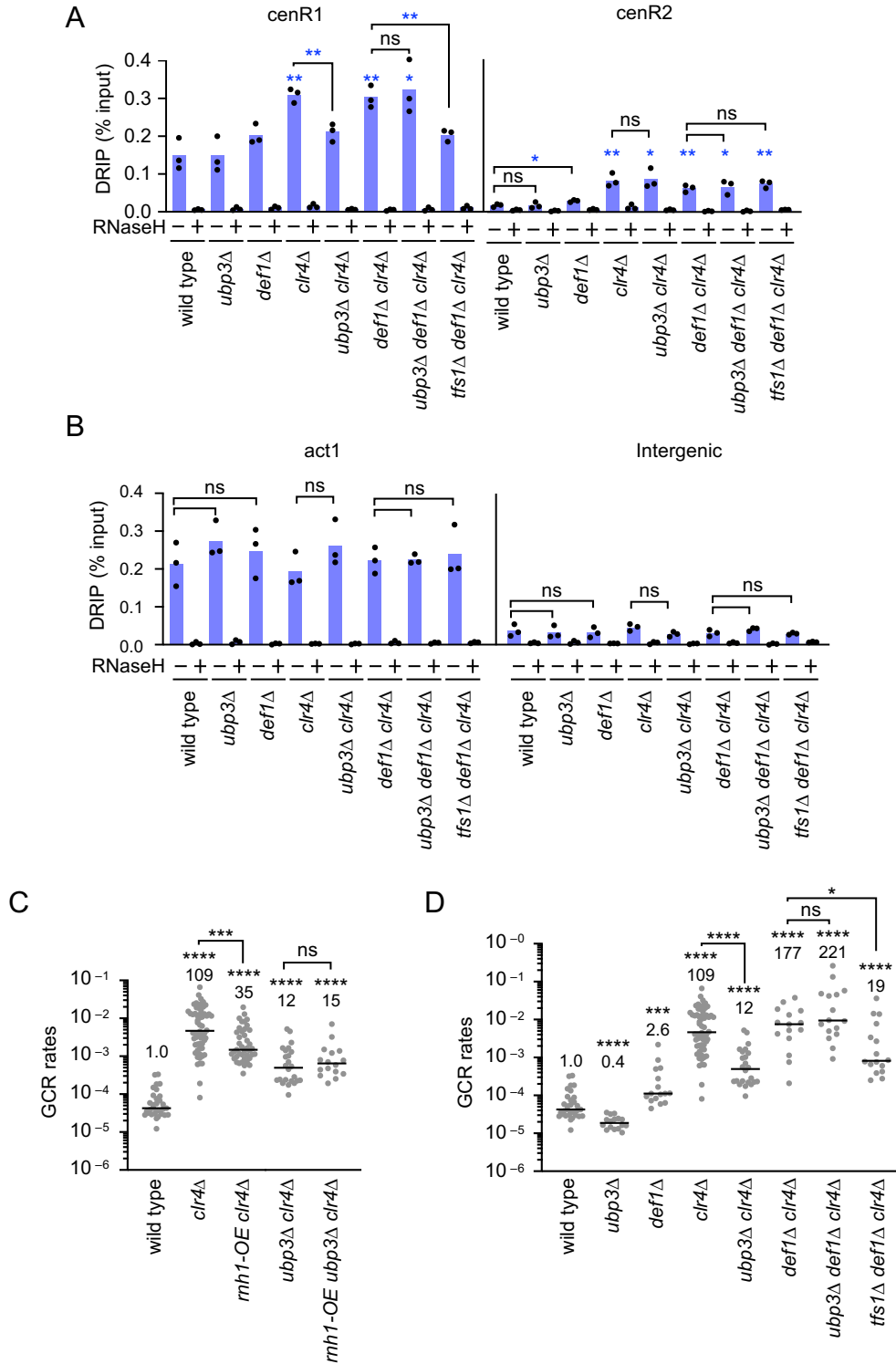


Fig. I-8 Ubp3 antagonizes Def1, facilitating DNA-RNA hybrids and GCR formation in *clr4Δ* cells.

(A-B) DNA-RNA hybrid levels at (A) cenR1 and cenR2 and (B) non-centromeric act1 and Intergenic sites in wild-type, *ubp3Δ*, *def1Δ*, *clr4Δ*, *ubp3Δ clr4Δ*, *def1Δ clr4Δ*, *ubp3Δ def1Δ clr4Δ*, and *tfs1Δ def1Δ clr4Δ* strains. Each dot represents a biologically independent experiment ($n = 3$). Bars show the mean. (C) GCR rates of wild-type, *ubp3Δ*, *def1Δ*, *clr4Δ*, *ubp3Δ clr4Δ*, *def1Δ clr4Δ*, *ubp3Δ def1Δ clr4Δ*, and *tfs1Δ def1Δ clr4Δ* strains. Each dot represents a biologically independent experiment. Lines show the median. GCR rates relative to wild type are shown at the top of each column. (D) GCR rates of wild-type, *clr4Δ*, *rh1-OE clr4Δ*, *ubp3Δ clr4Δ*, and *rh1-OE ubp3Δ clr4Δ* strains.

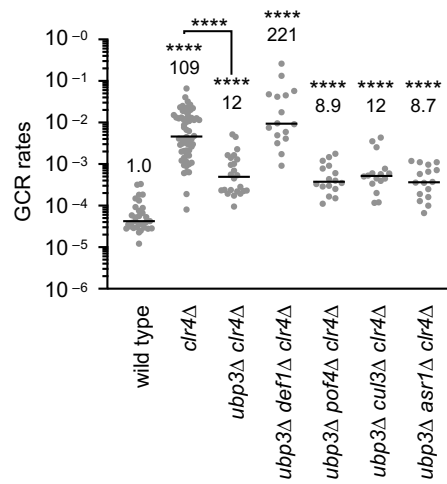


Fig. I-9 Loss of E3 ubiquitin ligases Pof4, Cul3, or Asr1 does not increase GCRs in *ubp3Δ clr4Δ* cells.

GCR rates of wild-type, *clr4Δ*, *ubp3Δ clr4Δ*, *ubp3Δ def1Δ clr4Δ*, *ubp3Δ pof4Δ clr4Δ*, *ubp3Δ cul3Δ clr4Δ* and *ubp3Δ asr1Δ clr4Δ* strains. Each dot represents a biologically independent experiment. Lines show the median. GCR rates relative to wild type are shown at the top of each column.

The transcription termination factor Seb1 has been shown to pause RNAPII at pericentromeric regions (Fig. I-6) (Wittmann et al. 2017; Parsa et al. 2018). It is possible that transcriptional restart following Seb1-mediated RNAPII pausing facilitates DNA-RNA hybrid accumulation. To test this, I introduced the *seb1-1* mutation (Marina et al. 2013), into wild-type and *clr4Δ* cells. Previous work showed that the *seb1-1* mutation did not globally alter transcription but specifically impairs RNAPII pausing at pericentromeres (Parsa et al. 2018). *seb1-1* reduced DNA-RNA hybrids at cenR1 in *clr4Δ* (Fig. I-10A and B), but not in wild-type cells, showing that Seb1 is required for DNA-RNA hybrid formation in *clr4Δ* cells. Like *ubp3Δ*, *seb1-1* did not affect DNA-RNA hybrid levels at cenR2. Importantly, *seb1-1* reduced GCR rates in *clr4Δ* cells (Fig. I-10C), demonstrating that Seb1-mediated RNAPII pausing contributes to the formation of DNA-RNA hybrids that cause GCRs.

To further understand the connection between transcription and DNA-RNA hybrid formation, I measured pericentromeric transcription levels by RT-qPCR (Fig. I-10D). Neither *tfs1Δ*, *ubp3Δ*, nor *seb1-1* greatly reduced pericentromeric RNA in *clr4Δ* cells. *tfs1Δ* and *ubp3Δ* caused only modest, partial reductions in cenR1 and cenR2 transcript levels, indicating that transcriptional restart accounts for only a subset of pericentromeric RNA production in *clr4Δ* cells. *seb1-1* did not significantly change cenR1 and cenR2 levels. Non-pausing transcription may persist in *seb1-1* cells, maintaining overall RNA levels. Despite minimal changes in RNA abundance, *tfs1Δ*, *ubp3Δ*, and *seb1-1* each reduced DNA-RNA hybrids to nearly wild-type levels. These observations demonstrated that elevated DNA-RNA hybrid formation in *clr4Δ* cells is not a simple consequence of increased transcription per se. Instead, they support a model in which the transcriptional pausing, backtracking, and restart (PBR) cycles promote DNA-RNA hybrid accumulation at pericentromeric repeats, thereby driving GCRs when heterochromatin is lost (Fig. I-11).

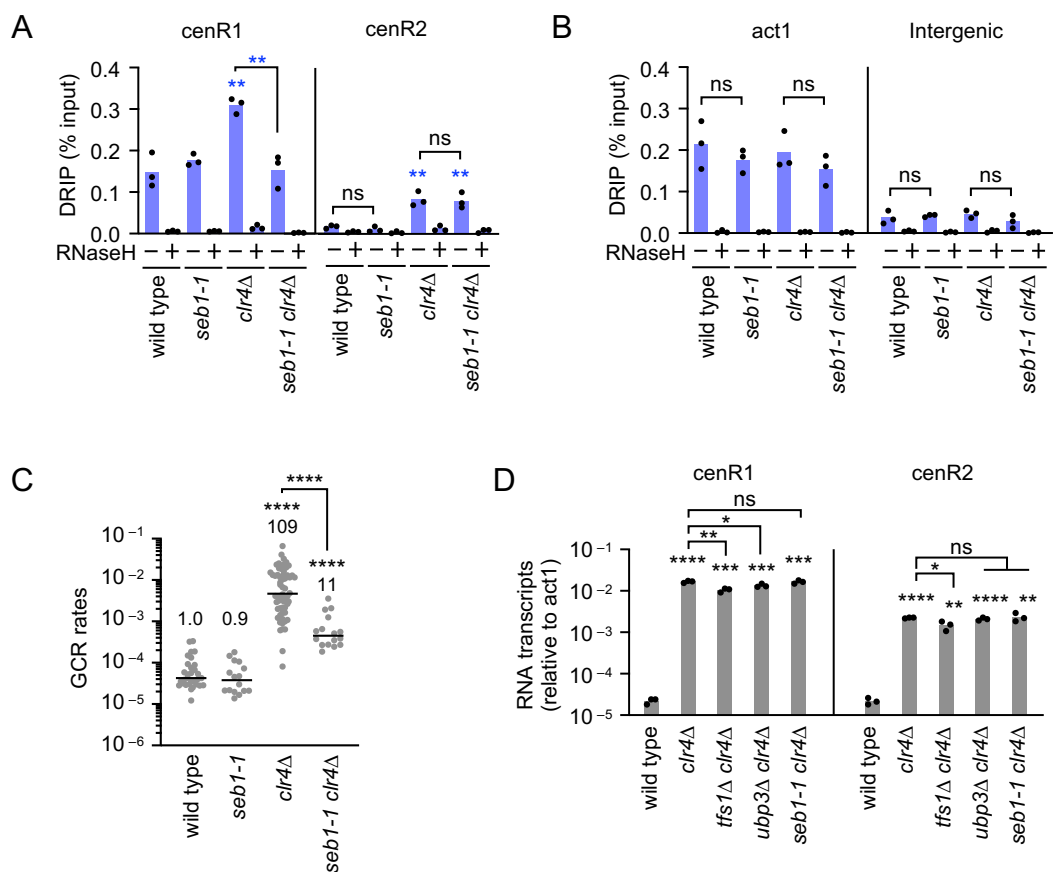


Fig. I-10 Seb1 promotes DNA-RNA hybrid accumulation and causes GCRs in *clr4Δ* cells.

(A-B) DNA-RNA hybrid levels at (A) cenR1 and cenR2 and (B) non-centromeric act1 and Intergenic sites in wild-type, *seb1-1*, *clr4Δ*, and *seb1-1 clr4Δ* strains. Each dot represents a biologically independent experiment ($n = 3$). Bars show the mean. (C) GCR rates in wild-type, *seb1-1*, *clr4Δ*, and *seb1-1 clr4Δ* strains. Each dot represents a biologically independent experiment. Lines show the median. GCR rates relative to wild type are shown at the top of each column. (D) RT-qPCR. RNA transcript levels of cenR1 and cenR2 relative to act1 are shown in wild-type, *clr4Δ*, *tfp1Δ clr4Δ*, *ubp3Δ clr4Δ*, and *seb1-1 clr4Δ* strains. Each dot represents a biologically independent experiment ($n = 3$). Bars show the mean.

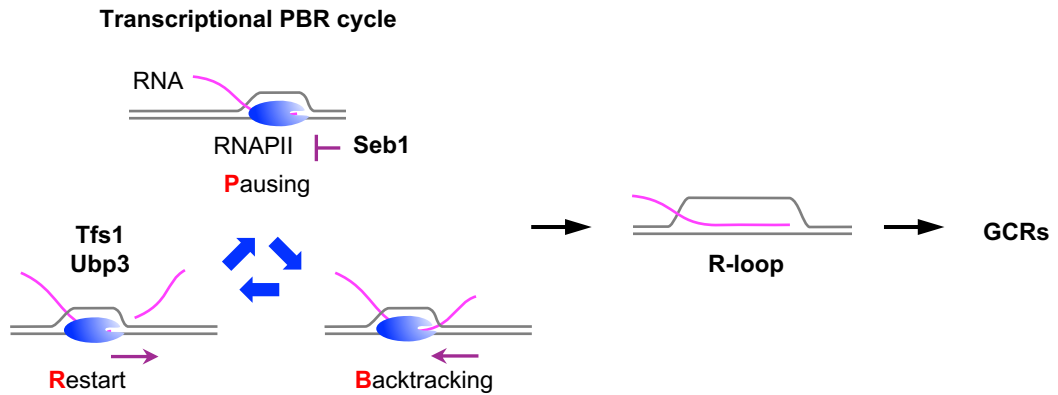


Fig. I-11 Model in which transcriptional PBR cycles causes genotoxic R-loops.

At pericentromeric repeats, Seb1 pauses and backtracks RNAPII for heterochromatin assembly. When heterochromatin is lost, Tfs1 and Ubp3 facilitate the restart of RNAPII, which is trapped by Seb1 again. Such transcriptional pausing, backtracking, and restart (PBR) cycles extend the half-life of DNA-RNA hybrids, forming the R-loops that cause GCRs in *clr4Δ* cells.

2.3. Discussion

In this part, I investigated how transcription dynamics causes GCRs at pericentromeres in the absence of heterochromatin. I demonstrate that loss of heterochromatin leads to accumulation of DNA-RNA hybrids at pericentromeric repeats, most likely in the form of R-loops composed of DNA-RNA hybrids and displaced ssDNA, and these R-loops promote GCR formation.

Remarkably, this study identifies a specific transcription mode, transcriptional PBR cycles, as a key driver of R-loop accumulation in the absence of heterochromatin (Fig. I-11). These findings establish a mechanistic framework connecting transcription dynamics and genome instability at centromeres.

Heterochromatin suppresses R-loop accumulation at pericentromeres

Heterochromatin, characterized by H3K9me_{2,3}, plays a conserved role in maintaining genome stability. In mice, knock out of Suv39h methyltransferase leads to chromosome aneuploidy and an increased risk of tumorigenesis (Peters et al. 2001). In *C. elegans*, loss of H3K9me_{2,3} leads to R-loop accumulation at heterochromatic repeat-rich regions and increased rates of repeat-specific insertion, deletion, and copy number variation (Zeller et al. 2016). Consistent with these observations, I show that loss of heterochromatin generates R-loops that cause GCRs at pericentromeric repeats in *S. pombe*. DRIP-seq data showed that *clr4Δ* cells accumulate R-loops at the pericentromeric cenR1 and cenR2 regions (Fig. I-3), and DRIP-qPCR confirmed that both *clr4Δ* and *rik1Δ* significantly increased R-loop levels at these loci (Fig. I-4). In contrast to *clr4Δ* and *rik1Δ*, deletion of *swi6*, in which substantial H3K9me_{2,3} and transcriptional silencing are maintained, did not elevate R-loop levels. Together, these results indicate that H3K9me_{2,3} and transcriptional silencing, rather than HP1 protein binding, are critical for suppressing R-loop accumulation at pericentromeres.

Notably, R-loop accumulation is not uniformly distributed across pericentromeric repeats but is instead enriched at specific sites in *clr4Δ* cells (Fig. I-3), suggesting that local DNA sequence features or chromatin context facilitate R-loop formation. The guanine-enriched sequences can promote R-loop formation by forming G-quadruplex (G4) structures on the displaced ssDNA (Duquette et al. 2004). However, I did not identify the canonical G4-forming motifs in the cenR1

and cenR2 surrounding regions, suggesting that G4 formation is unlikely to be the trigger of R-loop accumulation at these sites. Transcriptional pausing is known to promote R-loop accumulation (Petermann et al. 2022). A transcription pausing factor Seb1 is enriched at cenR1 but not at cenR2 (Parsa et al. 2018), and a DNA fragment containing cenR1 drives transcriptional silencing in a Seb1-dependent manner (Marina et al. 2013). These observations suggest that Seb1-dependent transcription pausing contributes to R-loop accumulation at cenR1. The L5 fragments containing cenR1 can nucleate heterochromatin at ectopic sites, consistent with the idea that transcription coupled to heterochromatin assembly is prone to R-loop accumulation. Nucleosome occupancy, which determines chromatin accessibility, can affect the R-loop accumulation (Castellano-Pozo et al. 2013). Chromatin surrounding the cenR2 regions contains fewer nucleosomes than other pericentromeric regions (Garcia et al. 2010), which may increase the accessibility of DNA template strand and thereby facilitate RNA rehybridization at cenR2.

Despite these findings, several important issues remain unsolved. Due to the resolution limitation of DRIP-seq, the precise DNA sequences and the structural features of these R-loops remain unclear. Recent studies showed that Dcr1, which cleaves dsRNA to produce siRNA, can directly bind and resolve R-loops (Camino et al. 2023; Wang et al. 2025). Further analysis of RNAi factors can help understand how heterochromatin regulates R-loops.

R-loop accumulation causes GCRs at centromeres

Several lines of evidence demonstrate that the accumulation of R-loops causes GCRs at centromeres. Loss of Clr4 or Rik1, but not Swi6, accumulated R-loops (Fig. I-4A and B), consistent with the fact that loss of Clr4 or Rik1, but not Swi6, increases isochromosome formation (Okita et al. 2019). Secondly, *rad51Δ* did not accumulate R-loops (Fig. I-4A and B), although *rad51Δ* increases isochromosome formation (Nakamura et al. 2008; Onaka et al. 2016). Thus, it is unlikely that R-loops are a byproduct of GCRs. Thirdly, overexpression of RNaseH1 reduced not only R-loops but also GCRs in *clr4Δ* cells (Fig. I-5). Previous work in our lab showed that loss of RNaseH1 and RNaseH2 increased isochromosome formation (Crystal Tang's Master's thesis). These data demonstrate that R-loop accumulation causes GCRs at centromeres.

Notably, RNaseH1 overexpression reduced R-loop levels in *clr4Δ* cells but not in wild-type cells (Fig. I-4A and B), showing that R-loops accumulated in *clr4Δ* cells are unique and hypersensitive to RNaseH1. RPA stably binds ssDNA ≥ 30 nt and facilitates RNaseH1-mediated R-loop disruption (Kim et al. 1992; Nguyen et al. 2017). It is possible that R-loops accumulated in *clr4Δ* contain longer ssDNA than those produced in wild-type cells. This idea is supported by the report showing that *clr4Δ* increases RPA localization at centromeres (He et al. 2022). Additionally, RNaseH1 preferentially digests R-loops containing RNA with N⁶-methyladenosine (m⁶A) modification (Zhang et al. 2024). Thus, it is also possible that R-loops accumulated in *clr4Δ* cells have such modifications.

Loss of Clr4 increased the GCR rate ~100 fold (Fig. I-5D), whereas DRIP-qPCR detected only a ~2-fold increase in R-loops (Fig. I-4A). This discrepancy is probably because the DRIP assay detects a mixture of physiological R-loops and genotoxic, GCR-prone R-loops. Therefore, the actual increase in GCR-prone R-loops in *clr4Δ* cells is likely far greater than 2-fold. Loss of Clr4 might accumulate R-loops that are longer, more stable, or structurally distinct. R-loop detection approaches with higher resolution, such as bisDRIP-seq, DRIPc-seq, or DRIP combined with proteomics, will help to distinguish these R-loops and quantify the genotoxic species elevated in *clr4Δ* cells.

Transcriptional PBR cycles form R-loops at pericentromeric repeats when heterochromatin is lost

Transcriptional PBR cycles appear to be the specific transcriptional process responsible for R-loop accumulation at pericentromeres. The R-loop accumulation in the absence of heterochromatin requires active transcription. *clr4Δ* and *rik1Δ* strongly induce *cenR1* and *cenR2* transcription and lead to R-loop accumulation (Fig. I-4A, B, and D), and treatment with the transcription inhibitor 1,10-phenanthroline reduces R-loop levels in *clr4Δ* cells (Fig. I-4E), indicating the essential role of transcription in R-loop formation. However, transcription level per se cannot account for the observed R-loop accumulation. Although *act1* transcripts are ~100-fold more abundant than *cenR1* transcripts in *clr4Δ* cells (Fig. I-4D), R-loop levels at *act1* and *cenR1* are comparable (Fig. I-4A and B). These data show that *cenR1* is prone to R-loop formation and suggest that additional transcription-associated factors promote R-loop

accumulation at this locus. Consistent with this idea, I found that Tfs1, Ubp3, and Seb1 are required for R-loop formation at cenR1 but not at act1 (Fig. I-7, 8, and 10). Notably, mutations in these factors did not greatly affect cenR1 RNA levels, suggesting that only certain steps of transcription are involved in R-loop formation. Mechanistically, Seb1 mediates RNAPII pausing at pericentromeres. Tfs1 and Ubp3 facilitate transcriptional restart following RNAPII pausing and backtracking. The cycles of transcriptional pausing, backtracking, and restart (PBR) may prolong the residence time of nascent RNA near its DNA template and repeatedly expose RNA and ssDNA through RNAPII pausing and restart, promoting the formation of R-loops. In the absence of heterochromatin, transcriptional PBR cycles become a dominant source of pathological R-loops at pericentromeric repeats.

Transcriptional PBR cycles may form R-loops in higher eukaryotes. As discussed in the introduction, in mammalian cells, transcription pausing is required for R-loop accumulation at the transcription promoter-proximal pausing site of protein-coding genes (Zhang et al. 2017). TFIIS localization has been detected at these regions (Sheridan et al. 2019). It is possible that TFIIS is involved in the R-loop formation. Comparing TFIIS occupancy with R-loop profiles will reveal whether TFIIS contributes to R-loop accumulation at promoter-proximal pausing sites in mammals.

A previous study reported that the dominant-negative TFIIS mutant, TFIIS-DEAA, increased R-loop levels (Zatreanu et al. 2019). This observation seems inconsistent with our finding that deletion of Tfs1/TFIIS or its conserved DE residues reduced R-loops. However, the distinct biochemical properties of these mutants may account for their opposing phenotypes. During transcriptional pausing and backtracking, RNAPII cleaves the extruded 3' RNA end to resume transcription elongation, and TFIIS promotes this process by stimulating RNAPII-mediated RNA cleavage (Kettenberger et al. 2003). In vitro assays of this RNAPII-TFIIS complex demonstrated that TFIIS-DEAA, the substitution of DE residues, not only abolishes the TFIIS-dependent cleavage activity but also inhibits the intrinsic transcript cleavage activity of RNAPII itself (Sigurdsson et al. 2010). As a result, TFIIS-DEAA results in a persistent stalled RNAPII. Such long-lived stalling RNAPII prolongs RNA exposure and favors R-loop formation. In contrast to TFIIS-DEAA, TFIIS deletion eliminates the stimulatory effect on cleavage but does not impair RNAPII's intrinsic cleavage activity (Sigurdsson et al. 2010). Under this condition, loss of TFIIS

leaves RNAPII to slowly resume transcription by its intrinsic, low-level cleavage activity. It is possible this slower transcription recovery provides time for cellular pathways to remove DNA-RNA hybrids. How these distinct RNAPII dynamics affect R-loop formation remains to be further investigated in the future.

3. Part II: Rad52-dependent annealing converts R-loops to ADR-loop

3.1. Introduction

3.1.1. Rad51-dependent HR maintains centromere integrity

DNA double-strand breaks (DSBs) represent one of the most deleterious forms of DNA damage and pose a severe threat to genome stability. Homologous recombination (HR) is a high-fidelity DSB repair mechanism and is essential for maintaining genome integrity. HR is initiated by resection of DSB ends, generating ssDNA. The Rad51 recombinase mediates the canonical HR pathway by binding to resected ssDNA to form a nucleoprotein filament. The Rad51 nucleoprotein filament searches for homologous duplex DNA, promotes DNA strand exchange, and initiates DNA repair through the formation of a displacement-loop (D-loop).

The importance of Rad51-dependent HR in genome maintenance is demonstrated by its relevance to human disease. Mutations in factors that promote Rad51-dependent HR, including BRCA1 and BRCA2, cause breast and ovarian cancers (Wooster et al. 1995). In addition, mutations in BRCA genes and Rad51 have been identified in patients with Fanconi anemia, a genetic disorder characterized by physical abnormalities, bone marrow failure, and increased risk of malignancy (Peake and Noguchi 2022).

Rad51 plays a critical role in maintaining centromere integrity. While the human centromere is a hotspot of DSBs, Rad51 is recruited to centromeres throughout the cell cycle and is critically important for preventing centromeric chromosome rearrangements (Yilmaz et al. 2021; Saayman et al. 2023). In fission yeast, loss of Rad51 greatly increased the spontaneous isochromosome formation at centromeres (Nakamura et al. 2008). When a DSB is artificially introduced adjacent to a centromere region, Rad51 also suppresses isochromosome formation (Tinline-Purvis et al. 2009). It is possible that the extensive degradation of DSB ends reaching a centromere repeat initiates isochromosome formation. The Mre11-Rad50-Nbs1 (MRN) complex binds to DSB ends and tethers broken ends to initiate DSB end resection. Supporting this idea, Rad51 paralogs Rad55 and Rad57, the MRN complex, and the exonuclease Exo1, which extends the end resection, suppress the DSB-induced isochromosome formation. In addition to isochromosome

formation, Rad51 suppresses chromosomal truncation with de novo telomere addition, both within and outside centromeres (Cullen et al. 2007; Onaka et al. 2016). Together, these findings establish Rad51-dependent HR as a protective pathway that suppresses centromere-associated genome instability and preserves chromosome integrity following DSBs.

3.1.2. Rad52-dependent SSA leads to GCRs at centromeres

Rad52 plays multiple roles in HR. In yeast, Rad52 is essential for Rad51-dependent HR, as it facilitates the loading of Rad51 onto ssDNA that is initially coated with the ssDNA-binding protein, RPA (Deveryshetty et al. 2025). This loading step is critical for the Rad51 nucleoprotein filament formation, which mediates strand invasion and D-loop formation. In mammalian cells, BRCA2, but not Rad52, mainly mediates Rad51 nucleoprotein filament formation (Feng et al. 2011). In addition to its Rad51-dependent role, Rad52 exhibits Rad51-independent recombination activities that are conserved from yeast to mammals. Rad52 promotes single-strand annealing (SSA) by catalyzing the annealing between complementary ssDNA (Liang et al. 2024). Moreover, Rad52 can also utilize single-stranded RNA (ssRNA) as a repair template (Mazina et al. 2017). Here, I refer to Rad51-independent but Rad52-dependent recombination as Rad52-dependent recombination for simplicity.

In chromosome arm regions, recombination between inverted DNA repeats can occur through either Rad51- or Rad52-dependent pathway. However, at a centromere in fission yeast, Rad52-dependent SSA is suppressed, probably because of the restricted lengths of single-stranded DNA formed during centromere replication (Zafar et al. 2017; Onaka et al. 2020). Genetic and biochemical analyses have demonstrated that Rad52 promotes centromeric isochromosome formation through its SSA activity (Onaka et al. 2020). Amino acid substitution of the conserved residue in the DNA-binding domain of Rad52, *rad52-R45K*, reduced Rad52-dependent SSA activity in vitro. The *rad52-R45K* did not affect Rad51 loading activity, as *rad52-R45K* did not increase GCR rate in the wild-type background. However, in the *rad51* deletion background, the *rad52-R45K* mutation greatly reduces isochromosomes but not chromosomal truncates, suggesting that Rad52-dependent SSA activity specifically causes centromeric GCR, resulting in isochromosome formation. Rad52-dependent SSA activity likely utilizes ssDNA produced during DNA replication as a substrate for annealing. Consistent with

this model, mutations in DNA replication elongation factors, including DNA Pol α , Pol ϵ , Swi1/Tof1/Timeless, and Pof3/Dia2/STIP1, induce Rad52-dependent centromere recombination and isochromosome formation. However, mutations in replication initiation factors such as Cdc18/Cdc6 do not affect the centromeric GCR rate. Overall, these studies demonstrate that Rad51-dependent HR acts as a protective pathway that suppresses centromeric genome instability, whereas Rad52-dependent SSA promotes centromere-associated GCRs and leads to isochromosome formation.

3.1.3. Break-induced replication and crossover cause isochromosome formation

After recombination intermediates between inverted repeats are formed, their downstream processing critically determines the nature of recombination products. Synthesis-dependent strand annealing (SDSA) mediated by Rad51 is a major pathway that maintains chromosome integrity. In this pathway, following D-loop formation and a short tract of DNA synthesis, the D-loop is dissociated, and the two DSB ends are reannealed, thereby preserving chromosome structure (Onaka et al. 2016). In contrast to SDSA, recombination intermediates can be processed through pathways that generate isochromosomes. Two major such pathways are crossover and break-induced replication (BIR). Crossover recombination causes the reciprocal or nonreciprocal exchange of chromosome regions. The Mus81-Eme1 endonuclease preferentially resolves joint molecules through crossover recombination (Boddy et al. 2001). In another pathway, DNA Pol δ promotes BIR, a conservative replication-based repair pathway that can copy extensive chromosomal regions from a homologous template (Lydeard et al. 2007). In fission yeast *rad51* Δ cells, Mus81 is required for the spontaneous formation of isochromosomes, indicating that isochromosomes are produced mainly through crossover recombination rather than BIR in *rad51* Δ cells (Onaka et al. 2016; Onaka et al. 2020). However, Cdc27/Pol32/PolD3, a subunit of Pol δ , is not essential for isochromosome formation in this context. When a DSB is introduced outside of the centromere region, extensive degradation of DSB ends can expose centromere repeats and trigger isochromosome formation (Tinline-Purvis et al. 2009). Under this condition, Cdc27 becomes essential for isochromosome formation, suggesting that BIR produces DSB-induced isochromosomes. What determines whether recombination intermediates are processed by crossover or BIR remains unclear. The structure of recombination intermediates might be a

key determinant. For instance, intermediates contain double Holliday junctions are preferentially resolved by structure-specific endonucleases such as Mus81-Eme1, leading to crossover products. In contrast, intermediates in which only one DSB end is engaged may favor a replication-based mode of repair, thereby promoting BIR.

Both crossover and BIR can occur following Rad52-dependent SSA. Rad52 can recruit both Mus81-Eme1 and PolD3 to common fragile sites (CFS) to facilitate mitotic DNA synthesis (MiDAS) in mammalian cells (Bhowmick et al. 2016). However, it remains unclear which downstream processing pathway is responsible for isochromosome formation when heterochromatin is lost.

3.1.4. R-loops facilitate recombination

Accumulating evidence indicates that R-loops are involved in HR. In Rad51-dependent HR, R-loops have been shown to enhance Rad51-mediated strand invasion (Ouyang et al. 2021). In vitro assays show that Rad51-ssDNA filaments preferentially invade DNA duplex adjacent to an R-loop, producing recombination intermediates that contain both DNA-DNA and DNA-RNA loops, referred to as DR-loop. R-loops can also initiate Rad51-independent recombination. At telomeres, Rad51AP1 and the telomeric repeat-containing RNA (TERRA) together form R-loops, which induce G-quadruplex formation on the displaced ssDNA strand (Yadav et al. 2022). This G-quadruplex stabilizes the DNA-RNA hybrid. Subsequent removal of RNA strand by RNaseH1 leaves an open DNA duplex that can provide a substrate for annealing. Together, these findings suggest that R-loops are the potential substrates for recombination during DNA damage repair.

3.2. Results

Rad52, but not Rad51, causes GCRs in *clr4Δ* cells

Loss of heterochromatin mainly leads to the formation of isochromosomes, which arise through recombination between centromere inverted repeats (Okita et al. 2019). In principle, such recombination might be induced by the two major recombination pathways in yeast: the Rad51-dependent HR and Rad52-dependent SSA. In the Rad51-dependent pathway, Rad51 binds ssDNA and facilitates DNA-strand exchange with the homologous duplex (Wright et al. 2018). R-loops can serve as the donor of Rad51-dependent strand exchange, forming the DR-loops (Fig. II-1A, Left) (Ouyang et al. 2021). In the Rad51-independent pathway, Rad52 promotes SSA between homologous sequences. The displaced ssDNA in R-loops might be captured by Rad52-dependent SSA, forming an Annealing-induced DNA-RNA loop (ADR-loop) (Fig. II-1A, Right). To determine which pathway is responsible for R-loop-induced GCRs in the absence of heterochromatin, I introduced the *rad51* or *rad52* mutation into yeast cells and determined the GCR rates (Fig. II-1B). *rad51Δ* increased GCR rates in both wild-type and *clr4Δ* cells, indicating that Rad51-dependent HR maintains centromere integrity even in the absence of heterochromatin. In contrast, the *rad52-R45K* mutation that specifically impairs SSA (Shi et al. 2009; Onaka et al. 2020) reduced GCR rates in *clr4Δ* cells, showing that Rad52-dependent SSA promotes GCR formation when heterochromatin is lost. Importantly, *rad52-R45K* and Rnh1 overexpression epistatically reduced GCRs in *clr4Δ* cells (Fig. II-1C), indicating that Rad52 and R-loops act in the same GCR pathway. Together, these data show that R-loops promote GCRs through Rad52-dependent SSA.

While the above data show that Rad52-dependent SSA is required for GCRs in *clr4Δ* cells, it remains unclear whether SSA activity is sufficient for GCR formation. Rad52 contains several functional domains, including DNA-binding, RPA-binding, Rad51-binding domains, and a nuclear localization sequence (NLS) (Fig. II-1D). The RPA-binding and Rad51-binding domains are dispensable for SSA (Hanamshet and Mazin 2020). Ectopic expression of the Rad52 full-length (Rad52-FL) and the truncates (Rad52-NM or Rad52-N) restored GCR rates to the same extent in *rad52-R45K clr4Δ* cells (Fig. II-1B), demonstrating that the RPA-binding and Rad51-binding domains of Rad52 are not required for GCRs. These results indicate that Rad52 SSA

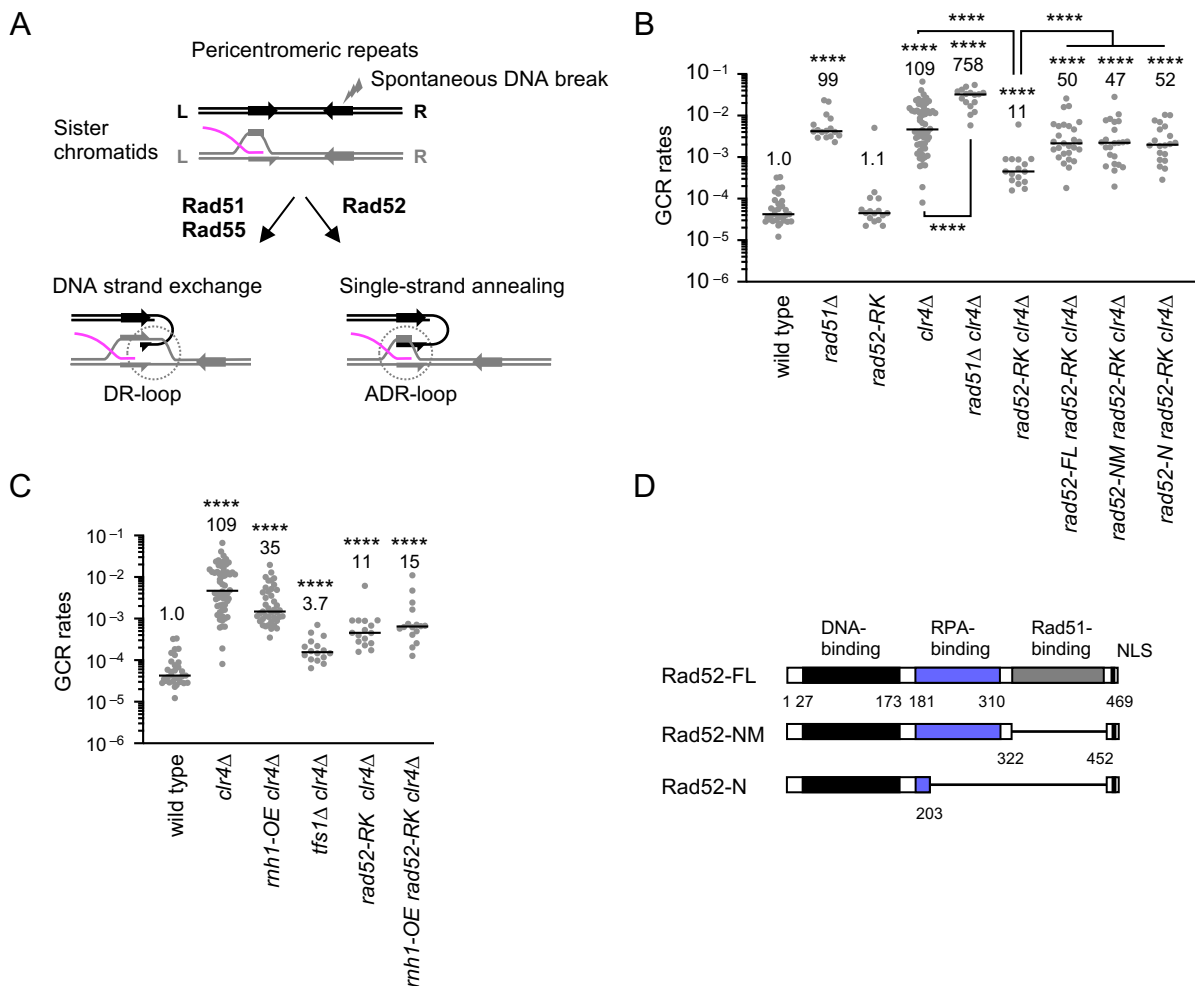


Fig. II-1 Rad52, but not Rad51, causes GCRs in *clr4*Δ cells.

(A) Homology-mediated recombination with R-loops. Rad51 promotes DNA strand exchange near, but not at, R-loops to form DR-loops. Rad52 promotes SSA with ssDNA region within R-loops to form ADR-loops. (B) GCR rates of wild-type, *rad51*Δ, *rad52-R45K*, *clr4*Δ, *rad51*Δ *clr4*Δ, *rad52-R45K clr4*Δ, *rad52-FL rad52-R45K clr4*Δ, *rad52-NM rad52-R45K clr4*Δ, and *rad52-N rad52-R45K clr4*Δ strains. Each dot represents a biologically independent experiment. Lines show the median. GCR rates relative to wild type are shown at the top of each column. (C) GCR rate of wild-type, *clr4*Δ, *mhl1-OE clr4*Δ, *tfs1*Δ *clr4*Δ, *rad52-R45K clr4*Δ, and *mhl1-OE rad52-R45K clr4*Δ strains. Each dot represents a biologically independent experiment. Lines show the median. GCR rates relative to wild type are shown at the top of each column. (D) Rad52-FL, Rad52-NM, and Rad52-N were expressed at an ectopic site on chr2.

activity is both necessary and sufficient for GCRs and that R-loops drive GCRs through the Rad51-independent but Rad52-dependent SSA pathway.

R-loops recruit Rad52 to pericentromeric repeats in *clr4Δ* cells

Multiple Rad52 proteins assemble at recombination sites, forming nuclear foci (Oshidari et al. 2020). To examine the effect of heterochromatin on Rad52 focus formation, I expressed Rad52-fmNeonGreen from the endogenous locus and detected Rad52 foci using fluorescence microscopy. *clr4Δ* increased the fraction of cells containing Rad52 foci (Fig. II-2A), showing that heterochromatin suppresses Rad52 focus formation. Because R-loops contain ssDNA, they may provide substrates for Rad52 binding at pericentromeric regions (Fig. II-2B). To determine whether Rad52 localizes to pericentromeres, I expressed Rad52-6His3Flag (Onaka et al. 2020) and performed ChIP using the anti-Flag antibody (Fig. II-2C). ChIP-qPCR showed that *clr4Δ* increased Rad52 localization at cenR1. Notably, Rnh1 overexpression, *tfs1Δ*, or *seb1-1* reduced the Rad52 localization at cenR1, showing that transcriptional PBR cycles and R-loops promote Rad52 localization at pericentromeres.

To test whether Rad52 affects DNA-RNA hybrids, I detected the DNA-RNA hybrid levels in *rad52-R45K* cells. DRIP-qPCR showed that *rad52-R45K* reduced DNA-RNA hybrids at cenR1 in *clr4Δ* cells (Fig. II-2D). Rad52 may play a direct role in annealing DNA-RNA hybrids (Mazina et al. 2017). It is also possible that the interaction between Rad52 and R-loops somehow stabilizes DNA-RNA hybrids. Together, these data demonstrate that R-loops recruit Rad52 to pericentromeric repeats, leading to GCRs in *clr4Δ* cells.

GCRs in the absence of heterochromatin occur through a pathway that is independent of Rad13 and Rnh26

How do Rad52 and R-loops cooperatively cause GCRs? In human ICF cells, which are deficient in heterochromatin formation, R-loops are accumulated at pericentromeric repeats and the XPG endonuclease cleaves R-loops to generate DNA breaks (Unoki et al. 2020; Shih et al. 2022). In

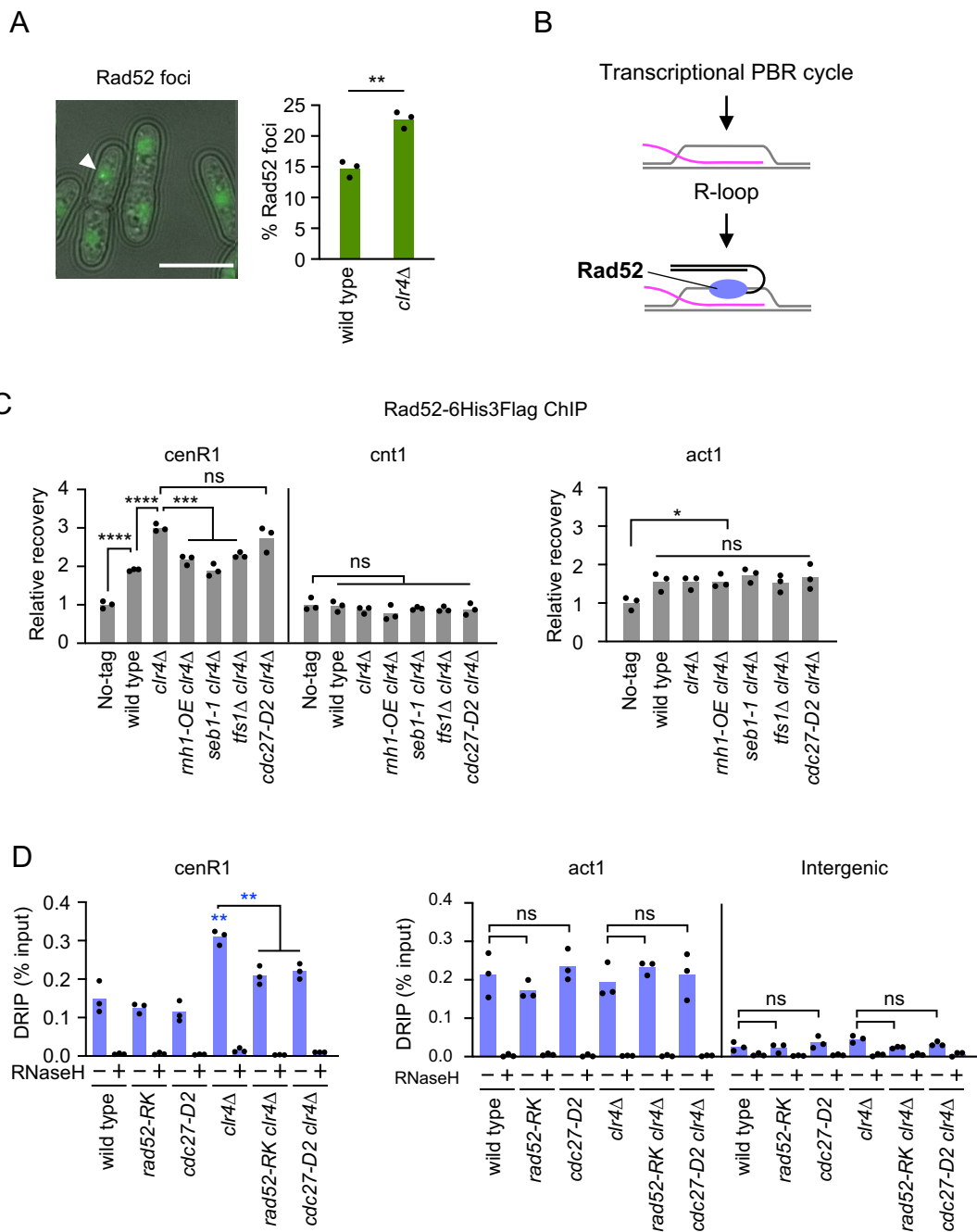


Fig. II-2 R-loops recruit Rad52 at pericentromeric repeats in *clr4Δ* cells.

(A) *Clr4* suppresses Rad52 focus formation. The image shows Rad52-fmNeonGreen foci in wild-type cells. Fluorescence and DIC images are overlaid. DIC, differential interference contrast. An arrow indicates Rad52 focus. A bar, 10 μ m. A bar graph shows the percentage of cells containing Rad52-fmNeonGreen foci. Each dot represents an independent experiment. Bars show the mean. (B) A model in which transcriptional PBR cycles form R-loops that recruit Rad52 proteins at pericentromeric repeats. (C) Rad52 localization at pericentromeric repeats. Rad52-6His3Flag ChIP experiments using no-tag strain and *rad52-6His3Flag* strains of wild-type, *clr4Δ*, *mh1-OE clr4Δ*, *seb1-1 clr4Δ*, *tfs1Δ clr4Δ*, and *cdc27-D2 clr4Δ*. The recovery relative to the no-tag control is shown. Each dot represents an independent experiment ($n = 3$). Bars show the mean. (D) Rad52 and Cdc27 stabilize DNA-RNA hybrids at pericentromeric repeats. DNA-RNA hybrid levels at *cenR1*, *act1*, and intergenic in wild-type, *rad52-R45K*, *cdc27-D2*, *clr4Δ*, *rad52-R45K clr4Δ*, and *cdc27-D2 clr4Δ* cells. Each dot represents an independent experiment ($n = 3$). Bars show the mean.

fission yeast, however, the XPG homolog Rad13 was dispensable for GCRs in *clr4Δ* cells (Fig. II-3), indicating that XPG-dependent DNA breaks are not the primary cause of GCRs.

Reactive oxygen species induce transcription-coupled HR (TC-HR) through R-loop formation in human cells, during which Cockayne syndrome protein B (CSB) binds R-loops and recruits Rad52 (Teng et al. 2018). However, the CSB homolog Rhp26 was dispensable for GCRs in *clr4Δ* cells (Fig. II-3), suggesting that R-loops and Rad52 at pericentromeric repeats cause GCRs in a manner different from TC-HR. These results suggest that Rad52-mediated GCRs occur through a pathway that is mechanistically distinct from known R-loop processing pathways in human cells.

Rad52-ssDNA complex and R-loop form ADR-loop in vitro

To directly test the model that Rad52 facilitates ADR-loop formation, I performed an in vitro assay. I expressed Rad52-6His3Flag protein in *E. coli* and purified it using TALON metal affinity resin that targets His-tagged protein (Fig. II-4A). The 90-nt D1 and D2 oligos are complementary, except for the central 30-nt region. I prepared R-loops using 30-nt R1 RNA that is complementary to the central region of D2. To compare R-loops with other structures, I prepared five distinct nucleic acid substrates (Fig. II-4B). In D-loops, 30-nt D3 DNA replaces the R1 RNA. All substrates contain the D1 oligo, whose central region is complementary to a 30-nt C1 ssDNA probe. The construction of substrates was confirmed by agarose gel electrophoresis (Fig. II-4C). Rad52 protein was incubated with ³²P-labeled C1 ssDNA at 30°C for 10 min, and the reaction was initiated by the addition of the substrate (Fig. II-4D). At the indicated period after adding substrates, aliquots were withdrawn from the reaction, deproteinized, and applied to non-denaturing polyacrylamide gel electrophoresis (PAGE). During the reaction, R1 RNA and D3 DNA remained bound to D2 oligo in R-loops and D-loops, respectively (Fig. II-4E). A phosphorimager showed that 20% of C1 ssDNA bound R-loops to form ADR-loops in the first 30 sec, increasing to 30% by 120 sec (Fig. II-4F). Both R-loops and D-loops served as more efficient substrates compared to bubble structures, though less efficient than D1 ssDNA. Importantly, no annealing products were formed using dsDNA, indicating that Rad52 does not catalyze strand exchange reaction under this condition. I further confirmed that ADR-loop

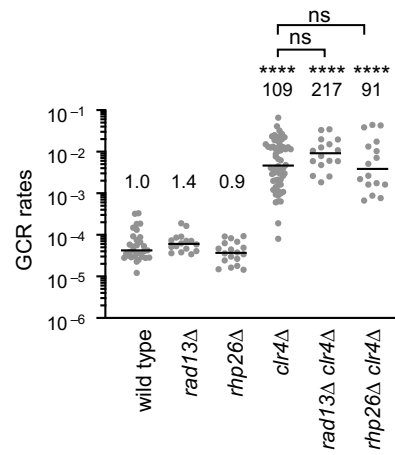


Fig. II-3 Rhp26 and Rad13 are not involved in GCRs in *clr4* Δ cells.

GCR rates of wild-type, *rad13* Δ , *rhp26* Δ , *clr4* Δ , *rad13* Δ *clr4* Δ , and *rhp26* Δ *clr4* Δ strains. Each dot represents a biologically independent experiment. Lines show the median. GCR rates relative to wild type are shown at the top of each column.

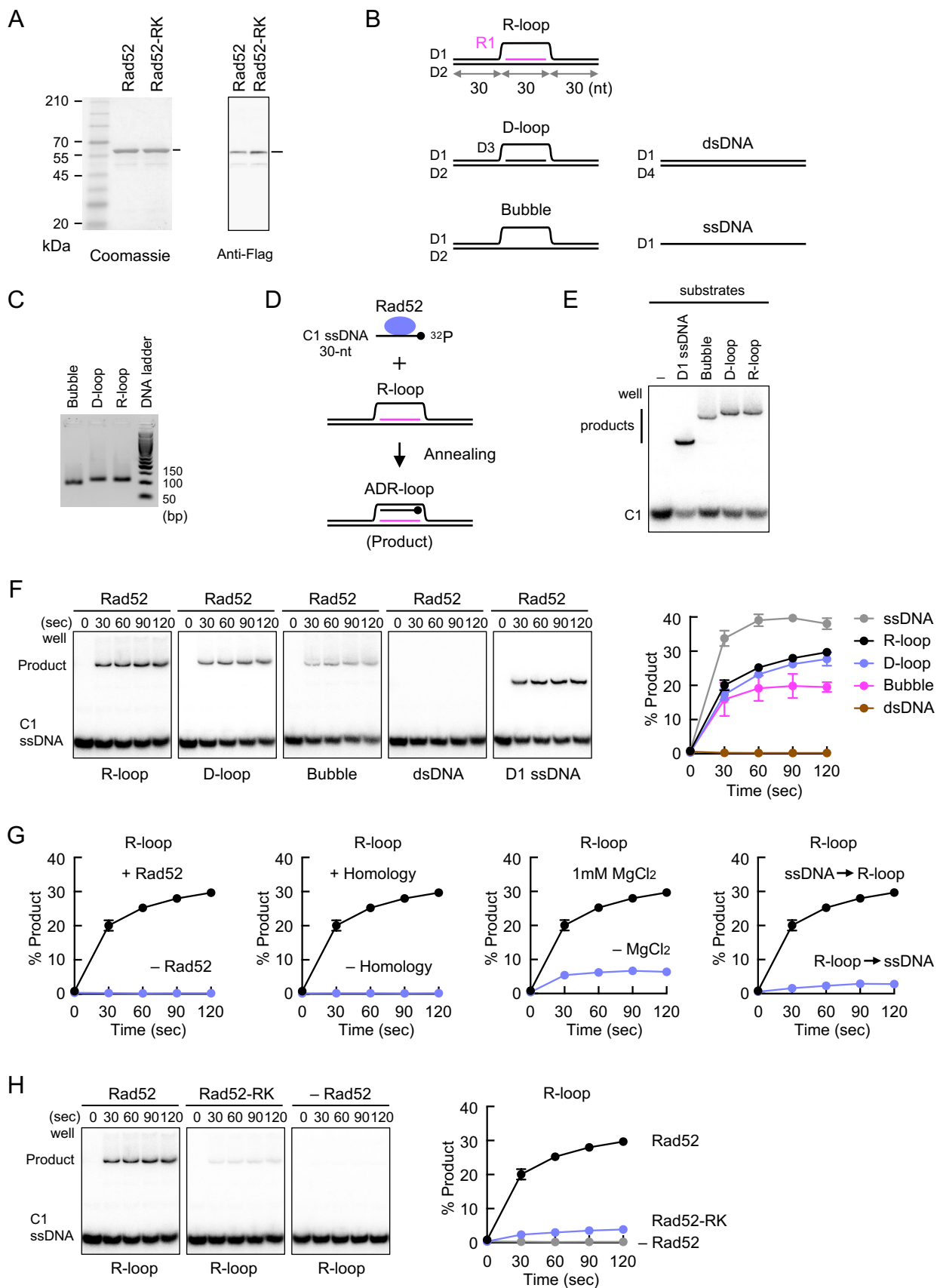


Fig. II-4 Rad52 protein converts R-loops into ADR-loops.

(A) Purified wild-type Rad52 and mutant Rad52-R45K proteins were separated by 10% SDS-PAGE and stained with Coomassie brilliant blue. Sizes of CLEARLY Stained Protein Ladder (Takara) are indicated on the left of the panel. (B) Illustrated are the substrates used in this assay. (C) Bubble, D-loop, and R-loop were separated by 3% agarose gel (Nacalai Tesque, 01153-22) in $1 \times$ TBE buffer and stained with EtBr. Sizes of the 50-bp DNA ladder are indicated on the right of the panel. (D) Schematic of the reaction. Rad52 was pre-incubated with C1 ssDNA labeled with ^{32}P at the 5'-end (a black circle). The reaction was initiated by adding substrates, such as the R-loop. The reaction mixture contains 0.3 nM of C1, 0.3 nM of substrates, and 1.35 nM of Rad52. (E) Rad52 promotes annealing between C1 and D1 ssDNA, Bubble, D-loop, or R-loop. The annealing products of Bubble showed higher mobility than R-loop and D-loop. The reaction mixture contains 0.3 nM of C1, 0.3 nM of the substrate, and 1.35 nM of Rad52. (F) The reaction product was separated by 8% non-denaturing PAGE in $1 \times$ TBE buffer. Radiation signals were detected using a phosphorimager FLA7000. Percentages of annealing products over time are shown in the graph. Mean $\pm$ SD of 3 independent experiments. (G) The ADR-loop formation under different conditions. In "– Homology", instead of C1, nC1 ssDNA that is not complementary to D1 was used. In "R-loop $\rightarrow$ ssDNA", Rad52 was pre-incubated with R-loops before adding C1 ssDNA. (H) Rad52-R45K hardly promotes ADR-loop formation.

formation requires Rad52 protein, indicating that ADR-loop formation does not occur spontaneously (Fig. II-4G). Sequence homology between C1 ssDNA and R-loop is essential for ADR-loop formation, demonstrating that ADR-loops are generated by homologous pairing. The presence of 1 mM MgCl₂ stimulated ADR-loop formation, probably by promoting Rad52 DNA-binding activity (Liang et al. 2024). Mixing Rad52 and R-loops before adding C1 ssDNA resulted in only marginal levels of ADR-loops, indicating that Rad52 initially associates with ssDNA rather than with R-loops during ADR-loop formation. Importantly, the Rad52-R45K mutant protein exhibited a 10-fold reduction in ADR-loop formation compared to wild-type Rad52 in the first 30 sec (Fig. II-4H). These biochemical results collectively indicate that the Rad52-ssDNA complex interacts with R-loops and forms ADR-loops.

Rad52 directly promotes DNA-RNA hybrid formation

Because the *rad52-R45K* mutation reduced DNA-RNA hybrid levels in *clr4Δ* cells, I examined whether Rad52 can directly promote annealing between DNA and RNA strands. Purified Rad52 or Rad52-R45K protein was incubated with ³²P-labeled C1 ssDNA at 30°C for 10 min, and the reaction was initiated by adding a complementary 30-nt ssDNA or ssRNA strand (Fig. II-5). Non-denaturing PAGE showed that dsDNAs formation occurred even in the absence of Rad52 protein, indicating spontaneous DNA-DNA annealing. Importantly, Rad52, but not Rad52-R45K, greatly enhanced dsDNA formation (Fig. II-5A), consistent with previous observations (Onaka et al. 2020). In contrast, DNA-RNA annealing did not occur spontaneously. However, the addition of Rad52 promoted the formation of DNA-RNA products, reaching approximately 15% of total C1 ssDNA substrate, whereas Rad52-R45K fails to do so (Fig. II-5B). Together, these results demonstrate that Rad52 can directly catalyze DNA-RNA hybrid formation, and that this activity depends on its DNA-binding domain.

Rad51 does not promote ADR-loop formation

rad51Δ did not reduce but increased GCRs in *clr4Δ* cells, indicating that Rad51 is not involved in GCR formation when heterochromatin is lost. Moreover, a member in our laboratory performed Rad51-6His3Flag ChIP assay and showed that loss of Clr4 did not alter Rad51

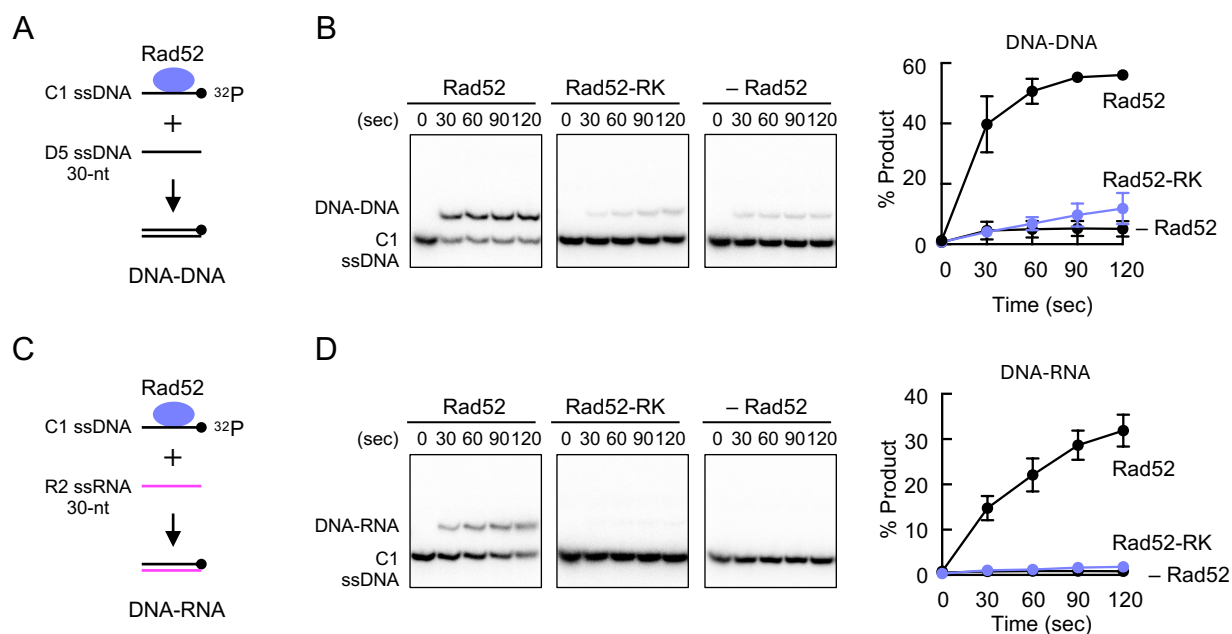


Fig. II-5 Rad52 promotes single-strand annealing in vitro.

(A) Rad52-dependent DNA-DNA annealing. Rad52 was pre-incubated with C1 labeled with ^{32}P at the 5'-end. The reaction was initiated by adding D5 ssDNA. The reaction mixture contains 0.3 nM of C1, 0.3 nM of D5 ssDNA molecules, and 1.35 nM of Rad52. The reaction product was applied to 12% non-denaturing PAGE in $1 \times \text{TBE}$ buffer. Radiation signals were detected using a phosphorimager FLA7000. (B) The gel image. Percentages of annealing products over time are shown in the graph. Mean $\pm$ SD of 3 independent experiments. (C) Rad52-dependent DNA-RNA annealing. The reaction mixture contains 0.3 nM of C1, 0.3 nM of R2 ssRNA molecules, and 1.35 nM of Rad52. (D) The gel image. Percentages of annealing products over time are shown in the graph.

localization at pericentromeric repeats. These results suggest that R-loops accumulated in *clr4Δ* cells do not interact with Rad51 protein. It is possible that Rad51 does not have the biochemical activity to promote ADR-loop formation. To address this, I performed ADR-loop assays using purified Rad51 protein (Fig. II-6). The Rad51 protein, provided by the Iwasaki laboratory, was previously used in an in vitro strand exchange assay (Tsubouchi et al. 2020). Each Rad51 monomer binds approximately three nucleotides to form a nucleoprotein filament (Subramanyam et al. 2018). In this assay, 4.5 nM Rad51 protein was preincubated with 0.3 nM ³²P-labeled 30-nt ssDNA (in moles of molecule) for 10 min, followed by the addition of 0.3 nM R-loop substrates to initiate the reaction. Rad51 hydrolyzes ATP to drive the strand exchange reaction. However, ADR-loop formation was not promoted by Rad51 regardless of ATP addition (Fig. II-6A). Like R-loop, D-loop is not annealed with the C1 ssDNA by Rad51 protein (Fig. II-6B). However, Rad51 appeared to weakly promote annealing between C1 ssDNA and a 90-nt D1 ssDNA (Fig. II-6C). A scaled-up graph confirms that, compared with the mock reaction, addition of Rad51 protein in the absence of ATP modestly increased dsDNA formation (Fig. II-6D), although its activity was much weaker than that of Rad52. Collectively, these results indicate that Rad51 does not catalyze ADR-loop formation.

DNA Pol δ causes GCRs in *clr4Δ* cells

After ADR-loops are formed between inverted repeats, crossover or BIR might form isochromosomes. Mus81 endonuclease promotes crossover rather than non-crossover recombination (Fig. II-7A, Left) (Boddy et al. 2001), whereas DNA Pol δ plays an essential role in BIR (Fig. II-7A, Right) (Lydeard et al. 2007). In *rad51Δ* cells, Mus81, but not a Pol δ subunit Cdc27/Pol32/PolD3, is required for the spontaneous formation of isochromosomes (Onaka et al. 2016). However, in *clr4Δ* cells, the *cdc27* mutation (Tanaka et al. 2004) but not *mus81Δ* reduced GCR rates (Fig. II-7B). Pol δ is also essential for chromosomal replication. However, *cdc27-D2* cells do not exhibit temperature-sensitive growth defects (Tanaka et al. 2004), showing no severe defects in chromosomal replication. The CMG helicase containing Cdc45, Mcm2-7, and GINS is involved in chromosomal replication (Pellegrini 2023). The *cdc45-928* mutation, which partially impairs DNA replication even at the same temperature 30°C (Nitani et al. 2006), did not reduce GCR rates in *clr4Δ* cells (Fig. II-7B). These results show that the Cdc27 function in BIR rather

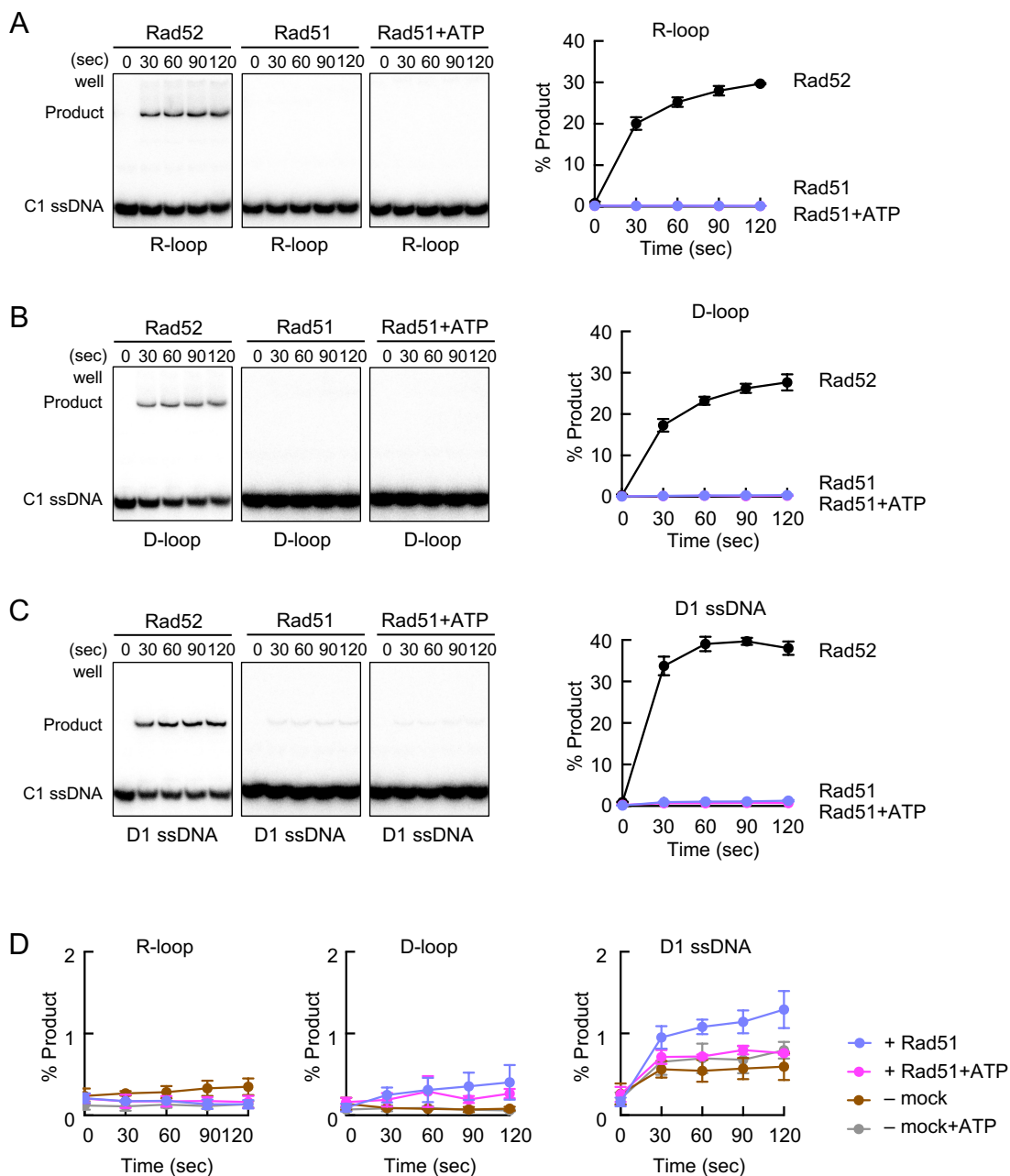


Fig. II-6 Comparison between Rad52 and Rad51 in DNA annealing.

(A) The ADR-loop formation assay using Rad51 instead of Rad52. Reactions were performed using 4.5 nM Rad51 in the presence or absence of 1 mM ATP. Percentages of annealing products over time are shown in the graphs. Mean $\pm$ SD of 3 independent experiments. (B) DNA annealing between C1 ssDNA and D-loop. (C) DNA annealing between C1 and D1 ssDNAs. (D) Scaled-up graphs showing percentage of Rad51-dependent annealing products.

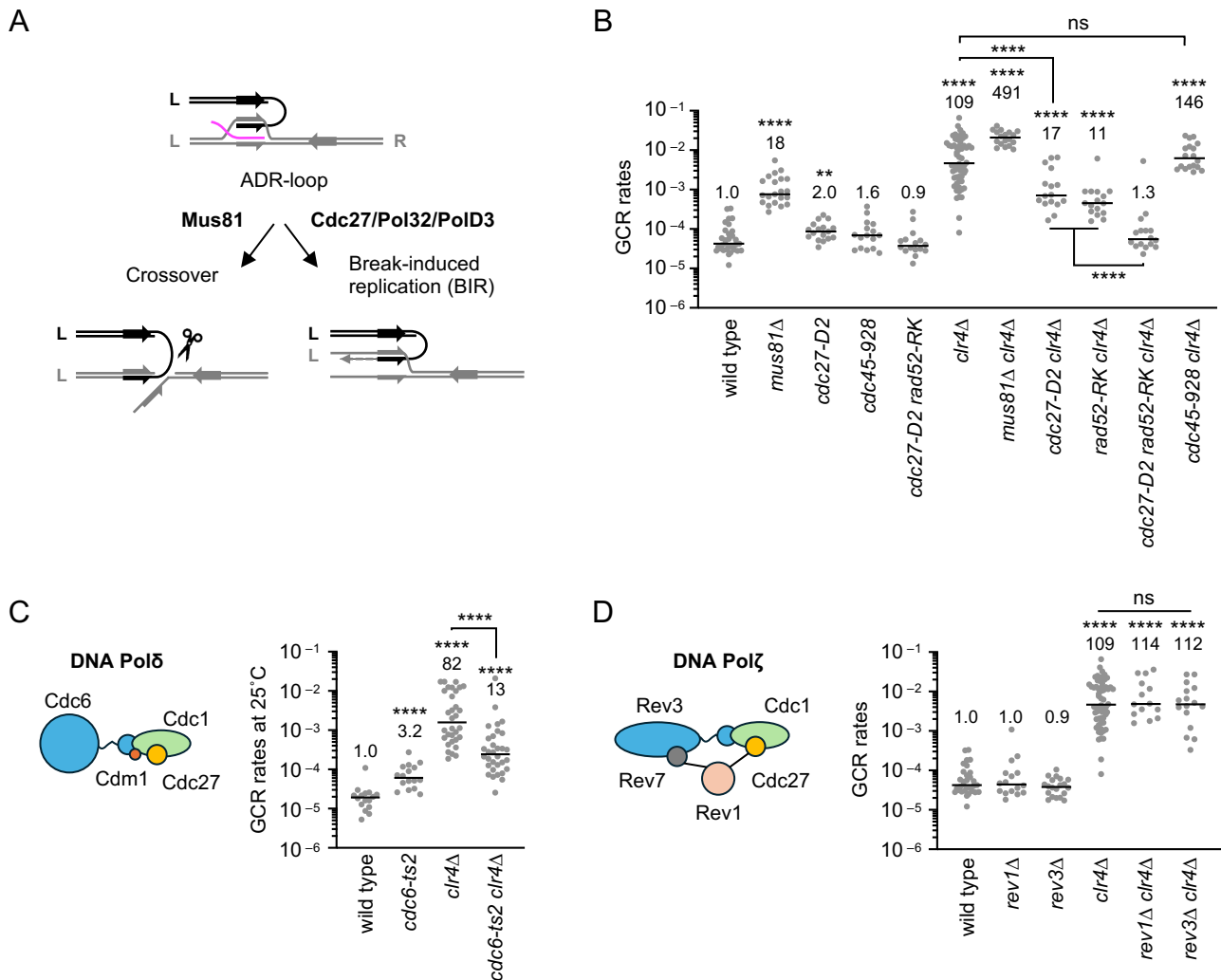


Fig. II-7 DNA Pol δ causes GCRs in *clr4*Δ cells.

(A) Following ADR-loop formation, crossover or BIR between pericentromeric inverted repeats can form isochromosomes. L, left; R, right. (B) Cdc27 is required for GCRs in *clr4*Δ cells. GCR rates of wild-type, *mus81*Δ, *cdc27-D2*, *cdc45-928*, *cdc27-D2 rad52-R45K*, *clr4*Δ, *mus81*Δ *clr4*Δ, *cdc27-D2 clr4*Δ, *rad52-R45K clr4*Δ, *cdc27-D2 rad52-R45K clr4*Δ, and *cdc45-928 clr4*Δ strains. (C) Cdc6, the catalytic subunit of Polδ, is required for GCRs in *clr4*Δ cells. GCR rates of wild-type, *cdc6-ts2*, *clr4*Δ, and *cdc6-ts2 clr4*Δ strains at 25°C. (D) Rev1 and Rev7, subunits of Polζ, are dispensable for GCRs. GCR rates of wild-type, *rev1*Δ, *rev3*Δ, *clr4*Δ, *rev1*Δ *clr4*Δ, and *rev3*Δ *clr4*Δ strains. Each dot represents a biologically independent experiment. Lines show the median. GCR rates relative to wild type are shown at the top of each column.

than chromosomal replication promotes GCRs. *cdc27-D2* did not significantly change Rad52 localization at centromeres, showing that Cdc27 acts downstream of Rad52 (Fig. II-2C). However, *cdc27-D2* reduced DNA-RNA hybrids in *clr4Δ* cells (Fig. II-2B). In addition, *rad52-R45K* and *cdc27-D2* synergistically reduced GCR rates to the wild-type level (Fig. II-7B). These data suggest that Rad52 and Cdc27 cooperatively form stable ADR-loops to cause GCRs.

Cdc27 is a common subunit of DNA polymerases Pol δ and Pol ζ . Pol ζ promotes translesion synthesis and microhomology-mediated BIR (Makarova et al. 2012; Sakofsky et al. 2015). Our previous lab member showed that the *cdc6-ts2* mutation (Francesconi et al. 1993) in a Pol δ -specific subunit Cdc6/PolD1 reduced GCR rates in *clr4Δ* cells at a semipermissive temperature of 25°C (Fig. II-7C). However, loss of Pol ζ -specific subunits Rev1 or Rev3 did not significantly change GCR rates (Fig. II-7D). These results show that Pol δ -dependent BIR, but not Pol ζ , promotes GCRs in the absence of heterochromatin.

3.3. Discussion

In this part, I elucidated how R-loops promote GCRs at pericentromeric repeats in the absence of heterochromatin. Genetic and biochemical analyses collectively support a model in which Rad52-dependent SSA, but not Rad51-dependent HR, initiates recombination using the R-loop substrate in *clr4Δ* cells. Furthermore, I show that Rad52 converts R-loops into a four-stranded recombination intermediate, termed ADR-loop, which is subsequently processed by DNA polymerase δ -dependent BIR to generate isochromosomes (Fig. II-8).

Rad52, but not Rad51, catalyzes R-loop-dependent recombination in *clr4Δ* cells

Homology-mediated DNA repair is generally initiated by end resection of DSB, which generates ssDNA as a substrate for recombination. In canonical HR, Rad51 assembles on resected ssDNA and promotes strand invasion into homologous duplex DNA, whereas Rad52 primarily catalyzes annealing reactions between complementary single-stranded nucleic acids. In *clr4Δ* cells, *rad52-R45K*, which specifically impairs Rad52 SSA, but not *rad51Δ*, reduced GCRs, indicating that Rad52-dependent SSA is the dominant pathway driving GCR formation in the absence of heterochromatin (Fig. II-1).

Several lines of evidence suggest that R-loops serve as substrates for Rad52-dependent SSA at pericentromeric repeats. Rad52 ChIP assay revealed increased Rad52 enrichment at pericentromeric repeats in *clr4Δ* cells. This enrichment was reduced by RNaseH1 overexpression, *seb1-1*, and *tfs1Δ*, indicating that R-loops contribute to Rad52 recruitment (Fig. II-2). R-loops contain the displaced ssDNA. However, in vitro reconstitution assays demonstrated that Rad52 does not directly interact with ssDNA within a R-loop (Fig. II-4G). Instead, Rad52 forms complex with a free ssDNA, and Rad52-ssDNA complex forms ADR-loop with the R-loop (Fig. II-4). Together, these results support a model in which R-loops act as recombinogenic platforms that preferentially engage Rad52-dependent SSA in heterochromatin-deficient cells.

Although these data establish Rad52 as the primary recombinase acting on R-loops in *clr4Δ* cells, the molecular basis for Rad52 selectivity over Rad51 remains incompletely understood.

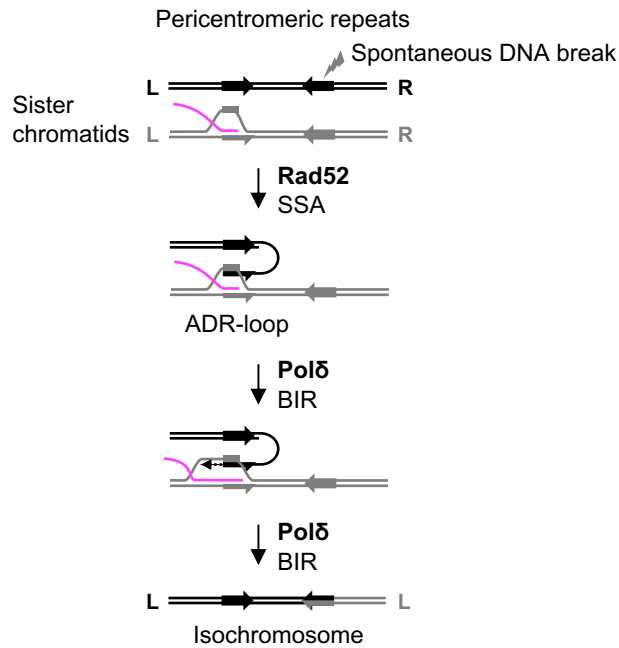


Fig. II-8 Model in which ADR-loop formation followed by Polδ-mediated BIR cause isochromosome formation.

Rad52 binds to ssDNA generated by resection of spontaneous double-strand break ends. R-loops formed at pericentromeric repeats provide a substrate for Rad52 binding, enabling the formation of ADR-loops that join inverted pericentromeric repeats. Subsequently, DNA Polδ catalyzes nascent DNA synthesis through break-induced replication (BIR), leading to isochromosome formation.

Rad52-dependent annealing is ATP-free and relatively faster than Rad51-dependent strand exchange, which may favor Rad52 engagement at transient R-loops. In addition, accessory factors, such as Rad59 (Feng et al. 2007), may modulate Rad52 activity or influence joint molecule formation. Further studies will be required to determine how R-loop sequence, topology, and associated proteins affect Rad52-dependent ADR-loop formation in vivo.

R-loops act as recombination-prone intermediates rather than sources of DNA damage

R-loops have been viewed as pathological structures that promote genome instability by inducing replication stress or DNA breaks. However, accumulating evidence suggests that R-loops can also function as direct recombination substrates. In fission yeast *clr4Δ* cells, disruption of the XPG endonuclease homolog Rad13 did not suppress GCR formation (Fig. II-3), against a model in which R-loop cleavage-induced DNA breaks are the primary drivers of genome instability. Partial impairment of DNA replication by the *cdc45-928* mutation failed to reduce GCR rates in *clr4Δ* cells (Fig. II-7B), suggesting that replication stress is not involved in GCR formation in this context. Instead, the data support a model in which R-loops act as recombination-prone intermediates. Consistent with this idea, Rad52 directly catalyzes annealing between ssDNA and R-loops in vitro, forming stable ADR-loop structures.

Rad52-dependent ADR-loop formation stabilizes recombination intermediates

The results presented here suggest that Rad52 converts R-loops into a more stable recombination intermediate, the ADR-loop. Rad52-R45K protein cannot convert R-loop into ADR-loop. Consistently, *rad52-R45K* reduced R-loop levels in *clr4Δ* cells, suggesting that the ADR-loop is a more stable structure than a R-loop. This interpretation is also consistent with previous biochemical studies showing that four-stranded DNA joint molecules, double D-loops, are more stable than three-stranded D-loops (Bugreev et al. 2007).

Notably, ADR-loop formation shows conceptual similarity to second-end capture during homologous recombination, in which a Rad52-ssDNA complex anneals to a displaced strand of a D-loop (Nimonkar et al. 2009). In the context of R-loops, however, Rad52-mediated annealing may occur at ectopic non-allelic sites, thereby promoting aberrant recombination between

inverted centromeric repeats. Rad52 also directly catalyzes DNA-RNA annealing, although it is less efficient than DNA-DNA annealing activity (Fig. II-5), raising the possibility that Rad52 directly contributes to formation of DNA-RNA hybrids. While biochemical assays support ADR-loop formation, the in vivo architecture of this intermediate remains unsolved, and direct visualization of ADR-loops in cells will be an important goal for future studies.

DNA Pol δ promotes extension and processing of ADR-loops

Genetic analyses indicate that DNA Pol δ , rather than Mus81-dependent crossover resolution, is required for GCR formation in *clr4 Δ* cells (Fig. II-7). The observation that *cdc27-D2* did not affect Rad52 localization suggests that Rad52 acts upstream of Pol δ during GCR formation. In human cells, Rad52 physically interacts with PolD3 (Tan et al. 2020), raising the possibility that Rad52 recruits Pol δ to ADR-loops to initiate DNA synthesis on the DNA strand opposite the DNA-RNA hybrid. Interestingly, *cdc27-D2* reduced R-loop levels in *clr4 Δ* cells. During BIR, DNA synthesis requires unwinding of the DNA duplex, which may generate additional single-stranded template DNA that can be captured by RNA, thereby promoting further extension of ADR-loops (Fig. II-8).

An alternative, but not completely exclusive, possibility is that Pol δ contributes to annealing between ssDNA and R-loop, creating ADR-loops. In *clr4 Δ* cells, *rad52-R45K* did not reduce GCR rates to the wild-type level (Fig. II-1B), suggesting that the initial step of GCR formation can occur through a Rad52-independent pathway. *cdc27-D2* and *rad52-R45K* synergistically reduced GCRs in *clr4 Δ* cells (Fig. II-7B), suggesting that Cdc27 and Rad52 may play redundant roles during the initiation stage of recombination. This may also explain why both *rad52-R45K* and *cdc27-D2* reduced R-loop levels in *clr4 Δ* cells. In human cells, Pol θ has the annealing activity that initiates recombination and is the key factor driving microhomology-mediated end joining (MMEJ) (Wyatt et al. 2016). Although MMEJ is evolutionarily conserved in yeast, Pol θ is absent in yeast (Sfeir and Symington 2015). Instead, Pol δ has been implicated in MMEJ in yeast. Whether yeast Pol δ has the intrinsic annealing biochemical activity needs to be further investigated.

Pol δ -dependent BIR creates isochromosomes following ADR-loop formation

In principle, both crossover recombination and BIR can form isochromosomes (Nakagawa and Okita 2019). Previous studies have shown that Mus81-mediated crossover recombination is the major pathway for spontaneous isochromosome formation in *rad51* Δ cells (Onaka et al. 2016). However, this study indicates that, in *clr4* Δ cells, isochromosome formation proceeds through Pol δ -dependent BIR rather than crossover recombination. One possible explanation for this difference is that distinct recombination intermediates favor different downstream processing pathway. The ADR-loops shares the structure features with a replication fork, including an RNA primer for lagging-strand synthesis and a nascent leading-strand. Such a fork-like architecture may render ADR-loop competent for initiating Pol δ -dependent BIR.

When ADR-loops form between non-allelic homologous sequences, Pol δ -dependent BIR would be expected to generate large-scale, homology-mediated GCR products, such as isochromosomes. However, whether ADR-loop can directly serve as the substrate for BIR initiation has not yet been tested. Future approaches, such as nascent DNA sequencing with thymidine analog EdU, or reconstitution of ADR-loop dependent BIR in vitro, would provide critical insights into the molecular details of this mechanism.

Conclusion

Overall, this study establishes the framework for transcription-induced GCRs at pericentromeric repeats when heterochromatin is compromised (Fig. III). I showed that transcription in the absence of heterochromatin causes the accumulation of R-loops, and that these R-loops are generated specifically through transcriptional PBR cycles. Rad52 interacts with R-loops and converts them to ADR-loops, thereby bridging the inverted pericentromeric repeats and promoting recombination that results in GCRs.

Repetitive sequences, including centromeric repeats and transposable elements, constitute approximately half of the human genome, and chromosomal translocation occurs through non-allelic recombination between repetitive elements (Pascarella et al. 2022). Most repetitive sequences are transcriptionally silenced by heterochromatin in human cells (Bickmore and van Steensel 2013; Haws et al. 2022). The PBR cycle and the ADR-loop formation might cause genome instability in human cells when epigenetic transcription silencing is compromised, such as during ageing (Zeller et al. 2016; Wu et al. 2024).

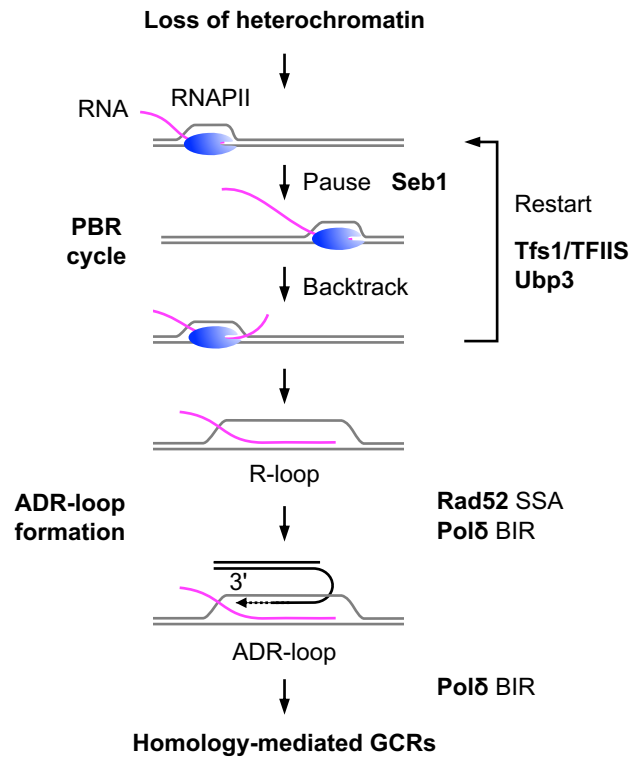


Fig. III Model in which transcriptional PBR cycles form R-loops and Rad52 converts R-loops into ADR-loops, resulting in homology-mediated GCRs.

Seb1 causes transcription pausing at pericentromeric repeats. Tfs1/TFIIS and Ubp3 promote transcriptional restart. Rad52 forms ADR-loops from R-loops and ssDNA, bridging a pair of pericentromeric inverted repeats. Polδ initiates BIR at the ADR loop, resulting in the isochromosome formation.

Material and methods

Yeast media

The fission yeast, *S. pombe*, was grown in Yeast Extract (YE) medium, Edinburgh Minimal Medium (EMM), Yeast Nitrogen Base (YNB), 5-Fluoroorotic Acid (5FOA), or Malt Extract (ME) medium supplemented with amino acids or bases at a final concentration of 225 mg/L unless otherwise indicated (Forsburg and Rhind 2006). The yeast cells were grown at 30°C unless otherwise indicated. YE medium contained 5 g/L yeast extract (Nacalai Tesque, 15838-45) and 30 g/L glucose (Nacalai Tesque, 16805-64). EMM medium contained 5 g/L ammonium chloride (Nacalai Tesque, 02424-55), 3 g/L potassium hydrogen phthalate (Nacalai Tesque, 28420-95), 5.55 g/L di-sodium hydrogen phosphate dodecahydrate (Nacalai Tesque, 31723-35), 20 g/L glucose, 20 mL/L 50× salt stock, 1mL/L 1,000× vitamin stock, and 0.1 mL/L 10,000× mineral stock (Forsburg and Rhind 2006). YNB medium contained 1.8 g/L yeast nitrogen base (BD Difco, 233520), 5.2 g/L ammonium sulfate (Nacalai Tesque, 02619-15), and 20 g/L glucose. To prepare 5FOA medium, YNB medium is supplemented with 1 g/L 5FOA (Apollo Scientific, PC4054) and 56 mg/L uracil (Tokyo Chemical Industry CO., Ltd., 66-22-8). Solid media, except ME, contained 15 g/L agar (Nacalai Tesque, 01028-85). ME media contained 30 g/L bacteriological malt extracts (Biokar, A1101HA), adenine (Nacalai Tesque, 06398-82), uracil, and 20 g/L Bacto agar (BD Difco, 214010).

Yeast strains and plasmids

The yeast strains and oligonucleotides used in this study are listed in Tables 1 and 2, respectively. Yeast strains were created by transformation using the lithium acetate/PEG method (Mongia et al. 2023) or tetrad dissection (Forsburg and Rhind 2006). The yeast transformation was confirmed by PCR analysis. The strains containing the kanamycin, hygromycin, or nourseothricin resistance gene were selected on the medium supplemented with 100 µg/mL of G418 (Nacalai Tesque, 09380-86), hygromycin B (Nacalai Tesque, 09287-84), or 50 µg/mL clonNAT (Werner BioAgents, 5.001.000).

The *Padh1-rnh1* gene was introduced at the Z locus (Yokobayashi and Watanabe 2005) near *spbp7E8.01* by yeast transformation using pTN1264 plasmid digested with *ApaI*. pTN1264 was created as follows. A 0.8 kb genomic region containing the *adh1* promoter was amplified using *Bgl*-*Padh1*/*Padh1*-*Hind* primers, digested with *Bgl*III and *Hind*III, and introduced between *Bgl*III-*Hind*III sites of pFA6a-natMX6 (Hentges et al. 2005) using T4 DNA ligase (Nippon gene, 311-00404), creating pTN1114. A 1.2 kb fragment containing the Z locus was amplified using Zlocus.FOR/Zlocus.REV primers and pNATZA21-cnp3C-CFP-TEV (Yokobayashi and Watanabe 2005), and introduced at the *Bst*XI site of pTN1114 using Gibson assembly master mix (New England Biolabs, E2611S), creating pTN1233. A 0.9 kb fragment containing *rnh1* cDNA with 6His3Flag at the C-terminal was amplified using *Rnh1*.FOR/*Rnh1*.REV primers and introduced between *Pvu*II-*Aat*II sites of pTN1233 using Gibson assembly master mix, creating pTN1264.

The *rad52-FL*, *rad52-NM*, and *rad52-N* genes were introduced into the Z locus by yeast transformation using *ApaI*-digested pTN1275, pTN1277, and pTN1276, respectively. pTN1275, 1277, and 1276 were created as follows. A 1.4 kb *Mlu*I-*Sac*I fragment containing the *hphMX6* gene from pFA6a-hphMX6 (Hentges et al. 2005) was introduced between *Mlu*I-*Sac*I sites of pTN1264, creating pTN1268. A 2.9 kb region containing the *rad52-6His3Flag* gene was amplified using TNF7567 genomic DNA and *rad52-N-F1*/*kanMX6-UP* primers. A 2.1 kb *Bam*HI-*Sal*I restriction fragment of the PCR product was introduced between *Bgl*III-*Sal*I sites of pTN1268, creating pTN1275 (*Rad52-FL*). A 1.4 kb *rad52-NM* and a 0.3 kb *rad52-NLS* regions were amplified from pTN1275 using *rad22-1/rad52-R_327* and *rad52-F_327/oligo211* primer pairs, respectively. To connect them, we performed a second PCR in the presence of the 1.4- and 0.3-kb fragments and *rad22-1/oligo211* primers. A 1.2 kb *Bgl*III-*Sal*I restriction fragment of the PCR product was introduced between *Bgl*III-*Sal*I sites of pTN1275, creating pTN1277 (*Rad52-NM*). A 1.0 kb *rad52-N* and a 0.3 kb *rad52-NLS* regions were amplified from pTN1275 using *rad22-1/rad52-R_209* and *rad52-F_209/oligo211* primer pairs, respectively. To connect them, we performed a second PCR in the presence of the 1.0- and 0.3-kb fragments and *rad22-1/oligo211* primers. A 0.9 kb *Bgl*III-*Sal*I restriction fragment of the PCR product was introduced between *Bgl*III-*Sal*I sites of pTN1275, creating pTN1276 (*Rad52-N*). All versions of *Rad52* were expressed under the endogenous *rad52* promoter at the Z locus.

The *rad52-6His3Flag* and *rad52-R45K-6His3Flag* genes were expressed in *E. coli* using pTN1287 and pTN1298, respectively. pTN1287 and 1298 were created as follows. The *rad52* gene, codon-optimized for expression in *E. coli*, was cloned into pUC-GW-kan, creating pTN1286 (Genewiz from Azenta Life Sciences). A 1.5 kb NdeI-AvrII fragment containing the *rad52* gene from pTN1286 was introduced between NdeI-AvrII sites of pTN1118(Onaka et al. 2020), creating pTN1287. To introduce the *rad52-R45K* mutation, we performed the first round of PCR using pTN1286 and *rad52p-F1/rad52p-R45K-R* and *rad52p-R45K-F/rad52p-R1* primer pairs, producing 0.4- and 0.5-kb fragments, respectively. To connect them, we performed a second PCR in the presence of the 0.4- and 0.5-kb fragments and *rad52p-F1/rad52p-R1* primers. A 0.6 kb MluI-HindIII restriction fragment of the PCR product was introduced between MluI-HindIII sites of pTN1286, creating pTN1289. A 1.5 kb NdeI-AvrII fragment containing the *rad52-R45K* gene from pTN1289 was introduced between NdeI-AvrII sites of pTN1287, creating pTN1298.

The *tfs1-D274, E275Δ* strain was produced by yeast transformation using NdeI-digested pTN1310, which was constructed as follows. A 2.7 kb genomic region containing the *tfs1* gene was amplified using *tfs1-1/tfs1-5* primers and digested using EcoRI. The 1.9 kb EcoRI restriction fragment was introduced between EcoRI-BsaAI sites of pTN782 containing *ura4+*(Okita et al. 2019), creating pTN1237. A 1.0 kb PCR fragment was prepared using pTN1237 and *ura4-chk2_F/tfs1-AcD* primers. We performed the second PCR in the presence of the 1.0 kb PCR product, pTN1237, and *ura4-chk2_F/tfs1-4* primers. A 1.5 kb NdeI-EcoRI restriction fragment of the PCR product was introduced between NdeI-EcoRI sites of pTN1237, creating pTN1310. After yeast transformation, *ura4+* clones were selected on EMM plates. Then, the *ura4+* pop-out clones were selected on 5FOA plates. DNA sequencing confirmed that no additional mutations were introduced into the PCR fragment during plasmid construction.

The *rad52-fmNeonGreen:hphMX6* strain was constructed by PCR-based gene targeting using pTN1252, which was created as follows. The *fmNeonGreen* gene, which encodes the monomeric NeonGreen codon-optimized to be expressed in fission yeast, was cloned into pUC57, creating pTN1250 (Genewiz from Azenta Life Sciences). A 0.7 kb AscI-BamHI fragment containing the *fmNeonGreen* gene from pTN1250 was introduced between AscI-BamHI sites of pFA6a-GFP(S65T)-hphMX6 (Bahler et al. 1998), creating pTN1252.

DNA-RNA immunoprecipitation (DRIP) assay

7×10^8 yeast cells were harvested from log-phase YE cultures, suspended in TE 10:25 (10 mM Tris-HCl (pH8.0), 25 mM ethylenediaminetetraacetic acid (EDTA)), and stored overnight at 4°C. To examine the effect of a transcription inhibitor, we added a 100 mg/mL stock solution of 1,10-phenanthroline (Nacalai Tesque, 26708) in ethanol to log-phase EMM cultures to a final concentration of 200 µg/mL. As a mock control, we added an equal volume of ethanol to cultures. The cultures were further incubated for 3 h before harvesting cells.

Cells were resuspended in 1 mL of SP1 buffer (20 mM sodium citrate, 20 mM Na₂HPO₄, 40 mM EDTA (pH5.6)). After adding 10 µL of β-mercaptoethanol (Nacalai Tesque, 21418-55), the cell suspension was incubated at 30°C for 20 min with rotation. After centrifugation at $3,200 \times g$ at 25°C for 1 min using a swing rotor TMS-21, cells were resuspended in 500 µL of SP1 buffer. After adding 50 µL of 3.5 mg/mL lyticase (Sigma-Aldrich, L4025), the cell suspension was incubated at 37°C for ≤ 50 min until ~30% of the cells became spheroplasts. After centrifugation at $800 \times g$ at 25°C for 2 min using the swing rotor, spheroplasts were suspended in 300 µL of TE 50:20 (50 mM Tris-HCl (pH8.0), 20 mM EDTA). After adding 100 µL of 10% sodium dodecyl sulfate (SDS) (Nacalai Tesque, 31607-65), the suspension was incubated at 50°C for 1 h. After adding 300 µL of 5 M potassium acetate (Nacalai Tesque, 28405-05), the tube was kept on ice for 10 min. After centrifugation at $19,900 \times g$ at 4°C for 5 min using the swing rotor, the supernatant was transferred to a new tube containing 750 µL of isopropanol and kept on ice for 10 min. After centrifugation at $17,900 \times g$ at 4°C for 5 min using a TOMY micro centrifuge Kitman with an angle rotor, the pellet was rinsed with 1 mL of 70% ethanol and resuspended in 300 µL TE 10:1 (10 mM Tris-HCl (pH8.0), 1 mM EDTA). Nucleic acid fragmentation was performed using a Sonifier 250 (Branson) at setting 2.5 for 10 s, repeated 4 times with 3-min intervals on ice, and confirmed by gel electrophoresis. After two rounds of phenol/chloroform extraction (Nacalai Tesque, 25967-74), 200 µL of the aqueous phase was recovered, mixed with 10 µL of 5 M NaCl (Nacalai Tesque, 31320-76), 4 µL of glycogen (Nacalai Tesque, 17110-11), and 600 µL of ethanol, and kept at -80°C for 1 h. After centrifugation at $17,900 \times g$ at 4°C for 15 min using an angle rotor, the pellet was rinsed with 500 µL of 70% ethanol and resuspend in 100 µL of TE 10:1. The concentration of nucleic acids was determined by NanoDrop One (Thermo Fisher Scientific) and adjusted to 800 ng/µL using TE 10:1. To prepare the input sample, the

nucleic acid solution was diluted 100-fold with Elution buffer (10 mM Tris-HCl (pH8.0), 1 mM EDTA, 1% SDS). 40 μ L of the nucleic acid solution (800 ng/ μ L) was transferred to each of two low protein-binding tubes (BIO-BIK, SC-0150). 50 μ L of H₂O and 10 μ L of 10 $\times$ RNaseH reaction buffer were added to each tube. After adding 0.5 μ L of 60 U/ μ L RNaseH (Takara, 2150A) to one of the two tubes, the tubes were incubated at 37°C for 2 h.

In a low protein-binding tube, 20 μ L of Dynabeads Protein G (Invitrogen, 10004D) was incubated at 4°C overnight with 2 μ L of 1 mg/mL S9.6 antibody (Kerafast, ENH001) in 400 μ L of 1 $\times$ PBS (137 mM NaCl, 27 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH7.4) supplemented with 2% bovine serum albumin (Sigma-Aldrich, A7906). The beads were washed with 400 μ L of 1% Lysis buffer (100 mM HEPES-KOH (pH7.4), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate) and suspended with 310 μ L of 1% Lysis buffer. After adding 90 μ L of the nucleic acid solution incubated in the presence or absence of RNaseH, the bead suspension was incubated at 4°C for 2 h with rotation. Beads were washed twice with 400 μ L of 1% Lysis buffer, once with 400 μ L of 1% Lysis buffer supplemented with 500 mM NaCl, twice with 400 μ L of Wash buffer (10 mM Tris-HCl (pH8.0), 1 mM EDTA (pH8.0), 250 mM LiCl, 0.5% NP40, 0.5% Na-deoxycholate), and once with 400 μ L of TE 10:1. To release DNA-RNA hybrids, beads were suspended in 60 μ L of Elution buffer and incubated at 65°C for 15 min. After recovering the supernatant containing DNA-RNA hybrids to a new tube, the beads were suspended in 40 μ L of Elution buffer again and incubated for an additional 10 min. The supernatants were combined into a single tube. After adding 97 μ L of TE 10:1 and 3 μ L of 20 mg/mL Proteinase K (Nacalai Tesque, 29442-85) to 100 μ L of the supernatant or the input sample, the mixture was incubated at 50°C for 2 h. Two rounds of phenol/chloroform extraction and one round of chloroform extraction (Nacalai Tesque, 08402-55) were followed by ethanol precipitation. The precipitate was resuspended in TE 10:1.

DRIP-sequencing (DRIP-seq) analysis

We prepared a DNA library using KAPA HyperPrep Kit (KK8504) in combination with IDT for Illumina-TruSeq DNA UD Indexes Set A (Illumina, Inc., 20027213) for sequencing on the Illumina NovaSeq6000 platform with 101-bp paired-end reads. From 12 to 30 million mapped

reads were obtained for each sequenced library (PRJDB20605). Raw reads were first trimmed to remove adapter sequences using cutadapt v2.7 (Martin 2011). After trimming, the reads less than 30 bp were discarded. The mean length of the remaining reads was 101 bp. The trimmed reads were then aligned to the *Schizosaccharomyces pombe* reference genome (ASM294v3) using bowtie2 v2.3.5.1 with default parameters (Langmead and Salzberg 2012). When a read matched a repetitive sequence, the read was evenly mapped to the repetitive region. DRIP-seq peaks were called using MACS2 v2.2.6, with the --qvalue option set to 0.01, using input DNA sequencing reads as a control (Zhang et al. 2008).

Reverse transcription-quantitative PCR (RT-qPCR)

5×10^8 yeast cells were harvested from log-phase YE cultures, suspended in 400 μ L of AE buffer (50 mM sodium acetate (pH5.3), 10 mM EDTA), and stored at -80°C . After adding 40 μ L of 10% SDS and 440 μ L of phenol, the cell suspension was incubated at 65°C for 4 min. The solution was quickly chilled in a dry-ice/ethanol bath and then thawed by incubation at 65°C . RNA was purified using phenol/chloroform extraction. 400 μ L of the aqueous phase recovered was mixed with 40 μ L of 3 M sodium acetate (pH5.3) and 1 mL of 100% ethanol. After centrifugation at $17,900 \times g$ at 4°C for 10 min, the pellet was rinsed with 1 mL of 80% ethanol and resuspended in 100 μ L of TE 10:1. RNA concentrations were determined using NanoDrop One. 50 μ L of RNA suspension was mixed with 125 μ L of Monarch StabiLyse DNA/RNA buffer and further purified using Monarch spin RNA isolation kit (New England Biolabs, T2110). RNA concentrations were determined and adjusted to 1.0 $\mu\text{g}/\mu\text{L}$ with RNase-free water. cDNA was synthesized from 1.0 μg of RNA using the LunaScript RT SuperMix kit (New England Biolabs, E3010), which contains random hexamer and poly (dT) primers. No-RT controls were used to detect DNA contamination. Details of quantitative PCR (qPCR) are explained below.

Gross chromosomal rearrangement (GCR) rates

A fluctuation assay determined spontaneous GCR rates (Nakamura et al. 2008; Okita et al. 2019). Yeast cells containing ChL were grown 6-7 days on EMM plates supplemented with adenine and uracil (EMM+AU). With a single colony formed on the EMM+AU plates, 10 mL of

EMM+AU liquid medium was inoculated. After 1-2 days of incubation, the cell culture was diluted in sterile deionized water and plated onto YNB and 5FOA plates supplemented with adenine and uracil (YNB+AU and 5FOA+AU, respectively). After 5-7 days, colonies formed on YNB+AU and 5FOA+AU plates were counted to determine the number of Leu⁺ and that of Leu⁺ Ura⁻ cells, respectively. Leu⁺ Ura⁻ colonies formed on 5FOA+AU were transferred to EMM plates supplemented with uracil (EMM+U) to examine adenine auxotrophy. The number of Leu⁺ Ura⁻ Ade⁻ cells indicative of GCR was determined by subtracting Leu⁺ Ura⁻ Ade⁺ from Leu⁺ Ura⁻ cells. The GCR rate per cell generation was determined as described previously (Lin et al. 1996). At least 15 biologically independent experiments were performed for each strain.

Fluorescent microscopy assay for Rad52-fmNeonGreen foci detection

Cells in log-phase EMM cultures were collected, stained with 2 µg/mL Hoechst 33342 (Nacalai Tesque, 19172-51) at room temperature for 1 h in the dark, and placed on glass-bottom dishes (Matsunami Glass, D11130H). Fluorescence images were observed using the DeltaVision Personal fluorescence microscopy system (GE Healthcare), which is based on an Olympus wide-field IX71 fluorescence microscope equipped with a CoolSNAP HQ2 CCD camera (Photometrics) and an oil-immersion objective lens (UAPO 40×; NA = 1.35; Olympus). An exposure time of 0.8 s was used for fmNeonGreen. In each biologically independent experiment, >290 nuclei were counted. Images were processed using Fiji v2.16.0. Three biologically independent experiments were performed for each strain.

Rad52 chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed, as previously described (Zafar et al. 2017). 1.5×10^8 cells from log-phase EMM cultures were collected. After adding formaldehyde (Nacalai Tesque, 16223-55) to a final concentration of 1%, the cell suspension was vigorously mixed for 15 min at room temperature. After adding 3 mL of 2.5 M glycine to neutralize the crosslinker, the cell suspension was further mixed for 5 min. Cells were washed with 0.1% Lysis buffer (100 mM HEPES-KOH (pH7.4), 140 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1% Na-deoxycholate) and stored at -80°C. Cell pellets were resuspended in 200 µL of 0.1% Lysis buffer

supplemented with 2 μ L of protease inhibitor cocktail (Sigma, P8215) and 4 μ L of 100 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma, P7626). After adding an equal volume of acid-washed glass beads, cells were disrupted at 5,000 rpm using a Micro Smash MS-100 (TOMY) for 30 s, 4 times with 3 min intervals on ice. We incubated 2 μ L of anti-Flag M2 antibodies (Sigma-Aldrich, F1804) with 30 μ L of Dynabeads M-280 sheep anti-Mouse IgG (Invitrogen, 11201D) in 400 μ L of 1 $\times$ PBS buffer supplemented with 2% bovine serum albumin at 4°C overnight. The beads were washed with 400 μ L of 1% Lysis buffer and suspended with 340 μ L of 1% Lysis buffer. After adding 60 μ L of the cell extract, the bead suspension was incubated at 4°C for 2 h with rotation. Beads were washed and eluted as described in DRIP assays. Three biologically independent experiments were performed for each strain.

qPCR analysis in DRIP-qPCR, RT-qPCR, and ChIP-qPCR

qPCR experiments in this work follow the MIQE guidelines (Bustin et al. 2009). qPCR was performed in 96-well plates (Bio-Bik, 3426-00) with sealing film (PlateSeal, qPCR pressure-activated sealing film, PS-PPO-100), in a StepOnePlus real-time PCR system (Applied Biosystems) with StepOne Software v2.3. 10- μ L reaction volume per well. Holding stage at 95°C for 20 s. Cycling stage at 95°C for 3 s and then 60°C for 30 s, for 40 cycles. For RT-qPCR and ChIP-qPCR, we used Fast SYBR Green Master Mix (Thermo Fisher Scientific, 4385612). For DRIP-qPCR, we used PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, A25742). The melting curve for each primer set was analyzed. Relative quantification of the samples was performed using a standard curve generated from serial dilutions of fission yeast genomic DNA. For a standard curve and RT-qPCR, three qPCR reactions (3 $\times$ technical replicates) were set up for each target locus. For DRIP- and ChIP-qPCR, two qPCR reactions (2 $\times$ technical replicates) were set up for each target locus. The mean of technical replicates was obtained. Three biologically independent experiments were performed for each strain ($n = 3$). qPCR primers listed in Table 2 were designed using Primer Express software (Applied Biosystems) or NCBI primer-BLAST (Ye et al. 2012). $T_m \geq 54^\circ\text{C}$. Amplicon sizes were 80–260 bp.

Western blot

Yeast cell extracts were prepared using the alkaline lysis method (Matsuo et al. 2006). 1×10^8 cells from log-phase YE cultures were collected, washed with H_2O , and suspended in 300 μL H_2O . After adding 300 μL of 0.6 M NaOH, the cell suspension was incubated at 30°C for 2.5 min with rotating. After centrifugation at $3,200 \times g$ for 2 min, cells were suspended in 140 μL of SDS sample buffer (60 mM Tris-HCl (pH6.8), 4% β -mercaptoethanol, 4% SDS, 0.005% bromophenol blue (BPB), 5% glycerol) and incubated at 95°C for 3 min. After centrifugation at $17,900 \times g$ for 1 min, cell extracts were recovered from the supernatant, separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (acrylamide:bis-acrylamide = 29:1), and transferred onto a Polyscreen PVDF transfer membrane (Perkin Elmer, NEF1002001PK). The membrane was blocked in Blocking one (Nacalai Tesque, 03953-95) for 1 h and incubated with anti-Flag M2 primary antibodies (1:1,000) at 4 °C overnight. The membrane was incubated with peroxidase AffiniPure goat anti-mouse IgG (heavy+light) (Jackson ImmunoResearch Laboratories, 115-035-146) (1:10,000) secondary antibodies at 32 °C for 1 h. The blot was developed using Supersignal West Femto substrate (Thermo Fisher Scientific, 34094). Images were acquired using ImageQuant LAS500 (GE Healthcare).

Rad52-6His3Flag protein expression and purification

Rad52-6His3Flag and Rad52-R45K-6His3Flag proteins were expressed in *E. coli* strain BL21-CodonPlus (DE3)-RIPL using plasmids pTN1287 and pTN1298, respectively. Cells were grown in 500 mL of LB medium (Lennox) (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with 50 $\mu g/mL$ of ampicillin at 30°C. When the optical density at 600 nm reached ~ 0.5 , 1 M of isopropyl- β -D-thiogalactopyranoside (IPTG) (Nacalai Tesque, 19742-94) was added to a final concentration of 1 mM. After 3 h incubation, the cells were collected by centrifugation at $6,000 \times g$ for 10 min at 4°C and stored at $-80^\circ C$. Cells were resuspended in 20 mL of buffer R (20 mM Tris-HCl (pH8.0), 500 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol) supplemented with 1 mM PMSF and 2 mM benzamidine, and disrupted by nine rounds of 20 s sonication using a Sonifier 250. After centrifugation at $40,000 \times g$ for 30 min at 4°C, the supernatant was mixed with an equal volume of buffer R containing 60% ammonium sulfate

(Nacalai Tesque, 02620-75). The mixture was stirred at 4°C for 30 min. After centrifugation at $20,000 \times g$ for 20 min at 4°C, the precipitate was recovered and suspended in 5 mL of Binding buffer (20 mM NaH₂PO₄ (pH8.0), 400 mM NaCl, 10% glycerol, 10 mM imidazole, 0.1% Triton X-100). The protein solution was applied to a column containing 1.5 mL of TALON metal affinity resin (Takara, 635502) to capture the His-tagged Rad52 protein. The column was washed 3 times with 5 mL of Washing buffer (20 mM NaH₂PO₄ (pH8.0), 400 mM NaCl, 20 mM imidazole, 0.1% TritonX-100), and the Rad52 protein was eluted in 0.5 mL fractions of Elution buffer (20 mM NaH₂PO₄ (pH8.0), 400 mM NaCl, 10% glycerol, 200 mM imidazole, 0.1% TritonX-100). The second and third elution fractions were combined and dialyzed against Storage buffer (20 mM Tris-HCl (pH7.5), 1 mM EDTA, 175 mM NaCl, 10% glycerol, and 1 mM dithiothreitol) using Dialysis membrane size 8 (FujiFilm, 046-30911) and stored at -80°C.

Preparation of nucleic acid substrates

To prepare R-loop, D-loop, DNA bubble, and dsDNA substrates, oligonucleotides listed in Table 2 were mixed at a final concentration of 625 nM in Duplex buffer (100 mM potassium acetate, 30mM HEPES (pH7.5)), denatured at 95°C for 2 min, and annealed by gradually reducing the temperature (-0.5°C per 30 s) to 4°C using the thermal cycler T100 (Bio-Rad) (Camino et al. 2023). The formation of the substrates was confirmed by 3% agarose gel (Nacalai Tesque, 01153-22) electrophoresis in 1× TBE buffer.

Rad52- and Rad51-mediated annealing assay

To label 5' termini of C1 or nC1 oligonucleotides (oligos) with ³²P, 5 pmol of C1 or nC1 oligos, 5 pmol of γ -³²P-ATP (Revvity, BLU002A, 3000 Ci/mmol), and 1 μ L of 10 unit/ μ L T4 polynucleotide kinase (New England Biolabs, M0201S) in 1× T4 PNK buffer were incubated at 37°C for 30 min. The ³²P-labeled C1 or nC1 oligos (0.3 nM in DNA molecules) and Rad52 proteins (1.35 nM) or Rad51 proteins (4.5 nM) were incubated in 200 μ L of Annealing buffer (25 mM Tris-acetate (pH7.5), 1 mM MgCl₂, 100 μ g/mL bovine serum albumin, 1 mM dithiothreitol, in diethylpyrocarbonate-treated H₂O) at 30°C for 10 min. 0.6 μ L of 100 nM substrates in Annealing buffer was added to the reaction, resulting in a final concentration of 0.3 nM. 20 μ L

aliquots were withdrawn at the indicated time and mixed with 20 μ L of 2 $\times$ Stop buffer (3% SDS, 0.1% BPB, 30 nM cold C1 or nC1 oligos). After adding 4 μ L of Proteinase K solution (Nacalai Tesque, 15679-64), the samples were incubated at 37°C for 1 h and loaded onto 8% non-denaturing PAGE in 1 $\times$ TBE buffer at 10 V/cm for 50 min. Gels were dried on DE81 ion exchange cellulose chromatography paper (Whatman, 3658-915) using a vacuum gel-drying apparatus at 65°C for 45 min. Radioactive signals were detected using a phosphorimager Typhoon FLA7000 (GE Healthcare) and quantified with Multi Gauge v3.2.

Statistics

A two-tailed Mann-Whitney test and a two-tailed Student T-test were performed with GraphPad Prism v10.4.1 for macOS and Microsoft Excel (version 16), respectively. For DRIP-qPCR, a two-tailed Student t-test was performed between RNaseH "-" samples of wild-type and mutant strains, as well as the indicated pairs of mutant strains. For ChIP-qPCR, RT-q PCR, and Rad52 focus formation, a two-tailed Student t-test was performed between wild-type and mutant strains, as well as the indicated pairs of mutant strains. For GCR rates, a two-tailed Mann-Whitney test was performed between wild-type and mutant strains as well as the indicated pairs of mutant strains. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.

Tables

Table 1. Strain list

Strain No.	Genotype
TNF7555	h- (smt0), mat2-3::natMX6
TNF7566	h- (smt0), mat2-3::natMX6, clr4::kanMX6
TNF8920	h- (smt0), mat2-3::natMX6, rik1::hphMX6
TNF8038	h- (smt0), mat2-3::natMX6, swi6::kanMX6
TNF9032	h- (smt0), mat2-3::natMX6, rad51::kanMX6
TNF8306	h- (smt0), mat2-3::natMX6, spbp7E8.01:Padh1-rnh1-6His3Flag:natMX6
TNF8291	h- (smt0), mat2-3::natMX6, spbp7E8.01:Padh1-rnh1-6His3Flag:natMX6, clr4::kanMX6
TNF7569	h- (smt0), mat2-3::natMX6, tfs1::kanMX6
TNF9028	h- (smt0), mat2-3::natMX6, tfs1-D274,E275Δ
TNF7624	h- (smt0), mat2-3::natMX6, tfs1::kanMX6, clr4::kanMX6
TNF9040	h- (smt0), mat2-3::natMX6, tfs1-D274,E275Δ, clr4::hphMX6
TNF8675	h- (smt0), mat2-3::natMX6, leo1::kanMX6, clr4::hphMX6
TNF7571	h- (smt0), mat2-3::natMX6, ubp3::kanMX6
TNF9051	h- (smt0), mat2-3::natMX6, def1::kanMX6
TNF8636	h- (smt0), mat2-3::natMX6, ubp3::kanMX6, clr4::hphMX6
TNF9077	h- (smt0), mat2-3::natMX6, def1::kanMX6, clr4::hphMX6
TNF9108	h- (smt0), mat2-3::natMX6, ubp3::kanMX6, def1::kanMX6, clr4::hphMX6
TNF9106	h- (smt0), mat2-3::natMX6, tfs1::kanMX6, def1::kanMX6, clr4::hphMX6
TNF9267	h- (smt0), mat2-3::natMX6, seb1-1
TNF9282	h- (smt0), mat2-3::natMX6, seb1-1, clr4::hphMX6
TNF8713	h- (smt0), mat2-3::natMX6, rad52-R45K
TNF8647	h- (smt0), mat2-3::natMX6, rad52-R45K, clr4::kanMX6
TNF7638	h- (smt0), mat2-3::natMX6, cdc27-D2
TNF8913	h- (smt0), mat2-3::natMX6, cdc27-D2, clr4::hphMX6
TNF8406	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL
TNF8415	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL, clr4::hphMX6
TNF6244	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL, rad51::kanMX6
TNF7898	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL, spbp7E8.01:Padh1-rnh1-6His3Flag:natMX6
TNF7927	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL, spbp7E8.01:Padh1-rnh1-6His3Flag:natMX6, clr4::hphMX6
TNF8024	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL, spbp7E8.01:Padh1-rnh1-6His3Flag:natMX6, rad51::kanMX6
TNF6688	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL, tfs1::kanMX6
TNF9045	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL, tfs1-D274, E275Δ
TNF6726	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL, tfs1::kanMX6, clr4::hphMX6
TNF9057	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL, tfs1-D274, E275Δ, clr4::hphMX6
TNF7154	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL, leo1::kanMX6, clr4::hphMX6
TNF7456	h- (smt0), mat2-3::natMX6, ade6Δ-D, ura4-D18, leu1-32, ChL, ubp3::kanMX6

TNF8878	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, def1::kanMX6
TNF7460	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, ubp3::kanMX6, clr4::hphMX6
TNF8976	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, def1::kanMX6, clr4::hphMX6
TNF9060	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, ubp3::kanMX6, def1::kanMX6, clr4::hphMX6
TNF9062	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, tfs1::kanMX6, def1::kanMX6, clr4::hphMX6
TNF8044	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, spbpb7E8.01:Padh1-rnh1-6His3Flag:natMX6, tfs1::kanMX6, clr4::hphMX6
TNF8694	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, spbpb7E8.01:Padh1-rnh1-6His3Flag:natMX6, ubp3::hphMX6, clr4::kanMX6
TNF9275	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, seb1-1
TNF9279	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, seb1-1, clr4::hphMX6
TNF7546	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rad52-R45K
TNF9025	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rad51::natMX6, clr4::hphMX6
TNF7819	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rad52-R45K, clr4::kanMX6
TNF8377	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rad52-R45K, spbpb7E8.01:Prad52-rad52-6His3Flag:hphMX6, clr4::kanMX6
TNF8393	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rad52-R45K, spbpb7E8.01:Prad52-rad52(1-321)-6His3Flag:hphMX6, clr4::kanMX6
TNF8396	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rad52-R45K, spbpb7E8.01:Prad52-rad52(1-202)-6His3Flag:hphMX6, clr4::kanMX6
TNF6746	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, mus81::hphMX6
TNF6637	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc27-D2
TNF8910	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc45-928
TNF8034	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc27-D2, rad52-R45K
TNF6845	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, mus81::hphMX6, clr4::kanMX6
TNF6670	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc27-D2, clr4::hphMX6
TNF8045	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc27-D2, rad52-R45K, clr4::hphMX6
TNF8958	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc45-928, clr4::hphMX6
TNF7410	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc6-ts2
TNF7426	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc6-ts2, clr4::kanMX6
TNF6749	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rev1::hphMX6
TNF7152	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rev3::hphMX6
TNF6830	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rev1::hphMX6, clr4::kanMX6
TNF7184	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rev3::hphMX6, clr4::kanMX6

TNF8224	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rad52-R45K, tfs1::kanMX6, clr4::hphMX6
TNF8221	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc27-D2, tfs1::kanMX6, clr4::hphMX6
TNF8051	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rad13::kanMX6
TNF8640	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, rad13::kanMX6, clr4::hphMX6
TNF8671	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL (rhp26::kanMX6), rhp26::kanMX6
TNF8678	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL (rhp26::kanMX6), rhp26::kanMX6, clr4::hphMX6
TNF6635	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc27-D1
TNF6639	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc27-D3
TNF6444	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc27-D1, clr4::kanMX6
TNF6671	h- (smt0), mat2-3::natMX6, ade6 Δ -D, ura4-D18, leu1-32, ChL, cdc27-D3, clr4::hphMX6
TNF9243	h- (smt0), mat2-3::natMX6, rad52-fmNeonGreen:hphMX6
TNF9265	h- (smt0), mat2-3::natMX6, rad52-fmNeonGreen:hphMX6, clr4::kanMX6
TNF7567	h- (smt0), mat2-3::natMX6, rad52-6His3Flag:kanMX6
TNF7581	h- (smt0), mat2-3::natMX6, rad52-6His3Flag:kanMX6, clr4::kanMX6
TNF9295	h- (smt0), mat2-3::natMX6, rad52-6His3Flag:kanMX6, clr4::hphMX6, seb1-1
TNF9291	h- (smt0), mat2-3::natMX6, rad52-6His3Flag:kanMX6, clr4::kanMX6, tfs1::kanMX6
TNF7754	h- (smt0), mat2-3::natMX6, rad52-6His3Flag:kanMX6, clr4::kanMX6, cdc27-D2

*ChL: A Ch16 derivative containing the ubc11::LEU2+, spc1322::ura4+, and ade6+ markers

Table 2. Oligonucleotide list

Name	Sequence (5'-3')	
DNA D1	5'-CATTGCATATTTAAAACATGTTGGATCCCACGTTGCAT GCTGATAGCCTACTAGAGCTGTATGAATTCAAATGACCTCT TATC AAGTGAC	Rad52 biochemical
DNA D2	5'-GTCACCTTGATAAGAGGTCATTTGAATTCATGGCTTAGA GCTTAATTGCTGAATCTGGTGGCTGGGATCCAACATGTTTTA AATATGCAATG	
DNA D4	5'-GTCACCTTGATAAGAGGTCATTTGAATTCATACAGCTCT AGTAGGCTATCAGCATGCAACGTGGGATCCAACATGTTTTA AATATGCAATG	
DNA C1	5'-ACAGCTCTAGTAGGCTATCAGCATGCAACG	
DNA nC1	5'-GAAGCATTTATCAGGGTTATTGTCTCATGA	
RNA R1	5'-GCACCAGAUUCAGCAAUUAAGCUCUAAGCC	
tRNAIle-F	5'-ATTTTGGCAGCATTTGTGGCGT	qPCR
tRNAIle-R	5'-GATGGACACCACTCTTGCCA	
chr1_inter2_F	5'-ATCCATTGTCTCTCCACCTC	
chr1_inter2_R	5'-TCAAAGCAAACCTCACCTAAAG	
RT-dg102-F	5'-TTGCACTCGGTTTCAGCTAT	
RT-dg102-R	5'-TGCTCTGACTTGGCTTGTCT	
dg2_F	5'-TTTTCAGCGAGACATGTACC	
dg2_R	5'-TCATAAAGCAACACTGGGTG	
cnt1/3-F	5'-CAACCACTGAAAGCGAATCTGTA	
cnt1/3-R	5'-ATTCTGTAAGTTTGCTGTGCTTTATATCA	
act1-F	5'-GGAAGATTGAGCAGCAGTTTGAA	
act1-R	5'-TTGTTTCGTGACATCAAGGAGAAA	
Bgl-Padh1	5'-GGGCAGATCTGGCATGCCCTACAACAATAAGAAAATG G	plasmid construction
Padh1-Hind	5'-CCGAAGCTTGGAATTCTCTTGCTTAAAGAAAAGC	
Zlocus.FOR	5'-CCGGGTCACCCGGCCAGCGACGCGGAGTGCATTAAAC TGATCAGA	
Zlocus.REV	5'-TCAAGGAGGGTATTCTGGGCCTCCATGTCG GACCGAAGTGCGAAAAGTTGC	
Rnh1.FOR	5'-AGAGAATTCCAAGCTTCAGCATATGGGTGG AAATAAGCG	
Rnh1.REV	5'-AGTGCCACCTGACGTGTCGACTCATTACTT GTCATCG	
rad52-N-F1	5'-CGCAAAGCAGATTGCAAAGG	
kanMX6-UP	5'-TCGCAGGTCTGCAGCGAGGAGCCG	
rad22-1	5'-CGTGGAAGACGAGAAGGC	
oligo211	5'-GAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACA TATTTGAAT	
rad52-R_209	5'-ATTTAATGTTTTCTGTTTTTTTATGAAAGACTCTC	
rad52-F_209	5'-CTTTCATAAAAAAACAGAAAACATTAAATAGG TCGCGAGAAAACACTACGATG	
rad52-R_327	5'-TTTTGGGGAAGTGTGTCAGTCACAGC	
rad52-F_327	5'-GCTGTGACTGACACTTCCCCAAAAGGTCGCGAGAAA ACTACGATG	

rad52p-F1	5'-AATACCGCACAGATGCGTAAGGAG	
rad52p-R1	5'-CCAGCGGCTGATTGTTACGGTG	
rad52p-R45K-R	5'-GCCCCGGGCGCTCTTGCGGCTCACATA	
rad52p-R45K-F	5'-TATGTGAGCCGCAAGAGCGGCCCGGGC	
tfs1-1	5'-TCACCTCGATACTTGGCAGG	
tfs1-5	5'-GGTTTCACAGTAGAAAGAGC	
ura4-chk2-F	5'-GGTTGGAAGGCATATCAGCAAAGAC	
tfs1-AcD	5'-GCTTACCGTTGTTCATAGGAGCAGATCTATACGG	
tfs1-4	5'-GAAGACGCTATCAGAGAGGC	

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References

- Aguilera A, Garcia-Muse T. 2012. R loops: from transcription byproducts to threats to genome stability. *Mol Cell* **46**: 115-124.
- Allshire RC, Madhani HD. 2018. Ten principles of heterochromatin formation and function. *Nat Rev Mol Cell Biol* **19**: 229-244.
- Altemose N, Logsdon GA, Bzikadze AV, Sidhwani P, Langley SA, Caldas GV, Hoyt SJ, Uralsky L, Ryabov FD, Shew CJ et al. 2022. Complete genomic and epigenetic maps of human centromeres. *Science* **376**: eabl4178.
- Asanuma T, Inagaki S, Kakutani T, Aburatani H, Murakami Y. 2022. Tandemly repeated genes promote RNAi-mediated heterochromatin formation via an antisilencing factor, Epe1, in fission yeast. *Genes Dev* **36**: 1145-1159.
- Bahler J, Wu JQ, Longtine MS, Shah NG, McKenzie A, 3rd, Steever AB, Wach A, Philippsen P, Pringle JR. 1998. Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14**: 943-951.
- Barra V, Fachinetti D. 2018. The dark side of centromeres: types, causes and consequences of structural abnormalities implicating centromeric DNA. *Nat Commun* **9**: 4340.
- Bhowmick R, Minocherhomji S, Hickson ID. 2016. RAD52 Facilitates Mitotic DNA Synthesis Following Replication Stress. *Mol Cell* **64**: 1117-1126.
- Bickmore WA, van Steensel B. 2013. Genome architecture: domain organization of interphase chromosomes. *Cell* **152**: 1270-1284.
- Boddy MN, Gaillard PHL, McDonald WH, Shanahan P, Yates JR, 3rd, Russell P. 2001. Mus81-Eme1 are essential components of a Holliday junction resolvase. *Cell* **107**: 537-548.
- Brickner JR, Garzon JL, Cimprich KA. 2022. Walking a tightrope: The complex balancing act of R-loops in genome stability. *Mol Cell* **82**: 2267-2297.
- Brown WR, Thomas G, Lee NC, Blythe M, Liti G, Warringer J, Loose MW. 2014. Kinetochores assembly and heterochromatin formation occur autonomously in *Schizosaccharomyces pombe*. *Proc Natl Acad Sci U S A* **111**: 1903-1908.
- Bruning JG, Mariani KJ. 2020. Replisome bypass of transcription complexes and R-loops. *Nucleic Acids Res* **48**: 10353-10367.
- Bugreev DV, Hanaoka F, Mazin AV. 2007. Rad54 dissociates homologous recombination intermediates by branch migration. *Nat Struct Mol Biol* **14**: 746-753.
- Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL et al. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**: 611-622.

- Camino LP, Dutta A, Barroso S, Perez-Calero C, Katz JN, Garcia-Rubio M, Sung P, Gomez-Gonzalez B, Aguilera A. 2023. DICER ribonuclease removes harmful R-loops. *Mol Cell* **83**: 3707-3719 e3705.
- Castellano-Pozo M, Santos-Pereira JM, Rondon AG, Barroso S, Andujar E, Perez-Alegre M, Garcia-Muse T, Aguilera A. 2013. R loops are linked to histone H3 S10 phosphorylation and chromatin condensation. *Mol Cell* **52**: 583-590.
- Chedin F, Hartono SR, Sanz LA, Vanoosthuyse V. 2021. Best practices for the visualization, mapping, and manipulation of R-loops. *EMBO J* **40**: e106394.
- Chen ES, Zhang K, Nicolas E, Cam HP, Zofall M, Grewal SI. 2008. Cell cycle control of centromeric repeat transcription and heterochromatin assembly. *Nature* **451**: 734-737.
- Chen L, Chen JY, Zhang X, Gu Y, Xiao R, Shao C, Tang P, Qian H, Luo D, Li H et al. 2017. R-ChIP Using Inactive RNase H Reveals Dynamic Coupling of R-loops with Transcriptional Pausing at Gene Promoters. *Mol Cell* **68**: 745-757 e745.
- Cristini A, Ricci G, Britton S, Salimbeni S, Huang SN, Marinello J, Calsou P, Pommier Y, Favre G, Capranico G et al. 2019. Dual Processing of R-Loops and Topoisomerase I Induces Transcription-Dependent DNA Double-Strand Breaks. *Cell Rep* **28**: 3167-3181 e3166.
- Crossley MP, Bocek M, Cimprich KA. 2019. R-Loops as Cellular Regulators and Genomic Threats. *Mol Cell* **73**: 398-411.
- Crossley MP, Song C, Bocek MJ, Choi JH, Kousouros JN, Sathirachinda A, Lin C, Brickner JR, Bai G, Lans H et al. 2023. R-loop-derived cytoplasmic RNA-DNA hybrids activate an immune response. *Nature* **613**: 187-194.
- Cullen JK, Hussey SP, Walker C, Prudden J, Wee BY, Dave A, Findlay JS, Savory AP, Humphrey TC. 2007. Break-induced loss of heterozygosity in fission yeast: dual roles for homologous recombination in promoting translocations and preventing de novo telomere addition. *Mol Cell Biol* **27**: 7745-7757.
- Deveryshetty J, Mistry A, Pangen S, Ghoneim M, Tokmina-Lukaszewska M, Gore SK, Liu J, Kaushik V, Karunakaran S, Taddei A et al. 2025. Mechanism of Rad51 filament formation by Rad52 and Rad55-Rad57 in homologous recombination. *Nat Commun* **16**: 6685.
- Dumelie JG, Jaffrey SR. 2017. Defining the location of promoter-associated R-loops at near-nucleotide resolution using bisDRIP-seq. *Elife* **6**.
- Duquette ML, Handa P, Vincent JA, Taylor AF, Maizels N. 2004. Intracellular transcription of G-rich DNAs induces formation of G-loops, novel structures containing G4 DNA. *Genes Dev* **18**: 1618-1629.
- Eissenberg JC, Elgin SC. 2000. The HP1 protein family: getting a grip on chromatin. *Curr Opin Genet Dev* **10**: 204-210.
- Feng Q, During L, de Mayolo AA, Lettier G, Lisby M, Erdeniz N, Mortensen UH, Rothstein R. 2007. Rad52 and Rad59 exhibit both overlapping and distinct functions. *DNA Repair (Amst)* **6**: 27-37.

- Feng Z, Scott SP, Bussen W, Sharma GG, Guo G, Pandita TK, Powell SN. 2011. Rad52 inactivation is synthetically lethal with BRCA2 deficiency. *Proc Natl Acad Sci U S A* **108**: 686-691.
- Feretaki M, Pospisilova M, Valador Fernandes R, Lunardi T, Krejci L, Lingner J. 2020. RAD51-dependent recruitment of TERRA lncRNA to telomeres through R-loops. *Nature* **587**: 303-308.
- Forsburg SL, Rhind N. 2006. Basic methods for fission yeast. *Yeast* **23**: 173-183.
- Francesconi S, Park H, Wang TS. 1993. Fission yeast with DNA polymerase delta temperature-sensitive alleles exhibits cell division cycle phenotype. *Nucleic Acids Res* **21**: 3821-3828.
- Fukagawa T, Earnshaw WC. 2014. The centromere: chromatin foundation for the kinetochore machinery. *Dev Cell* **30**: 496-508.
- Furuyama S, Biggins S. 2007. Centromere identity is specified by a single centromeric nucleosome in budding yeast. *Proc Natl Acad Sci U S A* **104**: 14706-14711.
- Garcia JF, Dumesic PA, Hartley PD, El-Samad H, Madhani HD. 2010. Combinatorial, site-specific requirement for heterochromatic silencing factors in the elimination of nucleosome-free regions. *Genes Dev* **24**: 1758-1771.
- Garcia-Muse T, Aguilera A. 2016. Transcription-replication conflicts: how they occur and how they are resolved. *Nat Rev Mol Cell Biol* **17**: 553-563.
- . 2019. R Loops: From Physiological to Pathological Roles. *Cell* **179**: 604-618.
- Grewal SIS. 2023. The molecular basis of heterochromatin assembly and epigenetic inheritance. *Mol Cell* **83**: 1767-1785.
- Hamperl S, Bocek MJ, Saldivar JC, Swigut T, Cimprich KA. 2017. Transcription-Replication Conflict Orientation Modulates R-Loop Levels and Activates Distinct DNA Damage Responses. *Cell* **170**: 774-786 e719.
- Hanamshet K, Mazin AV. 2020. The function of RAD52 N-terminal domain is essential for viability of BRCA-deficient cells. *Nucleic Acids Res* **48**: 12778-12791.
- Hartono SR, Malapert A, Legros P, Bernard P, Chedin F, Vanoosthuyse V. 2018. The Affinity of the S9.6 Antibody for Double-Stranded RNAs Impacts the Accurate Mapping of R-Loops in Fission Yeast. *J Mol Biol* **430**: 272-284.
- Haws SA, Simandi Z, Barnett RJ, Phillips-Cremins JE. 2022. 3D genome, on repeat: Higher-order folding principles of the heterochromatinized repetitive genome. *Cell* **185**: 2690-2707.
- He D, Guo Y, Cheng J, Wang Y. 2022. Chl1 coordinates with H3K9 methyltransferase Clr4 to reduce the accumulation of RNA-DNA hybrids and maintain genome stability. *iScience* **25**: 104313.
- Hentges P, Van Driessche B, Tafforeau L, Vandenhoute J, Carr AM. 2005. Three novel antibiotic marker cassettes for gene disruption and marker switching in *Schizosaccharomyces pombe*. *Yeast* **22**: 1013-1019.

- Hong EJ, Villen J, Gerace EL, Gygi SP, Moazed D. 2005. A cullin E3 ubiquitin ligase complex associates with Rik1 and the Clr4 histone H3-K9 methyltransferase and is required for RNAi-mediated heterochromatin formation. *RNA Biol* **2**: 106-111.
- Horn PJ, Bastie JN, Peterson CL. 2005. A Rik1-associated, cullin-dependent E3 ubiquitin ligase is essential for heterochromatin formation. *Genes Dev* **19**: 1705-1714.
- Iglesias N, Currie MA, Jih G, Paulo JA, Siuti N, Kalocsay M, Gygi SP, Moazed D. 2018. Automethylation-induced conformational switch in Clr4 (Suv39h) maintains epigenetic stability. *Nature* **560**: 504-508.
- Izban MG, Luse DS. 1992. The RNA polymerase II ternary complex cleaves the nascent transcript in a 3'---5' direction in the presence of elongation factor SII. *Genes Dev* **6**: 1342-1356.
- Jaco I, Canela A, Vera E, Blasco MA. 2008. Centromere mitotic recombination in mammalian cells. *J Cell Biol* **181**: 885-892.
- Jeon C, Yoon H, Agarwal K. 1994. The transcription factor TFIIS zinc ribbon dipeptide Asp-Glu is critical for stimulation of elongation and RNA cleavage by RNA polymerase II. *Proc Natl Acad Sci U S A* **91**: 9106-9110.
- Jia S, Kobayashi R, Grewal SI. 2005. Ubiquitin ligase component Cul4 associates with Clr4 histone methyltransferase to assemble heterochromatin. *Nat Cell Biol* **7**: 1007-1013.
- Johnson WL, Yewdell WT, Bell JC, McNulty SM, Duda Z, O'Neill RJ, Sullivan BA, Straight AF. 2017. RNA-dependent stabilization of SUV39H1 at constitutive heterochromatin. *Elife* **6**.
- Kabeche L, Nguyen HD, Buisson R, Zou L. 2018. A mitosis-specific and R loop-driven ATR pathway promotes faithful chromosome segregation. *Science* **359**: 108-114.
- Karakasili E, Burkert-Kautzsch C, Kieser A, Strasser K. 2014. Degradation of DNA damage-independently stalled RNA polymerase II is independent of the E3 ligase Elc1. *Nucleic Acids Res* **42**: 10503-10515.
- Kettenberger H, Armache KJ, Cramer P. 2003. Architecture of the RNA polymerase II-TFIIS complex and implications for mRNA cleavage. *Cell* **114**: 347-357.
- Khanduja JS, Joh RI, Perez MM, Paulo JA, Palmieri CM, Zhang J, Gulka AOD, Haas W, Gygi SP, Motamedi M. 2024. RNA quality control factors nucleate Clr4/SUV39H and trigger constitutive heterochromatin assembly. *Cell* **187**: 3262-3283 e3223.
- Kim C, Snyder RO, Wold MS. 1992. Binding properties of replication protein A from human and yeast cells. *Mol Cell Biol* **12**: 3050-3059.
- Kim HS, Roche B, Bhattacharjee S, Todeschini L, Chang AY, Hammell C, Verdel A, Martienssen RA. 2024. Clr4(SUV39H1) ubiquitination and non-coding RNA mediate transcriptional silencing of heterochromatin via Swi6 phase separation. *Nat Commun* **15**: 9384.
- Kim J, Guermah M, Roeder RG. 2010. The human PAF1 complex acts in chromatin transcription elongation both independently and cooperatively with SII/TFIIS. *Cell* **140**: 491-503.

- Kloeber JA, Chen B, Sun G, King CS, Wang Z, Wang L, Wu Z, Zhu S, Zhao F, Qin H et al. 2025. KCTD10 is a sensor for co-directional transcription-replication conflicts. *Nature*.
- Kvint K, Uhler JP, Taschner MJ, Sigurdsson S, Erdjument-Bromage H, Tempst P, Svejstrup JQ. 2008. Reversal of RNA polymerase II ubiquitylation by the ubiquitin protease Ubp3. *Mol Cell* **30**: 498-506.
- Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T. 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**: 116-120.
- Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9**: 357-359.
- Larson AG, Elnatan D, Keenen MM, Trnka MJ, Johnston JB, Burlingame AL, Agard DA, Redding S, Narlikar GJ. 2017. Liquid droplet formation by HP1alpha suggests a role for phase separation in heterochromatin. *Nature* **547**: 236-240.
- Li X, Manley JL. 2005. Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. *Cell* **122**: 365-378.
- Liang CC, Greenhough LA, Masino L, Maslen S, Bajrami I, Tuppi M, Skehel M, Taylor IA, West SC. 2024. Mechanism of single-stranded DNA annealing by RAD52-RPA complex. *Nature* **629**: 697-703.
- Lin M, Chang CJ, Green NS. 1996. A new method for estimating high mutation rates in cultured cells. *Mutat Res* **351**: 105-116.
- Lindahl T, Barnes DE. 2000. Repair of endogenous DNA damage. *Cold Spring Harb Symp Quant Biol* **65**: 127-133.
- Lydeard JR, Jain S, Yamaguchi M, Haber JE. 2007. Break-induced replication and telomerase-independent telomere maintenance require Pol32. *Nature* **448**: 820-823.
- Makarova AV, Stodola JL, Burgers PM. 2012. A four-subunit DNA polymerase zeta complex containing Pol delta accessory subunits is essential for PCNA-mediated mutagenesis. *Nucleic Acids Res* **40**: 11618-11626.
- Marina DB, Shankar S, Natarajan P, Finn KJ, Madhani HD. 2013. A conserved ncRNA-binding protein recruits silencing factors to heterochromatin through an RNAi-independent mechanism. *Genes Dev* **27**: 1851-1856.
- Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnetjournal* **17**: 10-12.
- Matsuo Y, Asakawa K, Toda T, Katayama S. 2006. A rapid method for protein extraction from fission yeast. *Biosci Biotechnol Biochem* **70**: 1992-1994.
- Mazina OM, Keskin H, Hanamshet K, Storici F, Mazin AV. 2017. Rad52 Inverse Strand Exchange Drives RNA-Templated DNA Double-Strand Break Repair. *Mol Cell* **67**: 19-29 e13.

- McCann TS, Guo Y, McDonald WH, Tansey WP. 2016. Antagonistic roles for the ubiquitin ligase Asr1 and the ubiquitin-specific protease Ubp3 in subtelomeric gene silencing. *Proc Natl Acad Sci U S A* **113**: 1309-1314.
- McKinley KL, Cheeseman IM. 2016. The molecular basis for centromere identity and function. *Nat Rev Mol Cell Biol* **17**: 16-29.
- Mongia P, Toyofuku N, Pan Z, Xu R, Kinoshita Y, Oki K, Takahashi H, Ogura Y, Hayashi T, Nakagawa T. 2023. Fission yeast Srr1 and Skb1 promote isochromosome formation at the centromere. *Commun Biol* **6**: 551.
- Motamedi MR, Hong EJ, Li X, Gerber S, Denison C, Gygi S, Moazed D. 2008. HP1 proteins form distinct complexes and mediate heterochromatic gene silencing by nonoverlapping mechanisms. *Mol Cell* **32**: 778-790.
- Motamedi MR, Verdel A, Colmenares SU, Gerber SA, Gygi SP, Moazed D. 2004. Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. *Cell* **119**: 789-802.
- Muller MM, Fierz B, Bittova L, Liszczak G, Muir TW. 2016. A two-state activation mechanism controls the histone methyltransferase Suv39h1. *Nat Chem Biol* **12**: 188-193.
- Musacchio A, Desai A. 2017. A Molecular View of Kinetochore Assembly and Function. *Biology (Basel)* **6**.
- Nakagawa T, Okita AK. 2019. Transcriptional silencing of centromere repeats by heterochromatin safeguards chromosome integrity. *Curr Genet* **65**: 1089-1098.
- Nakama M, Kawakami K, Kajitani T, Urano T, Murakami Y. 2012. DNA-RNA hybrid formation mediates RNAi-directed heterochromatin formation. *Genes Cells* **17**: 218-233.
- Nakamura K, Okamoto A, Katou Y, Yadani C, Shitanda T, Kaweeteerawat C, Takahashi TS, Itoh T, Shirahige K, Masukata H et al. 2008. Rad51 suppresses gross chromosomal rearrangement at centromere in *Schizosaccharomyces pombe*. *The EMBO journal* **27**: 3036-3046.
- Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A. 1998. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**: 386-389.
- Nguyen HD, Yadav T, Giri S, Saez B, Graubert TA, Zou L. 2017. Functions of Replication Protein A as a Sensor of R Loops and a Regulator of RNaseH1. *Mol Cell* **65**: 832-847 e834.
- Nimonkar AV, Sica RA, Kowalczykowski SC. 2009. Rad52 promotes second-end DNA capture in double-stranded break repair to form complement-stabilized joint molecules. *Proc Natl Acad Sci U S A* **106**: 3077-3082.
- Nitani N, Nakamura K, Nakagawa C, Masukata H, Nakagawa T. 2006. Regulation of DNA replication machinery by Mrc1 in fission yeast. *Genetics* **174**: 155-165.

- Okita AK, Zafar F, Su J, Weerasekara D, Kajitani T, Takahashi TS, Kimura H, Murakami Y, Masukata H, Nakagawa T. 2019. Heterochromatin suppresses gross chromosomal rearrangements at centromeres by repressing Tfs1/TFIIS-dependent transcription. *Commun Biol* **2**: 17.
- Onaka AT, Su J, Katahira Y, Tang C, Zafar F, Aoki K, Kagawa W, Niki H, Iwasaki H, Nakagawa T. 2020. DNA replication machinery prevents Rad52-dependent single-strand annealing that leads to gross chromosomal rearrangements at centromeres. *Commun Biol* **3**: 202.
- Onaka AT, Toyofuku N, Inoue T, Okita AK, Sagawa M, Su J, Shitanda T, Matsuyama R, Zafar F, Takahashi TS et al. 2016. Rad51 and Rad54 promote noncrossover recombination between centromere repeats on the same chromatid to prevent isochromosome formation. *Nucleic Acids Res* **44**: 10744-10757.
- Oshidari R, Huang R, Medghalchi M, Tse EYW, Ashgriz N, Lee HO, Wyatt H, Mekhail K. 2020. DNA repair by Rad52 liquid droplets. *Nat Commun* **11**: 695.
- Ouyang J, Yadav T, Zhang JM, Yang H, Rheinbay E, Guo H, Haber DA, Lan L, Zou L. 2021. RNA transcripts stimulate homologous recombination by forming DR-loops. *Nature* **594**: 283-288.
- Padeken J, Methot SP, Gasser SM. 2022. Establishment of H3K9-methylated heterochromatin and its functions in tissue differentiation and maintenance. *Nat Rev Mol Cell Biol* **23**: 623-640.
- Parsa JY, Boudoukha S, Burke J, Homer C, Madhani HD. 2018. Polymerase pausing induced by sequence-specific RNA-binding protein drives heterochromatin assembly. *Genes Dev* **32**: 953-964.
- Pascarella G, Hon CC, Hashimoto K, Busch A, Luginbuhl J, Parr C, Hin Yip W, Abe K, Kratz A, Bonetti A et al. 2022. Recombination of repeat elements generates somatic complexity in human genomes. *Cell* **185**: 3025-3040 e3026.
- Paulsen RD, Soni DV, Wollman R, Hahn AT, Yee MC, Guan A, Hesley JA, Miller SC, Cromwell EF, Solow-Cordero DE et al. 2009. A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. *Mol Cell* **35**: 228-239.
- Peake JD, Noguchi E. 2022. Fanconi anemia: current insights regarding epidemiology, cancer, and DNA repair. *Hum Genet* **141**: 1811-1836.
- Pellegrini L. 2023. The CMG DNA helicase and the core replisome. *Curr Opin Struct Biol* **81**: 102612.
- Petermann E, Lan L, Zou L. 2022. Sources, resolution and physiological relevance of R-loops and RNA-DNA hybrids. *Nat Rev Mol Cell Biol* **23**: 521-540.
- Peters AH, O'Carroll D, Scherthan H, Mechtler K, Sauer S, Schofer C, Weipoltshammer K, Pagani M, Lachner M, Kohlmaier A et al. 2001. Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* **107**: 323-337.
- Promonet A, Padioleau I, Liu Y, Sanz L, Biernacka A, Schmitz AL, Skrzypczak M, Sarrazin A, Mettling C, Rowicka M et al. 2020. Topoisomerase 1 prevents replication stress at R-loop-enriched transcription termination sites. *Nat Commun* **11**: 3940.

- Ribar B, Prakash L, Prakash S. 2007. ELA1 and CUL3 are required along with ELC1 for RNA polymerase II polyubiquitylation and degradation in DNA-damaged yeast cells. *Mol Cell Biol* **27**: 3211-3216.
- Saayman X, Graham E, Nathan WJ, Nussenzweig A, Esashi F. 2023. Centromeres as universal hotspots of DNA breakage, driving RAD51-mediated recombination during quiescence. *Mol Cell* **83**: 523-538 e527.
- Saini N, Roberts SA, Sterling JF, Malc EP, Mieczkowski PA, Gordenin DA. 2017. APOBEC3B cytidine deaminase targets the non-transcribed strand of tRNA genes in yeast. *DNA Repair (Amst)* **53**: 4-14.
- Sakofsky CJ, Ayyar S, Deem AK, Chung WH, Ira G, Malkova A. 2015. Translesion Polymerases Drive Microhomology-Mediated Break-Induced Replication Leading to Complex Chromosomal Rearrangements. *Mol Cell* **60**: 860-872.
- Saksouk N, Simboeck E, Dejardin J. 2015. Constitutive heterochromatin formation and transcription in mammals. *Epigenetics Chromatin* **8**: 3.
- Sanz LA, Chedin F. 2019. High-resolution, strand-specific R-loop mapping via S9.6-based DNA-RNA immunoprecipitation and high-throughput sequencing. *Nat Protoc* **14**: 1734-1755.
- Scott KC, Merrett SL, Willard HF. 2006. A heterochromatin barrier partitions the fission yeast centromere into discrete chromatin domains. *Curr Biol* **16**: 119-129.
- Sfeir A, Symington LS. 2015. Microhomology-Mediated End Joining: A Back-up Survival Mechanism or Dedicated Pathway? *Trends Biochem Sci* **40**: 701-714.
- Shang WH, Hori T, Martins NM, Toyoda A, Misu S, Monma N, Hiratani I, Maeshima K, Ikeo K, Fujiyama A et al. 2013. Chromosome engineering allows the efficient isolation of vertebrate neocentromeres. *Dev Cell* **24**: 635-648.
- Shang WH, Hori T, Toyoda A, Kato J, Pendorf K, Sakakibara Y, Fujiyama A, Fukagawa T. 2010. Chickens possess centromeres with both extended tandem repeats and short non-tandem-repetitive sequences. *Genome Res* **20**: 1219-1228.
- Sheridan RM, Fong N, D'Alessandro A, Bentley DL. 2019. Widespread Backtracking by RNA Pol II Is a Major Effector of Gene Activation, 5' Pause Release, Termination, and Transcription Elongation Rate. *Mol Cell* **73**: 107-118 e104.
- Shi I, Hallwyl SC, Seong C, Mortensen U, Rothstein R, Sung P. 2009. Role of the Rad52 amino-terminal DNA binding activity in DNA strand capture in homologous recombination. *J Biol Chem* **284**: 33275-33284.
- Shih HT, Chen WY, Wang HY, Chao T, Huang HD, Chou CH, Chang ZF. 2022. DNMT3b protects centromere integrity by restricting R-loop-mediated DNA damage. *Cell Death Dis* **13**: 546.
- Sigurdsson S, Dirac-Svejstrup AB, Svejstrup JQ. 2010. Evidence that transcript cleavage is essential for RNA polymerase II transcription and cell viability. *Mol Cell* **38**: 202-210.

- Sollier J, Stork CT, Garcia-Rubio ML, Paulsen RD, Aguilera A, Cimprich KA. 2014. Transcription-coupled nucleotide excision repair factors promote R-loop-induced genome instability. *Mol Cell* **56**: 777-785.
- Somesh BP, Reid J, Liu WF, Sogaard TM, Erdjument-Bromage H, Tempst P, Svejstrup JQ. 2005. Multiple mechanisms confining RNA polymerase II ubiquitylation to polymerases undergoing transcriptional arrest. *Cell* **121**: 913-923.
- Stork CT, Bocek M, Crossley MP, Sollier J, Sanz LA, Chedin F, Swigut T, Cimprich KA. 2016. Co-transcriptional R-loops are the main cause of estrogen-induced DNA damage. *Elife* **5**.
- Stunnenberg R, Kulasegaran-Shylini R, Keller C, Kirschmann MA, Gelman L, Buhler M. 2015. H3K9 methylation extends across natural boundaries of heterochromatin in the absence of an HP1 protein. *EMBO J* **34**: 2789-2803.
- Subramanyam S, Kinz-Thompson CD, Gonzalez RL, Jr., Spies M. 2018. Observation and Analysis of RAD51 Nucleation Dynamics at Single-Monomer Resolution. *Methods Enzymol* **600**: 201-232.
- Tan J, Duan M, Yadav T, Phoon L, Wang X, Zhang JM, Zou L, Lan L. 2020. An R-loop-initiated CSB-RAD52-POLD3 pathway suppresses ROS-induced telomeric DNA breaks. *Nucleic Acids Res* **48**: 1285-1300.
- Tanaka H, Ryu GH, Seo YS, MacNeill SA. 2004. Genetics of lagging strand DNA synthesis and maturation in fission yeast: suppression analysis links the Dna2-Cdc24 complex to DNA polymerase delta. *Nucleic Acids Res* **32**: 6367-6377.
- Teng Y, Yadav T, Duan M, Tan J, Xiang Y, Gao B, Xu J, Liang Z, Liu Y, Nakajima S et al. 2018. ROS-induced R loops trigger a transcription-coupled but BRCA1/2-independent homologous recombination pathway through CSB. *Nat Commun* **9**: 4115.
- Tinline-Purvis H, Savory AP, Cullen JK, Dave A, Moss J, Bridge WL, Marguerat S, Bahler J, Ragoussis J, Mott R et al. 2009. Failed gene conversion leads to extensive end processing and chromosomal rearrangements in fission yeast. *EMBO J* **28**: 3400-3412.
- Tsubouchi H, Argunhan B, Ito K, Takahashi M, Iwasaki H. 2020. Two auxiliary factors promote Dmc1-driven DNA strand exchange via stepwise mechanisms. *Proc Natl Acad Sci U S A* **117**: 12062-12070.
- Tubbs A, Nussenzweig A. 2017. Endogenous DNA Damage as a Source of Genomic Instability in Cancer. *Cell* **168**: 644-656.
- Unoki M, Sharif J, Saito Y, Velasco G, Francastel C, Koseki H, Sasaki H. 2020. CDCA7 and HELLS suppress DNA:RNA hybrid-associated DNA damage at pericentromeric repeats. *Scientific reports* **10**: 17865.
- Velazquez Camacho O, Galan C, Swist-Rosowska K, Ching R, Gamalinda M, Karabiber F, De La Rosa-Velazquez I, Engist B, Koschorz B, Shukeir N et al. 2017. Major satellite repeat RNA stabilize heterochromatin retention of Suv39h enzymes by RNA-nucleosome association and RNA:DNA hybrid formation. *Elife* **6**.

- Volpe TA, Kidner C, Hall IM, Teng G, Grewal SI, Martienssen RA. 2002. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**: 1833-1837.
- Vukic M, Daxinger L. 2019. DNA methylation in disease: Immunodeficiency, Centromeric instability, Facial anomalies syndrome. *Essays Biochem* **63**: 773-783.
- Wang Z, Zhang Y, Guo T, He M, Xu Y, Bhattacharjee S, Martienssen RA, Ren J. 2025. Dcr1 senses R-loops for RNAPII termination at sites of replication stress and repair pathway choice. *Mol Cell* **85**: 3947-3964 e3910.
- Wilson MD, Harreman M, Svejstrup JQ. 2013. Ubiquitylation and degradation of elongating RNA polymerase II: the last resort. *Biochim Biophys Acta* **1829**: 151-157.
- Wittmann S, Renner M, Watts BR, Adams O, Huseyin M, Baejen C, El Omari K, Kilchert C, Heo DH, Kecman T et al. 2017. The conserved protein Seb1 drives transcription termination by binding RNA polymerase II and nascent RNA. *Nat Commun* **8**: 14861.
- Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G. 1995. Identification of the breast cancer susceptibility gene BRCA2. *Nature* **378**: 789-792.
- Woudstra EC, Gilbert C, Fellows J, Jansen L, Brouwer J, Erdjument-Bromage H, Tempst P, Svejstrup JQ. 2002. A Rad26-Def1 complex coordinates repair and RNA pol II proteolysis in response to DNA damage. *Nature* **415**: 929-933.
- Wright WD, Shah SS, Heyer WD. 2018. Homologous recombination and the repair of DNA double-strand breaks. *J Biol Chem* **293**: 10524-10535.
- Wu Z, Qu J, Liu GH. 2024. Roles of chromatin and genome instability in cellular senescence and their relevance to ageing and related diseases. *Nat Rev Mol Cell Biol* **25**: 979-1000.
- Wyatt DW, Feng W, Conlin MP, Yousefzadeh MJ, Roberts SA, Mieczkowski P, Wood RD, Gupta GP, Ramsden DA. 2016. Essential Roles for Polymerase theta-Mediated End Joining in the Repair of Chromosome Breaks. *Mol Cell* **63**: 662-673.
- Yadav T, Zhang JM, Ouyang J, Leung W, Simoneau A, Zou L. 2022. TERRA and RAD51AP1 promote alternative lengthening of telomeres through an R- to D-loop switch. *Mol Cell* **82**: 3985-4000 e3984.
- Yan Q, Shields EJ, Bonasio R, Sarma K. 2019. Mapping Native R-Loops Genome-wide Using a Targeted Nuclease Approach. *Cell Rep* **29**: 1369-1380 e1365.
- Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**: 134.
- Yilmaz D, Furst A, Meaburn K, Lezaja A, Wen Y, Altmeyer M, Reina-San-Martin B, Soutoglou E. 2021. Activation of homologous recombination in G1 preserves centromeric integrity. *Nature* **600**: 748-753.
- Yokobayashi S, Watanabe Y. 2005. The kinetochore protein Moa1 enables cohesion-mediated monopolar attachment at meiosis I. *Cell* **123**: 803-817.

- Yu K, Chedin F, Hsieh CL, Wilson TE, Lieber MR. 2003. R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. *Nat Immunol* **4**: 442-451.
- Zafar F, Okita AK, Onaka AT, Su J, Katahira Y, Nakayama JI, Takahashi TS, Masukata H, Nakagawa T. 2017. Regulation of mitotic recombination between DNA repeats in centromeres. *Nucleic Acids Res* **45**: 11222-11235.
- Zatreanu D, Han Z, Mitter R, Tumini E, Williams H, Gregersen L, Dirac-Svejstrup AB, Roma S, Stewart A, Aguilera A et al. 2019. Elongation Factor TFIIS Prevents Transcription Stress and R-Loop Accumulation to Maintain Genome Stability. *Mol Cell* **76**: 57-69 e59.
- Zeller P, Padeken J, van Schendel R, Kalck V, Tijsterman M, Gasser SM. 2016. Histone H3K9 methylation is dispensable for *Caenorhabditis elegans* development but suppresses RNA:DNA hybrid-associated repeat instability. *Nat Genet* **48**: 1385-1395.
- Zhang J, Chen F, Tang M, Xu W, Tian Y, Liu Z, Shu Y, Yang H, Zhu Q, Lu X et al. 2024. The ARID1A-METTL3-m6A axis ensures effective RNase H1-mediated resolution of R-loops and genome stability. *Cell Rep* **43**: 113779.
- Zhang X, Chiang HC, Wang Y, Zhang C, Smith S, Zhao X, Nair SJ, Michalek J, Jatoi I, Lautner M et al. 2017. Attenuation of RNA polymerase II pausing mitigates BRCA1-associated R-loop accumulation and tumorigenesis. *Nat Commun* **8**: 15908.
- Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W et al. 2008. Model-based analysis of ChIP-Seq (MACS). *Genome Biol* **9**: R137.