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Emerging role for R-loops in chromosome segregation

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R-loops are evolutionarily conserved three-stranded nucleic acid structures formed when a nascent RNA transcript hybridizes with its complementary DNA strand, thereby exposing the non-template strand as single-stranded DNA. Traditionally viewed as a by-product of transcription, R-loops are now recognized as critical regulators of diverse cellular processes, including gene expression, DNA replication, damage and repair, telomere length regulation, mitochondrial function and chromosome segregation. Here, we review studies that define the role of R-loops in ensuring faithful chromosome segregation during cell division and discuss how defects in R-loop dynamics lead to kinetochore (centromeric DNA and associated proteins) dysfunction and chromosomal instability. Taken together, these studies suggest that R-loops function as double-edged swords necessary for accurate chromosome segregation under physiological conditions, yet are deleterious if not resolved and improperly regulated. Given that R-loops have been associated with various diseases, targeting R-loop dynamics may offer novel therapeutic strategies for the treatment of diseases such as cancer.

1. Introduction

R-loops, an evolutionarily conserved feature of DNA topology in prokaryotic and eukaryotic organisms, are formed when RNA hybridizes with a DNA template, displacing the non-template single-strand DNA [1–3]. R-loops are found across diverse genomic regions, such as rDNA, promoters, retrotransposons, telomeres, centromeres and open reading frames, where they play a crucial role in the regulation of various cellular processes (figure 1) [2,3]. This review describes the role of R-loops in chromosomal stability, primarily focusing on recent discoveries and molecular mechanisms through which R-loops influence kinetochore function and chromosome segregation.

R-loops have been shown to play a dual role (beneficial and detrimental) in biological processes such as transcription [4–6], DNA replication [7], DNA damage [4,8,9], mitochondrial function [10–13] and telomere maintenance [14,15]. We have briefly summarized how R-loops affect these cellular processes and provided a detailed discussion on the role of R-loops in chromosomal stability in subsequent sections. R-loops regulate transcriptional activity and have been detected at promoters, transcription start sites, gene bodies and terminal regions [2,6,16–18]. At these sites, R-loops act as a signal for the recruitment of regulatory proteins involved in transcription, such as helicase Senataxin and exonuclease Xrn2 [19–21], and/or influence epigenetic modifications including DNA methylation, histone phosphorylation and histone methylation [22–25]. Moreover, Senataxin plays a role in resolution of R-loops, as its absence, by either depletion or mutation, results in accumulation of R-loops, especially at the transcription termination sites [20,26,27]. R-loops also play a dual role in DNA replication, where they can initiate DNA replication by marking origins but also hinder replication progression by forming structures that block

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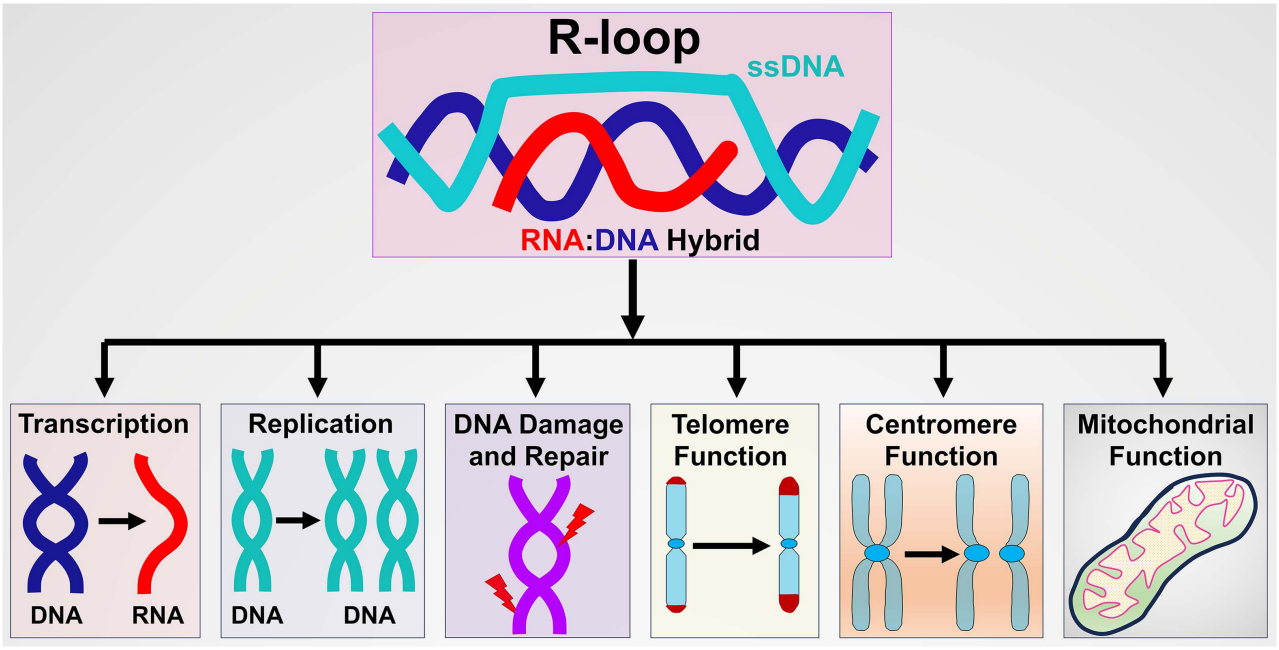


Figure 1. Impact of R-loops on different cellular processes.

replication forks [7,28–30], and are resolved by structure-specific enzymes such as XPF, XPG, MUS81 and Slx4 [31–35]. In addition to structure-specific enzymes, R-loop homeostasis is regulated by a tightly synchronized network of biochemical pathways in which mRNA biogenesis factors, helicases and ribonucleases work to prevent the accumulation or facilitate the removal of R-loops. The mRNA biogenesis factors, such as the THO/TREX complex, proactively prevent R-loop formation by facilitating the efficient removal of nascent transcripts from the nucleus [4,36–38]. Moreover, ATP-dependent helicases, such as Senataxin and DExH/DExD-box proteins, resolve R-loops by physically unwinding RNA:DNA hybrids [20,26,39–41]. Likewise, specialized ribonucleases, such as RNase H1 and RNase H2, degrade the RNA strand within the RNA:DNA hybrid, leading to the resolution of the R-loop structure [4,42,43]. Moreover, transcription–replication conflicts (TRCs), one of the major causes of R-loop-mediated DNA damage, occur when the transcriptional machinery collides with the replication forks on the same DNA strand, often leading to R-loop formation [44–46]. Studies have shown that DNA repair protein Rad52, replication-associated factors, such as FA proteins, chromatin remodeller FACT (*Fa*cilitates *C*hromatin *T*ranscription) complex and histone H3 modifications play crucial roles in the resolution of TRCs [47–51].

R-loops affect the replication of mitochondrial (mt) DNA both positively and negatively [52–54]. Although R-loops serve as primers and contribute to the replication of the light-strand origin (OriL), their excess can lead to oxidative stress and mitochondrial dysfunction [55,56]. Mitochondrial degradosome components such as SUV and PNPT1 help maintain R-loop homeostasis by degrading mtRNA, thereby preventing harmful build-up of R-loops and preserving the structural and functional integrity of mtDNA [57,58]. At telomeres, R-loops have been shown to regulate the telomeric length and modulate the maintenance of cohesion [59,60]. TERRA, a telomeric non-coding RNA, forms R-loops at telomeres that influence its length and stability; higher TERRA levels increase R-loops, which promote telomere elongation via ALT pathways involving proteins, for example, LSD1, RBM14 and MutS [60–64]. Together, these findings underscore the importance of maintaining a precise balance between R-loop formation and its resolution for proper cellular functions (figure 1).

2. R-loop-mediated regulation of chromosome segregation

Faithful chromosome segregation is essential for the equal transfer of genetic information between mother and daughter cells because uneven distribution of chromosomes results in aneuploidy, a hallmark of cancers and developmental disorders [65,66]. The kinetochore, which is required for chromosome segregation during mitosis and meiosis [67–72], is marked epigenetically by an evolutionarily conserved centromere-specific histone H3 variant (Cse4 in budding yeast, Cnp1 in fission yeast, CID in flies, CENH3 in plants and CENP-A in humans) [73–76]. Previous studies have shown that centromeric (*CEN*) association of CENP-A and its homologues is essential for faithful chromosome segregation, as their depletion from *CEN* chromatin results in mitotic defects [73–78]. The ordered assembly of kinetochore proteins and associated complexes allows for the establishment and maintenance of a proper kinetochore architecture and DNA topology [69,79–83].

R-loops represent an important regulator of DNA topology affecting the structural and functional integrity of kinetochores [24,84–88]. R-loops at the kinetochore form *in cis* through association of *CEN* RNAs with *CEN* DNA that is evolutionarily conserved across eukaryotic organisms ranging from yeast to human cells [85,86]. We provide an overview of studies from different

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systems including budding yeast, human cells, mouse and plants that have defined the molecular function of R-loops at the kinetochore and their physiological role in faithful chromosome segregation [24,84–86,88–90].

2.1. R-loops at *CEN* contribute to kinetochore dysfunction and chromosomal instability in budding yeast

Studies from budding yeast have shown that R-loops do not accumulate at the *CEN* chromatin in a wild-type strain [9,85]; however, deletion of *HPR1* (*hpr1*Δ), a member of the THO/TREX complex, results in significant enrichment of R-loops at the *CEN* chromatin [24,85]. Moreover, a previous study has reported that a deletion of *HPR1* exhibits a pause in replication fork progression at the centromeric and telomeric regions; however, the physiological significance of this observation was not defined [38]. Recent studies have shown that Hpr1 interacts *in vivo* with centromeric histone H3 variant, Cse4 [85], and the persistence of R-loops at *CEN* chromatin upon *HPR1* deletion correlates with a reduction in *CEN*-associated Cse4 and its chaperone Scm3, suggesting that R-loops either interfere with or alter the structural integrity of kinetochores in a manner that precludes the proper assembly and maintenance of kinetochore proteins, resulting in chromosomal instability (CIN) [85]. Support for this is based on defects in kinetochore biorientation in an *hpr1*Δ strain [85], the extent of which is similar to that observed for kinetochore mutants [91–94]. Proper biorientation of sister kinetochores modulates their attachment to microtubules from opposite poles, a process that is tightly regulated and a prerequisite for the equal division of chromosomes between mother and daughter cells [69,92,95–98]. The defective kinetochore biorientation in an *hpr1*Δ strain correlates with an increased kinetochore declustering phenotype indicative of weakened or altered kinetochore integrity and CIN phenotype [85,99,100]. Genome-wide analysis revealed that R-loops at *CEN* chromatin contribute to enrichment of ssDNA at the *CEN*, and this affects *CEN* DNA replication and cell cycle progression in an *hpr1*Δ strain [85]. Taken together, accumulation of R-loops at *CEN* chromatin results in kinetochore dysfunction contributed by reduced levels of *CEN*-associated kinetochore proteins, defects in geometric structure and biorientation of kinetochores, all of which in turn mediate CIN phenotype in an *hpr1*Δ strain (figure 2). Removal of R-loops from *CEN* chromatin by *RNH1* overexpression suppresses these phenotypes and restores the fidelity of chromosome segregation in budding yeast (figure 2) [85]. The R-loop-mediated alterations in the chromatin environment at the kinetochore likely affect the maintenance of Cse4 at *CEN* chromatin and likely facilitate the ectopic recruitment of canonical histone H3, as mislocalization and enrichment of histone H3 at *CEN* chromatin have been observed in an *hpr1*Δ strain [24,85]. Notably, these studies also defined a role for Hpr1 in preventing accumulation of R-loops at *CEN* chromatin [24,85] for the proper assembly and function of the kinetochore [85]. Moreover, the Cse4 interactome in budding yeast includes multiple proteins involved in RNA biogenesis and processing, including regulation of R-loops [85,101–104]. Hence, identifying R-loop regulators at the kinetochore and understanding how they affect chromosome segregation is an important direction for future research.

2.2. Depletion of CENP-A contributes to accumulation of R-loops at human *CEN* in S-phase and structural aneuploidy

Defining the role of R-loops in *CEN* function and chromosome segregation at different stages of the cell cycle is an area of active research. It has been reported that depletion of CENP-A in the late S-phase leads to a significant increase in the levels of R-loops at *CEN* chromatin, coincident with the increased levels of *CEN* RNA in human cells [86]. The increased accumulation of R-loops at *CEN* chromatin in late S-phase inhibits the progression of the replication fork through the *CEN* alpha-satellites region, resulting in increased frequency of DNA recombination at the *CEN* [86]. The mitotic entry of cells with under-replicated *CEN* DNA or unresolved recombination intermediates results in the formation of anaphase bridges and aberrant *CENs* [86]. The R-loop-mediated *CEN* instability promotes chromosomal breakage, rearrangements and translocations in the subsequent cell cycles, resulting in structural aneuploidy [86]. Removal of R-loops in CENP-A-depleted S-phase cells by *RNH1* overexpression rescued the aberrant *CEN* phenotypes. These studies provide an important insight into mechanisms by which CENP-A depletion during the S-phase contributes to defects in replication of *CEN* DNA and correlates with the dynamic regulation of R-loops at *CEN* chromatin for the maintenance of chromosomal stability in human cells (figure 3) [86].

2.3. R-loops at human *CEN* during mitosis contribute to faithful chromosome segregation

While increased accumulation of R-loops at *CEN* during the late S-phase in CENP-A-depleted cells exhibits deleterious effects and structural aneuploidy [86], the presence of R-loops in mitosis promotes high-fidelity chromosome segregation by facilitating correction of erroneous kinetochore-microtubule (KT-MT) attachments (figure 3) [84]. It has been reported that the ATR kinase, primarily involved in the regulation of DNA damage and replication stress in S-phase, has a novel role during mitosis [84]. Activation of the mitosis-specific ATR checkpoint pathway at human *CEN* chromatin is mediated by R-loops and serves to regulate KT-MT attachment in mitosis [84]. ATR kinase associates with human *CENs* in mitosis through its association with CENP-F regulated by Aurora A kinase, where it interacts with replication protein A-coated *CEN* R-loops [84]. The R-loop-regulated activation of ATR kinase stimulates the Checkpoint kinase 1 (Chk1) at the mitotic *CEN*, which in turn promotes the activity of Aurora B kinase. The activation of Aurora B kinase then facilitates the correction of erroneous KT-MT attachments and prevents the formation of lagging chromosomes [84]. Moreover, recent studies have begun to provide mechanistic insights into how ATR kinase modulates R-loop homeostasis at the *CEN* chromatin. Under replication stress, ATR kinase initiates the remodelling of *CEN* chromatin by recruiting the AAA+ ATPase (p97/VCP segregase) complex to the kinetochores [105]. Once recruited, this AAA+ ATPase extracts CENP-A from the *CEN* chromatin [105–107]. The depletion of CENP-A from *CEN* chromatin disrupts the structural integrity and transcriptional regulation of the *CEN* repeats, resulting in the accumulation of R-loops and CIN [86,105].

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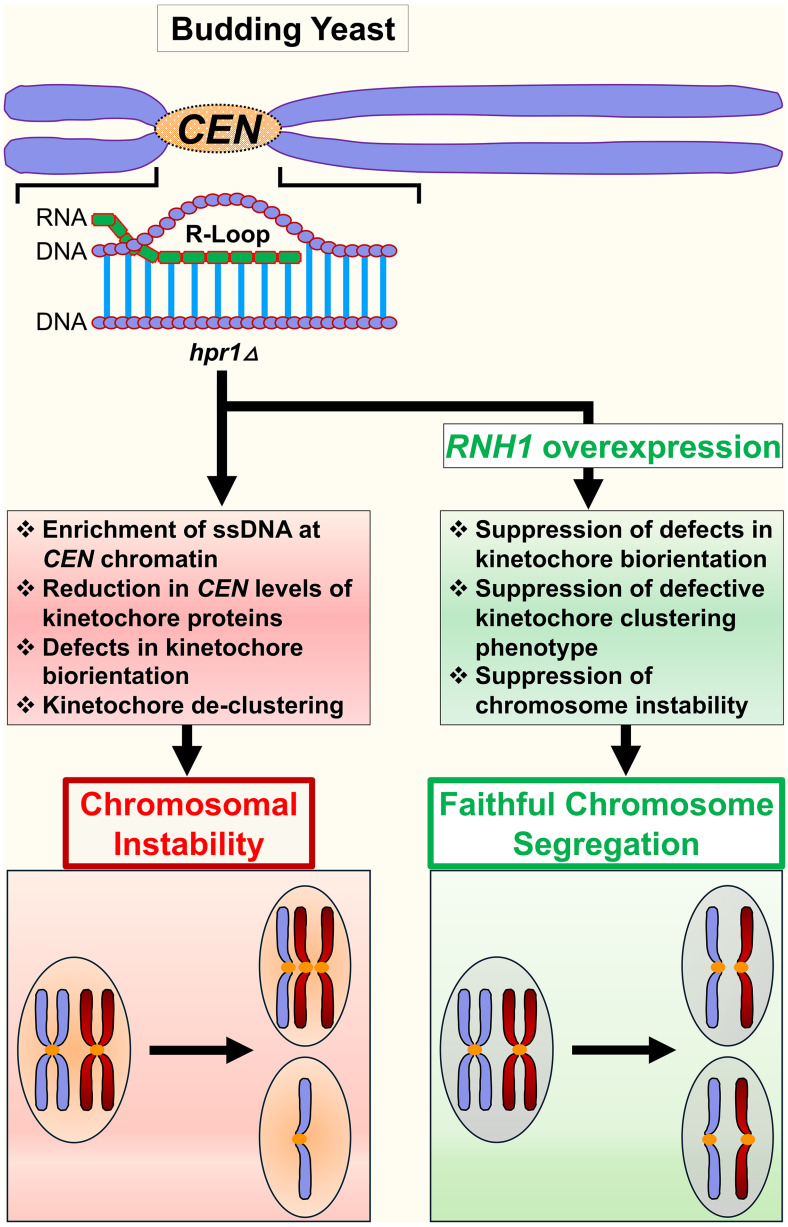


Figure 2. The accumulation of R-loops at *CEN* chromatin contributes to CIN in budding yeast. Research indicates that deleting *HPR1* leads to R-loop accumulation at *CEN* chromatin, resulting in increased ssDNA at *CEN*, decreased levels of kinetochore proteins at *CEN* chromatin, defects in kinetochore biorientation and kinetochore clustering, all of which contribute to CIN [24,85]. Moreover, overexpression of *RNH1* eliminates R-loop accumulation and alleviates defects in kinetochore biorientation, kinetochore clustering and chromosomal stability [85].

2.4. Balanced stoichiometry of *CEN* R-loops regulates chromosome segregation during meiosis in a mouse model

R-loops play an evolutionarily conserved role in meiotic cell division, where they have been linked with the resolution of double-strand breaks during meiotic prophase I [108–110]. During mouse oocyte meiotic cell divisions, *CEN* chromatin is decorated by R-loops and RNase H1 [90]. The oocyte-specific knockout of RNase H1 causes infertility in female mice due to abnormal spindle formation and misalignment of chromosomes caused by aberrant accumulation of R-loops at *CEN* chromatin during metaphase I [90]. The overexpression of RNase H1 in wild-type mouse oocytes also leads to spindle abnormalities and chromosome misalignment caused by a reduction in the levels of R-loops at the *CEN* [90]. During metaphase I, dysregulation of R-loops at *CEN* chromatin disrupts the deposition of RPA-ATRIP and activates the ATR-Chk1-Aurora B signalling pathway at the kinetochore [90]. Taken together, these findings indicate that balanced and dynamic R-loop homeostasis at *CEN* chromatin is important for ensuring accurate spindle formation and proper alignment of chromosomes during oocyte meiotic divisions (figure 4). Future studies should uncover how R-loop homeostasis affects the recruitment of kinetochore proteins at *CEN* chromatin during meiosis and how this is distinct from the mitotic cell division cycle.

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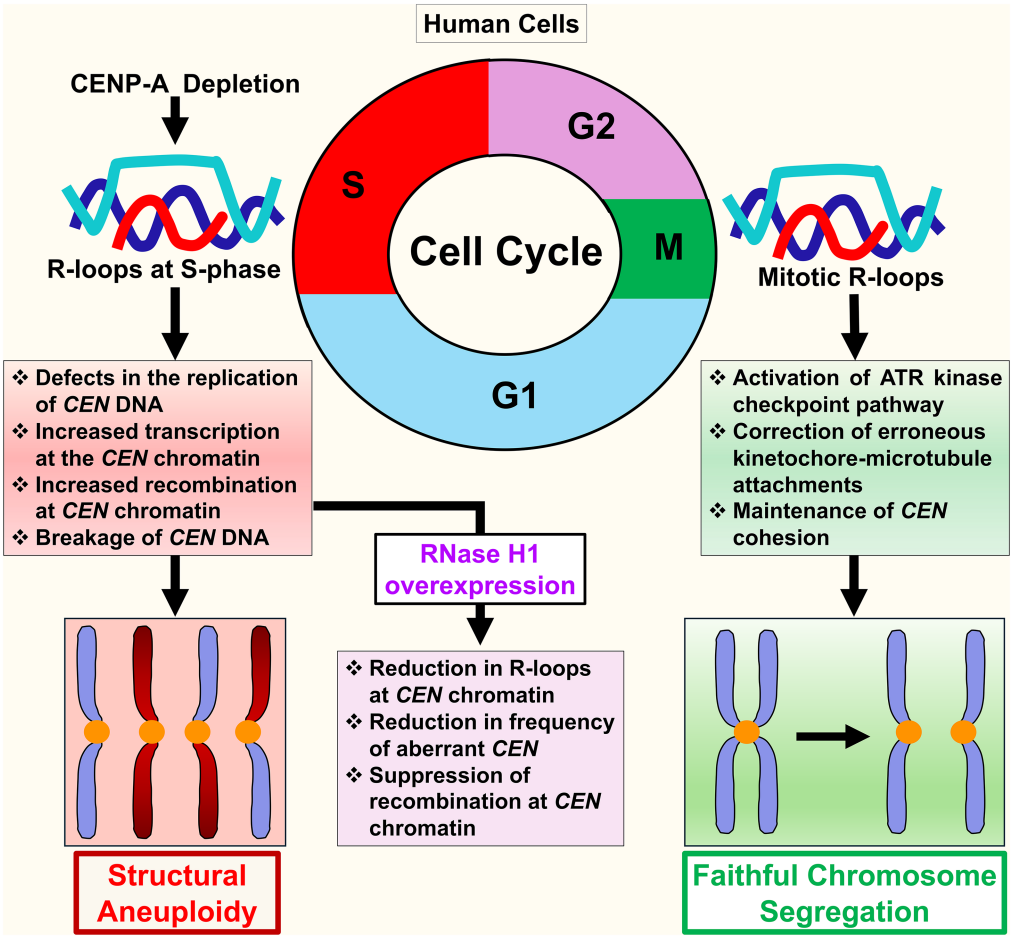


Figure 3. R-loops at human *CEN* chromatin during the S-phase lead to structural aneuploidy while contributing to faithful chromosome segregation during mitosis. During the late S-phase, upon CENP-A depletion, increased accumulation of R-loops at *CEN* chromatin causes defects in *CEN* replication, increased transcription and recombination of *CEN* DNA, which can lead to breakage of *CEN* and structural aneuploidy [86]. Conversely, mitotic R-loops at *CEN* chromatin are involved in correcting faulty kinetochore microtubule attachments by activating ATR kinase, thereby supporting chromosome stability [84].

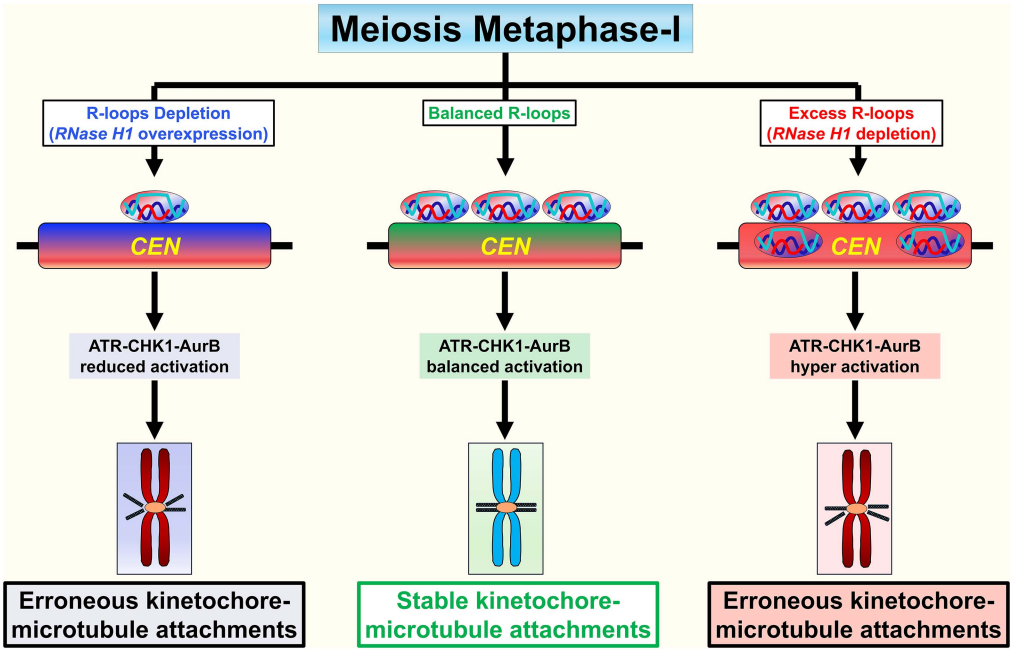


Figure 4. R-loops at *CEN* chromatin maintain meiotic fidelity in mouse oocytes. During metaphase I, R-loops regulate the balanced activity of the ATR-CHK1-Aurora B signalling pathway, ensuring proper kinetochore-microtubule attachments for faithful chromosome segregation. Both the depletion and increased accumulation of *CEN* R-loops result in erroneous kinetochore-microtubule attachments due to an imbalance in ATR-CHK1-Aurora B activity [90].

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2.5. Accumulation of R-loops in plants correlates with CENP-A depletion at *CEN* chromatin

Accumulation of R-loops at *CEN* chromatin has been reported in plants [89,111,112]. In maize, for example, two types of R-loops were detected at *CEN* chromatin: the first, formed by circular *CEN* RNAs, and the second, formed by linear *CEN* RNAs [89]. Upon depletion of circular RNAs by RNA interference, the levels of R-loops and chromatin loops formed by these circular RNAs were decreased at the *CEN* chromatin, whereas the levels of R-loops produced by linear RNAs with similar binding sites were significantly increased [89]. This increase in R-loops and subsequent decrease in the chromatin loops at the *CEN* resulted in reduced localization of CENP-A homologue CENH3 to the kinetochores [89]. These results are consistent with findings from budding yeast where accumulation of R-loops at *CEN* chromatin contributes to reduced levels of Cse4 at the kinetochore [85].

R-loops have also been reported at the *CEN* regions in rye and oat [111,112]. R-loops at rye *CENs* are observed at the core centromeric region, known as *CRS* (centromeric retrotransposons of *Secale cereale*), primarily enriched at CRS1, CRS2 and CRS3 regions [111]. Likewise, R-loop enrichment at *CEN* chromatin in oats was also observed at the centromere-specific retrotransposon (CRA), where CRA39 and CRA82 regions exhibited strong associations with R-loop formation [112]. However, the physiological relevance of R-loops at *CEN* chromatin in rye and oat and their impact on recruitment and/or maintenance of CENH3 require further investigation.

3. Centromeric R-loops and their clinical significance

Aberrant accumulation of R-loops at *CEN* chromatin has been detected in various human cancer cells [113–117]. R-loops have been implicated in cancer initiation and progression; for example, altered R-loop stability and dysregulated biogenesis contribute to leukaemogenesis [118]. Cells harbouring a mutation in SF3B1, an RNA splicing factor, show increased accumulation of R-loops at *CEN* chromatin, which results in defective mitotic spindles, increased chromosome oscillation, chromosome misalignment and CIN [118]. It has been shown that a mutation in SF3B1, when it occurs in combination with a deletion of ATM gene, results in chronic lymphocytic leukaemia (CLL) development [118]. Moreover, BRCA1, a tumour suppressor gene [119], associates with kinetochores in human cells and prevents the increased accumulation of R-loops at *CEN* chromatin [115]. Cells deficient in BRCA1 activity showed increased enrichment of R-loops at *CEN*, which resulted in reduced levels of *CEN*-associated CENP-A, increased frequency of *CEN* breakage and formation of acentric micronuclei [115]. Notably, BRCA1 depletion causes Rad52-mediated recombination between *CEN* DNAs, leading to kinetochore defects and CIN [115]. DNMT3B, a DNA methyltransferase, also regulates the integrity of kinetochores by controlling R-loop formation and preventing excessive DNA damage at *CEN* chromatin [120]. Human colon cancer cells deficient in DNMT3B function show enhanced susceptibility of *CEN* R-loops to cleavage by enzymes involved in DNA double-strand breaks such as XPG and XPF, resulting in defects in kinetochore integrity and errors in chromosome segregation [120]. Interestingly, DNMT3B has been linked with the inactivation of BRCA1 [121]; however, the functional interplay between DNMT3B and BRCA1 and how this relationship modulates R-loops at *CEN* chromatin and chromosome segregation require further investigation. Together, these results highlight the clinical importance of dynamic *CEN* R-loop homeostasis and could offer a potential therapeutic avenue targeting R-loop regulation.

4. Conclusions and perspectives

The formation, resolution and accumulation of R-loops at *CEN* chromatin profoundly influence chromosome segregation, underscoring their importance in the topological regulation of kinetochore function [84–86,88,89,122]. Studies from different eukaryotes (yeast, humans and plants) provide an evolutionarily conserved role for R-loops in chromosome segregation. Studies with budding yeast and plants suggest that R-loops may act as structural barriers, potentially disrupting the localization and/or maintenance of CENP-A (and its homologues) at the kinetochores. Based on these reports [85,89,111,112], we propose that R-loops play an important role in modulating the localization and/or maintenance of CENP-A and its homologues at the *CEN* chromatin. Studies from human cells show that accumulation of R-loops during S-phase leads to TRCs resulting in structural aneuploidy [86], while accumulation of R-loops during mitosis activates ATR-mediated mitotic checkpoint resulting in error corrections and faithful chromosome segregation [84]. We propose that the differential role of R-loops in S-phase and mitosis could also be influenced and/or modulated by the dynamic changes in chromatin and kinetochore structure that occur during replication and mitosis [96,97,123].

The emerging role of R-loops in chromosome segregation provides an excellent opportunity for future studies to address how R-loops are processed at *CEN* chromatin and which molecular factors govern this process. Recent advances in single-cell imaging technologies [124–127] offer powerful tools to elucidate how R-loops influence kinetochore dynamics and chromosome segregation throughout the cell cycle. Together, these studies will advance our understanding of R-loop biology at the kinetochore and may provide an avenue for the development of novel therapeutic strategies for diseases associated with R-loops such as cancer.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. This article has no additional data.

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Authors' contributions. P.K.M: conceptualization, data curation, formal analysis, resources, writing—original draft, writing—review and editing; M.A.B: conceptualization, data curation, formal analysis, funding acquisition, investigation, project administration, resources, supervision, writing—original draft, writing—review and editing.

Both authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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