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*REVISED UNMARKED VERSION*

## **CENTROMERE: A TROJAN HORSE FOR GENOME STABILITY**

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### **ABSTRACT**

Centromeres play a key role in the maintenance of genome stability to prevent carcinogenesis and diseases. They are specialized chromosome loci essential to ensure faithful transmission of genomic information across cell generations by mediating the interaction with spindle microtubules. Nonetheless, while fulfilling these essential roles, their distinct repetitive composition and susceptibility to mechanical stresses during cell division render them susceptible to breakage events. In this review, we delve into the present understanding of the underlying causes of centromere fragility, from the mechanisms governing its DNA replication and repair, to the pathways acting to counteract potential challenges. We propose that the centromere represents a “Trojan horse” exerting vital functions that, at the same time, potentially threatens whole genome stability.

## **CENTROMERE, THE KEY REGULATOR OF CHROMOSOME SEGREGATION**

Centromeres are loci of specialized chromatin serving as assembling point of the kinetochores, which in turn ensure correct attachment of pulling microtubules originating from the spindle pole<sup>1</sup>. Centromeric chromatin is biochemically defined by nucleosomes containing the histone H3 variant CENP-A, a highly conserved protein which allows kinetochore assembly and ensures the epigenetic propagation of centromere identity through cell divisions<sup>2</sup>. CENP-A is directly or indirectly required for the assembly of the Constitutive Centromere Associated Network (CCAN), which is essential for centromeric function<sup>3</sup>. Within this complex, CENP-C plays a key role in promoting chromosome segregation<sup>4</sup> and CENP-A incorporation and stability<sup>5,6</sup>.

In human, centromeric chromatin assembles on particular  $\alpha$ -satellite DNA sequences constituted by head-to-tail tandem repeats of AT-rich 171 bp-long divergent monomers<sup>7</sup>, overall accounting for ~3% of the genome<sup>8</sup>. These monomers are organized in tandem arrays whose length can span from 340 bp to 6 Kb<sup>9</sup> forming higher-order repeats (HORs) of about 0.3 – 5 Mb<sup>8</sup>. Centromeric sequences length vary among chromosomes due to variations in the number and organization of repeated tandem monomers; individual monomers are characterized by 50-70% of sequence homology, while HORs can be up to 95% similar owing to their homogeneous  $\alpha$ -satellite array<sup>8,10</sup>. HORs are also highly polymorphic among individuals and this is a sign of rapid evolution<sup>11</sup>. Despite the presence of different HORs, the kinetochore assembles uniquely on a single HOR per chromosome, which is then defined “active”. Other constituents of centromeric DNA are retroelements (REs), which have important roles in chromosome function and evolution<sup>12</sup>. The region flanking the centromeric region, named pericentromere, is less organized and more heterogeneous, but still keeps its repetitive nature, with DNA monomers intermingled with REs such as LINEs and SINEs (Long and Short Interspersed Nuclear Element, respectively) and other satellite sequences<sup>8,12</sup>.

Centromeric DNA is bound by a unique sequence-specific DNA binding protein named CENP-B that recognizes a 17bp motif named CENP-B box, present in every other  $\alpha$ -satellite repeat<sup>13</sup>. Despite its non-essential function<sup>14</sup>, CENP-B has been demonstrated to be important in CENP-A de novo deposition<sup>15</sup> and maintenance<sup>6</sup> as well as in centromere function<sup>4</sup> and architecture<sup>16</sup>.

## CENTROMERES ARE DAMAGE-PRONE GENOMIC LOCI

Given their distinctive function and complex nature, centromeres are inherently susceptible to a range of challenges that could potentially promote genome instability. Being the docking site for the kinetochore, centromeres must withstand the mechanical forces applied by the spindle microtubules during mitosis<sup>17</sup>. Altered microtubule dynamics and dysregulated kinetochore binding are well-known sources of chromosomal instability<sup>18</sup>. Indeed, prolonged microtubule pulling can enhance centromere fragility progressively over time ([dx.doi.org/10.2139/ssrn.4397184](https://doi.org/10.2139/ssrn.4397184)). The tension-induced stress can be heightened in cases of incorrect kinetochore/microtubule attachments such as merotelic attachments where the kinetochore is connected to microtubules from both spindle poles<sup>19</sup>. Disruption of the Polo-like kinase 1 (Plk1), which is vital for maintaining centromeric chromatin and kinetochore integrity, can also lead to centromere disruption via a microtubule-dependent mechanism<sup>20,21</sup>. Defects in spindle structure have been shown to trigger DNA damage at the centromere. For instance, primary murine cells with a mutated *Dido* gene, a structural component of the mitotic spindle pole, exhibit a high rate of DNA double-strand breaks (DSBs) localized specifically at the centromere; this is likely due to merotelic attachments, as indicated by the presence of centromere-containing micronuclei<sup>22</sup>. A similar phenotype is observed in cells with mutant Adenomatous Polyposis Coli (APC), a microtubule-associated protein that regulates spindle dynamics<sup>23</sup>.

In addition, the unique repetitive nature of its underlying DNA confers the centromere with a complex DNA topology, which makes it a potential fragile region of the genome<sup>24</sup>, especially during DNA replication (see next paragraph). Indeed, several topological structures, such as DNA catenates and DNA loops have been observed to accumulate at the centromere in physiological conditions<sup>9,16,25</sup>. Although these structures have been proposed to maintain centromere position<sup>16,25</sup>, they may create topological problems requiring the intervention of specific mechanisms to resolve them like DNA repair and recombination, overall making the centromere a hot-spot for DNA break events<sup>26</sup> (Figure 1). Intriguingly, centromere breaks have been observed even in unperturbed, physiological conditions and in quiescent cells ([dx.doi.org/10.2139/ssrn.4397184](https://doi.org/10.2139/ssrn.4397184),<sup>27</sup>).

This observed fragility is a plausible explanation for the rapid sequence diversification experienced by the centromere<sup>28</sup> and also for the so-called “centromere expansion”, according to which new active HORs emerge displacing old arrays towards pericentromeric regions<sup>8</sup>. Indeed,

recent *ad-hoc* in-depth genomic characterizations of these repetitive loci reveal structural variation taking place at centromeres. For instance, HOR rearrangements, inversions and retroelement insertions<sup>29</sup> support the relatively rapid evolution of centromeric sequences, where homologous recombination can favor mitotic unequal crossovers or gene conversion and sister chromatid exchange<sup>30,31</sup>. However, centromeres can tolerate a certain degree of variation while keeping their functionality, a phenomenon called the “centromere paradox”<sup>32</sup>. This vision of centromeres as recombinogenic regions is in sharp contrast with the longstanding definition of the centromere as “cold spot” for recombination during meiosis<sup>33</sup>: here it was proposed that meiotic recombination is suppressed by structural genetic polymorphisms and repressive heterochromatic marks, as observed in plants<sup>34</sup>, further facilitating centromere inheritance as large linkage blocks<sup>35</sup>. The reason behind DNA recombination taking place at the centromeres of somatic cells remains uncertain, and it will be explored to a certain extent in this review.

Further evidence of the consequences of recombination events at centromeres comes from pathologies. Extensive Genome-Wide Association Studies (GWAS) have discovered Single Nucleotide Polymorphisms (SNPs), which are generated by increased mutation rate, exclusively mapping at centromeric regions and associated to several human diseases (reviewed in<sup>11</sup>). Moreover, rearrangements and recombination at centromeres might be part of the cause of numerical and structural aneuploidies that characterize up to 90% of solid tumors and more than 50% of hemopoietic cancers (<sup>36,37</sup>, Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer). Specifically, functional analysis of copy number profiles of more than 8000 cancer genomes revealed a very recurrent landscape of whole-chromosome arm aneuploidies (gain, loss and translocation)<sup>38</sup> highlighting a high degree of fragility of the (peri-) centromeric regions. Copy number alterations, which have been sourced from The Cancer Genome Atlas (TCGA) database<sup>39</sup>, have been observed to occur within, or in close proximity to, centromeric domains. These occurrences have been recently identified in various cancer types and have been documented in a collection of 31 *ex-vivo* ovarian cancer models (<sup>40</sup>, [dx.doi.org/10.2139/ssrn.4397184](https://dx.doi.org/10.2139/ssrn.4397184)). The integration of cutting-edge genomic methodologies, particularly single-cell Whole Genome Sequencing (scWGS), combined with the successful completion of the human genome assembly, now enables the meticulous detection of aberrations within centromeres across diverse cancer forms. This includes the ability to map the rearrangements taking place within these genomic regions.

## DNA REPLICATION, A TENSE TIME FOR CENTROMERES

Owing to its repetitive nature, centromeric DNA harbours complex secondary structures originating from the repetitive elements themselves. These structures pose potential challenges for DNA replication machinery<sup>24</sup>. Such structures (as depicted in Figure 1) include hairpins, single-stranded DNA, nucleotide mismatches, and misaligned sequences. A notable phenomenon is the formation of R-loops – three-stranded nucleic acid structures characterized by the presence of a DNA:RNA hybrid. R-loops can emerge when replication and transcription processes converge at the centromere<sup>41</sup>. These structures might promote DNA replication stress (RS), generally characterized by prolonged replication timing and altered origin firings, eventually leading to fork stalling and consequent genomic instability<sup>42</sup>.

What is the relationship between centromeric components and DNA replication? CENP-A, besides its known role in centromere position and assembly, has been demonstrated to have a role in protecting  $\alpha$ -satellites from recombination<sup>31</sup>, promoting DNA repair<sup>43,44</sup>, and preventing replication stress at centromeres<sup>45</sup> (Figure 1). Conditional CENP-A depletion during S-phase led to slower replication fork progression through  $\alpha$ -satellite repeats, accumulation of converging forks with likely consequent increased topological stress, and delayed replication in mitosis in a subset of centromeres. This observed centromere dysfunction-induced RS drives centromere recombination and fragility, with further accumulation of DNA damage and chromosome translocations<sup>45</sup>. Specifically, the origin of RS at centromeres in the absence of CENP-A is suggested to arise from the formation of R-loops. These R-loops result from replication-transcription conflicts, a phenomenon observed at centromeres<sup>41,45-47</sup>, which is also recognized as a contributor to genetic instability<sup>48</sup>. Notably, centromeres exhibit active transcription, albeit at low levels, driven by RNA polymerase II<sup>49,50</sup>. The transcripts generated from these  $\alpha$ -satellite sequences might fulfil crucial roles in maintaining centromeric function and stability (reviewed in<sup>9,51</sup>). This accumulating evidence underscores the necessity for tight regulation of transcription and replication processes at centromeres, akin to other vulnerable regions within the genome.

Besides CENP-A, also CENP-B has a role in preventing RS by keeping a correct replication pace at centromeres (Figure 1). In fission yeast, CENP-B homologs were shown to maintain stability of stalled replicative forks by regulating the silencing of LTRs<sup>52</sup>. Human CENP-B was also shown to regulate the initiation of replication on selected alphoid repeats, as its depletion led

to an increased recruitment of pre-replication complex proteins, i.e. Treslin and Orc2<sup>53</sup>; here, the authors speculated that CENP-B can influence origin selection based on its ability to induce nucleosome positioning<sup>54</sup> or heterochromatin formation<sup>15</sup>. Recent findings provide further support for the involvement of CENP-B in regulation of both centromere transcription<sup>55</sup> and replication dynamics ([dx.doi.org/10.2139/ssrn.4397184](https://doi.org/10.2139/ssrn.4397184)). The latter work demonstrates that the absence of CENP-B leads to enhanced replication fork speed at centromeres, possibly due to the removal of CENP-B-mediated secondary structures<sup>16</sup>. This alteration in replication dynamics could potentially trigger RS and subsequent centromeric breakages ([dx.doi.org/10.2139/ssrn.4397184](https://doi.org/10.2139/ssrn.4397184)). This work also emphasizes that maintaining correct centromeric replication dynamics – encompassing fork velocity and the frequency of origin firing – is pivotal in averting RS, centromeric breaks and, on a broader scale, upholding genome stability ([dx.doi.org/10.2139/ssrn.4397184](https://doi.org/10.2139/ssrn.4397184)). However, the mechanisms that oversee replication dynamics regulation and timing within centromeric-specific chromosomes remain a subject of investigation. Studies have indicated that the timing of centromere replication is influenced by the type of cell and exhibits biases linked to satellite repeat families<sup>56</sup>. Investigating how this correlates with variation in length, sequence divergence of HOR elements, and the abundance of centromeric proteins such as CENP-A and CENP-B presents an intriguing avenue for further exploration.

Keeping a correct number of origins at the centromere was recently demonstrated to be crucial to avoid centromere instability, since perturbation of normal replication dynamics led to centromere breaks in human cell models ([dx.doi.org/10.2139/ssrn.4397184](https://doi.org/10.2139/ssrn.4397184)). Different mechanisms to control the number of origins at centromeres can be proposed; for instance,  $\alpha$ -satellites might have specific consensus sequences serving as replication initiation sites, and this could explain the higher frequency of replication origins at centromeres compared to the rest of the genome ([dx.doi.org/10.2139/ssrn.4397184](https://doi.org/10.2139/ssrn.4397184)). Additionally, specific proteins may control normal origin firing, as was shown for ATR and ATM that inhibit replication origins and maintain a slow replication rate even in the absence of any DNA damage<sup>57</sup>. At the centromeres, a proposed mechanism involves the inhibition of ATR activity by the positive supercoiling of centromeric DNA during replication of BAC-containing human centromeres<sup>58</sup>. This phenomenon might contribute to the elevated frequency of origin firings observed at centromeres. A comparable stress response mechanism is observed at telomeres, structures featuring positively supercoiled domains. In this context, the shelterin protein TRF2, through t-loop formation, inhibits both ATM activation

and the Non-Homologous End Joining (NHEJ) process <sup>59</sup>. Furthermore, alterations in replication origins firing and/or perturbations of the progression of the replisome also occur during the activation of oncogenes, a phenomenon called Oncogene-Induced Replication Stress (ORS) <sup>60</sup>. ORS has a profound deleterious effect on specific chromosome regions called Common Fragile Sites (CFSs), causing increased number of breaks and deletions in tumors <sup>61,62</sup>. However, the specific mechanisms by which ORS specifically threaten centromere stability is still under-investigated. A previous work reported recurrent centromere fragility upon oncogene Cyclin E overexpression <sup>62</sup>, probably by inhibition of pre-replication complex assembly <sup>63</sup> or by causing replication-transcription conflicts <sup>64</sup>.

Being characterised by multiple origin firings ([dx.doi.org/10.2139/ssrn.4397184](https://dx.doi.org/10.2139/ssrn.4397184)), centromeres are likely enriched in converging replicating forks with respect to the bulk genome. This could trigger the formation of replication-dependent topological stress that needs to be correctly resolved to prevent the arising of genome instability <sup>65</sup>. In budding yeast, point centromeres have already been observed as sites of converging forks, acting as pausing sites to facilitate DNA replication termination <sup>66,67</sup> along with Pif1 and Rrm3 DNA helicases <sup>66,68</sup>, Topoisomerase II (Top2) <sup>66,69</sup> and the SMC5/6 complex <sup>70</sup>. Interestingly, Top2 was found to play a role in converging forks also in vertebrates <sup>71</sup> along with other factors <sup>72,73</sup>, and TOP2A or SMC6 inhibition during DNA replication of human centromeres was demonstrated to cause an increase in centromere breakages ([dx.doi.org/10.2139/ssrn.4397184](https://dx.doi.org/10.2139/ssrn.4397184)) (Figure 1).

Centromere replication, due to its challenging nature, might require specialized polymerases alongside the canonical replication machinery. One group of enzymes that might play a role are the error-prone translesion synthesis (TLS) polymerases, known for their ability to handle stalled forks or intricate DNA structures. This family includes DNA polymerase eta (Pol $\eta$ ) and zeta (Pol $\zeta$ ), which employ a polymerase-switch mechanism triggered by the monoubiquitination of PCNA at Lys164 <sup>74</sup>. A recent mass spectrometry analysis of centromeric chromatin in human cells revealed a nearly complete set of proteins associated with the TLS pathway ([dx.doi.org/10.2139/ssrn.4397184](https://dx.doi.org/10.2139/ssrn.4397184)). These proteins appear to be more abundant in presence of RS caused by dysfunctional centromeres. REV3L (Pol $\zeta$  catalytic subunit) depletion leads to reduced replication velocity across centromeric repeats with consequent accumulation of centromeric breaks. This suggests a role for TLS in assisting unperturbed centromere replication in humans, which is further enhanced upon induced RS ([dx.doi.org/10.2139/ssrn.4397184](https://dx.doi.org/10.2139/ssrn.4397184)). In the context of



mouse pericentromeric loci, which also possess a repetitive nature, REV3L was found to facilitate replication through recruitment by HP1 proteins, readers of H3K9me3<sup>75</sup>. Depletion of REV3L in murine cells affects S-phase progression and replication timing and leads to pericentromeric double strand breaks (DSBs) and genomic deletions<sup>75</sup>. Given the presence and necessity of error-prone TLS to ensure complete centromere replication, which likely aids in preventing fork stalling, collapse or reversal, one might speculate that, overall, this mechanism can introduce mutagenic errors. These errors, in turn, might contribute to sequence divergence at centromeres over multiple rounds of replications. Increased rates of spontaneous mutagenesis are often typical of late-replicating genome regions<sup>76</sup>. Correlative analyses showed a bias for mutations occurring at A/T nucleotide-rich regions<sup>77</sup>, a characteristic feature of centromere sequences. The potential consequences of centromeric breaks caused by faults during DNA replication may lead to repair-dependent recombination events, further increasing the variability of centromeric repeats among individuals<sup>28</sup>. Given that centromeres lack genes, they might be able to tolerate the replication-dependent hypermutability of their repeats, which could also contribute to the gradual decay of HOR arrays.

Besides special polymerases, the smooth progression of the replisome across centromeric repeats might require specific helicases (Figure 1), such as hDNA2, FANCD1, BLM and WRN, which may have evolved to resolve complex structures related to replication issues (reviewed in<sup>78</sup>). The nuclease/helicase hDNA2<sup>79</sup>, an enzyme that resolves DNA secondary structures<sup>80</sup>, is enriched at centromeric regions in unperturbed conditions<sup>79</sup>. hDNA2 depletion elicited DNA replication defects, stalled forks and consequent activation of the DNA damage checkpoint ATR kinase, ultimately leading to arrest in S/G2 phase. Noteworthy, loss of hDNA2 impaired proper CENP-A deposition onto centromere nucleosomes, and cells escaping the cell cycle arrest showed high missegregation frequency<sup>79</sup>. BLM along with FANCD2, the Plk1-interacting checkpoint helicase (PICH) and other key factors are involved in the resolution of ultra-fine anaphase bridges (UFBs)<sup>81,82</sup>. UFBs, a category of structures not readily detectable with conventional DNA dyes and devoid of histones, manifest under normal conditions from different genomic sites, including centromeres (c-UFBs). Of the five distinct UFB categories, cUFBs constitute the most prevalent subset<sup>83</sup>. These formations are expertly resolved during anaphase by dedicated factors, and this is essential to avoid massive breakage during mitosis, which could give rise to complex chromosomal rearrangements like those observed in cancer karyotypes. For example, deregulation of BLM

activity *via* inhibition of the Polo- like kinase 1 (Plk1) leads to centromere disruption and consequent structural aneuploidy<sup>20,21</sup>. c-UFBs are mainly the result of the sister chromatids DNA entanglement at centromeres, where the persistence of cohesin at mitotic centromeres could impair efficient DNA decatenation<sup>84,85</sup>. DNA decatenation at the centromere relies on the activity of the Topoisomerase II $\alpha$  (TOP2A)<sup>86</sup>, known to be abundant at mitotic centromeres<sup>87,88</sup> (Figure 1). c-UFBs can also originate from replication stress, similarly to those arising from Common Fragile Sites where UFBs threads consist of under-replicated DNA or unresolved replication intermediates as a consequence of prolonged replication in mitosis<sup>81</sup>. Further disentanglement of un-replicated DNA and a gap-filling repair process are ensured by an end-joining mechanism mediated by 53BP1 in the ensuing interphase<sup>89</sup>.

The replication process of repetitive DNA at centromeres can result in the contraction or expansion of these repeats, influencing their function and consequently impacting genome stability by promoting aneuploidy. Indeed, fork stalling, which could be more prevalent at centromeres, can induce DNA polymerase slippage through mismatch base pairing. This phenomenon might contribute to the expansion or excision of repeats across multiple replication cycles (reviewed in<sup>90</sup>), as observed at certain CFSs and fragile X loci (reviewed in<sup>91</sup>). Repeats expansion and contraction have been linked to human disorders<sup>91</sup>, and to avoid such phenomena, cells have evolved mechanisms relying on the activity of mismatch repair proteins (MMR). These proteins bind and resolve aberrant structures caused by mismatches. Mutations in these factors result in microsatellite instability (MSI), which is associated to common forms of cancers, and altered developmental and neurological conditions<sup>92</sup>. Members of MMR, like MSH2-6, have been identified at centromeric chromatin in Bacterial Artificial Chromosome (BAC) reconstituted with *Xenopus laevis* egg extracts<sup>58</sup> and in human ([dx.doi.org/10.2139/ssrn.4397184](https://doi.org/10.2139/ssrn.4397184)). In the context of centromeric chromatin, MSH6 was found to be essential for efficient centromere replication of BAC-containing  $\alpha$ -satellite sequences<sup>58</sup>. Here, the authors proposed that the structures arising from the process of centromere replication did not involve the production of ssDNA, normally rapidly bound by RPA-1. Instead, the MSH2-6 complex seems to be recruited to act as a “spellchecker”, identifying and correcting any misincorporation errors (Figure 1). However, how MMR proteins contribute to the overall genome stability by acting specifically at centromeres will be an intriguing area for further investigation.

## DNA REPAIR PROTEINS SURVEIL THE CENTROMERIC REGIONS

Based on the events illustrated in the previous paragraphs, it is plausible that basal DNA damage is present at centromere triggering a low, but constant, activity of factors involved in DNA damage repair to maintain centromere integrity and replication fidelity (Figure 2).

Pioneering proteomics experiments, performed on chromatin reconstitution of BACs containing human centromeric  $\alpha$ -satellite DNA with *Xenopus laevis* egg extracts, have identified DNA damage repair (DDR) proteins, despite the apparent absence of DNA damage and defective replication<sup>58</sup>. Of particular significance are MRE11-RAD50 (involved in the homologous recombination repair system), PARP1, Ku80, the mismatch repair MSH2-6 complex (as mentioned above), XRCC1, XRCC5/DNAPK, ERCC6L/PICH helicase and MUS81 endonuclease<sup>58</sup>. Some of these centromere-associated proteins (e.g. PARP1, XRCC6, MSH2) were independently identified by pericentromeric major satellite-specific or  $\alpha$ -satellite-specific pull down coupled to mass spectrometry analysis in both mouse embryonic stem cells<sup>93</sup> and human cells ([dx.doi.org/10.2139/ssrn.4397184](https://doi.org/10.2139/ssrn.4397184)). This underscores a conserved mechanism in higher eukaryotes. Noteworthy, the accumulation of DNA repair factors was replication dependent, as chemical inhibition of fork assembly led to reduced levels of centromere-bound proteins<sup>58</sup>. For example, ERCC6L2 has been identified as centromere-bound component through PCNA interaction in independent analyses and its depletion impairs centromere chromatin maintenance<sup>94</sup>. ERCC6L2 also alleviates RS at centromere and by nascent DNA synthesis, authors show an increased replication at centromere repeats compared to chromosome arms leading to chromosome alterations<sup>94</sup>. Also, ERCC6L2 exerts a protective function by limiting DSBs end resection independently from the 53BP1-RIF1-REV7-Shieldin axis.

PARP-1 (Poly(ADP-ribose) polymerase-1) and PARP-2<sup>95</sup> were shown to interact with CENP-A and CENP-B<sup>95,96</sup> and other centromere-related factors such as CENP-E, CENP-F<sup>97</sup> and with the spindle checkpoint factor Bub3<sup>95,96</sup> in unperturbed conditions. Notably, PARP-1 was also identified at centromere chromatin by unbiased approaches<sup>58,93</sup>. Upon DNA damage, these identified centromeric PARP interactors underwent PARylation (polyADP-ribosylation), suggesting a role for PARP-1/-2 in centromere assembly and checkpoint control. Indeed, *PARP-2*<sup>-/-</sup> cells experienced chromosome mis-segregation due to DNA damage-induced kinetochore defects, impaired centromeric chromatin and errors in spindle formation<sup>98,99</sup>. Overall, this evidence accounts for important roles of PARP-1/-2 at centromeres, likely in ensuring proper

chromosome segregation through regulation of DNA repair. PARP-1 was also shown to interact with the SMC2/4 condensin complexes. Here, it was demonstrated that condensin I complex (having SMC2 as core subunit) interacts with PARP-1/XRCC1 to resolve DNA single strand breaks (SSB)<sup>100,101</sup>. The condensin complex II (where SMC4 is the core component) was shown to be involved in genome integrity maintenance through homologous recombination (HR) repair of DSBs, thus suggesting that condensins sustain centromere integrity preventing breakages. Condensin II complex further preserves centromere identity by favouring CENP-A deposition through interaction with its chaperone HJURP (Holliday Junction Recognition Protein)<sup>102-104</sup>. Interestingly, centromere localization of condensin II complex, as well as cohesins, depends on the retinoblastoma (pRB) protein<sup>105</sup>, a well-known tumor suppressor gene. This could account for the high rate of DNA damage, genomic instability, centromeric dysfunction and aneuploidy observed in pRB deficient cells<sup>105</sup>. The Phosphatase and TENsin homolog (PTEN), another well-established tumor suppressor gene fundamental for DNA repair<sup>106</sup>, is also tethered to centromeres through direct interaction with CENP-C *via* its C-terminal domain<sup>107</sup>. PTEN depletion causes spontaneous DSBs, centromere breakage and chromosomal translocations, suggesting that it can protect centromeres from dysfunction through different mechanisms. Indeed, it regulates RAD51 expression<sup>107</sup> and recruitment to stalled replication forks sustaining their restart<sup>108</sup>. Further, PTEN has a function in completing DNA decatenation in G2 and M phases through the regulation of Topoisomerase II $\alpha$  activity, whose loss of function, as mentioned before, potentially enhances DNA damage at centromeres.

The canonical nonhomologous end joining Ku80 protein has been demonstrated to be present at centromeres under physiological conditions<sup>58</sup>. Interestingly, recent evidence has shown Ku80's involvement in the ICF3 (Immunodeficiency with Centromere instability and Facial anomalies) syndrome. This rare disorder, stemming from a CDC7 mutation, is characterised by primary immunodeficiency and developmental delay, and centromere instability on a cytological level<sup>109</sup>. Ku80 co-immunoprecipitates in an ICF-mutation sensitive manner with CDCA7<sup>110</sup>. The ICF3 cellular model exhibited hyper susceptibility to DNA breaks, possibly due to compromised Ku80 function. Indeed, deficiencies in NHEJ-mediated DSBs repair and instability in centromeric repeats were observed; this is likely attributed to an increase in DNA replication stress at satellite sequences<sup>110</sup>. As a result, the authors suggest that chromosome missegregation in this context might be attributed to NHEJ defects<sup>110</sup>. This reinforces the notion that constant basal DNA damage

occurs at the centromere, prompting a collaborative effort among various factors to effectively counter these challenges.

Noteworthy, some CCAN proteins (constitutive centromere-associated network), have been demonstrated to take part in the DNA damage response at centromeres. This is the case for CENP-X and CENP-S, also known as MHF1 (FANCM-associated histone-fold protein) and MHF2, respectively, which were purified along with the FANCM DNA translocase <sup>111,112</sup>. CENP-T-W-S-X complex scaffolds centromeric DNA and outer kinetochore <sup>113</sup>, and FANCM (Fml1 in fission yeast) is a member of the DNA repair network whose defects lead to the clinical manifestation of the rare genetic disease Fanconi's anaemia (FA) <sup>114</sup>. CENP-X and CENP-S help FANCM to exert its function in DNA repair and recombination through reversal of stalled forks <sup>111,112</sup>. On the other hand, FANCM localizes at centromere through association with CENP-X/S tetramer in human cells <sup>115</sup>, where it may have a role in maintaining proper centromere function through its capability to resolve DNA damage-prone R-loops <sup>116</sup> and crossover suppression <sup>117</sup>. Whether other members of the FA pathway are enriched at centromeres and show functional interaction with CCAN components is an interesting issue to address, as it would further strengthen the idea of a basal DNA damage response acting at the centromere.

Interestingly some other DDR proteins like RNF8, RNF168, BRCA2 and 53BP1 show kinetochore localization even in the absence of DNA damage during mitosis <sup>118-120</sup>. The function of these proteins in this context is still unclear. It is reported that besides its role in DDR, 53BP1 contributes to spindle checkpoint function <sup>120</sup> and to the mitotic stopwatch (doi.org/10.1101/2022.11.14.515741). Similarly, phosphorylated BRCA2 was described to play an unexpected role in chromosome alignment <sup>118</sup>, and depletion of BRCA1 led to impaired centromeric cohesion and increased frequency of merotelic kinetochore attachments <sup>121</sup>. Interestingly, ATR is also present at centromeres during mitosis, mechanistically regulated by CENP-F, AURORA A and R-loops <sup>46</sup> and could maintain centromere identity by protecting CENP-A chromatin <sup>122</sup>. Alternatively, the presence at centromeres of these DDR factors during mitosis may suggest that they exert a surveillance mechanism to rapidly sense and respond to eventual breaks, given that centromeres are damage-prone regions.

In summary, these DNA damage repair factors potentially act both under stable conditions and in response to DNA damaging events. The specific circumstances determining their

engagement encompass various variables, including the nature of the lesion and/or the cell cycle phase, as discussed in the subsequent section.

## **MECHANISMS AND DYNAMICS OF REPAIR OF DAMAGED CENTROMERES**

To maintain genome stability in response to DNA insults, a multitude of specialized pathways for DNA repair are in place, including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) (reviewed in <sup>123</sup>). While the mechanisms of DNA repair at euchromatic regions are relatively well understood, the repair processes within heterochromatic regions, including (peri-)centromeres, remain poorly investigated. Recent efforts have been directed towards unravelling these intricate processes <sup>124</sup>. It is firmly established that the choice of the DNA repair pathways is contingent upon the specific chromatin environment in question. In the context of centromeres and their inherent repetitive nature, it is plausible that two predominant repair pathways operate extensively: MMR, prompted by DNA polymerase slippage (refer to centromere replication section) and HR, particularly when DSBs are present. Indeed, tandem repeats can favour repair processes as many donor templates are available to guide DNA synthesis, facilitating repair through the HR pathway <sup>125</sup>. However, long-standing inquiries persist regarding whether HR and NHEJ occur with comparable frequency or if one is favoured over the other depending on the type of DSBs, along with questions on the relative efficiency of repair compared to other genomic regions. Repair processes at centromeres could be hampered by their compact chromatin conformation, which conceivably restricts chromatin accessibility <sup>126</sup>. Nonetheless, a more recent genomic perspective on centromeres challenges this notion, highlighting that their underlying chromatin is notably accessible, especially at sites of kinetochore formation <sup>34</sup>. Chromatin relaxation, depending on KAP1 activity stimulated by ATM signalling, seems to be necessary for efficient repair of heterochromatin <sup>127,128</sup>; notably, KAP1 was shown to localize at pericentromeres along with HP1 <sup>129</sup>. Induced DSBs have been demonstrated to be rapidly and efficiently repaired by NHEJ at centromeres with faster kinetics compared to other genomic loci, suggesting that the condensed chromatin state of centromeres physically restricts the mobility of broken DNA ends, thus increasing the probability to correctly re-join them <sup>130</sup>. Other studies showed how heterochromatin is refractory to DDR, which occurs with slow kinetics and peculiar dynamics at damaged pericentromeric chromatin in human and mouse cells <sup>131,132</sup>.

As discussed in the previous sections, centromeres are susceptible to breaks and their repetitive nature poses a significant challenge for DNA repair mechanisms to mend the lesions without generating ectopic recombination among the repeats, which can occur if the HR pathway is employed<sup>133</sup>. HR at repetitive regions can generate genetic anomalies typically found in human pathologies, including deletions and translocations. Repetitive sequences, being redundant donor templates, are prone to generate such lesions<sup>134</sup>. Despite this potential risk, HR appears to be the predominant DNA repair process taking place at heterochromatic pericentromeric regions albeit with slower kinetics compared to euchromatin<sup>135</sup>. Notably, specialized mechanisms such as single-strand annealing (SSA), and synthesis dependent strand annealing (SDSA) are engaged in these repair pathways<sup>136</sup>, with the latter exploiting the ssDNA extruded from a broken end as a primer for DNA synthesis on a homologous template, often a repetitive sequence in the case of centromeres.

A recent work demonstrated that DSBs induced at mouse and human centromeres using CRISPR/Cas9 recruit HR proteins such as RAD51, RPA and BRCA1 in a CENP-A-mediated manner even during G1 phase when sister chromatids are not yet present<sup>43</sup>. The authors proposed that DNA end resection is favoured by R-loops, which form as a consequence of the DSBs-induced centromeric transcription promoted by SETD1A-dependent H3K4me2. RAD51 activity is crucial in preventing chromosomal abnormalities by hindering the action of RAD52 or other mutagenic pathways, like microhomology-mediated end-joining catalysed by DNA polymerase  $\theta$ <sup>43</sup>. The role of RAD51 at centromeric regions is not limited to a specific cell cycle phase; it extends to mitosis and even to non-cycling cells. In mitosis, RAD51 associates with chromatin following replication stress in a PLK1-dependent manner, promoting mitotic DNA synthesis (MiDAS) and safeguarding centromeres from erroneous recombination<sup>137</sup>. Subsequent investigations using innovative END-seq analyses revealed that RAD51 protects centromeres against spontaneous TOP2-mediated breaks in quiescent cells. This safeguarding function relies on RAD51's strand exchange capability. Moreover, RAD51 sustains centromere identity at damaged sites by facilitating CENP-A loading in both cycling and quiescent cells<sup>27</sup>.

Studies assessing HR repair mechanisms at pericentromeric regions in *Drosophila* in response to ionizing radiation have revealed distinct dynamics similar to those observed at repetitive ribosomal DNA (rDNA) in yeast<sup>138</sup>. Notably, resulting DSBs undergo specific processing patterns. These damaged foci re-localize outside heterochromatic domains and become

tethered to the nuclear pore complex (NPC) before Rad51 recruitment and HDR activation. The initiation of HDR is paused through a SUMOylation-dependent mechanism<sup>131,139</sup>. Subsequently, the HR block is lifted, potentially by proteasome-dependent degradation of inhibitory factors, allowing the repair process to continue at the NPC<sup>131</sup>. This process is regulated by the Smc5/6 complex, which prevents Rad51 binding to heterochromatin before being relocalized, thus restricting aberrant recombination events<sup>131</sup>. It remains to be investigated whether a similar mechanism operates at human centromeres. A comparable mechanism has been observed in mouse cells, where damaged centromeric and pericentromeric regions exhibit distinct spatial and temporal regulation of DNA damage repair<sup>140</sup>. Specifically, DSBs at pericentromeres occurring in G1-phase are maintained physically constrained and repaired through NHEJ. Conversely, breaks taking place in the S/G2 phase are directed to the nuclear periphery in a DNA resection-dependent manner, mediated by RAD51/BRCA2, facilitating the recruitment of HR proteins<sup>140</sup>. Breakages at centromeric regions recruit RAD51 independently of the cell cycle phase and are also extruded from the heterochromatic environment<sup>140</sup>. Furthermore, in yeast, the enhanced chromatin dynamics triggered by DNA damage are influenced by chromatin stiffening and relaxation elicited upon C-terminal serine phosphorylation of histone H2A, a hallmark of damaged loci<sup>141,142</sup>. Upon completion of the repair process, the normal closed chromatin conformation is re-established<sup>143</sup>. Notably, despite these dynamic changes during DDR, centromeres remain attached to the spindle axis, but kinetochore declustering occurs<sup>144</sup>. Given that this mechanism appears conserved, the extrusion of DSBs from damaged (peri)centromere regions may serve as an effective strategy to protect these loci from ectopic recombination and chromosomal translocation during repair processes, potentially involving unidentified factors.

A possible role in DNA repair at centromeres could be exerted by CENP-A itself and other canonical centromere components, although this concept remains highly contentious. CENP-A, along with CENP-N and CENP-U, was shown to bind at induced DSBs in both mouse and human cells independently of phosphorylation of H2A.X histone<sup>44</sup>, and *de novo* CENP-A incorporation has been detected at centromeric DSBs<sup>43</sup>. However, conflicting data also indicate a disassembly of centromeric chromatin during persistent DDR activation<sup>145</sup>. Notably, the protein HJURP, originally identified as a protein binding to DSBs<sup>146</sup>, is associated with CENP-A deposition. Another protein, RSF-1, has also been shown to contribute to CENP-A deposition<sup>147</sup>, and plays a critical role in NHEJ<sup>148</sup>, suggesting a potential non-centromeric role for CENP-A in DNA damage



resolution. Interestingly, damage-induced recruitment of RSF-1 resulted in the accumulation of the centromeric proteins CENP-S and CENP-X, but not CENP-A<sup>148</sup>. In human pluripotent stem cells (hPSC), DSBs induction did not lead to the colocalization of CENP-A with CENP-C, indicating the presence of a dysfunctional centromere that requires reassembly before mitosis can resume after DNA repair. This aligns with the observed elevated apoptosis rate upon DSBs generation in CENP-A depleted hPSC. This phenomenon is thought to arise from reduced CENP-A availability to rebuild the centromere post-damage<sup>149</sup>. Moreover, centromeric proteins can undergo poly(ADP-ribosylation) in response to damage by Poly(ADP-ribose) polymerase-1 (PARP-1) and -2 (PARP-2)<sup>95,96</sup>. However, the extent to which this signal remodels centromeric chromatin to facilitate proper DNA repair remains an area of limited investigation.

## **CONCLUDING REMARKS**

The centromere has captured the attention of many biologists since its initial discovery as the primary site of chromosome constriction and microtubule anchoring. In recent years, we have witnessed remarkable advancements in our comprehension of centromere assembly and sequence, and regulation of its architecture, altogether leading to the recognition that the centromere has a broader role in genome inheritance rather than just being the site for kinetochore assembly. It has now become evident that the centromere's significance extends beyond a mere chromosomal constriction, positioning it as a vital player in genome integrity. Today, centromere research is entering a new phase, propelled by refined investigative techniques enabling high-resolution studies of its sequence and structure. The aim is now to establish novel connections between genome instability and centromeres, going beyond their well-known role in chromosome segregation. This endeavour involves exploring the genetic, epigenetic, and transcriptomics landscapes of centromeres across different aspects, spanning from evolution to human populations genetics, and from aging to diseases. Delving into centromere (in)stability within pathological contexts, like cancer, offers a promising uncharted research direction that could yield innovative concepts, therapeutic strategies, and potentially novel diagnostic tools. Overall, it remains imperative to undertake further investigations to unveil the intricacies of finely tuned DNA replication and repair mechanisms that prevent ectopic recombination and genetic lesions. Understanding the regulatory balance between HR and NHEJ in resolving centromeric damage is vital to know how genetic integrity is preserved, while also promoting centromere evolution. The

question whether centromeric DNA damage adheres to the same repair principles at heterochromatin and the exploration of yet unidentified factors binding exclusively to centromeres upon DNA damage, are intriguing prospects for future inquiry.

In conclusion, the study of centromeres is poised to become a central focus for researchers in the upcoming years. This pursuit aims to unravel the key pathways that govern the delicate balance between tolerable genetic instability and the vital maintenance of centromere functions – qualities that can be likened to a metaphorical "Trojan horse" within the realm of genetics.

## **DECLARATION OF INTERESTS**

The authors declare no competing interests.

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## **FIGURE LEGENDS**

### **Figure 1. Challenges of Centromere DNA replication.**

Schematic representation of DNA replication of repetitive alphoid arrays (HORs) at the centromere ensured by canonical DNA replisome and Trans Lesions Synthesis (TLS) polymerases, regulated by polymerase switch. Peculiar structures present at centromeres (red box) challenge DNA replication, generating replication stress, which can be further enhanced by Oncogene activation. To avoid the insurgence of genetic instability derived from replication, different factors act at centromeres (green box). The illustrated pathways and factor activities are detailed in the text.

### **Figure 2. DNA repair factors acting at broken centromeres.**

Centromeres are breakage-prone loci due to pulling forces exerted by microtubules and peculiar structures (e.g. DNA secondary structures like DNA loops and R-loops) that can impair DNA replication (left). Different factors involved in either Homologous Recombination (HR, depicted

in blue), Non-Homologous End Joining (NHEJ, depicted in orange) or Mismatch Repair (MMR, depicted in purple), supported by common components (depicted in black; MRN denotes the MRE11-RAD50 complex), have been shown to repair centromeric DNA damage. Some of these factors are regulated by centromere components (as CENP-A, depicted in green). Faults in the repair process could lead to centromere alterations like centromeric sequence expansion/contraction or whole arm chromosome translocation that in turn can fuel aneuploidy and chromosomal instability. The highlighted processes are detailed in the text.

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