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3 **Homologous recombination and the formation of complex genomic** 4 **rearrangements**

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15 **Keywords:** chromothripsis, copy number variation, multi-invasion, non-allelic homologous
16 recombination, structure-selective endonuclease, structural variant

17

18 Abbreviations: BIR, break-induced replication; CNV, copy number variant; dsDNA: double-
19 stranded DNA; DSB, double-strand break; EJ, endjoining; hDNA, heteroduplex DNA; HR,
20 homologous recombination; MI, multi-invasion; MIR, MI-induced rearrangement; MMEJ,
21 microhomology-mediated end-joining; NE, nuclear envelope; NHEJ: non-homologous end-
22 joining; ssDNA, single-strand DNA; SSE, structure-selective endonuclease; SV, structural
23 variant.

1 **Abstract**

2 The maintenance of genome integrity involves multiple independent DNA damage avoidance
3 and repair mechanisms. Yet, the origin and pathways of the focal chromosomal reshuffling
4 phenomena collectively referred to as chromothripsis remain mechanistically obscure. Here ,we
5 discuss the role, mechanisms, and regulation of HR in the formation of simple and complex
6 chromosomal rearrangements. We emphasize features of the recently characterized Multi-
7 invasions Induced Rearrangement (MIR) pathway, which uniquely amplifies the initial DNA
8 damage. HR intermediates and cellular contexts at risk for genomic stability are discussed along
9 with the emerging roles of various classes of nucleases in the formation of genome
10 rearrangements. Long-read sequencing and improved mapping of repeats should enable better
11 appreciation of the significance of recombination in generating genomic rearrangements.

12

1 **Complex chromosomal rearrangements and chromothripsis**

2 Chromosomal rearrangements encompass any structural variation (SV) of the genome,
3 regardless of its association with copy number variation (CNV). The advent of high throughput
4 sequencing technologies revealed massive and complex clustered structural variations [1],
5 which have been proposed to constitute a novel genomic instability phenomenon found in
6 cancer genomes, congenital diseases, as well as in asymptomatic individuals [1-4]. Although
7 the terminology varies with the precise nature of the alterations and possibly its etiology, this
8 phenomenon is widely referred to as *chromothripsis*, and we will use this umbrella term here
9 **(Box: Mutational Phenomena)**. Formal criteria based on the CNV pattern and the physically
10 confined nature of the rearrangement junctions have been proposed to define chromothripsis
11 ([1, 5] but see also [6] for a different perspective). The key underlying feature of chromothripsis
12 is the abrupt acquisition of the associated rearrangements. The number of junctions in localized
13 rearrangements suggests a continuum of complexity, with chromothripsis being potentially an
14 extreme expression of mechanism(s) also responsible for simpler SVs [7].

15 **Pathways for complex chromosomal rearrangements as studied in *S. cerevisiae***

16 A number of experimental systems have been developed in tractable model organisms to
17 decipher the origin of both simple and complex SVs as well as the pathways promoting and
18 preventing their occurrence in various sequence contexts. Here, we will focus on work
19 performed in *Saccharomyces cerevisiae*, in which the conserved double-strand break (DSB)
20 repair mechanisms and the consequences of their defect have been best understood thanks to
21 the exquisite genetic and molecular tools in that organism [8].

22 Most strikingly, and despite their relatively low abundance in the *S. cerevisiae* genome,
23 repeated DNA elements were the predominant mediators of SVs [9-12]. Beyond obvious
24 pathological consequences, these repeat-mediated CNVs also contributed to rapid adaptation
25 upon artificial gene dosage imbalances [13, 14] or nutrient-limiting conditions [15, 16].

26 Genetic screens revealed the complex networks of proteins involved in genome maintenance in
27 yeast with implications for human cancer [17]. Kolodner and co-workers identified an
28 astounding 182 genes (3%) playing a primary role and 438 genes (7%) playing a supporting
29 role in suppressing genomic instability in the absence of exogenous DNA damage [17]. These
30 genes participate in two broad functions whose simultaneous inactivation synergizes to
31 destabilize the genome: 1) the prevention or removal of structural DNA damage or aberrant
32 structures, and 2) the promotion of accurate repair of the damage, with certain functions

1 intersecting both categories (*e.g.* mismatch repair). Structural DNA lesions at risk for genomic
2 stability are varied in nature and origin. In unchallenged cells they are believed to mainly
3 originate from replication errors in the form of a persistent ssDNA gaps or broken replication
4 fork, *i.e.* a single-ended **DSB** (see **Glossary**) [8, 18, 19]. These two types of lesion are
5 recombinogenic substrates. For simplicity we will focus here on DSBs, which have been best
6 studied in their double-ended form upon site-specific induction [20].

7 **Homologous recombination pathways and their associated risks to genomic stability**

8 The DSB repair strategies can be broadly separated based on their homology requirements and
9 sub-categorized based on their genetic dependencies, as (i) a homology-independent end-
10 joining (NHEJ) mechanism, (ii) single strand annealing mechanisms relying either on micro-
11 homology (Alt-EJ/MMEJ) or extensive homology (SSA), and (iii) homology-dependent DNA
12 strand invasion mechanisms collectively referred to as HR (**Fig. 1**). While SSA is a homology-
13 dependent process, it does not involve DNA strand invasion and may be responsible for
14 homology-directed repair independent of Rad51 [21]. We briefly review here the risks to
15 genomic stability inherent to the HR pathway, and refer the reader interested in the role of EJ
16 and annealing mechanisms to other recent reviews [22-24].

17 HR templates DSB repair by locating and copying an extensive identical (homologous) or near-
18 identical (homeologous) sequence present in intact duplex DNA (**Fig. 1**). Hence, HR uniquely
19 entails homology search and DNA strand invasion of the broken molecule to identify and invade
20 a homologous template. The DNA strand invasion reaction results in a **D-loop** (**Fig. 1**)
21 containing heteroduplex DNA (hDNA). These reactions are catalyzed by a helical filament of
22 Rad51 (RecA in bacteria) and associated proteins assembled on the resected ssDNA flanking
23 the DSB [25]. Upon pairing of the 3' extremity of the broken molecule, DNA synthesis restores
24 the sequence information disrupted by the DSB. The HR sub-pathways branch based on the
25 differential processing of this extended D-loop intermediate (**Fig. 1**). Two features of this
26 pathway have important consequences for genomic stability: the unstable nature of the DNA
27 synthesis occurring in the context of the D-loop and the potential of various types of DNA joint
28 molecules generated throughout HR to be aberrantly processed by structure-selective
29 endonuclease (SSE) (see below). Additionally, HR-mediated rearrangements can lead to
30 dicentric chromosome formation and subsequent chronic instability by **Breakage-Fusion-**
31 **Bridge** cycles [26, 27].

1 Disruption of the extended D-loop funnels the pathway towards Synthesis-Dependent Strand
2 Annealing (SDSA), which leads to a **non-crossover** outcome with minimal associated gene
3 **conversion**. As such, it is the most conservative sub-pathway of HR, even when using a donor
4 at an ectopic locus.

5 Alternatively, the displaced strand in the extended D-loop can anneal to the second end of the
6 DSB leading to the formation of a double Holliday Junction (dHJ), as part of the pathway
7 historically coined Double-Strand Break Repair (DSBR) [28]. This step may depend on more
8 extensive DNA synthesis than SDSA [29]. This covalently linked intermediate, detected
9 physically both in somatic and meiotic cells [30, 31], can either be topologically dissolved [32]
10 or resolved endonucleolytically [33]. While dissolution always yields a non-crossover outcome
11 with minimal gene conversion, resolution can lead to a **crossover** outcome. If occurring at an
12 allelic locus, crossovers will cause a loss of heterozygosity at the next cell division in half of
13 the cases. If occurring between non-allelic loci, a crossover will lead to a reciprocal
14 translocation (**Fig. 1**).

15 Alternatively to this second end annealing scenario, a defect in extended D-loop disruption or
16 the absence of a second end to anneal to (*e.g.* in the case of a single-ended DSB) leads to a long-
17 range displacement DNA synthesis mechanism known as Break-Induced Replication (BIR)
18 until stabilization of the broken chromosome by capture of a telomeric sequence or the merging
19 with a convergent replication fork [34, 35]. BIR is a physiological pathway of broken fork
20 recovery also prone to generate various types of rearrangements (reviewed in ref. [36]).
21 Replication forks can break through direct cleavage by structure-selective endonucleases, such
22 as MUS81-EME1, or by replicating through an existing single-stranded nick in the template
23 [19]. Indeed, the massive gene conversion resulting from its conservative nature [37, 38] will
24 lead either to loss of heterozygosity upon repair templated by the homologous chromosome or
25 an unbalanced SV if initiated at an ectopic site. Ectopic BIR is also associated with copy number
26 (CN) gain of the template molecule. The overall mutagenic potential of BIR depends on the
27 extent of synthesis it can achieve, which can be several hundreds of kilobases [37]. Finally, the
28 long-lived single-stranded DNA associated with BIR [38, 39] is at risk for increased
29 mutagenesis and *kataegis* [40].

30 A consequence of the unstable displacement DNA synthesis during HR is the occasional
31 occurrence of template switches at regions of extensive homo- and homeologies over rounds of
32 extended D-loop dissociation and re-invasion, both as part of BIR and SDSA [41-45]. Template
33 switches can also occur at nearby micro-homologies when D-loop extension during BIR is

1 impaired in a *PIF1* mutant [46]. These switches thus provide opportunities for multiple CN
2 gain-associated SVs from a single DSB repair event. This propensity to switch decreases at a
3 distance from the invasion point, as the migrating synthesis bubble is converted by **structure-**
4 **selective endonucleases (SSEs)** to a stable replication fork [35, 44, 47, 48]. Hence, BIR-
5 mediated SVs are predicted to be found clustered at one end of the gained region, where the
6 initial invasion and synthesis have occurred. Moreover, several HR intermediates are substrates
7 for SSEs, and a combination of strand cleavage and ligation can convert a D-loop to a **half-**
8 **crossover (Figs. 1, 2A)** [44, 49-51]. It is predicted to transfer a co-oriented 3'-protruding single-
9 ended DSB on the donor, thus maintaining the initial amount and orientation of DSB extremities
10 (**Fig. 2A**). Such DSB transfer on the donor have been physically detected in a related
11 mechanism (MIR, see below) and shown to be dependent on both Rad51 (*i.e.* DNA strand
12 invasion) and SSEs [52].

13 **Multi-invasions-Induced Rearrangement (MIR): an HR-based mechanism for the** 14 **formation of complex rearrangements**

15 Biochemical work revealed that a single presynaptic filament can pair and invade (*i.e.* form
16 hDNA with) multiple dsDNA donors at once, resulting in a multi-invasion intermediate (MI;
17 **Fig. 2B**) [52, 53]. MI joint molecules are readily formed by long presynaptic filaments made
18 with bacterial RecA, yeast Rad51-Rad54, and human RAD51-RAD54, and are stimulated by
19 increasing homology length ([52, 53] and Wright & Heyer, unpublished). These results suggest
20 that MIs are byproducts of basic activities of the presynaptic filament: the inter-segmental
21 homology sampling process [54] and hDNA formation.

22 It is noteworthy that terminal homologies are not required for MI formation [52, 53]. It suggests
23 that ssDNA regions, such as replication-associated gaps, distant from or devoid of a freely
24 rotating end could form MI joint molecules, despite the topological constraints for strand
25 intertwining [55]. This possibility greatly expands the pathological and physiological contexts
26 conducive to MI beyond the DSB-induced and -proximal situation studied so far [52] (see below
27 and **Fig. 3**).

28 MI joint molecules form in *S. cerevisiae* cells and induce the formation of chromosomal
29 rearrangements (**Fig. 2C**) [52]. Specifically, the endonucleolytic processing of MI by SSEs
30 leads to a translocation of the donors that inserts the intervening sequence of the invading
31 molecule between the two invaded regions, in a mechanism referred to as MI-Induced
32 Rearrangement (MIR; Fig. 2B) [52]. In addition to the translocation, MIR generates additional

1 single-ended DSBs on each donor (**Fig. 2C**; mechanisms detailed in ref. [56]). Moreover, the
2 initiating DSB is not repaired during the MIR process (**Fig. 2C**). Hence, while D-loop cleavage
3 leading to half-crossover keeps the total amount of DSB ends constant, MIR generates two
4 additional single-ended DSBs (compare **Figs. 2A** and **2C**). In both cases, the propensity to
5 generate additional and sometimes complex rearrangements upon attempted repair of the
6 remaining DSBs (initial and newly formed) depends on their respective sequences [52, 56].
7 Hence, MIR is a HR pathway that uniquely amplifies the initial damage, and is thus at risk of
8 runaway cascades of rearrangements.

9 **Reversibility of HR intermediates by HR regulators guards against MIR and repeat-** 10 **mediated genomic instability**

11 HR accuracy relies in part on kinetic proof-reading, which is enforced by the reversal of several
12 non-covalent intermediates of the pathway as a safeguard against HR-mediated rearrangements
13 [57]. Distinct but possibly overlapping reversal activities in yeast are supported by a diverse set
14 of HR regulators including the Srs2 helicase (putative functional human homologs are FBH1,
15 RTEL, PARI and RECQ5), the Mph1 helicase (human FANCM), the Sgs1-Top3-Rmi1
16 helicase-topoisomerase complex (STR; human BLM-TOPO3 α -RMI1/2), and mismatch repair
17 (MMR) factors (reviewed in [57]). These regulators operate at multiple possible steps during
18 the HR pathway [57]. Srs2 dissociates Rad51 filaments [58, 59] likely formed on ssDNA gaps
19 generated during replication that are otherwise toxic [60]. Nascent and extended D-loops are
20 nodes for two radically different decisions: anti-recombination and anti-crossover, respectively
21 (**Fig. 1**). Srs2, Mph1, and STR disrupt D-loops in reconstituted biochemical reactions with
22 Rad51, Rad54 and RPA [61-63]. In cells, they inhibit homeologous recombination in
23 coordination with MMR [9], promote and/or bias the HR repair outcome towards non-crossover
24 [61, 64-66], and in the case of Mph1 and Srs2 promote template-switch during BIR [43].
25 Additionally, the STR complex uniquely catalyzes dHJ dissolution [32, 67]. Hence, Srs2, Mph1
26 and STR are believed to operate throughout the HR pathway at the presynaptic (resection and
27 Rad51 filament), synaptic (nascent D-loop formation and stability) and post-synaptic (extended
28 D-loop stability and dHJ processing) steps, which results in conservative repair products and
29 mitigates the risk of HR-induced genomic instability.

30 Consistent with their proposed roles in D-loop disruption, Srs2, Mph1 and STR also suppress
31 MIR [52]. Additionally, the Rad1-Rad10 endonuclease (human XPF-ERCC1) suppresses MIR
32 by clipping the 3'-flap of the internal invasion, thus preempting formation of the MI

1 intermediate (**Fig. 2D**) [52]. Analysis of combinations between these MIR suppressive
2 pathways suggested that these inhibitory activities are exerted at different steps or substrates
3 [52]. Conversely, physical analysis of BIR intermediates suggested that Srs2 inhibits MI
4 formation presumably by preventing Rad51 filament assembly on and/or by disrupting internal
5 D-loops formed by the trailing ssDNA upstream of the extending D-loop [39]. Hence, multiple
6 independent activities prevent the formation and/or accumulation of HR byproducts at risk for
7 genomic instability. Their precise substrate(s), mechanisms and interactions have not yet been
8 deciphered.

9 **Long single-stranded DNAs and nucleases are instrumental in MIR**

10 Various endo- and exonucleases are instrumental for both the early and late steps of MIR, by
11 generating the recombinogenic substrates and by processing the subsequent MI joint molecules,
12 respectively (**Fig. 3**). Long ssDNAs are key precursors of MIR: the amount of MI species in
13 reconstituted D-loop reaction *in vitro* and the frequency of MIR *in vivo* are greatly stimulated
14 by increasing ssDNA and homology length, respectively [52, 53]. By exposing long ssDNAs,
15 extensive resection presumably enable the concomitant encounter of independent donors by the
16 Rad51-ssDNA homology search engine [52-54] and exposes repeated sequences located at a
17 distance from the DSB site, thus enabling SSA [12, 68]. Consequently, various cellular
18 processes that generate long ssDNA are potential instigators of repeat-mediated genomic
19 instability by MIR (**Fig. 3**). DSB resection is a regulated process that involves several endo-
20 and/or exonucleases [69], and which has recently been shown to be reversible [70]. Briefly, the
21 Sae2-MRX (human CtIP-MRN) endo- and exonuclease complex initiates DSB resection at
22 short range (~1 kb), while the Exo1 exonuclease (human EXO1) and Sgs1/Dna2 (human
23 BLM/DNA2) redundantly generate ssDNA of up to several tens of kilobases [69]. Single-strand
24 breaks as well as gaps on the lagging strand of replication forks can also be exonucleolytically
25 processed in a similar fashion [71, 72] (**Fig. 3**). Long ssDNAs can also be generated in a
26 nuclease-independent fashion at the replication fork, such as upon uncoupling between the
27 leading strand DNA polymerase and the replicative helicase [73-75] or when DNA primase
28 becomes limiting [74, 76]. The endonucleolytic processing of replication forks by SSEs is also
29 expected to yield single-ended DSBs exhibiting varying degrees of ssDNA, especially upon
30 uncoupling (**Fig. 3**). Broken forks can be rescued by BIR, which generates persistent, kilobases-
31 long newly synthesized ssDNA trailing behind the extending D-loop [38], which was physically
32 shown to invade *in cis* upstream of the extending D-loop [39]. Previously observed BIR-

1 induced recombination events between donor chromosomes may result from the
2 endonucleolytic processing of such MIs [43].

3 As discussed earlier, DNA strand invasion intermediates generated during HR exhibit a variety
4 of branch points that can be recognized and cleaved by SSEs with varying efficiency [33].
5 Consistently, three SSEs in *S. cerevisiae* (Mus81-Mms4, Slx1-Slx4, and Yen1) are required in
6 a redundant fashion for MIR [52]. Functional redundancy between these SSEs in cleaving
7 other DNA structures had been previously reported [44]. Importantly, while moving the
8 pathway towards the completion of the donor translocation, MI cleavage by SSEs propagates
9 additional single-ended DSBs onto the donors (**Fig. 2C**).

10 In conclusion, nucleases contribute to HR-mediated genomic stability and specifically play a
11 two-fold role in MIR by: 1) generating the initiating recombinogenic substrate and 2)
12 processing DNA joint molecules into both genomic rearrangements and additional
13 recombinogenic damage [44, 52].

14 **Chromothripsis as a consequence of a spatial and temporal failure to separate nucleases** 15 **from their potential substrate?**

16 Recent inducible experimental systems of chromothripsis in mammalian cells started to shed
17 light on cellular contexts prone to generate massive and localized structural DNA damage and
18 their commonalities, and the repair mechanisms involved in the formation of the rearrangement.

19 Nuclear compartmentalization defects are associated with DNA damage formation and 20 chromothripsis

21 Cellular compartmentalization and cell cycle regulation ensure that mutually exclusive
22 metabolic activities are separated spatially and/or temporally [77]. For instance, failure to
23 temporally separate DNA replication and mitotic entry results in mild chromosomal fragility
24 up to full chromosome shattering [78, 79]. The breakage results from replication fork collapse
25 triggered by the activation of SSEs [80-82], which normally act at mitosis as a last resort
26 mechanism for disentangling various types of DNA joint molecules that dissolution and
27 decatenation have failed to process [83, 84]. From a spatial point of view, one role of the nuclear
28 envelope (NE) is to isolate the genomic DNA from the nucleases that patrol the cytosol as part
29 of the cellular immune system (**Fig. 4**) [85]. In fact, NE disruption and/or programmed entry of
30 endo- and exonucleases (endoG, TREX1 and NM23-H1) into the nucleus are integral to the
31 genomic DNA elimination program of apoptosis induced during viral infection or in tumor cells

1 [86-88]. The cytosol also contains Yen1/GEN1, an SSE involved in the elimination of various
2 persistent replication and HR intermediates upon NE breakdown at mitosis [89]. Its activity is
3 subjected to a dual inhibitory control in human: a spatial exclusion from its potential substrates
4 and a cell-cycle regulated inactivation of the enzyme by phosphorylation [90, 91].
5 Consequently, NE rupture will accidentally expose genomic DNA to various nucleases, thus
6 fulfilling one of the prerequisite for chromothripsis: the formation of massive DNA damage
7 (**Fig. 4**).

8 Two independent chromothripsis-inducing contexts that both entail nuclear
9 compartmentalization defects have recently been investigated (**Fig. 5**) [92-94]. In two studies,
10 chromothripsis arose from the isolation of whole or pieces of chromosome within **micronuclei**
11 consecutive to their mis-segregation at the previous mitosis (**Fig. 5A**) [92, 93]. A consequence
12 of this disrupted nuclear homeostasis in micronuclei is the massive formation of DNA damage
13 containing, or coinciding with, long ssDNA (as revealed by colocalized phosphorylated RPA
14 and γ H2AX staining) specifically during or following S-phase, but not in G1 (**Fig. 5A**) [95].
15 Their S-phase-dependent nature suggested that defective replication itself or the exposure of
16 DNA structures generated during replication to cytoplasmic components such as SSEs leads to
17 DNA damage (**Fig. 4**).

18 In another study, chromothripsis was initiated by the attempted segregation of a dicentric
19 chromosome (**Fig. 5B**) [94]. The force exerted by the spindle at metaphase (≈ 1 nN) [96] is
20 expected to cause structural transitions and partial melting of protein-free dsDNA [97, 98], but
21 remains insufficient to break the molecules, as rupture of covalent bonds occurs only above 2
22 nN [99]. Instead, resolution of various types of DNA bridges involved the action of nucleases,
23 either at the mid-body during cytokinesis or upon breakdown or rupture of the NE [83, 84, 94].
24 In the case of chromatin bridges, the NE reformed around the segregated chromosomes as well
25 as the bridge that persisted between the two daughter cells, which remains subjected to robust
26 antagonistic forces (**Fig. 5B**) [94]. These forces ultimately lead to localized NE rupture at the
27 base of the bridge, exposing the intervening chromatin to cytoplasmic components. The TREX1
28 exonuclease, either by exploiting pre-existing nicks or aided by an uncharacterized
29 endonuclease, resects the stretched bridge until its resolution, causing the snapback of massive
30 amounts of RPA-coated ssDNA into the daughter cells (**Fig. 5B**). This damaged DNA prompted
31 the formation of 53BP1, γ H2AX and Mre11 foci in the daughter cells, where it induced
32 chromothriptic rearrangements [94]. Notably, these micronuclei-independent chromothripsis

1 events were uniquely found associated with kataegis, consistent with long-lived ssDNA as an
2 initiating substrate for chromothripsis (see below).

3 Hence, the commonality of these two experimentally distinct chromothripsis-inducing contexts
4 is a defect in nuclear compartmentalization, and in one case the demonstrated involvement of a
5 cytoplasmic nuclease and long ssDNAs.

6 Repair mechanisms generating chromothriptic rearrangements

7 Initial examination of the genomic characteristics of chromothriptic rearrangements, such as
8 the SV junction sequences and the limited and oscillating copy number variation, suggested
9 that chromothripsis resulted either from the EJ-mediated stitching of numerous DSBs and/or
10 of microhomology-mediated template-switching during replicative or repair processes [1, 4].
11 Experimental evidence indicated that the DNA damage generated in both micronuclei during
12 S-phase and upon bridge resolution in G1 or S phase stains with RPA antibodies, and certain
13 chromothripsis events were found associated with kataegis over kilobases-long regions [94,
14 100, 101]. These observations suggested the involvement of long ssDNAs in chromothripsis, a
15 recombinogenic substrate that is not readily processed by EJ mechanisms. Furthermore, HR-
16 proficient breast cancer cells exhibit clustered rearrangements of various types while HR-
17 deficient cells do not [102]. Moreover, chromothripsis was found mutually exclusive with
18 biallelic BRCA2 mutations in metastatic prostate cancers [103]. Finally, p53-deficient mice
19 inactivated for EJ (XRCC4 or LIG4) or HR factors (BRCA2) still exhibited complex
20 rearrangements consistent with chromothripsis or *chromoanaysynthesis*, suggesting the
21 involvement of both pathways in chromothripsis etiology [104].

22 Ly and colleagues experimentally addressed whether DNA damage, in addition to be generated
23 in micronuclei, could also be repaired there [92]. Depletion of canonical NHEJ factors (LIG4
24 and DNA-PKcs), but not HR (BRCA2 and RAD51) or MMEJ factors (LIG3 and PARP1), led
25 to an increase of the fragmentation of the micronuclei-contained Y chromosome observed at
26 the subsequent mitosis. It suggests that a significant fraction of the micronuclei-induced
27 damages are substrate for the NHEJ machinery, and that this type of repair is active in the
28 context of the micronuclei while HR repair is not. The remaining damages are subsequently
29 repaired upon reincorporation of the micronuclear DNA into the main nucleus at mitosis (**Fig.**
30 **5A**) [93], the nature of which remains unknown. Although not yet demonstrated by sequencing,
31 this NHEJ-dependent minimization of micronuclear DNA fragmentation suggests that the
32 mechanisms generating chromothriptic rearrangements could occur in the micronuclei in

1 addition to the main nucleus. The involvement of NHEJ is consistent with the analysis of
2 junction sequences in chromothripitic genomes [1].

3 In contrast to the Y-chromosome system, bridge-induced chromothripsis originated from
4 extensive ssDNA and did not undergo partial repair in micronuclei [94]. How could a single
5 long ssDNA or multiple ssDNA gaps that are not readily substrate for EJ repair trigger
6 chromothripsis in the daughter cell? Since long ssDNAs are suitable for Rad51 filament
7 assembly, bridge-induced chromothripsis potentially resulted from attempted HR repair. This
8 repair is expected to be independent of factors required for resection initiation and extension,
9 thus bypassing an important (although not unique [105]) G1-specific block to HR, at the
10 resection initiation level (reviewed in ref. [69]). Defects in p53 in the strain used may also have
11 potentiated HR [106] by failing to repress *RAD51* expression [107]. This situation is
12 nonetheless relevant as p53 is inactivated in the majority of human cancers [108]. Furthermore,
13 the prevalence of chromothripsis is higher in p53-deficient tumors [109]. Given the concomitant
14 NE integrity defect, we suspect that HR intermediates generated along this ssDNA may lead to
15 damage transfer and amplification similar to what is observed during half-crossover and MIR,
16 respectively (**Fig. 2**).

17 Hence, the etiology of chromothripsis is complex and likely influenced by the damage-inducing
18 context, with different DSB and/or ssDNA gap repair pathways involved in the formation of
19 the rearrangements.

20 **Why is there no extensive homology at the chromothripitic SV junctions? The need for**
21 **long-read genome assemblies.**

22 It is striking that prior to the advent of high-throughput DNA sequencing the finely-mapped SV
23 junctions observed in human genomes involved repeated elements [110, 111], in agreement
24 with yeast studies [8]. Then how is it that the vast majority of the SV junctions documented
25 since then exhibit significant micro-homology (1-10 nt) but rarely longer homologies [112],
26 despite the massive repeat content of the human genome? Instead of a biological reality, we
27 suspect that this bias originates from technical limitations of the dominant paired-end
28 sequencing technology and analysis pipelines. First, reads ambiguously mapped (*i.e.* in repeats)
29 are usually discarded. Second, paired-end sequencing of <400 bp fragments used to detect SV
30 poses an absolute upper threshold to the repeat size that can be detected at a SV junction.
31 Consistently, germline and somatic cancer rearrangements in humans identified by such
32 methods are found enriched near or at short repeats (SINEs) but strongly depleted at long

1 repeats (LINEs) [113]. Accordingly, the exhaustive establishment of mutational profiles
2 (particularly SVs) in *S. cerevisiae* required the use of additional molecular techniques in
3 addition to paired-end sequencing [114]. We anticipate that the improved mappability of long
4 repeated regions thanks to long-read technologies, optical mapping or Hi-C approaches will
5 reveal more SV formed at repeats [115-118]. The recent application of long-read sequencing
6 and across-platform comparisons reported a dramatic increase in the identification of SVs [115,
7 119-121], and improved SV calling algorithms could detect significant enrichment of junctions
8 within LINEs [112]. These technical advances will produce a more comprehensive picture of
9 the patterns of SVs in genomic rearrangements and will enable to test the involvement of HR
10 and especially MIR in chromothripsis.

11 **Concluding remarks**

12 Far from an error-free pathway, HR can generate a variety of rearrangements due to the
13 repetitive nature of the genome. The HR pathway entails the formation of DNA joint molecules
14 at risk to induce rearrangements when cleaved inappropriately. Consequently, dysregulation of
15 SSEs and other nucleases puts the cell at risk of a HR-mediated runaway cascade of damage
16 and rearrangement, as demonstrated with the MIR pathway. Given that the integrity of the NE
17 is compromised in experimental models for chromothripsis, we suspect that a key aspect of
18 both SV and damage formation of chromothripsis reside in the accidental exposure of
19 replication and HR intermediates to various nucleases. Finally, we highlight the need for
20 genomes assembled from long-read DNA sequencing techniques to allow evaluating the
21 contributions of HR between repeated DNA in the formation of genomic rearrangements and
22 chromothripsis.

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52

53

1 **Highlights**

- 2 • Homologous recombination generates genome rearrangements involving repeated DNA
3 elements with identical (homologous) or near identical (homeologous) sequences that
4 can be located anywhere in the genome.
- 5 • Multi-invasions are recombination byproducts that physically bridge two copies of a
6 repeated DNA element that can be processed by structure-selective endonucleases into
7 genome rearrangements and additional DNA double-stranded breaks.
- 8 • Different chromothripsis-inducing contexts in mammalian cells feature defective
9 isolation of genomic DNA from cytoplasmic components, including various types of
10 nucleases.
- 11 • Long DNA sequence read assemblies paired with additional approaches and improved
12 bioinformatic pipelines are needed to fully evaluate the contributions of homologous
13 recombination between repeated DNA to generate genomic rearrangements.

14

15 **Glossary**

16 **Breakage-Fusion-Bridge cycle:** Breakage-Fusion-Bridge cycle denotes a genetic
17 phenomenon first described by Barbara McClintock of telomere fusions generating a dicentric
18 chromosome that cannot be properly segregated during anaphase leading to breakage and a new
19 cycle of fusions [122]. Any genome rearrangements between sister chromatids, homologs or
20 different chromosomes resulting in a dicentric chromosomes will lead to chronic genomic
21 instability until stabilization of the broken fragments.

22 **Conversion/Crossover/half-crossover/non-crossover:** Conversion is a non-reciprocal
23 exchange of genetic information. Crossover is the reciprocal exchange of genetic markers
24 during HR, where both reciprocal products are recovered. In somatic cells, crossover is actively
25 avoided by using the SDSA pathway and dissolution of double Holliday junctions, leading to
26 non-crossovers which often involve conversion (**Fig. 1**). Half-crossover is an event where one
27 of the expected reciprocal products is lost.

28 **D-loop (Displacement loop):** D-loops are a central HR intermediate, whose processing
29 determines the HR subpathway (SDSA, DSBR, or BIR) and crossover/non-crossover outcome
30 (**Fig. 1**). D-loops are formed by Rad51-ssDNA filament through DNA strand invasion. Nascent

1 and extended D-loop denotes D-loops prior and after extension of the 3'-OH of the invading
2 DNA strand by DNA polymerase, respectively.

3 **Double-strand break (DSB):** A DSB consists in the interruption of the phospho-diester
4 backbone of two complementary DNA strands at the same or nearby position. DSBs come in
5 two flavors: either single-ended such as upon replication fork breakage or frank (two-sided)
6 (**Fig. 1**). Single-ended DSBs can be accurately repaired only by HR.

7 **Micronucleus:** Micronuclei are a classic hallmark of genotoxic stress and form when a
8 chromosome or chromosome fragment is not incorporated into the two daughter nuclei after
9 anaphase. Micronuclei are nuclear-like structures that features defective isolation of the
10 nucleoplasm from the cytoplasm (**Fig. 4**) [93, 95, 123]. Micronuclei are depleted for nuclear
11 pore complexes and other NE proteins, which is suspected to dysregulate nuclear protein
12 homeostasis and underlie micronuclei-specific NE fragility (**Fig. 4**) [124].

13 **Structure-selective endonucleases (SSE):** Non-linear DNA structures including intermediates
14 generated during HR (flaps, D-loops or HJs) and stalled replications forks are substrates for
15 SSEs. Mostly studied for their role in genome maintenance are Mus81-Mms4, Slx1-Slx4, Rad1-
16 Rad10 and Yen1 in *S. cerevisiae*, respectively MUS81-EME1/2, SLX1-SLX4, XPF-ERCC1
17 and GEN1 in humans.

18

19 **Outstanding Questions**

- 20 • What is the role of SSEs and cytoplasmic nucleases in generating massive DNA damage
21 and the complex rearrangements of chromothripsis?
- 22 • What are the DNA repair pathways involved in different damage-inducing contexts?
23 Especially for the repair of long ssDNAs?
- 24 • Are HR regulators involved in suppressing chromothripsis?
- 25 • Does MIR operates in human cells? In which context? Is there a mutational signature
26 unique to MIR?
- 27 • What additional types of SVs and junction sequences will be uncovered by long-read
28 assemblies of genomes with simple and complex rearrangements?

29

30 **Box: Mutational Phenomena**

1 **Chromothripsis** was originally described by Campbell and colleagues as massive focal
2 genome rearrangements in a patient with Chronic Lymphocytic Leukemia [1] and is
3 associated with a significant and variable proportion of cancer genomes depending on cancer
4 type [2] . Independently, chromothripsis was identified as a constitutional genetic change in a
5 child with severe congenital abnormalities [125]. The term derives from Greek (chromos for
6 chromosome; thripsis, shattering into pieces) describing the interpretation that chromothripsis
7 results from catastrophic chromosomal breakage into many individual DNA fragments.

8

9 **Chromoanasythesis** is a constitutionally acquired complex genome arrangement
10 phenomenon that was discovered in individuals with developmental delay and cognitive
11 anomalies [126]. The patterns of CNVs and breakpoint junctions differ from chromothripsis,
12 and the breakpoint sequence analysis suggested repeated template switching at
13 microhomologies as the process leading to the observed rearrangements.

14

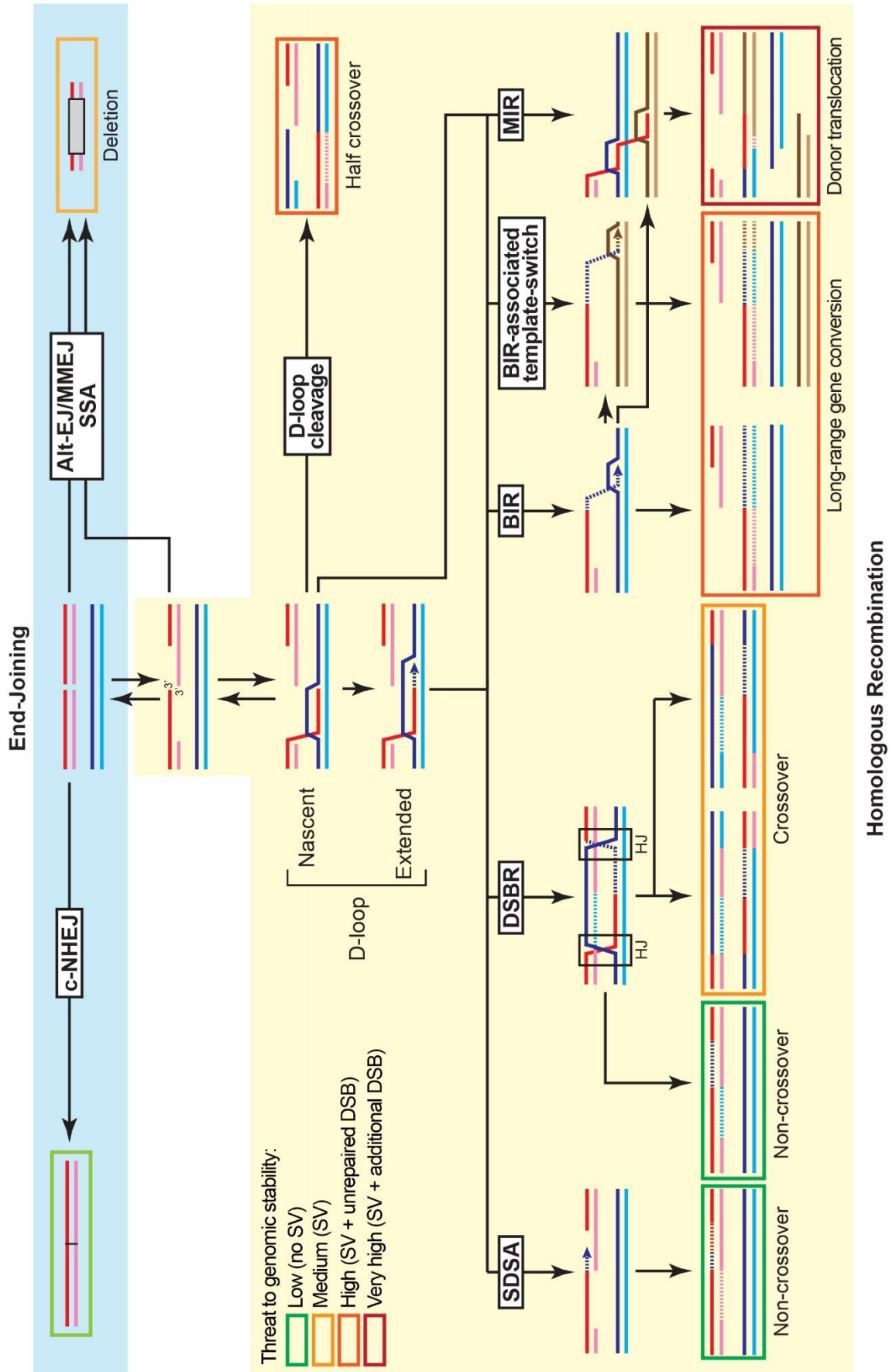
15 **Chromoplexy** describes yet another pattern of complex genome rearrangements involving a
16 connected chain of translocations which first discovered in prostate tumors [127].

17

18 **Kataegis** describes localized hypermutation of single nucleotide changes identified in mice
19 and in some cancer genomes [101, 128]. Kataegis is the C-to-T mutagenic signature of
20 APOBEC deaminases that act on single-stranded DNA, suggesting that kataegis signals
21 regions of long-lived single-stranded DNA intermediates [40, 101, 129].

22

1 **Figures**

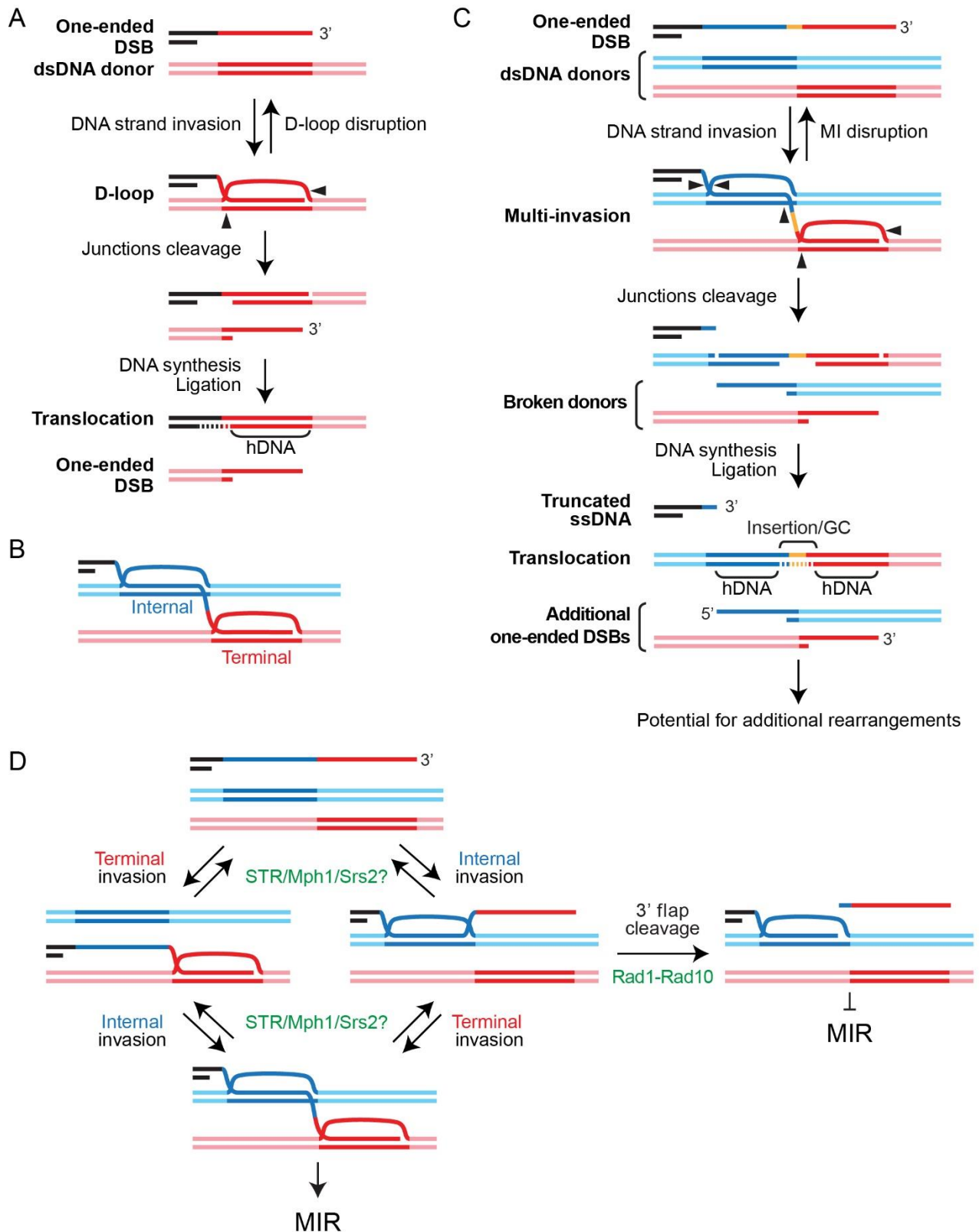


2

1 **Figure 1: Overview of the DSB repair pathways and their associated risk for genomic**
2 **stability.** The repair products are boxed. The box color (from green to red) indicates the threat
3 to genomic stability posed by the product of each pathway and sub-pathway. DNA synthesis is
4 indicated by an arrow and newly-synthesized DNA by a dotted line. The long ssDNA associated
5 with BIR has the potential to undergo MIR. More detailed mechanisms for D-loop cleavage
6 and MIR are provided in **Figures 2A** and **2C**, respectively. The reversibility of DSB resection
7 provided by the Shieldin complex with fill-in by the DNA polymerase α -primase complex
8 provides an unanticipated degree of flexibility in the choice of DSB repair between HR and EJ
9 mechanisms [130]. Hence, EJ pathways are available not only on unresected or minimally
10 resected DSB, they can also be engaged after extensive resection and subsequent fill-in.

11

12



1

2 **Figure 2: Single and multi-invasion joint molecules are at-risk HR intermediates for SSE-**
 3 **mediated genomic instability.** (A) Model for half-crossover formation from cleavage of a
 4 single D-loop. (B) Multi-invasions (MI) joint molecules are formed when a Rad51-ssDNA
 5 filament invades two independent donors along its length. It features an internal and a terminal

1 D-loop. (C) Model for multi-invasion-induced rearrangement (MIR). (D) Several activities
2 inhibit MIR in a reversible (Sgs1-Top2-Rmi1 (STR), Mph1, Srs2) or irreversible (Rad1-Rad10)
3 fashion. The specificity of STR, Mph1 and Srs2 as well as the precise nature of their substrates
4 has not been established.

5

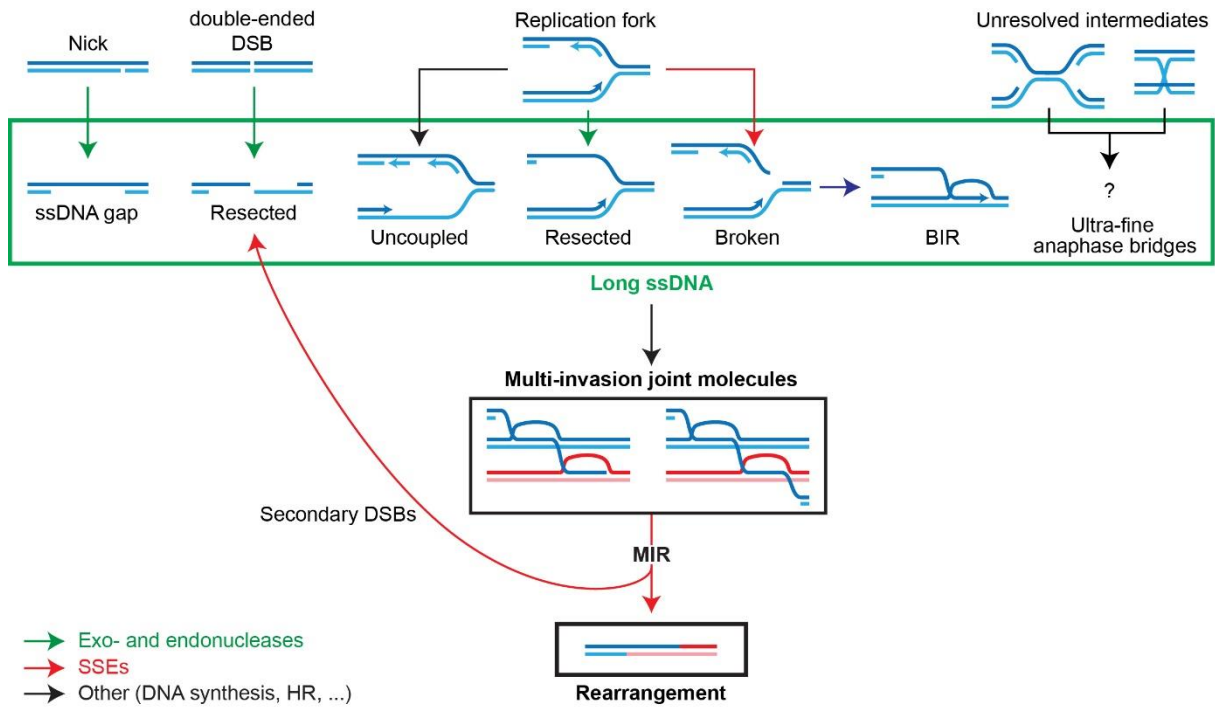
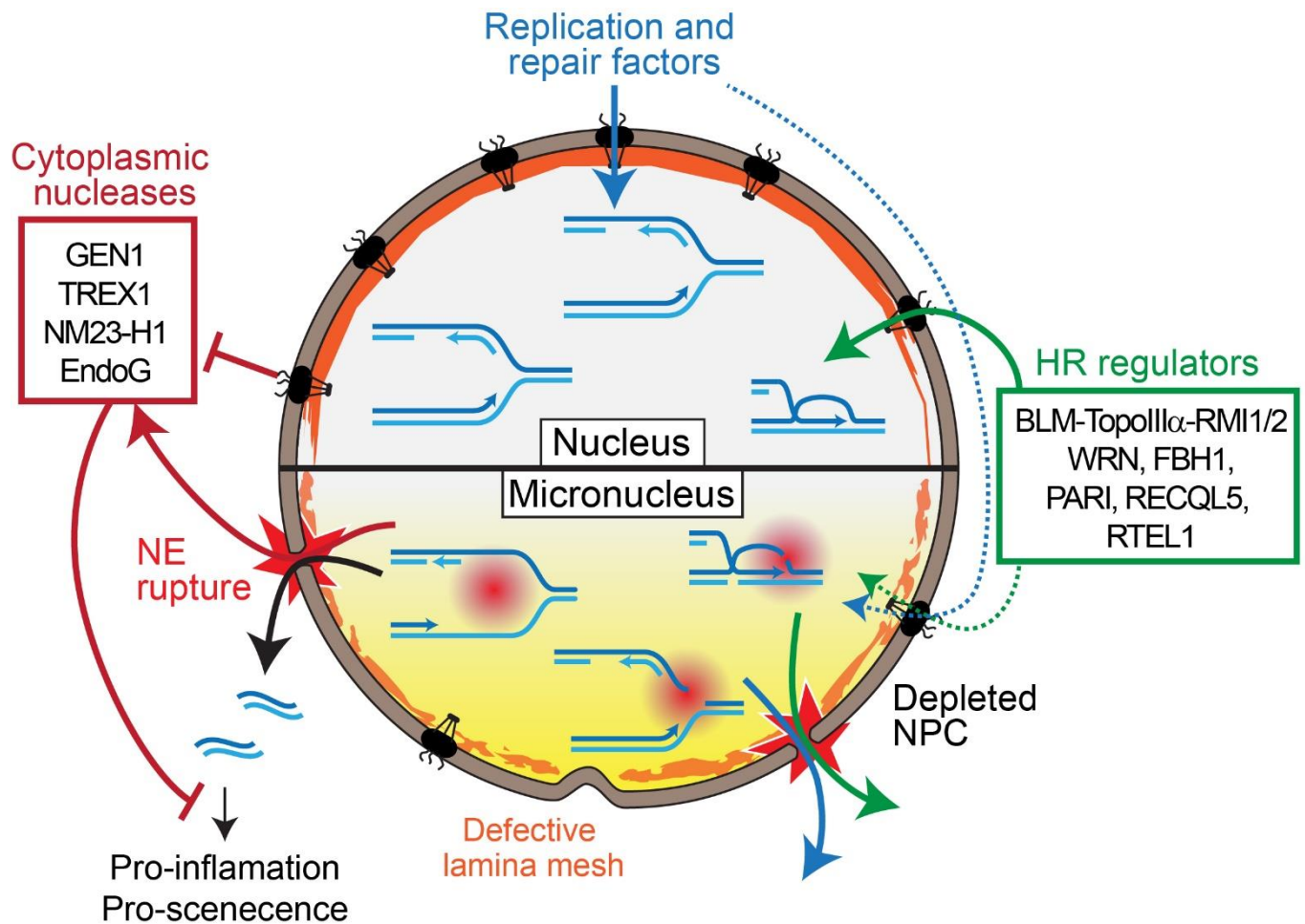


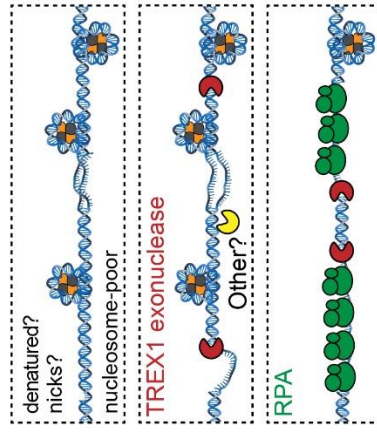
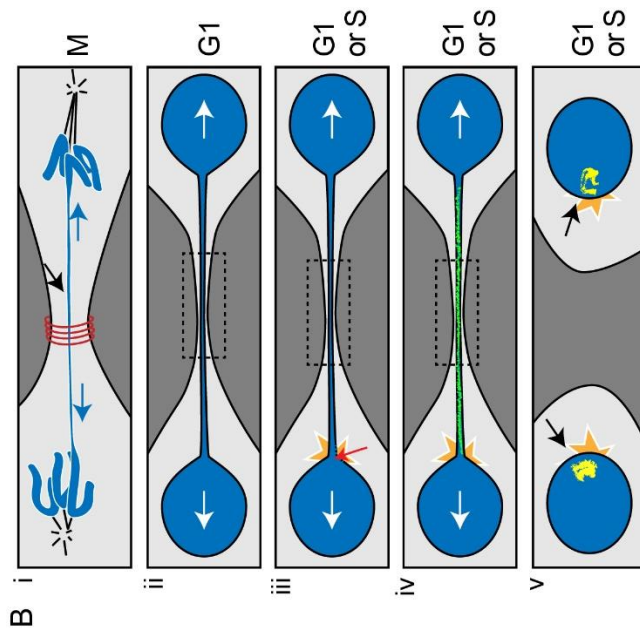
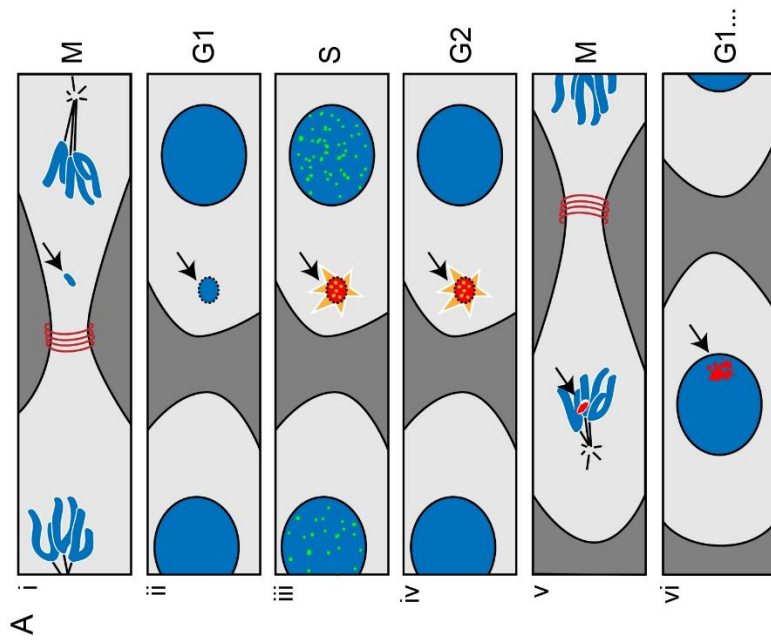
Figure 3: Nuclease involvement in MIR. Exo- and endonucleases involved in resection (green) generate long recombinogenic substrates, while SSEs (red) process MI joint molecules into rearrangements and additional resected secondary single-ended DSBs (indicated by backwards arrow to resected DSBs).



1

2 **Figure 4: S-phase-specific DNA damage from defective nucleoplasm isolation and DNA**
 3 **metabolism in micronuclei.** The nuclear envelope (NE) of micronuclei is depleted for nuclear
 4 pore complex and features abnormal lamina deposition. These dysregulations lead to abnormal
 5 nuclear protein import and frequent NE rupture, respectively. NE rupture leads to the
 6 penetration of cytoplasmic components to the micronucleus and the leakage of soluble nuclear
 7 components and DNA.

8



1 **Figure 5: Cellular models for chromothripsis.** (A) Chromothripsis by isolation and
2 breakage of chromosomes in micronuclei [92, 93]. (i-ii) Mis-segregation and physical
3 separation of a large chromatin fragment leads to micronucleus formation. (ii-iii) Micronuclei
4 are defective for the nuclear import and barrier function (**Fig. 4**). (iii) Upon S-phase entry, NE
5 rupture leads to the formation of massive DNA damage, which features co-localizing DSBs
6 (green) and γ H2AX foci [95] (iv-v) The micronuclear DNA reaches mitosis under-replicated
7 and fragmented. (v-vi) Micronuclear DNA fragments can be re-incorporated into the main
8 nucleus at subsequent mitosis where it may undergo final repair. (B) Chromothripsis originating
9 from attempted segregation of dicentric chromosomes [94]. (i) Spindle-exerted tensions can
10 stretch but not break the central portion of a dicentric chromosome. (ii) NE forms around the
11 daughter nuclei and the intervening chromatin bridge, depleted for lamin and nuclear pore
12 complex. Right inset: the bridge is partially depleted for nucleosomes. (iii) Tensions exerted on
13 the bridge cause NE rupture at the base of the bridge in G1/S phase. NE rupture leads to leaking
14 of nuclear components and penetration of cytoplasmic proteins such as the TREX1 exonuclease
15 (right inset). (iv) TREX1 exploits nicks or ssDNA gaps present in the bridge or generated by
16 other cytoplasmic endonuclease to resect the chromatin bridge (right inset). This leads to
17 accumulation of RPA foci (green) and (v) culminates in the resolution of the bridge. In addition
18 to RPA, the resected DNA causes the formation of 53BP1, γ H2AX and Mre11 foci (yellow) in
19 the daughter nuclei.

20

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